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CHARACTERIZATION AND ANTIBIOGRAM PROFILE OF BACTERIA ISOLATED FROM BURIGANGA RIVER

MD ABDUL KARIM* AND REHENA NASRIN HAPPY

Department of Botany, University of Dhaka, Dhaka-1000, Bangladesh

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Abstract

Selected bacterial isolates from the surface water of Buriganga river were characterized by morphological, biochemical characteristics and sequence-based PCR-amplified fragments of 16S rRNA. All isolates were rod shaped and Gram-positive. The isolates were confirmed as *Chryseobacterium arthrosphaerae* strain FDAARGOS 519, *Bacillus cabrialesii* strain TE 3, *Bacillus tequilensis* KCTC strain 13622(T), *Bacillus amyloliquefaciens* DSM 7 strain ATCC 23350, *Bacillus subtilis* strain E20 and *Bacillus subtilis* subsp. *subtilis* strain 168 based on sequence analysis. A phylogenetic tree was constructed that showed only one major cluster comprising of two sub-clusters grouping *Chryseobacterium arthrosphaerae* in one and *Bacillus tequilensis* in another. *Chryseobacterium arthrosphaerae* strain FDAARGOS 519 was susceptible to all antibiotics at different ranges, while *Bacillus tequilensis* KCTC strain 13622(T) and *Bacillus amyloliquefaciens* DSM 7 strain ATCC 23350 were found to be resistant to polymyxin only but sensitive to other antibiotics. However, *Bacillus cabrialesii* strain TE 3 was resistant to polymyxin and neomycin, while *Bacillus subtilis* subsp. *subtilis* strain 168 was resistant to polymyxin, vancomycin and rifampicin.

Introduction

The river Buriganga is considered a water resources and its contribution to the biodiversity of the area as well as environmental balance must be recognized. Water resources of Dhaka city is the most important and is the burning issue in terms of extreme degradation of water quality of the surrounding water bodies. The chief sources of water pollution are sewage and other wastes, industrial effluents, agricultural discharges and industrial wastes from chemical industries, fossils fuel plants and other power plants. Each day 3,500 m^3 of wastes from other industrial areas are being discharged through 22 large outlets along the banks into the Buriganga (Khan 2005). The discharge of such pollutants into the river Buriganga is causing deterioration of water quality of this river for about two decades (Ahmed *et al.* 1988, Rahman *et al.* 1996). They create great problem of water pollution rendering water no longer fit for drinking, agriculture, and as well as for aquatic life. Research studies showed that pollution affects not only on fish, but also on various aquatic species throughout the food chain.

The water pollution problems are very complex, invisible and difficult to investigate. Thousands of chemicals are present in the water and sediments - some are toxic, some build up in the food chain and become toxic, and some are toxic only when combined with other chemicals. Many are present in such small amounts that they are hard to measure (EPA 1997). The World Health Organization (WHO) estimated that about 80% of ill health especially in developing countries are water related (Cheesbrough 2000). The access of fecal matter to water through direct contamination of surface run-off or sewage may add a variety of pathogens (Karim *et al.* 2012). Atiribom *et al.* (2007) reported that high concentrations of bacteria and nitrates discharged into water can occur from animal husbandry operations. With the increase in the interest in the microbial ecology of aquatic environments, it is important to understand the autochthonous microbial flora. Since the bacteria are well known agents of mineralization and transformation

*Author for correspondence: <akarim@du.ac.bd>.

of organic and inorganic matters in aquatic ecosystem, the prevalence and characterization of some selected heterotrophic bacteria in the river Buriganga were investigated.

Materials and Methods

Water samples were collected into sterile glass bottles (Schott, Duran, Germany) from 30 cm below the surface at two selected sites of the river Buriganga and were kept into an ice box. After collection, the samples were labeled properly and brought back to the laboratory as soon as possible. Collected water samples were preserved in a refrigerator at 4ºC before and after the analyses. Nutrient agar (NA) medium was used for the enumeration and isolation of aerobic heterotrophic bacteria. The pH of the medium was adjusted to 7.0 since most of the samples were within the range of 6.6 - 7.1. Serial dilution plate technique (Greenberg *et al.* 1998) was used for the isolation of microorganisms. Plating in duplicate plates was made for each diluted sample. One ml of each of the diluted sample was taken in a sterilized Petri plate by micropipette with sterile tips. Then molten agar medium was poured and mixed thoroughly by rotating the Petri plate clockwise and anticlock wise. After solidifying the medium the plates were inverted and incubated at 37°C for 24 hrs in an incubator (Memmert GmbH + Co kg 8540 Sehwabach). After 24 hrs of incubation the plates, based on colonial morphology, different well discrete aerobic bacterial colonies were isolated and were purified through streak plate method. The purified isolates were then transferred on nutrient agar slant and preserved as stock culture in a low temperature incubator at 4°C for further study. The bacterial colonies on plating medium were morphologically studied as their form, elevation, margin, surface, pigmentation, opacity, whether grown inside, at the bottom or on the surface of the medium and their rate of growth.

Important cultural, morphological and biochemical characteristics were studied for the identification of the selected isolates. Different biochemical tests, *viz*., catalase, oxidase, carbohydrate fermentation, deep glucose agar, tyrosine degradation, arginine hydrolysis, casein hydrolysis, starch hydrolysis, gelatin hydrolysis, protease, urease, Levan formation, methyl red, nitrate reduction, indole production, phenylalanine deamination, citrate utilization, utilization of propionate, Voges-Proskauer (VP) test etc. were carried out.

All the bacterial isolates were cultured in LB agar to grow single colony and one colony for each was resuspended in 50 µl sterile miliQ water and subject to heat lysis by heating at 100°C water bath for 5 min. The lysed cell suspension were centrifuged for 1 min at 13000 rpm in microcentrifuge and then the supernatants were used as the source of template DNA for PCR amplification of 16S rRNA gene, it was stored at –20°C for further use.

The primer of following sequences CC [F] 5′- CCAGACTCCTACGGGAGGCAGC and CD [R] 3′-CTTGTGCGGGCCCCCGTCAATTC were used for the partial amplification of 16S rRNA gene and for automated sequencing (Moghaddam *et al*. 2018).

Twenty five µl of PCR master mixture (PCR buffer, dNTPs, DNA Taq polymerase, sterile miliQ water, primers and template DNA) was added to an oil free thermal cycler (UNO II). The amplifications were repeated 30 times for initial denaturation 5.0 min at 95°C, denaturation 1.0 min at 94ºC, primer annealing 30.0 sec at 55ºC, polymerization 1.0 min at 72°C, final extension 10.0 min at 72ºC and the final product was stored at 4ºC for further process. The PCR product was run in 1.0% agarose gel electrophoresis under UV light after ethidium bromide staining (Borst 2005). DNA bands were observed on UV-transilluminator and photographed by a Gel Documentation system (Microdoc DI-HD, MUV2I-254/365, Cleaver Scientific, UK). The amplified bands were gel purified using Gel purification kit (Invitrogen) and sequenced from Macrogen, South Korea.

The isolates were identified based on alignment of partial sequence of 16S rRNA gene with the existing sequences available in the database. The sequence generated from automated sequencing of PCR amplified DNA was analyzed through NCBI-BLAST (http://blast. ncbi.nlm.nih.gov/ blast.cgi) and rRNA BLAST (http://bioinformatics.psb.ugent.be/cgi-bin/rRNA/ blastform.cgi) programs to find out possible similar organisms in the databases. A phylogenetic tree of the isolates was constructed using neighbor joining (NJ) distance-based algorithm of phylogenetic analysis. Sequences obtained from BLASTN (nucleotide blast) were in FASTA format and relation between each sequence could be known by multiple sequence alignment using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). The data were analyzed to determine the descriptive statistics *viz.*, statistical mean and standard deviation (Sd) with SPSS v.16.0 for Windows (SPSS, SAS Institute Inc. Cary, USA).

The selected isolates were grown in different flasks containing media with various initial pH (4.5, 6.5 and 8.5) at 37°C and at various cultivation temperature (5, 10, 30, 40, 50, 55 and 65°C) maintaining initial pH of the medium at 7.0 to check the optimum pH and temperature for growth, respectively.

Antibacterial sensitivity test with gentamycin (CN-120), streptomycin (S-10), penicillin G (P-10), polymyxin B (PB 300), neomycin (N 30), vancomycin (VA 30) and rifampicin (RD 5) against the selected bacteria were tested for their ability to grow in the presence of different antibiotics at concentration selected for diagnostic value. The filter paper discs placed on the surface of Muller Hinton Agar (Atlas 1997) plates were inoculated with 0.1 ml of bacterial suspension. Inoculated plates were incubated at 37ºC for 24 hrs. Development of a clear zone around the disc indicated sensitivity while antibiotic disc without clear zone indicated resistance to the antibiotic.

Results and Discussion

The morphological characteristics of the selected isolates presented in Table 1 show that all selected isolates are rod shaped and all are spore former except BW-2. All isolates were found to be positive in oxidase, catalase, starch hydrolysis and casein hydrolysis test and negative in Voges-Proskauer (VP), methyl red (MR) and indole product test (Table 2). In nitrate reduction and

Table 1. Microscopic studies of the bacterial isolates.

Isolate No.	Vegetative cell	Spore	Sporangia	Diameter (μm)
BW-2	Short rod, rounded ends, occur singly	Non spore former		2.0 - 3.1 × 0.33 - 0.66
BW-7	Long rods, rounded ends, occur singly, non- motile.	Ellipsoidal	Not swollen	3.3 - 5.00 × 0.66
BW-18	Rods, rounded ends, occur singly, motile.	Ellipsoidal	Not swollen	3.63 - 4.62 × 0.66
BW-24	Short rods, rounded ends, occur in pair	Ellipsoidal	Swollen	1.99 - 4.29 × 0.66 - 0.99
BW-25	Rods, rounded ends, occur singly, motile.	Ellipsoidal	Swollen	2.64 - 3.63 × 0.66 -1.32
BW-37	Long rods, rounded ends, occur singly or in chain, motile	Ellipsoidal	Not swollen	1.2 - 2.4 × 0.4

hydrolysis of L-Arabinose test, only BW–2 showed negative and others were positive. The isolates BW-2 and BW-34 were found not to produce acid from D-Glucose, while others could do that, but

all isolates could not produce gas in the experiment. Except BW-2 and BW-7, all the isolates from other samples showed positive results in citrate utilization test (Table 2).

Identification of the isolates conducted by amplified DNA bands were found to be approximately 600 bp (Fig. 1). The isolates were identified as *Chryseobacterium arthrosphaerae* strain: FDAARGOS 519, *Bacillus cabrialesii* strain: TE3, *Bacillus tequilensis* KCTC strain: 13622(T), *Bacillus amyloliquefaciens* DSM 7 strain: ATCC 23350, *Bacillus subtilis* strain E20 and *Bacillus subtilis* subsp. *subtilis* strain 168 based on sequence analysis (Table 3).

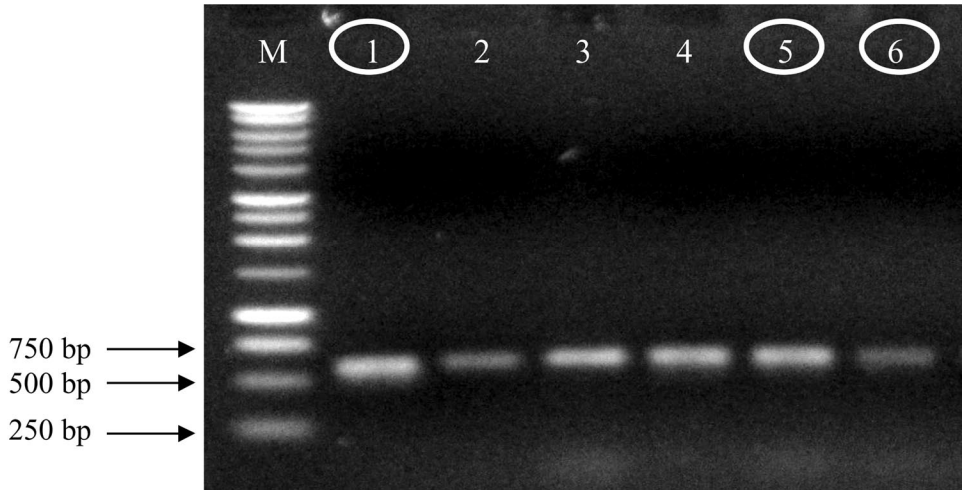

Fig. 1. Partial amplification of the 16S rRNA gene. Lane M = 1.0 kb ladder, lanes 1 - 6 are representing six different bacterial isolates *viz.*, BW-2, BW- 7, BW-18, BW-24, BW-25 and BW-37, respectively.

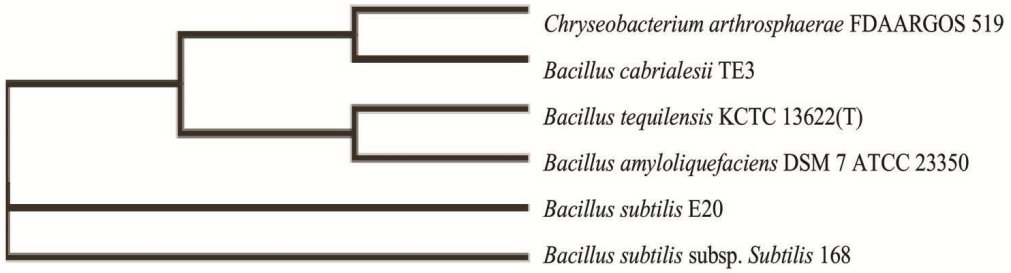

Fig. 2. Evolutionary relationships of the taxa. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987).

A phylogenetic tree of the six isolates was constructed using Neighbor Joining (NJ) distance-based algorithm (Fig. 2). Only one cluster comprising of two sub-clusters was detected. These sub-clusters were grouped *Chryseobacterium arthrosphaerae* in one cluster and *Bacillus tequilensis* in other. This observation suggested the evolutionary divergence of these bacteria and indicated their evolutionary trend.

Table 2. Biochemical characteristics of the bacterial isolates.

Isolate no.	Gram reaction	Oxidase	Catalase	Starch hydro-lysis	Casein hydro-lysis	Tyrosine degra-dation	VP	MR	Indole produc-tion	Nitrate reduc-tion	D-glucose		L-arabi-nose	Citrate utilization	Levan formation
											Acid	Gas			
BW-2	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–
BW-7	+	+	+	+	+	+	–	–	–	+	+	–	+	–	+
BW-18	+	+	+	+	+	–	–	–	–	+	+	–	+	+	–
BW-24	+	+	+	+	+	+	–	–	–	+	–	–	+	+	–
BW-25	+	+	+	+	+	–	–	–	–	+	+	–	+	+	–
BW-37	+	+	+	+	+	–	–	–	–	+	+	–	+	+	+

+ indicates the positive and – indicates the negative result.

Table 3. Sequence based identification of the selected bacterial isolates using bioinformatics.

Name of the isolates	Name and accession number of the bacterial isolates having highest similarity		Basis of the bioinformatics parameters		
	Accession No.	Scientific name	Strain	Max. coverage score	Identities match (%)
BW-2	NZCPO33811.1	*Chryseobacterium arthrosphaerae*	FDAARGOS 519	905	98.27
BW-7	NZRJVS01000032.1	*Bacillus cabrialesii*	TE3	933	98.49
BW-18	NZAYTO01000043.1	*Bacillus tequilensis* KCTC	13622(T)	806	95.07
BW-24	NC014551.1	*Bacillus amyloliquefaciens* DSM 7	ATCC 23350	773	93.22
BW-25	EU722405.1	*Bacillus subtilis*	E20	765	92
BW-37	NC000964.3	*Bacillus subtilis* subsp. *subtilis*	168	651	91.68

Selected bacterial isolates were allowed to grow within a varied range of temperature from 5 to 65°C; of which 40°C was most preferred by all isolates, while BW 7 and BW 24 both showed highest growth at 50°C and BW 25 was at 55°C (Fig. 3). It was found that four bacterial isolates

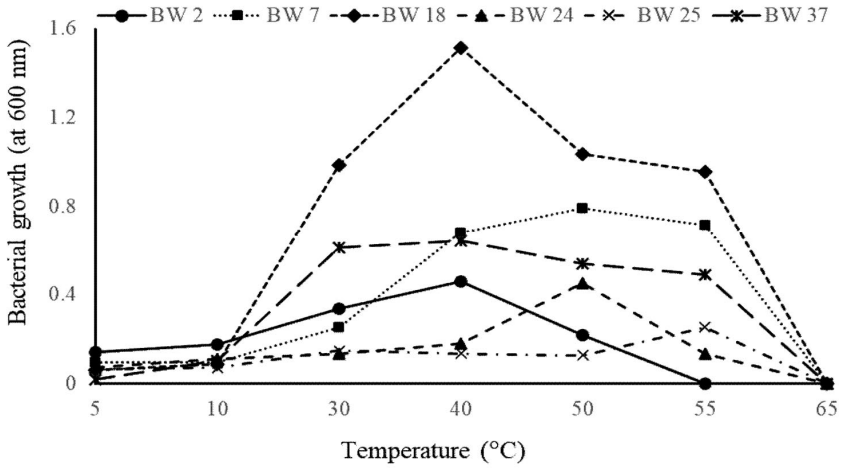

Fig. 3. Effects of temperature on bacterial growth.

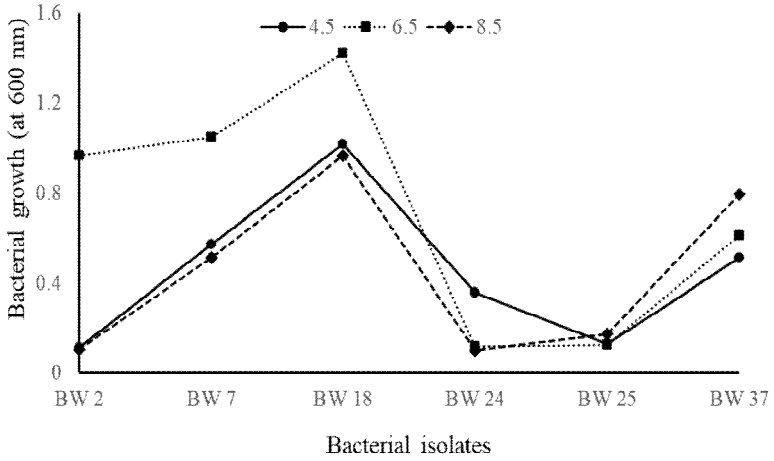

Fig. 4. Effects of pH on bacterial growth.

isolates grew better at pH 6.5, whereas BW 24 grew better at pH 4.5 and BW 37 grew better at pH 8.5 (Fig. 4). These differences were probably attributed by the pH differences in the aquatic habitat caused by the effluents changing the pH of the river water. Isolate BW 24 was found to be acidophilic in nature and grew well at pH 4.5, while BW 37 was found to be alkalophilic in nature and grew well at pH 8.5 (Fig. 4).

Table 4. Antibiogram of the selected isolates.

Isolate No.	Inhibition zone measured in diameter (mm)						
	Name of the antibiotics						
	CN 120	S 10	P 10	PB 300	N 30	VA 30	RD 5
BW-2	S (30)	S (21)	S (36)	S (4)	S (13)	S (13)	S (16)
BW-7	S (16)	S (13)	S (56)	R	R	S (7)	S (16)
BW-18	S (21)	S (16)	S (9)	R	S (15)	S (10)	S (18)
BW-24	S (18)	S (11)	S (8)	R	S (7)	S (9)	S (8)
BW-25	S (20)	S (13)	S (3)	R	S (10)	S (10)	R
BW-37	S (23)	S (4)	S (2)	R	S (15)	R	R

S = Sensitive, R = Resistant, CN 120 = Gentamycin, S 10 = Streptomycin, P 10 = Penicillin G, PB 300 = Polymyxin B, N 30 = Neomycin, VA 30 = Vancomycin, RD 5 = Rifampicin.

Chryseobacterium arthrosphaerae strain FDAARGOS 519 was susceptible to all antibiotics at different ranges (Table 4). *Bacillus tequilensis* KCTC strain 13622(T) and *Bacillus amyloliquefaciens* DSM 7 strain ATCC 23350 were found to be resistant to polymyxin only but sensitive to other antibiotics, while *Bacillus cabrialesii* strain TE 3 was resistant to polymyxin and neomycin. *Bacillus subtilis* strain E20 was resistant to polymyxin and rifampicin, but *Bacillus subtilis* subsp. *subtilis* strain 168 was resistant to polymyxin, vancomycin and rifampicin (Table 4).

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