Biodegradation of a Textile Mono Azo Dye: Reactive Violet 5 by a Novel Isolated Bacterial Strain

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Abstract: Aim: In this study we intended to isolate and identify Azo dye biodegrading and decolorizing bacterial strains as well as investigation of biodegradation mechanism. Different bacterial isolates were isolated from dye contaminated soils behind textile factories and tested against Reactive Violet5 (RV5) dye. The most potent isolate was identified as Bacillus *cereus* via partial sequencing of 16srRNA 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 *B. cereus* 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 *Bacillus cereus* was achieved with FTIR spectroscopy, ^{1}H NMR and GC-MS. The results showed two main compounds, benzenesulfonic acid and hydroxy-benzenesulfonic acid. as degradation products.

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Key words: *Bacillus cereus* Reactive Violet5, Biodegradation, FTIR spectroscopy, , 1H NMR, GC-MS spectral analysis

1. Introduction

Dye wastewater from textile and dyestuff industries is one of the most difficult industrial wastewaters to be treated. Wastewater from these industries is characterized by high alkalinity, biological oxidation demand, chemical oxidation demand, total dissolved solids with dye concentrations generally below 1 g/dm^3 (Kaushik and Malik, 2009). The synthetic origin and complex aromatic structures of dyes make them stable and persistant (Fewson, 1998). The chromophores in anionic and nonionic dyes mostly consist of azo groups or anthraquinone types. Azo dyes are the most widely used and account for 60% of the total dye structures known to be manufactured (Allen, 1971). Azo dyes comprise a diverse group of synthetic chemicals that are widely used by the textile, leather, food, cosmetics, and paper product industries. The annual world production of azo dyes is estimated to be around one million tons (Stolz, 2001), and more than 2000 structurally different azo dyes are currently in use. Azo dyes are generally recalcitrant to biodegradation due to their complex structures and xenobiotic nature and typically require an anaerobic–aerobic process to achieve complete mineralization. Many azo dyes and their degradation intermediates are mutagenic and carcinogenic (Chung and Cerniglia, 1992) and contribute to the mutagenic activity of ground and surface waters that are polluted by textile effluents (Umbuzeiro *et al*., 2005). There are a variety of physicochemical methods for color removal from effluents containing dyes (Wang *et al*., 2004). Both the physical and chemical methods have many disadvantages in application, such as high-energy costs, high-sludge production and formation of the secondary toxic by-products (Ghodake *et al*., 2011) . Several studies have focused on biomaterials that are capable of biodegrading and biosorbing dyes from wastewaters. Biological materials such as peat, chitosan, yeast, fungi and bacterial biomass, are used as biosorbents to concentrate and remove dyes from solutions (Manivannan *et al*., 2011).

Several studies have been reported the Laccases degradation of azo dyes (Adosinda *et al.,* 2003). These enzymes are multicopper phenol oxidases that decolorize azo dyes through a highly nonspecific free radical mechanism forming phenolic compounds, thereby avoiding the formation of toxic aromatic amines (Wesenberg *et al.,* 2003). Molecular identification of azo dyes biodegrading bacteria is useful in future manipulation and utilization. Bacterial 16S-rRNA gene is a common target for taxonomic purposes and identification, largely due to the mosaic composition of phylogenetically conserved and

variable regions within the gene (Gurtler and Sanisich, 1996). However, few studies reported the use of a single adaptable microorganism in a sequential anaerobic/aerobic treatment (Isik, 2004). Moreover, the available literature on sequential anaerobic/aerobic treatment with a single microorganism is extremely limited (Xu *et al.,* 2007). Apparently there is a need to develop novel biological decolorization processes leading to the more effective clean up of azo dyes using a single and adaptable microorganism that is efficient under both anaerobic and aerobic conditions. The aim of the present study is to isolate and identify azo dye biodegrading bacteria strain and investigation of biodegradation mechanism.

2. Materials and Methods

Chemicals:

Commercially important and commonly used reactive azo dye for cotton dyeing. Reactive Violet5 (RV5) (C.I. no 18097 (RV5) Trisodium 5-acetamido-4-hydroxy-3-[2- ydroxy-5-(2-sulfonatooxyethylsulfonyl) phenyl]azo- naphthalene-2,7-disulfonate; reactive violet 5, Figure. 1) was obtained from Ciba – Gigi chemical company , Cairo, Egypt. All other reagents were purchased from Sigma-Aldrich, St. Louis, MO, USA and used without further purification.

Dye stock solutions were prepared and used in all experiments.

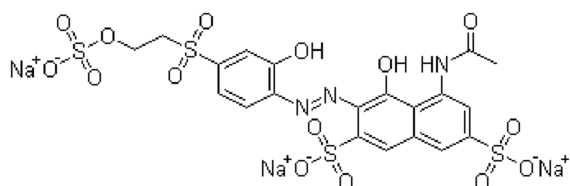

Figure.1 The chemical structure of Reactive Violet5 (RV5)

Bacterial strains :

Different bacterial isolates were isolated from wastewater generated by the Keswet El kaabaa Textile Company, Makkah, K.S.A. 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 16srRNA 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.

Identification of the isolate

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.

Dye decolourization experiment

Culture media: The mineral salt medium used in the degradation study contained (g l-1): K_2HPO_4, 7.00; KH_2PO_4, 2.00;$MgSO_4$. $7H_2O$, 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 Violet5 (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 15minutes. 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 0h(control) and 6 hrs intervals for observation.10 ml of the dye media and control medium was filtered and centrifuged at 5000 rpm for 20 minutes .Decolourization was assessed by measuring absorbance of the supernatant with the help of UV Spectrophotometer at wave length maxima (λm) of Reactive Violet5 dye (548 nm) .

Decolourization assay

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

$$\textbf{\% Decolourization} = \frac{\text{InitialOD} - \text{FinalOD}}{\text{InitialOD}} \textbf{X100}$$

Biodegradation analysis:

The biodegradation and biodegradation analysis was done using TLC, FTIR spectroscopy, ^{1}H 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 ^{1}H 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 (Bellefonte, PA). 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.

Molecular genetics analysis

DNA Extraction:

Genomic DNA was extracted from most potent isolate using Easy Quick DNA extraction kit (Genomix) 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. 16srRNA 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 .

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 (Bioshop; Canada) for 30 min using Tris-borate-EDTA Buffer. The gels were stained with 0.5ug/ml of ethidium bromide (Bioshop; Canada), visualized under the UV light and documented using a GeneSnap 4.00- Gene Genius Bio Imaging System (Syngene; Frederick, Maryland, USA).

Sequencing of 16S-rRNA and laccase genes :

The 990bp PCR-product for16srRNA gene and for laccase gene of most potent isolate were purified from excess primers and nucleotides by the use of AxyPrep 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

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 *Bacillus cerues*.

Molecular identification

The isolate was selected based on their ability to form a high dye decolorization zone on agar plates under aerobic or anaerobic conditions. Isolate was identified using 16S rDNA sequencing. The obtained data from 16S- rRNA gene sequencing were aligned at the National Center for Biotechnology Information (NCBI),

These sequence was compared to those in GenBank to obtain the most similar 16S rDNA fragments, which is isolate with a high efficiency rates to decolorize azo dyes under aerobic conditions were identified. The sequences of isolate (MLTF1) accession number KC255387 showed 99% similarity with Bacillus cereus ATCC 14579 strain accession number NR074540, The sequences of isolate (MLTF1) showed maximum similarity with Bacillus sp. However, the isolate had the closest relationship with Bacillus cereus Several studies were reported that potentiates of Bacillus cereus to decolorize and degrade different azo dye (Ola *et al.,*2010 ; Pahlaviani *et al.,*2011; Tripathi & Srivastava 2011)

Decolorization and biodegradation:

All the decolorization experiments were done under static conditions. Bacillus cereus successfully resulted in the decolorization of the dye, Reactive Violet5. The UV-VIS spectrum, as shown in Figure2

corresponds to initial and final samples of decolorization experiments. The absorbance values were analyzed from 300 to 800 nm. The initial dye solution, before decolorization showed high peak at . 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). Bacillus cereus completely decolorized Reactive Violet5 (200mg/L) within 72hrs under static condition. These observations suggest that the decolourization performance Bacillus cereus 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).

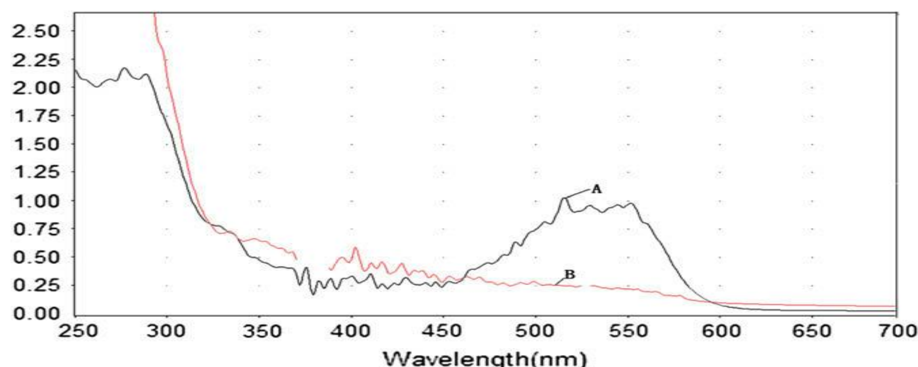

Figure2. Variation in the UV–vis spectra of Reactive Violet5 before and after decolorization by *Bacillus cereus* (A, 0 h; B, 72 h).

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).

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 **Bacillus cereus** (Figures 3a,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 SO_3 group antisymmetric and symmetric vibrational adsorption peaks can be assigned to the peaks at 1184 cm-1and 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 SO_3 group .

Figures.4a ,b and c shows the ^{1}H 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 ^{1}H 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 ^{1}H-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 5b).

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. MetaboliteI has been identified as benzenesulfonic acid and metabolite II as hydroxy-benzenesulfonic acid.

Proposed mechanism:

In this study 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 trans formations 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 slfon-ated 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 H_2O_2) 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.

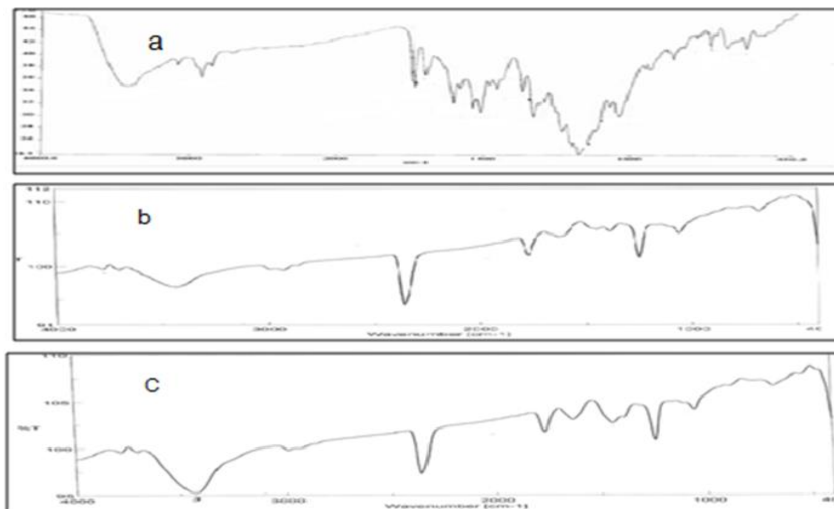

Figure 3. FT-IR spectra of (a)RV5, control dye, (b) Metabolite I and c Metabolite II.

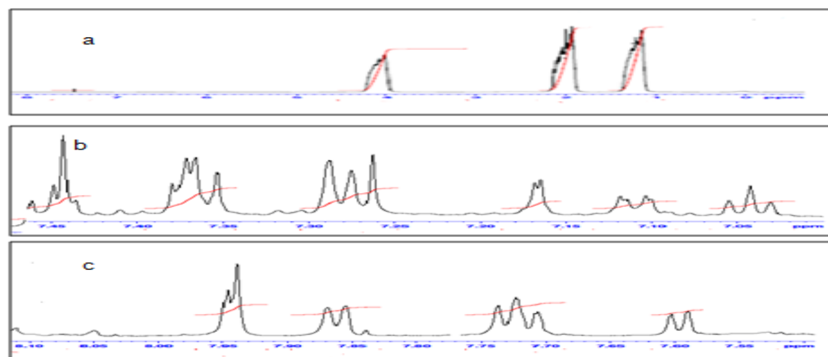

Figure. 4. The 1H NMR spectrum(a)RV5, control dye, (b) Metabolite I and c Metabolite II

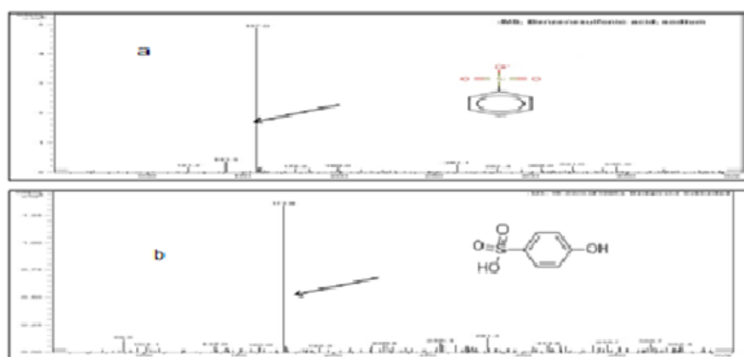

Figure 5 GC/MS spectra of (a) Metabolite Iand (b) Metabolite II.

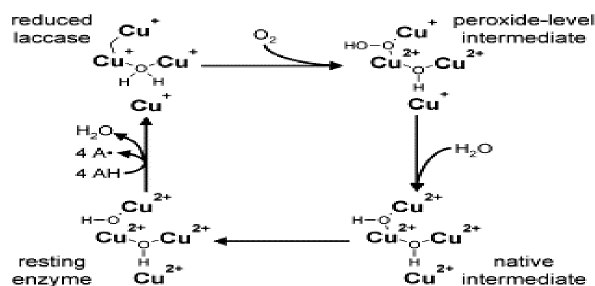

Figure 6. Illustration of the catalytic cycle of laccases (Wesenberg et al., 2003)

4. Conclusion

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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