David Hendricks Bergey was born on December 27, 1860 in Pennsylvania. In 1884 Dr. Bergey went to work in the laboratory of Dr. Henry Formad. It was here that Dr. Bergey was introduced to bacteriology. Dr. Bergey was responsible for numerous publications during his lifetime but he is best remembered for the manual of bacterial classification that is named after him. The first edition of the manual was published in 1923 by the Society of American Bacteriologists. The manual, which still bears his name, has been constantly revised and is still used today as a standard reference of bacterial classification.

Chapter 1

ISOLATION, SCREENING AND IDENTIFICATION OF BACTERIAL STRAIN CAPABLE OF DECOLORIZING SYNTHETIC AZO DYES

ABSTRACT

A screening for azo dye decolorizing microorganism resulted in *Pseudomonas spp.* strain isolated from the soil contaminated with untreated textile effluent drain of a textile company located at Tirupur Dt., Tamil Nadu, India. An enrichment culture technique was used to screen for bacteria decolorizing azo dye. Of four bacterial strains tested, one shows maximum decolorization of azo dye (Orange-II) under aerobic condition and was named as an isolate-1. The isolate-1 was found to degrade 56 % of 250 ppm orange II dye. Further characteristic analysis of isolate-1 shows a gram –ve, rod shape bacteria. Around 2000 bp of the isolate-1 16S rDNA was amplified by polymerase chain reaction (PCR) and the phylogenetic relationship was compared to the internal transcribed spacer (ITS) of the related sequences deposited in GeneBank database. The results of the present study reveal that the newly identified strain, isolate-1 was designated as *Pseudomonas oleovorans PAMD_1.*

1.1 INTRODUCTION

Synthetic azo dyes are widely used in textile, paper, printing and some cosmetic industries for their versatility. More than 2000 different azo dyes are currently being used in these industries (Mona *et al.,* 2008; Palaniappan *et al.,* 2002). These industrial effluents contain residual dyes or their metabolites, which are highly toxic, mutagenic, carcinogenic and highly resistant to degradation in nature (Vyas and Molitoris, 1995). Colors in water bodies reduce light penetration, alter the pH, increase the Biological Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) and thereby make aquatic life difficult (Olukanni *et al.,* 2009). Azo dyes are characterized by the presence of one or more azo groups substituted with aromatic amines. These dyes have structural diversity and are designed to resist the physical, chemical and microbial attack (Ramalho *et al.,* 2002).

There are several methods used in the treatment of textile waste effluent. These include physiochemical methods such as filtration, color adsorption by activated carbon, coagulation and flocculation (Southern, 1995; Churchley *et al.,* 1997). The suggested methods are not effectively applied due to high cost factor (Do *et al.,* 2002; Maier *et al.,* 2004), therefore color removal by biological adsorption has been suggested (Parkinson, 1996). In the current scenario, microbial or direct enzymatic treatment offers an indispensable, ecofriendly and cost-effective solutions towards azo dye pollution compared to physiochemical methods (Saratale, 2010).

Varieties of microorganisms including bacteria, fungi, yeasts, actinomycetes and algae are capable of degrading azo dyes, among which bacterial cells are inexpensive and

promising tool for the removal of different azo dyes from textile industrial effluent (Dafale *et al.*, 2008). Bacteria capable of decolorization either in pure cultures or in consortia (Telke *et al.*, 2008; Kalyani *et al.*, 2009) makes them invaluable tools in the biotreatment of the textile effluent (Yang *et al.*, 2003; Bella Devassy Tony, 2009).

It has been found that some microorganisms utilize the azo dyes as a sole carbon source and convert them into its colorless product. The anaerobic cleavage of azo bonds always favored over aerobic condition (Banat *et al.*, 1996). The important limitation of anaerobic degradation of azo dyes is releasing of aromatic amines. These amines are potentially carcinogenic and mutagenic compounds. The aerobic microorganisms may oxidize azo bonds further via deamination or hydroxylation reactions (Levine, 1991). The combination of anaerobic reduction with aerobic treatment may overcome the limitations of anaerobic degradation (Soltz, 2001). Now this interest is focused on the bacteria, as they can decolorize maximum under anoxic conditions and detoxify the aromatic products under aerobic environment by a cytoplasmic enzyme azoreductases (Robinson *et al.*, 2001; Stolz, 2001).

The specificity of azoreductase involves redox mediators with low molecular weight, such as flavins or quinines (Keck *et al.*, 1997). Many bacteria have been reported to readily decolorize azo dyes under anaerobic conditions, like *Bacteroides sp., Eubacterium sp., Clostridium sp., Proteus vulgaris* and *Streptococcus faecalis* (Bragger *et al.*, 1997; Rafii *et al.*, 1990) using azoreductases. The aerobic decolorization of azo dyes by *Pseudomonas sp., Bacillus sp., Sphingomonas sp.*, and *Xanthomonas sp.*, (Dykes *et al.*, 1994) has also been reported.

The objective of the present study was to isolate, identify and characterize bacterial strain capable of decolorizing the azo dye.

1.2 MATERIALS AND METHODS

1.2.1 Source of organism & Chemicals:

Soil samples were collected in the sterile bags from soil contaminated with untreated textile waste effluent from textile industry, Tirupur, Tamil Nadu, India. 10 g of soil sample was transferred into 500 ml Erlenmeyer flask containing 250 ml sterile physiological saline. The sample was acclimatized for 2 weeks at ambient temperature (Oranusi and Mbah, 2005).

Potato dextrose agar (PDA) and Orange II dye were purchased from Himedia, (Mumbai, India). Genomic DNA purification kit, PCR reagents, agarose gel electrophoresis consumables and primers were purchased from Chromous Biotech Pvt. Ltd., (Bangalore, India). Other chemicals were purchased from sd-fine chem. Limited, India.

1.2.2 Sterilization techniques

All glassware were washed with detergent, rinsed with distilled water and sterilized in oven at 80 °C for 2.5 hrs. All tips and polypropylene tubes used as well as media and solutions prepared were sterilized by autoclaving at 121 °C for 20 mins (Olukanni *et al.,* 2006). Inoculation was done with flame sterilized loops and all experiments were performed wearing sterile disposable hand gloves.

1.2.3 Isolation and screening

Principle of selective enrichment batch culture was used to select for dye decolorizers. 10 ml of acclimatized sample was withdrawn from 500 ml Erlenmeyer flask and inoculated to 100 ml dye broth containing (gl^{-1}): NaCl 2.0, $MgSO_4.7H_2O$ 0.42, KCl 0.29, K_2HPO_4 1.27, $NaNO_3.2H_2O$ 0.42, KH_2PO_4 0.85, EDTA 0.5 ml of 0.5 mM and deionized water (pH 7.0). 10 ml of 0.5 % dye (Orange-II) were separately autoclaved and added. Incubation was maintained at 30 °C in orbital shaker (Oranusi and Mbah, 2005).

Cultures were observed daily for increased turbidity (evidence of growth) and decolorization of the medium. 10 ml of decolorized cultures were inoculated into fresh dye broth (100 ml) and incubated. After 4 similar serial transfers, cultures were streaked on nutrient agar plates incorporated with Orange II azo dye and incubated at 30 °C ±2 for 5 days. Single colonies developed were picked based on their ability to produce clear and distinct zone of clearance on dye agar medium. Simultaneously the decolorizing ability was also observed in dye broth (Oranusi and Mbah, 2005).

1.2.4 Identification of dye decolorizing bacteria

The isolate-1 was initially subjected to gram staining and biochemical tests, the results were recorded. Further, molecular characterization of the isolate-1, having best decolorization potential was carried out.

1.2.4.1 Isolation of Genomic DNA

The chromosomal DNA of the strain was isolated according to the procedure described by Rainey *et al.,* (1996) with minor modifications, using Qiagen DNA isolation kit (Qiagen Cat no. 51304). 1 ml of the overnight culture was taken and centrifuged at

6000 rpm for 10 mins and the supernatant was discarded. The pellet was suspended in 400 µl of elution buffer (150 mM NaCl, 0.1 mM Tris-HCl (pH 8), and 10 mM EDTA). The resulting preparation was incubated at 37 °C for 30 mins, after 5 µl of lyzoyme solution (10 $mgml^{-1}$) was added. Then 5 µl of a proteinase K solution (15 $mgml^{-1}$) and 10 µl of a sodium dodecyl sulfate solution (25 %, wv^{-1}) were added, this was followed by incubation at 65 °C for 30 mins. The lysate was extracted with equal volume of phenol, and this was followed by centrifugation at 15000 rpm for 15 mins. An equal volume of chloroform was added to the aqueous layer, and the preparation was mixed and centrifuged at 15000 rpm for 15 mins. DNA was recovered from the aqueous phase precipitation. The collected DNA was quantified by measuring optical density (OD) at 260 nm and the quality was checked by 1 % agarose gel electrophoresis.

1.2.4.2 PCR amplification and analysis

The 16S rDNA gene amplification was performed using the reaction mixture containing 100 ng of template DNA, 20 µl of 16s rRNA Primers, FP (5'-AGAGTTTGATCCTGGCTCAG-3') and RP (5'-ACGGCTACCTTGTTACGACTT-3') (Primer Explorer V3), 200 µM of dNTPs, 1.5 mM of $MgCl_2$, 1U of proofreading *Taq* polymerase. The final volume of the mixture was made up to 200 µl by adding nuclease free water. The amplification was carried out with an initial denaturation at 95°C for 5 mins followed by 35 cycles of denaturation at 94 °C for 45 sec, extension at 72 °C for 1 min and then final extension at 72 °C for 5 mins using a thermocycler (iCycler, Bio-Rad Laboratories). Sequencing of the PCR products were carried out by Priority Life sciences (Hyderabad, India) on an ABI Prism Model-3100 (Applied Biosystems, USA) sequencer.

1.2.4.3 Analysis of PCR product

The amplified PCR products were analyzed on 1 % agarose gel in 1X TBE buffer (Tris–borate–EDTA buffer: 100 mM Tris–HCll^{-1}, pH 8.3, 83 mM boric acidl^{-1}, 1 mM EDTA) at 100 V. The patterns of bands were observed in UV transilluminator visualized by ethidium bromide staining. Sizes of the amplified products were determined using different size of standard DNA ladder. The reproducibility of DNA profiles was tested by repeating twice the PCR amplification with each of the selected primers. Only reproducible bands were considered for analysis. The amplified product was sequenced using automated DNA sequencer.

1.2.4.4 Analysis of sequence data

By using Basic Local Alignment Search Tool (BLAST) (Altschul *et al.,* 1997) from the website http://www.ncbi.nlm.nih.gov/blast, the partial 16S rRNA sequence of the isolated strain generated was compared with the 16S rRNA sequence of other closely related species from the Gen Bank database. The sequence was compared against the sequence available from Gen Bank using the BLASTIN program and was aligned using CLUSTALW software (http://www.ebi.ac.uk/tools/clustalw2) (Thompson *et al.,* 1994).

Distances were calculated according to Kimura's two parameter correction (Kimura, 1980). Phylogenetic tree was constructed according to neighbour-joining method (Saitou *et al.*, 1987) with the parameters of 65 % similarity (5.0/-4.0) cost matrix, gap open penalty=1.2, gap extension penalty=3. A bootstrap analysis was inspected closely to ensure that there were no PCR chimera events. The MEGA3 package (Kumar *et al.,* 2004) was used for all analysis.

1.2.5 Determination of decolorizing capability of isolate-1 for different dyes

Industrial effluent consists of a mixture of dyes, the decolorization ability of our isolate-1 to different dyes were studied. A sample of ~0.02 g dry biomass was added to a 150 ml conical flask containing 100 ml of 100 mgl^{-1} dye solution. The cultures were grown in individual dye components at 30 °C ± 2 for 5 days. Uninoculated dye solutions were used as the control for the dyes. Decolorization of the individual dyes was monitored at their respective maximum absorption wavelength in culture supernatants using a UV-Vis spectrophotometer (Shimadzu UV-2450).

1.2.6 Decolorization potential assay

The most promising isolate identified as *Pseudomonas oleovorans PAMD_1* was tested for decolorization of substrate specific azo dye (Orange II) in submerged culture. Decolorization experiment was done in 150 ml conical flask containing 100 ml of solution; three flasks were inoculated asceptically with ~0.02 g dry biomass using 10 different concentrations of dye (50 ppm – 500 ppm). The flasks were plugged with sterile cotton wool and incubated at 30 °C ±2 for 5 days. At the completion of incubation period, the culture broth was tested for its capacity to utilize the dye as sole carbon and nitrogen source by decolorization assay.

The percentage decolorization of the culture broth was determined at their respective maximum absorption wavelength of 482 nm using a spectrophotometer as described earlier. Decolorization was expressed in terms of percentage of decolorization.

This was calculated using the following formula (Zimmermann *et al.*, 1982; Sudharkar *et al.*, 2002; Chen *et al.*, 2003).

$$\% \text{ Decolorization} = \frac{\text{Initial absorbance value} - \text{Final absorbance value}}{\text{Initial absorbance value}} \times 100$$

1.3. RESULTS AND DISCUSSION

1.3.1 Isolation and characterization of dye degrading microbe

Among the 10 samples collected, four strains were selected as positive organisms which formed clear zone of azo dye around the bacterial colonies. Based on the clarity of the zone formation, strains were designated as isolate-1, isolate-2, isolate-3 and isolate-4. From the four isolated positive organisms, hyperactive strain was selected based on the complete degradation of Orange II dye in the agar medium and dye broth. Table 1 shows the decolorization capability of the four isolates in the dye broth. Among the four strains, isolate-1 showed highest percentage of dye decolorization (around 94 % for 100 ppm).

This isolate also formed and decolorized the dye broth containing the same (Fig 1a and 1b). The selected most active strain was designated as isolate-1. Isolate-1 was selected or further studies because of their superiority in decolorizing azo dyes when compared to other strains. The characteristic features of the isolate-1 are listed in Table 2. The observed morphological and biochemical properties reveal the characteristics criteria of *Pseudomonas spp.*, (Holt *et al.*, 1994). Further confirmation of isolated bacterial strain was done by 16S rDNA molecular method which was used successfully for the identification of strain (Pant and Adholeya, 2007).

However previous reports indicate that though several microorganisms may seem to have a potential for dye degradation, very few strains can withstand the conditions of dyeing effluents (Maeir *et al.,* 2004). Bacteria have been reported to be highly substrate specific to the dye which they are adopted (Pasti-Grisgby *et al.,* 1992, 1996). But the bacterium (isolate-1) isolated in this study decolorizes different dyes, this may due to source which contain various dyes (Oranusi and Mbah, 2005).

In earlier report, there are five bacterial constituents *B.Pumilus, B.megaterium, B.cereus, B.vallismortis* and *B.subtilis* were screened for their ability to decolorize Congo red, Bordeaux, Ranocid Fast Blue and Blue-BCC individually (Bella Devassy Tony *et al.,* 2009) and all isolates decolorized about 72 %. In conclusion, as a preliminary step in the development of textile effluent biotreatment processes, we have discovered textile effluent adapted strains of *Pseudomonas spp.* with potential use in effluent treatment.

1.3.2 Analysis of PCR product and Taxonomy of the strain

Fig 2 shows the 16S rDNA amplified PCR products that were analyzed on 1 % agarose gel observed under UV-transilluminator. Comparison of the partial 16S rDNA gene sequence (1495 bp) obtained from isolate-1 was done with the other bacterial sequences from the NCBI gene bank database. The PCR amplified sequence of isolate-1 was closer to other similar sequences reported in NCBI database as shown in Table 3. The highest degree of identity (<99 %) was obtained with *P. oleovorans* (gene bank No. DQ122200.1). Similarly, another organism D84018.1 (*P. oleovorans*) shared the maximum query coverage of above 99 % with the ITS sequence of the isolate-1. Organism showing more than 90 % similarity in the 5.8S sequence can be considered as

same species (Sugita and Nishikawa, 1999), whereas for identifying the same genus ≥ 99 % sequence identity is needed in the 16S rDNA sequence analysis (Guarro *et al.,* 1999).

The partial 16S rRNA gene sequence obtained for isolate-1 was directly deposited in the NCBI Gen Bank Database under the **Accession No. GU357740** (Fig 3). The phenogram reflecting the phylogenetic relationship of isolate-1 was constructed using data from the BLAST analysis of the rDNA region (Fig 4). The constructed phylogenetic tree using neighbor-joining method calculated by Tamura-Nei model shows that the strains *Pseudomonas oleovorans DQ122200.1 and U61* were very close to the isolate-1, moreover these strains also shares more than 99 % of query coverage and identity to the isolated selected strain. Based on the above study the isolate-1 was given the name *Pseudomonas oleovorans PAMD_1.*

1.3.3 Decolorization capability of isolate-1 on different dyes

Table 4 depicts the decolorization ability of the newly isolated isolate-1 for a number of azo dyes. Orange II, Reactive Yellow FG, Reactive Black B and Blue 4 showed decolorization in the range of 70–94 %. Whereas Free AB45, Procion Red H7B, and Direct Black 22 showed decolorization in the range of 54–65 %. This disparity in decolorization efficiency with different dyes may be attributed to their structural variations (Moosvi *et al.,* 2005). The reduction in decolorization efficiency might be due to the toxic effect of dyes or the blockage of the active sites of enzymes by dye molecule with different structures (Khehra *et al.,* 2005).

1.3.4 Decolorization potential Assay

The isolate *Pseudomonas Oleovorans PAMD_1* was found to degrade Orange II to a greater extent up to 66.60 % for 200 ppm. The percentage decolorization obtained in 120 hrs was highly significant. This result was supported with results of Zimmerman *et al.* (1982) and Sudharkar *et al.* (2002).

This isolate decolorized the dye substrate to a maximum extent of 300 ppm, while decolorizing efficiency varied when compared with the other isolates. Bacteria have been reported to be highly substrate specific to the dye to which they are adopted (Pasti-Grigsby *et al.,* 1992). The *Pseudomonas oleovorans PAMD_1* isolated in this study decolorize Orange –II due to the soil source which contained dyes of various chromophoric groups. Reductive cleavage of the azo bond is the initial step of the bacterial degradation of azo dyes. The existence of azo reductases in obligate aerobic bacteria was first proven in *Pseudomonas K22* and *Pseudomonas KF46* (Zimmermann *et al.,* 1982, 1984). Decolorization of azo dyes occurs by anaerobic, anoxic and aerobic conditions by different groups of bacteria (Anajali Pandey *et al.,* 2006). Earlier, various extensive studies were also carried out to determine the role of bacteria in the decolorization of azo dyes (Talarposhti *et al.,* 2001; Yoo *et al.,* 2001; Isik and Sponza, 2005, Van der Zee and Villaverde, 2005).

1.4. CONCLUSION

This preliminary identification and characterization of the isolate regarding its morphological and biochemical characters as well as decolorization activity and molecular identity gives useful information with regard to the further application of strain

PAMD_1 for various purposes. Results of this research also provide an evidence to the potential application of *Pseudomonas oleovoransPAMD_1* in the biological treatment of azo dye contaminated industrial effluents. Further investigations will be carried out with this isolate for azo dye decolorization which would help for solving the problems dumped into the ecosystems.

Table 1: Efficiency of dye decolorization by the isolates in dye broth after 120 hrs

Sl. No	Dye Concentration (ppm)	% Decolorization *			
		Isolate-1	**Isolate-2**	**Isolate-3**	**Isolate-4**
1	50	100	91.38	82.30	76.50
2	100	93.90	62.18	46.10	39.00
3	150	88.76	44.32	33.70	25.62
4	200	66.60	31.64	22.12	12.50
5	250	56.86	26.50	17.40	10.00
6	300	44.64	18.00	13.80	2.90
7	350	38.71	12.16	8.26	0
8	400	26.32	7.23	3.00	0
9	450	23.40	3.14	0	0
10	500	19.87	2.04	0	0

*Average of the triplicate

Table 2: Taxonomic characteristics of bacterial Isolate strain *Pseudomonas oleovorans PAMD_1*

Test name	Results
Gram stain	G -ve
Cell shape	Rod
Motility	+
Catalase	+
Oxidase	-
MR VP test	-
Soluble pigment	-
Gelatin test	-
Oxygen	+
Starch	+
Galactose	+
Nitrate reduction	-
Citrate utilization	+
Glucose utilization	+
Fructose utilization	+
Indole test	-

+ Positive result

- Negative result

Table 3: List of Sequences showing significant alignments with *Pseudomoans oleovorans PAMD_1* 16S rRNA

Accession No.	Description	Max score	Total score	Query coverage	E value	Max identity
DQ122200.1	*Pseudomonas oleovorans* strain iDCIII12 16S ribosomal RNA gene, partial sequence	183	183	100%	1e-43	100%
D84018.1	*Pseudomonas oleovorans* 16S rRNA gene, complete sequence	180	180	97%	1e-42	100%
HQ264094.1	Uncultured *Pseudomonas spp.* clone SL34 16S ribosomal RNA gene, partial sequence	180	180	100%	1e-42	98%
HM989986.1	Bacterium C04(2010) 16S ribosomal RNA gene, partial sequence	178	178	100%	5e-42	98%
GQ478265.1	*Pseudomonas spp.* B4M-K 16S ribosomal RNA gene, partial sequence	178	178	100%	5e-42	98%
GQ921386.1	Uncultured bacterium clone DF5IPCant16a11 16S ribosomal RNA gene, partial sequence	178	178	100%	5e-42	98%
FN298516.1	Uncultured bacterium partial 16S rRNA gene, clone paPseu1_PV_1	178	178	100%	5e-42	98%
FJ005061.1	*Pseudomonas spp.* enrichment culture clone Guo7 16S ribosomal RNA gene, partial sequence	178	178	100%	5e-42	98%
JF417772.1	Uncultured bacterium clone QQSB53 16S ribosomal RNA gene, partial	172	172	100%	2e-40	97%
HQ324140.1	*Pseudomonas spp.* MBYD-1 16S ribosomal RNA gene, partial sequence	172	172	100%	2e-40	97%
HM779585.1	Uncultured bacterium clone JFR0701_jaa46h07 16S ribosomal RNA gene, partial sequence	172	172	100%	2e-40	97%
HQ445717.1	Uncultured bacterium clone Luq_GN470_006 16S ribosomal RNA gene, partial sequence	172	172	100%	2e-40	97%
HM545333.1	Uncultured bacterium clone BM1-36 16S ribosomal RNA gene, partial sequence	172	172	100%	2e-40	97%
GU726583.1	*Pseudomonas spp.* IRH26 16S ribosomal RNA gene, partial seq	172	172	100%	2e-40	97%

Table 4: Spectrum of dyes decolorized by the isolate-1 in dye broth after 120 hrs

Sl. No	Name of the Dye	λ_{max} (nm)	% Decolorization*
1	Orange II	482	93.90
2	Reactive Yellow FG	418	83.25
3	Blue 4	606	71.29
4	Free AB 45	598	61.00
5	Procion Red H7B	512	65.42
6	Reactive Black B	598	70.25
7	Direct Black 22	470	53.90

*** Average of triplicate**

Figure 1a: *Pseudomonas oleovorans PAMD_1* forming peripheral halo zone on Orange-II dye agar medium

Figure 1b: *Pseudomoans oleovorans PAMD_1* on Orange-II dye broth.

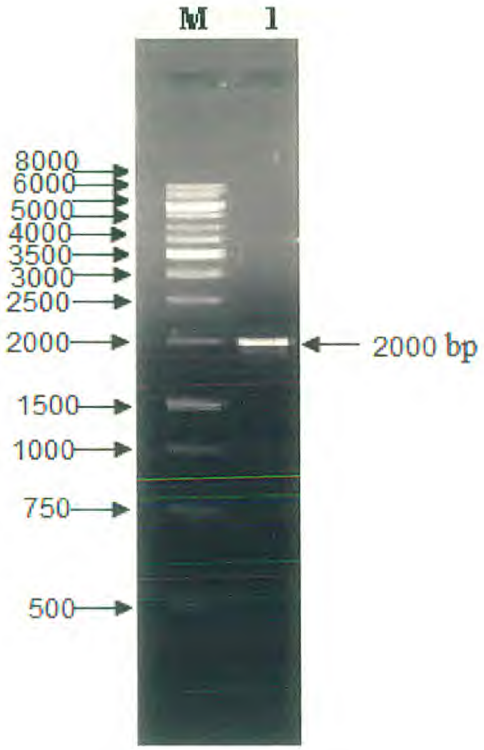

Figure 2: PCR amplification profile of 16S rDNA from the bacterial isolate-1.

LANE M: 1 Kb marker (Fermentas);
LANE 1: PCR Amplification of 16s rDNA.

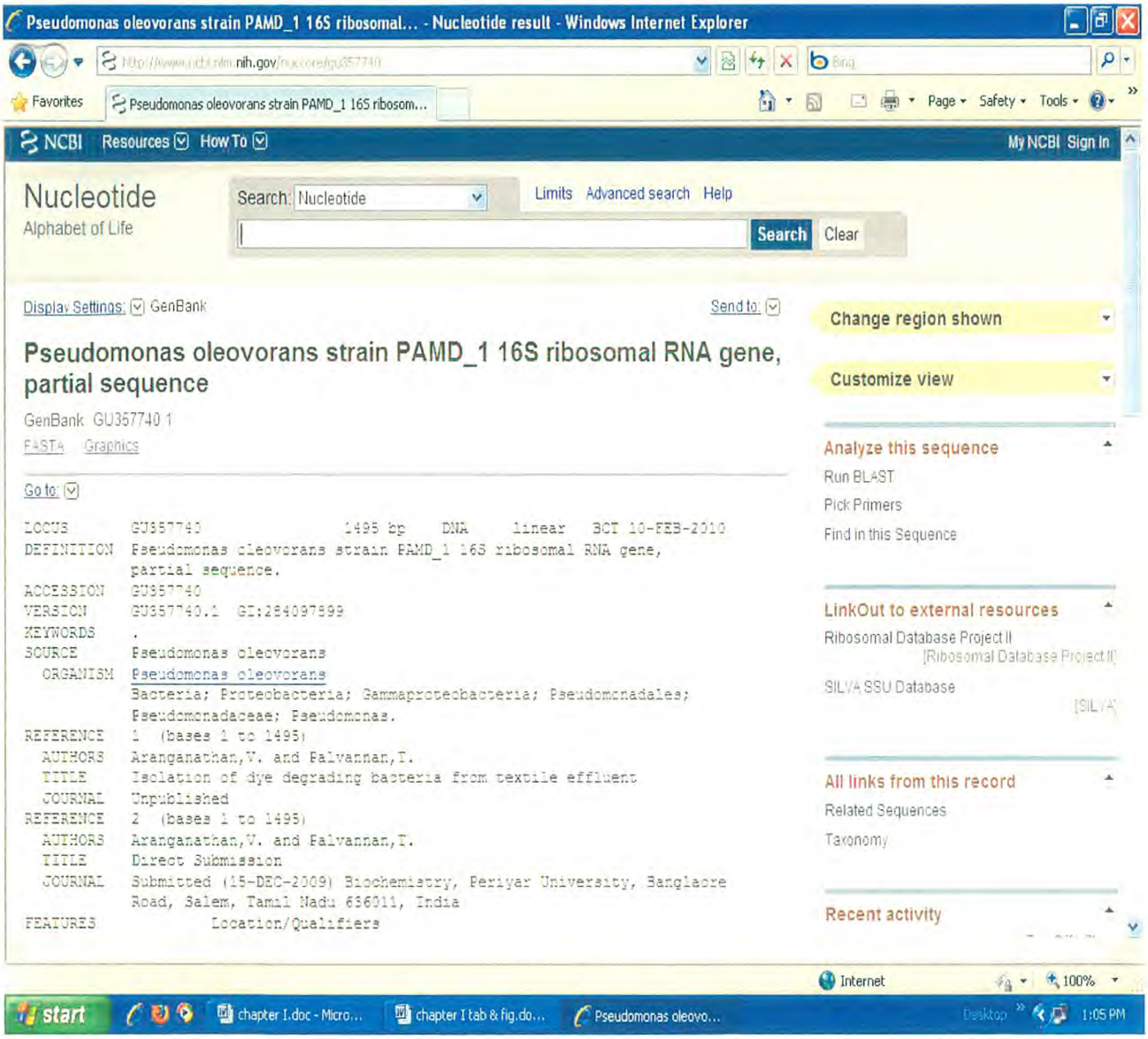

Figure 3: 16S rDNA sequence of the isolate-1 (*Pseudomonas oleovorans PAMD_1*) Submitted in NCBI web site

Figure 4: Average distance phylogenetic tree based on 16S rRNA sequences from *Pseudomoans oleovorans PAMD_1* isolate.