Isolation, characterization and cataloguing of microbes from the soil of Gangetic plain of Vaishali district (North Bihar).

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Abstract : The soil acts as a reservoir for millions of microorganisms. Already isolated and screened few of the bacterial strains namely, *Brevibacillus borstelensis, Paenibacillus dendritiformis* whose MTCC NO. are 10644 and 10642 and their plant growth promoting potentials have been established. This further opens the way for future research in this field. A few strains of microbial population of the study were isolated and their pure culture was prepared and maintained in the laboratory. Of the isolated species, two strains of microbes were screened, identified and characterized (Morphological, Biochemical and Molecular parameters). This has provided the basic resource material, which will be further utilized for different fields including agriculture and agro-based industries. These two strains are identified as two different varieties of *Aneurinibacillus migulanus,* which produce Antibiotic Gramicidin S. Few strains, were screened and characterized. All strains have shown almost +ve result of biochemical tests. The two strains isolated from Gangetic region of Vaishali district of North Bihar have been identified and confirmed by IMTECH, Chandigarh. These strains are *Aneurinibacillus migulanus* with different MTCC No. and are preserved and catalogued there. Some isolated strains are being purified and culture has been maintained in the laboratory. These strains were sent to IMTECH, Chandigarh for molecular characterization. The ability of *Aneurinibacillus migulanus* is to produce the Antibiotic Gramicidin S is correlated with Phenotype variation.

Keywords: *Microorganisms, Molecular characterization, Brevibacillus borstelensis, Paenibacillus dendritiformis, Aneurinibacillus migulanus,* Gramicidin S, Phenotype.

I. INTRODUCTION

Microbiological techniques, especially in suppression of crop diseases, production of plant growth promoting substances and augmentation of nutrient recycling, offer a powerful tool in modern agriculture. Biofertilizer has been identified as an alternative to chemical fertilizer to increase soil fertility and crop production in sustainable farming. Biofertilizers are products containing living cells of different types of microorganisms, which have an ability to convert nutritionally important elements from unavailable to available form through biological processes [1]. Numerous species of soil bacteria which flourish in the rhizosphere of plants stimulate plant growth by a variety of mechanisms. Searching novel sources of the microbes having plant growth promoting potential are therefore necessary to meet the escalating demand of sustainable farming. Properly developed biocontrol agents are not considered harmful to ecological processes or the environment [2]. The application of microbial fertilizers in practice, somehow, has not achieved constant effects. The mechanisms and interactions among these microbes still are not well understood, especially in real applications [3]. The fertile Gangetic plane of north Bihar is one such source where the possibilities of finding these microorganisms are more apparent. Thus, the present work focuses on the isolation, characterization and screening of soil microbes having plant growth promoting potentials, from Gangetic planes of North Bihar and their further cataloguing for their future application as biofertilizer or for other agro-based industries. Currently, I have already isolated and screened few of the bacterial strains namely, *Brevibacillus borstelensis, Paenibacillus dendritiformis* whose **MTCC NO. are 10644 and 10642** and their plant growth promoting potentials have been established, which further opens the way for future research in this field.

This survey demonstrates the trend of an increasing rate of research on PGPR, but is not an absolute measure of the activity in the area. Although there is evidence of PGPR having biofertilizing effects on forest tree species [4]. Modern methods for analysing microbial community structures may prove particularly valuable to help define the key organisms or groups of organisms responsible for natural disease control mechanisms as well as for monitoring the spread and impact of introduction of specific biocontrol agents or other management practices on natural microbial populations [5]. A large part of South Bihar is, nevertheless, rain fed and the soil is especially suited for paddy and pulses. Although a lot of efforts have been made by scientists of different Agricultural University to develop varieties that best suit the soil of Bihar, very little effort has been made to investigate the soil micro flora that supports and promotes the growth of crops even without addition of fertilizers, especially of the most fertile

Indo-Gangetic plains. Thus the Gangetic planes of Bihar need immediate attention to isolate and screen microbes that help in enhanced soil fertility and decomposition of agricultural wastes. This will not only help generating the database of indigenous microbes but also play a major role in poverty alleviation through involvement of rural masses.

Fig. 1.1. The district map of Bihar showing the Gangetic regions selected for the microbial diversity and their PGPR potential studies.

In the light of exploring the utility and importance of bacteria, the present investigation was aimed towards isolation and purification of bacterial strains obtained from soil, their characterization and identification. Soil samples were collected from five different sites: S1-Ganga river, S2-Banana field, S3-Wheat+Mustard field, S4-Guava field and S5-Stagnant pond.

II MATERIALS AND METHODS

Sources of sample collection

The isolates were obtained from the soil of Gangetic region of North Bihar. The next step was the screening of isolates to select bacterial strains with biofertilizer potential. They are mostly differentiated on the basis of structure and development of cells, morphology, responses to culturing, physiology and biochemistry. Their colony on agar media produce spores, the later representing the preparative phase. The majority of microorganisms, when grown on solid media, grow into colony representing a massive cluster of cells of organisms which are capable of growing together in a single complex colony. Microscopic examination of the organisms from a colony should reveal only a single type of cells. Differential staining procedures, such as Gram stains are useful for establishing that the colony does not contain a mixture of different microbial types.

A total of 83 microbial strains (bacteria) were isolated from the five soil sites. Most of the strains grew luxuriantly on Nutrient Agar within the temperature range30 to 37°C. Various isolates of bacterial strains showed a notable array of macroscopic features like diversity in spore colour, colony morphology and presence of diffusible exo-pigments.

Experimental approaches

- **Sampling for isolation of microbes**

✓ Sampling was done from the soil of Vaishali district of North Bihar.

✓ Sampling included variation of time, duration and season. Sampling Periods are

  - Pre-Monsoon,
  - Monsoon,
  - Post monsoon and winter season.

- ✓ The sampling site is near river Ganga which is wet land area that abounds in banana plantation fields, with soil rich in microbial population.

- **Microbiology**

- ✓ Microbes were isolated by serial dilution method, sub-culturing and pure culture method.
- ✓ Various types of selective and non selective media were utilized for isolation of large number of isolates.
- ✓ The isolated strains were then characterized as per the standard protocol stated in'Bergey's Manual of Determinative Bacteriology' and 'IMTECH laboratory manual'.
- ✓ Further, the selected strains were identified on the classical as well as modern parameters including biochemical and molecular biological techniques.

Morphological Parameters:

- Colony Morphology: Configuration, Margin, Surface, Density, Pigment.
- Grams Reaction: Shape, Size, Arrangement.
- Spore: Endospore, Position, Shape, Motility.

Biochemical Parameters:

i)Growth onMacConkey Agar, ii) Indole test, iii) Methyl red test, iv) Citrate utilization, v)Casein hydrolysis, vi)Starch hydrolysis, vii) Urea hydrolysis, viii)Gelatin hydrolysis, ix) Cellulase etc.

Molecular parameters: (Done with the help of IMTECH, Chandigarh)

i) Phenotypic Confirmation

ii) Sequencing of 16s rRNA

iii) Blast Analysis

- Data analysis: Analysis of variance was performed on all experimental data and mean values were compared.

Results and Discussion

- In the continuity few strains of microbial population of the study were isolated and their pure culture was prepared and maintained in the laboratory.
- Of the isolated species, two strains of microbes were screened, identified and characterized (Morphological, Biochemical and Molecular parameters). This has provided the basic resource material, which will be further utilized for different fields including agriculture and agro-based industries.
- These two strains are identified as two different varieties of *Aneurinibacillus migulanus,* which produce Antibiotic Gramicidin S.
- Few more strains were screened and characterized.
- All strains have shown almost +ve result of biochemical tests.
- All test isolates grew on NA showing morphology of *Bacillus* colonies. The pigment of the different colonies varied. The optimum temperatures for luxuriant growth of all the tested isolates were found to be between 25˚C - 37˚C which suggests that all the isolates are mesophilic in nature. The shapes, sizes and the pattern of distribution of cells varied among different isolates. All the selected bacterial isolates were motile and produced spores.
- The two test isolates namely, S1 (V) 23 and S2 (V) 12 were selected from this site on the basis of their luxuriant growth on Nutrient Agar. Yellow and creamish yellow pigment was produced by isolate S1 (V) 23 and whereas, creamish white pigment was produced by isolate S2 (V)12.Appearance of purple colored bacteria under microscope led to the conclusion that the tested organisms were Gram +ve.
- However, these isolates showed a wide range of temperature for growth between 4˚ C to 42˚ C which is quite interesting property and suggests its potential as biofertilizer in field conditions where the organisms have to survive under wide range of natural temperature fluctuations. Both the test isolates S1 (V) 23 and S2 (V) 12 showed good growth in the pH range 5-9.These isolates showed –ve growth on MacConkey agar, V-P test and Nitrate reduction. They did not form deep golden red ring in Indole test. Both they utilized Citrate and hydrolised Urea. They showed +ve Catalase test and produced lactose in Carbohydrate test.
- The molecular identification of the two isolates was carried out by16s rRNA sequencing. On the basis of BLAST alignment of the sequenced nucleotides the isolates S1 (V) 23 and S2 (V) 12 were identified as different strains of *Aneurinibacillus migulanus* respectively.
- Following are the sequencing of the isolated strains S1(V)23 and S2(V)12

III. FIGURES AND TABLES

S1(V)23

GCTCTTCGGCGGTTAGCGGCGGACGGGTGAGTAACACGTAGGCAACCTGCCTGTACGACTGGGATAA
CTCCGGGAAACCGGAGCTAATACCGGATACTTCTTTCAGACCGCATGGTCTGAAAGGGAAAGACTTT
TGGTCACGTACAGATGGGCCTGCGGCGCATTAGCTAGTTGGTGGGGTAACGGCCTACCAAGGCGACG
ATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGG
AGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAACGATGA
AGGTTTTCGGATCGTAAAGTTCTGTTGTTAGGGAAGAACCGCCGGGATGACCTCCCGGTCTGACGG

TACCTAACGAGAAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGGGCAAGCGTT
GTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGCTTCTTAAGTCAGGTGTGAAAGCCCACGGCT
CAACCGTGGAGGGCCACTTGAAACTGGGAAGCTTGAGTGCAGGAGAGGAGAGCGGAATTCCACGTG

TAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCCGTGGCGAAGGCGGCTCTCTGGCCTGTAACTG
ACGCTGAGGCGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACG
TTGAGTGCTAGGTGTTGGGGACTCCAATCCTCAGTGCCGCAGCTAACGCAATAAGCACTCCGCCTGG

GGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGT
GGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGGCTTGACATCCCGCTGACCCTCCTAGAGATA
GGAGCTCTCTTCGGAGCAGCGGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTT

GGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCTTAGTTGCCAGCATTTGGTTGGGCACTCTAGGGA
GACTGCCGTCGACAAGACGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGTCCTGGG
CTACACACGTGCTACAATGGATGGAACAACGGGCAGCCAACTCGCGAGAGTGCGCGAATCCCTTA

AAACCATTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCATGAAGCCGGAATCGCTAGTAATCGC
GGATCAGCATGCCGCGGTGAATACGTTCCCGGGTCTTGTACACA

RESULT OF IDENTIFY ANALYSIS

Rank	Name/Title	Authors	Strain	Accession	Pairwise Similarity	Diff/Total nt	megaBLAST score	BLASTN score
1	Aneurinibacillus migulanus	(Takagi et al. 1993) Shida et al. 1996	DSM 2895(T)	X94195	99.771	3/1311	2575	2575
2	Aneurinibacillus aneurinilyticus	(Shida et al. 1994) Shida et al. 1996	DSM 5562(T)	X94194	99.390	8/1311	2535	2535
3	Aneurinibacillus danicus	Goto et al. 2004	NCIMB 13288(T)	AB112725	97.477	33/1308	2315	2309
4	Aneurinibacillus terranovensis	Allan et al. 2005	LMG 22483(T)	AJ715385	95.795	55/1308	2173	2143
5	Aneurinibacillus thermoaerophilus	(Meier-Stauffer et al. 1996) Heyndrickx et al. 1997	DSM 10154(T)	X94196	95.115	64/1310	2105	2056
6	Bacillus fortis	Scheldeman et al. 2004	R-6514(T)	AY443038	92.037	104/1306	0	1649
7	Bacillus indicus	Suresh et al. 2004	Sd/3(T)	AJ583158	91.749	108/1309	0	1582
8	Bacillus benzoevorans	Pichinoty et al. 1987	DSM 5391(T)	D78311	91.609	109/1299	1637	1562
9	Bacillus cibi	Yoon et al. 2005	JG-30(T)	AY550276	91.597	110/1309	0	1588
10	Bacillus fordii	Scheldeman et al. 2004	R-7190(T)	AY443039	91.590	110/1308	1695	0

S2(V)12

TGCAGTCGAGCGGACCAATGAAGAGCTTGCTCTTCGGCGGTTAGCGGCGGACGGGTGAGTAACACGT
AGGCAACCTGCCTGTACGACTGGGATAACTCCGGGAAACCGGAGCTAATACCGGATACTTCTTTCAG
ACCGCATGGTCTGAAAGGGAAAGACCTTTGGTCACGTACAGATGGGCCTGCGGCGCATTAGCTAGTT
GGTGGGGTAACGGCCTACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGG
GACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGT
CTGACGGAGCAACGCCGCGTGAACGATGAAGGTTTTCGGATCGTAAAGTTCTGTTGTTAGGGAAGAA
CCGCCGGGATGACCTCCCGGTCTGACGGTACCTAACGAGAAAGCCCCGGCTAACTACGTGCCAGCAG
CCGCGGTAATACGTAGGGGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGCTT
CTTAAGTCAGGTGTGAAAGCCCACGGCTCAACCGTGGAGGGCCACTTGAAACTGGGAAGCTTGAGTG
CAGGAGAGGAGAGCGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCCGTG
GCGAAGGCGGCTCTCTGGCCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCGAACAGGATTA
GATACCCTGGTAGTCCACGCCGTAAACGTTGAGTGCTAGGTGTTGGGGACTCCAATCCTCAGTGCCGC
AGCTAACGCAATAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACG
GGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGGCTT
GACATCCCGCTGACCCTCCTAGAGATAGGAGCTCTCTTCGGAGCAGCGGTGACAGGTGGTGCATGGT
TGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCTTAGTTG
CCAGCATTTAGTTGGGCACTCTAGGGAGACTGCCGTCGACAAGACGGAGGAAGGTGGGGATGACGTC
AAATCATCATGCCCCTTATGTCCTGGGCTACACACGTGCTACAATGGATGGAACAACGGGCAGCCAA
CTCGCGAGAGTGCGCGAATCCCTTAAAACCATTCTCAGTTCGGATTGCAGGCTGCAACTCGCCTGCAT
GAAGCCGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGTCTTGTACAC
ACCGCCCGTCACACCACGAGAGTTTGCAACACCCGAAGTGGTGAGGTAACCGCAAGGAGCCAGCCGC
CGAAGGTGGGGCAGATGATTGGGGTGAAGTCGTAACAA

RESULT OF IDENTIFY ANALYSIS

Rank	Name/Title	Authors	Strain	Accession	Pairwise Similarity	Diff/Total nt	megaBLAST score	BLASTN score
1	Aneurinibacillus migulanus	(Takagi et al. 1993) Shida et al. 1996	DSM 2895(T)	X94195	99.931	1/1444	2849	2849
2	Aneurinibacillus aneurinilyticus	(Shida et al. 1994) Shida et al. 1996	DSM 5562(T)	X94194	99.446	8/1443	2783	2777
3	Aneurinibacillus danicus	Goto et al. 2004	NCIMB 13288(T)	AB112725	97.571	35/1441	2466	2460
4	Aneurinibacillus terranovensis	Allan et al. 2005	LMG 22483(T)	AJ715385	95.812	58/1385	2274	2244
5	Aneurinibacillus thermoaerophilus	(Meier-Stauffer et al. 1996) Heyndrickx et al. 1997	DSM 10154(T)	X94196	95.149	70/1443	2297	2248
6	Bacillus fortis	Scheldeman et al. 2004	R-6514(T)	AY443038	92.415	109/1437	0	1840
7	Bacillus fordii	Scheldeman et al. 2004	R-7190(T)	AY443039	91.562	121/1434	1847	0
8	Bacillus ginsengi	Qiu et al. 2009	ge14(T)	EF371375	91.528	122/1440	0	1717
9	Bacillus drentensis	Heyrman et al. 2004	LMG 21831(T)	AJ542506	91.459	120/1405	1824	0
10	Bhargavaea cecembensis	Manorama et al. 2009	DSE10(T)	AM286423	91.458	123/1440	0	1740

The reported literatures suggest that *Aneurinibacillus migulanus* is capable of producing the Antibiotic Gramicidin S.

Out of 38 microbial strains, 15 test isolates, that was selected for biochemical tests from this site on the basis of their luxuriant growth on Nutrient Agar. The results of biochemical tests of all the 15 test isolates were shown in the following table:

	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15
Malonate	+	-	-	+	-	+	+	-	+	+	+	-	+	-	-
V-P test	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-
Citrate	+++	-	-	++	+	+	+	+	+	+	+	-	+	-	-
ONPG	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-
Nitrate	++	-	+	+	+	+	+	+	-	+	+	+	+	+	-
Catalase	+	+++	++	-	+	+	+	+	++	+	+	+	+	+	+
Arginine	++	-	-	-	-	++	++	++	++	+	+	+	-	+	+
Sucrose	++	+	+	+	+	+	+	-	-	+	+	-	+	+	+
Mannitol	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glucose	++	+	+	+	+	+	+	+	-	+	+	+	+	+	+
Arabinose	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Trehalose	++	+	+	+	+	+	+	+	+	+	+	+	+	+	+

IV CONCLUSION

The two strains isolated from Gangetic region of Vaishali district of North Bihar have been identified and confirmed by IMTECH, Chandigarh. These strains are *Aneurinibacillus migulanus* with different MTCC No. and are preserved and catalogued there. Some isolated strains are being purified and culture has been maintained in the laboratory. These strains will be sent to IMTECH, Chandigarh for molecular characterization. The ability of *Aneurinibacillus migulanus* is to produce the Antibiotic Gramicidin S is correlated with Phenotype variation.

Innovations: This work has yielded primary data on microbial species existing in Bihar having prospects of being used as biofertilizers. The isolates have also revealed their antibiotic property that is subject for further research by pharmaceutical sector. The currently identified strains will be used in consortium with other strains to explore their biofertilizer potential. It has also proved the possibilities of inter-institutional collaboration in Bihar as exemplified

Application Potential:

Long Term

These isolated microbes having plant growth promoting potential and can be used successfully as biocontrol agent are therefore necessary to meet the escalating demand of sustainable farming. Thus, the present work focuses on the need of further isolation, characterization and screening of soil microbes having plant growth promoting potentials, from Gangetic planes of North Bihar and their further cataloguing for their future application as biofertilizer or for other agro-based industries.
The antibiotic property of the two isolates discovered (Gramacidin S) has further implications in the field of pharmacology and pharmaceutical industry in Bihar. Thus these isolates have a dual role as promoters of plant growth and retardents of plant diseases.

Immediate:

by the role of IMTECH, Chandigarh where the microbial strains are stored with MTCC No.

The biofertilizer potential of the isolated microbes immediately enhances the prospects of organic farming in Bihar. It also showcases the possibilities of adoption and implementation of green technology in the field of agriculture and agro-based industries.

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