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Isolation and Identification of Naphthalene Degradation Bacteria

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Abstract:-Seven Naphthalene degrading bacteria were isolated from contaminated soil by enrichment medium utilize naphthalene as source of carbon and energy. Strains NS1A and MM3 grew best on enriched media were selected for biodegradation. The isolates were morphological and biochemically characterized as NS1A (Staphylococcus aureus) and MM3 (Pseudomonas fluorescens). Degradation of naphthalene efficacy of Staphylococcus aureus and Pseudomonas fluorescens is 63.7% and 50.17 % respectively after seven days incubation. The optimum pH and temperature for growth of isolates is 7.00 and 37^{0}C on 15-20% salt concentration. This potency of naphthalene degradation will increase the possible action to develop role model for hydrocarbon pollutant degradation from our surrounding environments.

I. INTRODUCTION

The exponential growth of human population and industrial progress has led to the accumulation of huge amounts of non-degradable pollutants across our planet. Living conditions in the biosphere are changing dramatically in such a way, whichin presence of non- biodegradable residues, is affecting the potential survival of many species (Vingesh et al., 2011).Now days, major environmental problem ispolycyclic aromatic hydrocarbon (PAHs) contamination. It's composed of carbon and hydrocarbon, where carbon atoms are being arranged in a series of adjoining six-member benzene rings (Chaudhry, 1994). Their biochemical resolution in environment arises from dense clouds ofπ-electrons on both sides of the ring structures, making them resilient to nucleophilic attack (Jonsen et al., 2005). PAHs are common environment pollutantsin air, soil and water. These are carbon-based contaminants that are resilient to degradationand can continue in the environment for long periods and have potential to cause adverse environmental effects (Wania and Mackay, 1996).

PAHs are produced in all processes of incomplete combustion of organic substances and the fate of PAHs in nature is abundantand environmental anxiety due to their toxic, mutagenic and carcinogenic properties (Antrup, 1990). In addition, in the 1997 report on carcinogens, the U.S. Department of Health and Human Services cited evidence that more than 15 PAHs caused various types of cancer in experimental animals (NIEHS, 1997). According to (Phimister et al., 2004), PAHs arelargest class of chemical carcinogens, and it has been also reported in animals (Norzila Othman et al., 2009). Some of PAH metabolites bind to protein, DNA, and RNA, and adducted compounds may cause damage to cells and cause carcinogenic effects (Pahlmann et al., 1987).Higher molecular weight PAHs such as those containing four or more benzene rings are considered to be responsible for majority of potential hazards of these compounds to environment and human health (EPA, 1986). Lower molecular weight PAHs(naphthalene, anthracite and phenahthrene) are known to effects comparatively mild but could be potentially hazardous (Klaasen., 2001).

To remediate petroleum contaminants from environments, bio-stimulation and bio-augmentation are considered as environmental friendly techniques. Hydrocarbon utilizing microorganisms are ubiquitously distributed in the environment following oil spills. These microorganisms are naturally degrading numerous petroleum hydrocarbons and cleaning the oceans of oil pollutants (Medina-Bellveret al., 2005). Bioremediation and Biodegradation using microorganism is usually preferredas cost effectiveness and more complete cleanup (&Sarma., 2011). The microorganisms should possess all necessary enzymes needed to degrade PAHs. It is known that selection or adaptation of PAHs degrading microorganisms occur as a result of their previous exposure to these substances in the environment (Jain et al. 2005).

Petroleum hydrocarbons such as Naphthalene can be degraded by microorganisms such as bacteria, fungi, yeast and microalgae (Atlas RM, 1981 & 1984). The biological treatment of industrial waste usually depends upon the oxidative activities of microorganisms. Many fungal species are involved in degradation of naphthalene 2003). Naphthalene ($C_{10}H_8$) is an aromatic hydrocarbon with two ortho-condensed benzene rings. Systematic exposure of naphthalene and its derivatives has been shown to cause several diseases and disturbance to human metabolism. Naphthalene is commonly used as a model studying PAHs metabolism by bacteria because it is simplest and most soluble constituent of PAHs contaminated environment. Common naphthalene degrading bacteria includes Pseudomonas, Vibrio, Mycobacterium, Staphylococcus and Sphingomonasspecies (Atlas., 1984).

Since physiochemical remedial strategies to clean up petroleum contaminated site are not cost effective. Therefore, research is being focused to use of

microorganisms for bioremediation of PAHs contaminated environment as it seems to be an attractive technology for restoration of polluted sites. The present study was aimed at isolation and identification of microorganisms from petroleum contaminated sites which can degrade naphthalene with higher efficiency.

II. MATERIAL AND METHOD

A. Collection of Samples

For isolation of naphthalene degrading bacteria, all soil samples were collected from fertilizer industries and nearby different motor-market in Chandigarh region. The entiresamples were collected in sterile polythene bags and store at 4^{0C} for isolation of microbes.

B. Isolation of Naphthalene Degrading Bacteria

Bacterial strain isolation was carried out bymodified enrichment media (Komet al., 2012) and contain yeast extract (0.05%), peptone (0.05%), NaCl (0.05%), NH_4NO_3 (1.0%), KH_2PO_4 (0.5%), K_2HPO_4 (0.5%), $MgSO_4.7H_2O$ (0.02%). After sterilization of media, added previously dissolved naphthalene (500mg/l) in acetone. After evaporation of acetone, 1.0g of each soil sample was inoculated in flasks containing 50ml enrichment medium and incubated at 37^{0C} at 120rpm in an incubator shaker for 7-days. After that 1.0ml of sample was taken from each culture and transferred into fresh enrichment medium and incubate for 7-days again and repeat it for three times. Samples showing microbial growth in enrichment medium were inoculated on enrichment agar medium at 37^{0C} for 48hrs and repeat this till get pure culture. After obtained, pure cultures were stored in slants of enrichment medium with agar.

C. Screening of Isolates for Naphthalene Degradation

A loop-full of each isolate was inoculated into flasks containing 50 ml of screening medium. The screening medium was same as enrichment medium, except 150.0mg of naphthalene dissolved in acetone was added to each flask after autoclaving, as sole source of carbon. Thereafter, the test tubes were incubated by keeping on the laboratory bench at room temperature (20-25°C) for three days. The ability of each isolate to utilize naphthalene was indicated by an increase in turbidity of the medium measured at 600 nm using UV-Visible spectrophotometer.

D. Morphological and Biochemical Characterization of Isolates

Out of isolated cultures, two cultures (MM3 and NS_1A) were selected, have highest OD reading and characterized by a series of biochemical test according to Bergey'sManual of determinative Bacteriology (1957) and Cappucino and Shermen (1999). Biochemical identification of pure cultures was performed withgram stain, catalase activity, indole production, nitrate reduction, gelatineliquefaction, H_2S production, MR-VP, starch hydrolysis and carbohydrate utilization.

E. Determination of Time Course of Growth of the Isolates

The isolates were inoculated in Enrichment medium containing naphthalene (150mg/l). The cultures were incubated in incubator shaker at 37° C at 120 rpm. After 12 hrs intervals, each culture was shifted to a test tube under sterile conditions and measured optical density at 600nm using UV–Visible spectrophotometer. Similarly the growth curve of both isolates was also made by using the medium containing glucose as carbon source.

F. Effect of Different Concentration of Naphthalene on Growth of the Isolates

Afterward, the enrichment medium with different concentration of naphthalene in parts per million (PPM) such as 25, 50, 75, 150, 300, 450 were prepared. Then bacterial isolates were inoculated into 50ml of enrichment medium containing different concentration of naphthalene. The flasks were incubated at 37^0C for 72hrs at 120rpm. During incubation period, 2ml samples were collected from each flask at 6 hrs intervals for assayed level of microbial growth by measuring optical density at 600 nm using UV-Visible spectrophotometer.

G. Effect of Incubation Temperatures on Growth

Inoculated flasks containing enrichment media with bacterial cultures were incubated with control flasks at different temperatures (10°C, 20°C, 30°C, 37°C and 45°C) for 24 to 48 hours in a rotary shaker at 120 rpm. Examine the cultures after 2 days of incubation for presence or absence of growth. The degree of growth was determined by using spectrophotometer at wavelength 600 nm.

H. Effect of pH on Growth

Inoculate each of a series of flasks with bacterial cultures by adding 0.1 ml of the culture. Incubate the flasks with different pH (4.00 to 9.00) for 48 hr. at 37°C in a rotary shaker at 120rpm. Examine the growth by using spectrophotometer at wavelength 600 nm.

I. Effect of Salt Concentrations on Growth

Different salt concentrations (0.5%,10%,15%, 20% and 25% of NaCl) were added to the enrichment medium flasks. Bacterial cultures are inoculated with 0.1ml of culture to each tube and incubated for 24-48 hours at 37°C in a rotary shaker at 120 rpm. Growth was examined after 2 days with Spectrophotometer at wave-length of 600 nm.

J. Effect of Different Antibiotics on the Growth

All the strains were tested for antimicrobial resistance by the method of (Bauer et al. 1966) with antibiotic impregnated discs (Hi-Media) with concentration of drugs as Ampicillin 2mcg, Cephalothin 5mcg, Erythromycin 5mcg, Cloxacillin

5mcg, Novobiocin 5mcg, Lincomycin 2mcg, Penicillin-g 1mcg and 25mcg. After that strains were characterized as non-resistant, intermediate or resistive basis of diameter of inhibition zones around disc.

K. Biodegradation of Naphthalene with HPLC

Naphthalene degradationwas confirmation with HPLC. For this, 1.0ml of bacterial culture was inoculated in 100ml of enriched media containing 150mg/l naphthalene and incubated for 7-days at rotatory shaker at 37^{0C}. Afterward, aliquots (10ml) were centrifuged at 6000rpm for 15min. and collected the supernatant and filter with 0.2µm filter for analysis (Coraland Karagoz, 2005). Analysis was performed by chromatogram LC-20AT, analytical column C18 (150×4.6mm, Algient) at 0.7ml/min flow rate with injection volume of 20µl.The concentration of naphthalene was determined at λ-254nm using UV-Vis detector (Shimadzu) (Tom Kupiec, 2004).

III. RESULT

A total no. of seven bacteria were isolated and screened on enrichment medium containing 500ppmnaphthalene from oil containing sites as sole source of carbon and energy. Out of seven, two strains (MM_3 and NS_1A) showed the highest optical density and selected for further study.

Fig 1. Isolates MM3(Pseudomonas Fluorescens) and NS1A(Staphylococcus aureus)Recovered After Screening

IV. IDENTIFICATION OF SELECTED BACTERIAL ISOLATES

The morphological and biochemical characterization of the bacterial isolates were identified NS1A as Staphylococcus aurous and MM3 as Pseudomonas fluoresces. Colony of NS1A is large, greenish-yellow, opaque, gram positive and coccus shaped whereas MM3 is faintly cream in colour, smooth surface, gram negative and rod shaped. The biochemical test revealed that both showed positive result for gelatin hydrolysis, nitrate reduction and catalase reaction, which show that both isolates are producingexoenzyme known as gelatinaseand catalase. Similarly both showed negative results for starch hydrolysis, H_2S production and indole test which confirm that they can't produce amylase for starch hydrolysis, reduce thiosulphates and don't produce try ptophanase enzyme to reduce tryptophan. NS1A showed positive results for carbohydrate utilization by producing acid in the medium containing different carbohydrate while MM3 showed negative results. (Daaneet al., 2001)

S.No.	Morphological& Biochemical Characterization	NS1A	MM3
1.	Gram Stain	+Ve	-Ve
2.	Shape	Coccus	Rod
3.	Gelatin hydrolysis	+Ve	+Ve
4.	Starch hydrolysis	-Ve	-Ve
5.	Lactose	+Ve	-Ve
6.	Dextrose	+Ve	-Ve
7.	Sucrose	+Ve	-Ve
8.	H2S Production	-Ve	-Ve
9.	Nitrate Reduction	+Ve	+Ve
10.	Indole production	-Ve	-Ve
11.	Catalase	+Ve	+Ve
12.	MR-	+Ve	-Ve
13	VP	+ Ve	- Ve
14	Cetrimide agar	-Ve	+Ve
	Identified organism	Staphylococcus aureus	Pseudomonas fluorescent

Table 1: Morphological and Biochemical Characterization of Isolates

A. Time Course of Growth of Isolates NS1A and MM3

The time course of employment of naphthalene with two isolates for growth showed in figure. It's find that both isolates grew comparatively well in naphthalene. It's observed that isolate NS1A (Staphylococcus aureus) grew better showing OD value of 0.513 after 72hrs and MM3 (Pseudomonas fluorescens) peaked an OD of 0.493 after 96hrs at 600nm wavelength. With glucose as carbon employment, both isolate expressed highest growth at 42hrs at 600nm wavelength. During growth on naphthalene, isolates produced secondary metabolites, which were found greenish yellow for NS1A and faintly cream for MM3 isolate.

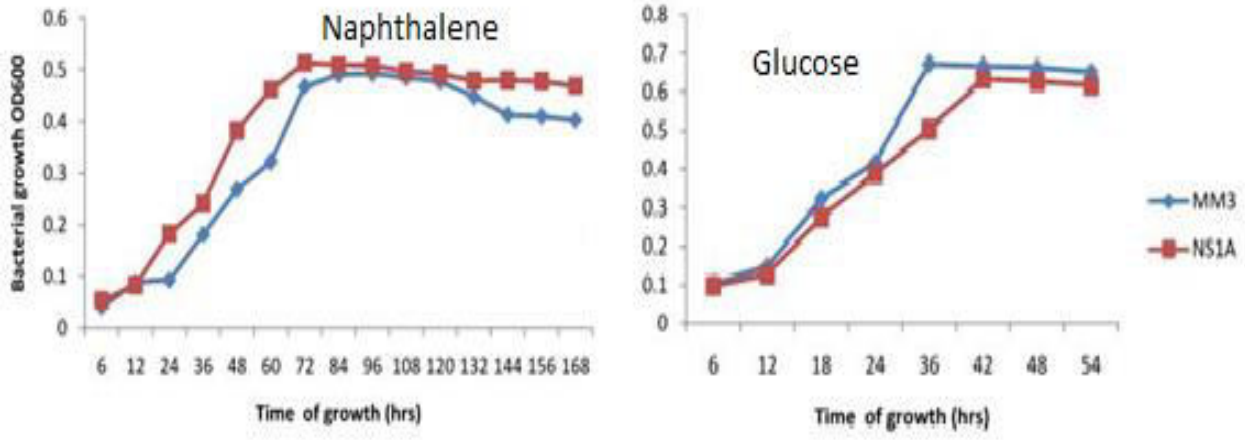


Fig 2. Time Course of Growth of Isolates NS1A and MM3

B. Effect of Naphthalene Concentration on Growth of Isolates

There was comparable growth of isolates were increased from 25ppm to 450ppm of naphthalene concentration. The corresponding growth of both isolates increased as the level of naphthalene increased from 25ppm to 150ppm. In isolates, NSIA and MM3 growth were observed as 0.193 and 0.162 respectively at 600nm wavelength when naphthalene compactness was 25ppm. Similarly optimum growth of NS1A and MM3 were observed as 0.431 and 0.423 respectively at 600nm wavelength when naphthalene compactness was 150ppm. Therefore the optimum degradation of naphthalene was best at 150ppm concentration and used further for other parameters.(Fig - B)

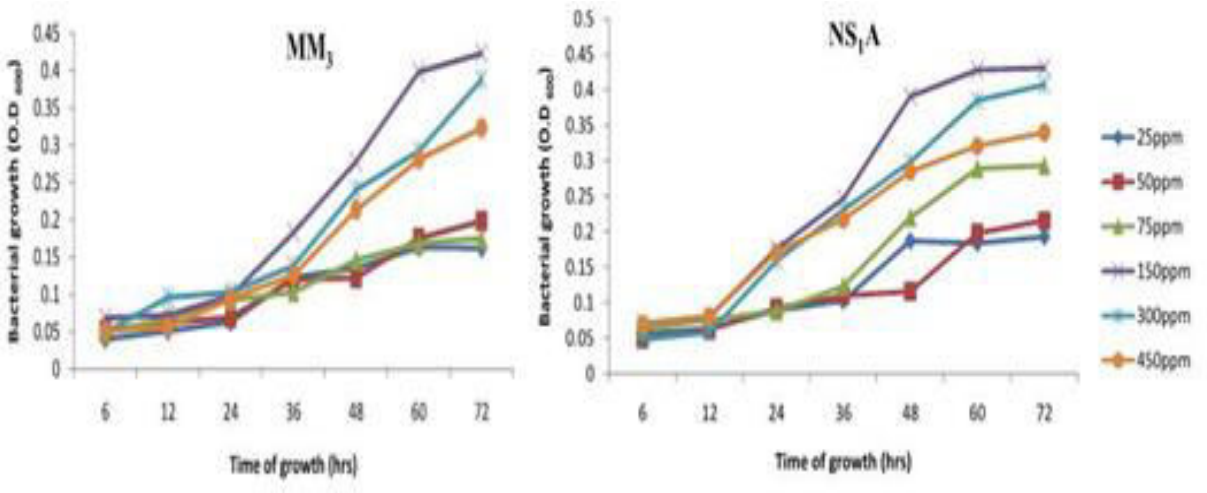


Fig 3. Effect of Naphthalene Concentration on Growth of Isolates

C. Effect of Temperature on Growth

Figure D demonstrate degradation of naphthalene by NS1A and MM3 as a function of temperature. The result evaluate that NS1A expressed best level at 37^{0C} whereas MM3 expressed best level at $30\text{-}37^{0C}$ after 48hrs. It indicates that both bacteria are mesophilic in nature.

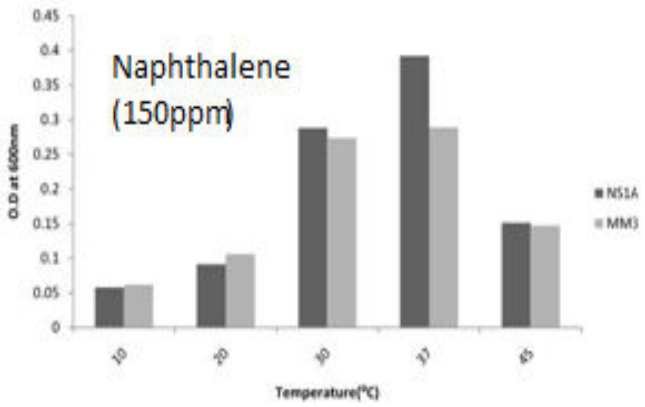


Fig 4. Effect of Temperature on Growth

D. Effect of pH on Growth

Biodegradation of naphthalene on NS1A and MM3 as a function shown in figure E, which suggest that best level growth of both isolates were at pH-7.0 and less growth at pH- 4.0 after 48hrs. This result confirmed that optimum pH for growth of both isolates is 7.0.

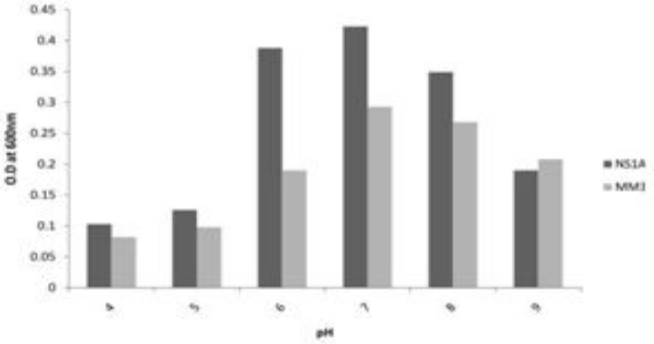


Fig 5. Effect of pH on Growth

E. Effect of Salt Concentration

As shown in figure F, result expressed that naphthalene degradation of NS1A has optimum growth at 15% of NaCl whereas MM3 has optimum growth at 20% on 600nm. Both isolates have minimum range of growth at 25% of NaCl. So, it is considered that optimum concentration of salt is 15-20% for growth of NS1A and MM3.

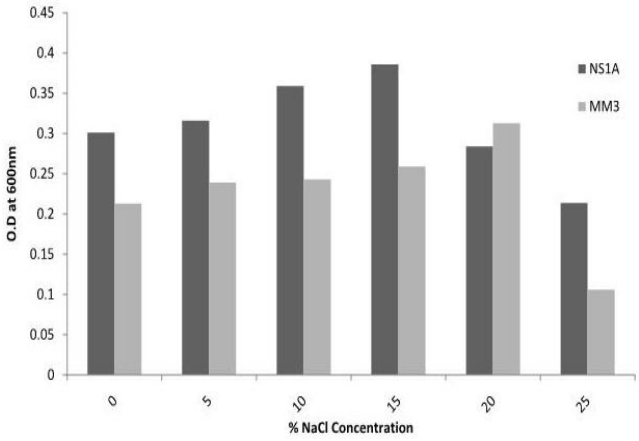
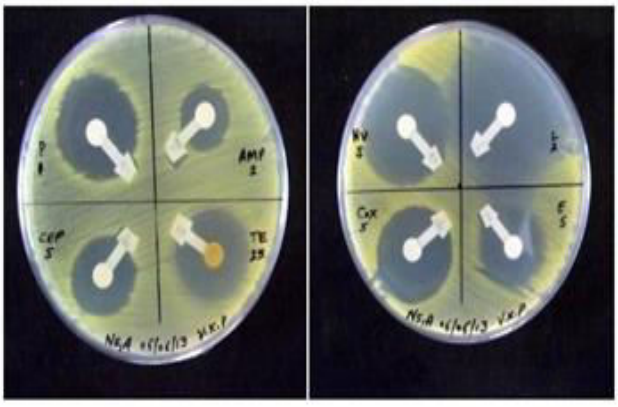


Fig. 6 Effect of Salt Concentration

F. Effect of Different Antibiotics on Growth

Figure G demonstrate that NS1A and MM3 were examined for antibiotic sensitivity or resistant based on diameter of inhibition zone around disc. The result expressed that NS1A is sensitive to all antibiotics whereas MM3 is sensitive to only three antibiotics as erythromycin, tetracycline and novobiocin.



Zone of inhibition (in mm) and resistant/sensitivity of NS_1A towards different antibiotics

Zone of inhibition (in mm) and resistant/sensitivity of MM_3 towards different antibiotics

Fig 7. Effect of Different Antibiotics on Growth

V. BIODEGRADATION OF NAPHTHALENE

We evaluate potential of naphthalene metabolites with HP-LC of NS1A and MM3. The samples were extracted after seven days of incubation of isolates with standard (150ppm). The concentration of both samples was compared with standard and observed that NS1A and MM3 had degraded naphthalene. It's concluded that NS1A has high degradation of naphthalene than MM3. The naphthalene concentration remained after 7 days of incubation in NS1A and MM3 is 54.45ppm and 74.73ppm respectively. Therefore biodegradation efficiency of $NS1_A$ is 63.7% and MM_3 is 50.17 %.

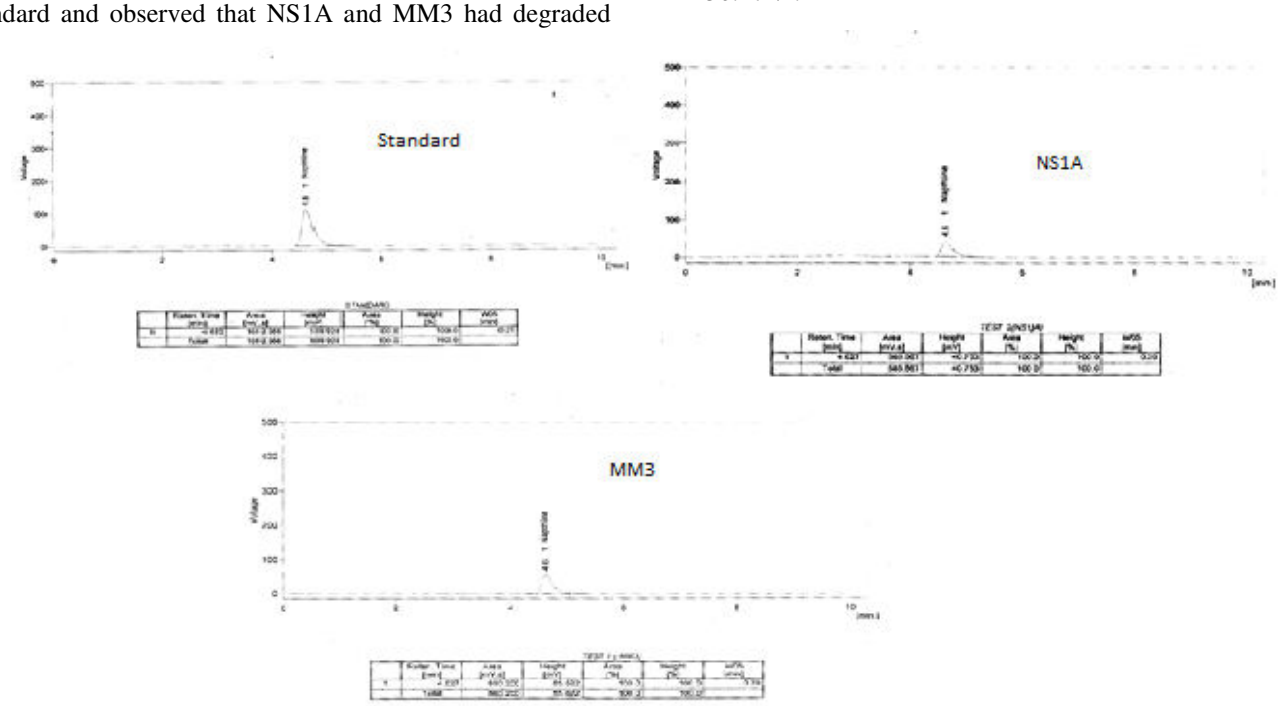


Fig 8. HPLC of Naphthalene Degradation by NS1A and MM3

VI. DISCUSSION

In the current study, total of eight isolated bacteria, only two species were selected for their ability to utilize naphthalene as carbon and energy source. The isolated strains (NS1A and MM3) have provided good chances for investigate the physiology of bacteria which linked to in vitro biodegradation of naphthalene in polluted region. The morphological and biochemical characterization of naphthalene degrading bacteria isolated from different sites resulted following genera as NS1A (Staphylococcus aureus) and MM3 (Pseudomonas fluorescens).

We have shown that NS1A and MM3 are susceptible to degradation of naphthalene. The effect of different concentration of naphthalene on growth showed the degradation ability of isolates. It was observed that maximum growth was observed with naphthalene concentration of 150ppm and similar results were obtained by Nnamchi et al, 2006, and suggested that with increasing the concentration of naphthalene, growth will increase but high concentration inhibit the growth of isolates. This is due to threshold concentration is reached and therefore growth stops. The isolates (NS1A & MM3) were studied for their degradation capability with HPLC. After seven days incubation, HPLC results reveal that NS1A & MM3 were found to degrade 63.31% and 51% of naphthalene respectively. Degradation study suggested strong evidence in PAHs with common bacterial species.

In antibiotic sensitivity test with eight antibodies, the result express that strain NS1A is sensitive to all eight antibodies whereas MM3 is sensitive to only three antibodies as Erythromycin, Tetracycline and Novobiocin. () have also reported that PAHs degrading microorganism susceptibility and showed resistance to various antibiotics. The antibiotic sensitivity tests assist in determination of sensitivity and resistance of bacteria in order to degradation of naphthalene and PAHs in environment.

Furthermore physiological testing including temperature, pHand salt concentration of both the isolates was also done in order to check the optimum conditions required for the isolates to degrade naphthalene and other PAHs. The isolate MM3 has optimal range of 15- 20% NaCl while NS1A showed optimum range of 10-15%. Similarly pH variation study showed that pH 7 is the optimum pH for both the isolates.

VII. CONCLUSIONS

Soils with hydrocarbon are proficient reservoir for naphthalene degrading bacteria isolation that can be used for takeout such components from our surrounding environments. In the present study, two bacterial strains were isolated and identified as Staphylococcus aureusand

Pseudomonas fluorescens with biochemical characterization. The isolates can best degrade 150ppm naphthalene concentration and their optimum pH and temperature is 7.00 and 37°C respectively. The biodegradation efficiency of Staphylococcus aureusand Pseudomonas fluorescensis 63.7% and 50.17% respectively after seven days incubation. The obtained study suggested that Staphylococcus aureusand Pseudomonas fluorescens could be applied in reduction of naphthalene and pesticides and PAHs.

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