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ORIGINAL PAPER

Efficacy of bacterial consortium-AIE2 for contemporaneous Cr(VI) and azo dye bioremediation in batch and continuous bioreactor systems, monitoring steady-state bacterial dynamics using qPCR assays

Chirayu Desai · Kunal Jain · Bharat Patel · Datta Madamwar

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Abstract Bacterial consortium-AIE2 with a capability of contemporaneous Cr(VI) reduction and azo dye RV5 decolourization was developed from industrial wastewaters by enrichment culture technique. The 16S rRNA gene based molecular analyses revealed that the consortium bacterial community structure consisted of four bacterial strains namely, *Alcaligenes* sp. DMA, *Bacillus* sp. DMB, *Stenotrophomonas* sp. DMS and *Enterococcus* sp. DME. Cumulative mechanism of Cr(VI) reduction by the consortium was determined using in vitro Cr(VI) reduction assays. Similarly, the complete degradation of Reactive Violet 5 (RV5) dye was confirmed by FTIR spectroscopic analysis. Consortium-AIE2 exhibited simultaneous bioremediation efficiencies of (97.8 ± 1.4) % and (74.1 ± 1.2) % in treatment of both 50 mg l^{-1} Cr(VI) and RV5 dye concentrations within 48 h of incubation at pH 7 and 37°C in batch systems. Continuous bioreactor systems achieved simultaneous bioremediation efficiencies of (98.4 ± 1.5) % and (97.5 ± 1.4) % after the onset of steady-state at 50 mg l^{-1} input Cr(VI) and 25 mg l^{-1} input RV5 concentrations, respectively, at medium dilution rate (D) of 0.014 h^{-1}. The 16S rRNA gene copy numbers in the continuous bioreactor as determined by real-time PCR assay indicated that *Alcaligenes* sp. DMA and *Bacillus* sp. DMB dominated consortium bacterial community during the active continuous bioremediation process.

Keywords Bacterial consortium · Biodegradation · Azo dye · Cr(VI) · Real-time PCR · FTIR

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Introduction

Industrial effluents are frequently co-contaminated with toxic chromium and aromatic compounds (Shen et al. 1996). Chromium and synthetic dyes co-exist in waste waters of paint, ink, wood preservative manufacturing and textile dyeing industries, and also in common effluent treatment plants (CETPs). Chromium compounds are applied as mordant for fixation of dye color especially in wool, leather, silk and paper dyeing industries. Chromium ions also form a part of metal complex azo dyes (Sadettin and Donmez 2007). Hexavalent chromium Cr(VI) is considered as a priority pollutant by the US-EPA and environmental authorities of many other countries (Kamaludeen et al.

2003; Desai et al. 2009). Likewise, azo dyes comprise nearly 60–70% of the synthetic dyestuffs employed in textile dyeing (Khehra et al. 2005). More than 10–15% of the applied dye is released to waste streams posing a serious concern in effluent treatment plants due to their color, bio-recalcitrance and potential toxicity to pristine ecosystems (Stolz 2001; Pourbabaee et al. 2006). Both Cr(VI) and azo dyes are water soluble, persistent environmental pollutants with carcinogenic and mutagenic properties (Cervantes et al. 2001; Desai et al. 2008b; Moosvi et al. 2007). In the current scenario, several microbial bioremediation strategies are sought after as an indispensable, ecofriendly and cost-effective solution towards restoring azo dye and chromium polluted ecosystems. Application of mixed microbial consortium offers an advantage of complemented catabolic versatility of different micro-organisms in bioremediation of xenobiotic anthropogens (Moosvi et al. 2007). It has been previously demonstrated that syntrophic interactions of mixed microbial consortium can lead to complete mineralization of azo dyes and reduction of hexavalent chromates (Fude et al. 1994; Khehra et al. 2005; Asgher et al. 2007). However, simultaneous treatment of Cr(VI) and synthetic dyes remains less investigated, with only a few recent reports on concurrent bioremediation in batch culture systems (Sadettin and Donmez 2007; Cetin et al. 2008; Kilic et al. 2007). There have been recent reports on development of continuous bioreactors for treatment of either dye (Blanquez et al. 2008) or Cr(VI) (Cabrera et al. 2007; Sen et al. 2007), but not both simultaneously. Therefore, simultaneous as well as continuous treatment of mixed pollution of Cr(VI) and azo dye remains poorly understood. In this study, we have addressed these issues, by developing an enriched bacterial consortium-AIE2 and testing its efficiency for concurrent Cr(VI) reduction and azo dye degradation in batch and continuous culture systems. Real-time PCR (also referred to as qPCR) is considered as an effective tool to monitor bacterial community dynamics, owing to its sensitivity in robust detection and quantification of bacteria (Yu et al. 2006; Kindaichi et al. 2006; Da Silva and Alvarez 2007). Therefore, in this study we have also designed and employed 16S rRNA gene based real-time PCR assays to investigate bacterial community dynamics before and after the onset of steady-state condition in the continuous process of concurrent Cr(VI) and azo dye bioremediation.

Materials and methods

Analytical reagents and culture media

Reactive Violet 5 (commonly abbreviated as RV5—a monoazo copper complex dye, C. I. No. 18097), with a chemical structure as shown in Fig. 1, was used as a model azo dye in this study. RV5 dye was procured from Mantung Dyestuff Industry, Vatva GIDC, Gujarat, India. A stock of RV5 dye (500 mg l^{-1}) was prepared and diluted in Milli-Q water and sterilized by autoclaving. Potassium chromate (K_2CrO_4) and Cr(VI) complexing reagent 1,5-diphenylcarbazide were procured from Sigma (St. Louis, MO, USA). Potassium chromate stock solution of 1,000 mg l^{-1} was used as a source of Cr(VI) and filter sterilized using 0.2 μm filters (Millipore, Bedford, CA). A modified inorganic broth containing all components of Bushnell–Hass medium except $FeCl_3$ (known to abiotically reduce chromium ions) was used throughout this study. The modified inorganic broth consisted of (g l^{-1}); $MgSO_4$ (0.2); K_2HPO_4 (1.0); $CaCl_2$ (0.02); $(NH_4)_2HPO_4$ (1.0); NH_4NO_3 (1.0), added with (0.1% w/v) glucose and yeast extract (0.4% w/v). After autoclaving the medium was amended with respective dilutions of sterile Cr(VI) and RV5 dye to reach the required concentrations.

Enrichment and isolation of bacterial consortium AIE-2

Effluent samples were collected from the Amalakhadi rivulet receiving mixed waste waters from chemical units and industries manufacturing dyes, paints, fertilizers, pharmaceuticals in the Ankleshwar Industrial Estate, Bharuch District, Gujarat, India. These effluent samples were inoculated (10% v/v final concentration) in 500 ml Erlenmeyer flasks containing

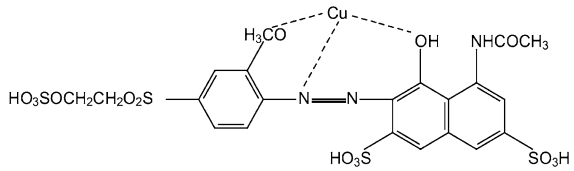

Fig. 1 Structure of reactive violet 5R; C·I. no. 18097; $\lambda_{max} = 558$ nm

modified Bushnell–Hass medium (300 ml) amended with varying concentrations 50–200 mg l^{-1} of Cr(VI) and RV5 dye and incubated at 37°C with shaking of 120 rpm for 3 days. These primary enrichment cultures thus obtained were given 50 consecutive transfers with 20% (v/v) inoculum's size in modified Bushnell–Hass broth amended with 150 mg l^{-1} initial Cr(VI) and azo dye RV5 concentrations. The enriched bacterial consortium-AIE-2 thus obtained was then tested for simultaneous treatment of Cr(VI) and RV5 azo dye.

Phylogenetic analysis of bacterial community structure in consortium AIE-2

Total bacterial community DNA of the initial consortium was extracted directly from the enrichment culture flasks containing 100 mg l^{-1} concentrations of Cr(VI) and RV5 azo dye using a previously published protocol (Desai and Madamwar 2007). In addition, the enrichment consortium was serially diluted and screened for bacterial isolates on Plate Count Agar (PCA) media (Desai et al. 2008a). The consortium community DNA and genomic DNA of isolated strains were used as templates in 16S rRNA gene amplifications using eubacterial universal primers, Fd1 (5′-AGA GTT TGA TCC TGG CTC AG-3′) and Rd1 (5′-AAG GAG GTG ATC CAG CC-3′) (Weisburg et al. 1991). Amplification program was performed with initial denaturation of 5 min at 94°C; followed by 35 cycles of 2 min at 94°C, 1 min at 55°C and 1.5 min at 72°C and a final extension at 72°C for 15 min in a Corbet Thermocycler (Corbet Life Sciences, Sydney, Australia). The 16S rRNA gene amplicons of the consortium bacterial community DNA were gel-purified using QIAquick gel-extraction kit (Qiagen, Australia). Mixtures of purified 16S rRNA gene amplicons were cloned into pGeneJET™ PCR cloning vector (Fermentas Life Sciences, USA) and transformed into *E. coli* DH5α to generate clone libraries. A total of 50 positive clones were randomly selected from the clone libraries using blue-white screening, their plasmids were isolated, 16S rRNA genes were re-amplified, purified and subjected to sequencing. The gel-eluted 16S rRNA gene amplicons retrieved from the bacterial isolates and 16S rRNA gene clone libraries were sequenced using Fd1, Rd1 primers using BigDye Terminator Cycle Sequencing v3.1 DNA sequencing chemistry in an Applied Biosystems 3130xl Genetic Analyser (Applied Biosystems, USA). The almost full-length 16S rRNA gene sequences of 1,500 nucleotides retrieved from each of the clones and isolates in this study were analyzed using BLASTn program to identify and download the nearest neighbor sequences from the NCBI database. Multiple sequence alignments were performed using Clustal W 1.6 program at (http://www.ebi.ac.uk/clustalw/) and the alignment files were analyzed using BioEdit software (Hall 1999). Phylogenetic analyses was performed using aligned sequences by the neighbor joining algorithm with Kimura 2 parameter distance and bootstrapping of 1,000 replicates in Molecular Evolutionary Genetics Analysis MEGA 4.0 software (Tamura et al. 2007). Representative sequences retrieved in this study have been assigned with GenBank accession numbers from EU522076–EU522079.

In vitro Cr(VI) reduction assay and FTIR analysis of reactive violet 5 degradation

The mechanism of Cr(VI) and RV5 dye bioremediation by the consortium was identified by performing in vitro Cr(VI) reduction assays (Desai et al. 2008a) and FTIR analysis of RV5 dye degradation (Kalyani et al. 2008). Consortium-AIE2 cells grown overnight in 100 ml modified Bushnell–Hass medium culture flasks were harvested at 4,000*g* for 10 min at 4°C, washed and resuspended in 0.1 M potassium phosphate buffer pH 7.0. Resultant cell suspensions were disrupted using an Ultrasonic Probe (Sonics Vibra Cell 500, USA) with amplitude of 35% at 50 W with 9 s pulses at 1 s intervals for 35 min. Sonicates thus obtained were centrifuged at 32,000*g* for 40 min at 4°C to yield cell-debris and supernatants. Resultant supernatants were filtered through 0.22 μm filters (Millipore, Bedford, USA) to obtain the cell-free extracts devoid of membrane fractions. These cell-free extracts were then used for Cr(VI) reductase assays as described previously (Desai et al. 2008a). In order to estimate the decolourization of azo dye RV5, overnight grown consortium-AIE2 (10% v/v) was inoculated in Erlenmeyer flasks containing 100 ml modified Bushnell–Hass broth, amended with 50 mg l^{-1} RV5 dye final concentrations and incubated at 37°C with 120 rpm shaking. Samples were withdrawn after 10 min (control sample) and after 6 h (experimental sample) to check for metabolites

produced during the biodegradation of RV5 dye. The metabolites within the control and experimental samples were extracted with equal volumes of ethyl acetate and evaporated to dryness in a Savant SpeedVac (Thermoscientific, Waltham, MA). Extracted metabolites were resuspended in HPLC grade potassium bromide KBr in a ratio of 5:95 and analysed at mid IR region (400–4,000 per cm) using Fourier Transform Infrared Spectroscopy (FTIR) in a Perkin Elmer, Spectrum GX spectrophotometer (Perkin Elmer, USA).

Simultaneous bioremediation of Cr(VI) and RV5 dye in batch systems

Bacterial consortium-AIE2 with 10% (v/v) inoculum's size was inoculated in Erlenmeyer flasks containing modified Bushnell–Hass medium (100 ml) amended with respective final concentrations of Cr(VI) and azo dye RV5. Samples were withdrawn periodically every 6 h and centrifuged at 6,000*g* for 20 min. The remaining Cr(VI) concentrations in the media supernatant samples were measured using 1,5-diphenyl carbazide (DPC) method (Desai et al. 2008a). The residual dye concentrations were estimated at the absorption maxima of Reactive Violet 5 at 558 nm, using Cintra UV–VIS spectrophotometer (GBC Scientific Equipments, Australia) (Moosvi et al. 2007). The bioremediation efficiencies were calculated using following equation $E_{[\mathrm{Cr\ or\ dye}]}(\%) = [(C_\mathrm{i} - C_\mathrm{t})/C_\mathrm{i}] \times 100$, where, C_i is the starting or initial concentration of Cr(VI) and dye and C_t is the concentration of Cr(VI) and dye at the end of time interval (t). Biomass dry weight were estimated gravimetrically and specific growth rates were measured using the following equation $\mu(t_x - t_{xi}) = \ln x/x_\mathrm{i}$, where x_i and x are the biomass concentrations at beginning time (t_{xi}) and at end of exponential phase (t_x) as described previously (Sen et al. 2007). The effect of pH and temperature on simultaneous bioremediation efficiencies was determined at a pH range of 4–9 and a temperature range of 25–60°C with shaking of 120 rpm. The pH was adjusted using either 0.1 M NaOH or 0.1 M HCl. The effect of initial azo dye concentrations on reduction of Cr(VI) was tested by adding variable RV5 dye concentrations (50–150 mg l^{-1}) to the batch culture flasks containing 50 mg l^{-1} Cr(VI) concentrations. Likewise, the effect of initial Cr(VI) concentrations on RV5 dye decolourization was tested by adding varying Cr(VI) concentrations (50–150 mg l^{-1}) to the batch culture flasks containing 50 mg l^{-1} RV5 dye concentrations. Time-course of respective samples was conducted by estimating Cr(VI) reduction and RV5 dye decolourization efficiencies after every 6 h upto 48 h as described above. All the estimations were carried out in triplicates.

Simultaneous bioremediation of Cr(VI) and RV5 in continuous systems

A continuous stirred tank reactor made up of a 2 l fermentor jar (New Brunswick, USA) with an assembly set-up as shown in Fig. 2, was employed for simultaneous bioremediation studies. The major components of the reactor assembly consisted of a cylindrical glass reactor connected through a Masterflex peristaltic pump (Cole-Parmer, Illinois, USA) to a media holding carboy (20 l capacity) at the inlet and to a liquid collection system through another peristaltic pump at the outlet using siliconized masterflex tubings (0.5 cm diameter). The jacketed reactor was connected to a water bath set at 37°C with a temperature controller module. The reactor was equipped with a NewBrunswick automated pH controller module with a sensory pH electrode immersed in to the reactor and connected to a pump assembly attached to 0.1 M HCl and 0.1 M NaOH inputs for automated influx to maintain a constant pH of 7 in the reactor. The reactor baffles were connected to a motor with 50 rpm for constant mixing of culture suspension and influent media. All the reactor components and accessories were pre-fitted (except the water bath, motor and pH electrode) and autoclaved together along with attached tubings at 121°C for 30 min. After autoclaving the reactor assembly was mobilized to a laminar air-flow using a trolley, for additions of respective Cr(VI) and azo dye RV5 stock dilutions to the reactor and the media holding carboy. An inoculum's size of 20% (v/v) was added from the pre-enriched batch culture flasks into the reactor media. The reactor was operated in the continuous mode by feeding the media into the reactor at the desired dilution rates (D) through the peristaltic pump. The reactor run in continuous mode under steady state condition was monitored for 168 h (7 days) for each run of different input Cr(VI) and dye concentrations and different dilution rates. The

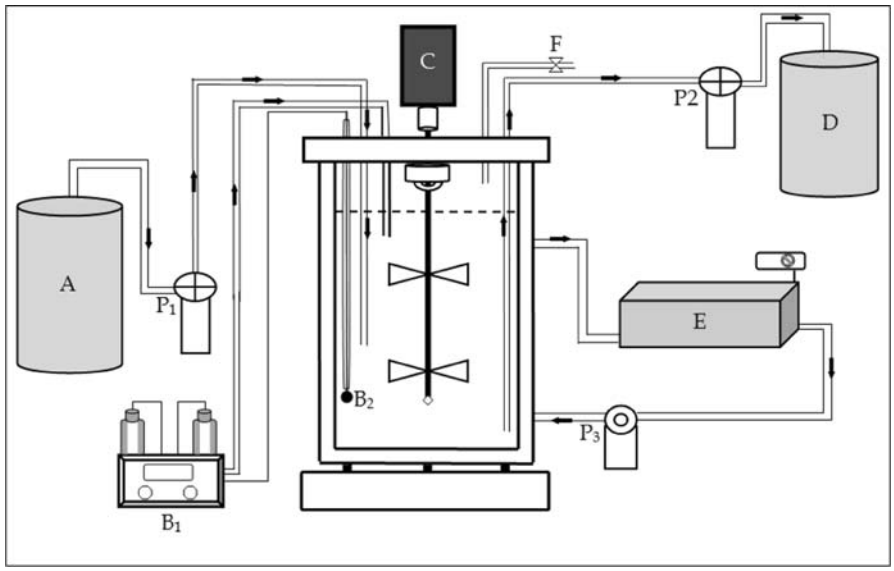

Fig. 2 Schematic representation of continuous bioreactor assembly. Reactor assembly components: *A* media holding carboy, *B1* and *B2* pH controller and pH meter probe, *C* motor attached to baffles, *D* wash-out collection carboy, *E* water bath with temperature regulator, *F* gas/air outlet, *P1, P2, P3* peristaltic pumps

flow-through samples (10 ml) were collected periodically for determination of remaining Cr(VI) and azo dye RV5 concentrations and estimation of biomass as described above. The continuous operation of the reactor was carried out using different media dilution rates (0.014–0.056 h^{-1}) and different initial Cr(VI) (50–150 mg l^{-1}) and RV5 dye (25–120 mg l^{-1}) concentrations to determine the simultaneous Cr(VI) reduction and dye decolourization efficiencies of consortium-AIE2 in the reactor. The flow-through samples were collected before and after the onset of steady-state to assess the bacterial community composition of the consortium-AIE2 using species-specific real-time PCR assay.

Real-time PCR assay of bacterial dynamics in the continuous bioreactor

Real-time PCR assay was performed for quantification of 16S rRNA gene copies of each of the consortial bacteria in the continuous bioreactor at different time intervals. For designing species-specific primers, the 16S rRNA sequences of the identified consortium strains and sequences from a number of related phyla of the domain *Bacteria* were obtained from the Ribosomal Database Project (RDP-II). All sequences were then aligned in BioEdit software to identify species-specific signature sequences for designing forward primers specific for individual consortium bacteria using the criteria outlined previously (Woo et al. 1999). The species-specific forward primers DMA-F (5′-GCG GGG GAC CTT CGG GCC TCG C-3′), DMB-F (5′-CCG AAT AAT CTC TTT TAC TTC ATG G-3′), DME-F (5′-CCG TAT AAC AAT CAA AAC CGC ATG G-3′), DMS-F (5′-TAA GAG CTT GCT CTT ACG GGT GGC G-3′) and universal reverse R1 (5′-CTG CTG CCT CCC GTA G-3′) primers were used in the real-time PCR assays generating expected 16S rRNA gene amplicons of ~145, ~192, ~279 and ~172 bp for *Alcaligenes* sp. DMA, *Bacillus* sp. DMB, *Stenotrophomonas* sp. DMS and *Enterococcus* sp. DME, respectively. The real-time PCR reaction mixtures for each template with a system volume of 25 µl consisted of 12.5 µl of 2× QuantiTect® SYBR® Green PCR Master Mix (including HotStarTaq® DNA polymerase, 5 mM $MgCl_2$, dNTP mix, QuantiTect SYBR Green PCR Buffer (containing SYBR Green I and ROX passive reference dye), 1 µl of each (10 µM) forward species-specific primers and reverse universal primers, 7.5 µl of Nuclease/RNase-free water and 3 µl template DNA (0.5 ng/µl). Real-time PCR assays were conducted in MicroAmp Optical 96-well reaction plates covered with optical-quality sealing tape and monitored with an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Fostercity, CA). The cycling program consisted of initial denaturation at 94°C for 5 min, followed by 40 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min and final extension at 72°C for 10 min. Consequently, a dissociation curve analysis

was performed by measuring loss of fluorescence over a temperature range of 55–95°C to detect the specificity of each amplicon. The plate set-up contained negative controls as reaction mixtures without primers and without template DNA in triplicates during each run and the plate was centrifuged briefly to bring all contents to the bottom of the wells prior to amplification. Standard curves were generated using triplicate 10-fold dilutions of plasmids containing ~145, ~192, ~279 and ~172 bp, 16S rRNA gene amplicons of *Alcaligenes* sp. DMA, *Bacillus* sp. DMB, *Stenotrophomonas* sp. DMS and *Enterococcus* sp. DME, respectively. The ng DNA concentrations within the plasmids and subsequent dilutions were measured using NanoDrop ND-1000 spectrophotometer prior to amplification for accurate quantification. All the experiments were performed using standard curves and negative controls during each run to nullify any variation from reaction to reaction. Appropriate dilutions of the samples prior to the real-time PCR amplification were made to fit the cycle threshold (C_T value) in the linear range of each standard curve. Amplification efficiencies for each run were calculated using following formula: $\varepsilon_c = [10^{(-1/\text{slope})} - 1]$ from the slope of graphs of cycle threshold (C_T values) versus log C_o (where, C_o = concentration of template plasmid DNA), as described previously (Kindaichi et al. 2006; An et al. 2006). Amplification efficiencies (ε_c) in a range of 0.99–1.2 were achieved in different real-time PCR amplifications. Target copy numbers for each template were calculated from the standard curves, assuming that the average molecular mass of a double-stranded DNA molecule is 660 g mol^{-1} using following formula: Target 16S rRNA gene (copies ml^{-1}) = [16S rRNA gene concentration (g ml^{-1}) × 6×10^{23} (copy $mole^{-1}$)]/[16S rRNA gene amplicon size (bp) × 660 g mol^{-1}] as described previously (Yu et al. 2006).

Results and discussion

Phylogenetic characterization of bacterial community composition in the consortium-AIE2

The bacterial community composition of the initial consortium-AIE2 in presence of 100 mg l^{-1} concentrations of RV5 and Cr(VI) was determined by cloning and sequencing of 16S rRNA genes (~1.5 kbp). Moreover, this data was matched with the 16S rRNA gene sequence data of distinct colony morphotypes screened on PCA media plates. As depicted from the phylogenetic tree (Fig. 3), consortium bacteria comprised of four different species namely, *Alcaligenes* sp. DMA, *Bacillus* sp. DMB, *Enterococcus* sp. DME and *Stenotrophomonas* sp. DMS representing three different bacterial phyla amongst *β-Proteobacteria*, *Firmicutes* and *γ-Proteobacteria*, respectively. BLASTn search revealed that bacterial strains *Alcaligenes* sp. DMA exhibited 96% sequence homology with *Alcaligenes feacalis* (AF155147), *Bacillus* sp. DMB exhibited 99% sequence homology with *Bacillus fusiformis* (DQ333300). Likewise, the *Enterococcus* sp. DME exhibited 99% sequence homology with *Enterococcus faecium* (AB326300) and *Stenotrophomonas* sp. DMS exhibited 98% sequence homology with *Stenotrophomonas maltophila* (AJ131780). Phylotype distribution of 16S rRNA genes inferred based on culture-independent analysis indicate that the enriched bacterial consortium is composed of (43%) *Alcaligenes* sp. DMA, (24%) *Bacillus* sp. DMB, (19%) *Stenotrophomonas* sp. DMS and (14%) *Enterococcus* sp. DME. Bacterial strains of the genera *Bacillus* and *Enterococcus* have been reported previously to possess aerobic azo reductases (Suzuki et al. 2001; Chen et al. 2004). Likewise, the genera of *Stenotrophomonas* has been reported previously as a part of bacterial consortium capable of decolourizing several azo dyes (Khehra et al. 2005; Mohana et al. 2008) and *Alcaligenes* genus has been reported to mineralize sulfonated aromatics, benzene and toluene sulfonates (Pandey et al. 2007). In addition different genera of *Bacillus* and *Alcaligenes* have also been reported to exhibit in vitro Cr(VI) reduction (Desai et al. 2008b; Peitzsch et al. 1998).

Determination of Cr(VI) and RV5 dye bioremediation mechanism by the consortium-AIE2

Hexavalent chromium reduction mechanism was evident in the cell-free extracts prepared from the bacterial consortium-AIE2. Cell-free extracts of consortium-AIE2 exhibited 87% reduction of (10 ± 0.5) mg l^{-1} Cr(VI) which was reduced to (1.3 ± 0.6) mg l^{-1} within 60 min incubation time in potassium phosphate buffer pH 7.0. Similar, reduction of Cr(VI)

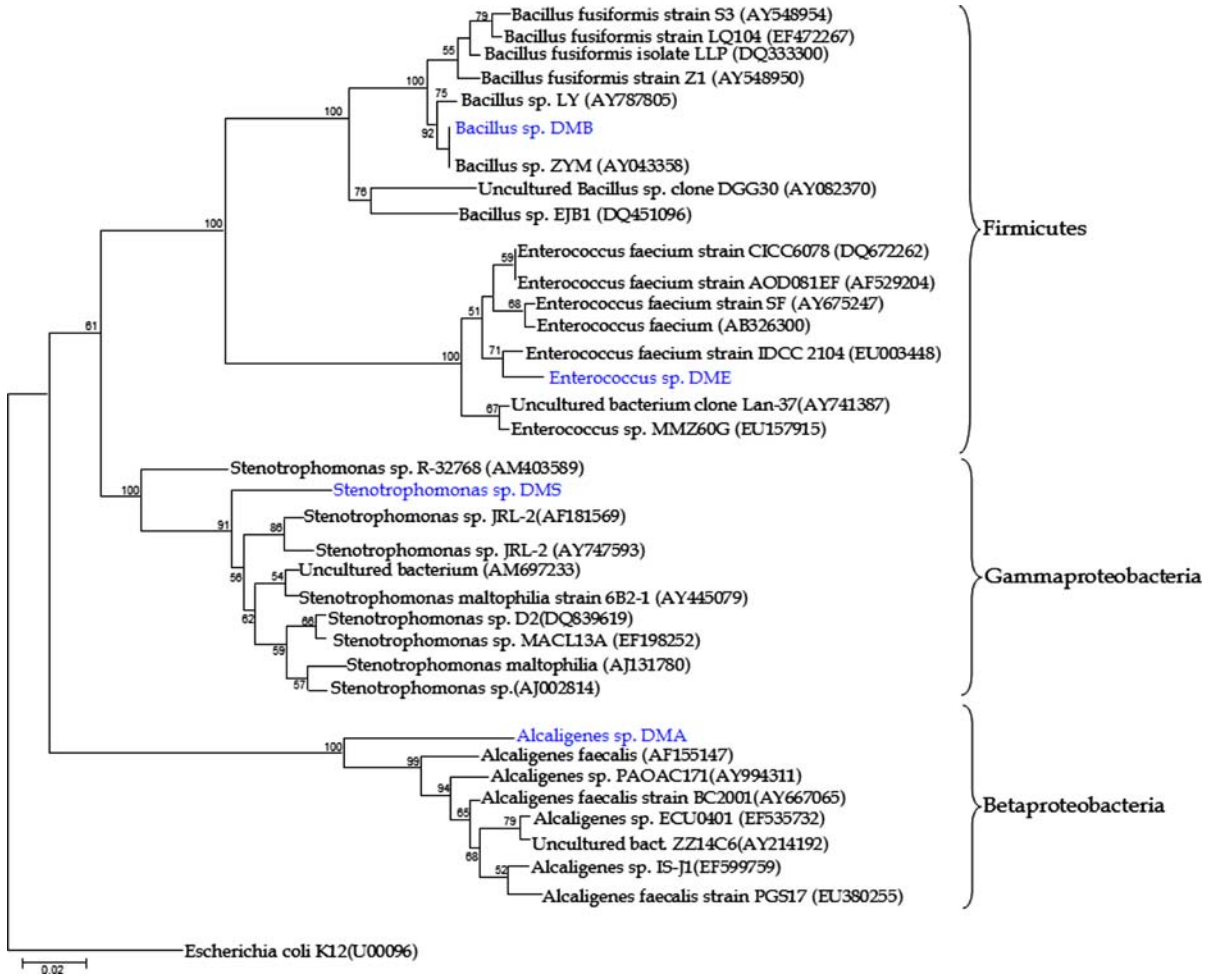

Fig. 3 Phylogenetic characterization of the consortium (AIE-2) bacterial community structure. The tree was constructed using 16S rRNA gene sequences (1.5 kb) retrieved from the consortium-AIE2 and sequences of closest phylogenetic neighbors obtained by NCBI BLAST(*n*) analysis. *Numbers* in the parenthesis indicate accession numbers of corresponding sequences. The tree was constructed using neighbor joining algorithm with Kimura 2 parameter distances in MEGA 4.0 software. *E. coli* K12 has been taken as an out-group. *Numbers at nodes* indicate percent bootstrap values above 50 supported by more than 1,000 replicates. The *bar* indicates the Jukes-Cantor evolutionary distance

to Cr(III) has also been reported in other studies employing bacterial consortiums (Fude et al. 1994; Cetin et al. 2008). Biodegradation of RV5 dye by the consortium-AIE2 was confirmed by scanning the control and decolourized samples using FTIR spectroscopy as shown in Fig. 4. Results obtained from FTIR analysis of control and decolourized samples indicated prominent differences in specific peaks of RV5 dye fingerprint region (400–4,000 cm^{-1}). FTIR spectra of control Reactive Violet 5 shows specific peaks for multi-substituted benzene ring, where peaks at 1,142.50, 1,339.19, 1,186.07 and 1,548.56 cm^{-1} corresponds to two SO_3H groups, symmetric SO_2 and –N=N– (azo bond), respectively. FTIR analysis of extracted metabolites of degraded RV5 showed peaks at 1,118, 1,637 and 3,434 cm^{-1} which indicates the production of primary amines and secondary amide, respectively, during catabolic degradation of RV5 dye. Absence of peaks at 671, 720, 763 and 817 cm^{-1} indicates the break down of benzene ring or the loss of aromatic nature of the compound. Jadhav et al. (2007) reported similar kind of benzene ring fission in Methyl Red by *Saccharomyces cerevisiae* cells. Correspondingly, break down of azo bond was

confirmed by the absence of spectral peaks at 1,437 and 1,548 cm^{-1}. While the absence of the peaks around 1,300 and 1,165–1,150 cm^{-1} clearly indicates the degradation of S=O bonds. Peaks at 2,922 and 2,925 cm^{-1} in control RV5 and degraded metabolites, respectively, shows the asymmetrical stretching of C–H in CH_3. Similar result of asymmetrical C–H stretching was observed in degradation of disperse dye Brown 3REL (Dawkar et al. 2008).

Simultaneous bioremediation of Cr(VI) and RV5 dye in batch systems

Simultaneous bioremediation of Cr(VI) and RV5 dye by the consortium-AIE2 was studied in different batch conditions prior to its scale-up into the continuous bioreactor. Results obtained from flask-level batch experiments performed at different pH and temperature conditions indicated maximum efficiency of chromium reduction ($E_{[Cr]}$ (%) = 97.6 ± 1.4) and RV5 dye decolourization ($E_{[dye]}$ (%) = 74.1 ± 1.2) at pH 7 and 37°C. The Cr(VI) reduction by the consortium-AIE2 was significantly inhibited at lower pH 4 ($E_{[Cr]}$ (%) = 6.4 ± 1.1), whereas simultaneous dye decolourization was more inhibited at a higher temperature of 60°C ($E_{[dye]}$ (%) = 3.3 ± 1). Previous studies have reported maximum simultaneous Cr(VI) and dye bioremediation efficiencies at pH 8 and 30°C using bacterial consortiums isolated from industrial wastewaters in batch systems (Kilic et al. 2007; Cetin et al. 2008). In, our previous studies we have determined a maximum RV5 decolourization rate at pH 7.5 and 8.5 using bacterial consortiums JW-2 and RVM 11.1, respectively isolated from textile effluents. Likewise, bacterial consortiums JW-2 and RVM 11.1 also exhibited temperature optima of 37 and 30°C, respectively for maximum RV5 dye decolourization (Moosvi et al. 2005; Moosvi et al. 2007). The specific growth rate of the consortium-AIE2 in absence of dye and Cr(VI) was 0.065 h^{-1}. Higher concentrations of 150 mg l^{-1} Cr(VI) and 120 mg l^{-1} RV5 dye, when added in batch cultures significantly decreased the growth rate of the consortium to 0.042 h^{-1}. Similar phenomenon has been reported in previous studies which suggest that the sulphonic groups of reactive dyes at higher concentrations act as

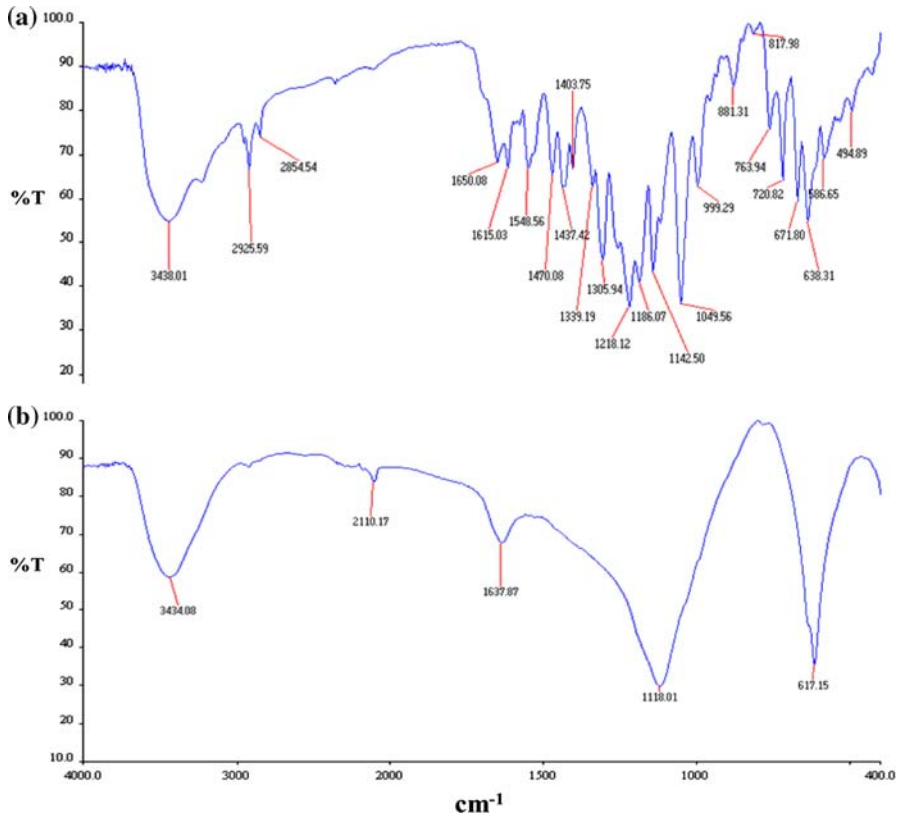

Fig. 4 FTIR spectral analysis of **a** control Reactive Violet 5 dye **b** extracted metabolites produced after 6 h (experimental sample) of RV5 decolourization by consortium-AIE2

detergents and exert inhibitory effect on microorganisms resulting in a decreased growth rate (Kalyani et al. 2009). Likewise, at higher initial concentrations, Cr(VI) has been reported to decrease the growth rate by exerting morphological changes and oxidative stress within microbial cells (Desai et al. 2008a). Effect of initial RV5 dye concentrations on Cr(VI) reduction efficiencies and similarly the effect of initial Cr(VI) concentrations on RV5 dye decolourization efficiencies were studied in batch systems. As shown in Fig. 5a with an increase in initial RV5 dye concentration, the simultaneous Cr(VI) reduction efficiency decreases. The lower initial RV5 dye concentration (50 mg l^{-1}) had a negligible effect on reduction of Cr(VI) (50 mg l^{-1}) yielding a simultaneous Cr(VI) reduction efficiency of $E_{[Cr]}$ (%) = 97.8 ± 1.4 upon 48 h of incubation in batch systems. Whereas, adding higher initial RV5 dye concentration (150 mg l^{-1}) resulted in 22% decrease in Cr(VI) reduction efficiency yielding $E_{[Cr]}$ (%) = 76 ± 0.8 as shown in Fig. 5a. As shown in Fig. 5b, the decolourization efficiency of 50 mg l^{-1} RV5 in presence of 50 mg l^{-1} Cr(VI) was $E_{[RV5]}$ (%) = 74.1 ± 1.2, which was slightly affected by 7.6% at higher Cr(VI) concentration of 150 mg l^{-1} yielding $E_{[RV5]}$ (%) = 66.3 ± 1.2. In general, higher initial RV5 concentrations were more inhibitory towards simultaneous Cr(VI) reduction, while higher initial Cr(VI) concentrations had a minute effect on RV5 decolourization. However, in a similar study by Sadettin and Donmez (2007) it was observed that bioaccumulation of Reactive Black B and Remazol Blue dyes by *Phormidium* sp. was significantly inhibited in presence of Cr(VI) ions. Furthermore, it can be observed from Fig. 5 that Cr(VI) reduction efficiencies of the consortium-AIE2 were higher as compared to RV5 decolourization, which is congruent with the results obtained in other studies on simultaneous Cr(VI) and dye bioremediation in batch systems (Cetin et al. 2008; Kilic et al. 2007). Kilic et al. (2007) demonstrated an increase in dye bioaccumulation in presence of Cr(VI) ions. However, in this study we observed that the dye decolourization by consortium-AIE2 was neither affected nor stimulated in presence of Cr(VI) ions. Likewise, Kilic et al. (2007) observed that 98% of 108.8 mg l^{-1} Cr(VI) and 62% of 88.5 mg l^{-1} dye were bioaccumulated in a mixed consortium after 7 days of incubation at respective concentrations. Whereas, the consortium-AIE2 was expedient in treatment of higher concentrations (50–100 mg l^{-1}) of both Cr(VI) and azo dye within 2 days of incubation time in batch systems. Given, these results of batch studies, combinations of lower initial RV5 concentrations with higher initial Cr(VI) concentrations were chosen for effective simultaneous treatment in continuous culture systems.

Simultaneous bioremediation of Cr(VI) and RV5 in a continuous bioreactor

Continuous stirred tank reactors (CSTR) are ideally designed for controlling synchronized growth of bacterial populations in a metabolically active steady-state, this offers an advantage in bioremediation of large quantities of industrial effluents (Sen et al. 2007). Therefore, simultaneous bioremediation experiments were carried out in a continuous stirred tank bioreactor using different combinations of Cr(VI) and RV5 dye concentrations, with a medium dilution rate (D) of 0.014 h^{-1} at pH 7 and 37°C. Furthermore, the effect of different medium dilution rates (0.014–0.056 h^{-1}) on the simultaneous Cr(VI) reduction and RV5 decolourization process was investigated. The consortium-AIE2 was effective in continuous treatment of Cr(VI) and RV5 dye as shown in Fig. 6a, b, respectively. As observed from Fig. 6, the input concentrations of Cr(VI) and RV5 dye exhibited minor increase, followed by a consistent decrease after the onset of respective steady-states in the continuous bioreactor. As, shown in Table 1, the highest simultaneous bioremediation efficiencies ($E_{[Cr]}$ = 98.4 ± 1.5, $E_{[RV5]}$ = 97.5 ± 1.4) were achieved after the onset of steady-state at 50 mg l^{-1} input Cr(VI) and 25 mg l^{-1} input RV5 concentrations. As observed in Table 1, the biomass concentrations decreased with an increase in the input Cr(VI) and RV5 concentrations in the continuous bioreactor operated at 0.014 h^{-1} dilution rates. The specific growth rate (μ) of the consortium in presence of 50 mg l^{-1} Cr(VI) and RV5 dye was 0.059 h^{-1}, hence, the dilution rates (0.014 h^{-1}, 0.028 h^{-1}) below the specific growth rate (μ) were more efficient in simultaneous treatment of Cr(VI) and RV5 dye as compared to the dilution rates (0.056 h^{-1}) near to specific growth rates. Similar, observations were made in a previous study by Sen et al. (2007) suggesting that the media dilution rates lower than

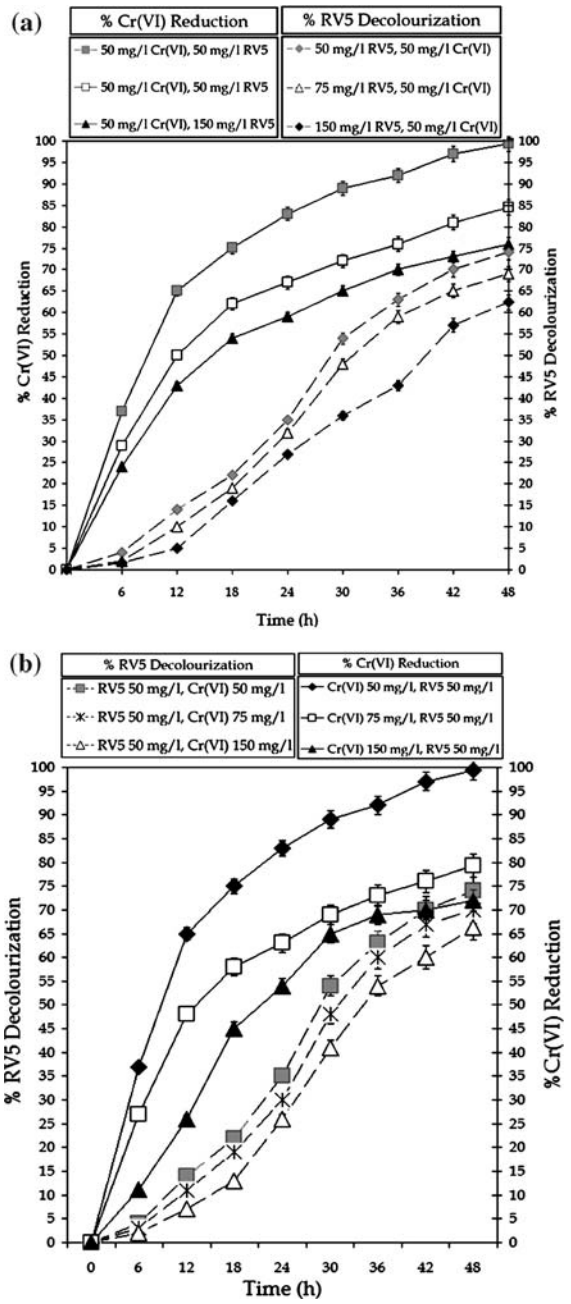

Fig. 5 Batch studies **a** time-course of Cr(VI) reduction and RV5 decolourization, at constant RV5 (50 mg l^{-1}) and varying Cr(VI) (50–150 mg l^{-1}) concentrations. **b** Time-course of Cr(VI) reduction and RV5 decolourization, at constant Cr(VI) (50 mg l^{-1}) and varying RV5 (50–150 mg l^{-1}) dye concentrations

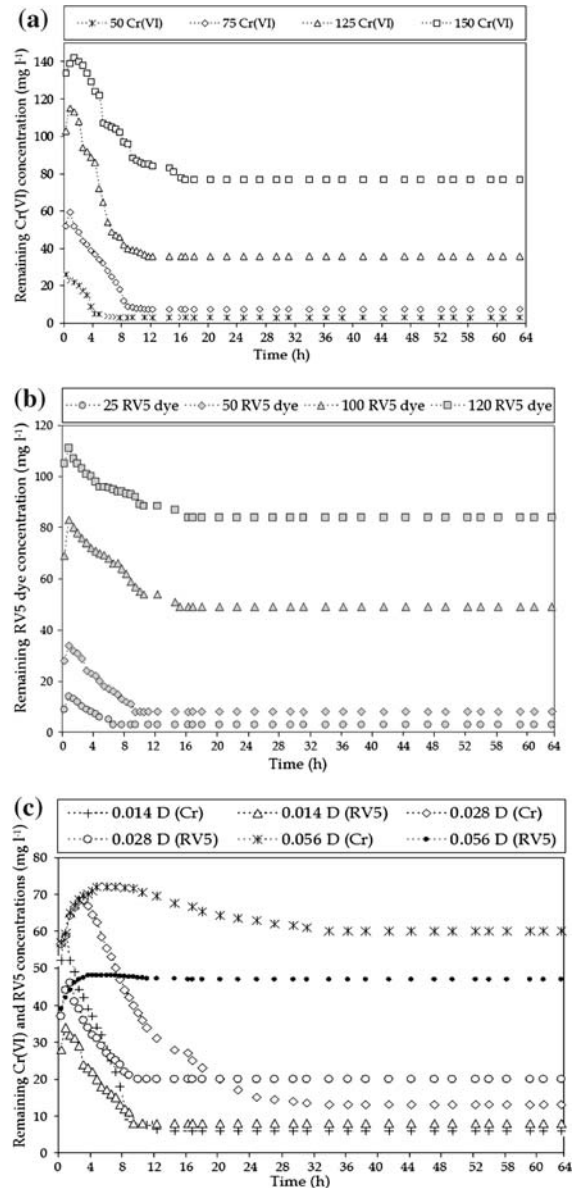

Fig. 6 **a** Continuous and simultaneous removal of Cr(VI) in presence of 25, 50, 100 and 120 RV5 dye mg l^{-1} input concentrations. **b** Continuous and simultaneous removal of RV5 dye in presence of 50, 75, 125 and 150 mg l^{-1} Cr(VI) input concentrations. **c** Simultaneous removal of Cr(VI) and RV5 dye by operating the continuous bioreactor at three different dilution rates (0.014, 0.028 and 0.056) using 75 mg l^{-1} Cr(VI) and 50 mg l^{-1} RV5 dye input concentrations

the specific growth rate were more efficient in removal of higher Cr(VI) concentrations. As shown in Fig. 6c, the decrease in simultaneous Cr(VI) reduction and RV5 dye decolourization at higher dilution rate (0.056 h^{-1}) can be due to increased cell-washout at higher dilution rate as compared to lower dilution rates (0.014 h^{-1}, 0.028 h^{-1}). The results obtained here corroborate with previous studies on

Table 1 Cr(VI) reduction and RV5 decolourization efficiencies in the continuous bioreactor (after 24 h or respective steady-state, $D = 0.014\ h^{-1}$)

Input Cr(VI) concentration (mg l^{-1})	Input RV5 dye concentration (mg l^{-1})	Biomass (mg l^{-1}) concentration in reactor effluent	$E_{[Cr]}$ (%) Cr(VI) reduction efficiency	$E_{[dye]}$ (%) RV5 dye decolourization efficiency
50 ± 0.6	25 ± 0.6	103.2 ± 1.4	98.4 ± 1.5	97.5 ± 1.4
75 ± 0.8	50 ± 0.5	98.6 ± 1.3	90.1 ± 0.5	84.2 ± 1.4
125 ± 0.5	100 ± 1.1	72.5 ± 1.5	71.3 ± 1.8	51 ± 0.7
150 ± 0.5	120 ± 0.9	69.8 ± 1.6	48.6 ± 1.2	30.4 ± 1.3

Cr(VI) bioremediation in continuous systems (Sen et al. 2007; Cabrera et al. 2007).

Real-time PCR assay of bacterial community dynamics in the continuous bioreactor

Species-specific real-time PCR assays have been used for precise determination of changes in bacterial community structures during active bioremediation processes (Da Silva and Alvarez 2007). Here, we used real-time PCR assay to check the bacterial species distribution and determine whether the steady-state condition was successfully achieved during the continuous bioremediation process. The steady-state condition was monitored in terms of 16S rRNA gene copy numbers of each consortial bacteria present in the bioreactor community DNA. As observed from Table 2, the bioreactor community DNA sample before the onset of steady-state exhibits relatively lower 16S rRNA gene copy numbers of the consortium bacteria as compared to the community DNA samples collected after the onset of steady-state. However, the relative abundance of different bacteria within the consortium remains unchanged before and after the onset of steady-state condition. As, observed from Table 2, the *Enterococcus* sp. exhibits highest C_T values and hence least 16S rRNA gene copy numbers in the bioreactor community DNA samples. Likewise, *Alcaligenes* sp. exhibits lowest C_T values and hence highest 16S rRNA gene copy numbers in the bioreactor community DNA samples. In general, the abundance of different bacterial strains (determined as 16S rRNA gene copy numbers) in the active bioreactor community can be stated as, *Alcaligenes* sp. DMA > *Bacillus* sp. DMB > *Stenotrophomonas* sp. DMS > *Enterococcus* sp. DME. Furthermore, *Alcaligenes* sp. DMA and *Bacillus* sp. DMB dominated the consortium bacterial community because they might be more tolerant to the combined stress exerted by Cr(VI) and azo dye RV5 and, also to the cell-wash out rates exerted in a continuous system. It can be deduced from Table 2 that steady-state condition was successfully achieved in the continuous bioreactor. As observed in 72 and 120 h samples, the proportion of the *Alcaligenes* sp. DMA, *Bacillus* sp. DMB, *Stenotrophomonas* sp. DMS and *Enterococcus* sp. DME after the onset of steady state remained more or less unchanged. These, results are similar to those obtained by cloning and sequencing of 16S rRNA genes from the initial bacterial consortium-AIE2 growing in presence of Cr(VI) and azo dye RV5 within enrichment culture flasks. Therefore, it can be concluded that the relative abundance of bacterial species within the consortium-AIE2 does not significantly change when applied to the continuous bioremediation process from batch systems.

Conclusions

The newly isolated bacterial consortium-AIE2 was expedient in contemporaneous bioremediation of Cr(VI) and RV5 dye under batch and continuous culture systems. The mechanism of bioremediation by the consortium-AIE2 was determined to be Cr(VI) reduction and RV5 dye degradation, respectively. Batch studies revealed that the consortium-AIE2 reduced Cr(VI) more efficiently, as compared to RV5 dye decolourization. Moreover, the Cr(VI) reduction by the consortium-AIE2 was inhibited in presence of higher RV5 dye concentrations, whereas higher Cr(VI) concentrations had a minor effect on RV5 dye decolourization. Previous studies have reported isolation and characterization of bacterial consortia in simultaneous bioremediation of Cr(VI) and synthetic

Table 2 Determination of 16S rRNA gene copy numbers of bacterial strains before and after the onset of steady-state condition in the continuous bioreactor using real-time PCR assay

Bacterial strain in the continuous bioreactor	Before the onset of steady-state		After the onset of steady-state			
	6 h sample		72 h sample		120 h sample	
	Cycle threshold (C_T)	16S rRNA gene (copy number ml^{-1})	Cycle threshold (C_T)	16S rRNA gene (copy number ml^{-1})	Cycle threshold (C_T)	16S rRNA gene (copy number ml^{-1})
Alcaligenes sp.	14.57 ± 0.26	$4.16 \times 10^9 \pm 2.27 \times 10^9$	12.71 ± 0.48	$5.54 \times 10^{11} \pm 1.87 \times 10^{11}$	12.56 ± 0.22	$5.91 \times 10^{11} \pm 2.33 \times 10^{11}$
Bacillus sp.	17.25 ± 0.21	$4.89 \times 10^8 \pm 1.83 \times 10^8$	15.64 ± 0.23	$4.85 \times 10^9 \pm 1.55 \times 10^9$	15.42 ± 0.12	$6.87 \times 10^9 \pm 1.57 \times 10^9$
Stenotrophomonas sp.	19.84 ± 0.36	$1.12 \times 10^7 \pm 1.23 \times 10^7$	16.38 ± 0.33	$2.62 \times 10^8 \pm 1.13 \times 10^8$	16.19 ± 0.21	$3.27 \times 10^8 \pm 1.41 \times 10^8$
Enterococcus sp.	33.26 ± 0.38	$3.89 \times 10^5 \pm 0.99 \times 10^5$	19.52 ± 0.17	$1.91 \times 10^7 \pm 1.08 \times 10^7$	19.64 ± 0.32	$1.71 \times 10^7 \pm 1.28 \times 10^7$

dyes in batch systems, but to the best of our knowledge continuous as well as simultaneous treatment has not been described in the literature. The continuous system was efficient in treatment of large-scale simultaneous inputs of different initial Cr(VI) (50–150 mg l^{-1}) and RV5 dye (25–120 mg l^{-1}) concentrations upto 168 h. Bioremediation efficiencies of the consortium after the onset of steady-state in a continuous bioreactor were comparatively higher than those obtained in batch systems. Greater than 97% of 50 mg l^{-1} of both Cr(VI) and RV5 dye were continuously treated by the consortium-AIE2 in the continuous bioreactor. The most efficient media dilution rate for simultaneous treatment was 0.014 h^{-1} which is much lower than the specific growth rate of the consortium. The bacterial species distribution during the steady-state condition as determined by real-time PCR suggests that *Alcaligenes* sp. DMA, *Bacillus* sp. DMB might be the key bacterial population essential for concurrent bioremediation of Cr(VI) and RV5 dye in the continuous bioreactor system. The results obtained here, endow with significant findings pertaining to the use of indigenous bacterial consortium in amelioration of mixed contamination of Cr(VI) and azo dyes commonly present in industrial effluents.

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