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Elucidation of biodegradation mechanism of Reactive Red 35 by *Pseudomonas aeruginosa* ARSKS20

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Abstract: *The current work is aimed to evaluate the degradation of Reactive Red 35 (RR35) by Pseudomonas aeruginosa ARSKS20 that decolorized 100 mg/L dye at 8.27 mg/L/h rate under optimum conditions. Induction of laccase, lignin peroxidase, tyrosinase, Veratryl alcohol oxidase, NADH-DCIP reductase, azoreductase enzymes during dye decolorization suggested their active participation in dye degradation. Metabolites obtained after decolorization were analyzed with HPTLC and FTIR studies confirmed the biodegradation of dye. Metabolites produced after biodegradation of RR35 were identified by GC-MS analysis. Predicted pathway of biodegradation of RR35 by bacteria elucidated for first time. The results showed efficient biodegradation of RR35 to low molecular weight compounds, namely, 1-Amino-2-hydroxy-5-(2-sulfooxy ethyl) benzene , 1-amino-2-methoxy benzene and naphthalene.*

Keywords: *Biodegradation, Pseudomonas aeruginosa ARSKS20, Reactive Red 35, FTIR, GCMS*

I. Introduction

Azo dyes are the major class of synthetic dyes and pigments, constituting 60–70% of all organic colorants [1]. They are widely used as substrates in textile, leather, plastics, papers, hair, mineral oils, waxes, food, and cosmetics industries [2]. In textile wastewater, reactive azo dyes concentration was reported in ranges from 5-1500 mg/L [3]. Effluents of these industries are complex and xenobiotic in nature and sometimes carcinogenic, tumorigenic or mutagenic to various lives. They present serious environmental problems in the receiving water bodies [4]. It was estimated that about 1,000-3,000 m^3 of water discharged after processing of 12-20 tones of textiles per day [5]. Considering this effluent load, physicochemical methods viz, membrane filtration, reverse osmosis, ozonation, electrochemical destruction, photocatalysis, ion exchange, coagulation, use of activated carbon, Fenton's reagent, and chemical flocculation adopted widely by industries. These methods are expensive and beget high amount of chemical-containing secondary sludge [6, 7].

Microbial treatment is an ecofriendly and cost competitive strategy for elimination of xenobiotic and or anthropogenic compounds from the environment [8]. The bacterial biodegradation due to the versatile metabolism and adaptive nature has widely exploited. Involvement of various oxidoreductase enzymes, viz, lignin peroxidase, laccase, tyrosinase, azoreductase, DCIP reductase are well documented during bacterial degradation of textile azo dyes [9, 10]. Different *Pseudomonas* sp. were reported to degrade and decolorize a wide range of azo dyes [10-12]. *Pseudomonas aeruginosa* BCH was reported to degrade Amaranth dye [3]; Remazol Orange 3R [13], into low molecular weight compounds.

Only few studies attempting biodecolorization of Reactive Red 35 are available. Reactive Red 35 (RR35), a vinyl sulfone based monoazo dye are proved hazardous to human as it is responsible for causing skin, eyes and respiratory irritation. RR35 was reported to decolorize 90.7% by a fungal species *Trametes versicolor* at 680 mg/L concentration [14]. But biodegradation of RR35 by bacteria with a detail investigation of intermediates and pathway prediction has not been reported. Thus, the aim of this work was exploring the potential of isolated *Pseudomonas aeruginosa* ARSKS20 for degradation of RR35. The study included the identification of intermediate of RR35 degradation and elucidation of degradation pathway. Involvement of oxidoreductase enzymes responsible for dye degradation was also studied. The ability of an isolate to decolorize several other azo dyes was also attempted.

II. Materials And Methods

2.1 Dyes and chemicals

Textile dyes were procured from Meghmani Dyes and Pigment, Ganesh Dyes Chem and Shriji Dyes, Vatva, Ahmedabad, Gujarat (India). o-tolidine, DCIP, NADH, catechol, ascorbic acid, veratryl alcohol, n-propanol, tartaric acid, and other chemicals are of analytical grade and purchased from Himedia, Merck and SRL.

2.2 Isolation and identification of dye-degrading bacteria

Bushnell Hass Broth (BHB; g/L: magnesium sulfate 0.20, calcium chloride 0.20, monopotassium phosphate 1.00, dipotassium phosphate 1.00, ammonium nitrate 1.00, ferric chloride 0.05, pH 7.0 ± 0.2) containing 2.5 g/L yeast extract (BHBY) and 100 mg/L RR35 was inoculated with 1% (w/v) soil contaminated with effluent discharge of dye-manufacturing industries. The system was incubated under static condition for 48 h at 35°C. Repetitive transfers were carried out in the fresh medium to assess consistent dye decolorization and growth of organisms. Serially diluted decolorized samples were spread on BHBY agar medium to obtain morphologically distinct colonies. Isolated bacterial colonies were further examined for their RR35 decolorizing ability at 100 mg/L concentration, and the most efficient isolate was selected accordingly. The efficient isolate was designated as ARSKS20, identified by 16s rRNA sequence using primer sets 27F (5´-AGAGTTTGATCCTGGCTCAG-3´) and 1492R (5´-GGTTACCTTGTTACGACTT-3´). Phylogenetic tree was constructed using the Neighbor joining method [15].

2.3 Culture maintenance

ARSKS20 was routinely maintained on BHBY medium containing 100 mg/L RR35 and stored at 4°C. Decolorization experiment was set up from acclimatized culture (grown for 24h) with 6 x 10^8 cells/mL culture density (1.0 O.D. at 600 nm) as inoculum.

2.4 Decolorization experiment

Decolorization was performed with 5% (v/v) active culture in 100 mL of BHBY medium amended with 100 mg/L RR35 at pH 8, 40°C under static condition. After decolorization, 2 mL sample was withdrawn and centrifuged at 8000×g for 15 min. Absorbance was measured by UV-visible spectrophotometer (Shimadzu 1800, Japan) at 512 nm. All experiments were conducted in triplicates along with abiotic control. Percentage decolorization and average decolorization rate were calculated as previously quoted method [1]. Decolorization of various dyes, such as Reactive Red 198, Reactive Red 106, Reactive Red 120, Reactive Red 111, Reactive Black 5, Reactive Violet 12, Reactive Blue 160, Reactive Blue 221, Reactive Blue 222, by *P. aeruginosa* ARSKS20 was measured at their respective $\lambda_{max.}$

2.5 Preparation of cell-free extract

The cells were harvested from the decolorized broth and control flask (without dye) by centrifugation at 12000×g at 4ºC for 20 min. Cell pellets were suspended in 50 mM potassium phosphate buffer (pH 7.4), homogenized and sonicated at 60 amplitude, seven strokes of 30s each with 2 min interval (Sonics-Vibracell VC750 Ultrasonic Processor) was given at 4ºC. This lysate was centrifuged at 12000×g for 20 min at 4ºC. The resulting supernatant was used as a crude enzyme source.

2.6 Enzyme assays

Laccase activity was determined in 2 mL reaction mixture containing 5 mM o-tolidine in acetate buffer (20 mM, pH 4.2). The reaction was initiated with 0.2 mL enzyme. The formation of oxidized product was measured at 366 nm [16]. Lignin peroxidase activity was determined by monitoring the formation of propanaldehyde at 300 nm in 2.5 mL reaction mixture containing 100 mM n-propanol, 250 mM tartaric acid and 10 mM H_2O_2 [17]. Veratryl alcohol oxidase (VAO) activity was measured by estimating oxidation of veratryl alcohol at 310 nm in reaction mixture containing 1 mM veratryl alcohol in 0.1M citrate phosphate buffer (pH 3.0) and 0.2 mL enzyme [2]. Tyrosinase activity was measured in 3 mL reaction mixture containing 50 mM of catechol and 2.1 mM ascorbic acid in 50 mM potassium phosphate buffer (pH 7.4), and 0.1 mL enzyme solution. Formation of o-benzoquinone, dehydroascorbic acid and decrease in optical density were measured at 265 nm. One unit of tyrosinase activity was equal to a ΔA 265 nm of 0.001 per minute at pH 7.4 at 25ºC [18]. NADH-DCIP reductase activity was measured by earlier reported method [19]. The assay mixture contained 50 µM DCIP, 50 µM NADH in 50 mM potassium phosphate buffer (pH 7.4) and 0.1 mL enzyme solution to make 5.0 mL total volume. From this, 2 mL reaction mixture was assayed at 590 nm using extinction coefficient of 19 mM/cm. Azoreductase activity was assayed in 2 mL reaction mixture containing 4.45 µM of Methyl red, 50 µM NADH in potassium phosphate buffer (20 mM, pH 7.5). Reduction of Methyl red was measured at 430 nm and calculated using a molar extinction coefficient of 0.023 µM/cm [20]. One unit of enzyme activity was defined as the amount of enzyme required to reduce 1 µM of substrate per min per gram of protein. All enzyme assays were done in triplicates at room temperature (25 ± 2ºC) along with their respective reference blank containing all components of the assay except the enzyme. The protein content was determined by Lowry method with bovine serum albumin as the standard [21].

2.7 Biodegradation analysis

The extraction of metabolites produced after degradation was carried out by centrifugation of decolorized broth at 12000×g for 20 min at 4°C. The metabolites produced were extracted with an equal volume of ethyl acetate, dried over anhydrous sodium sulfate and evaporated to dryness in a vacuum evaporator. The crystals obtained were dissolved in minimal volumes of HPLC grade methanol for High Performance Thin Layer Chromatography (HPTLC) and Gas Chromatography–Mass Spectroscopy (GCMS) analysis. Dried samples were used for Fourier Transform Infrared (FTIR) analysis.

To study the biodegradation profile of RR35 by HPTLC, 3 µL of sample was applied on a silica gel plate (HPTLC Silica gel 60 F_{254}, Merck, Germany) using micro syringe sample applicator and by spraying with nitrogen gas (Linomat V, CAMAG, Switzerland). Isopropyl alcohol:Acetone:Ammonia (4:3:3 v/v) mixture was used as the solvent system to resolve metabolites on TLC plate. The system was developed in pre-equilibrated twin trough chamber and scanned by TLC scanner (CAMAG TLC scanner 4, Switzerland) at 254 nm with slit dimension of 4×0.30 mm. The chromatograms were analyzed using HPTLC Win-CATS 1.4.7 software [10].

FTIR analyses (Perkin Elmer, Spectrum GX) of RR35 and its biodegradation products were carried out in the mid infrared region 400-4000/cm at 16-scan speed.

GCMS analysis of metabolites was carried out by the method described earlier [4] using QP 5000 Turbo mass spectrophotometer (Autosyztem XL GC^+, Perkin Elmer, USA). The ionization voltage was 70 eV. Gas chromatography was conducted in temperature programming mode with a Resteck column (0.25 mm × 30 mm; XTI-5). The initial column temperature was kept 70°C for 4min, and then it was increased linearly at 10°C/min to 270°C and kept for 4min. The temperature of injection port was 275°C and GCMS interface was maintained at 250°C. Helium was used as a carrier gas with a flow rate of 1 mL/min for 30min run time. The compounds were identified on the basis of mass spectra and using the NIST library.

2.8 Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) using Tukey–Kramer multiple comparison test.

III. Results And Discussion

3.1 Isolation and identification of bacterial isolate

The bacterial isolate that efficiently decolorized RR35 designated as ARSKS20, was identified by 16s rRNA partial sequence (1454bp). The phylogenetic analysis (Fig. 1) showed its close relationship to *P. aeruginosa* NCMG1179. Thus, the isolate ARSKS20 was identified as *Pseudomonas aeruginosa* strain ARSKS20. The 16s rRNA sequence was deposited in GenBank with the accession number JN817386.1.

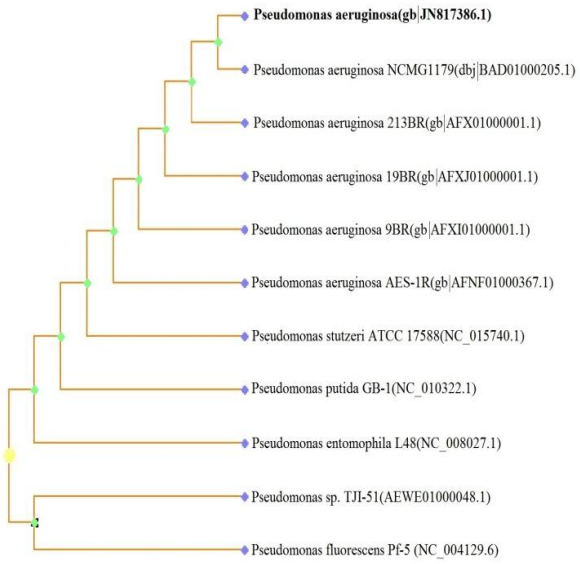


Fig. 1 Phylogenetic tree of *Pseudomonas aeruginosa* ARSKS20

3.2 Decolorization analysis

P. aeruginosa ARSKS20 decolorized 95% RR35 (with 8.27 mg/L/h decolorization rate) at pH 8 and 40°C under static condition. The UV–visible spectral scanning (400–800 nm) during the course of decolorization of RR35 revealed a decrease in the absorbance at various time intervals, indicating decrease in the dye

concentration (Fig. 2). After 12 h, complete disappearance of peak at 512 nm reflected the removal of RR35 from the media.

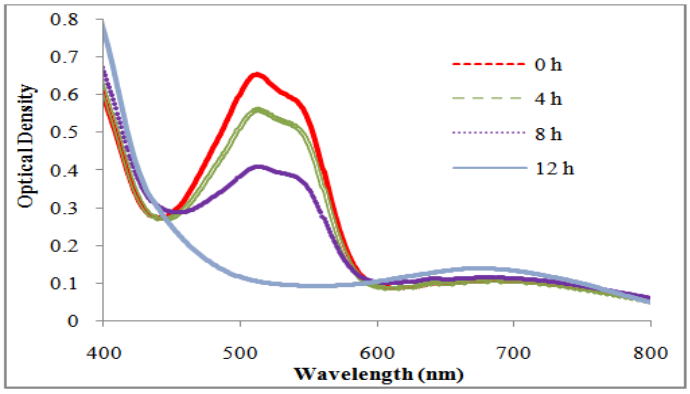


Fig. 2 UV-visible spectra of RR35 during decolorization study

Effluent from textile industries consists of mixture of structurally different dyes, so bacterial strain that adapted ability to decolorize wide spectrum range of dyes are to be selected for decolorization study. *P. aeruginosa* ARSKS20 could also decolorize various other azo dyes from 83 to 97% at 100 mg/L concentration as described in Table 1. Different strains of *P. aeruginosa* were reported earlier to decolorize various azo dyes [11, 22]. The obtained results show the decolorization ability of isolate over a broad range of structurally different azo dyes , which could be exploited for treatment of waste containing mixture of multiple dyes.

Table 1 Decolorization of various textile azo dyes by *P. aeruginosa* ARSKS20

Name of dye [a]	λ_{max} (nm)	Decolorization (%)	Rate of Decolorization (mg/L/h)
Reactive Red 198	515	93.58	5.50
Reactive Red 106	510	94.71	4.74
Reactive Red 120	520	90.54	3.94
Reactive Red 111	520	89.32	4.96
Reactive Black 5	602	84.71	3.68
Reactive Violet 12	550	97.77	6.11
Reactive Blue 160	610	95.05	6.34
Reactive Blue 221	611	93.66	5.85
Reactive Blue 222	613	83.86	3.49

a Dye concentration 100 mg/L

3.3 Enzyme analysis

During the decolorization of RR35 significant induced activity of various oxidoreductase enzymes was observed in *P. aeruginosa* ARSKS20. Induction of these enzymes in bacterial culture grown in presence of dye (test) compared to that without dye (control) suggests their active role in dye degradation. Activities of laccase, lignin peroxidase, tyrosinase, VAO, NADH–DCIP reductase, and azoreductase in *P. aeruginosa* ARSKS20 were found to be induced by 20, 24, 106, 260, 33, and 139%, respectively in presence of RR35 at decolorization when compared with control (Table 2). The increase in oxidoreductases strongly indicates that they were induced only after exposure of the organism to the dye. Inductive patterns of these enzymes were also documented in *P. aeruginosa* and other bacterial strains [3, 7, 22, 23].

Table 2 Enzyme activities of control cells and cells obtained after decolorization of RR35

Enzyme	Control	Test (After decolorization)
Laccase [a]	0.5151 ± 0.0092	0.6172 ± 0.0.0230**
Lignin peroxidase [a]	0.3182 ± 0.250	0.3934 ± 0.0042*
Tyrosinase [a]	2.9627 ± 0.1065	6.094 ± 0.0613***
Veratryl alcohol oxidase [a]	1.8652 ± 0.0712	6.7150 ± 0.2073***
NADH-DCIP reductase [b]	28.3891 ± 0.5751	37.8154 ± 0.3280***
Azoreductase [c]	1.1337 ± 0.1777	2.7108 ± 0.1880**

Values are mean of three experiments (±) SEM. Significantly different from control cells at *P < 0.05, **P < 0.01, and ***P < 0.001 by ANOVA with Tukey- Kramer comparison test.

a Enzyme activity in units/min/mg protein, b µg of DCIP reduced/min/mg protein, c µM methyl red reduced min/ mg protein

3.4 Biodegradation studies

Biodegradation of RR35 was studied using HPTLC, FTIR and GCMS analysis. HPTLC chromatogram of RR35 showed only two major peaks (0.49 and 0.57 R_f), whereas five major peaks, unlike from control at 0.29, 0.47, 0.54, 0.59 and 0.64 R_f value were observed in metabolites after degradation (Fig. 3). The increase in number of peaks distinct from control after treatment with *P. aeruginosa* ARSKS20 supports the dye RR35 is not only decolorized but also degraded into various metabolites.

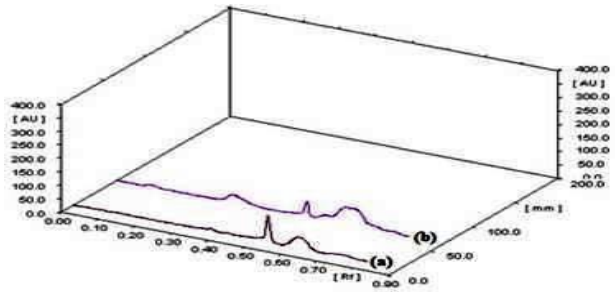


Fig. 3 HPTLC chromatogram of RR35(a) and metabolite(b)

Comparison between FTIR spectrum of RR35 and products obtained after decolorization confirmed the biodegradation of dye into different metabolites. Spectra of control RR35 (Fig. 4) showed the presence of different peaks. The peak at 3438/cm was observed for N–H stretching, whereas the peak at 1595/cm represented N=N stretching, which indicates the presence of azo group of RR35, peak representing O–H stretching was observed at 1410/cm, S=O stretching was observed at the peak of 1313/cm, 1117/cm, and 1049/cm, indicating presence of sulphonated compound. C–H stretching was observed at 2926/cm and 2852/cm, and N–H deformation was observed at the peak of 1636/cm. FTIR spectra of metabolites showed disappearance of sharp peak at 1595/cm, strongly supports predicted symmetric cleavage of azo bond in the dye molecule carried out by induced azoreductase activity observed during dye decolorization. The peaks at 3427/cm and 1289/cm represent N–H stretching and C–N stretching of secondary amines, respectively. The disappearance of some major peaks, like 1564/cm, 1507/cm, 1278/cm, 1206/cm, 1144/cm, and 1117/cm, from spectrum of parent dye and appearance of new peaks at 2959/cm, 1632/cm, 1458/cm, 1240/cm, and 1089/cm in spectrum of the metabolites support the biotransformation of RR35 into metabolites.

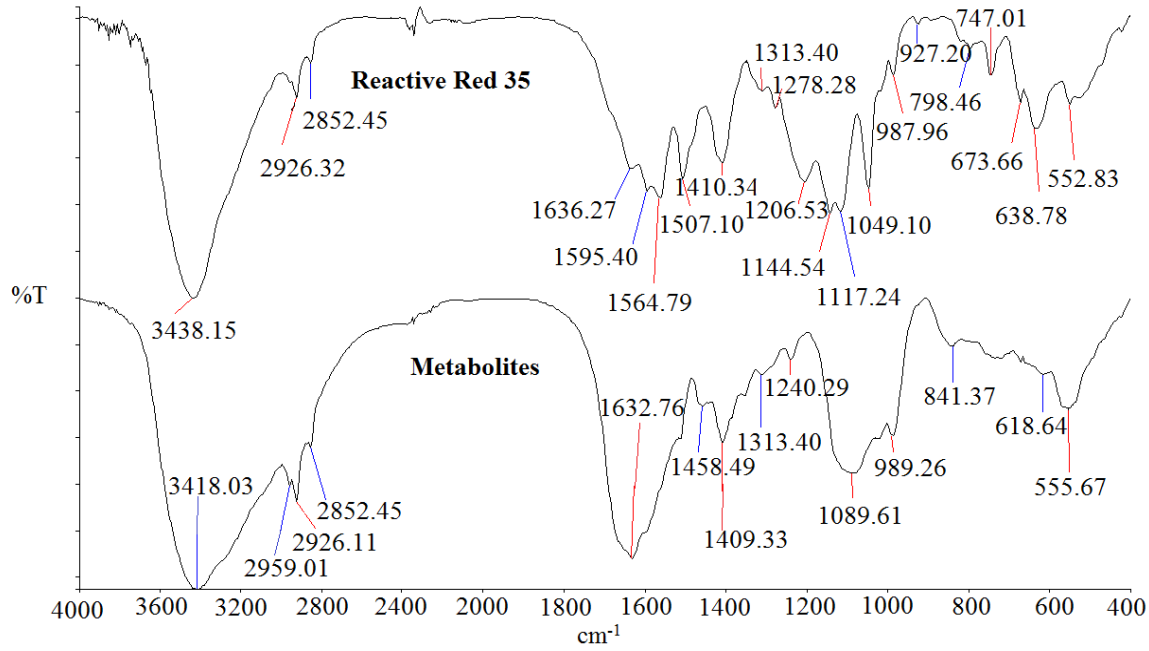
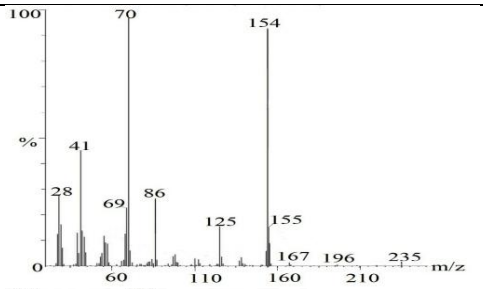
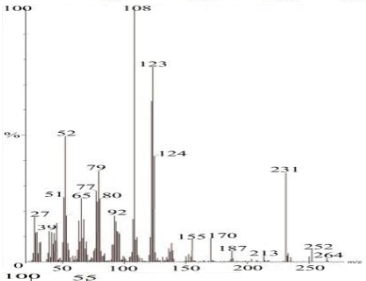
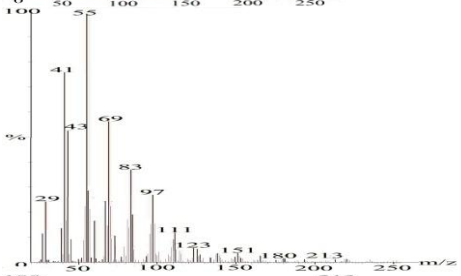
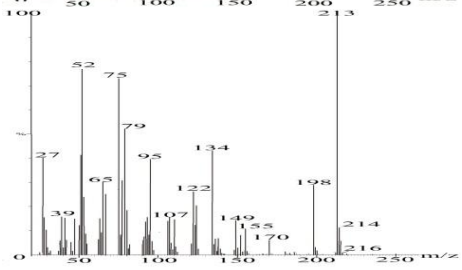
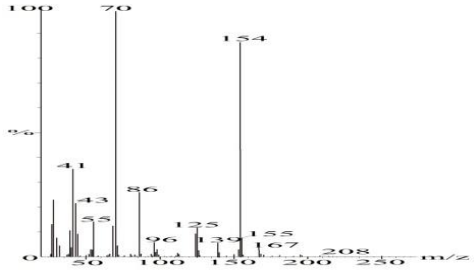
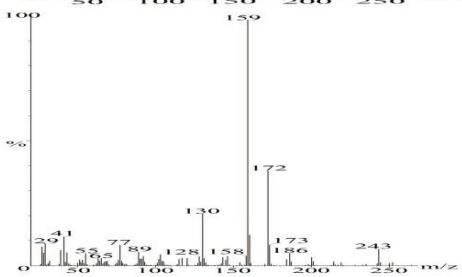
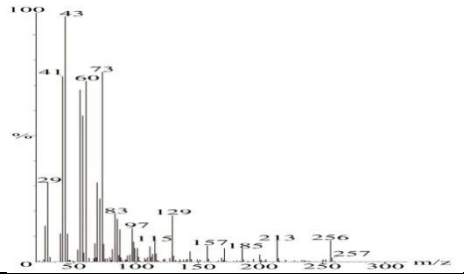


Fig. 4 FTIR spectrum of RR35dye and its metabolites obtained after decolorization by *P. aeruginosa* ARSKS20

GCMS analysis was carried out to investigate the identity of probable metabolites formed during the biodegradation process. Low molecular weight aromatic compounds produced during degradation of RR35 by *P. aeruginosa* ARSKS20 were identified on basis of fragmentation pattern and m/z values (Table 3).

Table 3 GC-MS spectral datasheet of metabolites formed after degradation of RR35 by *P. aeruginosa* ARSKS20

Sr. no.	Molecular weight of metabolite	Retention time (min)	Name of metabolite	Mass Spectra
1	235	19.755	1-Amino-2-hydroxy- 5-(2-sulfonyl- 2-sulfooxy ethyl) benzene	
2	231	23.342	1-Amino- 2-methoxy- 5-(2-1-sulfonyl ethane) benzene	
3	123	21.871	1-Amino- 3-(1-sulfonyl- 2-sulfooxy ethane) benzene	
4	216	21.386	2-Amino- 8-(acetyl amino) -1-naphthol	
5	155	19.935	Anion of naphthalene 1,7-diamine	
6	159	25.763	2-Amino- 1-naphthol	

7	129	20.155	Naphthalene	

According to the identity of metabolites, in the proposed biodegradation pathway (Fig. 5) RR35 undergoes symmetric cleavage resulting in formation of two intermediates, 1-Amino-2-methoxy-5-(1-sulfonyl-2-sulfooxyethane) benzene (intermediate-A) and 2-Amino-8-(acetyl amino)-1-hydroxy 3-6 naphthalene disulfonic acid (intermediate-B). In present study decolorization of RR35 by *P. aeruginosa* ARSKS20 was observed under microaerophilic condition, therefore preliminary degradation could be initialized by reductive symmetric cleavage of azo bond carried out by azoreductase enzyme. Induced activity of reductase strongly comprehends this catabolic reaction. These intermediates were not detected in GCMS, as they might be formed during the primary step for cleavage of dye and these transiently undergo further degradation by various enzyme systems. Primary reductive cleavage by azoreductase in *P. aeruginosa* BCH, *Lysinibacillus* sp. RGS, and *Providencia* sp. SRS82 were reported during biodegradation of Reactive Red 120, Remazol Red and Acid Black 210, respectively [1, 4, 9].

The involvement of lignin peroxidase for symmetric cleavage of azo bond in Remazol Orange 3R by *P. aeruginosa* BCH was also documented [13]. NADH–DCIP reductase was reported as a key enzyme for reduction of the azo bond during decolorization of Methyl Red by *Brevibacillus laterospores* MTCC 2298 [24], *Galactomyces geotrichum* [25] and *Bacillus* sp. UN2 [26].

Intermediate-A undergoes demethylation by laccase to produce 1-Amino-2-hydroxy-5-(2-sulfooxy ethyl) benzene (compound-1), and by desulfonation with oxidases to form 1-amino-2-methoxy-5-(2-hydroxy-1-sulfonyl ethane) benzene (compound-2). Compound-2 is further hydrolyzed to form 1-amino-2-methoxy benzene (compound-3).

Involvements of laccase and oxidases to carry out deamination and desulfonation reaction, respectively during bacterial degradation of various azo dyes were well documented [12, 13, 16]. As assumed in several studies, laccase play an important role in biodegradation of the dye by catalyzing deamination reaction [26-28] and demethylation reaction [2, 29] during the dye degradation, whereas VAO is assumed to carry out desulfonation reaction [7].

Intermediate-B undergoes desulfonation to produce 2-amino-8-(acetyl amino)-1-naphthol (compound-4), which is further transformed by deacetylation and dehydroxylation into anion of naphthalene-1-7-diamine (compound-5). In other way, compound-4 undergoes deacetylation to form 2, 8-diamino-1-naphthol (unidentified intermediate-C), which is further deaminated to form 2-amino-1-naphthol (compound-6). Compound-5 and 6 undergo deamination reaction to form naphthalene (compound-7) as end product of biodegradation of RR35 by *P. aeruginosa* ARSKS20. Thus, it transforms RR35 with high molecular weight compound to lower molecular weight compounds during degradation.

During degradation of Reactive red 2 by *Pseudomonas* sp. SUK1, 1-amino-8-naphthol 2, 5-disulfonic acid an identified intermediate undergo desulfonation reaction to 1-amino-2-naphthol (MW: 159), which further degraded via deamination reaction to form 2-naphthol [23]. Naphthalene as an end product was reported to produce from naphthalene-1, 5- disulfonic acid during degradation of Reactive Orange 13 by *Alcaligenes faecalis* PMS-1 [30]. Naphthalene as a one of the end product during degradation of Acid Black 210 by *Providencia* sp. SRS82 was reported earlier [4].

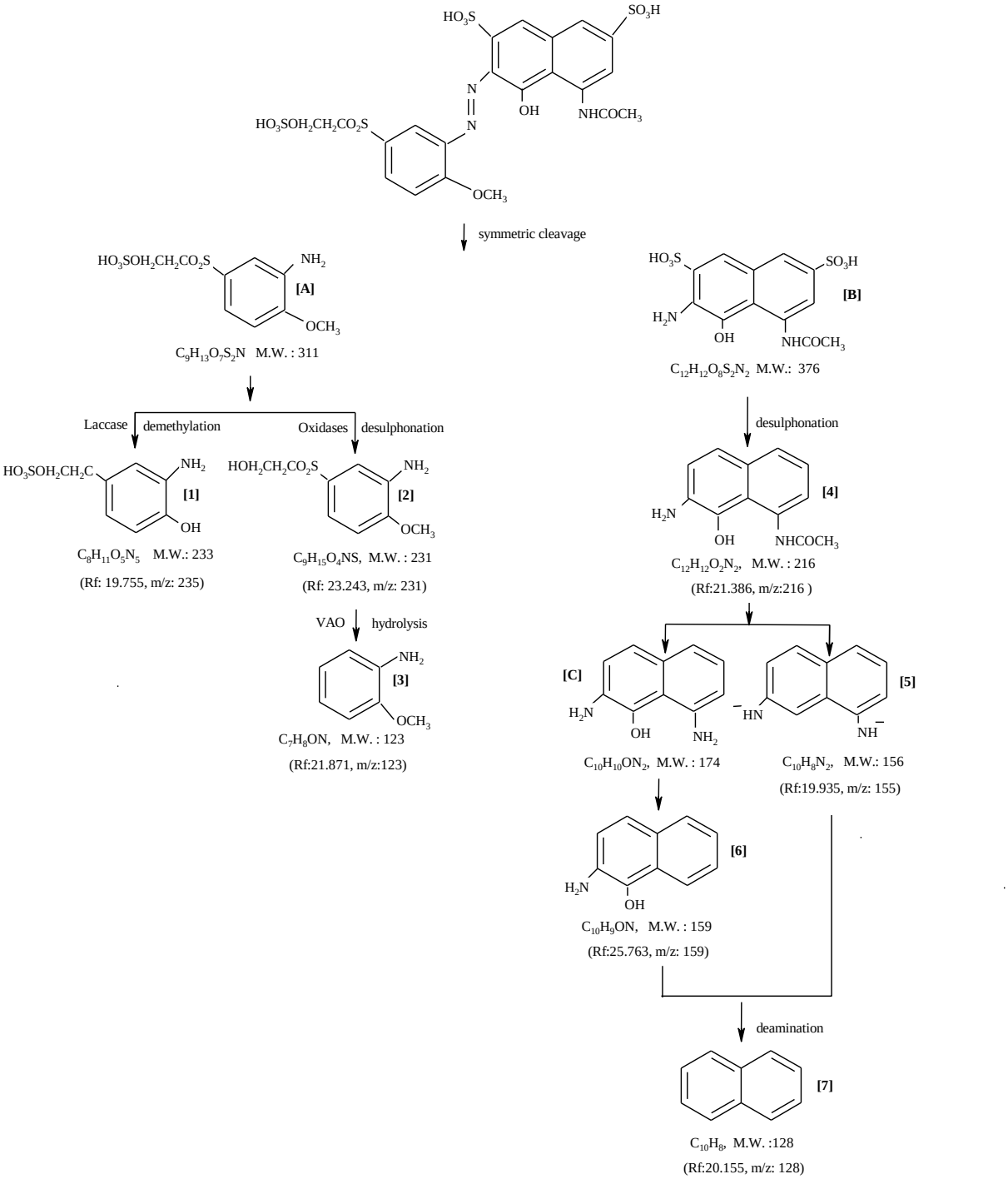


Fig. 5 Proposed pathway for the biodegradation of RR35 by *P. aeruginosa* ARSKS20. The compounds represented by alphabets have not been found in GCMS peaks, but their existence is rationalized as necessary intermediates for the final product found. The compounds represented in numbers have been found in GCMS peaks

IV. Conclusion

P. aeruginosa ARSKS20, isolated from dye-contaminated soil, efficiently decolorized various azo dyes and efficiently degraded RR35 under static condition. This microbe and its ability to decolorize wide spectrum azo dyes hold a tremendous potential to be applied for biodegradation of dye-contaminated effluents. Induced activities of different oxidoreductases in *P. aeruginosa* ARSKS20 shows its active participation in biodegradation of RR35. As revealed by GCMS analysis of metabolites, *P. aeruginosa* ARSKS20 has a high potential to degrade high molecular weight dye to low molecular weight compounds. Study promotes the

treatment of dye-containing waste water with potential bacterial strains as ecofriendly management of environmental pollution caused by textile dyes.

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