ISSN 0026-2617, Microbiology, 2009, Vol. 78, No. 2, pp. 227–233. © Pleiades Publishing, Ltd., 2009.
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EXPERIMENTAL ARTICLES

Cellulolytic Streptomycetes from *Sphagnum* Peat Bogs and Factors Controlling Their Activity

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Received March 19, 2008

Abstract—Two strains of *Actinobacteria,* ACTY and ACTR, were isolated from cellulolytic microbial communities obtained from an ombrotrophic *Sphagnum* peat bog. The strains were able to degrade cellulose, the main component of plant phytomass in this ecosystem. On the basis of their phenotypic and phylogenetic characteristics, the strains were identified as members of the genus *Streptomyces.* The isolates developed on media without available nitrogen sources and hydrolyzed cellulose within a temperature range of 5–25°C and in the pH interval from 4.5 to 6.0; they also exhibited acetylene reduction activity. Comparative analysis of the rates of cellulose degradation by the peat-inhabiting streptomyces at 5, 15, and 25°C and at pH values of 4.5 and 6.0, with and without a source of available nitrogen in the medium, indicated that high acidity and low temperatures, typical for boreal *Sphagnum* peat bogs, are the main factors limiting the growth and hydrolytic activity of these bacteria.

Key words: cellulose decomposition, *Sphagnum* bogs, *Streptomyces* spp., regulation of cellulolytic activity, N_2 fixation.

DOI: 10.1134/S0026261709020143

On Earth, about one third of the organic carbon stock is accumulated in *Sphagnum*-dominated wetlands [1]. The reasons for the low intensity of decomposition of plant debris in these ecosystems are still debatable. It is assumed that slow degradation of organic matter, including cellulose, the major natural biopolymer, is the result of high acidity (pH 3.5–5.5) [2, 3], low concentration of biogenic elements (N, 0.01–0.13 mg/l; P, 0–0.05 mg/l) [4], as well as of the toxic effect of the phenol compounds produced by *Sphagnum* mosses [3]. It has been previously demonstrated that low temperatures and the low content of available nitrogen are the factors limiting the growth and cellulolytic activity of micromycetes inhabiting *Sphagnum* peat bogs [5, 6]. Data on the mechanisms involved in the regulation of activity of cellulolytic bacteria inhabiting *Sphagnum* peat bogs are lacking in literature.

Analysis of the species composition of the hydrolytic prokaryotic communities of *Sphagnum* peat bogs by means of fluorescence in situ hybridization (FISH) revealed that bacteria belonging to the phylogenetic group *Actinobacteria* are the main constituents of these communities [7, 8]. For instance, in laboratory simulation experiments exploring *Sphagnum* phytomass degradation, the microorganisms belonging to this group constituted 20% of the total number of bacteria [8]. *Actinobacteria* were also the main metabolically active component of the prokaryotic cellulolytic community revealed in acidic *Sphagnum* peat amended with cellulose [7]. Isolation of the key microbial agents from these hydrolytic communities, as well as analysis of their functional characteristics, may improve our understanding of the reasons for the low intensity of the phytomass decomposition in oligotrophic *Sphagnum* peat bogs.

The purpose of this work was to isolate the representatives of the *Actinobacteria* from the cellulolytic community of *Sphagnum* peat, as well as to study the dependence of their growth and hydrolytic activity on temperature, pH, and availability of nitrogen.

MATERIALS AND METHODS

The objects of study were enrichment cultures of cellulolytic microorganisms obtained from the peat samples from the Obukhovskoe ombrotrophic peat bog, Yaroslavl oblast (58°14′ N, 38°12′ E) and described previously in [7]. The predominance of actinobacteria in these cultures was revealed by the FISH method.

Isolation of cellulolytic bacteria was performed by spread-plating of aliquots of the enrichments on agar medium CM1 (pH 4.2–4.6) containing the following (g/l): crystalline cellulose (Aldrich, 20 μm), 1; K_2PO_4, 0.1; $MgSO_4 \cdot 7H_2O$, 0.04; $CaCl_2 \cdot 2H_2O$, 0.02; yeast extract, 0.1, trace element solution SL6 ($ZnSO_4 \cdot 7H_2O$, 0.1; $MnCl_2 \cdot 4H_2O$, 0.1; H_3BO_3, 0.3; $CoCl_2 \cdot 6H_2O$, 0.2; $CuCl_2 \cdot 2H_2O$, 0.01; $NiCl_2 \cdot 6H_2O$, 0.02; $Na_2MoO_4 \cdot 2H_2O$, 0.03; distilled water, 1l), 10 ml/l; agar, 15;

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pH 4.2–4.6. The ability of the isolates to hydrolyze crystalline cellulose was assessed by detecting clear areas in agar medium around their colonies. Purification of the bacterial cultures exhibiting cellulolytic activity was performed by successive transfers onto agar media with starch or glucose (0.5 g/l).

Identification of the isolates was performed by the 16S rRNA gene analysis. For DNA extraction from the isolates, a modification of the previously described method based on the use of sodium dodecyl sulfate (SDS) as a lysing agent was applied [9]. PCR amplification of the 16S rRNA genes was carried out with the universal eubacterial primers 9f and 1492r [10] on a PE GeneAmp PCR System 9700 thermocycler (Perkin-Elmer Applied Biosystems, United States). The nucleotide sequences of the amplified fragments were determined using an ABI 377A sequencer (Perkin-Elmer Applied Biosystems, United States). Comparison of the 16S rRNA gene sequences of the isolates from *Sphagnum* peat bogs with the sequences in the GenBank database was performed using the Blast2 software package (http://www.ebi.ac.uk/blast2/). The obtained nucleotide sequences of the 16S rRNA genes of the *Streptomyces* isolates were deposited in the GenBank under the accession numbers FM163174 (ACTY) and FM163175 (ACTR).

Cellulose degradation was assayed in 500-ml serum vials containing 100 ml of medium. To study the cellulolytic activity of the isolates, the CM1 medium was used. We modified the medium (a) by replacing yeast extract with NH_4NO_3 (0.2 g/l) or (b) by eliminating the sources of available nitrogen. The cultures grown on crystalline cellulose were used as inocula. Suspensions of these cultures (2 ml) homogenized by intense agitation were transferred into the vials containing fresh media. The vials were hermetically sealed with silicon rubber stoppers and incubated in static conditions at 5, 15, or 25°C. Homogeneity of the inoculum aliquots was tested by determining the protein by the Lowry method [11]. The analyses were performed in triplicate. In the course of the experiment, the purity of the cultures was monitored by microscopic examination under an Axioplan 2 epifluorescence microscope (Zeiss, Germany) at ×1000 magnification.

Microbial degradation of cellulose was monitored by detecting the increase in the CO_2 concentration in the gas phase of the experimental vials using an INFRALYT 4 infrared gas analyzer (Dessau, Germany). The CO_2 concentration in the samples collected from each vial was determined by comparison with the peak value of the standard gas. The respiration rate was expressed in μg C-CO_2 /ml culture liquid per day. CO_2 accumulation in the vials was expressed in μg C-CO_2/ml gas phase.

The protein content was determined by the Lowry method [11]. The calibration curve was obtained using a series of standard albumin solutions in 0.1% SDS.

The nitrogen-fixing activity of the isolates was determined by the acetylene method. The cultures were grown in liquid starch- or glucose-containing media without any available nitrogen sources. Exponential-phase cells were harvested by centrifugation and transferred into 40-ml vials containing 20 ml of sterile CM1 medium with glucose (1 g/l) as a carbon source. The vials were hermetically sealed with rubber stoppers, supplemented with acetylene (10 vol %) and incubated under slow agitation (50 rpm) on a shaker (Heidolph, Germany) for 1 day at 25°C. The amount of ethylene formed from the acetylene added to the gas phase was determined on a Model 3700 gas chromatograph equipped with a flame ionization detector. A mixture of ethylene (0.01%) and nitrogen was used as the standard gas. Acetylene reduction activity was expressed per unit of protein in the biomass produced by the studied cultures.

PCR amplification of the *nifH* gene encoding the dinitrogenase reductase component, was performed using three pairs of degenerate oligonucleotide primers that targeted the maximum possible diversity of known diazotrophic bacteria [12–14]. The nitrogen-fixing methanotrophic bacterium *Methylocystis echinoides* IMET 10491^T was used as a positive control for PCR amplification with the primers described in [12, 13].

RESULTS

Isolation of cellulose degrading bacteria. A number of isolates forming clear areas around their colonies (which demonstrated their ability to hydrolyze cellulose) (Fig. 1a) were obtained by direct plating of the cell suspensions from enrichment cultures on agar medium (pH 4.5) with crystalline cellulose. Two strains of mycelial actinobacteria, ACTY and ACTR, which formed the largest and clearest zones around their colonies, were selected for subsequent identification and analysis of their cellulolytic activity. On starch- and glucose-containing media, strain ACTY produced colonies 2–20 mm in diameter, with white aerial and brownish-yellow substrate mycelia. Strain ACTR produced colonies up to 10 mm in diameter; the color of the aerial mycelium varied from yellowish to brown; the substrate mycelium was red, and the pigment diffused into the agar. On cellulose-containing media, both strains formed colonies 2–5 mm in diameter. The isolates grew within a temperature range from 3 to 32°C and in a pH range of 4.0–7.8. The morphology of the mycelium, the presence of the substrate and aerial mycelium with branching hyphae (0.8–1.0 μm in diameter), as well as the presence of nonmotile exospores arranged as chains of sporangia, suggested that these cultures belong to the genus *Streptomyces*.

Phylogenetic analysis. To determine the phylogenetic position of strains ACTY and ACTR, analysis of their 16S rRNA genes was carried out. Comparison of the obtained sequences with the sequences in the GenBank database confirmed that the isolates belong to the

Actinobacteria and revealed a high similarity between their 16S rRNA gene sequences and those of representatives of the genus *Streptomyces*. Analysis of the 16S rRNA gene sequences demonstrated that strain ACTY is closely related to *Str. venezuelae* (GenBank accession number AB045890) [15] (97% similarity), whereas strain ACTR was found to be close to *Str. purpurascens* (GenBank accession number AB045888) [16] (99% similarity).

Determination of the capacity for dinitrogen fixation. Removal of the sources of available nitrogen from the liquid CM1 medium did not inhibit the growth of the peat-inhabiting streptomycetes ACTY and ACTR. Their exospores germinated into vegetative cells and produced mycelium and microcolonies. The cultures survived multiple transfers onto nitrogen-free medium, even when grown with cellulose as the sole carbon and energy source. Since growth on nitrogen-free media is not typical of the members of the genus *Streptomyces*, we tested the new isolates for acetylene reduction activity and for the presence of the *nifH* gene in their DNAs.

The tests for acetylene reduction activity were positive. The rates were 0.17 and 2.03 mmol C_2H_2 mg^{-1} protein h^{-1} (for strains ACTY and ACTR, respectively) (table). This activity is high enough and can be compared to that of the nitrogen-fixing bacteria of the genus *Azospirillum*, which were previously isolated from *Sphagnum* peat bogs [17]. Despite these results, PCR amplification with the *nifH*-specific primers described in [12, 13] yielded amplicons of the expected length only in the positive control with DNA from the nitrogen-fixing methanotrophic bacterium *Methylocystis echinoides* IMET 10491^T, but not in the case when the DNA of strains ACTY and ACTR was used. The application of an alternative primer set used for amplification of the *nifH* gene in some actinobacteria [14] gave no results as well.

Cellulose degradation. The process of cellulose degradation by the strains isolated from *Sphagnum* peat bogs was monitored for 18 days. During this time, the development of mycelium and its hydrolytic activity caused aggregation of crystalline cellulose and a reduction in its volume. The results of microscopic examination confirmed disintegration of the microcrystalline structure of cellulose in the areas of the mycelium development (Fig. 1b).

Figure 2 shows the dynamics of CO_2 accumulation in the gas phase of the experimental vials with the culture of strain ACTY in a liquid cellulose-containing medium in the absence of available nitrogen source. It was a linear process and depended on the incubation temperature and the medium pH. The lowest value of CO_2 accumulation in the vials, (0.77 μg C-CO_2 ml^{-1} gas phase), was observed at 5°C and pH 4.5 (Fig. 2a), while the highest level of CO_2 accumulation (7.32 μg C-CO_2 ml^{-1} gas phase), at 25°C and pH 6.0 (Fig. 2b).

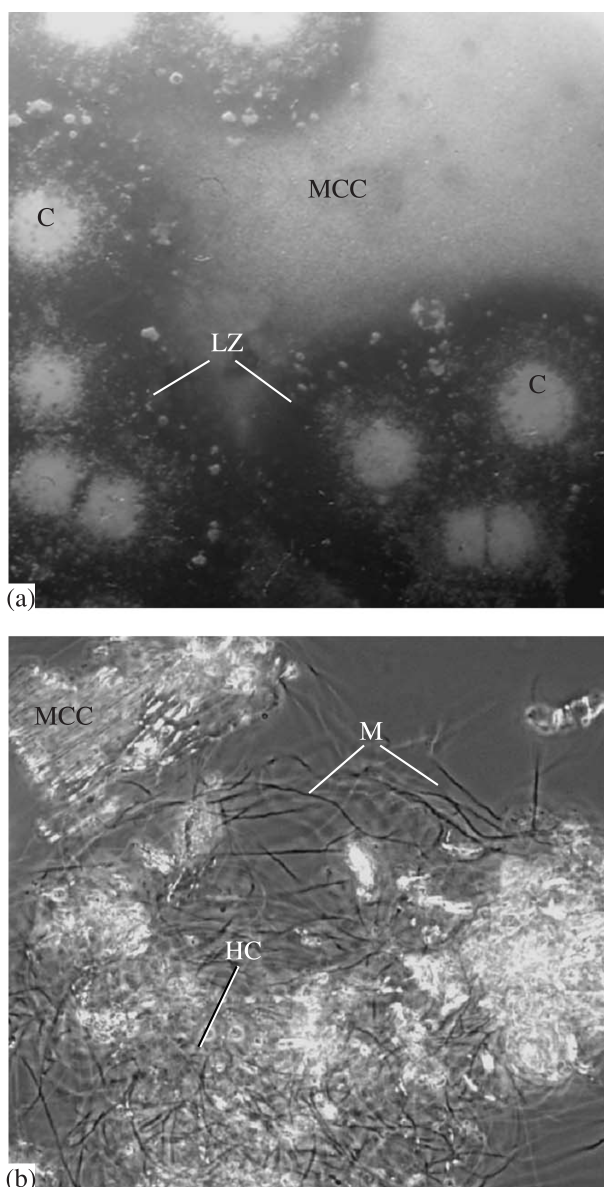

Fig. 1. Growth of *Streptomyces* sp. ACTY on crystalline cellulose: (a) hydrolysis zones formed around the colonies; (b) mycelium grown on crystalline cellulose and cellulose degradation. Designations: MCC, microcrystalline cellulose; LZ, lysed zone; C, colonies; M, bacterial mycelium; HC, hydrolized cellulose

The respiration rate (RR) of the studied cultures was used as a criterion to assess the rate of cellulose degradation. In the absence of a source of available nitrogen, the RR of both strains was proportional to the incubation temperature (table, Fig. 3). At an incubation temperature of 5°C, the respiration rate of strain ACTY was equally low at pH 4.5 and 6.0. At 15°C, the respiration rate at pH 6.0 was twice as high as that at pH 4.5, while at 25°C, this difference was fourfold. The activity of cellulose degradation by strain ACTR was much lower

Respiration rate and biomass yield of the isolates ACTY and ACTR grown on the nitrogen-free cellulose-containing medium depending on the incubation temperature and pH values

Strain	Respiration intensity (μg C-CO_2 ml^{-1} day^{-1}) / Biomass obtained after 18-day incubation (protein, μg ml^{-1})						Acetylene reduction activity, mmol C_2H_2 mg^{-1} protein h^{-1}
	pH 4.5			pH 6.0			
	5°C	15°C	25°C	5°C	15°C	25°C	25°C, pH 6.0
ACTY	0.05 ± 0.01 / 0.02 ± 0.01	0.42 ± 0.01 / 0.06 ± 0.01	0.47 ± 0.01 / 0.11 ± 0.01	0.06 ± 0.01 / 0.18 ± 0.01	1.02 ± 0.03 / 0.26 ± 0.02	2.02 ± 0.15 / 0.35 ± 0.03	0.17 ± 0.05
ACTR	0.00 / 0.16 ± 0.02	0.11 ± 0.02 / 0.19 ± 0.03	0.14 ± 0.01 / 0.31 ± 0.03	0.05 ± 0.01 / 0.12 ± 0.01	0.10 ± 0.01 / 0.21 ± 0.02	0.52 ± 0.02 / 0.28 ± 0.02	2.03 ± 0.08

than that by strain ACTY both at pH 4.5 and pH 6.0 (table, Fig. 3).

Similar to RR, the biomass yield of both strains grown on cellulose for 18 days was proportional to the incubation temperature and peaked at 25°C. Strain ACTY showed the maximum biomass accumulation (0.35 μg protein per 1 ml of medium) at pH 6.0. Strain ACTR was found to be more acid-tolerant; its biomass yield was 0.31 and 0.28 μg protein per 1 ml of medium at pH 4.5 and 6.0, respectively.

The presence of a source of available nitrogen in the cultivation medium stimulated cellulose degradation; however, for the cultures grown under acidic or semi-neutral conditions this effect was significantly different. Thus, this stimulating effect was insignificant at pH 4.5, while at pH 6.0, the rate of cellulose decomposition was 1.5–2.5 times higher in the presence of a source of available nitrogen (Fig. 3).

DISCUSSION

Numerous publications have reported the isolation of representatives of the genus *Streptomyces* from peat bogs [2, 18, 19]. Nutrient-rich media containing a source of available nitrogen were usually applied for the isolation of these microorganisms. However, the ability of the isolated cultures to grow and degrade biopolymers on nitrogen-deficient media, at low temperatures, and at pH values typical of their natural habitats has not been analyzed yet.

This study resulted in the isolation of two members of the phylogenetic group *Actinobacteria* from an acid-tolerant cellulolytic community of an ombrotrophic *Streptomyces* peat bog. The new isolates were identified as representatives of the genus *Streptomyces*. The isolated cultures grew on media with a low salt content (100 mg/l) at relatively low temperatures and pH, and were able to hydrolyze cellulose; these features characterize them as indigenous members of the hydrolytic communities of oligotrophic and acidic ecosystems. Hence, they were found to be convenient models for

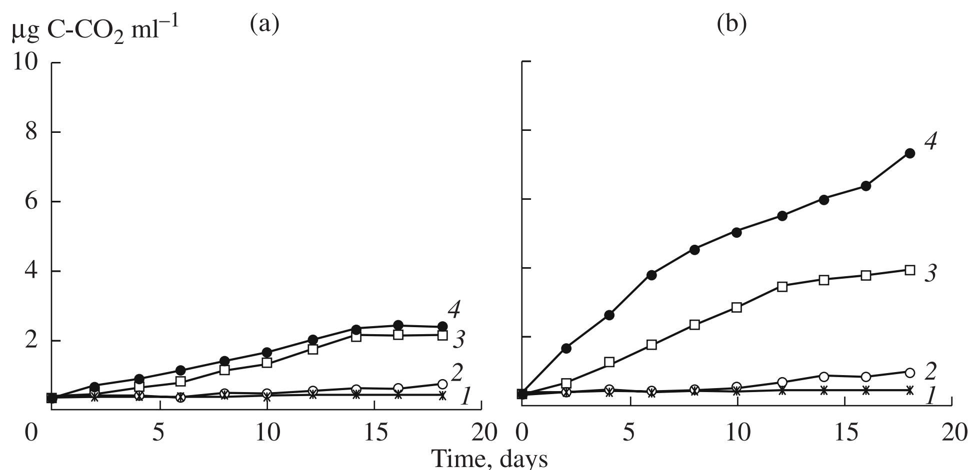

Fig. 2. Dynamics of CO_2 accumulation in the gas phase of the experimental vials during growth of *Streptomyces* sp. ACTY on nitrogen-free medium supplemented with crystalline cellulose: (a) pH 4.5; (b) pH 6.0; control (*1*); 5°C (*2*); 15°C (*3*); 25°C (*4*).

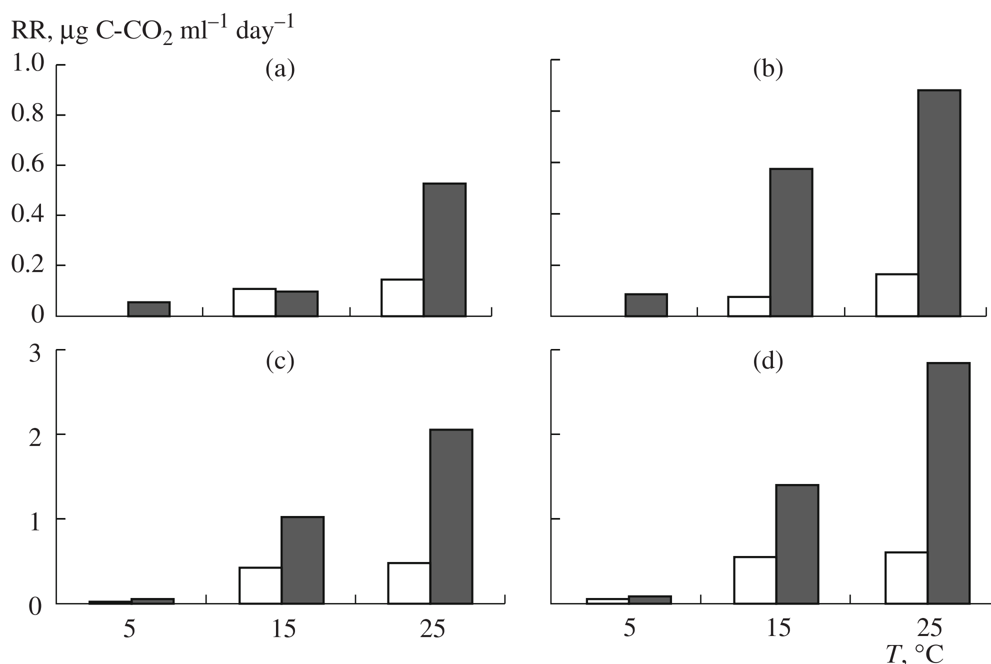

Fig. 3. Respiration rates of strains ACTR (a, b) and ACTY (c, d) grown on the medium with crystalline cellulose at various temperatures, pH values, and concentrations of available nitrogen in the media: pH 4.5 (a, b); pH 6.0 (c, d). White and black bars represent the presence of a nitrogen source (0.2 g NH_4NO_3 l^{-1}) or its absence, respectively.

studying the mechanisms regulating cellulose degradation in *Sphagnum* peat bogs.

The ability of the *Streptomyces* strains isolated from peatlands to grow on media without an available nitrogen source is a unique feature. Moreover, rather high rates of acetylene reduction in these bacteria were recorded. Data on the ability of members of the genus *Streptomyces* to fix dinitrogen are scarce; this characteristic is therefore not included in the descriptions of this genus [20]. It was previously demonstrated that the thermophilic streptomycete *Str. thermoautotrophicus* UBT1 is able to assimilate N_2 [21]; however, in this organism, the process of nitrogen reduction (in which an unusual, oxygen-dependent nitrogenase is involved) is coupled to the oxidation of CO by molybdenum-containing CO-dehydrogenase. The acetylene reduction activity was not detected in this thermophilic organism. Thus, the results obtained expand the spectrum of physiological characteristics of bacteria belonging to the genus *Streptomyces* and demonstrate that cellulolytic peat-inhabiting streptomycetes do not depend on the presence of available nitrogen sources in their natural environment. It seems likely that existing primers were not suitable for amplification of the *nifH* gene from the DNA of strains ACTY and ACTR. This suggestion is supported by the absence of the *nifH* gene sequences of streptomycetes in the GenBank database. Further investigations are required to gain insight into the nature of nitrogenase and the presence of the structural genes encoding it in the DNA of novel isolates from peat bogs.

The data on microbial degradation of cellulose have demonstrated the ability of streptomycetes from wetlands to assimilate molecular nitrogen. This process occurred in the absence of a source of available nitrogen; the addition of mineral forms of nitrogen had a significant stimulatory effect only at pH 6.0. In acidic environments typical of the studied ecosystem, the availability of nitrogen was not of great importance. The rate of cellulose degradation is known to increase twofold in peat samples supplemented with nitrogen mineral salts [22]. However, these data do not contradict our results, since, in addition to nitrogen-fixing streptomycetes, other hydrolytic microorganisms, such as mycelial fungi (whose activity depends on the availability of nitrogen), were detected in the cellulolytic community of *Sphagnum* peat bogs.

The study of the effect of temperature on the cellulolytic activity of streptomycetes from bog ecosystems confirmed the previous observation that the production of hydrolytic enzymes by these organisms slows down at temperatures below optimum [23]. It is obvious that the *Streptomyces* strains isolated from peat samples are represented by psychroactive organisms with a growth optimum at 25°C; however, they are able to grow at or below 15°C (Fig. 3). For instance, the biomass yield of both studied cultures grown at 25°C was comparable to that of the mesophilic cellulolytic *Streptomyces* sp. AT7 isolated from soil [24]. However, unlike typical mesophilic organisms, streptomycetes from bog ecosystems grew and hydrolyzed cellulose at temperatures ranging from 5 to 15°C. Strain ACTY exhibited the highest

resistance to low temperatures (Fig. 3). Thus, temperature is an important, but not the sole factor determining the growth of the *Streptomyces* strains isolated on cellulose from peat bogs.

The acidity of the medium was the key factor controlling the growth and cellulolytic activity of the studied isolates. Despite the fact that strains ACTY and ACTR grew within a wide pH range (4.0–7.8), the pH optimum for their physiological and hydrolytic activity was 6.0; pH values lower than 4.5 significantly suppressed their cellulolytic activity.

Hence, this study has confirmed the suggestion that members of the phylum *Actinobacteria*, in particular streptomycetes, are capable of decomposing cellulose under acidic conditions, at low temperatures, and in the absence of available nitrogen in the medium. Temperature and pH are the main factors that control the hydrolytic activity of these organisms; therefore the process of cellulose degradation is significantly inhibited in cold, acidic, and oligotrophic *Sphagnum* peat bogs.

ACKNOWLEDGMENTS

This work was supported by the Russian Foundation for Basic Research, project no. 07-04-91561, as well as by the program of the Presidium of the Russian Academy of Sciences “Molecular and Cell Biology” and by the Russian Science Support Foundation.

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