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Identification and Optimization of Novel NAR-1 Bacterial Consortium for the Biodegradation of Orange II

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Abstract: Background: Decolourisation of azo dyes has been an area of great research interest since three decades ago. However, there is much limitation in microbial potential for mineralization of aromatic compounds resulted from decolourisation of various azo dyes. Hence, we aim to identify microbes which can rapidly decolourise and further biotransform the aromatic compounds from azo dye decolourisation. **Results:** A novel bacterial consortium (NAR-1) which could decolourise Orange II was developed. These bacterial strains, identified as *Enterococcus casseliflavus* and *Enterobacter cloacae* by 16S rRNA gene sequence analysis were formulated as NAR-1 bacterial consortium. *E. cloacae* worked synergistically with *E. casseliflavus* to enhance Orange II decolourisation. Within 15 min under microaerophilic incubation, over 95% of Orange II decolourisation was observed and complete colour removal was achieved at an amazing rate of 60 min. Analyses of biodegradation products of Orange II with Thin Layer Chromatography and High Performance Liquid Chromatography showed that decolourisation of Orange II by individual isolates and bacterial consortium resulted in the formation of sulfanilic acid. After 5 days of subsequent aerobic incubation, NAR-1 bacterial consortium was able to further biotransform sulfanilic acid. Neither *E. casseliflavus* nor *E. cloacae* can further biotransform sulfanilic acid individually. Total Organic Carbon analysis was employed to ascertain mineralization potential of Orange II by NAR-1 bacterial consortium. **Conclusion:** From this study, we can conclude that the novel NAR-1 bacterial consortium has demonstrated great potential in decolourisation of Orange II and further biotransformation of sulfanilic acid.

Key words: Decolourisation, azo dyes, *Enterococcus casseliflavus*, *Enterobacter cloacae*, sulfanilic acid

INTRODUCTION

Azo dyes are synthetic organic compounds with azo bonds (-N = N-) and having criterions that are necessary for their various applications in textile dyeing and many other industries. As they are designed to be stable and long-lasting colourants, they are usually recalcitrant in natural environment. The release of these dyes into the environment without proper treatment may lead to potential risk of bioaccumulation that may eventually incorporate into food chain and affect human health. In 2006, Dubey and coworkers reported on the interaction of some p-substituted azo dyes with mammalian calf thymus DNA and such interactions has lead to cytotoxicity or cell death (Dubey *et al*., 2006). Azo dyes inhibit the activity of tyrosinase enzyme, thus inhibiting melanin synthesis and resulting in hypopigmentation (Dubey *et al*., 2006, 2007). Therefore, it is important to treat the effluent containing azo dyes before discharging them into the nature. The emission of azo dyes into the natural environment had been a concern due to their negative aesthetic effects and the toxicity, mutagenicity and carcinogenicity of these dyes and their partial degradation products (Olukanni *et al*., 2009; Saratale *et al*., 2011).

Biological techniques for treating dyes in effluent include bacterial and fungal biosorption and biodegradation in aerobic, anaerobic or combined anaerobic/aerobic system. Biological dye removal techniques based on microbial transformation of dyes hold promises in providing a lower treatment cost and a more efficient mean to treat the effluent (Dos Santos *et al*., 2007). Various microorganisms, either aerobic or anaerobic bacteria, fungi, yeast and algae have been reported to catalyze azo dye decolourisation (Dos Santos *et al*., 2007; Saratale *et al*., 2011). Notably, bacterial decolourisation is relatively faster but may require a mixed community to mineralize azo dyes and their degradation products (Khehra *et al*., 2005; Moosvi *et al*., 2007; Joshi *et al*., 2008; Khalid *et al*., 2008).

In the present study, we reported a novel bacterial consortium, designated as NAR-1, having decolourizing ability of azo dyes under microaerophilic condition. Effect of different parameters, such as inoculum size, dye and nutrient concentration, temperature and pH on

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decolourisation were studied. Orange II was used as model azo dye. It contains a sulfonic acid group as the substituents and therefore is categorized as sulphonated azo dyes. Reduction of Orange II under anaerobic condition is predicted to form two aromatic amines: sulfanilic acid and 1-amino-2-naphtol (Nam and Tratnyek, 2000). Sulfanilic acid is a typical representative of sulfonated aromatic amines. As the sulfonyl group is a xenobiotic structural element, it resists biodegradation and is considered to be toxic. The strongly charged anionic moiety of sulfanilic acid prevents it from penetrating through bacterial membrane. Sulfanilic acid is also noted for its strong bactericidal effect. Thus, their elimination from wastewater is of great importance (Perei *et al.*, 2001). Another reduction product, 1-amino-2-naphtol is also a known carcinogen. However, this compound is a transient species as it undergoes rapid autoxidation (Coughlin *et al.*, 1999; Nam and Tratnyek, 2000).

MATERIALS AND METHODS

Chemicals: Orange II (C.I. Acid Orange 7, $C_{16}H_{11}O_4SN_2Na$) was purchased from Sigma-Aldrich, USA and was used as the model dye for all the optimization experiments at final concentration of 0.1 g L^{-1}. All chemicals used in this study were of analytical grade.

Medium: P5 medium containing K_2HPO_4 (35.3 g L^{-1}), KH_2PO_4 (20.9 g L^{-1}), NH_4Cl (2 g L^{-1}), glucose (10 g L^{-1}), nutrient broth (20 g L^{-1}) and trace elements was used. Mixture of K_2HPO_4, KH_2PO_4 and NH_4Cl were dissolved in distilled water (750 mL) and the pH was adjusted to pH 7.0±0.2 and autoclaved at 121°C at 101.3 kPa for 20 min. Glucose, nutrient broth and trace element stock, which were prepared and autoclaved separately, were aseptically adde2d to the medium. The medium was finally topped up to 1000 mL with sterile distilled water.

Isolation of novel dye-decolourizing bacteria: Both isolates, namely C1 and L17 were lab contaminants. These bacterial colonies were subcultured onto nutrient agar plate to obtain single colonies. After isolation, a single colony was picked and grown at 37°C in conical flask (250 mL) containing 100 mL of P5 medium in a shaking incubator for about 8 h. The starter cultures were then inoculated individually into P5 medium containing Orange II (0.1 g L^{-1}) to confirm its decolourisation ability under microaerophilic condition at 37°C.

16S rDNA-based identification of NAR-1 bacterial consortium: Genomic DNA was isolated from C1 and L17 isolates by using Wizard Genomic DNA Isolation Kit (Promega) according to the manufacturer's instruction. Universal primers of bacterial 16S rRNA gene that correspond to *Escherichia coli* position 8f (pA: 5'- AGA GTT TGA TCC TGG CTC AG) and 1542r (pH': 5'-AAG GAG GTG ATC CAG CCG CA) were used for 25-cycle PCR amplification (Edwards *et al.*, 1989). The PCR bands were viewed under UV transillumination in 1% agarose and purified by QIAquick PCR Purification Kit (QIAGEN). Sequencing of the 16S rRNA genes were carried out using pA and pH' primers, as well as designed internal overlapping primers. The sequences were analyzed at NCBI server (http://www.ncbi.nlm.nih.gov/) using BLASTn tool (Altschul *et al.*, 1997). The sequences were aligned with sequences from related bacteria available from GenBank database by using ClustalW. MEGA version 4.1 (Beta 3) was used for construction of Neighbor-Joining phylogenetic tree with bootstrap values calculated based on 1000 replicates.

Optimization studies of dye decolourisation: P5 medium with Orange II (0.1 g L^{-1}) was inoculated with different inoculum sizes in the range of 1 to 10% (v/v) of bacterial culture and incubated at 37°C under static microaerophilic condition in universal bottles which was filled up to the brim. In order to determine the effect of dye concentration on decolourisation, bacterial culture was added into P5 medium containing final concentration of Orange II in the range of 0.1 to 0.3 g L^{-1} and incubated at 37°C under static microaerophilic condition. Decolourisation of Orange II was carried out at temperature ranging from 30 to 50°C. Concentration of nutrients in P5 medium can affect the rate of Orange II decolourisation. Therefore, modified P5 medium with concentrations of nutrient broth and glucose at (I) 5.0, 2.5; (ii) 10.0, 5.0; (iii) 20.0, 10.0; (iv) 30.0, 15.0 and (v) 40.0, 20.0 (g L^{-1}) were prepared, respectively. Modified P5 medium that contains 5.0 g L^{-1} nutrient broth and 2.5 g L^{-1} glucose was used for further optimization. This medium was prepared at different pH values of 6.5, 7.0, 7.5, 8.0 and 8.5 by varying the composition of K_2HPO_4 and KH_2PO_4.

Dye decolourisation studies in modified P5 medium: C1 and L17 isolates were individually inoculated into modified P5 medium containing glucose (2.5 g L^{-1}), nutrient broth (5 g L^{-1}) and Orange II (0.1 g L^{-1}) and grown for 9 h to achieve OD_{600nm} ~ 5 prior to use as inoculum. Modified P5 medium was inoculated with 10% (v/v) of NAR-1 bacterial consortium. Decolourisation was carried out at 45°C under microaerophilic condition. Samples were drawn at 15 min time intervals and analyzed for growth and decolourisation. The rate of

decolourisation was determined by using a scanning spectrophotometer (UV-Vis 1601, Shimadzu) and the absorbance of Orange II was analyzed at 482 nm. Decolourisation occurred when absorbance of the characteristic wavelength decreased. Modified P5 medium with dye was used as control. The decolourisation activity was calculated as follows:

$$\text{Decolourisation (\%)} = \frac{\text{Initial absorbance} - \text{Sample absorbance}}{\text{Initial absorbance}} \times 100\%$$

Further biotransformation of decolourised samples: Decolourised samples were transferred to a conical flask. Fresh inoculum of NAR-1 (10% v/v) was added and incubated at 37°C for 5 days under aerobic condition. The degradation products were analyzed using Thin Layer Chromatography (TLC), High Performance Liquid Chromatography (HPLC) and the mineralization potential was studied by determination of Total Organic Carbon (TOC).

Analysis of degradation product by thin layer chromatography (TLC): Decolourised and further biotransformed samples were centrifuged at 5,000 rpm for 15 min. The supernatant was transferred to a separating funnel and overnight extraction was done by using methanol. The extracted product was concentrated to about 2 mL using a rotary evaporator. TLC plate coated with F254 silica gel (Merck) was repeatedly spotted with tiny drop of sample and placed inside a Chromatographic Chamber for elution. The developing solvent used was isopropanol : methanol : chloroform at 65 : 25 : 10 ratio. Visualization was carried out under the ultraviolet (UV) light at wavelengths of 254 and 312 nm. All spots representing specific compounds were examined based on the retention factor (R_f) values.

Analysis of degradation product by High Performance Liquid Chromatography (HPLC): HPLC equipped with UV-Vis detector (W600-2487, Waters) was used to determine the presence of sulfanilic acid in decolourised and further transformed samples. The supernatant was transferred to a separating funnel and equal volume of organic solvent, ethyl acetate was added. The top layer was collected and concentrated to about 3 mL by using rotary evaporator and equal volume of methanol was added to it. All samples were filtered using 0.2 µm nylon filter and 10 µL was injected for HPLC analysis. Samples were eluted isocratically at flow rate of 0.7 mL min^{-1} using Hypersil C18 column (5 µm, 4.6 mm × 250 mm, Waters). The mobile phase consisted of 50% (v/v) methanol. The detection wavelength was set at 254 nm.

Analysis of degradation product by Total Organic Carbon (TOC): Total organic carbon (Shimadzu TOC 500) was used to study the mineralization of Orange II by NAR-1 bacterial consortium. Samples were centrifuged at 5,000 rpm for 15 min, filtered with 2 µm nylon membrane and diluted 10 times prior to analysis. The reduction of carbon in each sample was checked by TOC analysis. The percentage of mineralization was calculated as follows (Feng *et al*., 2003):

$$\text{Mineralization (\%)} = \left(1 - \frac{\text{TOC value of sample}}{\text{TOC value of modified P5} + \text{Orange II}}\right) \times 100\%$$

RESULTS

Isolation and 16S rRNA-based identification of novel dye decolourizing bacteria: During the initial study of Orange II decolourisation by bacterium C1, an extraordinary rapid rate of decolourisation was achieved immediately after the inoculation of bacterium into the dye-containing medium. After the decolourisation was completed, culture from the decolourised medium was streaked on nutrient agar plates and incubated at 37°C for 24 h. Two types of bacterial colonies were obtained and they could be distinguished by their colony morphology. Bacterium C1 produced small round and clear colonies whereas bacterium L17 gave larger, round and turbid colonies.

BLASTn analysis of 16S rRNA sequence obtained revealed that bacterium C1 has 99% similarity with *Enterococcus casseliflavus*. *Enterococcus* spp. with close similarity were used to contruct the phylogenetic tree (Fig. 1). The position of bacterium C1 in the phylogenetic tree suggests that C1 is closely related to *Enterococcus casseliflavus* and is most possibly belonged to another strain of the same species. Bacterium L17 was also identified by 16S rRNA typing. The results from BLASTn analysis showed that bacterium L17 has 99% similarity with *Enterobacter cloacae*. The phylogenetic tree, (Fig. 2) shows the relationship between 6 species of *Enterobacter* spp. and rooted with *Yersinia pestis*. This indicates that bacterium L17 is closely related to *Enterobacter cloacae* and most possibly belongs to this species. The 16S rDNA sequences of C1 and L17 was deposited at GenBank with accession numbers GQ995669 and GQ995670, respectively.

Orange II decolourisation studies by individual isolates and NAR-1 bacterial consortium: Investigation of growth curve of single bacterium was not sufficient. The inoculation of separately grown cultures of C1 and L17 decelerated the dye decolourisation rate compared to NAR-1 bacterial consortium that were grown together.

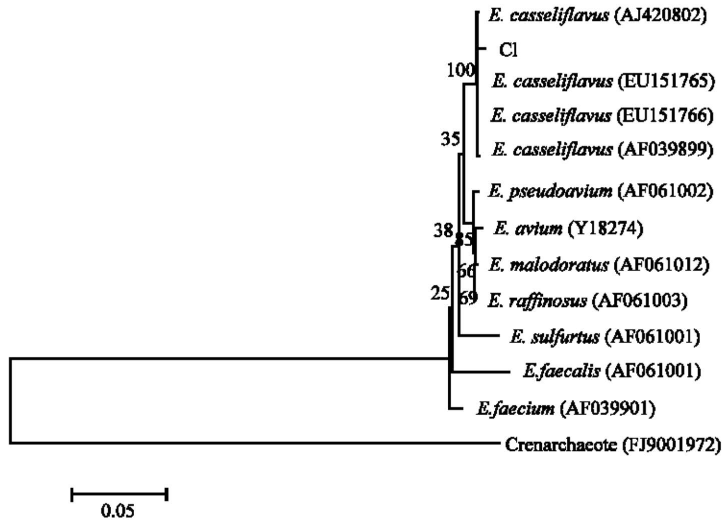

Fig. 1: Phylogenetic tree based on 16S rRNA gene sequence comparison showing the relationship between bacteria of genus *Enterococcus* and isolate C1. Numbers in bracket indicate the GenBank accession numbers

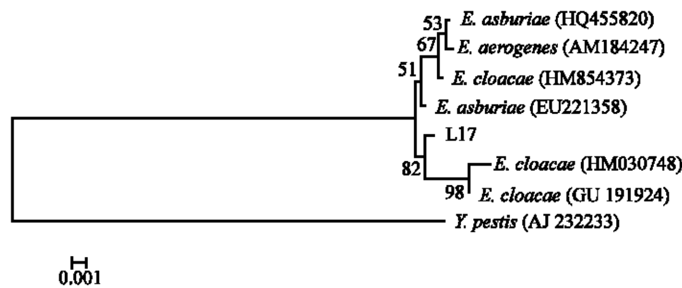

Fig. 2: Phylogenetic tree based on 16S rRNA gene sequence comparison showing the relationship between bacteria of genus *Enterobacter* and isolate L17. Numbers in bracket indicate the GenBank accession numbers

Therefore, the growth profile of NAR-1 bacterial consortium was also determined. Mixed culture of C1 and L17 which was incubated for 8 to 10 h until OD_{600nm} ~ 5 appropriately served as inoculum for dye decolourisation experiments. The influence of inoculum size on Orange II reduction by NAR-1 bacterial consortium was determined inoculating P5 medium with inoculum sizes in the range of 1-10% (v/v). It was observed that Orange II decolourisation peaked when 10% (v/v) of inoculum was used. Samples tested achieved 85% of Orange II decolourisation within 20 min at 37°C in P5 medium.

The performance of NAR-1 bacterial consortium in decolourisation of increasing concentration of Orange II was investigated and the result is shown in Fig. 3a. After 15 min of incubation at 37°C, it was observed that Orange II concentration lower than 200 mg L^{-1} had resulted in increased rate of decolourisation by NAR-1 bacterial consortium. Orange II decolourisation rate at concentrations of 100, 150 and 200 mg L^{-1} did not vary significantly. However, there was a drastic drop in decolourisation rate when the concentration of Orange II was higher than 200 mg L^{-1}. Dye removal for Orange II at concentration of 250 and 300 mg L^{-1} decelerated and only reached 55.47 and 33.32%, respectively.

Figure 3b indicates that active decolourisation of Orange II by NAR-1 bacterial consortium can be carried out at temperature ranging from 30 to 50°C. Better reduction followed by the elevated temperature from 30 to 45°C may due to higher respiration and substrate metabolism at this temperature range. The optimal temperature for the removal of Orange II by NAR-1 bacterial consortium was found to be at 45°C. At 50°C, NAR-1 bacterial consortium's decolourisation capability decreased slightly and reached 87.33% of decolourisation within 15 min. It was observed that rate of decolourisation decreased drastically as temperature reached 60°C.

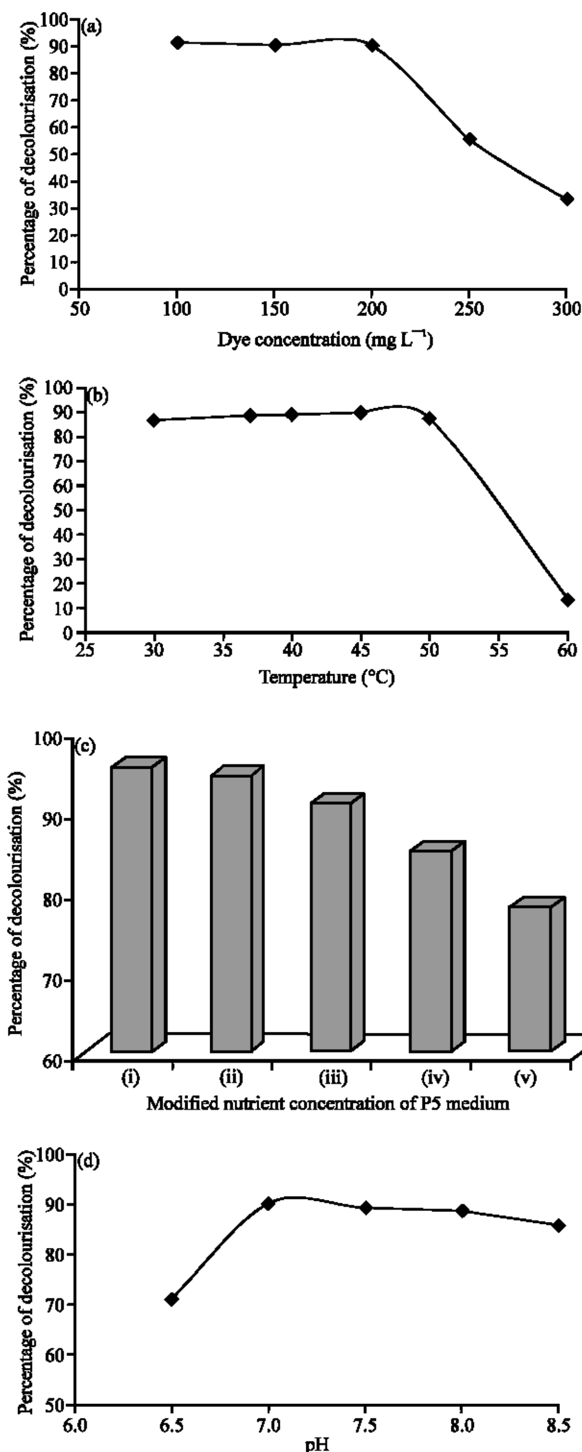

Fig. 3: Effect of (a) dye concentration; (b) temperature; (c) concentration of nutrient broth and glucose: (i) 5.0, 2.5, (ii) 10.0, 5.0, (iii) 20.0, 10.0, (iv) 30.0, 15.0 and (v) 40.0, 20.0 (g L^{-1}) and (d) pH on decolourisation of Orange II by NAR-1 bacterial consortium

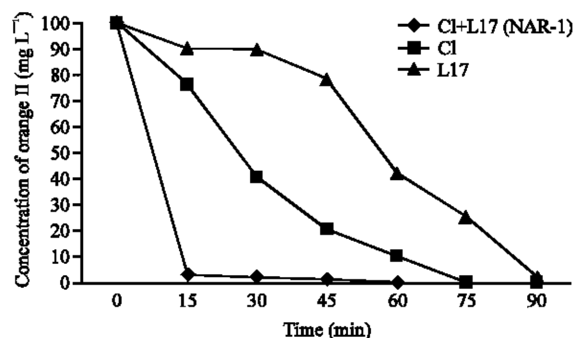

Fig. 4: Comparison of decolourisation of Orange II (0.1 g L^{-1}) by C1 and L17 individual isolates and NAR-1 bacterial consortium

Figure 3c shows that NAR-1 bacterial consortium had greater ability for azo dye removal at lower nutrient concentration. The result indicates that decreasing glucose and nutrient broth concentration enhanced the decolourisation of Orange II by NAR-1 bacterial consortium. In this study, modified P5 medium, which contains 5 g L^{-1} of nutrient broth and 2.5 g L^{-1} of glucose were found to be very suitable for rapid decolourisation of Orange II by NAR-1 bacterial consortium.

Figure 3d shows that neutral or slightly basic pH is preferred in the decolourisation of Orange II by NAR-1 bacterial consortium. Media with initial pH values in the range of 7.0 to 8.5 gave similarly good results of approximately 85 to 90% dye removal. At pH 7, Orange II decolourisation was optimum and reached 90% in less than 15 min.

Orange II decolourisation under microaerophilic condition of C1 and L17 individual isolates as well as NAR-1 bacterial consortium was compared and the result is shown in Fig. 4. Orange II was decolourised rapidly by NAR-1 bacterial consortium in the first 15 min of incubation. The overall rate of Orange II decolourisation by NAR-1 bacterial consortium was significantly higher than the rate of decolourisation by either C1 or L17 individual isolates.

Analysis of orange II degradation product with TLC, HPLC and TOC: Figure 5 shows TLC plate illustrating biodegradation products of Orange II. Spots for Orange II in P5 medium, sulfanilic acid standard and catechol standard were observed in lanes 2, 3, and 4 with R_f values of 0.75, 0.625 and 0.78, respectively. Spots with R_f value of 0.625 which is the same as the R_f value of sulfanilic acid standard were observed in lanes 5 and 7. This indicates that sulfanilic acid was probably formed after the decolourisation of Orange II by C1 and L17. An unidentified spot with R_f value of 0.425 was also observed

Table 1: TOC analysis of organic compounds mineralization in bacterial decolourized medium

Samples	Carbon concentration (ppm)	Percentage of mineralization (%)
Modified P5 medium	1200.15	-
Modified P5 medium +Orange II	1437.30	-
C1 microaerophilic decolourisation and further addition of C1 for further aerobic biotransformation	881.73	38.65
C1 microaerophilic decolourisation and further addition of NAR-1 for further aerobic biotransformation	825.93	42.54
NAR-1 microaerophilic decolourisation and further addition of NAR-1 for further aerobic biotransformation	865.53	39.78

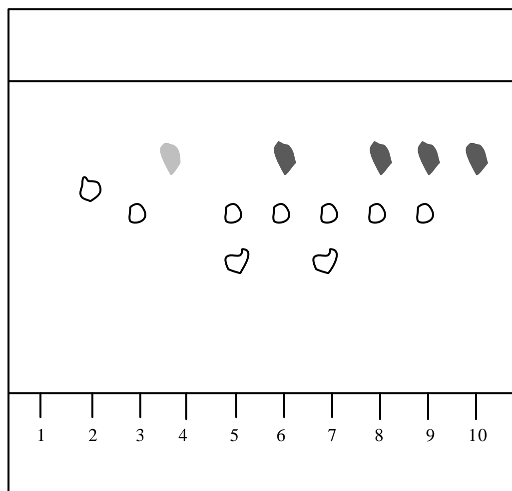

Fig. 5: TLC plate illustrating biodegradation products of Orange II in modified P5 medium at pH 7.0, 45°C after decolourisation followed by aerobic incubation for 5 days, 1: Modified P5 medium, 2: Modified P5 medium + Orange II (R_f: 0.75), 3: Sulfanilic acid (R_f: 0.625), 4: Catechol (R_f: 0.78), 5: Completely decolourised product by C1 (R_F: 0.625 and 0.425), 6: Further biotransformed product by C1, 7: Completely decolourised product by L17 (R_f: 0.625 and 0.425), 8: Further biotransformed product by L17, 9: Completely decolourised product by NAR-1 bacterial consortium, 10: Further biotransformed product by NAR-1 bacterial consortium

at lanes 5 and 7. This may suggest that 1-amino-2-napthol was formed due to the reduction of Orange II by C1 and L17. Lanes 6 and 8 show the formation of further biotransformed products after 5 days of aerobic incubation following Orange II decolourisation by C1 and L17, respectively. Spots with R_f value of 0.625 which represented sulfanilic acid were observed in lane 6 and 8. This indicates that sulfanilic acid was yet to be completely biotransformed either by C1 or L17 after 5 days of further incubation. However, the spot with R_f value of 0.425 was not observed. This may indicate that 1-amino-2-napthtol had been auto-oxidized or transformed into other metabolites after aerobic incubation. An extra spot with R_f value of 0.78 was observed at both lane 6 and 8. The R_f values of these spots were similar to the catechol standard but the colour shading was slightly different. 4-sulfocatechol may be the possible compound represented by these spots. Lane 9 shows the decolourised products and lane 10 illustrates the presence of further biotransformed products after 5 days of aerobic incubation following decolourisation by NAR-1 bacterial consortium. It was found that the spot representing sulfanilic acid (R_f value of 0.625) appeared in lane 9 but not in lane 10. This indicates that sulfanilic acid was formed as a result of Orange II reduction. Again, spot with R_f value of 0.78 was observed in both lanes. This may suggest that the sulfanilic acid formed may have been further degraded to 4-sulfocatechol by NAR-1 bacterial consortium after 5 days of aerobic incubation following microaerophilic decolourisation process.

From Fig. 6a, it was observed that the retention time for sulfanilic acid standard was at 3.161 min, where a sharp peak was formed. Peak with similar retention time as sulfanilic acid standard was found in chromatograms (b), (c) and (d) in Fig. 6. This suggests that sulfanilic acid was generated as a result of Orange II reduction by C1, L17 and NAR-1 bacterial consortium. These results coincide with the findings from TLC analysis reported earlier. Chromatograms (e) and (f) in Fig. 6 illustrate the metabolites formed after 5 days of further aerobic incubation of decolourised Orange II by C1 and L17 individually. The peak for sulfanilic acid was retained. This may suggest that sulfanilic acid was not completely biotransformed by either C1 or L17 individually even after 5 days of further incubation under aerobic condition. Two extra sharp peaks however appeared on chromatogram (e) and one extra peak was observed on chromatogram (f). This may indicate that some new metabolites formed after the reduction of azo bond and the reduction products were further transformed by C1 and L17. Chromatogram (g) in Fig. 6 shows the metabolites resulted from further aerobic biotransformation of decolourised Orange II by NAR-1 bacterial consortium. The peak represents sulfanilic acid could not be detected on this chromatogram. However, more new peaks were formed.

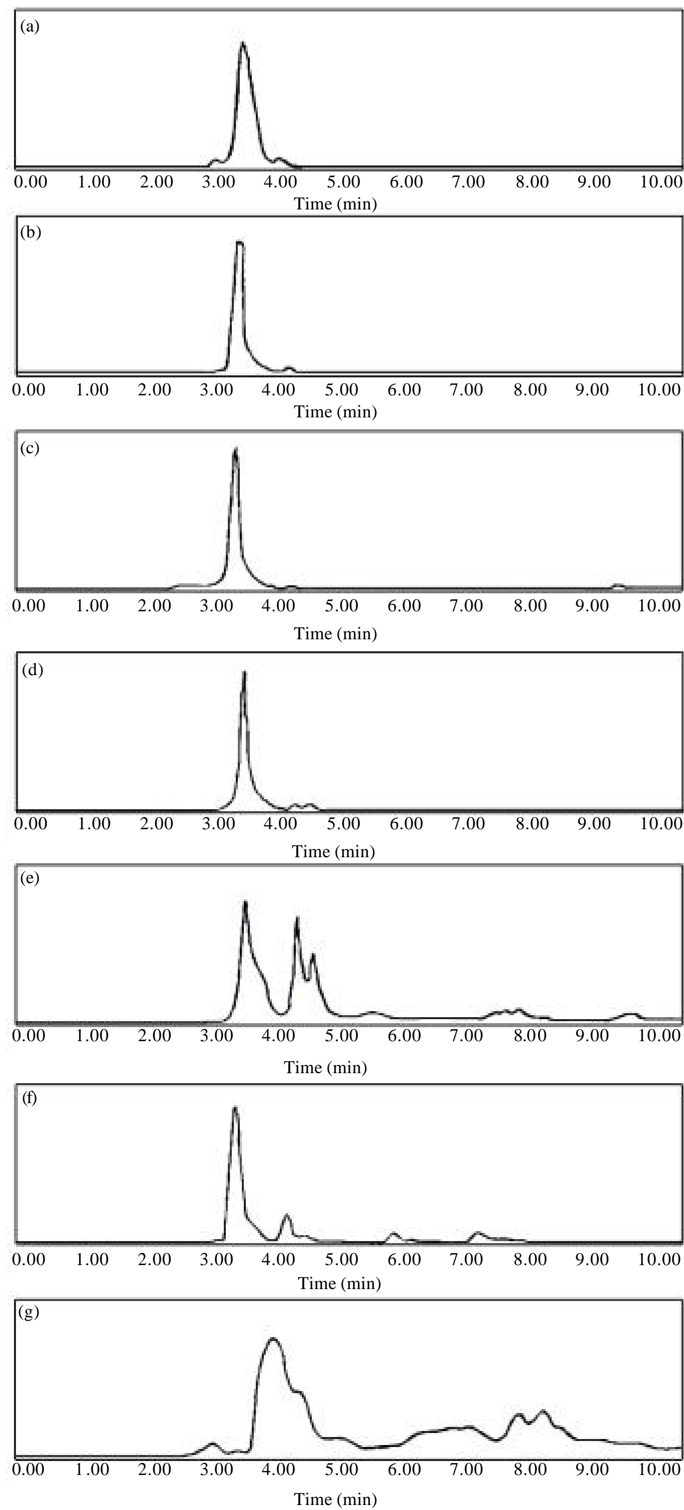

Fig. 6: Chromatograms of (a) sulfanilic acid standard; metabolites resulting from decolourised Orange II by (b) C1, (c) L17 and (d) NAR-1 bacterial consortium; metabolites resulting after 5 days of further biotransformation of decolourised Orange II by (e) C1, (f) L17 and (g) NAR-1 bacterial consortium

This may indicate that sulfanilic acid has been further biotransformed into other metabolites after 5 days of aerobic incubation with NAR-1 bacterial consortium.

Results from TOC analysis in Table 1 show that the organic carbon was reduced in all decoloursised sample tested. This suggests that the mineralization of organic compounds could have occurred after 5 days aerobic incubation and had resulted in more than 35% of mineralization. An addition of 10% fresh NAR-1 bacterial consortium inoculum following decolourisation by bacterium C1 appeared to be most effective in mineralization of organic compound, which achieved 42.54% of mineralization.

DISCUSSION

To the best of our knowledge, this is the first time *Enterococcus casseliflavus* and *Enterobacter cloacae* are reported for decolourisation of azo dyes. *E. casseliflavus* has never been applied in biodegradation studies. *E. cloacae* had been reported for filter paper biodegradation (Ramin *et al.*, 2008) and was isolated as contaminant in herbal medicine (Alwakeel, 2008). In this study, these isolates were formulated as NAR-1 bacterial consortium and had demonstrated great capability in decolourisation of azo dye. More than 95% of Orange II was decolourised by NAR-1 bacterial consortium in 15 min. This seems to be the fastest ever reported. Synergism of bacterial interaction among these isolates is obvious as the Orange II decolourisation rate of NAR-1 bacterial consortium was higher than individual isolates. Pearce *et al.* (2000) pointed out that higher degree of biodegradation could be expected when co-metabolic activities within a microbial community complement each other. Joshi *et al.* (2008) observed that more than 90% decolourisation could be achieved by bacterial consortium of *Aeromonas caviae*, *Proteus mirabilis* and *Rhodococcus globerulus* at 200 mg L^{-1} Orange II within 16 h. Bras *et al.* (2001) reported 94% decolourisation of 100 mg L^{-1} Orange II by mixed bacterial culture in 12 h. Coughlin and coworkers (2002) reported on complete degradation of 50 mg L^{-1} Orange II by *Sphingomonas* sp. strain ICX and unidentified bacterial strain SAD4i within 1 h in a rotating drum bioreactor containing the biofilm.

All the decolourisation processes in this study were carried out under microaerophilic condition which denotes the static incubation in universal bottle filled up to its brink. Azo dyes under aerobic condition are essentially considered recalcitrant to bacterial degradation as oxygen often inhibits azo reduction. The electrons liberated from the oxidation of electron donors by the cells are preferentially used to reduce oxygen rather than azo dyes (Coughlin *et al.*, 1999). Decolourisation under static microaerophilic condition had been reported by Joshi *et al.* (2008). Some groups reported on static incubation, instead of anaerobic condition for azo dye decolourisation (Moosvi *et al.*, 2007; Pearce *et al.*, 2000).

From the results shown, NAR-1 bacterial consortium could decolourise high concentration - that is up to 200 mg L^{-1} of Orange II. The significant decrease in decolourisation rate may be due to inhibition of bacterial cell growth by sulfonic group attached to Orange II. Pearce *et al.* (2000) suggested that the higher the dye concentration, the longer the time required to decolourise. This may due to the toxicity of the metabolites formed during dye reduction (Pearce *et al.*, 2000). The ability of NAR-1 bacterial consortium to decolourise at higher temperature of 45°C would facilitate its future application in treating dyeing industrial waste effluent that is normally discharged at high temperature. This study also showed that Orange II decolourisation was enhanced under nutrient limiting conditions though the presence of glucose and nutrient broth were still necessary for microbial survival and decolourisation. Published reports had frequently indicated that electron-donating co-substrates such as glucose and yeast extract were required for azo reduction by bacteria (Isik and Sponza, 2003; Pearce *et al.*, 2000; Moosvi *et al.*, 2007; Joshi *et al.*, 2008). These compounds provided electrons for the reductive cleavage of azo dyes (Isik and Sponza, 2003). Nutrient broth consists of major nutrient elements such as nitrogen from protein hydrolysate and extract (Dimitrova *et al.*, 2004). Nitrogen is a major constituent of cells and is essential for bacterial growth and enzyme production. However, some studies showed that nitrogen rich culture may inhibit the colour removal ability by bacteria (Banat *et al.*, 1996). Zissi and Lyberators (1996) pointed out that ammonium ion may compete with azo bond for electrons and hence inhibits the reduction of azo chromophore. Therefore, lower nutrient concentration is enough to act as co-metabolite and provides necessary nutrient for bacterial growth and enzyme production. It was noted that neutral and basic pH is more suitable for decolourisation of Orange II by NAR-1 bacterial consortium. In this study, phosphate buffer was used in P5 medium to provide pH control during growth and dye decolourisation. Review by Pearce *et al.* (2000) showed that biological reduction of azo bond could result in pH increase due to the formation of aromatic amines which were more basic than the original azo compound. Without buffering capacity, pH would fluctuate following bacterial metabolism and thus decreasing the dye decolourisation rate.

We also note the synergistic bacterial interaction between *E. casseliflavus* C1 and *E. cloacae* L17 in biotransformation of Orange II decolourisation products. The reduction of azo bond of Orange II was expected to produce two compounds: 1-amino-2-napthol and sulfanilic acid. 1-amino-2-napthol is a known carcinogen, however, it is a transient species as it undergoes rapid autoxidation and results in darkly coloured compound which is also highly recalcitrant and toxic (Coughlin *et al.*, 1999, 2002; Nam and Tratnyek, 2000).

In this study, we will discuss about the further biotransformation of sulfanilic acid. Sulfanilic acid is a typical representative of aromatic sulfonated amines that is hard to degrade due to the xenobiotic nature of sulfonyl group (Perei *et al.*, 2001). Degradation products such as aromatic amines are regarded as recalcitrant to anaerobic environment. Under aerobic incubation, many aromatic amines could be degraded and some could be mineralized (Isik and Sponza, 2003; Coughlin *et al.*, 2002). In TLC analysis, catechol was chosen as standard for comparison as the expected product 4-sulfocatechol is a derivative from catechol (Kulla *et al.*, 1983). From this study, NAR-1 bacterial consortium was able to complement each other by giving a synergistic effect to further biotransform sulfanilic acid, which has been known to be a dead end product. According to Rajaguru *et al.* (2000), the degradation of recalcitrant compounds, such as sulfonated aromatics is often accomplished by mixed culture. They also pointed out that compounds with xenobiotic characteristic require unusual catabolic activities, which may not be found in a single microorganism.

In the study done by Feng *et al.* (2003), mineralization of Orange II was detected by using TOC. Some of the intermediates formed during decolourisation could be more persistent and even more toxic than the parent compounds to aquatic animals and human beings (Feng *et al.*, 2003). Therefore, it is necessary to study the mineralization of Orange II simultaneously with the study on decolourisation. TOC is a method used to assess the extent of mineralization of organic compound. TOC removal indicates that the organic intermediates have been oxidized to carbon dioxide and water (Feng *et al.*, 2003). TOC analysis in determining the mineralization of Orange II intermediates in this study is only acceptable if the carbon removal is more than 83.5%. The total carbon removal achieved in all samples was less than 45% as the organic carbon mineralized may be either from modified P5 medium or Orange II. Thus, the TOC analysis results obtained was not conclusive and possible total mineralization of Orange II by NAR-1 bacterial consortium in this study is still questionable. Application of TOC to determine azo dyes mineralization is more suitable for studies with decolourisation medium that contain very little or exclude carbon source other than azo dyes as in Feng *et al.* (2003). Nevertheless, this study had ascertained that NAR-1 bacterial consortium could further biotransform Orange II degradation products into other compounds.

CONCLUSION

Two morphologically distinct bacterial isolates, namely, C1: *Enterococcus casseliflavus* and L17: *Enterobacter cloacae* were identified. These bacterial strains formed a novel bacterial consortium, NAR-1 which achieved better biodegradation potential of Orange II and sulfanilic acid as compared to pure cultures. This signifies synergistic interaction between the strains. NAR-1 bacterial consortium achieved over 95% of decolourisation during the first 15 min of microaerophilic incubation and complete decolourisation was amazingly achieved at 60 min. This bacterial consortium was found to be able to further biotransform sulfanilic acid under aerobic condition.

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