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Novel bacterial biofilm consortia that degrade and detoxify the carcinogenic diazo dye Congo red

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Abstract

Free-living planktonic single bacterial strain can decolorize Congo red (CR) but often produces the carcinogenic, mutagenic and genotoxic aromatic amines. Planktonic single and bacterial consortia are more susceptible to toxic pollutants than their biofilm counterparts. In the present study, four biofilm consortia (C1 = *Vitreoscilla* sp. ENSG301, *Acinetobacter lwoffii* ENSG302, *Klebsiella pneumoniae* ENSG303 and *Pseudomonas fluorescens* ENSG304, C2 = *Escherichia coli* ENSD101, *Enterobacter asburiae* ENSD102 and *E. ludwigii* ENSH201, C3 = *E. asburiae* ENSD102, *Vitreoscilla* sp. ENSG301 and *Bacillus thuringiensis* ENSW401, and C4 = *E. coli* ENSD101, *E. ludwigii* ENSH201 and *B. thuringiensis* ENSW401) were prepared and assessed for bioremediation of CR. All these biofilm consortia remarkably decolorized (96.9 to 99.5%) the CR (100 mg/L) in static condition within 72 h incubation at 28 °C. These consortia also synthesized significantly more intracellular azoreductase and laccase enzyme than extracellular of these enzymes. UV–Vis spectral analysis revealed that the major peak at 478 nm wavelength of CR was completely disappeared. FTIR analysis showed several major peaks along with azo bonds are completely or partly disappeared, deformed or widened. Chemical oxygen demand was reduced by 86.4, 85.5, 87.0 and 86.2% by C1, C2, C3 and C4, respectively. Accordingly, biodegraded metabolites of CR by different biofilm consortia did not inhibit the germination of wheat seeds and bacterial growth. Thus, these biofilm consortia can be applied in bioremediation of wastewater containing CR for safe disposal into the environment. To our knowledge, this is the first report on degradation and detoxification of aqueous solution containing CR by bacterial biofilm consortia.

Keywords Congo red · Biofilm consortia · Decolorization · Degradation · FTIR · Detoxification

Introduction

Congo red (CR) is a diazo dye, extensively used in textile, printing, food and biomedical industries worldwide. In microbiology, it is used in revealing of curli fimbriae (Haque et al. 2012, 2017). The wastewater containing CR is highly colored. Excessive release of such raw wastewater into aquatic ecosystems, such as rivers, ponds and cannels, remarkably decreases the photosynthetic ability of the aquatic plants by lowering the light penetration. The wastewater containing CR also deteriorates the water quality by depletion of dissolved oxygen, changing the pH, lowering the gas solubility, and increasing the biological oxygen demand, chemical oxygen demand and total organic carbon values (Forgacs et al. 2004; Chengalroyen and Dabbs 2013). CR-mixed wastewater is evident to diminish the seed germination, biomass production and soil fertility (Ghodake et al. 2009; Saratale et al. 2011; Sarim et al. 2019). Therefore, it is essential to treat the CR-containing wastewater before its final discharge into the environment.

The physicochemical methods, such as adsorption, coagulation–flocculation, filtration, ozonation, electrochemical oxidation, Fenton oxidation, sonochemical oxidation, advanced oxidation, ion exchange, reverse osmosis, and electrolysis, are used for the removal of CR from wastewater in many countries worldwide (Robinson et al. 2001; Huang et al. 2014; Ahmad et al. 2015; Ayati et al. 2016;

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Sathishkumar et al. 2019). However, most of these methods are expensive (Anjaneyulu et al. 2005; Vikrant et al. 2018), require high energy (Saratale et al. 2011), and produce a huge amount of sludge along with hazardous by-products (Robinson et al. 2001; Chengalroyen and Dabbs 2013; Kumar et al. 2019). Bioremediation is a microbial-based method, uses naturally occurring microorganisms to breakdown toxic substances into less toxic or non-toxic substances (Saratale et al. 2011). Compared with the physicochemical methods, bioremediation is less expensive, eco-friendly, requires less energy, produces less amount non-toxic sludge and mineralizes the dye completely (He et al. 2017). Hence, this method has been recommended to degrade and detoxify the azo dyes including CR from wastewaters (Rai et al. 2005; Pandey et al. 2007; Saratale et al. 2011).

A large number of fungi (Bhattacharya et al. 2011; Chakraborty et al. 2013; Wang et al. 2017; Asses et al. 2018), yeasts (Jadav et al. 2007; Jafaria et al. 2014), actinomycetes (Eun-Hee et al. 2005; Sathish and Joshua 2015) and bacteria (Gopinath et al. 2009; Saratale et al. 2011; Prasad and Aikat 2014; Abo-state et al. 2017; Kuppusamy et al. 2017) are effective to decolorize CR from aqueous solutions. Among them, bacterial decolorization is faster than other microorganisms (Saratale et al. 2011). Generally, single bacterial strain cannot achieve satisfactory removal of CR from wastewater due to the low substrate spectrum, adaptability and low enzyme activity (Gopinath et al. 2009). Furthermore, biodegraded metabolites of CR by free-living planktonic single bacterial strain were often found to be carcinogenic, mutagenic and genotoxic in nature due to the presence of aromatic amines (Joshi et al. 2008; Gopinath et al. 2009). Conversely, biodegraded metabolites of CR by planktonic bacterial consortia are almost non-toxic than the parent dye (Joshi et al. 2008; Asad et al. 2007; Lade et al. 2015a). In a bacterial consortium, the individual bacterial strain was shown to attack the dye molecule at different positions or utilized metabolites produced by the co-existing strain for further decomposition (Forgacs et al. 2004; Saratale et al. 2011). Thus, the degradation rate of a bacterial consortium is usually higher than that of a single bacterial strain. However, information regarding the uses of bacterial consortia to degrade and detoxify the CR is very limited in the literature (Asad et al. 2007; Joshi et al. 2008; Saratale et al. 2011; Abo-State et al. 2017). Free-living planktonic bacterial consortia are reportedly susceptible to toxic environments due to decreased protection and low metabolic activity combined with low bioavailability of the pollutants (McDougald et al. 2012; Koechler et al. 2015). Thus, more studies are required to find out bacterial consortia capable of degrading CR into less toxic metabolites even under toxic environments.

Biofilms are highly organized surface-associated microbial cells, encased in self-produced extracellular polymeric substances (EPS). EPS comprises curli fimbriae,

cellulose-rich polysaccharides and lipids (McDougald et al. 2012; Haque et al. 2017; Mosharaf et al., 2018). It was reported that bacteria associated with biofilms are resistant to environmental stressors, including high concentration of toxic chemicals (including heavy metals and dyes), pH, temperature and salinity as compared to their planktonic counterparts (Haque et al. 2012, 2017; McDougald et al. 2012; Koechler et al. 2015). Despite these advantages, the significance of enabling bacterial biofilms to enhance detoxification has just recently been appreciated and applied (Edwards and Kjellerup 2013). Recently, we isolated metal-adapted biofilm-producing bacteria, such as *Escherichia coli* ENSD101, *Enterobacter asburiae* ENSD102, *E. ludwigii* ENSH201, *Vitreoscilla* sp. ENSG301, *Acinetobacter lwoffii* ENSG302, *Klebsiella pneumoniae* ENSG303, *Pseudomonas fluorescens* ENSG304 and *Bacillus thuringiensis* ENSW401 from different wastewaters of Bangladesh (Mosharaf et al. 2018). In this study, we developed four biofilm consortia using these bacterial strains. The assumption is that these consortia might degrade and detoxify the CR completely. The variation of operational and physicochemical parameters, such as carbon and nitrogen sources, temperature, pH, dye concentration, agitation and incubation period, plays a vital role in the decolorization of azo dyes in bioremediation processes (Gopinath et al. 2009; Saratale et al. 2011; Prasad and Aikat 2014). Therefore, we studied the effect of these important physicochemical operational parameters on decolorization of CR in bioremediation processes. Various oxidoreductive enzymes have been shown to synthesize by different bacteria to degrade azo dyes (Saratale et al. 2011; Telke et al. 2015; Sarkar et al. 2017; Bhatia et al. 2017). Among them, azoreductase and laccase are the principal enzymes in degradation of CR (Imran et al. 2014; Lade et al. 2015a, b). Hence, we also quantified the production of azoreductase and laccase by different biofilm consortia. The biodegraded metabolites of CR were analyzed by UV–Vis spectrophotometer and Fourier-transform infrared (FTIR) spectroscopy. This study will contribute toward understanding the mechanism of decolorization of CR by bacterial biofilm consortia. To our knowledge, this is the first report, so far, on degradation and detoxification of CR by bacterial biofilm consortia under various nutritional and environmental conditions.

Materials and methods

Dyes and chemicals

Congo red (CR), Methyl red, 2, 2′-azono-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), nicotinamide adenine dinucleotide (NADH) were obtained from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals used were of

an analytical grade and were purchased from Wako Pure Chemical Industries, LTD (Japan), Bio Basic Canada Inc. (Canada) and HiMedia (Mumbai, India).

Bacterial strains and growth conditions

Escherichia coli ENSD101, *Enterobacter asburiae* ENSD102, *E. ludwigii* ENSH201, *Vitreoscilla* sp. ENSG301, *Acinetobacter lwoffii* ENSG302, *Klebsiella pneumoniae* ENSG303, *Pseudomonas fluorescens* ENSG304 and *Bacillus thuringiensis* ENSW401 used in this study were collected from our laboratory stock. All these bacterial strains were reported to produce biofilms in the SOBG broth at 28 °C in static condition (Mosharaf et al. 2018). These strains were routinely grown in yeast extract peptone (YP) medium at 28 °C. The optical density (OD at 660 nm) of the culture was measured by an absorption spectrophotometer (Intertech, Inc. Tokyo, Japan).

Compatibility tests

Compatibility tests were carried out as described in Furuya et al. (1997) with a few modifications. In brief, a single colony of each producer bacterium was spotted onto YP agar plate then incubated at 28 °C in static condition for 36 h. To kill the producer bacterium, a sheet of sterilized Whatman™ filter paper containing 0.5 mL chloroform was placed in Petri dish lid, then incubated at room temperature (25 ± 2 °C). After 2 h incubation, producer bacteria were covered with 5 mL melted water agar (1.5% at 50 °C) containing a suspension of indicator bacterium [ca. 10^8 cfu (colony forming unit)/mL] and incubated at 28 °C for 48 h. If no inhibition zone was observed around producer bacteria, then these bacteria were considered as compatible.

Preparation of consortia

Initially, a single colony of each compatible bacterium was inoculated in 50 mL Erlenmeyer flask containing 10 mL YP broth and incubated at 28 °C in shaking (160 rpm) condition until optical density (OD_{660}) reached at 1.0. Then, each culture was centrifuged at 10,000 rpm for 10 min. After discarding the supernatant, the cell pellet was re-suspended in 10 mL salt-optimized broth [(SOB) per liter, 20 g of tryptone, 5 g of yeast extract, 0.5 g of NaCl, 2.4 g of MgSO4, 0.186 g of KCl plus 2% glycerol (SOBG)]. Each consortium (10 mL, ca. 10^8 CFU/mL) was prepared using equal volume of compatible bacterial suspension (ca. 10^8 CFU/mL). Based on compatibility tests, four consortia were prepared as follows: C1 composed of *Vitreoscilla* sp. ENSG301, *A. lwoffii* ENSG302, *K. pneumoniae* ENSG303 and *P. fluorescens* ENSG304, C2 consisted of *E. coli* ENSD101, *E. asburiae* ENSD102 and *E. ludwigii* ENSH201, C3 contained *E.*

asburiae ENSD102, *Vitreoscilla* sp. ENSG301 and *B. thuringiensis* ENSW401, and C4 comprised *E. coli* ENSD101, *E. ludwigii* ENSH201 and *B. thuringiensis* ENSW401. All these consortia were used for the decolorization of CR.

Decolorization and process optimization studies

For decolorization experiment, 10 mL culture (ca. 10^8 CFU/mL) of each consortium was inoculated in 250 mL Erlenmeyer flasks containing 90 mL broth with 100 mg/L CR. The flasks were then kept at 28 °C in stationary condition. Within 72 h, 3 mL suspension was collected and centrifuged at 14,000 rpm for 15 min. The resultant supernatant was carefully collected in fresh test tubes. Decolorization was checked by measuring the change in absorbance of cultured supernatants at the maximum absorption wavelength (λmax) of 478 nm in UV–Vis spectrophotometer (Ultrospec-3000, Pharmacia Biotech, Cambridge, England). Abiotic control was also included. The decolorization efficiency was calculated as described (Prasad and Aikat 2014).

Decolorization (%)

$$= \frac{\text{Initial absorbance} - \text{Final absorbance}}{\text{Initial absorbance}} \times 100$$

CR decolorization in different nutritional conditions [nutritional strength (e.g., full strength SOB plus 2% glycerol, ½ strength SOB plus 2% glycerol and ¼ strength SOB plus 2% glycerol), carbon sources (glycerol and glucose) and nitrogen sources (tryptone and yeast extract)], incubation conditions (shaking and static), CR concentrations (50, 100, 150 and 200 mg/L), temperatures (20, 28, 37 and 42ºC), pH (5, 6, 7, 8 and 9), and incubation periods (24, 48, 72, 96, 120, 144 and 168 h) were also studied.

Quantification of oxidoreductive enzyme produced by different biofilm consortia

Each biofilm consortium (10 mL) was inoculated in ¼ strength SOB containing 2% glycerol and 100 mg/L CR then incubated at 28 °C in static condition. Within 72 h incubation, each biofilm culture was collected, then centrifuged at 14,000 rpm for 15 min at 4 °C. For extracellular enzyme production, the supernatant was carefully collected and assessed. For intracellular enzyme production, the cell pellet was re-suspended in sterile deionized water, then broken down the cells using ultrasonic cell disrupter (Ultrasonic Disrupter UD-200, Tommy, Tokyo, Japan). After sonication, the suspension was centrifuged at 14,000 rpm for 5 min at 4 °C and supernatant was collected. Production of extracellular and intracellular laccase (an oxidative enzyme) and azoreductase (a reductive enzyme) by different biofilm consortia were measured using UV–Vis spectrophotometer

(Ultrospec-3000, Pharmacia Biotech, Cambridge, England). Laccase specific activity was measured as described in Welfenden et al. (1982) by monitoring the increase in optical density at 420 nm. On the other hand, azoreductase specific activity was determined by monitoring the reduction in concentration of Methyl Red at 430 nm (Chen et al. 2005). Lowry method (Lowry et al. 1951) was used to determine protein with bovine serum albumin as standard.

Analysis of biodegraded metabolite by UV–Vis spectrophotometer

Biodegraded metabolite from each biofilm consortium was prepared as follows: each biofilm consortium was inoculated in ¼ strength SOB containing 2% glycerol and 100 mg/L CR, then incubated at 28 °C in static condition. Within 72 h incubation, 1.5 mL broth of each consortium was collected, then centrifuged at 13,000 rpm for 20 min. The pellets were discarded. The supernatant was carefully collected and again filtered through Einmalfilter [CHROMAFIL® Xtra PTFE-45/25, 0.45 µM (Macherey–Nagel, GmbH and Co. KG, Germany)]. Each biodegraded metabolite was analyzed using UV–Vis spectrophotometer (Ultrospec-3000, Pharmacia Biotech, Cambridge, England) within 350 to 700 nm wavelength. For control, ¼ strength SOB containing 2% glycerol and 100 mg/L CR was used.

Fourier-transform infrared (FTIR) spectroscopy

The biodegraded metabolite was prepared as described earlier section. Perkin Elmer FTIR (Spectrum-2) instrument operated by CPU32M software was used to acquire the IR spectra. Triglycine sulphate (TGS) detector was applied for FTIR scanning within 450 to 4000 cm^{-1} (16 scans at 4 cm^{-1} at 0.2 cm/sec scanning speed). The ¼ strength SOBG without CR was also scanned. Perkin Elmer's proprietary software (Version 10.05.03) was used to analyze the baseline subtracted spectra.

Toxicity tests

Removal of COD

For COD removal studies, biodegraded metabolite by each biofilm consortium was prepared as mentioned in earlier section. The COD removal by each biofilm consortium was determined by the dichromate closed reflux titrimetric method (APHA 2012) using the following equation:

$$\text{COD removal}(\%) = \frac{\text{Initial COD} - \text{Final COD}}{\text{Initial COD}} \times 100$$

Seed germination tests

For phytotoxicity tests, wheat (*Triticum aestivum* L.) seeds were collected from Bangladesh Wheat and Maize Research Institute, Bangladesh. Seeds were surface-sterilized with 1% sodium hypochlorite for 3 min, then rinsed 3–4 times with sterile distilled water. Three sterilized Whatman™ filter papers were soaked in cell-free biodegraded metabolite and placed in Petri plates. Then 20 seeds were placed in each plate. A total of 200 seeds were tested in each case. The seeded plates were kept at 20 °C and checked regularly. To maintain moisture content, adequate amount of biodegraded metabolites or 1/4 strength SOBG containing 100 mg/L CR was added every alternate day. At day 8, germinated seeds were counted and compared.

Bacterial growth inhibition tests

Two plant-growth-promoting rhizobacterial strains, including *Pseudomonas chlororaphis* ESR15 and *Bacillus aryabhattai* ESB6 used in this study were collected from our laboratory stock. For growth, a single colony of each bacterial strain was inoculated in glass test tubes containing 5 mL cell-free biodegraded metabolite containing 2% glycerol then incubated at 28 °C in shaking (160 rpm) condition. For control experiment, each strain was inoculated in 5 mL SOBG broth. Bacterial growth (OD$_{660}$) was measured by spectrophotometer after 24 h incubation.

Statistical analysis

All the experiments were laid out in a complete randomized design with three replications and repeated at least twice. The "agricolae" of R software version 3.3.3 was used to calculate analysis of variance and comparison of means. The means were compared using Fisher's least significance difference (LSD) test.

Results and discussion

Nutritional conditions on CR decolorization

All the bacterial strains used in the preparation of consortia were reported to produce biofilms on SOBG broth after 72 h incubation at 28ºC in static condition (Mosharaf et al. 2018). In this study, all these biofilm consortia i.e., C1, C2, C3 and C4 were also formed the air–liquid (AL) biofilms on full strength SOB (data not shown), ½ strength SOB (data not shown) and ¼ strength SOB containing 2% glycerol plus 100 mg/L CR (Fig. 1a) during biodegradation process. The clear biodegraded solutions were found after discarding the bacterial cells from the broths (Fig. 1b). In static condition,

Fig. 1 Decolorization of CR by different biofilm consortia within 72 h incubation at 28 °C in static condition. **a** All these consortia formed the air–liquid biofilms on the flasks containing 100 mL 1/4 strength SOB plus 2% glycerol along with 100 mg/L CR as shown by arrows. **b** Decolorized biodegraded metabolites of CR by different biofilm consortia along with non-biodegraded sample (red color) (color figure online)
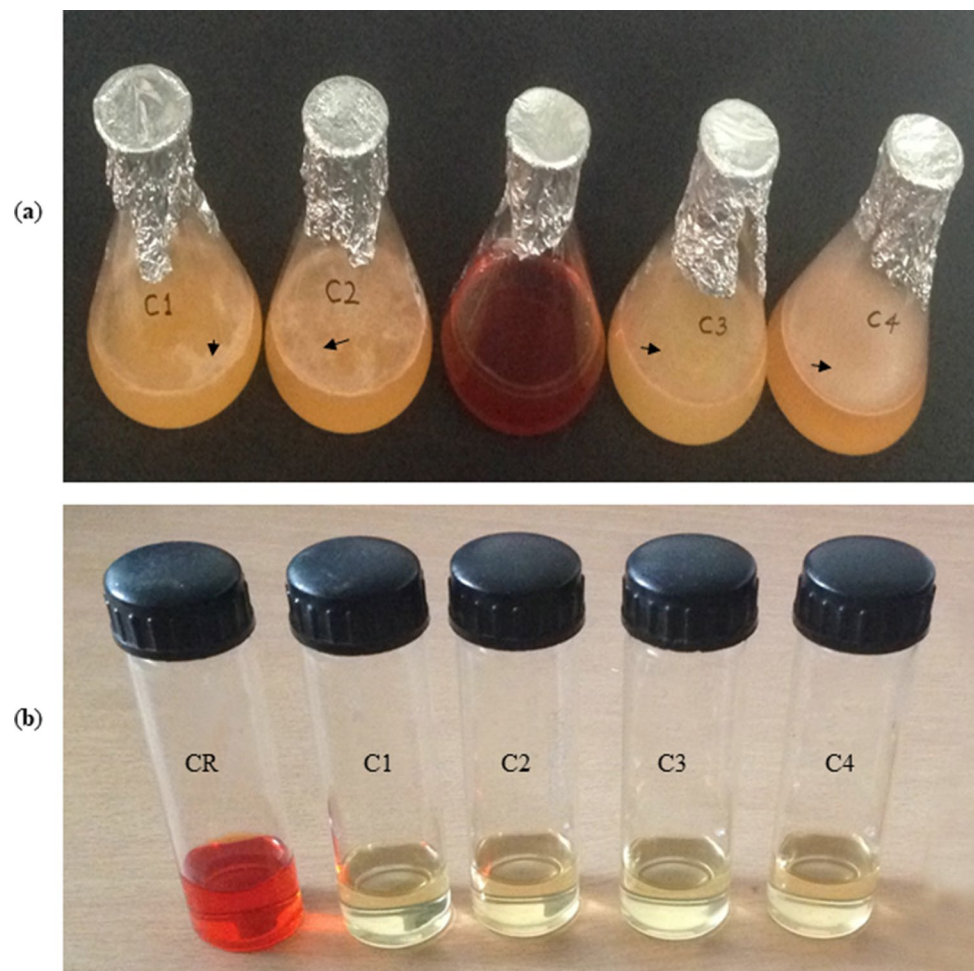
the rate of decolorization within 72 h incubation varied from 98.06 to 99.48%, 98.02 to 98.41% and 96.91 to 97.71% in full strength SOB, ½ strength SOB and ¼ strength SOB containing 2% glycerol, respectively (Fig. 2a). Decolorization percentage was found to be only slightly lessened in ¼ strength SOB containing 2% glycerol than the full strength SOB supplemented with 2% glycerol in static condition (Fig. 2a). Compared to static condition, CR removal by these consortia was decreased by 21.95–34.08%, 25.96–38.33% and 25.66–44.99% in full strength SOB, ½ strength SOB and ¼ strength SOB containing 2% glycerol, respectively, in agitated condition (Fig. 2a). Lade et al. (2015a) observed that only 14% CR was removed by the microbial consortia composed of eight bacterial strains in aerobic (120 rpm) condition, while 99% CR removal was achieved in microaerophilic condition. Removal of Reactive blue 160 (a diazo dye) was also sturdily inhibited by oxygen-rich conditions (remove only 24% after 4 h) than under microaerophilic (remove 90% after 4 h) conditions (Balapure et al. 2014). Furthermore, Babu et al. (2015) reported a vividly increased decolorization (93.10%) of CR by *Dietzia* sp. DTS26 in static condition

compared to the shaking condition (28.56%) within 30 h. Availability of molecular oxygen strongly hindered azo dye removal (Chang et al. 2001), due to competition of NADH with oxygen or azo groups as the electron receptor leading to shrink dye removal (Chung et al. 1992).

To examine the role of carbon source on CR removal in static condition, 2% glycerol was replaced by 2% glucose. The similar results were found within 72 h incubation period (Fig. 2b). However, when 2% glycerol was excluded from the 1/4 strength SOB containing 2% glycerol, decolorization percentage in C1, C2, C3 and C4 was reduced by 63.6, 63.9, 56.2 and 59.5% as compared to 1/4 strength SOB containing 2% glycerol (Fig. 2b). The similar results were also observed when 2% glucose was discarded from 1/4 strength SOB containing 2% glucose (Fig. 2b). Thus, a carbon source is required for the enhancement of the rate of the decolorization of CR. It was stated that reducing equivalents from various carbon sources are transferred to the dye during decolorization of azo dyes via reduction of azo bonds (Saratale et al. 2011). Conversely, Saratale et al. (2009a) remarked that carbon sources only stimulated the microbial growth
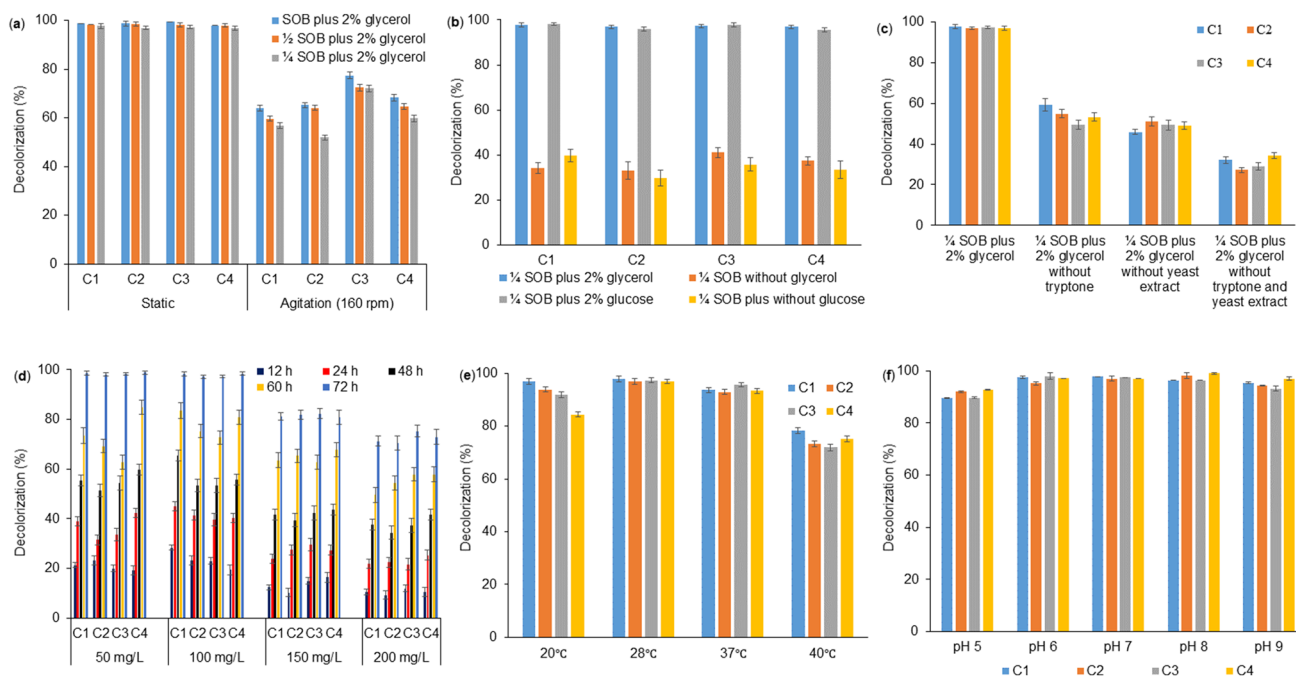
Fig. 2 Nutritional and environmental conditions on decolorization of CR by different biofilm consortia: **a** nutritional strength, **b** carbon sources (2% glycerol/glucose), **c** nitrogen sources (tryptone/yeast extract), **d** concentrations of CR (50 to 200 mg/L), **e** temperature, and **f** pH. The 1/4 strength SOB plus 2% glycerol was used for all the experiments and incubated in static conditions (except nutri- tional strength) at 28 °C (except for the effect of temperatures). The 1/4 strength SOB plus 2% glycerol was supplemented with 100 mg/L CR (except for the concentrations studies). The pH of the broth was always adjusted to 7.0 (except for the effect of pH). Data represent averages from triplicate assays and error bars showing standard devia- tion

but not decolorize the azo dyes. In this study, we found that 2% glycerol stimulated the microbial growth (Fig. 1a), thus, enhanced the rate of decolorization of CR (Fig. 2a).

To evaluate the effect of nitrogen sources on CR decol- orization, we excluded tryptone and/or yeast extract or both from 1/4 SOB plus 2% glycerol. Bacterial growth (data not shown) and decolorization rate were harshly abridged by exclusion of tryptone and/or yeast extract or both (Fig. 2c). It was reported that peptone, tryptone, beef extract and yeast extract played a pivotal role in generation of NADH, which has been shown to act as an electron donor for the reduction of azo dyes by microorganisms (Chang et al. 2000).

Effect of concentrations of CR on decolorization

To order to quantify the impact of different concentrations of CR (50–200 mg/L) on decolorization, a time-course experi- ment was carried out (Fig. 2d). The decolorization increased significantly as the time increased and reached maximum within 72 h incubation in static condition responding to the concentrations of CR. Conversely, CR removal was notably decreased as the concentrations increased. The C4 consortium displayed the highest decolorization (98.91%) which was identical with C1 (98.75%), C2 (97.95%) and C3 (98.23%) responding to 50 mg/L CR. Interestingly, CR

removal efficiencies by these biofilm consortia were found to be alike in the concentrations of 50 and 100 mg/L CR. On the other hand, CR removal was reduced only 16% and 26% in response to 150 and 200 mg/L, respectively, as compared to 100 mg/L of CR within 72 h incubation period (Fig. 2d). However, bacterial growth rate was indistinguishable in these concentrations (50–200 mg/L) of CR (data not shown). Generally, textile wastewater contains 10–200 mg/L of struc- turally different dyes (Pandey et al. 2007). It was reported that increasing the dye concentration gradually decreased the decolorization rate due to inadequate cell biomass, ratio of cell biomass and dye, toxic effect of the dyes and obstruction of active sites of azoreductase by dye molecules with dif- ferent structures (Jadhav et al. 2008; Saratale et al. 2009b). However, this toxic effect was reported to be compromised using bacterial co-culture than pure culture (Lade et al. 2015a; Bhatia et al. 2017; Saratale et al. 2009a).

Temperature on CR decolorization

Figure 2e depicts that different biofilm consortia are able to remove the CR (100 mg/L) over a wide range of tempera- tures (20–40 °C). However, the growth rate of different bio- film consortia was considerably reduced at 40 °C but not at temperature tested (data not shown). At 28 °C, a maximum

of 97.23% color removal was recorded followed by 93.83% (37 °C), 91.76% (20 °C) and 74.6% (40 °C) within 72 h incubation. Similarly, decolorization rates were reported to be only marginally affected when incubated at lower and higher temperatures than the optimum (Lade et al. 2015b). However, several researchers stated that incubation at high temperatures markedly reduced both microbial growth and enzymatic activities leading to poorer dye decolorization (Saratale et al. 2011; Phugare et al. 2011; Selvakumar et al. 2013).

pH on CR decolorization

The effect of different pHs on decolorization of CR dye (100 mg/L) is shown in Fig. 2f. At pH 8, the maximum CR removal (97.45%) was achieved followed by pH 7 (97.23%), pH 6 (96.89%), pH 9 (94.89%) and pH 5 (90.91%) within 72 h incubation at 28 °C in static conditions. The results suggested that all these biofilm consortia are able to decolorize CR in acidic to alkaline conditions. The maximum decolorization (90.81%) of CR by *Dietzia* sp. DTS26 was reported at pH 8 within 30 h (Chang et al. 2001). Maximum color removal was stated to accomplish at pH 6–10 (Chen et al. 2003; Kilic et al. 2007). The pH was shown to play an important role in transportation of dye molecules across the cell membrane (Kodam et al. 2005) and biological reduction of azo bonds (Willmott 1997). Dye removal rate was reported to increase 2.5-fold when pH raised to 7 from 5, however, increment of pH from 7 to 9 seemed to be less effective (Chung et al. 1992). According to another report, a microbial consortium composed of 15 different bacteria was proficient in decolorization of Trypan blue, a diazo dye, at pH 7.0, while a slight decrease was reported at pH 6.0, however, the increase of pH 8.5 did not have significant on dye removal (Lade et al. 2015b). Textile effluents generally possess the alkaline pH (Kuhad et al. 2004). Thus, all these biofilm consortia used in this study might be applied to treat the dye-containing wastewater with variable pH ranges.

Mechanism of biodegradation

Production of oxidoreductive enzymes by different biofilm consortia

We measured the specific activity of azoreductase and laccase enzyme produced by different biofilm consortia (Table 1). There were no significant variations found amongst the extracellular and intracellular specific activities of azoreductase and laccase enzymes synthesized by these biofilm consortia (Table 1). However, as presented in Table 1, both the enzymes showed significantly higher intracellular specific activities than those of extracellular ones (Table 1). As reported in the literatures, azoreductase

Table 1 Specific activity of oxidoreductive enzymes secreted by different biofilm consortia within 72 h incubation at 28 °C in static condition

Consortia	Azoreductase[1]		Laccase[2]	
	Extracellular	Intracellular	Extracellular	Intracellular
C1	1.26 ± 0.02	3.58 ± 0.08*	1.43 ± 0.02	2.39 ± 0.05*
C2	1.23 ± 0.02	3.61 ± 0.09*	1.49 ± 0.01	2.43 ± 0.07*
C3	1.28 ± 0.02	3.54 ± 0.07*	1.41 ± 0.02	2.38 ± 0.05*
C4	1.25 ± 0.02	3.57 ± 0.07*	1.43 ± 0.02	2.45 ± 0.07*

Vales are mean of three independent experiments

± Indicate standard deviation

*Indicate $P \leq 0.001$ by one-way ANOVA with Tukey-Kramer comparison test

[1] µM of Methyl red reduced min^{-1}mL of $enzyme^{-1}$mg of $protein^{-1}$

[2] µM of ABTS oxidized min^{-1}mLof $enzyme^{-1}$mg of $protein^{-1}$

enzyme plays an active role in the reductive cleavage of azo bonds (Lade et al. 2015a; Olukanni et al. 2013), while laccase enzyme was reported to oxidize the secondary metabolites formed after decolorization (Imran et al. 2014; Olukanni et al. 2013; Telke et al. 2009). Thus, combined action of azoreductase and laccase enzyme might be the responsible for the decolorization and degradation of CR.

UV–Vis spectral analysis

The extent of decolorization of CR by different biofilm consortia was measured by the UV–Vis spectrophotometer (350–700 nm) and the observations are presented in Fig. 3. The untreated CR erected the major peak was found in 478 nm wavelength. However, this peak was completely disappeared when the dye solutions were treated by different biofilm consortia within 72 h incubation at 28 °C. The disappearance of peak suggests the success of CR decolorization using the studied microbial consortia (Chen et al. 2003).

FTIR analysis

FTIR analysis was carried out to explain the possible changes of functional groups during decolorization of CR. The shifting or alteration of the structural bonds indicated in the FTIR analyses confirmed the ability of the bacterial biofilm consortia to degrade CR (Fig. 4). The standard CR diluted in 1/4 strength SOB containing 2% glycerol produced several characteristic peaks near to 925.83 cm^{-1}, 995.05 cm^{-1}, 1042.82 cm^{-1}, 1112 cm^{-1}, 1226 cm^{-1}, 1368 cm^{-1}, 1406.95 cm^{-1}, 1575 cm^{-1}, 1630 cm^{-1}, 1673.43 cm^{-1}, 1706.43 cm^{-1}, 2845.32 cm^{-1}, and 2945.52 cm^{-1} (Fig. 4a) which are the documented IR peak regions for CR (Olukanni et al. 2013; Prasad and Aikat 2014; Lade et al. 2015a). The peak at 1042.82 cm^{-1} of the

Fig. 3 UV–Vis spectra of non-biodegraded (control) and biodegraded metabolites of CR by different biofilm consortia within 72 h incubation at 28 °C in static condition
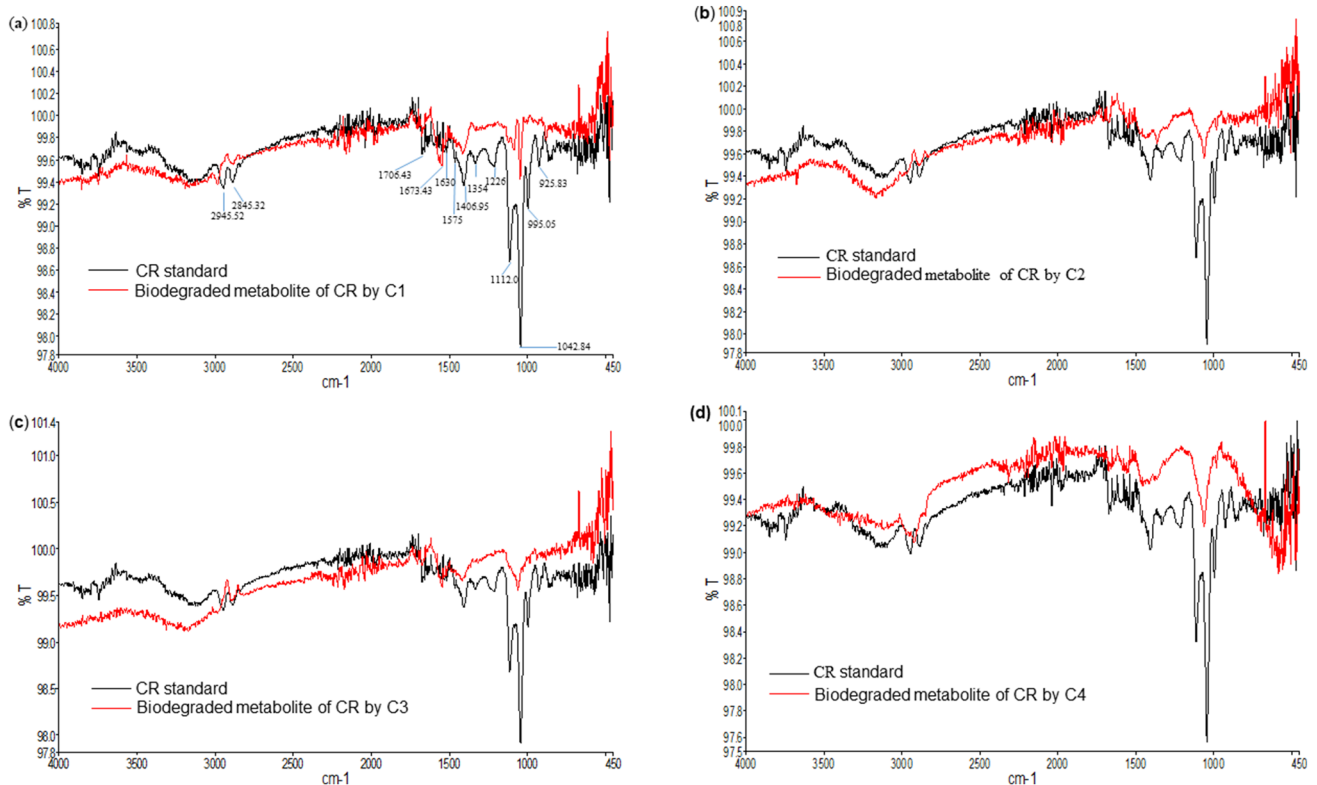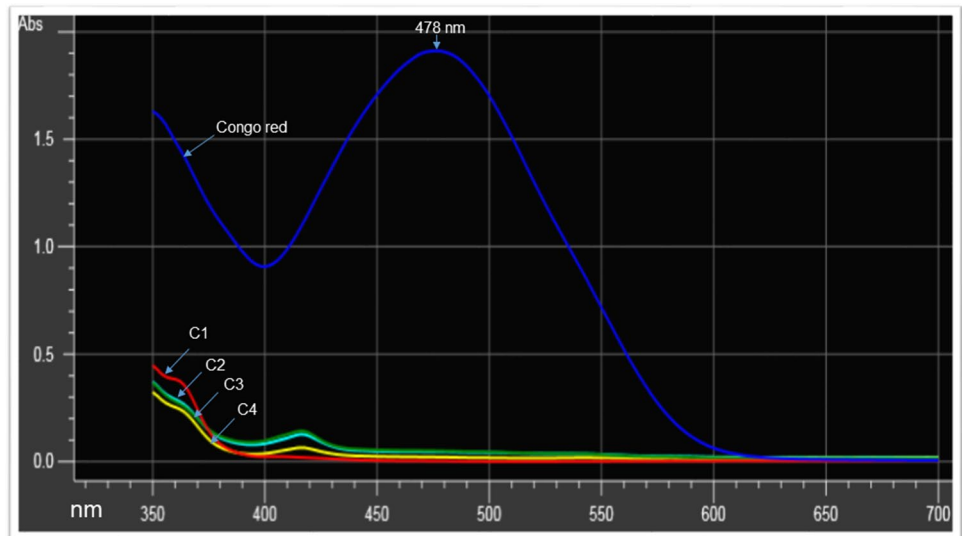

Fig. 4 Fourier-transform infrared spectroscopy (FTIR) of non-biodegraded (control) and biodegraded metabolites of CR by different biofilm consortia, such as C1 (**a**), C2 (**b**), C3 (**c**) and C4 (**d**) consortium within 72 h incubation at 28 °C in static condition

CR represents stretching vibration of S＝O, 1226 cm^{-1} for C–N stretching vibrations, 1368 cm^{-1} for C–N bending vibrations, 1575 cm^{-1} for N＝N stretching vibrations, while the peak at 2945.52 cm^{-1} is produced from the symmetric stretching (C–H). In this study, we observed that the principal characteristic peaks are completely or partly disappeared, deformed or widened. However, degradation of

CR by different bacterial biofilm consortia might vary. The cleavage of azo bond was confirmed by the absence of peak at 1575 cm^{-1} for N＝N stretching vibrations in the FTIR spectra (Fig. 4a–d) of biodegraded CR samples. Telke et al. (2010) reported that the absence of peak at 1578 cm^{-1} corresponds to N＝N stretching vibrations in the FTIR spectrum for degraded metabolites of CR. Absence of N＝N indicates

Table 2 COD before and after treatment by different biofilm consortia

Samples	COD (mg/L)	% COD removal
Untreated control	934.7 ± 6.19	–
Biodegraded metabolite by C1	126.8 ± 3.22	86.4
Biodegraded metabolite by C2	134.7 ± 5.24	85.5
Biodegraded metabolite by C3	121.3 ± 4.61	87.0
Biodegraded metabolite by C4	129.3 ± 5.18	86.2

Values are mean of three independent experiments

± Indicate standard deviation

the cleavage of azo bond by the different biofilm consortia. Our UV–Vis and FTIR results suggested that secondary metabolites are generated after degradation of CR by different biofilm-producing consortia. Gas chromatography-mass spectrometry (GC–MS) and High-performance liquid chromatography (HPLC) are also used for the determination of secondary metabolites of azo dyes formed after bacterial treatment. Based on GC–MS and HPLC, 4-amino-3-diazenylnaphthalene-1-sulfonate and phenylbenzene were identified as the secondary metabolites of CR degraded by *B. thuringiensis* (Olukanni et al., 2013). On the other hand, Lade et al. (2015b) have found biphenyl-1,4´-diamine, biphenyl and naphthalene are the secondary metabolites of CR degraded by a microbial consortium. Thus, secondary metabolites of CR might vary in response to different bacterial strains or consortia. Thus, future studies should focus on identifying the secondary metabolites of CR degraded by bacterial biofilm consortia.

Detoxification studies

COD removal

To confirm mineralization, we compared COD removal before and after treatment (72 h incubation at 28 °C in static conditions) by different biofilm consortia (Table 2). The decolorized CR solutions by C1, C2, C3 and C4 consortia showed 86.4, 85.5, 87.0 and 86.2% reduction of COD,

respectively. The enhanced reduction of COD indicates the efficient biodegradation of the CR dye. *Dietzia* sp. was found to reduce COD by 86.4% within 30 h at 100 mg/L of CR dye (Babu et al. 2015).

Biodegraded metabolites are non-toxic

Germination percentage varied from 87.5 to 93.8% when biodegraded metabolites used as irrigation water, while 73.6% germination was noted in control (i.e., ¼ SOBG containing 2% glycerol plus 100 mg/L CR used as irrigation water) (Table 3). Toxicity tests were also done using plant-growth-promoting rhizobacteria because of their agricultural importance. In this study, growth rate of *P. chlororaphis* ESR15 and *B. aryabhattai* ESB6 were indistinguishable in biodegraded metabolites and SOBG broth after 24 h incubation in shaking condition (Table 3). After that, bacterial treatment of extract of degraded metabolites was also reported to less toxic than the parental compound (Saratele et al. 2011; Babu et al. 2015). These results indicated that biodegraded metabolites are non-toxic. Thus, all these biofilm consortia can be used to degrade and detoxify the CR from wastewater.

Conclusion

In this study, we developed four novel bacterial biofilm consortia. All these biofilm consortia remarkably decolorized the CR (100 mg/L) in static condition within 72 h incubation at 28 °C. These consortia synthesized significantly more intracellular azoreductase and laccase enzyme than those of extracellular of these enzymes. UV–Vis spectral analysis revealed that the major peak at 478 nm wavelength of CR was completely disappeared. FTIR analysis showed several major peaks are completely or partly disappeared, deformed or widened. Toxicity tests revealed that biodegraded metabolites of CR are non-toxic. Thus, these biofilm consortia can be applied in bioremediation of CR from wastewater for safe disposal into environment.

Table 3 Effect of biodegraded metabolites on germination of wheat seeds and bacterial growth

Samples	Germination (%)	OD_660	
		P. chlororaphis	*B. aryabhattai*
Untreated control	73.6 ± 1.0	1.53 ± 0.13	1.49 ± 0.18
Biodegraded metabolite by C1	93.8 ± 2.0	1.69 ± 0.21	1.62 ± 0.20
Biodegraded metabolite by C2	89.6 ± 1.0	1.56 ± 0.17	1.53 ± 0.19
Biodegraded metabolite by C3	87.5 ± 1.5	1.52 ± 0.14	1.50 ± 0.13
Biodegraded metabolite by C4	91.2 ± 2.0	1.65 ± 0.22	1.58 ± 0.18

Values are mean of two independent experiments

± Indicate standard deviation

Compliance with ethical standards

Conflicts of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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