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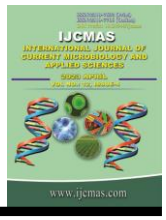

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Isolation and Identification of Bacteria from Pharmaceutical Site

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A B S T R A C T

Antibiotic pollution is an important and extensive environmental problem and even threat to human health. Biodegradation is a major mechanism which removes the pollutants from the environment. Therefore, the present study aimed to screen and isolate antibiotic degrading bacteria which can effectively degrade azithromycin, clarithromycin, cefadroxil and cephalexin antibiotic. Six antibiotic degrading bacteria were isolated from pharmaceutical waste site and the antibiotic degradation rate of each strain was determined by TMSUS method. Bacterial strains were morphologically identified using gram staining reaction and biochemical tests. The investigation revealed the pharmaceutical waste consist of bacterial community which useful for mankind and might be used for the biodegradation of pharmaceutical waste site to recover the resource.

Introduction

Pharmaceutical wastes refers to all waste, biological or non-biological from hospitals, that is discarded and not intended for further use and these include: pathological, hazardous chemicals and pharmaceutical wastes. These are in addition to antibiotics, clinical bandages, gauze, cotton, cotton and other miscellaneous wastes (Singh *et al.*, 2007). As regards live pathogens found in hospital wastes, the most predominant (80 -90%) is the genus *Bacillus* with *Staphylococci* and *Streptococci* whereas the most common pathogens is *Staphylococcus aureus* (from 2 - 10 colonies per gram of waste). *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans* are also common along with varying numbers of other common nosocomial pathogens such as *Klebsiella pneumoniae* and *Enterobacter* species (Razdan and Cheema, 2009).

Antibiotics are natural or synthetic molecules capable of inhibiting the growth of bacteria (bacteriostatic) or killing some bacteria (bactericidal) (Catteau *et al.*, 2018). Antibiotics exert a specification on certain structures or functions of the microorganism.

Additionally, they can be classified according to their action spectrum as a broad- or limited-spectrum, and according to their pharmacokinetics, concerning gastrointestinal, subcutaneous or

muscular absorption, distribution and elimination (hepatic or renal) in the human or animal body (Spagnolo *et al.,* 2021). Various methods have been explored to remove antibiotics from wastes, including chemical hydrolysis, membrane separation, activated carbon adsorption, and photodegradation.

However, there are a number of drawbacks associated with these methods, including the formation of secondary toxic byproducts and high operational costs, when using the conventional physical and chemical techniques (Ben *et al.,* 2011).

Biodegradation may provide alternative tools to degrade antibiotics in wastes. Adel *et al.,* (2015) reported that significant degradation of cephalexin antibiotics was produced by the *Bacillus subtilis* strain. Al-Gheethi *et al.,* (2014) investigated the biodegradation by *Bacillus subtilis* of four β-lactam antibiotics (amoxicillin, ampicillin, cefalexin, cefuroxime) and found that *Bacillus subtilis* treatment was especially efficient for removing amoxicillin, ampicillin, and cefalexin at a concentration of 1 mg/L, with removal rates up to 25.03%, 22.59%, and 10.62%, respectively.

Scientists have long been aware of potential problems from the presence of antibiotics in soil. Determined antibiotic concentrations in soil matrices have ranged from a few nanograms to milligrams per kg of soil. The highest concentrations are usually found in areas treated with manure or used for livestock (Zhao *et al.,* 2016).

The goal of this study is to find bacteria, naturally existing in soil for degradation of antibiotics. The specific objectives are to: (1) Isolate and characterize bacteria from soil on pharmaceutical waste for degradation of antibiotics and (2) evaluate the effectiveness of the isolated bacterial strains.

Materials and Methods

The enrichment and acclimatization method was used for isolating and screening antibiotic-degrading bacterial strains. The isolated bacterial strains were then quantitatively evaluated for their effectiveness in degrading azithromycin, clarithromycin, cefadroxil and cephalexinby using spectrophotometry method. The selected high-performance strains were then identified by 16S rRNA gene sequencing and phylogenetic analysis.

Collection of soil samples

The soil samples for microbiological analysis were collected in clean polythene bags. The soil samples were collected from pharmaceutical waste site of March to July, 2020. Soil sample was gathered from top area and 10 to 20 cm in deep.

Isolation of antibiotics degrading microbes

Isolation and purification were performed after enrichment and acclimatization. Specifically, 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.

Enrichment and Acclimatization of Bacterial Strains

Enrichment of the bacterial strains was carried out through a series of dilution and incubation. First, one gram (1 g) of collected soil was suspended in 9 mL of sterile distilled water and agitated for one minute.

This homogenized soil extract solution was then diluted by adding 1 mL of the solution to 9 mL of sterile purified water, and the process was repeated six times to achieve a 10^6-fold dilution. 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 (Liu *et al.,* 2013). 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

Gram staining was used to observe the morphology of the isolated bacterial strains as a means of preliminary identification, following the method reported (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.

Motility test

Prepare a semisolid agar medium in a test tube. Sulphideindole motility (SIM) medium or motility test medium with or without TTC (triphenyltetrazolium chloride) or motility nitrate medium can be used. Inoculate with a straight wire, making a single stab down the center of the tube to about half the depth of the medium. Incubate under the conditions favoring motility and Incubate at 37°C Examine at intervals, e.g. after 6 h, and 1 and 2 days (depends on generation time of bacteria). A freshly prepared medium containing 1% glucose can be used for motility tests on anaerobes.

Biochemical characterization of Isolated Strains

Oxidase test

Tested bacterial colony was smeared on the filter paper previously saturated with freshly prepared oxidase reagent. Positive oxidase test was recorded as the development of a blue-purple colour within 10 s (Cheesbrough, 1991).

Catalase test

Gas bubbles detecting within 10 s after added purified bacterial culture to 5 ml of hydrogen peroxide solution, considered as a positive catalase test (Cheesbrough, 1991).

Urease test

Slanted two millilitres of urea medium which placed in bijou bottles applied for the incubated bacterial colony at room temperature. Red-pink colour in the medium was considered as a positive test for urease induction (Cheesbrough, 1991).

Indole test

Appearance of bright red and yellow color which composed after added 0.5 ml of Kovac's reagent to incubated bacterial culture at 35°C for 24 h on SIM media indicated a positive and negative results respectively (Cheesbrough, 1991).

Simmons Citrate test

Simmons Citrate test was performed via inculcate Simmons Citrate Agar plates (TSBA, Himedia) surface with bacterial cultures then, incubated at 37°C up to 48 h. changing media colour from green to bright blue indicate positive reaction.

Methyl red (MR) test

After adding methyl red indicator solution (TSBA, Himedia) to inoculated culturing media and incubation at 35°C for up to 4 days, changing color

to red indicate MR test positive- appearance of tested bacteria (Allen *et al.,* 2016).

Gelatin hydrolysis

Nutrient gelatin stab method was applied according to Edison *et al.,* (2012). Heavy inoculums of a test bacterium inoculated into tubes containing nutrient gelatin, gelatin liquefaction is the positive results for bacterial gelatin hydrolysis.

Evaluation of Degrading Bacteria

The isolated bacterial strains that had shown the ability of degrading above-discussed antibiotics. Qualitative screening were further evaluated quantitatively for their effectiveness by using the thiol mercuric salt UV spectrophotometry (TMSUS) (Feng, 2009). 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 (Liu *et al.,* 1991). 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.

Results and Discussion

Isolation and Initial Screening of Azithromycin, Clarithromycin, Cefadroxil and Cephalexin - Degrading Bacteria

A total of six Azithromycin, Clarithromycin, Cefadroxil and Cephalexin 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. The inhibitory zone diameter was significantly found for the isolated strains ($p < 0.05$) (Figure 1). 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 Azithromycin, Clarithromycin, Cefadroxil and Cephalexin antibiotic with degradation rate 67.7 ± 1.9%, 63.3 ± 1.5%, 65.3 ± 3.0% and 62.3 ± 2.4% respectively (Fig. 2).

Similarily isolate B showed better degradation of Azithromycin, Clarithromycin, Cefadroxil and Cephalexin antibiotic with 53.4 ± 1.3%, 59.7 ± 1.3%, 57.1 ± 2.4% and 50.4 ± 2.4% respectively. Whereas Azithromycin, Clarithromycin, Cefadroxil and Cephalexin antibiotic in control showed higher degradation with 92.3 ± 1.6%, 90.9 ± 1.0%, 93.4 ± 1.2% and 91.6 ± 1.8% respectively.

Statistical analysis indicated that the degradation rates of amoxicillinin isolate A, significantly higher than of Clarithromycin, Cefadroxil and Cephalexin antibiotic ($p < 0.05$). Therefore Azithromycin antibiotic degraded isolate A, B and C were elected and further analysis was performed to characterize the selected isolates.

Table.1 Morphological colony features for three bacterial isolates

Isolates	Size	Gram stain	Color	Shape	Motility
A	Rods	+	Beige	Circular	Motile
B	Rods	+	Beige	Circular	Motile
C	Rods	_	White	Irregular	Non-motile

Table.2 Three bacterial isolates biochemical test

Isolates	Oxidase test	Catalase test	Simmons citrate test	Indole test	Methyl red test	Urease test	Gelatin hydrolysis Test
A	(+/-)	(+)	(+)	(-)	(-)	(-)	(+)
B	(+)	(+)	(+)	(-)	(-)	(-)	(-)
C	(+)	(+)	(+)	(-)	(-)	(-)	(+)

Fig.1 Antibiotic susceptibility test (Inhibitory zone diameters in mm)

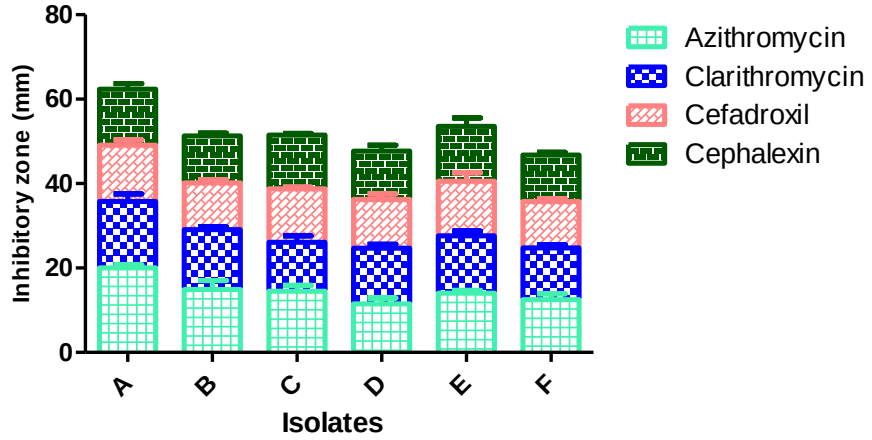


Fig.2 Measured degradation rates of antibiotics (control and test) for isolated strains

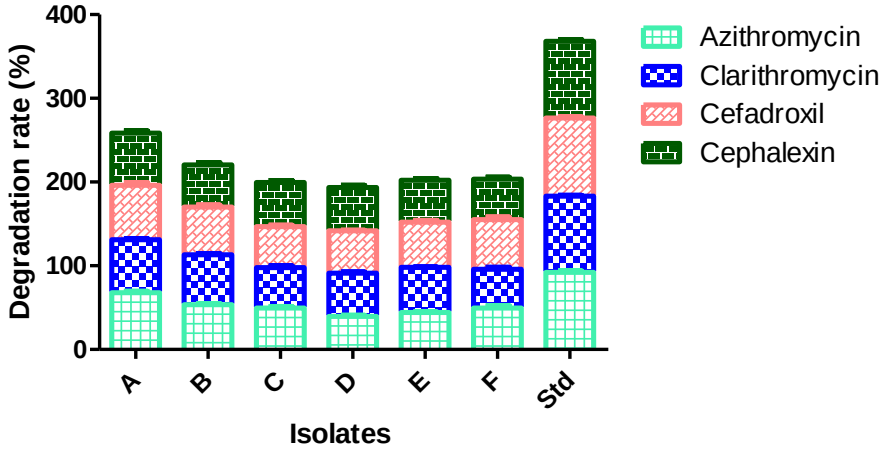
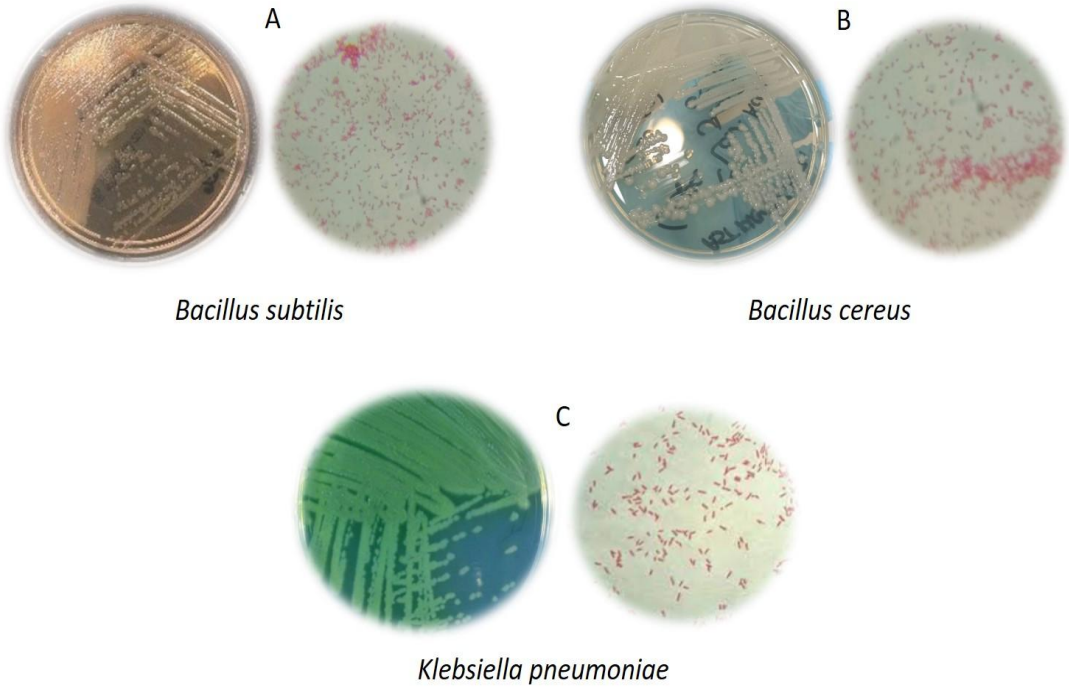


Fig.3 Morphological characters and gram stain for three bacterial strains isolated from pharmaceutical site



Strain isolation and identification

Three different antibiotic degrader's bacteria were isolated from contaminated soil of biomedical waste and initially labeled as A, B and C. Fig. 3 and Table 1illustrated 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. C isolates characterized by cylindrical rods size, white, Irregular large form and negative gram stain. Based on biochemical assays (as shown in Table 2) and previous morphological examination, three targeted isolates reflected different biochemical features. Positive for Oxidase test and negative for Catalase, Indole and Urease tests identified isolate A as *Bacillus* sp.

On the other hand, positive for Oxidase and Catalase tests and negative for Indole and Urease tests refer to *Klebsiella* sp. negative oxidase, Indole and Urease tests and positive Catalase test remarked isolate as *Bacillus cereus*. Performed morphological colony characteristics examination (Gram stain, mobility and motility, shape and color) and biochemical tests (catalase, urease, oxidase activities, nitrate reduction, Indol production, acid/gas production from carbohydrates and fermentation of sugars) to identify our antibiotic degradation isolates were considered a traditionally identification methods (Udgire *et al.*, 2015).

The Gram positive and gram negative bacteria were identified as, rods and cocci. These rods are motile and cocci are non-motile in nature. The motility test is not a biochemical test since we are not looking at metabolic properties of the bacteria. Rather, this test can be used to check for the ability of bacteria to migrate away from a line of inoculation. Heterotrophic strains isolated from pharmaceutical waste are subjected to different biochemical tests were given in table 2. It was further observed that the catalase and nitrate reduction were for *K. pneumoniae.*

These results are similar to the currently isolated

strains from Bergey's manual of Determinative of Bacteriology, 90% of results showed the similarity in characteristics with of isolated bacteria (Liu *et al.*, 2016). The pharmaceutical wastes could have contributed immensely in the increase of these bacteria might be due to the fact that pharmaceutical wastes is very rich in biological material. Microbes will adapt and grow at sub zero temperatures, as well as extreme heat, desert conditions, in water, with an excess of oxygen, and in anaerobic conditions, with the presence of hazardous compounds or on any waste stream. Forsberg *et al.*, (2012) reported that truly pathogenic forms may survive in waste. We isolated bacterial strains from pharmaceutical wastes. These strains could used for biodegradation and therefore may be suitable for industrial applications. After isolation and identification of microorganisms, a proper disposal of pharmaceutical waste is needed.

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