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# Chapter 3

## Materials and Methods

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Gosai., H., G. (2024). Assessment of pollution load of coastal mudflats along the western bank of Gulf of Khambhat with special reference to microbial community structure as bioindicator.

### 3.1 Description of the study area and location of study sites

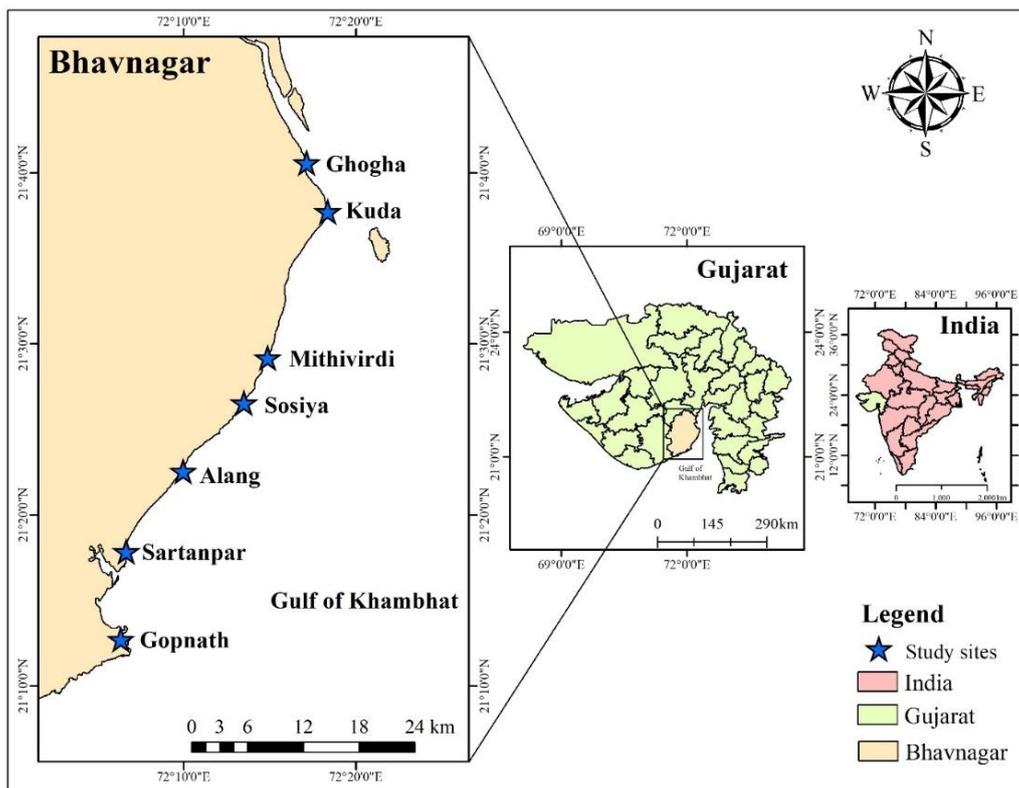
Over 8000 km of coastline make up India, which is home to nine coastal states, 60 districts, and two major island groups where the majority of the country's population makes their living from fishing (Senapati & Gupta, 2014). India is a developing nation that is industrializing and growing economically very quickly. As a result, the nation is coping with environmental contamination in the ocean and climate change, which puts human and marine biota health at danger. Gujarat, one of India's nine coastal states, has the longest coastline (about one-fifth of the nation's total length), 49 ports, and industries such as the automotive, energy, chemical, and pharmaceutical sectors that sustain people's livelihoods (MM&FICCI, 2019; Rabari *et al.*, 2022). The average elevation of the Gulf of Khambhat above mean sea level is 8 meters. The Gulf's lengths approximately 130 km long by 70 km wide, with an average depth of 30 meters. The district of Bhavnagar has experienced the development of several diamond-cutting and polishing facilities, as well as agro-based and salt-marine chemicals, cotton textile, woollen, silk, artificial thread-based, chemical, mineral-based, plastics, shipbuilding, and ship-breaking operations (MSME-GOI, 2011). The industries around the study region included energy, petrochemical, agrochemical, metal-mining, auto components, textile, pharmaceutical, effluent treatment, and port (MM&FICCI, 2019; Rai, 2020).

In the Gulf of Khambhat, a number of rivers come together to form estuarine deltas, including the Narmada, Tapi, Mahi, Sabarmati, Shetrunji, Ambika, and Purna. In the Gulf of Khambhat, the highest tidal current during spring tide can reach 10 meters, and at high tide, the tidal current can exceed 3 m/s (Gosai & Mankodi, 2023). The Gulf of Khambhat coastline features a semi-arid zone with an average temperature range of around 12°C, a hot bioclimate, and 900 mm of annual rainfall. It varies in temperature from 10°C to more than 43°C. The intertidal zone is muddy, clayey, and silty, while the soil is brackish, sandy, and salty to hypersaline. Due to the Gulf of Khambhat's semi-diurnal tidal characteristics, there are two almost equal-sized high and low tides each lunar day. In the Gulf of Khambhat, tides rise in different amplitudes from south to north. The bottom structure and substratum friction coefficient are responsible for the observed tide elevation. Due to this region's complex hydrodynamics and the potential for the construction of multiple industrial establishments, scientists and researchers are interested in it (Mitra *et al.*, 2020; Singh *et al.*, 2022).

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Along with the Gulf of Kutch, the Gulf of Khambhat is one of the most active macro-tidal regimes in the northeastern Arabian Sea. The movement of fronts and suspended sediments is greatly influenced by tides. The wide tidal range of the Gulf of Khambhat creates strong tidal currents and provides a means of moving suspended sediments. Large mudflats are evidence that sediments are moving netherward toward land. Research on suspended sediments reveals that throughout the monsoon, sediments brought in by several river systems stay suspended and start to settle as winter approaches (Nayak & Shetye, 2003). A lot of companies discharge a significant amount of treated wastewater into the Gulf of Khambhat on both sides of the waterway. It's unclear right now how the particles discharged into the Gulf are responding. Pre-monsoon, monsoon, and post-monsoon Lagrangian circulation in the Gulf of Khambhat were simulated using a 2D numerical model. The model results indicated the existence of residual eddies over the Gulf during the SW monsoon. The Lagrangian track results show that particles emitted inside the Gulf do not exit the area, with the exception of a small number of particles from the southern Gulf of Khambhat. Relative eddies may be to blame for this, as they keep particles from exiting the Gulf (Mitra *et al.*, 2020).



**Figure 3.1** Geographical distribution of the sampling sites in the Bhavnagar coastal region, Gulf of Khambhat

The research area is located in Gujarat, on the western coast of the Gulf of Khambhat, on India's west coast in the Arabian Sea. Gujarat's coastal district of Bhavnagar is situated at 21.7645°N 72.1519°E. The current investigation includes seven monitoring sites for the evaluation of coastal water from the Bhavnagar coast, Gulf of Khambhat. The sampling points includes Ghogha (21°40'36.0"N, 72°17'07.1"E), Kuda (21°37'44.7"N, 72°18'21.1"E), Mithivirdi (21°29'11.6"N, 72°14'51.2"E), Sosiya (21°26'34.0"N, 72°13'29.2"E), Alang (21°22'34.0"N, 72°09'58.3"E), Sartanpar (21°17'52.6"N, 72°06'39.3"E), and Gopnath (21°12'44.3"N, 72°06'20.5"E) (Figure 3.1).

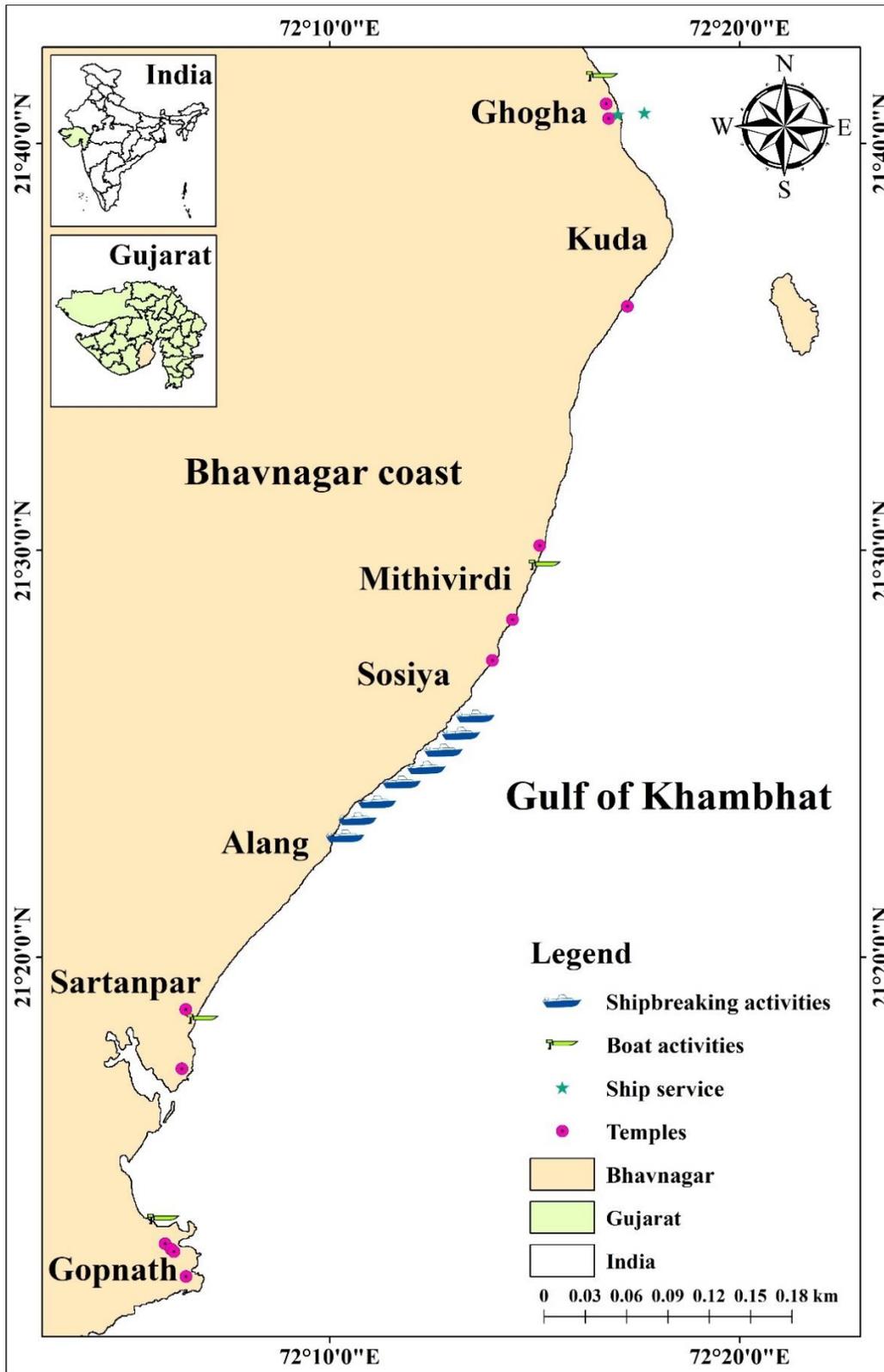
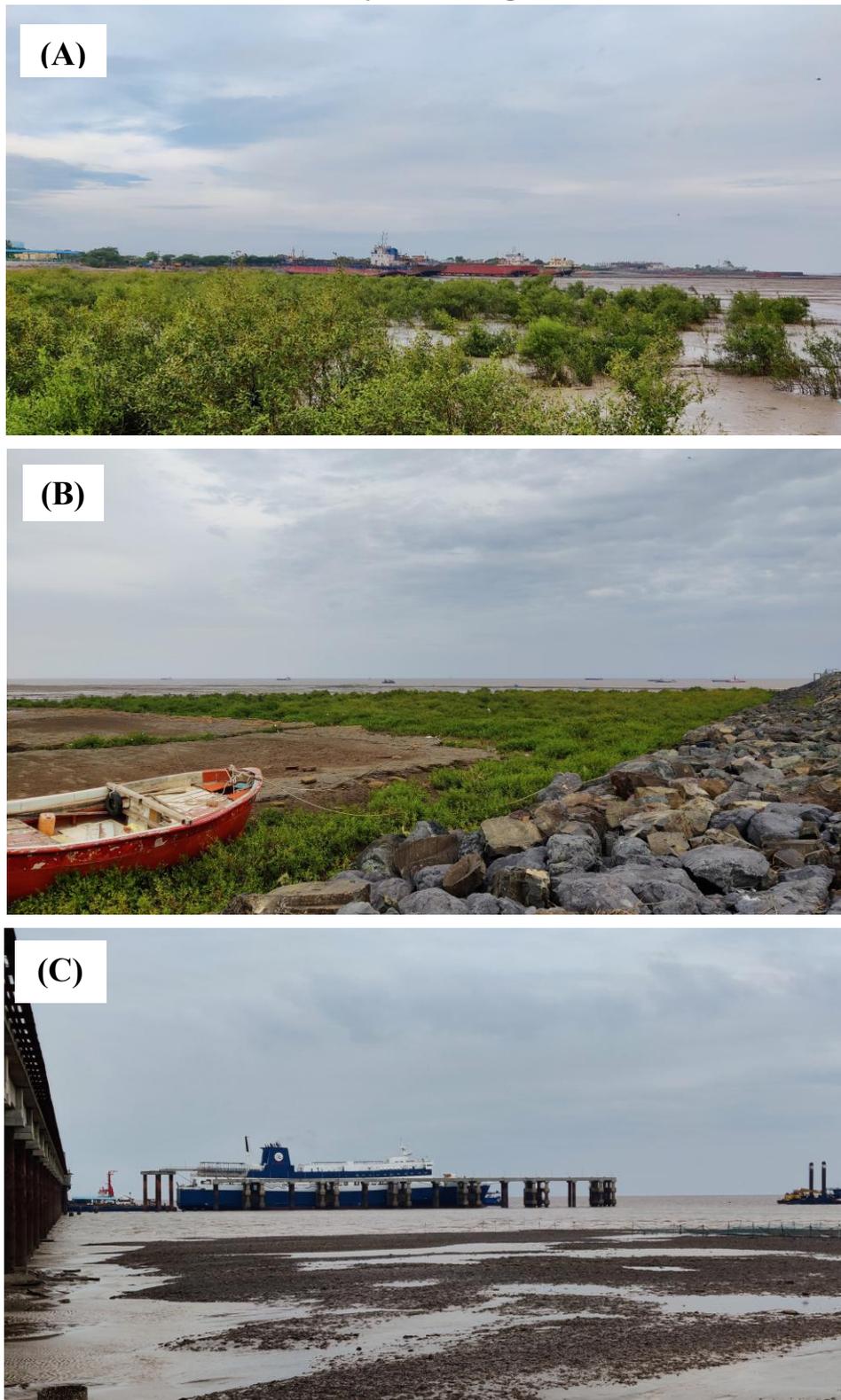


Figure 3.2 Coastal activities along the studied region, Bhavnagar coast

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## Study site - Ghogha



**Figure 3.3** On-site observation of Ghogha coast (A) Ship maintenance & muddy coast (B) Fishing boats (C) Shipping service

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## Study site - Kuda



**Figure 3.4** On-site observation of Kuda coast (A) Sandy coast, (B) Tourist, (C) Waste litter

## Study site – Mithivirdi



**Figure 3.5** On-site observation of Mithivirdi coast – sandy beach with tourist activity can be seen (A) Rocky and muddy coast during low tide, (B) Sandy coast, (C) Tourist activity

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## Study site – Sosiya



**Figure 3.6** On-site observation of Sosiya coast (A) Rocky and muddy coast, (B) Shipbreaking activity, (C) During low tide

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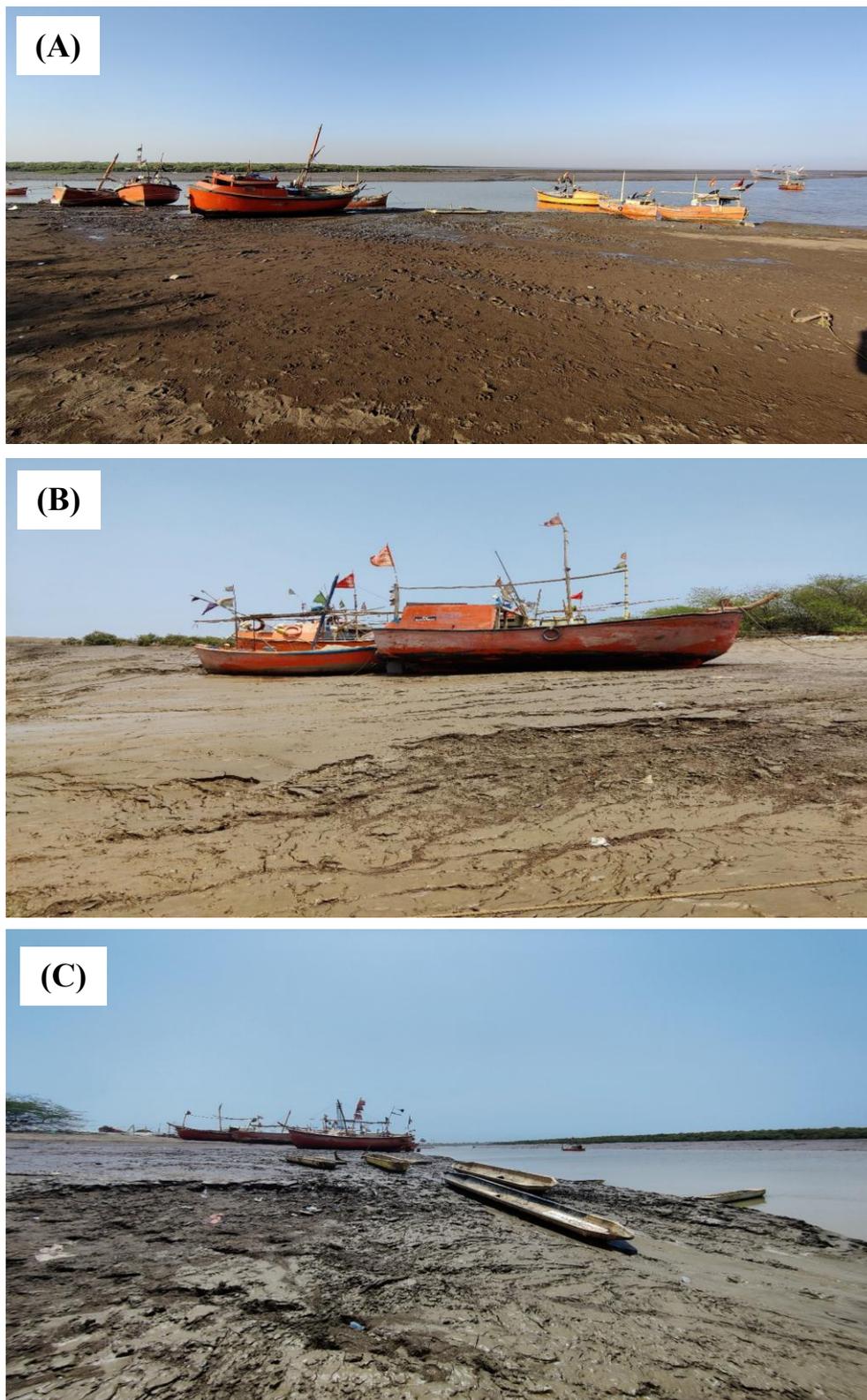
## Study site – Alang



**Figure 3.7** On-site observation of Alang coast (A) Shipbreaking activity, (B) Fishing boats (C) Rocky and muddy coast during low tide.

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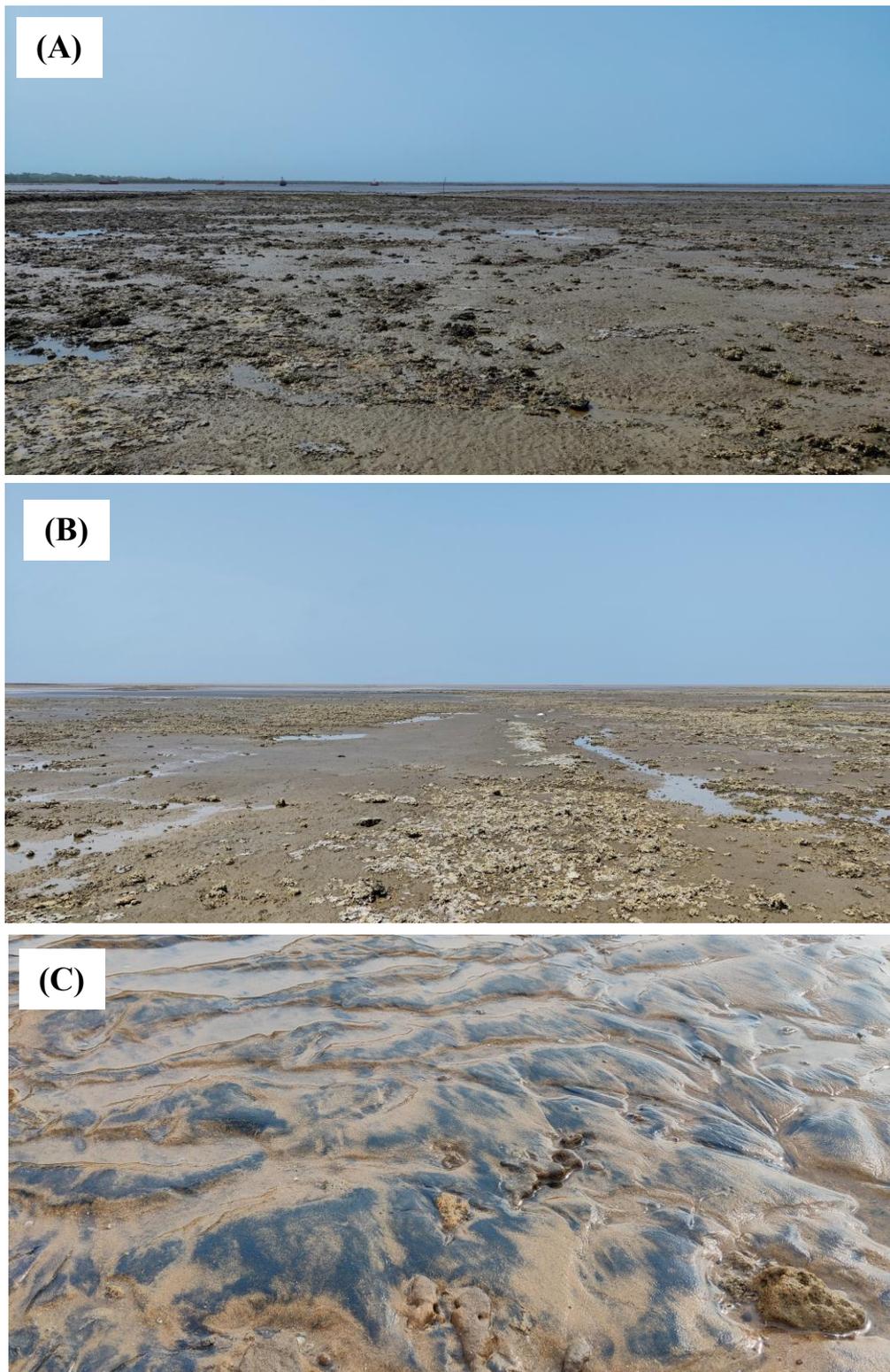
## Study site – Sartanpar



**Figure 3.8** On-site observation of Sartanpar coast (A) Fish landing centre, (B) Muddy coast (C) Catamarans

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## Study site – Gopnath



**Figure 3.9** On-site observation of Gopnath coast (A) Fishing boats, (B) Rocky and muddy coast (C) Black organic carbon layer deposition

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### 3.2 Spatio-temporal assessment of physico-chemical characteristics and heavy metals content of coastal surface water

A total of 63 coastal water samples (21 samples × 3 seasons), collected at low tide and 250-500 m apart, were analyzed for hydrochemistry and dissolved heavy metal content along the Bhavnagar coast in the Gulf of Khambhat. The study aimed to assess the impact of industrial activities and human influence across seasons, including pre-monsoon (PRM), monsoon (M), and post-monsoon (POM). Triplicates of water samples were collected from each location using properly cleansed and rinsed bottles. The samples were then stored at -20°C and analyzed within 48 hours at the laboratory of the Department of Environmental Studies, The Maharaja Sayajirao University of Baroda, Vadodara.

The data quality was ensured by implementing careful standardization, conducting procedural blank measurements, and performing triplicate sample analysis. The study includes various physico-chemical parameters of coastal water including temperature, pH, conductivity, total dissolved solids (TDS), total suspended solids (TSS), total hardness (TH), calcium hardness ( $\text{Ca}^{+2}$ ), chloride (Cl), salinity, dissolved oxygen (DO), 5<sup>th</sup> day biochemical oxygen demand (BOD<sub>5</sub>), and chemical oxygen demand (COD). The analysis of these parameters was carried out in accordance with the standard method (APHA, 2012; Grasshoff *et al.*, 1999; Strickland & Parsons, 1972).

To determine the concentration of dissolved heavy metals, we utilized the method outlined in the APHA (2012). Initially, coastal water samples were filtered through 0.45 µm pore glass fiber filters (GF/F, Whatman) and then acidified to pH 1.5–2.0 using nitric acid (HNO<sub>3</sub>). The filtered coastal water samples were refrigerated until analysis. To ensure precise and dependable analysis, we prepared standard solutions using high-quality analytical-grade chemicals from Merck and deionized water. Before use, precisely cleaned plasticware and glassware by immersing them in 14% (v/v) HNO<sub>3</sub> for a minimum of 24 hours, followed by rinsing with distilled water (Jones & Laslett, 1994).

### 3.3 Spatio-temporal assessment of physico-chemical characteristics and heavy metal contents of coastal surface sediment

To evaluate the physico-chemical characteristics and the presence of heavy metals along the Bhavnagar coast, Gulf of Khambhat, a total of 63 sediment samples (21 samples × 3 seasons) were collected. Three distinct seasons were observed for the sediment sampling: pre-monsoon (PRM), monsoon (M), and post-monsoon (POM). Sediment was gathered from four corners of the quadrat and one from its center, representing lower, middle, and high intertidal areas, as part of a composite sample process (Saher & Siddiqui, 2019). After the sediments were collected, each sample weighed at least 150-200g, and they were quickly placed into plastic zipper bags that had been previously cleaned.

Physico-chemical characteristics of coastal sediments such as pH, organic matter (OM), total available phosphate (TAP), total sulphur (TS), and available nitrogen were done using the standard method (APHA, 2012). The sediment samples that were obtained were dried prior to undergoing geochemical analysis. Following that, the tiny particles (<74µm) were separated by screening because of their greater affinity for metals. Additionally, by removing unwanted debris like stones and plastic fragments, the sifting procedure helped separate the desired sediment fraction for metal analysis (Hou *et al.*, 2023; Morillo *et al.*, 2004). For acid digestion modified method was used (Baird *et al.*, 1999). Using a top-loading scale, 1g of the previously dried sediment sample was meticulously weighed before the chemical analysis got underway. After each sample was put in its own 250 ml beaker, the samples were subjected to a digestion process employing 50 ml of a 3:1 ratio of high-purity concentrated Merck hydrochloric acid (HCl) and HNO<sub>3</sub>. The concentration of metals in the sample solution was ascertained using Atomic Absorption Spectroscopy (PerkinElmer PinAAcle-500 AAS) in the Department of Environmental Studies' advanced instrument laboratory at the Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India.

**Table 3.1** Sites with their sampling location and zone distribution

Site	Sampling site	Intertidal Zone
Gogha	S1	Lower intertidal zone
	S2	Middle intertidal zone
	S3	Higher intertidal zone
Kuda	S4	Lower intertidal zone
	S5	Middle intertidal zone
	S6	Higher intertidal zone
Mithivirdi	S7	Lower intertidal zone
	S8	Middle intertidal zone
	S9	Higher intertidal zone
Sosiya	S10	Lower intertidal zone
	S11	Middle intertidal zone
	S12	Higher intertidal zone
Alang	S13	Lower intertidal zone
	S14	Middle intertidal zone
	S15	Higher intertidal zone
Sartanpar	S16	Lower intertidal zone
	S17	Middle intertidal zone
	S18	Higher intertidal zone
Gopnath	S19	Lower intertidal zone
	S20	Middle intertidal zone
	S21	Higher intertidal zone

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### 3.3.1 Methods for assessing heavy metal pollution in sediment

For assessing the degree of heavy metal contamination in sediment samples, pollution indices might be a helpful tool. When there are no locally established natural reference levels, average crust values are usually used as representative background values in the sediments under analysis. By revealing how heavy metal concentrations differ from background values, these indices provide a comprehensive evaluation of sediment contamination (Taylor, 1964). Some of the frequently used single pollutant indices for evaluating sediment quality include enrichment factor ( $E_f$ ), contamination factor ( $C_f$ ), and geo-accumulation index ( $I_{geo}$ ). These indices offer helpful data on the degree of contamination, enrichment, and geochemical accumulation of various elements in sediments. These indices offer trustworthy quantitative techniques for evaluating and keeping track of sediment quality when contaminants are present. (Hakanson, 1980). With the use of these indices, it is possible to determine the extent and amount of metal pollution in a certain coastal region, which is useful information for environmental monitoring and management strategies meant to minimize any risks. Table 3.2 lists all of the applied indices, and Table 3.3 provides the different categories.

One popular technique for estimating the possible ecological impacts of heavy metal pollution is the ecological risk factor ( $E_r$ ) and potential risk index (RI). It provides a thorough evaluation of the overall risk related to every heavy metal discovered in sediments. By quantifying the susceptibility of biological communities to toxic chemicals,  $E_r$  and RI offer crucial insights into the likely ecological consequences of heavy metal poisoning. This information is essential for risk assessment and conservation programs that protect the integrity and health of ecosystems contaminated by heavy metals (Hakanson, 1980). The equation for the assessment of  $E_r$  and RI is given in Table 3.2.

**Table 3.2** Various pollution indices for a comprehensive analysis utilized in the present study

Pollution Indices	Equation	Significance	References
<b>Enrichment factor (E<sub>f</sub>)</b>	$E_f = \frac{[\text{Element}]_{\text{Sample}} / [\text{Fe}]_{\text{Sample}}}{[\text{Element}]_{\text{Crust}} / [\text{Fe}]_{\text{Crust}}}$	Distinguish between natural and human sources of metal concentration in sediment.	Hakanson (1980)
<b>Contamination factor (C<sub>f</sub>)</b>	$C_f = \frac{[\text{Element}]_{\text{Sample}}}{[\text{Element}]_{\text{Crust}}}$	Metal enrichment in sediments can be quantified. This factor is calculated by dividing the metal concentration found in the sediment sample by the appropriate background concentration levels of that specific element.	Grant and Middleton (1990)
<b>Geo-accumulation index (I<sub>geo</sub>)</b>	$I_{\text{geo}} = \log_2 \frac{[(\text{Element})_{\text{Sample}}]}{[1.5 \times (\text{Element})_{\text{Crust}}]}$	The geo-accumulation index was used to examine the pollution level in sediments.	Muller (1969)
<b>Ecological risk factor (E<sub>r</sub>)</b>	$E_r = T_r \times C_f$	E <sub>r</sub> and RI represent a factor that comprehensively evaluates the potential ecological risks that heavy metals pose in the sediment.	Hakanson (1980)
<b>Risk index (RI)</b>	$RI = \sum E_r = \sum T_r \times C_f$		

Here, [Element]<sub>sample</sub> is the concentration of a particular metal in a sediment sample; [Fe]<sub>sample</sub> is the concentration of Fe in a sample; [Element]<sub>Crust</sub>/[Fe]<sub>Crust</sub> is the background concentration value of a particular metal and iron; factor 1.5 is used to mitigate the effects of possible differences in background values that may be attributed to lithogenic effects; T<sub>r</sub> is a toxic response of the particular element i.e., Ni (5), Cu (5), Mn (1), Cr (5), Pb (5), Zn (1), Co (5) and Cd (30), respectively.

**Table 3.3** Classification of pollution indices and contamination levels

<b>Pollution indices</b>	<b>Classification</b>	<b>Quality</b>
<b>E<sub>f</sub><sup>1</sup></b>	$E_f < 1$	No enrichment
	$E_f = 1 - 3$	Minor enrichment
	$E_f = 3 - 5$	Moderate enrichment
	$E_f = 5 - 10$	Moderate to high enrichment
	$E_f = 10-25$	High enrichment
	$E_f = 25 - 50$	Very high enrichment
	$E_f > 50$	Extreme enrichment
<b>C<sub>f</sub><sup>2</sup></b>	$C_f < 1$	Low contamination
	$C_f = 1 - 3$	Moderate contamination
	$C_f = 3 - 6$	Considerable contamination
	$C_f > 6$	High contamination
<b>I<sub>geo</sub><sup>3</sup></b>	$I_{geo} \leq 0$	Unpolluted
	$I_{geo} = 0 - 1$	Unpolluted to moderately polluted
	$I_{geo} = 1 - 2$	Moderately polluted
	$I_{geo} = 2 - 3$	Moderately to strongly polluted
	$I_{geo} = 3 - 4$	strongly polluted
	$I_{geo} = 4 - 5$	Strongly to very strongly polluted
<b>E<sub>r</sub><sup>4</sup></b>	$\leq 40$	Low ecological risk
	$\leq 40 - < 80$	Moderate ecological risk
	$\leq 80 - < 160$	Considerable ecological risk
	$\leq 160 - < 320$	High ecological risk
	$\geq 320$	Very high ecological risk
<b>RI<sup>5</sup></b>	$\leq 95$	Low potential ecological risk
	$\leq 95 - < 190$	Moderate ecological risk
	$\leq 190 - < 380$	Considerable ecological risk
	$\geq 380$	Very high ecological risk

<sup>1,4,5</sup> Hakanson (1980), <sup>2</sup> Grant and Middleton (1990), <sup>3</sup> Muller (1969).

### 3.4 Spatio-temporal assessment of microbial diversity of coastal surface sediment

#### 3.4.1 Isolation media and cultivation condition for marine microbes

Zobell marine broth (ZMB) contains the nutrients required for the growth of marine bacteria. These media have minerals as in seawater and peptone and yeast extract as the sources of nutrients for the marine bacteria. The high amount of salt content is used to simulate seawater. Other minerals are used to mimic the mineral composition of seawater. Zobell Marine Broth (Himedia Pvt Ltd, Mumbai M385- Marine Broth 2216) was used to isolate marine microbes. The isolation media consisted following composition Peptone (5 g), Yeast extract (1 g), Ferric chloride (0.1 g), Sodium chloride (19.45 g), Magnesium chloride (8.8 g), Calcium chloride (1.8 g), Potassium chloride (0.55 g), Sodium bicarbonate (0.16 g), Potassium bromide (0.08 g), Strontium chloride (0.034 g), Boric acid (0.022 g), Sodium silicate (0.004 g), Ammonium nitrate (0.0016 g), Disodium phosphate (0.008 g), Sodium fluoride (0.0024 g), Final pH  $7.6 \pm 0.2$  (at  $25^{\circ}\text{C}$ ) in 1L. For preparation of media, suspend 40.25 g in 1000 ml distilled water. Heat if necessary to dissolve the medium. Dispense into tubes or flasks as desired. Sterilize by autoclaving at 15 lbs pressure ( $121^{\circ}\text{C}$ ) for 15-20 minutes.

#### 3.4.2 Isolation, enrichment and taxonomic studies

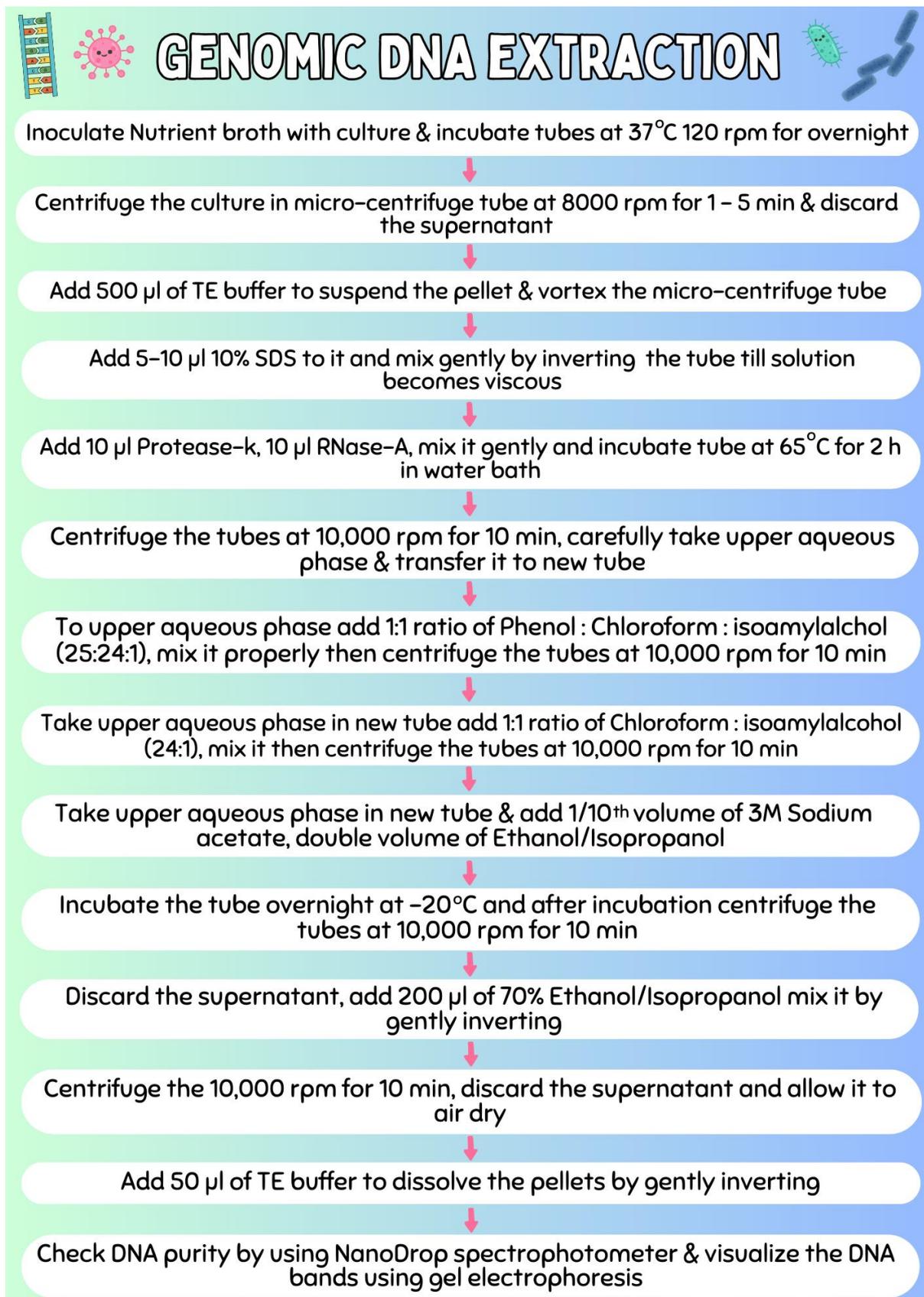
Cultivable marine bacterial isolates were isolated and enumerated (Brown & Smith, 2014). Isolation of bacteria was carried out with Zobell marine broth (ZMB) adopting serial dilution technique and incubated overnight at  $37 \pm 2^{\circ}\text{C}$  for 24h - 36 h in a BOD incubator. After incubation, single pure colonies from each of the selected isolates of mixed isolates growth on the plate were transferred by streaking in a new plate and incubated overnight at  $37 \pm 2^{\circ}\text{C}$  for 24 h - 36 h in a BOD incubator for enrichment. After incubation pure isolates were stored at  $4^{\circ}\text{C}$  until further analysis. Culture, morphophysiology and biochemical characters of the microbial isolates were studied following standard methods (MacFaddin, 1999; Ventosa *et al.*, 1998).

### 3.4.3 DNA extraction of bacterial colonies

The reagents required for the extraction of genomic DNA are mentioned in Table 3.4 and the extraction method is shown in Figure 3.10.

**Table 3.4** Reagents required for the extraction of DNA

Reagent	Concentration	Description
Tris-EDTA (TE) Buffer	-	Buffer solution used to store purified DNA comprised of 100 mM Tris (pH 8.0), 10 mM EDTA (pH 8.0)
Sodium dodecyl sulfate (SDS)	10%	Solubilisation of cell membrane lipids
RNase A (ThermoFisher Scientific)	100 µg/mL	Degrades single-stranded RNA. Buffer P1 is a resuspension buffer comprising 50 mM Tris-Cl (pH 8.0), 10 mM EDTA
Proteinase K (ThermoFisher Scientific)	20 mg/mL	Digestion of proteins
Phenol: Chloroform: Isoamyl alcohol (PCI) solution (25:24:1)	-	Separation of DNA from other cellular components
Chloroform: Isoamyl alcohol (CI) solution (24:1)	-	
Ethanol / Isopropanol	100%	Precipitates DNA from solution
Sodium acetate	5M	Used to dissolve the DNA precipitate and create a suitable buffer for further analysis



**Figure 3.10** Flow chart for DNA extraction from bacterial isolates

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#### 3.4.4 16s rRNA amplification

Polymerase chain reaction (PCR) was conducted using the primers **27F** (5'-AGAGTTTGATCCTGGCTCAG-3') and **1492R** (5'-TACGGCTACCTTGTTACGACTT-3'), targeting the 16S rRNA region of the domain Bacteria (Srinivasan *et al.*, 2015). For PCR amplification ThermoFisher Scientific Platinum™ Hot Start PCR Master Mix (2X) was used. The final **PCR reaction mixture (50 µl)** was as follows: Platinum™ Hot Start PCR Master Mix 2x (25 µl), Platinum™ GC Enhancer (10 µl), 20 pmol Forward Primer 27F (1 µl), 20 pmol Reverse Primer (1 µl), isolated template genomic DNA (1 µl). 1X master mix contains a final concentration of 1.5 mM MgCl<sub>2</sub> and 0.2 mM of each dNTP.

The **cycling conditions** for PCR were: initial denaturation at 94°C for 2 min; 35 cycles of 94°C for 30 seconds (denaturation), 55°C for 30 seconds (anneal), 72°C for 1 min (extend), and final extra extension at 72°C for 7 min followed by 5°C for 10 min (hold). 1-2 µl of PCR amplified gene products were visualised on agarose gel (1%) to check the amplification.

#### 3.4.5 BLAST search and phylogenetic study

Sanger sequencing results of forward and reverse primer sequences were trimmed and consensus sequences were made. The identification of bacteria was done using the National Center for Biotechnology Information (NCBI) website using the Nucleotide Basic Local Alignment Search Tool (Nucleotide BLAST). BLAST finds regions of similarity between biological sequences. The program compares nucleotide or protein sequences to sequence databases and calculates the statistical significance. The results are based on sequence alignment of the highest percentage of identification generated by nucleotide BLAST search.