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ANTIBACTERIAL AND CYTOTOXIC ACTIVITIES OF BIOACTIVE COMPOUNDS FROM BACTERIA ISOLATED FROM BURIGANGA RIVER WATER

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Abstract

A study was conducted to assess the water quality of the Buriganga River, focusing on bacterial screening, antimicrobial activity, and cytotoxicity effects. The bacterial load was significantly high, whereas pH levels were satisfactory. Out of 65 bacterial colonies 5 isolates were provisionally identified as *Bacillus* sp., *Aeromonas* sp., *Bacillus subtilis*, *B. cereus*, and *Enterobacter aerogenes* exhibiting antimicrobial activity against six pathogenic food bacteria. Based on the 16S rRNA gene sequencing method, 3 bacterial isolates were identified as *Enterobacter* sp., *Priestia aryabhattai*, and *Klebsiella pneumoniae*. In the cytotoxicity test, these 5 isolates revealed no toxic effects on HeLa and Vero cell lines. Biochemical tests revealed the presence of starch, casein, and pectinase.

Introduction

Antibacterial activity refers to the ability of a substance to inhibit the growth or kill bacteria. This concept is fundamental to understanding how various agents can be utilized to control bacterial infections (Ligon 2004). For the pharmaceutical sector, microorganisms have proven to be a valuable source of bioactive natural compounds (Sekurova *et al.* 2019).

Numerous secondary metabolites produced by microorganisms are essential to human health. These bioactive substances, including mycotoxins and bacteriocins made by bacteria, have been used to make significant medications, including actinomycin, vancomycin, streptomycin, and erythromycin (Falkinham *et al.* 2009). Distinct chemicals with novel structures were produced by river bacteria (Boobathy *et al.* 2009, Gram *et al.* 2010).

Bacterial cytotoxic activity is an important subject of study having applications in biotechnology, medicine, and the environment. Understanding bacterial pathogenesis, antibiotic resistance, and the creation of novel therapeutic approaches all depend on this activity (Martins *et al.* 2008). Therefore, the current study looks at the process of separating bioactive potential bacteria from the water of the Buriganga River, characterizing and identifying the bacteria, figuring out the *in vitro* antibacterial activities of specific bacterial species and assessing the cytotoxicity of cancer and non-cancer cell lines.

Materials and Methods

Water samples were collected from the ten areas of the Buriganga River and were gathered in plastic bottles that had been disinfected with ethanol. The sampling area were Wize Ghat, Bina Smriti Ghat, Lalkuthi Ghat, Nawabpur Kheya Ghat, Ispahani Ghat, Nagormohol Ghat, Alom Tower Ghat, Khaja Market Ghat, Tel Ghat and Shyambazar Ghat.

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The pH of the collected samples was measured by a pH meter (HANNA HI 8424) immediately after the samples were brought into the laboratory. Collected samples were preserved in a refrigerator at 4ºC. Nutrient Agar (NA) medium having pH 7.0 ± 0 was used for the enumeration and isolation of aerobic heterotrophic bacteria present in water samples. Serial dilution (Clesceri *et al.* 1998) and spread plate techniques (Sharp and Lyles 1969) were used to enumerate and isolate bacteria. After spreading the sample, the inoculated plates were placed invertedly and incubated at 37°C for 24 hrs in an incubator. After 24 hrs of incubation, the plates having well-discrete colonies were selected for counting. After counting, individual bacterial colonies were immediately isolated. Based on morphological characteristics, primary selection was done, and the selected bacterial isolates were purified by the streak plate method. Significant physiological and biochemical tests including Gram staining were performed for the provisional identification of the selected isolates. Provisional identification of Gram-positive bacteria was performed according to Bergey's Manual of Systematic Bacteriology (Sneath *et al.* 1986) and Gram-negative bacteria were identified following the Manual of WHO (Krieg and Holt 1984). The selected isolates were subjected to antimicrobial tests by giant colony technique against six pathogenic bacteria, such as *Escherichia coli*, *Staphylococcus aureus*, *Listeria* sp., *Enterococcus faecalis*, *Klebsiella pneumonia* and *Vibrio cholerae*.

For the cytotoxicity test, overnight cultures of the selected bacterial isolates were suspended in phosphate buffer saline (PBS) and pelleted by centrifugation at 12,000 rpm for 10 min at 4°C. The pellets were resuspended in 1.5 ml of PBS and to prevent overheating, the resuspended pellets were sonicated on ice for 5 min. After sonication, samples were centrifuged at 12,000 rpm for 10 min at 4°C. Cell-free supernatants (CFS) were collected and filter-sterilized via 0.2 µm filters (McCoy *et al.* 2021, Mondal *et al.* 2024). The Vero cells are kidney epithelial cells of African green monkeys, which were collected from the Cell and Tissue Culture Research Laboratory, Centre for Advanced Research in Sciences (CARS), University of Dhaka, Bangladesh and maintained in DMEM (Dulbecco's Modified Eagles' medium). Trypsin was used for the detachment of monolayer cells and the preparation of cell suspensions. Viable cells were counted by a hemocytometer, and the cell suspension was diluted with DMEM containing 10% FBS in order to obtain a final density of $15x10^4$ cells/ml. 48-well plates were seeded with 200 microliters of cell suspension per well and incubated for cell attachment at 37°C and 5% CO_2. The Vero cells were treated with the test sample CFS after 24 hrs. Bacterial growth medium served as a control. After the addition of the bacterial cell-free extracts, plates were incubated for an additional 48 hrs at 37°C, 5% CO_2 and 100% relative humidity. After 48 hrs of incubation, cytotoxicity was examined under an inverted light microscope (Optika, Italy). Duplicate wells were used for each sample.

Molecular techniques have also been used to identify the bacteria more specifically. Techniques utilizing the 16S rRNA gene sequence data have been developed for use in the field of molecular-level identifications. For the partial amplification of 16S rRNA gene, the following primer pairs were used: 27F- AGAGTTTGATCCTGGCTCAG (Lillo *et al.* 2006) and 1492R-GGTTACCTTGTTACGACTT (Lillo *et al.* 2006). The bacterial isolates were cultured to grow as a single colony and subjected to heat in 50 µl sterile deionized water in a dry bath incubator for 5 min. The lysed cell suspension was centrifuged (1 min, 13000 rpm) in a microcentrifuge (LABOGENE, SCAN SPEED 1524), and the supernatants were used as the source of template DNA for PCR amplification of the 16S rRNA gene. The amplified products were separated electrophoretically on 1% agarose gel. The gel was prepared using 1.0 g agarose powder containing 100 ml 1xTAE buffer. Agarose gel electrophoresis was conducted in 1xTAE buffer at 100 volts for 20 min. One molecular weight marker, 1 kb DNA ladder, was electrophorised along

side the amplified sample DNA. DNA bands were observed on a UV-transilluminator and photographed by a gel documentation system (MS Major Science UVDI, Taiwan).

The bacterial isolates can be identified using an alignment of the partial sequence of the 16S rRNA gene with the existing sequences in the database. Different samples were used to amplify their 16S rRNA gene, and the PCR-amplified DNA of the bacterial isolates was purified using a commercial purification kit (PROMEGA, Wizard SV Gel and PCR CleanUp System) and sent for automated sequencing.

Results and Discussion

The physico-chemical properties of the collected water samples are presented in Table 1. The mean temperature of the water samples ranged between 23°C and 28°C, with the highest temperature (28°C) recorded during the rainy season and the lowest (23°C) in winter. Therefore, in the present study, the observed temperature values were close to those reported by Saha *et al.* (2017).

Table 1. Physico-chemical parameters and bacterial load (cfu/ml) of the collected samples.

Sl. No.	Sampling site	Sampling date	Sampling season	pH	Temperature (°C)	Aerobic bacteria (cfu/ml)
01	Wize Ghat	14.08.23	Rainy	7.38	28	1.03×10^{8}
02	Bina Smriti Ghat	12.09.23	Rainy	6.94	28	2.88×10^{8}
03	Lalkuthi Ghat	01.10.23	Rainy	7.71	26	1.54×10^{8}
04	Nawabpur Kheya Ghat	16.12.23	Winter	7.43	27	1.4×10^{8}
05	Ispahani Ghat	18.01.24	Winter	6.88	23	8×10^{7}
06	Nagormohol Ghat	22.01.24	Winter	7.59	23	5.9×10^{7}
07	Alom Tower Ghat	31.01.24	Winter	6.94	24	4.5×10^{7}
08	Khaja Market Ghat	31.01.24	Winter	7.53	24	7×10^{7}
09	Tel Ghat	07.02.24	Winter	7.75	24	4.8×10^{7}
10	Shyambazar Ghat	07.02.24	Winter	7.77	26	2×10^{8}

The pH is a key variable that indicates pollution levels in any aquatic environment and the pH ranged between 6.88 and 7.77. In the rainy season, the highest pH (7.71) was recorded in Lalkuthi Ghat and the lowest in Bina Smriti Ghat. In the winter season, the highest pH (7.77) was recorded in Shyambazar Ghat and the lowest (6.88) in Ispahani Ghat. Saha *et al.* (2009) reported the similar result in the Buriganga River during the rainy season and winter season. The pH range of the river water was normal according to the Bangladesh standard, and no seasonal variation was found in the case of pH during both the rainy and winter seasons.

The present investigation for the enumeration of the bacterial load in water samples showed that the total aerobic heterotrophic bacterial load ranged from 4.5×10^{7} to 2.88×10^{8} cfu/ml in the Buriganga River, which is not satisfactory. Bina Smriti Ghat showed the highest count of bacteria at 2.88×10^{8} cfu/ml, expressing a serious threat for public health due to the excessive level of bacterial contamination. Saha *et al.* (2009) reported more or less similar results. Saha *et al.* (2009) also reported the maximum count of heterotrophic bacteria in the Buriganga River varied from 1.0×10^{5} to 42.0×10^{5} cfu/ml.

In the present investigation, 65 bacterial colonies were isolated from the selected water bodies, out of which 15 were finally chosen based on their size, shape, elevation, margin, texture, appearance, color and optical density. 15 isolates were tested against 6 pathogenic bacterial species, and 5 were selected after the antimicrobial test. The tested bacteria were *Escherichia coli*, *Staphylococcus aureus*, *Listeria* sp., *Enterococcus faecalis*,*Klebsiella pneumonia*,and *Vibrio cholerae*. These bacteria were provided by the Food Microbiology Lab, CARS, Dhaka University. F/3 isolate exhibited the maximum antagonism activity against all the tested bacteria and F/1 also demonstrated significant activity against 5 bacteria by inhibiting their growth on culture medium. The highest inhibition of F/8 and F/11 to *Enterococcus faecalis* was 18 mm. *Escherichia coli* and *Enterococcus faecalis* were more susceptible, and *Listeria* sp. was less susceptible to all selected isolates, expressing potential antagonistic activities of the isolates against harmful food pathogens. The result of this test is shown in Table 2.

Table 2. Antagonism of selected bacterial isolates against different pathogenic bacteria.

Pathogenic bacteria	Antagonism of selected bacterial isolates (mm)				
	F/1	F/3	F/5	F/8	F/11
Escherichia coli	9	9	8	6	6
Staphylococcus aureus	-	3	3	3	2
Listeria sp.	13	3	-	-	-
Enterococcus faecalis	13	12	16	18	18
Klebsiella pneumoniae	3	3	-	-	6
Vibrio cholerae	11	6	4	8	-

"sign indicates negative results".

Among the 5 isolates, 2 isolates were Gram-negative rods, while 3 were Gram-positive rod-shaped bacteria. These were identified provisionally based on biochemical and physiological tests according to Bergey's Manual of Systematic Bacteriology and the Manual of WHO. Among the 5 isolates, 3 isolates showed clear bands after agarose gel electrophoresis and purification of extract DNA. The PCR-amplified DNA (Fig. 1) of the 3 isolates (1 kb ladder = lane 7, F/3 = lane 2, F/8 = lane 5, F/11 = lane 6) was purified using a commercial DNA purification kit (PROMEGA, Wizard SV Gel and PCR Clean-Up System, USA) and sent for automated sequencing. These bands were subjected to molecular identification based on 16s rRNA sequencing using a pair of bacterial universal primers. The identified bacteria were closely similar to *Enterobacter* sp. (F/3), *Priestia aryabhattai* (F/8),and *Klebsiella pneumoniae* (F/11).

A previous study, according to Gilliland and Speck (1977), showed that crude cell-free supernatant separated from *Lactobacillus* broth exhibited antagonistic activity against Gram-positive isolates, *Bacillus cereus, B. subtilis*,and *Staphylococcus aureus*, while could not inhibit the growth of *Escherichia coli* and *Pseudomonas aeruginosa.* The present study of biochemical tests has revealed the presence of three significant bioactive compounds, such as starch, casein and pectinase, which are the key enzymes produced by the above 5 bacterial isolates, whereas four (F1, F3, F5 and F11) demonstrated the ability to produce pectinase, respectively. In the case of casein production, three isolates (F5, F8 and F11) were able to generate this bioactive compound. However, this finding highlights the potential of these bacterial isolates to produce valuable bioactive compounds, which could be explored for various industrial or therapeutic applications, depending on the specific bioactivity and enzyme capabilities of each isolate.

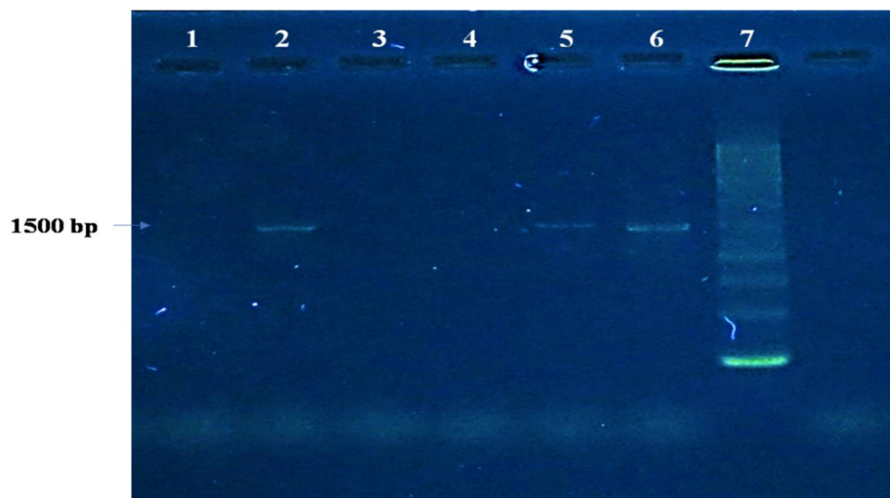

Fig. 1. PCR amplification part of the 16s rRNA gene. (1 kb ladder = lane 7, F/3 = lane 2, F/8 = lane 5, F/11 = lane 6).

In the cytotoxicity test, Vero and HeLa cell lines treated with the cell-free extract of all the selected isolates demonstrated a negative effect of cytotoxin production, and they almost looked like the solvent. As all bacterial extracts showed negative results in the Vero cell line, indicating that they are non-toxic to these cells. Furthermore, the cell morphology remained normal, suggesting that the bacteria under study are not harmful to both humans and animals. This suggests that if antibiotics or bioactive compounds could be isolated from these bacteria, they may have potential therapeutic value for human use (Fig. 2).

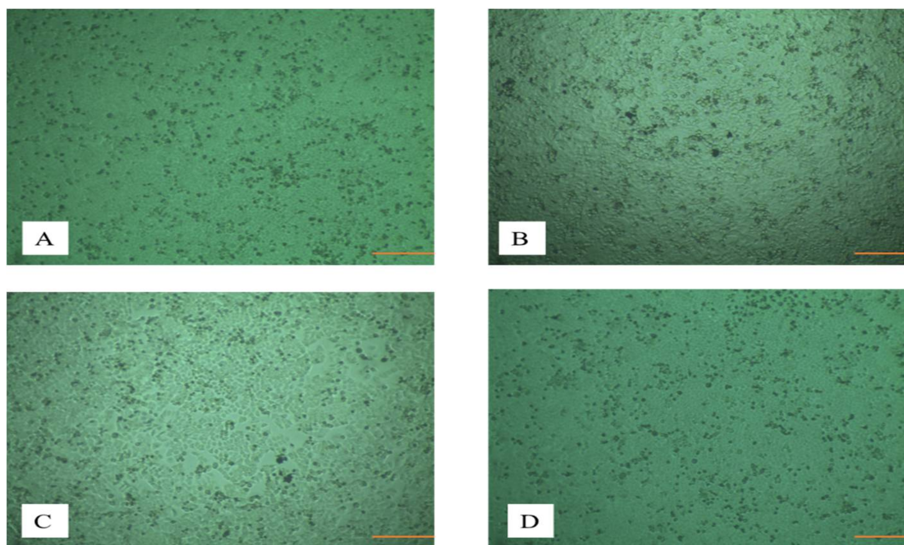

Fig. 2. Cytotoxic activity of cell free extract of selected bacteria on Vero cell line: A. Control, B. F/1, C. F/3, and D. F/5.

In addition, the bacterial extracts also produced negative results in HeLa cells, with no observed changes in cell morphology. This indicates that the bacterial extracts are non-toxic to these human cancer cells. While the extracts did not affect HeLa cells, this could suggest the potential for selective activity against other cancer cell lines (Fig. 3).

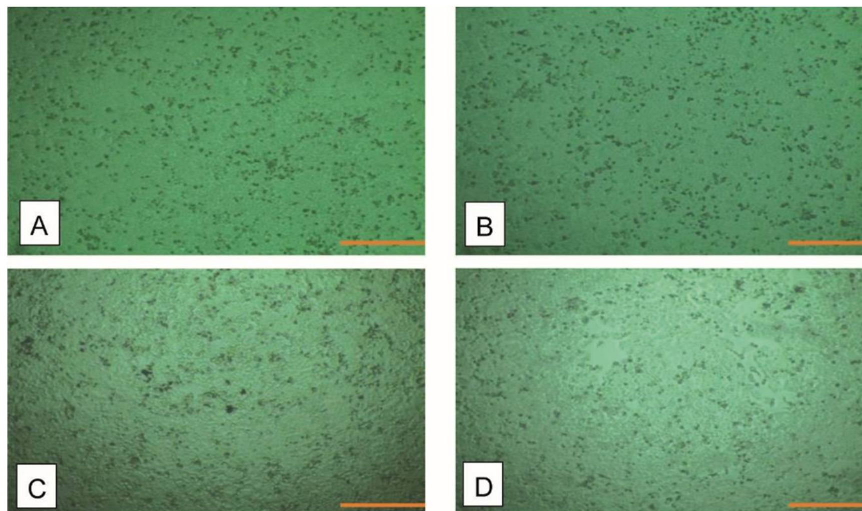

Fig. 3. Cytotoxic activity of cell free extract of selected bacteria on HeLa cell line: A. Control, B. F/1, C. F/3 and D. F/5.

The findings of this study highlight the bioactive potential of bacterial isolates from the Buriganga River in producing valuable enzymes and exhibiting antimicrobial activity. These isolates may hold promise for future pharmaceutical or industrial applications, especially given their non-toxic nature and antagonistic effects against pathogenic bacteria.

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