

Microremediation Of Tannery Wastewater By Siderophore Producing Marine Bacteria

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Abstract

The present study evaluated the microremediation potential of nine siderophore producing marine bacteria for hazardous raw tannery wastewater from common effluent treatment plant (CETP). Most of the pollutants detected in the wastewater were diminished after the bioremediation process. Further, among the three potent isolates selected for aerobic and anaerobic bioremediation study, *Marinobacter hydrocarbonoclasticus* demonstrated the highest bioremediation aerobically with a reduction in chromium (88%), sulphate (71%), phosphate (68%) and nitrate (57%). Notably, *Nitratireductor kimnyeongensis* could attack the effluent under both aerobic and anaerobic conditions as substantiated by statistically significant ($p < 0.05$) reduction in the pollutants [chromium (85%), sulphate (63%), Chemical Oxygen Demand (COD) (69%), phosphate (76%)]. From the study it is evident that the pollutant load reduction was achieved under both aerobic and anaerobic conditions however aerobic environment was more effective in reducing chromium, Biochemical Oxygen Demand (BOD), sulphate, nitrate and phosphate. The bioremediation efficiency was further confirmed by the bioassay experiments with plant and animal models where higher seed germination, greater plant length and biomass, as well as improved survival rate of *Artemia nauplii* for bioremediated wastewater was observed as compared to the untreated effluent indicating a significant reduction in toxicity. The results for simultaneous removal of multiple-toxicants thus signify effectiveness and ease of using the robust properties of these marine bacterial strains suggesting their potential application for bioremediation. Hence this could pave a promising way for an environment-friendly and economically feasible clean-up strategy for safer disposal of tannery wastewater.

Keywords: Bioremediation, Marine bacteria, Multiple-toxicants, Tannery, Toxicity bioassay

1. Introduction

India is the second largest producer of footwear and leather garments which process 7,00,000 tons of hides and skins to produce 2 billion square feet of leather annually. India holds approximately 3,000 tanneries which collectively employ more than 2.5 million people. Ironically tannery industry is one of the dirtiest industries as it produces hazardous wastes due to the usage of large quantities of water and chemicals to transform animal hides into leather which are detrimental to the environment [1,2].

Nearly 90% of water and 80% of the chemicals used in the tanning process are discharged as wastewater which amounts to more than 30 million m³ per year [3]. Most of the tanneries are engaged in chrome tanning and thus carry heavy pollution loads due to massive presence of basic chromium sulphate, highly coloured compounds, sodium chloride and wetting agents [4, 5]. It also contains various organic substances, different types of biologically oxidizable tanning materials and large quantities of putrefying suspended matter thus resulting in high levels of chemical oxygen demand (COD) and biochemical oxygen demand (BOD). Discharge of effluent containing high levels of these contaminants poses serious threats to human health as these chemicals find their way to the food chain. Metal contaminant such as chromium is well known for its mutagenicity and carcinogenicity in humans. Disposal of untreated wastewater makes the land infertile and renders ground and surface water unfit for irrigation and drinking [6]. As only a limited amount of natural water resource is available, treatment of wastewater becomes a crucial step in wastewater management sector.

Conventionally, tannery effluent is treated by physico-chemical techniques such as adsorption, coagulation-flocculation, precipitation, radiation, oxidation, reverse osmosis and ultra-filtration either individually or in combination [2, 7]. However these methods have inherent drawbacks; they are economically unfeasible, cannot remove contaminants completely and further lead to secondary pollution and involve complicated procedures [8, 9]. For the durative development of the leather-making industry, the effluents are required to be treated with new effective, feasible and economically viable methods urgently.

In this context, bioremediation is an attractive alternative that offers the possibility to destroy or render various contaminants harmless using metabolic activities of naturally occurring bacteria, algae, fungi or plants [10]. Microbes, the natural recyclers, are an ideal alternative as they are inexpensive and eco-friendly. Numerous bacteria and fungi have been investigated for the bioremediation of tannery effluent specifically *Cellulosimicrobium* sp. [11], *Stenotrophomonas* sp. [12], *Bacillus* sp. [13, 14], *Brachymonas denitrificans* [15], *Thiobacillus* sp. [16] and *Vibrio* sp. [17].

Notably, most of the tannery wastewater bioremediation studies are pollutant specific, aimed at removal of only one or two pollutants mostly chromium [18]; however, tannery wastewaters contain various salts, metals and organic pollutants. Therefore, it is apparent that organism which can degrade a mixture of different pollutants efficiently would be preferable for bioremediation of complex wastewaters.

Marine bacteria are a lucrative option for bioremediation of tannery wastewater as they are adapted to adverse conditions of marine environment including varying temperature, salinity, pH, currents and precipitation. Hence marine bacteria which are suitably adapted to the adverse conditions possess naturally occurring metabolic capability to degrade, transform and/or accumulate several pollutants like toxic metals, organic load, recalcitrant hydrocarbons, heterocyclic compounds and pharmaceutical substances [19, 20]. Production of siderophore is one of such metabolic capabilities of bacteria which can be harnessed for bioremediation. Siderophores are iron binding proteins with low molecular weight having the ability to bind a variety of metals in addition to iron, such as chromium, manganese, magnesium and gallium [21]. Hence they hold great potential to be employed for bioremediation which can be beneficial from both environmental and economic point of view [22].

Several studies have addressed the bioremediation of tannery wastewater using bacteria; nonetheless, related studies on use of marine bacteria are scanty. Therefore, the current study aimed to investigate the bioremediation capacity of the siderophore producing marine bacteria. The experiments were carried out using strains from coastal and oceanic waters which were tested for their capacity to reduce pollutants from the effluent of leather processing industries under aerobic and anaerobic environments. Toxicity assays were further performed to validate reduction in toxicity after the bacterial treatment of tannery wastewater. The data obtained from the present study would pave a way to efficient tannery wastewater treatment using marine bacteria thus leading to a cleaner environment.

2. Materials and methods

2.1. Isolation and identification of marine bacteria

Water and sediment samples (55 and 98, respectively) collected at different depths from Equatorial Indian Ocean (EIO; at 1° intervals across the equator along 83°E from 1°N to 5°S) and Arabian Sea coastal waters (Off Goa along 15°N between 72 and 73°E) were used for the isolation of bacteria. In brief, appropriately diluted samples were spread plated on isolation medium (M622- HiMedia) and incubated for 3–5 days at 32±2°C as reported in our previous paper [23]. For screening of

siderophore production bacterial cultures were spot inoculated in Chrome Azurol Sulphonate (CAS) plate [24] where appearance of orange/yellow zone around the colony showed positive results. Colony characteristics including size, shape, colour, margin, texture, Gram and spore staining were also examined for the strains.

The bacterial strains were identified by molecular techniques as described by Rajasabapathy et al. [23]. Briefly, the genomic DNA was extracted from the isolates by using DNeasy kit (Qiagen) and 16S rRNA gene of the bacterial isolates was amplified using universal 16S rDNA primers namely 27F [5'-AGAGTTTGATCCTGGCTCAG-3'] and 1492R [5'-GGTTACCTTGTTACGACTT-3'] [25]. Nucleotide sequencing of the amplified products was performed using a 3130xl genetic analyzer (Applied Biosystems-Foster City, USA) at National Institute of Oceanography, Goa, India. The sequences were compared with the available sequences in public database using BLAST software (www.ncbi.nlm.nih.gov) and a phylogenetic tree was constructed by Neighbor-joining method using MEGA5 software [26].

2.2. Collection and analysis of tannery wastewater

The tannery wastewater used in this study was collected from common effluent treatment plant (CETP), Pallavaram, Chennai, India (12°57'44"N and 80°8'8"E) in pre-sterilized bottles, brought to the laboratory and maintained at 4 °C until further analysis. Various physical and chemical parameters of the wastewater were analyzed according to the standard methods described in American Public Health Association [27]. The undiluted raw tannery effluent was used throughout the study.

2.3. Experimental design

The nine siderophore producing bacterial isolates were screened for their bioremediation capacity. Cells in 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 inoculum (approximately 1×10^6 cells/mL) was inoculated into an Erlenmeyer flask containing 100 mL tannery effluent. The flasks were incubated in the dark at 32 ± 2 °C under static condition. Preliminary studies showed a decline in bacterial growth after 5 days and hence this duration was used for the bioremediation study. As most of the tanneries are engaged with chrome tanning, the wastewater is mainly laden with chromium and sulphate. Hence these two parameters along with bacterial growth were considered for the selection of the potent strain.

The 3 strains demonstrating effective bioremediation from the above screening were used for tannery effluent treatment under aerobic and anaerobic conditions. For aerobic treatment, exponentially

growing cells (approximately 1×10^6 cells/mL) were inoculated into 125 mL of tannery effluent and the flasks were incubated in the dark at 32 ± 2 °C. The flasks were manually swirled regularly to avoid floc formation. For anaerobic condition, the tannery effluent was anoxically dispersed in to 125 mL serum bottles which were tightly closed with butyl rubber stopper and sealed using aluminium crimps [28]. One millilitre of the bacterial culture (approximately 1×10^6 cells/mL) was added anoxically using a sterile syringe and bottles were incubated in the dark at 32 ± 2 °C for 5 days under static condition. Tannery effluent without bacterial inoculum served as a control for all the experiments. Evaluation of physico-chemical parameters and biomass estimation was carried out after 5 days of treatment to examine the growth as well as the bioremediation capacity of the isolates in tannery effluent under both the conditions.

2.4. Biomass estimation

Biomass of bacteria was estimated by measuring the Total Viable Count (TVC). For TVC, day 0 and day 5 samples were serially diluted from 10^{-2} to 10^{-6} and 100 μ l was spread plated on Nutrient agar (M001; Hi-Media, India). The plates were incubated at 32 ± 2 °C for 24 to 72 h and the colonies were counted.

2.5. Analytical procedures

The physico-chemical parameters including sulphate, pH, chromium, phosphate, nitrate, COD and BOD were measured as per standard methods by APHA [27]. All the chemicals used were of analytical grade and the experiments were performed at least three times to minimize analytical error.

Estimation of sulphate was done by Barium chloride turbidometric method [27]. Chromium 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 [27]. The ultraviolet spectrophotometric method was used for the estimation of nitrate in treated and untreated samples [27]. Detection of phosphate was done by ascorbic acid method [27]. The COD of the raw and treated effluent was determined by open reflux oxidation method. The COD reduction rate was calculated for treated effluent [27]. For BOD estimation, airtight DO bottles were filled with samples 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 [27]. The pH and salinity of the effluent was recorded using pH analyzer (LABINDIA) and digital salinity refractometer (PAL-06S).

2.6 Toxicity assay

To evaluate the ecotoxicological risks and the environmental safety of bioremediated tannery wastewater, toxicity assays are the most attractive, ideal and recommended tools. To accomplish this, phytotoxic and cytotoxic effect of bioremediated and raw tannery effluent was analysed.

2.6.1 Phytotoxic effect

In the present study seed germination and seedling growth (plant length and dry weight) of seven different varieties of commonly used grains and pulses (*Vigna radiata*, *Pisum sativum*, *Brassica juncea*, *Cicer arietinum*, *Zea mays*, *Lens culinaris* and *Triticum aestivum*) was assessed. Twenty seeds from each variety were surface sterilised using 2% mercuric chloride solution for 2 min followed by three times washing with sterile distilled water [29]. Subsequently the seeds were placed in clean sterile petri plates containing Whatman filter paper No.1 discs which were moistened with 2 ml of tap water for control and same volume of raw and treated effluent were used for the samples. The seeds were incubated at 32 ± 2 °C under light for 7 days [30] wherein seeds were considered germinated when both the plumule and radicle grew to more than 2 mm [31]. To further measure the seedling growth, 10 seedlings were picked from each set except from untreated tannery effluent were 5 seedlings were chosen and planted in to plastic pots (10 × 7 cm) containing garden soil for further 10 days. The soil was moistened everyday with either tap water or respective treated/untreated effluent. The parameters of seedling growth i.e., total plant length and biomass (dry weight), were documented.

2.6.2 Cytotoxicity Assay

Cytotoxicity assay was performed to assess the effect of bioremediated and raw tannery effluent (aerobic and anaerobic) on *Artemia salina* nauplii. 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, 3mL capacity with 106 mm width) containing 1.5 mL of either treated or untreated wastewater samples or seawater (positive control). The multi-well plates were incubated at room temperature (30 ± 2 °C) and 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 [32].

2.7. Statistical analysis

Data were expressed as the mean \pm standard deviation (SD) (n=3). All the data were first checked for normality using Shapiro–Wilk's test. Data that failed to meet the normal distribution were transformed by log (x+1) transformation prior to statistical analysis. The significance of variability,

among groups with oxygen level and different 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 among different toxicants of tannery effluents under presence and absence of oxygen for the potent strains. Statistical significance of the data was measured at $p < 0.05$ level. All the tests were performed using SPSS 16.0 (SPSS Inc., USA).

3. Results and Discussion

3.1. Characteristics of tannery wastewater

The tannery wastewater collected from CETP, Chennai was dark brown in colour with salinity of 9 g/L. The pH of tannery effluents is generally basic, and the value varies from 6-9 [33]. The untreated effluent used for the study was alkaline in nature with pH of 8.09. The physico-chemical analysis of raw tannery wastewater revealed high levels of toxicants such as chromium (55.15 ± 1.76 mg/L), sulphate (2015.2 ± 45.2 mg/L), COD (3300 ± 53.25 mg/L) and BOD (945 ± 25.16 mg/L) represented in Table 1. These parameters were found to be higher than the permitted levels by the Bureau of Indian Standards (BIS) [34]. Alkaline pH and high toxicants concentrations in wastewater could affect the aquatic flora and fauna of the receiving water bodies. Moreover, the settling particles of wastewater can cause unfavourable changes in soil properties and thus affect soil organisms [11].

3.2. Selection of potent marine bacteria

The nine siderophore producing marine bacteria used in this study were facultative anaerobes, Gram-negative, and non-spore forming (Table 2). Strains were identified based on 16S rRNA gene sequencing [23] and the phylogenetic relationship of the isolates is shown in the Fig. 1 (other sequences used in the tree were retrieved from GenBank database).

Among the nine strains tested for bioremediation of tannery effluent, three bacterial cultures namely, *Pseudoalteromonas mariniglutinosa*, *Marinobacter hydrocarbonoclasticus* and *Nitratireductor kimnyeongensis* showed better growth and adaptability in tannery effluent. Fascinating observation about this data was more than two order raise in the viable count for the above strains. *P. mariniglutinosa* showed maximum viability and growth (1.19×10^9 cfu/mL) in the effluent compared to other strains (Fig. 2a). The bacteria showing better growth in tannery wastewater simultaneously also reduced the levels of toxicants significantly. The strains, *M. hydrocarbonoclasticus*, *N. kimnyeongensis* and *P. mariniglutinosa*, demonstrated higher percentage of reduction in chromium and sulphate concentrations by more than 75 and 60% respectively (Fig. 2b, c). Hence, after considering the growth of the strains and their adaptation to the effluent condition above three strains

which showed highest toxicant removal ability were further tested. As these marine bacterial isolates were facultative anaerobes, a comparative study of their bioremediation and detoxification capacity under aerobic and anaerobic environment was carried out.

3.3. Growth of bacteria under aerobic and anaerobic condition

During aerobic treatment, *P. mariniglutinosa* grew well, whereas *M. hydrocarbonoclasticus* and *N. kimnyeongensis* demonstrated better growth in both aerobic and anaerobic treatments (Fig. 3a). The TVC count for *N. kimnyeongensis* increased from of 2×10^6 to 5.5×10^8 cfu/mL under aerobic condition, whereas, it grew up to 7.9×10^8 cfu/mL in anaerobic environment. *Marinobacter* is previously reported to be halotolerant and also grow under anaerobic condition [35], while *Nitratireductor* species are noted to be facultative aerobes [36]. The TVC count varied significantly ($p < 0.05$) between the three strains reflected by two-way ANOVA (Table 3).

3.4. Reduction in different toxicants during aerobic and anaerobic treatment

The results revealed a significant reduction in physico-chemical parameters analysed after the bacterial treatment of tannery wastewater. The concentrations of different toxic parameters analyzed before and after treatment under aerobic and anaerobic conditions are summarised in Fig. 3. In the present investigation, the tannery wastewater was found to contain high levels of chromium as most of the tanneries (>90%) are engaged with chrome tanning process. Serious concern about the toxicity of chromium compounds necessitates recovery and reuse of chromium from tanneries and other industrial wastes or rendering it to a less toxic form [37]. To survive in the presence of chromium, bacteria have to detoxify it, which is achieved by either reduction or accumulation inside the bacterium or adsorption of chromium on its surface [38, 39].

All the three bacterial isolates in this study were efficient in detoxifying chromium and demonstrated more than 79% removal under both aerobic and anaerobic treatment (Fig. 3b). The chromium reduction capacity of the bacterial isolates was not affected significantly by the presence or absence of oxygen. The initial concentration of chromium was 55.15 mg/L; *M. hydrocarbonoclasticus* reduced it maximally by 88 and 89% in the presence and absence of oxygen thus bringing down the levels to 5.9 and 5.8 mg/L respectively. Hence, it proved to be the most efficient strain for chromium reduction. Higher chromium removal efficiency of *Marinobacter* sp. could be related to its metabolic capacity to produce metal binding siderophores [40] as well as presence of exopolysaccharides [41]. Siderophores form a part of metal detoxification processes wherein the ligand exhibits metal-binding characteristics, often reducing metal toxicity [21]. In anaerobic respiration, bacterial cells detoxify

chromium or utilize it as a terminal electron acceptor to derive energy and thus leading to decreased levels of chromium in the effluents [42].

Sulphate is utilized by bacteria for various purposes through two different mechanisms; firstly, by assimilation pathway wherein it is incorporated into the cell components like amino acids, and secondly, it is used as a terminal electron acceptor for respiration described as sulphate dissimilation wherein it is released as sulphide [43].

The tannery effluent collected from CETP had an initial sulphate concentration of 2015.2 mg/L (Fig. 3c). All the three strains showed enhanced sulphate removal in the presence of oxygen. After aerobic treatment, *M. hydrocarbonoclasticus* could remove the sulphate by 71% whereas, *N. kimnyeongensis* and *P. mariniglutinosa* demonstrated 63 and 61% of sulphate reduction respectively. *M. hydrocarbonoclasticus* showed a significant difference in sulphate removal when compared to other strains. Results of two-way ANOVA also revealed significant ($p < 0.05$) differences in sulphate removal for aerobic and anaerobic process and among the bacterial strains (Table 3). Earlier studies by Chandra et al. [44] reported only 54% aerobic sulphate reduction from tannery wastewater in aeration lagoon of CETP dominated by *Stenotrophomonas* sp. and *Burkholderiales* sp. The results of our study showed that maximum sulphate removal efficiency achieved was 572 mg/L which is well below the standard permissible limits (1000 mg/L) of BIS [34].

On the other hand, under anaerobic condition, *N. kimnyeongensis* showed highest sulphate reduction (62%). Earlier studies by Sigalevich et al. [45] demonstrated a maximum of 50% sulphate removal under 5% of oxygen by *Desulfovibrio oxyclinae*. Comparatively our strain showed better results by lowering the initial sulphate concentration of 2015 to 764 mg/L after the bacterial treatment under anaerobic condition. The difference in percentage removal of sulphate between different treatments are statistically different and significant ($p < 0.05$) as determined by ANOVA (Table 3). Sulphate removal under anaerobic condition suggests that the bacteria may have utilized sulphate mainly as a terminal electron acceptor for oxidation of organic substrate when oxygen is not available; similar findings were reported earlier by Pfennig et al. [46]. *N. kimnyeongensis* thus stands out to be the most potent strain to treat the tannery effluents for removal of sulphate under both aerobic and anaerobic conditions. Simultaneous sulphate and chromium removal could be explained by sulfidogenesis-induced chromium reduction pathway, in which chromium is reduced by sulphide generated by biological sulphate reduction [47]. Hence when sulphate is reduced it also leads to decrease in chromium concentration from tannery effluent which is evident from a close correlation between sulphate and chromium at the 0.01 level of significance (Table 4).

High levels of nitrate and phosphate lead to eutrophication thus deteriorating the quality of water. The bacteria *en route* bioremediation utilize nitrogen and phosphorus to fulfil its nutritional and energy requirements thus removing these contaminants from the effluent [48]. The initial nitrate concentration of 5.34 mg/L in this study was reduced to 3 mg/L by all three strains under anaerobic treatment which accounted for more than 40% reduction (Fig. 3d). Under aerobic condition, highest nitrate reduction of 57% was shown by *M. hydrocarbonoclasticus* which is known for denitrification of saline wastewater [49]. A comparable result of nitrogen removal from tannery effluent was reported by Chandra et al. [44]. It has been reported that under anaerobic condition the amount of nitrate reduction decreases when higher chromium concentrations prevail [50, 51]. Removal of nitrate under the aerobic condition is dominated by assimilative metabolism; on the contrary under anoxic condition electron rich organic matter donates electron and nitrate act as a terminal electron acceptor for generation of energy [52]. *N. kimnyeongensis* demonstrated highest reduction percentage for phosphate, 76 and 73%, under aerobic and anaerobic condition respectively (Fig. 3e). The other strains *M. hydrocarbonoclasticus* and *P. mariniglutinosa* also demonstrated considerable reduction in phosphate level aerobically from 5.5 to 1.2 and 1.5 mg/L respectively.

3.5. Reduction of organic load after treatment

All the organic and inorganic constituents contribute towards high BOD and COD which are important parameters for assessing the water quality. These are determined by the amount of oxygen needed to oxidize the organics in the wastewater [6]. Wastewaters with high BOD and COD can lead to deleterious effects to the organisms of receiving water bodies due to the depletion of oxygen. Tannery effluents are generally characterised by high COD and BOD originating from liming process of the hides as well as from chemicals added during the process [53].

High COD/BOD₅ ratio in the collected effluent suggested that the effluent is not completely biodegradable. Table 3 shows significant variations in COD reduction with respect to different strains and oxygen level. During aerobic treatment, *N. kimnyeongensis* reduced the initial COD level of 3300 mg/L by 52%, whereas under anaerobic treatment it reduced by 69%. All the strains demonstrated better COD reduction under anaerobic condition with more than 55% removal (Fig. 4a). This could be related to the utilization of organic load by the bacteria as a source of carbon as well as an electron donor [54].

The decrease in BOD level showed significant difference under the aerobic and anaerobic condition with different strains (Table 3). *N. kimnyeongensis* efficiently reduced BOD by 50% irrespective of the treatment conditions (Fig. 4b). Strains *P. mariniglutinosa* and *M. hydrocarbonoclasticus* showed

higher BOD reduction under aerobic treatment as compared to anaerobic. Reduction in BOD by *N. kimnyeongensis* was found to be significantly higher ($p < 0.05$) as compared to other strains. The reduction in BOD and COD can be associated with the bacterial degradation of complex organic and inorganic pollutants to fulfil the nutritional requirements [44].

3.6. Toxicity bioassay

Several researchers have reported efficient bioremediation; however they lack evaluation of the actual effect of the treated effluent on the biota. Chemical analyses of complex wastewater and disappearance of parent compounds may not always indicate detoxification of the effluent [55]. The practical implication and effectiveness of microremediation on the environment can be previewed by testing it on model plants and animals. The magnitude of toxicity reduction is therefore more reliable when analyzed with living organisms. Toxicity tests are thus crucial tools to assess the efficiency of bioremediation by understanding the effect of treated wastewater on the indigenous biological communities of the receiving bodies.

3.6.1 Phytotoxicity

Toxic pollutants are known to hinder seed germination and early seedling growth; hence these sensitive parameters are used to examine the phytotoxic activity [29, 44]. The effect of treated and raw tannery effluent on germination and plant growth parameters of the seven different commonly used grains and pulses are shown in Table 5. The bioremediated effluent displayed higher seed germination percentage for all types of seeds which was comparable to tap water control; on the other hand germination percentage was found to be the least for raw untreated tannery effluent. On an average 76, 90 and 75% seed germination was documented for *P. mariniglutinosa*, *M. hydrocarbonoclasticus* and *N. kimnyeongensis* treated effluent respectively whereas raw effluent showed only 35%. Tap water control displayed maximum number of seed germination (96%). Further plant growth parameters i.e. plant length and dry weight also showed higher values for treated as compared to raw effluent. Plant length of seedlings sprinkled with bioremediated effluent was on par with tap water control. *Pisum sativum* (peas) showed two times increase in plant length for *N. kimnyeongensis* treated effluent (52.1 cm) as compared to untreated tannery wastewater (24.8 cm). Similarly a maximum increase in dry weight for *Vigna radiata* (moong) from 20.5 to 31.0 mg was noted for wastewater treated with *M. hydrocarbonoclasticus*. Tannery effluent bioremediated by all three strains demonstrated one and half times increase in plant biomass of *V. radiata*. Overall, plants irrigated with treated effluent demonstrated higher growth than the untreated wastewater.

Similar phytotoxic studies were carried out by researchers to validate the detoxification of wastewater. A significant percent reduction in inhibition of seed germination and plant growth when irrigated with treated samples implies effective bioremediation [29, 30]. Seed germination and plant growth tests in the present study thus revealed a substantial detoxification of the tannery wastewater post-treatment which further confirms reduction of toxic pollutants.

3.6.2 Cytotoxicity

The brine shrimp (*Artemia salina*) lethality bioassay was employed to analyze the toxicity effect of the treated and untreated tannery wastewaters. *Artemia* is one of the most valuable test model organisms available for ecotoxicity testing due to its sensitivity for a wide range of compounds, ease of handling, commercial availability of dormant eggs (cysts) which can be easily stored for long duration and hatched in short period of 24 h. *Artemia*-based assays are hence rapid, simple and inexpensive [56].

In the present study, the mortality rate of *Artemia* in effluent treated aerobically with *M. hydrocarbonoclasticus* was least, 7, 16 and 27% while, anaerobically treated effluent showed 20, 37 and 50% after 24, 48 and 72 h respectively (Fig. 5a, b). On the other hand 17, 37, 46% and 16, 23, 47% of mortality rate was noted for wastewater treated with *N. kimnyeongensis* in the presence and absence of oxygen respectively. The data presented in Fig. 5 clearly demonstrate that the untreated raw tannery wastewater caused significant mortality (97%) during the 72 h of experiment. Our results thus indicate that microremediation of tannery wastewater reduced the mortality rate of *A. salina* to a significant level in both the conditions. Aerobically treated effluent with *M. hydrocarbonoclasticus* showed more than 3 times increase in the survival rate of nauplii after 72 h.

Tannery wastewaters are characterised by high levels of pollutants like chromium, sulphate and organic load thus rendering it highly toxic [2]. Removal of these contaminants makes the effluent less detrimental [57, 58] thus increasing the survival rate of *Artemia* as compared to untreated effluents [59]. Hence the present toxicity study confirmed that the treatment of tannery effluent with marine bacteria remediated the toxicants without production of any harmful intermediates or remediation products during the process.

4. Conclusions

Environmental pollution caused due to the release of noxious tannery effluent is one of the serious concerns associated with leather industry. Based on the results of this study, it can be concluded that the isolated siderophore producing marine bacteria are capable of reducing heavy pollution load from tannery wastewater with high efficiency. The same has been experimentally proved by bioassay

wherein enhanced plant growth and higher survival rate of *Artemia* nauplii in the post-remediated wastewater was observed. In addition, our findings have revealed the possibility of successful application of these marine bacteria for aerobic microremediation of raw tannery wastewater without further dilution thus making the treatment process simpler, cost effective and sustainable. Although the availability of easily metabolizable nutrients and energy sources are scanty in tannery effluent, our isolates adapted quickly and survived in the wastewater due to their innate capabilities to thrive in harsh natural habitat. To the best of our knowledge, this work is one of the first few efforts to utilize these capabilities of siderophore producing marine bacteria for the efficient simultaneous bioremediation of multiple toxicants. Marine bacteria can thus be considered as a key agent for reliable and economic *in situ* tannery wastewater clean-up strategy for safer environment.

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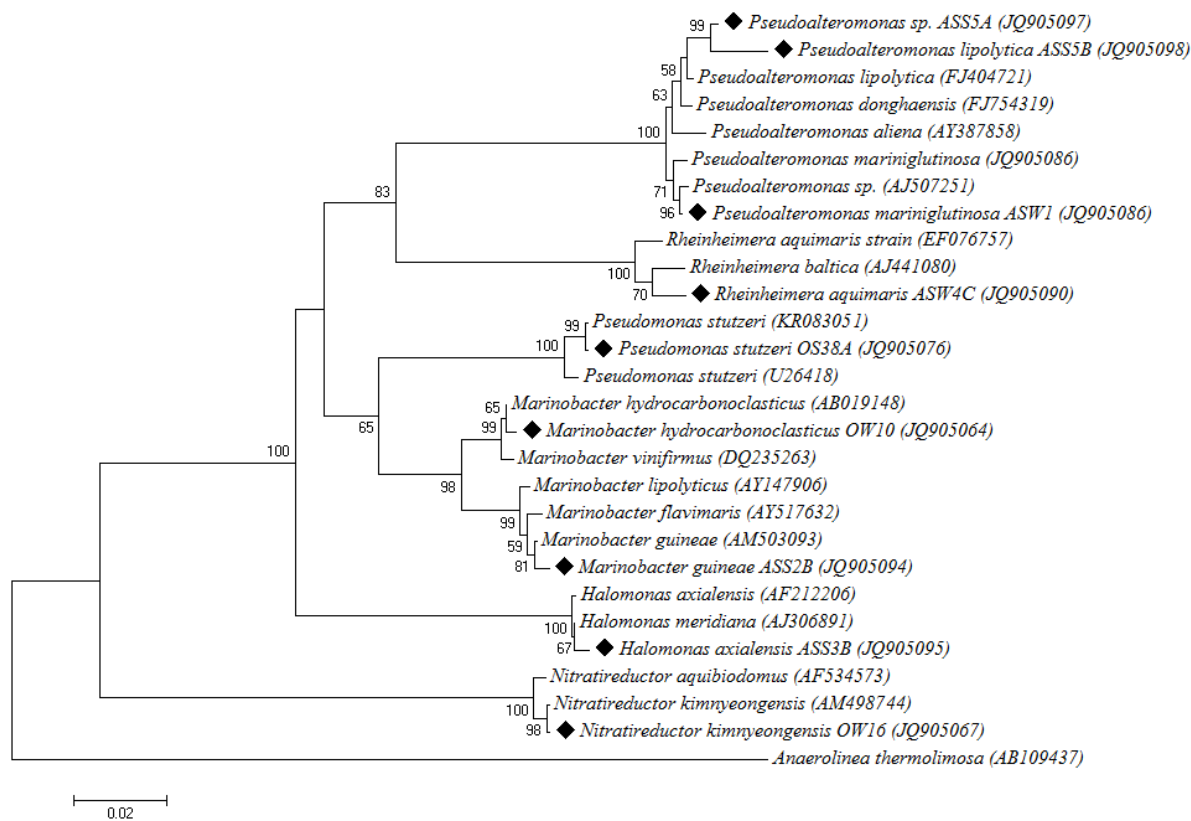


Fig. 1 The Neighbor-Joining tree constructed based on evolutionary distances and computed using the Maximum Composite Likelihood method representing relationship between the 16S rRNA sequence of marine bacteria. Bootstrap values (expressed as percentages of 1000 replications) greater than 50% are shown at the tree branch points. *Anaerolinea thermolimosa* is used as an out group

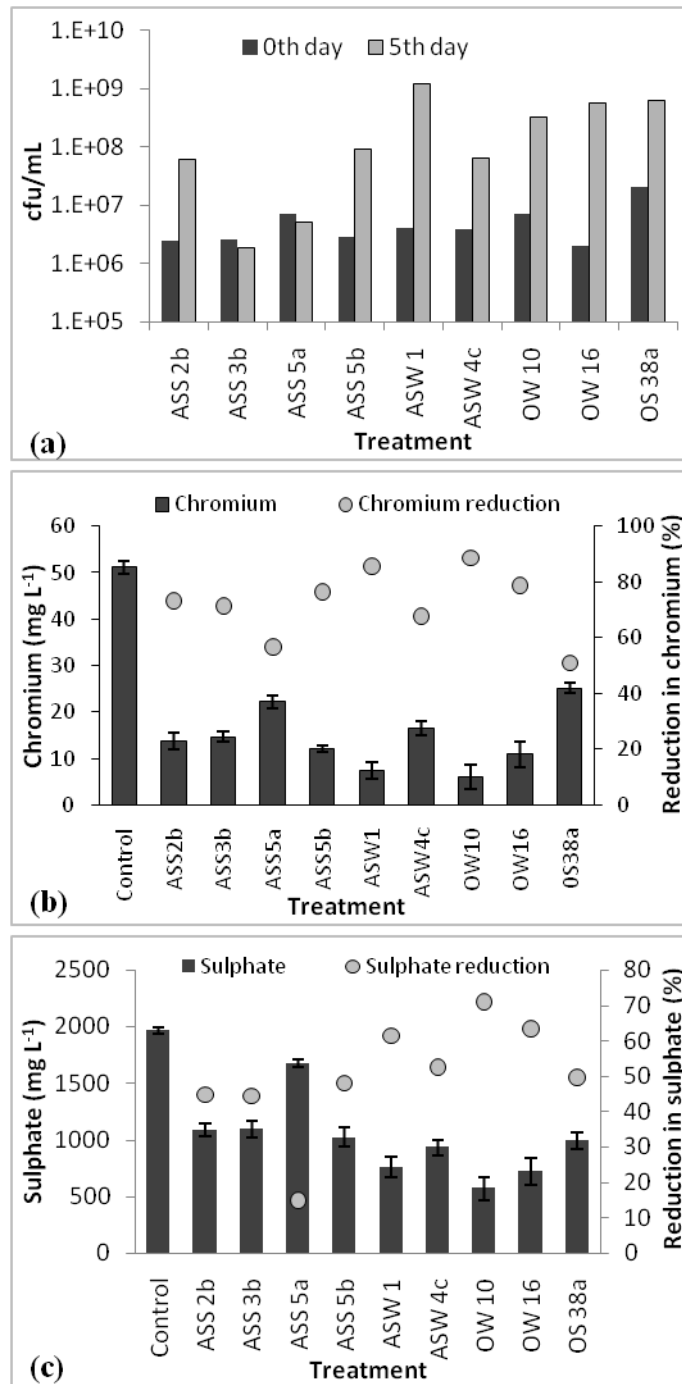


Fig. 2 Screening of marine bacteria for acclimatization and efficient bioremediation measured in terms of growth (TVC) in tannery wastewater (a) with simultaneous reduction in levels of chromium (b) and sulphate (c) post treatment. Values are mean \pm SD (n = 3)

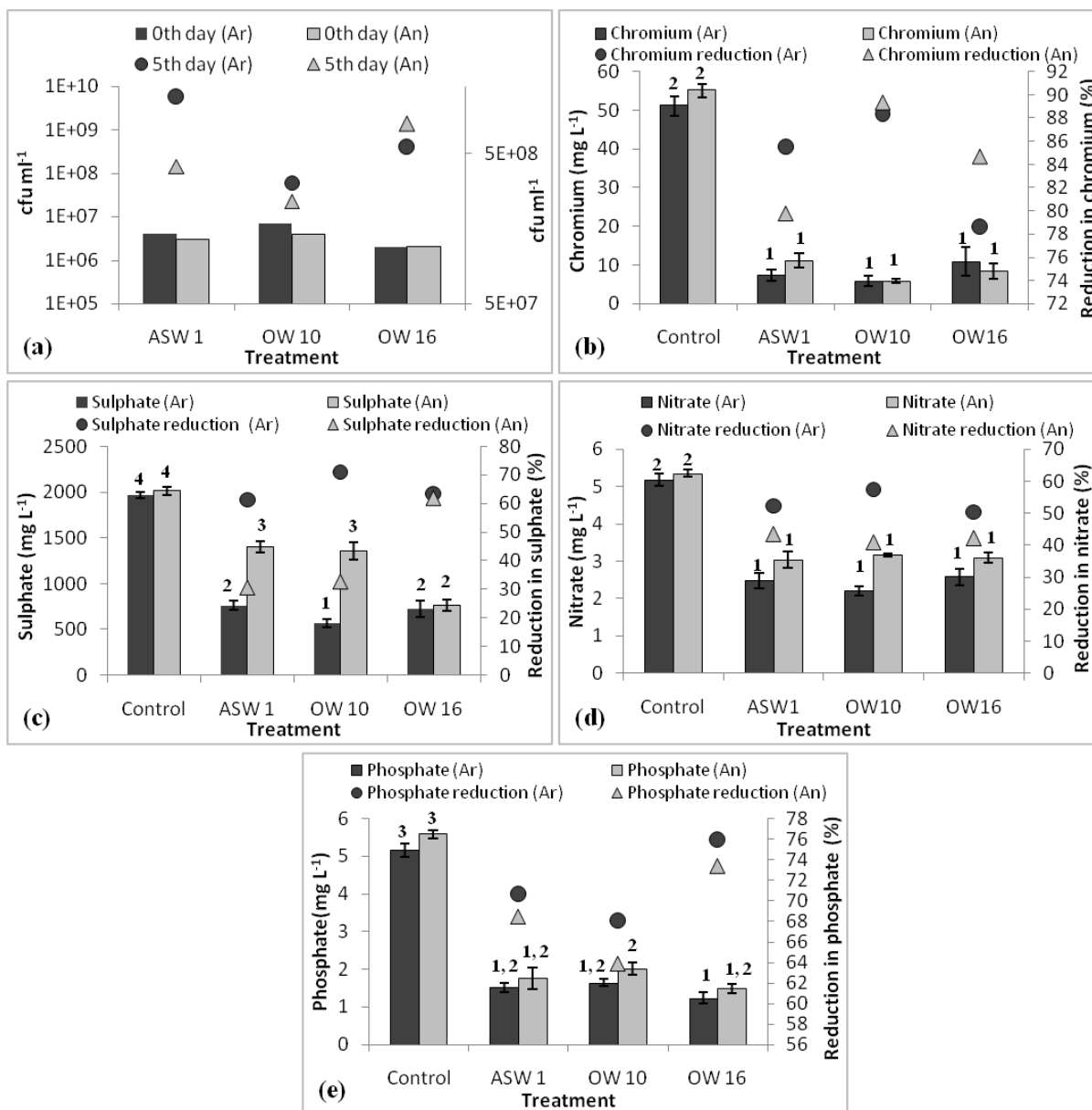


Fig. 3 Comparison of remediation capacity of marine bacteria under aerobic (Ar) and anaerobic (An) conditions. Graphs represent biomass (TVC) (a), reduction of chromium (b), sulphate (c), nitrate (d) and phosphate (e) in tannery wastewater. The superscript number 1, 2, 3, 4 and 5 on the bars denote groups which are significantly different ($p < 0.05$) formed by Post Hoc Tukey HSD test

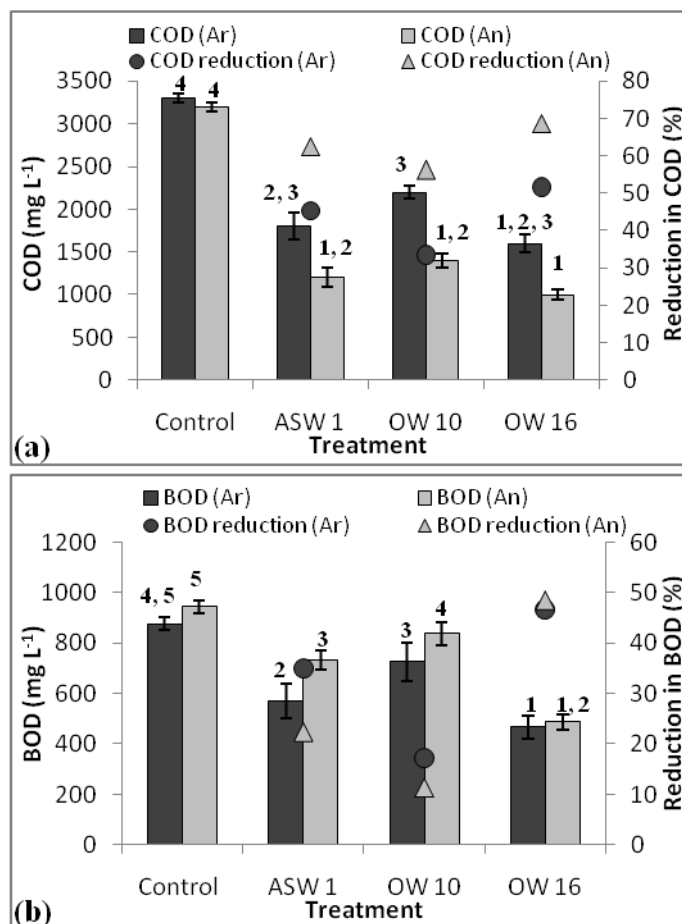


Fig. 4 Measurement of organic load by COD (a) and BOD (b) after treatment under aerobic (Ar) and anaerobic (An) condition. The superscript number 1, 2, 3, 4 and 5 on the bars denote groups which are significantly different ($p < 0.05$) formed by Post Hoc Tukey HSD test

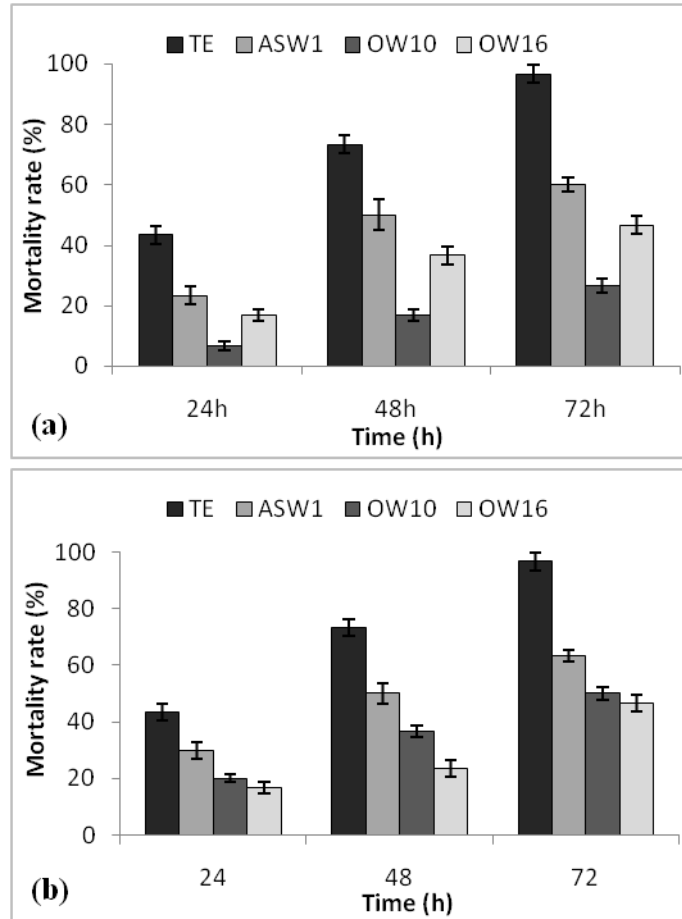


Fig. 5 The mortality rate of *Artemia* nauplii after exposure to raw (TE) and microremediated tannery wastewater at different time intervals under aerobic (a) and anaerobic (b) condition

Table 1: Characteristics of raw and treated tannery wastewater

Parameter	Values		Permissible limits (BIS, 1994)
	Before treatment	After treatment	
Colour	Dark brown	–	–
Odour	Pungent	–	–
pH	8.09	7.7	5.5-9.0
Salinity (ppt)	9	8.6	–*
Total solids (mg/L)	6187	–	2200
Total suspended solids (mg/L)	631	–	100
Total dissolved solids (mg/L)	4124	–	2100
Sulphate (mg/L)	2015.2	572	1000
Sulphide (mg/L)	5.6	–	2
Chromium (mg/L)	55.15	5.9	2
Nitrate (mg/L)	5.19	2.2	–*
Phosphate (mg/L)	5.15	1.2	–*
COD (mg/L)	3300	1600	250
BOD (mg/L)	876.84	467	30

– Not detected; –* No set limit

Table 2: Identity, accession number and colony characteristics of marine bacteria

Strain	Strain identity	Accession number	Size	Shape	Colour	Margin	Texture	Gram staining	Spore staining
ASS 2b	<i>Marinobacter guineae</i>	JQ905094	Punctiform	Rods	Watery	Rhizoidal	Smooth	Negative	Non-spores
ASS 3b	<i>Halomonas axialensis</i>	JQ905095	1mm	Rods	White	Circular	Smooth	Negative	Non-spores
ASS 5a	<i>Pseudoalteromonas</i> sp.	JQ905097	1mm	Rods	White	Circular	Smooth	Negative	Non-spores
ASS 5b	<i>Pseudoalteromonas lipolytica</i>	JQ905098	4mm	Rods	White	Circular	Rough with papillae	Negative	Non-spores
ASW 1	<i>Pseudoalteromonas mariniglutinosa</i>	JQ905086	Punctiform	Rods	Watery	Circular	Smooth	Negative	Non-spores
ASW 4c	<i>Rheinheimera aquimaris</i>	JQ905090	Punctiform	Rods	White	Circular	Smooth	Negative	Non-spores
OW 10	<i>Marinobacter hydrocarbonoclasticus</i>	JQ905064	1mm	Rods	White	Circular	Smooth with papillae	Negative	Non-spores
OW 16	<i>Nitratireductor kimnyeongensis</i>	JQ905067	Punctiform	Cocci	Watery	Circular	Smooth	Negative	Non-spores
OS 38a	<i>Pseudomonas stutzeri</i>	JQ905076	1mm	Cocci	White	Circular	Smooth	Negative	Non-spores

Table.3: Results of two-way ANOVA on chemical parameters of tannery effluent measured for different oxygen level and bacterial strains during effluent treatment

		F value	<i>p</i> value
TVC	Oxygen level	2.10	0.167
	Species	17260.00	<i>0.000</i>
	Oxygen level × Species	46.98	<i>0.000</i>
Sulphate	Oxygen level	1370.00	<i>0.000</i>
	Species	2890.00	<i>0.000</i>
	Oxygen level × Species	360.04	<i>0.000</i>
Chromium	Oxygen level	0.22	0.645
	Species	49.83	<i>0.000</i>
	Oxygen level × Species	0.91	0.458
Nitrate	Oxygen level	6.75	<i>0.019</i>
	Species	35.64	<i>0.000</i>
	Oxygen level × Species	0.58	0.638
Phosphate	Oxygen level	9.65	<i>0.007</i>
	Species	168.30	<i>0.000</i>
	Oxygen level × Species	0.22	0.884
COD	Oxygen level	22.81	<i>0.000</i>
	Species	64.47	<i>0.000</i>
	Oxygen level × Species	1.85	0.180
BOD	Oxygen level	55.55	<i>0.000</i>
	Species	232.60	<i>0.000</i>
	Oxygen level × Species	6.30	<i>0.005</i>

Italic values denotes the significant level at $p < 0.05$

Table 4: Correlation (Pearson) between different parameters of tannery effluent and the bacterial population (TVC) under aerobic and anaerobic condition

Aerobic								Anaerobic							
ASW1	Sulphate	Sulphide	Chromium	Nitrate	Phosphate	COD	BOD		Sulphate	Sulphide	Chromium	Nitrate	Phosphate	COD	BOD
Sulphide	-0.953**							Sulphide	-0.609						
Chromium	0.984**	-0.889*						Chromium	0.971**	-0.61					
Nitrate	0.874*	-0.875*	0.850*					Nitrate	0.945**	-0.495	0.958**				
Phosphate	0.995**	-0.926**	0.988**	0.888*				Phosphate	0.989**	-0.586	0.957**	0.963**			
COD	0.949**	-0.864*	0.929**	0.786	0.961**			COD	0.964**	-0.441	0.950**	0.987**	0.978**		
BOD	0.987**	-0.962**	0.959**	0.834*	0.974**	0.928**		BOD	0.973**	-0.564	0.914*	0.943**	0.991**	0.967**	
TVC	-1.000**	0.955**	-0.983**	-0.884*	-0.995**	-0.945**	-0.987**	TVC	-0.998**	0.569	-0.976**	-0.956**	-0.991**	-0.975**	-0.972**
OW10	Sulphate	Sulphide	Chromium	Nitrate	Phosphate	COD	BOD		Sulphate	Sulphide	Chromium	Nitrate	Phosphate	COD	BOD
Sulphide	-0.993**							Sulphide	-0.708						
Chromium	0.981**	-0.976**						Chromium	0.981**	-0.801					
Nitrate	0.900*	-0.880*	0.904*					Nitrate	0.958**	-0.717	0.965**				
Phosphate	0.996**	-0.994**	0.976**	0.919**				Phosphate	0.987**	-0.674	0.965**	0.980**			
COD	0.911*	-0.919**	0.823*	0.783	0.917*			COD	0.980**	-0.651	0.954**	0.983**	0.996**		
BOD	0.964**	-0.970**	0.931**	0.773	0.957**	0.899*		BOD	0.883*	-0.38	0.823*	0.911*	0.931**	0.946**	
TVC	-1.000**	0.990**	-0.982**	-0.909*	-0.995**	-0.906*	-0.958**	TVC	-0.999**	0.715	-0.981**	-0.961**	-0.990**	-0.981**	-0.885*
OW16	Sulphate	Sulphide	Chromium	Nitrate	Phosphate	COD	BOD		Sulphate	Sulphide	Chromium	Nitrate	Phosphate	COD	BOD
Sulphide	-0.955**							Sulphide	-0.894*						
Chromium	0.964**	-0.966**						Chromium	0.973**	-0.955**					
Nitrate	0.874*	-0.860*	0.880*					Nitrate	0.964**	-0.902*	0.959**				
Phosphate	0.991**	-0.942**	0.957**	0.885*				Phosphate	0.991**	-0.892*	0.963**	0.980**			
COD	0.955**	-0.970**	0.905*	0.839*	0.950**			COD	0.983**	-0.852*	0.939**	0.982**	0.990**		
BOD	0.995**	-0.970**	0.960**	0.849*	0.981**	0.970**		BOD	0.988**	-0.905*	0.963**	0.965**	0.997**	0.976**	
TVC	-0.999**	0.960**	-0.969**	-0.882*	-0.994**	-0.957**	-0.994**	TVC	-0.999**	0.897*	-0.976**	-0.965**	-0.993**	-0.981**	-0.991**

**Correlation is significant at the 0.01 level (2-tailed). * Correlation is significant at the 0.05 level (2-tailed).

Table 5: Phytotoxicity test for bioremediated tannery effluent

	<i>Vigna radiata</i>	<i>Pisum sativum</i>	<i>Brassica juncea</i>	<i>Cicer arietinum</i>	<i>Zea mays</i>	<i>Lens culinaris</i>	<i>Triticum aestivum</i>
Seed germination (%)							
TE	50	35	40	35	25	30	30
TPW	100	95	100	100	100	85	90
ASW1	90	80	70	75	65	80	70
OW10	100	95	80	90	85	95	85
OW16	80	90	65	70	75	80	65
Plant length (cm)							
TE	22.78 ±1.47	24.85 ±4.22	14.00 ±4.72	25.35 ±4.80	32.30 ±6.28	18.97 ±5.01	23.85 ±2.04
TPW	32.50 ±3.50	34.20 ±5.87	20.73 ±3.23	29.05 ±8.80	45.05 ±4.76	26.20 ±3.47	31.05 ±4.97
ASW1	33.65 ±3.19	50.90 ±3.89	17.14 ±2.35	35.40 ±4.61	50.10 ±3.37	24.05 ±4.33	34.22 ±3.49
OW10	34.25 ±2.85	51.15 ±3.21	18.00 ±4.24	35.20 ±7.80	48.00 ±3.48	25.40 ±2.28	30.25 ±4.33
OW16	34.35 ±1.94	52.15 ±3.87	19.00 ±5.50	29.35 ±6.12	46.65 ±7.96	25.05 ±2.20	30.50 ±4.97
Dry weight (mg)							
TE	20.50 ±3.52	54.60 ±4.46	4.20 ±0.23	129.93 ±9.36	202.07 ±29.09	19.14 ±2.15	30.53 ±4.18
TPW	28.88 ±7.31	70.40 ±6.71	6.45 ±0.34	181.54 ±12.37	236.85 ±32.65	23.53 ±1.93	37.10 ±5.67
ASW1	28.70 ±4.92	68.80 ±9.34	5.33 ±0.63	163.89 ±28.02	248.50 ±16.91	23.85 ±1.88	42.83 ±6.33
OW10	31.00 ±2.38	69.40 ±4.61	5.14 ±0.44	170.23 ±22.02	243.38 ±14.63	23.34 ±4.12	38.00 ±7.14
OW16	30.50 ±6.82	74.40 ±2.44	6.02 ±0.22	165.14 ±13.51	258.88 ±22.19	25.57 ±2.29	37.90 ±9.16

TE, Tannery effluent; TPW, Tap water

Scale: Low

High

