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Petroleum Hydrocarbon Degradation and Treatment of Automobile Service Station Wastewater by Halophilic Consortia Under Saline Conditions

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ABSTRACT

The halophilic consortia were enriched from water samples of Abhor, Red Sea, Jeddah, Saudi Arabia for the degradation of phenanthrene, fluorene, hexadecane, pyrene, and treatment of automobile service station wastewater under saline conditions (4%). Complete degradation of phenanthrene and fluorene was recorded up to a concentration of 500 mg.L^{-1} in 12 days, when the concentration was raised to 800 mg.L^{-1}, the percentage of degradation of the two compounds was recorded by 84 and 90% within 14 days, while when the concentration increased to 1000 mg.L^{-1}, a significant decline was recorded. Pyrene degradation was studied under saline conditions, where it recorded a degradation rate of 92 and 81% at a concentration of 50 and 100 mg.L^{-1} in 10 and 12 days, respectively, while when increasing the concentration, a severe decrease in the percentage of degradation was recorded that reached 57 and 44% at concentration 200 and 300 mg.L^{-1}, respectively .Hexadecane recorded complete degradation at a concentration of 0.5 and 1%, within 4 and 6 days, respectively, while at a concentration of 1.5%, the rate of deterioration was 88% in 10 days. Record 93% removal of COD in CSTR within 40th day, when treatment of automobile service station wastewater with halophilic bacterial consortia. The existing bacterial strains were classified as potentially responsible for petroleum hydrocarbon degradation and treatment of automobile service station wastewater such as *Ochrobactrum*, *Propionispira*, *Martelella*, *Bacillus*, *Marinobacter*, and *Azospira*. The present study recommends that the hydrophilic consortia can be used in the treatment of automobile service station wastewater under saline conditions.

INTRODUCTION

Pollution by petroleum hydrocarbons is one of the most serious issues that occupy the world at the present time, as it is considered one of the most dangerous pollutants in the water and terrestrial environments (Isiodu et al. 2016, Logeshwaran et al. 2018, Imron et al. 2019, Khalid et al. 2021). During the past century, the production and transportation of the use of petroleum compounds witnessed great progress, as this led to more concerns towards both humans and the environment, as oil spills in the marine environment caused many devastating environmental problems (Ivshina et al. 2015, Pereira et al. 2019, Khalid et al. 2021).

Due to human activities in the environment, many toxic compounds and pollutants entered marine ecosystems, and this led to changes in the composition and life of these aquatic ecosystems (Chen et al. 2017). Pollution of the seas with petroleum compounds occurs through oil spills, naturally or artificially, as natural spills occur through oil spills through volcanic processes and natural leaks from reservoirs in the depths of the oceans, synthetic spills, including many processes, including spills that occur during oil extraction and transportation, oil loading operations, and transportation accidents. These operations are among the main problems of pollution of the seas and oceans with hydrocarbons. As well as oil spills from oil transportation pipelines, vehicles, spills from sites, and drilling and exploration operations, in addition to wrong practices for the disposal of petroleum waste (Simister et al. 2013, Hewelke et al. 2018, Xu et al. 2018).

The automobile service station is considered one of the basic components in the service sector industry, as the most important environmental impacts that result from these services are the leakage of motor oils into the soil, as well as water used in washing and other wastes resulting from these stations. This pollutant release leads to many problems in the soil such as loss of fertility, permeability, the ability of the soil to hold water, and other important biological properties of the soil.

At present, the interest of researchers and the world has become focused on finding renewable and environmentally friendly energy sources (Pereira et al. 2019). Crude oil is a complex compound (Xue et al. 2015), it contains thousands of different hydrocarbon compounds (Cheng et al. 2014) and due to the toxicity of these compounds for both humans and the environment. Bioremediation of hydrocarbon pollutants has been proposed as a promising solution to get rid of various environmental pollutants, in addition to the natural biodegradation operations that naturally occur in the polluted environment (Thapa et al. 2012, Ron & Rosenberg 2014).

Many previous studies and research have focused on the ability of single or combined bacterial strains, as these cannot biodegrade hydrocarbon pollutants in the presence of different concentrations of salt (Jamal & Pugazhendi 2018, 2021). The present study aims to determine the ability and efficiency to degrade the petroleum hydrocarbon compounds (Phenanthrene, Pyrene, Fluorene, and Hexadecane) by halophilic consortia, which was, enriched from the coast of Abhor, Jeddah, Saudi Arabia (SA) under 4% salty conditions.

The halophilic consortia were also used to treat the automobile service station wastewater under salty conditions and to define the existing bacterial strains that have the capability to degradation of hydrocarbons under these conditions.

MATERIALS AND METHODS

Sampling

Marine water samples were gathered from Abhor, Red Sea, Jeddah, SA. Samples were gathered in sterilized Schott glass bottles (250 ml) and transferred to the laboratory for further research experiments.

Chemicals

Fluorene, phenanthrene, pyrene, and hexadecane were purchased from Sigma Aldrich® with a purity of 98-99%, while all chemicals and reagents used were purchased from Himedia®.

Medium Composition

The medium used in this study was mineral salts medium (MSM), which was described by Liu et al. (1995), and this medium consists of the following mg/L: 435 K_2HPO_4, 85 NH_4Cl, 170 KH_2PO_4, 27.5 $CaCl_2$, 668 $Na_2HPO_4.7H_2O$, 0.25 $FeCl_3.6H_2O$, 22.5 $MgSO_4.7H_2O$, as it was modified by adding up to 40 g/L of NaCl, and then it was sterilized by autoclave (JSR, South Korea), at 121 °C for 15 min at 15 lbs. In this study, 4 compounds of petroleum hydrocarbons (Fluorene (FLU), Phenanthrene (PHE), Pyrene (PY), and Hexadecane (HXD)) were used, to evaluate the biodegradation capacity of a halophilic consortia that were enriched from in Abhor, Jeddah, SA. Different concentrations of stock solution were prepared for the petroleum hydrocarbon compounds used in this study and then stored in the refrigerator at 4 °C.

Enrichment of Consortia

Samples were enriched after collection in mineral salts medium (MSM) in an Erlenmeyer flask (250 mL), with phenanthrene (PHE) compound as the sole carbon source, and incubated in an IKA ™ orbital shaker at 150 rpm.

Biodegradation of Petroleum Hydrocarbons

All biodegradation experiments of petroleum hydrocarbons were carried out using an experimental design. This design consisted of two controls (C1, C2) = (MSM + hydrocarbon as carbon source), and two test samples (T1, T2) = (MSM + hydrocarbon as carbon source + Bacterial Culture (BC), where the degradation process was studied at 4% NaCl concentration under a saline condition in MSM.

All biodegradation experiments were performed in duplicate and then kept in IKA ™ orbital shaker at 150 rpm at 30 °C. For the extraction of hydrocarbons remaining from the biodegradation process, ethyl acetate ($C_4H_8O_2$) v/v, was utilized for the process of separating hydrocarbons from the media and the halophilic consortia in experimental flasks, in which the samples were filtered by anhydrous sodium sulfate to remove the aqueous phase. After extracting, the samples were condensed to 1 mL after the filtration process by a PTFE syringe filter (0.2 µm) in a vial for GCMS (Gas chromatography-mass spectrometry)/HPLC (High-Performance Liquid Chromatography) analysis. The extraction process was performed twice to ensure a high recovery of hydrocarbons.

High-Performance Liquid Chromatography (HPLC) Analysis

Mineralization of PAHs (PHE-FLU-PY) was analyzed in HPLC (Agilent, USA). The mobile phase (acetonitrile-1mL.min^{-1}) and stationary phase (C_{18} column, Zorbax Eclipse plus) was used in HPLC for PAH degradation analysis. UV detector (254 nm) was utilized to disclose the PAHs present in the sample. Standards with high purity (>99%) were used to attain the linearity line for the calibration process.

Gas Chromatography-Mass Spectrometry (GCMS) Analysis

Hexadecane degradation by the consortia was analyzed in GCMS (Shimadzu, Japan). The temperature program for the analysis was used as detailed by Pugazhendi et al. (2017). Helium was utilized as the carrier gas with a flow rate of 1 $mL.min^{-1}$. Hexadecane high pure grade obtained from sigma was used for standard graph calibration.

Bioreactor Study

A double casing acrylic bioreactor continuous stirred tank reactor (CSTR) with a total amplitude of about 10 L. About 7 L were utilized in the experiment, samples were collected from automobile service station wastewater (6.5 L), running the reactor under continuous stirring, and 0.5 L of halophilic consortia. The air pump was linked to preserving the oxygen standard in the reactor and the temp was also controlled at 30°C by the water circulation unit (temp controlled) under salty conditions (Fig.1). Organic loading rates were determined as 0.084, 0.112, and 0.168 kg COD / m^3day. It was found that the improved OLR is 0.112 kg COD / m^3day with Hydraulic Retention Time (HRT) for 15 days. The bioreactor study took about 40 days during which the analysis of chemical oxygen demand (COD), mixed liquor volatile suspended solids (MLVSS), and mixed liquor suspended solids (MLSS), measurement was done according to the standard methods (APHA 2005).

Phylogenetic Analysis

Bacterial strains present in the consortia during the PAHs degradation and bioreactor study were analyzed using high throughput sequencing technique. The samples collected during biodegradation and operation of CSTR succumbed to DNA extraction (DNA Extraction Kit, Qiagen, Germany). Initial DNA was amplification was performed in PCR (Polymerase Chain Reaction) machine with universal primer (27F and 1492R). High throughput sequencing was executed with primers (515-532U and 909-928U) targeting the V4-V5 region in the 16S rRNA nucleotide sequence (Pugazhendi et al. 2017). The sequence obtained was analyzed in BLAST (Basic Local Alignment Search Tool) to recognize the bacterial strains.

RESULTS AND DISCUSSION

The biodegradation of petroleum hydrocarbons was studied by a halophilic bacterial consortia under salty conditions (40 g/L) of NaCl concentration, which was enriched from the coast of Abhor, Red Sea, Jeddah, SA, where degradation and treatment of automobile service station wastewater were used. An initial study of PHE degradation at 50 mg/L con-

Table 1: Degradation of polycyclic aromatic hydrocarbons at different concentrations under salty conditions (4%).

Petroleum Hydrocarbons	Concentration	Degradation [%]	Time (day)
Phenanthrene [$mg.L^{-1}$]	50	100	6
	100	100	7
	200	100	8
	500	100	12
	800	84	14
	1000	62	16
Fluorene [$mg.L^{-1}$]	50	100	6
	100	100	7
	200	100	8
	500	100	12
	800	90	14
	1000	71	16
Pyrene [$mg.L^{-1}$]	50	92	10
	100	81	12
	200	57	14
	300	44	16
Hexadecane [%]	0.5	100	4
	1	100	6
	1.5	88	10

centration in MSM confirmed the capability of the halophilic bacterial consortia to fully degrade PHE and FLU within 6 days see Table 1.

Degradation of Various Petroleum Hydrocarbons

Phenanthrene (PHE) and Fluorene (FLU)

The biodegradation of PHE and FLU were studied by the halophilic consortia at various concentrations (50, 100, 200, 500, 800, and 1000 mg.L^{-1}) under salty conditions. PHE and FLU were degraded at concentrations of 50, 100, 200, and 500 mg.L^{-1} completely within 6, 7, 8, and 12 days, respectively as shown in Fig.1 & 2. When the PHE and FLU concentration was raised to 800 mg.L^{-1} the proportion of degradation was about 84% and 90% within 14 days, respectively. In the concentration of 1000 mg.L^{-1} for each of the two compounds, a significant decrease in the percentage of biodegradation was recorded, reaching about 62% for PHE and 71% for FLU within 16 days, where a diminution in the percentage of degradation and a raise in the time taken for degradation of these compounds were observed. Table 1 shows the concentrations and degradation rates of hydrocarbons and the days to degradation. The results revealed a high agreement with studies conducted by Jamal and Pugazhendi (2018, 2021) - a degradation of more than 92% of PHE and FLU to a concentration of 800 mg.L^{-1} under salty conditions by halophilic consortia.

Pyrene

The biodegradation of PY by a halophilic consortia was studied under salty conditions. The degradation of PY was recorded at a concentration of 50 m.L^{-1}, where the percentage of degradation was about 92% within 10 days as shown in Fig. 3. Whereas when the PY concentration augmentation was 100 mg.L^{-1}, a degradation of about 81% was recorded within 12 days. When the concentricity was raised to 200 and 300 mg.L^{-1}, a degradation ratio of about 57% and 44% were recorded within 14 and 16 days, respectively. A severe decrease in the percent degradation was observed due to the suppression of the compound and its high toxicity, as it works to inhibit bacterial growth and stop biodegradation (Vaidya et al. 2017).

Hexadecane

The biodegradation of HXD was studied at a concentration of 0.5 and 1%, and complete degradation of HXD was registered within 4 and 6 days, respectively (Fig.4). When the HXD concentration was raised to 1.5%, the compound registered 88% degradation in 10 days by the halophilic bacterial consortia. Jamal and Pugazhendi (2021) recorded a complete degradation of hexadecane at different concentrations (0.5 and 1%), and at a concentration of 1.5%, the degradation rate reached 93% within 6 days under saline conditions (4%).

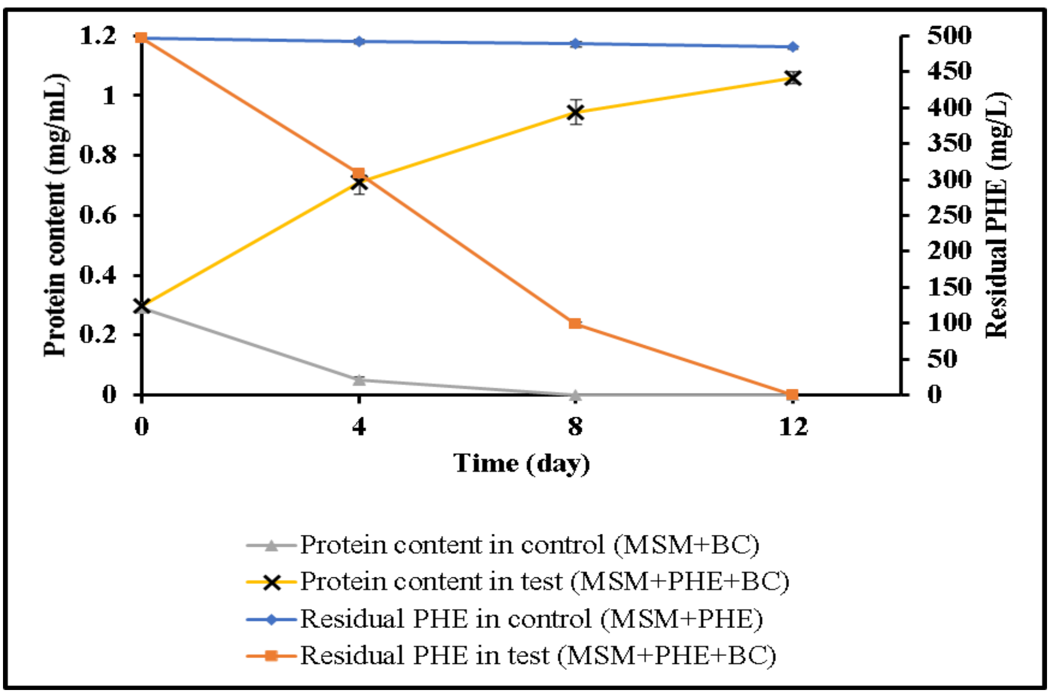

Fig. 1: Degradation of PHE at 500 ppm by the halophilic consortia under salty conditions (4%).

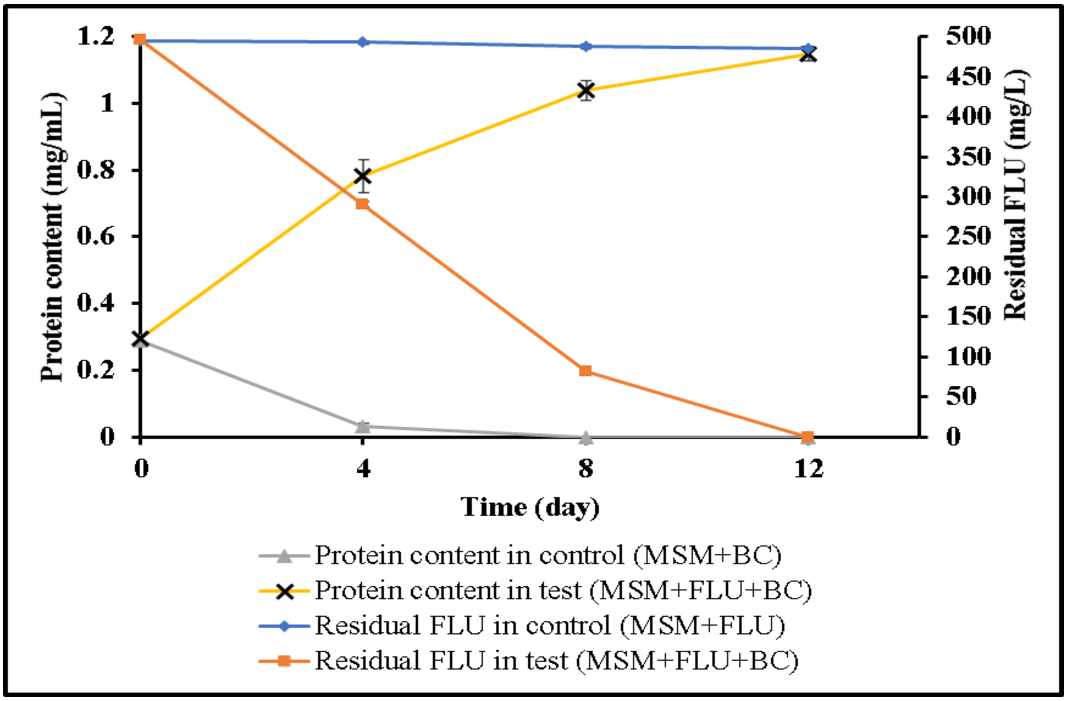

Fig. 2: Degradation of FLU at 500 ppm by the halophilic consortia under salty conditions (4%).

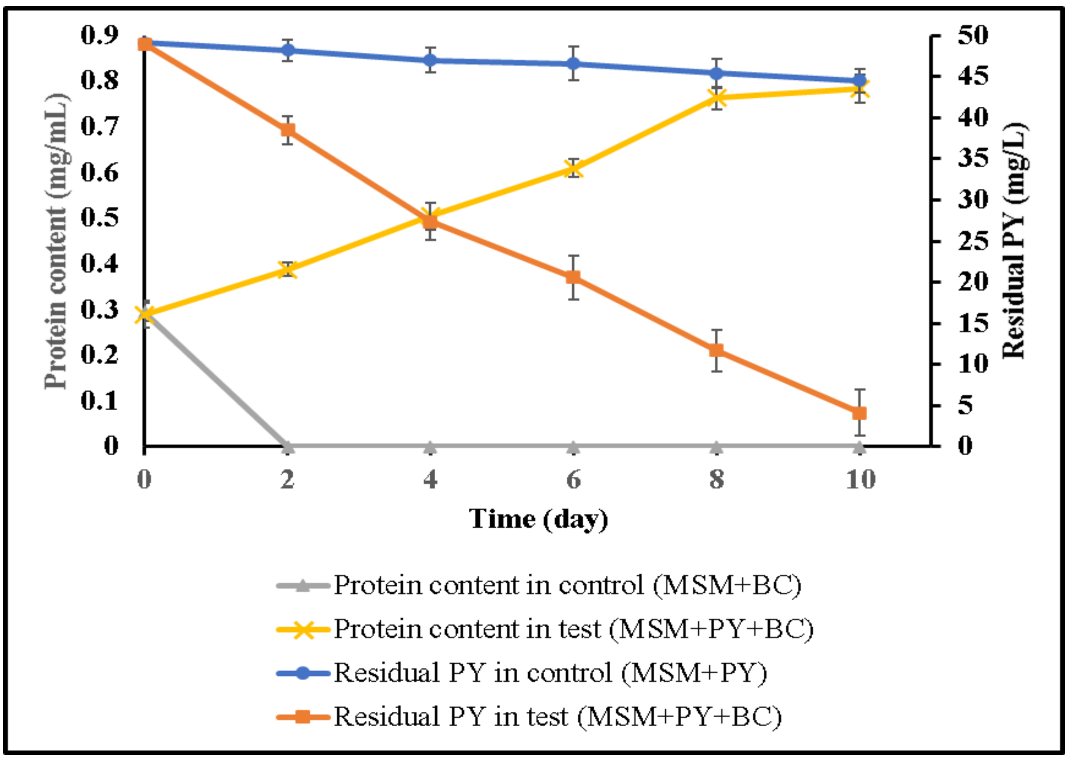

Fig. 3: Degradation of PY at 50 ppm by the halophilic consortia under saline conditions (4%).

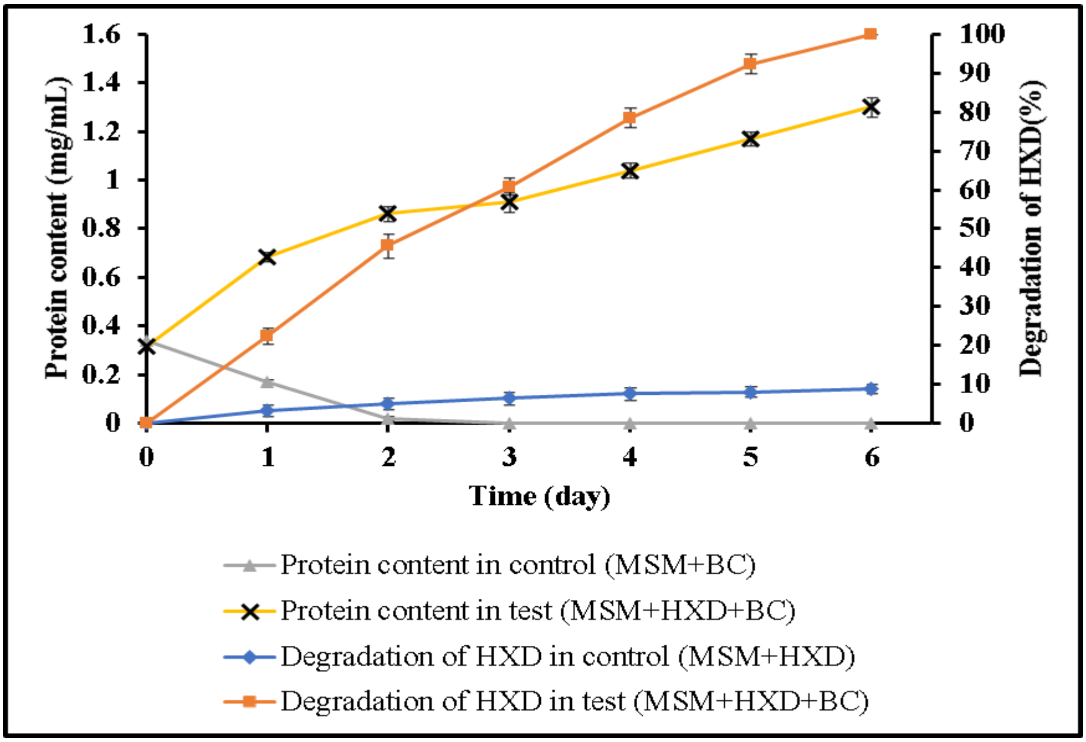

Fig. 4: Degradation of HXD at 1% concentration by the halophilic consortia under saline conditions (4%).

Bacterial Society Analysis

Initial analysis of the bacterial communities detected the presence of different groups of bacteria and archae bacteria from the samples, as this consortia degraded the PHE and used it as the sole carbon source. Several bacterial strains found in the consortia were dominated by *Martelella*, *Microbacterium*, *Propionispira*, *Ochrobactrum*, *Azospira,* and *Gordonia*, in addition to the presence of a trace amount (≤1%) of different bacterial genera (*Bacillus, Rhodococcus, Mycobacterium, Fusobacteria, Aeromonas,* and *Flavobacterium*), which were present in the consortia during the biodegradation of PHE.

Interestingly, the bacterial consortia exhibited high growth when HXD was used as a carbon source and recorded different lineage of and dominated bacterial strains such as *Bacillus*, *Microbacterium*, *Martelella*, *Gordonia*, *Ochrobactrum,* and *Mycobacterium*. Through the aforementioned results, it was found that the presence of halophilic bacterial consortia has the potential to degrade the various hydrocarbons and the strains' occupation percent change instituted on the hydrocarbons used as the sole carbon source.

In the studied samples from CSTR, several bacterial strains were found in the reactor where *Martelella* was dominated, followed by *Propionispira*, *Bacillus*, *Microbacterium*, *Mycobacterium*, *Ochrobactrum*, *Azospira*, *Pseudomonas* and *Gordonia*, in addition to a small number estimated at ≤0.5 of other bacterial strains such as *Rhodococcus, Sphingomonas, Fusobacteria, Pseudomonas, Sphingomonas, Flavobacterium, Stenotrophomonas,* and *Aeromonas*. Table 2 shows the percentage of bacterial strains that were dominant in this study. There are several studies revealed the efficiency of *Martelella* on the degradation of different PAHs that have proven its ability to degrade these compounds under saline conditions (Al Farraj et al. 2020, Chen et al. 2020, Li et al. 2020, Jamal & Pugazhendi 2021). Several studies have demonstrated the ability of several bacterial strains to degrade petroleum hydrocarbons, such as *Martelella, Bacillus, Ochrobactrum, Gordonia*, and *Marinobacter* under saline conditions (Adam 2016, Varjani 2017, Huang et al. 2019, Al-Mur et al. 2021).

Treatment of Petroleum Hydrocarbon from Automobile Service Station Wastewater in the Bioreactor (CSTR)

In this study, a bioreactor (CSTR) was operated to treat automobile service station wastewater collected from Jeddah, SA by a halophilic consortium under saline conditions, which is

Table 2: The percentage of bacterial strains that were dominant in this study.

Petroleum Hydrocarbons	Bacterial strain	Percentage [%]
PHE	*Propionispira*	35.6
	Martelella	28.5
	Microbacterium	15
	Azospira	10
	Ochrobactrum	6
	Gordonia	1.5
	Bacillus, Rhodococcus, Mycobacterium, Fusobacteria, Aeromonas and *Flavobacterium*	≤1
HXD	*Bacillus*	56
	Martelella	26
	Microbacterium	12
	Ochrobactrum	4.4
	Gordonia	1.2
	Mycobacterium	0.34
CSTR	*Martelella*	24.5
	Propionispira	21.4
	Bacillus	18.5
	Microbacterium	11.6
	Ochrobactrum	9.3
	Mycobacterium	5.2
	Azospira	4
	Gordonia	1.1
	Pseudomonas	1
	Rhodococcus, Sphingomonas, Fusobacteria, Pseudomonas, Sphingomonas, Flavobacterium, Stenotrophomonas, and Aeromonas.	≤0.5

prospective to be able to treat and degrade hydrocarbons in the wastewater. The reactor was operated at different organic loading rates (OLR) (0.168, 0.112, and 0.084 $kg.m^{-3}$day), and the OLR was optimal (0.112 $kg.m^{-3}$ day), with a hydraulic retention time (HRT) of 15 days (Fig. 5). Maximal COD removal of 93% was recorded in CSTR on 40^{th} day, while MLSS and MLVSS were maintained at 3.8 $g.L^{-1}$ and 3.2 $g.L^{-1}$ in the optimized conditions as shown in Fig.6. Al-Mur et al. (2021) in a study conducted on petroleum refinery wastewater treatment in CSTR, where COD removal recorded about 92%, this is consistent with the current study.

CONCLUSIONS

In this study, halophilic bacterial consortia enriched from Abhor, Red Sea, Jeddah, SA, showed its ability to degrade petroleum hydrocarbons under salty conditions (4%). Halophilic consortia's ability to degrade PHE and FLU compounds is up to 92% up to 1000 $mg.L^{-1}$ effectively, while PY degradation has a degradation rate of less than 70%, while HXD recorded a complete degradation at the concentration (0.5 and 1%), while at 1.5 % concentration the degradation recorded 88% under salty conditions. The CSTR study clearly demonstrated the efficiency and potential of the halophilic consortia in the treatment of automobile service station wastewater with about 93% removal of COD on the 40^{th} day under salty conditions. Thus, the halophilic bacterial consortia are a promising candidate that we can employ in the treatment of automobile service station wastewater and the degradation of petroleum hydrocarbons in our various ecosystems.

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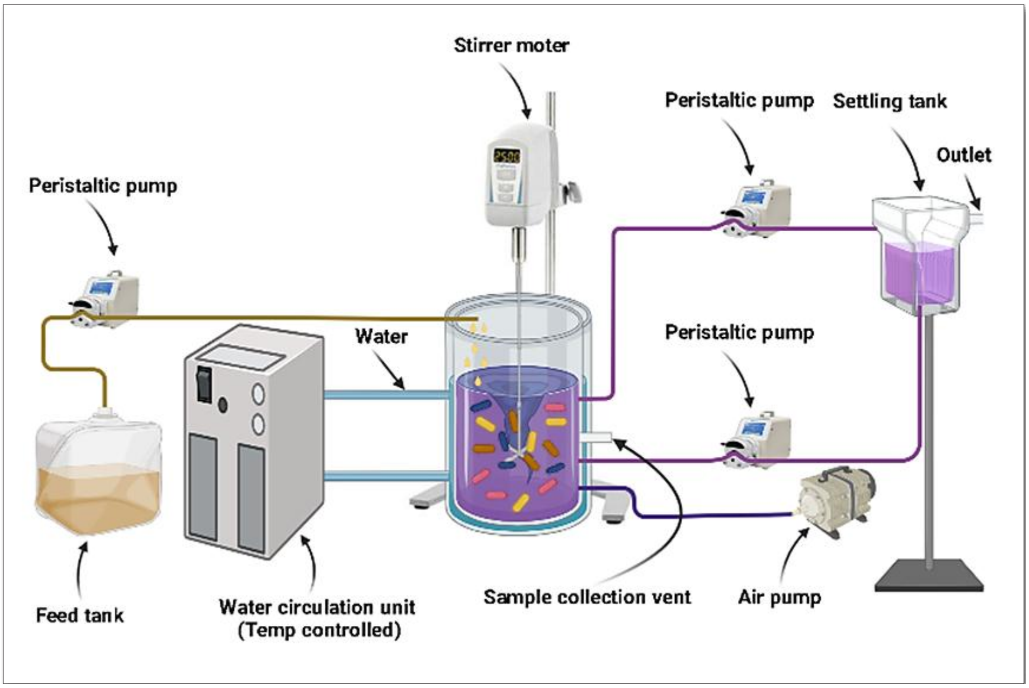

Fig. 5: Bioreactor (CSTR) design for the treatment of automobile service station wastewater by halophilic consortia.

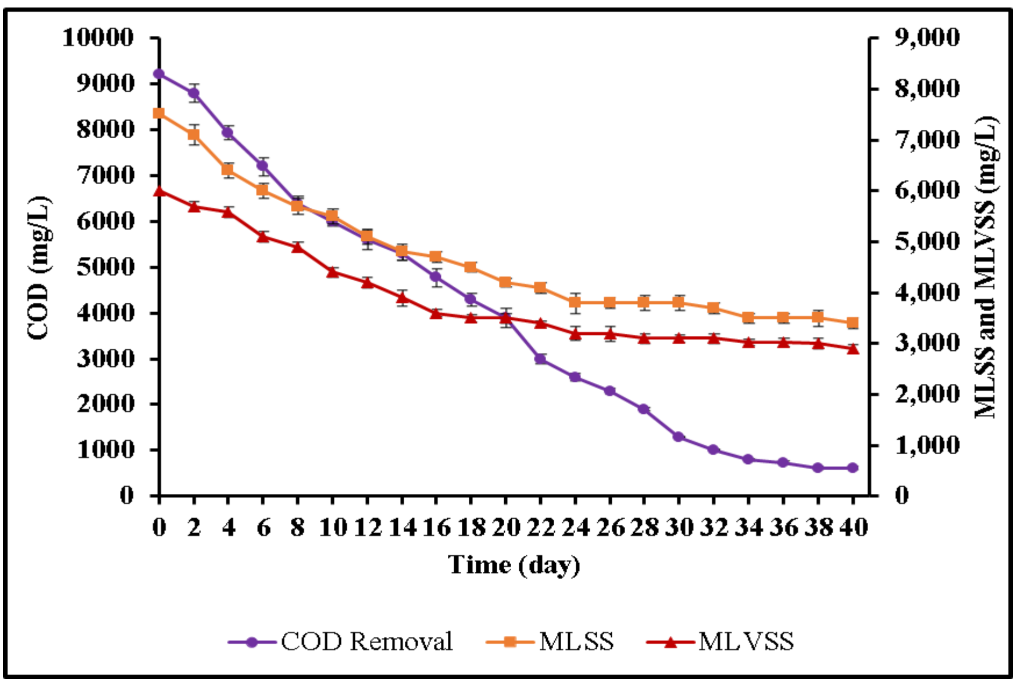

Fig. 6: Treatment of automobile service station wastewater in CSTR using halophilic bacterial consortia under saline conditions (4%).

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