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ORIGINAL ARTICLE

Effective bioremediation and toxicity assessment of tannery wastewaters treated with indigenous bacteria

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Abstract

This study evaluated the bioremediation capacity of indigenous bacteria isolated from tannery sludge for two different tannery wastewaters collected from Kanpur and Chennai. To identify bacteria which can efficiently degrade a mixture of different pollutants, the isolates were grown in hazardous 100% tannery wastewaters. The reductions in toxicants such as chromium, sulphate, biochemical oxygen demand (BOD) and chemical oxygen demand (COD) of the wastewater were analysed post-bioremediation. Amongst the isolates, *Citrobacter freundii* was able to reduce the concentration of multiple toxicants such as chromium by 73% and sulphate was reduced by 68% bringing down the level much below the permissible limit stipulated by Bureau of Indian Standards (BIS). Notably, the organic load characterized by BOD and COD was also lowered by 86 and 80%, respectively. The indigenous isolates, not only bioremediated the Kanpur effluent but, also significantly detoxified the Chennai effluent having higher toxicant load. An interesting observation made during the study was better survival and growth along with the development of appendages of *Artemia* nauplii in the treated wastewaters which thus further confirmed reduction in toxicity of the effluents. The results thus demonstrate that the tested indigenous strains are promising for bioremediation of tannery wastewater and effectively improve the water quality for safe discharge.

Keywords Bioremediation · Tannery wastewater · *Citrobacter* · COD · Toxicity removal · *Artemia*

Introduction

From the olden days to modern day, leather has been an integral part of human life. India is the second largest producer of footwear and leather garments with Kanpur and Chennai being the main centres, which process 7,00,000 t of hides and skins to produce 2 billion sq feet of leather annually. On an average, the conversion of 1 t of raw hide reportedly generates 30–35 m^3 wastewaters (Lofrano et al. 2008; Saxena et al. 2016). Only a limited amount of wastewater is being re-used in the tanning process whereas majority of it containing large quantities of salt, high levels of biochemical oxygen demand (BOD), chemical oxygen demand (COD), total dissolved solids (TDS) and other pollutants such as chromium, sulphate, synthetic tannins and azo dyes is discharged into the soil, rivers and oceans (Singh et al. 2013). Discharge of tannery effluent loaded with such toxic pollutants into the environment leads to serious soil and water pollution which further affects the health of humans and other living organisms. It causes various effects in humans ranging from nasal and skin irritation to lung carcinoma (Khan et al. 2015; Dhanarani et al. 2016). Therefore, it is essential that the tannery effluents be necessarily treated before it is let into the environment.

High concentrations of pollutants with the complex composition of organic and inorganic chemicals pose a huge challenge to the treatment methods (Schrank et al. 2009; Modenes et al. 2012). Over the years, various physical and chemical processes such as ultra-filtration and advanced oxidation processes have been developed (Lofrano et al. 2013) but these are expensive, complicated and not very efficient. Hence to resolve these problems bioremediation strategies

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have evolved as a promising tool which is environment-friendly, safe and cost-effective.

Various microorganisms have been investigated for the bioremediation of tannery effluent, specifically *Cellulosimicrobium* sp. (Bharagava and Mishra 2018), *Bacillus* sp. (Bachate et al. 2013; Yusuf et al. 2013; Kumari et al. 2016), *Thiobacillus* sp. (Mandal et al. 2010), *Planococcus* sp. (Behera et al. 2016), *Stenotrophomonas* sp. (Raman et al. 2018), *Penicillium* sp. and *Fusarium* sp. (Sharma and Malaviya 2016). However, most of these studies are pollutant-specific, aimed at the removal of only one or two pollutants, mainly chromium (Sharma and Malaviya 2016). In spite of the availability of techniques as well as organisms, identifying bacteria which can degrade a mixture of different pollutants efficiently is the need of the hour for superior bioremediation of complex wastewaters.

Indigenous microorganism-based technology is widely applied in the eastern part of the world for the waste management (Kumar and Gopal 2015). These organisms are a group of innate microbial consortium that inhabits the soil and the surfaces of living things having the capability of biodegradation, bioleaching and biocomposting. Hence the use of these indigenous microorganisms is inherently attractive and an efficient way to protect the environment (Cai et al. 2013). Therefore, in the present study, we have investigated the bioremediation capacity of the indigenous bacterial strains isolated from the tannery areas of Jajmau for their efficiency to reduce the toxicity of Kanpur and Chennai effluents. Further, the reduction in the pollutants level and bioremediation potential of the bacterial isolates was validated through toxicity assay using *Artemia salina* as a model organism.

Materials and methods

Isolation and characterisation of bacteria

The tannery effluent used for this study was collected from Jajmau, Kanpur (KE), Uttar Pradesh and from Common Effluent Treatment Plant (CETP), Pallavaram, Chennai (CE), India. The bacterial strains were isolated from the sludge samples collected from sludge dumping site at Kanpur industrial area. Briefly, the sludge sample (1 g) was serially diluted in sterile saline (0.85% NaCl) from 10^{-1} to 10^{-5} and then plated on nutrient agar (NA) (M001-HiMedia) and plates were incubated at 30 ± 2 °C for 24 h. Morphologically distinct isolates were further checked for their growth on effluent-containing plates. The method of Prakasam and Dondero (1978) was followed for preparing tannery wastewater agar where tannery effluent was the sole carbon source. Briefly, the tannery wastewater (1 L) was filtered through glass wool and 15 g of agar powder (HiMedia, Mumbai) was added for solidification. This was autoclaved at 121 °C for 20 min; on cooling, the plates were prepared which were later inoculated with bacterial isolates and incubated for growth. Colony characteristics including size, shape, colour, elevation, margin, texture opacity and Gram staining were examined for the strains. The strains were maintained in nutrient agar slants stored in sealed tubes at 4 °C as well as in glycerol vials at − 80 °C.

Molecular identification of the strains

The genomic DNA was extracted from the isolates using the DNeasy kit (Qiagen). 16S rRNA gene of the bacterial isolates was amplified using universal 16S rDNA primers namely 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) (Lane 1991). Nucleotide sequencing for these samples was performed using a 3130xl genetic analyzer (Applied Biosystems, Foster City, USA) at National Institute of Oceanography, Goa, India. The nucleotide sequences of the isolates obtained were compared with the sequences available in the public database using BLAST software (http://www.ncbi.nlm.nih.gov). The sequences obtained from this study were submitted to GenBank with accession numbers KX058027–KX058029. Neighbour-joining method was employed to construct the phylogenetic tree using MEGA5 software (Tamura et al. 2011) and the maximum likelihood method was adopted for calculating the evolutionary distance.

Experimental setup

In this study, three selected isolates were tested for their ability to grow in both KE and CE without any nutritional amendment for further experiments. The growth was evaluated by measuring the optical density (OD) at 600 nm for every 12 h interval until a decline in the growth was observed. The bacterial cultures were then tested for their bioremediation capacity for KE and CE. Cells in the exponential phase (in Nutrient broth-M088, Himedia) were harvested by centrifugation at 10,000 rpm for 10 min at 4 °C and the pellet was washed and re-suspended in 0.85% of NaCl. One millilitre of the above bacterial inoculum (approximately 1×10^6 cells/mL) was inoculated into a 250-mL Erlenmeyer flask containing 100 mL of autoclaved raw tannery effluents and the initial OD after inoculation was 0.2. The flasks were incubated in the dark at 30 ± 2 °C for 5 days under static condition. Same experimental setup was used for both the effluents, i.e. KE and CE. Bioremediation capacities of the strains were tested by estimating chemical and biochemical oxygen demand, sulphate, chromium, nitrate and phosphate levels in treated and untreated samples after 5 days of incubation.

Analytical measurements

Treated and untreated effluents were centrifuged at 10,000 rpm for 10 min at 20 °C and the supernatant was used for all the analyses. Estimation of sulphate was done by turbidometric method (APHA 2005). The COD of the untreated and treated effluent was determined by open reflux method and the COD reduction rate was calculated for treated effluent (APHA 2005).

$$\text{COD reduction } (\%) = \frac{\text{Initial COD} - \text{Final COD}}{\text{Initial COD}} \times 100.$$

The BOD was estimated following APHA (2005) protocol wherein airtight DO bottles were filled and incubated at the room temperature for 5 days. Dissolved oxygen was measured initially and after incubation, and the BOD was computed from the difference between initial and final DO. Total chromium [Cr (VI)] was determined spectrophotometrically by 1, 5-diphenyl carbazide method. The effluents were digested with concentrated nitric acid and the chromium content was estimated. The absorbance was measured at 540 nm using Cary 300/Agilent spectrophotometer (APHA 2005). The ultraviolet spectrophotometric screening method was used for the estimation of nitrate in treated and untreated samples whereas phosphate was determined by the ascorbic acid method (APHA 2005).

Scanning electron microscopy (SEM) analysis

Bacteria were pelleted by centrifugation before and after treatment and used for SEM analysis. Glutaraldehyde-fixed and ethanol-dehydrated bacteria were mounted on stubs and sputter coated with gold under vacuum in an argon atmosphere. The morphology of the coated samples was visualized by a scanning electron microscope (JEOL make).

Toxicity assay

Toxicity assay using *Artemia salina* was performed to assess the reduction in toxicity of the bioremediated tannery effluents (KE and CE). Briefly, the cysts of *Artemia* were hatched in seawater kept under constant light and aeration for 24 h. Ten nauplii per well were transferred to 24-multiwell plates (Costar USA, 3 mL capacity with 106 mm width) containing 1.5 mL of either treated or untreated effluent samples or seawater (positive control). The multiwell plates were incubated at room temperature (30 ± 2 °C) and the number of dead nauplii in each well was counted after 24, 48 and 72 h. The assay was conducted in triplicates. The percentage of mortality in treated and untreated effluents was calculated (Babu et al. 2015).

Statistical analysis

Data were expressed as the mean ± standard deviation (SD) ($n = 3$). All the data were first checked for normality using Shapiro–Wilk's test. The significance of variability, among groups with different effluents and strains, was evaluated for various toxicants of tannery effluents by two-way analysis of variance (two-way ANOVA). For the multiple comparisons among groups post-hoc test (Tukey's HSD test) was performed. To find out the significant relationship, Pearson correlations were performed for growth and different toxicants of both tannery effluents treated with three strains. Statistical significance of data was measured at $p < 0.05$ level. All the tests were performed using SPSS 16.0 (SPSS Inc., USA).

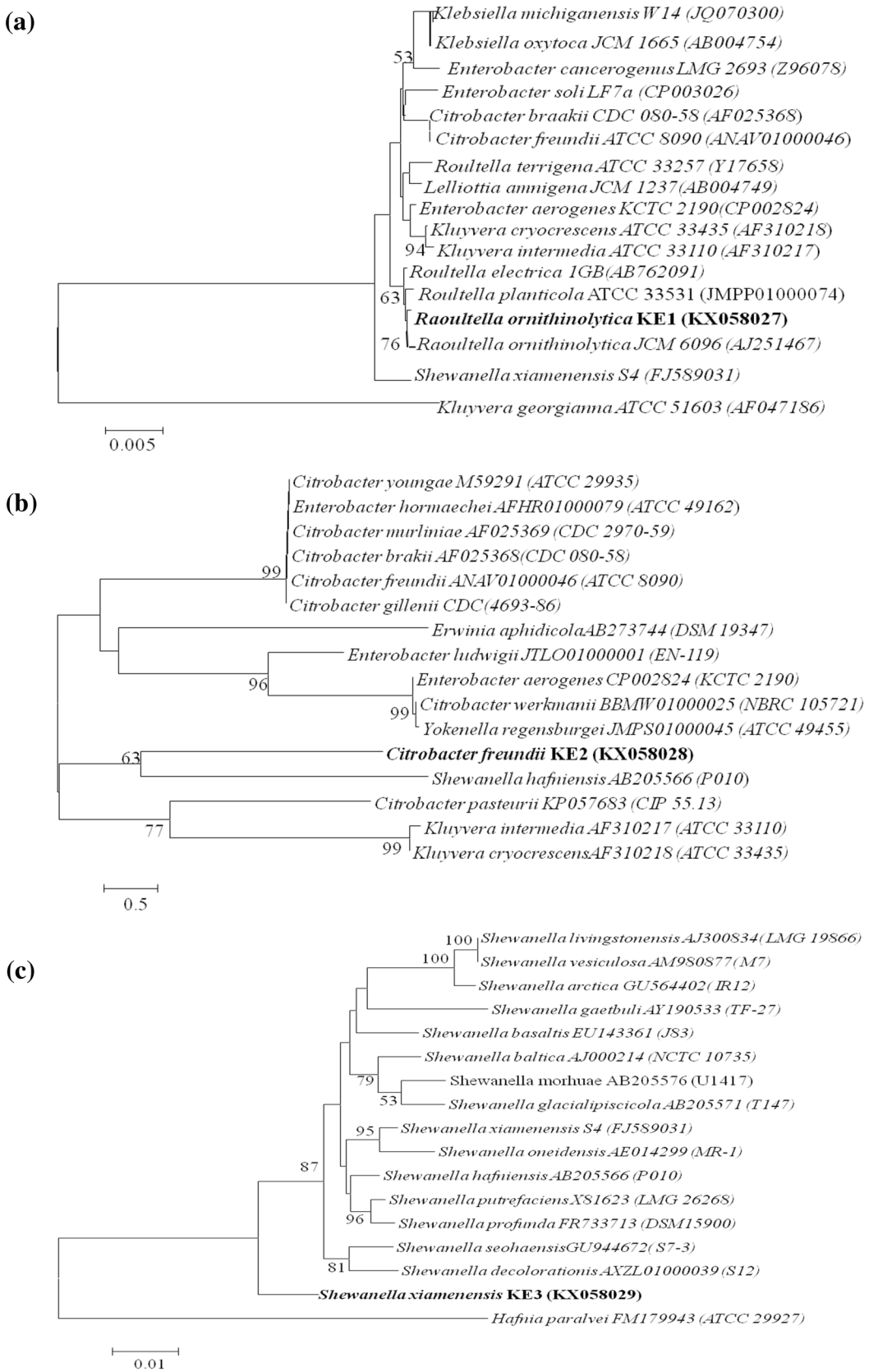
Results and discussion

Characteristics of tannery wastewater

The tannery effluent collected at the discharge point of tannery industry located at Jajmau, Kanpur was greenish grey, whereas, the effluent collected from CETP Chennai was yellowish brown in colour. KE sample collected was diluted before discharging whereas CE was straightway collected from the CETP; hence, the physicochemical parameters of KE were comparatively lower than CE (Table 1). Both the effluents contained high amounts of sulphate, COD, BOD, chromium, nitrate and phosphate.

Table 1 Tannery effluent characteristics

Parameters	Kanpur effluent	Chennai effluent	Maximum permissible limits (BIS 1994)
COD (mg/L)	2200 ± 100	2800 ± 173	250
BOD (mg/L)	833 ± 20	905 ± 24	100
Chromium (mg/L)	44.6 ± 2.3	50.9 ± 2.7	2
Sulphate (mg/L)	1661.2 ± 31.3	1867.6 ± 27.6	1000
Nitrate (mg/L)	7.60 ± 0.25	9.17 ± 0.21	10
Phosphate (mg/L)	5.65 ± 0.23	6.26 ± 0.26	5


(a)
Klebsiella michiganensis W14 (JQ070300)
Klebsiella oxytoca JCM 1665 (AB004754)
53
Enterobacter cancerogenus LMG 2693 (Z96078)
Enterobacter soli LF7a (CP003026)
Citrobacter braakii CDC 080-58 (AF025368)
Citrobacter freundii ATCC 8090 (ANAV01000046)
Roultella terrigena ATCC 33257 (Y17658)
Lelliottia amnigena JCM 1237(AB004749)
Enterobacter aerogenes KCTC 2190(CP002824)
Kluyvera cryocrescens ATCC 33435 (AF310218)
94
Kluyvera intermedia ATCC 33110 (AF310217)
Roultella electrica 1GB(AB762091)
63
Roultella planticola ATCC 33531 (JMPP01000074)
Raoultella ornithinolytica KE1 (KX058027)
76
Raoultella ornithinolytica JCM 6096 (AJ251467)
Shewanella xiamenensis S4 (FJ589031)
Kluyvera georgianna ATCC 51603 (AF047186)
0.005
(b)
Citrobacter youngae M59291 (ATCC 29935)
Enterobacter hormaechei AFHR01000079 (ATCC 49162)
Citrobacter murliniae AF025369 (CDC 2970-59)
Citrobacter brakii AF025368(CDC 080-58)
99
Citrobacter freundii ANAV01000046 (ATCC 8090)
Citrobacter gillenii CDC(4693-86)
Erwinia aphidicolaAB273744 (DSM 19347)
Enterobacter ludwigii JTLO01000001 (EN-119)
96
Enterobacter aerogenes CP002824 (KCTC 2190)
99
Citrobacter werkmanii BBMW01000025 (NBRC 105721)
Yokenella regensburgei JMPS01000045 (ATCC 49455)
63
Citrobacter freundii KE2 (KX058028)
Shewanella hafniensis AB205566 (P010)
Citrobacter pasteurii KP057683 (CIP 55.13)
77
Kluyvera intermedia AF310217 (ATCC 33110)
99
Kluyvera cryocrescensAF310218 (ATCC 33435)
0.5
(c)
100
Shewanella livingstonensis AJ300834 (LMG 19866)
100
Shewanella vesiculosa AM980877 (M7)
Shewanella arctica GU564402 (IR12)
Shewanella gaetbuli AY190533 (TF-27)
Shewanella basaltis EU143361 (JS3)
Shewanella baltica AJ000214 (NCTC 10735)
79
Shewanella morhuae AB205576 (U1417)
53
Shewanella glacialipiscicola AB205571 (T147)
95
Shewanella xiamenensis S4 (FJ589031)
Shewanella oneidensis AE014299 (MR-1)
87
Shewanella hafniensis AB205566 (P010)
Shewanella putrefaciens X81623 (LMG 26268)
96
Shewanella profunda FR733713 (DSM15900)
Shewanella seohaensisGU944672 (S7-3)
81
Shewanella decolorationis AXZL01000039 (S12)
Shewanella xiamenensis KE3 (KX058029)
Hafnia paralvei FM179943 (ATCC 29927)
0.01

◂**Fig. 1** Neighbour-Joining phylogenetic tree showing the relationship of strain *Raoultella ornithinolytica* represented as KE-1 (**a**), *Citrobacter freundii* represented as KE-2 (**b**) and *Shewanella xiamenensis* represented as KE-3 (**c**) based on 16S rRNA gene sequence. Bootstrap percentages (1000 replications) are shown at branching points (values > 50%). Scale bar represents substitutions per nucleotide position

Molecular identification and characterisation of the isolates

In this study, a total of nine morphologically different bacterial strains were isolated from sludge samples collected at sludge dumping site Kanpur. The three isolates were further selected on the basis of their growth on effluent-containing agar plates for further evaluation of their bioremediation capacity. The strains were identified as *Raoultella ornithinolytica* (KE1, 99%), *Citrobacter freundii* (KE2, 98%) and *Shewanella xiamenensis* (KE3, 97%) by 16S rRNA gene sequencing. Phylogenetic relationship of the isolates and its closest neighbouring sequences in GenBank database is given in Fig. 1.

The growth study of three strains in tannery effluents revealed a gradual increase in the growth represented by OD which showed a decline after 120 h; hence, 5 days of incubation period was maintained for bioremediation study throughout the experiment. On an average, all the three strains showed better growth in KE than CE. The growth curves of strains in the tannery effluents are given as Supplementary Figure.

Reduction of various toxicants after bioremediation

Tannery wastewaters are characterized mainly by high levels of BOD, COD, chromium, sulphate, suspended solids (SS) and several other salts (Dixit et al. 2015). In the present study, bioremediation by the three bacterial isolates showed a considerable reduction in the toxicant levels of tannery effluents after 5 days of treatment. The decrease in pollutants was observed with an increase in bacterial growth having a significant correlation ($p < 0.01$) (Table 2).

Chromium is one of the serious contaminants in tannery wastewater having the ability of biomagnification and can easily accumulate in the food chain (Khan et al. 2015; Dhanarani et al. 2016). It subsequently can cause eardrum perforation, ulceration and impaired foetal development in humans due to its genotoxic and mutagenic effect (Verma et al. 2001; Srinath et al. 2002; Cheung and Gu 2003). In this study *C. freundii* could bring down the concentration of chromium from 44.61 and 50.85 mg/L to 12.17 and 17.19 mg/L for KE and CE, respectively (Fig. 2b). Chromium bioremediation by *C. freundii* is known to involve both biosorption due to the production of extracellular polymeric substances as well as bioreduction mechanism (Zarasvand and Rai 2016). An increase in the size of *C. freundii* was observed in our study during the bioremediation process which suggests the involvement of chromium biosorption as a mechanism of bioremediation. This is further elaborated in the following section on changes in cell morphology of the bacterial isolates.

Earlier studies using a combined treatment of fenton oxidation with aerobic biological treatment using *Thiobacillus* sp. showed 52% of chromium reduction in leather industry wastewater (Mandal et al. 2010). Previous bioremediation studies by Singh et al. (2011) have reported 78 and 21% of chromium and sulphate removal, respectively, in the presence of an external carbon source. However, our results demonstrate comparable removal efficiencies by bacteria without any additional carbon source.

Sulphate is another major toxicant which affects the quality of water and soil by reducing the pH thus directly affecting the health of people; it can be successfully treated by biological processes (Li 2005). All the three indigenous strains in this study showed more than 40% reduction in sulphate from both the tannery effluents (Fig. 2a). Strain *C. freundii* could bring down sulphate concentration to 531 mg/L (with 68% reduction) while *S. xiamenensis* and *R. ornithinolytica* could minimize it to 732 and 797 mg/L, respectively, for KE. Similarly, *C. freundii* showed the highest sulphate removal from CE as well. Higher sulphate removal performance by all the strains was observed for KE compared to CE. Previous studies by Chandra et al. (2011) and Genschow et al. (1996) reported overall sulphate removal up to 55–58% during biological treatment of tannery wastewater.

The level of sulphate was brought down much below the Bureau of Indian Standards (BIS 1994) permissible limit by *C. freundii* within 5 days of treatment. *C. freundii* belonging to Enterobacteriaceae family is a facultative anaerobe known to be non-traditional sulphate-reducing bacteria (Zarasvand and Rai 2016). Zhou et al. (2015) have reported sulphate reduction by *C. freundii* isolated from the sludge of a paper mill. Another isolate from the mining area was also reported to be a potent sulphate reducer (Qiu et al. 2009). Our study using *C. freundii* strain revealed better sulphate reduction in tannery effluent as well and thus it showed its versatile role in bioremediation. Moreover, *R. ornithinolytica* known for bioremediation of hydrocarbons is also reported to be a sulphate-reducing bacteria (Huang et al. 2015). Our results hence demonstrated better bioremediation efficiencies by the isolates in two different effluents within the shorter treatment period. This was further supported by results of two-way ANOVA which revealed a significant ($p < 0.05$) reduction in toxicant levels for both the effluents by all the three bacterial species (Supplementary Table 1).

Although nitrate and phosphate are necessary nutrients for organisms, their presence in higher concentration can lead to eutrophication of the receiving water body. *S.*

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Table 2 Correlation (Pearson) between different parameters for Kanpur and Chennai effluent

	Chennai effluent											
	Growth	Sulphate	Chromium	Nitrate	Phosphate	COD	Growth	Sulphate	Chromium	Nitrate	Phosphate	COD
R.O.												
Sulphate	− 0.997**						− 0.999**					
Chromium	− 0.961**	0.972**					− 0.884*	0.869*				
Nitrate	− 0.996**	0.994**	0.968**				− 0.996**	0.994**	0.892*			
Phosphate	− 0.991**	0.995**	0.961**	0.983**			− 0.990**	0.986**	0.930**	0.983**		
COD	− 0.995**	0.990**	0.949**	0.984**	0.990**		− 0.994**	0.990**	0.883*	0.982**	0.989**	
BOD	− 0.996**	0.994**	0.967**	0.996**	0.979**	0.987**	− 0.996**	0.998**	0.847*	0.990**	0.978**	0.984**
C.F.												
Sulphate	− 0.999**						− 0.998**					
Chromium	− 0.995**	0.998**					− 0.993**	0.986**				
Nitrate	− 0.997**	0.993**	0.985**				− 0.994**	0.997**	0.982**			
Phosphate	− 0.988**	0.990**	0.991**	0.975**			− 0.993**	0.993**	0.985**	0.992**		
COD	− 0.995**	0.995**	0.992**	0.989**	0.996**		− 0.988**	0.987**	0.983**	0.977**	0.991**	
BOD	− 0.991**	0.994**	0.993**	0.983**	0.974**	0.977**	− 0.991**	0.994**	0.977**	0.987**	0.978**	0.983**
S.X.												
Sulphate	− 0.998**						− 0.994**					
Chromium	− 0.994**	0.996**					− 0.972**	0.943**				
Nitrate	− 0.998**	0.997**	0.991**				− 0.996**	0.998**	0.956**			
Phosphate	− 0.996**	0.993**	0.995**	0.991**			− 0.996**	0.986**	0.971**	0.987**		
COD	− 0.982**	0.977**	0.972**	0.972**	0.983**		− 0.979**	0.966**	0.980**	0.971**	0.973**	
BOD	− 0.980**	0.985**	0.987**	0.986**	0.973**	0.930**	− 0.994**	0.985**	0.966**	0.988**	0.986**	0.959**

R.O. Raoultella ornithinolytica, C.F. Citrobacter freundii, S.X. Shewanella xiamenensis

*Correlation is significant at the $p < 0.05$ level (two-tailed)

**Correlation is significant at the $p < 0.01$ level (two-tailed)

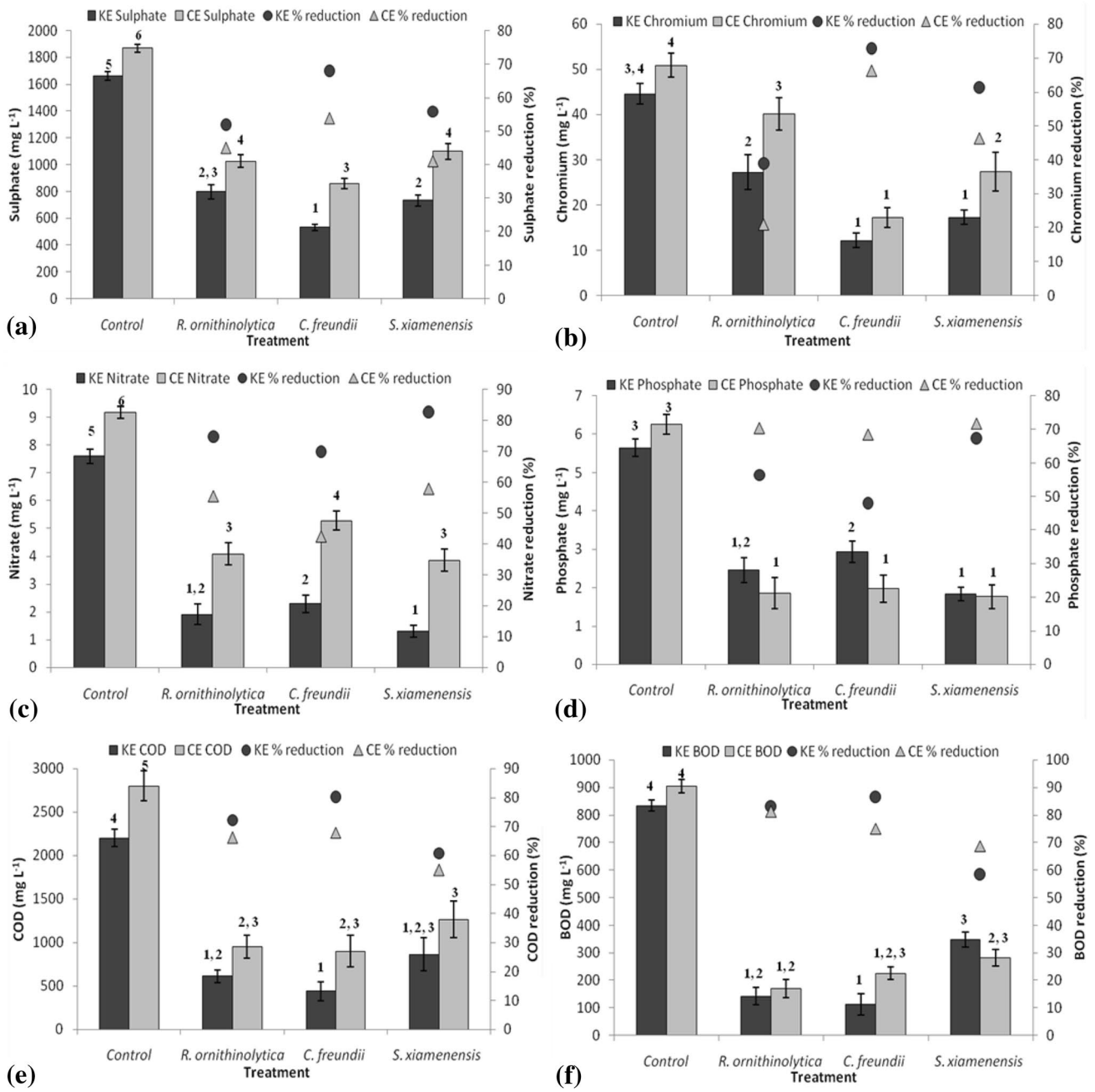

Fig. 2 Reduction in levels of sulphate (**a**), chromium (**b**), nitrate (**c**), phosphate (**d**), COD (**e**) and BOD (**f**) for Kanpur and Chennai effluent after the treatment. The superscript number 1, 2, 3, 4 and 5 on the bars denote groups which are significantly different ($p < 0.05$) formed by post-hoc Tukey HSD test

xiamenensis reduced the initial nitrate concentration maximum by 83% for KE whereas for CE phosphate level was brought down by 72% (Fig. 2c, d). Studies by Chandra et al. (2011) demonstrated a 70% reduction in phosphate level from bacterially treated tannery wastewater collected from aeration lagoon. Our results are superior to those reported above and the concentrations were reduced much below the permissible limits within 5 days. A table showing removal percentage of toxicants from present study and literature data is provided in Supplementary Table 2.

Reduction of organic load after bioremediation

Tannery effluent with high organic load characterized by COD and BOD leads to depletion of oxygen in the receiving water body thus causing deleterious effects on in situ flora

and fauna. It forms an important carbon and electron source for bioremediation and is regarded as a crucial parameter to determine water quality. Reasonable reduction in organic load for KE and CE was exhibited by *R. ornithinolytica* and *C. freundii* bringing down the COD level to as less as 440 mg/L by the latter (Fig. 2e). Highest BOD reduction was observed for *C. freundii* with 86% removal efficiency for KE wherein the BOD level was lowered to near the permissible limit (Fig. 2f). Furthermore, *R. ornithinolytica* showed above 80% of BOD reduction in both the tannery effluents. These removal efficiencies were much higher than the earlier reports by Singh et al. (2011) (36%) and Lefebvre et al. (2006) (77%). Comparable results were also demonstrated by Mandal et al. (2010) with 77 and 80% of COD and BOD reduction, respectively, for leather industry wastewater treated by *T. ferrooxidans*.

All the strains were capable of reducing the contaminant level which is evident from significant negative correlation ($p < 0.01$) between growth and sulphate, chromium, nitrate, phosphate and organic load (Table 2). For both the effluents treated with *C. freundii* and *S. xiamenensis*, reduction in sulphate and chromium was positively correlated. This could be attributed to sulphate-transporting membrane protein which also allows chromium to cross the membrane barrier thereby reducing the concentration of both the pollutants (Vaiopoulou and Gikas 2012). From the overall results, *R. ornithinolytica* and *S. xiamenensis* were more efficient in reducing pollutant load from KE whereas *C. freundii* demonstrated greater bioremediation capacity for both Kanpur and Chennai effluents. Levels of some of the toxicants in the effluents for example sulphate were reduced to 531 mg/L after the treatment which is well below the permissible levels given by BIS (1994).

Changes in cell morphology

The morphology of the bacterial cells was studied before and after the bioremediation using SEM (Fig. 3). An interesting observation was made in the cell size of studied bacteria post-treatment. The SEM images of the strains revealed that in the absence of toxicants, cells appeared to be discrete and individually clear. After the effluent treatment, the cell size of *R. ornithinolytica* and *C. freundii* was almost doubled in CE whereas cells in KE were wider, elliptical and bulged at ends. The increase in size may be attributed to the presence of the metals like chromium in tannery wastewater (Fernandez et al. 2017; Panda and Sarkar 2012; Sundar et al. 2011) or due to secretion of EPS (Kumari et al. 2016; Batool et al. 2012). Interactions such as absorption, transformation or accumulation may be responsible for the decrease in chromium concentration from effluent with chromium being accumulated inside the bacterial cell in some complex form (Panda and Sarkar 2012). *C. freundii* is reported to sustain and grow at elevated chromium concentration as high as 100 mg/L (Sharma et al. 2002). As the effluents used for

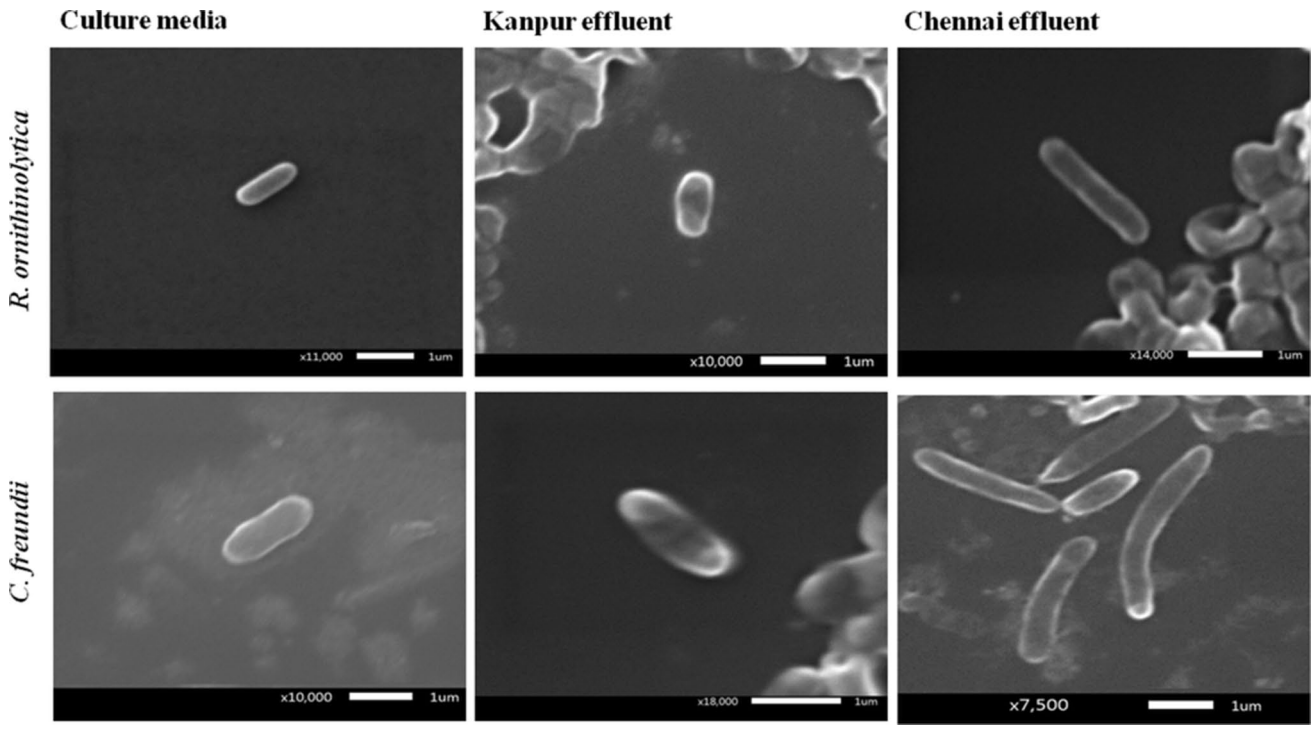

Fig. 3 SEM images of bacterial cells grown in normal media and after treatment of Kanpur and Chennai effluents

this study had a much lower concentration (50 mg/L) of chromium, luxuriant growth of the bacteria was observed.

Toxicity assay on *A. salina*

Toxicity tests are crucial tools to assess the effect of effluent on the indigenous biological communities of the receiving waters. Toxicity of the treated and untreated tannery wastewaters was analysed with bioassay experiments using *A. salina* as a model organism. *Artemia* is one of the most valuable test organisms available for ecotoxicity testing as it is sensitive for a wide range of compounds; its eggs can be easily stored for long duration and hatched in short period of 24 h. *Artemia*-based assays are hence rapid, convenient and cost-effective (Nunes et al. 2006).

In the present study, the mortality rate of *Artemia* in KE treated with *C. freundii* was much lower, 3, 13 and 43% while, the untreated effluent showed 37, 77 and 97% after 24, 48 and 72 h, respectively (Fig. 4). On the other hand 20, 33, 63% and 7, 17, 43% of the mortality rate was noted for CE treated with *R. ornithinolytica* and *C. freundii*, respectively. Bacteriological treatment reduced the mortality rate of *A. salina* to a significant level in both the effluents. More than five times increase in the survival rate after 24 h in treated Kanpur effluent was noticed. The further fast growth of nauplii and development of appendages was also witnessed in treated effluent within 72 h (Fig. 5).

Tannery wastewaters are characterized by high levels of pollutants like sulphate, chromium and organic load thus rendering it highly toxic (Suthanthararajan et al. 2004). Removal of these contaminants makes the effluent less detrimental (Hasegawa et al. 2014; Piccin et al. 2016) thus increasing the survival rate of *Artemia* as compared to in untreated effluents. Increase in the size of *Artemia* nauplii was also reported by Shakir et al. (2012) while studying the toxicity assay of tannery wastewater. The better survival and faster growth of *Artemia* nauplii, when introduced to treated effluent, can be attributed to sub-lethal exposure to metals like chromium from tannery wastewater termed as hormesis (Stebbing 1982; Shaojie and Wenli 2012; Sanchez et al. 2016). Hormesis is described as any process in which a cell or organism exhibits a biphasic response to exposure to increasing amounts of a toxic substance or condition; typically, low-dose exposures elicit

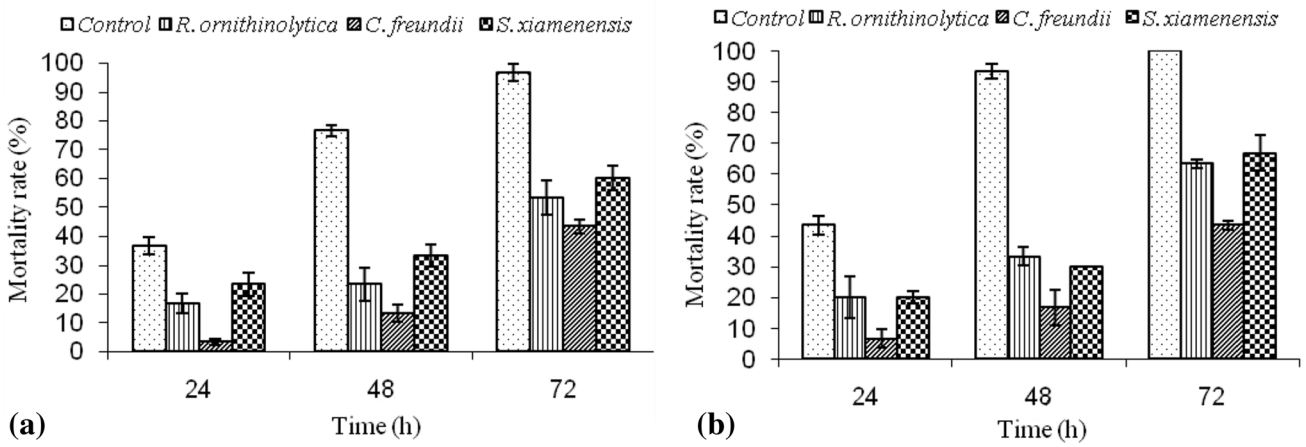

Fig. 4 Toxicity assay using *Artemia* for untreated (control) and bacterially treated tannery wastewater from Kanpur (**a**) and Chennai (**b**)

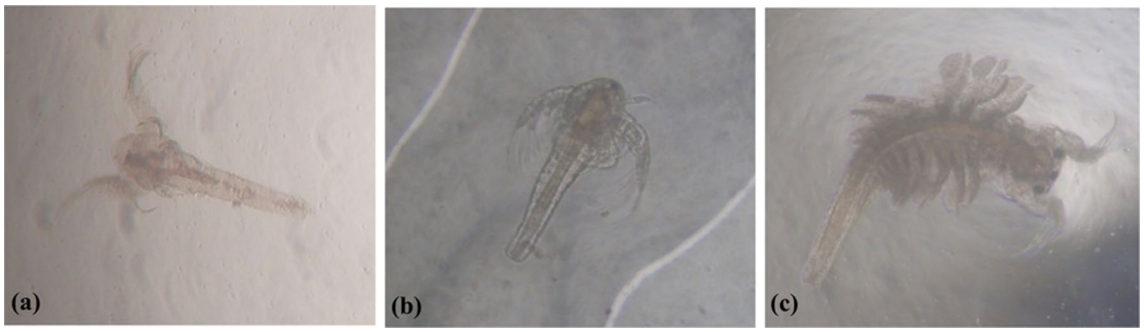

Fig. 5 Light microscope images of *A. salina* after 72 h in seawater (**a**), KE treated with *R. ornithinolytica* (**b**) and *C. freundii* (**c**) showing enhanced size and development of appendages

a stimulatory or beneficial response, whereas high doses cause inhibition or toxicity (Mattson and Calabrese 2010). The present toxicity study revealed that the treatment of tannery effluent with *C. freundii* not only remediated the toxicants, but also supported better survival rates of the nauplii. It can be concluded that the treated tannery effluents were comparatively less harmful to be discharged into the aquatic ecosystem.

Conclusion

The present study reports the isolation of three indigenous γ-proteobacteria from the Kanpur tannery sludge demonstrating their great potential for bioremediation by reducing the concentration of multiple toxicants from the tannery wastewater of Kanpur and Chennai. Amongst the various toxicants reduced during bioremediation, sulphate levels were lowered much below the permissible limit by the indigenous bacteria in Kanpur effluent whilst BOD was brought down to near the permissible limit within 5 days of treatment. The present work revealed *C. freundii* as one of the potential strains for the treatment of tannery effluents due to a significant reduction in sulphate, chromium, BOD and COD levels. Although several studies have focussed on bioremediation of effluents, very few have dealt with the toxicity reduction assessment using bioassays involving *Artemia*. Thus, this study highlights better survival and faster growth rate of crustaceous larvae, *Artemia* nauplii, in bacterially treated tannery effluents. Reduction in toxicant levels and enhanced survival rates of *A. salina* also suggest improved properties of biologically treated effluent. The present study thus implies a significant role of these bacterial strains as prospective bioremediators with cost-effective and eco-friendly treatment methods for tannery effluent for their safer disposal and avoidance of future risks to organisms and ecosystem. Further field studies using these promising strains for bioremediation of contaminated sites would be required for developing efficient bioremediation strategies and its practical implication.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical statement This article does not contain any studies with human participants performed by any of the authors. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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