



Decolorization of two azo dyes using marine *Lysobacter* sp. T312D9

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ABSTRACT

Aims: Novel azo dye-degrading bacterium T312D9 strain has been isolated from Abou Quir Gulf, Alexandria, Egypt.

Methodology and Results: The identification of the isolate by 16S rRNA gene sequencing revealed to be *Lysobacter* sp. This marine ecofriendly isolate was exploited for its ability to degrade two synthetic azo dyes considered as detrimental pollutants from industrial effluents: congo red and methyl red. Using different dye concentrations showed the highest metabolic activity for complete degradation obtained from 100 to 500 mg/L within 30 h under static condition, also, sustaining higher dye loading of 1 g/L was carried out. The significant induction of enzymes NADH - 2,6-dichloroindophenol (NADH-DCIP) reductase and tyrosinase indicated their prominent role in dye degradation. The biodegradation of two azo dyes were analyzed by gas chromatographic-mass spectrum analysis (GC-MS) and Fourier transform infrared spectroscopy (FTIR) before and after treatment. Toxicity study revealed the much less toxic nature of the metabolites produced after complete decolorization.

Conclusion, significance and impact of study: *Lysobacter* sp T312D9 represent an inexpensive and promising marine bacteria for removal of both methyl and congo red. High sustainable metabolic activity for biodegradation under static condition. NADH-DCIP reductase and tyrosinase were significantly induced during biodegradation of dyes. The obtained metabolites revealed to be less toxic in nature which offers a practical biological treatment.

Keywords: *Lysobacter* sp, decolourization, azo dyes, enzyme activities, GC-MS, FTIR, toxicity

INTRODUCTION

Synthetic azo dyes have a wide application in the food, pharmaceutical, textile, leather, cosmetics and paper industries (Adedayo *et al.*, 2004). These synthetic dyes are extremely stable under exposure to light and washing and also resistant to aerobic biodegradation. The discharge of these industrial colored effluents results in creating undesirable conditions that are lethal to resident organisms (Khehra *et al.*, 2006). Several methods are used in the treatment of textile effluents to achieve decolorization but they have many disadvantages and limitations (Figueroa *et al.*, 2009).

Biological processes provide alternative technologies that are more cost-effective and environmentally friendly (Asad *et al.*, 2007). Many microorganisms belonging to the different taxonomic groups of bacteria have been reported for their ability to decolorize azo dyes (Telke *et al.*, 2008; Mendes *et al.*, 2011a; Feng *et al.*, 2012). Many marine microbes are capable of degrading azo dyes, including bacteria (Priya *et al.*, 2011), fungi (Saritha *et al.*, 2010), actinomycetes (Mane *et al.*, 2008) and algae (Ergene *et al.*, 2009). In recent years, there has been an increasing interest in bacteria that have potentiality to degrade or mineralize several azo dyes without shaking

condition (Kumar *et al.*, 2006; Kalyani *et al.*, 2009; Mansour *et al.*, 2011).

Bacterial decolorization was associated with the involvement of oxidoreductive enzymes, including azoreductase, NADH-DCIP reductase, tyrosinase, lignin peroxidase and laccase. The ability for dye detoxification using these enzymes was reported using different *Bacillus* strains including *Bacillus velezensis* strain AB (Bafana *et al.*, 2008), *Bacillus subtilis* (Pereira *et al.*, 2009) and *Bacillus* sp. ADR (Telke *et al.*, 2011). A new *Pseudomonas putida* strain shows a broader specificity of azoreductase for decolorization of some azo dyes (Mendes *et al.*, 2011b).

The genus *Lysobacter* was first proposed by Christensen and Cook (1978) to be ubiquitous inhabitants of soil and water. Strains of *Lysobacter* were isolated from diverse sources in different parts from world, including; groundwater of a basement tile drain in Michigan, USA (Sullivan *et al.* 2003); upflow anaerobic blanket sludge reactors, Korea (Bae *et al.* 2005) and deep-sea sponge in Philippine Sea (Romanenko *et al.* 2008). *Lysobacter* spp. have been shown to be non-fastidious as to their nutrient requirements using Luria Bertani (LB) agar, nutrient broth (NB), Difco R2A medium (Ikner *et al.*, 2007) and 10% tryptic soy agar (TSA) (Giesler and Yuen 1998).

Novel hydrocarbon-degrading *Lysobacter* strains have been isolated from seawater and studied their ability to utilize recalcitrant compounds as its sole carbon and energy source. Maeda *et al.* (2009) reported the ability of marine *Lysobacter* to degrade carbasol to anthranilic acid when subjected to GC-MS analysis. Another *Lysobacter* sp. strain OC7 isolated from onshore sites was found to carry carbasol (CAR) - degrading genes (Maeda *et al.*, 2010). *Lysobacter* was, recently, of great interest for taxonomical research as well as for bioremediation technologies and industrial applications (Zhang *et al.*, 2011; Lou *et al.*, 2012; Brito *et al.*, 2012).

Thus, the main objective of this work was to study the ability of a new marine *Lysobacter* strain to degrade two azo dyes: methyl red and congo red under static condition. Enzymes involved in the degradation: DCIP reductase, tyrosinase, liginin peroxidase and azoreductase were assayed and metabolites formed after degradation were analysed using GC-MS and FTIR. Further, the toxicity potential of the two dyes was investigated during microbial degradation.

MATERIALS AND METHODS

Microorganism and culture conditions

Lysobacter sp was isolated from Abou Quir Gulf (Mediterranean Sea) near El Amya industrial pumping station considering enriched location contaminated with various dyes. Pure culture was maintained on nutrient agar slants. This organism was selected according to its ability to use commercial azo dyes as a sole carbon source. Composition of nutrient medium used for decolorization process was (g/L): NaCl 5, peptone 5, beef extract 3, dyes were used in a concentration of 100 mg/L. The medium was adjusted at pH 7.5; 100 mL medium in 250 mL Erlenmeyer flasks was inoculated with 3 mL bacterial suspension (10^7 cell/mL) and incubated at 28 ± 2 °C until complete decolorization was achieved under static condition. Two controls were made; the first one was inoculated in dye free sterile medium, while the second control was made containing heat killed cells in concentration as that of culturing processes indicated the effect of medium components on the decolorization assay.

Dyestuff and chemicals

Some commercial dyes were obtained from El-Beada Dyers Company, Borg El-Arab, Alexandria, Egypt, including: black 5, reactive turquoise blue, and cibacron violet, congo red (rosalmine), methyl red were tested for the decolorization by *Lysobacter* sp. The best wavelength (λ_{max}) for each dye (555, 520, 542, 506 and 502 respectively) was measured after centrifuge of medium and the supernatant containing dyes was measured at the maximum wave length.

16S rRNA sequence analysis

DNA was extracted from overnight pure culture of marine isolate using Qiagen DNeasy kit (QIAGEN-Inc., Germany)

and Genomic DNA purification kit (Promega). The purified DNA was amplified using a set of 16S rRNA universal primer fD1 (5'-AGAGT TTGAT CCTGG CTCAG-3') and rP2 (5'-ACGGC TACCT TGTTA CGACTT-3') (Lee, *et al.*, 2003) (f: forward; r: reverse; D: distal; P: proximal). The PCR product was purified and sequenced using BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) and model 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Then, sequences were searched on BLAST tool (blastn) towards nr/nt nucleotide sequence database in NCBI server (www.ncbi.nlm.nih.gov) and downloaded.

Nucleotide sequence accession number

The GenBank accession number for the sequences generated in this study is [HM438544.1](https://www.ncbi.nlm.nih.gov/nuclot/HM438544.1), the isolate was identified as *Lysobacter* sp. T312D9. This process was carried out at City for Scientific Research and Technology Applications, Genetic Engineering and Biotechnology Research Institute, the Environmental Biotechnology Department, New Borg El-Arab City, Alexandria, Egypt.

Decolorization at different dye concentration

Lysobacter sp. T312D9 was grown at 28 ± 2 °C in 250 mL Erlenmeyer flasks containing 100 mL nutrient broth to study the effect of initial dye concentration on the decolorization in static condition, nutrient medium was added with 100, 300, 500, 700 and 1000 mg/L of the dyes. The aliquot (3 mL) of the culture media was withdrawn at different time intervals (0, 6, 12, 24, 48, 60, 72 h), centrifuged at 5000 rpm for 10 min. Decolorization was monitored by measuring the absorbance of cell free supernatant. Growth of microorganism was determined by the gravimetric method after drying at 80 °C until constant weight was reached. All decolorization experiments were performed in three sets.

The percentage decolorization was calculated as follows:

$$\% \text{ Decolorization} = \frac{\text{Initial absorbance} - \text{Observed absorbance}}{\text{Initial absorbance}} \times 100$$

Enzyme assays

All enzyme activities were assayed in cell free culture media at room temperature. Bacterial culture was centrifuged at 5000 rpm for 30 minutes and the culture supernatant was further filtered through 0.45 mm pore size (Grid sterile cellulose membrane, Gelman laboratory). NADH-DCIP reductase assay was carried out using procedure of Salokhe and Govindwar (1999). Mixture composition (5.0 mL) was 25 μ M DCIP, 4.75 mL of potassium phosphate buffer (20 mM, pH 7.5) and 0.1 mL of enzyme solution. The reaction was initiated by addition of 50 μ M NADH. The decrease in color intensity of DCIP was observed at 595 nm. Azoreductase assay was carried out using procedure of Chen *et al.* (2005). Composition of the assay mixture (2.0 mL) was 4.45 μ M of methyl red (MR), 100 μ M NADH, 1.7 mL of potassium phosphate buffer (20 mM, pH 7.5). The reaction mixture was pre-

incubated for 4 min followed by the addition of NADH and monitored for the decrease in color absorbance (430 nm). The reaction was initiated by addition of 0.1 mL of the enzyme solution. Tyrosinase activity was determined in a reaction mixture of 2 mL, containing 0.01% catechol in 0.1 M phosphate buffer (pH 7.4) at 495 nm (Zhang and Flurkey, 1997). Lignin peroxidase (LiP) was determined by monitoring the formation of propanaldehyde at 300 nm in a reaction mixture of 2.5 mL containing 100 mM *n*-propanol, 250 mM tartaric acid, 10 mM H₂O₂ (Shanmugam *et al.*, 1999). One unit of enzyme activity was defined as amount of enzyme required to reduce 1 μ M of substrate min⁻¹. All the enzyme assays were run in triplicates and average rates were calculated.

Biodecolorization analysis

Decolorization was monitored by UV-Vis spectroscopic analysis (Hitachi U-2800) whereas biodegradation was monitored by FTIR spectroscopy. Also, identification of metabolites was carried out by GC/MS. One hundred mL samples were taken after complete degradation, centrifuged at 10,000 rpm and extraction of metabolites was carried from supernatant using equal volume of ethyl acetate. The extracts were dried over anhydrous Na₂SO₄ and evaporated to dryness in rotary evaporator. The biodegraded methyl red and congo red was characterized by Fourier Transform Infrared Spectroscopy (Perkin Elmer, Spectrum one) and compared with control dye. The FTIR analysis was done in the mid IR region of 400–4000 cm⁻¹ with 16 scan speed. The samples were mixed with spectroscopically pure KBr in the ratio of 5:95, pellets were fixed in sample holder, and the analyses were carried out.

Rotary vacuum evaporated sample was dissolved in methanol and GC/MS analysis of metabolites was carried out using a Hewlett Packard 989 B MS Engine, equipped with integrated gas chromatograph with a HP1 column (30 m long, 0.25 mm id, nonpolar). Helium was used as carrier gas at a flow rate of 1.1 mL/min. The injector temperature was maintained at 300 °C with oven conditions as 100 °C kept constant for 2 min then increased up to 250 °C with 10 °C/min, raised up to 280 °C with 30 °C/min rate. The compounds were identified on the basis of mass spectra and using the NIST library. Control with dye free sterile medium showed no peaks similar to those obtained in the presence of dye decolorizing medium.

Toxicity study

Toxicity bioassay was carried out using the brine shrimp *Artemia salina*. Two hundred mg ethyl acetate extract were dissolved in 2 mL DMSO. Different extract concentrations (100, 200, 500, 1000, 1500, 2000, 4000 and 6000 μ g/mL) were prepared, then 10 mL of sterile brackish water was added in 20 mL glass vials (three vials /concentration). Ten *Artemia salina* nauplii were transferred to each vial. Control vials free from extract with 10 nauplii were carried out. The number of the viable biomarker was counted after 24 h of application. The

percentage of mortality and the half lethal concentration (LC₅₀) were determined using the probit analysis method (Mayer *et al.*, 1982).

Statistical analysis

Data were analyzed by one-way analysis of Standard error using Origin lab 6.

RESULTS AND DISCUSSION

Marine bacteria are vital in recycling nutrients and could be useful for innovative applications, which are helpful to human beings. This study showed the isolated marine bacterial strain that able to decolorize two azo dyes, identified as *Lysobacter* sp T312D9 on the basis of 16S rRNA sequence where the phylogenetic relationship between this strain and other related microorganisms is shown in (Figure 1). The blast assay result indicated this strain belong to γ -proteobacteria group.

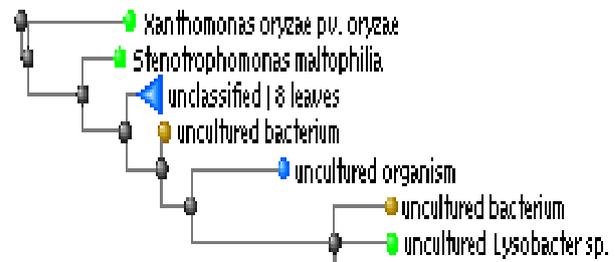


Figure 1: Phylogenetic analysis of 16s rRNA sequence of *Lysobacter* sp. T312D9. Tree view for RID: N7EK26G901S, query ID: lcl|45967, database: nr.

Decolorization of different azo-dyes

As preliminary study, *Lysobacter* sp. was able to decolorize various azo dyes at 100 mg/L in the nutrient broth. Complete removal of dye was observed for congo red and methyl red under static condition within 30 h. Similar results using *Comamonas* sp VS-MH2 showed high metabolic activity towards mixture of reactive azo dye by degrading them completely at 100 mg/L within 30 h (Pathak *et al.*, 2011). Ola *et al.* (2010) studied the ability of *Bacillus cereus* to decolorize two azo dyes cibacron black PSG and cibacron red P4B under aerobic conditions within 5 days. Another *Bacillus* sp. ADR also showed maximum decolorization of C.I. reactive orange 16 (100 mg/L) under static condition whereas less decolorization at shaking condition (Telke *et al.*, 2009). The other examined dye: reactive turquoise, cibacron violet and black5 showed a lower decolorization efficiency compared with congo and methyl red at 28, 32 and 70%, respectively, after 72 h of incubation.

Effect of different dye concentrations

Decolorization percent of congo red and methyl red by *Lysobacter* sp T312D9 was varied with initial dye

concentrations: 100–1000 mg/L (Figure 2). Dye degradation showed progress till 500 mg/L with efficiently decolorization activity 90-100% for methyl red and 95-100% for congo red within 30 h. Gradual decline of dyes removal above this concentration was observed till 1000 mg/L which exhibited decolorization percent at 80% for congo and 88% methyl red within 72 h. This gradually decline may occur due to the change in metabolic enzymes or accumulation of some by-products which have anti-decolorizing enzyme activity. Similar result was observed by Kalme *et al.* (2007) showing complete

decolorization of 50 and 100 mg/L Direct Blue-6 dye by *Pseudomonas desmolyticum* took 60 and 72 h, respectively, while, more dye concentrations (150–200 mg/L) resulted in 80–40% dye removal within 4 – 6 days and no more exceed than 15% dye removal was observed at 250 mg/L dye concentration. Two *Bacillus* strains AK1 and AK2 were capable of complete decolorizing Metanil Yellow azo dye (200 mg/L) within 27 and 12 h, respectively, whereas, maximum dye concentration (1000 mg/L) was decolorized within 84 and 78 h, respectively (Anjaneya *et al.*, 2011).

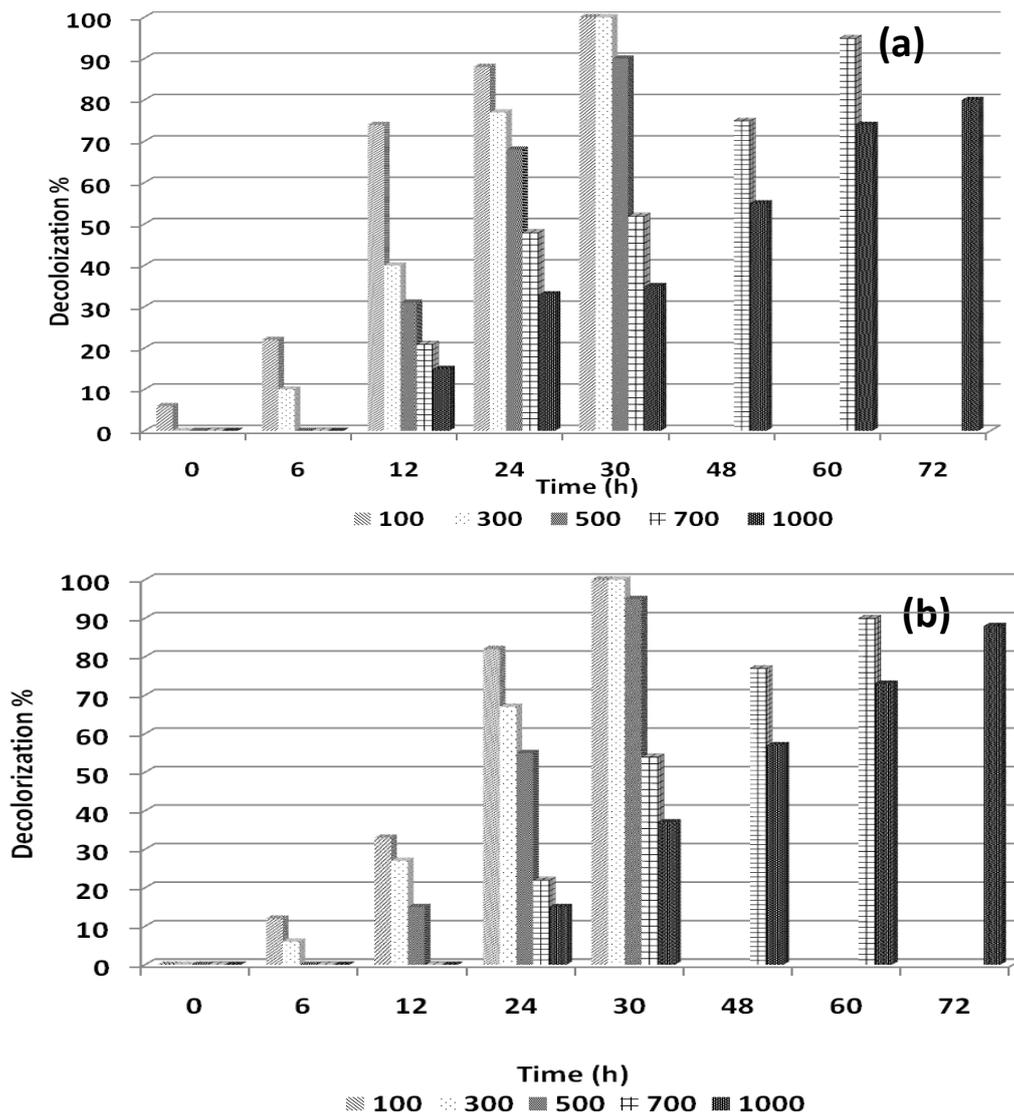


Figure 2: Decolorization percent of different methyl red (a) and congo red (b) concentrations at interval time using *Lysobacter* sp. T312D9

Enzymatic analysis

Azo-enzyme, NADH- DCIP reductase and tyrosinase, showed gradual increase in activities during the time course of methyl red and congo red decolorization by *Lysobacter* sp. T312D9 (Table 1). High significant activities of NADH- DCIP reductase and tyrosinase at 24 h were recorded at 152 and 200% for methyl red and 239 and 200% for congo red as compared to 0 time. This is followed by lignin peroxidase induction activity, 150% for methyl red and 157% for congo red at 12 h. Whereas, no significance was observed for azoreductase activity. These observations demonstrated that the significant

induction of enzymes NADH-DCIP reductase and tyrosinase indicated their important role in the dye degradation. In contrast, Yang *et al.* (2011) studied the significant induction of NADH-DCIP reductase and azoreductase for the acid yellow 199 treatment by *Shewanella oneidensis* MR-1 at 192% and 248%, respectively. Another report by Telke *et al.* (2010) showed no change in NADH-DCIP reductase activity, and decrease in azoreductase activity by 26% after 12 h of incubation for congo red decolorization by *Pseudomonas* sp. SU-EB.

Table 1 : Azo-Enzyme activities in mg/mg/min during the time course of *Lysobacter* sp. T312D9 decolorizing methyl red and congo red

Dyes	Enzyme activity (mg/mg/min)					
	Time (h)	0	6	12	24	30
Methyl red	DCIP activity	17.3±0.87	19.7±0.99	20.7±1.0	26.3±1.3	22.0±0.95
	Azo reductase	0.8 ± 0.04	0.8 ± 0.04	0.8 ± 0.04	0.8 ± 0.04	0.8± 0.04
	Tyrosinase	0.2	0.2 ± 0.3	0.3 ± 0.6	0.4 ±1.5	0.2 ± 1.2
	Lignin Peroxidase	0.8± 0.04	1.0 ± 0.05	1.2 ± 0.06	0.9 ± 0.04	0.9 ± 0.04
Congo red	DCIP activity	25.2±1.3	34.6±1.7	44.2±2.2	3±60.2	1.9±. 39.4
	Azo reductase	0.7±0.03	0.7±0.04	0.7±0.04	0.7±0.04	0.7±0.03
	Tyrosinase	0.3±0.01	0.4±0.02	0.4±0.02	0.6±0.03	0.3±0.01
	Lignin Peroxidase	0.7± 0.03	0.7± 0.04	1.1 ± 0.06	0.9 ± 0.05	0.9 ± 0.05

Values are mean of three experiments ± SEM (Standard error of mean), significantly different from control cells at P>0.05 using ORGIN LAB analysis

Metabolites analysis

For the biodegradation of azo dye, destruction of dye is a necessary because the removal of color is associated with the breaking of the chromophores i.e. conjugated unsaturated bond (-N=N-) in molecules (Merici and Kaptan, 2004). Comparison of FTIR spectrum of methyl red and congo red "parent dyes" with their extracted metabolites clearly indicated the biodegradation by *Lysobacter* sp T312D9 (Figure 3a&b). The FTIR spectrum of the two parent dyes showed peak at 1540 and 1578 cm⁻¹ for N=N stretching vibrations. Absence of these peaks after decolorization indicates cleavage of azo bond. This result was supported by Tekel *et al.*, (2010), Kalme *et al.*, (2007) and Kalyani *et al.* (2008) that reported the breakdown of azo bond due to the biodegradation of several azo dyes.

GC-MS analysis was carried out to investigate the metabolites formed after the biodegradation process (Table 2). The most suggested structures given with the NIST database were characterized as 1-alanine, N-(O-anisoyl)-methyl ester and benzamine 2-ethoxy o-phenetidine obtained from methyl red and congo red

biodegradation, respectively. The two metabolites were different than N,N dimethyl aniline that found by Hsueh and Chen (2007), and 3-Hydroperoxy 8-nitrosophthol that found by Telke *et al.* (2010). According to the biodegradation route(s) in decolorization of methyl red and congo red by *Lysobacter* sp T312D9 that differ from the previous published works at least in the final steps (s) and this will be subjected for more studies.

Metabolites toxicity

The end products after dye biodegradation are of concern due to their environmental toxic effect. This experiment was carried out to detect the toxicity of different extracted metabolites concentrations. The results in Table 3 indicated low toxicity effect with maximum metabolites concentration at 2000 and 1500 µg/mL and the LC₅₀ was found to be 3.3 and 3.2 after 24h for methyl and congo red, respectively. This indicates that the decolorization process yielded less toxic metabolites, which may be safely discharged. Similar results were reported using phytotoxicity studies for azo dye biotreatment that revealed the less toxic nature of Reactive Red 2 (Kalyan

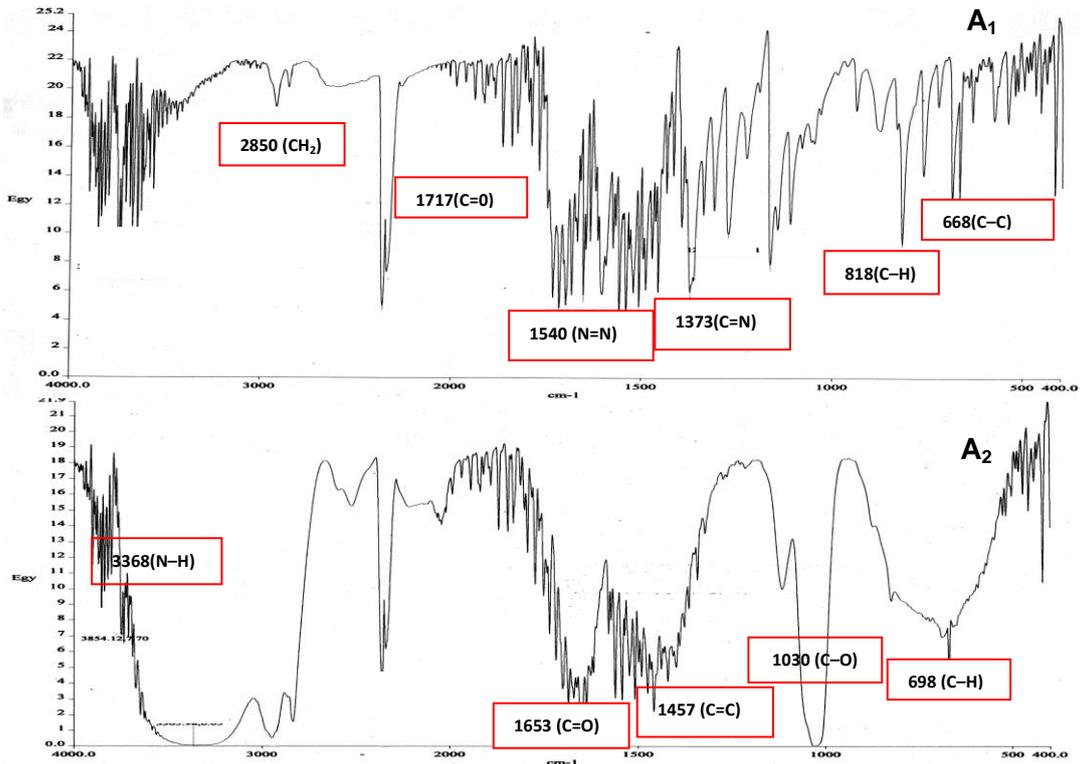


Figure 3a: FTIR spectrum of parent dye methyl red (A₁) and its metabolite (A₂) showing the absence of azo bond (–N=N–) after decolorization process.

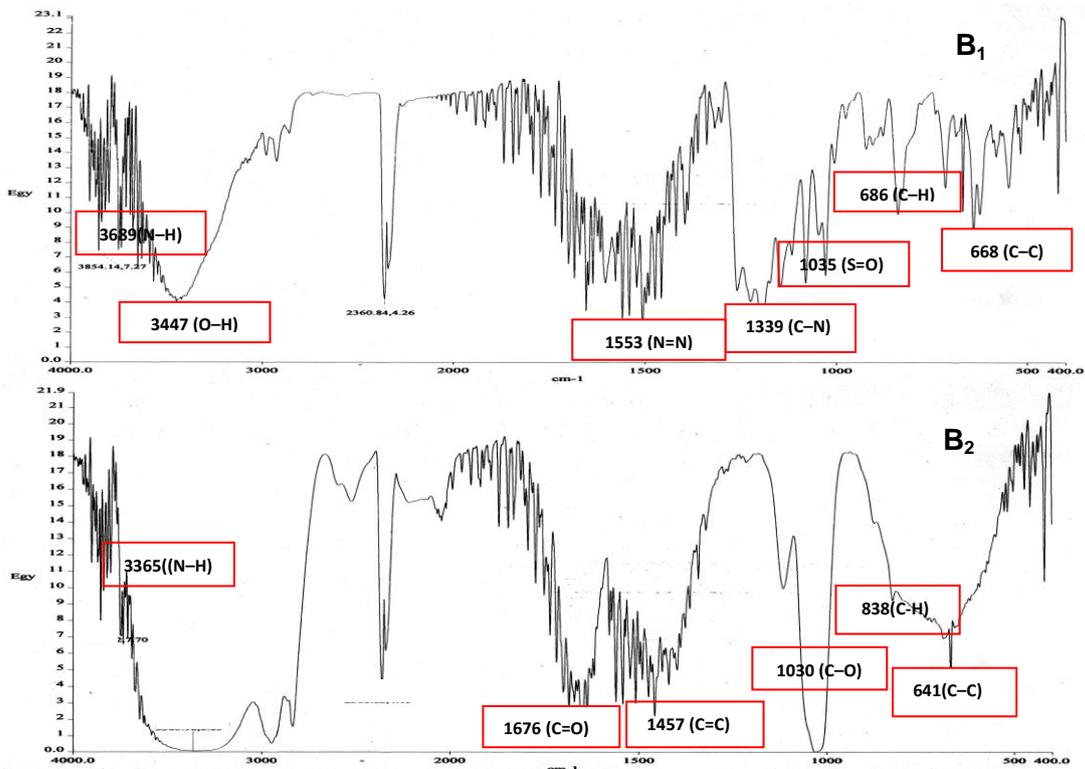
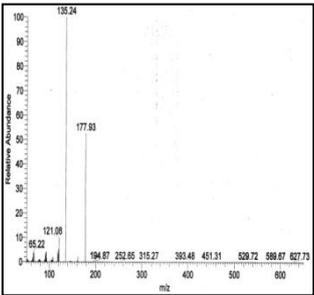
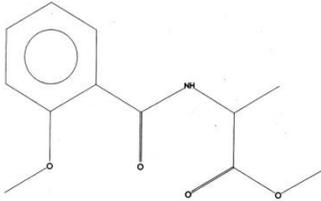
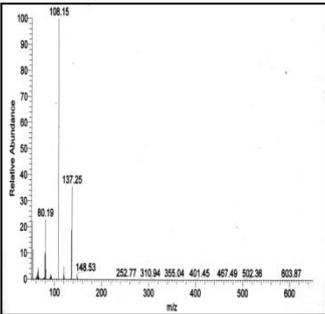
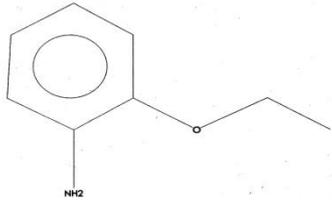


Figure 3b: FTIR spectrum of parent dye methyl red (B₁) and its metabolite (B₂) showing the absence of azo bond (–N=N–) after decolorization process.

Table 2: Gas chromatogram-Mass spectrum and suggested chemical structures of the two metabolites obtained after dye decolonization at different retention time

Dye	Metabolites	Mass spectrum	Rt. Time (min)	Suggested chemical structure
methyl red	1-alanine, N-(O-anisoyl)-, methyl ester		18.04	
congo red	Benzenamine, 2-ethoxy-O-phenetidine		10.75	

- (a) metabolite from methyl red biodegradation
- (b) metabolite from congo red biodegradation

Table 3: Toxicity of metabolites obtained after decolorization process using *Artemia salina* as biomarker

Metabolite extracts concentration (µg/mL)	Log concentration	Mortality % after 24h	
		Metabolite (a)	Metabolite (b)
100	2.0	0	15
200	2.3	10	20
500	2.6	10	35
1000	3.0	20	47
1500	3.2	45	50
2000	3.3	50	65
4000	3.6	60	80
6000	3.8	80	95

et al., 2008), Reactive Yellow 84A (Dhanve *et al.*, 2009), Congo Red (Telke *et al.*, 2010) and seven commercial textile azo dyes (Kurade *et al.*, 2011) metabolites. Also, *Artemia salina* biotoxicity tests were achieved for many degradable products from reactive textile dye showed the lowest toxicity level (Palácio *et al.*, 2009; da Silva *et al.*, 2011).

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