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ORIGINAL PAPER

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Biodegradation of acid blue-15, a textile dye, by an up-flow immobilized cell bioreactor

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Abstract Acid blue-15, a complex and resonance-stabilized triphenylmethane (TPM) textile dye, resistant to transformation, was decolorized/degraded in an up-flow immobilized cell bioreactor. A consortium comprised of isolates belonging to *Bacillus* sp., *Alcaligenes* sp. and *Aeromonas* sp. formed a multispecies biofilm on refractory brick pieces used as support material. The TPM dye was degraded to simple metabolic intermediates in the bioreactor with 94% decolorization at a flow rate of 4 ml h^{-1}.

Keywords Triphenylmethane dye · Biodegradation · Decolorization · Immobilized cell bioreactor · Biofilm

Introduction

The triphenylmethane (TPM) group of textile dyes has a complex aromatic molecular structure which resists degradation in the environment, resulting in direct and indirect exposure of the human population to high concentrations of these dyes. Many reports are available in the literature on the toxic and carcinogenic potential of different TPM dyes. Gentian violet is reported to be a thyroid and liver carcinogen [5]. Similarly, pararosaniline has the potential to cause urinary bladder cancer in humans [3]. Based on their potential for adverse human health effects, the United States Food and Drug Administration listed several TPM dyes as priority chemicals for carcinogenicity testing by the National Toxicology Program in 1993 [3]. Studies of the biodegradation of TPM dyes, like crystal violet, malachite green and pararosaniline, focused primarily on the decolorization of dyes via reduction reactions [1, 2, 6, 7, 10]. The TPM dyes used in industry (acid violet-17, acid violet-49, acid blue-15, etc.) are more complex, due to the presence of additional phenyl rings in their molecular structure. These resonance-stabilized ring structures impart intense color to fabrics but make the dyes highly recalcitrant and less susceptible to microbial attack. Textile mill effluent (TME) is a complex mixture, having high concentrations of suspended solids, metals, chemical surfactants and dyes. There are reports that, on average, 16.8% of textile dyes are lost in effluents [9]. The discharge of large volumes of colored untreated TME into the environment is aesthetically displeasing, impedes light penetration, damages the quality of the receiving streams and is toxic to flora and fauna [12].

Available effluent treatment processes focus on the removal of dyes by physical and chemical methods, thus leading to the generation of highly toxic sludge/solid wastes that require special disposal techniques, as per existing laws. The focus is now shifting to the development of environment-friendly biological treatment systems. In wastewater treatment, systems based on biofilms are successful due to their ability to work at different dilution rates and to sustain shock loading. Such systems have been developed and tested successfully for treating TME-containing azo dyes [8, 15]. However, there are no such reports available for the treatment of effluents containing TPM dyes. The present study is part of an effort to develop a treatment system based on immobilized cells for the treatment of effluents containing acid blue-15 (AB-15), a resonance-stabilized TPM dye, commonly used in the textile industry of this region.

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Materials and methods

Chemicals

The AB-15 dye used (Fig. 1), was a generous gift from Punjab Rang Udyog, Amritsar (Punjab, India). The medium components and other chemicals used were of analytical grade.

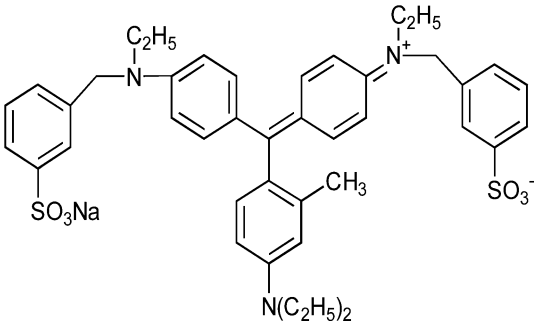

Fig. 1 Chemical structure of acid blue-15 (AB-15; CI 42645)

Medium

Mineral salts medium (MSM) contained (per liter): 3.6 g Na_2HPO_4, 1.0 g $(NH_4)_2SO_4$, 1.0 g KH_2PO_4, 1.0 g $MgSO_4$, 0.01 g $Fe(NH_4)$ citrate, 0.1 g $CaCl_2{\cdot}2H_2O$ and 10.0 ml of trace element solution. The trace element solution had the composition (per liter): 10 mg $ZnSO_4{\cdot}7H_2O$, 3.0 mg $MnCl_2{\cdot}4H_2O$, 1.0 mg $CoCl_2{\cdot}6H_2O$, 2.0 mg $NiCl_2{\cdot}6H_2O$, 3.0 mg $Na_2MoO_4{\cdot}2H_2O$, 30.0 mg H_3BO_3 and 1.0 mg $CuCl_2{\cdot}2H_2O$. The final pH of the medium was adjusted to 7.0. Stock solutions of 50% (w/v) glucose and 25% (w/v) yeast extract (HiMedia Laboratories, Mumbai, India) were sterilized separately and were added to MSM to final concentrations of 5.6 mM glucose and 0.25% (w/v) yeast extract. MSM was used in all studies unless stated otherwise.

Enrichment and isolation of dye-decolorizing microorganisms

Soil and sludge samples collected from the waste disposal sites of textile dyeing industries were enriched on MSM. The sterilized MSM containing 10 mg l^{-1} of AB-15 dye was inoculated with soil/sludge samples (10% w/v) and incubated at 30 ± 1 °C at 100 rpm. Plating of enriched populations was carried out on MSM/agar plates containing 10 mg l^{-1} of AB-15. A 10% (v/v) sample from the flask was further transferred to fresh MSM containing 10 mg l^{-1} of AB-15 every 15 days for a period of 6 months. A consortium of five morphologically distinct isolates showing clear zones around their colonies on MSM/dye plates was selected and used for further studies. The bacterial isolates were identified by the Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology, Chandigarh, India.

Immobilized cell bioreactor

The consortium composed of selected bacterial isolates was used as seed inoculum for the development of an up-flow immobilized cell bioreactor.

Immobilization support

Pieces (7–10 mm) of refractory bricks obtained from a local brick-kiln were used as an immobilization support because of their high porosity and inertness. The brick pieces were incubated overnight in 0.1 N HCl, followed by thorough washing with tap-water to remove salts/soil deposited in the cavities prior to use.

The bioreactor

The bioreactor was built from a glass column (20 cm length, 3.34 cm diameter) having a 9-cm bed of refractory brick pieces

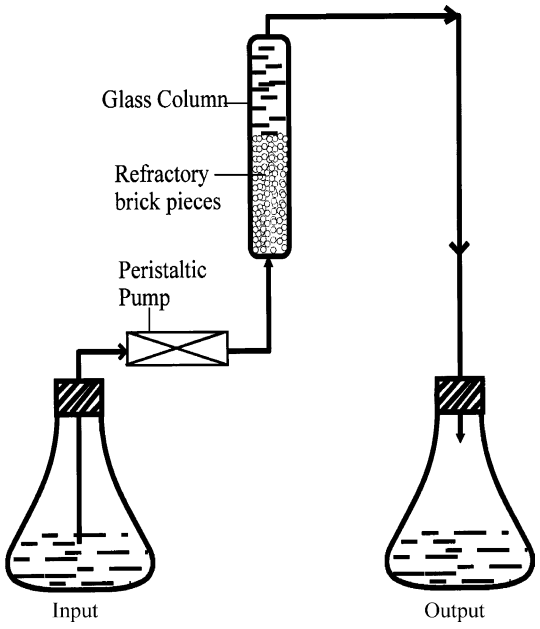

Fig. 2 Schematic diagram of the up-flow fixed-film bioreactor, with a mixed bacterial consortia immobilized on refractory brick pieces

(Fig. 2). The total volume of the reactor was 148 ml with a void volume of 45 ml. To initiate biofilm development, 500 ml of a nutrient broth-grown cell suspension of the consortium (initial cell concentration $6.3{\times}10^8$ colony-forming units ml^{-1}) was fed to the bioreactor in a loop for 7 days as inoculum for the refractory brick pieces. This was followed by a feed of MSM supplemented with glucose (5.6 mM), yeast extract (0.25% w/v) and 10 mg l^{-1} of AB-15 dye at a flow rate of 4 ml h^{-1} for a period of 1 month to allow the development of the biofilm on support particles. The dye concentration was gradually increased to a final concentration of 25 mg l^{-1} within a period of 1 month. The output of the bioreactor collected from successive 24-h intakes was used for further analytical studies. The bioreactor was in operation under these conditions for 18 months. Another bioreactor was operated under the same conditions as an abiotic control to determine the abiotic loss of dye during the process.

Decolorization assay

Decolorizing activity was expressed in terms of percentage decolorization and was determined by monitoring the decrease in absorbance at 560 nm, the λ_{max} of the dye, against the medium. Decolorization activity (%) was calculated according to the formula:

$$\text{Decolorization activity (\%)} = \frac{[(\text{initial absorbance}) - (\text{observed absorbance})]}{\text{initial absorbance}} \times 100$$

Aliquots (5 ml) of the cell-free supernatant of the output (collected every 24 h during the operation of the bioreactor) were scanned in the range of 200–800 nm to observe shifts in peaks.

Scanning electron microscopy

Support particles removed from the bioreactor after 3 months of operation were processed as described by Deya et al. [4] for SEM. SEMs were taken using a JSM- 6100, (JEOL, Tokyo, Japan).

Analytical studies

Thin layer chromatography

The feed of the bioreactor containing AB-15 dye (25 mg l^{-1}) was extracted with *n*-butanol. The cell-free supernatant of the output collected every 24 h of operation was extracted first with ethyl acetate to extract the biotransformed products and the aqueous phase was further extracted with *n*-butanol to extract the residual dye. The developing solvent systems used were ethyl acetate:hexane (2:3, v/v) for biotransformed intermediates/products and ethyl acetate:methanol (7:3, v/v) for residual dye. The bands of aromatic components were observed under UV light (365 nm) and other bands were observed by exposing the plates to iodine vapor in an iodine chamber.

NMR spectroscopy

The ^{1}H NMR spectra of the feed, ethyl acetate-extracted output and *n*-butanol-extracted output of the bioreactor were done using a 200 MHz, Brucker AMX 300 NMR spectrometer.

Results

Isolation of dye-decolorizing microorganisms

Selective enrichment of soil and sludge samples led to the isolation of a microbial consortium consisting of five bacterial isolates. The isolates were able to decolorize dye, as evident by clearing zones around the colonies on MSM/agar plates supplemented with 10 mg l^{-1} of AB-15 dye. The MTCC identified these isolates as *Bacillus* sp., *Alcaligenes* sp. and *Aeromonas* sp.

Development of immobilized cell bioreactor

The bacterial consortium in the bioreactor was initially fed with 10 mg l^{-1} of AB-15 dye, for acclimatization of the population. The concentration of AB-15 in the feed was progressively increased by increments of 5 mg l^{-1} $week^{-1}$ to a concentration of 25 mg l^{-1}. An increase in dye concentration beyond 25 mg l^{-1} and a flow rate of 4 ml h^{-1} resulted in a lowering of the decolorization efficiency of the bioreactor (data not shown). SEMs of an uninoculated brick piece showed the porous surface of the support material (Fig. 3a), which can provide a large surface area for bacterial growth and attachment. The surface of a support particle after 3 months of bioreactor operation was covered with rod-shaped bacterial cells (Fig. 3b, c).

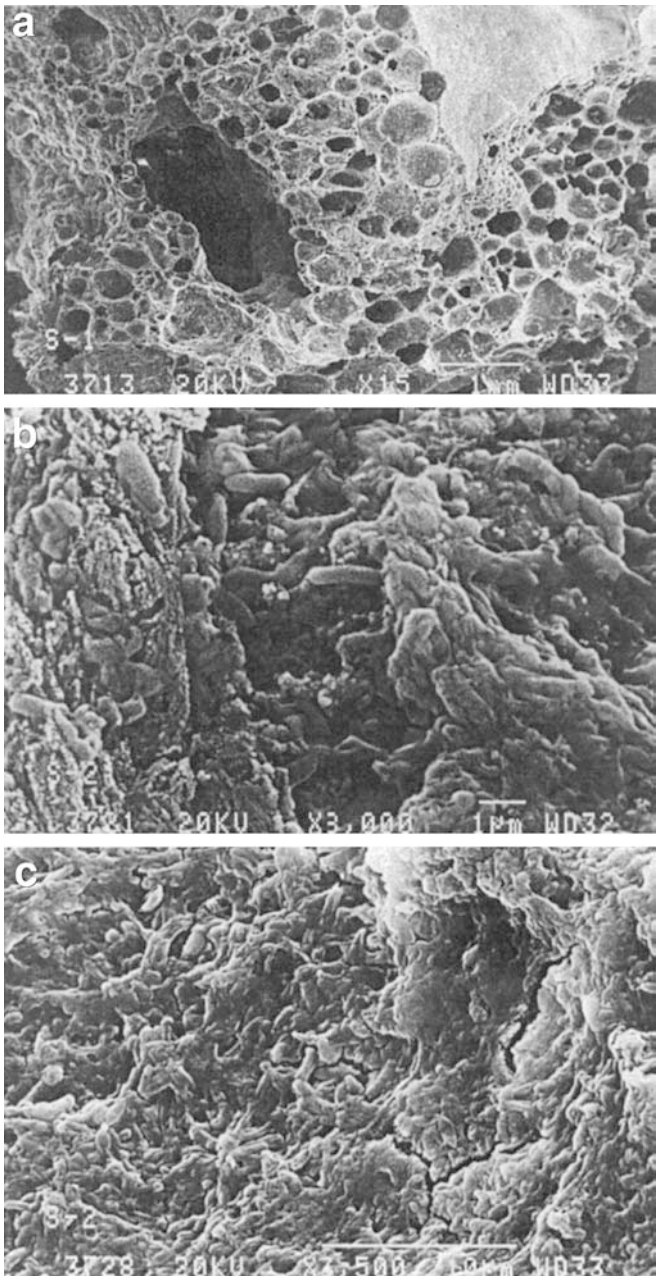

Fig. 3a–c Scanning electron micrographs. **a** Sterile support particle (refractory brick piece) showing different surface zones: smooth open regions and a cavity (×1,500). **b** Support particle completely covered with a layer of bacterial cells (×7,500). **c** A colonized support particle from the dye-fed reactor. Most of the surface has been colonized with rod-shaped cells (×3,000)

Decolorization of acid blue-15 dye

A comparison of absorbance profiles of feed containing 25 mg l^{-1} of AB-15 dye and the output of the bioreactor showed a 94% reduction in the peak at 560 nm. A significant shift in the peak from 306 nm to 360 nm was also observed, indicating decolorization and degradation of the dye molecule into metabolic intermediates (Fig. 4a, b). Moreover, no dye was found adsorbed, after extraction of the support material with *n*-butanol in the abiotic control bioreactor, suggesting that dye-decolorization was mainly due to the biological activity of the microbial consortium immobilized in the bioreactor.

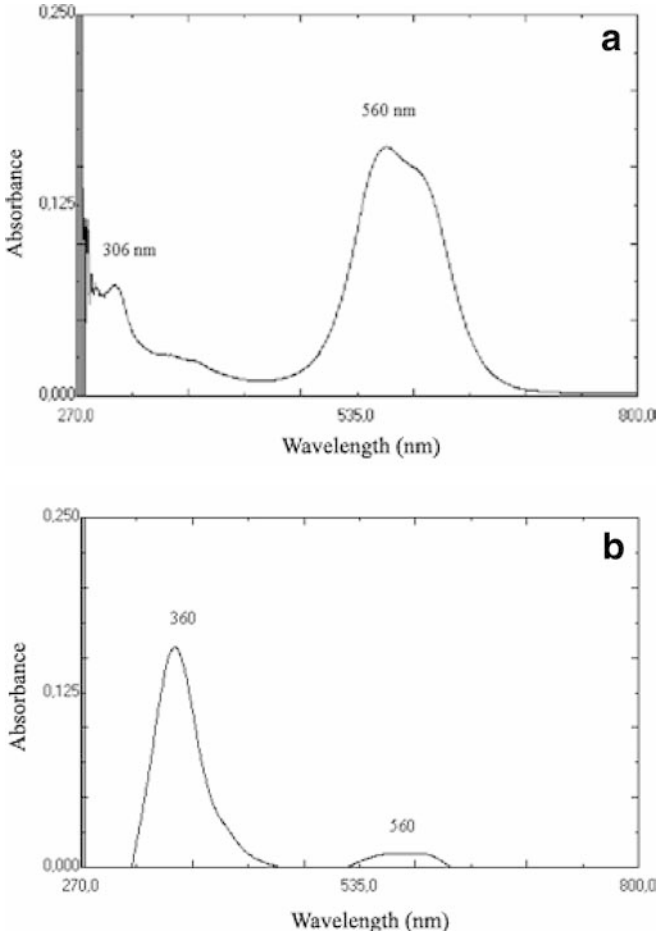

Fig. 4 UV-visible scans of: **a** input of bioreactor containing AB-15 (25 mg l^{-1}) and**b** output of bioreactor

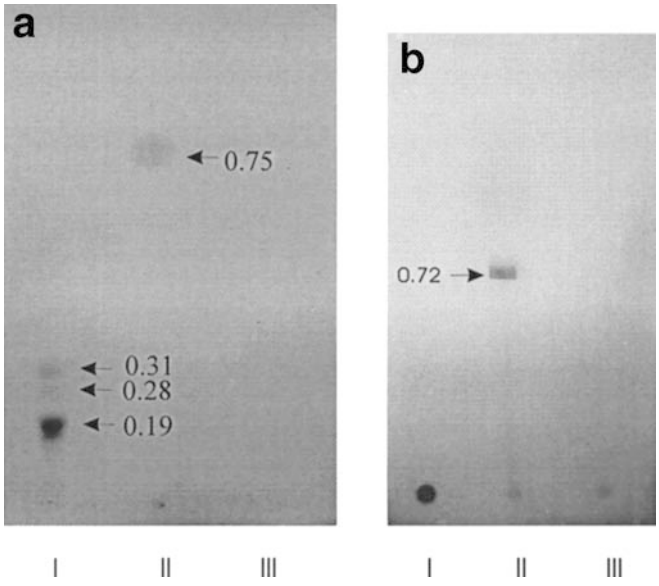

Fig. 5a, b Thin layer chromatography (TLC). **a** TLC resolution of: (*lane I*) input feed containing AB-15 dye, (*lane II*)*n*-butanol-extracted output of bioreactor and (*lane III*) ethyl acetate-extracted output of bioreactor, using the solvent system ethyl acetate:methanol (7:3, v/v). **b** TLC resolution of: (*lane I*) input feed containing AB-15 dye, (*lane II*) ethyl acetate-extracted output of bioreactor and (*lane III*)*n*-butanol-extracted output of bioreactor, using the developing solvent system ethyl acetate:hexane (2:3, v/v)

Analytical studies

Thin layer chromatography

The comparison of chromatograms of the *n*-butanol-extracted feed (Fig. 5, lane I) with the ethyl acetate- and *n*-butanol-extracted output of the bioreactor (Fig. 5, lanes II, III), respectively, indicated that the bands appearing in the feed were due to the parent dye (with R_f values of 0.31, 0.28, 0.19) and were completely absent in the output of the bioreactor. A band having an R_f value of 0.72 in the ethyl acetate-extracted output of the bioreactor and visible under UV light was the only intermediate evident in the chromatograms of organic extracts of the bioreactor output (Fig. 5b, lane II).

NMR spectroscopy

Comparison of the ^{1}H NMR spectra of the input feed (Fig. 6a) with that of *n*-butanol-extracted bioreactor output (Fig. 6c) indicated a complete breakdown of the aromatic portion of the parent dye ($\delta = 6.5$–8.0) into a non-aromatic intermediate that had signals ($\delta = 3.0$–5.0) different from the parent dye. Similarly, the peaks appearing in the ^{1}H NMR spectra of the ethyl acetate-extracted output of the bioreactor had signals ($\delta = 6.75$–7.75) different from the input feed containing the parent dye (Fig. 6b), indicating biotransformation of the complex AB-15 dye structure into simpler intermediates.

Discussion

In this study, we examined the decolorization and degradation of acid blue-15, a commercially available TPM dye, by a bacterial consortium immobilized on refractory brick pieces in an up-flow cell bioreactor. AB-15 has a more complex structure than other well studied model TPM dyes, like crystal violet and malachite green, etc., due to the presence of additional phenyl rings on the basic triphenyl structure (Fig. 1). These substituents, although they give intense color and dye-fastness, result in a resonance-stabilized structure recalcitrant to transformation in the environment. This results in biomagnification, leading to toxic responses in target populations.

The individual bacterial isolates showed less than 30% decolorization of 20 mg dye l^{-1} MSM during shake-flask studies, whereas the consortium of these isolates resulted in 70% decolorization of AB-15 (data not shown). Thus, the consortium of cultures was used as an inoculum for the bioreactor. The highly porous nature of the brick pieces allowed the formation of a biofilm of the members of the consortium, as evident from SEMs of the support pieces. The concerted

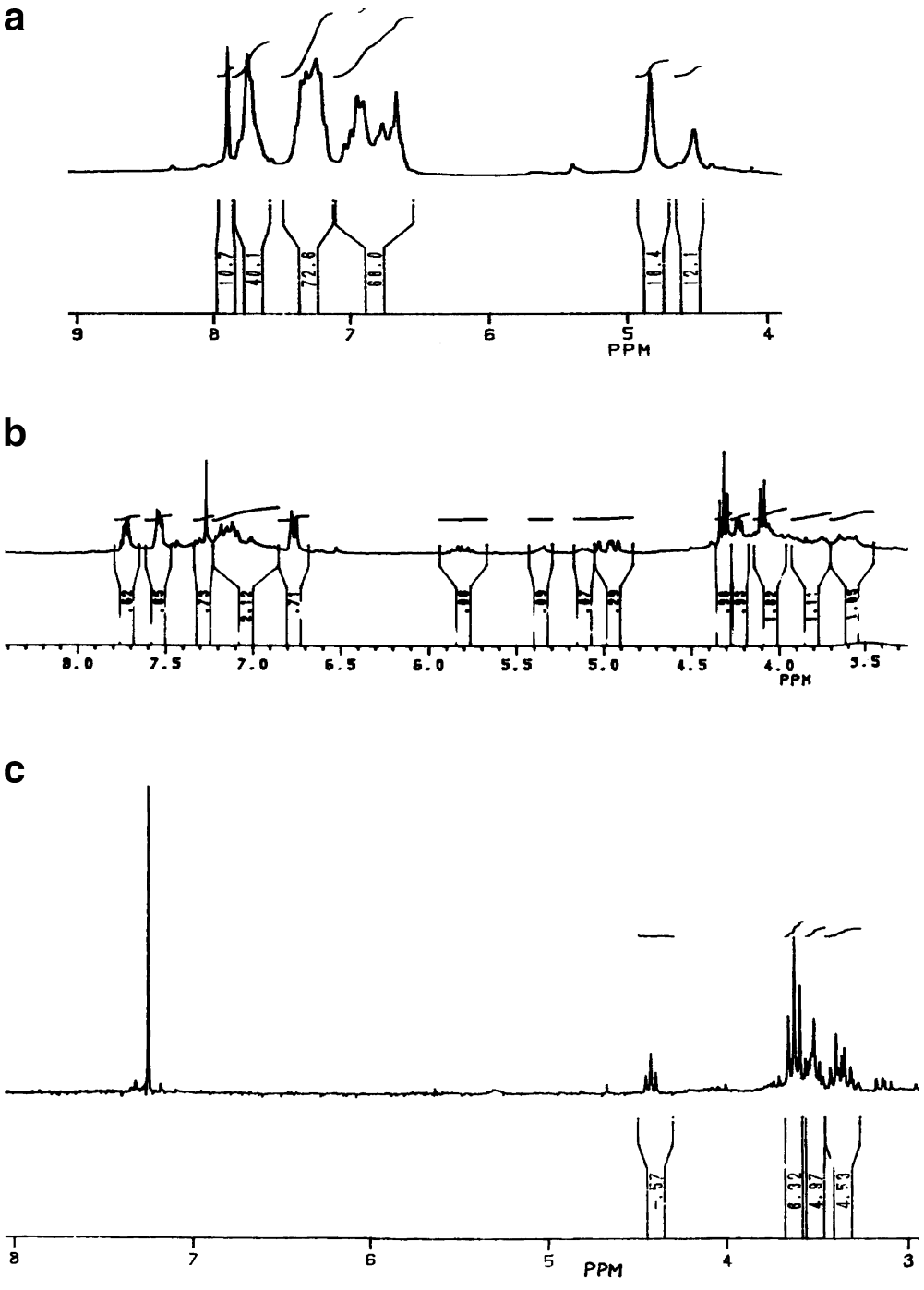

Fig. 6 ^{1}H NMR spectra of: **a** *n*-butanol-extracted feed containing AB-15 dye, **b** ethyl acetate-extracted output of bioreactor and**c** *n*-butanol-extracted output of bioreactor

metabolic potential of the immobilized cells led to more than 94% decolorization of AB-15 (25 mg l^{-1}). Analytical studies indicated the accumulation of aromatic metabolites formed by the biotransformation of AB-15. Sarnaik and Kanekar [11] reported similar studies on the biodegradation of methyl violet into three unknown intermediate metabolites by *Pseudomonas mendocina* MCM B-402. These metabolites were further degraded to phenol by a phenol-utilizing *Pseudomonas* sp. Yatome et al. [14] also reported the degradation of crystal violet by *P. pseudomallie* to an unidentified metabolite. Although there are reports on the decolorization of the azo dyes red-533 [13] and remazol black [8] using immobilized cell systems, there is no report available on using similar systems for the treatment of TPM dyes.

The results of this study show that the developed upflow immobilized cell bioreactor can enhance the ability of the microbial consortium to decolorize and degrade industrially important textile dyes. The data obtained from the study will be used for up-scaling the process to a 1.5-l laboratory-scale bioreactor.

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