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Bacterial Degradation and Corrosion of Naphtha in Transporting Pipeline

A. Rajasekar · S. Ponmariappan · S. Maruthamuthu · N. Palaniswamy

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Abstract Five naphtha hydrocarbon-degrading bacteria including representative strains of the two classified species (*Serratia marcescens*AR1, *Bacillus pumilus*AR2, *Bacillus carboniphilus* AR3, *Bacillus megaterium* AR4, and *Bacillus cereus* AR5) were identified by 16S rDNA gene sequence in a naphtha-transporting pipeline. The naphtha-degrading strains were able to be involved in the corrosion process of API 5LX steel and also utilized the naphtha as the sole carbon source. The biodegradation of naphtha by the bacterial isolates was characterized by gas chromatography–mass spectrometry. Weight-loss measurement on the corrosion of API 5LX steel in the presence/absence of consortia grown in naphtha–water aqueous media was performed. The scanning electron microscope observation showed that the consortia were able to attack the steel API 5LX surface, creating localized corrosion (pit). The biodegradation of naphtha by the strains AR1, AR2, AR3, AR4, and AR5 showed biodegradation efficiency of about 76.21, 67.20, 68.78, 68.78, and 68.15, respectively. The role of degradation on corrosion has been discussed. This basic study will be useful for the development of new approaches for the detection, monitoring, and control of microbial corrosion in a petroleum product pipeline.

Keywords Microbiologically influenced Corrosion · Carbon steel API 5LX · Naphtha · Biodegradation

A. Rajasekar (✉) · S. Ponmariappan · S. Maruthamuthu · N. Palaniswamy
Biocorrosion, Corrosion Protection Division, Central Electrochemical Research Institute, Karaikudi 630 006, India
e-mail: raja76sekar@rediffmail.com

Many studies have indicated the importance of microbial tampering of stored hydrocarbon fuels, which leads to the blocking of pipelines and filters, reducing fuel quality and resulting in corrosion of the pipeline [7, 21]. Corrosion, the major hallmark of pipeline failure, is the main component affecting the operation and maintenance costs of petroleum industry pipelines [3, 17]. It has been anticipated that 40% of all internal pipeline corrosion in the gas industry can be attributed to microbial corrosion [8]. It has been estimated that microbially influenced corrosion (MIC) causes damage of the order of hundreds of millions of dollars in the production, transport, and storage of oil every year in the U.S. oil industry alone [4]. The major bacteria involved in the MIC of oil- and gas-producing facilities as well as in transportation and storage facilities are anaerobic sulfate-reducing bacteria (SRB) [9, 23, 30]. However, aerobic bacteria and fungi might also participate in the corrosion process [2, 20, 26]. The microorganisms influence the corrosion by altering the chemistry at the interface between the metal and the bulk fluid [15, 18]. The most extensively studied microorganisms in relation to biocorrosion are the SRB, whose participation in corrosion was evidenced decades ago [30] in oil- and gas-producing facilities as well as in transportation and storage facilities. Therefore, most of the research on MIC has focused on SRB. However, recent studies suggest that SRB need not be present in abundance in all microbial communities responsible for MIC [13, 34]. We recently noticed the domination of chemolithotrophic bacteria in a diesel-transporting pipeline on corrosion in the absence of SRB in diesel and naphtha pipelines [20, 22, 25, 26, 27]. The types and ability of microorganism to degrade petroleum hydrocarbons have been widely documented [5, 10]. Jana et al. [12] carried out a failure analysis study in oil pipelines at Mumbai offshore and concluded that the combined effect of CO_2, SRB, and

chloride in the low-velocity area caused the severe corrosion and failure of the pipeline. Muthukumar et al. [22] reported the degradation of diesel in the presence of microbes and noticed the role of degradation on corrosion. Rajasekar et al. [25] proposed the microbial corrosion mechanism in a naphtha-transporting pipeline and storage tank in South West India. The length of the pipeline was 5.5 km and corrosion products (about 10 kg) were collected from the pipeline every 2 months. The microbial growth in the sludge often causes severe turbidity and cloudiness of naphtha. Moreover, sludge often changes the actual chemical properties of naphtha in the storage tank and in transporting pipelines. Biocorrosion studies involving the use of natural individual species obtained from industrial systems are scarce. However, such studies would better address the actual problem and increase the understanding of the microbial species involved in microbial corrosion and their interactions with metal surfaces. In the present study, the nature of degradation of naphtha by five bacterial species in a pipeline and its effect on corrosion have been assessed and discussed.

Materials and Methods

Collection of Samples and Characterization of Bacteria

The bacteria were isolated from corrosion product, sludge, and naphtha–water interface as described by Rajasekar et al. [25]. The isolated bacteria were grouped into various genera as per *Bergey's Manual of Determinative Bacteriology* [11]. These cultures were characterized depending on their morphology, Gram staining, spore staining, motility, oxidase, catalase, oxidation fermentation, gas production, ammonia formation, nitrate and nitrite reduction, indole production test, methyl-red and VOGES-PROSKAUER test, citrate and mannitol utilization test, hydrolysis of casein, gelatin, starch, urea, and lipid,

16S rRNA Gene Sequencing, and Phylogenetic Analysis

Genomic DNA was isolated according to the method described by Ausubel et al. [1]. Amplification of the gene encoding for small subunit ribosomal RNA was done using eubacterial 16S rDNA primers [forward primer: 5′ AGAGTTTGATCCTGGCTCAG 3′ (*E. coli* positions 8 to 28); reverse primer: 5′ ACGGCTACCTTGTTACGACTT 3′ (*E. coli* positions 1477 to 1498)] [31]. Polymerase chain reaction (PCR) was performed with a 50-μL reaction mixture containing 2 μL (10 ng) of DNA as the template, each primer at a concentration of 0.5 μM, 1.5 mM $MgCl_2$, and each deoxynucleoside triphosphate at a concentration of 50 μM, One microliter of *Taq* polymerase and buffer were used as recommended by the manufacturer (MBI Fermentas). PCR was carried out using a programmable Mastercycler Personal (Eppendorf, Germany) with the following temperature profile: initial denaturation at 95°C for 4 min; 35 cycles of denaturation (1 min at 95°C), annealing (1 min at 50°C), and extension (2 min at 72°C), followed by a final extension at 72°C for 8 min. The amplified PCR product was loaded on a low-melting agarose gel and subjected to electrophoresis. A band of approximately 1.5 kb in length was cut, eluted, and purified using the GFX^{TM} PCR DNA and Gel Band Purification kit (Amersham Biosciences). The purified PCR products were quantified by gel estimation and used for the sequencing reaction. The sequencing reaction was carried out using an ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems); for the sequencing reaction, the Big Dye Ready Reaction DyeDeoxy Terminator Cycle Sequencing kit (Perkin-Elmer) was employed. Six internal primers were used to generate large overlapping sequences that are useful for careful editing of the sequences. The deducted sequences were subjected to a BLAST search of the NCBI database for sequence homology and closest match. The 16S rDNA sequences are deposited in the Gene Bank (Accession Nos. DQ207558, DQ207559, DQ207560, DQ207561, and DQ207562). The sequences were aligned with closely related sequences in the database by clustal W (http://www.ebi.ac.uk/clustalw/). The pairwise evolutionary distances were computed using the DNADIST program with the Kimura 2–parameter model as developed by Kimura [16]. The phylogenetic trees were constructed by using four tree–making algorithms, the UPGMA, KITSCH, FITCH, and DNAPARS of the PHYLIP package [6]. The stability among the clads of a phylogenetic tree was assessed by taking 1000 replicates of the dataset and it was analyzed using the programs SEQBOOT, DNADIST, UPGMA, and CONSENSE of the PHYLIP package. Amplification of gene encoding for small subunit ribosomal RNA of all the naphtha-degradative strains (NDSs) was done using eubacterial 16S rDNA primers. The 16S rDNA amplicons derived from NDSs were cloned in the pTZ57R/T vector. The recombinant plasmid (pNDS, harboring the 16S rDNA insert) was partially sequenced. The sequence obtained was matched with the previously published sequences available in the NCBI database using BLAST.

Preparation of Inoculum

Serratia marcescens AR1, *Bacillus pumilus* AR2, *Bacillus carboniphilus* AR3, *Bacillus megaterium* AR4, and *Bacillus cereus* AR5 were selected for biodegradation study and

were isolated from a naphtha pipeline in southwest India. A loopful of culture was inoculated into 100 mL of sterile nutrient broth. The flasks were incubated and shaken using an orbital shaker at 200 rpm, for 12 h at 30°C. A 1-mL volume of the culture broth from each of the above five isolates were mixed to prepare a mixed bacterial consortium for the corrosion study.

Biodegradation of Naphtha

Bushnell–Hass (BH) broth (0.20 g/L magnesium sulfate, 0.02 g/L calcium chloride, 1 g/L monopotassium phosphate, 1 g/L dipotassium phosphate, 1 g/L ammonium nitrate, 0.5 g/L ferric chloride; Hi-Media, Mumbai) was used for biodegradation studies. Bacterial cultures AR1, AR2 AR3, AR4, and AR5 were precultured overnight at 30°C in BH broth medium. The individual bacterial cultures were transferred (initial load about 2.1×10^4 CFU/mL) to a 250-mL Erlenmeyer screw-cap flask (to prevent loss of volatile naphtha hydrocarbon), each containing 100 mL of sterile defined mineral salts medium (BH) with 10 g/L of naphtha fuel as a sole carbon source. An uninoculated control flask was also incubated in parallel to monitor abiotic losses of the naphtha fuel substrate. The flasks were incubated at 27°C and kept in an orbital shaker at 150 rpm for 15 days. All of the experiments were done in triplicate. At 2-day intervals, a set of flasks was used for the enumeration of microbial population by the pour plate technique using plate-count agar (HI-MEDIA, Mumbai, India).

Gas Chromatographic–Mass Spectrometry Analysis

Biodegradation of naphtha was monitored by quantitative gas-chromatographic (GC) analysis. At the end of the 15 days of the incubation period, the residual naphtha for each system of the entire flask was extracted with an equal volume of dichloromethane. A fixed quantity of 1 μL of the resultant solution (naphtha) was analyzed by GC–mass spectrometry (MS) by means of Thermo Finnigan GC-MS. A trace MS equipped with a RTX-5 capillary column (30 m long × 0.25 mm internal diameter) and flame ionization detector (FID) was used with high-purity nitrogen as the carrier gas. The oven was programmed between 80°C and 250°C at a heating temperature of 10°C/min. The degradation of naphtha oil as a whole was expressed as the percentage of naphtha oil degraded in relation to the amount of the remaining fractions in the appropriate abiotic control samples. The biodegradation efficiency (BE), based on the decrease in the total concentration of hydrocarbons, was evaluated by using the following expression as described by Michaud et al. [19]: BE (%) = $100 - (A_s \times 100/A_{ac})$, where A_s is the total area of peaks in each sample, A_{ac} is the total area of peaks in the appropriate abiotic control, and BE (%) is the efficiency of biodegradation

Corrosion Studies: Weight-Loss Method

API 5LX steel grade (C-0.29 max, S-0.05 max, P-0.04 max, Mn-1.25 max, balance Fe) coupons of size $2.5 \times 2.5 \times 0.5$ cm were mechanically polished to a mirror finish and then degreased using trichloroethylene. In the present study, 500 mL of naphtha with 2% of water (collected from the naphtha storage tank, which contains about 120 ppm chloride) has been used as the control system, whereas 500 mL naphtha with 2% of water and inoculated mixture cultures (2.1×10^5 CFU/mL) of NDSs were used as the experimental system. After 7 days, the coupons were removed and pickled, washed in water, and dried using an air-dryer. Final weights of the six coupons in each system were taken and the average corrosion rates were also calculated. The standard deviations for each system are presented. All of the experiments were performed in duplicate. A computer-controlled X-ray diffraction technique (XRD) was used for determining the nature of the oxides present in the corrosion product. The dried corrosion products were collected from the experimental system and crushed into a fine powder and used for XRD analysis. JEOL Model JDX-8030 was used to scan between 10° and 85° – 2θ with copper $K\alpha$ radiation (Ni filter) at a rating of 40 kV, 20 mA.

Enumeration of Bacteria by Acridine Orange Direct Counts (AODCs)

After the corrosion study, the metal API 5LX coupon was stained with 1 mL of 0.1% (w/v) acridine orange, for 15 min. Green- and red-fluorescing cells were counted in 20 randomly selected field with a Nikon epifluorescent microscope (E200; Nikon, Tokyo; equipped with a 10×/0.25 numerical aperture and 100×/1.25 numerical aperture) using a 100× objective and a 10× eyepiece. Plan objectives lens and Nikon filter set for green excitation were used Microscopic images were captured using a digital CCD. The numbers of bacteria were enumerated from counts of 10 microscopic fields (at 1000×) using replicates for each sample to confirm the bacterial attachment on the metal coupons

Results and Discussions

Phylogenetic characterization and environmental scanning microscopy analysis of a corrosive consortium of a sour

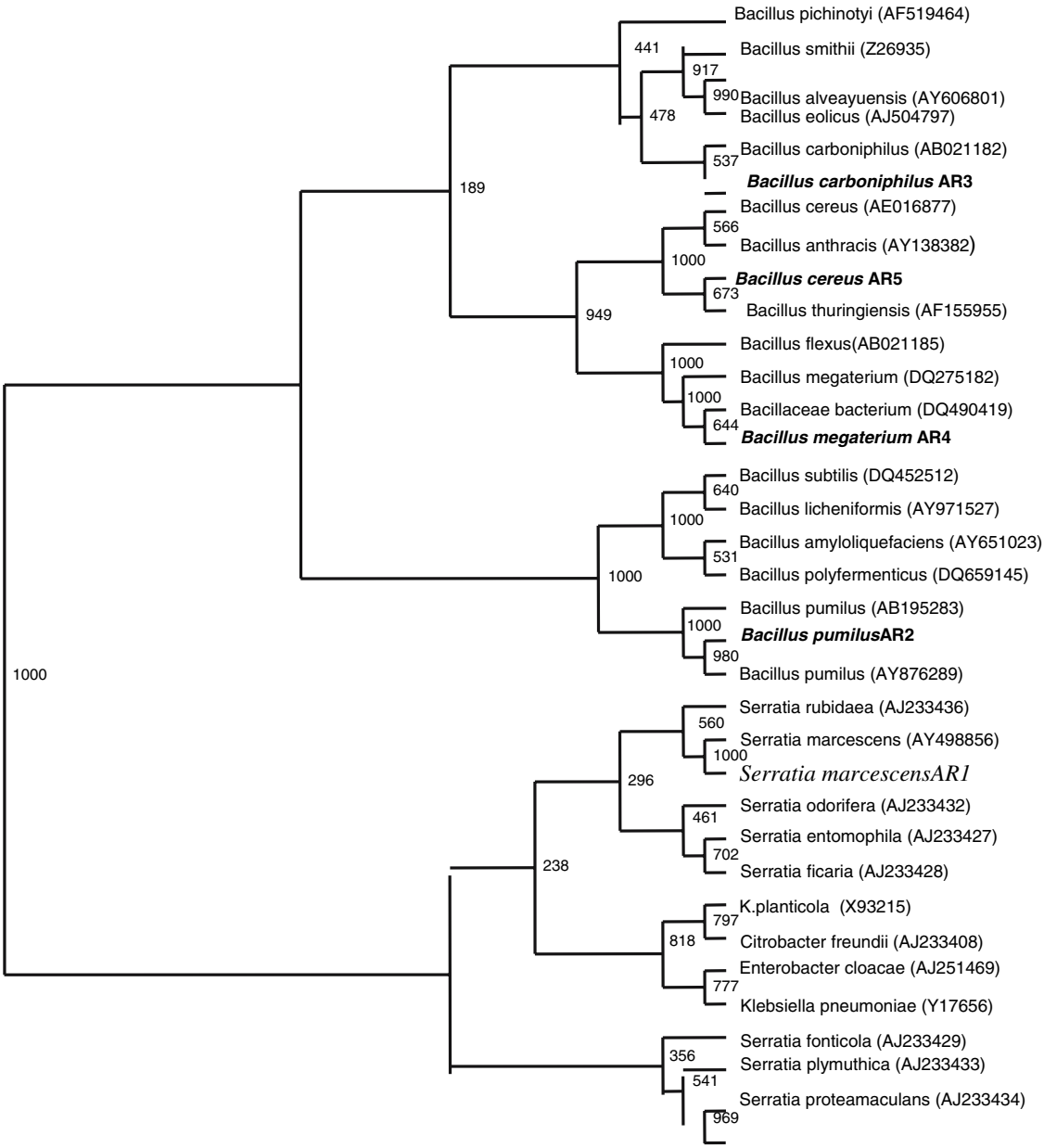

Fig. 1 UPGMA phenogram showing the phylogenic position of strain AR1 to AR5 within the genus Bacillus and Serratia based on 16S rRNA gene sequence analysis. Bootstrap values are given at the nodes

gas (H_2S containing natural gas) pipeline revealed the low abundance of SRB in the sour gas pipeline [13]. Rajasekar et al. [25] also noticed the involvement of aerobic bacteria on corrosion, whereas SRB could not be found in the naphtha pipeline. It was explained that the flow velocity might create a uniform distribution of oxygen, which might suppress the distribution of SRB [20]. Additionally, Maruthamuthu et al, [20] suggested that the water-soluble corrosion inhibitor addition in the petroleum product pipeline reduced the pH and suppressed the SRB proliferation in the petroleum product pipeline. Hence, an investigation of the role of naphtha-degradative bacteria on the corrosion process is worthwhile in the naphtha pipeline in the absence of SRB. The naphtha hydrocarbon-biodegradative strains were isolated from the corrosion product of the naphtha-transporting pipeline in southest India. The preliminary identification of NDSs by the biochemical test indicated that the isolate belonged to the genus *Bacillus* sp. and *Serratia* sp. Sequence alignment and comparison revealed more than 99% similarity with *S. marcescens* AR1, *B. pumilus* AR2, *B. carboniphilus* AR3, *B. megaterium* AR4, and *B. cereus* AR5, The nucleotides sequences data have been deposited in GenBank under the sequence numbers DQ207558, DQ207559, DQ207560, DQ207561,

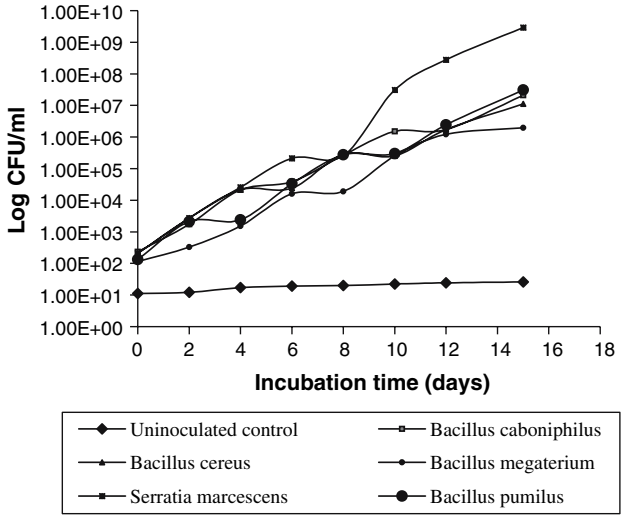

Fig. 2 Bacterial consortia in BH medium with naphtha as a sole carbon source

and DQ207562, respectively. The UPGMA phenogram of the 16S rRNA gene sequences of the bacterial isolates revealed 90% similarity of the hydrocarbon-degrading bacterial community present in the naphtha pipeline (Fig. 1). The four isolates (AR2, AR3, AR4, and AR5) belong to the Bacillaceae family and one isolate (AR1) was in the Enterbacteriacae family.

The bacterial isolates exhibited excellent growth in naphtha as a sole carbon source during biodegradation study. All five individual isolates showed maximum activity and the bacteria *S. marcescens* AR1 was increased up to 10^9 CFU/mL during biodegradation (Fig. 2). The naphtha oil degradation and population also correspondingly increased. Viable count data indicated that naphtha had a positive effect on microbial growth (Fig. 2). The GC retention data of the naphtha fuel corresponding to structural assignations done after a library search of a database and by mass spectra interpretation are presented in Table 1. From the GC-MS analysis, it was observed that the naphtha (uninoculated system) consists of aliphatic hydrocarbons, including cyclopentane, 1-3-dimethyl (1-3-dimethyl cyclopentane), heptane, cyclohexylmethene, 1-nonanol (nonyl alchol), cyclopentane, 1,2,3-trimethyl, heptane, 2-methyl, decane-1-chloro cyclohexane, 1-3, dimethyl – (cic-1,3-dimethyl cyclohexane), cyclohexane, 1-2-dimethyl, cyclohexane, 1-2-dimethyl, 1,3-dimethyl cyclohexane, octane, 2-methyl ethyl cyclohexane, 2,3-dimethyl heptane, 4-methyl octane, ethyl benzene, *p*-xylene, *o*-dimethyl benzene ethyl benzene, and ethyl benzene, and the major

Table 1 Percentage of biodegradation of naphtha hydrocarbon in presence of NDSs

RA	Compounds	RA	AR1	BE (%)	AR2	BE (%)	AR3	BE (%)	AR4	BE (%)	AR5	BE (%)
0.61	Cyclopentane, 1-3-dimethyl (1-3-dimethyl cyclopentane)	17	0	100	0	100	11	35	11	35	0	100
0.63	Heptane	36	0	100	0	100	36	100	22	39	0	100
0.71	Cyclohexylmethene	75	66	12	66	12	49	35	45	40	45	40
0.74	1-Nonanol (nonyl alcohol)	22	0	100	0	100	0	100	0	100	0	100
0.78	1,2,3-Trimethyl cyclopentane	18	0	100	0	100	0	100	0	100	0	100
0.85	2-Methyl heptane,	50	0	100	25	50	0	100	0	100	40	20
0.89	1-Chloro decane	62	0	100	39	37	59	5	52	18	43	31
0.92	Cyclohexane, 1-3, dimethyl – (cic-1,3-dimethyl cyclohexane)	80	65	49	60	25	65	19	63	79	79	1
0.97	1-2-Dimethyl cyclohexane,	29	0	100	0	100	0	100	0	100	0	100
1.02	1-2-Dimethyl cyclohexane,	100	72	28	62	38	0	100	81	19	79	23
1.06	1,3-Dimethyl cyclohexane	24	0	100	0	100	0	100	0	100	0	100
1.20	2-Methyl octane,	30	22	27	29	3	24	25	0	100	0	100
1.26	Ethyl cyclohexane	53	39	26	48	9	40	20	50	6	41	22
1.44	2,3-Methyl heptane	8	0	100	0	100	0	100	0	100	0	100
1.53	4-Methyl octane	4	0	100	0	100	0	100	0	100	0	100
1.59	Ethyl benzene	13	0	100	0	100	0	100	0	100	0	100
1.64	*p*-Xylene	49	26	53	42	14	33	33	34	30	20	59
1.79	*o*-Dimethyl benzene ethyl benzene	8	0	100	0	100	0	100	0	100	0	100
1.95	Ethyl benzene	10	0	100	0	100	0	100	10	100	0	100
	Total biodegradtion effeciency (%)			76.21		67.20		68.78		68.78		68.15

Note: RA = relative abundance (%), AR1 = *Serratia marcescens*, AR2 = *Bacillus pumilus*, AR3 = *Bacillus carboniphilus*, AR4 = *Bacillus megaterium*, AR5 = *Bacillus cereus*

Table 2 Corrosion rate of carbon steel API 5LX in presence/absence of mixed cultures

System	Weight loss (g)	Corrosion rate (mm/year)	Forms of corrosion
Control: 500 mL naphtha + 2% water (120 ppm chloride).	0.0784 ± 2	0.0676	Uniform
Test: 500 mL naphtha + 2% water (120 ppm chloride) + mixed cultures	0.1580 ± 1	0.1362	Blisters

component is cyclohexane, 1-2-dimethyl. The result indicates that *S. marcescens* AR1 utilizes all of the aliphatic and aromatic components, including nonyl alchol, cyclopentane, 1,2,3-trimethyl heptane, 2-methyl, decane-1-chloro, 2,3-dimethyl heptane, 4-methyl octane, ethyl benzene, *o*-dimethyl benzene ethyl benzene, and ethyl benzene completely. *B. pumilus* AR2 utilizes cyclopentane, 1-3-dimethyl (1-3-dimethyl cyclopentane), heptane, cyclohexylmethene, nonyl alchol, cyclopentane, 1,2,3-trimethyl, cyclohexane, 1-2-dimethyl, 1,3-dimethyl cyclohexane, octane, 2-methyl, 2,3-dimethyl heptane, 4-methyl octane, ethyl benzene, *o*-dimethyl benzene, and ethyl benzene. *B. carboniphilus* AR3 utilizes cyclopentane, 1-3-dimethyl (1-3-dimethyl cyclopentane), heptane, nonyl alchol, cyclopentane, 1,2,3-trimethyl, cyclohexane, 1-2-dimethyl, 1,3-dimethyl cyclohexane, octane, 2-methyl, 2,3-dimethyl heptane, 4-methyl octane, ethyl benzene, *o*-dimethyl benzene, and ethyl benzene. *B.megaterium* AR4 utilizes cyclohexylmethene, cyclo hexane, 1-3, dimethyl-(cic-1,3-dimethylcyclohexane), cyclohexane, 1-2-dimethyl, ethyl cyclohexane, and *p*-xylene. *B. cereus* AR5 utilizes cyclopentane, 1-3-dimethyl (1-3-dimethyl cyclopentane), heptane, cyclohexylmethene, nonyl alchol, cyclopentane, 1,2,3-trimethyl, cyclohexane, 1-2-dimethyl, 1,3-dimethyl cyclohexane, octane, 2-methyl, 2,3-dimethyl heptane, 4-methyl octane, ethyl benzene, *o*-dimethyl benzene, and ethyl benzene. All of the strains degrade almost all of the hydrocarbon present in the naphtha, which is a common feature of many other alkane-degrading microorganisms [24, 26, 28, 29, 32, 33, 34]. The identified species AR1, AR2, AR3, AR4, and AR5 show the total biodegradation efficiency of about 76.21, 67.20, 68.78, 68.78, and 68.15, respectively (Table 1). Bacteria *S. marcescens* AR1 showed maximum (76.21%) degradation and reached a population size of about 2.92×10^9 CFU/mL. The finding suggests that the strain *S. marcescens* AR1 has a high probability to degrade both aliphatic and aromatic components present in the naphtha.

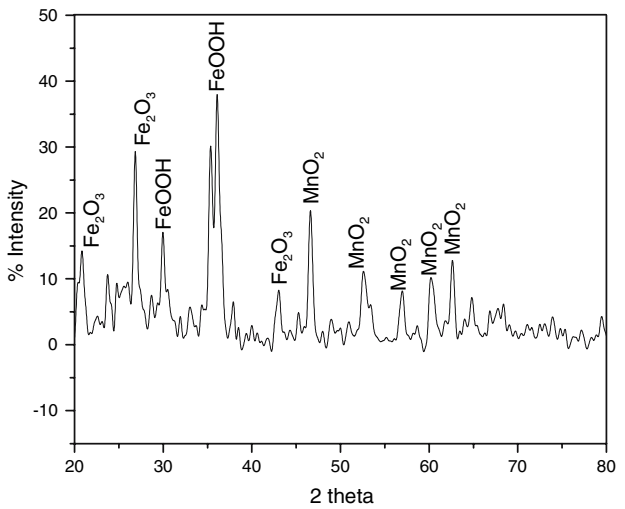

Fig. 3 XRD pattern of corrosion product of experimental system, inoculated with Naphtha degrading bacteria (NDA)

The corrosion rate of API 5LX in the presence/absence of the consortium is presented in Table 2. The corrosion rate for API 5LX was in the range of 0.0676 mm/year in the control system. In the presence of mixed cultures, the corrosion rate was higher in the range of 0.1362 mm/year when compared to the absence of bacteria. Additionally, blisters were noticed in the presence of bacterial consortia, whereas uniform corrosion was noticed in the control system [25]. After the weight-loss study, the number of total bacteria on the metal surface was determined by employing an epifluorescence microscope. The total bacteria count was in the range of 10^4 cells/cm^2 Jobson [14] also reported that intermediate hydrocarbon-degradation products make energy sources available for the physiological activities of the corrosion bacterium *Desulfovibrio* sp. Rajasekar et al. [25] also noticed the utility of naphtha as the carbon source by the bacterial community present in the field study. The present study supports the observation made by Rajasekar et al. [25]. NDSs were facultative anaerobic. Biochemical characteristics indicate the presence of catalase and cytochrome oxidase. Because NDSs have a peroxidase enzyme, they produce hydrogen peroxide for the degradation of petroleum products. Moreover, they produce catalase for overcoming the toxic nature of hydrogen peroxide. During the respiratory process, oxygen is consumed by NDSs and converted into water, wherein H^+ is utilized from the degraded product and electrons are supplied by a cytochrome oxidase enzyme [22, 25]:

$$^1/_2O_2 + H + + 2e^- \rightarrow H_2O.$$

It can be concluded that Fe^{2+} comes from the metal surface and combined with organic degraded products,

whereas hydrocarbon is degraded by identified strains and Fe^{2+} converted to Fe^{3+} by the addition of oxygen:

$$Fe^{2+} + {}^{1}/_{4}O_2 + H^{+} \rightarrow Fe^{3+} + {}^{1}/_{2}H_2O.$$

Figure 3 presents the details of XRD data corresponding to the phases present in the corrosion product sample collected from experimental systems. Higher-intensity peaks of ferric oxide (Fe_2O_3), iron oxide hydroxide [FeO(OH)], ferrous hydroxide [$Fe(OH)_2$], and manganese-di-oxide (MnO_2) were noticed, indicating the role of NDSs on manganese/iron deposition during the formation of the corrosion product and accelerating the microbial corrosion directly in the pipeline.. NAD converts ferric to ferric oxides by the inclusion of oxygen from the degraded products and forms organic complex [25, 26]. Because ferric/ manganese has a high affinity to oxygen, it takes oxygen from the degraded product and encourages the formation of ferric/manganese oxides and accelerates the corrosion.

It can be concluded that the degraded organic compounds in naphtha encourages the growth of bacteria and enhances the formation of corrosion products like ferric oxide and manganese oxide. Subsequently, all of the strains encourage the corrosion process by the formation of Fe_2O_3. Degraded carbon (diesel) acts as a good nutrient for bacteria, which increases the proliferation of bacteria on the steel and determines the nature of degradation and corrosion in the petroleum product pipeline. The present laboratory study support the observation made in the field study by Rajasekar et al. [25].

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