Sponge-associated bacterium, *Yangia pacifica*: a potential candidate for bioremediation of azo dyes

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The bioremediation potential of bacteria associated with the sponge *Cinachyrella cavernosa* on azo dyes, Amido black and Congo red is reported. Twenty four percent of the bacteria decolourized these dyes at 50 mg l^{-1} concentration. The isolate, *Yangia pacifica*, which showed higher tolerance and decolourization potential, was subjected to detailed studies. The strain showed >70% decolourization on day 3 and >96% on day 7. Decolourization was dependent on growth, medium, pH, temperature and dye concentration. Although decolourization reduced the toxicity of both dyes, the mechanism leading to decolourization of the two dyes was different.

Keywords: Azo dyes, decolourization, sponge-associated bacteria, *Yangia pacifica*.

AZO dyes are most widely used in textile industries and the effluent discharge from these industries has been reported[1] to contain around 300 mg l^{-1} dyes. The effluent containing ≥1 mg l^{-1} dye is known to be a potential threat to aquatic flora and fauna[2–4]. This has prompted dye waste management using bioremediation as an alternate strategy to economically non-viable chemical methods[5]. Considering the serious toxic effects of these dyes, it is imperative to develop a systematic procedure for eradicating them from aquatic environment. Most of the studies on decolourization and degradation of these dyes were carried out using individual bacterium and consortia of micro-organisms from soil, decomposed plant parts and industrial effluents[6–11]. However, microbes associated with sponges, which are known to produce potent bioactive compounds[12], have not been explored for degradation of the toxic and complex azo dyes. Very few studies have been carried out on *Y. pacifica*, which was first reported from the sediment of China Sea in 2006 (refs 13–14). In this communication we report for the first time the decolourization of Amido black (AB) and Congo red (CR) dye by *Y. pacifica*, a bacterium isolated from the sponge *Cinachyrella cavernosa*.

Sponge *C. cavernosa* was collected from the intertidal rocks of Anjuna beach, Goa (15°00′N, 73°00′E). The sponge was washed free of debris and sediment with filtered autoclaved seawater. Homogenized tissues were serially diluted in filtered autoclaved seawater up to 10^{-5} dilution. From the serially diluted tubes, 100 µl was inoculated onto Zobell marine agar medium. After incubation for 24–48 h, 76 morphologically different bacterial colonies were isolated, purified and tested for decolourization of the dyes. For decolourization studies, the bacterial isolates were inoculated into Zobell marine broth and incubated for 24 h in a shaker incubator at 25°C. The culture broth was centrifuged at 10,000 *g* for 10 min. The pellets were re-suspended in physiological saline and a cell density of 10^6 cells ml^{-1} was used as the inoculum.

Decolourization studies were carried out following Babu *et al.*[15] with modification. The bacterial suspension (100 ml l^{-1}) was inoculated into Zobell marine broth containing the dye (50 mg l^{-1}) and incubated for 2 days. Controls used were the medium and the medium with heat-killed cells. All experiments were performed in triplicate. The shift in wavelength or decrease in peak height was considered as positive for decolourization of dyes. The absorbance of the supernatants was measured at λ_{max} at 618 nm and 498 nm for AB and CR respectively, using microplate reader (FLUOstar Omega Microplate Reader-BMG LABTECH). The percentage of decolourization was calculated using the formula

$$\text{Decolourization (\%)} = \frac{\text{Initial absorbance} - \text{final absorbance}}{\text{Initial absorbance}} \times 100.$$

All readings were normalized with the controls.

The bacterium was identified as *Yangia pacifica* (Roseobacter clade) based on 16S rRNA gene sequencing (EMBL accession no: LK022801). It showed >50% decolourization of both dyes and was selected for detailed studies. Growth study was carried out at different pH, temperature and concentrations of dye before the decolourization assay. Growth was measured at 600 nm. The decolourization potential was determined as mentioned above. For viable cell count, 2 ml of the culture broth was removed and incubated with a cocktail containing piromedic, pipemedic, nalidixic acid and yeast extract for 6 hours[16] and fixed with formaldehyde (4% final concentration). The number of viable cells was counted[17] and expressed as cells ml^{-1}. Growth and decolourization were monitored daily for 7 days. Toxicity of the decolourized supernatant was tested by checking the growth of an azo dye sensitive, non-degrading bacterium, *Alteromonas* sp. in the supernatant amended with nutrient.

The factors affecting decolourization of the dyes were tested based on Plackett–Burman design[18]. The variables were different concentrations of nutrient in the medium (0.6, 1.2, 3.0 and 6.0 g l^{-1} of peptone and yeast extract in 5 : 1 ratio), different pH (6, 8 and 9), dye concentrations

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(50 and 100 mg l^{-1}) and temperature (27° ± 2°C and 37° ± 2°C). A total of 48 tests with different combinations of parameters were carried out for each dye. Cytoscape was used to make network to visualize the best conditions for decolourization.

To understand the mode of decolourization, clean glass pieces (6 × 6 × 1 mm) were incubated for 12 h in the decolourization culture flasks. The glass pieces were retrieved and subjected to stepwise dehydration in increasing ethanol concentration (30%, 50%, 70%, 90% and 100%). The samples were then air-dried, mounted on a stub and sputter coated with Au/Pd. The specimens were then visualized with a scanning electron microscope (JEOL JSM-5800). The shift in absorption spectrum of the dye was measured from 300 to 850 nm using a microplate reader. The shift in absorption due to transformation was confirmed using thin layer chromatography (TLC). For TLC, the culture broth was centrifuged at 10,000 *g* for 10 min after the decolourization experiment to separate the bacterial cells. The supernatant was lyophilized and the methanol extract of the lyophilized supernatant was passed through a silica gel column (Merck 60F 254), with 30% methanol in chloroform as mobile phase. The degradation was confirmed by comparing the *Rf* values with that of the *Rf* values of dyes present in the culture medium before the experiment.

The sponge associated bacteria showed 23.7% decolourization and 33% of this showed decolourization for both AB and CR. The decolourization potential for AB (7–77%) was higher than that of CR (1–61%), in the first 2 days. The relatively low decolourization of CR may be attributed to its high molecular weight, structural complexity and an additional azo bond[19]. Azo dye colour reduction is a ubiquitous capacity of many microorganisms under anaerobic conditions[20], as azoreductase is oxygen-sensitive[21]. However, some aerobic bacteria have developed the ability to reduce the azo group by special oxygen-tolerant azo reductases[22]. Thus it appears that the aerobic, microaerophilic and anaerobic niches within the sponge augment the development of strains with specialized oxygen-tolerant azoreductases.

Y. pacifica, an aerobic strain, showed 77% and 61% decolourization of AB and CR respectively. However, there are no reports available on the decolourization potential of azo dyes by *Yangia* sp. The percentage of decolourization by *Y. pacifica* in our study was comparatively higher than that reported for *E. coli* and *Pseudomonas* sp. under microaerophilic condition within 5 days[19]. The bio-decolourization of dye depends on the organism, its growth conditions and concentration of the dye[2,15]. The presence of specific nutrients in the culture medium can enhance the growth of bacteria[19,23] with a concomitant increase in decolourization[24]. *Y. pacifica* decolourized >80% of AB and CR when the nutrient concentration was 0.6 g l^{-1}. Decolourization of AB and CR showed only 8% and 14% increase (from 87.9% to 96.5% and 82.2% to 96.2% respectively) when the nutrient concentration was increased by 10-fold (Figure 1). Hence from an economic point, it will not be feasible to increase the nutrient concentration, though concentration of 6 g l^{-1} of nutrient gave the highest decolourization. *Y. pacifica* decolourized 50 and 100 mg l^{-1} of both AB and CR (Figure 1), but with a lower percentage in 100 mg l^{-1}. Such reduction in decolourization has been attributed to the loss of cell viability or negligible oxido-reductive enzyme activities[7]. Although the bacterium could tolerate up to 1 g l^{-1} of both dyes, the growth reduced with increased dye concentration (Supplementary Table 1), thereby creating inadequate biomass for dye uptake[25–27]. On the other hand, increased cell number brought about increased decolourization. Another factor that enhanced decolourization was temperature. It was observed that when the temperature was increased to 37° ± 2°C, decolourization also increased (Figure 1). Such changes in decolourization rate with temperature variations have been reported earlier[10]. Transport of dye molecules across the cell membrane may be affected by pH, which is considered as the rate limiting step for decolourization[28]. However, most of the decolourization studies have been

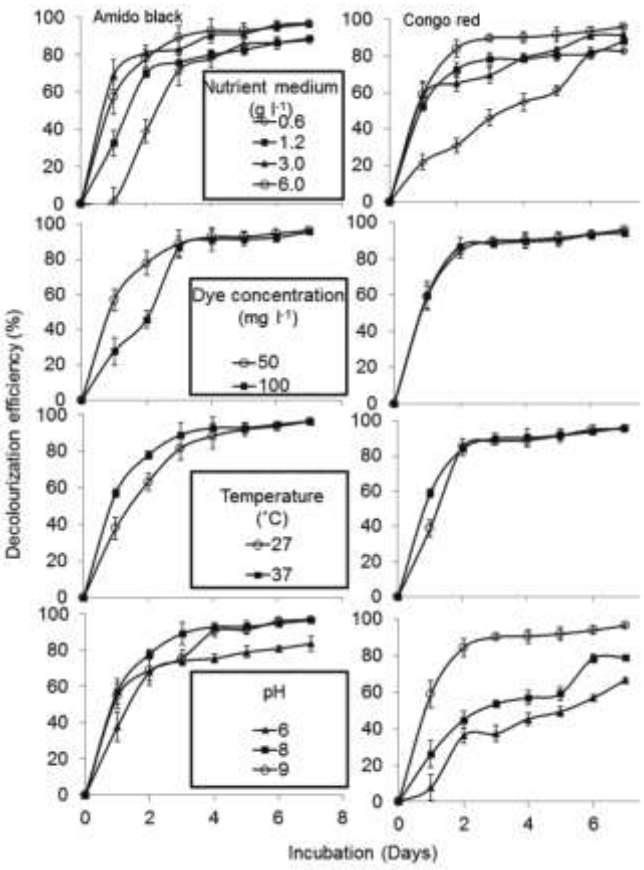

Figure 1. Effect of different parameters on decolourization efficiency. Data points indicate the mean of three independent replicates, standard error of mean is indicated by error bars. (Symbols are same for both the dyes).

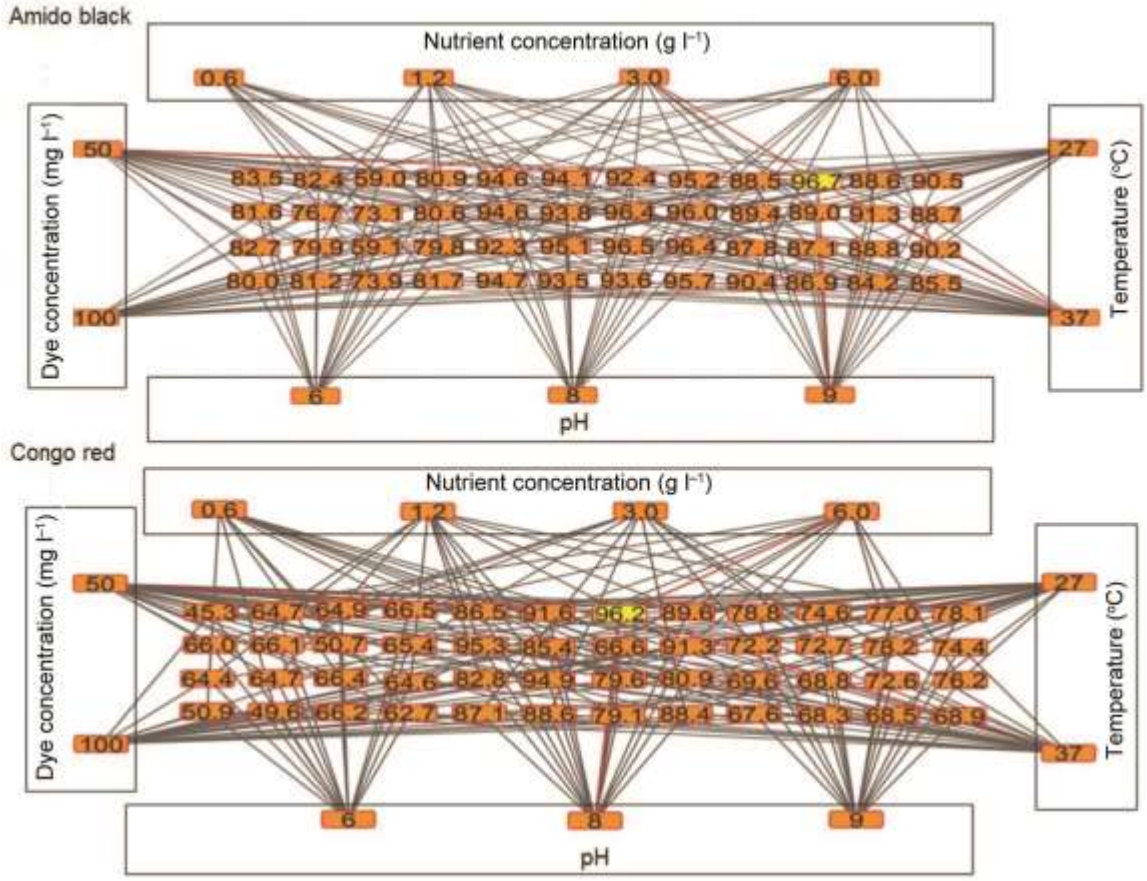

Figure 2. Snap shot of the network showing percentage of decolourization for different parameters.

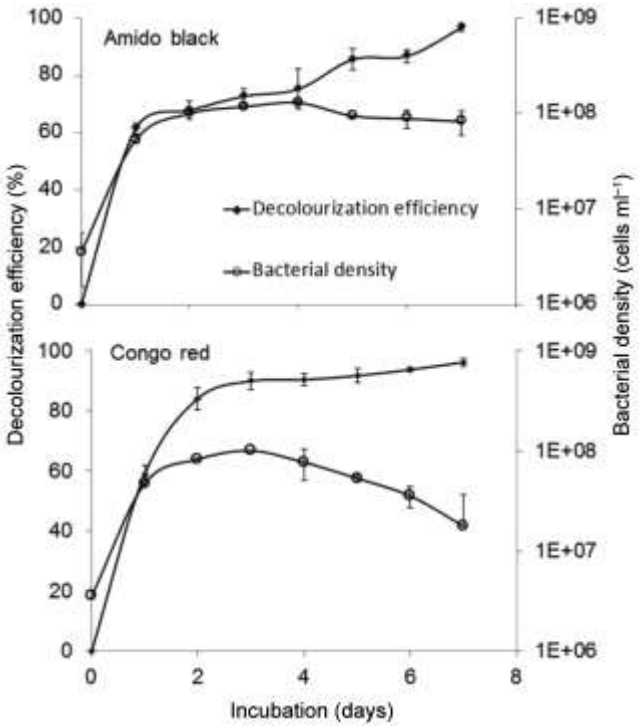

Figure 3. Dye decolourization efficiency and growth of *Y. pacifica* at optimum condition.

carried out at neutral pH[2] except for a recent report of decolourization of CR at pH 8 by *Dietzia* sp[29]. *Y. pacifica* exhibited better growth and decolourization properties in the alkaline range. The efficient functioning of this strain in alkaline pH makes it a potential candidate for biotreatment of alkaline textile effluents[30]. Among the different factors studied, the network analysis showed that the most influencing factors for decolourization of azo dyes by *Y. pacifica* were pH and nutrient concentration (Figure 2 and Supplementary Table 2). *Y. pacifica* decolourized 96.7% and 96.2% of 50 mg l^{-1} of AB and CR respectively, at the optimum conditions of 6 g l^{-1} nutrient medium, 37° ± 2°C temperature and pH 9 (for AB) and 8 (for CR) (Figure 3). Growth-related decolourization of 4-chloro-2-nitrophenol has been reported for *Bacillus subtilis*[31] which is similar to the present strain. There was a decrease in growth of *Y. pacifica* after 3 days which may be due to the byproducts of the dye and/or lack of nutrient. Studies have shown that degradation products of azo dyes are toxic[32]. However, the byproducts of the azo dye, decolourization by *Y. pacifica* was detoxified, as the azo dye non-decolourizing bacterium, *Alteromonas* sp. showed growth in the decolourized supernatant of *Y. pacifica* (Supplementary Table 3). This detoxification of the dye may be due to biodegradation (transformation) of

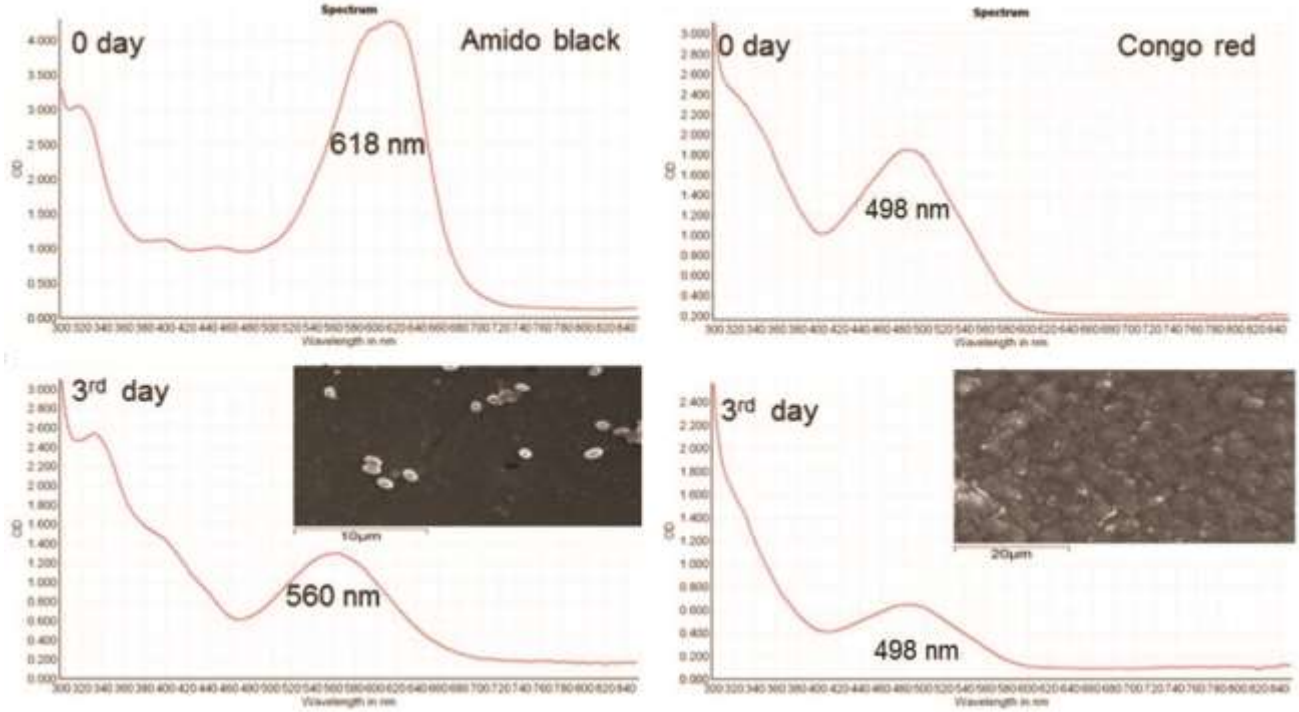

Figure 4. Mode of decolourization of the dye. Inset showing SEM image of *Y. pacifica* cells after decolourization.

the azo dye by *Y. pacifica* or the adsorption of the dye or its byproducts on the bacterial cells[31].

A shift in λ_{max} from 618 to 560 nm was observed in AB (Figure 4) at day 3, suggesting that there was a transformation of the dye[33]. Re-colourization of the supernatant occurred on addition of fresh dye, ruling out the possibility of the effect of redox potential on decolourization of AB, as opined by Isilk and Sponza[19]. Unlike AB, there was no shift in λ_{max} for CR, but a decrease was noted in the peak height (Figure 4). SEM image and Supplementary Figure 1 showed no AB adherence on the cell or pellet whereas CR was visible on the surface of bacterial cells and a red colour was seen on the pellet. The possible mode of decolourization by *Y. pacifica* may be by absorption of extracellular polymeric substances forming a trap for dissolved dyes[33] and subsequently transforming the dye. The dye degradation was also supported by TLC analysis. In both AB and CR-treated bacterial cultures, the *Rf* values of methanolic extracts of the supernatant after decolourization experiment were lower than that of the extracts before the experiment (0.516 and 0.645 for AB and 0.138 and 0552 for CR). Based on this, it can be deduced that decolourization of AB was by degradation while that of CR was more by adsorption.

The present study reiterates that sponge associated bacteria are promising source for bio-prospecting. The potentiality of *Y. pacifica* in degrading the azo dyes is reported for the first time. The high decolourization ability of the natural bacterium under alkaline and low nutrient conditions shows that it is a promising candidate for application either independently or in a consortium for decolourization of the textile effluents.

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Hearing impairment of Indian agricultural tractor drivers

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Noise is an occupational hazard affecting the health and safety of the tractor drivers. The hearing impairment of Indian tractor drivers has been assessed in the present study. Sixty healthy male subjects of similar age, height and weight were selected and divided into two groups of 30 subjects each, viz. tractor drivers with more than 10 years of driving experience and office workers as control. Audiometric testing of both the ears of the selected subjects was conducted at ten frequencies, i.e. 0.125, 0.25, 0.5, 1, 1.5, 2, 3, 4, 6 and 8 kHz. It was observed that the hearing threshold levels of office workers at measured test frequencies were less than 25 dB(A) and exceeded 25 dB(A) for

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