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Biodegradation of azo dye C.I. Acid Red 88 by an anoxic–aerobic sequential bioreactor

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Abstract

The potential of a sequential anoxic–aerobic bioreactor to decolorize and degrade azo dye C.I. Acid Red 88 (AR-88) was evaluated. An up flow fixed-film column reactor (UFCR) having polyurethane foam (PUF) as immobilization support was built using a consortium based on four acclimatized bacterial strains belonging to *Stenotrophomonas* sp., *Pseudomonas* sp. and *Bacillus* sp., isolated from waste disposal sites of textile processing industries (TPI) as inoculum. The UFCR was operated at flow rate of 7 mL h^{-1} with hydraulic retention time (HRT) of 12 h. The effluent of anoxic UFCR was fed to continuously stirred aerobic reactor (CSAR). The sequential anoxic–aerobic treatment of synthetic dye wastewater (SDW) feed having 100 mg L^{-1} of AR-88 dye resulted in the 98% color and 95% COD removal. The aromatic metabolic intermediates produced under anoxic conditions were transformed to non-aromatic metabolites during aerobic treatment. The UV–visible spectrophotometry, TLC and NMR analyses confirmed the biotransformation of parent dye into unrelated non-aromatic compounds. The azo dye AR-88 was transformed to non-aromatic metabolic intermediates after treatment in sequential anoxic–aerobic bioreactor.

Keywords: Azo dye; Decolorization; C.I. Acid Red 88; Bacterial consortium; Anoxic–aerobic sequential bioreactor

1. Introduction

Synthetic dyestuffs are extensively used in textile, paper, printing industries and dye houses [1] due to their ease of production, fastness and variety of colors compared to natural dyes. More than 100,000 commercially available dyes are known and approximately one million tons of these dyes are produced annually worldwide [2]. It has been estimated that more than 10% of the total dyestuff used in dyeing processes is released into the environment [3]. Azo dyes are the largest group of dyes used in textile industry [4] constituting 60–70% of all dyestuffs produced [5]. They have one or more azo groups ($R_1-N=N-R_2$) having aromatic rings mostly substituted by sulfonate groups. These complex aromatic substituted structures make conjugated system and are responsible for intense color, high water solubility and resistance to degradation of azo dyes under natural conditions [6,7]. Color in the effluent is one of the most obvious indicators of water pollution and the discharge of highly colored synthetic dye effluents is aesthetically displeasing and can damage the receiving water body by impeding penetration of light. Moreover, azo dyes as well as their breakdown products are cytotoxic [8] or carcinogenic [9]. The new environment regulations concerning textile products have banned the discharge of colored waste in natural

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water bodies [4]. Therefore, an effective and economic treatment of effluents containing diversity of textile dyes has become a necessity for clean production technology for textile industries. The treatment systems based on physical and chemical methods for removal of dyes from effluents are being widely used [10]. However, these procedures have inherent drawbacks as they generate a significant amount of sludge or cause secondary pollution due to formation of hazardous by-products [11,12]. The new guidelines for effluent and sludge disposal have generated interest in the wider use of biological treatment of TPI wastewaters, as they are known to achieve complete mineralization without producing any toxic sludge.

The decolorization of azo dye by microorganisms invariably starts by reductive cleavage of azo bond under anaerobic conditions [5,13]. Although, this step leads to decolorization of dye but it generates amines of the dye related structures that are not degraded under anaerobic conditions [14] and tend to accumulate to toxic levels [5,15]. However, such amines are reported to be readily biotransformed under aerobic conditions [16,17]. There are some reports on complete degradation of sulfonated aromatic amines by enriched bacterial communities [16–18].

In light of these facts, a sequential anoxic–aerobic bioreactor was designed for the decolorization and degradation of C.I. Acid Red 88, a sulfonated azo group textile dye commonly used in textile dyeing industries installed in and around the city of Amritsar.

2. Experimental

2.1. Materials

2.1.1. Dyes and chemicals

The azo dye C.I. Acid Red 88 (Fig. 1), commonly used in local textile processing industries (TPI) was obtained from Punjab Rang Udyog, Amritsar, Punjab (India) a dye-manufacturing unit. The media components and chemicals were purchased from Himedia Labs, Bombay (India). All chemicals used were of analytical grade. Polyurethane foam (PUF), used as immobilization support, was purchased from local department store (Amritsar, Punjab, India).

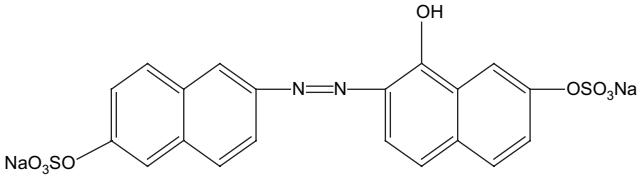

Fig. 1. Chemical structure of dye C.I. Acid Red 88 (CI 15625; λ_{max} 505 nm; COD of 100 mg of AR-88 per liter was 570 mg L^{-1}).

2.1.2. Composition of synthetic dye wastewater (SDW)

Mineral salts medium (MSM) of following composition (g L^{-1}): Na_2HPO_4 (3.6), $(NH_4)_2SO_4$ (1.0), KH_2PO_4 (1.0), $MgSO_4$ (1.0), Fe (NH_4) citrate (0.01), $CaCl_2 \cdot 2H_2O$ (0.10) and 10.0 mL of trace element solution per liter was used for all the studies. The trace element solution used was of following composition (mg L^{-1}): $ZnSO_4 \cdot 7H_2O$ (10.0), $MnCl_2 \cdot 4H_2O$ (3.0), $CoCl_2 \cdot 6H_2O$ (1.0), $NiCl_2 \cdot 6H_2O$ (2.0), $Na_2MoO_4 \cdot 2H_2O$ (3.0), H_3BO_3 (30.0), $CuCl_2 \cdot 2H_2O$ (1.0). The final pH of the medium was adjusted to 7.0. The MSM was supplemented with 0.05% (w/v) of yeast extract, 2.8 mM glucose and dye AR-88 (100 mg L^{-1}) from their respective filter sterilized stock solutions to prepare synthetic dye wastewater (SDW) feed for the bioreactor.

2.2. Inoculum for bioreactor

A consortium based on four bacterial strains viz. *Bacillus cereus*, *Pseudomonas putida*, *Pseudomonas fluorescence* and *Stenotrophomonas acidaminiphila* was used as inoculum for the UFCR. The bacterial strains were isolated from soil and sludge samples collected from waste disposal sites of local TPI. The consortium HM-4 was developed by mixing axenic cultures of the individual isolates, grown to their respective late exponential phase in MSM under shaking conditions, in equal proportions volume so that each isolate contributed optical density (O.D.) at 540 nm of 1.0 [19].

2.3. Experimental lab-scale sequential bioreactor

The laboratory scale sequential bioreactor (Fig. 2) consisted of an upflow fixed-film column reactor (UFCR) and a continuously stirred aerobic reactor (CSAR).

2.3.1. UFCR

The UFCR reactor was built from borosilicate glass column of 28 cm height and 3.4 cm internal diameter. The glass column was filled with PUF to a bed height of 26 cm. The void volume of the PUF filled reactor was 87 ml. The UFCR was fed in upflow mode by a peristaltic pump (Miclins, India) at an average flow rate of 7.0 mL h^{-1} with an average hydraulic retention time (HRT) of 12 h. The activated cell suspension of nutrient broth grown consortium was fed to UFCR in a loop for three days for the entrapment of microbial cells on the support particles. This was followed by a feed of MSM broth supplemented with 0.1% (w/v) yeast extract and 2.8 mM glucose for the development of biofilm on support particles. The reactor was then fed with SDW containing 20 mg L^{-1} of AR-88. The dye concentration in feed was progressively increased up to 100 mg L^{-1} over a stabilization period of three months.

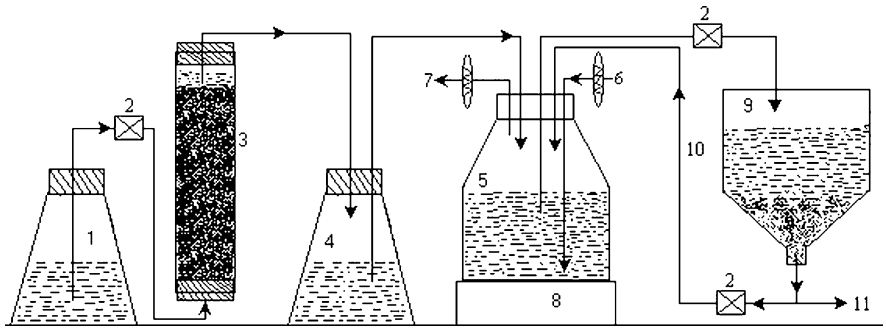

Fig. 2. Schematic diagram of anoxic–aerobic sequential bioreactor. 1, Feed tank; 2, peristaltic pump; 3, UFCR; 4, sedimentation tank; 5, CSAR; 6, air from compressor; 7, gases out; 8, magnetic stirrer; 9, sludge settling tank; 10, sludge reloading; and 11, effluent/sludge out.

2.3.2. CSAR

The CSAR was designed using a reagent bottle having inner dimensions of 13.3 cm diameter and height of 19 cm with working volume of 2.0 L. The contents of the reactor were agitated with a magnetic stirrer and were aerated continuously so as to maintain the dissolved oxygen (DO) level at $5.0 \pm 1\ mg\ L^{-1}$. The CSAR was directly fed in continuous mode with the output of UFCR during stabilization so as to enrich the cultures capable of degrading the intermediates formed during UFCR treatment. After the stabilization period, the CSAR was operated in batches of 22 days, including 20 days of continuous stirring of contents and two days for settling of sludge. The treated sample was removed by aspiration and fresh effluent from UFCR was added to CSAR to start the next batch. The system was operated at ambient temperature that varied from 20 to 45 °C and pH was between 6 and 7.

2.4. Scanning electron microscopy (SEM)

The samples of uninoculated PUF support and that from the bioreactor after biofilm development were processed as described previously [20]. The scanning electron micrographs were taken on a JSM-6100, JEOL (Japan) scanning electron microscope.

2.5. Analytical methods

Samples collected from UFCR and CSAR at regular time intervals were centrifuged at 10,000 rpm for 15 min and supernatant were used for further analysis.

2.5.1. Spectrophotometric analysis

Cell-free supernatant of UFCR reactor was read at 505 nm (λ_{max} of AR-88) using Shimadzu UV-1601 (Kyoto, Japan) spectrophotometer. The dye free medium was used as blank and SDW containing $100\ mg\ L^{-1}$ AR-88 dye was used as reference for calculating percentage of decolorization as per following equation:

$$\text{Decolorization}(\%) = \frac{(F-O)}{F} \times 100$$

where F = absorbance of feed and O = absorbance of UFCR output.

Aliquots (5 mL) of the cell-free supernatant of feed, UFCR output and CSAR output samples were scanned in the range of 200–800 nm to observe shifting of peaks due to transformations of dye after UFCR and CSAR treatment.

2.5.2. Thin layer chromatography (TLC)

The cell-free supernatant of output of the UFCR and CSAR were extracted twice with an equal volume of *n*-butanol to extract the residual dye and biotransformed products of AR-88. The SDW was also extracted in the same way. The pooled extracts were concentrated on a rotary vacuum evaporator (Buchii R-114, Switzerland). The concentrated samples were resolved on Silica gel HF_{254} TLC plates using hexane/ethyl acetate/methanol (5:3:2 v/v) as developing solvent. The resolved chromatograms were observed under UV light (254 nm) and by exposure to iodine vapors.

2.5.3. Nuclear magnetic resonance spectroscopy (NMR)

The *n*-butanol extracted samples of SDW and output of UFCR and CSAR were completely dried using rotary evaporator. The dried samples were dissolved in CD_3OD (Aldrich, USA) and transferred to NMR tubes (Wilmad, USA). The ^1H NMR spectra of the dried samples were recorded using a 200-MHz, Brucker AMX 300 NMR spectrometer to observe the structural transformations in dye molecule during bioreactor treatment.

2.5.4. Estimation of chemical oxygen demand (COD)

COD of SDW, UFCR and CSAR output was determined according to standard methods [21].

3. Results and discussion

The biodegradation of an azo dye C.I. Acid Red 88 was studied using anoxic–aerobic sequential bioreactor. The AR-88 is an intensely colored dye having two naphthalene rings with sulfonate functional groups joined by azo bond and is recalcitrant to biological degradation (Fig. 1). A consortium of four acclimatized bacterial strains belonging to *Stenotrophomonas* sp., *Pseudomonas* sp. and *Bacillus* sp., isolated from waste disposal sites of TPI was used as inoculum for the sequential bioreactor. The microbial populations immobilized on support pieces were progressively exposed to increasing concentrations of AR-88 from 20 mg L^{-1} to final concentration of 100 mg L^{-1} over a period of three months to avoid toxic shock to microorganisms. The UFCR showed 98% decolorization of SDW supplemented with 100 mg L^{-1} AR-88. The dye was not adsorbed on support material as evident from the extraction of support particles with *n*-butanol. The anoxic conditions in the column provided suitable environment for reduction of azo bond as the later is reported to be readily reduce under anaerobic conditions [22].

3.1. Scanning electron microscopy (SEM)

The PUF was selected as support material because of its high porosity, inertness, easy availability and low cost. The polygonal sacs with honeybee comb architecture of PUF provided large surface area for biofilm development (Fig. 3A). As evident from scanning electron micrographs shown in Fig. 3B, heterogeneous microbial populations comprising of rods and cocci have formed contiguous biofilm on the surface of support material bound by matrix of extracellular polysaccharide (EPS) fibers (Fig. 3B). Kim et al. [23] evaluated the development of microbial biofilms on the support media by SEM studies and reported up to 95.4% reduction in COD load and 98.5% removal of color of the textile effluent from a textile dyeing industry using a combined biological and chemical treatment process.

3.2. Analytical methods

3.2.1. Spectrophotometric analysis

The comparison of UV–visible absorption spectra (200–800 nm) of the SDW and output of UFCR and CSAR indicated complete disappearance of peak at 505 nm corresponding to λ_{max} of dye in the output of UFCR (Fig. 4). However, a new peak was observed at 320 nm, which was significantly reduced after 20 days of treatment in CSAR. Similar observations have been reported by Hayase et al. [24] regarding accumulation of

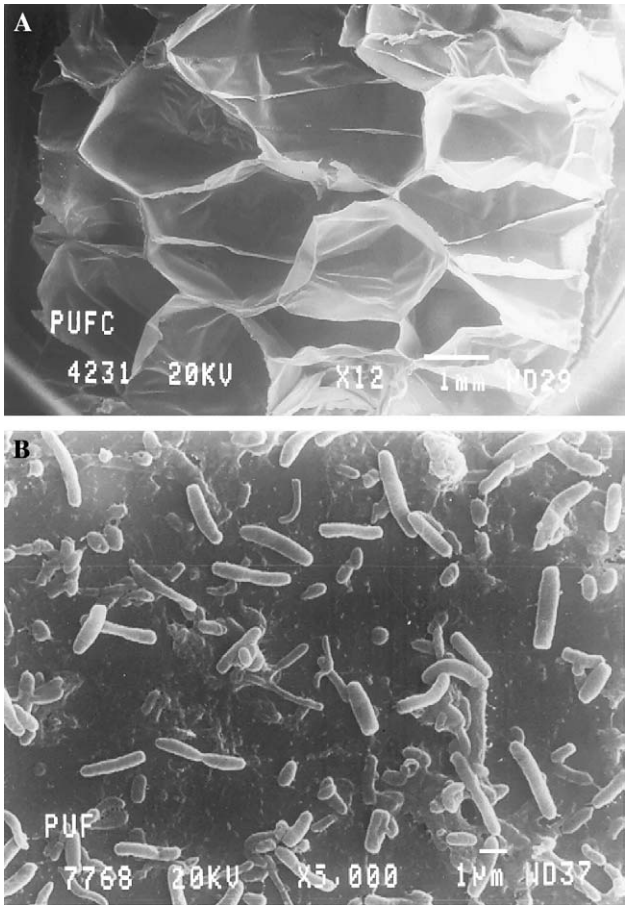

Fig. 3. Scanning electron micrographs of support particle ×12 (A) and after biofilm development ×5000 (B).

4-amiono-1-naphthalene sulfonic acid after decolorization of azo dye Bordeaux S. However, these amino derivatives/intermediates are reported to be degraded/transformed under aerobic conditions [17].

3.2.2. TLC analysis

The chromatogram of *n*-butanol extracted samples of SDW, UFCR, CSAR and 6-amino naphthalene sulfonic acid (6-ANS) showed the disappearance of band corresponding to parent dye (Rf value 0.04; Fig. 5A) in output of UFCR with concomitant appearance of two other bands with Rf values 0.42 and 0.54 (Fig. 5B). It was observed that one of the bands (Rf 0.54) in output of UFCR was comparable to that of 6-ANS (Fig. 5C) indicating to formation of aminonaphthalene derivative of dye during UFCR treatment. The chromatogram of CSAR output sample resolved into a single spot of Rf value 0.68 (Fig. 5D). The spot was not visible in UV light but could only be visualized after exposure to iodine vapors, which indicated its non-aromatic nature. These observations indicated the conversion of aromatic metabolites produced after UFCR treatment into non-aromatic intermediates. Complete mineralization of azo dyes based on substituted benzene rings by

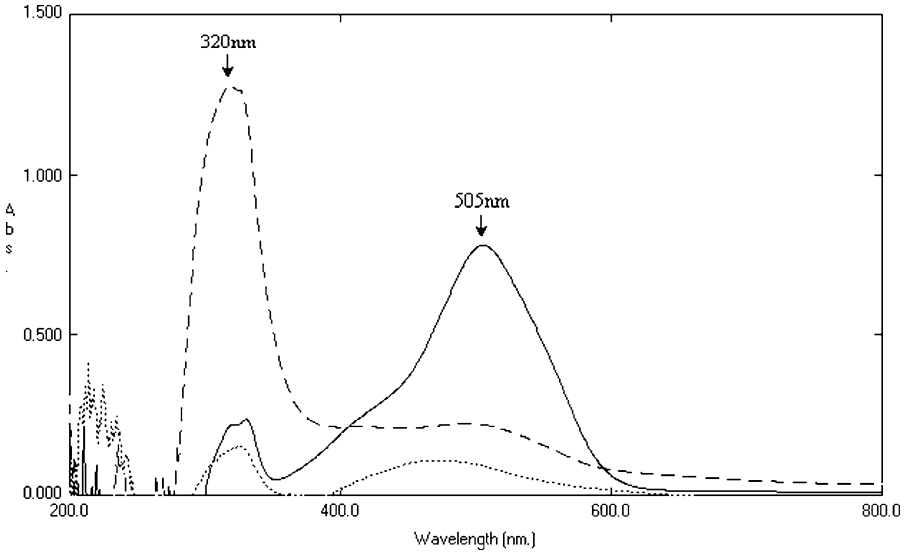

Fig. 4. UV–visible scans of SDW feed (—), UFCR output (- - -), CSAR output (…).

anaerobic/aerobic sequential has been reported in literature [17]. However, there are very few reports on degradation of sulfonated naphthalene rings based azo dyes. Haug et al. [13] reported complete degradation of Mordant Yellow 3, a naphthalene and benzene rings based azo dye, using co-culture of *Sphingomonas* sp. BN6 and *Pseudomonas* sp. BN9 at shake flask level.

Fig. 5. Thin layer chromatogram of SDW (A), UFCR output (B), 6-Amino naphthalene sulfonic acid (6-ANS) (C) and CSAR output (D).

3.2.3. ^1H NMR analysis

The ^{1}H NMR spectrum of dye AR-88 showed downshift doublet signal at δ 9.20 from hydrogen of naphthalene ring adjacent to thionate substituent. The doublet signals centered at δ 8.66, δ 8.63, δ 8.34, δ 8.26, δ 8.15 and δ 6.93 and triplet signals centered at δ 7.82, δ 7.43 and δ 7.62 are from the other naphthylic hydrogens of dye molecule (Fig. 6A). The ^{1}H NMR spectrum of UFCR output sample showed doublets centered at δ 8.62, δ 7.97, δ 7.69 and δ 6.93 and complex signals centered at δ 7.42 from aromatic hydrogens of the dye metabolites produced after UFCR treatment. A signal at δ 5.33 might be from some aromatic/olefenic C–OH group. The singlet, triplet and multiplet signals in the range of δ 1.0–3.0 are from saturated/unsaturated aliphatic compounds (Fig. 6B). As the signals in the aromatic region of UFCR output are comparable to those shown in the NMR spectrum of 6-amino naphthalene-2-sulfonic acid (Fig. 6C), the metabolite(s) formed after UFCR treatment of dye AR-88 might be some amino naphthalene sulfonate derivative. The treatment of UFCR output sample with CSAR resulted in the further degradation of aromatic metabolites as evident from ^{1}H NMR spectrum of the CSAR output sample. The singlet signal at δ 7.28 is of deutrated chloroform ($CDCl_3$) used as solvent for NMR analysis. The doublet signal centered at δ 4.21, triplet signal centered at δ 3.92 and multiplet signals centered at δ 4.52, δ 3.53, δ 3.42, δ 1.31 and δ 0.69 are from hydrogen of olefenic/aliphatic compounds (Fig. 6D). The ^{1}H NMR spectrum of the solvent extracted samples of CSAR output showed disappearance of signals in aromatic region (δ 7.0–9.0) indicating complete loss of aromaticity from dye AR-88 after sequential anoxic–aerobic treatment.

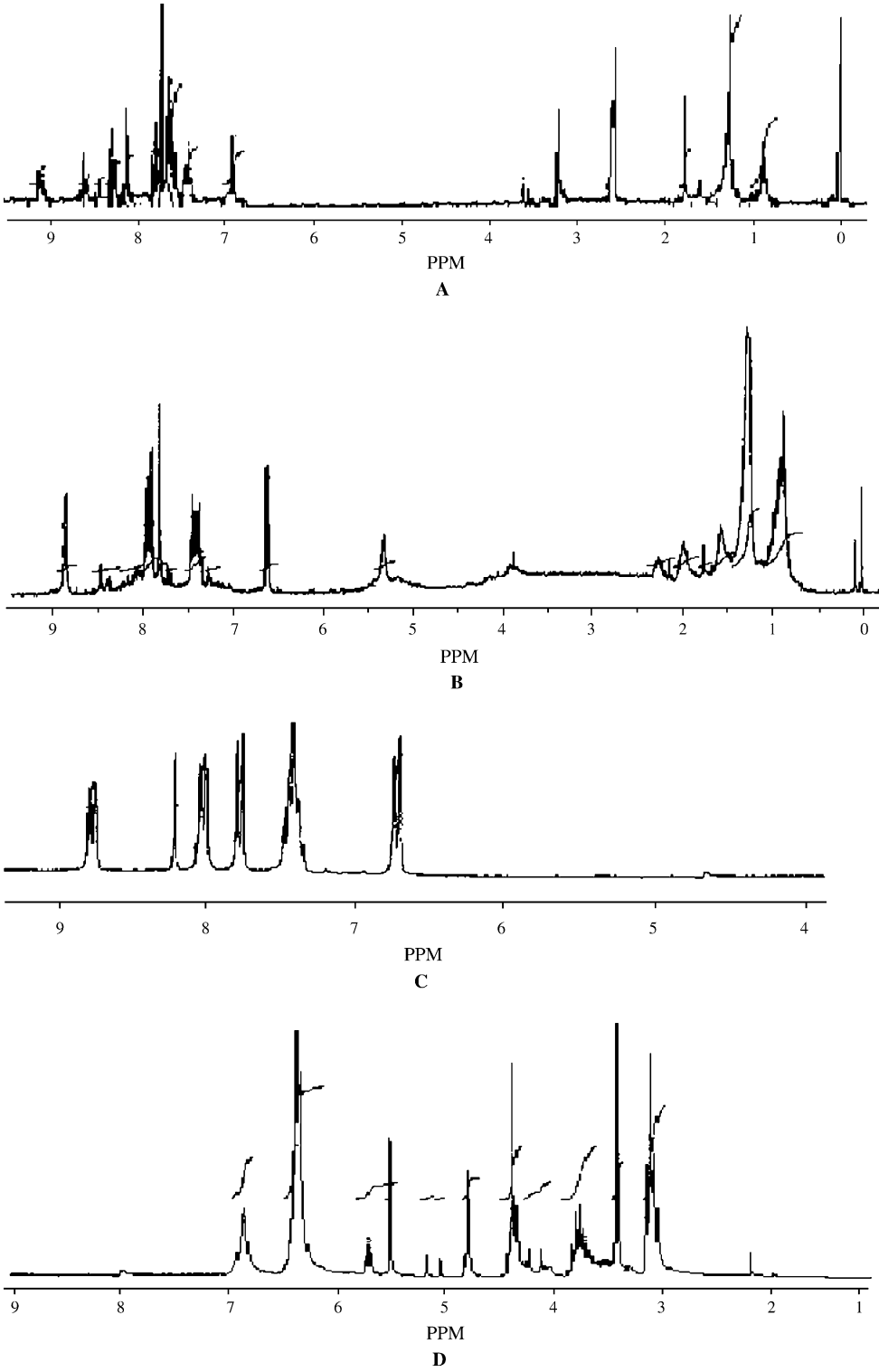

Fig. 6. ^{1}H NMR spectra of SDW feed (A), UFCR output (B), 6-aminonaphthalene sulfonic acid (C) and CSAR output (D).

3.2.4. Estimation of COD

The COD load of SDW supplemented with 100 mg L^{-1} of AR-88 was 1630 mg L^{-1}. The COD of 100 mg of AR-88 dye was 570 mg L^{-1}. After UFCR treatment, COD was reduced to 302 mg L^{-1}, resulting in 82% reduction in COD. It was further decreased to 30 mg L^{-1} (90% reduction) after CSAR treatment. Thus, the overall reduction of COD load by 98% was achieved after the sequential treatment of SDW.

Thus, the consortium has capability of working under both anoxic as well as aerobic conditions resulting in complete breakdown of aromatic moieties of dye structure. Biodegradation of some other textile dyes viz. C.I. Acid Red 97, C.I. Acid Red 119, C.I. Reactive Red 120, C.I. Acid Blue 113 and C.I. Acid Brown 100 using sequential bioreactor is being carried out.

4. Conclusions

1. The sequential anoxic–aerobic bioreactor achieved complete decolorization and up to 98% removal of COD load of synthetic dye wastewater containing 100 mg L^{-1} of C.I. Acid Red 88 dye.
2. The AR-88 dye was transformed to aminonaphthalene derivative after anoxic treatment in UFCR that was degraded to non-aromatic metabolites as evident from TLC and NMR analyses.
3. Further research is being carried out to improve the activity of the microbial populations in CSAR so as to enhance aerobic degradation of the intermediates formed after UFCR treatment. The toxic potential of the effluent generated at different stages will be compared to validate the efficiency of the sequential bioreactor.

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