Efficiency of *Pseudomonas stutzeri* Strain M15-10-3 in the Treatment of Leather Tanning Industrial Wastewater Using Gravel Biofilm System

E. El.Bestawy*[*] *and R. Aburokba**

[*] ***Corresponding Author**, Department of Environmental Studies, Institute of Graduate Studies and Research, Alexandria University, 163 Horria Ave. El-Shatby, P.O. Box 832, Alexandria, Egypt. Phone: +203-4295007 Fax:+203-4285793, E-mail: ebtesamelbestawy@yahoo.com*

*****Department of Biological Sciences, Faculty of Science, King Abdul Aziz University. P.O. Box 42805 Jeddah 21551, Kingdom of Saudi Arabia, E-mail: raburokba@windowslive.com*

Abstract

The study aimed to investigate the ability of ***Pseudomonas stutzeri* (PS)** as biofilm (fixed mode) for decontaminating tannery wastewater based on the remarkable efficiency that planktonic ***P. stutzeri*** exhibited in the removal of all the contaminants during the batch treatment in a previous study **(El-Bestawy *et al.*, 2013)**. ***P. stutzeri*** was fixed on white stone as supporting material and tested as continuous treatment biofilm system for the tannery effluent at different flow rates (30-200 ml/h) for 5 working hours where samples were collected on hourly interval. Extremely high contaminants concentration (total suspended solids (TSS), total dissolved solids (TDS), biochemical oxygen demand (BOD), chemical oxygen demand (COD), fat, oil and grease (FOG), ammonia (NH_3), nitrates (NO_3), chromium (Cr) and hydrogen sulphide (H_2S) were determined in the raw tannery wastewater. The highest achieved removal efficiencies {RE(s)} by the proposed biofilm system after 5 h were 78.98, 93.44 and 76.19% for TSS, BOD and FOG respectively at 30 ml/h while 82.60, 25.09, 97.67 and 38.30% were achieved for COD, NH_3, Cr and H_2S respectively at 200 ml/h. On the other hand, TDS, NO_3 and Total Viable Count (TVC) of ***P. stutzeri*** were increased with time (49.95, 41.4% and 26.4 fold at 50, 30 and 200 ml/h respectively) due to bacterial metabolic activity reaching their highest levels after 5 h. Results also revealed huge variations in the RE of all the tested parameters achieved by ***P. stutzeri*** biofilm system compared to those obtained by the control (bacteria-free system) confirming the efficient role of ***P. stutzeri*** in removing effluent contaminants. Fixation of ***P. stutzeri*** enhanced contaminants removal from the highly polluted tannery effluent, protected biofilm bacteria from effluent toxicity, death and the wash out as shown by higher RE (s) for all the tested parameters coupled with shortening of the treatment time (5 h) instead of 7 days in the batch treatment. Therefore, it is evident that the proposed biofilm system with the highly active bacterium ***P. stutzeri*** represents a very promising, renewable and cheap biotechnology for the treatment of wide range of contaminated effluents not only in the industrial sector but also for domestic and agricultural wastewater.

Key Words: Biofilm; *Pseudomonas stutzeri*; Tannery Wastewater; Treatment

INTRODUCTION

Tanning industry consumes large amount of water, chemicals (at least 300 kg per ton of hides) and producing wastewater with very high pollution loads **(Verheijen *et al.*, 1996)**. One ton of skin generally leads to the production of 20 to 80 m^3 of turbid and foul-smelling wastewater. This effluent includes high levels of biochemical oxygen demand (BOD), chemical oxygen demand (COD: 6200 mg/l), suspended solids (SS: 5300 mg/l), total dissolved solids (TDS: 37,000 mg/l), chromium (100–400 mg/l), sulfide (200–800 mg/l), total Kjeldah nitrogen (TKN: 273 mg/l), N-NH_3 (153 mg/l), PO_4^{-3} (21 mg/l) fat, solid wastes and notable pathogen contamination leading to considerable environmental pollution **(Leta *et al.*, 2004; Kongjao *et al.*, 2008)**. Great variability was observed in the effluent characteristics, depending on the type of hides and skins and the region from which they came, at the time of sampling **(Lefebvre *et al.*, 2005)**. Different ranges of tannery pollutants included total nitrogen (927 to 2140 mg/l), and COD (9583 to 13515 mg/l), ammonium N (149-178 mg/l), sulfide (466.3-794 mg/l) and total chromium (23.3-42.5 mg/l) during the feeding stages **(Wiemann *et al.*, 1998; Wiegant *et al.*, 1999; Stoop, 2003)**. Several problems (inhibition of biodegradation) have been encountered during the biological treatment of tannery wastewater because of the high toxicity of chromium and sulphides.

The untreated release of tannery effluents with the high concentrations of pollutants leads to eutrophication and other adverse environmental effects in the receiving water bodies **(*Leta et al.*, 2004; Someda *et al.*, 2005; Durai** and **Rajasimman, 2011)**. Being highly toxic, they affect flora and fauna of the ecosystem and increase the health risk of human beings **(Chattopadhyay *et al.*, 2000; Igwe** and **Abia, 2003; Uberoi, 2003; Horsfall** and **Spiff, 2005; Kolomaznik *et al.*, 2008)**.

Microbial cells growing in a biofilm are physiologically distinct from planktonic cells of the same organism, which, by contrast, are single-cells that may float or swim in a liquid medium. A biofilm is a complex, organized and heterogeneous aggregation of living microorganisms (bacteria, fungi, algae, and protozoa) in which cells are embedded into a self-produced matrix of extracellular polymeric substance (EPS) and adhere to each other and to a surface **(Characklis** and **Marshall, 1990)** and associated with wide biotic and abiotic surfaces **(Wimpenn, 2000)**. Biofilm EPS (slime) is a polymeric substance composed of extracellular DNA, proteins, and polysaccharides in various configurations **(Hall-Stoodley *et al.*, 2004)**. EPS matrix is a dynamic system that fills and forms the space between cells and is responsible for organization and communication of the biofilm community **(Lewandowski *et al.*, 1994)**. It is primarily excreted by bacteria colonizing various surfaces **(Flemming, 1993)**. Biofilms can accumulate on various types of substances (inorganic and organic solutes and particles) from the surrounding water through different processes such as sorption, adhesion, cohesion, uptake of ions, and mechanical entrapment of particulate matters **(Neu** and **Marshall, 1990; Costerton *et al.*, 1994; Schorer** and **Eisele, 1997)**. Biofilms may also be formed on living or non-living surfaces representing a prevalent mode of microbial life in natural, industrial and hospital settings **(Hall-Stoodley *et al.*, 2004)**.

Planktonic microbes form a biofilm in response to many factors, which may include cellular recognition of specific or non-specific attachment sites on a surface, nutritional cues, or exposure to sub-inhibitory concentrations of antibiotics **(Hoffman *et al.*, 2005; Karatan** and **Watnick, 2009)**. A biofilm community begins initially when a clean surface is exposed to an aqueous environment and becomes conditioned by the chemical constituents present. Inevitably, microorganisms become associated with the surface, adhere, and then attach. Once cells are firmly bound, the activity of the community is dependent on the metabolism

(substrate consumption, cellular growth and replication, and synthesis of exopolymers) of each member species under local surface conditions **(Watnick** and **Kolter, 2000)**. After formation, biofilms are more stable and extremely resistant against antibiotics **(França *et al.*, 2016)**, heavy metals **(Golby *et al.*, 2014)**, temperature increase **(Ylla *et al.*, 2014)** due to the complexity of biofilm communities as well as immobilization of biofilm members onto the EPS matrix **(Gonzales *et al.*, 1990; Stams** and **Oude, 1997; Watnick** and **Kolter, 2000)**.

Bacteria fixed on biofilm systems are well known for their efficient role in treating tannery wastewater. For example, methanogenic biomass used in anaerobic fixed biofilm reactor (UAFBR) to treat tannery wastewater achieved 60-75% COD removal and methane yield (0.36 m3 CH4/kg CODrem) **(Song *et al.*, 2003)**. Similarly, Cr^{3+} tolerant ***Acenetobacter* sp., *Aeromonas salmonicida*** and ***Pseudomonas matophilia*** isolated from tannery wastewater were fixed in a constructed sand-biofilm in repetitive units system and used to reduce high Cr^{3+}, BOD and COD under the hazardous threshold concentration prior to the safe discharge into the environment as also found by other workers **(El-Bestawy *et al.*, 1999; El-Masry *et al.*, 2004)**. Solid substrates used for the fixation of the biofilm directly affecting its efficiency as treatment system. **Sundar *et al.* (2011)** used a consortium of ***Bacillus subtilis*** and ***B. cereus*** biofilm on different substrates (glass beads, pebbles and coarse sand) as continuous a system for the bioremoval of trivalent chromium from tannery effluents at 20 ml/min flow rate, 30°C, and pH 4. Biofilm biomass on the substrates was in the following sequence: coarse sand > pebbles > glass beads (4.8×10^7, 4.5×10^7 and 3.5×10^5 CFU/cm^2). Biofilms on coarse sand had more surface area and was able to remove 98% of Cr (III) of which 92.60% were adsorbed onto sand biofilms and considered better option for tannery industry, especially during post chrome tanning operation. ***Pseudomonas aeruginosa***, a Gram-negative bacterium, commonly isolated from soil and water, is known for its nutritional and ecological variety. ***P. aeruginosa*** is also able to escape many stress factors, such as, heavy metals **(Kavamura** and **Esposito, 2010)** antibiotics **(Ceri *et al.*, 2010; Ciofu** and **Tolker-Nielsen, 2010)** and ultraviolet (UV) light **(Harrison *et al.*, 2006 & 2007)**.

The main aim of the present study was to investigate the ability of ***Pseudomonas stutzeri* (PS) M15-10-3** fixed biofilm to decontaminate tannery wastewater in a proposed continuous system

MATERIALS AND METHODS

Bacterium

***P. stutzeri* (PS)** strain **M15-10-3** originally isolated and identified from heavily contaminated tannery effluent was selected based on the remarkable efficiency that its planktonic form exhibited in the removal of all contaminants during batch treatment **(El-Bestawy *et al.*, 2013)**. The selected bacterium was maintained on nutrient agar (NA) medium and prior to each experiment, the culture was reactivated overnight.

Biofilm system construction

Two separating funnels (1 litre each) were sealed at the bottom by a porous net (d<1mm) and supplied with a flow controller (tap) at the outlet. They were sterilized by immersing in 75% ethyl alcohol overnight, rinsed twice with absolute ethanol, and five times with sterile distilled water, and then dried in a sterile condition. White stone (with average particle size 1.6 mm in diameter) was used as supporting material after thorough washing, rinsing and sterilization 4 times at 121°C for 15 min. Each funnel was packed with sterile stone up to 80% of their height leaving the top 20% free. After packing, one column was used as a control

where only wastewater was supplied during the treatment stage, while the other column was inoculated with 800 ml dense overnight **PS** liquid culture (density 5.5×10^9 CFU/ml) and left 10 days to allow bacterial cells adhesion forming the biofilm. The two columns were connected with an up flow air supply, which was adjusted to operate alternately for 1 h and pause for 2 h.

Determination of population dynamics

The seeded column was left as a batch culture for 10 days at pH 7 and temperature ranged between 20 and 24 °C (room temperature). After 10 days, a sample from the biofilm column was collected every 24 hrs, serially diluted (up to 10^{-8}) and 1000 µl of the appropriate dilution was cultured under aseptic conditions on NA plates and incubated for 24 hrs at 37°C. Bacterial plate counts were recorded every day till constant count was obtained for three consecutive days.

Operation Conditions

Raw samples were treated using the biofilm at different flow rates (30, 50, 100 and 200 ml/h). At each flow rate, samples were collected from both the biofilm and bacteria-free (control) columns every hour for five consecutive hours. After treatment, all samples were characterized for the same parameters as for the raw water and the efficiency of the treatment using the proposed biofilm for these contaminants was calculated.

Characterization of the raw and treated industrial effluent

Characterization of the wastewater before and after the proposed treatment included its pH, temperature, dissolved oxygen content (DO), total suspended solids (TSS), total dissolved solids (TDS), biochemical oxygen demand (BOD), chemical oxygen demand (COD), fat, oil and grease (FOG), sulfides (H_2S), ammonia (NH_3), nitrate (NO_3), total viable count of bacteria (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)**. Removal efficiency was calculated to determine the effectiveness of the remediation process according to the following equation:

$$\text{Removal Efficiency (RE \%)} = C_0 - RC/\ C_0 \text{ X } 100$$

Where C_0 = Initial Concentration before Treatment (Zero Time);

RC= Residual Concentration after Treatment at each Exposure Time.

Temperature, pH & total dissolved solids (TDS)

Temperature, pH and TDS were determined by using digital thermometer and laboratory Bench Meter.

Total suspended solids (TSS)

TSS was determined using simple, direct spectrophotometer (Hach Dr 5000) method (630 Suspended Solids) other than gravimetric one that require filtration or ignition and weighing steps which is often used for checking in-plant processes. TSS was measured at 810 nm.

Fat oil and grease (FOG)

Determination of oil and grease content Total content of grease and oily substances were determined using the partition gravimetric method described by **Clesceri et al. (1999)**. Fatty acid composition of the oily content was extracted using n-hexane followed by methylation and then determined using gas chromatograph model 8400GC, fitting with FID detector and fused silica capillary column. Oil and grease content were calculated according to the standard procedure **(Clesceri *et al.*, 1999)**.

Biochemical oxygen demand (BOD_5)

Method 5210 B was used for BOD_5 determination as described in the Standard Methods for Examination of Water and Wastewater (Clesceri *et al*. 1999). BOD_5 can be calculated as follows:

$$BOD_5, \text{ mg/l} = \frac{D1 - D2}{P}$$

Where D1 = DO of diluted sample immediately after preparation in mg/l,
D2 = DO of diluted sample after 5- day incubation at 20 °C in mg/l,
P = Decimal volumetric fraction of sample (300 ml).

Chemical oxygen demand (COD)

Closed Reflux Colorimetric Method 5220 D was used for COD determination using potassium dichromate as chemical oxidant as described in the Standard Methods for Examination of Water and Wastewater **(Clesceri *et al*., 1999)**. Colour developed was measured at 620 nm using DR/5000 HACH spectrophotometer DR/2010 HACH spectrophotometer and the concentration was calculated from the slope of the standard curve.

Determination of chromium

Chromium in tannery wastewater was digested using conc. HNO3 and determined following colorimetric method described by **Clesceri *et al.* (1999)** using spectrophotometer (HACH DR 5000) at 357.9 nm wave length.

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 colour 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-}.

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- colour compound. The blue colour is masked by the yellow colour from the excess reagent to give a final green- colour 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.

Nitrate

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 coloured 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.

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.

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.

RESULTS

PS population dynamics was determined to define the maturity of the biofilm. Biofilm considered mature after 16 days when bacterial total viable count recorded 3 consecutive readings as shown in **Table 1**. After maturation, raw samples were treated using the biofilm at different flow rates (30, 50, 100 and 200 ml/h) for 5 h with 1 h interval sampling.

Table 1: Population dynamics of the PS in the medium used for the biofilm formation

Time (day)	Total Viable Count (CFU/ml)
0	5.5×10^{9}
9	1.1×10^{10}
10	1.3×10^{10}
12	1.4×10^{10}
13	1.4×10^{10}
14	1.5×10^{10}
15	1.5×10^{10}
16	1.5×10^{10}

DO and pH levels

Very low DO concentrations (0.32-0.35 mg/l) were recorded in the raw effluents. These values were gradually reduced with increasing exposure time in biofilm and control systems reaching their lowest levels (0.29 and 0.32 mg/l) after the 5^{th} h especially in the biofilm system at the flow rate 200 and 30 ml/h respectively (data are not shown). This may be attributed to consumption of DO by biofilm bacterium during biodegradation of the included organic contaminants. On the other hand no significant variations were noticed in the pH values among the tested flow rates, exposure times in the biofilm (7.50-7.76) or control (7.51-7.77) systems before or after the remediation process.(data are not shown). It is well known that pH is a very important factor controlling microbial activity especially under heavy metals stress.as in the present study where the highly toxic Cr exists at very high levels. Moreover, metals biosorption processes on microbial dead cells are particularly sensitive to changes in physical conditions such as pH or ionic strength **(Malik, 2004)**.

Total dissolved solids (TDS)

Significant TDS increases were recorded during treatment using batch mode (free living bacteria) where biodegradation resulted in breaking down complex contaminants into simple dissolved salts. However, using biofilm system decreased TDS increases except at the flow rates 50 and 100 ml/h **(Figure 1A)**. Raw tannery effluent had TDS range of 10400-10430 mg/l. TDS increased with time recording the highest TDS additions (49.95 and 49.28%) after 5 working at 50 and 100 ml/h respectively resulting 15610 and 15560 mg/l RC respectively. Much lower TDS additions (0.63 and 0.26%) were recorded using the control system at 30 and 200 ml/h flow rate after 5 working hours yielding 10465 and 10457 mg/l RC respectively. Levels of TDS before and after the treatment are far exceeding the maximum permissible limit (MPL) of 2000 mg/l.

Increasing TDS concentration in the tannery effluent after biofilm remediation is an expected result due to oxidation of the available ammonia (NH_3) to dissolved nitrate (NO_3) salt which increases TDS concentration in treated wastewater. It is well known that organic matter

removal is more affected by changes in salinity than the changes in hydraulic retention time or organic loading rate **(Lefebvre *et al.*, 2005)**. So, it is essential to keep TDS changes to a minimum through a technique which converts nitrates into nitrogen gas.

Nitrate (NO_3)

Raw tannery effluent had NO3 range of 34.0-37.3 mg/l. As shown with the TDS, residual concentration (RC) of nitrate showed general increases with time due to nitrification process where ammonia available in the tannery effluent oxidized into NO_3 reached the highest levels (41.4%) after 5 h at 30 ml/h. Much lower NO_3 addition (6.39%) was obtained using the control system at flow rate of 50 ml/h after 5 working hours **(Figure 1B)**. But fortunately, levels of NO_3 before and after the treatment are below the MPL (50 mg/l) which is good for safe discharge.

Total suspended solids (TSS)

Raw tannery wastewater recorded very high TSS level (15610-15700 mg/l). Treatment using the proposed biofilm system revealed high TSS removals regardless the flow rate **(Figure 2A)**. The highest RE recorded 78.98% compared to 0.57% achieved by the control both at 30 ml/h after 5 h. The lowest obtained RC of the TSS was 3300 mg/l which is 55 folds increase than MPL (60 mg/l) of the TSS. Accordingly, TSS could not reach safe limit for discharging during the experiment duration (5 h).

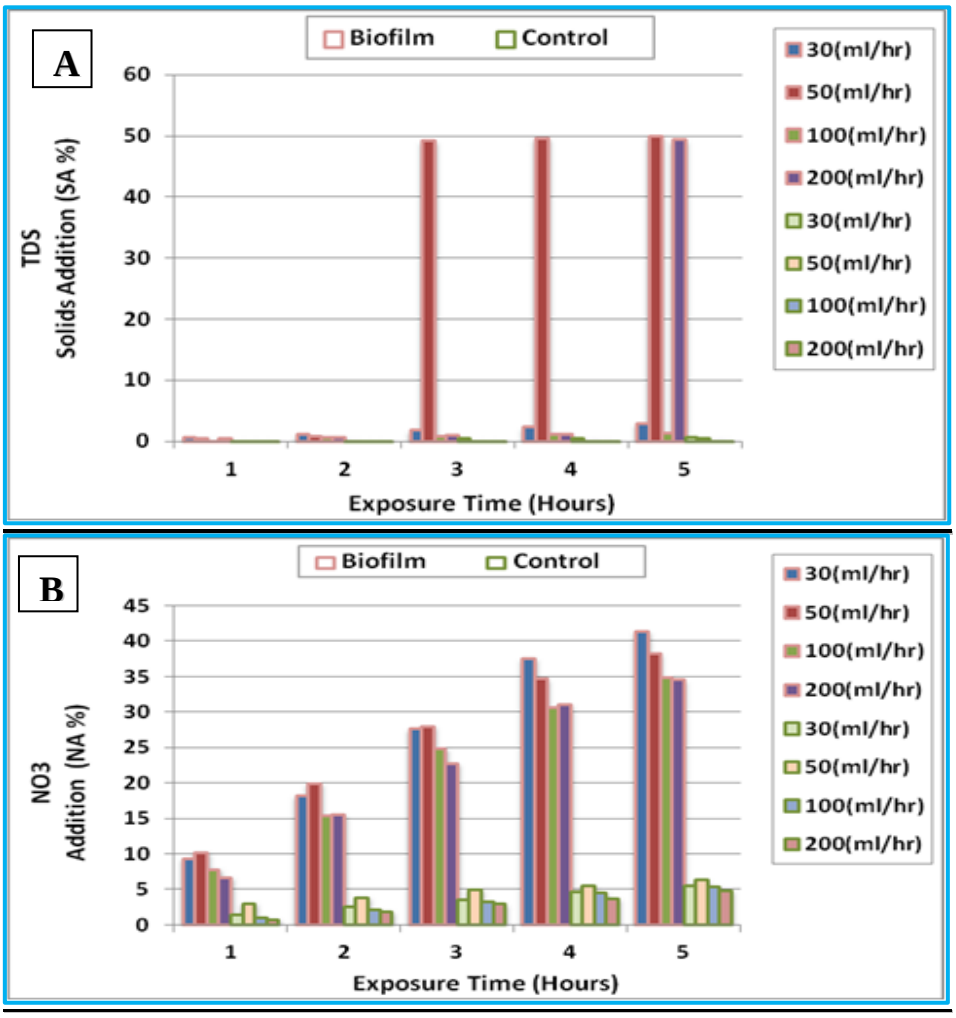

Figure 1. TDS Increases% (A) and NO_3 (B) in the treated tannery wastewater using biofilm and control systems at different flow rates and exposure times.

Biochemical oxygen demand (BOD)

High BOD levels (3170-3200 mg/l) were recorded in the raw tannery wastewater and both systems (biofilm and control) showed positive relation between RE of the BOD and exposure times. Biofilm system achieved the highest achieved BOD removal (93.44%) compared to the extremely low (2.19%) removal obtained by the control system both at 30 ml/h flow rate after 5 running hours **(Figure 2B)**. This is mainly attributed to the high capability of ***P. stutzeri*** for organic matter degradation. Treatment using biofilm resulted in TSS residues of 210 mg/l which is 3.5 folds higher than the MPL of BOD (60 mg/l).

Chemical oxygen demand (COD)

Extremely high COD levels (22410-22500 mg/l) were recorded in the raw tannery wastewater. COD removal followed a general increasing trend with increasing exposure time in the biofilm and control systems. The biofilm recorded highest COD removal of 82.60% at the highest flow rate (200 ml/l) after 5 exposure hours compared to almost the negligible activity (0.33% RE) recorded by the control after the same time at 30 ml/l due to the lack of specialized bacterial activity **(Figure 2C)**. However, no significant variation in the RE of COD (79.6, 82.17 and 82.1) at the other tested flow rates (30, 50 and 100 ml/l respectively) after 5 h. Results of the biofilm system showed huge reduction in the COD (from 22410 to 3900 mg/l) but levels still 39 fold higher than MPL of COD (100 mg/l) for the safe discharge.

Fat, oil and grease (FOG)

Extremely high FOG levels (4165-4200 mg/l) were recorded in the raw tannery wastewater. FOG removal in both biofilm and control systems positively related with increasing exposure time The biofilm achieved the highest (76.1%) FOG removal at 30 ml/l after 5 h with nearly no activity (0.71% RE) was recorded by the control system **(Figure 3A)**. Results of the biofilm system showed significant reduction in the FOG (from 4200 to 1000 mg/l) but levels still 10 fold higher than MPL (10 mg/l) of FOG for the safe discharge.

Ammonia (NH_3)

Raw tannery effluent had NH_3 range of 29.1-30.0 mg/l that showed general trend of increasing RE of NH_3 with increasing exposure time. Biofilm achieved the highest RE of NH_3 (25.09%) after 5 working h at 200 ml/h with lower efficiencies at other tested flow rates compared to much lower NH_3 removal (6.33%) obtained using the control system at 30 ml/h after 5 h **(Figure 3B)** which indicated the important role of biofilm bacteria in the bioremediation process. Levels of NH_3 before and after the treatment are above the Egyptian MPL (3 mg/l).

Chromium (Cr)

Raw tannery wastewater recorded contained high and dangerous Cr level (2185-2200 mg/l). The bulk RE% of Cr was achieved after the first h at all the tested flow rates in both biofilm and control systems. The highest RE of Cr (97.67%) was recorded by biofilm bacteria at the highest flow rate (200 ml/h) which is equivalent to 51 mg/l **(Figure 3C)**. On the other hand extremely minor RE of Cr (1.91%) was achieved by the control at the lowest flow rate (30 ml/h). According to the Cr MPL (2 mg/l) the treated effluent still has 25.5 fold increases in the Cr content which required longer exposure to reach the safe limits.

Hydrogen Sulfide (H_2S)

H_2S concentration in raw tannery wastewater recorded a range of 18.5-19.2 mg/l. The highest RE of H_2S (38.30%) was recorded by biofilm bacteria (equivalent to 11.6 mg/l) while minor H_2S removal (5.21%) was achieved by the control both after 5 working h at 100 and 30 ml/h

flow rate respectively **(Figure 3D)**. According to the H_2S MPL (1 mg/l), the treated effluent still has 11.6 fold increases in the H_2S content which required longer exposure and more aeration to help oxidizing H_2S and to reach the safe limits.

Total viable count of bacteria (TVC)

Fixation of bacteria on solid medium as a biofilm has many advantages. It enhances the bacterial growth, reduces wastewater toxicity and increase bacterial resistance towards the involved contaminants. Bacteria either in the biofilm or control systems had general trend of growth stimulation with time (5 h) with no inhibition at all which is opposite to their growth in a batch mode. Growth of biofilm bacteria stimulated reaching 26.4 fold increases at the highest flow rate (200 ml/h) after 5 working h **(Table 2)**. The growth of the bacteria in the control system was much lower than that of the biofilm but behaves in a similar way where it is gradually stimulated reaching the highest growth density (1.25 f) after 5 h but at the lowest flow rate (30 ml/h).

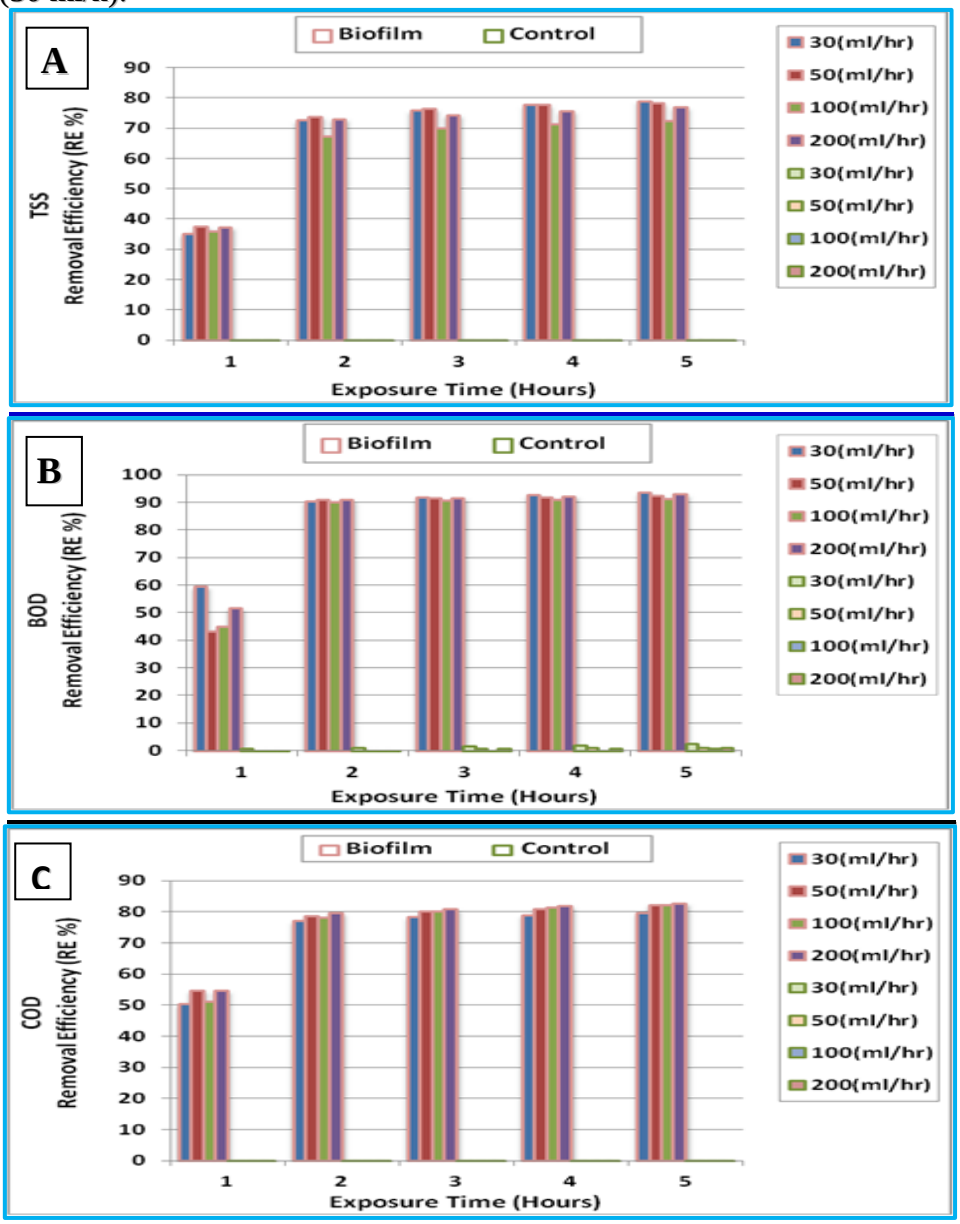

Figure 2. Removal efficiency (RE %) of TSS (A), BOD (B) and COD (C) from the tannery wastewater using biofilm and control systems at different flow rates and exposure times.

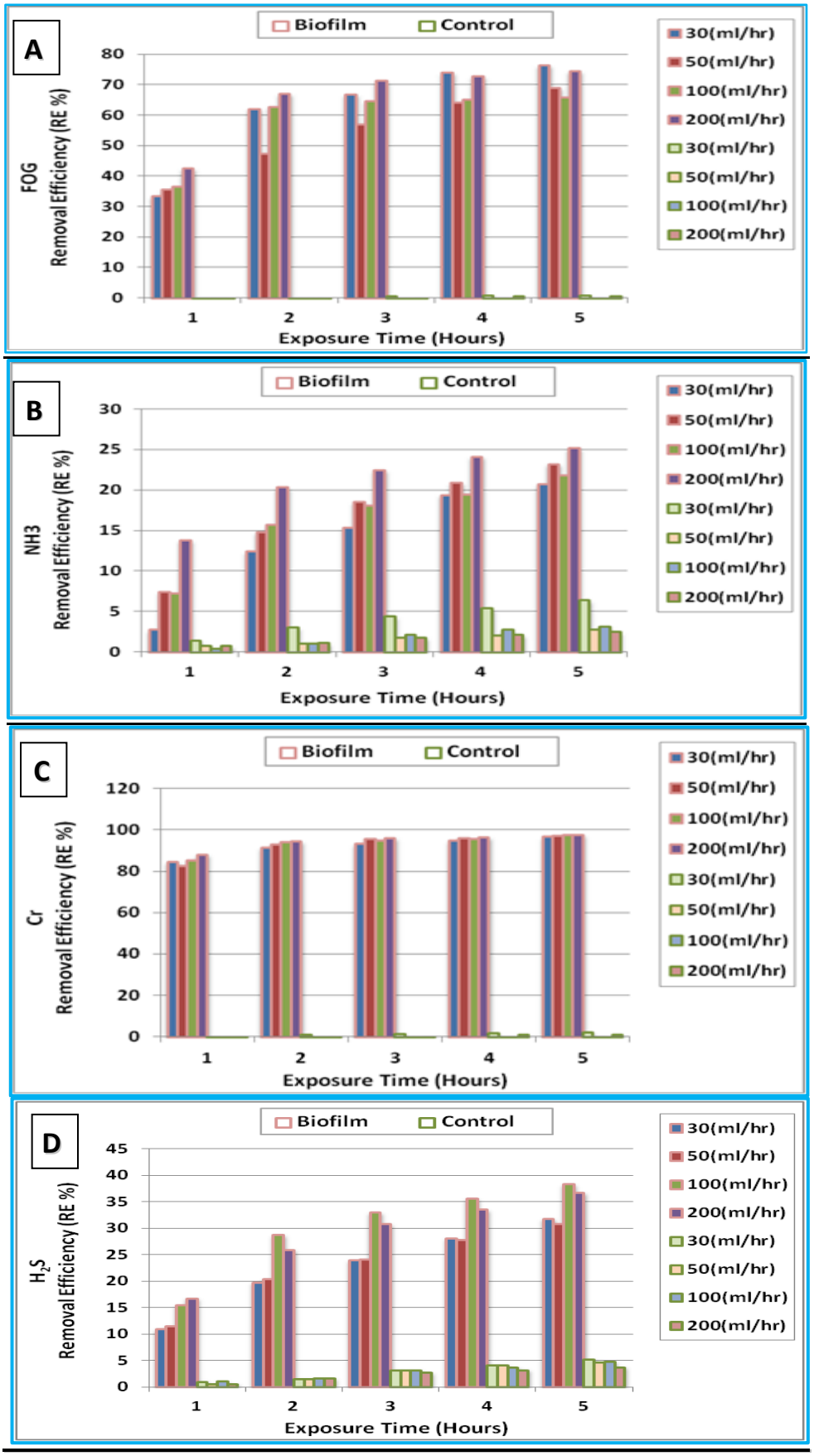

Figure 3. Removal efficiency (RE %) of FOG (A), NH_3 (B), Cr (C) and H_2S (D) from the tannery wastewater using biofilm and control systems at different flow rates and exposure times.

Table 2: Stimulatory and/or Inhibitory Effects of Tannery Effluents on Biofilm and Control Bacteria at the Different Flow Rates.

Exposure Time (h)	A. Biofilm System							
	30 (ml/h)		50 (ml/h)		100 (ml/h)		200 (ml/h)	
	RC	S&I%	RC	S&I%	RC	S&I%	RC	S&I%
Zero	0.5×10^{9}		0.5×10^{9}		0.5×10^{9}		0.5×10^{9}	
1	7.5×10^{9}	15 f	7.5×10^{9}	15 f	8.2×10^{9}	16.4 f	8.4×10^{9}	16.8 f
2	8.5×10^{9}	17 f	8.1×10^{9}	16.2 f	9.5×10^{9}	19 f	10.5×10^{9}	21 f
3	9.2×10^{10}	18.4 f	8.9×10^{9}	17.8 f	10.4×10^{9}	20.8 f	11.7×10^{9}	23.4 f
4	10.4×10^{9}	20.8 f	9.5×10^{9}	19 f	11.5×10^{9}	23 f	12.5×10^{9}	25 f
5	11.3×10^{9}	22.6 f	10.4×10^{9}	20.8 f	12.1×10^{9}	24.2 f	13.2×10^{9}	26.4 f
Exposure Time (h)	B. Control							
	30 (ml/h)		50 (ml/h)		100 (ml/h)		200 (ml/h)	
	RC	S&I%	RC	S&I%	RC	S&I%	RC	S&I%
Zero	4.97×10^{8}		5.13×10^{8}		5.24×10^{8}		5.31×10^{8}	
1	5.10×10^{8}	1.03 f	5.34×10^{8}	1.04 f	5.42×10^{8}	1.03 f	5.48×10^{8}	1.03 f
2	5.36×10^{8}	1.08 f	5.48×10^{8}	1.07 f	5.58×10^{8}	1.06 f	5.64×10^{8}	1.06 f
3	5.50×10^{8}	1.11 f	5.67×10^{8}	1.11 f	5.71×10^{8}	1.09 f	5.82×10^{8}	1.10 f
4	5.86×10^{8}	1.18 f	5.82×10^{8}	1.13 f	5.88×10^{8}	1.12 f	5.99×10^{8}	1.13 f
5	6.21×10^{8}	1.25 f	6.02×10^{8}	1.17 f	6.03×10^{8}	1.15 f	6.24×10^{8}	1.18 f

The Lowest Growth Stimulation GS%, The Highest Growth Stimulation GS

DISCUSSION

Biological treatment using microbial biofilms considered highly efficient, eco-friendly and an economic alternative to the conventional physicochemical processes **(Eccles, 1999; Mulligan *et al*., 2001; Malik, 2004; Harrison *et al*., 2007)**. Biofilms are excellent pollution indicators due to their (a) ubiquity on almost any surface in the water, (b) sessile mode of growth that reflects the actual habitat conditions, (c) short life cycle that enables a more rapid response to environmental changes than in higher level organisms, (d) species diversity in the community with various environmental tolerances, and (e) the relative ease of collecting biofilm samples **(McCormick** and **Cairns, 1994; Fuchs *et al*., 1996)**.Biofilm provides biological treatment with many advantages over their free living counterparts. These advantages include enhancement of contaminants removal, reduction of treatment time, protection of biofilm bacteria from effluent toxicity, death and the wash out of bacterial cells. In the present study these advantages were clearly shown where higher RE (s) for all the tested parameters coupled with shortening of the treatment time (5 h) instead of 7 days.

P. stutzeri* (PS)** was selected to be fixed in a biofilm system based on its great performance in the removal of all the contaminants during the batch treatment. This finding is supported by many workers who documented the superior resistance and ability of the genus ***Pseudomonas in degradation and accumulation of environmental pollutants. In a recent study ***P. aeruginosa*** was able to escape many stress factors, such as, heavy metals **(Kavamura** and **Esposito, 2010)**. Two general trends were noticed while treating tannery effluent using the proposed biofilm. The first trend was increasing the RE of all the tested parameters with time and the second was the huge variations in the RE of all the tested parameters achieved by ***P. stutzeri*** biofilm system compared to those obtained by the control (bacteria-free system) confirming the efficient role of ***P. stutzeri*** in removing effluent contaminants. Also, clear variations but no specific trends were noticed in the RE of all the tested parameters at the different flow

rates where the highest REs were achieved at the lower flow rates for some parameters and at the higher flow rates for other parameters.

Extremely high contaminants levels were determined in the raw tannery wastewater. Surprisingly, as high as 78.98, 93.44, 82.60, 76.19, 25.09, 97.67 and 38.30% were achieved as highest RE % for TSS, BOD, COD, FOG, NH_3, Cr and H_2S respectively by the proposed biofilm system after only 5 h which considered superior achievements. Three parameters were increased with time due to bacterial metabolic activity reaching their highest levels after 5 h. These included TDS (49.95% at 50), NO_3 (41.4% at 30 ml/h) and the TVC of ***P. stutzeri*** (26.4 f at 200 ml/h). Huge amounts of the tested contaminants were removed in such short time leaving residues (mg/l) of 15570-15610 (TDS); 3300 (TSS); 210 (BOD); 3900 (COD); 1000 (FOG); 21.8 (NH_3); 48.1 (NO_3); 51 (Cr); 11.6 (H_2S) and 13.2×10^9 CFU of ***P. stutzeri*** /ml. However, extremely lower RE(s) of the tested contaminants were removed by the control system (0.57, 2.19, 0.33, 0.71, 6.33, 1.91 and 5.21% for TSS, BOD, COD, FOG, NH_3, Cr and H_2S respectively at the same exposure time. Moreover much lower increases were achieved for TDS (0.63%), NO_3 (6.39%) and TVC (1.25 f) in the tannery wastewater treated with the control system confirming the remarkable ability of ***P. stutzeri*** biofilm. This is mainly attributed to the great characteristics that the genus ***Pseudomonas*** possesses including resistance against heavy metals **(Kavamura** and **Esposito, 2010),** antibiotics **(Ceri *et al.*, 2010; Ciofu** and **Tolker-Nielsen, 2010)** and ultraviolet (UV) light **(Harrison *et al.*, 2006)**.

Similarly and in consistent with the present study ***P. matophilia*** isolated from tannery wastewater were fixed in a constructed sand-biofilm system and used to reduce high Cr^{3+}, BOD and COD contamination load in that effluent. Results indicated the advantage of using the biofilm system in repetitive units to reduce chromium contamination in tanning wastewater to a level under the hazardous threshold concentration prior to the safe discharge into the environment. In addition, this type of bioremediation has already proven itself to be a cost-effective and more beneficial compared to chemical and physical methods for managing wastes and environmental pollutants **(Bonaventura** and **Johnson, 1997; El-Bestawy *et al.*, 1999; El-Masry *et al.*, 2004)**. It was reported also that through the metabolic activities of the biofilm system, degradable organic matter present in the surrounding water is gradually broken down **(Cammarota** and **Annajr, 1998)** as shown by high biodegradation for the BOD, organic nitrogen compounds and FOG in the present study.

Biofilm systems have proved to play an important role in the removal of contaminated industrial wastewater **(Gantzer *et al.*, 1989; Fogarty** and **Kelly, 1990; Gebara, 1999).** Metabolic activities of the biofilm system gradually degrade organic matter and accumulate/absorb inorganic contaminants contained in the surrounding wastewater **(Cammarota** and **Annajr, 1998)**. Biofilm can efficiently be used to monitor the impact of pollution on the biofilm community (i.e. biomass, diversity, presence or absence of species) and monitoring the self-accumulation of toxic elements in biofilm dry mass **(Gold *et al.*, 2003; Mages *et al.*, 2004; Kröpfl *et al.*, 2006)**, therefore they represent an important part in the aquatic ecosystem and wastewater treatment systems. Pollutants are processed and eliminated by means of the complex food chain established within the biofilm **(Liu** and **Tay, 2002)**. Different types of biofilm reactors have been used for biological treatment of water and wastewater **(Vı'lchez *et al.*, 2007)**.

Conclusion

Tannery wastewater showed extremely high levels of all the tested parameters that make it one of the strongest industrial effluents that has high pollution potential and dangerous effects on the receiving environments and also creates many difficulties in the treatment. Fixation of

bacteria on solid medium as a biofilm showed many advantages over their planktonic free living counterparts. It enhances the bacterial growth, reduces wastewater toxicity and increases bacterial resistance towards the involved contaminants. Considering the very short time that biofilm runs for (5 h), it seems that the proposed biofilm system is very efficient for treating the tannery effluents. This system could reach higher removal for all the tested parameters reaching acceptable limits for safe discharge by applying longer exposure time, using 2 or 3 biofilm units in sequence and using a preliminary oxidation step before biological treatment that could reduce levels of contaminants. The proposed biofilm system with the highly active bacterium ***P. stutzeri*** represents a very promising, renewable and cheap biotechnology for the treatment of wide range contaminated effluents not only in the industrial sector but also for domestic and agricultural wastewater.

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