5.1 Introduction

Industrial estates, situated within the ‘Golden Corridor’ (a highly industrialized zone from Vapi to Mehsana in Gujarat, India) manufacture dyes, paints and pigments, pharmaceuticals, textiles and many other commercial products. These industries release liquid wastes containing dyes, xenobiotic compounds and many other unnatural products in the environment and thus are the major cause of ground and surface water pollution in these areas. Dyestuff effluents are one of the major pollutants that are released into the environment. Even very low concentrations of dyes (less than 1 mg/l) can be highly visible in solutions. Synthetic dyes are very soluble in water and are recalcitrant to microbial degradation because they contain substituents such as azo, nitro or sulfo groups. They are frequently found in a chemically unchanged form even after waste-water treatment and hence they are regarded as recalcitrant pollutants (Pagga and Brown, 1986; Shaul et al., 1991). Azo dyes are the largest class of dyes and are widely used in the textile, leather, food, cosmetic and dyestuff manufacturing industries (Anliker, 1979; Reisch, 1996; Blumel et al., 2002) because of their chemical stability, ease of synthesis and versatility (Nakanishi et al., 2001). In year 2000, more than $7 \times 10^5$ tons of these dyes were produced worldwide (Suzuki et al., 2001). Azo dyes, characterized by the presence of one or more azo groups (-N=N-), have become a great concern in effluent treatment due to their colour, bio recalcitrance and potential toxicity to animals and human.

The treatment system of effluents based on physico-chemical methods is effective but suffers from shortcomings such as high cost, formation of hazardous by-products, intensive energy requirements and production of a large amount of sludge. In contrast, biological degradation of these dyes does not have similar problems. To establish biological waste-water treatment of azo dyes, it is essential to discover the microorganisms that encode azo dye-degrading enzymes (Sugiura et al., 2006). Diverse set of enzymes like azoreductases and peroxidases have so far been reported in dye degradation. Azoreductases of microorganisms are favourable for the development of biodegradation systems for azo dyes, because these enzymes catalyze reductive cleavage of azo groups (-N=N-) under mild conditions. In addition, bacterial enzymes can be readily overproduced (Nakanishi et al., 2001). The most generally
accepted hypothesis is that many bacterial strains possess cytoplasmic enzymes, which act as ‘azoreductases’ when under the pressure of azo dyes.

Azo bonds in azo dyes are reduced, under anaerobic conditions, leading to formation of corresponding amines. Intermediate aromatic amines are further mineralized under aerobic conditions (Nakayama et al., 1983; Blumel at al., 2002; Chen et al., 2005). Till now mainly combined anaerobic-aerobic microbial treatments of dye wastes have been used, due to limited knowledge about microorganisms having oxygen tolerant enzymes that are involved in decolourization of azo dyes. Aerobic treatment of dyestuff containing wastewaters possess significant potential, however, the aerobic metabolism of azo dyes requires specific enzymes (aerobic azoreductases), which catalyze the NAD(P)H-dependent reduction of azo compounds to the corresponding amines (Blumel et al., 2002; Chen et al., 2005). Very few aerobic bacteria which can grow on/with azo compounds have been reported such as azoreductase from Bacillus sp. OY1-2 (Suzuki et al., 2001) and Xenophilus azovorans (Blumel et al., 2002). Moreover, Flavobacterium can aerobically degrade 4,4’-dicarboxyazobenzene ring (Overney, 1979; Blumel et al., 2002) and Sphingomonas sp. azoreductases can cleave several sulphonated naphthol and benzol rings (Stolz, 1999).

Any individual microorganism is incapable of processing all the metabolic reactions to degrade any of the environmental pollutants, however a group of diverse organisms form a community and collectively process all the metabolic reactions for bioremediation. The individual strains may attack the dye molecule at different positions or may use decomposition products produced by another strain for further decomposition. To gain insights into these processes, it becomes inevitable to identify and characterize the dye decolorizing genetic machinery.

One approach is metagenomics or community genomics, which aims to access the genomic potential of an environmental sample either directly or after enrichment for specific purpose, respectively. Soil metagenome can be enriched in various ways keeping in mind specific gene targets that have potential applications in bioremediation, industry, medicine or agriculture. Bacteria capable of xenobiotic degradation are widely distributed in the environment. These bacteria have evolved to
utilize a variety of compounds that are present in the environment. Another approach, direct cloning of gene fragments from dye degrading bacterial consortia wherein genes can be targeted by carrying out PCR using gene specific/degenerate primers, may also give better insights. Moreover, transformation of azoreductase expressing clones to an easily manipulative host can serve for large-scale enzyme production or setting of a bioreactor with known parameters.

In order to search for azoreductases which can withstand the aerobic conditions and possess broad range of substrate specificity, attempts were made to clone oxygen tolerant azoreductases from dye decolourizing enriched mixed cultures.

5.2 Materials and methods

5.2.1 Bacterial strains, plasmids and growth conditions

*E. coli* strains DH5α, DH10B and BL21(DE3) were cultured at 37°C in Luria Broth (Himedia, Mumbai, India). Recombinant strains were also cultured under similar conditions with appropriate antibiotics (ampicillin-100 µg/ml and kanamycin-50 µg/ml). The pGEM-T kit (Promega, Madison, USA) was used for cloning of the azoreductase gene. The plasmids pUC19 and pET28a+ were used for expression studies of the azoreductase gene. All plasmid isolations were carried out using plasmid preparation kit obtained from Qiagen (Hilden, Germany).

5.2.2 Chemicals

Co-factors (FAD, FMN, NAD, NADP.Na₂, NADH.DPNH and NADPH.Na₄) were obtained from Sigma-Aldrich (Missouri, USA); restriction and modification enzymes were obtained from NEB (Ipswich, USA); antibiotics were obtained from Himedia (Mumbai, India); agarose and DNA molecular weight markers from Bangalore Genei (Bangalore, India); primers from MWG Biotech (Edersburg, Germany) and dNTPs from Bioron (Ludwigshafen, Germany). Reactive Violet 5 (RV5) was of commercial grade. All other essential chemicals were of molecular biology grade.

5.2.3 Contaminated site

The soil samples were collected from contaminated banks of the Khari-cut canal (N 22° 57. 878′; E 072° 38. 478′), flowing through GIDC (Vatva, Ahmedabad, Gujarat, India) and into the Khari river.
5.2.4 Development of V9 consortium

Soil samples were inoculated (10%) in Bushnell Haas Minimal medium (BHM: 1.7 mM MgSO₄, 0.2 mM CaCl₂, 7.4 mM KH₂PO₄, 5.7 mM K₂HPO₄, 12.5 mM NH₄NO₃ and 0.3 mM FeCl₃) supplemented with glucose and yeast extract and under selective pressure of the azo dye (Reactive Violet 5). For dye degradation under anoxic (static) condition, BHM with Reactive Violet 5 (100 ppm) was supplemented with glucose (0.1% w/v) and yeast extract (0.1% w/v). For dye degradation under aerobic (120 rpm) condition, BHM with Reactive Violet 5 (100 ppm) was supplemented with glucose (0.1% w/v) and yeast extract (0.5% w/v). Once the dye was decolourized, parts of these cultures were used as inoculum (10%) in fresh medium. Fifty transfers were carried out and each time dye decolourization was observed. The stabilized consortia were used for further studies. Dye degradation was monitored visually and also by determining the absorbance of the supernatant at 558 nm (λmax of RV5) using an UV-visible spectrophotometer (Analytik Jena AG Specord® 210, Jena, Germany).

5.2.5 Characterization of V9 consortium

5.2.5.1 Determination of carbon source

BHM supplemented with yeast extract (0.1% w/v) along with different carbon sources such as glucose, sucrose, lactose, pyruvate and starch (0.1% w/v) and Reactive Violet 5 (100 ppm) were inoculated with V9 consortium (10% v/v) and incubated at 37°C. A control, without any carbon source, was kept under similar conditions. To find the minimum amount required for dye decolourization, various concentrations (0.01, 0.05, 0.1, 0.2, 0.3 and 0.5% w/v) of the selected carbon source were studied.

5.2.5.2 Determination of nitrogen source

BHM supplemented with glucose (0.1% w/v) along with different nitrogen sources such as yeast extract, peptone, ammonium nitrate, sodium nitrate, potassium nitrate and urea (0.1% w/v) and Reactive Violet 5 (100 ppm) were inoculated with V9 consortium (10% v/v) and incubated at 37°C. A control, without any nitrogen source, was kept under similar conditions. To find the minimum amount required for dye decolourization, various concentrations (0.01, 0.05, 0.1, 0.2, 0.3 and 0.5% w/v) of the selected nitrogen source were studied.
5.2.5.3 Optimum temperature for dye decolourization
BHM supplemented with glucose (0.1% w/v), yeast extract (0.1% w/v) and Reactive Violet 5 (100 ppm) was inoculated with V9 consortium (10% v/v) and incubated at different temperatures such as 4°C, 20°C, 30°C, 37°C, 45°C and 55°C.

5.2.5.4 Effect of salinity on dye decolourization
BHM supplemented with glucose (0.1% w/v), yeast extract (0.1% w/v) and Reactive Violet 5 (100 ppm) along with various NaCl concentrations (0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20 and 30 g/l) were inoculated with V9 consortium (10% v/v) and incubated at 37°C.

5.2.5.5 Spectrum of dyes as substrates
BHM supplemented with glucose (0.1% w/v), yeast extract (0.1% w/v) and twenty four kinds of dyes (100 ppm), respectively were inoculated with V9 consortium (10% v/v) and incubated at 37°C. Decolourization was determined at respective absorbance maxima of dyes.

5.2.6 Isolation and identification of cultivable bacteria constituting the V9 consortium
The appropriate dilutions of enriched consortium were spreaded on rich medium (Luria agar) as well as minimal media (BHM agar and R2A) and incubated at various temperatures (20°C, 28°C and 37°C) for various growth periods (1 day, 2 days, 5 days, 10 days and 20 days). Based on morphological characteristics different colonies were picked and subsequently maintained on Luria agar plates.

The 16S rRNA genes were amplified from the bacterial cultures by colony PCR. Amplification was carried out in a 30 μl PCR reaction consisting of 1X buffer (10 mM Tris pH 9.0, 50 mM KCl, 1.5 mM MgCl₂, 0.1% Triton X-100), 0.33 mM each of dNTPs, 0.66 pmoles each of custom synthesized universal primers 27F (5’-GAGTTTGATCCTGCTCA-3’) and 1385R (5’-CGGTGTGTRCAAGGCCC-3’), and 1.5 U of Taq DNA polymerase. Amplification program was performed with initial denaturation step at 94°C for 5 min; followed by 30 cycles of 1 min denaturation step at 94°C, 1 min annealing step at 55°C, and 1.2 min elongation step at 72°C and a final extension step at 72°C for 20 min using Biorad iCycler version
Chapter 5: Community genomics

4.006 (Biorad, CA, USA). The ~1.5 kb PCR product was sequenced by automated DNA Analyzer 3730 using BigDye™ Terminator 3.1 sequencing chemistry (Applied Biosystems, Foster City, CA). Full length 16S rRNA gene sequences were analyzed using SEQMATCH tool of the Ribosomal Database Project (RDP-II) to identify the bacteria. The 16S rRNA gene sequences have been submitted to GenBank and the obtained accession numbers have been described in section 5.3.2.

5.2.7 Isolation of consortial DNA
The V9 consortium, after dye decolourization, was used for total community DNA preparation using Zhou et al. (1996) protocol with some modifications. The precipitation was done differently by adding polyethylene glycol (PEG) 10,000 at a final concentration of 5% followed by incubation at 4°C overnight. DNA concentration was measured using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Delaware, USA) and further analysed by gel electrophoresis.

5.2.8 Amplification, cloning and sequencing of azoreductase gene
In order to clone the azoreductase gene, three sets of primers were designed from the sequences available in NCBI database [(1) AZR1F 5'-ATGAAACTAGTCGTATTTA-3' and AZR1R 5'-TCACTCCAACCTAGTTT-3'; (2) AZR2F 5'-TAGCAAATTTAAGG-3' and AZR2R 5'-CGTATCATCTTGACG-3'; (3) AZR3F 5'-GGAATTCATATGAACATGTTAGTCTATAA-3' and AZR3R 5'-CGGATCCAACAAATCCCGTGY-3']. Amplification of the azoreductase gene from consortial DNA was carried out as described in section 5.2.6. The amplified products were purified by adding a solution (20% PEG 8000 in 2.5 M NaCl) to the reaction mixture in a ratio of 1:0.66 and incubating at 37°C for 1 hour followed by two 70% ethanol washes. All the centrifugations (Kubota 6500, Bunkyo-Ku, Tokyo, Japan) were carried out at 20,000 x g for 20 minutes at 4°C. The pellet was air-dried and resuspended in Milli-Q water (Millipore, Billerica, USA). The electrocompetent cells were prepared as described in section 2.2.4.4.2 of chapter 2. Purified amplified products were ligated into pGEM-T vector (according to manual of pGEM-T vector) and transformed into *E. coli* DH5α competent cells by
electroporation as described in section 2.2.4.5.2 of chapter 2. Recombinant plasmids were isolated and subjected to sequencing using M13 primers by automated DNA Analyser 3730 using ABI PRISM® BigDye™ Terminator Cycle Sequencing 3.1 (Applied Biosystems, CA, USA). The sequence has been submitted to GenBank and the accession number is described in section 5.3.4. DNA and protein sequences with homology to the sequenced gene were searched using BLASTn and BLASTx in NCBI databases, respectively.

5.2.9 Phylogenetic analysis

Three hundred sequences of each of azoreductase genes and proteins were downloaded from NCBI database in February 2010. The sequenced gene and ORF of its translated product were aligned with downloaded azoreductase genes and proteins sequences, respectively using Clustal W 1.6 program at (http://www.ebi.ac.uk/clustalw/). Phylogenetic analyses was performed (for both DNA and protein sequences) using aligned sequences by the neighbour joining algorithm (Saitou and Nei, 1987) using Kimura 2 parameter distance and bootstrapping with 550 replicates (Felsenstein, 1985) in MEGA 4.0 software (Tamura et al., 2007). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The resulting tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and the Poisson correction method (Zuckerlandl and Pauling, 1965) for DNA and protein sequences, respectively. All positions containing alignment gaps and missing data were eliminated only in pair wise sequence comparisons.

5.2.10 Expression of an azoreductase gene in E. coli

To facilitate cloning of azoreductase gene in expression vectors, BamHI and HindIII restriction site sequences were added in set 1 primers (AZR1FB 5’-GGCGGATCCATGAAACTAGTCGTTATTAAC-3’ and AZR1RH 5’-GGCAAGCTTTCACTCCACTCCTAGTTGTTTTTT-3’). The underlined bases in AZR1FB and AZR1RH represent sites for BamHI and HindIII, respectively. Amplified azoreductase gene and vectors pUC19 and pET28a+ were digested with
BamHI and HindIII enzymes. The ligations were performed in 10 μl reactions containing 50 ng vector, 150-200 ng insert DNA and 3 units of T4 DNA ligase in T4 DNA ligase buffer at 16°C overnight. Two μl of the reaction was used for transformation of 100 μl E. coli DH10B competent cells by electroporation (Bio-Rad MicroPulser, Hercules, California, USA) at a voltage of 1.8 kV and 2.5 kV for 0.1 cm and 0.2 cm cuvettes, respectively. The preparation of competent cells and electroporation are described in detail in section 2.2.4.4.2 and 2.2.4.5.2, respectively of chapter 2. For facilitating expression of the azoreductase gene, a second transformation of recombinant plasmid isolated from E. coli DH10B clone pET1 was carried out in E. coli BL21(DE3) competent cells.

5.2.11 Whole cell extract preparation
Cells were harvested and washed with 20 mM sodium phosphate buffer (pH 7.0). All the centrifugations were carried out at 20,000 x g for 20 minutes at 4°C. The cells were resuspended in the same buffer and two freeze-thaw cycles at -20°C and 4°C were carried out followed by disruption using an Ultrasonic probe (Sonics Vibra Cell 500, USA) at 35% amplitude and 9 second pulses with 5 second intervals for 15 times or till complete cell lysis (clear lysate). The lysate was then centrifuged and the obtained supernatant was collected and termed as crude extract.

5.2.12 Azoreductase activity assay
Enzyme assay was carried out in 2 ml 20 mM sodium phosphate buffer system containing 2 mg protein (crude extract), 50 ppm Reactive Violet 5 dye and 1 mM co-factor. The experimental reactions were incubated at 37°C till dye degradation and control reactions under similar conditions for three hours. Dye degradation was measured by the absorbance of the supernatant at 558 nm (λ.max). The amount of protein was calculated according to the method of Lowry et al. (1951).

5.2.13 Studies on suitable co-factor
To find out the co-factor required by the cloned azoreductase gene, enzyme assays were carried out with different co-factors (FAD, FMN, NAD, NADP.Na₂, NADH.DPNH and NADPH.Na₄) at a concentration of 1.5 mM. To determine the
minimum amount of the selected co-factor required for expression, enzyme assays were carried out with different concentrations (0 mM, 0.5 mM, 1 mM and 1.5 mM).

5.2.14 Azoreductase assay of whole cell crude extracts of *E. coli* strains, strains with vectors and strains with recombinant vectors
Azoreductase assay were performed for cell extracts of *E. coli* DH10B clone pUC1, *E. coli* DH10B clone pET1 and *E. coli* BL21(DE3) clone pET1. As controls, assays were also performed for cell extracts of *E. coli* DH5a, *E. coli* DH10B, *E. coli* BL21(DE3) and *E. coli* strains with vectors pUC19 and pET28a+. The assays were carried out as described in section 5.2.12.

5.3 Results and discussion

5.3.1 Contaminated site
The persistent release of toxic pollutants from industrial units (situated in GIDC, Vatva, Ahmedabad, Gujarat, India) manufacturing dyes, chemicals, solvents and other xenobiotic compounds have contaminated the surrounding water and soil bodies. The soil samples collected from contaminated banks of the Khari-cut canal, flowing through Vatva and into the Khari river were used for developing consortium, enriched with bacterial cultures capable of either degrading dye or growing in presence of dye stress.

5.3.2 V9 consortium
Reactive Violet 5 was chosen as a model dye on account of its complexity. It contains three aromatic rings, three sulphonated groups as well as copper as a metal ion. The structure of Reactive Violet 5 is shown in Fig. 5.1.

![Chemical structure of Reactive Violet 5 (RV5) dye.](image)
The developed bacterial V9 consortium was able to decolourize 100 ppm of Reactive Violet 5 (RV5) within 24 h at 37°C. The degradation of dye was observed visibly (Fig. 5.2a) as well as by the decrease in the absorbance at 558 nm (λ_{max} of RV5) (Fig. 5.2b).

![Image of dye decolourization profile](image)

**Fig. 5.2:** Dye decolourization profile depicted by (a) photograph and (b) UV-Visible overlay spectra. (Inoculated and Dye degraded)
To achieve efficient dye decolourization, various biotic and abiotic parameters were studied. V9 consortium was characterized for carbon source as well as nitrogen source and their concentrations. Various carbon sources such as glucose, sucrose, lactose, pyruvate and starch were screened for dye degradation studies and amongst them glucose was found to be the best (Fig. 5.3a). As observed from Fig. 5.3a, 95% of dye was decolourized within 24 h in presence of glucose. To find the minimum amount of glucose required for dye decolourization, various concentrations (0.01, 0.05, 0.1, 0.2, 0.3 and 0.5% w/v) were studied (Fig. 5.3b). From Fig. 5.3b, it can be observed that when glucose concentration was 0.1% w/v, 95% of dye was decolourized within 24 h.

Various nitrogen sources such as yeast extract, peptone, ammonium nitrate, sodium nitrate, potassium nitrate and urea were screened for dye degradation studies and amongst them yeast extract was found to be the best (Fig. 5.4a). As observed from Fig. 5.4a, 95% of dye was decolourized within 24 h in presence of yeast extract. It was observed that peptone leads to even faster rapid degradation of dye, however the dye decolourization is temporary and after further 24 h, colour reappears. Consequently, yeast extract was preferred over peptone. To find the minimum amount of yeast extract required for dye decolourization, various concentrations (0.01, 0.05, 0.1, 0.2, 0.3 and 0.5% w/v) were studied (Fig. 5.4b, c). Amount of nitrogen source requirement varied for anoxic and aerobic conditions. In anoxic conditions, 90% of dye was decolourized within 24 h in presence of 0.1% yeast extract (Fig. 5.4b). Conversely, in aerobic conditions 90% of dye was decolourized within 30 h in presence of 0.5% yeast extract (Fig. 5.4c). Lesser concentrations of yeast extract did not lead to optimum dye degradation in aerobic conditions. Thus, the minimum essential amount of yeast extract required was higher in aerobic (0.5%) than in anoxic (0.1%) conditions. Reasons can be attributed to the fact, that oxygen might be competing for the electrons. Moreover, one peculiar phenomenon was observed that for dye degradation, presence of yeast extract in medium is inevitable whereas, absence of glucose did not hamper the metabolic process.
Fig. 5.3: (a) Effect of various carbon sources on decolourization of RV5 by V9 consortium under anoxic condition at 37°C; (b) Effect of different glucose concentrations on decolourization of RV5 by V9 consortium under anoxic condition at 37°C.
Fig. 5.4: (a) Effect of various nitrogen sources on decolourization of RV5 by V9 consortium under anoxic condition at 37°C; (b) Effect of different yeast extract concentrations on decolourization of RV5 by V9 consortium under anoxic condition at 37°C; (c) Effect of different yeast extract concentrations on decolourization of RV5 by V9 consortium under aerobic condition at 37°C.

Moreover, optimum temperature required for efficient and faster dye decolourization was characterized. Optimized BHM medium with Reactive Violet 5 (100 ppm) was inoculated with V9 consortium (10% v/v) and incubated at different temperatures such as 4°C, 20°C, 30°C, 37°C, 45°C and 55°C (Fig. 5.5). The decolourization ability of V9 consortium was highest at 37°C showing 93% decolourization of RV5 within 24 h.

Fig. 5.5: Effect of different temperatures on decolourization of RV5 by V9 consortium under anoxic condition.
Fig. 5.6 shows overall decolourization profile of RV5 and growth pattern of V9 consortium under anoxic (Fig. 5.6a) and aerobic (Fig. 5.6b) conditions. Under anoxic conditions V9 consortium was able to decolourize 95% of RV5 within 24 h, while in aerobic conditions (120 rpm) 85-90% of RV5 was decolourized within 30-36 h at 37°C.

![Graph showing decolourization and growth profile](image-a.png)

![Graph showing decolourization and growth profile](image-b.png)

**Fig. 5.6**: Dye decolourization and growth profile of V9 consortium under (a) anoxic and (b) aerobic conditions at 37°C.
The V9 consortium was able to efficiently decolourize dye up to 500 ppm concentration and tolerate dye up to 2000 ppm. Moreover, generally contaminated sites are reported to have high salinity. Consequently, dye decolourization efficiency of V9 consortium in presence of high salinity was determined. BHM supplemented with glucose (0.1% w/v), yeast extract (0.1% w/v) and Reactive Violet 5 (100 ppm) along with various NaCl concentrations (0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20 and 30 g/l) were inoculated with V9 consortium (10% v/v) and incubated at 37°C (Fig. 5.7). The consortium showed more than 90% dye decolourization even at higher concentrations of NaCl, which demonstrated its ability of decolourizing dye RV5 over a wide range of saline conditions.

Fig. 5.7: Effect of salinity on decolourization of RV5.

To determine whether developed V9 consortium can decolourize broad range of substrates, various kinds of azo dyes (having different - groups, metal ions, ring formations, etc.) were used as substrates in the study. BHM supplemented with glucose (0.1% w/v), yeast extract (0.1% w/v) and twenty four kinds of dyes (100 ppm), respectively were inoculated with V9 consortium (10% v/v) and incubated at 37°C (Fig. 5.8). Decolourization was determined at respective absorbance maxima of dyes. Decolourization in the range of 85-95% within 24 h was observed for 22 out of 24 dyes used in the study.
Determination of azo-dye degrading organisms is essential for developing new bioremediation strategies in waste-water treatment plants (Suzuki et al., 2001). Consequently, cultivable organisms constituting the V9 consortium and playing a role in dye degradation were identified. Based on morphological characteristics seven different cultures were isolated. Subsequently their 16S rRNA genes were amplified (Fig. 5.9) and sequenced.

**Fig. 5.8:** Spectrum of dyes decolourized by the V9 consortium.

**Fig. 5.9:** 16S rRNA genes amplified by colony PCR. Lanes 1 to 7: 16S rRNA genes from seven different pure cultures; Lane 8: Supermix DNA ladder.
These 16S rRNA gene sequences were analyzed using SEQMATCH tool of Ribosomal Database Project (RDP-II). The organisms were identified as *Pseudomonas citronellolis* V91DM (JN400328), *Lysinibacillus fusiformis* V92DM (JN400329), *Gordonia cholesterolivorans* V93DM (JN400330), *Ochrobactrum pseudintermedium* V94DM (JN400331), *Stenotrophomonas sp.* V95DM (JN400332), *Enterococcus casseliflavus* V96DM (JN400333) and *Citrobacter sp.* V97DM (JN400334). The genera *Pseudomonas*, *Stenotrophomonas* and *Citrobacter* belong to class *Gammaproteobacteria* of phylum *Proteobacteria*. The genus *Ochrobactrum* belongs to class *Alphaproteobacteria* of phylum *Proteobacteria*. The genera *Enterococcus* and *Lysinibacillus* belong to class *Bacilli* of phylum *Firmicutes*. The genus *Gordonia* belongs to class *Actinobacteria* (class) of phylum *Actinobacteria*.

Many reports are available describing the roles of strains, belonging to genera identified in V9 consortium, in azo dye degradation. Azo reduction activity by strain *Pseudomonas putida* MET94 (Mendes et al., 2011) and decolourization of adsorbed textile dyes by mixed cultures of *Pseudomonas* sp. SUK1 and *Aspergillus ochraceus* NCIM-1146 (Kadam et al., 2011) have been reported. *Lysinibacillus* sp. strain AK2 is capable of decolourizing sulfonated azo dye Metanil Yellow (Anjaneya et al., 2011). *Ochrobactrum intermedium* ANKI is able to decolourize azobenzene (Vakkerov-Kouzova, 2007). Azoreductase (AzoA) from *Enterococcus faecalis* is a very active enzyme with broad spectrum of substrate specificity (Chen et al., 2008). Macwana et al. (2010) have isolated and identified an azoreductase from *Enterococcus faecium* which is 67% similar to azoreductase from *Enterococcus faecalis* but uses different co-factors NADH and NADPH instead of FMN. A bacterial strain *Stenotrophomonas maltophilia* AAP56 isolated from a polluted soil was able to decolorize recalcitrant dyes of an industrial effluent: SITEX Black (Said et al., 2010). Petty et al. (2010) sequenced complete genome of *Citrobacter rodentium* and they have identified an azoR gene (FMN dependent NADH-azoreductase).

### 5.3.3 Consortial DNA

The stabilized V9 consortium (transferred 50 times and each time dye was decolourized) was used for extraction of total consortial DNA. The DNA isolation was carried out according to protocol described by Zhou et al. (1996) with some
modifications. The consortium besides bacterial cultures also contained aromatic amines and other dye degradation products. Thus it was essential that these contaminants were removed from the extracted DNA as they would have interfered in further molecular experiments. Consequently, the precipitation was done using 5% PEG 10,000 and incubated at 4°C overnight. The advantage of using PEG 10,000 was that no additional purification step such as gel permeation chromatography was required. The extracted DNA (Fig. 5.10) was free of impurities and amenable to PCR, ligation and other molecular studies. The quantity and quality of extracted DNA is described in Table 5.1.

![Image](image_url)

**Fig. 5.10:** Consortial genomic DNA electrophoresed on 0.8% (w/v) agarose gel. Lane 1: Supermix DNA ladder; Lanes 2 and 3: Consortial genomic DNA.

**Table 5.1:** Details of consortial genomic DNA.

<table>
<thead>
<tr>
<th>Description</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>V9 consortium after dye degradation</td>
<td>200 ml</td>
</tr>
<tr>
<td>Extracted consortial genomic DNA</td>
<td>3.2 µg</td>
</tr>
<tr>
<td>A260</td>
<td>0.188</td>
</tr>
<tr>
<td>A280</td>
<td>0.098</td>
</tr>
<tr>
<td>A230</td>
<td>0.093</td>
</tr>
<tr>
<td>260/280</td>
<td>1.92</td>
</tr>
<tr>
<td>260/230</td>
<td>2.02</td>
</tr>
</tbody>
</table>
5.3.4 Azoreductase gene

Three sets of primers were designed from the sequences of azoreductases available in the NCBI database. Azoreductase gene was amplified (Fig. 5.11), by set 1 primers – AZR1F and AZR1R, from consortial DNA as template. The other two sets of primers could not amplify azoreductase gene from V9 consortium. The amplified azoreductase gene was cloned into the pGEM-T vector and sequenced. Amplified azoreductase gene (JN400335) had a length of 537 bp (Fig. 5.12) containing an ORF of 178 amino acids. BLASTn analysis of the sequence showed 97% identity to azoreductase gene of *Rhodobacter sphaeroides*, while BLASTx analysis showed 98% identity to azoreductase of *Bacillus cereus* G9241.

![Fig. 5.11: Azoreductase gene amplified from consortial genomic DNA. Lanes 1 and 2: Azoreductase gene; Lane 3: Supermix DNA ladder.](image)

![Fig. 5.12: Nucleotide sequence of the amplified azoreductase gene (JN400335).](image)
To find out the phylogenetic affiliation of the gene, 300 sequences each of azoreductase gene and protein were downloaded from the NCBI database. However, similar sequences from each cluster were removed to make the resulting trees less complex. The phylogenetic tree for azoreductase genes (Fig. 5.13a) and proteins (Fig. 5.13b) showed that the sequenced gene was clustered with the azoreductases of phylum *Firmicutes*. The gene is closely related to azoreductase of *Bacillus cereus* Q1. Within the phylogenetic tree of azoreductase genes, genes from *Gammaproteobacteria* class were not near to each other and were interspersed with azoreductase genes from *Firmicutes*. Conversely, within the phylogenetic tree of azoreductase proteins, those of *Gammaproteobacteria* and *Firmicutes* were not interspersed and cluster clearly apart.

The above observation can be explained by the fact that several enzymes are endowed with promiscuous activities, azoreductases being one of them. The term 'catalytic promiscuity' describes the capability of an enzyme to catalyse different chemical reactions, called secondary activities, at the responsible active site. Many of the enzymes active in degradation pathways are linked from their protein phylogeny and not strictly linked to the taxonomical affiliation of the bacteria (Perez-Pantoja et al., 2009), indicating that the genes encoding those catabolic enzymes are involved in very dynamic events. To characterize the catabolic potential for biodegradation it is necessary to take into consideration the broad diversity of catabolic routes evolved by microorganisms and also the diversity of enzymes of a given gene family or even between gene families.
**Fig. 5.13:** Phylogenetic relationship of the cloned gene sequence (a) and its deduced amino acid sequence (b) with gene and protein sequences of azoreductases available in database, respectively. The trees were constructed using neighbour joining algorithm with Kimura 2 parameter distances in MEGA 4.0 software. Numbers at nodes indicate percent bootstrap values above 50 supported by 550 replicates. The bar indicates the Jukes-Cantor evolutionary distance. The names of the downloaded sequences are as described in GenBank. The cloned gene is named as cloned azoreductase gene in (a) and as ORF of cloned azoreductase gene in (b).

### 5.3.5 Expression of the azoreductase

In order to clone the azoreductase gene, BamHI and HindIII sites were added in forward and reverse primers of set 1, respectively. The azoreductase gene, amplified using modified set 1 primers, was digested with BamHI and HindIII enzymes. The vectors pUC19 and pET28a+ were isolated and digested with Bam HI and Hind III enzymes (**Fig. 5.14**). The digested products were ligated into digested vectors. The constructs were transformed in *E. coli* DH10B. The presence of inserts in pUC19 was verified by carrying out amplification of azoreductase gene using M13 primers as shown in **Fig. 5.15**.

The resulting recombinant clones were named as *E. coli* DH10B clone pUC1 (pUC19 with azoreductase gene) and *E. coli* DH10B clone pET1 (pET28a+ with azoreductase gene). Moreover, for higher expression of the azoreductase gene, a second transformation of recombinant plasmid, isolated from *E. coli* DH10B clone pET1, was carried out in *E. coli* BL21(DE3) cells and the recombinant clone was named as *E. coli* BL21(DE3) clone pET1. The recombinant strains were checked for their *in vivo* azo dye degradation capabilities. However, no degradation was observed and reason can be attributed to the fact that recombinant *E. coli* strains were unable to uptake sulphonated azo dyes. This result corroborates with earlier similar observations by Blumel et al. (2002). Moreover, they had also suggested that besides uptake other limitation factor could be availability of NADPH, essential for the reduction of azo dyes. Consequently, for measuring *in vitro* enzyme activity, assays were carried out with 2 mg crude cell extracts (enzyme) in presence of co-factor and 50 ppm substrate (Reactive Violet 5 dye). The experimental reactions were incubated till dye degradation and control reactions were incubated for an extended period of three hours.
Fig. 5.14: Agarose gel (1%) analysis of vectors. Lane 1: Supermix DNA ladder; Lane 2: Isolated pUC19 vector; Lane 3: pUC19 vector digested with BamHI and HindIII enzymes; Lane 4: Isolated pET28a+ vector; Lane 5: pET28a+ vector digested with BamHI and HindIII enzymes.

Fig. 5.15: Amplification of azoreductase gene using M13 primers. Lane 1: Azoreductase gene; Lane 2: 100 bp ladder.
Among several reported azoreductases, the enzymes of *X. azovorans* KF46F (Blumel et al., 2002), *P. kullae* K24 (Blumel and Stolz, 2003), *E. coli* (Nakanishi et al., 2001), *Ent. faecalis* (Chen et al., 2004) and *R. sphaeroides* AS1.1737 (Bin et al., 2004) use NADH as electron donors. However, the enzymes of *E. coli* (Nakanishi et al., 2001) and *Ent. faecalis* (Chen et al., 2004) are also FMN-dependent. On the other hand, enzymes of *B. sp. OY1-2* (Suzuki et al., 2001) and *S. aureus* (Chen et al., 2005) use NADPH as electron donor. Consequently, to find out the co-factor required by the cloned gene product, enzyme assays were carried out with different co-factors as shown in **Fig. 5.16**. Co-factors FAD (**Fig. 5.16a**), FMN (**Fig. 5.16b**), NAD (**Fig. 5.16c**) and NADP.Na₂ (**Fig. 5.16d**) did not activate the enzyme as only 6%, 4.7%, 8% and 8.5% dye degradation was observed, respectively. Co-factor NADH.DPNH (**Fig. 5.16e**) did facilitate dye degradation but only 56%. On the other hand, co-factor NADPH.Na₄ (**Fig. 5.16f**) facilitated 85-90% dye degradation. A similar kind of fact has also been reported earlier (Zimmerman et al., 1982; Blumel et al., 2002). They had found azoreductases, which preferably used NADPH as co-factor and NADH only when present in significantly higher Kₘ values. Consequently, co-factor NADPH.Na₄ was best amongst all and was selected for further studies. To determine the minimum amount of NADPH.Na₄ required by the gene product, enzyme assays with different concentrations of co-factor were carried out (**Fig. 5.17**). As we can see, 6.8%, 46.7%, 83% and 85.3% dye degradation was observed with 0 mM (**Fig. 5.17a**), 0.5 mM (**Fig. 5.17b**), 1 mM (**Fig. 5.17c**) and 1.5 mM (**Fig. 5.17d**) NADPH.Na₄ concentrations, respectively. Thus it was clear that the cloned gene product requires at least 1 mM of NADPH.Na₄ for the desired activity.
Fig. 5.16: Dye degradation by pET1 *E. coli* BL21(DE3) clone with different cofactors: (a) FAD, (b) FMN, (c) NAD, (d) NADP.Na2, (e) NADH.DPNH and (f) NADPH.Na4. (–– 0 min, △ 10 min, ™ 20 min and ● 30 min)
Fig. 5.17: Dye degradation by pET1 E. coli BL21(DE3) clone at different concentrations of NADPH·Na₄ (a) 0 mM, (b) 0.5 mM, (c) 1 mM and (d) 1.5 mM. ( 0 min, 10 min and 20 min)
Azoreductase assays were carried out for cell extracts of clones carrying azoreductase gene - *E. coli* DH10B clone pUC1, *E. coli* DH10B clone pET1 and *E. coli* BL21(DE3) clone pET1. As controls, assays were also carried out for cell extracts of *E. coli* DH5α, *E. coli* DH10B, *E. coli* BL21(DE3) and *E. coli* strains with vectors pUC19 and pET28a+. The comparison of dye degradation capabilities of the recombinant strains with the control strains is shown in Fig. 5.18. Strains such as *E. coli* BL21(DE3) (Fig. 5.18a), *E. coli* DH5α (Fig. 5.18b) and *E. coli* DH10B (Fig. 5.18c) could degrade only 26.1%, 32.6% and 22.2% dye, respectively after 3 hours. Strains with vectors pUC19 (Fig. 5.18d) and pET28a+ (Fig. 5.18e) could also degrade only 23.5% and 24.4% dye, respectively after 3 hours. Whereas, recombinant pUC19 with azoreductase gene transformed in *E. coli* DH10B named as *E. coli* DH10B clone pUC1 (Fig. 5.18f) and recombinant pET28a+ with azoreductase gene transformed in *E. coli* DH10B named as *E. coli* DH10B clone pET1 (Fig. 5.18g) could degrade 24.4% and 59% dye, respectively after 3 hours. On the other hand, recombinant plasmid, isolated from *E. coli* DH10B clone pET1, transformed in *E. coli* BL21(DE3) named as *E. coli* BL21(DE3) clone pET1 (Fig. 5.18h) was able to degrade 90% of dye within 7 minutes.

Thus it can be observed that only 20-30% of total dye was degraded with cell extracts of strains such as *E. coli* DH5α, *E. coli* DH10B, *E. coli* BL21(DE3) and *E. coli* strains containing vectors pUC19 and pET28a+ after 3 hours. Even cell extracts of *E. coli* DH10B clone pUC1 and *E. coli* DH10B clone pET1 could degrade 24.4% and 59% dye, respectively after 3 hours. In comparison to these controls and recombinant clones in *E. coli* DH10B, *E. coli* BL21(DE3) clone pET1 was able to degrade 90% of dye and that too within 7 minutes only. Moreover, this also shows that the selection of right host strain is very important. Because when recombinant pET28a+ was transformed in *E. coli* DH10B it could degrade only 59% of total dye in 3 hours. Whereas, when the same was transformed in *E. coli* BL21(DE3) it was able to 90% of dye and that too within 7 minutes only.
Fig. 5.18: Comparison of dye degradation by cloned gene product with experimental controls: (a) *E. coli* B21(DE3), (b) *E. coli* DH5α, (c) *E. coli* DH10B, (d) Strain with vector pUC19, (e) Strain with vector pET28a+, (f) pUC1 *E. coli* DH10B clone, (g) pET1 *E. coli* DH10B clone and (h) pET1 *E. coli* BL21(DE3) clone. [−□− 0 min and △ 3 h for (a-g) / 7 min for (h)]

5.4 Conclusion

The present study helps us to understand the importance of consortium (mixed cultures) in bioremediation processes. The cloned azoreductase gene, having a length of 537 bp and containing an ORF of 178 amino acids, on phylogenetic analysis clustered with azoreductase of *Bacillus cereus* Q1 (phylum *Firmicutes*). The recombinant strains possessed *in vitro* azoreductase activities. However, they did not show *in vivo* activities and reason can be attributed to the fact that recombinant *E. coli* strains were unable to uptake azo dyes. The cloned azoreductase requires at least 1 mM of co-factor NADPH.Na+. In comparison to control strains (20-30% of total dye in 3 hours) and *E. coli* DH10B recombinant clones (25-60% of total dye in 3 hours), *E. coli* BL21(DE3) clone pET1 was able to degrade 90% of dye within 7 minutes only. The expression analysis of azoreductase gene will be of immense help in developing novel recombinant strains for application in dye contaminated waste-water treatment technologies.
Chapter 5: Community genomics

5.5 References


