International Journal of Environmental Bioremediation & Biodegradation, 2014, Vol. 2, No. 2, 62-68
Available online at http://pubs.sciepub.com/ijebb/2/2/3

DOI:10.12691/ijebb-2-2-3

Eco-Friendly Treatment of Acid Red by an Application of *Pseudomonas spp*

Maulin P Shah*

Industrial Waste Water Research Laboratory Division of Applied & Environmental Microbiology Enviro Technology Limited Plot GIDC, Ankleshwar Gujarat, India
*Corresponding author: shahmp@uniphos.com

Received April 14, 2014; Revised April 16, 2014; Accepted April 17, 2014

Abstract Acid red decolorizing bacteria was isolated and identified as *Pseudomonas spp*. The effect of operation parameters such as medium composition, pH, temperature, dye concentration on the decolorization of acid red was studied and the products of degradation were analyzed and confi rmed using LC–MS analysis. The reductive cleavage of azo bond was catalyzed by azoreductase, the key enzyme for the azo dye degradation. The catalytic reduction of acid red 37 by purifi ed 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: *acid red, pseudomonas, LC-MS, azo reductase*

Cite This Article: Maulin P Shah, "Eco-Friendly Treatment of Acid Red by an Application of *Pseudomonas spp*." *International Journal of Environmental Bioremediation & Biodegradation*, vol. 2, no. 2 (2014): 62-68. doi: 10.12691/ijebb-2-2-3.

1. Introduction

Dyes are intensely colored organic compounds having widespread applications; in textile industries for dyeing nylon, polyacylonitrile, modified nylon, wool, silk, cotton etc. Over 10,000 dyes with an annual production of over 7×105 metric tons are commercially available [1]. Approximately 50% of these dyes are released in the industrial effluents [2]. It is estimated that 10-70 L of water is required for processing one meter of cloth and that produces about 20-30 mgL-1 of BOD (Biochemical Oxygen Demand). An industry having production capacity of approximately 35,000 md^{-1} consumes 1000 KL water d^{-1} and about 800 KL dyes containing effluent d-1 [3]. Dyes are designed in such a way that they are resistant to light, water and oxidizing agents and, therefore, these dyes cannot be treated by conventional treatment processes such as an activated sludge [4]. Dye colors are visible in water concentration as low as 1 mg/l, whereas textile processing waste water normally contains more than 10-200 mg/l of dye [5]. A major environmental problem facing the textile dyeing industry is that the industry produces large volumes of high strength aqueous waste continuously. Wastewater containing recalcitrant residues into rivers and lakes lead to higher biological oxygen demand posing a serious threat to native aquatic life [6] and have toxic and carcinogenic effect in mammals [7]. The problem of environmental pollution is increasing day by day due to the release of xenobiotic substances into water, soil and air. These substances include organic compounds (pesticides, dyes, polymers etc.) and heavy metal ions. The damage caused by these pollutants to plants, animals and humans cannot be neglected and hence strategies must be developed to solve the problem of environmental pollution on the priority basis. Removal of dyes from the effluents or their degradation before discharge is a great environmental challenge for the industries [8]. Generally used physiochemical methods adsorption, floatation, coagulation, incineration, neutralization, reduction, oxidation, electrolysis and ion-exchange; are effective and efficiently remove dyes from the effluents but produced large sludge and by products which are more toxic. Biological processes like aerobic, anaerobic and bioaugmentation and biostimulation are getting more attention as they are cost effective, easy to use, ecofriendly and convert organic compounds completely into water and carbon dioxide [9]. Microbial communities are of primary importance in degradation of dye contaminated soils and water, as microorganisms alters to dye chemistry and mobility through reduction, accumulation, mobilization and immobilization. A number of biotechnological approaches have been suggested as of potential interest towards combating this pollution source in an eco-efficient manner by use of bacteria, fungi and algae for various bioremediation processes. Keeping in view the above points, the present study was undertaken to optimize various parameters and evaluate the potential of the isolate. Various parameters such as agitation, temperature, pH and different dye concentration required to achieve maximum dye decolorization were standardized. The organism effectively degrades the dye by the partially purified enzyme azoreductase. The enzyme and whole cell culture tested for their efficiency to degrade acid red and the intermediates products formed were analyzed by liquid chromatography and mass spectroscopy (LC–MS).

2. Materials and Methods

2.1. Dyes and Chemicals

Acid red was a generous gift from local textile industry of Ankleshwar, Gujarat, India, used to prepare the stock solution, which was filter sterilized and added to the growth medium at required concentrations. All the chemicals used were of analytical grade and procured from Himedia Pvt. Limited, Mumbai. The materials required for PCR amplification (Taq polymerase, dNTPs, primers) were obtained from Bio-Red, USA.

2.2. Isolation and Screening of Dye Decolourising Bacterium

Soil samples contaminated with textile dye effluent were collected from different vicinities in Ankleshwar and were used as a source to isolate morphologically distinct bacterial colonies capable for decolourising acid red. The soil samples were subjected to enrichment culture technique using nutrient broth amended with increasing concentrations of dye (100 mg/L 500 mg/L) with incubation time of 24 h at 37°C. Repeated transfers were carried out to isolate stable dye decolourising strains and the isolated strains were stored at 15°C for further use. The high decolourising bacteria were screened by performing a decolourisation assay with acid red using UV- VIS spectrophotometer (UV-1800, Shimadzu, Japan) at its respective λmax 502 nm. The percentage decolourisation was calculated using the following formula

$$\%Decolourisation = \frac{(Lnitial\ absorbance - Final\ absorbance)\times 100}{Lnitial\ absorbance}$$

$$Lnitial\ absorbance - Absorption\ at\ 0\ h\ of\ incubation$$

$$Final\ absorbance - Absorption\ at\ the\ time\ of\ incubation$$

2.3. Molecular Identification of the Isolate

Genomic DNA from the isolated organism was isolated and its presence was checked by running in 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). 16S r DNA universal primers used for PCR reaction [10]. The reaction mixture of total volume of 30µL consisted of 3 µL of 10X 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 19µL 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 [11]. 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 100 V and documented. The amplified product was subjected to cycle sequencing using ABI 3130 XL (Genetic Analyser, Applied Biosystems, USA). 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 V 1.82 at European bioinformatics site (http://www.ebi.ac.uk/clustalw). The Phylogenetic tree was constructed by the neighbour joining method using Kimura-2-parameter distances in mega 2.1 software [12].

2.4. Effect of Physico-Chemical Factors on Decolourisation

Mid log phase culture of the isolate was inoculated into LB media supplemented with acid red. The effect of various physicochemical factors (dye concentration, temperature, pH, oxygen) on the decolorization of acid red by the isolate was studied in detail. Decolorization of acid red by the isolate was carried out at various dye concentrations (50, 100, 200, 400 mg/L), temperature (20°C, 40°C, 60°C, 80°C) and pH (2-9). Decolorization experiment was also carried out under aerobic and anaerobic conditions. All decolorization experiments were performed in triplicates. Abiotic controls with bacteria were always included.

2.5. Characterization and Purification of the Intracellular Azoreductase Enzyme

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 50 mM 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 [13]. The fractionated sample was concentrated using protein purification column. Active fractions were collected and stored as the purified enzyme preparation.

2.6. Assay for Azoreductase Activity

Assays were carried out in cuvettes with a total volume of 1mL using Ultrospec 2100 UV-VIS Spectrophotometer (Shimadzu, Japan, UV-1800). The reaction mixture consists of 400 µL of potassium phosphate buffer with 200 µL of sample and 200 µL of acid red (100 mg/L). The reaction was started by addition of 200 µL of NADH (7 mg/mL) and was monitored photometrically at 502 nm. The linear decrease of absorption was used to calculate the azoreductase activity [14]. One unit of azoreductase can be 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.

2.7. 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. The cartridges were conditioned with pure acetonitrile, washed with deionized water (0.1% Formic acid) and the 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 3500 V, 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 and Discussion

3.1. Screening and Isolation of Dye Decolorizing Bacteria from Soil

From the serially diluted soil sample, 20 morphologically distinct bacterial colonies with decolourizing zones were isolated. All the 20 bacterial isolates were tested for their dye decolourising efficiency by liquid decolorization assay. Using 500 mg/L of acid red, five Bacterial isolates - A, B, C, D and E showed decolorization percentage of more than 70% at 48 h of incubation. The highest decolorization percentage was showed by E (87.2 ± 0.45). This bacterial isolate was selected and used for further studies.

3.2. Molecular Characterization and Identification of the Isolate

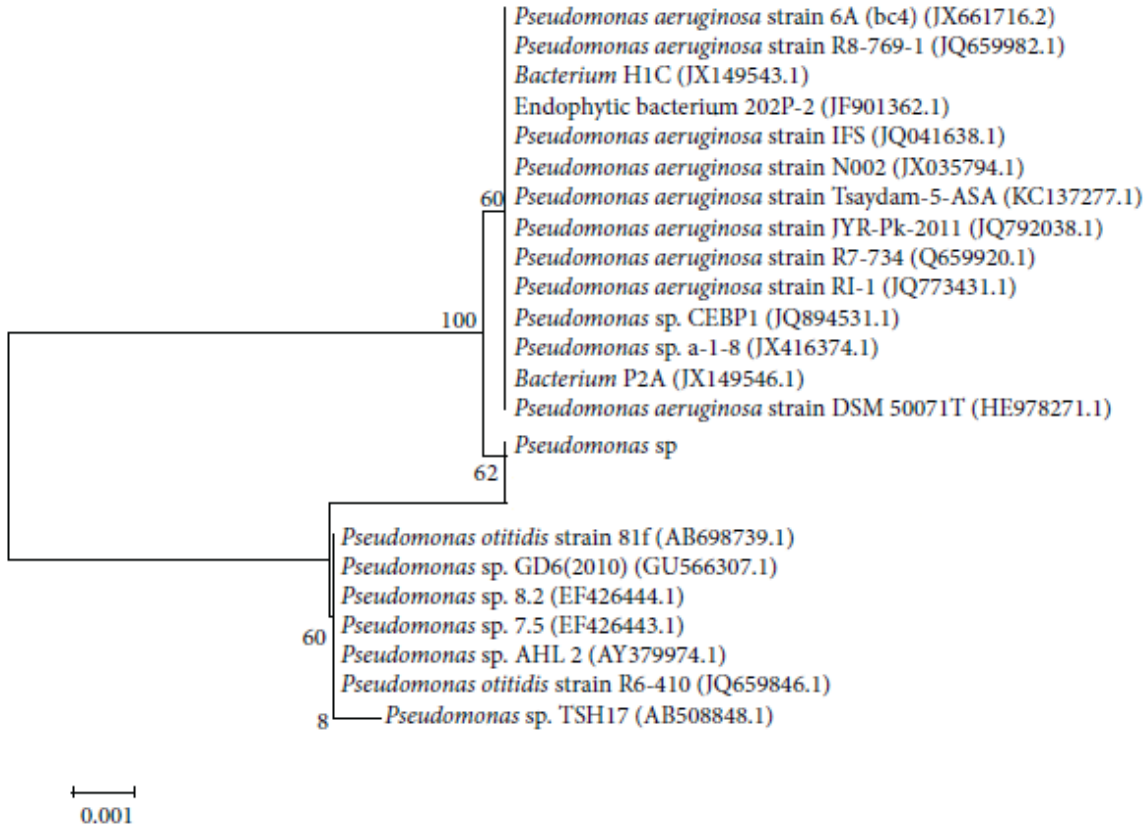

Figure 1. Phylogenetic tree of *Pseudomonas spp*.

DNA was isolated from the screened bacterial isolate E. 16S rDNA sequence of the colony E was amplified using universal primers. The size of the amplified band was determined to be approximately 1.5 Kb. Each of the amplified DNA was further purified by DNA Elution kit (Bioserve Biotechnologies India Private Limited). The purified bands were subjected to cycle sequencing and BLAST to determine the organism. The isolate E was identified as *Pseudomonas* by BLAST analysis (www.ncbi.nlm.nih. gov/BLAST/) in the nucleotide sequences database. The phylogenetic tree shown in Figure 1 clearly shows that the isolated strain was closely related to *Pseudomonas*. Phylogenetic analysis using other methods of tree building also supported this grouping with high bootstrap values.

3.3. Effect of Physico-Chemical Factors on Decolourisation

Table 1. Effect of different physicochemical parameters on decolorization: (LB medium, incubated at various time intervals with different tempertature, pH, oxygen condition)

Condition	Decolorization (%) 24 h	Decolorization (%) 48 h
Temperature		
20°C	32±0.2	51.3±0.5*
30°C	68±0.6	71.7±0.6***
40°C	85±0.7	95±0.5*
50°C	59±0.1	83.5±0.3*
60°C	41±0.3	54.31±0.06*
70°C	35±0.6	47.2±0.6*
80°C	21±0.08	43.2±0.2*
pH		
2	Nil	Nil
3	Nil	Nil
4	42±0.4	61±0.2*
5	48±0.2	66±0.2*
6	74±3.2	92±1.3*
7	95±0.8	99±0.43***
8	47±0.1	74±0.3*
9	8±0.2	10±0.2*
Oxygen		
Aerobic	86±7.6	91±2*
Anaerobic	73.96±3.2	92.1±1.3***
Dye concentration		
50 mg/L	62±1.1	88±2.1*
100 mg/L	48±3.1	97±1.8***
200 mg/L	50±1.1	84±2.2***
400 mg/L	35±2.1	86±2.1*

Values are mean of three experiments (±) SEM.
Significantly different from control cells at *P< 0.05, ***P< 0.001 by t test.
Oneway analysis of bariance (ANOVA) with TurkeY-Kramer comparison test.

Maximum decolorization of 90% was observed in pH from 6 to 7 (Table 1). Rate of decolorization decreased at lower pH (4-5) and at higher pH (8-9). No decolorization was obtained at lesser pH 1-2. *K. pneumoniae* RS-13 was found to completely degrade Methyl Red at pH 6.0-8.0 while *Acetobacter liquefaciens* S-1 completely degraded Methyl Red at pH 6.5 [15]. *E. coli* and *P. luteola* both exhibited best decolorization rate at pH 7.0 with constant decolorization rates up to pH 9.5 [16]. The isolate showed higher decolorization percentage when the medium was incubated at 40-50°C. The isolate is capable of decolorizing the dye up to 400mg/L. Lee et al. [17] stated that the increase in the concentration of the dye, the ability of the organism to decolorize decreased. In contrast to this decolorization was not affected by increasing the dye concentration (50 mg-400 mg/L). As indicated in previous studies [18], the chemical structure characteristics (e.g., resonance and inductive effects) and reactivity of dyes strongly determined color removal efficiency of bacterial decolorizers.

3.4. Characterization and Purification of Azoreductase

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 sulfate 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 2. Purification of azoreductase from *Pseudomonas 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

3.5. 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 [19].

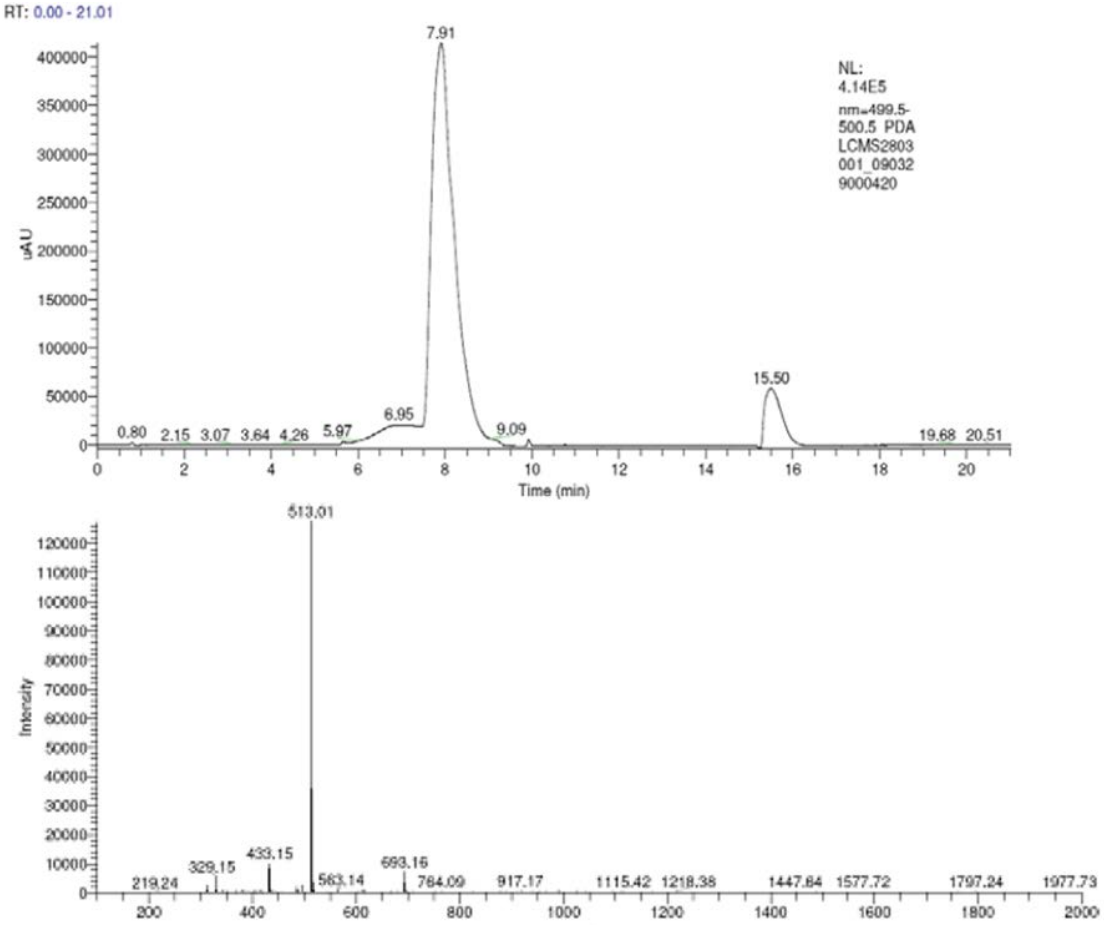

Figure 2a. Chromatogram and mass spectra of acid red ai 0 h.

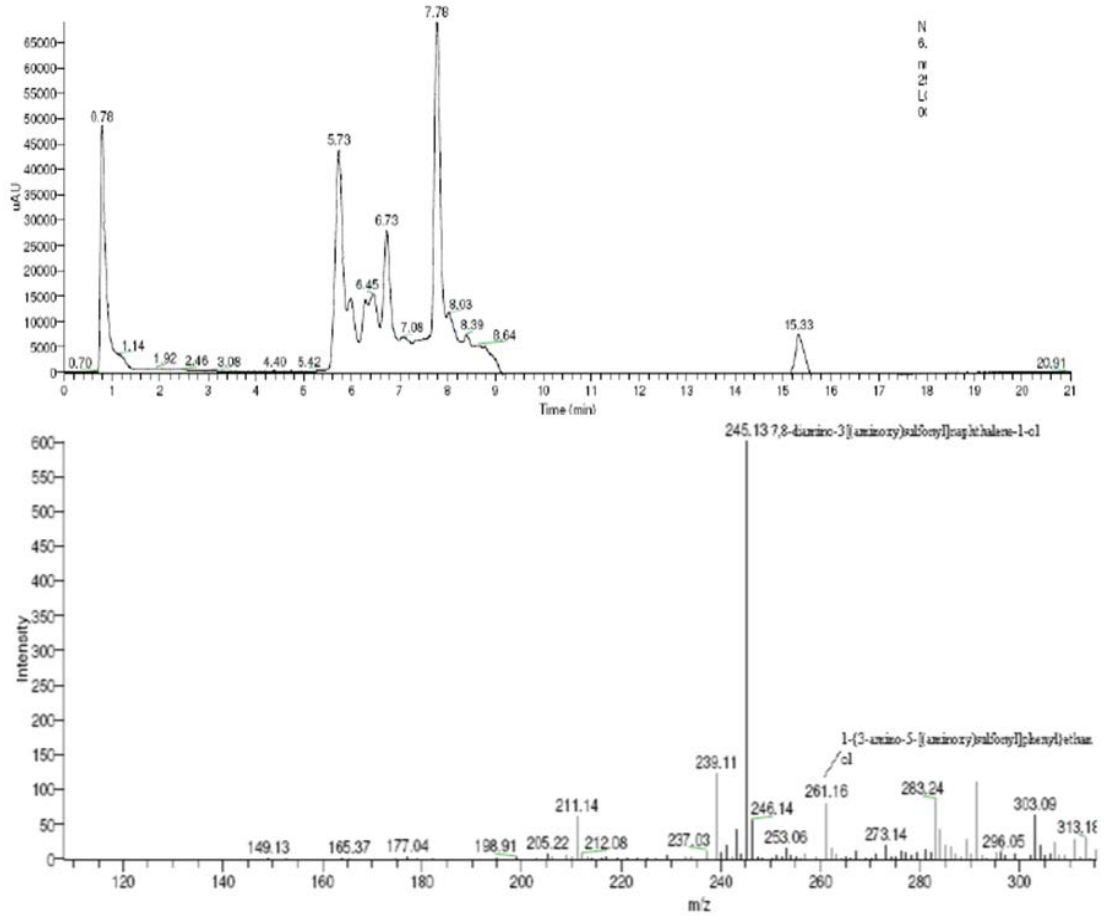

Figure 2b. Chromatogram and mass spectra of acid red at 24 h

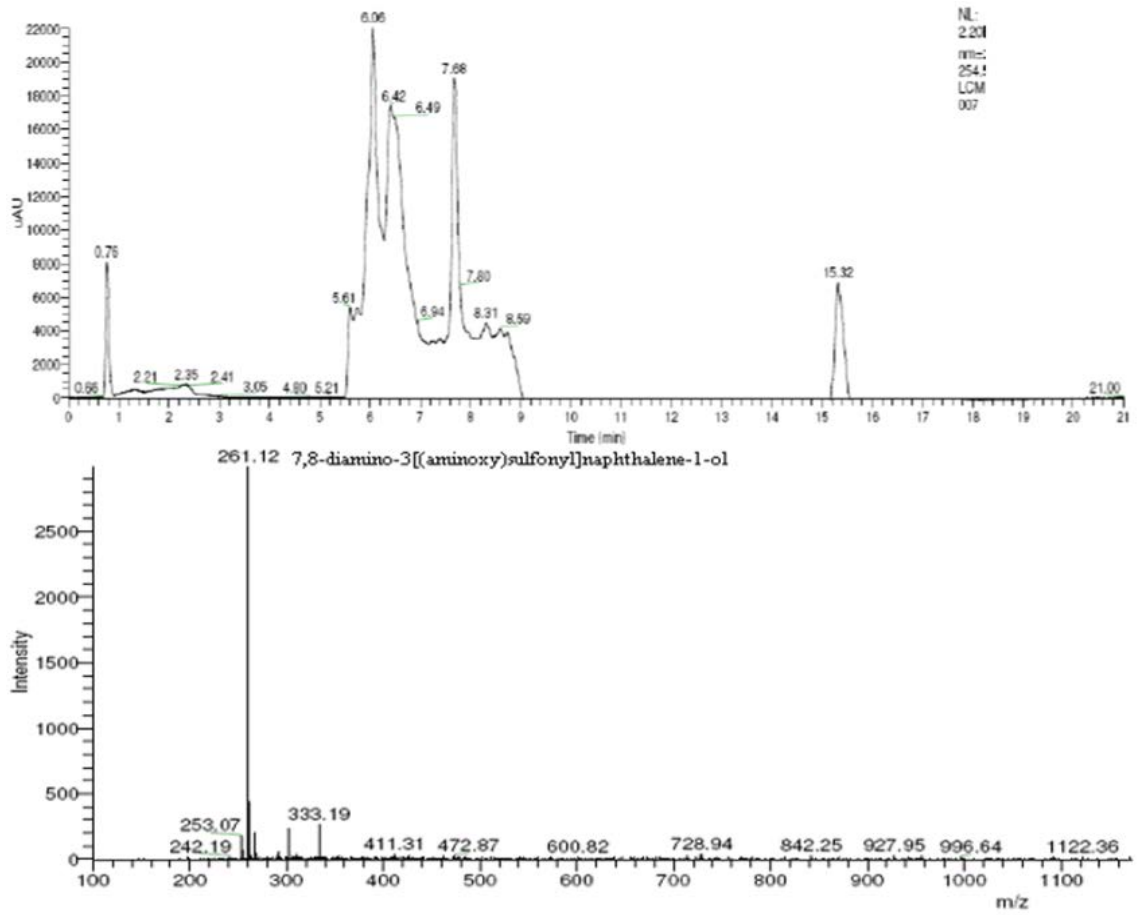

Figure 2c. Chromatogram of acid red treated with enzyme (3 h) (b) Mass spectra of acid red after 3 h.

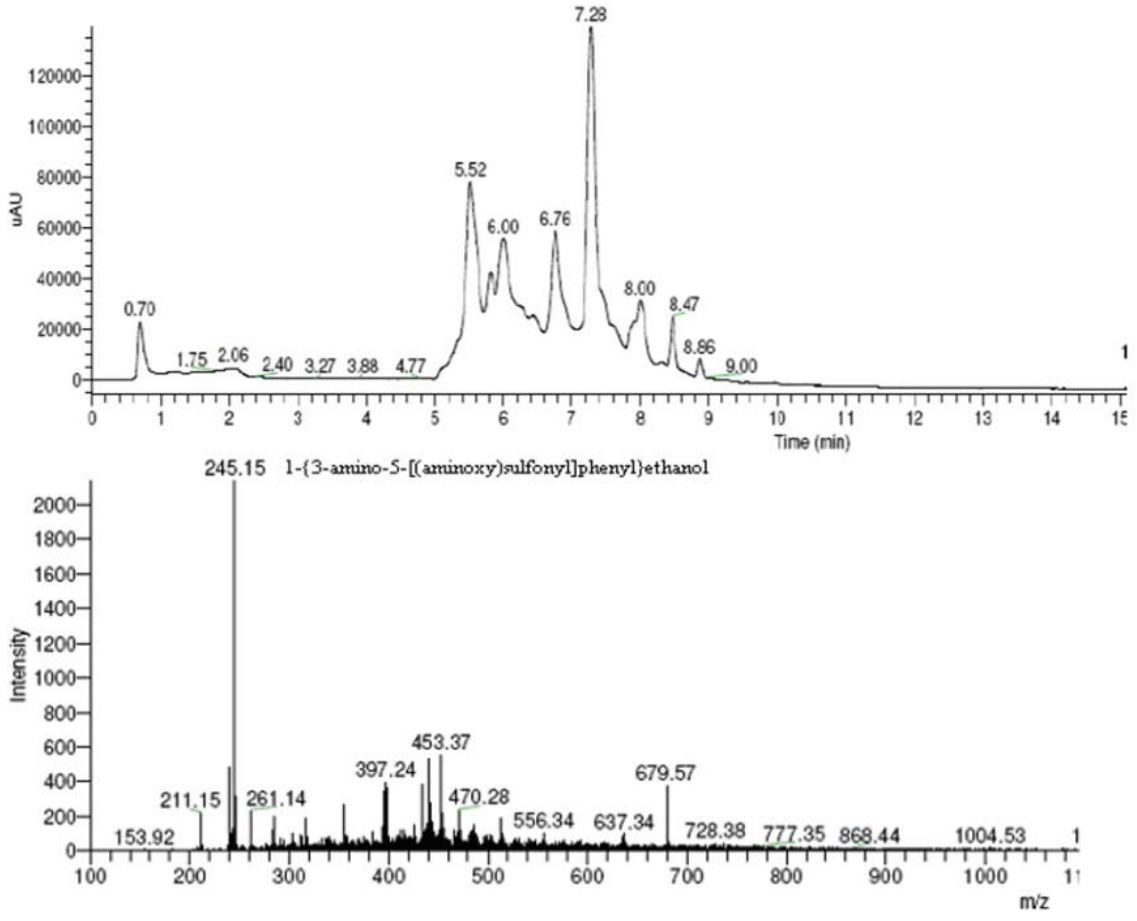

Figure 2d. Chromatogram of acid red treated with enzyme (5 h) (b) Mass spectra of acid red after 5 h

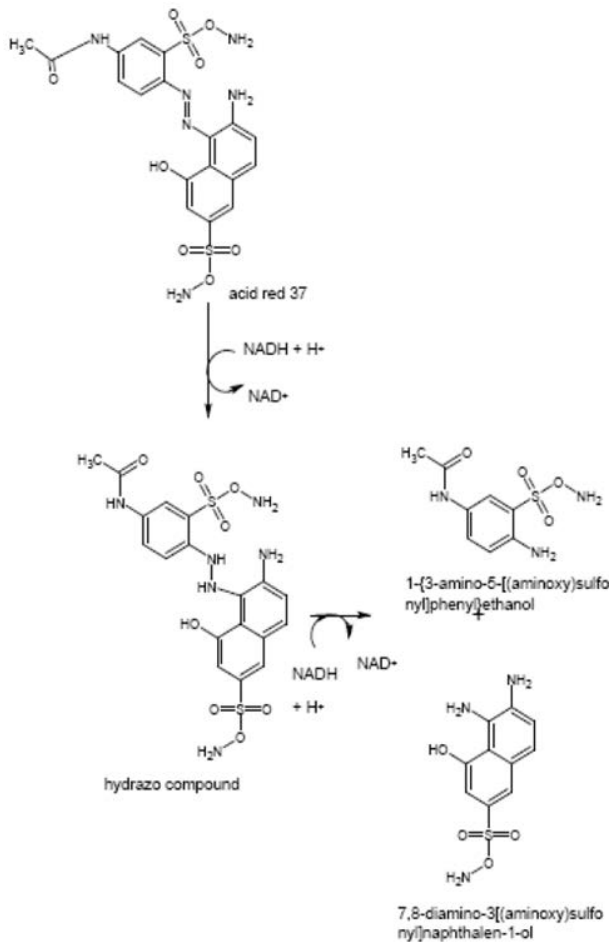

Figure 3. Proposed degradation pathway of acid red

4. Conclusion

The results obtained in this paper were very promising since azoreductase from *Pseudomonas 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°C 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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