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Reactive green dye remediation by *Alternanthera philoxeroides* in association with plant growth promoting *Klebsiella* sp. VITAJ23: A pot culture study

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ABSTRACT

Contamination of soil by textile effluent is a major threat found worldwide. These pollutants have diverse range of negative effects on the ecosystem, therefore restoration through cost effective biological strategy is the need of the hour. The aim of the current study was to enhance the decolourization of reactive green dye (RGD) using phytoremediation coupled with augmentation of effective bacteria to the rhizosphere. The isolate *Klebsiella* sp. VITAJ23 was isolated from textile effluent polluted soil and was assessed for its plant growth promoting traits (PGP) and the PGP functional genes were amplified. The soil was artificially polluted with RGD concentration ranging from 1000 to 3000 mg kg^{-1} and *Alternanthera philoxeroides* plantlets were planted in phyto and rhizoremediation treatments, the setup was maintained upto 60 d. The isolate VITAJ23 was augmented in the rhizoremediation setup and the morphological parameters were assessed at regular interval. There was a significant increase in the chlorophyll content as well as root and shoot length of the plant when treated with the bacterial suspension. Decolourization study revealed 79% removal of reactive green dye with an enhanced oxido-reductase enzyme activity in the setup bioaugmented with bacteria. The biodegraded metabolites were identified as 2-allylnapthalene, l-alanine, n-acetyl-and propenoic acid by GC–MS analysis and a plant-bacteria degradation pathway was predicted using computational tools. Inoculation of PGP-*Klebsiella* sp. VITAJ23 enhanced the rate of plant growth and dye degradation.

1. Introduction

The potential toxicity of azo dye has raised a major concern to all lifeforms since most of the dyes are xenobiotic and recalcitrant compounds and hence, can lead to several mutagenic effects (Saratale et al., 2011). Azo dye represents a major class of recalcitrant synthetic compound (Telke et al., 2010; Lade et al., 2012; Sinha et al., 2018). The release of dye containing wastewater pollute the water resources and leads to reduction in sunlight penetration, hence affecting photosynthesis rates (Saratale et al., 2011). Textile effluent discharge consists of array of compounds such as heavy metals, aryl phenols, chlorinated compounds, aromatic amines, volatile organic compounds (VOC) etc., which are toxic to the environment when exposed for a prolonged period. In addition, the toxicity of azo dyes is one of the major concerns since most of the dyes are xenobiotic and carcinogenic in nature and hence can lead to the formation of tumor, allergies, water borne disorders such as nausea, ulceration of skin and mucous membrane (Khandare and Govindwar, 2015). Conventional strategies are ineffective in removal of dye, moreover they are not cost effective, hence limiting its applicability especially in developing countries (Tamboli et al., 2010).

Bioremediation involving the use of plants have emerged as an eco-friendly, attractive and cost-effective strategy for the decolourization and detoxification of recalcitrant synthetic dyes (Khandare and Govindwar, 2015). Phytoremediation involves the usage of plants to extract, sequester, and/or detoxify xenobiotic organic and inorganic pollutants (Padmavathiamma and Li, 2007; Rascio and Navariizzo, 2011). Plant tend to show higher bio-removal capabilities towards contaminants either via their own enzymatic machineries or in association with microorganisms residing in the rhizosphere (Glick, 2010). Plant species such as *Phragmites australis*, *Typhonium fagelliforme*, *Aster amellus, Glandularia pulchella, Zinnia angustifolia, Portulaca grandifora* and *Ipomoea hederifolia* etc. have been extensively used for textile dye removal (Davies et al., 2005; Kagalkar et al., 2009; Ong et al., 2011; Rane et al., 2015). As plants are self-sustained systems, there on-site applicability requires low inputs and maintenance. However, extensive research is required as relatively meagre information is available about the enzymatic machinery involved in the transformation of most

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xenobiotic compounds. The use of plants in association with plant growth promoting rhizobacteria (PGPR) offers enhanced potential for the removal of toxic contaminants (Liu et al., 2014). Rhizoremediation combines two biological clean-up machinery, namely plant and microbes, which helps to ehnace the removal of toxic compounds from the soil as well as promotes plant growth through direct and indirect methods.

Plant growth promoting rhizobacterium (PGPR) belongs to the category of free-living bacteria that actively colonizes in the rhizosphere region and are considered as a potential tool capable of prompting plant growth (De-Bashan et al., 2012). The various mechanisms of PGPR includes (i) production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase, increases the root length and thereby leading to higher uptake ability of the plant (Penrose et al., 2001) (ii) production of growth hormone like indole acetic acid (IAA) and gibberellic acid (Patten and Glick, 2002) (iii) increasing the bioavailability of phosphorus for plant uptake (Gulati et al., 2010) (iv) sequestration of iron for plant by siderophore production (Dimkpa et al., 2008) and (v) asymbiotic nitrogen fixation. Plant–bacteria association have been extensively investigated to explore either plant–pathogen interactions or nitrogen fixation, however remediation strategy involving PGPR augmentation with plants are less explored (Kadam et al., 2014). The aim of the present study was to investigate the potential of bacterium-plant synergism for the removal of reactive green dye (RGD) by phyto and rhizoremediation strategy. *Alternanthera philoxeroides* plant augmented with PGP-*Klebsiella* sp. was used for enhanced uptake of dye.

2. Materials and methods

2.1. Dyes and chemicals

Reactive green dye (C. I. Reactive Green 6; CAS number: 12225-75-1; Mw Wt: 783.97 g/mol; Formulae: $C_{29}H_{16}C_{l2}N_8Na_2O_8S_2$) was obtained as a courteous gift from a textile industry in Tirupur industrial estate, TN, India. Stock solution of the dye was prepared using sterile deionized water and was filter sterilized using 0.22 µm syringe filter. All the chemicals used in the study were of highest purity available, chemicals like indole acetic acid, L-tryptophan, L-asparagine and chrome azurol S were purchased from Sigma-Aldrich Corporation. The chemicals methyl red, 2,6-Dichlorophenolindophenol, Nicotinamide adenine dinucleotide, vertryl alcohol and the salts used in the study were purchased from Hi-Medium, India.

2.2. Isolation and identification of microorganism

The effective strain was isolated from dye effluent polluted soil samples of Tirupur district, India (Fig. S1). Soil samples were collected by randomized sampling procedure and were serially diluted, 10^{-5} dilution was plated onto MSM agar media ($MgSO_4$ 0.1 g L^{-1}, $(NH_4)_2SO_4$ 0.6 g L^{-1}; NaCl 0.5 g L^{-1}; K_2HPO_4 1.36 g L^{-1}; $CaCl_2$ 0.02 gL^{-1}; $CuSO_4$ 0.2 gL^{-1}; $MnSO_4$ 1.1 g L^{-1}; $ZnSO_4$ 0.2 gL^{-1}; $FeSO_4$ 0.14 g L^{-1}; glucose 10 gL^{-1} and yeast extract 5 g L^{-1}) supplemented with 100 mgL^{-1} of reactive green dye (Ayed et al., 2011). Colony showing the highest decolourization zone was purified after 24 h of incubation at 28 ± 2 °C and decolourization study was performed at λ_{max} of the dye (420 nm) (Sinha and Osborne, 2016). Molecular identification was carried out by 16S rRNA ribosomal gene sequencing using universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) with the following PCR condition initial denaturation at 95 °C for 7 min, followed by denaturation at 95 °C for 1 min, and annealing at 35 °C, and an extension step of 5 min at 72 °C; followed by final extension at 72 °C for 10 min for 40 cycles (Dereeper et al., 2008).

2.3. Assessment of plant growth promoting traits (PGPR)

Indole acetic acid (IAA) production was detected using the standard protocol described by Gordon and Weber (1951). Seed culture of VITAJ23 (0.5 OD, and 3×10^8 cfu/ml) was inoculated in 10 mL MS media supplemented with 5 mM L-tryptophan and incubated at 28 ± 2 °C for 72 h. Supernatant was recovered by centrifugation and salkowski's reagent was added. After incubation in dark for 30 min, the development of pink coloration indicates the production of IAA. The amount of IAA produced was quantified spectrophotometrically at 530 nm using indole acetic acid as standard. Standard range of 0, 5, 10, 20, 50, and 100 µg/ml (ppm) was prepared of indole acetic acid dissolved in acetone (IAA is not soluble in water but it is in acetone). Upon incubation at 28 ± 2 °C, the absorbance reading were taken at 530 nm and a standard graph was plotted. The experiment was repeated with VITAJ23 supernatant to find the amount of IAA produced and the value was expressed in mgL^{-1} (Patten and Glick, 2002). Siderophore production was detected by observing the appearance of orange halos around bacterial growth on CAS agar plates (Schwyn and Neilands, 1987) after 72 h of incubation. Seed culture of VITAJ23 was prepared in 10 mL of modified FISS media (KH_2PO_4 (5.03), L-asparagine (5.03), glucose (5), $MgSO_4$ (0.04), $MnSO_4$ (0.001), $ZnCl_2$ (0.005); $FeSO_4$ (0.005)). Upon incubation for 72 h, culture supernatant was recovered by centrifugation at 10,000 rpm at 4 °C and was added onto CAS agar plates for the appearance of halozone. Production of ammonia was detected after inoculation of VITAJ23 seed culture to peptone water (10.0 g peptone; 5.0 g NaCl; 1000 ml distilled water; 7.0 pH) and incubated at 28 ± 2 °C for 4 days, the accumulation of ammonia was detected by adding Nessler's reagent (500 µL). The appearance of yellow/brownish color indicates the production of ammonia. Solubilization of tri-calcium phosphate was determined in Pikoskaya's agar media and NBRIP media. VITAJ23 was patched on the plates and incubated for 7 days at 28 ± 2 °C for the appearance of halozone. The amount of tri calcium solubilised was calculated by the formulae given below (Eq. 1) (Gulati et al., 2010).

$$Solubilization\quad Index = \frac{Total\quad diameter\quad of\quad the\quad halozone}{Colony\quad diameter} \qquad (1)$$

2.4. Molecular amplification of PGPR candidate gene

Bacterial cells were harvested from the overnight broth (0.5 Abs), genomic DNA was extracted using phenol chloroform extraction method (Oh et al., 2011). Target genes for the production of PGPR traits such as indole acetic acid production (*ipdc* gene), siderophore production (*sid* gene) and nitrogen fixation (*nif*H gene) were amplified using gene specific primers. The primer and amplification conditions are mention in the Tables 1 and 2 (Patten and Glick, 2002; Moelling et al., 2007; Chowdhury et al., 2009).

2.5. Phyto and rhizoremediation study for the removal of RGD

2.5.1. Preparation of soil

Red loamy soil (without manure) was collected from the nursery of Vellore Institute of Technology, Vellore and were sieved into uniform particle size using a 4 mm particle sieve. The sieved soil samples (2 kg)

Table 1
List of primers used for the detection of PGPR traits by molecular analysis.

Target	Primer Name	Sequence (5'–3')
IAA	*ipdc*-F	CTGGGGATCCGACAAGTAATCAGGC
	ipdc-R	GAAGGATCCCTGTTATGCGAACC
Siderophore	*sid*-F	GGCCGAGGCCGACTGGTACCTCTACGGGGCGCAG
	sid-R	CCGGAGCGGGCACGGATCCCTTCTTCTTCA
Nitrogen fixation	*nif*H-F	TGCGAYCCSAARGCBGACTC
	*nif*H-R	ATS GCCATCATYTCRCCGGA

Table 2
Primer conditions used for the detection of PGPR traits by molecular analysis.

Conditions	*ipdc* primer	*sid* primer	*nif*H primer
Initial denaturation	94 °C, 2 min	95 °C, 1min	94 °C, 1 min
Denaturation	94 °C, 30 s	95 °C, 30 s	94 °C, 30 s
Annealing	59 °C, 30 s	50 °C, 30 s	57 °C, 30 s
Extension	72 °C, 40 s	72 °C, 1 min	72 °C, 2 min
Final extension	72 °C, 10 min	72 °C, 10 min	72 °C, 5 min
No. of Cycle	30	30	30

were autoclaved and packed into sterile low density polyethylene bags (LDPE) (Pouyat et al., 2015). The complete physicochemical parameter of the experimental soil was analysed (Table 3).

2.5.2. Preliminary study for selection of effective model plant

Experimental design consisted of three plant species namely *Alternanthera philoxeroides* (Alligator weed), *Chrysopogon zizanioides* (Vetiver) and *Cyperus Alternifolius* (Umbrella grass). The plantlets used in the study were obtained from the nursery of Vellore Institute of Technology, Vellore. The plantlets were washed thoroughly with running tap water to remove the adherent soil particles, plants were pruned into root and shoot length of 5 cm and were used for planting (three plants for each pot). The plants were acclimatized in green house for 2 d (18.4 °C to 39.4 °C with 79–89% relative humidity) and were watered regularly (Wang and Qin, 2006). Upon acclimatization, reactive green dye was supplemented at concentrations ranging from 100-10,000 mg kg^{-1} dry weight of soil. The experiment was maintained for a period of 30 d and the ability of the plant to tolerate RGD was evaluated by examining the changes in the physiological parameters (root and shoot length). The setup without dye supplementation served as control.

2.5.3. Pot culture study for the removal of RGD

Fresh plantlet of *Alternanthera philoxeroides* were collected and rinsed thoroughly to remove soil and dust particles and were pruned to 5 cm of root/shoot length prior to planting. Sterile LDPE bags were packed with 2 kg of garden soil. The pots were differentiated into two sets, phytoremediation (without PGP augmentation) and rhizoremediation (with PGP-VITAJ23 supplementation), the experiment was conducted for a period of 60 d under greenhouse conditions. After 2 d of acclimatization, experimental pots were artificially contaminated with various concentration of RGD (1000, 2000 and 3000 mg kg^{-1}) in the soil. The pots without dye supplementation served as control. For PGPR bioaugmentation treatment, the overnight grown 0.5 absorbance VITAJ23 culture (cell density of 10^9 cfumL^{-1}) was used (Liu et al., 2015). Cell suspension of 10 mL (4.0×10^{11} – 1.0×10^{12} CFUmL^{-1}) was added to 2 kg of the soil after every 10 d and mixed thoroughly for uniform distribution. Plants were harvested every 15 d for the assessment of various morphological and biochemical parameters (Xun et al., 2015). The experimental flowchart is depicted in Fig. 1.

2.5.4. Analysis of various parameters

2.5.4.1. Measurement of growth parameters. Upon uprooting the plant after equal interval of 15 d, the root and shoot length was measured for each plant along with control. Stastical analysis by one way analysis of variance (ANOVA) was performed.

2.5.4.2. Photosynthetic pigment analysis. The chlorophyll (*a* and *b*) pigment was determined spectrophotometrically using Arnow's equation (Arnon, 1949; Moran, 1982). Fresh leaves (100 mg) was homogenized with 80% acetone and filtered. Absorbance reading of the supernatant was measured at 645 and 663 nm. Absorbance measurements were converted to mg $Chlg^{-1}$ leaf tissue using Arnow's equation (Eqs. 2–4).

$$\text{Chla (g/L)} = 0.0127\,\text{X A663} - 0.00269\,\text{X A645} \quad (2)$$

$$\text{Chlb (g/L)} = 0.0029\,\text{X A663} - 0.00468\,\text{X A645} \quad (3)$$

$$\text{Total Chl (g/L)} = 0.0202\,\text{X A663} + 0.00802\,\text{X A645} \quad (4)$$

2.5.4.3. Decolourization experiment. Amount of dye present in the soil was estimated by calculating the absorbance reading at λ_{max} of the dye (420 nm) using UV–vis spectrophotometer. Soil (25 g) was mixed with 50 mL of distilled water and was kept in shaking condition at 150 rpm for 1 h. The mixture was centrifuged at 10,000 rpm for 20 min. The percentage removal of RGD was calculated at λ_{max} of the dye using the formulae Eq. 5 (Balapure et al., 2014)

$$\%\,Decolourization = \frac{Initial\ \ absorbance - Final\ \ absorbance}{Initial\ \ absorbance} \times 100 \quad (5)$$

2.5.4.4. Enumeration of soil microbial load. The bacterial load in the rhizoplane of both phyto and rhizomediation plants were scraped in test tube containing 10 ml of distilled water and was plated onto nutrient agar. The appearance of bacterial colonies was determined after overnight incubation and colony-forming units per millilitre was calculated (Zhao et al., 2011).

Table 3
Total soil analysis before and after remediation treatment.

Parameter	Control soil	Artificially contaminated soil	After phytoremediation treatment	After rhizoremediation treatment
pH	7.01	8.7	7.56	7.16
EC (ms/cm)	0.62	9.24	0.73	0.69
Organic Matter (%)	12.1	42.66	21.5	16.1
Nitrate (ppm)	48.6	32.73	40.5	44.7
Ava P (ppm)	28.6	22.33	26.1	27.7
Exc Ca (ppm)	845	1589	977	840
Exc K (ppm)	108	143	122	110
Ava S (ppm)	37.6	366.80	40.1	39.7
Exc Mg (ppm)	238	294.66	240	238
Exc Na (ppm)	158	732	230	172
Ava Zn (ppm)	3.59	4.63	4.01	3.78
Ava Mn (ppm)	29.3	56.26	35.7	30.34
Ava Fe(ppm)	19.55	53.70	28.6	22.4
Ava Cu(ppm)	2.82	8.52	3.67	3.01
CEC (by addition)# (Meq/100 g)	7.15	19.12	7.34	7.23
K Saturation # (%)	2.87	8.88	2.67	3.10

a) EC = electrical conductivity; Exc. = exchangeable; Ava. = available; CEC = cation exchange capacity.

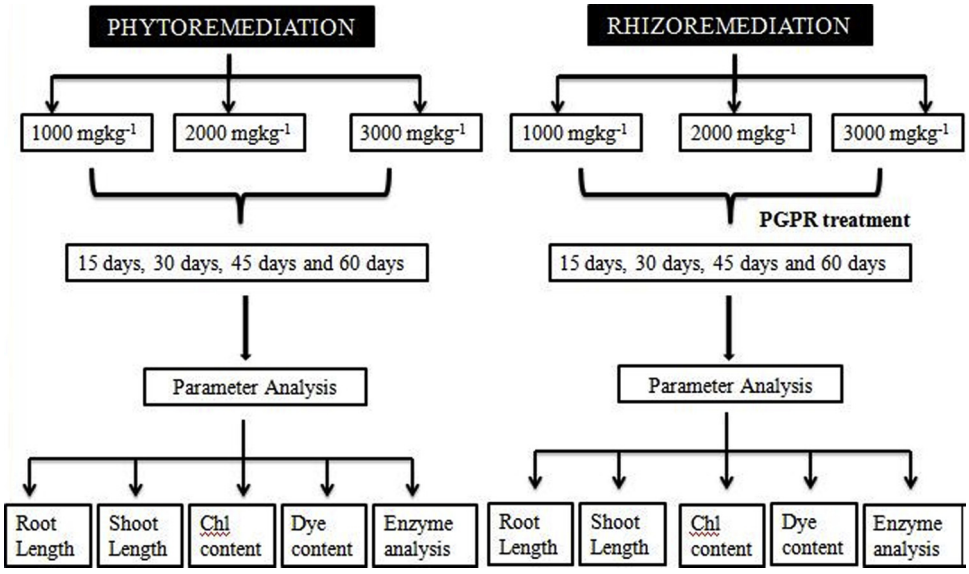

Fig. 1. Workplan for phyto and rhizoremediation studies.

2.6. Determination of enzyme activity by the plant

Synthesis of various oxido-reductase enzyme was monitored.

2.6.1. Enzyme source

The plants root, stem and leaves samples (2 g each) of the control *A. philoxeroides* and plantlets after 60 days of phyto and rhizoremediation treatments were chopped and suspended in 50 mM potassium phosphate buffer (pH 7.4) before grinding. The ground samples were centrifugation at 8000 rpm for 20 min at 4 °C and the supernatant was analyzed for extracellular enzyme (Khandare and Govindwar, 2015).

2.6.2. Enzyme activity

The enzymes involved in the degradation of azo dye such as azo reductase, lignin peroxidase (LiP), laccase and DCIP - NADH reductase were determined spectrophotometrically at 28 ± 2 °C (Lade et al., 2012; Chen and Yien, 2015). All the assay procedures were conducted at room temperature in triplicate. The change in absorbance unit per minute per mL of the enzyme was termed as one unit of enzyme activity.

2.7. Analysis of bio-transformed products by analytical procedures

The decolourized supernatant of the bacterial system, plant system and the plant-microbe association was obtained by centrifugation at 10,000 rpm for 25 min. The obtained supernatant was mixed with equal volume of ethyl acetate. The metabolites formed after decolourization of RGD was concentrated in a rotary evaporator and was used for HPLC, FTIR and GC–MS analysis. HPLC analysis was performed using an isocratic Perkin-Elmer instrument C18 system at a wavelength of 420 nm in order to monitor the degradation process. The gradient of methanol was used as mobile phase; the flow rate of 1 $mlmin^{-1}$ was maintained during the process (Waghmode et al., 2011). FTIR was performed to identify the presence or peak shift before and after treatment using a Nicolet FTIR (AVATAR- 330) model. The changes were visualized in the mid-IR region (400 - 4000 cm^{-1}) with 16 scan speed and 4 cm^{-1} resolutions (Telke et al., 2010). The identification of metabolites formed after decolourization process was detected using Perkin Elmer GC model (30mX0.25min X 0.25 µm) equipped with Clarus 680 EZ Mass spectrometer. Mass spectra were visualized with Turbo Mass Version 5.4.2 software and fragment ranging from 50 to 600 Da size were analysed (Waghmode et al., 2011).

2.8. Computational analysis and pathway prediction

The predicted metabolites obtained after GC–MS analysis of the bacterial broth, and the decolourized products by the plant system and the crude reactive green dye was converted into its corresponding SMILE format (Simplified molecular input line entry). With the help of computational analysis software, PathPred: Pathway Prediction server (www.genome.jp/tools/pathpred/) the various plausible pathways involved in the degradation of RGD was generated (Moriya et al., 2010).

2.9. Statistical analysis

Data were analysed by one-way analysis of variance (ANOVA) with Tukey–Kramer multiple comparisons test with $P < 0.05$ significance level.

3. Results

3.1. Isolation and identification of microorganism

Upon incubation of the MSM plates, a clear halozone was observed around the isolate VITAJ23, hence it was purified and maintained. Phylogenetic analysis by 16S rRNA gene sequencing revealed the bacterial strain as the closest neighbour of *Klebsiella variicola* with 98% sequence similarity. The sequence was further submitted in NCBI GenBank with an accession number KX7707 (Fig. 2). Dye decolourization assay revealed 90.03% decolourization of reactive green dye within 96 h (S2 (a)). UV–vis spectral analysis (400–900 nm) of the decolourized broth showed decrease and shift in λ_{max} (Fig. S2 (b)). Control dye exhibited peaks at 433, 635 and 679 nm, whereas a significant decrease or absence in the major peaks was observed in the decolourized broth. The appearance of new peaks and disappearance of major peaks supports the degradation of dye.

3.2. Assessment of plant growth promoting traits

The effective bacterial isolate VITAJ23 was tested positive for all the PGPR traits. It was found to produce siderophore, indole acetic acid and ammonia strongly. The isolate also solubilized inorganic Ca-phosphate. The characteristic pink colour in the bacterial broth indicated production of IAA (Fig. S3A). Quantitative assessment revealed 21.77 µg mL^{-1} of indole acetic acid production when 5 mg mL^{-1} tryptophan was supplemented (Table 4). The production of ammonia was confirmed by the

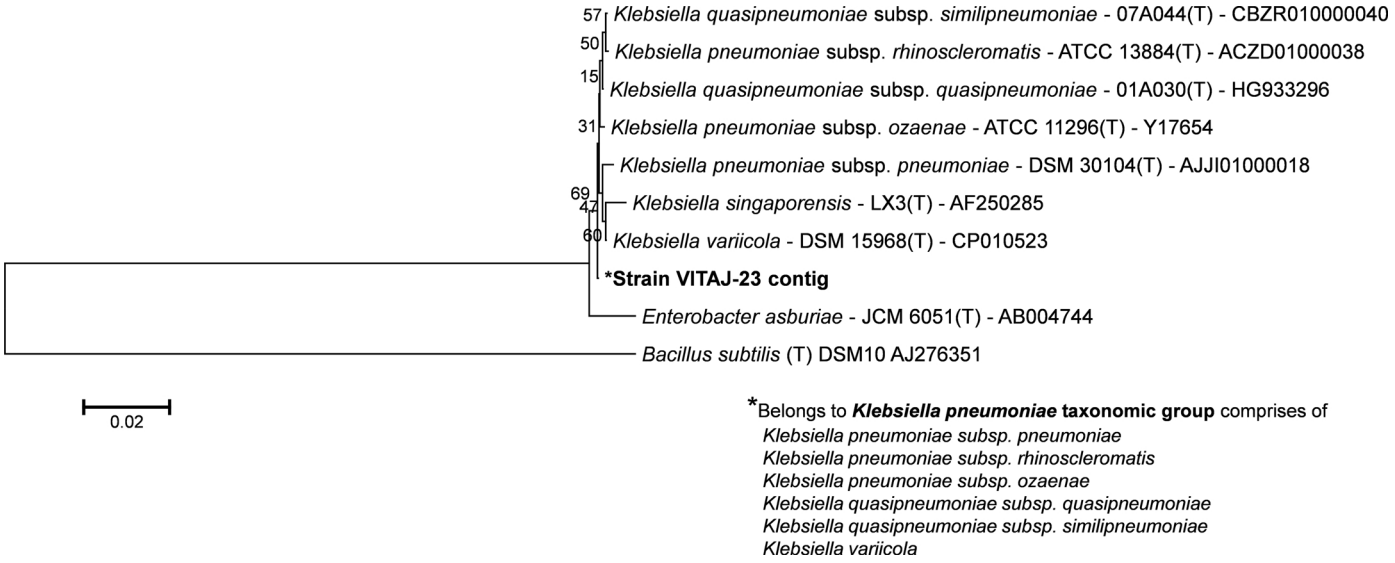

Fig. 2. Phylogenetic analysis of 16S rRNA gene sequence of effective bacterial isolate VITAJ23 (The percent numbers at the nodes indicate the levels of bootstrap support based on neighbour-joining analyses. The scale bar (0.02) indicates the genetic distance.

Table 4
Quantification data of PGPR traits exhibited by VITAJ23.

Isolate	Indole acetic acid (μgml^{-1})[a]	Phosphate solubilisation index	Siderophore production (μgml^{-1})
VITAJ23	21.77 μgml^{-1}	1.33	630 μgml^{-1}

[a] Indole acetic acid production with 5 $mgml^{-1}$ treatment of tryptophan.

appearance of deep yellow to brownish colour in the peptone media upon addition of Nessler's reagent (Fig. S3D). Quantitative analysis by microtiter plate revealed 630 μgmL^{-1} of siderophore in the broth (Table 4).

3.3. Molecular amplification of PGPR candidate gene

Agarose gel electrophoresis was performed for the PGPR target genes using 1.2% gel. PCR amplified products of VITAJ23 yielded the

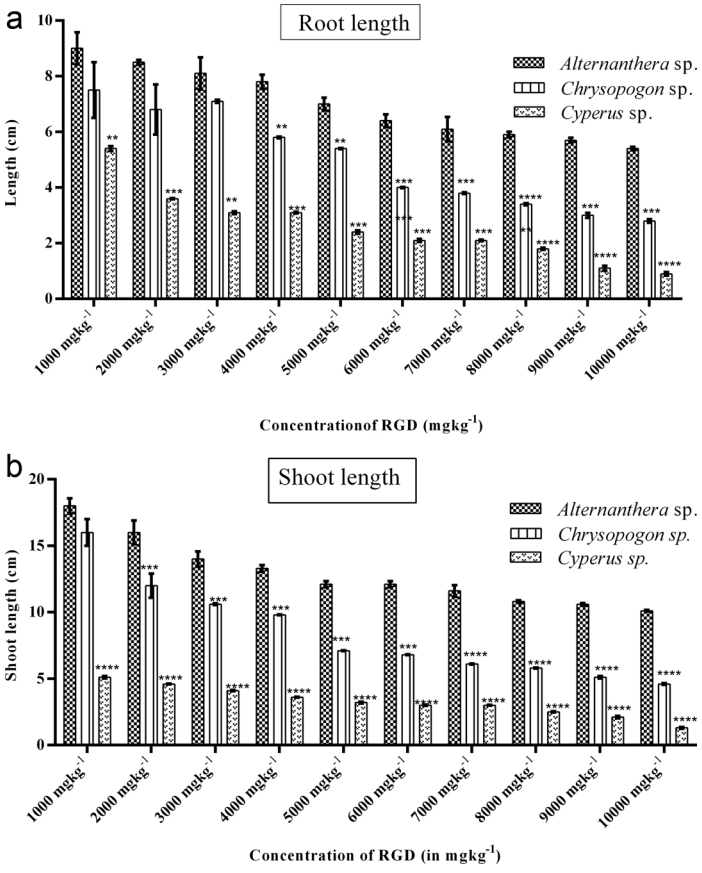

Fig. 3. a). Root length in preliminary setup using *Alternanthera philoxeroides*, *Chrysopogon zizanioides* and *Cyperus Alternifolius* plantlets at different RGD concentration (**P < 0.05, ***P < 0****, P < 0.0001), (b) Shoot length in preliminary setup using *Alternanthera philoxeroides*, *Chrysopogon zizanioides* and *Cyperus Alternifolius* plantlets at different RGD concentration (***P < 0.05, ****P < 0.0001).

expected 530bp, 300bp and 360bp amplicons indicating the presence of *ipdc*, *sid* and *nif*H candidate gene (Fig. S3E (a), (b)).

3.4. Phyto and rhizoremediation study

3.4.1. Preliminary study for selection of effective model plant

Preliminary screening of the plant species was carried out to determine the tolerance level of the plants towards RGD toxicity. Reactive green dye supplementation at higher concentrations reduced the growth of the plant, major impact of RGD toxicity was observed in *Cyperus Alternifolius* plant, followed by *Chrysopogon zizanioides* and *Alternanthera philoxeroides*. A linear decrease in root and shoot length was observed in *Cyperus Alternifolius* plant with increasing RGD concentration from 750 mg kg^{-1} (data not shown). Reactive dyes are recalcitrant xenobiotic compounds and are known to reduce the growth of the plant and disturbs their physiological functioning. *Chrysopogon zizanioides* was able to tolerate RGD upto 6000 mg kg^{-1} concentration, however the root and shoot length was greatly affected at elevated dye concentrations ($P < 0.001$) (Fig. S4 (a)). The preliminary study revealed *Alternanthera philoxeroides* as an effective plant model for the removal of RGD since it was capable of tolerating RGD upto 10,000 mg kg^{-1} concentration with minor variation in the root and shoot length at increasing dye concentration (data shown till 6000 mg kg^{-1}) (Fig. S4 (b)). Compared with *Chrysopogon zizanioides*, there was 45.71% and 54.54% increase in root and shoot length of *Alternanthera philoxeroides* at 10,000 mg kg^{-1} RGD concentration (Fig. 3a, 3b).

3.4.2. Pot culture study for the removal of RGD

The experimental setup was maintained using *A. philoxeroides* as a model plant to monitor its phyto and rhizoremediation potential for the removal of RGD.

3.4.3. Analysis of various parameters

3.4.3.1. Measurement of growth parameters. The plants in both the setup were capable of surviving tested reactive green dye concentrations. The plants augmented with bacteria had positive effects on their biomass production from 15th day onwards, and no significant difference was observed in root length as compared to control plants ($P > 0.05$). However, minor variation in the shoot length was observed at higher concentration of dye (3000 mgL^{-1}) at 30th and 45th interval which was observed to be restored by the end of 60th day interval (Fig. 4a). In phytoremediation setup, significant difference in root and shoot length was observed in the plants as compared to control set ($P < 0.001$) (Fig. 4b).

3.4.3.2. Photosynthetic pigment analysis. There was a significant decrease in the total chlorophyll content in the phytoremediation setup at higher concentration of reactive green dye (2000 and 3000 mg L^{-1}) as compared to the control set ($P < 0.01$). The total chlorophyll content in the rhizoremediation setup was found to be similar to that of control group (Fig. 4c).

3.4.3.3. Decolourization experiment. The phytoremediation setup with *A. philoxeroides* displayed a decolourization potential of 48.76%, 41.33% and 35.31% for 1000, 2000 and 3000 mg L^{-1} of RGD after a period of 60 d, whereas the plant and microbe association was found to be more efficient in dye decolourization. The plantlets of *A. philoxeroides* in augmentation with *Klebsiella* sp. VITAJ23 showed upto 75.53%, 66.90% and 59.01% decolourization for 1000, 2000 and 3000 mg L^{-1} of dye after 60 d (Fig. 4d).

3.4.3.4. Enumeration of soil microbial load. The rhizosphere region of the rhizoremediation setup plants indicated an increase in microbial load as compared to phytoremediation setup after 60 days (Table 6). The bacterial colonies isolated from the rhizosphere region of the rhizoremediation setup plants were of similar morphology as the effective strain VITAJ23.

3.5. Determination of enzyme activity by the plant

The enzymatic analysis of rhizoremediation plantlets revealed induction of azoreductase (45.50%), laccase (46.70%), lignin peroxidase (51.52%) and DCIP - reductase activity (57.49%) as compared to the control plants (Table 5). In the treated setup, maximum induction for oxidoreductase enzyme was observed in the root tissue region as compared to leaf and shoot. In phytoremediation plants the activity of azoreductase (26.05), laccase (30.61%), lignin peroxidase (31.94%) and DCIP-reductase (22.64%) was also found to be induced as compared to control plant set, however the rhizoremediation setup showed an enhanced activity (Table. 5).

3.6. Analysis of bio-transformed products by analytical procedures

HPLC analysis of crude RGD exhibited the presence of two peaks at 6.14 and 7.4 retention value (Fig. 5a), whereas peaks at retention value of 1.32, 1.72, 3.3, 4.8 and 6.87 were observed in the bacterial decolourized broth (Fig. 5b). The decolourized broth after phyto and rhizoremediation treatment showed similar peaks at retention value of 1.7, 2.52 and 4.4 min. Additional peaks at 2.03, 2.27 and 2.9 min was observed in the rhizoremediation decolourized broth, which was absent in the phyto setup (Fig. 5c, d).

The FTIR spectrum of control reactive green dye was compared with the extracted metabolites obtained after decolourization by the bacterial and plant system. Control dye represented the characteristic N═N stretching of the azo bond at 1597 cm^{-1}, whereas peaks at 1253.73-1226.73 corresponds to the presence of C−N stretch of aromatic amines (Fig. 6a). IR peak 1186 cm^{-1} confirmed the presence of sulfur compound and S═O stretching. The FTIR spectra of decolourized metabolites by the bacterium VITAJ23 presented vibrational peaks at retention value of 3300.20 representing N−H vibration, stretch of 2943.37 and 2831.50 corresponds to presence of $=CH_3$ or methyl group. IR peak at 1450.47 indicates presence of cyclic ring and peaks at 667.37 and 653.87 represents −C−H stretching (Fig. 6b). The decolourized broth obtained after phytoremediation and rhizoremediation treatment presented similar peaks at retention value of 2926.01, 1265.30 and 705.93 corresponding to presence of $=CH_3$ or methyl group, C−N stretching and C−N vibration respectively (Fig. 6c and d). Peaks at 1653.00, 1301.95 in the rhizo treated broth suggests the presence of N−H and O−H stretching. GC–MS analysis was performed to identify the metabolites produced during the biodegradation process and the chromatogram revealed the presence of several compounds (data not shown). The major intermediate found was 2-allylnapthalene (*m/z* 168, Rf value 12.55) by the VITAJ23 system whereas, l-alanine, n-acetyl and propenoic acid were found to be the probable metabolites produced by the plant system.

3.7. Computational analysis and pathway prediction

A pathway for the degradation of RGD was proposed using PathPred software. We propose that initially in the bacterial system, azoreductase enzyme acts on the N = N linkage of reactive green dye leading to the formation of intermediate (A) sodium 3-[(dichloro-1,3,5-triazin-2-yl) amino] benzene-1-sulfonate (Mw Wt- 341.93) and intermediate (B) sodium 1-amino-4-[(4-aminophenyl) amino]-9,10-dioxo-9,10-dihydroanthracene-2-sulfonate (Mw Wt- 431.39). Further, de-aromatization occurred in the cyclic structure and series of aromatic amines were formed ultimately leading to the formation of 2-allylnapthalene. In case of decolourization by the plant system, initially laccase enzyme acts on the dye molecule resulting in the asymmetric cleavage and formation of two unknown intermediates (A) and (B), later azoreductase enzyme acted on intermediate (A) which led to the formation of unknown

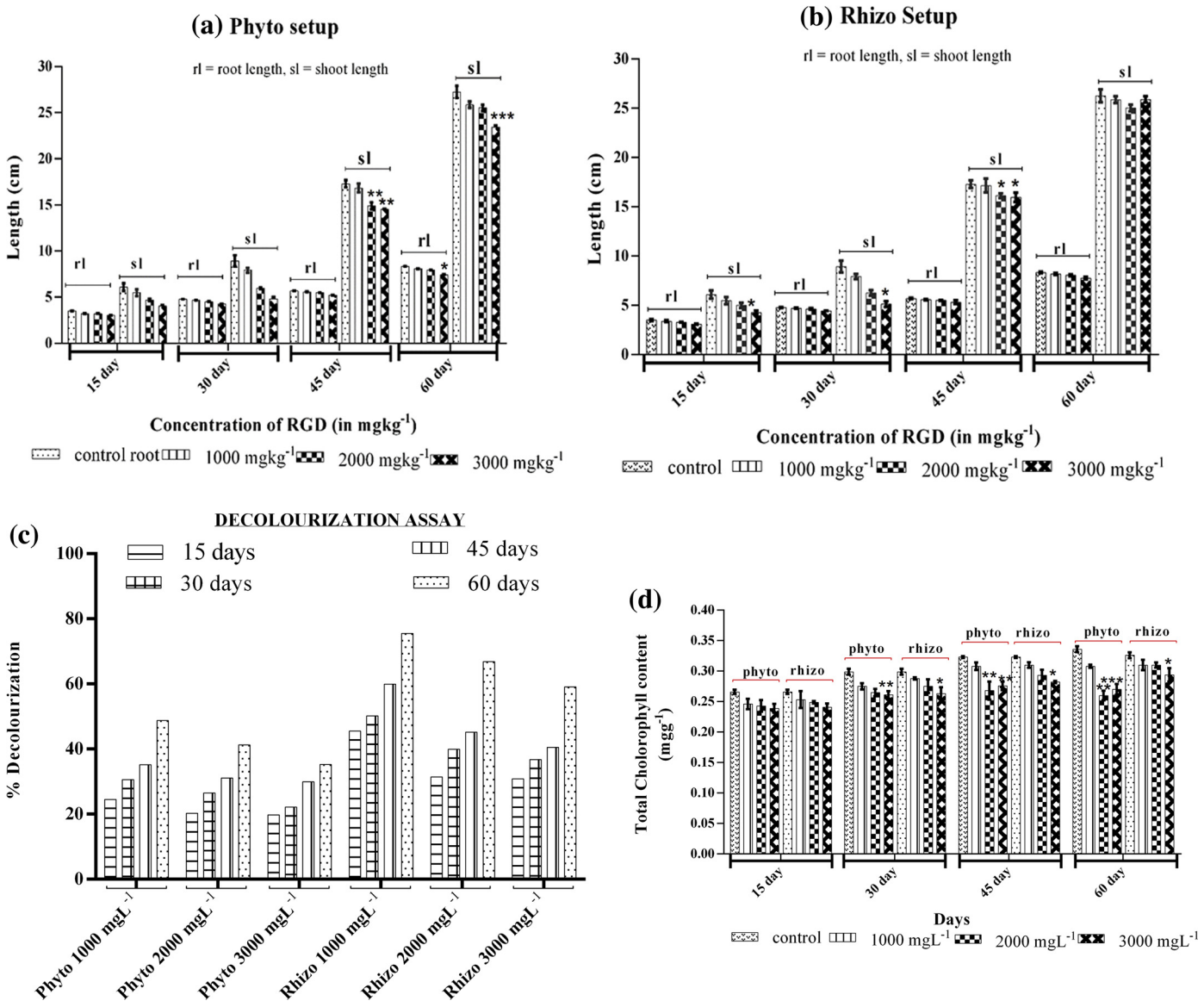

Fig. 4. a). Root and shoot length in phytoremediation setup, (b) root and shoot length in rhizoremediation setup (Values are mean of three experiments, standard error of mean (SEM), analysed by one-way ANOVA with Turkey Kramer multiple comparisons *P < 0.05, **P < 0 and ***P < 0.001), (c) Decolourization assay after each interval for phyto and rhizoremediation setup, (d) Total Chlorophyll content in phytoremediation and rhizoremediation setup.

intermediate (C), undergoes major conformational changes ultimately resulting in the formation of L-alanine, n-acetyl-and propenoic acid, as detected by GC–MS analysis (Fig. 7).

4. Discussion

Biological degradation of dyes has gained researcher's attention in the past few decades due to its cost effectiveness, formation of non-toxic by-products and eco-friendly nature (Waghmode et al., 2011). Plant-microbial synergism has established its effectiveness for the treatment of various pollutants (Glick, 2010; Khandare et al., 2013; Wojtera-Kwiczor et al., 2014). The root exudates of the plant provides primary carbon source which helps to nourishes and increases the viability of diverse microbial communities residing in the rhizosphere region, hence improving the *in-situ* remediation procedure for the removal of wide range of contaminants (Rentz et al., 2005). Preliminary screening of the plant species was performed to determine the tolerance level of the selected plants towards RGD toxicity. Vetiver grass or *Chrysopogon zizanioides* (L.) belongs to the Poaceae family and are characterized by the presence of tall, straight stem and narrow leaves with extensive root system. Vetiver was selected as a plant model since it has a great threshold levels for heavy metal (As, Cd, Cu, Cr, and Ni) and organic pollutant removal in the soil (Danh et al., 2009). *Cyperus Alternifolius* is also known to play a prominent role in the clean-up of wastewater (Leto et al., 2013). *Alternanthera philoxeroides*, the effective plant used in the study possesses extensive mat forming root system which imparts a higher rhizofilteration ability, hence making it an attractive and effective tool for wastewater treatment. Rane et al. (2015) reported the applicability of *A. philoxeroides* for the removal of Remazol red dye and textile effluents. The applicability of *A. philoxeroides* is also reported for the removal of several heavy metal such as Cr, Cu, Zn and Pb (Naqvi and Rizvi, 2000; Gu et al., 2003).

The 16S rRNA phylogenetic analysis revealed the isolated bacterium VITAJ23 to be the closest neighbour of *Klebsiella* sp. Previous studies by Cui et al. (2014) has reported *Klebsiella* sp. to have promising dye removal potential. In the present study, VITAJ23 was found to survive upto a concentration of 3500 mg L^{-1}, the strain showed an enhanced ability to withstand the toxic nature of the dye as compared to other strains reported previously (Ayed et al., 2011). The plant growth promoting trait of the isolate was tested prior to its use in remediation of

Table 5
Enzyme activities of *A. philoxeroides* plant control tissue at 0 h and after 60 days of reactive green dye exposure in the phyto and rhizoremediation setup.

	Azo reductase		LiP		Laccase		DCIP reductase	
	Control	Test	Control	Test	Control	Test	Control	Test
Phyto leaf	$0.87 \pm 0.08 \times 10^{-5}$	$1.27 \pm 0.08 \times 10^{-5*}$	$1.03 \pm 0.3 \times 10^{-4}$	$1.83 \pm 0.8 \times 10^{-4}$	$2.08 \pm 0.2 \times 10^{-5}$	$2.88 \pm 0.2 \times 10^{-5}$	$2.54 \pm 0.4 \times 10^{-7}$	$3.04 \pm 0.8 \times 10^{-7}$
Phyto shoot	$1.27 \pm 0.11 \times 10^{-3}$	$1.37 \pm 0.11 \times 10^{-3}$	$1.27 \pm 0.88 \times 10^{-4}$	$1.77 \pm 0.8 \times 10^{-4}$	$2.04 \pm 0.3 \times 10^{-5}$	$2.44 \pm 0.1 \times 10^{-5}$	$2.78 \pm 0.02 \times 10^{-7}$	$2.08 \pm 0.2 \times 10^{-7}$
Phyto root	$2.27 \pm 0.3 \times 10^{-4}$	$3.27 \pm 0.3 \times 10^{-4*}$	$2.13 \pm 0.7 \times 10^{-4}$	$3.13 \pm 0.8 \times 10^{-4*}$	$1.44 \pm 0.0 \times 10^{-5}$	$2.54 \pm 0.4 \times 10^{-5*}$	$1.04 \pm 0.8 \times 10^{-7}$	$2.54 \pm 0.2 \times 10^{-7*}$
Rhizo leaf	$1.27 \pm 0.18 \times 10^{-5}$	$1.77 \pm 0.08 \times 10^{-5}$	$1.47 \pm 0.03 \times 10^{-4}$	$2.93 \pm 0.04 \times 10^{-4*}$	$1.13 \pm 0.4 \times 10^{-4}$	$2.53 \pm 0.4 \times 10^{-4*}$	$1.07 \pm 0.1 \times 10^{-3}$	$3.24 \pm 0.1 \times 10^{-3**}$
Rhizo shoot	$2.27 \pm 0.18 \times 10^{-5}$	$4.27 \pm 0.1 \times 10^{-5*}$	$1.97 \pm 0.06 \times 10^{-3}$	$2.23 \pm 0.04 \times 10^{-3}$	$1.03 \pm 0.4 \times 10^{-4}$	$2.13 \pm 0.8 \times 10^{-4}$	$2.04 \pm 0.3 \times 10^{-3}$	$2.94 \pm 0.3 \times 10^{-3}$
Rhizo root	$5.67 \pm 0.18 \times 10^{-5}$	$9.56 \pm 0.38 \times 10^{-5***}$	$2.27 \pm 0.88 \times 10^{-4}$	$4.27 \pm 0.08 \times 10^{-4***}$	$2.02 \pm 0.02 \times 10^{-5}$	$3.79 \pm 0.12 \times 10^{-5**}$	$2.07 \pm 0.11 \times 10^{-3}$	$4.87 \pm 0.11 \times 10^{-3**}$

Values are mean of three experiments, standard error of mean (SEM), significantly different from control cells at *P < 0.05, at **P < 0 and at ***P < 0.001 by one-way ANOVA with Tukeye Kramer multiple comparisons test. (value are given as specific activity mg^{-1} of protein).

Table 6
Enumeration of bacteria in the rhizoplane region of the plants.

Sample	No. of colonies in the phyto setup (cfu/mL)	No. of colonies in the rhizo setup (cfu/mL)
Control	1×10^3	1.2×10^3
1000 mg L^{-1}	0.64×10^3	0.74×10^3
2000 mgL^{-1}	0.57×10^3	0.63×10^3
3000 mg L^{-1}	0.39×10^3	0.49×10^3

RGD. VITAJ23 produced siderophore, indole acetic acid and ammonia and was capable of solubilizing inorganic Ca-phosphate. Previously Liu et al. (2015, 2014) has established the potential of *Klebsiella* sp. for enhancing the growth and metabolism of plant. Synthesis of IAA had profound effect on the plant growth and production of siderophore increased the survivability of the bacteria in iron deficient conditions (Patten and Glick, 2002; Dimkpa et al., 2008). Previous reports have reported the use of PGP strains like *R. erythropholis* and *R. meliloti* for the bioremediation of oil aromatic and chloroaromatic compounds (Huang et al., 2008; Hou et al., 2015). The gene encoding the PGP traits were also amplified by the bacterial system. The *nif*H gene is the most widely sequenced marker gene responsible for encoding enzyme involved in atmospheric nitrogen fixation (Chowdhury et al., 2009). The *sid* gene is responsible for enhancing siderophore uptake by the bacterial system (Moelling et al., 2007), whereas *ipdc* gene encodes for indole-3-pyruvate decarboxylase involved in the biosynthesis of indole-3-acetic acid (IAA) (Patten and Glick, 2002). Molecular amplification of *nif*H, *sid* and *ipdc* gene by the bacterial system confirmed its role as a PGP strain.

The pot culture study demonstrated *A. philoxeroides* to be tolerant to reactive green dye as the growth was confluent upto 3000 mg L^{-1} of dye. The biomass and other growth parameters were enhanced when bioaugmentated with VITAJ23. Study by Dey et al. (2004) reported an increase in the root length of *Arachis hypogaea* seedling after application of PGPR strain. There was significant reduction observed in the total chlorophyll content of the phyto setup plants at higher concentration of the dye, which can be due to various factors including oxidative stress and disorientation of chloroplast. Similar pattern was observed for the removal of acid blue dye by *Hydrocotyle vulgaris* plant (Vafaei et al., 2013). However, the chlorophyll content was found to be restored after augmentation of *Klebsiella* sp. VITAJ23 from 15th d onwards. Pot culture study revealed that an increase in exposure time of dye and augmentation of PGP-VITAJ23 strain, enhanced the uptake efficiency of the plant. Dye decolourization study revealed 75.53% (1000 mg L^{-1}) and 48.76% (1000 mg L^{-1}) removal of RGD in rhizo and phytoremediation setup. Reports by Kabra et al. (2013); Khandare et al. (2013) and Khandare and Govindwar (2015) have demonstrated efficient removal of azo dye by plant system in an *in-vitro* culture based approach, however this is the first report which shows the application of plant-bacterial synergism in pot culture based removal study. It is an established fact that the success of pot culture study depends on the survival and growth of plants used in contaminated sites, as well as the capability of active microbial population to survive and adapt in the rhizosphere region (Cook and Hesterberg, 2013). Enzymatic analysis revealed significant induction of oxidoreductase enzymes in the root tissue of plant, as compared to leaf and shoot. The induction in the activity of enzymes was found to be enhanced after the augmentation of VITAJ23, hence suggesting its presumable role in the degradation of dye into simpler compounds by synergistic approach. Rane et al. (2015) also observed similar pattern in the induction of oxido-reductase enzyme in the degradation of azo dye.

The possible mechanism of RGD degradation was proposed by HPLC, FTIR and GC–MS analysis. The use of analytical tools for confirming the degradation of dyes have been documented by (Lade et al., 2012; Waghmode et al., 2011). HPLC analysis confirmed the conversion of crude dye into various metabolites. The FTIR spectrum revealed an

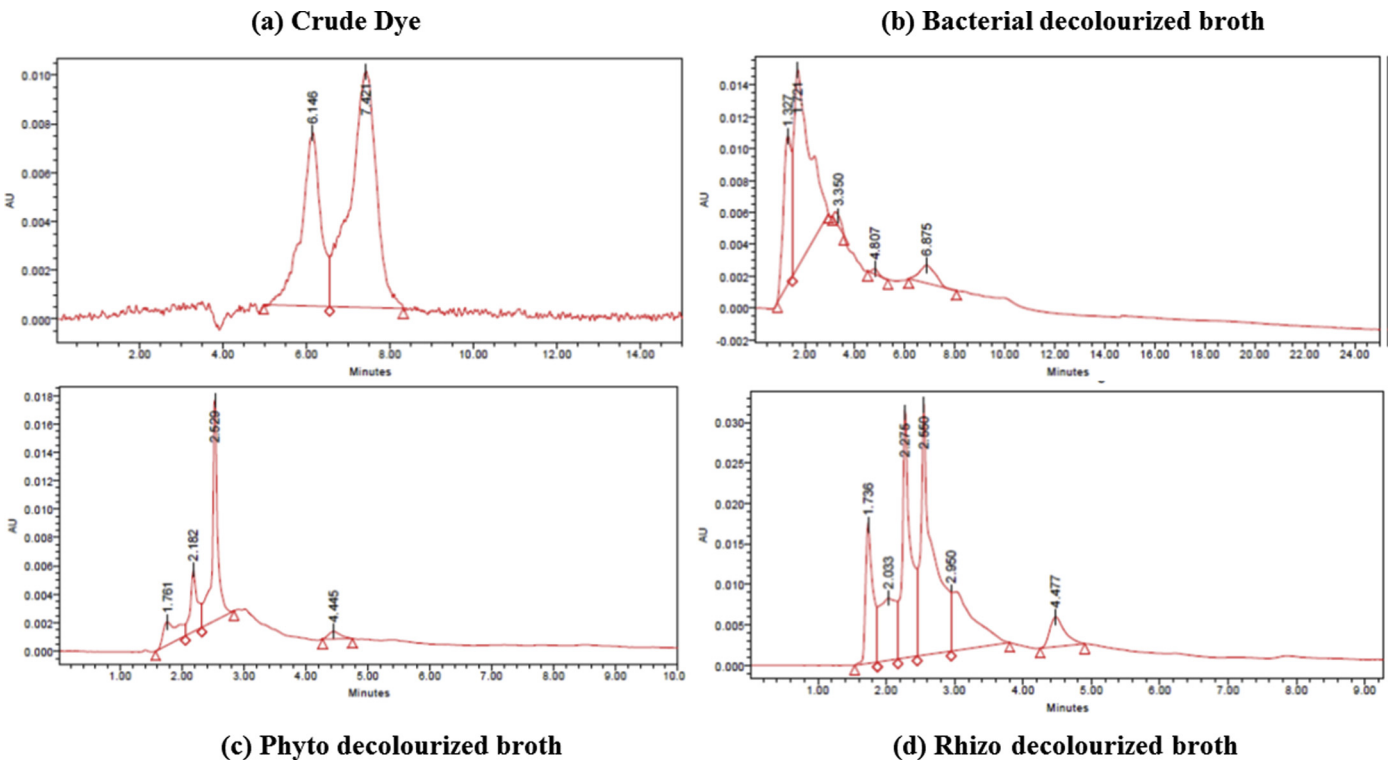

Fig. 5. a) HPLC chromatogram of control dye reactive green dye, (b) Chromatogram of *Klebsiella* sp. VITAJ23 decolourized metabolites after 96 h of incubation, (c) decolourized metabolites of phytoremediation setup (d) decolourized metabolites of rhizoremediation setup.

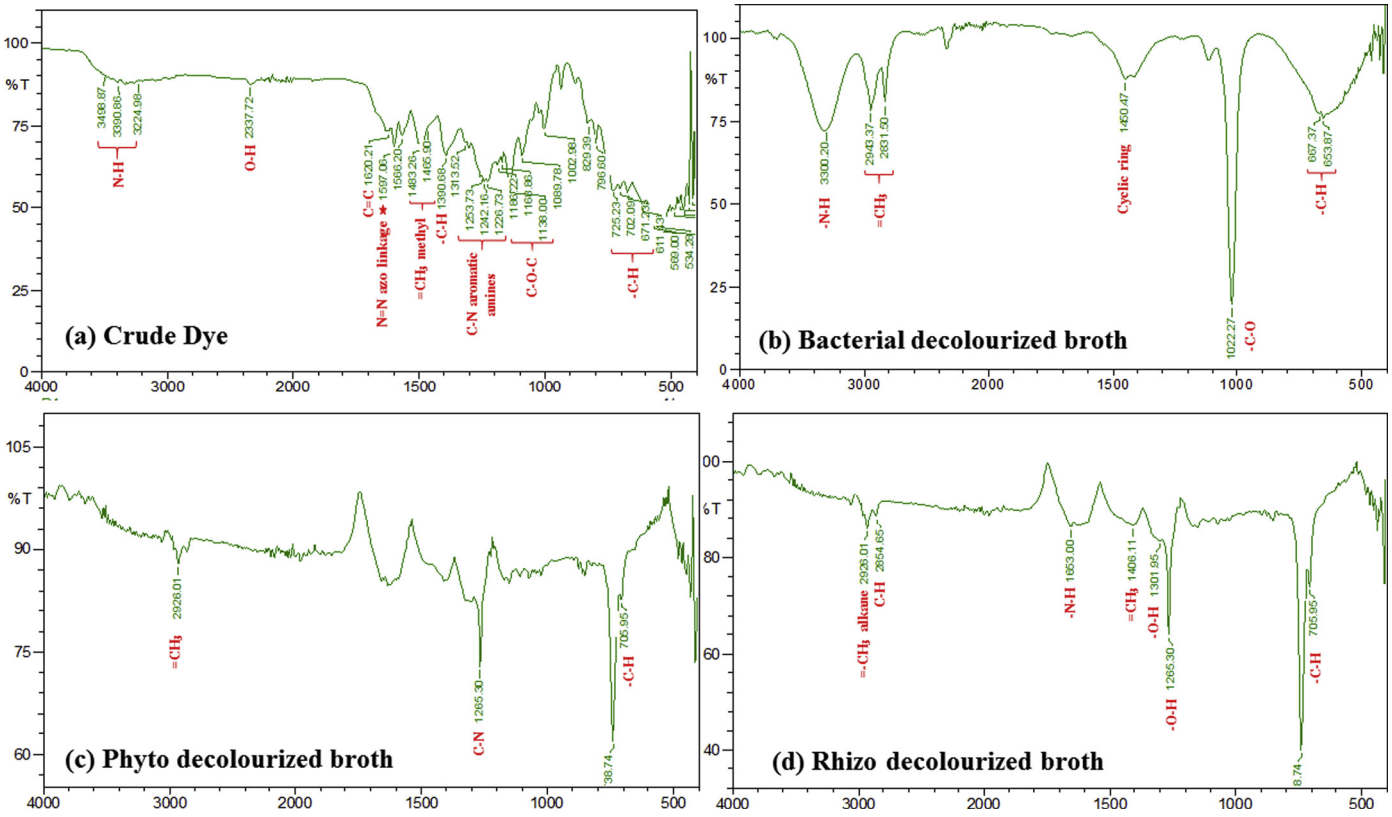

Fig. 6. a) FTIR spectrum of control reactive green dye, (b) spectra of *Klebsiella* sp. VITAJ23 decolourized metabolites after 96 h of incubation, (c) decolourized metabolites of phytoremediation setup (d) decolourized metabolites of rhizoremediation setup.

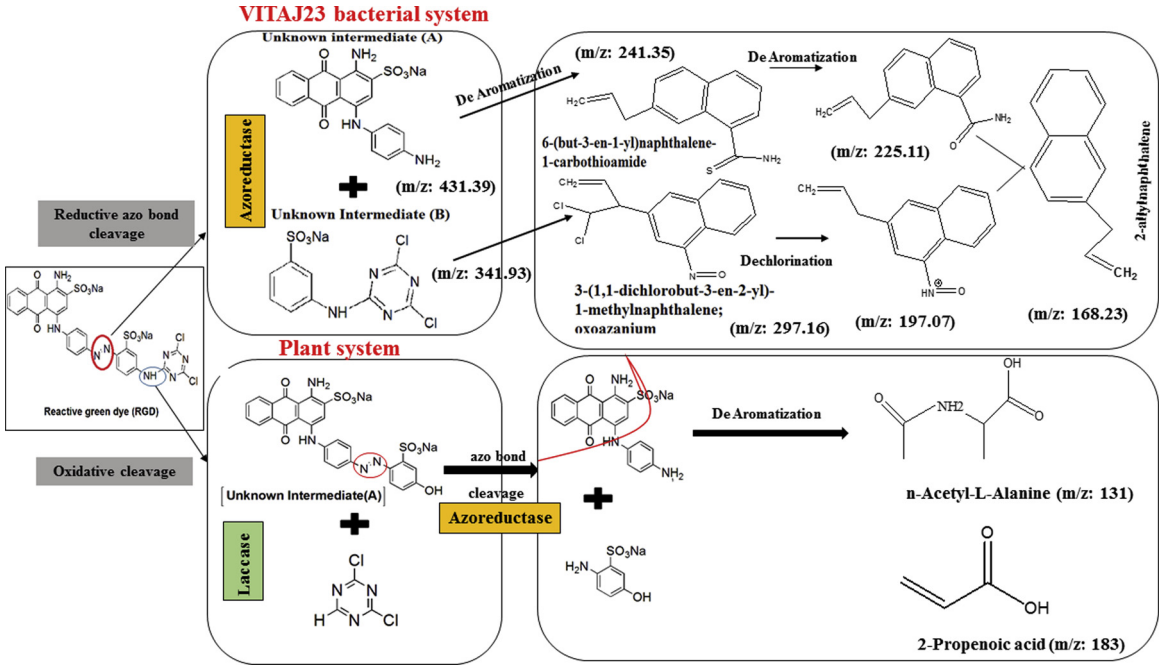

Fig. 7. Proposed pathway for the degradation of reactive green dye by *Klebsiella* sp.- VITAJ23 and the plant system.

absence in N═N azo bond suggesting reductive cleavage of the azo bond in reactive green dye. The disappearance of 829.39 cm^{-1} peak proves the removal of sulfur containing groups of RGD. The FTIR spectra of the bacterial decolourized broth revealed the presence of cyclic aromatic ring at 1450.47 cm^{-1} and ═CH_3 ring at 2943.37 cm^{-1}, which confirms the identification of 2-allynapthelene (*m/z* 168.23) by GC–MS analysis. Similarly, the appearance of 1663.00 and 1265.30 cm^{-1} vibrational peaks in phyto and rhizoremediation decolourized broth confirmed the formation of L-alanine, n-acetyl-and propenoic acid and a pathway was derived. It can be concluded that the plant system in association with the augmented bacteria VITAJ23 was effective in removal of reactive green dye. The study also emphasized the degradation of RGD into non-toxic products. Therefore, the current study could be employed on a larger scale for effective degradation.

Conflict of interest

The authors declare no conflict of interest

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.micres.2018.12.004.

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