Study on Polycyclic Aromatic Hydrocarbon Degrading Bacteria from Coastal Wetlands

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I hereby declare that the data presented in the dissertation report entitled, **"Study on Polycyclic Aromatic Hydrocarbon-Degrading Bacteria from Coastal Wetlands"** is based on the results of investigations carried out by me in the Discipline of Biotechnology at the School of Biological Sciences and Biotechnology, Goa University under the supervision of Dr. Sanika Samant (Assistant professor) and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will not be responsible for the correctness of observations/ experimental or other findings given the dissertation.

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This is to certify that the dissertation report **"Study on Polycyclic Aromatic Hydrocarbon Degrading Bacteria from Coastal Wetlands"** is a bonafide work carried out by Ms. Simran Shailesh Fatarpekar under my supervision in partial fulfillment of the requirements for the award of the degree Master of Science in the Discipline of Biotechnology at the School of Biological Science and Biotechnology, Goa University.

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PREFACE

Coastal wetlands, delicate ecosystems teeming with biodiversity, play a crucial role in maintaining environmental balance. However, these ecosystems face increasing threats from human activities, including the release of pollutants such as polycyclic aromatic hydrocarbons (PAH). PAHs are toxic compounds commonly found in oil spills, industrial effluents and urban runoff, posing serious risks to both aquatic life and human health.

In response to these challenges, the study presented in this work delves into the fascinating realm of PAH degrading bacteria thriving within coastal wetlands. By harnessing the natural remediation capabilities of these microorganisms, we aim to shed light on potential strategies for mitigating PAH contamination and restoring the health of these invaluable ecosystems.

Through this exploration, we hope to not only deepen our understanding of PAH degradation mechanism but also inspire conservation efforts aimed at safeguarding the ecological integrity of coastal wetlands for generations to come.

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LIST OF ABBREVIATIONS

Entity	Abbreviation
Atmosphere	atm
Carbon	C
Colony forming unit	cfu
Centimetre	cm
Deoxyribonucleic acid	DNA
Distilled water	DW
Gram	g
Gram per mol	g/mol
Gas Chromatography-Mass Spectrophotometer	GC-MS
Hydrochloric acid	HCl
Hydrophobic Organic Contaminant	HOC
High performance liquid chromatography	HPLC
Kelvin	K
Litre	L
Laminar air flow	LAF
Milligrams per litre	mg/L
Minimal media	MM
Milli molar	mM
Milli Litre	mL
Millimetre of mercury	mmHg

Nanometre	nm
Sodium hydroxide	NaOH
Phosphorus	P
Pascal	Pa
Polycyclic aromatic hydrocarbon	PAH
Phosphate buffer saline	PBS
Polymerase chain reaction	PCR
Potential of hydrogen	pH
Parts per thousand	ppt
Pounds-force per square inch	Psi
Revolutions per minute	rpm
Scanning electron microscopy	SEM
Ultraviolet visible	UV-VIS
Volume per volume	v/v
Degree celcius	^{O}C
Percentage	%

ABSTRACT

Naphthalene, a Polycyclic Aromatic Hydrocarbon has significant health implications when it serves as the sole source of carbon in the environment. The health hazard associated with naphthalene exposure are diverse and potentially severe, affecting multiple body system. Present study aimed for isolation and characterization of naphthalene degrading bacterial isolate named as GUSSF. It was identified as *Staphylococcus* sp. Studies were conducted to access factors that influences the growth rate and biodegradation of PAHs from Betim Verem Saligao road, Bardez, Penha De Franca, India, 403521. (Lat 15.504639 long 73.834740). Physicochemical parameters were assessed at various temperatures and pH for degradability**,** the maximum rate at which degradation observed was 64 % at day 16. SEM (Scanning electron microscopy) images of GUSSF showed the presence of aggregation and simultaneously compared to *Staphylococcus* sp. and observed that there was a change in the morphology. The results indicated that the isolate GUSSF as *Staphylococcus* sp. that might be useful in biodegradation of sites contaminated with PAHs.

CHAPTER 1: Introduction

1.1. Background

1.1.2. Polycyclic aromatic hydrocarbons

PAHs are classed based on the number of aromatic benzene rings: compounds with a high molecular weight are having more than three rings, while low molecular weight ones include two or three rings. Recent years have seen an increase in the volume of publications on PAH elimination. Bioremediation, which employs specific microorganisms such as yeast, fungi, and bacteria is one of the most widely utilized and promising technology for pollutant removal. Bacteria have been thoroughly examined, and there is information available on their ability to break down xenobiotics, including PAHs (Darmawan et al.,2015).

Gathering soil or water samples from wetlands along the coast supplementing the medium with hydrocarbons as a carbon source in order to find hydrocarbon-degrading microbes, and then isolating PAH degrading bacteria. Hydrocarbons including naphthalene, phenanthrene, and anthracene are frequently employed in the breakdown of PAHs. These hydrocarbons can be used as simulated compounds to investigate the breakdown mechanisms of PAHs. One of the most convenient and effective hydrocarbons to research PAH breakdown is naphthalene. Due to its frequent occurrence in tar from coal and petroleum, it is easily obtainable for scientific investigations. It is also a good model compound to study how more complicated PAHs degrade due to its comparatively basic structure.

While some PAHs like acenaphthene, acenaphtylene, anthracene, benz(a)anthracene, benzo(a)pyrene are utilized in the production of detergents, plastics, fungicides, and

insecticides (Elsalam et al.,2009). Inhaled air contaminated with PAHs is one of the most prevalent ways these compounds can enter the body. When people of all ages breathe in PAHs, they enter their lungs. Moreover, if people consume food or water that has been tainted with PAHs. Additionally, skin contact with PAH-contaminated items or soil, such as creosote, roofing tar, coal tar, or heavy oils, can result in exposure to PAHs. Wood is preserved with greasy liquid called creosote, which is a component of coal tar, they can spread and target fat tissues. After entering the body, PAHs have the ability to spread and target adipose tissues. The liver, fat, and kidneys are among the target organs. But the PAHs will exit an individual's body through the urine and feces in a short period of time (Elsalam et al.,2009; Bansal et al.,2015)

1.1.2. Scope of PAH Compound Contamination

Anthropogenic chemical input brought on by humankind's escalating energy demands has made chemical pollution of the soil environment a significant environmental health concern in recent years. In addition to natural sources like oil drains and water from the surface run-off, forests and prairie grass fires, and ash from volcanic eruptions, as PAHs, are released into the environment through a variety of means, including direct emissions from vehicles, chronic leaks of commercial or sewage effluents, power generation and heating, unintentional discharges during transportation, consumption and elimination of petroleum-based products, fossil fuel liquefaction and gas production techniques, refuse burning, and cigar (Grosser et al.,1995; Zhang et al., 2017).

1.1.3. PAH Biodegradation

Microorganisms are primarily important because they recycle components such as carbon, Sulfur, nitrogen, oxygen, and phosphorus, which helps break down organic compounds into carbon dioxide and water. After the organic matter is transformed into carbon dioxide, integrated metabolic processes are involved and molecular oxygen reduction occurs. There has been a spike in interest due to the amazing catalytic activity of microbes in breaking down a variety of organic compounds (Vijayanand et al.,2023).

Many hydrocarbons have a low biodegradation rate, so their concentration will not vary as much as it does with more easily soluble organic materials. High hydrocarbon concentrations can be caused by massive, undispersed oil accumulations in water, inhibiting biodegradation due to nutrient or oxygen constraint or the toxic effects of volatile hydrocarbons (Leahy et al.,1990).

1.1.4. PAHs sources and types

The class of organic pollutants known as PAHs is regarded as dangerous because of their poisonous, mutagenic, and carcinogenic qualities. Generally speaking, pyrogenic, biogenic and petrogenic sources produce PAHs. Biogenic refers to the production or emergence of biological creatures, such as biogenic sediments. As it comes to the production or genesis of rocks, particularly igneous rocks, it is referred to as petrogenic. When something is formed under extremely hot settings, it's called pyrogenic (Darmawan et al.,2015). Natural disasters such as lightning-induced moorland fireplaces, volcanic eruptions, and forest fires are negligible or small sources of PAH emissions (Vijayanand et al.,2023).

Under aerobic conditions, PAHs can be broken down by bacteria through the dioxygenase enzyme-catalyzed first oxidizing of the aromatic ring. Understanding the significance of PAHs in environmental restoration requires research on the bacteria that breakdown PAHs in coastal wetlands. These bacteria are essential for the breakdown of complex hydrocarbons, which reduces pollution and preserves the integrity of ecosystems. The effects of industrialization and urbanization have led to the discovery of about 400 variety of PAHs and their effects. The study may shed light on possible bioremediation techniques and how well they work in these delicate environments (Vijayanand et al.,2023).

1.1.5. Naphthalene

One prevalent environmental pollutant is naphthalene. Moreover, some industrial workplaces have elevated naphthalene levels. Since 2000, naphthalene exposure has been a serious concern for occupational and environmental medicine because to the significant carcinogenic implications associated with exposure to the chemical in animal studies. As a result, it is assumed that naphthalene significantly contributes to the increased risk of cancer in people. Its broad emission into the environment is caused by insufficient processes of combustion of commercial, natural, and home sources, including airline and other motor vehicle traffic, petrol burning, forestry fires, and domestic heating utilizing fossil fuels (Preuss et al.,2003).

Naphthalene is a white, crystalline substance with a boiling and melting point of 218℃ and 80.5°C respectively along with 31.7 mg/L water solubility. Its vapor has a partial pressure of 0.087 mmHg at 25°C. Naphthalene gradually sublimates and releases a distinct

odor at room temperature. Naphthalene (Molecular formula $C_{10}H_8$, CAS Registry Number 91-20-3) is easily understood and the most soluble. The primary method utilized in the industrial production of naphthalene involves the separation and condensation of coal tar from coke oven emissions, followed by its crystallization and distillation. Naphthalene can be recovered from particular petroleum fractions or from the fluid stream of methylnaphthalenes produced during cracking processes.

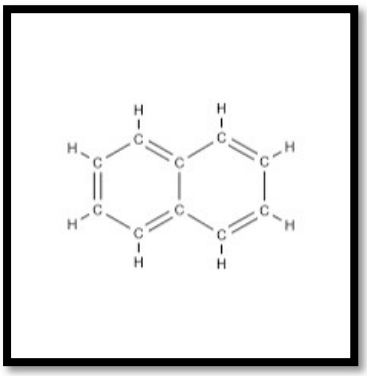

Fig 1. Chemical structure of Naphthalene

Naphthalene, this is a two-ring aromatic hydrocarbon **(Fig.1)** The terms antimite, naphthalin, naphthaline, naphthene, and tar camphor are used as synonyms. The highly volatile PAH is naphthalene, which has a gas-phase portion of 90–100% and a brief atmospheric half-life of 3–8 hours. The molecular weight of the substance is 128.17 g/mol, with melting and boiling points of 80.2 °C and 218 °C respectively, relative vapor density of 4.42 g/cm^{3}at 20 °C and 1 atm, vapor pressure of 10 Pa at 25 °C, and diffusion coefficient of 7.20 × 10^{-2}cm^2/s at 298 K. It dissolves in acetate and alcohol but not in water (Buckpitt et al.,2010).

Azo-dyes are also synthesized using naphthalene. It also acts as a feeder for naphthalene sulphonic acids, which are used extensively in the rubber and leather industries, as well as plasterboard components, dispersants, tanning agents and concrete plasticizers (Preuss et al.,2003).

The process of naphthalene degradation commences with the attack of the multi-component enzyme naphthalene dioxygenase on the aromatic ring, resulting in the formation of cis-dihydroxy-1,2-dihydronaphthalene. A cis-dihydrodiol dehydrogenase then converts the cis-naphthalene dihydrodiol that naphthalene dioxygenase produced into 1,2-dihydroxynaphthalene. The next steps in the metabolism of 1, 2-dihydroxynaphthalene is cis-o-hydroxybenzalpyruvate, 2-hydroxy-2H-chromene-2-carboxylic acid, and 2-hydroxybenzaldehyde, which result in Salicylate. Additionally, 1,2-dihydroxynaphthalene is converted to 1,2-naphthaquinone via non-enzymatic oxidation. Normally, salicylate is decarboxylated to catechol that is then further broken down in Meta- and Ortho-pathways by ring fission. Salicylate-5-hydroxylase transforms salicylate into gentisate (Seo et al.,2009).

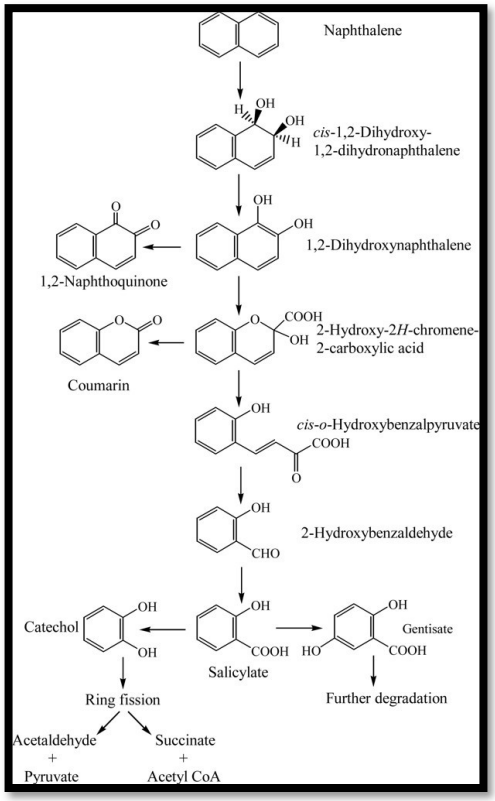

Fig 2. Proposed catabolic pathway of Naphthalene by bacteria (Seo et al.,2009)

1.1.6. Sources of PAHs in marine ecosystems.

Worldwide research has been done on PAH pollution. On the basis of PAH diagnostic ratios, the majority of the research were carried out to determine the routes to PAH contamination. It has been determined that the primary causes are pyrogenic and petrogenic, resulting from human activities such as the combustion of fuel such as oil and diesel, which releases emissions and directs petroleum input. The primary ways that pyrogenic PAHs, which are regarded as chronic pollutants, enter marine ecosystems are by atmospheric deposition and river runoff (Tobiszewski et al., 2012).

Oil spills release the most astounding amounts of petrogenic PAHs into marine environments. Oil spills are regarded as severe hydrocarbon pollution and depending on the type of oil, the aromatic component might make up 3% to 30% of the total. Naturally occurring oil seeps account for approximately 47% of all crude oil that enters the marine environment and are the main source of PAHs in oceans. The remaining portion is a result of anthropogenic activities like transportation. Oil seeps are frequently seen as naturally occurring chronic pollution because of their age (Duran et al., 2016).

Despite the fact pyrogenic PAHs are less bioavailable than petrogenic PAHs. Benthic organisms have specific habitats created by the hydrocarbons circulated by oil seeps, and the microbial community is continuously under selection pressure to shape their organization (Duran et al.,2016).

1.1.7. Coastal ecosystems

Diverse habitats can be found along coasts, such as coral reefs, bays, lagoons, salt marshes, mangroves, and estuaries. In addition to offering priceless ecosystem services, these ecosystems are crucial to the carbon cycle, especially when it comes to the mineralization of organic matter. In coastal ecosystems, salinity is one of the main environmental factors influencing microbial diversity and dispersion. This is because soil salinity can cause misinterpretation with other soil properties like water availability, nutrient conversions, and pollutant degradation. Furthermore, the variety and structure of microbial communities are restricted by the soil carbon supply, which supplies bacteria with primary nutrients. Microbial activity in coastal soil also has a significant impact on these microbial communities (Duran *et al.,*2016).

Furthermore, due to the widespread disruption caused by human activities, the influence of contaminants on microbial communities, such as PAHs, has received greater consideration in coastal ecosystems. PAHs are common organic contaminants that are highly persistent in the environment. PAHs are very harmful to all forms of life, being carcinogenic, mutagenic and immune-toxicogenic. The effects of PAH invasion on water microbial populations may vary depending on the water, with research showing that different PAHs promote different microbes in soil. Estuaries are complex hydrodynamic environments, and the activities of humans that take place there are so diverse that each estuary may serve as a case study with some unique characteristics. Estimating PAH fluxes from various sources is an important part of determining the PAH input processes and establishing the river contribution on a worldwide scale (Duran et al.,2016).

1.2. Aim and objectives

1.2.1. Aim

To study polycyclic aromatic hydrocarbon degrading bacteria from coastal wetlands.

1.2.2. Objectives

i. Isolation and characterization of PAH degrading bacteria
ii. Effects of physicochemical parameters on PAH degradation by bacteria
iii. Morphological and enzyme analysis of PAH degrading bacteria
iv. Estimation of PAH biodegradation

1.3. Hypotheses

The theories surrounding PAH degrading bacteria Centre on how these microbes degrade contaminants. For effective degradation, they may co-operate metabolic pathways, or operate within microbial communities. In addition to environmental factors and microbial competition, genetic adaptation may allow them to flourish in contaminated environments. Evolutionary forces may eventually lead to determination of more potent PAH degraders.

1.4. Scope

The PAHs can able to enter directly into the water environment via air or through rainfall mainly from urban areas. PAHs that are released in the atmosphere are known to reach the ground level through wet or dry deposition and can also be deposited in the soils (Wade, 2002). PAHs are primarily found in runoff from urban areas, as well as wastewater treatment and industry treatment. The latter from sewage sludge usually contains PAHs which is also directly deposited on soils (Vijayanand et al.,2023).

Bacteria that breakdown PAHs is essential to bioremediation because they help remove dangerous contaminants from the environment. Their range of usage includes the remediation of water and soil, the treatment of industrial waste, and even the restoration of harmed environments. These microorganisms may help with sustainable development initiatives and reduce environmental Pollution.

CHAPTER 2:Literature Review

Some members of the category of environmentally persistent chemicals known as PAHs are regarded as environmental contaminants (Zaidi et al.,1999). Taking into consideration their carcinogenic and mutagenic qualities the majority of PAHs tend to collect in sediments in marine environments because they are poorly dissolved in water. Sources of PAHs in coastal marine habitats include rivers, petrochemical industry, atmospheric deposition, and ship-spilled petroleum products (Readman et al., 2002). Urbanized estuaries may have marine sediment concentrations of PAHs more than 100,000 ng/g (Zaidi et al.,1999). As mentioned previously, its existence in coastal areas could be dangerous for both marine life (Hylland et al.,2006) and human health that leads to chronic diseases like cancer, respiratory diseases and cardiovascular diseases. The main biological process that breaks down PAHs is transformation. Thus, microbial decomposition has the ability to eliminate PAHs coming from the environment as a whole. Despite the availability of research on the breaking down of PAHs by only pure cultures, determining the rate of biodegrading processes in coastal regions sediments and water has been difficult. PAHs decay slower in open waterways and sediments than in pure cultures. However, physicochemical factors may influence their decomposition in natural environments (Zaidi et al.1999).

The existence, persistence, and fate PAHs in the environment are of concern to toxicologists; the majority of a high molecular weight PAHs are genetically toxic, and there is a chance that they will accumulate into food chains (Heitikamp et al., 1988). Some low molecular weight PAHs are critically toxic. Prolonged exposure to PAHs has been linked to elevated mutagenicity in sediments and malignant illnesses in aquatic animals. 16 PAHs

have been designated as priority pollutants by the U.S. Environmental Protection Agency due to their widespread distribution, toxicity, and carcinogenicity (Heitikamp et al.,1988).

PAH degradation appears to be connected with specific taxonomic groups of bacteria, including *Sphingomonas, Burkholderia*, and *Pseudomonas* among Gram-negative species and *Rhodococcus* and *Mycobacterium* across Gram-positive species. Given that high and fluctuation salinity facilitates the deterioration of wall of the cell integrity, protein denaturalization, as well as changes in osmotic pressure, conventional microorganisms cannot be used to treat industrial hyper saline waste waters or to remediate polluted hyper saline environments (Erdoğmuş et al. 2013).

There are few research using natural microbial consortium, and the majority of studies giving estimates of biotransformation and decomposition of PAH and related chemicals have been conducted on isolated micro-organisms. Microbial consortia are generally more effective than isolated microorganisms at removing PAH from contaminated areas. This is mostly due to the significant presence of several enzyme activities and synergistic PAH promotion, which enhances biodegradation capacities (Molina et al. 2009).

When phenanthrene was incorporated to water samples taken from Guayanilla Bay, the pace and amount of degradation by the native microbial flora in the samples were very sluggish. The modification of PAH molecules in the environment occurs mostly through microbial processes, although it is also impacted by a variety of environmental variables. Phenanthrene in saltwater samples decomposed rapidly after being prepared with hydrogen peroxide (H_2O_2) and subsequently infected with *Alteromonas* sp., a recognized indigenous phenanthrene degrading bacterium. The presence of a readily available carbon source, glucose did not promote phenanthrene breakdown in Bay water, demonstrating that carbon

cannot be a limiting factor. Phenanthrene may build in sediments where nutrients are not a limiting factor due to lack of nitrogen, which would slow down the chemical biodegradation. Guayanilla, in Puerto Rico's South, experiences little rainfall and is mostly hot and dry throughout the year. Thus, any pH variation greater than 8.0 may have the effect of delaying the breakdown of PAHs in the Bay waters. In addition, the high pH and scarcity of accessible nitrogen in Bay water may lengthen the period that PAHs remain in the water (Zaidi *et al.* 1999).

Further, compounds having two, three, and four rings—namely naphthalene, phenanthrene, and pyrene were employed as PAHs. By using HPLC measurement, degradation rates were found to be 98%, 92%, and 57% for naphthalene, phenanthrene, and pyrene, respectively, which all decomposed in 5 days, and 95% for pyrene after 10 days. *Cycloclasticus*. sp grew most slowly with pyrene and most quickly with naphthalene (Wang et al.2018).

Using an orthogonal approach, the impacts of multiple parameters were examined on the decomposition of phenanthrene, a 3-rings model PAH, in polluted sediment slurry by a bacterial strain of *Sphingomonas* sp. that had been collected from surface mangrove sediment. Salinity and inoculum size were the most important variables, but temperature, nutrient addition, and phenanthrene concentrations had little bearing. In polluted mangrove sediment slurry, the ideal conditions for biodegradation were 30°C, 15 ppt salinity, a carbon/nitrogen ratio of 1:1. Under ideal circumstances, the first order rate model could provide the best description of the biodegradation of phenanthrene. The kinetic model's accuracy in forecasting *Sphingomonas* sp. biodegradation at different phenanthrene concentrations was confirmed (Jianlinet al*.,* 2008).

Using the Degrading Bacterial Consortium C2PL05, the biodegradation process of naphthalene, phenanthrene, and anthracene was optimized. The carbon/nitrogen/phosphorous (C/N/P) molar ratio, pH, iron concentration, iron source, nitrogen source and carbon source were the factors that were optimized. Using three distinct treatments over the course of 168 hours, each factor was optimized. Cell density was assessed using Spectrophotometry absorbance at 600 nm, and PAH depletion was measured using HPLC (Simarro et al.,2011).

Certain acid-resistant Gram-positive bacteria, such *Mycobacterium* sp., exhibit enhanced PAH breakdown capabilities in acidic environments due to their increased permeability to hydrophobic substrates at low pH levels. Other *Pseudomonas* microorganisms, on the other hand, favor pH levels that are neutral. Our findings supported earlier research in confirming that the ideal pH for PAH biodegradation is neutral (Simarro et al.,2011).

Pyrene is a 4-ringed PAH that is thought to be recalcitrant. It was found that a strain of *Pseudomonas stutzeri* used pyrene as its only source of carbon and energy, with a generation span of 30 hours. Under non-growth conditions, the bacteria broke down pyrene within 24 hours, going from its water-soluble limit of 0.132 mg/L to non-detectable levels of 0.001 mg/L. Because *Pseudomonas* is widely distributed in the environment and a wealth of genetic information regarding potential engineered systems is accessible, this first report of their ability to grow exclusively on pyrene is crucial. The impacts of non-ionic surfactant on HOC biodegradation were examined using naphthalene. Only at concentrations over CMC did TritonX-100 improve the breakdown of naphthalene by *Pseudomonas fluorescens* (McNally et al.,1998).

Naphthalene enhanced phenanthrene and pyrene degradation, while phenanthrene hindered pyrene degradation for various systems and conditions. This combination of naphthalene, phenanthrene, and pyrene has been employed to prove that low molecular weight PAHs influence the degradation of high molecular weight PAHs, and not vice versa. This is likely due to the greater concentration and biodegradability of low molecular weight PAHs (McNally et al.,1998; Mackie et al.,2015)

According to Coral et al. (2005) At a petroleum refinery in Mersin, Turkey, 50 strains of the genus *Pseudomonas* (ARP) were identified from soil contaminated by crude oil using selective enrichment of cultures in modified M9 minimal medium (MM9). All of the isolates were determined to be members of the *Pseudomonas* genus, and due to certain biochemical traits, there may have been some similarities to *P. putida* strains. The strains ARP26 and ARP28 were chosen for biodegradation testing because they grew most well on MM9 agar media. HPLC demonstrated the biodegradation rates, detecting efficiencies as high as 93% and 98% in ARP26 and ARP28 cultures respectively, during the first 7 days of incubation (Guo et al.,2005).

Aagh et al. (2016) reveals that both the petrogenic and pyroletic inputs were found to be the primary sources of PAHs in water and sediment samples in the examined location, with petrogenic sources predominating because of the recent oil spill. The levels of PAHs in the water samples examined in this research were from 42 to 180 times greater than those found in earlier investigations, endangering human health as well as the life of aquatic biota.

Rhodococcus, *Sphingomonas*, and *Paracoccus* were the bacterial isolates that were able to break down combination PAHs (Phe + Fla + Pyr). According on the consortium and the

kind of PAH chemicals involved, their degradation percentages may be lower, equal, or even higher than those of their corresponding enriched consortia. These findings imply that PAH-degrading bacteria that have been concentrated in mangrove sediments may be employed for PAH bioremediation, either as a single isolate or as a mixed culture (Guo et al.,2005).

Kumari et al. (2018) reported that capacity to break down various PAHs found in crude oil by *Stenotrophomonas maltophilia*, *Ochrobactrum anthropi, Pseudomonas mendocina*, *Microbacterium esteraromaticum*, and *Pseudomonas aeruginosa.* The estimated percentage of PAHs in the crude oil sample from the Digboi oil refinery in India was naphthalene, fluorene, phenanthrene, and benzo(b)fluoranthene. Individual bacteria exposed to crude oil had an elevated level of biodegradation of particular PAHs after 45 days, *M esteraromaticum* biodegraded 81.4% of naphthalene, *P. aeruginosa* 67.1% of phenanthrene and 61.0% of benzo(b)fluoranthene, and *S. maltophilia* 47.9% of fluorene. But a group of these bacteria demonstrated improved biodegradation of the crude oil's 89.1% naphthalene, 63.8% fluorene, 81% phenanthrene, and 72.8% benzo(b)fluoranthene. The consortium's inclusion of biosurfactant improved the degradation by an additional 10%. These findings imply that the bacterial consortium that was formed has a great deal of promise for PAH cleanup.

CHAPTER 3: Methodology

3.1. Sample collection

Collection of sample was carried out on 22nd December 2023 from Betim Verem Saligao road, Bardez, Penha De Franca, India, 403521. (Lat 15.504639 long 73.834740)**. (Fig. 5 a)**

3.2. Physicochemical Analysis

3.2.1. Temperature

A glass thermometer was used to find out the temperature of the water sample at the sample collection site. This analysis was carried immediately after the sample collection in order to avoid errors in the results.

3.2.2. pH

pH of the water sample was measured using pH paper at the sampling site in order to avoid errors. The pH was again checked using pH probe and making sure that the pH meter (Eutech instruments) was standardized using pH buffer standards in acidic, neutral and alkaline pH range that is 4, 7 and 9 respectively. The probe was washed before using it. The sample was taken in a 100mL glass beaker into which the pH probe was placed and the reading was observed on the display shown. Triplicate readings were recorded of the sample.

3.2.3. Salinity

The salinity of the sample was measured using a refractometer. The sample stage was wiped using 70% ethanol. A drop of the sample was added on the stage using a dropper. The lid was closed and reading was observed.

3.3. Enrichment of culture medium

Enrichment method was performed by adding 10mL of water sample in 90mL minimal media (MM) Broth without dextrose supplemented with Naphthalene as a source of hydro-carbonate a concentration of 100mg/L Appropriate control flask was maintained containing sample and media only. The enrichment medium was incubated at 37ºC at 100rpm for 7 days and the growth was observed at 0^{th}day and on 7^{th}day of incubation.

3.4. Serial dilution

0.1mLof culture from enriched media was added to 0.9mLof 0.85% Saline in a glass vial (5 ml) and mixed thoroughly using 1mL pipette. Glass vials were placed on the surface in Laminar Air Flow (LAF). The glass vials were labeled as 10^0, 10^{-1},10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}. 10^0 were obtained by adding 0.1mL of enriched culture and 0.9mL of saline and mixing thoroughly. From 10^0 glass vial 0.1mL of culture was serially transferred to 10^{-1} glass vial and continued till 10^{-6} dilution.

3.4.1. Preparation of MM medium for bacterial isolation.

MM media (Himedia, Mumbai) (10.675g) was weighed for preparing 1L of media. The powder was added to the flasks and gradually dissolved using 1L of distilled water. 25g of agar was added to the flask and dissolved thoroughly. The contents of the conical flask and the media were autoclaved at 15psi at 121ºC for 15 minutes. Media was cooled at room temperature and Naphthalene (100mg/L) was added to autoclaved flask, stirred and poured into autoclaved Petri plates aseptically.

3.4.2. Spread plating

0.1mL of sample was spread plated from dilutions 10^0 to 10^{-6} using L-shaped spreader on MM plates. The plates were labeled properly. Plates were incubated at room temperature for 2 days and checked for growth on a daily basis.

3.4.3. Colony counting

The viable count of the bacteria on MM agar was determined using the method Colony forming unit (cfu/ml). According to (Joux et al.,1997) the formula;

$$\text{Viable count} = \frac{\text{Number of colonies}}{\text{Volume of inoculum} \times \text{dilution factor}}$$

3.4.4. Isolation of Morphologically distinct colonies

Based on the growth observed on the incubated plates post-serial dilution, morphologically unique colonies were picked. The colonies were selected and streaked onto MM agar plates bearing 100 mg/L of naphthalene in an aseptic manner. The plates were incubated for 5d at room temperature.

3.5. Characterization of bacterial isolates

Purified bacterial isolates were characterized by the following standard bacterial characterization assays.

3.5.1. Colony characterization

Parameters in accordance with the American Society of Microbiology's colony morphology protocol. Fundamental attributes such as shape, magnitude, hue, altitude, transparency, and feel were noted (Breakwell et al., 2007).

3.5.2. Gram staining

The visually distinct isolates were Gram stained and observed under a compound microscope (Lawrence and Mayo XSZ-N107T). The isolated species was thinly smeared onto a sterile glass slide. Heat was used to repair the smeared slides. After applying crystal violet, each smear was held for one minute. After that, distilled water was used to wash the crystal violet until the extra colour was removed. After applying Gram's iodine solution and letting the smears sit for a minute, the iodine residue was carefully removed by giving them a gentle wash with distilled water. After a minute, the decolorizer was added and rinsed. After using Safranin as a counterstain for 30 seconds, the area was cleaned. After air drying, the stained slides were examined using an oil immersion objective at a magnification of 100X. (Gram staining protocols | ASM.org,n.d).

3.5.3. Biochemical Characterization Studies

IMViC biochemical test was performed using KB001 kit from Himedia. Using reagents available from the kit including Kovac's reagent, Methyl red (MR) reagent, Barritt's A and Barritt's B for Indole test, MR test, Voges Proskauer (VP) test and citrate utilization test respectively.

3.5.3.1. Indole test

50µL of culture was inoculated into well no.1 and incubated for 48 hours at room temperature. After incubation, 2 drops Kovac's reagent was added to well no.1 and checked for Cherry red colour appearance for a positive test and colourless for a negative test.

3.5.3.2. Methyl Red test

50µL of culture was inoculated into well no.2 of the kit and incubated for 48hours at room temperature. After incubation, 2 drops of MR reagent were added to well no.2 and checked for colour change to red indicating a positive test and yellow/orange for a negative test.

3.5.3.3. Voges Proskauer test

50µL of culture was inoculated into well no.3 of the kit and incubated for 48 hours at room temperature. After incubation, 2 drops of Barritt's A and 2 drops of Barritt's B was added to well no.3 and checked for colour change to red colour indication for a positive test and colorless or slight copper for a negative test.

3.5.3.4. Citrate utilization

50µL of culture was inoculated into well no.4 of the kit and incubated for 48 hours at room temperature. After incubation, the colour change of green colour to blue from well no.4 was an indication of a positive test and green colour for negative test.

3.5.3.5. Glucose

50µL of culture was inoculated into well no.5of the kit and incubated for 48 hours at room temperature. After incubation, the colour change from pinkish red/red to yellow for a positive test.

3.5.3.6. Adonitol

50µL of culture was inoculated into well no.6 of the kit and incubated for 48 hours at room temperature. After incubation, the colour changes from pinkish red/red to yellow for a positive test.

3.5.3.7. Arabinose

50µL of culture was inoculated into well no.7 of the kit and incubated for 48 hours at room temperature. After incubation, the colour change from pinkish red/red to yellow for a positive test.

3.5.3.8. Lactose

50µL of culture was inoculated into well no.8 of the kit and incubated for 48 hours at room temperature. After incubation, the colour changes from pinkish red/red to yellow for a positive test.

3.5.3.9. Sorbitol

50µL of culture was inoculated into well no.9 of the kit and incubated for 48 hours at room temperature. After incubation, the colour changes from pinkish red/red to yellow for a positive test.

3.5.3.10. Mannitol

50µL of culture was inoculated into well no.10 of the kit and incubated for 48 hours at room temperature. After incubation, the colour changes from pinkish red/red to yellow for a positive test.

3.5.3.11. Rhamnose

50µL of culture was inoculated into well no.11 of the kit and incubated for 48 hours at room temperature. After incubation, the colour changes from pinkish red/red to yellow for a positive test.

3.5.3.12. Sucrose

50µL of culture was inoculated into well no.8 of the kit and incubated for 48 hours at room temperature. After incubation, the colour changes from pinkish red/red to yellow for a positive test.

3.5.4. Carbohydrate utilization test

Carbohydrate utilization test was performed using KB009 kit from Himedia. A combination of 35 tests for utilization of carbohydrate tests. Kit contains Part A, Part B each having12 carbohydrates utilization tests and Part C containing 11 sugars and 1 control.

3.5.4.1. Lactose

50µL fresh culture was inoculated into well no.1 of Part A kit and incubated for 48 hours. After incubation, the colour change was observed from pinkish red/red to yellow for a positive test and red/pink for negative test.

3.5.4.2. Xylose

50µL fresh culture was inoculated into well no.2 of Part A kit and incubated for 48 hours. After incubation, the colour change was observed from pinkish red/red to yellow for a positive test and red/pink for negative test.

3.5.4.3. Maltose

50µL fresh culture was inoculated into well no.3 of Part A kit and incubated for 48 hours. After incubation, the colour change was observed from pinkish red/red to yellow for a positive test and red/pink for negative test.

3.5.4.4. Fructose

50µL fresh culture was inoculated into well no.4 of Part A kit and incubated for 48 hours. After incubation, the colour change was observed from pinkish red/red to yellow for a positive test and red/pink for negative test.

3.5.4.5. Dextrose

50µL fresh culture was inoculated into well no.5 of Part A kit and incubated for 48 hours. After incubation, the colour change was observed from pinkish red/red to yellow for a positive test and red/pink for negative test.

3.5.4.6. Galactose

50µL fresh culture was inoculated into well no.6 of Part A kit and incubated for 48 hours. After incubation, the colour change was observed from pinkish red/red to yellow for a positive test and red/pink for negative test.

3.5.4.7. Raffinose

50µL fresh culture was inoculated into well no.7 of Part A kit and incubated for 48 hours. After incubation, the colour change was observed from pinkish red/red to yellow for a positive test and red/pink for negative test.

3.5.4.8. Trehalose

50µL fresh culture was inoculated into well no.8 of Part A kit and incubated for 48 hours. After incubation, the colour change was observed from pinkish red/red to yellow for a positive test and red/pink for negative test.

3.5.4.9. Melibiose

50µL fresh culture was inoculated into well no.9 of Part A kit and incubated for 48 hours. After incubation, the colour change was observed from pinkish red/red to yellow for a positive test and red/pink for negative test.

3.5.4.10. Sucrose

50µL fresh culture was inoculated into well no.10 of Part A kit and incubated for 48 hours. After incubation, the colour change was observed from pinkish red/red to yellow for a positive test and red/pink for negative test.

3.5.4.11. L- Arabinose

50µL fresh culture was inoculated into well no.11 of Part A kit and incubated for 48 hours. After incubation, the colour change was observed from pinkish red/red to yellow for a positive test and red/pink for negative test.

3.5.4.12. Mannose

50µL fresh culture was inoculated into well no.12 of Part A kit and incubated for 48 hours. After incubation, the colour change was observed from pinkish red/red to yellow for a positive test and red/pink for negative test.

3.5.4.13. Inulin

50µL fresh culture was inoculated into well no.1 of Part B kit and incubated for 48 hours. After incubation, the colour change was observed from pinkish red/red to yellow for a positive test and red/pink for negative test.

3.5.4.14. Sodium gluconate

50µL fresh culture was inoculated into well no.2 of Part B kit and incubated for 48 hours. After incubation, the colour change was observed from pinkish red/red to yellow for a positive test and red/pink for negative test.

3.5.4.15. Glycerol

50µL fresh culture was inoculated into well no.3 of Part B kit and incubated for 48 hours. After incubation, the colour change was observed from pinkish red/red to yellow for a positive test and red/pink for negative test.

3.5.4.16. Salicin

50µL fresh culture was inoculated into well no.4 of Part B kit and incubated for 48 hours. After incubation, the colour change was observed from pinkish red/red to yellow for a positive test and red/pink for negative test.

3.5.4.17. Dulcitol

50µL fresh culture was inoculated into well no.5 of Part B kit and incubated for 48 hours. After incubation, the colour change was observed from pinkish red/red to yellow for a positive test and red/pink for negative test.

3.5.4.18. Inositol

50µL fresh culture was inoculated into well no.6 of Part B kit and incubated for 48 hours. After incubation, the colour change was observed from pinkish red/red to yellow for a positive test and red/pink for negative test.

3.5.4.19. Sorbitol

50µL fresh culture was inoculated into well no.7 of Part B kit and incubated for 48 hours. After incubation, the colour change was observed from pinkish red/red to yellow for a positive test and red/pink for negative test.

3.5.4.20. Mannitol

50µL fresh culture was inoculated into well no.8 of Part B kit and incubated for 48 hours. After incubation, the colour change was observed from pinkish red/red to yellow for a positive test and red/pink for negative test.

3.5.4.21. Adonitol

50µL fresh culture was inoculated into well no.9 of Part B kit and incubated for 48 hours. After incubation, the colour change was observed from pinkish red/red to yellow for a positive test and red/pink for negative test.

3.5.4.22. Arabitol

50µL fresh culture was inoculated into well no.10 of Part B kit and incubated for 48 hours. After incubation, the colour change was observed from pinkish red/red to yellow for a positive test and red/pink for negative test.

3.5.4.23. Erythritol

50µL fresh culture was inoculated into well no.11 of Part B kit and incubated for 48 hours. After incubation, the colour change was observed from pinkish red/red to yellow for a positive test and red/pink for negative test.

3.5.4.24. Alpha-Methyl-D-glucoside

50µL fresh culture was inoculated into well no.12 of Part B kit and incubated for 48 hours. After incubation, the colour change was observed from pinkish red/red to yellow for a positive test and red/pink for negative test.

3.5.4.25. Rhamnose

50µL fresh culture was inoculated into well no.1 of Part C kit and incubated for 48 hours. After incubation, the colour change was observed from pinkish red/red to yellow for a positive test and red/pink for negative test.

3.5.4.26. Cellobiose

50µL fresh culture was inoculated into well no.2 of Part C kit and incubated for 48 hours. After incubation, the colour change was observed from pinkish red/red to yellow for a positive test and red/pink for negative test.

3.5.4.27. Melezitose

50µL fresh culture was inoculated into well no.3 of Part C kit and incubated for 48 hours. After incubation, the colour change was observed from pinkish red/red to yellow for a positive test and red/pink for negative test.

3.5.4.28. Alpha-Methyl-D-Mannoside

50µL fresh culture was inoculated into well no.4 of Part C kit and incubated for 48 hours. After incubation, the colour change was observed from pinkish red/red to yellow for a positive test and red/pink for negative test.

3.5.4.29. Xylitol

50µL fresh culture was inoculated into well no.5 of Part C kit and incubated for 48 hours. After incubation, the colour change was observed from pinkish red/red to yellow for a positive test and red/pink for negative test.

3.5.4.30. ONPG

50µL fresh culture was inoculated into well no.6 of Part C kit and incubated for 48 hours. After incubation, the colour change was observed from colorless to yellow for a positive test and colourless for negative test.

3.5.4.31. Esculin

50µL fresh culture was inoculated into well no.7 of Part C kit and incubated for 48 hours. After incubation, the colour change was observed from cream to black for a positive test and cream for negative test.

3.5.4.32. Citrate

50µL fresh culture was inoculated into well no.8 of Part C kit and incubated for 48 hours. After incubation, the colour change was observed from green to blue for a positive test and green for negative test.

3.5.4.33. Malonate

50µL fresh culture was inoculated into well no.9 of Part C kit and incubated for 48 hours. After incubation, the colour change was observed from light green to blue for a positive test and light green for negative test.

3.5.4.34. Sorbose

50µL fresh culture was inoculated into well no.10 of Part C kit and incubated for 48 hours. After incubation, the colour change was observed from pinkish red/red to yellow for a positive test and red/pink for negative test.

3.5.5. Growth Profile

A conical flask containing MM Broth supplemented with/without 10µL of Naphthalene (100mg/L) was prepared aseptically. An inoculum of 5% (v/v) was inoculated to this flask in aseptic conditions. Readings were recorded on 0 d. The flask was incubated at room temperature at 160 rpm. Readings were further recorded from day one till constant reading was observed. The absorbance was examined using a UV-visible spectrophotometer (Toshvin Analytical pvt ltd) at 600nm (Molina et al.,2009).

3.6. Effect of Physicochemical parameters on PAH degradation by bacteria

3.6.1. Effect of pH on GUSSF

For this MM broth was made as mentioned earlier and the desirable varying pH was obtained using pH probe at pH 5, 6, 7, 8, and 9 values, respectively, using 1N HCl and 1N

NaOH. The flasks were autoclaved, cooled and 100 mg/L naphthalene was added to the same aseptically. An inoculum of 5% (v/v) was inoculated to the flask in aseptic conditions. Readings were recorded at 0 d. The flasks were incubated at room temperature at 160 rpm. Using a glass cuvette, the absorbance was observed in a UV-visible spectrophotometer at 600nm and the readings were documented.

3.6.2. Effect of temperature on GUSSF

After preparation, MM Broth was autoclaved. Naphthalene was introduced in an aseptic condition at a concentration of 100 mg/L, inoculum of 5% (v/v) was inoculated to the flask with a control in aseptic conditions. Day 0 readings were recorded. The flasks were incubated at desirable temperatures at 12ºC, 30ºC, 37ºC and 42ºC respectively. Using glass cuvette, the absorbance was observed in a UV-visible spectrophotometer at 600nm and readings were recorded on daily basis.

3.7. Morphological and enzyme analysis of PAH degraders

3.7.1. SEM Analysis

To ensure that the purified culture grew effectively, 5%(v/v) of inoculum was inoculated into MM broth with 100 mg/L naphthalene. The culture broth was centrifuged at 10,000 rpm for 8 minutes following incubation of 4 days. The supernatant was discarded and the resulting pellet was washed twice with 1X PBS. The pellet was suspended in PBS and using a sterile nichrome loop, a drop of the pellet suspension was placed on a clean grease free glass slide. The slide was air-dried. The slide was fixed using 2.5% Glutaraldehyde

overnight. The solution was drained off, and the slide was immersed in a PBS buffer. The slide was then exposed to ethanol series using 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 100% ethanol. This was then followed by air drying and further processing for SEM including sputter coating the slide and mounting on gold stubs.

3.7.2. Manganese Peroxidase Assay

To ensure that the purified culture grew effectively, 5% (v/v) of inoculum was inoculated into MM broth containing 100 mg/L naphthalene. After 48 h of incubation, NA medium containing casein and methyl red was added for use as an indicator. pH was maintained by pH probe using 1N HCl or 1N NaOH, the plates were streaked aseptically and incubated at 37ºC for 72 h (Kumari et al.,2022).

3.7.3. Lipase assay

Simmon's Citrate agar plates amended with 0.01g methylene blue, were autoclaved and supplemented with 100mg/L Naphthalene. The plate was streaked aseptically and incubated for 3 days at room temperature. The plate is examined for production of lipase enzyme, which degrades methylene blue. A clearance zone indicates positive lipase activity (Kumari et al.,2022).

3.8. Estimation of PAH Biodegradation

3.8.1. Biodegradation assay

To ensure that the purified culture grew effectively, 5% (v/v) of inoculum was inoculated into MM broth containing 100 mg/L naphthalene. Readings were documented at 2-day

intervals, and the degradation rate was estimated. The biodegradable efficacy was estimated using the formula given by Kumari et al. (2022) as

$$\% \text{ of degradation} = \frac{\text{Absorbance of test} - \text{Absorbance of control}}{\text{Absorbance of test}} \times 100$$

Where *Absorbance of test*= flask containing culture media with Naphthalene

Absorbance of control= Flask containing Naphthalene without incubated culture

Chapter 4:Analysis and conclusions

4.2. Analysis of physicochemical parameters of water sample

4.2.1. Measurement of temperature of water sample

Table 1: Measurement of temperature of water sample.

Sample	Temperature	
Colourless	31ºC	

A glass thermometer was used to record the temperature of the water sample (Lat 15.504639 long 73.834740. This analysis was carried out during the time of collection of the water sample to avoid errors in the result. The sample temperature was found to be 31ºC **(Table 1**), which favored the growth of bacteria. The appropriate temperature provides information on the conditions required for the survival and proliferation of bacterial species. A higher temperature provides a more suitable environment and amplifies the growth rate of bacteria as an increase in temperature increases enzyme activity that causes cells to grow faster (Bamford et al.,2009).

4.2.2. Determination of pH of water sample

The water sample had a pH of 7.75, which is nearly neutral **(Fig. 5b).** The pH scale has a reading ranging from 0 to 14, wherein 0 represents an extremely acidic environment and 14 represent an extremely alkaline environment. Considering pH 7 to be neutral (Smith metal., 1961).

4.2.3. Determination of Salinity of water sample

The salinity of the sample was observed to be 2.9% (**Fig. 5c)**. Refractometer is now the most frequently used instrument for estuarine salinity determination (Possetti et al.,2009).

4.2.4. Analysis of colour and odour of the sample

No coloration was observed in the water sample. No traceable odour was traced.

4.3. Enrichment of culture medium

On enrichment, the flask supplemented with Naphthalene (100 mg/L) incubated at room temperature, 160rpm for 7d showed an absorbance reading of 0.359 at 600 nm using UV-visible spectrophotometer **(Fig.3c)**. The flask kept as a control failed to show any turbidity on analysis at same wavelength (Wu et al.,2013; Dhar et al. 2022).

4.4. Serial dilution

4.4.2. Spread plating

Colonies were obtained from dilutions 10^{-2} to 10^{-6} that were spread plated on MM agar plates and incubated at 37ºC **(Fig. 4)**. Distinct colonies were observed in 10^{-5} and 10^{-6} dilution **(Fig.4 d, e)**.

4.4.3. Isolation of Morphologically distinct colonies

Morphologically distinct colonies, as mentioned above, were picked up for further purification and processed for further use.

4.4.4. Colony counting

Table 2: Viable count on Naphthalene amended growth medium

Hydrocarbon	Average colony count (cfu/ml)
Naphthalene	362×10^6 cfu/mL

Average colony count was 362×10^6 cfu/ml **(Table 2).** According to Garcia-Armesto et al.,1993, colony-counting methods, such as plate count, are conventional methods of estimating microbial populations. They are, by definition, not rapid because of incubation periods necessary for the growth of colony-forming units to colonies and also because they are time-consuming in terms of sample preparation as well as labor intensive. Over the last few years, there have been many innovations in this method to address saving time, effort, and materials. Kästner et al. (1998) reveal that the breakdown of phenanthrene and anthracene by the indigenous microflora was completely suppressed. Only minor degradation was seen in sterilized soil. However, 10^8cfu/g viable BP 9 pyrene-degrading colonies were identified after 5 days, with 10^6cfu/g remaining after 35 days in the native soil. Strain BP 9 had a faster fall in CFU in sterilized soil, with 3×10^4cfu/g after 5 days, indicating improved survivability in native soil. Similar findings were achieved with the anthracene-degrading bacteria BA 2.

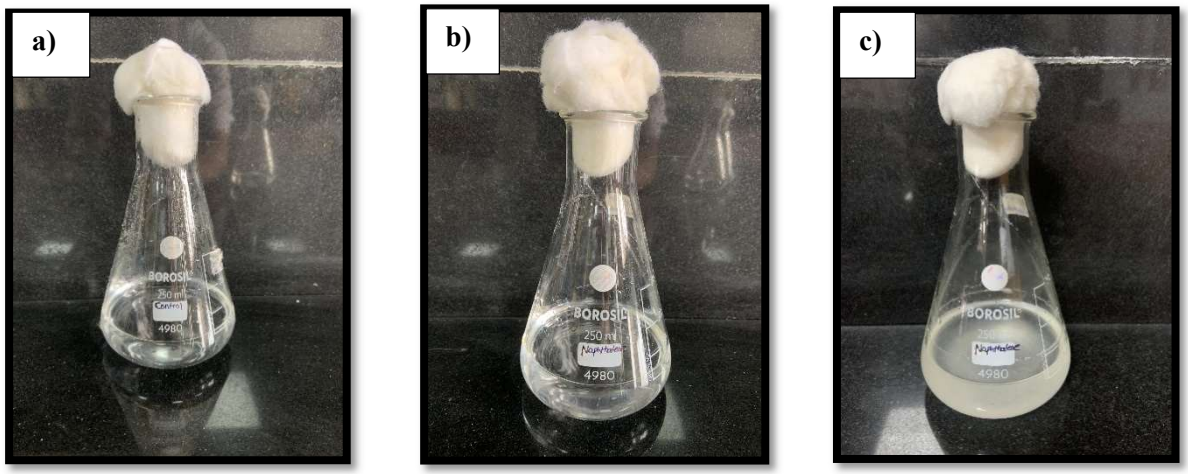

Fig 3. a) control flask before enrichment. B) Flask with sample and Naphthalene before enrichment C) Flask after 7 d enrichment showing turbidity

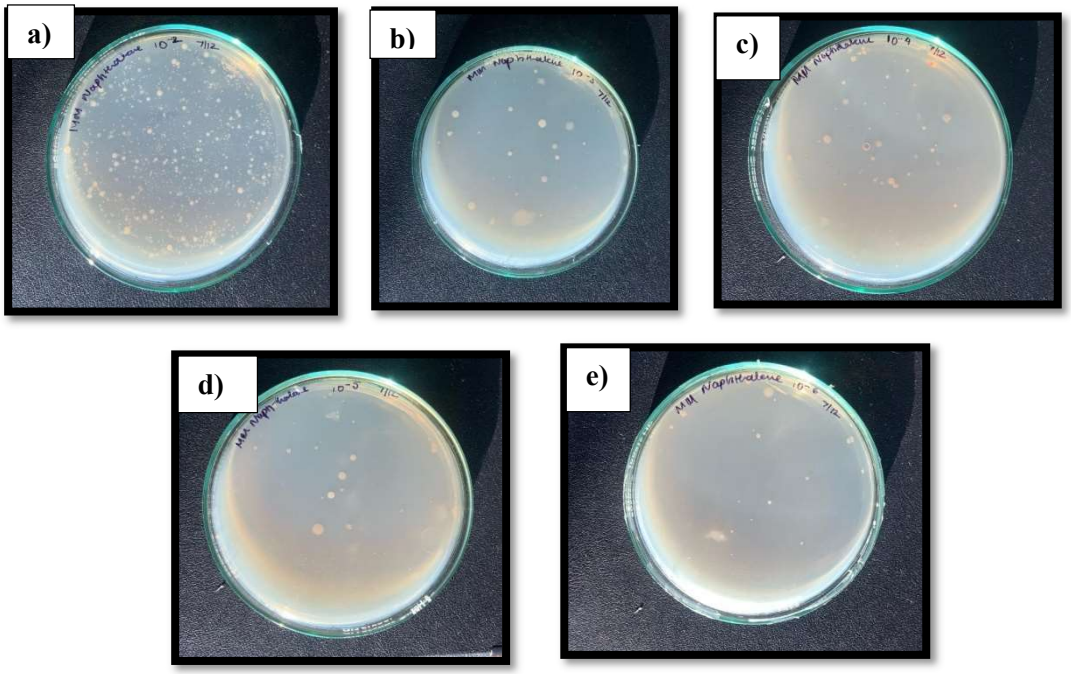

Fig 4. Serially diluted water sample (a) 10^{-2} (b) 10^{-3} (c) 10^{-4} (d) 10^{-5} e) 10^{-6} dilutions spread plated on MM plates

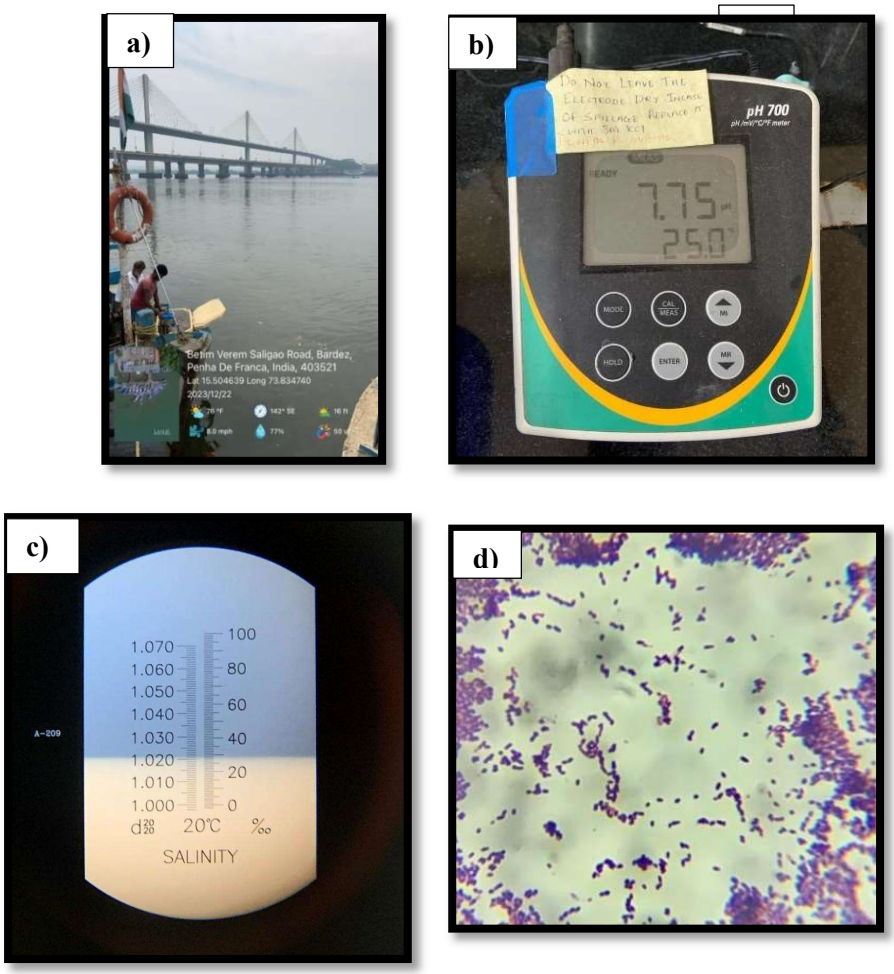

Fig 5. a) Sample collection site b) The pH of the water sample collected c) Salinity of the water sample d) Gram staining image showing Gram positive cocci.

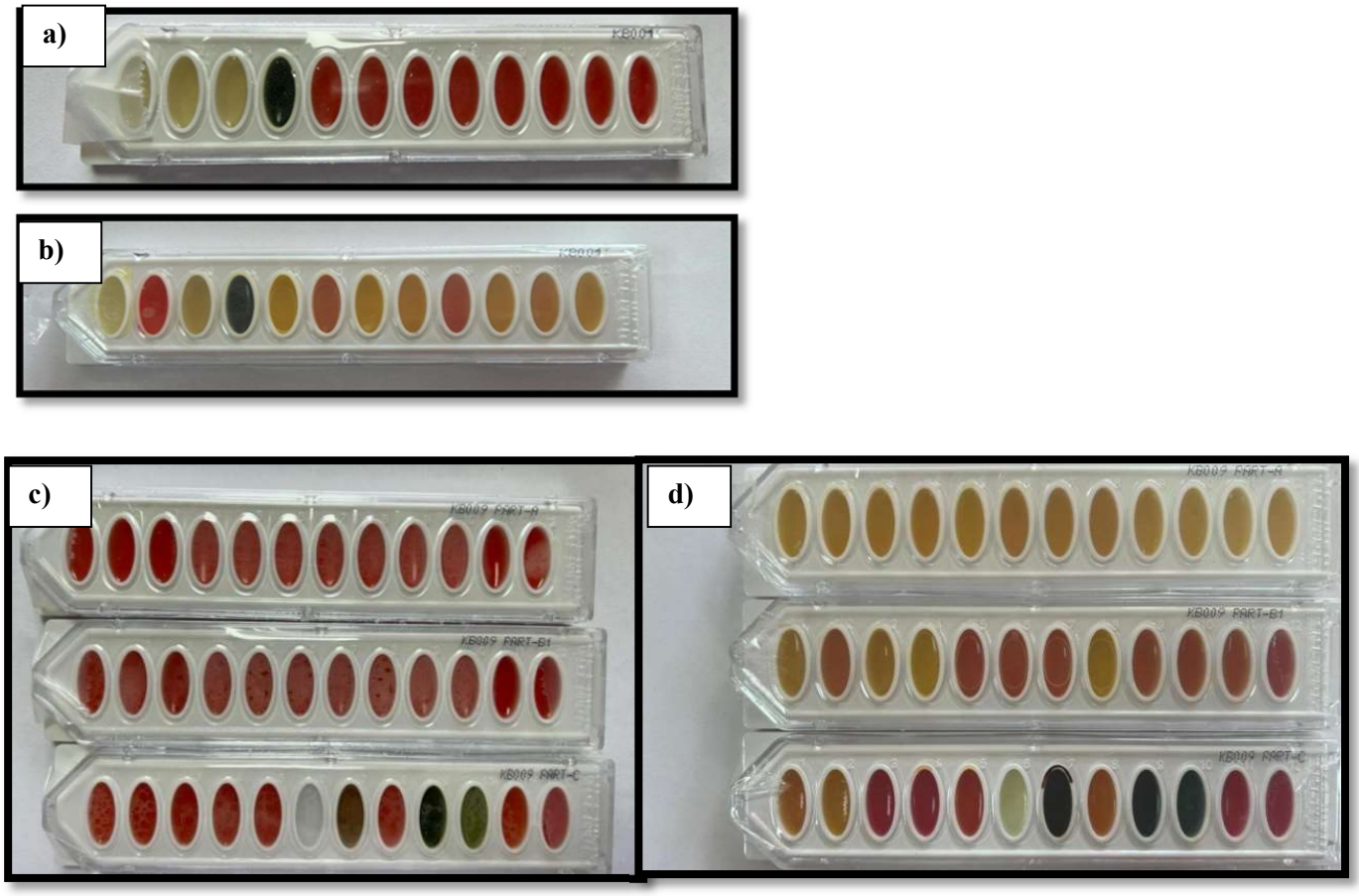

Fig 6. Biochemical characterization studies using Himedia kit: IMViC a) Control b) Test; Carbohydrate utilization c) Control d) Test

4.5. Characterization of bacterial Isolates

4.5.1. Colony characterization

The colony characteristics of bacterial isolates are tabulated as below

Table 3: Colony characteristics of bacterial isolate GUSSF

Characters of the bacteria	**Bacterial Isolate**
Form: Punctiform/round/wriled/concentric/	Round

Irregular/filamentous/rhizoid/spindle	
Size of isolated colony	0.2cm
Colour	Cream white
Diffusing pigments: Yes/No	No
Elevation: Flat/raised/convex/pulvinate/ Umbonate/hilly/crateriform	Convex
Margin: Entire/undulate/lobate/erose/ Filamentous/wavy/irregular	Entire
Surface: Smooth/shiny (glistening)/dull/dry	Smooth+ shiny
Opacity: Opaque/translucent/transparent	Opaque
Texture: Buttery/gummy/dry	Buttery

4.5.2. Gram Staining

Gram staining was performed successfully and viewed under a microscope. It was observed that bacterial GUSSF was Gram-positive having coccoid morphology (**Fig 5, d)**

4.5.3. Biochemical characterization study

The IMViC Test was performed and the results obtained are tabulated in **Table 4**

Table 4: IMViC Test of kit KB001

Sr. No	Test	Control	Result
1	Indole	-	-
2	MR	-	+

3	VP	-	-
4	Citrate	-	-
5	Glucose	-	+
6	Adonitol	-	-
7	Arabinose	-	+
8	Lactose	-	+
9	Sorbitol	-	-
10	Mannitol	-	+
11	Rhamnose	-	+
12	Sucrose	-	+

(+) Positive (-) Negative

It was observed that the culture was positive for the MR test that determines the ability of bacteria to perform mixed acid fermentation of glucose, producing stable acids such as lactic, acetic, and formic acid. A positive MR test was indicated by a red colour after the addition of Methyl red reagent. It indicates that the bacterial culture is capable of utilizing Glucose, Arabinose, Lactose, Mannitol, Rhamnose and Sucrose. A positive result for the mentioned sugars suggests metabolic versatility in the bacteria **(Fig 6 a, b).**

4.5.4. Carbohydrate utilization test

Table 5: Carbohydrate utilization test part A of kit KB009

Sr. No	**Test**	**Control**	**Result**
1	Lactose	-	+
2	Xylose	-	+

3	Maltose	-	+
4	Fructose	-	+
5	Dextrose	-	+
6	Galactose	-	+
7	Raffinose	-	+
8	Trehalose	-	+
9	Melibiose	-	+
10	Sucrose	-	+
11	Arabinose	-	+
12	Mannose	-	+

(+) Positive (-) Negative

Table 5 suggests, the sugars present in part A kit are all positive assessing the metabolic activity of the bacterial culture in the media containing different sugars.

Table 6: Carbohydrate utilization test part B of kit KB009

Sr. No	**Test**	**Control**	**Result**
1	Inulin	-	+
2	Sodium gluconate	-	-
3	Glycerol	-	+
4	Salicin	-	+
5	Dulcitol	-	-
6	Inositol	-	-
7	Sorbitol	-	-

8	Mannitol	-	+
9	Adonitol	-	-
10	Arabitol	-	-
11	Erythritol	-	-
12	Alpha-Methyl-D-glucoside	-	-

(+) Positive (-) Negative

As mentioned in **Table 6**, we can conclude that sugars alcohols such as Inulin, Glycerol, Salicin, and Mannitol show positive tests indicating the presence of growth and metabolic activity and ability to utilize sugars. Whereas sugar alcohols like Sodium gluconate, Dulcitol, Inositol, Sorbitol, Adonitol, Arabitol, Erythritol, and Alpha-Methyl-D-glucoside show negative test without metabolic activity and the ability to grow due to which there is no color change observed.

Table 7: Carbohydrate utilization test part C of kit KB009

Sr. No	**Test**	**Control**	**Result**
1	Rhamnose	-	+
2	Cellubiose	-	+
3	Melezitose	-	-
4	Alpha-Methyl-D-Mannoside	-	-
5	Xylitol	-	-
6	ONPG	-	-
7	Esculin	-	-

8	Citrate	-	-
9	Malonate	-	-
10	Sorbose	-	-
11	Control	-	-

(+) Positive (-) Negative

As given in **Table 7** it depicts that only Rhamnose and Cellubiose indicates positive test that are able to show metabolic activity while the rest sugars in part C shows negative tests.

The growth and metabolic activity were observed visually, indicated by a colour change in sugar-containing test media and appropriate positive and negative controls. Positive tests demonstrated known sugar utilized by the bacteria of interest. While a negative test indicated no growth and metabolic activity exhibited by no colour change or less colour change. From the results obtained by biochemical tests **(Table 4)**, Sugar utilization test: part A **(Table 5**), part B **(Table 6)** and part C **(Table 7)** it can be concluded that the organism is tentatively identified to be *Staphylococcus* sp

(Fig. 6c, d)

4.5.5. Growth Profile

Growth profile of isolate GUSSF was studied in media supplemented with Naphthalene (100mg/L) and without naphthalene **(fig. 10).** On day zero and on day two the cell density was 0.213 and 0.364, respectively. On day 4, the cell density was 0.523. Due to the bacteria's ability to break down PAHs and use the breakdown product, there was a sharp rise in cell density from day four till day 12. The culture attained an absorbance of 1.198 on day 14. This was followed by a decrease in absorbance on day sixteen exhibiting a reading of 1.206 A_{600nm}.

4.6. Effect of physicochemical parameters on PAH degradation by bacteria

4.6.1. Effect of pH on GUSSF

The rate and degree of utilization of naphthalene by GUSSF was relatively slow when naphthalene was added to the flasks as the only source of carbon **(Fig. 12)**. The utilization of naphthalene as the sole carbon source by the test culture GUSSF was indicated by the change in turbidity that was not observed in control flask kept without culture GUSSF. By changing the pH of the media from 5 to 9, there was very little to no effect observed for pH 6 and pH 7. Nevertheless, the impact was more pronounced, and the utilization of naphthalene was decreased at pH values of 5, 8, and 9. Consequently, any pH variation above pH 8 may potentially slow down the utilization of GUSSF culture. According to Kumari et al. (2022) study it stated that the LOP-9 strain showed maximum growth on pH 9, and the OD obtained was 0.254 at 600nm by spectrophotometer. The strains GWP-2 and LOP-9 showed the highest degradation efficacy of pyrene. Also stated that the Optimum pH range is 7-8 for biodegradation. As compared to LOP-2, GWP-2 showed the highest degradation rate at pH 9. As compared to other reported studies, the maximum degradation rate was observed at pH 7.

4.6.2. Effect of temperature on GUSSF

Temperature influences the chemical, physical and content of media and metabolic processes involved in PAH breakdown. Temperature is a key component in biodegradation and microbial development because microbial enzymes perform optimally at optimal temperatures that are relevant to their natural habitat. Lower and higher temperatures are not favorable for PAH decomposition (Kumari et al.,2022). **Fig. 11** illustrates the influence of temperature (12ºC, 30ºC, 37ºC, and 42ºC) at pH 7. At 12ºC, the bacterial strain GUSSF exhibited minimal growth due to a

reduction of chemical reaction rate, while at 30º C and 37º C, it showed optimal growth as it favored the conditions. 42ºC could be a stressful temperature affecting the growth. According to Kumari et al. (2022) different species have varying levels of sensitivity to temperature influences. Temperatures below 25ºC were insufficient for the growth of bacteria and naphthalene breakdown, while temperatures above 25ºC were optimal. In a similar study with *Corynebacterium roalyticum*, pyrene was utilized at 30ºC with pyrene. This also corroborates with our study wherein the optimal temperature for PAH degradation is between 30º and 40ºC.

4.7. Morphological and Enzyme analysis of PAH degraders

4.7.1. SEM analysis

According to BERGEY'S MANUAL of Systematic Bacteriology culture GUSSF is tentatively identified as *Staphylococcus* sp. SEM micrographs reveal the surface characteristics and morphology. According to Boudjemaa et al. (2019), *Staphylococcus* sp. has a regular and smooth surface, hence also known as a bald cell **(Fig. 7c)**. It appears as round or ovoid cocci grouped in irregular clusters. This is suggestive of morphological alterations of culture GUSSF when exposed to Naphthalene **(Fig.7a, b).** The aggregation of *Staphylococcus* sp. indicates that aggregation is possible when degradation occurs. According to Naik et al. (2011) *Pseudomonas aeruginosa* strain 4EA cell showed normal morphology when grown in the absence of lead while there was significant modifications observed in cell morphology when cells were exposed to 0.8 mM lead nitrate along with cell size shrinkage.

4.7.2. Manganese Peroxidase assay

The degradation of PAH by bacteria is reported to be via the activities of some enzymes such as oxygenases and peroxidases. These enzymes are known to initiate aerobic

metabolism via oxidation of the benzene ring by introducing two hydroxyl groups to aromatic compounds. This was substantiated by a manganese peroxidase assay that revealed a yellow hue formed on bacterial growth in methyl red agar media after three days of incubation **(Fig. 8b).** Culture GUSSF thus exhibits the ability to degrade naphthalene as peroxidases are known to catalyze oxidation-reduction reactions (Xu et al., 2021).

4.7.3. Lipase assay

Hydrolytic enzymes such as esterases and lipases are able to split the ester bond of recalcitrant pollutants like naphthalene to relieve their toxicity (Xu et al., 2021). This is also evident from the positive lipase activity indicated by a zone of clearance surrounding GUSSF **(Fig. 9b)**.

The change in colour observed during lipase enzyme activity was also reported by Kumari et al. (2022) in GWP-2 strain which showed less zone of clearance around the growth. Similarly, Hadibarata et al. (2009) also reported the presence of lipase in aromatic degrading bacterial species.

4.8. Estimation of PAH Biodegradation

4.8.1. Biodegradation assay of GUSSF

Staphylococcus sp. had the highest Naphthalene degrading capability (64%), with a bacterial growth rate of 0.678 at 600nm after 16 days at 37ºC and 160rpm. On day 14, the degradation rate was observed to be 50.6% with an absorbance of 0.552 under identical conditions. Similar studies with *Bacillus* sp. had a degradation capacity of 35% in 120 days

due to exposure to hydrocarbons, while in *Alcaligenes* and *Pseudomonas* sp., it was 58.4% degradation for pyrene in 7 days.

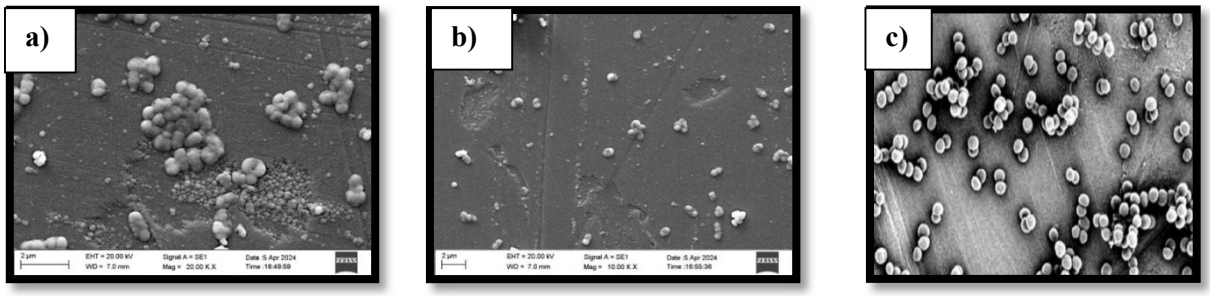

Fig 7. SEM micrographs of isolate GUSSF grown in naphthalene containing MM broth a)20,000 X and b) 10,000 X; c) Referred micrograph (Boudjemaa et al.,2019)

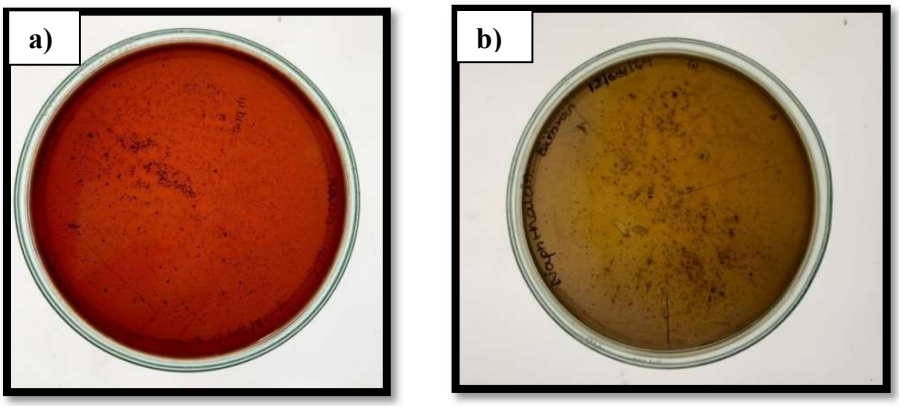

Fig 8. Manganese Peroxidase assay for GUSSF a) Control b) Test

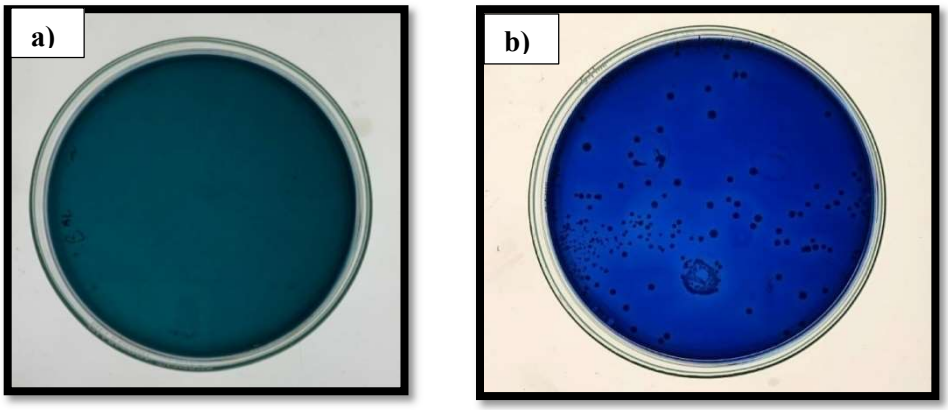

Fig 9. Lipase assay for GUSSF a) Control b) Test

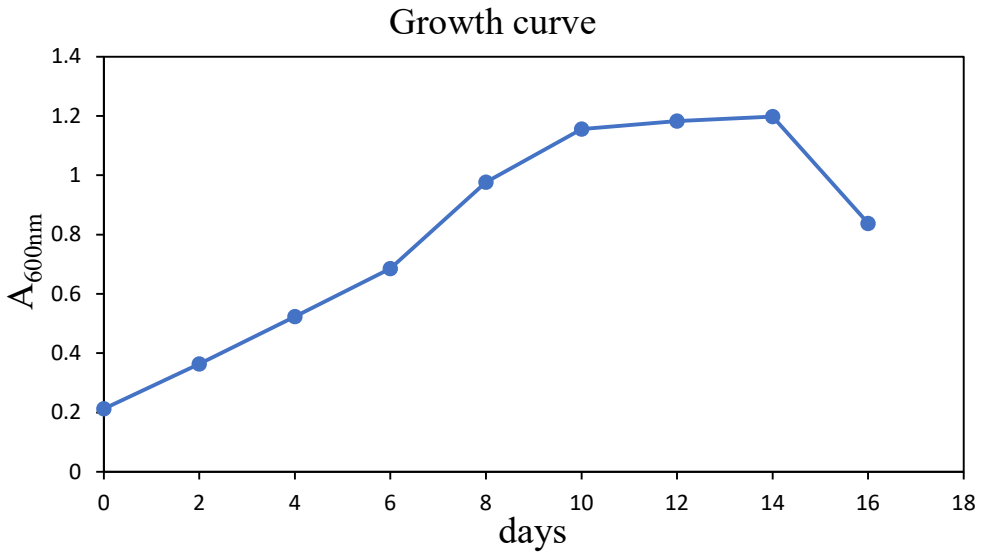

Fig 10. Growth profile of GUSSF culture in MM broth containing naphthalene (100mg/L).

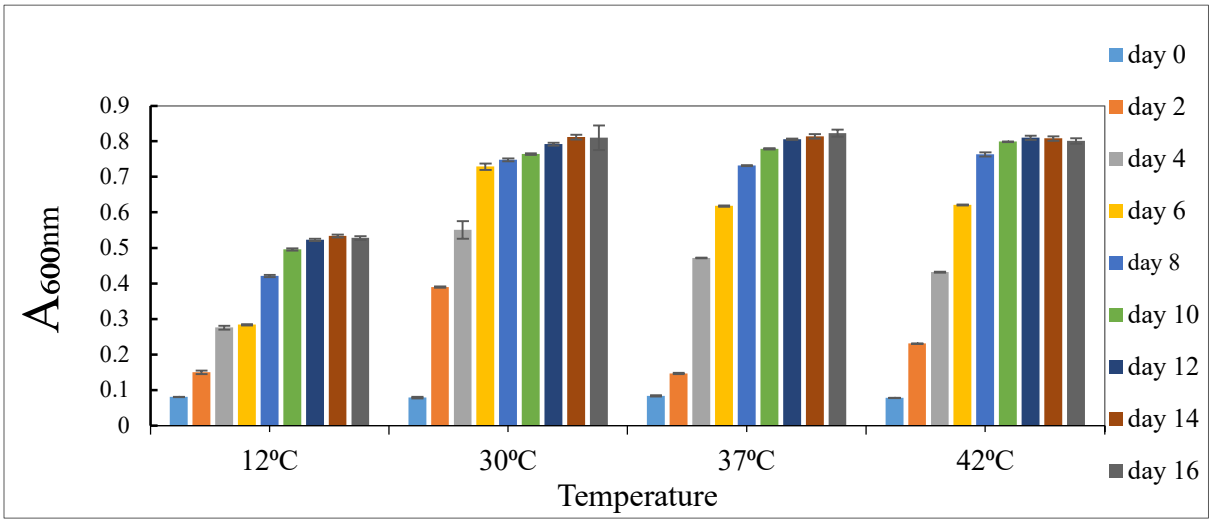

Fig 11. Effect of temperature on PAH degradation by GUSSF culture grown in MM broth containing naphthalene

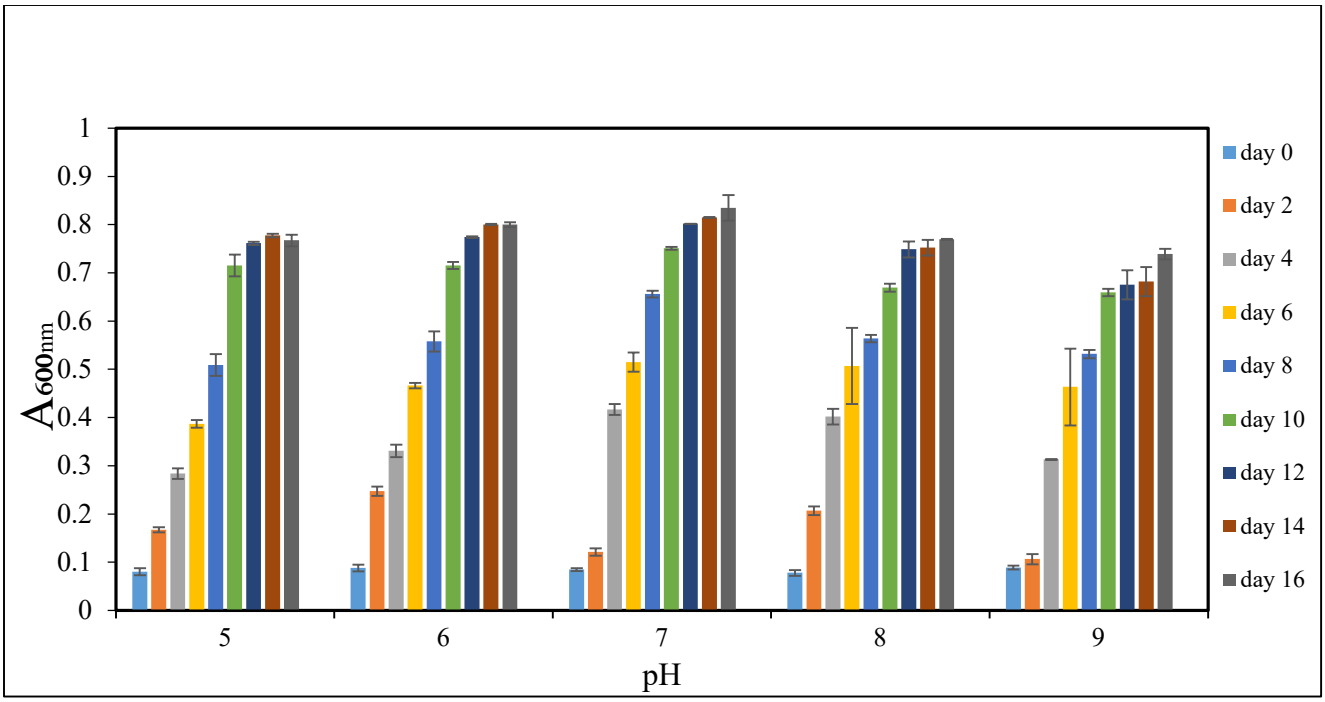

Fig 12. Effect of pH on PAH degradation by GUSSF culture grown in MM broth containing naphthalene

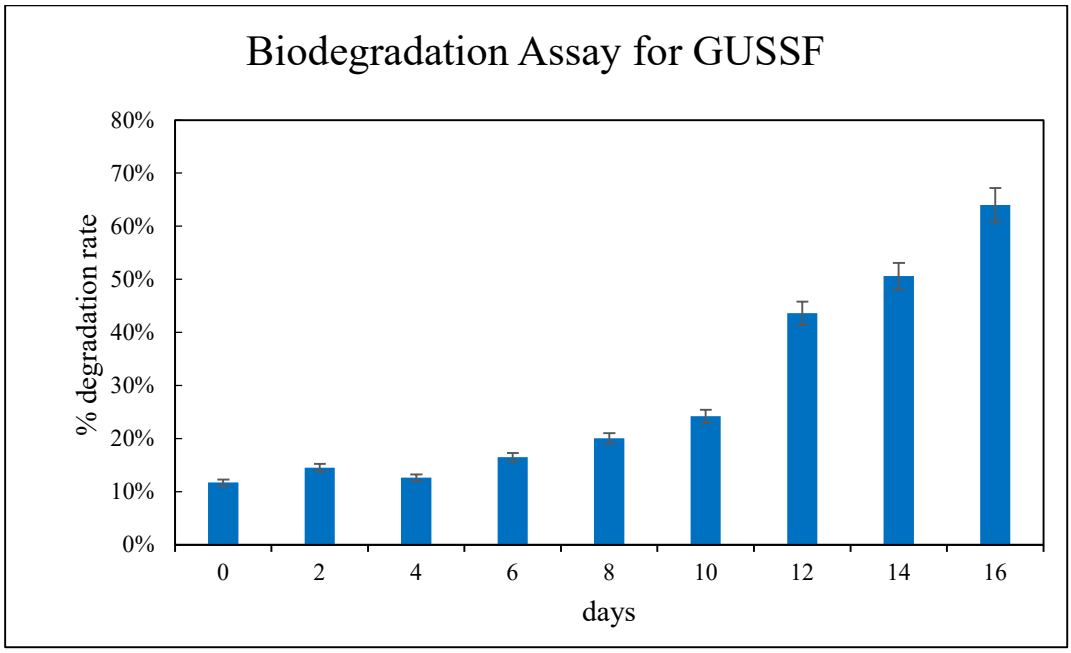

Fig 13. % Biodegradation of Naphthalene by GUSSF culture grown in MM broth containing naphthalene

This study mainly focuses on the isolation of polycyclic aromatic hydrocarbon-degrading bacteria from coastal wetlands. The water sample was collected from Betim, Goa. 362×10^6 cfu/mL colonies were obtained on MM media. The isolate obtained was studied for its Gram character and revealed to be a Gram-positive coccoid. The growth profile was studied followed by the optimization studies such as temperature and pH. The biodegradation ability was also assessed using a percentage biodegradation assay along with enzyme assays specific for lipase and manganese peroxidase activity, showing a positive reaction. Biochemical characterization revealed the culture to be *Staphylococcus sp*. The culture degraded naphthalene at optimal temperatures of 30℃ and 37℃ in a pH 7 environment with a degradation rate of 64% in 16 days. Thus, the culture has the potential to degrade polyaromatics like naphthalene in marine environments contaminated with such pollutants. This culture can be further explored for molecular studies of PAH degraders, along with the characterization of metabolites through HPLC and GC-MS. The isolate GUSSF can also be further used for biomass production, and study the degradation pathways.

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APPENDIX I

Preparation of media

- Minimal Broth Davis without Dextrose

Ingredients	g/L
Dipotassium phosphate	7.000
Monopotassium phosphate	2.000
Sodium citrate	0.500
Magnesium sulphate	0.100
Ammonium sulphate	1.000
Final pH (25°C)	7.2±0.2

- Nutrient agar

Ingredients	g/L
Peptone	5.000
Sodium chloride	5.000
HM peptone B#	1.500
Yeast extract	1.500
Agar	15.000
Final pH (at 25°C)	7.4±0.2

- Simmon's citrate agar

Ingredients	g/L
Ammonium dihydrogen phosphate	1.000
Magnesium sulphate	0.200
Dipotassium phosphate	1.000
Sodium citrate	2.000
Sodium chloride	5.000
Bromo thymol blue	0.080
Agar	15.000
Final pH (at 25°C)	6.8±0.1

APPENDIX II

Reagents preparation

1. **Naphthalene**

   0.1g of naphthalene powder in 1L of 90% ethanol

2. **Saline**

   0.85g of sodium chloride in 100mL of DW to attain 0.85%

3. **PBS**

Ingredients	**g/L**
NaCl	8.000
KCl	0.200
Na_2HPO_4	1.420
KH_2PO_4	0.240
pH	7.4

4. **Gram's staining**

(a) Gram's crystal violet

(b)Gram's iodine

(c) Gram's decolorizer

(d) safranin