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MOLECULAR DIVERSITY AMONG CELLULOLYTIC BACTERIAL CULTURES ISOLATED FROM RURAL AND URBAN WASTES

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Abstract- A total of 34 samples of decomposing rural and urban wastes were collected from different parts of Western Maharashtra from which 64 bacteria, 49 fungi and 63 actinomycetes strains were isolated. These isolates were screened for cellulase activity and the efficient cellulolytic microorganisms were identified on the basis of morphological, cultural and biochemical characters. Among cellulolytic bacterial isolates, based on cultural, morphological and biochemical characteristics, 11 isolates were identified as *Bacillus* spp., 3 as *Pseudomonas* spp., while the remaining three belonged to *Cellulomonas*, *Staphylococcus* and *Micrococcus* genus. The *Bacillus* isolates were further studied for their molecular diversity within group. The PCR amplification products of the 12 isolates of *Bacillus* spp. including standard *Bacillus* strain with 16 random primers produced 343 bands, out of which 182 bands were polymorphic and 158 bands were unique. Majority of the primers showed 100% polymorphism. The average polymorphism observed was 99.13%. The genetic similarity index based on the pooled data of RAPD profiles from all the 16 primers ranged from 0.11 to 0.76 among *Bacillus* isolates. The dendrogram constructed from the pooled data showed four major groups showing wide genetic variability between the isolates. There was wide genetic variability in *Bacillus* spp. at genus level from the same ecological niche.

INTRODUCTION

The decomposition process is carried out by various microorganisms including bacteria, fungi and actinomycetes. Different communities of microorganisms predominate during the different composting phases. There is practically no substance existing in nature that is not used by one microorganism or another (Iranzo *et al*., 2001). Initial decomposition is carried by mesophilic microorganisms, which rapidly biodegrade the soluble and easily degradable compounds. As temperature increases, on oxidation of carbon compounds, thermophiles take over. Temperature in a compost pile typically follows a pattern of rapid increase from 49 °C to 60 °C within 24 to 72 hours of pile formation and is maintained for several weeks. This is the active phase of composting, in which easily degradable compounds and oxygen are consumed, pathogens *viz*., *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, *Clostridium botulinum* and weed seeds are killed, and phytotoxins are eliminated. As the active composting phase subsides, temperature gradually declines to around 38°C and mesophilic microorganisms once again take over the other types of microorganisms and the curing phase begins (Fourti *et al*., 2008).

Microorganisms that populate substrates during composting reflect the evolution and the performance of the composting process. Their metabolic paths lead to significant changes in the physical and chemical parameters of the composting substrate, and that, in turn, leads to changes in the microbial community structure. In addition, the microbial community structure is of interest because composting, if not properly managed, might sustain potential pathogenic factors and emit gases such as CH_4 that contribute to the greenhouse effect (Wei *et al*., 2007). Although, the microbial community naturally present in the wastes usually carries out the process satisfactorily, the inoculation of wastes with microorganisms that each of them produces one of the several polymer degrading extra-cellular enzymes at high level is a strategy that could potentially enhance the way the process takes place

or the properties of the final product.

Microbiology of composting has been studied for decades, but there are still open questions regarding the diversity of microbiota in composting processes. Although fungi have been widely studied in recent years, researchers have also been paying attention to various bacteria that produce cellulases because of their fast growth, expression of multienzyme complexes, and resistance to extreme environments (Soares Jr., 2012). Cellulase-producing cellulolytic bacteria are often found from the genus of *Pseudomonas*, *Cellulomonas*, *Bacillus*, *Micrococcus*, *Cellvibrio*, and *Cytophaga* (Lamid *et al*. 2011). It is well documented in the literature that changing in the ecological niche or geographical region there is definite genetic variability at genus level, species level, etc. But, it is interesting to study whether there is genetic variability occurs at genus level or species level from the same ecological niche or not? Many of the examples of diversity studies have been of bacteria inhabiting extreme environments, and hence present study was designed to explore the diversity in cellulolytic *Bacillus* spp. isolated from the rural and urban wastes with the help of molecular tools.

MATERIALS AND METHODS

A total of 34 samples of decomposing rural and urban wastes were collected from different places of Western Maharashtra to isolate cellulolytic microorganisms. Isolation of microorganisms was carried out on specific medium. These isolates were screened for cellulase activity and the efficient cellulolytic microorganisms were identified on the basis of morphological, cultural and biochemical characters. The molecular diversity among twelve cellulolytic bacterial isolates which were identified as *Bacillus* spp. including the MPKV standard *Bacillus* strain 'BNF/BS-32' was analyzed by employing random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) technique. Initially, the bacterial isolates were grown in 50 ml LB broth for 24-48 hrs. 1.5 ml bacterial culture was taken in microcentrifuge tube and centrifuged at 12000 rpm for 2 min. This procedure was repeated for six times. The supernatant was discarded and the cell pellets were used for DNA extraction. The isolation of genomic DNA was carried out by following the methodology of Shiva Reddy *et al.* (2010) with certain modifications. The DNA pellet was dried and suspended in 100 µl of TE buffer. Quantification was done by gel electrophoresis and NanoDrop ND-1000 USA, UV visible spectrophotometer. The DNA with ratio of OD's (260/280nm) near 1.8 was used for further studies.

The molecular diversity and polymorphism of the selected cellulolytic *Bacillus* isolates along with the standard strain were determined by the RAPD-PCR technique. The purified genomic DNA samples from the individual isolates were diluted to working concentration of 20 ng/µl and used as template DNA. The DNA sequences of sixteen bacterial RAPD primers used for DNA amplification are given in Table 2. One primer at a time was used to study polymorphism within each group of selected *Bacillus* isolates, with genomic DNA extracts from all the strains as template DNA. The master mix required for each isolate was freshly prepared. The master mix 19 µl per tube and 1 µl of template DNA from the respective isolates was added to make total reaction volume in each tube equal to 20 µl. The PCR amplification was done by following the method proposed by Castrillo and Brooks (1998)

Table 1. Characteristics and geographic origin of the isolates

Isolate	Origin district	Source	Nature of waste	Colony characters
B-11	Satara	Open heap	Rural waste	Circular, flat
B-13	Satara	Pit	Rural waste	Circular, flat
B-15	Satara	Pit	Rural waste	Circular, flat
B-27	Pune	Open heap	Rural waste	Circular, flat
B-28	Pune	Open heap	Urban waste	Circular, flat
B-37	Ahmednagar	Open heap	Urban waste	Circular, flat
B-38	Nashik	Open heap	Rural waste	Circular, flat
B-42	Nashik	Pit	Rural waste	Circular, raised
B-46	Nashik	Open heap	Rural waste	Circular, flat
B-51	Dhule	Open heap	Rural waste	Circular, umbonate
B-57	Jalgaon	Open heap	Rural waste	Circular, flat
BNF/ BS-32	—	—	—	Circular, flat

with certain modifications as per following profiles: predenaturation at a temperature of 94 °C for 5 min, followed by 40 cycles of denaturation stages at a temperature of 94 °C for 1 min, annealing at a temperature of 36 °C for 1 min, elongation at 72 °C for 2 minutes, and finalizing at a temperature of 72°C for 10 minutes.

Twenty µl of the amplified products from each tube along with 4 µl of loading dye were separated on 1.2 per cent agarose gel using 1x TBE buffer of pH 8.0 along with l00 bp DNA ladder as DNA molecular weight marker. Electrophoresis was performed for 3 hrs at 80 volts in submarine electrophoresis apparatus. The gel was stained with ethidium bromide (0.1%) and photographed using gel documentation system. The amplification profiles for all the primers were compared with each other and the bands of DNA fragments were scored as present (1) or absent (0) generating the binary matrices. The binary data was analyzed under the SIMQUAL module of NTSYS PC-2.0 software programme (Rohlf, 1998) using DICE coefficient (Nei and Li, 1979). A dendrogram based on the UPGMA clustering method (SAHN) (Sneath and Sokal, 1973) was used to generate a tree. Principle coordinate analysis was performed to estimate the genetic distance between each group of the isolates by using NTSYS software. Then analyzing the product matrix by EIGEN to get Eigenvectors and Eigenvalues, and finally getting its 2D scatter plot graph. The per cent polymorphism was computed by dividing total number of polymorphic bands with total number of bands and multiplying resultant with 100.

RESULTS AND DISCUSSION

The PCR amplification product of twelve isolates of *Bacillus* spp. generated 343 bands with 16 random primers on agarose gel electrophoresis (Table 2). The number of bands produced per primer varied from 4 to 35. Out of 343 bands, 182 were polymorphic, 158 were unique while, 3 were monomorphic bands. Most of the primers produced polymorphic bands varied from 2 to 19. Maximum polymorphic bands (19) were observed in primer RBA-5, RBA-13 and RBA-15. The average polymorphism observed was 99.13%. Genetic variation among different strains can be documented by using different molecular markers (Sabir *et al*., 2013).

The genetic similarity index based on the pooled data of RAPD profiles from 16 primers ranged from 0.11 to 0.76 among all the isolates (Table 3). The genetic similarity matrix revealed highest genetic similarity index of 0.76 between the isolates B-42 and BNF/BS-32. The minimum genetic similarity index was observed in the isolates B-15 and B-51 (0.11).

On the basis of RAPD analysis, the dendrogram constructed from the pooled data had four major groups (Fig. 1). The first group consisted of two distinct isolates, i.e. B-11 and B-37 with a genetic similarity value of 0.46. The second group was divided into four subgroups. The subgroup IIa

Table 2. RAPD analysis of *Bacillus* spp. isolates

Primer Name	Primer sequence	Total No. of bands	No. of monomorphic bands	No. of unique bands	No. of polymorphic bands	% poly - morphism
RBA 1	5′ AAAACCGGGC 3′	20	1	7	12	95.00
RBA 2	5′ ACAGGGCTCT 3′	29	0	15	14	100.00
RBA 3	5′ ACAGGGGTGT 3′	15	0	8	7	100.00
RBA 4	5′ ACCGGGTTTC 3′	19	0	14	5	100.00
RBA 5	5′ AGGGGCGGCA 3′	35	1	15	19	97.14
RBA 6	5′ ATCCTGCCTG 3′	12	0	7	5	100.00
RBA 7	5′ ATCGGGTCCT 3′	4	0	2	2	100.00
RBA 8	5′ ATCGGGTCGA 3′	22	0	16	6	100.00
RBA 9	5′ ATCTGCGAGC 3′	9	0	5	4	100.00
RBA 10	5′ CCCGCCTTCC 3′	21	0	10	11	100.00
RBA 11	5′ CCGGCCCCAA 3′	21	0	9	12	100.00
RBA 12	5′ CCGGCCTTAA 3′	27	0	14	13	100.00
RBA 13	5′ CCGGCCATAC 3′	31	0	12	19	100.00
RBA 14	5′ CCGGCCTTCC 3′	25	0	9	16	100.00
RBA 15	5′ CCGGCTGGAA 3′	29	1	9	19	96.55
RBA 16	5′ CCGGGGAAAC 3′	24	0	6	18	100.00

consisted of two isolates, i.e. B-13 and B-15 having genetic similarity coefficient 0.70. The subgroup IIb consisted of three isolates, i.e. B-38, B-46 and B-57. The subgroup IIc consisted of B-42 and BNF/BS-32 having highest similarity coefficient 0.76 while subgroup IId consisted of single isolate B-27 with genetic similarity value of 0.40. The third group consisted of single B-28 isolate having genetic similarity coefficient 0.33. The fourth group consisted of a most distinct single isolate B-51 having similarity coefficient 0.16. Similar clustering was observed in 2D PCO scatter plot which was also helpful to make up the group of isolates on the basis of presence of points on particular axis (Fig. 2). From the genetic similarity coefficient, it was observed that the isolates B-51, B-37 and B-11 showed wide genetic diversity while the remaining isolates showed less genetic diversity among the *Bacillus* sp. isolated from decomposing wastes. Isolate B-51 was found most divergent, followed by B-11 and B-37 and then B-28. Most divergent isolate B-51 has umbonate colonies while the others have flat colonies with an exception of B-42 with raised colonies. Most divergent B-51, B-11, B-37 and B-28 isolates were all isolated from wastes from open heaps. Comparatively both the isolates from urban waste were more divergent than isolates from rural waste (Table 1).

Similar results were reported by Desai and Varadaraj (2010) who evaluated genetic relatedness among 12 native isolates of *Bacillus cereus* using 4 selected arbitrary primers in RAPD-PCR. The total amplified products of the 4 primers were 168 of 100-1000 bp. The genetic similarity coefficient for these isolates ranged from 0.04 to 0.47. The data analysis generated a dendogram with 3 clusters covering 11 isolates and the remaining one isolate was of non-cluster type. Shiva Reddy *et al.* (2010) also examined

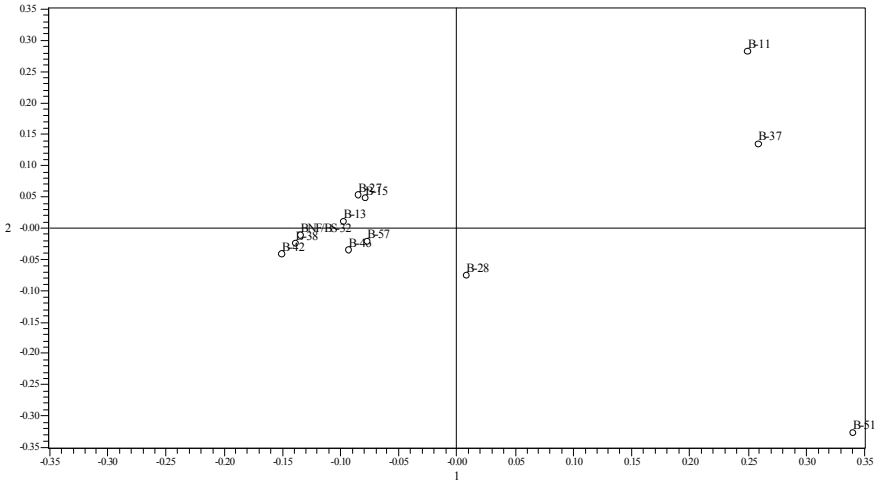

Fig. 1. Dendrogram representing the clustering among different isolates of *Bacillus* spp.

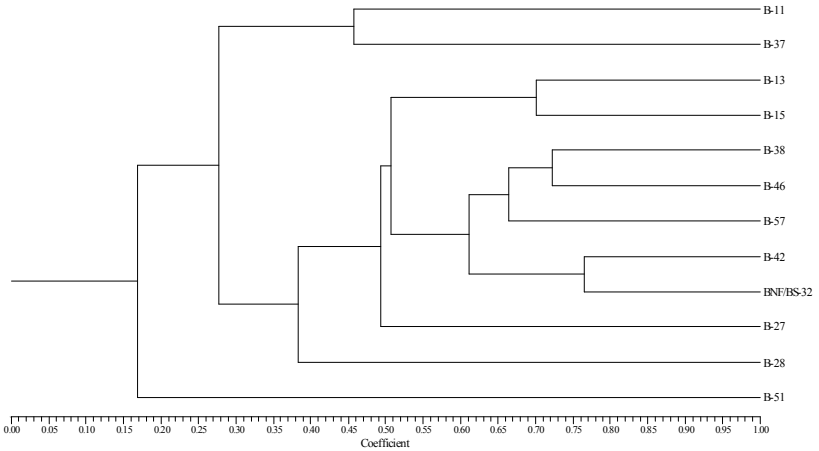

Fig. 2. RAPD 2D PCO scatter plot representing the phylogenetic relationship among different isolates of Bacillus spp.

Table 3. Genetic similarity as DICE coefficient based on pooled data of RAPD profiles for *Bacillus* spp.

Isolate	B-11	B-13	B-15	B-27	B-28	B-37	B-38	B-42	B-46	B-51	B-57	BNF/ BS-32
B-11	1.00											
B-13	0.36	1.00										
B-15	0.29	0.70	1.00									
B-27	0.36	0.52	0.45	1.00								
B-28	0.26	0.38	0.34	0.34	1.00							
B-37	0.46	0.24	0.25	0.22	0.19	1.00						
B-38	0.31	0.61	0.48	0.58	0.46	0.24	1.00					
B-42	0.27	0.62	0.52	0.49	0.39	0.19	0.70	1.00				
B-46	0.33	0.53	0.41	0.45	0.41	0.22	0.72	0.60	1.00			
B-51	0.23	0.15	0.11	0.17	0.17	0.21	0.15	0.15	0.17	1.00		
B-57	0.34	0.49	0.38	0.44	0.35	0.30	0.62	0.58	0.71	0.18	1.00	
BNF/ BS-32	0.31	0.55	0.47	0.51	0.39	0.27	0.61	0.76	0.54	0.16	0.63	1.00

ten *Bacillus megaterium* isolates on the basis of cluster analysis of 20 RAPD bands. It was found that all the 10 isolates formed two major clusters. Kumar *et al.* (2010) evaluated 70 isolates of *Bacillus thuringenesis* for molecular diversity using RAPD-PCR technique. The RAPD banding pattern data subjected to dendrogram construction showed two main clusters which were further divided into four subclusters at Eucledian distance of 150 and 80% similarity index. High local genotypic diversity in the cases of *Bacillus* spp. can be attributed to recombination, which appeared to be frequent (Istock *et al.*, 1992).

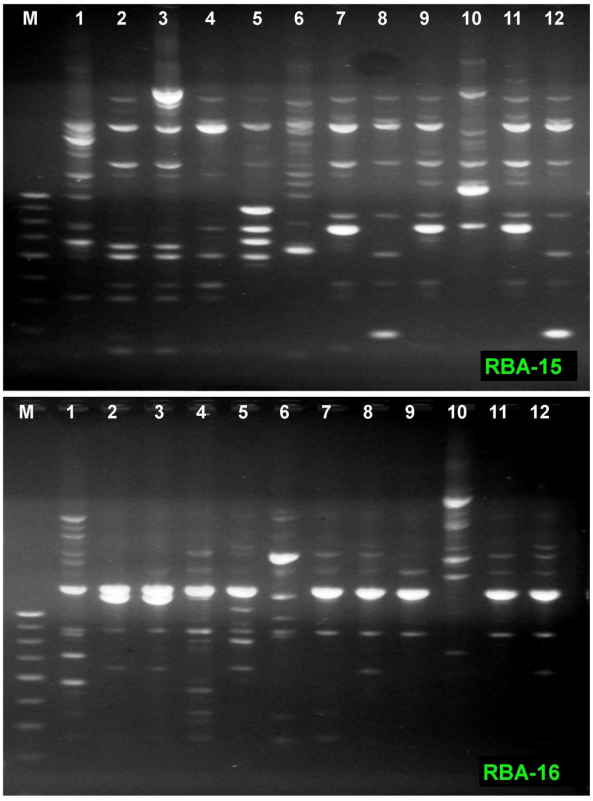

Plate 1 Amplification profiles of different isolates of *Bacillus* spp. with RBA-15 and RBA-16 primer

Lane M : Marker 100 bp (StepUp Low Range DNA Ladder)
Bacillus spp. isolates: 1. B-11 2. B-13 3. B-15 4. B-27
5. B-28 6. B-37 7. B-38 8. B-42 9. B-46 10. B-51
11. B-57 12. BNF/BS-32 (MPKV strain)

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