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Bioremediation of Waste Water from Pharmaceutical Industry by Bacteria (*Bacillus subtilis*)

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Abstract

This work aimed to characterize pharmaceutical waste generated in Sam Ace Pharmaceutical in Ede, Osun-state, and assess wastewater treatment plant performance by bacteria (Bacillus subtilis) and the feasibility of wastewater reuse. Freshly discharged pharmaceutical wastewater was collected and analyzed for the physicochemical parameters such as Biological Oxygen Demand (BOD), Chemical Oxygen Demand (COD), Total Dissolved Solid (TDS), Nitrate, Phosphate, Magnesium, Calcium, Zinc, and Copper by standard methods. 190ml of the sterilized FPWWS was inoculated with pharmaceutical wastewater for two weeks and determined the physicochemical parameters at a 7-day interval. The results observed for raw, bio-treated and removal efficiency showed: BOD(200 mg-1, 90 mg-1 , 45 mg-1 and 55%, 75.5%), COD(395 mg-1, 330 mg-1, 150 mg-1 and 16.46 %, 62.03%), TDS(11200 mg-1, 250 mg-1, 130 mg-1 and 79.17%, 89.17%), Nitrate (165 mg-1, 88 mg-1, 43 mg-1 and 46.67%, 73.94%) phosphate (31 mg-1, 18 mg-1, 6 mg-1, and 41.94%, 80.65%), magnesium (75 mg-1, 55 mg-1, 17 mg-1, and 22.67%, 77.33%), calcium (80 mg-1, 57 mg-1, 25 mg-1, and 28.75%, 68.75%), zinc (0.05 mg-1, 0.03 mg-1, 0.001 mg-1, and 40%, 98.6%), copper (0.06, 0.02, 0.001) Bacteria (Bacillus sp.) showed a potential removal of pollutants and other wastes from the fish pond wastewater.

1. Introduction

The vitality of water is well known throughout the world and the issue of sustainable water management is a critical issue of discussion in all sections of society, but the water resources are still at the risk of either being depleted or polluted raising an alarming situation (Ahmad et al., 2021). The reasons behind this overwhelming condition are the tremendous increase in population, industrialization, urbanization, and economic growth (Abdel-Raouf et al., 2012). Pollution of surface or groundwater bodies or the mother environment is caused mainly by human activities increasing the concentrations of the substances (natural or synthetic) above their prescribed limits which may cause harm to the humans and environment (Sousa *et al*., 2018). Environmental pollution is one of the major challenges of today's civilization (Spina *et al*., 2012). Industrial wastewater presents a potential hazard to the natural water system (Deepali 2012). This wastewater contains many inorganic and organic matters, which are toxic to the various life forms of the ecosystem (Spina *et al*., 2012). Several research investigations have shown the widespread occurrence of these pollutants in wastewater, surface water, and groundwater (Debska *et al*. 2004). The increasing pollution load of pollutants from industrial water streams has also caused great harm to the rivers, posing major health risks on either direct bathing or drinking in the river water (Seth *et al*. 2013). Environmental pollution caused by industrial effluents results in adverse effects on the general health of the workers, as well as the habitants, who live near the chemical synthesis industries and farmers along with field workers (Asamudo *et al*., 2005). Worldwide growth and expeditious industrialization have led to the recognition and increasing understanding of the interrelationship between pollution, public health, and the environment. Presently, 3.4 million people die each year in the world from waterborne diseases owing to rapid industrialization (Rajaram and Das, 2008). Surface water is the main source of industries for wastewater disposal (Kar *et al*., 2008). Untreated or allegedly treated industrial effluents have enhanced the level of surface water pollution up to 20 times the safe level in 22 critically polluted areas of the country. It is found that almost all rivers are polluted in most stretches by some industries (Lokhande *et al*., 2011). The level of wastewater pollution varies from industry to industry depending on the type of processes and the size of the industries. Currently, there is worldwide concern about the presence in the surface, marine, and underground waters of substances such as surfactants, plasticizers, additives, surfactants, epoxy resins, pharmaceutical, and personal care products (PPCP), and different chemical compounds, which have been named as emerging pollutants (EP). Known effects of some of these compounds include alteration of the endocrine system in animals, estrogenic and antiandrogenic effects in rats, complications in pregnancy, affecting gill and kidney tissue in freshwater fish, affecting the immune system of the seal, and resistance to bacterial pathogens to antibiotics (Petrie *et al*., 2015). Wastewater is generated from three major sources *i.e.*, industrial, agricultural, and municipal which contain pollutants such as xenobiotics, microplastics, and heavy metals and are augmented by a high amount of carbon, phosphorus, and nitrogen compounds (Ahmad et al., 2021). Wastewater treatment is one of the most pressing issues since it cannot be achieved by any specific technology because of the varying nature and concentrations of pollutants and the efficiency of the treatment technologies (Eerkes-Medrano et al., 2019). The degradation capacity of these conventional treatment technologies is limited, especially regarding heavy metals, nutrients, and xenobiotics causing the accumulation of these substances in water bodies (Farmer, 2018). Pharmaceuticals include any substance or mixture of substances for use in the diagnosis, treatment, mitigation, or prevention of a disease, disorder, abnormal physical state, or its symptoms in human beings or animals (Enick & Moore, 2007). Thus, these products include a wide range of structures, functions, behaviors, and activities. The presence of pharmaceutical residues in the environment was reported for the first time in the late 1970s (Jones *et al*., 2005). Most of the pharmaceuticals produced ultimately make their way directly or indirectly into the environment polluting the flora and fauna to different extents (Singh et al., 2017). It has been shown that pharmaceuticals are introduced into the environment by a diverse range of pathways. The main one is human excretion after consumption. When a medication has expired; people dispose of it in a toilet or the garbage (Ternes et al., 2002). Leaching from landfill sites to groundwater has been demonstrated in some studies (Jones *et al*., 2004). Often the pharmaceuticals pass into sewage and end up in water bodies without any treatment (Singh et al., 2017). Wastewater treatment systems implement physical, chemical, or biological mechanisms, and are the quintessential alternative for the elimination of organic and inorganic compounds. However, the removal rates determined for pharmaceutical products vary widely, as removal efficiency depends on the compound's physicochemical properties, reactor design, and operating conditions (Wang and Wang, 2016). It is considered that wastewater treatment systems should be improved to remove Emerging Pollutants. Some authors show that the use of aerobic, anaerobic, or anoxic biological treatments can influence the removal of substances such as ibuprofen, and naproxen, among others, with effectiveness processes between 20 and 80 %, as well as, they remain efficient in the removal of organic matter, nutrients and solids above 80 % (Alvarino *et al*., 2018). Biological treatments attract attention as degradation processes where microorganisms such as bacteria, algae, and fungi are used that can mineralize high molecular weight molecules and lead them to simple compounds such as water or carbon dioxide, by using pollutants as a source of carbon or energy, inducing the production of enzymes for their assimilation, in addition to having the ability to tolerate the toxicity of different substances (Ahmed *et al*., 2017). The microorganisms find the appropriate characteristics in terms of tolerance and consumption of pollutants, metabolic routes, and proteins. A bioprospecting process must be a systematic search like microorganisms by classical or modern laboratory techniques, such as the use of metagenomics, which make it possible to take advantage of the metabolic potential, as in this case, to remove pollutants and reduce the impact on ecosystems (de Pascale *et al*., 2012). Biological degradation processes based on microorganisms have always been a viable alternative for the treatment of different pollutants (Wang and Wang, 2016). However, the pharmaceutical industry presents variability of substrates taking into account the different production lines, which affects the removal of organic matter that may have values above the regulations. There is a lack of microbial isolates that can be used in bio-augmentation processes to increase degradation rates, together with the study of their enzymatic activities that allow improving the treatment systems and tolerate the changes that occur according to the process of manufacture. The current study focuses on the use of bacteria (*Bacillus sp*) for remediation of wastewater collected from Same Ace pharmaceutical company, Ede, Osun State

2. Methodology

2.1 Sample Collection

Raw pharmaceutical wastewater samples were aseptically collected in laboratory clean containers from Sam ace pharmaceutical in Ede, Osun-state. The samples collected were then corked and transferred to the laboratory for analysis after 1-2hrs of sample collection. The organism used in this study was *Bacillus subtilis* obtained from the Biology Unit, Department of Applied Science, Osun State College of Technology, Osun State.

2.2 Sterilization of Apparatus

All apparatus used in this study were thoroughly washed with detergent, rinsed with water, air-dried, and sterilized in a hot air oven at 160^oC for two hours. Materials such as the mouth of the test tube, inoculating loop, and inoculating needle were sterilized by flaming with a bursen burner before and after inoculation to prevent contamination.

2.3 Determination of Physicochemical Characteristics of Waste Water Samples

The Physico-chemical parameters of wastewater from Sam ace pharmaceutical in Ede; Osun-state were analyzed immediately using standard analytical procedure (APHA, 1998). The Physico-chemical parameters analyzed include; Biochemical oxygen demand (BOD), chemical oxygen demand (COD), Total dissolved solids, Nitrate, Phosphates, Magnesium, Calcium, Zinc, and Copper were also analyzed. The procedures involved in carrying out the Physico-chemical processes are discussed below:

2.3.1 Biochemical Oxygen Demand

To determine the biological oxygen demand (BOD), two 100 ml bottles were obtained with lids and cleaned well. 25 ml sample was taken in each bottle and 75 ml of distilled was added to the two bottles and were tightly closed. One bottle was kept in the incubator at 20-22°C for 5 days. The 10 ml of Manganese sulfate solution and 2 ml of alkali- iodide solution were added to the other bottle below the surface of the liquid by using a syringe. Thereafter the bottles were closed and mixed by inverting the bottle several times.

When the precipitate settles it leaves a clear supernatant above, the precipitate was shaken again slowly by inverting the bottle, and when the setting has produced at least 50 ml supernatant 8 ml of conc. H_2SO_4 was added. The bottle was closed and mixed by gentle inversion until dissolution was completed. 100 ml of the sample was titrated with 0.05M $Na_2S_2O_3$ solution until a pale yellow solution is reached, 2 ml of freshly prepared starch solution was added and titration continued until a blue color appeared. The procedure was repeated using 100 ml distilled water (blank) and this was repeated for incubated sample after 5 days. The BOD was calculated as follows:

BOD as mg/L = $16(V1 - V2)$

Where:

$V1$ = ml of $Na_2S_2O_3$ was used for the sample before incubation;

$V2$ = ml of $Na_2S_2O_3$ was used for the sample after incubation.

2.3.2 Chemical oxygen demand (COD)

COD analysis was performed using pre-packaged mercury-free and premixed COD vials based on Section 5220 of *Standard Methods* (APHA, 1998, 2012). Three types of COD vials with the ranges 5- 150, 20-900, and 100-4,500 mgCOD/L were used accordingly. A COD reactor was preheated to 150°C before testing.

During every test, a 2.5 mL sample was carefully added to one COD vial of ranges 5-150 or 20- 900 mgCOD/L, and a 0.5 mL sample was carefully added to one COD vial of range 100 4,500 mgCOD/L. Then, the vial was thoroughly shaken by hand. COD standards and a DW blank were processed the same as the samples. COD vials containing the sample, COD standard, and blank, were heated in the COD reactor for 2 h at 150±2°C, and then they were removed from the reactor and placed in a rack until they cooled and any suspended precipitate in the vials settled down.

After the outsides of vials were wiped to remove dust, the vials were placed into the Orbeco Hellige MC500 Multi-Parameter Colorimeter one by one, to measure their COD concentrations under a standard curve covering the expected range of sample concentrations. The wavelength of 440, 600, and 600 nm was set for the ranges 5-150, 20-900, and 100-4,500 mg COD/L, respectively. According to the requirements of the test method for using the COD vials, blanks of the ranges 20-900 and 100-4,500 mgCOD/L were used to set the zero in the colorimeter before sample testing.

2.3.3 Determination of Total Dissolved Solids

Total Dissolved Solids (TDS) for each water sample were determined using TDS meter (APHA, 1985).

2.3.4 Determination of Nitrate

The test tube was filled with the sample to the 20ml mark and one level spoonful (~ 1.5 ml) of nitratest powder (containing zinc dust 60% and barium sulphate 40%) and one nitratest tablet (ammonium chloride) were added and shaken for a minute. The tube was allowed to stand for a minute and was inverted 3-4 times to aid flocculation and was allowed to stand for two minutes to ensure complete settlement. The clear solution was dispersed into 10 ml mark and one nitricol tablet (Sulfanilic acid, acting as the aromatic amine), was added, crushed, and mixed to dissolve, then it was allowed to stand for 10 minutes for color development, and readings were taken on the Photometer (Wagtech) at 570 nm wavelength.

2.3.5 Determination of Phosphate

25ml of the sample was added to 0.5ml of ammonium molybdate and 2 drops of stannous chloride and mixed by swirling. A blue color developed within an hour and the intensity was measured using a spectrophotometer (21D) at 690 nm (APHA, 1998).

The concentration of the phosphate was calculated

Phosphate (mg/l) = A - B X C

Where; A = Absorbance of sample;

B = Absorbance of a blank sample,

C = Volume of standard phosphate

2.3.6 Determination of Magnesium

Ten ml of the sample was measured, a pinch of hydroxylamine hydrochloride was added and 5ml of mono-ethanol buffer or (buffer 10) was added, then two drops of Eriochrome black T indicator were added. This was titrated with 0.01 EDTA. The color changes from purple to blue-black.

2.3.7 Determination of Calcium

Ten ml of water sample was measured into a beaker; a pinch of potassium cyanide was added together with a pinch of hydroxylamine hydrochloride. Five ml of eight molar potassium hydroxides was added then a pinch of indicator (Putton and readers reagent) was added and titrated with 0.01M EDTA using a burette. Color changes from brown to green.

2.3.8 Determination of Heavy Metals (Zinc and Copper)

The following heavy metals; Copper (Cu), Zinc (Zn) were determined for each water sample using Test kits.

2.4 Experimental Set-Ups for conventional bioremediation of Pharmaceutical wastewater

To study the role of Bacteria (*Bacillus subtilis)* in pharmaceutical wastewater treatment method described by Adekanmi *et al*., (2020) was employed where they treated slaughterhouse wastewater with micro algae for a period of 14 days

- Pharmaceutical wastewater treatment wastewater + Bacteria (*Bacillus subtilis*)

The experiment was conducted and incubated under the same condition in 250 mL Erlenmeyer flask for a period of 7 and 14 days.

2.4.1. Inoculation and Sampling

- 10 mL of exponential growth of Bacteria (*Bacillus subtilis*) was inoculated into 250 mL Erlenmeyer flask containing 190ml of Sterilized Pharmaceutical wastewater samples. Samples were taken for physicochemical analysis at an interval of 7 days after inoculation.

3. Results and Discussions

3.1 Biochemical Oxygen Demand (BOD)

Biochemical Oxygen Demand (BOD) recorded in raw pharmaceutical wastewater is found to be lower (90 and 45 mg/L) after 7 and 14 days of bio-treatment compared with 200 mg/L obtained for raw pharmaceutical wastewater (Figure 1a). They had reduction efficiencies of 55 and 75.5 % after 7 and 14 days of biotreatment with *Bacillus subtilis* (Figure 1b). A high degradation rate during week two(day=14) could possibly be a result of the acclimatization of the microorganisms to the prevailing conditions High organic material present in pharmaceutical wastewater is an indication of higher BOD and COD. This is in conformity with the finding of del Pozo *et al*. (2003). This fact had a great influence on the rest of the parameters and the nature of the wastewater. Some information on the wastewater biodegradability can be gained by comparing different measures, for example, BOD and COD where a high ratio of BOD to COD shows a relatively high biodegradability whereas a low ratio indicates that the wastewater is more slowly biodegraded (Vollertsen and Hvitved-Jacobsen, 2002).

3.2 Chemical Oxygen Demand

The COD observed in this study showed that raw pharmaceutical wastewater was reduced to 330 and 150 mg/L respectively from the initial raw wastewater value of 395 mg/L after 7 and 14 days of treatment with *Bacillus subtilis* (Figure 2a) at removal efficiencies of 16.46 and 62.03 % respectively (Figure 2b). The rate of reduction of COD raw pharmaceutical wastewater confirms the effectiveness of the degradation process to reduce the pollutant load contained in the wastewater.

3.3 Total Dissolved Solid (TDS)

Total Dissolved Solid (TDS) recorded for raw pharmaceutical wastewater is 1200 mg/L (Figure 3a). TDS values obtained were generally beyond 1000 mg/l the upper limit set by WHO (WHO, 2011). The value was later reduced to 250 and 130 mg/L with removal efficiencies of 79.17 and 89.17% respectively after 7 and 14 days of treatment with *Bacillus subtilis* (Figure 3b). Chemical Oxygen Demand (COD) is considered the amount of oxygen consumed by the chemical breakdown of organic and inorganic matter.

3.4 Nitrate

The results obtained in this study showed a significant reduction of nitrate in raw pharmaceutical wastewater after bio-treatment with *Bacillus subtilis* for a period of 14 days with 88 and 43 mg/L on days 7 and 14 against 165 mg/L recorded for raw pharmaceutical wastewater (Figure 4a). The higher percentage reduction efficiencies were recorded after 7 and 14 days of bio-treatment (44.67 and 73.94 %) respectively (Figure 4b).

3.5 Phosphate

Phosphate and nitrate are among the prominent compounds in pharmaceutical wastewater. A relatively higher rate of phosphate decrease (18 and 6 mg/L Figure 5a) with reduction efficiencies of (41.94 and 80.65 % Figure 5b at day 7 and 14 respectively) was recorded in phosphate concentration after bio-treatment against the value observed for raw pharmaceutical wastewater 31 mg/L. High phosphate levels will result in the eutrophication of the river.

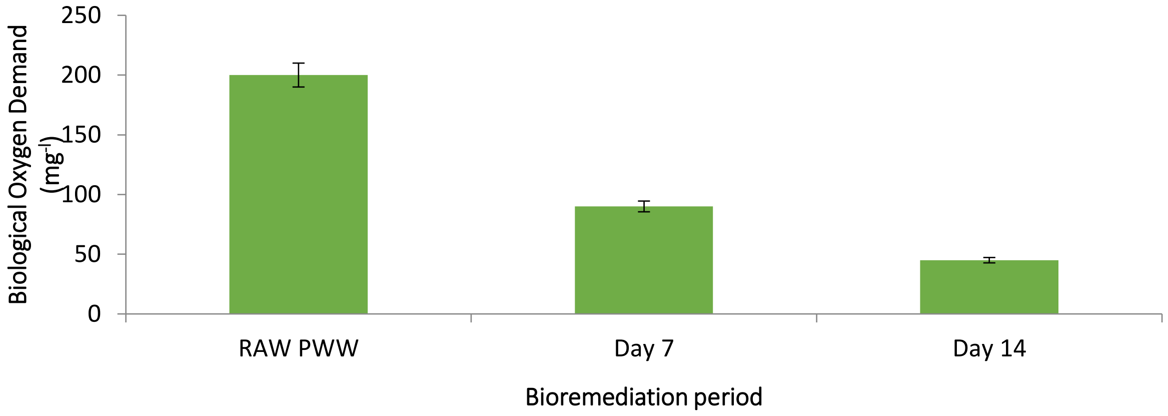

Figure 1a: Biological Oxygen Demand of the Bio-treated Pharmaceutical Wastewater with *Bacillus subtilis* after 7th and 14th day of treatment

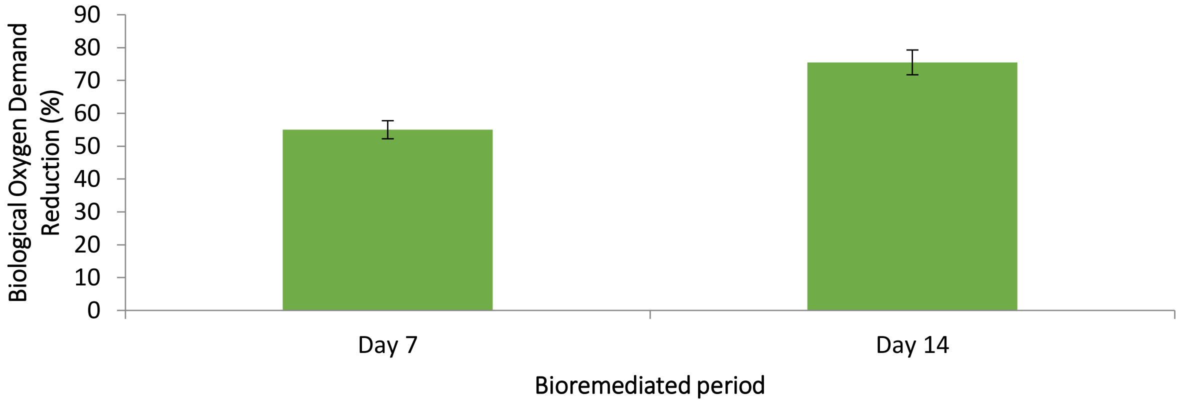

Figure 1b: Biological Oxygen Demand Removal efficiencies of Bio treated Pharmaceutical Wastewater after 7 and 14 days

Note: PWW= Raw Pharmaceutical Wastewater

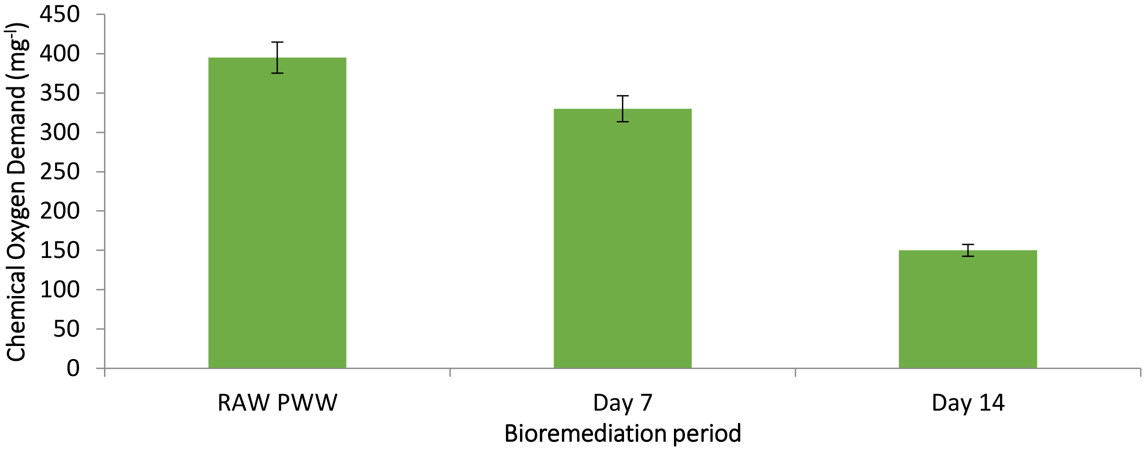

Figure 2a: Chemical Oxygen Demand of the Bio-treated Pharmaceutical Wastewater with *Bacillus subtilis* after 7th and 14th day of treatment

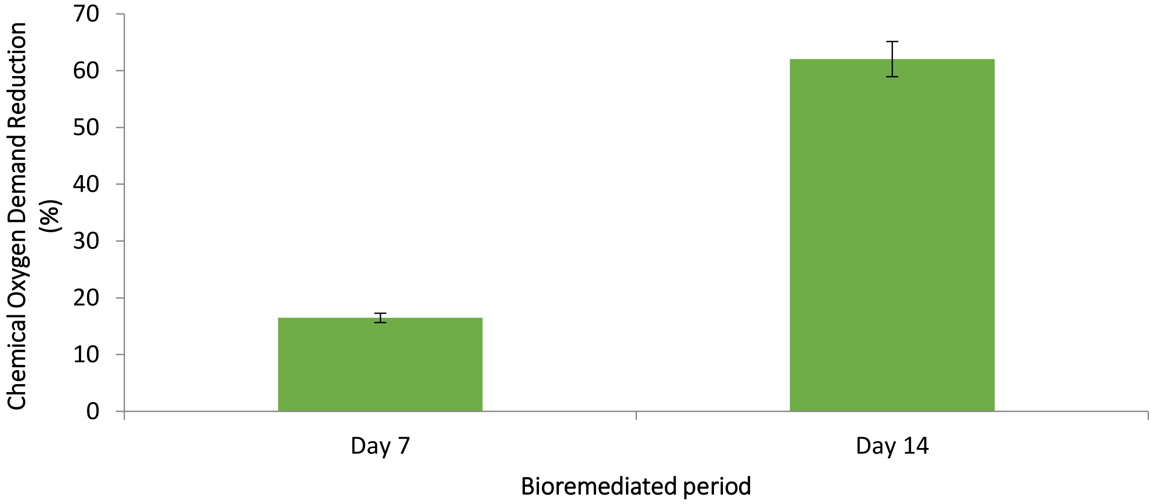

Figure 2b: Chemical Oxygen Demand Removal efficiencies of Bio-treated Pharmaceutical Wastewater after 7 and 14 days

Note: PWW= Raw Pharmaceutical Wastewater

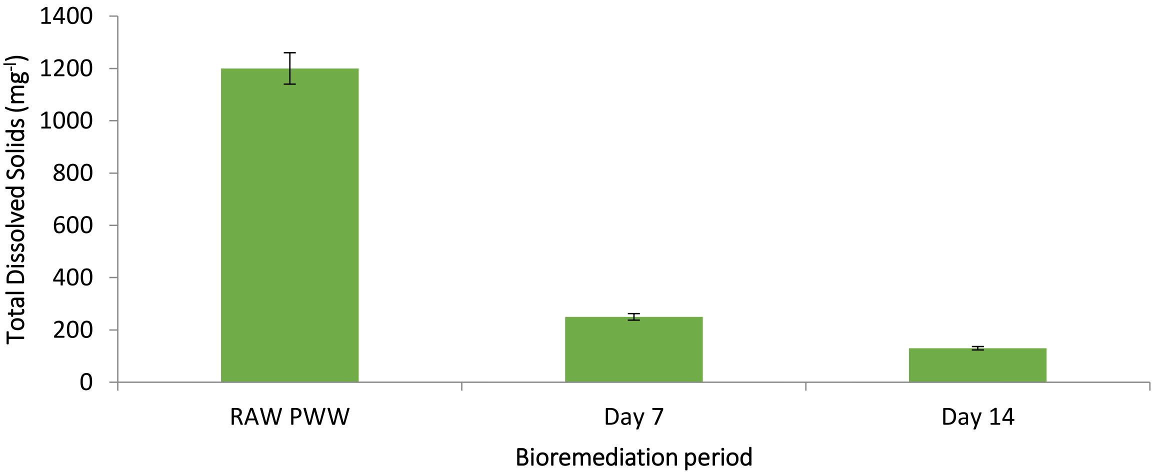

Figure 3a: Total Dissolved Solids of the Bio-treated Pharmaceutical Wastewater with *Bacillus subtilis* after 7th and 14th day of treatment

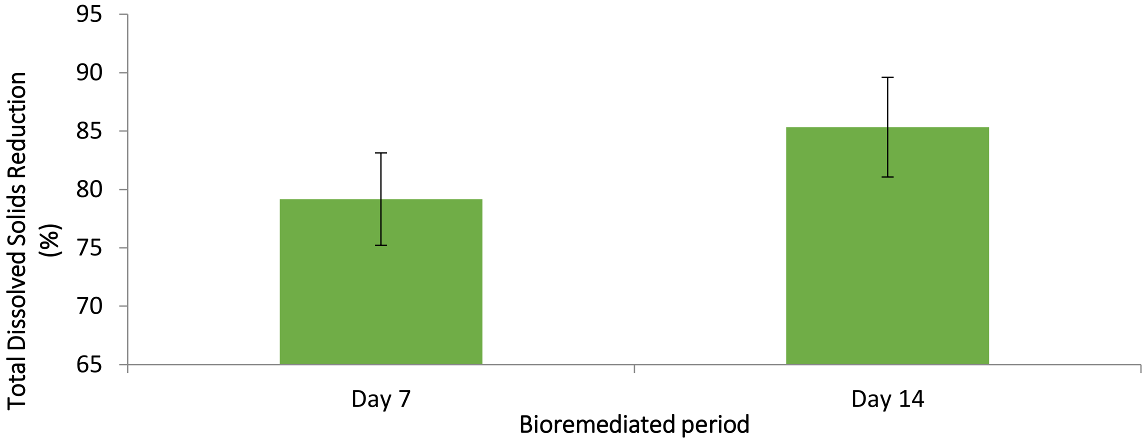

Figure 3b: Total Dissolved Solids Removal efficiencies of Bio-treated Pharmaceutical Wastewater after 7 and 14 days

Note: PWW= Raw Pharmaceutical Wastewater

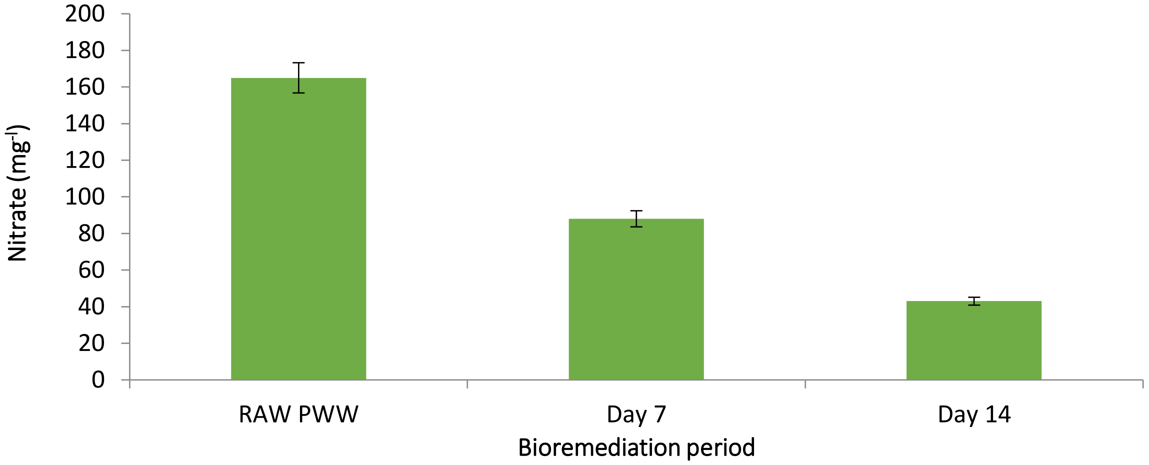

Figure 4a: Nitrate of the Bio-treated Pharmaceutical Wastewater with *Bacillus subtilis* after 7th and 14th day of treatment

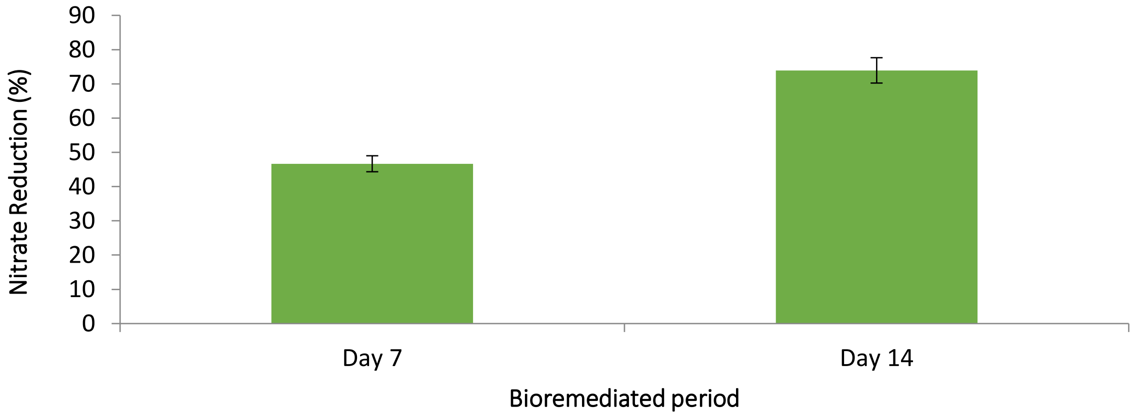

Figure 4b: Nitrate Removal efficiencies of Bio treated Pharmaceutical Wastewater after 7 and 14 days

Note: PWW= Raw Pharmaceutical Wastewater

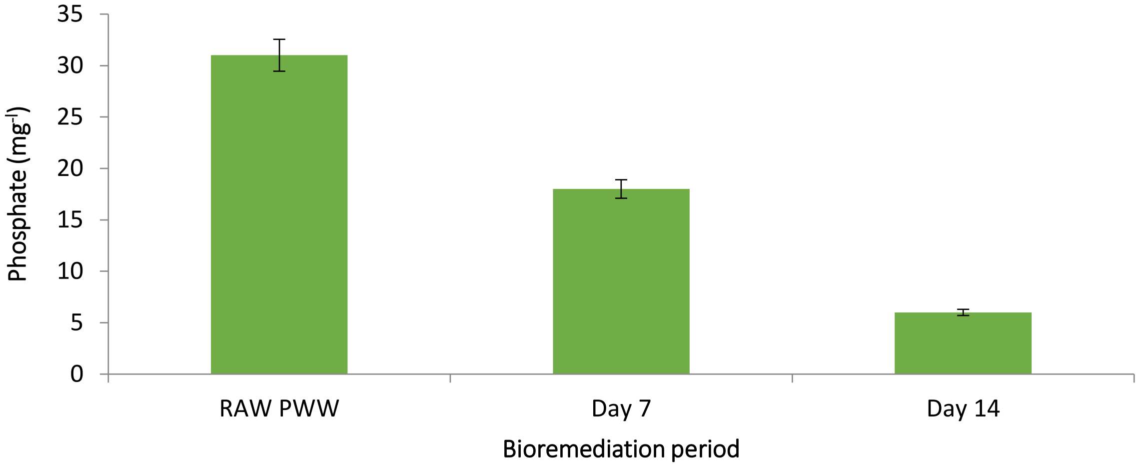

Figure 5a: Phosphate of the Bio treated Pharmaceutical Wastewater with *Bacillus subtilis* after 7th and 14th day of treatment

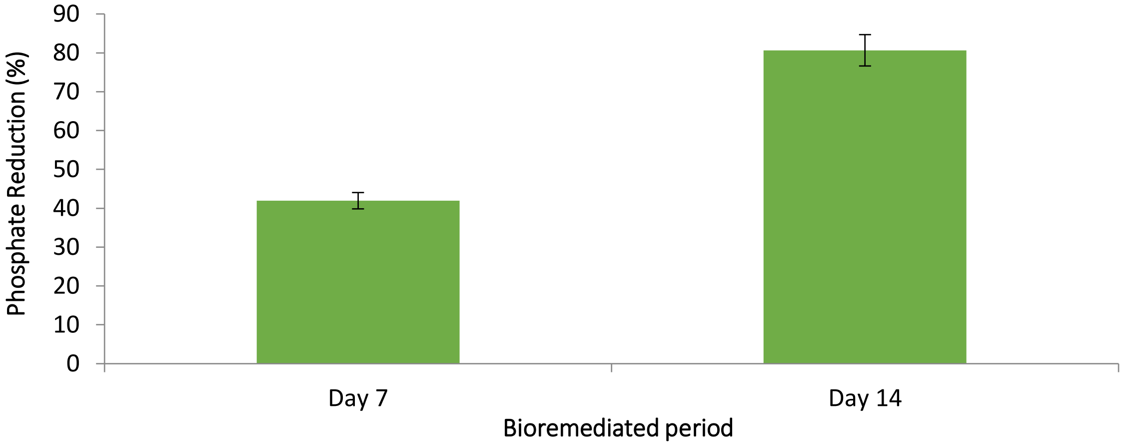

Figure 5b: Phosphate Removal efficiencies of Bio treated Pharmaceutical Wastewater after 7 and 14 days
Note: PWW= Raw Pharmaceutical Wastewater

3.6 Magnesium

The results obtained in this study showed a significant reduction of magnesium in raw pharmaceutical wastewater after bio-treatment with *Bacillus subtilis* for a period of 14 days with 55 and 17 mg/L on day 7 and 14 against 75 mg/L recorded for raw pharmaceutical wastewater (Figure 6a). The higher percentage reduction efficiencies were recorded after 7 and 14 days of bio-treatment (26.67 and 77.33 %) respectively (Figure 6b).

3.7 Calcium

The results of this study revealed a significant reduction of calcium in raw pharmaceutical wastewater after bio-treatment with *Bacillus subtilis* for a period of 14 days with 57 and 25 mg/L on day 7 and 14 against 80 mg/L recorded for raw pharmaceutical wastewater (Figure 7a). The higher percentage reduction efficiencies were recorded after 7 and 14 days of bio-treatment (28.75 and 68.75 %) respectively (Figure 7b).

3.8 Zinc

A relatively higher rate of Zinc decrease (0.03 and 0.001 mg/L Figure 8a) with reduction efficiencies of (40 and 98 % Figure 8b on day 7 and 14 respectively) was recorded in Zinc concentration after bio-treatment against the value observed for raw pharmaceutical wastewater 0.05 mg/L.

3.9 Copper

A higher rate of copper decrease (0.02 and 0.001 mg/L Figure 9a) with reduction efficiencies of (66.67 and 98.33 % Figure 9b at day 7 and 14 respectively) was recorded in copper concentration after bio-treatment against the value observed for raw pharmaceutical wastewater 0.06 mg/L.

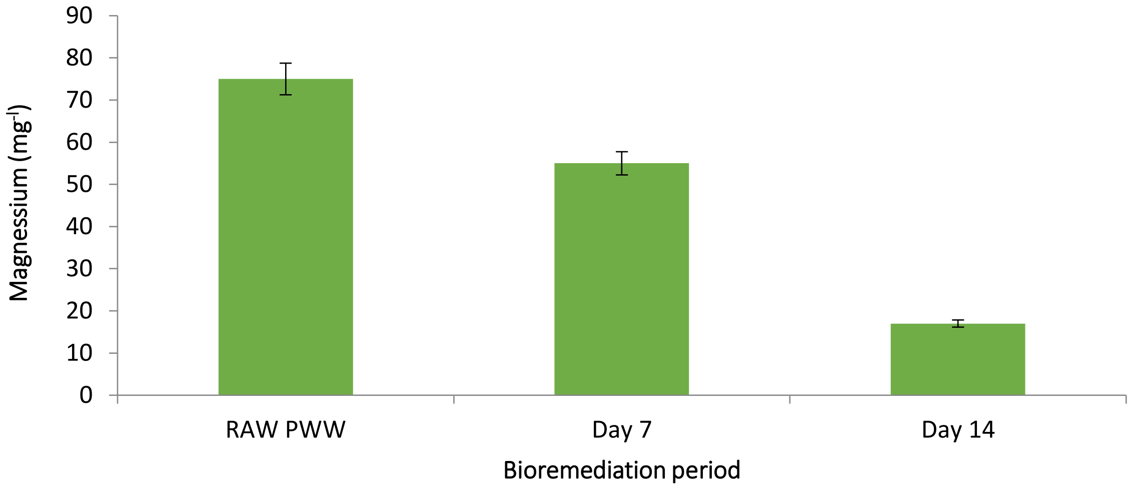

Figure 6a: Magnesium of the Bio-treated Pharmaceutical Wastewater with *Bacillus subtilis* after 7th and 14th day of treatment

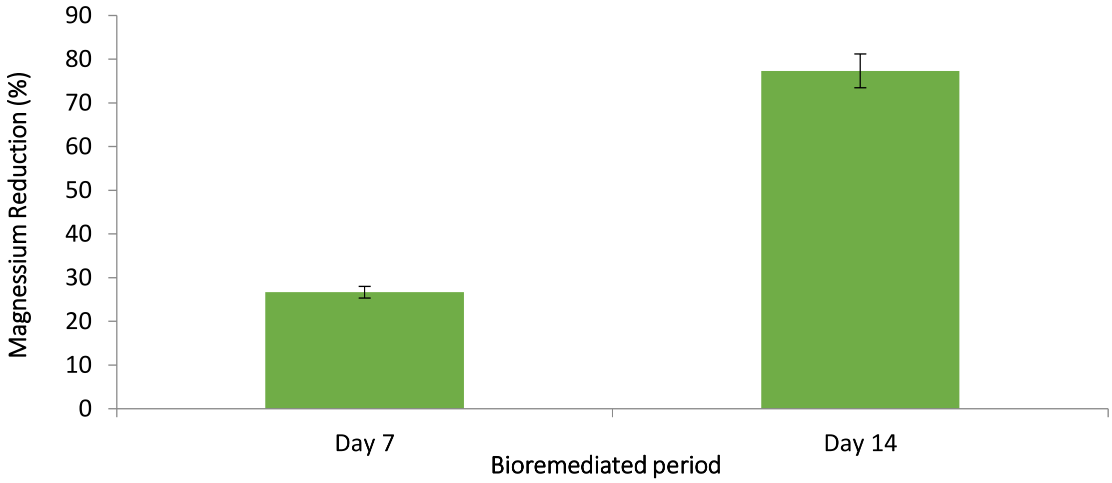

Figure 6b: Magnesium Removal efficiencies of Bio treated Pharmaceutical Wastewater after 7 and 14 days
Note: PWW= Raw Pharmaceutical Wastewater

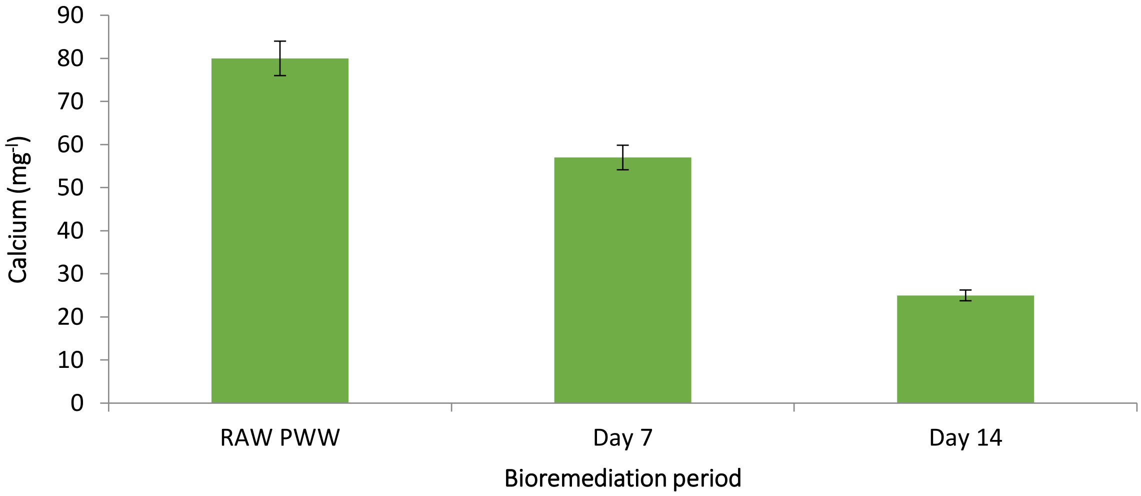

Figure 7a: Calcium of the Bio-treated Pharmaceutical Wastewater with *Bacillus subtilis* after 7th and 14th day of treatment

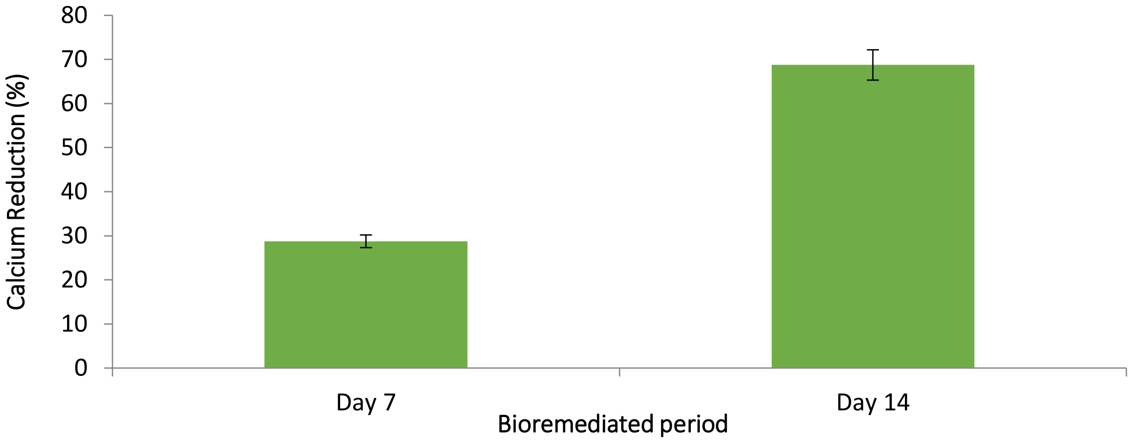

Figure 7b: Calcium Removal efficiencies of Bio treated Pharmaceutical Wastewater after 7 and 14 days
Note: PWW= Raw Pharmaceutical Wastewater

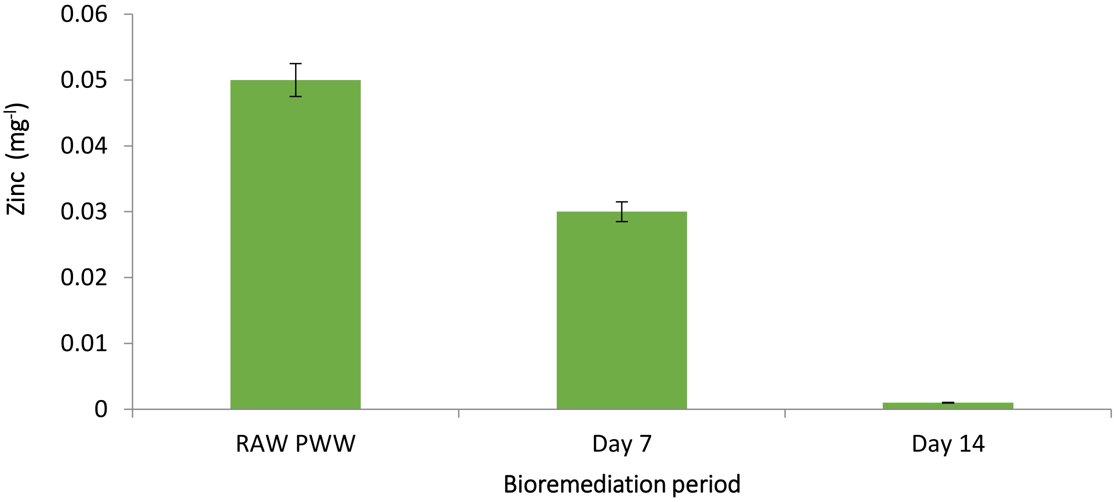

Figure 8a: Zinc of the Bio treated Pharmaceutical Wastewater with *Bacillus subtilis* after 7th and 14th day of treatment

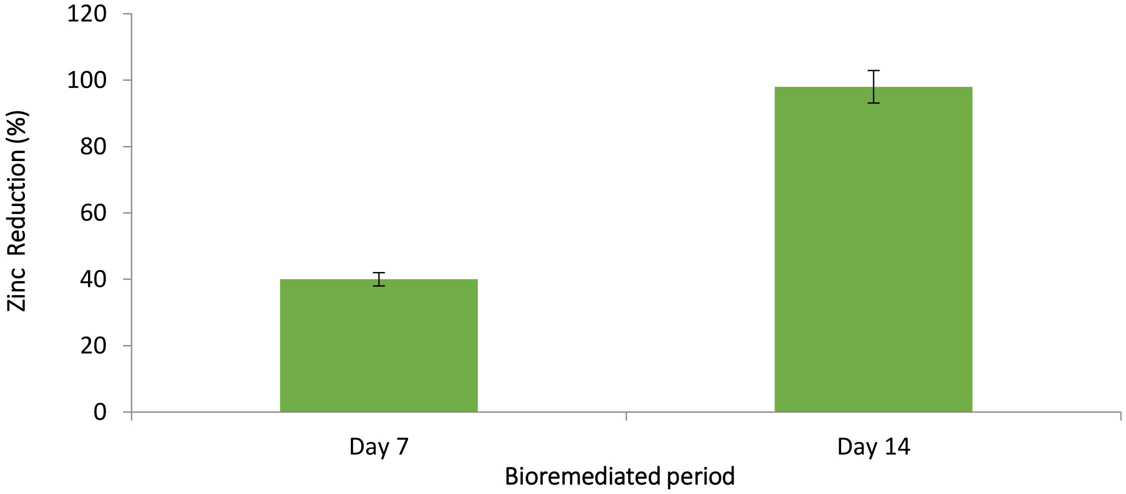

Figure 8b: Zinc Removal efficiencies of Bio treated Pharmaceutical Wastewater after 7 and 14 days
Note: PWW= Raw Pharmaceutical Wastewater

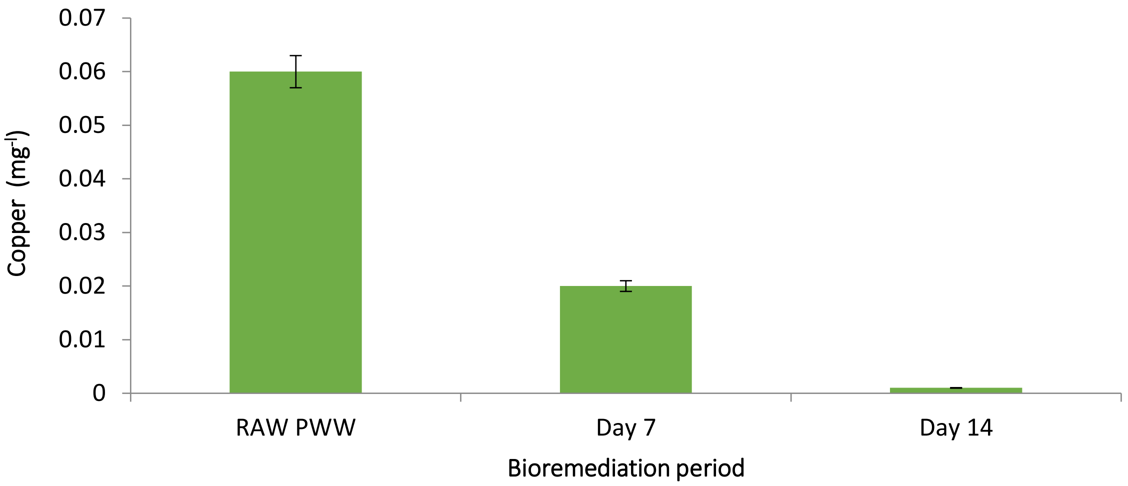

Figure 9a: Copper of the Bio treated Pharmaceutical Wastewater with *Bacillus subtilis* after 7th and 14th day of treatment

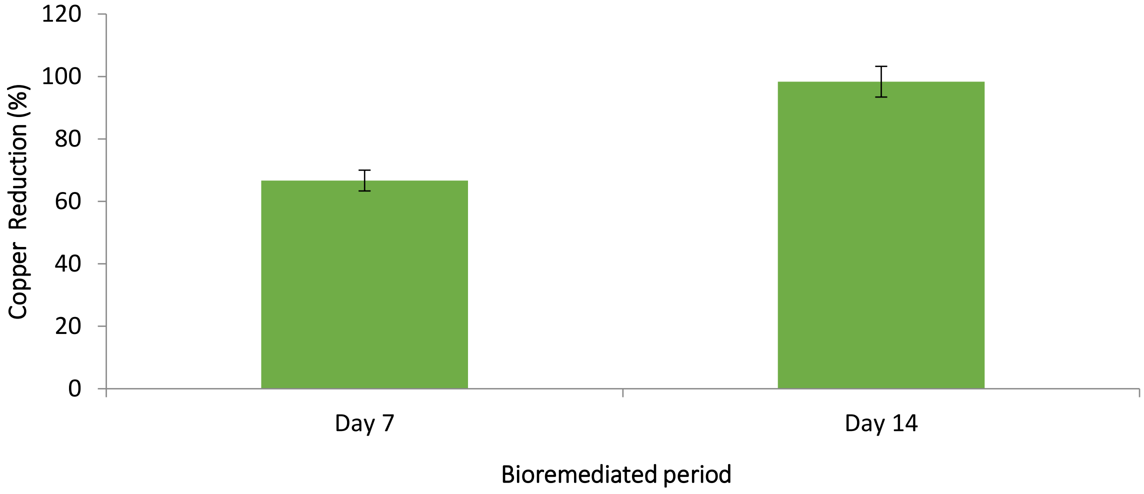

Figure 9b: Copper Removal efficiencies of Bio treated Pharmaceutical Wastewater after 7 and 14 days
Note: PWW= Raw Pharmaceutical Wastewater

4. Conclusion

In recent times very little attention was given to the environmental problem arising from pharmaceutical effluents so there was a constant need to find an alternative low-cost solution for the treatment of pharmaceutical effluent. Address bacterial isolate was evaluated for their bioremediation efficiency against pharmaceutical effluent taken from Sam ace pharmaceutical industry. In this project, we have collected raw effluents from the pharmaceutical industry and these effluents were transferred in plastic polythene bags and then examinations are conducted to detect the parameters like BOD, COD, TDS, nitrate, phosphate, magnesium, calcium, total hardness, Zinc, and copper. After that, the sample was inoculated with bacteria as detailed above. Then the results before treatment and after treatment were compared and have shown a drastic change in the degradation of the above-listed parameters. By examining all the effluents, we conclude that by applying the technique of Bio-remediation by using microorganisms there was a change in the degradation of effluent parameters. In conclusion, results confirmed that Bacillus sp. is effective species for BOD, COD, TDS, nitrate, phosphate, magnesium, calcium, total hardness, Zinc, and copper removal from the effluents of the pharmaceutical industries. Zn and Cu both are toxic heavy metals. Effluent discharge in water bodies can affect the living organism. The use of bacteria in the removal of environmental contaminants is beneficial since, due to their characteristics, they are a relatively simple model of a biological system that permits to conduct of multidimensional studies on adaptation, growth, behavior, and metabolic as well as growth regulation pathways and networks.

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