Studies on diversity of bacteria associated with tarballs in Goa and ability of selected species to degrade tarballs

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By

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DECLARATION

I, Varsha Laxman Shinde hereby declare that this thesis represents work which has been carried out by me and that it has not been submitted, either in part or full, to any other University or Institution for the award of any research degree.

Place: Taleigao Plateau.
Date : 05-01-2023 Varsha Laxman Shinde

I/We hereby certify that the work was carried out under my/our supervision and may be placed for evaluation.

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Acknowledgment

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Dedicated to my beloved father
Late Shree Laxman Bapu Shinde

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Chapter 1

Introduction and Review of Literature

1.1 Marine pollution

Marine pollution is "the entry of any substance/ energy into the marine water, mainly due to human activities, which results in hazardous effects to marine organisms, changes the quality of water, and hinders marine activities" (Odeku and Paulos, 2017). Anthropogenic activities are mainly responsible for the pollution in marine environments. Heavy metals, gases, crude oil, plastic, pesticides, excess nutrients, industrial wastewater, and sewage water are well-known marine pollutants (Willis et al., 2022). Some of these marine pollutants are responsible for coral bleaching, biomagnification of heavy metals, death of fishes and other marine life forms, and accumulation of microplastics in the tissues of marine organisms. Interestingly, marine organisms such as turtles and fish ingest tarballs and plastics as food.

Crude oil is one of the important marine pollutants. As per a global estimate, every year the oceans are contaminated with 0.47–8.40 million tons of crude oil (petroleum hydrocarbons) (Global Marine Oil Pollution Information Gateway, http://oils.gpa.unep.org/facts/sources.htm#naturalsources). This has reportedly resulted in habitat destruction and loss, mass mortality of marine life forms, physiological dysfunctions such as decreased feeding and growth, and problems with breathing, locomotion, balance, and swimming (Adzigbli and Yuewen, 2018). There are reports of seabirds dying from crude oil getting stuck on their feathers (Shinde et al., 2017; Landrigan et al., 2020).

1.2 Crude oil and its biodegradation in the oceans

Crude oil is a type of fossil fuel found in the earth's crust. The consumption of crude oil and related petroleum products is growing steadily worldwide (https://www.iea.org/reports/oil-market-report-august-2022). As most oil transport is done via sea, occasional

accidental spillages introduce crude oil into marine environments. However, there are other ways, such as releasing ballast water from ships, operational discharges during offshore drilling, pipeline ruptures, and seepage from the seabed, by which crude oil can enter our oceans (Harayama et al., 1999; Das and Chandran, 2011).

Crude oil comprises a mixture of thousands of saturated aliphatic (straight-chain alkanes) and aromatic compounds (ring form) (Figure 1.1a and b), asphaltenes, and resins. It is mainly composed of carbon and hydrogen-containing compounds and low amounts of nitrogen, sulphur, and oxygen (NSO compounds) in resins and asphaltenes (Harayama et al., 1999).

a. b.

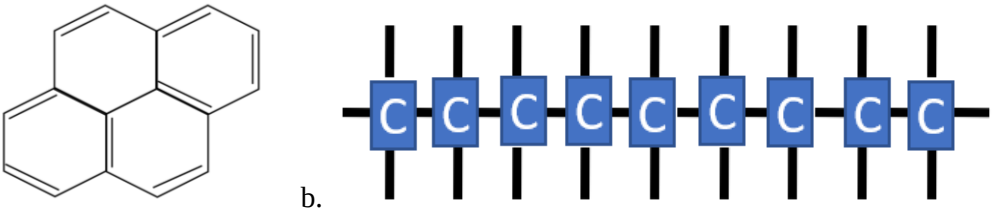


Figure 1.1: a) Ring structure of polycyclic aromatic hydrocarbon (PAH) compound, b) Chain structure of n-alkane compound.

Microorganisms play a vital role in remediating crude oil-polluted environments. Different groups of bacteria utilise crude oil for their growth (Xue et al., 2015). It is quite possible that some species of microorganisms from marine environments could have developed this ability over the years. More than 200 microbial genera, 200 species of bacteria (79 genera), cyanobacteria (9 genera), fungi (103 genera), and algae (19 genera) are reportedly involved in petroleum degradation (Xue et al., 2015).

The process of hydrocarbon breakdown by oil-eating microbes is shown in Figure 1.2. Hydroxylases such as mono-oxygenase and di-oxygenase are the key enzymes that are oxygen-dependent and used by bacteria in the biodegradation of alkanes and PAHs present in crude oil (Harayama et al., 1999). These enzymes are present in the integral membrane of bacterial cells. Bacterial enzymes can break down alkanes more easily than PAHs due to the complex ring structure of the latter. Low molecular weight alkanes and PAH compounds are more readily biodegradable than high molecular weight compounds. Byproducts such as volatile fatty acids, carbon dioxide, and hydrogen are

formed after hydroxylation and fermentation of crude oil hydrocarbons (Harayama et al., 1999).

Conceptually, while a population of hydrocarbon-degrading bacteria is initially less, it will rise once a pollutant like crude oil is introduced into the water body (Atlas, 1981). During this episode, microbial diversity declines (Aislabie et al., 2004) as oil-degrading bacteria dominate the environment. This rise in the bacterial community depends on the extent of hydrocarbon pollutants (Atlas, 1981).

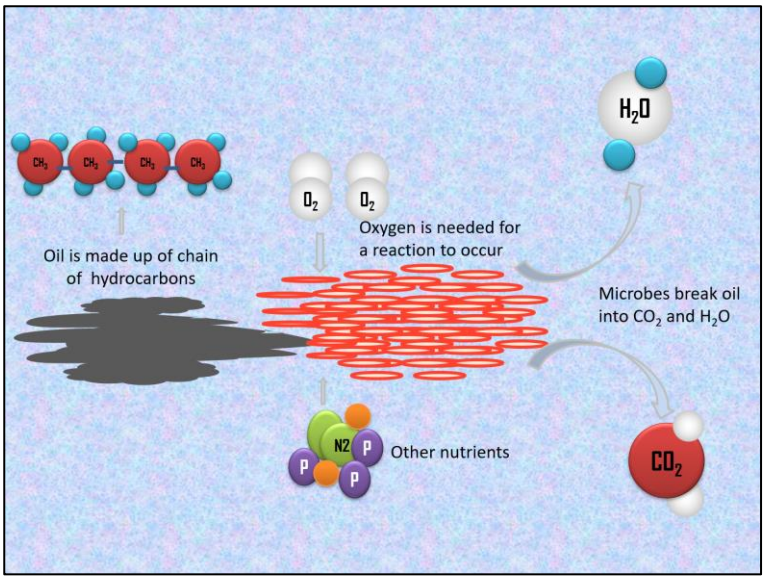


Figure 1.2: Depiction of hydrocarbon breakdown by bacteria [The image was prepared following the concept presented in https://education.gulfresearchinitiative.org/).

The following hydrocarbon-degrading bacterial genera have been reported from marine environments: *Achromobacter*, *Acinetobacter*, *Arthrobacter*, *Alcaligenes*, *Arcobacter*, *Bacillus*, *Brevibacterium*, *Chromobacterium*, *Cycloclasticus*, *Coryneforms*, *Corynebacterium*, *Flavobacterium*, *Micrococcus*, *Microbacterium*, *Mycobacterium*, *Nocardia*, *Pseudomonas*, *Sarcina*, *Serratia*, *Streptomyces*, *Vibrio* and *Xanthomonas* (Atlas, 1981; Xue et al., 2015). Some of them are obligate hydrocarbon degraders. These obligate hydrocarbonoclastic bacteria such as members of *Alcanivorax*, *Cycloclasticus*, *Marinobacter*, *Oleispira* and *Thalassolituus* might have evolved indigenous to the marine environment. These bacteria, however, may not be easily detected in unpolluted

waters, but they dominate after a crude oil pollution event (Yakimov et al., 2007; Brooijmans et al., 2009).

Interestingly, high molecular weight fractions of crude oil are not degraded easily and remain in the environment as tars. Tars are the most recalcitrant state of crude oil. When tars aggregate, they form tarballs. Tarballs serve as a substrate for several microbial life forms.

1.3 Tarballs and their formation

Tarballs are blobs of crude oil remnants (Figure 1.3). Unlike terrestrial environments, oceans have environmental conditions supporting tarball formation. Tarballs have solid or semi-solid consistency. Their size varies from a few millimetres to several centimetres. They can be black to brown in colour. Their texture can be brittle, hard, or soft, and very sticky, depending on their formation conditions (Chandru et al., 2008; Wang et al., 2011). Some tarballs have a uniform structure throughout, while others may be solid externally and liquid internally (Goodman, 2003).

As high molecular weight components of crude oil, tar residues can be tar mats, tarballs, or tar patties (Warnock et al., 2015). Tar residues accumulate on various marine substances. When they accumulate on mollusc shells, they are referred to as tar shells (Tran et al., 2019). Tarballs are grouped into pelagic, benthic, and beached tar residues. Pelagic tar residues float on the sea surface. In contrast, benthic tar residues are submerged on the seafloor (Iliffe and Knap, 1979), and beached tar residues are deposited on beaches due to sea currents and wave action.

Figure 1.3: A photograph of tarballs scattered on the seashore (Photo credit: Dr. Suneel Vasimalla, CSIR-NIO, Goa, India).

There are two theories on tarball formation (Warnock et al., 2015; Figure 1.4). In these theories, tarballs are referred to as 'sedimented tar' and 'weathered tarballs'. Sedimented tarballs are formed 1) due to the erosion of oiled sand or 2) when thick fresh oil is directly submerged into the bottom of the sea without going through any physical, chemical, or biological changes, getting mixed with sand and shells and breakdown to form tarballs.

According to the 'surface weathering' theory, tar residues are formed when the crude oil released into the marine environment by anthropogenic or natural activities changes over time (Chandru et al., 2008). It involves various physical, chemical, and biological processes. The physical processes include spreading, evaporation, dispersion, dissolution, sedimentation, and emulsification. The chemical and biological processes include photo-oxidation, photo-degradation, and biodegradation (Harayama et al., 1999; Wang et al., 2011).

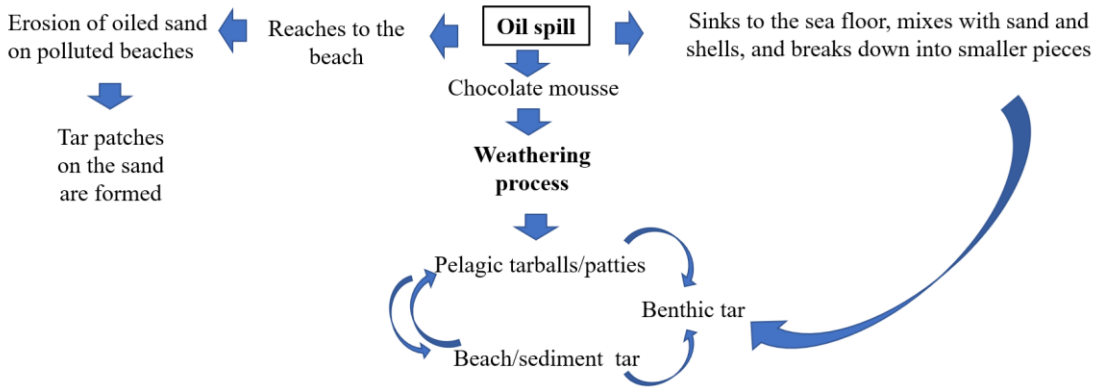


Figure 1.4: Depiction of tarball formation as described in Warnock et al. (2015).

Though the exact mechanisms of tarball formation are unknown, the weathering processes presumably co-occur. The weathering depends on environmental conditions such as temperature, wind velocity, ocean current dynamics, dissolved oxygen concentration, type and source of oil, and time and amount of oil spillage (Jordan and Payne, 1980).

According to Chandru et al. (2008), the following events occur during weathering: The crude oil initially loses its low molecular weight compounds, which are water-soluble, biodegradable, and can evaporate. Then, water in oil emulsions is formed due to strong ocean currents and wind. However, large molecular weight compounds are not easily affected by the above processes and remain in water bodies for a long time. They aggregate to form blobs. These blobs are known as tarballs. The weathering of crude oil is depicted in Figure 1.5. Warnock et al. (2015) reviewed the research conducted on marine tars since the 1970s. Their review focused on the formation, distribution, transportation, chemical composition, source tracking, and degradation of tarballs.

Chemically, tarballs differ from their parent, crude oil. This depends on the formation conditions (Wang et al., 1998). If one type of weathering process becomes dominant, it will influence the composition of resulting tarballs (Chandru et al., 2008). Heavy hydrocarbons such as PAHs are enriched in tarballs after lighter hydrocarbon fractions dissolve/ evaporate (Harayama et al., 1999; Nemirovskaya, 2011). Meta-toluic acid, a toxic tarball compound, persists in marine environments (Prakash et al., 2008).

Interestingly, oxygen from the atmosphere gets mixed with hydrocarbons present in crude oil, resulting in oxygenated hydrocarbons (oxyhydrocarbons). Oxyhydrocarbons are recalcitrant in nature (Kimes et al., 2014).

Tarballs have high metal concentration than the parent crude oil. High molecular compounds in weathering crude oil act as chelating groups on which metal ions bind from seawater (Liu et al., 2012). Metals such as vanadium, nickel, copper, iron, magnesium, and cobalt have been reported from tarballs (Wong et al., 1976; Hegazi, 2009; Zare-maivan, 2010; Liu et al., 2012), but their concentration varies across different reports. Metals in tarballs can facilitate the transformation of hydrocarbons through oxidation, resulting in environmentally persistent free radicals (Kimes et al., 2014).

1.4 Implications of tarballs

The adverse effects of tarballs on surrounding environment are poorly understood compared to that of pollution caused by fresh oil spillage. Possible adverse effects of tarball pollution are depicted in Figure 1.6 (Shinde et al., 2017). Available studies suggest that weathered oil is less toxic than crude oil (Warnock et al., 2015). Newly formed tarballs are considered more harmful than older or highly weathered ones because the former contain abundant water-soluble and low molecular weight hydrocarbon compounds, which are considered more toxic (Harayama et al., 1999).

Tarball pollution results in economic and ecological losses. The seawater contaminated with tarballs has a petroleum-like odour, making it less suitable for swimmers. Tarballs pose a high risk to human health as some individuals can be allergic to tarballs (https://cr4.globalspec.com/thread/56411/Is-Crude-Oil-Hazardous-To-Your-Health). Tarballs that travel towards the coast can stick to the fishing nets installed in the sea by fishermen, making them difficult to clean.

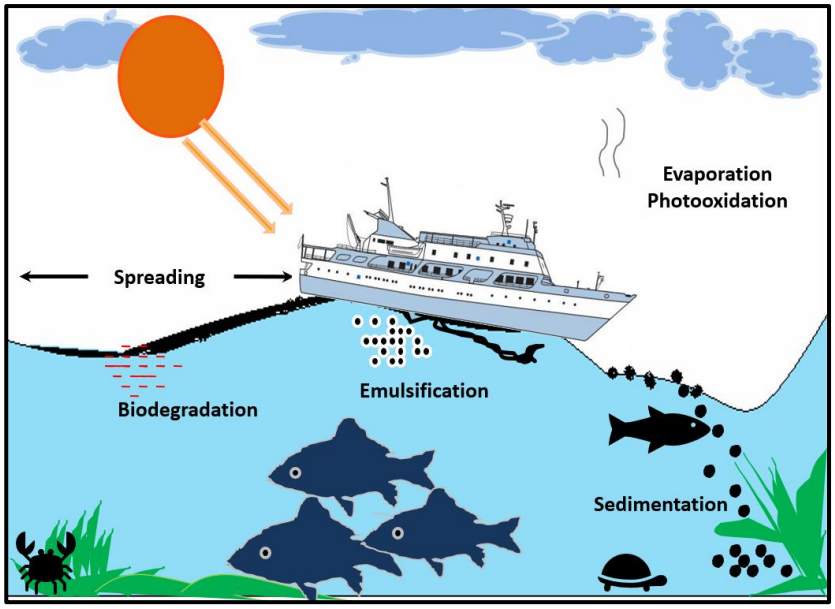


Figure 1.5: Depiction of weathering of crude oil in open ocean [(The image was prepared based on Ezeh et al. (2019)].

As residual crude oil, tarballs contain abundant carcinogenic PAHs, potentially affecting humans and marine organisms on long-term exposure (Shukla and Cameotra, 2012). Tarball surface serves as a substrate for bacteria, fungi, unicellular algae, and other microbes (Nair et al., 1972; Tao et al., 2011). Tao et al. (2011) analysed tarballs for total aerobic bacterial counts and the presence of *Vibrio vulnificus*, a human pathogen. They recorded higher total bacterial counts in tarballs than in sand and seawater. The presence of *V. vulnificus* was 10–100 times higher in tarballs than in sand and seawater. It needs to be clarified why the tarball surface supports a high concentration of microbes. It is suggested that microbes feed on the by-products formed by hydrocarbon-degrading bacteria in/on tarballs (Tao et al., 2011).

Animals and plants may die because of the exposure to fumes released by tarballs. Sea animals, such as turtles, can accidentally consume tarballs as food, leading to their death (Goodman, 2003; Warnock et al., 2015). Horn et al. (1970) reported tar residues from the stomach of an epipelagic fish, *Scomberesox saurus*, and stated that the toxic components of tarballs could easily enter the food chain.

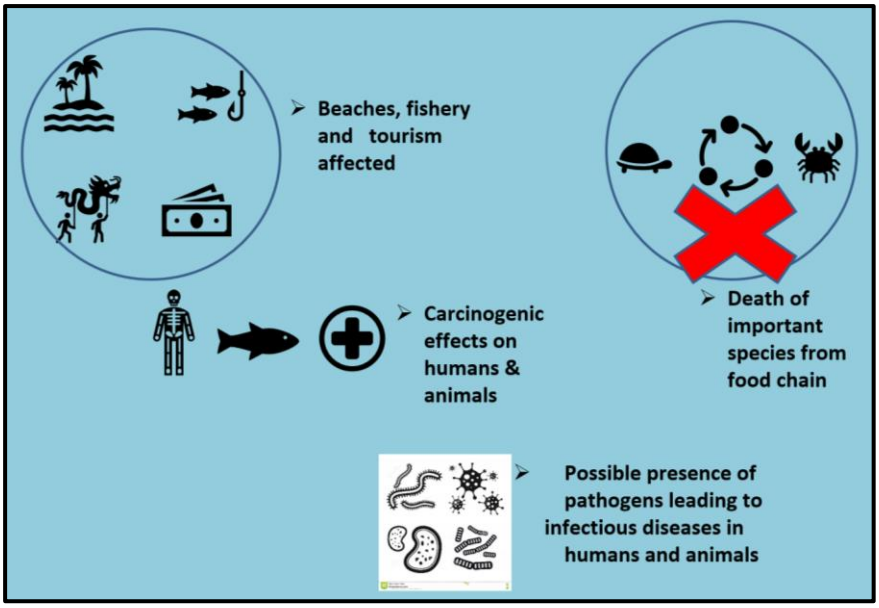


Figure 1.6: Implications of tarball pollution.

Tarballs become heavier after getting mixed with sand and sink to the bottom of the sea, disturbing the living conditions of benthic organisms, including nursing grounds of fish and shellfish (http://oils.gpa.unep.org/facts/habitats.htm). The toxic contents of less-weathered tarballs can be lethal to marine organisms such as bivalves and shrimps, which serve as food for pelagic fishes, leading to a decrease in food to the next level animals in the food chain, and thus indirectly affecting fisheries (https://timesofindia.indiatimes.com/india/tar-balls-hit-beaches-fauna-and-goa-tourism/articleshow/37675282.cms). The Parish government reported more than 12-meter land loss of salt marshes by suffocation caused by tar-like clumps (Bruckner, 2011). In addition, the cleaning drive of tarballs using heavy equipment can disturb marine niches.

1.5 Bacterial association with tarballs

Microorganisms from seawater attach to the tarball surface mainly to gain nutrients and colonise it in large numbers. As tarballs are moved from their origin to distant places due to wind and oceanic currents, microbes from diverse marine waters can associate with them. Tarballs can contribute to the change in bacterial diversity of marine sites. As a substrate, tarballs support phylogenetically diverse microorganisms, including human pathogens (Tao et al., 2011; Itah and Essain, 2005). These pathogens enter the rivers via various wastes and then reach the oceans. Their number is high in estuaries and coastal line areas (Sudha Rani et al., 2018).

1.6 Bioremediation aspects with tarball-associated microbes

In bioremediation, life forms, including microorganisms, remediate a polluted environment. It is low-cost, effective and environment-friendly. Microorganisms can degrade toxic pollutants, such as crude oil, metals, pesticides, and other substances, that are deliberately released into our environment due to various anthropogenic activities. Microbes can reduce the toxicity level of these pollutants and recycle them. Crude oil is a common pollutant of significant concern to water bodies, including marine waters. Some species of marine bacteria might be adapted to thrive in crude oil-contaminated sites. These bacteria could be involved in partial degradation of crude oil after an oil spill event. A bacterial species may not be capable of efficiently utilising thousands of different crude oil hydrocarbons. A bacterial consortium is probably better suited for such endeavours (Shinde et al., 2017, 2020).

Biodegradation, however, is slow in the water column due to the limited availability of oxygen and micronutrients such as nitrogen and phosphorus. There are a few well-known examples where bioremediation has been successfully employed to clean up crude oil-contaminated sites on the land. Oil-zapper, an innovative product developed by The Energy and Resources Institute (TERI), New Delhi, India (Gupta et al., 2011), is a bacterial consortium of *Acinetobacter baumannii, Alcaligenes odorans, Bukhardica cepacer, Pseudomonas aeruginosa* and strain S-30. Various industries such as Reliance and ONGC in India have used this product (https://www.teriin.org/technol%20ogy/oilzapper-and-oilivorous, http://www.otbl.co.in/pdf/December-2018. pdf).

Bioremediation in marine environments, however, is challenging. Environmental factors such as temperature, available nutrients, salinity, dissolved oxygen concentration, and physical state affect the biodegradation of crude oil in marine environments. High temperature favours the growth and degradation activity of microbes with the added benefit of lower viscous oil (Liu et al., 2017). Indigenous microbes play an essential role in crude oil degradation in marine environments. Generally, these microbes are present in low numbers. However, their population size increases in oil-spill areas. It is suggested that a wide array of chemical compounds in crude oil enable microbes to opt

for resource partitioning. This results in the temporal succession of microbial communities in the surrounding ecosystem (McGenity et al., 2012).

In the case of tarballs, the low surface area to volume ratio of tarballs limits their microbial degradation (Salleh et al., 2003). It is suggested that tarballs and oil mousses are less viscous at higher temperatures and have a greater surface area for physical breakdown and microbial degradation within sediments (Beazley et al., 2012). It has also been suggested that increased metal concentration enhances hydrocarbons' toxicity and affects microbial activity (Shukla and Cameotra, 2012).

There are limited studies on microbial degradation of tarballs. Raikar et al. (2001) studied thraustochytrid protists isolated from coastal waters, sediments, crude oil, and tarballs collected from Goa, India, for their ability to degrade tarballs. The growth of thraustochytrids was found to be very high in Oppenheimer-Zobell-Gaertner (OZG) and Modified Vishniac's (MV) media containing crude oil than in the basal salt medium and basal mineral salt medium. The addition of tarball to peptone broth was found to improve the growth of thraustochytrids. The authors claimed that the thraustochytrids isolated from coastal waters could degrade about 71 % of tarballs in a month.

Though numerous reports on hydrocarbon-degrading microbes are available, two reports are mentioned here. Prakash et al. (2008) reported *Pseudomonas* sp. strain GUI13 capable of degradation of meta-toluic acid, an essential component of tarballs. Rodrigues et al. (2010) reported a marine bacterium of the genus *Alkaligenes* capable of degradation of dibenzothiophene, sulphur-containing polycyclic aromatic hydrocarbons of tarballs.

In a recent study, Shinde et al. (2020) screened 38 tarball-associated bacteria from Betul beach, Goa, India, for crude oil degradation. Four bacterial strains, *Alcanivorax* sp. Betul-O, *Marinobacter* sp. Betul-26, *Pseudomonas* sp. Betul-14, and *Pseudomonas* sp. Betul-M were selected for preparing bacterial consortia (eleven consortia). These eleven bacterial consortia were studied for degradation of n-alkanes and PAHs of tarballs based on gravimetric and GC–MS-MS analyses. In their study, the bacterial consortia depleted 53.69–97.78 % and 22.78–61.98 % of n-alkanes and PAH compounds, respectively, within 45 days. A bacterial consortium comprising *Pseudomonas* sp. Betul-14, *Pseudomonas* sp. Betul-M, and *Alcanivorax* sp. Betul-O exhibited promising tarball

degradation abilities with 97.78 % and 61.98 % degradation of n-alkanes and PAH, respectively, within 45 days.

1.7 Global studies on tarball-associated bacteria

Tarballs form in the open ocean and float on the ocean's surface till they are moved to nearby shorelines due to wind and wave currents. Pollution is a severe problem, as many global coastlines are frequently affected by the deposition of tarballs. In tarball microbiology, there have been more studies on tarball-associated bacteria than on the fungi (Shinde et al., 2017; https://tarballs.in). Tarballs provide nutrients to various bacteria that colonise them in good numbers. These tarball-associated bacteria may be indirectly involved in the degradation of tarballs (Shinde et al., 2017, 2020). Till now, 74 bacterial genera have been reported from tarballs (https://tarballs.in). The diversity of tarball-associated bacteria was reviewed by Shinde et al. (2017). Figure 1.7 shows the locations from where tarballs were collected for microbiological studies.

Figure 1.7: Tarball sampling sites for microbiological studies. (This map was created using the tools available at https://www.visme.co/map-generator).

Table 1.1: Colony-forming units (CFUs) of bacteria reported from tarballs

Sl. No.	Reference	Location	CFU/g of tarball
1	Itah and Essien (2005)	Nigeria	$3(\pm0.02)\times10^5$
			$3(\pm0.1)\times10^5$
			$3(\pm0.26)\times10^4$
			$4(\pm0.13)\times10^4$
			$3(\pm0.02)\times10^4$
			$3.3(\pm0.16)\times10^5$
			$3(\pm0.3)\times10^5$
			$3(\pm0.11)\times10^5$
			$3(\pm0.22)\times10^5$
			$3(\pm0.21)\times10^4$
			$4(\pm0.23)\times10^4$
			$3.2(\pm0.18)\times10^5$
			$3(\pm0.4)\times10^5$
			$5(\pm0.2)\times10^4$
			$4(\pm0.05)\times10^4$
			$3.1(\pm0.18)\times10^5$
			$3.18(\pm0.22)\times10^6$
			$4.2(\pm0.04)\times10^5$
			$4.4(\pm0.14)\times10^4$
			$3.6(\pm0.21)\times10^5$
2	Tao et al. (2011)	Gulf of Mexico	$5.1 \pm 1.2\times10^6$
			$8.3 \pm 0.2\times10^6$
			$7.1 \pm 1.2\times10^6$
			$5.4 \pm 0.2\times10^6$
			$8.0 \pm 1.5\times10^6$
3	Petra et al. (2012)	Australia	4.1×10^4

Historically, in the early 1970s, microbes such as blue-green algae, diatoms, yeast, protists, and other forms of life such as barnacles and cirripede were reported from tar-like material from India (Nair et al., 1972). Bacterial counts in the form of colony-

forming units (CFUs) per gram of tarballs reported by Itah and Essien (2005), Tao et al. (2011) and Petra et al. (2012) are shown in Table 1. Itah and Essien (2005) reported heterotrophic bacteria associated with tarballs from Ibeno beach on the Nigerian coast in the range of 3.0×10^4–3.2×10^6 CFU/g of the tarball. They suggested that only 0.001 % of the total bacterial counts obtained could degrade the oil. Tao et al. (2011) reported heterotrophic bacterial counts of 5.1×10^6–8.3×10^6 CFU/g of tarball from the Gulf of Mexico. They observed a greater number of human pathogenic *V. vulnificus* in tarballs than in seawater and sand.

Petra et al. (2012) investigated the oil-degrading bacterial community from previously contaminated seawater of the Gulf St. Vincent (Southern Australia (SA)) and weathered oil (=tarballs) from the refinery of SA, Australia. They reported 6.6×10^1 CFU/ml total hydrocarbon-degrading population from seawater and 4.1×10^4 CFU/ml from weathered crude oil. In a study involving oil mousses (the predecessor of tarballs), Liu and Liu (2013) conducted a metagenomic analysis to study the bacterial community structure. They reported 54–86 oil-mousse-associated bacterial genera representing five phyla from three locations. In their study, most bacterial taxa (95–99 %) belonged to the *Proteobacteria*.

In a study unravelling the diversity of tarball-associated microorganisms using a culture-independent approach, Bacosa et al. (2016) analysed 44 tarball samples from Galveston and Mustang Island in Texas, United States. *Alcanivorax* and *Psychrobacter* were found to be the dominant genera in the tarballs of Galveston Island. These two genera are known as oil degraders (Harayama et al., 2004; Giudice et al., 2010). *Pseudoaltermonas* was dominant in the tarballs of Mustang Island, followed by *Psychrobacter, Oceanospirillales* and *Alcanivorax*. The tarballs from Galveston Island were rich in alkane-degrading bacteria, while those from Mustang Island were rich in PAH-degrading bacteria. The depletion of alkanes in tarballs of Galveston and Mustang Island was 21 % and 24 %, respectively. In comparison, the per cent depletion of PAH was 55 on Galveston Island, which increased to 63 % when travelling to Mustang Island.

Nkem et al. (2016) isolated and reported two bacteria, *Cellulosimicrobium cellulans* and *Acinetobacter baumannii* from tarballs of Rhu Sepuluh beach, Terengganu, Malaysia, using an enrichment technique. These bacteria could utilise 64.4 % and 58.1 % of diesel,

respectively. Shinde et al. (2018) reported forty-five bacteria from tarballs from Goa, India. The CFUs of bacteria reported in this study were 8.3×10^6 and 5.07×10^5 CFUs per gram of tarball. Of the 20 bacterial genera identified from the tarball samples in Betul beach, 16 are new records for tarball-associated bacteria. *Alcanivorax, Bhargavaea, Nitratireductor, Oceanimonas* and *Tistrella* were recovered from the enrichment culture technique.

In a metagenomic approach, Fernandes et al. (2019) determined tarball-associated bacterial diversity from the Vagator and Morjim beaches of north Goa. The tarball samples were analysed based on V_3–V_4 regions of 16S rRNA gene data using the Illumina Miseq Platform. They concluded that Proteobacterial members were dominant in both Vagator (≥85.5 %) and Morjim (≥94.0 %) tarball samples. Tran et al. (2019) recently investigated bacterial communities from tar shells (tar accumulated in shells), tarballs, and sand samples on the Alabama Gulf Coast, USA, at intertidal and supratidal zones using PCR-DGGE and DNA sequencing. Tar shells at intertidal and supratidal zones were dominant in *Desulfovibrio* species and *Actinobacteria* species, whereas these species were absent in tarballs at the supratidal zone. *Pseudoalteromonas* spp. were found to be prevalent in both tarballs and tar shells.

1.8 Identification of tarball-associated bacteria

1.8.1 Bacteria

Domain *Bacteria* include prokaryotic life forms that lack a true nucleus. They are characterised by bacterial-specific ribosomal ribose nucleic acid (rRNA) of the 70S, ester-linked membrane lipids, diacylglycerol diethers, and lack of cell organelles such as chloroplast and mitochondria. Size of a typical bacterial cell varies between 1 to 5 µm. They replicate by binary fission (Stuart, 2005). They are found in rod, cocci, spiral, and comma shapes (Figure 1.8). They are grouped as Gram-positive and Gram-negative based on Gram staining of the peptidoglycan and lipid content of the cell wall (Smith and Hussey, 2005). They can be autotrophs, heterotrophs and chemolithotrophs based on how they obtain energy.

1.8.2 Traditional approach to bacterial identification

Observation and recording of bacterial colony characters such as size, shape, colour, margin, and elevation is the primary step towards bacterial identification. The traditional approach to bacterial identification was based on morphotaxonomic characters such as Gram nature, size, shape, and presence/ absence of cell organelles (spores/ capsule/ pili/ flagella) of an individual bacterial cell. Traditionally, variations in the production of enzymes, pigments, co-metabolites, bi-products, end products and secondary metabolites produced by bacteria were used as identification criteria (Prakash et al., 2007).

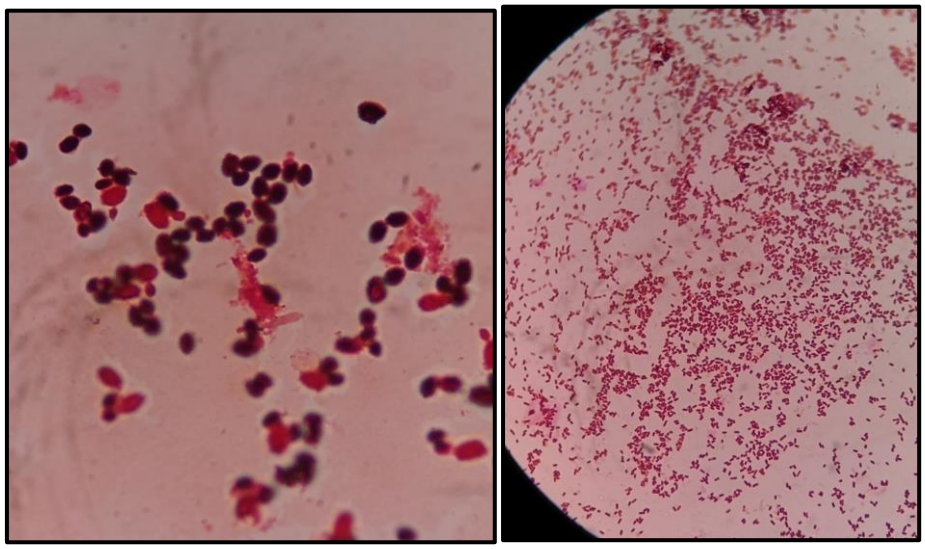


Figure 1.8: Bacterial cells under compound microscope after Gram staining.

1.8.3 Modern methods for bacterial identification

In the modern approach to bacterial identification, bacteria are identified based on G+C content, DNA-DNA hybridisation, 16S rRNA gene sequencing, cell wall lipid profiles (FAME), protein profiles (MALDI) and API profiles (Vandamme et al., 1996). Of these, 16S rRNA gene sequencing is a widely accepted and used method of bacterial identification.

1.8.4 16S rRNA gene sequencing-based bacterial identification

With the advent of DNA sequencing method, 16S rRNA gene sequencing has been extensively used in bacterial identification (Woo et al., 2008). This method is considered the most reliable method for bacterial identification in microbiology. The variation in gene sequence data is used for phylogenetic analysis to decipher the evolutionary relationships among the bacteria. The structures of 70S ribosome and 16S rRNA gene are shown in Figure 1.9.

The advantages of using the 16S ribosomal RNA gene in molecular biology techniques are as follows: In the process of evolution, the 16S rRNA gene is a highly conserved gene among bacterial phyla. It is ubiquitous, i.e., present in all bacteria. The function of this gene has not changed over time (Janda and Abbott, 2007). Matching the 16S rRNA gene sequences with publicly available nucleotide databases will identify organisms. Closely related species will have maximum similarity in sequences than distantly related species.

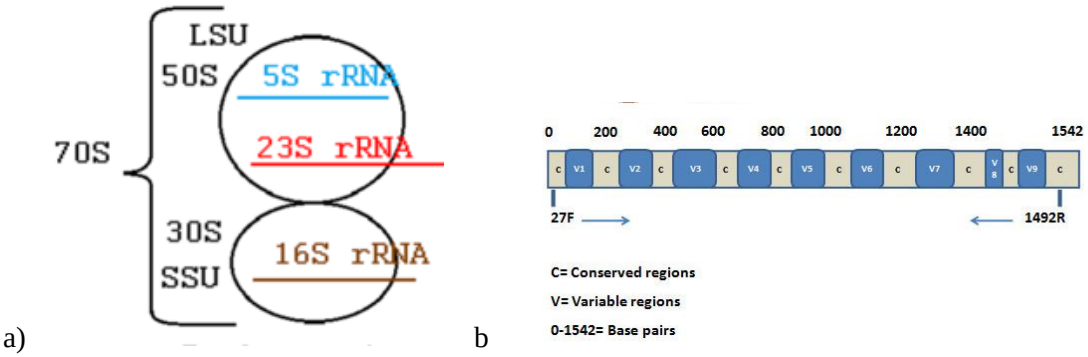


Figure 1.9: a) Structure of prokaryotic 70S ribosome (Recreated based on https://commons.wikimedia.org/wiki/File:Ribosome_Structure.png), b) Structure of 16S rRNA gene (Recreated based on https://help.ezbiocloud.net/16s-rrna-and-16s-rrna-gene/).

1.8.5 Polyphasic approach to bacterial identification

Describing new species or new genera based on a single identification method is error-prone. Hence the polyphasic approach is used for more accuracy (Das et al., 2014). The term ‘polyphasic’ was first coined by Colwell (1970). This approach combines all genotypic and phenotypic methods for identifying and grouping organisms.

1.9 Scenario of tarball pollution in Goa state, India

The west coast of India is regularly affected by tarball deposition, as the Arabian Sea is a major route for oil tankers (Suneel et al., 2016). According to Ingole and Sivadas (2007), oil spills along the Indian coast are quite frequent, and 71 % of such incidents occur in the Arabian Sea and the west coast of India. Constant coast monitoring shows that tarball depositions are more frequent than ever and tarballs are deposited along with the west coast of India regularly during pre-monsoon and monsoon seasons (Suneel et al., 2014). The coasts of Gujarat (Tithal, Maroli, Umbergam, and Nargol), Maharashtra (Mumbai, Ratnagiri, Malvan), Goa, Karnataka (Karwar, Kundapur) and Kerala (north of Cochin, Cherai, Kaipamangalam and Chavakad (Kaladharan et al., 2004; Suneel et al., 2014; https://www.newindianexpress.com/states/karnataka/2022/jun/13/tar-balls-surface-on-kodi-beach-2464867.html) have been in the news for tarball pollution. As Goa state is a major global tourist destination situated on the west coast of India, tarball deposition on its tourist beaches is a significant concern to the state's authorities.

Goa has a coastline of 101 km. It is known for its tourist destinations and is famous worldwide for its beautiful tourist places and pristine beaches. Tourism and fisheries are the main livelihood activities of the local community. Tarball deposition along the Goan coast has been reported since the 1970s (Nair et al., 1972; Dwivedi and Parulekar, 1974; Qasim, 1975; Dhargalkar et al., 1977; Oostdam, 1984; Kadam, 1988; Sengupta et al., 1993; Kadam and Rokade, 1996; Kaladharan et al., 2004; Shinde et al., 2017).

Tarballs are deposited on Goan beaches from May to October almost every year. The beaches of Goa that commonly get affected by tarball pollution are Arambol, Ashvem, Calangute, Candolim, Colva, Miramar, Mobor, Velsao etc. (Rekadwad and Khobragade, 2015). Coastal ecology and the local economy are possibly affected by tarball deposition (Rekadwad and Khobragade, 2015). The current scenario of tarball deposition along the Goa coast is illustrated in Figure 1.10.

At present two coastguard pollution control vessels are being built in Goa shipyard (https://pib.gov.in/PressReleasePage.aspx?PRID=1877732) equipped with advanced technology to monitor the pollution occurs in Indian water. This could help in tackling the tarball pollution. India has proposed regional contingency plan for oil spills in the

the National Pollution Response Exercise (NATPOLREX) conducted in 2022 at Marmugoa, Goa (https://timesofindia.indiatimes.com/city/goa/india-presses-for-regional-contingency-plan-for-oil-spills-tests-natl-response-off-goa/articleshow/90966292.cms).

1.10 Research gaps

When this thesis work was initiated in the year 2015, there were limited reported studies on tarball-associated microbes. The reported studies were primarily morphology-based. There were a few studies available on 16S rRNA gene sequence data of culturable bacterial diversity from tarballs from India as well as foreign countries. The presence of human pathogens on tarballs, other than *Vibrio* species, was not well-studied in India and around the globe. Indigenous bacterial populations from tarballs were rarely checked for their hydrocarbon degradation abilities. There were few reports on quantitative and qualitative analysis of hydrocarbon degradation by tarball-associated bacteria/ microbes. Documentation of tarball-associated microbes and their diversity in the form of a website was unavailable.

1.11 Objectives of the thesis and significance of this work

Majority of Earth's microbes are yet to be explored. Many untapped places, substrates, and niches are available for exploration. There are countless microbes waiting to be isolated, characterised, correctly identified and documented for biotechnological purposes. Tarballs are one of such microbial substrates formed and found in our oceans. Studying the diversity of tarball-associated bacteria is important to improve our understanding of their taxonomy, bioremediation potential and their ability to cause diseases in human and marine life forms.

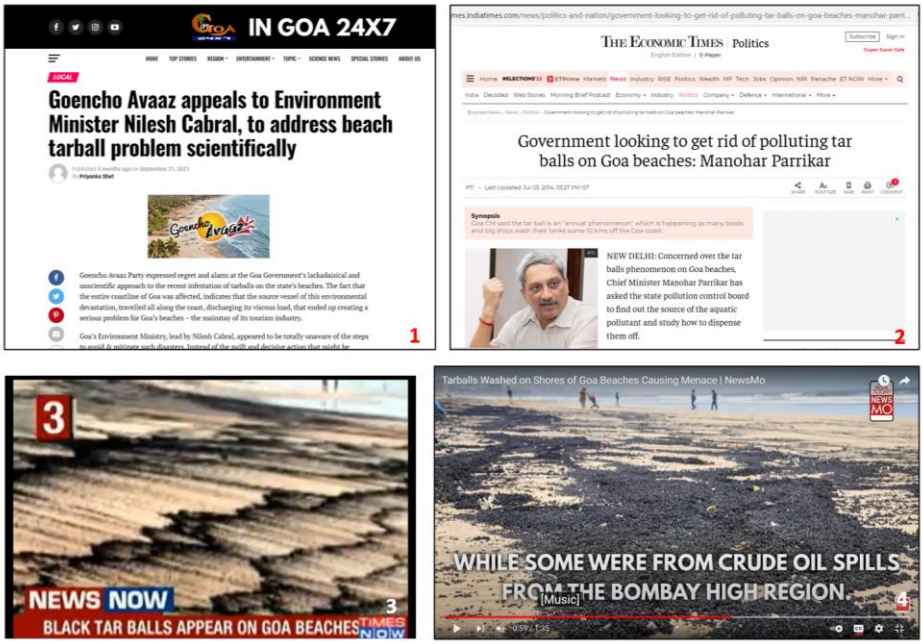


Figure 1.10 Screenshots of news items published in newspapers and news channels depicting tarball pollution menace in Goa state, India.

Sources:
1. https://ingoanews.com/goencho-avaaz-appeals-to-environment-minister-nilesh-cabral-to-address-beach-tarball-problem-scientifically/
2. https://timesofindia.indiatimes.com/videos/news/black-tar-balls-appear-on-goa-beaches/videoshow/6468118.cms?from=mdr
3. https://economictimes.indiatimes.com/news/politics-and-nation/government-looking-to-get-rid-of-polluting-tar-balls-on-goa-beaches-manohar-parrikar/articleshow/37703160.cms?from=mdr
4. https://www.youtube.com/watch?v=na_8TWzlihY

This thesis work is focused on culturable diversity of bacteria associated with tarballs, their identification using modern molecular biology tools such as 16S rRNA gene sequencing, their role in hydrocarbon degradation in tarballs, and documentation of these bacteria for future studies. To accomplish these goals, the four research objectives were framed.

The first two objectives of the thesis involve enrichment and isolation of tarball-associated bacteria, including pathogenic groups and performing their phylogenetic characterization based on 16S rRNA gene-data. The third objective deals with evaluating the crude oil and tarball degradation properties of select isolates of tarball-associated bacteria. The fourth objective deals with creation of an online database of bacteria reported from tarballs.

Research objectives:

1. **To collect tarball samples from Goan beaches and isolate bacteria associated with them**

   To characterise the diversity of bacteria from tarballs, tarball samples were collected from two beaches in South Goa (Betul) and North Goa (Candolim). The samples were processed for bacterial isolation using serial dilution and enrichment culture techniques. Isolates were phylogenetically analysed using 16S rRNA gene sequences. Objective 1 is covered in Chapter 2 of the thesis.

2. **To assess the role of tarballs as carriers of human pathogens**

   To investigate the presence of pathogenic groups of bacteria from tarballs, tarball samples were collected from two beaches in South Goa (Betul) and North Goa (Candolim). The samples were processed for isolation of pathogenic groups of bacteria using pathogenic specific growth media. The method used for isolation was serial dilution. Isolates were phylogenetically analysed using 16S rRNA gene sequences. Objective 2 is covered in Chapter 3 of the thesis.

3. **To investigate the degradation of tarballs by bacteria**

   To investigate the role of tarball-associated bacteria in hydrocarbon degradation, 38 tarball-associated bacterial isolates from Betul beach in Goa were screened for crude oil degradation. Based on the results obtained after two screening methods, an experiment was designed to study the degradation of tarballs using bacterial consortia. Eleven combinations of bacterial consortia were prepared using four bacterial isolates. Tarball degradation by these consortia was examined after 45 days of incubation. Results were analysed using GC-MS-MS analysis. Objective 3 is covered in Chapter 4 of the thesis.

4. **To perform taxonomic analysis of select marine bacteria associated with tarballs based on a polyphasic approach and development of an online database**

   For the documentation of tarball-associated bacteria, https://tarballs.in was designed using www.wordpress.com. Tarball-associated bacteria documented on this website belong to 75 genera (https://tarballs.in). Objective 4 is covered in Chapter 5 of the thesis.

Chapter 2

Isolation, Enumeration, and Phylogenetic Analysis of Heterotrophic Bacteria from Tarballs

2.1 Introduction

Tarballs are semisolid lumps of weathered crude oil formed in the open ocean. They get advected long distances by ocean currents and waves to reach beaches and cause pollution in the coastal marine environment. Microbes from seawater, sea sediments, or beach sand get attached to tarballs and affect the biodegradation of hydrocarbon-rich tarballs. Tarballs act as a substratum for the physical attachment of various microbes, and the by-products formed during their degradation support microbial growth (Shinde et al., 2017).

Only a few studies on bacterial enumeration from tarballs were available when this thesis work was initiated in 2015 (Itah and Essien, 2005; Tao et al., 2011). The taxonomy of tarball-associated microbes needed improvement as DNA-based identification and phylogenetic characterisation was scarcely incorporated into it. To the best of our knowledge, Nkem et al. (2016) were the first to report 16S rRNA gene-sequencing of culturable tarball-associated bacteria. In the same year, Bacosa et al. (2016) reported tarball-associated bacteria from Texas beaches following the 2014 Texas City "Y" spill, using a culture-independent method. This study provided interesting insights into the diversity of unculturable tarball-associated bacteria.

Nevertheless, information on diversity and DNA sequence-based identification of tarball-associated bacteria from beaches of Goa state in India was scarce and patchy, though Goan beaches have faced tarball pollution since the 1970s (Nair et al., 1972; Suneel et al., 2013, 2016). The first objective, therefore, was to isolate and enumerate heterotrophic bacteria from tarballs collected from touristic Betul beach (South Goa) and

Candolim beach (North Goa) and perform their taxonomic identification based on 16S rRNA gene sequence data analysis. Protocols were standardised to isolate the maximum number of bacteria from tarballs. Heterotrophic bacteria were isolated from tarballs using two different isolation methods: serial dilution method and enrichment culture method. Colony-forming units (CFUs) of heterotrophic tarball-associated bacteria were determined on Zobell Marine Agar (ZMA) medium.

2.2 Methodology

2.2.1 Sampling

The literature review (Chapter 1) revealed that tarballs were regularly deposited on Goan beaches from May to October, mostly in pre- and post-monsoon seasons. As per the local media reports (https://www.daijiworld.com/news/newsDisplay?newsID=885409, https://timesofindia.indiatimes.com/travel/travel-news/goa-beaches-flooded-with-tarballs-once-again/articleshow/86255800.cms), Anjuna, Arambol, Ashwem, Baga, Betalbatim, Benaulim, Betul, Candolim, Calangute, Colva, Cavelossim, Miramar, Mandrem, Mobor, Morjim, Salcete, Sinquerim, Varca and Velsao beaches were affected by tarball pollution. In this study, tarball sampling was done from Candolim beach in North Goa and Betul beach in South Goa, India (Figure 2.1). Betul is a coastal town in South Goa, India, situated at the delta of the Sal River. The GPS coordinates for Betul are 15.144837 °N 73.958244 °E. Candolim is a coastal town in North Goa, India. The GPS coordinates for Candolim are 15.515447 °N 73.768402 °E. Candolim is a much more crowded beach than Betul. As per Google Maps, the distance between the two beaches is 58.8 km.

Tarball samples were collected in August 2014, July 2016, August 2017, and May 2018 from Betul beach, while sampling was done from Candolim beach in June 2015 and May 2018 (Table 2.1). Tarballs were randomly hand-picked wearing sterile nitrile gloves and collected in zip-lock bags. Approximately 100–200 g of tarballs were collected from both beaches during each sampling time. The zip-lock bags with proper labels (date and location) were kept in an icebox and carried to the laboratory. A part of the tarball samples was processed immediately for bacterial enumeration and isolation. The remaining samples were stored at 4°C until further work.

2.2.2 Isolation of heterotrophic bacteria

Initially, tarball samples were processed for bacterial isolation using a protocol standardised in the lab after improving the procedure described in Tao et al. (2011). In the modified protocol, 1 g of tarball was weighed aseptically and washed with sterile seawater. The washed tarball samples were added to a test tube containing 9 ml sterile seawater. The sterile cotton swab was used to break tarballs and detach bacteria. The tubes were vortexed vigorously for 5–10 min. 1 ml of this was serially diluted further up to 10^{-5} dilution. 100 µl of each dilution was spread plated on ZMA (2216 M384, Hi-Media, India). The plates were then incubated at room temperature. After 48 h of incubation, CFUs were calculated.

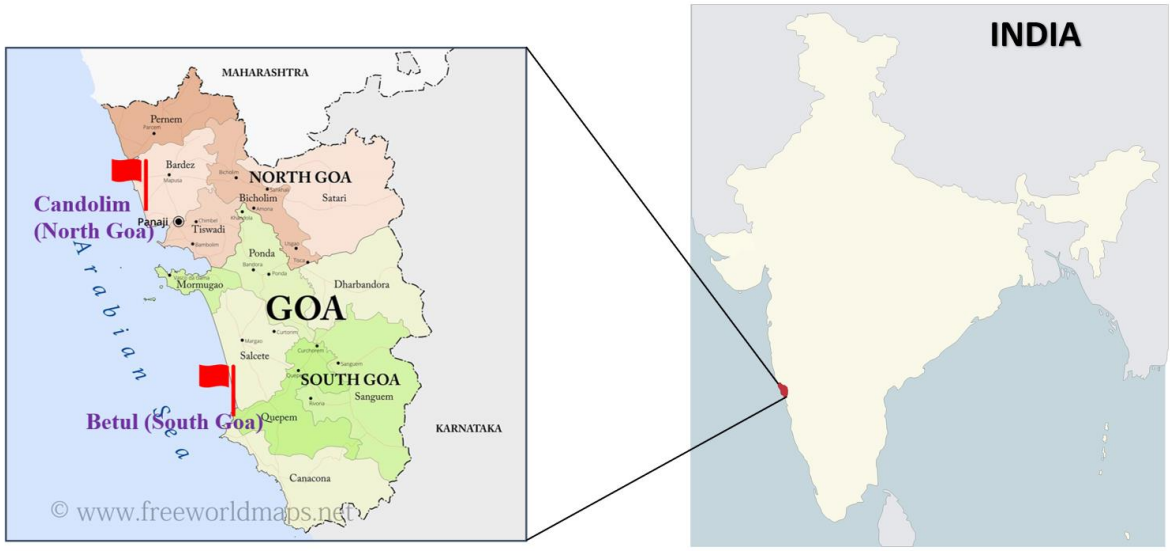


Figure 2.1: Tarball sampling sites at the coast of Goa, India (The India map was retrieved from https://www.freeworldmaps.net/asia/india/goa/where-is-goa.jpg. The map of the Goa state was retrieved from https://www.freeworldmaps.net/asia/india/goa/goa-political-map.jpg).

Table 2.1: Details of tarball sampling

Sl. no.	**Sampling location, including GPS data**	**Sampling date**
1	Betul beach, South Goa, India, 15.144837 °N, 73.958244 °E	13.08.2014
2	Candolim beach, North Goa, India, 15.515447 °N, 73.768402 °E	05.06.2015
3	Betul beach, South Goa, India, 15.144837 °N, 73.958244 °E	13.07.2016
4	Betul beach, South Goa, India, 15.144837 °N, 73.958244 °E	18.08.2017
5	Candolim beach, North Goa, India, 15.515447 °N, 73.768402 °E	02.05.2018
6	Betul beach, South Goa, India, 15.144837 °N, 73.958244 °E	08.05.2018

To increase the recovery of diverse groups of bacteria, one more bacterial isolation protocol was standardised in the lab after improving the isolation procedure described by Bayat et al. (2015). In this method, 1 g of the freshly collected tarball from the July 2016 sample collection was washed with sterile seawater to remove the particles adhered to it (as much as possible) and suspended immediately in 100 ml of Zobell Marine Broth (ZMB) containing tarball (1 g/100 ml) as a source of hydrocarbon to promote the growth of hydrocarbon-utilizing bacteria.

As the tarballs were insoluble in water, a sterile cotton swab was used to break them into smaller pieces so that maximum possible number of bacteria could get into the culture medium. That culture medium was incubated on a rotary shaker at 120 rpm, and 35 °C for 48 h. 1 ml of enriched medium was then transferred to ZMB containing tarball (1 g/100 ml) and further incubated on a rotary shaker at 120 rpm and 35 °C for 48 h. The final transfer was made in 100 ml of Bushnell Hass medium containing tarball as the only carbon source.

After the incubation, 1 ml from the final medium was serially diluted in sterile seawater and spread plated on ZMA medium. After 48 hours of incubation, the number of colonies was counted on each agar plate and multiplied by the dilution factor. Colony morphology characters were noted down, and Gram staining was performed for isolates to confirm their purity. Morphologically diverse bacterial colonies were further isolated by the streak plate method. Purified bacterial cultures were maintained as 80 % glycerol stocks and stored at −80 °C.

2.2.3 Gram staining

All bacterial isolates were subjected to Gram staining to check the purity of culture. Gram staining kit from Hi-Media was used. Gram staining was performed as follows: The bacterial suspension was prepared in a sterile saline solution. A thin smear was prepared on a clean and dry glass slide using a sterile wire loop. This was followed by air-drying the smear and heat-fixing it using a burner for a few seconds. The smear was then flooded with Crystal Violet (S012) (primary stain) for 2 minutes and washed under running tap water. Further, the smear was flooded with Gram's Iodine (S013) for 1 minute and again washed with water. The smear was decolourised with Gram's Decolourizer (S032) until the blue dye stopped coming off from the smear. The smear was washed under running tap water. This was followed by flooding the smear with Safranin (S027) (Counterstain) for 4 minutes and later washing with tap water. The smear was air-dried and examined under an oil immersion objective (100X).

2.2.4 DNA extraction and PCR amplification

DNA from bacterial cultures was extracted using the Fungal and Bacterial DNA isolation kit (Zymo Research, USA) according to the manufacturer's protocol. 16S rRNA gene of bacteria was amplified by Polymerase Chain Reaction (PCR) using primer-pair 27F and 1492R (Piterina et al., 2010). PCR for bacterial 16S rRNA gene amplification was performed in a 50 µl of volume, comprising 3 µl of template DNA (~50 ng/µl), 1 µl of each primer (20 pM) (Bioserve, India), 1 µl of dNTPs (10 mM) (Genaxy, India), 5 µl of Taq buffer A (10× with 15mM MgCl2) (GeNei, Bangalore), 1.5 µl of Taq polymerase (1 U/µl) (Chromous Biotech, India) and 37.5 µl of nuclease-free water (HiMedia, India). The reaction was carried out at 95 °C for 5 min (initial denaturation), followed by 35 cycles at 95 °C for 1 min (denaturation), 50 °C for 1 min (primer annealing), 72 °C for

2 min (elongation), and a final extension at 72 °C for 10 min. PCR products were purified using a QIAquick PCR purification kit (QIAGEN) following the manufacturer's protocol. After purification, PCR products were sequenced by ABI 3130 XL DNA sequencer using the primers mentioned above, at the Biological Oceanography Division, CSIR-NIO, Goa, India.

2.2.5 Phylogenetic analysis

The raw sequences obtained from forward and reverse primers were checked for quality in DNA Dragon (SequentiX, Germany). Whenever possible, consensus sequences were prepared in the same software using both forward and reverse sequences. A 16S rRNA gene sequence data set was prepared in MEGA version 11 (Tamura et al., 2021) using the newly-generated sequences and reference sequences retrieved from NCBI-GenBank (Table 2.3; Figure 2.2).

The Maximum Parsimony (MP) method was used to infer the evolutionary history among the isolates. The most parsimonious tree with length = 4245 is shown in Figure 2.2. The consistency index is 0.451119 (0.392596), the retention index is 0.895088 (0.895088), and the composite index is 0.403791 (0.351408) for all sites and parsimony-informative sites. The bootstrap test was performed (500 replicates) to check the reliability of the associated taxa clustered together and the percentage of replicates which are shown next to the branches in Figure 2.2. The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates).

This analysis involved 158 nucleotide sequences, comprising 65 newly-generated sequences from this study and 93 reference sequences obtained from NCBI-BLAST (Figure 2.2). The 93 sequences retrieved from NCBI included sequences from both type strains and non-type strains. There were a total of 1584 positions in the final dataset. *Aquifex pyrophilus* (GenBank accession number: M83548) was the designated outgroup.

2.3 Results

2.3.1 Enumeration of heterotrophic bacteria on tarballs

This study revealed a high number of tarball-associated heterotrophic bacteria on the ZMA medium. The bacterial CFUs on ZMA are presented in Table 2.2. Except for a few cases, CFUs were in the magnitude of 10^5 to 10^6. Numbers and alphabets are used to differentiate between the cultures isolated by serial dilution method and those isolated by enrichment culture technique, respectively (Table 2.3). A list of bacterial cultures isolated in this study, GenBank accession numbers for 16S rRNA gene sequences and Gram staining details are presented in Table 2.3.

2.3.2 Phylogenetic analysis of tarball-associated heterotrophic bacteria

In the bacterial phylogenetic tree shown in Figure 2.2, 65 newly-generated sequences of tarball-associated heterotrophic bacteria from Betul and Candolim beaches clustered within four bacterial phyla [*Proteobacteria*: 51 (*Gammaproteobacteria*: 41, *Alphaproteobacteria*: 10), *Actinobacteria*: 6, *Firmicutes*: 7 and *Bacteroidetes*: 1]. The newly generated 65 sequences were found clustering with 23 bacterial genera, namely *Alcanivorax*, *Bacillus*, *Bhargavaea*, *Brevibacterium*, *Cellulomonas*, *Enterobacter*, *Jonesia*, *Klebsiella*, *Labrenzia*, *Marinobacter*, *Marispirillum*, *Nitratireductor*, *Oceanimonas*, *Owenweeksia*, *Pantoea*, *Pelagibaca*, *Pseudomonas*, *Pseudoxanthomonas*, *Serratia*, *Shewanella*, *Thalassospira*, *Tistrella* and *Vibrio* (Figure 2.2). Sixteen bacterial isolates out of 65 were retrieved from the July 2016 tarball samples by the enrichment culture technique. Bacteria belonging to *Alcanivorax*, *Bhargavaea*, *Nitratireductor*, *Oceanimonas*, and *Tistrella* were exclusively recovered from the enrichment culture technique.

In the *Gammaproteobacteria* clade, the newly generated sequences from this study clustered with 11 bacterial genera. Two isolates from this study clustered with *Klebsiella* sequences. B-Betul-5 and B-Betul-8 clustered with *K. pneumoniae* strain DSM30104 (NR 117683) and *K. pneumoniae* strain L1 (KY630543) isolated from unknown sources.

Table 2.2: Colony forming units (CFUs) of heterotrophic bacteria from tarball samples on Zobell marine agar (ZMA) medium

Sl. No.	Sampling Date	Sample ID	Sampling location	Medium used	CFU/g of tarball
1	13.08.2014	VLS-B01	Betul beach	ZMA	8.3×10^6
2	05.06.2015	VLS-C01 (T01)	Candolim beach	ZMA	2.86×10^6
3	05.06.2015	VLS-C02 (T02)	Candolim beach	ZMA	1.16×10^6
4	05.06.2015	VLS-C03 (T03)	Candolim beach	ZMA	8.7×10^5
5	05.06.2015	VLS-C04 (T11)	Candolim beach	ZMA	1.87×10^5
6	05.06.2015	VLS-C05 (T12)	Candolim beach	ZMA	2.44×10^5
7	05.06.2015	VLS-C06 (T21)	Candolim beach	ZMA	4.63×10^5
8	05.06.2015	VLS-C07 (T22)	Candolim beach	ZMA	6.97×10^5
9	05.06.2015	VLS-C08 (T31)	Candolim beach	ZMA	3.94×10^5
10	05.06.2015	VLS-C09 (T32)	Candolim beach	ZMA	1.11×10^5
11	05.06.2015	VLS-C10 (T33)	Candolim beach	ZMA	6.07×10^6
12	05.06.2015	VLS-C11 (T41)	Candolim beach	ZMA	6.88×10^5
13	05.06.2015	VLS-C12 (T42)	Candolim beach	ZMA	6.58×10^5
14	13.07.2016	VLS-B02	Betul beach	ZMA	5.07×10^5
15	18.08.2017	VLS-B03/a	Betul beach	ZMA	5.8×10^5
16	18.08.2017	VLS-B03/b	Betul beach	ZMA	1.4×10^6
17	02.05.2018	VLS-C13	Candolim beach	ZMA	1.16×10^5
18	08.05.2018	VLS-B04	Betul beach	ZMA	Too many to count

Three isolates clustered with *Enterobacter* sequences. B-Betul-2 and B-Betul-10 clustered with uncultured *Enterobacter* sp. clone OD-3 (KX379247) isolated from aerobic granular sludge, *E. cloacae* subsp. dissolvens strain ATCC 23373 (NR 118011) isolated from maize plant, and *Enterobacter* sp. strain GSTE2 (OL701306) isolated from tellurite-reducing granules. Can-23 clustered with *E. ludwigii* strain WL1302 (KT328449) isolated from the intestine of wild common carp (*Cyprinus carpio* L.) (fish) and *E. ludwigii* strain Gol9 (MT263024) isolated from goldfish.

B-Betul-3 formed a cluster with *Pantoea dispersa* strain AA9 (MT275631) isolated from the midgut of *Aedes albopictus* (mosquito) (Sri Lanka), *P. dispersa* strain DSM 30073 (AB907780) isolated from black tea, and *P. dispersa* strain CBR2-BLDN (MK156736) isolated from a cotton boll.

Can-21 clustered with *Serratia rubidaea* strain DSM 4480 (114716) from an unknown source and *S. rubidaea* strain FC25279 (MW011784) isolated from vegetables.

B-Betul-9 clustered with *Vibrio fluvialis* strain NBRC 103150 (NR 114218) and *V. furnissii* strain 9119-82 (NR 037067) isolated from unknown sources. Can-14 clustered with *V. natriegens* strain Xmb012 (KT986142) isolated from ocean water, *V. alginolyticus* strain CZN-16 (KR347254) isolated from *Apostichopus japonicus* (sea cucumber), and *V. alginolyticus* strain NBRC 15630 (MH368083) isolated from coral, *Acropora formosa* (Mauritius).

B-Betul-Q clustered with *Oceanimonas doudoroffii* strain NIOT-SN (MK478944) isolated from marine sediment and *O. doudoroffii* strain MBIC1298 (NR027198) isolated from an unknown source.

Mob V1 clustered with *Shewanella* algae strain A59 (MH361590) isolated from the flamingo, *S. algae* strain SF7 (MH361600) from human urethral catheter insertion exudate and *S. algae* strain NBRC103173 (NR114236) isolated from an unknown source.

Ten bacterial isolates were found clustering within the *Marinobacter* clade. Can-17 clustered with *Marinobacter* strain (AJ292528) isolated from submarine hydrothermal vents and *M. lutaoensis* strain Kaz22 (MT277027) from oil fields. Can-7B clustered with *M. salsuginis* strain Xmb040 (KT986168) isolated from seawater from China and *M. salsuginis* SD-14B (NR 044044) from the red sea. Can-4 and Can-13 clustered with *M. oulmenensis* strain CECT 7499 (MT760273) isolated from an unknown source and *M. aquaticus* strain M6-53 (NR 157760) from a saline water source (South-West Spain). Can-9, B-Betul-23, B-Btul-26, B-Betul-27, Mob-V5, and Mob-V6 clustered with *Marinobacter* sp. MBTDCMFRIWC24 (KU554471) isolated from microalga, *M.*

hydrocarbonoclasticus strain NIOSSD026A50 (MH660329) from Arabian sea OMZ water column, and *M. hydrocarbonoclasticus* strain ATCC 27132 (MZ276315) from seagrass sediment.

Can-19 clustered with *Pseudomonas* sp. strain 065 (MK801627) isolated from coral, *Pseudomonas* sp. SJH-003 (KC335137) from marine sediment and *P. oceani* strain DSM 100277 (NR152090) from deep seawater (Okinawa Trough, north-western Pacific Ocean). B-Betul-4, B-Betul-7, B-Betul-19, MOB-V2, and B-Betul-M grouped with *P. oleovorans* strain NIOSSD020290 (KY616466) isolated from OMZ water column (Indian Ocean), uncultured bacterium clone BG1E01 (KC208272) from polluted aquatic environments of Brazil, *P. mendocina* strain ATCC 25411 (MK265746) from *Tinospora cordifolia* (Plant), *Pseudomonas* sp. strain PrP1085 (MF948928) isolated from an unknown source and *Pseudomonas* sp. H-11 (KF021702) isolated from an unknown marine source. B-Betul-14, B-Betul-15, B-Betul-F, and B-Betul-13 clustered with *P. aeruginosa* strain PAY (MN700178) isolated from the hospital (host- Homo sapiens), *Pseudomonas* sp. AG8 (KU860465) isolated from heavy metal-contaminated soil, India, and *P. aeruginosa* strain JCM5962 (KX946966) isolated from an unknown source. B-Betul-11 and B-Betul-G clustered with *P. stutzeri* ATCC 17588 = LMG 11199 (MT027239) isolated from Rugose spiraling whitefly and *P. stutzeri* strain IMB16-092 (MG190657) isolated from marine sediment. Can-25 clustered with *P. balearica* strain OAct420 (KC514126) isolated from South China sea sediment and *Pseudomonas* sp. strain YP07 (MT914226) of deep-sea hydrothermal vent.

B-Betul-P, B-Betul-O, B-Betul-H, and B-Betul-I clustered with *Alcanivorax dieselolei* strain CGMCC1.3690 (MT759958) isolated from an unknown source, *Alcanivorax* sp. strain KX64203 (KU954765) isolated from deep-sea sediment (Iheya North, Okinawa Trough), *A. dieselolei* strain S1025 (MF139136) from oil and water mixture in Qinghai oilfield and *Alcanivorax* sp. strain Voy50th32-4 (MT588672) from an unknown source.

B-Betul-24, B-Betul-1, and B-Betul-22 clustered with *Pseudoxanthomonas* sp. strain VITHBRA040 (MZ057740) isolated from High background natural radiation area sand, *P. indica* strain P15 (Type) (NR 116019) isolated from hexachlorocyclohexane dumpsite, and *Pseudoxanthomonas* sp. strain KNUSS-AIA5 (KY425600) isolated from Ganges River, India.

Of the 10 newly-generated sequences clustering within the *Alphaproteobacteria* clade (Figure 2.2), *Nitratireductor* was represented by four isolates, *Thalassospira* was represented by two isolates while *Marispirillum*, *Pelagibaca*, *Labrenzia*, and *Tistrella* included one isolate each.

B-Betul-C clustered with *Tistrella mobilis* strain JCM 21370 (NR 028632) isolated from wastewater (Thailand), *T. mobilis* strain DSD-PW4-OH23 (HQ425697) isolated from seawater of the South China Sea, and *T. mobilis* strain ORSB2 (MG786627) isolated from oil refinery sludge, Oran (Algeria).

Can-29 and Can-24 clustered with *Thalassospira profundimaris* strain mj01-PW1-OH20 (H Q425693) isolated from south China seawater, *T. australica* strain NP 3b2 (NR 146708) isolated from seawater (Australia), and *T. profundimaris* WP0211 (NR 042766), deep-sea sediment (China).

Mob-V7 clustered with *Marispirillum indicum* strain B142 (NR 044545) isolated from a deep-sea environment and *Marispirillum* sp. strain LB15 (MG386659) isolated from an unknown source.

Mob-V8 clustered with an unnamed hydrothermal vent strain TB66 (AF254109), *Pelagibaca abyssi* strain JLT2014 (NR 148263) isolated from deep seawater, and *Pelagibaca* sp. KU26E1 (AB636142) isolated from a marine source.

Can-10 clustered with *Labrenzia* sp. strain 1334-011 (KY770036) isolated from phycosphere (*Synechococcus* sp.), *L. aggregata* strain NBRC 16684 (NR 113861) from an unknown source (Japan), and *L. alba* strain 5OM6 (NR 042378) from oyster meat (Spain).

B_BETUL_R, B_BETUL_N, B_BETUL_A, and B_BETUL_D, recovered solely using the enrichment culture method, clustered within the *Alphaproteobacteria*. B-Betul-R clustered with *Nitratireductor aquimarinus* strain SY-2-4 (MZ268154) from an unknown source and *N. aquimarinus* strain CL-SC21 (NR 117929) from diatom *Skeletonema costatum* (Korea). B-Betul-N clustered with *N. kimnyeongensis* strain KY

101 (NR 042613) isolated from seaweed (Korea). B-Betul-D and B-Betul-A clustered with *N. aquibiodomus* strain NIOSSD020279 (KY616455) isolated from oxygen minimum zones of the northern Indian Ocean and *N. kimnyeongensis* strain NIOSSD026A71 (MH660350) isolated from Arabian Sea OMZ.

In the *Actinobacteria* clade, which comprised three genera, *Cellulomonas* and *Brevibacterium* were represented by three and two isolates, respectively, while *Jonesia* included only one isolate.

B-Betul-21(1) and B-Betul-21(2) clustered with *Brevibacterium pigmentatum* YB235 (KJ534269) isolated from sediment from the Atlantic Ocean and *B. iodinum* strain Sl-3 (KU894794) isolated from the rhizosphere of Siroy lily (plant).

Mob-V9 clustered with *Jonesia denitrificans* strain H43T7 (MK253318) isolated from the gut of *Ciconia boyciana* (bird), *J. denitrificans* strain DSM 20603 (NR 074806) isolated from an unknown source, and *Jonesia* sp. strain MGB 1190 (MT598060) isolated from a marine sponge.

B-Betul-28, B-Betul-29, and B-Betul-30 clustered with *Cellulomonas hominis* strain ZJY-507 (KP282823) isolated from crude oil samples, *Cellulomonas* sp. MCCC 1A11193 (KU560419) isolated from an unknown source, and *C. hominis* strain CE40 (NR 029288) isolated from an unknown source.

In the *Firmicutes* clade, which comprised two genera, *Bhargavaea* and *Bacillus*, each was represented by three isolates. B-Betul-K clustered with *Bhargavaea beijingensis* strain MSSRFBP26 (MW577390) isolated from mangrove rhizosphere. B-Betul-L clustered with *B. beijingensis* strain ge10 (NR 044192) isolated from ginseng root. B-Betul-J was paraphyletic to *B. beijingensis* strain M61 (KT720202) isolated from Viking Spacecraft Teflon Ribbon surfaces.

B-Betul-6 clustered with *Bacillus cereus* strain FJAT-46988 (MG651594) isolated from an unknown source and *B. cereus* strain NBRC 15305 (MZ151704) isolated from sand crab juice. B-Betul-5B clustered with *Bacillus australimaris* strain FORCN231 (MW402873) isolated from the digestive tract of giant borer larvae. Can-11 and Can-26

clustered with *B. australimaris* strain MCCC 1A05787 (MN077148) isolated from plant (*Waltheria indica*) and *Bacillus* sp. strain R352 (KX618403) isolated from seeds (*Paullinia cupana*) (Brazil).

Phylum *Bacteroidetes* was the least represented with one isolate in the genus *Owenweeksia*. Mob-V4 clustered with *O. hongkongensis* DSM 17368 strain C42 (MW540647) isolated from a coral (*Acropora humilis*) (Germany), uncultured bacterium clone BJGMM-1s-383 (JQ800822) isolated from soil of Yellow River Delta, China, and *Owenweeksia* sp. strain SC180 (MK271733) isolated from an unknown source.

2.4 Discussion

In this study, ZMA medium was used to count colony-forming units of heterotrophic tarball-associated bacteria (Table 2.2). ZMA is a specific growth medium used to isolate heterotrophic bacteria from seawater. This study used two bacterial isolation protocols (serial dilution and enrichment culture) to get a maximum number of bacteria from oily tarballs. In both methods, tarball samples were priorly processed to detach bacteria from tarballs into the solution, i.e., in seawater.

The number of bacterial CFUs recovered in our study (1.11×10^5–8.3×10^6 CFUs per gram of tarball sample) is in agreement with previous findings. Itah and Essien (2005) reported 3.00×10^4–3.18×10^6 CFUs of heterotrophic bacteria per gram of tarball. Mineral salt medium (MSM) and Tryptone soya agar (TSA) were used by Itah and Essien (2005) for heterotrophic bacterial counts. Tao et al. (2011) reported numbers in the range of 5.1×10^6–8.3×10^6 CFUs per gram of tarball. Petra et al. (2012) reported 4.1×10^4 CFU/ml of weathered crude oil.

In the enrichment culture technique, which is known for favouring particular organisms' growth, tarball was used as the only carbon source in the final medium to favour the growth of hydrocarbonoclasticus bacteria. Sixteen bacteria were isolated by the enrichment culture technique, while the rest 49 bacteria were isolated using the serial dilution plate method. Interestingly, *Nitratireductor* and *Alcanivorax* species were predominantly isolated by the enrichment culture technique, followed by species of

Bhargavaea, *Oceanimonas*, and *Tistrella*. *Pseudomonas* species, however, were recovered in both isolation methods (Table 2.3).

Tarballs and tarball-like substrates are known to support phylogenetically diverse bacteria (Shinde et al., 2018). The studies reported till now on tarball-associated bacteria are as follows. In culture-dependent studies, Itah and Essien (2005) isolated, enumerated, and characterised eight bacterial species from tarballs collected from the Nigerian coast based on morphology. They reported the following tarball-associated bacteria: *Bacillus submarinus, Corynebacterium glutamicum, Chromobacterium violaceum, Escherichia coli, Micrococcus varians, Nocardia marina, Pseudomonas aeruginosa*, and *Vibrio parahaemolyticus*. Nkem et al. (2016, 2020) characterised four bacterial species, *Acinetobacter baumannii, Cellulosimicrobium cellulans, Pseudomonas stutzeri*, and *Pseudomonas balearica* associated with tarballs from Malaysia based on a polyphasic approach. They used the enrichment culture technique using diesel oil as a carbon source.

In culture-independent studies, Petra et al. (2012) reported *Pannonibacter phragmitetus*, *Pseudomonas* sp., *Thalassospira* sp., *Xanthobacter autotrophicus*, and *Rhodobacteraceae* members from weathered crude oil from Australia based on DGGE profiles. Liu and Liu (2013) reported 10 bacterial clades based on metagenomic data from the Gulf of Mexico. Their results suggested that *Proteobacteria* was the dominant clade found in three different oil mousse (=tarballs), ranging from 95–99 % of the total OTUs. Also, *Alphaproteobacteria* and *Gammaproteobacteria* were dominant in the *Proteobacteria* phylum. They also reported *Bacteroidetes* and *Actinobacteria*. Similar results are observed in this study.

Liu and Liu (2013) reported bacteria from the *Actinobacteria* clade, *Alphaproteobacteria* clade, *Bacteroidetes* clade, *Chloroflexi* clade, *Cyanobacteria* clade, *Firmicutes* clade, *Flavobacteriales* clade, *Gammaproteobacteria* clade, *Planctomycetes* clade in oil mousse collected from the northern Gulf of Mexico after Deepwater Horizon oil spill. The genera they reported are *Alcanivorax, Arcobacter*, *Bartonella*, *Erythrobacter*, *Marinobacter*, *Pseudomonas*, *Vibrio*, *Rhodovulum*, *Stappia*, *Thalassospira*, and *Vibrio*. Bacosa et al. (2016) reported *Alcanivorax, Oceanospirillales*

clade, *Pseudoaltermonas*, and *Psychrobacter* from tarballs collected from Galveston and Mustang Islands in the USA.

Fernandes et al. (2019) reported 83 bacteria belonging to 55 genera from tarball samples collected from Vagator and Morjim beaches of North Goa, India, based on NGS analysis of the 16S rRNA gene region. They reported nine genera common to this study. Those are *Alcanivorax, Brevibacterium, Bacillus, Klebsiella, Marinobacter, Pseudomonas, Serratia, Shewanella, Thalassospira* and *Vibrio*. These nine genera include potential hydrocarbon degraders and possible pathogens. Many other bacterial genera are not found in the NGS analysis (Fernandes et al., 2019) but found in our culture-dependent studies from Goan tarball samples. These are *Bhargavaea*, *Cellulomonas*, *Enterobacter*, *Jonesia*, *Labrenzia*, *Marispirillum*, *Nitratireductor*, *Oceanimonas*, *Owenweeksia*, *Pantoea*, *Pelagibaca*, *Pseudoxanthomonas*, and *Tistrella*, while *Enterobacter* and *Tistrella* were common from both the sampling sites (Betul and Candolim beaches). Their absence in NGS studies (Vagator and Morjim beaches) of Fernandes et al. (2019) is interesting. This suggests that not one method is efficient/ sufficient in characterising the microbial diversity of tarballs. A combination of culture-based and culture-independent methods is expected to provide interesting insights.

In an interesting study, Tran et al. (2019) analysed bacterial communities using PCR-DGGE and DNA sequencing from tarballs and tarshells (Tar accumulated in shells) collected from the Alabama coast at intertidal and supratidal zones. They found that the bacterial communities, *Desulfovibrio* spp. and *Actinobacteria* members were dominant in tar shells at intertidal and supratidal zones. At the same time, *Pseudoalteromonas* spp. were dominant in tarballs and tar shells.

Table 2.3: A list of bacterial cultures isolated in this study, along with GenBank accession numbers, taxon name, and Gram staining details

Sl. No.	**Sampling location**	**Sample ID**	**Isolate no.**	**Taxon name**	**Gram staining results**	**GenBank Acc. No.**
1	Betul beach	VLS-B02	B_Betul_O	*Alcanivorax* sp.	Gram-negative, short rods	MG664223
2			B_Betul_2	*Enterobacter* sp.	Gram-negative, coccobacilli	MG664235
3			B_Betul_I	*Alcanivorax* sp.	Gram-negative, short rods	MG664222
4			B_Betul_P	*Alcanivorax* sp.	Gram-negative, coccobacilli	MG664224
5			B_Betul_H	*Alcanivorax* sp.	Gram-negative, coccobacilli	MG664221
6			B_Betul_6	*Bacillus* sp.	Gram-positive, short rods	MG664225
7			B_Betul_K	*Bhargavaea* sp.	NA	MG664227
8			B_Betul_L	*Bhargavaea* sp.	Gram-positive, short rods	MG664228
9			B_Betul_J	*Bhargavaea* sp.	Gram-negative, rods	MG664226
10			B_Betul_21(1)	*Brevibacterium* sp.	NA	MG664229
11			B_Betul_21(2)	*Brevibacterium* sp.	NA	MG664230

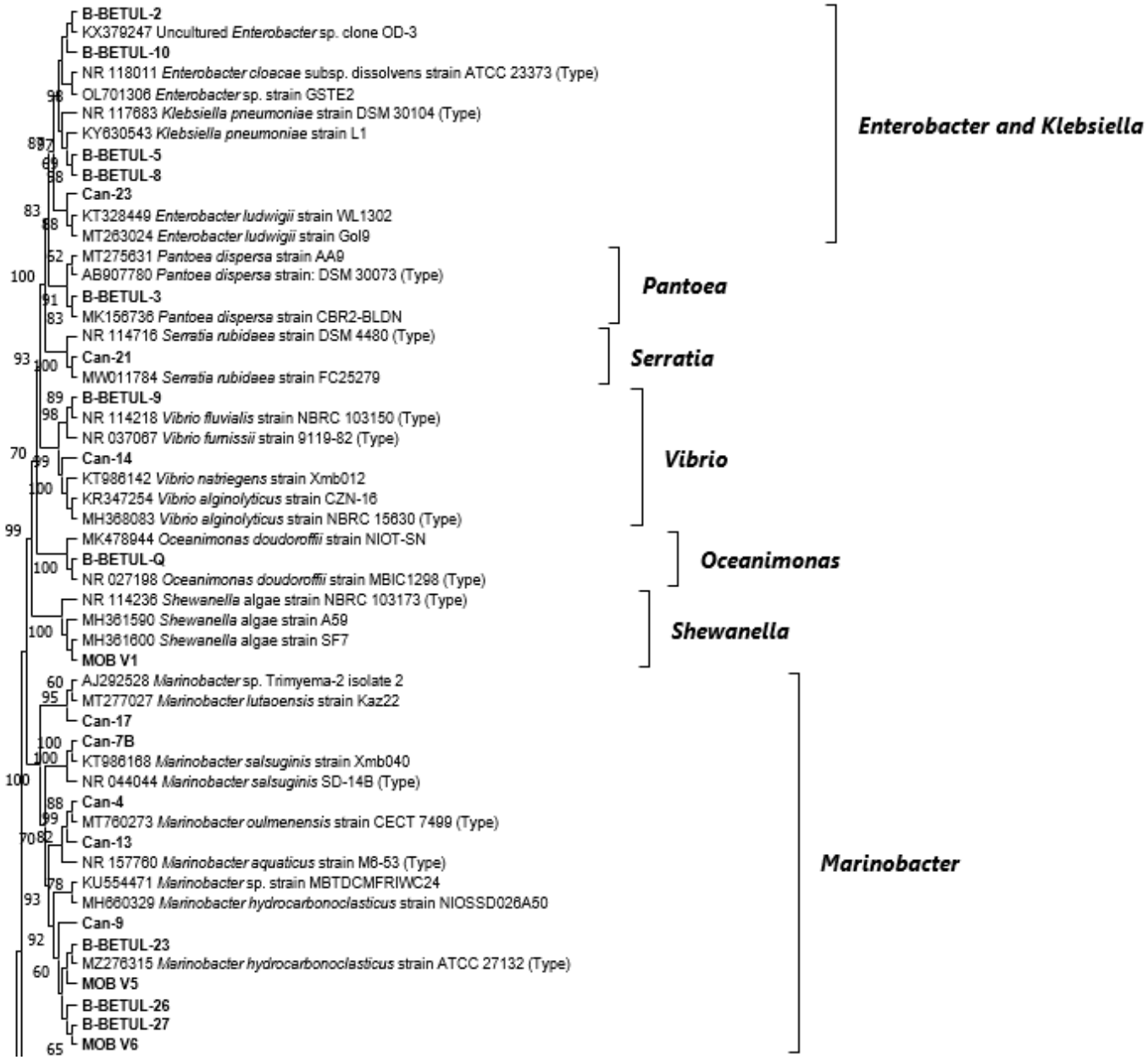
12			B_Betul_28	*Cellulomonas* sp.	Gram-negative, short rods	MG664231
13			B_Betul_30	*Cellulomonas* sp.	Gram-negative, short rods	MG664233
14			B_Betul_29	*Cellulomonas* sp.	Gram-negative, short rods	MG664232
15			B_Betul_10	*Enterobacter* sp.	Gram-negative, coccobacilli	MG664234
16			B_Betul_8	*Klebsiella* sp.	Gram-negative, cocci	MG664237
17			B_Betul_5	*Klebsiella* sp.	Gram-negative, cocci	MG664236
18			B_Betul_23	*Marinobacter* sp.	Gram-negative, short rods	MG664238
19			B_Betul_27	*Marinobacter* sp.	NA	MG664240
20			B_Betul_26	*Marinobacter* sp.	Gram-negative, rods	MG664239
21			B_Betul_R	*Nitratireductor* sp.	Gram-negative, short rods	MG664244
22			B_Betul_N	*Nitratireductor* sp.	Gram-negative, short rods	MG664243
23			B_Betul_D	*Nitratireductor* sp.	Gram-negative,	MG664242

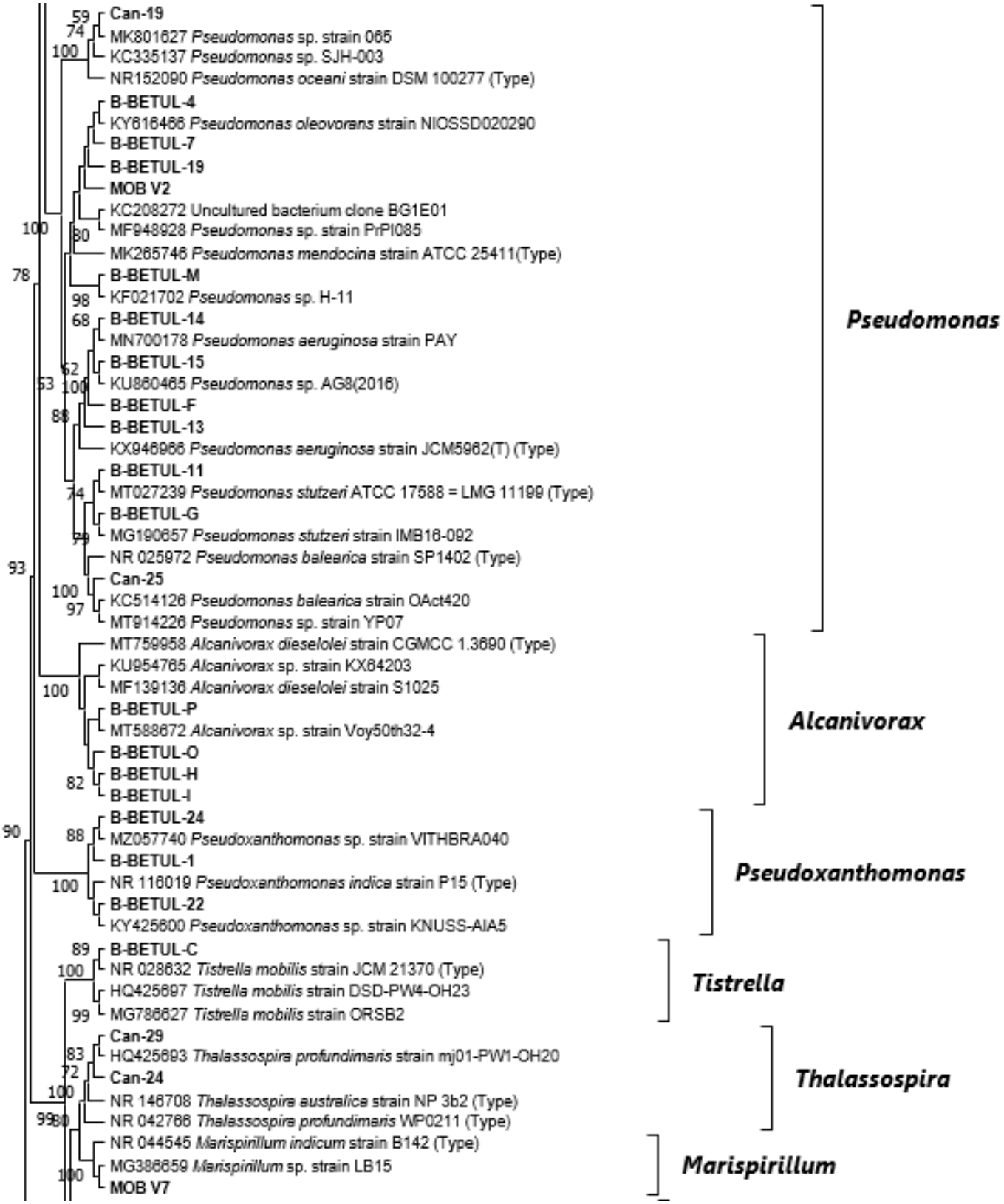
					rods	
24			B_Betul_A	*Nitratireductor* sp.	Gram-negative, thin short rods	MG664241
25			B_Betul_Q	*Oceanimonas* sp.	Gram-negative, coccobacilli	MG664245
26			B_Betul_3	*Pantoea* sp.	Gram-negative, coccobacilli	MG664246
27			B_Betul_4	*Pseudomonas* sp.	Gram-negative, thin short rods	MG664252
28			B_Betul_19	*Pseudomonas* sp.	Gram-negative, short rods	MG664251
29			B_Betul_7	*Pseudomonas* sp.	Gram-negative, short rods	MG664253
30			B_Betul_M	*Pseudomonas* sp.	Gram-negative, short rods	MG664256
31			B_Betul_G	*Pseudomonas* sp.	Gram-negative, coccobacilli	MG664255
32			B_Betul_11	*Pseudomonas* sp.	NA	MG664247
33			B_Betul_F	*Pseudomonas* sp.	Gram-negative, short rods	MG664253
34			B_Betul_14	*Pseudomonas* sp.	Gram-negative,	MG664254

					short rods	
35			B_Betul_15	*Pseudomonas* sp.	Gram-negative, coccobacilli	MG664250
36			B_Betul_13	*Pseudomonas* sp.	Gram-negative, short rods	MG664248
37			B_Betul_1	*Pseudoxanthomonas* sp.	Gram-negative, rods	MG664257
38			B_Betul_22	*Pseudoxanthomonas* sp.	Gram-negative, rods	MG664258
39			B_Betul_24	*Pseudoxanthomonas* sp.	Gram-negative, short rods	MG664259
40			B_Betul_C	*Tistrella* sp.	Gram-negative, rods	MG664260
41			B_Betul_9	*Vibrio* sp.	Gram-negative, short rods	MG664261
42		VLS-B01	MOB_V1	*Shewanella* sp.	Gram-negative, rods	MG797560
43			MOB_V2	*Pseudomonas* sp.	Gram-negative, rods	MG797561
44			MOB_V4	*Owenweeksia* sp.	NA	MG797562
45			MOB_V5	*Marinobacter* sp.	Gram-negative, rods	MG797563

46			MOB_V6	*Marinobacter* sp.	Gram-negative, rods	MG797564
47			MOB_V7	*Marispirillum* sp.	Gram-negative, rods	MG797565
48			MOB_V8	*Pelagibaca* sp.	Gram-negative, coccobacilli	MG797566
49			MOB_V9	*Jonesia* sp.	NA	MG797567
50	Candolim beach	VLS-C13	Can-4	*Marinobacter* sp.	Gram-negative, rod	MK503518
51			Can-5B	*Bacillus* sp.	Gram-positive, rod	MK503519
52			Can-7B	*Marinobacter* sp.	Gram-negative, long rods	MK503520
53			Can-9	*Marinobacter* sp.	Gram-negative, long rods	MK503521
54			Can-10	*Labrenzia* sp.	NA	MK503522
55			Can-11	*Bacillus* sp.	Gram-positive, short rods	MK503523
56			Can-13	*Marinobacter* sp.	Gram-negative, long rods	MK503524
57			Can-14	*Vibrio* sp.	Gram-negative, short rods	MK503525

58			Can-17	*Marinobacter* sp.	Gram-negative, long rods	MK503526
59			Can-19	*Pseudomonas* sp.	Gram-negative, rod	MK503527
60			Can-21	*Serratia* sp.	Gram-negative, rod	MK503528
61			Can-23	*Enterobacter* sp.	Gram-negative, short rods	MK503529
62			Can-24	*Thalassospira* sp.	Gram-negative, short rods	MK503530
63			Can-25	*Pseudomonas* sp.	Gram-negative, short rods	MK503531
64			Can-26	*Bacillus* sp.	Gram-positive, rods	MK503532
65			Can-29	*Thalassospira* sp.	Gram-negative, short rods	MK503533


B-BETUL-2
KX379247 Uncultured Enterobacter sp. clone OD-3
B-BETUL-10
NR 118011 Enterobacter cloacae subsp. dissolvens strain ATCC 23373 (Type)
OL701306 Enterobacter sp. strain GSTE2
NR 117683 Klebsiella pneumoniae strain DSM 30104 (Type)
KY630543 Klebsiella pneumoniae strain L1
B-BETUL-5
B-BETUL-8
Can-23
KT328449 Enterobacter ludwigii strain WL1302
MT263024 Enterobacter ludwigii strain Gol9
Enterobacter and Klebsiella
MT275631 Pantoea dispersa strain AA9
AB907780 Pantoea dispersa strain: DSM 30073 (Type)
B-BETUL-3
MK156736 Pantoea dispersa strain CBR2-BLDN
Pantoea
NR 114716 Serratia rubidaea strain DSM 4480 (Type)
Can-21
MW011784 Serratia rubidaea strain FC25279
Serratia
B-BETUL-9
NR 114218 Vibrio fluvialis strain NBRC 103150 (Type)
NR 037067 Vibrio furnissii strain 9119-82 (Type)
Can-14
KT986142 Vibrio natriegens strain Xmb012
KR347254 Vibrio alginolyticus strain CZN-16
MH368083 Vibrio alginolyticus strain NBRC 15630 (Type)
Vibrio
MK478944 Oceanimonas doudoroffii strain NIOT-SN
B-BETUL-Q
NR 027198 Oceanimonas doudoroffii strain MBIC1298 (Type)
Oceanimonas
NR 114236 Shewanella algae strain NBRC 103173 (Type)
MH361590 Shewanella algae strain A59
MH361600 Shewanella algae strain SF7
MOB V1
Shewanella
AJ292528 Marinobacter sp. Trimyema-2 isolate 2
MT277027 Marinobacter lutaoensis strain Kaz22
Can-17
Can-7B
KT986168 Marinobacter salsuginis strain Xmb040
NR 044044 Marinobacter salsuginis SD-14B (Type)
Can-4
MT760273 Marinobacter oulmenensis strain CECT 7499 (Type)
Can-13
NR 157760 Marinobacter aquaticus strain M6-53 (Type)
KU554471 Marinobacter sp. strain MBTDCMFRIWC24
MH660329 Marinobacter hydrocarbonoclasticus strain NIOSSD026A50
Can-9
B-BETUL-23
MZ276315 Marinobacter hydrocarbonoclasticus strain ATCC 27132 (Type)
MOB V5
B-BETUL-26
B-BETUL-27
MOB V6
Marinobacter


Can-19
MK801627 Pseudomonas sp. strain 065
KC335137 Pseudomonas sp. SJH-003
NR152090 Pseudomonas oceani strain DSM 100277 (Type)
B-BETUL-4
KY616466 Pseudomonas oleovorans strain NIOSSD020290
B-BETUL-7
B-BETUL-19
MOB V2
KC208272 Uncultured bacterium clone BG1E01
MF948928 Pseudomonas sp. strain PrPI085
MK265746 Pseudomonas mendocina strain ATCC 25411(Type)
B-BETUL-M
KF021702 Pseudomonas sp. H-11
B-BETUL-14
MN700178 Pseudomonas aeruginosa strain PAY
B-BETUL-15
KU860465 Pseudomonas sp. AG8(2016)
B-BETUL-F
B-BETUL-13
KX946966 Pseudomonas aeruginosa strain JCM5962(T) (Type)
B-BETUL-11
MT027239 Pseudomonas stutzeri ATCC 17588 = LMG 11199 (Type)
B-BETUL-G
MG190657 Pseudomonas stutzeri strain IMB16-092
NR 025972 Pseudomonas balearica strain SP1402 (Type)
Can-25
KC514126 Pseudomonas balearica strain OAct420
MT914226 Pseudomonas sp. strain YP07
Pseudomonas
MT759958 Alcanivorax dieselolei strain CGMCC 1.3690 (Type)
KU954765 Alcanivorax sp. strain KX84203
MF139136 Alcanivorax dieselolei strain S1025
B-BETUL-P
MT588672 Alcanivorax sp. strain Voy50th32-4
B-BETUL-O
B-BETUL-H
B-BETUL-I
Alcanivorax
B-BETUL-24
MZ057740 Pseudoxanthomonas sp. strain VITHBRA040
B-BETUL-1
NR 116019 Pseudoxanthomonas indica strain P15 (Type)
B-BETUL-22
KY425600 Pseudoxanthomonas sp. strain KNUSS-AIA5
Pseudoxanthomonas
B-BETUL-C
NR 028632 Tistrella mobilis strain JCM 21370 (Type)
HQ425697 Tistrella mobilis strain DSD-PW4-OH23
MG786627 Tistrella mobilis strain ORSB2
Tistrella
Can-29
HQ425693 Thalassospira profundimaris strain mj01-PW1-OH20
Can-24
NR 146708 Thalassospira australica strain NP 3b2 (Type)
NR 042766 Thalassospira profundimaris WP0211 (Type)
Thalassospira
NR 044545 Marispirillum indicum strain B142 (Type)
MG386659 Marispirillum sp. strain LB15
MOB V7
Marispirillum

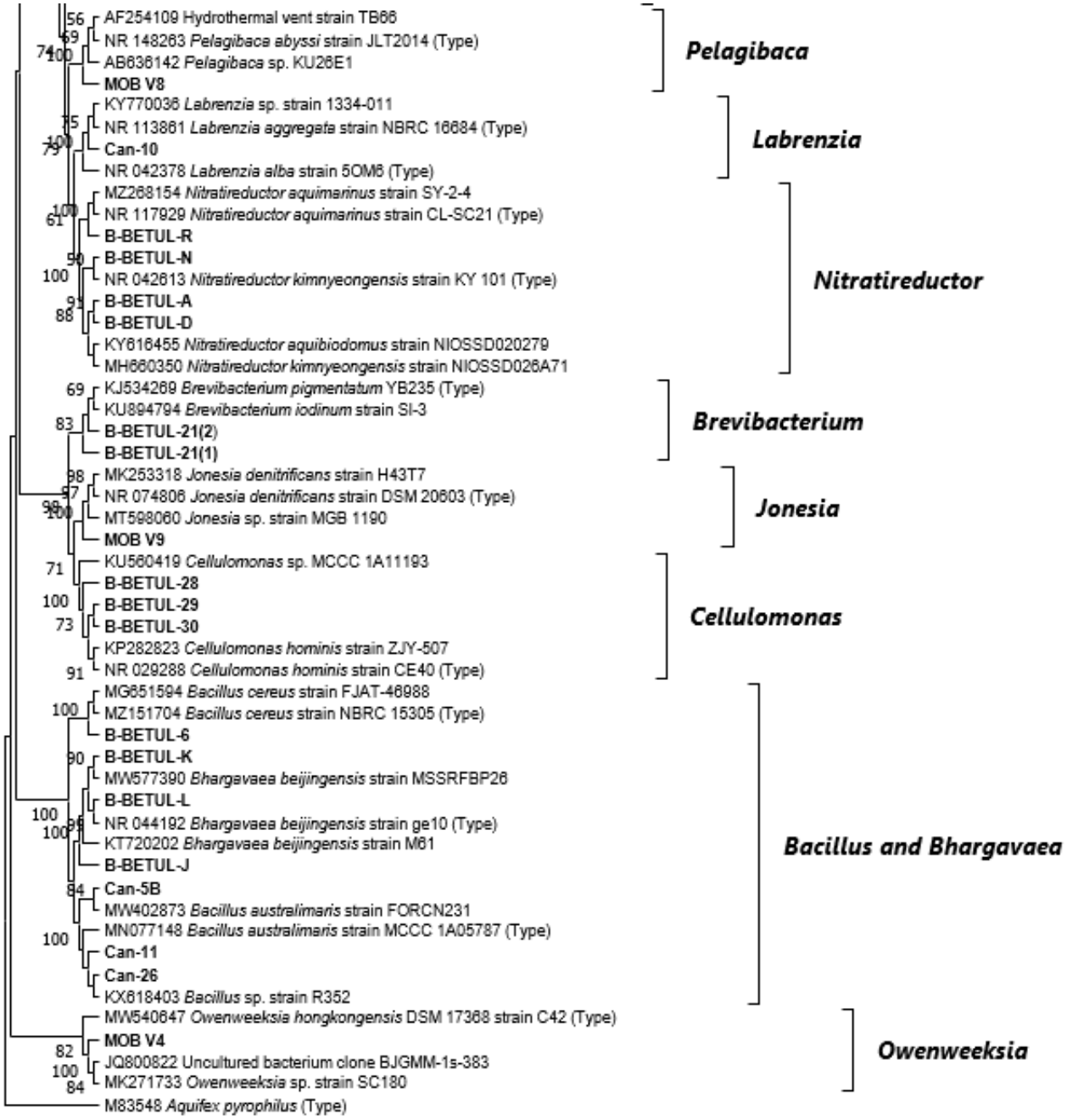


Figure 2.2: Phylogenetic relationships of heterotrophic tarball-associated bacteria from Betul and Candolim beaches of Goa, India, based on 16S rRNA gene sequence analysis.

Table 2.4: Possible roles of identified bacterial taxa as hydrocarbon degraders or pathogens

Sl. No.	Name of the closest taxon on the tree (Figure 2.2)	eport on gradation	Reference	Report on role as pathogen	eference
1	*Enterobacter cloacae*	Crude oil degradation	Ejaz et al. (2021)	Human pathogen	Davin-Regli and Pages (2015)
2	*Enterobacter ludwigii*	Diesel oil degradation	Yousaf et al. (2011)	Human pathogen	Davin-Regli et al. (2019)
3	*Klebsiella pneumoniae*	Hydrocarbon degradation	Rodrigues et al. (2009), Ozyurek and Bilkay (2018), You et al. (2018)	Human pathogen	Chang et al. (2022)
4	*Pantoea* sp.	Biosurfactants production, Hydrocarbon metabolism	Tonkova and Gesheva (2007)	Plant pathogen, Human pathogen	Walterson and Stavrinides (2015)
5	*Serratia* spp.	Biosurfactant production	Pendse and Aruna (2020), Semai et al. (2021)	Human pathogen	Ursua et al. (1996)
6	*Vibrio furnissii*	Biosurfactant production	Hassanshahian (20 14)		Ballal et al. (2017)
7	*Vibrio fluvialis*			Human pathogen	Igbinosa and Okah (2010)
8	*Vibrio alginolyticus*	Diesel oil degradation	Imron and Titah (2018)	Human pathogen	Fu et al. (2016)
9	*Oceanimonas* sp.	PAH degradation compounds	Lee et al. (2018)	Presence of genes for virulence and pathogenici ty genes	Yeganesh et al. (2015)
10	*Shewanella algae*	Biosurfactant production, alkane degrader	Joe et al. (2019), Gharaei et al. (2022)	Human pathogen	Holt et al. (2005)
11	*Shewanella algae*	Crude oil degradation	Bayat et al. (2015)	Human pathogen	Holt et al. (2005)
12	*Marinobacter salsuginis*	Degradation of n-hexane, n-decane, heptane, hexane, and petroleum ether	Antunes et al. (2007)		
13	*Marinobacter hydrocarbonoclasticu s*	Hydrocarbon degrader	Gauthier et al. (1992)		

14	*Pseudomonas mendocina*	Alkanes from crude oil	Mohanram et al. (2015), Medjahed et al. (2020)	Human pathogen	Gani et al. (2019)
15	*Pseudomonas aeruginosa*	Oil degradation	Muriel-Millan et al. (2019)	Human pathogen	Iglewski (1996)
16	*Pseudomonas stutzeri*	Crude oil degradation	Parthipan et al. (2017)	Human pathogen	Iglewski (1996)
17	*Alcanivorax dieselolei*	Alkane degradation	Liu and Shao (2005)		
18	*Pseudoxanthomonas* sp.	Diesel oil degradation	Nopcharoenkul et al. (2013)		Kuo and Lee (2018)
19	*Tistrella mobilis*	PAH degradation	Cui et al. (2009)		
20	*Thalassospira profundimaris*	Carbazole-PAH compound	Othman and Inayatullah (2021)	Human pathogen	Marchese et al. (2019)
21	*Thalassospira australica*	Plastic degradation	Perez et al. (2014)		
22	*Marispirillum* sp.	PAH degradation	Wang et al. (2018)		
23	*Pelagibaca* sp.	Oil degradation	Wang et al. (2014)		
24	*Labrenzia aggregata*	Oil degradation	Yetti et al. (2018)		
25	*Nitratireductor* sp.	Crude oil degradation	Gao et al. (2015)		
26	*Brevibacterium* sp.	Oil degradation	Ebadi et al. (2021)	Human pathogen	Shweta (2021)
27	*Cellulomonas hominis*	Phenanthrene degradation	Hegazi et al. (2007)	Human pathogen	Funke et al. (1995)
28	*Bacillus cereus*	Crude oil degradation	Christova et al. (2019)	Human pathogen	Tewari and Abdullah (2015)

2.5 Conclusion

Tarballs collected in this study are found to contain phylogenetically diverse groups of bacteria. The taxonomic information of these bacteria can be linked to possible functions. Most of the tarball-associated bacteria taxa identified in this study have been previously reported in hydrocarbon degradation studies (Table 2.4). Hence their presence on/in hydrocarbon-rich tarballs is quite interesting. Many of these taxa have also been reported for their pathogenicity (Table 2.4). These bacteria may get aggregated on tarballs for nutrition purposes and potentially cause diseases in marine life.

Chapter 3

Isolation, Enumeration and Phylogenetic Analysis of Pathogenic Groups of Bacteria from Tarballs

3.1 Introduction

Human activities are responsible for introducing various substances (chemicals, metals, oil, etc.) into seawater, which affects the marine environment. Pathogenic bacteria are the common biological pollutants introduced into seawater through contaminated water resources such as river run-off water and land run-offs (sewage water). These bacterial pathogens adversely affect marine biota and the overall ecosystem, consequently affecting human health (Zielinski et al., 2009). The survival and proliferation of these pathogens in the saline environment depend on their adaptability to the extremes of environmental factors such as salinity, temperature, nutrients, light, and other environmental factors (Stewart et al., 2008). In the marine environment, these pathogenic microbes are primarily associated with biotic forms such as fishes, plankton, etc., and abiotic components including sediments, organic matter, etc. (Thompson et al., 2005). In the recent past, tarballs (the oily weathered blobs of crude oil) have been reported to contain pathogenic bacteria (Tao et al., 2011).

Tarballs support the growth of diverse bacteria, including pathogenic ones. These pathogenic bacteria probably are risky to surrounding life forms and human health. Tao et al. (2011) reported the presence of pathogenic *Vibrio vulnificus* on tarballs, ten times higher than in sand and 100 times higher than in seawater. Though a few studies reported bacteria and fungi on tarballs, to my knowledge, Tao et al. (2011) were the first to report the presence and abundance of pathogenic bacteria on tarballs. It is suggested that different kinds of bacteria get associated with tarballs during weathering, a process where crude oil undergoes changes due to physical, chemical, and biological factors.

The Goa state in India has been in news for tarball deposition on its beaches. This specific issue of pathogenic microbes on tarballs has been poorly studied. While our published papers (Shinde et al. 2017, 2018) focused on the diversity of bacteria and fungi associated with tarballs, there were limited studies on the issue of presence of pathogenic microbes on/ in tarballs. Goa state is popular amongst regional, national, and international tourists for its beautiful beaches. Millions of tourists visit Goa every year (Nagvenkar and Ramaiah, 2009), and there is a possibility of a high number of different pathogenic groups of microbes in Goan seawater. It is believed there are greater chances of pathogenic groups of microbes associating with tarballs that end up on Goan beaches during pre-and post-monsoon seasons. There have been interesting studies that reported the presence of pathogens such as *Escherichia coli*, *Enterococcus faecalis*, *Pseudomonas* spp., *Salmonella* spp., *Shigella* spp., *Vibrio cholerae*, *V. parahaemolyticus* and *V. alginolyticus* in the Zuari estuary of Goa (Nagvenkar and Ramaiah, 2009; Gardade and Khandeparker, 2017).

There are limited reports on pathogenic microbes associated with tarballs from touristic beaches of Goa, India. After carefully considering the results of Tao et al. (2011), this study was initiated to quantify select pathogenic groups of bacteria on tarballs, followed by their taxonomic identification based on 16S rRNA gene sequence analysis. This study investigated the presence of *Vibrio* spp. and enteropathogenic bacteria on tarballs from Betul (South Goa), and Candolim (North) beaches of Goa, India, and enumerate them on selective media. In total, twenty-nine newly-generated 16S rRNA gene sequences of these pathogenic groups of bacteria were subjected to phylogenetic analysis to determine their taxonomic identity.

3.2 Methodology

3.2.1 Sampling

Tarball samples were collected from Candolim beach (North Goa) in May 2018 and from Betul beach (South Goa) in August 2014, August 2017, and May 2018 (Chapter 2, Figure 2.1, Table 2.1).

3.2.2 Isolation of pathogenic groups of bacteria

For *Vibrio* spp., 5 g of tarball was weighed aseptically and added to 5 ml of phosphate-buffered saline (PBS). The tarballs were broken into pieces using a sterile cotton swab and subjected to vortexing for 5–10 min. 1 ml from this was then serially diluted up to 10^{-5}. Serial dilutions were made in PBS buffer, and 100 µl from each dilution was spread plated on Thiosulfate-Citrate-Bile Salts-Sucrose agar (TCBS) (M189, Hi-Media, India) and Sodium Dodecyl Sulphate Polymyxin Sucrose Agar (SDS) (M1155, Hi-Media, India) and Tryptone Salt agar (T1N1) (Hi-Media, India).

For enumeration and isolation of enteropathogenic bacteria, 1 g of tarball was weighed aseptically and washed with sterile seawater. The washed tarball samples were added to a test tube containing 9 ml sterile seawater. The sterile cotton swab was used to break tarballs and detach bacteria. The tubes were vortexed vigorously for 5–10 min. 1 ml of this was serially diluted further up to 10^{-5} dilutions. 100 µl of each dilution was spread plated on MacConkey Agar (MA) (M081B Hi-Media, India) and Eosin Methylene Blue agar (EMB) (M317, Hi-Media).

Mannitol Salt agar (MSA) (M118, Hi-Media, India) medium was used for *Staphylococcus* and Micrococcaceae groups of bacteria. The plates were incubated at room temperature. After 48 h of incubation, the colony-forming units (CFUs) were calculated. Pure cultures were stored in 80 % glycerol at -80 °C until further processing.

Morphologically unique colonies were isolated and subjected to further processing.

3.2.3 Gram staining

All isolates were subjected to Gram staining to check the purity of cultures as per the protocol described in Chapter 2, Section 2.2.3.

3.2.4. DNA extraction and PCR amplification

DNA extraction from 29 bacterial cultures (Table 3.2) and PCR amplification of the 16S rRNA gene was performed as detailed in Chapter 2, Section 2.2.4.

3.2.5. Phylogenetic analysis

The raw sequences obtained from the forward and reverse primers were checked for quality in DNA Dragon (SequentiX, Germany). Whenever possible, consensus sequences were prepared in the same software using both forward and reverse sequences. A 16S rRNA gene sequence dataset was prepared in MEGA version 11 (Tamura et al., 2021) using the newly-generated sequences and reference sequences retrieved from NCBI-GenBank (Figure 3.1). The evolutionary history among isolates was inferred using the Maximum Parsimony method. The most parsimonious tree with length = 1547 is shown in Figure 3.1. The consistency index is 0.636716 (0.587977), the retention index is 0.936173 (0.936173), and the composite index is 0.596076 (0.550448) for all sites and parsimony-informative sites. The bootstrap values (percentage of replicate trees) in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches in Figure 3.1. The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates).

This analysis involved 73 nucleotide sequences, including 29 newly generated sequences from this study and 44 homologous sequences obtained from NCBI-BLAST for each organism. There were a total of 1503 positions in the final dataset. Evolutionary analyses were conducted in MEGA. *Aquifex pyrophilus* (GenBank accession number: M83548) was designated as an outgroup.

3.3 Results

3.3.1 Enumeration of pathogenic groups of bacteria on tarballs

The counts of bacterial CFUs on various growth media are presented in Table 3.1. This study reports a high number of pathogenic bacterial groups on the selective media. The findings in this study are in agreement with the previous study by Tao et al. (2011) who used T1N1 agar for the enumeration of *Vibrio* spp. from tarballs and reported the CFUs in the range of 5.1×10^6–8.3×10^6 CFUs per gram of tarball. In our study, CFUs were in the magnitude of 10^5 to 10^6, except in a few cases (Table 3.1, sample ID no. VLS-B03/a and VLS-C13) on the growth-specific media used for pathogens.

3.3.2 Phylogenetic analysis of tarball-associated pathogenic groups of bacteria

Details of bacterial cultures isolated in this study, along with their taxonomic identity, Gram nature and GenBank accession numbers for 16S rRNA gene sequence data are presented in Table 3.2. A total of 73 sequences, including the reference and outgroup sequences retrieved from NCBI and 29 newly generated sequences from this study were used to construct the phylogenetic tree (Figure 3.1). Out of twenty-nine isolates, most of them clustered within the *Gammaproteobacteria* (24 isolates), and a few of them clustered within the *Alphaproteobacteria* (5 isolates). In the phylogenetic tree shown in Figure 3.1, 29 newly-generated sequences of tarball-associated bacteria from Betul and Candolim beaches clustered within nine genera, namely *Alcanivorax*, *Enterobacter*, *Klebsiella*, *Pseudomonas*, *Serratia*, *Shewanella*, *Thalassospira*, *Tistrella* and *Vibrio*.

Eight isolates from this study clustered within the *Vibrio* clade (Figure 3.1). Betul-MA2, Betul-MA5 and Betul-TV6 formed a cluster with *V. alginolyticus* strain ATCC 17749 (NR118258) isolated from an unknown source and *V. alginolyticus* strain YWO3-29 (MT368021) isolated from shrimp PL nursery of Bangladesh. Betul-SV20 and Betul-MA6 clustered with *V. orientalis* strain 2-16 (MN847678) isolated from an unknown source and *V. parahaemolyticus* strain ATCC 17802 (NR117893) isolated from a marine sponge, *Scleritoderma cyanea* (west coast of Curaçao). Can-44 formed a clade with *Vibrio* sp. strain 201707CJKOP-Y132 (MG593696) and *V. fluvialis* strain NBRC 103150 (NR114218) isolated from unknown sources. Betul-MA9 and Betul MA10 clustered with *V. proteolyticus* strain PH8 (AF513463) isolated from unknown sample from The Hawaiian Archipelago and *V. proteolyticus* strain MSSRF QS36 (KP640642) isolated from *Avicennia marina* (plant).

Serratia clade was represented by one isolate from this study. Can-41 formed a cluster with *S. rubidaea* strain PDLF4S (Type strain) (JN934386) isolated from leaves palm date tree, *Phoenix dactylifera* (Tunisia).

Enterobacter clade was represented by three isolates from this study. Can-53 and Can-54 formed a cluster with *E. cloacae* strain IAE120 (MK414813) isolated from an

unknown source. Betul MA-8 grouped with *E. cloacae* strain DSM 30054 (NR117679) isolated from an unknown source.

Klebsiella clade comprises three isolates from this study. Can-42 clustered with *Klebsiella* sp. strain PI-22 (MK165124) isolated from freshwater fish. Betul-E2 clustered with *Klebsiella* sp. HF2 (DQ998840), *K. quasipneumoniae* subsp. *similipneumoniae* strain (MT898529) isolated from an unknown source and *Klebsiella* sp. SR-143 (KC455430) isolated from the rhizosphere of sugarcane. Betul-MA14 formed a separate cluster with *Klebsiella* sp. strain K-21 (MF592279) isolated from lake sediment (China), uncultured *Klebsiella* sp. clone GDKp15 (MH767067) isolated from rice and *K. pneumoniae* subsp. *rhinoscleromatis* strain SISX20 (MK780048) isolated from an unknown source.

Shewanella clade was represented by two isolates from this study. Can-31 and Betul-MA15 clustered with *S. algae* strain QC39 (JN384129) isolated from the coastal bay of China, *S. algae* strain ATCC 51192 (NR117771) isolated from an unknown source, *S. haliotis* strain Sh4 (KX271667) isolated from *Halimione portulacoides* (plant) and S. *algae* strain AAG1 (MH040957) isolated from *Abdopus aculeatus* (Octopus).

Can-43 clustered with *Alcanivorax* sp. strain KX64203 (KU954765) isolated from deep-sea sediment of Iheya North, Okinawa Trough (China), and *A. dieselolei* strain RMR60 (MN974176) isolated from oil spill of Chennai (India).

Pseudomonas clade was represented by six isolates from this study. Betul-E7 clustered with *P. aestusnigri* strain TSA1 (LT673837) isolated from deep-sea sediment and *P. aestusnigri* strain VGXO14 (NR126210) isolated from crude-oil-contaminated intertidal sand (Spain). Betul-E8 clustered with *Pseudomonas* sp. strain DT5000-1 (MK368453) isolated from deep-sea. Betul-E4 clustered with *P. khazarica* (KX712072) isolated from sea sediment, and *Pseudomonas* sp. enrichment culture clone BQN21 (KC151263) isolated from Quang Ninh coast (Viet Nam). Can-50 formed a cluster with *Pseudomonas* sp. PCSAS2-27 (GQ284546) isolated from mangrove sediment. Betul-E12B formed a cluster with *Pseudomonas* sp. strain LB56 (MT176182) isolated from decomposed Sargassum seaweed (Barbados) and *P. stutzeri* strain IRQNWYF2 (MT261835) isolated from an unknown source. Can-46 formed a cluster with *P. balearica* DSM 6083

(MZ276325) isolated from seagrass sediment and *Pseudomonas* sp. BAB-4354 (KM388724) isolated from pesticide-contaminated soil.

Thalassospira clade was represented by two isolates from this study. Betul-A and Betul-E5A formed a cluster with *Thalassospira* sp. MCCC 1A00370 (EU440790) isolated from Oceanic region, *Thalassospira* sp. SCS800m-1 (J X533661) isolated from South China sea, and *T. profundimaris* strain mj01-PW1-OH20 (HQ425693) isolated from seawater.

Three isolates from this study clustered within *Tistrella* clade. Betul E14A, Can-52, and Can-57 clustered with *Tistrella* sp. f-1-2 (EU306603) isolated from oil field ooze (China), *T. mobilis* strain DSD-PW4-OH20 (HQ425696) isolated from South China seawater, uncultured bacterium clone LHJB-30 (JF741939) isolated from an oil reservoir, *Tistrella* sp. JC366 (LN846828) isolated from water, and *T. mobilis* strain JCM 21370 (NR028632) isolated from an unknown source.

Table 3.1: Colony forming units (CFUs) of pathogenic groups of bacteria from tarball samples on selective culture media

Sl. No.	Sample ID	Sampling location	Sampling Date	Medium used	CFU/ g of tarball
1	VLS-B01	Betul beach	13.08.2014	T1N1	5.7×10^{6}
2	VLS-B03/a	Betul beach	18.08.2017	MA	1.86×10^{4}
3	VLS-B03/b	Betul beach	18.08.2017	MA	1.6×10^{6}
4	VLS-B03/a	Betul beach	18.08.2017	TCBS	8.8×10^{4}
5	VLS-B03/b	Betul beach	18.08.2017	TCBS	4.6×10^{5}
6	VLS-B03/a	Betul beach	18.08.2017	EMB	1.36×10^{6}
7	VLS-B03/b	Betul beach	18.08.2017	EMB	9.3×10^{5}
8	VLS-B03/a	Betul beach	18.08.2017	MSA	2.31×10^{5}
9	VLS-B03/b	Betul beach	18.08.2017	MSA	2.22×10^{5}
10	VLS-B04	Betul beach	08.05.2018	MA	2.36×10^{6}
11	VLS-B04	Betul beach	08.05.2018	EMB	1.08×10^{5}
12	VLS-B04	Betul beach	08.05.2018	TCBS	2.18×10^{5}
13	VLS-B04	Betul beach	08.05.2018	SDS	1.07×10^{5}
14	VLS-C13	Candolim beach	02.05.2018	MA	8×10^{3}
15	VLS-C13	Candolim beach	02.05.2018	TCBS	1.4×10^{4}

Legend: MA-Mac-Conkey agar, EMB-Eosin methylene blue agar, TCBS- Thiosulfate-citrate-bile salts-sucrose agar. SDS-Sodium Dodecyl Sulphate Polymyxin Sucrose, MSA-mannitol salt agar

Table 3.2: Details of isolates from this study including taxon name, Gram nature, and GeneBank accession numbers

Sl. No.	Sampling location	Sample ID	Isolate no.	Gram staining results	Taxon name	GenBank acc. no.
1	Betul beach	VLS-B04	Betul-MA2	Gram negative	*Vibrio* sp.	MK503503
2		VLS-B04	Betul-MA5	Gram negative short rods	*Vibrio* sp.	MK503504
3		VLS-B04	Betul-MA6	Gram negative	*Vibrio* sp.	MK503505
4		VLS-B04	Betul-MA8	Gram negative, coccobacilli	*Enterobacter* sp.	MK503506
5		VLS-B04	Betul-MA9	Gram negative, comma shaped	*Vibrio* sp.	MK503507
6		VLS-B04	Betul-MA10	Gram negative, coccobacilli	*Vibrio* sp.	MK503508
7		VLS-B04	Betul-MA14	Gram negative	*Klebsiella* sp.	MK503509
8		VLS-B04	Betul-MA15	Gram negative, short rods	*Shewanella* sp.	MK503510
9		VLS-B04	Betul-TV6	Gram negative, short rods	*Vibrio* sp.	MK503511
10		VLS-B04	Betul-E2	Gram negative, Rods	*Enterobacter* sp.	MK530099
11		VLS-B04	Betul-E4	Gram negative, short rods	*Pseudomonas* sp.	MK503512
12		VLS-B04	Betul-E5A	Gram negative, short rods	*Thalassospira* sp.	MK503513
13		VLS-B04	Betul-E7	Gram negative, short rods	*Pseudomonas* sp.	MK503514
14		VLS-B04	Betul-E8	Gram negative, Rods	*Pseudomonas* sp.	MK503515
15		VLS-B04	Betul-E12B	Gram negative, short rods	*Pseudomonas* sp.	MK503516
16		VLS-B04	Betul-E14A	Gram negative, Rods	*Tistrella* sp.	MK503517
17		VLS-B04	Betul-A	Gram negative, short rods	*Thalassospira* sp.	MK503545
18		VLS-B04	Betul-SV 20B	Gram negative, short rods	*Vibrio* sp.	MK503546
19	Candolim beach	VLS-C13	Can-31	Gram negative, Rods	*Shewanella* sp.	MK503534
20		VLS-C13	Can-41	Gram negative,	*Serratia* sp.	MK503535

				rods		
21		VLS-C13	Can-42	Gram negative, Rods	*Klebsiella* sp.	MK503536
22		VLS-C13	Can-43	Gram negative, short rods	*Alcanivorax* sp.	MK503537
23		VLS-C13	Can-44	Gram negative, short rods	*Vibrio* sp.	MK503538
24		VLS-C13	Can-46	Gram negative, Rods	*Pseudomonas* sp.	MK503539
25		VLS-C13	Can-50	Gram negative, short rods	*Pseudomonas* sp.	MK503540
26		VLS-C13	Can-52	Gram negative, short rods	*Tistrella* sp.	MK503541
27		VLS-C13	Can-53	Gram negative, short rods	*Enterobacter* sp.	MK503542
28		VLS-C13	Can-54	Gram negative, short rods	*Enterobacter* sp.	MK503543
29		VLS-C13	Can-57	Gram negative, short rods	*Tistrella* sp.	MK503544

3.4 Discussion

Tarballs are hydrocarbonaceous marine pollutants. They support the growth of bacteria, including pathogenic ones on them (Snellman et al., 1988; Itah and Essien, 2005; Elshafie et al., 2007; Tao et al., 2011). In this study, the tarball samples were collected immediately after their deposition on beaches. Though all the tarball samples processed were found to be high in bacterial load (CFU counts), there was a slight difference in CFU counts in the duplicate samples processed (Sample ID: VLS-B03, Table 3.1). This can be attributed to the oily and uneven structure of the tarballs. There was a matted growth on some media at desired dilution, and hence CFUs could not be counted. The maximum number of CFUs recorded on each medium are as follows: 2.36×10^6 (Mac-Conkey's agar), 1.36×10^6 (EMB agar), 4.6×10^5 (TCBS agar), and 1.07×10^5 (SDS agar), 2.28×10^6 (T1N1 agar) and 2.31×10^5 (MSA agar) (Table 3.1).

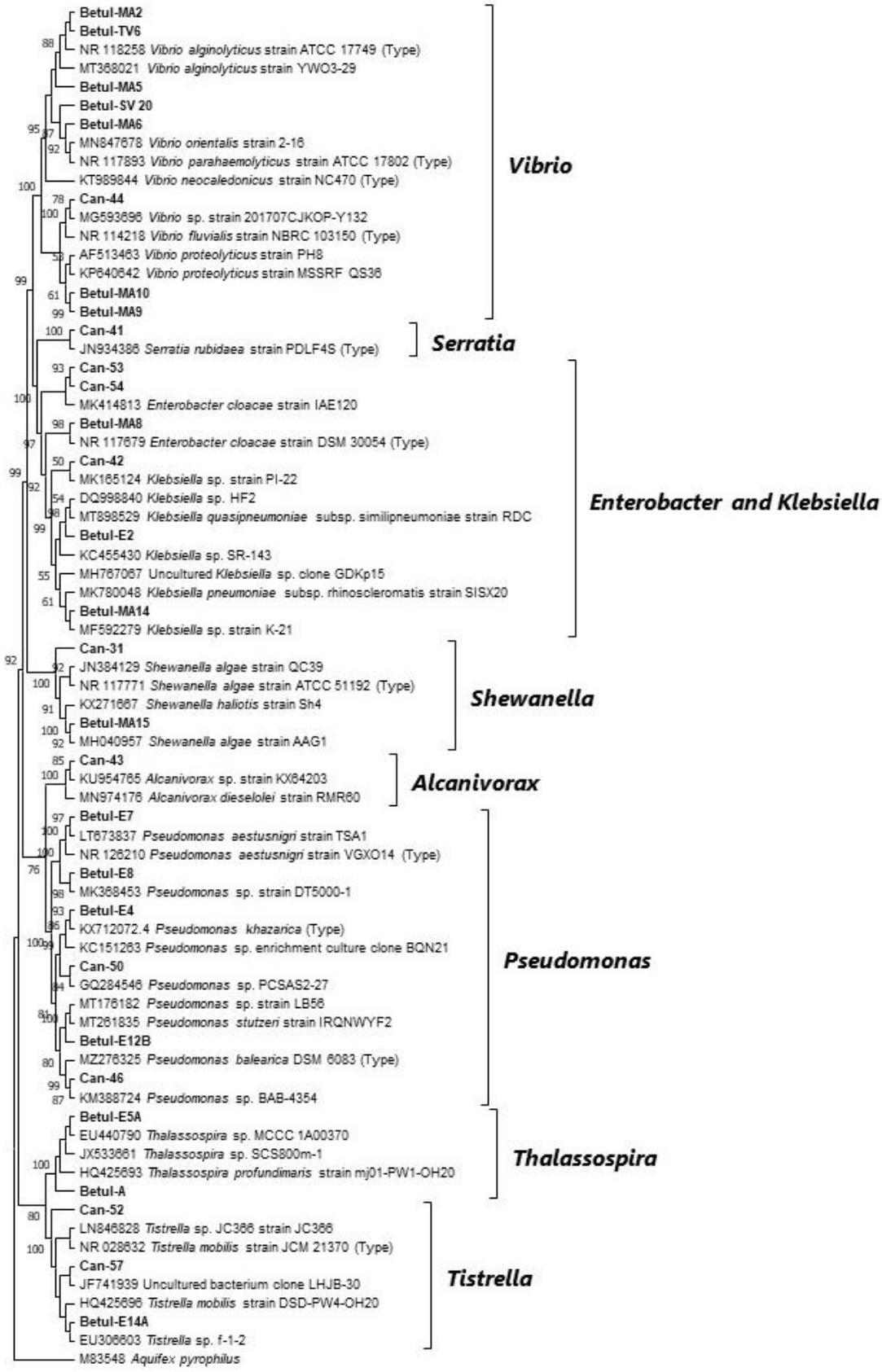


Figure 3.1: Phylogenetic relationships of pathogenic groups of bacteria isolated from tarballs, collected from Betul and Candolim beaches of Goa, based on 16S rRNA gene sequence analysis.

Tao et al. (2011) used T1N1 agar for the enumeration of *Vibrio* species and reported $2.8\pm1.0\times10^6$, $3.5\pm0.7\times10^5$, $2.8\pm0.4\times10^6$, $2.7\pm0.6\times10^5$ CFU/g per tarball from 5 samples collected from beaches of Gulf of Mexico, USA. *Enterobacter*, *Pseudomonas*, *Klebsiella*, *Shewanella* and *Vibrio* were reported in our previous study that investigated the general bacterial diversity associated with tarballs from Goa (Shinde et al., 2018). In this thesis work, a high number of *Vibrio* species and *Pseudomonas* species were found. Out of the nine bacterial genera identified in this study (Figure 3.1), seven are known for their pathogenic lifestyle. These are *Enterobacter*, *Klebsiella*, *Pseudomonas*, *Serratia*, *Shewanella*, *Thalassospira* and *Vibrio*.

Pathogenic bacteria such as *Vibrio* species and coliforms were previously determined from various seafood samples and seawaters (Edun et al., 2015) but rarely studied from tarballs. Freshly formed tarballs float on surface ocean water due to buoyancy. It is speculated that microbes are in an optimum growth atmosphere on tarballs that float on surface seawater. This is because oxygen required for growth is derived from the air; the temperature is obtained from the sunrays, while the nutrients are derived from the tarballs. According to Thompson et al. (2005), the population of *Vibrio* increases with increased organic nutrients. *Vibrio* species have been previously reported in oil-contaminated environments (Kostka et al., 2011). According to Vezzulli et al. (2016), with increasing climate temperatures, *Vibrio*-associated diseases are also increasing worldwide. It is suggested that components of crude oil can lower immunity in marine organisms and make them susceptible to bacterial/ viral infections (Bayha et al., 2017). In this scenario, the high numbers of pathogenic groups of bacteria on tarballs warrant serious consideration.

Recently, Fernandes et al. (2019) conducted an NGS analysis to unravel the unculturable diversity of bacteria from tarballs collected from North Goa. They reported 36 bacterial genera having the potential to cause disease either in humans or in animals or both. *Acinetobacter*, *Klebsiella*, *Rhodococcus*, *Staphylococcus* and *Vibrio* are the major pathogenic taxa reported by them. In a subsequent NGS study, Fernandes et al. (2020) analysed seawater samples from tarball-contaminated and tarball-non-contaminated periods from Vagator beach of Goa. They reported a greater number of *Vibrio* species (16.16 % OTUs) in tarball-contaminated seawater than in non-contaminated seawater (0.17 % OTUs). They identified three *Vibrio* species: *V. shilonii*, *V. fortis*, and *V.*

harveyi. An interesting study from the USA by Liu and Liu (2013) investigated three weathered oil mousse samples (=tarballs) collected from the salt marshes and surface seawater after the Deepwater Horizon oil spill and reported 57 % abundance of *Vibrio* in one of the samples (based on culture-independent pyrosequencing method). They suggested that *Vibrio* species are involved in hydrocarbon degradation. Itah and Essien (2005) reported pathogens like *E. coli* and *V. parahaemolyticus* from tarball samples collected from Ibeno beach in Nigeria.

Most tarball-associated bacteria taxa identified in this study have been previously reported for their pathogenic lifestyle. *Vibrio alginolyticus* is reportedly associated with seafood poisoning, and it causes intestinal infections in humans, leading to bacteremia, septic shock, and multiple organ failures (Fu et al., 2016). *Vibrio parahaemolyticus* is pathogenic, while *V. orientalis* from the Orientalis clade does not cause any disease (Chen et al., 2017; Restrepo et al., 2018). *Vibrio fluvialis* was declared as an emerging human pathogen from coastal regions by Ramamurthy et al. (2014), and this species was responsible for diarrhoea outbreaks and extraintestinal infections in humans. A virulent factor present in *V. proteolyticus* is reported to cause damage to eukaryotic cells which leads to cell lysis (Ray et al., 2016).

Among *Enterobacter* species, *E. cloacae* is a common opportunistic pathogen that causes nosocomial infections and has been reported from many clinical samples (Davin-Regli and Pages, 2015). *Klebsiella quasipneumoniae* was reportedly associated with bloodstream infections (Imai et al., 2019). Nosocomial infections are frequently caused by *K. pneumoniae* which is an important opportunistic pathogen (Chang et al., 2022). *Shewanella* is a human pathogen frequently found in clinical specimens of immunocompromised patients (Holt et al., 2005). *Shewanella haliotis* which causes bacteremia in humans was isolated from shellfish in Taiwan. *Shewanella* species are associated with human hepatobiliary disease (Wu et al., 2018). *Shewanella* is found in infections like otitis, pneumonia, sepsis, and soft tissue (Torri et al., 2018).

Marchese et al. (2019) for the first time reported *Thalassospira profundimaris* as an invasive infection-causing bacterium. *Thalassospira* sp. was discovered as a pathogen in marine agar-producing algae (Sun et al., 2011). While Noble and Overman (1994) reported that *Pseudomonas stutzeri* is not commonly found in patients and rarely causes

disease. Recently it was found to cause infective endocarditis in a 52-year-old woman (Alwazzeh et al., 2020). Ursua et al. (1996) described *Serratia rubidaea* as an invasive pathogen that caused cholangitis and septicemia in a 64-year-old woman-debilitated patient

The above reports suggest that the pathogenic bacterial groups investigated in this study can lead to diseases, especially in people frequently visiting beaches and sea animals. Microbes that are hydrocarbon degraders as well as known pathogens are mostly saprophytes having a versatile metabolic capacity (Rojo and Martinez, 2010). For example, *Vibrio* comprises known human pathogens and is involved in hydrocarbon degradation (Smith et al., 2012). It is stated that the reason behind most hydrocarbon-degrading bacteria being pathogens is that the traits which are useful in hydrocarbon utilisation such as surface adhesion, biosurfactant production, and efflux pump also play a role in pathogenicity (Rojo and Martinez, 2010). Seven bacterial genera including *Enterobacter*, *Klebsiella*, *Pseudomonas*, *Serratia*, *Shewanella*, *Thalassospira* and *Vibrio* reported in this study are known as both hydrocarbon degraders and pathogens. Further studies are needed to confirm their pathogenicity and degradation capabilities.

3.5 Conclusion

Tarballs do carry different groups of pathogenic bacteria. The CFUs of pathogenic groups of bacteria on the selective media were in the magnitude of 10^3–10^6. Phylogenetic analysis based on 16S rRNA gene sequence data revealed that the 29 tarball-associated bacteria belong to nine genera, namely, *Alcanivorax*, *Enterobacter*, *Klebsiella*, *Pseudomonas*, *Serratia*, *Shewanella*, *Thalassospira*, *Tistrella* and *Vibrio*. Seven of the nine bacterial genera have been reported as pathogens in previous studies (Chapter 3). Most of the newly generated bacterial sequences clustered with *Vibrio* and *Pseudomonas*. The analysis of diverse groups of pathogenic groups of bacteria is subject to future studies.

Chapter 4

Degradation of Tarballs Using Associated Bacterial Consortia

4.1 Introduction

Transportation of crude-oil and petroleum products is mostly done by sea route. Incidents like tanker accidents and the release of ballast water occasionally result in introduction of crude oil and petroleum products into marine waters (Kvenvolden and Cooper, 2003). Due to weathering, oil spill leads to the formation of tarballs, which get transported to nearby coastal areas by ocean currents and waves. Tarballs are semi-solid remnants of crude oil, and they are frequently reported from many coastal areas of the world (Warnock et al., 2015). They are rich in high molecular weight n-alkanes and polycyclic aromatic hydrocarbons (PAHs), which pose a great danger to algae and phytoplankton, the primary producers of the marine food chain. They can disturb the food chain, accumulate in tissues of living organisms, and some may even cause carcinogenic effects in marine organisms (Perelo, 2010; Hassanshahian et al., 2014).

Goa state in India has been facing tarball pollution on its tourist beaches since the 1970s (Suneel et al., 2015). Presently, the stakeholders are considering various mitigation methods. Biodegradation of tarballs is being considered as it is an environment-friendly option (Hassanshahian et al., 2014). It involves complete removal or partial conversion of toxic hydrocarbons of tarballs to less toxic products through mineralisation, using microorganisms such as bacteria and fungi (Bamforth and Singleton, 2005; Dellagnezze et al., 2014). As microorganisms tend to saturate on tarballs for nutrition and use it as a substratum to colonise (Shinde et al., 2017), microbial load on tarballs has been reported to be high (Snellman et al., 1988; Itah and Essien, 2005; Tao et al., 2011; Lotfinasabasl et al., 2012; Shinde et al., 2018). Tarballs are a serious threat to the marine environment (Warnock et al., 2015; Shinde et al., 2017).

Microbial degradation of tarballs, however, is poorly understood. Previous studies reported isolation of bacteria and fungi from tarballs and provided information on their abilities to degrade crude-oil and crude-oil derivatives such as kerosene, diesel, petrol, and fuel oil (Nair and Lokabharathi, 1977; Snellman et al., 1988; Lotfinasabasl et al., 2012; Nkem et al., 2016; Barnes et al., 2018). There are, however, few studies reported on degradation of tarballs by associated microbes. Itah and Essien (2005) reported the degradation of tarballs by associated bacteria and fungi from Nigeria, but their study was qualitative. There is a need to unravel possible roles played by associated microbes in tarball degradation.

In the present study, 38 tarball-associated bacteria isolated from Betul beach, Goa, India during our previous study (Shinde et al., 2018) were investigated for their ability to degrade tarballs. This study initially employed the dye-decolourization screening method (DCPIP method), followed by the characterisation of n-alkane degradation profiles of crude oil by GC-MS-MS analysis. This led to the selection of four bacterial strains for consortia preparation. Eleven bacterial consortia were prepared and studied for their abilities to degrade tarballs. After 45 days of incubation of tarballs with the bacterial consortia, n-alkanes and PAH compounds of control and residual samples were extracted and quantified by both gravimetric and GC-MS-MS methods.

4.2 Methodology

4.2.1 Materials used in GC-MS-MS analysis

For GC-MS-MS analysis, n-alkanes (C8–C40) and polycyclic aromatic hydrocarbons (PAHs; 16 compounds) standards were procured from Sigma Aldrich (USA), while the surrogate internal standards were procured from Chiron AS (Norway). A 100–200 mesh silica gel was baked at 400 °C for 4 h, cooled, then activated at 200 °C overnight and deactivated with 5 % of distilled water, and used for column preparation. Hexane and Dichloromethane used were of high purity, SupraSolv grade from Sigma Aldrich. All glassware was washed with detergent and distilled water, followed by the serial rinse with methanol, acetone, and hexane.

4.2.2 Screening of bacterial strains for crude oil degradation

In our previous study, 49 tarball-associated bacteria were isolated from Betul beach, Goa, India (Shinde et al., 2018), out of which 38 bacteria were screened in the present study for their crude-oil degradation abilities (Table 4.1). This was done following the method described in Bidoia et al. (2010) and adopted by Varjani et al. (2013). Briefly, 500 µl of dichloro-phenol-indophenol (DCPIP) from 1 µg/µl of stock solution was added into 50 ml of Bushnell Hass medium containing 500 µl of sterile crude oil. A known volume of bacterial suspension was added into the medium and incubated for 24–48 h. A control consortium was maintained without the inoculum. The change in colour of DCPIP from blue to colourless was observed. The colour change indicated the utilisation of crude oil by bacteria as a sole carbon source. Spectrophotometric analysis was performed to determine the colour difference in each consortium. After 72 h of incubation, the medium was centrifuged, and optical density was measured at 600 nm. The bacterial strains showing visible colour differences were considered having crude oil degradation ability.

4.2.3 Screening of bacterial strains for *n*-alkane degradation profiles

Based on their crude oil degradation abilities inferred through the DCPIP method (Table 4.1), four bacterial strains, *Pseudomonas* sp. Betul-14, *Nitratireductor* sp. Betul-D, *Pseudomonas* sp. Betul-M and *Alcanivorax* sp. Betul-O were selected and processed for characterisation of n-alkane degradation profiles by GC-MS-MS analysis. *Marinobacter* sp. Betul-26 was based on a literature survey that suggested *Marinobacter* is a good hydrocarbon degrader (Head et al., 2006; Hassanshahian et al., 2012; Xue et al., 2015). The taxonomic identity and GenBank accession numbers of 16S rRNA gene sequences are presented in (Shinde et al., 2018; Table 4.1). Each bacterial culture was separately inoculated in 25 ml of Bushnell Hass medium containing 500 µl of crude oil (2 %) as a sole carbon source. The consortia were kept on a rotary shaker for 30 days at 100 rpm and 35 °C. Optical density at 600 nm at a regular time interval and colony-forming units (CFUs) were measured to monitor bacterial growth. Controls without bacterial inoculum were used to quantify the abiotic loss during and incubation. Residual crude oil was extracted from the culture medium by shaking vigorously with an equal volume of 3:1 (v/v) hexane: dichloromethane (DCM) mixture for 15 min. The solvent extract was transferred to a clean beaker. This procedure was repeated twice. Sodium sulphate was

added to the hexane: dichloromethane extracts to remove the moisture, and the supernatants were concentrated to approximately 1 mL in a rotary evaporator. Initially, the sample was passed through the deactivated silica gel column (1 cm i.d. × 9 cm) to separate the impurities and transferred to the activated silica gel column (0.47 cm i.d. × 18 cm) for the fractionation. Briefly, 1 ml of concentrated sample was passed through 5 % H_2O-deactivated silica gel column. Aliphatic hydrocarbons were eluted with 30 ml of hexane: DCM (3:1 v/v). The eluent was subjected to roto-evaporation to decrease the volume to ~2 ml and then transferred to the fully activated silica gel column. Total n-alkanes were eluted with 10 ml of hexane. Eventually, the fraction was again concentrated to dryness using a nitrogen cycle purge to make the final volume before injecting it into GC-MS-MS.

4.2.4 Degradation of tarballs by bacterial consortia

Bacterial strains, *Pseudomonas* sp. Betul-14, *Marinobacter* sp. Betul-26, *Pseudomonas* sp. Betul-M and *Alcanivorax* sp. Betul-O were selected for consortia preparation. Eleven different combinations were prepared as shown in Figure 4.1 and Table 4.2. Though tarballs are in a semi-solid state and easy to weigh as compared to viscous crude oil, they contain sand particles, which leads to an error during weighing, hence in the quantitative values. To remove sand particles, tarballs were dissolved in hexane: DCM (3:1 v/v), and the upper portion of the solvent was carefully removed after all the particles settled at the bottom. This fraction containing dissolved tarball was kept at room temperature till the complete evaporation of the solvent. Bushnell Hass medium was prepared in 50 % seawater and distributed 25 ml in each of the 11 consortia. Tarballs were weighed (~150 mg) and added into the medium, sterilised at 121 °C for 20 min, and inoculated with respective bacterial strains. They were incubated on a rotary shaker at room temperature at 100 rpm. The bacterial growth was monitored by streaking on agar plate and Gram staining. After 45 days of incubation, residual tarball was extracted from the growth medium. First, the bacterial growth was halted by acidifying the medium at pH 2 (Hassanshahian et al. 2012). Dichloromethane was used to extract the residual tarball. The extract was passed through anhydrous Sodium sulphate to remove water molecules and collected in a pre-weighed empty beaker and kept at room temperature for complete evaporation. % degradation (Gravimetric analysis) was calculated using the formula:

$$\% \text{ degradation} = \frac{\text{Amount of tarball degraded}}{\text{Amount of tarball added in medium}} \times 100$$

Next, fractionation of tarball was achieved using the protocol described for crude oil. Pre-dried residual tarball was dissolved in 1 mL of Hexane: DCM (1:3 v/v) and the surrogate internal standard mixture (d8-Naphthalene, d10-Phenanthrene, and d12-Chrysene) was added to the sample before the extraction process to check the recovery percentage and passed through the column. Total alkanes were eluted with 10 mL of hexane, whereas PAH compounds were eluted using 30 ml of hexane-DCM mixture (3:1 v/v). Both fractions were quantified on GC–MS–MS. Analyses for n-alkane and PAHs were performed on a Thermofisher Scientific Trace 1300 Gas Chromatograph, coupled with Thermo TSQ8000 Mass Spectrometer with a Thermo AS 1310 auto-sampler under selected ion monitoring (SIM) mode. The DB-5 column had 60 m length × 0.25 mm internal diam. with 0.25 μm film thickness with helium (1.2 ml/min) as a carrier gas used for the sample analysis. For alkane and PAH analysis, the injector and detector temperatures were set to 260 °C and 300 °C, respectively. The oven temperature program for alkane analysis was initially 50 °C, held for one minute, then ramped to 140 °C at 10 °C/min, ramped to 320 °C at 6°C/min and finally a 28-min hold at 320 °C. The oven temperature program for PAH analysis was initially 70 °C with a hold of 2-min, then ramped to 150 °C at 30 °C /min and then ramped to 310 °C at 4 °C /min, then finally a 10-min hold at 310 °C. The n-alkanes C8–C40, including pristane and phytane and 16 PAH compounds such as Naphthalene (Nap), Acenaphthylene (Acy), Acenaphthene (Ace), Fluorene (Fl), Phenanthrene (Phe), Fluoranthene (Fluo), Pyrene (Py), 1-Methyl fluoranthene (1-M-Fluo), B(a)anthracene (BaA), Chrysene (Chr), 1-Methyl chrysene (1-M-Chr), B(a)fluoranthene (BaF), B(k) fluoranthene (BkF), B(a)pyrene (BaP), Dibenzo anthracene (D(B)Ant), and Benzoperylene (BgP) were analysed in this study. The recovery was confirmed through three replicate analyses of the samples which were spiked with surrogate standards. The percentage depletion of n-alkane/PAH present in the tarball was calculated using the formula:

$$Percent\ degradation\ (n-alkanes) = 100 - \left(\frac{\Sigma\ n-alkanes\ (\ nC8–nC40)\ from\ sample}{\Sigma\ n-alkanes\ (\ nC8–nC40)\ from\ control)} \times 100 \right)$$

$$Percent\ degradation\ (PAH) = 100 - \left(\frac{\Sigma\ concentration\ of\ PAH\ compounds\ from\ sample}{\Sigma\ concentration\ of\ PAH\ compounds\ from\ control)} \times 100 \right)$$

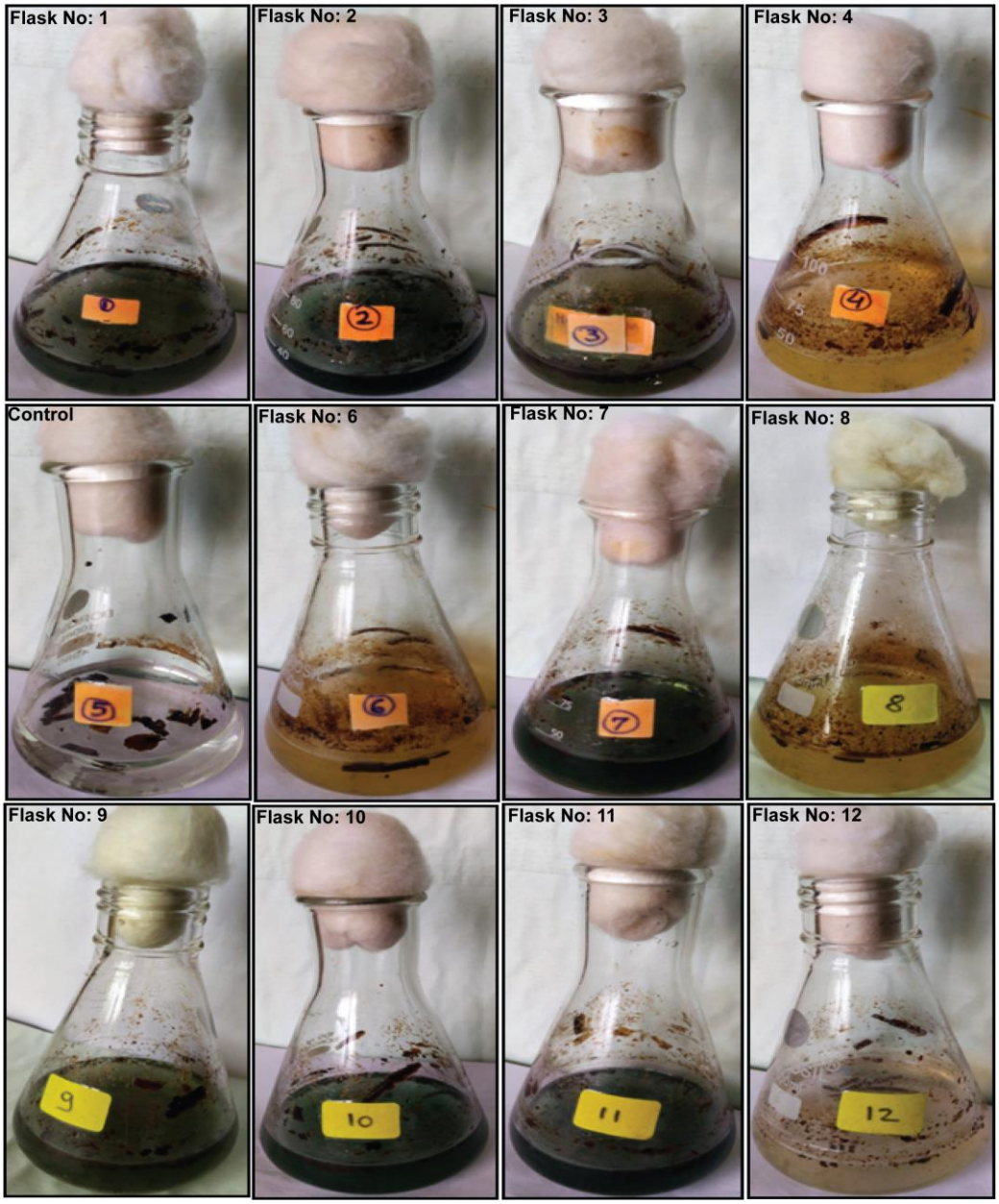


Figure 4.1: Bacterial consortia used for studying the degradation of tarballs in the laboratory.

4.3 Results

4.3.1 Screening of bacterial strains for crude oil degradation

The optical density (OD) measured at a specific time interval during the DCPIP screening is shown in Table 4.1. The OD of control for set A and set B was 0.088 and 0.079, respectively. In total, six bacterial strains decolourized the medium, the ODs of which were 0.022 *Pseudomonas* sp. (Betul-13), 0.017 *Pseudomonas* sp. (Betul-14), 0.036 *Nitratireductor* sp. (Betul-D), 0.026 *Pseudomonas* sp. (Betul-M), 0.04 *Nitratireductor* sp. (Betul-N), and 0.04 *Alcanivorax* sp. (Betul-O). *Pseudomonas* sp. (Betul-14), *Nitratireductor* sp. (Betul-D), *Pseudomonas* sp. (Betul-M), and *Alcanivorax* sp. (Betul-O) were selected for further processing. In the screening step, a sudden drop in colour intensity of DCPIP was observed for *Klebsiella* sp. (Betul-8), *Nitratireductor* sp. (Betul-N), and *Bhargavaea* sp. (Betul-L) after 24 h of incubation but was found to be constant afterwards. *Pseudomonas* sp. (Betul-14) and *Pseudomonas* sp. (Betul-15) showed late decolourization but were found to be consistent till the medium turned colourless. This can be attributed to different lag phases for different bacteria. A gradual colour change was observed for *Alcanivorax* sp. (Betul-O) and *Alcanivorax* sp. (Betul-H). Feeble dye decolourization was observed for *Brevibacterium* sp. (Betul-21(2)), *Cellulomonas* sp. (Betul-29), and *Enterobacter* sp. (Betul-10). These bacterial strains were excluded from further processing.

4.3.2 Characterization of n-alkane degradation profiles of crude oil by GC-MS-MS analysis

The results of % degradation of n-alkanes of crude oil are presented in Figure 4.2. Almost all alkanes, i.e., from C15 to C40, analysed were found to be degraded by *Pseudomonas* sp. Betul-14, *Marinobacter* sp. Betul-26, *Nitratireductor* sp. Betul-D, *Pseudomonas* sp. Betul-M and *Alcanivorax* sp. Betul-O. Hydrocarbons in the range of C8–C14 were either found none or in very low detectable levels and considered as an abiotic loss. Alkanes in the range of C35– C40 were also barely detected. *Alcanivorax* sp. Betul-O, *Pseudomonas* sp. Betul-14, and *Pseudomonas* sp. Betul-M exhibited maximum degradation of 32.72 %, 27.06 %, and 24.10 %, respectively. Comparatively, less degradation of crude-oil total alkanes was exhibited by *Nitratireductor* sp. Betul-D (13.26 %) and *Marinobacter* sp. Betul-26 (9.75 %).

4.3.3 Degradation of *n*-alkanes of tarballs by bacterial consortia

In this study, the bacterial consortia exhibited maximum degradation of tarballs than as an individual strain (Figure 4.3). The range of total alkanes present in the flasks varied between 6744 and 140,867 μg/g out of 304,156 μg/g present in the control. A significant amount of n-alkane was depleted. The calculated per cent degradation of n-alkanes from tarballs revealed that a minimum 53.69 % of total n-alkanes were depleted in almost all consortia, with a maximum degradation of 97.78 % in consortium 7 (*Pseudomonas* sp. Betul-14 + *Pseudomonas* sp. Betul-M + *Alcanivorax* sp. Betul-O), 97.21 % in consortium 11 (*Pseudomonas* sp. Betul-14 + *Marinobacter* sp. Betul-26 + *Pseudomonas* sp. Betul-M + *Alcanivorax* sp. Betul-O), 96.34 % in consortium 10 (*Pseudomonas* sp. Betul-14 + *Marinobacter* sp. Betul-26 + *Alcanivorax* sp. Betul-O), 95.74 % in consortium 9 (*Pseudomonas* sp. Betul-14 + *Marinobacter* sp. Betul-26 + *Pseudomonas* sp. Betul-M), 95.01 % in consortium 8 (*Alcanivorax* sp. Betul-O + *Pseudomonas* sp. Betul-M + *Marinobacter* sp. Betul-26), and 92.57 % for consortium 6 (*Marinobacter* sp. Betul-26 + *Pseudomonas* sp. Betul-M).

The lowest degradation was observed for the consortium comprising *Pseudomonas* sp. Betul-26 + *Alcanivorax* sp. Betul-O (consortium 4), i.e., 22.78 % of PAH compounds and 53.69 % of alkanes (Table 4.2 and Figure 4.3). The low L/H ratios in almost all the consortia further suggested that low molecular compounds were degraded more than the high molecular compounds (Table 4.3).

4.3.4 Degradation of PAH compounds of tarballs by bacterial consortia

The range of total PAHs present in the consortia varied between 113.6 and 230.7 μg/g out of 299.8 μg/g present in the control, suggesting that a significant amount of PAHs was degraded (Figure 4.4, Table 4.2). The severe depletion of naphthalene and acenaphthylene in both control and treatment was observed (data not shown), indicating that the high vapour pressure of low molecular weight (LMW) PAHs may cause the decrease of these compounds during the incubation period. Therefore, the degradation percentage was not considered for these compounds. The calculated percentage degradation of other PAHs showed that a minimum 22.78 % of total PAHs was degraded in almost all consortia, with the maximum degradation of 61.98 % occurring in consortium 7 within 45 days (Table 4.2, Figure 4.4). Maximum PAH depletion, i.e.,

61.98 % and 60.86 % was found in consortium 7 (*Pseudomonas* sp. Betul-14 + *Pseudomonas* sp. Betul-M + *Alcanivorax* sp. Betul-O) and consortium 11 (*Pseudomonas* sp. Betul-14 + *Marinobacter* sp. Betul-26 + *Pseudomonas* sp. Betul-M + *Alcanivorax* sp. Betul-O) respectively.

Table 4.1: Spectrophotometric analysis of dye decolorization of DCPIP measured at 600 nm

Set A							
Strain No.	O.D.	Genus Name	GenBank Accession No.	Strain No.	O.D.	Genus Name	GenBank Accession No.
Betul 1	0.082	*Pseudoxanthomonas* sp.	MG664257	Betul 23	0.086	*Marinobacter* sp.	MG664238
Betul 2	0.078	*Enterobacter* sp.	MG664235	Betul 24	0.088	*Pseudoxanthomonas* sp.	MG664259
Betul 3	0.09	*Pantoea* sp.	MG664246	Betul 26	0.088	*Marinobacter* sp	MG664239
Betul 4	0.065	*Pseudomonas* sp.	MG664252	Betul 28	0.069	*Cellulomonas* sp.	MG664231
Betul 5	0.085	*Klebsiella* sp.	MG664236	Betul 29	0.049	*Cellulomonas* sp.	MG664232
Betul 6	0.075	*Bacillus* sp.	MG664225	Betul 30	0.089	*Cellulomonas* sp.	MG664233
Betul 9	0.066	*Vibrio* sp.	MG664261	Betul D	0.036	*Nitratireductor* sp.	MG664242
Betul 11	0.078	*Pseudomonas* sp.	MG664247	Betul I	0.062	*Alcanivorax* sp.	MG664222
Betul 19	0.066	*Pseudomonas* sp.	MG664251	Betul K	0.084	*Bhargavaea* sp.	MG664227
Betul 21(1)	0.056	*Brevibacterium* sp.	MG664229	Betul M	0.026	*Pseudomonas* sp.	MG664256
Betul 21(2)	0.052	*Brevibacterium* sp.	MG664230	Betul Q	0.062	*Oceanimonas* sp.	MG664245
Betul 22	0.085	*Pseudoxanthomonas* sp.	MG664258	Control	0.09		
Set B							
Strain No.	O.D.	Genus Name	GenBank Accession No.	Strain No.	O.D.	Genus Name	GenBank Accession No.
Betul 8	0.066	*Klebsiella* sp.	MG664237	Betul H	0.062	*Alcanivorax* sp.	MG664221

Betul 10	0.053	*Enterobacter* sp.	MG664234	Betul J	0.076	*Bhargavaea* sp.	MG664226
Betul 12	0.071	Unidentified bacteria	Not available	Betul L	0.073	*Bhargavaea* sp.	MG664228
Betul 13	0.022	*Pseudomonas* sp.	MG664248	Betul N	0.04	*Nitratireductor* sp.	MG664243
Betul 14	0.017	*Pseudomonas* sp.	MG664249	Betul O	0.04	*Alcanivorax* sp.	MG664223
Betul 15	0.075	*Pseudomonas* sp.	MG664250	Betul P	0.067	*Alcanivorax* sp.	MG664224
Betul F	0.046	*Pseudomonas* sp.	MG664254	Betul R	0.063	*Nitratireductor* sp.	MG664244
Betul G	0.066	*Pseudomonas* sp.	MG664255	Control	0.079		

The experiment was conducted in two sets, Set A and Set B. The bold values are the lowest ODs observed in test flasks compared to respective control flasks in each set.

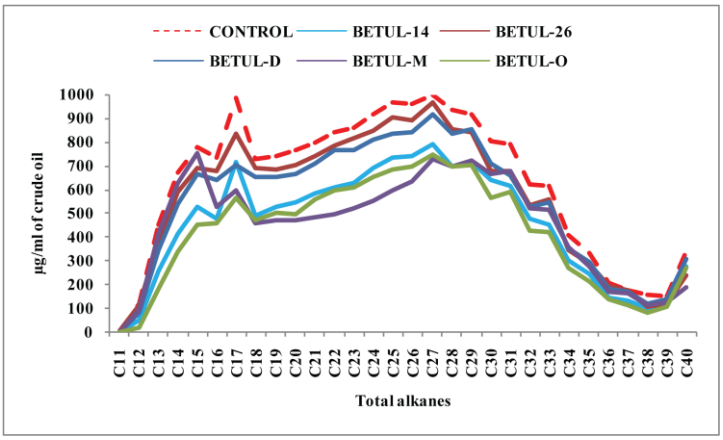


Figure 4.2: Graph depicting individual n-alkane degrading profiles of select bacterial strains (*Pseudomonas* sp. Betul-14, *Marinobacter* sp. Betul-26, *Nitratireductor* sp. Betul-D, *Pseudomonas* sp. Betul-M, and *Alcanivorax* sp. Betul-O).

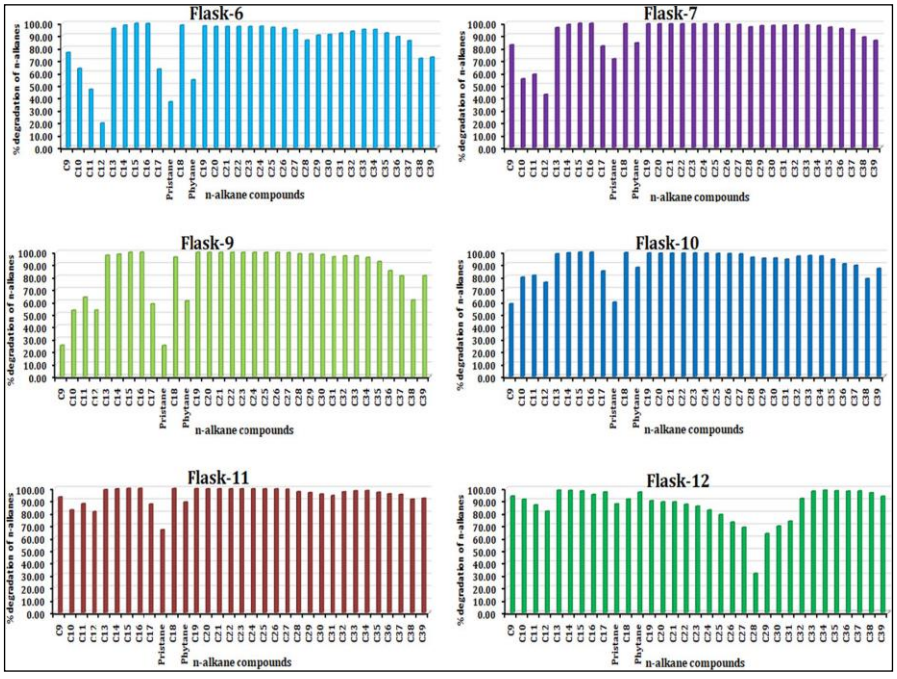


Figure 4.3: Graph depicting degradation of individual n-alkane compounds (%) of tarballs by select bacterial consortia.

Flask (consortium) no. 6: Bushnell Hass Medium+ Crude oil (*Marinobacter* sp. Betul-26 + *Pseudomonas* sp. Betul-M. **Flask (consortium) no. 7**: Bushnell Hass Medium + Crude oil (*Pseudomonas* sp. Betul-14 + *Pseudomonas* sp. Betul-M + *Alcanivorax* sp. Betul-O). **Flask (consortium) no. 9**: Bushnell Hass Medium + Crude oil (*Pseudomonas* sp. Betul-14 + *Marinobacter* sp. Betul-26 + *Pseudomonas* sp. Betul-M). **Flask (consortium) no. 10**: Bushnell Hass Medium + Crude oil (*Pseudomonas* sp. Betul-14 + *Marinobacter* sp. Betul-26 + *Alcanivorax* sp. Betul-O). **Flask (consortium) no. 11**: Bushnell Hass Medium + Crude oil *Pseudomonas* sp. Betul-14 + *Marinobacter* sp. Betul-26 + *Pseudomonas* sp. Betul-M + *Alcanivorax* sp. Betul-O. **Flask (consortium) no. 12**: Bushnell Hass Medium + Crude oil (*Marinobacter* sp. Betul-26 + *Alcanivorax* sp. Betul-O).

4.4 Discussion

Tarballs support diverse forms of bacteria. To check their role in hydrocarbon degradation, two different screening methods were employed in this study. The first method is based on dye decolourization of DCPIP and is a simple and rapid method to screen oil-degrading bacteria from high numbers of bacteria (Hanson et al., 1993). Mariano et al. (2008) applied the same method to screen diesel-degrading bacteria. Bidoia et al. (2010) reported different time spans for *Bacillus subtilis* to reduce dye as at 138 h, 125 h, 75 h, and 87 h in synthetic, semisynthetic, mineral, and oil medium, respectively. Varjani et al. (2013) suggested that DCPIP along with Bushnell Hass medium may not hamper the growth of microorganisms and is a reliable screening method.

To check the efficiency of bacteria for utilisation of hydrocarbons, they were checked individually for n-alkane degradation profiles from crude which were analysed by GC-MSMS analysis. While we observed less degradation of n-alkane compounds by *Nitratireductor* sp., Ma et al. (2021) reported *Nitratireductor* as the major oil-degrader from deep-sea hydrothermal sediments of the South Mid-Atlantic Ridge.

The degradation of C8 to C40 was determined in this study. Hassanshahian et al. (2012) determined the efficiency of 25 bacterial strains isolated from oil-contaminated areas of the Persian Gulf to degrade n-alkanes from crude oil by GC-MS analysis and reported that *Alcanivorax dieselolei* to degrade n-alkanes from crude oil to be 98 %.

It is believed that indigenous bacteria can probably degrade a wide range of target constituents of a complex mixture such as oily sludge. Mishra et al. (2001), however, stated that a single bacterial species has only limited capacity to degrade all the fractions of hydrocarbons present in crude oil. A bacterial consortium, therefore, can be more effective in degrading crude oil and tarballs. Santisi et al. (2015), who tested a consortium of *Pseudomonas* and *Alcanivorax* to degrade alkanes from crude oil, reported the depletion of C12–C30 alkanes in the range of 50–90 %. In India, the Tata Energy Research Institute (TERI), New Delhi had launched a successful product named ‘oil-zapper’, a patented product to remediate lands dumped with oily sludge. The product is a consortium of bacteria (Randhwa and Kaushal, 2014).

In a previous study, *Pseudomonas aeruginosa* from oil-contaminated soils could degrade 40 mg/L of phenanthrene and fluorene completely within 7 days and fluoranthene and pyrene more than 30 % within 12 days (Zhang et al., 2011). Fu et al. (2014) reported the biodegradation of phenanthrene, anthracene, fluoranthene, and pyrene at 100 mg/l is 99 %, 65 %, 99 %, and 79 %, respectively, by the consortium Y12. It could degrade 7 % of benzo[a]pyrene at 10 mg/l within 10 days. Our results indicate that Benzo(a)Fluoranthene and Benzo(a) Pyrene were highly degraded compared to other PAHs in almost all the consortia. Despite recent research developments in HMW PAH degradation, the studies on the biodegradation of HMW PAHs associated with other hydrocarbons in mixtures are limited (Peng et al., 2008; Seo et al., 2009; Kanaly and Harayama, 2010). Our results showed that the L/H ratio of PAHs (Table 4.3) ranged between 0.986 and 1.336, which is higher than the ratio of the control (0.937). It revealed more depletion of high molecular compounds than the low molecular compounds. Both high and low molecular compounds were almost equally degraded by the bacterial consortia, *Pseudomonas* sp. Betul-14 + *Pseudomonas* sp. Betul-M + *Alcanivorax* sp. Betul-O (consortium 7) and *Pseudomonas* sp. Betul-14 + *Marinobacter* sp. Betul-26 + *Pseudomonas* sp. Betul-M + *Alcanivorax* sp. Betul-O in (consortium 11) (~66 %, Figure 4.4). The consortium comprising *Pseudomonas* sp. Betul-14, *Pseudomonas* sp. Betul-M, and *Alcanivorax* sp. Betul-O (consortium 7) was found to be the most effective combination to degrade alkanes and PAH compounds, followed by the combination of *Pseudomonas* sp. Betul-14, *Marinobacter* sp. Betul-26, *Pseudomonas* sp. Betul-M and *Alcanivorax* sp. Betul-O (consortium 11).

Itah and Essien (2005) suggested that only 0.001 % of the total bacterial count obtained from tarballs could degrade tarballs. Bacosa et al. (2016) analysed 44 tarball samples from Galveston and Mustang Island in Texas, United States. *Alcanivorax*, *Psychrobacter*, *Pseudoaltermonas* and *Oceanospirillales* species were the dominant genera reported by them. *Alcanivorax* and *Psychrobacter* genera are known for their oil degradation abilities (Harayama et al., 2004; Giudice et al., 2010). The tarballs from Galveston Island were rich in alkane-degrading bacteria, while those from Mustang Island were rich in PAH-degrading bacteria. Nkem et al. (2016) isolated and reported two bacteria, *Cellulosimicrobium cellulans* and *Acinetobacter baumannii* using an enrichment technique from tarballs of Rhu Sepuluh beach, Terengganu, Malaysia; which could utilise 64.4 % and 58.1 % of diesel, respectively. In their extended work, in an

optimised experiment, Nkem et al. (2020) tested consortia of bacteria *Pseudomonas stutzeri*, *Cellulosimicrobium cellulans*, *Acinetobacter baumannii* and *Pseudomonas* balearica isolated from tarball in Terengganu Beach, Malaysia, which could degrade more than 93.6 % of diesel oil in 30 days of incubation.

79 bacterial genera, 9 cyanobacterial genera, 103 fungal genera, and 19 algal genera from marine niches have been reported to be involved in petroleum degradation (Xue et al., 2015). Bacteria have developed metabolic machinery that can degrade complex hydrocarbons. In the aerobic way of degradation, enzymes such as alkane monooxygenase and aromatic ring dioxygenase hydroxylases (ARDHs) are involved in the alkanes and aromatic compounds, respectively (Dellagnezze et al., 2014). In both enzymatic reactions, molecular oxygen is used as a co-substrate which helps the terminal or sub-terminal hydroxylation of alkane chains or the mono or dihydroxylation of aromatic rings (Al-Sayegh et al., 2016). *Alcanivorax* is known for its hydrocarbonoclastic abilities (Yakimov et al., 2007). *Alcanivorax dieselolei* is a principal alkane degrader that was first isolated from surface seawater and sediments of Bohai Sea (Liu and Shao, 2005). Hassanshahian et al. (2012) reported the efficiency of *Alcanivorax dieselolei* isolated from oil-contaminated areas of the Persian Gulf to degrade n-alkanes from crude oil to be 98 %. Santisi et al. (2015), who tested a consortium of *Pseudomonas* and *Alcanivorax* to degrade alkanes from crude oil, reported the depletion of C12–C30 alkanes in the range of 50–90 %.

The ratios C17/ Pristane and C18/Phytane are widely used as an indicator to detect biodegradation effects as n-alkanes are more likely to get biodegraded than isoprenoids (Wang et al., 1998, 1999). Our results also show that compound C17 was depleted relatively more than Pristane. The highest ratios of C18/ Ph and Pr/Ph (~ 10 and 3 times, respectively higher than the other consortia) were observed for consortium 12, which revealed the isoprenoid alkane, Phytane, was more degraded than the n-alkanes only for that consortium. The bacterial consortia, numbered 1, 2, 3, 6, 7, 8, 9, 10, and 11, were able to degrade the n-alkane compounds. Still, it is interesting to note that consortium no. 12 (*Marinobacter* sp. Betul-26 + *Alcanivorax* sp. Betul-O) was able to degrade isoprenoid alkanes considerably.

Table 4.2: Degradation of tarball by bacterial consortia (%)

Flask No.	**Consortium**	**Gravimetric analysis**	**GC analysis**	
			PAH	**Alkane**
1	*Pseudomonas* sp. Betul-14 + *Marinobacter* sp. Betul-26	45	46.27	85.94
2	*Pseudomonas* sp. Betul-14 + *Alcanivorax* sp. Betul-O	46	42.01	84.59
3	*Pseudomonas* sp. Betul-14 + *Pseudomonas* sp. Betul-M	47.44	36.24	89.78
4	*Pseudomonas* sp. Betul-M + *Alcanivorax* sp. Betul-O	33.69	22.78	58.29
6	*Marinobacter* sp. Betul-26+ *Pseudomonas* sp. Betul-M	48.06	36.23	95.22
7	*Pseudomonas* sp. Betul-14 + *Pseudomonas* sp. Betul-M+ *Alcanivorax* sp. Betul-O	66.41	61.98	98.97
8	*Marinobacter* sp. Betul-26 + *Pseudomonas* sp. Betul-M + *Alcanivorax* sp. Betul-O	47.51	25.4	98.70
9	*Pseudomonas* sp. Betul-14 + *Marinobacter* sp. Betul-26+ *Pseudomonas* sp. Betul-M	47.0	28.8	98.96
10	*Pseudomonas* sp. Betul-14 + *Marinobacter* sp. Betul-26+ *Alcanivorax* sp. Betul-O	48.48	43.24	97.94
11	*Pseudomonas* sp. Betul-14 + *Marinobacter* sp. Betul-26 + *Pseudomonas* sp. Betul-M + *Alcanivorax* sp. Betul-O	49.94	60.86	98.49
12	*Marinobacter* sp. Betul-26 + *Alcanivorax* sp. Betul-O	Not available	50.87	79.37

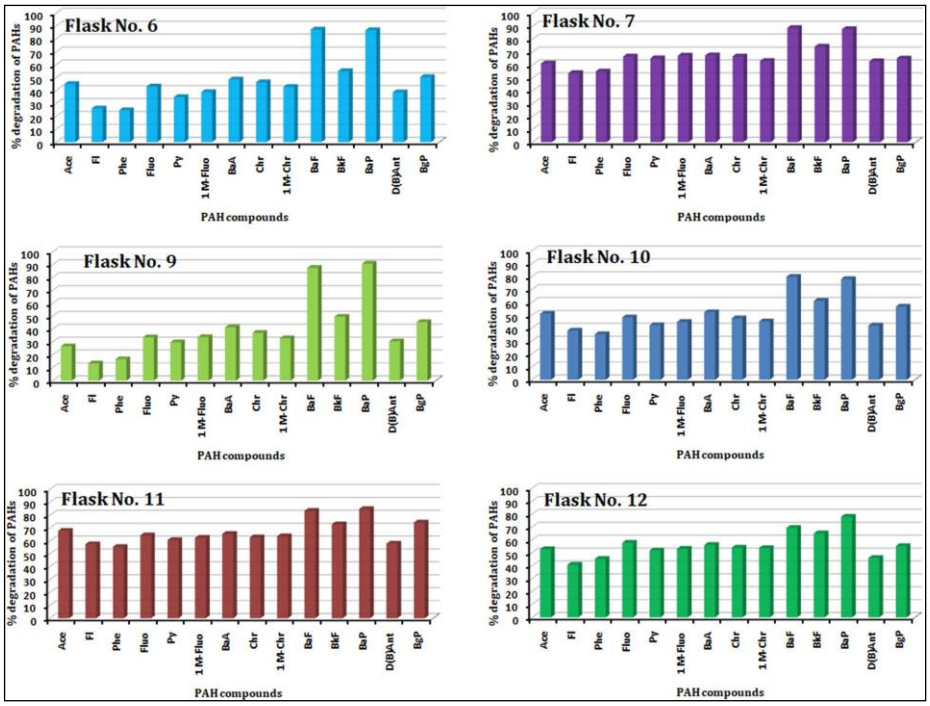


Figure 4.4: Graph depicting degradation of individual PAH compounds (%) of tarballs by select bacterial consortia.

Flask (consortium) no. 6: Bushnell Hass Medium + Crude oil (*Marinobacter* sp. Betul-26 + *Pseudomonas* sp. Betul-M. **Flask (consortium) no. 7**: Bushnell Hass Medium + Crude oil (*Pseudomonas* sp. Betul-14 + *Pseudomonas* sp. Betul-M + *Alcanivorax* sp. Betul-O). **Flask (consortium) no. 9**: Bushnell Hass Medium + Crude oil (*Pseudomonas* sp. Betul-14 + *Marinobacter* sp. Betul-26 + *Pseudomonas* sp. Betul-M). **Flask (consortium) no. 10**: Bushnell Hass Medium + Crude oil (*Pseudomonas* sp. Betul-14 + *Marinobacter* sp. Betul-26 + *Alcanivorax* sp. Betul-O). **Flask (consortium) no. 11**: Bushnell Hass Medium + Crude oil *Pseudomonas* sp. Betul-14 + *Marinobacter* sp. Betul-26 + *Pseudomonas* sp. Betul-M + *Alcanivorax* sp. Betul-O. **Flask (consortium) no. 12**: Bushnell Hass Medium + Crude oil (*Marinobacter* sp. Betul-26 + *Alcanivorax* sp. Betul-O).

Table 4.3: The weathering diagnostic ratios of n-alkane and PAH compounds of tarballs

	n-alkane DRs					PAH-DRs	
Flask No.	C_{17}/Pr	C_{18}/Py	Pr/Py	L/H-alkane	Σ n-alkanes (µg/g)	L/H-PAH	Σ PAH (µg/g)
1	NF	0.040	8.1521	0.052	42053	0.9867	160.53
2	NF	0.2446	8.6188	0.2222	46082	1.2264	173.2
3	NF	0.0852	9.8812	0.2026	30553	1.1762	190.51
4	0.0894	1.9262	9.5555	0.6535	124766	1.2139	230.72
6	NF	0.3555	12.636	0.3904	14290	1.2859	190.53
7	NF	0.2156	15.788	0.3499	3067	1.3364	113.60
8	NF	0.015	16.029	0.172	3866	1.3116	222.90
9	NF	0.0211	17.023	0.1242	3109	1.2985	212.72
10	NF	0.2751	23.971	0.2578	6143	1.180	169.58
11	NF	0.1191	27.038	0.1280	4495	1.1781	116.92
12	0.4794	75.542	97.217	0.6174	61715	1.1685	146.78
Control	**1.0743**	**10.015**	**9.2642**	**1.1667**	**299166**	**0.9370**	**298.81**

L/H = Sum of nC16–nC26 n-alkane concentration relative to sum of nC27–nC36 n-alkane concentration.

Σ n-alkanes = sum of total n-alkanes

L/H-PAH = Ratio of sum of concentration of Nap, Acy, Ace, Fl, Phe relative to sum of concentration of Fluo, Py, MFluo, BaA, Chr, 1M-Chr, BaF, BkF, BaP, D(B)Ant, BgP.

Σ PAH =sum of total PAHs

4.5 Conclusion

Advanced gas chromatography combined with mass spectrometry, GC-MS-MS was used for quantitative and qualitative analysis of the degradation of hydrocarbons present in tarballs by associated bacterial consortia. Bacterial consortia prepared from tarball-associated microbes showed promising results for hydrocarbon degradation and can be used in future for bioremediation purposes.

Chapter 5

Development of Online Database of Microbes Associated with Tarballs

5.1 Introduction

It would require a good amount of time to individually access a diverse and large amount of experimental data from published articles related to a particular field of research (Majhi et al., 2013). Systematically arranging this data and rechannelling it through a web interface will help researchers in analysing and interpreting the data easily. https://tarballs.in/ is a website on tarball-associated microbes. It deals with the microbial diversity of tarballs from the world. Tarballs are in news from various parts of the world for their deposition on beaches and the presence of various forms of microbes on them. The database includes basic to advanced information on tarball-associated microbes. The website intends to create awareness about tarball pollution among the general public and non-experts.

5.2 Methodology

5.2.1 Data collection and storage

Information on tarball-associated microbes was gathered from literature and our published works in which tarballs were collected from Goa, the associated microbes were cultured, preserved, and characterised based on morphology, microscopy, biochemical features and 16S rRNA gene data for bacteria (and ITS for fungi) and the gene sequences were submitted to National Centre for Biological Information (NCBI) – GenBank (Figure 5.1).

The website https://tarballs.in/ was created using https://wordpress.com/ (Figure 5.2). It comprises information on tarballs and tarball-associated bacteria. Researchers and the general public can submit information about tarball-associated microbes using the link: https://forms.gle/JfrweRZso5XrT3jr8. A dataset of tarball-associated bacteria reported from various countries of the world, including India, is available in the form of an MS

Excel file (Figure 5.2). This list will be updated to include tarball-associated fungi and other microbial forms. Each detail about organisms such as taxonomic rank and references was entered manually. Data was curated for accuracy to avoid errors. Phylum-wise distribution of isolates is shown in Table 5.1.

This polyphasic data for representative isolates from this study is compiled and presented in Table 5.2. This includes the Gram nature of bacteria, colony morphology details on the medium from which they were isolated, results of the biochemical tests performed such as IMViC test (Indole production, Methyl red test, Voges-Proskauer, and Citrate utilisation), Starch hydrolysis, Gelatine liquefication, Nitrate reduction, Urease, Catalase, Oxidase, Caseinase, Lipase, carbohydrate (Dextrose, Fructose, Sucrose, Lactose, Arabinose, Mannose, Mannitol, Maltose, Xylose, Salicin) utilisation tests with acid and gas production. This is the first study combining the gene sequence data with traditional identification methods for bacteria associated with tarballs.

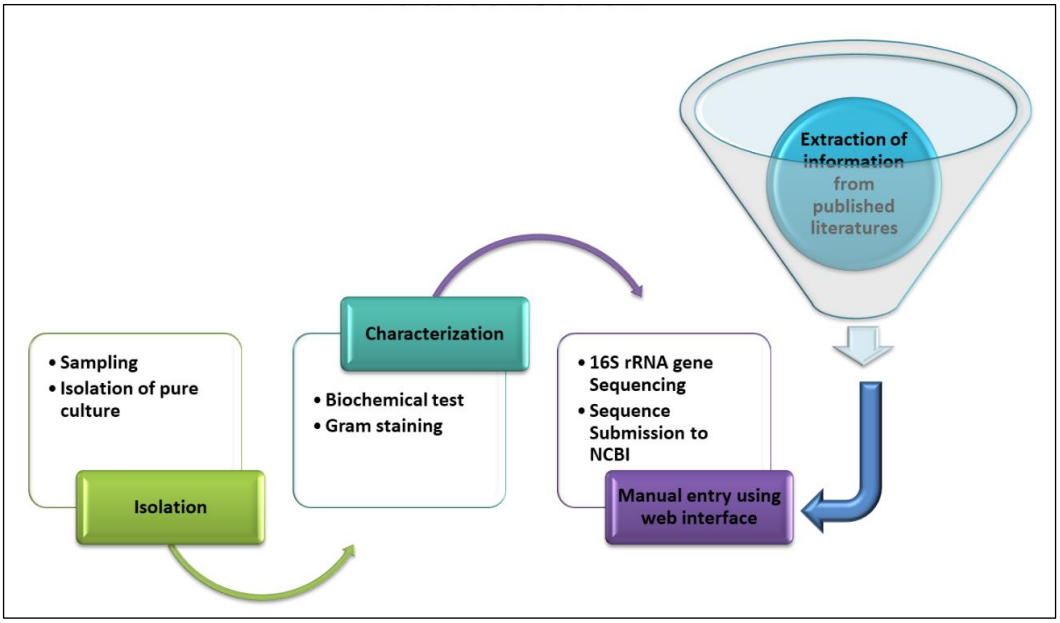


Figure 5.1: Data collection.

5.3 Results and Discussion

Information on tarballs and tarball-associated bacteria is freely available at https://tarballs.in/ without any login requirement or subscription. There are five pages on the website (Figure 5.2). The first page is the Home page, which contains all the links

and a cover photo. The second page titled “About” contains introductory information on tarballs with an image of tarballs. The third page titled “TAM database” contains a link to the Google form using which one can submit information on tarball-associated microbes, and another link to download the latest dataset of tarball-associated microbes. Currently, records for 209 tarball-associated bacteria are available on this website. Taxonomy has been included for each species.

Table 5.1: Phylum-wise species distribution

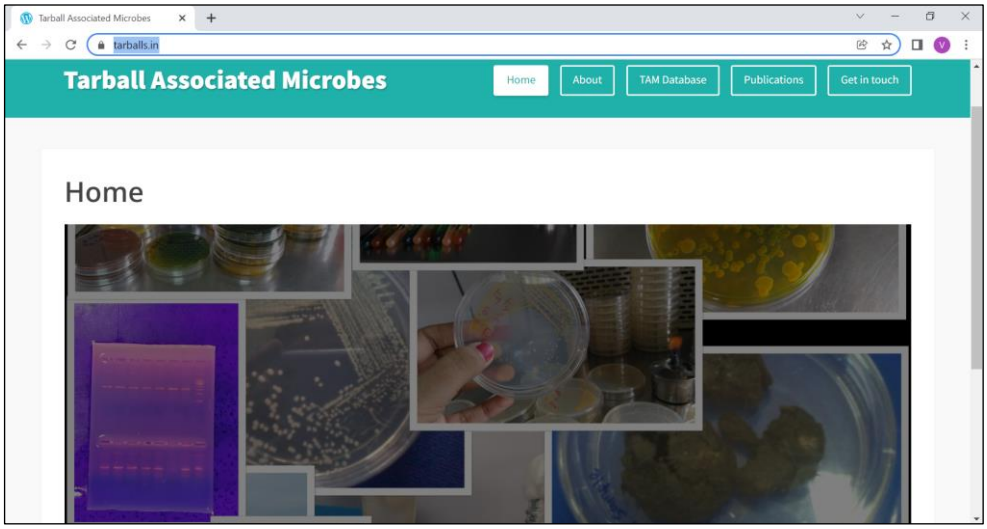
Kingdom	**Phylum**	**Class**	**Species**
Bacteria	*Proteobacteria*	*Gammaproteobacteria*	108
		Alphaproteobacteria	25
		Betaproteobacteria	5
		Deltaproteobacteria	1
		Epsilon proteobacteria	2
		Hydrogenophilalia	1
Bacteria	*Firmicutes*		23
Bacteria	*Actinobacteria*		28
Bacteria	*Bacteroidetes*		10
Bacteria	*Chloroflexi*		1
Bacteria	*Cyanobacteria*		1
Bacteria	*Fusobacteria*		1
Bacteria	*Planctomycetes*		1
Bacteria	*Spirochaetes*		1

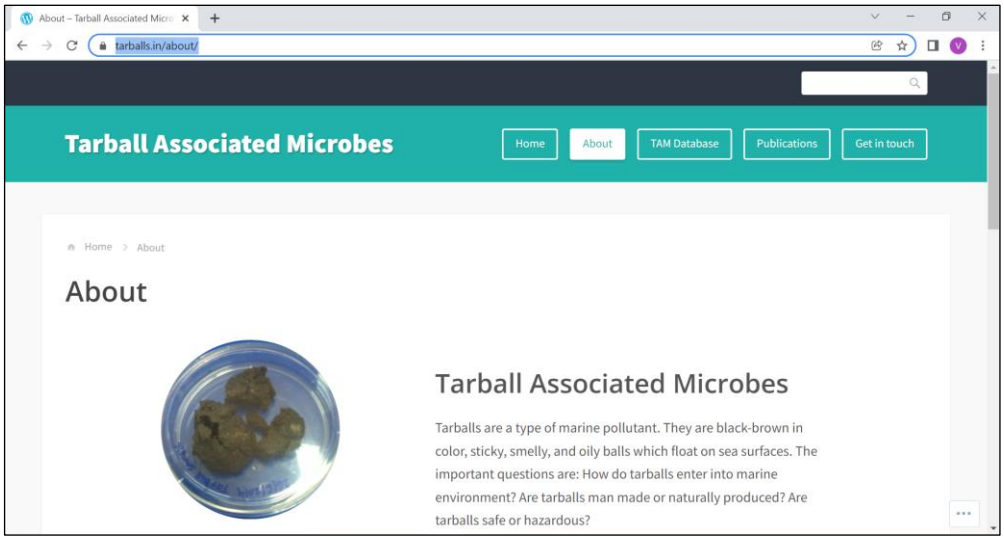
The outcome of worldwide studies on tarball microbes has been included in the database. There are also entries from unpublished work from this thesis. The columns in the excel sheet are 1) Domain, 2) Kingdom, 3) Phylum, 4) Class, 5) Order, 6) Family, 7) Genus, 8) Species, 9) Strain number, 10) Sampling country, 11) Sampling location, 12) Gene accession number, 13) Description, 14) Publication Year, 15) Full citation of the paper, 16) Other information if available, 17) Name of the person who has entered the data and 18) Data entry date.

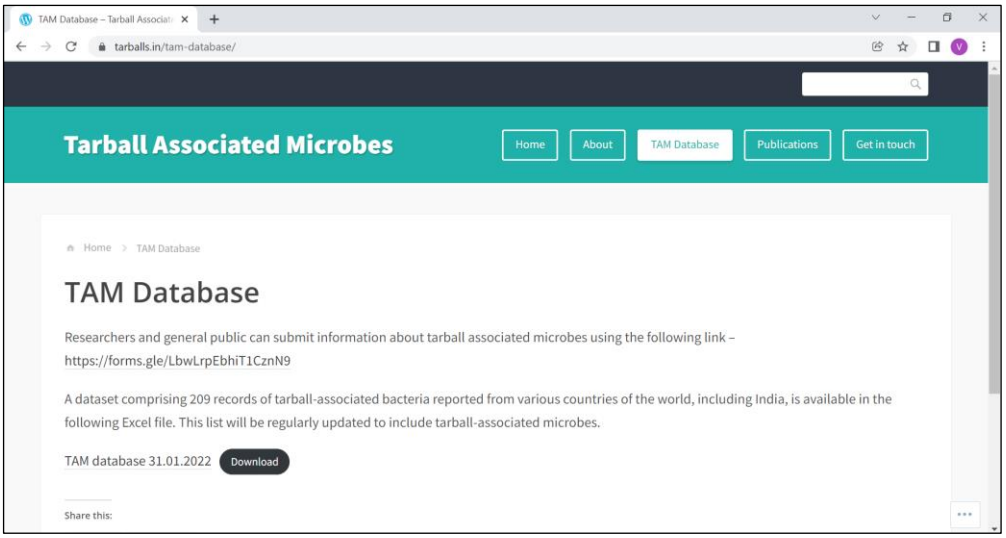
The fourth page of the website titled “Publications” contains links to the publications from this study. The available studies reveal that tarball-associated bacteria belong to 9 phyla, 29 different orders, and 75 genera (https://tarballs.in/). The website will be updated with fungal records from tarballs. Researchers worldwide can easily access information on tarballs and the microbes associated with tarballs and can use this

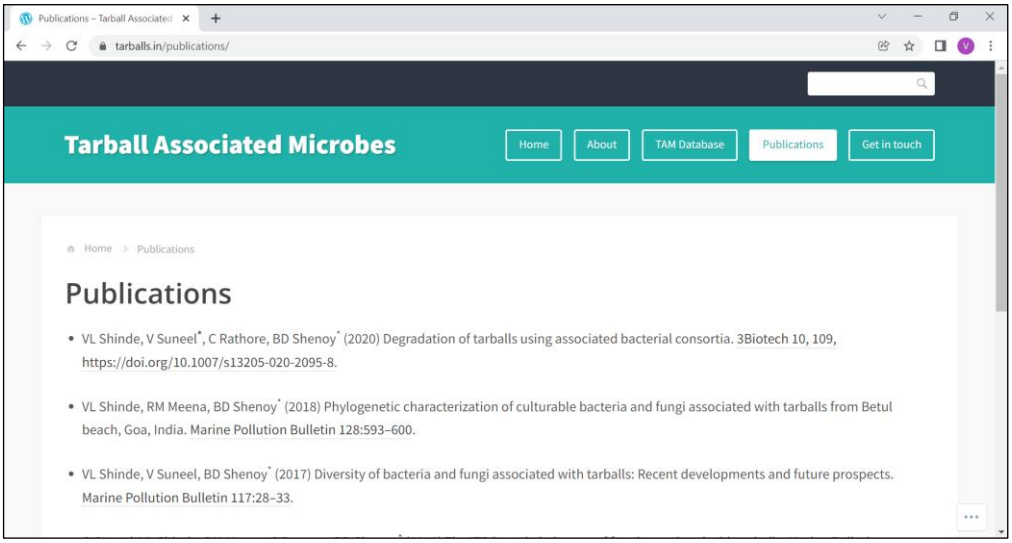
information for several purposes, such as devising a bioremediation strategy. The fifth page, “Get in touch” facilitates submitting messages to the web owners.

Anthropogenic activities are responsible for altered microbial diversity in a particular environment (Bhattacharjee and Joshi, 2014). Tarballs are a pollutant on which several microbial life forms reside including algae (Shinde et al., 2017). Exploring the diversity and maintaining the records of discovered species can be useful for future research. The field of microbiology needs to be supported with computational resources (Bhattacharjee and Joshi, 2014). Developing a database of microbes associated with marine pollutants is the need of the hour. Documenting these microbes in the form of online databases has a great significance for human welfare.









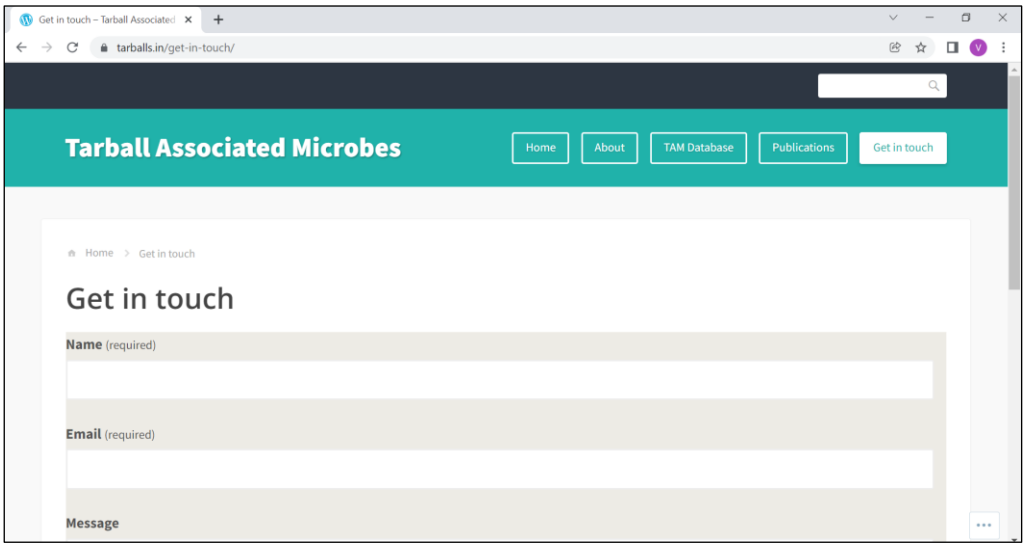


Figure 5.2: Screenshots of the website - https://tarballs.in/

Table 5.2: Polyphasic data for representative isolates

Sl. No.	Strain no.	Cultural features on ZMA	Micro morphology	Biochemical features	GenBank accession number of 16S RNA gene data	Closet taxon and % similarity (in bracket) in EZTaxon
1	B_Betul_2	1 mm, Circular, off-white, entire, convex, Translucent, Moist, Shiny	Gram negative, coccobacilli	Id:-ve, Mr:+ve, Vp:-ve, Cu:-ve, Sh:-ve, Gl:-ve, Nr:-ve, Us:+ve, Ct:+ve, Ox:-ve, Cs:-ve, Lp:-ve, Dx:O & F(G), Su: O & F(G), Lc:-ve, Ab: O & F(G), Mn: O & F(G), Man: O & F(G), Ml: O & F(G), Xy: O & F(G), Sc: O & F(G)	MG664235	*Enterobacter cloacae* LMG 2683 (99.06 %)
2	B_Betul_3	1 mm, Circular, off-white, entire, convex, Translucent, Moist, Shiny	Gram-negative, coccobacilli	Id:-ve, Mr:+ve, Vp:-ve, Cu:+ve, Sh:-ve, Gl:-ve, Nr:+ve, Us:-ve, Ct:+ve, Ox:-ve, Cs:-ve, Lp:-ve, Dx:N&F, Su:O&F, Lc:N, Ab:N&F, Mn:O&F, Man:N&F, Ml:O&F, Xy:O&F, Sc:O&F,	MG664246	*Pantoea dispersa* LMG 2603 (100 %)
3	B_Betul_8	1 mm, Circular, milky white, entire, convex, Opaque, Moist	Gram-negative, cocci	Id: -ve, Mr:+ve, Vp: -ve, Cu: -ve, Sh: -ve, Gl: -ve, Nr: -ve, Us:+ve, Ct:+ve, Ox: -ve, Cs: -ve, Lp: -ve, Dx: O & F(G), Su: O&F, Lc: O&F, Ab: O & F(G), Mn: O&F, Man: O & F(G), Ml: O & F(G), Xy: O & F(G), Sc: O & F(G),	MG664237	*Klebsiella pneumoniae* subsp. *Rhinoscleromatis* ATCC 13884 (99.57 %)

4	B_Betul_9	3 mm, Circular, Pale yellow, center white, entire, flat, semitransparent, Moist	Gram-negative, short rods	Id:-ve, Mr: +ve, Vp:-ve, Cu: +ve, SH: +ve, Gl: +ve, NR:-ve, Us: +ve, Ct: +ve, Ox: +ve, Cs: +ve, Lp:-ve, Dx:O&F, Su: O&F, Lc:N, Ab: O&F, Mn: O&F, Man:N&F, Ml: O&F, Xy:N, Sc: O&F,	MG664261	*Vibrio fluvialis* NBRC 103150 (97.67 %)
5	B_Betul_14	3 mm, Circular, centre dark greenish black, regular, centre raised, Semi-translucent Moist, Shiny	Gram-negative short rods	Id:-ve, Mr:+ve, Vp:-ve, Cu:-ve, SH:-ve, Gl:+ve, NR:-ve, Us:+ve, Ct:+ve, Ox:+ve, Cs:+ve, Lp:-ve, Dx:O&N, Su:N, Lc:N, Ab:O&N, Mn:O&N, Man:N, Ml:N, Xy:O&N, Sc:N,	MG664249	*Pseudomonas aeruginosa* JCM 5962 (99.78 %)
6	B_Betul_22	4 mm, circular, yellowish, entire, centre dome-shaped, translucent, moist	Gram-negative, rods	Id:-ve, Mr:+ve, Vp:-ve, Cu:-ve, SH:-ve, Gl:-ve, NR:+ve, Us:-ve, Ct:+ve, Ox:+ve, Cs:-ve, Lp:-ve, Dx:N, Su:N, Lc:N, Ab:N, Mn:O&N, Man:N, Ml:N, Xy:N, Sc:N&F,	MG664258	*Pseudoxanthomonas indica* P15 (99.21 %)
7	B_Betul_26	2 mm, Circular, centre yellow, colourless margin, entire, centre dome-shaped, Semi-transparent, Moist	Gram-negative, rods	Id:-ve, Mr:-ve, Vp:-ve, Cu:-ve, SH:-ve, Gl:-ve, NR:+ve, Us:+ve, Ct:+ve, Ox:+ve, Cs:-ve, Lp:-ve, Dx:N, Su:O&N, Lc:N, Ab:N, Mn:N, Man:N, Ml:N, Xy:N, Sc:N,	MG664239	*Marinobacter nauticus* ATCC 27132 (99.64 %)

8	B_Betul_30	1mm, Circular, off-white, entire, convex, Translucent, Moist, Shiny	Gram-negative, short rods	Id:-ve, Mr:+ve, Vp:-ve, Cu:-ve, SH:-ve, Gl:-ve, NR:-ve, Us:-ve, Ct:+ve, Ox:+ve, Cs:-ve, Lp:-ve, Dx:O&F, Su: O&F, Lc: O&F, Ab: O&F, Mn: O&F, Man: O&F, Ml: O&F, Xy: O&F, Sc: O&F	MG664233	*Cellulomonas hominis* JCM 12133 (98.98 %)
9	B_Betul_C	2 mm, Circular, off-white, entire, raised, opaque, dry	Gram-negative, rods	Id:+ve, Mr:+ve, Vp:-ve, Cu:-ve, SH:-ve, Gl:-ve, NR:+ve, Us:+ve, Ct:+ve, Ox:+ve, Cs:-ve, Lp:-ve, Dx:N, Su:N, Lc:N, Ab:O&N, Mn:N, Man:N, Ml:N, Xy:N, Sc:N	MG664260	*Tistrella mobilis* TISTR 1108 (99.63 %)
10	B_Betul_H	2 mm, Circular, white, entire, flat, opaque, Moist	Gram negative, coccobacilli	Id:-ve, Mr:-ve, Vp:-ve, Cu:-ve, SH:-ve, Gl:-ve, NR:+ve, Us:-ve, Ct:+ve, Ox:+ve, Cs:-ve, Lp:-ve, Dx:N, Su:N, Lc:N, Ab:O&N, Mn:N, Man:N, Ml:N, Xy:N, Sc:N	MG664221	*Alcanivorax dieselolei* B-5 (99.86 %)
11	B_Betul_L	4 mm, Circular, Brown, entire, flat, Opaque, Moist	Gram-positive, short rods	Id:-ve, Mr:-ve, Vp:-ve, Cu:-ve, SH:-ve, Gl:-ve, NR:-ve, Us:+ve, Ct:+ve, Ox:-ve, Cs:+ve, Lp:-ve, Dx:N, Su:N, Lc:N, Ab:N, Mn:N, Man:N, Ml:N, Xy:N, Sc:N	MG664228	*Bhargavaea beijingensis* ge10 (99.46 %)
12	B_Betul_Q	5 mm, Circular, Brown, serrated, flat, translucent, Moist	Gram negative, coccobacilli	Id:-ve, Mr:-ve, Vp:-ve, Cu:-ve, SH:-ve, Gl:-ve, NR:-ve, Us:+ve, Ct:+ve, Ox:+ve, Cs:-ve, Lp:-ve, Dx:N, Su:N,	MG664245	*Oceanimonas marisflavi* 102-Na3 (99.88 %)

				Lc:N, Ab:N, Mn:N, Man:N, Ml:N, Xy:N, Sc:N		
13	B_Betul_R	3 mm, Circular, Cream, entire, flat, translucent, Moist	Gram-negative, short rods	Id:-ve, Mr:+ve, Vp:-ve, Cu:-ve, SH:-ve, Gl:-ve, NR:-ve, Us:+ve, Ct:+ve, Ox:+ve, Cs:-ve, Lp:-ve, Dx:O&N, Su:O&F, Lc:N, Ab: O&F(G), Mn: O&F, Man: O&F(G), Ml: O&F(G), Xy: O&F(G), Sc: O&F(G)	MG664244	*Nitratireductor aquimarinus* CL-SC21 (99.25 %)
14	B_Betul_21(1)	NA		Id:-ve, Mr:+ve, Vp:-ve, Cu:+ve, SH:-ve, Gl:-ve, NR:-ve, Us:-ve, Ct:+ve, Ox:-ve, Cs:-ve, Lp:-ve, Dx:N, Su:N, Lc:, Ab:N, Mn:, Man:, Ml:, Xy:N, Sc:N	MG664229	*Brevibacterium epidermidis* NBRC 14811 (95.05 %)
15	Mob V1	8 mm, Circular, Pale yellow, Rhizoid, Umbonate, Transparent, Moist, Shiny/Glistening	Gram-negative, rods	Id:-ve, Mr:-ve, Vp:-ve, Cu:-ve, SH:-ve, Gl:+ve, NR:+ve, Us:, Ct:+ve, Ox:+ve, Cs:+ve, Lp:, Dx:, Su:, Lc:-ve, Ab:-ve, Mn:-ve, Man:-ve, Ml:, Xy:-ve, Sc:,	MG797560	*Shewanella indica* KJW27 (99.45 %)
16	Mob V4	1 mm, Circular, Orange, entire, convex, Transparent, Moist, Shiny		Id:-ve, Mr:-ve, Vp:-ve, Cu:-ve, SH:-ve, Gl:-ve, NR:-ve, Us:, Ct:+ve, Ox:+ve, Cs:-ve, Lp:, Dx:, Su:, Lc:-ve, Ab:-ve, Mn:-ve, Man:-ve, Ml:, Xy:-ve, Sc:,	MG797562	*Owenweeksia hongkongensis* DSM 17368 (96.05 %)
17	Mob V7	Punctiform, Circular, pale yellow, entire,	Gram-negative, rods	Id:-ve, Mr:-ve, Vp:-ve, Cu:-ve, SH:-ve, Gl:-ve, NR:-ve, Us:, Ct:+ve, Ox:+ve, Cs:-ve,	MG797565	*Marispirillum indicum* B142 (100 %)

		convex, Transparent, Moist, Shiny		Lp:, Dx:, Su:, Lc:-ve, Ab:-ve, Mn:-ve, Man:, Ml:, Xy:-ve, Sc:,		
18	Mob V8	2 mm, Circular, white, entire, convex, Opaque, slimy/mucoid, dull	Gram negative, coccobacilli	Id:-ve, Mr:-ve, Vp:-ve, Cu:-ve, SH:-ve, Gl:-ve, NR:-ve, Us:, Ct:+ve, Ox:+ve, Cs:-ve, Lp:, Dx:, Su:, Lc:, Ab:-ve, Mn:-ve, Man:-ve, Ml:, Xy:-ve, Sc:,	MG797566	*Pelagibaca abyssi* LT2014 (100 %)
19	Mob V9	5 mm, Circular, Pale yellow in centre, white in margin, entire, Umbonate, Translucent, Moist, Shiny		Id:-ve, Mr:-ve, Vp:-ve, Cu:-ve, SH:+ve, Gl:-ve, NR:-ve, Us:, Ct:+ve, Ox:+ve, Cs:-ve, Lp:, Dx:, Su:, Lc:+ve, Ab:+ve, Mn:+ve, Man:+ve, Ml:, Xy:+ve, Sc:,	MG797567	*Jonesia denitrificans* DSM 20603 (100 %)

Legends: MR: Methyl red, VP: Voges Proskauer, CU: Citrate utilization, SH: Starch hydrolysis, GL: Gelatine liquefication, NR: Nitrate reduction, +:Positive, _: Negative, N: Negative, O: Oxidative, F: Fermentative, F(G): Fermentative with gas production, ND: Not done

5.4 Conclusion

https://tarballs.in/ is the first web resource in the world that documents microbes associated with tarballs from different coasts of the world. The website is work in progress and promises advanced features in near future.

Chapter 6

Summary, Conclusion, and Future Directions

6.1 Summary of the work done

This thesis focussed on the cultivable portion of bacterial diversity associated with tarballs collected from two beaches in Goa state, India, and subsequent investigations into select bacterial cultures for their ability to degrade tarballs.

Under objective 1, sixty-five bacteria were isolated from tarball samples collected from Betul and Candolim beaches in Goa, India. The colony-forming units (CFUs) of heterotrophic bacteria found on ZMA medium were in the magnitude of 10^5–10^6. The 16S rRNA gene-based phylogenetic analysis revealed that the 65 tarball-associated bacteria belong to 23 genera, namely, *Alcanivorax*, *Bacillus*, *Bhargavaea*, *Brevibacterium*, *Cellulomonas*, *Enterobacter*, *Jonesia*, *Klebsiella*, *Labrenzia*, *Marinobacter*, *Marispirillum*, *Nitratireductor*, *Oceanimonas*, *Owenweeksia*, *Pantoea*, *Pelagibaca*, *Pseudomonas*, *Pseudoxanthomonas*, *Serratia*, *Shewanella*, *Thalassospira*, *Tistrella* and *Vibrio*. Thirteen bacteria clustered with *Pseudomonas* and ten bacteria formed a strongly supported clade with *Marinobacter*. A literature review revealed that many of the identified taxa could be potential hydrocarbon degraders or possible human pathogens.

Under objective 2, twenty-nine bacteria were isolated from tarball samples collected from Betul and Candolim beaches in Goa, India using selective media. The CFUs of pathogenic groups of bacteria on the selective media were in the magnitude of 10^3–10^6. Phylogenetic analysis based on 16S rRNA gene sequence data revealed that the 29 tarball-associated bacteria belong to nine genera, namely, *Alcanivorax*, *Enterobacter*, *Klebsiella*, *Pseudomonas*, *Serratia*, *Shewanella*, *Thalassospira*, *Tistrella* and *Vibrio*. Seven of the nine bacterial genera have been reported as pathogens in previous studies

(Chapter 3). Most of the newly generated bacterial sequences under Objective 2 clustered with *Vibrio* and *Pseudomonas*.

Ninety-four newly-generated 16S rRNA gene sequences under Objective 1 and Objective 2 belong to 23 bacterial genera. The CFU data of bacteria found on tarballs both for heterotrophic and pathogenic groups is in agreement with the previous studies of Itah and Essien (2005) and Tao et al. (2011).

Under objective 3, advanced gas chromatography combined with mass spectrometry (GC-MS/MS) was used for qualitative and quantitative analysis of the degradation of tarball hydrocarbons by associated bacterial consortia. Initially, five tarball-associated bacterial cultures were selected based on primary screening for crude oil degradation. Based on secondary screening, the four bacterial cultures were employed in consortia preparation. Among the eleven consortia, the consortium of *Pseudomonas* sp. Betul-14 + *Pseudomonas* sp. Betul-M + *Alcanivorax* sp. Betul-O exhibited the highest n-alkane and PAH degradation. Other than this, a few more bacterial consortia showed promising results for hydrocarbon degradation and can be studied in the future for bioremediation purposes.

Under the final objective, the website https://tarballs.in was created using https://wordpress.com/. This website includes information on tarballs and the tarball-associated bacteria including taxonomy. The information was retrieved from published studies worldwide. Researchers and the general public can submit information about tarball-associated microbes using the link - https://forms.gle/JfrweRZso5XrT3jr8. The information on 209 tarball-associated bacteria reported from the world is maintained in a Google Sheet. Additionally, polyphasic data for representative genera was compiled. This online database of tarball-associated microbes will not only assist microbiologists from the world with a depth of knowledge on tarball microbiology but also provide timely information to deal with tarball pollution.

6.2 Conclusion

Phylogenetically and functionally diverse microbes colonise the biotic and abiotic surfaces in marine environments for their survival, including accessing nutrients,

enhancing interactions, and stabilizing the ambient conditions in dynamic aquatic environments (Dang and Lovell, 2016). Tarballs are nutrient-rich abiotic surfaces. Over the years, marine microbes probably have developed the ability to utilise and degrade hydrocarbons in crude oil. So, it is not unusual to find certain species of microorganisms high in number on substrates such as crude oil and tarballs. Tarballs can be found floating on sea surfaces, lying on beaches, or submerged in sea sediments. Tarballs might influence the microbial diversity in these niches. There are reports on pathogenic bacteria surviving on plankton, bi-valves, particles, and organic matter (Brettar et al., 2007) but there is limited knowledge on survival of pathogenic microbes on tarballs in the marine environment. Some authors have expressed possible health risks from tarballs (Tao et al., 2011), while some studies recommend or reveals importance of using autochthonous or indigenous microbial populations from sediment/ tarballs/ pollution site for their bioremediation (Nkem et al., 2016, Perdigao et al., 2021).

Petra et al. (2012) stated that indigenous micro-flora of weathered oil is a great source of hydrocarbonoclastic microorganisms. This thesis work suggests tarballs are a promising source for isolating crude-oil and tarball-degrading microbes. The ecology of microbial colonisation of tarballs, however, is poorly understood. Therefore, further research is required in this direction. As tarballs comprise various complex organic compounds of high molecular weight, microorganisms utilizing them possibly contain powerful degradative enzyme systems. Further research is required to obtain insights into degradation products and possible pathways involved in the microbial degradation of tarballs.

In India, the ‘oil zapper’ has been used to remediate contaminated soils of oil refineries dumping oil sludge. Oil zapper comprises *Acinetobacter bauminii, Alcaligenes odorans, Bukhardica cepacer*, *Pseudomonas aeruginosa*, and strain s-30 (Gupta et al., 2011). Development of microbial consortia to degrade beached tarballs using both indigenous and non-indigenous microbes is subject to future studies.

Not all microorganisms from environmental samples are culturable in laboratory conditions. In the case of tarballs, their insoluble nature and ball-like structure limit bacterial cells from getting into the culturing medium. Therefore, there is less assurance of the growth of all cultivable bacteria on the nutrient medium during isolation

procedures. To detach these bacteria from oily tars and recover maximum CFUs, one can use different techniques, such as breaking tarballs into smaller pieces, vortexing, mild sonication and biosurfactants.

To find out the true microbial diversity associated with tarballs, one can opt for culture-independent methods. However, they are not a preferred method in the case of subsequent application in bioremediation. Tarballs can be a source of microbial consortia for bioremediation of oil-contaminated environments, which can only be possible by culture-dependent methods. As there are limited studies on the diversity of tarball-associated microorganisms, both culture-based and culture-independent methods will help.

The current studies are restricted to beached and pelagic tarballs. There are few studies on benthic tars. It is desirable that culture-based and culture-independent methods are used to investigate microbial diversity associated with benthic tars. The high concentration of metals in tarballs can be toxic not only to microorganisms that degrade them but also to other marine animals which are in proximity. Further research is required on this topic.

The changing ecosystem of bacteria due to tarballs and other marine pollutants need to be studied in detail. This will provide insights into the quality of the marine ecosystem. The web portal, https://tarballs.in/, can assist researchers on the biology of microbes associated with tarballs and their ability to degrade tarballs.

Future studies on tarball microbiology can consider the following issues. Isolation protocols with further modifications need to be implemented. The presence of pathogenic groups of bacteria needs detailed investigations. The presence of microbes other than bacteria needs to be investigated thoroughly. How harmful are tarballs? This needs further research. The pathogenicity of tarball-associated microbes is another area of future research. Studying genes responsible for hydrocarbon degradation by tarball-associated microbes can take this area of research to the next level. Modeling biodegradation as suggested by Geng et al. (2022), by studying different oil phases, reaction kinetics, microbial interactions and activities, environmental conditions, and beach hydrodynamics would be milestones in oil bioremediation in future.

References

Adzigbli L, Yuewen D (2018) Assessing the impact of oil spills on marine organisms. Journal of Oceanography and Marine Research 6:179. doi:10.4172/2572-3103.1000179

Aislabie JM, Balks MR, Foght JM, Waterhouse EJ (2004) Hydrocarbon spills on Antarctic soils: effects and managements. Environmental Science and Technology 38:1265–1275. https://doi.org/10.1021/es0305149

Al-Sayegh A, Wahaibi Y, Joshi S, Al-Bahry S, Elshafe A, Al-Bemani A (2016) Bioremediation of heavy crude oil contamination. Open Biotechnology Journal 10: 301–311. doi:10.2174/1874070701 610010301

Alwazzeh MJ, Alkuwaiti FA, Alqasim M, Alwarthan S, El-Ghoneimy Y (2020) Infective endocarditis caused by *Pseudomonas stutzeri*: A case report and literature review. Infectious Disease Report 12(3):105–109. doi:10.3390/idr12030020

Antunes A, França L, Rainey FA, Huber R, Nobre MF, Edwards KJ, da Costa MS (2007) *Marinobacter salsuginis* sp. nov., isolated from the brine-seawater interface of the Shaban Deep, Red Sea. International Journal of Systematic and Evolutionary Microbiology 57:1035–1040. doi:10.1099/ ijs.0.64862-0

Atlas RM (1981) Microbial degradation of petroleum hydrocarbons: An environmental perspective. Microbiological Reviews 45:180–209.

Bacosa HP, Thyng KM, Plunkett S, Erdner DL, Liu Z (2016) The tarballs on Texas beaches following the 2014 Texas City "Y" Spill: Modeling, chemical, and microbiological studies. Marine Pollution Bulletin 109(1):236–244. doi:10.1016/j.marpolbul.2016.05.076

Ballal M, Shetty V, Bangera SR, Prabhu M, Umakanth S (2017) *Vibrio furnissii*, an emerging pathogen causing acute gastroenteritis: A case report. JMM Case Reports 4(9):e005111. doi:10.1099/jmmcr.0.005111

Bamforth SM, Singleton I (2005) Bioremediation of polycyclic aromatic hydrocarbons: Current knowledge and future directions. Journal of Chemical Technology and Biotechnology 80:723–736. doi:10.1002/jctb.1276

Barnes NM, Khodse VB, Lotlikar NP, Meena RM, Damare SR (2018) Bioremediation potential of hydrocarbon-utilizing fungi from select marine niches of India. 3 Biotech 8(1):21. https://doi.org/10.1007/s13205-017-1043-8

Bayat Z, Hassanshahian M, Hesni MA (2015) Enrichment and isolation of crude oil degrading bacteria from some mussels collected from the Persian Gulf. Marine Pollution Bulletin 101:85–91. doi:10.1016/j.marpolbul.2015.11.021

Bayha KM, Ortell N, Ryan CN, Griffitt KJ, Krasnec M, Sena J (2017) Crude oil impairs immune function and increases susceptibility to pathogenic bacteria in southern flounder. Public Library of Science ONE 12(5):e0176559. https://doi.org/10.1371/journal.pone.0176559

Beazley MJ, Martinez RJ, Rajan S, Powell J, Piceno YM, Tom LM, Andersen GL, Hazen TC, Nostrand JV, Zhou J, Mortazavi B, Sobecky PA (2012) Microbial community analysis of a coastal salt marsh affected by the Deepwater Horizon oil spill. Public Library of Science ONE 7(7):e41305. https://doi.org/10.1371/journal.pone.0041305

Bhattacharjee K, Joshi SR (2014) NEMiD: A web-based curated microbial diversity database with geo-based plotting. Public Library of Science ONE 9(4):e94088. doi:10.1371/journal.pone.0094088

Bidoia ED, Montagnolli RN, Lopes PRM (2010) Microbial biodegradation potential of hydrocarbons evaluated by colorimetric technique: a case study. In: Mendez-Vilas

A (ed). Current research, technology, and education topics in applied microbiology and microbial biotechnology. Formatex Research Center, Badajoz, Pp 1277–1288.

Brettar I, Guzman CA, Hofle MG (2007). Human pathogens in the marine environment - an ecological perspective. CIESM Workshop Monographs No.31: Marine Sciences and Public Health 59–68.

Brooijmans RJW, Pastink MI, Siezen RJ (2009) Hydrocarbon-degrading bacteria: the oil-spill clean-up crew. Microbial Biotechnology 2(6):587–594. doi:10.1111/j.1751-7915.2009.00151.x

Bruckner M (2011) Comparison of *in situ* bioattenuation, biostimulation, and bioaugmentation and their application to marine oil spills. EM: Air and Waste Management Association's Magazine for Environmental Managers, Pp 20–23.

Chandru K, Zakaria MP, Anita S, Shahbazi A, Sakaria M, Bahry PS, Mohamed CAR (2008) Characterization of alkanes, hopanes, and polycyclic aromatic hydrocarbons (PAHs) in tarballs collected from the east coast of Peninsular Malaysia. Marine Pollution Bulletin 56(5):950–962. https://doi.org/10.1016/j.marpolbul.2008.01.028

Chang EK, Miller M, Shahin K, Batac F, Field CL, Duignan P, Struve C, Byrne BA, Murray MJ, Greenwald K, Smith WA, Ziccardi M, Soto E (2022) Genetics and pathology associated with *Klebsiella pneumoniae* and *Klebsiella* spp. isolates from North American Pacific coastal marine mammals. Veterinary Microbiology 265:109307. doi:10.1016/j.vetmic.2021.109307

Chen AJ, Hasan NA, Haley BJ, Taviani E, Tarnowski M, Brohawn K, Johnson CN, Colwell RR, Huq A (2017) Characterization of pathogenic *Vibrio parahaemolyticus* from the Chesapeake Bay, Maryland. Frontiers in Microbiology 8:2460. doi:10.3389/fmicb.2017.02460

Christova N, Kabaivanova L, Nacheva L, Petrov P, Stoineva I (2019) Biodegradation of crude oil hydrocarbons by a newly isolated biosurfactant producing

strain. Biotechnology & Biotechnological Equipment 33(1):863–872. doi:10.1080/13102818.2019.1625725

Colwell RR (1970) Polyphasic taxonomy of the genus *Vibrio*: numerical taxonomy of *Vibrio cholerae*, *Vibrio parahaemolyticus*, and related *Vibrio* species. Journal of Bacteriology 104(1):410–33. doi:10.1128/jb.104.1.410-433.1970

Cui Z, Shao Z (2009) Predominant strains of polycyclic aromatic hydrocarbon-degrading consortia from deep sea of the Middle Atlantic Ridge. Wei sheng wu xue bao= Acta Microbiologica Sinica 49(7): 902–909.

Dang H, Lovell CR (2016) Microbial surface colonization and biofilm development in marine environments. Microbiology and Molecular Biology Reviews 80:91–38. doi:10.1128/ MMBR.00037-15

Das N, Chandran P (2011) Microbial degradation of petroleum hydrocarbon contaminants: An overview. Biotechnology Research International, Article ID 941810. doi:10.4061/2011/941810

Das S, Dash HR, Mangwani N, Chakraborty J, Kumari S (2014) Understanding molecular identification and polyphasic taxonomic approaches for genetic relatedness and phylogenetic relationships of microorganisms. Journal of Microbiological Methods 103:80–00. https://doi.org/10.1016/j.mimet.2014.05.013

Davin-Regli A, Lavigne JP, Pagès JM (2019) *Enterobacter* spp.: Update on taxonomy, clinical aspects, and emerging antimicrobial resistance. Clinical Microbiology Reviews 32(4):e00002-19. doi:10.1128/CMR.00002-19

Davin-Regli A, Pagès JM (2015) *Enterobacter aerogenes* and *Enterobacter cloacae*; versatile bacterial pathogens confronting antibiotic treatment. Frontiers in Microbiology 6:392. doi:10.3389/ fmicb.2015.00392

Dellagnezze BM, De Sousa GV, Martins LL, Domingos DF, Limache EE, de Vasconcellos SP, da Cruz GF, de Oliveira VM (2014) Bioremediation potential of microorganisms derived from petroleum reservoirs. Marine Pollution Bulletin 89:191–200. https://doi.org/10.1016/j.marpolbul.2014.10.003

Dhargalkar VK, Kureishy TW, Bhandare MV (1977) Deposition of tar balls bibliography 116 (Oil residue) on beaches along the west coast of India. Mahasagar - Bulletin of the National Institute of Oceanography 10(3&4):103–108.

Dwivedi SN, Parulekar AH (1974) Oil pollution along the Indian coastline. IOC/WMO Symposium and Workshop on Marine Pollution (Petroleum) Monitoring, Maryland, USA, 101–105.

Ebadi K, Zarei M, Sanati AM (2021). Optimization of crude oil biodegradation by *Brevibacterium* sp. isolated from the native sponges of the Persian Gulf. Iranian Journal of Biotechnology 19(2):e2690. doi:10.30498/IJB.2021.2690

Edun OM, Akinrotimi OA, Makinde OO (2015). Seasonal changes of microbial load in some sea foods from Buguma and Ekerekana Creeks, Niger Delta, Nigeria. Peertechz Journal of Environmental Science and Toxicology 1(1):001–007. doi: https://dx.doi.org/10.17352/aest.000001

Ejaz M, Zhao B, X, Bashir S, Haider FU, Aslam Z, Khan MI, Shabaan M, Naveed M, Mustafa A (2021) Isolation and characterization of oil-degrading *Enterobacter* sp. from naturally hydrocarbon-contaminated soils and their potential use against the bioremediation of crude oil. Applied Sciences 11(8):3504. https://doi.org/10.3390/app11083504

Ezeh GN, Agunwamba JC, Ezugwu CN, Onyelowe KC, Chinonso U, Tien B, Truong TH (2019) Modelling crude oil spread in aquatic environment. Electronic Journal of Geotechnical Engineering 24(2):561–604.

Elshafie A, AlKindi AY, Al-Busaidi S, Bakheit C, Albahry SN (2007) Biodegradation of crude oil and n-alkanes by fungi isolated from Oman. Marine Pollution Bulletin 54 (11):1692–1696. doi:10.1016/j.marpolbul.2007.06.006

Fernandes C, Kankonkar H, Meena RM, Menezes G, Shenoy BD, Khandeparker R (2019) Metagenomic analysis of tarball-associated bacteria from Goa, India. Marine Pollution Bulletin 141:398–403. https://doi.org/10.1016/j.marpolbul.2019.02.040

Fernandes C, Khandeparker RDS, Shenoy BD (2020) High abundance of *Vibrio* in tarball-contaminated seawater from Vagator beach, Goa, India. Marine Pollution Bulletin 150:110773. doi: 10.1016/j.marpolbul.2019.110773

Fu B, Li QX, Xu T, Cui ZL, Sun Y, Li J (2014) *Sphingobium* sp. FB3 degrades a mixture of polycyclic aromatic hydrocarbons. International Journal of Biodeterioration and Biodegradation 87:44–51. http://dx.doi.org/10.1016/j.ibiod.2013.10.024

Fu K, Jun L, Yuxiao W, Jianfei L, He Y, Lei S, Lijun Z (2016) An innovative method for rapid identification and detection of *Vibrio alginolyticus* in different infection models. Frontiers in Microbiology:7. doi:10.3389/fmicb.2016.00651

Funke G, Ramos CP, Collins MD (1995) Identification of some clinical strains of CDC coryneform group A-3 and A-4 bacteria as *Cellulomonas* species and proposal of *Cellulomonas hominis* sp. nov. for some group A-3 strains. Journal of Clinical Microbiology 33(8):2091–2097. doi:10.1128/jcm.33.8.2091-2097.1995

Gani M, Rao S, Miller M, Scoular S (2019) *Pseudomonas mendocina* bacteremia: a case study and review of literature. American Journal of Case Reports. 20:453–458. doi:10.12659/AJCR.914360

Gao X, Gao W, Cui Z, Han B, Yang P, Sun C, Zheng L (2015) Biodiversity and degradation potential of oil-degrading bacteria isolated from deep-sea sediments of south Mid-Atlantic Ridge. Marine Pollution Bulletin. 97(1–2):373–380. doi:10.1016/j.marpolbul.2015.05.065

Gardade L, Khandeparker L (2017) Spatio-temporal variations in pathogenic bacteria in the surface sediments of the Zuari estuary, Goa, India. Current Science 113(9):1729–1738. http://www. jstor.org/ stable/26493313

Gauthier MJ, Lafay B, Christen R, Fernandez L, Acquaviva M, Bonin P, Bertrand JC (1992) *Marinobacter hydrocarbonoclasticus* gen. nov., sp. nov., a new, extremely halotolerant, hydro carbon-degrading marine bacterium. International Journal of Systematic Bacteriology 42(4):568–576. doi:10.1099/00207713-42-4-568

Geng X, An C, Lee K, Boufadel MC (2022) Modeling oil biodegradation and bioremediation within beaches. Current Opinion in Chemical Engineering 35:100751. https://doi.org/10.1016/j.coche.2021.100751.

Gharaei S, Ohadi M, Hassanshahian M, Porsheikhali S, Forootanfar H (2022) Isolation, optimization, and structural characterization of glycolipid biosurfactant produced by marine isolate *Shewanella* algae B12 and evaluation of its antimicrobial and anti-biofilm activity. Applied Biochemistry and Biotechnology 194(4):1755–1774. doi:10.1007/s12010-021-03782-8

Giudice AL, Casella P, Caruso C, Mangano S, Bruni V, De Domenico M, Michaud L (2010) Occurrence and characterization of psychrotolerant hydrocarbon-oxidizing bacteria from surface seawater along the Victoria Land coast (Antarctica). Polar Biology 33:929–943. doi:10.1007/s00300-010-0770-7

Goodman R (2003) Tarballs: The end state. Spill Science & Technology Bulletin 8:117–121. https://doi.org/10.1016/S1353-2561(03)00045-8

Gupta S, Rao PVC, Lal B, Choudary VN (2011) Process and composition for bioremediation of oily sludge. Patent publication number WO2011074007A2. Accessed from https://www.google.com/ patents/WO2011074007A2?cl=en.

Hanson KG, Desai JD, Desai AJ (1993) A rapid and simple screening technique for potential crude oil degrading microorganisms. Biotechnology Techniques 7:745–748. https://doi.org/10.1007/BF00152624

Harayama S, Kasai Y, Hara A (2004) Microbial communities in oil-contaminated seawater. Current Opinion in Biotechnology 15:205–214. doi: 10.1016/j.copbio.2004.04.002

Harayama S, Kishira H, Kasai Y, Shutsubo K (1999) Petroleum biodegradation in marine environments. Journal of Molecular Microbiology and Biotechnology 1:63–70.

Hassanshahian M, Emtiazi G, Cappello S (2012) Isolation and characterization of crude-oil-degrading bacteria from the Persian Gulf and the Caspian Sea. Marine Pollution Bulletin 64:7–12. doi:10.1016/j.marpolbul.2011.11.006

Hassanshahian M, Emtiazi G, Caruso G, Cappello S (2014) Bioremediation (bioaugmentation/ biostimulation) trials of oil polluted seawater: a mesocosm simulation study. Marine Environmental Research 95:28–38. http://dx.doi.org/10.1016/j.marenvres.2013.12.010

Head IM, Jones DM, Roling WFM (2006) Marine microorganisms make a meal of oil. Nature Review Microbiology 4:173–182. doi:10.1038/nrmicro1348

Hegazi AH (2009) Utilization of trace metals in source diagnosing of tarballs from the Mediterranean city of Alexandria, Egypt. Petroleum Science and Technology 27:386–395. https://doi.org/10.1080/ 10916460701783928

Hegazi RM, Sh N, Moustafa YM, El-Gemaee GH (2007) Impact of heavy metals on biodegradation of phenanthrene by *Cellulomonas hominis* strain N2. Journal of Pure and Applied Microbiology 1(2):165–175.

Holt HM, Gahrn-Hansen B, Bruun B (2005) *Shewanella algae* and *Shewanella putrefaciens*: clinical and microbiological characteristics. Clinical Microbiology and Infection 11(5):347–352. https://doi.org/10.1111/j.1469-0691.2005.01108.x

Horn MH, Teal JM, Backus RH (1970) Petroleum lumps on surface of sea. Science 168:245–246.

Igbinosa EO, Okoh AI (2010) *Vibrio fluvialis*: an unusual enteric pathogen of increasing public health concern. International Journal of Environmental Research and Public Health. 7(10):3628–3643. doi:10.3390/ijerph7103628.

Iglewski BH (1996) *Pseudomonas*. In: Baron S, editor. Medical Microbiology. 4th edition. Galveston (TX): University of Texas Medical Branch at Galveston; 1996. Chapter 27. https://www.ncbi.nlm.nih.gov/books/NBK8326/.

Iliffe TM, Knap AH (1979) Fate of stranded pelagic tar on a Bermuda beach. Marine Pollution Bulletin 10(7):203–205.

Imai K, Ishibashi N, Kodana M, Tarumoto N, Sakai J, Kawamura T, Takeuchi S, Taji Y, Ebihara Y, Ikebuchi K, Murakami T, Maeda T, Mitsutake K, Maesaki S (2019) Clinical characteristics in blood stream infections caused by *Klebsiella pneumoniae*, *Klebsiella variicola*, and *Klebsiella quasipneumoniae*: a comparative study, Japan, 2014-2017. BMC Infectious Diseases 19(1):946. doi:10.1186/s12879-019-4498-x

Imron MF, Titah HS (2018) Optimization of diesel biodegradation by *Vibrio alginolyticus* using Box-Behnken design. Environmental Engineering Research 23:374-82. http://dx.doi.org/10.4491/eer. 2018.015

Ingole BS, Sivadas SK (2007) The slippery coastline. Voice Green Globe 1(2):32–34.

Itah AY, Essien JP (2005) Growth profile and hydrocarbonoclastic potential of microorganisms isolated from tarballs in the Bight of Bonny, Nigeria. Journal of

Molecular Microbiology and Biotechnology 21:1317–1322. doi:10.1007/s11274-004-6694-z

Janda JM, Abbott SL (2007) 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. Journal Of Clinical Microbiology 45(9):2761–2764. doi:10.1128/JCM.01228-07

Joe MM, Gomathi R, Benson A, Shalini D, Rengasamy P, Henry AJ, Truu J, Truu M, Sa T (2019) Simultaneous application of biosurfactant and bioaugmentation with rhamnolipid-producing *Shewanella* for enhanced bioremediation of oil-polluted soil. Applied Sciences 9(18):3773. https://doi.org/10.3390/app9183773

Jordan RE, Payne JR (1980) Fate and weathering of petroleum spills in the marine environment: a literature review and synopsis. Marine Technology Society Journal 18:24–42.

Kadam AN (1988) Some studies on tar pellets at Veraval coast (Gujarath). Mahasagar Bulletin National Institute of Oceanography 21:173–175.

Kadam AN, Rokade MA (1996) Source Identification of tar residue from Mumbai beach. Indian Journal of Marine Sciences 25:379–380.

Kaladharan P, Prema D, Nandakumar A, Valsala KK (2004) Occurrence of tarball and waste material on the beaches along Kerala coast of India. Indian Journal of the Marine Biological Association of India 46 (1):93–97.

Kanaly RA, Harayama S (2010) Advances in the field of high-molecular-weight polycyclic aromatic hydrocarbon biodegradation by bacteria. Microbial Biotechnology 3:136–164. doi:10.1111/j.1751-7915.2009.00130.x

Kimes NE, Callaghan AV, Suflita JM, Morris PJ (2014) Microbial transformation of the Deepwater Horizon oil spill - past, present, and future perspectives. Frontiers in Microbiology 5:603. doi:10.3389/fmicb.2014.00603

Kostka JE, Prakash O, Overholt WA, Green SJ, Freyer G, Canion A, Delgardio J, Norton N, Hazen TC, Huettel M (2011) Hydrocarbon-degrading bacteria and the bacterial community response in Gulf of Mexico beach sands impacted by the Deepwater horizon oil spill. Applied Environmental Microbiology 77(22):7962–7974. doi:10.1128/AEM.05402-11

Kuo SF, Lee CH (2018) An oil refinery worker at Kaohsiung, with *Pseudoxanthomonas kaohsiungensis* bloodstream infection presenting as chronic pericarditis and masquerading as tuberculosis pericarditis. Journal of Microbiology, Immunology and Infection 51(4):575–577. doi:10.1016/ j.jmii.2017.12.003

Kvenvolden KA, Cooper CK (2003) Natural seepage of crude oil into the marine environment. Geo-Marine Letters 23:140–146. doi:10.1007/s00367-003-0135-0

Landrigan PJ, Stegeman JJ, Fleming LE, Allemand D, Anderson DM, Backer LC, Brucker-Davis F, Chevalier N, Corra L, Czerucka D, Bottein MD, Demeneix B, Depledge M, Deheyn DD, Dorman CJ, Fénichel P, Fisher S, Gaill F, Galgani F, Gaze WH, Giuliano L, Grandjean P, Hahn ME, Hamdoun A, Hess P, Judson B, Laborde A, McGlade J, Mu J, Mustapha A, Neira M, Noble RT, Pedrotti ML, Reddy C, Rocklöv J, Scharler UM, Shanmugam H, Taghian G, van de Water JAJM, Vezzulli L, Weihe P, Zeka A, Raps H, Rampal P (2020) Human health and ocean pollution. Annals of Global Health 86(1):151. doi:10.5334/aogh.2831

Lee DW, Lee H, Kwon BO, Khim JS, Yim UH, Park H, Park B, Choi IG, Kim BS, Kim JJ (2018) *Oceanimonas marisflavi* sp. nov., a polycyclic aromatic hydrocarbon-degrading marine bacterium. International Journal of Systematic and Evolutionary Microbiology 68:2990–2995. doi:10.1099/ ijsem.0.002932

Liu C, Shao Z (2005) *Alcanivorax dieselolei* sp. nov., a novel alkane-degrading bacterium isolated from sea water and deep-sea sediment. International Journal of Systematic and Evolutionary Microbiology. 55(3):1181–1186. doi:10.1099/ijs.0.63443-0

Liu J, Bacosa HP, Liu Z (2017) Potential environmental factors affecting oil-degrading bacterial populations in deep and surface waters of the northern Gulf of Mexico. Frontiers in microbiology 10(7):2131. doi:10.3389/fmicb.2016.02131

Liu Z, Liu J (2013) Evaluating bacterial community structures in oil collected from the sea surface and sediment in the northern Gulf of Mexico after the Deepwater Horizon oil spill. Microbiology Open 2:492–504. doi:10.1002/mbo3.89

Liu Z, Liu J, Zhu Q, Wu W (2012) The weathering of oil after the Deepwater Horizon oil spill: insights from the chemical composition of the oil from the sea surface, salt marshes and sediments. Environmental Research Letter 7:035302. http://dx.doi.org/10.1088/1748-9326/7/3/035302

Lotfinasabasl S, Gunale VR, Rajurkar NS (2012) Assessment of petroleum hydrocarbon degradation from soil and tarball by fungi. Bioscience Discovery 3:186–192.

Ma M, Gao W, Li Q, Han B, Zhu A, Yang H, Zheng L (2021) Biodiversity and oil degradation capacity of oil-degrading bacteria isolated from deep-sea hydrothermal sediments of the South Mid-Atlantic Ridge. Marine Pollution Bulletin 171:112770. https://doi.org/10.1016/j.marpolbul.2021.112770

Majhi MC, Behera AK, Kulshreshtha NM, Mahmooduzafar D, Kumar R (2017) Correction: ExtremeDB: A unified web repository of extremophilic archaea and bacteria. Public Library of Science ONE 12(6):e0179119. https://doi.org/10.1371/journal.pone.0063083

Marchese A, Santoriello L, Riccio G, Coppo E, Carrega G (2019) First description of human invasive infection due to *Thalassospira profundimaris*. Clinical Microbiology and Infection 25(9):1162–1163. doi:10.1016/j.cmi.2019.05.023

Mariano AP, Bonotto DM, Angelis DF, Pirôllo MPS, Contiero J (2008) Biodegradability of commercial and weathered diesel oils. Brazilian Journal of Microbiology 39:133–142. doi:10.1590/S1517-838220080001000028

McGenity TJ, Folwell BD, McKew BA, Sanni GO (2012) Marine crude-oil biodegradation: a central role for interspecies interactions. Aquatic Biosystems 8(issue):10. https://doi.org/10.1186/2046-9063-8-10

Medjahed FB, Hamed MB, Abi Ayada SME (2020) Hydrocarbon-degrading bacterial strain *Pseudomonas mendocina* newly isolated from marine sediments and seawater of Oran Harbor (Algerian coast). Archives of Ecotoxicology 2(2):22–29. https://doi.org/10.36547/ae.2020.2.2.22-29

Mishra S, Jeevan J, Kuhad RC, Lal B (2001) Evaluation of inoculum addition to stimulate in situ bioremediation of oily-sludge-contaminated soil. Applied and Environmental Microbiology 67:1675–1681. doi:10.1128/AEM.67.4.1675-1681.2001

Mohanram R, Jagtap CB, Pradeep K (2015) Isolation and characterization of crude oil degrading *Pseudomonas mendocina* strain MR21 from oil polluted Mumbai harbor. doi:10.13140/RG.2.2.29298.12481

Muriel-Millán LF, Rodríguez-Mejía JL, Godoy-Lozano EE, Rivera-Gómez N, Gutierrez-Rios RM, Morales-Guzmán D, Trejo-Hernández MR, Estradas-Romero A, Pardo-López L (2019) Functional and genomic characterization of a *Pseudomonas aeruginosa* strain isolated from the southwestern Gulf of Mexico reveals an enhanced adaptation for long-chain alkane degradation. Frontiers in Marine Science:6. doi:10.3389/fmars.2019.00572

Nagvenkar GS, Ramaiah N (2009) Abundance of sewage-pollution indicator and human pathogenic bacteria in a tropical estuarine complex. Environmental Monitoring and Assessment 155(1–4):245–56. doi:10.1007/s10661-008-0432-1

Nair S, Lokabharathi PA (1977) Degradation of hydrocarbons by fungus, *Fusarium* sp. Indian Journal of Marine Science 6:173–175.

Nair SA, Devassy VP, Dwivedi SN, Selvakumar RA (1972) Preliminary observations on tarlike material observed on some beaches. Current Marine Science 41:766–767.

Nemirovskaya IA (2011) Tarballs in Baltic Sea beaches. Water Resources 38:315–323. https://doi.org/10.1134/S0097807811020114

Nkem BM, Halimoon N, Yusoff FM, Johari WLW (2020) Use of Taguchi design for optimization of diesel-oil biodegradation using consortium of *Pseudomonas stutzeri*, *Cellulosimicrobium cellulans*, *Acinetobacter baumannii* and *Pseudomonas balearica* isolated from tarball in Terengganu beach, Malaysia. Marine Pollution Bulletin, https://doi.org/10.21203/rs.3.rs-107265/v1

Nkem BM, Halimoon N, Yusoff FM, Johari WLW, Zakaria MP, Reddy S, Kannan N (2016) Isolation, identification, and diesel-oil biodegradation capacities of indigenous hydrocarbon- degrading strains of *Cellulosimicrobium cellulans* and *Acinetobacter baumannii* from tarball at Terengganu beach, Malaysia. Marine Pollution Bulletin 107:261–268. http://dx.doi.org/10.1016/j.marpolbul. 2016.03. 060

Noble RC, Overman SB (1994). *Pseudomonas stutzeri* infection. a review of hospital isolates and a review of the literature. Diagnostic Microbiology and Infectious Disease 19(1):51–6. doi:10.1016/ 0732-8893(94)90051-5

Nopcharoenkul W, Netsakulnee P Pinyakong O (2013) Diesel oil removal by immobilized *Pseudoxanthomonas* sp. RN402. Biodegradation 24:387–397. https: //doi. org/ 10.1007 /s10532-012-9596-z

Odeku KO, Paulos BM (2017) Prohibition of pollution of marine environments: challenges and prospects. Environmental Economics 8(3):127–136.

Oostdam BL (1984) Tar pollution of beaches in the Indian Ocean, the south China sea and the south Pacific Ocean. Marine Pollution Bulletin 15:267–270.

Othman PNAM, Inayatullah O (2021) Kinetic study of carbazole degradation by free and immobilized *Thalassospira profundimaris*. Sustainable Engineering and Innovation 3(1):29–37. https://doi.org/ 10.37868/sei.v3i1.129

Ozyurek SB, Bilkay IS (2018) Biodegradation of petroleum by *Klebsiella pneumoniae* isolated from drilling fluid. International Journal of Environmental Science and Technology 15:2107–2116. https://doi.org/10.1007/s13762-017-1581-y

Parthipan P, Elumalai P, Sathishkumar K, Sabarinathan D, Murugan K, Benelli G, Rajasekar A (2017) Biosurfactant and enzyme mediated crude oil degradation by *Pseudomonas stutzeri* NA3 and *Acinetobacter baumannii* MN3. 3 Biotech 7(5):278. doi:10.1007/s13205-017-0902-7

Pendse AS, Aruna K (2020) Physicochemical and analytical characterization of biosurfactant produced by *Serratia rubidaea* KAP. International Journal of Scientific Research in Biological Sciences, 7(2):25–40.

Peng RH, Xiong AS, Xue Y, Fu XY, Gao F, Zhao W, Tian YS, Yao QH (2008) Microbial biodegradation of polyaromatic hydrocarbons. Federation of European Microbiological Societies Microbiology Review 32:927–955. doi:10.1111/j.1574-6976.2008.00127.x

Perelo LW (2010) Review: *In situ* and bioremediation of organic pollutants in aquatic sediments. Journal of Hazardous Materials 177:81–89. doi:10.1016/j.jhazmat. 2009.12.090

Perez LM, Valera RF, Webb HK, Crawford RJ, Ivanova EP (2014) Genome sequence of "*Thalassospira australica*" NP3b2T isolated from St. Kilda beach, Tasman Sea. Genome Announcements. 2(6):e01139-14. doi:10.1128/genomeA.01139-14

Perdigão R, Almeida CMR, Santos F, Carvalho MF, Mucha AP (2021) Optimization of an autochthonous bacterial consortium obtained from beach sediments for bioremediation of petroleum hydrocarbons. Water 13:66. https://doi.org/10.3390/ w13010066

Petra S, Simons KL, Kadali KK, Patil SS, Ball AS (2012) The importance of weathered crude oil as a source of hydrocarbonoclastic microorganisms in contaminated seawater. Journal of Microbiology and Biotechnology 22:1185–1192. doi:10.4014/jmb.1201.01049

Piterina AV, Bartlett J, Pembroke JT (2010) Molecular analysis of bacterial community DNA in sludge undergoing autothermal thermophilic aerobic digestion (ATAD): Pitfalls and improved methodology to enhance diversity recovery. Diversity 2:505–526. https://doi.org/10.3390/ d2040505

Prakash D, Raushan RK, Sangodkar UMX (2008) Isolation and characterization of meta toluic acid degrading marine bacterium. Indian Journal of Marine Science 37:322–325.

Prakash O, Verma M, Sharma P, Kumar M, Kumari K, Singh A, Kumari H, Jit S, Gupta SK, Khanna M, Lal R (2007) Polyphasic approach of bacterial classification – An overview of recent advances. Indian Journal of Microbiology 47(2): 98–108. doi:10.1007/s12088-007-0022-x

Qasim SZ (1975) Oil pollution of the seas around India. Journal of the Institution of Marine Technologists 19:15–19.

Raikar MT, Raghukumar S, Vani V, David J, Chandramohan D (2001) Thraustochytrid protists degrade hydrocarbons. Indian Journal of Marine Sciences 30:139–145.

Ramamurthy T, Chowdhury G, Pazhani G P, Shinoda S (2014) *Vibrio fluvialis*: an emerging human pathogen. Frontiers in microbiology 5:91. https://doi.org/ 10.3389/fmicb.2014.00091

Randhwa M, Kaushal J (2014) Bioremediation through oilzapper and oilvorous-S technologies: A review. Engineering Sciences International Research Journal 2:29–32.

Ray A, Kinch LN, de Souza Santos M, Grishin NV, Orth K, Salomon D (2016) Proteomics analysis reveals previously uncharacterized virulence factors in *Vibrio proteolyticus*. mBio. 7(4):e01077-16. doi:10.1128/mBio.01077-16

Rekadwad BN, Khobragade CN (2015) A case study on effects of oil spills and tar-ball pollution on beaches of Goa (India). Marine Pollution Bulletin 15:567–570. doi:10.1016/j.marpolbul.2015.08.019

Restrepo L, Bayot B, Arciniegas S (2018) PirVP genes causing AHPND identified in a new *Vibrio* species (*Vibrio punensis*) within the commensal Orientalis clade. Scientific Reports 8:13080. https://doi.org/10.1038/ s41598-018 -30903-x

Rodrigues DF, Sakata SK, Comasseto JV, Bícego MC, Pellizari VH (2009) Diversity of hydrocarbon-degrading *Klebsiella* strains isolated from hydrocarbon-contaminated estuaries. Journal of Applied Microbiology 106(4):1304–1314. doi:10.1111/j.1365-2672.2008.04097.x

Rodrigues J, Madhukar A, Kumar N, Sangodkar UMX (2010) Isolation and characterization of a marine bacterium belonging to the genus *Alkaligenes* capable of the complete mineralization of the dibenzothiophene. Indian Journal of Geo-Marine Sciences 40:391–397. doi:10.1111/j.1365-2672.2008.04097.x

Rojo F, Martínez JL (2010) Oil degraders as pathogens. In: Timmis K.N. (eds) Handbook of Hydrocarbon and Lipid Microbiology. Springer, Berlin, Heidelberg. https://doi.org/10.1007/ 978-3-540-77587-4_250

Salleh AB, Ghazali FM, Rahman R, Basri M (2003) Bioremediation of petroleum hydrocarbon pollution. Indian Journal of Biotechnology 2:411–425.

Santisi S, Cappello S, Catalfamo M, Mancini G, Hassanshahian M, Genovese L, Giuliano L, Yakimov M (2015) Biodegradation of crude oil by individual bacterial strains and a mixed bacterial consortium. Brazilian Journal of Microbiology 46:377–387. doi: http://dx.doi.org/10.1590/S1517-838246120131276

Semai A, Plewniak F, Charrié-Duhaut A, Sayeh A, Gil L, Vandecasteele C, Lopez-Roques C, Leize-Wagner E, Bensalah F, Bertin PN (2021) Characterisation of hydrocarbon degradation, biosurfactant production, and biofilm formation in Serratia sp. Tan611: a new strain isolated from industrially contaminated environment in Algeria. Antonie Van Leeuwenhoek. 114(4):411–424. doi:10.1007/s10482-021-01527-5

Sengupta R, Fondekar SP, Alagarsamy R (1993) State of oil pollution in the northern Arabian Sea after the 1991 Gulf oil spill. Marine Pollution Bulletin 27:85–91.

Seo JS, Keum YS, Li QX (2009) Bacterial degradation of aromatic compounds. International Journal of Environmental Research and Public Health 6:278–309. doi:10.3390/ijerph6010278

Shinde VL, Meena RM, Shenoy BD (2018) Phylogenetic characterization of culturable bacteria and fungi associated with tarballs from Betul beach, Goa, India. Marine Pollution Bulletin 128:593–600. https://doi.org/10.1016/j.marpolbul.2018.01.064

Shinde VL, Suneel V, Rathore C, Shenoy BD (2020) Degradation of tarballs using associated bacterial consortia. 3 Biotech, 10(3):1–10. doi:10.1007/s13205-020-2095-8

Shinde VL, Suneel V, Shenoy BD (2017) Diversity of bacteria and fungi associated with tarballs: recent developments and future prospects. Marine Pollution Bulletin 117:28–33. http://dx.doi.org/10.1016/j.marpolbul.2017.01.067

Shukla A, Cameotra SS (2012) Hydrocarbon pollution: effects on living organisms, remediation of contaminated environments, and effects of heavy metals co-contamination on bioremediation. In: Romero-Zerón L (ed.) Introduction to enhanced oil recovery (EOR) processes and bioremediation of oil contaminated sites. Intech Publishers. doi: 10.5772/48014

Smith AC, Hussey MA (2005) Gram Stain Protocols. Published by American Society for Microbiology, Downloaded from https://asm.org/getattachment/5c95a063-326b-4b2f-98ce-001de9a5ece3/ gram- stain-protocol-2886.pdf

Smith CB, Johnson CN, King GM (2012) Assessment of polyaromatic hydrocarbon degradation by potentially pathogenic environmental *Vibrio parahaemolyticus* isolates from coastal Louisiana, USA. Marine Pollution Bulletin 64(1):138–143. doi:10.1016/j.marpolbul.2011.10.007

Snellman EA, Collins RP, Cooke JC (1988) Utilization of fuel oils by fungi isolated from oceanic tarballs. Letters of Applied Microbiology 6:105–107.

Stewart JR, Gast RJ, Fujioka RS, Solo-Gabriele HM, Meschke JS, Amaral-Zettler LA, Castillo ED, Polz MF, Collier TK, Strom MS, Sinigalliano CD, Moeller PD, Holland F (2008) Proceedings: The coastal environment and human health: microbial indicators, pathogens, sentinels and reservoirs. Environmental Health, 7(Suppl 2):S3.

Stuart, H. (2005). Essential Microbiology. John Wiley & Sons Ltd, The Atrium, Southern Gate, Chichester, West Sussex PO19 8SQ, England, ISBN 0 471 49753 3.

Sudharani P, Kumar SG, Mukherjee J, Srinivas TNR, Sarma VVSS (2018) Perennial occurrence of heterotrophic, indicator, and pathogenic bacteria in the coastal Bay of Bengal (off Visakhapatnam) - Impact of physical and atmospheric processes. Marine Pollution Bulletin 127:412–423. https://doi.org/10.1016/j.marpolbul.2017.12.023

Sun X, He Y, Xu N, Xia Y, Liu Z (2011) Isolation and identification of two strains of pathogenic bacteria and their effects on the volatile metabolites of *Gracilariopsis lemaneiformis* (Rhodophyta). Journal of Applied Phycology. 24:277–284. https://doi.org/10.1007/s10811-011-9677-0

Suneel V, Ciappa A, Vethamony P (2016) Backtrack modeling to locate the origin of tar balls depositing along the West Coast of India. Science of the Total Environment 569–570:31–39. https://doi.org/10. 1016/j.scitotenv.2016.06.101

Suneel V, Vethamony P, Naik BG, Krishna MS, Jadhav L (2015) Identifying the source of tar balls deposited along the beaches of Goa in 2013 and comparing with historical data collected along the West Coast of India. Science of the Total Environment 527–528:313–321. https://doi.org/10.1016/j.scitotenv.2015.04.102

Suneel V, Vethamony P, Naik BG, Kumar KV, Sreenu L, Samiksha SV, Tai Y, Sudheesh K (2014) Source investigation of the tar balls deposited along the Gujarat coast, India, using chemical fingerprinting and transport modeling techniques. Environmental Science and Technology 48(19):11343–51. doi:10.1021/es5032213

Suneel V, Vethamony P, Zakaria MP, Naik BG, Prasad KVSR (2013) Identification of sources of tar balls deposited along the Goa coast, India, using fingerprinting techniques. Marine Pollution Bulletin 70:81–89. doi:10.1016/j.marpolbul.2013.02.015

Shweta FNU, Gurram PR, O'Horo JC, Khalil S (2021) *Brevibacterium* species: An emerging opportunistic cause of bloodstream infections. Mayo Clinic Proceedings 96(4): P1093–1094. https://doi.org/10.1016/j.mayocp.2021.01.022

Tamura K, Stecher G, Kumar S (2021) MEGA11: Molecular evolutionary genetics analysis version 11. Molecular Biology and Evolution 38(7):3022–3027. https://doi.org/10.1093/molbev/msab120

Tao Z, Bullard S, Arias C (2011) High numbers of *Vibrio vulnificus* in tarballs collected from oiled areas of the north-central Gulf of Mexico following the 2010 BP Deepwater horizon oil spill. Ecohealth 8:507–511. doi:10.1007/s10393-011-0720-z

Tewari A, Abdullah S (2015) *Bacillus cereus* food poisoning: international and Indian perspective. Journal of Food Science and Technology. 52(5):2500–2511. doi:10.1007/s13197-014-1344-4

Thompson JR, Marcelino LA, Polz MF (2005) Diversity, sources, and detection of human bacterial pathogens in the marine environment. In Oceans and Health: Pathogens in the Marine Environment (pp. 29–68). Springer US. https://doi.org/10.1007/0-387-23709-7_2

Torri A, Bertini S, Schiavone P, Congestrì F, Matteucci M, Sparacino M, Testa G, Pedna MF, Sambri V (2018) *Shewanella algae* infection in Italy: report of 3 years' evaluation along the coast of the northern Adriatic Sea. New Microbes and New Infections 23:39–43. doi:10.1016/j.nmni.2018.01.002

Tran MH, Yu K, Park JW (2019) Tar-filled shells and tarballs on the Alabama Gulf coast host distinctive bacterial communities. Bios 90(2):122–127. doi:10.1893/0005-3155-90.2.122

Ursua PR, Unzaga MJ, Melero P, Iturburu I, Ezpeleta C, Cisterna R (1996) *Serratia rubidaea* as an invasive pathogen. Journal of Clinical Microbiology 34(1):216–217. doi:10.1128/jcm.34.1.216-217.1996

Vandamme P, Pot B, Gillis M, de Vos P, Kersters K, Swings J (1996) Polyphasic taxonomy, a consensus approach to bacterial systematics. Microbiological Reviews 60(2):407–438. https://doi.org/10.1007/s12088-007-0022-x

Varjani SJ, Rana DP, Bateja S, Upasani VN (2013) Isolation and screening for hydrocarbon utilizing bacteria (HUB) from petroleum samples. International Journal of Current Microbiology and Applied Sciences 2:48–60.

Tonkova V, Gesheva E (2007) Biosurfactant production by antarctic facultative anaerobe *Pantoea* sp. during growth on hydrocarbons. Current Microbiology 54(2):136–41. doi:10.1007/ s00284-006-0345-6

Vezzulli L, Grande C, Reid PC, Hélaouët P, Edwards M, Höfle MG, Brettar I, Colwelle RR, Pruzzo C (2016) Climate influence on *Vibrio* and associated human diseases during the past half-century in the coastal North Atlantic. Proceedings of the National Academy of Sciences 113 (34):E5062-E5071. https://doi.org/10.1073/pnas.1609157113

Walterson AM, Stavrinides J (2015) *Pantoea*: insights into a highly versatile and diverse genus within the *Enterobacteriaceae*. Federation of European Microbiological Societies Microbiology Reviews 39(6):968–984. https://doi.org/10.1093/femsre/fuv027

Wang C, Du J, Gao X, Duan Y, Sheng Y (2011) Chemical characterization of naturally weathered oil residues in the sediment from Yellow River Delta, China. Marine Pollution Bulletin 62: 2469–2475. https://doi.org/10.1016/j.marpolbul.2011.08.021

Wang C, Huang Y, Zhang Z, Wang H (2018) Salinity effect on the metabolic pathway and microbial function in phenanthrene degradation by a halophilic consortium. AMB Express 8(1):67. doi:10.1186/s13568-018-0594-3

Wang W, Zhong R, Shan D (2014) Indigenous oil-degrading bacteria in crude oil-contaminated seawater of the Yellow Sea, China. Applied Microbiology and Biotechnology 98:7253–7269. https://doi.org/10.1007/s00253-014-5817-1

Wang Z, Fingas M, Landriault M, Sigouin L, Castle B, Hostetter D, Zhang D, Spencer B (1998) Identification and linkage of tarballs from the coast of Vancouver Island and Northern California using GC/MS and Isotopic techniques. Journal of High Resolution Chromatography 21:383–395. https://doi.org/10.1002/(SICI)1521-4168(19980701)21:7<383::AID-JHRC383>3.0.CO;2-3

Wang ZD, Fingas M, Page D (1999) Oil spill identification. Journal of Chromatography 843:369–411. https://doi.org/10.1016/S0021-9673(99)00120-X

Warnock AM, Hagen SC, Passeri DL (2015) Marine tar residues: a review. Water Air Soil Pollution 226:68. doi:10.1007/s11270-015-2298-5

Willis KA, Serra-Gonçalves C, Richardson K (2022) Cleaner seas: reducing marine pollution. Reviews in Fish Biology and Fisheries 32:145–160. https://doi.org/10.1007/s11160-021-09674-8

Wong CS, Green DO, Cretney WJ (1976) Distribution and source of tar on the Pacific Ocean. Marine Pollution Bulletin 7:102–106. https://doi.org/10.1016/0025-326X(76)90183-1

Woo PCY, Lau SKP, Teng JLL, Tse H, Yuen KY (2008) Then and now: Use of 16S rDNA gene sequencing for bacterial identification and discovery of novel bacteria in clinical microbiology laboratories. Clinical Microbiology and Infection 14(10):908–934. https://doi.org/10.1111/j.1469-0691.2008.02070.x

Wu ZY, Liu PY, Tseng SY, Lee YH, Ho SP (2018) Characteristics and phylogeny of *Shewanella haliotis* isolated from cultivated shellfish in Taiwan. Canadian Journal of Infectious Diseases and Medical Microbiology 2018:9895148. doi:10.1155/2018/9895148

Xue J, Yu Y, Bai Y, Wang L, Wu Y (2015) Marine oil-degrading microorganisms and biodegradation process of petroleum hydrocarbon in marine environments: A review. Current Microbiology 71(2): 220–228. https://doi.org/10.1007/s00284-015-0825-7

Yakimov MM, Timmis KN, Golyshin N (2007) Obligate oil-degrading marine bacteria. Current Opinion in Biotechnology 18:257–266. doi:10.1016/j.copbio.2007.04.006

Yeganeh LP, Azarbaijani R, Mousavi H, Shahzadeh Fazeli SA, Amoozgar MA, Salekdeh GH (2015) Genome-wide analysis of *Oceanimonas* sp. GK1 isolated from Gavkhouni wetland (Iran) demonstrates presence of genes for virulence and pathogenicity. Cell Journal 17(3):451–460. doi:10.22074/cellj.2015.6

Yetti E, A'la A, Mercuriani IS, Yopi Y (2018) Potency of microbial consortium F14 from three selected bacterial strains (*Labrenzia aggregata* LBF-1-0016, *Pseudomonas balearica* LBF-1-0062, and *Lysobacter concretionis* LBF-1-0080) for oil degradation. AIP Conference Proceedings 2024(1) 020043. https://doi.org/10.1063/1.5064329

You Z, Xu H, Zhang S, Kim H, Chiang P-C, Yun W, Zhang L, He M (2018) Comparison of petroleum hydrocarbons degradation by *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. Applied Sciences 8(12):2551. https://doi.org/10.3390/app8122551

Yousaf S, Afzal M, Reichenauer TG, Brady CL, Sessitsch A (2011) Hydrocarbon degradation, plant colonization, and gene expression of alkane degradation genes by endophytic *Enterobacter ludwigii* strains. Environmental Pollution 159(10): 2675–2683. doi:10.1016/j.envpol.2011.05.031

Zare-maivan H (2010) Distribution of heavy metals associated with petroleum in the Northern Persian Gulf: Bushehr and Nayband Bay area. Journal of the Persian Gulf (Marine Science) 1:1–5.

Zhang Z, Houb Z, Yang C, Ma C, Tao F, Xu P (2011) Degradation of n-alkanes and polycyclic aromatic hydrocarbons in petroleum by a newly isolated *Pseudomonas aeruginosa* DQ8. Bioresource Technology 102:4111–4116. doi:10.1016/j.biortech.2010.12.064

Zielinski O, Busch JA, Cembella AD, Daly KL, Engelbrektsson J, Hannides AK, Schmidt H (2009) Detecting marine hazardous substances and organisms: sensors for pollutants, toxins, and pathogens. Ocean Science 5:329–349. https://doi.org/10.5194/os-5-329-2009

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Phylogenetic characterization of culturable bacteria and fungi associated with tarballs from Betul beach, Goa, India

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ABSTRACT

Tarballs are semisolid blobs of crude oil, normally formed due to weathering of crude-oil in the sea after any kind of oil spills. Microorganisms are believed to thrive on hydrocarbon-rich tarballs and possibly assist in biodegradation. The taxonomy of ecologically and economically important tarball-associated microbes, however, needs improvement as DNA-based identification and phylogenetic characterization have been scarcely incorporated into it. In this study, bacteria and fungi associated with tarballs from touristic Betul beach in Goa, India were isolated, followed by phylogenetic analyses of 16S rRNA gene and the ITS sequence-data to decipher their clustering patterns with closely-related taxa. The gene-sequence analyses identified phylogenetically diverse 20 bacterial genera belonging to the phyla *Proteobacteria* (14), *Actinobacteria* (3), *Firmicutes* (2) and *Bacteroidetes* (1), and 8 fungal genera belonging to the classes *Eurotiomycetes* (6), *Sordariomycetes* (1) and *Leotiomycetes* (1) associated with the Betul tarball samples. Future studies employing a polyphasic approach, including multigene sequence-data, are needed for species-level identification of culturable tarball-associated microbes. This paper also discusses potentials of tarball-associated microbes to degrade hydrocarbons.

1. Introduction

Tarballs are semisolid blobs of crude oil, normally formed due to weathering of crude oil in the sea after any kind of oil spills. They get advected long distance by ocean currents and waves to reach beaches, and cause pollution in coastal marine environment. Microbes originating either from sea-water or beach-sand get attached to tarballs and possibly affect the biodegradation of hydrocarbon-rich tarballs. Tarballs act as a substratum for physical attachment of various microbes and the byproducts formed during their degradation possibly support microbial growth (Shinde et al., 2017).

The taxonomy of tarball-associated microbes, however, needs improvement as DNA-based identification and phylogenetic characterization have been scarcely incorporated into it (Bacosa et al., 2016; Nkem et al., 2016; Sanyal et al., 2016; Shinde et al., 2017; Barnes et al., 2018). To the best of our knowledge, Nkem et al. (2016) were the first to report 16S rRNA gene-sequencing of culturable tarball-associated bacteria. They characterized two bacterial species, *Acinetobacter baumannii* and *Cellulosimicrobium cellulans* associated with tarballs from Malaysia based on a polyphasic approach. Recently, tarball-associated bacteria were studied using a culture-independent method from Texas beaches following the 2014 Texas city "Y" spill (Bacosa et al., 2016) which provided insights into their unculturable diversity in the context of tarball chemistry.

In case of tarball-associated fungi, Sanyal et al. (2016) were the first to report phylogenetically diverse filamentous-fungi and yeasts, based on ITS sequence-data, from touristically important Candolim beach in Goa. Recently, Barnes et al. (2018) reported two tarball-associated fungal species, *Fusarium equiseti* and *F. circinatum* from Betul beach, Goa and provided their multigene sequence-data. Nevertheless, information available on molecular diversity and DNA sequence-based identification of tarball-associated bacteria and fungi is scarce and patchy (Bacosa et al., 2016; Nkem et al., 2016; Sanyal et al., 2016; Shinde et al., 2017; Barnes et al., 2018), especially from tourist beaches of Goa state which has been facing tarball pollution since the 1970s (Nair et al., 1972; Suneel et al., 2013, 2016). This study, therefore, aimed to isolate both bacteria and fungi from tarballs collected from touristic Betul beach in south Goa and characterize their phylogenetic relationships based on 16S rRNA gene (for bacteria) and the ITS sequence data (for fungi) analyses.

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2. Material and methods

2.1. Sampling of tarballs

Betul is a coastal town located in the south Goa, India. The GPS coordinates for Betul are 15.144837°N 73.958244°E. The Betul beach was surveyed twice for tarball sampling, initially in October 2014 and subsequently in July 2016. Tarballs were handpicked randomly along a stretch on the beach wearing sterile nitrile gloves and collected in zip-lock bags. The bags were kept in ice box and carried to the laboratory. Samples were stored at 4 °C until further processing.

2.2. Isolation of bacteria

Tarball samples collected during October 2014 were processed for bacterial isolation using an isolation protocol, which was standardized in the lab after improving the procedure described by Tao et al. (2011). In the modified protocol, 1 g of tarball was weighed aseptically and washed with sterile sea water. The washed tarball samples were then added into a test tube containing 9 ml of sterile seawater. The sterile cotton swab was used to break tarballs and detach bacteria. The tubes were vortexed vigorously for 5–10 min. 1 ml of this was serially diluted further up to 10^{-5} dilution. 100 µl of each dilution was spread plated on Zobell Marine Agar (ZMA). The plates were then incubated at room temperature. After 48 h of incubation, the colony forming units (CFUs) were calculated.

Tarball samples collected during July 2016 were also processed for isolation of bacteria using the afore-mentioned procedure. To increase the recovery of diverse groups of bacteria, one more bacterial isolation protocol was standardized in the lab, after improving the isolation procedure described in Bayat et al. (2015). In this method, 1 g of the freshly collected tarball (from the July 2016 sample collection) was washed with sterile seawater to remove the particles adhered to it as much as possible and suspended it immediately in 100 ml of Zobell Marine Broth (ZMB) containing tarball (1 g/100 ml) as a source of hydrocarbon to promote the growth of hydrocarbon-utilizing bacteria. As tarballs were insoluble in water, sterile cotton swab was used to break them, so that maximum possible number of bacteria could get into the culture medium. The medium was then incubated on a rotary shaker at 120 rpm and 35 °C for 48 h. 1 ml of enriched medium was then transferred to ZMB containing tarball (1 g/100 ml) and further incubated on a rotary shaker at 120 rpm and 35 °C for 48 h. Final transfer was made in 100 ml of Bushnell Hass medium containing tarball as only carbon source. After the incubation, 1 ml from the final medium was serially diluted in sterile sea-water and spread plated on ZMA. Morphologically diverse bacterial colonies were further isolated by streak plate method.

The bacterial cultures were selected based on their colony morphology and purified for further studies (Table 1). Colony morphology characters were noted down, and Gram staining was performed for each isolate to confirm its purity. Purified bacterial cultures were maintained as 80% glycerol stocks and stored at −80 °C.

2.3. Isolation of fungi

Fungi were isolated from tarballs collected during July 2016 using the method described by Sanyal et al. (2016). Fungal colonies with unique culture morphology were selected for further characterization (Table 2) and their purified cultures were maintained at 4 °C.

2.4. DNA extraction and PCR amplification

DNA from both bacterial and fungal cultures was extracted using the Fungal and Bacterial DNA isolation kit (Zymo Research, USA) according to manufacturer's protocol. From fungal DNA, the ITS region was amplified by PCR using the primer-pair ITS1 and ITS4 (White et al., 1990), following the method detailed in Sanyal et al. (2016).

16S rRNA gene of bacteria was amplified by PCR using primer-pair 27F and 1492R (Piterina et al., 2010). The reaction for bacterial 16S rRNA gene amplification was performed in a 50 µl of volume, comprising 3 µl of template DNA (~50 ng/µl), 1 µl of each primer (20 pM) (Bioserve, India), 1 µl of dNTPs (10 mM) (Genaxy, India), 5 µl of Taq buffer A (10× with 15 mM $MgCl_2$) (GeNei, Bangalore), 1.5 µl of Taq polymerase (1 U/µl) (Chromous Biotech, India), 37.5 µl of nuclease-free water (HiMedia, India). The reaction was carried out at 95 °C for 5 min (initial denaturation), followed by 35 cycles at 95 °C for 1 min (denaturation), 50 °C for 1 min (primer annealing), 72 °C for 2 min (elongation), and a final extension at 72 °C for 10 min.

PCR products were purified using QIAquick PCR purification kit (QIAGEN) following manufacturer's protocol. After purification, PCR products were sequenced by ABI 3130 XL DNA sequencer, using primers mentioned above, at the Biological Oceanography Division, CSIR-NIO, Goa.

2.5. Phylogenetic analysis

The raw sequences obtained from the forward and reverse primers were checked for quality in DNA Dragon (SequentiX, Germany). Consensus sequences were prepared in the same software using both forward and reverse sequences, wherever possible. Two different DNA sequence datasets were analyzed, one for 16S rRNA gene sequence data-set (for bacteria) and the ITS sequence data-set (for fungi), in MEGA 7 (Kumar et al., 2016) using the newly-generated sequences and reference sequences retrieved from NCBI-GenBank.

3. Results

3.1. Phylogeny of tarball-associated bacteria

A list of bacterial cultures isolated in this study, along with information on their Gram nature and GenBank accession numbers for 16S rRNA gene sequences is presented in Table 1. The CFUs of bacteria were found to be 8.3×10^6 per gram of tarball for the samples collected during October 2014, while the samples collected during July 2016 yielded 5.07×10^5 CFUs per gram of tarball. Eight bacterial isolates were retrieved by the dilution plate method from the October 2014 tarball samples (Table 1, prefixed with “MOB-”). Twenty-five and sixteen bacterial isolates were retrieved from the July 2016 tarball samples by the dilution plate method and the enrichment culture technique, respectively. Numbers and alphabets are used to differentiate between the cultures isolated by serial dilution method and those isolated by enrichment culture technique, respectively (Table 1). Bacteria belonging to *Alcanivorax, Bhargavaea*, *Nitratireductor*, *Oceanimonas* and *Tistrella* were exclusively recovered from the enrichment culture technique (Fig. 1).

A Maximum Likelihood tree generated using the 16S rRNA gene sequence data-set in MEGA is shown in Fig. 1. *Aquifex pyrophilus* (M83548) was the designated outgroup. The system output details generated by MEGA for the bacterial tree construction are presented in Supplementary Document 1. In the bacterial phylogenetic tree shown in Fig. 1, 49 newly-generated sequences of tarball-associated bacteria from Betul beach clustered within four bacterial phyla [*Proteobacteria*: 38 (*Gammaproteobacteria*: 31, *Alphaproteobacteria*: 7), *Actinobacteria*: 6, *Firmicutes*: 4 and *Bacteroidetes*: 1].

In the *Gammaproteobacteria* clade (Fig. 1), 11 bacterial isolates clustered within *Pseudomonas* clade. B_BETUL_19 and B_BETUL_7 clustered with *Pseudomonas* sp. (MF948939, a pyrene degrader), coralline algae-associated bacterium (MG551789) and uncultured bacterium clone isolated from a polluted aquatic environment (KC208430). B_BETUL_4 exhibited proximity with the type of *P. sihuiensis* (NR 148251). MOB_V2 clustered with *Pseudomonas* sp. from the soil of Liangshui river of Beijing (KT380612). B_BETUL_G and B_BETUL_11 grouped with uncultured bacterium clone (JX047093), *P. stutzeri*

Table 1
List of tarball-associated bacteria included in this study, along with details of their cultural morphology and 16S rRNA gene sequence accession numbers.

Sl. no.	Taxon name	Isolate number	Culture morphology	GenBank acc. no.
1	*Alcanivorax* sp.	B_BETUL_O	Gram negative, Short rods	MG664223
2	*Alcanivorax* sp.	B_BETUL_I	Gram negative, Short rods	MG664222
3	*Alcanivorax* sp.	B_BETUL_P	Gram negative, Coccobacilli	MG664224
4	*Alcanivorax* sp.	B_BETUL_H	Gram negative, Coccobacilli	MG664221
5	*Bacillus* sp.	B_BETUL_6	Gram positive, Short rods	MG664225
6	*Bhargavaea* sp.	B_BETUL_K	Data unavailable	MG664227
7	*Bhargavaea* sp.	B_BETUL_L	Gram positive, Short rods	MG664228
8	*Bhargavaea* sp.	B_BETUL_J	Gram negative, Rods	MG664226
9	*Brevibacterium* sp.	B_BETUL_21(1)	Data unavailable	MG664229
10	*Brevibacterium* sp.	B_BETUL_21(2)	Data unavailable	MG664230
11	*Cellulomonas* sp.	B_BETUL_28	Gram negative, Short rods	MG664231
12	*Cellulomonas* sp.	B_BETUL_30	Gram negative, Short rods	MG664233
13	*Cellulomonas* sp.	B_BETUL_29	Gram negative, Short rods	MG664232
14	*Enterobacter* sp.	B_BETUL_2	Gram negative, Coccobacilli	MG664235
15	*Enterobacter* sp.	B_BETUL_10	Gram negative, Coccobacilli	MG664234
16	*Jonesia* sp.	MOB_V9	Data unavailable	MG797567
17	*Klebsiella* sp.	B_BETUL_8	Gram negative, Cocci	MG664237
18	*Klebsiella* sp.	B_BETUL_5	Gram negative, Cocci	MG664236
19	*Marinobacter* sp.	B_BETUL_23	Gram negative, Short rods	MG664238
20	*Marinobacter* sp.	B_BETUL_27	Data unavailable	MG664240
21	*Marinobacter* sp.	B_BETUL_26	Gram negative, Rods	MG664239
22	*Marinobacter* sp.	MOB_V5	Gram negative, Rods	MG797563
23	*Marinobacter* sp.	MOB_V6	Gram negative, Rods	MG797564
24	*Marispirillum* sp.	MOB_V7	Gram negative, Rods	MG797565
25	*Nitratireductor* sp.	B_BETUL_R	Gram negative, Short rods	MG664244
26	*Nitratireductor* sp.	B_BETUL_N	Gram negative, Short rods	MG664243
27	*Nitratireductor* sp.	B_BETUL_D	Gram negative, Rods	MG664242
28	*Nitratireductor* sp.	B_BETUL_A	Gram negative, Thin short rods	MG664241
29	*Oceanimonas* sp.	B_BETUL_Q	Gram negative, Coccobacilli	MG664245
30	*Owenweeksia* sp.	MOB_V4	Data unavailable	MG797562
31	*Pantoea* sp.	B_BETUL_3	Gram negative, Coccobacilli	MG664246
32	*Pelagibaca* sp.	MOB_V8	Gram negative, Coccobacilli	MG797566
33	*Pseudomonas* sp.	B_BETUL_4	Gram negative, Thin short rods	MG664252
34	*Pseudomonas* sp.	B_BETUL_19	Gram negative, Short rods	MG664251
35	*Pseudomonas* sp.	B_BETUL_7	Gram negative, Short rods	MG664253
36	*Pseudomonas* sp.	B_BETUL_M	Gram negative, Short rods	MG664256
37	*Pseudomonas* sp.	B_BETUL_G	Gram negative, Coccobacilli	MG664255
38	*Pseudomonas* sp.	B_BETUL_11	Data unavailable	MG664247
39	*Pseudomonas* sp.	B_BETUL_F	Gram negative, Short rods	MG664254
40	*Pseudomonas* sp.	B_BETUL_14	Gram negative, Short rods	MG664249

(*continued on next page*)

Table 1 (*continued*)

Sl. no.	Taxon name	Isolate number	Culture morphology	GenBank acc. no.
41	*Pseudomonas* sp.	B_BETUL_15	Gram negative, Coccobacilli	MG664250
42	*Pseudomonas* sp.	B_BETUL_13	Gram negative, Short rods	MG664248
43	*Pseudomonas* sp.	MOB_V2	Gram negative, Rods	MG797561
44	*Pseudoxanthomonas* sp.	B_BETUL_1	Gram negative, Rods	MG664257
45	*Pseudoxanthomonas* sp.	B_BETUL_22	Gram negative, Rods	MG664258
46	*Pseudoxanthomonas* sp.	B_BETUL_24	Gram negative, Short rods	MG664259
47	*Shewanella* sp.	MOB_V1	Gram negative, Rods	MG797560
48	*Tistrella* sp.	B_BETUL_C	Gram negative, Rods	MG664260
49	*Vibrio* sp.	B_BETUL_9	Gram negative, Short rods	MG664261

Table 2
List of tarball-associated fungi included in this study, along with details of their ITS sequence accession numbers.

Sl. no.	Taxon name	Isolate no.	GenBank acc. no.
1	*Aspergillus* sp.	F_BETUL_F1	MG664262
2	*Aspergillus* sp.	F_BETUL_G2	MG664263
3	*Byssochlamys* sp.	F_BETUL_E1	MG664272
4	*Monascus* sp.	F_BETUL_L	MG664269
5	*Monascus* sp.	F_BETUL_U	MG664270
6	*Monascus* sp.	F_BETUL_I1	MG664267
7	*Monascus* sp.	F_BETUL_K2	MG664268
8	*Paecilomyces* sp.	F_BETUL_P	MG664264
9	*Paecilomyces* sp.	F_BETUL_B1	MG664271
10	*Penicillium* sp.	F_BETUL_I2	MG664274
11	*Penicillium* sp.	F_BETUL_M	MG664275
12	*Penicillium* sp.	F_BETUL_A1	MG664273
13	*Talaromyces* sp.	F_BETUL_J1	MG664276
14	*Talaromyces* sp.	F_BETUL_J2	MG664277
15	*Talaromyces* sp.	F_BETUL_O	MG664278
16	*Trichoderma* sp.	F_BETUL_R	MG664265
17	*Trichoderma* sp.	F_BETUL_S1	MG664266
18	*Xylogone* sp.	F_BETUL_H	MG664279

originated from soil of Xumen coral reef nature reserve (KT986148), the type of *P. stutzeri* (NR113652) and *Pseudomonas* sp. (KP730605, involved in Arsenite oxidation). B_BETUL_M clustered with *Pseudomonas* sp. (KF021702) of marine origin. B_BETUL_15, B_BETUL_13, B_BETUL_14 and BETUL_F formed a strongly-supported clade, with 97% bootstrap support, with the type of *P. aeruginosa* (LN681564); and *P. aeruginosa* from the estuary of Siak River (MG706126) and fish of Arabian Gulf region (KT005274). Three *Pseudomonas* isolates, B_BETUL_G, B_BETUL_M and B_BETUL_F, were recovered using the enrichment culture method.

Five bacterial isolates were found to cluster within *Marinobacter* clade. B_BETUL_23 and MOB_V5 exhibited proximity with the types of *M. alkaliphilus* (NR112223) and *M. halophilus* (NR137207) but this received poor statistical support. B_BETUL_27, MOB_V6 and B_BETUL_26 formed a strongly supported cluster (with 78% bootstrap support) with *M. hydrocarbonoclasticus* (NR074619, PAH degrader); and *Marinobacter* isolates from crude-oil contaminated surface water/sponge (JX119040) and microalga (KU554471). MOB_V1 formed a strongly supported a clade with the type of *Shewanella algae* (NR114236), *S. upenei* from aquaculture farm water (KY176382) and uncultured *Shewanella* sp. from marine hot spring (JX047073). Similarly, B_BETUL_Q formed a clade with *Oceanimonas* sp. (MG456888, PAH degrader), uncultured bacterium clone from marine sediment from jetty (JQ257780) and the type of *O. doudoroffii* (NR114185). The *Oceanimonas* isolate B_BETUL_Q was recovered using the enrichment culture method.

In a major clade with strong bootstrap support, 6 bacterial isolates from the Betul tarball samples exhibited proximity with members *Vibrio*, *Pantoea, Klebsiella* and *Enterobacter*. B_BETUL_9 exhibited a strong clustering pattern with the type of *V. fluvialis* (NR114218), *Vibrio* sp. involved pyrene degradation (MF948987), *V. fluvialis* from shell-fish (KT163389) and *V. furnissii* from seawater (KU321330). B_BETUL_3 formed a strongly supported clade with the type of *Pantoea dispersa* (AB907780) and *Pantoea* sp. from straw (KU986664). B_BETUL_8 and B_BETUL_5 clustered with the type of *Klebsiella pneumoniae* subsp. *Rhinoscleromatis* (NR037084) and *K. pneumoniae* from river (KJ806340). *Enterobacter* was found to be paraphyletic, with one clade sister to *Klebsiella* and including B_BETUL_2, uncultured *Enterobacter* sp. from aerobic granular sludge (KX379247), *Enterobacter* sp. from mangrove sediment (GQ284538) and endophytic bacterium of *Spartina alterniflora* (KP757600). The second *Enterobacter* clade included B_BETUL_10, the type of *E. ludwigii* (NR042349) and bacterium isolated from borewell (MG650114).

B_BETUL_O, B_BETUL_H, B_BETUL_I and B_BETUL_P, recovered solely in the culture enrichment method, formed a well-supported with the type of *Alcanivorax xenomutans* (NR133958), *A. dieselolei* from sea lettuce (KJ849833) and deep-sea sediment (LT673801), and uncultured *Alcanivorax* sp. from Gujarat coastal water (LC269892). Similarly, B_BETUL_1, B_BETUL_24 and B_BETUL_22 clustered with the type of *Pseudoxanthomonas indica* (NR116019), *P. mexicana* (AB246768, chemosynthetic resin degrader), enrichment culture clone from soil sample (JN867356), *Pseudoxanthomonas* isolate from oil-contaminated soil (MF 405126), uncultured bacterium clone from PAH contaminated soil (HQ218747) and *P. indica* capable of degrading alachlor (KX682025).

Of the 7 newly-generated sequences clustered within the *Alphaproteobacteria* (Fig. 1), *Nitratireductor* was represented by four isolates, while *Marispirillum, Pelagibaca* and *Tistrella* included one isolate each. B_BETUL_C formed a well-supported clade with the type of *Tistrella mobilis* (NR114036), *T. mobilis* from water of South China sea (HQ425697), uncultured bacterium clone from water sample from oil well (KC442816), *T. mobilis* isolate involved in PAH degradation (EU440998) and *Tistrella* sp. from oil field ooze (EU306603). *Tistrella* isolate B_BETUL_C was retrieved using the enrichment culture method. Similarly, MOB_V7 formed a well-supported clade with the type of *Marispirillum indicum* (NR044545), phenanthrene degrading *Marispirillum* isolate (MG386659) and uncultured *Marispirillum* sp. from oil well-water (KF733613). MOB_V8 grouped with the type of *Pelagibaca abyssi* (NR148263), *P. abyssi* of marine origin (JX878396) and bacterium originated from hydrothermal vent (AF254109). B_BETUL_N, B_BETUL_A, B_BETUL_D and B_BETUL_R, recovered solely using the enrichment culture method, formed a well-supported clade within the

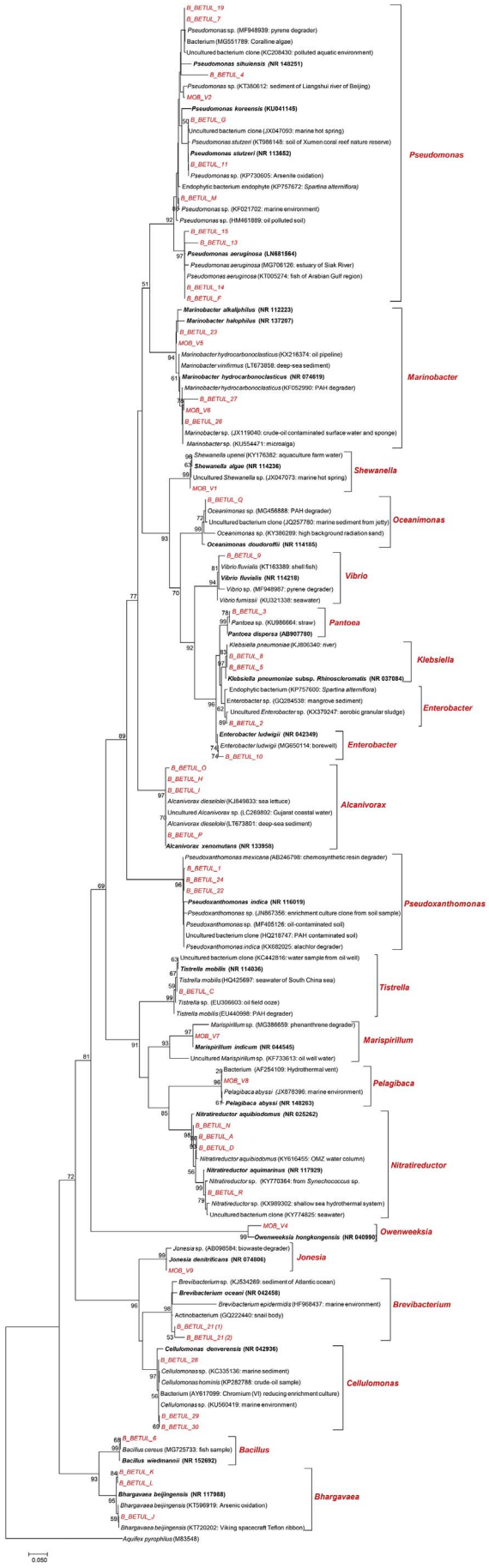


Fig. 1. Phylogenetic diversity of tarball-associated bacteria from Betul beach, Goa, India based on 16S rRNA gene sequence analysis. The newly-generated sequences from this study are highlighted in red italics. The sequences from type materials are represented in bold. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

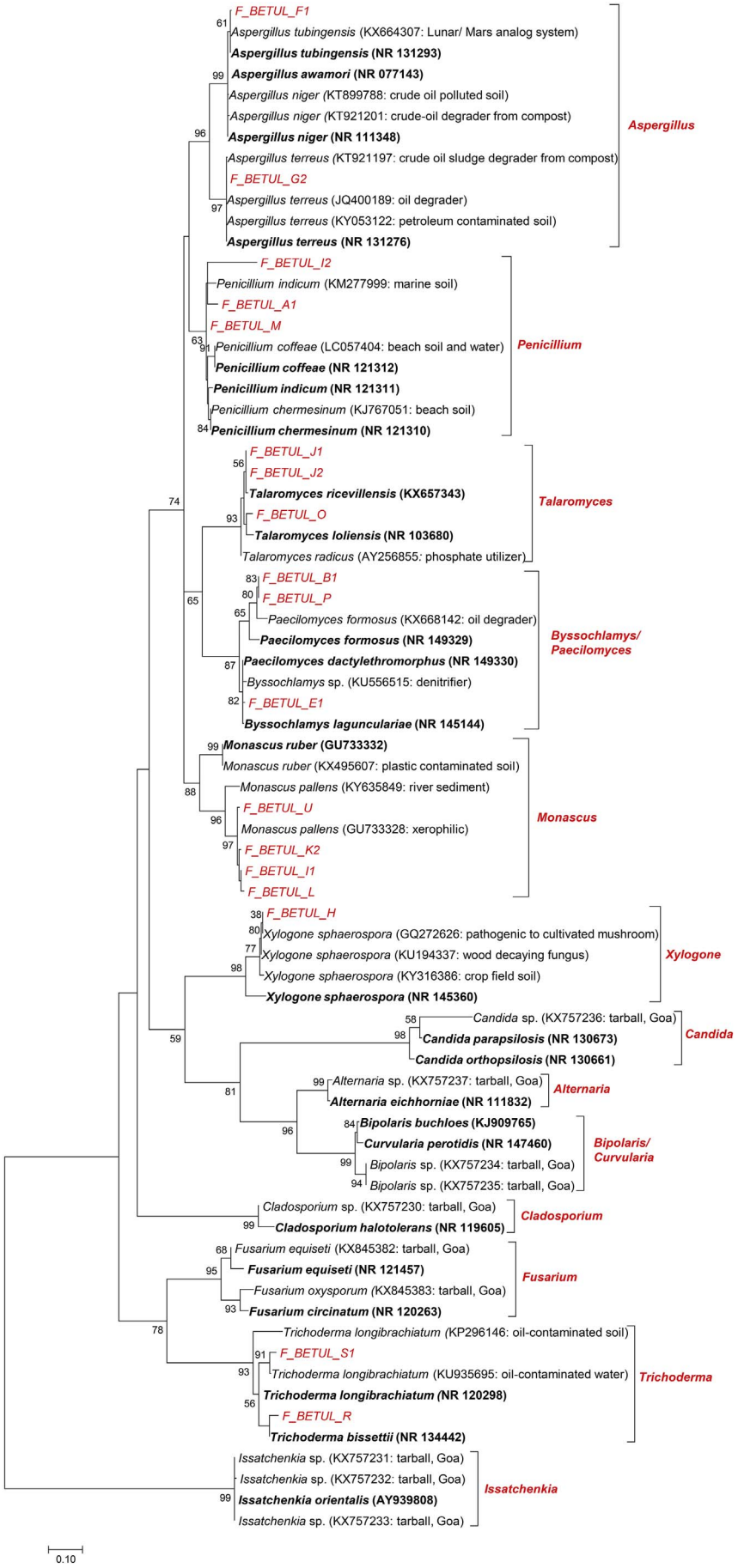
Alphaproteobacteria. B_BETUL_R clustered with the type of *Nitratireductor aquimarinus* (NR117929), *Nitratireductor* isolate associated with *Synechococcus* sp. (KY770364), uncultured bacterium clone from seawater (KY774825), and *Nitratireductor* sp. from shallow sea hydrothermal system (KX989302). B_BETUL_A and B_BETUL_D grouped with *N. aquibiodomus* from OMZ water column (KY616455).

Phylum *Bacteroidetes* was the least represented with one isolate in the genus *Owenweeksia*. MOB_V4 clustered with the type of *Owenweeksia hongkongensis* (NR040990) with 99% bootstrap support. Among the *Actinobacteria*, *Cellulomonas* and *Brevibacterium* were represented by three and two isolates, respectively, while *Jonesia* included only one isolate. MOB_V9 formed a strongly supported cluster with the type of *Jonesia denitrificans* (NR074806) and *Jonesia* sp. involved in biowaste degradation (AB098584). B_BETUL_21(1) and B_BETUL_21(2) formed a clade with the type of *Brevibacterium oceani* (NR 042458), *B. epidermidis* of marine origin (HF968437), *Brevibacterium* sp. from sediment of Atlantic ocean (KJ534269) and Actinobacterium from snail body (GQ222440). Similarly, B_BETUL_28, B_BETUL_29 and B_BETUL_30 clustered, with a strong bootstrap support, with the type of *Cellulomonas denverensis* (NR042936), *C. hominis* from crude-oil sample (KP282788), *Cellulomonas* spp. of marine origin (KC335136 and KU560419) and bacterium from Chromium (VI) reducing enrichment culture (AY617099). Among the *Firmicutes*, *Bhargavaea* was represented by three isolates, while *Bacillus* included only one isolate. B_BETUL_6 formed a well-supported clade with the type of *Bacillus wiedmannii* (NR152692) and *B. cereus* from fish sample (MG725733). B_BETUL_K, B_BETUL_L and B_BETUL_J, recovered solely in the culture enrichment method, formed a strongly supported clade with the type of *Bhargavaea beijingensis* (NR117988), *B. beijingensis* involved in arsenic oxidation (KT596919) and *B. beijingensis* from Viking spacecraft Teflon ribbon (KT720202).

3.2. Phylogeny of tarball-associated fungi

A list of the fungal cultures included in this study, along with information on the GenBank accession numbers for ITS sequences is presented in Table 2. Colony morphology of select tarball-associated fungal cultures is presented in Supplementary Figs. 1 and 2. A Maximum Likelihood tree generated using the ITS sequence dataset in MEGA is shown in Fig. 2. *Issatchenkia* clade was the designated outgroup. The system output details generated by MEGA for the fungal tree construction is presented in Supplementary Document 1.

In the fungal phylogenetic tree shown in Fig. 2, 18 newly generated sequences of tarball-associated fungi from Betul clustered within three classes of phylum *Ascomycota* [*Eurotiomycetes*: 15, *Sordariomycetes*: 2, and *Leotiomycetes*: 1]. Among the *Eurotiomycetes*, *Monascus* was represented by four isolates, *Penicillium* and *Talaromyces* included three isolates each, *Aspergillus* and *Paecilomyces* had two isolates each and *Byssochlamys* included only one isolate. F_BETUL_F1 and F_BETUL_G clustered within the *Aspergillus* clade. The former grouped with the types of *Aspergillus tubingensis* (NR131293), *A. awamori* (NR077143) and *A. niger* (NR111348); *A. tubingensis* from Lunar/Mars analog system (KX664307); and two *A. niger* isolates (KT921201, crude-oil degrader; KT899788, from crude-oil polluted soil). The latter clustered with the type of *A. terreus* (NR131276); and three *A. terreus* isolates (KT9211197, crude-oil sludge degrader; JQ400189, oil-degrader; KY053122, from petroleum contaminated soil). F_BETUL_I2, F_BETUL_A1 and F_BETUL_M formed a moderately supported cluster with the types of *Penicillium coffeae* (NR121312), *P. indicum* (NR121311) and *P. chermesinum* (NR121310); *P. indicum* from marine soil (KM277999), *P. coffeae* from



(caption on next page)

Fig. 2. Phylogenetic diversity of tarball-associated fungi from Betul beach, Goa, India based on ITS sequence analysis. The newly-generated sequences from this study are highlighted in red italics. The sequences from type materials are represented in bold. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

beach soil water (LC057404) and *P. chermesinum* from beach soil (KJ767051). F_BETUL_J1, F_BETUL_J2 and F_BETUL_O clustered with the types of *Talaromyces ricevillensis* (KX657343) and *T. loliensis* (NR 103680); and *T. radicus* (AY256855, phosphate solubilizer). F_BETUL_B1 and F_BETUL_P grouped with the type of *Paecilomyces formosus* (NR149329) and *P. formosus* (KX668142, oil-degrader). F_BETUL_E1 formed a moderately supported clade with the type of *Byssochlamys laguncullariae* (NR145144), *Byssochlamys* sp. (KU556515, denitrifier) and the type of *P. dactylethromorphus* (NR145144). F_BETUL_U, F_BETUL_K2, F_BETUL_I1 and F_BETUL_L clustered with *Monascus pallens* (GU733328: Xerophilic), in the proximity of *M. pallens* from river sediment (KY635849).

In the *Leotiomycetes* clade, *Xylogone* was represented by one isolate. F_BETUL_H formed a well-supported clade with the type of *Xylogone sphaerospora* (NR145360) and three nontype isolates of *X. sphaerospora*, including pathogen of cultivated mushrooms (GQ272626), wood decaying fungus (KU194337) and isolate from crop field soil (KY316386). Among the *Sordariomycetes*, *Trichoderma* included two isolates, F_BETUL_S1 and F_BETUL_R, which grouped with the types of *T. longibrachiatum* (NR 120298) and *T. bissettii* (NR 134442); and *T. longibrachiatum* isolates from oil-contaminated sites (KP296146 and KU935695). The phylogenetic tree also included the ITS sequences of tarball-associated fungi from Candolim, Goa (*Candida*, *Alternaria*, *Biploaris/Curvularia, Cladosporium* and *Issatchenkia*) (Sanyal et al., 2016) and that of *Fusarium* species associated with tarballs from Betul beach, south Goa (Barnes et al., 2018).

4. Discussion

4.1. Tarball-associated bacteria

Tarballs are oily residues and therefore difficult to dissolve in water. Two bacterial isolation protocols used in this study have been standardized after improving the known methods from the literature to retrieve phylogenetically diverse bacteria. *Nitratireductor* and *Alcanivorax* species were predominantly isolated in the enrichment culture technique, followed by species of *Bhargavaea, Oceanimonas* and *Tistrella*. *Pseudomonas* species, however, were recovered in both the isolation methods (Table 1). The number of bacterial CFUs recovered in our study (8.3×10^6 CFUs per gram of tarball for the October 2014 samples and 5.07×10^5 CFUs per gram of tarball for the July 2016 samples) is in agreement with previous findings. Itah and Essien (2005) reported 3.00×10^4–3.18×10^6 CFUs of heterotrophic bacteria per gram of tarball, while Tao et al. (2011) reported the numbers in the range of 5.1×10^6–8.3×10^6 CFUs per gram of tarball.

Tarballs and tarball-like substrates are known to support phylogenetically diverse bacteria from 31 identified genera Itah and Essien (2005), Tao et al. (2011), Petra et al. (2012), Liu and Liu (2013), Bacosa et al. (2016), Nkem et al. (2016) and Supplementary Table 1. Among them, only *Alcanivorax, Bacillus, Pseudomonas, Psychrobacter* and *Vibrio* have been reported from multiple locations. Of the 20 bacterial genera identified to be present in Betul tarball samples in this study, 16 genera are new records for tarball-associated bacteria, while *Alcanivorax*, *Bacillus*, *Pseudomonas* and *Vibrio* have been reported earlier from other sampling location/s (Supplementary Table 1). Some species of *Enterobacter, Klebsiella, Pantoea, Pseudomonas* and *Vibrio* exist as pathogens (Delétoile et al., 2009), and their association with tarballs will have implications in human and animal health issues (Tao et al., 2011).

The 16S rRNA gene-based tree (Fig. 1) provides indications about ecology and potential abilities to degrade xenobiotics of tarball-associated bacteria reported in this study. These indications can be utilized to devise an effective screening strategy for tarball-associated microbes. For example, *Pseudomonas* species are known to be present in marine habitats such as from Coralline algae (MG551789), marine hot spring (JX047093), soil of coral reef nature reserve (KT986148), *Spartina alterniflora* (endophyte) (KP757672) and fish of Arabian Gulf region (KT005274). They are also known from oil polluted environments such as soil (HM461889) and have a history of involvement in PAH degradation (KF052990), specifically pyrene degradation (MF948939), and Arsenite oxidation (KP730605). Similar observation and analysis for other hydrocarbonoclastic bacteria such as members of *Alcanivorax, Marinobacter, Nitratireductor, Tistrella* (Hassanshahian et al., 2012; Gao et al., 2015; Rahman et al., 2002) can provide hints about their hydrocarbon-degradation history and potentials. Most hydrocarbonoclastic bacteria are reported to have *nif H* gene and supposed to be diazotrophic in nature (Dashti et al., 2015). This feature can help in dealing with the issue of nitrogen availability, which is a major growth factor for bacteria and its scarcity limits biodegradation process in oceans (Das and Chandran, 2011).

4.2. Tarball-associated fungi

Fungal diversity associated with tarballs has already been reviewed in our previous study (Sanyal et al., 2016). Tarballs (and tarball-like substrates) are known to support phylogenetically diverse 31 fungal genera Nair et al. (1972), Nair and Lokabharathi (1977), Snellman et al. (1988), Itah and Essien (2005), Elshafie et al. (2007), Lotfinasabasl et al. (2012), Sanyal et al. (2016), Barnes et al. (2018) and Supplementary Table 1. Of the 33 reported fungal genera, 15 genera are known to be associated with tarball samples from Goan beaches. This study summarizes 14 fungal genera, identified based on DNA sequence-data, from tarballs collected from Goa (Fig. 2, Sanyal et al., 2016, Barnes et al., 2018). Among the known tarball-associated fungal genera, *Aspergillus, Paecilomyces, Penicillium, Talaromyces* and *Trichoderma* have been extensively studied for their hydrocarbon-degrading potential (Dawoodi et al., 2015) and Barnes et al. (2018) have summarized recent studies on bioremediation potentials of hydrocarbon-degrading fungi. Some species of *Trichoderma* and *Candida* are potential human pathogens (Sanyal et al., 2016) and further studies are required to assess the risks of tarballs as carriers of human and animal pathogens in the coastal environment.

4.3. Future perspectives

This study reveals the presence of phylogenetically diverse 28 microbial genera (20 bacterial genera and 8 fungal genera), characterized based on gene sequence-data, in the tarball samples collected from Betul beach in south Goa, India. The DNA datasets (i.e. 16S rRNA gene and the ITS) generated in this study will strengthen future studies on DNA sequence-based identification and phylogenetic characterization of tarball-associated microbes. Future studies employing a polyphasic approach for species-level identification of tarball-associated bacteria and fungi are required, as the monophasic approach based solely on morphology or single gene sequence-dataset is prone to errors. It is imperative that in future studies the uncultivable microbial fraction is also studied along with culturable tarball-associated microbes, especially to obtain insights into the diversity of anaerobic bacteria and fungi in tarballs.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marpolbul.2018.01.064.

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References

Bacosa, H.P., Thyng, K.M., Plunkett, S., Erdner, D.L., Liu, Z., 2016. The tarballs on Texas beaches following the 2014 Texas City "Y" spill: modeling, chemical, and microbiological studies. Mar. Pollut. Bull. 109, 236–244.

Barnes, N.M., Khodse, V.B., Lotlikar, N.P., Meena, R.M., Damare, S.R., 2018. Bioremediation potential of hydrocarbon-utilizing fungi from select marine niches of India. 3 Biotech 8 (21). http://dx.doi.org/10.1007/s13205-017-1043-8.

Bayat, Z., Hassanshahian, M., Hesni, M.A., 2015. Enrichment and isolation of crude oil degrading bacteria from some mussels collected from the Persian Gulf. Mar. Pollut. Bull. 101, 85–91.

Das, N., Chandran, P., 2011. Microbial degradation of petroleum hydrocarbon contaminants: an overview. Biotechnol. Res. Int. 2011, 941810 (13 pages). https://doi.org/10.4061/2011/941810.

Dashti, N., Ali, N., Eliyas, M., Khanafer, M., SorKhoh, N.A., Radwan, S.S., 2015. Most hydrocarbonoclastic bacteria in the total environment are diazotrophic, which highlights their value in the bioremediation of hydrocarbon contaminants. Microbes Environ. 30, 70–75.

Dawoodi, V., Madani, M., Tahmourespour, A., Golshani, Z., 2015. The study of heterotrophic and crude oil-utilizing soil fungi in crude oil contaminated regions. J. Bioremed. Biodegr. 6, 270. http://dx.doi.org/10.4172/2155-6199.1000270.

Delétoile, A., Decré, D., Courant, S., Passet, V., Audo, J., Grimont, P., Arlet, G., Brisse, S., 2009. Phylogeny and identification of *Pantoea* species and typing of *Pantoea agglomerans* strains by multi-locus gene sequencing. J. Clin. Microbiol. 47, 300–310.

Elshafie, A., AlKindi, A.Y., Al-Busaidi, S., Bakheit, C., Albahry, S.N., 2007. Biodegradation of crude oil and n-alkanes by fungi isolated from Oman. Mar. Pollut. Bull. 54, 1692–1696.

Gao, X., Gao, W., Cui, Z., Han, B., Yang, P., Sun, C., Zheng, L., 2015. Biodiversity and degradation potential of oil-degrading bacteria isolated from deep-sea sediments of south mid-Atlantic ridge. Mar. Pollut. Bull. 97, 373–380.

Hassanshahian, M., Emtiazi, G., Cappello, S., 2012. Isolation and characterization of crude-oil-degrading bacteria from the Persian Gulf and the Caspian Sea. Mar. Pollut. Bull. 64, 7–12.

Itah, A., Essien, J., 2005. Growth profile and hydrocarbonoclastic potential of microorganisms isolated from tarballs in the bight of bonny, Nigeria. World J. Microbiol. Biotechnol. 21, 1317–1322.

Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger data sets. Mol. Biol. Evol. 33, 1870–1874.

Liu, Z., Liu, J., 2013. Evaluating bacterial community structures in oil collected from the sea surface and sediment in the northern Gulf of Mexico after the Deepwater Horizon oil spill. Microbiology 2, 492–504.

Lotfinasabasl, S., Gunale, V.R., Rajurkar, N.S., 2012. Assessment of petroleum hydrocarbon degradation from soil and tarball by fungi. Biosci. Discov. 3, 186–192.

Nair, S., Lokabharathi, P., 1977. Degradation of hydrocarbons by fungus, *Fusarium* sp. Indian J. Mar. Sci. 6, 173–175.

Nair, S., Devassy, V., Dwivedi, S., Selvakumar, R., 1972. Preliminary observations on tarlike material observed on some beaches. Curr. Sci. 41, 766–767.

Nkem, B.M., Halimoon, N., Yusoff, F.M., Johari, W.L.W., Zakaria, M.P., Reddy, S., Kannan, N., 2016. Isolation, identification and diesel-oil biodegradation capacities of indigenous hydrocarbon-degrading strains of *Cellulosimicrobium cellulans* and *Acinetobacter baumannii* from tarball at Terengganu beach, Malaysia. Mar. Pollut. Bull. 107, 261–268.

Petra, S., Simons, K.L., Kadali, K.K., Patil, S.S., Ball, A.S., 2012. The importance of weathered crude oil as a source of hydrocarbonoclastic microorganisms in contaminated seawater. J. Microbiol. Biotechnol. 22, 1185–1192.

Piterina, A.V., Bartlett, J., Pembroke, J.T., 2010. Molecular analysis of bacterial community DNA in sludge undergoing autothermal thermophilic aerobic digestion (ATAD): pitfalls and improved methodology to enhance diversity recovery. Diversity 2, 505–526.

Rahman, K., Thahira-Rahman, J., Lakshmanaperumalsamy, P., Banat, I., 2002. Towards efficient crude oil degradation by a mixed bacterial consortium. Bioresour. Technol. 85, 257–261.

Sanyal, O., Shinde, V.L., Meena, R.M., Damare, S., Shenoy, B.D., 2016. The ITS-based phylogeny of fungi associated with tarballs. Mar. Pollut. Bull. 113, 277–281.

Shinde, V.L., Suneel, V., Shenoy, B.D., 2017. Diversity of bacteria and fungi associated with tarballs: recent developments and future prospects. Mar. Pollut. Bull. 117, 28–33.

Snellman, E.A., Collins, R.P., Cooke, J.C., 1988. Utilization of fuel oils by fungi isolated from oceanic tarballs. Lett. Appl. Microbiol. 6, 105–107.

Suneel, V., Vethamony, P., Zakaria, M.P., Naik, B.G., Prasad, K.V.S.R., 2013. Identification of sources of tar balls deposited along the Goa coast, India, using fingerprinting techniques. Mar. Pollut. Bull. 25, 81–89.

Suneel, V., Ciappa, A., Vethamony, P., 2016. Backtrack modeling to locate the origin of tar balls depositing along the west coast of India. Sci. Total Environ. 569–570, 31–39.

Tao, Z., Bullard, S., Arias, C., 2011. High numbers of *Vibrio vulnificus* in tarballs collected from oiled areas of the north-Central Gulf of Mexico following the 2010 BP Deepwater horizon oil spill. EcoHealth 8, 507–511.

White, T., Bruns, T., Lee, S., Taylor, J., 1990. Amplification and direct sequencing of fungalribosomal RNA genes for phylogenetics. In: MA, I., DH, G., JJ, S., TJ, W. (Eds.), PCR Protocols: A Guide to Methods and Applications. Academic Press, New York, pp. 315–322.

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ORIGINAL ARTICLE

Degradation of tarballs using associated bacterial consortia

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Abstract
Tarballs are semi-solid blobs of crude-oil formed in marine environment. Microbial degradation of tarballs is poorly understood, though there are indications that tarball-associated microbes can degrade recalcitrant hydrocarbons present in tarballs. In this study, 38 tarball-associated bacteria from Betul beach, Goa, India were initially screened for crude oil degradation. Based on preliminary studies and literature survey, four bacterial strains, *Alcanivorax* sp. Betul-O, *Marinobacter* sp. Betul-26, *Pseudomonas* sp. Betul-14, and *Pseudomonas* sp. Betul-M were selected for bacterial consortia preparation. Eleven bacterial consortia were prepared and studied for degradation of *n*-alkanes and polycyclic aromatic hydrocarbon compounds (PAHs) of tarballs based on gravimetric and GC–MS–MS analyses. The bacterial consortia depleted 53.69–97.78% and 22.78–61.98% of *n*-alkanes and PAH compounds, respectively, within 45 days. Bacterial consortium comprising *Pseudomonas* sp. Betul-14, *Pseudomonas* sp. Betul-M, and *Alcanivorax* sp. Betul-O exhibited promising tarball degradation abilities with 97.78% and 61.98% degradation of *n*-alkanes and PAH, respectively, within 45 days. Further research is required to obtain insights into degradation products and possible pathways involved.

Keywords Alkanes · Biodegradation · Crude oil · Oil pollution · Polycyclic aromatic hydrocarbons (PAHs)

Introduction

Transportation of crude-oil and petroleum products is mostly done through sea-route. Incidents like tanker accidents and release of ballast water occasionally result in introduction of crude oil and petroleum products into marine waters (Kvenvolden and Cooper 2003). Due to weathering, oil spill leads to the formation of tarballs, which get transported to nearby coastal areas due to ocean currents and waves. Tarballs are semi-solid remnants of crude oil and they are frequently reported from many coastal areas of the world (Warnock et al. 2015). They are rich in high molecular weight *n*-alkanes and polycyclic aromatic hydrocarbons (PAHs), which pose a great danger to algae and phytoplankton, the primary producers of marine food chain. They can disturb the food chain, accumulate in tissues of living organisms, and some may even cause carcinogenic effects in marine organisms (Perelo 2010; Hassanshahian et al. 2014).

Goa state in India has been facing tarball pollution on its tourist beaches since 1970s (Suneel et al. 2015). Presently, the stakeholders are considering various mitigation methods. Biodegradation of tarballs is being considered as it is an environment-friendly option (Hassanshahian et al. 2014). It involves complete removal, or partial conversion of toxic hydrocarbons of tarballs to less toxic products through mineralization, using microorganisms such as bacteria and fungi (Bamforth and Singleton 2005; Dellagnezze et al. 2014). As microorganisms tend to saturate on tarballs for nutrition and use it as a substratum to colonize (Shinde et al. 2017), microbial load on tarballs has been reported to be high (Snellman

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et al. 1988; Itah and Essien 2005; Tao et al. 2011; Lotfinasabasl et al. 2012; Shinde et al. 2018).

Tarballs are a serious threat to marine environment (Warnock et al. 2015; Shinde et al. 2017). Microbial degradation of tarballs, however, is poorly understood. Previous studies reported isolation of bacteria and fungi from tarballs and provided information on their abilities to degrade crude-oil and crude-oil derivatives such as kerosene, diesel, petrol, and fuel oil (Nair and Lokabharathi 1977; Snellman et al. 1988; Lotfinasabasl et al. 2012; Nkem et al. 2016; Barnes et al. 2018). There are, however, few studies reported on degradation of tarballs by associated microbes. Itah and Essien (2005) reported the degradation of tarballs by associated bacteria and fungi from Nigeria, but their study was purely qualitative. There is a need to unravel possible roles played by associated microbes in tarball degradation.

In the present study, 38 tarball-associated bacteria isolated from Betul beach, Goa, India during our previous study (Shinde et al. 2018) were investigated for their ability to degrade tarballs. This study initially employed dye-decolorization screening method (DCPIP method), followed by characterization of *n*-alkane degradation profiles of crude oil by GC–MS–MS analysis. This led to selection of four bacterial strains for consortia preparation. Eleven bacterial consortia were prepared and studied for their abilities to degrade tarballs. After 45 days of incubation of tarballs with the bacterial consortia, *n*-alkanes and PAH compounds of control and residual samples were extracted and quantified by both gravimetric and GC–MS–MS methods.

Materials and methods

Materials used in GC–MS–MS analysis

For GC–MS–MS analysis, *n*-alkanes (C_8–C_{40}) and polycyclic aromatic hydrocarbons (PAHs; 16 compounds) standards were procured from Sigma Aldrich (USA), while the surrogate internal standards were procured from Chiron AS (Norway). A 100–200 mesh silica gel was baked at 400 °C for 4 h, cooled, then activated at 200 °C overnight and deactivated with 5% of distilled water, and used for column preparation. Hexane and Dichloromethane used were of high purity, SupraSolv grade from Sigma Aldrich. All glassware was washed with detergent and distilled water, followed by the serial rinse with methanol, acetone, and hexane.

Screening of bacterial strains for crude oil degradation

In our previous study, 49 tarball-associated bacteria were isolated from Betul beach, Goa, India (Shinde et al. 2018), out of which 38 bacteria were screened in the present study for their crude-oil degradation abilities (Table 1). This was done following the method described in Bidoia et al. (2010) and adopted by Varjani et al. (2013).

Briefly, 500 µL of dichloro-phenol-indophenol (DCPIP), from 1 µg/µL of stock solution, was added into 50 mL of Bushnell Hass medium containing 500 µL of sterile crude oil. A known volume of bacterial suspension was added into the medium and incubated for 24–48 h. A control consortium was maintained without the inoculum. The change in color of DCPIP from blue to colorless was observed. The change in color indicated utilization of crude-oil by bacteria as a sole carbon source. Spectrophotometric analysis was performed to determine the color difference in each consortium. After 72 h of incubation, the medium was centrifuged, and optical density was measured at 600 nm. The bacterial strains showing visible color differences were considered to be having crude oil degradation ability.

Screening of bacterial strains for *n*-alkane degradation profiles

Based on their crude oil degradation abilities inferred through the DCPIP method (Table 1), four bacterial strains, Betul-14, Betul-D, Betul-M, and Betul-O were selected and processed for characterization of *n*-alkane degradation profiles by GC–MS–MS analysis. *Marinobacter* sp. Betul-26 was also selected based on the literature survey which suggested that *Marinobacter* is a good hydrocarbon degrader (Head et al. 2006; Hassanshahian et al. 2012; Xue et al. 2015).

The taxonomic identities and GenBank accession numbers of 16S rRNA gene sequences (Shinde et al. 2018) are presented in Table 1. Each bacterial culture was separately inoculated in 25 mL of Bushnell Hass medium containing 500 µL of crude oil (2%) as a sole carbon source. The consortia were kept on a rotary shaker for 30 days at 100 rpm and 35 °C. Optical density at 600 nm at a regular time interval and colony-forming units (CFUs) were measured to monitor the bacterial growth. Controls without bacterial inoculum were used to quantify the abiotic loss during sterilization and incubation period. Residual crude oil was extracted from the culture medium by shaking vigorously with an equal volume of 3:1 (v/v) hexane: dichloromethane (DCM) mixture for 15 min and the solvent extract transferred to a clean beaker. This procedure was repeated twice. Sodium sulfate was added to the hexane: dichloromethane extracts to remove the moisture in it, and the supernatants were concentrated to approximately 1 mL in a rotary evaporator.

Initially, the sample was passed through the deactivated silica gel column (1 cm i.d. × 9 cm) to separate the impurities and transferred to activated silica gel column (0.47 cm i.d. × 18 cm), for the fractionation. Briefly,

Table 1 Spectrophotometric analysis of dye decolorization of DCPIP measured at 600 nm

Strain no.	O.D	Genus name	GenBank accession number	Strain no.	O.D	Genus name	GenBank accession number
Set A							
Betul 1	0.082	*Pseudoxanthomonas* sp.	MG664257	Betul 23	0.086	*Marinobacter* sp.	MG664238
Betul 2	0.078	*Enterobacter* sp.	MG664235	Betul 24	0.088	*Pseudoxanthomonas* sp.	MG664259
Betul 3	0.09	*Pantoea* sp.	MG664246	Betul 26	0.088	*Marinobacter* sp.	MG664239
Betul 4	0.065	*Pseudomonas* sp.	MG664252	Betul 28	0.069	*Cellulomonas* sp.	MG664231
Betul 5	0.085	*Klebsiella* sp.	MG664236	Betul 29	0.049	*Cellulomonas* sp.	MG664232
Betul 6	0.075	*Bacillus* sp.	MG664225	Betul 30	0.089	*Cellulomonas* sp.	MG664233
Betul 9	0.066	*Vibrio* sp.	MG664261	Betul D	**0.036**	*Nitratireductor* sp.	MG664242
Betul 11	0.078	*Pseudomonas* sp.	MG664247	Betul I	0.062	*Alcanivorax* sp.	MG664222
Betul 19	0.066	*Pseudomonas* sp.	MG664251	Betul K	0.084	*Bhargavaea* sp.	MG664227
Betul 21(1)	0.056	*Brevibacterium* sp.	MG664229	Betul M	**0.026**	*Pseudomonas* sp.	MG664256
Betul 21(2)	0.052	*Brevibacterium* sp.	MG664230	Betul Q	0.062	*Oceanimonas* sp.	MG664245
Betul 22	0.085	*Pseudoxanthomonas* sp.	MG664258	**Control**	**0.09**		
Set B							
Betul 8	0.066	*Klebsiella* sp.	MG664237	Betul H	0.062	*Alcanivorax* sp.	MG664221
Betul 10	0.053	*Enterobacter* sp.	MG664234	Betul J	0.076	*Bhargavaea* sp.	MG664226
Betul 12	0.071	Not identified	Not available	Betul L	0.073	*Bhargavaea* sp.	MG664228
Betul 13	**0.022**	*Pseudomonas* sp.	MG664248	Betul N	**0.04**	*Nitratireductor* sp.	MG664243
Betul 14	**0.017**	*Pseudomonas* sp.	MG664249	Betul O	**0.04**	*Alcanivorax* sp.	MG664223
Betul 15	0.075	*Pseudomonas* sp.	MG664250	Betul P	0.067	*Alcanivorax* sp.	MG664224
Betul F	0.046	*Pseudomonas* sp.	MG664254	Betul R	0.063	*Nitratireductor* sp.	MG664244
Betul G	0.066	*Pseudomonas* sp.	MG664255	**Control**	**0.079**		

The experiment was conducted in two sets, set A and B. The bold values are lowest ODs observed in test flasks as compared to respective control flasks in each set

1 mL of concentrated sample was passed through 5% H_2O-deactivated silica gel column. Aliphatic hydrocarbons were eluted with 30 mL of hexane:DCM (3:1 v/v). The eluent was subjected to roto-evaporation to decrease the volume to ~ 2 mL and then transferred to the fully activated silica gel column. Total *n*-alkanes were eluted with 10 mL of hexane. Eventually, the fraction was again concentrated to dryness using a nitrogen cycle purge to make final volume before injecting into GC–MS–MS.

Degradation of tarballs by bacterial consortia

Bacterial strains, Betul-14, Betul-26, Betul-M, and Betul-O, were selected for consortia preparation. Eleven different combinations were prepared as shown in Fig. 1 and Table 2. Though tarballs are in semi-solid state and easy to weigh as compared to viscous crude-oil, they contain sand particles, which leads to an error during weighing, hence in the quantitative values. To remove sand particles, tarballs were dissolved in hexane:DCM (3:1 v/v), and the upper portion of the solvent was carefully removed after all the particles settled at the bottom of the consortium. This fraction containing dissolved tarball was kept at room temperature till the complete evaporation of the solvent. Bushnell Hass medium was prepared in 50% sea water and distributed 25 mL in each of 11 consortia. Tarballs were weighed (~ 150 mg) and added into the medium, sterilized at 121 °C for 20 min, and inoculated with respective bacterial strains. They were incubated on a rotary shaker at room temperature at 100 rpm. The bacterial growth was monitored by streaking on agar plate and by Gram staining. After 45 days of incubation, residual tarball was extracted from the growth medium. First, the bacterial growth was halted by acidifying the medium at pH 2 (Hassanshahian et al. 2012). Dichloromethane was used to extract the residual tarball. The extract was passed through anhydrous Sodium sulfate to remove water molecules and collected in a pre-weighed empty beaker and kept at room temperature for complete evaporation. Percentage depletion of *n*-alkane/PAH present in the tarball was calculated using the formula:

$$\% \text{ degradation} = \frac{\text{Amount of tarball degraded}}{\text{Amount of tarball added in medium}} \times 100$$

Fig. 1 Bacterial consortia used for studying the degradation of tarballs in the laboratory

Next, fractionation of tarball was achieved using the protocol described for crude oil. Pre-dried residual tarball was dissolved in 1 mL of Hexane:DCM (1:3 v/v) and the surrogate internal standard mixture (d8-Naphthalene, d10-Phenanthrene, and d12-Chrysene) was added to the sample prior to the extraction process in order to check the recovery percentage and passed through the column. Total alkanes were eluted with 10 mL of hexane, whereas PAH compounds were eluted using 30 mL of hexane-DCM mixture (3:1 v/v). Both fractions were quantified on GC–MS–MS. Analyses for *n*-alkane and PAHs were performed on a Thermofisher Scientific Trace 1300 Gas Chromatograph, coupled with Thermo TSQ8000 Mass Spectrometer with a Thermo AS 1310 auto-sampler under selected ion monitoring (SIM) mode. The DB-5 column had 60 m length × 0.25 mm internal diam. with 0.25 µm film thickness with helium (1.2 mL/

Table 2 Degradation of *n*-alkanes and PAH compounds of tarball by 11 bacterial consortia (%)

Flask no.	Consortia	Gravimetric analysis	GCMS analysis	
			PAH	Alkane
1	Betul-14 + Betul-26 (*Pseudomonas* sp. + *Marinobacter* sp.)	45	46.27	81.86
2	Betul-14 + Betul-O (*Pseudomonas* sp. + *Alcanivorax* sp.)	46	42.01	79.9
3	Betul-14 + Betul-M (*Pseudomonas* sp. + *Pseudomonas* sp.)	47.44	36.24	84.97
4	Betul-M + Betul-O (*Pseudomonas* sp. + *Alcanivorax* sp.)	33.69	22.78	53.69
6	Betul-26 + Betul-M (*Marinobacter* sp. + *Pseudomonas* sp.)	48.06	36.23	92.57
7	Betul-14 + Betul-M + Betul-O (*Pseudomonas* sp. + *Pseudomonas* sp. + *Alcanivorax* sp.)	66.41	61.98	97.78
8	Betul-26 + Betul-M + Betul-O (*Alcanivorax* sp. + *Pseudomonas* sp. + *Marinobacter* sp.)	47.51	25.4	95.01
9	Betul-14 + Betul-26 + Betul-M (*Pseudomonas* sp. + *Marinobacter* sp. + *Pseudomonas* sp.)	47.0	28.8	95.74
10	Betul-14 + Betul-26 + Betul-O (*Pseudomonas* sp. + *Marinobacter* sp. + *Alcanivorax* sp.)	48.48	43.24	96.34
11	Betul-14 + Betul-26 + Betul-M + Betul-O (*Pseudomonas* sp. + *Marinobacter* sp. + *Pseudomonas* sp + *Alcanivorax* sp.)	49.94	60.86	97.21
12	Betul-26 + Betul-O (*Marinobacter* sp. + *Alcanivorax* sp.)	Not available	50.87	79.16

$$\% \text{ degradation (Gravimetric analysis)} = \frac{\text{Amount of tarball degraded}}{\text{Amount of tarball added in medium}} \times 100$$

min) as a carrier gas used for the sample analysis. The injector and detector temperatures were set to 260 °C and 300 °C, respectively, for alkane and PAH analysis. The oven temperature program for alkane analysis was initially 50 °C, held for a minute, then ramped to 140 °C at 10 °C/min, and ramped to 320 °C at 6 °C/min and finally a 28-min hold at 320 °C. The oven temperature program for PAH analysis was initially 70 °C with a hold of 2-min, then ramped to 150 °C at 30 °C /min and then ramped to 310 °C at 4 °C / min, then finally a 10-min hold at 310 °C. The *n*-alkanes C_8–C_{40}, including pristane and phytane and 16 PAH compounds such as Naphthalene (Nap), Acenaphthylene (Acy), Acenaphthene (Ace), Fluorene (Fl), Phenanthrene (Phe), Fluoranthene (Fluo), Pyrene (Py), 1-Methyl fluoranthene (1-M-Fluo), B(a)anthracene (BaA), Chrysene (Chr), 1-Methyl chrysene (1-M-Chr), B(a)fluoranthene (BaF), B(k) fluoranthene (BkF), B(a)pyrene (BaP), Dibenzo anthracene (D(B)Ant), and Benzoperylene (BgP) were analyzed in this study. The recovery was confirmed through three replicate analyses of the samples which were spiked with surrogate standards.

Results and discussion

Screening of bacterial strains for crude oil degradation

The optical density (OD) measured at a specific interval of time during the DCPIP screening is shown in Table 1. The OD of control for set A and set B was 0.088 and 0.079, respectively. In total, six bacterial strains decolorized the medium, the ODs of which were 0.022 (Betul-13), 0.017 (Betul-14), 0.036 (Betul-D), 0.026 (Betul-M), 0.04 (Betul-N), and 0.04 (Betul-O). Among them, Betul-14, Betul-D, Betul-M, and Betul-O were selected for further processing.

In the screening step, a sudden drop in color intensity of DCPIP was observed for Betul-8, Betul-N, and Betul-L after 24 h of incubation but found to be constant afterwards. Betul-14 and Betul-15 showed late decolorization but were found to be consistent till the medium turned colorless. This can be attributed to different lag phase for different bacteria. The gradual color change was observed for Betul-O and Betul-H. Feeble dye decolorization was observed for Betul (21)-2, Betul-29 and Betul-10. These bacterial strains were excluded from further processing. This is a simple and rapid method to screen oil degrading bacteria (Hanson et al., 1993). Mariano et al. (2008) applied the same method to screen diesel degrading bacteria. Bidoia et al. (2010) reported different time spans for *Bacillus subtilis* to reduce dye as at 138 h, 125 h, 75 h, and 87 h in synthetic, semi-synthetic, mineral, and oil medium, respectively. Varjani et al. (2013) suggested that DCPIP along with Bushnell Hass medium may not hamper the growth of microorganisms and is a reliable method for screening.

Characterization of *n*-alkane degradation profiles of crude oil by GC–MS–MS analysis

The results of % degradation of *n*-alkanes of crude oil are presented in Fig. 2. Almost, all alkanes, i.e., from C_{15} to C_{40}, analyzed were found to be degraded by Betul-14, Betul-26, Betul-D, Betul-M, and Betul-O. Hydrocarbons in the range of C_8–C_{14} were either found none or in very low detectable levels and considered as an abiotic loss. Alkanes in the range of C_{35}–C_{40} were also barely detected. Betul-O, Betul-14, and Betul-M exhibited maximum degradation of 32.72%,

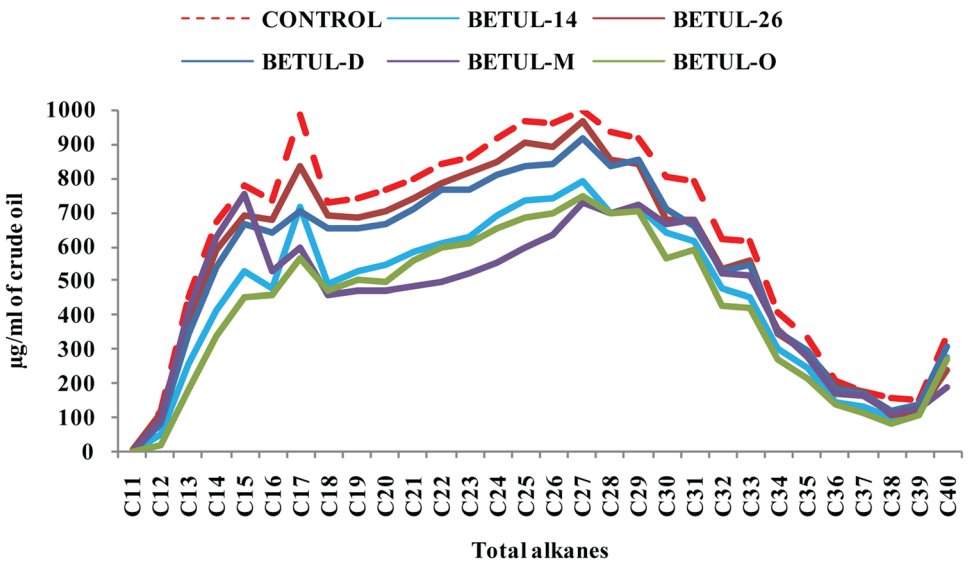

Fig. 2 Graph depicting individual *n*-alkane degrading profiles of select bacterial strains (Betul-14, Betul-26, Betul-D, Betul-M, and Betul-O)

27.06%, and 24.10%, respectively. Comparatively, less degradation of crude-oil total alkanes was exhibited by Betul-D (13.26%) and Betul-26 (9.75%).

Degradation of *n*-alkanes of tarballs by bacterial consortia

In this study, the bacterial consortia exhibited maximum degradation of tarballs than as an individual strain (Fig. 3). The range of total alkanes present in the flasks varied between 6744 and 140,867 µg/g out of 304,156 µg/g present in the control. Apparently, a significant amount of *n*-alkane was depleted. The calculated percent degradation of *n*-alkanes from tarballs revealed that minimum 53.69% of total *n*-alkanes were depleted in almost all consortia, with the maximum degradation of 97.78% in consortium 7 (*Pseudomonas* sp. + *Pseudomonas* sp. + *Alcanivorax* sp.), 97.21% in consortium 11 (*Pseudomonas* sp. + *Marinobacter* sp. + *Pseudomonas* sp + *Alcanivorax* sp.), 96.34% in consortium 10 (*Pseudomonas* sp. + *Marinobacter* sp. + *Alcanivorax* sp.), 95.74% in consortium 9 (*Pseudomonas* sp. + *Marinobacter* sp. + *Pseudomonas* sp.), 95.01% in consortium 8 (*Alcanivorax* sp. + *Pseudomonas* sp. + *Marinobacter* sp.), and 92.57% for consortium 6 (*Marinobacter* sp. + *Pseudomonas* sp.). The lowest degradation was observed for the consortium comprising *Pseudomonas* sp. + *Alcanivorax* sp. (consortium 4), i.e. 22.78% of PAH compounds and 53.69% of alkanes (Table 2 and Fig. 3). The low *L/H* ratios in almost all the consortia further suggested that low molecular compounds were degraded more than the high molecular compounds (Table 3). Hassanshahian et al. (2012) also determined efficiency of 25 bacterial strains from Persian Gulf to degrade *n*-alkanes from crude oil by GC–MS analysis.

It is believed that indigenous bacteria probably can degrade a wide range of target constituents of a complex mixture such as oily sludge. Mishra et al. (2001), however, stated that a single bacterial species has only limited capacity to degrade all the fractions of hydrocarbons present in crude oil. A bacterial consortium, therefore, can be more effective in the degradation of crude oil and tarballs. In India, the Tata Energy Research Institute, (TERI), New Delhi had launched a successful product named 'oil-zapper', a patented product to remediate lands dumped with oily sludge. The product is a consortium of bacteria (Randhwa and Kaushal 2014). A total of 79 bacterial genera, 9 cyanobacterial genera, 103 fungal genera, and 19 algal genera from marine niches have been reported to be involved in petroleum degradation (Xue et al. 2015). Bacteria have developed metabolic machineries that can degrade complex hydrocarbons. In aerobic way of degradation, enzymes such as alkane monooxygenase and aromatic ring dioxygenase hydroxylases (ARDHs) are involved in the alkanes and aromatic compounds, respectively (Dellagnezze et al. 2014). In both of these enzymatic reactions molecular oxygen is used as a co-substrate which helps the terminal or sub-terminal hydroxylation of alkane chains or the mono or dihydroxylation of aromatic rings (Al-Sayegh et al. 2016). *Alcanivorax* is known for its hydrocarbonoclastic abilities (Yakimov et al. 2007). *Alcanivorax dieselolei* is a principal alkane degrader that was first isolated from surface seawater and sediments of Bohai Sea (Liu and Shao 2005). Hassanshahian et al. (2012) reported efficiency of *Alcanivorax dieselolei* isolated from oil-contaminated areas of Persian Gulf to degrade *n*-alkanes from crude oil to be 98%. Santisi et al. (2015), who tested consortium of *Pseudomonas* and *Alcanivorax* to degrade alkanes from crude oil, reported the depletion of C_{12}–C_{30} alkanes in the range of 50–90%.

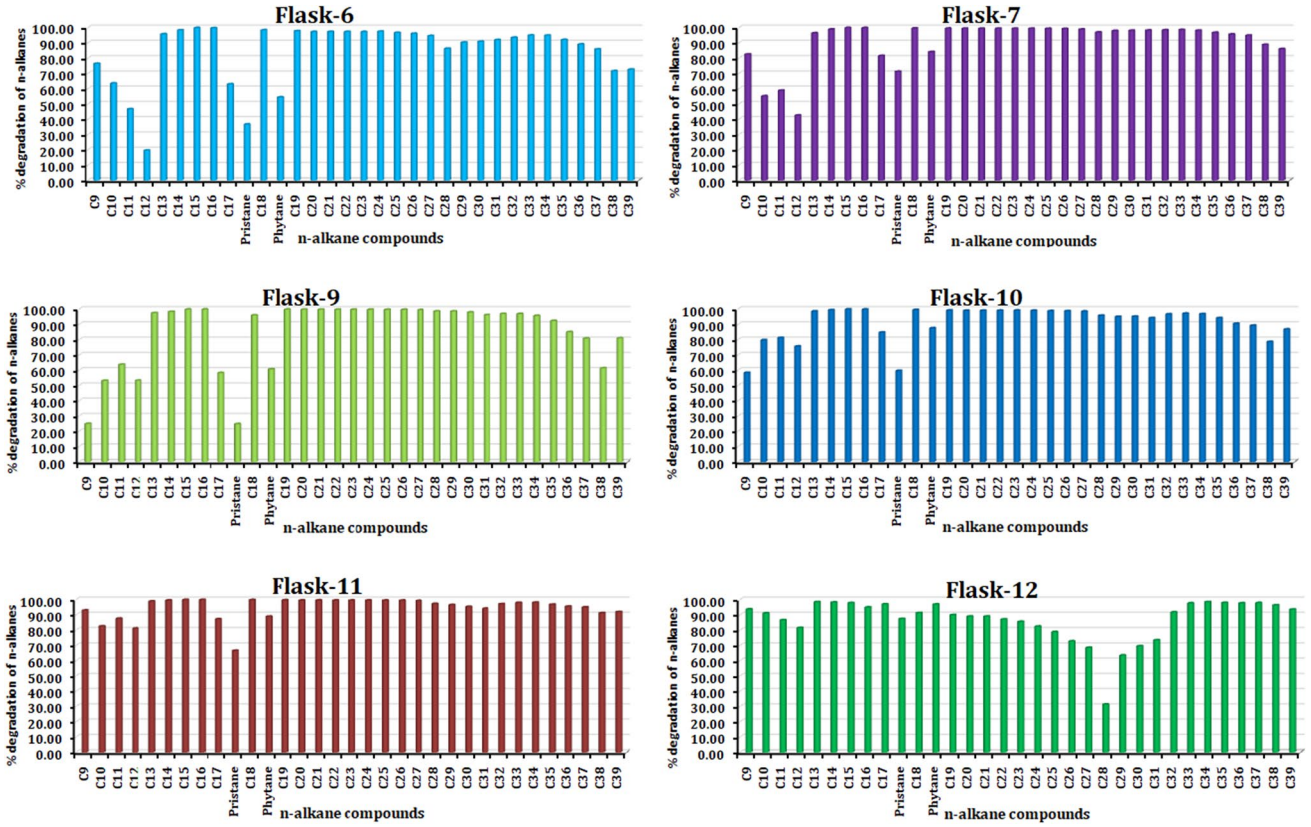

Fig. 3 Graph depicting degradation of individual *n*-alkane compounds (%) of tarballs by select bacterial consortia. Flask (consortium) no. 6: Bushnell Hass Medium + Crude oil (Betul-26 + Betul-M (*Marinobacter* sp. + *Pseudomonas* sp.). Flask (consortium) no. 7: Bushnell Hass Medium + Crude oil Betul-14 + Betul-M + Betul-O (*Pseudomonas* sp. + *Pseudomonas* sp. + *Alcanivorax* sp.). Flask (consortium) no. 9: Bushnell Hass Medium + Crude oil Betul-14 + Betul-26 + Betul-M (*Pseudomonas* sp. + *Marinobacter* sp. + *Pseudomonas* sp.). Flask (consortium) no. 10: Bushnell Hass Medium + Crude oil Betul-14 + Betul-26 + Betul-O (*Pseudomonas* sp. + *Marinobacter* sp. + *Alcanivorax* sp.). Flask (consortium) no. 11: Bushnell Hass Medium + Crude oil Betul-14 + Betul-26 + Betul-M + Betul-O(*Pseudomonas* sp. + *Marinobacter* sp. + *Pseudomonas* sp + *Alcanivorax* sp.). Flask (consortium) no. 12: Bushnell Hass Medium + Crude oil Betul-26 + Betul-O (*Marinobacter* sp. + *Alcanivorax* sp.)

The ratios C_{17}/Pristane and C_{18}/Phytane are widely used as an indicator to detect biodegradation effects as *n*-alkanes are likely to get biodegraded than isoprenoids (Wang et al. 1998, 1999). Our results also show that the compound C_{17} was depleted relatively more than Pristane. The highest ratios of C_{18}/Ph and Pr/Ph (~ 10 and 3 times, respectively, higher than the other consortia) were observed for the consortium 12, which revealed that the isoprenoid alkane, Phytane, was more degraded than the *n*-alkanes only for that particular consortium. The bacterial consortia, numbered 1, 2, 3, 6, 7, 8, 9, 10, and 11, were able to degrade the *n*-alkane compounds, but it is interesting note that the consortium no. 12 (*Marinobacter* sp. + *Alcanivorax* sp.) was able to degrade isoprenoid alkanes considerably.

Degradation of PAH compounds of tarballs by bacterial consortia

The range of total PAHs present in the consortia varied between 113.6 and 230.7 µg/g out of 299.8 µg/g present in the control, suggesting that a significant amount of PAHs was degraded (Fig. 4, Table 2). The severe depletion of naphthalene and acenaphthylene in both control and treatment was observed (data not shown), indicating that the high vapor pressure of low molecular weight (LMW) PAHs may cause the decrease of these compounds during the incubation period. Therefore, the degradation percentage was not considered for these compounds. The calculated percentage degradation of other PAHs showed that minimum 22.78%

Table 3 The weathering diagnostic ratios of *n*-alkane and PAH compounds of tarballs

Flask no.	*n*-alkane DRs					PAH-DRs	
	C_{17}/Pr	C_{18}/Py	Pr/Py	*L*/*H*-alkane	Σ *n*-alkanes (µg/g)	*L*/*H*-PAH	Σ PAH (µg/g)
1	0.11	1.47	9.30	0.13	55,167	0.9867	160.53
2	0.11	0.28	9.85	0.26	61,120	1.2264	173.2
3	0.12	0.08	9.24	0.26	45,721	1.1762	190.51
4	0.13	1.98	8.30	0.69	140,867	1.2139	230.72
6	0.07	0.37	12.90	0.46	22,613	1.2859	190.53
7	0.08	0.22	16.85	0.47	6744	1.3364	113.60
8	0.06	1.03	16.45	0.51	15,166	1.3116	222.90
9	0.07	1.05	17.78	0.50	12,966	1.2985	212.72
10	0.05	0.34	30.38	0.31	11,147	1.180	169.58
11	0.05	0.12	28.18	0.18	8497	1.1781	116.92
12	0.03	30.53	38.78	0.65	63,380	1.1685	146.78
Control	0.12	10.63	9.29	1.27	304,156	0.9370	298.81

L/*H* = Sum of *n*C16–*n*C26 *n*-alkane concentration relative to sum of *n*C27–*n*C36 *n*-alkane concentration

Σ *n*-alkanes = sum of total *n*-alkanes

L/*H*-PAH = Ratio of sum of concentration of Nap, Acy, Ace, Fl, Phe relative to sum of concentration of Fluo, Py, MFluo, BaA, Chr, 1 M-Chr, BaF, BkF, BaP, D(B)Ant, BgP

Σ PAH = sum of total PAHs

of total PAHs was degraded in almost all consortia, with the maximum degradation of 61.98% occurring in consortium 7 within 45 days (Table 2, Fig. 4). Maximum PAH depletion, i.e., 61.98% and 60.86% was found in consortium 7 (Betul-14 + Betul-M + Betul-O) and consortium 11 (Betul-14 + Betul- 26 + Betul-M + Betul-O), respectively.

In a previous study, *Pseudomonas aeruginosa* from oil-contaminated soils could degrade 40 mg/L of phenanthrene and fluorene completely within 7 days, and fluoranthene and pyrene more than 30% within 12 days (Zhang et al. 2011). Fu et al. (2014) reported the biodegradation of phenanthrene, anthracene, fluoranthene, and pyrene of each at 100 mg/L is 99%, 65%, 99%, and 79%, respectively, by the consortium Y12. It could degrade 7% of benzo [a] pyrene at 10 mg/L, within 10 days. Our results indicate that the compounds Benzo(a)Fluoranthene and Benzo(a) Pyrene were highly degraded compared to other PAHs in almost all the consortia. In spite of recent research developments in HMW PAH degradation, the studies on biodegradation of HMW PAHs associated with other hydrocarbons in mixtures are limited (Kanaly et al. 2010; Peng et al. 2008; Seo et al. 2009). Our results showed that the *L*/*H* ratio of PAHs (Table 3) ranged between 0.986 and 1.336, which is higher than the ratio of the control (0.937). It revealed more depletion of high molecular compounds than the low molecular compounds. Both high and low molecular compounds were almost equally degraded by the bacterial consortia, Betul-14 + Betul-M + Betul-O (consortium 7) and Betul-14 + Betul-26 + Betul-M + Betul-O in (consortium 11) (~ 66%, Fig. 4). The consortium comprising Betul-14, Betul-M, and Betul-O (consortium 7) was found to be the most effective combination to degrade both alkanes and PAH compounds, followed by the combination of Betul-14, Betul-26, Betul-M, and Betul-O (consortium 11).

Future prospects

Tarballs are a promising source to isolate crude-oil and tarball-degrading microbes. The ecology of microbial colonization on tarballs is poorly understood. Therefore, further research is required in this direction. As tarballs comprise complex organic compounds of high molecular weight, microorganisms utilizing them possibly contain highly degradative enzyme systems. Further research is required to obtain insights into degradation products and possible pathways involved in microbial degradation of tarballs.

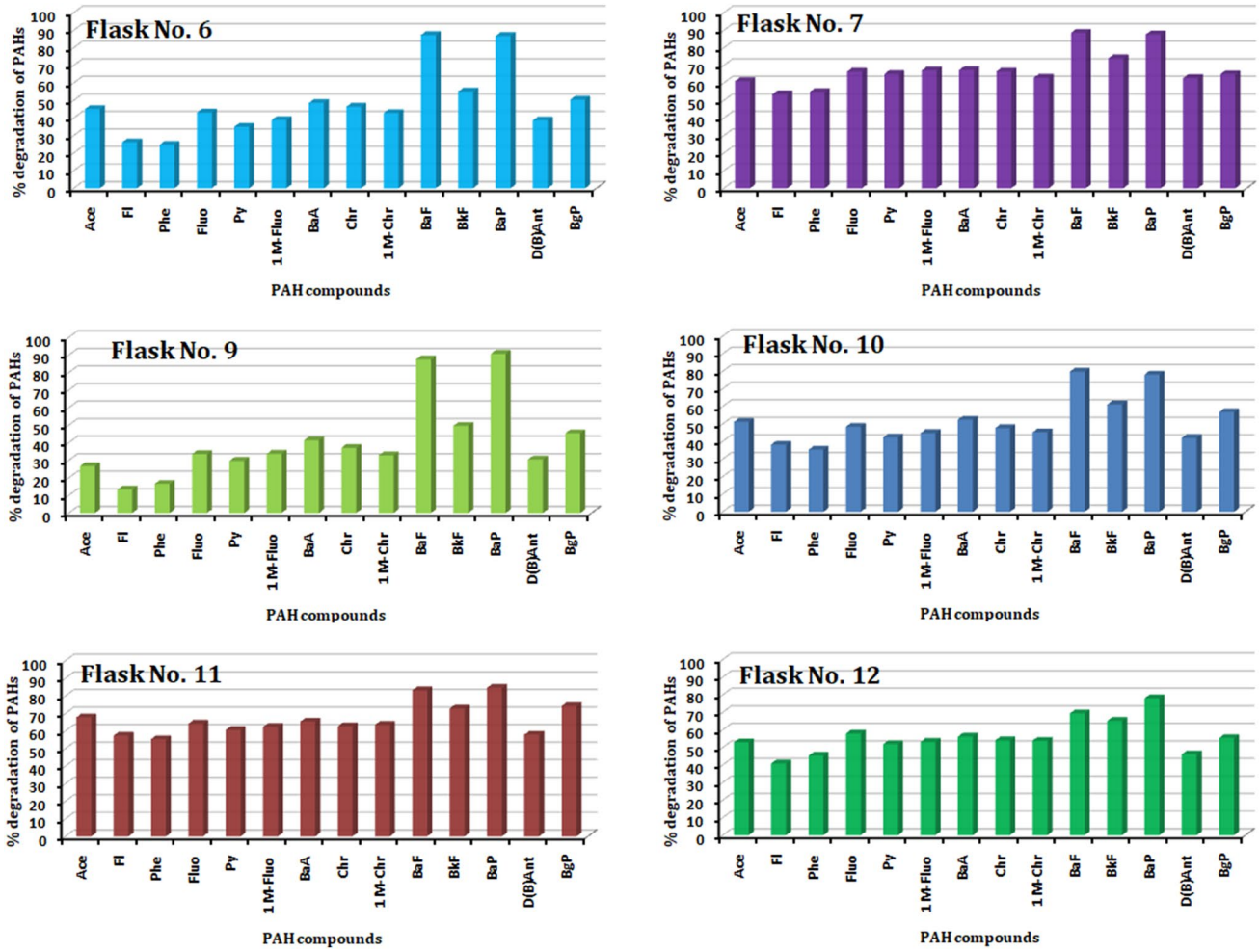

Fig. 4 Graph depicting degradation of individual polycyclic aromatic hydrocarbon (PAH) compounds (%) of tarballs by select bacterial consortia. Flask (consortium) no. 6: Bushnell Hass Medium + Crude oil (Betul-26 + Betul-M (*Marinobacter* sp. + *Pseudomonas* sp.). Flask (consortium) no. 7: Bushnell Hass Medium + Crude oil Betul-14 + Betul-M + Betul-O (*Pseudomonas* sp. + *Pseudomonas* sp. + *Alcanivorax* sp.). Flask (consortium) no. 9: Bushnell Hass Medium + Crude oil Betul-14 + Betul-26 + Betul-M (*Pseudomonas* sp. + *Marinobacter* sp. + *Pseudomonas* sp.). Flask (consortium) no. 10: Bushnell Hass Medium + Crude oil Betul-14 + Betul-26 + Betul-O (*Pseudomonas* sp. + *Marinobacter* sp. + *Alcanivorax* sp.). Flask (consortium) no. 11: Bushnell Hass Medium + Crude oil Betul-14 + Betul-26 + Betul-M + Betul-O (*Pseudomonas* sp. + *Marinobacter* sp. + *Pseudomonas* sp + *Alcanivorax* sp.). Flask (consortium) no. 12: Bushnell Hass Medium + Crude oil Betul-26 + Betul-O (*Marinobacter* sp. + *Alcanivorax* sp.)

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Author contributions VLS, SV, BDS conceived the idea. All authors conducted experiments. VLS, SV and BDS analysed the results. All authors reviewed the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

References

Al-Sayegh A, Wahaibi Y, Joshi S, Al-Bahry S, Elshafie A, Al-Bemani A (2016) Bioremediation of heavy crude oil contamination. Open Biotechnol J 10:301–311

Bamforth SM, Singleton I (2005) Bioremediation of polycyclic aromatic hydrocarbons: current knowledge and future directions. J Chem Technol Biotechnol 80:723–736. https://doi.org/10.1002/jctb.1276

Barnes NM, Khodse VB, Lotlikar NP, Meena RM, Damare SR (2018) Bioremediation potential of hydrocarbon-utilizing fungi

from select marine niches of India. 3 Biotech 8:21. https://doi.org/10.1007/s13205-017-1043-8

Bidoia ED, Montagnolli RN, Lopes PRM (2010) Microbial biodegradation potential of hydrocarbons evaluated by colorimetric technique: a case study. In: Mendez-Vilas A (ed) Current research, technology and education topics in applied microbiology and microbial biotechnology. Formatex Research Center, Badajoz, pp 1277–1288

Dellagnezze BM, De Sousa GV, Martins LL, Domingos DF, Limache EE, de Vasconcellos SP, da Cruz GF, de Oliveira VM (2014) Bioremediation potential of microorganisms derived from petroleum reservoirs. Mar Pollut Bull 89:191–200. https://doi.org/10.1016/j.marpolbul.2014.10.003

Fu B, Li QX, Xu T, Cui ZL, Sun Y, Li J (2014) *Sphingobium* sp. FB3 degrades a mixture of polycyclic aromatic hydrocarbons. Int Biodeterior Biodegrad 87:44–51. https://doi.org/10.1016/j.ibiod.2013.10.024

Hanson KG, Desai JD, Desai AJ (1993) A rapid and simple screening technique for potential crude oil degrading microorganisms. Biotechnol Tech 7:745–748. https://doi.org/10.1007/BF00152624

Hassanshahian M, Emtiazi G, Cappello S (2012) Isolation and characterization of crude-oil-degrading bacteria from the Persian Gulf and the Caspian Sea. Mar Pollut Bull 64:7–12. https://doi.org/10.1016/j.marpolbul.2011.11.006

Hassanshahian M, Emtiazi G, Caruso G, Cappello S (2014) Bioremediation (bioaugmentation/biostimulation) trials of oil polluted seawater: a mesocosm simulation study. Mar Environ Res 95:28–38. https://doi.org/10.1016/j.marenvres.2013.12.010

Head IM, Jones DM, Roling WFM (2006) Marine microorganisms make a meal of oil. Nat Rev Microbiol 4:173–182. https://doi.org/10.1038/nrmicro1348

Itah AY, Essien JP (2005) Growth profile and hydrocarbonoclastic potential of microorganisms isolated from tarballs in the Bight of Bonny. Nigeria World J Microbiol Biotechnol 21:1317–1322. https://doi.org/10.1007/s11274-004-6694-z

Kanaly RA, Harayama S (2010) Advances in the field of high-molecular-weight polycyclic aromatic hydrocarbon biodegradation by bacteria. Microb Biotechnol 3:136–164. https://doi.org/10.1111/j.1751-7915.2009.00130.x

Kvenvolden KA, Cooper CK (2003) Natural seepage of crude oil into the marine environment. Geo-Mar Lett 23:140–146. https://doi.org/10.1007/s00367-003-0135-0

Liu C, Shao Z (2005) *Alcanivorax dieselolei* sp. nov., a novel alkane-degrading bacterium isolated from sea water and deep-sea sediment. Int J Syst Evol Microbiol 55:1181–1186. https://doi.org/10.1099/ijs.0.63443-0

Lotfinasabasl S, Gunale VR, Rajurkar NS (2012) Assessment of petroleum hydrocarbon degradation from soil and tarball by fungi. Biosci Discov 3:186–192

Mariano AP, Bonotto DM, Angelis DF, Pirôllo MPS, Contiero J (2008) Biodegradability of commercial and weathered diesel oils. Braz J Microbiol 39:133–142

Mishra S, Jeevan J, Kuhad RC, Lal B (2001) Evaluation of inoculum addition to stimulate in situ bioremediation of oily-sludge-contaminated soil. Appl Environ Microbiol 67:1675–1681. https://doi.org/10.1128/AEM.67.4.1675-1681.2001

Nair S, Lokabharathi PA (1977) Degradation of hydrocarbons by fungus, *Fusarium* sp. Ind J Mar Sci 6:173–175

Nkem BM, Halimoon N, Yusoff FM, Johari WLW, Zakaria MP, Reddy S, Kannan N (2016) Isolation, identification and diesel-oil biodegradation capacities of indigenous hydrocarbon-degrading strains of *Cellulosimicrobium cellulans* and *Acinetobacter baumannii* from tarball at Terengganu beach. Malays Mar Pollut Bull 107:261–268. https://doi.org/10.1016/j.marpolbul.2016.03.060

Peng RH, Xiong AS, Xue Y, Fu XY, Gao F, Zhao W, Tian YS, Yao QH (2008) Microbial biodegradation of polyaromatic hydrocarbons. FEMS Microbiol Rev 32:927–955. https://doi.org/10.1111/j.1574-6976.2008.00127.x

Perelo LW (2010) Review: in situ and bioremediation of organic pollutants in aquatic sediments. J Hazard Mater 177:81–89. https://doi.org/10.1016/j.jhazmat.2009.12

Randhwa M, Kaushal J (2014) Bioremediation through oilzapper and oilvorous-S technologies: a review. Eng Sci Int Res J 2:29–32

Santisi S, Cappello S, Catalfamo M, Mancini G, Hassanshahian M, Genovese L, Giuliano L, Yakimov M (2015) Biodegradation of crude oil by individual bacterial strains and a mixed bacterial consortium. Braz J Microbiol 46:377–387. https://doi.org/10.1590/S1517-838246120131276

Seo JS, Keum YS, Li QX (2009) Bacterial degradation of aromatic compounds. Int J Environ Res Public Health 6:278–309. https://doi.org/10.3390/ijerph6010278

Shinde VL, Suneel V, Shenoy BD (2017) Diversity of bacteria and fungi associated with tarballs: recent developments and future prospects. Mar Pollut Bull 117:28–33. https://doi.org/10.1016/j.marpolbul.2017.01.067

Shinde VL, Meena RM, Shenoy BD (2018) Phylogenetic characterization of culturable bacteria and fungi associated with tarballs from Betul beach, Goa, India. Mar Pollut Bull 128:593–600. https://doi.org/10.1016/j.marpolbul.2018.01.064

Snellman EA, Collins RP, Cooke JC (1988) Utilization of fuel oils by fungi isolated from oceanic tarballs. Lett Appl Microbiol 6:105–107

Suneel V, Vethamony P, Naik BG, Krishna MS, Jadhav L (2015) Identifying the source of tar balls deposited along the beaches of Goa in 2013 and comparing with historical data collected along the West Coast of India. Sci Total Environ 527–528:313–321. https://doi.org/10.1016/j.scitotenv.2015.04.102

Tao Z, Bullard S, Arias C (2011) High numbers of *Vibrio vulnificus* in tarballs collected from oiled areas of the north-Central Gulf of Mexico following the 2010 BP Deep water horizon oil spill. EcoHealth 8:507–511. https://doi.org/10.1007/s10393-011-0720-z

Varjani SJ, Rana DP, Bateja S, Upasani VN (2013) Isolation and screening for hydrocarbon utilizing bacteria (HUB) from petroleum samples. Int J Curr Microbiol Appl Sci 2:48–60

Wang Z, Fingas M, Landriault M, Sigouin L, Castle B, Hostetter D, Zhang D, Spencer B (1998) Identification and linkage of tar balls from the coast of Vancouver Island and Northern California using GC/MS and Isotopic techniques. J High Resolut Chromatogr 21:383–395

Wang ZD, Fingas M, Page D (1999) Oil spill identification. J Chromatogr 843:369–411

Warnock AM, Hagen SC, Passeri DL (2015) Marine tar residues: a review. Water Air Soil Pollut 226:68. https://doi.org/10.1007/s11270-015-2298-5

Xue J, Yu Y, Bai Y, Wang L, Wu Y (2015) Marine oil-degrading microorganisms and biodegradation process of petroleum hydrocarbon in marine environments: a review. Curr Microbiol 71:220–228. https://doi.org/10.1007/s00284-015-0825-7

Yakimov MM, Timmis KN, Golyshin PN (2007) Obligate oil-degrading marine bacteria. Curr Opin Biotechnol 18:257–266. https://doi.org/10.1016/j.copbio.2007.04.006

Zhang Z, Houb Z, Yang C, Ma C, Tao F, Xu P (2011) Degradation of *n*-alkanes and polycyclic aromatic hydrocarbons in petroleum by a newly isolated *Pseudomonas aeruginosa* DQ8. Bioresour Technol 102:4111–4116. https://doi.org/10.1016/j.biortech.2010.12.064

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Review

Diversity of bacteria and fungi associated with tarballs: Recent developments and future prospects

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ABSTRACT

Tarballs are formed by weathering of crude oil in marine environment. They are transported from open ocean to the shores by sea currents and waves. Tarball pollution is a major concern to global marine ecosystem. Microbes such as bacteria and fungi are known to be associated with tarballs. They presumably play an important role in tarball degradation and some are potential human and animal pathogens. This paper highlights the recent studies on tarball-associated bacteria and fungi. Future perspectives on diversity, ecology and possible applications of tarball-associated microbes in bioremediation of beached tarballs have been discussed.

Contents

1. Formation of tarballs in marine environment

Fossil fuels are precious, non-renewable energy sources. There has been a steady growth in the consumption of petroleum and its products all over the world (US Energy Information Administration, 2016). As most of oil transport is done *via* sea, occasional accidental spillages result in the introduction of crude oil into marine environment. However, crude oil can also enter marine environment through release of ballast water from ships, operational discharges during offshore drilling, pipeline ruptures and natural seepage from the seabed (Harayama et al., 1999; Das and Chandran, 2011). It is estimated that the oceans receive 0.47–8.40 million tonnes of oil (petroleum hydrocarbons) annually (Global Marine Oil Pollution Information Gateway, http://oils.gpa.unep.org/facts/quantities.htm) and some of it ends up on the shores as tarballs.

Tarballs are formed when the crude oil released into marine environment by anthropogenic or natural activities changes over time due to weathering (Chandru et al., 2008). The weathering involves various physical, chemical and biological processes. The physical processes include spreading, evaporation, dispersion, dissolution, sedimentation and emulsification. The chemical and biological processes include photo-oxidation or photo-degradation and biodegradation (Harayama

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et al., 1999; Wang et al., 2011), respectively. Though the exact mechanisms of tarball formation are poorly understood, the weathering processes are presumed to occur simultaneously. The weathering is believed to depend on environmental conditions (such as temperature, wind velocity, ocean current dynamics, and dissolved oxygen concentration), type and source of oil, and time and amount of oil spillage (Jordan and Payne, 1980). Chandru et al. (2008) provided a detailed explanation on tarball formation.

Tarballs have solid or semi-solid consistency. Their size varies from a few millimeter to several centimeters, while the color ranges from black to brown (Fig. 1). Their texture can be brittle, hard or soft and very sticky, depending on their formation conditions (Chandru et al., 2008; Wang et al., 2011). Some tarballs have a uniform structure throughout, while others may be solid externally and liquid internally (Goodman, 2003). Recently, Warnock et al. (2015) have reviewed the research conducted on marine tars since 1970s, including formation, distribution, transportation, chemical composition, source tracking and degradation of tarballs. The geographical locations of tarball pollution at the global level are presented in Fig. 2.

Tarballs chemically differ from their parent crude oil, depending on the formation conditions (Wang et al., 1998). For example, if a weathering process became dominant, it could influence the composition of resulting tarballs (Chandru et al., 2008). During weathering, if oxygen from the atmosphere gets mixed with hydrocarbons present in the crude oil, it can form oxygenated hydrocarbons ("oxyhydrocarbons"). Oxyhydrocarbons are known to be recalcitrant in nature (Kimes et al., 2014). It has been reported that heavy hydrocarbons such as polycyclic aromatic hydrocarbons (PAHs) are enriched in tarballs after lighter hydrocarbon fractions dissolve/evaporate during weathering (Harayama et al., 1999; Nemirovskaya, 2011). Meta-toluic acid, a toxic compound present in tarballs, is known to persist in marine environment (Prakash et al., 2008).

Tarballs have high metal concentration than the parent crude oil. It is quite possible that high molecular compounds present in weathering crude oil act as chelating groups on which metal ions bind from sea water (Liu et al., 2012). Metals such as vanadium, nickel, copper, iron, magnesium and cobalt have been reported from tarballs (Wong et al., 1976; Hegazi, 2009; Zare-maivan, 2010; Liu et al., 2012) but their concentration varies across different reports. Metals present in tarballs can facilitate transformation of hydrocarbons through oxidation, resulting in environmentally persistent free radicals (Kimes et al., 2014). It has been suggested that increased concentration of metals enhances the toxicity of hydrocarbons and affects microbial activity (Shukla and Cameotra, 2012).

2. Consequences of tarball pollution

The adverse effects of tarballs on environment are poorly understood as compared to pollution caused by fresh oil spillage. Available studies suggest that weathered oil is less toxic than the crude oil (Warnock et al., 2015). Newly formed tarballs are considered to be more toxic than older or highly weathered one's, because the former contains abundant water soluble and low molecular weight compounds (hydrocarbons) which are considered to be more toxic in nature (Harayama et al., 1999). Tarball pollution can result in economic and ecological losses. For example, Goa state located on the West Coast of India is a global tourist destination. Tarballs deposited on Goan beaches are likely to negatively affect the coastal ecology and local economy (Rekadwad and Khobragade, 2015). Sea water that gets contaminated with tarballs has petroleum-like odor, making it less-suitable for swimmers. Tarballs pose a high risk to human health as some individuals can be allergic to tarballs (http://www.emaxhealth.com/1275/bp-oil-spill-dangerous-chilrens-lungs). Tarballs that travel towards the coast can stick to the fishing nets installed in the sea by fishermen, making them difficult to clean.

Crude oil contains chemically diverse hydrocarbons, including PAHs. Tarballs, as residual crude oil, are expected to contain abundant carcinogenic PAHs, which may affect humans and marine organisms on long-term exposure (Shukla and Cameotra, 2012). Tarball surface serves as a substrate for bacteria, fungi, unicellular algae and other microbes (Nair et al., 1972; Tao et al., 2011). Tao et al. (2011) analyzed tarballs for total aerobic bacterial counts and for the presence of *Vibrio vulnificus*, a human pathogen. It was observed that total bacterial counts were higher in tarballs as compared to those in sand and sea water. The presence of *V. vulnificus* was 10 and 100 times higher in tarballs as compared to sand and sea water, respectively. It is not clear why tarball surface supports high concentration of microbes. It is believed that microbes feed on the by-products formed by hydrocarbon degrading bacteria present in/on tarballs (Tao et al., 2011).

Animals and plants may die because of exposure to fumes released by tarballs. Sea animals such as turtles can accidentally consume tarballs as food, leading to their death (Goodman, 2003; Warnock et al., 2015). Horn et al. (1970) reported tar residues from stomach of an epipelagic fish, *Scomberesox saurus* and stated that the toxic components of tarballs can easily enter into food chain. Tarballs become heavier after being mixed with sand and sink to the bottom of sea, disturbing living conditions of benthic organisms, including nursing grounds of fish and shellfish (http://oils.gpa.unep.org/facts/habitats.htm). The toxic contents of less-weathered tarballs can be lethal to marine organisms such as bivalves, shrimps, which serve as food for pelagic fishes, leading to decrease in food to the next level animals in the food chain, and thus indirectly affecting fisheries (http://timesofindia.indiatimes.com/india/Tar-balls-hit-beaches-fauna-and-Goa-tourism/articleshow/37675282.cms). Parish government reported more than 12 m land loss of salt marshes by suffocation caused by tar-like clumps (Bruckner, 2011). In addition, the cleaning drives of tarballs using heavy equipment can disturb marine niches.

3. Microbial diversity associated with tarballs

Table 1 presents a list of bacterial and fungal taxa reported till date from tarballs. In early 1970s, microbes such as blue-green algae,

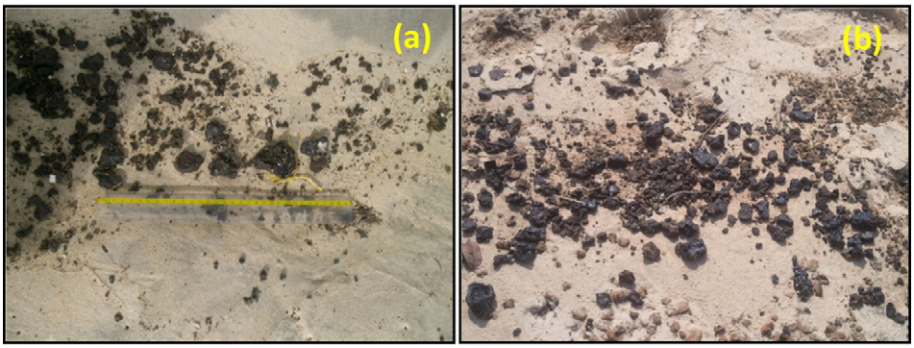

Fig. 1. Tarball deposits on beaches of Goa, India, a: Benaulim beach, b: Candolim beach.

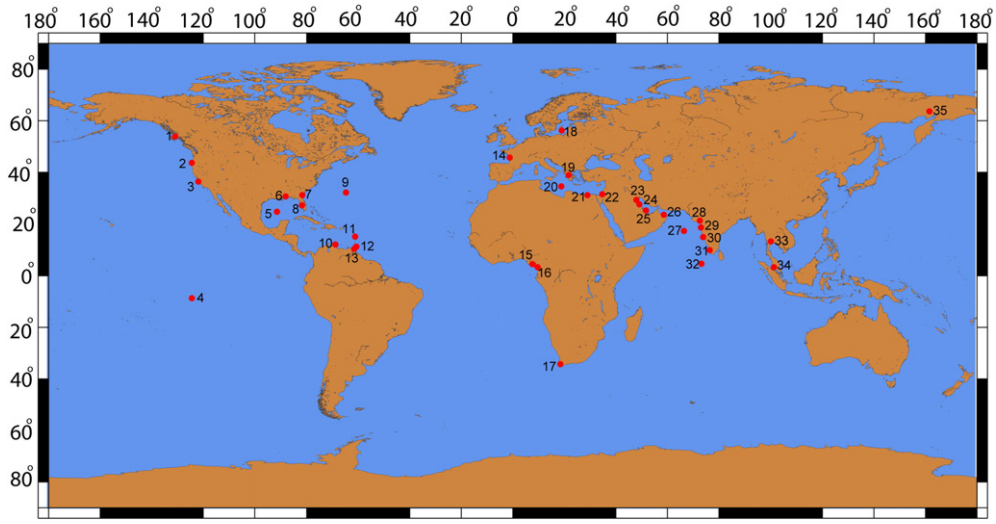

Fig. 2. Geographical locations of tarball pollution at the global level: 1. British Columbia; 2. Oregon; 3. California; 4. Pacific Ocean; 5. Gulf of Mexico; 6. Alabama 7. Georgia; 8. Florida; 9. Bermuda; 10. Curacao; 11. Eastern Caribbean; 12. Tobago; 13. Trinidad; 14. France; 15. South-East Nigeria; 16. Cameroon; 17. Cape of Good Hope; 18. Baltic Sea beaches; 19. Greece; 20. Meditterrian Sea; 21. Alexandria; 22. Israel; 23. Kuwait; 24. Saudi Arabia; 25. Qatar; 26. Muscat; 27. Arabian Sea; 28. Gujarat; 29. Mumbai; 30. Goa; 31. Kochi; 32. Maldives; 33. Thailand; 34. Malaysia; 35. Alaska (Adopted from Suneel, 2014).

diatoms, yeast, protists, and other forms of life such as barnacles, cirripede were reported from tar-like material from India (Nair et al., 1972). In the recent past, Itah and Essien (2005) reported heterotrophic bacteria associated with tarballs from Ibeno beach of Nigerian coast to be in the range of 3.0×10^4–3.2×10^6 CFU/g of tarball. They suggested that only 0.001% out of total bacterial count obtained could degrade oil. Tao et al. (2011) reported heterotrophic bacteria count of 5.1×10^6 – 8.3×10^6 CFU/g of tarball from the Gulf of Mexico. They observed higher number of human pathogen, *V. vulnificus* in tarballs than in sea water and sand.

Petra et al. (2012) investigated oil degrading bacterial community from previously contaminated sea water of the Gulf St. Vincent (Southern Australia (SA)) and weathered oil (=tarballs) from refinery of SA, Australia. They reported 6.6×10^1 CFU/ml total hydrocarbon degrading population from sea water and 4.1×10^4 CFU/ml from weathered crude oil. In a study involving oil mousses (the predecessor of tarballs), Liu and Liu (2013) conducted meta-genomic analysis to study the bacterial community structure. They reported 54–86 oil-mousse associated bacterial genera representing five phyla from three different locations. The majority (95–99%) of the bacterial taxa belong to the phylum *Proteobacteria*.

In a study unraveling the diversity of tarball associated microorganisms using metagenomics approach, Bacosa et al. (2016) analyzed 44 tarball samples of Galveston and Mustang Island of Texas, Unites States. *Alcanivorax* and *Psychrobacter* were found to be dominant genera in tarballs of Galveston Island. These two genera are known for their oil degradation abilities (Harayama et al., 2004; Giudice et al., 2010). *Pseudoaltermonas* was dominant in tarballs of Mustang Island followed by *Psychrobacter*, *Oceanospirillales* and *Alcanivorax*. The tarballs from Galveston Island were found to be rich in alkane-degrading bacteria, while those from Mustang Island were rich in PAH-degrading bacteria. The depletion of alkanes in tarballs of Galveston and Mustang Island was 21% and 24%, respectively, while percent depletion of PAH was 55% in Galveston Island and it increased to 63% when travelled to Mustang Island.

Recently, Nkem et al. (2016) isolated and reported two bacteria, *Cellulosimicrobium cellulans* and *Acinetobacter baumannii* using enrichment technique from tarballs of Rhu Sepuluh beach, Terengganu, Malaysia; which could utilize 64.4% and 58.1% of diesel, respectively. In an interesting recent study, Sanyal et al. (2016) isolated fungi from tarballs of Candolim beach in Goa, India and sequenced the fungal barcode region (ITS). Based on the ITS-phylogeny, they reported 5 fungal clades associated with tarballs. This was the first study which employed the DNA barcode region to characterize fungal diversity associated with tarballs. For a detailed review of fungal diversity associated with tarballs, please refer to Sanyal et al. (2016).

4. Microbial degradation of tarballs

Indigenous microbes play important roles in crude oil biodegradation in marine environment. Generally, they are present in low numbers. However, their population size increases in oil-spilled areas. Twenty-five marine bacterial genera are known to play active roles in oil degradation (Kimes et al., 2014). It is suggested that a wide array of chemical compounds present in crude oil enables microbes to opt for resource partitioning. This results in temporal succession of microbial communities in surrounding ecosystem (McGenity et al., 2012). Environmental factors such as temperature, available nutrients, salinity, dissolved oxygen concentration and physical state of crude oil are known to affect biodegradation of crude oil. High temperature favors the growth and degradation activity of microbes with added benefit of lower viscous oil. In case of tarballs, low surface area to volume ratio of tarballs limits their microbial degradation (Salleh et al., 2003). It is suggested that tarballs and oil mousses are less viscous at higher temperatures and have greater surface area available for physical breakdown and microbial degradation within sediments (Beazley et al., 2012).

There have been limited studies on microbial degradation of tarballs. Thraustochytrid protists isolated from coastal waters, sediments, crude oil and tarballs collected from Goa, India, were studied by Raikar et al. (2001) for their ability to degrade tarballs. The growth of thraustochytrids was found to be very high in OZG (Oppenheimr-Zobell-Gaertner) and MV (Modified Vishniac's) media containing crude oil than the basal salt medium and basal mineral salt medium. Addition of tarball to peptone broth was found to significantly improve the growth of thraustochytrids. The authors claimed that the thraustochytrids isolated from coastal waters could degrade about 71% of tarballs in a month. Though numerous reports on hydrocarbon-degrading microbes are available, two reports are mentioned here. Prakash et al. (2008) reported *Pseudomonas* sp. strain GUI13 capable of degradation of meta-toluic acid, a major component of tarballs. Rodrigues et al. (2010) reported a marine bacterium of genus *Alkaligenes* capable of degradation of dibenzothiophene, sulfur containing polycyclic aromatic hydrocarbons of tarballs.

Table 1
Taxonomy of bacteria and fungi reported till date from tarballs.

Authors	Sampling location	Taxon/clade	Lineage (as per NCBI)
Nair et al. (1972)	Ratnagiri beach, Maharashtra, India	*Rhodotorula* sp.	Fungi; Basidiomycota; Sporidiobolales; Sporidiobolaceae
	Calangute beach, Goa, India	*Rhodotorula* sp.	Fungi; Basidiomycota; Sporidiobolales; Sporidiobolaceae
		Unidentified fungi	Fungi
Nair and Lokabharathi (1977)	Undesignated beach, Goa, India	*Fusarium* sp.	Fungi; Ascomycota; Hypocreales; Nectriaceae
Snellman et al. (1988)	North Atlantic and the North Pacific Oceans	*Acremonium* sp.	Fungi; Ascomycota
		Acremonium kiliense (=*Sarocladium kiliense*)	Fungi; Ascomycota; Hypocreales; Hypocreales *incertae sedis*
		Acremonium strictum (=*Sarocladium strictum*)	Fungi; Ascomycota; Hypocreales; Hypocreales *incertae sedis*
		Alternaria sp.	Fungi; Ascomycota; Pleosporales; Pleosporaceae
		Aspergillus sp.	Fungi; Ascomycota; Eurotiales; Aspergillaceae
		Aspergillus chevalieri	Fungi; Ascomycota; Eurotiales; Aspergillaceae
		Aspergillus ficuum	Fungi; Ascomycota; Eurotiales; Aspergillaceae
		Aspergillus fumigatus	Fungi; Ascomycota; Eurotiales; Aspergillaceae
		Aspergillus heteromorphus	Fungi; Ascomycota; Eurotiales; Aspergillaceae
		Aspergillus niger	Fungi; Ascomycota; Eurotiales; Aspergillaceae
		Aspergillus repens (=*Aspergillus pseudoglaucus*)	Fungi; Ascomycota; Eurotiales; Aspergillaceae
		Aspergillus ruber	Fungi; Ascomycota; Eurotiales; Aspergillaceae
		Aspergillus sydowii	Fungi; Ascomycota; Eurotiales; Aspergillaceae
		Aspergillus terreus	Fungi; Ascomycota; Eurotiales; Aspergillaceae
		Aspergillus ustus	Fungi; Ascomycota; Eurotiales; Aspergillaceae
		Aspergillus versicolor	Fungi; Ascomycota; Eurotiales; Aspergillaceae
		Beauveria sp.	Fungi; Ascomycota; Hypocreales; Cordycipitaceae
		Cercosporidium sp.	Fungi; Ascomycota; Capnodiales; Mycosphaerellaceae
		Cladosporium cladosporioides	Fungi; Ascomycota; Capnodiales; Cladosporiaceae
		Cladosporium resinae (=*Amorphothe caresinae*)	Fungi; Ascomycota; Capnodiales; Cladosporiaceae
		Epicoccum purpurascens (=*Epicoccum nigrum*)	Fungi; Ascomycota; Pleosporales; Didymellaceae
		Graphium sp.	Fungi; Ascomycota
		Humicola fuscoatra	Fungi; Ascomycota; Sordariales; Chaetomiaceae
		Penicillium sp. 1	Fungi; Ascomycota; Eurotiales; Aspergillaceae
		Penicillium chrysogenum	Fungi; Ascomycota; Eurotiales; Aspergillaceae
		Penicillium citrinum	Fungi; Ascomycota; Eurotiales; Aspergillaceae
		Penicillium javanicum	Fungi; Ascomycota; Eurotiales; Aspergillaceae
		Penicillium herquei	Fungi; Ascomycota; Eurotiales; Aspergillaceae
		Penicillium oxalicum	Fungi; Ascomycota; Eurotiales; Aspergillaceae
		Scopulariopsis brevicaulis	Fungi; Ascomycota; Microascales; Microascaceae
		Tetracoccosporium sp.	Fungi; Ascomycota; Pezizomycotina incertae sedis
		Chaetomium sp.	Fungi; Ascomycota; Sordariales; Chaetomiaceae
		Chaetomium globosum	Fungi; Ascomycota; Sordariales; Chaetomiaceae
		Chaetomium murorum (=*Botryotrichum murorum*)	Fungi; Ascomycota; Sordariales; Chaetomiaceae
		Chaetomium spirale	Fungi; Ascomycota; Sordariales; Chaetomiaceae
		Lophotrichus ampullus	Fungi; Ascomycota; Microascales; Microascaceae
		Microascus cinereus	Fungi; Ascomycota; Microascales; Microascaceae
		Sordaria sp.	Fungi; Ascomycota; Sordariales; Sordariaceae
		Sordaria fimicola	Fungi; Ascomycota; Sordariales; Sordariaceae
		Cunninghamella elegans	Fungi; Mucoromycota; Mucorales; Cunninghamellaceae
		Mucor sp.	Fungi; Mucoromycota; Mucorales; Mucoraceae
		Syncephalastrum racemosum	Fungi; Mucoromycota; Mucorales; Syncephalastraceae
Itah and Essien (2005)	Ibeno beach, the Bight of Bonny, Nigeria	*Vibrio parahaemolyticus*	Bacteria; Proteobacteria; Vibrionales; Vibrionaceae
		Pseudomonas aeruginosa	Bacteria; Proteobacteria; Pseudomonadales; Pseudomonadaceae
		Corynebacterium glutamicum	Bacteria; Terrabacteria group; Corynebacteriales; Corynebacteriaceae
		Micrococcus varians (=*Kocuria varians*)	Bacteria; Terrabacteria group; Micrococcales; Micrococcaceae
		Escherichia coli	Bacteria; Proteobacteria; Enterobacterales; Enterobacteriaceae
		Cladosporium resinae (=*Amorphotheca resinae*)	Fungi; Ascomycota; Capnodiales; Cladosporiaceae
		Saccharomyces '*estuari*'	Fungi; Ascomycota; Saccharomycetales; Saccharomycetaceae
		Chromobacterium violaceum	Bacteria; Proteobacteria; Neisseriales; Chromobacteriaceae
		Nocardia marina	Bacteria; Terrabacteria group; Corynebacteriales; Nocardiaceae
		Penicillium citrinum	Fungi; Ascomycota; Eurotiales; Aspergillaceae
		Candida marina (=*Bandonia marina*)	Fungi; Basidiomycota; Trichosporonales; Tetragoniomycetaceae
		Cryptococcus albidus (=*Naganishia albida*)	Fungi; Basidiomycota; Filobasidiales; Filobasidiaceae
		Bacillus '*submarinus*'	Bacteria; Terrabacteria group; Bacillales; Bacillaceae
Elshafie et al.	Beaches of Oman	*Aspergillus niger*	Fungi; Ascomycota; Eurotiales; Aspergillaceae

(continued on next page)

Table 1 (*continued*)

Authors	Sampling location	Taxon/clade	Lineage (as per NCBI)
(2007)		*Aspergillus terreus*	Fungi; Ascomycota; Eurotiales; Aspergillaceae
		Penicillium chrysogenum	Fungi; Ascomycota; Eurotiales; Aspergillaceae
		Aspergillus nidulans	Fungi;Ascomycota; Eurotiales; Aspergillaceae
		Cladosporium cladosporoides	Fungi; Ascomycota; Capnodiales; Cladosporiaceae
		Rhizopus stolonifer	Fungi; Mucoromycota; Mucorales; Rhizopodaceae
		Penicillium sp. 2	Fungi; Ascomycota; Eurotiales; Aspergillaceae
		Paecilomyces sp.	Fungi; Ascomycota; Eurotiales; Thermoascaceae
		Aspergillus ochraceus	Fungi; Ascomycota; Eurotiales; Aspergillaceae
		Alternaria alternata	Fungi; Ascomycota; Pleosporales; Pleosporaceae
Tao et al. (2011)	Beaches of Alabama and Mississippi, USA	*Vibrio vulnificus*	Bacteria; Proteobacteria; Vibrionales; Vibrionaceae
Lotfinasabasl et al. (2012)	Akshi coastal area, Maharashtra, India	*Aspergillus terreus*	Fungi; Ascomycota; Eurotiales; Aspergillaceae
		Rhizopus sp.	Fungi; Mucoromycota; Mucorales; Rhizopodaceae
Petra et al. (2012)	The weathered crude oil from Australia	*Rhodobacteraceae* clade	Bacteria; Proteobacteria; Rhodobacterales
		Pannonibacter phragmitetus clade	Bacteria; Proteobacteria; Rhodobacterales; Rhodobacteraceae
		Thalassospira clade	Bacteria; Proteobacteria; Rhodospirillales; Rhodospirillaceae
		Pseudomonas clade	Bacteria; Proteobacteria; Pseudomonadales; Pseudomonadaceae
		Xanthobacter autotrophicus clade	Bacteria; Proteobacteria; Xanthobacteraceae; Xanthobacter
Liu and Liu (2013)	Sea surface oil mousses from the northern Gulf of Mexico	*Alphaproteobacteria* clade	Bacteria; Proteobacteria
		Gammaproteobacteria clade	Bacteria; Proteobacteria
		Other *Proteobacteria* clade	Bacteria; Proteobacteria
		Flavobacteriales clade	Bacteria; FCB group; Bacteroidetes/Chlorobi group; Bacteroidetes; Flavobacteriia
		Planctomycetes clade	Bacteria; PVC group
		Bacteroidetes clade	Bacteria; FCB group; Bacteroidetes/Chlorobi group
		Firmicutes clade	Bacteria; Terrabacteria group
		Actinobacteria clade	Bacteria; Terrabacteria group
		Chloroflexi clade	Bacteria; Terrabacteria group
		Cyanobacteria clade	Bacteria; Terrabacteria group; Cyanobacteria/Melainabacteria group
Bacosa et al. (2016)	Galveston Island, USA	*Alcanivorax* clade	Bacteria; Proteobacteria; Oceanospirillales; Alcanivoracaceae
		Psychrobacter clade	Bacteria; Proteobacteria; Pseudomonadales; Moraxellaceae
		Other bacteria	Bacteria
	Mustang Island, USA	*Pseudoalteromonas* clade	Bacteria; Proteobacteria; Alteromonadales; Pseudoalteromonadaceae
		Psychrobacter clade	Bacteria; Proteobacteria; Pseudomonadales; Moraxellaceae
		Oceanospirillales clade	Bacteria; Proteobacteria; Oceanospirillales
		Alcanivorax	Bacteria; Proteobacteria; Oceanospirillales; Alcanivoracaceae
		Other bacteria	Bacteria
Nkem et al. (2016)	Rhu Sepuluh beach, Terengganu, Malaysia	*Cellulosimicrobium cellulans*	Bacteria; Terrabacteria group; Micrococcales; Promicromonosporaceae
		Acinetobacter baumannii	Bacteria; Proteobacteria; Pseudomonadales; Moraxellaceae
Sanyal et al. (2016)	Candolim beach, Goa, India	*Curvularia/Biploaris* clade	Fungi; Ascomycota; Pleosporales; Pleosporaceae
		Alternaria clade	Fungi; Ascomycota; Pleosporales; Pleosporaceae
		Cladosporium clade	Fungi; Ascomycota; Capnodiales; Cladosporiaceae
		Candida clade	Fungi; Ascomycota; Saccharomycetales; Debaryomycetaceae
		Issatchenkia clade	Fungi; Ascomycota; Saccharomycetales; Saccharomycetaceae

5. Future perspectives

The beaches all over the world are frequently exposed to tarballs. Over the years, sea microflora have apparently developed abilities to utilize and degrade hydrocarbons present in crude oil. So, it is not unusual to find certain species of microorganisms to be high in number on substrates such as crude oil, tarballs. Some authors have expressed possible health risks from tarballs (Tao et al., 2011), while some studies recommend the use of indigenous microbial populations of tarballs for its bioremediation. Petra et al. (2012) have suggested that indigenous micro-flora of weathered oil are a great source of hydrocarbonoclastic microorganisms. In India, the 'oil zapper' has been used to remediate contaminated soils of oil refineries dumping oil sludge. It is a consortium of bacteria, including *Acinetobacter bauminii*, *Alcaligenes odorans*, "*Bukhardica cepacer*", *Pseudomonas aeruginosa* and strain s-30 (Gupta et al., 2011). Development of such microbial consortia to degrade beached tarballs, using both indigenous and non-indigenous microbes, is subject to future studies.

Not all microorganisms from environmental samples are culturable in laboratory conditions. In case of tarballs, their insoluble nature and ball-like structure limit all bacterial cells to get into the culturing medium. Therefore there is less assurance of growth of all cultivable bacteria on the nutrient medium during isolation procedures. To detach these bacteria from oily tars and recover maximum CFUs, one can use different techniques, such as breaking tarballs into smaller pieces, vortexing, mild sonication and use of biosurfactants. To find out the true microbial diversity associated with tarballs, one can choose to do metagenomics studies of tarballs. However it is not a preferred method in case of *in situ* bioremediation. Tarballs can be a source of microbial consortia for bioremediation of oil contaminated environment, which can only be possible by culture dependent methods. As there are limited studies on diversity of tarball associated microorganisms, both culture-based and culture independent methods will help in determining the microbial diversity associated with tarballs.

The current studies are restricted to pelagic tarballs. There are few studies on benthic tars. It is desirable that microbial diversity associated

with benthic tars are investigated by culture-based and culture independent methods. The high concentration of metals in tarballs can be toxic not only to microorganisms which degrade them but also to other marine animals which are in proximity. Further research is required on this topic. Our web portal, www.tarballs.in (Shinde and Shenoy, 2016) aims to assist researchers on biology of microbes associated with tarballs and their ability to degrade tarballs.

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References

Bacosa, H.P., Thyng, K.M., Plunkett, S., Erdner, D.L., Liu, Z., 2016. The tarballs on Texas beaches following the 2014 Texas City "Y" spill: modeling, chemical, and microbiological studies. Mar. Pollut. Bull. 109, 236–244.

Beazley, M.J., Martinez, R.J., Rajan, S., Powell, J., Piceno, Y.M., Tom, L.M., Andersen, G.L., Hazen, T.C., Nostrand, J.V., Zhou, J., Mortazavi, B., Sobecky, P.A., 2012. Microbial community analysis of a coastal salt marsh affected by the Deep water Horizon oil spill. PLoS One 7, e41305.

Bruckner, M., 2011. Comparison of *in-situ* bioattenuation, biostimulation, and bioaugmentation and their application to marine oil spills. EM 20–23.

Chandru, K., Zakaria, M.P., Anita, S., Shahbazi, A., Sakaria, M., Bahry, P.S., Mohamed, C.A.R., 2008. Characterization of alkanes, hopanes, and polycyclic aromatic hydrocarbons (PAHs) in tarballs collected from the East Coast of Peninsular Malaysia. Mar. Pollut. Bull. 56, 950–962.

Das, N., Chandran, P., 2011. Microbial degradation of petroleum hydrocarbon contaminants: an overview. Biotechnol. Res. Int. Article ID 941810, 13 pages. 10.4061/2011/941810.

Elshafie, A., AlKindi, A.Y., Al-Busaidi, S., Bakheit, C., Albahry, S.N., 2007. Biodegradation of crude oil and *n*-alkanes by fungi isolated from Oman. Mar. Pollut. Bull. 54, 1692–1696.

Giudice, A.L., Casella, P., Caruso, C., Mangano, S., Bruni, V., De Domenico, M., Michaud, L., 2010. Occurrence and characterization of psychrotolerant hydrocarbon-oxidizing bacteria from surface seawater along the Victoria Land coast (Antarctica). Polar Biol. 33, 929–943.

Goodman, R., 2003. Tarballs: the end state. Spill Sci. Technol. Bull. 8, 117–121.

Gupta, S., Rao, P.V.C., Lal, B., Choudary, V.N., 2011. Process and composition for bioremediation of oily sludge. Patent Publication Number WO2011074007 A2 Accessed from. https://www.google.com/patents/WO2011074007A2?cl=en.

Harayama, S., Kishira, H., Kasai, Y., Shutsubo, K., 1999. Petroleum biodegradation in marine environments. J. Mol. Microbiol. Biotechnol. 1, 63–70.

Harayama, S., Kasai, Y., Hara, A., 2004. Microbial communities in oil-contaminated seawater. Curr. Opin. Biotechnol. 15, 205–214.

Hegazi, A.H., 2009. Utilization of trace metals in source diagnosing of tarballs from the Mediterranean city of Alexandria, Egypt. Pet. Sci. Technol. 27, 386–395.

Horn, M.H., Teal, J.M., Backus, R.H., 1970. Petroleum lumps on surface of sea. Science 168, 245–246.

Itah, A.Y., Essien, J.P., 2005. Growth profile and hydrocarbonoclastic potential of microorganisms isolated from tarballs in the Bight of Bonny, Nigeria. World J. Microbiol. Biotechnol. 21, 1317–1322.

Jordan, R.E., Payne, J.R., 1980. Fate and weathering of petroleum spills in the marine environment: a literature review and synopsis. Mar. Technol. Soc. J. 18, 24–42.

Kimes, N.E., Callaghan, A.V., Suflita, J.M., Morris, P.J., 2014. Microbial transformation of the Deepwater Horizon oil spill - past, present, and future perspectives. Front. Microbiol. 5:603. http://dx.doi.org/10.3389/fmicb.2014.00603.

Liu, Z., Liu, J., 2013. Evaluating bacterial community structures in oil collected from the sea surface and sediment in the northern Gulf of Mexico after the Deepwater Horizon oil spill. Microbiol. Open 2, 492–504.

Liu, Z., Liu, J., Zhu, Q., Wu, W., 2012. The weathering of oil after the Deepwater Horizon oil spill: insights from the chemical composition of the oil from the sea surface, salt marshes andsediments. Environ. Res. Lett. 7:035302. http://dx.doi.org/10.1088/1748-9326/7/3/035302.

Lotfinasabasl, S., Gunale, V.R., Rajurkar, N.S., 2012. Assessment of petroleum hydrocarbon degradation from soil and tarball by fungi. Biosci. Discov. 3, 186–192.

McGenity, T.J., Folwell, B.D., McKew, B.A., Sanni, G.O., 2012. Marine crude-oil biodegradation: a central role for interspecies interactions. Aquat. Biosyst. 8, 10.

Nair, S., Lokabharathi, P.A., 1977. Degradation of hydrocarbons by fungus, *Fusarium* sp. Ind. J. Mar. Sci. 6, 173–175.

Nair, S.A., Devassy, V.P., Dwivedi, S.N., Selvakumar, R.A., 1972. Preliminary observations on tarlike material observed on some beaches. Curr. Mar. Sci. 41, 766–767.

Nemirovskaya, I.A., 2011. Tarballs in Baltic Sea beaches. Water Res. 38, 315–323.

Nkem, B.M., Halimoon, N., Yusoff, F.M., Johari, W.L.W., Zakaria, M.P., Reddy, S., Kannan, N., 2016. Isolation, identification and diesel-oil biodegradation capacities of indigenous hydrocarbon-degrading strains of *Cellulosimicrobium cellulans* and *Acinetobacter baumannii* from tarball at Terengganu beach, Malaysia. Mar. Pollut. Bull. http://dx.doi.org/10.1016/j.marpolbul.2016.03.060.

Petra, S., Simons, K.L., Kadali, K.K., Patil, S.S., Ball, A.S., 2012. The importance of weathered crude oil as a source of hydrocarbonoclastic microorganisms in contaminated seawater. J. Microbiol. Biotechnol. 22, 1185–1192.

Prakash, D., Raushan, R.K., Sangodkar, U.M.X., 2008. Isolation and characterization of meta toluic acid degrading marine bacterium. Indian Journal of Marine Science 37, 322–325.

Raikar, M.T., Raghukumar, S., Vani, V., David, J., Chandramohan, D., 2001. Thraustochytrid protists degrade hydrocarbon. Ind. J. Mar. Sci. 30, 139–145.

Rekadwad, B.N., Khobragade, C.N., 2015. A case study on effects of oil spills and tar-ball pollution on beaches of Goa (India). Mar. Pollut. Bull. 15, 567–570.

Rodrigues, J., Madhukar, A., Kumar, N., Sangodkar, U.M.X., 2010. Isolation and characterization of a marine bacterium belonging to the genus *Alkaligenes* capable of the complete mineralization of the dibenzothiophene. Ind. J. Geo-Mar. Sci. 40, 391–397.

Salleh, A.B., Ghazali, F.M., Rahman, R., Basri, M., 2003. Bioremediation of petroleum hydrocarbon pollution. Indian J. Biotechnol. 2, 411–425.

Sanyal, O., Shinde, V.L., Meena, R.M., Damare, S., Shenoy, B.D., 2016. The ITS-based phylogeny of fungi associated with tarballs. Mar. Pollut. Bull. 113, 277–281.

Shinde, V., Shenoy, B.D., 2016. www.tarballs.in - An Information Portal for Tarball Associated Microbes (Accessed on 28th September 2016).

Shukla, A., Cameotra, S.S., 2012. Hydrocarbon pollution: effects on living organisms, remediation of contaminated environments, and effects of heavy metals co-contamination on bioremediation. In: Romero-Zerón, L. (Ed.), Introduction to Enhanced Oil Recovery (EOR) Processes and Bioremediation of Oil Contaminated Sites. Intech Publishers, http://dx.doi.org/10.5772/48014.

Snellman, E.A., Collins, R.P., Cooke, J.C., 1988. Utilization of fuel oils by fungi isolated from oceanic tarballs. Lett. Appl. Microbiol. 6, 105–107.

Suneel, V., 2014. Transports and Finger Printing of Tarballs Deposited Along the West Coast of India. Ph.D. thesis submitted to. Andhra University, Visakhapatnam, India.

Tao, Z., Bullard, S., Arias, C., 2011. High numbers of *Vibrio vulnificus* in tarballs collected from oiled areas of the North-Central Gulf of Mexico following the 2010 BP Deepwater Horizon Oil Spill. EcoHealth 8, 507–511.

US Energy Information Administration, . Short-term Energy Outlook (STEO): Independent Statistics and Analysis. Published in June 2016. Accessed on 4th July 2016 from https://www.eia.gov/forecasts/steo/pdf/steo_full.pdf.

Wang, Z., Fingas, M., Landriault, M., Sigouin, L., 1998. Identification and linkage of tarballs from the coasts of Vancouver Island and Northern California using GC/MS and isotopic techniques. J. High Resolut. Chromatogr. 21, 383–395.

Wang, C., Du, J., Gao, X., Duan, Y., Sheng, Y., 2011. Chemical characterization of naturally weathered oil residues in the sediment from Yellow River Delta, China. Mar. Pollut. Bull. 62, 2469–2475.

Warnock, A.M., Hagen, S.C., Passeri, D.L., 2015. Marine tar residues: a review. Water Air Soil Pollut. 226:68. http://dx.doi.org/10.1007/s11270-015-2298-5.

Wong, C.S., Green, D.O., Cretney, W.J., 1976. Distribution and source of tar on the Pacific Ocean. Mar. Pollut. Bull. 7, 102–106.

Zare-maivan, H., 2010. Distribution of heavy metals associated with petroleum in the Northern Persian Gulf: Bushehr and Nayband Bay area. J. Pers. Gulf (Mar. Sci.) 1, 1–5.

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The ITS-based phylogeny of fungi associated with tarballs

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ABSTRACT

Tarballs, the remnants of crude oil which change into semi-solid phase due to various weathering processes in the sea, are rich in hydrocarbons, including toxic and almost non-degradable hydrocarbons. Certain microorganisms such as fungi are known to utilize hydrocarbons present in tarballs as sole source of carbon for nutrition. Previous studies have reported 53 fungal taxa associated with tarballs. There is apparently no gene sequence-data available for the published taxa so as to verify the fungal identification using modern taxonomic tools. The objective of the present study is to isolate fungi from tarballs collected from Candolim beach in Goa, India and investigate their phylogenetic diversity based on 5.8S rRNA gene and the flanking internal transcribed spacer regions (ITS) sequence analysis. In the ITS-based NJ tree, eight tarball-associated fungal isolates clustered with 3 clades of *Dothideomycetes* and 2 clades of *Saccharomycetes*. To the best of our knowledge, this is the first study that has employed ITS-based phylogeny to characterize the fungal diversity associated with tarballs. Further studies are warranted to investigate the role of the tarball-associated fungi in degradation of recalcitrant hydrocarbons present in tarballs and the role of tarballs as carriers of human pathogenic fungi.

1. Introduction

Tarballs are the remnants of crude oil which change into semi-solid phase due to various weathering processes in the sea (Suneel et al. 2013). There have been tarball deposits on the beaches of Goa, especially during the southwest monsoon (Dhargalkar et al. 1977, Suneel et al. 2013). Tarballs are rich in hydrocarbons, including toxic and almost non-degradable hydrocarbons (Suneel et al. 2013). Certain microbes such as fungi are capable of sustaining and increasing their population on hydrocarbon-contaminated surfaces. These fungi can utilize hydrocarbons as sole source of carbon for nutrition (e.g. Snellman et al. 1988, Das and Chandran 2011, Al-Nasrawi, 2012, Jawhari, 2014). Previous studies from India and other parts of the world have so far reported 53 fungal taxa that are associated with tarballs (Table 1). It is to be highlighted here that the reported tarball-associated fungal taxa are apparently not represented by gene sequence-data. It is essential to improve our understanding of diversity and genetic capabilities of tarball-associated microbes before these microbes are tested for their suitability in bioremediation of hydrocarbon contaminated beaches. This study is therefore initiated as part of our ongoing investigations into microbial diversity associated with tarballs along the coastline of Goa, India. The objective of the present study is to collect tarball samples from one of the popular tourist beaches of Goa: Candolim, and isolate fungi from the collected tarballs, followed by characterization of their phylogenetic diversity based on ITS sequence analysis.

2. Materials and methods

2.1. Sampling

Tarball samples were collected from Candolim beach located on the northern part of Goa during the monsoon period on 23rd July 2015. They were handpicked wearing gloves and put into sterile zip-lock bags. The samples were brought to the lab and kept in refrigerator at 4 °C until further processing.

2.2. Isolation of fungi

The tarball samples were processed aseptically in a laminar airflow in the laboratory. They were kept in a petri-dish and washed twice with filtered sea-water using a sterile cotton swab to remove the excess sand adhering to their surface. The almost sand-free tarballs were surface-sterilized using 1% sodium hypochlorite and then immersed in sea-water, taken off and kept aside. A sterilized toothpick was used to prick-out a small portion of the tarball onto potato dextrose agar (PDA) (prepared with filtered sea-water) amended with Chloramphenicol (100 mg/l), followed by streaking and incubation at 28 ± 2 °C for

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Table 1
List of fungi reported from tarballs.

Sl. no.	Taxon name	Sampling location	Ref.[a]
1	*Acremonium* sp.	North Atlantic and north Pacific oceans	1
2	*Alternaria alternata*	Muscat coastline, Gulf of Oman	2
3	*Alternaria* sp.	North Atlantic and north Pacific oceans	1
4	*Amorphotheca* (=*Cladosporium*) *resinae*	North Atlantic and north Pacific oceans; Ibeno beach, near Akwa Ibom State, Nigeria	1, 3
5	*Aspergillus ficuum*	North Atlantic and north Pacific oceans	1
6	*Aspergillus fumigatus*	North Atlantic and north Pacific oceans	1
7	*Aspergillus heteromorphus*	North Atlantic and north Pacific oceans	1
8	*Aspergillus niger*	North Atlantic and north Pacific oceans; Muscat coastline, Gulf of Oman	1, 2
9	*Aspergillus ochraceus*	Muscat coastline, Gulf of Oman	2
10	*Aspergillus pseudoglaucus* (=*A. repens*)	North Atlantic and north Pacific oceans	1
11	*Aspergillus* sp.	North Atlantic and north Pacific oceans	1
12	*Aspergillus terreus*	North Atlantic and north Pacific oceans; Muscat coastline, Gulf of Oman; Akshi coastal area, Maharashtra, India	1, 2, 4
13	*Aspergillus ustus*	North Atlantic and north Pacific oceans	1
14	*Aspergillus versicolor*	North Atlantic and north Pacific oceans	1
15	*Beauvaria* sp.	North Atlantic and north Pacific oceans	1
16	*Chaetomium globosum*	North Atlantic and north Pacific oceans	1
17	*Chaetomium murorum*	North Atlantic and north Pacific oceans	1
18	*Chaetomium* sp.	North Atlantic and north Pacific oceans	1
19	*Chaetomium spirale*	North Atlantic and north Pacific oceans	1
20	*Cladosporium clasdosporoides*	North Atlantic and north Pacific oceans; Muscat coastline, Gulf of Oman	1, 2
21	*Cryptococcus albidus*	Ibeno beach, near Akwa Ibom State, Nigeria	3
22	*Cryptococcus marinus* (=*Candida marina*)	Ibeno beach, near Akwa Ibom State, Nigeria	3
23	*Cunninghamella elegans*	North Atlantic and north Pacific oceans	1
24	*Emericella (Aspergillus) nidulans*	Muscat coastline, Gulf of Oman	2
25	*Epicoccum purpurascens*	North Atlantic and north Pacific oceans	1
26	*Eurotium (Aspergillus) chevalieri*	North Atlantic and north Pacific oceans	1
27	*Eurotium rubrum* (*Aspergillus ruber*)	North Atlantic and north Pacific oceans	1
28	*Fusarium* sp.	Undesignated beach in Goa, India	5
29	*Graphium* sp.	North Atlantic and north Pacific oceans	1
30	*Humicola fuscoatra*	North Atlantic and north Pacific oceans	1
31	*Microascus cinereus*	North Atlantic and north Pacific oceans	1
32	*Mucor* sp.	North Atlantic and north Pacific oceans	1
33	*Paecilomyces* sp.	Muscat coastline, Gulf of Oman	2
34	*Passalora* (=*Cercosporidium*) sp.	North Atlantic and north Pacific oceans	1
35	*Penicillium chrysogenum*	North Atlantic and north Pacific oceans; Muscat coastline, Gulf of Oman	1, 2
36	*Penicillium citrinum*	North Atlantic and north Pacific oceans; Ibeno beach, near Akwa Ibom State, Nigeria	1, 3
37	*Penicillium herquei*	North Atlantic and north Pacific oceans	1
38	*Penicillium javanicum*	North Atlantic and north Pacific oceans	1
39	*Penicillium oxalicum*	North Atlantic and north Pacific oceans	1
40	*Penicillium* sp. 1	Muscat coastline, Gulf of Oman	2
41	*Penicillium* sp. 2	North Atlantic and north Pacific oceans	1
42	*Pseudallescheria* (=*Lophotrichus*) *ampullus*	North Atlantic and north Pacific oceans	1
43	*Rhizopus* sp.	Akshi coastal area, Maharashtra, India	4
44	*Rhizopus stolonifer*	Muscat coastline, Gulf of Oman	2
45	*Rhodotorula* sp.	Goa, India	6
46	*"Saccharomyces estuary"*	Ibeno beach, near Akwa Ibom State, Nigeria	3
47	*Sarocladium* (*Acremonium*) *kiliense*	North Atlantic and north Pacific oceans	1
48	*Sarocladium* (*Acremonium*) *strictum*	North Atlantic and north Pacific oceans	1
49	*Scopulariopsis brevicaulis*	North Atlantic and north Pacific oceans	1
50	*Sordaria fimicola*	North Atlantic and north Pacific oceans	1
51	*Sordaria* sp.	North Atlantic and north Pacific oceans	1
52	*Syncephalastrum racemosum*	North Atlantic and north Pacific oceans	1
53	*Tetracoccosporium* sp.	North Atlantic and north Pacific oceans	1

[a] Legend: 1 = Snellman et al. (1988); 2 = Elshafie et al. (2007); 3 = Itah and Essien (2004); 4 = Lotfinasabasl et al. (2012); 5 = Nair and Lokabharathi (1977); 6 = Nair et al. (1972).

48–72 h. The exuberant fungal mycelia were sub-cultured on the PDA medium and maintained at 28 ± 2 °C to allow optimal growth of fungi.

2.3. DNA extraction, PCR amplification and sequencing of the ITS region

Fungal DNA was extracted from mycelia using ZR Fungal/Bacterial DNA MiniPrep kit (Zymo Research, catalogue number D6005) following the manufacturer's protocol. The ITS region was amplified by polymerase chain reaction (PCR). The reactions were carried out in a 50 μl of volume, containing 5 μl of (10×) PCR buffer (supplemented with 15 mM $MgCl_2$) (Merck Biosciences), 38 μl of nuclease-free water, 0.5 μl of 10 mM dNTPs, 1 μl of (20 pM) forward primer ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′), 1 μl of (20 pM) reverse primer ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al. 1990), 1 μl of (3 units/μl) Taq polymerase (Chromous) and 1.5 μl (~50 ng) of DNA template. The following PCR cycling conditions were followed: initial

denaturation at 95 °C for 2 min, denaturation at 95 °C for 1 min, annealing at 52 °C for 30 s, extension at 72 °C for 1 min and final extension at 72° for 10 min. The denaturation, annealing and extensions steps were repeated 35 times (White et al. 1990). The PCR products were purified using QIAquick PCR purification kit (QIAGEN, Catalogue number 28106) following manufacturer's protocol. The sequencing of ITS gene

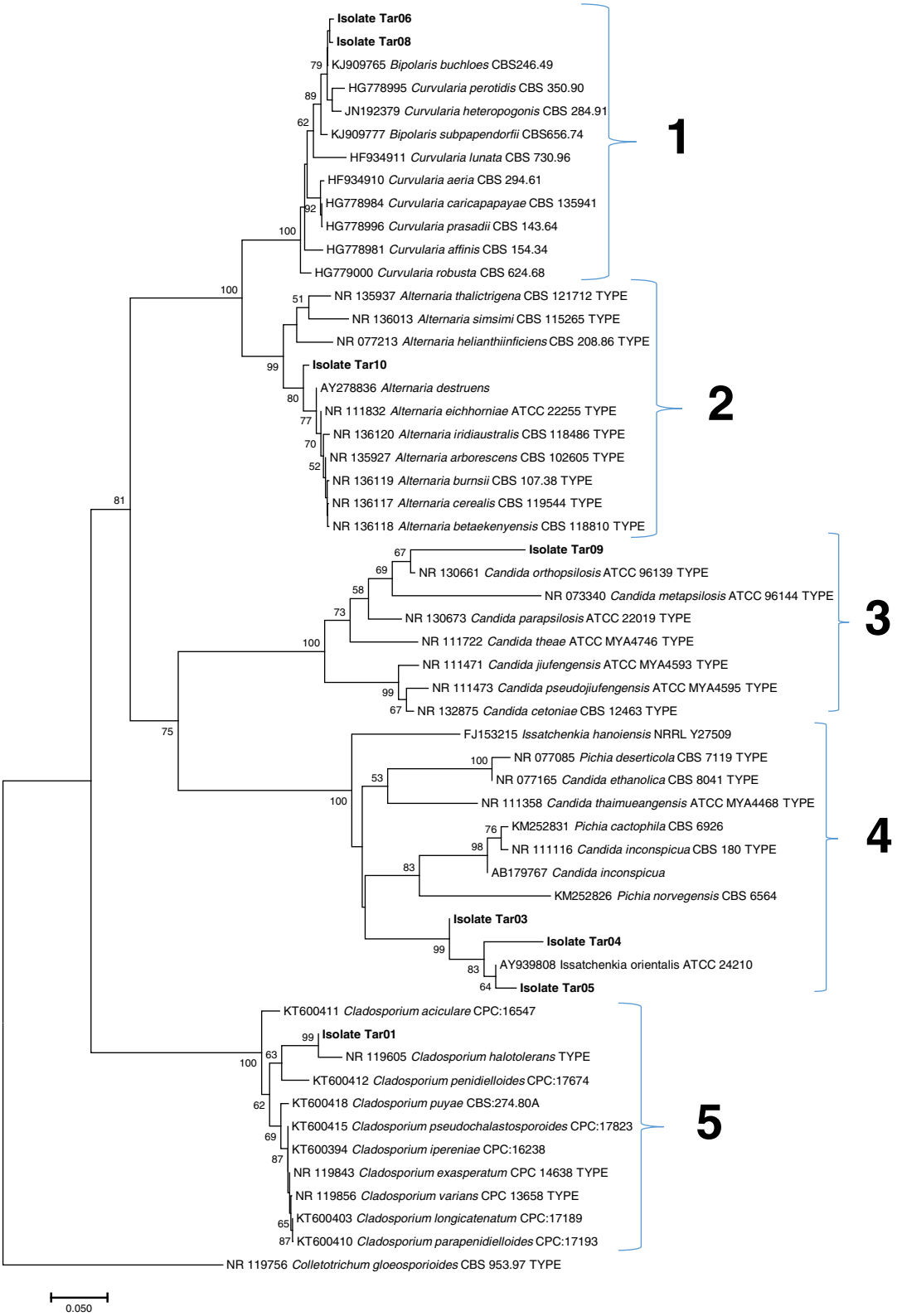

Fig. 1. Phylogenetic relationships of fungi associated with tarballs from Candolim beach, Goa, inferred from NJ analysis of the ITS sequences using MEGA. The tree is rooted with *Colletotrichum gloeosporioides* (NR_119756).

region was done using Genetic Analyzer 3130*xl* (ABI) based on the Big Dye terminator v 3.1 (Chain terminator) chemistry.

2.4. Sequence alignment and phylogenetic tree construction

The raw sequences obtained from the forward primer were checked for quality in MEGA version 7 (Kumar et al. 2015) and trimmed to obtain good quality sequences. A multiple sequence alignment was prepared in MEGA using the newly-generated ITS sequences (Fig. 1, in bold) and the reference sequences retrieved from NCBI-GenBank (Fig. 1). The evolutionary tree was inferred in MEGA using Neighbour-Joining method (Saitou and Nei 1987). The sequences generated in this study have been deposited in NCBI-GenBank (Table 2).

3. Results

The culture morphology of eight tarball-associated fungal isolates from Candolim beach is presented in Fig. 2 and described in Table 2. The evolutionary relationships of the fungal isolates are presented in the NJ tree (Fig. 1). The tree details as retrieved from MEGA software are reproduced here, with minor modifications: "The phylogenetic analysis involved 55 nucleotide sequences, including 8 ITS sequences from this study. All ambiguous positions were removed for each sequence pair. There were a total of 773 positions in the final dataset. The optimal tree with the sum of branch length = 2.67369481 is shown (Fig. 1). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004) and are in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). The differences in the composition bias among sequences were considered in evolutionary comparisons". *Colletotrichum gloeosporioides* is the designated outgroup (Fig. 1).

In the NJ tree (Fig. 1), the eight fungal isolates associated with tarballs collected from Candolim clustered with 3 clades each of *Dothideomycetes* (Clades 1, 2 and 5) and *Saccharomycetes* (Clades 3 and 4). In the class *Dothideomycetes*, Clade 1 consisted of isolate Tar06, isolate Tar08 and *Curvularia*/*Biploaris* sequences, while isolate Tar10 clustered with *Alternaria* sequences in the Clade 2. Isolate Tar01 clustered with *Cladosporium* sequences in Clade 5. In the class *Saccharomycetes*, isolate Tar09 clustered with the *Candida* sequences (Clade 3), while the isolates Tar03, Tar04 and Tar05 clustered with *Issatchenkia orientalis* (AY939808) in Clade 4.

4. Discussion

Though there have been a few studies on tarball-associated fungi (Table 1), our understanding of fungal diversity associated with tarballs needs improvement. *Rhodotorula* sp., a yeast species, was apparently the first report on fungus from tarballs (Nair et al. 1972). Later, Nair and Lokhabharati (1977) isolated *Fusarium* sp. from tarballs collected from Goa and studied the effects of glucose, kerosene and petrol on its growth. They observed that the addition of up to 4% of kerosene and petroleum in the growth medium could enhance the fungal growth, above which it actually declined. They concluded that kerosene was a better substrate for growth of the *Fusarium* sp., in comparison with petrol.

In late 1980s, Snellman et al. (1988) isolated fungi from tarballs collected from certain areas in the north Atlantic and north Pacific Oceans and investigated their ability to degrade fuel oil. They reported 42 filamentous fungi associated with the tarballs samples (Table 1). Majority of the isolates from their study belonged to fungal genera *Aspergillus* and *Penicillium*. Later, Itah and Essien (2005) isolated fungi and bacteria from tarballs collected from Ibeno beach in Nigeria and tested their ability to degrade tarballs. They reported mycological counts in the range of $1\ (\pm 0.3) \times 10^2$ to $2\ (\pm 0.4) \times 10^4$ c.f.u. g^{-1} of tarball. In their study, *Cladosporium resinae*, *Candida marina* and "*Saccharomyces estuari*" were found to be the most efficient utilizers and biodegraders of tarballs. Elshafie et al. (2007) isolated fungi from tarballs collected from the beaches of Oman and tested these fungi for their abilities to grow and degrade n-alkanes and crude oil. In their study, *Aspergillus* and *Penicillium* were the most dominant taxa, while *A. niger*, *A. terreus* and *P. chrysogenum* were found to be active bio-degraders of selected hydrocarbons.

Recently, Lotfinasabasl et al. (2012) isolated fungi from soil and tarball samples collected from mangrove forest of Alibaug and Akshi coastal area, Maharashtra, India and tested their ability to degrade kerosene. They reported *Aspergillus terreus* and *Rhizopus* sp. from the tarball samples and observed that *Rhizopus* sp. exhibited the highest growth diameter in 5% kerosene. Though, so far 53 fungal taxa have been reported to be associated with tarballs collected from various parts of the world, we believe that the present report is the first study on generation of sequence-data for tarball-associated fungi, followed by ITS-based phylogenetic analysis to place them in closest fungal clades. There are already two such reports on tar-ball associated bacteria (Bacosa et al. 2016, Nkem et al. 2016).

In our study, four fungal isolates (Tar03, Tar04, Tar05 and Tar09) were found to belong to the *Saccharomycetes.* While the placement of isolate Tar09 within the *Candida* clade (Fig. 1, Clade 3) is strongly supported, the placement of isolates Tar03, Tar04 and Tar05 within *Issatchenkia orientalis* subclade (Fig. 1, Clade 4) is tentative for the reason that limited ITS sequences from the type materials could be retrieved for the genus *Issatchenkia* from the NCBI BLAST search analysis. Though the placements of isolates Tar06 and Tar08 in the *Bipolaris*/*Curvularia* clade (Fig. 1, Clade 1), isolate Tar10 in the *Alternaria* clade (Fig. 1, Clade 2), and isolate Tar01 in the *Cladosporium* clade (Fig. 1, Clade 5) are statistically well-supported, we refrain from giving species names to the fungi just based on monophasic data (i.e. ITS sequence-data).

Further studies employing a polyphasic approach are required to accurately identify these fungi to species level. Additional studies are required from India to obtain better insights into genetic diversity of

Table 2
Colony morphology of fungal cultures and the GenBank accession numbers of the ITS region.

Sl. no.	Strain no.	Fungal colony morphology after 21 days of inoculation on the PDA medium	GB# of the ITS
1	Tar01	Dark green; powdery texture; mycelial growth; colonies raised and uplifted from the surface; colony diam. 5.6 mm	KX757230
2	Tar03	Off white; non-mycelial growth; smooth yeast like texture; colonies were not raised from the surface; colony diam. 46 mm	KX757231
3	Tar04	White; non-mycelial growth; rough texture; colonies not raised above the surface; colony diam. 32.1 mm	KX757232
4	Tar05	White; rough textured; non mycelial growth; colonies raised a bit from the surface; colony diam. 40 mm	KX757233
5	Tar06	Light grey; mycelial growth; cottony texture; colonies not raised from the surface; colony diam. 68.7 mm	KX757234
6	Tar08	Whitish grey; mycelial growth; shrinkable cottony texture; colonies not raised from the surface; colony diam. 52.2 mm	KX757235
7	Tar09	White; non-mycelial growth; rough texture; colonies not raised from surface; colony diam. 34.5 mm	KX757236
8	Tar10	Whitish on outer surface and dark grey on inner surface; mycelial growth; shrinkable feathery texture; colonies raised from the surface; colony diam. 63 mm	KX757237

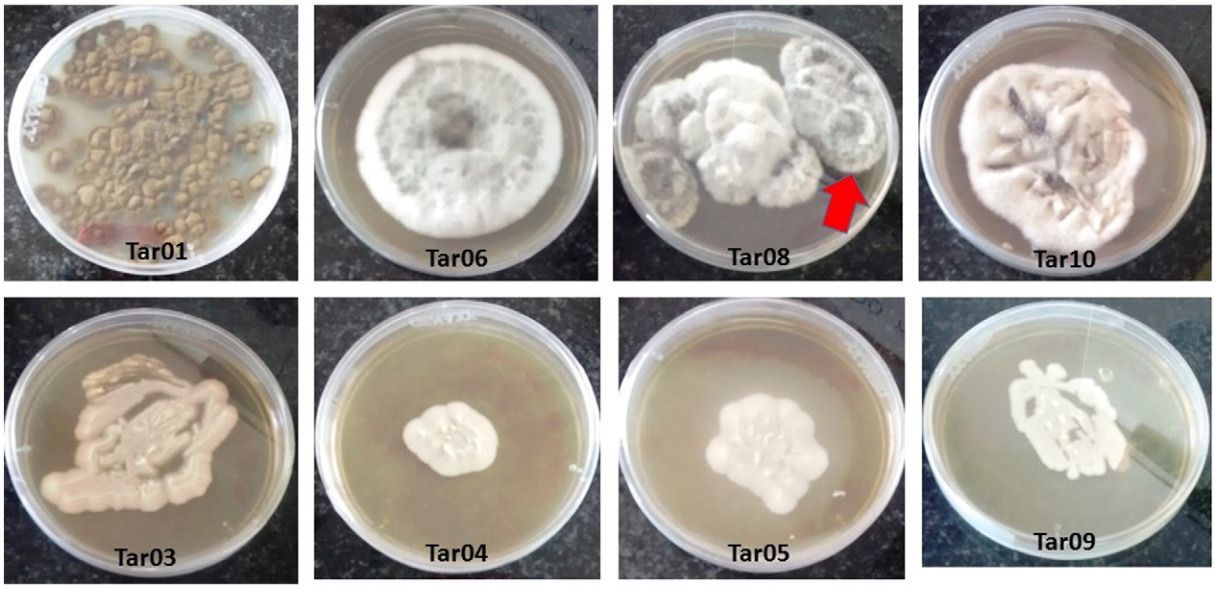

Fig. 2. Culture morphology of tarball-associated fungi from Candolim, Goa, India (Tar01, Tar06, Tar08, Tar10 - Dothideomycetes; Tar03, Tar04, Tar05, Tar09 – Saccharomycetes, red arrow indicates the colony of fungal isolate Tar08 in a mixed culture).

fungi and other microbes involved in different stages of degradation of tarballs. Detailed assessment of ability of tarball-associated fungi and other microorganisms to degrade toxic hydrocarbons present in tarballs is expected to assist in bioremediation of tarballs (Nadapdap et al., 1996).

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References

Al-Nasrawi, H., 2012. Biodegradation of crude oil by fungi isolated from Gulf of Mexico. J. Bioenerg. Biomembr. 3, 147. http://dx.doi.org/10.4172/2155-6199.1000147.

Bacosa, H.P., Thyng, K.M., Plunkett, S., Erdner, D.L., Liu, Z., 2016. The tarballs on Texas beaches following the 2014 Texas City "Y" Spill: modeling, chemical, and microbiological studies. Mar. Pollut. Bull. 109, 236–244.

Das, N., Chandran, P., 2011. Microbial degradation of petroleum hydrocarbon contaminants: an overview. Biotechnol. Res. Int., 941810 http://dx.doi.org/10.4061/2011/941810.

Dhargalkar, V.K., Kureishy, T.W., Bhandare, M.V., 1977. Deposition of tar balls (oil residue) on beaches along the west coast of India. Mahasagar: Bulletin of the National Institute of Oceanography 10, 103–108.

Elshafie, A., AlKindi, A.Y., Al-Busaidi, S., Bakheit, C., Albahry, S.N., 2007. Biodegradation of crude oil and n-alkanes by fungi isolated from Oman. Mar. Pollut. Bull. 54, 1692–1696.

Jawhari, I.F.H.A., 2014. Ability of some soil fungi in biodegradation of petroleum hydrocarbon. J. Appl. Environ. Microbiol. 2, 46–52.

Itah, A.Y., Essien, J.P., 2005. Growth profile and hydrocarbonoclastic potential of microorganisms isolated from tarballs in the Bight of Bonny, Nigeria. World J. Microbiol. Biotechnol. 21, 1317–1322.

Kumar, S., Stecher, G., Tamura, K., 2015. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33, 1870–1874.

Lotfinasabasl, S., Gunale, V.R., Rajurkar, N.S., 2012. Assessment of petroleum hydrocarbon degradation from soil and tarball by fungi. Biosci. Discov. 3, 186–192.

Nadapdap, H., Smith, J.P., Broadmeadow, S., 1996. Bioremediation of crude oil tarballs in a mangrove environment in the Maxus Field. 25th Annual Convention Proceedings of Indonesian Petroleum Association. 3, pp. 127–133.

Nair, S., Lokabharathi, P.A., 1977. Degradation of hydrocarbons by fungus, *Fusarium* sp. Indian Journal of Marine Science 6, 173–175.

Nair, S.A., Devassy, V.P., Dwivedi, S.N., Selvakumar, R.A., 1972. Preliminary observations on tarlike material observed on some beaches. Current Marine Science 41, 766–767.

Nkem, B.M., Halimoon, N., Yusoff, F.M., Johari, W.L.W., Zakaria, M.P., Reddy, S., Kannan, N., 2016. Isolation, identification and diesel-oil biodegradation capacities of indigenous hydrocarbon-degrading strains of *Cellulosimicrobium cellulans* and *Acinetobacter baumannii* from tarball at Terengganu beach, Malaysia. Mar. Pollut. Bull. 107, 261–268.

Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425.

Snellman, E.A., Collins, R.P., Cooke, J.C., 1988. Utilization of fuel oils by fungi isolated from oceanic tar balls. Lett. Appl. Microbiol. 6, 105–107.

Suneel, V., Vethamony, P., Zakaria, M.P., Naik, B.G., Prasad, K.V.S.R., 2013. Identification of sources of tar balls deposited along the Goa coast, India, using fingerprinting techniques. Mar. Pollut. Bull. 70, 81–89.

Tamura, K., Nei, M., Kumar, S., 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. Proc. Natl. Acad. Sci. U. S. A. 101, 11030–11035.

White, T.J., Bruns, T., Lee, S., Taylor, J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: MA, I., DH, G., JJ, S., TJ, W. (Eds.), PCR Protocols: a Guide to Methods and Applications. Academic Press, New York, pp. 315–322.

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This is to certify that Prof./Dr./Ms./Mr. Varsha L. Shinde *has attended and presented poster/oral titled* "Degradation of crude-oil by tarball-associated bacteria from Goa" *at the Seminar "New Perspectives in Biosciences", held on 7th December, 2017 at Department of Microbiology, Goa University.*

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