

Isolation and antibiotic sensitivity of *Bacillus thuringiensis* strain from dump soil

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ABSTRACT

Bacillus thuringiensis (or Bt) is a commonly used as a pesticide. *B. thuringiensis* is a naturally-occurring soil bacterium, also occurs naturally in the gut of caterpillars of various types of moths and butterflies, as well as on the dark surface of plants. The xylanase producing bacterial strains were isolated from dump soil. The strains were isolated on xylan agar media and screening was carried out by xylanolysis method. To test the sensitivity of the isolates, ten different antibiotics were used. The strains were tested for resistance to doxycyclin, erythromycin, chloramphenicol, cephalaxin, kanamycin, ampicillin, streptomycin, vancomycin, amoxicillin and neomycin. The strains showed sensitive to doxycyclin, erythromycin, chloramphenicol, cephalaxin, kanamycin, ampicillin, streptomycin and vancomycin and also showed resistance to amoxicillin and neomycin, when tested by disc diffusion method on nutrient agar plate confirmed by antibiotic spread plate method. The inhibitory effect of *B. thuringiensis* strains against the test bacteria *Bacillus subtilis*, *Sarcina lutca*, *Shigella dysenteriae*, *Shigella sonnei* and *Pseudomonas aeruginosa* examined. It was found that, *B. thuringiensis* S1, *B. thuringiensis* S2 and *B. thuringiensis* S3 strains showed an inhibitory effect on all of the test bacteria.

Keywords: antibiotics, sensitivity, resistant, *Bacillus thuringiensis*

INTRODUCTION

B. thuringiensis (Bt) is a Gram-positive, soil-dwelling, spore-forming, rod-shaped bacteria. It is approximately 1 µm in width and 5 µm in length (Madigan and Martinko, 2005; Sakai *et al.*, 2007). It grows at body temperature and produces a diamond-shaped crystal from its crystal proteins (Cry proteins) and uses it to fend off insects, predators, and other pathogens (Jimenez-Juarez *et al.*, 2007). Microorganism that produces chemicals toxic to insects (Schnepf *et al.*, 1998; Whalon and Ganghey, 1998) common insect targets are "moths, mosquitoes, blackflies, beetles, hoppers, aphids, wasps and bees as well as nematodes (Vadlamudi *et al.*, 1995; Dorsch *et al.*, 2002; Zhang *et al.*, 2005). *Bacillus thuringiensis* occurs naturally in the environment, isolated from soil, insect and plant surface (Meadows, 1993; Schnepf *et al.*, 1998; Aslim *et al.*, 2002). *B. thuringiensis* has been implicated in burn wound infections and food-poisoning (Jackson *et al.*, 1995; Damgaard *et al.*, 1997). Little interest was shown in antimicrobial susceptibility profiles of *Bacillus* species until very recently. This was due to a combination of reasons: the low recognition of the ability of *Bacillus* species (Turnbull *et al.*, 2004).

While many antibiotics are known to exist, efforts to discover new antibiotics still continue. Therefore, many species such as *Streptomyces*, *Bacillus* and *Penicillium* have been studied continuously for their ability to produce antibiotics (Brock and Madigan, 1991). In addition, due to the fact that *Bacillus* species have produced antibiotics in

the soluble protein structure and that these antibiotics have been found to be cheaper and more effective in studies conducted to date, these microorganisms are preferable for commercial production. Currently, the target is to produce antibiotics such as polymyxin and bacitracin from *Bacillus* (Debavov, 1982; Priest, 1989). It was reported that members of the species *Bacillus* generally produced polypeptide type bacteriocines, and that these antibiotics generally affect Gram positive bacteria (Huck *et al.*, 1991; Marahiel *et al.*, 1993). The apparent increase of the occurrence of antibiotic resistance among bacteria during the past years and its possible implication in public health has led to an intensified surveillance of bacterial resistance in many countries.

While biomedical scientists are discovering newer and most potent anti-microbial drugs, the pathogenic bacteria with their demonstration to survive, are gaining resistance them in a curious way. In a sensitive bacterial population, there may be a small number of drug resistant bacteria which develop resistant spontaneously as a result of mutation. More frequently resistance is due to the presence of additional gene(s) as extra chromosomal DNA known as R-factors (plasmids) (Choudhury, 1995).

The aim of this work was to investigate the changes occurring in the bacterial their possible relation to antibiotic susceptibility, resistance to antimicrobial agents, information on the frequency and distribution of multidrug resistance patterns and mproduction of bioactive compounds by strains.

The primary purpose of the study was to determine the susceptibilities of these species to a set of antibiotics selected to have the greatest guidance value to clinicians encountering possibly *B. thuringiensis* infections. In determining some features of the microorganisms, which play an important role in soil development and biological control, our aim is to provide information for further biotechnical studies.

MATERIALS AND METHODS

Bacterial strain

For the screening of xylanase producing *Bacillus thuringiensis* strains, dump soil samples were collected from Alam nagar of Rangpur, Bangladesh.

Media and culture conditions

Nutrient agar media, Czapek-dox agar media and Yeast extract xylan agar were used as a solid medium throughout the work. Yeast extract xylan agar plates were used for the isolation and identification of the bacteria and the bacteria were cultured at 37 °C.

Isolation and characterization of bacteria

All the xylanase producing bacterial strains which were isolated by their growth on xylane agar media as clear zones and xylanolytic properties were characterized according to the biochemical tests described in the "Berger Manual of Determinative Bacteriology". Finally, *Bacillus thuringiensis* strains were identified by 16S rRNA.

Antibiotic resistance and sensitivity

The antibiotic resistant *Bacillus thuringiensis* strains were isolated from the selected strains containing of xylanolytic activity using the disc diffusion method (Bauer *et al.*, 1966). A 16 h broth cultures of the collected strains when grown at 37 °C and was spread on both nutrient agar plate using sterilized glass spreader. Then ampicillin (10 µg/disc), amoxycillin (10 µg/disc), chloramphenicol (30 µg/disc), kanamycin (30 µg/disc), cephalixin (30 µg/disc), doxycycline (30 µg/disc), erythromycin (15 µg/disc), neomycin (30 µg/disc) and streptomycin (10 µg/disc) antibiotics were distributed on plate and kept the plates at 4 °C for 4-6 h, so that the antibiotic can diffuse on the agar media.

The plates were then incubated at 37 °C for 16 h and the growth of the bacteria was observed. The presence of a clear zone around the disc was the index of sensitivity to the antibiotic. The test results of antibiotic sensitivity were determined according to the inhibition zone diameter (Barry, 1986; Çetin and Gürler, 1989). The absence of such a clear zone or the presence of some colonies within the clear zone indicated that the collected strains were resistant to that antibiotic. The drug resistance bacteria tested by disc diffusion methods were again confirmed by

spreading its culture on the selected antibiotic plates of different concentrations. The plates were then incubated at 37 °C and observed on next day. The clear plate indicated that the strains sensitive to this selective concentration and presence of colonies on the plate indicated that the strains were resistant to that selective concentration.

Antibacterial activity

The primary assay performed *in vitro* by disc diffusion assay (Bauer *et al.*, 1966). Five pathogenic bacteria were selected for the antibacterial studies, three of which were Gram negative and the other two were Gram positive (Table 1). The test organisms were collected from the Institute of Biological Sciences (IBSc), University of Rajshahi, Bangladesh.

Table 1: Bacteria used for antibacterial study

Serial No.	Test Organisms	Strain Number
Gram positive		
1.	<i>Bacillus subtilis</i>	QL-40
2.	<i>Sarcina lutca</i>	QL-166
Gram negative		
3.	<i>Shigella dysenteriae</i>	AL-35587
4.	<i>Shigella sonnei</i>	AJ-8992
5.	<i>Pseudomonas aeruginosa</i>	CRL

The test organisms were inoculated in the medium to test tube and were agitated to ensure uniform dispersion of the organism into the medium. The medium was poured into the sterile Petridis and agitated clockwise, anticlockwise, left to right, right to left for uniform dispersion. 2 mg of crude extract (*B. thuringiensis*) was dissolved in 1 mL of ethyl acetate extract that served as a stock solution. Filter paper discs were taken in a petridish and sterilized in oven at 110 °C for 1 h. Then 15 µL and 100 µL of the stock solution were placed on the paper discs separately by micropipette to obtain 30 µg/disc and 200 µg/disc respectively. The discs were then air-dried. Here, kanamycin (30 µg/disc) used as a standard discs. The sample impregnated discs and standard disc were placed gently on the solidified agar plates seeded with the test organisms to ensure contact with the medium. Then the plates were kept in a refrigerator at 4 °C for 4-6 h in order to provide sufficient time to diffuse the compounds into the medium.

After incubation, the antibacterial activities of the test samples were determined by measuring the radius of the inhibition zones (Barry, 1986; Reinheimer *et al.*, 1990).

RESULTS

Isolation of bacteria

Bacterial strains were isolated from dump soil, which was described in materials and methods. In a preliminary experiment of this research, three bacterial strains were

spread on the two different agar plates for isolation and rapid identification of the xylanase-producing bacteria and



Figure 1: Xylanolysis colonies on xylan agar plate are surrounded by a clear, colourless zone after 48 h incubation at 37 °C

xylanolytic properties. The plate was then incubated at 37 °C for 48 h. the colonies which formed clear zone on the xylan agar plates were picked, and then further purified by pure colonies producing bacteria on the xylan agar plate were shown in Figure 1.

Antibiotic resistance and sensitivity test

The three drug resistant strains were isolated by disc diffusion method on nutrient agar plates against some commonly used antibiotics to see the resistance pattern of these strains. All strains showed multiple resistance to the antibiotic used .Resistant and sensitive strains were separated according to their diameter of zone of inhibition produced around the antibiotic disc and growth type and number of colonies on clear zone and comparative study with control strains (sensitive). The result of drug resistance pattern on nutrient agar showed that the three strains were resistant to amoxicillin and neomycin. Whereas, sensitive to doxycyclin, erythromycin, chloramphenical, cephalaxin, kanamycin, ampicillin, steptomycin and vancomycin (Figure 2). The results of overall antibiotic sensitivity tests on the agar plates have shown in the Figure 3.

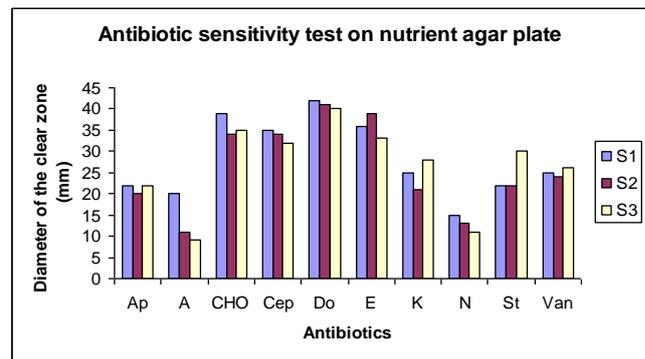
Antibiotic spread plate method against different selection concentration of antibiotics

Antibiotic resistance strains through the disc diffusion method were also reisolated and again the drug resistant were confirmed by antibiotic spread plate method with different selective antibiotic concentration (10, 20, 30, 50,100 µg/mL). The antibiotics used were amoxicillin and neomycin for confirmation of drug resistance of those strains was isolated from disc diffusion method. The bacterial samples of *B. thuringiensis* was remarked as highly resistant colonies on these antibiotic plates indicated that all these strains were resisstant to these selective concentrations of antibiotics. While no growth at next other concentrations indicated that these strains were

sensitive to these selective to these concentrations of antibiotic.



Figure 2: Antibiotic sensitivity and resistant pattern strain (S1) on xylan agar plate



Ap = Ampicillin, E = Erythromycin
 A = Amoxicillin, K = Kanamycin
 CHO = Chloramphenical, N = Neomycin
 Cep = Cephalaxin, St = Steptomycin
 Do = Doxycycline, Van = Vancomycin
 S1: Strain 1; S2:Strain 2; S3: Strain 3

Figure 3: Antibiotic sensitivity test on nutrient agar plate

Antibacterial activity

Generally, antibacterial screening is for primary selection of the compounds as therapeutic agent. The antibacterial screening is done by primary assay, a qualitative assay to determine whether the compounds are antibacterially active or not. As shown in Table 2. A 30 µg/mL of the ethyl acetate extract of the cultural broth of *B. thuringiensis* bacteria strains, showed antibacterial activity against the tested pathogenic bacteria. The maximum antibacterial activities were observed against *Pseudomonas aeruginosa* for the tested extract. The strains showed a relatively lower inhibitory effect on *Shigella dysenteriae*.

DISCUSSION

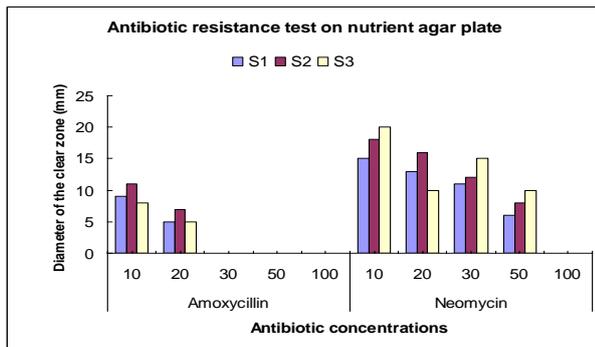
In this study, bacterial strains were isolated from dump soil, which degraded α-1,4 xylan and to belong *B.*

thuringiensis. It was observed that, the strains were resistant to two antibiotics i.e. amoxicillin and neomycin (Figure 4). The antibiotic resistance in each of these three

strains was confirmed by antibiotic spread plate method using amoxycillin (0.5 mg/mL) and neomycin (0.5 mg/mL)

Table 2: Antibacterial activity of the ethyl extracts (30 µg disc⁻¹) of *B. thuringiensis* strains against standard kanamycin (30 µg/disc)

Strain No.	<i>Bacillus subtilis</i>	<i>Sarcina lutca</i>	<i>Shigella dysenteriae</i>	<i>Shigella sonnei</i>	<i>Pseudomonas aeruginosa</i>
S1 (EA 30 µg/disc)	9±0.05	11±0.10	1±.05	4±.20	13±.30
S2(EA 30 µg/disc)	6±0.02	10±0.10	1±.05	3±.1	10±.20
S3(EA 30 µg/disc)	10±0.05	11±0.10	2±.05	5±.02	14±.30



S₁=Strain 1, S₂=Strain 2 and S₃=Strain 3

Figure 4: Antibiotic resistance test on nutrient agar plate

in 10, 20, 30, 50 and 100 µg/mL concentration on nutrient agar plates.

Present investigation suggests that the ethyl acetate extract of the cultural broth of *B. thuringiensis* bacteria (strain1) contained some antibacterial components, which have activity against both Gram positive and Gram-negative bacteria [Figure 5 (a, b, c, d and e)]. All of the *B. thuringiensis* isolates were resistant to amoxicillin, ampicillin, ceftriaxone, penicillin and oxacillin while susceptible to the remaining antimicrobials (Luna *et al.*, 2007). Xylanase producing *Aeromonas* strains were resistant to amoxycillin, ampicillin, cotrimazole, and sensitive to erythromycin, tetracycline and doxycyline (Roy and Abedin, 2002; Roy *et al.*, 2003).

The high level of drug resistance in tested bacterial strains could be related to the production of antimicrobial compounds found in some fish-farm isolates belonging to the genus *Bacillus*. The antimicrobial activity and resistance to antimicrobials of bacteria from a sediment habitat can help explain the selection and persistence of such strains in this particular ecology (Chelossi *et al.*, 2003).

Strains of *B. thuringiensis*, *B. subtilis*, *B. stearothermophilus*, *B. licheniformis*, *B. megaterium* and *B. cereus* have been reported to produce substances like bacteriocin. Bacteriocins are ribosomally synthesized antimicrobial peptides produced by a number of different bacteria (Parker and Collier, 1990). As for the inhibitor

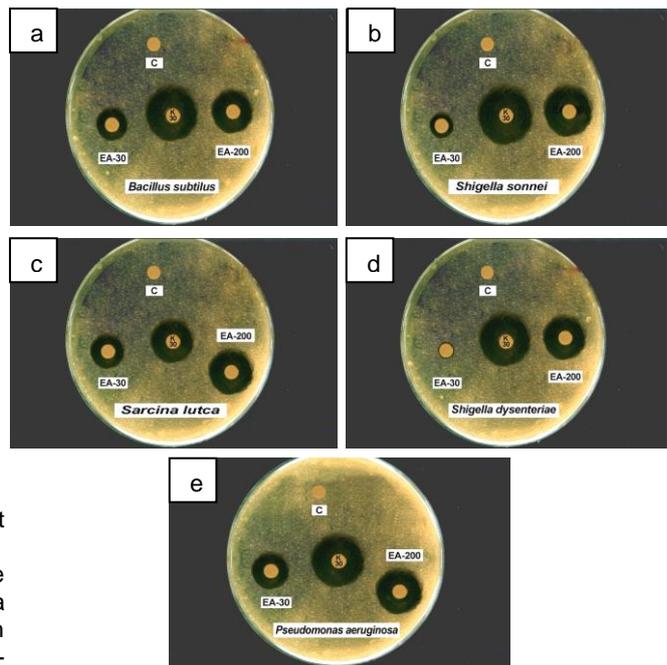


Figure 4: Antibacterial activity of the ethyl acetate extracts of strain S₁ and standard kanamycin (30 µg/disc)

effect of isolates; it was observed that *B. thuringiensis* D1, *B. thuringiensis* D3, had an inhibitory effect on all of the four test bacteria (Aslim *et al.*, 2002).

B. thuringiensis showed an antimicrobial effect on gram (+) bacteria and *B. thuringiensis* produced thuricin. The *Bacillus* strains isolated from soil were tested on the antibiotics ampicillin, kanamycin, streptomycin, chloramphenicol, tetracycline, erythromycin, gentamicin and novobiocin proved to be resistant to four different antibiotics (Bernhard *et al.*, 1987), it is recommended that this strain be used in certain biotechnological studies (Chelossi *et al.*, 2003).

A large variety of specific biochemical functions, such as fertility, resistance to antimicrobial drugs, production of bacteriocins, and production of toxins, have been attributed to some plasmids (Cohen, 1976; Bernhard *et al.*, 1978).

For the treatment of *B. thuringiensis* and other *Bacillus* infections, there is little advice found for treatment. Yet various *Bacillus* species demonstrated small populations with some form of resistance to clindamycin and erythromycin. Therefore the wider choice of newer antimicrobials can be useful in treating an infection.

In conclusion, this paper has broadened the number of antimicrobials potentially useful against *B. thuringiensis*. Sensitive to doxycycline, erythromycin, chloramphenicol, cephalaxin, kanamycin, ampicillin, streptomycin and vancomycin testing by disc diffusion method. Resistance in these species to amoxicillin and neomycin was confirmed by antibiotic spread plate method.

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