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Treatability of pharmaceutical effluents using free-living bacteria in a batch mode

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ABSTRACT

The present study aimed to investigate the ability and efficiency of biological treatment to decontaminate pharmaceutical wastewater using indigenous and/or exogenous bacteria. One indigenous bacterium isolated from final effluent of the pharmaceuticals processing wastewater and five exogenous bacteria were molecularly identified and used as individual or mixed free-living cultures in a batch mode remediation process. Raw pharmaceutical effluents had high levels of all the tested parameters makes it one of the strongest industrial effluents. Treatment of pharmaceutical effluent for 6 d was time and bacterial species-dependent. *Pseudomonas fluorescens* (PF) was the most efficient in removing all the tested parameters except NO_3 which increased due to oxidation of NH_3 while *Bacillus amyloliquefaciens* (S1) and *Bacillus cereus* (L1) characterized by the lowest efficiency for contaminants removal. There was a general trend of increasing the removal efficiency (RE) of all parameters by all the tested bacteria with increasing the exposure time. However, bulk changes in all parameters were achieved within the first 24 h. The highest removals recorded were 94.63%, 79.44%, 75.07%, 81.03%, 90%, 93.75%, 55.2%, 94.29%, and 15.6% for total dissolved solids (TDS), total suspended solids (TSS), biochemical oxygen demand, chemical oxygen demand, fat, oil and grease (FOG), nitrate (NO_3), ammonia (NH_3), phenol, and total viable count of bacteria (TVC), respectively. Despite the highly efficient removals achieved for the tested parameters, their residual levels of all the parameters still above the maximum permissible limits for the safe discharge except FOG. 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; Organic matter; Nitrogen; Pharmaceuticals; Phenol; Pollution control; Wastewater

1. Introduction

Pharmaceutical industry manufactures a wide range of research based life-saving and life-enhancing medicines. Recently, there is a growing concern about the occurrence, fate, and toxicity of pharmaceutical discharges into the aquatic environment. Municipal wastewater is a significant source of pharmaceuticals and personal care products (PPCPs) in the environment because many of them are not removed completely in conventional wastewater treatment plants

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[1–4]. Pharmaceutical products (PPs) and their residues are receiving a lot of concern among all the emerging water contaminants, even at very low concentrations [5]. Characteristics of the pharmaceutical discharges are diverse according to the process operations, which gives rise to a wide variation in the liquid wastes. There is little similarity between effluents from different factories and individual effluent may differ continually as a result of process changes. In many cases, these effluents contain little or no biodegradable organic matters and the pollutant loads in terms of biological oxygen demand (BOD_5) may be negligible compared with the higher chemical oxygen demand (COD) [6]. Most substances found in a pharmaceutical industrial wastewater are structurally complex organic chemicals that are resistant to biological degradation [7], therefore, conventional biological treatment methods are usually inappropriate [8]. Microorganisms perform best under steady state environment but at suitable rates, environmental changes will allow the biomass to acclimate. Biotechnologists are striving to develop new strains of microorganisms and processes for destruction of organic toxics and hazardous materials to shorten time and retention time [9–12]. The occurrence of pharmaceuticals and their metabolites in surface water and aquatic sediment was a subject of numerous studies [13–15]. In addition to pharmaceutical effluents discharged into aquatic environments, significant quantities of antibacterial drugs used for bacterial fish infections in fish farms are released into the environment via different routes despite optimization of drug therapy. Several registered antibacterials are currently available for combating bacterial pathogens, including tetracyclines, (fluoro) quinolones, potentiated sulfa, penicillin, and chloramphenicol derivatives. Oxytetracycline (OTC) and quinolone drugs (oxolinic acid – OA and flumequine – FLU) are the most widely used in Mediterranean aquaculture. Antibacterial drugs used in aquaculture have been shown to persist in water and sediments in the vicinity of euryhaline fish farms, sometimes long after their use has ceased [16]. Their persistence prolongs their life in the water column, allows accumulation in marine sediments via high drug sorption; abiotic drug processing; development of resistance; drug complexing with marine cations. Moreover, their lipophilicity allows them to enter the aquatic food chain leading to contamination of non-target organisms. Their abiotic degradation in the water column (mainly by photolysis) of antibiotics can take several days or weeks.

More information on quality, quantity, and toxicity of pharmaceuticals and their metabolites are definitely needed especially when attempting to reuse wastewater and dispose sludge to agricultural areas and landfills [17]. The occurrence of pharmaceuticals was first reported in treated wastewater in the United States during 1976 [18], followed by 1981 in river waters in the United Kingdom [19] and then in 1986 in wastewaters in Canada [20]. Contamination of water and sediments of aquatic ecosystem poses hazardous effects on biotic and abiotic ecological elements. Aquatic populations greatly affected by the occurrence of pharmaceuticals and their metabolites even at low doses. For example, low-dose anthracene (1 mg L^{-1}) in lightly contaminated Bizerte lagoon sediments for 30 d severely affected biomass, activity, and community structure of the indigenous microbial communities. Anthracene contamination resulted in a significant reduction of bacterial abundance with an impact on cell integrity. Also, sediment oxygen consumption was strongly inhibited. Treatment using biostimulation (addition of nitrogen and phosphorus fertilizer) and/or bioaugmentation (inoculation of a hydrocarbon clastic bacterium) did not minimize the anthracene toxic effects. Changes observed in microbial population and structure suggested that the proposed treatments might be promising to promote bacterial growth [21]. In a recent study, the effects of penicillin G (3–700 mg L^{-1}) contaminating sediments of a Tunisian coastal zone (South-Western Mediterranean Sea) on free-living nematode communities were investigated for 30 d. Great adverse effects were observed in diversity, species richness, equitability, and number of species that were significantly decreased with increasing level of the antibiotic contamination [22,23].

Since wastewater treatment is the "last line of defense" against water pollution, it is the only way to clean and improve water quality so that it can be returned safely to our environment [24]. Concentrations of some antibiotics and other pharmaceutical compounds in wastewater may be reduced or eliminated in biological wastewater treatment systems using the activated sludge process, which is the most commonly used wastewater treatment process in the world. The key component in biological WWTP responsible for the removal of pollutants is the aeration basin containing the microorganisms (activated sludge). Sewage treatment plants (STPs) are designed to clean urban and industrial wastewater. The removal rate of pharmaceuticals in STPs can, indeed, vary to a large extent. The treatment efficiency in STPs is significantly affected by several factors [25], such as the physico-chemical properties of pharmaceuticals, treatment processes employed, age of the activated sludge [26], hydraulic retention time, and finally environmental conditions such as temperature and light intensity [27]. Knowing only that RE is not sufficient to understand if the pharmaceuticals are adsorbed into sludge (often used for soil amending), or whether they are biodegraded or abiotically degraded. Additionally, toxic degradation products occurring in the treated waste may not be identified if they are not explicitly addressed. Finally several pharmaceuticals are excreted as conjugates and can make a significant, but poorly understood, contribution after release of the active moiety by cleavage during treatment in STPs.

The main objective of the present study was to investigate potential minimization of pollutants generated from pharmaceutical industry through bioremediation technology. Bioremediation was carried using powerful bacterial species either indigenous or exogenous and either individually or in combinations, under optimum conditions.

2. Materials and methods

2.1. Sampling

Samples were collected from the final effluent of pharmaceutical company, Jeddah City, Saudi Arabia. Pharmaceutical 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

Six bacterial species were used included one indigenous (*Bacillus cereus* strain Kt7-14) out of four isolated from the contaminated pharmaceutical wastewater. The other five (S1, PS, TAQ, PF, and PQ) species were kindly provided from IGSR (Institute of Graduate Studies & Research, Alexandria University) collection. They were originally isolated from heavily polluted wastewater and environments. They also exhibited superior ability for organic matter and heavy metals remediation. All the species of *Pseudomonas* were isolated from the contaminated water of Mariut [28]. The six selected bacterial species were investigated as individual or mixture, for their ability to bioremediate contaminated wastewater from pharmaceuticals industry.

2.3. Media and culturing conditions

Dehydrated nutrient broth (NB) and nutrient agar (NA) were supplied by HiMedia (India) and used during the present study. NB medium contained (g L^{-1}) peptic digest of animal tissue, 5.0; NaCl, 5.0; yeast extract, 1.5 and beef extract, 1.5. NA medium contained the same NB ingredients plus 15.0 g agar. They were prepared by dissolving 13.0 and 28.0 g L^{-1} from NB and NA dehydrated media, respectively, pH was adjusted to 7.4, sterilized by autoclaving at 121°C for 20 min and freshly used for growth experiments as well as biodegradation assays. After culturing, the selected bacterial species were incubated at 30°C for 24 h.

2.4. Bacterial isolation, purification and identification

Heterotrophic bacterial colonies were purified by streaking on NA plates and incubated at 30°C. Pure isolates were inoculated onto NA slants, incubated under the previous conditions and kept as a stock in the fridge for further investigations. The pure isolates (four) from pharmaceuticals wastewater as well as the five exogenous bacteria were subjected to identification using cell and colony morphology, Gram staining, and biochemical profiling using the dehydrated media in the API kits (bioMérieux's API identification products are test kits for identification of Gram positive and Gram negative bacteria and yeast). This was followed by molecular characterization of the most promising isolates after the screening test.

2.5. Molecular identification

Total genomic DNA was extracted from 5 mL overnight NB culture of the purified isolates [29]. PCR was performed in a light cycler Eppendorf PCR machine. A 1,300 bp fragment was obtained by PCR amplification of the 16S rDNA gene [30] using the primers B341F (5′-CCTACGGGA GGCAGC), and 1392R (5′-ACGGGCGGTG TGTRC-3′). The PCR mixture was composed of 100 ng of genomic DNA, 30 pmol of each primer, 200 μM of dNTPs, 1 U 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, Holland). Each of these purified products was sequenced by the chain terminator method (ABI 3130XL system, DNA technology, Denmark) using the two corresponding PCR primers separately. The resulted DNA sequences were phylogenetically analyzed using the BLAST search program. Multiple sequence alignment and molecular phylogeny were performed using BioEdit software [31].

2.6. Bioremediation bioassays

2.6.1. Screening of bacterial isolates

All the pure isolates either indigenous from the pharmaceutical wastewater or the exogenous were tested visually for bioremediation of pharmaceutical effluent in order to select the most promising candidates. They were inoculated individually in 250 mL flasks containing pharmaceutical effluent and incubated at 37°C under 100 rpm agitation speed. Visual observations were taken daily for a week according to which the promising isolates for bioremediation process were selected.

2.6.2. Bioassays using free-living bacteria

Six promising bacterial candidates (L1, S1, PS, PF, TAQ, and PQ) were selected according to the preliminary screening of the all isolates and were employed as free-living individuals or mixture for remediation of the highly contaminated pharmaceutical effluent. Selected species were inoculated individually and as mixture in 100 mL NB medium and incubated till heavy growth was obtained. Then each culture was seeded into 900 mL pharmaceutical effluent reaching a final volume of 1 L each after counting the total viable count of bacteria (TVC) to define the density of the different inoculants. Pharmaceutical effluent was characterized immediately after sampling collection to define its pollution strength which considered zero time or raw readings. Effluent cultures, individual and mixed as well as a control sample (1 L un-inoculated pharmaceutical effluent) were incubated for 7 d where samples were aseptically drawn at 24 h interval.

2.7. Characterization of the raw and treated industrial effluent

Pharmaceutical wastewater was characterized before and after the proposed treatment. Characterization included its pH, temperature, dissolved oxygen (DO) content, TSS, TDS, BOD_5, COD, fat, oil and grease (FOG), phenol, ammonia (NH_3), nitrate (NO_3), and TVC, all of which were determined using the standard techniques described in the Standard Methods for the Examination of Water and Wastewater [32]. After treatment, the selected parameters were analyzed to determine their residual levels at each exposure time and their RE were calculated to determine the effectiveness of the remediation process according to the following equation:

$$\text{Removal efficiency}\left(\text{RE }\%\right) = C_0 - \frac{\text{RC}}{C_0} \times 100 \quad (1)$$

where C_0 = initial concentration before treatment (zero time); RC = residual concentration after treatment at each exposure time.

2.8. TDS and pH

The pH and TDS were determined using a laboratory bench meter.

2.9. Total suspended solids

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

2.10. Biochemical oxygen demand

Method 5210 B was used for BOD_5 determination as described in the Standard Methods for Examination of Water and Wastewater [32]. BOD_5 can be calculated as follows:

$$\mathrm{BOD}_5\left(\mathrm{mgL}^{-1}\right) = D1 - \frac{D2}{P} \tag{2}$$

where $D1$ = DO of diluted sample immediately after preparation in mg L^{-1}, $D2$ = DO of diluted sample after 5 d incubation at 20°C in mg L^{-1}, P = decimal volumetric fraction of sample (300 mL).

2.11. Chemical oxygen demand

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 [32]. Color 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.

2.12. Fat, oil and grease

Determination of total content of grease and oily substances was carried out using the partition gravimetric method described in the Standard Methods for Examination of Water and Wastewater [32]. Fatty acid composition was extracted using *n*-hexane followed by methylation and then determined using gas chromatograph model 8400GC, fitting with flame ionization detector and fused silica capillary column. Oil and grease content were calculated according to the following equation:

$$\mathrm{FOG}\left(\mathrm{mgL}^{-1}\right) = \frac{\left(A - B\right)}{\mathrm{sample}\left(\mathrm{mL}\right)} \tag{3}$$

where A = weight of the beaker with FOG; B = weight of the clean beaker.

2.13. Determination of ammonia

Ammonia was determined using phenate method (4500-NH_3 F) described in the Standard Methods for the Examination of Water and Wastewater [32]. Immediately after collection, samples were fixed and determined spectrophotometrically using the indophenol blue technique. Ammonia compounds combine with sodium hypochlorite and alkaline solution of phenol and disodium nitroprusside dihydrate to form monochloramine, which reacts with salicylate forming 5-aminosalicylate that is oxidized in the presence of a sodium nitroprusside catalyst to form indophenol blue. The blue color developed after 2 h was measured at 665 nm and results were expressed as mg L^{-1}.

2.14. Nitrate

Nitrates were determined using cadmium reduction method (4500-NO_3) described in the Standard Methods for the Examination of Water and Wastewater [32]. NO_3 was reduced almost quantitatively to nitrite (NO_2) in the presence of cadmium (Cd). This method uses commercially available Cd granules treated with copper sulfate ($CuSO_4$) and packed in a glass column. The NO_2 produced was converted into a reddish purple azo dye formed by adding 1 mL of sulfanilamide reagent to 50 mL of water sample followed by 1 mL of N-(1-naphthyl)-ethylenediamine dihydrochloride solution (NED) to form a highly colored azo dye after 30 min. Color density was measured spectrophotometrically at wavelength of 540 nm. Nitrite content was expressed in mg L^{-1}. Nitrate concentration in each sample was calculated after correction of nitrite concentration of the same sample.

2.15. Phenol

Phenol was measured using colorimetric method. By using 4-aminoantipyrine (4-amino-2,3-dimethyl-1-phenyl-3-pyoarolin-5-on) to estimate the residues of phenol through the color developed that was measured at 460 nm by using (Hach DR 5000) spectrophotometer.

2.16. Total viable count of bacteria

Changes in the total heterotrophic bacterial viable counts during wastewater treatment were determined using pour plate technique of the standard plate count method after sequential dilutions [32]. Samples serially diluted, cultured in NA medium and incubated at 37°C for 24 h. Colony forming units (CFUs) of the bacterial TVC were recorded and averages were calculated.

2.17. Statistical analysis

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

3. Results

3.1. Screening of bacterial isolates

Preliminary screening test was performed. The nine bacterial isolates (four indigenous and five exogenous) were

individually inoculated in 250 mL flasks containing pharmaceutical effluent and incubated at 37°C under 100 rpm agitation speed for 1 week. Visual observations (i.e., degree of clearness) were taken daily. Six isolates (L1, S1, PS, PQ, PF, and TAQ) showed turbidity reduction in the effluent while the other three (L2, L3, and L4) showed no ability to reduce the turbidity. Therefore, the six isolates that reduced the effluent's turbidity were selected for bioremediation assays supposing their ability to degrade the contaminants.

3.2. Classical identification

Traditional identification of the pure pharmaceutical isolates (L1–L4) (Table 1) led to exclusion of three pathogenic or environmentally harmful isolates. Moreover, the five exogenous bacteria (S1, PS, TAQ, PF, and PQ) were subjected to the same procedures in order to confirm their identification.

3.3. Molecular identification

Based on the most probable identification obtained, all pathogenic or harmful isolates were excluded resulting in six isolates (S1, PS, PQ, TAQ, PF, and L1) from which genomic DNA was prepared, except PF which was a reference strain. Genomic DNA was prepared and examined on 1% agarose gel. PCR amplification was then carried out using universal primers coding for 16S rDNA gene with specific PCR conditions. The resulted 1,300 bp amplicons were examined on 1% agarose gel electrophoresis. The PCR products were purified and the two strands of each amplicon were sequenced using chain terminator method (MWG Company, Germany). 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 2 compiles GenBank accession numbers of the highest sequence similarity as well as the closest neighbor(s) to the 16S rDNA gene partial sequences. Sequences of the four isolates S1, PQ, TAQ, and L1 were affiliated according to their 16S rDNA to members of genus *Bacillus*, whereas isolate PS was affiliated to genus *Pseudomonas* with sequence 96% similar to *Pseudomonas stutzeri* M15-10-3. On the other hand, isolate L1 and TAQ are related as they are similar to *Bacillus cereus* (*B. cereus* OPP5 (98%) and *Bacillus cereus* Kt7-14 (97%), respectively. PQ and S1 showed high similarity (98%) to *Bacillus* sp. SA-3 and (97%) to *Bacillus amyloliquefaciens* T004, respectively. The phylogenetic relationships of the experimental isolates and closely related species were analyzed using the multi-sequence alignment program (MEGA 5) and the results are presented in phylogenetic tree (Fig. 1).

Table 1
Most probable identification of the indigenous and exogenous bacteria based on their API biochemical profiles

Bacterial code	Most probable identification
L1	*Bacillus cereus* Id = 99.4%
L2	*Salmonella arizona* Id = 88.5%
L3	*Salmonella* sp. Id = 93%
L4	*Vibrio cholerae* Id = 66.9%
PF	*Pseudomonas putrefaciens* Id = 99%
TAQ	*Bacillus cereus* Id = 97%
S1	*Bacillus amyloliquefaciens* Id = 82.1%
PS	*Pseudomonas stutzeri* Id = 99.9%
PQ	*Bacillus* sp. Id = 95%

3.4. Treatability of pharmaceutical effluent using free-living bacteria (batch mode)

3.4.1. Variation in the pH and DO levels

Results revealed significant change in the pH values of either the control or the seeded wastewater before or after the remediation process (Fig. 2(a)). The untreated effluent showed nearly acidic pH (5.32) which was increased during the treatment reached the highest value of 7.44 by PF (*Pseudomonas putrefaciens*) after 1 exposure d. Concerning variation in DO levels (Fig. 2(b)), results revealed very low DO level (0.29 mg L^{-1}, zero time) in the raw wastewater. There was a general trend of gradual decreasing DO levels with increasing exposure time (till the 6th day) in all samples either seeded or not. After 6 exposure d, wastewater seeded by the PQ (*Bacillus* sp.) culture showed the lowest RL of DO (0.13 mg L^{-1}) reflecting the highest consumption rate compared with the unseeded wastewater (control) where highest RL of DO (0.22 mg L^{-1}) was recorded.

3.5. Effluent solids

3.5.1. Total dissolved solids

Raw wastewater had the lowest TDS (410 mg L^{-1}) at the starting point that increased due to remediation process and breaking down complex contaminants into simple dissolved salts (Fig. 3(a)). The highest increases in TDS

Table 2
Similarity percentages to the nearest neighbors of the selected isolates

Isolate no.	Nearest neighbor(s)	GenBank accession of the nearest neighbor	Similarity%
S1	*Bacillus amyloliquefaciens* T004	HQ840415.1	97
PS	*Pseudomonas stutzeri* M15-10-3	HM030751.1	96
PQ	*Bacillus* sp. OU-40	EM_PRO:FN663629	95
TAQ	*Bacillus cereus* OPP5	JQ308572.1	97
L1	*Bacillus cereus strain* Kt7-14	JF460754.1	98

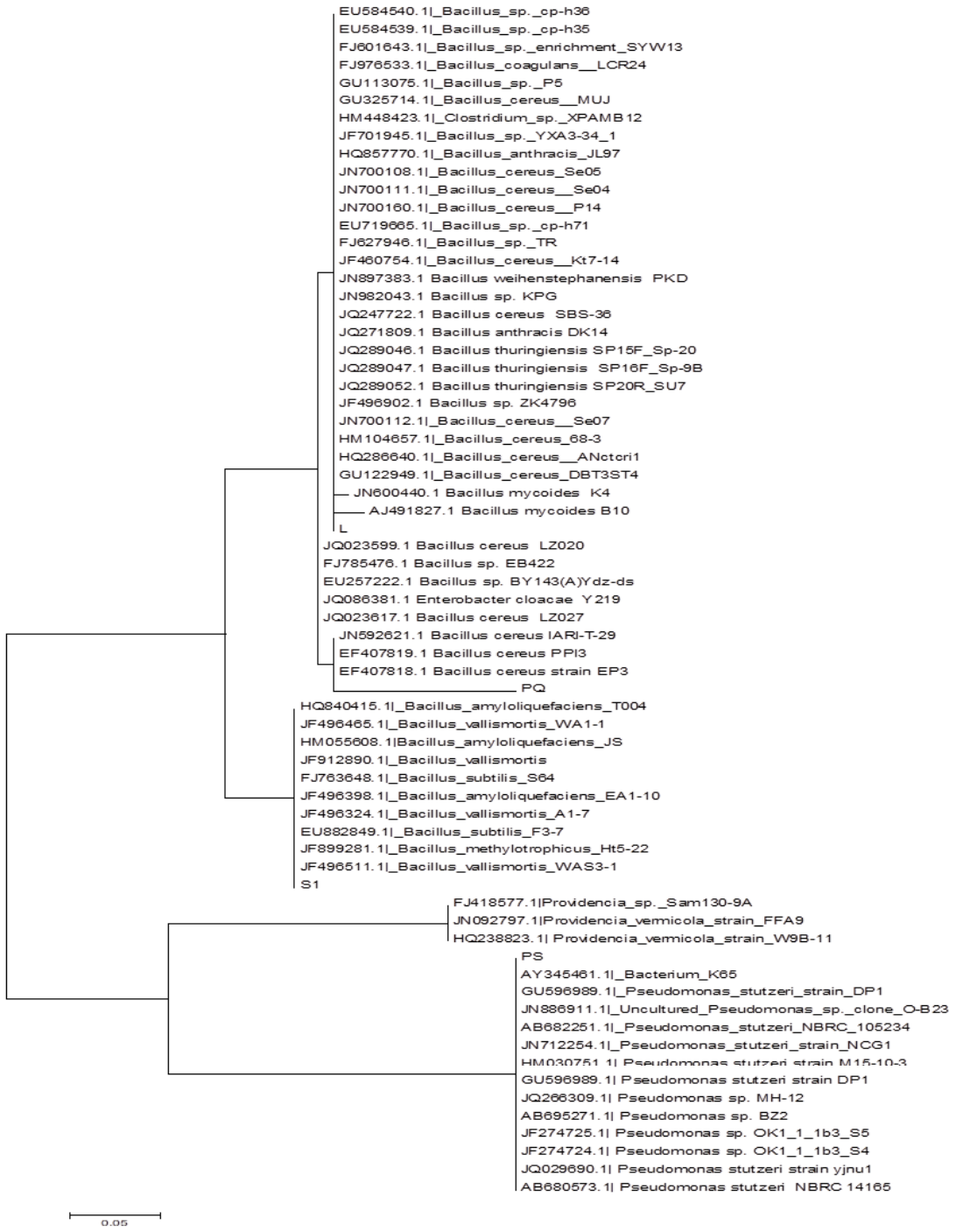

Fig. 1. Phylogenetic relationships among isolate S1, PQ, PS, L, and the most closely related bacterial species. The tree was generated by MEGA 5 Software.

concentration in all treatment cultures were achieved after the first 24 h followed by gradual but insignificant increases till the 6th exposure d. Highly significant variations were noticed among the tested bacteria depending on their ability to degrade and/or transform the available contaminants in pharmaceutical wastewater. In that respect, PF was the most active strain resulted in 82.92% increase in the TDS after the first 24 h followed by slight increases reaching 94.63% after the 6th exposure d. This was followed by PS, TAQ, mixed culture, PQ, S1, and L1 recording 73.17%, 68.29%, 67.07%, 65.85%, 60.97%, and 58.53%, respectively, after 24 h which slightly increased reaching 87.31%, 84.14%, 80.48%, 76.58%, 74.39%, and 68.29%, respectively, after 6 d. On the other hand, the control (unseeded wastewater) recorded only 3.65% and 8.78% TDS additions at the same exposures, respectively.

3.5.2. Total dissolved solids

Raw pharmaceutical wastewater recorded high TSS level (350 mg L^{-1}) at the starting point. Highly significant removals were detected by all cultures after the first 24 h of the treatment. Significant variations were recorded among the tested bacteria indicating different abilities for removing TSS. PF exhibited the highest efficiency (79.44%) followed by the mixed culture (57.05%), PQ (56.13%), L1 (53.37%), PS (47.54%), TAQ (45.39%) and finally S1 with lowest recorded efficiency (26.38%) after 6 d (Fig. 3(b)). On the other hand, very low RE of the TSS (1.71%) was recorded after the first 24 h treatment by the control (unseeded wastewater) confirming the high efficiency and role played by the tested bacteria for degradation and removal of pharmaceutical wastewater contaminants.

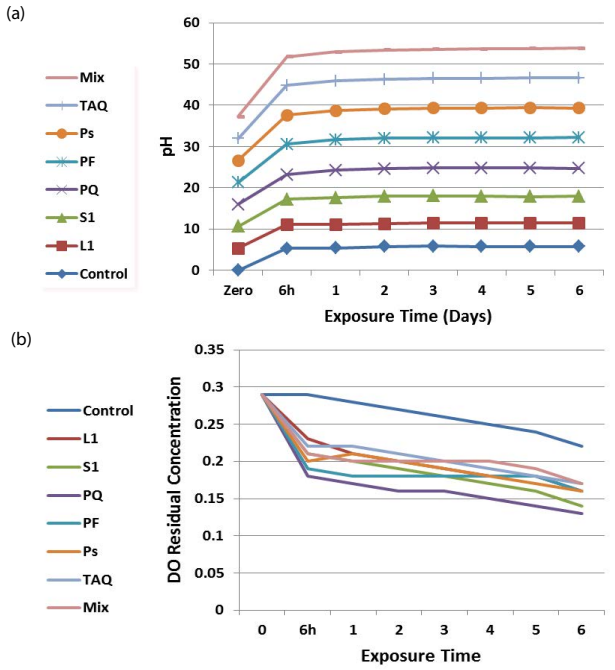

Fig. 2. Variations in the pH (a) and DO (b) levels in the raw and treated pharmaceuticals wastewater at different exposure times.

3.6. Organic matter

3.6.1. Biochemical oxygen demand

High BOD level (640 mg L^{-1}) characterized the raw pharmaceutical effluent at the starting point. PF exhibited the highest efficiency (75.07%) followed by the mixed culture (66.19%), PQ (48.28%), PS (42.52%), TAQ (37.85%), L1 (36.91%), and finally S1 with lowest recorded efficiency (25.70%) at the end of the experiment (Fig. 4(a)) compared with very low RE of the BOD (1.25%) recorded after the first 24 h treatment by the control that reached −0.31% (i.e., BOD increase) after 6 exposure d. This confirmed that BOD is removed mainly by the powerful ability of the augmented bacteria. Although as low as 160 mg L^{-1} residual concentration (RC) representing 75.07% RE was achieved by PF after 6 exposure d, this value still 2.7 folds higher the MPL of the BOD (60 mg L^{-1}) for safe discharge of pharmaceutical wastewater.

3.6.2. Chemical oxygen demand

Raw pharmaceutical effluent recorded very high COD level (5,400 mg L^{-1}). Again PF exhibited the highest efficiency

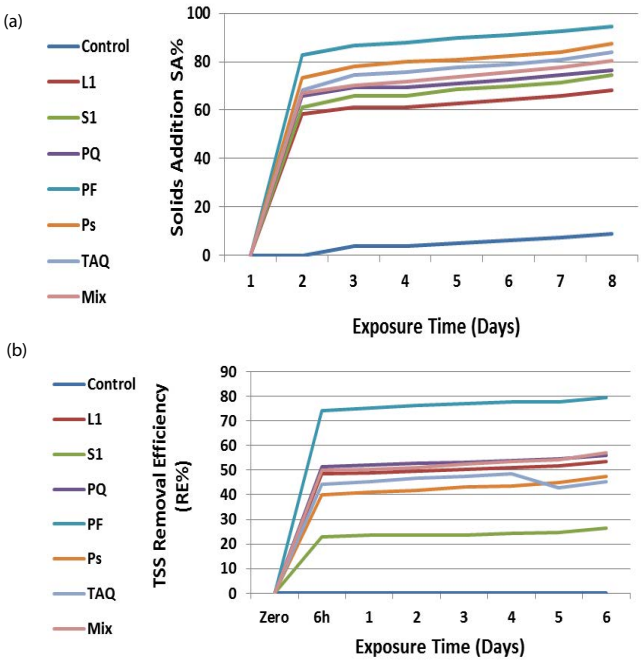

Fig. 3. TDS addition (a) and TSS removal efficiency (b) % in the raw and treated pharmaceuticals wastewater at different exposure times.

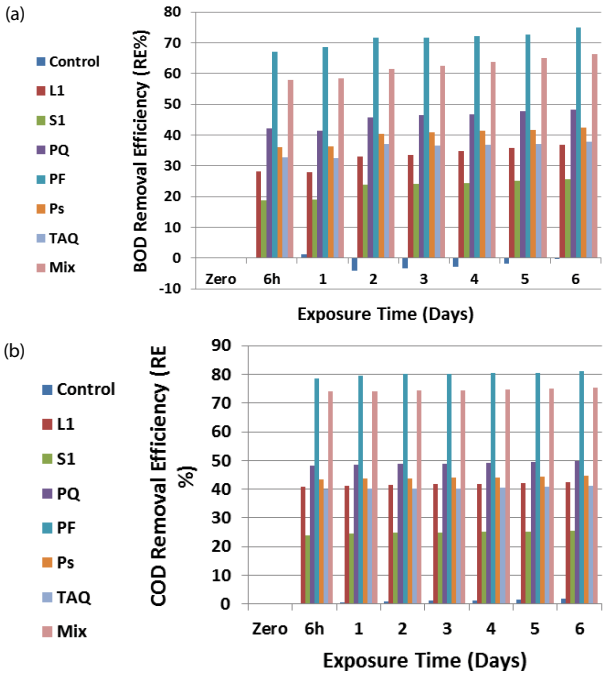

Fig. 4. Removal efficiency (RE%) of BOD (a) and COD (b) in the raw and treated pharmaceuticals wastewater at different exposure times.

(81.03%) followed by the mixed culture (75.28%), PQ (49.81%), PS (44.81%), L1 (42.59%), TAQ (41.07%), and finally S1 with lowest recorded efficiency (25.59%) after 6 d (Fig. 4(b)). However, extremely low RE of the COD (0.74%) was recorded after the first 24 h treatment by the control that reached 1.85% after 6 exposure d confirming again that COD is removed mainly by the metabolic activity of the augmented bacteria. The highest achieved RE of the COD (81.03%) resulted in 1,005 mg L^{-1} RC which still very high (≈10 folds) compared with the MPL of the COD (100 mg L^{-1}) for the safe discharge into the environment.

3.6.3. Fat, oil and grease

PF exhibited the highest efficiency (90%) while L1 with lowest recorded efficiency (35%) after the 6 treatment d (Fig. 5). Control culture recorded even lower REs of FOG (5% and 30%) after 1 and 6 treatment d, respectively. The lowest RC of FOG (2 mg L^{-1}) achieved by PF after 6 exposure d is lower than MPL (10 mg L^{-1}) for safe discharge of pharmaceuticals effluent.

3.7. Other pharmaceutical chemical pollutants

3.7.1. Nitrate (NO_3)

Opposite to other parameters, considerable increases in NO_3 concentration occurred with time by all the tested bacteria reached their highest levels at the end of the experiment (6 d). Considerable variation was shown by the tested bacteria toward formation of NO_3. Unlike other parameters, the order of bacterial efficiency after 24 h treatment was as follows: TAQ (43.75%), PF (40.63%), PS (37.5%), mixed culture (28.13%), PQ (25%), L1 (25%), S1 (12.5%), and finally the control with the lowest efficiency (3.13%). These figures were increased after 6 treatment d to record 93.75% (TAQ), 84.38% (PF), 87.5% (PS), 81.25% (mixed culture), 81.25% (PQ), 84.38% (L1), 68.75% (S1), and 40.63% (control) (Fig. 6). The lowest (3.13% and 40.63%) nitrate addition (NA %) was recorded by the unseeded wastewater after 1 and 6 treatment d, respectively, indicating the absence of nitrifying bacteria. NO_3 levels, either after 1 or 6 d, were higher than the Egyptian MPL (50 mg L^{-1}) and the Saudi MPL (15–20 mg L^{-1}) required for discharging into open water or to central treatment plant, respectively.

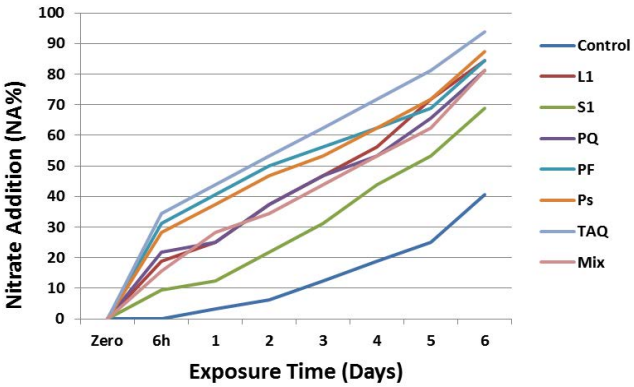

Fig. 6. NO_3 addition% in the raw and treated pharmaceuticals wastewater at different exposure times.

3.7.2. Ammonia (NH_3)

Level of ammonia in the pharmaceutical effluent (12.5 mg L^{-1}) was reduced due to its oxidation into nitrate during the remediation action by the tested bacteria (Fig. 7). Among the tested bacteria, PF was the most efficient recording the highest NH_3 RE % of 49.52% and 55.2% after the 1st and 6th treatment d, respectively, compared with S1 which recorded the lowest RE % of 9.68% and 15.44% at the same exposure times, respectively. The unseeded wastewater showed very low NH_3 removal reaching only 8% after 6 d treatment compared with samples seeded with the selected bacteria which indicated their important role in the bioremediation process.

3.7.3. Phenol

Raw pharmaceutical effluent recorded very high and dangerous phenol level (12.5 mg L^{-1}) that was considerably removed by all the selected bacteria after the first 24 h treatment after which minor insignificant REs % were recorded with increasing exposure time. PF was the most efficient achieving the highest phenol RE of 93.06% and 94.29% after 1 and 6 treatment d, respectively. This was followed by the L1 (72.68%), PQ (69.52%), mixed culture (56.34%), PS (43.67%), TAQ (35.21%), and finally S1 with the lowest achieved efficiency (26.41%) (Fig. 8). The control culture showed very low phenol removal reaching only 0.19% and 0.94% after 1 and

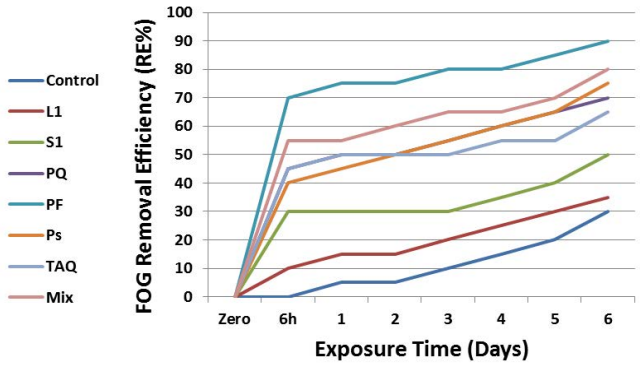

Fig. 5. Removal efficiency (RE%) of FOG in the raw and treated pharmaceuticals wastewater at different exposure times.

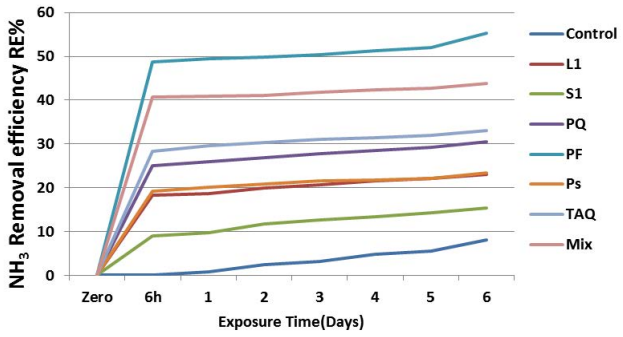

Fig. 7. Removal efficiency (RE%) of NH_3 in the raw and treated pharmaceuticals wastewater at different exposure times.

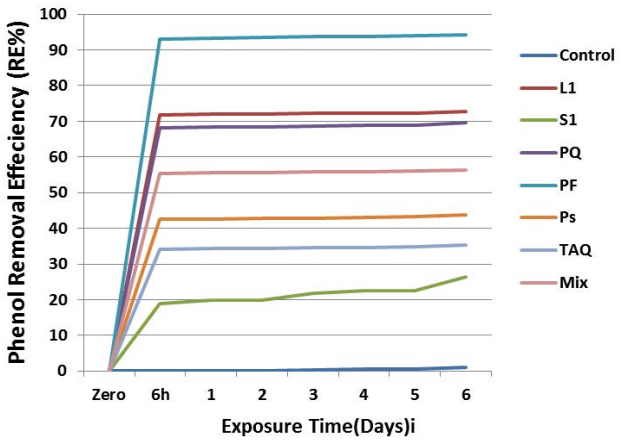

Fig. 8. Removal efficiency (RE%) of phenol in the raw and treated pharmaceuticals wastewater at different exposure time.

6 d compared with samples seeded with the selected bacteria which indicated their important metabolic role in phenol removal. Although high phenol removals were recorded, its level in the final effluent is still higher than the permissible levels (0.02 mg L^{-1}).

3.7.4. Biological contaminants

TVC was determined to define biological pollution extent in the pharmaceutical raw effluent. It is also an indication to stimulatory and/or inhibitory effects of the pharmaceutical effluent on the growth of the augmented tested bacteria. PQ, mixed culture, PS, L1, TAQ, and PF showed similar behavior where they were not affected by the toxicity of the contaminated effluent and their growth was even stimulated till the 6th exposure d (Table 3). These results indicated their high resistance against such kind of wastewater which was confirmed by their high ability to degrade and accumulate the involved contaminants. S1 and the control which showed the lowest ability to remove all the tested contaminants showed no responses toward effluent toxicity during the first 3 treatment d (0% GI). Then S1 growth was gradually inhibited till the 6th treatment d (15.6%). On the other hand, growth of the control bacteria was gradually inhibited reaching the highest inhibition (9.3%) after the 6th d.

Table 4 illustrates the maximum permissible limits (MPLs) of the tested parameters stated by Saudi, Egyptian, and Syrian Environmental Organizations. Saudi limits were set not for the final discharge into open environments but for the primary treated wastewater before disposing into the central treatment point. Egyptian limits are stated by the environmental laws (48/1982 & 4/1994) that regulate discharging of domestic and industrial wastewater into fresh and saline open water.

3.8. Statistical analysis

Correlation coefficients (Pearson's r) among the different contaminants present during treated pharmaceutical effluents using the selected bacteria in the free-living mode are presented in Table 5.

Table 3
Stimulatory (S) and/or inhibitory (I) effect of the contaminated effluent on the growth of the selected bacteria (TVC, CFU/mL) at different exposure times

Culture		Exposure time (d)							
		Zero	6 h	1	2	3	4	5	6
Control	TVC	3.2×10^7	3.2×10^7	3.2×10^7	3.4×10^7	3.4×10^7	3.2×10^7	3.2×10^7	2.9×10^7
	S & I%	0	1.0 f	1.0 f	1.1 f	1.1 f	1.0 f	1.0 f	9.3
L1	TVC	3.2×10^7	3.2×10^7	4.1×10^7	4.3×10^7	4.2×10^7	4.4×10^7	4.1×10^7	3.8×10^7
	S & I%	0	1.0 f	1.2 f	1.3 f	1.3 f	1.4 f	1.2 f	1.1 f
S1	TVC	3.2×10^7	3.2×10^7	3.0×10^7	3.1×10^7	3.2×10^7	3.3×10^7	3.1×10^7	2.7×10^7
	S & I%	0	1	6.25	3.1	0	1.0	3.1	15.6
PQ	TVC	3.2×10^7	3.2×10^7	3.9×10^7	3.9×10^7	4.0×10^7	4.1×10^7	3.9×10^7	3.5×10^7
	S & I%	0	1	1.2 f	1.2 f	1.25 f	1.28 f	1.21 f	1.09 f
PF	TVC	3.2×10^7	3.2×10^7	3.5×10^7	3.9×10^7	3.9×10^7	4.0×10^7	3.9×10^7	3.5×10^7
	S &I%	0	1	1.09 f	1.21 f	1.21 f	1.25 f	1.21 f	1.09 f
Ps	TVC	3.2×10^7	3.2×10^7	4.0×10^7	4.1×10^7	4.2×10^7	4.3×10^7	4.0×10^7	3.7×10^7
	S & I%	0	1	1.25 f	1.28 f	1.31 f	1.34 f	1.25 f	1.15 f
TAQ	TVC	3.2×10^7	3.2×10^7	4.2×10^7	4.2×10^7	4.3×10^7	4.3×10^7	4.0×10^7	3.6×10^7
	S & I%	0	1	1.31 f	1.31 f	1.34 f	1.34 f	1.25 f	1.13 f
Mix	TVC	3.2×10^7	3.2×10^7	3.7×10^7	3.8×10^7	3.9×10^7	3.9×10^7	3.7×10^7	3.3×10^7
	S & I%	0	1	1.15 f	1.18 f	1.21 f	1.21 f	1.15 f	1.03 f

S: Stimulation, I: Inhibition, GS: Growth stimulation, GI: Growth inhibition, f: fold.

Table 4
Correlation coefficients (Pearson's r) among the different microorganism during bioremediation using free-living bacteria

	L1	S1	PQ	PF	PS	TAQ	MIX
L1		0.784**	0.922**	0.847**	0.825**	0.818**	0.785**
S1	0.784**		0.871**	0.733**	0.974**	0.976**	0.818**
PQ	0.922**	0.871**		0.953**	0.943**	0.902**	0.929**
PF	0.847**	0.733**	0.953**		0.844**	0.789**	0.948**
PS	0.825**	0.974**	0.943**	0.844**		0.980**	0.910**
TAQ	0.818**	0.976**	0.902**	0.789**	0.980**		0.877**
MIX	0.785**	0.818**	0.929**	0.948**	0.910**	0.877**	

**Correlation is significant at *P* value= 0.01 (2-tailed).

Table 5
Maximum permissible limits for the tested contaminants based on national environmental regulations for safe discharge

Parameter	Saudi limits[a]	Egyptian limits[b]	Syrian limits
pH	5–10/6–9	6–9	9.5–6.5
Temperature °C	60	35	10
DO (mg L^{-1})	–	4	4
BOD_5 (mg L^{-1})	25	60	60
COD (mg L^{-1})	150	100	200
TSS (mg L^{-1})	15	60	60
TDS (mg L^{-1})	–	2,000	–
FOG (mg L^{-1})	8.0	10	15
NO_3 (mg L^{-1})	15–20	50	50
NH_3 (mg L^{-1})	1.0	3	10
Phenol (mg L^{-1})	0.1	0.005	0.5

[a]Limits Stated by the Saudi Presidency of Meteorology and Environment (PME) for direct Discharge.
[b]Limits Stated by the Egyptian Environmental Laws (No 48/1982 & 4/19).

4. Discussion

Among the four indigenous bacteria isolated from pharmaceuticals effluent only L1 showed ability to clarify pharmaceuticals wastewater indicating that it has the required degrading enzymes. The five exogenous strains (S1, PS, PF, TAQ, and PQ) used in the present study are known for their ability to degrade environmental pollutants since they were isolated originally from highly contaminated environment of Lake Mariut, a hypereutrophic lake south of Alexandria, so they are highly resistant. Molecular characterization of the six most active bacteria identified them as to *Pseudomonas stutzeri* M15-10-3 (96% similarity with PS), *Bacillus cereus* strain Kt7-14 (98% similarity with L1), *Bacillus* sp. SA-3 (95% similarity with PQ), *Bacillus amyloliquefaciens* T004 (97% similarity with S1) and *Pseudomonas aeruginosa* (TAQ) while PF was supplied as a reference strain and identified as *Pseudomonas fluorescens* [28] all exhibited superior ability for organic matter and heavy metals remediation. These strains were kindly provided from the IGSR Bacterial Collection.

Results revealed that *Pseudomonas fluorescens* (PF) is the most efficient strain used in the present study. The marvelous resistance and superior potentiality of PF for biodegradation of toxic organic pollutants could be effectively used in degrading phenol ranging from 100 to 1,000 mg L^{-1} in waste containing phenol converting the toxicant to nutrient, biomass, and CO_2 [33]. Therefore, performance of PF during the present study toward contaminants of pharmaceuticals wastewater is expected and logical. The two strains (S1 and PQ) belong to the genus *Bacillus* which is well known as highly resistant spore-forming bacterium. It was reported that microorganisms such as *Pseudomonas* and *Bacillus* are well known natural degraders to aromatic compounds such as aromatic amino acids, phenols, or quinones [34–36]. This is not surprising since they have evolved catabolic pathways to degrade aromatic compounds such as phenol in the present study. Pharmaceutical effluents used in the present study can be classified as strong since it was highly polluted and contained extremely high levels of the tested contaminants that required powerful treatment to minimize its pollution load and discharge it safely. It was subjected to treatment in a batch experiment using free-living individual and mixed bacteria that was time and species dependent and accordingly resulted in varied levels of contaminants REs. Environmental laws in Egypt, Saudi Arabia, and Syria (Table 4) stated MPL for the different water contaminants for the safe discharge into the open environment. These limits are set to minimize the ecological disturbances and protect aquatic as well as soil environments from hazardous discharges. Batch treatment of pharmaceutical effluent confirmed that PF considered the most efficient for removing all the tested parameters. However, residual levels of all the parameters still above the MPL for the safe discharge except FOG. Therefore, another treatment step or technology must be adopted to bring the effluent to the safe limits for the environment as also found by other workers [27]. There are some evidences that many of substances of pharmaceutical origin are not degraded in STPs and are also not biodegradable in the natural environment. Much more exhaustive studies are required to bioremediate the active pharmaceutical agents especially the ones which are non-biodegradable and persistent in nature. Thus, the adverse effects of these chemicals on flora and fauna can be minimized for a healthy and safe future. These effects can be further studied and validated as per modern research methodology [21,33].

Phenol compounds constitute a family of pollutants particularly toxic to the aquatic fauna, flora, and man. These compounds are released to the environment by a considerable number of industries, such as pharmaceutical plants. Pharmaceutical effluent that was used in the study was heavily loaded with the phenol and nitro aromatic compounds. The presence of the nitro group makes these compounds resistant to biodegradation and the microbial conversion often leads to the production of harmful metabolites. This was shown in the batch treatment of phenol-contaminated effluent by PF for 6 d.

There was a general trend of increasing the RE of all parameters by all the tested bacteria with increasing the exposure time. The bulk changes in all parameters were achieved in the first 24 h followed by gradual increase (in the case of TDS and NO_3) or decrease (other parameters) in their concentration. Significant variations were detected among the tested bacteria toward the different contaminants. In that respect, PF was the most efficient in removing all the tested

parameters with only one exception (NO_3) which increased due to oxidation of NH_3. On the other hand, S1 and L1 were characterized by the lowest efficiency for contaminants removal. The control (unseeded wastewater) showed the lowest recorded RE for all the tested parameters indicating very low biodegradation activity compared with the seeded wastewater. Growth of PQ, mixed culture, PS, L1, TAQ, and PF was not affected by the effluent toxicity and even stimulated till the 6th exposure d as also found by other workers [21]. S1 and the control showed no responses toward effluent toxicity during the first 3 treatment d (0% GI). Then S1 and the control growth were gradually inhibited reaching the highest inhibition (15.6% and 9.3%) after the 6th d.

5. Conclusion

Pharmaceutical 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 its treatment. The batch treatment of pharmaceutical effluent was time and bacterial species dependent with PF considered the most efficient for all the tested parameters. However, residual levels of all the parameters still above the MPL for the safe discharge except for the FOG. This system could reach higher removal for all the tested parameters reaching acceptable limits for safe discharge if longer exposure time was used or with the aid of another treatment step or technology such as biofilm with the most active bacterial strain.

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