# BIOSTIMULATION AND BIOAUGMENTATION OF DIESEL CONTAMINATED ENVIRONMENT (SOIL)

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# CERTIFICATE

This is to certify that the work entitled, "**Biostimulation and Bioaugmentation of diesel contaminated environment"** submitted by Shalini Kanwar (101720) and Mehak Puran(101563) in partial fulfillment for the award of degree of Bachelor of Technology in Biotechnology, Jaypee University of Information Technology, Waknaghat has been carried out under by supervision. This work has not been submitted partially or wholly to any other university or institute for the award of this or any other degree or diploma.

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## LIST OF ABBREVIATIONS

- **RPM- Rotation per minute**
- **BTEX- Benzene, Toulene, Ethybenzene, Xylne**
- **NAPL-non- aqueous phase liquid**
- **PAHs - polycyclic aromatic hydrocarbons.**
- **XYL- xylene degraditive plasmid.**
- **NAH- napthlene degradativi plasmid.**
- **CMC- critical micelle concentration**
- **QSS- quorum sensing system**
- **AHLs - N- acylholoserine lactones**
- **NRPSs- non ribosomal peptide synthetases**
- **US EPA- United States Environment Protection Act**
- **BH- Bushnell - Hass medium**
- **EMB – Eosin Methylene Blue**

## ABSTRACT

Contamination of soils with diesel hydrocarbons has always been an important worldwide issue. Among all the available remediationmethods, bioremediation is widely considered to be a cost-effective and environmentally friendly approach. For bioremediationto be effective, the overall rate of intrinsic biodegradation and subsequent removal of hydrocarbons must be accelerated,which can be done through biostimulation and bioaugmentation. Spillage of hydrocarbons is also a source of contamination for soil and water ecosystem. It provides efficacy, safety on long term use, cost and simplicity of administration with promising opportunity for creating better environment. The goal of our study was to check the diesel degradation potential at low temperature. We conducted our experiments on a carbon free medium and only carbon source provided was the diesel(5%). Microbes (bacteria in our study) utilized diesel as a sole carbon source and degraded it. A set of follow up experiments were conducted to check the diesel degrading potential of the potent isolates. Experiments at $15^0$C and $30^0$C were performed and biomass and biosurfactant activity was monitored. Microbial activity was highest at seventh day of incubation i.e 0.2521mg/ml and 0.1509 mg/ml, resecptively at both the temperature. Biosurfactant activity and biomas activity was observed higher at $30^0$C as compared with $15^0$C.But these results showed that the isolate was able to grow at low temperature and consumed 83.45% of diesel at $15^0$C as compared to 93.58% at $30^0$C. A set of experiments was also performed at extreme pH conditions(4, 5, 10 and 11) with 5% diesel and the bacterial isolate was found to be alkaliphile. BTEX degradation potential was also measured but the selected bacterial isolate was not able to grow on aromatic compounds. Various Biochemical tests were performed of the isolates which gave indications of isolate belonging to *Acinetobacter*genus.

# CHAPTER 1

## 1.1 Introduction

Due to the rising population of the world and daily life demands supplied through industries and modern industrialized agricultural systems, the need for the preservation of ecosystems is important. Hydrocarbon spillage has the most common occurrence and the potential to cause the greatest environmental impact. Hydrocarbons in soil are of concern because of their potential for detrimental effects on soil properties. Understanding these effects is important to manage or remediate contaminated soil. One of the most economical and stable approaches to cope with this vital task is the use of the techniques developed through progresses is bioremediation. Bioremediation as a branch of environmental biotechnology takes advantage of various living organisms including bacteria, fungi, algae, and plants in order to remediate the contaminated ecosystems.

## 1.2 Principles of bioremediation

Bioremediation is the use of microorganisms to degrade the environmental contaminants into less toxic forms. It involves the use of naturally occurring bacteria and fungi or plants to degrade or detoxify substances hazardous to human health and/or the environment. Contaminant compounds are transformed by living organisms through reactions that take place as a part of their metabolic processes. So for bioremediation to be effective it is very important that these organisms should be metabolically active and should carry different enzymes required for the biodegradation.

Bioremediation, involving bioaugmentation and biostimulation is an economical and eco-friendly approach, that has emerged as the most advantageous soil and water clean-up technique for contaminated site containing hydrocarbon pollutants (Meenutyagi et al. 2010).

**(i)** **Biostimulation -**Biostimulation involves the addition of nutrients and oxygen to help indigenous microorganism to induce propagation at faster rate. Its goal is to increase the metabolism and population growth of hydrocarbon degrading microorganism through addition of limiting growth factors, primarily nitrogen (N) and phosphorous (P) (Margesin and Schinner 1999). The longer the oil persists in the environment, the more resistant to biodegradation certain components of the petroleum mixture become (Margesin and Schinner 1997b, Mitchell 1999). It follows that a major goal of hydrocarbon bioremediation is to increase

biodegradation rates as soon as possible following a spill. In addition, because oil on beaches and sediment surfaces is detrimental to wildlife and unsightly (to humans), faster and more complete microbial degradation than would occur without manipulation is desired. This can be attained through the addition of various forms of N and P.

**(ii) Bioaugmentaion -** Bioaugmentation is the addition of pre grown microbial culture to enhance the degradation of unwanted compounds. The decision to implement either or both of these techniques for bioremediation largely depends on the degrading capability of the indigenous microbes and the extent of contamination of the site to be treated. (Meenu Tyagi et al. 2010). The principle of bioaugmentation is that the indigenous microbes may not be able to degrade all of the compounds in a given petroleum mixture. If the non-native or genetically modified microbes can survive in the different habitat and compete the indigenous microbes, they should bring about efficient degradation of the petroleum (Atlas 1995). In the lab, cold-adapted microbes may efficiently degrade target hydrocarbon compounds over wide ranges of temperature and nutrient level. The *in situ* competitive interaction between novel and introduced species generally seems, however, to favor the indigenous species. When the added microbes do have a favorable effect, that effect appears to lessen considerably over time (Margesin and Schinner, 1997b). The reasons for failure of bioaugmentation at any temperature include concentration of the carbon source may be too low to support growth, introduced microbes may be adversely affected by toxins in the water or sediments, other available sources of food may "distract" microbes from the pollutant, and microbes may have trouble moving through the soil to contaminated microsites (Goldstein et al. 1985). Despite these hurdles, there are times when bioaugmentation may be the only way to stimulate hydrocarbon biodegradation (Margesin and Schinner 1997a, 1997b).

Hydrocarbon-contaminated environments are characterized by low or high temperatures, acidic or alkaline pH, high salt concentrations, or high pressure. Hydrocarbon-degrading microorganisms, adapted to grow in these environments, play an important role in the bioremediation. The biodegradation of a wide range of hydrocarbons, including aliphatic and aromatic compounds have been shown to occur in various extreme habitats.

Biosurfactant or microbial surfactants produced by microbes are structurally diverse and heterogeneous groups of surface-active amphipathic molecules. They are capable of reducing surface and interfacial tension and have a wide range of industrial and environmental applications. Biosurfactant increases the bioavailability of low solubility hydrocarbons during bioremediation. Permeability of the microbial cell membrane might be adversely affected by the use of synthetic surfactant, which would interfere with the capacity of a microorganisms to biodegrade. Microbial surfactants are generally much less toxic than chemical surfactants, but are as effective and more readily biodegradable. Using microorganisms that produce their own biosurfactants capable of degrading pollutants can further lower treatment costs.

# CHAPTER 2

## Review of Literature

### 2.1- Hydrocarbon Spillage

Diesel spills take place during the process of manufacturing, storage and transportation. Major spills, such as pipeline, tanker or storage tank accidents, create an acute problem of pollution.Therefore, diesel hydrocarbons create a world-wide problem of contaminated water and soil that require decontamination.

Diesel oil contains low molecular weight compounds that are usually more toxic than long chain hydrocarbons, because long chain ones are less soluble and less bioavaliable. Light oils contain high proportion of saturated hydrocarbons, hence these can be more toxic than heavy oils(Dorn et al.1998).Diesel oil hydrocarbons are derived from crude oil refining and is a complex mixture of saturated and aromatic hydrocarbons.Cold regions have been considered to be especially sensitive to oil pollution,because of the prolonged degradation time of oil hydrocarbons(Horel And Schiewer 2009).

The Exxon Valdez oil spill in 1989 in Prince William Sound, Alaska, was the genesis of global attention to this process. About 11 million gallons (42 million liters) of crude oil was spilled into the vast sea. The history of bioremediation in response to the Exxon Valdez oil spill was effective. Polycyclic aromatic hydrocarbons (PAHs) are a minor constituent of crude oils; however, they are among the most toxic to plants and animals. Bacteria can convert PAHs completely to biomass, $CO_2$, and $H_2O$, but they usually require the initial insertion of $O_2$ via dioxygenase enzymes. *Exxon Valdez* spill, involved adding fertilizers containing nitrogen (N) nutrients to speed up the rates of oil biodegradation.

The ***Deepwater Horizon*** **oil spill** also called as the **BP oil spill**, the **BP oil disaster**, the **Gulf of Mexico oil spill**, and the **Macondo blowout** began on 20 April 2010 in the Gulf of Mexico.It leaked about 5000 ft (1500 m) from the well ,below the ocean surface and spred to about more than 50 mi (80 km) from the nearest shore.considerable amounts of natural gas (methane ($CH_4$) was released into the shore. But it was estimated that BP *Deepwater Horizon* spill was a light crude and more inherently biodegradable initially than the *Exxon Valdez* heavy crude.

## 2.2- Historical perspective

Bioremediation was invented by George M. Robinson in1960s. He pioneered the idea of making custom mixtures of dried bacteria cultures for commercial use. His "bug-brew" recipes gained acceptance and notoriety after several well publicized demonstrations. As Bug Brew was so effective when used on various forms of pollutants in a degraded system he then went on and formed US Microbiotics Incorperated (nicknamed 'BUGS') in 1997. Later in 1972 ,the first commercial in situ bioremediation system was installed to cleanup a Sun Oil pipeline spill in Ambler, Pennsylvania (National Research Council, National Academy Press, 1993).

In 1979, Anand Mohan Chakrabartyfor the first time, obtained a strain of *Pseudomonas putida* that contained the XYL and NAH plasmid as well as a hybrid plasmid derived by recombinating parts of CAM and OCT (these are incompatible and cannot co-exist as separate plasmids in the same bacterium). As these strains were capable of metabolizing hydrocarbons more efficiently than any other single plasmid they grew rapidly on crude oil. Considering this in mid-1980s emphasis went on bioengineering organisms for bioremediation.

**Pre 1989**-"Courtship" Period, was a period of research. This was when bioremediation was little known. During the 1970s and 1980s several scientific papers and articles were published on bioremediation. Several studies following major oil spills like the Amoco Cadiz spill measured oil degradation in the environment and confirmed laboratory research.

**Between 1989-1991** Between this period bioremediation was widespread and gained attention and interest. During the first weeks after the spill, responders flooded in with offers to help clean up the oil that had spread over 500 km of coastline in Prince William Sound. Bioremediation agents of all kinds were used but the testing and evaluation was not conducted. Prior to 1989, there were no documented uses of this technology on marine oil spills. During the 1990s, bioremediation was used (on a trial basis) at a total of four US spills: Prall's Island in New Jersey, Seal Beach in California, and the Apex barges and Mega Borg spills in the Gulf of Mexico.

**After 1992**

After 1992 "Establishment" period started for bioremideation. During this time, bioremediation has achieved a certain level of acceptance, with more realistic expectations than earlier, but the level of interest and attention has decreased considerably because the toxicity profile of various fertilizer formulations or microbial products were not known. In

the case of a large spill, such as the Exxon Valdez, a pilot test was conducted before the responsible authorities commit to the use of bioremediation on a large scale. However, the expense and effort required to establish a monitoring program may prevent the use of bioremediation at smaller spills

But this technology did not produce better results. So by 1990s scientists switched to greater reliance on natural microorganismsand techniques to enhance their performance.(Sheetal Sonawdekar,2012)

### 2.3- Bioremediation

The term bioremediation has been introduced to describe the process of using biological agents to remove toxic waste from environment. Bioremediation is the most effective management tool to manage the polluted environment and recover contaminated soil. It is an attractive and successful cleaning for polluted environment. Bioremediation uses microorganisms to degrade organic contaminants in soil, groundwater, sludge, and solids. The microorganisms break down contaminants by using them as an energy source.

Bioremediation is a “treatment that uses naturally occurring organisms to break down hazardous substances into less toxic or non toxic substances”. It has also been described as “ a treatability technology that uses biological activity to reduce the concentration or toxicity of a pollutant. It commonly uses processes by which microorganism transfom or degarde chemicals in the environment”

Bioremediation is an important alternative for soil and industrial wastes clean-up. Industrial and environmental biotechnology are going to new paths, resulting in processes with "clean technologies", with the maximum production and the less residues. Technologies of remediation and bioremediation are continuously being improved using genetically modified microorganisms or those naturally occurring, to clean residues and contaminated areas from toxic organics.

#### 2.3.1- Factors that Affect Bioremediation

**(i) Contaminant concentrations** directly influence microbial activity. When concentrations are too high, the contaminants may have toxic effects on the present

bacteria. In contrast, low contaminant concentration may prevent induction of bacterial degradation enzymes.

**(ii) Contaminant bioavailability** depends on the degree to which they sorb to solids or are sequestered by molecules in contaminated media, are diffused in macropores of soil or sediment, and other factors such as whether contaminants are present in non-aqueous phase liquid (NAPL) form. Bioavailability for microbial reactions is lower for contaminants that are more strongly sorbed to solids, enclosed in matrices of molecules in contaminated media, more widely diffused in macropores of soil and sediments.

**(iii) Site characteristics** have a significant impact on the effectiveness of any bioremediation strategy. Site environmental conditions important to consider for bioremediation applications include pH, temperature, water content and nutrient availability.

**(iv) pH** affects the solubility and biological availability of nutrients, metals, and other constituents; for optimal bacterial growth, pH should remain within the tolerance range for the target microorganisms. Bioremediation processes preferentially proceed at a pH of 6-8.

**(v) Temperature** directly affects the rate of microbial metabolism and consequently microbial activity in the environment. The biodegradation rate, to an extent rises with increasing temperature and slows with decreasing temperature

### 2.3.2- Bioremediation advantages

1.-It is helpful in complete destruction of a wide variety of contaminants. Compounds which are known to be legally hazardous can be transferred to harmless productswhich eliminates the chance of future liability associated with treatment and disposal of contaminated material.

2.-It has been proved less expensive than the other technologies which are used for clean-up of hazardous waste.

3.-Can be carried out on the site, without causing disruption of normal activities. This eliminates the potential threat to human health and environment duringtransportation.

**Table 1.1:** Different types of Bioremediation strategies and their advantages and limitations.

| **Technolgies** | **Examples** | **Benefits** | **Limitations** | **Factors to be considered** |
|---|---|---|---|---|
| In Situ | In Situ bioremediation<br>Biosparging<br>Bioventing<br>Bioaugmentation | Most cost efficient<br>Noninvasive<br>Relatively passive<br>Natural attenuation processes<br>Treats soil and water | Environmental constraints<br>Etended treatment time<br>Monitoring difficulties | Biodegradative abilities of indigenious microorganisms<br>Presence of metals and other inorganics<br>Environmental parameters<br>Biodegradability of pollutants<br>Chemical solubility<br>Geological factors<br>Distribution of pollutants |
| Ex Situ | Landfarming<br>Composting<br>Biopiloes | Cost efficient<br>Low cost<br>Can be done on site | Space requirements<br>Extended treatment time<br>Need to control abiotic loss<br>Mass transfer problem<br>Bioavailability limitation | |
| Bioreactors | Slurry reactors<br>Aqueous reactors | Rapid degradation kinetic Optimized environmental parameters<br>Enhances mass transfer effective use of inoculants and surfactants | Soil requires excavation<br>Relatively high cost capital<br>Relatively high operating cost | Bioaugmentation<br>Toxicity of amendments<br>Toxic concentrations of contaminants |

## 2.4- Hydrocarbon degrading Microbes

Hydrocarbon degrading microorganisms are mostly found in all places in nature but are found at relatively higher densities in petroleum contaminated sites. Hydrocarbon degrading bacteria both aerobic and anaerobic and fungi are widely distributed in these hydrocarbon contaminated sites**.** In environment hydrocarbons are biodegraded primarily by bacteria, yeast, and fungi. It has been reported that biodegradation ranged from 6% to 82% for soil fungi, 0.13% to 50% (Pinholt et al ,1979) for soil bacteria, and 0.003% to 100% for marine bacteria.(Phillips et al, 1974 )A consortia of microbes with broad enzymatic capacities are required to degrade complex mixtures of hydrocarbons such as crude oil in soil , fresh water , and marine environments **.**Some hydrocarbon degrading microorganisms are as listed in Table1.2.

Bacteria are considered as primary degraders of spilled oil in environment as they work active agents in petroleum degradation.(Brooijmans et al, 2009). Floodgate isolated 25 genera of hydrocarbon degrading bacteria and 25 genera of hydrocarbon degrading fungi from marine environment(Floodgate et al, 1984). In earlier days, the study at which bacteria, yeast, and filamentous fungi participate in the biodegradation of petroleum hydrocarbons was the subject of limited, but was the part of the ecosystem (Leathy et al, 2001). Crude petroleum oil from petroleum contaminated soil from North East India was reported by Das and Mukherjee (Mukhrjee et al,2007). It was found that *Acinetobacter sp.* Were capable of utilizing n-alkanes of chain length $C_{10}$–$C_{40}$ as a sole carbon source (Holst et al,2007).

Many fungal species are studied which particpete in hydrocarbon degredation .some belongs to the genera *Amorphoteca, Neosartorya, Talaromyces, and Graphium* and yeast genera, namely,*Candida, Yarrowia, and Pichia* . They were isolated from petroleum-contaminated soil and proved to be the potential organisms for hydrocarbon degradation (Chaillal et al, 2004). Some group of terrestrial fungi, namely,*Aspergillus, Cephalosporium, and Pencillium* were also found to be the potential degrader of crude oil hydrocarbons. The yeast species, namely, *Candida lipolytica, Rhodotorulamucilaginosa, Geotrichum sp,* and *Trichosporonmucoides* isolated from contaminated water were noted to degrade petroleum compounds (Boguslawska et al,2001)

**Table 1.2:** Different types of petroleum hydrocarbon degrading bacteria (Desai and Vyas, 2006, Microbewiki, 2011)

| Sr no. | Components of crude oil | Microorganisms |
|---|---|---|
| **1** | Staturated hydrocarbons | *Arthrobacter sp., Acinetobacter sp., Candida sp., Pseudomonas sp., Rhodococcus sp., Streptomyces sp., Bacillus sp., Aspergillusjaponicu* |
| **2** | Monocyclic aromatic hydrocarbons | *Pseudomonas sp., Bacillus sp. B. stereothermophilus, Vibrio sp., Nocardia sp., Corynebacterium sp., Achromobacter sp.* |
| **3** | Polycyclic hydrocarbons | *Arthrobactersp, Bacillus sp., Burkholderiacepacia., Pseudomonassp., Mycobacterium sp., Xanthomonas sp., Phanerochaetechrysosporium, Anabena sp., Alcaligenes* |
| **4** | Resins | *Pseudomonas sp., Members of Vibrionaceae.,Enterobacteriaceea.,Moraxella sp.* |

### 2.5- Role of biosurfactant in bioremediation

Due to the low solubility of hydrocarbons in water, and the first step in hydrocarbon degradation which involves first membrane-bound oxygenase, makes it essentialfor bacteria to come into direct contact with their hydrocarbon substrates. This can be achieved through adhesion mechanisms or emulsification of the hydrocarbon. After an oil spill, low molecular weight hydrocarbons rise to the surface and come into contact with air and get volatized remaining are metabolized by microorganisms, which take up soluble hydrocarbons. Residual high molecular-weight hydrocarbons degradation involves microorganisms with high-cell-surface hydrophobicity, which can adhere to them via hydrophobic fimbriae, fibrils, outer membranelipids and proteins and certain small cell-surface molecules, such as gramicidin Sand prodigiosin. Bacterialcapsules and other anionic exo polysaccharides appear to inhibit adhesion to hydrocarbons.(Ron et al,2014)

The rhamnolipid biosurfactant produced by *P. aeruginosa* stimulates the uptake of hydrophobic compounds finally leading to its degradation (Terpstra et al, 1989).

Das and Mukherjee have demonstrated the crude petroleum-oil biodegradation efficiency of biosurfactant producing *B. subtilis*DM-04 and *P. aeruginosa* strains which have been isolated from the petroleum oil contaminated soil from North-East India (Rodregues et al, 2004). The study shows that all the three bacteria are effective biosurfactant producers in petroleum oil-contaminated soil which offers the advantage of continuously supplying natural, nontoxic and biodegradable biosurfactants by bacteria at low cost for solubilizing the hydrophobic oil hydrocarbons before biodegradation. Other studies, shows that the biosurfactant secreted by the *B. subtilis*and*P .aeruginosa* strains enhanced the apparent solubility of pyrene (a toxic polyaromatic hydrocarbon), and also influenced the bacterial cell surface hydrophobicity resulting in higher uptake and utilization of pyrene by bacteria.( Rodregues et al, 2004)

Biosurfactant are amphiphillic molecules having hydrophobic moiety and a hydrophilic moiety. Hydrophilic moiety consist of amino acids or peptides anions or cations, mono, di or polysaccharides whereas hydrophobic moiety consist of unsaturated or saturated fatty acids. The presence of surfactant at air- water interface reduces the surface tension of solution and leads to stabilization of foam. Biodegradation of hydrophobic organic compounds in pollute soil is a process involving interactions among soil particles, pollutants, water, and microorganisms. Surfactants are compounds that may affect these interactions by stimulation of mass transport of the pollutant from the soil to the aqueous phase and hence helps in overcoming the problem of limited bioavailability. This can be achieved by three mechanism-emulsification of liquid pollutant, micellar solubilization and facilitated transport. Biosurfactant activity in the culture can be measured through hemolytic activity, drop collapsing test, emulsification measurement (using xylene), oil displacement test (Maneerat et al.2007).

Biosurfactants are easily biodegraded and more useful than chemically derived synthetic biosurfactants like lower toxicity, better environment compatibility, higher foaming higher selectivity and specific gravity at extreme pH and temperature due to these propriety they have wide industrial application in remediation of organics and metals enhanced transport of bacteria an oil recovery, in cosmetic additives, in emulsification, foaming, detergency, wetting, foaming and solublization. Major disadvantage of biosurfactant is they do not compete economically with synthetic surfactants. To reduce production cost different approach has been experimented like increase of yield and product accumulation, use of

economical engineering process, use of cost free and cost credit feed stock for microbial growth.

Molecular weights of microbial biosurfactants generally range from 500-1500Da. Among biosurfactants, the lower molecular weight glycolipids and glycopeptides are typically more effective in reducing surface and interfacial tensions while high molecular weight amphipathic polysaccharides and proteins as well as lipopolysaccharides and lipoproteins are oil-in-water stabilizers. The minimum biosurfactant concentration required to form micelles, the critical micelle concentration (CMC) ranges from 1-200mg/l(Ramakrishna Sen.2010)

Biosurfactants are complex molecules of different structure peptides, glycolipids, glycopeptides, fatty acids and phospholipids different types of biosurfactants is shown in table3 (Ramakrishna Sen.2010). Several bacteria and yeast produce fatty acids and phospholipids surfactants during growth on n-alkanes. Hydrophobic lipophillic balance (HLB) is directly related to length of hydrocarbon chain in their structure. *Acinetobactersp* strain H01-N phosphatidylethanalamine, forms rich vesicles, these extracellular membrane vesicles forms partition hydrocarbon to form a microemulsion, which plays an important role in alkane uptake by microbial cells(Kappeli etal, )

### 2.5.1- Biosurfactant synthesis in microorganisms

Biosurfactant production by microbe is still unknown. One of the reason can be their survival on hydrophobic substrates and desorption from the hydrophobic substrates allowing direct contact with cell, thereby increasing the bioavailability of insoluble substrates. Synthesis take place by de novo pathway. Based on four assumption proposed by Syldatk and Wagner diagrammatic representation is shown in Fig 1. (Syldatk et al, 1987).Introduction and repression are dependent on the presence of carbon, nitrogen, phosphate, trace elements and multivalent cations (Kitametoetal 2002,Weiyh et al, 2002). Literature survey suggests that complex pathways are involved in biosurfactant production and genes for biosurfactant production are present on chromosomal DNA. Intracellular communication and production of enzymes pigments and biosurfactant by Quorum sensing system(QSS) which depend on the production of diffusible signal molecules termed as auto inducers. The regulatory machinery is different for different biosurfactantproducers. (Bobgelmezet al,2003) . Table 1.3 shows the types of biosurfactants produced by various microorganism and their applications.

Polymer synthsis in *Acinetobacter*species is controlled by an intricate operon system and its further excretion being controlled byenzymes. Quorum sensing system (QSS) plays a fundamental role in rhamnolipid and surfactin synthesis. Depending upon the cell density, signal molecules (autoinducers) of regulatory pathways accomplish the biosynthesis of BioSurfactant. the regulation of serrawettin production by *Serratia* is believedto be through non ribosomal peptide synthetases (NRPSs) and N-acylhomoserine lactones (AHLs) encoded by QSS located on mobile transposon. this regulation is under positive as well as negative control of QSS operon products. In case of yeast and fungi, glycolipid precursor production is catalyzed by genes that encode enzyme cytochrome P450 monooxygenase(Ramakrishna Sen.2010)

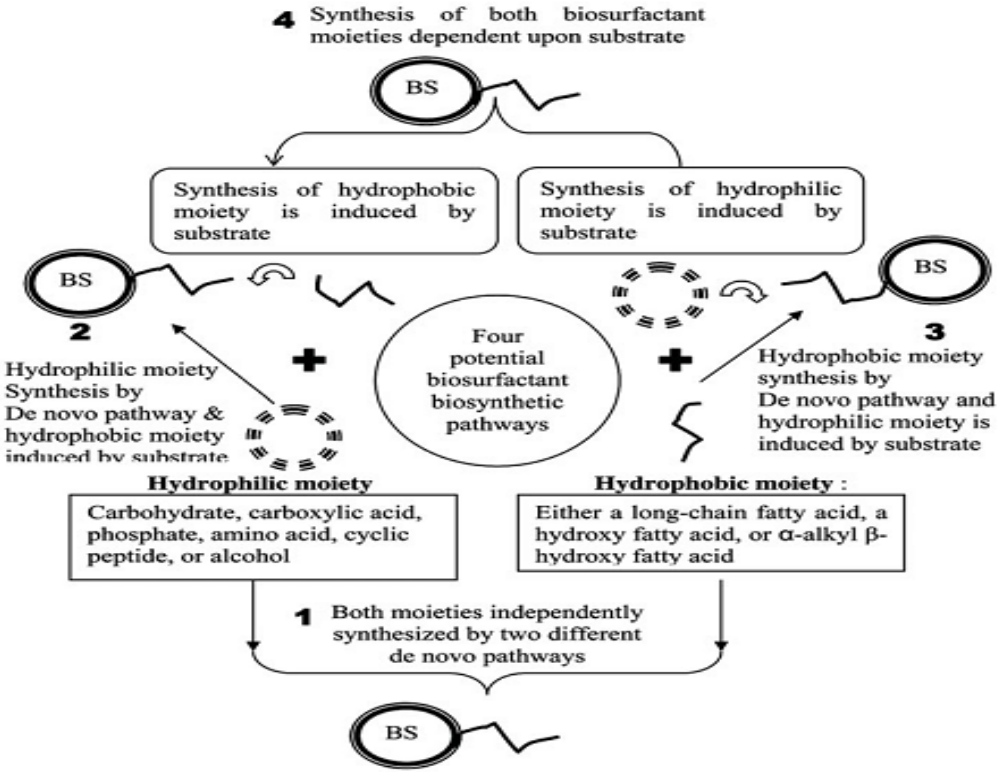


**Figure1**. Potential biosurfactant biosynthetic pathways operating in different microorganisms. Based on Syldatk and Wagner four assumption (1987): BS: Biosurfactant molecule.

**Table 1.3-** Types of biosurfactants produced by various microorganism and their applications.

| Sr no. | | Biosrfactant type | Miroorganism | Application |
|---|---|---|---|---|
| **1.1** | **Type of glycolipids produced by microorganisms** | Sophorolipids | *Candida bombicola*<br>*Rhodococcussps.*<br>*Tsukamurella sp.*<br>*Arthrobacter sp.*<br>*Rhodococcusruber* | Act as an Emulsifier, also used in Alkane dissimilation |
| **1.2** | | Rhamnolipids | Different strains of *Pseudomonas aeruginosa* and *Renibacterium salmoninarum*<br>*P. putida*<br>*Bacillus subtilis.* | Act as a Biocontrol agent,Surface active agent and Antifungal activity |
| **1.3** | | Trehalose lipid | *Rhodococcussps.*<br>*Tsukamurella sp.*<br>*Arthrobacter sp.*<br>*Rhodococcusruber* | Bioremediation Antimicrobial properties and Oxidise the gaseous alkanes |
| **1.4** | | Liposan | *Candida lipolytica* | Emulsifier |
| **1.5** | | Rubiwettins R1 and RG1 | *Serratiarubidaea* | Swarming and spreading |
| **2.1** | **Type of lipopeptides produced** | Amphomycin | *Streptomyces canus* | Antibiotic, inhibitor of cell wall synthesis |
| **2.2** | | Surfactin | *B. subtilis* | Antifungal, antibacterial and antiviral agent |
| **2.3** | | Arthrofactin | Arthrobacter | Oil displacement agent, antimicrobial agent |

### 2.5.2 - Various factors effecting biourfactant production

1. **Effect of fatty acid substrate in synthesis of ester by lipase**

   It is been observed that lipase shows reduced activity towards shorter chain fatty acids, or fatty acid with double bond near the carboxyl group.

2. **Effects of different carbon and nitrogen source**

   Three types of carbon source are provided that is carbohydrates, hydrocarbons and vegetable oils. Hydrocarbons and Vegetable oils are hydrophobic which is useful only to some microbes. Nitrogen source like ammonium salts and urea are been used.

   Environment factors like pH, temperature, agitation, oxygen availability. These factors effect the cellular growth and activity of microbes.

### 2.5.3- Types of extraction of Biosurfactant

Extraction process depend upon ionic charge, water solubility and location (intracellular, extracellular and cell bound)There are two types of process-

1. Batch mode- ammonium sulfate precipitation, acetone precipitation, acid precipitation, solvent extraction and crystallization.
2. Continuous mode- centrifugation, adsorption, foam separation, and precipitation tagential flow filteration, diafilteration and precipitation ultrafilteration.

### 2.6- Reason for choosing Diesel in the present study

Diesel fuel is prepared by fraction distillation of crude oil, with a boiling range of about 149°C to 371°C. The components of diesel fuel include paraffinic and naphthenic hydrocarbons, naphthalene and cracked gas oils. Important properties of diesel include:-

1. **Aromatics** These compound are added to diesel fuel to increase the density of the fuel and its heating value. But aromatics also decrease the cetane number to the diesel fuel and contribute to NO and PM emissions, especially the ploynuclear-aromatics. Diesel mainly consist of **BTEX compounds as aromatics that is benzene, toluene, ethylebenzene and xylene.** Ethanol is used as a gasolineadditive to meet renewable fuel and Clean Air Act requirements. So, ethanol in groundwatercontaminated with the gasoline constituents benzene, toluene, ethylbenzene, and xylenes (BTEX) are increasing everyday(Dooher et al,2001). Gasoline consist of 52% xylene, 11% benzene, 11%

ethylebenzene and 26% toluene. According to US.EPA benzene is considered carcinogen and they have found increased cases of leukemia patients.( Kirk Riley,1989)

**Table 1.4**:- **Health effects of diesel and BTEX compounds.**

| Sr no. | | Exposure | Health effects |
|---|---|---|---|
| **1** | **Diesel** | Inhalation of diesel vapours and absorption of diesel fuel | Exposure to vapours causes drowsiness, dizziness blood pressure elevation, headache, nausea and lung damage whereas diesel fuel causes skin irritation long term exposure causes severe redness, pain and chemical blisters. |
| **2** | **BTEX compounds** | Ingestion(consuming BTEX contaminated water)<br>Inhalation and absorption through skin. Severe effect is due to occupational exposure | Sensory and skin irration, central nervous system(CNS) depression, can effect respiratory system, kidney, liver and blood systems. |

## 2.7- Objective

i. In vitro diesel degradation studies by native bacterial strains.

ii. To study Diesel degradation at low temperature.

iii. Characterization of diesel degradating bacterial strain.

# CHAPTER 3

## Materials and Methods

### 3.1- Materials

#### 3.1.1-Diesel sample

The diesel used in the present study was procured from HPCL Dealer Motor Barrier, Shimla.

#### 3.1.2 -Chemicals

The chemicals used in the present study used were Nutrient Agar, Nutrient Broth, Bushnell-Hass Medium , Kings Medium, Xylene , Biuret Reagent, protease peptone-, K2HPO4, MgSO4.7H2O, agar, glycerol, peptone, leaf extract, gelatin, Simmons citrate agar medium.

### 3.2- Methods

#### 3.2.1– Hydrocarbon utilization Studies

##### 3.2.1.1-Experiment set up at $30^0$C and $15^0$C with pH 7

A set of experiments were conducted to check the diesel degrading potential of the microbe. An experiment was set up at $30^0$C and $15^0$C with pH 7 to check the growth activity of the microbe. Over-night grown culture was prepared in Nutrient Medium (50ml). It was inoculated into 4 flasks of Bushnell-Hass(BH) medium (100ml each) with filtered diesel(0.2µm filter) samples from HPCL Dealer Motor Barrier, Shimla as the sole carbon source(5%).The culture flasks were incubated at $30^0$C and $15^0$C for 9 days. The growth pattern were obtained by measuring the pH, protein estimation and Xylene emulsification test of the isolates. All experiments were performed in duplicates.

##### 3.2.1.2- Experiment set up at $30^0$C and $22^0$C with different pH

An experiment was set up at $30^0$C and $22^0$C with different pH (4, 5, 10 and 11) to check the growth activity of the microbe. Over-night grown culture was prepared in Nutrient Medium (50ml). It was inoculated into 4 flasks of Bushnell-Hass (BH) medium (100ml each) at different pH 4, 5, 10 and 11 respectively with filtered diesel(0.2µm filter) samples from

HPCL Dealer Motor Barrier, Shimla as the sole carbon source(5%).The culture flasks were incubated at $30^0$C and $22^0$C for 6 days. The growth pattern were obtained by measuring the pH, protein estimation and Xylene emulsification test of the isolates. All experiments were performed in duplicates.

### 3.2.2- BTEX Degradation study

The bacterial isolates were tested for their ability to grow on aromatic compound benzene, toluene, ethyl benzene and xylene. Over-night grown culture was prepared in Nutrient Medium (50ml). It was inoculated into 4 flasks of Bushnell-Hass (BH) medium (100ml each) at pH 7 with benzene, toluene, ethyl benzene and xylene as the sole carbon source(5%) respectively. The culture flasks were incubated at $30^0$C for 6 days. The growth pattern were obtained by measuring the pH.

### 3.2.3 -Study pH of medium, cell biomass and emulsification activity

Ph estimation, Biuret test and Xylene emulsification test were done in duplicates for the next 12 days and 6 days respectively for the experiments performed. The effect of cell growth was seen on both pH of the medium and in the emulsification activity.

#### 3.2.3.1–Biosurfactant assay

**Biosurfactant assay was performed using Xylene emulsification test**. A sample of culture (35µl), xylene (35µl) and 5 ml of Tris buffer (2.0mM, pH 8.0) was added in a glass test tube. After vortexing for 45 seconds and was incubated for 20 min at room temperature, absorbance was taken at 660nm. 35µl of Tris buffer was added instead of the sample in a control test tube. Emulsification index was measured for experiment set up at 15C. 2ml of culture media was taken in a test tube and equal amount of diesel sample was added. The mixture was vortexed for 2 min and kept overnight. Emulsification index was calculated.

#### 3.2.3.2 – Protein aasay

Biuret test was performed for measuring the protein content of the bacterial cultures. A sample of overnight grown culture (1ml) was centrifuged at 10,000 rpm for 10 min. The pellet was washed with distilled water and dissolved in 1 ml of distilled water.1 ml of dissolved culture sample, distilled water and biuret reagent were added in glass test tube respectively.After vortexing for 45 seconds and the reaction mixture was incubated for 10

min at room temperature. The concentration of the protein was calculated by taking the absorbance at 310nm.

### 3.2.3.3 - Study the amount of diesel being consumed

The level of diesel degradation was determined using the gravimetric analysis both in samples and control. Hexane(40ml) was added to the medium and kept in the rotary shaker for 30 minutes. After mixing it was allowed to withstand in a separating funnel for few minutes. The aqueous phase containg bacterial cells was separated from the remaining diesel mixed with hexane. Medium was removed and hexane + diesel was collected in a pre weighted dish and kept overnight. Amount of diesel left was calculated next day.

### 3.2.4- Morphological characterization

Gram nature of the isolates was studied using streaking and KOH string test.

### 3.2.4.1- Gram staining and KOH string test

Gram nature of the isolate was studied using conventional gram staining and KOH method. Gram staining was done by taking a culture drop on a slide and leaving it for air dry. Crystal violet was added on the slide and left for 1 min. it was then rinsed with water for 5 seconds. Grams iodine was then added on to the smear for 1 min and again rinsed with water for 5 seconds to remove the excessive strain. The smear was then decolorized with 90% etanol for 15-30 seconds. The slide was again rinsed for 5 seconds with water. Counter stain safaranin was added for 1 min and rinsed with water for 5 seconds. It was then blot dried with filter paper and viewed under the microscope using oil emulsion.

For KOH string test,a colony was picked and mixed continuously with a drop of 3% aqueous KOH on a glass slide. If suspension becomes viscous and string formation is observed, the isolate is gram negative as gram positive cells do not have string formation.

### 3.2.4.2- Streaking on EMB agar and Kings medium

Streaking of the single colony isolate was perfomed on the selective and differential Eosin methylene blue medium which inhibits the growth of gram positive bacteria. Bacterial isolate was tested for fluorescence activity by plating them on Kings media(protease peptone-20g/l, K2HPO4-1.5g/l, MgSO4.7H2O-1.5g/l, agar-15g/l, glycerol-10g/l at pH-7.2(King *et al*.1954) and visualized in U.V light. A single colony was taken and streaked on Kings medium.

### 3.2.5- Biochemical identification of microbial isolate.

Various biochemical tests were performed to identify the microbial isolate.

#### 3.2.5.1- HIMEDIA HicarbohydrateTM- Kit and HIMEDIA HiAssorted tm Biochemical Test Kit

3 strips (part A,B,C) and each part containing 12 carbohydrates and their utilization was studied. 0.5ul of culture media was added into each well of the strips and it was incubated at $37^0$C overnight. Color change was observed.

#### 3.2.5.2 - Gelatin- Hydrolysis test

An overnight grown culture was inoculated into four tubes containing autoclaved 10 ml Nutrient Gelatin medium(peptone-5g/l, leaf extract-3g/l, gelatin-120g/l at pH-6.8) and incubated at 25°C for 1 week, checking everyday for gelatin liquefaction. Anuninoculted test tube was kept as a control. After 7 days the tubes are immersed in an ice bucket for 15-30 minutes. Tubes are tilted to observe if gelatin has been hydrolyzed.

#### 3.2.5.3 - Citrate utilization test

10 mlSimmons citrate medium was added in 4 test tubes respectively and was autoclaved. Slants were prepared, streaking it with overnight grown culture and incubated at 35C for 48 hours. Anuninoculted test tube was kept as a control. Color change was observed.

### 3.2.6- Molecular Characterization

Molecular characterization of the bacterial strains was done by isolating their genomic DNA. For further characterization of the isolates, 16s rRna universal primer were used for carrying out PCR.

#### 3.2.6.1- Bacterial DNA isolation

Overnight grown culture was centrifuged and the DNA was isolated using the PROMEGA kit. DNA concentration was measured on nano drop.

#### 3.2.6.2- PCR Reaction using 16s rRNA universal primers

Universal 16s rRNA of 10pm/µl stock concentration, Forward primer 8F 5'-AGAGTTTGATCCTGGCTCAG- 3',tm-54.3 and Reverse primer 1492R(L) 5'-

GGTTACCCTTGTTACGACTT-3',tm- 49.4 were used to prepare working concentration(5pm/ µl) of primers. 10µl of stock concentrationwastaken and dissolved it in 10 µl of autoclaved water. For 50 µl of PCR reaction mixture, 25 µl of master mix was prepared by adding autoclaved distilled water-19 µl, forward primer- 2 µl, reverse primer-2 µl, DNA sample-2 µl. This master mix was divided into two aliquots containing 12.5 µl master mix and 12.5 µl PCR ready mix (2X stock) and added with a total of 25 µl reaction mixture in each aliquot. PCR amplification of reaction mixture using the program: 35 cycles each consisting of initial denaturation of 5 min at 94C, final denaturation for 1 min at 94C, annealing for 1 min at 51.8C, elongation for 1 min at 72C and final elongation for 10 min at 72C was done.

**3.2.6.3- Gel Electrophoresis**

50 ml of 1% agarose gel was prepared using 1X TAE buffer. 2µl ethidium bromide was added to it after melting the solution. The gel was allowed to solidify in casting tray. The PCR products were loaded into the well, 5µl sample was mixed with 2µl loading dye and 1kb of ladder was run alongside in an electrophoresis tank. The gel was visualized under U.V light.

# CHAPTER 4

## Results and Discussions

### 4.1- Results

### 4.1.2- Hydrocarbon utilization Studies

The purpose of the study was to find the growth of isolate at low temperature ,pH and with different carbon sources. Flasks containing Bushnell Hass media were supplemented with 5% diesel sample, 5% benzene,5% toluene,5% xylene. It showed that biomass activity was dependent on biosrfactant activity. As the biosurfactant activity decreased bacterial isolate also went into the death phase(Fig 2.2). The bacterial isolate showed growth in the presence of diesel but was not able to utilize benzene, toluene and xylene. Growth was also observed at 15°C and at pH 10 and pH 11. Emulsification index was measured for the culture media at 15°C using formula - height of emulsion/ total height*100% which came out to be 28.57%.(Fig 2.1)

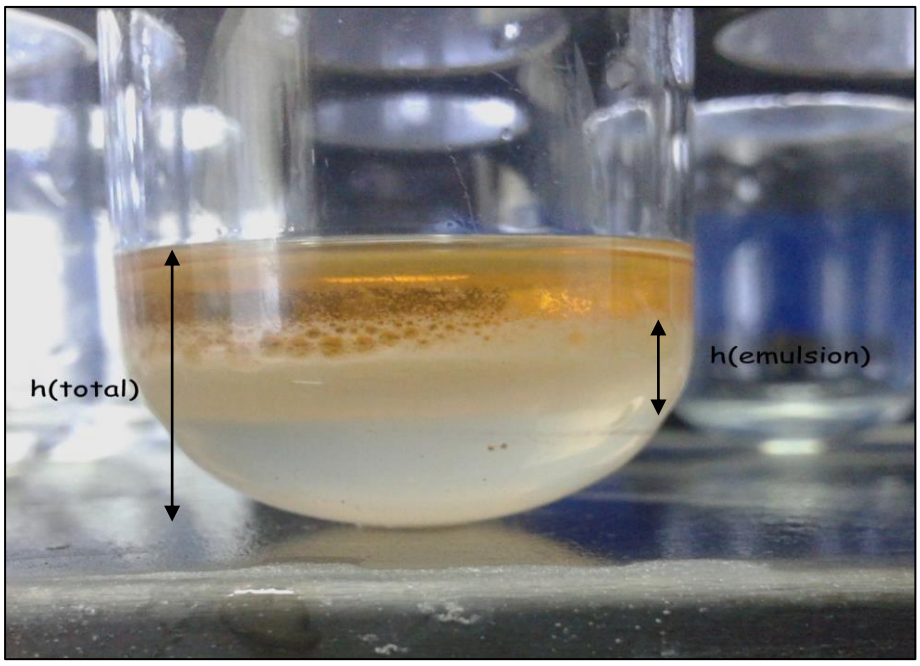


**Fig 2.1 -Measurement of the Emulsification index of 5% diesel sample at 15C=28.57%**

**Table2.1 –** Reading of protein activity and emulsification activity at 30°C, pH 7 for a time period of 9 days.

| No of days | **pH** | **Protein activity(mg/ml)** | **Emulsification activity (absobance 660nm)** |
|---|---|---|---|
| 1 | 7.2 | **0.00** | **0.00** |
| 2 | 7.38 | 0.0722 | 0.1784 |
| 3 | 7.76 | 0.1152 | 0.03735 |
| 4 | 8.34 | 0.1791 | 0.07975 |
| 5 | 8.19 | 0.205 | 0.13195 |
| 6 | 8.43 | 0.2143 | 0.3123 |
| 7 | 8.66 | 0.2521 | 0.3953 |
| 8 | 8.56 | 0.2011 | 0.08325 |
| 9 | 8.98 | 0.1784 | 0.05115 |

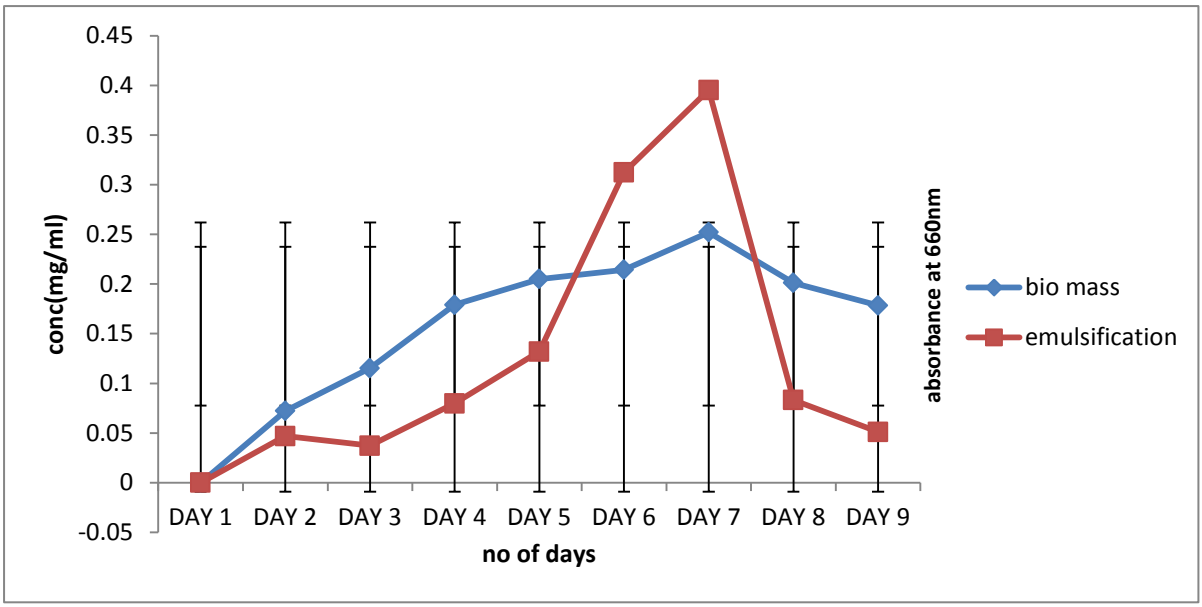


**Fig 2.2** - Comparison of protein activity and biosrfactant activity at 30$^{o}$C , pH 7 for a time period of 9 days.

### 4.1.1.1- Experiment set up at 30$^{o}$C and 15$^{o}$C with pH 7

Comparison of growth and emulsification at 15$^{o}$C and 30$^{o}$C is seen in Fig 3.1 and Fig 3.2 at both temperature, bacterial isolate was able to grow in the presence of 5% diesel sample. It was observed that growth at emulsification activity was better at 30$^{o}$C compared at 15$^{o}$C but pH trend was same at both temperature with initial decrease in ph then increased as biosurfactant production increased (Fig 3.3).

**Table2.2 -**Reading of protein activity and emulsification activity at 15°C, pH 7 for a time period of 9 days.

| **No of days** | **pH** | **Protein activity(mg/ml)** | **Emulsification activity (absobance 660nm)** |
|---|---|---|---|
| 1 | 6.94 | **0.00** | **0.00** |
| 2 | 6.87 | **0.00** | **0.00** |
| 3 | 6.3525 | **0.00** | **0.00** |
| 4 | 6.7775 | 0.0872 | 0.0814 |
| 5 | 7.01 | 0.1259 | 0.0865 |
| 6 | 7.275 | 0.1296 | 0.0925 |
| 7 | 7.8175 | 0.1509 | 0.0619 |
| 8 | 7.875 | 0.1295 | 0.0366 |
| 9 | 7.8375 | 0.0969 | 0.0301 |

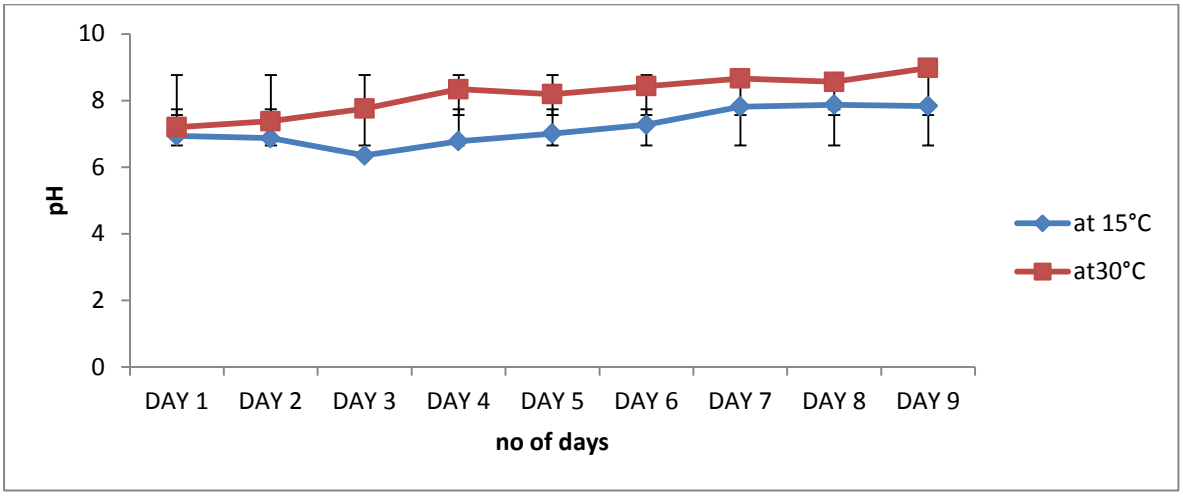


**Fig 3.1**- Comparison of pH at 30°C and 15°C for a time period of 9 days.

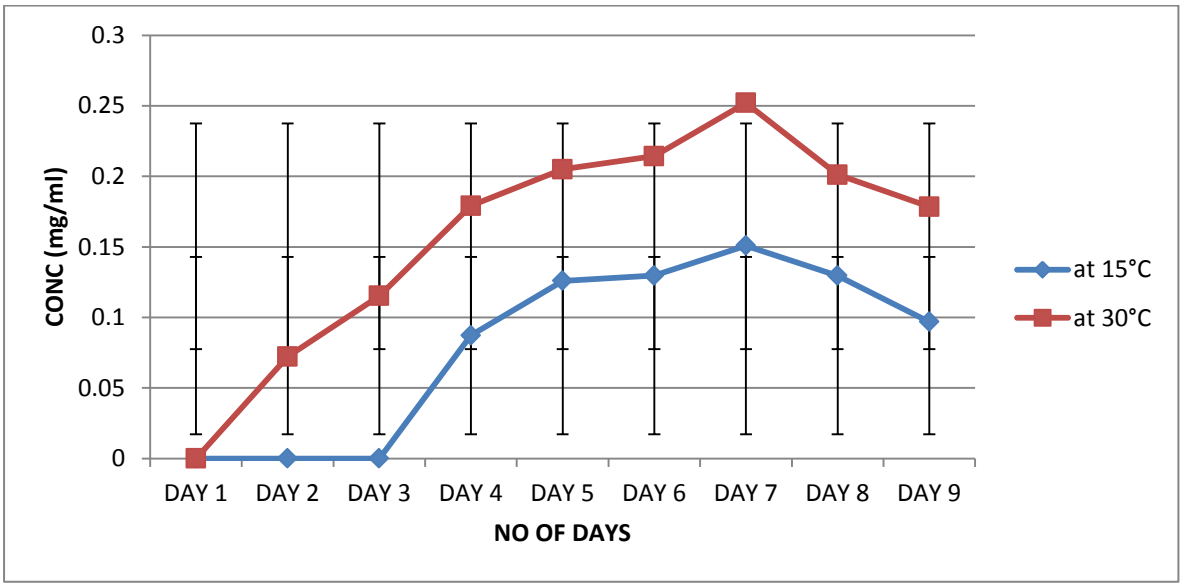


**Fig 3.2** Comparison of protein activity at 15°C and 30°C for a time period of 9 days using biuret test.

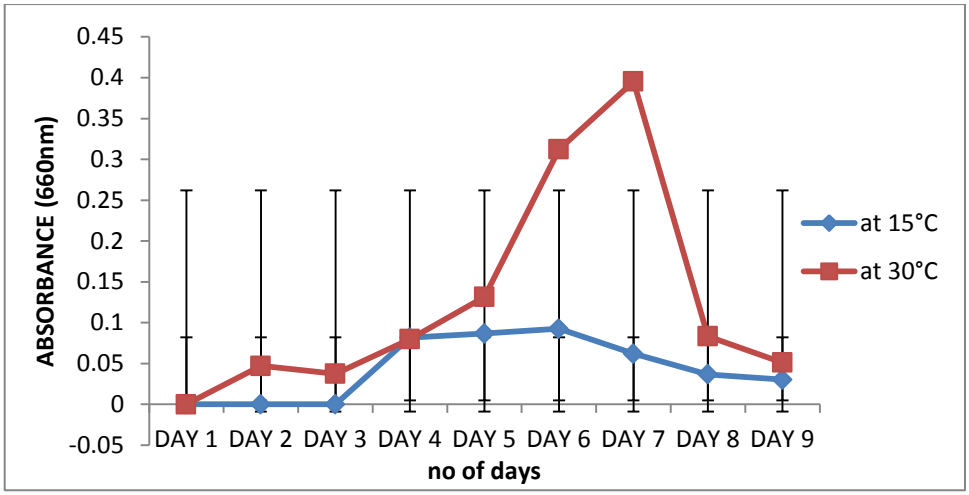


**Fig 3.3** Comparison of biosurfactant activity at 15°C and 30°Cfor a time period of 9 days using xylene emulsification test.

### 4.1.1.2-Experiment set up at 30°C and 22°C with different pH

At pH 4 and 5 no growth was observed but at pH 10 and 11 good biosurfactant and protein activity was seen at both the temperature (Fig4.1- Fig5.3) showing the isolate is not acidophilic but can grow at extreme alkaline pH.

**Table2.3** Reading of protein activity and emulsification activity at 30°C, pH 10 and pH11 for a time period of 6 days. No growth was observed at pH 4 and Ph 5

| **Initial Ph of culture media** | **No of days** | **Ph change** | **Protein activity (mg/ml)** | **Emulsification activity (absorbance 660nm)** |
|---|---|---|---|---|
| **At pH 10** | 1 | 9.78 | 0.044018 | 0.00655 |
| | 2 | 9.11 | 0.056431 | 0.0081 |
| | 3 | 9.11 | 0.157178 | 0.0226 |
| | 4 | 9.54 | 0.222682 | 0.02765 |
| | 5 | 9.51 | 0.231057 | 0.0168 |
| | 6 | 9.43 | 0.157777 | 0.0174 |
| **At pH 11** | | | | |
| | 1 | 9.23 | 0.068843 | **0.00395** |
| | 2 | 8.68 | 0.09337 | 0.0052 |
| | 3 | 8.95 | 0.112866 | 0.005295 |
| | 4 | 9.07 | 0.168495 | 0.01075 |
| | 5 | 9.35 | 0.179412 | 0.01705 |
| | 6 | 9.46 | 0.088584 | 0.01675 |

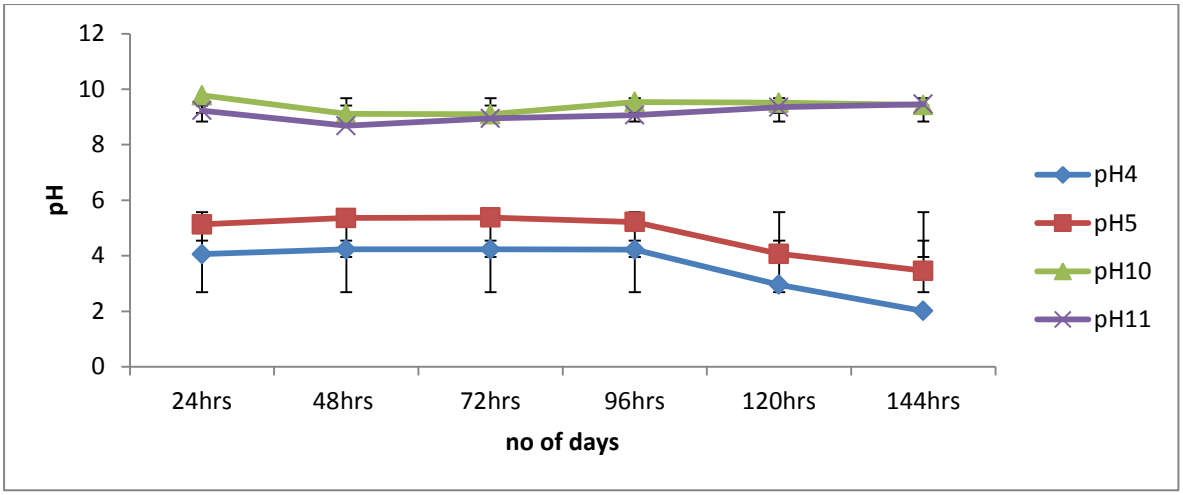


**Fig 4.1**-ph study of culture medium initially at ph 4,5 ,10 and 11 and temperature 30°C.

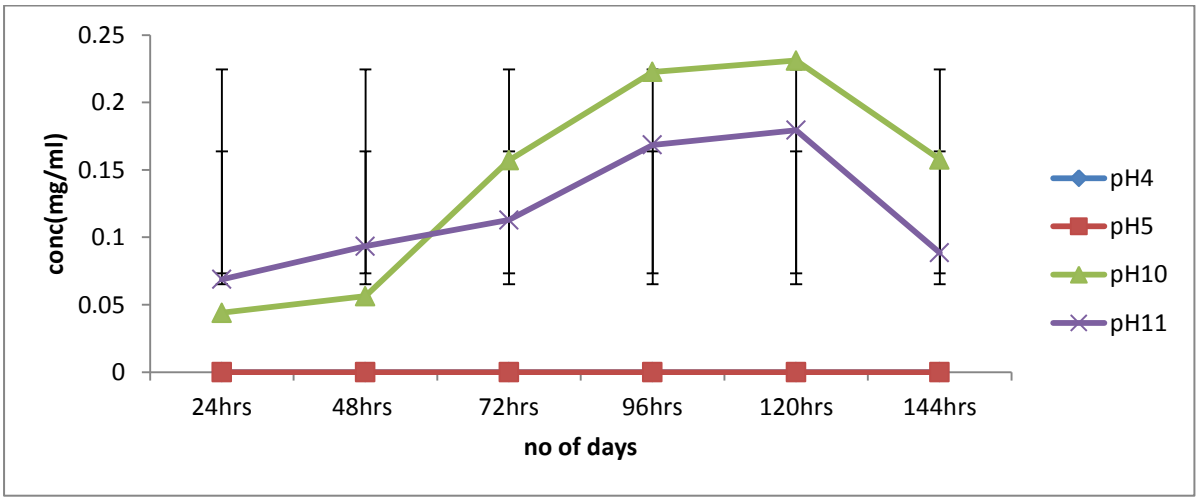


**Fig 4.2-** protein activity of culture medium initially at ph 4,5 ,10 and 11 and temperature 30°C using biuret test

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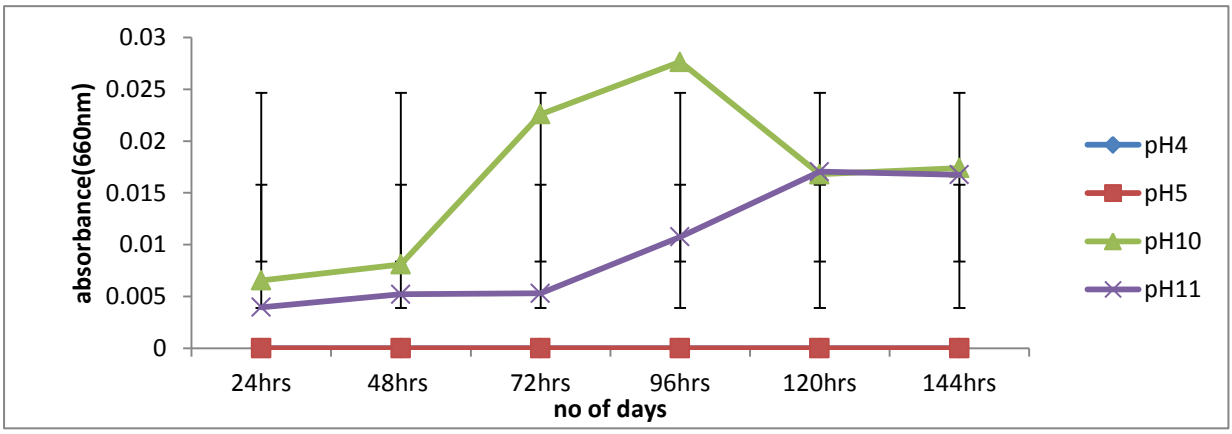


**Fig 4.3**-biosurfactantactivity of culture mediuminitially at ph 4,5 ,10 and 11 and temperature 30°C using xylene emulsification test.

**Table2.4-** Reading of protein activity and emulsification activity at 22°C, pH 10 for a time period of 6 days. No growth was observed at pH 5.

| **No of days** | **Ph change** | **Protein activity (mg/ml)** | **Emulsification activity (absobance 660nm)** |
|---|---|---|---|
| 1 | 9.67 | 0.016002 | 0.00285 |
| 2 | 8.98 | 0.017498 | 0.00595 |
| 3 | 9.02 | 0.011815 | 0.0099 |
| 4 | 9.06 | 0.194566 | 0.005 |
| 5 | 9.05 | 0.295115 | 0.0186 |
| 6 | 9.5 | 0.30329 | 0.041 |

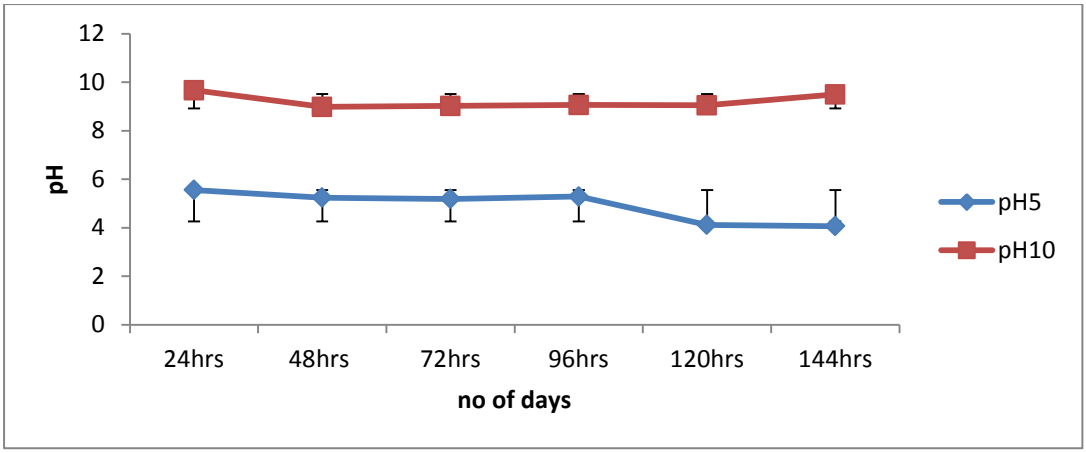


**Fig 5.1**-ph study of culture medium initially at ph 5 and 10 and temperature 22°C.

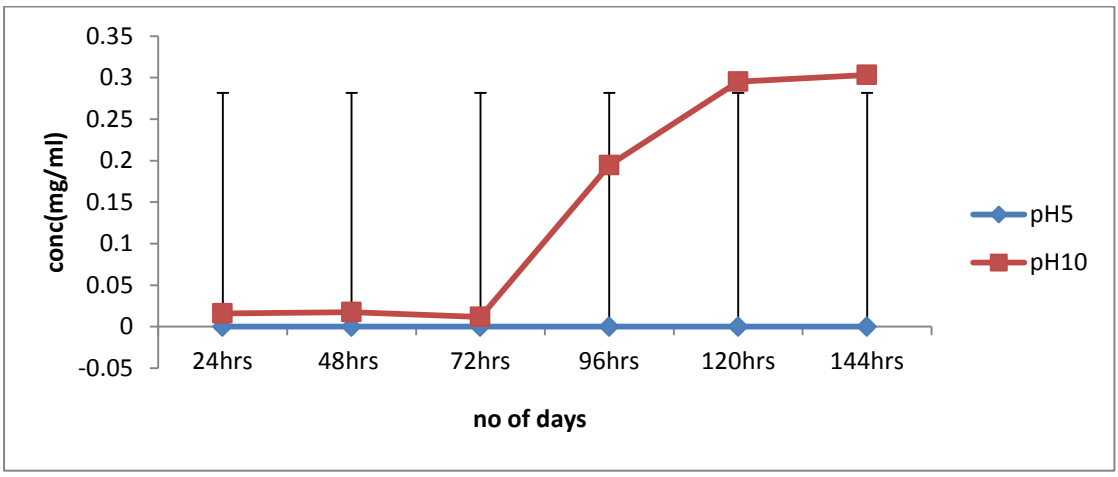


**Fig 5.2-** protein activity of culture medium initially at ph 5 and 10 and temperature 22°C using biuret test.

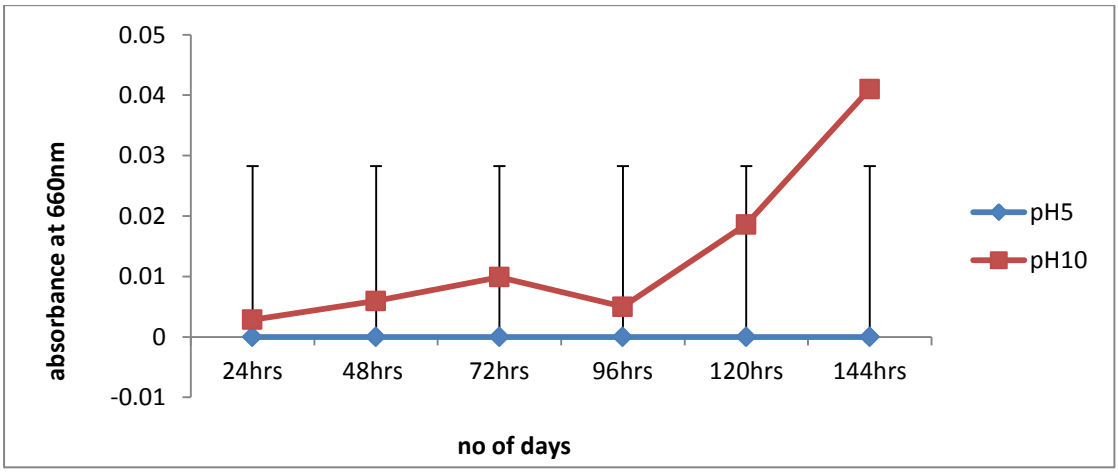


**Fig 5.3**-biosurfactant activity of culture medium initially at ph 5 and 10 and temperature 22°Cusing xylene emulsification test.

**4.1.1.3- Study the amount of diesel being consumed**

Gravimetric analysis was done to determine the amount of diesel being left. 40ml of hexane was mixed with the culture media and separated via separator funnel . Hexane is non polar compound, it get mixed with oil separate from the culture media which is eluted out. Oil and hexane mixture is collected in a pre weighted dish and kept overnight. Hexane in volatile in nature and gets evaporated , leaving the diesel left in the dish(Fig 8.1)

**Fig 8.1 –Gravimetric analysis-** Separatory funnel showing separation betweenculture media and diesel sample

**Table3.1**Gravimetric analysis of diesel samples at 30°C and 15°C for a time period of 9 days.

| **Sr no.** | **Diesel Samples at 30°C** | **Diesel left** | **Diesel consumed** |
|---|---|---|---|
| **1.1** | Sample | **6.41%** | **93.58%** |
| **1.2** | Control | **31.54%** | **68.45%** |
| **2** | **Diesel Samples at 15°C** | | |
| **2.1** | Sample | **16.575%** | **83.45%** |
| **2.2** | Control | **78.37%** | **21.63%** |

**Table3.2** Gravimetric analysis of diesel samples at 30°C and 22°C for a time period of 9 days. At pH 4 and pH 5 no growth was observerd.

| **Sr no** | **Diesel Samples at 30°C** | **Diesel left** | **Diesel consumed** |
|---|---|---|---|
| 1.1 | Sample at pH10 | 25.17% | 74.82% |
| 1.2 | Control at pH10 | 92.81% | 6.706% |
| 1.3 | Sample at pH 11 | 36.188% | 63.811% |
| 1.4 | Control at pH 11 | 32.122% | 67.8785 |
| | **Diesel Samples at 22°C** | | |
| 2.1 | Sample at pH10 | 37.40% | 62.59% |
| 2.2 | Control at pH10 | 79.78% | 20.21% |

### 4.1.3- Morphological characterization

#### 4.1.2.1- Gram staining and KOH string test

Gram nature of the strain was studied using gram straining procedures and KOH method. Pink colored coccobacilli shape colonies were observed under 100X light microscope (Fig 9.1) and string formation was observed in KOH method indicating the strain is gram negative.

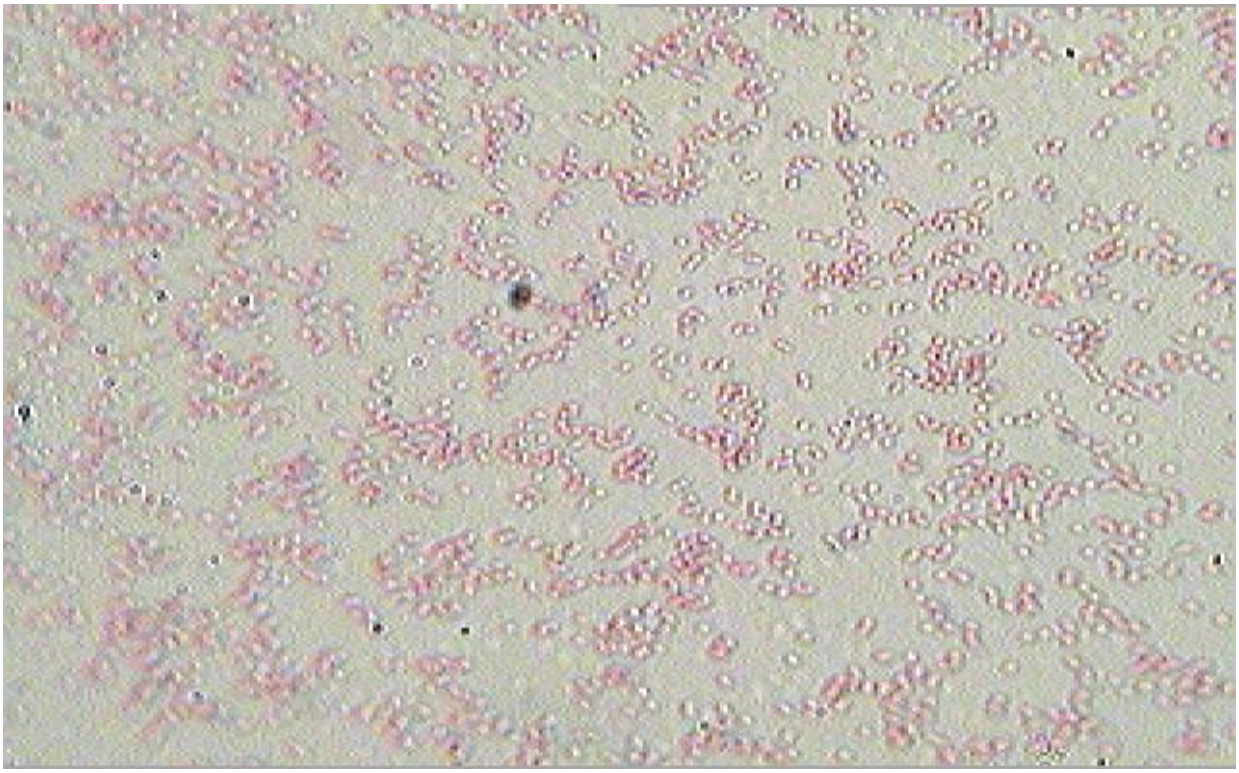

**Fig9.1** Gram staining showing gram negative coccobacilli bacteria

#### 4.1.2.2- Streaking on EMB agar and Kings medium

On streaking on Kings medium non fluorescence colonies were observed (Fig9.2). Indicating the strain does not belong to *Pseudomonas* species as they show fluorescence. Blue centered colonies were observed on EMB agar plate (Fig 9.3) which is one of the characteristics of *Acinetobacter*species . (ASM Microbial Library 2007)

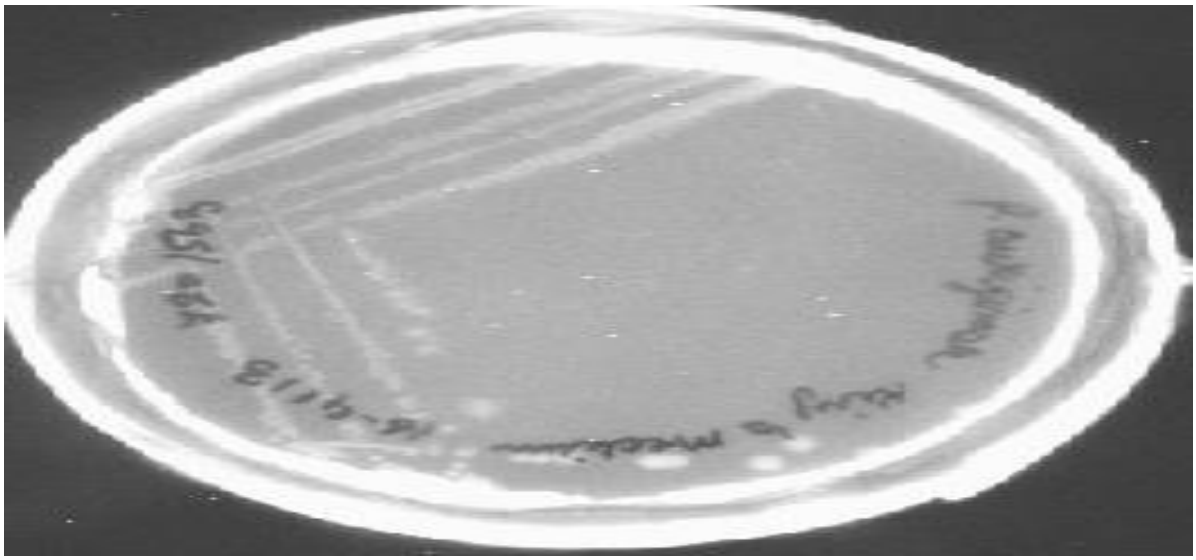

**Fig 9.2-** Bacterial colonies on Kings medium plate showing no fluorescence under UV light

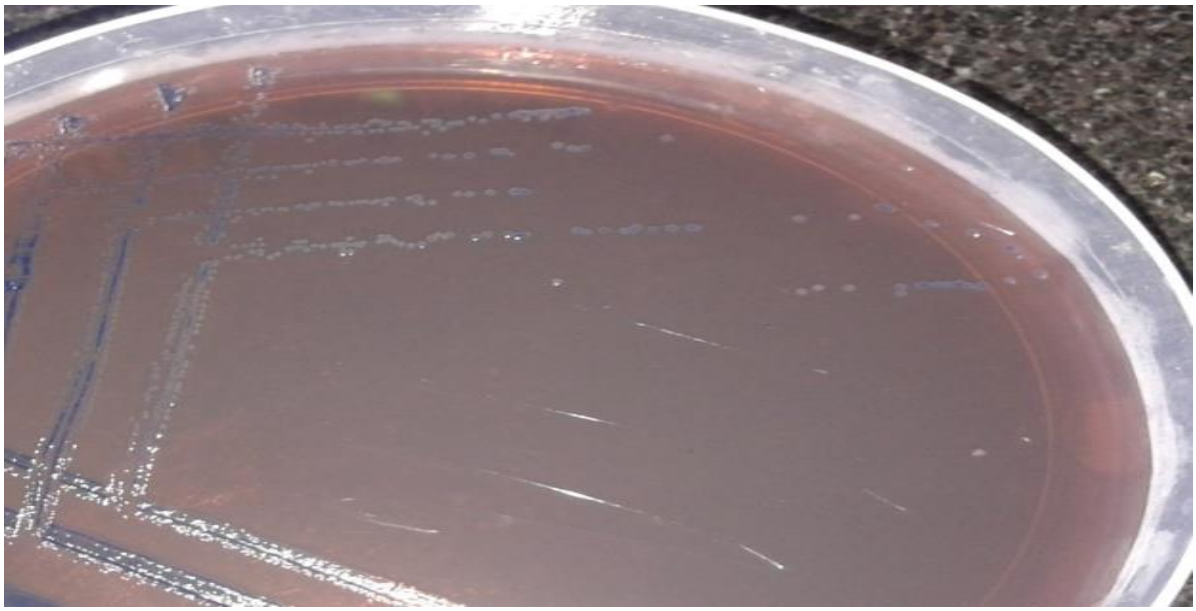

**Fig 9.3-** Bacterial colonies in EMB agar plate showing blue-grey center.

**4.1.3.1- Biochemical identification of microbial isolate**

Isolate was not identified using both HIMEDIA HicarbohydrateTM- Kit and HIMEDIA Hi Assorted tm Biochemical Test Kit. And showed negative result for gelatin hydrolyze testas gelatin remained solidified after 1 week of incubation (Fig 10.1). Color change from green to blue was observed in citrate utilization test (Fig 10.1.2). No color change was observed in control(Fig 10.2.2)

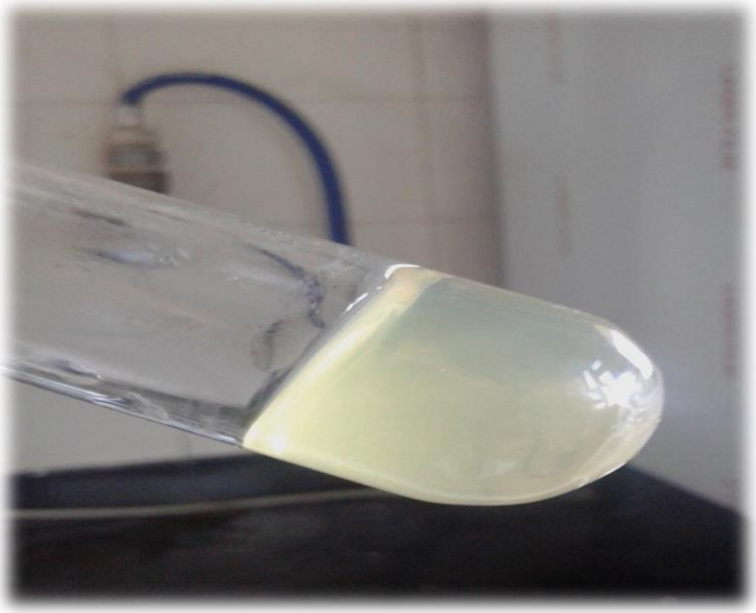

**Fig 10.1**-Gelatin hydrolysis test-showing negative result

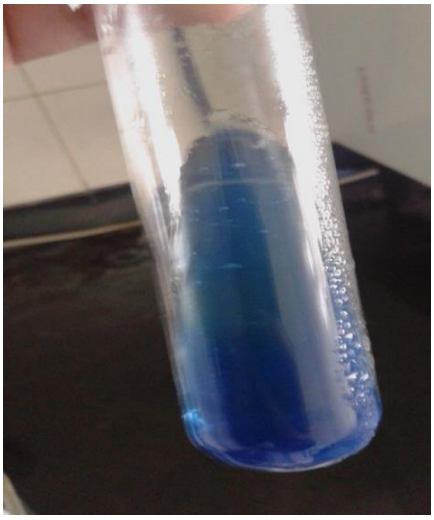

**Fig 10.2.1**- Green to Blue colourization ofcirate simmon agar medium

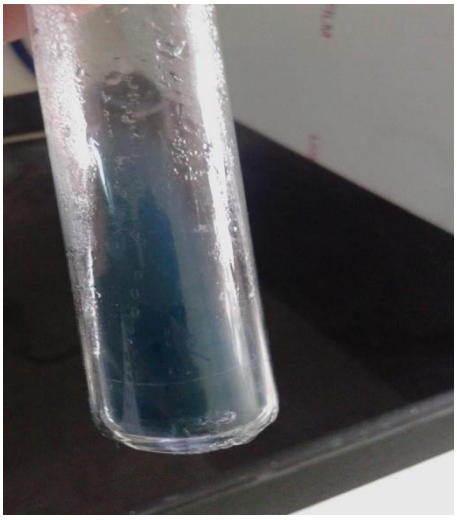

**Fig 10.2.2**- no color change in citrate simmon agar

### 4.1.5- Molecular Characterization

#### 4.1.4.1- Bacterial DNA isolation

Bacterial DNA isolation was done in duplicates using PROMEGA kit and concentration was measured on nano drop: 628.4mg/µl, 260/280=1.89 and 464.9ng/ µl, 260/280 =1.96 respectively. It showed a good concentration with no protein or RNA contamination.

#### 4.1.4.2 - PCR Reaction using 16s rRNA universal primers

16sRNA sequence are conserved which are present in all bacterial domains. On carrying Gel Electrophoresis of the PCR product ,it was confirmed that isolate belongs to bacterial domain and a band formation was observed near the well , which showed that the PCR product size was greater than 1kb ladder.

**4.2- Discussion**

Environmental conditions can have a major impact on bacterial growth and production of secondary products like biosurfactant In this study, the effect of different conditions like low temperature condition, extreme pH and BTEX degradation was studied. Initial decrease pH was observed due to the production of carboxyl ion in the medium then as the biosurfactant production increased in the medium pH value also increased due to alkaline nature of biosurfactant. It has been observed that the biosurfactant production is an important survival tool in hydrocarbon contaminated environment. Form the graphs it is visualizedthat with decreases in biosurfactant activity, biomass activity was also reduced.Gravimetric analysis for amount of diesel being consumed. Hexane which is a volatile non polar compound get mixed with diesel in medium and separates culture medium. After leaving for 24hrs in an open air hexane gets evaporated leaving diesel behind.

Gram staining showed the isolate to be gram negative but gram stain reaction is not always truly indicative of the organisms true cell wall structure. Poorly controlled decolorizing can be a source of misleading results. It was further confirmed by KOH(3%) string test where string formation was observed.Dilute alkali solutions (3% KOH) lyses gram negative cell walls while the cell walls of gram positive bacteria are not disrupted. When gram negative bacteria are lysed the DNA is released causing the mixture to become viscous and string formation is observed.

It is been observed that most of the hydrocarbon degrading microbes belong to *Pseudomonas species*which shows fluorescence when plated on King's medium. But our isolate showed absence of fluorescence. EMB inhibits the growth of Gram-positive bacteriaand provides a color indicator distinguishing between organisms that ferment lactose and those that do not. On plating on EMB agar medium *Pseudomonas species* show colorless colonies due to lack of fermentation of sugars or acid production. (NaowaratCheeptham and CarolynneFardy, Thompson Rivers University, Kamloops, BC, Canada). Our isolate showed colonies with blue-grey center which is a characteristic feature of *Acinetobacter species*(ArchanaLal, Independence Community College, Independence, KS). On referring to Bergeysmanualbiochemical test were performed with respect to *Acinetobacter species.*

Gelatin hydrolysis test was done to detect the ability of an isolate to produce gelatinase, a proteolytic enzyme that liquefy gelatin. Hydrolysis of gelatin indicates the presence of gelatinases enzyme. These enzymes degrade gelatin to polypeptides which are further converted into amino acids. The bacterial cells can then take up these amino acids for metabolic processes. Our isolate showed absence

of gelatinase enzyme as nutrient gelatin medium remained solidified even after the seven days of incubation.

In Citrate utlilization test color change is observed indicating that the isolate can utilize sodium citrate as its only carbon source and alkaline carbonates and bicarbonates are produced ultimately. Due to these alkali production by the isolate color change is visualized in the medium.( Tankeshwar Acharyav, 2004)

Futher molecular characterization is performed for idnentification of isolate by sequencing its DNA product.

## Conclusion

Compared to a number of other remediation treatments, the biological methods are relatively inexpensive, uncomplicated and enable effective degradation of pollution without great interference in the environment. In our study, degradation potential of isolate was tested under various condition like at temperature 15°C and under extreme pH condition. 5% diesel as the only carbon source was provided. Aromatic degradation was also tested using BTEX compounds, but isolate showed negative result for its degradation. Biosurfactant activity was found at low temperature and showed it effect on protein activity There are several advantages of biosurfactants when compared to chemical surfactants, mainly biodegradability, low toxicity, biocompatibility and ability to be synthesized from renewable feedstock. Isolate was able to grow at low temperature but had lower biosurfactant activity compared at mesophilic temperature (30°C). Biochemical test were performed for the isolate which showed a close relation with *Acinetobacter species*. Bioremediation is an option that offers the possibility to destroy or render harmless various contaminates using natural biological activity. It uses relatively low cost, low technology techniques which is accepted by the general public and can be carried out on contaminated site itself. Research has been conducted to degrade diesel contamination at mesophillic temperature. At high temperature diesel contamination is not much concern at its gets evaporated due to its volatile nature. At low temperature diesel fuel becomes more dense which becomes difficult to eliminate, raising some serious health and environment issues. As our isolate can tolerate 5 % diesel at 15°C temperature is an important result for bioremediation of hydrocarbons at low temperature.

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