Isolation and characterization of soil bacteria from the petrol pump station of Madurai town

Dr. B. Sadhana* and J. S. Saranya
Assistant Professor
Centre for Research and P.G Department of Botany,
Thiagarajar College ,
Madurai-625009, Tamil Nadu, India.

Abstract

The microbial degradation is the major mechanism for the elimination of used petroleum products from the environment. The present investigation was carried out to determine the bacterial flora of soils contaminated with used petroleum products in Madurai town. The serial dilution and pour plate method was employed for the isolation of soil bacteria from the contaminated soil. The *Bacillus* and *Proteus* species were isolated and identified based on the morphological, microscopic observation and biochemical tests carried out in microbiology laboratory. The present study suggested that the isolated bacterial species could be employed for bioremediation in environments polluted with petroleum and its products.

Introduction

Crude oil or petroleum hydrocarbon exploration and exploitation which came after industrial revolution stems from advances in science and technology which have enabled humans to exploit their natural resources, although not without a cost, as it has generated unprecedented disturbances in global elemental cycles [31].

Bioremediation is the application of biological treatment to clean up hazardous chemicals. This process involves detoxification where the pollutant may be converted to less toxic substances and mineralization, where the waste material can be converted into inorganic compounds such as carbon dioxide, water, methane and sometimes fatty acids [23].Contamination of the environment with petroleum hydrocarbons has caused critical health defects and therefore increasing attention has been focused on developing and implementing innovative technology for cleaning up this contamination ([24],[32]).

Bioremediation methods therefore come in handy and have correctly received favorable publicity as promising environmentally friendly technique for the remediation of hydrocarbon contaminated ecosystem [9]. This is possible because microorganisms have enzyme system to degrade and utilize different hydrocarbon as a source of carbon and energy [7]. A number of gram positive and negative microbes have been reported to be capable of utilizing a wide variety of hydrocarbons as carbon and energy [10].

Hydrocarbon utilizing microorganisms are ubiquitously distributed in the environment following oil spills. They can naturally degrade numerous contaminating petroleum hydrocarbons and cleans the

oceans of oil pollutants. Biodegradation by natural population of microorganisms is the most reliable mechanism by which thousands of xenobiotic pollutants including crude oil are eliminated by the environment.

The microorganisms include bacteria of the genera *Klebsiella, Proteus, Bacillus, Escherichia, Pseudomonas, Streptomyces, Nocardia, Serratia, Xanthomonas, Micrococcus,* etc. and fungi of the genera- *Rhizopus, Fusarium, Pencillium, Cladosporium* and *Aspergillus,* etc. [3]. The hydrocarbon degrading microbes have an inherent capacity to assimilate hydrocarbons and/or its products [2]. The process is therefore regarded as a complex biological oxidation process involving mostly aerobic organisms which may be enhanced by supplementation with fixed nitrogen, phosphate and other nutrients [11].

The objective of this work is to assess the effective distribution of various microbes in the hydrocarbon contaminated soil in the petrol pump station in Madurai, Tamil Nadu, India. The soil bacterial population was measured using serial dilution technique and was characterized by using morphological, microscopic observation and biochemical tests carried out in the microbiology lab.

Materials and methods

Soil study: The soil sample was collected from the petroleum oil spill region of Petrol pump station from Madurai, Tamil Nadu, India. The collected soil was air dried, mixed thoroughly and analyzed for soil pH and soil moisture.

Soil pH : Ten grams of air dried soil were added to 100 ml of distilled water and made to a suspension of 1: 10 (W/V) solution. Then the pH of the suspension was determined using pH meter.

Soil moisture: Soil moisture content was determined by taking samples between 11 am and 12noon and drying them in an oven at 120^{o}C.

Isolation of soil bacteria

Sample Collection

The Petroleum oil spilled soil was collected from Villapuram area, Madurai, Tamil Nadu, India. The collected soil was packed in polythene bags and stored at room temperature.

Isolation and enumeration of soil microbes

The soil sample was collected from the study site. 10g of sample of pulverized, air dried soil was weighed and were serially diluted as from 10^{-1} to 10^{-7}. The dilution was shaking vigorously for few min. for obtaining uniform suspension of micro-organisms. 1 ml of aliquots each from each dilution is transferred to each sterile petri plate. To this approximately 15 ml of cooled nutrient agar medium (45^{o}C) was added to each petri plate and mixed the inoculam by gentle rotation of the petri plate.

All the inoculated plates were incubated in at 25^{o}C for 2-7 days. The number of microorganism per gram of the soil was calculated by applying the formula:

$$\text{Viable cells/g dry soil} = \frac{\text{Mean plate count} \times \text{dilution factor}}{\text{Dry weight of the soil}}$$

After incubation, the isolated bacterial colonies were observed and identified based on the morphological, microscopic observation and biochemical tests [6] carried out in microbiology lab.

Morphological tests for bacterial isolates

- Gram staining technique
- Motility test
- Observing endospores

Biochemical tests for bacterial isolates

- Starch hydrolysis (Amylase activity)
- Gelatin liquefaction
- Sugar fermentation (Glucose, lactose and sucrose)
- Catalase activity
- Hydrogen sulphide production.

Table: 1

Morphological Characteristics of isolated bacteria from petrol pump station of Madurai

Sl. No	Bacterial Isolate	Gram nature and morphology	Motility	Endospore staining
1	A (*Bacillus sp.*)	Gram positive rods	Motile	+
2	B (*Proteus sp.*)	Gram negative rods	Motile	-

+ = Endospore former - = Non-Endospore former

Table: 2

Biochemical Tests for isolated bacteria from petrol pump station of Madurai

Sl. No	Biochemical tests	Isolate A: *Bacillus sp.*	Isolate B: *Proteus sp.*
1	Starch hydrolysis - Amylase activity	+	-
2	Gelatin liquefaction	+	+
3	Sugar fermentation (Glucose, lactose and Sucrose)	$+^{AG}$ - -	+ - +
4	Catalase activity	+	+
5	Hydrogen sulphide production	-	+

+ = Positive reaction; - = Negative reaction
$+^{AG}$ = Positive reaction with Acid and Gas production

Result and Discussion

Polyaromatic hydrocarbons (PAHs) have been identified as hazardous chemicals by different state and central pollution control Boards, because of their toxic, carcinogenic and tetragenic effects on living body. At present, hydrocarbon fuels (mainly diesel) contain an excessive quantity of PAHs, causing abundant distribution of the same in the ecosphere. In order to protect environment from such PAH emission from diesel oil, a stringent EURO III standard has recently been enforced. This specifies that the maximum allowable concentration of PAH in diesel oil to be used as automobile fuel should be 11% by weight.

As the usage of Petroleum hydrocarbon products increases, soil contamination with diesel and engine oil is becoming one of the major environmental problems. There are so many bacterial strains that can degrade or transform the components of crude oil products to the non-toxic, non-hazardous, biodegradable and environmentally friendly compounds. This is known as Biodegradation. Many oil-degrading bacteria have been isolated and their degradation potential is investigated. Most of bioremediation studies have been carried out using pure cultures and the roles of these bacteria in a natural environment remain substantially unknown ([13], [26]).

Generally, the pH of the soil determines the mineral contents as well as microbial composition. Fungi are predominant in the rhizosphere under low pH conditions (<5.5) and beneficial nitrogen fixing micro organisms are favored at neutral pH. High pH releases K^{+},Mg^{2+}, Ca^{2+}, Mn^{2+}, Cu^{2+} and Al^{3+} by weathering processes of soil where as low pH favors solubility of salts including carbonates, phosphates and sulphates [28].

In the present study, soil analysis showed the neutral pH-7.61±0.03 and the soil moisture – 0.96. There are two bacterial species isolated separately and pure cultured under laboratory conditions. The bacterial **Isolate – A** was observed as white slimy colonies on the nutrient agar medium. They are gram-positive, motile, rod shaped and endospore former. They have shown positive reaction in starch hydrolysis, gelatin liquefaction, glucose fermentation and catalase reaction. And this bacterium showed negative results for hydrogen sulphide production, lactose and sucrose fermentation. Thus, the **Isolate - A** bacterium was confirmed as *Bacillus sp.* (Table: 1 and 2; Plate:1-3).

The bacterial colony of **Isolate-B** formed while colonies with concentric zones and were spread in a uniform film over the moist surfaces of nutrient agar medium. They are gram negative rods with swarming motility and non-endospore former. This bacterium showed positive reaction in gelatin liquefaction, glucose (Acid and gas production) and sucrose fermentation and Hydrogen sulphide production. And the negative result was observed in starch hydrolysis and lactose fermentation. Thus, the **Isolate B** was confirmed as *Proteus sp*. (Table:1 and 2; Plate:4 and 5).

The release of contaminants to the environment, including petroleum and petroleum-derived products, is one of the main causes of soil and water contamination which causes a risk for human and animal health, since many of these contaminants have demonstrated to be toxic and carcinogenic. Hydrocarbon molecules that are released into the environment are hard to remove, as they adsorbs to surfaces and are trapped by capillarity in a water- immiscible phase.

Biosurfactant are biological surface active microorganisms in order to metabolize water-immiscible substrates, allowing its adsorption, emulsification or dispersion. Hydrocarbon contaminated sites can be considered as enrichment environment for isolation of hydrocarbon degrading/ biosurfactant producing microbial strains.

Biosurfactants and bioemulsifier production by the soil microbes provide them with an advantage in contaminated site, since they uses water insoluble carbon sources for growth. They play a key role in the emulsifying the hydrocarbons. They are thought to be very suitable alternatives to chemical surfactants due to their properties like eco-friendly, less toxicity and biodegradability.

In recent years, many microbial ecologists have identified various microbial species that are effective degraders of hydrocarbons in natural environments. Many of they were isolated on their ability to metabolize various carbon sources, such as aliphatic and aromatic compounds and their chlorinated derivatives. The microorganisms were obtained originally by enrichment culture procedures, where maximum specific growth rate or maximum final cell concentration was used as the selection criterion.

Pseudomonas appears to be the most ubiquitous bacteria found in oil contaminated soils and soil in general. This bacterium is able to adapt too many different hydrocarbons. They are responsible for degrading most of aromatics in gasoline, although the efficiency in degrading aromatics hydrocarbons can vary among strains.

Petroleum oil is one our most important natural resources, the major fossil fuel of our industrial society and the source of feedstocks for the petrochemical industry. It is also for most life forms, and episodic and chronic pollution of the environment by oil causes major ecological perturbances and constitutes a critical impediment to its sustainable use by Mankind. Hydrocarbonoclastic bacteria occupy a unique tropic niche among heterotrophic bacteria participating in the global carbon cycle, in that they preferentially consume aliphatic and aromatic hydrocarbons that are relatively difficult to use by bacteria.

Refinery products, which continually pollute the natural environment, are toxic to microorganisms, causing the changes in their quantitative and qualitative composition. As a result, the biological balance of soil disturbed. Microbial degradation is used for detoxification of soils contaminated by petroleum products. The efficiency of this process relies on the adaptability of

microbes which use petroleum substances as a source of energy and carbon for living in contaminated soils.

References [21] have showed that autochthonous microorganisms present in contaminated soils are more efficient in degrading petroleum compounds than microbes existing in non-contaminated soils. Since many naturally occurring microorganisms have the ability to utilize hydrocarbons as the sole source of carbon and are widely distributed [25], the biodegradation of these compounds is common in nature.

Bacterial degradation of petroleum has been known for over 50 years, responsible bacteria have mostly been isolated from areas, such as soils, petroleum storage tanks and oil spills [15]. There are numerous reports of isolation of petroleum hydrocarbon degrader bacteria from oil exposed areas ([17], [30]). The ability to isolate high numbers of certain oil-degrading microorganisms from petroleum contaminated environment is commonly taken as evidence that these microorganisms are the active degraders of that environment [22].

Biodegradation of individual hydrocarbon compounds by pure bacterial strains has been studied extensively and metabolic pathways have been described ([14], [12], [20], [29]). Reference [18] showed the ability of native bacteria to utilize diesel fuel as the sole carbon and energy source. They isolated the ten bacterial strains from the oil refinery field in Tehran of Iran.

Bioremediation is not new to human race but new approaches that stem from advances in molecular biology and process engineering are emerging. Microbes bioremediate the environment as they biodegrade the pollutant to obtain carbon and energy, Biodegradation specifically refers to chemical breakdown or mineralization of materials facilitated by biological organisms or products [5].

The biodegradation of crude oil by microorganisms is one of the primary ways for eliminating crude oil from contaminated sites and appears to be the most environmentally friendly method of removed oil pollutant ([19], [16], [8], [4]). A bioprocess engineering study was carried out with the degradation of polyaromatic hydrocarbons by mixed culture isolated from oil contaminated soil [27]. *Pseudomonas putida*, *P. alcaligenes*, *Alcaligenes sp.* and *Acinetobacter sp.* were isolated from the petrol pump soil of Delhi city. Reference [1] studied the bacterial diversity in crude oil contaminated sites in Ahmadabad. They isolated the following microorganisms namely, *Micrococcus, Pseudomonas, Acinetobacter, Bacillus* and *Staphylococcus.*

The present study revealed that the bacterial species (*Bacillus* and *Proteus* sp.) richness in the petroleum oil contaminated soil favored the degradation of hydrocarbons by their enzyme activities.

References

[1] C. Afuwale, and H. A. Modi, "Study of bacterial diversity of crude oil degrading bacteria isolated from crude oil contaminated sites," *Life Sciences Leaf lets*, Vol .6,pp.13-23, 2012.

[2] A. Amadi, "An Assessment of the performance of some petroleum degrading microorganisms," *J. Appl. Bacterilo,*Vol. 4,pp. 61 – 67, 1988.

[3] O. O. Amund, "Observation on the degradation of crude mineral oil by estuarine Microbial community," *Nig .J. Microbial*, Vol. 4, pp. 134-143,1984.

[4] S. Bharathi and N. Vasudevan, "Utilization of petroleum hydrocarbons by *Pseudomonas fluorescens* isolated from petroleum contaminated soil," *Environ. Int,* Vol.26, pp.413-416,2001.

[5] C. Bonaventure and F.M. Jonson, "Healthy Environments for Healthy people: Bioremediation today and tomorrow," *Environ. Health. Perspect*, Vol.1, pp. 5-20,1997.

[6] J. G. Cappuccino and N. Sherman, Microbiology: A Laboratory Manual, State University of New york, The Benjamin/Cummings Publishing Company, New York, 1987.

[7] N. Das and P. Chandran, " Microbial degradation of petroleum hydrocarbon contaminants; An overview" *Biotechnol .Res. Int*,Vol. 2011,pp. 941810, 2011.

[8] J. P. Del Arco and F. P. De Franca, "Influence of oil contamination levels on hydrocarbon biodegradation in Sandy Sedimen,"*Environ. Pollut,*Vol.110,pp.515 519,2001.

[9] J. D. Descai and I. M. Banat, "Microbial production of surfactants and their commercial potential," *Microbiol. Mol. Biol. Rev,* Vol.61.PP.47–64,1997.

[10] J. M. Fought and D. W. S. Westlate, "Biodegradation of hydrocarbons in fresh water," *Pergamon. Press. Co,* New York,pp.217-230,1987.

[11] W. T. Frankenberger, "Use of Urea as a Nitrogen fertilizer in Bio-reclamation of petroleum hydrocarbons in soil," *Bull .Environ. Contam. Toxicol,*Vol. 40.pp.66-68,1988.

[12] D. T. Gibson and V. Subramanian, Microbial degradation of aromatic hydrocarbons. In: Gibson, D,T,(Ed.). Microbial degradation of organic compounds, Mascel Dekkar., New york.pp.181-252,1984.

[13] S. Harayama, H. Kishira, Y. Kasai and K. Shutsubo, "Petroleum Biodegradation in Marine Environments," *J.Mol. Microbiol. Biotechnol*, Vol.1(1),pp.63-70,1999.

[14] M. A. Heitkamp and C. E. Cerniglia, "Mineralization of polycyclic aromatic hydrocarbons by a bacterium isolated from sediment below an oil field," *Appl. Environ. Microbiol,*Vol. 54(6),pp.1612-1614,1988.

[15] N. G. Huy, K. Amanda, M. Haruki, N. B. Hun, D. T. Hang, D. T. C. Ha, T. Imanaka, M. Morikawa and S. Kanaya, "Characterization of petroleum-degrading bacteria from oil contaminated sites in Vietnam," *J. Biosci. Bioeng*,Vol. 88(1),pp.100-102,1999.

[16] A. Kapley, J. Heman, J. P. Chhatre, R. Shankar and P. Chakrabarti Khan, "Osmotolerance and hydrocarbon degradation by a genetically engineered," *Microbial Consortium. Bioresour*. *Technol,* Vol.61,pp.241-245,1999.

[17] Y. Kasai, H. Kishira, K. Syutsubo and S. Harayama, "Molecular detection of marine bacterial populations on beaches contaminated by the Nakhodka tanker oil-spill accident," *Environ. Microbiol*, Vol. 3(4),pp.246-255,2001.

[18] D. J. Kebria, A. Khodadadi, H. Ganjidoust, A. Badkoubi, and M. A. Amoozegar, " Isolation and characterization of a novel native *Bacillus* strain capable of degrading diesel fuel," *Int. J. Environ. Tech*, Vol.6(3),pp.435-442, 2009.

[19] A. Korda, P. Santas, A. Tenente and R. Santas, "Petroleum hydrocarbon bioremediation," *Appl. Microbiol. Biotechnol*, Vol.48,pp.677-689,1997.

[20] O. Koren, V. Knezevic, Z. R. Eliora, E. Rosenberg, "Petroleum pollution bioremediation using water-insoluble uric acid as the nitrogen source," *Appl.Environ. Chem*, Vol.16(4),pp.341-451,2003.

[21] S Lies Indah, "Hydrocarbon degrading bacteria: Isolation and identification," *Makara Sams*, Vol.11(2),pp.98-103,2007.

[22] P. O. Okerentugba, O. U. Ezeronye, "Petroleum degrading potentials of single and mixed microbial cultures isolated from rivers and refinery effluents in Nigeria," *Afr. J.Biotech*, Vol. 2(9),pp.288-292,2003.

[23] A. Martello, " Bioremediation: cleaning up with Biology and Technology".Vol.5, pp.7,1991.

[24] K. S. M. Rahman, I. M. Banat, J. Thahira, T. Thayamanavan and P. Lakshmanaperumalsamy, "Bioremediation of gasoline contaminated soil by a bacterial consortium amended with poultry litter, coir pith and rhamnolipid biosurfactant," *Bioresour. Technol*, Vol. 81,pp. 25–32,2002.

[25] H. T. Rehm and I. Reif, "Mechanisms and Occurrence of microbial oxidation of long-chain alkanes," 1st Ed. Springer Berlin/Heidelberg.pp.175-215, 1981.

[26] V. Riis, D. Miethe and W. Babel," Degradation of refinery products and oils from pollutant sites by the autochthonous microorganisms of contaminated and pristine soils," *Microbiol. Res*, Vol.150,pp.323,1995.

[27] R. Ruma, R. Raja, C. Ranjana and B. Pinaki, "Degradation of polyaromatic hydrocarbons by mixed culture isolated from oil contaminated soil-A bioprocess engineering study," *Indian Journal of Biotechnology*, Vol. 6,pp.107-113,2007.

[28] N. S. Subba Rao, "Soil microorganisms and plant growth,"4th edn. In: Organic matter decomposition under-degradation of hydrocarbon,pp.269,1995.

[29] L. A. Stirling and R. J. Watkinson, "Microbial metabolism of alicyclic hydrcarbons: Isolation and properties of a cyclohexane - degrading bacterium," *J. Gen. Microbiol,* Vol.99(1),pp.119-125, 1977.

[30] K. Tazaki, S. K. Chaerun, R. Asada and K. Kogure, "Interaction between clay minerals and hydrocarbon-utilizing indigenous microorganisms in high concentration of heavy oil: Implications for bioremediation,"*Clay Miner*, Vol.40(1),pp.105-114,2005.

[31] J. R. Trabalka and D. E. Retchle, "The changing carbon cycles: A global Analysis", Springe Berlin,1986.

[32] P. Y. Young, R.L. Johnson and J. G. Xu, "Biodegradation of Petroleum hydrocarbons in soil as affected by heating and forced aeration," *J. Environ. Qual*, Vol. 26,pp.1511-1516,1996.

PLATE:1

A) Nutrient Agar plate showing *Bacillus* Colonies

B) Microscopic Observation of Gram Positive Rod Shaped *Bacillus* sp.

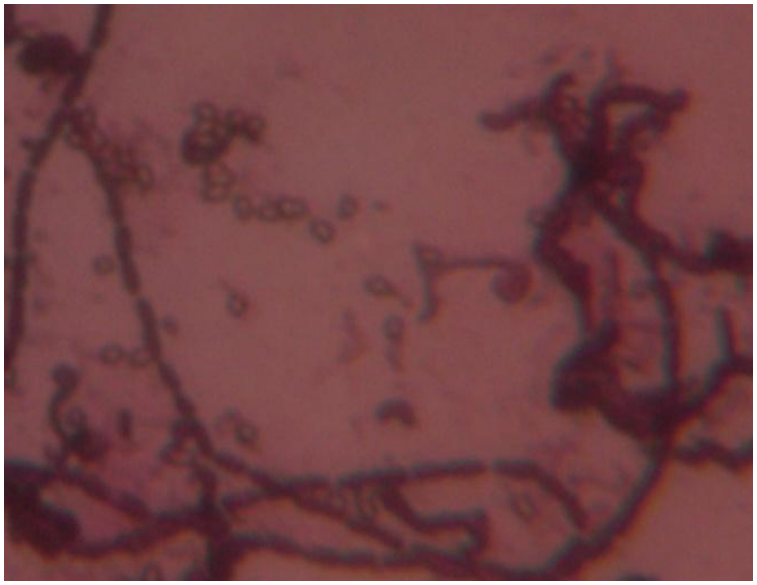
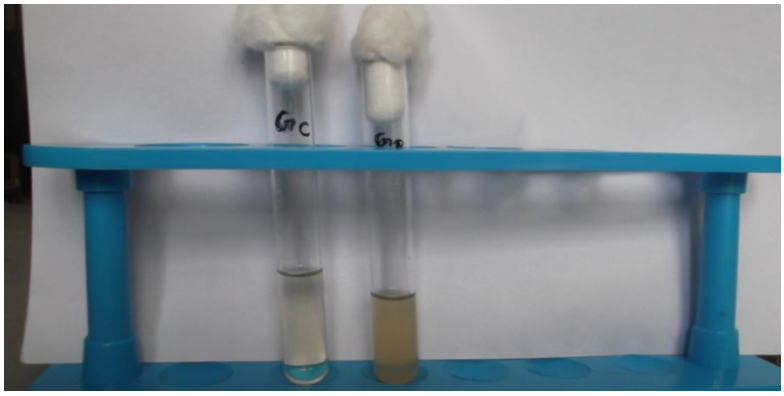


PLATE:2

A) Positive Result for Gelatin Liquefaction of Isolate A



1 2

1- Control 2-Test

B) Positive Result for Sugar Fermentation of Isolate A

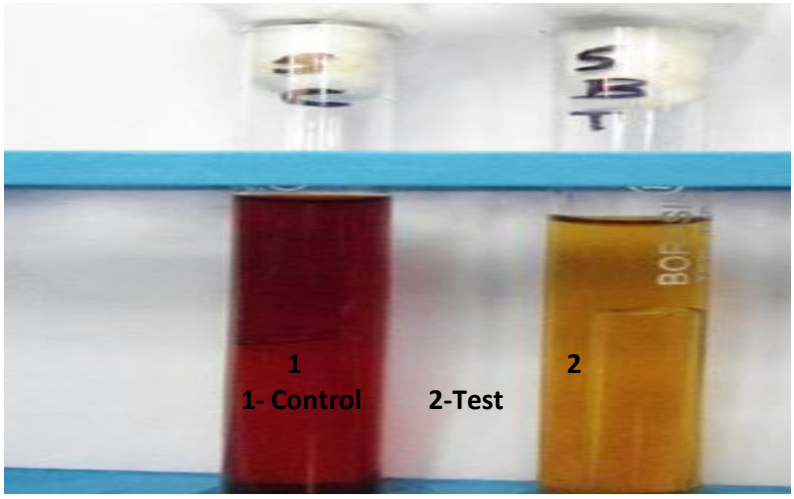


PLATE:3

Positive Result for Catalase Activity of Isolte A

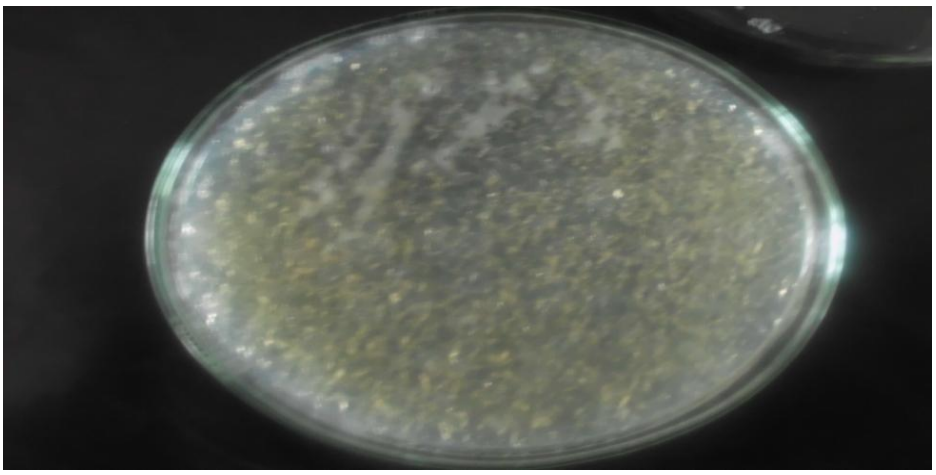
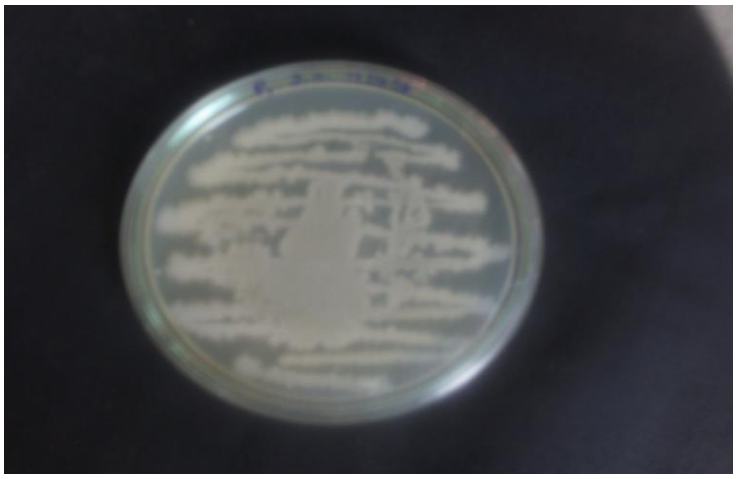


PLATE: 4

A) Nutrient Agar Plate Showing *Proteus* Colonies



B) Microscopic Observation of Gram Negative Rod Shaped *Proteus* sp.

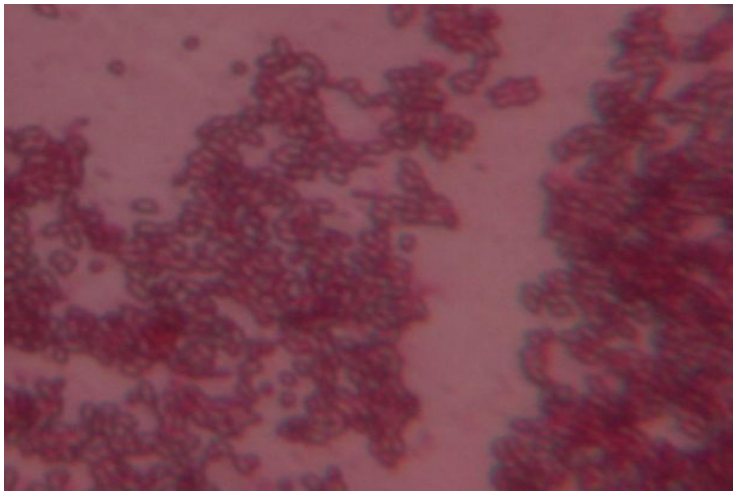
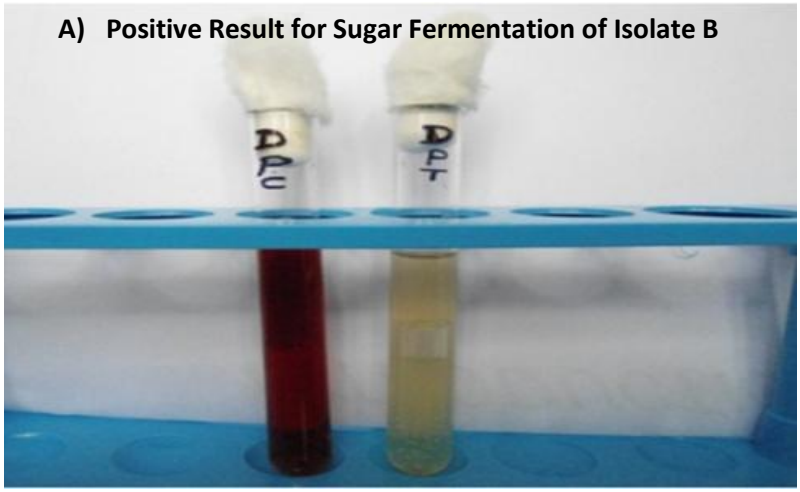


PLATE: 5

A) Positive Result for Sugar Fermentation of Isolate B



1 2

1-Control 2-Test

B) Positive Result for Gelatin Liquefaction of Isolate B