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Aerobic Biodecolorization of Mixture of Azo Dye Containing Textile Effluent using Adapted Microbial Strains

R. Rajendran, S. Karthik Sundaram and K. Uma Maheswari
PG and Research Department of Microbiology, PSG College of Arts and Science, Coimbatore 14, India

Corresponding Author: S. Karthik Sundaram, PG and Research Department of Microbiology, PSG College of Arts and Science, Coimbatore-14, India

ABSTRACT

The current study aimed at developing a microbial consortium using indigenously adapted microbial populations which could be used in the treatment of azo dye containing textile effluent under aerobic conditions. From about 265 different bacterial isolates, 3 organisms (*Bacillus* sp., *Pseudomonas* sp. and *Alcaligenes* sp.) and from 35 different fungal isolates and two fungal strains (*Aspergillus* sp. and *Penicillium* sp.) were selected. Around 26 different combinations were developed using permutation and combination and the efficient combination was identified based on the reduction of various physico-chemical parameters (Chemical Oxygen Demand, Hardness, Total Solids, Total Suspended Solids, Total Dissolved Solids, pH, Color and Turbidity). There was a gradual increase in the percentage of reduction in all the parameters from the first day onwards and after the fifth day there was no considerable reduction in the parameters. One of the combinations A13 was found to be the efficient combination capable of reducing all the parameters to a significant extent. Also the toxic end product in the treated effluent was found to be negative which was confirmed by HPLC analysis. It could thus be ascertained from this study that the recalcitrant nature of the azo dye could be over come even in an aerobic condition with the enrichment of selected combination of microbes in the treatment plant. Usage of such a consortium helps us in treating the azo dyes in a cost effective manner.

Key words: Aerobic treatment, azo reduction, microbial consortium, synthetic dyes, decolorization

INTRODUCTION

Waste stream generated from the textile industry is essentially based on water-based effluent generated in the various activities of wet processing of textiles either in the processing stage or in the pre-processing stage (Karthikeyan and Mohan, 1999). It has been estimated that more than 200 tonnes/day of textile effluent is generated at Tirupur, in Coimbatore. According to Tamilnadu State Pollution Control Board, there are more than 830 dyeing units in Tirupur alone (Balasubramanian *et al*., 2006). With a wide variety of dyes used on a commercial basis, most of the dyeing units in and around the city are making use of the azo dyes due to their cheap cost and the intensity of the color that is applied on to the fabric. These azo dyes have poor exhaustion properties and as a result more dyes end up in the effluent of the dyeing industries (Omar, 2008). When this waste water is discharged in to the natural water body, it contaminates the entire system with its color and the organic load it possess reduces the dissolved oxygen level in the water system making it toxic for the natural inhabitants (Igwe *et al*., 2007). The azo dyes are biorecalcitrant under aerobic conditions but could be cleaved under oxygen limiting conditions leading to the

formation of toxic aromatic amines. These aromatic amines could not be further reduced under the oxygen limiting but are auto-oxidizable or could be mineralized by microbes at a faster rate under aerobic condition (Olukanni *et al.*, 2009). But this treatment procedure is time consuming and is not feasible commercially also the cost of treating the effluent under anaerobic situation demands more money (Libra *et al.*, 2004).

The use of a microbial consortium could very well put an end to this problem. Enzymes which could act without any substrate specificity like laccase, manganese peroxidase and lignin peroxidase could be used in bioremediation (Khammuang and Sarnthima, 2009). These enzymes are also present in the fungus like *Aspergillus* sp., *Penicillium* sp., *Trichoderma* sp., etc could be used in the treatment of effluent system (Raju *et al.*, 2007). These organisms are ubiquitous and are also present in the effluent system. When the consortium developed contains any of these organisms there is a greater chance that the chromophore of the azo dye would be reduced under aerobic condition following a different pattern of reduction without the formation of the toxic intermediates (Kumar *et al.*, 2011; Buitron *et al.*, 2004).

The current study involves in the isolation of indigenously adapted efficient microbial strains capable of bioremediation textile effluent containing mixture of azo dyes. These efficient isolates were used in the designing of different microbial combinations. The efficient combination capable of reducing the organic load and the dye component to a significant extent without the formation of the toxic end products was identified by analyzing different physico-chemical parameters.

MATERIALS AND METHODS

Sample collection: Effluent sample was collected from a common effluent treatment plant, in Coimbatore which was involved in the treatment of textile effluents from more than 10 dyeing units containing a mixture of azo dyes. This sample was used in isolation of microbes and in treatment trials.

Enrichment and isolation of indigenously adapted predominant microbial strains: The effluent sample was enriched in culture media for bacteria [Enrichment Media 1 (in g L^{-1}): peptone 5, yeast extract 2.5, NaCl-5, (pH 7.0, 2% agar)] and fungi [Enrichment Media 2 (in g L^{-1}): peptone-5, glucose- 5 (pH 7.0, 1.5% agar)] for 72 h. The enriched culture was then serially diluted and plated in the respective solid media and incubated for 24-48 h, respectively. The predominant microbial colonies were selected for further screening.

Selection of adapted bacterial strains: Those bacterial colonies which were predominant on the respective solid media were selected and inoculated in the same media amended with 0.01 g of eight different mono and di azo dyes (Mono azo dyes - Reactive Red BSID, Reactive Yellow merl, Remazol Brilliant Violet, Orange merl, Red M5B and Reactive Red 120; Diazo dyes - Amido Black 10B and Reactive Black 5) (Khelifi *et al.*, 2009). The colonies which exhibited decolorization of the dye expressed as zone of clearance were chosen for further screening.

Selection of adapted fungal strains based on laccase production: The predominant fungal strains isolated through enrichment were inoculated onto the screening medium (g L^{-1}): 3.0 peptone, 10.0 glucose, 0.6 KH_2PO_4, 0.001 $ZnSO_4$, 0.4 KH_2PO_4, 0.0005 $FeSO_4$, 0.05 $MnSO_4$, 0.5 $MgSO_4$, 20.0 agar (pH-6) supplemented with 0.02% guaiacol) and the plates were incubated

at the room temperature for 72 h. Following incubation, the plates were then observed for reddish brown zone formed around their colonies, as laccase catalyzes the oxidative polymerization of guaiacol to form a reddish brown color. The selected fungal strains were subjected for further screening (Coll *et al*., 1993).

Screening of selected microbes based on decolorization: The selected microbes were screened quantitatively in broth cultures amended with 0.01 g of each of the 8 dyes separately. The percentage of reduction was calculated based on the formula depending on the difference in the absorbance of the dyes in the UV-visible spectrophotometer (Phetsom *et al*., 2009).

Identification of selected microbial strains: The bacterial strains were identified by standard biochemical and microscopic tests (Cappuccino and Sherman, 1999). The fungal strains were identified using lactophenol cotton blue staining (Klich, 2002; Rafi and Sajjad-Ur-Rahman, 2002).

Compatibility analysis for the selected microbes: Microbes that are involved in a consortium should not exhibit any antagonistic effect over the other microbes that are in the consortium, only when the organisms are compatible with each other the remediation capacity of the consortia would be efficient. The compatibility analysis was done with the selected efficient microbial strains. Mueller hinton agar plates were swabbed with one of the selected microbe. Four wells were cut and 10 μL the culture supernatant (after 72 h of incubation) of the other organisms selected were added to the well. The test was repeated by differing the swabbed organism with the five selected cultures used in the study and the culture supernatants of the four other cultures which were not swabbed. The organism was said to be incompatible with the other if a zone of clearance was observed around the well (Umechuruba and Nwachukwu, 1997).

Design and development of microbial consortia: Various combinations of microbes were developed using the selected three bacterial isolates (Designated 1, 2 and 3) and the two fungal isolates (Designated A and B) by applying it in the Cayley's table. Accordingly A1, A2, A3, A12, A13, A23, A123, B1, B2, B3, B12, B13, B23, B123, AB, AB1, AB2, AB3, AB12, AB13, AB23, AB123, 123, 12, 13 and 23 were the twenty six different combinations that were built. These 26 combinations were used in treatment trials in identifying the efficient microbial consortium based on the reduction in the physico-chemical parameters.

Characterization of untreated effluent: Various physical and chemical parameters (Chemical Oxygen Demand [COD], Hardness, Total suspended solids, Total solids, Total dissolved solids, Color, turbidity and pH) were assayed for the untreated effluent by standard methods (APHA, 1992).

Treatment trials using the developed consortia: An initial inoculum concentration of 5% for all the 26 different microbial combinations were inoculated in 200 mL of the untreated textile effluent and was incubated under room temperature in a metabolic shaker at 120 rpm for a period of 7 days. The reductions in the parameters were observed on a daily basis for all the 26 combinations. The 5% initial inoculum concentration of the consortium was built by mixing each organism in equal volumes in a combination making up to 5 mL for every 100 mL of the effluent sample (Asgher *et al*., 2007).

Analytical methods

Physico chemical analysis: The parameters such as COD, total hardness, TS, TSS, TDS, pH, color and turbidity were the different parameters estimated to analyze the bioremediation efficiency of the different microbial combinations as per TNPCB (Tamil Nadu Pollution Control Board) and CPCB (Central Pollution Control Board). COD, Total Hardness, TS, TDS, TSS and pH were analyzed based on standard protocols (APHA, 1992). The color intensity and the turbidity of the effluent sample were measured at 490 and 620 nm, respectively (Telke *et al*., 2010).

Toxicity analysis by HPLC for the detection of aromatic amines: The textile effluent sample treated using the combination A13 for a period of 5 days was centrifuged and 50 mL of supernatant was extracted with equal volume of dichloromethane and concentrated in a rotavapour evaporator at 50°C. The concentrated products were separated using HPLC (Shimadzu, reverse phase C-18 column, 4.6 mm diameter and 25 cm length), 25°C, Eluent-acetonitrile: water (80:20V/V) flow rate was 1 mL min^{-1}. The products were monitored by their absorbance at 254 nm with a UV detector (Senan and Abraham, 2004).

Statistical analysis: Organisms selected through plate decolorization technique were selected through their decolorization on broth cultures (secondary screening) using 6 different synthetic dyes. The patterns of decolorization of the various dyes in relation the isolated microbe were analyzed using a two way Analysis of Variance (ANOVA).

RESULTS AND DISCUSSION

Isolation and selection of indigenously adapted microbial strains: About 265 bacterial strains and 35 fungal strains which were predominant and distinct were isolated from the effluent source through enrichment technique. From the selected bacterial strains based on the zone of clearance on eight different dyes, 7 strains were selected. From about 35 different fungal strains isolated only 2 organisms were selected based on the formation of a reddish brown zone around their colonies indicating the production of laccase through the oxidation of guaiacol. These 9 organisms were capable of reducing the mixture of all the dyes creating a zone of clearance around them.

From the 9 selected microbes, 5 organisms (Bacterial isolates 2, 6 and 7; fungal isolates - A and B) were found to be efficient in reducing all the dyes by more than 60% (Table 1) in liquid media. However, there exists a significant difference between the decolorization percentage of different isolates upon the various dyes used in the study (ANOVA, $p>0.05$). There was no significant difference between the decolorization percentage of the different dyes used by a single isolate (ANOVA, $p<0.05$) (Table 2). These 5 organisms were then applied in the Cayleys table and about 26 different combinations were developed which were used in treatment trials.

Identification of selected microbial strains: The bacterial isolates were identified as *Bacillus* sp., *Pseudomonas* sp. and *Alcaligenes* sp., based on their microscopical morphology and biochemical analysis (Table 3). Isolate 2 was found to be a rod shaped Gram positive bacterium which was found to be positive for Voges-Proskauer and oxidase test. The isolate 6 on the other hand was found to be a rod shaped Gram negative bacterium which utilized citrate, dextrose, lactose, mannitol and was found to produce hydrogen sulphide. The organism was also found to be positive for catalase and oxidase test. The isolate 7 was found to be Gram negative coccobacilli which gave

Table 1: Screening of selected microbes based on decolorization

	Decolorization (%)							
Isolates	Re red BSID	Remazol brilliant violet	Orange merl	Red M5B	Reactive red 120	Re yellow merl	Amido black-10B	Reactive black 5
1	14.6	26.3	46.3	2.9	12.1	34.5	6.0	8.3
2	78.0	60.4	19.5	92.4	53.5	82.6	66.7	80.4
3	12.2	39.9	12.2	13.7	2.8	20.8	6.2	25.9
4	4.9	26.9	4.9	3.8	25.0	29.9	8.0	50.5
5	2.4	18.9	2.4	20.7	21.7	2.3	13.8	49.8
6	80.5	52.0	61.0	83.6	69.3	78.0	66.7	86.8
7	85.4	79.3	65.9	93.6	91.5	77.3	68.7	86.5
A	78.9	76.5	90.4	83.6	80.6	80.6	77.6	81.2
B	89.4	78.0	93.0	89.7	82.2	89.4	79.2	82.8

Bacterial isolates: 1 to 7, Fungal isolates: A and B

Table 2: Two way ANOVA analysis for Screening of selected microbes based on decolorization

Sources of variation	Sum of squares	Degrees of freedom	Mean sum of squares	F-value
Between different synthetic dyes on decolorization	2176.614	8	272.0768	1.53
Between isolates on decolorization	64827.580	7	9261.0830	52.17
Residual error	9941.564	56	177.5279	

Table 3: Identification of bacterial isolates based on biochemical and microscopic analysis

	Bacterial isolates		
Microscopic/Biochemical characters	Isolate 2	Isolate 6	Isolate 7
Gram Stain	+	-	-
Shape	Bacilli	Bacilli	Coccobacilli
Indole	-	-	-
MR	-	-	-
VP	+	-	-
Citrate	-	+	-
Dextrose	Acid formed	+	-
Lactose	-	+	-
Mannitol	-	+	-
Sucrose	Acid formed	-	-
H_2S	-	+	-
Urease	-	-	-
Catalase	-	+	+
Oxidase	+	+	+
Nitrate	-	-	-
Gelatin	-	-	-
Organism tentatively identified as	*Bacillus* sp.	*Pseudomonas* sp.	*Alcaligenes* sp.

(+): Positive result, (-): Negative result

positive results for oxidase and catalase tests. Based on their microscopical observations and the biochemical characteristics exhibited by the isolates, these bacterium were found to be identified as *Bacillus* sp., *Pseudomonas* sp. and *Alcaligenes* sp., respectively on comparing it with the Bergey's manual of determinative bacteriology (Bergey and Breed, 1957).

Table 4: Physico-chemical characterization of untreated effluent

Parameters	Pollution control norms	Initial values
Hardness (mg L^{-1})	Not objectionable	325.125
COD (mg L^{-1})	400<	1819.13
TS (mg L^{-1})	50	33530
TSS (mg L^{-1})	3000<	12100
TDS (mg L^{-1})	3000<	21430
pH	5-9	10.5
Color at 490 nm	Nil	1.096
Turbidity at 620 nm	Not objectionable	0.976

The fungal isolates were identified as *Aspergillus* sp. and *Penicillium* sp., based on its microscopical morphology. On microscopic examination the first fungal isolate showed a septate and dichotomous hyphae, at 45° angle branching. Conidiophores were coarsely roughened, uncolored, vesicles spherical, metulae covering nearly the entire vesicle in biseriate species. Conidial heads were radiate, uni- and biseriate and was confirmed as *Aspergillus* sp., the second fungal isolate showed the terverticillate hyphae and the conidia were spherical to elliptical in shape. Conidia were smooth and had a green color reflection in the mass and was confirmed to be *Penicillium* sp.

Compatibility analysis: The organisms under study were found to be compatible with each other as there was no zone of inhibition around the wells in all the plates tested. This was because the co-existence of the isolated organisms in a common environment for a longer period.

Characterization of untreated effluent: The initial values of the estimated parameters for the untreated effluent sample and that of the permissible limits for the safety release of the effluents in the natural water body as laid down by Central Pollution Control Board (CPCB) and Tamil Nadu State Pollution Control Board (TNPCB) has been provided in Table 4. From the results analyzed it could be noted down that the untreated effluent sample was high in both the organic content (COD-1819.13 mg L^{-1}) and it has dyes exhausted from the fiber during dyeing process. The high TS (33530 mg L^{-1}), TSS (12100 mg L^{-1}) and TDS (21430 mg L^{-1}) content of the sample adds up to the COD of the effluent.

The organic content in the effluent sample was found to be very high. The color of the sample also was found unacceptable to be discharged in to the natural water body. Similar results were observed for Manu and Chaudhari (2003) where the organic content load of the dye effluent was found to be similar to that of the current study.

Treatment trials using the developed consortia: There was a reduction in all the parameters observed for all the microbial combinations from the first day onwards till the fifth day of incubation (Fig. 1). The average percentages of reduction for each of the parameter for all the combinations were first calculated on a daily basis. Based on the average values for each parameter on a day to day basis, a graph was constructed to analyze the retention time required for the efficient bioremediation of the textile effluent. From the results observed it was concluded that there was a reduction in the physico-chemical parameters from the first day onwards till the fifth day of incubation. Not more than 2% reduction in the parameters was observed after five days of incubation. Hence it was found out that the optimal time required for the efficient bioremediation of the textile effluent by the consortium was found to be 5 days interval.

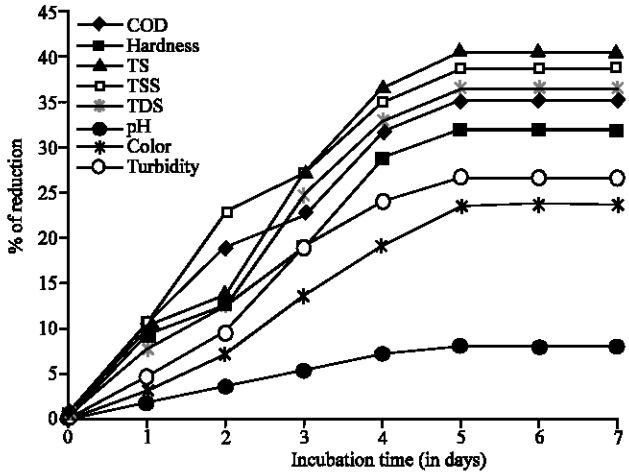

Fig. 1: Reduction pattern shown by microbial combinations on different physico-chemical parameters (Average pattern)

A maximum of 75% reduction was observed in the COD of the sample for the combination A13. About 4 other combinations (A2, A3, B1 and B23) showed a reduction in COD by more than 50%. About 5 different combinations (A3, A12, A13, A23 and B23) showed more than 50% reduction in color of the effluent sample after 5 days of incubation with A13 having a reduction of more than 71%. The hardness of the sample was reduced to a maximum of about 57% by the combination A13 and the combination B3 was able to reduce the total hardness by more than 50%. The pH of the sample was found to be very much under the control after treatment for most of the combination. A maximum of 15% reduction in pH was observed for the combination A13. About 2 combinations (123 and AB3) were able to reduce the turbidity of the sample by more than 50% with AB3 able to reduce the TS by upto 56%. Around 10 different combinations (A2, A3, A12, A13, A23, A123, 123, B23, B123 and AB) were efficient enough in reducing the TS of the sample by more than 50%. The combination A13 was efficient in reducing the TSS by about 94%. About seven other combinations were able to reduce the TSS by more than 50%. A12 combination was efficient in reducing TDS by upto 61% and about six other combinations were able to reduce the TDS by more than 50%. Though there were a few combinations (AB123, AB2, 23) efficient in reducing a particular parameter to a significant extent they were not able to reduce most of the physico-chemical parameters under study to a significant extent. The results observed showed that the combination A13 was efficient in reducing almost all the parameters under study by a significant margin. Effluent sample treated with this efficient combination was used in analyzing the toxicity of the sample through HPLC analysis (Table 5).

About 90% of reduction was observed in the organic load of the sample by a combined biological, physical and chemical method according to the study of Kim *et al.* (2003). Decolorization time taken by the cultures to achieve 66% decolorization compares favorably with reports on dye decolorization by most of the white rot fungi which require 7-20 days period for 90% decolorization of a diverse range of synthetic dyes as per Kirby *et al.* (2000) and other mixed microbial cultures according to the study of Senan and Abraham (2004) and Adedayo *et al.* (2004). Microbial components of mixed microbial cultures are capable of decolorizing dyes via biotransformation and biodegradation (Banat *et al.*, 1996). The efficiency of the decolorization process depends on the survival,

Table 5: Percentage of reduction of various physico-chemical parameters for microbial consortia developed after 5 days of incubation

	Physico-chemical parameters analyzed (% reduction)							
Consortia	COD	Hardness	TS	TSS	TDS	pH	Color	Turbidity
A1	46.4	43.8	9.4	9.4	54.1	12.6	9.5	3.8
A2	50.1	28.6	73.1	14.3	50.0	13.6	36.8	37.9
A3	50.0	42.9	65.4	67.8	57.5	12.3	71.5	45.3
A12	37.6	31.3	87.5	30.0	61.4	13.6	54.8	35.0
A13	75.0	57.1	75.0	94.3	59.5	15.7	71.3	45.1
A23	16.7	28.6	90.0	37.5	29.6	13.6	53.0	44.2
A123	9.1	21.4	89.3	21.4	23.6	13.4	48.2	33.3
123	16.7	26.7	78.6	14.3	35.0	13.6	37.4	51.9
B1	50.0	35.7	11.0	7.7	31.5	5.9	5.4	21.6
B2	29.2	14.3	24.3	15.4	25.5	2.0	21.4	17.2
B3	46.7	56.3	26.1	33.3	35.8	1.1	2.4	10.4
B12	28.6	38.5	48.1	50.0	30.3	1.6	12.7	6.6
B13	30.0	33.3	24.3	45.5	32.3	7.8	1.4	16.7
B23	50.1	42.9	54.7	77.8	54.0	11.4	53.8	42.2
B123	12.6	28.6	50.5	58.3	20.0	7.3	1.7	2.7
AB	45.0	21.4	51.8	84.6	51.6	7.4	1.5	4.0
AB1	38.0	33.3	12.2	6.0	36.0	3.9	3.3	18.9
AB2	45.5	35.7	32.5	55.7	43.1	5.8	8.7	22.9
AB3	36.0	30.8	24.5	16.4	34.2	3.1	7.2	56.9
AB12	38.0	25.0	5.0	26.9	39.5	4.9	17.2	30.8
AB13	33.4	30.8	28.2	40.0	30.7	5.8	7.7	36.0
AB23	33.0	16.7	29.6	20.6	34.4	3.1	3.0	38.2
AB123	20.1	25.0	23.2	54.1	12.6	9.5	3.8	35.3
12	26.0	26.7	12.2	42.0	23.0	4.2	26.0	6.6
13	25.0	26.7	14.6	38.6	18.2	5.6	24.2	17.2
23	24.0	26.7	16.2	44.2	22.4	6.2	25.8	10.4
Average	35.11	31.88	40.67	38.70	36.38	7.88	23.45	26.58

adaptability and activities of enzymes produced by microorganisms present in the mixed cultures (Senan and Abraham, 2004). Dye removal efficiency of about 61-76% reduction of RR141 dye was found in case of non acclimatized cultures and about 68-96% reduction was obtained for the same dye for acclimatized cultures. More than 70% reduction in the organic load was observed for the acclimatized cultures where as only about 18% reduction was observed in non-acclimatized cultures according to the study of Pasukphun *et al.* (2010). Similarly for the study of Saif-Ur-Rehman *et al.* (2008) around 72% reduction in color was observed in the decolorization of turquoise blue dye as a result of combination of ozonation and Fenton process. These results indicate that the results observed for the combination A13 was found to be promising in the bioremediation of textile effluent treatment.

Toxicity analysis by HPLC: The chromatogram of the treated effluent sample showed 3 peaks in it with one major peak and 2 minor peaks. The retention time (1.460, 2.120, 2.317) and the % of area (5.6, 14.1, 80.4) obtained for the three compounds (peaks) in the chromatogram were not that of the aromatic amines and as the retention time of all the amines were more than 3 min which was confirmed by comparing the retention time and the % of area obtained in the work of

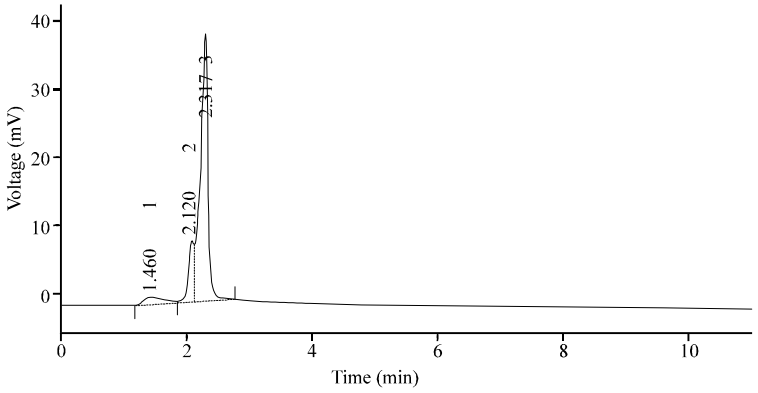

Fig. 2: HPLC chromatogram for A13 treated textile effluent

Zhang *et al.* (2003). It was thus confirmed that the textile effluent sample treated with the combination A13 under shaking condition was not found to produce any toxic aromatic amines at the end of five days of incubation (Fig. 2).

CONCLUSION

Treating azo dye containing textile effluents using a single pure culture of microbe is both time consuming and biorecalcitrant under aerobic conditions. The use of a microbial consortium helps in reducing the azo dyes under aerobic conditions at a faster rate. The consortium of microbes with its wide spectral enzymatic system, with and without substrate specificity helps in reducing any number of dyes containing aromatic rings present in the effluent. Only such treatment should be employed in a common effluent treatment plant as the dyeing units deal with more number of dyes on a daily basis. This idea could very well be adopted by any industry in developing a green environment.

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