

A Rapid Two-Step Bioremediation of the Anthraquinone dye, Reactive Blue 4 by a Marine-derived Fungus

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Abstract

A rapid two-step technique for bioremediation of the anthraquinone dye, the Reactive Blue 4 (RB4) is reported here. In the first step, 1000 mg L⁻¹ of this dye treated with partially purified laccase from a marine-derived fungus resulted in 61% color removal and two-fold decrease in COD by 12 h. The metabolites formed during the enzymatic degradation were characterized by MS, UPLC, and UV-Visible spectroscopy. These analyses confirmed changes in the aromatic character of the parent dye and formation of low molecular weight phenolic compounds as the final products of the enzymatic degradation. Based on these results the probable degradation products of RB4 were 2-formylbenzoic acid, 1,2,4,5-tetrahydroxy-3-benzoic acid, 2,3,4-trihydroxybenzenesulfonic acid and 1,2,3,4-pentahydroxybenzene. In the second step, the enzyme-transformed dye solution when subjected to sorption on the powdered fungal biomass resulted in a further reduction in color up to 93% within 10 min. Sorption of the degraded dye was confirmed by the changes in the pattern of FTIR spectrum. The two-step treatment resulted in a decrease of 29% in total carbon accompanied by two-fold decrease in toxicity. This is the first report on decolorization, detoxification and mineralization of RB4 by laccase from a marine-derived fungus.

Key words: bioremediation, biosorption, degradation products, detoxification, marine-derived fungus, reactive blue 4

1 Introduction

The annual worldwide production of dyes and pigments is more than 0.7 Mt. About 10-15% of this amount is discharged into the environment without any proper treatment (Vinu and Madras 2009). Pollution caused by dye effluent is mainly due to long durability of dyes in wastewater, stability and resistance to degradation. The dyeing process results in the production of large amounts of wastewater having intense coloration. Improper disposal of dyes leads to the reduction in sunlight penetration in the water bodies causing a decrease in photosynthetic activity, and the increased heterotrophic activity lowers dissolved oxygen levels, adversely affecting the aquatic life.

Synthetic dyes used are recalcitrant to the removal by conventional wastewater treatments such as adsorption, photo-oxidation, coagulation, flocculation, photo- and chemical degradation. Moreover, the main disadvantage related with chemical methods is the formation of toxic compounds resulting from the cleavage of the chromophoric groups (Robinson et al. 2001). Biological treatment is often economical alternative in comparison with physical and chemical processes. Bioremediation methods such as microbial decolorization and degradation, adsorption by living or dead microbial biomass are commonly recommended for the treatment of industrial effluents (Fu and Viraraghavan 2001; McMullan et al. 2001).

Biological treatments involving white rot fungi (WRF) have attracted increasing interest since several studies revealed their potential to combine decolorization and toxicity reduction (Fu and Viraraghavan 2001). With the virtue of extra-cellular enzymes, they have abilities for remarkable biotransformation or biodegradation of a broad variety of xenobiotics (Wesenberg et al. 2003). The enzyme-based methods consume low energy as well as cause minimal adverse impact on ecosystems. Among these, laccases has aroused interest because of their ability to transform a wide variety of hazardous chemicals (Majeau et al. 2010). They are useful biocatalysts for a wide range of biotechnological applications because of their nonspecific oxidation capacity, and ability to use available molecular oxygen as an electron acceptor (Baldrian 2006).

Dead fungal biomass is a suitable alternative for bioremediation as it is not affected by toxic wastes, do not require nutrients and do not release toxins or propagules in the environment (Tigini et al. 2011).

Biodegradation using whole fungus culture system is known to be a relatively slow process and there is always a necessity to create an optimal favorable environment, maintenance and nutrition

requirements (Crini 2006). In view of this, the present study was focused on developing a quick two-step methodology for remediation of Reactive Blue 4 (RB4). This is one of the commercially important reactive dyes widely used in dyeing cotton fibers (Carneiro et al. 2007). Reactive dyes are highly water soluble and are non-degradable in the conventional, biological treatment systems, adsorb poorly to biological solids and therefore remain in the discharged effluents (Epolito et al. 2005). Besides coloring the water bodies wherein they are discharged, they also adversely impact the aquatic ecosystem and cause acute toxicity. Biological removal of reactive dyes is reported to be low (Greaves et al. 2001). Very slow environmental degradation of RB4 resulting in long residual time in the environment may lead to potentially chronic long-term dye exposure. Therefore, our aim was to eliminate RB4 by biological processes.

A marine-derived basidiomycetous fungus having 99% homology to 18SrDNA and ITS gene sequences of *Cerrena unicolor* (Verma et al. 2010) was chosen in the present study for the following reasons. It produces high titre of thermostable metal-tolerant laccase (D'Souza Ticlo et al. 2009b). It was efficient in decolorization and sorption of several dyes and colored effluents (D'Souza et al. 2006, Verma et al. 2011). Conditions for optimum growth and laccase production in this fungus were standardized by D'Souza-Ticlo et al. (2009a).

In the present study degradation of RB4 dye was attempted in a two step- process using this fungus. In the first step it was subjected to degradation using partially purified laccase and the metabolites were identified. In the subsequent step the powdered biomass of the same fungus was used for sorption of the degraded products.

2 Materials and Methods

2.1 Chemicals

Commercial Reactive Blue 4 (RB4), an anthraquinone dye (with 33% purity) was obtained from Sigma-Aldrich, St. Louis, MO, USA and was used without any further purification. All other chemicals were of analytical grade.

2.2 Organism and Culture conditions

The basidiomycetous fungus NIOCC #2a isolated from decaying mangrove wood (D'Souza et al. 2006) was used for the present study. It is a non-sporulating fungus and therefore microscopic identification was not possible. The fungus showed 99% homology to *Cerrena unicolor* by analysis of 18S rRNA and

ITS gene sequences (Verma et al. 2010). It was grown in Boyd & Kohlmeyer broth (Kohlmeyer and Kohlmeyer 1979) for 7 days. The fungal biomass after rinsing was homogenized in sterile sea water in Omni Macro-homogenizer (Marietta, GA, USA) for 5 s. The resulting mycelial suspension (10%, v/v) was used for inoculating 20 ml of low nitrogen (LN) medium (Tien and Kirk 1988) which was modified to replace glucose with fructose (10%) and ammonium tartrate with glycine (3%) as the carbon and nitrogen source respectively (C:N ratio 7.7:1.0). The culture was raised at 30°C under static conditions. On day six, CuSO₄ at a final concentration of 2 mM was added to the culture under aseptic conditions to stimulate laccase production (D'Souza-Ticlo et al. 2009b).

2.3 Partial Purification of laccase

After 12 days, when laccase production reached its maximum, the culture supernatant was obtained by filtering through Whatman GF/C filter paper and subsequently through 0.22 µm filters (Millipore, USA). It was frozen at -20 °C to precipitate the exopolymeric substance. The precipitate was removed after thawing the culture supernatant followed by centrifugation at 11,000 x g for 15 min. The culture supernatant was then concentrated by Ultra-filtration using YM3 membrane (Millipore, USA). The concentrate after filtering through 0.22 µm sterile filter was mixed appropriately with Bio-Lyte (3/10) ampholyte (Bio-Rad, USA) and 10% (v/v) glycerol. The proteins in the concentrated culture filtrate were separated on the basis of their pIs (Isoelectric points) in a mini-Rotofor system (Bio-Rad, USA) maintained at ~ 4°C. The typical initial voltage and current were 300 V and 25 mA with the voltage stabilizing in 3-4 h at approximately 1500 V and 8 mA. After focusing, 20 separate fractions were rapidly collected by vacuum aspiration. Aliquots containing laccase activity were pooled and applied to High Load 16/60 Superdex 75 preparative grade column in a fast protein liquid chromatography system (Amersham Biosciences, Sweden). The sample was eluted with 0.2 M Na acetate buffer (pH 4.5) containing 1 M KCl at a flow rate of 1 ml min⁻¹ (D'Souza-Ticlo et al. 2009b). The fractions showing maximum absorbance at 280 nm and laccase activity were collected and concentrated using a 10 kDa cut off Amicon Ultra centrifugal filter device (Millipore, USA). This whole procedure was repeated several times to get a sufficient amount of enzyme.

2.4 Enzymatic degradation of RB4

Degradation reactions were carried out in 100 ml Erlenmeyer flasks containing 10 ml of RB4 at a concentration of 1000 mg l⁻¹, dissolved in 0.1 M Na acetate buffer (pH 5.0). Partially purified laccase

(10 μ l) having 100 U of activity was added and incubated on a rotary shaker at 100 rpm, 30 °C. Analytical studies were carried out at regular intervals during incubation.

2.5 Analytical studies

Color was determined by calculating the total area under the plot by integration of the absorbance between 400 and 800 nm of the spectrum (Shimadzu UV-2450, Japan). Percentage decolorization was calculated with respect to the color at 0 h. The dye solution without any enzyme was maintained as a control.

Laccase was assayed by measuring oxidation of 1 mM ABTS (2, 2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonate) at 405 nm (Niku-Paavola et al. 1988). Units were calculated as μ M of the substrate transformed $\text{min}^{-1} \text{ l}^{-1}$ and expressed as U l^{-1} .

Chemical oxygen demand (COD) was estimated by open reflux method (APHA, AWW, WEF, 2005). The values calculated in $\text{mg O}_2 \text{ l}^{-1}$ were expressed in percentage.

Total carbon was analyzed by combustion of the lyophilized samples at 1200 °C in an oxygen atmosphere and detected with the aid of CN elemental analyzer (Thermo Fisher Scientific, UK, Model No. 2000 MARS). Aspartic acid was used as the internal standard as per the manufacturer's recommendation. The analytical precision for carbon was ± 0.38 %.

The reaction products formed due to enzymatic activity were analyzed by UPLC (Ultra Performance Liquid Chromatography, Waters Corporation, MA, USA). The UPLC conditions were modified from that of Gomas et al. (2010). It was performed on a 5 cm x 2.1 mm, 1.7- μ m particle, Acquity C₁₈ column. The mobile phase was a gradient prepared from acetonitrile and H₂O. Elution was initiated with 2% acetonitrile for 1 min then the proportion of acetonitrile was changed stepwise to 10% at 1 min, 50% at 3 min, 20% at 5 min and 0% at 7 min. The scan was taken for the retention time of 0 to 7 min at the flow rate of 500 μ l min^{-1} . Detection was monitored at wavelength of 254 nm.

Electrospray ionization mass spectrometry (ESI-MS) of freeze-dried samples, was performed on a Quadrupole-time of flight (Q-TOF) mass spectrometer (Model Qstar XL, Applied Biosystems, Rotkreuz-Switzerland). The lyophilized samples were dissolved in methanol:water (1:1) and directly analyzed as described earlier (Verma et al. 2011). The samples were introduced at a constant flow rate into the electro spray source using an integrated syringe pump. The mass/charge (m/z) range was 0-1000

in negative ion mode. On the basis of this qualitative study, probable transformation products formed during the different time intervals of enzymatic treatments are proposed.

2.6 Fungal biomass preparation and biosorption

After 12 h of enzymatic treatment, RB4 solution was further subjected to sorption using the fungal biomass of NIOCC #2a, prepared as follows. The fungal biomass after collecting the culture supernatant was rinsed several times with distilled water. It was inactivated in NaCl solution (9 g l^{-1}) by autoclaving at $121 \text{ }^\circ\text{C}$ for 15 min (Prigione et al. 2008). After washing repeatedly with distilled water to remove the salts and other precipitated components, it was lyophilized and powdered. Powdered biomass of 100-200 μm particle size (0.2 g) was introduced in 100 ml Erlenmeyer flask and 10 ml of untreated or enzyme-treated RB4 was added. The flasks were incubated at 100 rpm, 30°C . After centrifugation at $11,000 \times g$ for 5 min, changes in the UV/Visible spectra were recorded. Thus, color was measured at regular intervals until no further change was noticed.

FTIR spectra were recorded between 4000 and 400 cm^{-1} using FTIR (model 8201PC, Shimadzu, Japan) with 1 cm^{-1} resolution. Pellets were prepared by mixing the lyophilized samples with 50 mg KBr using diffused reflectance spectroscopy accessory.

FTIR spectra of lyophilized fungal biomass before and after sorption were recorded.

2.7 Toxicity test with *Artemia* larvae

Bioassay was carried out using nauplii of *Artemia salina* (Verma et al. 2011). Treated and untreated RB4 samples were serially diluted with $0.22 \mu\text{m}$ -filtered seawater in disposable multiwell test plate with 24 (6 x 4) test wells. Nauplii (10 organisms) were incubated in the suitably diluted effluents at room temperature in the dark and mortality was estimated after 24 h. Lethal concentration that resulted in 50% mortality (LC_{50} value) by 24 h was calculated with 95% confidence limits with the aid of computer program EPA Probit analysis, version 1.5 (Finney, 1971).

2.8 Phytotoxicity test

Phytotoxicity tests can be carried out using several seed species as there are no standardized norms for using any special seed species (Osma et al. 2010). The phytotoxicity of the original and treated dye solution was assessed by the seed germination of green gram, *Vigna radiata*. This species was selected as it is one of the major and widely consumed leguminous plants of tropical countries. Moreover, the seeds are sufficiently large and have short germination period. The length of the radicle of the

germinating seeds which are mostly straight can be measured accurately. Three replicates of 10 seeds each were used for all the tests. After 40 h of incubation in 10 ml of dye solution (1:10) at 30° C in dark, the percentage of seed germination and the length of radicle were determined. The values obtained for the seeds incubated in de-ionised water were used as control. Germination index was calculated according to Osma et al. (2010). Besides, the root was excised from the cotyledons and the total dry weight of each replicate was determined.

3 Results and discussion

Reactive Blue 4 ([2-anthracenesulfonic acid, 1-amino-4-(3-((4, 6-dichloro-s-triazin-2-yl) amino)-4-sulfoanilino)-9, 10-dihydro-9.10-dioxo, disodiumsalt]) is an anthraquinone dye with dichlorotriazine group as a reactive site. It has a molar mass 637.4 and λ_{\max} is at 595 nm. The same dye with similar content (33%) was used in the photo-degradation studies by (Carneiro et al. 2007). Its degradation by physical and chemical methods has been extensively investigated (Carneiro et al. 2005; 2007; Gözmen et al. 2009). A two-step remediation of this dye using biological methods is described here.

3.1 Enzymatic degradation of RB4 – Step 1

A partially purified laccase of the fungus NIOCC #2a was used to degrade RB4. Reactive dyes used in the dyeing industries do not bind completely to the fibers and up to 50%, (sometimes exceeding 800 mg l⁻¹) remains unutilized in the dye bath and ultimately ends up in the effluent (Gözmen et al. 2009). Therefore in the initial studies, RB4 at a concentration of 1000 mg l⁻¹ was used in the submerged culture of this fungus. It was found to be decolorized up to 80% by day 6 (data not shown). Therefore in all the subsequent studies our aim was to speed up the process of decolorization using the same concentration. Spectral analysis of RB4 solution showed decrease in the absorbance between 500-760 nm resulting in 61% color removal after 12 h of incubation (Fig. 1). The color was changed from dark blue to pale brown as a result of increase in absorbance at 400-500 nm (see the insets depicting color change after each step in Fig. 1). Similar change of color in RB5 treated with dye decolorizing peroxidase was observed (Sugano et al. 2009). No further change in the spectral pattern was observed after 12 h although more than 60% of the enzyme activity was retained, indicating that the degradation products did not serve as substrates for the enzyme. Zeng et al. (2011) reported 70% reduction in color of RB4 at the concentration of 35 mg l⁻¹ using crude laccase of *Trametes trogii* in 30 min. An another anthraquinone dye, remazol brilliant blue R was decolorized up to 90% by purified laccase of *Trametes* sp. in 3 days (Yang et al. 2009). However, the concentration of the dye used was 200 mg l⁻¹ and the

reduction in color was measured at its absorbance maximum. Hence these results cannot be compared with the present study where we have measured the color by calculating the total area under the plot by integration of the absorbance between 400 and 800 nm of the spectrum. In another study, using purified laccase the same dye at a concentration of 300 mg l⁻¹ was 100% decolorized within 3 days (Hadibarata et al. 2012).

In order to examine the environmental fate, the study of the degradation products is extremely necessary. The detailed characterization of the intermediates and metabolites produced during biodegradation must be carried out to ensure the absence of toxic intermediates such as aromatic amines, which are known to be carcinogenic (Ali 2010). The increase of absorbance in the UV region during this period (Fig. 1) suggests the formation of smaller phenolic compounds resulting from the degradation of parent dye molecule. Further analysis of degradation was carried out using UPLC technique. This method is recognized as a tool with high specificity and accuracy for the determination of degradation products (Gomas et al. 2010). In UPLC analysis, the peaks at retention time 4.9 and 3.7 min represented the dye content (Online Resource 1a). Several new peaks appeared after the dye was mixed with buffer and enzyme solution (Online Resource 1b). The UPLC elution profile of the dye was considerably altered during the incubation period supporting a change in the aromatic character of the parent dye (Online Resource 1c-f). The peaks corresponding to the dye content were reduced in intensity. The number of peaks at lower retention times increased gradually (see the boxes with dotted lines in Online Resource 1c-f) as the incubation period progressed, indicating the formation of more polar oxidation products.

The ESI-MS spectra were recorded after 0, 2, 4, 6, and 12 h of laccase treatment. The dichloro dye, RB4 showed doubly charged characteristic quartet at *m/z* 354, 356, 358 and 360, indicating the presence of three halogen atoms. Therefore, the original dye exists as an amine hydrochloride salt (spectrum not shown). After 2 h of treatment the presence of quartet with reduced intensity and additional peaks at *m/z* 793, 657, 521, 443, 385, 302, 249, 205, and 199 were noticed (Fig. 2a). However, no characteristic isotopic halogenated fragment ions appeared. This indicated the elimination of halogen atoms initially followed by enzymatic oxidation of different functional groups. Gradual disintegration of the dye solution into smaller and simpler molecules occurred as indicated by the spectra at 4, 6 and 12 h (Fig. 2b, c, and d). Based on these results the probable degradation pathways are shown in Fig. 3. The enzyme treatment resulted in formation of several degradation products such as 2-formylbenzoic acid (*m/z* 149), 1,2,4,5-tetrahydroxy-3-benzoic acid (*m/z* 185), 1,2,3,4-

pentahydroxybenzene (m/z 157) and 2,3,4-trihydroxybenzenesulfonic acid (m/z 205). Polymerization was evident as certain intermediates (such as m/z 185, 233) were detected at 4 h but not at 6 h and these reappeared at 12 h (Fig. 2, 3). Laccase is known to generate free radicals (Baldrian 2006), which can act randomly as evident from the present study. Therefore no particular pathway of degradation is suggested here (Fig. 3). The characterization of degradation products derived from the oxidation of anthraquinone dyes other than RB4, using laccase has been reported (Osma et al. 2010; Hadibarata et al. 2011). The degradation products of RB4 generated by advanced oxidation processes (AOPs) have been characterized (Gözmen et al. 2009). The degradation intermediates obtained by AOPs were totally different from those obtained by using laccase in the present study.

The COD content in the untreated dye solution was 2791 mg O₂ l⁻¹. A 47% reduction was observed after 12 h of enzymatic treatment of RB4 indicating a partial mineralization of the dye. This is the first report showing reduction in COD after enzymatic treatment of RB4.

3.2 Further remediation of the enzyme-treated RB4 – Step 2

Enzyme treatment alone did not result in complete decolorization (Fig. 1) and therefore laccase-treated dye solution was further subjected to sorption over the powdered biomass of NIOCC #2a. The sorption equilibrium was reached within 10 min and this resulted in further decrease in color up to 93% (Fig. 1). Low molecular weight phenolic compounds appeared after the enzymatic degradation (Fig. 2). These were removed during the step-2 as was evident from a significant decrease in absorbance in the UV region (Fig. 1). Treatment of dyes and dye-containing effluents by sorption using fungal biomass has been reported (Bayramoğlu et al. 2006; Tigini et al. 2011). The dye solution directly subjected to sorption (without the enzyme treatment) was also decolorized up to 83% in the present study, but the pattern of UV spectrum did not change considerably (Fig. 1). Direct sorption took longer time to reach equilibrium than enzyme-treated dye solution. The phenomenon of sorption is only a phase transfer (Gadd 2009). Our aim was to degrade the dye solution to the maximum and therefore, we subjected it to sorption after enzymatic degradation.

The lyophilized biomass makes sorption process quick and facilitates the treatment of large volumes of effluents (Tigini et al. 2011). Dried, lyophilized and granular biomass may help to concentrate the pollutants in a small volume of solid material which can easily be eluted or desorbed into small volume of eluant for disposal (Aksu and Cağatay 2006). Bayramoğlu et al. (2006) suggested that physical and chemical modification methods can be used to maximize the dye removal efficiency of

the fungal biomass. They have reported maximum sorption of RB4 at 600 mg l⁻¹ concentration using heat-treated fungal biomass of *Phanerochaete chrysosporium*. Based on this available information heat-treated lyophilized biomass for sorption was used in the present studies.

A comparison of FTIR spectra of unloaded biomass (control) with that of dye-loaded biomass (direct sorption) showing shifts and appearance of new peaks in the region of 1600-700 cm⁻¹ confirmed sorption of the dye on the fungal biomass (Online Resource 1a, 2b). Peaks in the unloaded biomass spectrum represented its functional groups in the form of bending vibrations of C-H (629 cm⁻¹), stretching vibrations of C-OH (1061 cm⁻¹), C-N stretching vibrations (1148 cm⁻¹) and CH₃ deformation (1375 cm⁻¹). CH₂ deformation at 1413 and amino groups stretching vibrations at 1537 cm⁻¹ were observed. The stretching vibrations of C-H bonds were observed at 2924 and 2852 cm⁻¹. The stretching vibration between C=C was seen at 1647 and 1660 cm⁻¹ (Online Resource 2a). FTIR spectrum of the dye loaded biomass (direct sorption) showed significant change in the position of the peaks. The appearance of new peaks at 799 and 1341 cm⁻¹ represented C-H deformation of aromatic compounds (Online Resource 2b). Most of these peaks were absent when the enzyme-treated dye was loaded over the biomass (Online Resource 2c). The appearance of several new peaks such as at 741 cm⁻¹ represented sorption of the degraded components of the dye. In conclusion, the chemical shift and lowering of bands was more prominent in the biomass after step-2 than in the dye directly loaded on the biomass (Online Resource 2b and 2c).

The peaks in the region 1200-900 cm⁻¹ indicate the presence of mycelial bound polysaccharides which play a major role in sorption (Gadd 2009). In the earlier study, the role of EPS present in the biomass preparation of NIOCC #2a in sorption of melanoidin pigments from molasses-based effluents was discussed (Verma et al. 2011).

The UV-Visible and FTIR spectra confirmed that the smaller polar compounds resulting due to enzymatic degradation were better candidates for sorption. Decolorization and degradation of textile dyes by enzymatic process (Osma et al. 2010) and biosorption (Bayramoğlu and Arica 2007) have been demonstrated individually. Our study reports a combination of these two techniques wherein enzymatic breakdown of larger molecules is followed by effective sorption of the resultant degradation products. However, step-2 did not contribute in further reduction of COD. Tigini et al. (2011) and Verma et al. (2011) also reported that biosorption does not help in reducing COD. This might be due to the leakage

of cellular components from the fungal biomass into the liquid phase. An overall 22% reduction of total carbon occurred after the two-step treatment.

3.3 Toxicity assessment

The toxicity of many reactive dyes is known to be enhanced after remediation steps. Reaction intermediates of anthraquinone dyes following photo modification, in particular have been found to be more toxic (Lizama et al. 2002). Biological treatment may also produce more toxic intermediates (Hu 2001; Ali 2010). In order to evaluate the toxicity changes in RB4 solution, the viability of *Artemia nauplii* was assessed. A comparison of LC₅₀ values for untreated and treated samples indicated more than two-fold decrease in toxicity after the two-step treatment of the dye (Table 1). There was no significant change in toxicity in either of the single step treatments. Enzymatic treatment did not result into the complete mineralization of the initial dye but lead to the formation of smaller one or two chain phenolic compounds (Fig. 2) which are known to be toxic. Tsioulpas et al. (2002) noted that phenolics and some of the oxidation products of the laccase reaction in the treated olive mill wastewater were more toxic than the original effluent. In the present study, sorption of such toxic low molecular weight polar compounds in the step-2 enabled a significant reduction in toxicity.

It is also necessary to assess the phytotoxicity of the dye after remediation because dyeing effluents are discharged in agriculture land or into water bodies which are used for agriculture. Therefore, the toxicity to plants was assessed by the seed germination test. Enzyme treatment alone did not reduce phytotoxicity. The decrease in phytotoxicity was significant only after the step 2-treatment (Table 1). Osma et al. (2010) on the other hand reported that the degradation products of a reactive dye Remazol Brilliant Blue R after laccase treatment showed decreased phytotoxicity.

Reactive dyes such as RB4 are highly water soluble, not easily biodegradable and are adsorbed very poorly to biological solids (Vandevivere et al. 1998). In contrast, present results demonstrate that the two step-process, combining enzyme treatment and biosorption yielded a substantial and rapid decolorization, detoxification, reduction in COD and total carbon. In earlier studies using submerged culture of the same fungus at the same concentration of dye, about 80% of the color was removed only by day six (data not shown). In submerged cultures, possibility of growth inhibition by synthetic dyes will hamper the process of decolorization and therefore its use on a large scale is not feasible (Zeng et al. 2011). Here we achieved more than 90% decolorisation in ~12 h, proving the efficiency of the above strategy for the process of rapid remediation on an industrial scale.

4 Conclusion

A two step technique resulted in effective decolorization (93%) and degradation of RB4 accompanied by 47% reduction in COD. In the first step, partially purified laccase from the ligninolytic marine-derived fungus NIOCC #2a was used for degradation. Probable degradation pathways were proposed based on the products formed and identified. In the second step, sorption of the laccase-treated dye over the fungal biomass resulted in a significant reduction in toxicity and total carbon. These findings indicate that the enzymatic oxidation followed by sorption of the degraded products on the fungal biomass can be adopted for rapid decolorization and detoxification of textile dyes.

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Legends to the Figures

Fig. 1 Visible and UV spectra of the Reactive Blue 4; Untreated (———), after direct biosorption (———), after enzymatic treatment (———) and after biosorption following enzyme treatment (———). The insets indicate colors of untreated dye and the dye after each step of treatment

Fig. 2 Mass spectra finger printing of laccase treated Reactive Blue 4 solution at the time intervals of a) 2 h, b) 4 h, c) 6 h, d) 12 h

Fig. 3 The proposed degradation pathways of RB4 by enzymatic treatment (step-1) based on negative ion m/z values observed at different time intervals. The time of appearance of the degradation intermediates/products are indicated within the boxes .

Legends to the Supplementary Figures

Online Resource 1. The Ultra performance liquid chromatograms of; a) Untreated Reactive Blue 4 and at different intervals of enzymatic treatment; b) 0 h, c) 2 h, d) 4 h, e) 6 h, f) 12 h Increasing numbers of peaks at lower retention time are marked with broken line boxes

Online Resource 2 FTIR spectra of a) unloaded fungal biomass, b) dye-loaded fungal biomass, c) enzyme-treated dye, loaded on the fungal biomass (step-2)

Table 1 Toxicity analysis of Reactive Blue 4, before and after treatment.

Toxicity tests	Control (Distilled water)	Untreated	Direct Sorption	Enzyme treated (Step-1)	Sorption following enzyme treatment (Step-2)	
<i>Artemia</i> test (24 h-LC ₅₀) [#]	--	48 (43-95) ^a	57 (48-68) ^a	51 (45-59) ^a	99 (79-135) ^b	
Phytotoxicity (48h) ^{\$}	Germination index (%)	--	57.6 (0.4) ^a	63.9 (1.7) ^{ab}	66.9 (1.7) ^b	91.4 (4.5) ^c
	Dry weight in g (root)	2.6 (0.1) ^a	0.6 (0.03) ^b	1.0 (0.02) ^c	1.0 (0.0) ^c	1.8 (0.04) ^d

[#] LC₅₀ values are in $\mu\text{l ml}^{-1}$. Upper and lower confidence limits are within brackets.

^{\$} Standard deviation values are within brackets.

Different letters indicate significant differences between the methods of treatment for a particular test (Tukey's test at the level $\leq 0.05\%$).

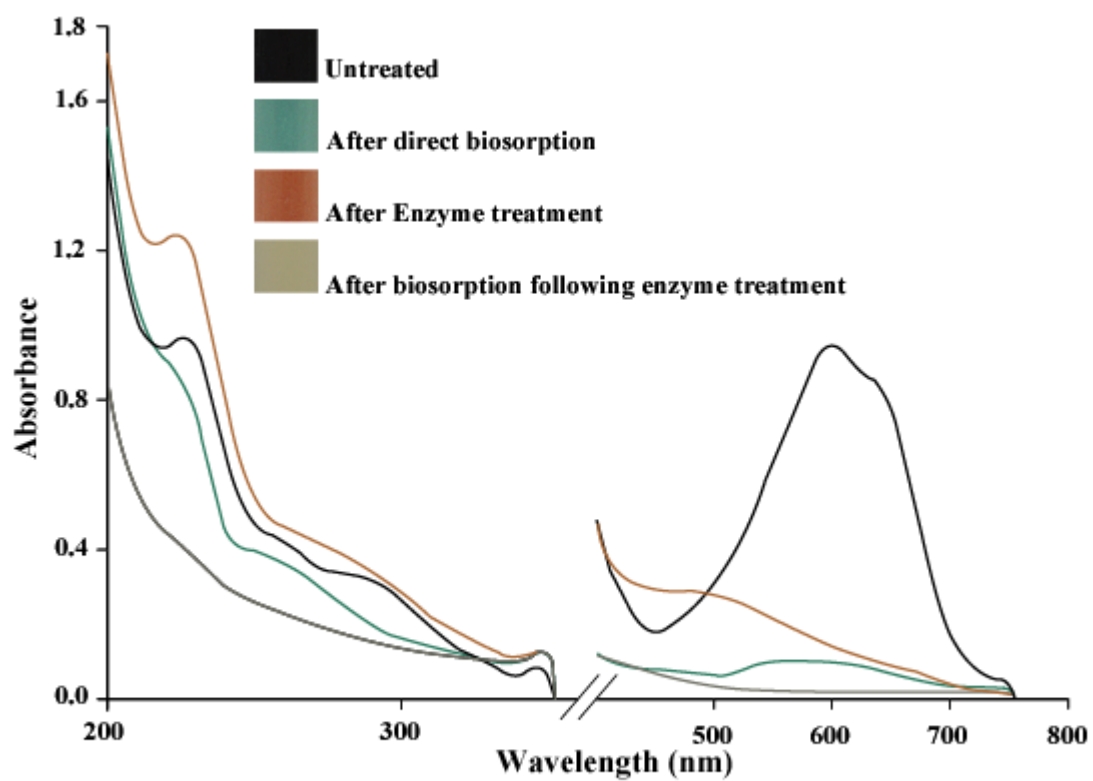


Fig. 1

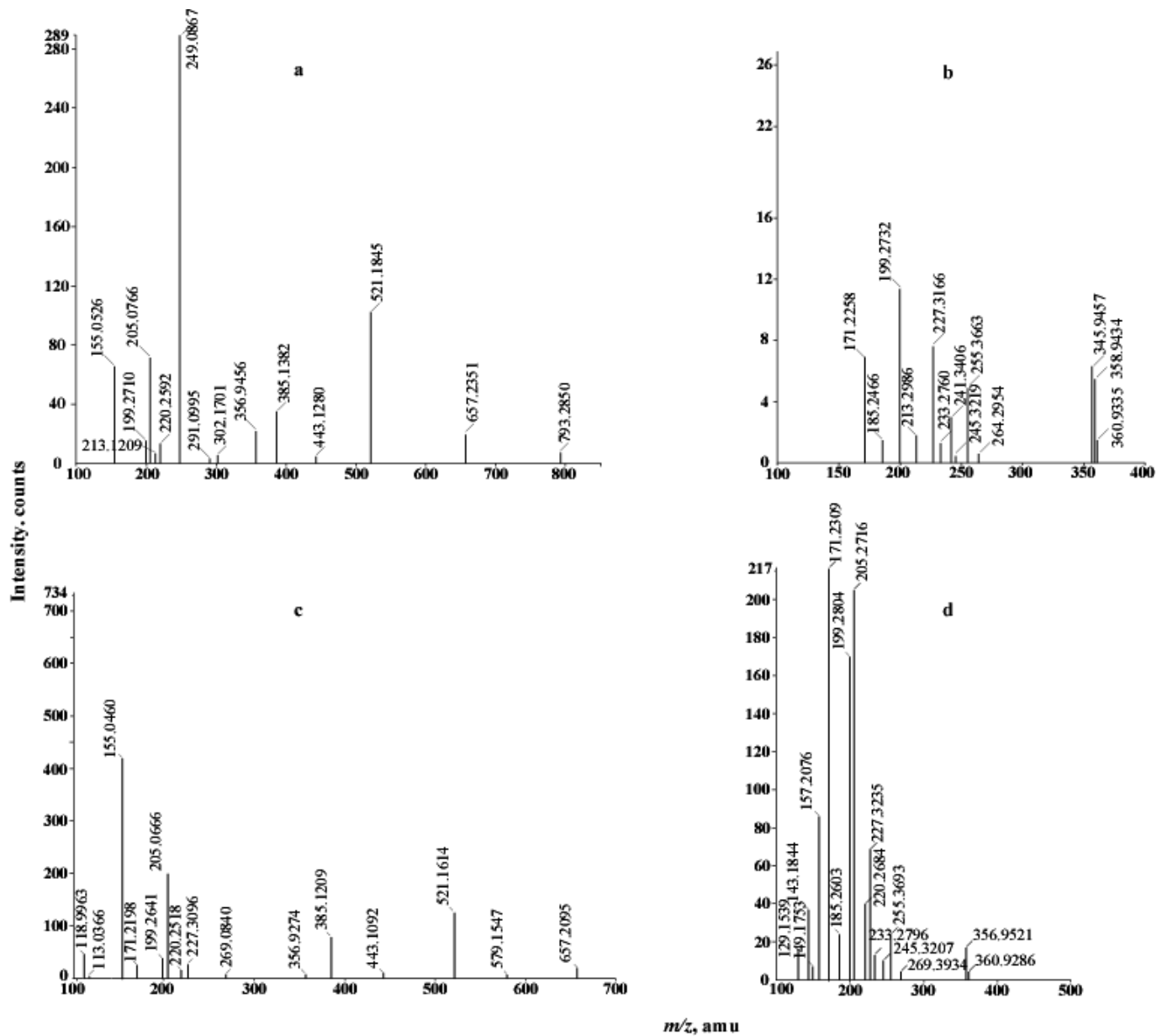


Fig. 2

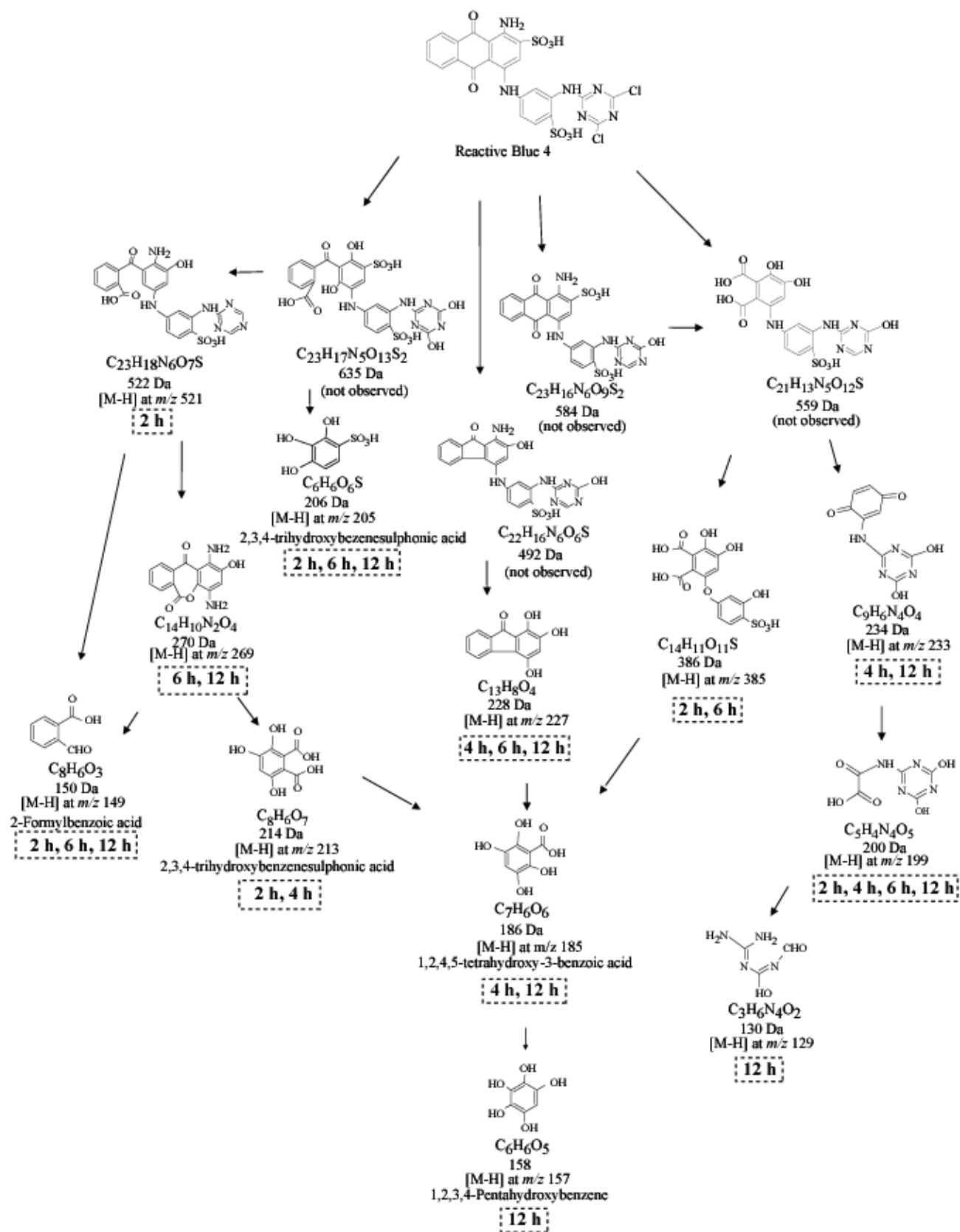
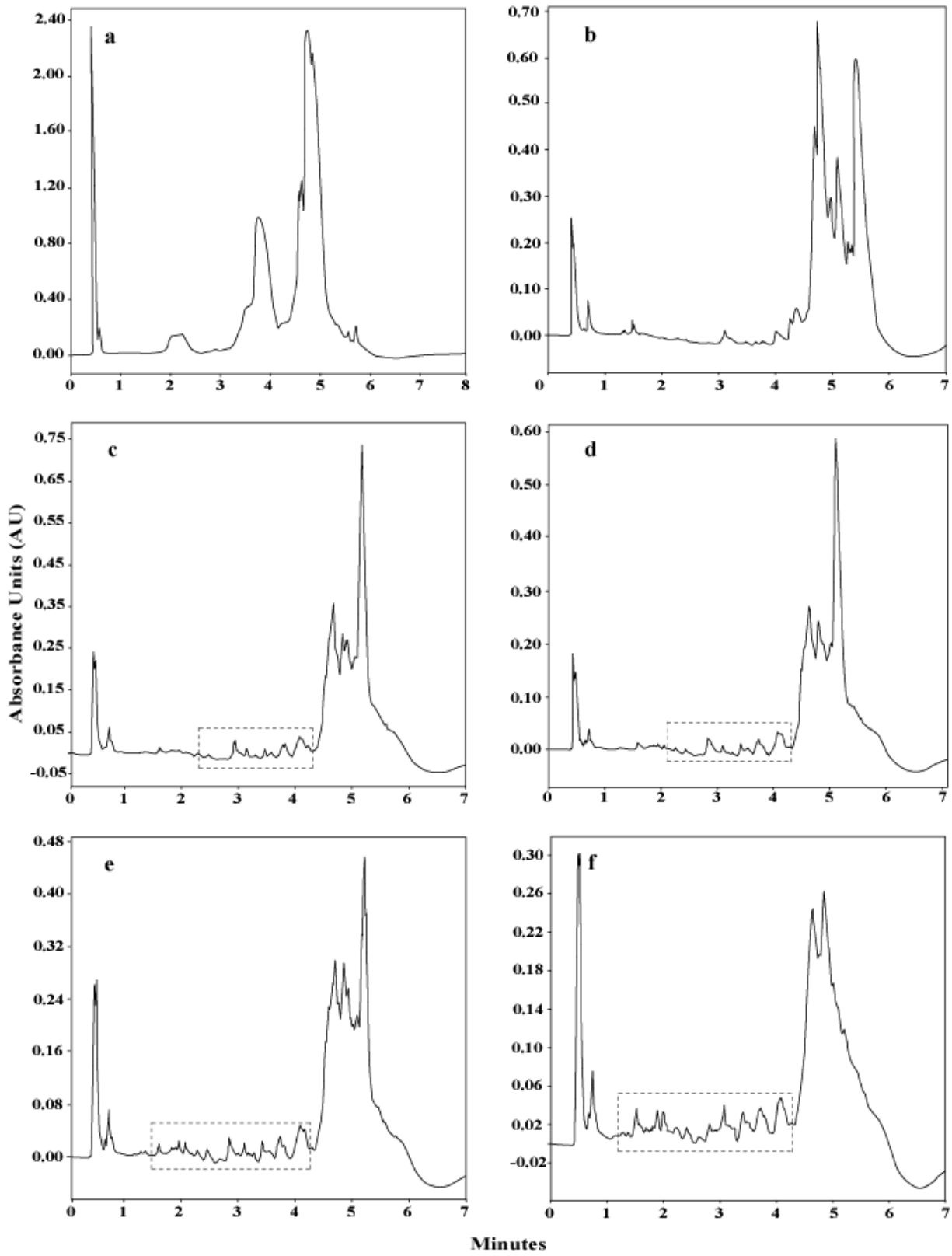
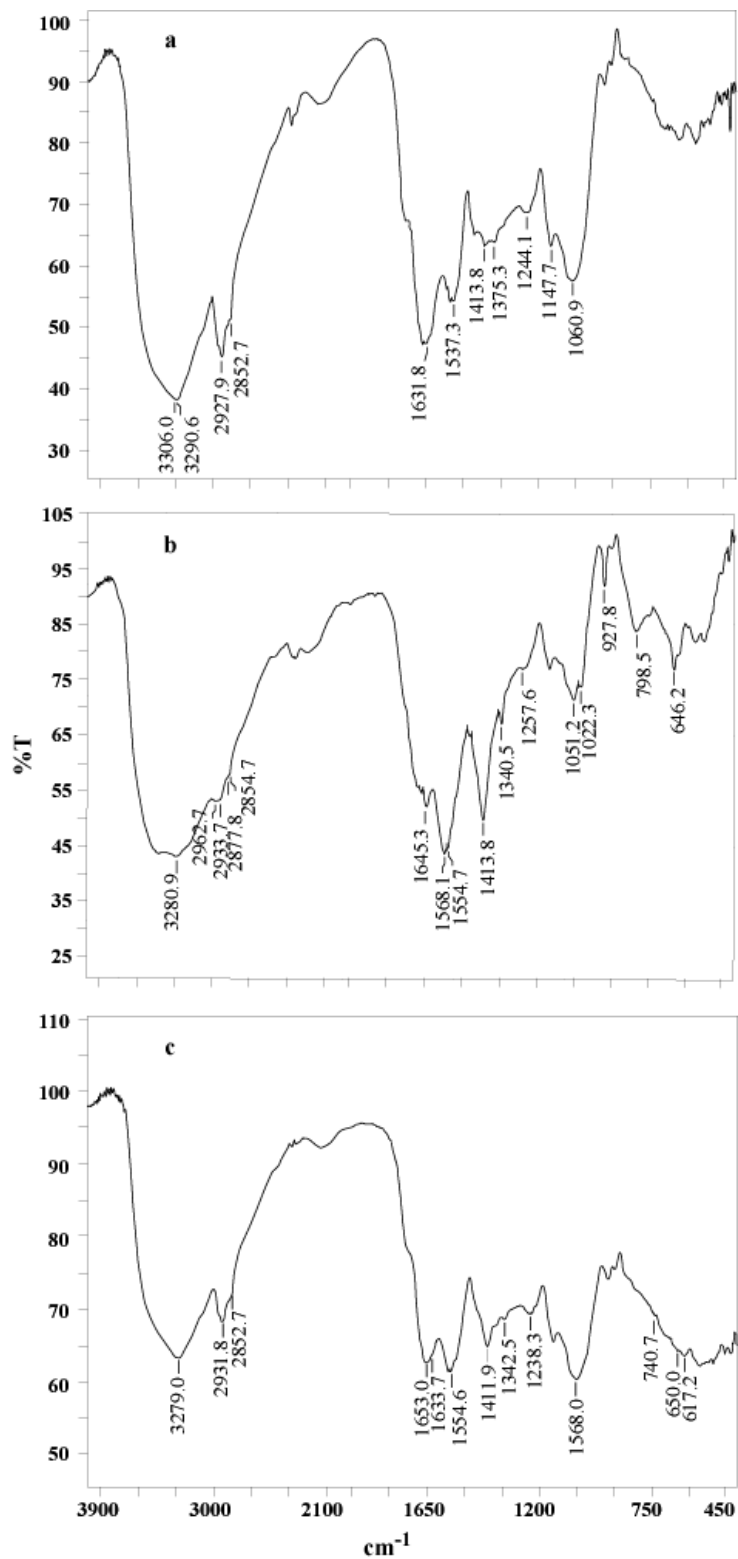


Fig. 3



ESM_1



ESM_2