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Research Paper

Isolation and characterization of humic substances-degrading bacteria from the subarctic Alaska grasslands

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Humic substances (HS), an important fraction of soil organic carbon, are distributed widely throughout cold environments. A total of cold-adapted 122 bacterial strains were isolated from 66 Alaska grassland soil samples based on their ability to grow on humic acids (HA), a main fraction of HS, as a carbon and energy source. These isolates were identified based on 16S rRNA gene sequencing, with class *Bacilli* (79.5%) and *γ-Proteobacteria* (17.1%) comprising the largest groups. Among them, 45 strains, mainly *Paenibacillus* (27 strains) and *Pseudomonas* (15 strains), were selected for further screening. Two strains (*Pseudomonas* sp. PAMC 26793 and *Paenibacillus* sp. PAMC 26794) most efficiently degraded HA, but showed significant differences in their ability to grow on various monocyclic aromatics, which are putative degradative metabolites of HS. Fourier transform infrared spectra also showed substantial but different changes in HA chemical structure after incubation with each strain. Gel permeation chromatography demonstrated that depolymerization and polymerization of HA occurred during HS degradation by these newly isolated microbes.

Abbreviations: HA – humic acids; HS – humic substances; FA – fulvic acids

Keywords: Humic substances / Low temperature / Soil bacteria / Biodegradation

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Introduction

Humic substances (HS) are ubiquitous natural components found throughout the environment, including cold polar regions (the Arctic and Antarctic), and HS represent the main carbon reservoir in the biosphere [1]. HS are formed by secondary synthesis reactions (humification) during the decomposition of plant and other organic material, in which a variety of other biomolecules are condensed with the decomposing matter. HS are considered modified lignins, which are macromolecular structures consisting of high molecular weight compounds. HS are based on aromatic nuclei with phenolic and carboxylic substitutions and thus could be more recalcitrant to soil microorganisms. However, recent studies suggest that HS are aggregates of relatively low molecular weight building blocks, resulting in a significantly smaller organic complex [2–4]. Based on their solubility in acids and alkalis, HS can be divided into two main fractions, humic acids (HA) and fulvic acids (FA), and one minor fraction, humin [2].

Substantial research has focused on the structure, distribution, and reaction chemistry of HS as an attractive new source of fine chemicals, energy, and carbon/nitrogen for microorganisms [5]. However, knowledge of the role played by microorganisms in forming and decomposing HS is not sufficient. Current evidence suggests that soil bacteria play a critical role in the HS degradation process due to their prevalence, diversity, and catabolic versatility [3, 6]. Several different soil bacteria have been isolated and characterized based on their capacity to metabolize HA, a principal component of HS, and lignin, a suitable surrogate of biopolymer HS

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in lab experiments. It was reported that biodegradation of HA and lignin by soil *Streptomyces* strains and a *Novosphingobium* sp., respectively, is associated with extracellular peroxidase [7, 8] and/or laccase activity [9]. However, bacterial degradation of HS in cold environments remained unexplored and was the impetus for the present study.

Materials and methods

Sample collection

Soils containing decaying plant and moss debris were collected during August 2011 from the top layer (0–20 cm) of 33 grassland sites (64°50.596′–64°50.820′N, 163°42.663′–163°42.746′W; pH 4.3–4.5; soil organic carbon, 35.9–39.9%; moisture content of above 400%) in Nome, Alaska. For isolation of HA-degrading bacteria, each soil sample was divided into two depth regions (0–10 cm and 10–20 cm depth) and 1.0 g of each fraction was stored in 20% glycerol at −20 °C until used. For extraction of natural HA from the soil sample, the remainder of the sample was stored at −20 °C.

Isolation of cultivatable HA-degrading bacteria

Soil samples in 20% glycerol were homogenized and placed at 4 °C for 1 h. The supernatant (100 μl) was spread on minimal medium (0.5 g Na_2HPO_4, 1.71 g KCl, 0.05 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.02 g $CaCO_3$, 15.0 g agar per liter; pH 7.2 ± 0.2 at 25 °C) containing 1 g L^{-1} of commercial HA dissolved in 0.2 N NaOH (MB-H0754; MB Cell, CA, USA) and incubated at 15 °C for 7 days. The commercial HA was used as a substitution for natural HA to study microbial degradation of HS. The bacterial cells grown on HA plates were considered HA-degrader isolates and preserved as cell suspensions in 20% glycerol at −80 °C.

Extraction of natural HA

Soil samples containing HS were completely dried at 45 °C overnight and passed through a 230 mesh-testing sieve (63 μm sieve pore) to remove coarse plant debris and small stones. One gram of the soil was treated with 25 ml 0.5 N NaOH for 3 h under continuous shaking and incubated at 4 °C for 12 h. The NaOH extract was separated by centrifugation at 5500*g* for 15 min and acidified to pH 2.0 with 5.0 N HCl. The insoluble fraction containing HA was separated from the FA solution by centrifugation and re-dissolved in 0.1 N NaOH. Finally, the HA solution was prepared by centrifugation (4000*g* for 7 min).

Utilization tests for HA or lignin-derived aromatic compounds

Bacterial isolates having the ability to degrade HA were tested for their ability to metabolize an array of aromatic compounds, including benzoic acid, cinnamic acid, coumaric acid, vanillic acid, ferulic acid, coniferyl alcohol, and phenol, which are assumed to be derived from HA or lignin biodegradation. Bacteria from glycerol stocks of each isolate was transferred into 96-well plates and used to inoculate MSB agar plates [10] supplemented with each aromatic compound as the sole carbon source (5 mM final concentration), except for phenol which was provided in the vapor phase. During incubation at 15 °C, the isolates were scored for their growth rate as follows: score 4, 3, 2, and 1 indicate the formation of a dense colony of 5.0-mm-diameter within 3, 6, 9, and 12 days, respectively. Score 0 indicates no cells grown until 13 days.

Identification and diversity analyses of bacterial isolates

Colony PCR for part of the 16S rRNA gene was performed using the cell suspensions in 20% glycerol and eubacteria universal primers, 27F (5′-AGAGTTT-GATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACC-TTGTTACGACTT-3′). After sequencing using the PCR primers (27F and 1492R) and inter-primers (518F, 5′-CCAGCAGCCGCGGTAATACG-3′ and 800R, 5′-TAC-CAGGGTATCTAATCC-3′), the DNA sequence of each isolate was assembled with minimum of 2-fold cover-age and deposited under accession no. KF011585–KF011706 in GenBank. Finally, the 16S rRNA gene sequence was compared with those of type strains available in the EzTaxon database (www.eztaxon.org) to determine taxonomic affiliation.

Growth of PAMC 26793 and PAMC 26794 on HA

Pseudomonas sp. PAMC 26793 or *Paenibacillus* sp. PAMC 26794, which were selected for further characteriza-tion of HS metabolism owing to its remarkable ability to degrade HA, were inoculated into MSB broth containing 5 mM glucose and incubated for 3 days at 15 °C with shaking. After culturing, the remaining glucose in cell culture was removed by washing the cells with glucose-free MSB. The cell suspension in MSB was transferred to 50 ml MSB or MSB containing HA (1 g L^{-1}) to an optical density measured at a wavelength of 600 nm (OD_{600}) of 0.1. During culturing for 23 days at 15 °C, at the indicated time intervals, 100-μl samples of the culture were diluted to 10^{-4} in MSB and 100 μl diluent was cultivated on MSB plate containing 5 mM glucose. After incubation

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for 5 days at 15 °C, CFU ml^{-1} for each strain was calculated.

Transformation of HA by PAMC 26793 and PAMC 26794

PAMC 26793 or PAMC 26794, grown in MSB with 5 mM glucose under the above conditions, was transferred to 250 ml MSB containing HA (1 g L^{-1}) to an OD_{600} of 0.1. After culturing for 28 days at 15 °C, the cells were pelleted by centrifugation for 10 min at 12,000*g* (4 °C). To extract HA from the supernatant, the solution was acidified to pH 2.0 with 5 N HCl and incubated to precipitate HA overnight. The insoluble HA fraction was separated by centrifuging (4000*g* for 7 min) and re-dissolved in 0.1 N NaOH.

Gel permeation chromatography was used to detect the changes in molecular mass distribution of HA during the incubation with PAMC 26793 or PAMC 26794. The HA solution (8 mg HA ml^{-1}) was filtered through 0.2-μm-membrane filter and 20 μl filtrates was loaded onto Ultrahydrogel-500 column (7.8 mm ID × 300 mm, Waters, Inc.) linked to a Shodex OHpak SB-804 HQ column (8.0 mm ID × 300 mm, Showa Denko America, Inc.) attached to a Hewlett Packard 1100 HPLC. The flow rate of the mobile phase (degassed water) was 0.5 ml min^{-1} and the absorbance was monitored at OD_{254} with a diode array detector.

To examine the structural changes of HA by PAMC 26793 or PAMC 26794, the Fourier transform infrared (FTIR) spectrum was obtained from 2% freeze-dried HA in a KBr disc, which was pressed at 250 atm, with a Scinco Nicolet 6700 FT-IR spectrophotometer (Thermo Fisher Scientific Inc.).

Results

Isolation and diversity analysis of HA-degrading bacteria

Each soil sample from 33 grassland sites in Nome, Alaska was divided into two parts (0- to 10-cm and 10- to 20-cm depth). The soil samples from 0- to 10-cm depth were black and those from 10- to 20-cm depth were grayish. Generally, grassland soil in Alaska has been divided into a superficial organic layer and an underlying mineral layer, which was based on a 10- to 15-cm depth [11]. Thus, these black soils from the 0- to 10-cm depth are considered the organic layer, with the grayish layer from 10- to 20-cm depth being the mineral layer.

All of the soil samples were incubated on HA plates for 7 days at 15 °C and the number of grown bacterial colonies counted. On average 75 ± 70 colonies from 1.0 g soil from the 0- to 10-cm depth grew and 22 ± 14 colonies from the 10- to 20-cm soil sample grew. Among them, a total of cold-adapted 122 bacterial colonies were isolated for their ability to grow well on HA-based media plates. When the 16S rRNA gene from each isolate was sequenced and analyzed using the EzTaxon database, the 122 sequences (GenBank accession no. KF011585–KF011706) were taxonomically associated with two major taxa: *Bacilli* with major genus *Paenibacillus* (79.5%) and *γ-Proteobacteria* with major genus *Pseudomonas* (13.9%). Unclassified bacteria with no class affiliation accounted for 6.6% of all the 16S rRNA gene sequences (Fig. 1A).

Substrate utilization of HA-degrading bacteria

The 122 strains, which were preliminarily isolated as degraders of commercial HA, were re-examined in detail

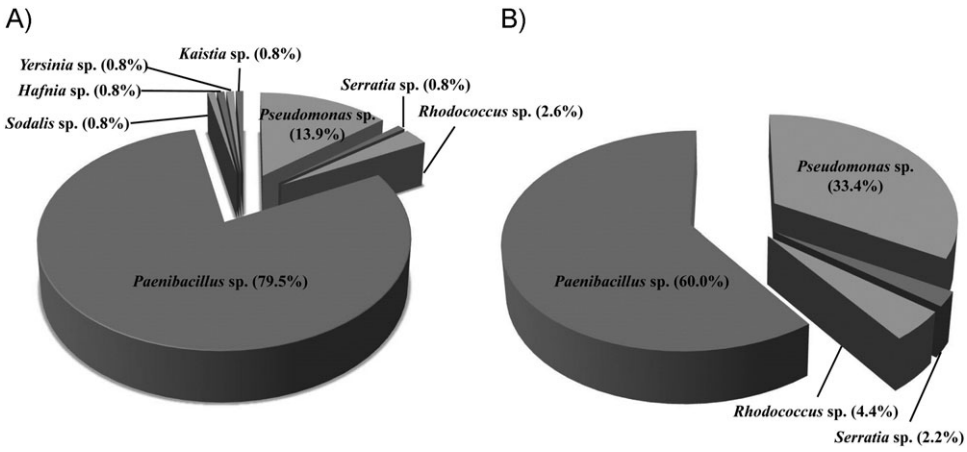

Figure 1. Taxonomic distribution of humic acids-degrading bacterial isolates. (A) Initial 122 isolates from grassland soils in Alaska; (B) 45 isolates, which were assigned PAMC number, were selected from the 122 isolates.

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for the ability to utilize HA as a carbon and energy source. Based on these analyses, 45 strains were selected as putative HS-degraders due to their efficient metabolism of HA and subsequently assigned Polar and Alpine Microbial Collection (PAMC) accession numbers.

HA is thought to be complex aromatic macromolecules with amino acids, amino sugars, peptides, and aliphatic compounds involved in linkages between the aromatic groups. Thus, these 45 isolates were tested for their ability to metabolize various aromatic compounds, which are supposed to be derived from soil HA or lignin by microbial catabolism (Fig. 2). Although all 45 strains were able to utilize glucose as a carbon source, they displayed substantial differences in their ability to utilize these putative HA metabolites as a carbon and energy source. For example, of the 45 strains, only 8 were able to utilize coniferyl alcohol, whereas 20 and 25 strains were able to utilize benzoic acid and phenol, respectively. When the 16S rRNA genes were analyzed using the EzTaxon database, the 45 strains were taxonomically classified as follows: *Paenibacillus* spp., 27 strains (60%); *Pseudomonas* spp., 15 strains (33.4%); *Rhodococcus* spp., 2 strains (4.4%); *Serratia* sp., 1 strain (2.2%; Fig. 1B). Although more *Paenibacillus* spp. strains were isolated based on HA degradation, the overall growth rate on HA plates, which could be directly correlated with HA degradation rate, appears to be higher in *Pseudomonas* spp. than *Paenibacillus* spp.

Characterization of HA bacterial degradation

Two strains, *Pseudomonas* sp. PAMC 26793 and *Paenibacillus* sp. PAMC 26794, were finally selected based on their excellent ability to grow on HA plates and/or utilize a broad range of aromatic metabolites as carbon and energy sources. The selection of these two strains is consistent with the fact that genus *Pseudomonas* and *Paenibacillus* are dominant group among HA-degrading strains. As shown in Fig. 2, *Paenibacillus* sp. PAMC 26794 could not utilize ferulic acid, coniferyl alcohol, benzoic acid, coumaric acid, and vanillic acid, whereas *Pseudomonas* sp. PAMC 26793 could metabolize these compounds. Among the aromatic compounds tested, ferulic acid, coumaric acid, and vanillic acid are known to be lignin-derived [9, 12].

To confirm the ability to use HA as the sole carbon source, PAMC 26793 and PAMC 26794 were inoculated in MSB containing HA and the growth was measured by calculating CFU values over time (Fig. 3). Both PAMC

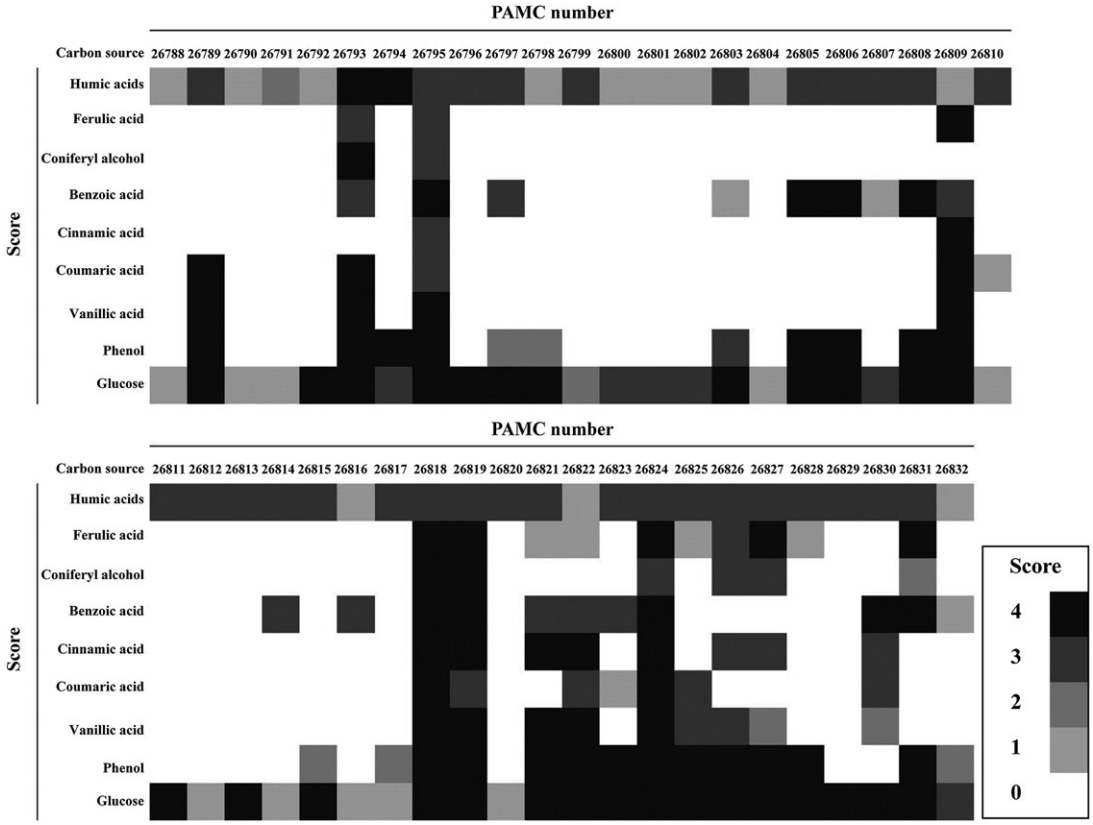

Figure 2. Growth tests for PAMC strains on various aromatic compounds derived from humic acids or lignin biodegradation.

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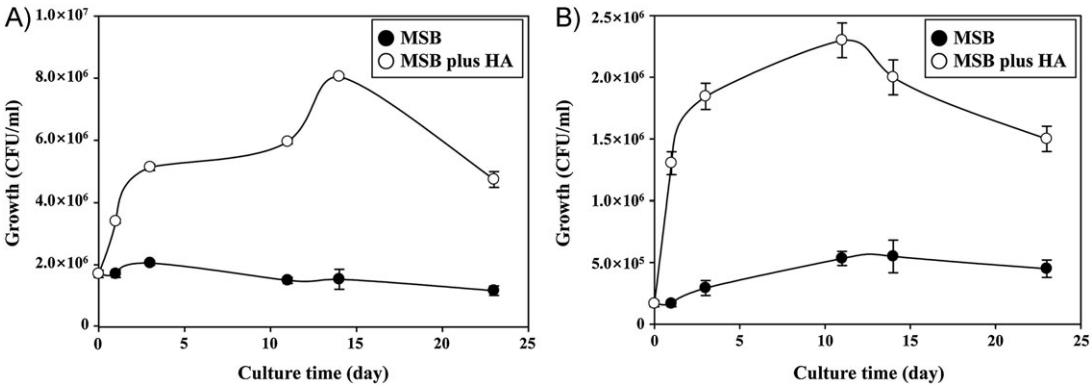

Figure 3. Growth of humic acids (HA)-degrading bacterial isolates in MSB medium and MSB plus HA (1 g L^{-1}). (A) *Pseudomonas* sp. PAMC 26793; (B) *Paenibacillus* sp. PAMC 26794.

26793 and PAMC 26794 showed growth at 15 °C using HA as a substrate, respectively, but did not grow in MSB media without HA. Specifically, the growth of PAMC 26793 and PAMC 26794 increased up to 14 days after incubation, showing a maximal CFU value of $80.5 \pm 0.7 \times 10^5$ and $23.0 \pm 1.4 \times 10^5$ CFU ml^{-1}, respectively. After 14 days, the CFU counts of both strains decreased. These results again demonstrated the ability of PAMC 26793 and PAMC 26794 to utilize HA as a sole carbon source.

To further confirm the ability of PAMC 26793 and PAMC 26794 to degrade natural HS, their growth was tested on natural HA, which was extracted from the Alaska grassland soil (0- to 10-cm depth) from which both strains were originally isolated. On the natural HA plates, PAMC 26793 and PAMC 26794 grew well and showed a faster growth rate than on plates supplemented with commercial HA, suggesting that the natural HA had been more extensively subjected to *in situ* microbial degradation and was therefore a better substrate for PAMC 26793 and PAMC 26794 growth (data not shown).

Structural changes of HA during incubation with bacterium

To characterize the ability of these bacterial isolates to depolymerize (i.e., degradation) and/or polymerize (i.e., further humification) natural HS, commercial HA was used in this study as a model compound. FTIR spectroscopy was used to obtain insight into the structural changes that occurred in the HA degradation process during incubating with PAMC 26793 or PAMC 26794 for 28 days at 15 °C (Fig. 4). As compared with the negative control (spectrum A, no cells), substantial changes in the FTIR spectra of the final HA structures were observed. The spectra of HA from PAMC 26793 (spectrum B) and PAMC 26794 (spectrum C) cultures showed appearance of a new absorption peak at 3359.8 cm^{-1}, which is assigned the H-bonded OH groups from phenols and alcohols in HA. In the PAMC 26794 culture, a peak at 1709.8 cm^{-1}, assigned to the CO stretching of COOH, ketones and aldehydes, showed reduced absorption, which had been previously reported to occur during HA metabolism by microbes [13, 14]. Additionally, several changes in absorption peaks from 1378.4 to 538.2 cm^{-1}, corresponding to functional groups of aromatic compounds, were detected. These structural changes are similar to the FTIR spectra reported after HA biodegradation by *Streptomyces* sp. strains [15]. Although we did not detect a decrease in the absorption peaks from 2930 to 2850 cm^{-1}, corresponding to CH stretching vibration in CH_3 and CH_2 groups of aliphatics, structural changes of HA by PAMC 26794 were confirmed through the analysis of spectral patterns on FTIR. In contrast to PAMC 26794, spectral changes during the HA degradation process by PAMC 26793 were thought to be related to a decrease in the SiO peak (669.1 cm^{-1}) from the silicates and a disappearance of polysaccharide moieties in HA (963.8 cm^{-1}). The FTIR spectral analysis showed that obvious structural changes in HA are observed during degradation of HA and the extent and sites of changes are different between these two bacterial strains.

To detect the changes in molecular weight distributions of HA during the incubation with PAMC 26793 and PAMC 26794, gel permeation chromatography (GPC) analysis was performed on HA isolated from PAMC 26793 and PAMC 26794 cultures during a 28-day growth cycle at 15 °C (Fig. 5). When compared with control HA, in which no bacteria were added, HA from PAMC 26793 showed depolymerization, in which the high molecular weight fraction (retention times 21.2–22.1 min) and the low

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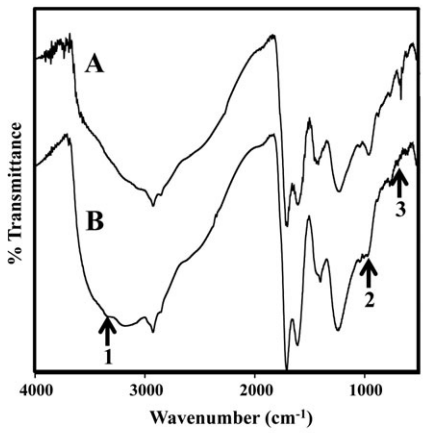

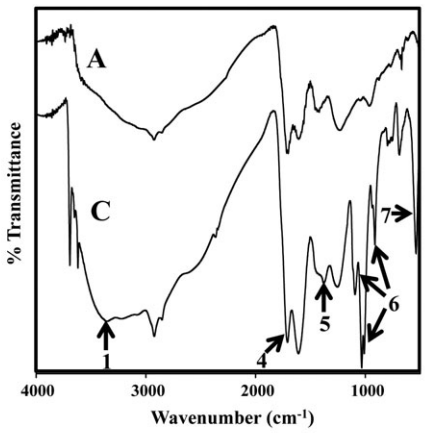

Arrow number	Wavenumber (cm^{-1})	Proposed assignment	PAMC 26793
1	3500-3300	O-H vibrations of the hydroxyl groups (phenols and alcohols) and N-H vibrations (amides and amines)	3359.8
2	1040	C-O stretching (polysaccharides) Si-O	963.8
3	770	Si-O	669.1

Arrow number	Wavenumber (cm^{-1})	Proposed assignment	PAMC 26794
1	3500-3300	O-H vibrations of the hydroxyl groups (phenols and alcohols) and N-H vibrations (amides and amines)	3359.8
4	1719	C=O stretching in COOH, aldehydes and ketones	1709.8
5	1388	OH-deformation and C-O stretch (phenolic)	1378.4
6	1130-1000	C-OH stretch (aromatics) and C-O-C stretch (aryl esters and phenols)	1032.6 1009.9 912.6
7	570-536	O-Me groups (C-O-C bending and deformation in aromatics)	538.2

Figure 4. Fourier transform infrared (FTIR) spectra of humic acids (HA) after incubation with bacterial isolates. Spectrum A, HA with no bacteria added; spectrum B, HA incubated with *Pseudomonas* sp. PAMC 26793; spectrum C, HA incubated with *Paenibacillus* sp. PAMC 26794. The arrows indicate the changes in absorption peaks compared with a negative control (no bacteria). Below are assignments of the FTIR peaks that showed changes in their absorption spectra.

molecular weight fraction (>23.3 min) decreased. Interestingly, a new higher molecular weight peak appeared (20.0 min), which is thought to be a more polymerized form. This combined reaction between depolymerization and polymerization process was also observed for PAMC 26794.

Discussion

Extracellular manganese peroxidase, lignin peroxidase, and laccase enzymes are all able to biotransform high molecular weight HS to unstable compounds, which can undergo either polymerization or depolymerization [2, 16–18]. In

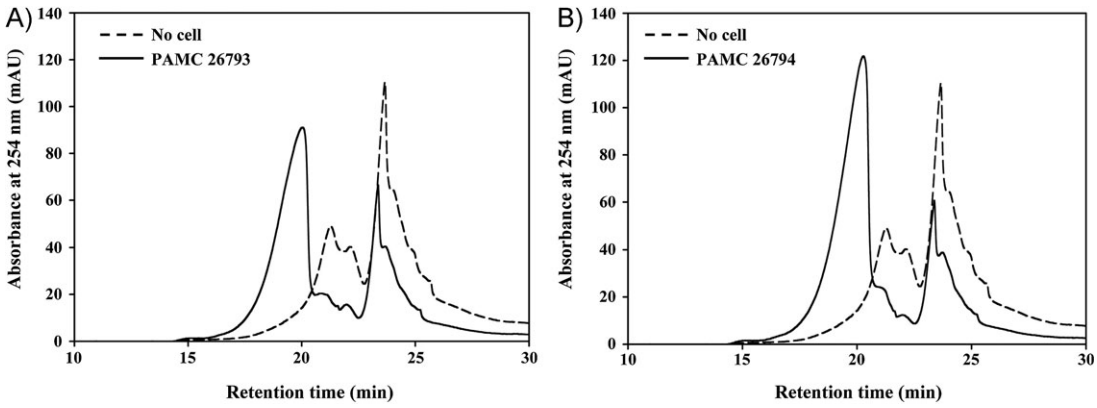

Figure 5. Gel permeation chromatograms for changes in molecular weight distribution of humic acids after incubation with bacterial isolates. (A) *Pseudomonas* sp. PAMC 26793; (B) *Paenibacillus* sp. PAMC 26794.

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detail, these enzymes catalyze nonspecific oxidization of the phenolic nucleus, resulting in a phenoxy free radical, which undergoes spontaneous polymerization or further degradation [18]. Based on the above experimental results from growth tests and structural analysis with HA and its metabolites, it is assumed that *Pseudomonas* sp. PAMC 26793 and *Paenibacillus* sp. PAMC 26794 degrade natural HS to some metabolic intermediates through their extracellular degradative enzyme(s), and these intermediates are further metabolized to support the cell growth of themselves and other surrounding microorganisms and at the same time can be polymerized to produce higher molecular weight polymers.

Although the present study is limited to cultivatable bacteria, it appears that microorganisms with a variety of phylogenetic classifications are capable of degrading HS in low-temperature environments. Interestingly, it was found in this study that *Paenibacillus* was dominant group among the HS-degrading bacterial strains isolated on HA plates. Since aerobic endospore-forming *Paenibacillus* spp. strains are essentially ubiquitous in and can be readily cultured from bulk and rhizosphere soils, it now seems likely that the chance of isolation of HS-degrading *Paenibacillus* increased [19]. However, the ecological interactions and the distribution, abundance, and diversity of HS-degrading bacteria should be estimated in future. In conclusion, we believe that many cold-adapted microbes containing soil bacteria play a crucial role in HS degradation in the grassland soil. Further study will enable further insight into the relationship between bacterial HS degradation and soil organic carbon cycling in cold environments. Also, after the biocatalytic breakdown of HS is fully understood, natural HS could be used as a major renewable source of aromatic and phenolic bio-products [20].

Acknowledgments

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