Metabologically active hydrolytical microbial communities of soil ecosystems under influence of soil physical factors

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Abstract. Application of molecular biological techniques in environmental studies provides a more complete information concerning the taxonomic diversity and potential hydrolytic activity of soil microbial complexes that exist in a wide range of environmental factors. Among the key environmental parameters that determine the functional activity of the hydrolytic complex of soil layer, the most significant one is moisture. Moisture levels providing maximum activity of a hydrolytic microbial complex depend on the soil type. At high levels of moisture and temperature, the role of prokaryotic organisms, mainly actinomycetes, in the microbial complex significantly increases. It was discovered a new functional activity of actinomycetes in the hydrolytic prokaryotic complex: their controlling influence on the respiratory level of the complex in a wide range of parameters (moisture, organic matter, successional time). At the optimum for the life of most microorganisms levels of moisture (60% of field capacity) and temperature (27°C), representatives of Firmicutes and Actinobacteria phylums stand out among chitinolytic and pectinolytic dominants of the studied soils within the Bacteria domain. With increasing moisture and decreasing temperature the proportion of Proteobacteria increases. With decreasing moisture and increasing temperature, there is an increase in the amount of unicellular actinobacteria.

1. Introduction
The rate of the microbial hydrolytic complex development and the efficiency of microbial destruction of biopolymers, polysaccharides specifically, are different in soils of different climatic zones [1,2]. The intensity of microorganisms-destroyers strongly depends on environmental factors, including temperature, moisture, redox conditions, osmotic pressure of soil solutions, etc. [3]. Identification of parameters of stability and functional activity of hydrolytic prokaryotic communities in a wide range of key environmental factors is essential for solving the problems of soil fertility and addressing the issues of biotechnology related to processing of renewable raw materials. Polysaccharides of different structures entering the ecosystem are extremely widespread in nature, and are an important source of organic matter. Such polysaccharides as chitin, pectin, cellulose, constantly present in litter, make up a renewable pool of organic carbon and nitrogen [4]. Content of polysaccharides in different types of soils varies from 0.06% to 3.0% of the mass of soil, and from 1% to 14% of the mass of organic matter in soils. Soils with plant residue receive from 2 to 14 t/ha of polysaccharides, a considerable part of which are mineralised or participate as structural elements in the formation of humic acids [5]. The inclusion of elements of feeding into the biological cycle requires the degradation of polysaccharides by respective enzyme complexes of microorganisms. Both in Russia and abroad, this area of research is still largely unexplored. However, numerous scientific and technological publications have covered the issues of
synthesis of microbial hydrolases in laboratories and industrial sites, and of their application in various industries, medicine and agriculture. World scientific literature on the issue of the present thesis contain, as a rule, the results of research of microbial depolymerase (chitinase, cellulase, ligninase etc.) not in natural systems, but derived from cultures of microorganisms incubated on artificial growth media. Processes of microbial destruction of polysaccharides, and the activity of chitinases specifically, can become the basis for development of effective methods of soil bioremediation and detoxication. However, the problem of microbial degradation of polysaccharides in natural systems, especially soils is hitherto little studied, despite its immense relevance. The study of structural and functional diversity of microbial hydrolytic complexes localized in soil will provide a more complete picture of gradual destruction of biopolymers in terrestrial biogeocenoses, and help assess the role of different groups of organisms at different stages of the process.

2. Materials and Methods

2.1. The objects of study
The objects of study were samples of soils, which are: the upper humus horizons of Voronic Chernozems (N 51°1’41”, E 40°43’31”), Haplic Arenic Kashtanozem (N 49.39936º, E 46.81083º), Haplic Luvisols (Abruptic) (N 53°58’23”, E 37°10’34”). Samples of substrates were repeatedly selected, sufficiently so to account for the spatial and temporal variations in the structure of hydrolytic microbial communities. Special attention is paid to the analysis of dynamics of structural and functional parameters in succession of hydrolytic (chitinolytic and pectinolytic) microbial communities of terrestrial ecosystems. To determine the structure of hydrolytic microbial complexes in the samples of terrestrial ecosystems, we used the method of microcosms with microbial succession initiation through moisturising and introduction of purified polysaccharides (chitin (“ICN Biomedicals”, Germany), pectin (“Sigma”, Germany), or cellulose (“Sigma”, Germany)). The mass of added substrates in all cases did not exceed tenths of a percent of the mass of the sample. Incubation of soil microcosms was carried out in a wide range of values of soil moisture pressure matrix, P (0 to -808 kPa), redox potential and temperature (5, 27 and 50°C).

2.2. Evaluation of the abundance and taxonomy for the dominant groups of hydrolytic prokaryotic complexes microorganisms was performed by molecular-biological methods of FISH (fluorescence in situ hybridisation)
Evaluation of the amount of bacteria, the length of the mycelium of actinomycetes and fungi was performed using the method of fluorescence microscopy of preparations stained with a number of different fluorochromes (microscope “Axioskop2 +”). Using the method of fluorescent hybridisation in situ (FISH) [6], that we had modified to work with the soil, we revealed the specifics of prokaryotic metabolically active community destroying polysaccharides. We applied a range of probes specific for members of Archaea and Bacteria domains, as well as certain phylogenetic Bacteria groups. Total biomass and the proportion of metabolically active microorganisms were determined by calculation [7].

2.3. Emissions of greenhouse gases
Emissions of greenhouse gases (carbon dioxide, nitrous oxide and methane) from samples of litter and soil tiers with and without the introduction of polysaccharides were evaluated by gas chromatography [8]. Statistical analysis was conducted by multivariate methods of mathematical statistics using STATISTICA-6 and Statgraphics Plus software.

3. Results

3.1. The functional activity of microbial soil hydrolytic complexes under influence of soil moisture
The study of the influence of soil moisture on the emission of carbon dioxide from soils with the introduction of various polysaccharides has shown that the pressure of soil moisture affects the dynamics
and intensity of carbon dioxide emissions to a greater extent with the introduction of chitin than of other tested substrates (pectin, glucose and cellulose). The mass of introduced substrates in all samples contained the equal number of moles of carbon. As the soil moisture pressure increases from -800 kPa to -0.5 kPa, CO₂ emission intensity with the introduction of chitin in microcosms with chernozem samples increases more than by two times (from 4 to 18 µg CO₂/g of soil per hour), which is not observed with the introduction of other substrates. Similar results were observed for the microcosms of all studied soils. Since polysaccharides occur in soils in both aerobic and anaerobic microzones, we studied their transformation in microaerobic conditions. As an indicator of transformation intensity we used methane emissions from soil samples with and without the introduction of chitin. When moisture level is close to full capacity, intense emission of methane was observed for all tested samples of soil, which indicates the presence of strictly anaerobic microzones in all these soils. The difference in methane emissions between soil samples with the introduction of chitin and without it (control) was apparent at the early stages of soil incubation, which proves that it was being used by microorganisms already during the first day of the experiment. The highest methane emission values and the differences in the values of emission between the experimental and control variants were recorded on 20-30th days of the experiment, which is clearly connected with the development of methanogens in hydrolytic (chitinolytic) metabolism products. It should be noted that the emission of methane from soils was observed at all tested levels of matrix soil moisture pressure: from -800 to -0.5 kPa. It was found out that the anaerobic transformation of chitin occurs in soils with different intensity at the studied levels of moisture and the matrix potential. Intervals of moisture values for anaerobic transformation of chitin in different soils are different. Thus, in the weakly sandy soil the most active work of hydrolytic anaerobes was registered in a fairly narrow range of moisture content: from 15% of the soil mass (matrix pressure -1.16 kPa) to 25% of the soil mass (full moisture capacity 0 kPa), whereas in chernozem that interval increased from 36% of the soil mass (matrix pressure -32.6 kPa) to 64% of the soil mass (full moisture capacity 0 kPa). In soils with a high content of clay fractions (EPCH <1 mm - 28%) the transformation ratio of chitin increased in areas with high moisture values, and for the sandy soil — with lower values (EPCH <1 mm - 5%). There is a correlation between the rate of transformation of chitin in anaerobic conditions and the radius of pores occupied by water. With the radius of pores (water-filled) is less than 6 micrometers, the activity of anaerobic chitinolytic microbial complex, determined by the values of transformation ratios of chitin, was minimal, indicating impediments in development of anaerobic microorganisms due to the small pore diameter. Thus, the upper moisture limit for the studied process is determined by the full moisture capacity of soils, and bottom limit - by the size of pores, where the bacteria growth is still possible.

3.2. The functional activity of soil hydrolytic microbes under influence of temperature

The study of the respiratory activity in soil microcosms with the introduction of chitin and pectin showed that CO₂ emission in these cases exceeded emission in control, on average, by 2-6 times for all the soils. The rate of CO₂ production in the experimental variants with the introduction of polysaccharides varied depending on incubation temperature. At the minimum temperature (5 °C), the peak of activity was observed at the end of the third week of the experiment, at 27°C - by the end of the first week, at 50°C - on the first day of the experiment. In the control variants such activity was not observed, indicating a high functional ability of microbial complexes to hydrolyse polysaccharides.

We used emission of carbon dioxide as a functional indicator of microbial chitinolytic complex in the experiments based on mathematical methods of experimental design in order to optimise conditions (optimal values of factors), providing microbial decomposition of chitin in soils. The experiment was planned according to the scheme of the central composite design "2²+star."

It has been revealed that the optimal values of factors differ for different types of soils. For chernozem, the maximum of respiration is detected approximately on the 20th day at 25 °C; for the sandy soil - on the 10th day at 30 °C.
Thus, thanks to experimental design we demonstrate the principal possibility of solving an optimisation problem by defining the optimal values of factors (temperature and successional time) for the functional indicator (respiration) of microbial hydrolytic complex evolving in soils of different types.

3.3. Correlation of biomass accumulation and activity of chitinolytic and pectinolytic microbial complexes of soils developed at different parameters of temperature and soil moisture pressure

Determining the amount of microorganisms and the biomass of hydrolytic microbial complex we registered their increase for all groups of microorganisms in cases of the complex developing with the introduction of polysaccharides, as compared to control options. Experiments on the dynamics of abundance and biomass of different groups of microorganisms during microbial succession in soil samples incubated at different temperatures, generally, confirmed the data obtained as a result of the study of respiratory activity dynamics in all soils. Thus, the activity of chitinase in wet chernozem in situ even without the introduction of chitin at 27°C reached its maximum on the 8th day of the experiment and was six times higher than the activity of chitinase in the same soil, incubated at 5°C. Evaluation of the ratio of biomass of individual groups of microorganisms (prokaryotes, fungi) in the samples with and without the introduction of the substrate showed that regardless of the type of the substrate and the type of soil, fungi (unlike prokaryotes) tend to decompose polysaccharides more actively at low temperatures. The activity of prokaryotic complex was largely related to the type of soil. The activity of actinomycetes at increased temperatures was especially great in the chernozem with the introduction of chitin. Thus, the phylogenetic structure of the natural hydrolytic microbial complex depends on climatic conditions and soil properties.

3.4. Analysis of structural and functional parameters of the hydrolytic microbial complex development

We have obtained some new and interesting results during the analysis of basic information concerning the dynamics of structural (biomass of fungi, bacteria and actinomycetes) and functional (carbon dioxide emissions) indicators of the hydrolytic complex development conducted by methods of multivariate mathematical statistics. Stepwise multiple regression to determine the relationship between functional and structural indicators reveals that minor biomass components - the filamentous and unicellular bacteria, especially actinomycetes, play a significant role in the functioning of soil microbial complex regardless of the temperature. The resulting regression model based on two variables has a high statistical significance (explains up to 90% of the variance of the functional value). Dependence is described by the following equations for the Chernozem soil (equation 1) and Haplic Luvisols (equation 2):

\[
R = 52.87 \times B + 89.27 \times A, \quad (1)
\]
\[
R = 37.77 \times B + 555.49 \times A, \quad (2)
\]

where \( R \) – CO₂ emission (μg C-CO₂/g·h), \( A \) – actinomycetes biomass (mg/g), \( B \) – bacteria biomass (mg/g).

Thus we have established a high importance of minor biomass components of the microbial complex in the hydrolysis of polysaccharides, regardless of the temperature of the process; these components - filamentous and unicellular prokaryotes - have been shown to contribute significantly to the control of microbial complex respiration in a wide range of environmental parameters (temperature, moisture, organic matter, successional time). In the process of chitin degradation a particularly significant role is played by actinomycetes.

Discriminant functions \( F_1 \) and \( F_2 \) for the natural values of structural (equation 3) and functional characteristics (equation 4) are as follows:

\[
F_1 = -1.41 - 9.73 \times A + 5.71 \times B - 0.07 \times F - 0.03 \times R, \quad (3)
\]
\[
F_2 = -1.49 + 17.54 \times A + 1.71 \times B - 0.49 \times F + 0.12 \times R, \quad (4)
\]

where \( A \) – actinomycetes biomass (mg/g), \( B \) – bacteria biomass (mg/g), \( F \) – fungi biomass (mg/g), \( R \) – CO₂ emission (μg C-CO₂/g·h).

Of great interest is the search for connections between functional and structural indicators of natural microbial complexes.
Stepwise multivariate regression analysis of the data obtained for a variety of microbial successions (with the introduction of chitin and pectin and without the introduction of polymers) in a wide range of soil moisture pressure, allowed to identify and describe certain connection with the regression model equation 5:

$$R = 399.03 * A + 2.37 * F,$$

where $R$ – CO$_2$ emission (µg C-CO$_2$/g·h), $A$ – actinomycetes biomass (mg/g), $F$ – fungi biomass (mg/g).

Analysis of variance indicates the high quality of the model ($p < 0.01$, the level of 99%), which generally has enough information capacity at the coefficient of determination of 69%. The connection between respiration and fungal biomass was revealed to be as expected and quite traditional, but what turned out to be very new and unexpected was the determining influence of the complex of actinomycetes on this functional parameter. The degree of influence of actinomycetes on respiration, according to analysis of variance, is 87%.

Thus, we demonstrate that the role of a minor component of the biomass (actinomycetes) in the functioning of soil microbial complex is essential. Respiration as a key functional indicator of natural microbial complexes can be substantially controlled by actinomycetes, whose role seems to be determined not so much by their direct contribution to the stoichiometry of the processes of organic matter decomposition, but rather by their contribution to the regulation of the functioning of the microbial complex as a whole. Regulatory role of actinomycetes, apparently, is provided for by the mechanisms based on the production of biologically active substances, which is typical of actinomycetes. It is essential, that this result was obtained under conditions simulating a wide range of environmental situations (moisture, organic matter, successional time), which may occur in situ.

It was also revealed that the level of soil moisture in a given range of certain structural parameters may be a more important factor than those essential for microbial complex factors, such as nutrient supply and time. Moisture has a more significant effect on the activity of chitinolytic complexes than on pectinolytic ones.

In the process of degradation of polysaccharides, with the increase of soil moisture in the microbial complex, the role of prokaryotic microorganisms perceptibly increases, too, especially that of actinomycetes. Respiration in a wide range of conditions (moisture, organic matter, successional time) can be significantly controlled by actinomycetes, whose role seems to be determined by their contribution to the regulatory control of the microbial community functioning.

### 3.5. Molecular and biological analysis (method FISH) of component composition of hydrolytic prokaryotic chitinolytic and pectinolytic complexes of soils developed at different values of moisture and temperature

The abundance of metabolically active cells of prokaryotes in microbial hydrolytic complexes of the studied soils was estimated by the method of in situ-hybridisation with rRNA-specific fluorescent-labeled oligonucleotide probes (FISH-method) in the time frames of the experiment corresponding to the maximum accumulation of microbial biomass, as measured by luminescence microscopy, and the greatest respiration rate in the process of soil incubation at 5°C, 27°C and 50°C.

The experiments showed that the total accumulated amount of cells in the soil samples (for members of Bacteria and Archaea domains) comprised from 26% (for Chernozem) to 90% (for Haplic Luvisols) of the total number of cells detected by hybridisation with universal probes and identified by acridine orange staining. A particularly low amount of cells was registered by FISH in the control variants in chernozem samples incubated at 5°C. It should be noted that during the development of the microbial complex of this sample at all temperatures, and especially at 5°C, most of the bacteria were represented by unidentified cells of small sizes. These data suggest that a significant proportion of prokaryotic organisms of chernozem occur in the form of dormant or metabolically inactive cells. The variants of different types of soils incubated at all studies temperatures with chitin and pectin, as compared to control variants, showed the increase (by 1.5-3 times) in the amount of metabolically active members of Bacteria and Archaea domains: their number was from 4 to 20.5·10$^8$ cells/g of soil for Bacteria and
from 9 to 29 \times 10^7 \text{ cells/g of soil} for Archaea. Participation of archaea in the mineralization of nitrogen-containing organic matter in soils is discussed in many papers \cite{9, 10}.

In chitinolytic and pectinolytic complexes of chernozem the total amount of metabolically active forms of Bacteria varied slightly at different temperatures, which is apparently related to the high buffer capacity of chernozem and a high content of organic matter in the soil.

Component analysis of the composition of cells within the Bacteria domain in the hydrolytic complex of typical chernozem samples revealed differences in the response of different groups of bacteria to the soil incubation temperature change, which determined the number of dominants.

It has been shown that members of phylogenetic groups \textit{Actinobacteria}, \textit{Firmicutes} and \textit{Bacteroidetes} responded with significant increase in the number of metabolically active forms to the introduction of chitin and pectin in all the studied soils regardless of temperature, indicating the active participation of the representatives of the mentioned phylogenetic groups in the decomposition of polysaccharides in a wide temperature range. These groups of organisms are most frequently mentioned among the main agents of destruction of organic matter in natural ecosystems \cite{1}. When the temperature was decreased to 5°C, the hydrolytic microbial soil complex showed the increase in the number of \textit{Firmicutes} and \textit{Betaproteobacteria} and the decrease of gram-positive bacteria belonging to the phylogenetic group of \textit{Actinobacteria}, with high content of G + C pairs in the DNA, as compared to their amount in soils at 50 °C. The number of representatives of the \textit{Actinobacteria} phylogenetic group increased considerably in the experimental variants and made up to 35% of the identified unicellular units within the \textit{Bacteria} domain in the hydrolytic complex of soils. It should be noted that the amount of mycelial forms of actinobacteria increased, too; their biomass in the desert-steppe soil and chernozem with the introduction of chitin increased by almost an order. Also, in variants with polysaccharides and incubation at 50°C (in certain soils at 27°C, as well), the number of metabolically active cells of \textit{Gammaproteobacteria} was bigger than in the control samples. The increase in the number of \textit{Verrucomicrobia} in variants with pectin indicates their possible involvement in the process of degradation of this substrate. Nowadays, among the described and legitimized representatives of \textit{Verrucomicrobia}, there have been identified bacteria capable of growing on cellulose, pectin, starch \cite{11}. The result of the next set of experiments was identification of characteristics of the phylogenetic structure of pectinolytic and chitinolytic complexes in soil samples of varying moisture degrees. With the development of pectinolytic and chitinolytic complexes within the \textit{Bacteria} domain, under the average soil moisture pressure (3.2 kPa) the dominants are the representatives of \textit{Firmicutes} and \textit{Actinobacteria} phylogenetic groups, which was registered for all the studied soils. With the increase of moisture, the number of gram-negative forms (proteobacteria) showed increase, as well. With the decrease of matrix pressure to the values close to the wilting point, the number of gamma- and alpha-proteobacteria and unicellular actinobacteria in the hydrolytic complex increases.

Thus, the application of FISH method, modified by us for the purposes of soil microbiology, enabled us to identify certain characteristics in the structure of microbial hydrolytic complex of the studied soils under various environmental conditions (temperature and moisture). Within the Bacteria domain, with an average moisture content (60% of full capacity) and temperature (27°C), the representatives of \textit{Firmicutes} and \textit{Actinobacteria} phylogenetic groups dominate over pectinolytic and chitinolytic complexes. The increase of moister and the decrease of temperature lead to the increase in the number of gram-negative alpha- and beta-proteobacteria, whereas the decrease of moisture and the increase of temperature result in the increase in the number of actinobacteria in the studied prokaryotic microbial complexes, which deserves special attention.

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