Characterization of Crude Oil Degrading Marine Bacterium Bacillus licheniformis

Article *in* Indian Journal of Microbiology · March 2024

DOI: 10.1007/s12088-024-01222-9

6 authors, including:

Ramprasath Chandrasekaran
EUKPRO BIOTECH PRIVATE LIMITED
21 PUBLICATIONS **77** CITATIONS

Indian J Microbiol
https://doi.org/10.1007/s12088-024-01222-9

ORIGINAL RESEARCH ARTICLE

Characterization of Crude Oil Degrading Marine Bacterium *Bacillus licheniformis*

M. Srimathi[1] · M. Suganthi[1] · S. Sugitha[1] · K. Ashok Kumar[1] · C. Ramprasath[2] · G. Abirami[1]

Received: 19 October 2023 / Accepted: 7 February 2024

Abstract The spillage of petroleum hydrocarbons, one of the most versatile energy resources, leads to disastrous environmental pollution. The present study aims to degrade oil using enzymes from bacterial strains. A total of 39 bacteria were isolated from six different soil samples collected from Ullal Beach, Mangalore, Karnataka, located at 12°52′N latitude and 74°49′E longitude, India. All 39 bacterial isolates were screened for the production of four industrially important extracellular enzymes. Among these isolates, ten showed the highest lipase production. These cultures were further screened for bio-surfactant assays, including oil displacement and drop collapse assay and Emulsification Index. EBPL0613-F2 exhibited the best reaction in crude oil degradation. A polyphasic taxonomical approach identified the crude oil-degrading bacterium EBPL0613-F2 as *Bacillus licheniformis* and submitted in NCBI and the Accession Number is PP059616. It was then cultivated in ocean water media with tween 20 and 1% crude oil as the sole carbon and energy source. The strain was screened for lipase quantitative and qualitative assay and the protein content was also estimated. The identified bacterial strain *Bacillus licheniformis* EBPL0613-F2 demonstrated moderate lipase activity, with 76 U/ml and 24 U/ml, respectively, after 48–72 h of incubation in the crude oil substrate. For Tween 20 substrates, it exhibited 36 U/ml and 34 U/ml, respectively. FTIR analysis was used to examine the properties of crude oil following the biodegradation. The results suggest that, EBPL0613-F2 recorded the highest degradation rate so this culture has the potential for use in the degradation of crude oil in a greener manner.

Keywords Lipase · Petroleum hydrocarbon · Biosurfactant · Biodegradation · Crude oil

Introduction

India has a coastline of about 5500 km on the mainland and approximately 2000 km on the offshore islands. The biodiversity in these coastal waters is significantly high. Growing attention is being directed towards marine bacteria as unique reservoirs of extracellular substances, including polysaccharides, lipids, glycoproteins, and lipopolysaccharides Enzymes from organisms grown in salt environments have proven to be useful for industrial processes. The industrial demand for enzymes with suitable specificity and stability concerning pH, temperature, metal ions, surfactants, and organic solvents tends to drive the quest for new enzyme sources [1, 2].

The hydrolysis and synthesis of esters are conducted using glycerol and long-chain fatty acids, a process accelerated by enzymes known as lipases. The majority of commercially useful extracellular lipases are produced by a diverse range of bacteria. Only 2% of the microorganisms on earth have been investigated as potential enzyme suppliers. Enzymes with high activity and stability at higher temperatures are desirable for utilization in bioengineering and biotechnology [3, 4].

Due to their widespread and durable nature, crude oil hydrocarbons are among the organic contaminants that warrant the highest level of concern. Alkanes, monocyclic hydrocarbons, and polycyclic aromatic hydrocarbons are just

✉ G. Abirami
drabirami.cas@gmail.com

[1] Department of Biotechnology, School of Life Sciences, Vels Institute of Science Technology and Advanced Studies, Pallavaram, Chennai, Tamil Nadu, India

[2] Eukpro Biotech Private Limited, Chrompet, Chennai, Tamil Nadu, India

Published online: 07 March 2024

a few examples of the complex molecules found in crude oil [5, 6]. Despite its complexity and inertness, alkane, one of the major components of crude oil, poses hazards to the environment and human health. Over the past decades, there has been extensive research on crude oil biodegradation. Numerous microbes and their enzymes have been identified and described as a result of these studies [7, 8].

Consequently, there is a compulsion to safeguard the coastline and the ocean domain from all artificial calamities. The oil spill that occurs during the emission from refineries, mishaps of super tankers, their landings, and spills along the shore cause irremediable destruction to the diversity of life. Therefore, urgent research is needed to remediate oil spills, identify effective degrading microbes, isolate them, and develop strategies to expedite biodegradation [9].

Materials and Method

Soil and Crude Oil Sample Collection

The six different soil samples were collected randomly from soil surfaces in and around Ullal Beach, Mangalore, Karnataka, located at India. The marine soil samples were collected aseptically in clean, sterile bags from the beach. The collected samples were immediately transferred to the laboratory in an ice box for bacteriological examination. Engine oil from a vehicle was collected and used throughout the study.

Isolation of Bacteria

Aqueous dilution of the soil suspension was added to Soybean Casein Digest Medium (SCDM), and the plates were incubated at 37 °C for 24 h to isolate the bacteria. One gram of dried soil was dissolved in 9 ml of distilled water to create the suspension. Bacterial colonies were then selected from the mixed culture plate and cultured for 24 h at 37 °C. Until examination, plates containing pure cultures were kept at 4 °C.

GC–MS Analysis of Collected Crude Oil

Chromatograph interfaced to a mass spectrometer (GC–MS) equipped with a Elite-1, fused silica capillary column (30 m 0.25 mm ID 1 m df, composed of 100% Dimethyl poly siloxane). For GC/MS detection, an electron ionization system with ionizing energy of 70 eV was used. Helium gas (99.999%) was used as the carrier gas at constant flow rate 1 ml/min and an injection volume of 2 ml was employed (Split ratio of 10:1) injector temperature 250 °C; ion-source temperature 280 °C. The oven temperature was programmed from 110 °C (isothermal for 2 min) with an increase of 10 °C/min to 2000 °C, then 5 °C/min to 280 °C, ending with a 9 min isothermal at 280 °C. Mass spectra were taken at 70 eV; a scan interval of 0.5 s and fragments from 45 to 450 Da. Total GC running time was 36 min. The relative % amount of each component was calculated by comparing its average peak area to the total areas, software adopted to handle mass spectra and chromatograms was a Turbo mass.

Extracellular Enzyme Screening on Solid Agar Media

The 39 bacterial isolates were screened for the production of industrially important Enzyme viz amylase, cellulase, protease, and lipase by streaking the isolates on SCDM (Soyabean Casein Digest Medium) supplemented with 1% of the respective substrate for each extracellular enzyme and incubated at 37 °C for 3 days. [10].

Bio Surfactant Assay

Drops Collapse Test

A drop of crude oil was put on a glass slide, followed by a drop of cell-free culture broth, and drop-collapse activity was observed. A culture that produces bio surfactants shows flat drops. Triton X-100 solution (1 mg/ml), a chemical surfactant, was employed as a positive control, and deionized water as a negative control [11].

Oil Spreading Test

The oil spreading assay is also called oil displacement activity, where 20 ml of distilled water and 20 ml of crude oil were put on the surface of Petri dish. The oil surface was then covered with 10 ml of cell culture broth. The diameter of the clearing zone reveals the surfactant activity. No clear zone or oil displacement was noticed while using distilled water as the negative control (zero surfactant). The positive control was X-100 [12, 13].

Emulsification Activity

The Emulsification Index of the bio surfactant was determined using a crude oil sample. A mixture of 5 ml of crude oil and 5 ml of the filtered culture was stirred for 5 min. Subsequently, the sample underwent centrifugation and homogenization for 2 min. After 24 h of incubation, the Emulsification Index $E_{24} = (H_{EL}/H_S) \times 100\%$ [14].

Identification of Crude Oil Degrading Bacteria

Morphological and Biochemical Analysis of Selected Isolate

Light microscopy was used to evaluate the cell morphology and motility of an exponentially increasing liquid culture on the newly constructed wet mount. Gram staining was employed to determine the isolate's classification. Experiments for indole synthesis, MR-VP, citrate utilisation, triple sugar ion agar, nitrate reduction, and urease, carbohydrate Utilization Test have been done [15].

16 s rRNA Sequencing

The selected EBPL0613-F2 cultures were produced to 16 s rRNA sequencing and the sequence was searched against the BLAST tool (https://blast.ncbi.nlm.nih.gov) for the identification of the selected strain. Multiple sequence alignment was performed using CLUSTALW and a phylogenetic tree was constructed by the Maximum Parsimony method with software MEGA version 4.0. The resilience of connections was assessed using a bootstrap analysis involving 1000 bootstrap replications [16].

Quantitative and Qualitative Screening Method

Assessment of enzyme lipase regulation is found by observing the clear zone around the colonies called as a qualitative Assay. The best lipase-producing bacteria were chosen to degrade crude oil using the bio-surfactant analysis method. The total protein content is also estimated by Lowry Method [17]. Subsequently, they were inoculated into a modified ocean water medium broth, tween 20 as a substrate and then the culture was maintained at 37 °C with 100 rpm agitation for 3 days. Lipase activity was quantitatively assessed using a spectrophotometer at 380 nm with p-nitrophenyl laurate as the substrate. One unit of lipase activity was defined as the release of 1 µmol of free p-nitrophenol per minute by the enzyme [18].

FT-IR (Fourier Transform Infrared Spectroscopy Analysis)

The FTIR analysis of the crude oil was conducted at the Pondicherry Centre for Biological Science and Educational Trust (PCBS), which serves as a key tool for lubricant analysis in FTIR spectroscopy. This analysis reveals the functional groups present in the crude oil and helps determine the nature of the crude oil, indicating whether it is of good or poor quality. 1% of crude oil substrate and 1 ml of culture were added to the modified Ocean Water Medium Broth. The mixture was then incubated in a shaker at 37 °C, 100 rpm for 3 days. Following incubation, the sample underwent FT-IR analysis. Additionally, an FTIR analysis of crude oil without a culture was conducted to serve as the control [19].

Result

Collection of Soil Samples and Crude Oil Sample

The six different soil samples were collected randomly from the soil surface in and around Ullal Beach, Mangalore, Karnataka- 12°49′58.6 latitude and longitude, 74°49′60.0 E (Fig. 1). The soil samples were collected aseptically in clear zip-lock bags. The collected samples were immediately transferred to the laboratory for further bacteriological examination. Engine oil from a vehicle was collected as an oil sample (Fig. 2). Over the past three decades, the significant evolution in biotechnology development can be attributed to the careful choice of isolation sources and the formulation of appropriate selection criteria tailored to the intended activities.

Overall, 39 marine bacteria were isolated from the soil surface in and around Ullal Beach, Mangalore, Karnataka. The 39 marine bacteria were streaked separately on SCDM (Soyabean Casein Digest Medium). All the isolated marine bacteria were designated with alphabets and Arabic numerals EBPL0612 to EBPL0650. *Bacillus licheniformis* is a well-known environmentally friendly microorganism, now employed in oil degradation through enzymes produced by them. Marine bacteria have enormous applications in biotechnology for treating environmental pollution. In recent days, bioremediation is considered a greener approach for such kind of remediation. In this study, a total of 39 marine bacteria were isolated from six different soil samples collected in and around Ullal Beach, Mangalore, Karnataka, India. To the best of our knowledge, we are the first to exploit Ullal Beach soil samples for the isolation of microbes for such environmental applications. Some of the previous studies reported that petroleum hydrocarbon degrading potent bacteria was isolated from Bohai Bay china [20].

GC–MS Analysis of Collected Crude Oil

The GC–MS analysis of collected crude oil was done. Totally 74 compounds were found. The Major compound were Benzene, Naphthalene, Pentadecane, Octadecane, Cyclohexane, Pentacosane. (Fig. 3 and Table 1).

Extracellular Enzyme Screening on Solid Agar Medium

Screening of Marine Bacteria for Industrially Important Enzymes

The 39 marine bacterial isolates were screened for industrially important enzymes like amylase, cellulase, protease and

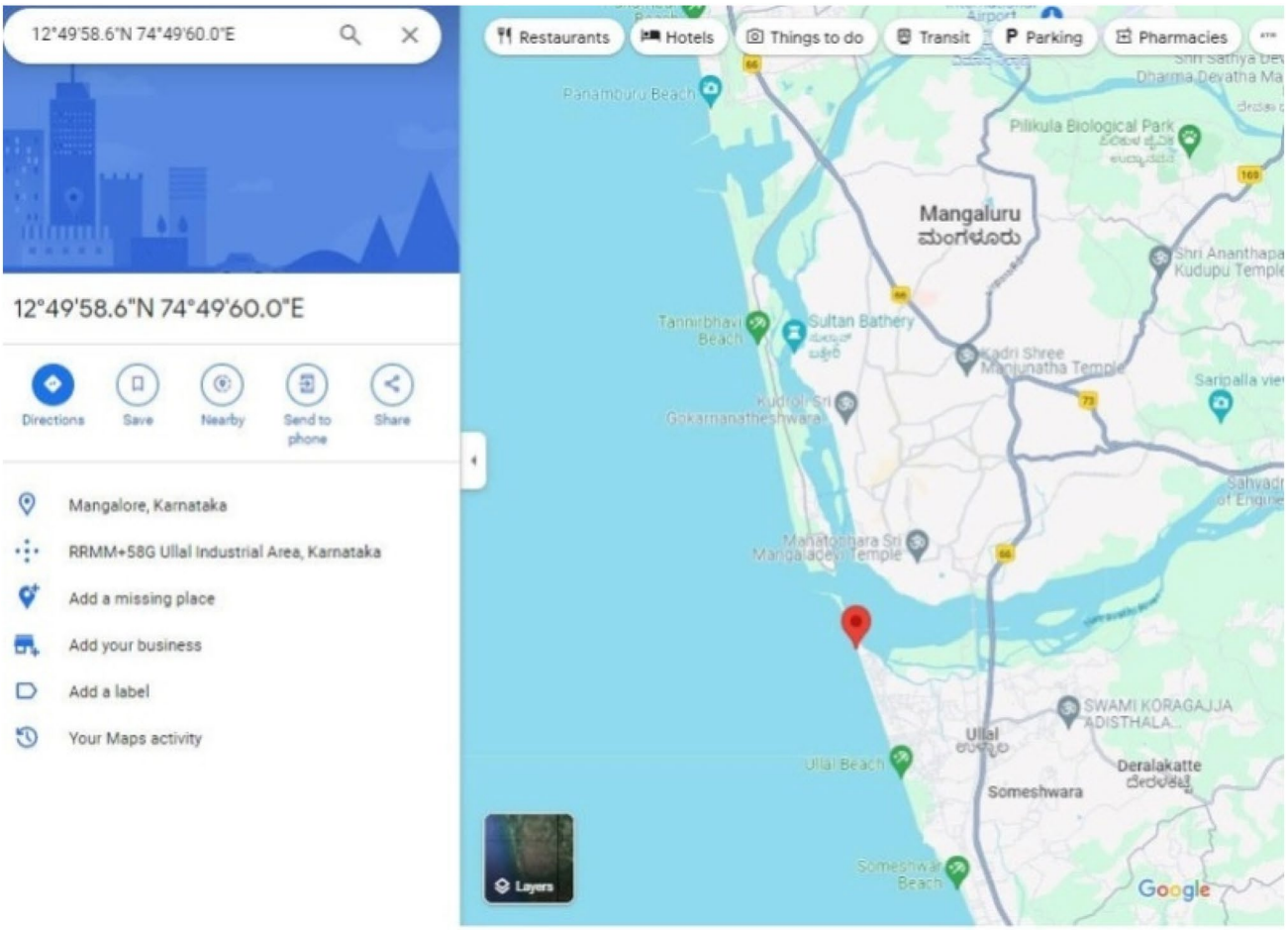


Fig. 1 sampling site

Fig. 2 Crude oil

lipasse enzyme production, of which EBPL0639 was the highest amylase-producing marine bacteria, EBPL0613-F2, EBPL0621, EBPL0623, and EBPL0639 were found to be the highest cellulase-producing marine bacteria, EBPL0613-F2, EBPL0617, EBPL0629, EBPL0639, and EBPL0647 were found to be the highest protease-producing marine bacteria, EBPL0613-F2, EBPL0617, EBPL0618, EBPL0621, EBPL0624, EBPL029, EBPL0631, EBPL0636, EBPL0649, EBPL0650 have utilized the tween 20 substrate and was found to be highest lipase producing marine bacteria. It is clear that EBPL0613-F2 is a potent candidate culture capable of producing four different enzymes which is listed in Fig. 4a–d and Table 2. Recent studies also indicate that the screening of enzymes is of utmost importance in identifying a potent culture for diverse applications. It unveils the activity or the sought-after product through qualitative and typically indirect selection criteria, without delving into specific details about the intended activity [21].

Bio surfactant Assays (Drop Collapse Test and Oil Spreading Test)

The best ten lipase-producing marine bacterial isolates were used in this method. A sensitive drop collapse test

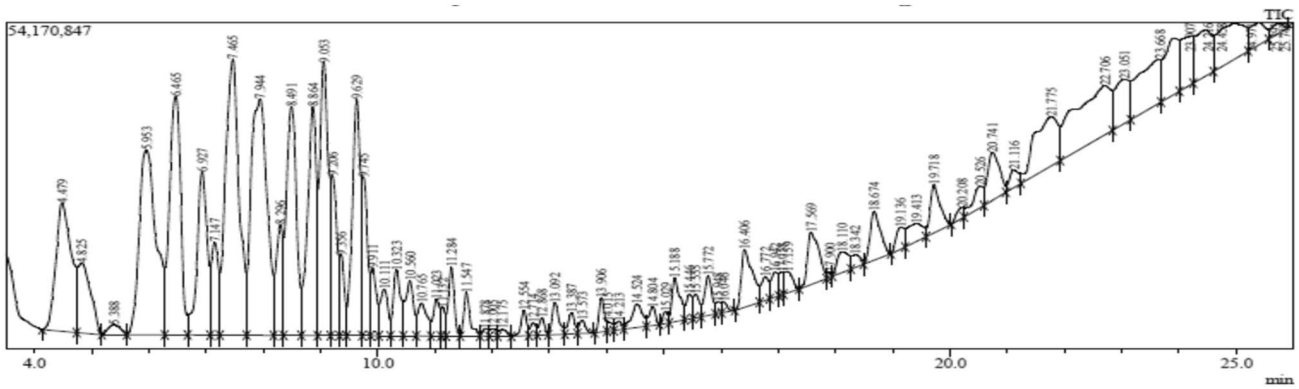


Fig. 3 GC–MS analysis of crude oil

can provide findings with just a trace quantity of surfactant. The test was conducted using cell-free culture broth and a substrate of 5–10 µl of crude oil with a flat droplet, EBPL0613-F2 delivered favorable results. Other strains produced spherical drops, indicating negative results in drops collapse test. The cell free culture broth of the organisms was put through the oil spreading procedure. One minute after the drop collapsed in order to further establish the formation of bio surfactant. The result states that EBPL0613-F2 gave positive results with a clearance zone within 20 s respectively and other strains gave negative results for the oil spreading test in Table 3 and Fig. 5a, b. Production of bio surfactant in the micro organism indicated that, only these microbes are capable of displace the oil. Both bio surfactant assays indicate surface and wetting activities; the larger diameter indicates the much higher surface activity of the bacteria [22, 23].

Circular drops were evaluated as negative result (−) indicating the absence of biosurfactant synthesis. Flat drops were scored as (+) to (+ + +) equating partial to total spreading on crude oil surface. A solution with a concentration of 1% tween 20 was used positive control (+ + +) whereas the sterilized standard medium was used as the negative control (−).

Emulsification Activity

The emulsification index after 24 h, where the height of the emulsified layer (H_{EL}) is, and the height of the total liquid column (H_S), was calculated in Table 4. The maximum emulsification activity was found in EBPL0613-F2-F2 as 61.20%. The Maximum emulsification activity was found to be 50–70% reported by [24]. Bacterial culture ADY2b indicate better emulsification activity of about 58.33% [25]

Identification of EBPL0613-F2- Colony Morphology and Biochemical Characteristics

Rod-shaped, creamy yellow, opaque colonies were observed on Nutrient Medium. EBPL0613-F2 was identified as Gram-positive, motile, spore-forming bacteria. Their ability to form spores when nutrients are limiting makes species of Bacillus self-sustainable bioremediation means [26]. The Catalase, Indole, VP, citrate, and Nitrate reduction tests showed positive results, while the oxidase, MR, Triple Sugar Iron agar, and urease tests showed negative results (Table 5). In the carbohydrate fermentation test, the bacterium utilized Glucose, Lactose, Maltose, Galactose, Mannitol, Sucrose, Arabitol, and Fructose sugars (Table 6). Nucleotide sequence data were obtained from the DNA sequencing software of the ABI 3730xl DNA analyzer (Model 373, Forster, CA, U.S.A.). The sequences were then compared through local alignment search of the GenBank database using the BLAST version 2.2.9 program of the National Center for Biotechnological Information (NCBI). The 16S rRNA sequence analysis revealed that the strain EBPL0613-F2 belongs to the genus Bacillus, with 99.9% identity to *Bacillus licheniformis* strain pb-HK09002, respectively. The phylogenetic relationship was constructed from a partial 16SrRNA nucleotide sequence through the missing gap deletion process (Fig. 6). Therefore, the BLAST result for a complete 16S rRNA nucleotide sequence of EBPL0613-F2 was determined and submitted in NCBI and the Accession Number is PP059616.

Quantitative and Qualitative Screening Method

The results revealed that the isolate exhibited high clear zones with crude oil and low clearance zones with tween20 showed in Fig. 7, the key consideration in choosing lipase-producing strain was the distinct appearance of the hydrolysis zone surrounding the colony. Lipolytic activity can

Table 1 List of compounds present in collected crude oil analyzed through GC–MS

Peak#	R.time	Area	Area%	Height	Name
1	4.479	398,587,245	4.02	21,276,420	Benzene, 1,3-dimethyl-
2	4.825	184,497,505	1.86	11,601,400	Benzene, 1,2-dimethyl-
3	5.388	25,109,852	0.25	1,697,632	Heptane, 5-ethyl-2-methyl-
4	5.953	634,186,473	6.40	30,566,227	Benzene, 1,3,5-trimethyl-
5	6.465	539,601,046	5.44	39,485,972	Benzene, 1,3,5-trimethyl-
6	6.927	330,863,373	3.34	27,036,540	Benzene, 1,2,3-trimethyl-
7	7.147	134,518,727	1.36	15,389,941	Indane
8	7.465	753,414,945	7.60	45,791,103	Spiro[3.5]nona-5,7-dien-1-one, 5,9,9-trimethyl-
9	7.944	763,789,485	7.71	39,220,500	Benzene, 1,2,4,5-tetramethyl-
10	8.296	154,457,277	1.56	18,432,144	Benzene, 2-ethyl-1,4-dimethyl-
11	8.491	460,896,778	4.65	37,874,635	Benzene, 1,2,4,5-tetramethyl-
12	8.864	411,752,449	4.15	37,948,870	Benzene, 1-methyl-2-(2-propenyl)-
13	9.053	467,433,169	4.72	45,248,176	Benzene, 1-methyl-2-(2-propenyl)-
14	9.206	194,953,127	1.97	26,511,180	9-methylheptadecane
15	9.356	74,028,288	0.75	13,377,009	1-sec-butyl-4-methylbenzene #
16	9.629	394,636,421	3.98	39,245,723	Naphthalene
17	9.745	165,222,161	1.67	26,284,129	Benzene, Pentamethyl-
18	9.911	75,243,824	0.76	11,267,980	Benzene, 1,4-dimethyl-2-(1-methylethyl)-
19	10.111	72,071,322	0.73	7,720,483	Benzene, 1,3,5-triethyl-
20	10.323	98,655,817	1.00	10,919,310	7-Ethylbicyclo[4.2.1]nona-2,4,7-triene
21	10.560	88,401,068	0.89	9,202,085	1H-indene, 2,3-dihydro-4,7-dimethyl-
22	10.765	62,475,429	0.63	5,411,499	1H-indene, 2,3-dihydro-4,6-dimethyl-
23	11.023	47,825,247	0.48	6,140,193	Benzene, pentamethyl-
24	11.132	23,188,254	0.23	4,770,740	Nonane, 3-methyl-5-propyl-
25	11.284	80,891,444	0.82	11,417,026	Naphthalene, 1-methyl-
26	11.547	54,992,146	0.55	7,252,700	Naphthalene, 1-methyl-
27	11.878	9,517,336	0.10	1,260,914	Pentadecane
28	12.005	9,708,030	0.10	1,213,417	Benzene, (2,2-dimethyl-1-methylenepropyl)-
29	12.175	10,460,757	0.11	1,143,236	Cyclopentanol, 3,3,4-trimethyl-4-p-tolyl-, (R,R)-(+)-
30	12.554	28,679,856	0.29	4,255,706	Tetradecane
31	12.714	14,440,510	0.15	2,085,812	Naphthalene, 2-ethyl-
32	12.868	19,148,465	0.19	2,873,060	Naphthalene, 2,6-dimethyl-
33	13.092	44,934,128	0.45	5,323,113	Naphthalene, 1,3-dimethyl-
34	13.387	27,528,914	0.28	3,481,725	Naphthalene, 1,6-dimethyl-
35	13.573	16,842,003	0.17	2,208,499	Naphthalene, 1,3-dimethyl-
36	13.906	37,083,295	0.37	5,701,482	Heptadecane
37	14.015	9,379,346	0.09	1,528,142	1,1′-biphenyl, 2,4′-dimethyl-
38	14.213	11,448,458	0.12	1,425,384	Naphthalene, 1,4,6-trimethyl-
39	14.524	51,015,087	0.51	3,901,230	3-(2-methyl-1-propenyl)-1H-indene
40	14.804	24,099,364	0.24	3,012,303	3-(2-methyl-1-propenyl)-1H-indene
41	15.029	12,606,529	0.13	1,996,980	Naphthalene, 2,3,6-trimethyl-
42	15.188	69,686,853	0.70	7,323,417	Heneicosane
43	15.446	30,338,420	0.31	4,079,958	1,1′-biphenyl, 3,4′-dimethyl-
44	15.555	28,092,433	0.28	3,936,583	Benzene, 1,1′-(1-methyl-1,2-ethanediyl)bis-
45	15.772	59,350,338	0.60	6,591,521	Tricosane
46	15.948	12,315,834	0.12	1,993,584	2-methyltetracosane
47	16.046	10,519,798	0.11	1,770,499	2-Methylheptadecane
48	16.406	128,041,339	1.29	9,631,277	Heptadecane
49	16.772	35,301,518	0.36	3,947,357	Benzene, 1,1′-(1,2-ethanediyl)bis[4-methyl-
50	16.942	36,623,895	0.37	4,167,163	Heneicosane, 10-methyl-
51	17.058	17,802,882	0.18	3,761,164	1,1′-biphenyl, 2,4,6-trimethyl-

Table 1 (continued)

Peak#	R.time	Area	Area%	Height	Name
52	17.159	35,858,032	0.36	3,555,281	1,1′-biphenyl, 2,4,6-trimethyl-
53	17.569	117,789,351	1.19	8,692,661	Eicosane
54	17.900	5,195,857	0.05	904,285	Benzenemethanol, 2-methyl-.alpha.-phenyl-
55	18.110	48,861,283	0.49	3,403,754	Octadecane
56	18.342	21,583,926	0.22	2,080,875	Cyclohexane, (1-octylnonyl)-
57	18.674	102,169,272	1.03	8,056,074	Eicosane
58	19.136	36,867,005	0.37	3,604,168	Tetratriacontane
59	19.413	58,198,707	0.59	2,966,216	Benzene, 1,1′-ethenylidenebis-[4-methyl-
60	19.718	94,651,873	0.95	7,978,698	Eicosane
61	20.208	16,371,999	0.17	1,715,607	Tetracosane
62	20.526	47,288,777	0.48	3,540,821	Octatriacontane, 1,38-dibromo-
63	20.741	114,858,968	1.16	7,931,594	Eicosane
64	21.116	34,518,376	0.35	2,950,251	Tetracontane
65	21.775	244,588,340	2.47	8,106,741	Docosane
66	22.706	393,054,760	3.97	8,118,080	Tetracosane
67	23.051	134,345,131	1.36	7,236,293	Heneicosane
68	23.668	219,405,360	2.21	7,194,577	Dotetracontane
69	23.907	161,628,556	1.63	9,398,088	2-tetradecyl-1-octadecene
70	24.216	121,248,031	1.22	7,984,243	3-methylhexacosane
71	24.458	154,390,876	1.56	7,702,641	Tetracosane
72	24.971	207,473,253	2.09	5,960,921	Di-n-octyl phthalate
73	25.393	69,515,109	0.70	3,723,929	Pentacosane
74	25.740	26,353,931	0.27	1,696,321	Pentacosane

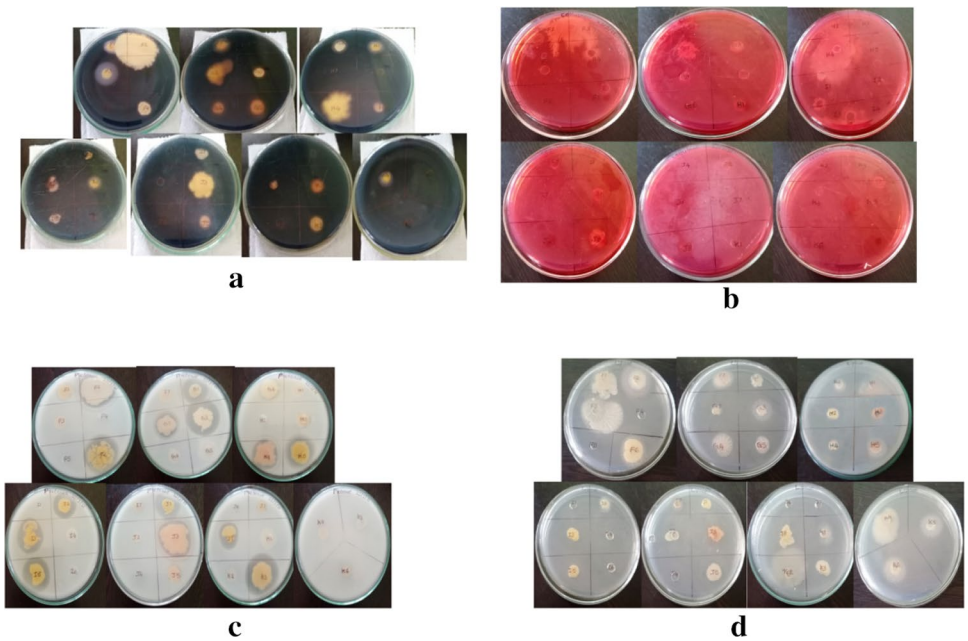


Fig. 4 **a** Amylase Enzymes Assay **b** Cellulase Enzymes Assay **c** Protease Enzymes **d** Lipase Enzymes Assay

be detected by the visual appeal of clear or murky zones encircling colonies or by the growth of crystals on the agar surface.

The amount of lipase produced was monitored at 48h intervals up to 72 h. The maximum lipase activity was observed at both 48 and 72 h for both crude oil and tween20 as substrates, as depicted in the figure. Subsequently, although the bacterial growth rate continued to increase, the specific growth rate decreased on both substrates. Beyond 48 h, the growth deviated from exponential, as

Table 2 Screening of marine bacteria for extracellular industrially important Enzymes

S. no.	Culture no.	Amylase	Cellulase	Protease	Lipase
1	EBPL0612-F1	+	–	+	+
2	EBPL0613-F2	+	+	+ + +	+ + +
3	EBPL0614-F3	+	+	–	+
4	EBPL0615-F4	–	+	–	–
5	EBPL0616-F5	–	+	–	–
6	EBPL0617-F6	+	+	+ + +	+
7	EBPL0618-F7	+	+	–	+
8	EBPL0619-G1	–	+	+ +	–
9	EBPL0620-G2	+	+	+ +	–
10	EBPL0621-G3	+	+ +	+ + +	+
11	EBPL0622-G4	+	–	–	–
12	EBPL0623-G5	+	+ +	–	–
13	EBPL0624-G6	+	+	+ +	+
14	EBPL0625-H1	+	+	–	–
15	EBPL0626-H2	–	+	–	–
16	EBPL0627-H3	–	+	–	–
17	EBPL0628-H4	+	–	+ +	–
18	EBPL0629-H5	+	+	+ + +	+
19	EBPL0630-I1	–	–	–	–
20	EBPL0631-I2	+	+	+ +	+
21	EBPL0632-I3	+	+	+ +	–
22	EBPL0633-I4	+	+	–	–
23	EBPL0634-I5	+	+	+ +	–
24	EBPL0635-I6	–	+	–	–
25	EBPL0636-I7	–	–	–	+
26	EBPL0637-J1	+	–	+ +	–
27	EBPL0638-J2	–	–	–	–
28	EBPL0639-J3	+ +	+ + +	+ + +	–
29	EBPL0640-J4	–	–	–	–
30	EBPL0641-J5	+	–	+	–
31	EBPL0642-J6	–	–	–	–
32	EBPL0643-J7	–	–	+	–
33	EBPL0644-J8	+	+	+ +	–
34	EBPL0645-K1	+	+	–	+
35	EBPL0646-K2	–	+	–	–
36	EBPL0647-K3	+	–	+ + +	+
37	EBPL0648-K4	+	+	–	–
38	EBPL0649-K5	–	+	–	+
39	EBPL0650-K6	–	+	–	+

bacterial pellets began to form, limiting nutrient and oxygen supply for homogeneous growth. Consequently, lipase yield decreased due to the depletion of nutrient materials. Using Lowry's technique, the total protein content was calculated (Fig. 8), Illustrates the protein composition of lipase observed as 1.46 U/ml & 1.37 U/ml (Fig. 9) respectively after 48–72 h of incubation in crude oil substrate and for tween 20 substrate it exhibits 2.64 U/ml & 1.90 U/ml

Table 3 Analysis of the bio surfactant assay

S. no.	Cultures	Drop collapse test	Oil displacement test
1.	EBPL0613-F2	+ + +	+ + +
2.	EBPL0617-F6	–	–
3.	EBPL0618-F7	–	–
4.	EBPL0621-G3	+ +	+
5.	EBPL0624-G6	+	+
6.	EBPL0629-H5	–	–
7.	EBPL0631-I2	+	+
8.	EBPL0636-I7	–	–
9.	EBPL0649-K5	–	–
10.	EBPL0650-K6	+	+ +

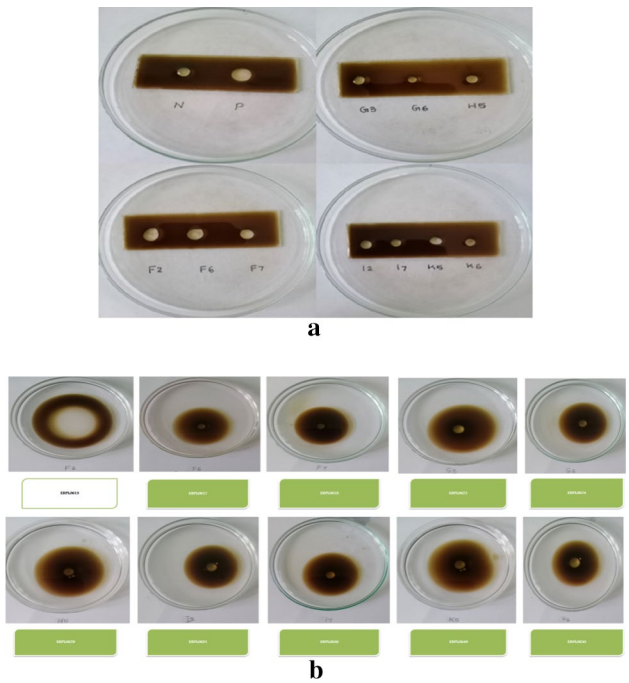


Fig. 5 **a** Drop collapse test. **b** Oil displacement Assay

respectively after 48–72 h of incubation (Fig. 10). It suggested that each bacterial strain has a different molecular weight of lipase enzyme and also a different amount of protein content. This is due to the genetic diversity of bacterial species. However, *Bacillus Licheniformis* possess moderate lipase activity, exhibited only 76 U/ml and 24 U/ml respectively after 48 & 72 h of incubation in crude oil substrate and for tween 20 substrate it exhibit only 36 U/ml & 34 U/ml respectively after 48 & 72 h of incubation (Fig. 11). Previously it was reported that using olive oil as a carbon source the lipase production was found to be better when compared

Table 4 Emulsification activity

S. no.	Bacterial cultures	Emulsification activity (%)
1.	EBPL0613-F2	61.20
2.	EBPL0617-F6	41.24
3.	EBPL0618-F7	38.12
4.	EBPL0621-G3	31.54
5.	EBPL0624-G6	22.35
6.	EBPL0629-H5	32.20
7.	EBPL0631-I2	38.20
8.	EBPL0636-I7	24.30
9.	EBPL0649-K5	35.33
10.	EBPL0650-K6	45.46

Table 5 Morphology, physiochemical and biochemical characterization of EBPL0613-F2

S. no.	Morphology, physiochemical and biochemical characterization	EBPL0613-F2
1.	Colony morphology	Serrate, transparent, flat, filamentous, and dry but light orange
2.	Microscopic observation	Small rods & spores are seen
3.	Gram staining	Gram positive
4.	Motility	Positive
5.	Oxidase	Negative
6.	Catalase	Positive
7.	Indole production test	+
8.	MR test	−
9.	VP test	+
10.	Citrate utilization test	+
11.	Urease test	−
12.	Triple sugar ion agar test	−
13.	Nitrate reduction test	+

Table 6 Carbohydrate fermentation test

S. no.	Carbohydrate fermentation	Properties
1.	Glucose	Positive
2.	Lactose	Positive
3.	Maltose	Positive
4.	Galactose	Positive
5.	Mannitol	Positive
6.	Sucrose	Positive
7.	Arabitol	Positive
8.	Fructose	Positive

to other carbon source [27]. The statistical analysis was conducted using triplicate samples, and both the standard deviation and standard error were calculated.

FT-IR (Fourier Transform Infrared Spectrophotometry) Analysis

The infrared spectra for some of the samples were plotted versus absorbance with the wave numbers in cm^{-1}. For sample EBPL0613-F2, the spectra display sizable carbonyl and aromatic peaks, indicating a degree of oil deterioration. As the sample EBPL0613-F2 proportion was lowered from 90 to 0% (100% 145 nm) the intensity of the carbonyl or aromatic peaks steadily decreased, indicating an increase in the percentage of saturated hydrocarbons. Even while overlapping absorption peaks possess a tendency to obscure the connection between IR peaks and oil composition, a rapid inspection of the spectra would still show some variances or trends in the 1645-668 cm^{-1} range. These may be seen in the aromatic ring peaks, the C=O absorption peak at around 1508 cm^{-1} both the long chain aliphatic hydrocarbon vibration, and the aromatic ring peak between 9668 and 504 cm^{-1}, accordingly these vibrations all can either increase or decrease depending on the crude oil nature or type (Fig. 12). FT IR extensively used to study chemical changes in the compounds. A recent study also employs FTIR to understand the effects of bioremediation in bacteria [28].

Conclusion

Microbes demonstrate resilience to rapid and repetitive fluctuations in environmental factors such as temperature, light, and salinity, enduring challenges like wave action, ultraviolet radiation, and periods of drought. Microorganisms originating from these harsh environments may possess advantageous properties applicable in various biotechnological contexts. Despite these potential benefits, additional studies are needed to effectively employ bioremediation for the restoration of habitats contaminated with petroleum oil. The current evaluation of knowledge suggests that Bacillus Licheniformis holds significant bioremediation potential. This study supports the notion that Bacillus Licheniformis can be harnessed to develop bioremediation agents for removing crude oil from marine-contaminated sites. This alternative approach to crude oil degradation has the potential to be cost-effective, environmentally safe, and versatile.

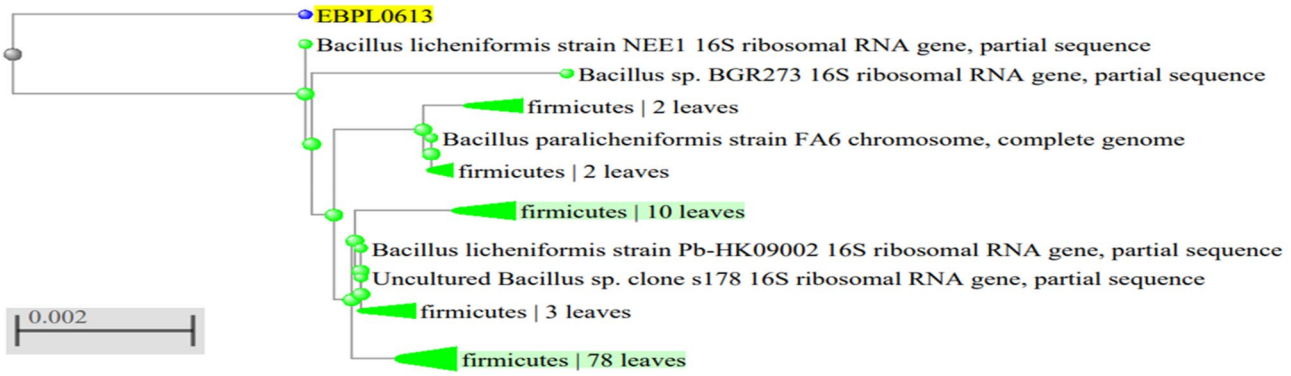


Fig. 6 Phylogenetic tree of EBPL0613-F2 isolate and the relationship of their 16S rRNA nucleotide sequence

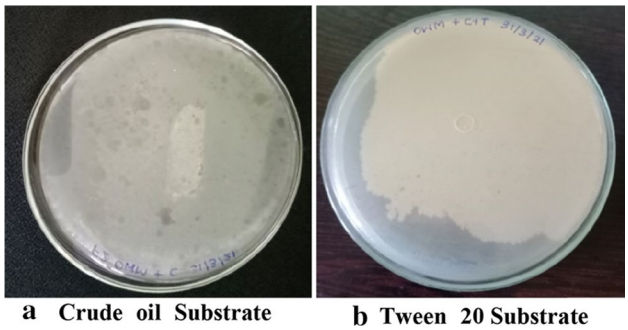


Fig. 7 Qualitative screening method using crude oil and tween 20 as a substrate

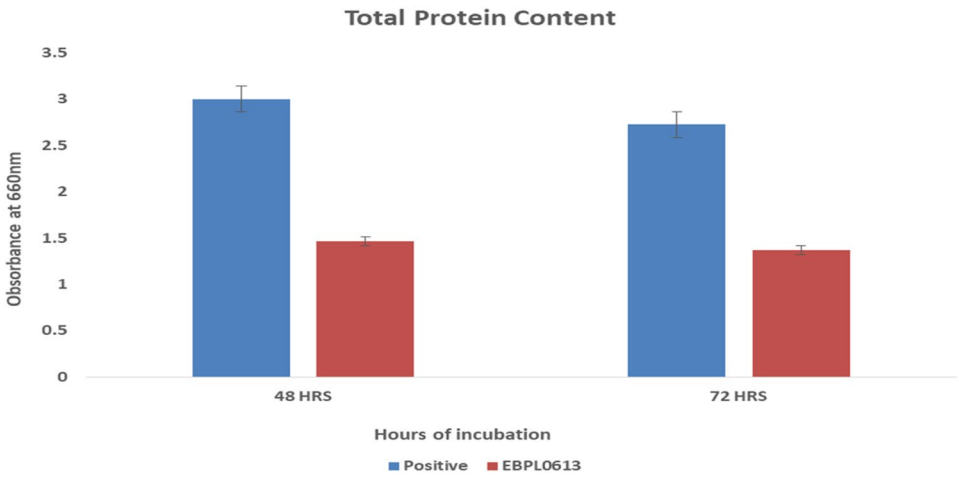


Fig. 8 Comparative analysis of crude oil degrading bacteria by total protein content using crude oil substrate

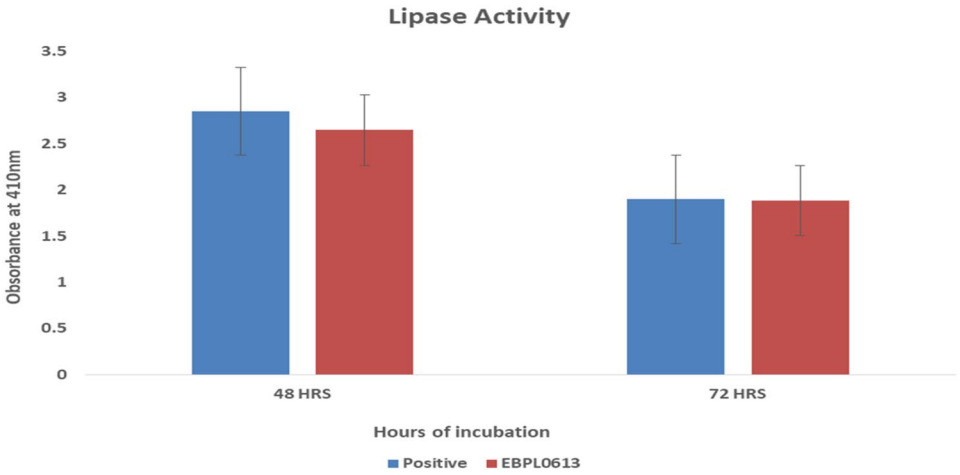


Fig. 9 Comparative analysis of crude oil degrading bacteria by lipase activity using crude oil substrate

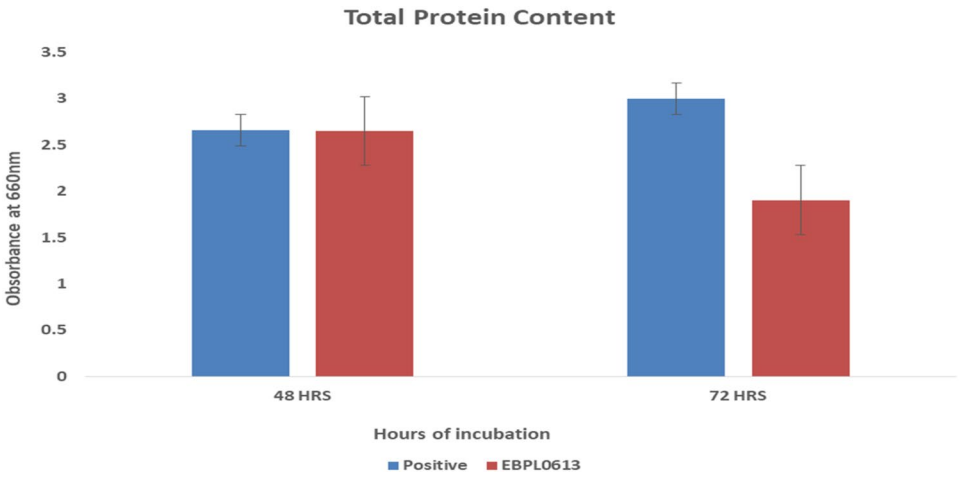


Fig. 10 Comparative analysis of crude oil degrading bacteria by total protein content using tween 20 substrate

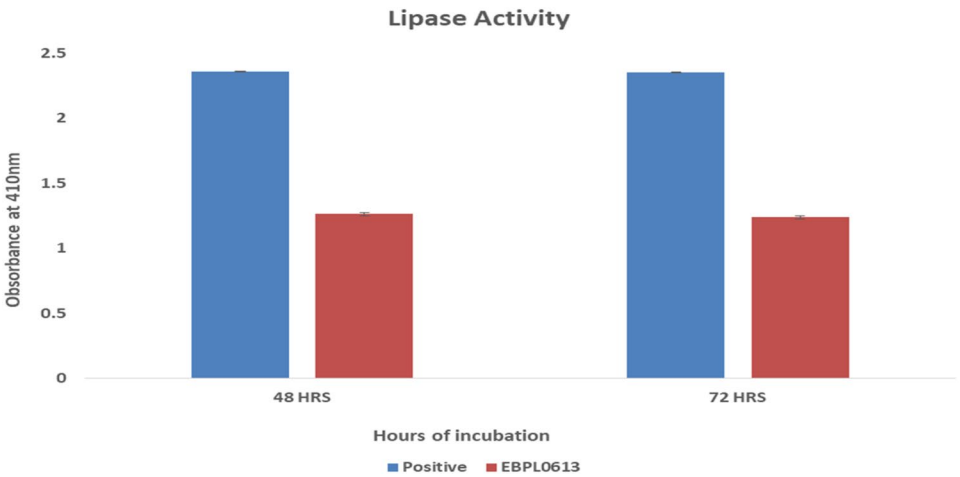
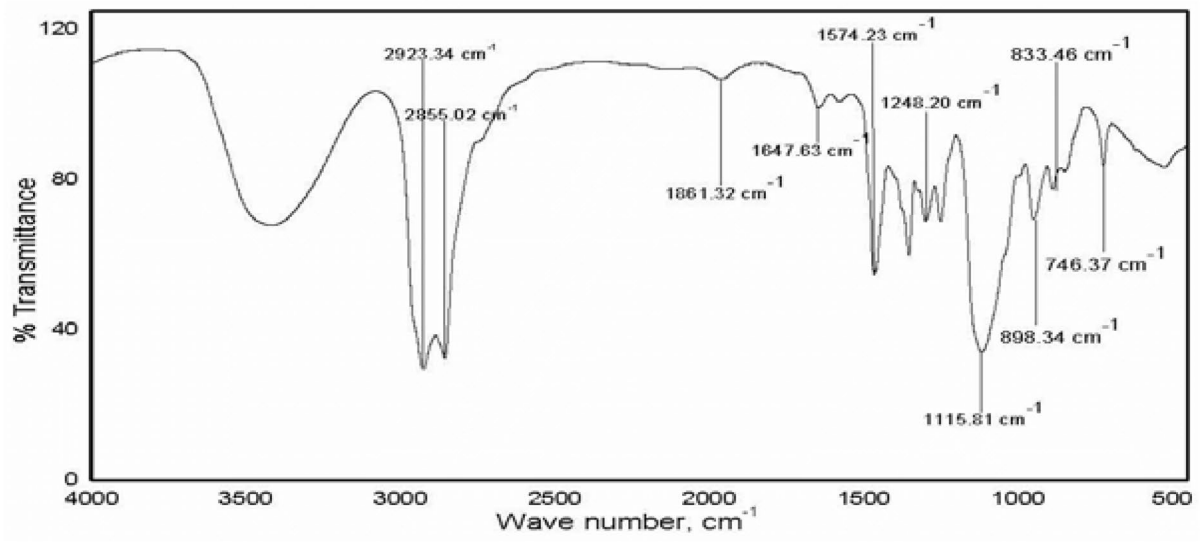
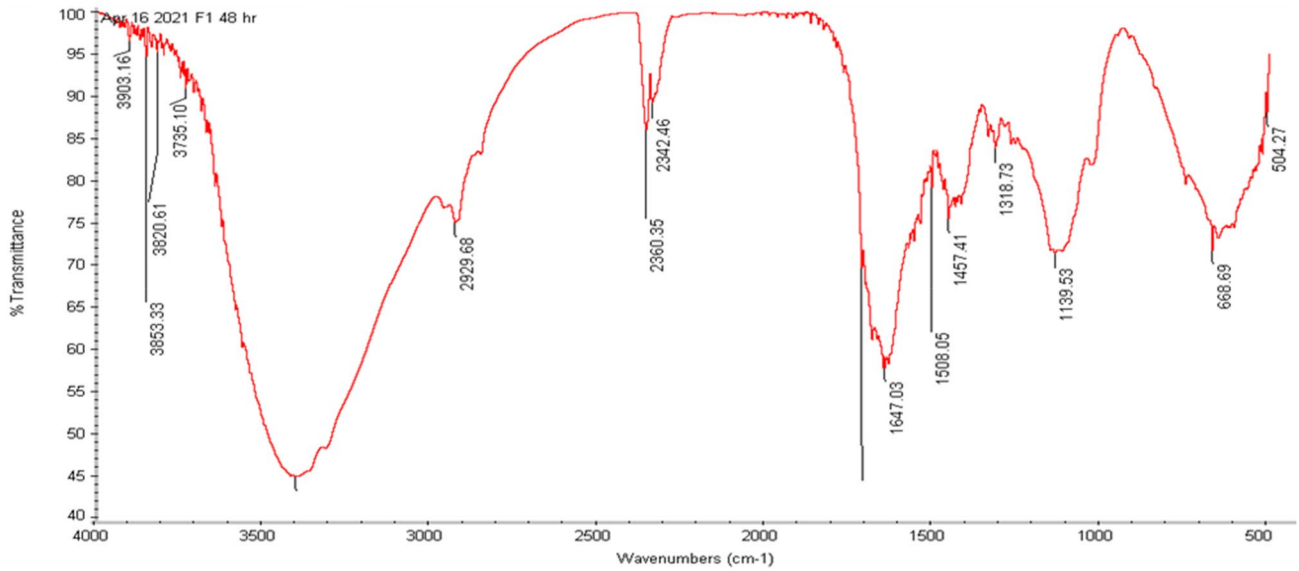


Fig. 11 Comparative analysis of crude oil degrading bacteria by lipase activity using tween 20 substrate



a



b

Fig. 12 **a** FT-IR (Control). **b** sample – EBPL0613-F2

Acknowledgements Expressing gratitude to Vels Institute of Science, Technology and Advanced Studies (VISTAS) and Eukpro Biotech Pvt Limited for generously providing the facilities and space to conduct our research.

Author Contributions SM—Responsible for experimental and writing the article, SM-Assisting in data plotting, SS—Assisting in the drafting of the article. AK—Data analysis and interpretation, RC—Critical revision of the article, AG—was responsible for conceptualization, writing and Editing the article.

Declarations

Conflict of interest The authors declare no competing interest.

Consent for Publication All authors agree to publish the following manuscript.

References

1. Sun H, Gao L, Xue C, Mao X (2020) Marine polysaccharide degrading enzymes: status and prospects. Compr Rev Food Sci Food Saf 19:2767–2796. https://doi.org/10.1111/1541-4337.12630
2. Sahoo RK, Swapnab Kumari K, Sahoo S, Das A, Gaur M, Dey S, Mohanty S, Subudhi E (2021) Bio-statistical optimization of lipase production by thermophilic *Pseudomonas formosensis* and

its application on oral biofilm degradation. Biocatal Agric Biotechnol 33:101969. https://doi.org/10.1016/j.bcab.2021.101969
3. Arnosti C, Wietz T, Bribkhoff I, Hejemann H (2021) The biogeochemistry of marine polysaccharides: sources, inventories, and bacterial drivers of the carbohydrate cycle. Ann Rev Mar Sci 13:81–108. https://doi.org/10.1146/annurev-marine-032020-012810
4. Birolli WG, Lima RN, Porto M (2019) Applications of marine-derived microorganisms and their enzymes in biocatalysis and biotransformation. Underexplor Potent Front Microbiol 10:1453. https://doi.org/10.3389/fmicb.2019.01453
5. Heidarrezaei M, Shokravi H, Huyop F, Rahimian Koloor SS, Petrů M (2020) Isolation and characterization of a novel bacterium from the marine environment for trichloroacetic acid bioremediation. Appl Sci 10:4593. https://doi.org/10.3390/app10134593
6. Zadjelovic V, Chhun A, Quareshy M, Silvano E (2020) Beyond oil degradation: enzymatic potential of *Alcanivorax* to degrade natural and synthetic polyesters. Environ Microbiol 22:1356–1369. https://doi.org/10.1111/1462-2920.14947
7. Khalid FW, LimZ S, Sabri S, Gomez-Fuentes C, Zulkharnain A, AhmadS A (2021) Bioremediation of diesel contaminated marine water by bacteria. A review and bibliometric analysis. J Mar Sci Eng 9:155. https://doi.org/10.3390/jmse9020155
8. Muthukumar B, Al Salhi MS, Narenkumar J, Devanesan S, Rao TN, Kim W, Rajasekar A (2022) Characterization of two novel strains of *Pseudomonas aeruginosa* on biodegradation of crude oil and its enzyme activities. Environ Pollut 304:119223. https://doi.org/10.1016/j.envpol.2022.119223
9. ElumalaiP PP, Alsalhi M, Huang M (2021) Characterization of crude oil degrading bacterial communities and their impact on biofilm formation. Environ Pollut 286:117556. https://doi.org/10.1016/j.envpol.2021.117556
10. Masi C, Tebiso A, Selva Kumar KV (2023) Isolation and characterization of potential multiple extracellular enzyme-producing bacteria from waste dumping area in Addis Ababa. Heliyon 4:12645. https://doi.org/10.1016/j.heliyon.2022.e12645
11. Meng Xu, Xinge Fu, Gao Yu, Duan L, Congchao Xu, Sun W, Li Y, Meng X, Xiao X (2020) Characterization of a biosurfactant-producing bacteria isolated from Marine environment: surface activity, chemical characterization and biodegradation. J Environ Chem Eng 8:1–9. https://doi.org/10.1016/j.jece.2020.104277
12. Ghasemi A, Moosavi-Nasab M, Setoodeh P, Mesbahi G, Yousefi G (2019) Biosurfactant production by lactic acid bacterium Pediococcus dextrinicus SHU1593 grown on different carbon sources: strain screening followed by product characterization. Scientific reports 9:5287. https://doi.org/10.1038/s41598-019-41589-0
13. Maitham A, Hawaj F, Mohammed M, Mohaini AJ (2022) Biosurfactant screening and antibiotic analysis of *Bacillus salmalaya*. Int J Curr Res Rev 14:56–64. https://doi.org/10.31782/IJCRR.2022.141209
14. Ron EZ, Rosenberg E (2019) Consequences of microbial interactions with hydrocarbons oils, and lipids: biodegradation and bioremediation. Handbook of hydrocarbon and lipid microbiology. Springer, Cham, p 50432
15. Din BU, Rafique M, Javed MT, Kamran MA, Mehmood S, Khan M, Sultan T, Munis MFH, Chaudhary HJ (2020) Assisted phytoremediation of chromium spiked soils by *Sesbania sesban* in association with *Bacillus xiamenensis* PM14: a biochemical analysis. Plant Physiol Biochem 146:249–258. https://doi.org/10.1016/j.plaphy.2019.11.010
16. Johnson JS, Spakowicz DJ, Hong BY, Petersen LM, Demkowicz P, Chen L, Leopold SR, Hanson BM, Agresta HO, Gerstein M, Sodergren E, Weinstock GM (2019) Evaluation of 16S rRNA gene sequencing for species and strain-level microbiome analysis. Nat Commun 610:5029. https://doi.org/10.1038/s41467-019-13036-1
17. Bharathi D, Rajalakshmi G (2019) Microbial lipases: an overview of screening, production and purification. Biocatal Agric Biotechnol 22:101368. https://doi.org/10.1016/j.bcab.2019.101368
18. OluwaseyeI AE, Japhel A, Efemena M, Adebayo L (2020) Isolation, optimization and molecular characterization of lipase producing bacteria from contaminated soil. Sci Afr 8:1–10. https://doi.org/10.1016/j.sciaf.2020.e00279
19. Shu-Bo Y, Jesús M, Zukui L (2022) Predicting crude oil properties using fourier-transform infrared spectroscopy (FTIR) and data-driven methods. Digit Chem Eng 3:1–9. https://doi.org/10.1016/j.dche.2022.100031
20. Tian X, Wang X, ShitaoPeng ZW, Zhou R, Tian He (2018) Isolation, screening, and crude oil degradation characteristics of hydrocarbons-degrading bacteria for treatment of oily wastewater. Water Sci Technol 78:2626–2638. https://doi.org/10.2166/wst.2019.025
21. Olicón-Hernández DR, Guerra-Sánchez G, Porta CJ, Santoyo-Tepole F, Hernández-Cortez C, Tapia-García EY, Chávez-Camarillo GM (2022) Fundaments and concepts on screening of microorganisms for biotechnological applications. Mini review. Curr Microbiol 27:79–373. https://doi.org/10.1007/s00284-022-03082-2
22. Youssef NH, Dunacn KE, Nagle DP, Savage KN, Knapp RM, McInerney MJ (2004) Comparison of methods to detect biosurfactant production by diverse microorganism. J Microbiol Methods 56:339–347. https://doi.org/10.1016/j.mimet.2003
23. Rodrigues LR, Teixeira JA, Van der Mei HC, Oliveira R (2006) Physiochemical and functional characterization of a biosurfactant produced by *Lactococcus lactis*53. Colloid Surf B 49:79–86. https://doi.org/10.1016/j.colsurfb.2006.03.003
24. Lázaro-Mass S, Gómez-Cornelio S, Castillo-Vidal M, Alvarez-Villagomez CS, Quintana P, De la Rosa-García S (2023) Biodegradation of hydrocarbons from contaminated soils by microbial consortia: a laboratory microcosm study. Electron J Biotechnol 61:24–32. https://doi.org/10.1016/j.ibiod.2004.02.002
25. Sethuramalingam B, Narasingam A, Marimuthu Ragavan R, Indu P, Nallusamy V, Khalid S, Saeedah Musaed A, Tse-Wei C (2022) Molecular characterization of biosurfactant producing marine bacterium isolated from hydrocarbon-contaminated soil using 16S rRNA gene sequencing. J King Saud Univ Sci 34:1–5. https://doi.org/10.3390/ijerph19020782
26. Oyetibo GO, Chien MF, Ikeda-Ohtsubo W, Suzuki H, Obayori OS, Adebusoye SA, Ilori MO, Amund O, Endo G (2017) Biodegradation of crude oil and phenanthrene by heavy metal resistant *Bacillus subtilis* isolated from a multi-polluted industrial wastewater creek. Int Biodeterior Biodegrad 120:143–151. https://doi.org/10.1016/j.ibiod.2017.02.021
27. Mobarak-Qamsari E, Kasra-Kermanshahi R, Moosavi-Nejad Z (2011) Isolation and identification of a novel, lipase-producing bacterium, *Pseudomnas aeruginosa* KM110. Iran J Microbiol 3:92–8
28. Raci G, Vukeli I, Kordi B, Radi D, Lazovi M, Nesi L, Pankovi D (2023) Screening of native trichoderma species for nickel and copper bioremediation potential determined by FTIR and XRF. Microorganisms 11:815. https://doi.org/10.3390/microorganisms11030815