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Biotransformation and detoxification of a greater tinctorial textile colorant using an isolated bacterial strain

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Abstract

The acclimated cells of *Achromobacter xylosoxidans* strain APZ wereemployed to decolorize a textile colorant, Reactive green 19A (RG19A) of greater tinctorial strength.The strain decolorized and simultaneously mineralized RG19Awith a high average decolorization rate of 208.33 µgmin^{-1} under normal operating (pH 7.0 and 35°C) and static conditions. The spectral and chromatographic data described that the decolorization was due to biodegradation facilitated by the extracellular oxidoreductases secreted by *A. xylosoxidans* strain APZ. The extent of RG19A mineralization during biotransformation was monitored by measuring the percentage reduction in the concentration of chemical oxygen demand and released aromatic amines. Plant growth parameters of seedlings of *Phaseolus mungo, Triticum aestivum* and *Sorghum bicolor* seedlings were assessed to monitor toxicity reduction.

Key words

Achromobacter xylosoxidans, Aromatic amines, Detoxification, Mineralization

Introduction

Discharge of textile waste water into river streams distorts the water quality to a larger extent and especially, the azo dyes and effluent are responsible for dual pollution; firstly due to their strong color index that causes aesthetic problems and secondly, obstruction of sunlight penetration and oxygen transfer into water bodies (Pinheiro *et al*., 2004). Dyes are highly recalcitrant in nature and are chemical mutagens that are toxic to the biotic components of the ecosystem (Martins *et al*., 2002; Sponza and Işik, 2005). The azo bond undergoes reductive cleavage to form carcinogenic intermediates (aromatic amines) and induces mutation in the biological systems.

The conventional physico-chemical treatments decolorize the effluent but fail to detoxify them; thus the situation demands the necessity to eliminate the color and toxicity of these carcinogenic intermediates (Karthikeyan *et al.,*2014; Vidhyadevi *et al*., 2014; Madhu *et al.,* 2009). Biological systems as a whole or enzyme formulations are known to be better option in all the fields related to biochemical engineering (Vinodhini *et al.,* 2015;Seenuvasan *et al*., 2014a,b,c,d; Seenuvasan *et al*., 2013a,b).Thus, dye bio-

degradation using microorganisms (fungi, bacteria) and enzymes show consistent performance to biotransform and detoxify the reactive dyes (Palanivelan *et al*., 2014; Biyik *et al.,* 2012). Bacterial degradation of azo dyes under aerobic and anaerobic mode can be achieved by inducing either pure or consortial forms, which renders good performance in mineralizing the aromatic amines released during reductive cleavage of azo bonds (Balaji *et al*., 2014; Prasad and Rao, 2014; Laowansiri *et al.,*2008; Kapdan *et al.,* 2003). Thus, microbial decolorization by biodegradation with concomitant detoxification is an environmentally reliable technique alternate to the conventional decomposition techniques (Selvanaveen *et al*., 2015; Dayaman and Dasgupta, 2008; Ambrósio and Takaki, 2004).

This paper reports the ability of *A. xylosoxidans* strain APZ in catalyzing the degradation and detoxification of a textile dye RG19A. The mineralization of RG19A facilitated by the various extracellular oxido-reductases were studied. RG19A biotransformation was revealed using the spectral and chromatographic techniques and phyto-toxicological assessments were done to determine the extent of detoxification.

Materials and Methods

Isolation and identification of RG19A acclimated cells : A mixed bacterial culture, previously reported to degrade and mineralize an azo dye; acid red 88 (Kumar *et al.,* 2015) was used as seed culture for isolating an acclimated strain. The culture was acclimated to RG19A in Bushnell-Haas medium (BHM) containing RG19A (100-500 mgl^{-1}) with a simultaneous decreas in glucose concentration (1.0 to 0.1 g l^{-1}) incubated at 37°C under static condition. The cells which were able to survive 300 mgl^{-1}RG19A in the absence of glucose were considered as acclimated cells. The isolate was identified using 16S rRNA gene sequencing, and the culture was maintained as glycerol stock preserved at −80°C.

RG19A biotransformation experiments : The ability of cells to decolorize RG19A was carried out by inoculating 1.0 ml of fully grown cells of the strain in an Erlenmeyer flask containing 100 ml of 100 mgl^{-1} RG19A in BHM under static condition. After complete decolorization, the biotransformed products were recovered following the method of Kumar *et al.* (2015).

Analytical methods : An aliquot of culture media was withdrawn after decolorization and centrifuged at 10,000 rpm for 15 min to separate the biomass. Decolorization was quantitatively analyzed and compared with abiotic control (without microorganism) in Shimadzu UV–1800 spectrophotometer (Tokyo, Japan) in the range 300–900 nm. The average decolorization rate, ADR_{RG19A} ($\mu g\ min^{-1}$) and percentage RG19A decolorization (%D) for RG19A concentration, C (mgl^{-1}) after decolorization time t (min) were calculated by the following formula given below;

$$ADR_{RG19A}(\mu g\ min^{-1}) = \frac{C \times \%D \times 1000}{100 \times t} \quad (1)$$

$$\%D = \frac{\text{Initial absorbance-final absorbance}}{\text{Initial absorbance}} \quad (2)$$

Characterization of biotransformed products : The initial RG19A and decolorized solutions were scanned in a UV-vis spectrophotometer to monitor the changes in the absorbance peak. The functional groups in RG19A and products were characterized in the mid-IR region (400–4000 cm^{-1}) using Perkin Elmer 237B Infrared spectrometer. HPLC profile were recorded using Shimadzu, Japan using HPLC grade methanol as mobile phase. The analytes (RG19A and products) were detected at 640 and 325 nm while maintaining the flow rate at 1.0 ml min^{-1} with 10 min run time in C_{18} column (symmetry, 4.6 × 250 mm). TLC analysis were carried out by dissolving the analytes in methanol: ethyl acetate: *n*-propanol: water: acetic acid (1:2:3:1:0.2, v/v) and were spotted on Aluchrosep Silica Gel 60/UV_{254} supported on aluminum sheets, SDFCL, Mumbai. GC–MS analysis was carried on Agilent 5975C Shimadzu; Japan equipped with an HP–5 capillary column. Helium was used as a carrier gas at a flow rate 1.0 ml min^{-1} for 30 min run time.

Assays for elucidating the activities of oxido-reductases : The cells were grown in BHM in the presence and absence (control) of RG19A for 8 hrs, and centrifuged at 10,000 rpm for 30 min at 4°C. The resulting supernatant was used as source for assaying activities of lignin peroxidases (Shanmugam *et al.,* 1999), veratryl alcohol oxidase (Bourbonnais and Paice, 1988), azo-reductase (Maier *et al.,* 2004), tyrosinase (Zhang and Flurkey, 1997) and laccase (Kumar *et al.,* 2012b) activity spectrophotometrically. The reference blanks contained all the components, except the assayed enzyme solution. All enzyme assays were carried out at room temperature in triplicate.

RG19A mineralization and detoxification analyses : The percentage reduction in the chemical oxygen demand and

liberated aromatic amines during oxidoreductive breakdown of RG19A was measured to estimate the mineralization (Kumar *et al*., 2015). The detoxification analyses were done using phytotoxicity assays by supplying 5 ml of 500 mg l^{-1} RG19A and products to seeds of *P. mungo*, *T. aestivum* and *S. bicolor* under controlled environment. The control sets were also included using distilled water and after 7 days of treatment, the percentage germination, length of plumule and radicle, along with the chlorophyll content (Hiscox and Israelstam, 1979; Arnon, 1949) of the seedlings were reported.

Chemicals : RG19A ($C_{40}H_{23}Cl_2N_{15}Na_6O_{19}S_6$, molecular weight, MW =1418.94and maximum wavelength, λ_{max}=640 nm),2,2'–azino–bis(3–ethyl–benzothiazoline–6 sulfonic acid, ABTS), p-chloranil, aniline-2-sulfonic acid and HPLC grade methanol were obtained from Sigma Aldrich (Bangalore, India) with the highest purity and were of analytical grade. RG19A stock solutions were prepared using double distilled water and stored in dark condition at room temperature.

Results and Discussion

Bacterial cells provide a viable and an inexpensive tool in aiding bioremediation of industrial effluent (Selvanaveen *et al.,* 2015; Kumar *et al.,* 2015,2014; Syed *et al.,*2009). The cells acclimated to RG19A Bushnelt completely decolorized 500 mg l^{-1} RG19A in the absence of glucose in the Bushnell-Haas medium (BHM). The acclimated strain was identified as *Achromobacter xylosoxidans* strain APZ using 16SrRNA gene sequencing and their phylogenetic relationship was studied (Figure not shown). The strain exhibited an ADR_{RG19A} value of 208.33 µgmin^{-1} when inoculated with 100 mg l^{-1} RG19A for 8 hrs under the static condition at pH 7.0 and 37°C. Decolorization was due to the bacterial action and not due to variation in pH (Kumar *et al.,* 2012a).

The UV-vis spectral profile displayed a decreased absorbance for the products when compared with RG19A; this explains that cells of *A. xylosoxidans* strain APZ catalyzed the degradationof RG19A. The spectrum displayed peaks at 640 and 325 nm for initial RG19A and products, illustrating that RG19A decolorization was due to biodegradation (Figure not shown).

FT-IR spectra of RG19A displayed peaks at different positions in the fingerprint region (1500-500 cm^{-1}), the peaks at 3434 and 3758 cm^{-1} for NH stretching vibrations describe ssecondary amines and 2098 cm^{-1} for NH_3 stretching for amino amide. A peak at 1583 cm^{-1} represented the N=N stretch of azo bond and 1280 cm^{-1} confirm the azoic nature of RG19A. A peak at 1487 cm^{-1}showed N=O stretching vibrations and values at 1418 and 1280 cm^{-1} showed OH deformation and C-N vibration of amines. A peak at 1179 cm^{-1} represents the aromatic N-CH_3 stretching vibrations. A peak at 1134 cm^{-1} describes the asymmetric C-OH stretching vibrations. A peak at 1134, 1045, 1078 and 841 cm^{-1} for S=O stretching vibrations confirmed the presence of sulfones and sulfoxide (sulfur containing nature of the benzene ring). The bands below 900 cm^{-1} indicated the aromatic nature of RG19A, the spectra had peaks at 912, 713 and 635 cm^{-1} for C-H and C-S stretching of vinyl (C=CH_2), aromatic (para-disubstituted benzene ring) and aromatic (mono-substituted benzene ring). A weak to medium bond at 547 cm^{-1}represented the C-X stretching of chloro–alkanes. A peak value of 2366 cm^{-1} represented the NH stretching of amines as shown in Fig.1(a).

FT–IR spectra of the products exhibited new peaks at 3902, 3852 and 3750 cm^{-1}representing weak to medium appearance of N-H stretching of secondary amines. A peak at 3435 cm^{-1} represent NH overtone and formation of amides. A broad peak at 2623 cm^{-1} for N-H stretching of ammonium ions and 2345 cm^{-1} represented the presence of charged amines. CN and C=O stretching vibrations were found at 1934 and 1653 $cm^{-1}$for carboxylic acid associated with amides. A peak 1653 cm^{-1}showed CH bending vibrations with asymmetric deformation of alkanes and formation of amides. A Peak at 1653 cm^{-1} represented the formation of benzene ring with C=O stretching and explained the reduction of azo bond. The absence of peak at 1583 cm^{-1} illustrated the breakdown of primary and secondary nitro compounds. The formation of nitro compounds was represented by a peak at 1569 cm^{-1}. A peak 1137cm^{-1} described C-O stretch as in case of secondary and tertiary alcohols. A peak 1213 cm^{-1} corresponds to C-O stretching of phenols, NO_2 stretch and OH deformation from primary to secondary alcohols. A weak to strong bond peak value 1413 cm^{-1} supported by 977 cm^{-1} represents N=O stretching vibrations, and CH deformation. A peak at 628 cm^{-1} represented C-H stretching vibrations, and disappearance of 1280 and 1583 cm^{-1}represented the breakdown of N=N and loss of azoic nature, respectively. A peak at 2367 cm^{-1} represented the NH stretch ofsecondary amines supported by peaks at 3852 and 3902 cm^{-1}. The absence of peak at 1487

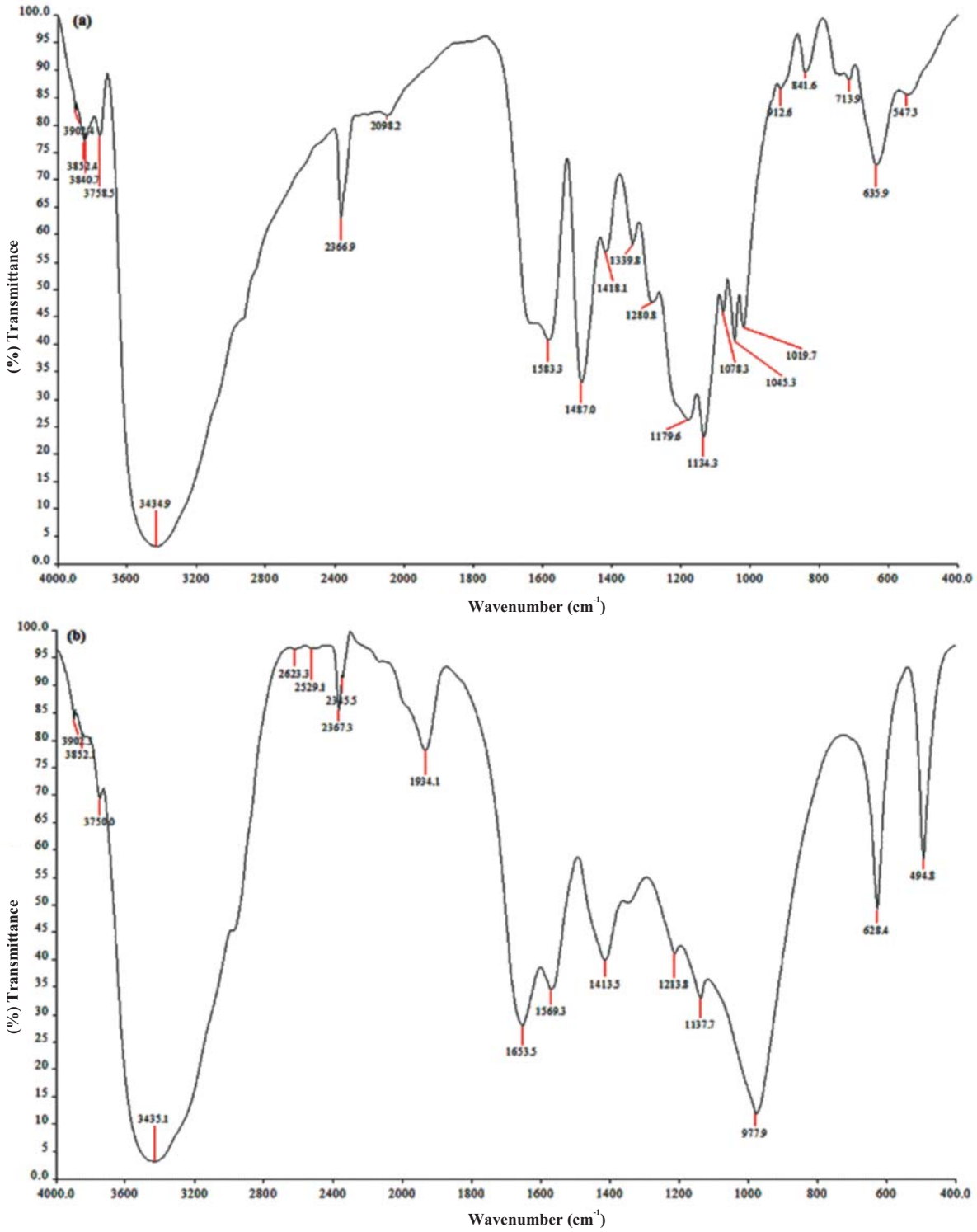


Fig.1 : FT-IR spectra showing different functional peaks and respective shifts for (a)initial RG19A and (b) biotransformed products

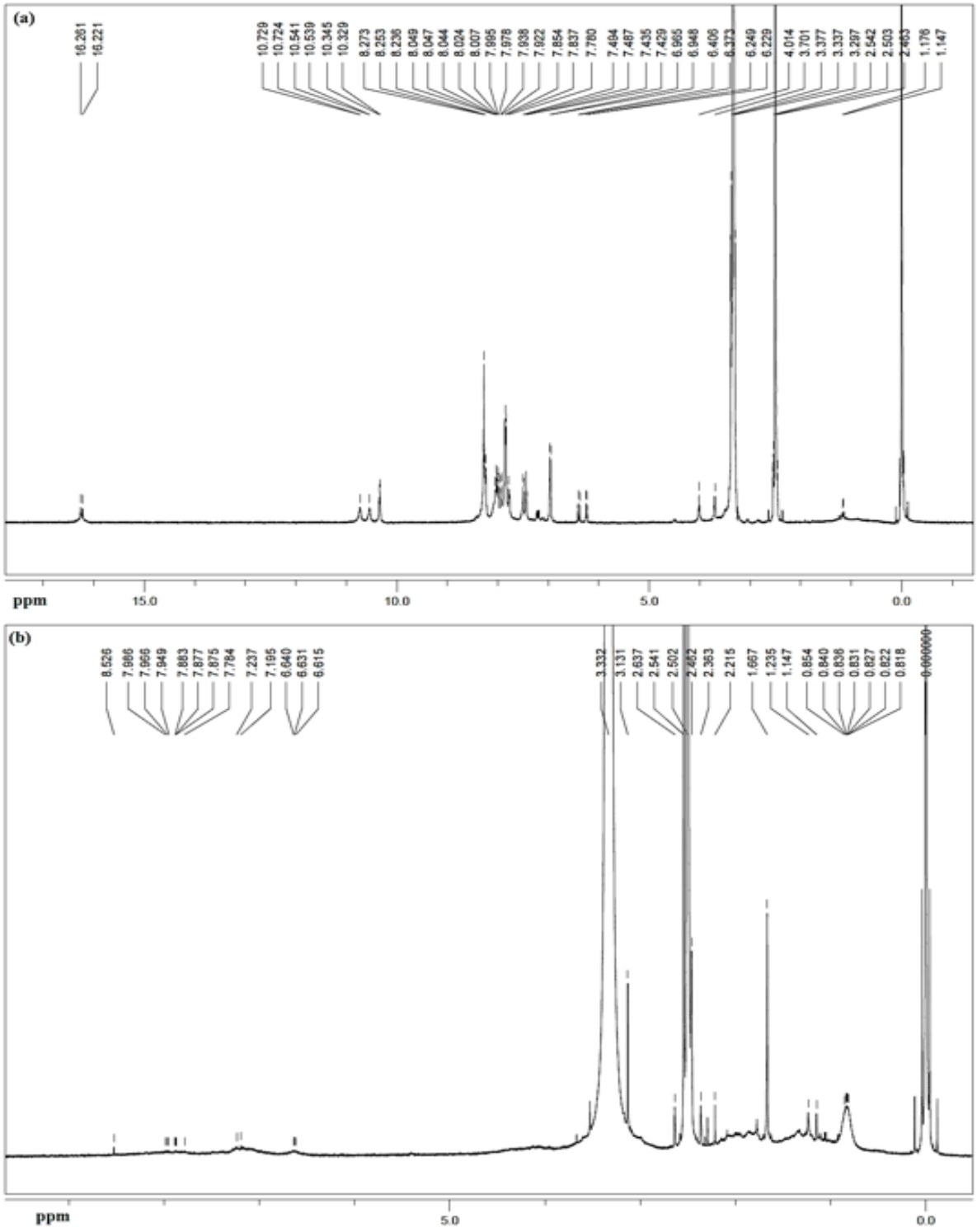


Fig.2 : ^{1}H-NMR spectra showing signals and peak shifts in (a) initial RG19A and (b) biotransformed products

cm^{-1} for ring vibration represented RG19A degradation (Fig.1b).

^{1}H-NMR spectra of RG19A showed peaks between 7.43 and 8.27 ppm representing protons of two aromatic rings. A peak at 7.99 ppm refered to the aromatic protons meta to dimethylamino group. The cluster of peaks observed in between 7.78 to 8.273 ppm were due to the aromatic protons present in the ring carrying sulfone group. Signals between 2.46 to 4.01 ppm were due to methylene and methyl protons as shown in Fig. 2(a).

^{1}H-NMR spectra of the products showed peaks between 2.21 to 2.64 ppm indicating the presence of methyl protons and CH_3-C=O functional moiety. Weak signals for aromatic protons in between 6.0 to 9.0 ppm indicate partial degradation of RG19A. The spectra also had peak between 0.82 and 2.637 ppm in the high field region indicating formation of aliphatic hydrocarbons of lower molecular weight as shown in Fig. 2(b).

HPLC elution profile of RG19A and products were compared and it was found that RG19A displayed a major

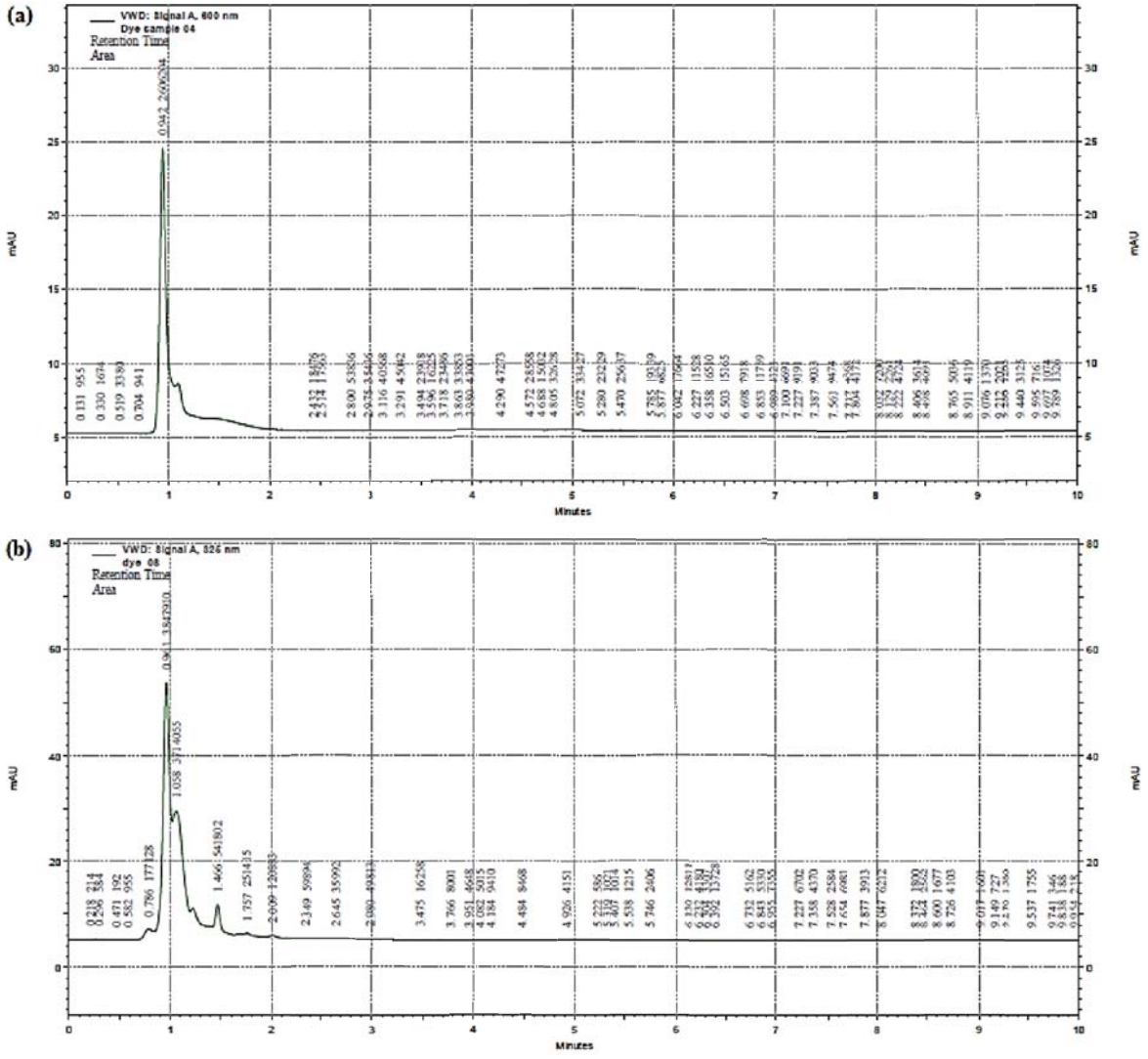
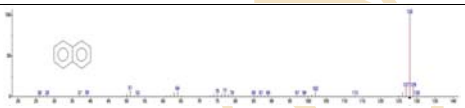
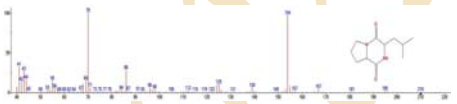
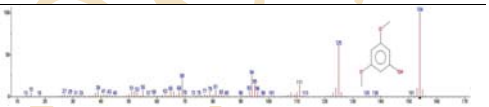
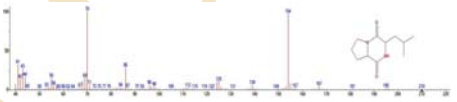
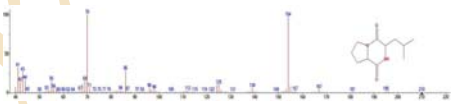
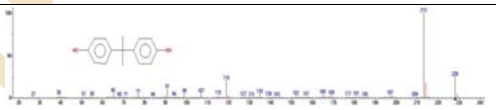
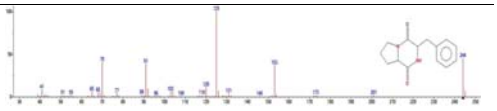


Fig.3 : HPLC elution profiles of (a) RG19A and (b) degradation products at different retention time

Table 1 : Mass spectral information of the biotransformed products obtained after RG19A degradation by A. xylosoxidans strain APZ

S.No.	Rt (min)	Area (%)	Molecular formula	MW (m/z)	Name of the products	Mass peak
1	8.859	8.67	$C_{10}H_8$	128	Naphthalene	
2	17.402	5.54	$C_{11}H_{18}N_2O_2$	210	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro -3-(2-methylpropyl)	
3	17.714	5.67	$C_8H_{10}O_3$	154	3,5-dimethoxyphenol	
4	18.650	4.43	$C_{11}H_{18}N_2O_2$	210	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro -3-(2-methylpropyl)	
5	18.754	1.24	$C_{11}H_{18}N_2O_2$	210	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro -3-(2-methylpropyl)	
6	21.153	68.76	$C_{15}H_{16}O_2$	228	4,4'-(1-methylethylidene) bisphenol	
7	22.669	1.49	$C_{14}H_{16}N_2O_2$	244	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro -3-	

peak at retention time (R_t) 0.942 min, whereas the products had peaks at (R_t) 0.786, 0.961, 1.058 and 1.466 min as shown in Fig.3.

TLC sheet had a spot for RG19A and the relative retardation factor (*Rf*) was 0.91, whereas *Rf* value of the products were 0.37 and 0.59, respectively. The formation of new peaks in HPLC profile and spots in TLC sheet explained RG19A biotransformation by *A. xylosoxidans* strain APZ. The compounds from the mass spectral profile were identified by standards available in NIST library for the products of RG19A biotransformation, explaining the nature of the compound produced by *A. xylosoxidans* strain APZ (Table 1).

The compound pyrrolo [1, 2-a] pyrazine-1, 4-dione, hexahydro-3-(2-methylpropyl) ($C_{11}H_{18}N_2O_2$; MW=210) eluted thrice at different R_t (17.402, 18.650 and 18.754 min). The compound pyrrolo [1, 2-a] pyrazine-1, 4-dione, hexahydro-3-(phenylmethyl) ($C_{14}H_{16}N_2O_2$; MW=244) eluted

Table 2 : Activity of oxidoreductases during RG19A biodegradation by *A. xylosoxidans* strain APZ

Enzyme assay	Induction	
	Control (0 h)	After decolorization (8 h)
Laccase[a]	0.11 ± 0.072	0.587 ± 0.072
LiP[a]	ND	0.28 ± 0.05
Tyrosinase[a]	ND	0.13 ± 0.02
VAO[a]	ND	0.107 ± 0.03
Azoreductase[b]	0.019 ± 0.023	0.905 ± 0.112

ND not detected, [a]Activity in unitsmin^{-1}mg protein^{-1}, [b]mM of methyl red reducedmin^{-1}mg protein^{-1}

Table 3 : Phytotoxicity assessment of RG19A and their biotransformed products against seedlings of *P. mungo, T. aestivum* and *S. bicolor*

No.	Treatment solution	GP (%)	Plumule length (cm)	Radicle length (cm)	Chlorophyll content (mgg^{-1} tissue)
			P. mungo		
1	Control	90	9.45 ± 0.67	13.55 ± 1.23	0.075
	RG19A	80	6.40 ± 0.37	7.30 ± 0.48	0.020
	Biotransformed products	90	7.80± 0.22	9.40 ± 1.19	0.057
			T. aestivum		
2	Control	90	8.60 ± 0.54	6.35 ± 1.10	0.015
	RG19A	70	4.40 ± 0.24	0.03 ± 0.01	0.009
	Biotransformed products	90	6.70 ± 0.63	1.12 ± 0.12	0.011
			S. bicolor		
3	Control	100	11.85 ± 0.21	6.15 ± 0.66	0.027
	RG19A	80	4.60 ± 0.23	1.27 ± 0.11	0.001
	Biotransformed products	100	8.90 ± 0.78	4.37 ± 0.27	0.012

at 22.669 min. The above said two compounds are poly unsaturated fatty acids (PUFA) possessing antibacterial property (Melo *et al.,*2014). Naphthalene (MW=128) was the final product to elute out at 8.859 min that was also observed during RG19A degradation using *Micrococcus glutamicus* NCIM2168 (Saratale *et al.,*2009) and Navy Blue HE2R using *Exiguobacterium* sp. RD3 (Dhanve *et al.,*2008).

The values of COD and aromatic amine reduction were 89 and 73%, respectively, for 100 mg l^{-1} RG19A which was considered as parameter to evaluate mineralization by the cells of A. *xylosoxidans* strain APZ. It is evident that cells consume intermediates as nutrients during biotransformation (Saratale et al., 2009). Elimination of S=O groups may be achieved by assimilation of sulfates for synthesis of cellular material required by *A. xylosoxidans* strain APZ. The azoic and sulfonic groups initially present in RG19A were not found in the FT–IR spectra of the products. The sulfonic acid derivatives were also absent in the mass spectra of products, which is an indication that the strain had mineralized RG19A to a greater extent.

Parshetti *et al.* (2010) demonstrated the formation of sulfonated aromatic amines during methyl orange degradation by *Kocuria rosea* MTCC 1532 that was supported by a peak at 1045 cm^{-1} for S=O stretching vibration in the products. This describes that the cells of *K. rosea* MTCC 1532 did not eliminate the sulfone group completely. Similarly, Saratale *et al.*(2009) demonstrated the degradation of RG19A using *Micrococcus glutamicus* NCIM 2168 and identified two intermediates; 2-aminobenzene sulfonic acid with 2-chloro-4, 6 diamine-(1, 3, 5) triazine-3-amino benzene sulfonic acid) and 3, 4, 6-triamino-5-hydroxy naphthalene-2,7-disulfonic acid. These two intermediates contained sulfone derivatives; but in the present investigation the cells of *A. xylosoxidans* strain APZ eliminated the sulfone groups. The FT-IR spectra of biotransformed products as shown in Fig. 3(b) did not have any specific peak value supporting the presence of sulfonic group, as well in the mass spectra as shown in Table 1. Thus, from the economical as

well as environmental point of view, A. *xylosoxidans* strain APZ could be a best a reliable catalyst for degrading and mineralizing sulfonated textile colorants.

The activities of extracellular oxido reductases of *A. xylosoxidans* strain APZ were elucidated to understand the cleavage of naphthalenyl azo ring of RG19A (Table 2). The ring cleavage is mediated by generation of free radicals which leads to spontaneous cleavage (Saratale *et al.,* 2011).

The reductive cleavage of N=N bond is initiated by azoreductase to release aromatic amines (Chang *et al.,* 2001; Chang *et al.,* 2000). LiP helps in oxidation of phenolic groups which is further attacked by water molecules to produce phenyl diazine and finally oxidizes to generate nitrogen (Chivukula and Renganathan, 1995). The conversion of *o*-phenols to *o*-quinone is catalyzed by tyrosinase (Kalme *et al.,* 2009). Conclusively, the reductase and oxidases were required for degradation and mineralization of RG19A by A. *xylosoxidans* strain APZ.

The result of phytotoxicity assay is tabulated in Table 3 and it was found that the seeds when treated with RG19A exhibited low germination percentage when compared with the seeds treated with control and biotransformed products.

The length of radicle and plumule, as well as total chlorophyll content was comparatively lesser in case of seeds treated with RG19A. The lengthening of plumule and radicle in the case of the products may be attributed to the reason that RG19A had been mineralized. The results of the phytotoxicity assessment indicate that RG19A was toxic but their products of biotransformation were of reduced toxic nature (Kumar *et al.*, 2016).

RG19A biotransformation was catalyzed by oxidoreductases induced by *A. xylosoxidans* strain APZ. The products of biotransformation were analyzed using spectral and chromatographic techniques. The phytotoxicity assays, confirmed the non-toxic nature of mineralized products. Furthermore, *A. xylosoxidans* strain APZ eliminated the sulfone from RG19A which is of greater importance to the industries.

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