



Patrycja Golińska, Hanna Dahm

Enzymatic activity of actinomycetes from the genus *Streptomyces* isolated from the bulk soil and rhizosphere of the *Pinus sylvestris*

Received: 29 March 2011; Accepted 16 May 2011

Abstract: The enzymatic activity (chitinolytic, proteolytic, pectolytic and cellulolytic) of twenty strains of *Streptomyces* isolated from soil and rhizosphere of Scots pine was analyzed. Most strains produced chitinases, catalyzing the degradation of chitin, the main component of fungal cell walls including fungi pathogenic for plants. This activity was about 4 times higher in the presence of *Fusarium oxysporum* than *Rhizoctonia solani* mycelium or chitin flakes. The number of the proteolytic strains was also significant. In general, rhizosphere and soil organisms synthesized comparable amounts of these enzymes. Over half of the analyzed *Streptomyces* strains produced pectolytic enzymes (polygalacturonase, pectin lyase and pectate lyase). This property was more common among rhizosphere than among soil strains. The *Streptomyces* strains also showed cellulolytic activity (endocellulases, exocellulases) – enzymes decomposing basic components of cell walls of plant and lower fungi (cellulose). The cellulolytic activity was differentiated and depended on the *Streptomyces* strain. Conclusion of our studies is that *Streptomyces* are the microorganisms more chitinolytic and proteolytic than pectolytic and cellulolytic.

Additional key words: Forest soil *Streptomyces*, chitinases, proteases, pectinases, cellulases

Address: P. Golińska, H. Dahm, Department of Microbiology, Nicolaus Copernicus University, Institute of General and Molecular Biology, Gagarina 9, 87-100 Toruń, Poland, e-mail address: golinska@umk.pl

Introduction

Actinomycetes inhabiting forest soils are still poorly known in comparison with actinomycetes of cultivated soils. Most of these microorganisms (approx. 90%) belong to the genus *Streptomyces*. These microorganisms are numerous in this environment and participate in the decomposition of various complex compounds (chitin, pectins, cellulose) using them as carbon and energy sources.

Cellulose and pectins are compounds commonly occurring in the natural habitat in considerable amounts as they are the main components of plant wall. Therefore enzymes degrading these components

(pectinases, cellulases) play an important role in the mineralization of these organic materials and in competition of microorganisms for nutrients. Another important problem is the participation of cellulases and pectinases in plant pathogenesis. Microorganisms which produce enzymes may be potential causes of plant diseases (Gołębiowska 1975; Dahm et al. 1987; Dahm and Strzelczyk 1995; Redlak et al. 2001).

Chitinolytic microorganisms on the other hand may play the role of biological plant protection factors, hydrolyzing the cell wall of most fungi, which are the main cause of plant diseases. A similar role may be played by cellulases degrading cellulose in the cell wall of some lower fungi (Mahadevan and Craw-

ford 1997; Manucharova 2004; Trejo-Estrada et al. 1998).

Proteinases also participate in the mineralization of organic substances of plant and animal origin. Proteins are the main nitrogen component of plant and animal remains and organic fertilizers leaching into the soil. The protein is also contained in cells of soil microorganisms and in metabolites released by them (Rodziewicz and Sobieszczanski 1988; Januszek 1999). Proteinases may participate in the degradation of live cells, including fungi pathogenic for plants, interacting with chitinases and cellulases.

The aim of the present work was to analyze the ability of *Streptomyces* isolated from the soil and rhizosphere of forest trees to produce some enzymes participating in the mineralization of high molecular weight compounds of great importance in this habitat.

It was also decided to stress the significance of some enzymes (mainly chitinases) in the biological protection of trees from fungal pathogens.

Materials and Methods

The analyzed actinomycetes from the genus *Streptomyces* were isolated from root-free soil and the rhizosphere of the Scots pine (*Pinus sylvestris* L.) (Lat 53°01'46"; Long 18°42'24"). The trees grew on sandy soils; C:N = 24; pH = 4.1.

Chitinolytic activity (EC 3.2.1.14)

To study chitinolytic enzyme production, streptomycetes strains were grown in Petri dishes containing medium with colloidal chitin according to Chen and Lee (1994). After 14 days of incubation at 26°C width of the zone of chitin hydrolysis was measured.

Chitinolytic activity was analyzed on N medium according to Nawani and Kapadnis (2004) with chitin flakes (Sigma) (2 g/l) and *Rhizoctonia solani* or *Fusarium oxysporum* mycelium (2.5 g dry mass/l) as chitinase inducers.

Fusarium oxysporum and *Rhizoctonia solani* mycelium – as a chitin source was grown on Czapek – Dox medium (Johnson et al., 1960). 200 ml medium were inoculated with 1 ml wash obtained from a 10-day fungal culture on slants with Potato Dextrose Agar (Difco). The thus prepared Erlenmeyer flasks were placed on a shaker for a period of 5 days for *F. oxysporum* and 12–14 days for *R. solani*. The cultures were centrifuged (3000 rpm for 20 minutes). The supernatant was removed and the mycelium was washed 2 times with distilled water. The thus obtained mycelium was transferred to a small amount of water (50–100 ml) and homogenized. The mycelial suspension was poured into 50 ml flasks and sterilized in an autoclave (121°C, 1 atm). The dry mass of mycelium in 1 ml suspension was determined.

Erlenmeyer flasks with N medium were inoculated with actinomycetes using a wire loop. The cultures were grown in a shaker for 7 days at 26°C. After incubation the cultures were centrifuged in a centrifuge with cooling at 4°C for 15 minutes at 8000 rpm. In the obtained post-culture liquid the chitinolytic activity of actinomycetes was determined.

The reaction mixture contained: 1 ml post-culture liquid, 1 ml 1% colloidal chitin in 0.05 M acetate buffer pH 5.5. Chitinolytic activity was determined by measuring reducing sugars by the method of Miller (1959). The enzymatic reaction was performed for 3 hours at 37°C. The produced N-acetyl-D-glucosamine was assayed in a spectrophotometer (UV-1202, SHIMADZU) at 530 nm. The amount of enzyme which under the experimental conditions released 1 μmol N-acetyl-D-glucosamine per 1 minute from the substrate was taken as the unit of activity.

Proteolytic activity

Studying proteolytic enzyme production the isolates were grown in Petri dishes with medium according to Rodina (1968) – modified (8-fold lower concentration of gelatin). After 7 days of incubation at 26°C the Petri dishes were flooded with Frazier's reagent for 5 minutes. The width of zone of protein hydrolysis was measured.

The activity of acid (EC. 3.4.23) and neutral (EC 3.4.24) proteinases was determined according to Hazen (1965). Microorganisms were grown for 7 days in liquid medium according to Nascimento et al. (2005), using 5 g/l yeast extract as a substrate.

The reaction mixture were 750 μl post-culture liquid and 750 μl 2% azocasein (Sulfanilamide Azocasein, Sigma) in 0.2 M acetate buffer pH 4.5 (for acid proteinases) or 750 μl 2% azocasein (Sulfanilamide Azocasein, Sigma) in 0.2 M Tris-HCl buffer pH 7.5 (for neutral proteinases).

The reaction mixes were placed in a water bath at 37°C for 4 hours of incubation. The reaction was stopped by adding 1.6 ml 7% perchloric acid (HClO₄). Then the samples were centrifuged at 15,000 rpm for 10 minutes at 4°C. 3 ml of the supernatant were added to 450 μl 10 N NaOH. The proteolytic activity was determined spectrophotometrically at 440 nm.

The amount of enzyme which under the experimental conditions released 1 μmol tyrosine per 1 minute from the substrate was taken as the unit of acid and neutral proteinase activity.

Pectolytic activity

In order to determine pectolytic activity actinomycetes were grown in Minimal medium, Beg et al. (2000).

100 ml medium were inoculated with actinomycetes using a wire loop. The cultures were grown for 14 days at 26°C. After this time the cultures were cen-

trifuged at 15000 rpm for 10 minutes at 4°C, and subsequently pectolytic activity was determined for 10 strains which had shown pectolytic activity in qualitative analysis. In preliminary studies on pectolytic enzyme production, strains were grown in Petri dishes with medium according to Strzelczyk and Szpotański (1989). After 7 days of incubation at 26°C the Petri dishes were flooded with 1% aqueous solution of “Cetrimide” (alkyltrimethylammonium bromide) and the width of zone of pectin hydrolysis was measured.

Polygalacturonase (EC 3.2.1.15; PG), pectate lyase (EC 3.1.1.11; PGL) and pectin lyase (EC 4.2.2.10; PL) – were determined by the thiobarbiturate method (TBA) of Sherwood (1969 cf. Johansson 1988).

The reaction mixture contained 2 ml post-culture liquid and 2 ml of 1% citrus pectin solution in 0.05 M Tris-HCl buffer pH 8.0 (for PL), 2 ml of 1% sodium polypectate solution in 0.05 M acetate buffer pH 5.0 (for PG) or 2 ml of 1% sodium polypectate solution in 0.05 M Tris-HCl buffer pH 8.0 (for PGL) and also 0.5 ml 0.01 M CaCl₂ (for PL and PGL).

After 3h incubation at 30°C the reaction was stopped by adding 0.3 ml 9% ZnSO₄ × 7H₂O and 0.3 ml 0.5 M NaOH. The samples were centrifuged at 14000 rpm for 10 minutes. Next 1 ml supernatant was added to a mixture of 0.6 ml 0.04 M thiobarbituric acid with 0.1 ml distilled H₂O. Finally 0.3 ml 1 M HCl was added.

The samples were incubated in a boiling water bath for 30 minutes. After cooling measurements were performed in a Shimadzu UV VIS-1202 spectrophotometer at 515 nm for PG and 548 nm for PL and PGL.

The amount of enzyme which under the experimental conditions released 1 μmol galacturonic acid per 1 minute from the substrate was taken as the unit of pectolytic enzyme activity.

Cellulolytic activity

In studies on cellulolytic enzyme production the isolates were grown in Petri dishes with medium according to Berg and Pettersson (1977). After 7 days of incubation the Petri dishes were flooded with 0.1% solution of Congo red for 15 minutes and the next 1 M NaCl for 15 minutes. The width of zone of carboxymethylcellulose hydrolysis was measured.

Analysis of cellulolytic activity was performed using a method according to Cai et al. (1999).

Actinomycetes strains were grown in liquid medium according to Grigorevski de Lima et al. (2005), enriched in carboxymethylcellulose (CMC, Sigma), and modified by the addition of yeast extract. The yeast extract content was decreased from 0.3% to 0.1%. 50 ml of medium was inoculated with actinomycetes (using a wire loop). The cultures were grown

at 26°C for 9 days. After this time the cultures were centrifuged at 15000 rpm for 10 minutes at 4°C, and then the activity of cellulolytic enzymes was measured in the supernatant.

Endo-β-1,4-glucanase (EC 3.2.1.4) and exo-β-1,4-glucanase (EC 3.2.1.91)

The reaction mixture contained 0.5 ml post-culture supernatant and 1.7 ml 0.01 M acetate buffer pH 5.5 and 0.8 ml 2% carboxymethylcellulose in 0.01 M acetate buffer pH 5.5 (for endo-β-1,4-glucanases) or 1% Awicel (microcrystalline cellulose 0.1 mm, Serva) (for exo-β-1,4-glucanases).

The samples were incubated in a water bath at 50°C for 50 minutes. The enzymatic reaction was stopped by placing the tubes in a boiling water bath for 10 minutes. In order to deproteinize the samples 1 ml of the incubation mix (1 ml 1% ZnSO₄, 1 ml 0.06 M Ba(OH)₂) was added to each test tube. The test tube contents were mixed and centrifuged.

Reducing sugars were determined colorimetrically by the method of Somogyi and Nelson (1944, cf. Deng and Tabatabai 1994).

Table 1. Actinomycete strains isolated from root-free soil and the rhizosphere of the Scots pine (*Pinus sylvestris* L.) used in the experiment

Strain symbol	Species
SG1	<i>Streptomyces griseoviridis</i>
SG2	<i>Streptomyces cyaneus</i>
SG3	<i>Streptoverticillium olivovertici*</i>
SG4	<i>Streptomyces cyaneus</i>
SG5	<i>Streptomyces varsoviensis</i>
SG6	<i>Streptomyces griseoviridis</i>
SG7	<i>Streptomyces exfoliatus</i>
SG8	<i>Streptomyces griseoviridis</i>
SG9	<i>Streptomyces cyaneus</i>
SG10	<i>Streptomyces chromogenus</i>
SR1	<i>Streptomyces exfoliatus</i>
SR2	<i>Streptomyces xanthochromogenes</i>
SR3	<i>Streptomyces phaeochromogenes</i>
SR4	<i>Streptomyces anulatus</i>
SR5	<i>Streptomyces exfoliatus</i>
SR6	<i>Streptoverticillium olivovertici*</i>
SR7	<i>Streptomyces griseoviridis</i>
SR8	<i>Streptomyces exfoliatus</i>
SR9	<i>Streptomyces exfoliatus</i>
SR10	<i>Streptomyces exfoliatus</i>

SG – bulk soil of Scots pine; SR – rhizosphere of Scots pine

*the genus *Streptoverticillium* is currently included in the genus *Streptomyces*

The analyzed strains were isolated and assigned to genus on the basis of 16S rRNA sequence analysis and species according to methods proposed by Shirling and Gottlieb (1966), Kutzner (1981) and Williams et al. (1983) by Golińska (2008, unpublished data).

The liberated reducing sugars were measured in a Shimadzu spectrophotometer UV VIS-1202 at 520 nm.

The amount of enzyme which under the experimental conditions released 1 μmol glucose per 1 minute from the substrate was taken as the unit of endo- and exoglucanase activity.

β -glucosidase (EC 3.2.1.21)

β -glucosidase activity was determined in homogenates of actinomycete mycelium. For this purpose actinomycete cultures were centrifuged three times in a centrifuge with cooling (4°C), washed two times with 20 ml of distilled H₂O and once with 20 ml of 0.01 M acetate buffer pH 5.5. Then the pseudo-mycelium was homogenized in a glass homogenizer in the presence of 30 ml of 0.01 M acetate buffer pH 5.5 and added to 5 ml 0.01 M acetate buffer pH 5.5. The obtained extract was centrifuged at 15000 rpm for 10 minutes at 4°C.

To 50 μl supernatant 0.9 ml of 0.01 M acetate buffer pH 5.5 and 50 μl of 40 mM p-nitrophenyl β -D-glucopyranoside (Sigma) were added.

The mixture was incubated at 40°C for 30 minutes. The reaction was stopped by adding 3 ml of 1 M Na₂CO₃ to the test tubes.

The presence of p-nitrophenol was measured in a Shimadzu UV VIS-1202 spectrophotometer at 400 nm. The amount of enzyme which under the experimental conditions released 1 μmol of p-nitrophenol per 1 minute from the substrate was taken as a unit of β -glucosidase activity.

Statistical analysis

Data were analyzed using analysis of variance in the Statistica 5.1. package (StatSoft). Significance of differences between means were evaluated using Newman-Keuls multiple range test and Student's t-test ($p=0.05$).

Results

Chitinolytic activity was found in strains of *Streptomyces* isolated both from root-free soil and from the rhizosphere. In both groups of *Streptomyces*, strains showing high chitinolytic activity (10–20 units) as well as those showing low activity (approx. 1 unit) were found. One of the analyzed strains (SG9) was not chitinolytic at all (independently on the chitin source). The most active strains belonged mainly to the species *Streptomyces exfoliatus*, and the least active ones to the species *S. cyaneus*. Some strains of *S. exfoliatus* were also characterized by a very low activity. This activity may thus be considered as the property of a strain (Fig. 1).

The type of enzymatic substrate also affected the chitinolytic activity of the analyzed actinomycetes. The highest activity was found in the presence of *Fusarium oxysporum* mycelium. This activity was about 4 times higher than in the presence of *Rhizoctonia solani* mycelium or chitin flakes (Table 2).

Almost all studied actinomycetes (with the exception of three) produced proteolytic enzymes. Depending on the strain, this activity was from about 10 units to 40 units (neutral proteinases) and from about 1 unit to 5 units (acid proteinases). Among the most proteolytic active strains were the species: *Streptomyces anulatus*, *S. phaeochromogenes*, *S. xanthochromo-*

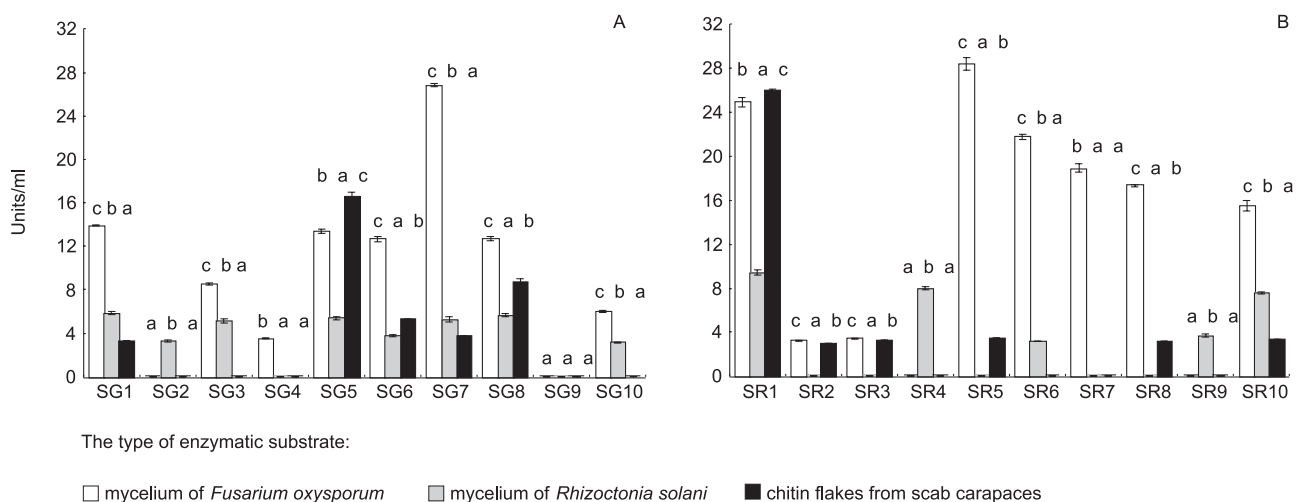


Fig. 1. Total chitinolytic activity of *Streptomyces* sp. strains isolated from bulk soil and rhizosphere of Scots pine (U/ml – μmol N-acetyl-D-glucosamine/ml per 1 minute) – average values ($n=6$) \pm standard deviations SG1-10 *Streptomyces* sp. strains isolated from bulk soil of Scots pine; SR1-10 *Streptomyces* sp. strains isolated from rhizosphere of Scots pine (Table 1) values marked with the same letter do not differ significantly ($p \leq 0.05$; Newman-Keuls multiple range test)

Table 2. Two-factor analysis of variance (ANOVA), comparing the effect of strains and chitin sources on chitinolytic activity (U/ml – μmol N-acetyl-D-glucosamine/ml per 1 minute) of *Streptomyces* sp. strains isolated from bulk soil and rhizosphere of Scots pine

Source of variation	Variance	Df (degrees of freedom)	F parametr	p (significance level)
1. strains	448.13	19	2597.31	0,0000*
2. chitin sources	2453.33	1	14219.11	0,0000*
Interaction	198.85	38	1152.53	0,0000*
Error	0,1725	300		

Newman-Keuls multiple range test ($p \leq 0,05$)

1. strains				
SG1	SG2	SG3	SG4	SG5
7.6811 i	1.0963 b	4.5482 f	1.1859 b	11.7749 l
SG6	SG7	SG8	SG9	SG10
7.2561 h	12.0251 l	9.0577 j	0 a	3.0591 e
SR1	SR2	SR3	SR4	SR5
20.1122 m	2.0769 c	2.2342 c	2.6707 g	10.6239 k
SR6	SR7	SR8	SR9	SR10
8.3186 j	6.3056 g	6.8579 g	1.2373 b	8.8250 j

2. chitin sources	
<i>Fusarium oxysporum</i>	<i>Rhizoctonia solani</i>
11.5601 c	3.4860 a
	chitin flakes
	3.9960 b

SG1-10 *Streptomyces* sp. strains isolated from bulk soil of Scots pine; SR1-10 *Streptomyces* sp. strains isolated from rhizosphere of Scots pine (Table 1)

*indicate the experimental factors, which affected significantly the chitinase activity

**mean values marked with the same letter do not differ significantly ($p \leq 0.05$)

genes (rhizosphere) and *S. olivovertici* and *S. cyaneus* (soil). One strain among the analyzed ones (*S. exfoliatus*) did not produce any proteinases.

Investigations of proteolytic activity showed statistically significant differences between the analyzed strains of *Streptomyces*. However, in general rhizosphere and soil organisms synthesized comparable amounts of these enzymes (Fig. 2).

Over half of the analyzed *Streptomyces* strains produced pectolytic enzymes. This property was more

common among rhizosphere strains than among soil strains. The enzyme produced by actinomycetes the most commonly and in the greatest amounts was pectate lyase (PGL) (1.4 units), and the least commonly and in the smallest amounts polygalacturonase (max approx. 0.03 units). However, some strains synthesized all three analyzed pectolytic enzymes (PG, PL, PGL) and they belonged to different species: *Streptomyces exfoliatus* (the most active), *S. olivovertici*, *S. cyaneus*.

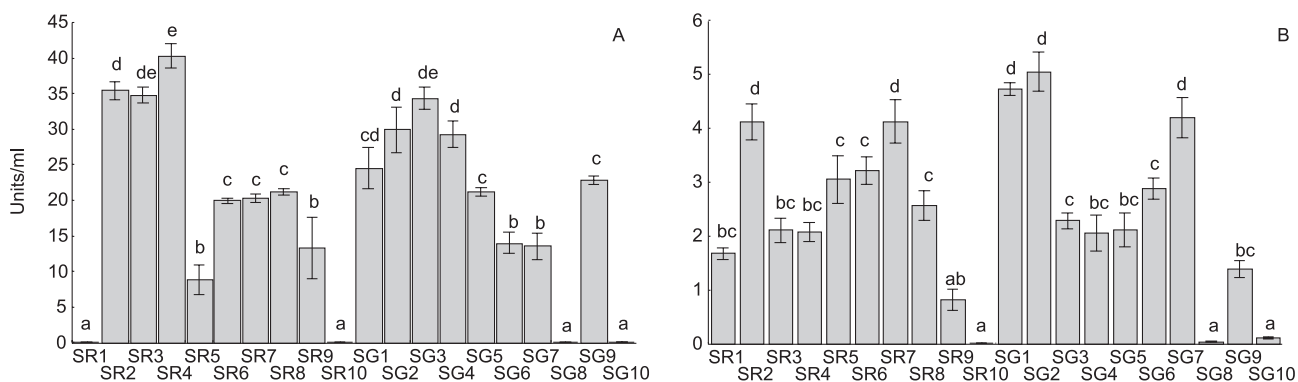


Fig. 2. Total proteolytic activity (U/ml – μmol tyrosine/ml per 1 minute) of neutral (A) and acid (B) proteinases from *Streptomyces* sp. strains isolated from bulk soil and rhizosphere of Scots pine – average values ($n=8$) \pm standard deviations

SG1-10 *Streptomyces* sp. strains isolated from bulk soil of Scots pine; SR1-10 *Streptomyces* sp. strains isolated from rhizosphere of Scots pine (Table 1)

Values marked with the same letter do not differ significantly ($p \leq 0.05$; Newman-Keuls multiple range test).

Half of the analyzed strains (both from the soil and from the rhizosphere) produced exo- and endocellulases (Table 3), but none of the strains synthesized β -glucosidase.

However, the cellulolytic activity of these microorganisms was low, from 0.011–0.1539 units/ml for exocellulases and 0.0072–0.1280 units/ml for endocellulases. Some strains produced both exocellulases as well as endocellulases, some, however, produced only one of these enzymes. The highest cellulolytic activity was found in the rhizosphere strain *Streptomyces exfoliatus* and the soil strain *S. varsoviensis* (Table 3).

Summing up the investigations on the enzymatic activity of the analyzed *Streptomyces* it can be stated that these organisms were characterized by a higher chitinolytic and proteolytic activity than a pectolytic and cellulolytic one. They were cellulolytic to a very small degree.

Discussion

So far the ability of actinomycetes to form pharmaceutical compounds used in medicine and veterinary medicine has been the focus of investigations. Studies on the physiology and enzymatic activity are less numerous, however some of the enzymes had a great ecological importance (Mehta et al. 2006).

Chitinases and proteases produced by actinomycetes, are considered as factors which may participate in the biological control of plant pathogens, mainly fungal pathogens (Ordentlich et al. 1988, cf. Strzelczyk et al. 1990).

The analysis of chitinolytic, cellulolytic and proteolytic activities in actinomycetes performed by us also concerned these aspects.

Chitinases are a group of complex chitinolytic enzyme systems capable of degrading chitin to its oligomers (chito oligosaccharides) and/or nanomers (N-acetylglucosamine) directly. Chitinolytic enzymes have been identified in various streptomyces including *Streptomyces plicatus* (Robbins et al. 1988), *S. lividans* (Miyashita et al. 1995), *S. viridificans* (Gupta et al. 1995).

Many factors affect enzymatic activity, the most important ones being temperature, pH and the type of enzymatic substrate. For this reason numerous scientists take such factors into consideration in their experiments. Similarly in our experiments the chitinolytic activity was analyzed in the presence of such substrates as colloidal chitin, chitin flakes of crab carapaces, mycelium or by enriching the chitin substrates with a small amount of glucose.

Only a few species of the genus *Streptomyces* are believed to be able to utilize cellulose, but many can use

Table 3. Total cellulolytic activity (U/ml – μ mol glucose/ml per 1 minute) *Streptomyces* sp. strains isolated from bulk soil and rhizoplane of Scots pine – average values (n=8) \pm standard deviations

Strain	Species	Exocellulases U/ml	Endocellulases U/ml
SR1	<i>S. exfoliatus</i>	0.0064 \pm 0.0063 a	0 a
SR2	<i>S. xanthochromogenes</i>	0 a	0 a
SR3	<i>S. phaeochromogenes</i>	0.0245 \pm 0.0337 ab	0.0077 \pm 0.0108 a
SR4	<i>S. anulatus</i>	0 a	0.1128 \pm 0.0887 b
SR5	<i>S. exfoliatus</i>	0.1539 \pm 0.0056 d	0.1280 \pm 0.0294 b
SR6	<i>St. olivovertici</i>	0 a	0 a
SR7	<i>S. griseoviridis</i>	0.016000 \pm 0.006746 ab	0.0363 \pm 0.0365 a
SR8	<i>S. exfoliatus</i>	0.0099 \pm 0.0016 ab	0.0072 \pm 0.0065 a
SR9	<i>S. exfoliatus</i>	0 a	0 a
SR10	<i>S. exfoliatus</i>	0 a	0 a
SG1	<i>S. griseoviridis</i>	0.0501 \pm 0.0790 c	0 a
SG2	<i>S. cyaneus</i>	0 a	0 a
SG3	<i>St. olivovertici</i>	0 a	0 a
SG4	<i>S. cyaneus</i>	0.0285 \pm 0.0191 b	0.0909 \pm 0.0418 b
SG5	<i>S. varsoviensis</i>	0.1509 \pm 0.0120 d	0.1096 \pm 0.0554 b
SG6	<i>S. griseoviridis</i>	0 a	0.0227 \pm 0.0093 a
SG7	<i>S. exfoliates</i>	0.0011 \pm 0.0011 a	0.0117 \pm 0.0149 a
SG8	<i>S. griseoviridis</i>	0 a	0.0109 \pm 0.0101 a
SG9	<i>S. cyaneus</i>	0 a	0 a
SG10	<i>S. chromogenus</i>	0 a	0 a

SG1-10 *Streptomyces* sp. strains isolated from bulk soil of Scots pine; SR1-10 *Streptomyces* sp. strains isolated from rhizosphere of Scots pine (Table 1)

Values marked with the same letter do not differ significantly ($p \leq 0.05$; Newman-Keuls multiple range test)

chitin. Chitin, which also contains nitrogen, may be both a valuable carbon source and a nitrogen source for these microorganisms. The *Streptomyces coelicolor* genome contains 8 genes encoding enzymes which degrade cellulose and as many as 13 responsible for chitin degradation (Hopwood 2007).

Detailed analysis of chitinase gene promoters in *Streptomyces* indicated that glucose as a preferred carbon source inhibits the expression of these genes, whereas chitin, presumably via a soluble breakdown products induced it (Hopwood 2007).

The highest chitinolytic activity was most commonly observed in the presence of *Fusarium oxysporum* mycelium and in the presence of colloidal chitin and glucose as a starter (Strzelczyk et al. 1990; Trejo-Estrada et al. 1998; Joo et al. 2005).

The higher chitinolytic activity observed by us in the presence of *F. oxysporum* mycelium than in the presence of *R. solani* mycelium may be due to a different meshing of chitin and the presence of other substances in the mycelium of both species.

The marine chitinase-producing *Streptomyces* sp. were isolated (El-Dein et al. 2010) on the selective medium containing colloidal chitin. Isolates forming the largest clear zone around the well developed colonies on the chitin agar plate was selected for further study. The strains produced up to 2.689 U/ml at 144 h incubation time. The secretion of chitinases was significantly influenced by nitrogen source incorporated into the growth medium.

Chitinases were highly increased in the presence of 6.9% chitin as a sole C and N source but inorganic N source $(\text{NH}_4)_2\text{SO}_4$ was not suitable for enzyme production (El-Dein et al. 2010).

In our studies the most active strain produced ca. ten times more chitinases, 28.38 U/ml per 1 minute.

The role of chitinolytic enzymes in antifungal activity and the capacity for biocontrol is underlined by many authors (Mahadevan and Crawford 1997). Such enzymes, besides antibiotics commonly produced by actinomycetes, increase the biocontrolling activity of these organisms. For biological control of diseases caused by *Phytophthora infestans*, *Streptomyces violaceusniger* (strain YCED-9) has been used, which shows a strong antagonism *in vitro* in respect to these fungal pathogens. This strain of *S. violaceusniger* may produce several compounds with an antibiotic character: AFA (with activity against *Fusarium*), a fungicidal polyene complex similar to guanidylfungin A or nigericin and geldanamycin, which strongly inhibit the growth of the *Pythium* and *Phytophthora* spp.

Our investigations on chitinolytic properties have shown that some strains isolated both from the rhizosphere of forest trees and bulk soil are characterized by a high chitinolytic activity and may play an active biocontrolling role in the forest environment.

Proteinases are the most common hydrolytic enzymes in nature. Localized in cells or secreted outside they play an important role both in the process of degradation of trophic proteins and in the changes in cellular proteins. Extracellular proteinases which depolymerize protein macromolecules play a significant role in interactions among organisms in natural habitats (Rodziewicz and Sobieszczanski 1988). The scientific literature indicates that actinomycetes are proteolytic organisms, whose activity depends on ma-

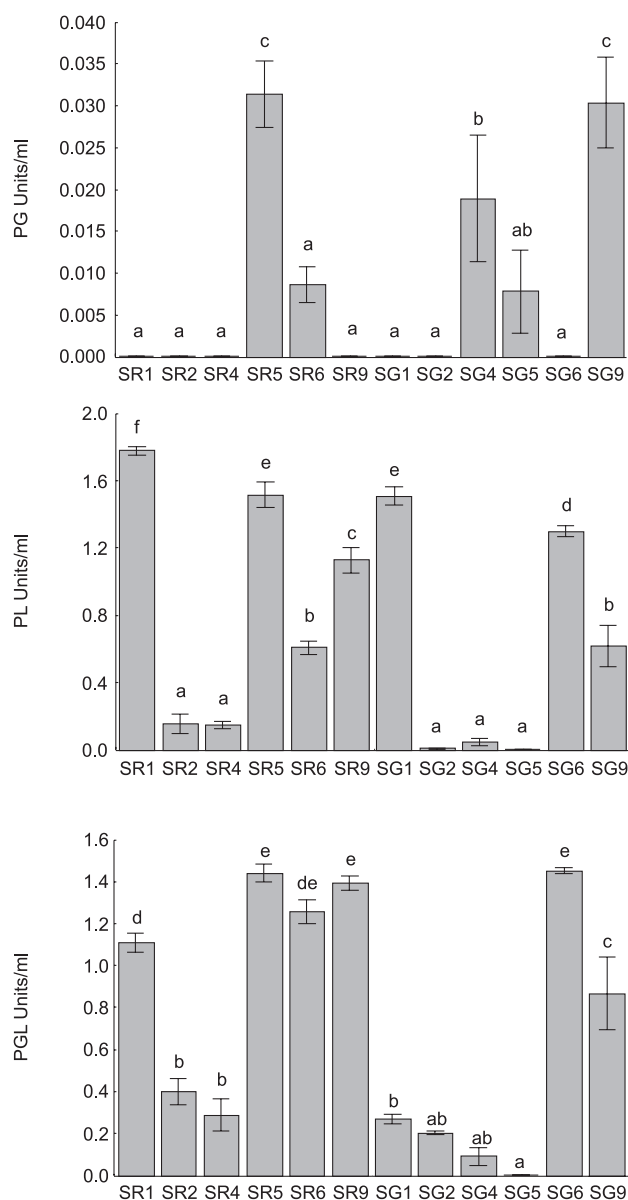


Fig. 3. Total pectolytic activity (polygalacturonase-PG; pectin lyase-PL; pectate lyase-PGL) of *Streptomyces* sp. strains isolated from bulk soil and rhizosphere of Scots pine (U/ml – μmol galacturonic acid/ml per 1 minute) – average values ($n=9$) \pm standard deviations

SG1-10 *Streptomyces* sp. strains isolated from bulk soil of Scots pine; SR1-10 *Streptomyces* sp. strains isolated from rhizosphere of Scots pine (Table1)

Values marked with the same letter do not differ significantly ($p \leq 0.05$; Newman-Keuls multiple range test)

ny environmental factors, mainly the temperature, pH and availability and type of nutrients, even though proteinases of microorganisms are believed to lack a distinct substrate specificity and are active over a broad range of pH (from pH 2-11) (Rodziewicz and Sobieszkański 1988; Strzelczyk et al. 1990; Kumar and Takagi 1999; Nascimento et al. 2005).

Our investigations have shown that even though most of the analyzed *Streptomyces* produced both neutral and acid proteinases, some were noted which were not proteolytic. Neutral proteinases showed a several times higher activity than acid ones, even though the actinomycetes were isolated from acid forest soils.

Strzelczyk et al. (1990) observed the ability to hydrolyze gelatin and sodium caseinate by all analyzed strains of *Streptomyces* spp. isolated from root-free soil, the rhizosphere and the mycorrhizosphere of the Scots pine. Enrichment of media in glucose and NH_4NO_3 caused a considerable stimulation of the proteolytic activity of soil strains and a strong inhibition of the activity of rhizosphere strains. In the conditions of our experiments proteolytic activity of the rhizosphere and soil actinomycetes was similar and this was a property of strains.

Apart from chitinases, cellulases are the enzymes most intensively studied in soil *Streptomyces*.

Cellulose and pectins are the main components of plant cell walls, therefore microorganisms with cellulolytic and pectolytic properties are important in natural environments, including forest ones, in mineralizing dead plant mass. Enzymes decomposing these linkages also are important in plant pathogenesis. Moreover, cellulases formed by soil microorganisms may also be a factor important for the biocontrol of fungal pathogens containing cellulose in their cell wall (*Phycomycetes*) (Jennings and Lysek 1996).

However, our investigations have shown a low cellulolytic activity of *Streptomyces*. Grigorevski de Lima et al. (2005) noted the activity of the cellulases in analysed actinomycetes in the presence of 1% CMC and 0.3% yeast extract. An increase in yeast extract concentration to 1% caused a decrease of this activity.

Enzymatic cellulose hydrolysis is a complicated process in which a complex of cellulolytic enzymes participate (endo-, exocellulases, β -glucosidases) (Wachinger et al. 1989, Bhat and Bhat 1997, Criquet 2002). The ability to synthesize β -glucosidase is the least common among bacteria.

None of the strains of actinomycetes analyzed by us produced this enzyme. Similarly Gierasimiuk and Strzelczyk (2003) analyzing cellulolytic activity of bacteria isolated from the bulk soil and rhizosphere of forest trees noted a trace activity of β -glucosidases. However, Pérez-Pons et al. (1995) have demonstrated β -glucosidase activity in *Streptomyces* strain QM-B814 in the presence of cellobiose.

Wachinger et al. (1989) noted formation of high levels of Avicellulase during growth of streptomycetes on citrate as the carbon source. This indicates that cellulolytic enzymes can be formed constitutively and the strong inhibition of enzyme production in the presence of glucose can be interpreted in terms of catabolite repression.

Pectolytic activity has been demonstrated in numerous bacteria and pathogenic, saprophytic and ectomycorrhizal fungi (Dahm and Strzelczyk 1995; Dahm et al. 1997; Redlak et al. 2001). Pectinases participate in the decomposition of dead organic plant matter and are important in interactions between microorganisms and living plants. They may participate both in pathogenesis and in establishment of symbiosis between micro- and macroorganisms.

The total decomposition of pectin compounds requires the activity of a complex of pectolytic enzymes. In general pectin lyases are synthesized mainly by fungi and pectate lyases by bacteria (Favela-Torres et al. 2006).

However, Gierasimiuk and Strzelczyk (2003) have demonstrated the activity of the pectinase complex (PG, PGL, PL) in the cultures of some strains of bacteria from the genus *Bacillus*. Similarly Dahm et al. (1987) noted the synthesis of pectolytic enzymes by actinomycetes, but their activity was low. Some researchers (Strzelczyk and Szpotański 1989) suggest that strains of rhizosphere actinomycetes are characterized by a higher pectolytic activity from those inhabiting root-free soil. Similarly in our investigations actinomycetes isolated from the pine rhizosphere were more pectolytic than those from bulk soil.

Acknowledgements

This research has been financed by a grant from Polish Ministry of Science and Higher Education (Grant No. 2 PO4 C 040 29)

References

- Beg Q.K., Bhushan B., Kapoor M., Honda G.S. 2000. Production and characterization of thermostable xylanase and pectinase from *Streptomyces* sp. QG-11-3. *Journal of Industrial Microbiology and Biotechnology* 24: 396–402.
- Berg B., Pettersson G. 1977. Location and formation of cellulases in *Trichoderma viride*. *Journal of Applied Bacteriology* 42: 65–75.
- Bhat M.K., Bhat S. 1997. Cellulose degrading enzymes and their potential industrial applications. *Biotechnology Advances* 15: 583–620.
- Cai Y.J., Chapman S.J., Buswell J.A., Chang S.-T. 1999. Production and distribution of endoglucanase, cellobiohydrolase and β -glucosidase com-

- ponents of the cellulolytic system of *Volvariella volvacea*, the edible straw mushroom. *Applied and Environmental Microbiology* 65: 553–559.
- Chen J.P., Lee M.S. 1994. Simultaneous production and partition of chitinase during growth of *Serratia marcescens* in an aqueous two-phase system. *Biotechnology Techniques* 8: 783–788.
- Criquet S. 2002. Measurement and characterization of cellulase activity in sclerophyllous forest litter. *Journal of Microbiological Methods* 50: 165–173.
- Dahm H., Strzelczyk E. 1995. Impact of vitamins on cellulolytic, pectolytic and proteolytic activity of mycorrhizal fungi. *Symbiosis* 18: 233–250.
- Dahm H., Strzelczyk E., Majewska L. 1987. Cellulolytic and pectolytic activity of mycorrhizal fungi, bacteria and actinomycetes associated with the roots of *Pinus sylvestris*. *Pedobiologia* 30: 73–80.
- Dahm H., Strzelczyk E., Mańka M. 1997. Production of cellulolytic and pectolytic enzymes by *Fusarium oxysporum* (Schlecht.), *Rhizoctonia solani* (Kühn) and *Trichoderma viride* (Pers. ex Gray). *Phytopathologia Polonica* 13: 19–30.
- Deng S.P., Tabatabai M.A. 1994. Cellulase activity of soils. *Soil Biology and Biochemistry* 26: 1347–1354.
- El-Dein A., Hosny M.S., El-Shayeb N.A., Abood A., Abdel-Fattah A.M. 2010. A potent chitinolytic activity of marine *Actinomycete* sp. and enzymatic production of chito oligosaccharides. *Australian Journal of Basic and Applied Sciences* 4: 615–623.
- Favela-Torres E., Volke-Sepúlveda T., Viniegra-González G. 2006. Production of hydrolytic depolymerising pectinases. *Food Technology and Biotechnology* 44: 221–227.
- Gierasimiuk J., Strzelczyk E. 2003. Cellulolytic and pectolytic activity of bacilli isolated from the root-free soil and the rhizosphere of different forest trees. *Folia Forestalia Polonica* 45: 15–26.
- Gołębiowska I. 1975. *Mikrobiologia rolnicza*. Warszawa: PWRiL
- Grigorevski-Lima A.L., Pires do Nascimento R., Pinto da Silva Bon E., Coelho R.R.R. 2005. *Streptomyces drozdowiczii* cellulase production using agro-industrial by-products and its potential use in the detergent and textile industries. *Enzyme and Microbial Technology* 37: 272–277. doi:10.1016/j.enzmictec.2005.03.016
- Gupta R., Saxena R.K., Chaturvedi P., Viridi J.S. 1995. Chitinase production by *Streptomyces viridificans*: its potential in fungal cell walls lysis. *Journal of Applied Bacteriology* 78: 378–383.
- Hazen G.G., Hause J.A., Hubicki J.A. 1965. An automated system for the quantitative determination of proteolytic enzymes using azocasein. *Annals of the New York Academy of Sciences* 130: 761–768.
- Hopwood D.A. 2007. *Streptomyces* in nature and medicine. The antibiotic makers. New York: Oxford University Press.
- Januszek K. 1999. Aktywność enzymatyczna wybranych gleb leśnych Polski południowej w świetle badań polowych i laboratoryjnych. *Zeszyty Naukowe AR. Kraków*.
- Jennings D.H., Lysek G. 1996. *Fungal Biology: understanding the fungal lifestyle*. London: Bios Scientific Publishers.
- Johansson M. 1988. Pectic enzyme activity of spruce and pine strains of *Heterobasidion annosum* (Fr.). *Bref. Physiological and Molecular Plant Pathology* 33: 333–349.
- Johnson L.F., Curl E.A., Bond J.H., Fribourg H.A. 1960. *Methods for studying soil microflora – plant disease relationship*. Minneapolis: Burgess Publishing Company.
- Joo G.J. 2005. Production of an anti-fungal substance for biological control of *Phytophthora capsici* causing phytophthora blight in red-peppers by *Streptomyces halstedii*. *Biotechnology Letters* 27: 201–205.
- Kumar C.G., Takagi H. 1999. Microbial alkaline proteases: from a bioindustrial viewpoint. *Biotechnology Advances* 17: 561–594.
- Kutzner H.J. 1981. The Family *Streptomycetaceae*, [In]: *The Prokaryotes. A Handbook on Habitats Isolation and Identification of Bacteria* (Eds. Starr M. P., Stolp H., Trüper H. G., Balows A., Schlegel H. G.). Springer-Verlag, 2028–2090.
- Mahadevan B., Crawford D.L. 1997. Properties of the chitinase of the antifungal biocontrol agent *Streptomyces lydicus* WYEC108. *Enzyme and Microbial Technology* 20: 489–493.
- Manucharova N.A., Belova E.V., Polyanskaya L.M., Zenova G.M. 2004. A chitinolytic *Actinomycete* complex in chernozem soil. *Microbiology* 73: 56–59.
- Mehta V.J., Thumar J.T., Singh S.P. 2006. Production of alkaline protease from an alkaliphilic actinomycete. *Bioresource Technology* 97: 1650–1654.
- Miller G.L. 1959. Use of dinitrosalicylic acid reagent for the determination of reducing sugar. *Analytical Chemistry* 31: 426–428.
- Miyashita K., Fujii T., Sawada Y. 1991. Molecular cloning and expression of a *Streptomyces lividans* 66. *Journal of General Microbiology* 137: 2065–2072.
- Nascimento R.P., d' Avila-Levy C.M., Souza R.F., Branquinha M.H., Bon E.P.S., Pereira-Jr N., Coelho R.R.R. 2005. Production and partial characterization of extracellular proteinases from *Streptomyces malaysiensis*, isolated from a Brazilian cerrado soil. *Archives of Microbiology* 184: 194–198.

- Nawani N.N., Kapadnis B.P. 2004. Production dynamics and characterization of chitinolytic system of *Streptomyces* sp. NK1057, a well equipped chitin degrader. *World Journal of Microbiology and Biotechnology* 20: 487–494.
- Pérez-Pons J.A., Rebordosa X., Querol E. 1995. Induction and preliminary characterization of intracellular β -glucosidases from a cellulolytic *Streptomyces* strain. *FEMS Microbiology Letters* 128: 235–239.
- Redlak K., Dahm H., Ciesielska A., Strzelczyk E. 2001. Enzymatic activity of ectendomycorrhizal fungi. *Biology and Fertility of Soils* 33: 83–90.
- Robbins P.W., Albright C., Benfield B. 1988. Cloning and expression of a *Streptomyces plicatus* chitinase (chitinase-63) in *Escherichia coli*. *The Journal of Biological Chemistry* 263: 443–447.
- Rodziewicz A., Sobieszczanski J. 1988. Pozakomórkowe proteiny drobnoustrojów. *Postępy Mikrobiologii* 27: 55–73.
- Shirling E.B., Gottlieb D. 1966. Methods for characterization of *Streptomyces* species. *International Journal of Systematic Bacteriology* 16: 313–340.
- Strzelczyk E., Gorlach K., Różycki H. 1990. Chitinolytic and proteolytic activity of *Streptomyces* isolated from root-free soil, rhizosphere and mycorrhizosphere of pine (*Pinus sylvestris* L.). *Biology and Fertility of Soils* 9: 268–272.
- Strzelczyk E., Szpotański T. 1989. Cellulolytic and pectolytic activity of *Streptomyces* isolated from root-free soil, rhizosphere and mycorrhizosphere of pine (*Pinus sylvestris* L.). *Biology and Fertility of Soils* 7: 365–369.
- Trejo-Estrada S.R., Paszczyński A., Crawford D.L. 1998. Antibiotics and enzymes produced by the biocontrol agent *Streptomyces violaceusniger* YCED-9. *Journal of Industrial Microbiology and Biotechnology* 21: 81–90.
- Wachinger G., Bronnenmeier K., Staudenbauer W.L., Schrempf H. 1989. Identification of mycelium-associated cellulose from *Streptomyces reticuli*. *Applied and Environmental Microbiology* 55: 2653–2657.
- Williams S.T., Goodfellow M., Alderson G., Wellington E.M.H., Sneath P.H.A., Sackin M.J. 1983. Numerical classification of *Streptomyces* and related genera. *Journal of General Microbiology* 129: 1743–1813.