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Exploited Application of *Lactobacillus* in Microbial Degradation and Decolorization of Acid Orange

Maulin P Shah*

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

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Abstract A bacterium identified as *Lactobacillus* was isolated from acclimated sludge from a dyeing wastewater treatment plant. This strain rapidly decolorized Reactive orange (RO) at 0.1% (w/v) concentration of both sucrose and peptone supplemented in Mineral Salt Medium (MSM) under static conditions at a temperature of 30°C with in 24 h with an initial dye concentration of 100 mg/L. The organism exhibited a remarkable color removal capability (95%) even at a high concentration of 1000 mg/L (RO16) dye within 24 h. The biodegradation products were analyzed by, FTIR spectroscopy and LC-MS analysis. The LC-MS analysis indicated the presence of 1-amino- 1-napthalene sulphonic acid in degraded product of the dye.

Keywords: *Lactobacillus, biodegradation, Reactive orange, decolorization*

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1. Introduction

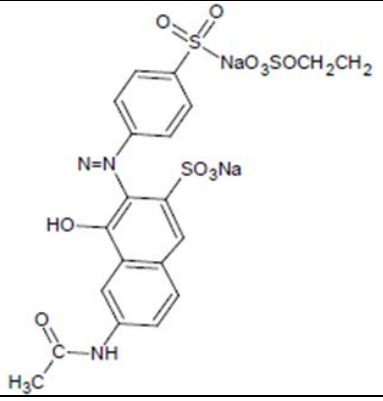
Azo dyes are the largest group of synthetic chemicals that are widely used by the textile, leather, cosmetics, food coloring and paper production industries. The chemical structure of these compounds features substituted aromatic rings that are joined by one or more azo groups (–N=N–). The annual world production of azo dyes is estimated to be around one million tons [1] and more than 2000 structurally different azo dyes are currently in use [2]. During the dyeing process, approximately 10-15% of the used dye is released into wastewater [3]. Moreover, many azo dyes and their degradation intermediates such aromatic amines are mutagenic and carcinogenic, and discharge of them into surface water leads to aesthetic problems and obstructs light penetration and oxygen transfer into bodies of water, hence affecting aquatic life [4]. The treatment of textile wastewater is essential before discharging the wastewater into a receiving water body [5]. During the past two decades, several physicochemical decolorization techniques have been reported; however, few have been accepted by the textile industries [6]. Biological methods are generally considered environmentally friendly as they can lead to complete mineralization of organic pollutants at low cost [1]. 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. To overcome this problem, studies included either using microbial consortia or combinations of anaerobic and aerobic steps in an attempt to achieve not only dye decolorization, but also degradation of the aromatic amines [7]. However, few studies reported the use of a single adaptable microorganism in a sequential anaerobic/aerobic treatment [8]. Moreover, the available literature on sequential anaerobic/aerobic treatment with a single microorganism is extremely limited [9]. 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. Although degradation of azo dyes by microorganisms has been extensively documented, little is known about the biodegradation of azo dyes by lactic acid bacteria [10,11]. To our knowledge, this was the first study on efficient azo dye decolorization using *Lactobacillus* strains and might help development of biological processes for treatment of dye-polluted wastewaters. In the present investigation, one of the prominent azo dyes Reactive orange 16 used in textile industry was decolorized using *Lactobacillus* a textile soil isolate.

2. Materials and Methods

2.1. Dyes, Chemicals & Media

All chemicals used in our experiment were of analytical grade. Reactive orange (RO), NADH were obtained from Sigma Aldrich Company (St. Louis, MO, USA). The physical and chemical characteristics of RO are listed in Table 1.

Table 1. Physical and chemical properties of Reactive orange

Molecular weight (g/mol)	6170.54
Molecular formula	$C_{20}H_{17}N_3Na_2O_{11}S_3$
Color index number	17757
λ_{max} (nm)	494
Chemical structure	

2.2. Culture Media

Nutrient broth and nutrient agar of Himedia Lab. Pvt. Ltd. (India) were used. Nutrient broth ingredients per liter peptone 10 g, sodium chloride 5 g, meat extract 3 g, pH 7.6. Nutrient agar ingredients per liter peptone 10 g, sodium chloride 5 g, meat extract 3 g, agar powder 30 g, and pH 7.6. Mineral salt medium (MSM) per liter; $(NH_4)_2SO_4$ 1 g, K_2HPO_4 6 g, KH_2PO_4 1 g, $MgSO_4.7H_2O$ 0.1 g, NaCl 5 g, pH 7.0.

2.3. Isolation and Screening of Dye Degrading Microorganism

The nutrient broth along with dye (Reactive orange RO, 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 in Ankleshwar (India). The flask was incubated at temperature 37°C under the static condition. After 48 h of incubation, 1.0 ml of culture was diluted and plated on the nutrient agar plate containing 200 mgl^{-1} Reactive orange. Bacterial isolate showing the clear zone around the colonies were screened for their ability to decolorize dye in the culture broth. Out of 75 isolates, the one showing faster and higher decolorization under static condition was chosen for further study. The pure culture was maintained on dye-containing nutrient agar slants at 4°C.

2.4. Molecular Identification of the Isolate

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). 16S rDNA universal primers used for PCR reaction [12]. 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), 3U/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). 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 5.1 software [13].

2.5. Decolorization at Different Dye Concentrations

All decolorization experiments were performed in triplicates. 100 ml MSM broth was inoculated at 1% v/v of microbial culture. The dye was added at concentrations of 100, 250, 500, 750 and 1000 mg l^{-1}. After 24 h aliquots (3 ml) of the culture media was withdrawn at different time intervals, centrifuged at 5000 rpm for 15 min to separate the bacterial cell mass. The clear supernatant was used to measure the decolorization at the absorbance maxima of the dye 600 nm. Un-inoculated medium used as control. Percentage of decolorization was calculated as mentioned by Dave and Dave [14].

$$\text{Decolorization } (\%) = (I - F/I) * 100$$

Where, I = Initial absorbance
F = Absorbance of decolorized sample.

2.6 Optimization of Parameters for Decolorization

The isolate was cultivated for 24 h in conical flasks containing 100 ml mineral salt medium (MSM) and was amended separately with 100 mg l^{-1} of RO. Decolorization was studied with various carbon sources (1%) (Sucrose, glucose, lactose, maltose and starch) and nitrogen sources (1%) (Yeast extract, urea, peptone, ammonium sulphate and ammonium chloride) and at different dye concentrations (100, 250, 500, 750, 1000 mg/l), pH values (5-9) and temperature (20°C-50°C). Decolorization experiments were also carried out under shaking and static conditions. Growth was monitored spectrophotometrically at 600 nm. At periodic time intervals an aliquot of 5 ml of culture media was withdrawn, centrifuged at 5000 X g for 5 min in a refrigerated centrifuge (Dupont Sorvall RC-5B) to separate the bacterial cell mass. The supernatant was used for analysis of decolorization percentage and all the experiments were repeated in triplicates and mean value was taken for analysis.

2.7. FTIR Analysis

The controls and samples obtained at varied time intervals were extracted with ethyl acetate were dried and mixed with KBr (1:20; 0.02 g of sample with KBr at a final weight of 0.4 g). The samples were then ground, desorbed at 60°C for 24 h and pressed to obtain IR-transparent pellets. The absorbance FT-IR spectra of the samples were recorded using an FT-IR Spectrum 2000

Perkin–Elmer spectrometer. The spectra were collected within a scanning range of 400-4000 cm- 1. The FT-IR was first calibrated for background signal scanning with a control sample of pure KBr and then the experimental sample was scanned. The FT-IR spectrum of the control was finally subtracted from the spectra of the non-degraded and degraded dyes.

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

About 100 ml of Reactive orange (200 mg/L) containing MSM Media treated with the isolate and the purified enzymes was extracted with equal volume of ethyl acetate at various time intervals (0,24 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. Identification and Phylogenetic Position of Bacterial Isolate

A bacterial strain having remarkable Reactive orange decolorization capacity was isolated from dye contaminated soil sample collected in Industrial estate of Ankleshwar, Gujarat, India. The identification of the strain was done on the basis of 16S rDNA gene sequences. Bacterial strain was identified as, *Lactobaillus*. 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 5.1 software [17] (Figure 1a).

3.2. Effect of Physico-chemical Factors on Decolorization

The bacterial isolate showed maximum dye decolorization at pH 7 (Table 2). At this optimum pH, the isolate showed 95% of decolorization of Reactive orange. At pH 8, the isolate showed 65% decolorization of Reactive orange. Whereas at pH 4.0, the isolate showed only 25% dye decolorization respectively. Rate of decolorization decreased at lower pH (4-6) and at higher pH (8-9). Chan and Kuo reported that the neutral pH would be more favorable for decolorization of the azo dyes and is suitable for industrial applications [18]. The temperature effect on the decolorization of Reactive orange was significant for the isolate *Lactobacillus*. When the decolorization of the dye was tested for a wide range of temperatures from 20 to 50°C, it was observed that the increase in decolorization of Reactive orange with increase in temperature and was optimum at 30°C. Further increase in the temperature increases the decolorization of dye up to 40°C and above this temperature a decreased dye decolorization was noticed (Table 2). To understand the effect of low temperature, room temperature and high temperature on the decolorization of dye, the assay was carried out at different temperature range from 20 to 50°C. The decrease in dye decolorization at high temperature can be attributed to the decline in microbial activity that led to the inactivation of the enzyme and eventually the loss of cells viability [19]. These results further showed that there is no thermal deactivation of decolorization activity under operational temperatures. Therefore, the isolate could acclimatize to broad range of temperature of practical dyeing wastewater. The isolate is capable of decolorizing the dye up to 1000 mg/L. Lee et al. [15] 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 (100 mg-1000 mg/L). As indicated in previous studies [16], the chemical structure characteristics (e.g., resonance and inductive effects) and reactivity of dyes strongly determined color removal efficiency of bacterial decolorizers. At 100 mg of dye concentration 92% dye removal was observed; 83-85% decolorization was observed at concentration of 250 mg and 80% of dye was removed at concentration 500 mg. The time required for decolorization of 100 mg dye was 24 h, whereas it was 48 h for 250mg dye concentration. As concentration was increased (750 mg), the time required for decolorization varied from 48 to 72 h. When the dye concentration was as high as 1000 mg, almost 80% of the dye was removed after 72 h. This means that an acceptable high color removal can be achieved by the isolate *Lactobacillus* in an extensive range of azo dye concentrations. This decrease in decolorization efficiency at high dye concentrations above 1000mg may be due to the toxicity of the dye to bacteria and/or inadequate biomass concentration for the uptake of higher concentrations of dye [20]. The observed gradual decrease in decolorization might be due to the culture entering into the stationary phase and subsequently into the death phase, resulting in the inhibition of enzyme systems gradually. *Lactobacillus* exhibited dye-decolorizing activity when incubated under stationary conditions, whereas agitated cultures grew well but showed negligible decolorization. To conform whether this decolorization was due to the microbial action or change in pH, the change in pH was recorded, which was in the range of 6-7 at static condition. This confirms the decolorization of dye was due to microbial action. Therefore, static conditions were adopted to investigate bacterial decolorization in the following experiments.

Table 2. Effect of different plysicochemical parameters on decolorization: (NB medium, Incubated at various time intervals with different temperature, pH, and oxygen condition)

Condition	Decolorization % 24 hrs	Decolorization % 48 hrs	Decolorization % 72 hrs
pH			
3	20.34 ± 1.34	23.08 ± 1.56	22.22 ± 0.45
4	25.34 ± 2.34	27.45 ± 1.34	25.58 ± 1.23
5	33.78 ± 5.02	50.68 ± 4.11	75.79 ± 0.45
6	65.99 ± 19.34	79.21 ± 2.67	80.45 ± 1.44
7	88.95 ± 1.29	87.22 ± 0.6	94.91 ± 0.66
8	51.98 ± 2.76	61.65 ± 0.52	69.77 ± 0.86
9	22.01 ± 1.91	28.28 ± 0.72	36.75 ± 0.54
Condition	Decolorization % 24 hrs	Decolorization % 48 hrs	Decolorization % 72 hrs
Temperration			
20	43.67 ± 2.3	57.47 ± 0.23	56.11 ± 1.15
25	46.66 ± 0.65	60.17 ± 1.77	69.31 ± 0.29
30	91.98 ± 1.46	90.43 ± 0.8	92.27 ± 0.7
35	86.20 ± 1.3	88.06 ± 0.32	87.42 ± 2.1
45	60.45 ± 1.67	62.23 ± 1.67	61.40 ± 1.67
50	41.33 ± 1.33	52.88 ± 0.88	50.66 ± 1.33
Condition	Decolorization % 24 hrs	Decolorization % 48 hrs	Decolorization % 72 hrs
Dye concentration (mg/l)			
100	92.72 ± 0.34	92.42 ± 1.02	92.93 ± 1.36
250	85.92 ± 0.33	85.58 ± 0.54	83.46 ± 0.27
500	82.64 ± 0.34	80.94 ± 3.07	80.25 ± 1.53
750	74.38 ± 0.44	74.26 ± 0.58	76.92 ± 3.5
1000	70.11 ± 0.42	71.04 ± 0.99	70.28 ± 0.85
Condition	Decolorization % 24 hrs	Decolorization % 48 hrs	Decolorization % 72 hrs
Static	95.13 ± 0.80	90.13 ± 0.34	92.17 ± 1.23
shaking	65.93 ± 1.36	64.25 ± 1.53	62.24 ± 1.45

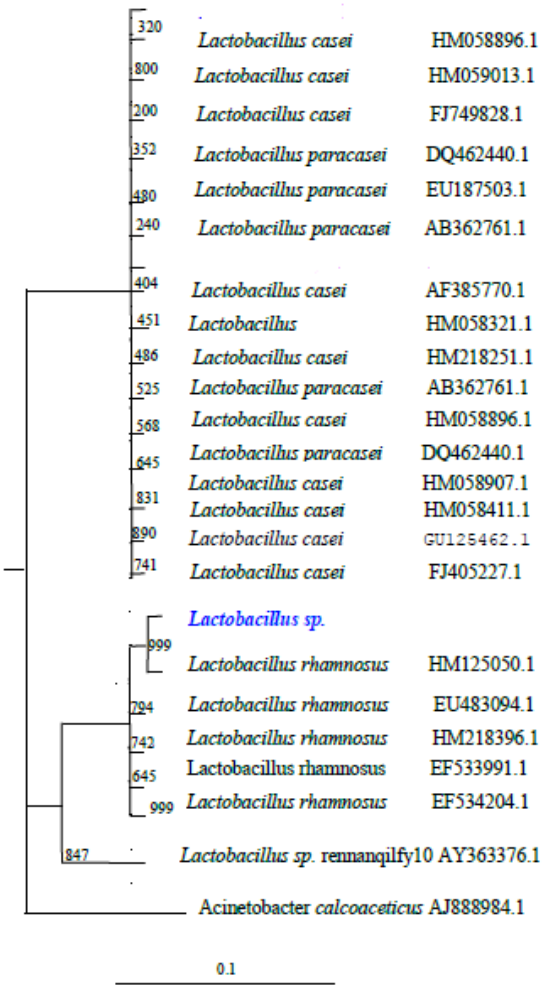


Figure 1a. Neighbor-joining tree showing the estimated phylogenetic relationships of the newly-isolated strains (shown in blue) and other closely-related strains of the genus *Lactobacillus*. Bootstrap values out of 1000 are given at the nodes

Earlier studies revealed that the dye could not be used as the sole carbon and nitrogen source by the organism and the organism required additional carbon and nitrogen sources to co-metabolize the dye. Hence, different carbon sources and nitrogen sources were evaluated for dye decolorization at 100 mg/ l dye concentration. The reduction of azo dyes depends on the presence and availability of co-substrate because it acts as electron donor for the azo dyes reduction. It was found that isolate *Lactobacillus* gave maximum decolorization with the combination of sucrose and peptone followed by glucose and yeast extract decolorization (above 70%) and with sucrose gave the highest decolorization (92%) (Table 1). The organic nitrogen sources are considered essential medium supplements for the generation of NADH that act as electron donor for the reduction of azo dyes by microorganisms [17]. The results showed that there is a moderate increase in cell growth and dye decolorization, when both carbon and nitrogen sources were added together each at 0.1% concentration.

3.3. Identification of Metabolic Intermediates

3.3.1. FTIR Analyses

The FTIR spectrum of a control dye and extracted metabolites (24 hours) by *Lactobacillus* was compared. The spectrum of the control dye (Figure 2a) displayed a peak 3418.11 cm^{-1} for –NH stretching. The stretching between C N was reported at 2271.30 cm^{-1} and amide, 5-membered ring peak at 1706.86 cm^{-1}. The peak at 1637.21 cm^{-1} showed carbonyl stretching vibration. Peak at 1374.15 cm^{-1} showed unsaturated nitrogen compounds. Peak at 1229.06 cm^{-1} showed S=O stretching vibrations. The peak at 1106.62 cm^{-1} indicates the aromatic nature. The peak at 616.95 cm^{-1} showed hydrocarbon chromophore-C-H bending. The FTIR spectrum of 24

hours extracted metabolites of *Lactobacillus* (Figure 2b) showed a significant change in positions of peak, when compared to the control dye spectrum. A new peak at 1636.59 cm-1 represented –N=N- stretching vibration. The C-H deformation showed at 1398.13 cm^{-1}. The peak at 3408.37 cm^{-1} showed N-H stretching vibration.

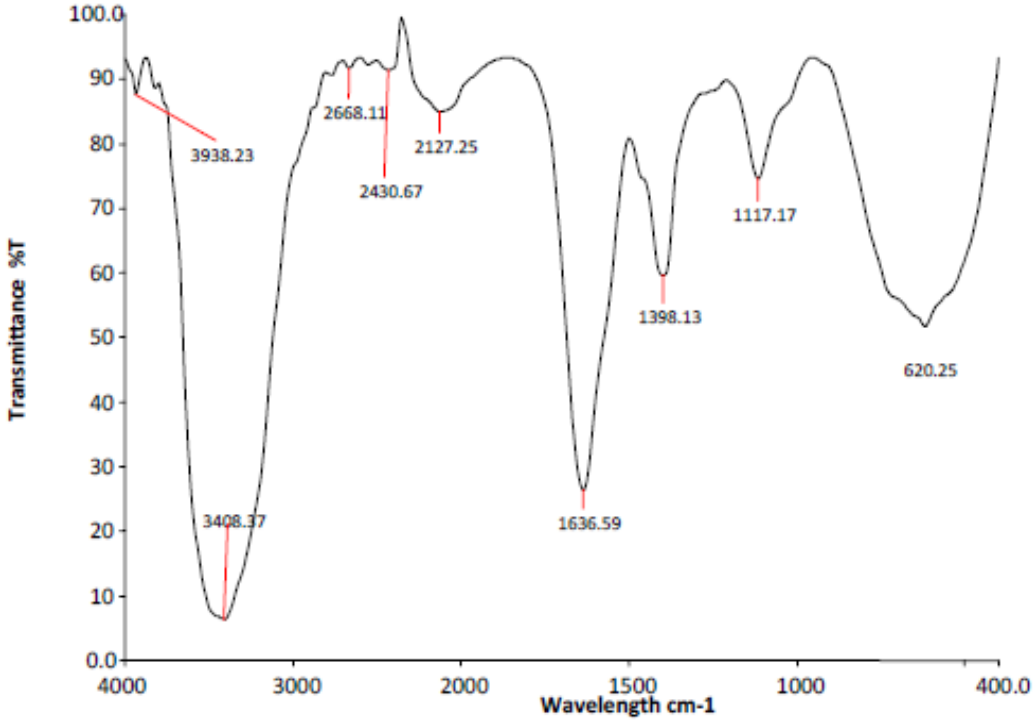


Figure 2a. The FTIR spectrum of a control dye FTIR Resultes

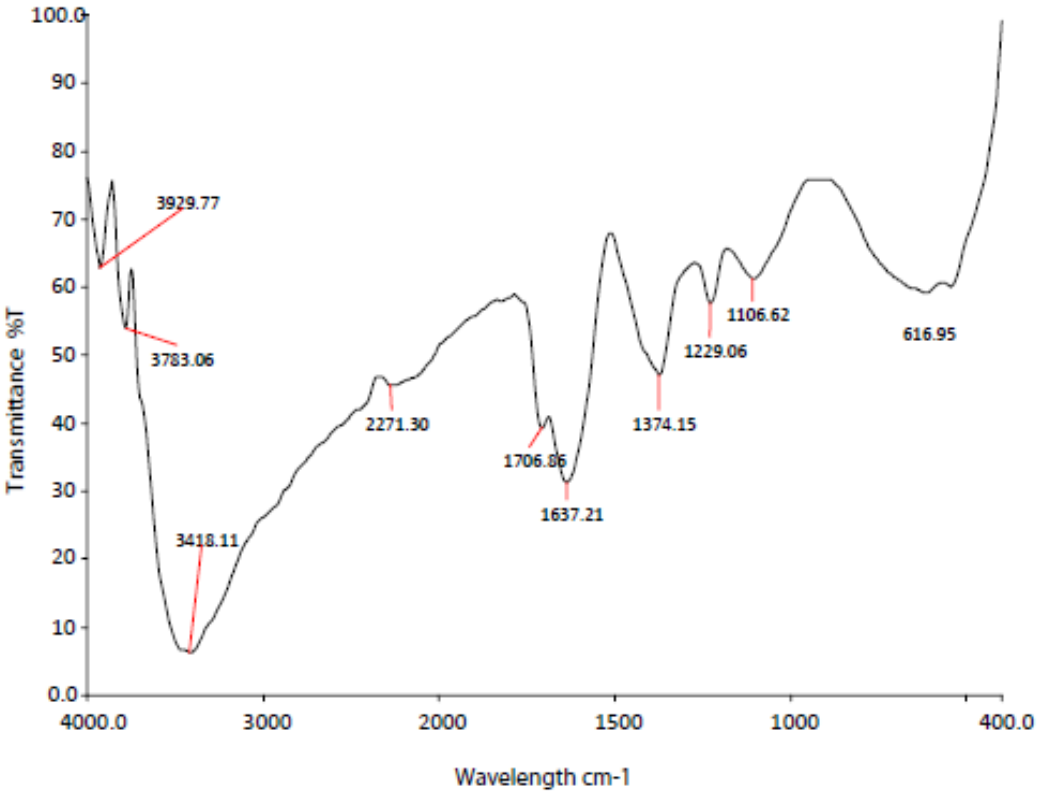


Figure 2b. The FTIR spectrum of extracted metabolites (24 hours)

3.3.2. LC-MS Analyses

During the degradation there is asymmetric cleavage of azo bond in RO (Figure 3a and Figure 3b) resulting in formation of 1-amino-1-napthalene sulphonic acid, which was confirmed by the standard library data, this is further, converted to aniline. While the naphthalene part of the dye was further biodegraded with opening of one ring, the formation of aldehyde as one of the intermediate is confirmed from the IR data.

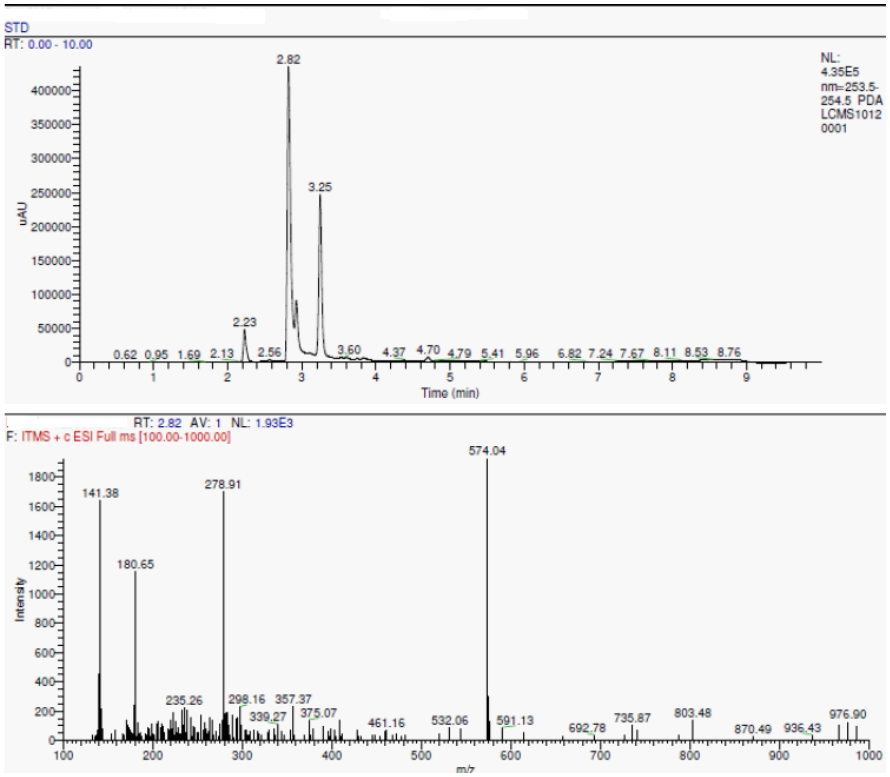


Figure 3a. Chromatogram and mass spectra of Reactive orange at 0h

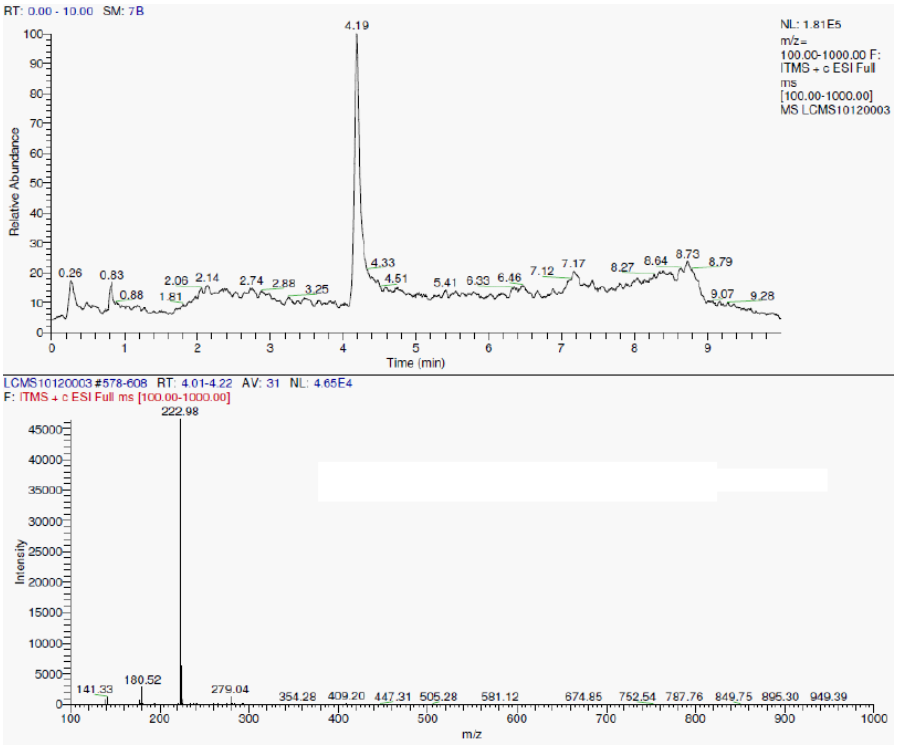


Figure 3b. Chromatogram of reactive orange treated with Lactobacillus after 24hrs

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