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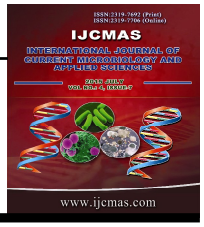

Original Research Article

Azo Dye Degrading Bacteria from Textile Effluent

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Keywords

Azo dyes, Textile effluents, Microorganisms

A B S T R A C T

Effluent was collected from different industries located at different places like Tirupur, Tamil nadu and Mumbai, Maharashtra. The sample was analyse physiochemical characteristics like color, smell, pH, COD was noted. Azo dye degradation was checked with supply of carbon and nitrogen source to bacteria present in effluent which stimulated the growth of azo dye degrading bacteria. Those bacteria were isolated. Morphological and biochemical characterization was done to identify isolates and was found to be *Pseudomonas spp*, *Citrobacter spp*, *E.coli* and *Micrococcus spp*. Optimum conditions (pH and temperature) were determined Plasmid screening was done and plasmid was found in all isolates. Extracted plasmids were transformed in *E. coli* DH5α. It was confirmed that *E. coli* was transformed and transformed cells showed good decolorization potential. Azo dyes are being degraded and it was confirmed by TLC analysis. Isolates were checked for effluent bioremediation for different effluent samples collected from different places. It was found that *Micrococcus spp.* worked well in adapted environment and *E. coli* showed same decolorization potential in adapted and non adapted environment.

Introduction

The textile industry accounts for the largest consumption of dyestuffs, at nearly 80%. The textile industry discharge millions of liters of effluents directly into public drains which ultimately reaches fresh water. These effluents contains high amounts of grease, dirt, nutrients from dye bath, dye additives, residual dyes etc., 50% textile effluent has residual azo dye which is very difficult to remove by conventional treatments like chemical flocculation, adsorption, oxidation, filtration, electrochemical method, use of activated carbon etc., although some process like MEE (Multiple Effect Evaporator), reverse osmosis, nanofiltration are effective but are very expensive due to complex structural set up, huge chemical and power consumption and most of these physical-chemical method used for treating textile effluents produce large volume of sludge, other toxic byproducts trouble for safe disposal, affecting directly and indirectly affecting aquatic flora and fauna and its quality. Biochemical characterization was done on the isolates degrading various mono-azo, di-azo, tri-azo and poly-azo

textile dyes used in dyeing and printing industries. They observed that *P. aeruginosa* PFK10 secretes a fluorescent yellow-green pigment (pyoverdine).Whole genome sequencing about the metabolic pathways of the strain revealed that initial ability to degrade textile azo dye, which can be used for bioremediation of contaminated sites (Faldu *et al.*, 2006).

Materials and Methods

Sample collection of effluents and its physicochemical analysis: Textile waste water samples were collected in a clean bottle from Tirupur, Tamil Nadu and Bhiwandi, Maharashtra. Different types of azo dyes were used for decolorization studies which are as follows: Napthol dye, Acidic dye, Direct dye, Nylon dye, Procine dye, Vat dye and Base dye. Out of these dyes, procine and direct dyes were used for decolorization test. Some Physiochemical parameters *viz.*, COD (Chemical Oxygen Demand), pH, color and smell of textile waste water was measured.

Isolation and identification of azo dye degrading bacteria: Azo dye degrading bacteria were isolated in half strength nutrient broth with 1% w/v Azo dye. The tubes were then incubated at 37^0C for decolorization.

The samples which got decolorized were separated. Minute volume (0.1 ml) from decolourized sample was plated on nutrient agar supplemented with azo dye by spread plate technique. The plates were then incubated at 37^0C for 24 hrs. Different bacterial cells grown on nutrient pate were then isolated and streaked on nutrient agar plate to get pure culture. The colony characteristic and biochemical characteristics was used for the identification of the organism.

Determination of optimum growth condition: Optimum pH and temperature was determined.

Determination of biodegradation activity: Biodegradation activity of all 4 bacteria along with different combinations of them was determined.

Plasmid screening: Plasmid was isolated from the organisms degrading the dyes and transformed the competent cells of *E. coli* DH5α.

Degraded product analysis: Whether the azo dyes are degraded or not was confirmed by TLC analysis. Sample was loaded on Silica plate, plate was placed in mobile phase solvent system - Isopropanol: Methanol: Ethyl acetate: Water: Glacial acetic acid in the ratio 3:2:2:1:0.5 (modified composition).

Bioremediation of Textile Effluent: Textile effluents collected from different textile industries were used to check the degradation capability of isolated bacteria. Effluents from Tirupur textile industry (I), Mumbai textile industry (II), Punjab, two different textile industries (III and IV).

Results and Discussion

Sample collection: Sample was collected from Tirupur and Mumbai textile effluent outlet. It has been highly polluted with different types of dyes, organic matter etc making it very harmful for the environment it is been exposed. Composition of textile effluent with its hazardous effect to the surrounding has been reported.

Estimated COD value is very high and shows that large amount organic matter is present. According to Indian Standard Institute (ISI)- IS:2490 (Part I) general limits

(2nd revision) permissible limits of COD for Industrial effluent is 250 mg/L for discharging it into inland surface water but both effluent has very high COD value hence, they cannot be discharged before treatment. Similarly high COD value, unpleasant smell and basic pH of effluent have been reported by Usman Aftab *et al.* (2011). Olukanni *et al.* (2006) also worked on COD determination which showed very high for textile effluent collected from drain.

Isolation and identification of dye degrading bacteria: From effluent I sample, two type of bacteria (**a** and **b**) was isolated from a decolorized media From effluent II sample, two type of bacteria (**c** and **d**) was isolated from a decolorized media. Textile effluent harbor large amount bacterial load of different types utilizing dyes in it as its carbon source. Here, a total of four bacterial strains were isolated showing decolorization activity similarly, Agarry *et al.* (2011) isolated six bacterial species from textile effluent; Hassan *et al.* (2013) reported 11 bacterial isolates showing decolorization activity. The colony characteristics and biochemical characteristics indicated that the organisms were *Pseudomonas* spp., *E. coli, Citrobacter* spp and *Micrococcus* spp. Similar results were reported by Usman Aftab *et al.* (2011), Olukanni *et al.* (2006) and Hassan *et al.* (2013) worked on same genus and results were confirmed with the help of Bergey's Manual of Determinative Microbiology

Determination of optimum condition

Optimum pH

According figure 2, optimum pH for all isolates was found to be **7**. Similar study was done by Tripathi and Srivastava (2011) where effect of pH on bacteria survival was checked and it was found that pH 7 was optimum pH for *Pseudomonas* and *Bacillus* species. It was also reported till pH 7.6 the decolorization property was not affected for *Pseudomonas* species. Similar results are reported by Rashid Mahmood *et al.* (2015) that organisms showed good growth and decolorization at pH 7.5 and drastically decreased below 6.5 and 8.0. Every organism have requirement of particular hydrogen ion concentration for their growth.

Optimum Temperature

According to figure 3, optimum temperature for all isolates was found to be 37^0C. Similar study was done by Tripathi and Srivastava (2011) where effect of temperature on bacteria survival was checked and it was found that 37^0C was optimum temperature for *Pseudomonas* and *Bacillus* species. Similar results are reported by Usman Aftab *et al.* (2011) that organisms showed good growth at temperature 37^0C. This may be due temperature sensitive property of bacteria. With temperature change, an enzyme starts losing its structural integrity and becomes inactive or is degraded ultimately affecting bacteria's growth.

Determination of potential biodegradation of isolates:

1. Orange dye:

a. Static condition

Figure 4 shows maximum degradation within 24 hrs. High potential decolorization was shown by 'd' up to 51% followed by 'a', c', and 'b' with potential decolorization of 49%, 43% and 34% respectively. In combination, 'bc' showed high decolorization potential (57%).

According to figure 4, *Micrococcus* spp. (d) has shown maximum decolorization activity when compared to others. In the previous studies, *Micrococcus* spp. was found to be a

good decolorizer. Hassan *et al.* (2013) reported that *Micrococcus* has decolorized novacron blue, novacron orange dye to almost 80% within 24 hrs but here *Micrococcus spp.* has shown less degradation rate because it is strictly aerobic bacteria and so with depletion in oxygen supply, hence after 24 hrs, decolorization rate also decreased and concentration used was 0.01% which is very less compared to concentration of dye (1%) used in current study.

When organisms are used in combination, decolorization rate is increased but for some combination decolorization rate is slow because when organisms of different sources or same source are present together they act on different bonds of same compound thus enhancing the degradation rate. Some organisms produce toxic byproducts or inhibitors for other organism which ultimately decreases degradation rate of compounds. Similar results are reported by Rashid Mahmood *et al.* (2015) where 8 different genus belonging bacteria was used in combination and its degradation potential was determined. The report says that as compared to individual organism in consortium degradation potential increased approximately 10% more.

Shaker condition: Maximum degradation was seen within 24 hrs (Fig. 5). High potential decolorization was shown by ‘c’ up to 51% followed by ‘d’, ‘a’, and ‘b’ with potential decolorization of 50%, 40% and 34% respectively. In combination, ‘bc’ showed high decolorization potential (51%). c (*Citrobacter* spp.) has shown maximum decolorization activity in shaker condition within 24 hrs in shaker, since *Citrobacter* is an aerobic organism. Agitation may have increased the oxygen and nutrient supply, thus enhancing its biodegradation ability. Hui Wang *et al.* (2009) also reported that *Citrobacter* sp. CK3 has shown maximum decolorization rate at pH 7 and 37^0C with dye concentration of 200 mg/l.

When organisms are used in combination, decolorization rate is increased but for some combination decolorization rate is slow because when organisms of different sources or same source are present together they act on different bonds on same compound thus enhancing the degradation rate. Some organisms produce toxic byproducts or inhibitors for other organism which ultimately decreases degradation rate of compounds. Similar results are reported by Rashid Mahmood *et al.* (2015) where 8 different genus belonging bacteria were used in combination and their degradation potential was determined. The report says that as compared to individual organism in consortium degradation potential increased approximately 10% more.

Degradation rate decreased in all isolates (individual and in combination) except a and b. This may be because of depletion of nutrients. Thus if supply of nutrients are constant biodegradation rate may increase or remain constant or this may be due to byproducts produced on degrading dyes which can act as inhibitors in degradation pathway/pathways of dye. Inhibitors can be removed continuously so as to improve degradation rate. German Buitr *et al.* (2004) has reported use of sequential batch reactor for improving the degradation rate by supplementing nutrients and removing used media intermittently. Thus constant supply of nutrients is maintained. In case of ‘a’ and ‘b’ biodegradation rate is approximately same even after 24 hrs, this can be due to absence in inhibitor production or low nutrient consumption rate. Initially 7 different types of dyes were used for decolorization except direct and porcine dye others showed very slow decolorization. It

took almost a week for partial decolorization. Different rate in decolorization of different dye may be due to; different molecule and bond are present and its structure. The similar report being reported by Susana Camarero *et al.* (2005) suggesting importance of structure of synthetic dyes and intermediate formed during decolorization mechanism's effect on potential decolorization rate.

Direct Dye

Static condition

Maximum degradation was seen within 48 hrs. High potential decolourization was shown by 'd' up to 24% followed by 'a', 'b', and 'c' with potential decolorization of 23%, 22% and 21% respectively. In combination, 'bc' showed high decolorization potential (80.4%). According to figure 6, *Micrococcus* spp. (d) has shown maximum biodecolorization activity when compared to others . In the previous studies, *Micrococcus* spp.was found to be a good decolorizer . Hassan *et al.* (2013) reported that *Micrococcus* has decolorized Novacron blue, novacron orange dye to almost 80% within 24 hrs but here *Micrococcuss spp.* has shown less degradation rate because it is strictly aerobic bacteria and so with depletion in oxygen supply, hence after 24 hrs, decolorization rate also decreased and concentration used was 0.01% which is very less compared to concentration of dye (1%) used in current study. Agarry *et al.* (2011) has reported that with increase in dye concentration bacterial decolorization ability decreases. When organisms are used in combination, decolorization rate is increased but for some combination decolorization rate is slow because when organisms of different sources or same source are present together they act on different bonds on same compound thus enhancing the degradation rate. Some organisms produce toxic byproducts or inhibitors for other organism which ultimately decreases degradation rate of compounds. Similar results are reported by Rashid Mahmood *et al.* (2015) where 8 different genus belonging bacteria were used in combination and their degradation potential was determined. The report says that as compared to individual organism in consortium degradation potential increased approximately 10% more.

Shaker condition

Maximum degradation was seen within 48 hrs. High potential decolourization was shown by 'a' up to 74% followed by 'c', 'd', and 'a' with potential decolorization of 22%, 22% and 19% respectively. In combination, 'bc' showed high decolorization of 30.3%. 'a' (*Pseudomonas*) as a potent decolorizer of azo dye. May be agitation and mixing have enhanced nutrient and oxygen supply, thus improving 'a' decolorization property. In earlier studies, *Pseudomonas* has been isolated and used by many research people for dye degradation. Agarry *et al.* (2011) isolated *Pseudomonas fluorescens*, *Pseudomonas nigificans*, and *Pseudomonas gellucidium* and assayed its dye decolorization property. The report concludes as *Pseudomonas fluorescens* as potent decolorizer of synthetic dyes. Tripathi and Srivastava (2011) also reported presence of *Pseudomonas putida* in textile effluent. Treatment with this bacterium showed large reduction in COD and BOD of textile effluent. Use of pseudomonas spp was also reported by Zimmermann *et al.* (1982) where enzyme called azoreductase was extracted and its high decolorization activity was determined.

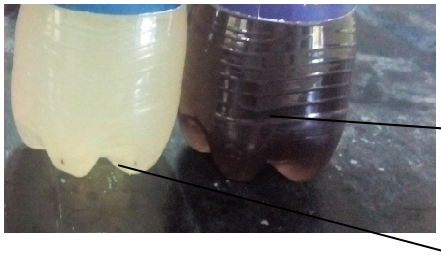
Table.1 Physiochemical characterization

	Color	**pH**	**COD (Chemical Oxygen Demand)**	**Smell**
Sample I	Dark Brown	5.6	15,000 Mg/L	Pungent
Sample II	Off White	10.6	25,000 Mg/L	Ammonia Like

Table.2 R_f value

	control	**a**	**b**	**c**	**d**
R_f value	0.85	0.71	0.77	0.51	0.55

Fig.1



Tirupur sample (effluent I)

Mumbai Sample (Effluent II)

Fig.2

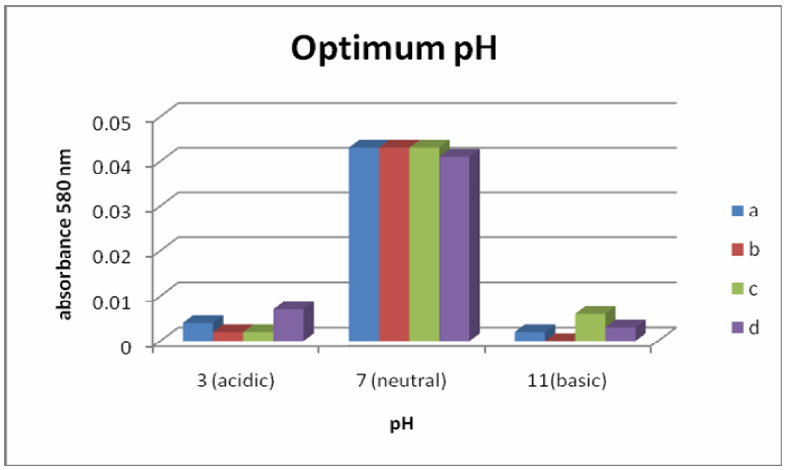


Fig.3

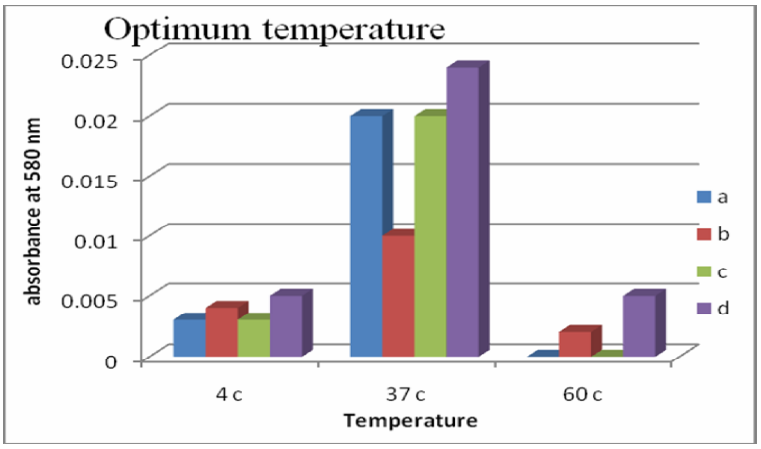


Fig.4 Comparison of potential decolorization within 24–72 hrs

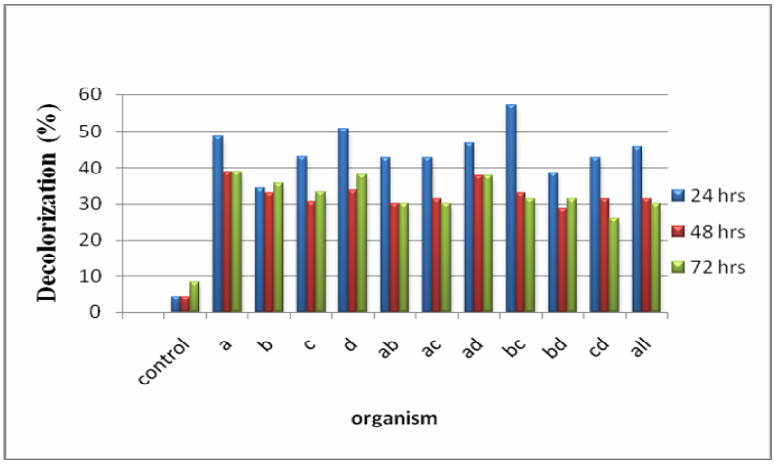


Fig.5

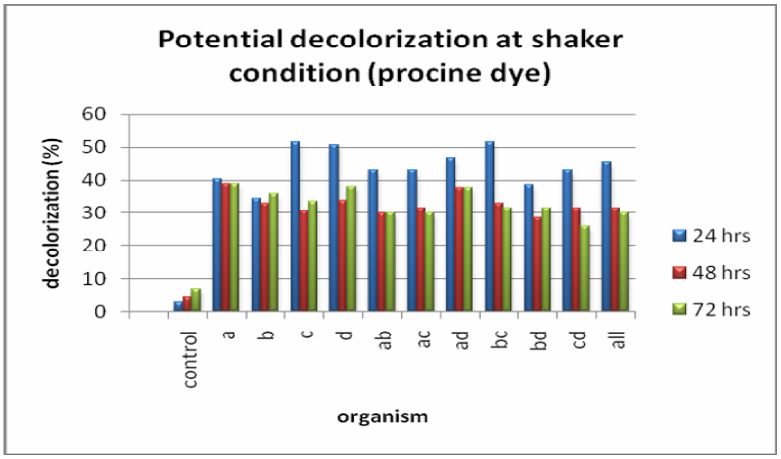


Fig.6

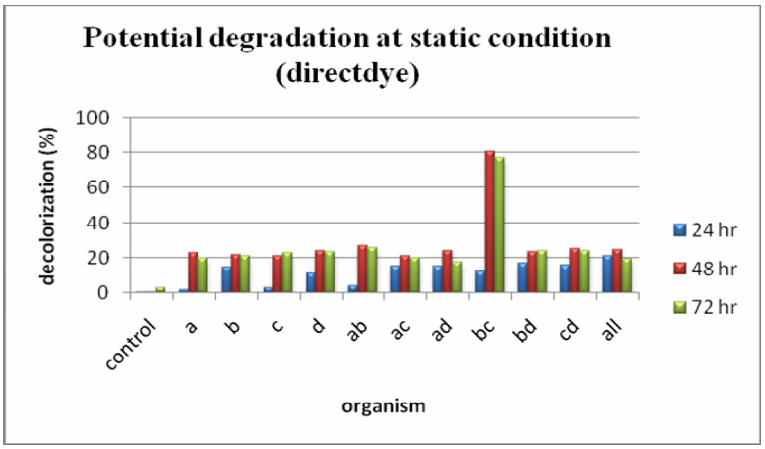


Fig.7

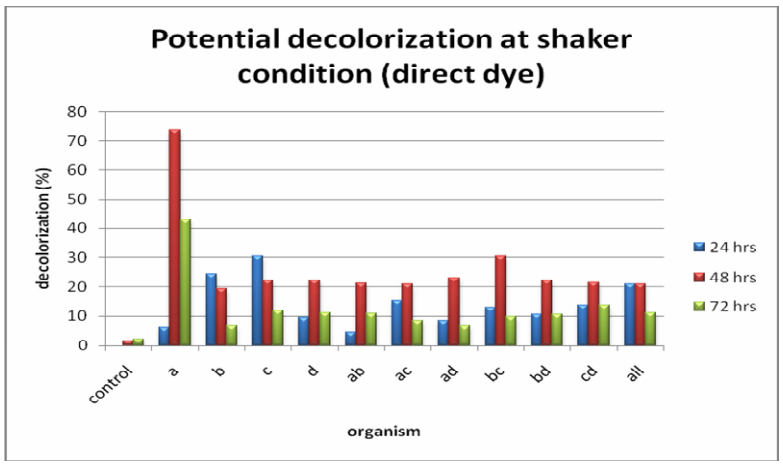


Fig.8

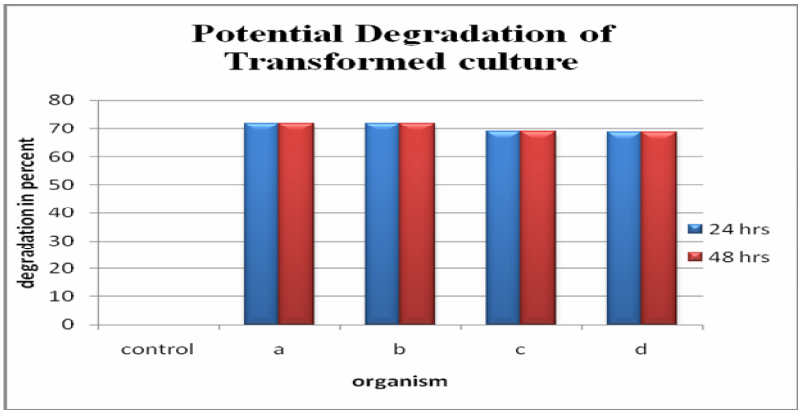


Fig.9

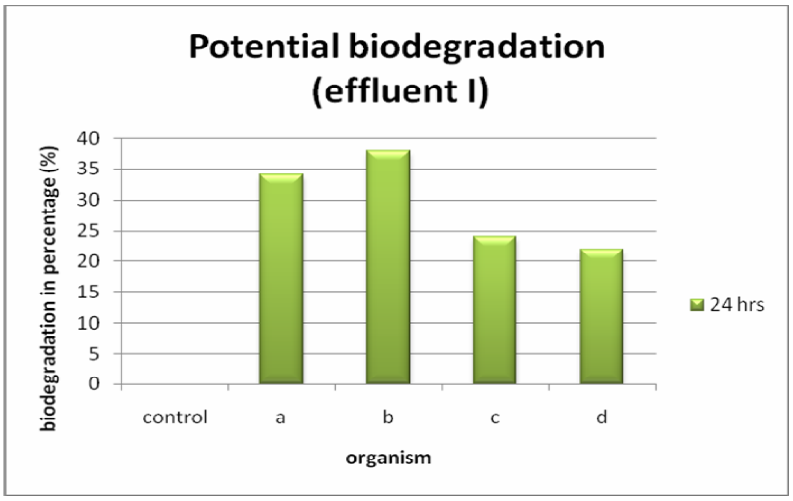


Fig.10

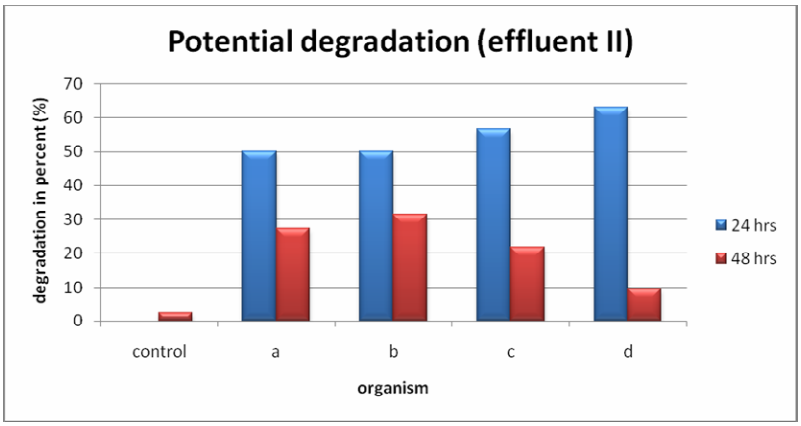


Fig.11

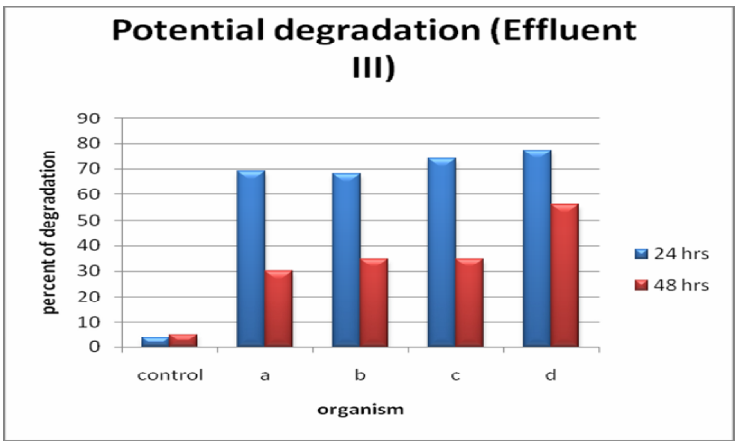
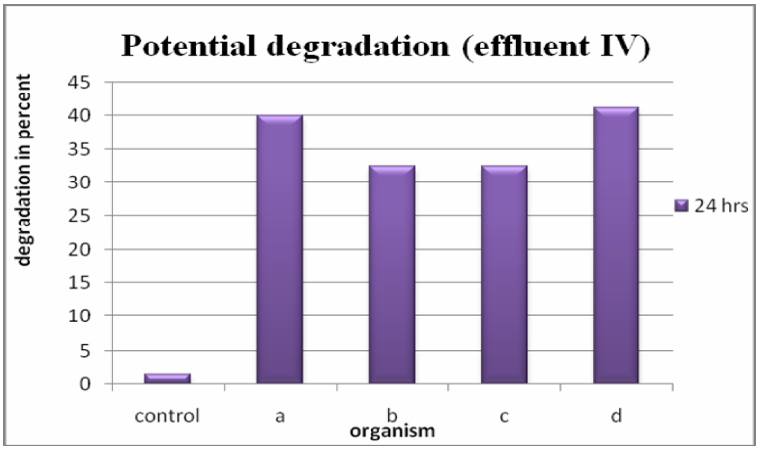


Fig.12



Plasmid screening: Plasmid was present in all bacterial isolates. Olukanni *et al.* (2006) screened for plasmid in his isolates by Kado and Lio method but no plasmid was detected. Hence, only genomic DNA was involved in dye degradation but in this case, all isolates have shown presence of plasmids. This indicates extra chromosomal involvement in decolorization process. Khaled Elbanna *et al.* (2010) reported presence of plasmid of 3 kb size having azo dye degrading gene isolated from lactic acid bacterial isolates.

Biodegradation potential of transformed culture

Transformed *E. coli* DH5α with plasmid from 'a' and 'b' bacteria showed high degradation potential of about 72% each and transformed bacteria with plasmid from 'c' and 'd' showed 68% degradation potential each within 24 hrs. After 24 hrs, degradation decreases to negligible rate (as shown in figure 8). This may be due to non availability of nutrients (especially carbon source) necessary for degradation of azo dyes.

Control (non transformed *E. coli* DH5α) does not shows decolorization ability whereas cells treated with transformed procedure with plasmid isolated from respective isolates have shown decolorization within 24 hrs. Results obtained of transformed cells are similar to isolates**.** Indicating same genes can be involved in azo dye degradation.

Degraded product analysis

A comparison between non treated dye sample and treated dye sample with isolates with the help of TLC analysis to confirm dye degradation. R_f value is different for control and test samples Similar R_f value of degraded product treated with 'c' and 'd' suggests that azo dyes are been degraded and same products are produced. Sheh and Dave (2009) have also confirmed the results where they did not get any spots on TLC chromatogram indicating that the dyes are degraded. Usman Aftab *et al.* (2011) confirmed azo dye decolorization by *Corynebacterium* spp. The degraded products were tested for confirmation by TLC chromatography in which control showed only a single band and treated samples showed more than a single band suggesting that dye is degraded. In this study, TLC chromatogram showed single point but at different distance confirming

that azo is is being degraded by isolates. This difference may be due to use of different extraction method used to extract byproducts or different nature of compound produced by different bacterial species. Usman Aftab *et al.* (2011) reported non toxic nature of degraded product separated by TLC using soil bacteria.

Bioremediation of textile effluent

Effluent I was decolorized maximum within 24 hrs and organism 'b' showed maximum potential decolorization up to 38%. It is followed by a, c and d with potential degradation rate of 34%, 24%, 28% respectively. Figure 9 shows that, bacteria in its adapted environment shows maximum biodegradation activity. This result is similar to results reported by Olukani *et al.* (2006), where isolates isolated from the effluent showed more activity in same effluent if supplied with some nutrients as compared to when added in different effluent sample.

Effluent II was decolorized maximally within 24 hrs and organism 'd' showed maximum decolorization rate of up to 63%. It is followed by c, a and b with potential degradation of 56%, 50%, 50% respectively (Fig. 10). This shows that biodegradation activity is more in adapted isolates as compared to non adapted isolates. When potential degradation is noted, adapted isolates shows approximately 10% more color removing activity is more than non adapted isolates. The above result is similar to results reported by Olukani *et al.* (2006).

Effluent III was decolorized maximally within 24 hrs and organism 'd' showed maximum decolorization rate of up to77%. It is followed by c, a and b with potential degradation of 74%, 69%, 68% respectively (Fig. 11). This shows that biodegradation activity is more in adapted isolates as compared to non adapted isolates. When potential degradation is noted, adapted isolates shows approximately 10% more color removing activity is more than non adapted isolates. The above result is similar to results reported by Olukani *et al.* (2006).

Effluent IV was decolorized maximally within 24 hrs and organism 'd'; showed maximum decolorization rate of up to 41%. It is followed by a, c and b with potential degradation of 40%, 32%, 32% respectively (Fig. 12). When figure 9, 10, 11 and 12 are compared, Bacteria a and b shows biodegradation activity more in non-adapted environment as compared to adapted environment. This is contradictory to the results reported by Olukanni *et al.* (2006). This may be due to stimulation of dye degrading gene when its environment is changed. The stress induction or supply of some components in non adapted effluent may lead to over expression of dye degrading gene leading to more decolorizing. Whereas c and d shows higher decolorization activity in adapted effluent which is similar to the results by Olukanni *et al.* (2006). Hence these organisms are adapted to their environment for their survival hence degrading azo dyes in effluent. Thus bacteria 'b' is showing similar degradation potential in adapted as well as non adapted environment when supplemented with nutrients.

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