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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\textbf{A B S T R A C T}

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 \textit{in vitro}. The anti-bacterial effect of the isolate was also evaluated against \textit{Pseudomonas} spp. and \textit{Xanthomonas} spp., the two deadliest plant pathogens, and \textit{Aeromonas veronii}, \textit{Pseudomonas fluorescens}, and \textit{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.

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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 \cite{1,2}. Various preventive measures, including heavy use of pesticides and chemical compounds, pose a severe challenge to food security globally. For instance, \textit{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 \cite{3}. Other bacterial species such as those belonging to \textit{Pseudomonas} cause a variety of plant diseases with diverse symptoms, such as cankers, spots, blight, rot, and galls, while \textit{Xanthomonas} spp. (especially \textit{X. euvesicatoria} and \textit{X. perforans}) cause spots and blights of leaf, stem, and fruits \cite{4,5}. In fish, \textit{Aphanomyces invadans} causes epizootic ulcerative syndrome (EUS) leading to similar damage \cite{6}. Other widely known and potential disease-causing agents in fish include \textit{Aeromonas veronii}, \textit{Pseudomonas fluorescens}, and \textit{Streptococcus iniae} that are responsible for motile aeromonad septicemia (AMS), bacterial hemorrhagic septicemia, and streptococcosis, respectively, in both carp and catfish \cite{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.
A biological control is an approach 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 pathogen 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, 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].

2.3. 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. (PS05), Xanthomonas spp. (Xp03), Aeromonas veroni (BN10), Pseudomonas fluorescens (PULK2), 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'-AGGAACTTATCCAGGCCAGC-3') for amplification of 16s rDNA universal sequence, 4 μL of template, and 15 μL of nuclelease-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).

| Isolate | Source | Shape | Motility | Hemolysis | Starch | Gelatin | VP | Citrate | Growth at 6% NaCl | Growth at different pH | Growth at different temperatures (°C) |
|---------|--------|-------|----------|-----------|--------|---------|----|---------|------------------|-------------------|----------------------|
| BC01-BC04 | Soil   | Rod   | +        | +         | +      | +       | +  |        |                  |                   |                      |

Table 1 Biochemical characterization of Bacillus sp. isolates.

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.
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, Table 2

| Name of the tested Fungus and Bacterium | Penicillum 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                       | 15                            | 9                        |
| Bacillus sp. BC02                      | 9                           | 8                           | 8                       | –                       | 7                        | 9                             | 10                       |
| Bacillus sp. BC03                      | 7                           | –                           | 7                       | –                       | –                        | –                             | –                        |

| Name of the tested Fungus and Bacterium | Penicillum 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                       | 15                            | 9                        |
| Bacillus sp. BC02                      | 9                           | 8                           | 8                       | –                       | 7                        | 9                             | 10                       |
| Bacillus sp. BC03                      | 7                           | –                           | 7                       | –                       | –                        | –                             | –                        |

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.

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whose closest neighbors (99% or greater homology match) have potent antagonistic activity against a range of pathogenic microbes, including plant pathogen Pythium irregular 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 mycotoxicogenic 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 entomological agent of EUS and BHS, consequently leading to high mortalities in the cultured carp and catfish [31]. The isolate also displayed intermediate effects on Aphanomyces invadans, Aeromonas veronii, and Pseudomonas sp., 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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