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Preliminary Studies On Biodecolorization Of Reactive Red 35 By *Pseudomonas Aeruginosa* Arsks20

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Abstract— Pseudomonas aeruginosa ARSKS20, an antibiotics sensitive strain could decolorize Reactive Red 35(RR35) dye up to 95% with 8.27 mg l^{-1} h^{-1} at 40°C, pH 8, under static condition at 100 mg l^{-1} concentration. Yeast extract at 5 g l^{-1}concentation and with 5% inoculum volume was proved as an optimum for dye decolorization. It can tolerate 50 g l^{-1} NaCl concentration and decolorized 90% RR35. Pseudomonas aeruginosa ARSKS20 could efficiently decolorized RR35 (100 mg l^{-1}) up to twelve repeated dosing without supplementation of new nutrients. HPLC analysis confirmed the biodegradation of RR35. The Less toxic nature of the dye degraded products was observed by microbial toxicity assay of agricultural important bacteria.
Keywords—Decolorization, Degradation, Pseudomonas aeruginosa ARSKS20, Reactive Red 35, Toxicity

I. INTRODUCTION

Due to wide structural diversity more than 10,000 different dyes are produced and depend on affinity to various matters they have been utilized in textile dyeing, paper printing, pulp, plastic, food, cosmetics, and tannery industries. It was estimated that over 7 x 10^5 – 1 x 10^8 tons of different dyes are produced worldwide per year [1]. During the production around 1–2% and in applications around 10–15% dyes are lost to environment [2]. For reactive azo dyes this loss may increase up to 50% [3]. Due to synthetic nature and complex aromatic structure of dyes which contributing stability and increase recalcitrance to biodegradation, wastewater from textile and dyes processing industries is the most difficult and poses a serious environmental problem. The biomagnifications of residual dyes to any ecological niche causes toxicity and ultimate destabilization it by inhibiting the activities of flora and fauna of the particular tropical region [4]. Various physicochemical methods for the treatment of dye containing wastewater was established [5], but due to their limitation and disadvantages as narrow range of application, high cost, produced large secondary hazardous sludge, intensive energy requirements; biological treatment has been increasing interest [6], [7]. Due to catabolic diversity, wide spectrum of microorganisms has been widely utilized in biodegradation of dyes containing waste water [1]. Various bacterial species have been reported to decolorize and degrade a wide range of azo dyes [4], [6], [8]–[10]. *Pseudomonas aeruginosa* NGKCTS decolorized 91% Reactive Red BS at 300 mg l^{-1} concentration in 5.5 h [3], *Pseudomonas* sp. SUK1 decolorized 96% Reactive Red 2 at 300 mg l^{-1} within 6 h [11]. *P. aeruginosa* BCH decolorized 98% of Remazol Orange 3R at 50 mg l^{-1} within 15 min [12], 97% Remazol Red at 50 mg l^{-1} within 20 min [13]. The present study portrays the decolorization of Reactive Red 35 (RR35) by *Pseudomonas aeruginosa* ARSKS20. Various physicochemical operational parameters have been optimized for enhanced decolorization. Degradation of RR35 has been evaluated with High Performance Liquid Chromatography (HPLC). The toxicity of the dye after treatment was also assessed by studying microbial toxicity.

II. MATERIALS AND METHODS

A. Dyes And Chemicals

Reactive Red 35, a vinyl sulfone based monoazo dye, also known as Reactive Red 5B, was procured from Meghmani dyes and Pigments, Vatva, Ahmedabad, (India). All bacteriological media and ingredients were purchased from Himedia. All other chemicals used in the study were of analytical grade and obtained from Himedia, Merck and SRL.

B. Biochemical Characterization Of Dye-Degrading Bacteria

Biochemical characterization of an isolate designate as ARSKS20 has been carried out by using various biochemical test kits as per

the SOP of manufacturer, Tulip and Himedia kit (KB002, KB003, and KB009) for RR35 dye degrading potent bacterial identification. Antibiotics susceptibility was assayed using multi disc Dodeca universal I (DE001) and V (DE013), Dedeca G1 minus (DE003) and G2 minus (DE010), Dodeca Pseudo 1 (DE020) using Mueller Hilton Agar.

C. Decolorization Experiment

Decolorization studies were performed by batch process in 250 ml Erlenmeyer conical flask containing 100 ml Bushnell Hass Broth (BHB) supplemented with 2.5 g l^{-1} yeast extract (BHBY) (BHB; g l^{-1}: 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) emended with 100 mg l^{-1} RR35. After decolorization, 2 to 3 ml sample was withdrawn and centrifuged at 8000×*g* for 15 min and absorbance was measured by UV-visible spectrophotometer (Elico, SL159) at 512 nm. All experiments were carried out in triplicates along with abiotic control. Percentage decolorization and average decolorization rate were calculated as previously reported methods [14], [15].

D. Establishment Of Optimum Operational Parameters

Dye decolorization performance of ARSKS20 was assessed under various environmental parameters such as static and shaking conditions, initial dye concentrations (50–500 mg l^{-1}), pH values (4–11) and temperatures (25–50°C), inoculum size (1–10 v v^{-1}).The effect of nutritional parameters such as carbon source *viz.*, dextrose, fructose, maltose, sucrose, lactose, malt extract, starch, sodium citrate and nitrogen source *viz.*, yeast extract, peptone, casein, soyabean, meat extract, tryptone, ammonium nitrate, sodium nitrate, ammonium sulphate, urea at 2.5 g l^{-1} concentration was evaluated under optimized conditions as mentioned above. Decolorization efficiency of culture in presence of higher salt concentration was assessed in presence of 5–50 g l^{-1} NaCl concentration. The decolorization of repeated addition of dye (100 mg l^{-1}) to culture media was studied under optimized conditions without the supplement of additional nutrients and procedure was repeated until the loss of effective decolorization. All experiments were conducted in triplicates with 100 mg l^{-1} RR35 concentration unless it was stated.

E. HPLC Analysis

Biodegradation of RR35 was evaluated by HPLC (Shimadzu, SCL-10AVP) equipped with C_{18} column (Princeton, 4.6 mm x 150 mm) and at dual wavelength by isocratic method. The mobile phase was methanol with flow rate of 1 ml min^{-1} for 10 min [12]. The 10 µl of sample was injected manually into injector port.

F. Toxicity Assessment

The toxicity of dye (1000 mg l^{-1}) and its biodegraded product was tested by performing microbial toxicity of agriculture important bacteria by the well diffusion assay according to the method reported earlier [3]. *Bacillus subtilis* (MTCC 1305), and *Azotobacter chroococcum* (MTCC 7724) procured from Microbial Type Culture Collection & Genebank, Institute of Microbial Technology (IMTECH), Chandigarh, India, were inoculated on Nutrient agar and Ashby's mannitol agar, respectively. Well of 6.0 mm diameter on respective media plate were filled with untreated dye (1000 mg l^{-1}) and its metabolites, and zone of inhibition were measured after 48 h.

III. RESULTS AND DISCUSSION

A. Biochemical Characterization And Antibiotic Susceptibility

Wide substrate specificity of *Pseudomonas aeruginosa* ARSKS20, gram negative, short rod was biochemically characterized as per Table 1. Microorganisms used for biodegradation purpose should not be pathogenic or not to be resistant to antibiotics, therefore antibiotics susceptibility of the culture towards wide range of antibiotics was evaluated and observed that it showed susceptibility to major antibiotics (Table 2).

B. Optimum Operational Conditions For Decolorization Of RR35

Microbial metabolism is sensitive to the presence of chemical substances, like dyes, carbon and nitrogen sources, pH and temperature, presence or absence of oxygen, salinity [4]. So in biodegradation processes for better performance of microorganisms isolated from a contaminated site must be exploited at their optimum growth conditions.

C. Effect Of Static Versus Shaking Condition On Decolorization Of RR35

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Decolorization of RR35 at static condition was observed 84% of at 12 h, whereas no decolorization was observed even after 48 h at shaking condition at 120 rpm, which suggest that the facultative anoxic condition was favorable for decolorization of RR35. It could be due to the involvement of azoreductase enzyme in decolorization of dye, as it carried out reductive cleavage under of azo dyes, similar finding was also reported earlier [3].

TABLE 1
BIOCHEMICAL CHARACTERIZATION OF *P. aeruginosa* ARSKS20

No.	Test	*P. aeruginosa* ARSKS20	No.	Test	*P. aeruginosa* ARSKS20
1	Methyl red	-	26	Sucrose	-
2	Voges Proskauer's	-	27	L-Arabinose	-
3	Citrate utilization	+	28	Mannose	-
4	Indole	-	29	Inulin	-
5	Glucuronidase	±	30	Sodium gluconate	-
6	Nitrate reduction	+	31	Glycerol	-
7	Lysine utilization	-	32	Salicin	-
8	Catalase	+	33	Dulcitol	-
9	Phenylalanine deamination	-	34	Inositol	-
10	H_2S Production	-	35	Sorbitol	-
11	Oxidase detection	+	36	Mannitol	+
12	Gelatin liquefaction	+	37	Adonitol	-
13	Growth at 41°C	+	38	Arabitol	-
14	Growth at 4°C	-	39	Erythritol	-
15	Motility	+	40	α-Methyl-D-glucoside	-
16	Urease	-	41	Rhamnose	-
17	Lactose	-	42	Cellobiose	-
18	Xylose	-	43	Melezitose	-
19	Maltose	-	44	α-Methyl-D-Mannoside	-
20	Fructose	+	45	Xylitol	-
21	Dextrose	+	46	ONPG	-
22	Galactose	-	47	Esculin hydrolysis	-
23	Raffinose	-	48	D-Arabinose	-
24	Trehalose	-	49	Malonate utilization	+
25	Melibiose	-	50	Sorbose	-

D. Effect Of Initial Dye Concentration On Decolorization Of RR35

Percentage decolorization of RR35 by *P. aeruginosa* ARSKS20 was evaluated with 50-500 mg l^{-1} at static condition. Dye decolorization was 88-85% at 50-100 mg l^{-1} dye concentration (Fig.1a). While 74- 57% decolorization was recorded at a concentration of 150-350 mg l^{-1}. Only 40-34% decolorization was found at 350-500 mg l^{-1} after 12 h. Increase in dye concentration might be inhibiting the enzyme system involve in dye decolorization which resulted into decreased in dye decolorization. A similar study was also reported earlier for decolorization of Remazol Orange by *P. aeruginosa* BCH [16]. Reactive azo dyes with sulfonic acid (SO_3H) groups on their aromatic rings increased toxicity and inhibited the growth of microbial cells at higher concentrations [10].

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E. Effect Of pH On Decolorization Of RR35

Medium pH play crucial role for microbial activity as a metabolic and enzymatic reaction was greatly influenced at varied pH [4]. Decolorization of RR35 was evaluated at pH range from pH 4 to 11. Dye decolorization was observed 84-87% at pH 7-8 with 7-7.22 mg l^{-1} h^{-1} rate (Fig. 1b). Decolorization was decreased to 39-13% at pH 9-11 and observed 28-14% at pH 6-5. There was no decolorization recorded at pH 4. The optimum pH for decolorization of dye is neutral or slightly alkaline, decolorization decreased at strongly acidic or alkaline pH [17]. Optimum decolorization of Remazol Red by *Lysinibacillus* sp. RGS was obtained at pH 7 [15].

F. Effect Of Temperature On Decolorization Of RR35

Temperature is an important factor associated with all microbial activity as microbial cell responds to change in environmental temperature and adapt it via biochemical and enzymatic mechanisms [10]. Decolorization of RR35 was assessed in temperature range from 25 to 50°C. Dye decolorization was observed 84-88% at 35-40°C with 7.01-7.32 mg l^{-1} h^{-1} rate (Fig. 1c).

TABLE 2
ANTIBIOTICS SUSCEPTIBILITY OF *P. aeruginosa* ARSKS20

No.	Antibiotics	Code	Concentration	Zone of inhibition (mm)	No.	Antibiotics	Code	Concentration	Zone of inhibition (mm)
1	Amikacin	AK	30 mcg	33	20	Erythromycin	E	15 mcg	36
2	Amoxycillin	AMX	10 mcg	26	21	Gentamicin	GEN	10 mcg	28
3	Ampicillin	AMP	10 mcg	12	22	Levofloxacin	LE	5 mcg	44
4	Ampicillin/ Sulbactam	A/S	10/10 mcg	32	23	Lomefloxacin	LOM	10 mcg	38
5	Augmentin	AMC	30 mcg	21	24	Lomefloxacin	LOM	30 mcg	46
6	Azithromycin	AZM	30 mcg	16	25	Meropenem	MRP	10 mcg	52
7	Cefadroxil	CFR	30 mcg	17	26	Netillin	NET	30 mcg	33
8	Cefepime	CPM	30 mcg	39	27	Ofloxacin	OF	5 mcg	42
9	Cefixime	CFM	5 mcg	0	28	Pefloxacin	PF	5 mcg	37
10	Cefoperazone	CPZ	75 mcg	35	29	Penicillin	P	10 units	18
11	Cefotaxime	CTX	30 mcg	32	30	Piperacillin	PI	100 mcg	38
12	Cefpodoxime	CPD	10 mcg	0	31	Rifampicin	RIF	5 mcg	11
13	Ceftazidime	CAZ	30 mcg	35	32	Roxithromycin	RO	30 mcg	30
14	Ceftriaxone	CTR	30 mcg	25	33	Sparfloxacin	SPX	5 mcg	46
15	Cefuroxime	CXM	30 mcg	18	34	Streptomycin	S	10 mcg	30
16	Chloramphenicol	C	30 mcg	20	35	Tetracycline	TE	30 mcg	10
17	Ciprofloxacin	CIP	5 mcg	50	36	Ticarcillin	TI	75 mcg	32
18	Clindamycin	CD	2 mcg	0	37	Tobramycin	TOB	10 mcg	37
19	Cloxacillin	COX	1 mcg	18	38	Vancomycin	VA	30 mcg	0

Decolorization was observed 82 and 68% at 30 and 25°C, respectively, at a lower temperature microbial activity was ceased and affects decolorization. Dye decolorization ability of *P. aeruginosa* ARSKS20 was affected adversely at 50°C; this might be attributed due to loss of cell viability and the denaturation of enzyme systems responsible for dye decolorization [9], [18].

G. Effect of inoculum size on decolorization of RR35

Rate of dye decolorization varies with inoculum size [3], as dye decolorization responds of *P. aeruginosa* ARSKS20 was assessed with 1-10% (v v^{-1}) inoculum size. Optimum decolorization rate was observed 7.42 mg l^{-1} h^{-1} with 5% inoculum (Fig. 1d), further increased in inoculum size were not significantly increased in decolorization. A similar observation was recorded for decolorization

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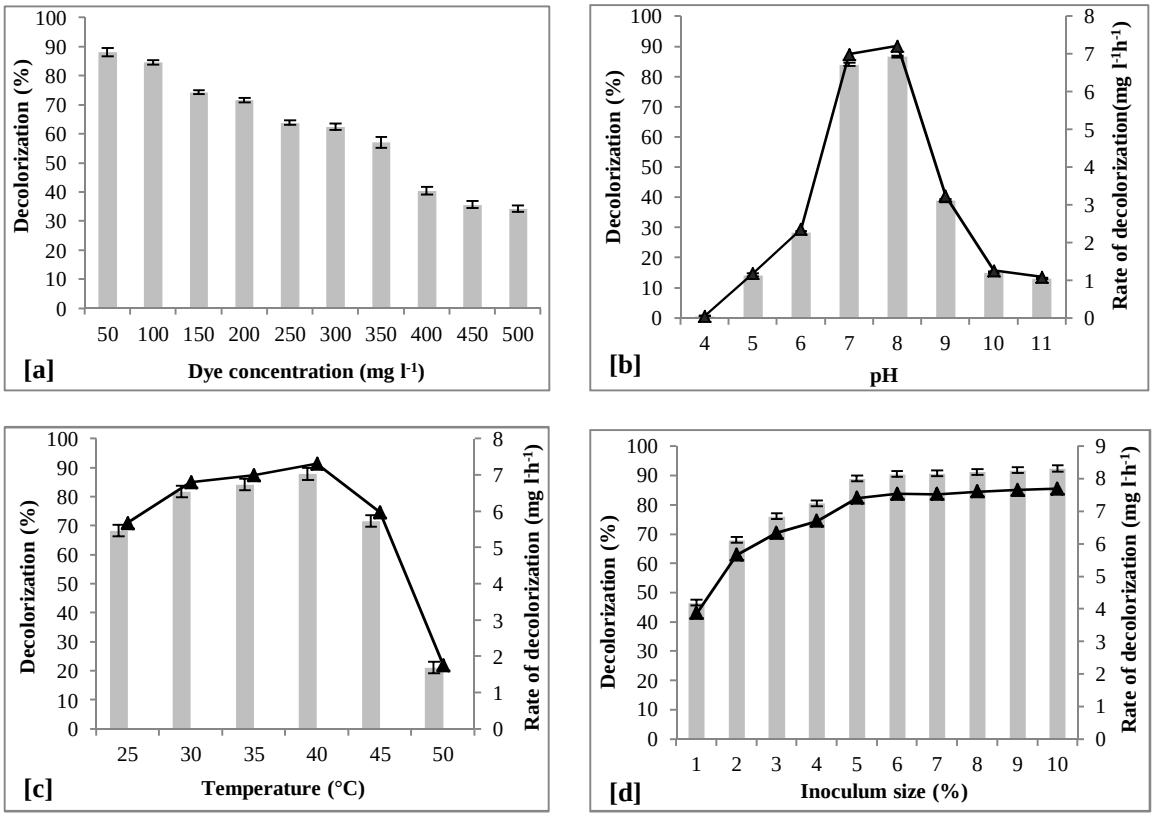
of Reactive violet 5 [19].

H. Effect Of Carbon And Nitrogen Source On Decolorization Of RR35

Presences of carbon and nitrogen sources are remarkably influence on decolorization of dye, as dyes are deficient in utilizable carbon and nitrogen source. Carbon sources are essential for growth and energy requirement of the microorganisms while metabolism of organic nitrogen is also important for regeneration of NADH required for reductive cleavage of azo bond of dyes [4]. Nitrogen source was found as an obligate requirement for decolorization of RR35 by *P. aeruginosa* ARSKS20 with BHB, so effect of carbon source (2.5 g l^{-1}) was evaluated with 2.5 g l^{-1} yeast extract. Results suggest that no effective decolorization was observed in presence of any carbon source (Fig. 1e). Thus, the addition of a carbon source seemed to be less effective in promoting decolorization of RR35, it probably due the bacteria assimilating the simple carbon source over using dye as a sole source of carbon [4]. These can be proved from the obtained results as decolorization was limited in presence of simple carbon source like glucose than compare to starch. Decolorization of RR35 was observed 90.33% with 7.53 mg l^{-1} h^{-1} rate in presence of 2.5 g l^{-1} yeast extract (Fig. 1f). Tryptone was found a second influencing nitrogen source with 75.54% decolorization at 6.3 mg l^{-1} h^{-1} rate. Ammonium nitrate, sodium nitrate, ammonium sulphate and urea were unable to fulfill obligatory requirement of nitrogen source for decolorization of RR35 by *P. aeruginosa* ARSKS20. Yeast extract was reported as an important nitrogen source for dye decolorization [20]. Yeast extract with 5 g l^{-1} concentration was found optimum for decolorization of RR35 (Fig. 1g).



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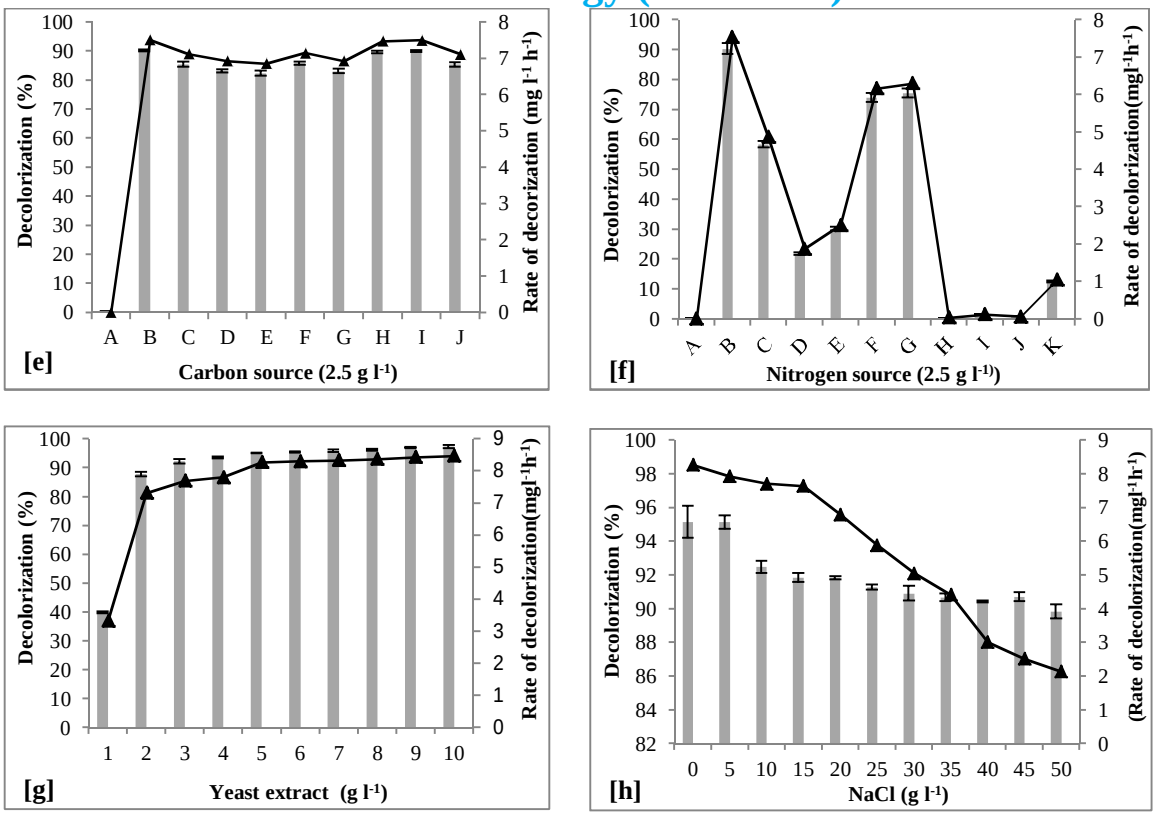

Fig. 1 Optimization of operational parameters; Initial dye concentration [a], pH [b], Temperature [c], Inoculum volume [d], Carbon source [e] (A: BHB; B: BHBY; C: dextrose, D: fructose; E: maltose; F: sucrose; G: lactose; H: malt extract; I: starch; J: sodium citrate), Nitrogen source **[f]** (A: BHB; B: yeast extract; C: peptone; D: casein; E: soyabean; F: meat extract; G: tryptone; H: ammonium nitrate; I: sodium nitrate; J: ammonium sulphate; K: urea), Yeast extract concentration**[g],** Effect of NaCl concentration[**h**].

I. Effect Of Salinity On Decolorization Of RR35

Effluent from dye processing industries contain various types of acids, alkali, metals and salt at higher concentration. High salts are needed for dying process and found to release up to 15- 20% salts in effluent. Sodium concentration above 3 g l^{-1} is responsible for moderate inhibition of most microbial activities. So microorganisms which can tolerate high salt concentration are suitable for bioremediation of dye containing waste water [9]. Decolorization of RR35 was observed 95-90% in presence of 5-50 g l^{-1} NaCl concentration (Fig. 1h). It was observed that the rate of decolorization was decreased by 4.11-7.50, 17.78-46.55, and 63.60- 74.12% at 5-15, 20-35, and 40-50 g l^{-1} NaCl concentration, respectively. Thus, the rate of decolorization was decreased with increased NaCl concentration; bacterial isolate *P. aeruginosa* ARSKS20 can tolerate 50 g l^{-1} NaCl concentration with 90% decolorization of RR35. Our observation and finding are in accordance with previously reported work [12].

J. Effect Of Repeated Addition Of Dye

The dyes containing waste are continuously discharged from dyeing and textile industries. Effect of repeated addition of RR35 at 100 mg l^{-1}concentration was evaluated to assess efficiency of *P. aeruginosa* ARSKS20 to sustain at larger scale. The obtained results shows that *P. aeruginosa* ARSKS20 decolorize RR35 at 8.27 mg l^{-1} h^{-1} rate with first cycle of 100 mg l^{-1} concentration (Fig. 2). Further dosing of RR35 at same concentration shows increased decolorization rate even without supplementation of new nutrients. In second cycle 30.93 mg l^{-1} h^{-1}decolorization rate was observed. Dye decolorization rate 30.07-27.00 mg l^{-1} h^{-1} was observed from three to eight cycles. Nine to eleven cycles showed 24.90-23.63 mg l^{-1} h^{-1} decolorization rate. Decolorization efficiency was decreased (16.51 mg l^{-1} h^{-1}) at twelve cycle. Decreased decolorization efficiency could be due to accumulation of the dye metabolites and exhaustion of nutrient as the experiment was continuous fed with dye only. Similar observations are in closed

accordance with earlier reports [3], [21].

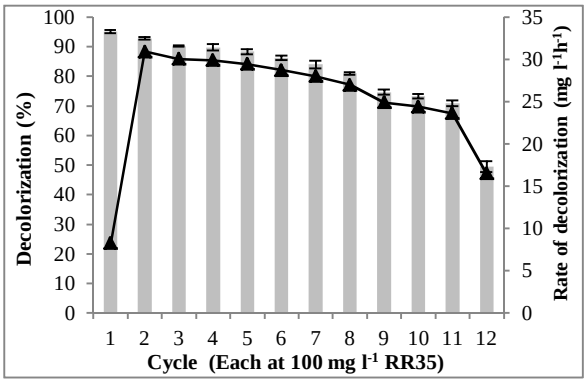

Fig. 2 Effect of continuous dye addition on decolorization of RR35

K. HPLC Analysis

The HPLC analysis of RR35 show single peak at retention time 1.187 min (Fig. 3a). After decolorization three new peaks were observed at retention time 1.447, 2.069, and 2.369 min (Fig. 3b). Dye decolorization and degradation was strongly supported in published research reports on HPLC analysis [11], [12], [17].

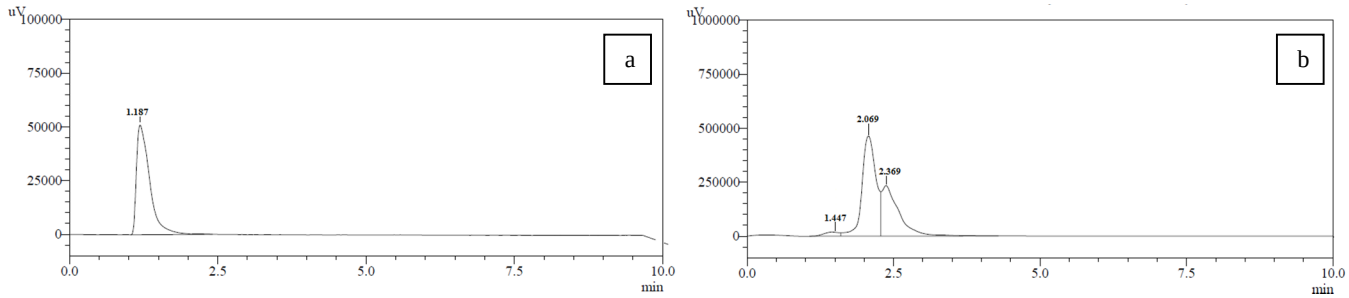

Fig. 3 HPLC chromatogram of RR35 (a), and its metabolites (b)

L. Microbial Toxicity

The toxicity of RR35 (1000 mg l^{-1}) and its metabolites obtained after degradation was evaluated by well diffusion method against agricultural important bacteria such as *B. subtilis* (MTCC 1305) and *A. chroococcum* (MTCC 7724) were responsible for starch hydrolysis and nitrogen fixation in soil, respectively. Toxicity of RR35 was reduced by 38.32 and 43.13% respectively, in *B. subtilis*, *A. chroococcum* after treatment with *P. aeruginosa* ARSKS20 (Table 3). Results are closed agreement with earlier report [5].

TABLE 3
MICROBIAL TOXICITY OF DYE AND ITS METABOLITES

Bacteria	Inhibitory zone diameter (cm) RR35	Degradation product
B. subtilis (MTCC 1305)	1.07 ± 0.03	0.66 ± 0.03*
A. chroococcum (MTCC 7724)	1.60 ± 0.06	0.91 ± 0.04*

Values are mean of three experiments (±) SEM. Significantly different from RR35 dye at *P < 0.001 by ANOVA with Tukey- Kramer comparison test.

IV. CONCLUSIONS

Bacterium *P. aeruginosa* ARSKS20 showed efficient decolorization of Reactive Red 35 dye with fewer nutrient requirements.

Efficiency to decolorize repeated addition of dye promotes it in real scale up applications. Ability to reduced toxicity of dye and being nonpathogenic nature *P. aeruginosa* ARSKS20 was proved to utilize for ecofriendly treatment of dye containing waste water.

V. ACKNOWLEDGMENT

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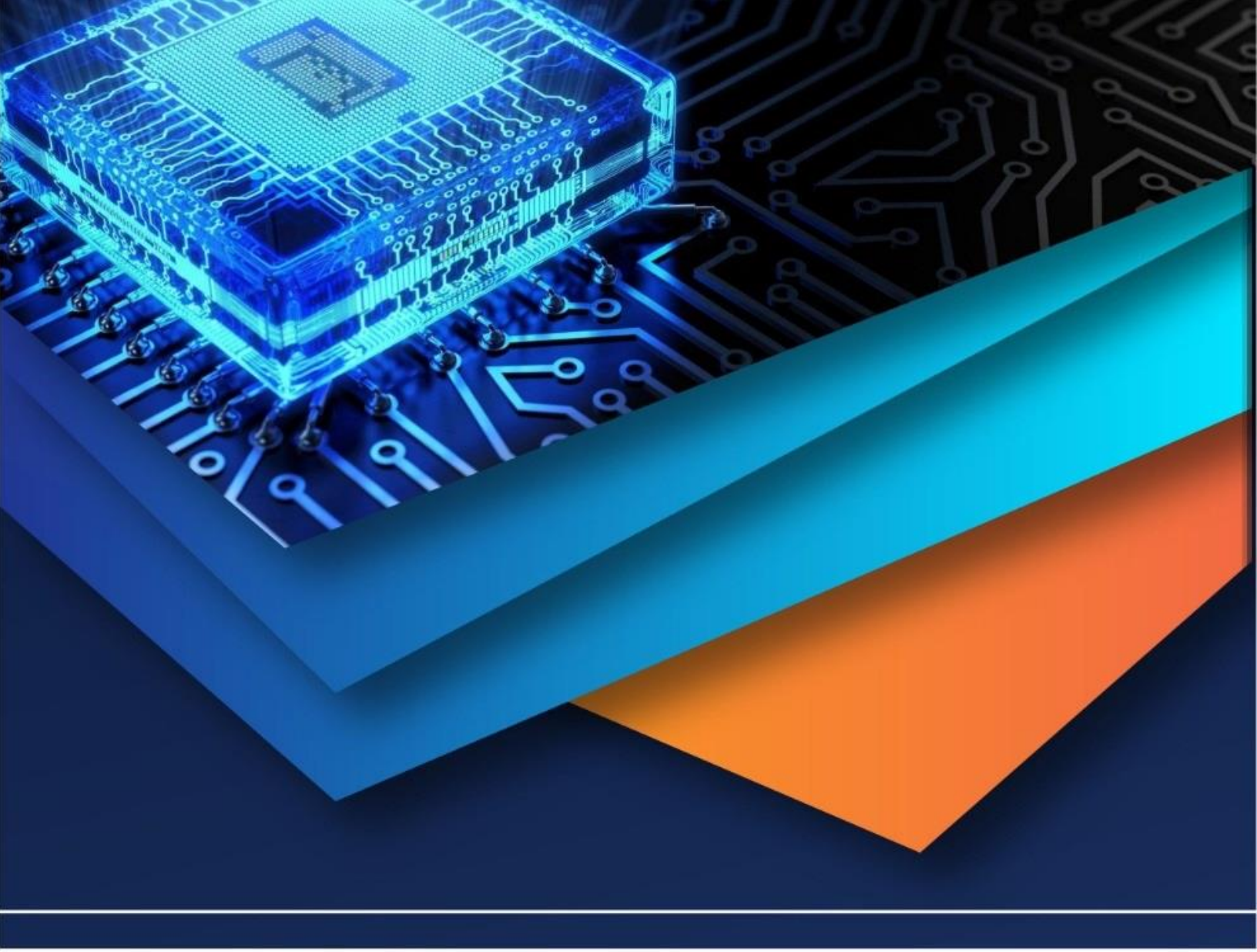

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