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EXPERIMENTAL ARTICLES

Population Dynamics of Cellulolytic Bacteria Depend on the Richness of Cellulosic Materials in the Habitat[1]

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Abstract—Bacterial diversity and hydrolytic enzyme activity of paddy soil, paddy soil mixed with cow dung, and paddy soil mixed with cow dung and rice straw samples were studied. A total of twelve different bacterial species were found by 16S rDNA sequence analysis, viz., *Bacillus subtilis*, *Streptomyces colombiensis*, *Paenibacillus polymyxa*, *Staphylococcus pasteuri*, *B. aquimaris*, *B. licheniformis*, *B. amyloliquefaciens*, *Microbacterium testaceum*, *P. illinoisensis*, *Micrococcus* sp., *B. subtilis*, *Rhizobium tropici*, *Micrococcus luteus*, *Bacillacea bacterium*, *Microbacteriaceae bacterium*, *B. megaterium*. The bacterial strains of the samples were divided into three phyla, namely *Firmicutes*, *Actinobacteria*, and *Proteobacteria*. The population of bacteria under the phylum *Firmicutes* was highest in all the samples. In addition, the population of bacteria under *Firmicutes* was gradually increased from the paddy soil sample (60%) to the paddy soil mixed with cow dung and rice straw samples (84%). On the other hand, the population of the bacteria under the phyla *Actinobacteria* and *Proteobacteria* gradually decreased from 28 and 12% of paddy soil to 11 and 5% of paddy soil mixed with cow dung and rice straw, respectively. The population of bacteria showing cellulase, xylanase, lichinase, and mannase activity were lowest in normal paddy soil (57, 53, 50 and 18%, respectively), except amylase were lowest in soil samples mixed with cow dung (39%); however, they gradually increased and were highest in the sample of paddy soil mixed with cow dung and rice straw (100, 100, 65, 54, and 61%, respectively). So it was observed that soil samples mixed with cow dung and rice straw may be a good source of plant cell wall degrading bacteria.

Keywords: bacterial diversity, lignocellulosic materials, rice straw, cow dung

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The role of cellulolytic enzymes in the degradation of structural carbohydrates of the plant cell wall into ready-to-fermentable sugar stream is inevitable. Cellulase synergistically acts upon plant cell wall polysaccharides to release glucose into the liquid media. Cellulase predominantly dominates all the plant cell wall degrading enzymes due to their vast and diverse range of applications [1]. Beside their uses in biofuel production, cellulases are used in the textile industry for cotton softening and in the detergent industry for anti-redeposition in washing powders [2]. Cellulases are also used in the pulp and paper industry with hemicellulases to improve the drainage and run ability of the paper machines and to enhance the deinking of recycled fibers [3]. All these processes require cellulases of different types of varying heat and pH stability and this has generated interest in exploring different environments to isolate hypercellulolytic microorganisms with diverse cellulase systems.

Different types of paddy soil samples are the best niches for the isolation of cellulase producers, and those cellulase producers can also be exploited for cellulases with specific properties. On the other hand, the activity of microbial biomass is commonly used as a measure of the microbiological status of a soil [4]. Furthermore, microbial populations in soil interact with each other and with soil. These interactions, in turn, affect major environmental processes, including biogeochemical cycling of nutrients, plant health, and soil quality [5]. As a consequence, some microbiological properties, such as bacterial extracellular enzyme activities, have been hypothesized to be potential indicators of soil quality due to their essential role in soil biology [6]. Besides, some researchers had been isolated bacterial microbes capable of degrade cellulolytic materials, such as a microorganism hydrolyzing rice hull had been isolated from soil and identified as *Bacillus amyloliquefaciens* by analysis of 16S rDNA and partial sequences of the gyrA gene, and named as *B. amyloliquefaciens* DL-3 [7]. Moreover, microorganisms from different extreme environments have been explored for the production of cellulases by some researchers [8, 9]; however, there is a paucity of information regarding the cellulase and other cell wall hydrolyzing enzyme producing abilities of these

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microbial resources from different types of soil samples mixed with cattle manure and cellulosic materials. Bacteria, having high growth rates as compared to fungi, have better potential for commercial cellulase production. Although the application of bacteria in producing cellulase is less investigated, cellulases produced by bacteria are often more effective catalysts, as they have less feedback inhibition. Moreover, bacteria can be genetically engineered to produce copious amounts of enzymes. Technologies are available which may allow the conversion of lignocellulose into fuel ethanol using genetically engineered bacteria. Imgram et al. [10] worked on the genetic engineering of enteric bacteria using a portable ethanol production pathway. They were integrated the decarboxylase and alcohol dehydrogenase genes into the chromosome of *Escherichia coli* B to produce strain KO11 for the fermentation of hemicellulose-derived syrups. This organism can efficiently ferment all hexose and pentose sugars present in the polymers of hemicellulose.

In view of the above facts, the experiment was conducted to observe the population dynamics of the bacterial community in paddy soil as well as in paddy soil mixed with cow dung and paddy soil mixed with cow dung and rice straw based on analysis of 16S rDNA. Moreover, the cell wall hydrolytic enzyme activity of the isolated bacterial strains was determined with the distribution pattern of the experimental samples. These kinds of bacteria can be the suitable gene source of potential enzyme synthesis for bioethanol production and other uses.

MATERIALS AND METHODS

Sample collection. There were three kinds of sample, namely paddy soil, paddy soil mixed with cow dung, and paddy soil mixed with cow dung and rice straw. Samples were collected with three random replications from the Research field of Gyeongsang National University, Jinju, Korea.

Isolation of bacteria. The population dynamics of cultivable bacteria of the soil samples were analyzed using a serial dilution and spread plating method. Each sample (1 g) was suspended in 9 mL distilled water. The suspension was serially diluted with distilled water (0.1%, w/v), where 0.1 g of sample was diluted in 100 mL of distilled water (1 : 1000). The diluted suspensions were spread on tryptic soya agar (TSA, Difco, NJ, USA) plates with a sterile glass spreader with three replications and the plates were kept in incubators at 28 and 37°C for 48 h. The bacterial colonies were initially screened and grouped by colony color and morphological characteristics.

Bacterial strains and growth conditions. *Escherichia coli* DH5α (TAKARA BIO INC., Shiga, Japan) was cultured in Luria-Bertani broth (LB broth, Difco, NJ, USA) at 37°C. Ampicillin (50 μg/mL) was added to the LB broth for the culture of recombinant *E. coli* DH5α.

Isolation of genomic DNA. The isolated bacteria were cultured in tryptic soy broth (TSB, Difco, NJ, USA) and centrifuged at 13.000 g for 5 min at 4°C. The pellet was subjected to DNA extraction using the G-spin™ Genomic DNA Extraction Kit (iNtRON Biotechnology, Suwon, Korea).

Cloning of 16S rDNA of isolated bacteria. Bacterial 16S rDNA was amplified by PCR using forward primer 5'-CGGAGAGTTTGATCCTGG-3' and reverse primer 5'-GGCTACCTTGTTACGACTT-3' with Super-Therm DNA polymerase (JMR, Side Cup, Kent, UK), 1.5 mM $MgCl_2$, 2 mM dNTP in a final volume of 50 μL for thirty cycles (denaturation at 95°C for 30 s, annealing at 50°C for 30 s and extension at 72°C for 90 s followed by final incubation at 72°C for 10 min) as described by different researchers [11–13]. The anticipated product of approximately 1,500 bp was extracted after agarose gel electrophoresis of PCR product by a gel extraction kit (NucleoGen, Seoul, Korea). 16S rDNAs were directly cloned into the pGEM-T Easy vector (Promega, WI, USA) and recombinant colonies were selected randomly. Plasmid DNAs of recombinant colonies were extracted using the NucleoGen Plasmid Mini Kit (NucleoGen, Seoul, Korea). Standard procedures for the restriction of endonuclease digestion, agarose gel electrophoresis, purification of DNA from agarose gels, DNA ligation and other cloning related techniques were used as described by Sambrook and Russel [14]. Restriction enzymes were obtained from Gibco-BRL (Gaithersburg, MD, USA), Promega (Madson, WT, USA) and Bohringer Mannheim (Indianapolis, IN, USA). Other chemicals were obtained from Sigma Chemical Co. (Louis, MO, USA).

16S rDNA sequencing and analysis. Nucleotide sequencing of 16S rDNA was conducted using the dideoxy-chain termination method with the PRISM Ready Reaction Dye terminator/primer cycle sequencing kit (Perkin-Elmer Corp., Norwalk, CN, USA). The samples were analyzed using an automated DNA sequencer (Applied Biosystems, Foster City, CA, USA). Assembly of the nucleotide sequences was performed using the DNAMAN analysis system (Lynnon Biosoft, Quebec, Canada). All reference sequences were obtained from the National Center for Biotechnology Information (NCBI) and Ribosomal Database Project (RDP) databases. Searches for similarity of 16S rDNA sequences were performed using the BLASTN and PSI-BLAST tools on the NCBI website. Sequences were aligned using the multiple sequence alignment program CLUSTAL W [15]. Phylogenetic analysis was performed using neighbor-joining methods [16]. Bootstrap analysis was performed using data resampled 1,000 times using the DNAMAN analysis system. Nucleotide sequences (16S rDNA) of the bacterial strain have been deposited in the GenBank database of NCBI under the accession numbers from KM226900 to KM226915 for the bacteria of paddy soil, KM226916 to KM226933

Table 1. The 16S rDNA sequences of clones of bacteria retrieved from paddy soil

Bacterial isolates	Clone, accession no.	No. of isolates	Phylum	Nearest relatives with accession number*	Similarity, %
PPL-S1	KM226900	8	*Firmicutes*	*Bacillus subtilis* KJB06-35 (HQ687501)	99
PPL-S2	KM226901	7	*Actinobacteria*	*Streptomyces colombiensis* 3026 (EF371424)	99
PPL-S3	KM226902	4	*Firmicutes*	*Paenibacillus polymyxa* DSM 36T (HG324066)	99
PPL-S4	KM226903	6	*Firmicutes*	*Staphylococcus pasteuri* SSL11(EU373323)	98
PPL-S5	KM226904	4	*Firmicutes*	*Bacillus aquimaris* GSP18 (AY505499)	99
PPL-S6	KM226905	5	*Firmicutes*	*Bacillus licheniformis* BPRIST006 (JF414759)	99
PPL-S7	KM226906	4	*Firmicutes*	*Bacillus amyloliquefaciens* FZB42 (CP000560)	99
PPL-S8	KM226907	6	*Actinobacteria*	*Microbacterium testaceum* StLB03 7 (APO12052)	99
PPL-S9	KM226908	8	*Firmicutes*	*Paenibacillus illinoisensis* JCM 9907 (NR_040884)	99
PPL-S10	KM226909	7	*Actinobacteria*	*Micrococcus* sp. JR5-3 (JQ229694)	99
PPL-S11	KM226910	7	*Firmicutes*	*Bacillus subtilis* SB 3130 (GU191916)	99
PPL-S12	KM226911	12	*Proteobacteria*	*Rhizobium tropici* CAF438 (EU399935)	99
PPL-S13	KM226912	3	*Actinobacteria*	*Micrococcus luteus* CV39 (AJ717368)	98
PPL-S14	KM226913	6	*Firmicutes*	*Bacillus licheniformis* 9945A (CP005965)	99
PPL-S15	KM226914	8	*Firmicutes*	*Bacillus subtilis* 6051-HGW (CP003329)	99
PPL-S16	KM226915	5	*Actinobacteria*	*Microbacteriaceae bacterium* YR2-6 (JQ229710)	98

*—Accession number of the nearest relative. When more than one sequence had the same similarity value, only the accession number of the first sequence is given.

for the bacteria of paddy soil mixed with cow dung and KM226934 to KM226952 for the bacteria of paddy soil mixed with cow dung and rice straw (Table 1).

Extracellular hydrolytic enzyme activity assay of isolated bacteria. The Agar diffusion method was used for the detection of extracellular hydrolytic enzyme activity of the bacterial strains of soil samples as described by Jung and Kim [17]. The bacterial isolates were grown on LB plates with 0.5% (w/v) of carboxyl methylcellulose, oat spelt xylan, lichen, locust bean gum, and starch for the detection of cellulase, xylanase, lichenase, mannase, and amylase activity, respectively. To visualize the yellow halo zone surrounded by a red background due to cellulase, xylanase, mannase and lichenase activity, the plates were flooded with 0.5% congo red solution for 30 min, then rinsed with water and washed twice with 1 M NaCl, and to visualize amylase activity. The α-amylase producing colonies were selected by flooding the plates with iodine solution (1% iodine in 2% potassium iodide w/v) and then washed with water [18]. The size of halos formed around bacterial colonies on agar media were grouped into four classes: 0 mm (no halo zone), $0 < 2$ mm, $2 < 4$ mm, $4 < 6$ mm diameter of the halo zone.

RESULTS AND DISCUSSION

Isolation and diversity of bacteria. In the paddy soil sample, sixteen different bacterial species were observed to be related to the sequences of databases (98 to 99% similar) and their diversity was assessed (Table 1). Twelve different bacterial species were found by 16S rDNA sequence analysis, viz., *Bacillus subtilis, Streptomyces colombiensis, Paenibacillus polymyxa, Staphylococcus pasteuri, Bacillus aquimaris, Bacillus licheniformis, Bacillus amyloliquefaciens, Microbacterium testaceum, Paenibacillus illinoisensis, Micrococcus* sp., *Bacillus subtilis, Rhizobium tropici, Micrococcus luteus*. Among the isolates, *Rhizobium tropici* PPL-S12 was found in the highest number (twelve) followed by *Bacillus subtilis* PPL-S1, *Paenibacillus illinoisensis* PPL-S9, and *Bacillus subtilis* PPL-S15 (eight of each). Similarly, in the paddy soil mixed with cow dung, eighteen different bacterial species were observed related to the sequences of databases (98 to 100% sim-

Table 2. The 16S rDNA sequences of clones of bacteria retrieved from paddy soil mixed with cow dung

Bacterial isolates	Clone, accession no.	No. of isolates	Phylum	Nearest relatives with accession number*	Similarity, %
PPL-SC1	KM226916	4	*Firmicntes*	*Bacillus subtilis* WJ05 (HM045829)	99
PPL-SC2	KM226917	5	*Firmicutes*	*Bacillus pumilus* SI (FJ768456)	99
PPL-SC3	KM226918	8	*Firmicutes*	*Paenibacillus polymyxa* DSM 36T (HG3 24066)	99
PPL-SC4	KM226919	10	*Firmicutes*	*Bacillus licheniformis* BPRIST006 (JF414759)	99
PPL-SC5	KM226920	6	*Actinobacteria*	*Streptomyces colombiensis* 3026 (EF371424)	99
PPL-SC6	KM226921	3	*Firmicutes*	*Bacillus pumilus* SB 3182 (GU191909)	99
PPL-SC7	KM226922	2	*Firmicutes*	*Bacillus subtilis* C12-1 (GU257448)	99
PPL-SC8	KM226923	3	*Actinobacteria*	*Microbacterium testaceum* StLB037 (AP012052)	99
PPL-SC9	KM226924	8	*Firmicutes*	*Bacillus subtilis* 168 (AL009126)	100
PPL-SC10	KM226925	7	*Proteobacteria*	*Rhizobium tropici* CAF438 (EU399935)	99
PPL-SC11	KM226926	6	*Firmicutes*	*Bacillus megaterium* DSM319 (CP001982)	100
PPL-SC12	KM226927	4	*Firmicutes*	*Bacillus licheniformis* ATCC 14580 (CP000002)	99
PPL-SC13	KM226928	7	*Firmicutes*	*Bacillus subtilis* KJB06-35 (HQ687750)	99
PPL-SC14	KM226929	9	*Firmicutes*	*Bacillus pumilus* S1 (FJ768456)	100
PPL-SC15	KM226930	5	*Firmicutes*	*Bacillacea bacterium* GYPB (JF34888)	99
PPL-SC16	KM226931	6	*Firmicutes*	*Bacillus subtilis* 6051-HGW (CP003329)	99
PPL-SC17	KM226932	4	*Firmicutes*	*Bacillus licheniformis* 9945A (CP005965)	99
PPL-SC18	KM226933	3	*Actinobacteria*	*Microbacteriaceae bacterium* YR2-6 (JQ229710)	98

*—Accession number of the nearest relative. When more than one sequence had the same similarity value, only the accession number of the first sequence is given.

ilar) and their diversity was assessed (Table 2). Ten different bacterial species were found by 16S rDNA sequence analysis viz., *Bacillus subtilis, Bacillus pumilus, Paenibacillus polymyxa, Bacillus licheniformis, Streptomyces colombiensis, Microbacterium testaceum, Rhizobium tropici, Bacillus megaterium, Bacillacea bacterium* and *Microbacteriaceae bacterium*. Among the isolates, *Bacillus licheniformis* PPL-SC4 was found in the highest number (ten), followed by *Bacillus pumilus* PPL-SC14 (nine). In the sample of paddy soil mixed with cow dung and rice straw, sixteen different bacterial species were observed related to the sequences of databases (98 to 99% similar) and their diversity was assessed (Table 3). Only nine different bacterial species were found by 16S rDNA sequence analysis viz., *Bacillus subtilis, Bacillus pumilus, Paenibacillus polymyxa, Bacillus licheniformis, Streptomyces colombiensis, Paenibacillus illinoisensis, Bacillus megaterium, Rhizobium tropici*. Among the isolates, *Bacillus pumilus* PPL-SSC8 was found in the highest number (fourteen), followed by *Paenibacillus polymyxa* PPL-SSC3 (eleven). More than sixty percent of the isolates described here belong to *Bacilli* and related genera, and this group is known to possess the ability to withstand extreme environments by producing endospores [19]. In this study, the number of *Bacilli* was increased gradually from the normal paddy soil sample (54%) to the paddy soil mixed with cow dung and rice straw (80%). It has been observed by some researchers that substrates available to bacteria seemed mainly to determine the composition of the bacterial members at the respective stages [20]. In addition, the degradation of macromolecular carbon compounds is related to the composition of the bacterial community because the enzymatic capacity for the initial steps of

Table 3. The 16S rDNA sequences of clones of bacteria retrieved from paddy soil mixed with cow dung and rice straw

Bacterial isolates	Clone, accession no.	No. of isolates	Phylum	Nearest relatives with accession number*	Similarity, %
PPL-SSC1	KM226934	2	*Firmicutes*	*Bacillus subtilis* WJ08 (HM045832)	100
PPL-SSC2	KM226935	3	*Firmicutes*	*Bacillus pumilus* MTCC (CP007436)	99
PPL-SSC3	KM226936	11	*Firmicutes*	*Paenibacillus polymyxa* DSM 36T (HG324066)	99
PPL-SSC4	KM226937	3	*Firmicutes*	*Bacillus licheniformis* DSM 13 (AE017333)	99
PPL-SSC5	KM226938	7	*Firmicutes*	*Bacillus subtilis* 41KBZ (FJ615523)	99
PPL-SSC6	KM226939	5	*Firmicutes*	*Bacillus licheniformis* YRL03 (EU373408)	99
PPL-SSC7	KM226940	5	*Actinobacteria*	*Streptomyces colombiensis* 3026 (EF371424)	99
PPL-SSC8	KM226941	14	*Firmicutes*	*Bacillus pumilus* BPT-18 (EF523475)	99
PPL-SSC9	KM226942	2	*Firmicutes*	*Bacillus subtilis* SB 3130 (GU191916)	99
PPL-SSC10	KM226943	7	*Firmicutes*	*Paenibacillus illinoisensis* JCM 9907 (NR_040884)	99
PPL-SSC11	KM226944	4	*Firmicutes*	*Bacillus subtilis* PV79 (CP006881)	99
PPL-SSC12	KM226945	2	*Firmicutes*	*Bacillus subtilis* XF-1 (CP004019)	99
PPL-SSC13	KM226946	7	*Firmicutes*	*Bacillus licheniformis* BPRIST006 (JF414759)	99
PPL-SSC14	KM226947	5	*Firmicutes*	*Bacillus megaterium* DSM319 (CP001982)	100
PPL-SSC15	KM226948	4	*Firmicutes*	*Bacillus subtilis* AU25 (EF032688)	99
PPL-SSC16	KM226949	3	*Firmicutes*	*Bacillus subtilis* 6051-HGW (CP003329)	99
PPL-SSC17	KM226950	5	*Firmicutes*	*Bacillus licheniformis* 9945A (CP005965)	99
PPL-SSC18	KM226951	5	*Proteobacteria*	*Rhizobium tropici* CAF438 (EU399935)	99
PPL-SSC19	KM226952	6	*Actinobacteria*	*Streptomyces colombiensis* CSB10 (FJ189760)	99

*—Accession number of the nearest relative. When more than one sequence had the same similarity value, only the accession number of the first sequence is given.

degradation occurs in a comparatively limited number of microbial populations [21, 22].

Phylogenetic placement of the bacterial isolates. In the paddy soil sample, sixteen different bacterial species were observed related to the sequences of databases (98 to 99% similar) and their diversity was assessed (Table 1). Phylogenetic placement of the isolated bacterial strains from paddy soil, paddy soil mixed with cowdung, and paddy soil mixed with cow dung and rice straw were conducted on the basis of 16S rDNA sequences (Figs. 1–3). The bacterial strains were divided into three phyla, namely *Firmicutes, Actinobacteria*, and *Proteobacteria*. The population of bacteria under the phylum *Firmicutes* was highest in all the samples. In addition, the population of bacteria under the phylum *Firmicutes* gradually increased from the paddy soil sample (60%) to the paddy soil mixed with cow dung and rice straw (84%). On the other hand, the population of bacteria under the phylum *Actinobacteria* gradually decreased from 28% in paddy soil to 11% in paddy soil mixed with cow dung and rice straw. Similarly, the population of bacterial under the phylum *Proteobacteria* also gradually decreased from 12% in paddy soil to 5% in paddy soil mixed with cow dung and rice straw (Fig. 4). Siddique et al. [23] mentioned that *Proteobacteria, Actinobacteria* and *Firmicutes* are the major phyla of Bacteria.

Evaluation of hydrolytic enzyme activities. Hydrolytic enzyme activity of the bacterial strains of paddy soil was evaluated for cellulase, xylanase, lichinase, mannase, and amylase activity (Table 4). Among the bacterial strains, *Paenibacillus polymyxa* PPL-S3, *Bacillus licheniformis* PPL-S14, and *Bacillus subtilis* PPL-S15 showed activity of all five tested enzymes,

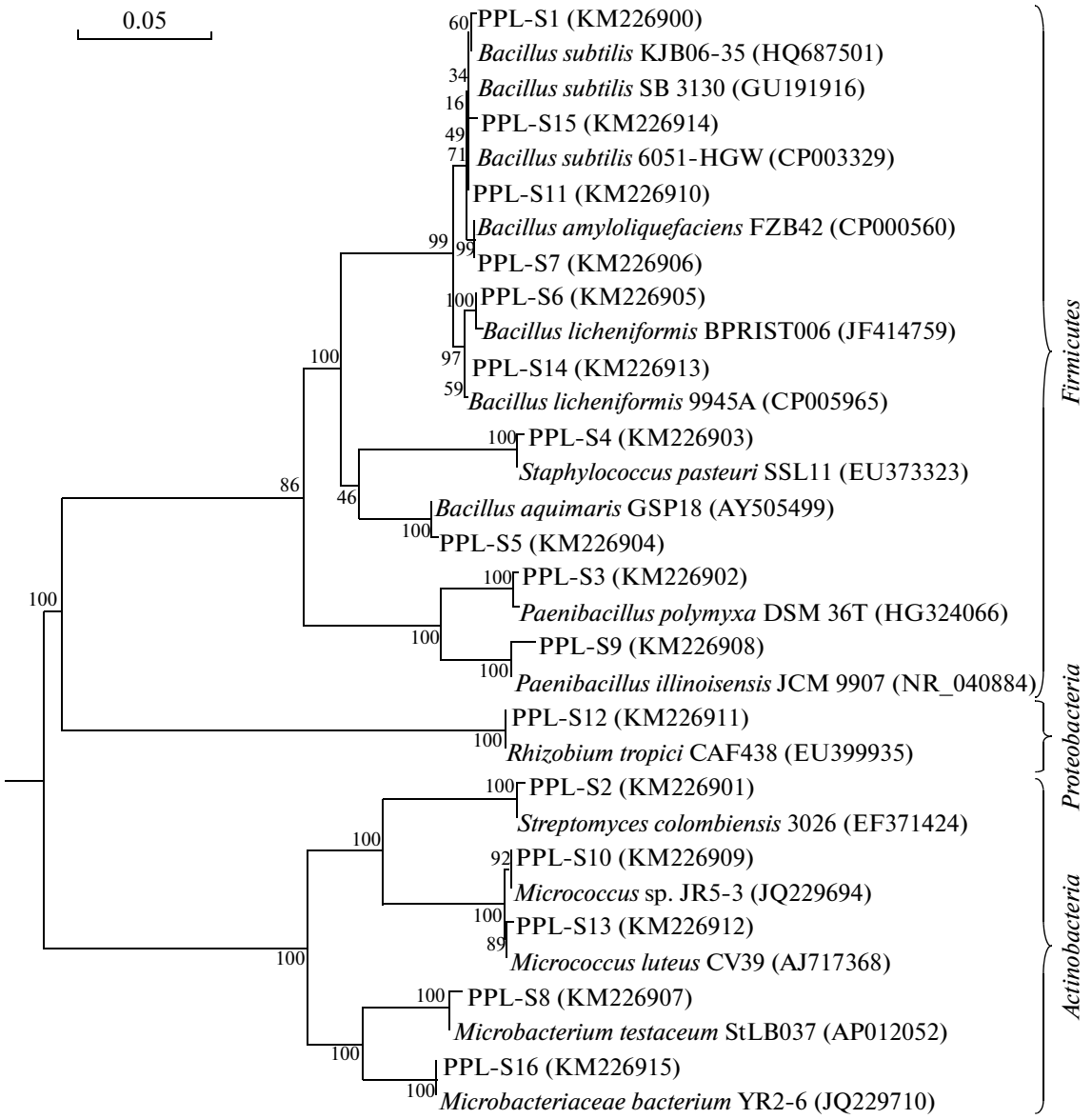

Fig. 1. Phylogenetic placement of 16S rDNA sequences from the bacteria collected from normal paddy soil. Numbers above each node indicate percentage of confidence levels generated from 1,000 bootstrap trees. The scale bar is in fixed nucleotide substitutions per sequence position.

but *Staphylococcus pasteuri* PPL-S4, *Microbacterium testaceum* PPL-S8, *Micrococcus* sp. PPL-S10, and *Microbacteriaceae bacterium* PPL-S16 did not show any activity of the five tested enzymes. Similarly, hydrolytic enzyme activity of the bacterial strains from the paddy soil mixed with cow dung was evaluated for cellulase, xylanase, lichinase, mannase, and amylase activity (Table 5). Among the bacterial strains, *Paenibacillus polymyxa* PPL-SC3, *Bacillacea bacterium* PPL-SC15, *Bacillus licheniformis* PPL-S14, *Bacillus subtilis* PPL-SC16, and *Bacillus licheniformis* PPL-SC1 showed activity for all five tested enzymes, but *Microbacterium testaceum* PPL-SC8 and *Microbacteriaceae bacterium* PPL-SC18 did not show any activity for these five enzymes. Moreover, the hydrolytic enzyme activity of the bacterial strains of paddy soil mixed with cow dung and rice straw was also evaluated for cellulase, xylanase, lichinase, mannase, and amylase activity (Table 6). All the bacterial strains showed cellulase and xylanase enzyme activity. Moreover, among the bacterial strains, *Paenibacillus polymyxa* PPL-SSC3, *Bacillus subtilis* PPL-SSC5, *Bacillus subtilis* PPL-SSC15, *Bacillus subtilis* PPL-SSC16, *Bacillus licheniformis* PPL-SSC17, and *Rhizobium tropici* PPL-SSC18 showed activity of all five tested enzymes.

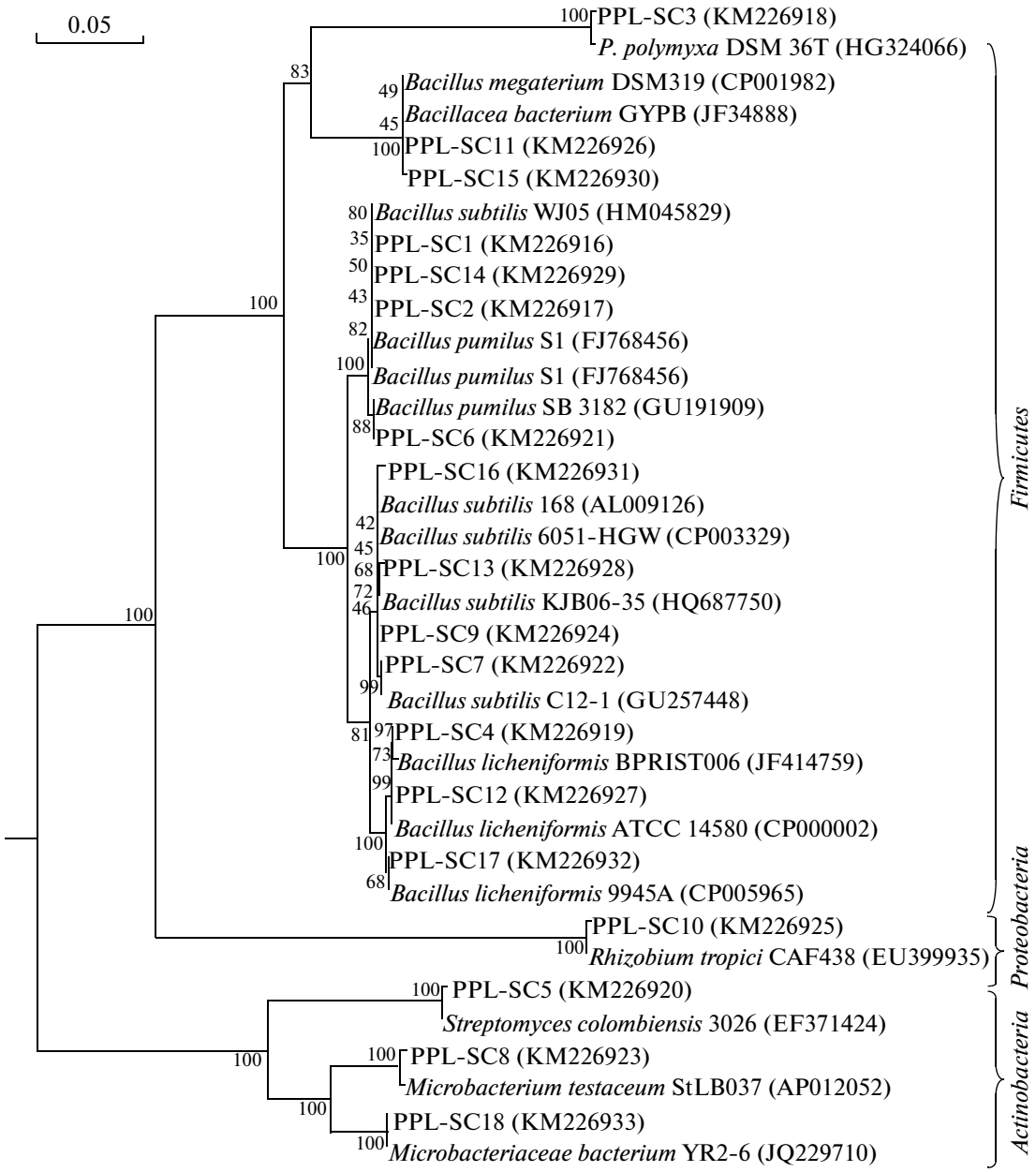

Fig. 2. Phylogenetic placement of 16S rDNA sequences from the bacteria collected from normal paddy soil with cow dung. Numbers above each node indicate percentage of confidence levels generated from 1,000 bootstrap trees. The scale bar is in fixed nucleotide substitutions per sequence position.

Bacillus and *Paenibacillus* have been reported by many researchers as cellulase producers [9, 24].

Distribution of bacterial isolates on the basis of hydrolytic enzyme activity. Distribution of bacteria isolated from the paddy soil, paddy soil mixed with cow dung, and paddy soil mixed with cow dung and rice straw on the basis of extracellular hydrolytic enzyme activity was evaluated (Fig. 5). The population of bacteria showing cellulase, xylanase, lichinase, and mannase activity was lowest in normal paddy soil (57, 53, 50 and 18%, respectively), but gradually increased and was highest in paddy soil mixed with cow dung and rice straw (100, 100, 65, 54, and 61%). On the other hand, the population of bacteria showing amylase activity was gradually decreased from the paddy soil sample (47%) to paddy soil mixed with cow dung (39%), and then increased to 61% in paddy soil mixed with cow dung and rice straw. The degradation of macromolecular carbon compounds is related to bacterial community composition because the enzy-

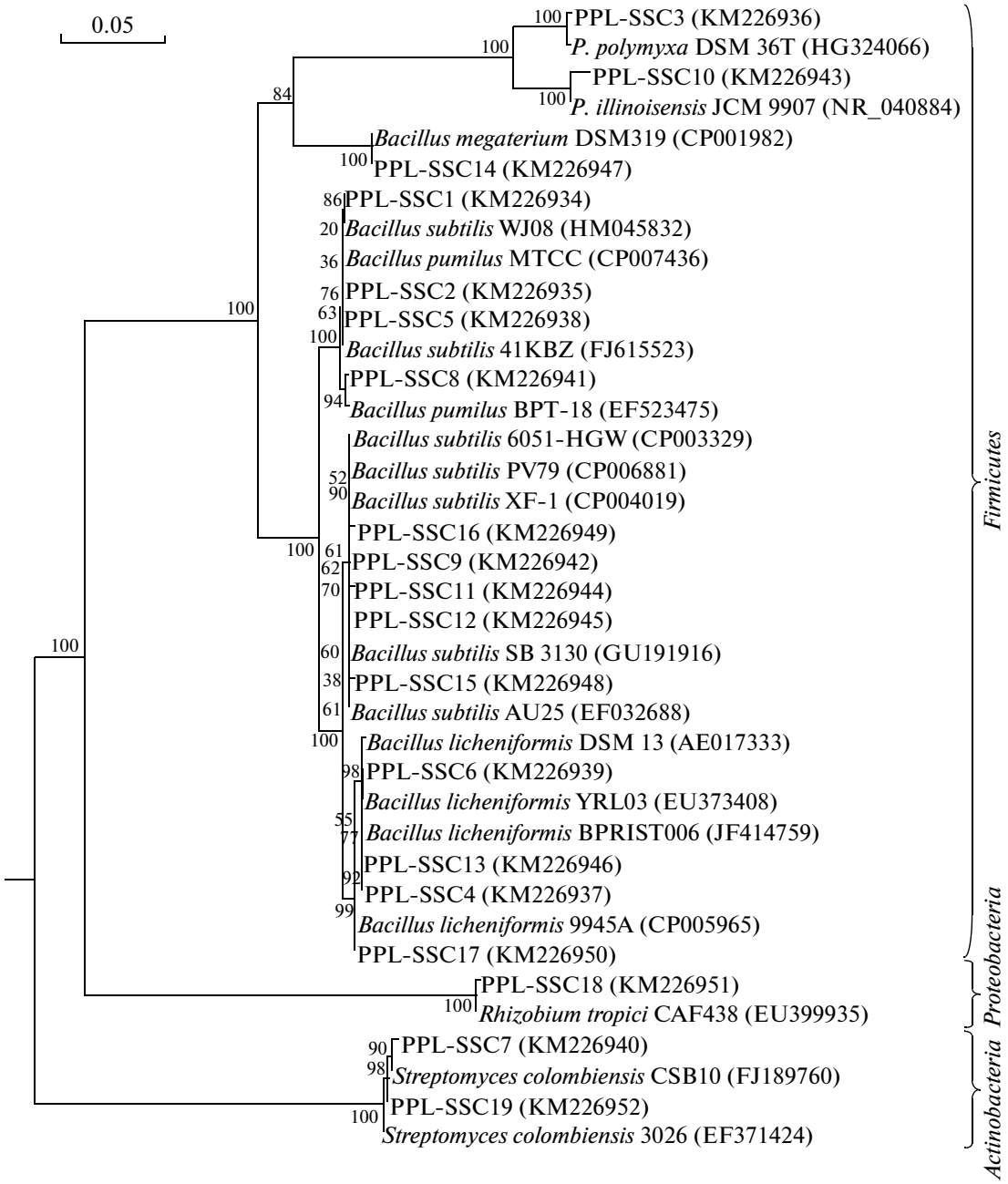

Fig. 3. Phylogenetic placement of 16S rDNA sequences from the bacteria collected from normal paddy soil with cow dung and rice straw. Numbers above each node indicate percentage of confidence levels generated from 1,000 bootstrap trees. The scale bar is in fixed nucleotide substitutions per sequence position.

matic capacity for the initial steps of degradation occurs in a comparatively limited number of microbial populations [21, 22].

In summary, in the current study in total twelve different bacterial species were found by 16S rDNA sequence analysis. The bacterial strains of the samples were divided into three phyla, namely *Firmicutes, Actinobacteria*, and *Proteobacteria*. The population of bacteria under the phylum *Firmicutes* was highest in all the samples. In addition, the population of bacteria under *Firmicutes* gradually increased, but *Actinobacteria* and *Proteobacteria* gradually decreased, from the paddy soil sample to the paddy soil mixed with cow dung and rice straw. The population of bacteria showing cellulase, xylanase, lichinase, and mannase activity also gradually increased from normal paddy soil to paddy soil mixed with cow dung and rice straw. Thus it was observed that soil samples mixed with cow dung

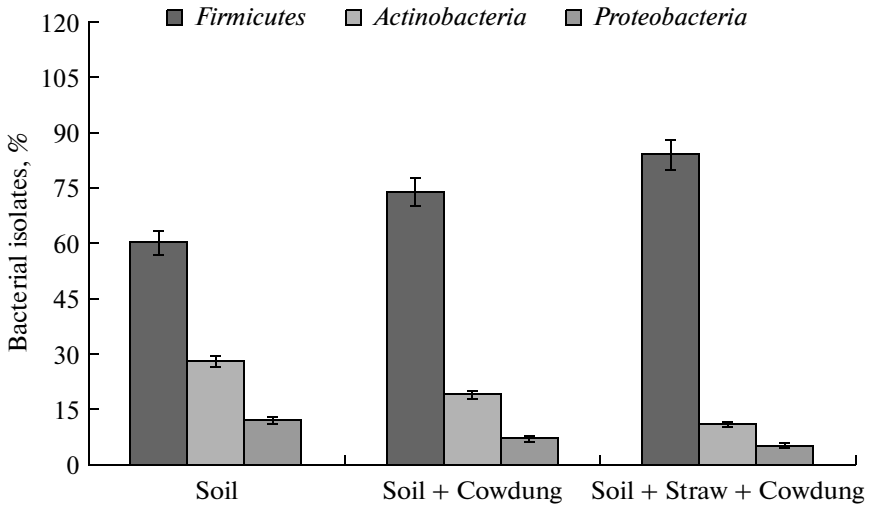

Fig. 4. Distribution of bacteria isolated from the paddy soil, paddy soil mixed with cow dung, and paddy soil mixed with cow dung and rice straw into phyla on the basis of 16S rDNA sequence. Symbols in square brackets mention the total percentage of each bacterial group in those samples. Values indicate the mean's of three replications. A P-value <0.05 was considered significant, vertical bar showing standard deviation.

Table 4. Evaluation of extracellular hydrolytic enzyme activity from the paddy soil

Bacterial isolates	Clone, accession no.	No. of isolates	Cellulase	Xylanase	Lichenase	Mannase	Amylase
Bacillus subtilis PPL-S1	KM226900	8	+++*	+++	+	–	++
Streptomyces colombientsis PPL-S2	KM226901	7	+	++	–	–	–
Paenibacillus polymyxa PPL-S3	KM226902	4	+++	+++	+++	++	+++
Staphylococcus pasteuri PPL-S4	KM226903	6	–	–	–	–	–
Bacillus aquimaris PPL-S5	KM226904	4	–	+	–	–	–
Bacillus licheniformis PPL-S6	KM226905	5	+++	+++	+++	–	–
Bacillus amyloliquefaciens PPL-S7	KM226906	4	++	+	–	–	–
Microbacterium testaceum PPL-S8	KM226907	6	–	–	–	–	–
Paenibacillus illinoisensis PPL-S9	KM226908	8	++	–	++	–	++
Micrococcus sp. PPL-S10	KM226909	7	–	–	–	–	–
Bacillus subtilis PPL-S11	KM226910	7	+++	+++	+	–	–
Rhizobium tropici PPL-S12	KM226911	12	–	–	+	–	–
Micrococcus luteus PPL-S13	KM226912	3	–	–	–	–	++
Bacillus licheniformis PPL-S14	KM226913	6	+++	+++	+++	++	+++
Bacillus subtilis PPL-S15	KM226914	8	+++	+++	+	+	++
Microbacteriaceae bacterium PPL-S16	KM226915	5	–	–	–	–	–

*—Size of halos formed around bacterial colonies on agar media.
Symbols: –, denotes no halo zone, indicating no enzyme activity; + denotes 2 mm diameter of the halo zone, indicating enzyme activity; ++ denotes 4 mm halo zone, indicating enzyme activity; +++ denotes 6 mm diameter halo zone, indicating enzyme activity.

Table 5. Evaluation of extracellular hydrolytic enzyme activity from the paddy soil with cow dung

Bacterial isolates	Clone, accession no.	No. of isolates	Cellulase	Xylanase	Lichenase	Mannase	Amylase
Bacillus subtilis PPL-SC1	KM226916	4	+++*	++	+	–	+++
Bacillus pumilus PPL-SC2	KM226917	5	+++	+++	+	–	–
Paenibacillus polymyxa PPL-SC3	KM226918	8	+++	+++	+++	++	+++
Bacillus licheniformis PPL-SC4	KM226919	10	+++	+++	+++	+++	–
Streptomyces colombiensis PPL-SC5	KM226920	6	+	–	–	+	+
Bacillus pumilus PPL-SC6	KM226921	3	++	++	–	–	–
Bacillus subtilis PPL-SC7	KM226922	2	+++	++	+	+	–
Microbacterium testaceum PPL-SC8	KM226923	3	–	–	–	–	–
Bacillus subtilis PPL-SC9	KM226924	8	+++	++	+	–	–
Rhizobium tropici PPL-SC10	KM226925	7	–	–	+	–	–
Bacillus megaterium PPL-SC11	KM226926	6	+++	++	–	+++	+
Bacillus licheniformis PPL-SC12	KM226927	4	+++	+++	+++	++	–
Bacillus subtilis PPL-SC13	KM226928	7	+++	+++	+	–	–
Bacillus pumilus PPL-SC14	KM226929	9	++	+	–	–	–
Bacillacea bacterium PPL-SC15	KM226930	5	++	++	++	+	++
Bacillus subtilis PPL-SC16	KM226931	6	+++	++	+	+	+
Bacillus licheniformis PPL-SC17	KM226932	4	+++	+++	+++	+++	++
Microbacleriaceae bacterium PPL-SC18	KM226933	3	–	–	–	–	–

*—Diameter of halos formed around bacterial colonies on agar media.
Symbols: –, denotes no halo zone, indicating no enzyme activity; + denotes 2 mm the halo zone, indicating enzyme activity; ++ denotes 4 mm halo zone, indicating enzyme activity; +++ denotes 6 mm diameter of the halo zone, indicating enzyme activity.

Table 6. Evaluation of extracellular hydrolytic enzyme activity from the paddy soil mixed with cow dung and rice straw

Bacterial isolates	Clone, accession no.	No. of isolates	Cellulase	Xylanase	Lichenase	Mannase	Amylase
Bacillus subtilis PPL-SSC1	KM226934	2	+++*	+++	+	+	–
Bacillus pumilus PPL-SSC2	KM226935	3	+++	+++	–	–	+
Paenibacillus polymyxa PPL-SSC3	KM226936	11	+++	+++	+++	+++	+++
Bacillus licheniformis PPL-SSC4	KM226937	3	++	+++	+++	+++	++
Bacillus subtilis PPL-SSC5	KM226938	7	+++	+++	+	++	+
Bacillus licheniformis PPL-SSC6	KM226939	5	+++	+++	+++	–	–
Streptomyces colombiensis PPL-SSC7	KM226940	5	+	++	–	–	+
Bacillus pumilus PPL-SSC8	KM226941	14	+++	++	–	–	–
Bacillus subtilis PPL-SSC9	KM226942	2	+++	+++	+	+	–
Paenibacillus illinoisensis PPL-SSC10	KM226943	7	++	++	++	–	++
Bacillus subtilis PPL-SSC11	KM226944	4	+++	+++	+	+	–
Bacillus subtilis PPL-SSC12	KM226945	2	+++	+++	+	–	+
Bacillus licheniformis PPL-SSC13	KM226946	7	+++	++	+++	+++	–
Bacillus megaterium PPL-SSC14	KM226947	5	+++	++	–	+++	–
Bacillus subtilis PPL-SSC15	KM226948	4	++	++	+	++	+
Bacillus subtilis PPL-SSC16	KM226949	3	+++	++	+	+	+
Bacillus licheniformis PPL-SSC17	KM226950	5	+++	+++	+++	+++	++
Rhizobium tropici PPL-SSC18	KM226951	5	++	+	+	+++	+++
Streptomyces colombiensis PPL-SSC19	KM226952	6	+	++	–	–	+

*—Size of halos formed around bacterial colonies on agar media.
Symbols: – denotes no halo zone, indicating no enzyme activity; + denotes 2 mm diameter of the halo zone, indicating enzyme activity; ++ denotes 4 mm halo zone, indicating enzyme activity; +++ denotes 6 mm diameter halo zone, indicating enzyme activity

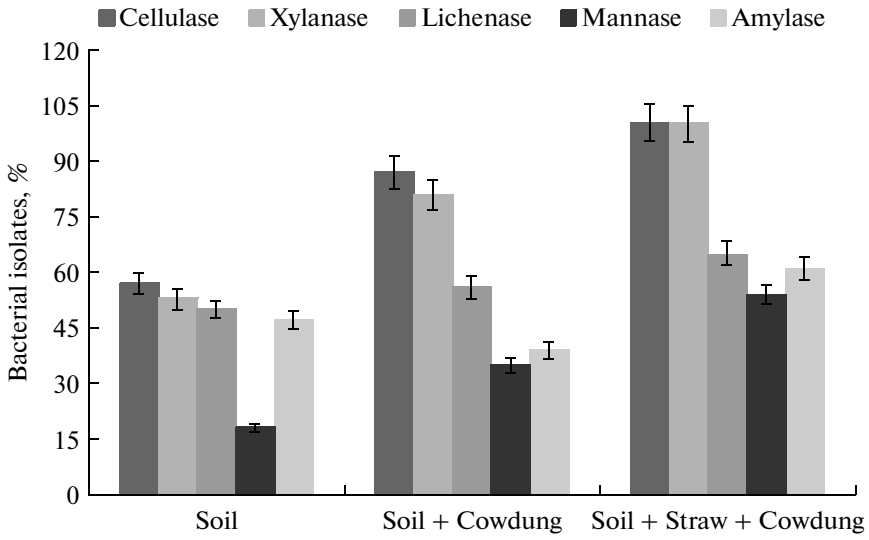

Fig. 5. Distribution of bacteria isolated from the paddy soil, paddy soil mixed with cow dung, and paddy soil mixed with cow dung and rice straw on the basis of extracellular hydrolytic enzyme activity. Symbols in square brackets mention the total percentage of each bacterial group in those samples. Values indicate the mean's of three replications. A P-value <0.05 was considered significant, vertical bar showing standard deviation.

and rice straw may be a good source of cellulolytic bacteria, which can be used in lignocellulose degradation and bioethanol production.

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