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Isolation and Molecular Characterization of Bacillus cereus Strain from the Soil Samples Collected from Rhizosphere Zone of Different Crops of Uttar Pradesh India

Mr. Riazuddin Riazuddin[1], Ms. Vanshika Vanshika[2], Mr. Manish Kumar Sharma[3], Mr. Jagpal Singh[4]

[1]Research Scholar, Biotechnology, FARMER Voluntary Centre - ICAR-AINP on SAP
[2]Project Trainee, Zoology, FARMER Voluntary Centre - ICAR-AINP on SAP
[3]Project Assistant, Agriculture, FARMER Voluntary Centre - ICAR-AINP on SAP
[4]Secretary, Agriculture, FARMER Voluntary Centre - ICAR-AINP on SAP

Abstract:
Bacteria are a very diverse group of organisms in soil, and major taxonomic groups are represented in most soils. The extent of the diversity of microorganisms in soil is seen to be critical to the maintenance of soil health and quality, because a wide range of bacteria are involved in the important soil functions. The present study dealt with the isolation and molecular characterization of *Bacillus cereus* strains isolated from the field of Mustard crop in Ghaziabad at GPS location (28.657351^0 Latitude 77.47841^0 Longitude). The total 173 soil samples were collected from rhizosphere zone of 14 different crops; Sugarcane, Pea, Lemon, Peach, Mustard, Okra, Wheat, Garlic, Alfalfa, Barley, Sorghum, Potato, Fenugreek and Mango covering 32 locations of 5 districts; Ghaziabad, Meerut, Baghpat, Hapur and Bulandshahr of Uttar Pradesh, India. The media used in this research were nutrient broth and agar. *Bacillus* species were identified by their phenotypic and biochemical characteristics. The colony morphology observed on Nutrient Agar were circular in shape, flat to slightly raised elevation, dull, dry to slightly mucoid in young colonies surface and Creamy white to orange colour. The results of Gram staining and 3% KOH test was Gram Positive and Negative respectively of the isolates. Based on the colony morphology, gram staining and 3% KOH test the isolate was tentatively identified as a *Bacillus* species. As per closest matching sequence identified through NCBI BLAST analysis and revealed that Blast analysis of the partial sequences is from the genus *Bacillus* within the Firmicutes in the domain bacteria with similarities between 95.84% to 99.58% and finally confirmed that the isolated bacteria closely related to the *Bacillus cereus strains PQ220154.1 (95.84), PP916647.1 (99.50) and OK335864.1 (99.58%)*.

Keywords: *Bacillus cereus,* Soil, Rhizosphere, Characterization

Introduction:

Rhizosphere encompasses the narrow zone of soil directly surrounding the root system of plants [1]. Gr-

oups of rhizosphere bacteria that are competent in colonizing the root environment are referred to as rhizobacteria [2]. The plants have evolved with a plenitude of micro-organisms with diverse functional potential [3]. *Bacillus* species are Gram-positive, endospore-forming, chemoheterotrophic rod-shaped bacteria which are usually motile with peritrichous flagella; they are aerobic or facultative anaerobic and catalase positive [4]. Members of the *Bacillus* genus are generally found in soil and represent a wide range of physiological abilities, allowing the organism to grow in every environment and compete desirably with other organisms within the environment due to its capability to form extremely resistant spores and produce metabolites that have antagonistic effects on other microorganisms [5].

Bacillus is described as aerobic or facultative anaerobic, gram positive, rod shaped, flagellated motile bacteria, catalase positive belonging to the division Firmicutes [6]. Soil microorganisms are important components of ecosystem functioning as they determine the mineralization of soil organic matter and energy flow [7,8]. Microbial recycling of crop residues provides an important component to improve the soil organic matter pool and soil productivity in agricultural management systems, particularly in the tropics, where microbial soil organic matter turnover time is usually shorter as opposed to temperate agro-ecosystems [9,10,11]. Majority of soil bacteria are heterotrophic in nature and derive their carbon and energy from complex organic substances/organic matter, decaying roots and plant residues [12]. Soil microorganisms also influence above-ground ecosystems by contributing to plant nutrition [13], plant health [14, 15], soil structure [17,18] and soil fertility [19,20].

The existence of a large microbial community in the soil is supported by an enormous group of organic mattersin the earth. Most of these microorganisms are bioactive and survive at the top few inches of the agricultural soils [21]. The microorganisms can live in several habitats along with humans and also in the utmost adverse conditions such as inside the rocks of the oceanic crust [22], cold temperature [23], hot springs [24], and miles deep in the ocean [25]. Abiotic and biotic factors are involved in regulating the activity and diversification of soil microorganisms.The presence of microbes in soil is based on the existence of ambient conditions provided by the types of vegetation, the texture and chemical nature of the soil, nutrients availability, pH, moisture content, climate, and temperature. The physiology of the soil is also determined by all these conditions as it varies across the same place between different seasons. Further, the dumping of the organic wastes from agricultural fields ensures the availability of the high nutrient content in soil for the growth of the microorganisms [26]. Some bacterial communities in the soil such as *Thiobacillus, Rhizobium, Nitrosomonas, Clostridium, Nitrobacter, Caulobacter, Pseudomonas*, and *Frankia* carry out an essential task in nutrient cycling [27].

The soil acts as a reservoir for millions of microorganisms, of which approximately more than 85% are beneficial for plant life. Thus, soil is a resilient eco system and soil microorganisms provide precious life to soil ecosystems catering to plant growth. Soil microorganisms play a vital role in the evolution of agriculturally useful soil conditions and in stimulating plant growth [28].

Material and methods:

Collection of Soil Samples:

173 soil samples were collected from rhizosphere zone of 14 different crops; Sugarcane, Pea, Lemon, Peach, Mustard, Okra, Wheat, Garlic, Alfalfa, Barley, Sorghum, Potato, Fenugreek and Mango covering 32 locations of 5 districts; Ghaziabad, Meerut, Baghpat, Hapur and Bulandshahr of Uttar Pradesh, India. Cross-sectional study involving stratified random sampling was used. The soil samples were taken from a depth of 0-20cm and 20-40cm. A zigzag format of sampling was used; soil samples were mixed

thoroughly to constitute a composite sample it was then transported intact at 4^0 C temperatures in sealed polyethylene bags with icepacks in polystyrene (Thermocol) box to the laboratory for processing. 1 gm of soil was serial diluted in normal saline. The location wise soil sample details are given in **Table – 1.**

Table 1. Location wise soil sample details

Sr. No.	District	Locations	Crops	Nos. of Soil Samples
1	Ghaziabad	Kakda, Rawli, Kunheda, Dabana, Jalalabad, Hisali, Badka arifpur , Dasna, Modinagar	Sugarcane	(20)
			Pea	(3)
			Lemon	(3)
			Peach	(5)
			Mustard	(3)
			Okra	(4)
2	Meerut	Mataur, Daulara, Bharala, Eklota, Panwari, Samoli, Siwaya, Dulhera, Machhri, Chirori, Surani, Baphawat	Wheat	(13)
			Sugarcane	(16)
			Mustard	(12)
			Garlic	(2)
			Fellow Field	(16)
			Alfalfa	(2)
			Garlic	(2)
			Barley	(4)
			Sorghum	(5)
			Potato	(3)
			Fenugreek	(2)
			Mango	(2)
3	Baghpat	Sunheda, Gurana	Sugarcane	(6)
4	Hapur	Hafizpur, Khadkhadi, Nizampur, Pootha Hussainpur	Sugarcane	(4)
			Wheat	(4)
			Sorghum	(5)
5	Bulandshahar	Kaithla, Ginora Saikh, Etmadpur,	Sugarcane	(27)
			Wheat	(10)
Total	**5**	**32**	**14**	**173**

Isolation of Soil Bacteria by Spread Plate Techniques

Nutrient Agar Granulated (Hi media) at a concentration of 28 g per litre of distilled water with pH 6.0 – 6.5 was used for the cultivation of microorganisms. The media were then autoclaved at 121° C temperatures under 15 psi pressure for 20 minutes after which it was then dispensed into 90-mm-diameter Hi media disposable sterile plastic Petri Plates under aseptic conditions. The freshly sieved soil was carefully mixed and pulverized with spatula on the larger piece of paper. 1 gram of soil was weighed on a sterile aluminium foil and transferred immediately to the conical flask containing 150 ml of normal saline. The flask was dispersed by stirring with Teflon-coated magnetic bars for 15 minutes.

The soil suspension was then serial diluted where 1ml of the soil suspension was added to 9 ml test tube of normal saline. Dilution ratios included: 10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}. These preparations were mixed with a vortex mixer at approximately 150 rpm for 1 minute and 1ml of aliquots was rapidly transferred to other 9 ml tubes. 200 µl aliquots from different dilutions were transferred to petri dishes containing Nutrient Agar and spread over the surface with a sterile glass spreading rod. This was followed by incubation at 28ºC for 24 to 72 hours in the dark in BOD. Sub culturing was done on Nutrient Agar Plates to isolate pure cultures. The pure culture isolated plate was submitted to the Plant Pathology Division of IARI for morphological and molecular characterization **(Figure1.)**.

Characterization of the Isolates:

Morphological Identification:

Preliminary characterization was performed using morphological and cultural characteristics and morphological identification of the isolate was done under the compound microscope to observe cell size, shape and arrangement characteristics after sub culturing, gram staining and 3% (w/v) KOH test of bacteria isolates.

Molecular Identification:

The identity of the isolates up to species level was confirmed through molecular characterization. Genomic DNA was extracted using the CTAB (Cetyltrimethylammonium bromide) method and subjected to 16S rRNA gene amplification using primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). Forward and reverse sequences were assembled into a single contig using BioEdit Sequence Alignment Editor (version 7.2.5). The assembled sequence was curated and aligned with reference sequences from NCBI using CLC Sequence Viewer 8.0.

Result and discussion:

Morphological characterization was based on classical macroscopic techniques of shape, elevation, form and colour of pure colonies. Most colonies were able to grow within 2-3 days of incubation at 28 ºC ± 2. The colony morphology observed on Nutrient Agar they were circular in shape, flat to slightly raised elevation, dull, dry to slightly mucoid in young colonies surface and Creamy white to orange colour. The results of Gram staining and 3% KOH test was Gram Positive and Negative respectively of the isolates. Based on the colony morphology, gram staining and 3% KOH test the isolate was tentatively identified as a ***Bacillus*** species **(Figure 2 and Table:2).**

Isolates further underwent molecular characterization. The amplified DNA Curated Sequence as show in **Figure 3** and the **Figure 4** show screenshot of the NCBI BLAST results, page displaying the sequence alignment output. The **Table 3** below show closest matching sequence identified through NCBI BLAST analysis and revealed that Blast analysis of the partial sequences from the genus *Bacillus* within the Firmicutes in the domain bacteria with similarities between 95.84% to 99.58% and finally confirmed that the isolated bacteria closely related to the *Bacillus cereus strains PQ220154.1 (95.84), PP916647.1 (99.50) and OK335864.1(99.58%)*.

Bacillus are described as aerobic or facultative anaerobic, gram positive, rod shaped, flagellated motile bacteria, catalase positive belonging to the division Firmicutes [6] with a wide ecological diversity mostly saprophytic they are commonly found in soil, dust, milk, plant surfaces, a few are animal or insect parasites or pathogens. Many *Bacillus* species have been described including *Bacillus anthracis*,

accusative agent of anthrax, *Bacillus cereus* which causes food poisoning, *Bacillus megaterium*, *Bacillus subtilis* and *Bacillus coagulans* notable food spoilers, causing ropiness in bread and related food. *Bacillus thuringiensis* is an important insect pathogen, and is sometimes used to control insect pests. Other species include; *Bacillus mycoides, Bacillus weihenstephanensis* among others [29]. *Bacillus* is the most abundant genus in the rhizosphere of soil, are naturally occurring soil bacteria that aggressively colonize plant roots and benefit plants by providing growth promotion [30].

Some of the *Bacillu*s species have been classified as plant growth promoting Rhizobacteria [31]. There are a number of metabolites that are released by these strains [31 which strongly affect the environment by increasing availability of the plants [32]. Soil bacteria isolates including *Bacillus cereus* UW 85 produces Siderophore and they can be used as efficient PGPR to increase the yield of the crop [33]. Many soil microorganisms are able to solubilize phosphate through their metabolic activities exuding organic acids, which directly dissolve the rock phosphate, or chelating calcium ions that release Phosphate to the solution. About 95% of Gram-positive soil *bacilli* belong to the genus *Bacillus* [34].

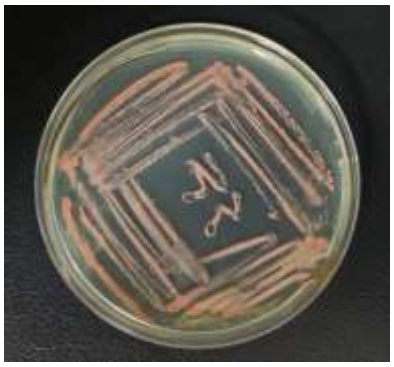

Fig. 1 Culture submitted to Indian Type Culture Collection(ITCC), IARI (New Delhi)

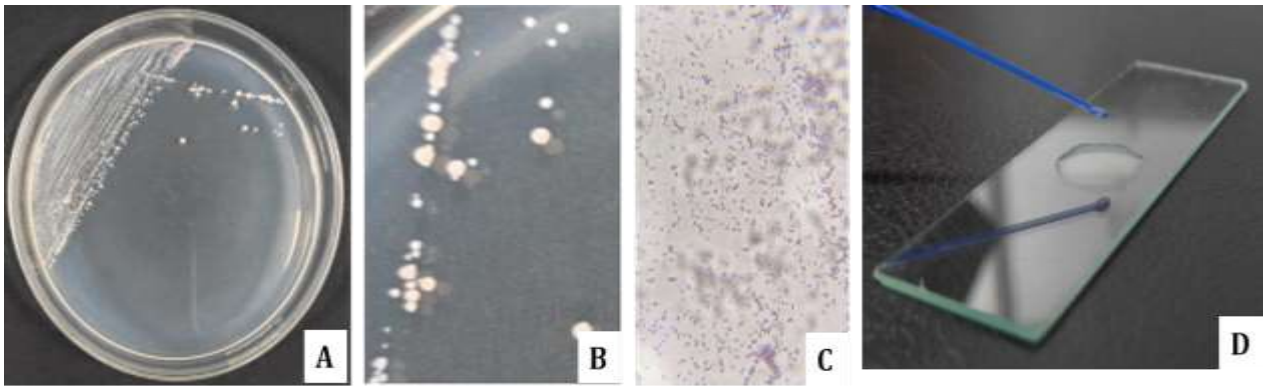

Fig. 2 (A)Colony morphology observed on Nutrient Agar, (B) well isolated single cell colony,(C)Gram staining (Gram positive), (D) 3% KOH test (Negative)

Table:2 Morphological Features of the Bacterial Isolate on Nutrient Agar

Characteristic	**Description**
Shape	Circular
Elevation	Flat to slightly raised

Characteristic	Description
Surface	Dull, dry to slightly mucoid in young colonies
Color	Creamy white to orange

TAATGCAGTCGAGCGAACGGATTGGAACCTGGCTCTTATGATTTTCGCGGCAGAGGGGTGAA
TAACACGTGGGTAACCTGCCCATAACAGTGGTATAACTCCGAAAAACCGGGGCTAATACCGG
ATAACATTTTGAACCGCGGGGTTCGGATTTGAAAGGCGGTTTCCGGTGTCTCTTATGGATGCC
CCCGCGTCACTATCTCTATTTGGAGATGTAACGGCTCACCAGGGACAATATGCATACCCCACC
TGAGAGGGAGATCGCCCACTGTGAGACTGACACACCGCCCAGACTCTACGGGGAGGCAGC
AATGGGGAATCTTCCGCAATGACGAAATCTCAGACAGAACAACGCCGCGAGAGTGATGAAG
GCTTTCAGGTCGTAAATCTCTGTTGTTAGGGAAAAACAAGTGCTAGTGAATAAGCTGGCACC
TTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAG
GTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTTCTTAAGTCTGA
TGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGA
AGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGT
GGCGAAGGCGACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAAC
AGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCC
GCCCTTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTG
AAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGC
AACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGAAAACCCTAGAGATAGGGCTTCTCCT
TCGGGAGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTT
AAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCATCATTAAGTTGGGCACTCTAAGG
TGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGA
CCTGGGCTACACACGTGCTACAATGGACGGTACAAAGAGCTGCAAGACCGCGAGGTGGAGC
TAATCTCATAAAACCGTTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCTGGAA
TCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGC
CCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGGGGTAACCTTTTTGGAGCCAGC
CGC

Fig. 3.Curated Sequence

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per Ident	Acc. Len	Accession
Bacillus cereus strain BAC75 16S ribosomal RNA gene, partial sequence	Bacillus cereus	2591	2591	100%	0.0	99.58%	1426	OK335864.1
Bacillus cereus strain OK335864 16S ribosomal RNA gene, partial sequence	Bacillus cereus	2564	2564	99%	0.0	99.50%	1410	PP916647.1
Bacillus cereus strain AUMC B-553 16S ribosomal RNA gene, partial sequence	Bacillus cereus	2283	2283	99%	0.0	95.84%	1418	PQ220154.1
Bacillus cereus strain FG104 16S ribosomal RNA gene, partial sequence	Bacillus cereus	2268	2268	100%	0.0	95.51%	1447	PQ764647.1
Bacillus cereus strain HBU10035 16S ribosomal RNA gene, partial sequence	Bacillus cereus	2265	2265	100%	0.0	95.44%	1483	MW453077.1
Bacillus sp. (in: firmicutes) strain PS25 16S ribosomal RNA gene, partial sequence	Bacillus sp. (in: f	2265	2265	100%	0.0	95.44%	1451	PV450042.1
Bacillus sp. hb54 16S ribosomal RNA gene, partial sequence	Bacillus sp. hb54	2265	2265	100%	0.0	95.44%	1461	KF863861.1
Bacillus spizizenii strain PP978433.1:5-1478 16S ribosomal RNA gene, partial sequence	Bacillus spizizenii	2265	2265	100%	0.0	95.44%	1474	PQ609715.1
Bacillus spizizenii strain PQ093564.1:9-1477 16S ribosomal RNA gene, partial sequence	Bacillus spizizenii	2265	2265	100%	0.0	95.44%	1469	PQ609713.1
Bacillus cereus strain LXYZB3 16S ribosomal RNA gene, partial sequence	Bacillus cereus	2265	2265	100%	0.0	95.44%	1450	MZ452375.1
Bacillus sp. (in: firmicutes) strain PF33 16S ribosomal RNA gene, partial sequence	Bacillus sp. (in: f	2265	2265	100%	0.0	95.44%	1454	PV450104.1
Bacillus cereus strain SRS-1 16S ribosomal RNA gene, partial sequence	Bacillus cereus	2265	2265	100%	0.0	95.51%	1438	PQ797086.1

Fig. 4. Screenshot of the NCBI BLAST results page displaying the sequence alignment output.

Table 3. Closest matching sequence identified through NCBI BLAST analysis.

Closest match in NCBI	Percent identity	E-value	Accession
Bacillus cereus	99.58	0.0	OK335864.1
Bacillus cereus	99.50	0.0	PP916647.1
Bacillus cereus	95.84	0.0	PQ220154.1

Conclusion:

Bacillus cereus strain isolated from the Mustard crop field of Ghaziabad with GPS location (28.657351^0 Latitude 77.47841^0 Longitude) out of total 173 soil samples were collected from rhizosphere zone of 14 different crops; Sugarcane, Pea, Lemon, Peach, Mustard, Okra, Wheat, Garlic, Alfalfa, Barley, Sorghum, Potato, Fenugreek and Mango in 5 districts covering 32 locations of Uttar Pradesh India.

Modernization has introduced intensive agricultural practices wherein the pesticides play an important role, both in stabilization and in increasing of agricultural products. As a consequence, humans and members of other ecosystems are exposed to increased levels of compounds that have detrimental effects on their health, thereby signifying the importance of microbial inoculants. *Bacillus* members have been widely studied as pathogens and environmental strains of industrial, ecological and medical relevance. Studies have reported the production of various valuable enzymes and metabolites that degrade different types of pollutants, and promote the growth of animals and plants by *B. cereus* group when used as probiotics. A comprehensive analysis, using a comparative genomics approach, pertaining to the characteristics of *B. cereus* related to agriculture had not been reported much.

Bacillus cereus is used in agriculture for plant growth promotion and biocontrol of plant diseases. It acts as a plant growth-promoting bacterium (PGPB) by producing growth hormones like IAA, solubilizing nutrients, and improving nutrient uptake in many crops. It also protects plants from a variety of bacterial, fungal, and nematode pathogens, and can be used for bioremediation of contaminated soils, such as those with heavy metals.We conclude that this isolated bacteria stain is potential candidate for further research on their plant growth-promoting properties.

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