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# Purification and Analysis of Norcadia spp .Azoreductase

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**Abstract** Nocardia spp. was isolated as Acid Red degrader. The reductive cleavage of azo bond was catalyzed by azo reductase, the key enzyme for the azo dye degradation. Azo reductase from the Nocardia spp. isolate was purified and characterized. Azoreductase was found to have the following features. The catalytic reduction of acid red 37 by purified azoreductase in the presence of NADH as electron donor was studied and the products of degradation were determined as 1-{3-amino-5-[(aminoxy)sulfonyl]phenyl}ethanol and 7,8-diamino-3[(aminoxy)sulfonyl]naphthalene-1-ol.

***Keywords:*** *Nocardia, Acid Red, LC-MS, NADH, Azoreductase*

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## 1. Introduction

Azo dyes account for about one-half of all dyes produced and are the most commonly used synthetic dyes in the textile, food, paper making, color paper printing, leather and cosmetic industries (Chang and Lin 2001). The amount of dye lost in industrial applications depends on the type of dye used and varies from 2% loss for basic dyes to about 50% loss for certain reactive sulfonated dyes when used with cellulosic fabrics due to the relatively low levels of dye fiber fixation (McMullan et al. 2001; Pearce et al. 2003; Hai et al. 2007). In addition to toxicity, textile dye wastewaters have high TOC, high salt content and extremes in pH, with reactive dye baths having high pH and acid dye baths have low pH (Golob et al. 2005). Dye wastewaters are treated physically and chemically by flocculation, coagulation, adsorption, membrane filtration, precipitation, irradiation, ozonization and Fenton's oxidation (Lodha and Choudhari 2007; Wong et al. 2007). Due to the inherent drawbacks of physical, chemical and photochemical approaches to dye removal, the use of biological methods for the treatment of textile wastewaters has received attention as a more cost effective alternative (Olukanni Olukanni et al. 2006; Dos Santos et al. 2007). Anaerobic microbial wastewater treatment can be very effective in removing color, primarily by the activity of azo reductases that cleave the azo bond yielding the corresponding amines, which are frequently toxic, mutagenic and carcinogenic and resist further degradation under anaerobic conditions (Gottlieb et al. 2003; O'Neill et al. 2000; Pinheiro et al. 2004; Van der Zee et al. 2001; Van der Zee and Villaverde 2005). In this process the azo dye acts as the terminal electron acceptor in anaerobic respiratory oxidation of carbon sources and other electron donors (Carliell et al. 1995; Ryan et al. 2010). In contrast, aerobic biological methods, such as activated sludge processes, are largely ineffective in the treatment of textile wastewaters as a standalone process, resulting in little or no color removal from azo dyes, with dye removal primarily occurring through adsorption to the sludge (Brik et al. 2006; Singh et al. 2007). Bacteria capable of aerobic decolorization and mineralization of dyes, specially sulfonated azo dyes, have proven difficult to isolate and the bacteria need to be specially adapted (McMullan et al. 2001; Pearce et al. 2003). However, both mixed and pure cultures of bacteria have been shown to be able to aerobically degrade and detoxify aromatic amines produced by anaerobic decolorization of azo dyes (Khalid et al. 2009). The first azo reductases identified from anaerobic microorganisms were found to be oxygen sensitive; however, recent work described an *Enterococcus gallinarum* isolate, obtained from the effluent of a textile industry wastewater treatment plant, capable of decolorizing azo dye DB38 by azoreductase enzyme action under aerobic conditions (Amit et al. 2008). A number of studies on the degradation of azo dyes by bacteria and fungi have indicated the involvement of extracellular oxidative enzymes such as, tyrosinase, lignin and manganese peroxidases and laccase (Fu and Viraraghavan 2001; Shanmugam et al. 2005; Zille et al. 2005; Ulson et al. Ulson de Souza et al. 2007; Kaushik and Malik 2009; Joshi et al. 2010; Kurade et al. 2011). The present study was focused on purification and analysis of Azoreductase enzyme from Nocardia spp.

## 2. Materials and Methods

### 2.1. Chemicals and Media

All chemicals used in our experiment were of analytical grade (Merck, Rankem, India). Acid red dye was obtained from local textile industry (Ankleshwar).

## 2.2. Sample Collection

The effluent samples were collected at the point of outlet from industry as per APHA (Kader et al., 2007) Standards. The samples were collected in clean containers for physico-chemical analysis and sterile borosil glass bottles covered with brown wrappers were used to collect samples for microbiological analysis.

## 2.3. Physico-chemical Analysis of Effluents

The samples of effluents were analysed for physico-chemical parameters. The parameters color, temperature, odour & Dissolved Oxygen fixation were observed during the collection of the samples prior to the other physico-chemical analysis. The other parameters like pH, conductivity, Total Dissolved Solvents (TDS), total alkalinity, total hardness, nitrate, chloride, sulphate, chromium, calcium, magnesium, Chemical Oxygen Demand & Biochemical Oxygen Demand were analysed as per the standard procedures prescribed by APHA (Eaton et al., 1995).

## 2.4. Isolation and Screening

The nutrient broth along with dye (Acid Red, 100 mg $L^{-1}$) was inoculated with 10% (w/v) of soil sample collected from the waste disposal site of textile processing and dye manufacturing units. The flask was incubated at 37°C under static condition. After 48 h of incubation, 1.0 ml of culture was diluted and plated on the nutrient agar plate containing 200 mg $L^{-1}$ Acid Red. Bacterial isolate showing the clear zone around the colonies were screened for their ability to decolorize dye in the culture broth. Out of 70 isolates, the one showing faster and higher decolorization under static condition was chosen for further study. Pure cultures were maintained on dye-containing nutrient agar slants at 4°C.

## 2.5. Molecular Identification

Genomic DNA was isolated from the organism isolated showing maximum decolorization and its presence was checked by agarose gel (0.8%) stained with ethidium bromide. Amplification of 16S rDNA sequence by polymerase chain reaction was done using thermal cycler (Gene Amp® 2720). (Grifoni et al., 1995). The reaction mixture of total volume of 30 µL consisted of 3 µL of 10 X Buffer, 1 µL of 10 mM dNTPs, 1 µL of 16S rDNA primer (5 picomole/µL), 3 U/µL of Taq Polymerase, 5 µL of template DNA (280 ng/ml) and 19 µL of sterile distilled water. The PCR reaction was set to initial denaturation of 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for one minute, annealing at 55°C for one minute, extension at 72°C for one minute and final extension at 72°C for 10 minutes. The amplified products were stained with 0.5 µg/ml ethidium bromide and loaded on 0.8% agarose gel, and the DNA fragments were separated at 100V and documented. The amplified product was subjected to cycle sequencing using ABI 3130 XL (Genetic Analyser, Applied Biosystems, USA) and resulting sequence was deposited to Genbank (http://www.ncbi.nlm.nih.gov/Genbank). The nucleotide sequence analysis of the sequence was performed at BlastN site at NCBI server (http://www.ncbi.nlm.nih.gov/BLAST). The alignment of the sequences was performed by using Clustalw program V1.82 at European bioinformatics site (http://www.ebi.ac.uk/clustalw). The Phylogenetic tree was constructed by the neighbour joining method using MEGA 2software (Kumar et al., 2001).

## 2.6. Purification of Azoreductase

Cells from the mid log phase culture were harvested by centrifugation at 10000 rpm for 10 minutes at 4°C. Pellets were disrupted by sonication at 40% power for 6 minutes. The cell lysate was subjected to fractionated ammonium sulfate precipitation at 40% saturation to remove impurities, followed by 70% saturation in a second step to precipitate the azoreductase. After 24 h, the precipitated protein is centrifuged for 10 minutes at 10000 rpm at 4°C and the pellet was dissolved in equal volume of 50mM potassium phosphate buffer (pH 7.2). Ammonium sulfate precipitated sample was then desalted by dialysis against phosphate buffer (50 mM, pH 7) overnight under room temperature. 2 mL of the resulting solution was fractionated by anion exchange chromatography using DEAE sephadex column installed in an Amersham Pharmacia Biotech AKTA purifier; pump P-900, monitor pH/C- 900, monitor UV-900, auto sampler Frac-950. Elution buffer (sodium phosphate buffer containing 1 M NaCl was set to a gradient of 100% for 150 minutes. Proteins were eluted at a flow rate of 1.5 mL/minute (Moutaouakkil et al., 2003)

## 2.7. Azoreductase Assay

Assays were carried out in cuvettes with a total volume of 1mL using Shimadzu UV-1800 (Shimadzu-Japan). The reaction mixture consists of 400 µL of potassium phosphate buffer with 200 µL of sample and 200 µL of acid red 37 (100mg/L). The reaction was started by addition of 200 µL of NADH (7mg/mL) and was monitored photometrically at 502 nm. The linear decrease of absorption was used to calculate the azo reductase activity (Meiyer et al., 2004). One unit of azo reductase defined as the amount of enzyme required to decolorize 1 µmol of acid red per minute. The effect of pH on azoreductase activity was determined by incubating the reaction mixture at pH values ranging from 4 to 9. The optimum temperature for enzyme activity was determined by conducting the assay at various temperatures from 20°C to 80°C in 50 mM potassium phosphate buffer (pH 7). The relative activity of azoreductase at each temperature was determined.

## 2.8. Liquid Chromatography and Mass Spectroscopy Analysis (LCMS Analysis)

About 100 ml of acid red (200 mg/L) containing LB Media treated with the isolate and the purified enzymes was extracted with equal volume of ethyl acetate at various time intervals (0, 24, 48 h).The extract was evaporated in a vacuum evaporator (Buchii R 124, Germany) and used for LC- MS analysis. The powdery residue was then dissolved in acetonitrile (HPLC Grade). LC-MS analysis was performed using Finnigan model Mass Spectrometer (Thermo Electron Corporation, USA) using C-18 column from Waters. Cartridges were conditioned with pure acetonitrile, washed with deionized

water (0.1% Formic acid) and elution took place with 70% acetonitrile, containing 0.1% formic acid. The flow rate was 0.8 ml/ min. The ion trap detector with atmospheric pressure electro-spray ionization (API-ESI) source was used for quantification in negative ionization mode. Operating conditions were dry with temperature of 325°C, Capillary voltage 3500V, Nebulizer 14 psi, dry gas Helium 5.0 l/min. Ion trap full scan analyses were conducted from m/z 200-1400 with an upper full time of 300 minutes. The nebulizer gas flow and the curtain gas flow (Nitrogen gas) were set at 10 and 8 psi. The ion spray, orifice and ring voltage were set at +4800, 40, +70 V respectively. Instrumentation control of data acquisitions were performed with data analysis MS (X caliber, USA).

# 3. Results & Discussion

## 3.1. Identification and Phylogenetic Position of Bacterial Isolate

A bacterial strain having remarkable Acid Red decolorization capacity was isolated from dye contaminated soil sample collected in and around the Dyeing Industry, India. The identification of the strain was done on the basis of 16S rDNA gene sequences. Bacterial strain was identified as, *Nocardiopsis spp*. The nucleotide sequence analysis of the sequence was performed at BlastN site at NCBI server (http://www.ncbi.nlm.nih.gov/BLAST). The alignment of the sequences was performed by using Clustalw program V1.82 at European bioinformatics site (http:// www.ebi.ac.uk/clustalw). To analyze the phylogenetic position, the Phylogenetic tree was constructed by the neighbour joining method using MEGA 2 software (Pearce et al., 2003) (Figure 1).


**Figure 1.** Evolutionary relationships of *Nocardiopsis spp*

## 3.2. Physico-Chemical analysis of effluents

The physico-chemical parameters of effluents are analyzed and recorded in Table 1.

## 3.3. Azoreductase Characterization and purification

The enzyme was assayed for extracellular activity. As there was no significant activity observed, intracellular release of the enzyme was performed. After each step of purification the activity was assayed and it was found that the specific activity of the enzyme increased after each step of purification. Enzyme activity in the crude cell extract was found to be 0.0009 U/mg. After ammonium sulphate precipitation and FPLC, the activity increased to 88.49 U/mg. Table 2 gives the activity of azoreductase after each step of purification. The optimum pH found for the activity of azoreductase enzyme was pH 7 with an activity of 0.0003 U/mg. The optimum temperature was found to be 40°C with an activity of 0.00072 U/mg.

**Table 1. Physico-Chemical Characterization of Effluent**

| Characteristics | Sampling site |
|---|---|
| Color | Dark Black |
| Temperature | 25°C |
| pH | 7.1 |
| Total Dissolve Solids (TDS) mg/L | 680 |
| Odour | Foul |
| Nitrate mg/L as $NO_3$ | 04 |
| Chloride mg/L as CL | 80 |
| Sulphate mg/L as $SO_4$ | 120 |
| COD mg/L | 95 |
| BOD mg/L | 22 |
| Cr mg/L | 0.284 |

**Table 2. Purification of Azoreductase from *Nocardia spp***

| Sample | Amount of Protein(mg) | Activity (nkat) | Specific activity (U/mg) | Purification Factor (fold) | Yield (%) |
|---|---|---|---|---|---|
| Crude enzyme | 833.8 | 7505 | 9 | 1 | 100 |
| Ammonium Sulfate precipitation | 147.8 | 6802 | 46 | 5.1 | 90.60 |
| Anion exchange chromatography | 2.4 | 900 | 375 | 41.6 | 11.9 |

## 3.4. Biodegradation of Acid Red by LC-MS

The products of degradation were analyzed and the spectrum of LC-MS of the 0 h, 24 h sample and 48 h sample is shown in the Figure 2. The chromatogram of acid red at 0th hour showed a peak at retention time of 8.01. The mass spectrometric peak at this retention time showed m/z ratio of 513.01 (Figure 2a). In the case of decolourized culture (24 h) of acid red, the mass spectrometric peak at retention time of 5.71, showed a significant m/z ratios of 261 (24 h), which corresponds to 1-{3-amino-5-[(aminoxy)sulfonyl] phenyl}ethanol (Figure 2b). A new peak with the retention time of 5.9, m/z ratios of 245.15 was formed at 48 h which corresponds to 7,8-diamino-3[(aminoxy)sulfonyl]naphthalene-1-ol (data not shown). In case of decolorization with the enzyme (Figure 2c) the mass spectrometric peak at retention time of 6.6(m/z ratio 245.16) was seen at 3 h of incubation with the enzyme. Figure 2d shows that at 5 h of incubation new peak with the retention time of 5.8(m/z ratio of 261.11) was formed. These mass values corresponds to 1-{3-amino-5-[(aminoxy) sulfonyl] phenyl} ethanol and 7,8-diamino-3[(aminoxy) sulfonyl]naphthalene-1-ol . In the degradation pathway, hydrogen ions are donated to the azo group of acid red 37 and a hydrazo compound is formed which further reduced to aromatic amines. As shown in Figure 3, the possible mechanism of degradation could be cleavage of azo bond resulting in the formation of degraded products like 7, 8-diamino-3[(aminoxy)sulfonyl]naphthalene-1-ol and 1-{3-amino-5-[(aminoxy)sulfonyl]phenyl}ethanol through two step NADH dependant reduction mechanism. The complete catabolic pathway of degraded products has to be studied. However, these amino derivatives/intermediates are reported to be degraded/ transformed under aerobic conditions (Sandhya et al., 2008).

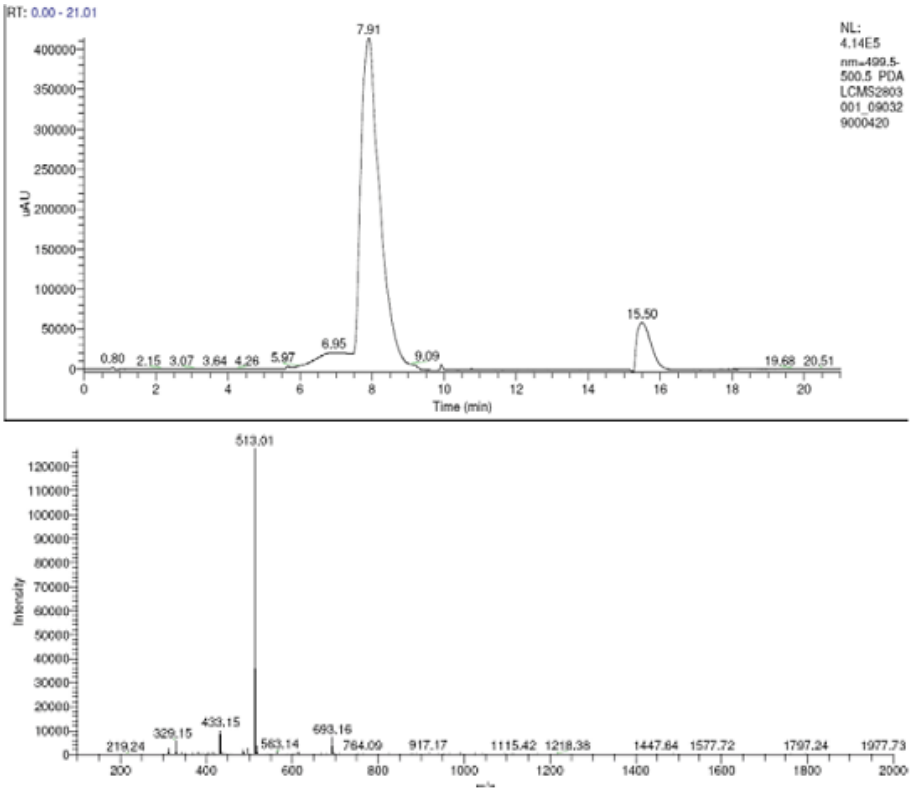


**Figure 2a.** Chromatogram and mass spectra of acid red at 0 h

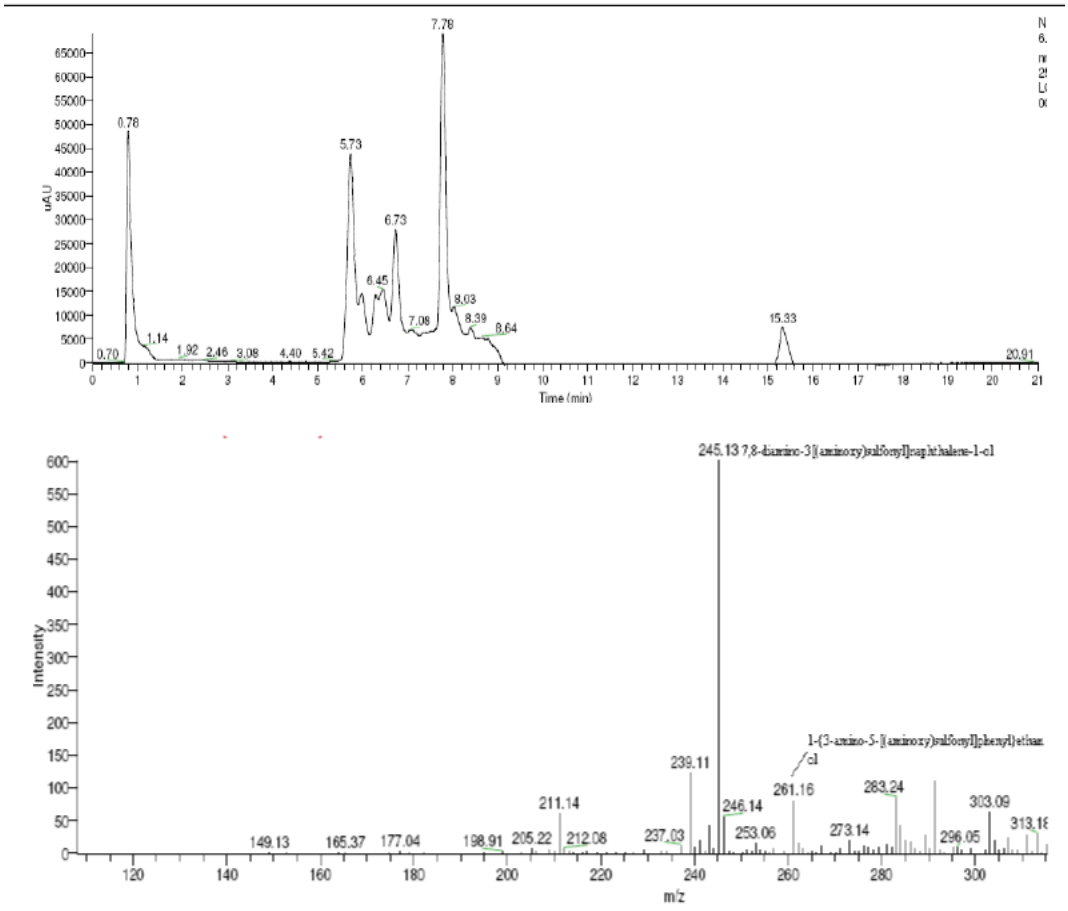


**Figure 2b.** Chromatogram and mass spectra od acid red at 24 h

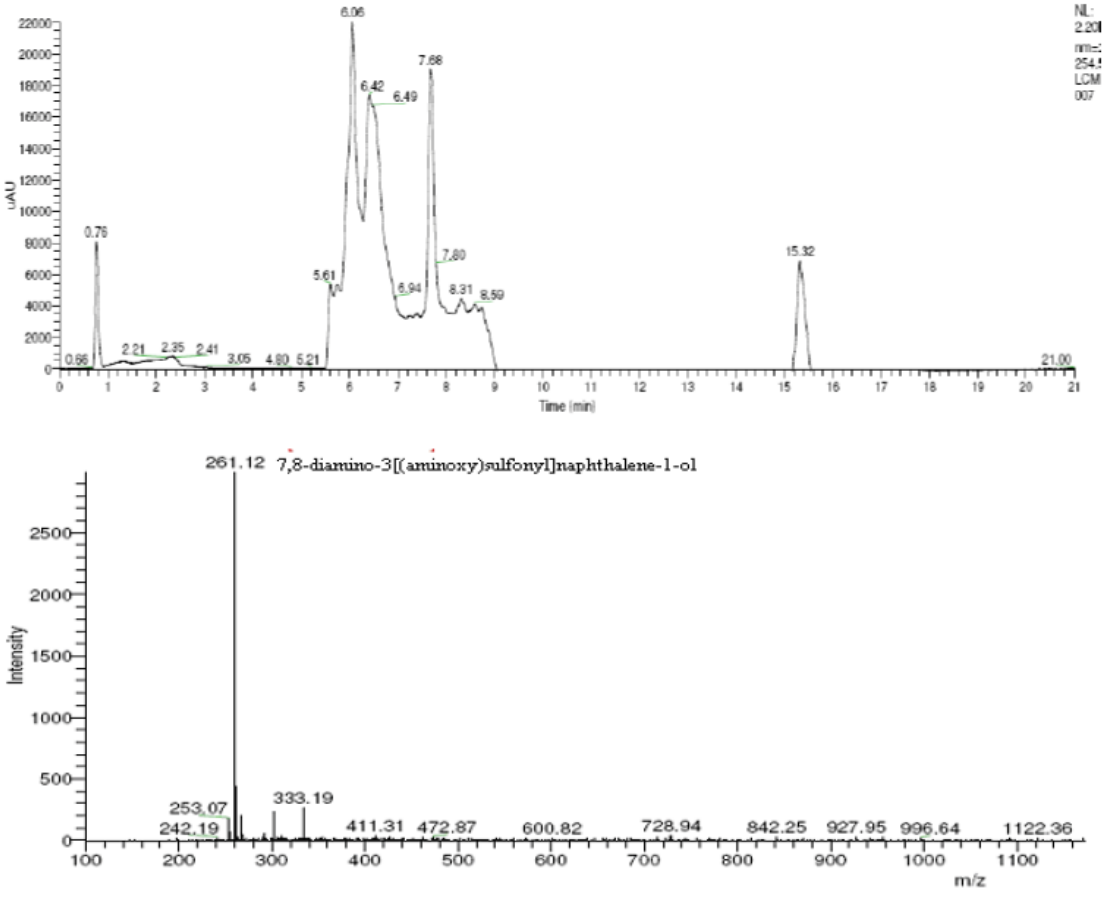


**Figure 2c.** Chromatogram of acid red treated with enzyme (3 h) (b) Mass spectra of acid red after 3 h

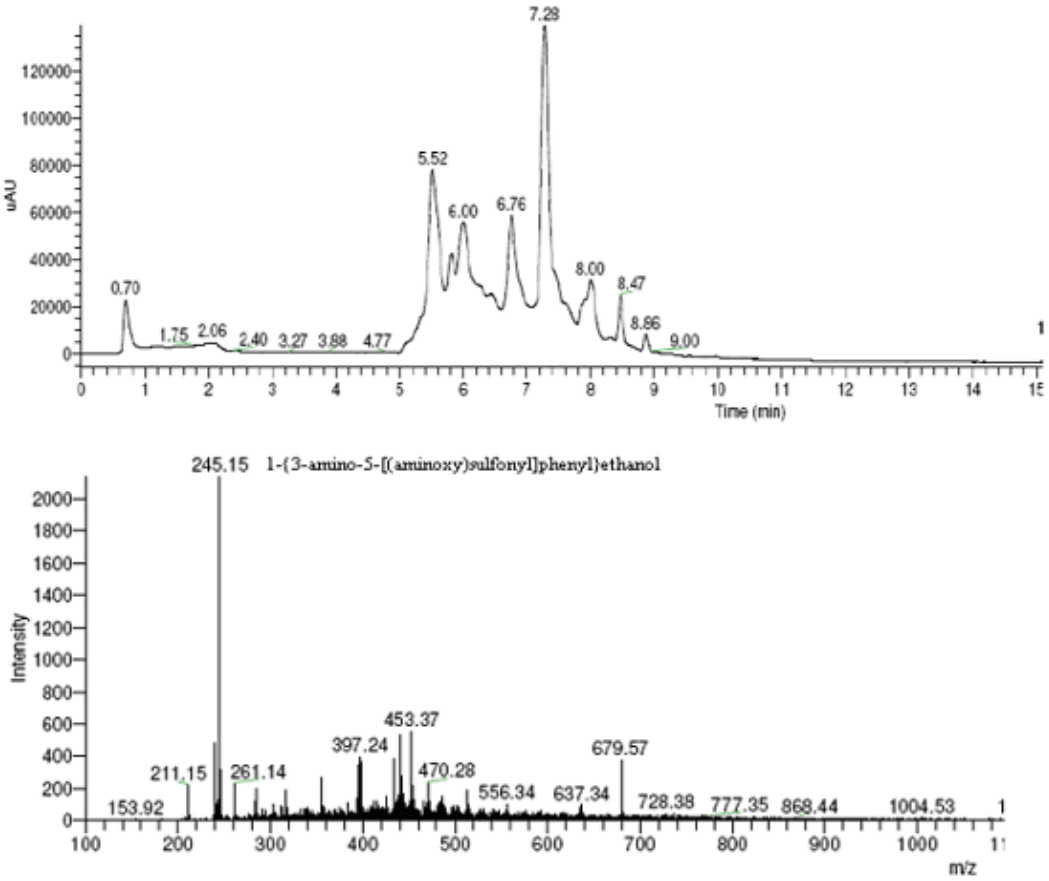


**Figure 2d.** Chromatogram of acid red treated with enzyme (5 h) (b) Mass spectra of acid red after 5 h

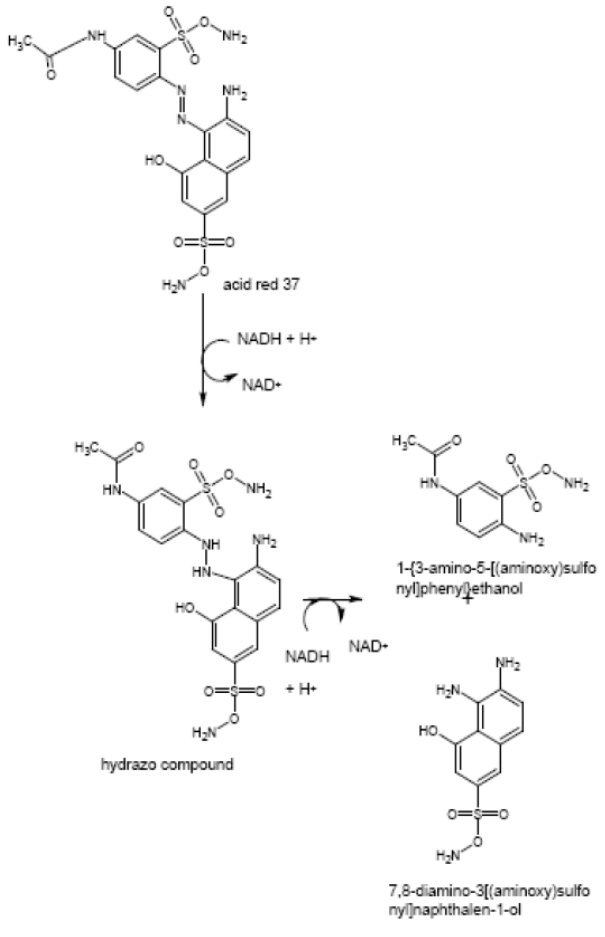


**Figure 3.** Proposed dehradation pathway of acid red 37 by *Nocardia spp*

# 4. Conclusion

The results obtained in this paper were very promising since azoreductase from *Nocardia spp.* could able to degrade the acid red. The partially purified enzyme azoreductase was found to degrade the dye via symmetric cleavage of its azo bond. The culture exhibited good decolorization ability at pH from 6 to 8 and temperature from 30 to 50°C. Decolorization efficiency was not affected by the concentration of the dye. However, more studies in order to study the heterologous expression of this enzyme are underway in our laboratory.

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