ISOLATION AND BIOCHEMICAL CHARACTERIZATION OF HALOPHILES FROM NADHI PAALAM BRINE PIT AND EXAMINING ITS EFFECTIVE UTILIZATION OF PETROLEUM HYDROCARBONS

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ABSTRACT

Halophiles ("salt-loving") constitute the natural microbial communities of hypersaline ecosystems, which are widely distributed around the world. The halophiles are also gaining environmental importance because of their greater use in several bioremediation processes. Petroleum hydrocarbons (PHCs) are an essential constituent of fossil fuel energy resources and most important raw materials for a wide range of products such as fuel, synthetic polymers, and petrochemicals. Along with their increasing consumption, the contamination of marine environments with PHCs has emerged as a significant environmental concern. The primary cause of contamination includes oil spills, transportation of petroleum products by the sea, waste burnouts, industrial effluent discharges, and sediment drilling activities. Saline and hyper saline environments are linked to oil exploration and active petroleum seeps, resulting in an additional challenge for bioremediation. The growing environmental concerns related to petroleum contamination have prompted the exploration of alternative, eco-friendly remediation strategies. One such approach is the use of halophiles, microorganisms that thrive in saline environments, due to their unique metabolic capabilities. This study investigates the petroleum degradation potential of halophilic bacteria isolated from the Nadhi paalam Brine Pit, a hyper saline ecosystem in India. These halophiles were screened for their ability to degrade petroleum hydrocarbons, two of the most persistent pollutants in the environment. Preliminary results indicate that several strains exhibited significant degradation of petroleum products, with a notable reduction in hydrocarbon concentrations over a period of weeks.

Keywords: Halophiles, Brine Pit, bioremediation, petroleum degradation, Environmental pollution.

INTRODUCTION

Halophiles belong to a diverse group of extremophilic microorganisms including Archaea, bacteria, and eukaryote domains. The main characteristic is their salinity requirement, halophilic "salt-loving". Halophilic microorganisms constitute the natural microbial communities of hypersaline ecosystems, which are widely distributed around the world. They require sodium ions for their growth and metabolism. Thus, based on the NaCl optimal requirement for growth the halophiles are classified in three different categories: slight (1–3%); moderate (3–15%); and extreme (15–30%). In contrast to halotolerant organisms, obligate halophiles require NaCl concentrations higher than 3% NaCl or above of seawater, with about 3.5% NaCl. The tolerance parameters and salt requirements are dependent on temperature, pH, and growth medium (Paulina Corral et al., 2019). The halophilic organisms are found in all three domains of classification i.e., Bacteria, Archaea and Eukarya (Shivanand and Mugeraya, 2011).The presence of NaCl over the accepted limit is critical for any organism to survive as it causes high osmolarity in the environment. As a result the organism loses its water content to the surrounding environment. To prevent this, halophiles carry over several mechanisms to equate the osmolarity in the inside and outside environment (Sarma et al., 2012). The halophiles are also gaining environmental importance because of their greater use in several bioremediation processes (Manjula et al., 2018).

Petroleum hydrocarbons (PHCs) are an essential constituent of fossil fuel energy resources, and their consumption has witnessed a substantial surge in recent decades because of the worldwide growth in population and industrialization. In addition, they are by far the most important raw materials for a wide range of products such as fuel, synthetic polymers, and petrochemicals. Along with their increasing consumption, the contamination of marine environments with PHCs has emerged as a significant environmental concern. The primary cause of contamination includes oil spills, transportation of petroleum products by the sea, waste burnouts, industrial effluent discharges, and sediment drilling activities. Saline and hypersaline environments are linked to oil exploration and active petroleum seeps. These environments are considered the largest and ultimate sink for PHC pollution due to nearby human activities. In broader terms, saline and hypersaline environments are classified by NaCl concentrations exceeding 1% and seawater (3–3.5%), respectively, resulting in an additional challenge for bioremediation

(Khalil et al., 2021). Therefore, the development of technologies for the efficient remediation of such sites is needed. Several attempts have been made to treat hypersaline wastewater contaminated with PHCs. The base-catalyzed dechlorination, UV oxidation, coagulation, adsorption, filtration, and dispersants have been used. Although these technologies are available, existing research recognizes several drawbacks in their practical applications including costs (Chen et al., 2020). Similarly, thermal methods such as in-situ burning oblige a toxicity assessment because incomplete combustion may result in the production of carcinogenic compounds. According to, Corexit dispersants contain varying amounts of hazardous compounds, including organic sulfonic acid salt (10–30% w/w) and propylene glycol (15% w/w). Bioremediation using microorganisms is a natural, economical, and efficient way to convert PHCs into less toxic compounds relying on biological processes (Gomes et al., 2018).

Although bioremediation is very promising, one of the main obstacles in recent research concerns about its application under saline and hypersaline conditions, which can adversely affect numerous microorganisms employed. The slow rate of biodegradation of petroleum hydrocarbons can be attributed to the extreme environmental conditions damaging mainly the cell membrane, with the concomitant loss of intracellular water and enzyme denaturation (Pereira.et al., 2024).

METHODOLOGY

Collection of Samples

Sediment sample was collected in a sterile container from the brine pit located at Nadhi paalam area, Ramanathapuram District, Tamil Nadu, India. The collected samples were processed immediately.

Isolation of halophiles

Halophilic bacteria were isolated from the salt pans water sample using nutrient agar medium with 20% Salt (Yeast extract - 2g/L, Peptone - 5g/L, Beef extract - 1g/L, Sodium chloride – 25% , Agar - 20g/L with final pH (at 25°C) 7.2±0.2. Ten grams of sediment sample was suspended in 90 ml of sterile distilled water blank and shaken vigorously for 15 minutes. It was serially diluted up to 10^{-5}. Then 100 µl of sample was taken from the dilution 10^{-1} to 10^{-5}. The plates were incubated at 37 ± 2°C for 7 days. After incubation, pigmented colonies were selected and sub cultured.

Phenotypic characteristics of halophiles

The isolates were examined for colony and cell morphology. Colony morphology was focused on pigmentation, surface and shape. Further, the isolates were stained by differential staining, The biochemical tests like indole, Methyl red, Voges-Proskauer, Citrate utilization, Urease, Catalase, Oxidase, Starch and Caesin utilization were performed

Determination of Growth Curve of Halophiles

The growth kinetics of the four halophilic bacterial isolates were assessed by measuring optical density (OD) at 600 nm (OD_{600}) at regular time intervals. The isolates were cultured in Nutrient broth with 20% Salt. A single colony of each isolate was inoculated into 50ml of the growth medium and incubated overnight to reach the logarithmic phase. Growth was monitored by measuring OD_{600} at regular intervals (24 hours) using a spectrophotometer. A growth curve was plotted by plotting OD_{600} against time to determine the lag phase, exponential phase, stationary phase, and decline phase of each isolate. Experiments were conducted in triplicates, and the mean values were used for analysis.

Petroleum degradation by halophiles

The tolerances to petroleum oil concentration (5 %) along with its degrading efficiency by the selected isolates were assessed. Each experiment was performed in triplicates and the growth was determined by using UV-Visible spectroscopy at OD600 nm in every 24 h. The synergic effect of all parameters was analysed to identify the optimal conditions for petroleum hydrocarbon degradation (Shukor et al., 2009 & Parach et al., 2017).

RESULT AND DISCUSSION

Collection of Sample

Halophiles are organisms that can tolerate and survive under moderate to high saline conditions. The microorganisms survives under those condition produce several coloured pigments, which in turn has several biotechnological potentials. In this study, totally 2 sediments samples were collected from different parts of the brine pit located at Nadhi paalam area, Ramanathapuram District, Tamil Nadu, India .

Fig. 1: Collection of sediment samples from Nadhi paalam area, Ramanathapuram District, Tamil Nadu, India

Isolation of halophiles

Halophilic bacteria were isolated using halophilic agar medium prepared with distilled water. The plates were incubated and observed after seven days, it showed morphologically different colonies. The serially diluted sample yielded approximately 50 × 106 CFU/g. The morphology of the isolated colonies was distinctive. Most of them were white, yellow, creamy and pale yellow in color.

Fig. 2: Pure cultures of pigment producing halophilic bacteria

The shape of the colonies was circular, irregular and the surface of the colonies was smooth, mucoid in nature. Four different yellow colored colonies (HA 1, HA 2, HA 3, HA 4) were taken for further studies.

Phenotypic characteristics of halophiles

The sediment samples of salt pan showed circular colonies. Most of them were smooth, shiny, mucoid. The gram staining results showed many gram negative rods.

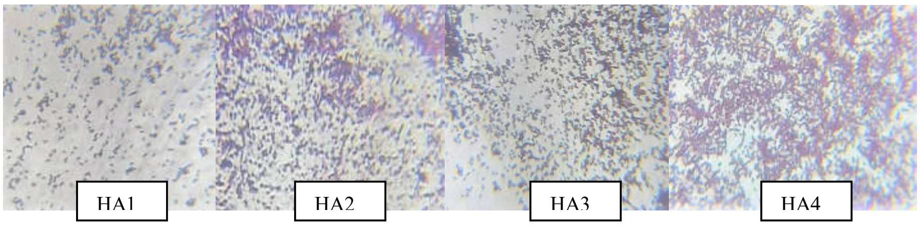

Fig. 3: Gram staining

Table 1: Morphology of isolated halophiles

Isolates	Colony color	Form	Surface	Gram stainig
H 1	Yellow	Circular	Slimy, Mucoid	Negative rods
H 2	Pale yellow	Circular	Slimy	Negative rods
H 3	Creamy yellow	Circular	Slimy	Negative rods
H 4	White	Circular	Mucoid	Negative rods

Biochemical characterization

The biochemical results showed in Table 2. The colony morphology, gram staining and biochemical results of the isolated halophilic bacteria were compared with bacterial identification key, Bergey's manual.

Table: 2 Biochemical results of pigmented halophilic bacteria.

Isolates	Indole	MR	VP	Citrate	Urease	Amylase	Catalase	Oxidase	Protease
HA 1	-	+	-	+	+	+	+	-	+
HA 2	-	+	-	+	+	+	+	-	+
HA 3	-	+	-	+	+	+	+	-	+
HA 4	-	+	-	+	+	+	+	-	+

Determination of Growth Curve of Halophiles

Among the four isolates HA4 demonstrated the highest growth rate on **Day 11**, indicating its superior adaptability to the given conditions. In contrast, HA3 and HA2 followed a similar trend but exhibited slightly lower OD_{600} values respectively. HA1 exhibited the slowest growth, indicating lower metabolic activity or adaptation potential under the experimental conditions.

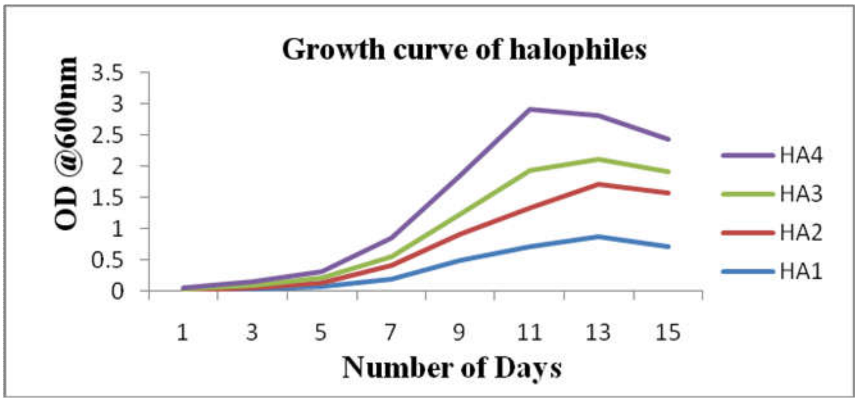

Fig. 4: Growth curve of halophiles

Petroleum degradation by halophiles

The results of this study demonstrate that among the four isolates tested, HA4 exhibited the highest efficiency in petroleum degradation**.** The degradation potential of HA4 was significantly greater than that of the other isolates, suggesting its superior capability in hydrocarbon breakdown.

The statistical analysis further confirmed the significant difference in degradation rates, with HA4 achieving [5%] degradation compared to other isolates.

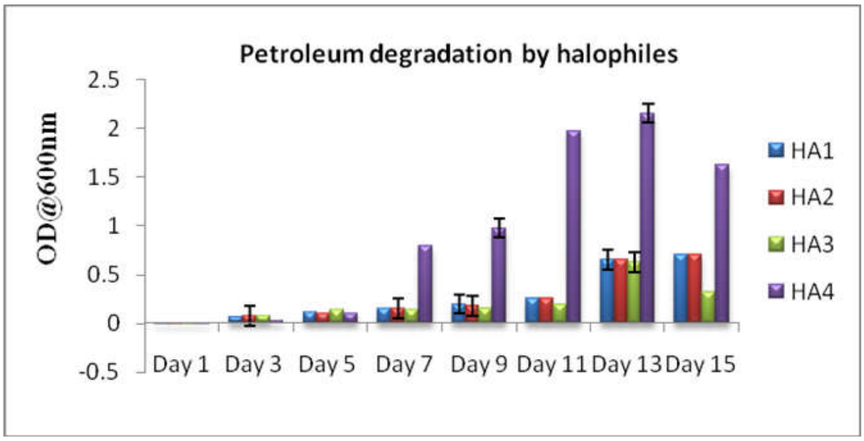

Fig. 5: Growth and petroleum degradation of halophiles

Discussion

Halophiles ("salt-loving") constitute the natural microbial communities of hypersaline ecosystems, which are widely distributed around the world which are classified in three different categories: slight (1–3%); moderate (3–15%); and extreme (15–30%). The halophiles are also gaining environmental importance because of their greater use in several bioremediation processes (Manjula et al., 2018).

Petroleum hydrocarbons (PHCs) are an essential constituent of fossil fuel energy resources in addition, they are by far the most important raw materials for a wide range of products such as fuel, synthetic polymers, and petrochemicals. Along with their increasing consumption, the contamination of marine environments with PHCs has emerged as a significant environmental concern. The primary cause of contamination includes oil spills, transportation of petroleum products by the sea, waste burnouts, industrial effluent discharges, and sediment drilling activities. Saline and hypersaline environments are linked to oil exploration and active petroleum seeps, resulting in an additional challenge for bioremediation (Khalil et al., 2021). Therefore, the development of technologies for the efficient remediation of such sites is needed. Several attempts have been made to treat hyper saline wastewater contaminated with PHCs. The base-catalyzed dechlorination, UV oxidation, coagulation, adsorption, filtration, and dispersants have been used. Although these technologies are available, existing research recognizes several drawbacks in their practical applications including costs (Chen et al., 2020). Similarly, thermal methods such as in-situ burning oblige a toxicity assessment because incomplete combustion may result in the production of carcinogenic compounds. Bioremediation using microorganisms is a natural, economical, and efficient way to convert PHCs into less toxic compounds relying on biological processes (Gomes et al., 2018). In this study halophiles showed a very good result in degrading petroleum oil. This research underscores the promising role of halophiles in the sustainable management of petroleum waste, contributing to the development of bioremediation technologies in saline environments. Further studies are needed to optimize these strains for large-scale application and to explore their molecular mechanisms of pollutant degradation.

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ETHICAL STATEMENT

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Ethical Approval: There is no ethical issue to do this Project.

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