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Remazol reactive dye degrading Bacteria from freshwater fish of River Cauvery, Pallipalayam of Namakkal District, South India

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Abstract

Background: Azo dye effluents cause severe pollution problems in soil and water and affect their flora and fauna throughout the world. The combination of dye degrading bacteria in the gut of freshwater fish has been considered a prospective approach towards the development of a sustainable environment. In the southernmost part of India, especially in the Namakkal District of Tamil Nadu state, urbanization and industrial development lead to various environmental issues. In lieu, most of the earlier works were carried out on the bacterial dye degradation from soil and water sources, least concentration on the dye degradation from fish gut flora. The objective of this study was to analyze the role of dye degrading bacteria in the gut of freshwater fish collected from the study area.

Results: We have studied the aerobic degradation of remazol yellow reactive dye by bacteria forming the gut flora of exotic fish *Oreochromis mossambica* from river Cauvery at Pallipalayam of Namakkal District, India. Eight dye degrading gut isolates viz. *Pseudomonas* sp. AD1, *Bacillus* sp. AD2, *Staphylococcus* sp. AD3, *Staphylococcus* AD4, *Enterobacter* sp. AD5, *Escherichia coli* AD6, *Streptococcus* sp. AD7 and *Clostridium* sp. AD8 were obtained capable of growing in azo dye incorporated in Basic mineral medium (BMM) medium. Optimization study was carried out for various parameters for the selected isolates. Based on the growth, maximum growth was seen in *Pseudomonas* sp. AD1 (in 0–24 h) and minimum in *Escherichia coli*.AD 6 (in 72–96 h). *Pseudomonas* sp. AD1, *Enterobacter sp.*AD5, and *Bacillus* sp. AD 2 exhibited efficient dye degradation during 0–96 h analysis which was confirmed by FTIR analysis. Loss of azo group stretch at 1572–76 cm^{-1} and 1429 cm^{-1} and presence of primary (–NH2–) and secondary amides (–NH2–), aliphatic –C–N– stretching and confirmed –C–S– stretching in FTIR analysis confirms the breaking down of the azo bond.

Conclusion: This study showed that there is a platform for using the bacterial flora of fish gut that paves a prominent way in the degradation of Azo dye.

Keywords: Azo dye, Remazol yellow, *Oreochromis mossambica*, Gut flora, Bacteria, FTIR analysis

Background

Environmental pollution has been considered as one of the serious problems throughout the World especially from the textile industries (Khan and Malik 2014). The known reasons behind this are industrialization, urbanization, and other man-made activities. Textile dyes have been used for many years for coloring and printing fabrics. The effluent contains recalcitrant and other hazardous contents, not only carcinogenic and toxic to humans and other biotic organisms (Mahmoud et al. 2010; Reddy Roja et al. 2020) especially the recalcitrant nature of azo dye and the various issues raised by the releasing of azo dye in aquatic systems (Sinha et al. 2019a, b; Lade et al. 2012) and various disorders to human health systems (Saratale et al. 2011; Chung and Cerniglia 1992) were reported already. The complex aromatic structure of textile dye resisted detergent, sunlight, and temperature

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a noteworthy point mentioned here. The effluent from dye-stuff industries has contaminated the soil as well as surface and underground water (Fu and Vijayaraghavan 2001) and responsible for a lot of problems in an aquatic environment. The prominent uses of Azo dyes in diversified industries like textile, leather, printing, plastic, pharmaceutical, and food. The dyeing industries used water as a principal medium for the application of dyes, and discharge the wastewater, as one among the important medium for an aquatic pollutant (Sweety 2018). Different types of dyes and additives are known which make this an organic as well as inorganic pollutant source in textile effluent. With 80% of total azo dye consumption in dying industries, India is ranked second in the world (Juwarkar et al. 1997). In India, textile industries are contributing to about 25% of total export earnings and employing almost the total labor force (MSME report 2013). Pallipalayam, Namakkal District, Tamil Nadu state is one of the important business domains for textile and dye industries (Olukanni et al. 2005). Hence we need a vital bio-mediated alternate option for remediated the polluted site to maintain a sustainable environment.

Though numerous soil and water based potential dye degrading bacteria were existing in the environment, the present study dealt with the discharged water from dye industry mixed with the river water, hence the fish gut dwelling bacteria from the polluted river was taken into account. Besides, fishes are continuously exposed to the microorganisms present in water and sediment. The intestinal tract of fishes is populated which harbors a high load of microorganisms due to the availability of more organic matter consumed by fish as food. The organisms may enter the mouth with water or food and pass through and/or colonize the digestive tract. The economic constraints as a hurdle in the existing dye effluent treatment process viz. adsorption, filtration, coagulation, and chemical flocculation insisted the need for alternate eco friendly approaches through biotic organisms (Sanmuga Priya et al. 2015). In addition with this, the recently published report revealed the novel utility of bacteria from fish gut wastes are used for the detoxification of textile dye (Reddy Roja et al. 2020).

Azo dyes are known to undergo reductive cleavage whereas the resultant aromatic amines are metabolized under aerobic conditions (Sathian et al. 2013). So for complete mineralization of azo dyes, the microbial population forming part of the treatment system should be able to work efficiently to find an alternative to developing a sustainable environment. Because of these problems, the most potent bacterial culture was taken from the fish gut was taken into consideration for the present study. In the present work, degradation of an azo dye- Remazol yellow was studied using gut flora of exotic freshwater fish *Oreochromis mossambica* and analyzed by Fourier transform infrared spectroscopy (FTIR).

Materials and methods

Bacterial isolates

O. mossambica fishes (20 nos.) (Barragan et al. 2007) were collected from river Cauvery at Vasantha Nagar of Pallipalayam of Namakkal district. The physico chemical parameters of water samples were measured and recorded at the site of fish collection. The foregut flora was eviscerated and contents were transferred aseptically into the nutrient broth and incubated overnight in a shaker for enrichment at 37 °C.

Isolation of azo dye degrading bacteria

After enrichment, the broth was spreaded on Basic mineral medium (BMM) [$NaNO_3$ (2.0 g/L), NaCl (0.8 g/L), $CaCl_2{\cdot}2H_2O$(0.1 g/L), KH_2PO_4 (2.0 g/L), $Na_2HPO_4.12H_2O$ (2.0 g/L), $MgSO_4$(0.2 g/L), $FeSO_4.7H_2O$ (0.001 g/L); Agar 18–20 g/L] containing an azo dye—Remazol yellow (0.5 g) as the sole carbon source for a period of 4 days at 37 °C (Ajaz et al. 2019). Based on the growth of the organism, the predominant isolates were taken for spectrophotometric analysis**.** (Nachiyar and Rajkumar 2003; Pandey et al 2007). The optimal growth conditions of various parameters (pH, temperature, concentration of dye, amount of bacteria and agitation speed) were determined as per standard procedure (Ajaz et al. 2019). Further the morphological parameters and and biochemical tests were performed for the predominant isolates as per standard procedure (Cappucino and Sherman 2008).

Optimization of various parameters:

Optimization of various parameters were carried out as per the standard procedure (Ajaz et al. 2019) ie. pH (5,6 and 7), temperature (30 °C, 37 °C and 45 °C), agitation speed (125 rpm, 150 rpm, 175 rpm and 200 rpm) [for *Clostridium* sp. AD8 static condition was maintained] and amount of bacteria (2%, 4%, 6%, 8% and 10%). 100 ml of Basic mineral medium (BMM) was taken and inoculated with 2% bacterial suspension and incubated. Stock solution of dye was added into the BMM to obtain a final dye concentration of 50 mg/L. Aliquot was taken at 0 h and after 4 days to measure the initial and final absorbance. Aliquot was centrifuged before measuring the optical density at 600 nm. Decolourization percentage was calculated as per following formula.

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

Dye decolorization assay by UV–Vis Spectrophotometer

Bacterial inoculums were freshly prepared by incubating bacterial suspension (6% v/v) in BMM broth with 0.5 g dye in an incubator shaker at 175 RPM for 20 h at 37 °C (Remi Model No. 1575). At defined intervals of 1st, 2nd, 3rd, and 4th day, the cultures were withdrawn, centrifuged at 10,000*g* and 10 °C for 15 min, and the supernatant was examined for absorbance at 600 nm under visible light range in a spectrophotometer (Perkins 1987) (Shimadzu-1800). Based on the results, the efficient organisms alone were taken for FTIR analysis. (Sanmuga Priya et al. 2016).

Biodegradation of dye by Fourier transform infrared spectroscopy (FTIR) analysis

For the characterization of functional groups, the dye was studied before and after decolourisation. The FTIR analysis was performed in the mid IRF region of 400–4000/cm. The effluent retained after biodegradation of Remazol yellow dye with the isolates were extracted with equal volumes of ethyl acetate. Then, the extract was dried over anhydrous Na_2 SO_4 and evaporated in a dry bath, and then dissolved in ethanol to use for FTIR analysis (Thermo Fisher) (Bisschops and Spanjers 2003; Phugare et al. 2011; Saratale et al 2009).

Statistical analysis

Statistical analyses were done by using Graph Pad PRISM version 6.01. For the analysis of the degradation ability of the isolates, colony statistics using one-sample t-test was employed.

Result and discussion

Bacterial isolates

The usage of different chemicals in the textile industry, a challenging task for the removal of dye from wastewater (Khan and Malik 2016; Moosvi et al. 2005). The pollutants from dye industry not only pollute the water, but they also pollute soil too (McMullan et al. 2001). In the present study, based on the biochemical tests and the growth on selective media, seven bacterial genera were obtained from the 76 isolates were isolated from the gut flora of exotic fish *O. mossambica* normally dwelling including polluted water bodies (Talwar and Jhingran 1991). The results are presented in Table 1. Biodegradation of xenobiotics involves an anaerobic, aerobic, or sequential combination of the two processes. Anaerobic degradation of azo dyes is known to yield only azo reduction and decolorisation (Melgoza et al. 2004). In general, complete mineralization of azo dyes requires both anaerobic and aerobic bacterial processes. The sequential anaerobic/aerobic treatment processes based on mixed culture of bacteria are widely used because the degradation products that result from anaerobic reduction of azo dyes have to be degraded by aerobic processes (Husseiny 2008; Kodam et al. 2005). In addition with this, though a number of bacteria in soil and water showed potentiality in dye degradation, however in a recent study revealed the firewater fish gut bacterial flora involved in the degradation of malachite green (Reddy Roja et al. 2020), further the bacteria residing in fish gut may played a potential role in dye degradation than the water and sediment sources.

Table 1 Bacterial genera isolated from the gut flora of *O. mossambica*

Name of the fish	No. of isolates obtained	Name of the bacterial isolates
O. mossambica	76	*Pseudomonas sp.*,AD1 *Bacillus sp.*, AD2 *Staphylococcus sp.*, AD3 *Staphylococcus sp.*, AD4 *Enterobacter sp.*, AD5 *Escherichia coli* AD6 *Streptococcus sp.*, AD7 *Clostridium sp.*, AD8

Isolation of azo dye degrading bacteria

The isolates ie. *Pseudomonas* sp. AD1 *Bacillus* sp. AD2, *Staphylococcus* sp. AD3 & AD4, *Enterobacter* sp. AD5, *Escherichia coli* AD6, *Streptococcus* sp. AD7 and *Clostridium* sp. AD8 were selected and grown in BMM with dye. Azo dyes are the most widely used and account for over 60% of the total number of dyes manufactured (Saratale et al. 2009; APHA 1992). The reduction of the azo bond by azo reductase under anaerobic conditions was already reported (Moutaouakkil, et al. 2003). However, there are certain oxygen insensitive or aerobic azo reductases that have been reported from aerobic microorganisms (Elisangela et al. 2009; Kalyani et al. 2008). In azo dyes, certain carboxylated analogs of sulfonated azo compounds are utilized aerobically as a sole source of carbon and energy by specifically adapted bacteria (Chivukula and Renganathan 1995), hence the present study was concentrated only on aerobic degradation of azo dye. Further Shah (2014) reported the degrading ability of dye was varied with different bacterial species. This finding was coincided with the current study findings were the maximum dye degradation was reported in *Staphylococcus* AD4 and it was confirmed by FTIR analysis.

Optimization of various parameters

Regarding the optimization of various parameters, the results revealed that the maximum decolourization at

pH 7, temperature 37 °C, agitation speed 175 rpm and amount of bacterium 6% were obtained (Fig. 1a–d). The optimization values of various parameters were determined by measuring the optical density at 600 nm. The present study findings were supported by various earlier studies. Ajaz et al (2019) reported the synazol red degradation by *Alcaligens aquatilis* at pH 7, temperature 37 °C with agitation speed 200 rpm. In another study, the optimal conditions for *B. cereus* and *B. megatarium* were found to be 37 °C, pH 7 and 8% of inoculums by the degradation of an azo dye (Shah et al. 2013). Further there was a study insisted the need of strong coordination between physico-chemical and microbiological parameters in the degradation of dye (Shah 2014).

Dye decolorization assay by UV–Vis Spectrophotometer

The dye decolorization efficiency of individual species was determined by UV–Vis spectrophotometric analysis at 600 nm for 0–96 h. The results were presented in Fig. 2. *Staphylococcus* sp. AD4 and *Streptococcus* sp. AD7 exhibited high absorbance at 24 h while it was reduced significantly ($p < 0.05$) after the determined incubation time of 96 h. Only *Bacillus* sp. AD2 reduced the dye absorbance significantly ($p < 0.01$) to near zero after the incubation period. In *Pseudomonas* sp. AD1 and *Staphylococcus* sp. AD3, the absorbance seems to be increasing owing to the production of pigments by the isolates in the medium. *Enterobacter* sp. AD5 and *Escherichia* sp. AD6 exhibited a non-significant reduction of absorbance. *Pseudomonas* sp. exhibited about 90% and 86% decolorization at 100 and 200 mg/l concentration and above this concentration decrease in degradation in *Mordant black* dye. *Pseudomonas* sp. is the microorganism that shows the highest activity on dyes selected. Pseudomonads are reported to grow considerably on plates since 96 h of incubation using different classes and chemical subclasses of textile

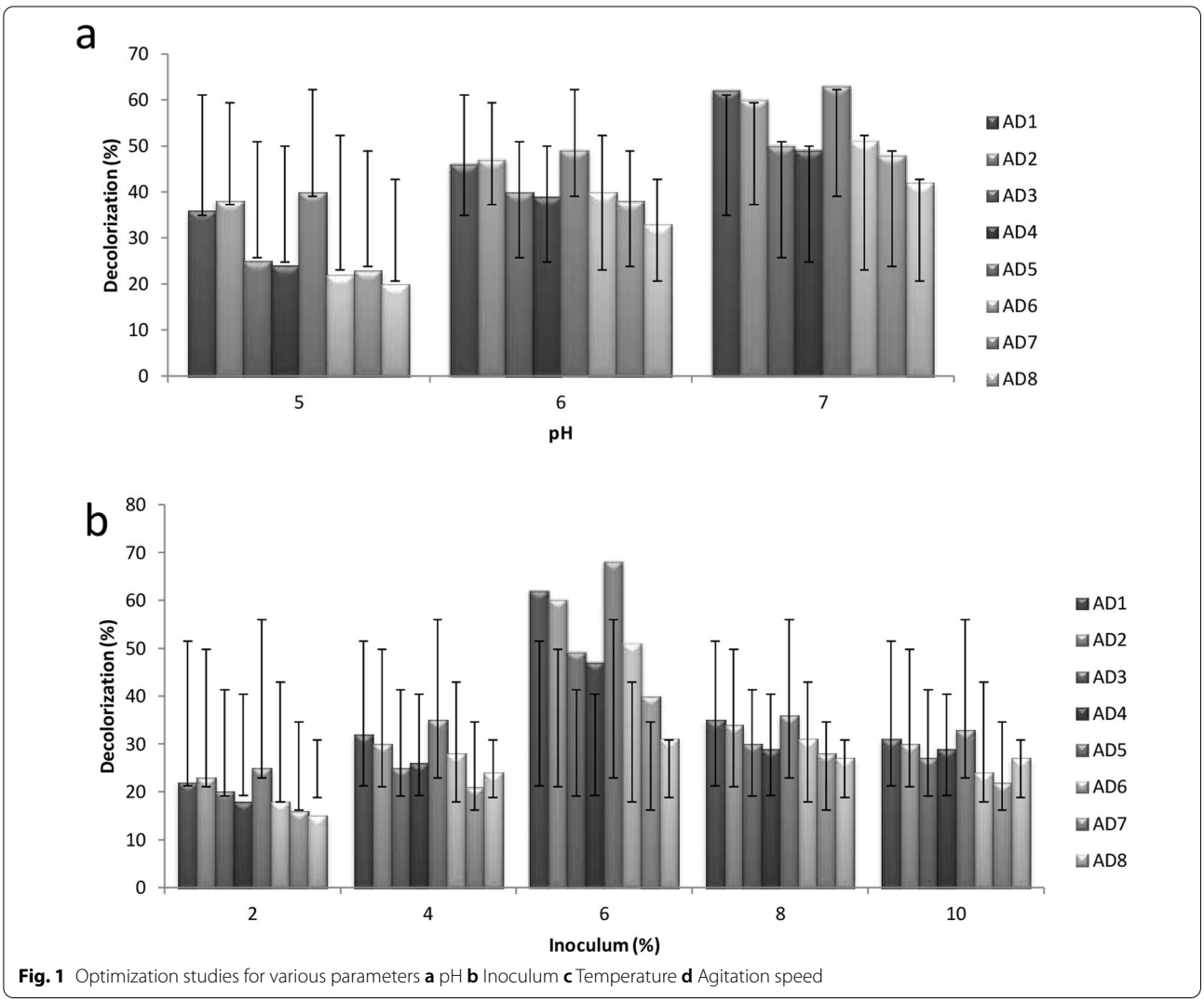

Fig. 1 Optimization studies for various parameters **a** pH **b** Inoculum **c** Temperature **d** Agitation speed

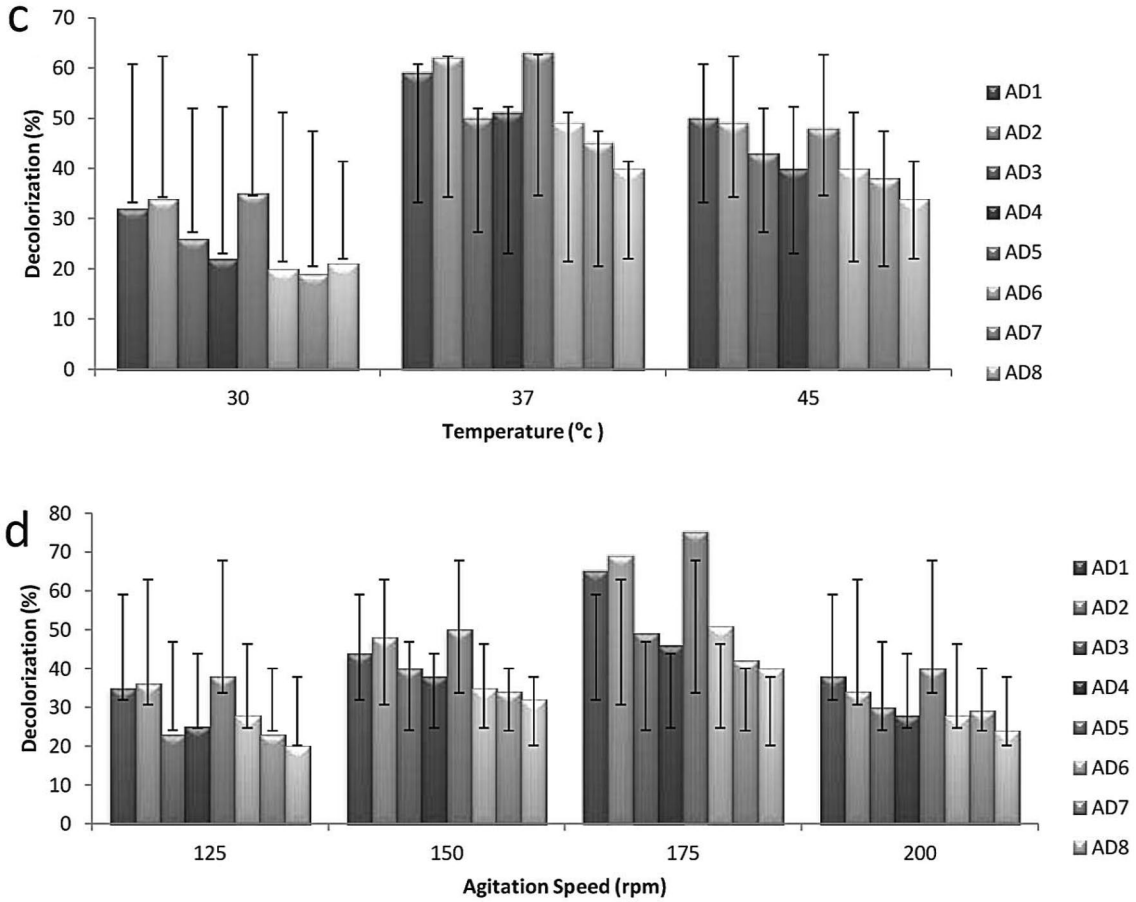

Fig. 1 continued

dyes, including azo, nitro, tri-phenyl methane, and anthraquinone types as the only source of carbon (BMM, dye, and agar) (Afreen et al. 2017). Earlier reports strongly revealed that the role of *Pseudomonas* sp. in decolorization and degradation (Moosvi et al. 2005). In the present study, findings were also supported the *Pseudomonas* sp. role in the decolorization of Remazol dye than the other organisms were isolated from the study area. Based on the results, the efficient dye decolorization organisms such as *Pseudomonas* sp. AD1, *Bacillus* sp. AD2 and *Enterobacter* sp. AD5, were taken for FTIR analysis. The results revealed that the decolorization was less in individual and consortium under aerobic conditions. This view was strongly supported by Moosvi et al. (2005) and only 12–22% performance was achieved within 48 h. On the other hand, individual cultures *Pseudomonas sp.* were able to completely decolorize the azo dyes. Although *Enterobacter sp*., and *Streptococcus* sp., do not show similar activity which the *Pseudomonas sp*. does, this is the first time in which growth on textile dyes of these microorganisms is reported (Perkins 1987). The chemical structural differences in textile dyes due to the substitution of various functional groups on the aromatic base greatly influence their decolorization rates (Pasti-Grigsby et al. 1992; Dhanve et al. 2009). These views were supported in the present study. *Pseudomonas* sp, *Streptococcus* sp, and *Staphylococcus* showed variations in the degradation of Remazol dye.

FTIR analysis

Fourier Transform Infrared Spectroscopy (FTIR) analyses were done for the control and the decolorized sample (Fig. 3) and from the chromatogram, the results showed various peaks. At 1496 cm^{-1}, aliphatic and at 1084 cm^{-1}, aromatic stretching were exhibited. The presence of C-S stretching has been seen at 650–675 cm^{-1} confirms the denaturation of the sulfur bonds in the Ramazol Yellow structure. At 1161 and 1358 cm^{-1}, symmetric and asymmetric SO2 stretching were seen respectively. Primary amide (–NH2–) bending was seen at 1630–37 cm^{-1}. Secondary amide (N–H) stretching and wagging (650–675 and 3280–85 cm^{-1}), and secondary amines were evident

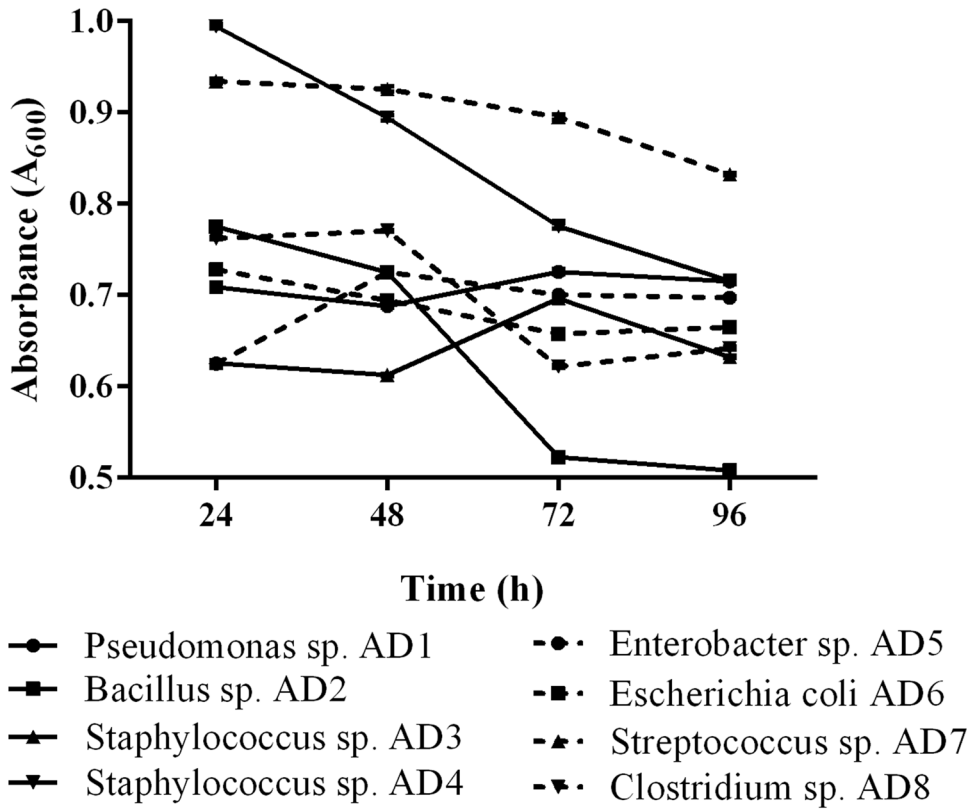

Fig. 2 Dye decolourization assay using selective bacterial isolates by UV–Vis Spectrophotometer

at 1545–1550 cm^{-1}. At 781 cm^{-1}, –NH2 wagging and twisting were seen. Aromatic/aliphatic isonitrile (–N≡C–) stretching or possible azide stretching was seen at 2119–25 cm^{-1}. Aromatic /aliphatic nitro compound (NO2) asymmetric stretching was seen at 1358, 1545–50 cm^{-1} and 1630–37 cm^{-1}. Aliphatic C–N stretching was seen at 1074 and 1161 cm^{-1} and aromatic C–N stretching was seen at 1358 cm^{-1}. The Oxime stretching (C=N–OH) was seen at 1630–37 cm^{-1}. In comparison to the control FTIR, the peaks observed in presence of strains tested exhibited new peaks. This may be due to azoreductase enzyme activity. In the earlier reports, azoreductase activity has been studied in detail after the remediation of azo dye by *Enterobacter* sp. (Prasad and Aikat 2014) and by *Pseudomonas* sp. (Zimmermann et al. 1982).

Spectrophotometric analysis data reveals the presence of aromatic amines in all the four azo dyes decolorized broths under microaerophilic conditions. FTIR results were analyzed according to published reports (Stuart 2004; Cheng and Cerniglia 1992). The formation of colorless aromatic amines in microaerophilic conditions can be a result of reductive cleavage of the azo bond (–N=N). Although the azo group (–N=N–) specific stretch at 1400–1450 cm^{-1} was not observed. The stretching and wagging of secondary amides (–NH2–); bending of primary amide (–NH2–), aliphatic (–C–N–) stretching, and –C-S- stretching were seen in all the four FTIR readings. Aromatic/aliphatic isonitrile (–N≡C–) stretching or possible azide stretching was seen only in *Clostridium* sp.AD1, *Enterobacter* sp.AD5, *Bacillus* sp.AD2 while *Pseudomonas* sp. AD1 exhibited anhydride (–C=O) stretching. The FTIR results exhibited that the azo group was degraded by all the isolates analyzed and S- and N- groups were found to be still present in the system after the degradation of Remazol yellow dye.

On contrary, the aerobic process doesn't show the formation of aromatic amines, but very little quantity of degradation was observed within the same time. This suggests the inability of bacterial consortium for aerobic degradation of selected azo dyes. The general approach of biodegradation is to mineralize the textile dyes using the natural capability of native microorganisms. But, most of the textile azo dyes are xenobiotics, and its biodegradation results in the formation of

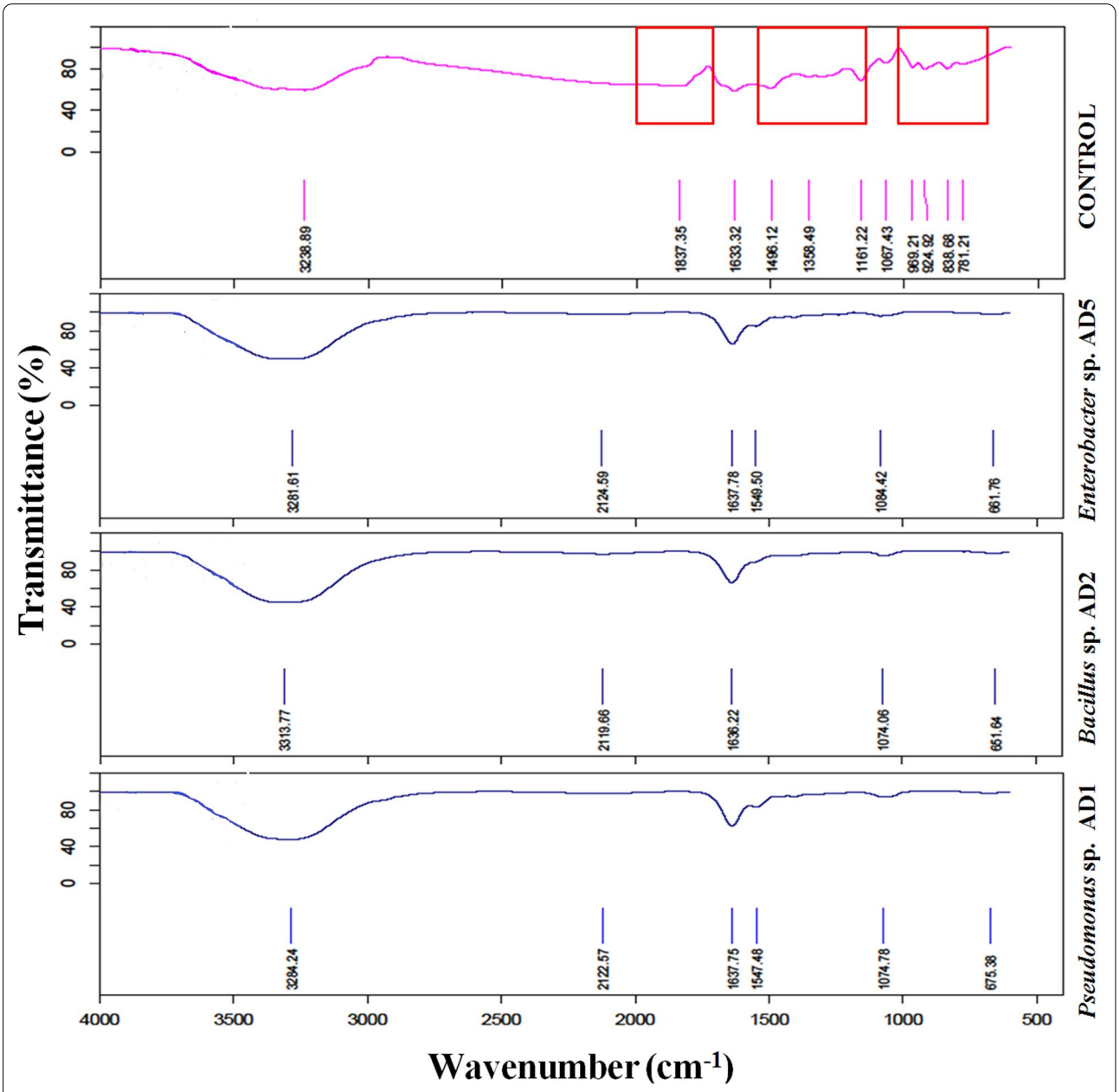

Fig. 3 FTIR analysis for decolourized and control samples

aromatic amines, which are more toxic than parent dyes and even carcinogenic or mutagenic (Levine 1991).

Conclusions

Pseudomonas sp. AD1 and *Enterobacter* sp. AD4 and *Bacillus* sp. AD2 are the most prominent bio decolourizing agents than the other isolated gut flora collected from exotic fish *O. mossambica*. The efficient degradation of dye by the above said organisms were confirmed by FTIR analysis within 96 h with the prominent loss of azo bonds seen in FTIR analysis. The present study gave a platform to further the approaches about the purification and quantification of the enzymes involved in the degradation by *S Pseudomonas* sp. AD1 and *Enterobacter* sp. AD4 and *Bacillus* sp. AD2 isolates.

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Authors' contributions
ASN made a substantial contribution in designing the work and concept development. SK has carried out the field study and acquisition of data. AM interpreted the results and helped in statistical analysis. ASN and AS reviewed, edited, and shaped the manuscript. All authors are read and approved the final draft of the manuscript.

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Availability of data and materials
The data used in the present study are available from the corresponding author based on reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
No conflict of interests reported by the authors.

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