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Aerobic bacterial consortium CN-1: Potential degrader of azo dyes

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Abstract

Environmental technologists working in the field of bioremediation face a great challenge in treating dye containing textile effluent as effluent contains diverse groups of dyes, which a single microorganism cannot handle. In the present study, aerobic bacterial consortium CN-1 containing 5 different bacterial strains with the ability to degrade 3 different azo dyes were checked for its ability to degrade the same 3 dyes mixed together. The consortium removed 91.5% of these dyes when mixed in the ratio of 65:65:20 (Acid Blue 113, Mordant Black 17and Acid Black 24). Plackett Burman screening design indicated the importance of low concentration of dye and low concentration of glucose for maximum degradation of dyes. The consortium began to degrade the mixed dye when they were in their mid lag phase, and the rate of degradation reached a maximum of 6.6 mg h^{-1} after 4 hr of incubation which started to decrease gradually to 4.4 mg h^{-1} by 16 hr after which the decrease in the rate of degradation was found to be quite slow. Degradation was found to follow the first order of kinetics with k value of 0.085 hr^{-1} and R^2 being 0.982. Salicylic acid and 2-naphthol were identified as intermediate compounds from GC-MS analysis.

Key words

Acid blue 113, Acid black 24, Biodegradation, Consortium CN-1, Mordant Black 17

Introduction

Aesthetically, beautiful colours originating from dyes and dyestuffs used in textile industries pose danger to the environment in long run as they are highly reactive and are mostly comprised of azo dyes. Azo dyes have been used increasingly in industries because of their ease and cost effectiveness in synthesis as compared to natural dyes. During dyeing processes about 10–90% of dye stuff do not bind to the fibres and therefore, is released into the waste water treatment system or the environment (Zollinger 1991; Abdullah, *et al.*, 2000). They are stable in acidic and alkaline conditions and are resistant to temperature and light. Most of azo dyes are toxic, carcinogenic, and mutagenic (Pinheiro *et al.*, 2004). Their structural complexity (–N≡ N–) makes them poorly degradable, with potential for persistence and accumulation of high-levels of dye in the environment. This demands consistent efforts to develop an effective method for elimination of these dyes.

Treatment of such dye containing effluent includes various physical and chemical treatment processes including adsorption, concentration, chemical transformation, but with time, potential hazards and disadvantages of these methods were noted as formation of toxic sludge and formation of even more toxic metabolites. Alternatively, approach is shifting towards the use of conventional biological processes that present eco-friendly and cost competitive alternatives in the treatment of dyes. *In situ* degradation of effluent is more convincing as it involves isolation of microorganisms from effluent and using the same for treatment of the effluent (Olukanni et al., 2006; Puvaneswari *et al.*, 2006; Vijaykumar *et al.*, 2007).

On the other hand, presence of azo, nitro and sulfo groups make dyes more resistant to microbial degradation and their residues accumulate in nature (Joe *et al.*, 2008). Yet, there have been numerous attempts to develop biological processes for treatment of textile effluents using bacteria, fungi and enzymes (McMullan *et al.*, 2001; Wesenberg *et al.*, 2003).

Dye degradation is a complex process that involves multiple steps. Even though a number of bacteria can bring about dye degradation efficiently yet, each one is specific for a particular group of dyes. But for the treatment of dye containing effluent they cannot be depended as the effluent will contain different groups of dyes. Under such condition microbial consortia with organisms having different specificities can perform the treatment of dye containing effluent more efficiently which is difficult or even impossible for individual strains or species (Tony *et al.,* 2007; Chan *et al.,* 2011).

In previous studies, bacterial consortium (using bacteria isolated from textile effluent and soil near tannery effluent) capable of removing Acid Blue 113 (Valli Nachiyar *et al.,* 2012), Mordant Black 17 (Karunya *et al.,* 2014 a) and Acid Black 24 (Anuradha *et al.,* 2014) individually in mineral salt medium, as well as, in effluent has been reported. In the present study, however microbial consortium was checked for its efficiency to degrade mixture of these dyes mixed in different concentrations.

Materials and Methods

Collection of effluent : Effluent samples were collected from Suntex processing mills, Gummidipoondi; Professional fabrics, Tirupur and Kafer Textile mills, Tirupur. Numerous colonies were obtained through serial dilution method.

Dyestuff and chemicals : Acid blue 113 (AB113) [CI No.26360], Acid black 24 (ABK24) [CI No.26370] dyes were obtained from Saujanya Dye Chemicals, Ahmedabad, (Gujarat, India) while Mordant black 17 (MB17) [CI No.15705] was obtained from Dynasty Chemicals (Ningbo) Co., Ltd., China.

Isolation and identification of microorganisms : Bacteria present in effluent were isolated by serial dilution method and their dye degrading ability was checked by pour plate method on nutrient agar containing AB 113, AB 24 and MB 17 separately. All the bacterial cultures were maintained on nutrient agar slants and were used for biodegradation studies after preculturing in nutrient broth for 12 hrs. The bacterial cells were cultivated in nutrient broth for 24 hr and cells after centrifugation were resuspended in 20% glycerol and stored at –20 ºC as stock cultures. Purity of glycerol stocks were checked on nutrient agar plates before sub culturing on nutrient agar slants for inoculum preparation. Organisms were identified using 16S rRNA analysis (Karunya *et al.,* 2014 a).

Culture medium: For degradation studies, mineral salt medium having following composition was used (g l^{-1}) 1.73 K_2HPO_4, 0.68 KH_2PO_4, 0.1 $MgSO_4.7H_2O$, 0.1 NaCl, 0.03 $FeSO_4$, 1.0 NH_4NO_3, 0.02 $CaCl_2.2H_2O$ and 5.00 glucose. Glucose was sterilized separately and added to medium at the time of inoculation.

Consortium development : The consortium was developed aseptically by transferring 2% inoculum containing approximately 2 x 10^8 cells per ml of 24 hr grown culture of each individual strains (10 isolates + *Pseudomonas aeruginosa* CLRI BL22 obtained from CLRI) in 15 different combinations in 250 ml Erlenmeyer flasks containing 50 ml of medium and 100 mg l^{-1} dye solution. Individual strains were inoculated with 10% (v/v) aliquots of 24 hr grown culture, respectively, to maintain the same cell count in pure culture and in the consortium. Selected isolates in combination of 3 to 10 were inoculated into NB for 8 hrs to form a consortium. Dye degradation procedure was repeated to screen the best consortium from 15 different combinations against mixture of all the three dyes.

Degradation studies : The optimum ratio of all three dyes (Mordant Black 17, Acid Blue113, and Acid Black 24) was found by mixing these dyes in different ratios (AB 113 : AB 24 : MB 17, 50:50:50, 60:60:30, 50:75:25, 70:50:25 and 65:65:20) followed by degradation with selected microbial consortium. Dye degradation studies were carried out with effective consortium (CN-1) in 250 ml conical flasks containing 50 ml mineral salt medium with 100 mg l^{-1} mixture of all the three dyes. The medium was inoculated with 12 hr old 2% inoculum, incubated in an incubator shaker at 200 rev min^{-1} at 30°C. Degradation of all the three azo dyes were monitored spectrophotometrically by reading n-butanol extract of the culture medium containing degradation product at 570 nm (Yatome *et al.,* 1981). Rate of decolorization was calculated from the difference between the initial and final absorption values of the supernatant at λ_{max} for mixed dye. Intracellular protein content, which is used as an index of bacterial growth, was estimated of Lowry method (1957).

Kinetics of biodegradation : Biodegradation rate of dyes was calculated by testing the kinetic data by first-order model (Lagergren, 1898).

Plackett burmann screening design for medium components : Plackett Burman methodology (PB) was used to screen (n) variables in (n+1) number of experiments (Plackett and Burman 1946). PB matrix was developed manually and the screening design was set up for 7 variables (A – G) in two levels, high and low. In the present study, seven independent variables were screened in eight combinations organized according to Plackett-Burman matrix. Each variable can be at a high level (+1), and a low level (-1). Agitation and incubation were dummy variables. The medium components used in PBSD trials are given in Table 1.

Table 1 : Medium components for Plackett Burman screening design

Variables	Medium component	Levels	
		High (+1)	Low(-1)
A	Dye concentration (mg l^{-1})	1000	100
B	Glucose concentration (g l^{-1})	5	1
C	Ammonium nitrate (g l^{-1})	1	0.5
D	Temperature (ºC)	40	20
E	Agitation (rpm)	150	0
F	Incubation time (hr)	24	48
G	pH	9	6

HPLC analysis : Culture supernatant containing degradation products was filtered through 0.22 m filters. 25 µl of this filtrate was subjected to HPLC analysis in Shimadzu SLC–6B model equipped with SPD – 6AV UV-Visible Spectrophotometric Detector and C – R4A Chromatopac using a C-18 reverse phase HPLC column with a solvent system consisting of methanol and water. A linear gradient from 20% methanol (isocratic for the initial 2 min) increased to 100% over 10 min and was then maintained at 100% concentration for 10 min with a flow rate of 1ml min^{-1}. The culture supernatant containing dyes, as well as, degradation products were analyzed at their corresponding λ_{max}.

GC-MS analysis : Culture medium after 48 hr of incubation (containing the degradation products) was centrifuged and supernatant was extracted thrice with equal volume of ethyl acetate, dried over anhydrous Na_2SO_4 and then the solvent was evaporated in a rotary evaporator. This extract was subjected to GC-MS analysis using GC-MS-QP 2010 [SHIMADZU] MS spectrometer. The column used was VF-5ms, 30 m x 0.250 mm dia with film thickness of 0.25 µm and the column oven was programmed between 70 and 300 ºC @ 10 ºC min^{-1} with injection temperature of 240ºC. Mass spectra was recorded under scan mode in the range of 40–1000 m z^{-1}. Compounds were identified using WILEY8.LIB.

Results and Discussion

A bacterial consortium CN-1 was constructed using five different bacterial strains for degradation of 3 different azo dyes namely Acid Blue 113, Acid Black 24 and Mordant Black 17. Isolation, identification and construction of consortium CN-1 and degradation of dyes separately by this consortium have been reported (Karunya *et al.,* 2014a).

Dye degradation is a complex process that involves multiple steps. Even though, there are number of bacteria which can bring about dye degradation efficiently; each one is specific for a particular group of dyes. But for the treatment of dye containing effluent they cannot be depended upon as

Table 2 : Dye degradation by constructed consortium CN^{-1}

Consortia No.	Isolates (SUB)	Decolorization (%)
CN1	2,3,4,6,7,10	93.34
CN2	1,2,3,4,6,7	86.72
CN3	1,2,3,4,6,9	89.12
CN4	1,2,4,6,7,9	85.23
CN5	1,2,3,4,6,9,10	82.14
CN6	1,2,3,4,5	84.74
CN7	1,2,3,4,6	86.32
CN8	1,3,4,6,9	83.27
CN9	1,2,4,6,7	86.45
CN10	1,2,3,4	84.57
CN11	2,3,4,6	88.63
CN12	1,2,4,5	84.39
CN13	2,3,4	81.78
CN14	1,2,4	86.54
CN15	1,3,6	82.36

the effluent contains different groups of dyes. Under such conditions, microbial consortia, with organisms having different specificities, can perform treatment of dye containing effluent more efficiently, which is difficult or even impossible for individual strains or species (Tony *et al.,* 2009). Initial screening studies have resulted in isolation of 10 different bacteria using 15 different bacterial consortia (CN-1–CN-15) and their ability to decolorize the dye mixture was checked at 100 mg l^{-1} in MSM (Table 2). The results clearly show that CN-1 was found to be comparatively efficient in bringing about maximum decolorization (93.34%) of dye mixture after 48 hr of incubation. Khehra *et al.,* (2005) reported decolorization of group of azo dyes by consortia HM-4 containing *Bacillus cereus*, *Pseudomonas putida*, *Pseudomonas fluorescens* and *Stenotrophomonas acidaminiphila*. Higher decolorization efficiency of consortium was to the concerted activities of the constituent strains. Individual cultures might have transformed the dye to intermediates, which acted on the redox mediators for efficient transfer of reducing equivalents from the strains, leading to enhanced decolorization potential of the consortium.

Three selected azo dyes were mixed in different ratios and the ability of the consortium CN-1 to degrade this mixed dye was checked. It is clear from Fig. 1 that the consortium degraded the mixed dye when they were mixed in 65:65:20 ratio (AB 113: MB17: AB 24). As the concentration of AB 24 decreased, degradation increased indicating the recalcitrant nature of AB 24 as compared to AB 113 and MB 17.

PBSD has been used to identify the factors affecting dye degradation by the microbial consortia. The experimental design for screening the medium components and the experimental values are given in Table 3. Screening of medium components by Plackett Burmann design showed

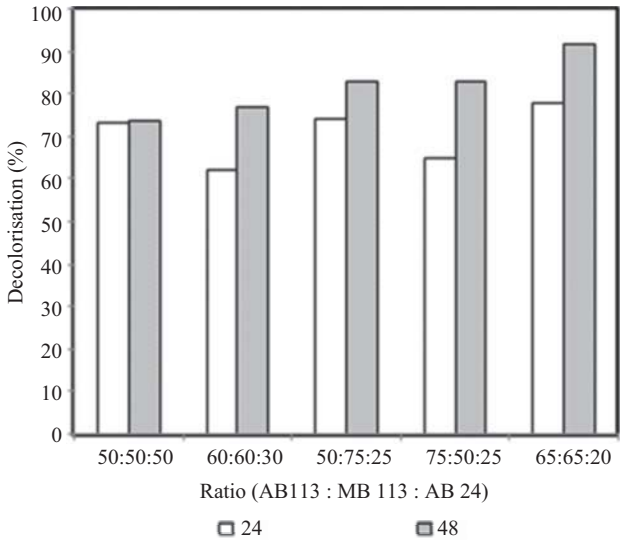

Fig. 1 : Optimization of mixed dye composition

wide variation from 10.29% to 92.07% which reflects the importance of medium components to attain higher degradation. Of all the variables tested, dye concentration was found to play an important role followed by temperature and concentration of ammonium nitrate and glucose (Table 4). It is clear from the table that dye degradation by the consortium was high with low concentration of dye and low concentration of glucose provided temperature and ammonium nitrate were held at high concentration and pH maintained at 9. Similar results were observed in degradation of AB 113 (Karunya *et al.,* 2014b) and MB 17 (Karunya *et al.,* 2014 a,c). Nanthakumar *et al.* (2013) have reported dye concentration as one of the significant variable in the degradation of Reactive Red 195 by a Termite Associated Bacterial Consortium BUTC7. In contrast, Usha *et al.* (2011) have reported yeast extract, aeration and temperature as significant variables affecting Reactive Red 120, and dye and pH on Reactive Black 5 dye degradation by *P. aeruginosa* and *A.punctata*. Glucose, yeast extract, aeration, inoculum, dye concentration and pH were reported as other main factors that showed significant positive effects on both dye degradation.

The consortium exhibited a very short lag phase reaching its log phase by 4 hrs of incubation. The growth rate increased up to 14 hrs of incubation, reaching maximum of 2.34 mg hr^{-1} after which it reached the stationary phase. The consortium reached its decline phase after 26 hr of incubation (Fig. 2a). Regarding degradation, the consortium started to degrade the mixed dye when they are in their mid lag phase. The rate of degradation reached maximum of 6.6 mg h^{-1} after 4 hrs of incubation which started decreasing gradually to 4.4 mg h^{-1} by 16 hrs after which the decrease in the rate of degradation was found to be quite slow. Biodegradation rate was calculated by testing the kinetic data with first-order model. From the plot of log (B_e - B_t) versus t, it was observed that this model fits the data well (Fig. 2b). The k value was found to be 0.085 hr^{-1} and R^2 being 0.982.

HPLC chromatograms of the media containing mixture of dyes at 100 mg l^{-1} were analyzed at 0^{th} hr and at different incubation periods (24hr and 48hr). Scanning was carried out at 3 different wavelengths (525nm, 565 nm and 618 nm for Mb 17, AB 113 and AB 24 respectively) to identify degradation of individual components of the mixture of dyes. At all the tested wavelengths, peaks with identical R_T

Table 3 : Plackett Burman design matrix with experimental values for mixed dye degradation

Trials	A	B	C	D	E	F	G	Decolorization (%)
1	H	H	H	L	H	L	L	10.29
2	L	H	H	H	L	H	L	62.01
3	L	L	H	H	H	L	H	92.07
4	H	L	L	H	H	H	L	13.75
5	L	H	L	L	H	H	H	12.25
6	H	L	H	L	L	H	H	13.82
7	H	H	L	H	L	L	H	19.64
8	L	L	L	L	L	L	L	56.1

Table 4 : Plackett Burmann Screening Design results and significant levels

	A	B	C	D	E	F	G
EH	57.5	104.19	178.19	187.47	128.36	101.83	137.78
EL	222.43	175.74	101.74	92.46	151.57	178.1	142.15
D	-164.93	-71.55	76.45	95.01	-23.21	-76.27	-4.37
MS	3400.238	639.9253	730.5753	1128.363	67.33801	727.1391	2.387113
MSE				397.2386			
F TEST	8.559688	1.610935	1.839135	2.840516	0.169515	1.830485	0.006009

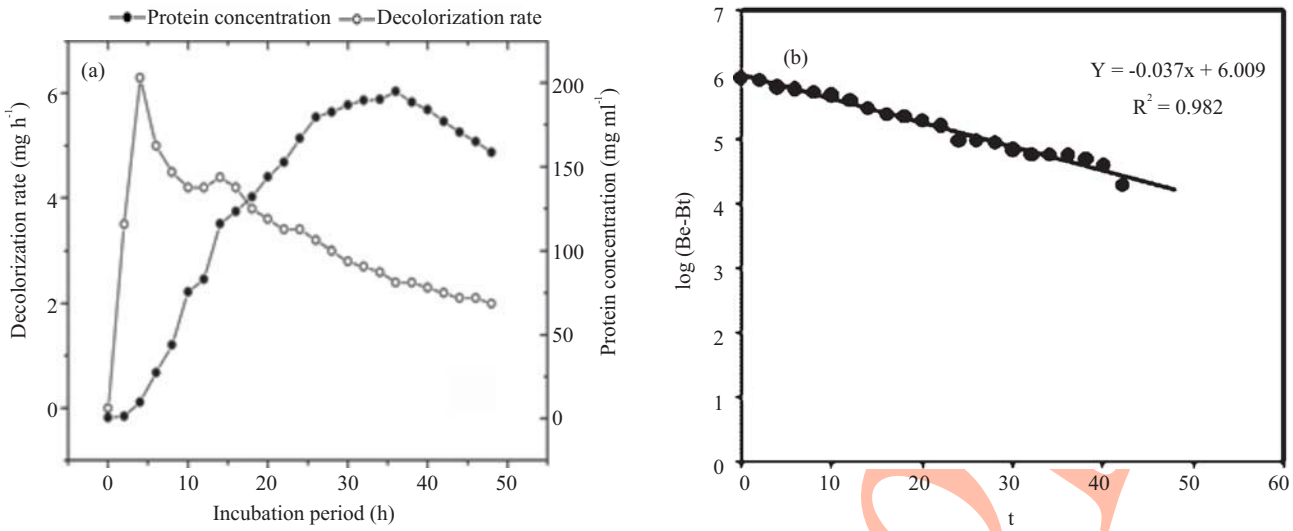

Fig. 2 : (a) Time course and of Mixed dye degradation (100 mg L-1) degradation by CN-1, (b) Kinetic study of Mixed dye degradation (100 mg L-1) degradation by CN-1

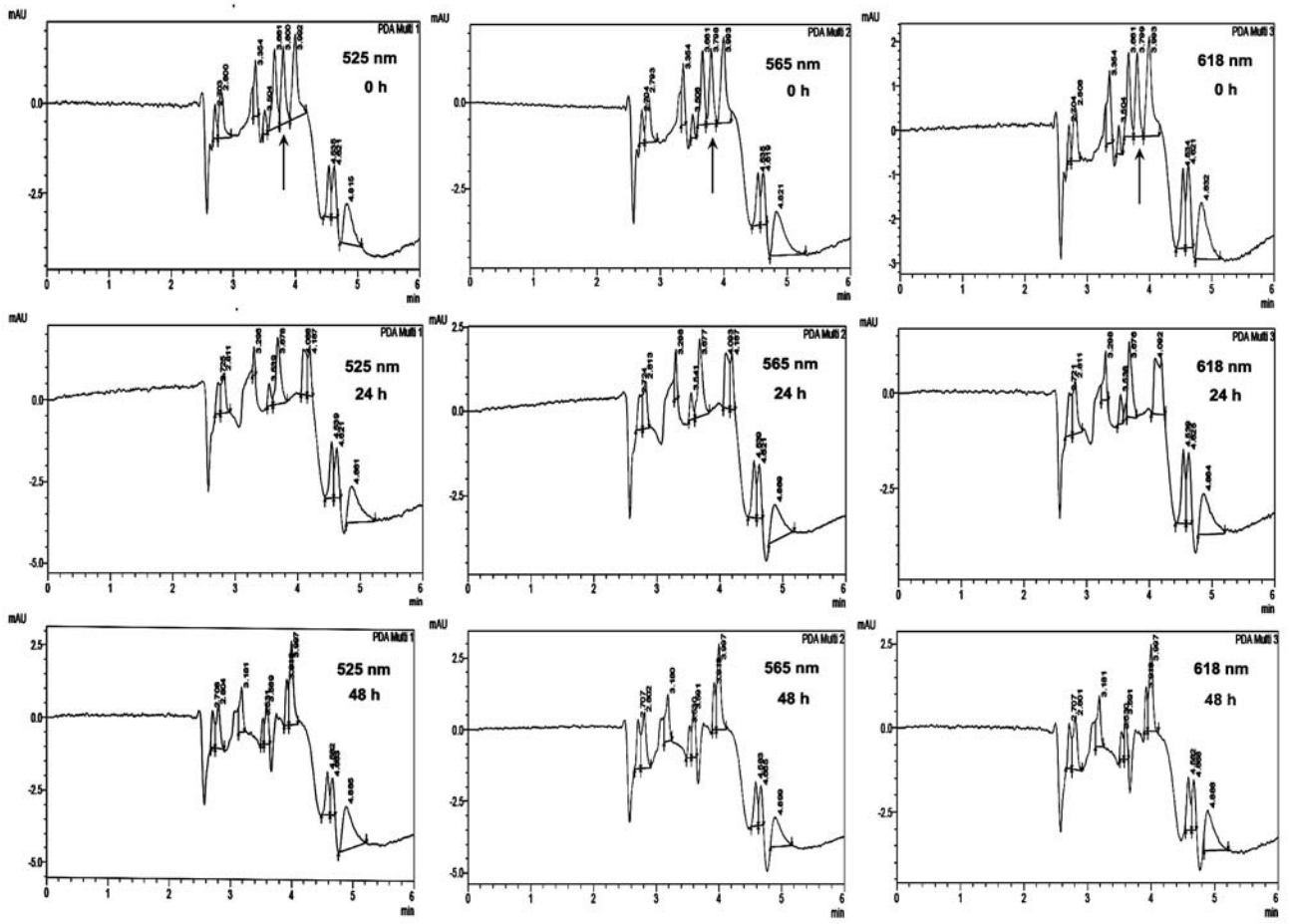

Fig. 3 : HPLC chromatogram showing the course of degradation of individual dyes in mixture. Acid Black 24 (525 nm), Acid Blue 113 (565 nm) and Mordant Black 17 (618 nm)

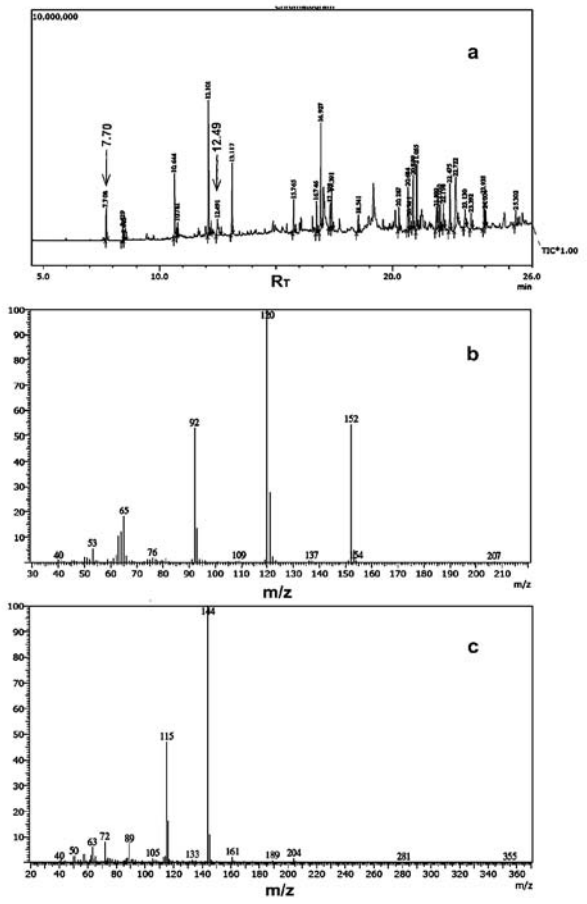

Fig. 4 : GC-Mass Spectra of ethyl acetate extract of culture filtrate containing degradation products of mixed dye (100 mg l^{-1}) by CN^{-1}. (a) gas chromatogram and mass spectra for peak corresponding to RT value of (b) 7.7 min (c) 12.5 min

values have been obtained making it difficult to identify the individual dyes. This might be due to overlapping of absorbance of all the three dyes. It is evident from Fig. 3 that the peaks with R_T value of 3.8 disappeared from samples after 24 hrs.

GC-MS spectra of ethyl acetate extract (Fig. 4) showed many peaks of which two peaks were identified as those of methyl salicylic acid and 2-naphthol with R_T values of 7.7 min and 12.5 min. Mass spectra corresponding to peak with R_T value of 7.7 min indicated fragmentation pattern with m z^{-1} values of 152, 120, 92 and 65 corresponding to methyl salicylic acid and the fragmentation pattern for R_T value 12.5 min gave m z^{-1} values of 144, 115, 89 and 72, confirming the presence of 2-naphthol.

All the three dyes studied were azo dyes in which AB 113 and AB 24 were diazo dyes and MB 17 a monoazo dye. While AB 113 and MB 17 contained 2 naphthalene rings in their structure, AB 24 had 3 naphthalene rings. The presence of 2-naphthol and salicylic acid in the culture medium clearly indicated that the naphthalene part of any or all of these dyes might have undergone degradation. 2-naphthol has been identified as one of the intermediate in degradation of MB 17 (Karunya *et al.,* 2014a) by consortium CN-1. Salicylic acid is one of the typical intermediates in bacterial degradation of naphthalene (Annweiler *et al.,* 2000; Pumphrey *et al.,* 2007) which can easily be assimilated easily by the bacteria.

The current decolorization/degradation process offers to be an advantageous bioremediation system for decolorization of dyes in textile effluent, more particularly, azo dyes in that it is an aerobic treatment system, whereby toxic intermediates like aromatic amines produced in effluent by abiotic and biotic system may be completely mineralized. This study was carried out to check the efficiency of constructed microbial consortium CN-1 to degrade a mixture of dyes (AB 113, AB 24 and MB 17), which has been found to be successful.

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