CHAPTER. II.

ISOLATION AND IDENTIFICATION OF POTENT XENOBIOTIC DEGRADING ALKALIPHILE

2.1 Introduction:

Bacteria are the primary source for degradation of organic contaminants in soil (201). Bacteria possess numerous biotransforming enzymes like azoreductase (202-204), cytochrome P-450 monooxygenase (205), dioxygenases (206) etc. by utilizing this enzyme machinery towards the xenobiotic compounds degradation to simple environmentally benign metabolites further consuming them as a carbon and energy source. These significant contaminated sites are now the reservoir of the well adapted microorganisms to degrade different organic xenobiotic compounds such as PAHs, azo dye, nitroaromatics. Moreover, it is widely recognized that contaminated land is a potential threat to human health, and its continual discovery over recent years has led to international efforts to remedy many of these sites. Therefore, it is necessary to isolate the pure microbial strain for the degradation and enhance its degradation potential by adaptation to xenobiotic compounds in laboratory.

Organic xenobiotic compounds degrading microorganisms require an environmental habitat that has a sufficient and preferably sustainable source of nutrients, water, air, mild ambient temperature, and a moderate or optimum pH (207). Therefore, soil is a perfect source of microorganisms for isolation (208). Hydrocarbon utilizing alkaliphile isolated (209) from Kuet Coast of Arebian Gulf. An aerobic mixed microbial culture, obtained from the aeration basin of the wastewater treatment plant (210). *Stenotrophomonas maltophilia* isolated from petroleum contaminated site (211) Jorden, oil destructing extremophilic actinomycetes isolated from various types of soil of Georgia (212). Similarly several microbes are isolated from various habitats like contaminated site (213), sulfidogenic estuarine sediment (214) activated sludge (215), coking plant (216), oil-reservoir water and oil sands (217) etc. The site of microbial habitat is considered for
their isolation and maintaining the same condition in laboratory for study. Therefore the present studies are planned to isolate and identify the potent xenobiotic degrading alkaliphilic bacteria from pristine Crater Lake of Lonar, (MS) India.

2.2 Material and Methodology:

2.2.1 Chemicals:

The nutrient media chemicals such as agar, yeast extract, peptone were purchased from Hi-media Laboratories (Mumbai, India), Sodium chloride, sodium carbonate, copper sulfate, ammonium persulphate, glacial acetic acid, methanol were obtained from SRL (Mumbai, India), camphor 99.9% (Loba-chem, India), K2HPO4, Na2HPO4, CaCO3, CuSO4, CoSO4, H3BO3, (NH4)2SO4, MgSO4, MgO, ZnSO4.7H2O, FeSO4.7H2O and NaOH from (Sd-Fine Chemicals, Mumbai), 500 bp DNA ladder (Bangalore Genei, India) DNAzol (Invitrogen, USA), ethanol (Gebsen & Gessen, GmbH & Co., Germany), dNTP (Vivantis, USA), 20F forward and 1540R primers (Integrated DNA Technologies, USA), protease-free DNAse/RNAse mixture (Sigma, USA), nuclease free water, Taq polymerase enzyme, Taq polymerase buffer A, gel loading buffer, Agarose, TE buffer, TAE buffer, 500 bp DNA ladder, Proteinase K (Bangalore Genei, India), methylene dichloride (MDC), methanol, were obtained from SRL (Mumbai, India).

2.2.2 Sampling sites and Sample Collection

The place of Lonar lake has a periphery of 1.7 km and is situated in a hallow, 0.14 Km below the ground level. The studied strains were isolated from soil sediments of the Lonar Lake. Water and sediment samples were collected at the surface and at depths of nearly 0.5 meter. Samples were collected with the scoop in to clean and sterile bottles labeled and stored in boxes and transported to the laboratory and stored at 4 °C until analysis. The pH was checked (with pH paper); temperature and depth of sampling were noted immediately at the site.
2.2.3. Media preparation:

The broth media used for biodegradation study consist of yeast extract, peptone, NaCl -5 g/L respectively. The micronutrients’ in mg /L were KH$_2$PO$_4$ -170, Na$_2$HPO$_4$ -290,(NH$_4$)$_2$SO$_4$ -100, MgSO$_4$,MgO-0.1,FeSO$_4$ -0.05,CaCO$_3$ 0.20,ZNSO$_4$ 0.08, CuSO$_4$ 0.016, CaSO$_4$ 0.016,Boric Acid 0.06, and pH 9. The pH was adjusted by sodium bicarbonate (0.1 M or 1 M) solution. The media was sterilized by autoclaving at 121$\degree$C for 15 pounds at 20 minutes. The solid media was prepared in same way by adding 2% agar. The same broth media of pH 9 was used for biodegradation study except the pH parameter changed. Likewise salinity, carbon and nitrogen sources changed without altering other components.

2.2.4 Isolation and maintenances of microorganisms:

The soil collected in the form of sediment from Lonar Lake Buldhana, MS. India dissolved in sterile sodium bicarbonate buffer pH 9 aseptically. Isolation of pure strains of microorganisms was done by serial dilution and pourat plate technique. During serial dilution of soil sample, ten small sterile test tubes were taken and labeled from 1 to 10. Then 4.5 mL sterile a physiological buffered saline was added to each test tube. By the pipette 0.5 mL of the original suspension transferred into test tube 1, this bacterial suspension was mixed thoroughly before proceeding to the next step. From first test-tube 0.5 mL of the diluted bacterial suspension withdrawn by clean sterile pipette and added to second test tube. It continued to proceed till the dilution in 10$^{th}$ test-tube. In test tube 1 the bacteria diluted 10 fold, as 1:10 or 1 x 10$^{-1}$. Similarly the dilution of the last testtube was 1 x 10$^{-10}$ followed. Using the pipette, 0.5 ml aliquots from each testtube was poured and spreaded by serile spreader to get isolated colonies on respected petri plate. The petriplates incubated at 37$\degree$C for 24 hours. Four isolated colony were observed on alkaline agar plate.

Among them one was having faint yellow colonies, while other was off white mucoid. The third one was red color and fourth was saffron colored. All four colonies separated from each other and maintained on agar slants by sub culturing aseptically.
2.2.5. Screening for degradation potential and adaptation:

All four alkaliphilic microbes isolated from Lonar Crater Lake Buldhana, M.S. India by serial dilution method and screened for aromatic amine degradation by growing it on aniline and other nitro aromatic amine or phenol amended alkaline agar plate separately. The agar plates were incubated at 37°C for 24 hr and observed for the growth. Higher concentration was used for adaptation study after screening the potential. Adaptation was carried by adding higher concentration to the broth possessing high potential of degradation (*Bacillus badius*).

2.2.6. Microscopic Analysis

2.2.6.1 Gram staining: Grams staining was done by using crystal violet flooding after smear fixation and putting Grams iodine as mordent followed by washing by distilled water. This further treated with safranine and washed with alcohol, water, and visualized under microscope.

2.2.6.2 Endospore staining: Endospore staining was carried by negative staining method.

2.2.6.3. Scanning Electron microscopic study (SEM): SEM was carried out by cell harvesting at 10000 x g for 10 minusing cold centrifuges. Bacterial sample prepared in 4% glutaraldehyde using distilled water. Specimen was fixed for SEM as per the protocol of Grekova- Vasileva (218). Cells were dehydrated thereafter with gradient alcoholic solution. After drying and mounting of specimen on aluminium sub gold coating was done for 5 min and image was examined using JEOL-JSM 5510 (219).
2.2.7 Biochemical tests:

Following biochemical tests were performed for high degradation potential bacterial strain.

2.2.7.1 Sugar fermentation: Sugar fermentation tests were performed for acid production. 0.08 mg/100 ml Bromothymol blue and 0.5% sugar was added in alkaline agar to observe the acid production.

2.2.7.2 Catalase, Protease, and Oxidase: Catalase test was carried by 1:10 diluting 30% H₂O₂ and dipping loopful bacterial culture in it showing bubbles indicating release of oxygen produced. Protease test was carried by using gelatin 0.3% in agar and growing bacteria on it. After 24 hr incubation 24 n at 37°C it flooded with 10% TCA found clear halo on agar plate. Cytocrome C oxidase test carried by using 1% Dimethyl paraphenylenediamine hydrochloride. Blue color changed blackish in a small while (220).

2.2.7.3 H₂S Production: Ammonium sulfate amended agar slants used to check H₂S production

2.2.8 Identification of potent xenobiotic degrading alkaliphile by 16s rRNA method

2.2.8.1 Extraction of bacterial DNA

The DNA from the bacterial cells was extracted using a typical DNAzol method (221) by Saitou, N., Nei M. 1987. Approximately 9 colonies of pure culture from petri plate were suspended in 1x TE buffer and washed twice in 500 µl TE by vortex mix followed by centrifuge at 10000 x g for 10 minutes at room temperature. Bacterial pellets then suspended to 100 µl Protease K (at the final concentration of 325µg/ml) at 65°C for 1 hour and 30 minutes. Lysis was done by DNAzol treatment as 500µl for bacterial pellets with Proteinase K mixture at 65°C for 5 minutes. Upper DNAzol phase was carefully separated onto 500µl of absolute chilled ethanol resulting in bacterial DNA
precipitation followed by two washing of 70% cold ethanol and DNA pellets were collected at 10000 x g for 10 minutes at room temperature. The resulting pallet was solubilized in 100µl of TE buffer. For each 100 PCR reaction 500ng templates DNA was used.

2.2.8.2 16S rRNA sequence amplification by PCR.

16S rDNA is DNA that encodes for 16S ribosomal (rRNA). 16S rDNA probes are often utilized because they hybridize to the high copy number of RNA loci in microbial cells (222). 16S rRNA method is generally employed for identification of bacteria at molecular level. DNA extracted from sub cultured colonies by DNAzol method was subjected to 16S rRNA PCR. Following primers were used for PCR and DNA sequencing (223), Forward primer 20F 5’ATGTTGATCATGGCTCA3; Reverse primer 1540R 5’AAGGAGGTGATCCAACCGCA 3’ generates a PCR product of approximately 1.5 kbp (E. coli numbering system (224). PCR mix was prepared by adding 10 µl 10X Taq polymerase buffer, 4 µl of 10 mM dNTP mix, 40 picomoles each reverse and forward primers, 1U of Taq Polymerase enzyme to 67 µl of nuclease free water. To this 90 µl master mix, 10 µl of DNA template was added, mixed, and immediately spinned and divided into five equal aliquots in 200 µl capacity PCR tubes. PCR was carried out with 400 µM final dNTP mix, 40 picomoles each primers. Following conditions were used for thermal cycling. Initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 1 min, primer annealing at 50.2°C for 1 min and primer extension at 72°C for 2 min, with final extension at 72°C for 5 min. An approximately 1.5 kbp amplicon was observed in 1% agarose gel.
2.2.8.3 DNA electrophoresis for visualization of PCR products

Briefly, 0.5 g agarose was added to 50 ml of 1X TAE buffer for preparing 1% gel, mixed without shaking and placed for boiling under microwave oven for 1 min. When temperature of the boiling gel was lowered up to 65 - 70°C, 20 µl of 1mg/ml Ethidium bromide was added and mixed to 50 ml warm gel solution, allowed to cool further and casted with 10 comb mini gel casting mechanism, without forming bubbles in a gel. Submarine gel electrophoresis for DNA visualization was performed in Bio-Rad mini sub cell GT submarine electrophoresis systems using 1X TAE buffer at 75 volts for 45 minutes with Bio-Rad universal power pack. When the tracking dye migrates to anodal end of the gel, electrophoresis was stopped and gel was observed and documented in gel doc XR system.

2.2.8.4 DNA sequencing and BLAST tree analysis

PCR product was sequenced by Sanger’s Dideoxy chain termination method and subjected to electrophoresis by automated DNA Sequencer Applied Bio system ABI 3100 genetic analyzer. After DNA sequence data interpretation, nucleotide data subjected to NCBI BLAST analysis. The phylogenic analysis was constructed by using a Neighbor-Joining (NJ) method in MEGA4.1 software (225). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed by 16S rRNA sequence analysis. The culture was deposited in National Collection of Industrial Microorganisms (NCIM), Pune, India with the accession number NCIM 5330.
The sequence of the 16S rDNA gene shown maximum similarity with *Bacillus badius* and the strain further identified as *Bacillus badius* D1 which is now available in the Gene Bank with accession number HQ015711.

This culture identified as *Bacillus badius* by 16 S rRNA sequence and phylogeny constructed by Neighbour joining method (226).

2.2.9 Growth study:

Growth curve of the isolated bacterial strains is checked over the time period of 0 to 72 hours. The nutrient medium was inoculated with the pure culture of these strains and growth of the cells was monitored by checking its optical density at 600 nm with JASCO V-630 UV-visible spectrophotometer.

2.3 Results:

2.3.1 Screening:

Out of four bacterial isolates saffron colored colonies and off white colored colonies only survived on such aniline amended agar. Off white coloured colonies were found bigger size than other, indicating higher potential for degradation and therefore it was selected for further study. All microbes isolated from Lonar Lake showed change in tinge of pigmentation by varying little media composition.

2.3.2. Photographic image of screened bacteria on 0.4 g/L aniline amended agar.

![Photographic image of screened bacteria](image)

Fig (2.3.2) Photographic image of aniline resistant bacteria
**Fig 2.3.2** depicts the resistant colony of *Bacillus badius* D1 Single bacterial colony found resistant to 0.4 g/L aniline on aniline amended alkaline agar medium.

**2.3.3 Gram’s nature of potent xenobiotic degrading alkali phile:**

![Image](image1)

**Fig 2.3.3** Gram’s nature of experimental bacteria

**Fig 2.3.3** indicates the Gram’s nature of isolate. Violet colored cylindrical rods were observed indicating Gram positive bacillary appearance.

**2.3.4 Morphological study by Scanning Electron Microscopy (SEM):**

SEM of purified isolate Bacillus badius was taken in lower as well as higher magnification. Serially dilution of culture and repeated study of SEM confirmed that the isolated strain is having typical morphology of cylindrical shaped bacillus with spongy appearance at magnification X 25000 separately spread having size of 30 µm length and 0.1 µm width as shown in (Fig. 2.6.4) was observed in higher magnification.

![Image](image2)

**Fig 2.3.4** Scanning electron micrograph (SEM) of Bacillus badius D1
2.3.5. Sugar Fermentation Tests:

The bacterial strain Bacillus badius D1 was incubated with 0.5% sugars like dextrose, lactose, sucrose, galactose, maltose raffinose, cellobiose, arabinose, starch etc. and bromothymol blue 0.08 mg/100 ml in petridish containing alkaline agar media. The change in color from blue to green and finally yellow on Drigalaski fermentation medium indicated formation of acid in (Fig. 2.3.5.1)

Fig.2.3.5.1 The Sequential changes observed on Drigalaski fermentation medium

2. 3.6 Catalase, protease and Cytochrome c oxidase tests:

Presence of catalase was confirmed by decomposition of H₂O₂ and formation of oxygen bubbles (not indicated by Figure.)

The presence of protease was confirmed by gelatin liquification test (Fig. 2.3.6.1)

Fig. 2.3.6.1 Gelatin liquification
Fig. 2.3.6.1 Gelatin liquification test clearly indicated halo around the grown culture suggesting that it has proteolytic potential also.

Presence of Cytochrome C oxidase was indicated by change in color of 1% Dimethyl paraphenylene -diamine hydrochloride. Blue color changed blackish in a small while.

2.3.6.2 Cytochrome C oxidase

![Image of Cytochrome C oxidase test]

Fig. 2.3.6.2 Cytochrome C oxidase test.

The blue color of reagent immedietly changing to balck showed in Fig. (2. 3.6.2) the redox potential of isolated off white colored cylidrical Gram positive alkaliphile.

2.3.6.3 Endospore staining: Endospore observed by negative staining. Negative staining requires acidic stain like eosin or nigrosin. Due to negative chromogen and negative charge on bacteria the stain does not penetrate in the cell and it remain unstained giving internal transperant picture against color background. This technique does not require heat fixation.

2.3.6.4 H2S Production: Ammonium sulfate amended agar slants observed blackish due to H2S production possessing composition 3 g/L each of peptone, beef extract and agar. Rest of others contain 0.25g/L Fe₂(NH₄)SO₄, 0.25 g/LNa₂S₂O₃ in 1000 ml distilled water.

2.3.6.5 DNA Extraction: The extacted DNA absorbed UV-vis light giving sholder peak at 260 nm confirmed its purity.
2.3.6.6 Sequence amplification:

Isolated Bacillus radius D1 was further subjected for identification and characterization by 16S rRNA.

2.3.6.7 16S ribosomal RNA gene, partial sequence (HQ015711.1) of potent xenobiotic degrader alkaliphile

The 1339 base pair sequence showed high GC content [55.29%] than AT content [44.71%] similar to archaeabacteria.
2.3.6.8 DNA PCR product electrophoretic visualization:

Fig.2.3.6.8.1 PCR product from 16S rRNA of *B. badius D1*, Lane 1: Mol. wt. marker (500 base Pair DNA ladder), Lane 2: 1.5 kb desired PCR product.

Desired PCR product observed around 1.5Kb. DNA blast analysis matched maximum with *Bacillus badius*.

2.3.6.9 DNA sequence and Blast tree analysis based Phylogeny:

Fig 2.3.6.9 DNA sequence based blast tree analysis phylogeny

The phylogenetic diversity of microorganisms living at high salt concentrations is surprising. Halophiles are found in each of the three domains: Archaea, Bacteria, and Eucarya (227). Phylogenetic analysis used to investigate the ORFs (open reading frames) which are highly diverged at gene level (228). Divergent genomic GC content has evolved repeatedly in widely separated bacterial taxa (229). The potent xenobiotic
degrading bacteria showed more similarity with *Bacillus badius* strain. P27G10 (Fig.No.-2.3.6.9) on constructing phylogenetic tree on the basis of Gene Blast.

2.3.6.10. Growth curve:
The growth of bacterial isolate *Bacillus badius* D1 was studied by using spectrophotometric observations of optical density. Data collected from approximately 72 hr. monitoring using six hr. interval sampling of bacterial suspensions plotted against the time on normal graph.

![Growth curve of Bacillus badius](image)

Fig 2.3.6.10 Growth curve of Bacillus badius
The 72 hr. growth study with 100 ml broth indicted that after 48 hr. the growth was retarded probably due to exhausting the nutrients.

2.4 Discussion:
Various types of aromatics and nitroaromatics are ubiquitously distributed in the environment. Many of them are proved to be mutagenic teratogenic and carcinogenic. Increase in the content of these nitroaromatics during industrial activities and other human activities could increase the risk of cancer and other adverse health effects. To treat the environmental contamination, various types of physicochemical methods like flocculation, adsorption, membrane filtration, ozonization, peroxidation, chlorination etc.
are applied. Biological system has been widely used for environmental cleanup due to various aspects. Bioremediation using suitable microorganism is convinient approach of waste treatment and detoxification of contaminants. This treatment is an ecofriendly, relatively simple and cost effective alternative to physicochemical cleanup option. Degradation of organic compounds like hydrocarbons and nitroaromatics in the soil involves the utility of microorganisms having specific metabolic capacities. In contaminated environment specific organisms are abandunt because of the adaptive capability of microorganism to chemical contamination. It has been well documented that bacteria are the main agents for degradation of wide variety of chemical contamination. In present studies, we isolated, characterized and identified the bacterial strain for the degradation of mono or di substituted organic compounds. An alkaliphilic bacteriatal strain *Bacillus badius* D1 was isolated from soil sediment of alkaline Crater Lake of Lonar (MS) India. This alkaliphilic isolate was identified as *Baiillus badius* by 16S rRNA analysis and was deposited in National Collection of Industrial microorganism NCIM Pune, India with (accession No.**HQ015711.1**) The 16 S rRNA sequencing and polygenetic anlalysis indicated that the strain isolated was releated to genera bacillus shared more than 99% identity with their closest pylogentic relatives confirmed the strain *Bacillus badius* D1. The morphological studies carried by light microscopy showed the Gram positive nature while SEM indicated the cylindrical shape of isolated bacteria. The biochemicals tests carried for the same bacteria cleared that it has other activities like catalase, cytochrome C oxidase and protease, and sugar fermentation.

Several lines of investigation have reported the degradation potential of various neutrophilic bacterial strains towards the environmental contamination (230). In present study an attempt has been made to exmine the degradative potential of isolated *Bacillus badius* towards aromatic and nitroaromatic compounds. The longer exponential phase of strains keeps its enzyme ability to perform the biocatalytic activity efficiently. The microbial enzymes involved in the biotransformation of these compounds are mono and dioxygenase, azoreductases, nitroreductases, hydrolases, and transferases. The isolated strain *Bacillus badius* D1 has a promising degradative potential towards nitroaromatics.
In 72 hr approximately 80% degradation of aniline was observed at the concentration of 1.5 g/L in shaking condition at 37 °C. The other investigators also observed the similar potential of neutrophilic bacteria (231). Since the strain confirmed was of wild sort, acclimatization results reciprocated and found strain is of immense use in biotechnological processes to clean up the environment. Some of the reactions catalyzed by this strain can also be used in certain synthetic organic chemistry. Bacterial chemotaxis might have evolved as selective advantage to the bacteria for searching for the chemicals that can be used as a source of carbon and energy to the cells (232). Experimental data has suggested that strain is having potential for bioremediation purpose and biotechnological applications in verities of nitro aromatic compound polluted area. Degradation potential of this bacterium increased the applicability of this microorganism for nitro aromatic removal applications particularly the industrial waste. The microorganism is a good candidate for the bioremediation of hyper saline environment and treatment of saline waste.