Enhancement of Microbial Biosolubilization and Biogenic Methane Potential of Low to High Grade Coal of Pakistan

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Enhancement of Microbial Biosolubilization and Biogenic Methane Potential of Low to High Grade Coal of Pakistan

Thesis Submitted by
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DEDICATED TO MY BELOVED PARENTS
BROTHERS AND MY HUSBAND

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Noureen Fatima

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Abbreviations

EC	Electrical conductivity
Ph	$-\log(H^+)$
HA	Humic Acid
FTIR	Fourier transform infrared spectroscopy
GC-MS	Gas Chromatography Mass spectroscopy
SEM	Scanning electron microscopy
TC	Thar Coal
BC	Baluchistan Coal
DNA	Deoxyribonucleic Acid
rRNA	Ribosomal ribonucleic acid
CFU	Colony forming unit
WGS	Whole Genome Sequencing
SDA	Sabaraud Dextrose Agar
SOM	Soil organic matter
NCBI	*National Center for Biotechnology Information*
BLAST	*Basic Local Alignment Search Tool*
NA	Nutrient agar
MSM	Minimal Salt Medium
GSP	Geological Survey of Pakistan
TH	Thar

Abstract

The process of bio solubilization depends on coal ranking and its oxidative state. Lignite coal being less complex in structure is easier to solubilize microbially by the production of extracellular enzymes, but bituminous coal has been less used for bioconversions due to its signature features, that are considered appropriate for thermal power generation resulting negative environmental impacts. Green energy procedures can encourage the efficacy of bituminous coal for multiple applications and sustainable environment. This research was aimed to inquire and reveal the enhanced bio solubilization extent of lignite and bituminous coal designated as low and high rank aerobically and anaerobically that contributes to extract and purify eco-friendly and bio active compounds.

In this concern, the bituminous coal sample from Baluchistan coalfield, Duki region of Pakistan and lignite coal samples from thar Sindh region of Pakistan, were subjected for chemical characterization to explore its vulnerability for biological transformation. The native coal solubilizing fungal isolates NF-1 and GB, identified as *Debaromyeces hansenii and Aspergillus ochraceus.* Respectively based on fungal ITS sequences were isolated. Major conditions were optimized for *Debaromyeces hansenii* NF-1 and *Aspergillus ochraceus* GB, 1% and 1.5% glucose with 0.5% of pulverized coal was investigated as an ideal ratio for maximum organic liberation within 7 days respectively for bituminous coal while for lignite coal, 2% glucose was utilized by *Debaromyeces hansenii* NF-1 and 1% glucose was utilized by *Aspergillus ochraceus* GB with 1% coal loading ratio and incubation time of day 11. Along bio-treatment, enhancing agents were also used to modify medium (sodium acetate) as well as coal structure (NaOH) that intensify liberation of humic acid, assessed through UV-Visible spectrophotometer. Conjugative effect of fungal isolates (NF-1 and GB) on pulverized pre oxidize coal residues was found effective in liberating aromatics. Qualitatively, ethyl acetate soluble extracts of bio-liquified black liquids were investigated through GC-MS. MS spectra showed organic pool, in that pool bio-treated pre oxidized solubilized liquids represented more diversity of organics than virgin. Gravimetrically, it was analysed that *Debaromyeces hansenii* NF-1 showed 52.4% and *Aspergillus ochraceus* GB 71% weight loss of pre oxidized coal residues within 2 days by increasing process efficiency. Bio-treatment showed significant differences in elemental composition (O/C, H/C and C/N) of solubilized pre oxidized coal residues. FT-IR spectrum indicated that NF-1 and GB fungal isolates intensify the peaks of phenolic, aldehydic, aromatic, carboxylic, aliphatic and humic acid functionalities in pre oxidized residual coal.

For quantitative estimation, gravimetric ratio of extracted humic acid from pre oxidized coal residues by using *Aspergillus ochraceus* was 78% and *Debaromyeces hansenii* showed 60%

while 52% and 42% was extracted from its bio-extracts respectively. Spectroscopic E_4/E_6 ratio, elemental and FTIR spectrum of biologically (NF-1 and GB) extracted humic acid from pre oxidized coal residues specify formation of condensed aromatics and low molecular weight, enhanced nitrogen/oxygen content and vibrational changes in phenolic, amines, carboxylic, aldehydic and aliphatic compounds of humic molecules respectively. The illumine miseq technique of next generation sequencing had shown similarity in bacterial diversity in 6 samples out of 8 while, 2 samples had shown different behaviour from other samples after determining their relative and read abundance, alpha and beta diversity and heat map. While the fungal alpha diversity of 4 samples had shown distinction from each other in their relative abundance. Another sample from Baluchistan Duki coal mine had shown the presence of more *Proteobacteria and Actinobacteria* than *Firmicutes.*

After solubilization of coal by fungal isolates different compounds that were produced after degradation of coal were extracted. These compounds were name as Humic and Fulvic acids. About 4.5% concentration of KOH had shown maximum yield of humic acid in both types of coal. The percentage of humic acid using NaOH for native lignite and Bituminous coal was 21.15% and 11.6% and for HNO3 treated coal percentage for lignite and Bituminous coal was 57.8% and 46.9% respectively. The FTIR results of HNO3 treated coal had clearly indicated the presence of N-H group peak at 2923.04cm in case of bituminous coal while introduction of nitro group in lignite coal at 2921.84cm, while both peaks were absent in native coal FTIR spectrum. The elemental analysis of HNO3 treated coal had shown the reduction in carbon content and slight change in sulphur and nitrogen and increase in oxygen content that confirms the oxidation process occurred. The O/C ratio was also increased in case of HNO3 treated coal. 60% and 78% humic acid was extracted from bituminous and lignite coal by potential fungal strains.

Both the low and high rank coal were allowed to be treated anaerobically in order to find out the extent of methane production. The raw bituminous (HRC) was capable of producing more methane and carbon dioxide as compared to LRC (Lignite). The co-digestion strategy is productive in stimulation of methane generation but also enhanced its yield. A higher methane potential was observed in the biogasification setup of bituminous coal as compared to lignite coal.

The co-substrate Grass peanut shell was able to produce more cumulative methane along with bituminous coal as compared to lignite coal. Grass and peanut shells comprised of lignin and celluloses which are degraded by anaerobic microbes releasing hydrogen which is limiting the reaction rate on the first hand for its deficiency in the reaction mixture. The maximum

yield of methane and carbon dioxide was obtained at optimum temperature of 35° C and pH 7.0 in both lignite and bituminous coal samples.

The whole genome sequencing of potential microbial strains had identified the presence of annotations with their nucleotide base data and gene rank. The circular and sequence view of both the potential fungal isolates *Debaromyeces hansenii* and *Aspergillus ochraceus* had shown the particular number of bases present in the nucleotide sequences. The total base pairs of isolate *Debaromyeces hansenii* were determined as 722,974bp and total number of sequences are 516. The total number of sequences lengths include 1781 sequences in *Aspergillus ochraceus*.

Chapter No 1

Introduction

Over the past few years, the incremental demand and consumption of energy due to successive progress in industrialization and urbanization leading the world towards energy insecurities. It is estimated that if the world's energy requirements remain continuous in future at such high ratio it will lead us towards depletion of energy. Utilization of fossil reserves for energy generation is perfect approach for sustainable energy. With the emergence of fresh biological systems for sustainable generation of petrochemical fuels and organic moieties from naturally occurring reserves help us to overcome the energy shortage and negative environmental impacts.

Coal being an abundant and imperative world's energy reserve, accounting approximately 71.4% of the total global fossil fuel assets. There are approximately 1.1 trillion tons of coal deposits in worldwide that would be enough for 150 years. These huge coal deposits are majorly found in USA, Canada, Indonesia, Australia, Russia, China and India making 74% of total share. Pakistan has ranked as seventh wealthiest nation due to coal assets. The native coal assets of Pakistan are approx. 185.5BT out of which 175.5BT of assets are in Sindh Monocline. By 2011 energy sector of Pakistan shares 6.7% from coal. Natural gas shares its 49.20%.in total energy generation of Pakistan. The global energy demand is anticipated up to 60% by 2030. To fulfill 40% of world's perpetual energy demand coal being second largest energy matrix, shares 24% in overall production of electricity. Remarkable abundance of coal deposits enables to meet energy crisis for millions of years than oil and gas.

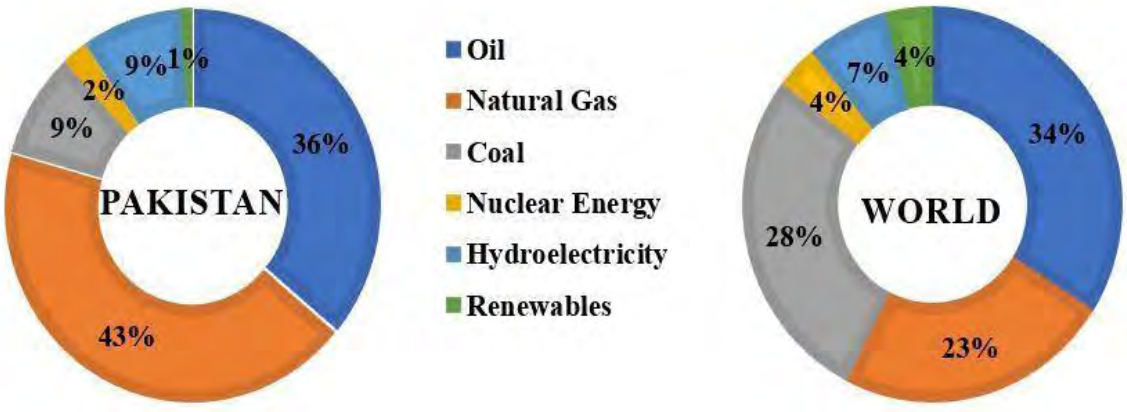

Figure 1.1 | Primary Energy Consumption of the World and Pakistan; A Comparison (BP Statistical Review, 2013)

The dissemination of coal basins around the globe is unequal due to formation and deposition of peat under different periods in geological records. In geological records carboniferous age (about 360 M to 290 M years ago) was important for the development of main coal basins than Devonian age. Biochemical processes acting on the remains of dead organic matter,

experiencing chemical, physical and morphological alterations under boggy and oxygen free environment under high temperature and pressure resulted the formation of peat and eventually converted into coal through a process called coalification. Major forces behind the coalification phenomena were physical forces. Coal is disparate organic rock, comprises of concentrated carbon and aromatic clusters linked by aliphatic and heteroaliphatic bonds having tremendous physicochemical properties depending on its types. Coal has wide range of both organic and inorganic components along water. Organically, major components are carbon, hydrogen, oxygen, discrete number of macerals, sulfur and nitrogen content. Inorganically, it comprises of variety of components that are involved in ash formation and originated from marshy water, dust, sad, silts and vegetative elements (Macerals). Number of changes occur during coal genesis. Chemical nature of coal is very diverse. Lignite and subbituminous coal, generally under the category of low rank coal (soft coal) retaining the macromolecular arrangement like coal-forming plants. Hard or high ranked such as bituminous and anthracite coal have more aromaticity, heating value carbon and volatile content.

Table 1.1 Types and properties of coal

PROPERTIES	PEAT	LIGNITE	SUB-BITUMINOUS	BITUMINOUS	ANTHRACITE
CARBON CONTENT	Less than 40%	45 to 60%	45 to 60%	45 to 85%	86 to 98%
RANK	Low	Low	Intermediate	Intermediate	High
TEXTURE	Soft	Soft	Soft-Hard	Hard	Highly hard
MOISTURE	High	High (35%)	Low	Low (15%)	Very low
VOLATILITY	High	High	Low-High	Low	Low

Coal is an organic entity, depending on its type it is quite vulnerable to microbial transformations mainly by bacteria and fungi. A unique and multifarious microbial system is required to depolymerize the coal complex polymer into monomers (precursors). Number of

researches are carried out to fully comprehend the biological system behind coal transformation, but the accurate procedure is not known due to complexity of such kind of system. Previous literature demonstrated that biological entities may utilize three possible pathways for coal transformation into fuel and non-fuel products. First pathway is the link cleavage and depolymerization of coal. Second pathway could be decarboxylation as it lowers oxygen containing groups of coal molecule resulting improved calorific value. Third pathway would be the removal of heavy metals, inorganic elements from the coal lowers undesired emissions prior to combustion. Major coal transforming pathways are solubilization and depolymerization that could be enzymatic and non-enzymatic.

Peat ⇨ Lignite ⇨ Sub-Bituminous ⇨ Bituminous ⇨ Anthracite

Coal Transformation (Coalification)

Coal bio solubilization is a non-enzymatic process that usually requires basic pH ranging from (pH 7 ± 10). It occurs by liberating chelating, alkaline substances and surface active agents through biological entities as a result pH of the system increases. Coal bio solubilization results into a significant increase in the molecular mass of coal humic constituents. Depolymerization is an enzymatic process (Lignin peroxidases, manganese peroxidases and laccases) in which pH values decreases (pH 3 ± 6) as a result complex bonding in coal carbon breaks up and produce monomeric products. Bio solubilizing ratio of coal in terms of time and percentage highly depends upon on its age/rank and solubilizers. There are number of agents that could be used to enhance the bio solubilization frequency. The rate of coal dissolution intensified by reducing coal particle sizes and increasing concentration of particles as a result fungal mediated coal bio solubilization becomes faster. There is a correlation exist between pH and bio-solubilization/liquefaction processes. The acetate amendments in growth media leads to enhance the bio solubilized products. Furthermore, microbial growth is enhanced by incorporating minerals (S, N, P metal ions) which shows that their presence is very limited in coal basins. Pre-treatment of coal polymer assisted in liberating huge variety of beneficial organic moieties through sequential breakdown of coal nuggets. There are two major categories of pretreatment for enhancing the production of coal derived organics and fuel.

- ♦ Biological based
- ♦ Chemical based pretreatments

During biological treatment naturally occurring highly oxidized coal particles (e.g., weathered lignite, leonardite) or oxidative coal particles undergoes transformation by the action of biological entities.

Organic nature of coal particles makes it susceptible to biological attack, which leads to the formation of mixture of organics in supernatant, eventually yielding fuel and value-added compounds. Previous literature showed that impact of wood eating/decaying fungus such as wood-rot fungus (*Coriolus versicolor)* and other fungal species were higher on lower range of oxygen containing coal as they are highly vulnerable to bio-solubilization than high range of coal. Refractoriness in bituminous coal could be overcome by using several agents. Chemical or Artificial oxidative pre-treatments based on utility of chemicals such as acids, bases, oxidants etc. for coal degradation. Coal derived functional
groups (high rank coal) could easily be targeted by chemicals such as nitric acid (HNO3), hydrogen peroxide (H2O2) and Sodium hydroxide (NaOH). Several studies have shown the importance of
chemical pretreatments for improved production of solubilized liquids from pretreated coal. Chemically modified coal particles would encourage the growth of biological entities. Usually, the microbial growth on coal particle is very slow. Moreover, pre oxidation of coal particles enhances the bioavailability of available amalgams.

Fractional degradation of organic material (coal) releases numerous microscopic and heterogeneous humic elements that self-assembles to form extensive supramolecular structure of humic acids (HA). There are numerous non-covalent forces such as π-π, CH-π and van der Waals forces that are involved in the stability of supramolecular arrangement of humic acid. Literature shows low rank coal frequently used to derive humic acid but using enhancing agents to modify the structure of bituminous coal will make it more feasible for biological attack to extract coal derive humic acid.

Humic acid perform significant role in maintaining soil physical and chemical property, stability of carbon, act as terminal electron acceptor to remediate the recalcitrant environmental pollutants such as heavy metals, contaminants, pesticides and encouraging plants physiology, promoting plant growth by modifying root system and microbial entities. Hence, HA are important for sustainable agriculture. Humic acid have been called as plant bio stimulants due to its benefactor nature for plant development. Thus, coal derived HA facilitates in maintaining sustainable organics and environment.

Amongst the major environmental issues are global warming, pollution, climatic changes etc. Besides the environmental hazard's energy crisis is also a major problem for human race as the non-renewable energy resources are depleting so quickly. In order to address both these problems simultaneously we need a clean energy source. The combustion of petrochemicals, diesel fuels, natural gas causes the release of Sox,NOx carbon mono oxide, carbon dioxide

etc into the environment which not only pollutes the environment but also pose serious health hazards. Although coal was always in use but in 1973 world was facing major oil crisis it was then when different countries of the world started exploiting full potential of coal (Berkowitz, 2012).

Bio gasification is a process to introduce clean energy to the world in the form of methane gas and it has various economical significances as well. In the bio gasification process the substrate is coal which act as a carbon source in the presence of microbial consortia and in a completely anaerobic environment. Methane can be produced from pretreated and presolubilized coal or can be produced directly from virgin coal. This is done in a single anaerobic step. This is done via the naturally occurring microbial consortia of coal.

A number of other related microbial consortia also reported to have methanogenic communities. The biogenic methane burns cleanly and can be produced from vast coal deposits (Al Jubori *et al*., 2009). Post depositional (secondary) and thermogenic processes are responsible for methane production and are collected in coal beds for years. This process can be taken to large scale production setups (Strapoc *et al*., 2011). As a result of bio-gasification mixture of gases are produced from coal comprising of carbon dioxide, sulphur dioxide, methane etc.

The energy output of coal during combustion is measured by the fixed carbon of its organic matter.While the behaviour of coal towards combustion is measured by the mineral matters present in coal. The reservoir properties of coal such as gas content, gas adsorption capacity, permeability and porosity are control by composition of coal. (Flores, 2014). The combustion characteristics of lignite coal shows its lower activation energy and ignition temperature than higher coal ranks. The coking value of lignite coal is almost zero. The mineral content of low rank coal i.e. lignite coal is greater (Guo *et al*., 2019).

To produce gas from coal consortia development and enrichment is an obligatory process. Basically, two diverse approaches are used to convert coal into gas. In the first approach the nutrients essential for microbial growth and development are added to coal to support and enhance the development of indigenous micro flora. While the other approaches are to add the already developed consortia prepared *ex-situ* into the coal (Fuertez *et al*., 2017).

The microbial consortium which is responsible for gas formation mainly consists of fermentative facultative and methanogenic microbes. These microorganisms colonize naturally in the coal and degrade the coal. These organisms act on the bio available compounds present in coal and produce methane as an end product (Green et al., 2008, Papendick et al., 2011, Strapoc et al., 2011, Tang et al., 2012, Ulrich and Bower, 2008).

For the enhancement of methane potential coal co-digestion strategy is introduced. Co-digestive materials provide an increased carbon source for anaerobic microorganisms and an increased methane production is observed (Navaneethan *et al*., 2011) Greenhouse gas emission is also significantly reduced using this strategy (Maeng *et al*., 1999).

There is very little work done on the gas production of different ranks of coal. Gas is produced under anaerobic conditions. Aerobic conditions are totally unfavourable for methanogens. The other unfavourable conditions include the low nutrient availability (sulphur, phosphorus, nitrogen). Another limiting factor in gas production is biodegradable compounds available in low quantity (Jones et al., 2010). The geological condition of coal mines also affects gas formation and microbial population of methanogens in coal. So, when the nutrients are added into the coal the microbial consortia acts differently in response to nutrients amendments and select their methanogenic pathway of choice feasible with the availability of nutrients. On observing the coalbed methane production three

different types of methanogenic pathways are reported. Hydrogenotrophic (CO_2 reduction), acetoclastic, methylotrophic (methanol utilization) are three different methanogenic pathways (Zinder, 1984; Zinder, 1993).

Microcosm is the main factor responsible for gas production in the coalbed methane. The lower rank coal usually contributes in higher bioavailability in producing gas. Studies reveal that the low rank coal produces more methane because of greater release of low molecular weight acids from the coal surface. The microorganisms associated with coal biodegradation are enriched with VFAs desorbed from coal. These enriched microorganisms then degrade low molecular weight acids and alcohols (Robbins *et al*., 2016).

Though various excellent studies have been conducted on the enhancement of methane production rate, e.g effect of temperature in cultures were detected, similarly effect of PH was studied. They only tried to evaluate an effect of single parameter on biogenic methane production from coal. But in present study combine effect from multiple parameters was conducted for enhancing the production of methane. In addition, to that very less data is available on the methane production from bituminous coal using co- substrates strategies. While up to our knowledge no work has been reported yet on the enhancement of biolosolubilzation extent of Balochistan Duki coal either aerobically and anaerobically.

The potential microbial strains isolated from indigenous Duki and Thar coal mine i.e. *Debaromyeces hansenii* and *Aspergillus ochracheus* had yet been unidentified for solubilization activity of coal.

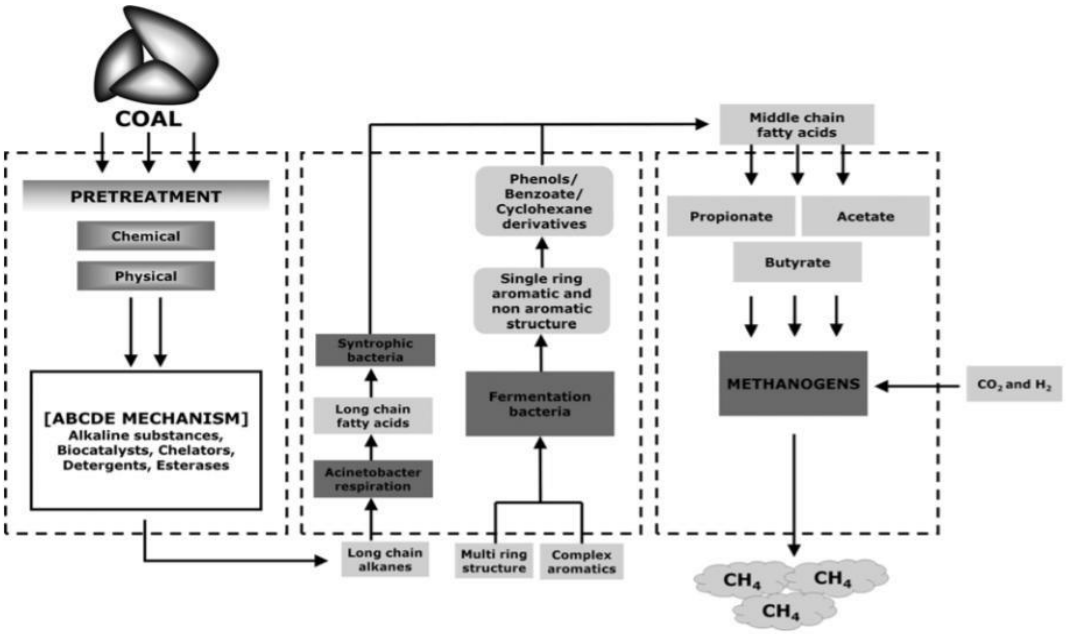

Fig 1.2 Coal methanogenesis mechanism by Pamidimarri D.V.N. Sudheer et al,2016

Aim and objectives

The study aimed to enhance the Process of Microbial Bio solubilization and Biogenic Methane Potential of Low to High grade Coal of Pakistan

Following were the objectives of current study for fulfilling the aim.

1. Isolation and characterization of indigenous coal samples and associated microbial fungal isolates.
2. Evaluation of indigenously present microbial communities (bacterial and fungal) from different coal ranks using illumina Miseq and evaluating it statistically using R.
3. Extraction of by products (humic and fulvic acid) from coal samples through fungal and chemical pretreatment of low and high rank coal.
4. Evaluation of methanogenic potential of selected native coal samples and enhancement of methanogenic process through co-digestion.
5. Whole genome sequencing of potential coal solubilizing fungal isolates and their annotation study.

Chapter No. 2
Literature Review

Presently life is totally dependent on energy resources, which increase the value of energy due to increase in rapid urbanization and modernization to meet the energy requirement. Being an important fossil fuel 2.5% of coal consumption was recorded in the previous year (British petroleum, 2013). Among fossil fuel as coal is ancient energy source but the use of natural gas and petroleum was more than coal because these were more economical and environmentally friendly sources from last few years. Additionally, statistical data of British Petroleum showed the energy consumption rates which is increased yearly. Oil increased by 1.9%, gas increased by 1.7%, coal increased by1.8% and worldwide consumption rates of coal, gas and oil was 29.9%, 23.8% and 32.9% respectively. Globally 87% energy consumption of oil, gas and coal had been recorded (British petroleum, 2015).

Huge coal reservoirs are now used to meet the energy requirements because oil and natural gas reservoirs depleted and pollute the atmosphere badly. 860 Billion tons coal reservoirs have been found around the world. 40% power requirement was fulfilled by coal reservoirs (WES, 2013). Coal as the cheapest source used for many purposes like in cement industries, power stations, iron and steel mill etc (Singh and Sinha, 2003). China, India and South Africa account 75% energy production from coal and 30% energy production for Europe (Aytar *et al*., 2013). In 2013 World Coal Authority reported 984 billion tons coal reservoirs which according to an estimate can be used for coming almost 200 years. Coal is found everywhere in the world but Huge coal reservoirs in top continent include Pakistan, Russia, Canada, U.S.A, India and China (Harres *et al*., 2008).

On the basis of abundant coal reservoirs, Pakistan is the 7th country having huge lignite type coal reservoirs which provide 40% electricity to the whole country. Pakistan all province i.e. Punjab, KP, Sindh, Baluchistan and Azad Kashmir have coal reservoirs and all types of coal i.e. anthracite, bituminous, sub-bituminous and lignite are present. Sindh consist of lignite type coal and other coal resources in Pakistan represent the sub-bituminous coal type (Pakistan coal power generation potential,2006). As huge reservoirs are present in Pakistan still there is need to import the coal for running steel and iron industries. To improve the mining and industrial process, indigenous coal reservoirs must be explored so that it helps in the country economy and use of imported coal could be lessened (Malkani and Shah, 2014).

Coal is a fossil fuel that is very useful for mankind from thousands of years ago. As the world population increases with the passage of time their demand for energy utilization also increases. In different parts of the world, due to its low price and high availability it gains importance for its use in iron industries, electric power generation and cement factories (Marinov *et al*., 2010). Coal is vulnerable energy source throughout the world because the non

renewable energy sources (crude oil, natural gas and hydroelectric power) are depleting due to their high demand (Meshram *et al*., 2015). Coal being ancient fossil fuel is an essential source of energy that is the biggest contributor to the development of world's industries (Singh and Sinha, 2003). World Coal Association estimates the 70% steel and 41% electricity production which totally depends on coal (British Petroleum statistical review of the world energy London,2012). Due to its high availability and low cost, coal plays a vital role in industrial sector but due to its combustion its cause many environmental problems especially pollute the air (Liu *et al*., 2015).

2.1 Coal worldwide distribution and consumption

Coal is a cheap substrate distributed in many countries in the world including U.S, China, Australia, India, Canada, Indonesia, Russia, England, Sweden, Japan, Korea, Brazil, Italy, France, Poland, Denmark, South Africa, Netherland, Turkey and Belgium (Robert *et al*., 2013).

Coal play important role in many industrialized countries as it is vulnerable energy source makes 80% of the assets which can be utilized for more than 800 years. Major power stations in Spain that produce 30% electricity consumed 90% coal (Martinez *et al*., 2003). In Turkey, 28% total energy requirement is meet by energy production (Aytar *et al*., 2013).
Currently, 25% world's initial energy consumption depends on coal (approximately 2200million tonnes petroleum comparable Mtpe) and likely 15% by European Union (about 241 Mtpe) while China and South Africa it is more than 75%.

About 860 billion tons coal discovered in the whole world in which bituminous coal makes 47% (450 billion), sub-bituminous coal makes 30% which is 260 billion and 23% coal is covered by lignite whose figure reaches 195 billion.

According to World Energy Resources Survey 2013, five top most coal reservoirs are United states of America followed Russian Federation, China, Australia and India. Percentage of coal that is used by countries to produce electricity are South Africa (94%), Poland (93%), China (81%), Australia (76%) and Germany (49%) (Gadonneix *et al.,* 2010).

2.2 Coal deposits in Pakistan

Pakistan is rich in energy sources. Almost, 186 million tonnes of coal are produced

which made it the 6 biggest country in the world has enormous coal reservoirs. Discovery of huge reserves of coal in Sindh highlighted the coal values early in 1980's. 185Bt coal resources have been found (Report by private sector and infrastructure board Pakistan, 2004). All provinces of Pakistan have huge coal reservoirs included Azad Kashmir. In Punjab,

amount of coal is 253 million tons, in Sindh amount of coal is 184,623 million tons, Khyber Pakhtunkhwa has 91 million tons of coal. Baluchistan having 217million tons of coal deposits. Azad Kashmir having good quality coal of 9 million tons (Pakistan coal power generation potential, 2004). In Pakistan there is need to produce kind of experts who have the capability to explore the use of bituminous, lignite and sub-bituminous coal. There is a lot of coal reservoirs consist of lignite in different regions of Pakistan (Malkani, 2012). Four districts of Punjab such as Chakwal, khushab, Mianwali and Jehlem rich in coal reservoirs that is present in a salt range which spread over a surface of 260 square kilometers that ranges from 0.3 to 1.5 meters in thickness. 97% coal in Pakistan is lignite leaving 3% coal as sub-bituminous to bituminous. On the basis of heating values in a different province of Pakistan, the values range such that in Sindh it ranges from 184,6235, 219-13, 555 Btu/Ib3. In Punjab it ranges from 2179,637-15,499 Btu/Ib3. In Baluchistan, it ranges from 2359,472-15801 Btu/Ib3. In KPK value ranges from 919,386-14,217 Btu/Ib3. In Azad Jammu Kashmir coal having a heating value of 97,336-12,338 Btu/Ib3 (Geological Survey of Pakistan/Pakistan Energy Year Book, 2003).

Along with its ideal geographical location Almighty has provided Pakistan with lots of energy resources. Some of them are explored so far and many are waiting to be rightly explored.

The hub of the coal resources i.e. 99% of Pakistan's coal reserves is located in Thar field, Sindh Pakistan which has about 175,506 Million tons coal, Baluchistan having 217 Million tons of coal. The coal reserves in Punjab are approximately 253 million tons. KPK have 91 Million tons and Azad Kashmir has 9 million tons of coal reserves.

This coal comprises of a very high content of sulphur and ash. With the direct association with climate the moisture content of Thar coal is also high.

As described above the largest coal resources of Pakistan are found in Thar Desert Sindh. It was first discovered in 1991 by British Overseas Development Agency (ODA) in collaboration with Sindh Arid Zone Development Authority (SAZDA) (Pakistan Coal Power Generation Potential, 2004). According to Geological Survey of Pakistan/ Pakistan Energy Year Book 2003(4) the provincial coal resources of country is given in table.

Table 2.1 Geological Survey of Pakistan

Province	Resources in	Heating
Value	Million tons	BTU
Sindh	184,623	5,219 - **13,555**
Baluchistan	217	9,637 - **15,499**
Punjab	235	9,472 - **15,801**
NWFP	91	9,386 - **14,217**
AJK	9	7,336 - **12,338**
Total	**185,175**	

2.3 The energy crisis in Pakistan

For economic growth of a country and sustainability of life huge amount of energy is required (Zaleski, 2001). Currently, the whole world faces energy crisis particularly Pakistan. Government shows its interest by developing techniques which utilized the coal reserves to produce electricity, cement and related industries. Pakistan has a diverse range of population consists 0f 181.3 million people facing a severe energy shortage. 60% electricity was produced from water. There is a shortage of electricity been 2 years which become a more severe due shortage of water since last 3 years. 40% people survive without electricity (Farah and Daniyal, 2014). These crises lead the country to enormous loss of economic, Socio political and strategic damage. To meet energy demands, Pakistan needs to learn lessons to cope up with this crisis. They must develop small dams in order to store the water which helps to get rid of these problems (Khan11*et al*., 2012). One factor for energy crises is due to low-rank coal which is most abundant in Pakistan having low heating values and high moisture content that's why cannot be used so far (Fassett*et al*., 1994). To overcome these problems there is a need to develop such modern techniques that help to exploit the lignite, sub-bituminous, bituminous coal as these are rich in Pakistan (Malkani *et al*., 2012).

2.4 Coal structure

Economically coal gained importance for more than hundred years as well as its structure is important too. Hard coal and soft coal are still under discussion process (Krevelin,1993). All coal ranks have different coal structure but only structure models are reported in the literature (Schumacher,1997). More than seven years ago, there is a lot of coal ranks have been discovered for molecular analysis (Methews and Chaffe, 2012). Lignite Coal having chemical formula C H O N S proposed by Weiminss.
180 195 31 5

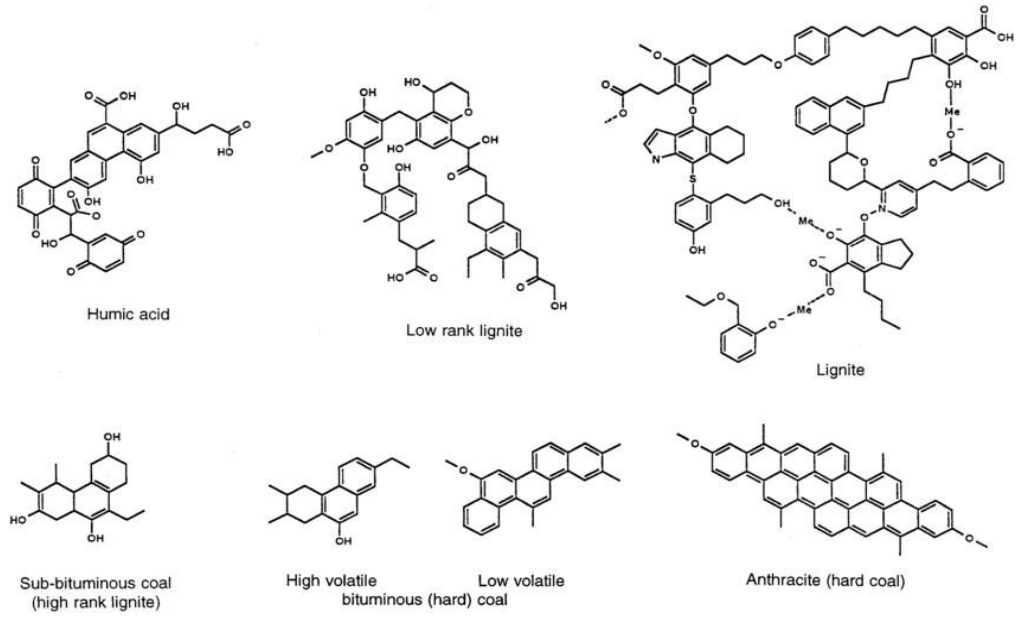

Figure 2.1: Structure models for typical coal substances of different rank (Fakoussoa *et al*., 1999).

2.5 Different types of coal

Coal has 4 major types:

1. **Anthracite**: It is considered as the king of the coal ranks. The distinguishing properties include brittleness, hardness giving black and lustrous shine. It has a very increased carbon content and low volatile matter making it hard coal. Its percentage of carbon is >85% which is considerably high as compared to other ranks having 25 million BTU/ton.
2. **Bituminous Coal:** This rank of coal lies between sub bituminous and anthracite. Being used in the generation of electricity in thermal power plants. The appearance of this coal is smooth and shiny having high heating value. The carbon content is 4585% and energy content of this coal is 24 million BTU/ton.

3. **Sub bituminous Coal:** This rank of coal is not so shiny and is blackish coloured. As compared to lignite it has high heating value.35-45% of carbon content whereas, energy content is 17 to 18 million BTU/ton.
4. **Lignite:** Lowest coal rank also known as brown coal having a very low percentage of carbon. Having energy content of 25-35% and energy content of 13 million BTU/ton.

2.6 Low rank Coal (Lignite Coal)

Lignite coal also known as low rank coal (LRC) is a dusty, chalky and soft coal that consists of high humidity (approx. 30 to 45%) and low carbon content (40 to 60%) (Lerato *et al*., 2013).It has been proved to produce much high content of humic substances due to the low coalification and easy actions of the chemical other substances on it. It is assumed that, because of the structural similarity of both lignite and lignin, these lignite coal can be degraded easily by the lignin degrading organisms (Kaiyi, Wang *et al*., 2019). Lignite coal consists of various irregular and regular parts. The irregular part consists of groups that contain oxygen and also side chains (phenolic hydroxyl, carboxyl, alcoholic hydroxyl along with carbonyl groups) are present. The regular part consists of aromatic, heterocyclic rings containing Sulphur, nitrogen and oxygen, includes aromatic rings (oxidized), Carbohydrates, alicyclic and benzene rings. However, because of the presence of much linkages in lignite coal followed by presence of side chains mark them mostly as irregular structures. Lignite coal has much more complicated structure as compared to other hard coals as they are composed of fulvic and humic acids (Alkali- soluble), hydrophobic and insoluble bitumen. Because of the much more moisture content present and high reactivity in lignite coal it is difficult to transport. Lignite coal consists of more active ingredients that make them reactive. As lignite coal possess much less laborious work than other coal so it is being preferred sometimes.

2.6.1 Lignite as source of by-products

By products or end products of coal are achieved due to the bio-solubilization of coal. Other than the physical or chemical process that are used to deal with the coal processing, many concerns are being dealt using the biological processing of coal that not only requires low temperature and pressure as compared to the chemical processing but also avoid unrequired by products. (Ghani *et al.*, 2015). Besides its advantages, lignite coal also has some disadvantages including high moisture, more volatility as well as high ash content. Therefore, it cannot be used direct or without doing some initial steps. However, besides the many disadvantages of using coal, low rank coal is utilized and can be studied through bio-solubilization as it is environmentally friendly method with no pollutant causing hazards by the coal as compared to the result of burning of coal. Because of the presence of low carbon content such coal can be degraded easily even by the biological and biotechnological method. However, many work and progress are being done in this field. As lignite that is taken for bio-solubilization purpose is low rank means it has low carbon content that means it will have low aromaticity, resulting in less hardness and hence easy degradation for study. Coal bio-solubilization has the capability to convert the raw or waste coal into a new useful industrial product. (Ghani et al., 2015). As a result of coal bio-solubilization, different chemically important liquid is derived by the specific microbes that are further utilized for production of other useful products.

2.7 Bituminous coal (Hard rank coal)

This rank of coal lies between sub bituminous and anthracite. Being used in the generation of electricity in thermal power plants. The appearance of this coal is smooth and shiny having high heating value. The carbon content is 45-85% and energy content of this coal is 24 million BTU/ton.

The coal mine of Duki is 320km from Quetta in east, it is the centre of activities from where all the mining area is approachable to fair roads. The geological survey of Pakistan (GSP) investigated the detailed geological mapping. The coal mine ranges in thickness from 0.15 to 2.30m. The four separate coal ones contain about 1080m thick coal bearing horizons of 15, 22, 36 and 92m in thickness. The coal of Duki coal field is black to brownish black in colour. It is fissile to laminated at some places as well. The roof and floor of coal mine is claystone with sand stone at certain places. The calorific value of coal per pound generally lies in the range between 10,131 to 14,164 BTU/lb. According to average BTU value the rank of Duki coal ranges from sub- bituminous B to C with few exceptions of sub- bituminous A and some coal beds are lignite.

2.8 Global diversity of coal microbiota

There is a huge microbial diversity present in coal that has the capability to solubilize the coal. These microorganisms are categorized on the basis of Kingdom, class and coal rank. The microorganism present in Low and high ranks of coal in kingdom Fungi under class of basidiomycetes are *Coriolusversicolor, Poriaplacenta, Piptoporusbetulins, Coprinus sclerotigenis* and microorganisms in low ranks of coal under the class of Deuteromycetes were *Fusarium oxysporum, Trichodermaatroviride, Aspergillus sp*. Microorganisms present in a high rank of coal in Kingdom Bacteria under the class of Actinomycetes are *Streptomyces badius, Streptomycessetonii, Streptomyces, Viridosporus*, *Rhodococcussp*, Class Firmicutes under low-rank coal type consist of *Bacillus sp, Clostridium sp*, Class Proteobacteria of kingdom bacteria categorized under low-rank coal consists of *Psedomonads sp, desulfovibrio, Geobacter*, the microorganism present in kingdom Archea under Class of Methanomicrobia in a low rank of coal were *methanosarcina*, *Methanosaeta Methanospirilum* (Fashion, 1991; Fernandez *et al*., 1997; Denizli *et al*., 2003; Leon *et al*., 2013; Sekhohola *et al*., 2013). Coal microbial diversity that efficiently removes the inorganic sulphur includes species, for example*, Acidithiobacillus ferroxidans* and *Acidithiobacillus thiooxidans* (Cardona and Marquex, 2009). Other species that have been found in biodesulfurization were *Acidithiobacillus sp*, *Sulfolobus acidocaldarius* and metabolites of fungi (Tao *et al*., 2012). *Aspergillus Niger* and *Penicillium sp* have been reported that efficiently demineralized the sub-bituminous coal with reduction of 73% ash production (Manoj, 2009). Currently, a thermophilic Bacterium name *Acidithio baciluscaldus* isolated which act as oxidizing bacteria and oxidized the sulphide minerals by attaching itself to the mineral surface (Tao *et al*., 2012). Further coal microbial diversity has been found that play an important role in removing sulphur from coal species are *Thiobacillus acidophilus*, *Thiobacillus novellus* and *Leptospirillum ferroxidans* etc (Aller *et al*., 2001). White rot fungi such as T*rametes versicolor* and P*hanerochaete chrysosporium* removes sulphur upto 40% (Ayter *et al*., 2008). *Pseudoxanthomonas* is an aerobic bacterium that can speed up the degradation process and removes 18.26% sulphur from coal (Nayak *et al*., 2005; Singh *et al*., 2013).

2.9 Fungi: An important degrader of coal

Microorganisms that are able to grow on coal, utilize it and bio-solubilize it can be found mostly in aerobic microorganisms. Microorganisms are used to break the chemical linkages in coal molecules and promote the extraction of valuable chemical and industrial products. For conversion of macromolecules into simpler and low molecular weight products,

degradation of coal through microorganisms is an efficient and economical way. Structural complexity of coal makes it difficult to degrade biologically because it is a complex polyaromatic molecule. Like other microbial species such as bacteria that are able to grow on the coal and solubilize it, fungi are also known to bio-solubilize coal. (Lerato *et al*., 2013). Up till now a number of fungal strains have been identified and studied to carry out the process of changing or degrading the coal compounds hence undergo bio-solubilization. These fungi act on the already solubilized coal by many oxidizing compounds and then easily convert them into low molecular weight compounds at the end by interacting with them. Naturally fungi are active agents against lignin specially many filamentous fungi degrade lignin much easily.

Many filamentous or mould fungi are most prominent in this treatment. Majorly saprophytic fungi as well as ectomycorrhizal fungi are known to undergo lignin degradation as well as degradation of complex coal structure such as hard coal but less study have been done. Most prominently three fungal phyla were considered as important in carrying out the phenomena of bio-solubilization because of its specific structural features as well as ability to degrade coal. They include phylum Ascomycota, Basidiomycota (litter and wood decomposing fungi) and Zygomycota. Earlier studies indicates that some imperfect fungi are also known to degrade complex structure of coal . (Lerato *et al.,* 2013). They include brown rot and white rot fungi. However, the degradation by ascomycetes and other phyla have studied to be limited. These fungi act on different groups of coal important for complex linkages such as hydroxyl and carboxyl groups. These fungi undergo this process by the help of certain oxidases and hydrolytic enzymes.

2.10 Fungal strains and their enzymes

Many filamentous fungi transform the coal by the help of bio-solubilization in the presence of specific fungi important in degradation. Certain species with the high or improved metabolic effect is much efficiently used for more production. It has been considered that the ability of these fungi is due to their habitat in lignin occupying areas where they naturally adapt themselves to that environment. The major risk of pollutants that pose serious hazard related to any organic matter that is source of energy is aromatic hydrocarbons that includes polyaromatic hydrocarbon (PAH) along with other complex groups as well as synthetic aromatics that are present in various coals. However, these highly complex compounds are degraded with the help of certain ligninolytic enzymes secreted by ligninolytic fungi.

2.11 Enzymatic Action of Fungal Strains

The active fungal strains that allow the degradation of lignin and lignite coal includes *Phanerochaete chrysosporium* as well as *Trametes versicolor*. These fungi belong to phylum basidiomycetes. Similarly many high rank coal solubilization fungi such as *Poria monticola* and *Polyporus versicolor* degrade complex coal structure. An ascomycetes fungus known *as Fusarium oxysporum* tends to secrete laccases resulting in coal degradation. Also P. c*hrysosporium* and many other strains produce lignin peroxidase enzyme as well as their production of manganese peroxidase is also investigated to degrade lignin. Also, humic acid derived from coal is used for the growth of fungi and utilizes carbon as a source of energy.

For example, *Pycnoporus cinnabarinus* is known to degrade the coal's humic acid as well as some Ascomycota species are involved. The amount of enzymes produced during degradation of coal highly depends upon the substances derived from coal and the type of coal. It has been observed that the use of lignite substances such as humic acids, bitumen and also lignite powder, after adding has increased the lignite coal solubilization up to many folds. Some of the detailed action of these enzymes from their active fungi on the coal activity are listed below:

2.11.1 Laccases

Laccases are polyphenol dioxygen oxidoreductase enzyme that belongs to multicopper family thus including copper (copper blue oxidase). Certain laccases from fungi are naturally proteins or glycosylated proteins. They perform their function like other enzymes by the process of oxidation in the presence of O_2 thus results in H_2O_2 formation. Four copper ions (Cu^{+2}) are present catalytic site of the copper blue oxidases. Fungal laccases are mostly used because of their property that they have high redox potential as compared to bacteria. The oxidation of both non-aromatic as well as aromatic compounds are known to done by the laccases. They are specific to greater no of substrates because of its low specificity thus considered important in many fields. These includes yellow and white laccases. By the aromatic, lignin degradation product, the conversion of blue laccase to yellow takes place by its reduction in specific copper site.

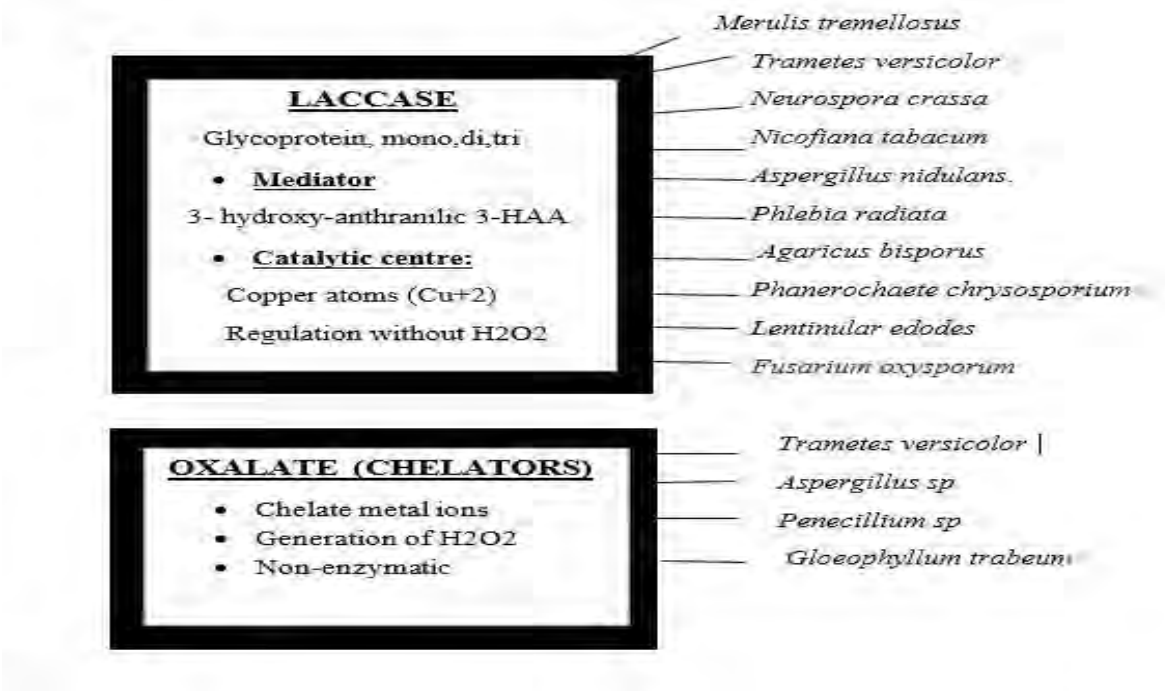

Figure 2.2. Various fungal strains in release of enzymes/ catalyst

2.11.2 Peroxidases

Fungal peroxidases are basically oxidoreductases that are known to undergo oxidation by utilizing hydrogen peroxide (H2O2) (Grzegorz *et al*., 2017). Much fungal peroxidases are known to perform function and have potential in transforming the highly polymerized substance into low molecular weight. By using different substrates that are reducing in nature, peroxidases derived from fungus undergo oxidation of both non-phenolic as well as phenolic compounds. These fungal peroxidases are also referred as lignin modifying peroxidases (LMP).

2.11.3 Lignin peroxidases

Lignin peroxidases are one of the most important degrading enzymes that are able to degrade lignin and are secreted by many fungal species most commonly by wood-rot fungi from basidiomycetes. It is glycoprotein in nature that contains haem group (iron protoporphyrin IX) / or ferric iron as a constituent of its prosthetic group. For their proper activity H2O2 is required. They are involved in changing the structure of coal that can be visualized. It was suggested that induction of peroxidases took place by the addition of lignite derived substances and hence by their release the coal solubilization was done. (Martin *et al*.,1999). By the action of peroxidases on aromatic groups of non-phenolic compounds, as a result of oxidation the cleavage of Carbon-Oxygen (C-O) and Carbon-Carbon (C-C) mediated bond breakage takes place thus forming aryl cations radicals..

2.11.4 Manganese peroxidase

Manganese peroxidase is an enzyme that resembles the lignin peroxidase it is known to be the enzyme that is produced extracellularly, is glycosylated in nature, prosthetic group as Heme is present and undergo lignite degradation as in case of lignin. Peroxidase and thus belong to lignin modifying peroxidases. (Grzegorz *et al*., 2017). However, the Heme is sandwich between alpha helices. Many other peroxidases are also known such as Horseradish peroxidases (HBr), Plant peroxidase. These plant peroxidases have also been investigated in conversion of high molecular weight coal thus modifying it. The difference lies here is that it used Mn (III) and Mn (II) as mediators as compared to lignin peroxidase. Thiol is also used as a mediator or provide improvement in efficiency as compared to lignin peroxidase in lignite degradation. These thiol as mediator attack that part of coal on which manganese peroxide are not able to perform function. They undergo oxidation and H2O2 results in glutathione oxidation. (Grzegorz *et al*., 2017).

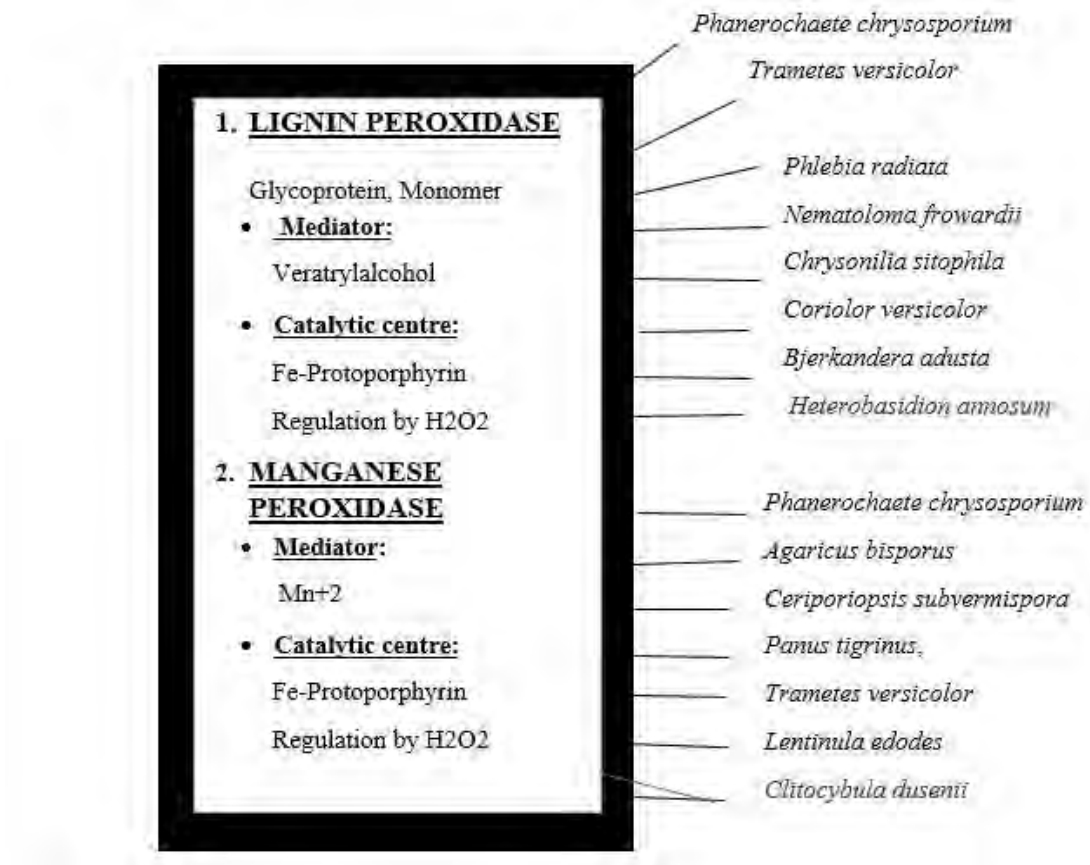

Figure 2.3 Fungal Strains Releasing Different Enzymes For Solubilization

2.11.5 Hydrolases

These are the hydrolytic enzymes that are excreted by the fungi extracellularly that include esterase enzymes. They undergo hydrolytic reactions by breaking of bonds. The esterase enzyme studied to be excreted by fungal strain T. versicolor. Similarly, a deuteromycete called *Trichoderma atroviride* is active in solubilizing activity (Martin *et al*., 1999). This fungus is known to undergo this activity by both non-enzymatic as well as enzymatic substances hence, referred as mixed mode of action. This fungus has found to secrete laccase and esterase enzyme thus, having some ability to solubilize the coal, thus produce substances similar to fulvic and humic acids.

2.12 Biological pretreatment

Coal is an outcome of vegetative organic matter, very sensitive to biological mediated solubilization or depolymerization as fungal mediated enzymes attack on it effectively and release variety of organic intermediates like humic components. Previous literature showed that hard coal bio-solubilizing tendency is much lower than oxidized soft coal. Extensive bio mediated pretreatments on different coal types such as bituminous coal, lignite (brown coal) and peat for liberating bio liquified products have been studied. Various studies have shown that in order to harvest low and high rank coal mediated black liquid products (humic acids) several wood- and litter-decomposing basidiomycetes (e.g. *Stropharia rugoso-annulata, Phanerochaete chrysosporium, Nematoloma frowardii*) were tried by growing them on solid media along coal that attack on the covalent linkages of polymeric coal molecule. The synergistic attack of biological communities on a coal macromolecular structure leads to the formation of mixture of organics in supernatant, eventually yielding fuel and value-added compounds.

Methanogenic property of some biological entities is taking part in the direct generation of petroleum substitutes, by transforming specific coal functional groups. Such biological treatment of coal is time taking processes and act as rate limiting factor due to refractoriness of coal. Biological mediated release of aromatic and aliphatic moieties is further fermented into value added products and fuel (methane, ethane) by biological entities. Up till now, scientist have discovered huge range of native bacterial as well as fungal coal isolates that act as mediator for solubilization process/biological benefaction. Among the various strains (*Phanerochaete chrysosporium, Colorius versicolor Penicillium* species and *Trichoderma atroviride)* gained more attention as coal eating fungi. Moreover, few bacterial strains also promoted coal degradation like *(Rhodococcus, Bacillus cereus, B. subtilis Bacillus pumilus,*

Streptomyces setonii 75Vi2 and Pseudomonas). Existing literature have shown the part of archaea, fungi and bacteria in subsurface anaerobic carbon recycling and methane production, but their mechanism is not yet fully established. However, dense mycelia growth of fungus along enzymes is an important factor of efficient benefaction of coal.

2.13 Chemical pretreatment

Complexity of coal confines its utility but with the help of novel techniques its bioavailability can be enhanced. However, to use chemical agents as an enhancer of various types of coals for encouraging the production of solubilized liquids has been reported. Refractoriness in coal could be overcome by using several techniques and strategies. As the name indicates chemical pretreatments involved the utility of chemicals such as acids, bases, oxidants etc. for coal degradation. Coal derived functional groups could easily be targeted by chemical processing. These pretreatments could be done directly and indirectly. In direct method, structural disruption of macromolecular coal is directly leading into smaller organic fractions that could be beneficial to produce petroleum related hydrocarbons, but it is an energy intensive process with a lot of structural fragmentation of coal. Several existing literatures have been encouraging the use of In-direct methods for enhanced microbial solubilization tendency that could accelerate solubility of hydrophobic substrates.. In 1994 Fakoussa also observed that oxidative pretreatments with hydrogen peroxide (H_2O_2) and Nitric acid (HNO_3) facilitated enhanced generation of solubilized liquid. These methods involve different concentrations of chemical oxidants, acids and bases including potassium permanganate ($KMnO_4$), hydrogen peroxide (H_2O_2) and sodium hydroxide and nitric acid. Methanogenic microorganisms effectively utilize coal having hydrophilic functional groups (e.g., ethers, ketone, hydroxyl, and carboxyl groups) after treatment with H_2O_2. Therefore, enhancing bioavailability of the hard coal. Numbers of researches are still going on but actual bioavailability enhancing mechanism is still not known. Fenton or quinone reactions considered as an important part for releasing radicals into coal nugget that convert coal into low molecular components and organic acids (acetic acids and formic acid). Currently, the exact pathway and nature of released organics after this reaction is not fully understood. Various studies have specified the presence of low molecular mass substances like benzoic acids, biphenyls (ethers), phenols in addition of numerous n-alkanes, cycloalkanes and n-alkanols.

2.14 Anaerobic Treatment of Coal

2.14.1 Anaerobic microbial process

In different biomass conversion process the most economical way for the production of biogas is biological anaerobic digestion. The methanogenic bacterial activity is crucial for methanogensis (Demirel and Scherer., 2008). The most appropriate presence of bicarbonate for electron acceptors and extremely reduced surface environment that showed accessible methanogenesis in surface coal beds (Steve H. Harris, Smith. et al., 2008). In deep surface environment, active methanogens are present and also present in organic rich, poor electron acceptor surrounding (Mesle, Dormart et al. 2013). Methanogens are extremely sensitive to pH and temperature fluctuations that are also inhibited by high volatile fatty acids (Mesle, Dormat et al., 2013). Both mesophilic and thermophilic methanogenesis consortium have been isolated but temperature shows deep structural effect on microbes community (Mesle, Dormart et al.,2013). Methanogenesis proceeds by three different pathways that depends upon carbon source that reduce into methane.

- **Hydrogenotrophic pathway** (in which carbon dioxide reduce to hydrogen as electron donor)
- **Methylotrophic pathway** (in which one carbon compounds are reduced).
- **Acetoclastic pathway** (in which acetate reduction occurs (Mesle, Dormart et al., 2013) Methyl type fermentation and carbon dioxide reduction both pathways played vital role in gas production, but gases that produce from methyl type fermentation overprinted gas from the carbon dioxide reduction pathway (Flores, Rice et al., 2008). Conversion of composite organic substances into methane need methanogens bacteria that are acetogenic and fermentative (Aikuan, Yong et al., 2010).

The anaerobic microbial digestion of organic matter needs at least four stages.

First Stage: During this stage cleavage of complex organic polymers to alcohols and organic acids occurs because of fermentative anaerobic organism activities.

Second Stage: During this stage the organisms converts simpler molecules in order to get energy under anaerobic conditions e.g. during anaerobic digestion H_2 generating acetogenic organisms that converts alcohols and organic acids into actetate, H_2 and HCOOH or CO_2.

Third Stage and Fourth Stage: Acetotrophic methanogens utilize acetate and in return they produce CO_2 and CH_4 (Flores, Rice et al. 2018). H -utilizing methanogens they utilize the 2 accessible H_2 in order to convert CO_2 or HCOOH to CH_4.

2.15 Gasification of coal

During this process coal is converted into gaseous fuel by heating in medium of gasification of coal i.e, oxygen or air, steam. In comparison with combustion the process of oxidation is completed in one process. Chemical energy of biomass is transferred into explosive gas in two different stages during this process. The more versatile and also easier to use the gas produced than original quality can be standardized. Furthermore, it can be used in gas turbines and power gas engines and also used in a chemical feedstock from which the production of liquid fuel is occurred. The process of coal gasification includes biochemical and also thermochemical processes, steam or oxygen, former uses air at higher temperature >800oC, moreover, later process involves the use of anaerobic microorganisms at ambient conditions of pressure and temperature (Moore 2012; Sekhohola, Igbinigie et al. 2013

2.16 Bio-methane potential of coal

The limitation of low coal to methane yield is directly proportional to surface area of coal and yield may increase due to increased surface area of coal particles (Samuel *et al*., 2011). On the other hand when the microbial consortia or nutrients are introduced into the coal beds it is converted into coal bed methane or more precisely microbially enhanced coal bed methane (Scott, 1999). As a result of thermogenic and biogenic processes methane is produced in underground coal seams and is a source of natural gas and it is termed as coal bed methane (CBM) (McGlade *et al*., 2013). The whole world is getting benefits from this coal bed methane and meeting their energy demands since decade. U.S is meeting approximately 4% of their annual gas demand by their proven coal bed methane reserve (U.S. Energy Information Administration. Natural Gas–U.S. Energy Information Administration (EIA). Nat Gas 2017). Basically, the methane production of coal is generally referred to low rank coal that is lignite and sub bituminous coal (Paul *et al*., 2013).

The highly branched compounds having a number of oxygen containing functional groups are available to indigenous microcosm of coal which are responsible in making methane (Harris *et al*., 2008; Jones *et al*., 2008; Strapoc *et al*., 2011). A higher proportion of recalcitrant compounds are present in high rank coal which is not so beneficial for methane production (Faiz and Hendry, 2006; Formolo *et al*., 2008). The bioavailability of low rank coal for methane generation is higher as compared to high rank coal. An unconventional natural gas is produced from anaerobic degradation of organic materials in the presence of micro-organisms. In the past natural gas was studied in the landfills, wetlands, municipal waste etc when the micro-organisms decompose the organic waste an-aerobically. It is a matter of recent times that studies have been conducted on methane production and it is going

on. Coalbed methane is one of the highlighted locations where biogenic methane production is deeply studied in the initial stages of coalification (Thomas, 2002). Coalbed methane is an advance biogas origin which is gradually produced by microbial decomposition of complicated carbon related materials (Scott and Kaiser, 1995; Butland and Moore, 2008; Flores *et al*., 2008; Formolo *et al*., 2008). Methane can be produced in laboratory conditions creating an anaerobic environment using coal as a substrate. Different enhancement factors can also be added in order to generate higher rates of methane (Jones *et al*., 2010; Ulrich and Bower, 2008).

2.17 Stimulation of methane generation from native coal samples

Methane is generated in the presence of consortia which acts on the substrate say coal and methane is produced along some other gases if the conditions are suitable. Mostly the consortia is either prepared or collected directly from coal mines sites. On these sites the conditions are perfectly favourable for the developments of micro-organisms which act on the substrate coal use them as a carbon source and converting this substrate into methane (Herbert *et al*., 1990). Most of the coal conversion to methane is under lab experimentation and are very less likely to be taken to the field levels because of the lack of research. The environment is facing challenges on daily basis so biogenic methane can be introduced as clean energy source. Using the bio-stimulation and bio-augmentation technique the enhancement of methane generation can be done. In both these processes nutrients and microbial consortia are added respectively into the set up (Elizabeth, 2010). Thermogenic and biogenic processes both are responsible for methane production. Biogenic production of methane from coal is generated at less than 100◦C temperature at near low depth locations (Clayton, 1998).

Firstly we understand about primary and secondary biogenic methane production. Primary methanogenesis is produced following peat degradation and is immediately lost before coal formation and its burial. On the other hand secondary methanogenesis of coal can be well understood using coal biogenetic gas composition (Scott *et al*., 1994). The factors which are important in methanogenesis are the substrate which is coal, consortia to convert coal into gas, favourable condition that are temperature, nutrient availability and absence of oxygenic to create an anoxic environment. Tight anoxic environment is favourable to grow consortia for methane production (Furmann, 2011). The components like aromatic hydrocarbons, long chain alkanes and humic compounds can be degraded using microbial consortia and methane is produced as a result alongside carbon dioxide (Zaixing *et al*., 2018). The future of energy demands is quite high while the energy resources are getting depleted so we must have to think about the alternate energy resources including shale oil and coalbed

methane etc. For the development of unconventional energy resources secondary biogenic methane production is an important field to be focused on. Hydrogen peroxide acts on organic matter and convert it into substrate for methanogenesis (Noritaka *et al*., 2017). The process of methanogenesis takes place in an environment which is quite diverse and it mostly occurs in subsurface environment. This process is utilized on global scale to get benefit from them (Strapoc *et al*., 2011). Methanogens use acetic acid, methanol and acetic acid as carbon source for the generation of methane (Jones *et al*., 2010). Various low ranks to high rank coal reservoir are found across the world and in Pakistan which are the subsequent source of coalbed biomethane production. The existing coalbed methane process can be enhanced by enriching it with nutrient and microcosm (Faiz *et al*., 2006).

2.18 Enhancement of methane generation using co-digestion strategy

In co-digestion process variety of wastes are treated simultaneously thus providing an increased carbon source which increases the efficiency of the process. By the addition of more organic carbon into the system makes it available for the anaerobic microbes. By the synergistic effect of co-digestive materials and coal an increased methane production is observed (Navaneethan *et al.,* 2011)

Greenhouse gas emissions can be reduced using co-digestion strategy. GHG which is mostly emitted from dairy manure directly into the environment can be minimized by the controlled emission of methane (Maeng *et al*., 1999). Carbon cycle fix CO_2 into the biomass carbon hence when the organic carbon is burnt $CO_2$is recycled back into the (Zitomer *et al*., 2008).

2.19 Health issues and Combat using clean energy source

When the air got polluted it become the cause of a number of disease which are lethal damaging brain, cells, heart, lungs, skin and eyes. The brain damaged due to increased cerebro-vascular ischemia and dementia. Apart from this bladder cancer, obesity, diabetes and skin cancer are some other gifts of air pollution. Cell got affected by altered rheology, increased coagulability, and peripheral thrombosis, translocated particles and reduced oxygen saturation. According to a report of World Health Organization the major health risk posing threat to human life was air pollution in the year 2016. WHO reported the death of 7 million locals as a result of air pollution.

As we all know that in the current scenario Pakistan is facing energy crisis. Load Shedding of electricity has adversely affected the industrial sector. According to an estimate annual expenditure on the import of petroleum products and crude oil is US $14.5 billion (HDIP, 2012).

2.20 Applications of coal by-products

There are several by-products of bio solubilization of lignite and bituminous coal which includes humic acid, fulvic acid, lower fatty acids, aromatic compounds. But main are the humic acid and fulvic acid and collectively they are known as humic substances. Through all processes the major goal is to achieve these by-products because they have several applications. For example, humic acid has applications in medicine, cosmetics, agriculture, health, wastewater treatment, soil remediation, industries and also used as water soluble fertilizer. Besides the production of humic acid as a by-product, fulvic acid is also produced as a result of bio-solubilization that is of prime importance and has gained much more attention in various fields in many useful applications regarding protection of environment, agriculture, health and industrial use and science life. Fulvic acid is brownish yellowish compound found in coal as well as other natural sources. Fulvic acid from humus can be obtained from coal and soil. Fulvic acid is characterized by low molecular weight, light colour, low carbon content and also low polymeric linkages but more level of oxygen and solubility. Compared with the humic acid, it is analysed that there is no such difference in their products formed as a result of decomposition. With the passage of time more research is being done to monitor the specificities of fulvic acid along with other by-products. Fulvic acid when used assures non-toxicity and hence is non expensive. Also, fulvic acid is known to produce a derived compound that is Heat stable, cationic, having therapeutic properties that is CHD-FA (Leighann et al., 2012).

2.21 Humic acid

2.21.1 Agricultural applications

Humic acid is very important from an agricultural point of view.

Improve soil properties

Humic acid accomplishes many functions in soil. It acts as a soil conditioner. It increases the crop production, enhances soil fertility, improves structure of soil including water retention and soil porosity, adhesin, compaction and aeration. Growth, yield and product quality increases due to humic acid which increases the nutrients and micronutrients uptake. To encourage the activity of microorganisms in soil, humic acid acts as a catalyst (M. M. Tahir., *et al*, 2010)

Metal chelation

Humic acid has a complexing property. Various micronutrients like sodium, zinc, potassium, iron, magnesium, calcium and copper make complexes with humic acid and form chelates.

These chelates are then used in soil where deficiency of these nutrients occurs. (Ali, Mohsen., *et al*, 2008)

As fertilizer

To boost up the agriculture production humic acid is used as organic fertilizer. Humic acid has Organic Carbon (51-57%), Nitrogen (4-6%) and Phosphorus (0.2-1%). Due to its ability to provide Nitrogen and P to the plants, it can improve the crop yield. Humic acid acts as an effective and slow releasing nitrogen fertilizer because it possesses very stable nitrogen content. (M. Sharif., *et al*, 2006)

Beneficial effect on plant

Humic acid is also used as a plant growth promoter. Humic acid had a beneficial effect on nutrients uptake by plants. Rate of absorption of ions on the surface of roots and their penetration into plant tissue increases with addition of humic acid to the soil. Humic acid contains quinone groups due to which plants show more active metabolism and increased respiratory activity. Growth of many plants including tomatoes is promoted by foliar spray of humic acid.

Humic acid increased the rate of photosynthesis of plants. With humic acid application dehydrogenase activity of roots and root mass regrowth enhanced significantly but chlorophyll content was unaffected (A. Kirn, S. R. Kashif., *et al*, 2010).

Enhanced enzymatic activity

With foliar spray of humic acid enzymatic activity of dehydrogenase, catalase and phosphatase is enhanced. Through enhancing the activities of various enzymes, humic acid improves the biochemical quality of the soil.

Hormone like activity

Humic acid has hormone-like activity in addition to increasing plant growth and nutrient uptake it also improves stress tolerance. It has gibberellins and auxin-like activity (A. Kirn, S. R. Kashif., *et al,* 2010).

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2.21.2 Medicinal / Medical application

Humic acid exists as a therapeutic agent and is used in the treatment of many diseases.

- In drug delivery system
- Influence on prion proteins
- Antiviral property
- Anticancer property
- Alleviate bronchitis
- Immune boosting property
- Effect on blood coagulation
- As solubilizing agent

2.22 Fulvic acid

2.22.1 In Agriculture

Fulvic acid is most importantly used for the improvement of physical and chemical properties of not only plants but also soil. They can be used as a pure bio-stimulant in increasing the plant growth as well as improving soil fertility these days. It is a good fertilizer, a good water binder that is known to provide growth to the plants. Fulvic acid being in solution used for plants always promote the growth by contributing its role in exchange capacity of cation and hence known to balance the PH of soil containing plants. In soil it keeps moisture by movement and attraction of water molecules and nutrients towards the roots. Also they are known to hold the water in the upper surface of soil. They can bind themselves with the mineral present in soil thus providing adequate quantity of minerals such as copper,

magnesium, iron, chromium, calcium, phosphorous. They are also known to increase the resistance of some plants against some pathogens and increase the growth of roots as well as promote a significant growth of tomato fields. They conserve the soil water thus prevent transpiration. It is also known to reduce the water stress and hot dry wind effects. In soil fulvic acid also play its role by preventing the leaching of fertilizers.

2.22.2 In Metal Chelation

Besides plant growth promotion, fulvic acids promote metal chelation in soil actively as they are known as good metal chelators. There are many heavy metals in soil that can not only make plants devoid of minerals but also prove harmful for the other living organisms not only in soil but also others that are plant consuming. Because of the structural arrangement of fulvic acid that it has, the functional group in fulvic acid that includes 1) Phenolic OH group 2) Carboxyl type I group analysing from IR/ UV spectroscopy, depending upon the chemical nature, behaviour of fulvic acid and its competing nature shows much more potential of fulvic acid for heavy metals (Robert *et al*., 1980). The problem area lies in the lacking of nutrient availability for the plants due to the formation of complex between metal ions and organic matter in soil. However, due to the absorption property of fulvic acid they can actively take metal ions and hence promote activity. These different groups help in controlling the mobility of heavy metals by binding them. By making complex with metals such as Cu, Fe and Zn their efficiency is increased thus promoting plant root growth much fast as compared to normal condition. Many essential micronutrients that are needed by plants for proper growth is provided by Zn in balanced amount and thus prevent zinc deficiency.

2.22.3 In Medicine

Besides its role in agriculture, Fulvic acid is equally proved to be beneficial in medical health. Over many years the biological significance of fulvic acid is being admired over other functions as it has helped the medical fields prominently. Many years ago extracts from plants were used to cure different diseases but now many organic compounds are used. Fulvic acids are known for its many functions including immunomodulation or inflammatory disease prevention such as diabetes, myocardial diseases, metabolic ones, also anti-oxidative properties for skin, Gut disorders, against apoptosis leading to cancer cells and many others. Beside this it is involved in immunomodulation, Gut disorder, antioxidant properties, prevention of cancer, cleaning and detoxification and antimicrobial resistance.

Chapter No. 3

Materials and Methods

3.1 Sampling and origin of coal

The focus of current study was to enhance the bio solubilizing extent of native fungal and bacterial strains obtained from two different types of coal i.e., Lignite and Bituminous. The bituminous coal samples were obtained from various depths (321 to 341m) of Baluchistan coalfield, Duki region of Pakistan. The Coal was sampled from different sites (43.61909° North and 105.54648° West) of coal mine. While the Lignite coal samples was obtained from Thar Coal Mine, Sindh region of Pakistan. The samples were taken from various depths i.e., 450ft, 650ft and 1000ft. All the samples were securely sealed in air-tight zipper polyethylene bags and delivered to research laboratory to store them in a sterile place for various research studies.

Map showing location and occurrence of coal samples

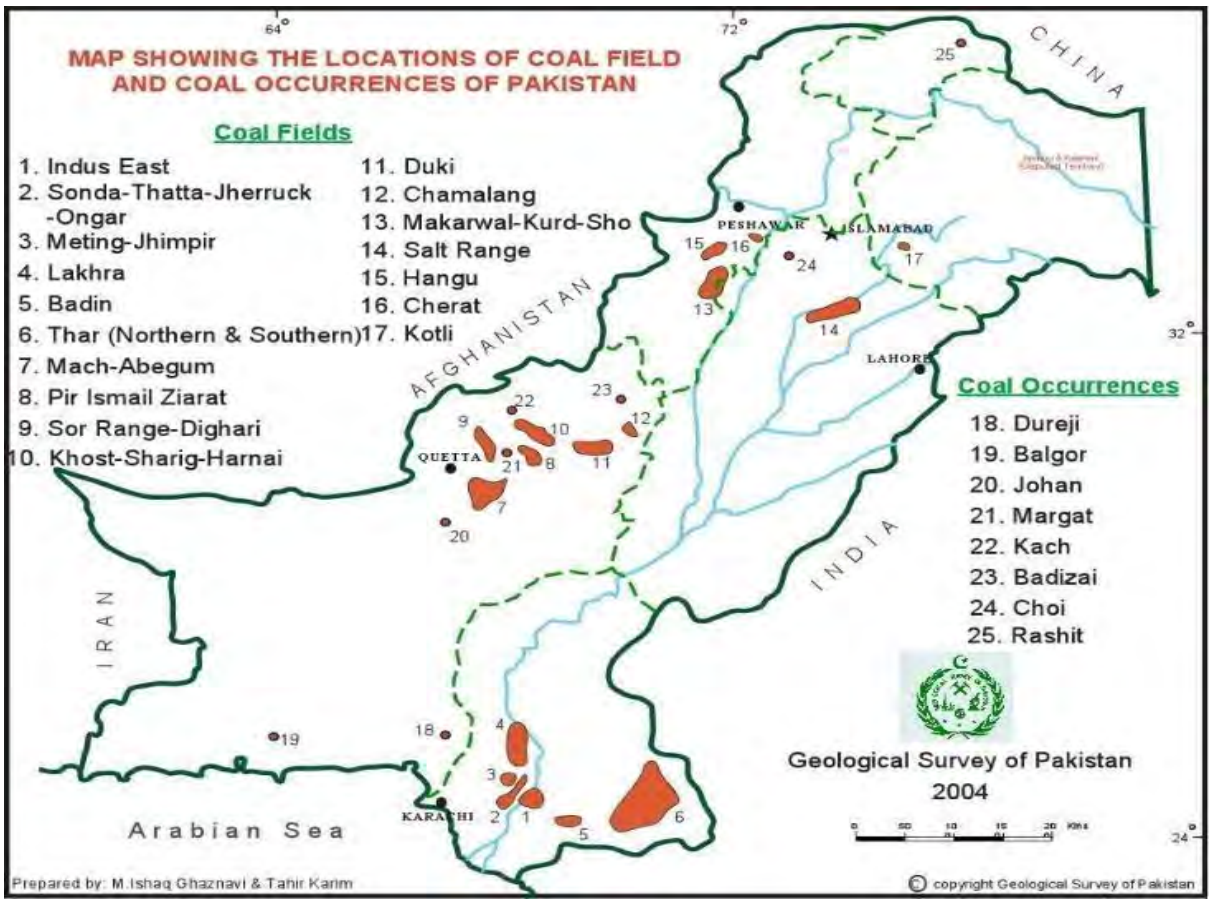


Fig 3.1 Geological survey of Pakistan

Table 3.1 Coal sample ID along with coal field

S. Nr	Sample Name and ID	Province	Coal Field
1.	Thar	Sindh	Thar desert coal fields
2.	Balo	Baluchistan	Duki Coal Mine

3.2 Coal sample preparation and preservation

The coal samples were broken down into small particles having size in mm and sub mm. The large coal particles were separated using quarter and conning method. The plant debris, resins shell fragments were also separated. Firstly, grinding of the coal samples were done and later on crushing process were performed. The samples were added in to the crusher and the size were further reduced to 60 mesh size which later crushed into smaller size for carrying out the experiments. All the grounded samples were used further for different investigations and findings that includes: proximate and ultimate analysis. The samples used for biological study that includes aerobic and anaerobic processes were preserved for further studies.

3.3 Ultimate Analysis of Coal Samples

The ultimate analysis of coal represents the presence of total carbon, nitrogen, hydrogen, oxygen and sulphur in a particular percentage by mass of coal. Except, all of the above, the total oxygen was determined with the help of CHNS Analyzer (LECO TruMac Series, Saint Joseph, Michigan) according to ASTM standard. The total oxygen content was determined by calculating the difference from values obtained from carbon, hydrogen, nitrogen and sulphur. The gross calorific values were estimated using LECO AC500 Isoperibol Calorimeter (Saint Joseph, Michigan, USA). All the characterization of samples was run in triplicates and mean value is calculated.

3.3.1 Total Hydrogen, Carbon and Nitrogen (ASTM D-5373)

The coal samples was prepared by using (60 mesh) sieve less than 250µm. About 600mg of coal sample was used for triplicate analysis to determine total carbon, hydrogen and nitrogen as percentage by mass using CHNS analyzer (LECO TruMac Series, Saint Joseph, Michigan). The CHNS Analyzer includes a furnace that is capable of maintaining a temperature (900-1050ºC) ensuring quantitative recovery of carbon, hydrogen, nitrogen in coal by use of their quantitative gases (CO_2 , H_2O and NO).

3.3.2 Total Sulfur (ASTM D-4239)

The pulverized coal sample was allowed to pass 60 mesh sieve 250 µm and mixed properly to determine the total sulphur percentage by mass with the help of CHNS analyzer (LECO TruMac Series, Saint Joseph, Michigan). A weighed small portion of each sample was burned in a tube furnace at 1350ºC in a course of oxygen. The Sulphur compounds and sulphur present in each sample were decomposed and oxidized to sulphur dioxide. After completing the analysis, the mass percent sulphur value was determined as indicated by the instrument.

3.3.3 Total Oxygen

The total oxygen was calculated after obtaining the percentage values of Sulphur, Nitrogen, Carbon, Hydrogen, and ash by using difference method

$$Percentage\ of\ Oxygen = 100 - (C + H + N + S + Ash)$$

3.4 Proximate Analysis of Coal Samples

Volatile matter, moisture, fixed carbon and ash content of coal were detected by proximate analysis. Except carbon all of these were determined in accordance of ASTM standard experimentally. After obtaining values of moisture content, volatile matter and ash content, the value of fixed carbon is determined by using difference method. All the experiments were run in triplicates and mean value is calculated.

3.4.1 Moisture Content (ASTM D-3302)

For calculating the total amount of moisture content present in all the coal samples, the blank containers were heated at the same conditions which was performed while drying, the containers were covered with lids, cool and allow to weigh. 1g of each coal sample was dip out with the help of spatula into the container, closed and weighed. The lid was removed, and containers were placed immediately in an oven having temperature 110ºC. It was heated for 1 hour. The weight of each sample was noted down until the difference between calculated weights remain constant. The difference in calculated weights was reported in the form of percentage (%) of moisture content and the moisture content percentage analysis in each sample was calculated by using the formula:

$$Moisture\ Percentage = \left(\frac{A-B}{}\right) \times 100$$

Where:

A = amount of coal samples in grams

B = amount of coal sample after heating in grams

3.4.2 Volatile Matter Content (ASTM D-3175)

About 1g of ground coal particle from each coal sample was taken and placed into pre weighed platinum container (capacity 10-20mL, diameter 25-35mm, and height 30-35mm) with the tight lid. It was then suspended in the furnace chamber under temperature 950 ± 20°C at the specified height. The loss in weight was calculated and reported as a % volatile matter content

$$Volatile\ matter = \frac{100(M2 - M3)}{M2 - M1} - M_0$$

Where:

M_0 = Moisture content M1 = Mass (g) of empty crucible with lid

M2 = Mass (g) before heating M3 = Mass (g) after heating

3.4.3 Determination of ash content (ASTM D-3174)

For determining the ash content present in coal samples, 1g of coal was heated in muffle furnace at 700-750°C temperature, till the difference in weight remained constant. Then, the difference in weight was calculated and the residual as was calculated as % ash

Where:

M1= Mass of empty container in grams M2 = Mass of container + coal in grams

M3 = Mass of container + ash in grams

M4 = Mass of container in grams after cleaning the ash

3.4.4 Fixed Carbon (ASTM D-5142)

After heating the coal particles from each coal sample, the volatile matter is removed and the remaining combustible solid residue is basically the fixed carbon (Speight, 2012).
The fixed carbon is determined by using the formula

$$Fixed\ Carbon = 100 - (Moisture + Volatile\ Matter + Ash)$$

3.5 Coal Biosolubilization

3.5.1 Isolation of pure culture

To isolate different microbes from both types of coal, shake flask experiment was conducted. In experimental protocol 10 % grinded coal was mixed in 100ml MSM which had 0.1% coal as sole source of carbon along with isolated microbes and placed in shaker incubator at 30°C. The rpm was set at 150 rpm /min for the duration of 15 days. After the incubation period of 15 days, increased concentration of coal from 0.1-0.5% was observed. Later using same protocol coal concentration was increased from 0.5%, 1%, 1.5%, 2%, 2.5%, 3%, 4%, 5% up to 6%. Upon every step, screening was observed on coal agar plates by pour plate method. Plates of coal agar were prepared with 2% agar in MSM. Sub culturing was observed upon nutrient agar and SDA plates for isolation of microbial colonies. And in turn bacterial and fungal isolation in nutrient agar and SDA slants in glycerol too was performed.

3.5.2 Selection of Isolates based on Biosolubilization Activity

Coal biosolubilization activity by isolated microbes was performed in petri dishes. In the initial step, fungal and bacterial isolates were grown on nutrient agar and SDA plates incubated at 30 °C. In the second step,1g coal chunks was placed (0.2mm - 0.5mm in diameter) on the above-mentioned plates and incubated in static incubator for 14 days. After 14 days of incubation period, each plate was observed with blackish liquid produced in surrounding areas of each coal particle. Isolates were selected on these parameters for further research work.

3.5.3 Purification of fungal isolates

MEA slants were prepared by culturing with pure colonies in sterile test tubes. Tubes were incubated in fungal incubator at 30 ˚C for 4 days. The purified fungal isolates were stored at 4 ˚C. Moreover, purified fungal isolates were also preserved in MSM liquid culture using shake flask experimentation.

3.5.4 Screening of coal solubilizing isolates

MEA plates after five days of incubation period were observed in laminar flow hood. It was observed that six fungal strains were most visible. By using sub-culturing technique these visible fungal colonies were carefully picked and placed on MEA plates via point inoculation or streaking proceeded for incubation. After incubation time of four to five days these colonies were rechecked to observe growth of pure colony. If not, same process was repeated. Plate assay was used to test the solubilizing tendency of these fungal strains. Out of six, four isolates were positive (coal solubilizer) and selected for confirmation of best solubilizing activity on a plate assay which is used to investigate the solubilizing potential of isolates, and only two best solubilizing isolates designated as NF-1 and GB were selected.

3.6 Microscopic study

Acridine Orange staining

To observe potential coal solubilizing bacterial and fungal strains based on their morphology acridine orange staining was used.

The procedure used for staining is as follows:

1. The bacterial and fungal smear was properly prepared and fixed prior to staining
2. The freshly prepared acridine orange stain were flooded on the slide and allowed to remain on slide for 2 minutes without drying.
3. The slides were rinsed with tap water and allow the moisture to drain from slide and air dried.
4. After air drying, immersion oil was added and covered with a cover slip.
5. The slides were observed under fluorescent microscope.

3.7 Sequencing of 16S rRNA and 18Sr RNA gene

The 16S r RNA and 18Sr RNA gene were purified after amplification using PCR purification kit (Nucleobond) and quantified using QUBIT. The samples were sent to Macrogen KOREA (Europe) for DNA sequencing. The sequenced data was analyzed by editing the sequence using Bioedit software version 3.1and complete sequence was BLAST in NCBI and unknown sequence was compared with sequences already present in NCBI.

Clustal W program was used to align the 16SrRNA and 18SrRNA gene of all the prokaryotic and eukaryotic genes of isolates with sequences similar from the NCBI database to construct the phylogenetic trees.

3.8 Taxonomic evaluation of fungal strains

The taxonomic evaluation of purified native fungal isolates NF-1 and GB was carried out based on their morphological features and Molecular identification.

3.9 Morphological identification of both bacterial and fungal strains

The major morphological characteristics like, mycelium and colony type, spore formation, nature and color were detected from purified native fungal isolates that were grown on MEA plates. The microscopic investigation was performed by observing cell morphology, shape of hyphae, conidia structures/ spore nature.

3.10 Optimization parameters or enhance release of organics:

The potential fungal isolates were optimized for enhanced release of solubilized products from thar and bituminous coal samples. The released organics were determined by 200 to 600 nm range of UV-Visible spectrophotometric scan. Major conditions were optimized such as the concentration of glucose, coal loading ratio and incubation time.

3.10.1 *Optimization of glucose concentration*

MSM media without glucose was prepared and poured in 50ml of flasks. (0.1%, 0.5%, 1.0%, and 1.5%) different glucose concentrations were added in each flask and pH (5.5) was balanced. 0.5g of coal was measured for each flask which is dried, grounded and sieved having size less than 650 µm. Media and coal were autoclaved for 15 minutes at 121˚C containing different concentration of glucose. Inoculum was prepared by adding fungal isolates in a sterile distilled water. Solution was homogenized by using vortex or homogenizer, 0.2 at 600 nm was the absorbance of suspension taken. About 2ml inoculum was added in each flask and incubated at 25◦C 110rpm in shaker for five days. After five days, 0.5g of autoclaved raw coal was added in each flask. Flasks were placed into the shaker at 25◦C, 110rpm for 7 days.

3.10.2 *Optimization of coal loading ratio*

MSM media was prepared that have optimized conc. of glucose for fungal isolates, pH (5.5) was balanced and poured into 50ml of flasks. Different concentrations of coal (0.1%, 0.5%, 1.0%, and 1.5%) were measured for each flask which were dried, grounded and sieved having size less than 650 µm. Media and different coal conc. were autoclaved for 15 minutes

at 121˚C. Inoculum was prepared by adding loop full of inoculums from potential fungal isolates in a sterile distilled water. Solution was homogenized by using vortex or homogenizer, 0.2 at 600 nm was the absorbance of suspension. About 2ml inoculum was added in each flask and incubated at 25◦C 110rpm in shaker for five days. After five days different conc. (0.1%, 0.5%, 1.0%, and 1.5%) of autoclaved raw coal were added in each flask. Flasks were placed into the shaker at 25◦C, 110rpm for 7 days.

3.10.3 *Optimization of incubation time*

MSM media was prepared that have optimized conc. Of fungal isolates, pH (5.5) was balanced and poured into 50ml of flasks. Optimized ratio of raw coal for potential fungal isolates was measured which is dried, grounded and sieved having size less than 650 µm.
Media and coal were autoclaved for 15 minutes at 121˚C. Inoculum was prepared by adding loop full of inoculum of fungal strains in a sterile distilled water. Solution was homogenized by using vortex or homogenizer, 0.2 at 600 nm was the absorbance of suspension. About 2ml inoculum was added in each flask and incubated at 25◦C 110rpm in shaker for four days.
After five days autoclaved coal was added in each flask and placed at 25◦C 110rpm in shaker for different time periods (7days,12days,17days and 21days).

3.11 Enhancing agents for coal bio solubilization

3.11.1 Chemical pretreatments

Baluchistan coal samples were dried, grounded and sieved, having particle size less than 650 µm with the help of sterile pestle and motel. About 1g of coal per 3ml was pretreated by using 8M concentration of sodium hydroxide (NaOH) in high-performance liquid chromatography (HPLC) vials for 24 h at room temperature. The pretreated samples were transferred into microcentrifuge tubes and the supernatant was separated by 20 minutes of centrifugation at 10,000 rpm. Sterile distilled water was used to wash 10 times the pre oxidized coal samples to neutralize pH and remove any residual chemical agents at the same speed and duration.

3.11.2 Sodium acetate amendments

Concentration and size of coal particles being used for solubilization has remarkable effects on accelerating solubilization activity. Size of dry bituminous coal particles were

reduced to (0.3-0.6) mm in diameter with the help of sterile pestle and motel and properly sieved. After optimization of major conditions additionally 0.25g sodium acetate ($C_2H_3NaO_2$) was added as an enhancing agent in MSM media along optimized glucose and coal conc. for both fungal isolates. Flasks were placed into the shaker at 25◦C, 110rpm for 7 days. In addition to that medium having sodium acetate ($C_2H_3NaO_2$) as a sole carbon source was also prepared to check its activity.

3.12 Coal solubilization extent

3.12.1 Bio solubilization percentage

For quantitative estimation or investigation of enhanced coal bio solubilized activity, petri plate assay was performed. NF-1 and GB coal derived fungal isolates were grown on malt extract media (MEA) and incubated for five days in order to completely develop mycelia on plates.0.5 g finally grounded (0.3-0.6) mm in diameter untreated and pre oxidized coal particles were spread on fungal mycelia. The controls were run along this i.e. plate having MEA agar and NF-1 and GB fungal mycelia but without coal, and plate having MEA agar and coal but without fungal mycelia. The plates were incubated at 30 ˚C for 5 days and collected black liquid products over virgin and oxidized coal particles by the action of NF-1 and GB fungal strains. The coal particles were weighed after incubation period. Bio solubilization percentage' P' was calculated by net weight loss of bituminous coal particles with the help of formula. $P = (1-W1/W0) \times 100\%$

where P is solubilization percentage (%)

W0 shows the initial weight of bituminous coal(g)

W1 shows the weight of the residual bituminous coal (g) after bio solubilization.

3.13 Coal degradation setup

3.13.1 Shake flask experiment of virgin coal

MSM media was prepared having additional 0.5g of sodium acetate source and optimized conc. of glucose for NF-1 and GB fungal isolates, pH (5.5) was balanced and poured into 100ml of flasks. Optimized ratio of virgin coal for NF-1 and GB fungal isolates were measured which is dried, grounded and sieved. Media and virgin coal samples were autoclaved for 15 minutes at 121˚C. Inoculum was prepared by adding loop full of inoculum from actively growing zones of the plates which is (NF-1, GB) in a sterile distilled water. Solution was homogenized by using vortex or homogenizer, the absorbance value of suspension was adjusted to 0.2 at 600 nm. About 2ml inoculum (suspension) was added in

each flask and incubated at 25◦C 110rpm in shaker for four days. After four days autoclaved virgin coal residues were added to determine parametric effects during solubilization experiments. Autoclaved and aerobic glass flasks were used for the experiments that was run in triplicate. Flasks were placed into the shaker at 25◦C, 110rpm for 7 days. Positive and negative controls were used under all studies i.e.:

1. Fungal inoculated media without coal

2. Media having coal but no fungal inoculum

3.13.2 Shake flask experiment of pre-oxidized coal

MSM media was prepared having additional 0.5g of sodium acetate source and optimized conc. of glucose for NF-1 and GB fungal isolates, pH (5.5) was balanced and poured into 100ml of flasks. Optimized ratio of pre oxidized coal for NF-1 and GB fungal isolates were measured which is dried, grounded and sieved. Media and pre oxidized coal samples were autoclaved for 15 minutes at 121˚C. Inoculum was prepared by adding loop full of inoculum from actively growing zones of the plates which is (NF-1, GB) in a sterile distilled water. Solution was homogenized by using vortex or homogenizer, the absorbance value of suspension was adjusted to 0.2 at 600 nm. About 2ml inoculum (suspension) was added in each flask and incubated at 25◦C 110rpm in shaker for four days. After four days autoclaved pre oxidized coal residues were added to determine parametric effects during solubilization experiments. Autoclaved and aerobic glass flasks were used for the experiments that was run in triplicate. Flasks were placed into the shaker at 25◦C, 110rpm for 7 days. Positive and negative controls were used under all studies i.e.:

1. Fungal inoculated media without coal

2. Media having coal but no fungal inoculum

3.14 Humic acid extraction

3.14.1 Extraction of Humic Acids from Raw thar and bituminous coal

Humic acids were extracted from raw and biologically oxidize bituminous (Baluchistan) coal by following alkali method:

1. 2g of raw thar(Sindh) and bituminous (Baluchistan) coal was measured and finally grounded with sterile pestle and motel having particle size of (0.3-0.6) mm in diameter.

2. Sterile flask was used to suspend coal particles in 100ml of 0.1M NaOH solution.

3. The suspension (coal +0.1M NaOH) was stirred at 20◦C, 300rpm for 24 h by using magnetic stirrer.
4. After reaction time (24 h) suspension was poured into 50ml falcons and separated by centrifugation at 8000 rpm for 20 minutes. (Solid phase as called bitum and supernatant (liquid phase) called as humic acid + fulvic acid.)
5. 6.0M HCL solution was prepared. pH of filtered supernatant was dropped from 12 to 1.8. The solution was settled for at least 12 h.
6. Precipitates were formed in supernatant and separated by 20 minutes of centrifugation at 8000 rpm.
7. The sterile distilled water was used to wash precipitated humic acids for three to four times and desiccating in desiccator at 60◦C.
8. Humic acid was stocked at 4◦C for further analysis.

3.14.2 Extraction of Humic Acids from biologically oxidized bituminous coal

1. After fungal biological pretreatment, both Thar and bituminous coal residues were proceeded further for humic acid extraction.
2. The biologically oxidized (pretreated) coal residues was added in 100 ml of 0.1M NaOH solution.
3. The suspension (coal +0.1M NaOH) was stirred at 20◦C, 300rpm for 24 h by using magnetic stirrer.
4. After reaction time (24 h) suspension was poured into 50ml falcons and separated by centrifugation at 8000 rpm for 20 minutes. (Solid phase as called bitumin and supernatant (liquid phase) called as humic acid + fulvic acid.)
5. 6.0M HCL solution was prepared. pH of filtered supernatant was dropped from 12 to 1.8. The solution was settled for at least 12 h.
6. Precipitates were formed in supernatant and separated by 20 minutes of centrifugation at 8000 rpm.

7. The sterile distilled water as used to wash precipitated humic acids for three to four times and desiccating in desiccator at 60◦C.

8. Humic acid was stocked at 4◦C for further analysis.

3.14.3 Extraction of Humic Acids from biologically oxidized bituminous coal supernatants

Humic acid was extracted from supernatants by following**:**

1. After fungal pretreatment by potential and fungal isolates(NF-1 and GB), the filtered supernatant was further preceded for humic acid extraction.

2. 6.0M HCL solution was prepared. pH of filtered supernatant was dropped from 12 to 1.8. The solution was settled for at least 12 h.

3. Precipitates were formed in supernatant and separated by 20 minutes of centrifugation at 8000 rpm.

4. The sterile distilled water as used to wash precipitated humic acids for three to four times and desiccating in desiccator at 60◦C.
5. Humic acid was stocked at 4◦C for further analysis.

Humic acid percentage determination

Gravimetrically humic acid percentage was calculated by:

Humic acid (W/V, %) = Weight of oven dry precipitates/Vol of humic acid sample taken × 10

3.15 Extraction of Fulvic acid

1. The supernatant left after extracting humic acid were taken and maximum (higher) pH was adjusted by adding KOH and allowed to be stirred on magnetic stand for 4-8 hours. The potassium fulvate was precipitated.

2. The supernatant was centrifuged at 10,000rpm for 5 minutes and liquid was discarded.

3. The liquid precipitates after removing the supernatant was rinsed almost 3 times with deionized water (resuspended and centrifuged) again and again to remove salts.

4. Then the produced fulvic acid from each sample was dried for 24-48 hours at 50ºC.

5. After drying, the approximate weight of the produced fulvic acid was calculated from each sample.

3.16 Analytical investigation

3.16.1 Spectrophotometric studies

UV-Visible spectral gave maximum intensity at 240 -250 nm on each optimization parameter, that was used for further analysis. For the qualitative assessment of enhanced release of organic moieties from bituminous and lignite coal in supernatant was determined in the range of 200 to 600 nm spectral scan of filtrate by using SPECORD-200 Analytic UV-Vis Spectrophotometer (Jena, Germany).

3.16.2 Elemental Analysis of bio solubilized coal and humic acid

Elemental analysis of bio solubilized residues and fungal transformed humic acid from both virgin and pretreated coal (bituminous) was determined by using a Vario-EL Elemental Analyzer (Germany). (N, S, C and H) ratio of all dried products obtained from bio solubilized coal and humic acid was analyzed. Each sample was mixed well and performed in triplicate. The total weight of Carbon, Hydrogen, Nitrogen and Sulphur was subtracted to determine Oxygen content.

3.16.3 Fourier Transform-Infrared Spectroscopy (FT-IR)

FT-IR analysis was carried out for the investigation or detection of functional groups and vibrational changes occurred in fungal treated (NF-1 and GB) coal and fungal transformed humic acid from both virgin and pretreated bituminous coal. KBr pellet was formed in the ratio of 1:100 by uniform mixing of virgin high rank and low rank coal that was used for bio-solubilization assay, with dried KBr powder. The IR spectra was carried out in the range of 3500-500 cm-1 as a reference.

After fungal (NF-1,GB) pretreatment, samples of residual coal were collected and rinsed with distilled water in order to remove all the fungal mycelia. Remaining coal was desiccated by using a desiccator for 24h. Homogenously mixed dried 200 mg KBr powder with approximately 2mg of each remaining coal to form KBr pellets. These pellets were analysed by using FTIR Spectrum-65 (Perkin Elmer, USA) in 4000-500 cm-1 region that used to superimpose with reference coal. IR spectra for determining the degrading ability of NF-1 and GB fungal strains on coal functional groups.

3.16.4 Gas chromatography-mass spectrometry (GC–MS) of bio solubilized products

Gas chromatography–mass spectrometry (GC–MS) is an operative system, utilized for estimating petroleum hydrocarbons. GC–MS is very selective, specific and sensitive

instrument that could be used for qualitative and quantitative estimation of targeted components in coal derive bio solubilized liquid products. Sometimes accurate recognition of volatile and low-molecular organic constituents in complicated methylated samples through MS detector is difficult due low concentrations and altered distribution patterns of these compounds. During coal solubilization experiment by NF-1 and GB fungal isolates liberated multiple organics such as amides, aliphatic, carboxylic, ethers, low molecular fatty acids and polycyclic aromatic hydrocarbons (PAHs). A vacuum rotary evaporator was used to remove water content from bio solubilized liquid products to get viscous black liquid at 70 °C. High viscosity of black liquid could clog GC column. A huge range of organic solvents including ethyl acetate, methanol, benzene, Dichloromethane (DCM) petroleum ether, chloroform, acetone and carbon tetrachloride was utilized to prevent this problem. Ethyl acetate was used in 1 (viscous black liquid) to 10 (organic solvent) ratio by volume in order to extricate organic constituents from thick black liquid by using 100 mL separating funnel for at least 6 h. Gas chromatogram (6890 N) was attached with MS (5973 MSD) and fitted with an Agilent
JW Scientific DB-5 fused- silica capillary column (30–0.32 mm, and 0.25 μm of film thickness with 5% diphenyl-95% dimethylpolysiloxane) was utilized for characterization and recognition of coal derived organics. The instrument was operated in split less mode with ratio of 10:1 by using (helium) as carrier gas with flow rate of 1.5 mL/min. Approximately 2 μl of sample was inserted manually by using an injection and inlet set at 280 °C. The fused- silica capillary column was heated from 120 °C to 280 °C at a rate of 10 °C /min and held at 280 °C for 10 mi. Based on molecular weight range 50–1000 amu. was specified. The MS system was run at electron impact ionization mode with (70 eV). Data was obtained and processed by (software), NIST Ver. 02 library for recognition of compounds.

3.17 Anaerobic solubilization of coal

3.17.1 Anaerobic Consortia

For the development of anaerobic consortia 500ml of serum bottles are used. The experiment was conducted in an anaerobic chamber where no air could get access into the reaction setup. The bottles were autoclaved prior to use. Then 250ml of autoclaved enrichment media was added to serum bottles. 10g of the coal was added into the bottles as a carbon source. N gas was sparged into the reaction medium (serum bottles). The bottle was 2 sealed with stoppers made of butyl rubber with an inlet pipe protruding out of the stopper and was closed with a clip. The nitrogen gas was 99.99% pure. 10ml of vitamin and mineral solutions were added into the reaction medium. 10% (v/v) of deep Thar and Balochistan coal

mine water was added into the bottle with the inlet pipe. 60ml of an injection was attached to the inlet pipe to examine the success of experiment. The bottle was wrapped with aluminum foil to provide dark condition to anaerobes. The whole setup was kept into an incubator for 12 days at a temperature of 30◦C. The amount of gas collected in the injection showed that consortia were developed. This anaerobic consortium was then used for the stimulation of methane generation in coal.

3.17. Vitamin solution

Vitamin solution was added into the medium. These vitamins assist the media for the growth of microorganisms. It was used for supporting the indigenous anaerobic growth and is given below:

Table 3.2 Chemical Composition of Vitamin solution

Vitamins	mg/l
Folic Acid	2
Biotin	2
B_{12}	0.1
Pyridoxine HCl	10
Thioacetic acid	5
4-aminobenzoic acid	5
Thiamine	5
Ca-pentothenate	5
Riboflavin	5
Nicotinic acid	5

3.18 Biogasification setup

The enriched anaerobic consortium was employed biogasification assay for checking the potential biogas generation from native coal along with optimization parameters. The anaerobic consortium was enriched from deep coal mine water sample. 250ml of enrichment media was added into serum bottles each and was autoclaved. The serum bottles were purged with N and 10% v/v anaerobic culture, vitamin solution and trace minerals solution were

2 added. 10ml of consortia was added into the reaction bottles in an anaerobic chamber. The bottles were tightly sealed by butyl rubber stopper with silicon sealant. The butyl rubber stopper was connected with syringe for bio-gas collection. Experiment was conducted in triplicates under different conditions and incubation period was extended to 45 days.

Figure 3.2 Methanogenesis setup

3.19 Methane potential of coal

10g of autoclaved Lignite and Bituminous coal was added in 500ml of serum bottles as a carbon source. N2 gas was sparged into the serum bottles. Anaerobic consortia of 10% v/v were added into the medium along with vitamin and mineral solutions. Experiment was run along with positive and negative control.

3.20 Methane Potential of Chemically pretreated coal

Both type of lignite and Bituminous coal was pretreated with hydrogen peroxide and hydrogen peroxide to observe the methane potential of pretreated coal. Chemical pretreatment causes the depolymerization of coal structure converting it into monomers. Experiment is run in triplicates. 2g of pretreated coal is added in 250ml of serum bottles along with a positive and negative control.

Figure 3.3 Anaerobic Consortia

Figure 3.4 Syringe Filled with biogas

3.21 Methane Potential of pretreated coal water

The coal water obtained from the washing step of chemical pretreatment was used to observe the methane potential. The coal water from first three washing badges of centrifugation was collected and was 80 ml. Vitamin and mineral solutions were added in it. N_2 gas was sparged in the headspace. Microbial consortia were added along with no coal. Injection was attached to observe the methane potential.

3.22 Optimization Parameters

3.22.1 Temperature and pH

For enhancing the process of coal solubilization anaerobically, two parameters that includes temperature and pH were optimized. The lignite and bituminous coal were allowed to produced methane at different temperatures including 25°C, 35°C, 45°C, and 55°C. Similarly, both types of coal were allowed to produce methane at different pH including 6.0, 6.5, 7.0, 7.5 and 8.0. Both the methane and CO_2 produced at optimum conditions were calculated. The reaction setup contains 10g of lignite and bituminous coal at each temperature and pH along with vitamin solution and trace mineral elements. Nitrogen gas were sparged at the head space of each bottle. Injection was attached to calculate the gas produced.

3.23 Enhancement of Methane Potential

3.23.1 Methane Potential using co-digestion strategy

The co-digestive materials used were grass, cow dung and peanut hull. Two combinations were used the one was grass and cow dung and the other was grass and peanut hull. All the materials were ground to powder form to increase the surface area as well as efficiency of experiment. In combination no.1, 2g of co-digestive powder of grass and 2g cow dung was added in a 250ml serum bottle along with autoclaved enrichment media. Vitamin and mineral solution were added. The bottle was sparged with N2 gas. 10%v/v of the microbial consortia was added in the end under anaerobic conditions. A positive and negative control was run along the experiment. In positive control no substrate i.e. no coal was added and all the other condition were same. While in negative control no consortia were added keeping all other reaction conditions similar.

In the second combination of co-digestive materials 5g of grass along with 2g of peanut hull (powdered) was added in 500ml of serum bottles. All the other conditions were same as of combination no.1.

3.24 Gas Scrubbing

The biogas produced in the syringe was scrubbed using a 3M NaOH solution. By scrubbing the gas samples we get the estimated methane produced in biogas sample. The injection of biogas was injected into the 3M NaOH solution. CO_2 got dissolved into the NaOH solution and CH_4 was left in the injection.

The reading of methane in the injection was noted down for every sample on daily basis. The gas in the injection in fig 3.4 is methane after scrubbing with 3M NaOH.

3.25 Molecular study

3.25.1 PCR (polymerase Chain Reaction)

DNA amplification using PCR

For amplifying DNA, PCR (Polymerase Chain reaction) were performed. The PCR mixture was prepared carefully in safety hood in order to avoid any sort of contamination.

For all the primer set PCR thermal profile were denatured at 95◦C for 45 seconds. Annealing at 58◦C for fungal universal primer and 60◦C for bacterial universal primers for almost 45 seconds and extending at 72◦C for about 60 seconds and final extension was carried out at 72◦C for 10 minutes. The PCR reaction was coupled both with taq polymerase and master mix respectively. The DNA fragment that were extracted were amplified with universal primer ITS1 and ITS4 for fungal strains while bacterial strains were amplified with universal primers 27F forward and 1429R as reverse primers.

3.25.2 Quantity of reaction mixture when PCR was performed with master mix Table 3.3 Ingredients of PCR reaction mixture

Ingredients	With master mix
DNA	3µl
Primer (forward+reverse)	5µl
PCR water	30µl
Master mix	10µl

After preparing the reaction mixture, they were spin for complete mixing and placed into PCR. The temperature of lid was kept at 98◦C. PCR was set for about 40 cycles after maintaining all the conditions and get started. After the completion of PCR, the products were analysed by electrophoresis using 1% agarose gel in TBE buffer (40mM trisborate, 1mM

EDTA pH 8.1) according to Sambrook and Russell., 2001. In order to find out the size of the product, DNA marker of 1kb (Gene Ruler) was applied while running the gel.

3.25.3 Agarose gel electrophoresis

For visualizing PCR products, and to check the presence of DNA, agarose 1.5 g was dissolved in 1 x TBE buffer and was heated in microwave oven for 60 seconds in order to dissolve the agarose completely. The agarose solution was allowed to cool and about 2 µL of ethidium bromide was added to the solution followed by well shaking for thoroughly mixing. and sample loading comb were placed in tray. The solution was poured into gel tray, allowed to solidify for 10 to 20 minutes. About 2 µL Loading dye was mixed with DNA samples followed by loading of samples into the wells. The agarose gel was run for 30 minutes at 110 V and 400mA and observed under UV illuminator.

3.25.4 DNA Extraction protocol (Bacterial and Fungal) using Qiagen KIT

1. About 1.8ml of bacterial and fungal culture was added to 2ml collection tube and allowed to centrifuge at 10,000rpm for 30 seconds at room temperature. The supernatant was discarded, and tubes were centrifuged again at 10,000rpm for 30 seconds at room temperature. Media supernatant was removed completely with the help of pipette tip.

2. About 300µl of power bead solution was added in cell pellets and vortexed gently to mix well. The supernatant with resuspended pellet were transferred to 0.1mm power bead glass tube.

3. In step 3, about 50µl of SL solution was added to the power bead tube.
4. The power bead tube was placed with proper balancing in power lyzer 24 homogenizers and allowed to homogenize for 5 minutes at 2000 rpm.

5. After completion of homogenization, the power bead tubes were centrifuged at maximum speed of 10,000rpm for 30 seconds at room temperature.

6. The supernatant after centrifugation were transferred to clean new 2ml collection tube provided.

7. In next step, 100µl of IRS solution were added and vortexed for about 5 seconds. Tubes were incubated at 4ºC for 5 minutes.

8. After 5 minutes of incubation, tubes were centrifuged at 10,000rpm for 1 minute.

9. Supernatant was transferred to new 2ml collection tube, avoiding the pellet.

10. About 900µl of SB solution were added to the supernatant and vortexed for 5 seconds gently.
11. In next step, about 700µl of solution were loaded into MB spin column and allowed to centrifuge at 10,000rpm for 30-60 seconds at room temperature. The flow through were discarded and the remaining supernatant were loaded again into MB spin column and centrifuged again at 10,000rpm for 1 minute at room temperature condition.
12. Further, 300µl of CB solution were added and allowed to centrifuge at 10,000rpm for 30 seconds.
13. The flow through were discarded and again the centrifugation was done at 10,000rpm for 1 minute at room temperature.
14. The MB spin column were placed into new 2ml collection tube. The liquid must not be splashed on the spin filter basket.
15. In last step, about 50µl of EB solution were added into the centre of white filter membrane.
16. The samples were allowed to centrifuge again at 10,000rpm for 30 seconds at room temperature.
17. The spin filter was discarded and DNA is now ready to use for further applications.
18. DNA was stored at -20ºC for further studies.

3.25.5 PCR Clean Up

1. For removing the primer dimers formed during PCR II along with PCR components, PCR clean up procedure were performed.
2. Reagent needed:

- Mag Bio Beads
- Freshly prepared 80% Ethanol

3. The PCR plates or tubes from PCR II were centrifuged to collect condensation.
4. The Mag Bio beads were allowed to vortex for about 30 second in order to homogenize them.
5. The lid was removed from the plates and tubes carefully.

6. About 20µl of Mag Bio Beads were added into each tube/well having PCR II products. Pipetting was done 10 times to mix the beads gently.
7. Incubation was done at room temperature for 5 minutes.
8. The tubes/ plate was gently placed on magnetic stand for 2 minutes until the supernatant got clear. The supernatant was removed carefully and discarded.
9. **Washing Steps:**

    - **1st wash:** About 200µl of 80% ethanol were added into tubes/wells without resuspending the beads.
    - Incubation was done at room temperature for 30 seconds.
    - Supernatant was carefully removed and discarded.

10. **2nd Wash:**

- About 200µl of 80% ethanol were added again into tubes and wells without disturbing the beads.
- Incubation was done again at 30 seconds at room temperature.
- Supernatant was discarded carefully.

11. Without removing tubes/plates from the magnetic stands with lids off, allow the beads to air dry for 15-30 minutes until all the ethanol was evaporated and beads become brittle.
12. The tubes/plates were allowed to remove from the magnetic stand.
13. About 27µl of resuspension buffer were added into each well and tube and mixed gently for about 10 times with pipette. Incubation was done at room temperature for 2 minutes.
14. Again, the tubes and plates were placed on the magnetic stand and incubated at room temperature for 2 minutes.
15. About 25µl of clear supernatant were carefully transferred to new labelled plates and tubes.

16. The clean products were again run-on gel electrophoresis with some slight increase in band size of PCR II products.

17. Samples were stored at -20ºC for further analysis.

3.25.5 DNA easy power lyzer power soil kit:

All the centrifugation steps were performed at room temperature.

1. About 0.5g of coal samples from both types of coal were taken and added into power bead tube provided in kit.

2. About 75µl power bead solution was added in power bead tube.

3. In addition to power bead solution, about 60µl of C1 solution were added and vortexed several times or briefly vortexed to mix well.

4. The power bead tube was placed in power layzer 24 Eppendorf holder with proper balancing. The samples were run at 4000rpm for 45 seconds to 1 minute with 2 repetitive cycles.

5. After bead beating, the tubes were placed in centrifuge and centrifugation was done at 10,000rpm for 3 minutes.

6. The supernatant was transferred to 2ml clean collection tube provided in the kit.
7. In next step, 250µl of C2 solution was added and vortexed briefly for 5 seconds. Later on, incubation was done at 2-8ºC for 5 minutes.

8. After incubating the samples, tubes were centrifuged at 10,000rpm for 1 minute. About 600µl of supernatant were transferred to new collection tube avoiding the pellet.

9. About 200µl of C3 solution were added and vortexed for 5 seconds briefly.
   Incubation was done again at 2-8ºC for 5 minutes.

10. After second incubation, tubes were centrifuged again at 10,000rpm for 1 minute. About 750µl of supernatant was transferred to new collection tube, avoiding the pellet.

11. In next step,1200µl of solution C4 were added to the supernatant and vortexed for 5 seconds.

12. The MB spin column was taken and about 675µl of supernatant from step 11were added in the column and centrifugation was done at 10,000rpm for 1 minute. The flow through was discarded and remaining 675µl of solution were added into the column.

13. The column was allowed to centrifuge again at 10,000rpm for 1 minute and remaining supernatant were loaded again into MB spin column and centrifugation were done at 10,000rpm for 1 minute. Total three loads for each sample to MB spin column were processed.
14. About 500µl of solution C5 was added into the column and centrifuged at 10,000rpm for 1 minute.
15. The flow through was discarded and column were allowed to spin again at 10,000rpm for 1 minute.
16. The MB spin column were placed into new collection tube carefully. By avoiding any kind of splashing created by C5 solution into the column.
17. In next step,100µl of solution C6 were added into the centre of membrane filter. Pin addition to solution C6, PCR water or TE buffer can also be added.
18. In last step, centrifugation was done again at 10,000 rpm for 1 minute. The spin column was discarded.
19. The extracted DNA was stored at -20ºC or -80ºC for carrying out further downstream applications.

3.26 Metagenomic studies:

3.26.1 16S amplicons protocol

For carrying out PCR amplification of 16S bacterial community, the DNA concentration were measured with the help of QUBIT. The concentration must be 1-10ng/µl in range. The reagents used were PCRBIO HiFI polymerase along with forward and reverse primers, BSA, template DNA and HiFi Buffer.

The conditions followed were 95°C for 2 minutes, 95°C of 15 seconds, 55°C for 15 seconds, 68C for 40 seconds (33 cycles) and 68°C for 4 minutes with infinite hold at 4°C .

PCR II:

After running PCR II, the PCR 1 products were allowed to run on gel.

For running PCR II, the index primers or Index barcodes were added to the samples. The reagents used in PCR II were PCRBIO HiFI polymerase along with primers and HiFi Buffer.

The reaction conditions followed were:

98°C for 1 minute, 98°C 10 seconds, 55°C for 20 seconds, 68°C for 40 seconds, 68 for 5 minutes and 4°C (infinite). Total cycles followed were 13.

3.26.2 PCR for 18S amplicons (For identifying fungal communities) For running PCR for 18S amplicons following conditions were used.

Step 1: Primers use for amplifying fungal communities are fITS7F-adp/ ITS4R-adp.

Negative control was also included The

reagents used were:

PCR water, PCRBIO HiFi polymerase along with buffer, forward and reverse primers, BSA and template DNA. The quantity of the reagents were adjusted according to manufacturer's manual

The PCR conditions followed were 94ºC for 5 minutes, 94ºC for 30 seconds, 57 ºC for 30 seconds, 72 ºC for 30 sec and 72 ºC for 10 minutes. The number of cycles followed were 30

3.26.3 Protocol for Preparation of FS (FUNGAL) DNA library using KIT:

Step 1: Fragmentation/ End prep:

Fragmentation of DNA occurs at 37ºC incubation step. As, the concentration of DNA was high and size of DNA was also high, the incubation was done for 15 minutes at 37ºC.

Fragmentation size	Incubation at 37ºC	Optimization
200bp-450bp	15-17 minutes	15-20 minutes

The kit used were the NEBNext Ultra II FS DNA library kit for Illumina.

It was ensured that the kit reagent Ultra II FS reaction buffer were completely thawed.

The Ultra II FS enzyme were also vortexed for 5-8 seconds before use and place on ice.

Following components listed below were added to 0.2ml thin walled PCR tubes placed on ice.

Table 3.4 Components used in PCR

Components	Volume of reagent per library
Genomic DNA	26µl
(yellow) NEB Next ultra II FS reaction Buffer	7µl
(yellow) NEB Next Ultra II FS enzyme Mix	2µl
Volume in total	35µl

The reaction mixture was vortexed for 5 seconds and spin gently on a microcentrifuge. In thermocycler PCR, the lid temperature was maintained to set at 75ºC by running the program mentioned below:

- 5-30 minutes at 37ºC
- 30 minutes at 65ºC
- Hold at 4ºC

Step 2: Adapter ligation:

The components listed in table was added directly to FS reaction mixture

Table 3.5 FS Reaction mixture

Component	Volume
FS reaction buffer	35µl
(Red) NEB Next Ultra II ligation master mix	30µl
(Red) NEB Next ligation Enhancer	1µl
(Red) NEB Next Adaptor for Illumina	2.5µl
Volume in total	68.5µl

- The Ultra II ligation master mix were mixed up and down by pipetting several times.
- After addition of all the components, the incubation at 20ºC for 15 minutes in a PCR thermocycler were done with the heating lid off.
- After 15 minutes about 3µl of (red) USER enzyme to the ligation mixture was added and mixed well.
- Later on, the reaction was incubated at 37ºC for 15 minutes with lid heating set at ≥ 47ºC.

STEP# 3

Size selection of adaptor ligated DNA for DNA input ≥100ng

- The fungal strains selected in the present study were of size 200-350bp, so the conditions selected for clean-up were according to the 200-350bp.
- The volume of reaction mixture was brought to 100µl by addition of 28.5µl 0.1X TE buffer.
- The NEB Next sample purification beads were vortexed to resuspend.
- About 30µl of resuspended beads were added to 100µl of sample. The mixture was mixed by pipetting up and down at least 10 times.
- The samples were incubated at room temperature for at least 5 minutes.
- The tubes/plates were placed on magnetic stand to separate the supernatant from beads.
- After incubating for 5 minutes, when the solution become clear, transfer the supernatant to clean new tube containing DNA. The unwanted beads were discarded.

- Again, 15µl of resuspended NEB Next beads were added to the samples and mix with the help of pipetting by 10 times at least. The samples were incubated at bench top at room temperature for 5 minutes.
- After incubating the tubes/plates were placed again on magnetic stand to separate the supernatant from beads.
- When solution become clear after five minutes the supernatant was clearly discarded containing unwanted DNA. It must be noted that the beads must not be disturbed as it contains desired DNA.

Washing steps:

- While the tubes are still on magnetic stand, about 200µl of 80% freshly prepared ethanol were added into the tubes. Incubation was done for 30seconds at room temperature, and then supernatant was discarded and removed carefully. The beads must not be disturbed as they contain the desired DNA.
- After washing twice or thrice, all the clear and visible liquid were removed carefully.
- The beads were air dried for up to 5 minutes while the tube was on magnetic stand with the lids open.
- The tube or plate were removed from the magnetic stand and target DNA was eluted by addition of 17µl of 0.1X TE buffer.
- The added TE buffer and DNA were mixed together by pipetting up and down at least 10 times. The mixture was incubated for 2 minutes at room temperature.
- The tubes were placed back on magnetic stand, after 5 minutes when solution becomes clear, transfer about 15µl of supernatant to new PCR tube.

Step#4 PCR-Enrichment adaptor ligated DNA

If forward and reverse primers are not combined already, the following components will be added to the solution.

Table 3.6 Adaptor ligation solutions

DNA fragments having adaptors	15µl
(blue) NEB Next ultra II Q5 master mix	25µl
(Blue) Index primers/i7 primer	5µl
Universal PCR primer i5	5µl
Volume in total	50µl

The tubes were placed in PCR thermocycler for performing PCR amplification using the following conditions:

PCR Cycle steps	Temperature	Time	Cycles
Initial Denaturation	98	30 seconds	01
Denaturation	98	10 seconds	3-7 cycles
Extension/Annealing	65	75 seconds	3-7 cycles
Final Extension	65	5 minutes	1
Hold	4	∞	∞

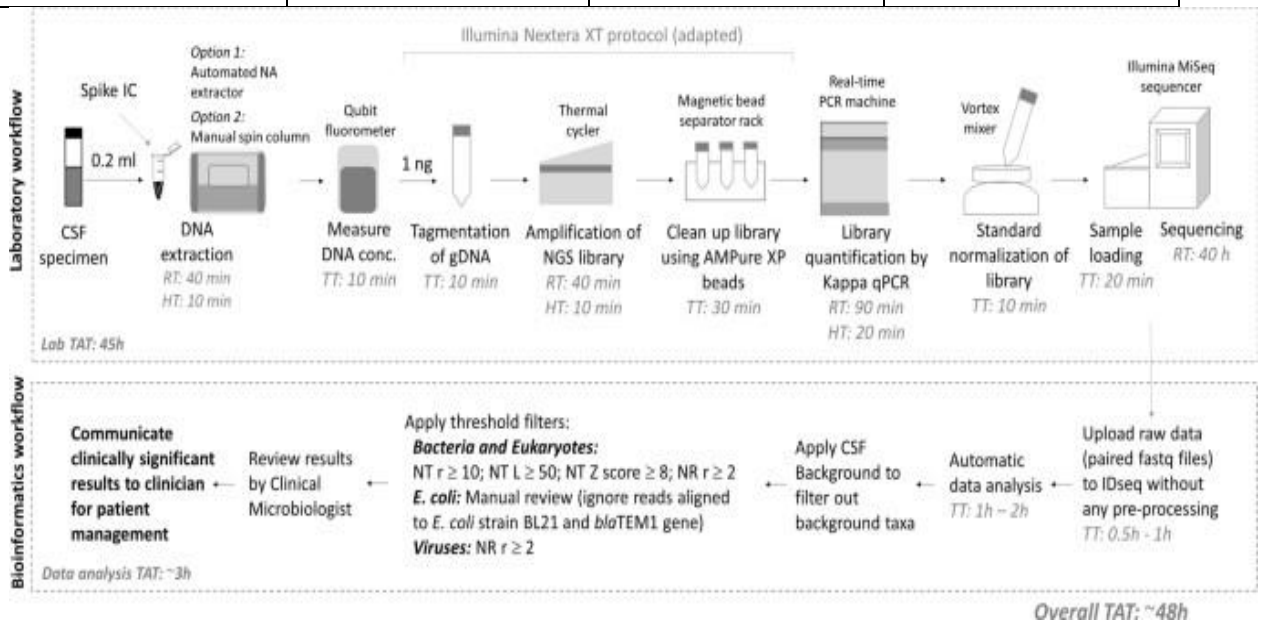


Fig 3.7 Illumina Nextera XT proctocol

For preparation of standard library, there are some certain number of cycles required according to the input DNA. In the present study, the input FS DNA reaction is 200ng, so, the library preparation requires 4 cycles of PCR.

3.26.4 Fungal ITS 2 Illumina Library preparation:

- ♦ The DNA extracted from the coal samples were transferred to the 96 well plates or PCR tubes.
- ♦ Each well or tube indicates the sample number, so it must be mentioned on the excel sheet that corresponds to the plate number or tube.

PCR 1 Master Mix was prepared according to instructions by the manufacturer that includes: PCR Buffer, dd water,dNTP,s, forward and reverse primers and Taq polymerase. Replicates were made during PCR 1

- About 24µl of master mix was added into each well and tube.
- About 2µl of template DNA from each sample were added into each well and tube.
- The plates or tubes was allowed to spin at 700rpm for 1 minute before placing them into thermocycler PCR machine.

Thermocycler conditions followed were 94 ºC for 30seconds, 57 ºC for 30 sec, 72 ºC for 30 seconds, final elongation at 72 ºC and hold at 4 ºC.

- After completion of PCR 1 cycle, the PCR products were stored at -20ºC for using it further.
- Before initiating PCR 2, the technical replicates from PCR 1 were pooled together into new tubes or plates.
- The pooled tubes or plates served as a template for PCR 2.
- The reaction mixture for PCR 2 were mixed, but at this time forward and reverse primers were not added.
- About 19µl of master mix was added into each tube or well.
- Index primers/ index combinations (2µl) were added manually into each tube and well in a right order.
- About 2µl of template DNA was added from pooled plate or tube to make a total volume of 25µl.
- The tubes or plates was allowed to centrifuge for 1 minute at 700 rpm and PCR reaction were run with thermocycler conditions mentioned below:

94ºC for 5 minutes
94ºC for 30 seconds
55ºC for 30 seconds
72ºC for 30 seconds
Final Elongation 72ºC for 10 minutes Hold
at 4ºC

- After completion of cycle of PCR 2, the 1.5% agarose gel were run to check the amplification of DNA.

3.27 Pooling, precipitation and cleaning

1. The DNA concentration was measured after PCR 2 clean up. About 5µl pool of PCR 2 product were added into 2ml Eppendorf tube.
2. The DNA was precipitated using salt and ethanol. About 1/10 volume of 100µl of pooled DNA, 10µl of 3M sodium acetate and 2 volume of 96%cold ethanol was added.

3. The precipitated mixture was stored at -20ºC overnight. The samples were spined at 13,000rpm for 15 minutes (visible pellet appeared).
4. Washing were done by using 75% ethanol and centrifuged at 13,000rpm for 5 minutes. Ethanol was discarded without disturbing the pellets. The steps were repeated twice. The pellet was allowed to dry at room temperature for 30 minutes.
5. The pellets were re dissolved in 100µl of DNase free water or TE buffer having pH 8.
6. The gel was run with freshly prepared gel electrophoresis buffer.
7. The two wells were allowed to combine using Sellotape on comb to make wells the bigger one to accommodate loading sample properly.
8. About half 50µl of washed DNA were allowed to run on 1.5% agarose gel for 45 minutes. The gel was viewed under blue light and cut the band and placed them into sterile 1.5ml Eppendorf tube.
9. The gel fragments were purified using QIAquick gel extraction kit.
10. The gel was weighed, and the size should not exceed 400mg.
11. According to protocol:
    a. About 3 volume of buffer and 1 volume of gel (if the gel weight were 200mg, we added 3×200 i.e 600µl of buffer in the Eppendorf tube.
12. The final concentration and size of DNA library was checked using Qubit fluorometer and nanodrop.

3.28 Tape Station Analysis:

3.28.1 Agilent D1000 Tape station assay protocol

1. All the reagents of D1000 Tape station were allowed to settle at room temperature for 30 minutes.
2. The tape station controller software was launched and D1000 tape station device was inserted into tape station nest of tape station instrument.
3. The sample positions required in the tape station controller software were selected.
4. The reagents were vortexed and spin down before making them in use.
5. Preparation of ladder:

- 3µl of sample buffer
- 1µl of D 1000 DNA ladder at position A1

Preparation of samples

1. The samples were prepared by pipetting 3µl of D1000 sample buffer and 1µl of DNA sample.
2. The sample tubes were mixed together by vortexing in an IKA MS3 at 2000rpm for 1 minute.
3. The ladder and sample tubes were spin down for at least 1 minute.

Sample Analysis

4. The samples were loaded into tape station instrument. The ladder tube was placed in A1 position on the tube strip holder.
5. The caps of the tube strips were removed and confirmed visually that liquid is positioned at the bottom.
6. Then, tape station is started, and it worked automatically and display the results.
7. Nextera XT modified protocol for bacterial whole genome:
8. Table from protocol:
9. The new PCR tubes were taken and labelled according to the samples.
10. The DNA concentration of the samples was adjusted to 0.5 (dilute 3X from 1ng/µl) by using Qubit. The PCR grade water was used for dilution.
11. About 5µl of TD buffer of input DNA i.e. 0.5ng/µl or 1.5ng in total was added.
12. The samples were vortexed briefly and spin down for 30 seconds for 1 minute.
13. The tubes were placed in thermocycler PCR and programme run was:
    - 55ºC for 5 minutes
    - Hold at 10 seconds
14. When the samples reach to hold 10ºC immediately NT buffer of 2.5µl were added.
15. The samples were voetexed again and spin down again by centrifugation
16. The samples were allowed to incubate at room temperature for 5 minutes.

17. About 7.5µl of NPM was added to PCR tube along with 2.5µl of index primers 1 and 2.
18. Again, vortex the samples and spin down for 30seconds to mix all the consumables well.
19. The tubes were allowed to place in thermocycler by running the programme mentioned below:

72ºC for 3 minutes
95ºC for 30 seconds
15(12-16) cycles of

- ♦ 95ºC for 10 seconds
- ♦ 55ºC for 30 seconds
- ♦ 72ºC for 30 seconds
- ♦ 72ºC for 5 minutes
- ♦ Hold at 4ºC

3.29 PCR Clean up

For cleaning the PCR products, AMPure beads were used to purify the DNA library. This clean up helps to select the size of DNA by removing short library fragments. The cleaning steps were followed as described by the manufacturers in protocol.

3.30 QUBIT Assay protocol

For running QUBIT assay, **QUBIT Flex Fluorometer** instrument was used.

1. The required number of tubes for carrying out the Qubit assay were set up for samples and standards. **Qubit 1X dsDNA HS** requires two standards for measuring DNA concentration in a sample.
2. The tubes with required samples were labelled properly.
3. About 10µl of each Qubit standards were added into appropriate tubes.
4. About 1-20µl of required DNA sample were added into the labelled tubes accordingly.
5. Qubit 1X dsDNA working solution was added to each tube to attain the final volume of 200µl.
6. After adding all the required reagents, the samples were vortexed for about 3-5 seconds.

7. The tubes were allowed to incubate at room temperature for 2 minutes.
8. On Qubit Flex Fluorometer instrument home screen, 1X ds DNA high sensitivity function was selected and **read standard** option was selected for knowing the standard concentration first.
9. The labelled tube with standard#1 was inserted in the instrument and lid was closed and standard# 1 value were noted.
10. Similarly, standard#2 value was also noted.
11. After reading the standards, **read sample** option was selected and DNA concentration present in each sample was recorded.
12. The final concentration of DNA from each sample was obtained in µl with respect to Qubit tube concentration.

3.31 Real Time qPCR protocol for library quantification:

Process workflow of qPCR:

(a) Reagent Preparation

5ml KAPA SYBR FAST qPCR master mix (2x) was combined with 1ml primer mix and 0.2ml ROX High and Low (50x)

(b) Sample preparation:

The DNA library is diluted by using library dilution buffer (10mM Tris HCL, pH (88.5)+0.05% Tween 20

(c) Reaction Setup:

For preparing 20µl reaction: 12µl of KAPA SYBR Fast+ primer mix + 4µl of Standard DNA library dilution+ PCR Grade water 4µl

(d) Cycling conditions:

Table 3.7 qPCR cycling conditions

Steps	Temperature	Duration (Time)	Cycles
Initial Denaturation	95ºC	5 min	1
Denaturation	**95 ºC**	**30 sec**	
Annealing/ Extension/ Data acquisition	**60 ºC**	**45 sec**	**35 cycles**
Melt Curve Analysis	65-95 ºC		

For libraries having average fragment length ≥ 700bp, the annealing/ Extension can be combined and increase up to 90 seconds.

(e) Data Analysis:

- ♦ Outliers are removed
- ♦ Reaction efficiency is confirmed 90-110%
- ♦ Confirmation of R2> 0.99 and ΔCq for DNA standard 3.1-3.6
- ♦ KAPA library quantification data analysis designed for library quantification

(f) Library concentration calculation:

- ♦ ΔCq were corrected and confirm for library dilution
- ♦ Size adjustment calculation was performed
- ♦ Undiluted library concentration was calculated by the formula:

qPCR conc x (452bp) average fragment length X dilution factor

Chapter No. 4

Results

4.1 Geology of coal samples

The coal samples from Sindh province used in this study was collected from depths, drilled in the range between 120m to 290m. Typically, the Sindh coals are less than 3 meters in thickness but the main seam intercepted in Thar was over 29m in thickness (San Filipo *et al* 1989; San Filipo *et al,* 1994). The coal fields of Thar were mainly composed of Eocene sedimentary rock, dune sand, alluvium and Paleocene. Mostly, the age of thickest coal in the Thar reported by Ahmed and Zaigham in 1993 were of Jurassic age, However, these coal stratas may belong to late Paleocene to early Eocene age based on Palynological studies in close vicinity of the sample used in present study TC (Ahmed *et al,* 1993). One of the studies conducted by Fasset and Durrani in 1994 also reported the age of Thar coal fields of Paleocene to early Eocene,

The coal from Balochistan DUKI coal mine was discovered firstly in 1877 during construction of railway line of Sibi HArnai. Due to increasing demand of energy the GSP (Geological survey of Pakistan) worked to explore coal resources timely. The coal fields of Duki- Anambar are found to be present in Loralai district of division Zhob. It is located in Duki- Anamber syndines. The host formation of coal bearing strata in Duki and its vicinity area is Toi which is 20m thick. In total 17 coal seams are known out of which 15 are mined (0.15-0.30m).

The coal seams of Baluchistan region are of Eocene age and are found in Ghazig formation. The Duki coal field of Pakistan are present between 29°45′ to 30°15′ to 69°0′ on Latitude and longitude respectively. A coal bearing horizon of about 1080m thick contains four separate coal zones of 15, 22, 36 and 92m in thickness. The coal of this field is brownish black, black in coloration. The floor and roof of Baluchistan coal seam is claystone with some sand stone at few places. The BRU value of most coal beds of Duki region in rank from sub-bituminous B to C with few of them as bituminous A and some beds are lignite. The calorific value ranges between 9500 to 14515 BTU/lb. The general range is between 10,131 to 14,164 BTU/lb. The sample used in the present study was extracted from Duki coal mine with depth of about 800ft.

Table 4.1 shows the type coal fields, depth and ASTM rank of coal fields.

Table 4.1 Origin of coal samples

Sample ID	Coal Field	Depth(m)	ASTM Rank
TH-1	Thar	170.58	ligB
TH-2	Thar	181.5	ligA
BC-1	Baluchistan	204.6	subC
BC-5	Baluchistan	354.9	subA
TC	Thar	118.5	ligA
BC	Baluchistan	191.7	bitA
BC-3	Baluchistan	160.1	bitB
BC-6	Baluchistan	195.9	subC

4.2 Chemical characterization of coal samples:

The chemical characterization of Duki coal samples and Thar region of Pakistan has been listed in table 4.2. The thar coal of Sindh region of Pakistan are particularly young in age and contains high moisture content with brown coloration and woody texture resembling dried peats. The moisture content of Thar coal samples is observed to be present in the range of 24% to 36% approximately. While the moisture content present in Baluchistan coal sample varies from 5% to 10% of moisture content. The sulphur content seems to be present between medium (1-3%) to higher > 3% respectively in Thar coal samples but it varies with < 1% for some thicker seams of coal specially ―Thar Sweet Pot‖ (Wood *et al*, 1983). Similarly, the sulphur content present in Baluchistan Coal field varies from <1 1% to 2%.

The moisture content in Thar coal samples named as (TH-1, TH-2 and TC) varies from 24% to 36%. The more amount of moisture is present in TH-2 coal sample, as Thar coal is designated as low rank coal so it contains more moisture content. While the Baluchistan coal were ranked as high rank so it contains very less moisture content. Sample BC contains about 10.01% moisture content while other samples from Baluchistan range contains less than 10% moisture content.

The nitrogen content in all the coal samples i.e both Thar and Baluchistan vary from <1 to 2.9% while the ash yield was quite variable between all the coal samples. It contains lower to medium range of ash content (6% to 17%), while the average ash content in all the thar coal samples were 14.2%. while the ash content from Baluchistan coal mine designated as high rank (Sub-bituminous and bituminous) contain somewhat lower values of ash content. One of the studies conducted by Rizwan et al, 2014, had shown average values of ash content for Thar coal fields while somewhat lower values for Baluchistan region coal mine.

The elemental analysis of coal excluding water from oxygen and hydrogen content of sample were determined. The carbon content in coal samples ranges from 35-73% in Thar coal mines while more carbon content is present sample BC-6 from Baluchistan coal. while other samples carbon content ranges from 44% to 58%. All these values for chemical analysis were compiled with other analytical investigations from coal fields of Pakistan conducted by various organizations of government like geological survey of Pakistan. (Warwick and Javed, 1990; Fasset and Durrani, 1994, Rizwan et al, 2014). While table 4.3 depicts the atomic ratios with respect to (O/C and H/C). The H/C ratios ranges from 0.07 to 0.11 in case of lignite Thar coal samples, while for Baluchistan bituminous coal sample the ratio lies in the range between 0.11 to 0.19. Similarly, O/C ratios varies from 0.45 to 0.59 for Thar coal while for Baluchistan it ranges from 0.27 to 0.44. These values are not following any trend as these seems to vary from each other. Generally, with the increase in coal rank these values decreases because of increase in carbon and decrease in oxygen and hydrogen content. Some of the samples from Thar region shows very less values as compared to bituminous Baluchistan region and these indices are present higher in this region which further confirms the coalification process.

Table 4.2 Elemental analysis of coal samples

Sample ID	C%	H%	N%	S%	O%	H/C	O/C
TH-1	35.21	4.01	0.71	0.53	30.87	0.12	0.55
TH-2	37.32	4.42	0.52	3.32	22.32	0.11	0.59
BC-1	44.42	4.91	0.94	7.92	17.12	0.11	0.38
BC-5	41.09	5.01	0.81	6.04	18.47	0.12	0.44
TC	62.2	4.44	1.97	3.31	28.07	0.07	0.45
BC	56.20	10.99	3.07	11.15	18.59	0.19	0.33
BC-3	57.32	8.09	2.91	3.21	20.21	0.14	0.35
BC-6	70.21	5.01	2.08	2.20	19.21	0.07	0.27

Table 4.3 Proximate analysis of coal samples

Sample ID	Moisture (%)	Ash (%)	Volatile matter	Fixed Carbon	
TH-1	31.15	6.0	42.1	36.9	
TH-2	35.92	13.5	19.9	64.6	
BC-1	8.57	12.01	23.1	52.4	
BC-5	8.92	16.04	21.4	43.8	
TC	24.01	9.37	11.47	31.15	
BC	10.01	12.0	37.1	40.89	

BC-3	5.91	12.21	36.2	31.32	
BC-6	6.32	10.41	37.9	32.13	

4.3 Primary screening of fungal isolates for coal solubilizing activity

The primary screening of coal solubilizing activity were based on the formation of black colour droplets from coal particles that were sprinkled on the surface of fungal spores or mycelium formed on malt extract agar (MEA) medium plates. The production of black droplets on solid surface were estimated by using UV-Visible spectrophotometry. Similarly, minimal salt medium (MSM) with added concentration of ammonium sulphate has been reported to be one of the best medium for undergoing better coal degradation activity. The fungal isolates were screened out both by using solid and liquid submerged medium for release of organic moieties estimated by UV-Visible spectrophotometry. In the presence of coal particles in liquid medium, the fungal mycelium in submerged liquid medium was characterized by entrapment of fungal mycelium around coal particles.

After screening of all the coal fungal isolates, the production of small black and brown colour droplets surrounding coal particles on fungal mycelia were estimated by UV-Visible spectrophotometer in the range between 200nm-250nm. Out of all the isolates, 4 isolates shows maximum release of organics named as NF-1, GB , DS and LP. But significant organic moieties were released by two potential isolates NF-1 and GB on the basis of UV-Visible scan. The absorbance intensity of isolate GB were more than NF-1. The fungal isolate GB was isolated from Bituminous coal environment from DUKI coal mine and has shown maximum absorption intensity and release of organics during 7 to 11 days of incubation time period.

By comparing all the absorption intensities of isolates, the two fungal isolates NF-1 and GB were selected for further experimentation of coal solubilization and these isolates seems to be efficient for subsequent transformation of coal into valuable by products.

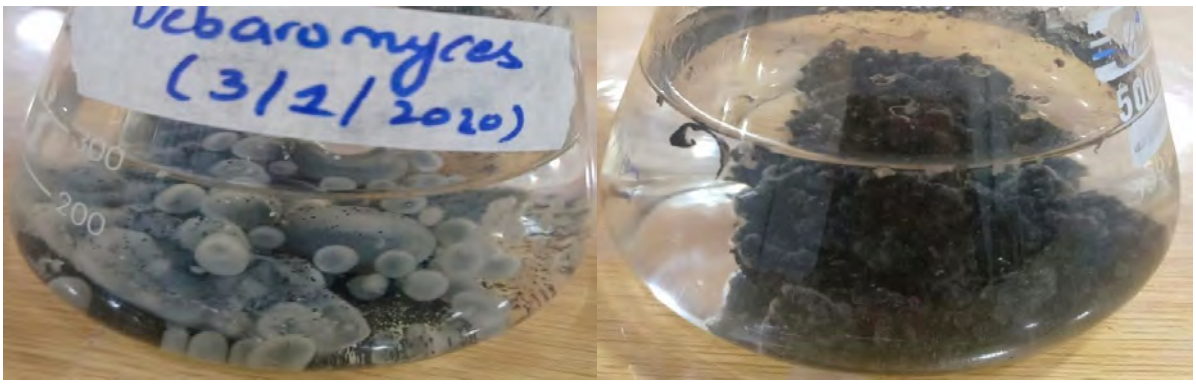


Fig 4.1 Fungal Mycelium of isolate NF-1 and GB Entrapping coal particles

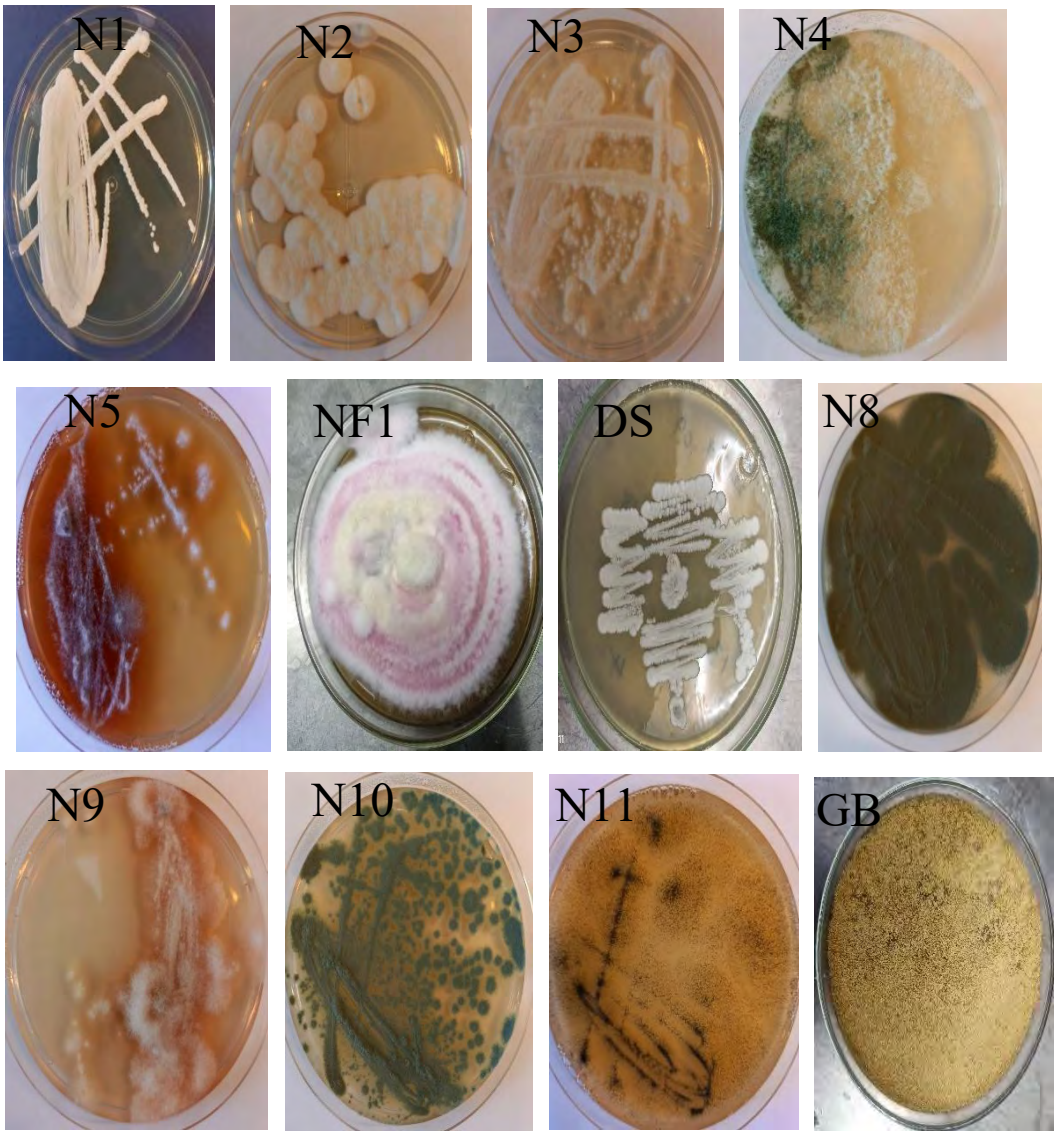


Fig 4.2 Isolated Fungal strains from DUKI And Thar Coal Mines

Table 4.4 Identification of isolated microorganisms

Code No.	Organism
N1	*Dichomitus squalens*
N2	*Rhizopus Delemar*
N3	*Aspergillus nidulans*
N4	*Trichoderma Sp.*
N5	*Penicillium Sp.*
NF-1	*Debaromyeces hansenii*
DS	*Picornia igniria*

N8	***Neurospora sp. Isolate***
N9	***Bassochlamys spectabilis***
N10	***Aspergillus niger***
N11	***Trametes versicolor***
GB	***Aspergillus ochraceus***

4.4 Microscopic studies using Acridine Orange stain

Acridine orange microscopy were performed to identify the mycelium and hyphae structure of fungal isolates morphologically. The prepared slides were observed by using acridine orange stain and observed under fluorescence microscope with green back ground. The morphological features of the two selected potential fungal isolates are listed in table below

Table 4.5 Morphological features of selected potential isolates

	Mycelium	**Hyphal type**	**Spore nature**
NF-1	Nerve like, peridium membranaceus, Ascomycota(ascus)	Immersed, perithecial, ostiolate, septate hyphae with sac like structure	Hairy, dense, dark in colour, fusiform, ellipsoidal, unicellular, conidiophore
GB	brush like, dense	Dense, pointed like hyphae, thick walled	Conidiophore, spherical rough pointed spores

NF -1 GB LP DS

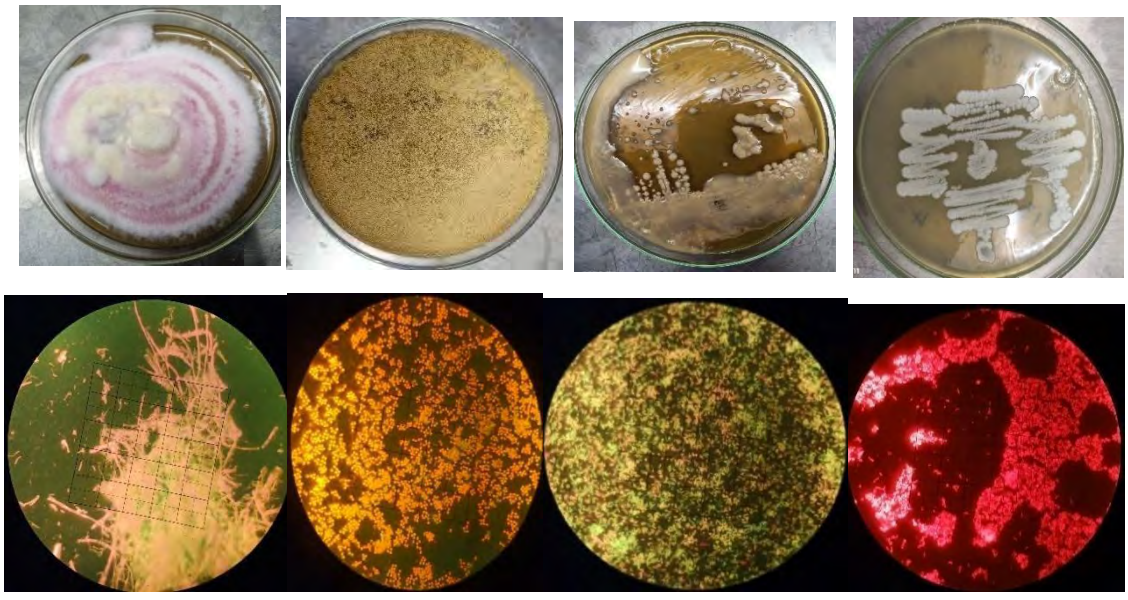


Fig 4.3 Potential Coal Degrading Fungal strains with their Acridine Orange microscopy

4.5 Phylogenetic analysis of the potential fungal isolates

For determining the relation among native coal solubilizing fungal strains NF-1 and GB phylogenetic tree was constructed. This phylogenetic relationship was established by using MEGA 5.0 software. Position of NF-1 and GB fungal isolate in phylogenetic trees was determined by aligning ITS gene sequences of seven fungal isolates by neighbour-joining tree shown in figure 4.4and 4.5. The constructed phylogenetic tree demonstrates that NF-1 fungal isolate made a clad with ***Debaromyeces hansenii*** and GB fungal isolate made a clad with ***Aspergillus ochraceus*** that have effective enzyme system for lignin degradation. With the help of this tree, we can conclude that NF-1 and GB fungal isolate may be useful for coal transformation and liberation of novel products.

Figure 4.4 Phylogenetic tree of *Aspergillus ochraceus*

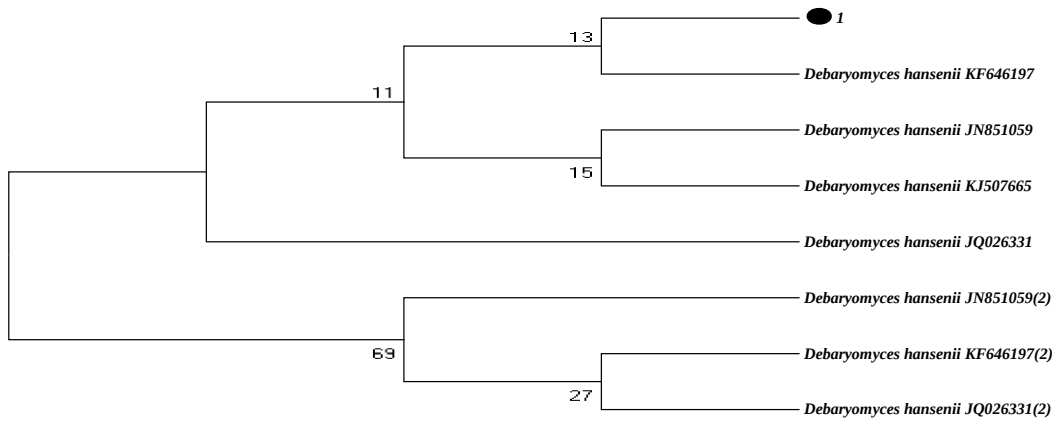


Figure 4.5 Phylogenetic tree of *Deberomyeces hansenii*

4.6 NF-1 and GB coal bio solubilization

For estimating the coal biosolubilization extent of native NF-1 and GB fungal strains, freshly prepared malt extract agar medium was point inoculated with fungal mycelia by using sterile loop and placed in fungal incubator for about 5 days at 30 ˚C. After completion of

incubation period 0.5g of autoclaved coal particles were scattered over dense growth of NF-1 and GB fungal mycelium hyphae and again subjected for 5 days incubation period. To check the activity of NF-1 and GB on coal particles, two references were used, mentioned below:

1. MEM plates having coal but without NF-1 and GB inoculation

2. MEM plates having NF-1 and GB inoculation but without coal

The highest coal biosolubilization extent of these native fungal isolates were determined on the basis of black liquid formation on the surface of fungal mats. Almost 3ml of black liquid was collected from 5 plates from NF-1 and 4ml of black liquid was collected from 5 plates of GB isolate using sterile syringe after 5 days of incubation time. A;The fungal isolates NF-1 and GB after 5 days on MEA plates, B: Autoclaved coal in MEA medium without inoculum as the control. C: coal solubilization by NF-1 and GB on MEA agar plates are shown in the figure below.

4.7 Optimization of NF-1 and GB for enhanced coal bio solubilization activity:

The optimized conditions for maximum activity of NF-1 and GB are necessary for the enhanced release of organic moieties from coal particles. The enhanced solubilization of coal by NF- and GB was dependent on some parameters that were optimized. The major parameters include was glucose concentration, incubation time and coal loading ratio, and apart from theses parameters the pretreatment incubation time have been reported as an effective key for release of organic moieties from coal particles during biosolubilization activity. The enhanced biosolubilization activity was calculated at 240nm. There are some points like pH, concentration and coal particles size that are directly involved to retard the process of coal solubilization. During pretreatment process of coal, the coal particles was finally grounded in to fine particle size of <250 to ≤300nm. The concentration and size of coal particles have been reported before for enhanced coal biosolubilization process. It was reported previously that biosolubilizatio n activity was significantly improved through nutrient amendments in MSM medium such as acetate and sulphates. MSM media was used for optimization and bio-solubilization studies. Two controls were:

1. MSM medium having coal but without NF-1 and GB inoculation
2. MSM medium having NF-1 and GB inoculation but without coal

During bio solubilization process the quantity of released organics through NF-1 and GB fungal pre-treatments were absorbed at 240nm in the range between 200-600nm under UVVisible spectral scan while no peaks were detectable in positive and negative controls.

4.7.1 Glucose effect

The coal fungal solubilization activity was enhanced by using optimized ratio of glucose supplement as a carbon source. Different glucose concentrations i.e. 0.1%, 0.5%, 1.0% and 1.5% (w/v) with 1.0% (w/v) coal loading ratio was supplemented for enhanced release of organics. The NF-1 and GB biological pretreatments were also performed without supplemental source of glucose. NF-1 had utilized 1% and GB had utilized 1.5% glucose concentration as an ideal ratio for maximum organics release by showing absorbance at 240nm as shown in figure 4.6 and 4.7. The glucose act as valuable source for maturation and enhancing activity of NF-1 and GB during coal solubilization. However, no peaks were detected in the absence of glucose in whole UV-Visible scan. It has been reported in various studies that glucose supplements are essential for enhancing the process of released organics from coal. Higher concentration of glucose i.e. 1.5% and 2% had also been reported. Moreover, various studies have been reported by increasing glucose ratio (0.5% (5g/L) that leads to encourage the growth as well as solubilization of higher coal ratio (10%). Different coal ranks depolymerize under variable concentration of glucose. Absence of glucose supplement in a media resulted in low solubilization of low rank of coal. However, the addition of glucose may encourage coal depolymerization process by increasing the growth and production of extracellular enzymes of fungal isolates.

It is also observed that the excessive concentration of glucose may leads to limit the oxygen content in medium that inhibit fungal growth and its enzyme activity, hence, resulted to low solubilization. It can be concluded that 1.0% (v/w) and 1.5% (v/w) glucose concentration was optimal for NF-1 and GB fungal isolates for enhanced solubilisation of lignite and bituminous coal.

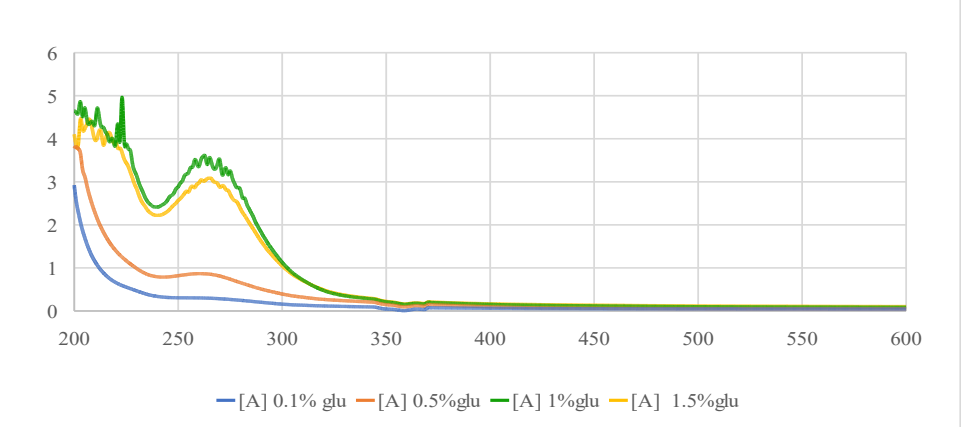


Figure 4.6 NF-1 glucose effect on release of organics using bituminous coal

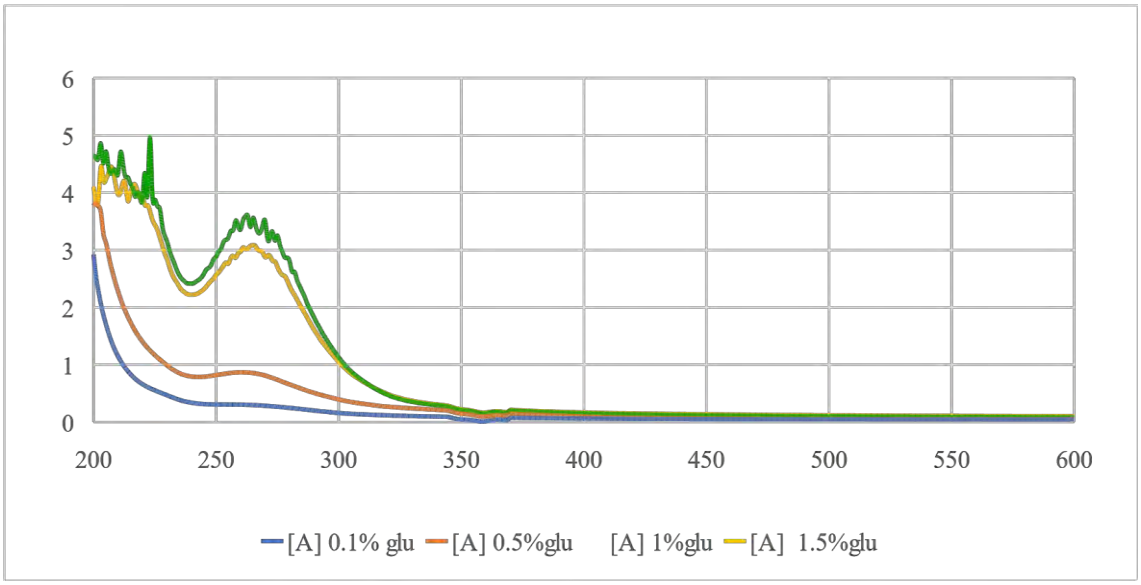


Figure 4.7 GB glucose effect on release of organics using bituminous coal

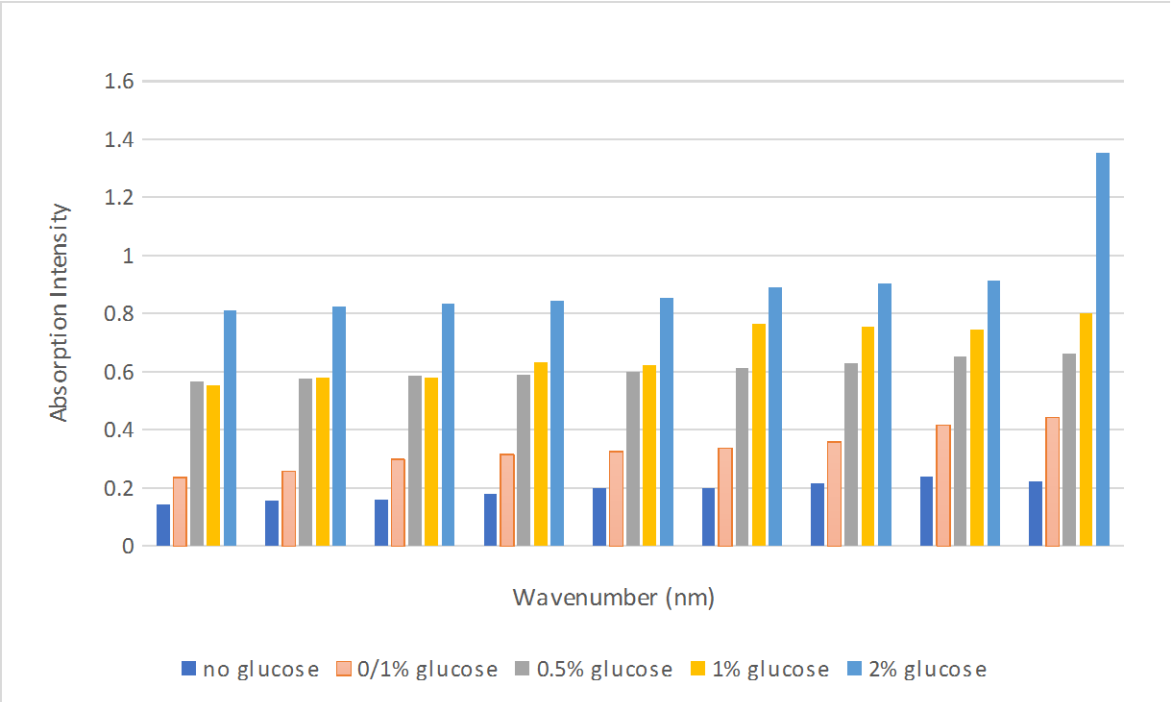


Fig 4.8 NF-1 Effect of Glucose concentration on release of organics using lignite coal

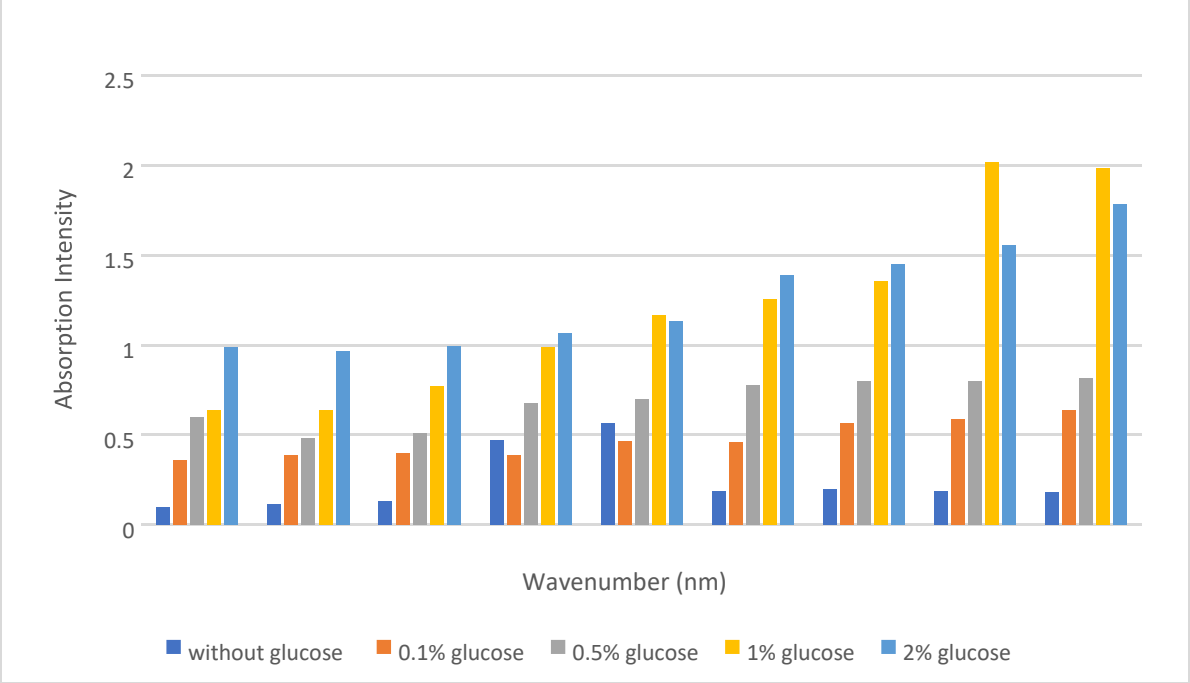


Fig 4.9 GB Effect of Glucose concentration on release of organics using lignite coal

4.7.2 Coal Loading Effect:

Size and concentration of coal particles used for solubilization has a remarkable effect on accelerating solubilization activity. Different percentages of lignite and bituminous coal 0.1%, 0.5%, 1.0% and 1.5% (w/v) were treated with NF-1 and GB fungal isolates. For enhanced release of organics moieties from coal matrix by both fungal isolates, 0.5% coal loading ratio was found optimum for bituminous coal (high rank coal), while 1.5% of coal loading ratio was found optimum my isolates NF-1 and GB in case of lignite coal as shown in

figure 4.10 and 4.11. However, the use of high percentage of coal during solubilization may suppress fungal growth and activity by lysing fungal mycelium.

During optimization studies for enhanced release of organic moieties from bituminous coal matrix by NF-1 and GB fungal isolates, 0.5% coal loading ratio for both isolates was found to be optimum, while in case of lignite coal 1.5% coal loading ratio was found optimum for enhance release of organics. However, usage of higher percentages of coal during solubilization process may suppress fungal growth and activity by lysing fungal mycelium. Humic acid was extracted from lower coal ranks by using 1.0% optimal coal value.

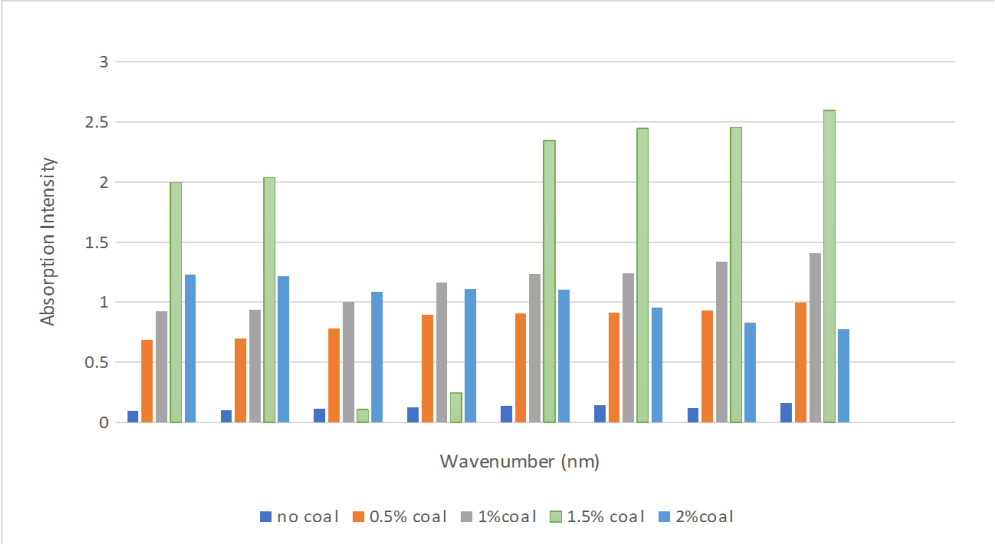


Fig 4.10 NF-1 coal loading Ratio effect on release of organics using lignite coal

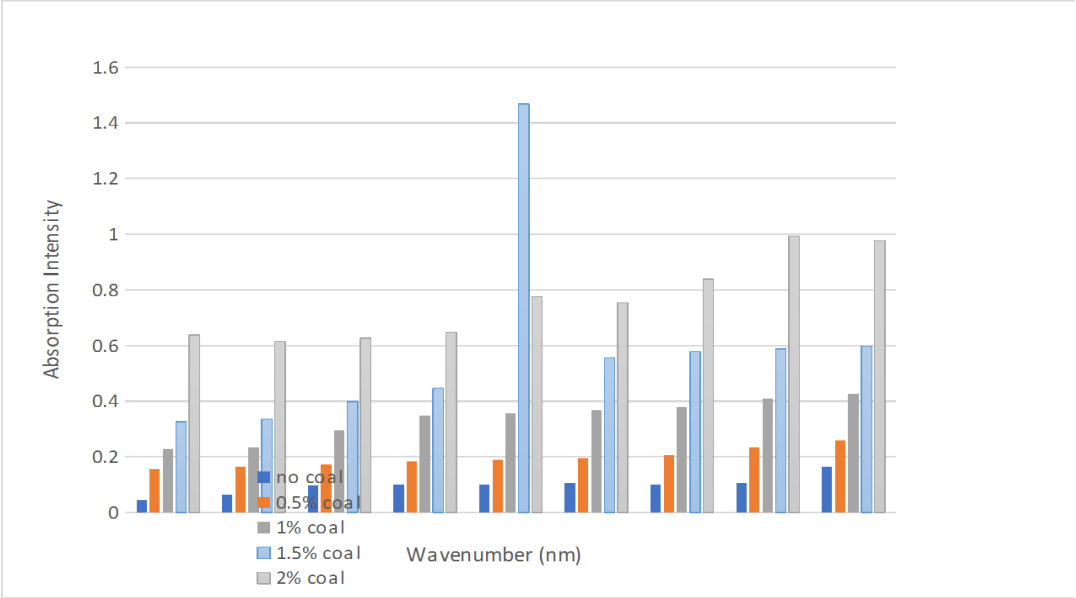


Fig 4.11 GB coal loading Ratio effect on release of organics using lignite coal

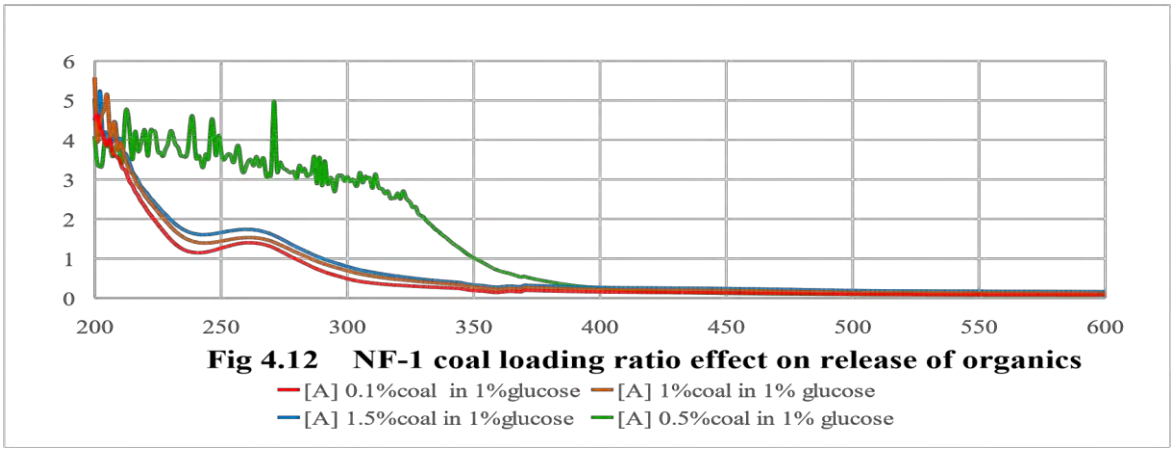


Fig 4.12 NF-1 coal loading ratio effect on release of organics

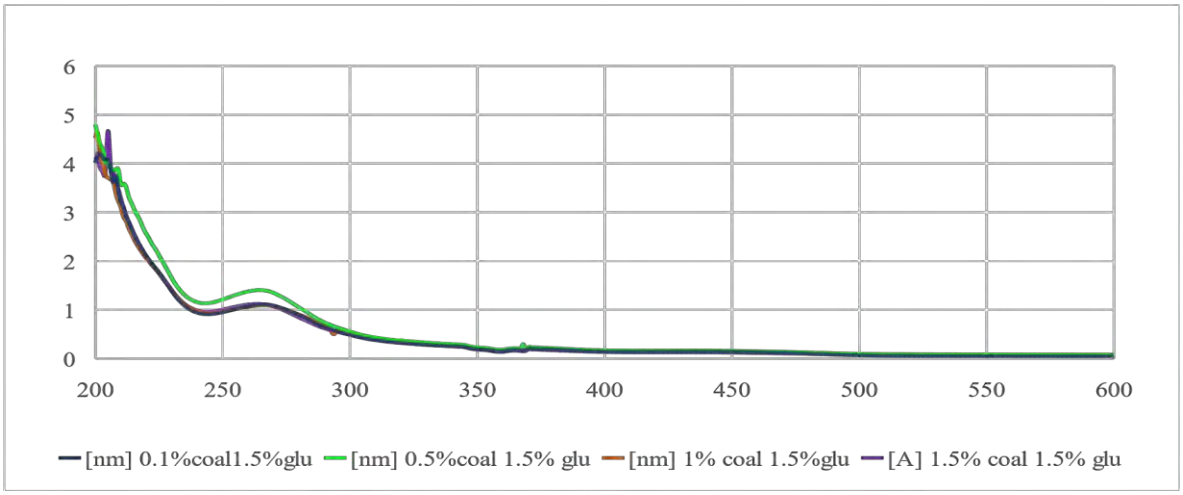


Fig 4.13 GB coal loading ratio effect on release of organics

4.7.3 Incubation Effect

Incubation time period is considered as an important factor for solubilization studies. For enhancing the release or organic moieties from coal matrix, the optimized ratio of bituminous and lignite coal (0.5% and 1.5%) and glucose (1.0% and 1.5%) was added in MSM liquid medium then allow to treat with NF-1 and GB fungal isolates for 7,12,17 and 21 days. NF-1 showed 7 days and GB showed 17 days using bituminous coal for maximum organic release while both isolates showed enhance release of organics at day 11 using lignite coal. Generally, stationary phase is involved efficiently in coal solubilization process. During this phase the fungal isolates releases extracellular enzymes that attacks the complex natural coal residues resulted in concentration of dense organic moieties in a medium. So, acceleration in incubation time period for maximum activity of enzymes and coal might be favourable for enhance release of organics. However, it is estimated that enzyme activity was limited in long incubation period of 14 days as absorbance reduces at 240-280nm.

The substantial increase in absorption intensity was observed in range of 240 nm. However, longer incubation periods, characterizes limited organics ratio that might be utilized by NF-1 and GB fungal isolates for their growth as a result lower their concentration in supernatant. Moreover, the intensity at 200-300 nm decreased continuously during 11,17 and 21 days of incubation with respect to 7 days incubation period, reported significant reduction of absorption intensity for longer incubations (9,15 and 19 days) at 240 nm range. Optimization of incubation period might be essential step during biological pre-treatments for enhanced and continuous release of organics from coal particles.

In our study NF-1 and GB showed 7 days using bituminous coal and 11 days using lignite coal for significant organic release as shown in figure 4.14 and 4.15. Stationary phase might be involved in coal solubilization process. During this phase fungal isolates releases extracellular enzymes that might attack on complex nature coal residues as a result concentration of dense organic moieties increases in medium. So, accelerating incubation period for maximum activity of enzymes and coal might be favourable for enhanced release of organics. However, it was also estimated that enzyme activity was limited during longer incubation periods (14 days) as absorbance reduces at 240-280nm. Previous studies have revealed that 7 days of incubation during fungal pretreatment were beneficial to amplify the extent of organic moieties. These moieties were continuously detected by UV-V spectrophotometer in range of 215-300 nm. Therefore, substantial increase in absorption intensity was also observed in range of 240 nm. Whereas longer incubation periods, represents limited ratio of organics that might be utilized by fungal isolates itself for their growth as a result lower their concentrations in supernatant. Moreover, the intensity at 200300 nm continuously decreased during 11,17 and 21 days of incubation with respect to 7 days incubation period.

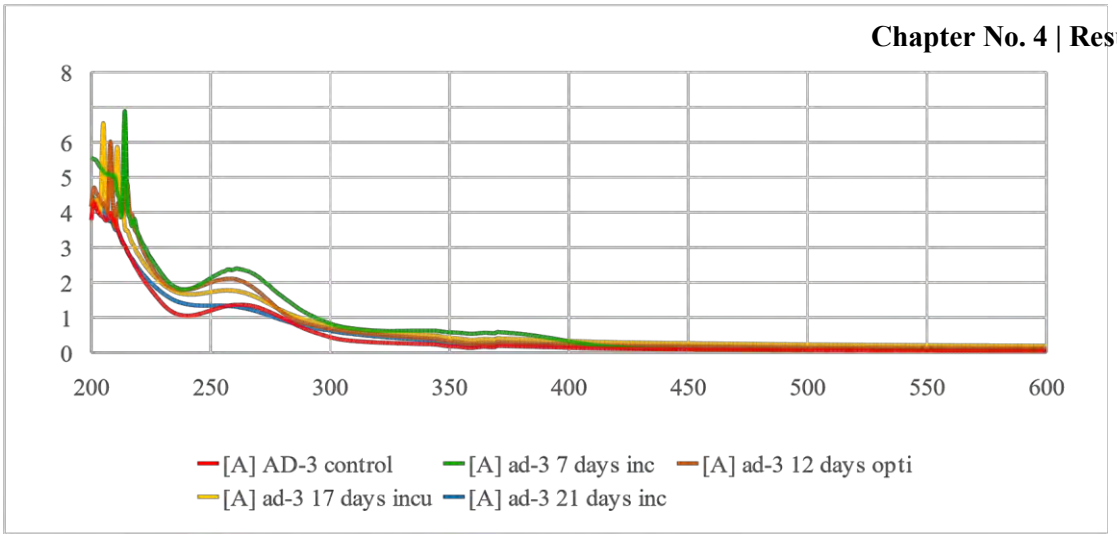


Fig 4.14 NF-1 Incubation effect on release of organics

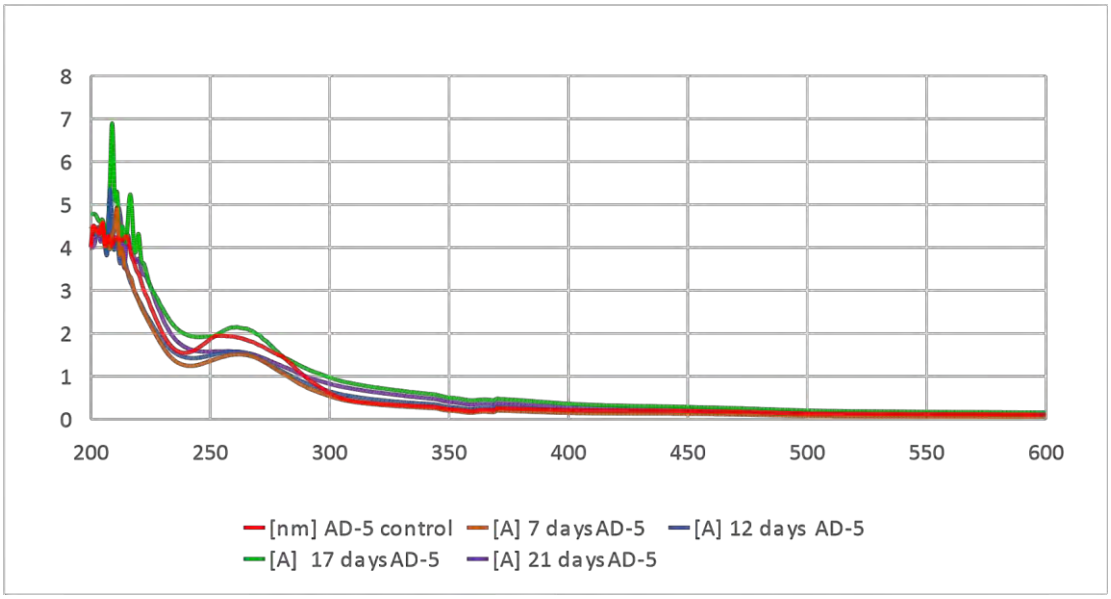


Fig 4.15 GB Incubation effect on release of organics

4.8 Stimulators for enhancing coal solubilization:

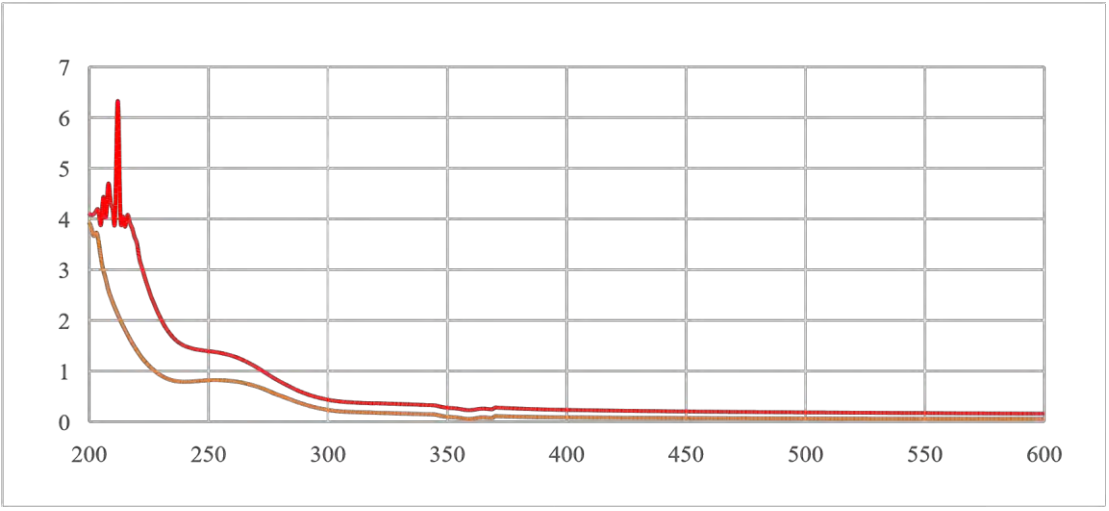
4.8.1 Chemical pretreatments

Refractoriness in bituminous coal, act as a major resistance in fuel sciences. Signature features of bituminous coal (Baluchistan coal) enforced its effective transformation into nonfuel. Generally, bituminous coal was less susceptible for biological attack due to its highly condensed polyaromatic components and complex chemical linkages. Pre-oxidation of bituminous coal by using strong oxidizing agent (NaOH) before biological reaction demonstrated that there is a great potential to produce coal derived organics moieties. Pre-oxidation of bituminous coal transform structure of coal by introducing oxygen and decreasing carbon content to make it vulnerable for biological attack. Biological entities efficiently utilized these partially distorted coal particles, since, NaOH has been used for

humic and fulvic acids production. Previous studies reported the efficiency of oxidative treatments for enhancing bio-solubilization time (10.5 days) and percentage (31.83%).

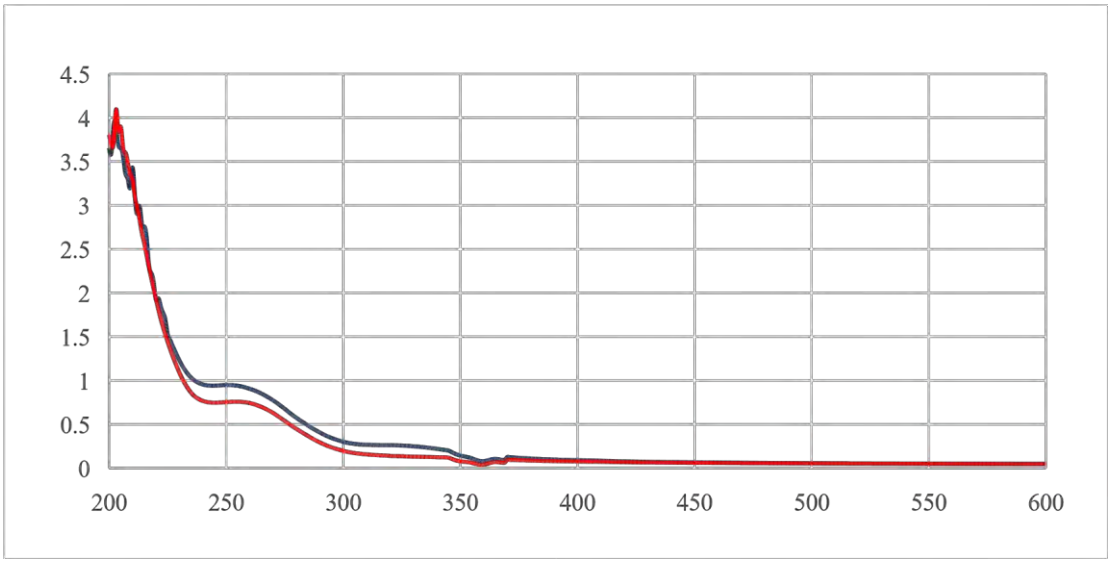
4.8.2 Sodium acetate modification

Nutrient supplements have been reported as stimulator to amplify microbial growth and organic moieties. After optimization of major conditions additionally 0.25g sodium acetate ($C_2H_3NaO_2$) was added as an enhancing agent in MSM media along with optimized glucose and coal conc. for both fungal isolates. The effect of acetate amendments for enhanced fungal strains activity was observed along glucose shown in figure 4.18 and 4.19 while it was observed that acetate as a carbon source has less activity shown in figure 4.16 and 4.17. Previous literature reveled that nutrient and acetate amendments significantly increase the number of microbial cells from (3.03 104 cells ml to 9.93 107). Moreover, concentration and size of coal particles (0.2-0.4 to 0.3-0.6) mm have remarkable effects on accelerating solubilization activity. It was reported that 0.2–0.5 mm was enough for enhanced organic release. Recent researches have shown that the surface area is also an important factor for enhanced bio-solubilization. Bio-solubilization/liquefaction percentage was enhanced four times by reducing size of particles (600–850 µm to 150–300 µm). Therefore, the chance for maximum bioavailability of coal particles was increased.



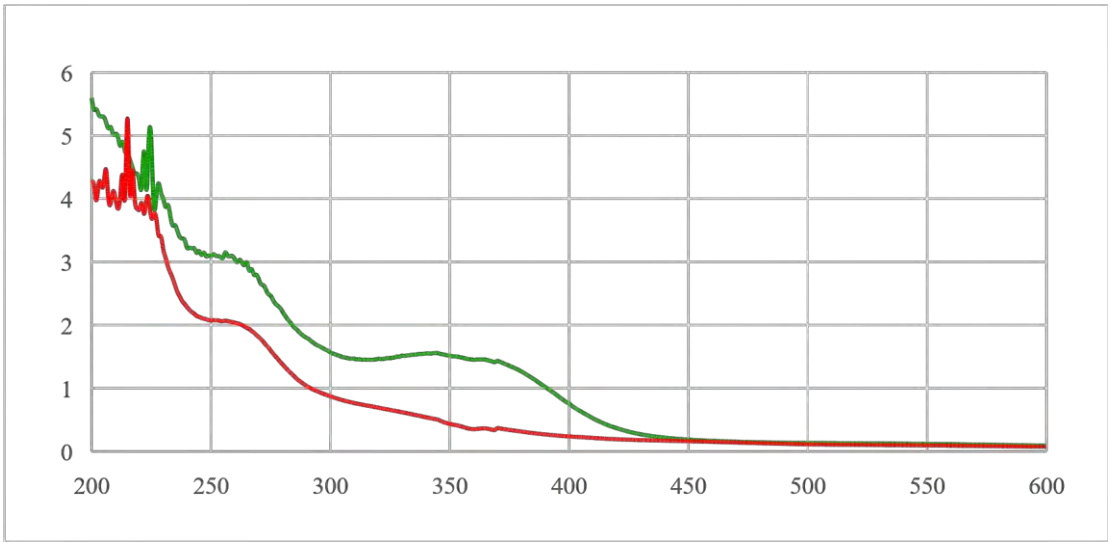
Red: NF-1 REF S.A Brown: NF-1 S.A 7 days

Fig 4.16 NF-1 acetate as a sole carbon source



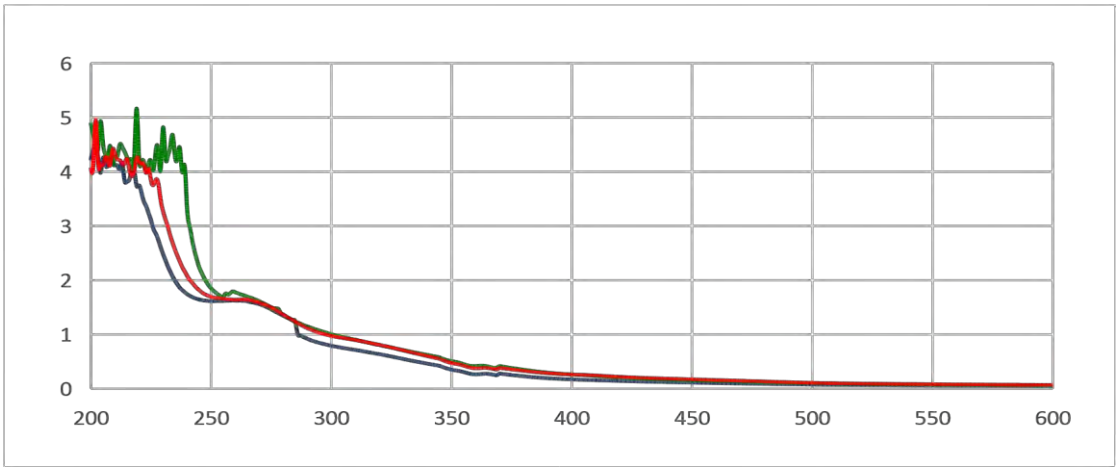
Red: GB Ref S.A, Blue: GB at day 7 (S.A)

Fig 4.17 GB acetate as a sole carbon source



Red: NF-1 Conc S.A and Glucose Green: NF-1 S.A and Glucose

Fig 4.18 NF-1 acetate as an enhancer along glucose



Red: GB conc. Acetate/Glu, Green: GB at day 7 Acetate/Glu, Blue: GB 17 days Acetate/ Glu

Fig 4.19 GB acetate as an enhancer along glucose

4.9 Reduction in coal particle size as a stimulator for lignite coal solubilization

For enhancing the coal solubilization potential of lignite coal by fungal isolates NF-1 and GB the strategy followed were the reduction in coal particle size. As lignite coal is ranked as low rank coal with very simple structure of aliphatic ring and side chains so no chemical pretreatment was applied. Being less complex as compared to bituminous (high Rank coal), the lignite coal was allowed to solubilized by fungal isolates directly. However, reduction in particle size were done. The particle size 0.1mm and 0.3mm were used. Then, the solubilization percentage from both the particle size of lignite coal was calculated. For running the solubilization experiment the optimized conditions were used. Both the isolated fungal strains were allowed to grow in solid medium, with 2% glucose concentration for NF1 and 1.5% glucose concentration for GB. Both the strains were allowed to incubate at 30°C for 11 days and the formation of black liquid on the plates were collected and their solubilization percentage were calculated as shown in table 4.6 below.

The isolate NF-1 had shown maximum solubilization percentage 32% with 0.1mm coal size particles as compared to 0.3mm coal size particles whose biosolubilization extent were just 20% while isolate GB had shown 35% solubilization extent with lignite coal at day 11 by utilizing 0.1mm coal particles as compared to particle size 0.3-0.5mm, which produces about 31% solubilized black liquid. Hence, the particle size 0.1mm was better than 0.3mm in enhancing the process of coal solubilization of lignite coal. It was reported earlier that lesser the size or coal particles more will be the surface area that will be easily available for the fungal isolates to uptake and release extracellular enzymes for carrying out the process of coal solubilization.

Table 4.6 Fungal solubilization Extent of lignite coal at day 11

Test	NF-1 isolate		GB Isolate	
	Ground coal (0.mm)	Coal Particles (0.3-0.5mm)	Ground coal (0.1mm)	Coal Particles (0.3-0.5mm)
Initial Mass (g)	1.03	1.01	1.04	1.02
Final Mass (g)	0.71	0.81	0.68	0.71
Soluilization(%)	32	20	35	31

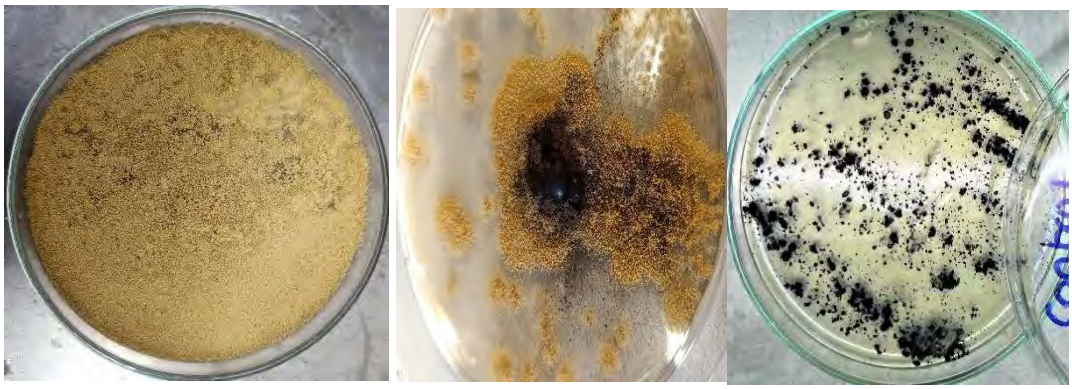


Fig 4.20 Biosolubilization extent of isolate GB (plate assay method)

Fig 4.21 Biosolubilization extent of isolate NF-1 (Plate assay method)

4.10 Percentage estimation of bio solubilization process

The enhanced bio solubilization extent of virgin and pre-oxidized coal particles was investigated by standard method. Particles of virgin and pre oxidized (NaOH) coal was measured before and after scattering on MEA plates of NF-1 and GB fungal isolates for both bituminous and lignite coal. NF-1 and GB fungal isolates showed solubilization time of

virgin coal residues 5 days and percentages were 31.8% and 45.6% respectively. NF-1 and GB fungal isolates showed solubilization time of pre oxidized coal residues 3 and 2 days and percentages were 52.4% and 71% respectively shown in table 4.7.

While using lignite coal the maximum solubilization percentage obtained were 22.46% by NF-1 isolate and 16.66 percent with particle size of 0.5mm-0.7mm. While other isolate GB had shown maximum solubilization percentage of 38.8% with particle size of 0.1mm and 28.12% with particle chunks of 05-0.7mm respectively.

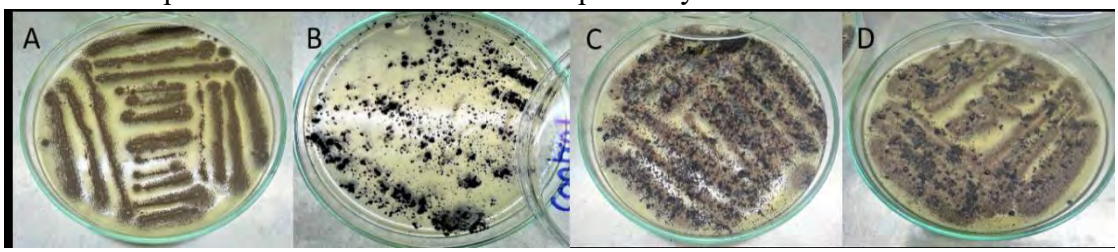


Figure 4.22 Bio-solubilization experiment on MEA plate. A: GB positive control after 4 days incubation B: virgin bituminous coal on MEA media negative control C: fungus and virgin coal D: GB fungal solubilization of coal after 5 days incubation. (Conditions, 30 ºC, 5 days incubation).

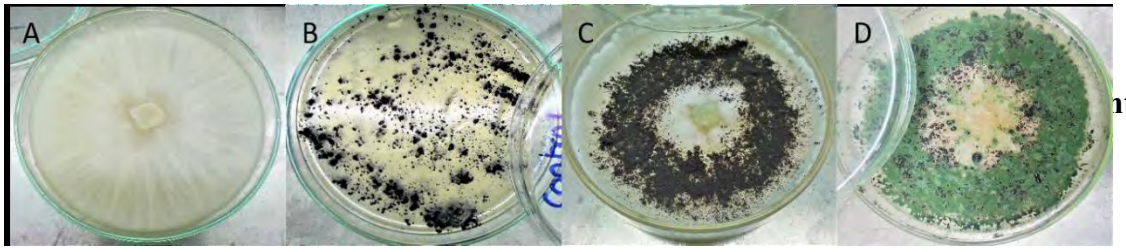


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Figure 4.23 Bio-solubilization experiment on MEA plate. A: NF-1 positive control after 4 days incubation B: virgin bituminous coal on MEA media negative control C: fungus and virgin coal D: NF-1 fungal solubilization of coal after 5 days incubation. (Conditions, 30 ºC, 5 days incubation)

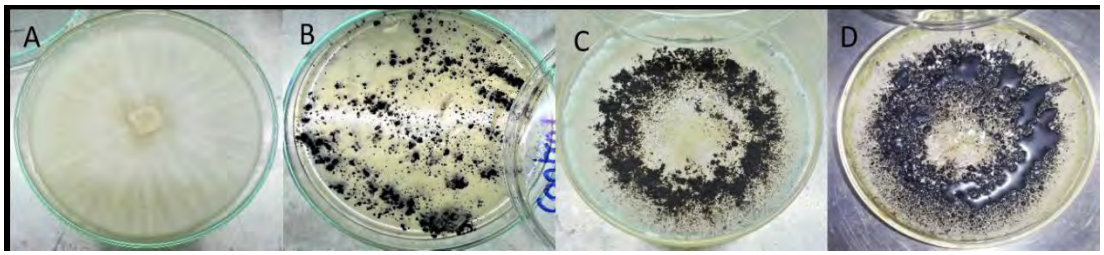


Figure 4.24 Bio-solubilization experiment on MEA plate. A: NF-1 positive control after 4 days incubation; B: pre oxidized bituminous coal on MEA media negative control C: fungus and virgin coal D: NF-1 fungal solubilization of coal after 5 days incubation.

(Conditions, 30 ºC, 5 days incubation).

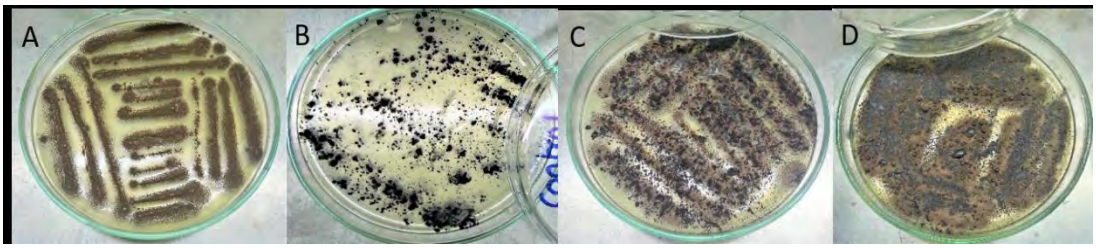


Figure 4.25 Bio-solubilization experiment on MEA plate. A: GB positive control after 4 days incubation; B: pre oxidized bituminous coal on MEA media negative control C: fungus and virgin coal D:GB fungal solubilization of coal after 5 days incubation. (Conditions, 30 ºC, 5 days incubation

Table 4.7 Biosolubilization extent of NF-1 and GB using Raw Bituminous Coal

Initial mass of raw coal sample (g)	**0.5**	**0.5**
Final mass of raw coal sample (g)	**0.341**	**0.272**
Solubilization time (days)	**5**	**5**
Solubilization percentage (%)	**31.8**	**45.6**

Table 4.8 Bio solubilization % from pretreated Bituminous coal

Initial mass of pretreated coal sample (g)	1.0 g	1.0 g
Final mass of pretreated coal sample (g)	0.658	0.719
Solubilization time (days)	5	5
Solubilization percentage (%)	52.4	71

4.11 Comparative analysis of virgin and pre oxidized coal in liquid culture setup

The intensive release of organics from virgin and pre-oxidized coal particles by the action of NF-1 and GB fungal isolates were investigated through liquid culture set up.

4.12 Optimized parameters for NF-1 solubilization experiment

Enhanced yield of coal derived organic compounds was obtained by running NF-1 fungal isolates in shake flask experiment. MSM medium was prepared by adding 0.5g of sodium acetate and optimized glucose concentration (1.0%), for NF-1 pH (5.5) was balanced

and poured into 100ml flasks. The optimized coal concentration of bituminous virgin and pre oxidized coal (0.5%) for NF-1 fungal isolate was measured, dried, grounded, sieved and incubated for 7 days. The experiment was run in triplicates for both virgin and pre oxidized coal along one positive and two negative controls shown in figure 4.26.

The UV-Visible spectral scan for controls was taken in the range of 200-600nm. The extent of organics in both negative and positive controls were not observed in UV-Visible scan. Whereas some peaks were noted in positive control by the action of GB fungal strain. The UV-Vis spectrophotometer analysis in case of untreated coal showed that the strength of peaks pattern in region of 200-300 nm was not stronger but conjugative effect of GB and pre oxidized coal was effective in liberating aromatics and humic materials in region of 220-350 nm. The enhanced bisolubilization activity was determined at 240nm. The untreated bituminous coal contains the complex and larger aromatic rings. After coal fungal pretreatment breakage took place at some linkages as a result of which few aromatic fractions were released in liquid culture medium. Peroxidation of raw bituminous coal results into alteration of coal structure by introduction of oxygen content and decrease in carbon content to make it susceptible for biological attack. The biological entities efficiently trapped partially distorted coal particles in submerged condition as shown in figure 4.25. The biosolubilization percentage was calculated by gravimetric analysis. Therefore, it was difficult to remove coal particles from fungal round shaped balls in order to determine the weight loss after NF-1 biosolubilization of bituminous (raw and oxygenated) coal.

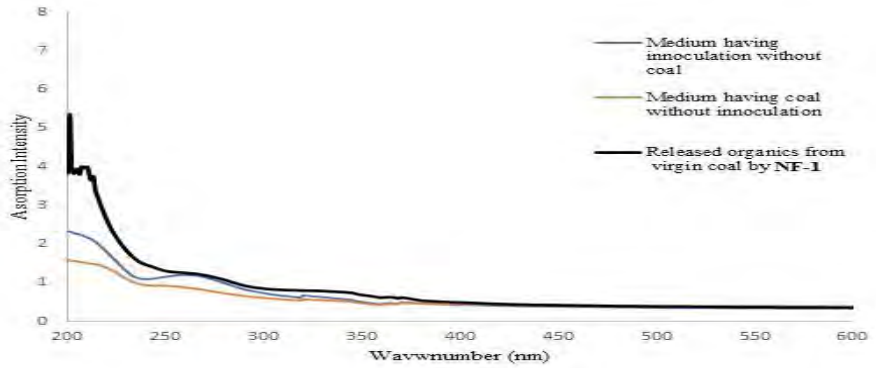


Fig 4.26 NF-1 enhanced release of organics from virgin coal

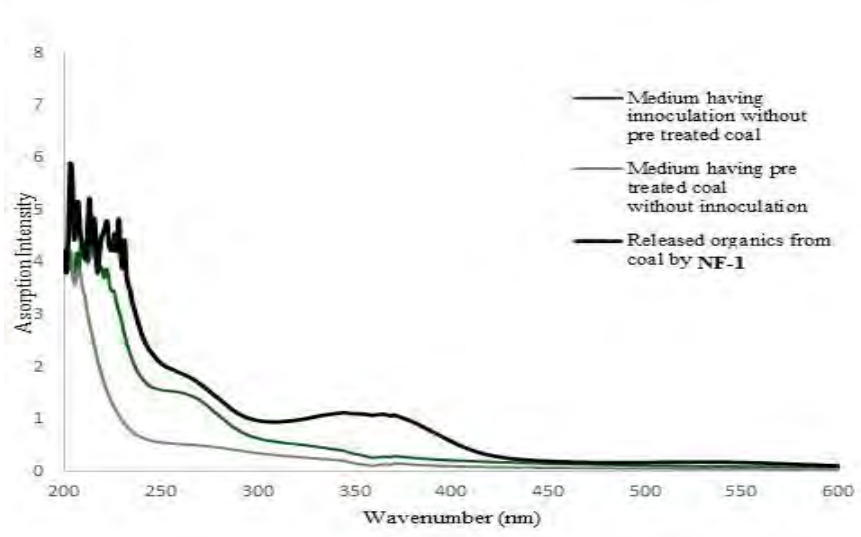


Fig 4.27 NF-1 enhanced release of organics from pre oxidized coal

4.13 Optimized parameters for GB solubilization experiment

The enhance release of coal derived organic moieties was obtained by the action of GB fungal isolates in shake flask experiment. MSM media was prepared by adding 0.5g of sodium aetate and optimized glucose concentration of (1.5%) for GB ,pH (5) was balanced and poured into 250ml flask. The optimized ratio of raw and pre oxidized coal ().5%) for GB fungal isolate was measured which is dried, grounded and sieved and incubated for 7 days. This experiment was run in triplicates for both virgin and pre oxidized coal along one positive and two negative controls. shown in figure 4.28.

The UV-Vis spectral scan for controls was taken in the range of 200-600 nm. The extent of organics in both negative controls were not observed in UV-Vis scan whereas some peaks were observed in positive control by the treatment with GB fungal isolate. UV-Vis spectrophotometer analysis in case of raw coal showed that the strength of peaks in region of 200-300 nm was not stronger but conjugative effect of GB and pre oxidized coal was effective in releasing aromatics and humic materials in region of 220-350 nm. shown in figure 4.29. The enhanced bio solubilization activity was determined at 240 nm. The raw bituminous coal contains complex connectivity as well as larger aromatic rings. After coal-fungal treatment breakage occurred at some linkages as a result fewer aromatic fraction was released in liquid culture system. Pre oxidation of virgin bituminous coal resulted into alteration of coal by introducing oxygen and decreasing carbon content to make it vulnerable for biological attack. Biological entities efficiently trapped these partially distorted coal particles in submerged condition. Bio solubilization percentage was investigated by gravimetric analysis. Therefore, it was difficult to remove coal particles from fungal round shaped balls in order to determine the weight loss after GB bio-solubilization of bituminous (virgin and

oxygenated) coal. Extensive research on solubilization of coal by biological entities have been reported

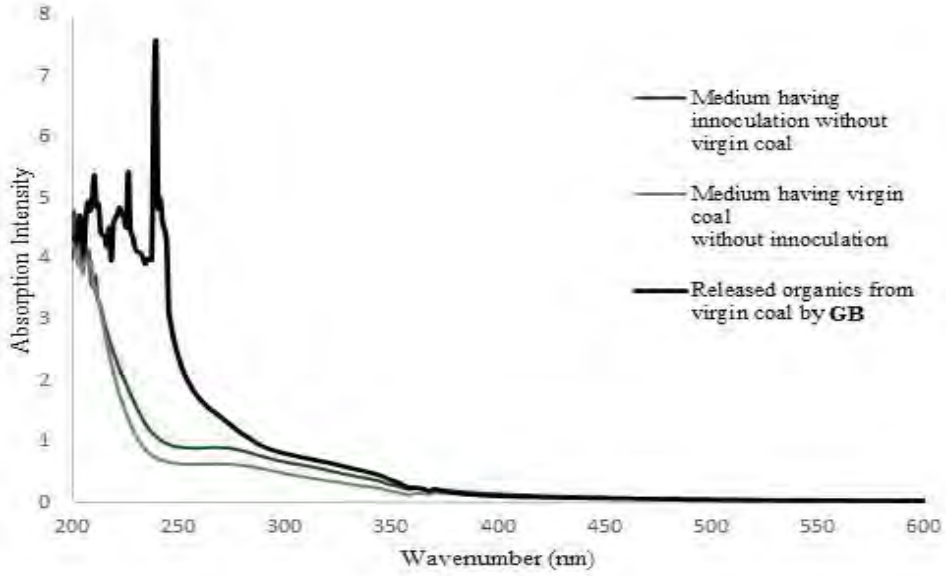


Fig 4.28 GB enhanced release of organics from raw coal

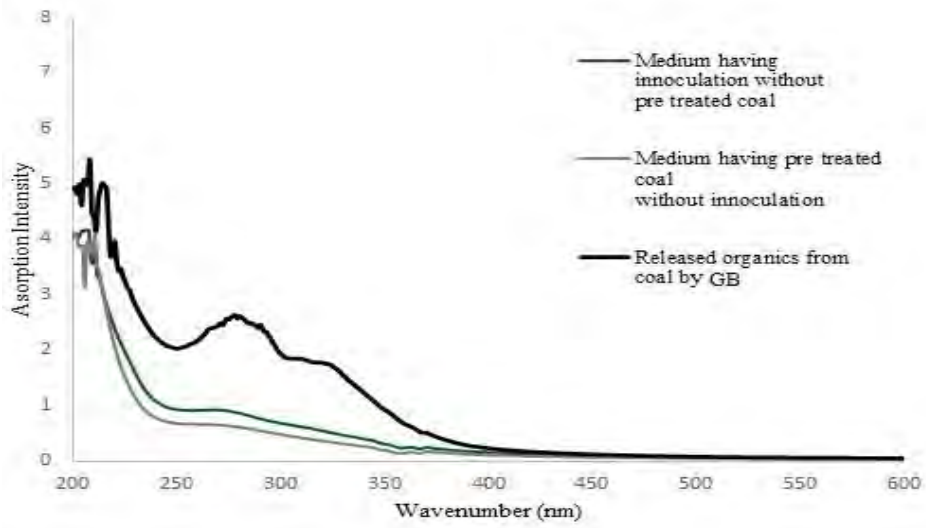


Fig 4.29 GB enhanced release of organics from pre-treated coal

Bio solubilized liquids obtained from virgin and pre oxidized bituminous coal after NF-1 and GB fungal treatment analysed by using UV-Vis spectrophotometer. In case of raw coal it were shown that the strength of peaks in region of 200-330 nm was not stronger but

conjugative effect of NF-1 and GB fungal isolates on pre oxidized coal was effective in liberating aromatics and humic materials in region of 220-400 nm..

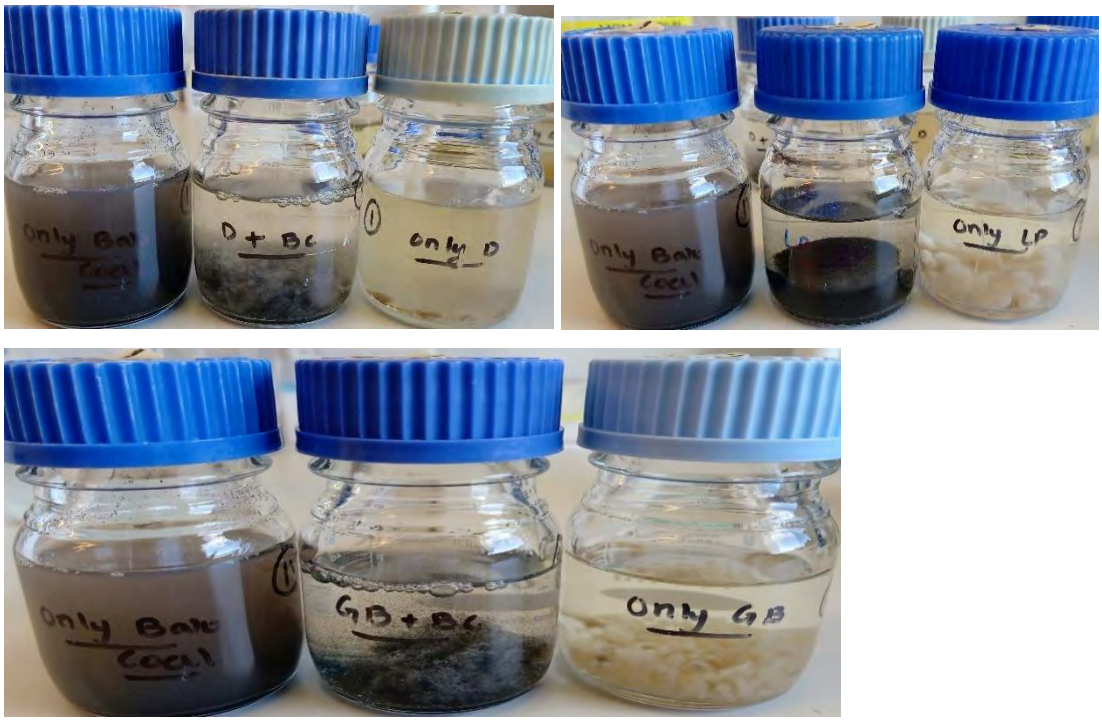


Fig 4.30 Coal Biosolubilization process by potential fungal isolates in Liquid medium using High rank Bituminous Coal

Fig 4.31 NF-1 and GB liquid medium coal solubilization process (Entrapment of coal Particles in fungal mycelium using Lignite coal

4.14 Elemental analysis of bio solubilized (virgin and pre oxidized) coal residues

Table shown below shows the effect of fungal isolates (NF-1 and GB) treatment on elemental composition such as (C,N,O,S and H) or raw and NaOH treated preoxidized coal residues of bituminous coal. The biological treatment has shown clear transformation of each elemental percentage. Pre oxidation of bituminous coal with NaOH used as an enhancer resulted to lower carbon/hydrogen content. The percentage of hydrogen and carbon was lower in coal residues treated with fungal isolates with respect to raw and pre oxidize bituminous coal

residues. The oxygen and nitrogen percentage were double as compare to raw and pre oxidize bituminous coal residues through fungal activity that might be involved in the enhanced humic released both from raw and pre oxidize bituminous coal residues. Components of sulphur has limited the use of bituminous coal but biological treatment (NF-1, GB) lower Sulphur content both in raw and pre oxidized coal.

Table 4.9 Elemental analysis of raw bituminous coal and residues from its bio solubilization using NF-1 and GB fungal isolates

Sample ID	Elemental Analysis Elements (%) C	H	N	O	S	Atomic ratios H/C	O/C	N/O
Coal Bituminous	55.1	4.99	1.07	17.69	9.15	1.08	0.24	0.06
Residues (NF-1)	49.10	3.80	2.45	24.37	7.99	0.92	0.37	0.11
Residues (GB)	42.08	3.50	2.99	29.84	6.43	0.99	0.53	0.11

Table 4.10 Elemental analysis of pre oxidize bituminous coal and residues from its bio solubilization using NF-1 and GB fungal isolates

Sample ID	Elemental Analysis Elements (%) C	H	N	O	S	Atomic ratios H/C	O/C	N/O
Pre oxidized coal (Bituminous)	51.5	4.07	1.86	21.87	9.01	0.94	0.31	0.09
Residues (NF-1)	45.60	3.41	3.18	26.56	6.32	0.89	0.43	0.13
Residues (GB)	37.03	3.01	3.98	33.68	5.78	0.97	0.68	0.13

The O/C, H/C and C/N ratios of solubilized coal residues were enhanced through biological pathways. Biological processes showed significant differences in elemental composition of solubilized coal. Treating virgin bituminous coal particles with NF-1 and GB fungal isolates showed low sulfur, carbon, and hydrogen content but high oxygen and nitrogen ratio. Treating pre oxidize (introduce oxygen and decrease carbon value) bituminous coal particles with NF-1 and GB fungal isolates showed greater reduction in sulfur, carbon and hydrogen ratio and higher content of oxygen and nitrogen. The reduction in carbon might be due to C–C, C–O–C and C–O bond breakage, higher content of oxygen could represent oxidize functional groups.

4.15 FT-IR analysis of virgin coal by NF-1

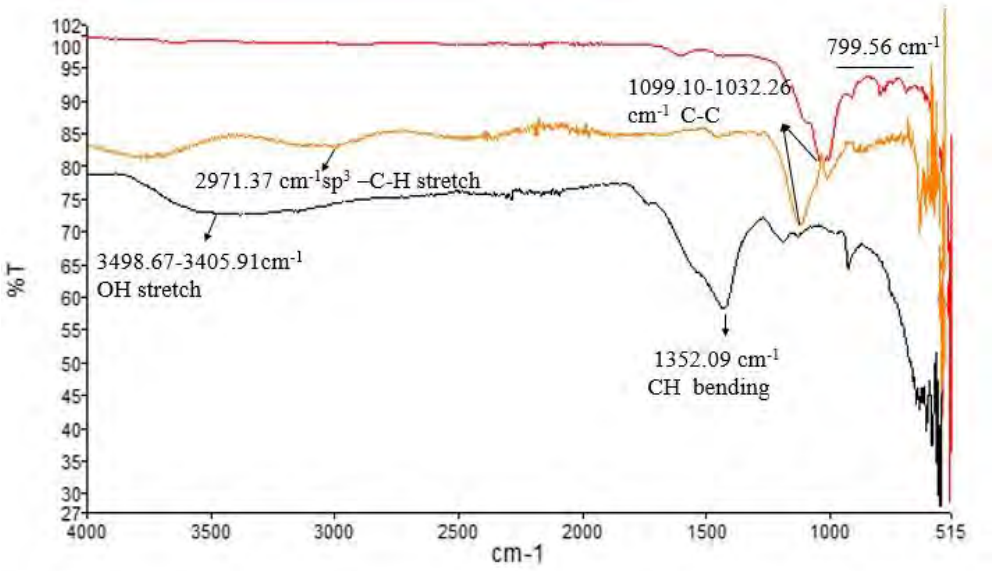


Fig 4.32 IR spectra of unmodified coal, bio solubilized liquid and coal residues. Red: reference coal; orange: NF-1 solubilized liquid; Black:NF-1 solubilized coal

FTIR analysis was carried out for detection of functional groups and vibrational changes occur in untreated coal (control sample). NF-1 fungal treated bio solubilized coal residue (modified with humic acid) and liquid in the range of 4000-400cm on Perkin Elmer, USA with (15 scan and cm^{-1}resolution rate) shown in figure 4.32. The characteristic broad peaks observed in the residual spectra at 3498.67-3405.91 cm^{-1} corresponds to -OH stretch respectively due to the presence of humic acid functionalities in residual coal which are absent in unmodified coal (control sample). The CH bending shows peak at 1352.09 cm^{-1} in residual coal to the presence of aldehydes. The sp –C-H stretch at 2971.37 cm^{-1} in solubilized liquid due to presence of aliphatic compounds. The peaks at 1099.10- 1032.26cm^{-1} corresponds to ether bonds which is present in control and coal solubilized liquid product but absent in residual coal. In addition, the remaining peaks that are observed in all spectra (control and residual coal and supernatant) in the region of 900-600cm^{-1}show the presence of inorganic moieties in coal sample. In the range of 1000–500 cm^{-1} absorbance of side chain aromatic was greater in unmodified coal control and bio solubilized liquid. This shows that NF-1 fungal isolate might be involved in the aromatic cleavage and release from bituminous coal into bio solubilized liquid. It is also documented aromatic liberation into liquid products from pre oxidized coal by the action of wood-decaying fungal isolate. These all above discussed peaks gives confirmation of formation of coal based humic compound by the action of NF-1 fungal isolate.

4.16.1 FT-IR analysis of pre oxidize coal by NF-1

FT-IR analysis was carried out for the investigation or detection of functional groups and vibrational changes occurs in pre oxidized coal (control sample), NF-1 fungal treated bio solubilized coal residue and liquid in the range of 4000-400cm^{-1} on Perkin Elmer, USA with (15 scan and 1cm resolution rate) shown in figure 4.33. The bands indicated due to at hydrocarbon species (2926 cm^{-1}), aromatic rings (1634 cm^{-1}), etheric bonding (1158 cm^{-1}, 1078 cm^{-1}), hydrogen in benzene rings (948 cm^{-1}, 859 cm^{-1}) and minerals (542 cm, 469$cm^{-1.}$ The characteristic peaks observed in the residual spectra at 2923.18cm^{--1}corresponds to sp_3 –C-H due to the presence of humic acid functionalities in pre oxidized residual coal which are absent in pre oxidized coal (control sample). The C=C, COO and C=O bond shows peak at 1635.34cm^{-1} and 1601.93 cm^{-1} in pre oxidized residual coal and bio solubilized liquid to the presence of carboxylic and aromatic rings present in compound. The C-H bending at 1436.07cm^{-1} in residues is also observed. The peak at1032.210cm^{-1} and 1034.92 cm^{-1} corresponds to ether bonds which is present in control and pre oxidized residual coal but absent in coal solubilized liquid product. The characteristic peaks observed in the coal solubilized liquid product at 1152.82 cm^{-1} correspond to C-O-C. In addition, the remaining peaks that are observed in all spectra (control and residual coal and supernatant) in the region of 900-600cm^{-1} show the presence of inorganic moieties in coal sample. Pre oxidation of raw

coal enhances humic production by increasing content of oxygen and nitrogen containing functional groups as well as the content of OH and N-H groups both in pre oxidized residual coal and liquid by the action of NF-1 fungal isolate. These all above discussed peaks gives confirmation of formation of coal based humic compound by the action of NF-1 fungal isolate.

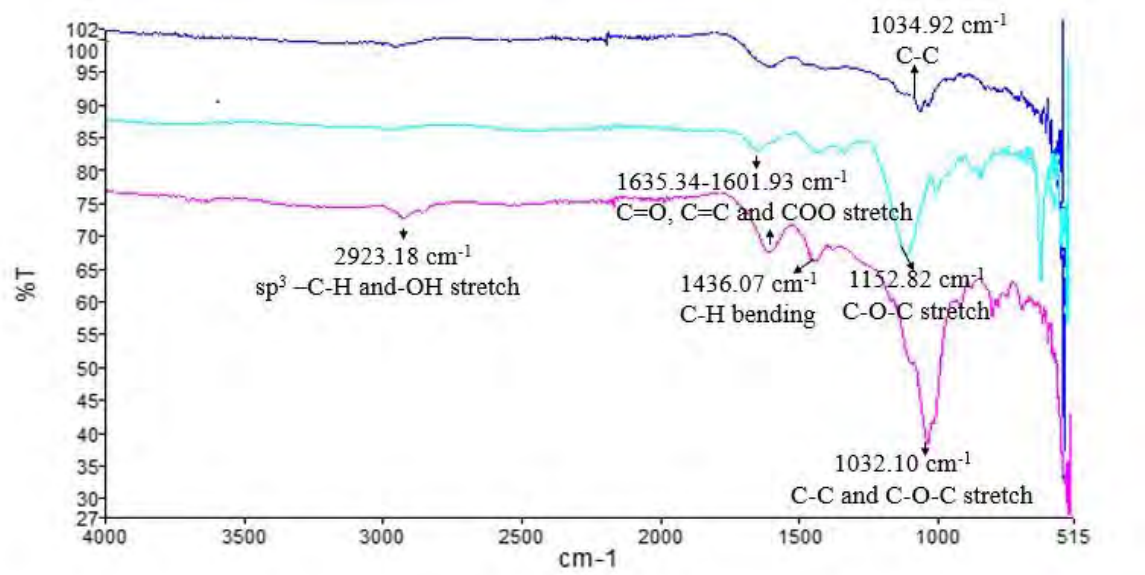


Fig 4.33 IR spectra of modified coal, bio solubilized liquid and pre oxidized coal residues. Blue: reference coal; light blue: NF-1 solubilized liquid; pink:NF-1 solubilized coal

4.17 FT-IR analysis of virgin coal by GB:

FT-IR analysis was carried out for detecting functional groups and vibrational change occurs in untreated coal (control sample), GB fungal treated bio solubilized coal residue (modified with humic acid) and liquid in the range of 4000-400cm^{-1} on Perkin Elmer, USA with (15 scan an 1cm resolution rate) by isolate shown in figure 4.34. The minimal characteristic peaks observed in the residual spectra at 2923.31 cm^{-1}, corresponds to sp –C-H stretch due to the presence of humi acid functionalities in residual coal which are absent untreated coal (control sample). The C=C bond shows peak at 1693.07cm^{-1}in residual coal to the presence of aromatic double bonds present in compound. The peak appears at 1496.07cm^{-1} corresponds to –C-H bending vibration which is absent in control sample. The peak observed in the region of 1300-1100 cm^{-1} due to ether and C -O stretch. In addition, the remaining peaks that are observed in both spectra (control and residual coal) in the region of 900-600cm^{-1} show the presence of inorganic moieties in coal sample. In the

range of 1000–500 cm^{-1} absorbance of side chain aromatic was greater in untreated coal control and bio solubilized liquid. This shows that GB fungal isolate might be involved in the aromatic cleavage and release from bituminous coal into bio solubilized liquid. It was also documented that aromatic liberation into liquid products from pre oxidized coal by the action of wood decaying fungal isolate. These all above discussed peaks gives confirmation of formation of coal based humic compound by the action of GB fungal isolate.

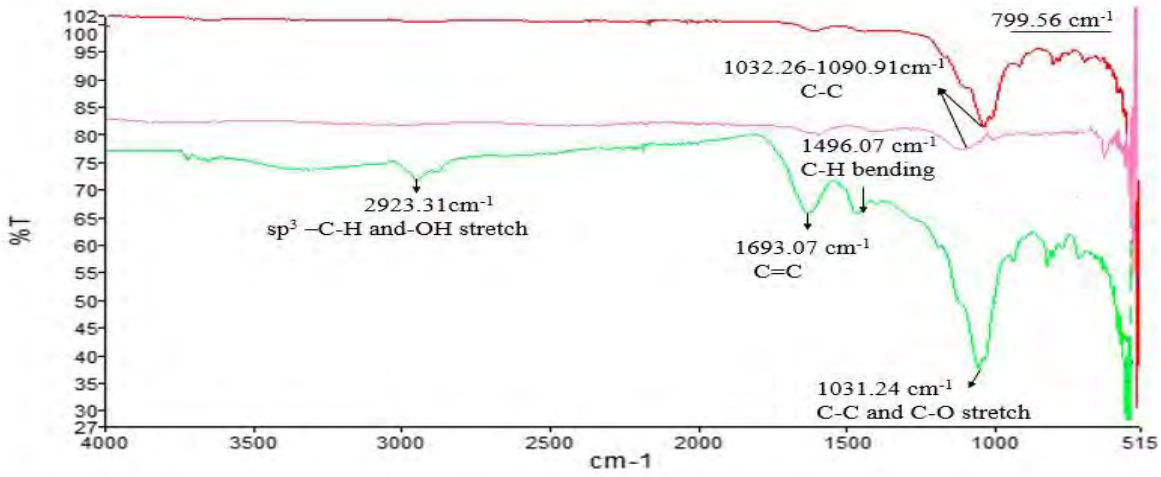


Fig 4.34 IR spectra of unmodified coal, bio solubilized liquid and coal residues

FT-IR analysis was carried out for the investigation or detection of functional groups and vibrational changes occurs in pre oxidized coal (control sample), GB fungal treated bio solubilized coal residue (modified with humic acid) and liquid in the range of 4000-400cm^{-1} on Perkin Elmer, USA with (15 scan and 1cm resolution rate) by GB fungal isolate shown in figure 4.35. The intensified peaks observed in the residual spectra at 3498.67 and 2923.1 cm^{-1} corresponds to sp_3 –C-H stretch and -OH stretch due to the presence of humic acid functionalities in pre oxidized residual coal which are absent in pre oxidized coal (control sample). The C=C, COO and C=O stretch shows peak at 1683.56 cm^{-1} and CH_3 and CH_2 bending at 1470.43 cm^{-1} in pre oxidized residual coal to the presence of methylene, alkane and methyl groups present in compound. The peak at1034.92 and 1032.39 cm^{-1} correspond to ether bonds which is present in control and pre oxidized residual coal but absent in coal solubilized liquid product. The peak at 1111.02 cm^{-1} corresponds to C-O-C stretching in coal solubilized liquid product. In addition, the remaining peaks that are observed in all spectra

-1

(control and residual coal and supernatant) in the region of 900-600cm show the presence of inorganic moieties in coal sample. Pre oxidation of raw coal enhances humic production by

increasing content of oxygen and nitrogen containing functional groups as well as the content of OH and N-H groups both in pre oxidized residual coal and liquid by the action of GB fungal isolate. These all above discussed peaks gives confirmation of formation of coal based humic compound by the action of GB fungal isolate.

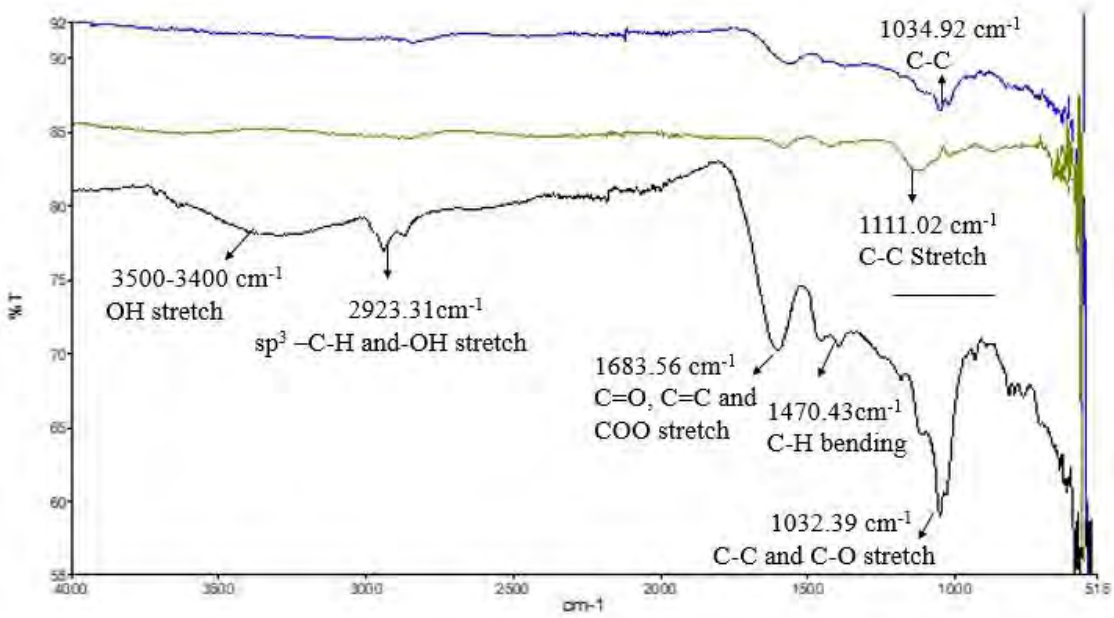


Fig 4.35 IR spectra of modified coal, bio solubilized liquid and pre oxidized coal residues. Blue: reference coal; Green: GB solubilized liquid; black: GB solubilized coals

4.18 FTIR spectra of coal residues by NF-1 Isolate using lignite coal:

The FTIR analysis is used to detect the presence of functional groups and any changes occurred at vibrational bonding present in coal samples. Figure 4.36 shows the IR spectra of untreated lignite coal (Control). The spectra shows presence or aromatic structure clearly in the range between (515-1000 $cm^{-1)}$.In between range of 800-850 cm^{-1} C-C and C-H stretching clearly shown while at 973 cm^{-} and 996 cm CH3 bending and C-C stretching is seen. While at 1023 cm^{-1} C=O bond is present.

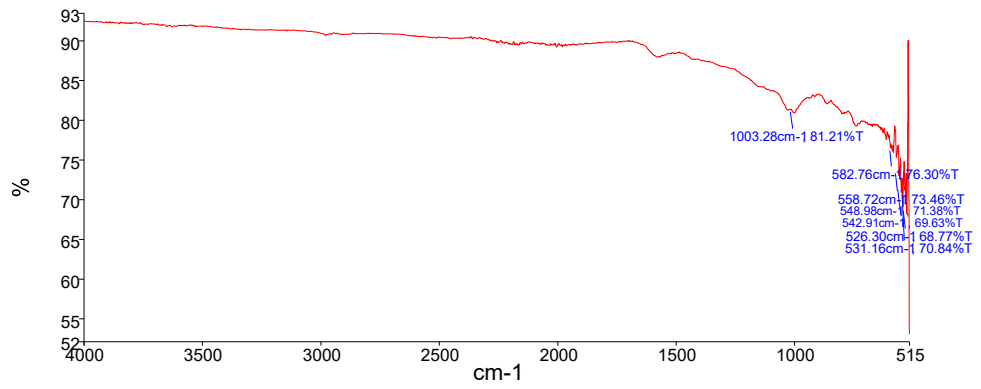


Fig 4.36 FTIR spectra of Lignite Coal (Untreated)

While figure 4.37 shows the IR spectra of lignite coal having particle size 0.1mm treated biologically with strain NF-1. This spectrum shows clear modifications in the functional groups present in Lignite Coal (control). The peaks in the range of 515-600 cm^{-1} CH out of plane aromatic bonding while a visible peak at 1027 cm^{-1} shows the -C-O bonding (inorganic carbonate) structure which shows that solubilization has occurred and shows visible solubilization peaks. A visible peak at 1611 cm shows C=C unsaturated compounds presence which is absent in control. It shows that new compounds are formed and are clearly detected in FTIR spectra as compared to untreated coal. Similarly, another peak at 3277.20 cm^{-1} shows the presence of some NH aminoacidic groups which shows that solubilization took place and produces significant compounds after coal solubilization by NF-1 by releasing extracellular enzymes.

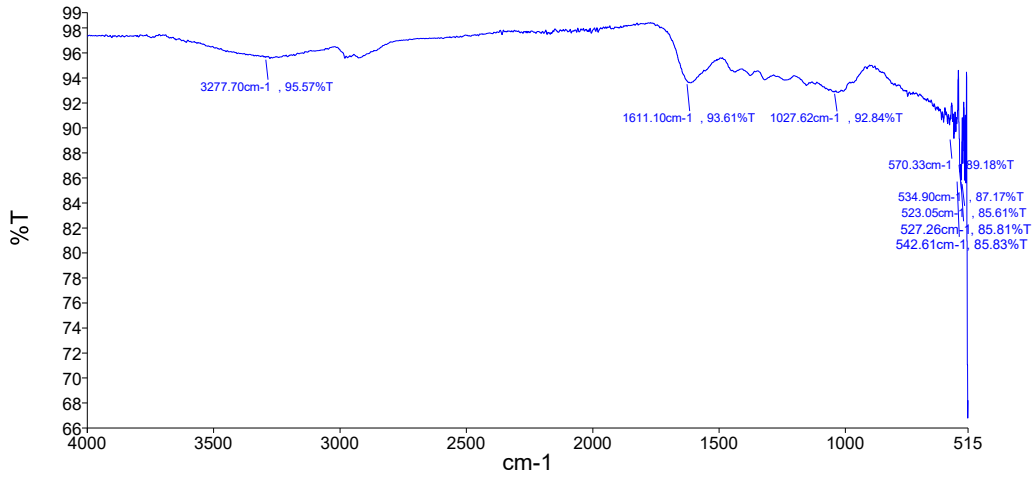


Fig 4.37 FTIR spectra of Lignite coal having particle size (0.1mm) treated with NF-1

The IR spectra shown in figure 4.38 shows the treatment of lignite coal having particle size (0.3-0.5mm) with NF-1 isolate. The strain NF-1 had shown the visible changes in coal structure by solubilizing aromatic side chains and rings detected in range between (515-600cm^{-1}. This range shows the clear C-H stretching and bending which means some deformation in structure of coal had occurred. While a detectable peak at 1391.90cm^{-1} which shows CH group deformation clearly after absorption and solubilization phenomenon. It 3 means NF-1 isolate have ability to utilize the coal as sole carbon source and converting it into new compounds or solubilized products.

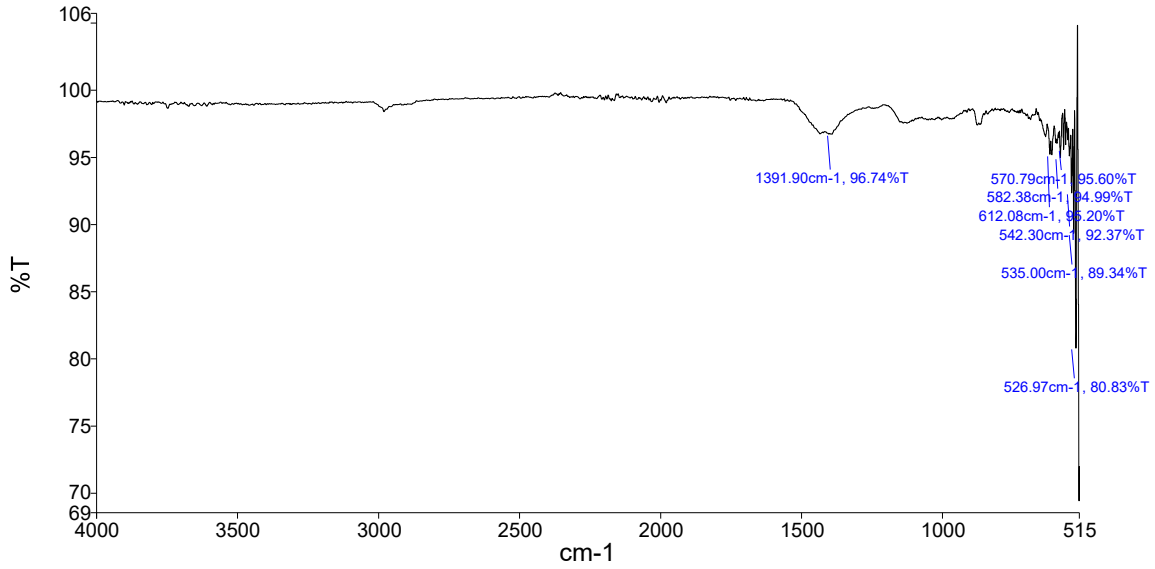


Fig 4.38 FTIR spectra of Lignite coal having particle size (0.3-0.5mm) treated with NF-1

4.19 FTIR spectra of coal residues by GB Isolate using lignite coal:

The FTIR spectra by fungal isolate GB utilizing 0.1mm particle size shows clear changes in coal structure and functional group. At 1031.01 it clearly detects the deformation of C-H bonding and out of plane stretching. At 613.95 cm^{-1}C-O-H twist occur which means breakage in bonding take place by fungal isolate GB. Similarly, a clear peak at 2922 cm^{-1}C-H asymmetric stretching of >CH bonding occur which shows clear solubilization of 0.1mm particle size of lignite coal by isolate NF-1.

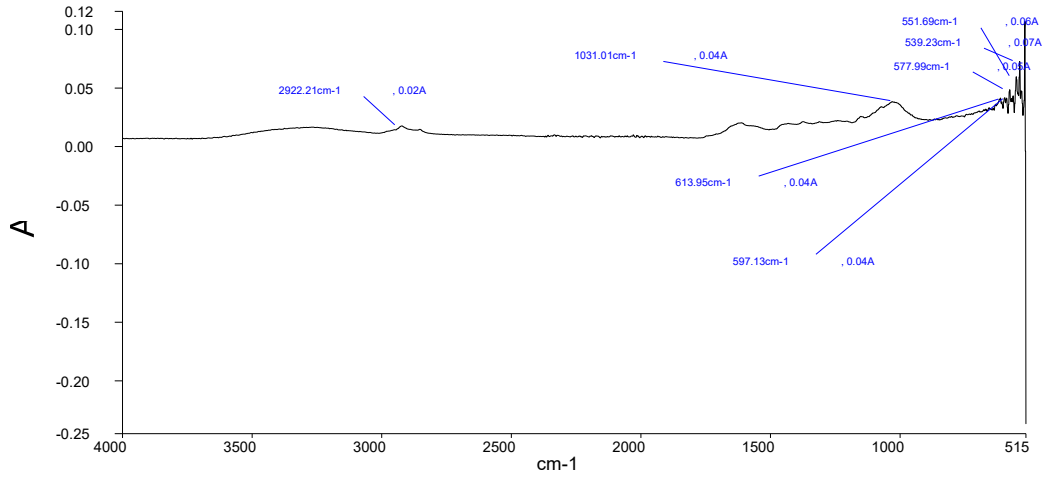


Fig 4.39 FTIR spectra of Lignite coal having particle size (0.1mm) treated with GB

Figure 4.39 shows the IR pattern of isolate GB with lignite coal utilizing particle size of 0.3-0.5mm. This spectrum shows clear changes in functional groups structure and very

sharp and visible peaks are detected. The pattern of aromatic functional group stretching is clearly shown within the range 515-600 cm^{-1}. While peak at 1404.05cm^{-1} shows C=O symmetric stretching of -COO groups present in coal structure. Comparing this spectrum with control no such kind of peak is detected, which means isolate GB have ability of utilizing coal particle size of 0.3-0.5mm and converting it into solubilized organic moieties. Similarly peaks at 1555.20 cm^{-1} and 1633.90 cm^{-1} shows the presence of Amides and C-) stretching in ester bonds. Another peak at 3278.65 cm^{-1} shows the pattern of molecular vibrations of N-H stretching which means after solubilizing coal particles new functional moieties and groups are detected and formed by the activity of isolate GB. This might be due to the fact as well that fungal strain GB requires specific functional groups for the attack.

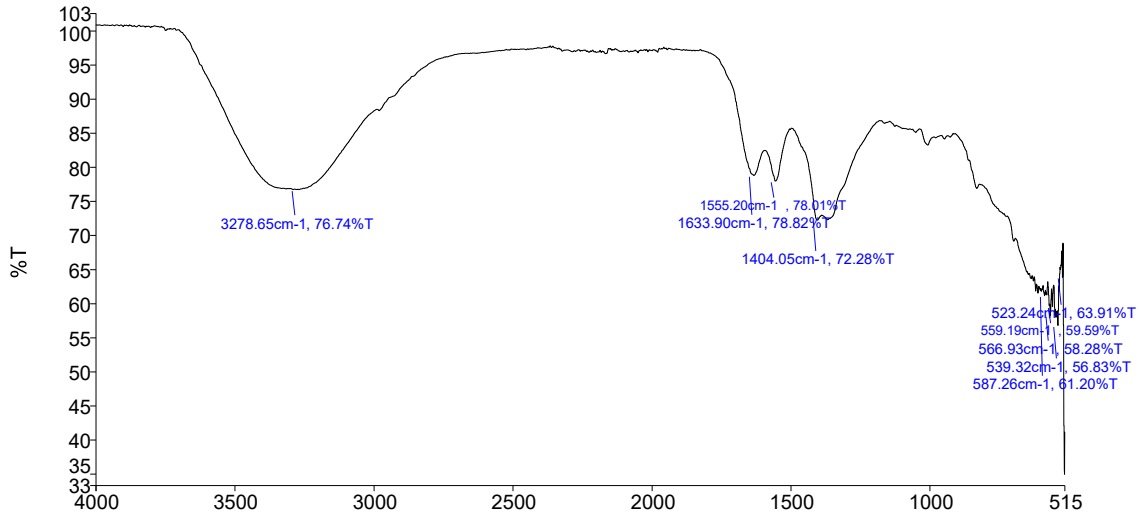


Fig 4.40 FTIR spectra of Lignite coal having particle size (0.1mm) treated with GB

4.20 GC-MS analysis

4.20.1 GC-MS NF-1 liquified products:

GC/MS spectrometry was utilized for evaluating diverse fragments and composition of organic constituents in solubilize product. The total ion chromatograms (TICs) of ethyl acetate extracts of black liquid products, from NF-1 solubilized untreated and pre oxidized coal were shown in Fig 4.41 and 4.42. GC–MS detected total 19 components in virgin coal, 9 components showed major peaks while in pre oxidize coal total 18 components were identified, 13 components showed major peaks that were identified by using NIST02 library as given in table 4.11 and 4.12.Compounds identified in virgin coal were assembled into alcohols, carboxylic, alkenes and polyaromatic hydrocarbons (PAHs) while ketones, acetic acids, esters, n-alkanes, branched alkanes, alcohols, fatty acids, carboxylic acids phenol and benzene derivatives were most prominent in pre oxidize coal.

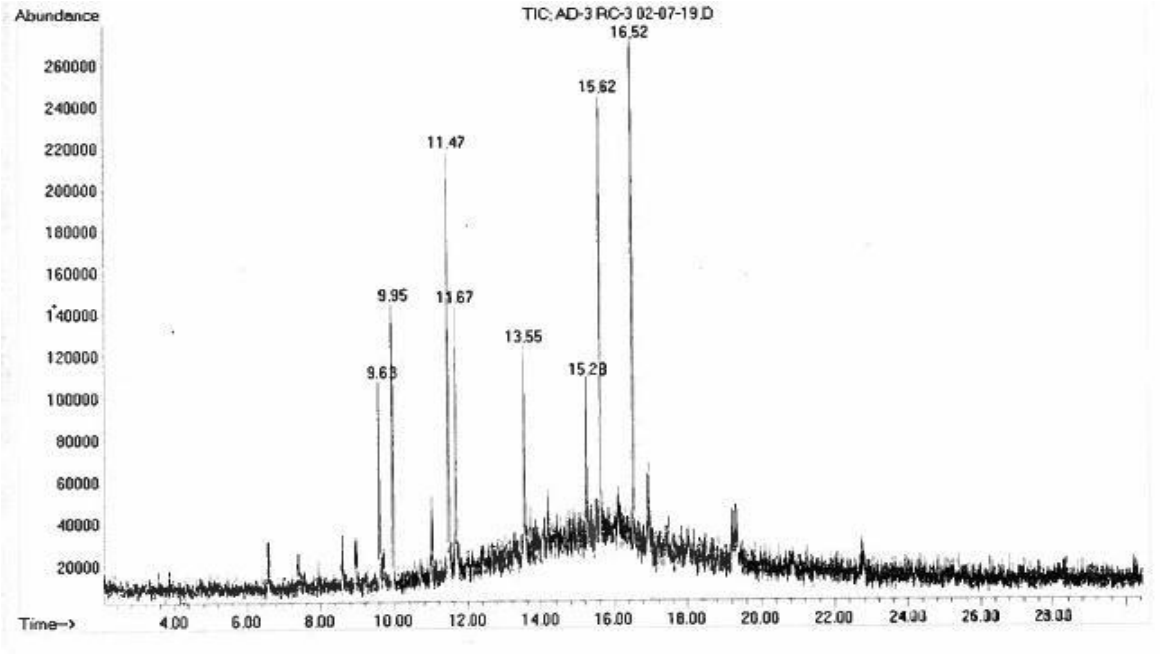


Fig 4.41 MS spectra of NF-1 liquified virgin coal products

Table 4.11 GC-MS detectable compounds of liquid virgin coal treated with NF-1 isolate

RT (min)	Compound name	Structural formula	CAS no.
9.626	1-Dodecanol, 3,7,11-trimethyl-	C H O 15 32	006750-34-1
9.630	Formic acid, decyl ester	C H O 11 22 2	5451-52-5
9.950	Isopropyl Myristate	C H O 17 34 2	000110-27-0
11.471	1,2-Benzenedicarboxylic acid, butyl 2-methylpropyl ester	C H O 16 22 4	017851-53-5
11.671	1 –Octadecene	C H 18 36	000112-88-9
13.551	Cyclooctane, methyl-	C H 9 18	001502-38-1
15.277	1-Octadecene	C H 18 36	000112-88-9
15.620	Phenol, 2,2'-methylenebis [6-(1,1dimethylethyl)-4-methyl-	C H O 23 32 2	000119-47-1
16.521	1,2-Benezenedicarboxylic acid, mono (2-ethylhexyl) ester	C H O 16 22 4	004376-20-9

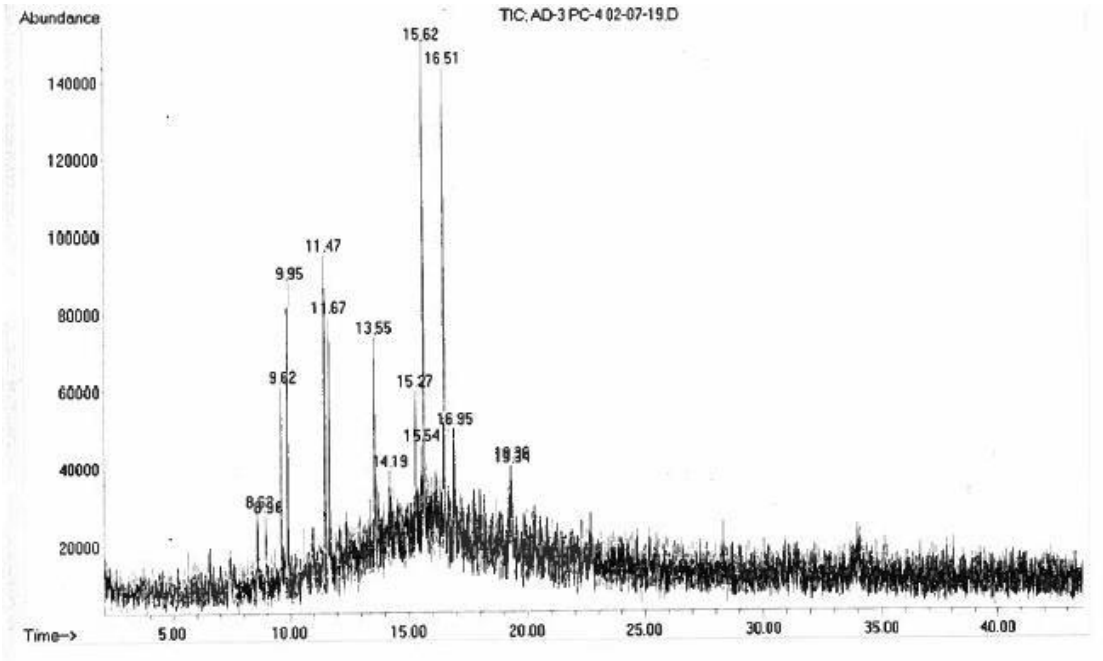


Fig 4.42 MS spectra of NF-1 liquified pre oxidize coal products

Table 4.12 GC–MS detectable compounds from NF-1 liquified pre oxidize coal products

RT (min)	Compound name	Structural formula	CAS no.
8.616	2, 4-Dimethyl-1-hexene	C H 8 16	016746-87-5
8.959	Methanone, (2-methylphenyl) phenyl-	C H O 14 12	000131-58-8
9.626	Pentafluoropropionic acid, pentadecyl ester	C18H31F5O2	100028-00-7
9.947	Isopropyl Myristate	C H O 17 34 2	000110-27-0
11.466	1,2-Benzenedicarboxylic acid, dicyclohexyl ester	C H O 20 26 4	000084-61-7
11.675	Cyclopentane, 2-isopropyl-1, 3dimethyl-	C H 10 20	032281-85-9
13.551	Ethanone, 1-cyclohexyl-	C H O 8 14	000823-76-7
14.185	2-Methyl-d-glucose	C H O 7 14 6	146-72-5
15.275	2-Aziridinone, 1-tert-butyl-3-(1methylcycloheptyl)-	C H NO 14 25	26944-18-3
15.535	Androstane-3,12,17-trione, (5. beta.)-	C H O 19 28 2	846-46-8
15.624	Phenol, 2,2'-methylenebis[6-(1,1dimethylethyl)-4-methyl-	C H O 23 32 2	000119-47-1

16.514	1,2-Benzenedicarboxylic acid, dicyclohexyl ester	C H O 20 26 4	000084-61-7
16.951	2-Nonanol	C H O 9 20	9-99-628

4.20.2 GC-MS GB liquified products

GC/MS spectrometry was utilized for evaluating diverse fragments and composition of organic constituents in solubilize product. The total ion chromatograms (TICs) of ethyl acetate extracts of black liquid products, from GB solubilized non treated and pre oxidized coal were shown in Fig 4.43 and 4.44. GC–MS detected total 11 components in virgin coal, 10 components showed major peaks while in pre oxidize coal total 22 components were identified, 19 components showed major peaks that were analyzed by using software (NO. NIST02) and given in table 4.13 and 4.14. Compounds identified in virgin coal were assembled into amides, esters, alcohols, formic acids, carboxylic, alkenes and aromatic acids while phenol derivatives, ketones, acetic acids, amines, amides methyl esters, esters, nalkanes, phthalate, aliphatics, alcohols, fatty and carboxylic acids amines, amides were most prominent in pre oxidize coal.

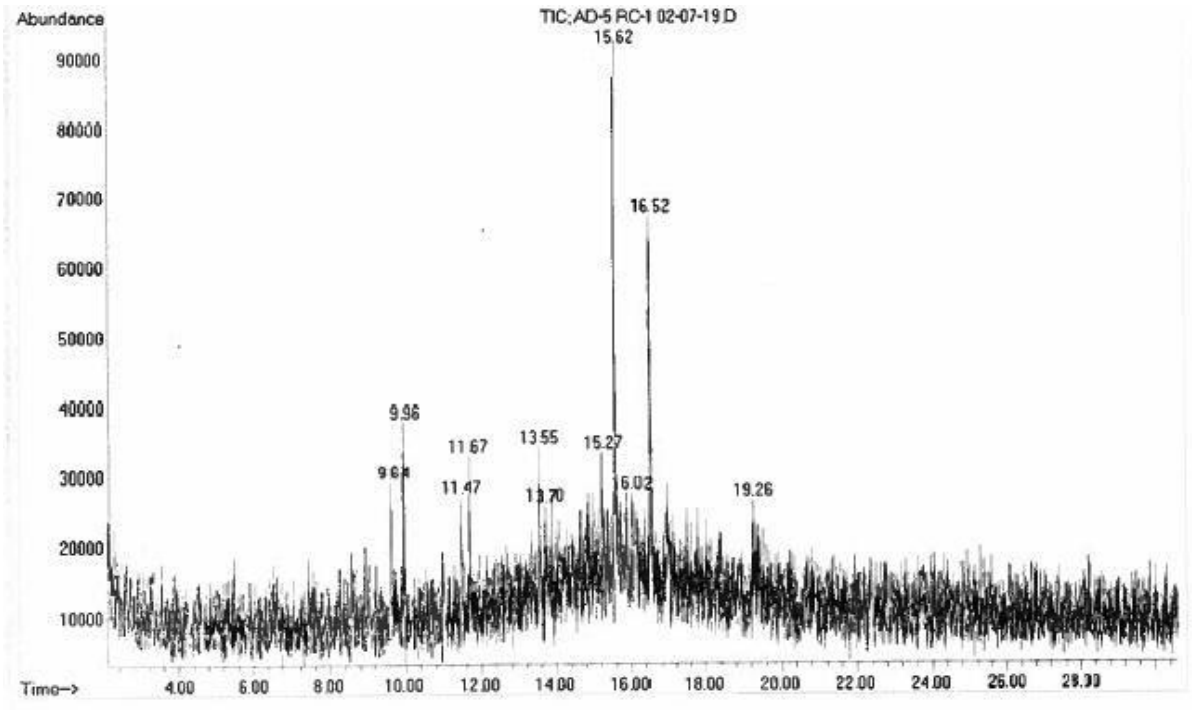


Fig 4.43 MS spectra of GB liquified virgin coal products

Table 4.13 GC–MS detectable compounds from GB liquified virgin coal products

RT (min)	Compound name	Structural formula	Case No.
9.635	5,18:9,14-Dietheno-6H-dibenzo [d, k] thiacyclotetradecin,8,15,16,17-tetrahydro	C H S 25 22	07800767-7
9.957	Isopropyl Myristate	C H O 17 34 2	00011027-0
11.473	Terephthalanilide,4',4''-dinitro-	C H N4 20 14 O 6	03406284-5
11.668	Benzene-1,3-dicarboxamide,5-nitro-N, N'-dicyclohexylN, N'-dimethyl-	C H N 22 31 3 O 4	29487476-3
13.551	N, N'-Dicyclohexylformamidine	C H N 13 24 2	00230389-1
15.272	Z-11-Tetradecen-1-ol trifluoroacetate	C H O 14 28	3401015-6
15.620	Phenol,2,2'-methylenebis[6-methoxy-3-(2-propenyl)-	C H O 21 24 4	05533455-9
16.519	1,2- Benzenedicarboxylic acid, mono (2-ethylhexyl) ester	C H O 16 22 4	4376-209
16.960	19-Norpregn-4-ene-3,20-dione	C H O 20 28 2	472-54-8
19.261	4-Oxocyclopentane-1,2-dicarboxylic acid	C H O 7 8 5	1703-613

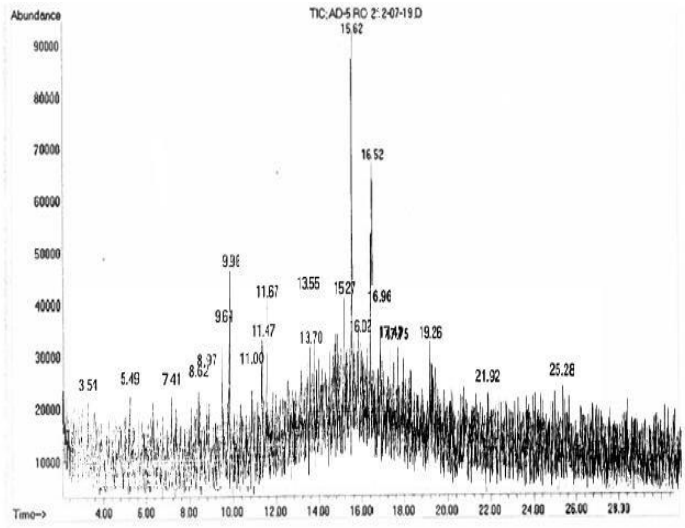


Fig 4.44 MS spectra of GB liquified pre oxidized coal products

Table 4.14 GC–MS detectable compounds from GB liquified pre oxidized coal products

RT (min)	Compound name	Structural formula	Case No.
3.547	2H-1-Benzopyran, 3,4-dihydro-6,7-dimethoxy-3-(2,3,4,5tetramethoxyphenyl (S)-	C11H13N*O2*	3382-18-1
5.486	Cresyl diphenyl phosphate	C H O P 19 17 4	026444-49-5
8.975	3,4-Dimethoxyphenyl isothiocyanate	C H NO S 9 9 2	33904-04-0
9.635	5,18:9,14-Dietheno-6H-dibenzo [d, k] thiacyclotetradecin,8,15,16,17-tetrahydro	C H S 25 22	078007-67-7
9.957	Isopropyl Myristate	C H O 17 34 2	000110-27-0
11.006	Butanal, (2.4-dinitrophenyl) hydrazone	C H N O 10 12 14 4	1527-98-6
11.473	Terephthalanilide,4',4''-dinitro-	C H N4O 20 14 6	034062-84-5
11.668	Benzene-1,3-dicarboxamide,5nitro-N, N'-dicyclohexyl-N, N'-dimethyl-	C H N O 22 31 3 4	294874-76-3
13.551	N, N'-Dicyclohexylformamidine	C H N 13 24 2	002303-89-1
15.272	Z-11-Tetradecen-1-ol trifluoroacetate	C H O 14 28	34010-15-6
15.610	Phenol,2,2'-methylenebis [6-(1,1dimethy	C H O 23 32 2	000119-47-1
15.620	Phenol,2,2'-methylenebis[6methoxy-3-(2-propenyl)-	C H O 21 24 4	055334-55-9
16.519	1,2- Benzenedicarboxylic acid, mono (2-ethylhexyl) ester	C H O 16 22 4	4376-20-9
16.521	Di-n-octyl phthalate	C H O 24 38 4	000117-84-0
16.960	19-Norpregn-4-ene-3,20-dione	C H O 20 28 2	472-54-8
17.470	Thionazin	C H N O3PS 8 13 2	297-97-2
19.261	4-Oxocyclopentane-1,2dicarboxylic acid	C H O 7 8 5	1703-61-3

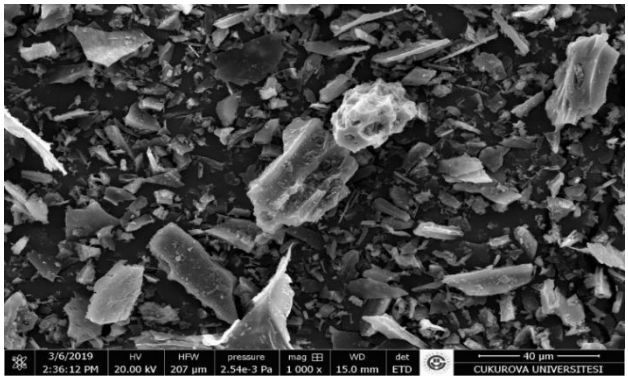
21.926	C(14a)-Homo-27-norgammacer14-ene,3. beta. -methoxy-	$C_{32}H_{52}O_3$	1260-05-5
25.288	3, 5-Dimethoxyphenethylamine	$C_{10}H_{15}NO_2$	3213-28-3

GC-MS spectra of ethyl acetate soluble extracts of bio liquified black liquids showed organic pool, but it was observed that bio treated pre oxidized solubilized liquid represented most variety of organics than virgin. Presence of high concentration of aromatics in pre oxidize solubilized liquids may indicated that bio treatment effectively decomposes condensed aromatics. Reliability of GC–MS results were compared it with other spectroscopic analysis. Aromatics and aliphatics were observed during UV–Visible analysis while aliphatics carboxylic groups, alkenes and polyaromatic hydrocarbons (PAHs) were detected by GC–MS. Aromaticity of coal related to released aliphatic concentration. Bituminous being high ranked coal showed more liberation of alcoholic and aromatic derivatives than aliphatic. FT-IR peaks characterized the presence of phenolics, ketones, amides, ethers and carboxylic groups meanwhile, GC–MS also confirmed their presence. Moreover, low molecular weight compounds also identified by MS spectra.

4.21 SEM Analysis of Lignite coal treatment with isolate NF-1 and GB

Scanning electron microscopy (SEM) model JSM 5910 lv (Jeol SEM, Japan) fitted with Energy Dispersive X-Ray Analyzer, energy dispersive spectroscopy (EDS) (Inca 200, Oxford Instruments, UK) were used to investigate the pattern of fungal isolates adherence and erosion on coal surface.

Figure 4.45 shows the lignite coal untreated (control). In comparison with control the figure 4.46 shows the presence of fungal strain NF-1 on lignite coal producing some extracellular polymer like structures that helps to attach the microorganisms on surface of coal that favors the attack of coal components and uptake of nutrients from coal particles. The fungus is found to adhere on the surface and producing cracks which means solubilization activity is performed by NF-1 Isolate. Figure 4.47 shows the presence of isolate GB on the surface of lignite coal. This figure clearly shows the breakage in structure of coal as well as visible cracks are observed which further confirms the solubilization potential of isolate GB..



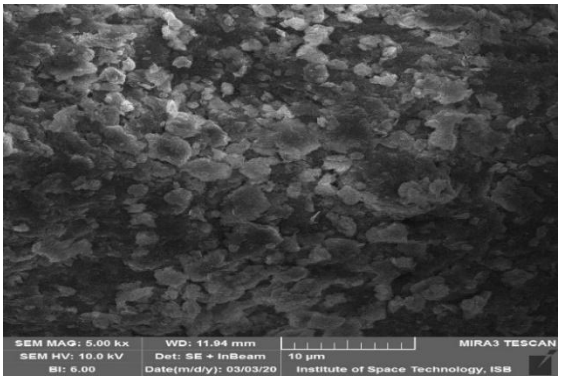


Fig 4.45 Lignite coal (control)

Fig 4.46 Lignite coal after treating with isolate NF1

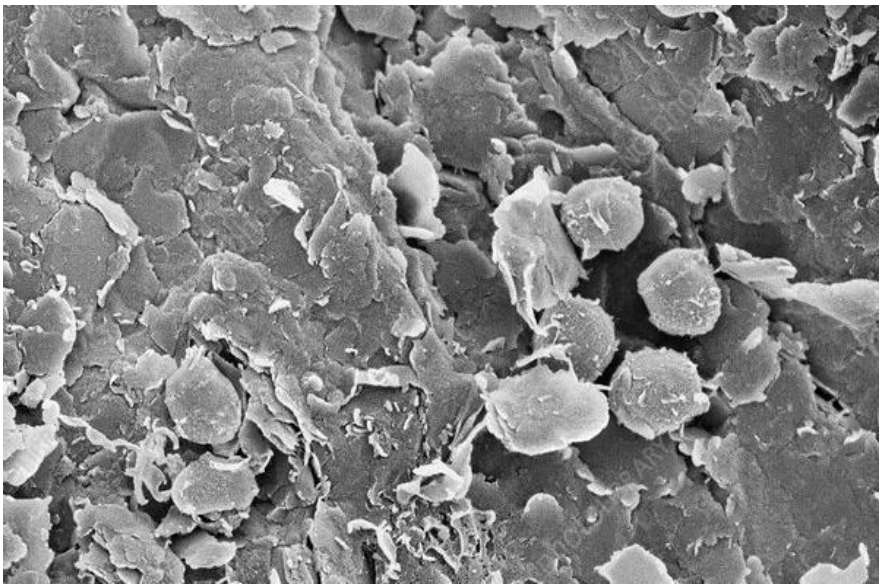


Fig 4.47 Lignite coal after treatment with Isolate GB

4.22 SEM analysis of Bituminous coal treated with fungal isolates:

SEM analysis of bituminous coal shown in figure 448 shows the structure of bituminous coal in control. While figure 4.49 shows some amorphous kind of structure of coal along with adherence of strain GB on the surface. The amorphic structure and morphological changes in coal structure confirms that NF-1 have potential of solubilizing coal particles and further cracks in structure means that some organic moieties and release organics are also produced by the release of extracellular enzymes.

Similarly, figure 4.50 shows the entrapment of coal particles with hyphae of fungal mycelium of isolate GB. Apart from entrapment of coal particles some cracks are also visible on the

surface of coal particles which detects the solubilization potential of isolate GB. It means both the isolates have ability of utilizing coal particles as sole carbon sole and releasing organic compounds.

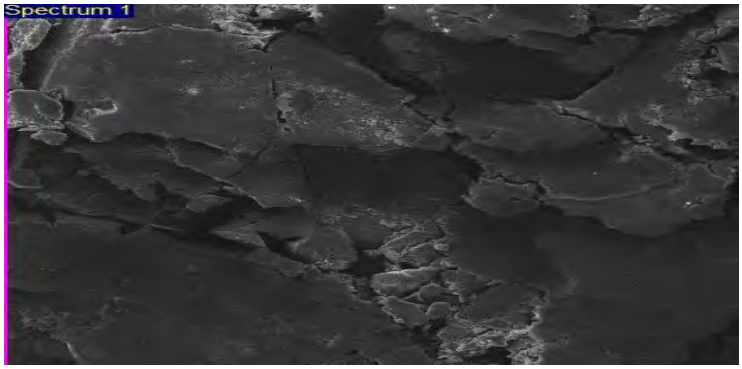


Fig 4.48 Bituminous Coal before Treatment (Control)

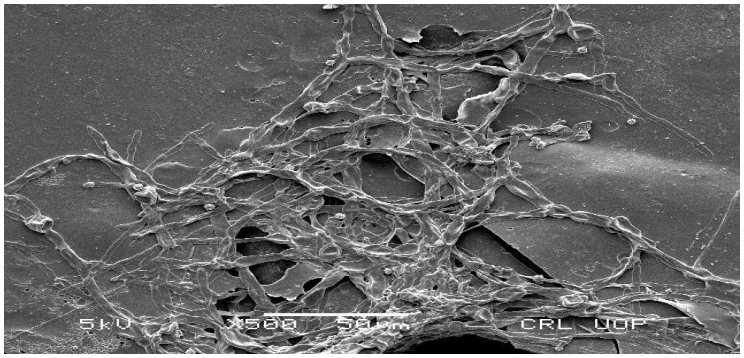


Fig 4.49 Bituminous coal after treatment with strain NF-1

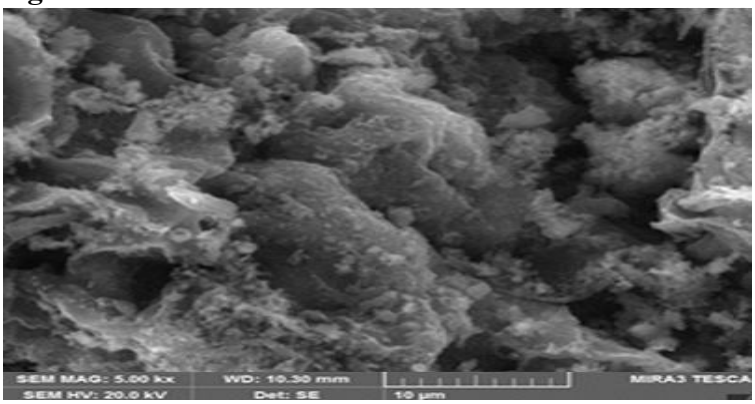


Fig 4.50 Bituminous coal after Treatment with isolate GB

(Results Phase 2)

Metagenomic Analysis of Baterial and Fungal Amplicions Present in Coal Samples

4.23 Metagenomic Analysis of Bacterial and fungal communities present in coal samples

4.23.1 DNA Extraction of bacterial and fungal communities

For analyzing the microbial communities (Bacterial and fungal) present in coal samples, the DNA was extracted using powerlyzer ultraclean Microbial isolation Kit (Qiagen), according to the manufacturer's instruction. Figure 4.51 shows the tape station of extracted DNA bands of all the fungal and bacterial communities along with their size compared with ladder.

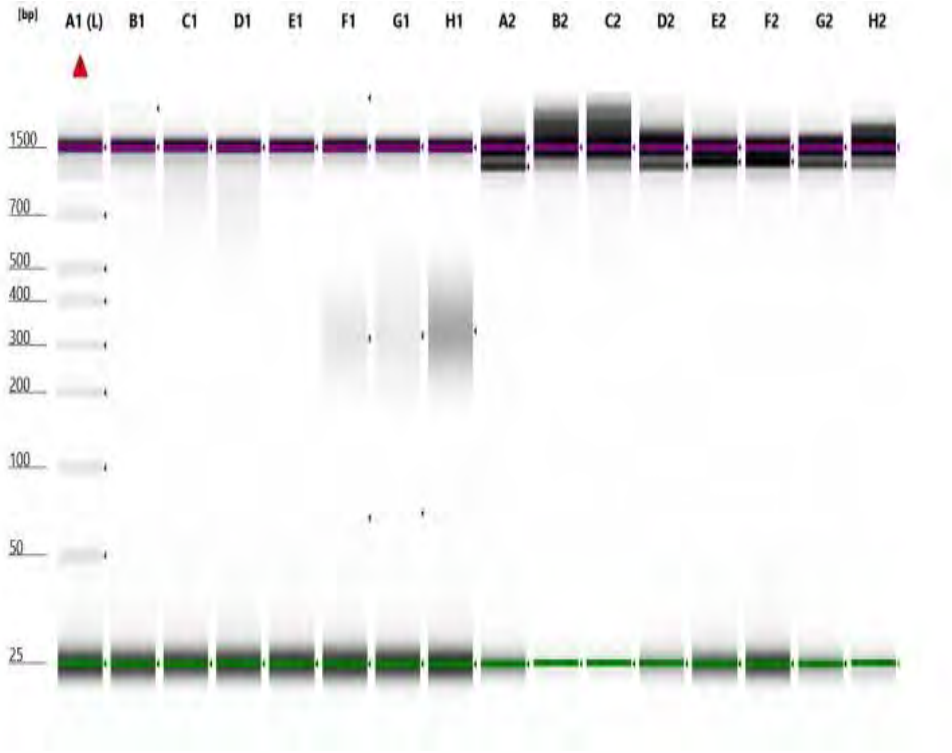


Fig 4.51 While A1= Ladder, B1= NOU-G1, C1=NOU-H1, D1= NOU-M1, E1=NOU-P1, F1=NOU-R1, G1=NOU-R2, H1=NOU-RT, H1= NOU-S (representing bacterial communities from coal samples)

While A2= NOU-5, B2= NOU-6, C2= NOU-7, D2= NOU-8 represents fungal communities DNA bands.

All the communities show their specific size of their DNA compared with the ladder.

4.23.2 PCR (Polymerase chain reaction 1 and 2):

After extraction of DNA, Polymerase chain reaction was performed. In polymerase chain reaction 1, the specific universal bacterial and fungal primers were used. For bacterial communities 27F and 1492 R were used, while for fungus ITS1 and ITS 4 were used. In PCR 2 adaptor primers were used. Both adaptor sequence primers and PCR primers are the

artificial DNA oligonucleotides. The adaptors contain the sequencing primers binding sites, index sequences and the particular sites that allows the library fragments to attach to the flow cell. The adaptors used for bacterial sequencing were 27F_adp and 1492R_adp and for sequencing fungal community's adaptor primers used were ITS1_adp and ITS4_adp. Total 8 samples for bacterial diversity were sequenced and 4 samples for fungal diversity were sequenced.

After PCR 2, the products were cleaned up and, after cleaning the increase in band size was observed which further confirms the cleanup had occurred.

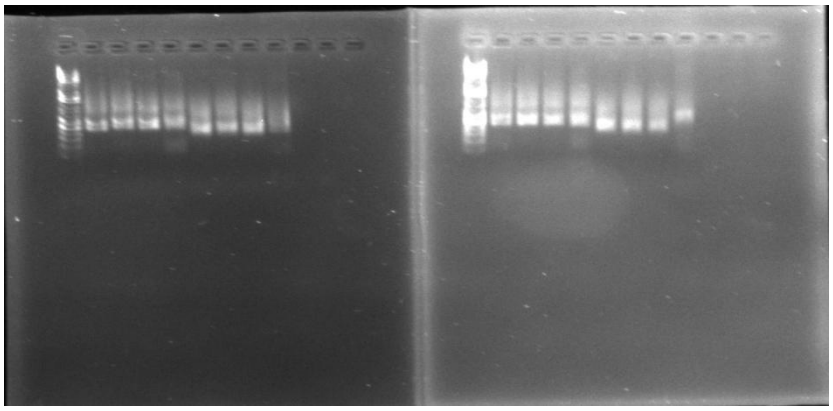


Fig 4.52 PCR 1 and PCR 2 of bacterial communities

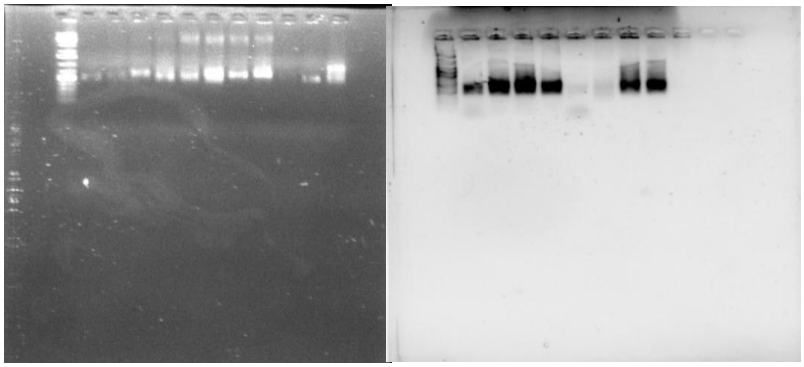


Fig 4.53 Fungal amplicons PCR 1 and PCR 2

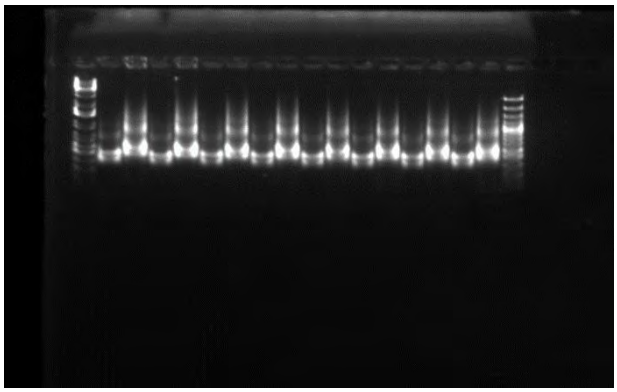


Fig 4.54 Clean up of bacterial PCR products

4.23.3 QUBIT analysis for determining the final concentration of DNA

Qubit analysis were performed for determining the presence of final concentration of bacterial and fungal DNA extracted from each coal sample. Sample NOU-P1 had shown more concentration of bacterial DNA, while sample NOU-7 from fungal community had shown more concentration of fungal community DNA. Similarly, their final pooling for sequencing were calculated and after pooling the final sequencing was performed using Illumina Miseq technique of next generation sequencing.

Table 4.15 QUBIT analysis of bacterial and fungal communities for Miseq

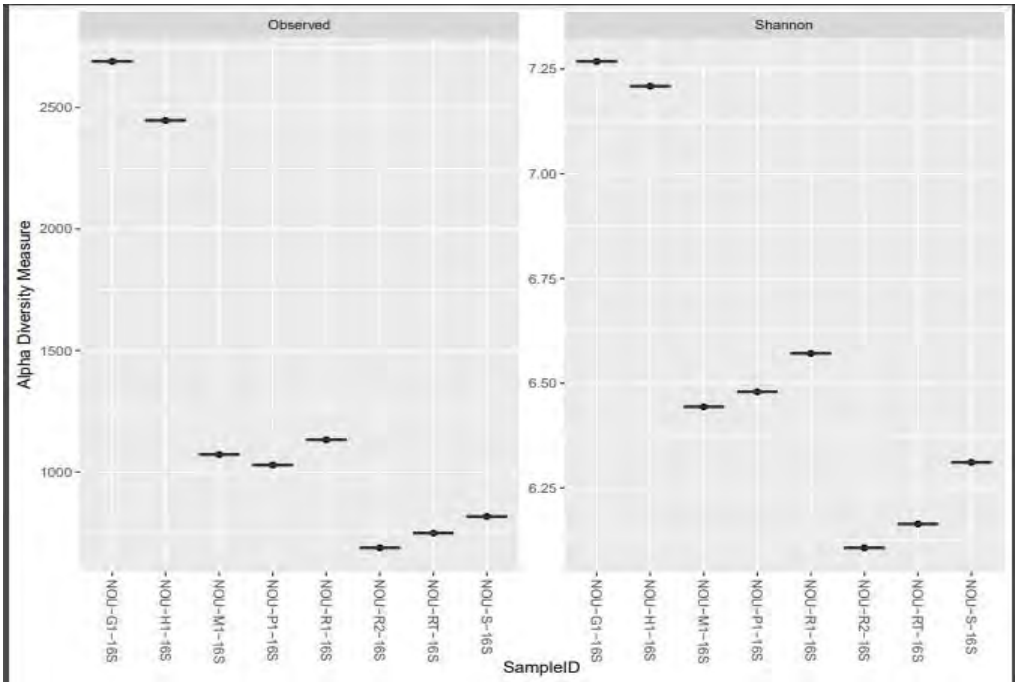
Sample ID	QUBIT Bacterial community concentration (ng/µl)	Final pooling for sequencing (µl)
NOU-G1	14.2	4.16
NOU-H1	15.4	3.2
NOU-M1	12.5	2.1
NOU-P1	16.31	1.78
NOU-R1	3.97	21.54
NOU-R2	23.6	1.01
NOU-RT	16.6	2.31
NOU-S	1.98	28.9

Sample ID	QUBIT Fungal community DNA concentration(ng/µl)	Final pooling for sequencing in (µl)
NOU-5	1.73	28.90
NOU-6	12.20	4.10
NOU-7	47.50	1.05
NOU-8	17.60	2.84

4.24 Statistical Analysis of Data:

4.24.1 Alpha Diversity of bacterial community

Measurement of alpha diversity for comparing the total diversity present in different communities is important. In present study, the total bacterial diversity from 8 different coal samples from Dukki and Thar coal mine was determined. Basically, alpha diversity measures the mean diversity of species present in different sites or habitats. The quantitative speciesbased measures such as Shannon diversity (Shannon & Weaver, 1949) were calculated. In the present study the bacterial diversity of sample NOU-M1, NOU-P1, NOU-R2, NOU-RT, NOU-S, NOU-R1 resembles with each other. The diversity of these 6 samples co relates with each other. While two samples NOU-G1 and NOU-H1 differs from each other on the basis of richness. The bacterial habitat of these samples showed different diversity on the basis of Shannon and observed richness. This might be because of the depth, the samples were taken from different depths, so might be diversity at different depths changes. It also depends on the coal environment; different coal environment shows different alpha-diversity.



4.55 Alpha diversity of bacterial communinties present in coal samples

4.24.2 Beta diversity of bacterial community

So far alpha diversity measures the diversity within a single sample. However, beta diversity was proposed by Whittaker, which is basically a measure of change in diversity across different environmental gradients (Whittaker, 1960). Beta diversity is used to evaluate the ratio between regional and local specie diversities. It helps to measure the extent to which two microbial communities' changes over time. (Koleff, et al., 2003). Specie based beta diversity evaluates whether similar environments contain the same specie despite of

geographic barriers and distance (Nougez et al, 2005). In the present study, out of 8 samples, 2 samples depicted very different behavior among species present in these microbial communities. A high beta diversity index indicates a low level of similarity, while a low beta diversity index shows a high level of similarity. The samples shown in figure 4.56 in blue shows low beta diversity which means that the bacterial species present in these 6 communities have high level of similarity, while the two samples showing orange color indicated high beta diversity index which means, these two samples have low beta diversity index as compared to other 6 samples. The two samples named as NOU-G1 and NOUH1depicted higher beta diversity index. The present study is reporting first time the beta diversity among coal habitats from Pakistani coal mines.

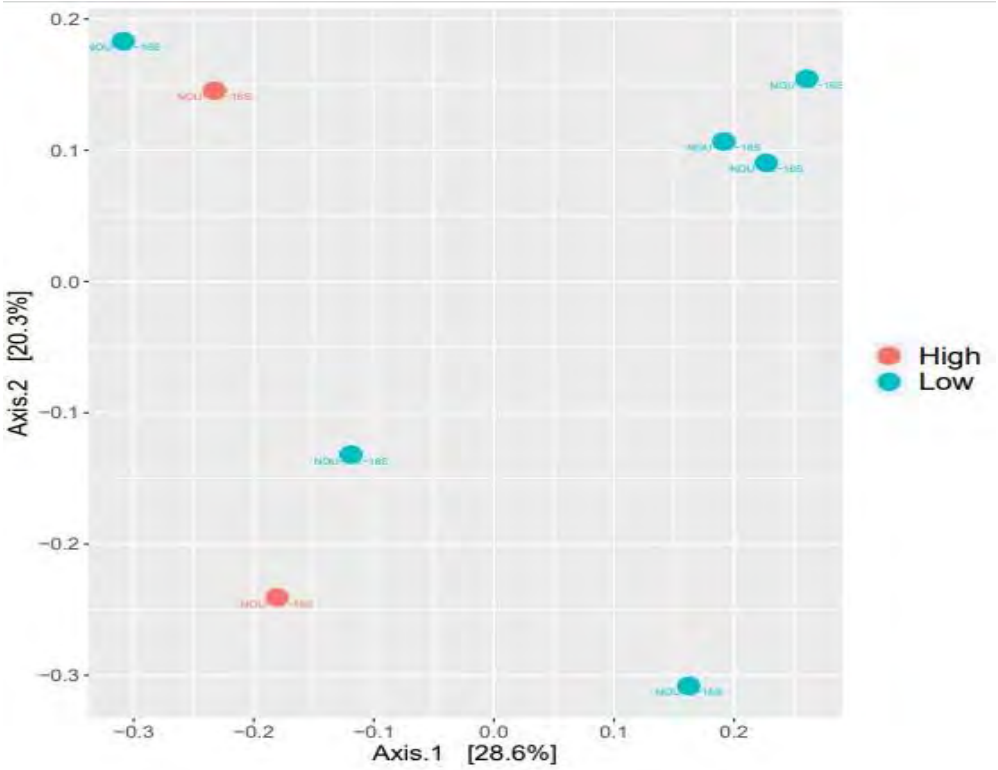


Fig 4.56 Beta diversity of bacterial communities

4.24.3 Heat Map of bacterial diversity in coal habitat

Heat map is considered to be an important tool designed for the visual display of data from NGS (next generation sequencing). It is basically a graphical representation of data in which color coding system is used for representing different values existing in a matrix. Its shows the data values for each row and column and the dendrograms along the sides depicts that how the variables and the designed rows are clustered independently. The heatmap shown below in the figure 4.57 describing the relative percentage of 16srRNA gene sequence

designated to each bacterial genus (y axis) crossing with the 8 coal mine samples analyzed (y axis). The colors representing in the map shows the relative percentage of the microbial assignments with each sample. The shifting in colors from light blue to bright orange indicates the higher abundance or particular genus in each sample.

In the figure below almost 10 genus from bacterial diversity are shown against the 8 samples along x axis. The light blue color squares indicates that the very low abundance of *Nitrospirae* and *Firmicutes* present in each coal sample. Whereas, the genus shown with medium color (light orange) indicates relatively higher abundance of *Plantomycetes, Chloroflexi, Thaumarchaeota* and *Verrucomicrobia* in each 8 coal samples. Whereas, the bright orange square boxes indicate the higher abundance of *Acidobactera, Bacteroidetes, Actinobacteria* and *Proteobacteria* in all the mentioned coal samples. It is analyzed through figure that the abundance of *Protebacteria* and *Actinobacteria* in all the coal samples is relatively higher as compared to all other genus's.

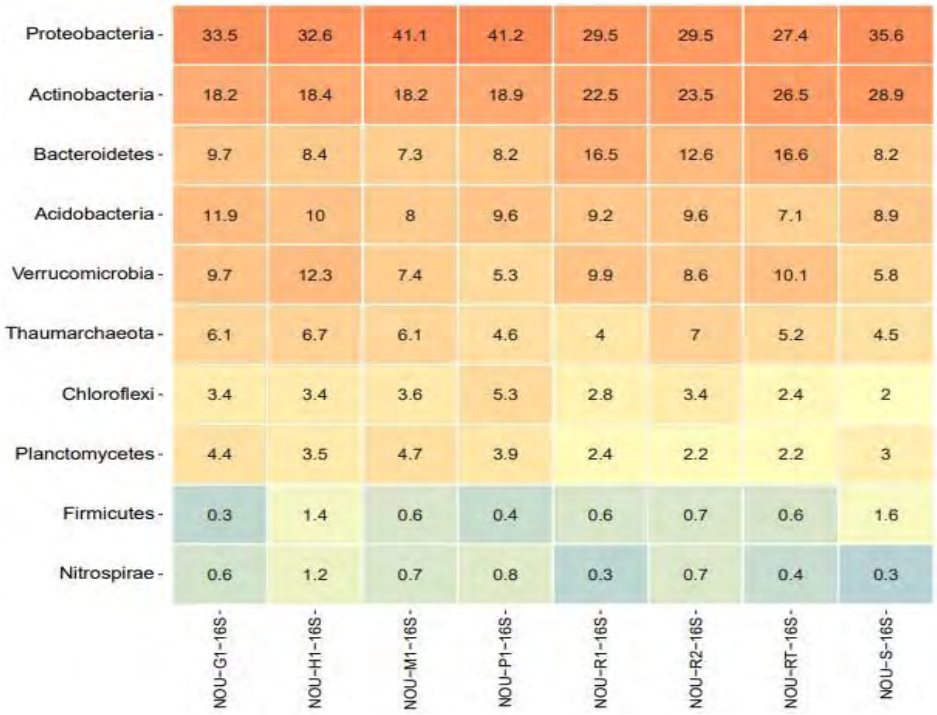


Fig 4.57 Heat map of bacterial communities present in coal habitat

4.24.4 Read abundance of top 20 bacterial genera

The read abundance depicts the taxa or phylotypes that are commonly interpreted as a measure of genic or taxon abundance of microbial community present in each sample. It is useful for comparing the similarity present in community.

In the present study the total read counts were calculated as the sum of assigned and unassigned reads. The ratio or proportion of read assigned to each bacterial genera was then

calculated on a sample-by-sample basis. The y axis in figure 4.58 shows the assigned and unassigned reads of top 20 bacterial genera present in 8 coal samples plotted against the percentage (read abundance) of these genera's. The high and low abundances are plotted in the figure shown in blue and orange boxes. The low abundance means that these bacterial groups are highly present and shows maximum similarity in each coal sample while high read abundance shown in orange means that these groups have low level of similarity present in each coal sample.

Bacteroidetes and *Actinobacteria* groups are showing low read abundance which means they are quite similar in each of 8 coal samples. While other groups like *Thaumarcheotal, Verrucomicrobia* and *Chloroflexi* had shown high abundance which means they are similar in each sample but with some low proportion. A positive and significant co relation and abundance exist between all these bacterial groups in each coal biomass.

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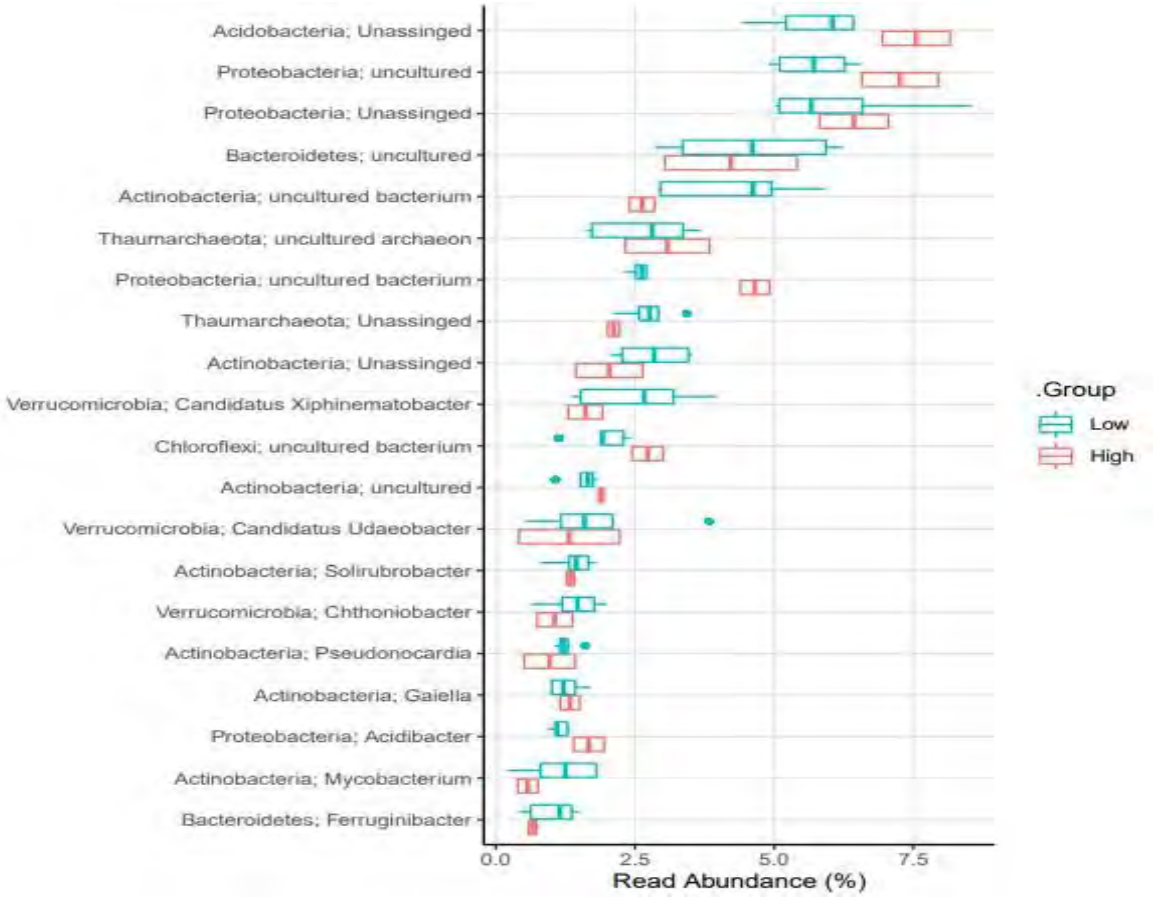


Fig 4.58 Read abundance of top 20 bacterial genera present in coal samples

4.24.5 Relative abundance of bacterial genera in coal samples:

The relative abundance refers to the evenness of individuals distribution among species in a particular habitat or community. It is defined as the total percentage of microbial sequences present in a sample.

In the present study, the relative abundance of different bacterial taxa varied across different coal samples. The relative percentage of different genera in different coal samples varies. The relative percentage of *Proteobacteria, Actinobacteria, Acidobacteria,*and *Bacteriodetes* covers large part of bacterial community in all the coal samples while some other bacterial genera like *Entothenellaeota, Firmicutes, Nitrospirae, Rokubacteria, cyanobacteria, chloroflexi and patoscibacteria* are present relatively in small proportion in all the respective coal 8 samples. The bacterial communities in sample NOU-R1, NOU-R2, NOU-RT, NOU-S relatively differs from other 4 coal samples from different coal mine locations.

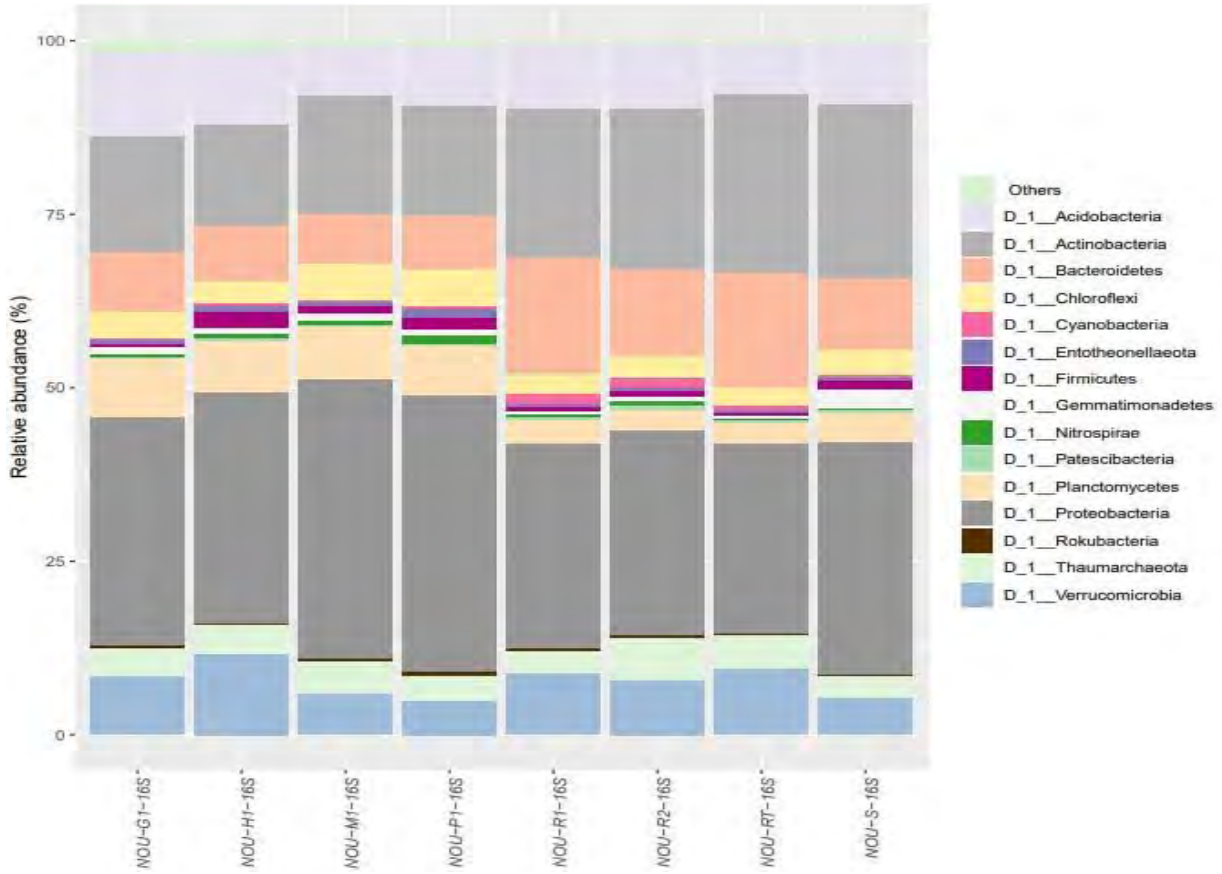


Fig 4.59 Relative abundance of bacterial communities in coal samples

4.24.6 Relative abundance of Fungal communities present in coal samples

Just like bacterial communities, the relative abundance of fungal communities was also determined. As fungus contain chitin in its wall so it was really very difficult to extract eukaryotic fungal DNA directly from hard rank coal. Only one high rank coal sample from

bituminous coal of Dukki coal mine were able to analyzed statistically, while other samples had not produced significant reads after sequencing. So out of 8 samples, the fungal communities of 4 samples were able to be analyzed statistically and relative abundance of these 4 samples was determined.

The relative abundance of NOU-5 sample from bituminous coal is showing higher abundance of *Apiotrichum* and *Blastobotrys* than other fungal communities. While the large part of fungal community in all the four samples were covered by Cladosporium, *Apiotrichum, Hebeloma* and *sterigmatomyeces* genera while other fungal genera like *Tetracladium, Tausonia, Coprinellus, Fusarium, Mollisia, Mycena, Hollermeinella* and some others had shown relatively less abundance in all the respective 4 samples. The 3 samples NOU-6, NOU-7 and NOU-8 relatively correlates with each other while NOU-5 differs from other three samples in relation to fungal communities present.

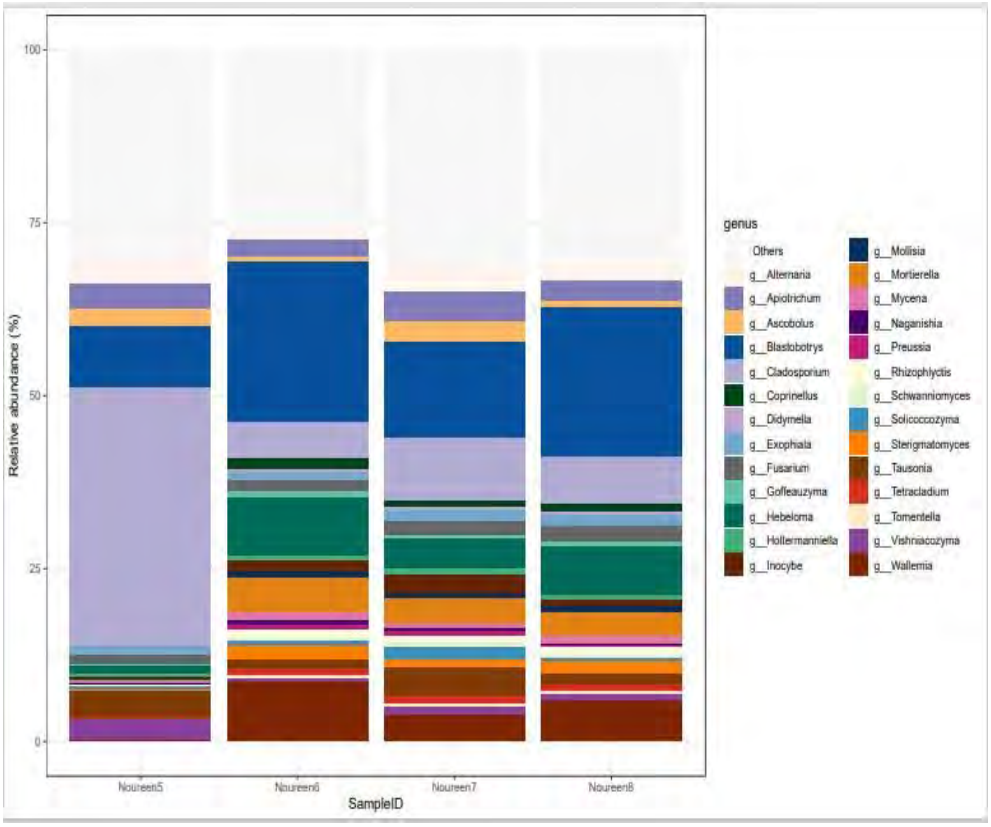


Fig 4.60 Relative abundance of Fungal communities in coal samples

4.24.7 Alpha diversity of fungal communities

Similarly, the alpha diversity on the basis of observed richness and Shannon diversity index was also determined for the fungal communities from 4 coal samples. The alpha diversity of all the four samples differs from each other. The three samples NOU-6, NOU-7 and NOU-8 correlates to each other while sample NOU-5 showed difference among all the

samples. The fungal communities of all the coal samples have shown significantly higher alpha diversity measured by observed richness. This might be because of the coal environment; different coal environment shows different alpha-diversity. The coal ranking might also be involved in different kind or diversity measurements. The high coal rank had shown different alpha measurements on the basis of richness as compared to low rank coal.
There is very less data reported on microbial communities' identification from Pakistani coal mines. Up to date, this is the first study on microbial community analysis from hard rank coal Bituminous from Dukki coal mine and low rank coal, Lignite, Thar coal mine.

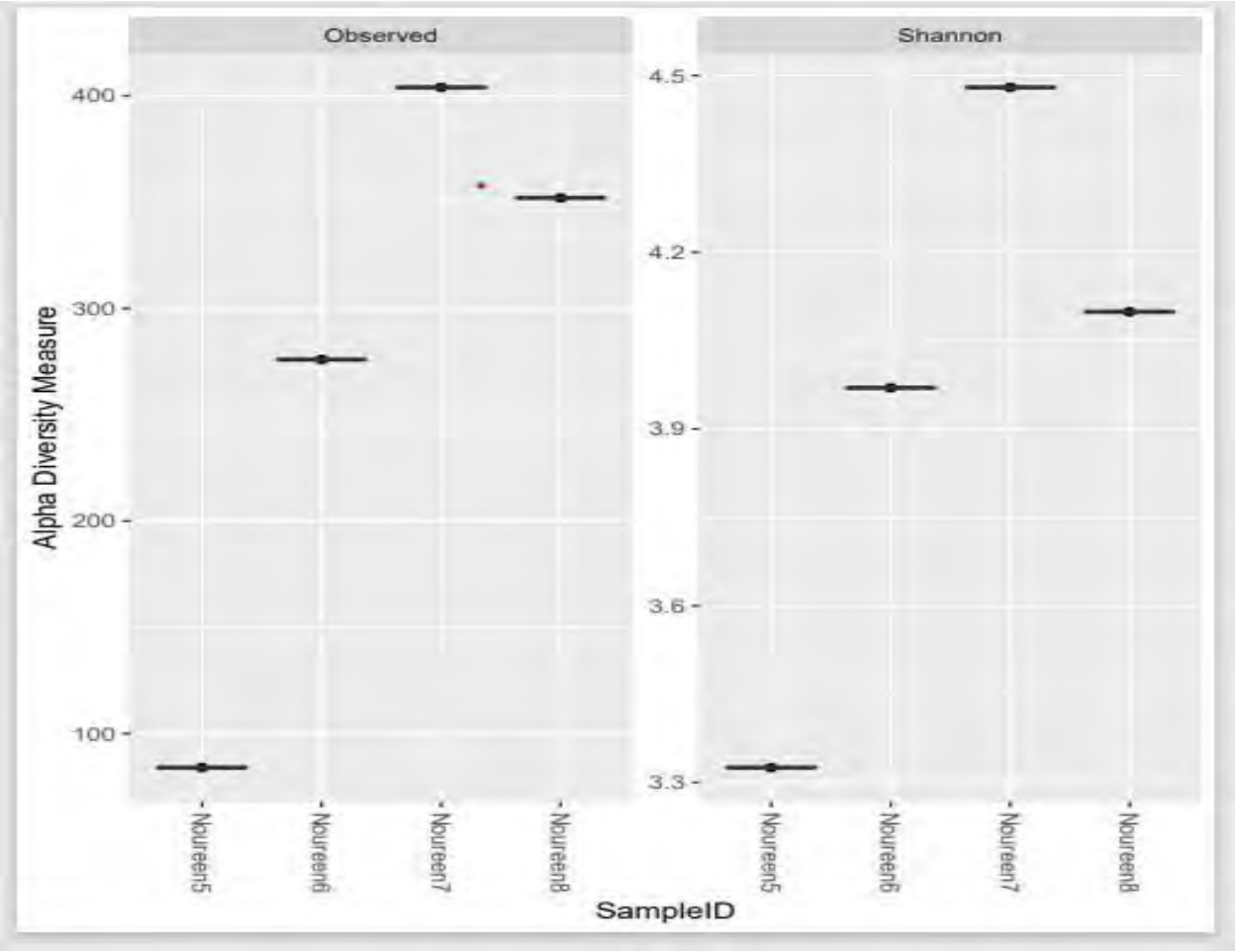


Fig 4.61 Alpha diversity of fungal communities

4.224.8 Taxonomy Tree of bacterial community from Dukki coal mine sample:

Specific species (showing the top 10 genus in high relative abundance by default) were selected to make the taxonomy tree by independently R&D software. Taxonomy tree in single Dukki coal sample is shown in figure below. Total counts that obtained after

sequencing were 49829. At 97% identity, 339 OTUs belonging to 43 species, 130 genera, 108 families, 74 orders, 47 classes and 23 phyla.

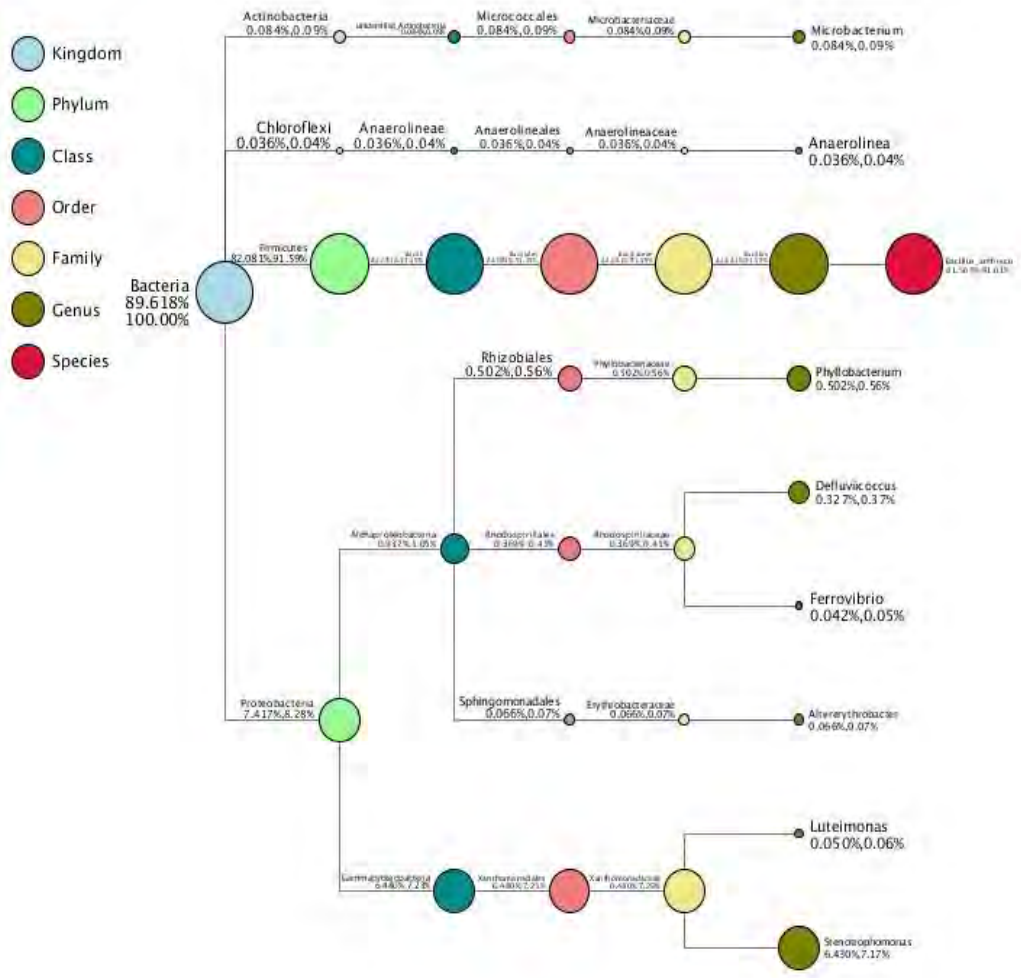


Fig 4.62 Taxonomic tree of Baluchistan sample

4.24.9 GraPhlAn Display

Tree graph of species annotation for each group were construct by GraPhlAn (Asnicar *et al*., 2015). The OTU tree of one group shows in figure 4.63. The OTU annotation tree in green indicates the presence of Actinobacteria. The blue indicates the presence of Firmicutes and red one display the presence of Proteobacteria. The Dukki sample contains more bacterial diversity of Proteobacteria group.

Fig 4.63 OTU annotation tree construct of B3 by GraPhlAn

4.24.10 Krona Display

KRONA visually displays the analysis result of species annotation. Circles from inside to outside stand for different taxonomic ranks, and the area of sector means respective proportion of different OTU annotation results (Ondov *et al*., 2011).

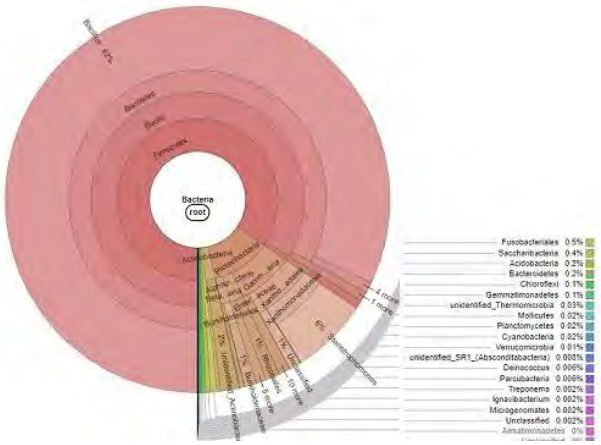
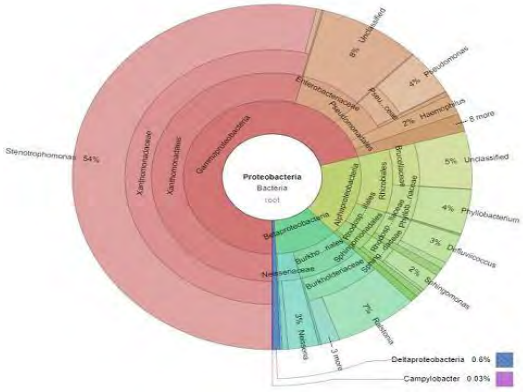
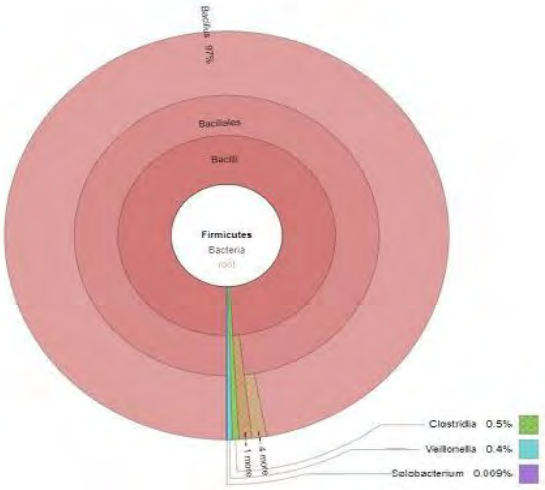


Fig 4.64 Krona display of baluchistan sample



Phylum Proteobacteria

Phylum Actinobacteria



Phylum Firmicutes

(Results Phase 3)
Anaerobic Treatment of Coal

4.25 Anaerobic treatment of Coal

4.25.1 Anaerobic consortium development

For the production of biogas and checking the methane potential of the low and high rank coal the anaerobic consortium was developed. The microorganisms responsible for the production of biogas and methane are anaerobic in nature and it is very difficult to isolate and culture them in laboratory conditions. Strict anaerobic conditions were required for the growth of anaerobic bacteria. Deep Duki and Thar mine water consists of plenty of anaerobic bacterial species which are responsible for coalbed methane generation (CBM). So, in order to develop the anaerobic consortium, the indigenous consortium of deep coal mine and soil water was enriched. Duki and Thar both coal types were added as a sole carbon source. Vitamin and mineral solutions were added to promote the steady growth and nutrient availability within the medium. The whole set up was kept in an anaerobic chamber where no air can get access. The anaerobic consortium set up bottle was kept in dark for an incubation period of 15 days. After the development of anaerobic consortium, it was used for biogas production and ultimately methane potential.

4.25.2 Methane potential of Native Lignite and Bituminous coal

The methane potential of native lignite and bituminous coal was calculated in ml and then converted into micromole/g. The setup was designed with the 10g native coal along with vitamin solution and indigenous anaerobic consortium for about 60 days. It was shown in the figure that the methane potential produced by bituminous coal were 1.7µmol/g at day 15 while its potential increases with time and finally about 2.4 µmol/g were calculated at day 60. Similarly, methane potential of native lignite thar coal was determined and its potential was lesser than bituminous coal at day 60, about 1.2 µmol/g of cumulative methane was generated by lignite coal while the production of gas was negligible in control setup. By analyzing concentration of methane it was observed that the indigenous anaerobic consortium from both coal types have potential of generating more methane with bituminous coal. The low production of methane from bituminous coal might be because of presence of mineral matter in hard rank of coal.

While figure 4.66 shows the potential of Carbon dioxide produced by the native coal samples lignite and bituminous coal. the produced carbon dioxide by lignite coal were lesser than

bituminous coal of Duki coal mine. At day 60 the carbondioxide potential drops while at day 5 increased potential of CO_2 were observed. The control having no coal also showed increase CO as compared to lignite coal but later its potential drops quickly as the time or days passes.

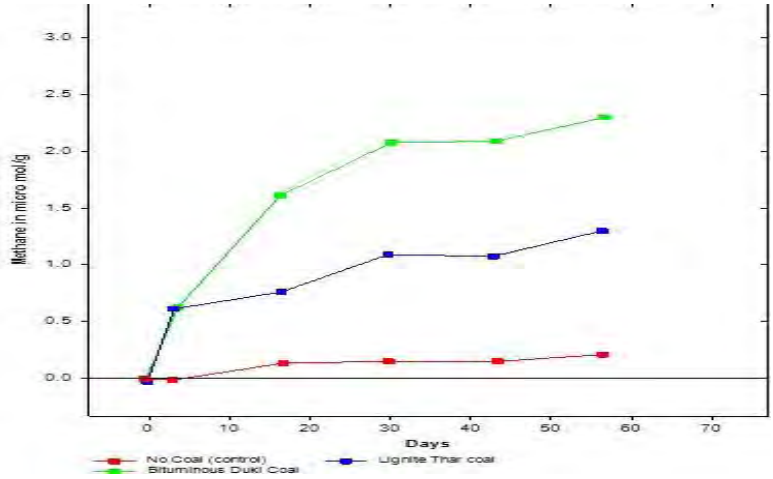


Fig 4.65 Methane potential of Lignite and Bituminous Coal

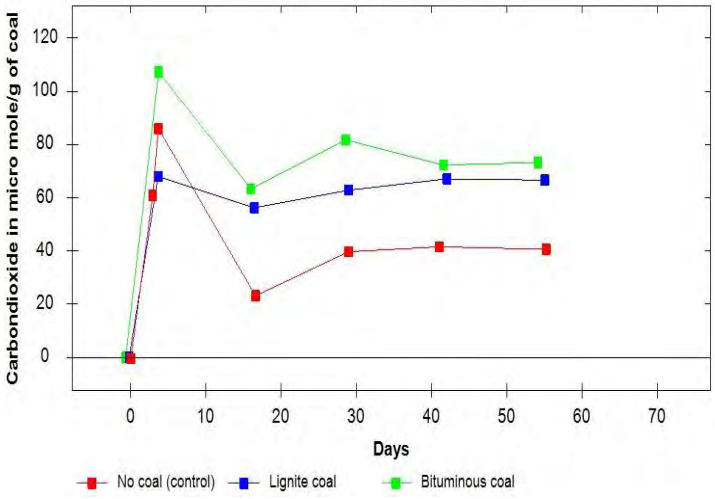


Fig 4.66 Carbondioxide potential of Lignite and Bituminous coal

4.26 Optimization parameters for enhancing the production of methane

4.26.1 Optimization of Temperature

Methanogenic microorganisms have ability to grow in variety of temperatures i.e in marine sediments at 2°C and at geothermal vents above 100°C. There exist a huge diversity of mesophilic and thermophilic microorganisms). Most of the mesophilic methanogens grow at temperatures between 30°C to 37°C while thermophilic methanogen grows above 50°C to 65°C (Bergey and Holt, 1994; Ferry, 1993).

For enhancing the process of biogenic methane generation, the produced indigenous anaerobic consortium was allowed to grow at different temperatures. Different temperatures used in the present study were 25°C, 35°C, 45°C and 55°C. The cumulative methane and produced CO2 were calculated in cc. The optimum temperature of maximum methane yield was recorded 35°C using bituminous coal while more CO2 was produced at 55°C. Overall between temperature 23°C to 37°C methane potential increases but it started to decrease at higher temperatures. Similarly, methane generation of lignite coal were optimized best at 35°C. The microbial community used in this study have potential of producing higher methane at mesophilic condition but above 45°C the methane yield was retarted. Using coal from Duki and thar coal mine the best incubation temperature was characterized between 25°C to 35°C. .

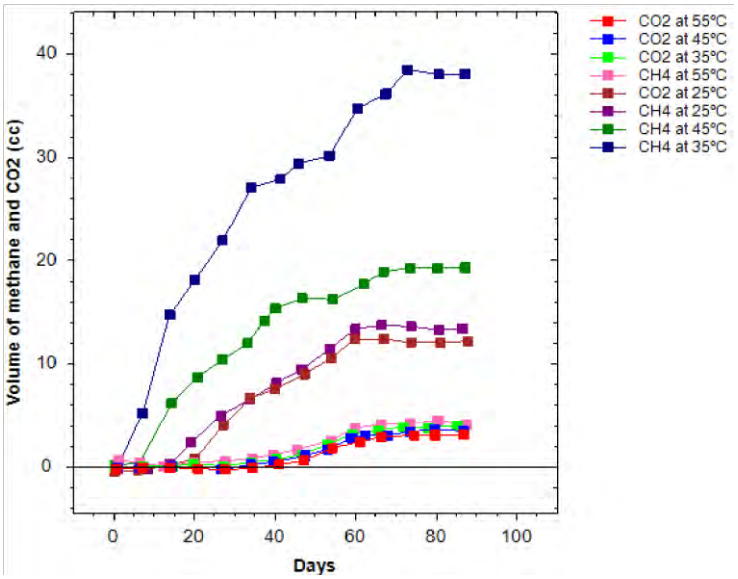


Fig 4.67 Methane and CO_2 produced at different temperatures (Bituminous Coal)

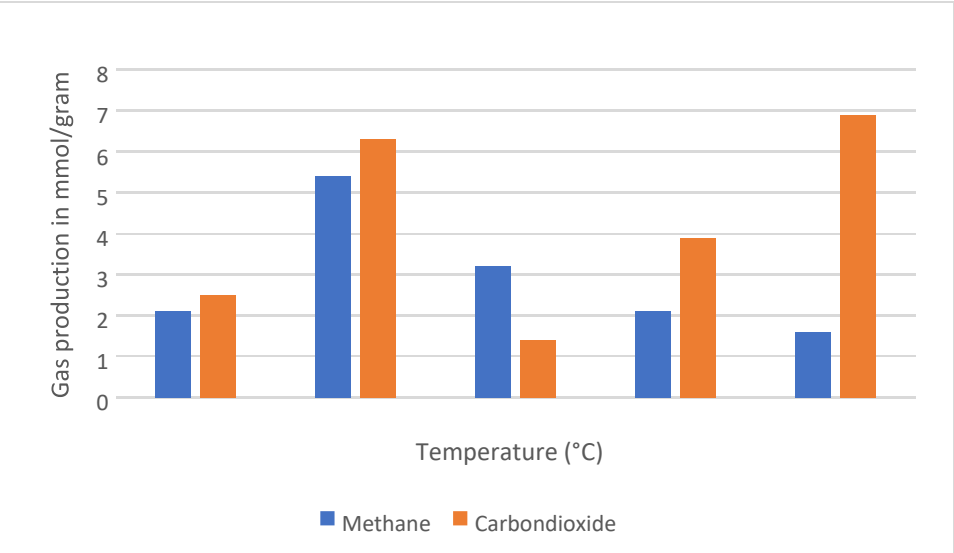


Fig 4.68 Methane and CO Produced by lignite coal at different temperatures

4.26.2 pH optimization

Like all the biochemical processes, pH also has an important effect on production of methane from complex organic matter (Green et al, 2008). When the conditions are far from optimum pH range then there will be a reduced biogenic activity that ultimately limits the gas production. In the microbial communities, methanogens are strongly affected by pH. (Zupancic and grilc, 2012). In the present study, the experiment carried out at various pH between 6.0 to 8.0. The various pH ranges used were 6.0, 6.5, 7.0, 7.5 and 8.0. The maximum methane by bituminous coal was produced at pH 7.5 (23cc). At pH higher than 7.5 there is a decrease in trend of methane production. Similarly, the CO trend at pH 7.0 was optimum 2 (25cc) but above this pH the yield of methane retarded. It is said that the methanogenic communities seem to dominate by neutrophilic species with decreased growth and methane production outside of above mentioned pH ranges (7.5-8.5).

Similarly, the lignite coal showed increase trend in methane production at optimum pH 7.0(19mmol/g) while there is a retard in methane yield above this pH. The CO produced 2 above pH is also low and started to decrease. So, both lignite and bituminous coal showed maximum methane yield between pH 7.0-7.5 while above this pH very less amount of methane was produced. Zhang et al, 2016a conducted a study in which low pH 6.0 and 8.0 was proved to be significant for bituminous coal biogasification by mesophilic microbial anaerobic community.

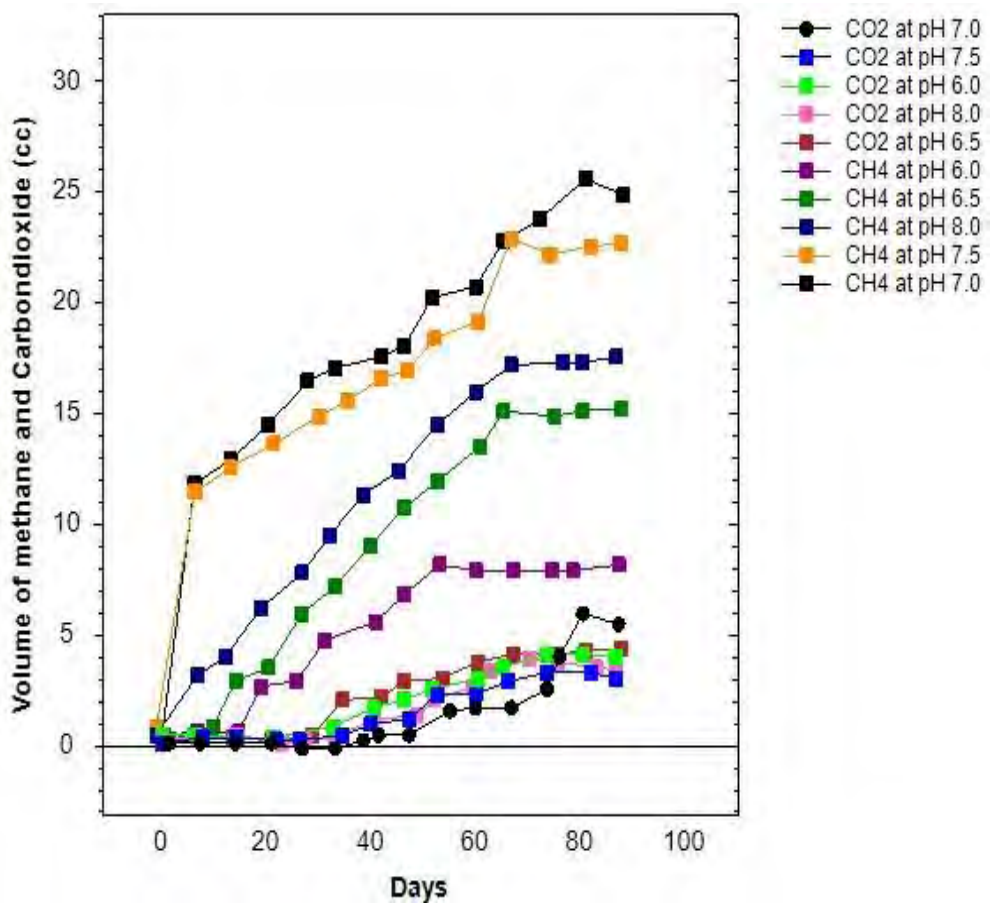


Fig 4.69 Methane and CO_2 produced at different pH (Bituminous coal)

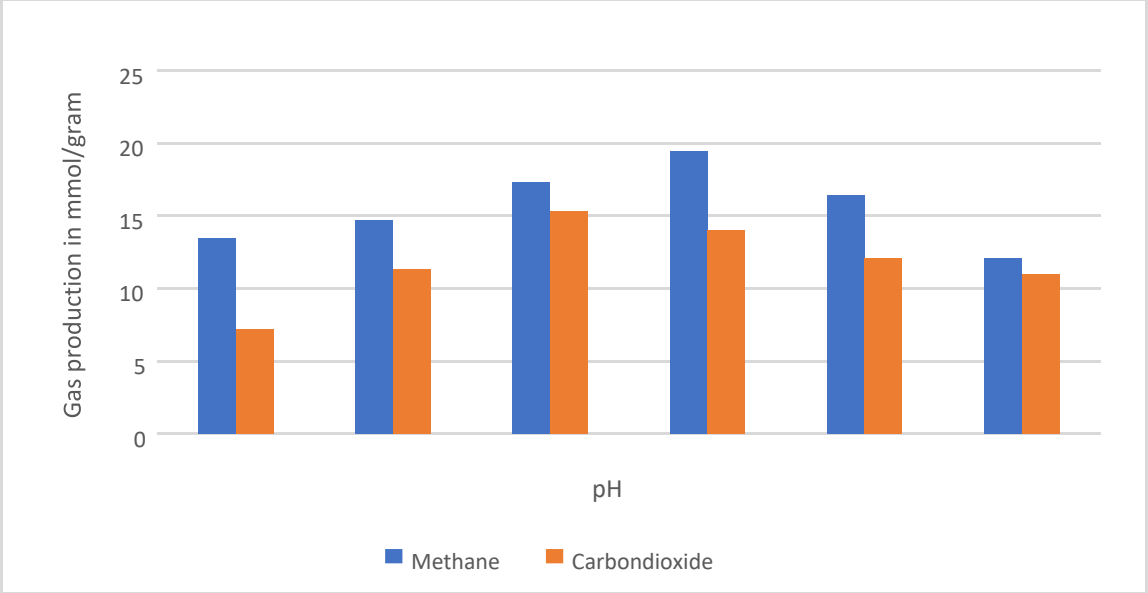


Fig 4.70 Methane and CO_2 Produced by lignite coal at different pH

4.27 Methane and CO production and optimum conditions 2

After evaluating the optimized conditions of temperature and pH the methane and CO2 potential were again checked out at these optimized temperature and pH. The optimum temperature and pH for lignite methanogenic consortium were 35°C and pH 7.5, while for bituminous coal the best optimized temperature was 37°C and pH 7.0. The setup was run on these optimized conditions for investigating out the enhanced methane and CO yield. Under 2 optimized conditions about 44mmol/g of cumulative gas yield were recorded by bituminous coal while the net CO2 production were 21mmol/g at day 60.

Similarly, the methane and CO yield by lignite methanogenic consortium at optimized 2 conditions were 48mmol/g at day 60, while very less yield of CO were recorded (4mmol/g) 2 at day 60 and after 60 days the yield of methane and CO was retarded.

These results at optimized and favorable conditions indicates the suitability of indigenous methanogenic microbial consortium to maximize the production of methane. A number of literature on wide range of methane yield at optimum and favorable conditions using different ranks and microbial consortiums to various strict anaerobic environment had been reported. (Green *et al*., 2008; Gupta and Gupta, 2014; Harris et al., 2008; Jones et al., 2010; Orem et al., 2010; Papendick et al., 2011; Park and Liang, 2016; Wawrik et al., 2012). John Fuertez et al, 2017 also reported the maximize yield of methane at optimized cultivated conditions between pH range (5.5 to 7) and mesophilic temperature 37°C.

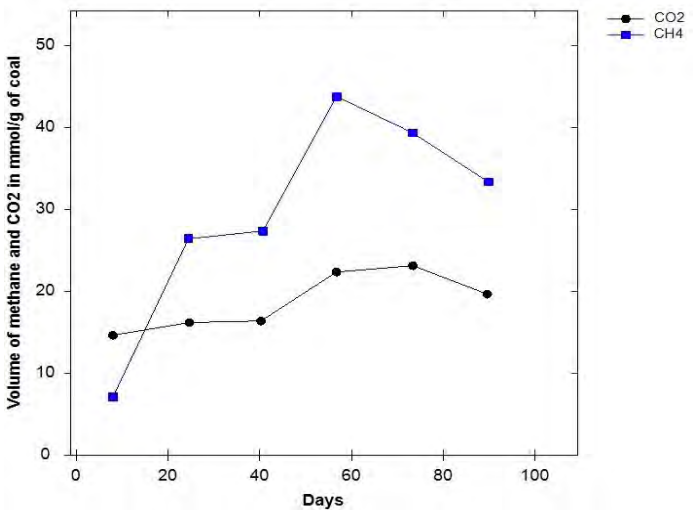


Fig 4.71 Methane and Co2 produced at optimum condition by Bituminous coal

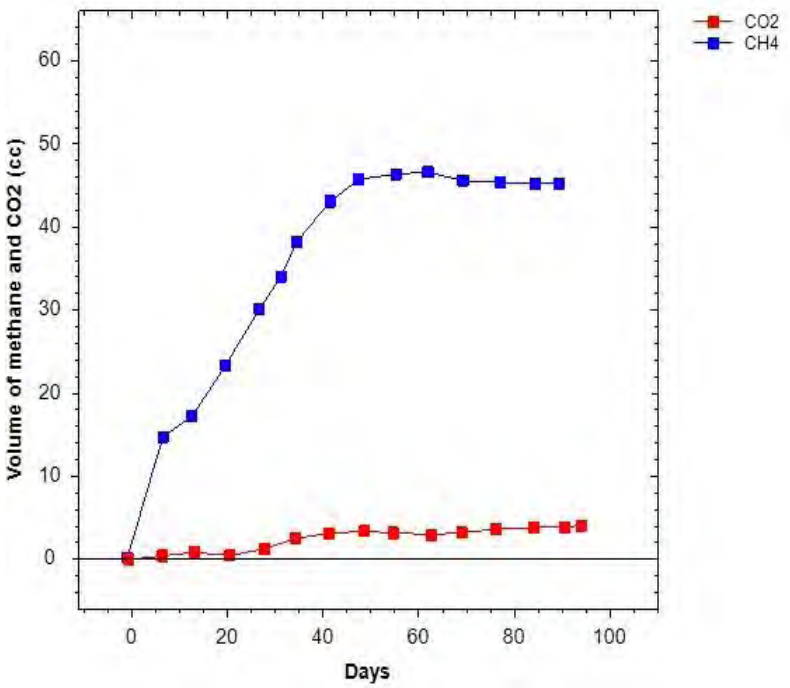


Fig 4.72 Methane and Co2 produced at optimum conditions by Lignite coal

4.28 Enhancement of biogas generation using Co-substrate strategies

For enhancing the process of methane generation, co- substrate strategies were used. The strategies used includes two combinations with each respective coal type i.e. lignite and bituminous.

Combination no. 1: Coal+Grass+Cow dung
Combination no. 2: Coal+ Grass+ peanut shell

4.28.1 Cumulative gas yield of lignite coal with different combinations

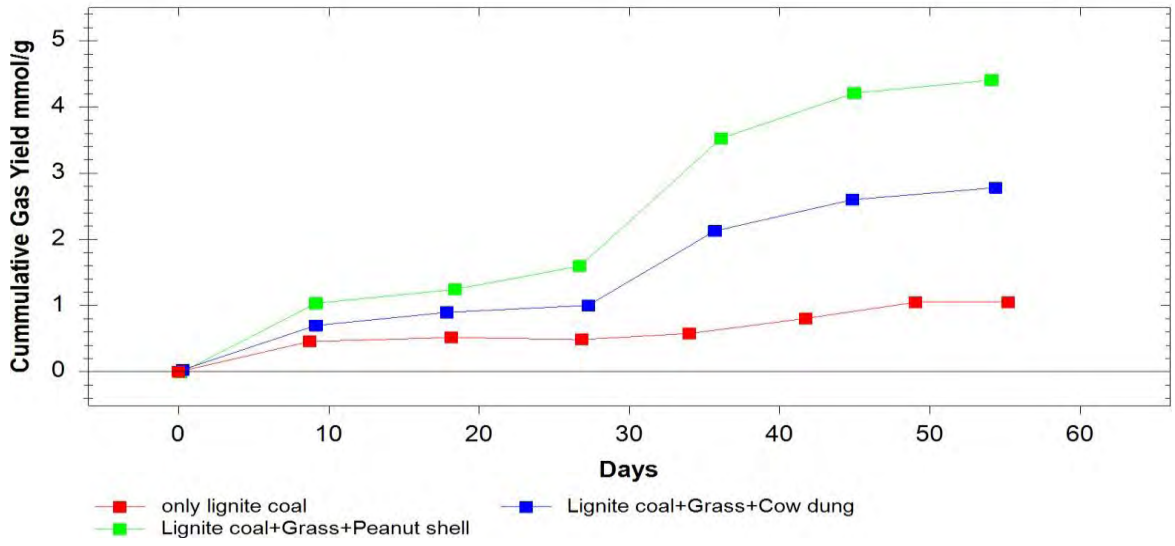


Fig 4.73 Bio gasification Potential of Lignite coal with Co-substrates

The above figure indicates the amount of cumulative gas yield in mmol/g of coal by using lignite coal with combination 1 and combination 2. While the control contains only lignite coal. At start there was very less amount of methane produced but at day 10, combination 2 produces about 1mmol/g of coal that exceeded to 4.9mmol at day 50. But combination 1 produced decrease amount of methane at day 50 which was 3mmol/g. while in control where there was only lignite coal along with vitamin and mineral solution, only small amount of gas was produced. The synergistic effect of combination 2 was more than combination 1.

4.28.2 Cumulative gas yield of bituminous coal with different combinations:

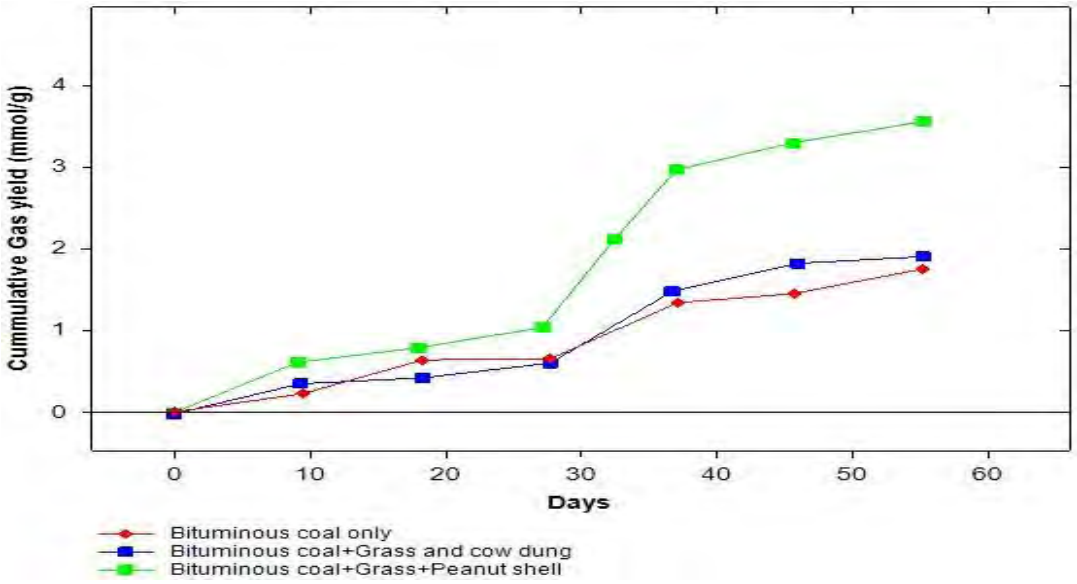


Fig 4.74 Bio gasification Potential of Bituminous coal with Co-substrates

The above figure indicates the amount of cumulative gas yield in mmol/g of coal by using bituminous coal with combination 1 (Grass cow dung) and combination 2 (Grass peanut shell). While the control contains only bituminous coal. At start there was very less amount of methane produced but at day 20, combination 2 produces about 0.9mmol/g of coal that exceeded to 3.5mmol at day 45. But combination 1 produces decrease amount of methane at day 50 which was 1.9mmol/g. while in control there was only bituminous coal along with vitamin and mineral solution, only small amount of gas was produced. The synergistic effect of combination 2 was more than combination 1. One of the studies by Hongyu Guo *et al*, 2018 reported the cumulative gas yield of bituminous coal along with corn straw as compared to control that contains only bituminous coal. The highest methane

production was 2.69 mmol/g with bituminous coal D and 1.35 mmol/g with bituminous coal C.

4.29 FTIR Analysis of the residual coal after co substrate strategies

A small peak is observed at 3300cm-1 which represents the hydroxyl group (OH) along this shoulder that shows variations in hydrogen bonded hydroxyl group in polymeric association. Another small peak was observed at 2800cm^{-1} which represents the asymmetric and symmetric C-H bonds that are CH_2 and CH_3 aliphatic stretching. At 1625cm^{-1} C=C aromatics was observed as symmetric stretching. The stretching at 1450cm represents carboxylic acids showing asymmetric aliphatic C-H deformation of methoxy and methylene groups. This stretching was deep in raw coal and loses its depression in the coal subjected to experimentation which proved that carboxylic acids were consumed by anaerobes to produce gas.

At 870cm shoulder C-H aromatics were present. These aromatics out of plane rings have neighboring two C-H groups. Furthermore, aromatics were detected in the region of 1030-140cm^{-1}. The shoulder of 1030-40cm^{-1} was deep in raw coal and peak became less deep in experimental coal showing the same result as anaerobes consumed the aromatics in gas production, the same was the case observed for 870cm^{-1}peak. While the region of 400-700cm^{-1} includes mineral matters and aromatics.

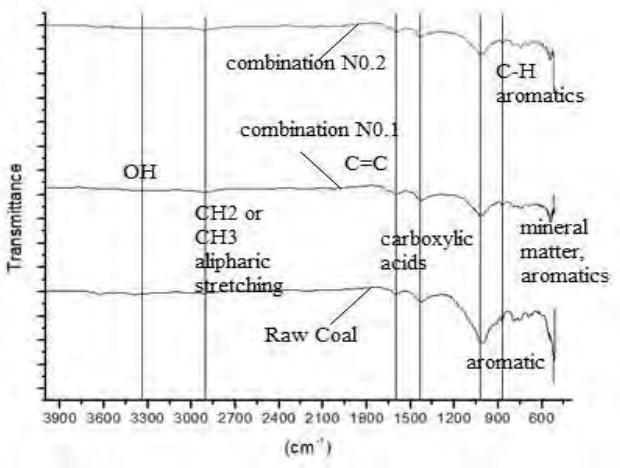


Fig 4.75 FTIR analysis of lignite coal after degrading anaerobically

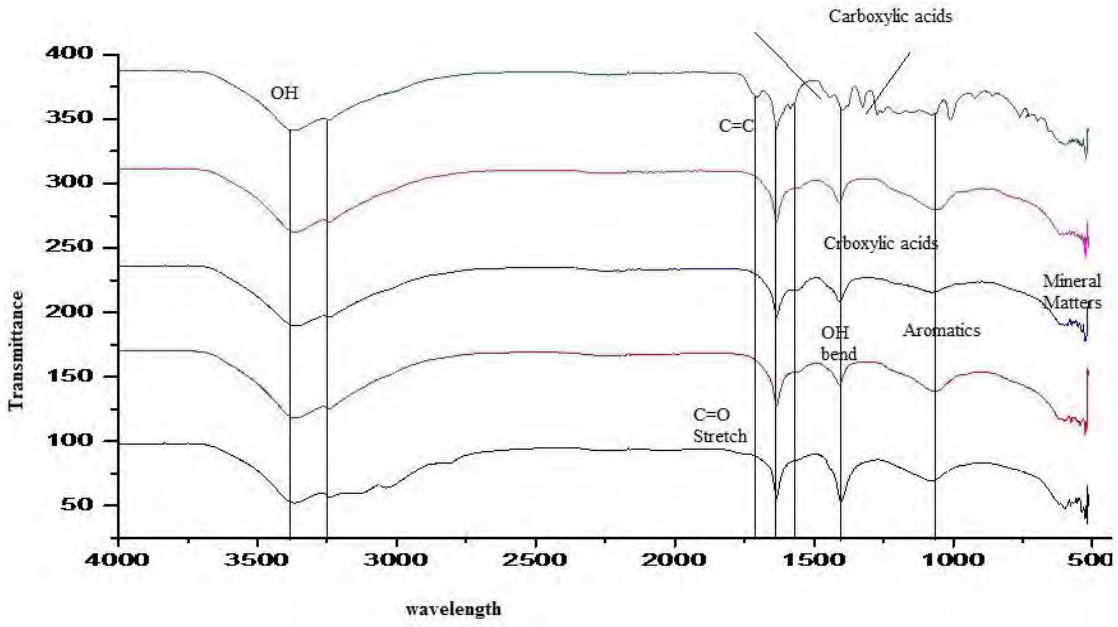


Fig 4.76 FTIR analysis of bituminous coal after degrading anaerobically

Furman found out the chemical compound classes present in residual coal supporting methanogenesis in coal (Furmann *et al*., 2013). At 3300cm peak was observed in raw coal and experimental coal but stretching across experimental coal is less because of the consumption of these groups by anaerobic bacteria. The stretching for asymmetric and symmetric C-H was observed at 2800cm^{-1} .

(Results Phase 4)

Extraction and purification of compound (Humic Acid) and whole genome sequencing of potential coal solubilizing fungal isolates

4.30 Gravimetric analysis of humic acid extracted from bituminous coal

Humic acid was extracted from biologically treated raw and pre oxidize bituminous (Baluchistan) coal supernatants as well as coal residues by following alkali method: 9.5% humic acid was directly obtained from 2g of raw bituminous coal which was used as a control. The ratio of humic acid by fungal (NF-1 and GB) treated pre oxidize coal residues was found to be maximum. A maximum 60% and 78% humic acid was extracted from fungal (NF-1 and GB) treated pre oxidize coal residues respectively. The extraction of humic acid from fungal (NF-1 and GB) treated raw coal was found to be 42% and 56% respectively. When 0.5 g of fungal (NF-1 and GB) treated raw and pre oxidize coal residues was treated with 0.1M alkali solutions these optimum extraction percentages of humic acid were obtained. The ratio of humic acid by fungal (NF-1 and GB) treated raw coal supernatants was found to be 30 and 36% and pre oxidize coal supernatants was found to be 42 and 52% respectively shown in table 4.16

Table 4.16 Humic acid percentage from Raw coal treated with NF-1 and GB

Initial weight of Raw coal residues (g)	0.5	0.5
HA from Raw coal supernatant	0.15	0.18
HA (%) from Raw coal supernatant	30	36
HA from Raw biological treated coal residues (g)	0.21	0.28
Humic acid percentage (%) from Raw biological treated coal residues (g)	42	56

Table 4.17 Humic acid (%) percentage from Pre oxidize coal

Initial weight of pre oxidize coal residues (g)	0.5	0.5
HA from pre oxidize coal supernatant	0.21	0.26
HA (%) from pre oxidize coal supernatant	42	52
HA from pre oxidize biological treated coal residues (g)	0.30	0.39
Humic acid percentage (%) from pre oxidize biological treated coal residues (g)	60	78

4.31 Elemental composition, E4/E6 ratio and Fourier-transform infrared spectroscopy (FTIR) analysis of humic acid

4.31.1 E4/E6 analysis

Structure and composition of humic acid are characterized by using absorbance ratio (E4/E6 at 465 and 665 nm) under alkaline conditions. The amount of humic acid molecules is not associated with E4/E6 ratio. Humic substances usually characterized by absorbances or optical densities of dilute solutions containing between 0.01 to 0.05% (wt/vol) of humic and fulvic acid at 465 and 665 nm E4/E6. UV-Vis spectra were obtained from 200 to 700 nm no characteristic absorption peaks of humic acid were observed until 0.05M NaHCO solution 3 was prepared to dissolve humic for getting constant ratio of E4/E6. Condensed aromatic rings structures, molecular weight and amount of carbon of humic substances has been linked with the (E4/E6) ratio. The E4/E6 ratio showed the degree of humification and regulated by molecular weight of particles, pH and oxygen carbon ratio.

High E /E ratio (2.2 and 3.2) was obtained by treating NF-1 and GB fungal isolates
4 6

with pre oxidize coal residues respectively shown in table 4.19.

Table 4.18 Elemental composition and E4/E6 ratio of alkali extracted HA (control) and HA extracted from raw coal by fungal (NF-1 and GB)

Samples	Elemental analysis Elements (%) C	H	N	O	S	**Atomic** H/C	**Ratio** O/C	UV/VIS E /E 4 6
HA (control)	52.08	3.84	1.26	19.56	6.25	0.88	0.28	1.0296
HA RC (resi) NF-1	51.79	3.49	2.09	22.85	5.94	0.80	0.33	1.1931
HA RC (sup) NF-1	48.01	3.10	2.45	29.25	5.55	0.77	0.45	1.7037
HA RC (resi) GB	50.32	3.33	2.84	28.76	5.23	0.79	0.42	1.7887
HA RC (sup) GB	46.84	3.05	2.43	33.76	5.01	0.78	0.54	2.1894

Table 4.19 Elemental composition and E4/E6 ratio of alkali extracted HA (control) and HA extracted from pre oxidize coal by fungal (NF-1 and GB)

Samples	Elemental analysis Elements (%) C	H	N	O	S	**Atomic** H/C	**Ratio** O/C	UV/VIS E /E 4 6
HA (control)	52.08	3.84	1.26	19.56	6.25	0.88	0.28	1.0296

HA PC (sup) NF-1	49.05	2.81	3.21	34.99	4.54	0.68	0.53	1.9570
HA PC (resi) NF-1	44.3	2.54	3.68	36.56	3.99	0.67	0.61	2.2764
HA PC (sup) GB	47.84	2.69	3.44	37.64	4.31	0.67	0.59	1.8924
HA PC (resi) GB	43.2	2.26	3.94	39.24	3.62	0.62	0.68	3.2068

4.32 Elemental analysis of humic acid

B. mycoides activity has effect on O/C, H/C and C/N ratio of extracted HA. reported increase in oxygen content of oxidized HA than control HA. Jiang *et al*. (2013) documented higher nitrogen ratio of *Bacillus* sp. Y7 HA than HA. Biological processes showed significant differences in elemental composition of extracted humic compounds. Humic acid (HA) after treating virgin bituminous coal particles with NF-1 and GB fungal isolates showed low carbon and hydrogen content but high oxygen and nitrogen ratio. HA obtained after treating pre oxidize (introduce oxygen and decrease carbon value) bituminous coal particles with NF1 and GB fungal isolates showed greater reduction in carbon and hydrogen ratio and higher content of oxygen and nitrogen shown in table 4.19.

4.33 FT-IR analysis of humic acid (HA) derive from virgin coal by NF-1

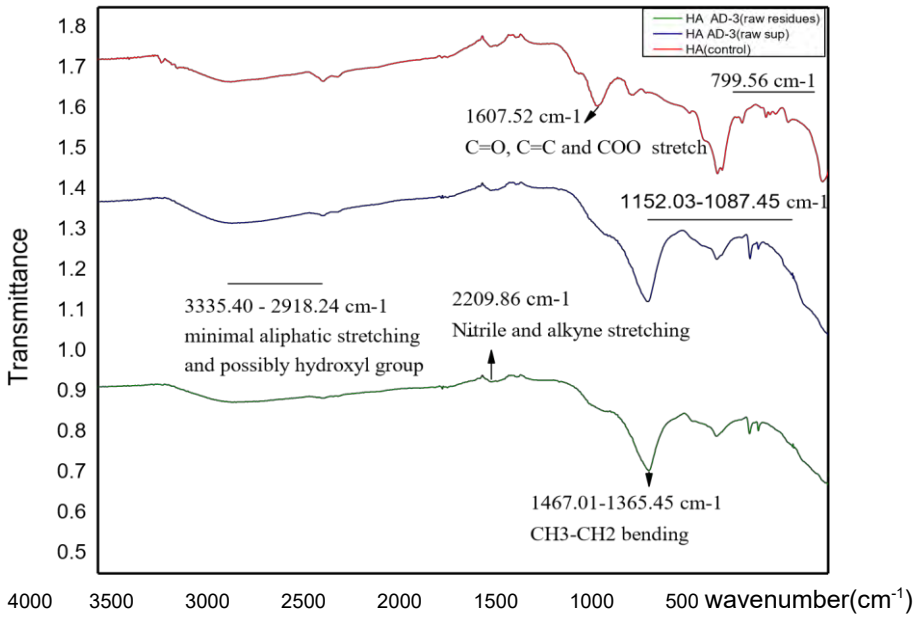


Fig 4.77 IR spectra of Alkali-solubilized humic acid HA using raw coal (control sample)

FT-IR analysis was carried out for the investigation or detection of functional groups and vibrational changes occurs in Alkali-solubilized humic acid extracted directly from Baluchistan coal designated as HA (control sample), NF-1 fungal treated humic acid in bio solubilized liquid designated as HAL and coal residues (modified with humic acid) designated as HAF in the range of 4000-400cm^{-1} on Perkin Elmer, USA with (15 scan and 1cm resolution rate) shown in figure 4.77. The characteristic peaks observed in the residual (HAF) spectra at 3335.40 and 2918.28 cm^{-1} corresponds to sp –C-H stretch and -OH stretch respectively due to the presence of humic acid functionalities. The peaks at this region was also observed in HA (control sample) and HAL (bio solubilized liquid) show similar intensity as residual (HAF). The C=C, COO and C=O stretch shows peak at 1607.52 cm^{-1} in HA (control) which is absent in HAF and HAL. The peak at 2209.86 cm^{-1} corresponds to nitrile and alkyne stretching which is present in all humic spectra. The CH3-CH2 bending shows peak at 1467.01cm^{-1} and 1365.45cm^{-1} in HAF and HAL but absent in HA (control) due to presence of methylene, alkane and methyl groups present in compound. The peak at 1152.03-1087.45 cm^{-1} shows ether compounds that are not observed in HA (control). In addition, the remaining peaks that are observed in HA control in the region of 900-600cm^{-1} show the presence of inorganic moieties. The absence of peaks at 799.56cm^{-1} in HAF and HAL may suggest aromatic breakdown. These all above discussed peaks gives confirmation of formation of coal based humic compound by the action of NF-1 fungal isolate.

4.34 FT-IR analysis of humic acid (HA) derive from pre oxidize coal by NF-1

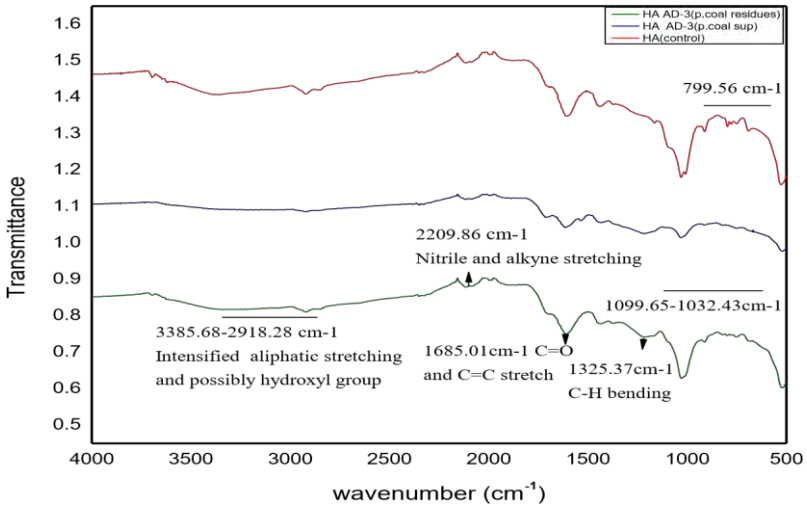


Fig 4.78 IR spectra of Alkali-solubilized humic acid HA (control sample), humic acid in bio solubilized liquid (HAL) and NF-1 fungal treated pre oxidize coal residues (HAPF) red: reference HA; blue: HAL green: HAPF

FT-IR analysis was carried out for the investigation or detection of functional groups and vibrational changes occurs in Alkali-solubilized humic acid extracted directly from Baluchistan coal designated as HA (control sample), NF-1 fungal treated humic acid in bio solubilized liquid designated as HAL and pre oxidize coal residues (modified with humic acid) designated as HAPF in the range of 4000-400cm^{-1} on Perkin Elmer, USA with (15 scan and 1cm resolution rate) shown in figure 4.34. The intensified characteristic peaks observed 2in the residual (HAPF) spectra at 3385.68cm^{-1} and 2918.28 cm^{-1} corresponds to sp2 –C-H stretch and -OH stretch respectively due to the presence of enhanced humic acid functionalities. The peaks at this region were also observed in HA (control sample) but spectra of HAL (bio solubilzed liquid) show low intensity. The C=C and C=O stretch shows peak at 1685.01 cm^{-1} in HAPF and HA (control sample). The peak at 2209.86 cm^{-1} corresponds to nitrile and alkyne stretching which is present in all humic spectra. The CH_2-CH_3 bending shows peak at 1325.37 cm^{-1} in HAPF and HAL (bio solubilized liquid) due to presence of carboxylic groups. In addition, the remaining peaks that are observed in (HAPF and HA control sample) in the region of 1099.65-1032.43cm^{-1} show the presence of ethers. The absence of peaks at 799.56cm^{-1} in HAL may suggest aromatic breakdown. Pre oxidation of raw coal enhances humic production by increasing content of oxygen and nitrogen containing functional groups as well as the content of OH and NH groups in both HAPF and HAL by the action of NF-1 fungal isolate. These all above discussed peaks gives confirmation of enhanced formation of coal derive humic compound by the action of NF-1 fungal isolate.

4.35 FT-IR analysis of humic acid (HA) derive from virgin coal by GB

FT-IR analysis was carried out for the investigation or detection of functional groups and vibrational changes occurs in Alkali-solubilized humic acid extracted directly from Baluchistan coal designated as HA (control sample), GB fungal treated humic acid in bio solubilized liquid designated as HAL and coal residues (modified with humic acid) designated as HAF in the range of 4000-400cm^{-1} on Perkin Elmer, USA with (15 scan and 1cm resolution rate) shown in figure 4.79.The minimal characteristic peaks observed in the residual (HAF) spectra at 3276.02 and 2918.28 cm corresponds to sp_2 –C-H stretch and OH stretch respectively due to the presence of humic acid functionalities. The peaks at this region were also observed in HA (control sample) but spectra of HAL (bio solubilized liquid) show

high intensity than residual (HAF). The C=C, COO and C=O stretch shows peak at 1629.91 cm^{-1} in HA (control) and HAL. The peak at 2209.86 cm^{-1} corresponds to nitrile and alkyne stretching which is present in all humic spectra. The CH bending shows peak at 1487.03 cm^{-1} which is more intense in HAL (bio solubilized liquid) due to presence of methylene, alkane and methyl groups present in compound than HAF. In HA (control) and HAL at 1099.65-1032.43 cm^{-1} peaks shows ether compounds that are not observed in HAF.

In addition, the remaining peaks that are observed in HA control and HAL in the region of 900-600cm^{-1} show the presence of inorganic moieties. The absence of peaks at 799.56cm^{-1} in HAF may suggest aromatic breakdown. Pre oxidation of raw coal enhances humic production by increasing content of oxygen and nitrogen containing functional groups as well as the content of OH and NH groups both in pre oxidized residual coal and liquid by the action of GB fungal isolate. These all above discussed peaks gives confirmation of enhanced formation of coal based humic compound by the action of GB fungal isolate.

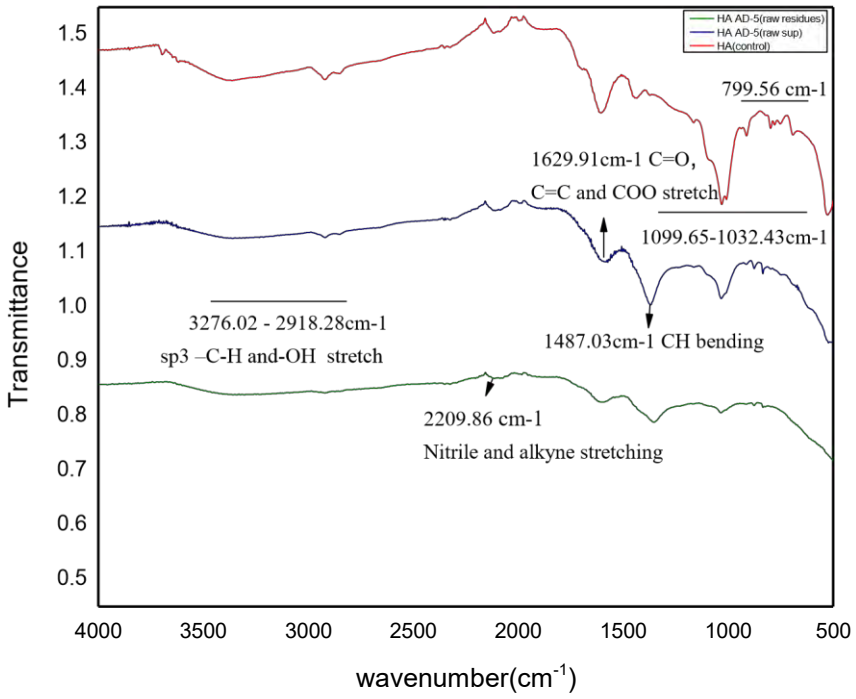


Fig 4.79 IR spectra of Alkali-solubilized humic acid HA (control sample), humic acid in bio solubilized liquid (HAL) and GB fungal treated coal residues (HAF) red: reference HA; blue: HAL green: HAF

4.36 FT-IR analysis of humic acid (HA) derive from pre oxidize coal by GB

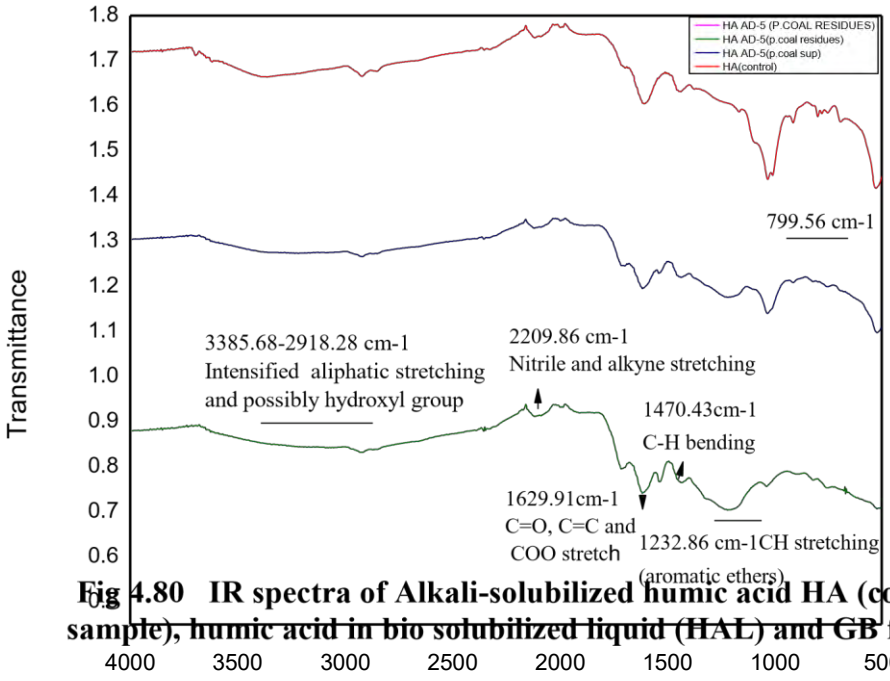


Fig 4.80 IR spectra of Alkali-solubilized humic acid HA (control sample), humic acid in bio solubilized liquid (HAL) and GB fungal treated pre oxidize coal residues (HAPF) red: reference HA; blue: HAL green: HAPF

FT-IR analysis was carried out for the investigation or detection of functional groups and vibrational changes occurs in Alkali-solubilized humic acid extracted directly from Baluchistan coal designated as HA (control sample), GB fungal treated humic acid in bio solubilized liquid designated as HAL and pre oxidize coal residues (modified with humic acid) designated as HAPF in the range of 4000-400cm^{-1} on Perkin Elmer, USA with (15 scan and 1cm resolution rate) shown in figure 4.80. The intensified characteristic peaks observe in the residual (HAPF) spectra at 3385.68 and 2918.28 cm corresponds to sp_2 –C-H stretch and -OH stretch respectively due to the presence of enhanced humic acid functionalities. The peaks at this region were also observed in HA (control sample) but spectra of HAL (bio solubilized liquid) show low intensity. The C=C, COO and C=O stretch shows peak at 1629.91 cm^{-1} in HAPF and HAL. The peak at 2209.86 cm^{-1} corresponds to nitrile and alkyne stretching which is present in all humic spectra. The CH bending shows peak at 1470.43 cm^{-1} in HAPF and HAL (bio solubilized liquid) due to presence of methylene, alkane and methyl groups present in compound. In HAPF spectra CH stretching at 1232.86 cm^{-1} shows aromatic ethers but absent in HA and HAL. In addition, the remaining peaks that are observed in HA

control and HAL in the region of 900-600cm^{-1} show the presence of inorganic moieties. The absence of peaks at 799.56cm in HAPF may suggest aromatic breakdown. Pre oxidation of raw coal enhances humic production by increasing content of oxygen and nitrogen containing functional groups as well as the content of OH and NH groups both in HAPF and HAL by the action of GB fungal isolate. These all above discussed peaks gives confirmation of enhanced formation of coal based humic compound by the action of GB fungal isolate.

4.37 Whole Genome sequencing of potential fungal isolates NF-1 and GB

4.37.1 DNA extraction of fungal isolates

For whole genome sequencing of potential coal solubilizing fungal isolates, DNA extraction was performed. The kit method was used for attaining maximum concentration of DNA. After extracting DNA using manufacturers instruction, the gel electrophoresis was performed for confirming the DNA bands. The ladder was run for confirming the actual size of DNA. The figure 4.81 below shows the DNA bands from potential coal solubilizing fungal isolates.

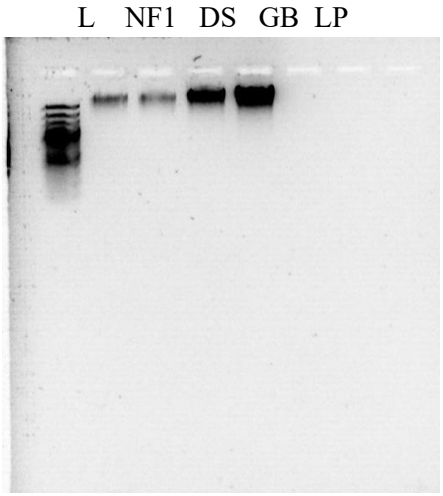


Fig 4.81 DNA extraction of potential fungal isolates

4.37.2 Tape station analysis

The agilent tapestation system is basically an automated electrophoresis solution for testing the sample quality control of DNA and RNA samples. The screen tape devices are used to confirm the DNA quality and concentration present in the sample. The figure 4.82 shows the tapestation of fungal isolates. A1 is the ladder, while B1 represents NF-1 and C1 represents isolate GB. The size and concentration of DNA is shown below in the table. The final concentration of extracted DNA of isolate NF-1 is 0.807 while GB shows the final concentration as 3.38. The band size of both the strains are shown in graph.

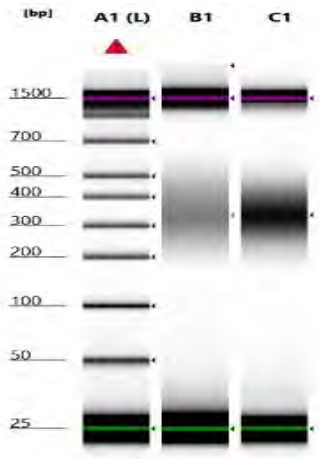


Figure 4.82 Tape station analysis of potential fungal isolates

4.37.3 Library Preparation

After confirming final concentration and size of DNA. Fungal library was prepared by using NEBNext Ultra II FS DNA kit. The manufacturer's instructions were followed for preparing the library. The steps followed were:

1.Fragmentation

2.Adaptor Ligation

3.Size selection of adaptor ligated DNA

4.PCR Enrichment of Adaptor ligated DNA

After following the above steps, the PCR was run using adaptor primers and later on PCR cleanup process was followed and final concentration of fungal libraries was calculate using Qubit Assay.

4.37.4 QUBIT assay analysis after preparing fungal libraries

After preparation of fungal libraries, QUBIT assay analysis was performed for quantifying the final DNA concentration of prepared libraries. The final concentration of isolate NF-1 was 1.79 ng/µl and GB had final concentration of 6.23 ng/µl.

Table 4.20 QUBIT Assay values of extracted DNA and concentration after library preparation

Sample ID	QUBIT values after extracting DNA (ng/µl)	QUBIT DNA concentration after preparing FS fungal libraries (ng/µl)
D (NF-1)	24.8	1.70
DS	25.2	1.89
GB	79.6	6.23

4.38 Sequencing and Data analysis

After determining final concentration of DNA by preparing libraries, then sequencing was performed using QIA seq whole genome sequencing kit at Aarhus University, Denmark. The machine used were illumina Miseq system. About 5-10ng of input material was used and library was sequenced at 10X sequencing coverage.

The raw data obtained after sequencing were further statistically analyzed using software Geneious prime.

4.39 Whole genome sequence of isolate NF-1 identified as *Debaromyeces Hansenii*

4.39.1 Circular view of sequence

The figure below shows the circular view of the ***Debaromyeces Hansenii*** sequences having 729 bases. The GC content is also shown inside the circular view of the sequence. The statistical analysis of the whole genome is shown below in the table.

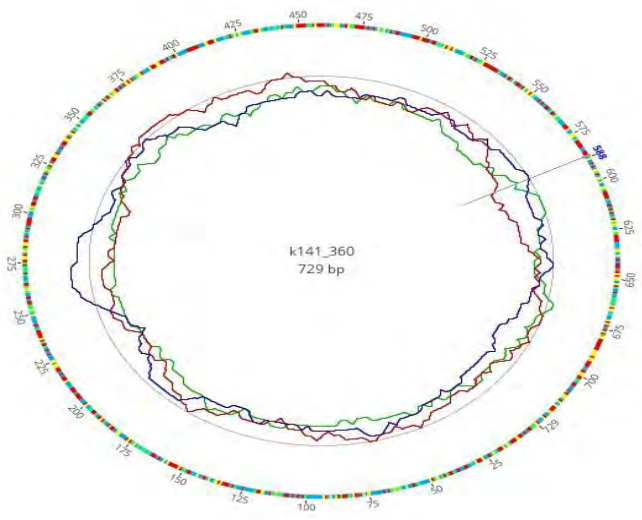


Figure 4.83 Circular view of genome sequence of isolate *Debaromyeces Hansenii*

Nucleotide statistics of all the sequences:

The total base pairs of isolate *Debaromyeces Hansenii* is 722,974bp and total number of sequence are 516. Length of 516 sequences:

- Mean: 23562.8
- Standard deviation:66503.1
- Minimum: 202
- Maximum: 722974

Table 4.21 Nucleotide statistics of sequences of *Debaromyeces Hansenii*

Nucleotide bases	Frequency	Percentage (%)

A	3,880,651	31.9
C	2,207,887	18.2
G	2,203,046	10.1
T	3,866,825	31.8
GC	4,410,933	36.3
All	12,158.409	100

The figure below shows the sequence view of isolate NF-1, the sequences containing all the bases are shown from nucleotides k141 – 528.

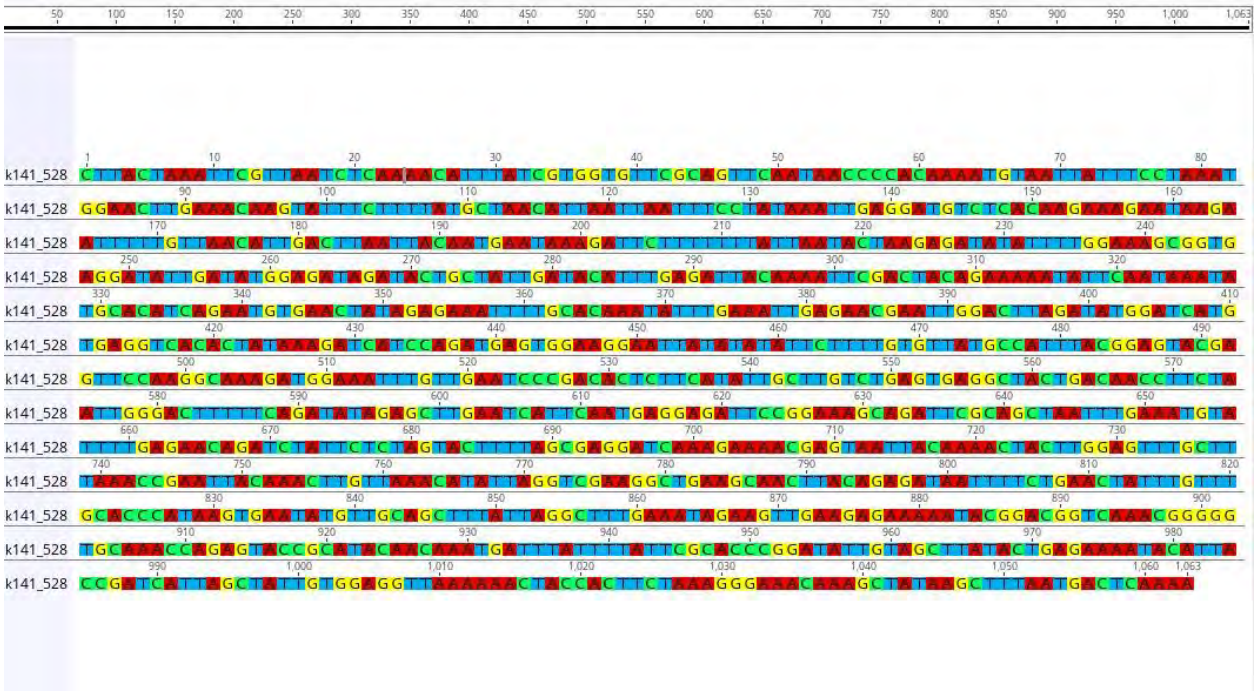


Figure 4.84 Sequence view of *Debaromyeces Hansenii*

4.40 Whole Genome sequencing of isolate GB identified as *Aspergillus Ochraceus*

4.40.1 Circular view of sequence of *Aspergillus Ochraceus:*

The total number of base pair shown are 301bp and the nucleotide sequence is mentioned from k141-869. The statistical analysis of the genome along with known GC content is shown below in the table:

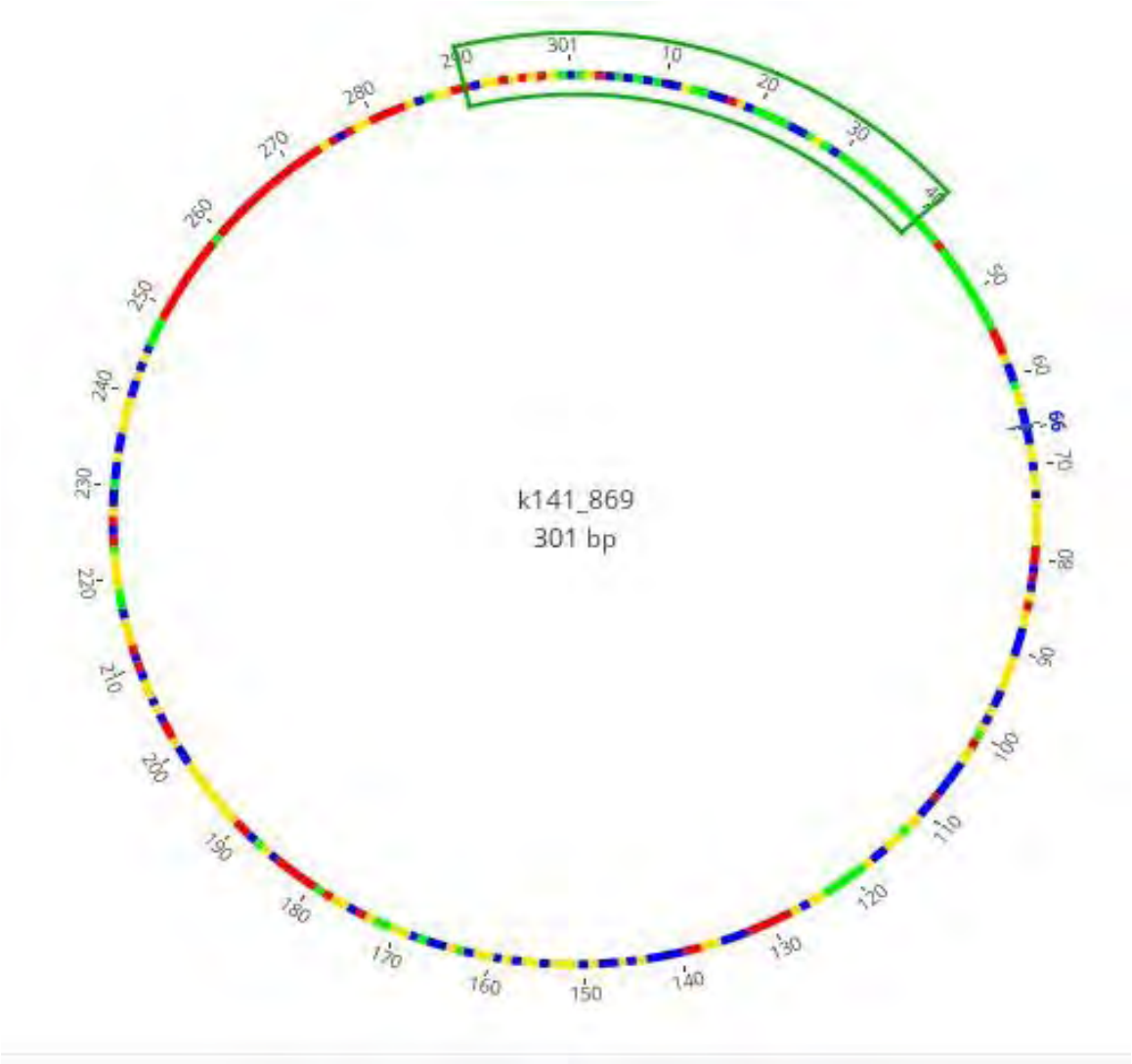


Figure 4.85 Circular view of genome sequence of isolate *Aspergillus Ochraceus*

The total number of sequences lengths include 1781 sequences.
Length of 1781 sequences: Mean:
20381.1
Standard deviation: 37039.3
Minimum sequences: 201
Maximum sequence: 334006

Table 4.22 Nucleotide statistics of sequences of *Aspergillus Ochraceus*

Nucleotide Bases	Frequency	Percentage
A	9,155,684	25.2
C	9,022,176	24.9
G	9,008,507	24.8
T	9,112,448	25.1
GC	18,030,683	49.7
All	36,298,815	100

4.40.2 Sequence view of *Aspergillus Ochraceus*

The sequence view of ***Aspergillus Ochraceous*** is shown below in the figure 4.86 along with the GC content.

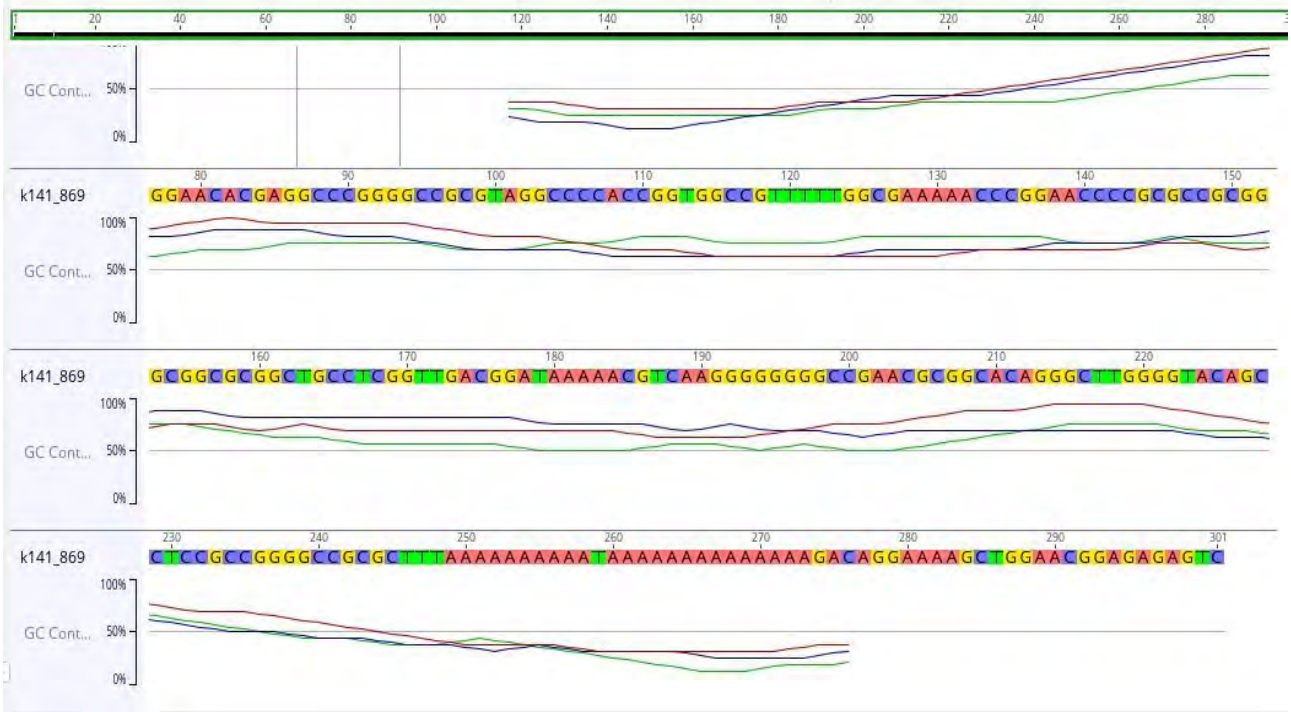


Figure 4.86 sequence view of isolate *Aspergillus Ochraceus*

4.41 Annotations

4.41.1 Annotated sequences of *Debaromyeces Hansenii*

The annotation and track study of *Debaromyeces Hansenii* predicted the following statistics of annotated genes.

Table 4.23 Annotation and track study of *Debaromyeces Hansenii*

CDS	842
No. of genes	762
Initial	72
Internal	8
Intron	80
Single	690
Start Codon	762
Stop codon	762
Terminal	72
Transcript	762

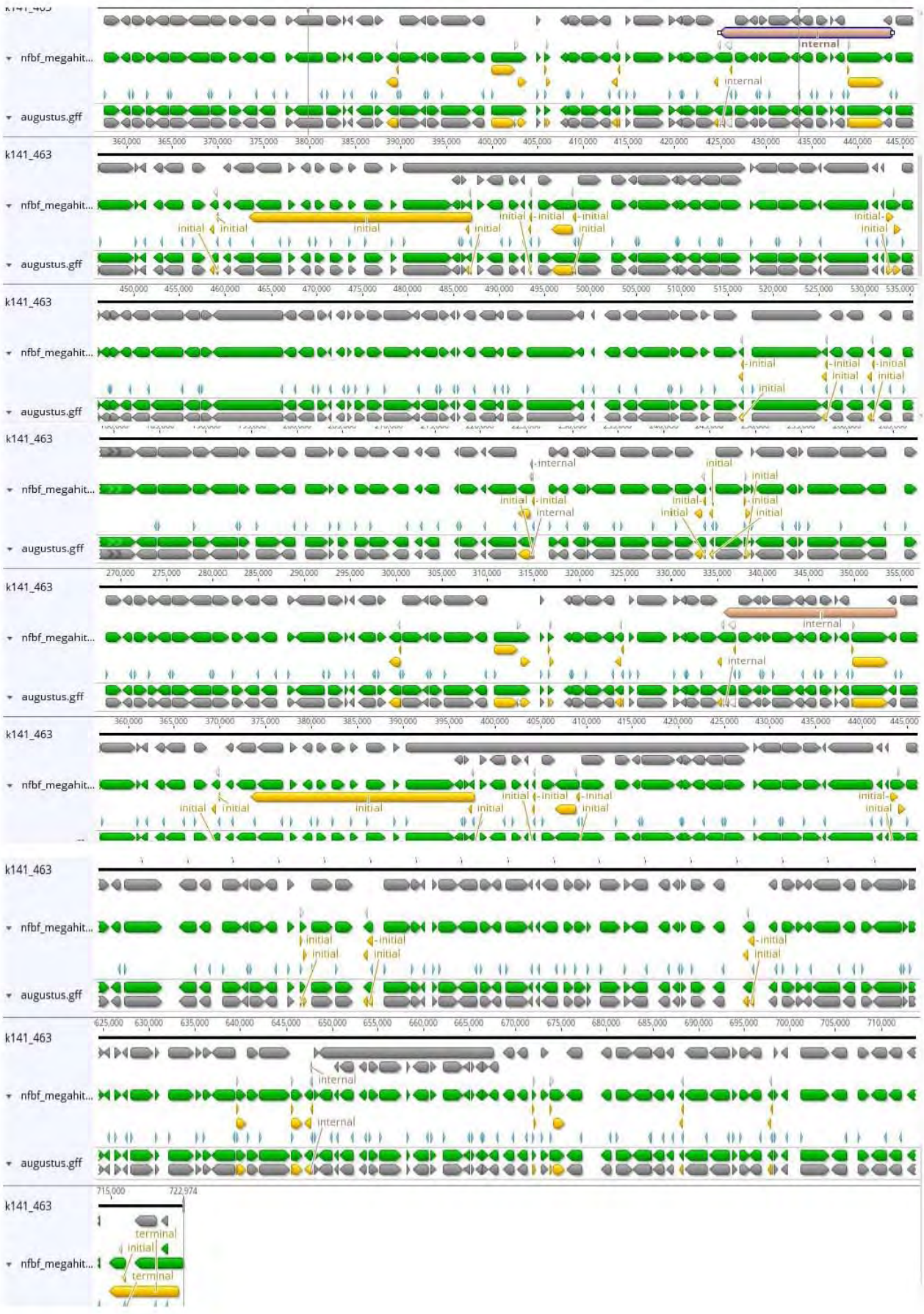

k141_463
nfbf_megahit...
augustus.gff
internal
initial
terminal

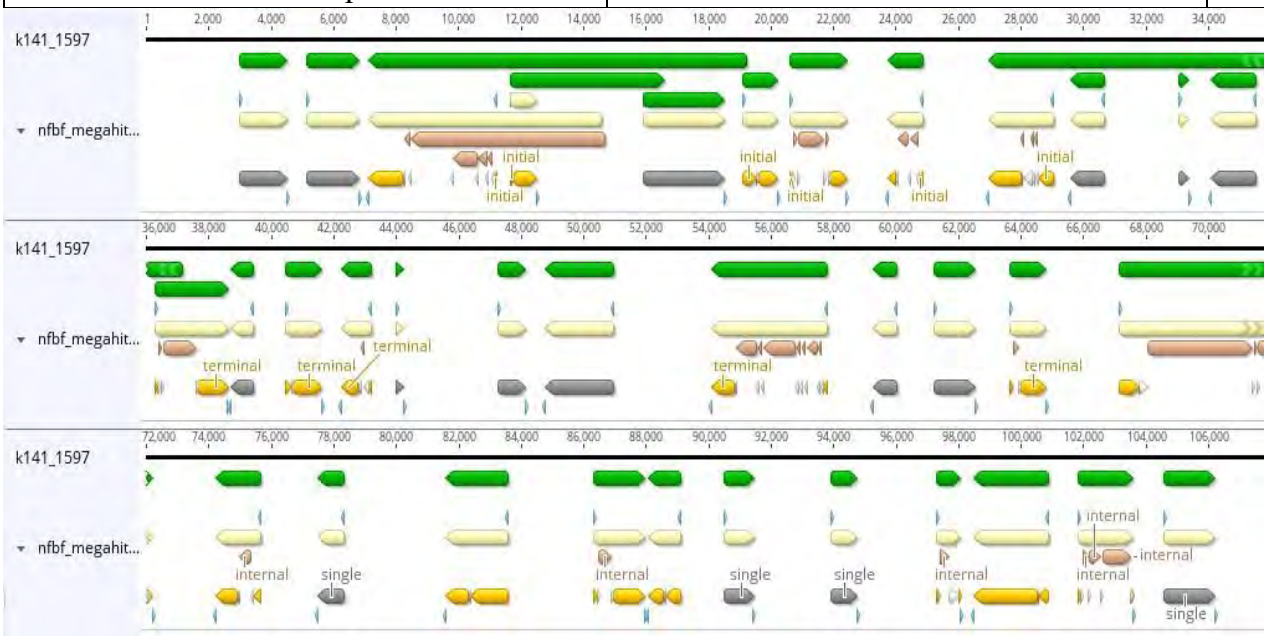
Figure 4.87 Annotations of *Debaromyeces Henseni*

4.41.2 Annotated sequence of *Aspergillus Ochraceus*

The annotation and track study of *Aspergillus Ochraceus* predicted the following statistics of annotated genes. The total length of gene predicted were 10,640.

Table 4.24 Annotation and track study of *Aspergillus Ochraceus*

CDS	421
Gene	381
Initial	36
Internal	4
Intron	40
Single	345
Start Codon	381
Stop codon	381
Terminal	36
Transcript	381



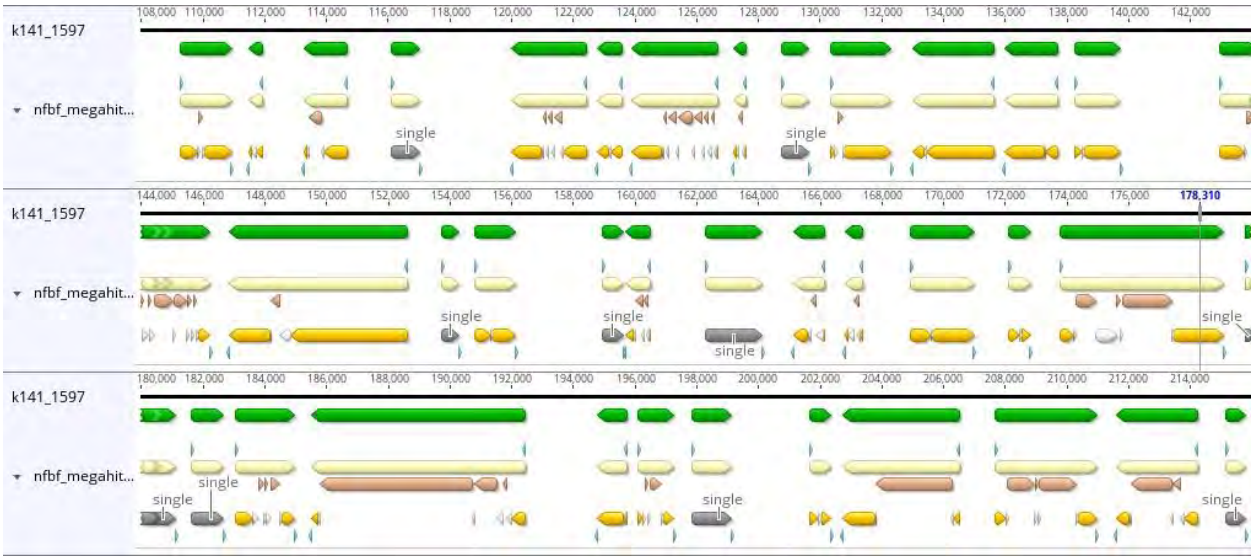


Figure 4.88 Annotations of *Aspergillus Ochraceus*

Chapter 5

Discussion

Pakistan has blessed with diverse range of indigenous coal assets (mostly low rank coal reserves). Soft coal poorly suited to direct combustion have been reported numerously for biological benefactions as it is very sensitive (similarities with lignin), highly hydrophilic and oxygenated. Hard coal has high fuel value as it lights on fire easily. Bituminous coal suited to direct combustion due it to heating value liberates more pollution into the air and its left-over cause adverse problems that could be biologically transformed. In Pakistan biological transformations of soft coal have been studied extensively. A unique and multifarious microbial system is required for the Conversion of hard (bituminous) coal into chemicals and variety of organic intermediates like Humic components.

In present study, the sampling of coal was conducted from the coal fields of Thar Sindh and Dukki Baluchistan region of Pakistan. Typically, the Sindh coals are less than 3 meters in thickness but the main seam intercepted in Thar was over 29m in thickness (San Filippo *et al* 1989; San Filipo *et al,* 1994). The coal fields of Thar were mainly composed of Eocene sedimentary rock, dune sand, alluvium and Paleocene (Rizwan *et al,* 2014). Mostly, the age of thickest coal in the Thar reported by Ahmed and Zaigham in 1993 were of Jurassic age, However, these coal strata may belong to late Paleocene to early Eocene age based on Palynological studies in close vicinity of the sample used in present study TC (Ahmed *et al,* 1993). One of the studies conducted by Fausset and Durrani in 1994 also reported the age of Thar coal fields of Paleocene to early Eocene.

After Khan *et al,* 1987 estimated about 49mt of sand stone and shale lithology of coal out of which 13.7 mt are measured and 10.8 indicated and 24.5mt as inferred reserves. Malkani in 2012 showed about 80.4mt Duki-Anamber coal fields reserves. Out of these, Takhri Anamber coal field shows 5.6mt and Duki coal field showed 74.8mt. The measured Duki coal field reserves are 21.6mt with indicated reserves of 43.2mt and inferred reserves at 20mt. Signature features of bituminous coal (Baluchistan coal) and Thar (Sindh coal) enforced its effective transformation into non-fuel. The characterization of coal samples was investigated on the basis of which their elemental and proximate analysis along with atomic ratios was calculated. In present study, the atomic ratio with respect to (O/C and H/C) of all the selected coal samples varies. The H/C ratios ranges from 0.07 to 0.11 in case of lignite Thar coal samples, while for Baluchistan bituminous coal sample the ratio lies in the range between 0.11 to 0.19. Similarly, O/C ratios varies from 0.45 to 0.59 for Thar coal while for Baluchistan it ranges from 0.27 to 0.44. Rizwan *et al*, 2014 reported the same pattern of O/C and H/C ratio for thar lignite coal and bit lesser indices for Baluchistan coal in his study.

After selection of coal samples from Thar and Duki region, isolation and screening studies of potential coal solubilizing fungal strains were conducted. After screening the production of black color surrounding coal particles on fungal mycelium were estimated by UV-Vis spectrophotometer in the range between 200nm-250nm. Rizwan *et al*, 2014 reported the significant release of organics in the range between 200-300nm that indicates the presence of chemical bonds liberating organics compounds. Out of all the isolates, 4 isolates show maximum release of organics named as NF-1, GB, DS and LP. But significant organic moieties were released by two potential isolates NF-1 and GB on the basis of UV-Visible scan. The absorbance intensity of isolate GB was more than NF-1. Tao *et al*. (2009) reported the presence of alkyl substituted unsaturated aldehyde and ketones and alkyl substituted, that showed maximum absorption peaks at 240nm incubation time of after 15 days. The maximum absorption intensity by isolate GB were shown at 240nm. However, the peak range of intensities of isolate NF-1, LP and DS were also observed in the range of 200nm-250nm but their absorption range was less than isolate GB.

Acridine orange microscopy were performed to identify the mycelium and hyphae structure of fungal isolates morphologically. NF-1 had shown nerve like peridium membranous sac like structure with dense hyphae. While GB had shown brush like conidiophore spherical round spores. One of the studies by Byung Cheon Lee *et al* in 2004 reported the use of acridine orange for staining the nuclei of the threadlike structure of femoral veins. This method had helped them to discern the thread like structures from fibrin strings. Daniel Palestrant *et al*, in 2009 also documented the use of acridine orange stain for detecting gut bacterial biofilms.

After identification of potential fungal isolates morphologically, their molecular identification was done. These two strains were identified as *Debaromyeces Hansenii* named as NF-1 and *Aspergillus Ochraceus* named as GB.

After identification of fungal isolates their optimization studies were conducted on the basis of Glucose concentration, Coal loading ratio and Incubation time.It has been reported in various optimization studies that glucose supplements are essential for enhanced release of organics from coal. *Penicillium chrysogenum* MW1 utilized 0.1% of glucose to solubilize low ranked within 7 days. Higher concentration of glucose (1.5%) was reported for enhancing coal solubilization by *Rhizopus oryzae*. Moreover, some studies had been reported by increasing glucose ratio 0.5% leads to encourage the growth *T. atroviride* ES11 but higher ratio (10%) may also suppress solubilization. Different ranks of coal either lignite and

bituminous depolymerize under variable concentration of glucose. Absence of glucose supplement in medium resulted in low solubilization. However, glucose addition may encourage coal depolymerization process by activating the growth and production of fungal extracellular enzymes. It is observed that excessive glucose (from 2%) may leads to limit oxygen content in medium that inhibit fungal growth and enzymatic activity. Hence, resulted to low solubilization. While in this study for NF-1 and GB fungal isolates to enhance solubilization of bituminous coal 1.0% (v/w) and 1.5% (v/w) glucose concentration was optimal for maximum release of organics by showing absorbance at 240nm.

Several studies showed the optimized coal ratio as important source for solubilizing and extracting organics from different coal ranks. Humic acid was extracted from lower coal ranks by using 1.0% optimal coal value. *Trichoderma* sp. was optimized with 1.5% (w/v) coal for enhanced coal solubilization activity. Previous researches have shown that the size and the surface area is also an important factor for enhanced bio-solubilization. *Bacillus spp.* Y7 showed maximum liberation of organics by using 1% coal particles after 12 days. Adnan *et al*, 2019 reported *Rhizopus oryzae* showed maximum degradation of 0.5 % low rank coal within11 days of incubation. *G. alkanivorans* S7 and *B. mycoides* NS1020 sp. were reported to solubilize 5% (w/v) of low rank coal. reported 5% (w/v) of coal, solubilized by fungal isolates. Aneela et al, 2017 reported, *Trichoderma* sp. optimization with 1.5% (w/v) coal for enhanced coal solubilization activity. In present research work the optimization studies for enhanced release of organic moieties from bituminous coal matrix by NF-1 and GB fungal isolates, 0.5% coal loading ratio for both isolates was found to be optimum, while in case of lignite coal 1.5% coal loading ratio was found optimum for enhance release of organics.

In previous studies it was revealed that 7 days incubation during fungal pretreatment were significant to amplify the extent of organic moieties. These moieties were continuously detected under UV-Visible spectrophotometer in range between 215-300nm. *Bacillus sp.* Y7 showed maximum liberation of organics after 12 days. reported 11 days were best in degradation of low rank coal. reported the enhanced bio solubilization of nitric acid pretreated chines lignite particles within 11 days and 14 days respectively.one of the studies reported significant reduction of absorption intensity (might be due to pH) for longer incubations (9,15 and 19 days) at 240 nm range. In our study NF-1 and GB showed 7 days using bituminous coal and 11 days using lignite coal for significant organic release.

Up till now, immense biological entities have been documented for estimating coal solubilization extent gravimetrically. Coal solubilization percentage depends upon the density

of particles being solubilized. Lignite particles with lower density (1.3 kg/L) showed 44.86% fungal solubilization and particles with higher density (1.7 kg/L) showed 6.94% fungal solubilization Yin et al. (2009) reported that 31.83% of a Chinese lignite was solubilized into black liquid by a fungus isolated from decaying wood. Obrien et al. (2008) documented 7.8% coal weight loss in case of 600-800 µm coal size and 28% in 150-300 µm coal size in a slurry bioreactor while Gokcay, Kolankaya, and Dilek (2001) reported 100% solubilization of 8 N HNO_3 pre-treated coal by *Coriolus versicolor* and *Pleurotus florida*.

Previous literature determined bio solubilization percentage of coal particles, using various native fungal and bacterial isolates *Phanerochaete sp*. with 66.482% § 0.318, *Bacillus sp*. with 36.896% § 0.626, *Trichoderma sp*. with 50.0313% § 0.337 and *Pseudomonas sp.* with 25.581% § 0.565. Yin et al, 2009 reported that bio solubilization time of pre-oxidized coal particles were reduced from (2–4 weeks to 10.5 days) and percentage increased from (<25% to 31.83%). NF-1 and GB fungal isolates showed solubilization time of virgin coal residues 5 days and percentages were 31.8% and 45.6% respectively. NF-1 and GB fungal isolates showed solubilization time of pre oxidized coal residues 3 and 2 days and percentages were 52.4% and 71% respectively

Extensive research on coal solubilization by biological entities have been reported. It was reported that 36.77% untreated lignite was solubilized into black liquid by indigenous fungus. It was also reported that 7.8% coal weight loss in case of 600-800 µm coal size and 28% in 150-300 µm coal size in a slurry bioreactor while reported 100% solubilization of 8N HNO3 pre-treated coal by *Coriolus versicolor* and *Pleurotus florida.*

It was reported that 36.77% untreated lignite was solubilized into black liquid by indigenous fungus.(Haider et al, 2014).It was reported earlier that 7.8% coal weight loss in case of 600-800 µm coal size and 28% in 150-300 µm coal size in a slurry bioreactor while reported 100% solubilization of 8N HNO3 pre-treated coal by *Coriolus versicolor* and *Pleurotus florida.*

It was reported by Aneela *et al,* 2017 that chemical bond in supernatant detected at 200-300 nm. reported aromatics at 260-450 nm. reported *Trichoderma sp*. AY6 and *phanerochaete sp.* AY5 showed maximum absorbance. One of the studies by Adnan *et al,* 2019 reported *Rhizopus oryzae* showed maximum absorbance at 220-330nm.

It was documented earlier that *Bacillus* sp. Y7 has effect on O/C, H/C and C/N ratio of solubilized coal. Kawiatos *et al*, 2018 reported gain of oxygen and nitrogen and loss of

carbon content in solubilized coal residues. *F. oxysporum* LOCK 1134 showed 32% of carbon, 10% oxygen in solubilized lignite.

By comparing IR spectrum of NF-1 and GB treated virgin/pre oxidize coal residues and liquids, significant changes were observed. Bio treated (NF-1 and GB) virgin residual coal and liquids showed minimal characteristic peaks of aliphatics, phenolics, ethers, aldehydic and aromatic groups but intensified phenolic, aldehydic, aromatic, carboxylic, aliphatic, methylene, alkane, methyl groups and humic acid functionalities were observed in biotreated (NF-1 and GB) pre oxidize residual coal. Pre oxidation of virgin coal enhances humic production by increasing content of oxygen and nitrogen containing functional groups as well as the content of OH and NH groups both in pre oxidized residual coal and liquid by the action of biological treatment. It was also documented that aromatic liberation into liquid products from pre oxidized coal by the action of wood-decaying fungal isolate documented for the enhanced release of contained phenolic, non-condensed aromatic rings, methylene, oxygen and sulfur bridges, and carboxylic from Elbistan lignite by the action of *Coriolus versicolor.* Cemil Koyunogl, and Hüseyin Karaca, 2017 reported ester and nitroso aromatic hydrolysis during solubilization. The decarboxylation of Elbistan lignite through fungal treatment reported the presence of phenolics and aliphatic stretches in biotreated lignite particles also reported the aliphatic and aromatic shifts in biotreated residues. These results agree with previous studies on coal biosolubilization by (Achi 1994a; Crawford and Gupta 1991; Cooke, Fuller and Gaikwad 1986).

T. atroviride ES11 involved in partial liberation of carboxylic acids, benzene derivatives and alcohols from coal reported the detectable compounds of ethers and aromatic acids in solubilized product. Black liquid released by *Rhizopus oryzae* by Adnan *et al* ,2019 contained number of organics such as amides alkanes, amines, aromatic and fatty acids from low rank coal elaborated that coal transformation does not involve in direct liberation of phenolics it produces mixture of polar organics. Phenolic release based upon structure of coal. *T. atroviride* ES11 liberates 97 mg l− 1 phenolics during bio solubilization of coal matrix Wood decaying fungus liberated variety of organics including carbonyl, aromatic acid, branched hydrocarbons, esters and hydroxyl from Chinese lignite documented the liberated organics nitrogen containing polyaromatic hydrocarbons (PAHs), aliphatics and aromatics by the action of MW1 fungal isolate Haider *et al,* 2015. A similar pattern of solubilization was discussed previously by aneela et al, 2017 which confirms the visible changes on coal

structure by bacterial isolates *Pseudomonas sp. AY2* and *Bacillus sp. AY3*. Similarly fungal isolates *Phanerochaete sp*. and *Trichoderma sp.*

Scanning electron microscopy (SEM) had shown the penetration of coal particles and causing surface erosion. One of the studies proposed by Manoj B. (2013) clearly explains the SEM analysis of bituminous coal which shows the presence of some crystalline structure and distribution of mineral content on the surface morphology by *Aspergillus niger.* Another study reported by stewart *et al*, 1990 also confirms the surface of air oxidized illions and exposure of fungal isolate *Penicillium* RWL-5 and appearance of fungal hyphae on coal surface causing physical changes on structure of bituminous coal. One of the studies documented by Xin Feng et al, 2021 confirms the rough structure and surface of lignite coal pretreated with H2O2, while significant amount of macro molecular structural changes had also be shown by fungal isolate *Trichoderma citrinoviride..*

Metagenomic studies of indigenous bacterial and fungal communities present in all coal samples was investigated. Previous studies conducted by Thompson *et al,* 2017, had reported diverse microbial communities from different habitats of soils and sediments. Similarly, another study by Torsvik *et al,*2002 reported the alpha diversity pattern from different habitats of water and air environment. The highly mixed water and air environment showed lower diversity. Hall-stoodley *et al,* 2004 reported very low alpha diversity from biofilms and mats despite being spatially structured.

There is very less data reported on microbial communities' identification from Pakistani coal mines. Up to date, this is the first study on microbial community analysis from hard rank coal Bituminous from Dukki coal mine and low rank coal, Lignite, Thar coal mine.

Kendra E. Walters *et al,* 2020 depicted the highest beta diversity from sediment, biofilm/mat and inland water habitat, while other habitats that includes soil, air and marine habitat showed 17% less beta diversity than others. Barberan *et al*,2010 reported the spatial variation of beta diversity in different environmental conditions. Similarly, Evans *et al,* 2016 reported the low beta diversity patterns in various environments.

One of the studies by Anne winding *et al*, 2017 reported the heatmap plot indicating the clear differences in the protist communities according to the method of different DNA extraction procedures applied. They also showed relatively higher abundance among different genus present in each soil sample. Santos *et al,*2015 documented the higher absolute abundance of 18srRNA genes recovered by qPCR in his study of microbial diversities extracted from

different soil habitats. Geisen *et al*, 2015 indicated the most divergent OTU according to DNA extraction method applied and higher abundance extracted from ecologically significant soils.

A study by Anthony *et al*, 2010 reported the relationship between abundance and biological abundance in a sample of house dust spike with known quantities and identities of fungi. Their result showed the difference in read abundance among species.

Another study by Cristina Di muri *et al,*2022 reported the fish species read counts that were significantly correlated with the biomass and read abundance with other fish species. The present study also reported the correlation between different bacterial genera present in each coal sample.

One of the studies conducted by Rumakanta *et al*, 2020 reported the Proteobacteria, Firmicutes and Actinobacteria covering higher relative abundance in gut microbiome. Liu *et al* 2018, Ma *et al*,2017 reported earlier the observed dominance of Proteobacteria, Firmicutes and Actinobacteria taxa in earthworm gut. Pass *et al,* 2017 reported the observed relative abundance of *Proteobacteria* and *Actinobacteria* in gut microbiome.

Similarly, another recent study by Gong *et al* ,2018 reported the same group of bacterial relative abundance of SAR group of eukaryotes in cultivated soil.

One of the studies by Rumakanta *et al*, 2020 indicated the higher abundance of fungal genera in gut microbiome of earthworm. Similarly, Byzov *et al,* 2007 reported the higher abundance of fungi in earthworm gut fluid. Medina-Sauza *et al,* 2019 indicated the dominance of fungal taxa in the presence of amino acid and organic carbon source in earthworm gut.

Previous studies conducted by Thompson *et al,* 2017, had reported diverse microbial communities from different habitats of soils and sediments. Similarly, another study by Torsvik *et al,*2002 reported the alpha diversity pattern from different habitats of water and air environment. The highly mixed water and air environment showed lower diversity. Hallstoodley et al, 2004 reported very low alpha diversity from biofilms and mats despite being spatially structured.

Rumakanta et al, 2020 had reported significantly higher alpha diversity for both prokaryotic and eukaryotic communities measured by observed richness. Similarly, winding *et al*, 2015 had documented higher alpha diversity measures while determining the eukaryotic diversity of different soil samples.

In agreement with the results of the present study, many investigators have used 97% identity to classify microorganisms at the species level (De Gannes *et al*., 2013; Lindeque *et al*., 2013). Other studies have explored the classification of oral microbiota. For instance, Pushalkar *et al* (2011) identified 860 species, 52 genera, and 8 phyla; Ling *et al*. (2010) identified 203 genera and 10 phyla; Keijser *et al*. (2008) identified 318 genera and 22 phyla; and Siqueira and Rôças (2009) identified 460 species, 100 genera, and 9 phyla. The diff erences in microbial taxonomy observed among these studies may be due to differences related to the selection process for subjects, the geographic location, oral sample collection sites, target sequencing regions of 16S rDNA, databases, and the data-analysis methods. 23 bacterial phyla were represented in Baluchistan samples. Three predominant phyla were Firmicutes (85%), Actinobacteria (2%), Proteobacteria (12%), constituting 99% of the total microbiota. These dominant phyla were largely the same as those previously described for childhood dental caries using pyrosequencing (Jiang et al., 2013). At the genus level, 130 diff erent genera were identified in Baluchistan sample. Most of them were in abundance higher than 2% of which the genera Actinomyces, Streptococcus, Rothia, Selenomonas and Veillonella have been detected in early caries studies (Tanner et al., 2011). Taxonomic tree, GraphIan tree, Krona display showed huge diversity at phylum, class, order, Family, genus and specie level in Baluchistan sample.

Uptill now the aerobic process of biosolubilization of lignite and bituminous coal was investigated. The anaerobic treatment process was also conducted. The indigenous anaerobic consortium was developed and co substrate technologies were followed for enhancing the process of coal.

In one of the studies by Fallgren et al, 2013 reported the higher methane yield from bituminous coal as compared to lignite and sub bituminous native coal samples. Another study reported the methane generation production by bituminous coal by using mixed consortium and pure cultures of *Methanococcus* and *Methanosarcina.* The use of higher concentration bituminous coal was found to show the inhibitory effect on methane generation. (Raudsepp *et al.* 2017). Haider *et al,* 2017 reported about 0.275 µmol/g of methane from bituminous coal samples, while Haider *et al*, 2014 reported more production of methane from lignite coal which was about 22.96 µmol/g.

Previous studies by Green et al, 2008 reported a similar kind of microbial consortium from powder river Basin WY U.S that is capable of producing higher methane between temperatures 22°C to 38°C. A similar trend was also reported by Levanian *et al*, 2014, a

thermophilic microbial consortium enriched from Jharia coal mine India. That consortium depicts the increasing trend of methane production between 37°C to 65°C and a visible decline were observed at 70°C. John Fuertez *et al*, 2018 reported the same trend for bituminous coal and 30- 37°C were the optimized temperature for methane yield but they showed a decline in methane production above 48°C. Zhang *et al*, 2016 documented an increasing trend in methane yield between 24°C to 32°C during transformation of bituminous coal. the production was declined at 40°C. the microbial community was obtained from Illinois basin of United states

John Fuertez *et al,* 2017 documented a higher methane yield at pH 5.2 while a decrease trend in methane production were observed above 7.2. Another study conducted by Levania *et al*, 2014 documented the effect of pH range between 4.0 to 9.0 and maximum methane was produced at pH 6.5 for bituminous coal. The pH higher than 6.5 showed decrease trend in methane production. Another study by Rathi et al, 2015 tested the methane yield along with carbondioxide between pH 6.0 to 7,5. The maximum methane was produced along with carbondioxide at pH 7.5 . Gupta and Gupta in 2014 also showed the maximum methanogenic yield between pH 7.0-7.5. While in 2010 Gao *et al,* 2010 mentioned an adverse effect on methane yield between range 7.0-7.5.

Hao *et al*, 2010 indicated very low pH 5,5 for extending the lag phase of methane generation from acetate and changes in methanogenic pathway was noted. In 1994, Wolkwein *et al* evaluated the production of methane using six different types of consortium on three different coal types i.e. bituminous, subbituminous and lignite coal) at pH 5.0 and 7.0. Out of 18 about 12 different coal-consortium combinations produced large methane yield at pH 5.0.

One of the studies by Hongyu Guo et al, 2018 reported the cumulative gas yield of lignite coal along with corn straw as compared to control that contains only lignite coal. The highest methane production was 2.05mmol/g while in present study it was 4.9mmol/g of coal along with grass and peanut shell combination. While in present study it was 3.5mmol/g along with grass and peanut shell combination.

An enhanced or higher methane production is observed by the co-degradation of coal with straws of wheat, grass corn (Yoon *et al*., 2016; Guo *et al*., 2018). The composition of straw includes celluloses, hemicelluloses and lignin being rich in hydrogen providing a substrate for micro-organisms to generate hydrogen anaerobically (Wang J and Yin Y, 2018).

Benkhedda and Landais studied the aliphatic and aromatic, symmetric and asymmetric C-H stretching in the Fourier transform infrared (FTIR) bands after the methanogenesis of coal (Benkhedda and Landais, 1992; Drobniak and Mastalerz, 2006).

During coal transformation Bacillus sp. Y7 was reported to alter the structure and composition of humic material. *F. oxysporum* was reported to liberate 1474 mg/g of humic acid in bio extract from coal. One of the syudies reported that humic acid extracted from raw lignite was 38.6% but fungal treatment (*Penicillium sp.* P6) maximizes humic acid content up to 55.1%. The findings of were, the content of humic acid extracted from coal (lignite) by different fungal strains was different. The ratio of humic acid extracted by the action of *Aspergillus fumigatus* (MTCC 4333) 1.00%, *Fusarium udum* (MTCC 4628) 1.56% and *Aspergillus fumigatus* (MTCC 4334) 1.85%. (Valero *et al*., 2014) reported that 25 to 37% of humic acid extracted from LRC by using *Bacillus mycoides*. *Penicillium* sp. P6 was reported to transform 1.0% of sterilized lignite into 72.2% humic acid and 37.5% of fulvic acids. However, its percentage from control was 5.0–6.0%.Haider et al, 2016 reported that humic acid extracted by the action of fungal isolate MW1 was 57.81% and its ratio directly from raw coal was 57.19%. demonstrated that by using catalyst (AC–NiSO4) during coal oxidation with nitric acid accelerates the amount of released humics up to 18.7%. The study showed that nitric acid pretreatment accelerates the humic content. Samples from Thar, Chakwal, and Quetta coals were treated chemically as a result 24.6, 13.6, and 18.0% humic obtained. Adnan et al, 2020 reported that treating lignite before biological attack with H_2O_2 increased humic content by 48.1% to 71.7%.

Various literature has reported the effect of molecular weight and condensation of aromatic rings on E4/E6 ratio coefficient. It was reported earlier that E4/E6 ratio of humic acid (HA) obtained through raw coal was 4.8 and it was 5.2 by the activity of *Bacillus mycoides* and have similar spectra as HA extracted by alkali treatment. demonstrated that by using catalyst (AC–NiSO4) during coal oxidation with nitric acid accelerates the amount of released humics as well as E4/E6 ratio up to 18.7%.

One of the studies reported earlier by Yan wang *et al*, 2018 predicted the genomic sequence of A. ochraceus fc-1 totaled 37.02 Mb. The genes predicted were 11,740 genes. The predicted protein coding sequence were 14.4% and GC content were 48.8%. while the present study predicted the gene length to be 10,640. They reported this strain to be a producer of OTA (Ochratoxin A). Another study conducted by Harris and Mantle predicted the genome sequence of Aspergillus Ochraceus and they also proposed an alternative pathway of OTB

with phenylalanine. Gallo et al ,2012 also suggested another third pathway of OTA biosynthesis by genome sequencing of *Aspergillus Ochraceus*.Vander *et al,* 1965 were the first one to predict the genome sequence of A. Ochraceus and they identified the production of Ochratoxin A, a toxic metabolite, by A. ochraceus.

In 2000, a study by Andree *et al*, reported the Genome of hemiascomycetous Yeast D*ebaromyeces Hansenii*, they analyzed about 2830 random sequence tag totalling 2.7MB size of genome. While the present study depicted the total size of genome of Debaromyeces Hansenii as 722,974bp and number of sequences as 1781.Another study by Shailesh kumar *et al*, 2012 reported the size of genome of *Debaromyeces Hansenii* strain MTCC234 as 11.46Mb. the total GC content reported were 35.4% while in present study it was 36.3%. Bansal PK in 2000 reported the sequence of HOG 1 homologue from *Debaromyeces hansenii* by identifying the whole genome sequence of the strain.

In 2021, Clara Navarrete et al investigated the transcriptomic and proteomic profile of D. hansenii (CBS 767). They indicated the specific genes that are involved in expression and regulation of protein activity using sodium and potassium.

Conclusion

Overall goal of study was the enhancement of microbial biosolubilisation process aerobically through biosolubilization and production of biomethane anaerobically using co- substrate strategies of low to high grade coal of Pakistan.

The two isolated potential indigenous fungal strains NF-1 and GB had ability of solubilizing low and hard rank of coal and releasing different kinds of useful organic compounds and showed efficient coal solubilization extent. The low and high rank coal had shown more potential of solubilization by fungal strain GB as compared to NF1. Up to our knowledge the strain NF1 *Debaromyeces hansenii* and GB *Aspergillus ochraceus* had been reported first time to be involved in degradation of low and hard rank of coal. For increasing the process of coal biosolubilization, the use of enhancers (sodium acetate and chemical pretreatment) had accelerated liquefaction as well as solubilization magnitude (52.4% & 71%) for lignite coal as compared to bituminous coal.

The illumina miseq technique of next generation sequencing had shown similarity in bacterial diversity in 6 samples out of 8 while, 2 samples had shown different behavior from other samples after determining their relative and read abundance, alpha and beta diversity and heat map. While the fungal alpha diversity of 4 samples had shown distinction from each other in their relative abundance. Another sample from Baluchistan Duki coal mine had shown the presence of more *proteobacteria and actinobacteria* than *Fermincutes.*

After solubilization of coal by fungal isolates different compounds that were produced after degradation of coal were extracted. These compounds were name as Humic and fulvic acids. About 4.5% concentration of KOH had shown maximum yield of humic acid in both types of coal. The percentage of humic acid using NaOH for native lignite and Bituminous coal was 21.15% and 11.6% and for HNO3 treated coal percentage for lignite and Bituminous coal was 57.8% and 46.9% respectively. The FTIR results of HNO3 treated coal had clearly indicated the presence of N-H group peak at 2923.04cm in case of bituminous coal while introduction of nitro group in lignite coal at 2921.84cm, while both peaks were absent in native coal FTIR spectrum. The elemental analysis of HNO3 treated coal had shown the reduction in carbon content and slight change in sulphur and nitrogen and increase in oxygen content that confirms the oxidation process occurred. The O/C ratio was also increased in case of HNO3 treated coal. 60% and 78% humic acid was extracted from bituminous and lignite coal by potential fungal strains.

Both the low and high rank coal were allowed to be treated anaerobically in order to find out the extent of methane production. The anaerobic consortium has a degradation ability which

produces labile products including short chain aliphatics and aromatics from the carbon source (coal and co-digestive substrates) and used them in the generation of gas.

The raw bituminous (HRC) was capable of producing more methane and carbon dioxide as compared to LRC (Lignite). The co-digestion strategy is productive in stimulation of methane generation but also enhanced its yield. A higher methane potential was observed in the biogasification setup.

The co-substrate Grass+peanut shell was able to produce more cummulative methane along with bituminous coal as compared to lignite coal. Grass and peanut shells comprised of lignin and celluloses which are degraded by anaerobic microbes releasing hydrogen which is limiting the reaction rate on the first hand for its deficiency in the reaction mixture. The maximum yield of methane and carbondioxide was obtained at optimum temperature of 35_{o} c and pH 7.0.

The whole genome sequencing of potential microbial strains had identified the presence of annotations with their nucleotide base data and gene rank. The circular and sequence view of both the potential fungal isolates *Debaromyeces hansenii* and *Aspergillus Ochraeus* had shown the particular number of bases present in the nucleotide sequences.

Future Prospects

Future Prospects

Recently application of coal to produce humic acid and manage soil problems is attaining significant attention because it offers environmental economical friendly and green energy solutions. Previous studies and researches focused on limited coal applications especially bituminous Hard rank coal. the humic acid produced from hard and low rank coal must be considered from commercial and economic point of view due to its cost effectiveness and countless capabilities. The coal biosolubilization technology is multifaceted having broad future prospects, some of them are listed below:

- ♦ The current study can be scaled up to large capital expenses on industrial level as only majority of lab scale trials had been conducted.
- ♦ Production of Humic derivates can be performed by designing reactors at large scale either aerobically and native anaerobes can also be explored.
- ♦ The isolated potential coal solubilizing fungal isolates can further be used for genetic and mutation studies for enhancing the production of produced compounds.
- ♦ Enhanced bio-gasification extent of bituminous coal through consortium and anaerobes can be improve for sustainable energy production.
- ♦ Non-fuel potential of Pakistani bituminous coal needs to be explored, in order to get the maximum quality and effectivity of potential organic assets.
- ♦ Liquid fuel production can be achieved through bioconversion of complex coal to simple organic liquid.
- ♦ Proteomics study of two potential fungal isolates could be done to get the information about microbial enzymes and those enzymes can be extracted and purified and their significance at industrial level can be determined.
- ♦ Availing biological processes on industrial scale have been limited but catalytic means can provide the opportunity to be utilize as having more potential than chemical.

References

References

- ACHARYA, C., SUKLA, L. B., &MISRA, V. N. 2005. Biological elimination of sulphur from from sulphur coal by Aspergillus-like fungi. *Fuel*, *84* (1213), 1597-1600.
- ADAMS, D.J., OPARA, A.O. 2015. Optimization of biogenic methane production from hydrocarbon sources. U.S. *Patent WO 2015013531* A1.
- AHMAD, A.; JINI, D.; ARAVIND, M.; PARVATHIRAJA, C.; ALI, R.; KIYANI, M.Z.; ALOTHMAN, A. 2020. A novel study on synthesis of egg shell based activated carbon for degradation of methylene blue via photocatalysis. *Arab. J. Chem,13*, 8717–8722.
- AHMAD, A.; MUBHARAK, N.M.; NASEEM, K.; TABASSUM, H.; RIZWAN, M.; NAJDA, A.; KASHIF, M.; BIN-JUMAH, M.; HUSSAIN, A.; SHAHEEN, A. 2020. Recent advancement and development of chitin and chitosan-based nanocomposite for drug delivery: Critical approach to clinical research. *Arab. J. Chem*,*13*, 8935– 8964.
- ALLER, ., MART NE , O., DE LINAJE, J. A., M ND E , R., MOR N, A. 2001. Biodesulphurisation of coal by microorganisms isolated from the coal itself. *Fuel processing technology*, *69*(1), 45-57.
- ALMAGRO, A., PRISTA, C., BENITO, B., LOUREIRO-DIAS, M.C., AND RAMOS, J. 2001. Cloning and expression of two genes coding for sodium pumps in the sal-tolerant yeast *Debaryomyces hansenii*. *J Bacteriol* ,*183*: 3251–3255.
- ALMAGRO, A., PRISTA, C., CASTRO, S., QUINTAS, C., MADEIRA-LOPES, A., RAMOS, J., AND LOUREIRO-DIAS, M.C. (2000) Effects of salt on *Debaryomyces hansenii* and *Saccharomyces cerevisiae* under stress conditions. *Int J Food Microbiol, 56*: 191–197.
- AMANN, R. I., LUDWIG, W., & SCHLEIFER, K. H. 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiological reviews*, *59*(1), 143-169
- ARAVIND, M.; AHMAD, A.; AHMAD, I.; AMALANATHAN, M.; NASEEM, K.; MARY, S.M.; PARVATHIRAJA, C.; HUSSAIN, S.; ALGARNI, T.S.; PERVAIZ, M. 2021. Critical green routing synthesis of silver NPs using jasmine flower extract for biological activities and photocatalytical degradation of methylene blue. *J. Environ. Chem. Eng.*, 9, 104877.

- ASIF, M. 2021. Comparative Study on Extraction of Humic Acid from Pakistani Coal Samples by Oxidizing the Samples with Hydrogen Peroxide. *ASEAN J. Sci. Eng. 2*, 1–8.
- ASNICAR, F., WEINGART, G., TICKLE, T. L., HUTTENHOWER, C., & SEGATA, N. 2015. Compact graphical representation of phylogenetic data and metadata with GraPhlAn. *PeerJ*, *3*, e1029.
- A TAR, P., KA , C. M., MUTLU, M. B., ABUK, A. 2013. Coal Desulfurization with Acidithio bacillus ferrivorans, from Balya Acidic Mine Drainage. *Energy & Fuels*, *27*(6), 3090-3098.
- BAKER, D. C. 1994. Projected emissions of hazardous air pollutants from a Shell coal gasification process-combined-cycle power plant. *Fuel*, *73*(7), 1082-1086.
- BANSAL PK, MONDAL AK. 2000. Isolation and sequence of the HOG1 homologue from *Debaryomyces hansenii* by complementation of the hog1Delta strain of Saccharomyces cerevisiae. Yeast. Jan 15;*16*(1):81
- BARHOUMI, A.; NCIB, S.; CHIBANI, A.; BRAHMI, K.; BOUGUERRA, W.; ELALOUI, E. 2019. High-rate humic acid removal from cellulose and paper industry wastewater by combining electrocoagulation process with adsorption onto granular activated carbon. *Ind. Crop. Prod. , 140*, 111715.
- BARNHART, E. P., DE LEÓN, K. B., RAMSAY, B. D., CUNNINGHAM, A. B., & FIELDS, M. W. 2013. Investigation of coal-associated bacterial and archaeal populations from a diffusive microbial sampler (DMS). *International Journal of Coal Geology*, *1125*, 64-70..
- BECKMANN, S., KRÜGER, M., ENGELEN, B., GORBUSHINA, A. A., &CYPIONKA, H. 2011. Role of bacteria, archaea and fungi involved in methane release in abandoned coal mines. *Geomicrobiology Journal*, *28*(4), 347-358.
- BECKMANN, S., LUEDERS, T., KRUGER, M., NETZER, F. V., ENGELEN, B., AND CYPIONKA, H. 2011. Acetogens and acetoclastic Methanosarcinales govern methane formation in abandoned coal mines. *Appl. Environ. Microbiol.* 77, 3749–3756. doi: 10.1128/AEM.02818-10
- BERGEY, D. H. 1994. *Bergey's manual of determinative bacteriology*. Lippincott Williams & Wilkins.

- BERLENDIS, S., BEYSSAC, O., DERENNE, S., BENZERARA, K., ANQUETIL, C., GUILLAUMET, M., & CAPELLE, B. 2014. Comparative mineralogy, organic geochemistry and microbial diversity of the Autun black shale and Graissessac coal (France). *International Journal of Coal Geology*, *132*, 147-157
- BIBI, S.; AHMAD, A.; ANJUM, M.A.R.; HALEEM, A.; SIDDIQ, M.; SHAH, S.S.; AL KAHTANI, A. 2021. Photocatalytic degradation of malachite green and methylene blue over reduced graphene oxide (rGO) based metal oxides (rGOFe3O4/TiO2) nanocomposite under UV-visible light irradiation. *J. Environ. Chem. Eng. , 9,* 105580.
- BLANDER, M., SINHA, S., PELTON, A., & ERIKSSON, G. 2011. Calculations of the influence of additives on coal combustion deposits. *Argonne National Laboratory*, *315*.
- BORAH, D. 2004. Electron-transfer-induced desulfurization of organic sulfur from sub-bituminous coal. *Energy & fuels*, *18*(5), 1463-1471.
- BORAL, P.; VARMA, A.K.; MAITY, S. 2021. Nitration of jharia basin coals, india: A study of structural modifications by XRD and FTIR techniques. *Int. J. Coal Sci.*
    - *Technol.* , *1*–12.
- BRITISH PETROLEUM COMPANY. (2013). *BP Energy Outlook 2030*. BP.
- BRODIE, E., EDWARDS, S., & CLIPSON, N. 2003. Soil fungal community structure in a temperate upland grassland soil. *FEMS Microbiology Ecology*, *45*(2), 105-114.
- BUGG, T. D. H., AHMAD, M., HARDIMAN, E. M., AND SINGH, R. 2011. The emerging role for bacteria in lignin degradation and bio-product formation. *Curr.*
    - *Opin. Biotechnol.* 22, 394–400.

- BURANGE, A.S.; AHMAD, A.; LUQUE, R. 2021. Electrophilicity in heterogeneous catalysis: Role of surface and sub-surface modification. *Catal. Sci. Technol.* **11**, 43154326.
- CAICEDO, G., PRADA, M., PELAEZ, H., MORENO, C., & MARQUEZ, M. 2012. Evaluation of a coal biodesulfurization process (semi-continuous mode) on the pilot plant level. *Dyna*, *79*(174), 114-118.
- CARA, J., VARGAS, M., MORÁN, A., GÓMEZ, E., MARTÍNEZ, O., &FRUTOS, F. G. 2006. Biodesulphurization of a coal by packed-column leaching. Simultaneous thermogravimetric and mass spectrometric analyses. *Fuel*, *85*(12-13), 1756-1762.
- CARDONA, I. C., & MÁRQUEZ, M. A. 2009. Biodesulfurization of two Colombian coals with native microorganisms. *Fuel Processing Technology*, *90*(9), 1099-1106.
- CARLSON, J. E., TULSIERAM, L. K., GLAUBITZ, J. C., LUK, V. W. K., KAUFFELDT, C., & RUTLEDGE, R. 1991. Segregation of random amplified DNA markers in F 1 progeny of conifers. *Theoretical and Applied Genetics*, *83*(2), 194-200.
- CHAPUT, D. L., HANSEL, C. M., BURGOS, W. D., & SANTELLI, C. M. 2015. Profiling microbial communities in manganese remediation systems treating coal mine drainage. *Applied and environmental microbiology*, AEM-03643.
- CHEN, G. Q., & WU, X. F. 2017. Energy overview for globalized world economy: source, supply chain and sink. *Renewable and Sustainable Energy Reviews*, *69*, 735749.
- CHEN, X.; XU, Y.; FAN, M.; CHEN, Y.; SHEN, S. 2019. The stimulatory effect of humic acid on the co-metabolic biodegradation of tetrabromobisphenol A in bioelectrochemical system. J. Environ. Manag. 2019, 235, 350–356.
- Cheng, G.; Niu, Z.; Zhang, C.; Zhang, X.; Li, X. 2019. Extraction of humic acid from lignite by KOH-hydrothermal method. *Appl. Sci. 9,* 1356.

- CHOWDHURY, S. P., KHANNA, S., VERMA, S. C., & TRIPATHI, A. K. 2004. Molecular diversity of tannic acid degrading bacteria isolated from tannery soil. *Journal of applied microbiology*, *97*(6), 1210-1219.
- COUCH, G. R. 1993. Biodesulfurization of coal. *London: Coal Research Report*.
- DAS, T.; BORA, M.; TAMULY, J.; BENOY, S.M.; BARUAH, B.P.; SAIKIA, P.;
- SAIKIA, B.K. 2021. Coal-derived humic acid for application in acid mine drainage
- (AMD) water treatment and electrochemical devices. *Int. J. Coal Sci. Technol*. , *1*–12.
- DE GANNES, V., EUDOXIE, G., & HICKEY, W. J. 2013. Insights into fungal communities in composts revealed by 454-pyrosequencing: implications for human health and safety. *Frontiers in microbiology*, *4*, 164.
- DONG, L.H.; YUAN, Q.; YUAN, H. 2006. Changes of chemical properties of humic acids from crude and fungal transformed lignite. *Fuel , 85*, 2402–2407.
- DOWNEY, R. 2010. Method for optimizing in-situ bioconversion of carbon-bearing formations. *U.S. WO2010002460*
- 2012. SHAILESH KUMAR, ANMOLDEEP RANDHAWA, KALIANNAN GANESAN, GAJENDRA PAL SINGH RAGHAVA, AND ALOK K. MONDAL.
- 2012. Draft Genome Sequence of Salt-Tolerant Yeast Debaryomyces hansenii var. hansenii MTCC 234, *ASM journal, 11*(7),
- FAISON, B. D. 1991. Biological coal conversions. *Critical reviews in biotechnology*, *11*(4), 347-366.
- FAKOUSSA, R. M., & HOFRICHTER, M. 1999. Biotechnology and microbiology of coal degradation. *Applied Microbiology and Biotechnology*, *52*(1), 25-40.
- FALLGREN, P. H., JIN, S., ZENG, C., REN, Z., LU, A., &COLBERG, P. J. 2013. Comparison of coal rank for enhanced biogenic natural gas production. *International Journal of Coal Geology*, *115*, 92-96.
- FALLGREN, P. H., S. JIN, C. ZENG, Z. REN, A. LU, AND P. J. S. COLBERG.
- 2013. Comparison of coal rank for enhanced biogenic natural gas production. *International Journal of Coal Geology 115*:92–96.

- FARAH HAYAT, D. D. P. 2014. Prevailing Energy Crisis in Pakistan. *Mechanics, Energy, Environment*, 251-258.
- FASSETT, J. E., &DURRANI, N. A. 1994. Geology and coal resources of the Thar coal field, Sindh Province, Pakistan . *US Geological Survey. 94-167.*
- FERRY, J. G. 2012. Methanogenesis: ecology, physiology, biochemistry & genetics.
- *Springer Science & Business Media*.
- FILIP, Z., &KUBÁT, J. 2001. Microbial utilization and transformation of humic substances extracted from soils of long-term field experiments. *European journal of soil biology*, *37*(3), 167-174.
- FONG, S.S.; SENG, L.; MAJRI, N.B.; MAT, H.B. 2007. A comparative evaluation on the oxidative approaches for extraction of humic acids from low rank coal of Mukah, Sarawak. *J. Braz. Chem. Soc. , 18,* 34–40.
- FRANKE, I.H., WALTER, A., EBNER, C., INSAM, H., 2014. Investigation into the effect of high concentrations of volatile fatty acids in anaerobic digestion on methanogenic communities. *Waste Manag. 34* (11), 2080–2089.
- FUERTEZ, J., BOAKYE, R., MCLENNAN, J., ADAMS, D.J., SPARKS, T.D., GOTTSCHALK, A. 2017. Developing methanogenic microbial consortia from diverse coal sources and environments. *J. Nat. Gas Sci. Eng In Press.* https://doi.org/10.1016/j.jngse.2017.07.
- FUNAKI, B., KOSTELIC, J. K., LORENZ, J., HA, T. V., YIP, D. L., ROSENBLUM, J. D., ... & ZALESKI, G. X. 2001. Super selective micro coil embolization of colonic haemorrhage. *American Journal of Roentgenology*, *177*(4), 829-836.
- GADONNEIX, P., DE CASTRO, F. B., DE MEDEIROS, N. F., DROUIN, R., JAIN, C. P., KIM, Y. D., & NAQI, A. A. 2010. Survey of energy resources: Focus on shale gas. *World Energy Council*.
- GALLAGHER, L. K., GLOSSNER, A. W., LANDKAMER, L. L., FIGUEROA, L. A., MANDERNACK, K. W., &MUNAKATA-MARR, J. 2013. The effect of coal oxidation on methane production and microbial community structure in Powder River Basin coal. *International Journal of Coal Geology*, *115*, 71-78.

- GALLO A, BRUNO KS, SOLFRIZZO M, PERRONE G, MULÈ G, VISCONTI A,
- BAKER SE. 2012. New insight into the ochratoxin A biosynthetic pathway through deletion of a nonribosomal peptide synthetase gene in *Aspergillus carbonarius*. *Appl Environ Microbiol 78:8208 –8218.*
- GENNARO, P. D., BARGNA, A., AND SELLO, G. 2011. Microbial enzymes for aromatic compound hydroxylation. *Appl. Microbiol. Biotechnol.*90, 1817–1827.
- GHANI, M. J., RAJOKA, M. I., & AKHTAR, K. 2015. Investigations in fungal solubilization of coal: Mechanisms and significance. *Biotechnology and bioprocess engineering*, *20*(4), 634-642.
- GONZALEZ, J. M., ORTIZ-MARTINEZ, A., GONZALEZ-DELVALLE, M. A., LAIZ, L., &SÁIZ-JIMÉNEZ, C. 2003. An efficient strategy for screening large cloned libraries of amplified 16S rDNA sequences from complex environmental communities. *Journal of Microbiological Methods*, *55*(2), 459-463.
- GREEN, M. S., FLANEGAN, K. C., &GILCREASE, P. C. 2008. Characterization of a methanogenic consortium enriched from a coalbed methane well in the Powder River Basin, USA. *International Journal of Coal Geology*, *76*(1-2), 34-45.
- GRÜNDGER, F., JIMÉNEZ, N., THIELEMANN, T., STRAATEN, N., LÜDERS, T., RICHNOW, H. H., &KRÜGER, M. 2015. Microbial methane formation in deep aquifers of a coal-bearing sedimentary basin, Germany. *Frontiers in microbiology*, *6*, 200.
- GUO, H., LIU, R., YU, Z., ZHANG, H., YUN, J., LI, Y., ... & PAN, J. 2012. Pyrosequencing reveals the dominance of methylotrophic methanogenesis in a coal bed methane reservoir associated with Eastern Ordos Basin in China. *International journal of coal geology*, *93*, 56-61.
- GUO, W., ZHAO, R., FU, R., BI, N., WANG, L., ZHAO, W. & ZHANG, J. 2014. Contribution of arbuscular mycorrhizal fungi to the development of maize (Zea mays L.) grown in three types of coal mine spoils. *Environmental Science and Pollution Research*, *21*(5), 3592-3603.

- GUPTA, P., & GUPTA, A. 2014. Biogas production from coal via anaerobic fermentation. *Fuel*, *118*, 238-242.
- HAIDER, R. 2014. Bisolubilization and biogasification of low rank coal for its applications. Pakistan: School of Biotechnology, National Institute for Biotechnology & Genetic Engineering (NIBGE), Quaid-i-Azam University.
- HAIDER, R., M. A. GHAURI, E. J. JONES, AND J. R. SANFILIPO. 2014. Methane generation potential of Thar lignite samples. *Fuel Processing Technology 126:*309–
- 14.
- HAIDER, R., M. A. GHAURI, J. R. SANFILIPO, E. J. JONES, W. H. OREM, C. A. TATU, K. AKHTAR, AND N. AKHTAR. 2013. Fungal degradation of coal as a pretreatment for methane production. *Fuel 104:*717–25.
- HAIDER, R.; GHAURI, M.A.; AKHTAR, K. 2015. Isolation of coal degrading fungus from drilled core coal sample and effect of prior fungal pretreatment on chemical attributes of extracted humic acid. *Geomicrobiol. J* , *32*, 944–953.
- HAIDER, R.; GHAURI, M.A.; SANFILIPO, J.R.; JONES, E.J.; OREM, W.H.; TATU, C.A.; AKHTAR, K.; AKHTAR, N. 2013. Fungal degradation of coal as a pretreatment for methane production. *Fuel , 104*, 717–725.
- HALES, B. A., EDWARDS, C., RITCHIE, D. A., HALL, G., PICKUP, R. W., & SAUNDERS, J. R. 1996. Isolation and identification of methanogen-specific DNA from blanket bog peat by PCR amplification and sequence analysis. *Applied and environmental Microbiology*, *62*(2), 668-675.
- HALLBERG, K. B., & JOHNSON, D. B. 2001. Biodiversity of acidophilic prokaryotes.
- HANDAYANI, S. 2007. Assessment of indigenous microorganism for natural attenuation of abandoned mine site. *Indonesian mining journal*, *10*(1), 38-46.
- HARRIS JP, MANTLE PG. 2001. Biosynthesis of ochratoxins by *Aspergillus ochraceus. Phytochemistry 58:*709 –716.
- HARRIS, S. H., SMITH, R. L., & BARKER, C. E. 2008. Microbial and chemical factors influencing methane production in laboratory incubations of low-rank subsurface coals. *International Journal of Coal Geology*, *76*(1-2), 46-51.

- HEROD, A. A., STOKES, B. J., & SCHULTEN, H. R. 1993. Coal tar analysis by mass spectrometry—a comparison of methods. *Fuel*, *72*(1), 31-43.
- HOFRICHTER, M.; FRITSCHE, W. 1997. Depolymerization of low-rank coal by extracellular fungal enzyme systems. II. The ligninolytic enzymes of the coal-humicacid-depolymerizing fungus Nematoloma frowardii b19. *Appl. Microbiol. Biotechnol*, *47*, 419–424.
- 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*(1), 57-59.
- IEA COAL INDUSTRY ADVISORY BOARD. 2002. Coal in the Energy Supply of
- India.
- IRAM, A., AKHTAR, K., &GHAURI, M. A. 2017. Coal methanogenesis: a review of the need of complex microbial consortia and culture conditions for the effective bioconversion of coal into methane. *Annals of Microbiology*, *67*(3), 275-286.
- JAMAL, Q., AHMED, I., REHMAN, S. U., ABBAS, S., KIM, K. Y., &ANEES, M. 2016. Isolation and Characterization of Bacteria from Coal Mines of Dara Adam Khel, Pakistan. *Geomicrobiology Journal*, *33*(1), 1-9.
- JIANG, Z., XIAO, T., KUZNETSOV, V. Á., & EDWARDS, P. Á. 2010. Turning carbon dioxide into fuel. *Philosophical Transactions of the Royal Society of London A: Mathematical, Physical and Engineering Sciences*, *368*(1923), 3343-3364.
- KASHIF, M.; JAAFAR, E.; BHADJA, P.; LOW, F.W.; SAHARI, S.K.; HUSSAIN, S.; LOONG, F.K.; AHMAD, A.; ALGARNI, T.S.; SHAFA, M.; ET AL. 2021. Effect of potassium permanganate on morphological, structural and electro-optical properties of graphene oxide thin films. *Arab. J. Chem., 14,* 102953.
- KEIJSER, B. J. F., ZAURA, E., HUSE, S. M., VAN DER VOSSEN, J. M. B. M., SCHUREN, F. H. J., MONTIJN, R. C., ... & CRIELAARD, W. 2008.
- Pyrosequencing analysis of the oral microflora of healthy adults. *Journal of dental research*, *87*(11), 1016-1020.

- KELLNER, H., LUIS, P., ZIMDARS, B., KIESEL, B., AND BUSCOT, F. 2008 .
- Diversity of bacterial laccase-like multicopper oxidase genes in forest and grassland cambisol soil samples. *Soil Biol. Biochem.* 40, 638–648. doi:
- 10.1016/j.soilbio.2007.09.013
- KHAN11, A. N., BEGUM, T., & SHER, M. 2012. Energy crisis in Pakistan: causes and consequences.
- KRÜGER, M., BECKMANN, S., ENGELEN, B., THIELEMANN, T., CRAMER, B., SCHIPPERS, A., & CYPIONKA, H. 2008. Microbial methane formation from hard coal.

- coal and timber in an abandoned coal mine. *Geomicrobiology Journal*, *25*(6), 315321.
- KUDANGA, T., NYANHONGO, G. S., GUEBITZ, G. M., AND BURTON, S. 2011. Potential application of laccase-mediated coupling and grafting reactions: a review. *Enzyme Microbiol. Technol.* 48, 195–208.
- LABORDA, F., FERNANDEZ, M., LUNA, N., & MONISTROL, I. F. 1997. Study of the mechanisms by which microorganisms solubilize and/or liquefy Spanish coals. *Fuel Processing Technology*, *52*(1-3), 95-10
- LANE, D. J. 1991. 16S/23S rRNA sequencing. *Nucleic acid techniques in bacterial systematics*, 115-175.
- LARENA, I., SALAZAR, O., GONZÁLEZ, V., JULIÁN, M. C., & RUBIO, V. 1999. Design of a primer for ribosomal DNA internal transcribed spacer with enhanced specificity for ascomycetes. *Journal of Biotechnology*, *75*(2-3), 187-194.
- LAVANIA, M., CHEEMA, S., SARMA, P. M., GANAPATHI, R., & LAL, B. 2014. Methanogenic potential of a thermophilic consortium enriched from coal mine. *International Biodeterioration & Biodegradation*, *93*, 177-185.
- LI, D., HENDRY, P., & FAIZ, M. 2008. A survey of the microbial populations in some Australian coalbed methane reservoirs. *International Journal of Coal Geology*, *76*(1-2), 14-24.
- LINDEQUE, P. K., PARRY, H. E., HARMER, R. A., SOMERFIELD, P. J., & ATKINSON, A. 2013. Next generation sequencing reveals the hidden diversity of zooplankton assemblages. *PloS one*, *8*(11), e81327.
- LING, Z., KONG, J., JIA, P., WEI, C., WANG, Y., PAN, Z.& XIANG, C. 2010. Analysis of oral microbiota in children with dental caries by PCR-DGGE and barcoded pyrosequencing. *Microbial ecology*, *60*(3), 677-690.

- LIU, Z., GUAN, D., WEI, W., DAVIS, S. J., CIAIS, P., BAI, J., ... & ANDRES, R. J. 2015. Reduced carbon emission estimates from fossil fuel combustion and cement production in China. *Nature*, *524*(7565), 335.
- MA, Q., QU, Y. Y., ZHANG, X. W., SHEN, W. L., LIU, Z. Y., WANG, J. W., ... &
  - ZHOU, J. T. 2015. Identification of the microbial community composition and structure of coal-mine wastewater treatment plants. *Microbiological research*, *175*, 1-
- 5.
- MACPHEE, J.M.; GIROUX, T.P.; CHARLAND, J.F.; PRICE, J.T. 2004. Detection of natural oxidation of coking coal by TG- FTIR mechanistic implications. *Fuel , 83,* 1855–1860.
- MADHAVI, V., AND LELE, S. S. 2009. Laccase: properties and applications. *BioResources* 4, 1694–1717.
- MALIK, A.Y.; ALI, M.I.; JAMAL, A.; FAROOQ, U.; KHATOON, N.; OREM, W.H.; BARNHART, E.P.; SANFILIPO, J.R.; HE, H.; HUANG, Z. 2020. Coal biomethanation potential of various ranks from Pakistan: A possible alternative energy source. *J. Clean. Prod, 255*, 120–177.
- MALKANI, M. S. 2012. A review of coal and water resources of Pakistan. *Journal of Science, Technology and Development*, *31*(3), 202-218.
- MALKANI, M. S., & SHAH, M. R. 2014. Chamalang coal resources and their depositional environments, Baluchistan, Pakistan. *Journal of Himalayan Earth Science*, *47*(1).
- MANOJ, B., KUNJOMANA, A. G., & CHANDRASEKHARAN, K. A. 2009.
- Chemical leaching of low rank coal and its characterization using SEM/EDAX and
- FTIR. *Journal of Minerals and Materials Characterization and Engineering*, *8*(10), 821.
- MANOJ, B.; NARAYANAN, P. 2013. Study of changes to the organic functional groups of a high volatile bituminous coal during organic acid treatment process by FTIR spectroscopy. *J. Miner. Mater. Characterisation Eng., 1,* 39–43.

- MARINOV, S. P., GONSALVESH, L., STEFANOVA, M., YPERMAN, J., CARLEER, R., REGGERS, G.,GADJANOV, P. 2010. Combustion behaviour of some biodesulphurized coals assessed by TGA/DTA. *ThermochimicaActa*, *497*(1-2), 46-51.
- MASTALERZ, M., &BUSTIN, R. M. 1993. Electron microprobe and micro-FTIR analyses applied to maceral chemistry. *International Journal of Coal Geology*, *24*(14), 333-345.
- Mathews, J. P., & Chaffee, A. L. 2012. The molecular representations of coal–a review. *Fuel*, *96*, 1-14.
- MESHRAM, P., PUROHIT, B. K., SINHA, M. K., SAHU, S. K., & PANDEY, B. D. 2015. Demineralization of low-grade coal–A review. *Renewable and Sustainable Energy Reviews*, *41*, 745-761.
- MESLÉ, M., DROMART, G., & OGER, P. 2013. Microbial methanogenesis in subsurface oil and coal. *Research in microbiology*, *164*(9), 959-972.
- MILICI, R. C., FLORES, R. M., & STRICKER, G. D. 2013. Coal resources, reserves and peak coal production in the United States. *International Journal of Coal Geology*, *113*, 109-115.
- MOCHIMARU, H., UCHIYAMA, H., YOSHIOKA, H., IMACHI, H., HOAKI, T., TAMAKI, H., ... & KAMAGATA, Y. 2007. Methanogen diversity in deep subsurface gas-associated water at the Minami-Kanto gas field in Japan. *Geomicrobiology Journal*, *24*(2), 93-100.
- MUNAWAR, N., ANWAR, M. A., SALEEM, M., GHAURI, M. A., & AKHTAR, K. 2007. Biodepyritization of indigenous coal using acidophilic, mesophilic and moderately thermophilic bacteria. *Pakistan Journal of Zoology*, *39*(3), 147.
- MUSTAFA, A.; AHMAD, T.; AKHTAR, J.; SHAHZAD, K.; SHEIKH, N.; MUNIR, S. 2016. Agglomeration of Makarwal coal using soybean oil as agglomerant. *Energy* , *38*, 733–3739.
- MUYZER, G., TESKE, A., WIRSEN, C. O., & JANNASCH, H. W. 1995.

- Phylogenetic relationships of Thiomicrospira species and their identification in deepsea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. *Archives of Microbiology*, *164*(3), 165-172.
- NASIR, S.; SARFARAZ, T.B.; VERHEYEN, T.V.; CHAFFEE, A.L.2011. Structural elucidation of humic acids extracted from Pakistani lignite using spectroscopic and thermal degradative techniques. *Fuel Process. Technol*. , *92*, 983–991.
- NAYAK, A. S., VIJAYKUMAR, M. H., & KAREGOUDAR, T. B. 2009.
- Characterization of biosurfactant produced by Pseudoxanthomonas sp. PNK-04 and its application in bioremediation. *International Biodeterioration &*
- *Biodegradation*, *63*(1), 73-79

- NAZEER, A., SHAH, S. H., ABBASI, S. A., SOLANGI, S. H., & AHMAD, N. 2017. Coal Bed Methane (CBM) Resources of Lower Indus Basin, Sindh-Pakistan. *Bahria Univ. Res. J. Earth Sci.*, *2*(1).
- NICOMRAT, D., DICK, W. A., & TUOVINEN, O. H. 2006. Assessment of the microbial community in a constructed wetland that receives acid coal mine drainage. *Microbial Ecology*, *51*(1), 83-89.
- OLSON, G. J., BRIERLEY, J. A., & BRIERLEY, C. L. 2003. Bioleaching review part B. *Applied microbiology and biotechnology*, *63*(3), 249-257.
- ONDOV, B. D., BERGMAN, N. H., & PHILLIPPY, A. M. 2011. Interactive metagenomic visualization in a Web browser. *BMC bioinformatics*, *12*(1), 385.
- OREM, W. H., VOYTEK, M. A., JONES, E. J., LERCH, H. E., BATES, A. L., CORUM, M. D., ... & CLARK, A. C. 2010. Organic intermediates in the anaerobic biodegradation of coal to methane under laboratory conditions. *Organic Geochemistry*, *41*(9), 997-1000.
- PRIVATE POWER AND INFRASTRUCTIVE BOARD RETRIEVED from www.ppib.gov.pk. 2014. Pakistan Coal Power Generation Potential, Islamabad.
- PARK, S. Y., & LIANG, Y. 2016. Biogenic methane production from coal: A review on recent research and development on microbially enhanced coalbed methane (MECBM). *Fuel*, *166*, 258-267.
- PATTI, A.F.; VERHEYEN, T.V.; DOUGLAS, L.; WANG, X. 1992. Nitrohumic acids from Victorian brown coal. *Sci. Tot. Environ*., *113*, 49–65.
- PENNER, T. J., FOGHT, J. M., AND BUDWILL, K. 2010. Microbial diversity of western Canadian subsurface coal beds and methanogenic coal enrichment cultures. *Int. J. Coal Geol.* 82, 81–93.
- PETROLEUM, B. 2012. British Petroleum Statistical Review of World Energy June 2012.
- PETROLEUM, B. 2015. BP Statistical Review of World Energy June 2015,
- PICCOLO, A.; CONTE, P.; COZZOLINO, A. 1999. Effects of mineral and monocarboxylic acids on the molecular association of dissolved humic substances.

- *Eur. J. Soil Sci , 50,* 687–694.
- PIKOLI, M. R., ADITIAWATI, P., & ASTUTI, D. I. 2013. Bacterial diversity in subbituminous coal and soil from coal mine of South Sumatra, Indonesia. *Int. Journal of integrative biology 2* (14).
- POKORN , R., OLEJN KOV , P., BALOG, M., I F K , P., H LKER, U., JANSSEN, M., ... VARE KA, L. U. 2005. Characterization of microorganisms isolated from lignite excavated from the Záhorie coal mine (southwestern Slovakia). *Research in microbiology*, *156*(9), 932-943.
- POSCH, S., & HAIDER, M. 2013. Dynamic modeling of CO2 absorption from coalfired power plants into an aqueous mono ethanolamine solution. *Chemical engineering research and design*, *91*(6), 977-987.
- PRAYUENYONG, P. 2002. Coal biodesulfurization processes. *Songklanakarin Journal of Science and Technology*, *24*(3), 493-507.
- PUROHIT, B. K., PRAMANIK, S., MESHRAM, P., SAHU, S. K., & PANDEY, B. D. 2013. Chemical beneficiation of Hingula coal. In *26th National Convention of Metallurgical and Materials Engineers & National Seminar* pp. 89-93.
- PUSHALKAR, S., MANE, S. P., JI, X., LI, Y., EVANS, C., CRASTA, O. R., ... & SAXENA, D. 2011. Microbial diversity in saliva of oral squamous cell carcinoma. *FEMS Immunology & Medical Microbiology*, *61*(3), 269-277.
- RATHI, R., PRIYA, A., VOHRA, M.H., LAVANIA, M., LAL, B., & SARMA, P. 2015. Development of a microbial process for methane generation from bituminous coal at thermophilic conditions. *International Journal of Coal Geology, 147*, 25-34.
- RAWLINGS, D. E. 2002. Heavy metal mining using microbes. *Annual Reviews in Microbiology*, *56*(1), 65-91.
- ROHWERDER, T., GEHRKE, T., KINZLER, K., & SAND, W. 2003. Bioleaching review part A. *Applied microbiology and biotechnology*, *63*(3), 239-248.

- SABAR, M.A.; ALI, M.I.; FATIMA, N.; MALIK, A.Y.; JAMAL, A.; LIAQUAT, R.; HE, H.; LIU, F.-J.; GUO, H.; URYNOWICZ, M.; ET AL. 2020. Evaluation of humic acids produced from Pakistani subbituminous coal by chemical and fungal treatments.
- *Fuel*, 278, 118301.
- SAHA, P.; SARKAR, S. 2019. Microbial degradation of coal into a value added product. *Int J. Coal Prep. Utiliz*. 39, 1–19.
- SALEEM, M.; IRFAN, M.; TABASSUM, S.; ALBAQAMI, M.D.; JAVED, M.S.; HUSSAIN, S.; PERVAIZ, M.; AHMAD, I.; AHMAD, A.; ZUBER, M. 2021.
- Experimental and theoretical study of highly porous lignocellulose assisted metal oxide photoelectrodes for dye-sensitized solar cells. *Arab. J. Chem., 14,* 102937.
- SANCHEZ, C. 2009. Lignocellulosic residues: biodegradation and bioconversion by fungi. *Biotechnol. Adv.* 27, 185–194.
- SANTELLI, C. M., PFISTER, D. H., LAZARUS, D., SUN, L., BURGOS, W. D., & HANSEL, C. M. 2010. Promotion of Mn (II) oxidation and remediation of coal mine drainage in passive treatment systems by diverse fungal and bacterial communities. *Applied and environmental microbiology*, *76*(14), 4871-4875.
- SARLAKI, E.; PAGHALEH, A.S.; KIANMEHR, M.H.; VAKILIAN, K.A. 2019. Extraction and purification of humic acids from lignite wastes using alkaline treatment and membrane ultrafiltration. *J. Clean. Prod. 235*, 712–723.
- SCHUMACHER, J. D. 1997. *Untersuchungen zur Oxidation kohlerelevanter Strukturanaloga durch bakterielle Monooxygenasen* (Doctoral dissertation).
- SCOTT, A. C., &GLASSPOOL, I. J. 2007. Observations and experiments on the origin and formation of inertinite group macerals. *International Journal of Coal Geology*, *70*(1-3), 53-66.
- SCOTT, A.R., GUYER, J.E. 2004. Method of generating and recovering gas from subsurface formations of coal, carbonaceous shale and organic-rich shales. U.S. Patent Application 20040033557 A1
- A. M. SCOTT, D. H., & CARPENTER 1996. *Advanced power systems and coal quality* (Vol. 87). IEA Coal Research.

- SEKHOHOLA, L. M., IGBINIGIE, E. E., & COWAN, A. K. 2013. Biological degradation and solubilisation of coal. *Biodegradation*, *24*(3), 305-318.
- Shakiba, N. Investigation of the Effective Parameters on Separation and Purification of Humic Acid from the Leonardite Humate Using a Proper Filter. Master's Thesis, University of Tehran, Tehran, Iran, 2016.

- SHARMA, P., GOEL, R., AND CAPALASH, N. 2007. Bacterial laccases. *World J. Microbiol. Biotechnol.* 23, 823–832. doi: 10.1007/s11274-006-9305-3
- SHI, K.; TAO, X.; HONG, F.; HE, H.; JI, Y.; LI, J. 2012 Mechanism of oxidation of low rank coal by nitric acid. J. Coal Sci. Eng. 18, 396–399.
- SHIMIZU, S., AKIYAMA, M., NAGANUMA, T., FUJIOKA, M., NAKO, M., AND ISHIJIMA, Y. 2007. Molecular characterization of microbial communities in deep coal seam groundwater of northern Japan. *Geobiology* 5, 423–433. doi: 10.1111/j.1472-4669. 2007 .00123.x
- SILVA-STENICO, M. E., VENGADAJELLUM, C. J., JANJUA, H. A., HARRISON, S. T., BURTON, S. G., & COWAN, D. A. 2007. Degradation of low rank coal by Trichoderma atroviride ES11. *Journal of industrial microbiology & biotechnology*, *34*(9), 625-631.
- Singh, D. N., Kumar, A., Sarbhai, M. P., & Tripathi, A. K. 2012. Cultivationindependent analysis of archaeal and bacterial communities of the formation water in an Indian coal bed to enhance biotransformation of coal into methane. *Applied microbiology and biotechnology*, *93*(3), 1337-1350.
- SINGH, P. K., SINGH, A. L., KUMAR, A., & SINGH, M. P. 2013. Control of different pyrite forms on desulfurization of coal with bacteria. *Fuel*, *106*, 876-879.
- SINGH, R., & SINHA, S. N. 2003. Beneficiation of high ash Indian coal-problems and prospects. *The Indian Mining and Engineering Journal*, 1-4.
- SINNATT, F. S., & BARAGWANATH, G. E. 1938. *The Hydrogenation of Victorian Brown Coals*. State Electricity Commission of Victoria.
- SIQUEIRA JR, J. F., & RÔÇAS, I. N. 2009. Diversity of endodontic microbiota revisited. *Journal of dental research*, *88*(11), 969-981.
- SKHONDE, M.P.; HEROD, A.A.; VAN DER WALT, T.J.; TSATSI, W.L.;

- Mokoena, K. The effect of thermal treatment on the compositional structure of humic acids extracted from South African bituminous coal. Int. J. Miner. Process. 2006, 81, 51–57
- SMIT, E., LEEFLANG, P., GLANDORF, B., VAN ELSAS, J. D., & WERNARS, K. 1999. Analysis of fungal diversity in the wheat rhizosphere by sequencing of cloned PCR-amplified genes encoding 18S rRNA and temperature gradient gel electrophoresis. *Applied and Environmental Microbiology*, *65*(6), 2614-2621.
- SOLEIMANI, M., BASSI, A., & MARGARITIS, A. 2007. Biodesulfurization of refractory organic sulfur compounds in fossil fuels. *Biotechnology advances*, *25*(6), 570-596.
- SRIVASTAVA, K.C., WALIA, D.S. 1997. Biological production of humidic acid and clean fuels form coal. U.S. Patent. 5670345 A.
- STARR, M. P., &SKERMAN, V. B. D. 1965. Bacterial diversity: the natural history of selected morphologically unusual bacteria. *Annual Reviews in Microbiology*, *19*(1), 407-454.
- STEPNIEWSKA, Z., PYTLAK, A., AND KUZNIAR, A. 2014. Distribution of the methanotrophic bacteria in the Western part of the Upper Silesian Coal Basin (Borynia-Zofiowka and Budryk coal mines). *Int. J. Coal Geol.* 130, 70–78. doi: 10.1016/j.coal.2014.05.003
- STEVENSON, F. J. 1994. *Humus chemistry: genesis, composition, reactions*. John Wiley & Sons. STRĄPOĆ, D., MASTALERZ, M., DAWSON, K., MACALADY, J., CALLAGHAN, A. V., WAWRIK, B., ... & ASHBY, M. 2011. Biogeochemistry of microbial coal-bed methane. *Annual Review of Earth and Planetary Sciences*, *39*, 617-656.
- SUDHEER, P. D., DAVID, Y., CHAE, C. G., KIM, Y. J., BAYLON, M. G., BARITUGO, K. A., ... & PARK, S. J. 2016. Advances in the biological treatment

➢

of coal for synthetic natural gas and chemicals. *Korean Journal of Chemical Engineering*, *33*(10), 2788-2801.

- SYAHREN, A.M.; WONG, N.C. EXTRACTION AND CHEMICAL
- CHARACTERISTICS OF NITRO-HUMIC ACIDS FROM COALS AND
- COMPOSTS. J. TROP AGRIC. FD. SC. 2008, 36, 269–279.

- TANG, Y. Q., JI, P., LAI, G. L., CHI, C. Q., LIU, Z. S., AND WU, X. L. 2012.
- Diversity microbial community from the coalbeds of the Ordos Basin, China. *Int. J.*
- *Coal Geol.* 90–91, 21–23. doi: 10.1016/j.coal.2011.09.009
- TANNER, A. C. R., MATHNEY, J. M. J., KENT, R. L., CHALMERS, N. I., HUGHES, C. V., LOO, C. Y., ... & PAPADOPOLOU, E. 2011. Cultivable anaerobic microbiota of severe early childhood caries. *Journal of clinical microbiology*, *49*(4), 1464-1474.

- TAO, S., WANG, Y., TANG, D., XU, H., LV, Y., HE, W., & LI, Y. 2012. Dynamic variation effects of coal permeability during the coalbed methane development process in the Qinshui Basin, China. *International Journal of Coal Geology*, *93*, 16-
- 22.

- TAYLOR, G. H., TEICHMÜLLER, M., DAVIS, A. C. F. K., DIESSEL, C. F. K., LITTKE, R., & ROBERT, P. 1998. Organic petrology: Berlin. *Gebrüder Borntraeger*.

- THE LAHORE CHAMBER OF COMMERCE AND INDUSTRY. 2014. Retrieved from http://lcci.com.pk/index.php?cal_events_month=11&cal_events_year=2013&cal_ev ents_focus=2015-11-26

- VALERO, N., GÓMEZ, L., PANTOJA, M., & RAMÍREZ, R. 2014. Production of humic substances through coal-solubilizing bacteria. *Brazilian Journal of Microbiology*, *45*(3), 911-918.

- VAN DER MERWE KJ, STEYN PS, FOURIE L, SCOTT DB, THERON JJ. 1965.
- Ochratoxin A, a toxic metabolite produced by Aspergillus ochraceus Wilh. Nature 205:1112–1113. https://doi.org/10.1038/2051112a0
- Van Krevelen, D. W. 1993. Coal: Typology–Physics–Chemistry–Constitution (Coal Science & Technology). *Elsevier Science, Amsterdam*.
- VARGA, J., TÓTH, B., KOCSUBÉ, S., FARKAS, B., SZAKÁCS, G., TÉREN, J., & KOZAKIEWICZ, Z. 2005. Evolutionary relationships among Aspergillus terreus isolates and their relatives. *Antonie Van Leeuwenhoek*, *88*(2), 141-150.
- VOLKWEIN, J. C., SCHOENEMAN, A. L., CLAUSEN, E. G., GADDY, J. L., JOHNSON, E. R., BASU, R., ... & KLASSON, K. T. 1994. Biological production of methane from bituminous coal. *Fuel Processing Technology*, *40*(2-3), 339-345.
- WANG, C.F.; FAN, X.; ZHANG, F.; WANG, S.Z.; ZHAO, Y.P.; ZHAO, X.Y.;
  - ZHAO, W.; ZHU, T.G.; LU, J.L.; WEI, X.Y. 2017.c haracterization of humic acids

- extracted from a lignite and interpretation for the mass spectra. *rsc adv. , 7,* 20677– 20684.
- WANG, L., NIE, Y., TANG, Y. Q., SONG, X. M., CAO, K., SUN, L. Z., ... & WU, X. L. 2016. Diverse bacteria with lignin degrading potentials isolated from two ranks of coal. *Frontiers in microbiology*, *7*, 1428.
- WEI, M., YU, Z., & ZHANG, H. 2015. Molecular characterization of microbial communities in bioaerosols of a coal mine by 454 pyrosequencing and real-time PCR. *Journal of Environmental Sciences*, *30*, 241-251.
- WEI, M., YU, Z., JIANG, Z., & ZHANG, H. 2014. Microbial diversity and biogenic methane potential of a thermogenic-gas coal mine. *International Journal of Coal Geology*, *134*, 96-107.
- WORLD ENERGY RESOURCES: 2013 Survey - World Energy Council https://www.worldenergy.org/publications/2013/world-energy-resources-2013survey/
- XAVIER, D.; SILVA, A.S.; SANTOS, R.P.; MESKO, M.; COSTA, S.; FREIRE, V.; CAVADA, B.; MARTINS, J. 2012. Characterization of the coal humic acids from the candiota coalfield, brazil. I*nt. j. agric. sci*. , 4, 238–242.
- XIAO, H. M., MA, X. Q., & LAI, Z. Y. 2009. Isoconversional kinetic analysis of cocombustion of sewage sludge with straw and coal. *Applied Energy*, *86*(9), 1741-1745.
- XU, W., WANG, H., ZHU, T., KUANG, J., & JING, P. 2013. Mercury removal from coal combustion flue gas by modified fly ash. *Journal of Environmental Sciences*, *25*(2), 393-398.
- YIN, S., TAO, X., & SHI, K. 2011. The role of surfactants in coal biosolubilisation. *Fuel processing technology*, *92*(8), 1554-1559.
- YIN, S., TAO, X., SHI, K., & TAN, Z. 2009. Biosolubilisation of Chinese lignite. *Energy*, *34*(6), 775-781.
- ZARA, M.; AHMAD, Z.; AKHTAR, J.; SHAHZAD, K.; SHEIKH, N.; MUNIR,

- S.2017. extraction and characterization of humic acid from pakistani lignite coals.
- *energy sources part a recovery util. environ. effects 39*, 1159–1166.
- ZHANG, J., LIANG, Y., HARPALANI, S., 2016a. Optimization of methane production from bituminous coal through biogasification. *Appl. Energy 183*, 31–42.
- ZHANG, J., LIANG, Y., PANDEY, R., HARPALANI, S., 2015. Characterizing microbial communities dedicated for conversion of coal to methane in-situ and exsitu. *Int. J. Coal Geol*. *146*, 145–154.
- ZHANG, J., PARK, S., LIANG, Y., HARPALANI, S., 2016b. Finding cost-effective nutrient solutions and evaluating environmental conditions for biogasifying bituminous coal to methane ex-situ. *Appl. Energy 165*, 559–568.
- UPAN I , G.D., GRILC, V., 2012. KUMAR, S., BHARTI, A. Anaerobic treatment and biogas production from organic waste. In: (Eds.), *Management of Organic Waste. InTech*, Rijeka, pp. 1–28.

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Extraction and Chemical Characterization of Humic Acid from Nitric Acid Treated Lignite and Bituminous Coal Samples

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Abstract: Currently, conversion of coal into alternative fuel and non-fuel valuable products is in demand and growing interest. In the present study, humic acid was extracted from two different ranks of coal, i.e., low rank and high rank (lignite and bituminous), through chemical pretreatment by nitric acid. Samples of lignite and bituminous coal were subjected to nitric acid oxidation followed by extraction using KOH and NaOH gravimetric techniques. The chemical pretreatment of both types of coal led to enhanced yields of humic acid from 21.15% to 57.8% for lignite low-rank coal and 11.6% to 39.6% bituminous high rank coal. The derived humic acid from native coal and nitric acid treated coal was analyzed using elemental analysis, E4/E6 ratio of absorbance at 465 nm and 665 nm using UV-Visible spectrophotometry and Fourier transformed infrared spectroscopy FTIR. The chemical characteristics of coal treated with nitric acid have shown increased molecular weight and improved aromaticity with more oxygen and nitrogen and lower C, H, and sulphur content. The E4/E6 ratio of nitric acid-treated low and high ranks of coal was high. The FTIR spectroscopic data of nitric acid-treated lignite coal indicates an intensive peak of carboxyl group at 2981.84 cm^{-1}, while bituminous coal was shown in cooperation with the N-H group at 2923.04 cm^{-1}. SEM was performed to detect the morphological changes that happen after producing humic acid from HNO_3 treatment and native coal. The humic acid produced from HNO_3 treated coal had shown clear morphological changes and some deformations on the surface. SEM-EDS detected the major elements, such as nitrogen, in treated humic acid that were absent in raw coal humic acid. Hence, the produced humic acid through HNO_3 oxidation showed a more significant number of humic materials with improved efficiency as compared to native coal. This obtained humic acid can be made bioactive for agriculture purposes, i.e., for soil enrichment and improvement in growth conditions of plants and development of green energy solutions.

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