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Original Article

Isolation and characterization of *Bacillus* sp. strain BC01 from soil displaying potent antagonistic activity against plant and fish pathogenic fungi and bacteria

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ABSTRACT

Fungal and bacterial pathogens infect a diverse range of hosts including various plant and animal species. Fungal and bacterial diseases, especially of plants and aquatic animals, such as fish, lead to significant damage to crops and aquaculture, respectively, worldwide. The present study was conducted to isolate and characterize potent *Bacillus* strains with significant antagonistic activity against the major plant and fish pathogenic fungi and bacteria. We randomly collected 22 isolates of *Bacillus* from the soil, rhizosphere, and sediment from different parts of Bangladesh. Initial characterization, based on *in vitro* antagonistic activity on the culture plate, resulted in the selection of four gram-positive *Bacillus* sp. isolates. Among these, the isolate BC01, obtained from soil demonstrated the highest broad-spectrum antibacterial and anti-fungal activities. We confirmed the genus of BC01 to be *Bacillus* by morphological and biochemical tests as well as using molecular data analysis tools, including the study of 16s rDNA, phylogenetic relationship, and evolutionary divergence scores. The isolate significantly inhibited the mycelial growth of the plant pathogen, *Penicillium digitatum* and fish pathogen, *Aphanomyces invadans in vitro*. The anti-bacterial effect of the isolate was also evaluated against *Pseudomonas* spp. and *Xanthomonas* spp., the two deadliest plant pathogens, and *Aeromonas veronii*, *Pseudomonas fluorescens*, and *Streptococcus iniae*, three major fish pathogens that are primarily responsible for global aquaculture loss. The results of the present study could pave the way for developing potent drugs to combat microbial infection of plants and fish.

1. Introduction

In recent years, a significant portion of agriculture and aquaculture production has incurred huge losses due to a disease outbreak. Diseases in plant and fish cause approximately 20% of crop damage and 15% of production loss in aquaculture annually [1,2]. Various preventive measures, including heavy use of pesticides and chemical compounds, pose a severe challenge to food security globally. For instance, *Penicillium digitatum* is the most dangerous pathogen of the citrus plant; it causes rot or mould and is recognized as a potential threat to the Rutaceae family [3]. Other bacterial species such as those belonging to *Pseudomonas* cause a variety of plant diseases with diverse symptoms, such as cankers, spots, blight, rot, and galls, while *Xanthomonas* spp. (especially *X. euvesicatoria* and *X. perforans*) cause spots and blights of leaf, stem, and fruits [4,5]. In fish, *Aphanomyces invadans* causes epizootic ulcerative syndrome (EUS) leading to similar damage [6]. Other widely known and potential disease-causing agents in fish include *Aeromonas veronii*, *Pseudomonas fluorescens*, and *Streptococcus iniae* that are responsible for motile aeromonad septicemia (AMS), bacterial hemorrhagic septicemia, and streptococcosis, respectively, in both carp and catfish [7,8]. The management of crops in the field and fish in the culture ponds by pesticides and disinfectants has proven to be difficult owing to their long-term adverse effects on the environment and biogeochemical cycle, thereby compelling to opt for more environment-friendly preventive approaches.

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A biological control is an approaches of controlling pest and pathogen in a natural way without affecting human and environment. An agent or organism which can kill or suppress the growth of any plant or animal pathogen, is called the biological control agent, BCA. BCA or natural enemy of the pathogens could be explored, as it either suppresses or reduces the effects of undesirable microbes and favors the growth of desirable and eco-friendly microbes and insects [9]. In the context of BCA application, *Bacillus* species have been in use for the past many years such that the genus is considered to be a prospective antibiotic-producing agent [10,11].

Species belonging to the genus *Bacillus* are gram-positive, spore-forming, rod-shaped, motile bacteria that are present in diverse environmental conditions but mostly in the soil. Their long-term survival in different harsh conditions is attributed to the production of endospores by the simple and rapid development of different reliable formulations [12]. These species provide protection to plants and fish against microbial infections through diverse mode of actions, e.g., through secretion of antibiotics, enzymes, volatile compounds, etc. [13–16]. The use of *Bacillus* as a BCA has also reported to cut down the cost of agriculture by suppressing the need for fertilizers and pesticides [17]. In addition, earlier reports reveal that antagonistic bacteria isolated from soil can be used as a source of potential antibiotics against fish diseases [18]. However, not a single study has reported an isolate that can potentially inhibit both plant and fish pathogens. Hence, any isolate that could considerably inhibit both kinds of pathogens would serve as a landmark reference in the field of BCAs.

Several molecular approaches are currently being utilized for the isolation of microbes. For instance, polymerase chain reaction (PCR) in combination with 16s rRNA sequencing tool is frequently used for rapid and sensitive identification of microbes [19]. This technique has been successfully employed for identification of *Bacillus* spp. from various environments including soil [20]. Moreover, many sequence analysis tools (i.e., phylogeny, p-distance analysis) enable precise determination of microbial clustering within a taxon and its functional role as a pathogen [21]. Therefore, the current study aimed to isolate and characterize the potent antibiotic-producing *Bacillus* spp. and elucidate the antagonistic spectrum properties against the major plant and fish pathogens.

2. Materials and methods

2.1. Isolation of bacterial isolates

Bacterial samples were collected from four different parts of Rajshahi and Sylhet, Bangladesh. These included the river Padma sediment, hill soil of Sylhet, rhizosphere soil of bean root from Saheb bazaar, and sediment of Chalan Beel during April and May 2016. From each place, the sample was collected from at least 10 cm of depth with a sterile inoculating loop/spoon. The sample was stored in a pre-sterilized Eppendorf and transported to the USDA-laboratory of the Shahjalal University of Science and Technology at ambient temperature. Then, the samples were air dried by heating at 70 °C for 1 h in a dryer.

2.2. Culture conditions

For the isolation of *Bacillus* sp., serial dilution technique was used considering different aqueous dilutions (10^{-1} to 10^{-4}) using phosphate buffered saline (PBS, pH 7.2). A sample from each dilution was then streaked on a nutrient agar (NA) plate amended with cycloheximide (100 μg mL^{-1}) to prevent fungal growth and incubated at 37 °C for 24 h [22].

2.3. Biochemical identification of Bacillus species

The suspected *Bacillus* colonies were identified on the basis of morphology and Gram staining. Subsequent identification tests included hemolysis, starch hydrolysis, gelatin hydrolysis, citrate test, Voges–Proskauer (VP) test, and growth at different pH and temperature [23].

2.4. Evaluation of antagonistic activity

The antagonistic activity of *Bacillus* sp. BC01 isolate was measured by cross streak method as described by Ran et al., 2012 [14]. A pure culture of *Bacillus* sp. BC01 isolate was first grown on the nutrient broth (NB) and incubated for 24 h at 37 °C. From the broth, 50 μl of *Bacillus* sp. culture was poured in 6 mm circular Whatman's filter paper prepared before using punching machine. The fungal and bacterial isolates, i.e., *Penicillium digitatum* (PGT01), *Aphanomyces invadans* (Aph01), *Pseudomonas* spp. (PSD05), *Xanthomonas* spp. (Xpp03), *Aeromonas veronii* (BN10), *Pseudomonas fluorescens* (PUKL2), and *Streptococcus iniae* (S20) were used from the stock sources of the USDA laboratory of the Shahjalal University of Science and Technology, Sylhet. Glucose-peptone (GP) broth and potato-dextrose broth (PDB) culture were used to grow *A. invadans* and *P. digitatum* respectively. While all other bacteria were grown on nutrient broth (NA) culture and incubated overnight at 37 °C for plant pathogenic fungi and bacteria and 25 °C for fish pathogenic bacteria. Then, 50 μl of bacterial culture was grown in nutrient agar (NA) medium by spread plate method and aseptically poured the previously prepared filter paper disc carefully on to the culture plate. In present study, we analyzed 12 h, 18 h and 24 h of bacterial culture for inhibition assay. After 24 h of incubation, the zone of inhibition was measured according to Foysal et al., 2011 [7].

2.5. Genomic DNA extraction and PCR preparation

The extraction of bacterial DNA was performed using a commercial genomic DNA extraction kit (Bio Basic Inc.; Ontario, Canada). Proteinase K and RNase A were added to remove protein and RNA contaminations and obtain good-quality DNA according to the manufacturer's instructions. The extracted DNA was quantified by gel electrophoresis using lambda (λ) DNA as the marker; it was then stored at −20 °C for further use. The PCR master mixture was prepared as a 50 μL solution containing 25 μL of 2 × concentra ted solution (Thermo Scientific, United States), 3 μL each of forward primer (8F, 5′-AGAGTTTGATCCTGGCTCAG-3′) and reverse primer (1492R, 5′-AGGAGGTGATCCAACCGCA-3′) for amplification of 16s rDNA universal sequence, 4 μL of template, and 15 μL of nuclease-free water [24].

2.6. Optimization of PCR

PCR amplification condition was optimized after several experimental trials using following parameters: An initial denaturation step at 94 °C for 4 min followed by a second denaturation step at 94 °C for 1 min, annealing for 1 min at 62 °C, an extension at 72 °C for 90 s, and a final extension step of 72 °C for 10 min. A total of 35 serial cycles of amplification reaction was performed.

2.7. Sequencing of 16s rDNA

The amplified PCR product was purified using PureLink PCR purification kit (Thermo Scientific) according to the manufacturer's guidelines. The purified PCR product was then sent to the first BASE sequencing center, Malaysia, for 16s rDNA sequencing. The

sequence received in raw format was then assembled for making contigs before further sequence analysis using MEGA 7.0.

2.8. Analysis of sequence data and phylogenetic relationship

The DNA sequences obtained from the NCBI nucleotide database were aligned using the muscle clustering program, MEGA 7.0. The phylogenetic tree was constructed using the neighbor-joining method with 1000 bootstrap replicates and using a *Streptomyces* sp. as the control strain. The scale bar indicated the number of differences in nucleotide substitutions per sequence. The GenBank accession numbers of the *Bacillus* species most similar to the bacterial isolates are indicated in parenthesis. Evolutionary divergence study was performed as a measurement of scoring similarity matrix; the lower score meant a close neighbor with a similar evolutionary origin. The total sequence variability was calculated as the average mean distance.

3. Results

3.1. Isolation and identification of bacteria

From NA culture, the colonies of prospective *Bacillus* sp. were identified according to Bergey's manual for the identification of *Bacillus* species modified by Wulff et al., 2002, a manual for the classification of *Bacillus* genus [23]. Based on the test results (Table 1), four *Bacillus* species, namely, BC01, BC02, BC03, and BC04, were selected for the further *in vitro* study.

3.2. Antagonistic activity of Bacillus sp. BC01 species

The *Bacillus* sp. BC01 reported potential inhibitory effect against all the tested microbes (plant and fish pathogens) in the *in vitro* antibiotic study. Study results revealed that overnight incubation culture (24 h) was most effective against tested isolates (Fig. 1). Other three isolates, however, failed to exhibit their potency against culture microbes. The highest zone of inhibition with *Bacillus* sp. BC01 was 16 mm for *Penicillium digitatum*, followed by 15 mm for *Xanthomonas* sp. *Aphanomyces invadans*, Pseudomonas sp., and *Aeromonas veronii*. *Streptococcus iniae* and *Pseudomonas fluorescens* moderately inhibited by the study isolate and showed 10 and 9 mm zone of inhibitions (Table 2).

3.3. DNA quantification and PCR product length

The amount of extracted DNA of *Bacillus* sp. BC01 isolate was approximately 20 ng as compared to the lambda DNA marker, and the protein-DNA ratio was around 1.8 measured using a spectrophotometer. This extracted DNA was used for PCR amplification. The PCR product of *Bacillus* sp. BC01 isolate after amplification by universal primers resulted in a band of 1484 bp on the agarose gel (Fig. 2).

3.4. Sequence analysis

After receiving the 16s rDNA purified product sequence from first BASE sequencing center, it was primarily edited using BioEdit (version 7.2) that removed the overlapping sequences and chimeras. Before editing, the NCBI homology BLAST showed a 99.9% similarity to *Bacillus subtilis* NBRC 13,719 and *Bacillus subtilis* BCRC10255. The phylogenetic tree analysis also supported the homology search and confirmed the closed positioning of *Bacillus* sp. BC01 to these two strains. Similarity score revealed a very low divergence of the study strain with *Bacillus subtilis* NBRC 13719 (0.0022) and *Bacillus subtilis* BCRC10255 (0.0030), indicating toward a same evolutionary origin (Fig. 2 and Table 3). *Bacillus amyloliquefaciens* BCRC 11601 also depicted a very high sequence similarity (0.0052) to *Bacillus subtilis* BC01 strain. The sequence had 99% homology and 100% coverage to over 100 strains of *Bacillus* sp. deposited in the NCBI data bank. The sequence is now available at the NCBI data bank under the accession number MF355364.1.

Here the numerical value corresponds to the following species: [1] *Bacillus* sp. BC01_(MF355364.1); [2] *B. subtilis* (NR_112629.1) NBRC_13719; [3] *B. cereus* (NR_112630.1) NBRC_15305; [4] *Bacillus* sp. (AJ704827.1) P9; [5] *B. filamentosus*

Table 1
Biochemical characterization of *Bacillus* sp. isolates.

Isolate	Source	Shape	Motility	Hemolysis	Starch hydrolysis	Gelatin hydrolysis	VP test	Citrate test	Growth at 6% NaCl	Growth at different pH				Growth at different temperatures (°C)			
										2	5	8	10	10	25	45	55
BC01-BC04	Soil	Rod	+	α	+	+	+	–	–	–	+	+	+	–	+	+	+

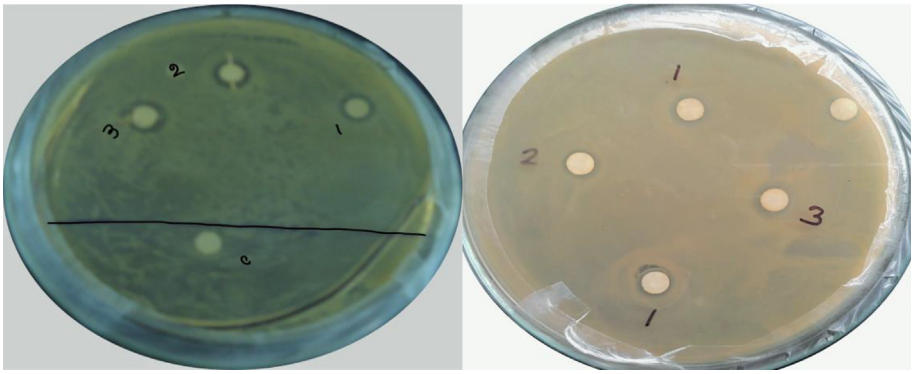

Fig. 1. Inhibitory effects of *Bacillus* sp. BC01 against plant pathogen *Penicillium digitatum* and fish pathogen *Aeromonas veronii* with three different incubation culture, 12 h (1), 18 h (2), and 24 h (3). Other disc used as a control for this study.

Table 2
Inhibitory effect of *Bacillus* sp. BC01 against various crop and fish pathogens (in mm).

Name of the tested Fungus and Bacterium	*Penicillium digitatum* (PGT01)	*Aphanomyces invadans* (Aph01)	*Xanthomonas* sp. (Xpp03)	*Pseudomonas* sp. (PSD05)	*Aeromonas veronii* (BN10)	*Pseudomonas fluorescens* (PUKL2)	*Streptococcus iniae* (S20)
Bacillus sp. BC01	16	15	15	15	15	9	10
Bacillus sp. BC02	9	8	8	–	7	–	–
Bacillus sp. BC03	7	–	7	–	–	–	–

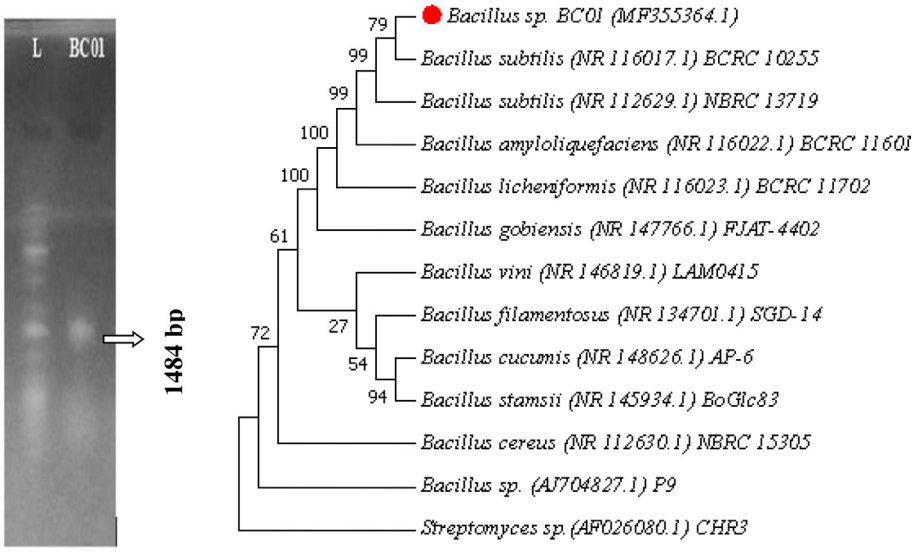

Fig. 2. The amplified 1484 bp PCR product of *Bacillus* sp. BC01 strain with universal sequencing primer (left) and phylogenetic relationship of strain *Bacillus* sp. BC01 (MF355364.1) with other *Bacillus* sp. isolates characterized from the soil, sediment, and rhizosphere samples having antimicrobial activity (right). The tree was constructed using the neighbor-joining method in MEGA7; the bootstrap consensus tree inferred from 1000 replicates was considered to analyze the taxa.

Table 3
Determining the evolutionary divergence between the sequences. The numbers of base differences per site from between sequences are shown. Much similar sequences marked in bold.

	1	2	3	4	5	6	7	8	9	10	11	12	13
1	–												
2	**0.0022**												
3	0.0582	0.0560											
4	0.0866	0.0843	0.0724										
5	0.0612	0.0590	0.0597	0.0799									
6	**0.0030**	**0.0007**	**0.0567**	**0.0851**	**0.0597**								
7	0.0149	0.0127	0.0552	0.0828	0.0590	0.0134							
8	**0.0052**	**0.0030**	**0.0582**	**0.0843**	**0.0582**	**0.0037**	**0.0112**						
9	0.0642	0.0619	0.0552	0.0731	0.0463	0.0627	0.0619	0.0619					
10	0.0373	0.0351	0.0612	0.0866	0.0545	0.0358	0.0299	0.0531	0.0619				
11	0.0530	0.0507	0.0500	0.0769	0.0455	0.0515	0.0478	0.0515	0.0448	0.0522			
12	0.0575	0.0552	0.0649	0.0843	0.0493	0.0560	0.0522	0.0552	0.0336	0.0567	0.0500		
13	0.1978	0.1955	0.1888	0.1985	0.1910	0.1963	0.1963	0.1933	0.1851	0.1970	0.1866	0.1873	–

(NR_134701.1) SGD14; [6] *B. subtilis* (NR_116017.1) BCRC_10255; [7] *B. licheniformis* (NR_116023.1) BCRC_11702; [8] *B. amyloliquefaciens* (NR_116022.1) BCRC_11601; [9] *B. cucumis* (NR_148626.1) AP–6; [10] *B. gobiensis* (NR_147766.1) FJAT–4402; [11] *B. vini* (NR_146819.1) LAM0415; [12] *B. stamsii* (NR_145934.1) BoGlc83; [13] *Streptomyces* sp. (AF026080.1) CHR3.

4. Discussion

The diseases of crop plants, fruits, and aquatic life, such as fish, pose one of the greatest challenges to and threaten the food security worldwide. These, in turn, have serious financial implications. Various alternative management approaches are in use to prevent the loss; one of them includes the use of natural enemies of microbial pathogens. These antagonistic microbes utilize natural mechanisms, such as predation, to overcome the damage caused by microbes to plants and aquatic life. The present study focused on screening antibiotic-producing *Bacillus* sp. that could be used as an alternative to commercial antibiotics and pesticides against a broad range of microbes, especially those responsible for the production losses in agriculture and fisheries.

The isolate, BC01, found in the present study belongs to the genus *Bacillus* and was confirmed by the identification method of Wang et al. 2009 (method for identification of eight *Bacillus* species and subspecies) [25]. The *Bacillus* sp. BC01 isolate depicted a sequence (>98%) similarity with two *Bacillus subtilis* species from Wang's study, namely *Bacillus subtilis* and *B. amyloliquefaciens*,

whose closest neighbors (99% or greater homology match) have potent antagonistic activity against a range of pathogenic microbes, including plant pathogen *Pythium irregulare* and human pathogen *Staphylococcus aureus* and *Escherichia coli* [26,27]. Most recent studies also reported successful use of soil *Bacillus* species as a BCA to control plant pathogen mycotoxigenic Fusarium species causing head blight [28], *Botryosphaeria berengeriana* associated with pear ring rot [29], and *Rhizoctonia solani* involved in wilt and root rot [30]. The close clustering of *Bacillus* sp. BC01 isolate to those strains makes it an ideal candidate as a BCA.

Chemical pesticides and antibiotics have long been used to control the spread of diseases; however, their effects on environment and ecosystem have always remained questionable. Moreover, the frequent use of antibiotics and chemical pesticides in farmland and culture ponds increases the chances of production of antibiotic-resistant strains [7,21]. Several studies exist on the use of *Bacillus* and *Streptomyces* genera for controlling plant and human pathogens [17,18]. In addition, reports on the use of *Streptomyces* sp. against bacterial pathogens of fish are available; however, molecular characterization based on 16s rRNA analysis has yet to be conducted to find out potential *Bacillus* sp. candidates that could be effective against both plant and fish pathogenic fungi and bacteria [18]. In the current study, the isolate *Bacillus* sp. BC01 showed a broad spectrum of antagonistic activity against both fungi and bacteria. *Penicillium digitatum* and *Xanthomonas* sp., the two most dangerous bacterial pathogens of fruits and vegetables, were potentially inhibited by the study isolate. The isolate also exhibited a high spectrum of antagonism against *Aeromonas veronii*, an etiological agent of EUS and BHS, consequently leading to high mortality in the cultured carp and catfish [31]. The isolate also displayed intermediate effects on *Aphanomyces invadans*, *Aeromonas veronii*, and *Pseudomonas* sp., in *in-vitro* inhibition assays. Therefore, the wide antagonistic spectrum of antibiotics produced by the isolate against several pathogenic microbes makes it a potential study strain. It can thus be utilized as a source of environment-friendly, prospective antibiotic-producing bacterial strain for controlling diseases in farmland and culture ponds. Further research on molecular characterization of active compounds or metabolites secreted by the isolate and study of its enzymatic pathways are warranted to utilize it as an effective biological control agent.

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Conflict of interest

The author declares no conflict of interest.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jgeb.2018.01.005.

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