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Biological Treatment of Leather-Tanning Industrial Wastewater Using Free Living Bacteria

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Abstract

The present study aimed to investigate decontamination of tannery wastewater in terms of removal capacity and efficiency using indigenous and/or exogenous bacteria. Wastewater samples were collected from leather tanning factory. Three out of 17 indigenous tannery wastewater plus 3 exogenous bacterial isolates were identified and used in a batch mode remediation process as individual or mixed free living cultures. The tested tannery effluents had extremely high levels of all the tested parameters indicating high pollution potential, dangerous effects on the receiving environments and creating many treatment difficulties. Treatment of the tannery effluent was a function of time and bacterial species. ***Pseudomonas stutzeri* (PS)** considered the most while ***Bacillus* sp**. (RE12) considered the least efficient in removing all the tested parameters. Removal efficiencies (REs) were significantly and proportionally correlated with time regardless of bacterial species or parameters. However, bulk changes in all parameters were achieved within the first 24 h. The highest removals recorded were 86.7, 94.14%, 79.16, 95.64, 36.33, 93.66 and 44.91% for total suspended solids (TSS), biochemical oxygen demand (BOD), chemical oxygen demand (COD), fat, oil and grease (FOG), ammonia (NH_3), chromium (Cr) and hydrogen sulphide (H_2S), respectively after only 24 h. On the other hand, total dissolved solids (TDS) and nitrates (NO_3) recorded the highest increase (97.68 and 45.87%) after one and 7 days, respectively. Despite the highly efficient removals achieved for the tested parameters, their residual levels were slightly higher than the maximum permissible limits (MPL) for the safe discharge. Therefore, it is highly recommended to use the most promising bacteria in a fixed form to bring the effluent to the safe limits for the environment.

Keywords: Bacteria, Chromium, Leather, Nitrogen, Organic Matter, Pollution Control, Tanning Industry, Wastewater

1. Introduction

Tanning is the process, which converts the protein of the rawhide or skin into a stable material, which will not putrefy and is suitable for a wide variety of end applications. The highly polluting chromium is the most commonly used tanning material producing leather that is more supple and pliable than vegetable-tanned leather, and does not discolor or lose shape in water as drastically as vegetable-tan (Elsheikh 2009). Tannery effluent is among the most hazardous industrial pollutants due to its huge organic and inorganic load, which is highly toxic to human life and environment (Horsfall & Spiff 2005; Kongjao *et al.* 2008). One ton of skin generally leads to the production of 20 to 80 m^3 of turbid and foul-smelling wastewater including chromium (100–400 mg/l), sulfide (200–800 mg/l), high levels of fat and other solid wastes, and notable pathogen contamination as well as pesticides added for skin conservation during transport. The quantity of effluent generated is about 30 L for every kilogram of hide or skin processed. In addition, solid wastes of the tanning process represent up to 70% of the wet weight of the original hides exerting considerable strain on water treatment installations (Doble & Kumar 2005). Great variability was observed in the influent characteristics, depending on the type of hides and skins and the region from which they came, at the time of the sampling (Lefebvre *et al.* 2005). The untreated release of tannery effluents containing high COD, BOD levels, trivalent chromium, sulfides, sodium chloride, Ca, Mg, organics and other toxic ingredients, to the natural water bodies severely affects flora and fauna of the

ecosystem, increases human health risk (Kolomaznik *et al.* 2008) and also leads to eutrophication (Durai & Rajasimman 2011).

Many conventional processes were used to treat tannery wastewater including oxidation (Schrank *et al.* 2003), chemical (Song *et al*. 2004) and biological processes (Farabegoli *et al.* 2004). Durai & Rajasimman (2011) listed microorganisms efficiently used in the treatment of tannery wastewater. Cr-tolerant species include ***Bacillus* spp** with ability to reduce hexavalent chromium to its trivalent form (Morales *et al.* 2007), ***Streptomycin*** sp., ***Pseudomonas aeruginosa, P. fluorescens, Micrococcus*** sp, ***Streptomyces*** as well as yeasts like ***Pichi guilliermondii*** and ***Aspergillus*** spp (Congeevaram *et al.* 2007). Mechanism of Cr-tolerance or resistance of selected microbes is of particular importance in the bioremediation of contaminated tannery wastewater (Polisak *et al.* 2009). Biological treatment involves stabilization of waste by decomposing them into harmless inorganic solids either by aerobic, anaerobic or combined process. Aerobic decomposition rate is more rapid than the anaerobic process and it is not accompanied by unpleasant odours but anaerobic treatment is less energy-intensive and, hence, preferable to aerobic treatment. Activated sludge process (Ramteke *et al.* 2010), shaft-type hybrid bioreactor (Mazumder *et al.* 2008), sequencing batch reactor (SBR) coupled with respirometry (Ganesh *et al.* 2006), membrane sequencing batch reactor (MSBR) (Goltara et al., 2003), membrane bioreactor with powdered activated carbon (PAC) (Munz *et al.* 2007), wetlands planted with ***Typha latifolia*** (Calheiros *et al.* 2008) and combined biological-biological treatment (Srivastava *et al.* 2007) are examples of aerobic decontamination of tannery wastewater**.** Anaerobic methods include denitrification-nitrification (Leta *et al.* 2004), hybrid upflow anaerobic sludge blanket (HUASB) (Banu and Kaliappan 2007) and single UASB reactor for treating both the solid (generated from fleshing) and liquid wastes (Rajasimman *et al.* 2007) were found superior for the treatment of tannery wastewater. In many cases it is more efficient and feasible and sometimes obligatory to apply a treatment sequence of aerobic-anaerobic, or vice versa, for strong industrial wastewater such as tannery effluents. Examples include using UASB combined with an aerobic post-treatment (Lefebvre *et al.* 2006 a, b), combination of the sulfur recovery unit integrated with a USAB reactor (Suthanthararajan *et al.* 2004) and anoxic denitrification combined with aerobic nitrification (Chung *et al.* 2004). In other cases, application of combined process of physical or chemical with biological process to treat tannery wastewater would give satisfactory results compared to individual treatment processes. Complementary physical methods include adsorption (Espantaleon *et al.* 2003), coagulation (Scholz & Lucas 2003), flocculation (Ryu *et al.* 2007), oxidation (Di Iaconi *et al.* 2002) and using Fenton's reagents (Mandal *et al.* 2010).

Factors affecting the maximum degradation of tannery effluents include pH, agitation and initial substrate (COD) concentration (Durai *et al.* 2010). The essential tannery effluent components, which represent difficult tasks during its treatment, include salinity (Sivaprakasam *et al.* 2008; Apaydin *et al.* 2009), sulphides (Schenka *et al.* 1999), chromium (Eastmon *et al.* 2008; Pandi *et al.* 2009; Vignati *et al.* 2010) and tannins (Munz *et al.* 2009).

The main aim of the present study was to investigate the ability of biological treatment to decontaminate tannery wastewater in terms of removal capacity and efficiency using free-living indigenous and/or exogenous bacteria. Also the study aims to investigate the synergistic and/or antagonistic effects of the tested bacterial species during the treatment process.

2. Materials and Methods

2.1. Sampling

Leather tanning wastewater samples were collected from the final drainage effluent of a leather tanning factory in **Jeddah City, Saudi Arabia** during the course of the study. Tannery wastewater samples were subjected to physicochemical as well as microbiological characterization to define their pollution strength and selecting the best treatment technology. In addition, post-treatment characterization took place in order to calculate the treatment efficiency**.**

2.2. Microorganisms

Three indigenous (RE4, RE6 and RE12) isolated from the contaminated tannery effluent among 17 isolates in addition to three exogenous (S1, PS and PQ) bacterial species kindly provided from Institute of Graduate Studies & Research, Alexandria University (IGSR) collection were selected and used during the present study. Exogenous bacteria were originally isolated from heavily polluted wastewater and environments, therefore, exhibiting superior ability for organic matter and heavy metals remediation (El-Bestawy *et al.* 2002; El-Bestawy 2005; El-Bestawy & Ibrahim 2005; El-Bestawy *et al.* 2005). Selected bacteria were investigated as individual or mixture for their ability to bioremediate Cr-contaminated wastewater from leather tanning industry.

2.3. Media and Culturing Conditions

Dehydrated nutrient broth (NB) and agar (NA) were supplied by HIMEDIA. NB medium contained (g/l) peptic digest of animal tissue, 5.0; sodium chloride, 5.0; yeast extract, 1.5 and beef extract, 1.5 with 15.0 g/l agar in case of NA medium. NB and NA were prepared by dissolving 13.0 and 28.0 g/l from corresponding dehydrated media respectively, pH was adjusted to 7.4, sterilized by autoclaving at 121ºC for 20 min and freshly used for growth experiments as well as biodegradation assays. Other media (HIMEDIA) were used in the identification of the purified isolates and included the following:

Starch medium: contained (g/l) peptic digest of animal tissue, 5.0; sodium chloride, 5.0; yeast extract, 1.5; beef extract, 1.5 and agar, 15.0. It was prepared by dissolving 28.0 g/l distilled water to which 2.0 g starch (Scharlau) was added and the pH was adjusted to 7.4.

Lipid medium: contained (g/l) peptic digest of animal tissue, 5.0; sodium chloride, 5.0; yeast extract, 1.5; beef extract, 1.5 and agar, 15.0. It was prepared by dissolving 28.0 g/l distilled water to which 10.0 g plant oil was added and pH was adjusted to 7.4.

Phenol Red agar: base medium contained (g/l) protease peptone, 10.0; sodium chloride, 5.0; beef extract, 1.0; phenol red 0.025 and agar, 15.0. It was prepared by dissolving 31.0 g/l distilled water with pH adjustment to 7.4.

EMB medium: contained (g/l) peptic digest of animal tissue, 10.0; dipotassium phosphate, 2.0; lactose, 5.0; sucrose, 5.0; Eosin-Y, 0.40; methylene blue, 0.065 and agar, 13.5. It was prepared by dissolving 36.0 g/l distilled water with pH adjustment to 7.2 ± 0.2.

Endo medium: contained (g/l) peptic digest of animal tissue, 10.0; dipotassium phosphate, 2.5; lactose, 10.0; sodium sulphite, 3.3; basic fuchsine, 0.3 and agar, 12.5. It was prepared by dissolving 38.6 g in one liter distilled water with pH adjustment to 7.4 ± 0.2.

CLED medium: contained (g/l) peptones, 11.9; L-Cystine, 0.128; bromthymol blue, 0.02; lactose, 10.0 and agar, 15.0, which were supplied by Biolab. It was prepared by dissolving 37.0 g in one liter distilled water with pH adjustment to 7.3.

After culturing, the selected bacterial species were incubated (Incubator WTS binder TUTTLINGEN/Germany) at 35°C for 24 hours.

2.4. Bacterial Isolation, Purification and Identification

Heterotrophic bacterial colonies from tannery wastewater were purified by streaking on NA agar plates, incubated at 35°C. Pure indigenous as well as exogenous isolates were classically identified using cell and colony morphology, Gram staining and biochemical profiling using the dehydrated media in the API kits. In addition to the API testing, some other confirmation biochemical tests were performed included starch hydrolysis, lipid hydrolysis, oxidase, catalase, growth on phenol red agar medium, EMB medium, Endo medium and finally on CLED medium. This was followed by molecular characterization of the most promising isolates.

2.5. Molecular Identification

Total genomic DNA was extracted from 5 ml overnight NB culture of the purified isolates (Sambrook *et al.* 1989). PCR was performed in a light cycler Eppendorf PCR machine. A 1300 bp fragment was obtained by PCR amplification of the 16S rDNA gene (Ausubel *et al.* 1999) using the primers F-start: 5'-AGAGTTTGATCMTGGCTCAG-3' and R-1387: 5'- CGGGCGGTGTGTACAAGG-3'. PCR mixture was composed of 100 ng of genomic DNA, 30 pmol of each primer, 200 µM of dNTPs, 1U of Taq polymerase and 10 µl of 10X PCR reaction buffer, the reaction volume was adjusted to 100 µl in 0.5 ml Eppendorf tube. The PCR amplification conditions were performed by an initial denaturation step at 94°C for 10 min followed by 30 denaturation cycles at 94°C for 1 min, annealing at 60 °C for 1 min and an extension at 72 °C for 1 min followed by a final extension step at 72°C for 10 min. Amplicons of 16S rDNA were purified using PCR purification kit (QIAGEN). Each of these purified products was sequenced by the chain terminator method (API model 3730xl, Bioneer, Germany) using the two corresponding PCR primers separately. The resulting DNA sequences were phylogenetically analyzed using the BLAST search program (Altschul *et al.* 1990). Multiple sequence alignment and molecular phylogeny were performed using MEGA 5.0 software (Hall 1999).

2.6. Bioremediation Bioassays

2.6.1. Screening of Bacterial Isolates

Seventeen indigenous and three exogenous pure isolates were tested visually for bioremediation of tannery

effluent in order to select the most promising candidates. They were individually inoculated in 250 ml flasks containing tannery effluent and incubated at 35°C under 100 rpm agitation speed (Shaking Incubator JSSI-100T). Visual observations were taken daily for a week according to which the promising isolates for bioremediation process were selected.

2.6.2. Bioassays Using Batch Cultures

Six promising bacterial candidates (3 indigenous; RE4, RE6 and RE12 and 3 exogenous; S1, PS and PQ) were selected according to the preliminary screening test and employed as free-living individuals or mixture for remediation of the highly contaminated tannery effluent. Seven cultures (6 individual and one mixed) were activated each in 100 ml (10%) NB medium (3 replica each) and incubated until heavy growth was obtained. Total viable count (TVC) of all cultures was counted to determine the initial densities of the different inocula. All bacterial inocula were seeded into to 900 ml (90%) raw tannery effluent, previously characterized (zero time or raw readings), reaching a final volume of 1 L. Effluent cultures, individual and mixed as well as a control sample (one liter un-inoculated tannery effluent) were incubated for 7 days under the previously mentioned conditions where samples were aseptically drawn at 24 h interval. Treated effluent' samples were re-characterized, residual levels of the selected parameters were determined at each exposure time and their removal efficiency were calculated to determine the effectiveness of the remediation process.

2.7. Characterization of the Raw and Treated Industrial Effluent

Wastewater was characterized before and after the proposed treatment. Characterization of the wastewater included its pH, temperature, DO, TSS, BOD, COD, FOG, H_2S, NH_3, NO_3, bacterial TVC and total chromium (Cr) all of which were determined using the standard techniques described in Standard Methods for the Examination of Water and Wastewater (*Clesceri et al.* 1999). Post- treatment characterization determined residual levels of the selected parameters at each exposure time. Removal efficiency was calculated to determine the effectiveness of the remediation process according to the following equation:

Removal Efficiency (RE %) = C0 - RC/ C0 X 100

Where C0 = Initial Concentration before Treatment (Zero Time);

RC= Residual Concentration after Treatment at each Exposure Time.

2.7.1. Determination of Chromium

Chromium in tannery wastewater was digested using conc. HNO_3 and determined following colorimetric method described by Clesceri *et al.* (1999) using spectrophotometer (HACH DR 5000) at 357.9 nm wave length.

2.7.2. Sulfides (H_2S)

Hydrogen sulfide and acid-soluble metal sulfides react with N, N- dimethyl-p-phenylenediamine sulfate to form methylene blue. The intensity of the blue color is proportional to the sulfide concentration. H_2S was determined using simple, direct method (690 Sulfide). Sample S^{2-} was measured at 665 nm and results obtained in µg/l S^{2-}.

2.7.3. Ammonia

Ammonia compounds combine with chlorine to form monochloramine, which reacts with salicylate forming 5-aminosalicylate that is oxidized in the presence of a sodium nitroprusside catalyst to form a blue- color compound. The blue color is masked by the yellow color from the excess reagent to give a final green- color solution. Ammonia was determined using simple, direct method (385 N Ammonia, Salic). NH_3-N (mg/l) in the samples was measured at 665 nm.

2.7.4. Nitrates

Cadmium metal reduces nitrates in the sample to nitrite which reacts in an acidic medium with sulfanilic acid forming an intermediate diazonium salt. The salt couples with gentisic acid to form an amber colored solution. Nitrates were determined using simple, direct method (355 N, Nitrate HR PP). NO_3^-N (mg/l) in samples was measured spectrophotometrically at 500 nm. Readings were converted into nitrate (NO_3^-) by multiplication in 4.4.

2.7.5. Total Viable Count of Bacteria (TBVC)

Raw and treated tannery samples were sequentially diluted, cultured (3 replicas each) using the pour plate technique of the standard heterotrophic plate count method (Clesceri *et al.* 1999) in NA- medium and incubated at 35ºC for 24 hours. Colony forming units (CFU) of the total viable bacterial counts (TVC) were recorded (Colony Counter Stuart colony counter protected by Bio Cote) and averages were calculated.

2.8. Statistical Analysis

Correlation coefficients (Pearson's r) were used to determine the relations among the different contaminants present in the raw and treated tannery effluents.

3. Results

3.1. Classical Identification

Traditional identification of tannery (RE1-RE17) isolates (Table 1) led to exclusion of nine pathogenic or environmentally harmful isolates. Moreover, exogenous (S1, PS and PQ) bacteria were subjected to identification using the same methods.

Table 1. Most Probable Identification of the Indigenous and Exogenous Bacteria Based on Their API Biochemical Profiles

Bacterial Code	Most Probable Identification
RE1	*Pseudomonas matophilia* Id=99%
RE2	*Klebsiella pneumonia* Id=95.9%
RE3	*Klebsiella pneumonia* Id=99%
RE4	*Pseudomonas aeruginosa* Id=46.9%
RE5	*Klebsiella pneumonia* Id=98.4%
RE6	*Kluyvera.spp* Id=92.3%
RE7	*Salmonella. spp* Id=98.2%
RE8	*Salmonella. spp* Id=99.4%
RE9	*Aeromonas hydrophila* Id= 99.9%
RE10	*Klebsiella pneumonia* Id=95.9%
RE11	*Enterobacter cloacae* Id=80.5%
RE12	*Pseudomonas fluorescens/ putida* Id=87%
RE13	*Aeromonas hydrophila* Id= 99.7%
RE14	*Aeromonas hydrophila* Id= 99.9%
RE15	*Aeromonas hydrophila* Id= 99.9%
RE16	*Klebsiella pneumonia* Id=94.2%
RE17	*Kluyvera.spp* Id=97.4%
S1	*Pseudomonas fluorescens /Putida* Id= 82.1%
PS	*Providencia rettgeri* Id=99.9%
PQ	*Pseudomonas fluorescens /Putida* Id= 62%

3.2. Screening of Bacterial Isolates

Clear variations were recorded among the eleven isolates (8 indigenous plus three exogenous) towards bioremediation of tannery effluent for one week in the screening test. In that test, visual observations (i.e. degree of clearness) were taken daily. Eight isolates (RE4, RE6, RE12, RE15, RE17, S1, PS and PQ) showed reduction in the tannery effluent turbidity while the other 3 (RE1, RE11 and RE14) showed no ability to reduce the turbidity. Among the first group, 6 isolates (RE4, RE6, RE12, S1, PS and PQ) were selected for bioremediation assays based on their high reduction of turbidity in the tannery effluent indicating their ability to degrade the included contaminants. Additional biochemical characteristics (Table 2) were determined for the 6 isolates to confirm their identification.

Table 2. Biochemical Characteristics of the Selected Bacteria for Tannery Wastewater Bioremediation

Bacterial Codes	Starch Hydrolysis	Lipid Hydrolysis	Growth on Phenol Red Agar Base	Oxidase	Catalase	Growth on EMB Medium	Growth on Endo Medium	Growth on CLED Medium
RE4	-	-	G	-	+	G	G	+
RE6	-	-	GCF	-	+	GF	G	+
RE12	+	+	G	-	+	-	-	+
S1	+	+	G	-	+	-	-	+
PS	-	-	GCF	+	+	G	G	+
PQ	+	+	G	-	+	-	-	+
GC: Growth with Medium Color Change, -: Negative, +: Positive, G: Growth, F: Fermentation								

3.3. Molecular Identification

Genomic DNA was prepared from the six selected bacterial isolates (RE4, RE6, RE12, S1, PS and PQ), purified and sequenced. The 16S rDNA sequences of the isolates were submitted to Gene Bank sequencing data and aligned against the 16S rDNA sequences of Ribosomal Database project. Table 3 compiles Gene Bank accession numbers of the highest sequence similarity as well as the closest neighbor (s) to the 16S rDNA gene partial sequences of the tested strains. Phylogenetic relationships of the experimental isolates and the closely related species were analyzed using the multi sequence alignment program (MEGA 5) and the results are presented in phylogenetic tree (Fig. 1). Sequences of the three isolates RE12, S1 and PQ were affiliated according to their 16S rDNA to members of genus ***Bacillus*****,** whereas isolate **PS** was affiliated to genus ***Pseudomonas***, RE4 was affiliated to ***Providencia*** and RE6 was affiliated to ***E. coli***. Order of descending similarity was as follow: high (99%) of RE4 to ***Providencia vermicola***, high (97%) of **S1** to ***Bacillus amyloliquefaciens*** strain **T004**, 96% similarity between RE6 and ***Escherichia coli* O7:K1 CE10** as well as between PS and ***Pseudomonas stutzeri* M15-10-3** and finally RE12 and PQ showed lower similarity (86%) to ***Bacillus*** sp. **SA-3** and ***Bacillus*** sp. **PL47** respectively.

Table 3. Similarity Percentages to the Nearest Neighbours of the Selected Isolates

Isolate Code	Nearest Neighbour (s)	GenBank Accession of the Nearest Neighbor	Similarity %
RE4	***Providencia vermicola* W9B-11**	**EM_PRO:HQ238823**	**99 %**
RE6	***Escherichia coli* O7:K1 CE10**	**CP003034.1**	**96 %**
RE12	***Bacillus* sp. 5-8**	**HM489982.1**	**86 %**
S1	***Bacillus amyloliquefaciens* T004**	**HQ840415.1**	**97 %**
PS	***Pseudomonas stutzeri* M15-10-3**	**HM030751.1**	**96 %**
PQ	***Bacillus* sp. PL47**	**HQ536222.1**	**86 %**

3.4. Treatability of Tanning Effluent Using Free Living Bacteria (Batch Mode)

No significant changes in the pH values (Fig. 2a) among raw effluent (7.08) and either the control or the seeded wastewater before or after the remediation process (highest value of 7.36 by RE4 after 5 exposure day). Very low DO level (0.38 mg/l, zero time) was recorded in the raw effluent which is far from the safe limit (4 mg/l) stated by the **Egyptian** environmental laws (No 48/1982 & 4/1994 for discharging into fresh and marine aquatic ecosystems) indicating strong effluent.

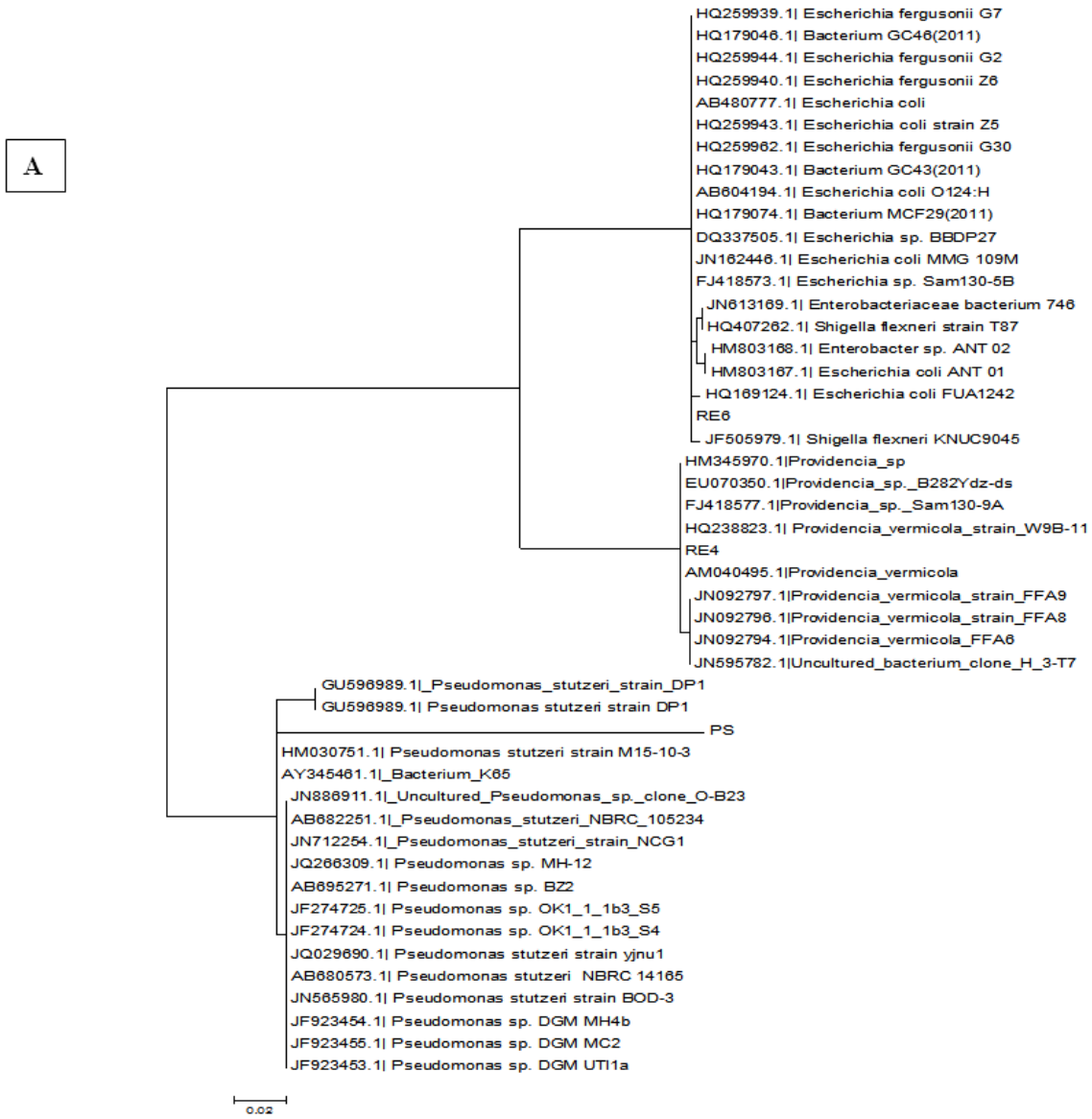
There was a general trend of gradual decreasing DO levels with increasing exposure time (till the 7^{th} day) in all

samples either seeded or not (Fig. 2b). Mixed culture seeded-effluent showed the lowest DO residue (0.24 mg/) reflecting the highest consumption rate compared to the control that recorded the highest DO residue (0.34 mg/l) after seven exposure days.

3.4.1. Effluent Solids

Raw wastewater recorded 10350 mg/l TDS that increased gradually in all samples (Fig. 2c) due to biodegradation and formation of dissolved salts. Generally the highest increases were achieved after the first 24 h followed by gradual but insignificant increases till the end of the experiment (7 days).

Highly significant variations were noticed among the tested bacteria depending on their ability to degrade and/or transform the available contaminants in tannery wastewater. PQ was the most active resulted in 97.68% and 97.91% TDS increase after one and 7 days respectively while RE12 was the least active recording only 12.27 and 12.51 % respectively after the same exposures. On the other hand, control wastewater showed the lowest recorded increase in the TDS concentration (0.09 and 0.29% after 24 h and 7 days respectively) indicating very low biodegradation activity compared to the seeded wastewater. The lowest recorded TDS (10380 mg/l) 5.19 fold higher than the MPL of TDS (2000 mg/l).



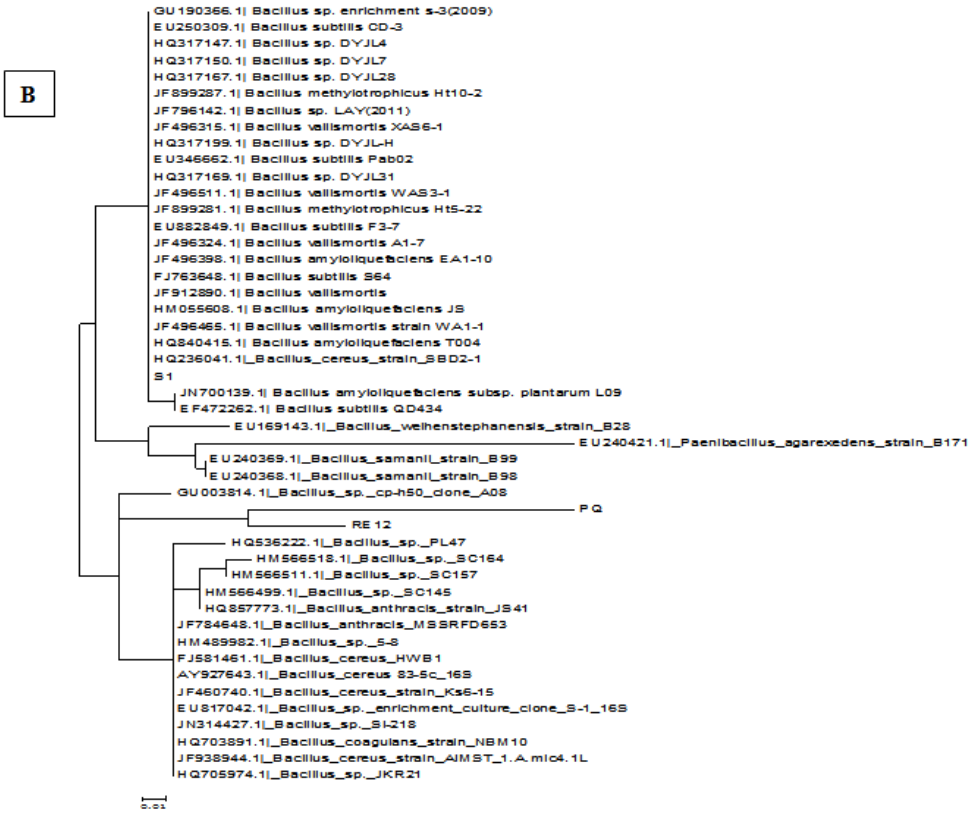


Figure 1. Phylogenetic Relationships among the Tested Isolates and the Most Closely Related Bacterial Species. The Tree was Generated by MEGA 5 Software

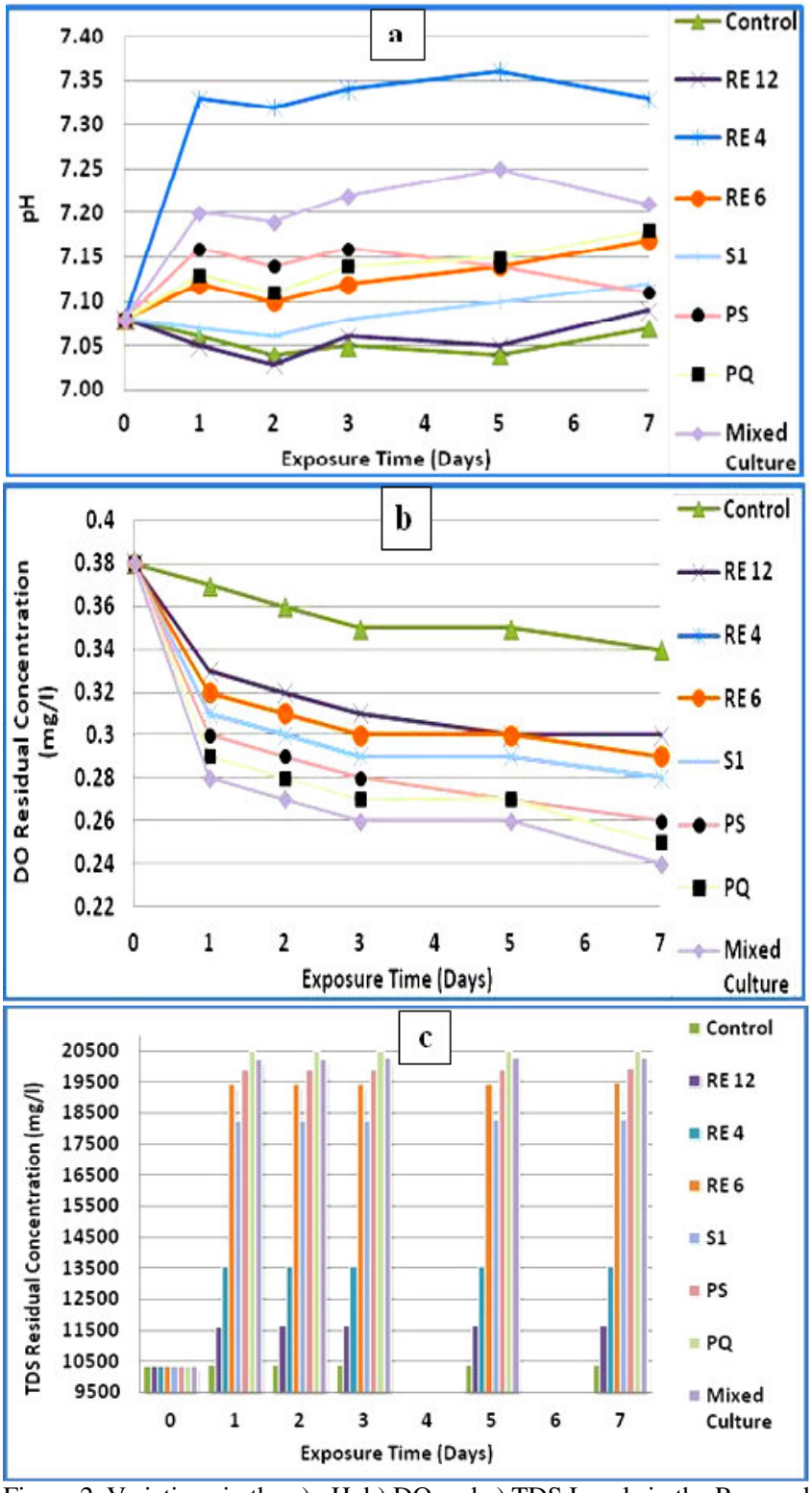


Figure 2. Variations in the a) pH, b) DO and c) TDS Levels in the Raw and Treated Tannery Wastewater at Different Exposure Times

Very high TSS level (14000 mg/l) was recorded in the raw tannery effluent. Highly significant removals were achieved by all the tested cultures after the first 24-treatment h. PS exhibited the highest efficiency (86.7%) while RE12 showed the lowest recorded efficiency (20.24%) after the first 24 h treatment. This was followed by slight insignificant increases in their removal of TSS until the end of the experiment (Fig. 3a). On the other hand, very low RE of the TSS (0.02%) was recorded by the control confirming the high efficiency and role played by

the augmented bacteria for degradation and removal of tannery wastewater contaminants. A range of 1835-11150 mg/l was recorded as the lowest RC of the TSS, which is 30.58-185.83 fold increase in the TSS content allowed for safe discharge (60 mg/l).

3.4.2. Organic Matter

Very high BOD level (3500 mg/l) characterized the raw tannery effluent at the zero time. PS exhibited the highest efficiency (94.71%) and RE12 recorded the lowest efficiency (65.77%) after the first 24 h treatment (Fig. 3b) compared to very low RE (0.090%) recorded by the control after the same exposure. Although as low as 185 mg/l RC was achieved by PS, it slightly higher (3.1 fold) than the MPL of the BOD (60 mg/l) for safe discharge of tannery effluent.

With the extremely high COD, level (25100 mg/l) recorded in the raw tannery effluent, generally lower COD REs (%) were achieved compared to those obtained for BOD removal. Again PS exhibited the highest efficiency (79.16%) and RE6 showed lowest recorded efficiency (58.47%) after the first 24 h treatment (Fig. 3c) compared to the extremely low RE of the COD (0.02 and 0.12%) recorded by the control after one and 7 exposure days respectively. Treatment resulted in 5188 mg/l RC representing 51.88 fold higher than the MPL of the COD (100 mg/l) for safe discharge of tannery effluent.

3.4.3. Fat, Oil and Grease (FOG)

PS exhibited the highest removal efficiency (95.64%) from the extremely high FOG level (2339 mg/l) in the raw tannery wastewater while RE12 showed the lowest recorded efficiency (6.80%) after the first 24 h treatment (Fig. 4a). The un-seeded wastewater recorded the lowest RE of the FOG (0.17 and 0.73%) after one and seven treatment days respectively. The lowest RC of FOG (81 mg/l) is 8.1 fold higher () than MPL (10 mg/l) for safe discharge of tannery effluent.

Other Tannery Chemical Pollutants

3.4.4. Ammonia (NH_3)

Removal efficiency of NH_3 from tannery effluent increased with increasing exposure time reaching the highest values after the seventh exposure day. PS was the most efficient recording the highest RE of NH_3 (29.50 and 36.33%) after the first and seventh treatment days respectively compared to RE 12 which recorded the lowest RE of 8.99 and 13.67% at the same exposure times respectively (Fig. 4b). Control wastewater showed very low NH_3 removal reaching only 3.24% after 7 days compared to samples seeded with the selected bacteria, which indicated their important role in the bioremediation process. The lowest recorded 17.7 mg/l represents 5.9 fold higher than MPL of NH_3 (3 mg/l).

3.4.5. Nitrate (NO_3)

Opposite to all the tested parameters, there were considerable increases in NO_3 concentration with time by all the tested bacteria reached their highest levels at the end of the 7^{th} day. The tested bacteria exhibited considerable variations towards formation of NO_3. The order of bacterial efficiency after 24 h treatment was as follows; PQ (38.23%), mixed culture (35.47%), RE6 (30.28%), PS (25.99%), S1 (20.18%), RE4 (11.31%) and finally RE12 with the lowest efficiency (7.34%). These figures were increased after 7 treatment days to record 45.87% (PQ), 44.65% (mixed culture), 42.20% (PS), 37.31% (RE6), 27.52% (S1), 20.18% (RE4) and 13.15% (RE12) (Fig. 4c). The lowest increase in the NO_3 levels (0.62 and 4.89%) were recorded by the control after one and seven treatment days respectively indicating the absence of nitrification bacteria. NO_3 recorded levels < 50 mg/l along the whole experiment duration, which is below the **Egyptian** MPL (50 mg/l), but higher than **Saudi** MPL (15-20 mg/l) required for discharging into the central treatment plant.

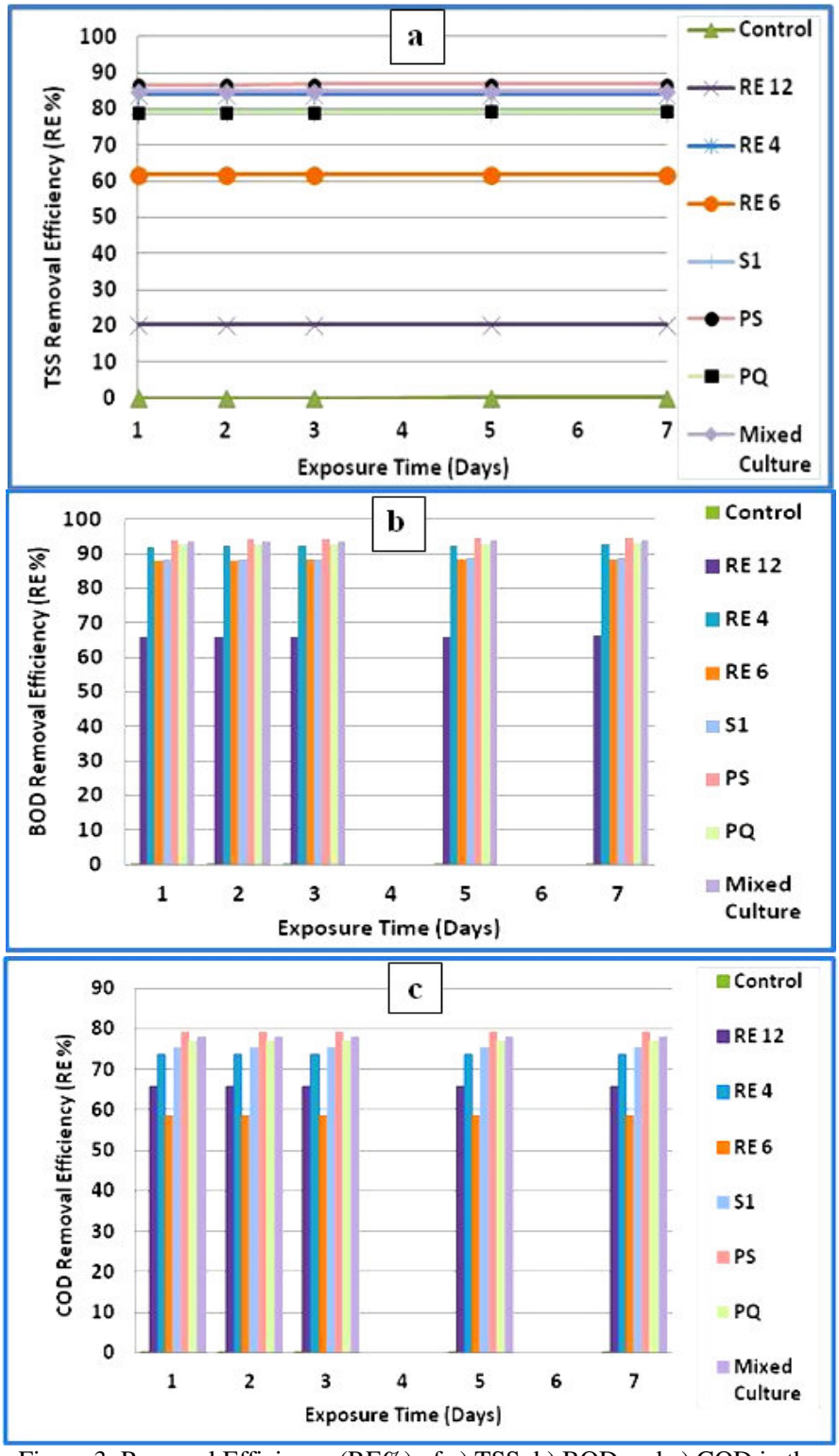


Figure 3. Removal Efficiency (RE%) of a) TSS, b) BOD and c) COD in the Raw and Treated Tannery

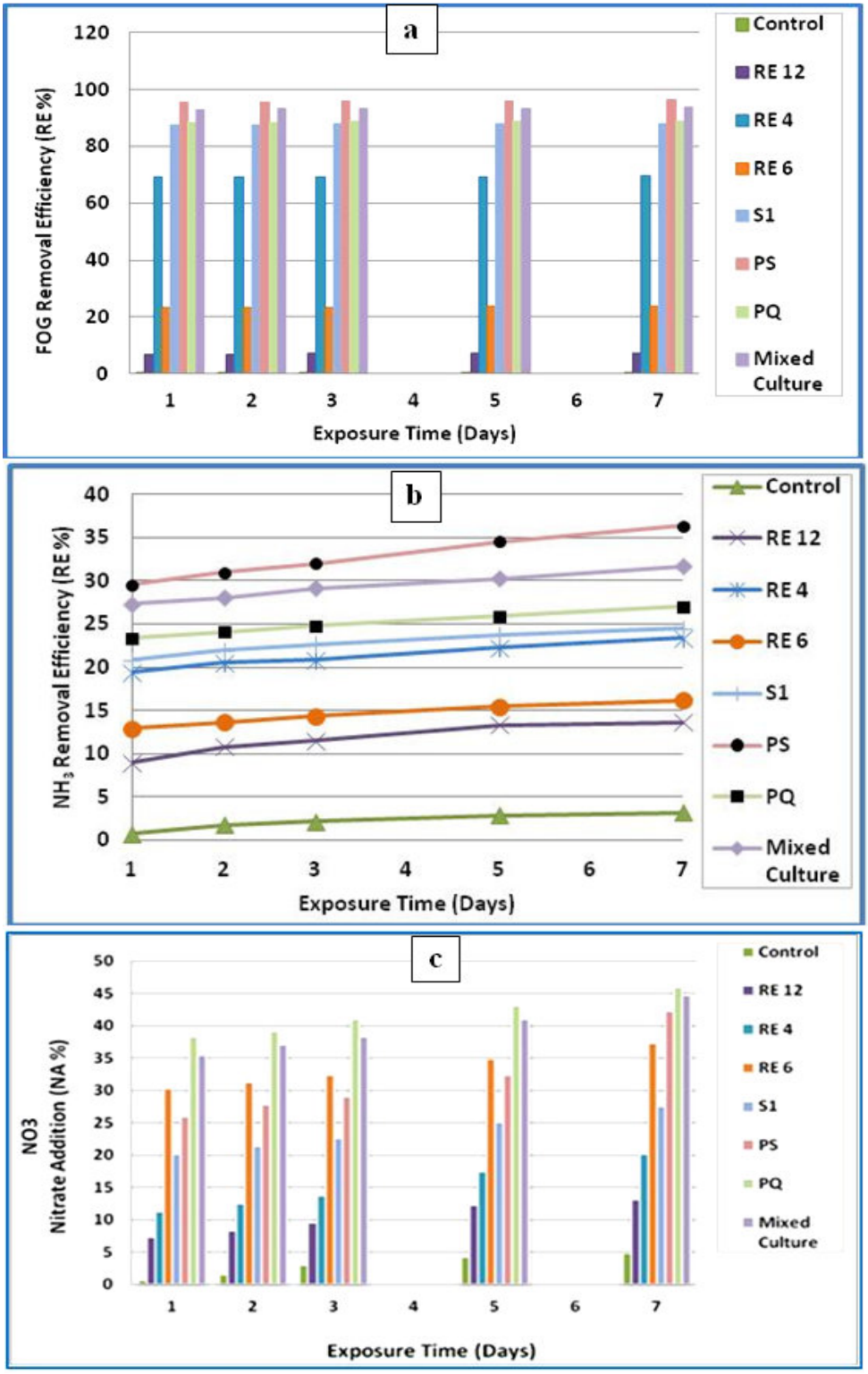


Figure 4. Removal Efficiency (RE%) of a) FOG, b) NH_3 and c) NO_3 Addition% in the Raw and Treated Tannery Wastewater at Different Exposure Times

3.4.6. Chromium (Cr)

Raw tannery wastewater recorded very high and dangerous Cr level (2100 mg/l) but treatment has led to considerable RE% of Cr due to the metabolic action of the selected bacteria within the first 24 h treatment followed by minor insignificant variations in the RE% with increasing exposure time. PS was the most efficient achieving the highest Cr RE of 93.66 and 93.88 % after one and seven treatment days respectively while RE12

showed the lowest achieved efficiency (58.13%) (Fig. 5a) compared to 0.05 and 0.57% removal achieved by the control wastewater after one and seven days. Although high Cr removal was recorded its level in the final effluent still higher than the permissible levels (0.01 mg/l)**.**

3.4.7. Hydrogen Sulfide (H_2S)

Considerable increases in the H_2S (initial concentration= 16.3 mg/l) removal occurred by all cultures with increasing the exposure time although their bulk removals occurred in the first 24 treatment hours reaching their highest after 7 exposure days. **PS** was the most active recording the highest RE (44.91%) and RE12 the lowest RE (10.43) after the 7th treatment day (Fig. 5b). As usual the control wastewater exhibited the lowest recorded RE of the H_2S (0.61 and 3.68% after the first and 7th exposure day) due to the absence of the specialized bacteria. The lowest achieved RC of the H_2S (8.98 mg/l) still higher than the MPL (1.0 mg/l) for **Egyptian** Limits.

3.4.8. Biological Contaminants

Total viable count of bacteria (TVC) was determined to define biological pollution extent in the tannery raw effluent. It is also an indication to stimulatory and/or inhibitory effects of the tannery effluent on the growth of the augmented tested bacteria.

PS, mixed culture, PQ, S1, RE4 and RE6 showed similar behaviour where they were not affected by the contaminated effluent and their growth (G) was even stimulated till the 3rd exposure day. Then the growth gradually reaching the highest inhibition (GI%) after 7 days (Table 4). These results indicated their high resistance against toxicity of such kind of wastewater, which was confirmed by their high ability to degrade and accumulate the involved contaminants. Finally, RE12 and the control, which showed the lowest ability to remove all the tested contaminants showed no responses towards effluent toxicity during the first 3 treatment days (0% GI). Then RE12 growth was gradually inhibited (58.6%) followed by recovery to record growth stimulation of 1.8 fold. Concerning the control, growth was gradually inhibited reaching the highest inhibition (82.5%) after the 7th day.

3.5. Statistical Analysis

Results concluded that during the bioremediation using the selected bacteria in the free-living mode process (Table 5); all the tested parameters (except pH) were strongly correlated to each other and cannot be ignored since they are all affecting the efficiency of the decontamination process.

4. Discussion

The six most active isolates were molecularly characterized as ***Providencia vermicola*** (RE4), ***Escherichia coli*** (RE6), ***Bacillus* sp.** (RE12), ***Bacillus*** sp. (S1), ***Bacillus* sp.** (PQ) and ***Pseudomonas stutzeri*** (PS) with the later being the most efficient in decontaminating tannery effluent. Many authors (i.e. El-Bestawy 2005; El-Bestawy & Ibrahim 2005; El-Bestawy et al. 2005; El-Bestawy & Hans-Jorgen Albrechtsen 2007) extensively prove the marvellous resistance and superior potentiality of Pseudomonas for biodegradation of toxic organic pollutants and biosorption of heavy metals. *Pseudomonas stutzeri* is a denitrifying bacterium(Lalucat et al., 2006) and efficiently used for bioremediation which explains its performance towards tannery wastewater contaminants. Three isolates (RE12, S1 and PQ) found belonging to the genus *Bacillus* which is well known as highly resistant spore-forming bacterium. *Bacillus* species possess excellent characteristics and are extremely efficient for many agricultural, environmental and industrial applications.

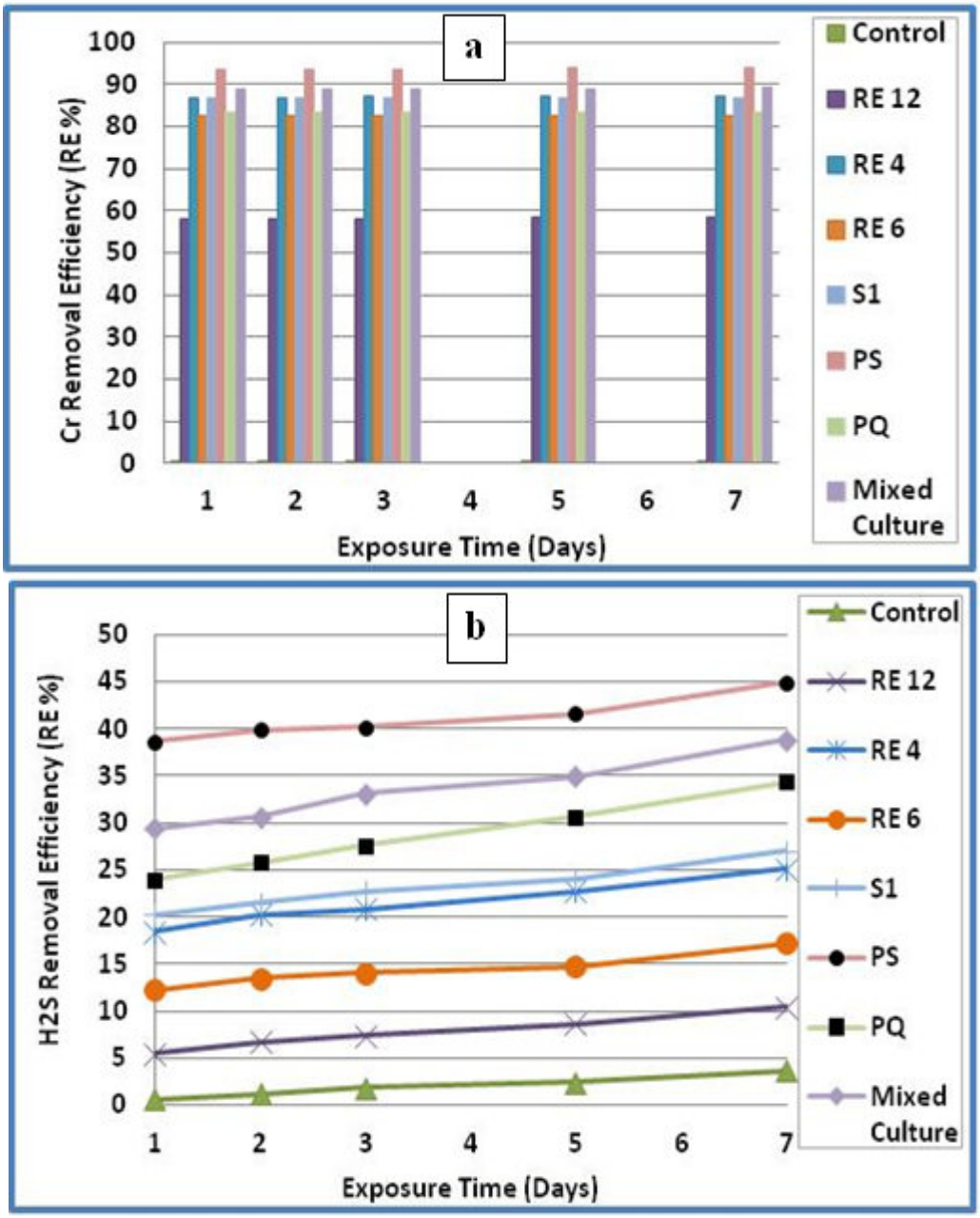


Figure 5. Removal Efficiency (RE %) of a) Cr and b) H_2S in the Raw and Treated Tannery Wastewater at Different Exposure Times

Table 4: Stimulatory or Inhibitory Effect of Tannery Effluents on the Growth of the Selected Bacteria

Exposure Time (Days)	Control		RE12		RE4		RE6		S1		PS		PQ		Mixed Culture	
	TVC	S & I%	TVC	GS & GI %	TVC	S & I%	TVC	S & I%	TVC	S & I%	TVC	S & I%	TVC	S & I%	TVC	S & I%
Zero	2.4×10^{9}		2.9×10^{9}		1.7×10^{9}		2.0×10^{9}		8.2×10^{8}		1.3×10^{9}		5.1×10^{8}		9.2×10^{8}	
1	2.4×10^{9}	0	2.9×10^{9}	0	1.8×10^{9}	1.1 f	2.1×10^{9}	1.1 f	8.4×10^{8}	1.02 f	1.4×10^{9}	1.1 f	5.6×10^{8}	1.1 f	9.5×10^{8}	1.03 f
2	2.4×10^{9}	0	2.9×10^{9}	0	1.8×10^{9}	1.1 f	2.1×10^{9}	1.1 f	8.8×10^{8}	1.1 f	1.4×10^{9}	1.1 f	5.8×10^{8}	1.1 f	9.8×10^{8}	1.1 f
3	2.4×10^{9}	0	2.9×10^{9}	0	1.8×10^{9}	1.1 f	2.1×10^{9}	1.1 f	8.9×10^{8}	1.1 f	1.4×10^{9}	1.1 f	5.9×10^{8}	1.2 f	9.9×10^{8}	1.1 f
5	1.1×10^{9}	54.2	1.2×10^{9}	58.6	1.0×10^{9}	41.2	1.0×10^{9}	50	4.2×10^{8}	48.8	8.6×10^{8}	33.8	4.2×10^{8}	17.6	7.2×10^{8}	21.7
7	4.2×10^{8}	82.5	5.3×10^{9}	1.8 f	3.9×10^{8}	77.1	4.7×10^{8}	76.5	4.6×10^{5}	99.9	8.5×10^{5}	99.9	9.2×10^{5}	99.8	8.9×10^{5}	99.9

Stimulation, I: Inhibition, GS: Growth Stimulation, GI: Growth Inhibition, f: fold

Table 5. Correlation Coefficients (Pearson's r) among the Different Parameters (Contaminants) During Bioremediation Using Free Living Bacteria

* Correlation is significant at the 0.05 ** Correlation is significant at the 0.01

	pH	DO	TDS	TSS	BOD	COD	FOG	NO_3	NH_3	Cr	H_2S
pH		-.448	.071	-.605	-.539	-.448	-.400	.262	-.357	-.520	-.324
DO	-.448		-.658	.878**	.866**	.870**	.872**	-.896**	.926**	.861**	.918**
TDS	.071	-.658		-.519	-.354	-.498	-.809*	.432	-.715*	-.373	-.741*
TSS	-.605	.878**	-.519		.904**	.842**	.912**	-.804*	.908**	.926**	.888**
BOD	-.539	.866**	-.354-	.904**		.965**	.738*	-.775*	.835**	.995**	.766*
COD	-.448	.870**	-.498	.842**	.965**		.749*	-.696	.851**	.957**	.768*
FOG	-.400	.872**	-.809*	.912**	.738*	.749*		-.750*	.932**	.768*	.943**
NO_3	.262	-.896**	.432	-.804*	-.775*	-.696	-.750*		-.821*	-.777*	-.853**
NH_3	-.357	.926**	-.715*	.908**	.835**	.851**	.932**	-.821*		.862**	.986**
Cr	-.520	.861**	-.373	.926**	.995**	.957**	.768*	-.777*	.862**		.795*
H_2S	-.324	.918**	-.741*	.888**	.766*	.768*	.943**	-.853**	.986**	.795*	

They exhibited superior pesticidal ability (Greene *et al.* 2001; Nunez-Valdez *et al.* 2001); used in enzyme immunoassays and in the bioremediation (El-Bestawy *et al.* 2002). Industrially, ***Bacillus* spp**. are widely used for the manufacture of extracellular enzymes (Sabir & El-Bestawy 2009). These characteristics of ***Bacillus* spp**. explain their occurrence in the highly contaminated and hypertrophic environment of **L. Mariut, Alexandria** as well as the very strong tannery wastewater (RE12). ***E coli*** (RE6) isolated from the tannery effluent is well known for the important role it plays in the modern biological engineering and industrial microbiology (Lee 1996; Cornelis 2000).

Tannery wastewater used in the present study can be classified as strong since it is highly polluted and contained extremely high levels of the tested contaminants that required powerful treatment to minimize its pollution load and discharge it safely. Batch treatment using free-living individual and mixed bacteria was found to be time and

species dependent and accordingly resulted in varied levels of contaminants removal efficiencies. Raw tannery effluent pH recorded almost neutral due to the mixing the high acidic and high alkaline discharges from all tanning processes in the main reservoir (Leta *et al*. 2004; Kongjao *et al.* 2008). Treated tannery effluent showed almost no change in its pH which lies in the permissible limits (6-9) The very low levels (near depletion) of DO in the raw and treated effluents indicating high pollution capability if disposed into an open environment. It also means that to stabilize this effluent, it requires, in addition to powerful bacterial strains, high amounts of oxygen for degradation of the contaminants.

TDS levels increased during treatment attributed to the high organic content and the potent nitrification bacterial activities. . Removal of organic matter was more affected by changes in salinity than the changes in hydraulic retention time or organic loading rate (Lefebvre *et al*. 2005). Although highly significant removals of TSS were achieved in very short time (24 h) by the tested bacteria but longer treatment time is required for the safe discharge.

Biodegradation of tannery effluents depends mainly on selecting the appropriate individuals organism(s) (Kimata *et al*. 2003) or mixed consortia (Sivaprakasam *et al.* 2008). The present bacterial selection showed excellent BOD removal almost within 24 h. *P. stutzeri* and the mixed culture showed the highest RE of BOD (94.71 and 94.03% respectively) compared to activated sludge process (ASP) that remove 90 to 97% (Haydar *et al.* 2007) and 84 to 92% (Orhon *et al.* 1999; Ram *et al.* 1999) of BOD_5 from tannery effluent. *Pseudomonas stutzeri* (PS) was highly efficient in removing huge COD load from the severely polluted (initial concentration= 25100 mg/l) wastewater which is also supported by Ramteke *et al.* (2010). . *Pseudomonas stutzeri* (PS) and the mixed culture achieved 79.16% and 78.04% RE respectively only after 24 h while COD residues in the treated effluent are attributed to the portions that are slowly or non-biodegradable (Ganesh *et al.* 2006). In contrast, sludge retention time (SRT) was increased to 15 days to achieve 75% COD removal (Ryu *et al.* 2007) while an aeration time of 12 h after 267 days settlement was required to achieve 80% COD removal using continuous ASP (Haydar *et al.* 2007) whereas 90% removal using membrane reactor (Goltara *et al.* 2003). Therefore, the present achievement considered excellent since it was obtained using batch treatment for short time (7 days) that may be remarkably enhanced if continuous treatment was used. In another study, to enhance COD removal, seawater and ferric salt flocculation were used as chemical integration step to enhance the biological treatment during the 110 days operation with seawater being more effective (Ryu *et al.* 2007). However, in the present study no pre- or post-treatments were used giving the proposed treatment another advantage. Moreover, *Pseudomonas stutzeri* followed by the mixed culture in batch mode showed high efficiency for FOG removal (95.64 and 93.07% respectively) after 24 indicating the potency of the selected organisms.

Adverse environmental effects of nitrogen content in the tannery effluent on water and air ecosystems are reported (Leta *et al.* 2004; Durai & Rajasimman 2011). Therefore, reduction of nitrates before final discharge using subsequent treatment is necessary to protect the environment. During the present study, ammonia, the common nitrogen form in tanning wastewaters, was transformed into nitrates and increased its levels in the treated effluent. Generally the low RE % of NH_3 achieved may be an indication to the deficiency of ammonia-oxidizing bacteria as found by Goltara *et al.* (2003) where 60 to 90% and 100% removal of total nitrogen and ammonium respectively using membrane reactor.

High sulfide concentration present in the wastewater may render aerobic biological treatment unsuitable. Sulfide removal is not an easy task and might require integration of different treatment units to be achieved (Sivaprakasam *et al.* 2008). Here again PS and the mixed culture showed the highest H_2S RE% (44.91 and 38.83% respectively) after 7 days. This low efficiency may be attributed to either the absence of sulfate oxidizing bacteria or deficiency of enough oxygen required for oxidation of sulfide into sulfate. To enhance removal of H_2S from tannery effluent longer time is needed providing that enough oxygen is available or otherwise oxidation post treatment step should be taken.

Bacillus, Enterobacter cloacae, Escherichia, Pseudomonas, Oscillatoria **sp,** ***Arthrobacter*** **sp** and ***Agrobacterium radiobacter***, sulfate-reducing bacteria and also some yeasts and fungi reported capable of reducing Cr (VI) to Cr (III), thus are promising in bioremediation of chromium through bio-absorption and bioaccumulation (Vermaa *et al*. 2001; Srinatha *et al*. 2002; Morales *et al*. 2007; Congeevaram *et al*. 2007; Pandi *et al*. 2009; Polisak *et al*. 2009). Dissolved oxygen (DO), pH, Cr (VI) initial concentration, biomass density, temperature, glucose content in the influent and contact time strongly influence chromium removal (Shakoori *et al*. 2000; Chen *et al.* Gu 2005). All the tested bacteria used in the present study showed resistance to Cr with various degrees and were able to remove huge amounts of Cr**.** ***P. stutzeri*** (exogenous) and the mixed culture (tannery and exogenous bacteria) reducing total Cr reaching RE of 93.66 and 89.01% after only 24 h. To reach

the MPL (0.01-0.1 mg/l), longer exposure time and larger biomass concentration are required (Srivastava *et al*. 2008).

Growth stimulation (GS) of the tested bacteria in the tannery wastewater occurred especially at the early exposures (till 3rd day) due to resistance they acquired followed by gradual inhibition reaching the highest GI% after the 7th day supported with the study of Shukla *et al*. (2007). RE12 showed the lowest ability to remove the tested contaminants while showing no responses towards effluent toxicity during the first 3 treatment days (0% GI) followed by inhibition (58.6%) at the fifth day and unexpectedly significant recovery reaching the highest recorded stimulation (1.8 fold) at the seventh exposure day. Its high resistance may be attributed to the presence of very high concentrations of Cr and other contaminants in the tannery effluent that has led to a defense mechanism prevents dealing with the involved contaminants either organic or inorganic and protect it from their toxicity.

5. Conclusion

Tannery effluent used in the present study with the extremely high levels of all the tested parameters considered one of the strongest industrial effluents with high pollution potential and dangerous effects on the receiving environments. Batch treatment of tannery effluent was time and bacterial species dependent with ***Pseudomonas stutzeri*** (PS) considered the most while ***Bacillus* sp** (RE12) considered the least efficient for all the tested parameters. There was a general trend of increasing the RE of all parameters by all the tested bacteria with increasing the exposure time but bulk changes in all parameters were achieved within the first 24 h. The highest removals recorded 86.7, 94.14%, 79.16, 95.64, 36.33, 93.66 and 44.91% for TSS, BOD, COD, FOG, NH_3, Cr and H_2S respectively mostly after only 24 h. On the other hand, TDS and NO_3 recorded the highest increase of 97.68 and 45.87% after one and 7 days respectively. Due to the short treatment time, residual levels of all the parameters were still above the MPL for the safe discharge. Therefore, increasing time, inoculums biomass or fixation of the selected bacteria must be considered to bring the effluent to the safe limits for the environment.

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