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Research Article

ELUCIDATING A PATHWAY FOR DEGRADATION OF AZO DYE REACTIVE RED 120 BY BACTERIAL CONSORTIUM

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ABSTRACT. A detailed characterization of degraded metabolites is essential for understanding the mechanisms of complex dye degradation, as a result of metabolic activity of micro-organisms. The resulting knowledge may prove insightful for designing microbial tools for the purpose of bioremediation. In the current study, an azo dye RR120, completely decolorized by a bacterial consortium RAR, was analysed for identification of degraded metabolites. For this purpose, the dye degradation was first confirmed using qualitative techniques like UV-Vis spectrophotometer and HPTLC. On confirmation of biodegradation, the metabolites thus produced were analysed using techniques like HPLC, GC-MS and FTIR to identify stable and/ or unstable intermediate products. Specifically, these studies indicated that more than 2 intermediates are produced on degradation of RR120 dye. The cleavage of –N=N–bond, degradation of aromatic rings, loss of sulphone groups and breakage of C–Cl bond was evident from FTIR spectrum. Based on HPTLC analysis, there occurred a high probability that the degraded metabolites were assimilated by the cells of pure cultures and consortium RAR. Finally, based on above information, a detailed azo dye RR120 degradation pathway was proposed. Thus, the current study provides complete information on the metabolic activity of bacterial consortium RAR and the degradation of complex azo dye RR120.

Keywords: *UV-Vis spectrophometer, HPTLC, HPLC, GC-MS, FTIR, consortium, metabolic activity.*

INTRODUCTION

The development of a country is marked by the extent of industrialization. Unfortunately, the same industrialization contributes to major environmental pollution caused due to generation of wastewaters by various industrial processes. A key composition of these effluents is dyes required for improving the appearance of finished goods. Azo dyes are examples of extensively used group of dyes that are utilised in the textile, cosmetics, paper, food and pharmaceutical industries [1]. Over 70% of the manufactured dyes are azo dyes, which are preferred due to its stability and cost effectiveness [2]. Especially the textile industries, share a major contribution, to over 100 tons of discarded effluents, globally, that pollute the soil and water bodies [3].

Among the various treatment methods available, bioremediation of dyes is the most promising approach- given the environmental safety of these techniques. However, factors like stability and toxicity of dyes may limit the applications of biological techniques. The chemical stability of synthetic azo dyes is due to the presence of nitrogen double bonds [4]. Many microbial species may not be able to degrade these stable bonds. Moreover, few reported instances describe the toxicity of metabolites produced on

degradation of dyes that may cause equal or more harm than the parent compound [5]. Hence, it is necessary to monitor the dye degradation process by characterizing the metabolites at different stages of bacterial metabolism.

Different microorganisms follow varied mechanisms through simple or complex pathways for degradation of dye molecules. The structure, stability, microbial strain and the surrounding environment are critical factors that affect the overall process of dye degradation by microorganisms [6]. However, since dyes are utilised by micro-organism by either reductive or oxidative metabolic pathways, the enzyme systems play a key role in biotransformation of dyes. The azoreductases are suggestive of logical as well as practical mechanisms in bacterial dye degradation that begins under anaerobic conditions by formation of colorless aromatic amines [7].

The metabolites produced on degradation of dye can be mapped using various analytical techniques. The UV-Vis spectroscopy is a basic technique that is primarily used to determine the degradation of dye, qualitatively. The dye degradation is confirmed based on disappearance of standard peaks of parent molecule at λmax, and formation of new peaks that indicate the presence of degraded metabolites. An increase in absorbance towards the UV region also confirms the degradation of dyes [8]. High Performance Thin Layer Chromatography (HPTLC) or a simple TLC also proves to be helpful in qualitative analysis of dye degradation process. It is based on the separation of spots on TLC plates to resolve the parent dye molecule and the breakdown products [9]. The High Performance Liquid Chromatography (HPLC) technique, like UV-Vis spectroscopy, also confirms dye degradation based on disappearance of standard peaks and appearance of new peaks. However, in this technique the different retention times compared to the original dye can be used to identify the structural analogues of metabolites [10]. When chromatographic techniques are coupled with Mass Spectrometry (MS), they provide the mass spectra of the mixture components and hence prove to be a very powerful analytical tool. The Gas Chromatography-Mass Spectrometry (GC-MS), and similar chromatographic techniques, are useful for the determination of molecular weights and structural formula of degraded metabolites of the dye molecule [11]. Another technique i.e., Fourier transform infrared spectroscopy (FTIR) is a widely used infrared spectroscopy, that can be exploited for the determination of functional groups of breakdown products of the parent dye molecule [12].

Collectively, the above techniques help in better understanding of metabolic processes/enzyme systems of micro-organisms that aid the process of bioremediation or biotransformation. This, in turn, can help in designing efficient bacterial consortium, by analysing the potential of individual isolates and the effect of their concerted metabolic activity on degradation of dyes. This is possible by co-inoculation of suitable bacterial isolates that produce enzymes that prevent the gaps in metabolism of a complex dye by a single bacterium. Moreover, it can help in genetic engineering process that may allow incorporation of transcription and translation genes that transcribe specific dye degrading enzymes [13].

In the current study, the metabolites produced on degradation of azo dye RR120 by a bacterial consortium RAR (optimised in a previous study) were characterised using analytical techniques and a metabolic pathway of the consortium was proposed for efficient degradation of dye.

MATERIALS AND METHODS

Optimization of dye decolorization by a bacterial consortium RAR

A bacterial consortium RAR of three isolates i.e., *Shewanella haliotis* RDB_1 (LK-1), *Shewanella putrefaciens* RDB_2 (DL-1) and *Aeromonas hydrophila* RDB_3 (LK-2) was optimised for decolorization of textile dye Reactive Red 120 (RR120) in previous studies [14-16]. The decolorised dye was evaluated using analytical techniques to confirm biodegradation of RR120 by bacterial consortium RAR in the current study.

Separation and analysis of degradation products of RR120 in aqueous supernatants

A 50ppm dye was decolorized by the consortium under optimised conditions [17], and the decolorized media was analysed for degraded metabolites in the current study. On partial and complete decolorization of RR120, the respective supernatants of the experimental set up were collected through centrifugation at 10000rpm/ 20min. Also, the supernatants of consortium/pure cultures grown in absence of RR120 (control) were collected, as control, for the analysis.

Qualitative analysis of degraded metabolites

The above supernatants were analyzed for spectrum scan using a wavelength range of 200-800nm by UV-VIS spectrophotometer for comparison of peaks obtained before and after dye degradation. In addition, the supernatant of completely decolorized RR120 was subjected to HPTLC analysis. For this purpose, pre-coated TLC plates (Alumina silica gel 60 F 254, Merck, Darmstadt, Germany) of dimensions 200.0 x 100.0 mm were used as stationary phase for separation of samples. The mobile phase comprising of n-propanol: ammonia (7:3 v/v) [18] was used with saturation for 20min in Camag twin trough glass tank (TTC 20x10). The sample volume of 4µL was spotted with a semiautomatic Linomat V band spotter from CAMAG (Switzerland). The solvent phase was allowed to run till 70mm of solvent front. The drying of these plates in a stream of air was performed at room temperature for 5min while the scanning of plates was carried out at 254 nm, 366 nm and 520 nm using CAMAG TLC scanner 4 to confirm separation of samples [19, 20].

Separation and analysis of organic solvent extracted degradation products of RR120

Sample preparation

The consortium RAR (10% v/v inoculum at 1.0 $OD_{540\ nm}$) was inoculated in 2 sets of 200 mL sterile 3%YE medium (pH 7.4) containing 500 ppm RR120. One flask was incubated under static (ST) conditions at 35°C/2h, and another under sequential static-shaker (ST-SH) condition (i.e., static until complete dye decolorization was observed and then on shaker for additional 24 h). Similarly, another set of flask was prepared with 3%YE medium without RR120 and incubated under above described conditions. After incubation, the above media were centrifuged at 10000 rpm/20 min. The supernatant was used for extraction of metabolites using two different solvents i.e., dichloromethane (DCM) and ethyl acetate. For extraction process, the supernatant of decolorized medium from each flask was mixed with above solvents, in equal proportion, using a separating funnel. The mixture was allowed to stand for extraction of degraded metabolites of RR120 with intermittent shaking. The organic extracts were collected and passed over anhydrous Na_2SO_4 and then dried at 35 ºC in a desiccator to evaporate the organic solvents [21]. Similarly, the extraction procedure was repeated with the supernatant of consortium grown in 3% YE without RR120. The DCM extracted dried sample from decolorized

broth incubated under ST condition and ST-SH were designated as A_1 and A_2 respectively. Similarly, DCM extracted dried sample from consortium grown in medium without RR120 incubated under ST and ST-SH conditions were designated as B_1 and B_2 respectively. All four samples were analysed by HPLC analysis, and the samples A_1 and A_2 were further analyzed by FTIR and GC-MS.

HPLC technique

The samples A_1, A_2, B_1 and B_2 were dissolved in HPLC grade methanol and 20µL volumes were injected in C18 column (Thermoscientific, model: Ultimate 3000; Acclaim, 5 µm x 250 mm x 4.6 id) with an autosampler (WPS-3000). The temperature of column was maintained at 28 ºC and the mobile phase was placed on a solvent rack (SR-3000) and isocratic composition of 55:45 ratio of methanol: water [22] at 1 mL/min flow rate was used with 15min as run time. The absorbance was recorded by using DAD 3000 detector at wavelengths 254 nm, 350 nm, 400 nm and 512 nm to analyze separation of degradation products. Chromeleon 6.0 software was used in interfacing and to carry out automated operations.

FTIR analysis

The analysis of samples A_1 and A_2 was performed on IR Spirit FTIR (Model: QATR-S, Shimadzu) at Shimadzu Customer Support Centre. The dried samples were placed on the sample platform of ATR unit and IR spectra of samples were obtained using software Labsolution IR.

GC-MS technique

The samples A_1 and A_2 were dissolved in chromatography grade methanol and were separated by GC-MS (QP2010, Shimadzu). The sample volume of 1 µL was injected in to the Restek column (0.25 mm×30 m) with 70eV as an ionization voltage. The column temperature was 80 ºC/2 min initially, then increased upto 140 °C at the rate of 15 °C/min and was maintained for 10min. The temperature was further increased upto 290°C at the rate of 25 °C/min and was upheld for 6min. The temperatures of injection port and interface were maintained at 275 °C and 250 °C respectively while the flow rate of 1.5 mL/min was set for carrier gas helium.

RESULTS AND DISCUSSION

Analysis of biodegraded RR120 metabolites by UV-Visible Spectrophotometry

The UV-Visible spectrum scan of intact dye molecule RR120 in 3% YE (abiotic control) exhibited the maximum absorbance of 1.367 at wavelength 516.8 nm. The breakdown of dye RR120 by consortium RAR, as well as in control, was monitored periodically to confirm its biodegradation (Fig. 1). No degradation was recorded in control samples. In partially decolorised samples, there was a decrease in the absorbance at 516.8 nm. Additionally, an increase in the absorbance, at a new wavelength in the UV region (Table 1), was also observed. In completely decolorised samples, absence of absorbance at 516.8nm was noted for pure cultures (in previous studies) as well as consortium RAR. However, for these samples, the pure cultures such as LK-2 and DL-1 demonstrated absorbance of 0.147 and 0.096 respectively at wavelength 375.2 nm. Similarly, consortium RAR exhibited absorbance of 0.128 and 0.135 at 375.2 nm and 447.2 nm respectively. However, LK-1 did not show absorbance at any new wavelength.

The spectral scan of decolorized samples of RR120 and abiotic control of the dye was compared to confirm decolorization of RR120 due to biodegradation and not by adsorption. The wide range (200 nm-800 nm) of wavelength used in our study helped to confirm the disappearance of peaks of the colored molecule in the visible range and to detect the presence of new peak, if any, due to the degradation by the pure cultures and the consortium. The disappearance of standard peaks and appearance of new peaks confirms biodecolorization with simultaneous biodegradation of a dye. New peaks are absent in case of dye adsorption [23]. Interestingly, the absorbance at wavelength 447.2 nm for completely decolorized RR120 by consortium RAR was not shown by pure cultures in our study. This proved that the metabolism of dye was carried out differently in consortium RAR as compared to individual cultures. The absence of aromatic amines, detected based on no absorbance at wavelength 270 nm (λmax) was also confirmed in the decolorized samples [24].

Similar to our study, Patil et al. [25] performed spectrophotometric analysis of standard RR120 dye molecule and the decolorized sample by the consortium PMB11 to confirm biodegradation of dye. In their study, maximum absorbance of RR120 was observed at 510 nm, and they confirmed biodegradation of RR120 based on difference in wavelength of the parent molecule and the degraded metabolites. During the biodegradation study carried out by Oturkar et al. [26], there were three absorption peaks for the dye RR120 at 240 nm, 291 nm and 525 nm. They observed a decrease in absorbance at 525 nm, complete disappearance of peak at 291 nm and appearance of new peak at 265 nm after 4h of decolorization by *Bacillus lentus* BI377. The peak at 265 nm was assumed to be a result of aromatic metabolites. However, after 12h, no absorbance at 525 nm and 265 nm led to the confirmation of RR120 degradation by *B. lentus* BI377. A different approach was followed by Su and Lin [24] to monitor the degradation of RR120 by a fungal-bacterial consortium. They reported a decrease in absorbance at 297 nm with simultaneous appearance of a new peak at 286 nm during the decolorization process, hence confirming biotransformation of RR120 into another compound.

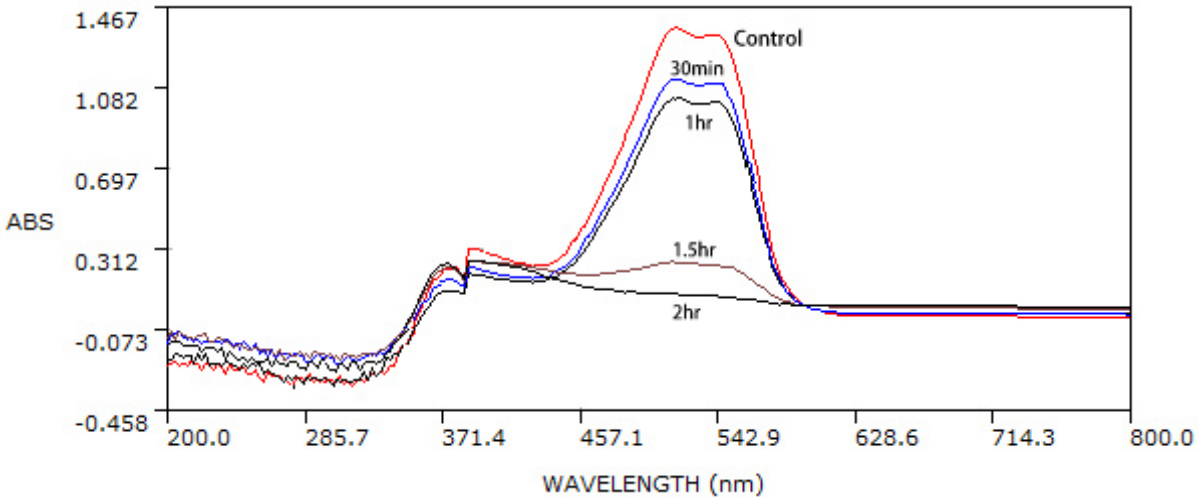

Fig. 1. *UV-VIS spectral scan of RR120 decolorization by consortium RAR*

Table 1. *UV-Visible spectral scan of controls and decolorized RR120 samples of individual isolates and consortium RAR*

Culture	Abiotic control		Biotic control		Partial decolorization of RR120		Complete decolorization of RR120	
	λmax	**Abs**	**λmax**	**Abs**	**λmax**	**Abs**	**λmax**	**Abs**
LK-1	516.8	1.367	No absorbance (Abs) recorded at all wavelengths.		516.8	0.822	-	-
					377.6	0.199	-	-
LK-2	516.8	1.367			516.8	0.976	-	-
					375.2	0.203	375.2	0.147
DL-1	516.8	1.367			516.8	0.931	-	-
					375.2	0.182	375.2	0.096
Consortium RAR	516.8	1.367			516.8	0.732	-	-
					375.2	0.173	375.2	0.128
					-	-	447.2	0.135

HPTLC analysis for the decolorization / degradation of RR120 by individual isolates and consortium RAR

The biodegradation of RR120 was further confirmed by HPTLC analysis. An attempt of separating partially decolorized RR120 samples by each pure culture and consortium was made, however it was not useful in interpretation, hence the respective data is not presented. The developed TLC plates were analysed using three different approaches as discussed below.

When evaluated in white light, the plate showed presence of red color band in tracks 6 and 7 loaded with 500 ppm and 2000 ppm RR120 in 3% YE respectively, which was not visible in tracks 2, 4, 9 and 11 having the decolorized samples of RR120 by LK-1, DL-1, LK-2 and consortium RAR respectively (Fig. 2a). The plate was further derivatized using heat as an agent and observed in white light as well as at 366 nm (Fig. 2b). On exposure to 366 nm, there was one new band developed in track 9 with sample of decolorized RR120 by the pure culture LK-2 when compared with the chromatogram observed under 254 nm prior to derivatization of the plate (Fig. 2c). This band was not seen in any of the other decolorized samples.

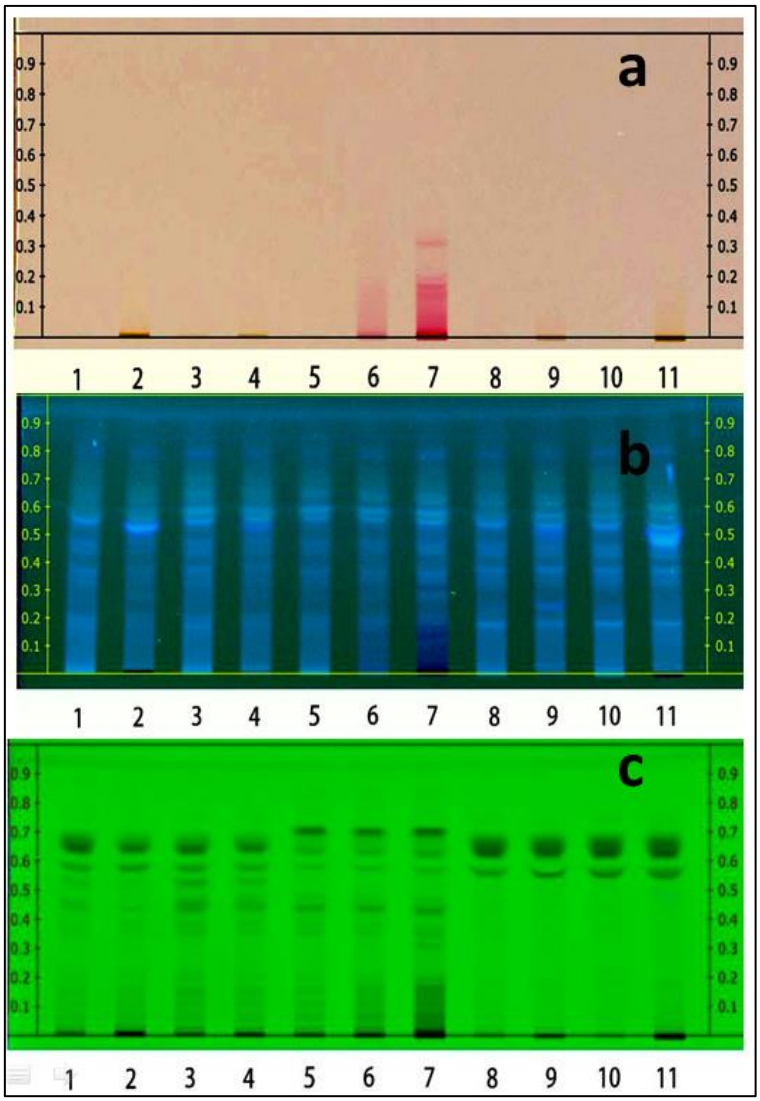

Fig. 2. *HPTLC plate with separated bands of control and degraded RR120 samples (a) under white light (b) at λ=366 nm and (c) at λ=254 nm*

The plate was subjected to a second evaluation which was carried out by observing the chromatogram at 520 nm. The two concentrations of the dye RR120 viz. 500 ppm and 2000 ppm in 3% YE medium showed a total of four peaks. The peaks were more prominent in 2000 ppm as compared to 500 ppm concentration of dye. As observed in Fig. 3, the chromatogram of 2000 ppm RR120 dye demonstrated peak 1 with Rf value 0.16 and 14.62% area; peak 2 with Rf value 0.19 and 13.25% area, peak 3 with Rf value 0.33 and 20.35% area, peak 4 with Rf 0.65 and 51.78% area. With concentration of 500 ppm of dye RR120, the peak with Rf 0.64 and 100% area was numbered 1 which may be same as peak 4 in the 2000 ppm concentration as both showed almost same Rf value. Further, when these peaks were compared with peaks of blank 3% YE medium, the same peak consisting of Rf 0.64 with 100% area was observed, indicating that it may be due to some component present in nutrient medium in all the control samples. Hence, peaks 1, 2 and 3 in the chromatogram of the standard 2000 ppm which were less prominent in the chromatogram of the standard 500 ppm and absent in blank 3% YE medium were confirmed for the presence of the dye RR120 in 3% YE medium. In 500 ppm RR120,

these peaks were not detected for their Rf value probably due to the lower concentration of the dye. All samples of the supernatants of the dye decolorized by pure cultures and the consortium did not show presence of any of the peaks numbered as 1, 2, 3 which were seen in the 3% YE medium with RR120, confirming the metabolism of the colored dye molecule to colorless degraded products. This evaluation confirmed the absence of colored molecule RR120 in the supernatants obtained from the media decolorized by the pure cultures and the consortium.

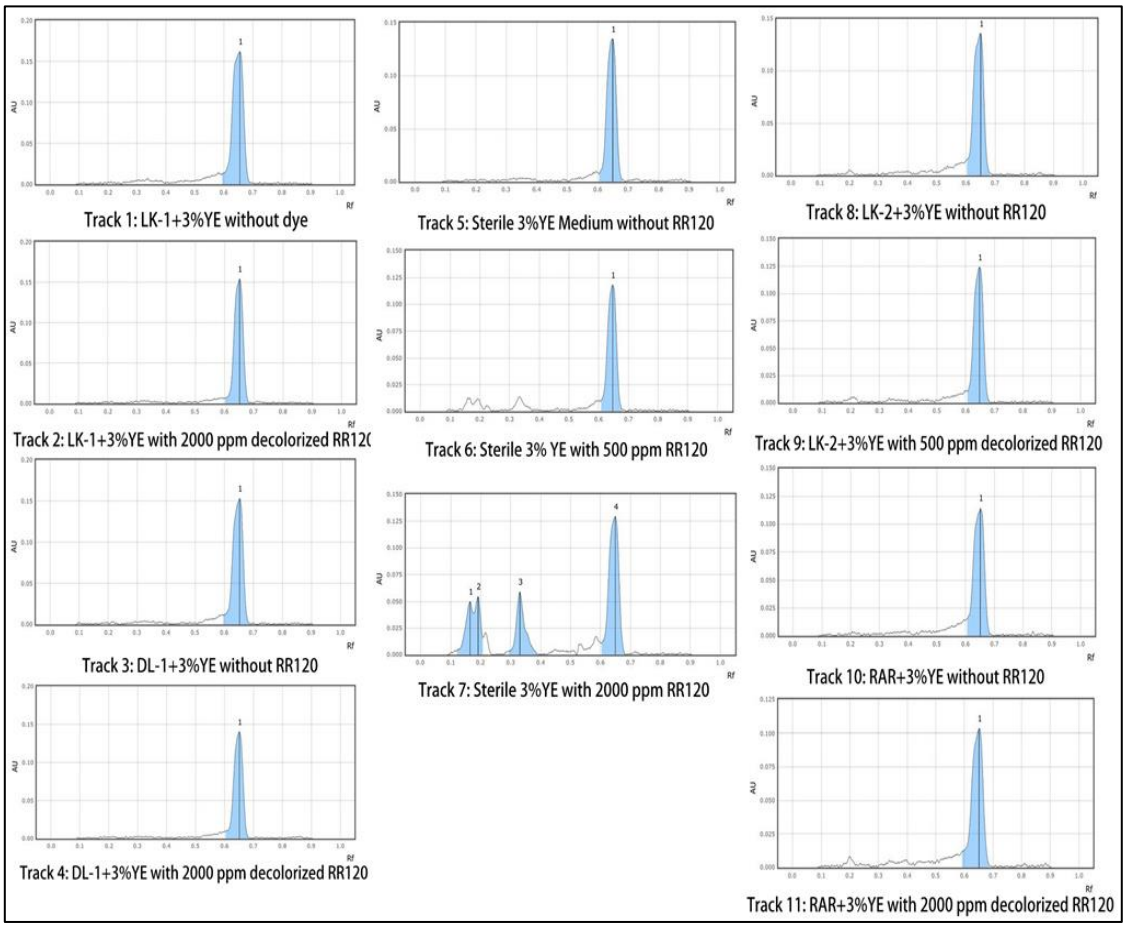

Fig. 3. *HPTLC chromatogram of control and degraded RR120 samples (2000 ppm and 500 ppm) at λ= 520 nm*

However, to detect the presence of any new colorless molecule in the supernatants due to the degradation of RR120, the same plate was further evaluated at 254 nm. To differentiate the bands due to the degradation of RR120 and normal metabolites of the growth, the three pure cultures and the consortium were grown in the 3% YE medium without RR120. The supernatants of each culture and the consortium when separated and visualized at 254 nm in comparison with the supernatant of each culture and the consortium grown in the 3% YE medium with RR120, yielded a comparative analysis of any degradation products. Table 2 indicates that majority of the peaks were same for pure cultures and consortium RAR grown in 3% YE with RR120 (tracks 2, 4, 9, 11) and in 3% YE without RR120 (tracks 1, 3, 8, 10). The peaks having Rf 0.32 and 0.53 in abiotic control of 2000 ppm dye were suggestive of presence of RR120 after comparing with Rf

of the peaks given by medium control. The former peak with Rf 0.32 had completely disappeared in tracks 2, 4, 9 and 11 of all the test samples confirming the degradation of dye RR120 by respective pure culture and the consortium RAR, while the latter showing Rf 0.53 was a common peak in abiotic control of 2000 ppm as well as in the biotic control samples. This indicated that bacteria under study were producing some metabolic intermediate in absence of RR120 having Rf same as one of the peaks given by RR120 present in abiotic control. This peak was also present in all decolorized test samples except the decolorized sample (track 11) by consortium RAR which confirmed complete degradation of RR120 by RAR. Another important observation supportive to this interpretation was the lesser number of peaks (3 in number) in decolorized RR120 sample by RAR in comparison with the number of peaks in the other decolorized test samples by the pure culture (Table 2).

All the above observations led to the conclusion that the colored molecule RR120 was degraded by each of the pure cultures and consortium RAR. The disappearance of dye RR120 band under 520 nm and appearance of no new band under 254 nm were seen in the tracks (2, 4, 9 and 11) in which supernatants of RR120 degraded medium were spotted in comparison with the bands seen in tracks in which supernatants of 3% YE grown cultures were spotted (Fig. 2c). It can be hypothesized that as the degradation of RR120 proceeded, the intermediates were assimilated by the cells of pure cultures when grown individually and in the consortium for their metabolism.

Various other researchers have used HPTLC as the only analytical method to confirm the azo dye degradation [6, 19, 27-30]. Mohana et al. [20] have confirmed the degradation of RR-BS 111 by *P. aeruginosa* using the samples such as 500 ppm dye in NB, NB without dye and decolorized broth. They confirmed the degradation of the major dye components with the development of one peak with Rf value of 0.35 and area of 5.56%. Modi et al. [19] have also confirmed the degradation of RR195 by the consortium by analysing the chromatogram of dye and its degradation products at 550 nm. The four peaks obtained in the standard dye track had disappeared completely in the track loaded with decolorized dye sample. The biodegradation of the simulated synthetic effluent by the yeast-bacterium consortium was similarly confirmed using HPTLC analysis [8]. Moosvi et al. [29] separated and analyzed various samples to confirm the degradation of RV5 using the consortium RVM 11.1 same as those performed in current study. They too compared the Rf values of various bands in the decolorized samples with Rf values of bands produced due to the separation of abiotic and biotic control (bacteria grown in a medium lacking dye) to confirm the degradation of RV5. The confirmation of degradation of DR81 by the consortium NBNJ6 was carried out by separating samples like decolorized medium, un-inoculated medium and abiotic control using HPTLC. It was found that under visible light, there was one band with Rf 0.48 which decreased in intensity in the track of decolorized medium and the degradation of dye was confirmed by visualizing plate in UV light [27]. Few more studies also attempted biodegradation of RR120, however, none of these studies have confirmed the biodegradation by employing the technique of HPTLC [24-26, 31-34].

Table 2. *HPTLC peaks for the decolorization / degradation of RR120 by Consortium RAR at 254 nm*

Track no.	**5**		**7**		**10**		**11**	
Sample	**Medium Blank**		**Abiotic control**		**Biotic control**		**Test**	
Sr. no.	**3% YE**		**Standard–2000 ppm**		**Consortium RAR**		**Consortium RAR + 2000 ppm**	
	Rf	**Area %**	**Rf**	**Area %**	**Rf**	**Area %**	**Rf**	**Area %**
1	0.12	3.48	0.13	3.15	-	-	-	-
2	0.17	3.98	0.17	6.10	-	-	-	-
3	-	-	-	-	0.19	1.33	0.19	6.00
4	0.21	2.16	0.21	1.84	-	-	-	-
5	-	-	**0.32**	**1.86**	-	-	-	-
6	0.36	4.34	0.36	4.82	-	-	-	-
7	0.39	5.38	0.38	4.85	-	-	-	-
8	0.45	16.32	0.44	16.28	-	-	-	-
9	0.49	4.55	0.48	4.17	-	-	-	-
10	-	-	**0.53**	**0.58**	-	-	-	-
11	0.59	7.80	0.58	7.35	0.57	27.36	0.57	42.52
12	0.65	14.16	0.64	13.26	0.65	69.14	0.66	51.48
13	0.71	36.08	0.71	33.90	-	-	-	-
14	-	-	-	-	0.76	2.17	-	-
15	0.83	1.75	0.83	1.83	-	-	-	-

Separation and analysis of organic solvent extracted degradation products of RR120

Further confirmation of biodegradation of RR120 was carried out using dried organic solvent extracted degraded metabolites. Though two solvents such as ethyl acetate and DCM were used during extraction, the consistent, reproducible results were obtained only with DCM extract of degradation products. Hence DCM extract of RR120 degradation products was analyzed further by HPLC, FTIR and GCMS.

HPLC analysis for the degradation of RR120 by consortium RAR

The HPLC chromatogram of each separated sample was analyzed at four electromagnetic wavelengths viz. 254 nm, 350 nm, 400 nm and 512 nm. The respective peaks and retention time are indicated in Table 3. The reference dye molecule RR120 showed single peak with retention time 2.28 min at 512 nm and 2.27 min at 254 nm. No peaks were detected at either 350 nm or 400 nm for reference dye molecule. To eliminate any peak in the degraded samples of RR120 due to metabolism of nutrients from YE by the consortium RAR, the samples B_1 and B_2 were also separated by HPLC. The B_1 sample showed the presence of one peak with retention time 2.26 min at 350nm and 5 peaks with retention time of 2.27 min, 2.58 min, 2.73 min, 3.29 min, and 5.03 min at 254 nm. However, there were no peaks at the other two wavelengths i.e. 400 nm and 512 nm.

Table 3. *Retention time (RT) values of separated components from RR-120 treated under static and sequential static shaker condition*

Sample	**Abiotic control**	**B_1 Biotic**	**A_1 Test**	**B_2 Biotic**	**A_2 Test**
		Static	**Static**	**Static-Shaker**	**Static-Shaker**
λ (nm)	**RT values (min)**				
254	2.27	2.27	**2.24**	-	-
	-	2.58	2.58	-	-
	-	2.73	2.73	2.71	2.71
	-	3.29	3.29	3.23	3.23
	-	5.03	**5.05**	5.01	-
	-	-	-	-	**8.96**
	-	-	-	-	**9.51**
	-	-	**9.99**	-	-
350	-	2.26	2.24	-	-
400	-	-	2.24	-	-
512	2.28	-	2.24	-	-

RT: *Retention time in min*

The methanol solubilized dried extract sample A_1 yielded fractions with retention time as 2.24 min, 2.58 min, 2.73 min, 3.29 min, 5.05 min and 9.99 min at 254 nm; and one peak with retention time of 2.24 min each at 350 nm, 400 nm and 512 nm. To confirm the peaks due to RR120 degradation under static condition, the peaks having common retention time obtained from two samples i.e. B_1 and A_1 were eliminated. Accordingly, the peaks having retention times 2.58 min, 2.73 min, 3.29 min, as seen in Table 3, were same for samples A_1 and B_1. It can be interpreted that the above three peaks may be due to the metabolic intermediates produced by the consortium RAR in absence of RR120. Hence, the only three peaks with retention time 2.24 min, 5.05 min and 9.99 min in sample A_1 could be due to the degradation intermediates / products of RR120 by consortium RAR under static condition. Similarly, sample A_2 showed a total of 4 peaks at 254 nm and none at 350 nm, 400 nm and 512 nm. The retention time of these peaks were 2.71 min, 3.23 min, 8.96 min and 9.51 min. On a similar elimination basis, as discussed earlier, the two peaks with retention time 2.71 min and 3.23 min of sample A_2 may be due to the intermediates of consortium RAR when grown in absence of RR120 (Table 3). Hence, the peaks having retention times 8.96 min and 9.51 min may be the only two components that could be considered as the RR120 degradation products under sequential static–shaker condition.

The sensitive technique of chromatography such as HPLC was used for separation of various chemical components from the mixture of degradation products of RR120 and the differentiation of separated fractions on the basis of retention time was used for confirmation of biodegradation of RR120. In this technique, the methanol solubilized intermediates/products of RR120 biodegradation produced by the consortium RAR under static and sequential static-shaker condition, extracted using DCM, were separated and assessed. There may be three probable products of RR120 degradation under ST and two probable products under ST-SH condition by the consortium RAR. This could be proved by the number of peaks based on their retention time. Additionally, the degradation

products of each treatment by RAR may be different chemically as evident from their retention time values. In conclusion, the degradation and not mere decolorization of RR120 by the consortium RAR can be confirmed based on above observations in our study.

Su and Lin, [24] attempted HPLC for studying the biodegradation of RR120 by fungal-bacterial consortium. They observed that multiple peaks with different retention times for RR120, but the degradation product with single peak of retention time 3.8min which was different from the retention times of the peaks of RR120. This new peak was identified by them as 3-methane sulfinylbut-3-en-2-one, on comparing with standard chromatogram. They also studied the elution profile of RR120 and its degradation products at 270nm- a wavelength specific for the detection of amines. However, they did not find a single peak in the degraded sample at 270 nm, thus confirming the degradation of RR120 to harmless products without presence of amines which are common intermediates of azo dye degradation. Similarly, Patil et al. [25] also performed HPLC of RR120 and its degradation products obtained due to the action of consortium PMB11. The control dye demonstrated a peak at RT 1.72 min while degradation products showed 5 peaks with different RTs. In another study on biodegradation of RR120 by consortium SDS, HPLC profile of the dye showed almost same retention time of 1.74 min for RR120 dye as the observation by Patil et al. [25]. However, the study conducted with the consortium SDS demonstrated only three peaks with retention time as 1.77 min, 2.43 min and 2.73 min for the degraded sample of RR120 [31]. One of the peaks having retention time of 2.73 min in degraded sample of RR120 in the above study matched with one peak having the same retention time in degraded RR120 under static condition by the consortium RAR of this study.

FTIR spectral analysis of static and sequential static-shaker treatment used for the decolorization / degradation of RR120 by consortium RAR

FTIR spectra of RR120 and dried DCM extracted decolorized samples from two treatments such as ST and ST-SH, using the consortium RAR, were determined using attenuated total reflection (ATR) sampling technique in conjunction with infrared spectroscopy. There were significant differences in spectra of RR120 and the two decolorized samples obtained from the treatment at ST and ST-SH condition by the consortium RAR (Fig. 4a and 4b).

The IR spectrum of the standard RR120 molecule showed intensity for O–H stretching in the range of wavenumber 3600-3200 cm^{-1}, more specifically at 3405.27 cm^{-1}. The intensity of C–H stretching was observed at wavenumbers 2924.11 cm^{-1} and 2854.14 cm^{-1}. The –N=N– stretching of azo linkage yielded the peak at 1590.55 cm^{-1}. The N–H deformation and C–N stretching had contributed a strong peak at ~1540 cm^{-1}. The peaks at 1482.04 cm^{-1} and 1426.36 cm^{-1} were contributed by the heterocyclic aromatic rings. C=N bond in pyrimidine rings was identified by the peak at 1406.37 cm^{-1}. The prominent peaks were seen at 1322.13 cm^{-1} and 1040.86 cm^{-1} suggestive of asymmetric and symmetric frequencies, respectively, of S–O stretching. The C–Cl stretch present in RR120 may be attributed by peaks at 910.93 cm^{-1} and 795.18 cm^{-1}.

In the decolorized sample from ST condition i.e. A_1; there was slight decrease in O–H stretching in the wavenumber range from 3600 cm^{-1}–3200 cm^{-1}. Similarly, the C–H stretching was also seen intact at the respective wavenumbers. The absence of a peak at 1590.55 cm^{-1} had confirmed that the azo linkages in the dye molecule were completely degraded. There were no peaks found at 1482.04 cm^{-1} and 1426.36 cm^{-1} in the decolorized

sample which was suggestive of complete degradation of heterocyclic aromatic rings. The presence of a peak at 1406.37 cm^{-1} suggested that C=N of pyridine rings was intact. The peaks at 1322.13 cm^{-1} and 1040.86 cm^{-1} had completely disappeared, which confirmed total loss of–SO_3^- group during the ST treatment of RR120 by consortium RAR. The C–Cl bond was also broken down with the loss of chlorine, indicated by absence of peak at 910.93 cm^{-1} and 795.18 cm^{-1}.

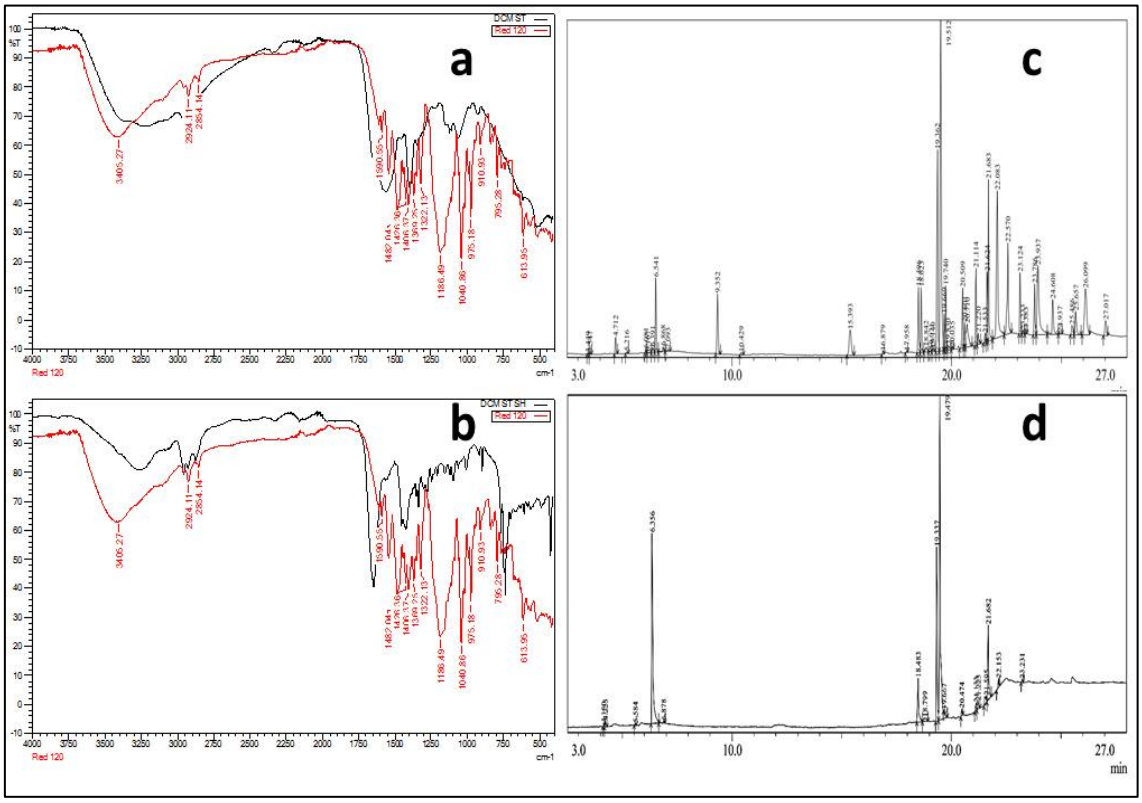

Fig. 4. *Analytical characterization of RR120 metabolites degraded by consortium RAR (a) FT-IR spectra under static condition (A_1 Sample) in comparison with standard RR120 dye (b) FT-IR spectra under sequential static and shaker (A_2 Sample) (c) GC-MS chromatogram under static condition (d)* GC-MS chromatogram under sequential static-shaker condition

The sample of decolorized RR120 under ST-SH condition (A_2) demonstrated similar IR spectra as that of ST decolorized sample with some exceptional observations at certain wavenumbers. The intensity of frequency of O–H stretching in the range of 3600 cm^{-1}–3200 cm^{-1} had decreased further as compared with ST treatment. The stretching of C–H at 2924.11 cm^{-1} and 2854.14 cm^{-1} showed the peaks same as those in the IR spectra of RR120. The peak at wavenumber 1590.55 cm^{-1} of azo bond was absent in this sample as well, however, there was a sharp peak between ~1600 cm^{-1}–1650 cm^{-1} which was not found in ST decolorized RR120. This peak may be due to presence of –C=O stretching because of secondary amides or –C=C stretching in the degradation products of RR120. The presence of C=N bond of pyridine was seen in this sample as well, but there was a slight shift of the peak of C=N from the wavenumber 1406.37 cm^{-1} as seen in IR spectrum of RR120.

Hence, from the comparison of all the above observations of IR spectra of RR120 and decolorized samples it was concluded that the consortium RAR was carrying out

degradation of the azo dye molecule proven by the evidences of cleavage of –N=N–bond, degradation of aromatic rings, loss of $–SO_3Na$ groups and breakage of C–Cl bond with dehalogenation in the decolorized sample. The best suited treatment for RR120 degradation by the consortium RAR was ST-SH process.

A similar type of degradation analysis of RR120 by FTIR technique was carried out by Su and Lin, [24]. They confirmed similar wavenumbers designating similar chemical characteristics of RR120. They could confirm–N =N–stretching at 1590 cm^{-1}, C–N stretching and N–H deformation at 1543.02 cm^{-1}, S–O stretching at 1323.33 cm^{-1} and C–Cl stretching at 1044.72 cm^{-1} in spectra of RR120. They also confirmed degradation of RR120 by three peaks at 1637.92 cm^{-1}, 1617 cm^{-1} and 618.94 cm^{-1} indicating the presence of –C=O stretching, –C=C– stretching and C–S stretching respectively. According to them, the appearance of these peaks indicated the absence of aromatic amines in the degraded samples. The research team of Phugare et al. [31] also confirmed the degradation of RR120 by the consortium SDS using the technique of FTIR. They too, observed the O–H stretching at 3435 cm^{-1}, –C–H stretching at 2926 cm^{-1}, –S=O stretching of sulphonic acid at 1047 cm^{-1} in IR spectrum of RR120 confirming the chemical features of the dye. The IR spectrum of degraded metabolites of their sample showed presence of –C–H stretching at 2866 cm^{-1}, stretching of secondary amides at 1635 cm^{-1}, somewhat similar to the IR spectra of the degraded samples of RR120 in the present study. Other studies of RR120 biodegradation by consortium PMB11 conducted by Patil et al. [25] and by *Bacillus lentus* BI377 performed by Oturkar et al. [26] also reported similar observations.

GC-MS analysis of degradation products obtained from ST and ST-SH treatment of RR120 by consortium RAR

The DCM extracted breakdown products of A_1 and A_2 were separated by gas chromatography. An attempt was made to characterize these products by their mass spectra. The probable compounds were shortlisted on the basis of their similarity index of more than 80 and also their chemical relatedness to the parent compound.

Various peaks with different RT were noted in the chromatogram of A_1 and A_2 samples. The number of peaks was more in GC analysis of A_1 sample than A_2 (Fig. 4c and 4d). One of the peaks having retention time of 5.216 min was suggestive of presence of naphthalene with molecular weight 128, in the degraded sample A_1. The peak of naphthalene was not detected in the sample A_2. Some of the probable compounds from mass spectra of separated samples by GC-MS showed presence of functional groups or chemical entities which were confirmed by FTIR analysis. Two such observations were probability of presence of C=N of pyridine rings according to IR analysis may be indicated by the evidence of 5H-1-pyridine (RT 6.38 min) and 5H-cyclopenta(b)-pyridine(RT 6.35 min) in A_1 and A_2 samples respectively and the presence of -C=O as per IR observations as in N-propyl-2-pyrrolidinone (RT 3.45 min in A_1), 3,6-diisopropylpiperazin-2,5-dione (RT 18.85 min in A_1), 3-isobutylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione (RT 19.51 min in A_1/ 19.48 min in A_2), 3-benzyl-6-isopropyl-2,5-piperazinedione(RT 21.21 min in both A_1 and A_2), 3-benzylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione (RT 21.68 min in both A_1 and A_2), 3-allyl-5-(1H-indol-3-ylmethyl)-2-thioxo-4-imidazolidinone(RT 25.48 min in A_1).

RR120 dye possesses 6 sulphonate groups, hence it cannot volatilize and therefore, it was not possible to analyze and study its mass spectra by GC-MS [35]. The number of peaks in the ST-SH degraded sample was comparatively lesser than static degraded

sample. This may be due to the further aerobic degradation of the intermediates of ST treatment under ST-SH treatment. The absence of naphthalene in ST-SH treatment was the evidence of above observation, indicating its further metabolism may be to catechol which could have been degraded via ortho or meta fission pathway and TCA cycle to CO_2 and H_2O [36]. Thus, the biodegradation of RR120 by the consortium RAR was confirmed by GC-MS analysis.

Many of the compounds as degradation intermediates in proposed pathway could not be detected during this analysis. One of the reasons may be the proposed intermediates being non-volatile could not be detected in GC-MS analysis. Additionally the presence of very low molecular weight compounds may be very difficult to determine by this technique or these intermediates may have got transformed into non-detectable CO_2 and H_2O molecules by sequential metabolism. The MS results of degradation of RR120 by RAR were compared with the results of other groups who have worked with the degradation of RR120 by different individual bacteria or consortia. The degradation product 2-aminobenzene sulfonic acid with calculated MW 173.01 has been confirmed by Oturkar et al. [26] but with exact mass of 170.01 while studying biodegradation of RR120 by *B. lentus* BI377. This was also detected by Phugare et al. [31] with MW of component as 173 with RT of 22.78 min. The intermediate naphthalene-1-0l with calculated MW of 144 and retention time of 21.86 min has been confirmed in their study, during the biodegradation of Red HE3B (RR120) using the consortium SDS. The presence of naphthalene with molecular weight 128 was detected with fraction having retention time of 5.217 min in this study and the same was confirmed but with differing retention time of 21.80 min by Patil et al. [25] using the consortium PMB11 for degradation of RR120. Similar to a study carried out by Oturkar et al. [26], there may be presence of probable low molecular weight metabolites in the degraded samples of this work. There were many different products of RR120 degradation detected by GC-MS analysis by others. For instance, Patil et al. [25] have confirmed the presence of 2–amino–5–chlorotriazine (MW 132/RT 21.18 min), 5–sulphonate aniline (MW 174/RT 22.28), 2, 5–diaminobenzene aminotriazene (MW 209/RT26.87) in addition to naphthalene as the degradation products of Red HE3B by PMB11. The metabolites like 2–aminobenzenesulphonic acid (MW 173 / RT 22.78 min), 3–amino–5–[(4–amino–6–chloro–1, 3, 5–triazin–2–yl) amino]–4–hydroxynaphthalene–2–sulfonic acid methanethiol (MW 463 / RT 22.94 min), 3–amino–4 hydroxynathalene–2, 7–disulfonic acid (MW 319 / RT 24.45 min), 6–chloro–1, 3, 5–triazine–2, 4–diamine (MW 146 / RT 28.07 min) and naphthalene–1–ol (MW 144 / RT 21.86 min) were confirmed as degradation products of RR120 by the consortium SDS [31]. Total of 9 different degradation products of RR120 were detected by Oturkar et al. [26] with the use of *B. lentus* BI377.

Description of proposed pathway for RR120 degradation by consortium RAR

From the results of all analytical techniques and enzymes profiling of pure cultures [14-16], the biodegradation of RR120 by the consortium RAR was confirmed and a pathway was proposed (Fig. 5). The proposed intermediates are indicated in Table 4.

The sodium salt of RR120 (A) while at the periplasmic side of the membrane may have been an ionized molecule in anionic form designated as compound B with the loss of Na^+. The presence of azoreductase was confirmed in all three isolates in consortium RAR [14-16]. With this evidence, in the foremost step of the pathway, the primary symmetrical attack by azoreductase was assumed to cleave two azolinkages of RR120 by

reduction mechanism [37, 38] to yield 2 molecules of compound D and a molecule of compound C. The former molecules may undergo desulfonation by a specific enzyme which catalyzes hydrolytic loss of H_2SO_4 yielding aniline as an intermediate (R). There may be presence of specific deaminase which catalyzes hydrolytic deamination to produce phenol with the loss of ammonia. Phenol can be metabolized due to hydroxylation mechanism [39] to catechol (J). The 2nd reduced product (C) may have undergone cleavage by peroxidase enzymes (LiP confirmed in all isolates and MnP in LK-1) to yield compound E.

On successive desulfonation and reductive deamination, it may be converted to naphthalene-1-ol (G). Naphthalene-1-ol by an unidentified mechanism can be transformed into naphthalene (H) which via salicylic acid (I) can be metabolized into catechol (J). In this study, presence of naphthalene was confirmed by GC-MS. The other degradation product of peroxidase action may be dehalogenated to produce the intermediate rich in substituted 1, 3, 5-triazine rings (M) which undergoes deamination and cleavage to yield 2 molecules of compound O and a molecule of benzene (Q) which can then be metabolized to catechol (J). The O intermediate can be further degraded by gammaproteobacteria such as genera *Shewanella* and *Aeromonas*. All the catechol molecules can be further oxidized to CO_2 by ortho or meta fission in association with TCA cycle and respiratory ETC.

After degradation of RR120 by the consortium, the pH of the medium was 8.0 but not too alkaline as the literature confirms [40]. One assumption can be the alkalinity produced due to the amines or ammonia might have been neutralized to some extent by the acid produced due to desulfonation mechanism as evident in the proposed pathway.

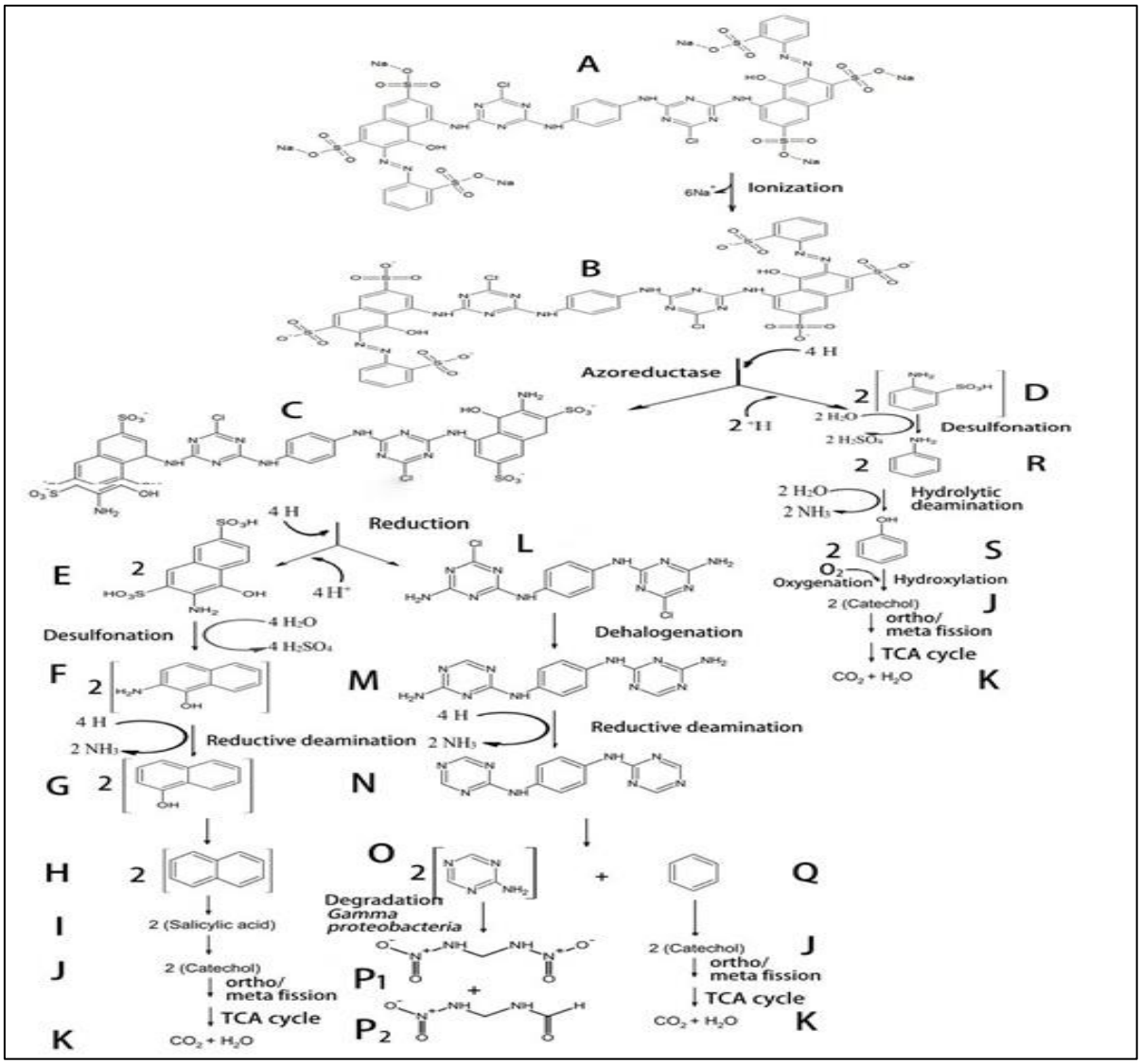

Fig. 5. *Proposed degradation pathway for breakdown of RR120 dye by consortium RAR*

The important structural features of RR120 supportive for its recalcitrant nature:

i. Molecular weight of the dye: lower MW dyes are easily degraded as compared to high molecular weight dyes [41]. The molecular weight of sodium salt of RR120 used in this study was of 1469.98 making it difficult to be degraded.
ii. Position of sulfo groups: These are strong electron withdrawing groups; thus, influencing the rate of decolorization / degradation. When sulfo groups are in para position to the azo bond, the decolorization is faster. But, when these groups are in ortho position to the azo bond, there is decrease in the rate of decolorization of dye [42]; like in case of RR120. There are 4 sulfo groups in ortho position to azo groups in RR120 structure.
iii. Carboxyl group or hydroxyl group in ortho position to azo bond, inhibit decolorization process but a weaker effect as compared to ortho position sulfo groups. There are two hydroxyl groups in ortho position to azo bond in case of RR120.
iv. No. of azo bonds: The more the number of azo bonds, it is difficult to degrade the molecule. In case of RR120 there are two azo bonds.

2. ***Table 4.*** *Proposed intermediates of RR120 degradation by RAR*

Code	Molecule name, Chemical formula, Molecular Weight	Reference
A	Reactive Red 120 Chemical Formula: $C_{44}H_{24}Cl_2N_{14}Na_6O_{20}S_6$ Molecular weight: 1469.98	Parent molecule
B	5-((4-chloro-6-((4-chloro-6-((8-hydroxy-3,6-disulfonato-7-((E)-(2-sulfonatophenyl)diazenyl)naphthalen-1-yl)amino)-1,3,5-triazin-2-yl)amino)-4-hydroxy-3-((E)-2-sulfonatophenyl)diazenyl)naphthalene-2,7-disulfonate Chemical Formula: $C_{44}H_{24}Cl_2N_{14}O_{20}S_6^{6-}$ Molecular weight: 1332.02	[26, 31]
C	5,5'–(((1, 4-phenylenebis(azanediyl))bis(6-chloro-1,3,5-triazine-4,2-diyl))bis(azanediyl))bis(3-amino-4-hydroxynaphthalene-2,7-disulfonate) Chemical Formula: $C_{32}H_{20}Cl_2N_{12}O_{14}S_4^{4-}$ Molecular weight: 995.72	
D	2-aminobenzenesulfonic acid Chemical formula: $C_6H_7NO_3S$ Molecular weight: 173.19	
E	3-amino-4-hydroxynaphthalene-2,7-disulfonic acid Chemical formula: $C_{10}H_9NO_7S_2$ Molecular weight: 319.30	[31]
F	2-amino-1-naphthol Chemical formula: $C_{10}H_8O$ Molecular weight: 144.17	
G	Naphthalen-1-ol Chemical formula: $C_{10}H_8O$ Molecular weight: 144.17	
H	Naphthalene Chemical formula: $C_{10}H_8$ Molecular weight: 128.17	[25, 36]
I	Salicylic acid Chemical formula: $C_7H_6O_3$ Molecular weight: 138.12	[36, 43]
J	Catechol Chemical formula: $C_6H_6O_2$ Molecular weight: 110.11	[26, 36]
K	By-products of Ring fission and TCA cycle: Carbon dioxide and Water	
L	N^2, $N^{2'}$-(1,4-phenylene)bis(6-chloro-1,3,5-triazine-2,4-diamine) Chemical formula: $C_{12}H_{10}Cl_2N_{10}$ Molecular weight: 365.18	Proposed in this work
M	N^2, $N^{2'}$-(1,4-phenylene)bis(1,3,5-triazine-2,4-diamine) Chemical formula: $C_{12}H_{12}N_{10}$ Molecular weight: 296.30	
N	N^2,$N^{2'}$-di(1,3,5-triazin-2-yl)benzene-1,4-diamine Chemical formula: $C_{12}H_{10}N_8$ Molecular weight: 266.27	

Table 4. *Continues*

Code	Molecule name, Chemical formula, Molecular Weight	Reference
O	1,3,5-triazin-2-amine Chemical formula: $C_3H_4N_4$ Molecular weight: 96.09	[44]
P_1	Methylenedinitramine Chemical formula: $CH_4N_4O_4$ Molecular weight: 136.07	[45, 46]
P_2	4-nitro-2,4-diazabutanal Chemical formula: $C_2H_5N_3O_3$ Molecular weight: 119.08	
Q	Benzene Chemical formula: C_6H_6 Molecular weight: 78.11	[31]
R	Aniline Chemical formula: C_6H_7N Molecular weight: 93.13	[26, 47]
S	Phenol Chemical formula: C_6H_6O Molecular weight: 94.11	Proposed in this work

CONCLUSION

The islet cell culture should be uniformed for optimized islet isolation and transplantation. Because of the range of islet sizes is similar in rats [33] and humans [34] (50-500 µm and 50-250 µm respectively) and beta cells make up rates are 70-80% and 50-70% of cells in islets in rat [34] and humans respectively; we thought that our findings can use for improving human islet cell culture. For this reason, studies for media optimization of islet culture will take a significant role in the performance of oncoming studies of human islet cell transplantation. In transplantation applications, extending the duration of islet cell culture after isolation may allow time to increase the viability and functionality of islets. In addition, long-term culture also provide time to prolong islet viability when selecting suitable islet donors matched to the recipient. However, in future studies, the results should be evaluated in terms of insulin major gene transcription factors.

Conflict of Interest. The authors declared that there is no conflict of interest.

Authorship Contributions. Concept: B.R., A.K., Design: B.R., A.K., Data Collection or Processing: B.R., A.K., Analysis or Interpretation: B.R., A.K., Literature Search: B.R., A.K., Writing: B.R., A.K.

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