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Detoxification of Distillery Effluent through *Bacillus thuringiensis* (MTCC 4714) Enhanced Phytoremediation Potential of *Spirodela polyrrhiza* (L.) Schliden

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Rapid growth of distilleries in India resulted into substantial increase in industrial pollutant load. There are 254 distilleries in India producing 1000 million litres of alcohol and 3.5 x 10^8 kilolitres of effluent each year (AIDA 1994). The industrial waste generated by various distillery units are posing serious threat to the adjoining aquatic and terristrial habitats due to practice of discharging them into nearby water courses and lands (Agarwal and Pandey, 1994). The distillery effluents have high BOD, COD, phenols & heavy metals (Chandra, 2003). The colour of the effluent persists even after the anaerobic treatment and poses a serious threat to the environment. The water bodies receiving coloured wastes got coloured and affect the penetration of light in aquatic ecosystems, which in turn affect the aquatic life (Kumar et al 1997). Therefore, it is essential to reduce the toxic level of various pollutants in the distillery effluent before discharging them into nearby watercourses or lands.

Phytoremediation of effluents is an emerging low cost technique for removal of toxicants including metals from industrial effluent and is still in experimental stage (Lasat et al 2002). Aquatic plants have excellent capacity to reduce the level of toxic metals (Cr, Pb, Cd etc.), Biochemical oxygen demand (BOD) and total solids from polluted waters (Kadlec and Kadlec 1979). Besides, various microorganisms are also reported to reduce the load of pollutants from industrial effluents (Miyata et al 2000; Knotek-Smith et al 2003). However, no study has been conducted with bacterial transformation of recalcitrant colouring compound of distillery effluent followed by subsequent reduction of the remaining load of pollutants by phytoremediation process. Hence, a commonly occurring free floating macrophyte, *Spirodela polyrrhiza* (L.) Schliden and a strain of *B. thuringiensis* (isolated from distillery sludge) have been selected for the bioremediation of the distillery effluent and results are being presented in this manuscript.

MATERIALS AND METHODS

Anaerobically treated distillery effluent (UT) was collected from K.C. Thapar & Bros. Ltd., Unnao (U.P.), India. The physico-chemical analyses (BOD: biochemical oxygen demand; COD: chemical oxygen demand, TS: total solids

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and phenols) of effluent were done by standard procedures (APHA 1992). Plants of *S. polyrrhiza* were collected from unpolluted water body from Lucknow, India and were acclimatized in hydroponic tubs in experimental field at Industrial Toxicology Research Centre, Lucknow. Fronds of the third generation were picked and placed in 5% Hoagland solution in growth chamber (Light:Dark, 14:10 h; Temperature 25±2°C and 115μ mol m^{-2} s^{-1} illumination provided through florescent tube light). Experiments were conducted in two phases. Under first phase distillery effluent was treated by *S. polyrrhiza* for 96 h (ST) and role of the plant in decolourisation of effluent and reduction of pollutants was studied. Various concentration of distillery effluent (5, 10, 20, 40, and 100) were prepared by adding required aliquot of the distilled water. Approximately 1 g plant material was placed in glass dishes (diameter 15.5 cm) containing 100 ml effluent of each concentration in triplicates. The petri plates were then placed in growth chamber under aforesaid conditions. Plants placed in 5% Hoagland solution served as control. The solution volume was constantly maintained at 100 ml by adding Hoagland solution. The plants were harvested after 96 h. for metal analysis and to study the physiological parameters of *S. polyrrhiza.*

Fresh weights of the plants were recorded. Biomass of the test plant was reported on dry weight (DW) basis. Reducing sugars and starch were estimated by the method of Dubois et al (1956). For metal analysis, dried (1g) plant samples (ground in a grinder) or one ml treated and untreated distillery effluent samples of different dilutions were digested in HNO_3: $HClO_4$ (3:1, v/v) at 80°C and various metal (Cd, Cu, Cr, Fe, Mn, Ni, Pb and Zn) concentrations were measured by Inductively Coupled Plasma Spectrophotometer (8440 Plasma Lab, Australia). Decolourisation of distillery effluent (decrease in melanoidin content) was measured at 475 nm as per procedure given by Ohmomo et al (1987). The decolourisation after phytoremediation, by *S. polyrrhiza* for 96 h, was expressed as percentage decrease in absorbance at 475 nm against initial absorbance at the same wavelength (Ohmomo et al 1987). The reduction in pollutant load (BOD, COD, TS, Phenols and heavy metal content) in *S. polyrrhiza* treated distillery effluent has been recorded as per procedures described above.

While in second phase, distillery effluent was treated by a strain of *Bacillus thuringiensis* (MTCC 4714, IMTECH, Chandigarh, India) isolated from distillery sludge for 7 d (BT) and *S. polyrrhiza* plants were exposed to the effluent treated by bacteria for 96 h (BT+ST). The pure culture of *B. thuringiensis* was inoculated to 50 ml modified glucose peptone yeast extract melanoidin broth (GPYM) containing 1% (w/v) glucose, 0.1% K_2HPO_4 (w/v) and 0.05% $MgSO_4.7\ H_2O$ (w/v). Inoculated flasks were incubated overnight on rotary shaker (125 rpm). The growth of the bacteria was monitored at 660nm. The different concentrations of distillery effluent (5, 10, 20, 40 and 100%) were prepared by adding required aliquots of the effluent to modified glucose peptone yeast extract melanoidin broth (GPYM: Ohmomo et al 1987). The inoculum (1 ml) was taken from log phase culture (1.1 x 10^7 cells/ml) of *B. thuringiensis* and inoculated to 500 ml conical flasks containing effluent of each concentration in triplicate. Flasks

containing 500 ml modified GPYM and inoculated by same size of inoculum of *B. thuringiensis* served as control. The cultures were allowed to grow at 35 ± 2^{0}C for 7 consecutive days. After 7 d cultures were centrifuged at 3000 rpm at 4^{o}C to separate the bacterial biomass. The supernatant was sterilized by passing through membrane filter (0.22 µm, Millipore).

The reduction in pollutants load including heavy metals of *B. thuringiensis* treated as well as *B. thuringiensis* followed by *S. polyrrhiza* remediated distillery effluent has been recorded as per aforementioned procedure. Besides, heavy metal accumulation in fronds of *S. polyrrhiza* and its impact on total biomass, reducing sugars and starch contents were also recorded as per procedures described above.

A one-way analysis of variance in complete randomized design was performed to assess the variability and validity of the data (Gomez and Gomez 1984). Duncan's multiple range test (DMRT) was used to find out the variation between means.

RESULTS AND DISCUSSION

The distillery effluent showed high concentration of various heavy metals (ANOVA, $p<0.05$). It contained maximum amount of Fe followed by Zn, Cu, Pb, Cd, Cr, Mn and Ni, respectively even after anaerobic treatment (Table 1). Further, significantly (ANOVA, $p<0.05$) high BOD, COD, total solids and phenols was observed in distillery effluent used in the present study (Table 3). It has been noted that *B. thuringiensis* significantly (ANOVA $p<0.05$) reduced the concentrations of various heavy metals in distillery effluent after 7 d (Table 2). Maximum reduction in levels of all the metals studied was observed from 5% distillery effluent followed by 10, 20, 40 and 100%.

It has been observed that when S. *polyrrhiza* was subjected to grow on various concentrations of distillery effluent for 96 h, accumulated significant amount (ANOVA $p<0.05$) of various heavy metals (Figure 1). *S. polyrrhiza* accumulated maximum amount of these metals from 100% distillery effluent followed by 40, 20, 10, and 5% (DMRT, $p<0.05$). It was interesting to note that *B. thuringiensis* treated (7 d) distillery effluent when subjected to phytoremediation by *S. polyrrhiza* for 96 h, resulted into increased removal of these metals in comparison to *S. polyrrhiza* or *B. thuringiensis* alone (Figure 1).

S. polyrrhiza significantly (ANOVA $p< 0.05$) decolorized the distillery effluent after 96 h (Table 3). But the decolourization potential of *S. polyrrhiza* was found to be increased when it was exposed to the effluent treated by aforesaid bacterium for 7 d (BT) for 96h (BT+ST). In this case maximum decolourization was 44.66% in 5% distillery effluent (DMRT, $p<0.5$) while minimum was 15.55% in 100% distillery effluent after 96 h of exposure. It has been observed that *B. thuringiensis* concomitantly reduced the toxicity of distillery effluent after 7d exposure. Hence, *S. polyrrhiza* could grow in 100% effluent (treated by *B. thuringiensis* for 7d) without any visual toxicity symptoms upto 96 h.

Table 1. Heavy metal concentrations (ppm) in distillery effluent prior to bioremediation.

Metals	Effluent concentration (%)				
	5	10	20	40	100
Cd	$0.040^{e}\pm0.002$	$0.082^{d}\pm0.003$	$0.162^{c}\pm0.01$	$0.328^{b}\pm0.01$	$0.830^{a}\pm0.03$
Cr	$0.036^{e}\pm0.001$	$0.074^{d}\pm0.004$	$0.153^{c}\pm0.01$	$0.226^{b}\pm0.01$	$0.796^{a}\pm0.03$
Cu	$0.140^{e}\pm0.005$	$0.284^{d}\pm0.012$	$0.575^{c}\pm0.03$	$1.150^{b}\pm0.04$	$2.920^{a}\pm0.13$
Fe	$0.840^{e}\pm0.036$	$1.688^{d}\pm0.075$	$3.378^{c}\pm0.15$	$6.762^{b}\pm0.32$	$16.94^{a}\pm0.73$
Mn	$0.032^{e}\pm0.001$	$0.060^{d}\pm0.003$	$0.121^{c}\pm0.01$	$0.250^{b}\pm0.01$	$0.644^{a}\pm0.03$
Ni	$0.022^{e}\pm0.001$	$0.052^{d}\pm0.003$	$0.101^{c}\pm0.01$	$0.198^{b}\pm0.01$	$0.526^{a}\pm0.02$
Pb	$0.080^{e}\pm0.003$	$0.171^{d}\pm0.006$	$0.348^{c}\pm0.02$	$0.692^{b}\pm0.03$	$1.76^{a}\pm0.070$
Zn	$0.380^{e}\pm0.016$	$0.775^{d}\pm0.028$	$1.550^{c}\pm0.07$	$3.101^{b}\pm0.12$	$7.84^{a}\pm0.22$

Mean (n=3) ± SD; ANOVA, $p<0.05$ (for each metal separately). Different superscripts (a-e) on means indicate significant difference ($p<0.05$) between means in a row (for each metal).

While in untreated effluent toxicity symptoms (yellowing of fronds, reduced growth) were visible in test plant after 96 h of treatment. Distillery effluent affected the biomass of *S. polyrrhiza* in concentration dependent manner (ANOVA, $p<0.05$). Further, it has been observed that biomass of *S. polyrrhiza* was not found significantly affected by 5% distillery effluent during phytoremediation (DMRT, $p>0.05$). However, 10, 20, 40, and 100% distillery effluent significantly reduced the biomass of *S. polyrrhiza* to 23.83, 39.02, 61.15 and 75.74%, respectively (Table 4). Results revealed that bioremediation of distillery effluent by *B. thuringiensis* reduced the toxicity of distillery effluent to *S. polyrrhiza.* Therefore, distillery effluent concentrations <40% have no toxicity to biomass of *S. polyrrhiza* (DMRT $p< 0.05$). However, 40 and 100% distillery effluent reduced the biomass to 17.3 and 39.58%, respectively (Table 4).

It has been observed that during phytoremediation of distillery effluent *S. polyrrhiza* fronds significantly (ANOVA, $p<0.05$) accumulated starch and reducing sugars (Table 5). However, 5% distillery effluent has no significant effect on these parameters of *S. polyrrhiza* (DMRT, $p>0.05$). Further, It has been observed that when *S. polyrrhiza* plants were exposed to *Bacillus* treated (BT) distillery effluent concentration < 40%, starch and reducing sugars contents were not found significantly altered (DMRT $p<0.05$). However, *S. polyrrhiza* grown in *Bacillus* treated 40% and 100% distillery effluent (BT) have shown enhanced levels of starch and reducing sugar contents (DMRT, $p<0.05$). Results indicated that both *B. thuringiensis* and *S. polyrrhiza* lowered the BOD, COD and total solids of distillery effluent in concentration dependant manner (Table 3). However, maximum reduction in these parameters was observed when *S. polyrrhiza* was exposed to *B. thuringiensis* treated distillery effluent for 96 hours.

In our study, bioaccumulation and biosorption probably achieved the heavy metal removal by *B. thuringiensis*. *Bacillus sp.* (*Bacillus* H9) has been reported to reduce metal toxicity (both by extracellular and intracellular processes) through

Table 2. Heavy metal concentrations (ppm) in distillery effluent treated by *B. thuringiensis* for 7 d.

Metals	Effluent concentration (%)				
	5	10	20	40	100
Cd	$0.01^{e}\pm0.0001$	$0.021^{d}\pm0.001$	$0.056^{c}\pm0.003$	$0.131^{b}\pm0.01$	$0.398^{a}\pm0.02$
Cr	$0.01^{e}\pm0.0001$	$0.022^{d}\pm0.001$	$0.058^{c}\pm0.002$	$0.09^{b}\pm0.003$	$0.472^{a}\pm0.02$
Cu	$0.03^{e}\pm0.001$	$0.071^{d}\pm0.003$	$0.173^{c}\pm0.005$	$0.40^{b}\pm0.02$	$1.24^{a}\pm0.058$
Fe	$0.16^{e}\pm0.009$	$0.422^{d}\pm0.018$	$1.013^{c}\pm0.042$	$2.57^{b}\pm0.12$	$7.47^{a}\pm0.273$
Mn	$0.01^{e}\pm0.0001$	$0.018^{d}\pm0.001$	$0.044^{c}\pm0.002$	$0.103^{b}\pm0.01$	$0.322^{a}\pm0.01$
Ni	ND	$0.017^{d}\pm0.001$	$0.039^{c}\pm0.002$	$0.090^{b}\pm0.01$	$0.275^{a}\pm0.01$
Pb	ND	$0.073^{d}\pm0.002$	$0.166^{c}\pm0.012$	$0.377^{b}\pm0.02$	$1.06^{a}\pm0.043$
Zn	$0.07^{e}\pm0.003$	$0.195^{d}\pm0.008$	$0.501^{c}\pm0.002$	$1.178^{b}\pm0.05$	$3.55^{a}\pm0.166$

Mean (n=3) ± SD; ND = not detected; ANOVA, $p<0.05$(for each metal separately). Different superscripts (a-e) on means indicate significant difference ($p<0.05$) between means in a row (for each metal) according to DMRT.

binding of metal to liposaccharides of extra cellular membrane and accumulation of metals inside cells (Roane et al 2001). Reduction in BOD, COD, solids and phenols by *B. thuringiensis* can be attributed to the degradation of complex inorganic and organic compounds present in distillery effluent to meet its nutritional requirements.

During present study, *S. polyrrhiza* removed significant amount (ANOVA $p<0.05$) of various metals (Cd, Cr, Cu, Ni, Mn, Zn, Fe and Pb) through accumulation in plant tissues (Figure 1) simultaneously reduced the COD, BOD, solids and phenols from distillery effluent. The removal of cadmium, copper and chromium by *S. polyrrhiza* through accumulation has been reported (Pandey et al 1999). It has been suggested that reduction in load of pollutants (organic and inorganic) other than heavy metals was probably for fulfillment of nutritional requirements (Kadlec and Kadlec 1979). However, the exact mechanism responsible for decolourisation of distillery effluent by *S. polyrrhiza* is still to be investigated. The microbial decolourisation of distillery effluent is well documented (Ohmomo et al 1987, Chandra 2003). Phenol is also present in distillery effluent (Chandra 2003). The test plant used in this study has significantly reduced the phenols in distillery effluent. Further, the reduction in phenol content by *S. polyrrhiza* was more when plants were exposed to *B. thuringiensis* treated (7 d) distillery effluent. This might be due to the reduced toxicity of distillery effluent. During present study, when *S. polyrrhiza* was exposed to distillery effluent concentration >5% accumulated starch and reducing sugars. It has been reported that nutrient deficiencies and heavy metal toxicity could induce accumulation of starch and reducing sugars (Lunáčkova et.al. 2003).

It has been observed that distillery effluent concentration >5% reduced the biomass of the *S. polyrrhiza*. This might be attributed to the toxicity of distillery effluent as it was rich in heavy metals (Cd, Cr, Cu, Zn, Ni, Pb, Fe & Mn). The phytotoxic effect of these metals to biomass is well documented (Van Assche and Clijesters 1990). However, the biomass was not found affected by the toxicity of

Table 3. Reduction in decolourisation, BOD, COD, TS and phenol of distillery effluent through bioremediation.

Parameters	Treatments	Effluent concentration (%)				
		5	10	20	40	100
Decolourisation (%)	ST	$26.8^{a}\pm1.2$	$21.32^{b}\pm1.0$	$15.68^{c}\pm0.7$	$7.63^{b}\pm0.32$	$2.69^{d}\pm0.12$
	BT	$18.82^{a}\pm0.8$	$16.65^{b}\pm0.8$	$2.48^{c}\pm0.11$	$0.35^{b}\pm0.02$	$0.26^{d}\pm0.01$
	ST+BT	$44.66^{a}\pm1.3$	$38.66^{b}\pm1.2$	$35.59^{c}\pm1.6$	$26.01^{b}\pm1.3$	$15.55^{d}\pm0.7$
BOD ($mg\ L^{-1}$)	UT	$1432^{a}\pm62$	$2859^{d}\pm132$	$5660^{c}\pm283$	$11435^{b}\pm560$	$28700^{a}\pm1430$
	ST	$853^{a}\pm41$	$1856^{d}\pm83$	$4250^{c}\pm201$	$9102^{b}\pm455$	$25560^{a}\pm1260$
	BT	$600^{a}\pm25$	$1383^{d}\pm60$	$3384^{c}\pm169$	$7945^{b}\pm360$	$22170^{a}\pm1109$
	ST+BT	$340^{a}\pm15$	$972^{d}\pm45$	$2592^{c}\pm118$	$6209^{b}\pm278$	$17995^{a}\pm788$
COD ($mg\ L^{-1}$)	UT	$2880^{b}\pm130$	$5759^{d}\pm250$	$11480^{c}\pm500$	$23020^{b}\pm1015$	$57600^{a}\pm2638$
	ST	$1723^{a}\pm80$	$3761^{d}\pm176$	$8679^{c}\pm420$	$18416^{b}\pm910$	$51552^{a}\pm2567$
	BT	$1261^{a}\pm52$	$2885^{d}\pm134$	$6968^{c}\pm336$	$16114^{b}\pm703$	$44640^{a}\pm2210$
	ST+BT	$706^{a}\pm30$	$1987^{d}\pm89$	$5338^{c}\pm250$	$10382^{b}416$	$37267^{a}\pm1850$
TS	UT	$1976^{c}\pm93$	$3878^{d}\pm185$	$7835^{c}\pm380$	$15330^{b}\pm660$	$39015^{a}\pm1550$
	ST	$431^{e}\pm18$	$1194^{d}\pm59.7$	$3094^{c}\pm130$	$10712^{b}\pm436$	$31290^{a}\pm1146$
	BT	$198^{e}\pm8$	$776^{d}\pm80$	$2351^{c}\pm117$	$8922^{b}\pm400$	$27016^{a}\pm1250$
	ST+BT	$494^{e}\pm2.5$	$407^{d}\pm15$	$1472^{c}\pm65$	$5319^{b}\pm200$	$20873^{a}\pm877$
Phenol ($\mu g\ ml^{-1}$)	UT	$17.48^{e}\pm0.63$	$36.6^{d}\pm1.23$	$73.1^{c}\pm3.12$	$147.2^{b}\pm6.4$	$369.7^{a}\pm17.2$
	ST	$9.04^{e}\pm0.40$	$23.7^{d}\pm1.12$	$60.4^{c}\pm2.78$	$133.5^{b}\pm5.5$	$360.4^{a}\pm16.2$
	BT	$6.87^{e}\pm0.30$	$19.6^{d}\pm0.88$	$54.2^{c}\pm2.10$	$120.5^{b}\pm5.5$	$346.4^{a}\pm15.3$
	BT +ST	$2.57^{e}\pm0.12$	$12.6^{d}\pm0.52$	$39.92^{c}\pm1.70$	$98.33^{b}\pm3.8$	$295.4^{a}\pm13.6$

Mean (n=3) ± SD; ANOVA, $p<0.05$ (for each treatment separately). Identical superscripts (a-e) on means indicate no significant difference ($p>0.05$) between means in a row. UT = untreated; ST treated by *S. polyrrhiza* (96 h); BT = treated by *B. thuringiensis* (7d); BT+ST = BT treated by *S. polyrrhiza* for 96 h.

Table 4. Amelioration of toxic effects of the distillery effluent to biomass of *S. polyrrhiza*.

Effluent concentration (%)	Biomass (g DW)	
	ST	ST+BT
0	$0.1179^{a}\pm0.004$	$0.118^{a}\pm0.004$
5	$0.1004^{a}\pm0.005$	$0.1225^{a}\pm0.005$
10	$0.0898^{b}\pm0.001$	$0.1200^{a}\pm0.003$
20	$0.0719^{c}\pm0.003$	$0.1126^{a}\pm0.004$
40	$0.0458^{d}\pm0.002$	$0.0980^{b}\pm0.003$
100	$0.0286^{e}\pm0.001$	$0.0716^{c}\pm0.003$

Mean (n=3) ± SD; ANOVA, $p<0.05$. Identical superscripts on means indicate no significant difference ($p>0.05$) between means vertically in a column for each parameter according to DMRT.

the distillery effluents upto 20% when exposed to *B. thuringiensis* for 7 d prior to 96 h phytoremediation of distillery effluent by *S. polyrrhiza*. While 40 and 100% distillery effluent have toxic effect on biomass but to the lesser extent. This might be due to the utilization of various pollutants by *B. thuringiensis* during 7 d bioremediation of distillery effluent. It could be concluded from present study that both *B. thuringiensis* and *S. polyrrhiza* bioremediated the distillery effluent by reducing the pollutant load (BOD, COD, phenol and heavy metals), accumulating

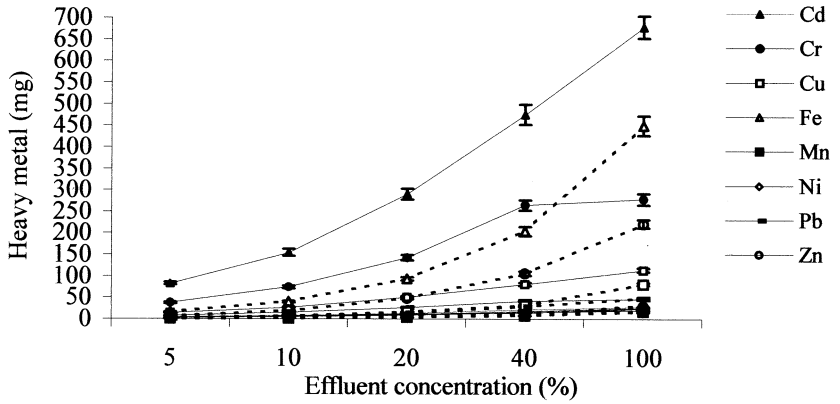

Figure 1 Heavy metal accumulation (mg g^{-1} DW) by *S. polyrrhiza* grown in bacterial untreated (solid line) and treated (dotted line) distillery effluent after 96 h

Table 5. Amelioration of toxic effects of the distillery effluent to starch and reducing sugars of *S. polyrrhiza*.

Effluent conc(%)	Starch (mg g^{-1}FW)		Reducing Sugar (mg g^{-1}FW)	
	ST	ST+BT	ST	ST+BT
0	95.17^{e}±3.7	92.98^{e}±3.6	10.85^{e}±0.38	10.96^{e}±0.50
5	93.24^{e}±3.2	94.31^{e}±3.5	11.25^{e}±0.52	11.25^{e}±0.52
10	108.24^{d}±4.1	93.64^{e}±4.3	20.18^{d}±0.88	11.13^{e}±0.48
20	120.23^{c}±4.4	91.78^{e}±3.5	29.72^{c}±1.28	12.56^{e}±0.58
40	135.53^{b}±5.6	105.8^{b}±4.25	38.52^{b}±1.60	20.12^{b}±0.98
100	157.67^{a}±6.4	110.5^{a}±3.78	48.72^{a}±1.88	30.32^{a}±1.21

Mean (n=3) ± SD; ANOVA, $p<0.05$(for each parameter separately). Identical superscripts on means indicate no significant difference ($p>0.05$) between means vertically in a column for each parameter according to DMRT.

the heavy metals (Cd, Cr, Cu, Ni, Mn, Fe, Zn and Pb) and decolorizing the brown colour of the effluent. Further, enhanced decolourisation was noted when *B. thuringiensis* (7 d) treated distillery effluent was subjected to phytoremediation (*S. polyrrhiza*). This was due to toxicity amelioration by *B. thuringiensis* through utilization of complex compounds present in distillery effluent. This has been confirmed by reduced toxicity to biomass, starch and reducing sugars. Thus, these findings revealed that phytoremediation, after bacterial treatment, would be an effective technique for decolourisation of anaerobically treated distillery effluent for its safe disposal in environment.

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