International Journal of Manures and Fertilizers Vol. 3 (1), pp. 449-456, January, 2014. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Cellulose bacteria biodiversity in long-term manure experiment site

Gaurav Kaushik, Jadav Vishnu and Ranjan Arslan

College of Agriculture, Faculty of Crop Physiology, Assam Agricultural University, Jorhat, Assam, India.

Accepted 16 December, 2013

To study the Influence of long-term application of manure on cellulolytic bacterial diversity, 54 efficient cellulolytic bacterial cultures were isolated from two long-term manure experimental field sites of Berlin-Dahlem (established in 1923) and BadLauchstädt (established in 1902). The sequence divergence of highly conserved region of 16S rDNA was exploited by restriction analysis of PCR-amplified 16S rDNA using restriction enzyme *Scr*FI to assay the evolutionary relatedness of isolates. Restriction analysis identified 10 genetically diverse pattern groups comprising five groups each of bacterial and actinomycetal domain. Irrespective of manuring, a dominant pattern group (H_2) was identified, containing 31.48% of total isolates. On the other hand, two site-specific pattern groups highly specific for the brown soil (H_2, 9.25% of total isolates) and for black soil (J_2, 11.11% of total isolates), respectively were identified. In general, the composition of cellulolytic isolates in two sites displayed differences with respect manure application and soil properties. Manure strongly influenced the abundance of cellulolytic bacterial diversity in brown soil. The terminal restriction fragment length polymorphism (T-RFLP) data revealed a distinct relationship of total bacterial diversity with long-term manure application. This influence is more prominent in nutrient poor brown soil. Based on 16S rDNA sequence analysis, isolates of the dominant as well as the specific pattern groups could be assigned to the genus *Streptomyces* comprising species of diverse phylogenetic affiliation. Furthermore, sequencing of 16S rDNA of isolates of five bacterial pattern groups revealed a high phylogenetic diversity among these isolates, including *Streptococcus, Paenibacillus, Bacillus, Bacillus megaterium* and *Bacillus pumilus*.

Key words: Cellulolytic bacteria, 16SrDNA gene, restriction analysis, restriction fragment length polymorphism (T-RFLP), terminal restriction fragment length polymorphism (T-RFLP), phylogenetic diversity.

INTRODUCTION

The process of photosynthesis is the main route for the acquisition of energy in plant biomass of which cellulose is the major component. Release of energy and return of bio-sequestered carbon to soil environment are primarily the concern of cellulose utilizing special group of microorganisms called cellulolytic organisms. The carbon cycle is closely associated with the activities of cellulose-utilizing microorganisms in soil. Thus, microbial cellulose utilization is responsible for one of the largest material flows in the biosphere and is of interest in relation to analysis of carbon flux at both local and global scales. Plant biomass is the only foreseeable sustainable source of fuels and materials available to humanity (Lynd et al., 2002). In this context, cellulosic materials are particularly attractive because of their relatively low cost and plentiful supply. The central technological impediment to more widespread utilization of this important resource for soil enrichment is the general absence of appropriate technology for overcoming the recalcitrance of cellulosic biomass (Halliwell, 1965) and a little knowledge about the

*Corresponding author. E-mail: Gaurav16@yahoo.com

participating bacterial communities. A promising strategy to overcome this impediment involves the utilization of native cellulolytic microbes producing wide array of cellulase enzymes. Notwithstanding its importance in various contexts, fundamental understanding of microbial cellulose utilization is in many respects rudimentary. Thus, interest in cellulose decomposition and in cellulolytic microorganisms has been stimulated by the need of greater understanding of this important process in nature (Coughlan and Mayer, 1992; Eriksson et al., 1990). In this context, cellulolytic bacteria play the pivotal role in the transformation of cellulosic residues in the soil ecosystem (Mullings and Parish, 1984; Szegi, 1988). The group of aerobic cellulolytic bacteria is rather diverse and affiliated to various taxonomic groups (Eriksson et al., 1990). There is a general interest in studying the diversity of indigenous bacteria capable of degrading cellulosic materials because of their inherent capacity to elaborate cellulase enzyme complex for sequential degradation of pure crystaline cellulose, more so, cellulose derivatives of higher resistance, hugely available in wastes of farm and industrial origin. Efforts have been made to isolate potential degraders and characterize bacterial communities as well as their response to the substrate cellulose, more so to identify the genes involved in the degradation process. A range of approaches is available for assessing the composition, diversity of soil microbial communities, including 16S rDNA analysis, assays of substrate utilization profiles (Ulrich and Wirth, 1999; Ulrich et al., 2008). While examining the distribution of cellulolytic species across taxonomic groups, it deems useful to consider microbial taxonomy based on phylogeny, rather than a set of arbitrary morphological or biochemical characteristics as used in classical taxonomy. Current views of the evolutionary relatedness of organisms are based largely on phylogenetic trees constructed from measurements of sequence divergence among chronometric macromolecules, particularly small-subunit - rRNAs (16S rRNA of procaryotes and 18S rRNA of eukaryotes Olsen and Woese, 1993; Woese, 2000)**.** An inspection of these trees reveals that the ability to digest cellulose is widely distributed among many genera in the domain *Eubacteria* and in the fungal groups within the domain *Eucarya*, although no cellulolytic members of domain *Archaea* have yet been identified. Within the eubacteria there is considerable concentration of cellulolytic capabilities among the predominantly aerobic order *Actinomycetales* (phylum *Actinobacteria*) and the anaerobic order *Clostridiales* (phylum *Firmicutes*) (Lynd et al., 2002).

Most studies of aerobic cellulolytic bacteria involve techniques based on pour-plate serial dilution, which is inexpensive and suboptimal, necessarily selective since an unknown part of the indigenous microbial population is considered to be non-cultured (Olson and Bakken, 1987). Moreover, a range of different cellulosic substrates is used to isolate and detect cellulolytic bacteria and their activities but not all are equally utilized by all cellulolytic bacteria lacking of wide array of cellulase system (Beguin and Aubert, 1994). Thus, knowledge of the diversity and community level physiological profile of cellulolytic bacteria remains obscure. Despite methodological constrains, evidence for distinct differences in community composition could recently be demonstrated by application of ribosomal RNA sequence analysis McCraig et al. (2001) offering new perspectives to the traditional phenotypic classification system. Existing scientific literatures show huge bacterial diversity under the influence of long term manure application in field scale (Stellwag et al., 1995; Teather and Wood, 1982; Pourcher et al., 2001; Sekiguchi et al., 2007; Ulrich et al., 2008). But evidences regarding the long term effect of farm yard manure on bacterial diversity in different soil types are sparse. Thus, the aim of the study was to explore the effect of long-term manure application on abundance of phylogenetically diverse culturable cellulolytic bacteria in two different agricultural soils.

MATERIALS AND METHODS

Study site and soil sampling

Over hundred year old long-term manure experimental sites were selected for the isolation and characterization of cellulolytic bacterial communities. Nutrient-poor Orthic Luvisol (Brown soil) and Haplic Chernozem (Black soil) were studied at long–term field experimental sites, located in Berlin-Dahlem and Bad Lauchstadt (Table 1), respectively. At both sites, winter wheat was grown in three replicated plots with or without farmyard (FYM) treatments and were sampled in April, 2003 at full growth stage of wheat. Ten samples were collected form plough layer of each plots using soil corer and pooled as composite sample, finally sieved (<5 mm) and stored at 4°C until processing.

Isolation of soil cellulolytic bacteria

The culturable soil cellulolytic bacteria were isolated using soil serial dilution and pour plate technique. Soil samples (1 g dry weight basis) were suspended in 99 ml sterile distilled water by magnetic stirring in order to dislodging bacterial cells for clay particles. Replica aliquots (1 ml) were poured and dispersed by swirling with Ken Knight's Agar Medium prepared form K_2HPO_4-1.0 g, $NaNO_3$-0.1 g, KCl-0.1 g, $MgSO_4$, $7H_2O$-0.1 g, Cellulose powder-10 g, Agar agar-15.0 g, and Distilled water 1 L. Cycloheximide (50 mg^{-1}) was added to suppress fungal growth. pH was adjusted at 7.0 prior to autoclaving. Congo red (0.2% w/v) was used as an indicator for the detection of cellulolytic bacteria as described by Teather and Wood (1982). In dilution plate, detection of cited bacteria was carried out after 3 and 5 days of incubation at 30°C. After an incubation of 2 to 5 weeks at 20°C, cellulolytic isolates were transferred to Tryptic Soya Agar, pH 7.0 (Difco).

DNA isolation and PCR amplification

For template preparation, DNA was isolated form bacteria grown in

Table 1. Site properties of the experimental plots.

Long-term land use experiment at Berlin-Dahlem (DIII)	
Location	Berlin, Deutschland; 52°28´N, 13°18´E
Established	1923
Climate	Semi-continental
Mean annual rainfall	547 mm
Mean annual temperature	9.2°C
Soil classification	Brown soil/Orthic Luvisol
Soil type	Silty sand
Crop rotation	Fodder beet- winter wheat –potato- winter wheat
Manure	30 ton ha^{-1} every second year (since1939)
Static, long-term fertilizer experiment at Bad Lauchstädt	
Location	Saxony-Anhalt, Deutschland, 51°24´N, 11°53´E
Established	1902
Climate	Semi-continental
Mean annual rainfall	483 mm
Mean annual temperature	8.7°C
Soil classification	Blach soil/ Haplic Chernozem
Soil type	Loan
Crop rotation	Sugar beet –spring barley-potato- winter wheat
Manure	30 ton ha^{-1} in every year (since 1902)

tryptic soy broth (Difco) for 1 to 4 days at 28°C. Cells were harvested (0.5 to 1.5 ml) by centrifugation, washed with 0.3% NaCl, resuspended in 50 µl 0.3% NaCl, and ground using mortar and pestle. Finally, 5 to 10 µl of the crushed cells were added to 20 µl of 25 mM NaOH/0.25% SDS and heated for 15 min at 95°C. Aliquots (0.2 µl) of the resulting lysate were directly used for PCR without further purification. The primers fDI and 926r (Ulrich and Wirth, 1999; Ulrich et al., 2008) used in this study are homologous to the consensus sequence of the 16S rDNA genes and are capable of amplifying almost the complete 16S rDNA of most eubacteria. Routinely, a 50 µl reaction mixture containing 1 µl of template DNA, 5 µl of 10 × reaction buffer (Applied BiosystemWeiterstadt, Germany), 3.5 µl of $MgCl_2$ 0.5 µl of deoxynucleoside triphosphate, 0.25 µl of each primer, 0.5 µl Taq-polymerase (PE Biosystem) and 39.5 µl water was used. The amplifications were performed in a GeneAmp PCR system 2400 (Perkin-Elmer Corporation, Norwaik, CT) with the following protocol: initial denaturation at 95°C for 2 min; 25 cycles of 30 s at 94°C, 40 s at 54°C, 1.3 min at 72°C, a single final extension at 72°C for 8 min and a final soak at 4°C (Ulrich and Muller, 1998). After the reaction, aliquots (3 µl) were mixed with 5V stopper and the PCR products were examined in a 1% agarose gel.

RFLP analysis

PCR product (2 to 8 µl) were digested with *Scr*FI (New England Biolabs, Beverly, MA) and separated in 2.5% Metaphor agarose gels (FMC Bioproducts, Rockland, ME). The DNA molecular weight markers V and VI, respectively, were used as a size standard (Boehringer Mannheim, Germany). The gels were stained with ethidium bromide and finally documented using video camera image system (EasyImage Plus, Herolab, Wiesloch, Germany). Isolates with identical restriction patterns were designated as a single 16S rDNA ribotype group.

Sequencing

Representative isolate of these pattern groups were used for 16S rDNA sequence determination. PCR products of 16S rDNA were purified by Qiaquick PCR Purification Kit (Quiagen, Santa Clarita, CA) according to the manufacturer's instructions. A cycle sequencing protocol was applied for sequencing both complementary strands using primers 346r, 399f and fdI with ABI Prism 310 Sequencer (Applied Biosystems) using a BigDye Terminator Cycle Sequencing Kit. The nearly complete 16S rDNA sequences were compared to sequences available from the Ribosomal Database Project and EMBL/Gene Bank database.

Analysis for phylogenetic tree

To understand the phylogenetic relationships among the pattern groups, a phylogenetic tree was constructed. The similarity values of isolate ribotype were based on a pairwise comparison of sequences and pairwise distance (DNADIST). For phylogenetic analysis, the DNA sequences were aligned using the W algorithm (program version Clustal X ,1.83) and trees were constructed using the Neighbour-Joining and Maximum-likelihood algorithms (PHYLIP Programme package 3.57) (Felsenstein, 1993). The tree topologies were evaluated by bootstrap analysis of the Neighbour-Joining tree using original dataset and 1000 bootstrap data sets.

Analysis of bacterial communities

Total DNA was extracted from 0.25 g of composite soil using a Fast DNA Spin Kit for Soil (Q BIOgene, Carlsbad, CA) following manufacturer's direction. Moreover, a wash procedure with 5.5 M guanidine thiocyanate was performed twice after the extracted DNA to completely remove humic acids which interferes during

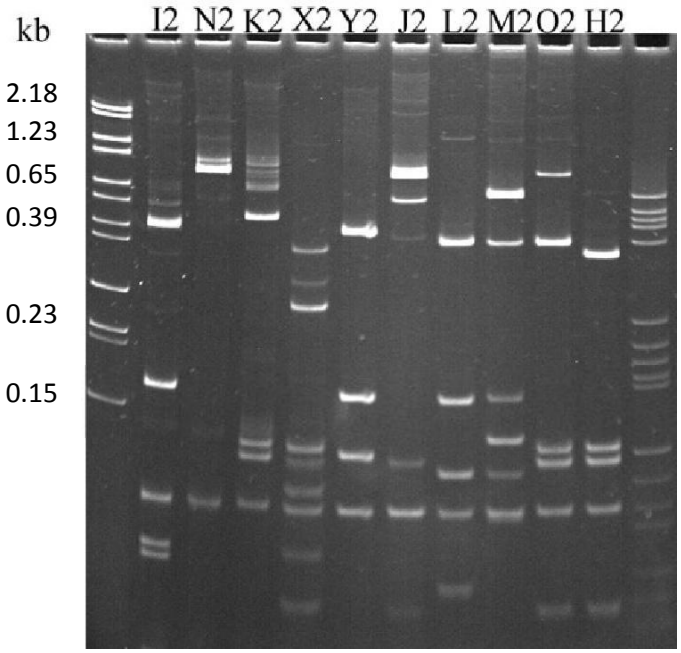

Figure 1. Restriction patterns of anplified 16S rDNA digested with endonuclease ScrFI. Lanes I2 to H2 corresponds to pattern groups as shown in Table 3. First and last lanes represent molecular weight markers V and VI, respectively (Boehringer Mannheim, Germany).

amplification in PCR. T-RFLP analysis was performed as described by Ulrich and Becker (2006). The 16S rRNA gene was amplified using eubacterial primers 8f labeled with 6-FAM and 926r. The polymerase chain reaction (PCR) products were digested with HhaI and subsequently separated with GeneScan 1000 Rox (Applied Biosystems, Foster City, CA) as an internal size standard on an ABI 310 DNA sequencer (Applied Biosystems) using POP6 polymer. Terminal fragments were evaluated by the GeneScan Analytical Software version 3.1.2. T-RFLP profiles were standardized in a similar way as suggested by Dunbar et al. (2001). After the standardization of the profiles, only peaks above a threshold of 50 fluorescence units were considered. Cluster analysis of the T-RFLP profiles was performed using the GelCompar II software v. 2.5 (Applied Maths, Saint-Martens-Latem, Belgium). The ABI files were converted into the Gel Compar curve format (Ulrich and Becker, 2006).. The optimization procedure of the peak tolerance resulted in a value of 0.01%. To consider both the presence and the relative abundance of the terminal restriction fragments (TRFs), densitometric curves of whole profiles were analyzed using Pearson's correlation coefficients. The resulting similarity matrix was the basis for clustering by the Ward algorithm (Ward, 1963). Densitometric curves in the range of 30 to 900 bp were used for the calculation.

RESULTS

Biodiversity of cellulolytic bacterial isolates

A great phenotypical variability among the isolates was observed when grown on SEA medium. Isolates were selected on the basis of phenotypic characteristic, subcultured and purified following standard microbiological protocol. The rDNA of the isolates were amplified using primer pair fdI and 926r, resulting in characteristic single band of about 1400 bp. Restriction analysis of PCR products of the 54 isolates using restriction enzyme *Scr*FI revealed various master patterns as demonstrated in Figure 1. DNA fragment smaller than 70 bp were not properly resolved by electrophoresis and therefore were not used for the comparison of the patterns. Ultimately, five to eight restriction fragments per pattern were used as tools to differentiate the isolates. Using the restriction enzyme *Scr*FI, 54 isolates were classified into 10 genetically diverse pattern groups.

Five isolates out of the 10 RFLP groups were assigned to the *Actinomycetales* branch, and rest to the non-actinomycetales as supported by high bootstrap values (Table 3). At all sampling positions whether manured or unmanured, a dominant pattern group (H_2) was identified, containing 31.48% of total isolates.

The restriction pattern of master group H_2 and O_2 are very similar in band pattern. Only two bands is different (Figure 1). Sequence data of the representative member of those groups showed 99.1 and 99.3% similarity with reference sequence of *Streptomyces avermitlis* and *Streptomyces* sp., respectively. Two site-specific pattern groups could be identified, representing a part of the total population, which was highly specific for the brown soil treated with FYM (H_2, 9.25% of total isolates) and for black soil treated with no FYM (J_2, 11.11% of total isolates), respectively. In general, the composition of cellulolytic isolates in two sites displayed differences with respect manure application and soil properties. Cellulolytic bacterial diversity in brown soil had been influenced by manuring while the same was not noticed in black soil treated with manure. But the composition of cellulolytic 16S rDNA RFLP groups was impacted by manure application with highest evidence in the brown soil. Based on 16S rDNA sequence analysis, isolates of the dominant as well as the specific pattern groups could be assigned to the genus *Streptomyces*. Furthermore, sequencing of 16S rDNA of isolates of five pattern groups of L_2, X_2, K_2, N_2 and I_2 revealed a high phylogenetic diversity among these isolates, representing sequence similarity of *Streptococcus* (98.8% similarity), *Paenibacillus* (98.0% similarity), *Bacillus* (99.1% similarity), *Bacillus megaterium* (98.0% similarity) and *Bacillus pumilus* (98.7% similarity), respectively. The genotypic group K_2 has a phylogenetic relationship with an unknown soil bacterium with a close relationship with bacilli. The master group Y_2 is phylogenetically very distant as compared to other groups and classified as Gram-negative genus of *Fateuria* (Figure 2). Phylo-genetic study reveals that the cellulose decomposing ability is widely distributed among many genera including

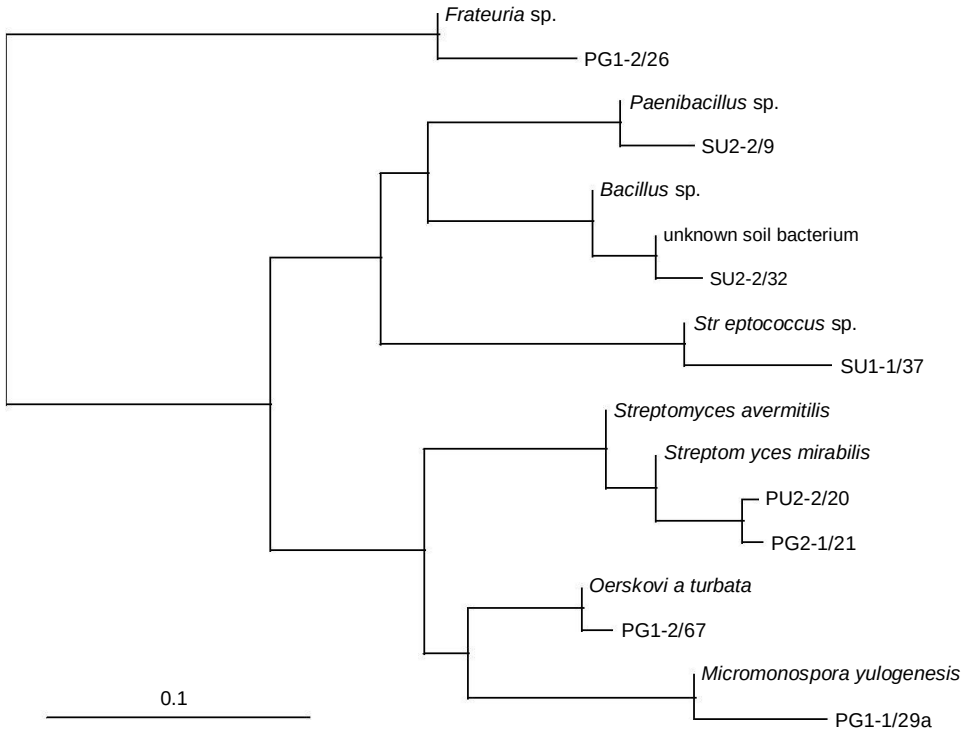

Figure 2. Phylogenetic tree showing the relationship of the cellulolytic isolates and related reference species. The tree constructed using neighbour-joining method is based on nearly complete 16S rDNA sequences. The tree was rerooted by outgroup of E.coli. The bar, 0.1 indicates the relative sequence divergence. SG - Schwarzerde stallmistgedüngt, SU - Schwarzerde Kontrolle, PU - Parabraunerde Kontrolle, PG - Parabraunerde stallmistgedüngt mean black soil manured,.black soil unmanured, brown soil unmanured and brown soil manured, respectively.

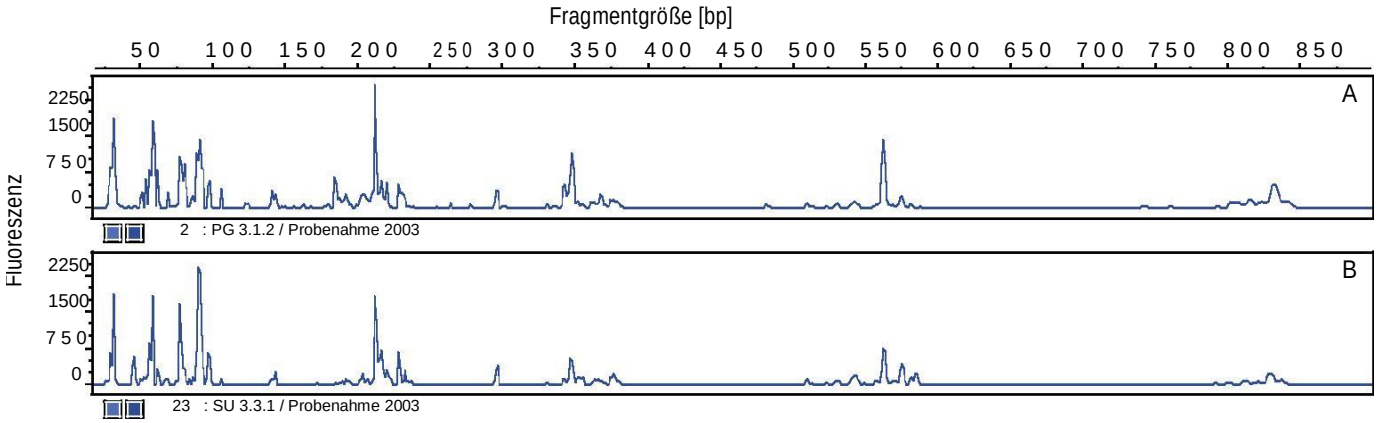

Figure 3. T-RFLP pattern of bacterial community under brown soil (A) and black soil (B).

the domain *Eubacteria* comprising aerobic order *Actinomycetales* (phylum *Actinobacteria*) largely comprises of *Streptomyces* species of diverse phylogenetic affiliation; and the aerobic genera of *Streptococcus, Paenibacillus* and *Bacillus*. The phylogenetic relationship between cellulolytic bacterial isolates and reference strains is demonstrated in Figure 2.

Terminal restriction fragment length polymorphism (T-RFLP) analysis of 16S rDNA for charcterization of total bacterial community of long-term manure experimental sites

The huge number of T-RFs in the individual T-RFLP profiles (Figure 3) of soil of experimental sites representing a greater abundance of bacterial groups.

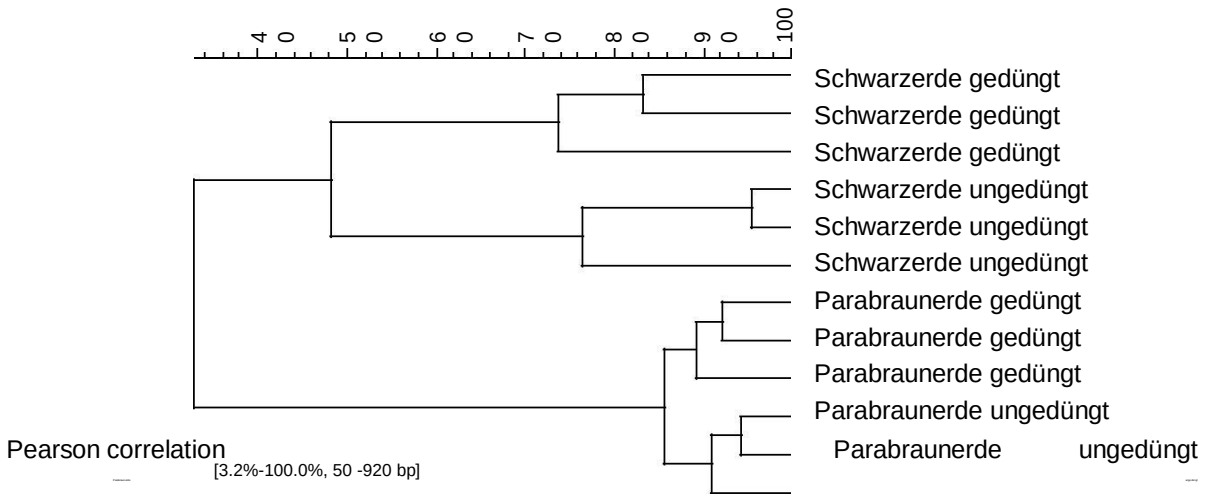

Figure 4. Dendogram showing the similarity between the T-RFLP profiles (community structure) derived from the manured (gedungt) and control (ungedungt) plots of the black soil (Schwarzerde) and brown soil (Parsbraunerde).

After recognizing the predominent T-RFs of the complete T-RFLP Profile, a hierarchical cluster analysis was performed to identify relatively homogeneous group of isolates based on T-RFLP profile characteristics, using an algorithm (complete linkage). Distance or similarity measures are generated by the proximities procedure following WARD-clustering. Cluster analysis of the T-RFs grouped the community and yielded a different basic dendogram topology representing different bacterial clusters. In agreement with the results from the cluster analyses, the combined dataset revealed very tight clustering of the soil bacterial community and a very clear separation among the community (Figure 4).

The primary branch differentiates the community structures of the two soils investigated. The secondary branch represents the T-RFLP profiles obtained from both manured and unmanured plots of experimental sites. In the tertiary branches, all profiles obtained form manured brown soils/and or black soils are distinguished from the control unmanured plots of respective soil (Figure 4).

DISCUSSION

Bibliography antecedent reveals that most of the studies so far conducted on cellulolytic bacteria in agroeco-system were based mostly on the enumeration of colony forming units on agar plate using serial dilution and pour plate technique where the community composition was rarely analyzed (Ulrich et al., 2008).

Moreover, phylogenetic studies of cellulolytic bacterial communities published so far have focused on microbial communities associated with terrestrial and marine herbivores (Stahl et al., 1988; Stellwag et al., 1995) or other highly specific environment (Marri et al., 1997; Rainey et al., 1993). This present study deals with cellulolytic bacterial diversity in two long-term manure experimental sites of different soil and environmental condition with a view to study the soil cellulolytic bacterial biodiversity along gradients of fertility where trends of diversity of bacteria can be explored (Giller et al., 1997). The experimental site in Berlin-Dhalem is nutrient poor sandy soil while in BadLauchstädt is highly fertile loamy soil. Thus, a broad range of different soil ecological situation is present as charcterised by soil properties (Tables 1 and 2). Therefore, distinct soil ecological conditions would expect to harbour diverse cellulolytic bacterial communities.

Single methodology is not sufficient to resolve the community composition in soil. So, in this study a polyphasic approach comprising of culture dependent and culture independent methodlogy were adopted. Culture dependent methods encopassing screening of cellulolytic bacteria and a subsequent classification using RFLP of amplified 16S rDNA was applied, revealing a simple community of culturable soil cellulolytic bacteria.Whereas, culture independent methodlogy consists of total soil DNA analysis and characterizing through T-RFLP profiling. Culture dependent methods provide insufficient information about soil cellulolytic bacterial community composition since a major part of the soil residing cellulolytic bacteria is considered to be nonculturable due to the lack of proper designing of media with appropriate composition. Thus, phylogenetic diversity on the basis of database obtained from RFLP profiling are considered to be representative for a culturable subset of the potentially cellulolytic bacterial community. Despite these methodological constraints, evidence for differences in community composition could be demonstrated.

RFLP profiling of 54 bacterial isolates using single

Table 2. Characteristic of experimental soil.

Parameter	Berlin-Dahlem (Orthic- Luvisol)		Bad Lauchstädt (Haplic- Chernozem)	
	Manured	Control	Manured	Control
C_{org} (%)	0.86 ± 0.16*	0.62 ± 0.03	2.27 ± 0.05*	1.68 ± 0.01
N_t (%)	0.07 ± 0.01	0.05 ± 0.00	0.19 ± 0.00	0.13 ± 0.00
CEC (cmol kg^{-1})	8.8 ± 0.34	9.6 ± 1.17	39.1 ± 3.81*	29.8 ± 1.26
pH	6.0 ± 0.11	5.6 ± 0.16	6.7 ± 0.06	6.3 ± 0.29
Texture				
Clay (%)	2.9		21.0	
Silt (%)	25.0		67.8	
Sand (%)	72.1		11.2	

Table 3. 16S rDNA genotype groups identified and their relative proportion at the four experimental plots.

Pattern group	Brown soil manured	Brown soil unmanured	Black soil manured	Black soil unmanured	Total isolate	Percent of similarity to the closest relative	Putative assignment
H_2	5 (9.25)	4 (7.40)	4 (7.40)	4 (7.40)	17 (31.48)	99	*Streptomyces avermitilis*
O_2	1 (1.85)	1 (1.85)	2 (3.70)	1 (1.85)	5 (9.25)	99.3	*Streptomyces*
M_2	0	1 (1.85)	2 (3.70)	0	3 (5.55)	98.5	*Streptomyces*
L_2	1 (1.85)	1 (1.85)	0	3 (5.55)	5 (9.25)	98.4	*Streptomyces lavendulae*
J_2	2 (3.70)	0	0	6 (11.11)	8 (14.81)	98.8	*Streptococcus*
Y_2	1 (1.85)	0	0	2 (3.70)	3 (5.55)	98	*Streptomyces cyaneus*
X_2	2 (3.70)	0	1 (1.85)	1 (1.85)	2 (3.70)	98.3	*Paenibacillus*
K_2	2 (3.70)	0	0	2 (3.70)	4 (7.40)	99.1	*Bacillus*
N_2	1 (1.85)	0	2 (3.70)	1 (1.85)	5 (9.25)	98.7	*Bacillus megaterium*
I_2	0	0	0	1 (1.85)	2 (3.70)	98	*Bacillus pumilus*

Pattern groups are based on the restriction analysis by the enzyme ScrFI.

endonuclease yielded in altogether 10 16S rDNA genotypic master groups. As single endonuclease digestion is not sufficient to resolve the bands of amplified gene, most of the pattern groups probably include more than one species. This corroborates the findings of earlier workers (Ulrich and Wirth, 1999; Ulrich and Becker, 2006; Ulrich and Muller, 1998). After sequencing of 16S rDNA, isolates of genotypic group H_2 were classified to genus *Streptomyces*. Thus, it may be concluded that group H_2 represents several *Streptomyces* species. Although there exists differences in community composition, the community structure of that functional group was rather simple. Only a single predominant RFLP group (H_2) that comprised 31.48% of the total isolates were identified. This is the line with the observation of Akasaka et al. (2003). They also found the predominance of *Cellulomonas* species during the degradation cellulose rich paddy straw. This phenomenon is further supported by the report of many scientists (Ulrich and Wirth, 1999; Lynd et al., 2002; Lynd et al., 1999; Ulrich et al., 2008) who observed site specific predominance of a single group of bacteria.

In order to evaluate the phylogenetic range of the cellulolytic bacterial isolates, single representatives of another five genotype groups were identified by 16S rDNA sequencing. Besides the predominance of *Streptomyces* species, the sequence data revealed non actinomyetes bacterial species of *Streptococcus* (98.8% similarity), *Paenibacillus* (98.0% similarity), *Bacillus* (99.1% similarity), *Bacillus megaterium* (98.0% similarity) and *Bacillus pumilus* (98.7% similarity), indicating a diversity of species within the Gram-positive cellulolytic soil bacteria. DNA sequencing in combination with RFLP analysis revealed instead of pattern group H_2, isolates of pattern groups O_2, M_2, L_2 and Y_2 also belong to the *Actinomycetales*. Thus, the results indicate the predominance of the *Actinomycetales* within the culturable cellulolytic bacteria. This result correspond to findings of Errikson et al. (1990) and Lynd et al. (2002) who discussed the striking role of actinomycetes in lignocellulosic materials decomposition in soil.

Although, the pattern of distibution of RFLP groups of culturable cellulolytic bacteria exhibited some similarities among the plots both manured and unmanured, for example, predominant occurance of the pattern group, H_2 several characteristic differences among the distribution of other RFLP groups were found. RFLP group X_2 was present in black soil under both the manured and

unmanured plots but was distinctly absent in brown soils under similar treatments. Thus, it may be concluded that such diversity of cellulolytic bacteria is related to the site properties. Similarly, the existence of greater number of RFLP groups in black soil as compared to that of brown soil might be related to higher organic carbon content and higher fertility status as well as better texture of soil. Results, thus, substaintiated the earlier findings of numerous authors that diversity of microorganisms largely depends on the soil properties, particularly soil texture (Ulrich and Becker, 2006).

A distinct site-specific bacterial community structure was indentified on the basis of T-RFLP analysis of 16S rDNA gene fragments. The community was further differentiated on application of manure. Thus, long-term manure application was proven to cause discribible impacts on the community structure. In general, site properties displayed stronger effects on the community structure than the long-term application of manure. This is the line with the observation of Girvan et al. (2003) who demonstrated soil type to be the primary determinant of community structure in arable soils under three different farm mangement practices. Soil parent material and texture were particularly proven to have a decisive influence on the composition of the bacterial communities (Ulrich and Becker, 2006). Other factors such as fertilizers, were also considered as potent drivers of soil microbia change and community structure (Marschner et al., 2003; O'Donnell et al., 2001). Long-term manure application in the present experiment caused a differentiation in the community structure of total bacterial community. This finding, thus, corroborates the earlier reports of Hartmann et al. (2006) that application of farmyard manure not only suppressed disease producing pathogens but also influenced on the bacterial community structure. This is the line with observation of Sekiguchi et al. (2007). This is further supported by Ulrich et al. (2008) who recorded a shift in bacterial communities in two contrasting soils under long-term manure application. This present experiment demonstrated the strong impact of both soil type and long-term manure application on the total bacterial community. This result has further supported by the unique distribution of RFLP genotypic groups of cellulolytic bacteria in two experimental sites as well as under the influence of manure application.

Contrasting soil property with a strong fertility gradient and long-term manure application as cellulose source is an idle experimental site to study the diversity of cellulolytic bacteria. In this present investigation application of molecular techniques followed by phylo-genetic analysis of sequence data adopted though differentiated the community structure of culturable cellulolytic bacteria, this analytical approach of using a single endonuclease could not explore the full range of diversity of this functional group. A finer resolution using a further combination of restriction enzymes is deemed necessary to revealed the diversity at a finer scale. Soil is a heterogenous system where the microbial load is subject to spatiotemporal variation. So, single assessment technique is not sufficient to work out the whole range of diversity. A polyphasic approach consisting of molecular approach coupled with functional attributes as well as structural diversity will be more effective to study the cellulolytic bacterial diversity in a rational way.

ACKNOWLEDGEMENT

The author (NS) gratefully acknowledges the financial assistance offered by German Govt. (InWEnt-International, Capacity Building) to carry out the programme in collaboration with ZALF (Center for Agriculture and Land Use Research, Institute for Primary Production, Muenceberg, Germany) during 2002-2003.

REFERENCES

Akasaka H, Izawa T, Ueki K, Ueki A (2003). Phylogeny of numerically abundant culturable anaerobic bacteria associated with degradation of rice plant residue in Japanese paddy field soil. FEMS Microbial. Ecol. 43:149-161.

Beguin P, Aubert LP (1994). The biological degradation of cellulose. FEMS Microbiol. Rev. 13:25-58.

Coughlan MP, Mayer F (1992). The cellulose-decomposing bacteria and their enzyme systems. In: Balows, A Truper, HG Dworkin, M Harder, W Schleifer KH (eds.) The Procaryotes, 2nd edition. Springer verlag, New York. pp. 460-515.

Dunbar J, Tick nor LO, Kuske CR (2001). Phylogenetic specificity and reproducibility and new method for analysis of terminal restriction fragment profiles of 16S rRNA genes from bacterial communities. Appl. Environ. Microbiol. 67:190-197.

Eriksson KEL, Blanchette RA, Ander P (1990). Microbial and enzymatic degradation of wood and wood components, Ch. 2.6. Cellulose degradation by bacteria, Springer Series in Wood Science (Timell TE, ed.). Springer, Berlin.

Felsenstein J (1993). PHYLIP (Phylogeny interference pachage), 3.5c edu.Seattle, University of Washington.

Giller KE., Beare MH, Lavelle R, Izac AMN, Swift JJ (1997). Agricultural intensification, soil biodiversity and agroecosystem fallow. Appl. Soil Ecol. **6**:3-16.

Girvan MS, Bullimore J, Pretty JN, Osborn AM, Ball AS (2003). Soil type is the primary determinant of the composition of the total andactive bacterial communities in arable soils. Appl. Environ. Microbiol. 69:1800–1809.

Halliwell G (1965). Hydrolysis of fibrous cotton and reprecipitated cellulose by cellulolytic enzymes from soil microorganisms. Biochem. J. 95:270-281.

Hartmann M, Fliessbach A, Oberholzer HR, Widmer F (2006). Ranking the magnitude of crop and farming systems on soil microbial biomass and genetic structure of bacterial communities. FEMS Microbiol. Ecol. 57:378–388

Lynd LR, Weimer PJ, van Zyl WH and Pretorius IS. (2002).Microbial cellulose utilization: fundamentals and biotechnology. Microbiol .Mol. Biol. Rev. 66:506–577

Lynd LR, Wyman CE, Gerngross TU **(**1999). Biocommodity engineering. Biotechnol. Prog. 15:777–793.

McCraig AE, Grayston SJ, Prosser JI, Glover LA (2001). Impact of cultivation on characterization of species composition of soil. International J. Food Microbiol. 4:315-325.

Marri I, Barboni E., Irdani T, Perito B, Mastromei G (1997). Restriction

enzyme and DNA hybridization analysis of cellulolytic Streptomuces isolates of different origin. Can. J. Microbiol. 43:395-399.
Marschner P, Kandeler E, and Marschner, B (2003). Structure and function of the soil microbial community in a long-term fertilizer experiment. Soil Biol. Biochem. 35:453–461
Mullings R, Parish JH (1984). Mesophilic aerobic Gram negative cellulose degrading bacteria from aquatic habitats and soils. J. Appl. Bacteriol. 57:455-468.
O'Donnell AG, Seasman M, Macrae A, Waite I, Davies JT (2001).Plants and fertilisers as drivers of change in microbial community structure and function in soils. Plant Soil 232:135–145
Olsen GJ,Woese CR (1993). Ribosomal RNA: a key to phylogeny. FASEB J. 7:113-123.
Olson RA, Bakken LR (1987).Variability of soil bacteria: optimization of plate counting technique and comparison between total counts and plate counts within different size groups. Microb. Ecol. 13:59-74.
Pourcher AM, Sutta L, Hebe L, Mogueder G, Bollet C, Simoneau P, Gardan L (2001). Enumeration and characterization of cellulolytic bacteria from refuse of landfill. FEMS Microbiol. Ecol. 34:229-241.
Rainey FA, Jansen PH, Morgan HW, Stuchebrandt E (1993). A biphasic approach to the determination of the phenotypic and genotypic diversity of some anaerobic, cellulolytic, thermophilic, rod-shaped bacteria. Antonie von Leeuwenhoek.64:341-355.
Sekiguchi H, Kushida A, Takanaka S (2007). Effects of cattle manure and green manure on the microbial community structure in upland soil determined by denaturing gradient gel electrophoresis. Microb. Environ. 22;327-335.
Stahl DA, Plesher B, Mansfield HB, Montgomery L (1988). Use of phylogenetically based hybridization probes for studies of ruminal microbial ecology. Appl. Environ. Microbiol. 54:1079-1084.
Stellwag EJ, Smith TD, Luczkovich B (1995). Characterization and ecology of carboxymethylcellulose-producing anaerobic bacterial communities associated with the intestinal tract of the pinfish, *Lagodon romboides*. Appl. Environ. Microbiol. 61:813-816.
Szegi J (1988). Cellulose decomposition and soil fertility. Academiai Kiado, Budapest. p. 186.
Teather RM, Wood PJ (1982). Use of Congo red-polysaccharide interactions in cnumeration and characterization of cellulolytic bacteria fron bovine rumen. Appl. Environ. Microbiol. 43:777-780.
Ulrich A, Wirth S (1999). Phylogenetic diversity and population densities of culturable cellulolytic soil bacteria across an agricultural encatchment. Microb. Ecol. 37:238–247
Ulrich A, Becker R (2006).Soil parent material is a key determinant of the bacterial community structure in arable soils. FEMS Microbiol. Ecol. 56:430–443
Ulrich A, Muller T (1998). Heterogeneity of plant-associated streptococci as characterized by phenotypic features and restriction analysis of PCR amplified 16S rDNA. J. Appl. Microbiol. 84:293-303.
Ulrich A, Klimke G, Wirth S (2008). Diversity and Activity of Cellulose-Decomposing Bacteria, Isolated from a Sandy and a Loamy Soil after Long-Term Manure Application. Microbiol. Ecol.55:512-522.
Ward JH (1963). Hierarchical grouping to optimize an objective function. J. Am. Stat. Ass. 58:236.
Woese CR **(**2000). Interpreting the universal phylogenetic tree. Proc. Natl. Acad. Sci. USA 97:8392–8396.