

Isolation and Identification of a New Bacterial Strains Degrading Paracetamol Isolated from Yemeni Environment

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Abstract

Paracetamol has emerged as an important environment pollutants due to its wide usage. This study carried out to isolate and identify of a novel paracetamol-degrading bacteria from the contaminated site of pharmaceutical wastewater at Sana'a City, Yemen. The enrichment media and selective media were used to isolate and purify the bacteria from wastewater samples. The isolated bacteria were identified according to phenotypic and genotypic characterization using 16S rRNA sequence analysis. The results showed that the three bacterial strains and signed as an STB1, STB3 and STB5.

The STB1 strain was identified as *Pseudomonas aeruginosa* strain DSM 50071 with similarity 92%, while the STB3 and STB5 were identified as *P. aeruginosa* strain NBRC 12689 with similarity 89% and 99%, respectively. The optimal conditions for paracetamol degradation by all stains were recorded at 30°C and pH 7.0. Also, the paracetamol degradation was found to be influenced by an increase in the bacterial cell concentration. The STB1, STB3, and STB5 strains were degraded 68.6%, 51.3% and 79.4% of 4000 mg/L paracetamol, respectively, in 120h. The results performed that the degradation potential of the three isolated bacterial strains possibly played a major active role in the paracetamol removal processes.

Key words: Bacteria Isolation; Biodegradation; Paracetamol; Physiological characterization

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Introduction

Paracetamol or acetaminophen is a common analgesic and an anti-inflammatory commonly used as a non-prescription drug and sold as over-the-counter drug worldwide [1]. The paracetamol consumption has increased throughout the world in the last decades. In England, it was consumed as one of the top three drugs prescribed, while in the USA, it was consumed as one of the top 200 prescriptions [2,3]. It was ranked as the second prescription drugs throughout the year 2008 in Kuwait [4]. In Yemen, it was also ranked as first of top ten drugs produced by local industries and one of the top ten drugs imported [5].

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Paracetamol is one of the most often detected drug products in the aquatic environment. It was 0.298 µg/L reported in drinking water [6], 6.5 µg/L in groundwater [7], 15.7 µg/L in surface water [8], 0.246 mg/L in sewage water [9], and 1.367 mg/L in wastewater [10]. Therefore, the public awareness about its potential risk and effect of paracetamol on the environment and human health has raised [11].

Previous studies on removal of paracetamol from wastewater mainly focused on chemical methods including oxidation processes [12]. The cost of the chemical agent, the energy source, and the generation of secondary pollutants due to the use of excessive chemicals are the major drawbacks to using these methods on removing the paracetamol from wastewaters [13]. So, the biodegradation of paracetamol compound is being considered as an environmentally friendly and low-cost option and has been demonstrated to have the potential to remove this substance by degrading it to nontoxic compounds [14].

This study describes the isolation, identification, and characterization of a new paracetamol degrading bacteria which isolated from wastewater contaminated site of a Yemen Drug Company for Industrial and Commercial (YEDCO) situated at Sana'a City, Yemen. The wastewater was generated by the factory containing a high concentration of paracetamol through the manufacturing processes.

Materials and Methods

Study Design

This study was conducted at YEDCO factory located at Sana'a City, Yemen. 50 samples were collected from different places in and around this factory such as production units wastewater, wastewater channels, and wastewater influent to the sewer. The experimental study carried out at the quality control department at YEDCO for chemical and microbiological analysis during 10 months, from period November 2016 to August 2017.

Chemicals and Cultivation Medium

The standard of paracetamol was purchased from Anqiu Lu'an Pharmaceutical Co. Ltd. (China) and all other chemicals were of the highest purity commercially available. The applied mineral salts medium (MSM) contained the following components (per liter): 0.5g of K_2HPO_4 , 0.5g of KH_2PO_4 , 0.01g of NaCl, 0.2g of $MgCl_2 \cdot 6H_2O$, 0.02g of $CaCl_2$, 0.339 mg of $MnSO_4$, 0.428 mg of $ZnSO_4$, 0.347 mg of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.4 mg of $CoCl_2 \cdot 6H_2O$, 10 mg of EDTA. The pH was adjusted to 7.2–7.4 with 8M NaOH, the solution and autoclaved at 121°C for 15 min. Paracetamol was sterilized by filtration paper (0.22 µm) and added to the medium as the sole energy and carbon sources in different concentration [3].

Isolation and Purification of Bacteria and Culture Conditions

One mL of each sample was transferred separately into bottle contained 90 mL of trypticase soy broth (TSB) and incubated at 30°C for 3 days. After the incubation period, ten mL of TSB media was inoculated into a bottle containing 90 mL of MSM with different concentration of paracetamol (500–2000 mg/L) and incubated at 30°C for 5 days [15,16].

Then, 1 mL of MSM was transferred to MSM agar containing 500 mg/L of paracetamol and incubated 5 days at 30°C. The colonies showing growth on the plate were purified on trypticase soy (TSA) and after several transfers on MSM agar containing paracetamol and checked again on TSA to be pure. Pure colonies isolated were tested for growth on higher concentrations of paracetamol (500–3000 mg/L) in MSM agar. The isolated strains showing best growth of the compounds under study were selected and characterized further [15].

Identification of Isolated Bacteria

The isolated bacteria were identified according to its phenotypic and genotypic characteristics. Preliminary characterizations were based on morphological, physiological and biochemical tests [17]. Further identification was completed by 16S rDNA sequencing. Genomic DNAs of the harvested cells were extracted using a DNeasy® Blood & Tissue kit (QIAGEN, Germany), and the 16S rRNA was amplified by means of PCR (Eppendorf, Germany) using the bacteria universal primer 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (I) (5'-GGTTACCTTGTTACGACTT-3').

PCR mixture contained 5 μL of 5X Q5 reaction buffer, 0.2 μL of 10 mM dNTPs, 100 pM of forward primer and 100 pM of reverse primer, 5 ng/ μL of DNA template, 0.01 $\mu\text{L}/\mu\text{L}$ of Q5 high-fidelity DNA polymerase (NEB, M041S) and the volume completed up to 25 μL by nuclease-free water [18].

Amplification was started with an initial denaturation at 98°C for 5 min, followed by 35 cycles at 98°C for 20 s, 58°C for 25 s, and 72°C for 30 s/kb, followed by 8 min at 72°C for a final extension. The resulting 16s rRNA gene sequences were compared to database NCBI using BLAST program [19]. The multiple alignments of the sequence were performed, and a neighbor-joining phylogenetic tree was constructed using the latest version (2.6.1) of the BLAST program.

Physiological Characterization

The physiological characterizations such as temperature, pH, glucose concentration, and bacteria cell count were investigated as the following:-

Temperature and pH Effect on Paracetamol Biodegradation

The optimum of temperature and pH effects on the biodegradation rate of paracetamol (1500 mg/L) was determined within 72h. For the temperature effect, each bacterium strain was cultivated individually in a volume of 100 mL of MSM media containing paracetamol and incubated at different levels of temperature (15, 20, 25, 30, 35, 40 and 45°C) for 72h.

However, in the pH effect study, the bacteria strains were cultivated independently in MSM media containing paracetamol and having different levels of pH (4, 5, 6, 6.5, 7, 7.5, 8, 9, and 10) and incubated at 30°C for 72h. For rate degradation, 5 mL of each culture medium was withdrawn and centrifuged individually at 8000 rpm 30 min. The supernatant was collected in separate clean test tubes and examined for residual paracetamol content [15,16].

Contact Time Effects on Paracetamol Biodegradation

The effect of contact time on paracetamol biodegradation was performed on MSM media for each bacterium strain. Each bacterium isolated was cultured separately on MSM media containing a different concentration of paracetamol (1000-4000 mg/L) and incubated in different contact time with a label (24, 48, 72, 96, and 120h) at 30°C. Sampling was done at regular time intervals for residual paracetamol measuring [20].

Glucose Concentration Effects on Paracetamol Biodegradation

The glucose effecting on paracetamol biodegradation was estimated with different concentration of glucose (0, 1, 3, and 5 mg/L). Each concentration of glucose was added independently to MSM media (100 mL) containing-paracetamol (2500 mg/L) inoculated by each bacterium strain and incubated at 30°C for 120h. After each time designated, the sample was analyzed for residual paracetamol [20].

Bacteria Concentration Effects on Paracetamol Biodegradation

The MSM medium containing 2000 mg/L of paracetamol was cultivated by each bacterial strain individually with different initial bacterial concentration (1.0×10^4 , 1.0×10^6 , and 1.0×10^8 CFU/mL) and incubated at 30°C for 72h. The residual paracetamol content was assessed for each experimental individually [20].

Chemical Analysis Methods

300 mg of working standard of paracetamol was weighed and dissolved in a volumetric flask containing 100 mL of methanol. 5 mL of this solution was transferred to a 100 mL volumetric flask containing 47 mL of methanol and 53 mL of distilled water and mixed well. Then, the prepared solution was filtered by 0.22 μm nylon membrane filter before used [21].

Sample Analyzed

The residual concentration of paracetamol was assessed by using HPLC (PerkinElmer, USA). The mobile phase with a flow rate of 1.0 mL/min was composed of acetonitrile: water (47: 53 v/v). The separation was performed at 30°C by RP-8 column with dimension (5 μ m, 4.6 \times 250 mm). The injection volume was 20 μ m and detection wavelength was fixed at 275 nm [21].

Statistical Analysis

The obtained data were performed by statistical analysis using IBM SPSS Statistics software (version 18.0, 2009). The statistical analysis of variance between biodegradation efficiency and isolated bacterial strains were compared by Kruskal-Wallis test. Also, the analysis of variance between biodegradation efficiency and physiological characterizations (glucose and microbial concentration) were compared by Friedman test. However, the correlation coefficient between degradation and physiological characterizations (pH and temperature) were performed by using Pearson test. Values ($P < 0.01$) was considered statistically significant.

Results

Three bacteria strain named STB1, STB3, and STB5 that capable of degrading paracetamol were isolated from the pharmaceutical wastewater samples. These bacteria were gram-negative, slimy colonies, and non-spore forming. All strains were motile rods and positive for the catalase, oxidase, and nitrate reduction test. While, the citrate utilization, Vogues Proskauer, indole production, H₂S production, urease, DNase, and methyl-red test were negative.

From the current results, Figure 1 showed the color change on the MSM broth containing 4000 mg/L of paracetamol by isolated bacteria strains. The change in media color indicated that there were new transformation products formed during the degradation.

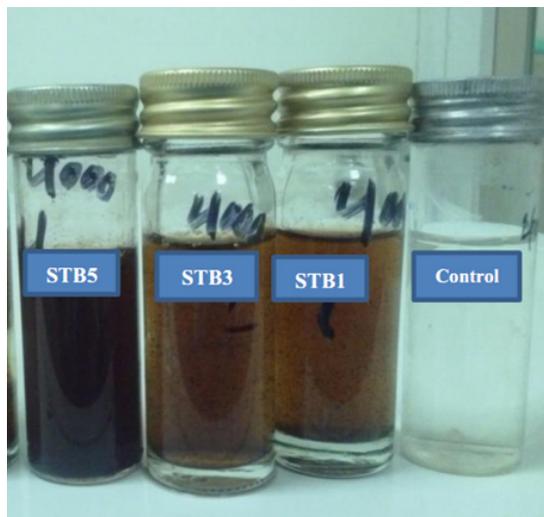


Figure 1: The changes on MSM broth containing 4000 mg/L of paracetamol.

The 16S rRNA was amplified from all samples specifically by the selected primers and the bands show the expected size (approximately 1.4 kb) according to the reference marker (Figure 2).

The results of 16S rRNA sequence alignment and phylogenetic tree analysis revealed that the 16S rRNA sequence of strains of STB1, STB3, and STB5 belonged to the genus *P. aeruginosa*. An 1174 base per (bp) 16S rRNA gene sequence that was obtained from strain STB1 and analyzed shows 92% similarity to the 16S rRNA gene sequence of *P. aeruginosa* strain DSM 50071 (Figure 3).

Moreover, a 1223 bp 16S rRNA gene sequence that was obtained from strain STB3 and analyzed shows 89% similarity to the 16S rRNA gene sequence of *P. aeruginosa* strain NBRC 12689 (Figure 4). A 749 bp 16S rRNA gene sequence that was obtained from strain STB5 and analyzed shows 99% similarity to the 16S rRNA gene sequence of *P. aeruginosa* strain NBRC 12689 (Figure 5).

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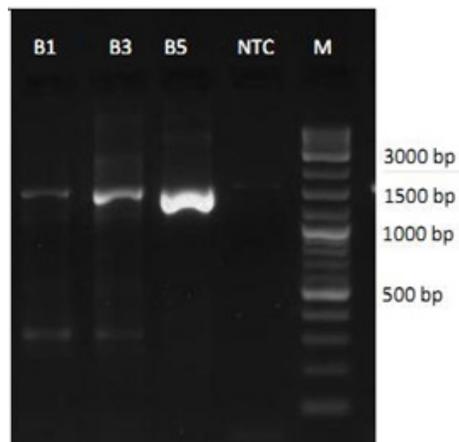


Figure 2: 1.7% agarose gel shows the resolved bands of the 16S rRNA from the bacterial strains; NTC = No Template Control.

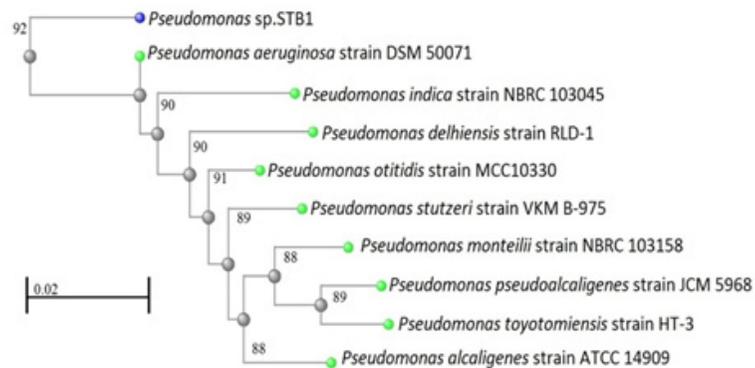


Figure 3: Neighbor-joining phylogenetic tree derived from 16S rRNA gene sequence data showing the positions of *Pseudomonas sp. STB1* and related organisms. Bar, 0.02 changes per nucleotide position.

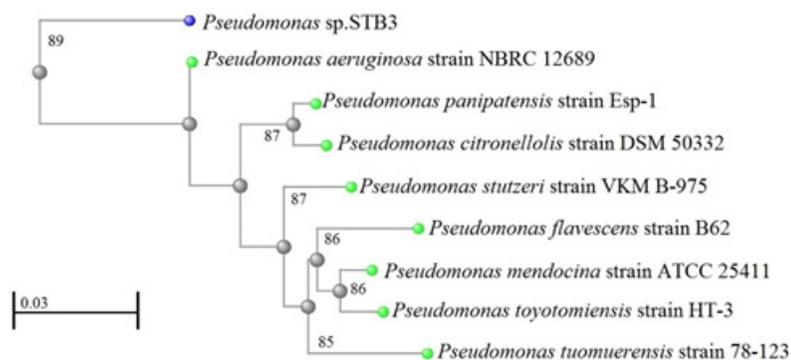


Figure 4: Neighbor-joining phylogenetic tree derived from 16S rRNA gene sequence data showing the positions of *Pseudomonas sp. STB3* and related organisms. Bar, 0.03 changes per nucleotide position.

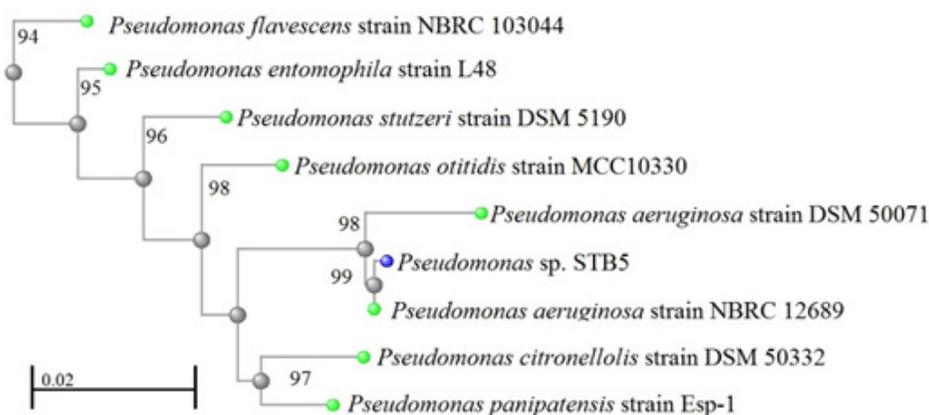


Figure 5: Neighbor-joining phylogenetic tree derived from 16S rRNA gene sequence data showing the positions of *Pseudomonas sp. STB5* and related organisms. Bar, 0.02 changes per nucleotide position.

Temperature and pH Effect on the Paracetamol Biodegradation

The results revealed that the optimum temperature was found to be at around 30°C for paracetamol biodegradation by all strains. The maximum biodegradation of paracetamol was 100% recorded at 30°C by bacteria strain STB1 and STB5 after 72h. Also, the maximum degradation by STB3 was 93.46%, respectively, reported of 1500 mg/L of paracetamol within 72h (Table 1).

However, it can still be considered to show relatively high degradation efficiency in a wide range of temperature (25–35°C). Whereas, the decreasing effects on paracetamol degradation was recorded at temperatures greater than 40°C or smaller than 20°C for all strains (Table 1).

Temperature (°C)	The paracetamol reduction (%) after 72h			P value
	STB1	STB3	STB5	
15	27.93	21	31.42	< 0.01*
20	49.2	40.57	53	
25	78.96	72.5	79.16	
30	100	93.46	100	
35	84.4	77.64	85.3	
40	47.25	42.81	51	
45	27.91	20.49	30.1	

Table 1: The effect of different temperatures on paracetamol biodegradation by isolated bacterial strains.

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

Moreover, the results in Table 2 showed that the optimum pH for paracetamol degradation was observed at pH 7.0 for all isolated bacterial strains. Also, it was reported that the complete degradation of 1500 mg/L of paracetamol was recorded by STB1 and STB5 after 72h. In addition, 93.46% of paracetamol was degraded by STB3, respectively, at the same pH and time as listed in Table 2. The degradation rate of paracetamol decreased in an experimental study at pH greater than 8.0 or smaller than 6.0. However, it can still be considered to show relatively high degradation efficiency in a wide range of pH (6.0–8.0) and performed the best in the pH range of 6.5–7.5 for all strains (Table 2).

pH	The paracetamol reduction (%) after 72h			P value
	STB1	STB3	STB5	
4	19	16	20	< 0.01*
5	42	38.14	43	
6	63.54	58	62	
6.5	97	89.53	96.86	
7	100	93.46	100	
7.5	96.31	88.79	95.92	
8	85.17	80.15	86.14	
9	37	32.7	38	
10	15	10	16	

Table 2: The effect of different levels of pH on paracetamol biodegradation by isolated bacterial strains.

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

Effect of Contact Time on Biodegradation of Different Concentration of Paracetamol

The STB1 strain has completely degraded the paracetamol at a concentration of 1500 mg/L within 72h. Also, it was recorded that the 84.73% and 68.6% of 3000 mg/L and 4000 mg/L, respectively, of paracetamol were degraded at 120h (Figure 6).

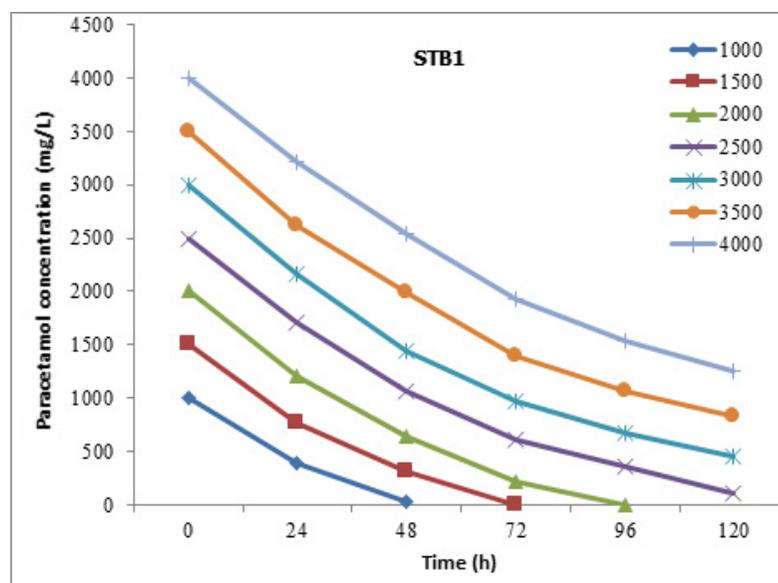


Figure 6: The paracetamol degradation by STB1.

Furthermore, the strain STB3 was reported to degrade 93.46% of 1500 mg/L paracetamol after 72h of incubation. At high concentration of paracetamol, the STB3 was recorded to degrade 86.92%, 63.22%, and 51.3% of 2500, 3000, and 4000 mg/L, respectively, of paracetamol within 120h of incubation (Figure 7).

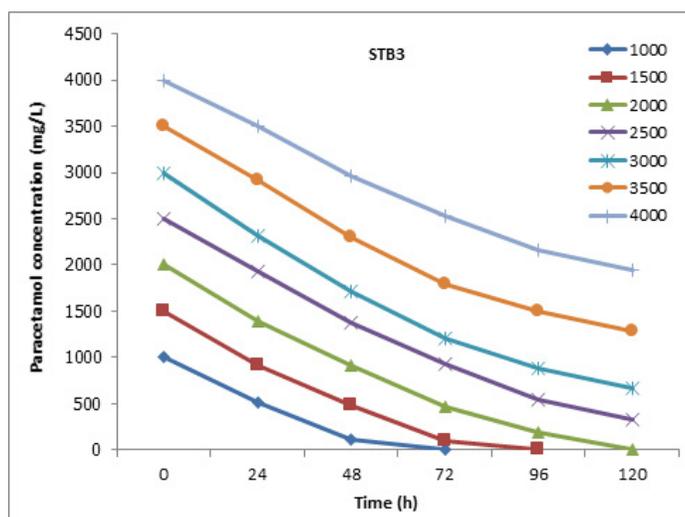


Figure 7: The paracetamol degradation by STB3.

The results showed that the STB5 was 100% degraded the 2000 mg/L of paracetamol at 72h. Also, 94.13% and 79.4% of 3000 and 4000 mg/L, respectively, of paracetamol were degraded at 120h (Figure 8).

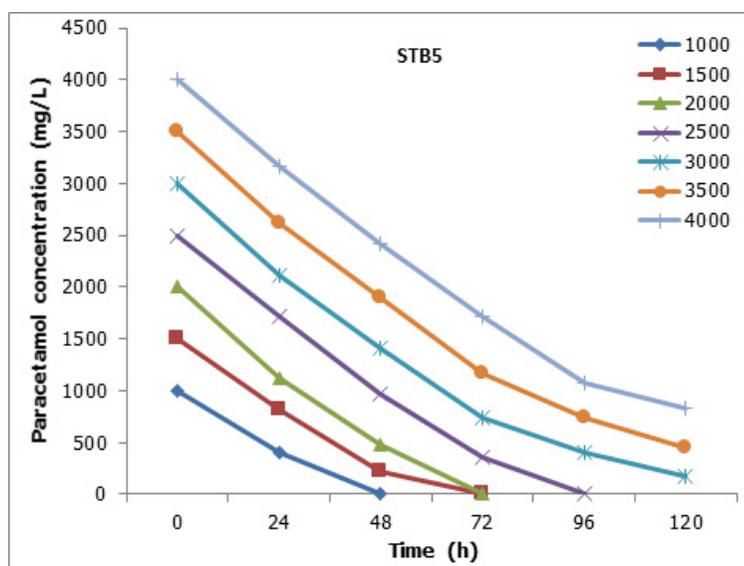


Figure 8: The paracetamol degradation by STB5.

The Kruskal-Wallis test showed that there is not a significant difference ($P > 0.01$) in the biodegradation efficiency between the STB1, STB3, and STB5 strains.

Effect of Glucose on Paracetamol Biodegradation

From the present results, the glucose was found to be accelerated the paracetamol biodegradation by all isolated bacterial strains. It was found that the STB1 was degraded 100% of 2500 mg/L of paracetamol when added 5 g/L of glucose to the media. Without glucose, the STB1 was degraded 75.40% of 2500 mg/L of paracetamol within 72h (Figure 9).

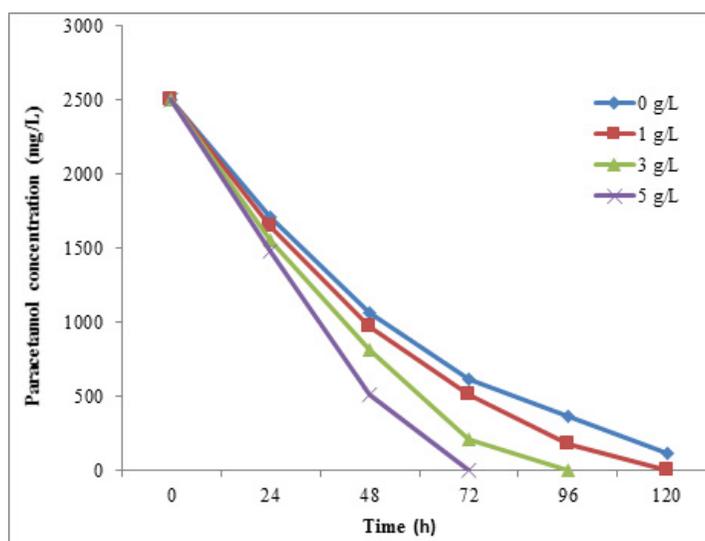


Figure 9: Effect of glucose on paracetamol biodegradation by STB1.

However, the results showed that the paracetamol biodegradation was increased with increasing the glucose concentration for BTS3. At 5 mg/L of glucose, 93.92% and 100% of 2500 mg/L paracetamol were degraded by BTS3 and STB5 strains, respectively, within 72h (Figure 10 and Figure 11). Also, the Friedman test showed a significant difference ($P < 0.01$) between the glucose concentration and biodegradation efficiency of all tested strains.

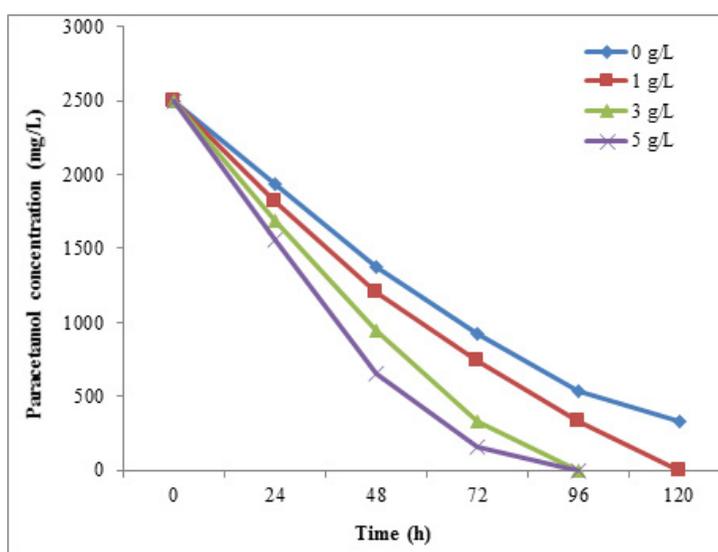


Figure 10: Effect of glucose on paracetamol biodegradation by STB3.

Effect of Bacterial Cells Concentration on Paracetamol Biodegradation

The results showed a different rate of paracetamol degradation with a different concentration of microbial cells. When the microbial concentration cells of STB1 were increased from 1.0×10^4 to 1.0×10^8 cells/mL, the paracetamol degradation was increased from 67.95% to 100%, respectively, after 48 h of incubation (Figure 12).

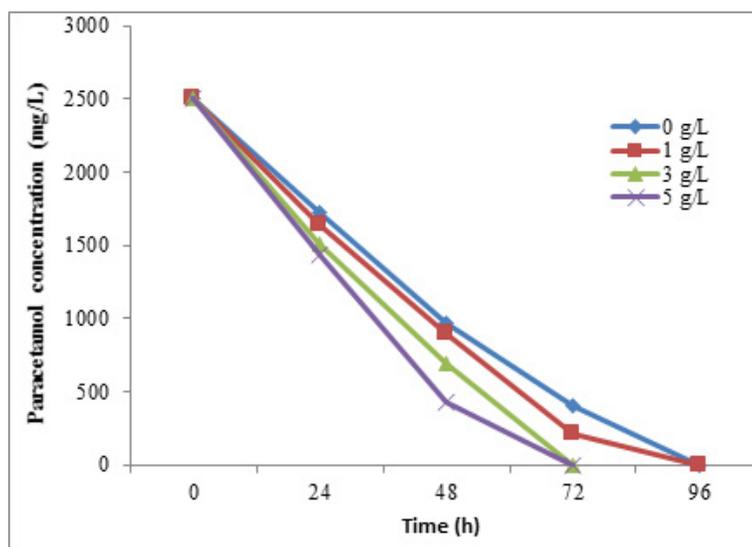


Figure 11: Effect of glucose on paracetamol biodegradation by STB5.

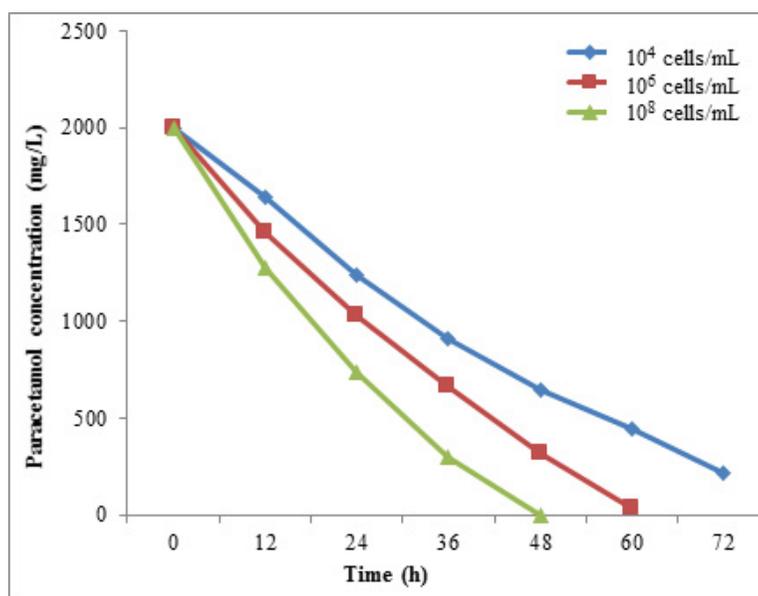


Figure 12: Effect of cells concentration on paracetamol biodegradation by STB1.

Furthermore, the Figure 13 showed that the STB3 degraded 67.85% of paracetamol at 1.0×10^4 cells/mL within 60h. In addition, the complete degradation occurred when microbial cells increased to 1.0×10^8 cells/mL. Also, the complete (100%) degradation of paracetamol occurred after 60 and 48h for the cell concentration 1.0×10^8 cells/mL STB5 (Figure 14).

However, the Friedman test showed a significant difference ($P < 0.01$) between the bacterial concentration and biodegradation efficiency of all tested strains.

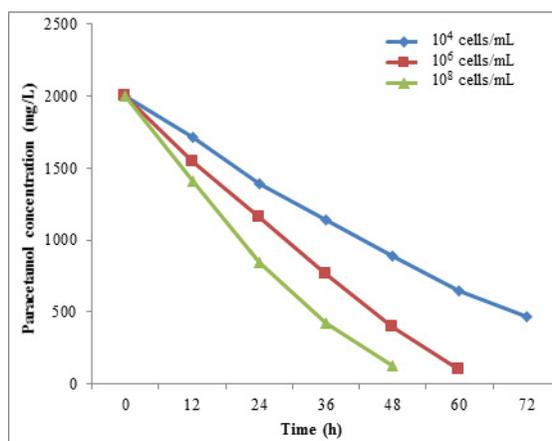


Figure 13: Effect of cells concentration on paracetamol biodegradation by STB3.

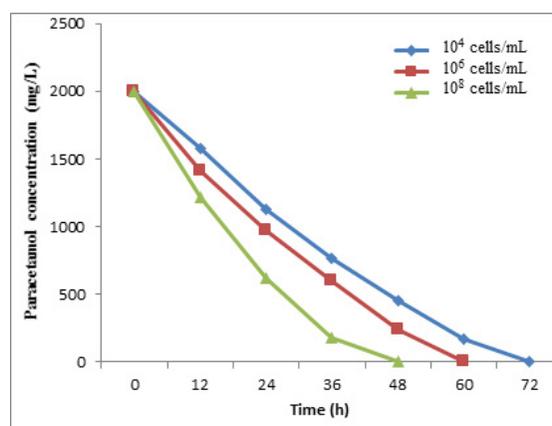


Figure 14: Effect of cells concentration on paracetamol biodegradation by STB5.

Discussion

Biodegradation is an important role in removing paracetamol from the environment. The results of the present study indicate that the site contaminated with the pharmaceutical effluent containing paracetamol is rich with variety microorganisms which tolerate and use it as the sole source of carbon and energy for growth. Three bacteria strain signed as an STB1, STB3, and STB5 were isolated from this contaminated site. The STB1 strain was identified as *Pseudomonas aeruginosa* strain DSM 50071 with similarity 92%, while the STB3 and STB5 were identified as *P. aeruginosa* strain NBRC 12689 with similarity 89% and 99%, respectively.

In similar, several reports were recorded that the *Pseudomonas* species degrading paracetamol were successfully isolated from a different site contaminated with pharmaceutical wastewater containing paracetamol [15,22,23]. The pH plays a vital role in biodegradation and gives an understanding of degradation process of paracetamol substance. The optimum pH for paracetamol degradation was found at 7.0 for all bacteria stains.

This result coincides with Hu., *et al.* [22] who revealed that the paracetamol (1200 mg/L) was completely degraded by *P. aeruginosa* strain HJ1012 at around pH 7.0 on 48h. Also, the optimum pH for the *Pseudomonas* sp. growth on media was recoded to be between 7.0-8.0 [14, 23]. A reduction in paracetamol biodegradation in more acidic and alkaline media is due to the extreme pH levels that inhibit the bacterial cells growth.

The temperature considers a significant role in microbial growth rate and can influence the degradation of organic compounds through direct effects on enzyme activity [24]. In this study, the effect of temperature was found on bacterial strains and 30°C was shown to be the most effective temperature for paracetamol biodegradation. This result is in agreement with the findings Hu., *et al.* [22] who found that the optimum temperature was appeared to be at around 30°C for paracetamol biodegradation by *P. aeruginosa* strain HJ1012.

Furthermore, a study by Fang., *et al.* [14] and Yun., *et al.* [23] observed that the optimum temperature for the *Pseudomonas* sp. growth on media was recorded at 30°C. It was documented that growth and metabolic activity of *Pseudomonas* sp. Ap-3 was maximum at 30°C [25]. Some of the experimental studies on the paracetamol biodegradation by *Pseudomonas* sp. were carried out at a temperature of 30°C and pH of 7.0 [22].

The present study was detected that the degradation rate of paracetamol was affected by cell concentrations which play a significant role in transport elements. These findings are similar to those of Akay and Tezel, [26] who revealed that the biotransformation of paracetamol was influenced at a different cell concentration of *R. erythropolis*. Biodegradation of organic substrates provides microorganisms with energy and building materials that are used for growth of new cells, cell maintenance and co-metabolism of other less degradable substances [27].

The result of this study was revalued that the biodegradation on paracetamol (2500 mg/L) in the presence of glucose was increased with increasing glucose amounts. A study by Ahmed., *et al.* [15] observed that the glucose enhance the *Pseudomonas* sp. strain ST1 growth in media containing paracetamol (500-5000 mg/L). The presence of glucose provides a source of carbon and energy for the microbes, thus enhancing their activity to utilize the resistant aromatic amines [28].

The result showed that the STB1 and STB5 could perform complete degradation of paracetamol at concentrations of 1500 and 2000 mg/L or below, respectively, within 72h. Whereas the STB3 was reported to degrade 93.46% of 1500 mg/L paracetamol after 72h of incubation. At 4000 mg/L, 68.6%, 51.3% and 79.4% of paracetamol concentration were degraded by STB1, STB3, and STB5, respectively, within 120h of reaction. In a similar study by Ahmed., *et al.* [15] who reported that the isolated *Pseudomonas* sp. strain ST1 was able to degrade 76.8% of 4000 mg/L paracetamol within 72h.

Another study by Fang., *et al.* [14] who observed that the *Cupriavidus* sp. strain F1, *Lysobacter* sp. strain F2 and *Pseudomonas* sp. strain (Figure 2) were able to degrade paracetamol up to 400 mg/L, 2500 mg/L, and 2000 mg/L, respectively. In a similar study by Hu., *et al.* [22] who reported that the *P. aeruginosa* strain HJ1012 could completely degrade paracetamol as high as 2200 mg/L within 75 h of reaction.

A study by Zhang., *et al.* [29] who recorded that the *Stenotrophomonas* sp. strain (Figure 2) and *Pseudomonas* sp. strain (F1 and F2) were able to complete degradation of paracetamol at concentrations of 2000, 400, and 2500 mg/L or below in 116h, 60h, and 45h, respectively. Also, it was reported that the *Pseudomonas* sp. degraded 96% of 1500 mg/L paracetamol in 12h [23]. *Pseudomonas* species are environmental organisms documented for their ability to degrade aromatic compounds of environmental concern [30]. The adaptability of *Pseudomonas* species to different organic substrates makes it an attractive organism for its use in biodegradation for wide ranges of organic substances that present in wastewater.

Conclusion

Pharmaceutical wastewater is the most potential sources to isolate microbes that degrading paracetamol. The isolated bacterial strains were showed a high degradation efficiency of paracetamol and could tolerate paracetamol at 4000 mg/L. The rate of paracetamol degradation was also affected by initial pH values, temperatures, and a glucose concentration of culture medium. Therefore, *P. aeruginosa* may prove to be important in bioremediation and wastewater treatment. A further investigation on paracetamol metabolic pathways involved in biodegradation, identification, and isolation of genes responsible for producing degradative enzymes and sequencing and cloning of such genes may increase further knowledge about the abilities of these bacterial strains towards biodegradation process.

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