International Journal of Environmental Bioremediation & Biodegradation, 2014, Vol. 2, No. 3, 133-138
Available online at http://pubs.sciepub.com/ijebb/2/3/6

DOI:10.12691/ijebb-2-3-6

Microbial Degradation of Azo Dye by *Pseudomonas spp* 2413 Isolated from Activated Sludge of Common Effluent Treatment Plant

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Received March 24, 2014; Revised May 21, 2014; Accepted May 21, 2014

Abstract In this study we intended to isolate and identify azo dye degrading and decolorizing bacterial strains as well as investigation its biodegradation mechanism. Different bacterial isolates were isolated from activated sludge of common effluent treatment plant and tested against Reactive Violet 5 (RV5) dye. The most potent isolate was identified as Pseudomonas spp via partial sequencing of 16s rRNA DNA. The decolorized sample showed lowering of peak to a smaller absorbance value for dye concentration of 200 mg/L, which informs that the decolorization is due to removal or degradation of dye. The comparison of TLC chromatograms before and after decolorization by Pseudomonas spp under UV light showed that the original dye was quite different from the supernatant obtained after dye decolorization, which was suggested by different values of retention factors obtained in the TLC experiment This difference confirms that decolorization was due to breakdown of dyes into unknown products. The identification of several degradation products from purified RV5 by Pseudomonas spp was achieved with FTIR spectroscopy, 1H NMR and GC-MS. The results showed four compounds, nitrobenzene, 4-nitrophenol, 4-nitroaniline, and 4-nitroanisole, as degradation products.

Keywords: *pseudomonas, Decolorization, GC-MS, FTIR, reactive violet*

Cite This Article: Maulin P Shah, "Microbial Degradation of Azo Dye by *Pseudomonas spp* 2413 Isolated from Activated Sludge of Common Effluent Treatment Plant." *International Journal of Environmental Bioremediation & Biodegradation*, vol. 2, no. 3 (2014): 133-138. doi: 10.12691/ijebb-2-3-6.

1. Introduction

Over $7x10^5$ metric tons of synthetic dyes are produced worldwide every year for dyeing and printing. A very small amount of dye in water (10-50 mg/l) is highly visible and affects the aesthetic merit, water transparency and gas solubility of water bodies, Dyeing units are found in most countries and their numbers have increased. These industries have shown a significant increase in the use of synthetic complex organic dyes as the coloring material. The annual world production of textiles is about 30 million tones requiring 700,000 tonnes of different dyes which causes considerable environmental pollution problems. Dyes include a broad spectrum of different chemical structures, primarily based on substituted aromatic and heterocyclic groups such as aromatic amine (C6H5-NH2), which is a suspected carcinogen, phenyl (C6H5-CH2) and naphthyl (NO2-OH), the only thing in common is their ability to absorb light in the visible region. The waste water from these industries are characterized by high alkalinity, biological oxygen demand, chemical oxygen demand, total dissolved solids and with dye concentrations usually less than 1 g/dm^3 [1]. Color is the first contaminant to be recognized in wastewater and has to be removed before discharging in to water bodies or on land. The inefficiency in dyeing processes has resulted in 10-15% of unused dyestuff entering the wastewater directly. Color present in dye effluent gives a straightforward indication of water being polluted and discharge of this highly colored effluent can damage directly the receiving water. Furthermore, it is difficult to degrade the mixtures of the wastewater from the textile industry by conventional biological treatment processes, because their ratio of Biochemical Oxygen Demand (BOD)/ Chemical Oxygen Demand (COD) is less than 0.3 [2]. The discharges of dye house wastewater into the environment is aesthetically displeasing, impede light penetration, damage the quality of the receiving streams and may be toxic to treatment processes, to food chain organisms and to aquatic life. The degradation of molecules of dyes in the environments by microorganisms is likely to be slow, which means that it is possible for high levels of dye to persist, and potentially accumulate. Due to the low biodegradability of dyes, conventional biological treatment processes are inefficient in treating dye wastewaters. In addition, numerous physical and chemical techniques such as flocculation combined with flotation, electro-flotation, flocculation with Fe(II), $Ca(OH)_2$, membrane filtration, precipitation, ionexchange, Ozonation and Katox treatment method involving the usage of activated carbon and air mixtures were also used

[3]. Even though some of the above mentioned methods are effective, most of them suffer from shortcomings such as excess usage of chemicals, sludge disposal, expensive operating cost, ineffective color reduction for sulfonated azo dyes and poor sensitivity towards shock load conditions. Biological decolonization is employed under either aerobic or anaerobic environment. A number of reports discourage the azo dye decolonization by microorganism under anaerobic conditions as it leads to the formation of corresponding aromatic amines. Even though their reductive cleavage is responsible for colour removal, the formation of aromatic amines is highly undesired as they are reported to be carcinogenic. In the presence of oxygen, aromatic amines can be degraded. Degradation of azo dyes has been studied under aerobic conditions using both pure and mixed microbial cultures. The restrictive environmental legislation, the ecological problem and the high cost of conventional technologies for dye house effluent treatment have resulted in the search of economically viable and technologically suitable wastewater treatment plants. The effectiveness of microbial decolorization depends on the adaptability and the activity of selected microorganisms. Over the past decades, many microorganisms are capable of degrading azo dyes, including bacteria, fungi and yeast [5,6]. However, the low pH requirement for an optimum activity of the enzymes and the long hydraulic retention time for complete decolorization are the disadvantages of using fungi. In addition, they may inhibit the growth of other useful microorganisms. Thus, large-scale applications of fungal decolorization have been limited. With the aforesaid details in the background, this investigation was aimed to study the feasibility of biodegradation of the dye house effluent by employing the bacterial strain and investigation of biodegradation mechanism.

2. Materials & Methods

Commercially important and commonly used reactive azo dye for cotton dyeing. Reactive Violet 5 was obtained from local textile industry, Ankleshwar, Gujarat, India. All other reagents were purchased from Sigma-Aldrich, USA & Hi-Media, India and used without further purification. Dye stock solutions were prepared and used in all experiments.

2.1. Isolation & Screening

Different bacterial isolates were isolated from wastewater generated by the local textile company, Ankleshwar, Gujarat, India. These isolates were used in order to determine the most potent one. Well grown bacterial colonies were picked and further purified by streaking. Identification of the bacterial isolates was carried out by 16s rRNA gene sequencing assay. Bergey's Manual of Systematic Bacteriology according to Krieg and Holt (1984). Screening of bacterial isolates for textile reactive azo dye degradation was carried out according to Manivannan *et al. (*2011) as follow.

2.2. Identification

The major physiological and biochemical tests were performed as described previously (Mata *et al*., 2002). Morphological and physiological characteristics of the best isolated strain were studied either on nutrient agar or in nutrient broth. Gram reaction, motility, shape and color of colony, catalase, urease, oxidase activities, nitrate reduction, esculin, tween 20 and 80 hydrolyzes and indol productions were checked as recommended by Smibert and Krieg (1994). Acid production from carbohydrates and sugars and utilization of carbon and nitrogen sources were evaluated as recommended by Ventosa *et al*. (1982). To determine the optimum temperature and pH for the growth of the strain, the cultures were incubated at a temperature range of 5 -55°C with intervals of 5°C and pH values of 5-11. pH values below and above 6 were adjusted by sodium acetate and TrisHCl buffer, respectively.

2.3. Dye Decolourization Experiment

Culture media: The mineral salt medium used in the degradation study contained (g l-1): K2HPO4, 7.00; KH2PO4, 2.00; MgSO4. $7H_2O$, 0.1; NH_2SO_4 1.00; Sod. Citrate 0.5; traces of yeast extract and glucose. Dye decolourization experiment was carried out in 100 ml flask containing 50 ml MSM amended with Reactive Violet 5 (200 mg/l), traces of yeast extract and glucose. The pH was adjusted to 7 ± 0.2 using Sodium hydroxide and Hydrochloric acid solution. Then, the flasks were autoclaved at 121°C for 15 minutes. The autoclaved flasks were inoculated with 2% inoculum containing approximately 2 x 108 cells and incubated at 37 ± 1°C at static condition for 72 hrs.. Samples were drawn at 0 h (control) and 6 hrs intervals for observation. 10 ml of the dye media and control medium was filtered and centrifuged at 7000 rpm for 10 minutes. Decolourization was assessed by measuring absorbance of the supernatant with the help of UV Spectrophotometer at wave length maxima (λm) of Reactive Violet 5 dye (548 nm).

2.4. Decolourization Assay

Decolourization assay was measured in the terms of percentage decolourization using UV Spectrophotometer. The percentage decolourization was calculated from the following equation,

$$\%\ Decolourization = \frac{InitialOD - FinalOD}{InitialOD} \times 100$$

2.5. Biodegradation Analysis

The biodegradation and biodegradation analysis was done using TLC, FTIR spectroscopy, 1H NMR and GC-MS. Th supernatants obtained after decolorization were extracted with dichloromethane and dried over anhydrous Na_2SO_4 and evaporated to dryness. The residue obtained was first examined by thin layer chromatography according to Kalyani *et al.,* 2008 on silica gel using mobile phase solvent system *n* propanol, methanol, ethyl acetate, water and glacial acetic acid (3:2:2:1:0.5) and results were observed under UV illuminator. Infrared spectra: were determined on Thermo NICOLET 5700 Spectrophotometer. The software used in spectrophotometer was OMNIC. Analysis was carried out at room temperature in the mid IR region of 400 to 4000cm -1 at a scan speed of 60. The H NMR spectra

were recorded with a Bruker Avance DRX500 spectrometer (Bruker, Germany), operating at 500 MHz for the 1H nucleus,. Experiments were performed in DMSO-d6 at 25°C in 5-mm NMR tubes. Chemical shifts σ in ppm are referred to TMS as the internal standard. GC-MS was performed using a QP5000 mass spectrometer from Shimadzu (Kyoto, Japan) fitted with a GC-17A gas chromatograph (Shimadzu; Kyoto, Japan). The ionization voltage was 70 eV. Gas chromatography was conducted in the temperature programming mode with a XTI-5 column (0.25 mm by 30 m) from Restek. The initial column temperature was held at 40°C for 4 min, then increased linearly to 270°C at 10°C/min, and held for 4 min at Helium was used as carrier gas with a flow rate of 1.0 ml/min. Injection was splitless to increase sensitivity.

2.6. Molecular Genetics Analysis

2.6.1. DNA Extraction

Genomic DNA was extracted from most potent isolate using Easy Quick DNA extraction kit (Bangalore Genei, India) following the manufacturer's instructions. PCR amplification of 16S-rRNA gene and laccase genes The amplification of 16s rRNA gene was performed with primer U1 [5CCA GCA GCC GCG GTA ATA CG3] and U2 [5ATC GG(C/T) TAC CTT GTT ACG ACT TC3] according to Kumar *et al.* (2006).The PCR primers used for amplification of laccase gene were F (AGTACGGGCTCCTTTCATGC) and R (AGCATGCGCAAGTCCTATCA). The reaction mixture was (10 Pmol. Of each primer, 50-100 ng of DNA template and 12.5 µl of 2x superhot PCR Master Mix).The Thermal cycler program was 94°C for 4 min., 94°C for 1 min., 55°C for 1 min. 16s rRNA gene and for laccase gene, 72°C for 1.5 min, the number of cycles was 35 and 40 cycles for 16srRNA and laccase genes respectively and the post PCR reaction time was 5 min at 72°C.

2.6.2. Analysis of the PCR Products

After the amplification, the PCR reaction products were fractionated with 100 bp ladder marker (Fermentas, Germany) on 10 x 14 cm 1.5%-agarose gel for 30 min using Tris-borate- EDTA Buffer. The gels were stained with 0.5 ug/ml of ethidium bromide, visualized under the UV light and documented using a GeneSnap 4.00- Gene Genius Bio Imaging System (Syngene; Frederick, Maryland, USA).

2.6.3. Sequencing of 16S-rRNA and Iaccase genes

The 990bp PCR-product for 16s rRNA gene and for laccase gene of most potent isolate were purified from excess primers and nucleotides by the use of Axy Prep PCR Clean-up kit (AXYGEN Biosciences, Union City, California, USA) and directly sequenced using the same primers as described for the amplification process. The products were sequenced using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (ABI Applied Biosystems, Foster City, California, USA) on a 3130XL Genetic Analyzer (Applied Biosystems). The bacterial 16S rDNA sequences obtained were then aligned with known 16S rDNA sequences in Genbank using the basic local alignment search tool (BLAST) at the National Center for Biotechnology Information, and percent homology scores were generated to identify bacteria.

3. Results and Discussion

3.1. Bacterial Identification

According to The Bergey's manual of systematic bacteriology and considering the physiological and biochemical tests performed, the strain was tentatively named as *Pseudomonas*.

3.2. Molecular Identification

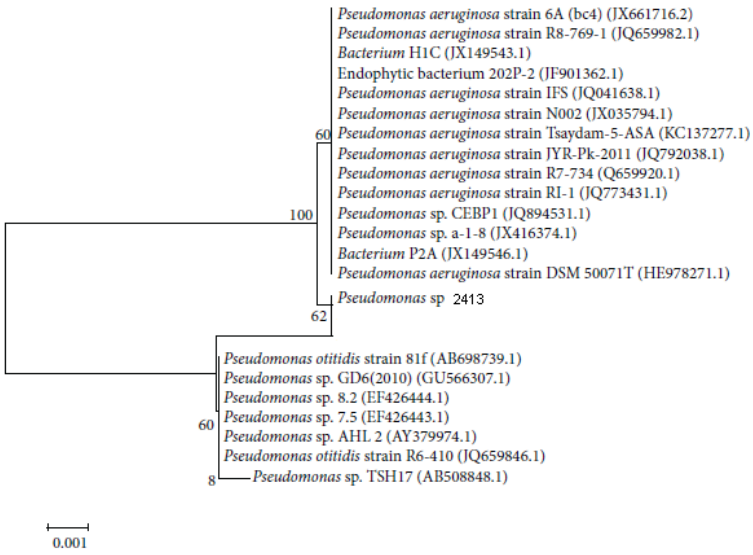

Figure 1. Phylogram (neighbor-joining method) showing genetic relationship between strain 2413 and other related reference microorganisms based on the 16S rRNA gene sequence analysis

The results of 16S rDNA sequence alignment and phylogenetic tree analysis revealed that 16S rDNA sequence of strain 2413 was 100% identical to that of Figure 1. The DNA–DNA hybridization between strain 2413 and a reference strain *P. aeruginosa* JCM 5962T was 96%. The taxonomic characteristics of strain 2413 were mostly the same as those of *P. aeruginosa* JCM 5962T, that is, tests for production of catalase and oxidase,

reduction of NO3 to NO2, and hydrolysis of casein and gelatin are positive, but o-nitrophenyl-b-D-galactopyranoside (ONPG) test and hydrolysis of starch were negative for the both strains. However, strain 2413 was able to hydrolyze neither lipids (supplied as tributyrin), maltose nor D-mannose, all of which were hydrolyzed by *P. aeruginosa* JCM 5962T. Several studies were reported that potentiates of Pseudomonas to decolorize and degrade different azo dye (Ola *et al.,* 2010; Pahlaviani *et al.,* 2011; Tripathi & Srivastava 2011).

3.3. Decolorization and Biodegradation

All the decolorization experiments were done under static conditions. Pseudomonas successfully resulted in the decolorization of the dye, Reactive Violet5. The UV-VIS spectrum, as shown in Figure 2 corresponds to initial and final samples of decolorization experiments. The absorbance values were analyzed from 300 to 800 nm. The decolorized sample showed lowering of peak to a smaller absorbance value for dye concentration of 200 mg/L, which informs that the decolorization is due to removal or degradation of dye (Figures 2a, b). Pseudomonas completely decolorized Reactive Violet5 (200 mg/L) within 72hrs under static condition. These observations suggest that the decolourization performance Pseudomonas isolate static anoxic condition where depletion in oxygen content followed. Decolourization decreased at shaking condition could be competition of oxygen and the dye compounds for the reduced electron carriers under aerobic conditions (Kalme *et al.,* 2007).

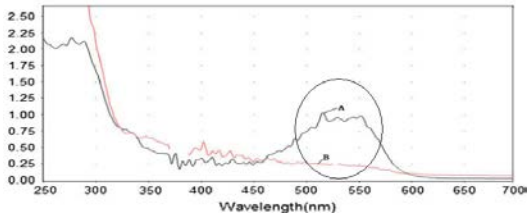

Figure 2. Variation in the UV–vis spectra of Reactive Violet5 before and after decolorization by *Pseudomonas* spp. (A, 0 h; B, 72 h)

3.4. Analysis of Degradation Products

TLC of extracted metabolites confirmed the degradation of RV5. The RF value of RV5 was noted as 0.65 where as extracted metabolites had shown two spots with the increased Rf values as 0.74 and 0.83 (data not shown).

3.5. Spectroscopic Characteristics

The chemical structures of isolated metabolites were clarified by the analysis of IR, NMR and GC-MS spectrometry. Comparison of FTIR spectrum of control dye with metabolites extracted after complete decolourization clearly indicated the biodegradation of the parent dye compound by Pseudomonas (Figures 3 a, b and c). The FT-IR spectra of RV5 control dye display peaks at 3535.9, 1634, 1600, 1198.2 and 1035.4. Results of FTIR analysis of both the samples obtained after decolourization showed absence of peak at 1600 cm^{-1} indicates breakdown of azo bond, might be due to action of azoreductase. The IR spectrum of metabolite I showed absorptions at 699 cm^{-1}, 759 cm^{-1}, 765 cm^{-1} and 827 cm^{-1} for substituted benzene. The SO3 group antisymmetric and symmetric vibrational adsorption peaks can be assigned to the peaks at 1184 cm^{-1} and 1042 cm^{-1}, respectively. Peaks at 1130 and 1011 cm^{-1} can be assigned to the in-plane skeleton vibration of benzene ring and in-plane bending vibration of benzene ring. Whereas the IR spectrum of metabolite II indicates the main characteristic bands 3360 cm^{-1} for hydroxyl group and 1130 cm^{-1} and 1011 cm^{-1} witch assigned to the in-plane skeleton vibration of benzene ring. 699 cm^{-1}, and 759 cm^{-1}. These two bands are characteristic bands of disubstitution structure out-of-plane skeleton bending vibrations of benzene ring. Peaks at 1184 and 1042 cm^{-1}, for the SO3 group. Figures.4a, b and c shows the 1H NMR spectrum of of RV5 dye and its metabolite products. RV5control dye 1H-NMR (D20) 8: 7.28 (δ, 2H, J=8.8 Hz), 7.60 (δ, 1H, J=1.5 Hz), 7.72 (δ, 2H, J=8.8 Hz), 7.84 (dd, 1H, J=8.8,1.5 Hz), 7.86 (s, 1H), 8.01(δ, 1H, J=8.8 Hz). MetaboliteI 1H NMR (500 MHz, CDCl3) δ = 7.447 (dd; J = J=8.035, 8. J=8.035; 1H; 5-H), 7.461 (d; J = 8.035; 1H; 4-H), 7.521 (d; J = 7.684; 1H; 2-H), 7.461 (dd; J = 8.035; 1H; 6-H), 7.448 (dd; J = 8.035; 1H; 3-H), 8.2 (1H, s, –SO3H). ppm. Metabolite II 1H-NMR (500 MHz, CDCl3) δ = 7.390 (6, 1H, J=8.034, J=0.546,), 6.896 (7, 1H, ddd; J=8.034, J=0.537), 6.896 (10, 1H, dd; J=8.034, J=0.546), 7.390 (11, 1H, dd; J=8.034, J=0.537) ppm. Mass spectrum of Metabolite I) (Figure 5a) showed M+ at M/Z 157 for molecular formula C6H5O3S. Mass spectrum of Metabolite II) showed M+ at M/Z 173 for molecular formula C6H6O4S (Figure 5 b). The above – mentioned results of spectral analysis and chromatography proved that the identified compounds are products of the cleavage of N-C-bond in the dye molecule. Metabolite I has been identified as benzenesulfonic acid and metabolite II as hydroxy-benzenesulfonic acid.

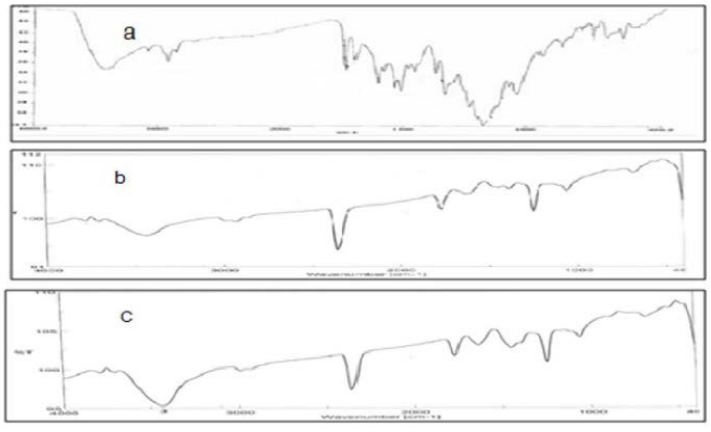

Figure 3. FT-IR spectra of (a) RV5, control dye, (b) Metabolite I and c Metabolite II

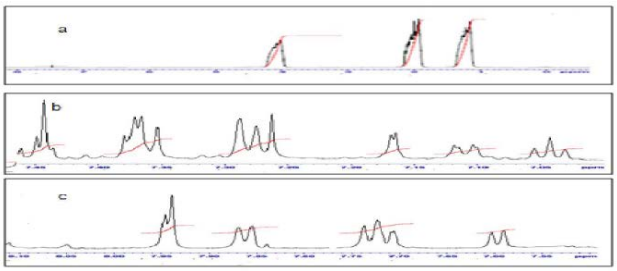

Figure 4. The 1H NMR spectrum(a)RV5, control dye, (b) Metabolite I and c Metabolite II

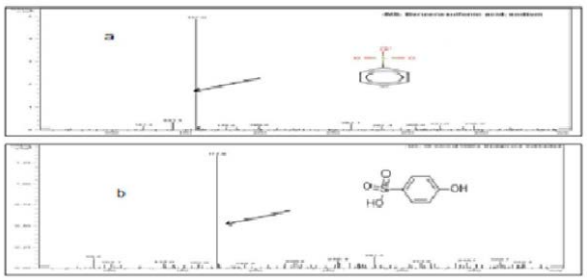

Figure 5. GC/MS spectra of (a) Metabolite Iand (b) Metabolite II

3.6. Proposed Mechanism

Based on study carried out, we presumed that azoreductase would have been responsible for the asymmetric cleavage of azo linkage to form two intermediate compounds. Different substituent present on the benzene ring are removed stepwise; however, sulfonate remained on the benzene ring and would have been removed as a last moiety and further transformations of the intermediate products would be carried by laccase. Degradation mechanism of Reactive Violet5 under aerobic environment by infrared (FTIR), NMR and gas chromatography-mass spectrometry (GC-MS) They described the formation of four intermediatory compounds 1-diazo-2-naphthol, 4- hydroxybenzenesulphonic acid, 2-naphthol and benzenesulphonic acid Previous studies reported that in azo dyes in which the whole molecule represents a fully conjugated electronic system, an access site with a lignin-like structure is sufficient to provide an enzyme-dependent excitation state, from which the stepwise propagation of cleavage processes usually resulted in the biodegradation of the entire molecule. (Pasti-Grigsby *et al*. 1993). Oxidative biodegradation takes place upon action of enzymes such as peroxidases and laccases. Bacterial extracellular azo dye oxidizing peroxidases have been characterized in *Streptomyces chromofuscus* (Pasti-Grigsby *et al*. 1996). Because very little is known about how bacteria break down the azo compounds and the characteristics by which different enzymes act in the decolorization, it is only possible to assume that the known extracellur enzymes are responsible for the degradation and use this assumption to explain the results at this time. Fortunately, the mechanism of biodegradation of phenolic azo dyes by peroxidases and laccase from white rot fungi has been investigated before. Goszczynski *et al*. (1994) suggested two different mechanisms for the degradation of slfonated azo dyes. The first based on asymmetrical cleavge of the azo group results in the formation of quinine monoamine and azo derivatives as direct oxidation products, The second is an asymmetrical cleavage and yield quinine compound diazen derevatives. These compounds finally undergo various spontaneous reactions result in the formation of secondary products. Laccase belongs to a group of enzymes called blue copper oxidases with a molecular weight of 60 to 390 KDa (Call and Muke, 1997). Laccase also has broad substrate specificity and is capable of oxidizing phenols and aromatic amines by reducing molecular oxygen (instead of H2O2) to water by a multicopper system (Wesenberg *et al*., 2003) Figure 6. Laccases are most diverse in their catalytic action and laccase catalyzed transformation of dyes depends on the chemical structure of dye molecules. Also, decolorization of malachite green by *B. laterosporus* was faster with the significant increase in laccase where biodegradation process involved deamination and opening of benzene ring structure to produce non toxic products (Gomare and Govindwar, 2007.

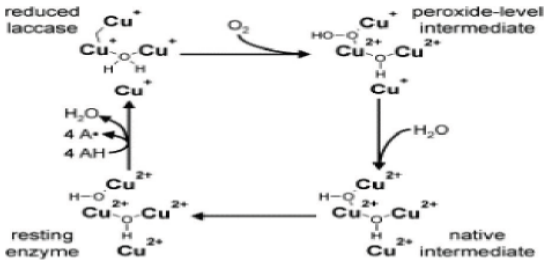

Figure 6. Illustration of the catalytic cycle of laccases (Wesenberg et al., 2003)

4. Conclusion

The present study confirms the ability of newly isolated bacterial culture *Pseudomonas spp* 2413 to decolorize the textile dye Reactive Violet with remarkable decolorization efficiency, thus suggesting its application for decolorization of dye bearing industrial wastewaters. Although decolorization is a challenging process to both the textile industry and the waste water treatment, the result of this findings and literature suggest a great potential for bacteria to be used to remove color from dye wastewaters. Interestingly, the bacterial species used in carrying out the decolorization of Reactive Violet in this study was isolated from the activated sludge of common effluent treatment plant. The ability of the strain to tolerate, decolorize azo dyes at high concentration gives it an advantage for treatment of textile industry waste waters. However, potential of the strain needs to be demonstrated for its application in treatment of real dye bearing waste waters using appropriate bioreactors. The results obtained in this study are very promising for the very effective. However, further work is needed to identify other gene(s) responsible for this kind of textile azo dyes decolorization.

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