Int.J.Curr.Microbiol.App.Sci (2023) *12*(04): 174-183

EXCELLENT PUBLISHERS

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 12 Number 4 (2023)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2023.1204.020

Isolation, Screening and Characterization of Antibiotic-Degrading Bacteria from Pharmaceutical Waste Site

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Keywords

Norfloxacin, Levofloxacin, Amoxicillin and Cloxacillin, unmetabolized parent

Article Info

Received:
03 March 2023
Accepted:
05 April 2023
Available Online:
10 April 2023

A B S T R A C T

Antibiotic is one of the most significant discoveries and have brought a revolution in the field of medicine for human therapy. In developing nations, antibiotics use have helped to increase the life expectancy by lowering the deaths due to bacterial infections, but the risks associated with antibiotics pollution is largely affecting people. Since antibiotics are released partially degraded and undegraded into environment creating antibiotic pollution, and its bioremediation is a challenging task. The present study aims to isolate and acclimatize antibiotic-degrading bacterial strains for Norfloxacin, Levofloxacin, Amoxicillin and Cloxacillin from the contaminated soil of pharmaceutical waste site. Bacterial strains were isolated and acclimatized by continuous enrichment of cultures with antibiotics as the sole carbon source. The antibiotic susceptibility test, thiol mercury salt ultraviolet spectrophotometry (TMSUS), morphological observations, and 16SrDNA sequence analysis were used to identify and characterize the isolated strains. Three bacterial isolates (A, B and F) were obtained, and two of them (A, B) with the highest degradation rates were identified to belong to the same genera as *Bacillus*. These two isolates were found to be resistant to antibiotic in an antibiotic sensitivity test. The TMSUS indicated that the strains A and B had good performance in amoxicillin degradation. Two bacterial strains isolated from the pharmaceutical waste site are effective in degrading antibiotic and can be potentially used for bioremediation of antibiotic-contaminated soils.

Introduction

Antibiotics widely used in prevention and therapy of human and animal diseases and as promoters for animal growth (Leong *et al.,* 2016). Because antibiotics cannot be absorbed or metabolized completely by the animals, 30–90% of antibiotics are excreted into the environment via urine and feces as the unmetabolized parent compound (Bound and Voulvoulis, 2004). Residues of antibiotic has increased the frequency of horizontal gene transfer (HGT) and resistance gene fixation in genomes, leading to the development of diverse resistance genes in genomic islands (Gillings and Stokes, 2012). Residues of antibiotic in the environment could cause the emergence and development of antibiotic-resistant genes and bacteria, which brings profound negative impacts on the health of human and animals (Zhu *et al.,* 2013). Compared with other conventional methods such as

hydrolysis and photolysis, microbial degradation of tetracycline is considered to be an inexpensive, eco-friendly, and high efficient technology for the removal of tetracycline from the environment (Migliore *et al.,* 2012). In recent years, there are only a few antibiotic-degrading microorganisms that have been reported. Zhang *et al.,* (2015) found that 57.8% tetracycline could be degraded by *Advenella kashmirensi* which was selected from the pharmaceutical site. Leng *et al.,* (2016) have isolated tetracycline-degrading bacteria *S. maltophilia* strain DT1 from a contaminated soil. Most antibiotic-degrading bacteria are drug-resistance bacteria because they need to survive in the presence of antibiotics to play their role of degradation.

In particular, the phylogeny, the genetic traits and the evolutionary history of specific resistance pathways can also be obtained through comparative genomic analysis (Wang *et al.,* 2017). Comparative genomics analyses can shed light on the genetic basis underlying the degradation ability to organic contaminants of bacteria. Therefore, investigating the resistance mechanisms in the antibiotic resistant bacteria at the molecular level is important to predicting the environmental fate of antibiotics and assessing the biological impacts of antibiotics on the microbes in the environment.

Materials and Methods

The Improvement method was used for isolating and screening antibiotic-degrading bacterial strains. The spectrophotometry method was used for quantitatively evaluation for degrading Norfloxacin, Levofloxacin, Amoxicillin and Cloxacillin from isolated bacterial strains. The selected high-performance strains were then identified by 16S rRNA gene sequencing and phylogenetic analysis.

Collection and Isolation of antibiotics degrading microbes from soil

The soil samples were collected in clean polythene bags for microbiological analysis. Soil sample was collected from top area and 10 to 20 cm in deep *from* pharmaceutical waste site of March to July, 2020. For the isolation and purification of antibiotic degraded microbes, the solution from the final flask of acclimatization was serially diluted with sterile distilled water for three times (i.e., 1:1000 dilution), spread onto the LB agar medium, and incubated at 30°C for 48 h.

One milliliter of the diluted solution was then added to 49 mL of sterile LB medium containing 20 µg/mL antibiotic as the sole carbon source, incubated at 30 ˚C, shaken at 150 rpm for 6 or 7 days until an optimal optical density (OD) is reached. The cultures were then exposed to gradually increased antibiotic concentrations (40 to 100 µg/ml) to acclimatize the bacterial strains to antibiotics. Specifically, 2 mL of enriched culture medium was transferred to the first flask containing 48 mL of LB medium with 40 µg/mL of antibiotics; then 3 mL of the solution was transferred from the first flask to a second flask containing 47 mL of LB medium with 60 µg/mL of antibiotic; next, 4 mL of solution was transferred from the second flask to a third flask containing 46 mL of LB medium with 80 µg/mL of antibiotic; and finally 5 mL of solution was transferred from the third flask to a fourth flask containing 45 mL of LB medium with 100 µg/mL of antibiotic. The above transfers were performed at 4-day intervals. In other words, the bacterial strains were given 4 days to acclimatize to each antibiotic concentration from 40 to 100 µg/mL (Fu *et al.,* 2015). The purpose of acclimatization was to obtain bacterial strains with high tolerance and degradation ability to antibiotics.

Identification of isolated bacteria

Qualitative Screening of antibiotic-Degrading Bacteria

To qualitatively determine if an isolated bacterial strain was capable of degrading antibiotics, a piece of filter paper containing 10 µg of antibiotics were applied to the surface of the agar which had been inoculated with a bacterial strain. As the diffusion

distance of antibiotics in the agar increased, the antibiotics concentration decreased logarithmically to a certain concentration below which the bacterium would not grow, thus forming a transparent antimicrobial circle on the filter paper. The size of this inhibition zone reflected the sensitivity of the test bacteria to antibiotics, i.e., the smaller the circle, the more effective the bacterium is in degrading antibiotics.

Morphological characterization of Isolated Strains

To observe the morphology of the isolated bacterial strains, Gram staining was used as a means of preliminary identification (Kumar *et al.,* 2007). The observed morphological features included color, opaqueness, and surface texture of the bacterial colony. These features would help visual identification of bacterial strains.

Evaluation of Degrading Bacteria

Qualitative screening were further evaluated quantitatively for their effectiveness by using the thiol mercuric salt UV spectrophotometry (TMSUS) (Liu *et al.,* 1991). Specifically, the purified strains were inoculated on an LB medium containing 100 μg/mL of antibiotics in a shaker culture and the concentration of survived in the medium was measured by TMSUS in 48 h.

To determine the maximum detection wavelength, 25 mL of antibiotics solution of 100 μg/mL was prepared in a 50 mL volumetric bottle. Following the procedure described in (Fu *et al.,* 2015), the absorbance was measured in the range from 310 to 340 nm. When a peak appeared, the scan gradient was narrowed in the wavelength range around the peak to find the exact maximum absorption wavelength. The measured result is shown in Figure 1 and it was found that the maximum detection wavelength was around 325 nm for antibiotics, which is consistent with the range of 324 to 345 nm reported in the literature for the maximum absorption of antibiotics (Feng, 2009).

To establish the standard curve, antibiotics solutions with concentrations of 0, 15, 30, 45, 60, 75, and 90 μg/mL were prepared and the absorbance of antibiotics was measured at the maximum absorption wavelength (325 nm). Distilled water was used as the blank control (0 concentration). There was a good linear relationship between the antibiotic concentration and the absorbance value.

Gene Sequencing and Phylogenetic Analysis of Isolated Strains

The antibiotic degrading isolates were cultivated on the LB agar medium at 30 ◦C for 48 h. The culture was used for the amplification of bacterial 16S rRNA gene by PCR. Two universal 16S rRNA gene primers (F27:5'-AGTTTGATCMTGGCTCAG-3' and R1492: 5'-GGTTACCTTGTTACGACTT-3') were employed. Culture samples of 25 μL were prepared and each sample was composed of 0.5 μLof bacterial culture as the template DNA, 7.5 μL of 2× Taq PCR Master Mix (containing 0.2 U Taq DNA polymerase/μL, 250 μM of deoxy-ribonucleoside triphosphate (dNTP), 2.5 μL of 10× PCR Buffer, 0.5 μL of primer (10 μM), and 19.8 μL of double-distilled H_2O. The PCR procedure was carried out as follows: Primary denaturation at 94 °C for 4 min; 30 cycles of denaturation at 94°C for 45 s; annealing at 55°C for 45 s; and extension at 72°C for 60 s; and an additional reaction for 10 min at 72°C. The PCR products were detected on 1.5% agarose gel to confirm its purity and size. The PCR products were further sent for sequencing.

The 16S rRNA gene sequences were compared with other 16S rRNA gene sequences available in Genbank by using the Basic Local Alignment Search Tool (BLASTN) program and aligned with similar sequences by using multiple sequence alignment software (Thompson *et al.,* 1994). The phylogenetic tree was constructed by applying the neighbor-joining method using Molecular Evolutionary Genetics Analysis (MEGA) 7.0 program based on Kimura-2 parameters with 1000 replicates of bootstrap values (Saitou and Nei, 1987).

Results and Discussion

Isolation and Initial Screening of Norfloxacin, Levofloxacin, Amoxicillin and Cloxacillin - Degrading Bacteria

A total of seven Norfloxacin, Levofloxacin, Amoxicillin and Cloxacillin degrading bacterial strains were isolated from the soil collected from medical waste area site. All the isolates were first subjected to preliminary screening which was carried out through antibiotic susceptibility tests against all above antibiotics as shown in fig 1. The inhibitory zone diameter was significantly found for the isolated strains. Therefore, isolated strain further subjected to quantitative degrading evaluation.

TMSUS Measurements

The measured absorbance values for the seven bacterial strains, along with the blank controls. Using the standard curve (Y = 0.011X - 0.004), these absorbance values were converted to antibiotics concentrations (survived antibiotics after exposing to the three degrading strains). Comparing with the corresponding control, it was deceptive that all three isolated strains were capable of degrading antibiotics, with high degradation rates. Isolate A was capable of degrading Norfloxacin, Levofloxacin, Amoxicillin and Cloxacillin antibiotic with degradation rate 64.1 ± 5.2 %, 63.8 ± 3.3 %, 69.3 ± 2.6 %, 69.3 ± 2.6 % and 62.8 ± 2.4% respectively (Fig. 2). Similarly isolate B showed better degradation of Norfloxacin, Levofloxacin, Amoxicillin and Cloxacillin antibiotic with 56.9 ± 3.6 %, 62.2 ± 0.7 %, 67.1 ± 2.4 % and 51.4 ± 3.8 % respectively. Whereas Norfloxacin, Levofloxacin, Amoxicillin and Cloxacillin antibiotic in control showed higher degradation with 87.3 ± 1.2 %, 89.9 ± 1.0 %, 95.4 ± 1.5 % and 89.6 ± 0.9 % respectively. In comparison to control group Isolate F was showed highest degradation for amoxicillin antibiotic with 58.0 ± 5.0%. Statistical analysis indicated that the degradation rates of amoxicillinin isolate A, isolate B and isolate F were significantly higher than Norfloxacin, Levofloxacin and Cloxacillin antibiotic ($p < 0.05$). Therefore amoxicillin antibiotic degraded isolate A, isolate B and isolate F were selected and further analysis was performed to characterize the selected isolates.

Strain isolation and identification

Three different antibiotic degrader's bacteria were isolated from contaminated soil of biomedical waste and initially labelled as A, B and F. Fig. 3 and Table 1 illustrated morphological characteristics for isolates. Isolate A was distinguished with rod, beige in color and Circular. Similarly Rods size, beige in color, Circular and positive gram stain features characterize isolate B. F isolates characterized by cylindrical rods size, white, Irregular large form and negative gram stain.

Gene sequencing and phylogenetic analysis of antibiotic-degrading isolate

The isolates were amplified using the primers 27F and 1492R, resulting in a characteristic single band of approximately 1450 bp (Fig. 4). The isolated strain was identified as *B. subtilis and B. pumilis* subgroup on the basis of 16S rDNA gene sequencing and the phylogenetic tree (Fig. 5-8). The phylogenetic trees of the isolates clearly demonstrated its evolutionary relationship with a group of *B.* species produced by a neighbor-joining method with the help of MEGA 7.0 program. The isolates belonged to the genus *Bacillus*, more than 50 species of which have been identified. The genus *Bacillus* is Gram-positive, and much research has demonstrated the wide distribution of the genus *Bacillus* in activated sludge, plants, soils, hospital waste and waste water (Chen and Wu, 2006), which can produce spores that are resistant to adverse conditions. Research has shown that *Bacillus* can produce antimicrobial substances that inhibit the reproduction of harmful microorganisms and degrade the nutrients in the soil.

Table.1 Morphological colony features for three bacterial isolates

Isolates	Size	Gram stain	Color	Shape
A	Rods	+	Beige	Circular
B	Rods	+	Beige	Circular
F	Cylindrical rods	_	White	irregular

Fig.1

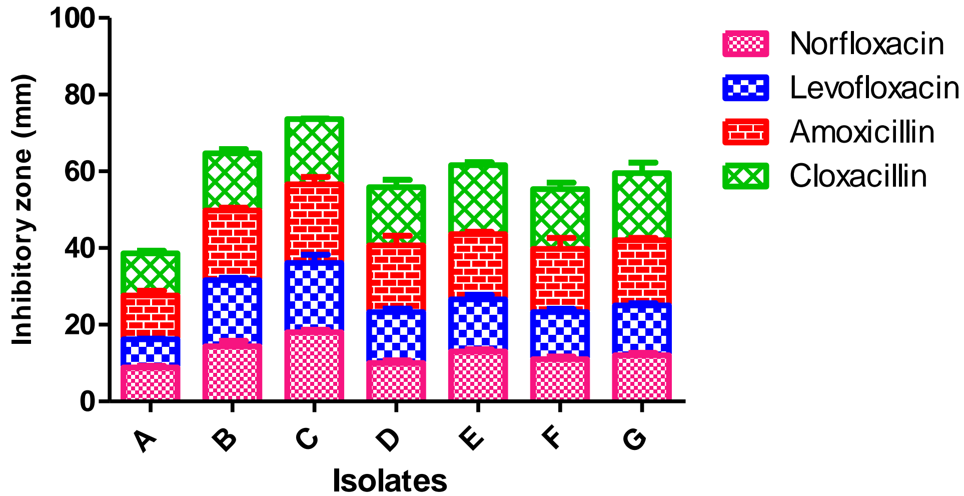

Fig.2

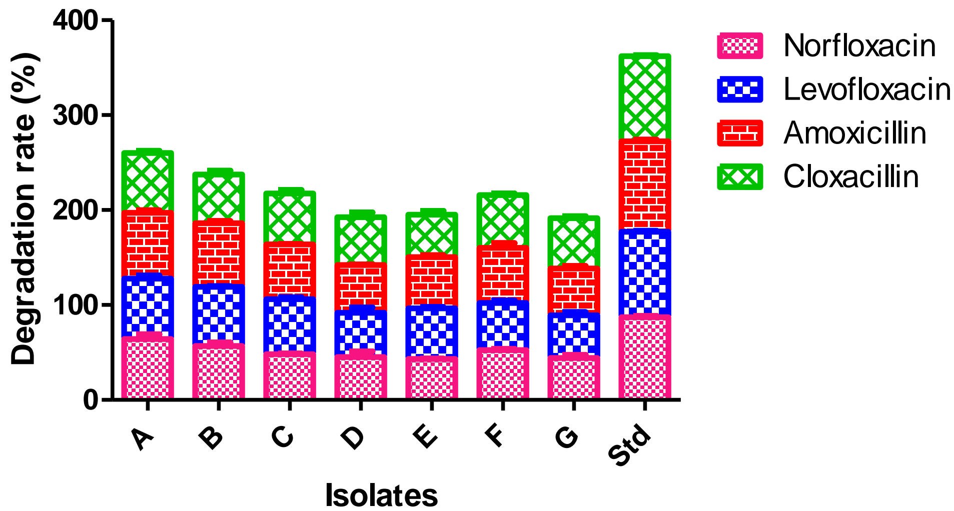

Fig.3 Morphological characters and gram stain for three bacterial strains isolated from hospital waste site

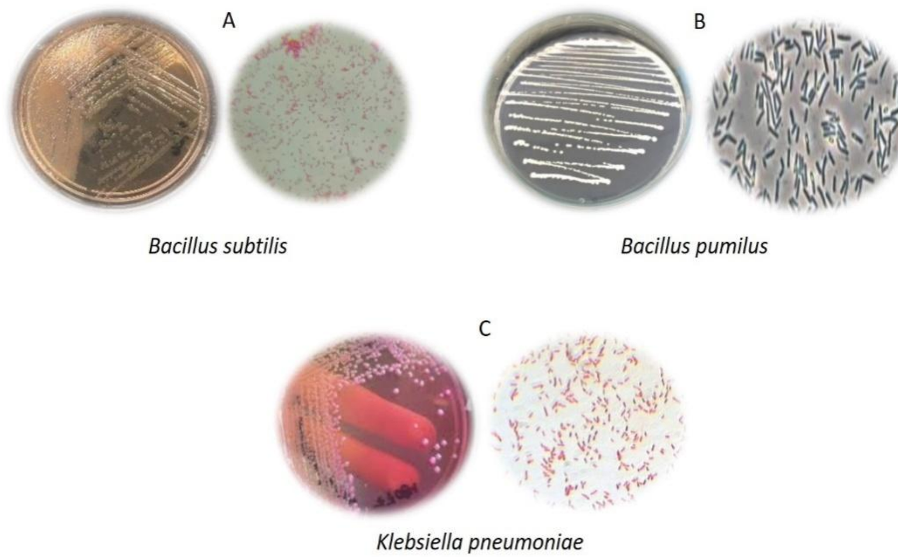

Fig.4 Results of PCR amplification of bacterial isolate (A)-*B. subtilis*(B)- *B. pumilis* M-marker (bp)

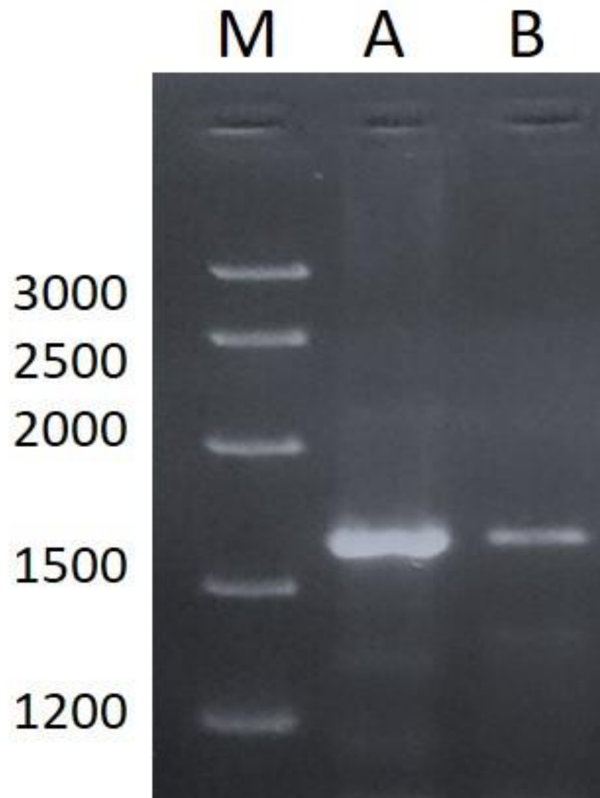

Fig.5 Phylogenetic analysis of isolated strain *B. subtilis* in the neighbor- joining tree

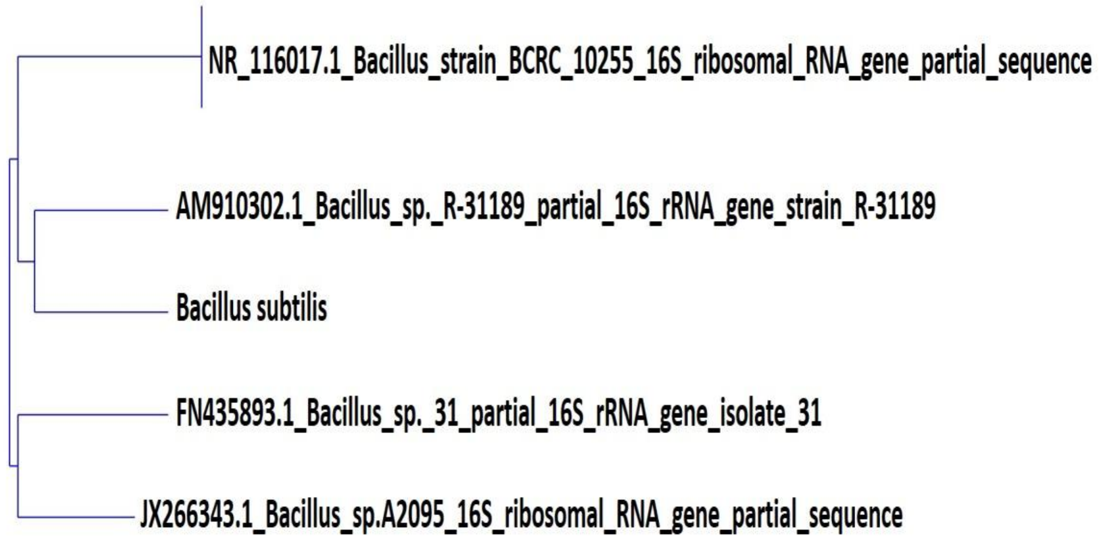

Fig.6 16S rDNA sequencing of bacterial isolate *B. subtilis*

TGCTAAAATGCCCCCCCAAAAATTGCTATGCCCCACTGTCAG
CGATCAGTTTAAACCCGGGGACGTCCTTGAATCCGGGTTAA
CGATCATTAGCAATCTTTCCAACGAATTAGCTAAAACCTTTAA
GATATCGTGCAACGTACGTACGTGGGCCAAAATTTGCGTACA
GTACGTAGCTGACGTCACGTCGATACGATCGATCGATTACGAC
CGATCATTAGCAATCTTTCCAACGAATTAGCTAAAACCTTTAA
GATATCGTGCAACGTACGTACGTGGGCCAAAATTTGCGTACA
CGATCATTAGCAATCTTTCCAACGAATTAGCTAAAACCTTTAA
GATATCGTGCAACGTACGTACGTGGGCCAAAATTTGCGTACA
TAGCTGACGTCACGTCGATACGATCGATCGATTACGTAGCTGA
CGTCACGTCGATACGATCGATCGATTACGTTTAAACCCGGGGA
CGTCCTTGAATCCGGGTTATCGGCATAGCTGCAGTCGCGAATT
ACTGCGCAAACCTTGCAATTCGTACGTAGTAGCGAATTCGAA

Fig.7 Phylogenetic analysis of isolated strain *B. pumilus* in the neighbor- joining tree

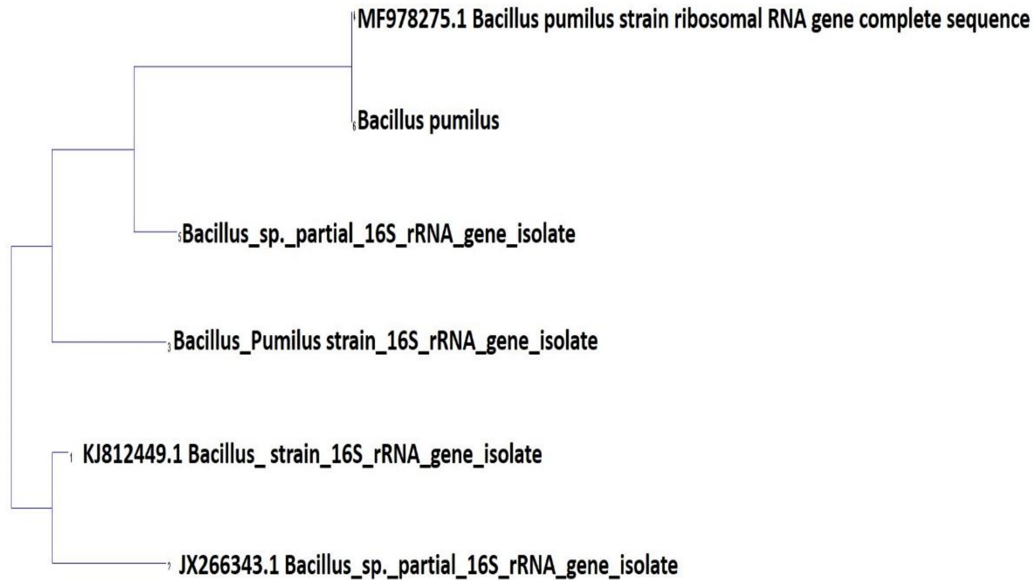

Fig.8 16S rDNA sequencing of bacterial isolate *B. pumilus*

GCATCGATCATTAGCAATCTTTCCAACGAATTAGCTAAAACCTT
TAAGATATCGTGCAACGTACGTACGTGGGCCAAAATTTGCGTA
CAGTACGTAGCTGACGTCACGTCGATACGATCGATCGATTACG
ACCGATCATTAGCCTTTTTTTTGGGGAAACCCCCCGCAATCTT
TCCAACGAATTAGCTAAAACCTTTAAGATATCGTGCAACGTAC
GTACGTGGGCCAAAATTTGCGTACACGATCATTAGCAATCTTT
CCAACGAATTAGCTAAAACCTTTAAGATATCGTGCAACGTACG
TACGTGGGCCAAAATTTGCGTACATAGCTGACGTCACGTCGAT
ACGATCGATCGATTACGTAGCTGACGTCACGTCGATACGATCGA
TCGATTACGTTTAAACCCGGGGACGTCCTTGAATCCGGGTTATC
GGCATAGCTGCACATGACGTACGTACGTACTGCGATACGATACG

For example, Peng (2013) isolated a strain of *Bacillus pumilis* 4D-14 from broiler farms, which had strong inhibitory effect on multiple strains of pathogenic bacteria. Yang *et al.,* (2016) isolated a strain *Bacillus cereus* XG1 from the watermelon leaves infected by watermelon bacterial fruit rot and they concluded that the strain XG1 had the potential to be developed as a microbe-herbicide.

Wang *et al.,* (2018) isolated a *Bacillus cereus* strain WHY-2 from the deep-sea surface sediments with an effective function of adsorbing thorium ions (Th), which could alleviate the environmental pollution caused by thorium ions as radioactive substances in groundwater. The antibiotic resistant bacteria play a major role in the microbial degradation of antibiotics. These bacteria can produce corresponding degrading enzymes, which destroy the molecular structure of antibiotics by modification or hydrolysis (Leng, 2017). Several studies (e.g., Liu *et al.,* 2016) have found that antibiotic degrading enzymes mainly include the following four types: β-Lactamase, aminoglycoside modifying enzyme, macrolide passivase, and

chloramphenicol inactivating enzyme, among which β-Lactamase can destroy the chemical bonds of cephalosporins and penicillins. Furthermore, there are ring-opening oxidases associated with fosfomycin resistance as well as esterases associated with macrolides resistance that can breach the structure of the corresponding antibiotics.

In conclusion, Antibiotic contamination is a major global threat and has increased the risk of antibiotic resistance in the microorganisms. Although the European Union has banned many antibiotics, they are still in use and creating a future global challenge for health issues. Solid waste treatment plant are not able to completely eliminate the antibiotic residues from the soil matrices; hence, more investigations on pharmaceutical and hospital waste capacities and capabilities are the calls of present conditions worldwide. Antibiotics have a short life span, but still, their hydrophobicity and lipophilic nature lead to their persistence in the environment. Measures should be adopted for the controlled use of antibiotics, and no antibiotic should be sold without being prescribed along with the safe removal of the discarded antibiotics along with the excreta. It is obvious that the complete removal of residual antibiotics is impractical but though, the observations lie at the core of mechanisms associated with their uptake and abolition. Finally, more in-depth studies are needed for better technology development for the clean environment and safer future for our next generations.

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How to cite this article:

Rita Yadav and Archana Shrivastav. 2023. Isolation, Screening and Characterization of Antibiotic-Degrading Bacteria from Pharmaceutical Waste Site. *Int.J.Curr.Microbiol.App.Sci.* 12(04): 174-183.
doi: https://doi.org/10.20546/ijcmas.2023.1204.020