

Decolorization of Congo red-mediated by marine *Alcaligenes* species isolated from Indian West coast sediments

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Running Head: *Alcaligenes* sp. mediated decolorization of Congo red

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Abstract:

Alcaligenes species capable of degrading highly recalcitrant, carcinogenic, water soluble dye – Congo red were isolated from Indian West coastal sediments. Individual strains showed decolorization rates ranging from 76.49-98.76% within 24-48h. Decolorization was most efficient at anoxic conditions catalyzed by intracellular azoreductase enzyme with an activity of 0.032 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ of protein. Degradation was confirmed by HPLC and FTIR analysis. LC/MS analysis of degraded metabolites established the cleavage of the Azo bond producing biphenyl diamine and 1,2'-diaminonaphthalene-4-sulfonic acid. These results signify the effectiveness and ease to engineer processes such as feed batch/immobilized-cell systems using these strains as biocatalysts to address the problem of global coastal water pollution caused by increased disposal of Azo dye containing industrial effluents.

Keywords: Congo Red (CR); Marine microbial decolorization; UV-Visible spectrophotometer; FTIR; HPLC; LC/MS

Introduction:

Azo dyes compose the major versatile subset of synthetic dyes expansively exploited in paper, wood, textile, cosmetics, food processing and pharmaceutical industries (Hiral et al. 2015; Kalyani et al. 2009; Robinson et al. 2001). Most of these are xenobiotics, extremely recalcitrant towards degradation amid the potential for accumulation and prolonged existence at elevated levels in surroundings (Talarposhti et al. 2001; Thakur et al. 2014). Congo red [CR], ($C_{32}H_{22}N_6Na_2O_6S_2$), one such anionic carcinogenic diazo dye with chemical name 3, 3'-[(1, 1'-biphenyl)-4, 4'-diylbis (azo)] bis-(4-amino-1-naphthalene acid) disodium salt, is a symmetrical linear molecule. It is composed of a central hydrophobic center consisting of two phenyl rings, linked through diazo bonds to two charged terminal naphthalene moieties. These terminal parts of CR contain sulfonic acid and amine groups (Romhanyi 1971). The color developed by infinitesimal amounts of highly dense untreated dyes notably decreases photosynthetic activity by lowering the light penetration besides having possible toxic and even carcinogenic effects, eventually affecting the biotic system adversely, including plants, animals and humans (Anjaneya et al. 2011). It is well established that pollution worsens the quality of life in various aspects such as health and life span. Overall circumstances dictate the necessity of bioremediation for reduction of pollutants.

Physico-chemical techniques for instance adsorption, coagulation-flocculation, filtration, radiation, oxidation, photocatalytic processes have gained favor to treat effluents to accomplish decolorization. However, execution of these methods has inherent drawbacks of being economically unfeasible coupled with incapability to remove the dye completely, generation of a considerable amount of sludge initiating secondary pollution problems and involvement of complicated procedures. Microbial remediation has been a considered ideal as it is inexpensive and eco-friendly. Microbial species of bacteria, actinomycetes, fungi and algae are proficient of eliminating Azo dye via biodegradation or liberalization, biotransformation, mineralization (Kaushik et al. 2015; Kumar et al., 2015) and their effectiveness varies with the survival, adaptability and activity of the selected organism and the oxidation potential of the Azo dye. The mechanism of microbial azo dye degradation involves reductive cleavage of azo bonds ($-N=N-$) catalyzed by azoreductase under anaerobic conditions resulting in wastewater by product. The resulting intermediates (e.g., aromatic amines) are subsequently broken down aerobically/anaerobically (Saratale et al. 2009). A wide variety of organisms has been testified for decolorization of CR and other textile dyes specifically *Aeromonas caviar*, *Proteus mirabilis*, *Rhodococcus globerulus* (Joshi et al. 2008), *Sphingomonas* spp., *Bacillus* spp., (Kumar et al. 2007; Gopinath et al. 2009) *Alternaria alternate*, (Chakraborty et al. 2013) *Shewanella sciamensis* (Ng et al. 2013). Most of the studies are focused on

fungi however long growth cycle, the requirement of limiting growth conditions, long retention time, these entire limit the decolorization process on the contrary to bacterial decolorization.

The current study deals with isolation of *Alcaligenes sp.* capable of decolorizing the industrial diazo dye CR within static anoxic condition. Seeing that the bacteria are proficient in the dye degradation optimization of various physicochemical conditions was studied to achieve faster decolorization. Intermediates formed have been examined using analytical techniques to establish the fate of CR metabolism by *Alcaligenes sp.*

Materials and method:

Dyestuff chemicals and microbiological media:

Congo red Certified ($C_{32}H_{22}N_6Na_2O_6S_2$) Mol.wt. : 696.68 ($\lambda_{max} = 498nm$) was procured from Himedia Laboratories Pvt. Ltd. Mumbai India. Culture media such as Nutrient broth and agar Luria-Bertani agar Biochemical test kit and stains for Gram nature analysis Mueller Hinton agar and Hexa disc I and II for antibiotic susceptibility tests were purchased from Hi-media Laboratory India. Chemicals for sample preparation for the UV-Visible spectrophotometer, FTIR, HPLC, LC-MS analysis such as methanol ethyl acetate (ultra-pure grade) were procured from Merck Specialties Pvt. Ltd. Mumbai India and were of analytical grade.

Sampling Sites:

Northwest coast of India stretches about 2130 km long with numerous west flowing river mouths creeks bays. Throughout the peninsular stretch Maharashtra-Gujarat coastal belt is a recipient of a variety of wastes. A pragmatic approach was undertaken to obtain potential CR decolorizing strains from metal and hydrocarbon contaminated sediments of Ulhas, Veraval and Mumbai as in Fig. 1 and Table 1.

Bio-decolorizing bacterium screening:

Sediment samples were amassed from coastal sites in and around Ulhas, Veraval and Mumbai of India using the Van Veen's Grab and were stored at 4°C until analysis. Ten grams of soil sample was agitated in 50mL of the saline (0.8% NaCl) solution and kept on Rotospin rotary mixer at 150 rpm for 30 min. Microorganisms comprising the capability to degrade dye were screened by inoculating 5mL of the supernatant obtained subsequently on settling the soil for 20 min. into nutrient broth with 50% sea water containing 100 ppm CR dye and incubated at 37°C under shaker conditions for a week. Colonies were decided on the basis of their ability to form clear zones on nutrient agar plates supplemented with 200ppm CR dye. The isolated organisms were principally adjudged based on phenotypic characteristics. The bacterial isolates showing positive implications of growth and decolorization with elevated

concentration of CR dye were sub-cultured regularly and preserved on Luria-Bertani (LB) agar slants stored at 4 °C and as glycerol stocks at -80 °C.

Extraction of DNA and 16S rRNA gene sequencing:

For molecular characterization genomic DNA was mined out using DNeasy kit (Qiagen) and 16S rRNA was amplified using universal bacterial primers; 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'CGGTTACCTTGTTACGACTT-3') and plasmid extraction was carried out. Amplification was carried out under standard conditions (initial denaturation at 94 °C for 3 min 30 cycles of denaturation at 94 °C for 30 s 30 cycles of annealing at 57 °C for 30 s extension at 72 °C for 1 min and final extension at 72 °C for 5 min hold at 4°C) using Eppendorf vapor.protect PCR. Amplified PCR products were purified using Qiagen QIAquick PCR Purification Kit and the amplicons were sequenced by Sanger's dideoxy method (SciGenom Labs Pvt. Ltd India.). Nucleotide sequences were analyzed by Blast-n site at NCBI server (www.ncbi.nih.gov/BLAST) and deposited in NCBI for requisite accession no. Sequences were aligned using CLUSTALW program V1.82 on European Bioinformatics site (www.ebi.ac.uk/clustalw) (Saralate et al. 2013). Phylogenetic tree was deduced using the aligned sequences by the Neighbor-joining method conducted in MEGA6 software and the maximum likelihood method was employed for calculating evolutionary distances (Tamura et al. 2013).

Antibiotic susceptibility testing:

Antibiotic resistance was done by an agar diffusion test as described by Bauer et al. (1996) using Mueller-Hinton agar according to Clinical and Laboratory Standards Institute (CLSI) recommendations (Bauer et al. 1996; CLSI. 2005). For antibiotic resistance determination isolates were grown in Luria-Bertani (LB) broth until the turbidity was 0.5Mc Farland standards. Isolates were swabbed on Mueller-Hinton agar and all isolates were tested against Hexa-discs of Hexa I, II. All plates were incubated for 16-18 hrs at 37°C and zone of inhibition was measured for each disc as the standard protocol (NCCLS 2002). Ten antimicrobial agents belonging to different groups viz aminoglycosides cephalosporin quinolones β -lactam surfactants macrolides and glycopeptides were used for sensitivity testing.

Bio-Decolorization quantification:

To determine dye decolorization efficiency cultured cells were amassed from the 6 h culture stock in 50mL LB medium at 37 °C with shaking speed of 150 rpm (Genetix GX-2102C). Bacterial cells were centrifuged at 8000g for 5 min and rinsed twofold with deionized water. The apposite biomass was inoculated into sterile LB broth along with dye for bio-decolorization test. The cell suspension was transferred to a 250 ml Erlenmeyer flask holding 100 ml fresh autoclaved nutrient broth supplemented with 100ppm of CR dye to reach an initial cell mass (OD 600nm) at 0.250 in triplicates. All the

decolorizing reactions were incubated on shaker 120 rpm at 37°C and aliquots of samples were withdrawn at 0, 6, 12, 18, 24 and 48 h respectively. Centrifuged at 8000g for 5 min (Eppendorf Centrifuge 5810R) and the absorbance of supernatants were measured using UV-vis Spectrophotometer (Shimadzu UV-1800 Japan) at 498 nm (λ_{\max}). Decolorization activity was computed according to the formula: Dye Decolorization activity (%D) = (A-B)/A×100 in which A corresponds to initial absorbance and B to final absorbance (Babu et al. 2015).

Effects of physicochemical operational parameters:

Individual strains cultured overnight in the nutrient broth were monitored for decolorization of CR at 37°C under static anoxic as well as shaking (120 rpm) condition. Decolorization rate with altered initial concentrations of CR (50–1000 ppm) was assessed by using individual strains at 37°C under stagnant condition. Studies on the upshot of temperature (4–75°C) and pH (3–9) were carried out in the nutrient broth (dye concentration 100ppm) under static anoxic condition. All decolorization tests were executed in triplicates. Abiotic controls (absence of microorganism) were integrated. The dye decolorization activity (%D) was computed as described by Babu et al. 2015. The average decolorization rate ($\text{mg L}^{-1} \text{h}^{-1}$) was calculated (Jadhav et al. 2008b) as follows: Average decolorization rate = $\Delta C(t) / \Delta t$ where $\Delta C(t)$ (mg L^{-1}) is the change in dye concentration over the time interval Δt (h); wherein the Standard Equation for concentration of the dye was given by $y = 0.0016x$ ($r^2=0.997$); where y is the absorbance and x is the concentration of the dye in ppm.

Enzyme activities:

These strains were screened for enzyme production namely urease oxidase catalase phosphatase protease DNase protease and lipase. The presence of azoreductase enzyme was determined by methods proposed by Vijaykumar et al. (2007) and Anjaneya et al. (2011) performed in triplicates. Bacterial cultures were grown in the Nutrient broth supplemented with Congo red (100ppm) at 37 °C for 24h; centrifuged at 8000g for 5 min at 4 °C. The filtered supernatant was exploited as the crude source of extracellular enzyme. The cells obtained was rinsed twofold with 50mM potassium phosphate buffer (pH 7.0), resuspended in the same. These cells were disrupted in cold condition by treating with 0.1g/mL of lysozyme and incubated at 37 °C for 15mins followed by sonication (30 s 70% output 5×) using Hielscher Ultrasonicator. The homogenate obtained was centrifuged at 8000g for 5 min at 4 °C and supernatant was utilized as a crude source of intracellular enzyme. To 50mM pH 7.0 phosphate buffer supplemented with 50ppm Congo red, 500 μL of crude enzyme was added, this reaction mixture (1 mL) was pre-incubated for 3min followed by the addition of 2mM NADH. Spectrophotometric reduction in the absorbance at 340 nm on incubation at room temperature in the dark was used as a measure to

determine azoreductase activity with Congo red having a molar extinction coefficient of $25.64 \text{ mM}^{-1} \text{ cm}^{-1}$. One unit of enzyme activity was termed as the amount of enzyme that catalyzes oxidation of $1 \mu\text{mol}$ of the substrate (NADH) per minute.

Bio-decolorization and Biodegradation analysis of CR:

To disclose the mechanism involved in decolorization the byproducts were qualitatively analyzed using UV–visible spectrophotometer (Shimadzu UV-1800 Japan); biodegradation was scrutinized by high-performance liquid chromatography (HPLC) and Fourier transform infrared spectroscopy (FTIR) spectroscopy (Saratale et al. 2009; 2013). Detection of metabolites was accomplished by liquid chromatography–mass spectroscopy (LC–MS). After complete decolorization (pH 7.0) at 37°C by *Alcaligenes* species the culture broth was centrifuged at $8000g$ for 5 min, an equivalent volume of ethyl acetate was employed to extract metabolites from the clear supernatant. Extracts were dried over anhydrous Na_2SO_4 and evaporated near to dryness in a rotary evaporator. Crystals obtained were dissolved in petite volume of HPLC grade methanol filtered through $0.22 \mu\text{m}$ membrane filter and used for analysis as suggested by Babu et al. (2013).

During UV–vis spectral analysis alterations in the absorption spectrum ($225\text{--}700 \text{ nm}$) were recorded and evaluated against the results from the control runs.

HPLC analysis carried out using Agilent technologies 1260 Infinity HPLC equipped with 1260 Quat Pump VL two C18 reversed-phase chromatographic columns (analytical ZORBAX Eclipse XDB 4.8x 250mm; particle size 5micron) and PDA- 1260DAD VL⁺ Detector. An aliquot of $10 \mu\text{L}$ was injected. Gradient elution in a mobile phase of methanol: water (90:10v/v) at a flow rate of 1 mL/min and the column temperature maintained at 20°C . The metabolites eluted were analyzed by UV– visible detector, wavelength of $200\text{--}700\text{nm}$.

The biodegraded CR was characterized by FTIR (FT-IR-8201 PC Shimadzu Japan) in comparison with that of the control dye. The FTIR analysis of the samples mixed in the ratio 5:95 with spectroscopically pure KBr; the pellets were fixed in a sample holder and then analyzed in the mid-IR region ($400\text{--}4000 \text{ cm}^{-1}$); 16 scan speed.

Waters Acquity Ultra Performance LC coupled with Xevo Waters MS-Zspray (Waters Corporation 34 Maple Street Milford USA-MA 01757) was used to carry out Liquid chromatography/mass spectrometry (LC/MS) analysis of degraded metabolites. The analysis was performed in the temperature programming mode with a UPLC BEH C18 ($2.1 \times 50 \text{ mm} \times 1.7 \mu\text{m}$) maintained at 35°C . The sample volume injected was $10 \mu\text{L}$ and a linear gradient elution was methodized using the mobile phase A -Methanol LC grade and mobile phase B- LC grade Water. The capillary voltage was maintained at 4.50 kV and the cone at

50V. The source desolvation temperature was 500°C and the collision energy MS was 3V. The nitrogen-Argon carrier gas flow rate was 1000L/hr and through the cone it was maintained at 20L/hr. The degraded metabolites were scanned at an MS range from 200-700E (m/z). LC-MS data obtained was subjected to comparison based on retention times and mass spectra of degradation metabolites based on their fragmentation pattern (Li et al. 2013; Telke et al. 2010).

Statistical analysis:

All statistical analysis was computed with Statistica7 software. Data expressed in terms of mean results \pm Standard deviation. Chem Draw Ultra 12 was used for chemical structure analysis.

Results and Discussions:

Characterization and phylogenetic positioning of isolates:

The CR decolorizing strains were isolated from various sites by step up culture enrichment technique and the isolates identified as *Alcaligenes faecalis* by phenotypic and genotypic-16S rRNA gene sequence analysis in depth. Fig. 2 showed the phylogenetic relationship of *Alcaligenes faecalis* isolates and other homologous sequences from the GenBank database. The numerals adjacent to nodes refer to statistical frequency of the indicated species. The numbers shown aside are accession numbers assigned as in NCBI GenBank database. This plasmid bearing *Alcaligenes* sp. showed maximum decolorization activity within a short residence time of 48h and exhibited multi-drug resistance against Bacitracin, Chloramphenicol, Penicillin G, Polymyxin B, Augmentin and Erythromycin (Table 2). Plasmids that confer antibiotic resistance may be associated in the decolorization process- metabolic plasmids. Studies documented by Akiyama et al. (2010) supports similar findings of broad-host-range (BHR) metabolic plasmids attained from horizontal gene transfer detected in wastewater treatment plants (WWTPs) that do not target genetic elements for destruction but confer additional properties beneficial in effluent treatment. However, limited reports are available on the role of the plasmid bearing *Alcaligenes* sp in bioremediation.

Effects of Physico-chemical operational parameters:

Bacteria are highly responsive to variation in physicochemical operational parameters. The effect of agitation temperature pH and initial dye concentration on individual strains was studied in depth (Table 3). CR decolorization under static anoxic conditions demonstrated complete decolorization (-93.66-98.75%) within 24-48h however the activity seemed to retard by ~20% in shaker conditions even after 48h incubation. Inhibition of CR % decolorization might be due to the Azo nature; generally resistant to attack by bacteria under aerobic conditions (Hu et al. 1998; Phugare et al. 2011). Azo decolorization by bacterial species is normally induced by oxygen sensitive enzymatic reduction of Azo bonds. The

prevalence of oxygen inhibits reduction activity hampering electron transfer from NADH to Azo bonds (Mendes et al.2011; Morrison et al.2012).

The study revealed the decolorization was functional over a wide range of temperature from 37 to 55°C. Extreme deviation in temperature seems to slow down CR decolorization. Optimum decolorization was attained in the mesophilic range. Khan et al. (2013) proposed that the degradation potential was proportional to an increase of temperature but within the optimum temperature range. Beyond the optimum temperature reduction in decolorization activity is due to lose of cell viability decreased the rate of reproduction and denaturation of enzymes responsible for degradation.

pH was found to be the vital parameter in this process. Studies disclosed neutral pH (6.5-7.2) most favourable for decolorization; extreme acidic (3.0-4.0) or alkaline pH (8.0-9.0) hindered the process. pH seemed to be directly linked to biochemical processes in addition to the growth of microbial systems. Color solubility and ionization state of the functional groups like carboxyl hydroxyl and amino groups of dye or fungal /bacterial cell wall is also affected by pH. Matter-of-fact even with growth at acidic and alkaline pH, cell density was inadequate to favor decolorization.

To assess the maximum decolorization individual strains were tested against altered concentrations of the dye. Dye concentration from 50 to 100 ppm showed -83.46-98.53% decolorization in a span of 48hrs. *Alcaligenes* sp. was found to be more efficient in removal of dye compared to *Dietzia* sp. DTS26 (Babu et al. 2015). Elevated dye concentrations, 500 and 1000ppm showed -12.67- 47.99% and -9.89 - 25.75% decolorization respectively with difference in the time. Decline in efficiency at high dye concentrations attributed to bactericidal response to dye and/or the culture entering into stationary phase and subsequently into death phase resulting in inhibition of enzyme systems gradually (Phugare et al. 2011). Similar studies were previously reported in case of individual bacterial strains by Kalyani et al. (2009).

Organisms dwell in environments that have countless dimensions (temperature, precipitation, chemical concentrations, prey availability etc.) each of which can vary temporally and/or spatially. The outcome of evolution under heterogeneous conditions depends on the nature of environmental variation. Although *Alcaligenes* species were persistent at all sampling sites; pockets of the population have evolved under variable conditions that might explain some of the diverse modes of phenotypic variation that have been observed. Of all *Alcaligenes faecalis* KT336452 from the region of Mumbai seems to show robustness in the process of decolorization even at 500 ppm achieving decolorization rate of 10.18 mgL⁻¹ h⁻¹ as compared to the other studied strains. The exposure to organic pollutants on a large scale might be considered as one such an enhancing factor.

In order to obtain additional insight of decolorization mechanisms other than oxidase and reductase tracking of several other enzymes were studied. These strains showed induction of enzymes such as urease oxidase catalase phosphatase protease DNase protease and lipase. The decolorization was mainly due to the induction of azoreductase enzyme. An intracellular reductase enzyme was found to be profoundly responsible for such decolorizing activity having an activity of $0.032 \mu\text{mol min}^{-1} \text{mg}^{-1}$ of protein as compared to extracellular reductase enzyme having an activity of $0.004 \mu\text{mol min}^{-1} \text{mg}^{-1}$ of protein. The summation of enzymes documented the key of effectiveness and survival of the organisms during decolorization process.

Biodegradation product analysis:

UV-Vis spectrophotometer, HPLC, FTIR and GC-MS analysis enabled to explore *Alcaligenes* sp. degrading mechanisms. Decolorization process attributed to biodegradation if λ_{max} peak completely disappears or shifts to an additional wavelength resulting in the formation of new peaks (Chen et al. 2003). UV-Vis spectral analysis (225-700nm) inferred that CR dye is composed of three component systems at λ_{max} of 502.00 339.00 and 237.50; however on degradation by the isolates; a single peak was obtained which is off track from the control CR dye with a λ_{max} at 280nm for all isolates except one which shows a λ_{max} at 257nm (Fig 3).

HPLC analysis of the dye showed a prominent peak at retention time 16.660 min (Fig 4A). As the decolorization advanced emergence of additional peaks were observed after 12h at retention time 2.120, 9.927, 10.883, 12.753, 16.593, (CR) and 25.787min. On further degradation of the parent dye (after 36h) a prominent metabolite peak was observed at retention time 17.720min. (Fig 4B) the decrease in the surface area cover of the control CR peak confirms the degradation of CR dye.

FTIR spectrum of dye and extracted metabolites was compared as shown in Fig 5A and 5B. The spectrum of dye exhibited a peak for $-\text{NH}_2$ with N-H bending at 3471cm^{-1} ; several weak bands from overtones and combination vibrations at $3027\text{-}2320\text{cm}^{-1}$; N=N stretching at 1604 and 1585cm^{-1} ; C=C conjugated with the aromatic ring at $1500\text{-}1400\text{cm}^{-1}$ and for aromatic amines at $1354\text{-}1180 \text{cm}^{-1}$. The FTIR spectrum of CR metabolites showed major peaks at 2918cm^{-1} and 2850cm^{-1} for $-\text{C}-\text{H}$ stretching reduction in the peak $-\text{NH}_2$ with N-H bending at 3462cm^{-1} due to deamination 1220.94cm^{-1} for C-N stretch aromatic amines $900\text{-}675 \text{cm}^{-1}$ for C-H "oop" aromatics reduction of the peak $1604\text{-}1585$ (1658.78) cm^{-1} . In the case of dye metabolites results give the evidence of Azo bond cleavage by the azoreductase as the reduction in the peak for aromatic amines was observed.

Based on the enzymes induced and MS spectrum the probable biodegradation pathway adapted by *Alcaligenes* sp. is illustrated in Fig 6. Preliminary cleavage occurs with reduction of Azo bonds by

azoreductase with successive oxidative and reductive enzymes. Azoreductase catalyzes primary reductive cleavage of Azo bond leading to the formation of biphenyl diamine with mass peak (m/z) 184 and 12'-diaminonaphthalene-4-sulfonic acid with mass peak (m/z) 260. Further these undergo deamination amine oxidation and desulfonation to produce intermediates resulting in the transformation of complex dye molecules to simpler metabolites.

Conclusions:

The concerted activities of drug-resistant *Alcaligenes sp.* to decolorize and degrade diazo dye CR were significant as compared to other identified strains in terms of relative time. The decolorization efficiency triggered by the intracellular enzymatic reduction of Azo bonds is strongly affected by physicochemical operational parameters. Based upon the analysis of biodegradation product decolorization of CR seems to involve a complete breakdown of the Azo bond to form simpler aromatic amines. Results of the study imply the budding uses *Alcaligenes sp.* as a cheap and capable biocatalyst for the removal of Azo dyes from industrial effluents.

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References:

- Akiyama, T, Asfahl, K.L., Savin, M.C., Broad-host-range plasmids in treated wastewater effluent and receiving streams. 2010, *J Environ Qual* 39: 2211–2215
- Anjaneya, O., Souche, S.Y., Santoshkumar, M., Karegoudar, T.B., Decolorization of sulfonated azo dye Metanil Yellow by newly isolated bacterial strains: *Bacillus sp.* strain AK1 and *Lysinibacillus sp.* strain AK2. *J Hazard Mater* 2011, 190:351–358.
- Babu, S., Mohandas, C., Vijayaraj, A.S., Dhale, M.A., Detoxification and color removal of Congo red by a novel *Dietzia sp.* (DTS26) – A microcosm approach. *Ecotox Environ Safe* 2015, 114:52–60.
- Babu, S.S., Mohandass, C., Raj, A.V., Rajasabapathy, R., Dhale, M.A., Multiple approaches towards decolorization and reuse of a textile dye(VB-B)by a marine bacterium *Shewanella decolorationis*.*Water Air Soil Pollut* 2013, 224: 1–12
- Bauer, A.W., Kirby, W.M.M., Sherris, J.C., Turck, M., Antibiotics susceptibility testing by a standardized single disk method. *AM J Clin Pathol* 1966, 45: 493- 496.
- Chakraborty, S., Basak, B., Dutta, S., Bhunia, B., Dey, A., Decolorization and biodegradation of congo red dye by a novel white rot fungus *Alternaria alternata* CMERI F6. *Bioresource Technol* 2013, 147: 662–666

Chen, K.C., Wu, J.Y., Liou, D.J., Hwang, S.C.J., Decolorization of the textile dyes by newly isolated bacterial strains. *J Biotechnol* 2003, 101: 57–68

CLSI., Performance standards for antimicrobial susceptibility testing; 15th informational supplement. CLSI/NCCLS M 100-S 15. Clinical and Laboratory standards Institute. Wayne P.A., 2005.

Gopinath, K.P., Murugesan, S., Abraham, J., Muthukumar, K., *Bacillus* sp. mutant for improved biodegradation of Congo Red: random mutagenesis approach. *Bioresource Technol* 2009, 100:6295–6300

Hiral, S., Kumar, J.I. Nirmal, Patel, K., Kumar, R.N., Photocatalytic decoloration of three commercial dyes in the aqueous phase and industrial effluents using TiO₂ nanoparticles. *Desalination and Water Treatment*. 2015, doi: 10.1080/19443994.2015.1005147

Hu, T.L., Degradation of azo dye RP2B by *Pseudomonas luteola*, *Water Sci Technol* 1998, 38: 299–306.

Jadhav, U.U., Dawkar, V.V., Ghodake, G.S., Govindwar, S.P., Biodegradation of direct red 5B, a textile dye by newly isolated *Comamonas* sp. UVS. *J. Hazard Mater* 2008b, 158:507–516.

Joshi, T., Iyengar, L., Singh, K., Garg, S., Isolation, identification and application of novel bacterial consortium TJ-1 for the decolorization of structurally different azo dyes. *Bioresource Technol* 2008, 99: 7115–7121

Kalyani, D.C., Telke, A.A., Dhanve, R.S., Jadhav, J.P., Ecofriendly biodegradation and detoxification of reactive red 2 textile dye by newly isolated *Pseudomonas* sp. SUK1. *J Hazard Mater* 2009, 163:735–742

Kaushik, P., Malik, A., Mycoremediation of Synthetic Dyes: An Insight into the Mechanism, Process Optimization and Reactor Design. *Microbial Degradation of Synthetic Dyes in Wastewaters, Environmental Science and Engineering* 2015, DOI 10.1007/978-3-319-10942-8_1

Khan, R., Bhawana, P., Fulekar, M.H., Microbial decolorization and degradation of synthetic dyes: a review. *Rev Environ Sci Biotechnol* 2013, 12: 75–97

Kumar, K., Devi, S.S., Krishnamurthi, K., Dutta, D., Chakrabarti, T., Decolorization and detoxification of Direct Blue 15 by a bacterial consortium. *Bioresource Technol* 2007, 98: 3168–3171

Kumar, C. G., Mongolla, P., Microbial degradation of Basic Dyes in wastewaters. *Microbial Degradation of Synthetic Dyes in Wastewaters, Environmental Science and Engineering* 2015, 85-110

Li, X., Zhang, J., Jiang, Y., Hu, M., Li, S., Zhai, Q., Efficient biodecolorization/ Degradation of Congo red and Alizarin Yellow R by chloroperoxidase from *Caldariomyces fumago*: catalytic mechanism and degradation pathway. *Ind. Eng. Chem.Res.* 2013, 52: 13572–13579, DOI: 10.1021/ie4007563.

Mendes, S., Pereira, L., Batista, C., Martins, L.O., Molecular determinants of Azo reduction activity in the strain *Pseudomonas putida* MET94. *Appl Microbiol Biot* 2011, 92:393–405

Morrison, J.M., Wright, C.M., John, G.H., Identification, isolation and characterization of a novel azoreductase from *Clostridium perfringens*. *Anaerobe* 2012, 18:229–234

NCCLS., Performance standards for antimicrobial disk susceptibility tests, 7th edn. In: Approved standard National Committee for Clinical Laboratory Standards document M2-A7, NCCLS, Villanova, P A., 2002.

Ng, I.S., Chen, T., Lin, R., Zhang, X., Ni, C., Sun, D., Decolorization of textile azo dye and Congo Red by an isolated strain of the dissimilatory manganese-reducing bacterium *Shewanella xiamenensis* BC01. *APPL Microbiol Biot* 2013, doi:10.1007/s00253-013-5151-z

Phugare, S.S., Kalyani, D.C., Patil, A.V., Jadhav, J. P., Textile dye degradation by bacterial consortium and subsequent toxicological analysis of dye and dye metabolites using cytotoxicity, genotoxicity and oxidative stress studies. *J Hazard Mater* 2010, 186: 713–723

Robinson, T., McMullan, G., Marchant, R., Nigam, P., Remediation of dyes in textile effluent: a critical review on current treatment technologies with a proposed alternative. *Bioresource Technol* 2001, 77:247–255

Romhanyi, G., Selective differentiation between amyloid and connective tissue structures based on the collagen specific topo-optical staining reaction with Congo red. *Virchows Arch A* 1971, 354: 209–222.

Saratale, R.G., Gandhi, S.S., Purankar, M.V., Kurade, M.B., Govindwar, S.P., Oh, S.E. and Saratale, G.D., Decolorization and detoxification of sulfonated azo dye CI Remazol Red and textile effluent by isolated *Lysinibacillus* sp. RGS. *Journal of bioscience and bioengineering*, 2013, 115: 658-667.

Saratale, R.G., Saratale, G.D., Kalyani, D.C., Chang, J.S., Govindwar, S.P., Enhanced decolorization and biodegradation of textile azo dye Scarlet R by using developed microbial consortium-GR. *Bioresource technology* 2009 May 31, 100(9): 2493-500.

Talarposhti, M.A., Donnelly, T., Anderson, G.K., Colour removal from a simulated dye waste water using a two-phase anaerobic packed bed reactor. *Water Res* 2001, 35:425–432

Tamura, K., Stecher, G., Peterson, D., Filipinski, A., Kumar, S., MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol Bio and Evol* 2013, 30: 2725-2729.

Telke, A.A., Joshi, S.M., Jadhav, S.U., Tamboli, D.P., Govindwar, S.P., Decolorization and detoxification of Congo red and textile industry effluent by an isolated bacterium *Pseudomonas* sp.SU-EBT. *Biodegradation* 2010, 21: 283–296.

Thakur, J.K., Paul, S., Dureja, P., Annapurna, K., Padaria, J.C., Gopal, M., Degradation of sulphonated azo dye Red HE7B by *Bacillus* sp. and elucidation of degradative pathways. *Current microbiology*, 2014, 69: 183-191.

Vijaykumar, M.H., Parag, A., Vaishampayan, Yogesh, S.S., Karegoudar, T.B, Decolorization of naphthalene-containing sulfonated azo dyes by *Kerstersia* sp. Strain VKY1, *Enzyme Microb Tech* 2007, 40: 204–211.

Figure Legends

Figure 1: Map showing the study area and sampling sites.

Figure 2: Molecular Phylogenetic analysis by Maximum Likelihood method. The evolutionary history was inferred using the Neighbor-Joining method Tamura-Nei model (14). The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The evolutionary distances were computed using the number of differences method and are in the units of the number of base differences per sequence. The analysis involved 24 nucleotide sequences. All positions with less than 95% site coverage were eliminated. That is fewer than 5% alignment gaps missing data and ambiguous bases were allowed at any position. There were a total of 768 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

Figure 3: Overlay spectrum graph report obtained after UV-Visible spectroscopy analysis of the decolorized metabolites at Wavelength Range (nm): 225.0 to 700.0

Figure 4A: Qualitative analysis of Congo red degradation as a function of time by *Alcaligenes faecalis* KT336455 after 12h (green) vs. the standard dye (blue) by comparing HPLC chromatograms. Peaks represent the scale of the PAD response of the respective metabolites at varying retention time.

Figure 4B: Qualitative analysis of Congo red degradation as a function of time by *Alcaligenes faecalis* KT336455 after 36h (green) vs. the standard dye (blue) by comparing HPLC chromatograms. Peaks represent the scale of the PAD response of the respective metabolites at varying retention time.

Figure 5A: FTIR spectrum of Congo red dye.

Figure 5B: FTIR spectrum of residual metabolites after degradation of Congo red by *Alcaligenes faecalis* KT336455

Difference in FTIR spectra of Congo red and residual metabolites obtained after decolorization of the dye by *Alcaligenes faecalis* KT336455 are indicative of degradation. Structural alterations are reflected by difference bands in the amide-I region most prominent at 1604 cm^{-1} .

Figure 6: Proposed pathway for Congo red degradation by *Alcaligenes* species based on mass peak (m/z) on LC/MS analysis and information obtained from the NCBI database. The pathway represents enzymatic conversions that involve different steps in degradation process.

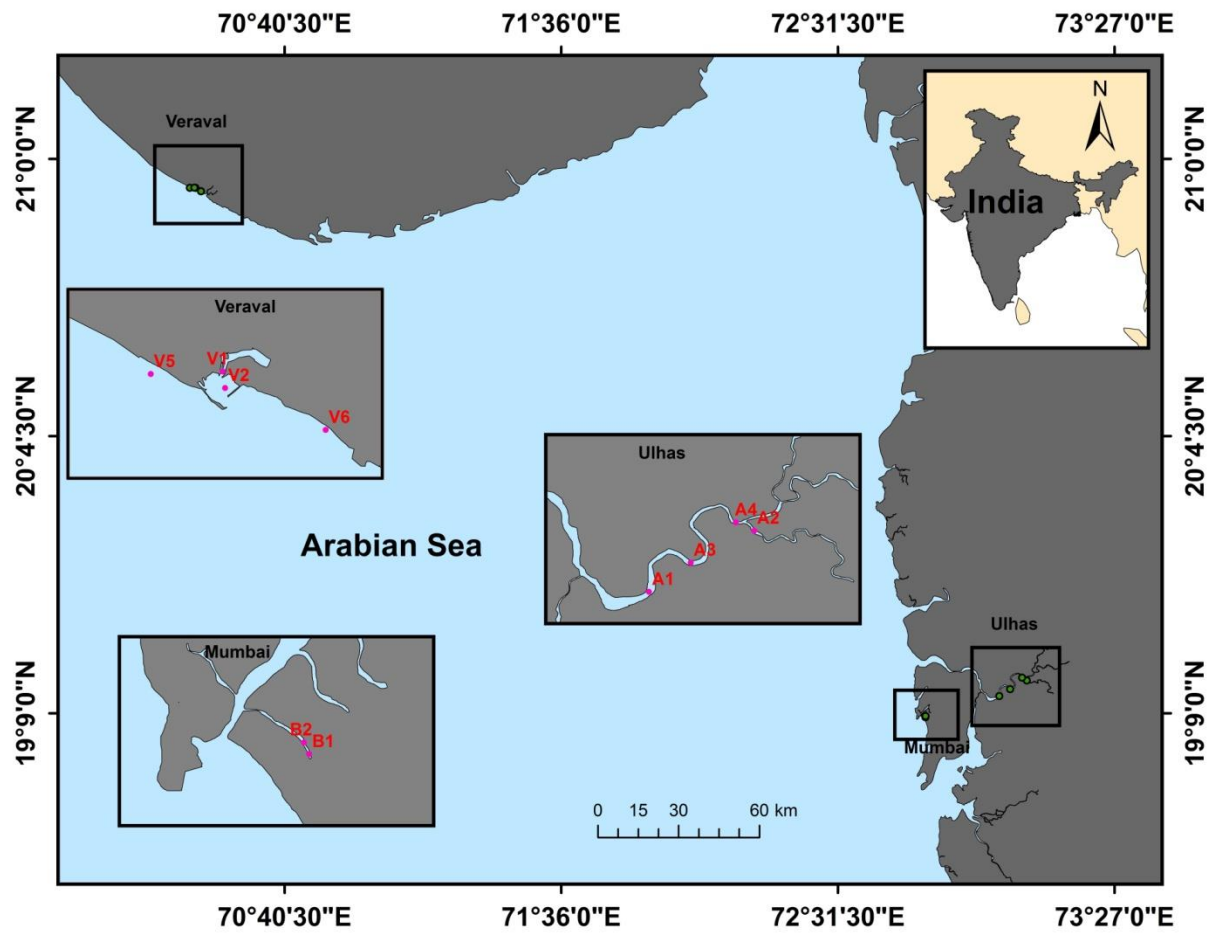


Figure 1

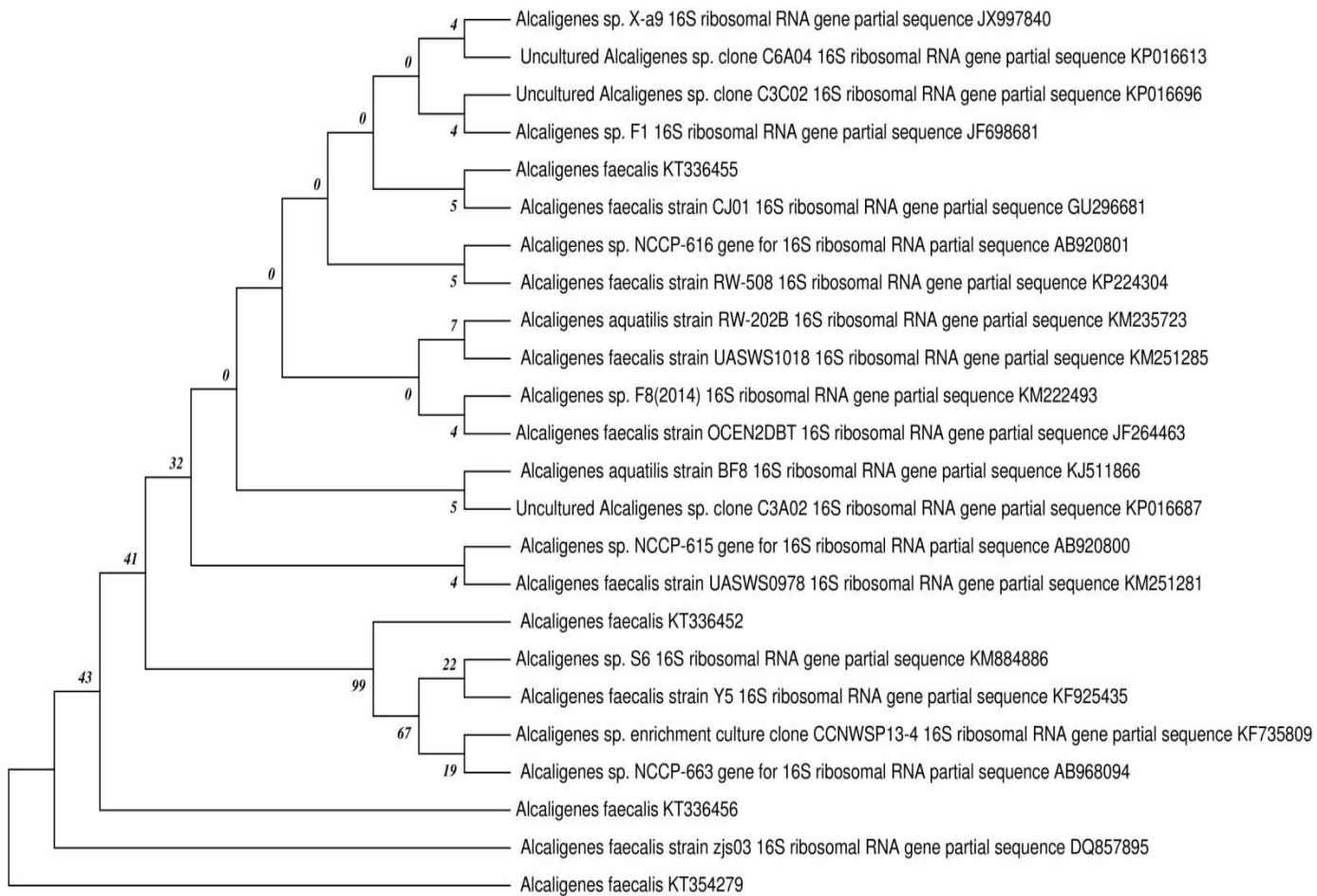


Figure 2.

Overlay Spectrum Graph Report

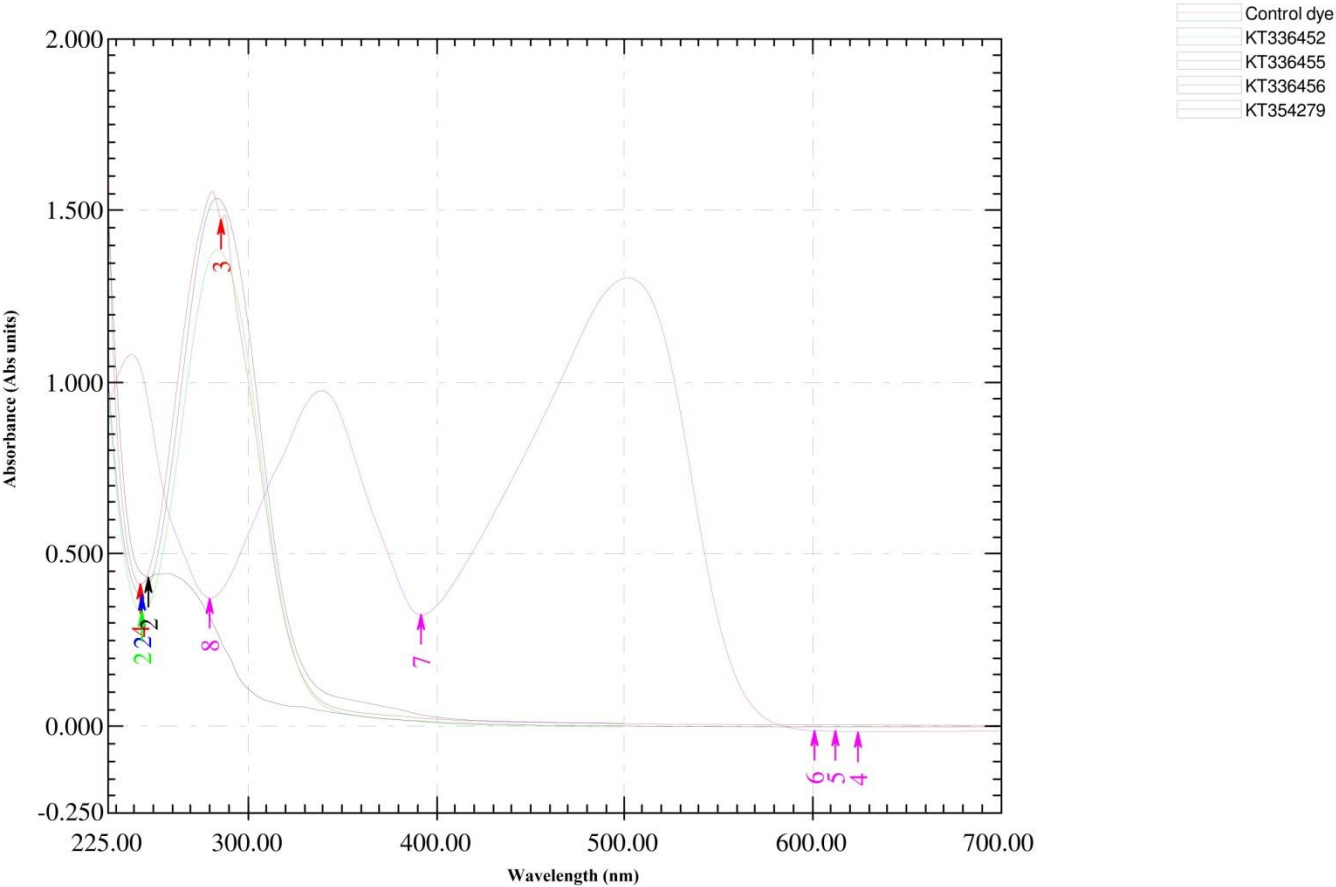
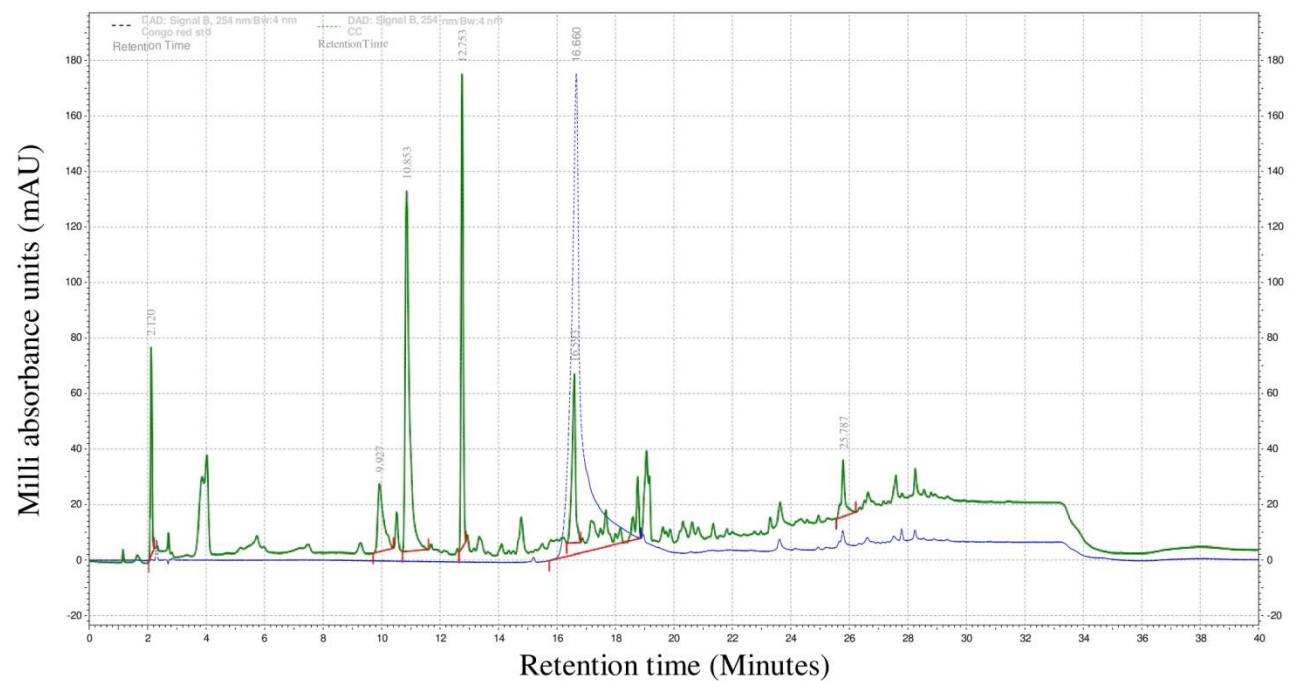


Figure 3.

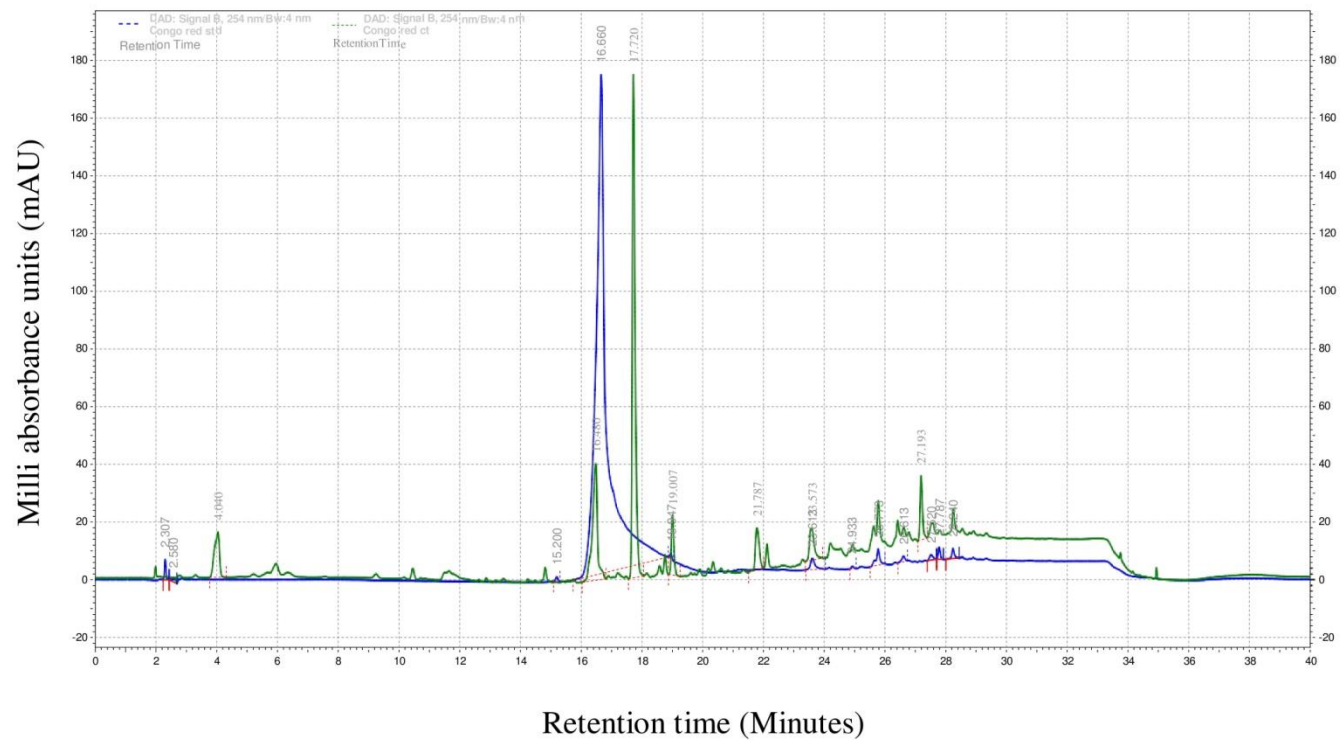


Key:

..... Congo red Dye

..... Degraded metabolites obtained after 12h.

Fig.4A



Key:

..... Congo red Dye

..... Degraded metabolites obtained after 36h.

Fig.4B

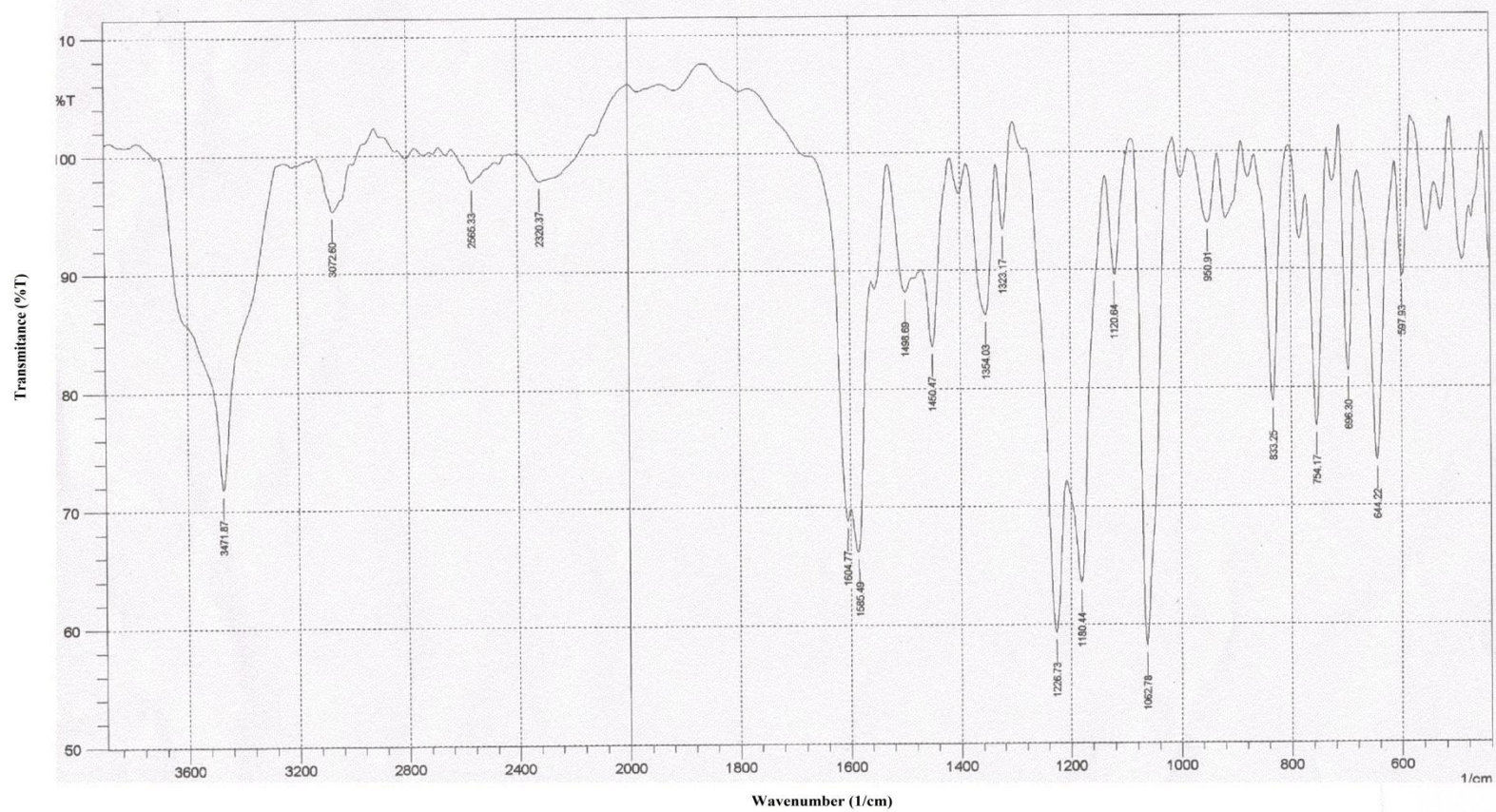


Figure 5A

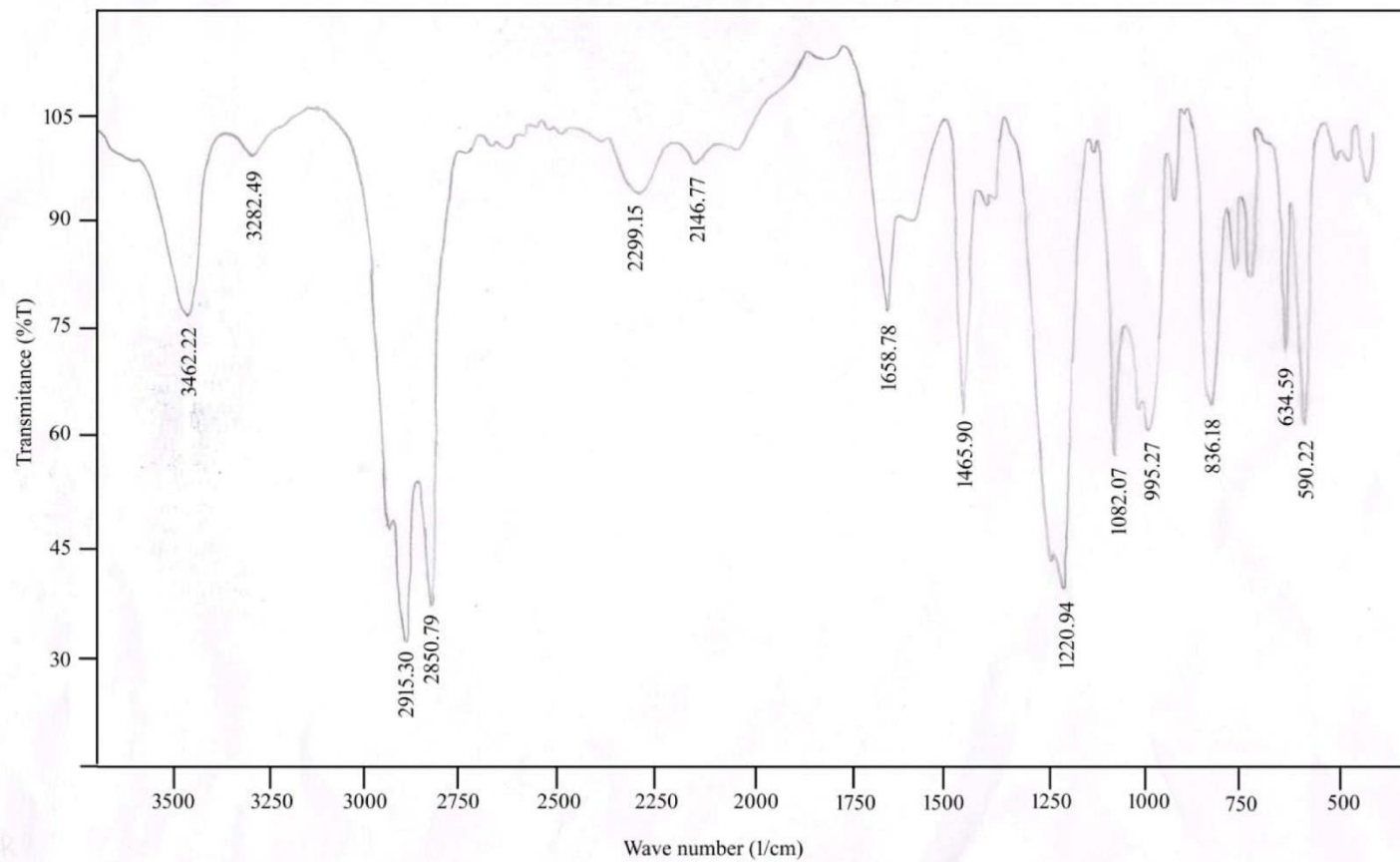
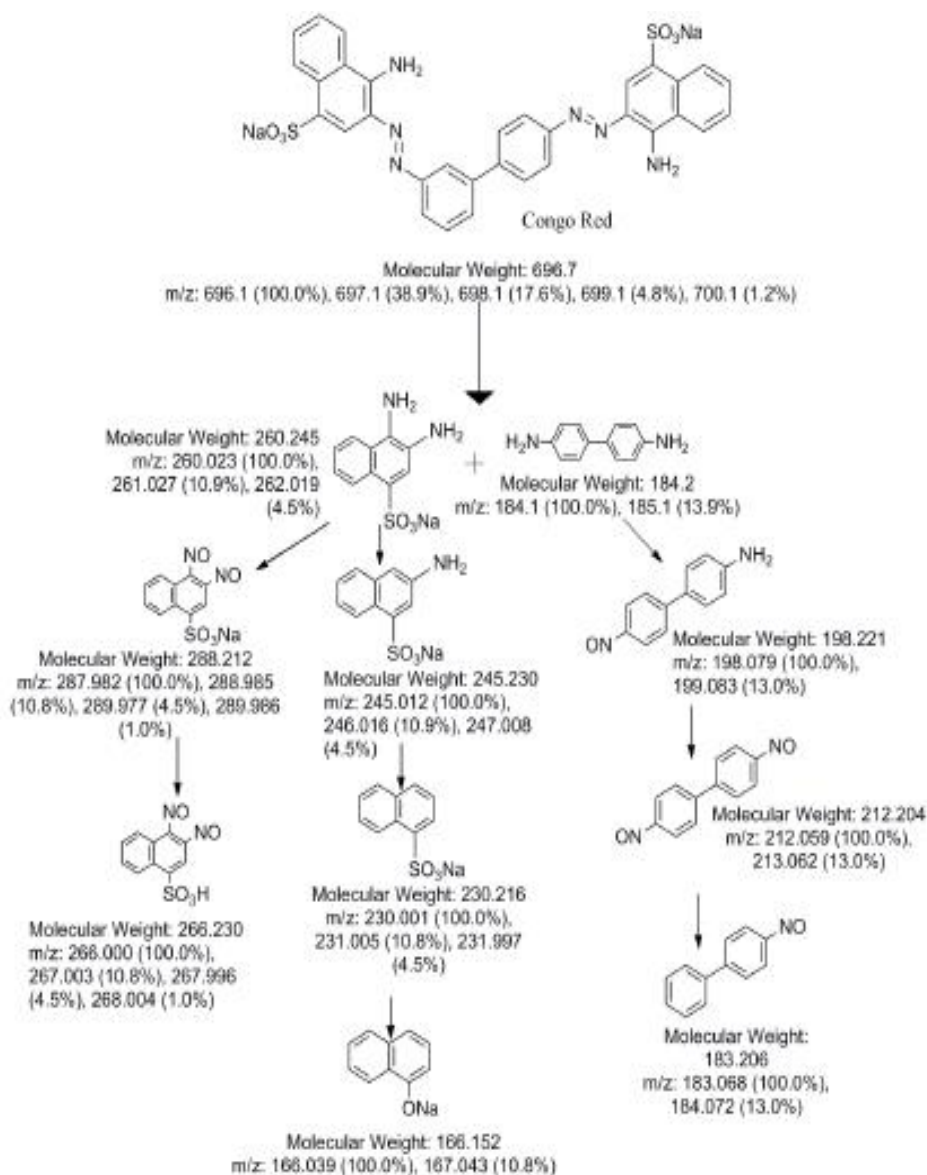


Figure 5b

Figure



intermediates:

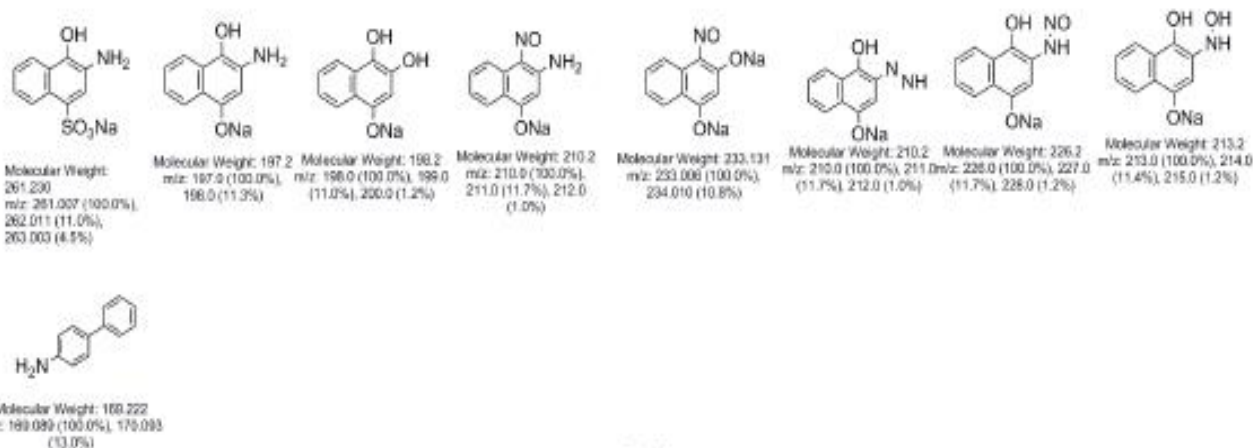


Fig.6.

Table 1: Background of the isolated marine CR degrading strains.

Sr. No.	Location	Latitude	Longitude	Code	Marine bacterial isolates identified as:
1	Mumbai	19°07'75.4" N	72°48'19.7"E	B1	<i>Alcaligenes faecalis</i> KT336452
2	Mumbai	19°08'11.0" N	72°47'93.9"E	B2	<i>Enterococcus</i> sp.
3	Veraval	20°53'47.73"N	70°22'38.93"E	V2	<i>Brucella</i> sp. KT336453
4	Veraval	20° 54'10.43"N	70°21'31.65"E	V5	<i>Bacillus</i> sp. KT357280
5	Veraval	20°58'27.97"N	70°23'45.07"E	V6	<i>Bacillus</i> sp. KT354281
6	Veraval	20°54'12.38"N	70°22'26.61"E	V1	<i>Bacillus megaterium</i> KT354282
7	Ulhas	19°12'24.84"N	73° 03'54.56"E	A1	<i>Pseudomonas</i> sp.
8	Ulhas	19°15'31.50"N	73° 09'26.20"E	A2	<i>Alcaligenes faecalis</i> KT336455
9	Ulhas	19°13'48.20"N	73° 06'06.20"E	A3	<i>Alcaligenes faecalis</i> KT354279
10	Ulhas	19°16'04.93"N	73° 08'30.11"E	A4	<i>Alcaligenes faecalis</i> KT336456

Table2: Antibiotic susceptibility test of CR degrading *Alcaligenes faecalis* isolates.

Antibiotic Susceptibility Test (AST)										
Antibiotic	Symbol	Conc.(mcg)	KT336452		KT336455		KT354279		KT336456	
Bacitracin	B	10 units	NI	R	6.75 ^a ±0.5 ^d	R	7 ^a ±0.00 ^d	R	6 ^a ±0.00 ^d	R
Chloramphenicol	C	30	6.5 ^a ±0.707 ^d	R	9.25±0.957	R	12.75±1.5	I	18.5±0.707	S
Penicillin G	P	10	NI	R	8.75±0.5	R	20.5±0.577	S	NI	R
Polymyxin B	PB	300	NI	R	11.5±0.577	I	7±0.00	R	9.5±0.707	R
Gentamicin	GEN	10	15.5±0.577	S	35.25±0.5	S	26.25±0.957	S	15.5±1.00	S
Neomycin	N	30	9.75±0.957	R	25±1.414	S	19.5±0.577	S	15±0.00	I
Cefotaxime	CTX	30	35.25±0.957	S	6.5±0.577	S	36±0.00	S	16±3.00	I
Augmentin	AMC	30	NI	–	6.75±0.5	–	15.5±0.577	–	NI	–
Erythromycin	E	15	6.375±0.577	R	7±0.00	R	7.75±0.957	R	29.5±2.121	S
Ofloxacin	OF	5	NI	R	31±3.605	S	37±1.414	S	21±1.732	S

a- Mean Zone diameter (mm).

NI-No Inhibition

d- Standard deviation

AST profile: R- Resistant, I-Intermediate, S- Sensitive

Table 3: Rate of Congo red dye decolorization by *Alcaligenes faecalis* at different temperatures, pH, dye concentrations and incubation conditions.

Accession no./ Location		Different parameters													
		pH			Temperature (°C ±2)				Dye concentration (ppm)				Incubation conditions		
		3-4	6.5-7.2	8-9	4	37	55	75	50	100	250	500	1000	Static anoxic	Shaker
KT33645 2 (Mumbai)	Decolorization (%)	9.09	76.48	57.63	16.1 9	75.4 9	52.9 4	9.21	85.35	76.49	57.24	47.99	25.75	98.76 (CD)	51.82
	Time (h)	12	24	24	18	36	24	24	36	36	24	24	36	36	24
	Rate (mgL ⁻¹ h ⁻¹)	0.9	3.21	2.44	0.99	2.11	2.25	0.46	1.16	2.14	6.02	10.18	7.04	2.75	2.2
KT33645 5 (Ulhas)	Decolorization (%)	12.82	93.34	24.16	13.3 3	93.3 4	66.0 1	8.55	96.50 (CD)	93.34	83.56	28.44	15.21	98.39 (CD)	72.38
	Time (h)	36	24	18	18	24	18	24	24	24	24	24	24	24	24
	Rate (mgL ⁻¹ h ⁻¹)	0.41	3.9	1.43	0.84	3.9	3.71	0.43	2	3.92	8.73	6.17	6.14	4.11	3.04
KT35427 9 (Ulhas)	Decolorization (%)	12.74	93.59	14.47	1.85	93.5 9	10.5 7	2.76	98.53 (CD)	92.75	73.54	31.33	13.17	97.45 (CD)	82.37
	Time (h)	24	48	24	48	48	24	18	36	36	36	36	36	36	24
	Rate (mgL ⁻¹ h ⁻¹)	0.6	1.96	0.67	0.08	1.96	0.52	0.26	1.37	2.59	5.13	4.51	3.53	2.71	3.45
KT33645 6 (Ulhas)	Decolorization (%)	1.69	83.46	1.69	0.69	83.4 6	0.32	1.76	91.45	83.46	41.27	12.66	9.89	93.66	75.37
	Time (h)	24	48	12	12	48	18	18	36	48	48	36	24	48	36
	Rate (mgL ⁻¹ h ⁻¹)	0.15	1.75	0.3	0.22	1.75	0.13	0.21	1.26	1.75	2.19	1.96	3.91	1.96	2.11

Standard Equation is given by: $y=0.0016x$; where y is the absorbance and x is the concentration of the dye in ppm
 CD – Complete decolorization.