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Studies on Biodegradation of Acetaminophen by *Bacillus subtilis subsp. subtilis* NCIB 3610(T)

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Abstract

Acetaminophen (paracetamol) has been classified as one of the emerging organic pollutants due to its entrance into the water bodies. Removal of pharma micropollutants using microorganisms or synthetic systems from the environment is desirable. In this study, acetaminophen degrading microorganism was isolated from Pharmaceutical industrial wastewater. Identification of the isolate was carried out using morphological, biochemical, and 16s rRNA sequencing. *Bacillus subtilis* subsp. *subtilis* NCIB 3610(T) showed 73.2% degradation of paracetamol (2500 ppm) based on colorimetric and reverse phase high performance liquid chromatography analysis. Based on the Computer Assisted Kinetic Evaluation (CAKE) program, it can be concluded that the strain exhibits Simple first -order model (SFO). The degradative product was identified as 4-aminophenol based on High Resolution Mass Spectrometry method. The chemotaxis assay reported that the strain understudy was found to be suitable for the bioaugmentation purposes.

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4 Aminophenol; Acetaminophen; *Bacillus Subtilis*; Biodegradation.

Introduction

The presence of pharmaceutical contaminants in the aquatic environment has become a subject of emerging concern.[1] The pharmaceuticals contaminants comprising drugs, heavy metals, dyes, and personal care products have been considered emerging environmental contaminants due to their occurrence in water bodies.[2,3] Now, many of these compounds are perceived worldwide ranging from ng/L to µg/L but due to their biologically active potential, it could affect the ecosystem. With the increased production and consumption, micropollutants and personal care products are occurring in the environment.

One of the most frequently detected pharmaceutical painkillers in treated and untreated waste water is acetaminophen (APAP, paracetamol, N-acetyl-para-aminophenol).[4] Acetaminophen is a heavily demanded over the counter medicine. Paracetamol

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is prescribed as fever reductant, pain reliever, antipyretic, and analgesic drug.[5] Acetaminophen is continuously entered into the aquatic environment by many emission routes starting from industrial wastewater, customer use and clearance and hospital discharged water.[6,7,8] Wastewater treatment plants aim to remove of toxicants leading to the production of intermediates or end products that are highly ecotoxic related to the parent molecule.

The COVID-19 pandemic is predicted to have a big effect on the use of acetaminophen and subsequently its occurrence in the environment. At the wastewater treatment plants, advanced methods like electrochemical oxidation,[9] H2O2/UV, H2O2/Fe2+/UV oxidation,[10] ozonation,[11] and photo-catalysis methods[12] are used to remove paracetamol like drugs . Physico-chemical methods have high operation costs and give the possibility of the generation of toxic intermediate or end products. Biological treatment can be employed for the removal of pharma micropollutants to reduce the risk factors associated with other methods. For the biological methods, the selection of microorganisms based on their degradative potential, biodegradative pathway and cultivation conditions, is the first step that must be studied. This studies was aimed to isolate *Bacillus subtilis subsp.subtilis* and using it for the biodegradation of acetaminophen.

Materials and Methods

Chemicals used and Collection of Samples

The standard of Paracetamol was purchased from Loba Co. Ltd. The pharmaceutical wastewater was used for the isolation of acetaminophen degraders. This sample contains untreated industrial waste water. The five samples were kept in the sterile containers during transportation to the laboratory and then samples were stored at 4 °C. The samples were kept for the settling of heavy particles like sand, and other debris. The sediments were discarded before use and the upper layer of water was used for the further experiment.

Enrichment, Screening, Isolation, and identification of APAP-degrading Bacteria

APAP degradative strains isolation was carried out as per the protocol of Chopra and Kumar, with little modification.[13] APAP biodegradation test was performed in 250-mL Bushnell Haas Medium (BHM) containing acetaminophen and waste water sample and was incubated at room temperature for 3 days. The microorganisms were isolated by culturing on a nutrient agar plate and Bushnell Haas medium with acetaminophen (100 -3000ppm). The isolate showing maximum tolerance to acetaminophen, was used for further studies. The isolated bacteria were classified using the phenotypic and genotypic characteristics. The characterizations have been grounded on morphological, biochemical tests and 16s rRNA sequencing method for the confirmation.

Fermentation Studies

The isolate was inoculated in Bushnell Haas broth (P_H 7) containing APAP at a rate of 2500 ppm. The flasks were incubated at room temperature in dark conditions for 4 days with an intermittent determination of cell concentration (OD 600nm), PH and residual concentration of APAP using colorimetric method.[14] The standard dose response curve of paracetamol 100-1000 ug/mL was used as a reference for the calculation. The biodegradation of APAP was calculated by Eq. 1:

$$\text{Rate of degradation (\%)} = (C0\text{-}Ct)/C0 \times 100 \quad ...(1)$$

Where C0 is the preliminary concentration of APAP; Ct is the concentration of APAP after incubation at time 't'. Kinetic parameters during biodegradation were evaluated using Computer Assisted Kinetic Evaluation (CAKE) program.[34]

Reverse-phase HPLC Based Analysis

The fermented broth was centrifuged and the supernatant was collected. Solvent extraction was carried out using methanol. After solvent evaporation, the sample was processed for reverse phase HPLC. The elution of paracetamol was carried out with the assistance of reverse phase HPLC by isocratic mode, JASCO (250 × 4.6 mm, packed with 5 µ). The solvent system used was methanol: water (15:85) with 1 ml/min flow rate and monitored at 240 nm.

Identification of APAP Degradative Metabolite

The analysis of APAP biodegradative metabolite was carried out using High-Resolution Mass Spectrometry (HR-MS). Bruker Impact HD instrument was used for the mass analysis of the standard and its biodegradative metabolite extracted from the fermented broth. HR MS was done with conditions

of a double electrospray ionization source in positive ion mode, scanning range of 50 to 1200 m/ z, 4.0 spectra/sec of scan rate, 200°C source gas temperature, 7.01 l.min-1 gas flow rate and 10 µl sample injection volume. The Nebulizer pressure was adjusted to 1.7 bars. Data were analyzed with the assistance of Bruker compass data analysis 4.2 Software.

Screening of Biodegradation of Acetaminophen Bioaugmentation by Employing Chemotaxis Assay

The chemotactic behavior of the strain towards acetaminophen was tested qualitatively with the help of drop plate assay according to the procedure described earlier.[15] The medium was smeared with bacterial inoculum on the agar surface of medium. A pinch (5-10 mg) of acetaminophen crystals were placed in the well (6 mm) of the plate. The plate was incubated at room temperature for 48 Hrs. and bacterial growth near the chemical was recorded as a positive chemotactic response.

Results

Enrichment, Screening, Isolation, and Identification of APAP-Degrading Bacteria

Enrichment of acetaminophen degraders was done using Bushnell Haas Medium containing acetaminophen (100-3000mg/L) concentration. Bacterial Cultures showing maximum tolerance to acetaminophen were sub cultured on nutrient agar plates. A total of 23 isolates were obtained but isolates showing high APAP tolerance were further studied.

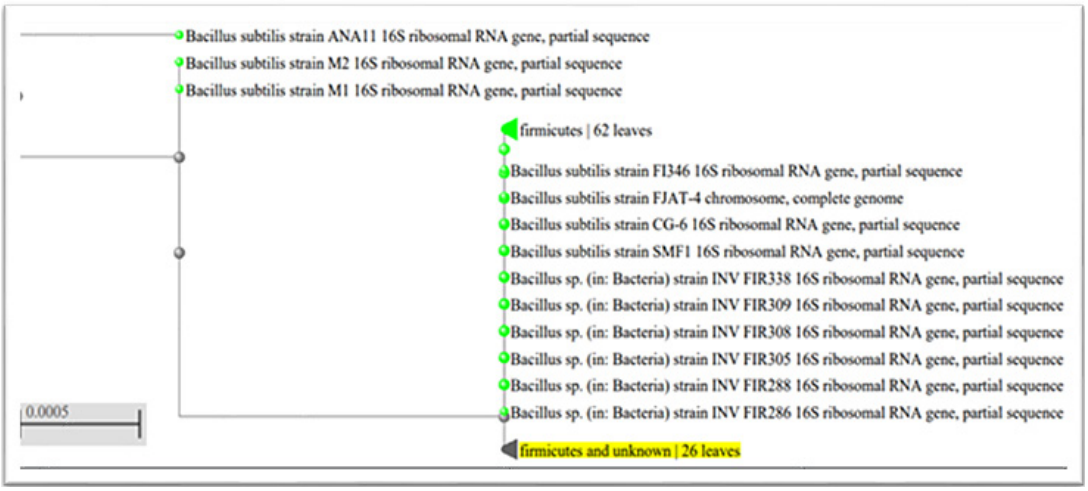
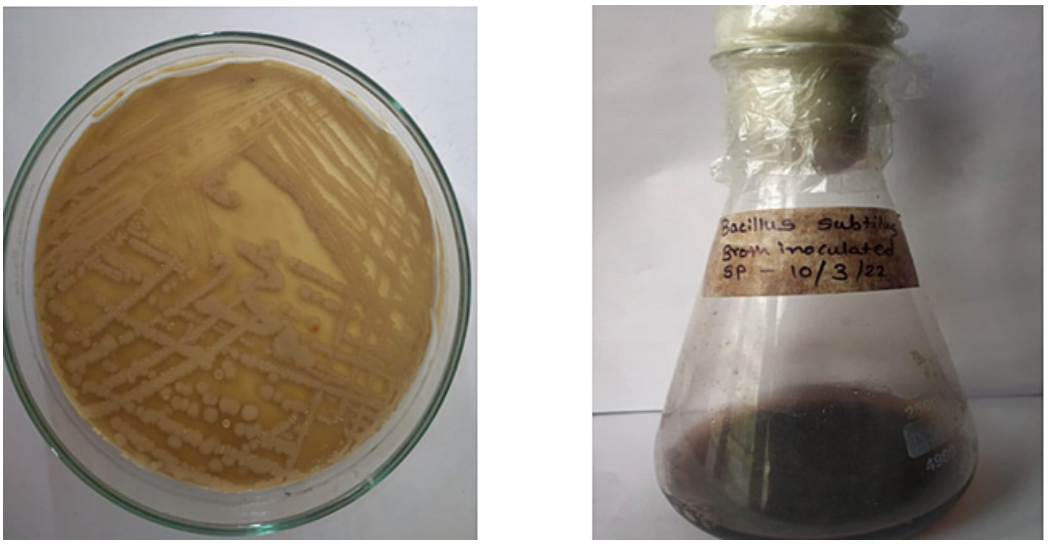


Fig. 1: Phylogenetic dendrogram using neighbor - joining analysis based on 16srRNA sequence

The isolate showing tolerance to 2500 ppm concentration of APAP was found to be Gram-positive rod-shaped bacteria with cream-colored colonies. Based on the biochemical tests, isolate found to be catalase positive, glucose fermentative, methyl red positive and non-spore forming. Molecular identification was done by 16S rDNA sequencing method. Isolate was found to be *Bacillus subtilis* subsp. subtilis NCIB 3610(T) with 99.86% similarity using EzBioCloud Database. Phylogenetic dendrogram (Fig.1) was plotted using the neighbor joining method.

Fermentation Studies

The isolate was inoculated in BHM medium (pH 7) containing acetaminophen (2500ppm) concentration and1% inoculum (10^9 cfu/ml).Flasks were incubated at room temperature for 04 days to evaluate the time course effect with the relationship between acetaminophen reduction associated with bacterial growth. With the increase in the incubation period, the broth showed brown to black coloration as shown in Fig.2.



2a **2b**

Fig. 2.2a: Growth of Bacillus subtilis on BH medium containing APAP. 2b: Bacillus subtilis fermented BH broth (2500 ppm APAP) showing black coloration due to aminophenol.

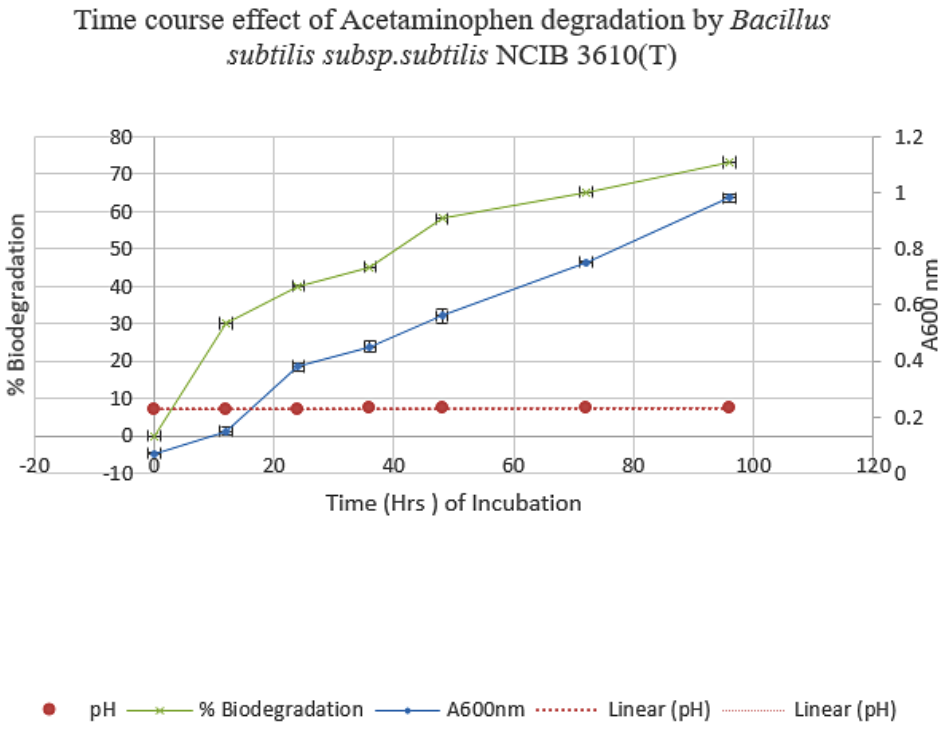


Fig .3: Time Course Effect of Biodegradation of Paracetamol by Bacillus Subtilis

The strain *Bacillus subtilis* degraded 73 % of acetaminophen with an increase in biomass and Ph as given in Fig.3. The specific growth rate of the *Bacillus subtilis* was calculated using Monod

model as 0.3499, when grown in the presence of acetaminophen. Kinetic parameters during biodegradation was evaluated using Computer Assisted Kinetic Evaluation (CAKE) program and results are given in Table.1.

Table 1: Kinetic parameters for APAP biodegradation by Bacillus subtilis

Model Kinetic	APAP concentration	K (d^{-1})	Chi-sq error	DT50 (days)	DT90 (days)
Simple First Order	2500 ppm	0.1	5.1	3.4	11.5

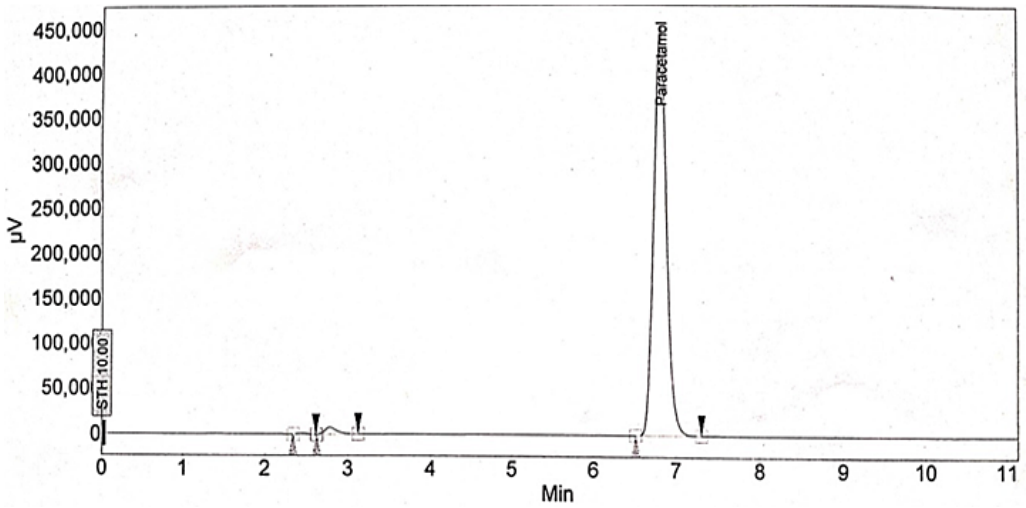


Fig.4a: HPLC Chromatogram of degradative metabolite extracted from Bacillus subtilis fermented broth (2500 ppm acetaminophen)

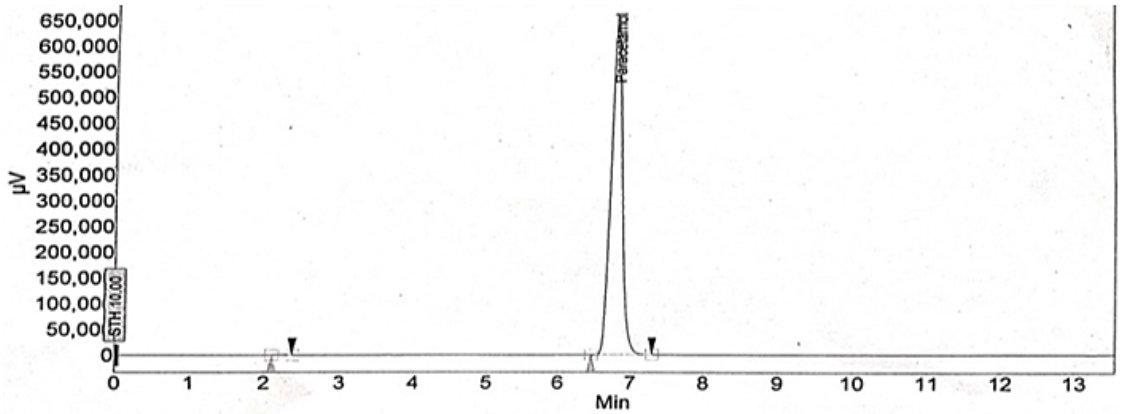


Fig 4b: HPLC chromatogram of standard acetaminophen (Paracetamol)

Reverse-phase HPLC- based Analysis

Reverse-phase HPLC was done with the standard paracetamol and biotransformed metabolite of paracetamol extracted from *Bacillus Subtilis* sp. fermented broth given in Fig.4a and 4b. In the HPLC chromatogram of the biotransformed metabolite

of paracetamol, alongwith the paracetamol (6.4 min retention time), one unknown metabolite was found with a retention time of 2.217 min. *Bacillus subtilis* degraded 73.4% acetaminophen (2500ppm) after 96 hours of incubation.

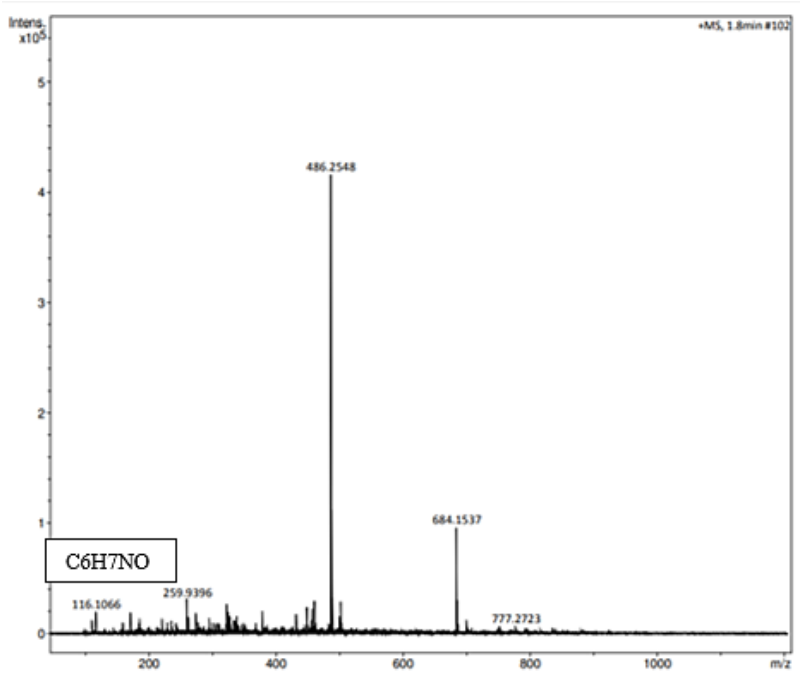


Fig.5: HRMS of Bacillus Subtilis mediated biodegradative metabolite of acetaminophen

Identification of APAP Degradative Metabolite

Methanol -extracted degradative metabolite of acetaminophen was identified using high -resolution mass spectroscopy. One peak of 116.10 of 4 aminophenol (C6H7NO) was identified as the biodegradative product of acetaminophen using Bruker compass data analysis 4.2 Software (Fig.5).

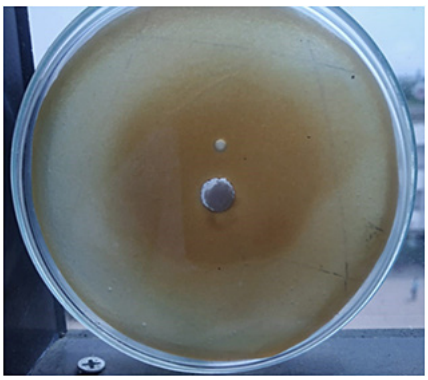


Fig. 6: Chemotaxis of *Bacillus Subtilis* towards acetaminophen

Screening of Biodegradation of Acetaminophen Bioaugmentation using Chemotaxis Assay

The positive - chemotactic behavior of *Bacillus subtilis* towards paracetamol was studied by using drop plate assay (Fig. 7). Drop plate assay result showed the development of bacterial ring near the crystals of acetaminophen after 48 Hrs. of incubation suggesting positive chemotactic behavior of the strain which is the primary requirement for bioaugmentation.

Discussion

Bacillus subtilis is a Gram-positive bacterium that is well known for its potential application in bioremediation. The biodegradation of pharmaceutical micropollutants like paracetamol has gained interest among researchers due to low cost and efficacy. Bacteria viz., *Rhodococcus ruber*, *Stenotrophomonas*, *Pseudomonas moorei, Delftia tsuruhatensis*, *Cunninghamella echinulate*, and *Shinella* has been reported to have paracetamol biodegradative potential.[16,17,18,19.] In *Bacillus genera*,

Bacillus drentensis and *Bacillus cereus* have been reported to degrade acetaminophen at a rate of 300 and 200 ppm respectively.[13,21] Compared to this reported strain, *Bacillus subtilis subsp.subtilis* NCIB 3610(T) was found to degrade paracetamol in a higher concentration of 2500 ppm ,giving a more ecotoxic end product 4 Aminophenol. Sewage wastewater isolate *Bacillus subtilis* strain DPP3 (MN744327) followed Haldanes growth kinetic model with the maximum specific growth rate of 0.259 (Hr^{-1}) in the presence of 593 ppm concentration of acetaminophen and 10 days incubation period for the degradation.[33] The paracetamol biodegradation pathway comprises the catalytic activity of hydroquinone 1,2-dioxygenase, aryl acylamidase and deaminase enzymes giving 4 aminophenol and hydroquinone as product.[22,23,24] Based on the fermentation studies and chemotaxis assay, *Bacillus subtilis* can be employed for the bioaugmentation of drug polluted area. Conductive polymer/multiwalled carbon nanotube based microbial biosensor using *Bacillus subtilis* has been reported to have the potential to detect paracetamol even in low concentration.[26] Considering the reported literature about the acetaminophen using microorganisms, their use is not just limited to degrade the pollutant but also aids in the determination of their concentration.

For the effective treatment of the pharmaceutical industries wastewater, bioaugmentation in a membrane reactor is generally employed where the microorganisms should have the potential to cope up with inhospitable environment.[27] Report is available on the applicability of the fluidized bed pelleted bioreactor augmented with the fungi *Trametes versicolor* and *Aspergillus luchuensis* for the elimination of ketoprofen, metoprolol and ibuprofen.[28] Bioaugmentation of APAP-degrading organism, *Ensifer* sp. POKHU to the activated sludge resulted in the reduction of acetaminophen concentration, followed by ecotoxicity level.[29]

For the biodegradation of acetaminophen in wastewater treatment plants, microorganisms adopted different strategies like internalization of the drugs, cell surface absorption, enzyme assisted mechanism, and biotic- abiotic coupling.[30,31,32] Due to the rapid and extended eco toxic effects of the pharmaceutical micro pollutants, immediate removal of this pollutants from the source site is needed. Combined approach of Physico and biological processes will ensure the detoxification of wastewater.

Conclusion

Acetaminophen is one of the emerging pharmaceutical micropollutants which must be removed from the water reservoir using an economical and ecofriendly biological approach. Based on the current study, it can be concluded that *Bacillus subtilis* has the potential to tolerate and degrade high concentrations of acetaminophen. Recovery of acetaminophen degradative product, 4 aminophenol, will be suitable due to its application in dye industries. In the pharmaceutical industrial wastewater treatment plant, emphasis should be given to the degradation of pollutants into the products with industrial applications.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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