



Original article

Decolorization, degradation and detoxification of carcinogenic sulfonated azo dye methyl orange by newly developed biofilm consortia

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ABSTRACT

Metabolites of azo dyes are often carcinogenic, teratogenic, mutagenic and recalcitrant in nature. In this study, four biofilm consortia such as 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 applied to degrade and detoxify methyl orange (MO), a carcinogenic, sulfonated mono azo dye, used in textile dyeing industry worldwide. The consortia of C1, C2, C3 and C4 showed 97.30, 98.75, 99.51 and 99.29% decolorization, respectively in yeast extract peptone (YEP) broth containing 200 mg L⁻¹ MO within 60 h of incubation in static condition. The optimum pH and temperature for decolorization was 7.0 and 28 °C, respectively. Some divalent metal ions including Mg²⁺, Ca²⁺, Zn²⁺ and Mn²⁺ could stimulate MO decolorization. UV–Vis spectral analysis showed that the absorption peak at 465 nm originated from the azo (N=N) bond was completely disappeared within 60 h of incubation. Fourier transform infrared spectroscopy (FTIR) results also revealed that several major peaks including azo bond peak at 1602.6 cm⁻¹ are completely or partly vanished, deformed or shifted. Activities of azoreductase, NADH-DCIP reductase and laccase were significantly increased in the bacterial cells within 60 h of incubation in comparison to that of control (0 h). The chemical oxygen demand was incredibly reduced by 85.37 to 91.44% by these consortia. Accordingly, plant (wheat seed germination) and microbial (growth of the plant probiotic bacteria such as *Pseudomonas cedrina* ESR12 and *Bacillus cereus* ESD3 on biodegraded products) toxicity studies showed that biodegraded products of MO are non-toxic. Thus, all these consortia can be utilized in bioremediation of MO from wastewater for safe disposal into environment. To our knowledge, this is the first report on degradation and detoxification of MO from wastewater by bacterial biofilm consortia.

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1. Introduction

Discharge of untreated wastewater from various industries including textile, paper, tannery, cosmetics, and pharmaceuticals, is one of the major causes of environmental pollution. Among them, textile dyeing industry generates a huge amount of wastewater every day (Saratale et al., 2011). In Bangladesh, textile dyeing

industry used on average 250 to 350 L of water to dye and wash a kg of fabric, and around 85% of this wastewater are discharged into the environment without any treatments (https://www.textilepact.net/pdf/publications/reports-and-award/an_analysis_of_industrial_water.pdf; Accessed 08 August 2020). Cui et al. (2016) have reported that 2 to 50% of the dyes remain unused and are present in the wastewater. In fact, textile dyeing wastewater contains 10 to 200 mg L⁻¹ of synthetic dyes (Pandey et al., 2007) which are highly colored (Singh et al., 2012). However, some dyes are visible in water even at very low (<1 mg/L) concentrations (Bell et al., 2000). It is forecasted that textile dyeing industries would be dumping a mind-boggling 20,300 crore liters of untreated wastewater into nearby waterbodies every year from 2021 in Bangladesh (<https://www.textiletoday.com.bd/invisible-price-pay-producing-garment-concerning-environment/>; Accessed: 08 August 2020).

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Thus, textile dyeing industries of Bangladesh has been listed as 'Red industries' (most polluting). Recently, Routoula and Patwardhan (2020) have reported that up to 84,000 tons of dye can be lost in water from textile and leather industry per year, responsible for about 20% of the industrial water pollution (Kant, 2012).

Among the synthetic dyes, azo dye accounts for approximately 80% of the total amount of organic dyes (Yang et al., 2019). Azo dye contains one or more azo ($-N=N-$) groups and sulfonic (SO_3^-) groups (Chang et al., 2004; Sarkar et al., 2017), linking phenyl, naphthyl rings usually substituted with triazine amine, chloro, hydroxyl, methyl, nitro and sulfonate (Bell et al., 2000). Disposal of dye-containing wastewater into agricultural fields, wetlands and rivers has the following disadvantages: (i) being human health hazards, (ii) reducing the soil fertility and crop quality, (iii) reducing the photosynthetic capacities of the aquatic plants leading to loss of biodiversity, (iv) deteriorating the water quality by reducing dissolved oxygen (DO) and the gas solubility, and increasing the biological oxygen demand (BOD), chemical oxygen demand (COD) and total organic carbon (TOC) values (Chengalroyen and Dabbs, 2013; Du et al., 2015; Alam et al., 2017; Afrad et al., 2020). Methyl orange [(MO) dimethylaminoazobenzenesulfonate] is a sulfonated azo dye, used not only in textile dyeing industry but also as pH indicator in biomedical. Azo dye metabolites including MO were reported to be carcinogenic, teratogenic, mutagenic and highly recalcitrant in nature (Chen et al., 2011; Prabhakar et al., 2019). Thus, MO-containing wastewater should be decolorized and detoxified before discharged into the environment.

Physicochemical methods are used for decolorization and detoxification of MO from wastewater (Huo et al., 2013; Youssef et al., 2016; Xie et al., 2016; Gómez-Obando et al., 2019). However, many of these methods have severe drawbacks including costly, high energy and reagent requirements, incomplete decolorization and production of sludge along with hazardous by-products. Advanced oxidation processes (AOP) utilize oxidizing agents and/or catalysts to decolorize and detoxify the dye MO (Haji and Al-Bastaki, 2011; Niu, 2013). This process is also highly expensive and energy consuming. Bioremediation technologies or biological methods use microbes (e.g., fungi, yeast, bacteria, actinomycetes and algae) and/or their enzymes and plants to degrade and detoxify the azo dyes from wastewater (Saratale et al., 2011). Compared to the physicochemical and AOP methods, biological methods have numerous advantages. They are cheap, environment friendly, produce less sludge, require less water and yield nontoxic metabolites (He et al., 2017). Among the microorganisms, bacterial decolorization is faster than fungal decolorization (Khan et al., 2014). Numerous MO removal bacterial strains have been reported by several researchers (Ali et al., 2005; Parshetti et al., 2010; Kalyani et al., 2011; Du et al., 2015; Akansha et al., 2019). Joshi et al. (2008) have shown that breakdown products of the azo dyes by a single bacterial strain are toxic and carcinogenic aromatic amines, while metabolites by mixed bacterial cultures i.e., consortia are nontoxic. In case of bacterial consortium, the individual bacterial strain attacked the dye molecule at different positions or utilized metabolites produced by the co-existing strain for further decomposition (Forgacs et al., 2004). However, limited studies are available for decolorization and detoxification of MO by bacterial consortia (Ayed et al., 2010; Masarbo et al., 2018). The planktonic bacterial consortia are often susceptible to toxic environments due to decreased protection and low metabolic activity combined with low bioavailability of the pollutants (von Canstein et al., 2002; McDougald et al., 2012). Thus, more studies are required to find out bacterial consortia capable of degrading MO into less toxic metabolites even under toxic environments with high metabolic activity.

Bacterial biofilms are surface-associated cells, enclosed in self-produced extracellular polymeric substances (EPS). EPS mainly composed of curli (a proteinaceous surface appendages), nanocellulose-rich polysaccharides, nucleic acids and lipids (McDougald et al., 2012; Mosharaf et al., 2018). Compared to the free living planktonic bacteria, biofilm-associated bacteria are resistant to adverse environmental cues [including high concentrations of chemicals (e.g., heavy metals and dyes), pH, temperature and salinity]. Production of signal molecules and enzymes, and exchange of genetic materials are also higher in biofilm cells. Moreover, biofilm cells are persistent to diverse metabolic states (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).

The present study is undertaken to investigate the ability of the newly developed biofilm consortia to decolorize MO as well as to examine the effect of various nutritional and environmental parameters on MO decolorization. Azoreductase, NADH-DCIP reductase and laccase enzymes played a crucial role in biodegradation of MO by bacterial consortia (Ayed et al., 2010; Masarbo et al., 2018). Thus, this study aims to quantify the production of these enzymes in response to MO. The metabolites generated during degradation of MO were also analyzed by UV-vis spectrophotometer and Fourier transform infrared (FTIR) spectroscopy. Furthermore, these metabolites were studied for their toxicity using phytotoxicity and microbial toxicity assays. This study will contribute towards understanding the mechanism of MO decolorization by bacterial biofilm consortia. To our knowledge, this is the first report, so far, on degradation and detoxification of MO by bacterial biofilm consortia under various nutritional and environmental conditions.

2. Materials and methods

2.1. Chemicals

Methyl orange [MO ($C_{14}H_{14}NaO_3S$), $\lambda_{max} = 465$ nm], 2,6-Dichloroindophenol (DCIP), nicotinamide adenine dinucleotide (NADH), and 2,2'-azono-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA). Yeast extract and peptone were obtained from HiMedia (Mumbai, India). Glycerol and glucose were purchased from Wako Pure Chemical Industries, LTD (Japan) and BDH® chemicals (UK), respectively. All other chemicals used were of an analytical grade and procured from Bio Basic Canada Inc. (Canada) and Wako Pure Chemical Industries, LTD (Japan).

2.2. Bacterial strains, culture media and growth conditions

In this study, *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 were used from the stock of the department of Environmental Science, Bangabandhu Sheikh Mujibur Rahman Agricultural University (BSMRAU), Bangladesh. We isolated these strains from different industry wastewaters of Bangladesh, and all these strains were shown to produce biofilms (Mosharaf et al., 2018). Yeast extract peptone [YEP (1% peptone, 0.5% yeast extract, pH 7.0)], Luria-Bertani [LB (1% tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.0)] and salt-optimized broth plus glycerol [SOBG (per liter: 20 g of tryptone, 5 g of yeast extract, 0.5 g of NaCl, 2.4 g of $MgSO_4 \cdot 7H_2O$, 0.186 g of KCl, and 50 mL of 40% glycerol, pH 7.0)] media were used in this study. The bacterial strains were routinely

grown in YEP at 28 °C with or without shaking conditions. To measure the optical density (OD at 660 nm) of the culture, an absorption spectrophotometer (Intertech, Inc. Tokyo, Japan) was used.

2.3. Inoculum preparation

Single colony of each bacterial strain was inoculated in YEP broth and incubated at 28 °C in shaking condition (150 rpm) until OD₆₆₀ reached at 0.6 to 0.8. Then, the cultures were aseptically collected and centrifuged at 10,000 rpm for 10 min. The resulted pellets were re-suspended in YEP broth and used for different experiments.

2.4. Assessment of compatibility of the bacterial strains and preparation of consortia

In order to prepare biofilm consortia, the bacterial compatibility was assessed as follows: 1 µL (ca. 10⁶ colony forming unit (CFU) mL⁻¹) suspension of each bacterium was spotted onto YEP agar plates. The spot to spot distance was 1 cm. The inoculated plates were then incubated at 28 °C in static condition for 72 h. If bacterial strains overlapped each other without any signs of inhibition, considered as compatible. Compatibility assays were also performed as described in [Furuya et al. \(1997\)](#) with a few modifications. In brief, 2 µL (ca. 10⁶ CFU mL⁻¹) culture of each producer bacterium was dotted onto YEP agar plate then incubated at 28 °C in static condition for 48 h. A sterilized filter paper containing 0.5 mL chloroform was placed in Petri dish lid then incubated at room temperature (25 ± 2 °C) to kill the producer bacteria. After 2 h incubation, 5 mL melted water agar (1.5% at 50 °C) containing a suspension of indicator bacterium [ca. 10⁶ CFU mL⁻¹] was overlaid and incubated at 28 °C for 48 h. If no inhibition zone was observed around producer bacteria, considered as compatible. Based on compatibility results, four consortia were prepared such as C1 (*Vitreoscilla* sp. ENSG301 *A. lwoffii* ENSG302, *K. pneumoniae* ENSG303 and *P. fluorescens* ENSG304), C2 (*E. coli* ENSD101, *E. asburiae* ENSD102 and *E. ludwigii* ENSH201), C3 (*E. asburiae* ENSD102, *Vitreoscilla* sp. ENSG301 and *B. thuringiensis* ENSW401) and C4 (*E. coli* ENSD101, *E. ludwigii* ENSH201 and *B. thuringiensis* ENSW401).

2.5. Decolorization experiments

Initially, 10 mL culture (ca. 10⁶ CFU mL⁻¹) of each consortium was inoculated in 90 mL YEP broth containing 100 mg L⁻¹ MO in the Erlenmeyer flasks and incubated at 28 °C in static condition. Within 60 h, 1.5 mL culture was withdrawn. To separate the bacterial cells, the culture was centrifuged at 12,000 rpm for 10 min. Clear suspension was used to measure the decolorization. MO decolorization was determined by measuring the change in absorbance of clear culture supernatants at the maximum absorption wavelength (λ_{max}) of 465 nm in UV-Vis spectrophotometer (Ultrospec-3000, Pharmacia Biotech, Cambridge, England). Abiotic control was also included. The decolorization percentage was calculated using following equation:

$$\text{Decolorization (\%)} = \frac{\text{Initial absorbance} - \text{Final absorbance}}{\text{Initial absorbance}} \times 100$$

Decolorization performance was studied in different growth media (e.g., YEP, ½ strength YEP, LB and SOB), carbon sources (½ strength YEP plus 2% glycerol or 2% glucose), nitrogen sources (peptone-deficient YEP broth or yeast extract-deficient YEP broth), metal ions [½ strength YEP containing 1 mg L⁻¹ Mg²⁺ (MgSO₄·7H₂O), Ca²⁺ (CaCl₂), Cu²⁺ (CuCl₂), Zn²⁺ (ZnCl₂), Mn²⁺ (MnCl₂), Ni²⁺ (NiCl₂), Cr²⁺ (K₂Cr₂O₇) or Pb²⁺ (PbCl₂)], oxygen tension (incubation

in shaking and static conditions), dye concentrations (100, 200, 300 and 400 mg L⁻¹), temperatures (20, 28, 32, 37 and 40 °C), pH (5, 6, 7, 8 and 9), salinity in terms of NaCl (1 to 10%) and incubation periods (12, 24, 36, 48, 60, 72 and 84 h). All decolorization experiments were performed three times with two replications

2.6. Enzyme assays

In order to quantify the intracellular enzymes, cell-free extract was prepared as follows: 10 mL culture (ca. 10⁶ CFU mL⁻¹) of each consortium was inoculated in 90 mL YEP broth containing 200 mg L⁻¹ MO in the Erlenmeyer flasks and incubated at 28 °C in static condition. For control, each biofilm culture was collected just after inoculation (0 h) then centrifuged at 12,000 rpm for 15 min at 4 °C. The supernatant was discarded and each pellet was suspended in 50 mM potassium phosphate buffer (pH 7.2). Then bacterial cells of each consortium were disrupted using ultrasonic cell disrupter (Ultrasonic Disrupter UD-200, Tommy, Tokyo, Japan) by giving 10 S of 20 s each for 2 min interval based on 40 amplitude output at 4 °C. Each suspension was immediately centrifuged at 15,000 rpm for 5 min at 4 °C. For enzyme assays, cell-free extract was collected. Same procedure was followed for complete degradation within 60 h of incubation.

Azoreductase activity was quantified as the method described in [Ayed et al. \(2010\)](#) with a slight modifications. In brief, we monitored the decrease of the MO concentration at 465 nm instead of 466 nm. The chemicals used in the reaction mixture (volume 2.2 mL) were 152 mM MO, 50 mM sodium phosphate buffer, pH 5.5 and 20 mM NADH. One unit azoreductase activity was expressed as a microgram of MO reduced min⁻¹ mg protein min⁻¹. The NADH-dichlorophenol indophenol (DCIP) reductase activity was determined as described in [Parshetti et al. \(2010\)](#). The reaction mixture contains the following chemicals (volume 5 mL): 50 µM DCIP, 28.57 mM NADH in 50 mM potassium phosphate buffer, pH 7.4 and 0.1 mL cell free extract. Production of laccase was estimated as the method of [Wolfenden and Willson \(1982\)](#), which monitors the increase of substrate concentration at a wavelength of 420 nm. Lignin peroxidase was assessed by monitoring the formation of propanaldehyde at 300 nm in a reaction mixture containing 100 mM *n*-propanol, 250 mM tartaric acid, 10 mM H₂O₂ ([Shanmugam et al., 1999](#)). To quantify the protein, the Lowry method ([Lowry et al., 1951](#)) was used with bovine serum albumin as standard. All these assays were performed in triplicate.

2.7. UV-Vis spectral analysis

For UV-vis spectral analysis, the metabolites were prepared as follows: each consortium was inoculated in YEP broth containing 200 mg L⁻¹ MO and incubated at 28 °C without shaking condition. Within 60 h of incubation, 1.5 mL culture of each consortium was collected and centrifuged at 14,000 rpm for 15 min. The supernatant was carefully collected then filtered through Einmalfilter [CHROMAFIL® Xtra PTFE-45/25, 0.45 µm (Macherey-Nagel, GmbH and Co. KG, Germany)]. Each metabolite was analyzed by UV-Vis spectrophotometer (Ultrospec-3000, Pharmacia Biotech, Cambridge, England) within 200 to 800 nm wavelength. For control, YEP broth containing 200 mg L⁻¹ MO was used.

2.8. Fourier transform infrared spectroscopy

The metabolites were prepared as described in earlier section. FTIR analysis was carried out using Perkin Elmer FTIR (Spectrum-2) instrument operated by CPU32M software. Triglycine sulphate (TGS) detector was applied for FTIR scanning within 600 to 2000 cm⁻¹ (16 scans at 4 cm⁻¹ at 0.2 cm/sec scanning speed) and changes in the percent transmission at different wavenumbers

were recorded. YEP without MO was also scanned. Perkin Elmer's proprietary software (Version 10.05.03) was used to analyze the baseline subtracted spectra.

2.9. Assessment of the detoxification

2.9.1. Quantification of COD

YEP broth was supplemented with 200 mg mL⁻¹ MO and COD was determined (i.e., initial COD). The COD removal study was carried out as follows: 10 mL culture (ca. 10⁶ CFU/mL) of each consortium was inoculated in 90 mL YEP broth containing 200 mg mL⁻¹ MO in the Erlenmeyer flasks and incubated at 28 °C in static condition. Within 60 h of incubation, each culture was centrifuged at 12,000 rpm for 15 min. The pellets were discarded and the supernatants were collected. The supernatants were further filtered through Einmalfilter [CHROMAFIL® Xtra PTFE-45/25, 0.45 µM (Macherey-Nagel, GmbH and Co. KG, Germany)]. For quantification of COD, dichromate closed reflux titrimetric method (APHA, 2012) was used. The COD removal was estimated using following equation:

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

2.9.2. Seed germination tests

Wheat (*Triticum aestivum* L.) seeds (variety: BARI Gom 25) were collected from Bangladesh Wheat and Maize Research Institute, Bangladesh. Seeds were surface sterilized with 1% sodium hypochlorite for 3 min then rinsed 5 times with sterile distilled water. Then 15 seeds (3 replications) were placed in each plate and incubated at 20 °C and checked regularly. To maintain moisture content, adequate amount of biodegraded metabolites or YEP containing 200 mg L⁻¹ CR was added every alternate day. Germination (%) was recorded at 5th day.

2.9.3. Microbial growth inhibition tests

Plant probiotic rhizobacterial strains including *Pseudomonas cedrina* ESR12 and *Bacillus cereus* ESD3 used in this study were collected from the stock of the department of Environmental Science, BSMRAU, Bangladesh. In order to check the growth, each strain was inoculated in the glass test tubes containing 5 mL cell-free metabolite of each consortium and incubated at 28 °C in agitate (150 rpm) condition. For control, each probiotic bacterium was grown in 5 mL YEP broth. Bacterial growth (OD₆₆₀) was measured using a spectrophotometer after 24 h incubation.

2.10. Statistical analysis

All the experiments were laid out in a complete randomized design with two replications and repeated at least three times. However, enzyme assays, UV–vis spectral analysis and FTIR spectroscopy studies were performed only twice. The “agricolae” package of R software version 3.3.6 was used to calculate analysis of variance and comparison of means. The means were compared by using Fisher's least significance difference (LSD) test.

3. Results

3.1. Effect of media composition, carbon and nitrogen sources and metal ions on decolorization of MO

The effect of media composition, nutritional condition, carbon and nitrogen sources and metal ions on decolorization of MO (100 mg L⁻¹) by different consortia including C1, C2, C3 and C4 were examined within 60 h of incubation at 28 °C, and the results are

depicted in Fig. 1. Growth rate of these consortia was indistinguishable in YEP (Fig. 1A), LB and SOBG (data not shown). However, the consortia of C3 and C4 grew slightly higher in comparison to that of C1 and C2 in all these media. Decolorization efficiencies of these consortia ranged from 96.99 to 98.47%, 90.39 to 91.98% and 92.59 to 94.36% in YEP, LB and SOBG media, respectively in static condition (Fig. 1B). The cell-free extract was found to be clear and transparent in YEP (Fig. 1D), LB and SOBG (data not shown). Furthermore, no dye was detected in the cell pellets after centrifugation (Fig. 1E). In shaking condition, MO decolorization was reduced by 36.20 to 43.07%, 37.18 to 42.46% and 32.03 to 42.32% in YEP, LB and SOBG, respectively than the static condition (Fig. 1A). Thus, oxygen tension might play a key role in decolorization of MO.

In order to study the effect of nutritional conditions on decolorization, ½ strength YEP broth was used instead of full strength YEP. The growth rate of these consortia was not diminished in ½ strength YEP broth (data not shown). However, MO removal was decreased by 21.77 to 35.66% in ½ strength YEP (Fig. 1F) in comparison to that of full strength YEP. Next, the efficacy of these consortia in decolorizing MO in response to different carbon and nitrogen sources was examined. The MO removal by these consortia was still lower in ½ strength YEP supplemented with 2% glycerol or 2% glucose, (Fig. 1F). In peptone/yeast extract-deficient YEP media, bacterial growth (data not shown) and the rate of decolorization (Fig. 1F) were drastically reduced than the standard YEP. Thus, nitrogen not only required for the bacterial growth but also essential for the decolorization of MO. When ½ strength YEP media containing 100 mg L⁻¹ MO and 1 g L⁻¹ of Mg²⁺, Ca²⁺, Cu²⁺, Zn²⁺, Mn²⁺, Ni²⁺, Cr²⁺ or Pb²⁺, Mg²⁺, Ca²⁺, Zn²⁺ and Mn²⁺ could remarkably increase MO decolorization, while Ni²⁺, Cr²⁺ and Pb²⁺ had no significant effect on decolorization (Fig. 1G).

3.2. Effect of pH, temperature and salinity on decolorization

We quantified the effect of pH, temperature and salinity on decolorization of MO by different biofilm consortia in static condition (Fig. 2). Overall, the consortia of C3 and C4 performed better in terms of MO removal as compared to that of C1 and C2 in response to pH, temperature and salinity. At 28 °C, the growth rate of these consortia was not diminished on YEP broth at pH 5.0 to 9.0 in shaking condition (data not shown). All these biofilm consortia decolorized 92.35 to 98.95% MO at pH 5.0 to pH 9.0 (Fig. 2A), suggesting good decolorizing potential of these consortia. The pH 7.0 was found optimum, which was not considerably varied with pH 8.0. Thus, all these consortia could be utilized in a wide range of pH level for decolorization of MO.

Next, we examined the effect of temperature vary from 22 to 40 °C on decolorization by different biofilm consortia. Initially, we checked the growth of these biofilm consortia on YEP broth at varying temperatures in shaking condition. The growth rate of these biofilm consortia was only slightly reduced at 40 °C in comparison to that of other temperatures (i.e., 22, 28, 32 and 37 °C) tested (data not shown). Within 60 h of incubation (static condition), the rate of decolorization varied from 88.27 to 90.85, 96.99 to 98.47, 96.24 to 97.70, 88.33 to 91.62 and 76.12 to 87.34% at 22, 28, 32, 37 and 40 °C, respectively (Fig. 2B). Thus, all these consortia exhibited good decolorizing capacity from 22 to 40 °C. The optimum temperature for the maximum decolorization was detected to be 28 °C followed by 32, 37, 22 and 40 °C. We also studied the effect of salinity in terms of different concentrations of NaCl (%) on decolorization of MO. For this experiment, YEP broth was supplemented with 1.0, 2.5, 5.0, 7.5 and 10.0% of NaCl, and the results are depicted in Fig. 2C. These consortia removed 90.11 to 96.13% MO from YEP containing 1.0 to 7.5% NaCl within 60 h of incubation. However, 68.34 to 81.34% MO was also removed by these consortia from YEP containing 10% NaCl.

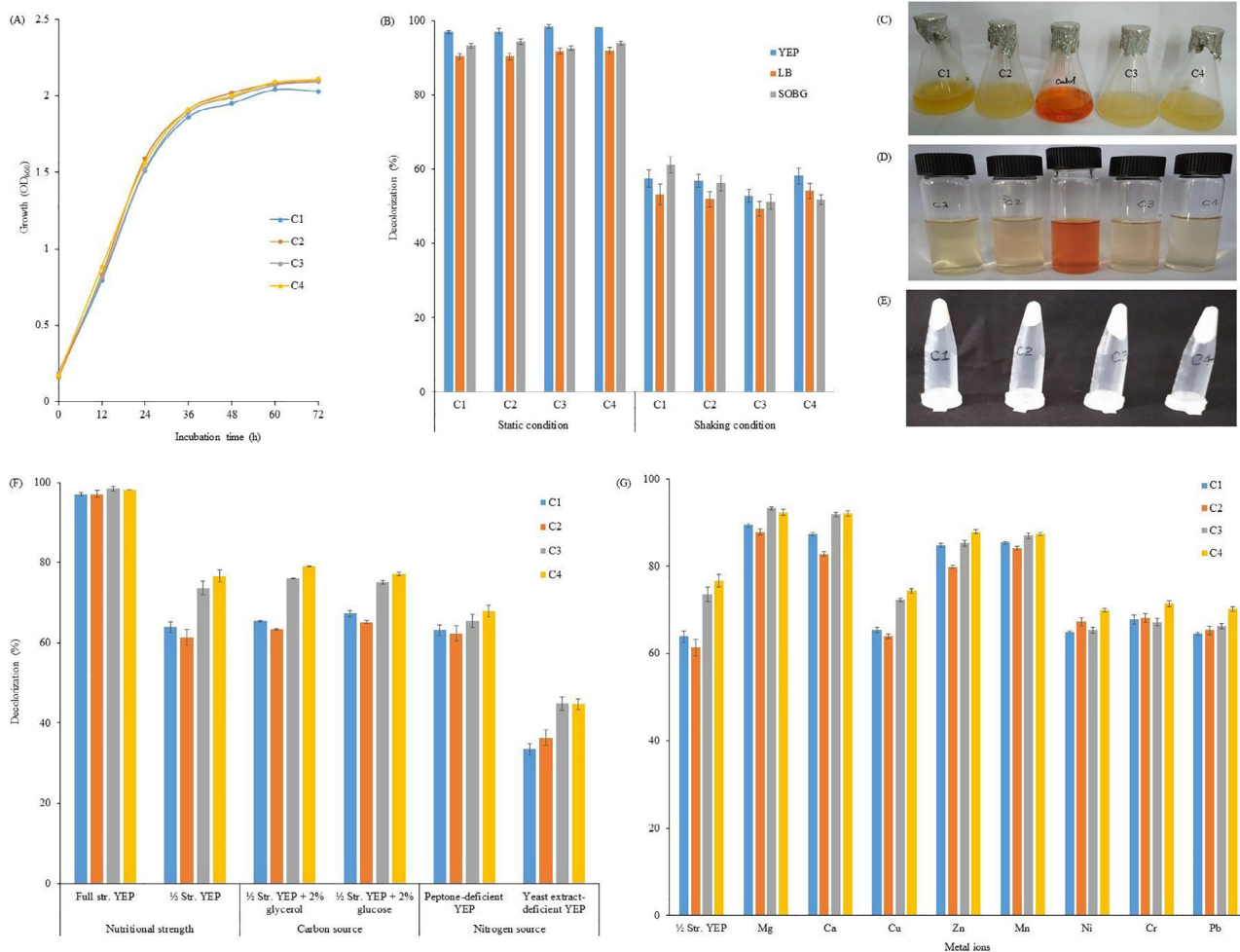


Fig. 1. Effect of nutritional conditions on decolorization of MO. (A) Growth rate of different consortia in yeast extract peptone (YEP) containing 100 mg L⁻¹ MO in agitate conditions at 28 °C. (B) Decolorization of MO within 60 h by different consortia in YEP, LB and SOB media (pH 7) at 28 °C in static and agitate conditions. (C) Decolorized YEP broth along with bacterial cells within 60 h. (D) Biodegraded products after discarding the bacterial cells by centrifugation. (E) No dye remain in the cell pellets. Effect of nutritional strength, carbon and nitrogen sources (F), and divalent metal (as indicated) ions (G) on decolorization within 60 h in static condition. Data represent averages from triplicate assays and error bars showing standard deviation.

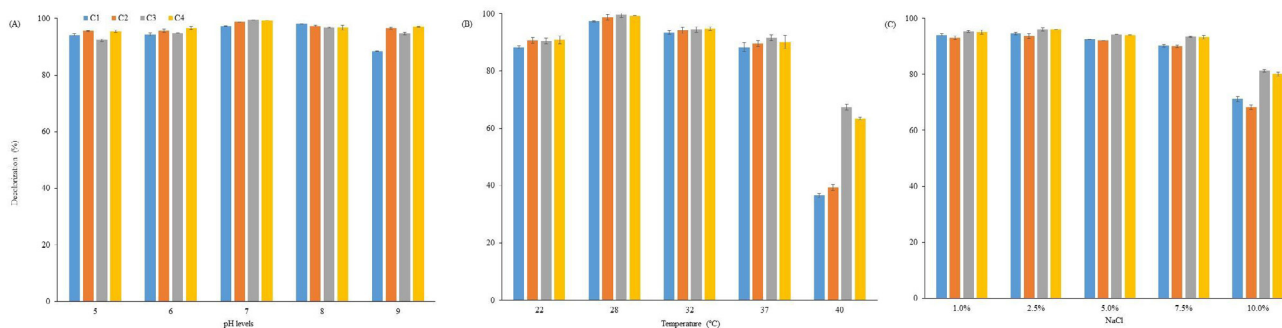


Fig. 2. Effect of pH (A), temperature (B) and salinity [in terms of NaCl] (C) on decolorization of MO. Each consortium was inoculated in the flasks containing YEP broth (pH 7) along with 100 mg L⁻¹ MO. The flasks were incubated at 28 °C in static conditions and decolorization was assessed within 60 h. Data represent averages from triplicate assays and error bars showing standard deviation.

3.3. Effects of dye concentrations and incubation times on decolorization

A time-course experiment was conducted to quantify the effect of different concentrations (100 to 400 mg L⁻¹) of MO on decolorization by different consortia (Fig. 3). MO removal was significantly influenced by the dye concentrations, incubation periods

and the consortia. Overall, as the time increased, the MO removal was also increased. For example, at 200 mg L⁻¹, decolorization efficiency increased from 59.34, 54.34, 82.89 and 84.89% within 36 h to 79.21, 81.21, 99.80 and 98.80% within 48 h, respectively by C1, C2, C3 and C4, respectively. Among these consortia, MO removal was faster in C3 and C4. It is worth mentioning that MO removal efficiency was not significantly varied at 100 mg L⁻¹ and

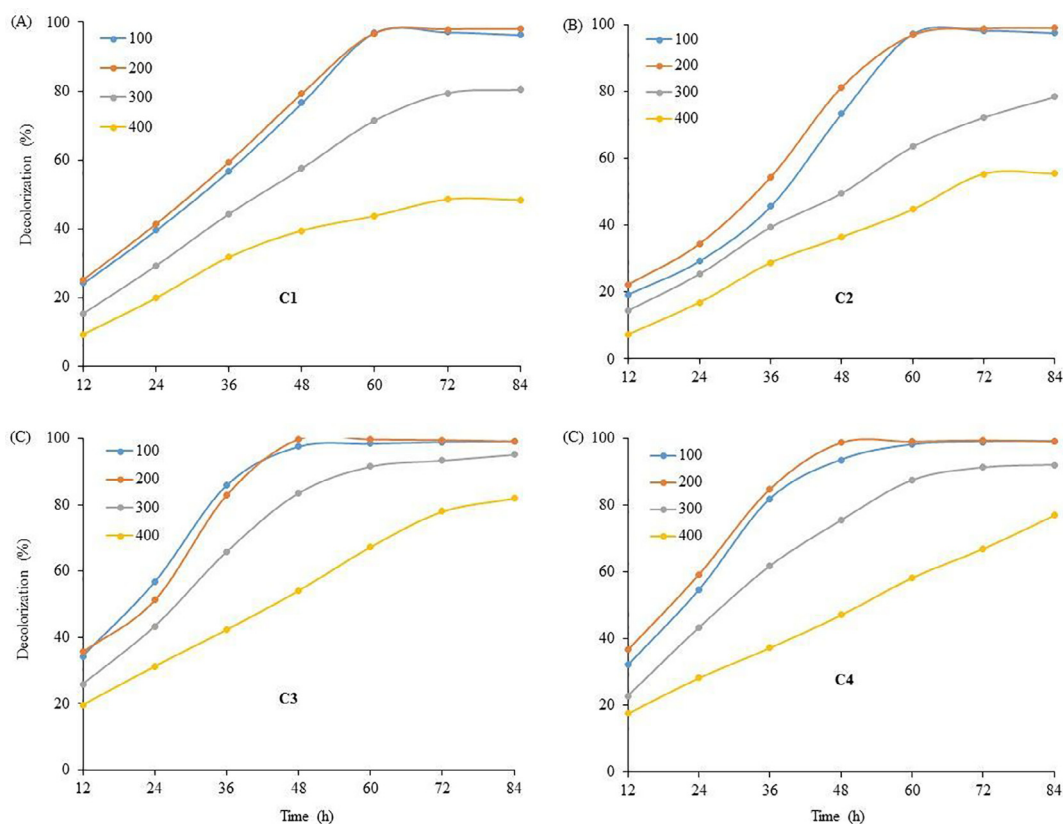


Fig. 3. Time-course effect of the concentrations (100–400 mg L⁻¹) of MO on decolorization by different consortia. Data represent averages from triplicate assays and error bars of standard deviations are too small to be represented.

200 mg L⁻¹. However, MO removal was decreased at 400 mg L⁻¹. Importantly, 81.99 and 76.99% removal by C3 and C4 were still observed at 400 mg L⁻¹ within 84 h. Growth rate of these consortia were also slightly reduced at 400 mg L⁻¹ as compared at 100 to 300 mg L⁻¹ MO (data not shown).

3.4. Production of azoreductase, NADH-DCIP reductase, lignin peroxidase and laccase

In order to get insight into the MO decolorization mechanism by the consortia of C1, C2, C3 and C4, activities of azoreductase, NADH-DCIP reductase, lignin peroxidase and laccase were determined before (just after inoculation i.e., 0 h) and after decolorization (i.e., within 60 h of incubation), and the results are depicted in Fig. 4. Except lignin peroxidase, intracellular activities of azoreductase, NADH-DCIP reductase and laccase were significantly induced in these biofilm consortia in optimum conditions (i.e., 200 mg L⁻¹ MO, pH 7.0, 28 °C and static condition) (Fig. 4). For example, azoreductase activity was induced by 2.46-, 2.52-, 3.62- and 3.57-fold by C1, C2, C3 and C4, respectively within 60 h of incubation as compared to that of 0 h (Fig. 4A). Thus, azoreductase, NADH-DCIP and laccase enzymes synthesized by these consortia might responsible for the degradation of MO in optimum conditions.

As decolorization rate by these consortia decreased when grown in (i) YEP containing 100 mg L⁻¹ MO and 10% NaCl at 28 °C, (ii) YEP containing 100 mg L⁻¹ MO at 40 °C and (iii) YEP containing 400 mg L⁻¹ MO at 28 °C, we quantified the intracellular oxidoreductase activities in these conditions. Within 60 h of decolorization, no NADH-DCIP and lignin peroxidase activities was detected in these consortia cultivated in the above mentioned conditions (data not shown). However, intracellular azoreductase

and laccase activities of these consortia were induced within 60 h of incubation in all the conditions tested (Fig. 4). Compared to the optimum conditions, intracellular azoreductase and laccase activities were significantly reduced in all these conditions. Thus, decreased MO decolorization by these biofilm consortia in the above mentioned conditions might be due to lower production of oxidoreductive enzymes.

3.5. UV-vis and FTIR analysis

Decolorization of MO was confirmed by UV-visible spectral analysis. The changes of the UV-vis spectra (from 200 to 800 nm) of MO within 60 h decolorization by consortia of C1, C2, C3 and C4 are shown in Fig. 5. The absorbance band of MO at 465 nm was attributed to azo bond. This band was disappeared within 60 h of incubation. The dissipation of the peak at 465 nm suggests that the success of MO removal by these consortia. Thus, decolorization of MO by these consortia may be largely attributed to biodegradation.

To confirm these results of MO dye degradation, FTIR analysis was done. Comparison of FTIR spectra between the parent dye (MO) and produced metabolites within 60 h of incubation indicates the biodegradation of the MO dye by these consortia (Fig. 6). FTIR spectra of parent dye MO (Fig. 6A) showed peak at 1602.6 cm⁻¹ for N=N stretch and peak at 1202 cm⁻¹ and 1123 cm⁻¹ for C-N confirms the azo nature of the dye (Ayed et al., 2010; Parshetti et al., 2010). The peaks at 1442 cm⁻¹ and 1420 for C=C-H in plane C-H bend, peaks 1045 cm⁻¹, 947 cm⁻¹ and 847 cm⁻¹ for ring vibrations and peak at 818 cm⁻¹ for distributed benzene ring, this confirms aromatic nature of the dye (Parshetti et al., 2010). Peaks at 698 cm⁻¹ and 624 cm⁻¹ for -C-S- stretching vibrations and peak at 1369 cm⁻¹ for S=O stretching vibrations indicates the

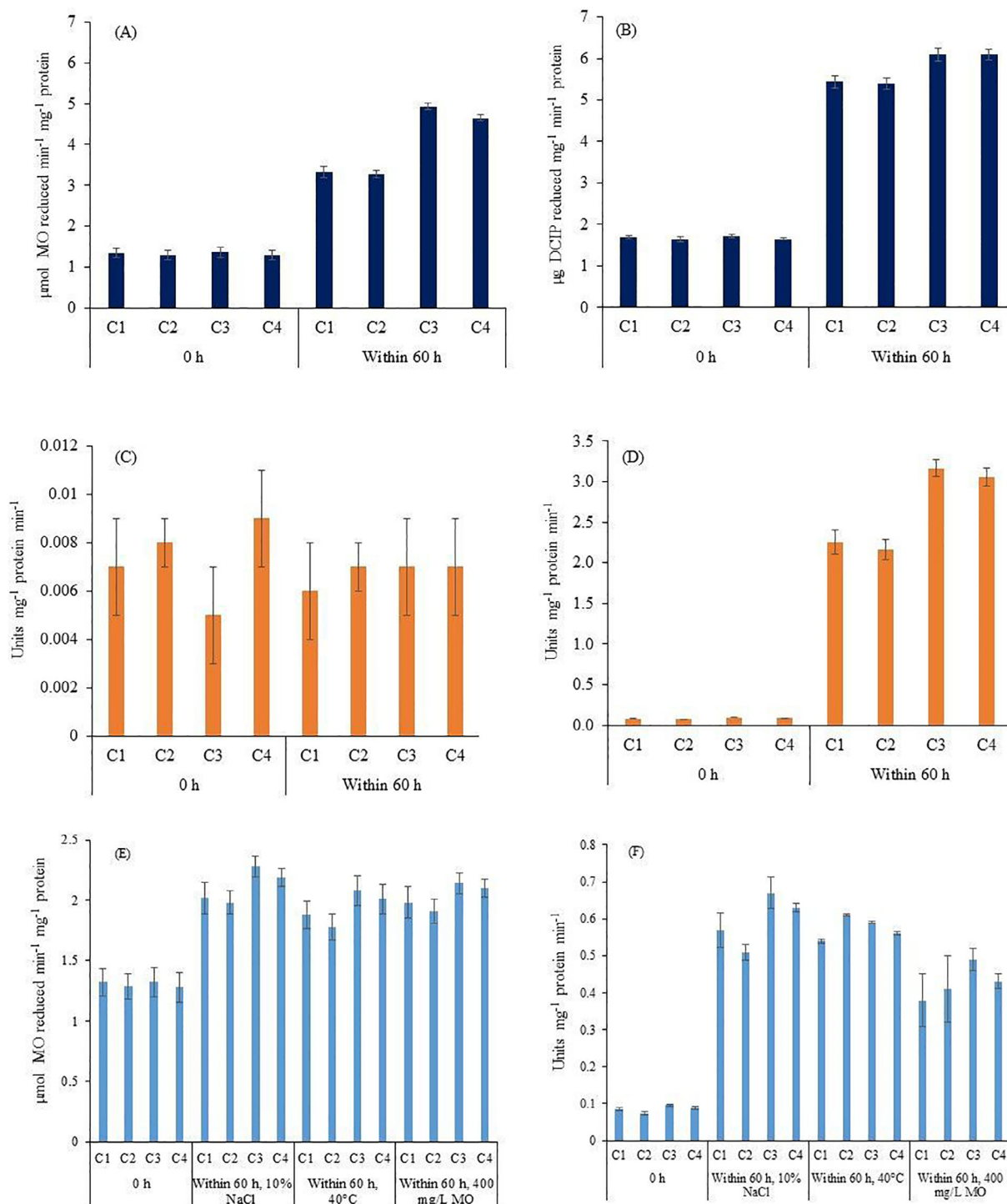


Fig. 4. Enzyme assays. Intracellular azoreductase (A), NADH-DCIP reductase (B), lignin peroxidase (C) and laccase (D) activities by different consortia at 0 h and 60 h incubation (complete decolorization). The inoculated flasks containing YEP broth (pH 7) along with 200 mg L^{-1} MO and incubated at 28°C in static condition. Intracellular activities of azoreductase (E) and laccase (F) were also determined as indicated conditions. Data represent averages from triplicate assays and error bars showing standard deviation.

sulfonated nature of the dye (Kalyani et al., 2011). The FTIR spectra within 60 h of incubation by these consortia displayed that most of the major peaks (e.g., 1602.6 cm^{-1} , 1202 cm^{-1} , 1123 cm^{-1} , 1045 cm^{-1} , and 947 cm^{-1}) were disappeared completely or partly, deformed and shifted (Fig. 6B–E), suggesting degradation of MO. However, the peak at 1369 cm^{-1} for S=O stretching vibration was widened or shifted the band locations (Fig. 6B–E), indicated the formation of sulfonated aromatic amines from MO degradation by these consortia.

3.6. Biodegraded metabolites are non-toxic

In the present study, 88.88, 85.37, 91.44 and 90.85% COD were removed by application of C1, C2, C3 and C4, respectively within 60 h (Fig. 7A). The enhanced reduction of COD indicates the efficient biodegradation of MO by these consortia. When *P. cedrina* ESR12 and *B. cereus* ESD3 were inoculated in the broth of biodegraded metabolite of each consortium, the growth rate of these probiotic bacteria were not significantly differed between

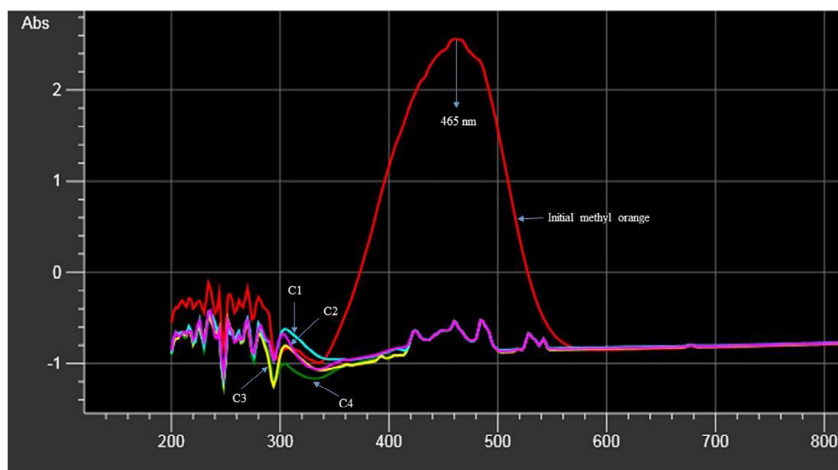


Fig. 5. UV-Vis spectra of non-biodegraded (control) and biodegraded metabolites of MO (200 mg L^{-1}) by different consortia within 60 h incubation at $28 \text{ }^\circ\text{C}$ in static condition.

biodegraded metabolites (Fig. 7B) and YEP (data not shown) in shaking condition. All the wheat seeds were germinated when YEP broth containing 200 mg L^{-1} MO applied as irrigation water (Fig. 7C). However, the growth of the shoots were severely inhibited (Fig. 7C). On the other hand, roots and shoots of the seedlings were normal when biodegraded metabolites used as irrigation water (Fig. 7E–H). Thus, biodegradation of MO by different consortia resulted by its complete detoxification.

4. Discussion

Unfavorable environmental conditions, toxic effects of the dyes, and low metabolic activity of the free living (planktonic) bacteria often restrict the decolorization of azo dyes (Saratale et al., 2011; Edwards and Kjellerup, 2013). In order to overcome these problems, we utilized biofilm producing bacteria as consortia because of their higher capacities to survive in adverse environmental conditions and toxic chemicals (Mosharaf et al., 2018; Koehler et al., 2015; Edwards and Kjellerup, 2013). In this study, nutritional (e.g., growth media, divalent metal cations and nitrogen sources) and environmental (e.g., temperature, pH, salinity, dye concentrations and oxygen tension) conditions significantly influence the rate of decolorization of MO (Figs. 1–3). Therefore, it is necessary to find out optimum condition for decolorization of MO to make the process faster and efficient.

Nitrogen sources (e.g., peptone and yeast extract) directly influence the decolorization of MO (Fig. 1F). Peptone and yeast extract were reported to trigger the expression of NADH, which acted as the electron donor and redox mediator for the bacterial degradation of azo dyes (Chang and Lin, 2000). Moreover, yeast extract acted as both carbon and nitrogen source for the decolorization of MY-G, a sulfonated mono azo dye (Guo et al., 2020). Consistent with Guo et al. (2020), carbon sources were found less effective for the decolorization of MO (Fig. 1F). Carbon sources seemed to be less effective in promoting decolorization due to the preference of the bacterial cells in assimilating the extra carbon sources over using the dye compound as the carbon source (Saratale et al., 2011). In this study, Mg^{2+} , Ca^{2+} , Zn^{2+} and Mn^{2+} but not Cu^{2+} , Ni^{2+} , Cr^{2+} and Pb^{2+} could enhance the rate of decolorization (Fig. 1H). It was reported that divalent metal ions affect decolorization by regulating the chemical transportation across the cell membrane or increasing the activity of enzymes – this causes the metal ions to have different effects on dye decolorization (Du et al., 2015;

Seesuriyachan et al., 2009). Thus, nitrogen sources and some divalent metal ions could be added for higher decolorization of MO.

MO decolorization by different biofilm consortia was drastically reduced in agitate condition as compared to that of static condition (Fig. 1B). MO removal was also reported to higher in static condition than in agitate condition by *Bacillus stratosphericus* SCA1007 (Akansha et al., 2019), *Kocuria rosea* MTCC 1532 (Parshetti et al., 2010) and a bacterial consortium composed of *Sphingomonas paucimobilis*, *B. cereus* ATCC14579 and *B. cereus* ATCC11778 (Ayed et al., 2010). In fact, no MO removal by *K. rosea* MTCC 1532 was found in shaking condition (Akansha et al., 2019). However, only 14% Congo red, a diazo dye, was reported to decolorize by a bacterial consortium composed of eight bacterial strains in aerobic (120 rpm) condition (Lade et al., 2015). Thus, oxygen tension might play an important role in decolorization. In order to prove this hypothesis, we measured the dissolved oxygen (DO) level in the cultures of these biofilm consortia within 30 min incubation. The DO level in these cultures ranged from 0.002 to 0.004 mg L^{-1} in static condition, while it was 1.7 to 1.9 mg L^{-1} in agitate condition. A positive correlation between DO and the decolorization of C.I. Reactive Red 22 by *E. coli* NO3 was also reported by Chang and Kuo (2000). Availability of oxygen inhibits the azo dye removal due to competition of NADH or azo groups as the electron receptor leading to decrease the dye removal (Chang et al., 2001; Chung et al., 1992). Saratale et al. (2011) have reported that under static conditions both reductive- and oxidative enzymes are induced leading to enhance the rate of degradation of azo dyes.

Environmental parameters including pH, temperature and salinity, and dye concentrations were also found to affect decolorization of MO (Fig. 2). Among the environmental parameters, pH plays a pivotal role in transfer of dye molecules across the cell membrane, and considered as the rate-limiting step for the decolorization of azo dye (Chang et al., 2001). The optimum pH for the decolorization of MO was reported to be between 6.0 and 10.0 (Chen et al., 2003; Kilic et al., 2007). In the present study, all these consortia decolorized 92.35 to 98.95% MO at pH 5.0 to pH 9.0 (Fig. 2A). This might be meaningful in the treatment of MO at wide range of pH levels. Thao et al. (2013) have reported that *P. putida* mt2 only decolorizes <40% MO at pH 5.0. These contradictory results might imply different decolorizing mechanism by different bacteria/consortia. Consistent with Akansha et al. (2019), Masarbo et al. (2018) and Du et al. (2015), the optimum pH for MO decolorization by these biofilm consortia was pH 7.0.

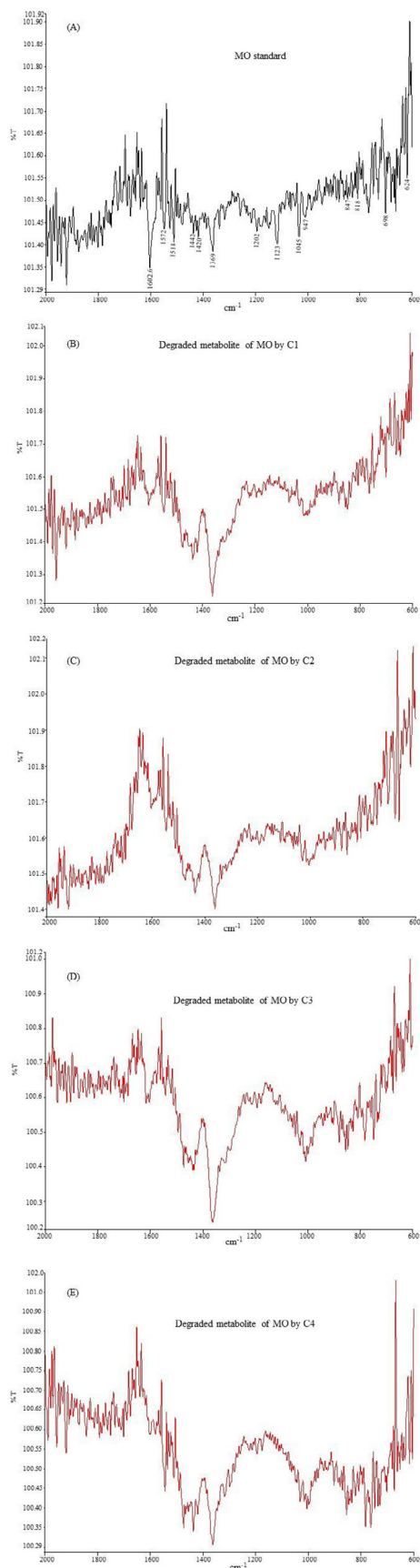


Fig. 6. Fourier transform infrared spectroscopy (FTIR) of non-biodegraded (control) (A) and biodegraded metabolites of MO (200 mg L^{-1}) by the consortia of C1 (B), C2 (C), C3 (D) and C4 (E) within 60 h incubation at $28 \text{ }^{\circ}\text{C}$ in static condition.

Under optimal temperature, decolorization rate is higher than the higher temperatures (Saratale et al., 2011). The rate of decolorization was shown to decrease both at higher temperatures and higher salinities due to decrease of cell viability or the denaturation of an azoreductase enzyme (Guo et al., 2020; He et al., 2017; Selvakumar et al., 2013; Saratale et al., 2011; Phugare et al., 2011). Furthermore, sulfonic acid group (e.g., MO) containing aromatic rings inhibit the growth of microbes at higher dye concentrations (Chen et al., 2003). The lower cell biomass and dye, toxicity of the dyes and obstruction of active azoreductase sites by dye molecules were also reported to decrease the rate of decolorization (Saratale et al., 2009; Jadhav et al., 2008). In the present study, all the biofilm consortia displayed good decolorizing capacity at 22 to $40 \text{ }^{\circ}\text{C}$ (Fig. 2B), 1 to 7.5% NaCl (Fig. 2C) and 100 to 300 mg L^{-1} of MO (Fig. 3). Accordingly, azoreductase, NADH-DCIP reductase and laccase activities were induced in these biofilm consortia (Fig. 4). Several researchers have also reported that oxidoreductive enzymes including azoreductase, NADH-DCIP reductase, lignin peroxidase and laccase played an important role in MO decolorization (Guo et al., 2020; Masarbo et al., 2018; Du et al., 2015; Ayed et al., 2010; Parshetti et al., 2010). Bacterial azoreductases are involved in the reductive cleavage of azo bonds ($-\text{N}=\text{N}-$) to produce colorless aromatic amine products (Chang et al., 2001). NADH-DCIP reductases are the marker enzymes for the reduction of azo bonds, and also responsible for the detoxification of xenobiotic compounds (Saratale et al., 2007). Furthermore, laccases are copper containing enzymes that catalyze the oxidation of aromatic and sulfonated azo dyes (Du et al., 2015; Ayed et al., 2010; Jadhav et al., 2008).

To identify the metabolites generated from MO after bacterial biofilm consortia treatment, UV-vis (Fig. 5) and FTIR (Fig. 6) spectroscopy were used. UV-vis spectral analysis revealed that the major peak at 465 nm was disappeared. The disappearance of the major visible light absorbance or generation of a new peak is associated with biodegradation of dyes (Chen et al., 2008). The FTIR spectroscopy results showed that most of the major peaks including the azo bond peak (1602.6 cm^{-1}) were disappeared completely or partly, deformed and shifted (Fig. 6B–E). Because the peak at 1369 cm^{-1} for $\text{S} = \text{O}$ stretching vibration widened or shifted (Fig. 6B–E) thus, sulfonated aromatic amines might be generated from MO degradation by these consortia. Sulfonated aromatic amines were also reported to produce by *K. rosea* from MO (Parshetti et al., 2010). Based on gas chromatography-mass spectrometry (GC-MS) and high liquid chromatography (HPLC), N,N-dimethyl-*p*-phenylenediamine and 4-aminobenzenesulfonic acid were identified as the main intermediates of MO (Masarbo et al., 2018; Du et al., 2015; Parshetti et al., 2010). In this study, we are unable to identify the produced metabolites of MO due to lack of research facilities. However, this study confirmed that biodegraded products of MO are non-toxic as reported earlier (Ayed et al., 2010; Parshetti et al., 2010; Du et al., 2015; Masarbo et al., 2018; Guo et al., 2020). Thus, all these consortia can be utilized to degrade and detoxify the MO from wastewater.

5. Conclusions

We observed that several nutritional (e.g., growth media, divalent metal cations and nitrogen sources) and environmental (e.g., temperature, pH, salinity, dye concentrations and oxygen tension) cues significantly influence the rate of decolorization of MO. All the studied biofilm consortia could effectively decolorize the dye MO (200 mg L^{-1}) in yeast extract peptone broth. The results of the spectroscopy and enzymatic studies confirmed that MO decolorization is due to degradation process. COD removal and toxicity

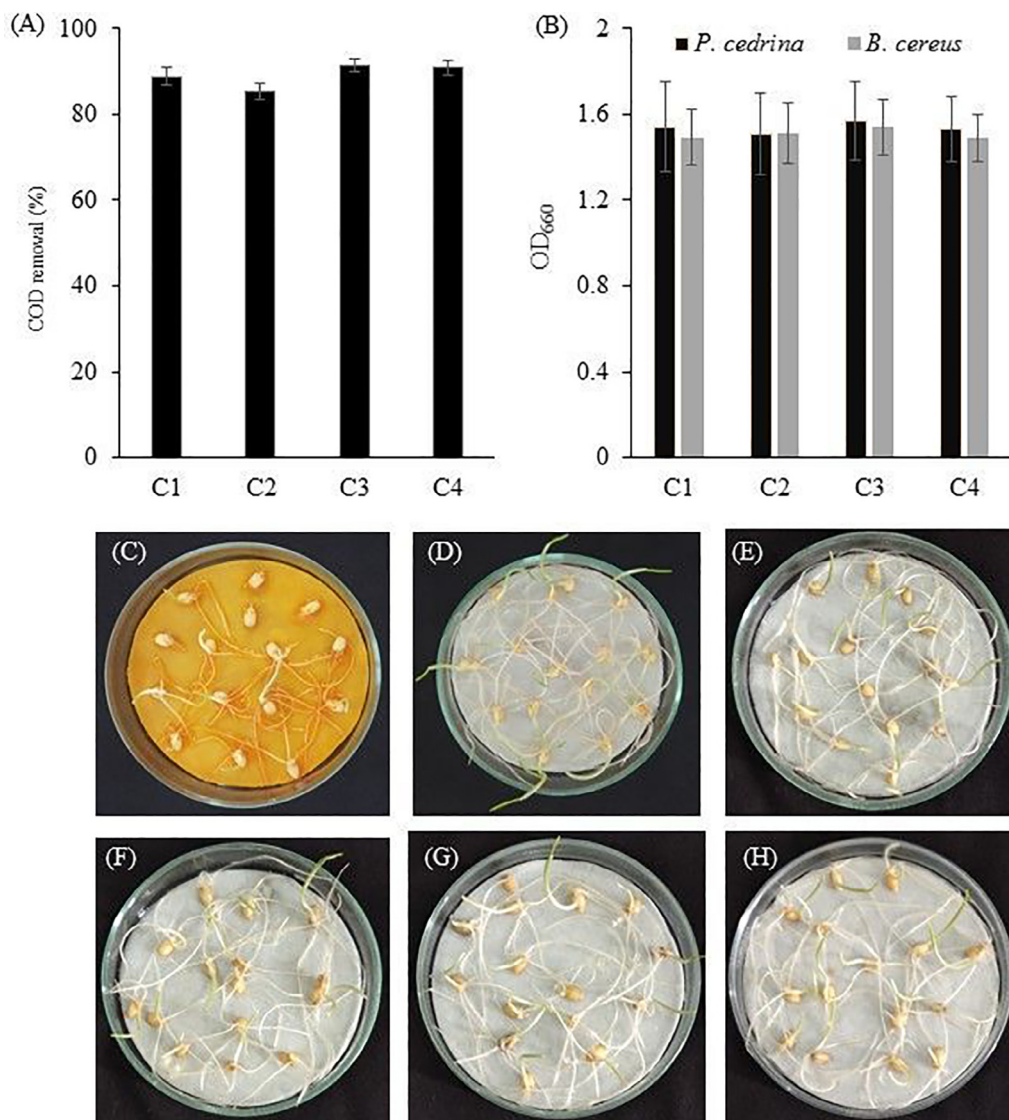


Fig. 7. Detoxification studies. (A) COD removal (%) by different consortia within 60 h incubation. (B) Growth rate of *P. cedrina* ESR12 and *B. cereus* ESD3 on biodegraded products of MO after 24 h incubation in agitate condition at 28 °C. Wheat seed germination irrigated with YEP broth containing 200 mg L⁻¹MO (C), tap water (D) and biodegraded products by different consortia (E-H) on 5th day. Data represent averages from triplicate assays and error bars of standard deviations.

studies revealed that biodegraded metabolites are non-toxic. Thus, these consortia can be utilized in bioremediation of MO from wastewater for safe disposal into environment.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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