

POLISH JOURNAL OF ECOLOGY (Pol. J. Ecol.)	57	2	229–238	2009
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*Regular research paper*

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## DEGRADATION OF CHITIN IN NATURAL ENVIRONMENT: ROLE OF ACTINOMYCETES

**ABSTRACT:** The actinomycetes in water samples and bottom sediments of lowland, eutrophic lake as well as in soil (farmland, sandy) of the lake basin were studied. Chitin-degrading actinomycetes were isolated (with a plate technique) from each habitat; subsequently, their chitinolytic activity (with the fluorometric method) was determined in relation to temperature (10–50°C) and the physical type of chitinous substance (colloidal chitin, chitin powder, and shrimp shells). This study demonstrated that actinomycetes were the most abundant in soil samples (average of  $18 \times 10^3$  CFU g<sup>-1</sup> in farmland soil,  $9 \times 10^3$  CFU g<sup>-1</sup> in sandy soil), and the least abundant in water samples (average of  $2.7 \times 10^1$  CFU mL<sup>-1</sup> in lake water at neutral pH,  $0.6 \times 10^1$  CFU mL<sup>-1</sup> in lake water with alkaline pH). The highest percentage of chitinolytic actinomycetes was observed in soil (average of 80% in sandy soil and 85% in farmland soil). Chitinolytic actinomycetes also made up a large fraction of total actinomycetes in water samples (average of 73%). In silt and sandy sediments, percentages of chitinolytic actinomycetes equaled 23 and 15%, respectively.

Actinomycetes collected in soil were characterized by the highest activity (average of 14 nmol MUF mg<sup>-1</sup> of protein h<sup>-1</sup> in farmland soil, 8.5 nmol MUF mg<sup>-1</sup> protein h<sup>-1</sup> in sandy soil). The lowest activity was observed among benthic actinomycetes (average of 5.4 nmol MUF mg<sup>-1</sup> of protein h<sup>-1</sup> in silt, 0.65 nmol MUF mg<sup>-1</sup> protein h<sup>-1</sup> in sandy sediments). The impact of temperature and the

type of chitinous substrate on the activity of chitinases produced by actinomycetes demonstrated that their activity peaked at 40°C and in the presence of colloidal chitin. Observed differences in actinomycetales number and activity in the lake and the soil may be explained by higher accumulation of chitin substances in the soil. This polymer allows microorganisms to continually synthesize chitinolytic enzymes and take active part in that compound decomposition.

**KEY WORDS:** actinomycetes, chitin, chitinases

### 1. INTRODUCTION

Chitin is one of the most common polysaccharides on Earth, and in addition to cellulose, is one of the most basic exoskeletal compounds, that is, homo-biopolymers of unbranched linear chains of β-D-glucosyl (Patil *et al.* 2000, Souza-Neto 2003). Degradation and recycling of chitin is an important component in maintaining the global cycle of carbon and nitrogen. These processes primarily include microbiological processes because chitin can be used by microorganisms as the only source of carbon (McCreath and Gooday 1992, Nielsen and Sørensen 1999).

Enzymatic hydrolysis of chitin to free N-acetylglucosamine (GlcNAc) is carried out by a system of chitinolytic enzymes. Functioning of this system is characterized by synergism and continuity (Patil *et al.* 2000), and requires combined action of at least two types of hydrolytic enzymes, endochitinases (EC 3.2.1.14) and chitobiasis (EC 3.2.1.29) (Wiwat *et al.* 1999) According to Nielsen and Sørensen (1999), chitinolytic activity is expressed through a synthesis of several types of enzymes, which hydrolyze  $\beta$ -1,4 bonds between N-acetylglucosamine radicals. Chitinases are produced by bacteria (Huang *et al.* 1996), actinomycetes (Mahadevan and Crawford 1997), fungi (Pinto *et al.* 1997), and also by yeast and plants (Bhushan and Hoondal 1998).

Microbiological degradation of chitin in natural environment is carried out by chitinolytic microorganisms. Our understanding of the role of bacteria in decomposition of chitin is much more complete than that of fungi and actinomycetes. According to Swiontek Brzezinska *et al.* (2008a) the participation of chitinolytic microorganisms in water and soil was greater than in bottom sediments. In lake water, chitinolytic bacteria on average constituted 13% of the total number heterotrophic bacteria, in soil – 18%, and in bottom sediments – only 5%. The share of fungi in decomposition of chitin was greater. In the water, they constituted no less than 50% all of fungi, in soil – 47%, and in bottom sediments – 42% all of fungi. Chitinolytic bacteria constitute on average 13% of total numbers of heterotrophic bacteria in the oligo-mesotrophic lake and 9% in the eutrophic lake (Swiontek Brzezinska and Donderski 2006). It is known that numer-

ous actinomycetes are capable of decomposing chitin, primarily in soil environment, but information regarding their chitinolytic activity levels and their role in breaking down chitin in aquatic environments is quite limited. Therefore, the purpose of this study was to determine chitinolytic activity and abundances of actinomycetes in water and bottom sediments of temperate, eutrophic lake typical for postglacial lowland region.

## 2. MATERIAL AND METHODS

For the microbiological surveys water and bottom sediments of alkaline reaction were sampled (in Mirakow, near farm tourism facility) as well as of neutral reaction (in the area of agricultural activity, where the arable lands directly border on the lake shoreline). All the sites are situated in the southeast part of the lake. The lake (Lake Chełmżyńskie) and its basin are situated in the southern part of the lakeland region in Central Poland. Morphometric parameters of the lake are presented in Table 1.

Water samples were obtained from the upper 5 cm thick water layer with an automatic pipettor PipeBoy (De Wille Biotechnology), while silt (neutral pH) and sand sediment (alkaline pH) samples were collected with a 5 cm  $\times$  0.75 m tube scoop. Sandy soils (alkaline pH) and farmland soils (neutral pH) were collected directly to sterile jars. All samples were placed on ice in an insulated container at  $\pm 7^\circ\text{C}$ , and were analyzed immediately after they arrived in the laboratory. The samples were collected seasonally in the spring (April 3, 2007), summer (August 21, 2007), and autumn (October 20, 2007). The pH of soil and bottom sediments was determined

Table 1. Morphometric and trophic characteristics of studied lake.

Area (ha) <sup>(1)</sup>	271.1
Maximal depth (m) <sup>(1)</sup>	27.1
Mean depth (m) <sup>(1)</sup>	6.0
pH <sup>(2)</sup>	7.3 – 8.7
TP (mg L <sup>-1</sup> ) <sup>(2)</sup>	0.05 – 0.07
TN (mg L <sup>-1</sup> ) <sup>(2)</sup>	1.0 – 1.98
Electrolytic conductivity ( $\mu\text{S cm}^{-1}$ ) <sup>(2)</sup>	601 – 703
Chlorophyll a ( $\mu\text{g L}^{-1}$ )	22.1 – 59.3

<sup>(1)</sup> data supplied by Provincial Inspectorate of Environmental Service in Bydgoszcz

<sup>(2)</sup> data supplied by Department of Environmental Microbiology and Biotechnology, Nicolaus Copernicus University (data for 0.5 m depth, spring, summer, autumn 2007)

with the potentiometric method (Bednarek *et al.* 2005).

The abundance (CFU – Colony Forming Units) of actinomycetes was determined with a plate technique by surface inoculation on medium containing colloidal chitin (Hsu and Lockwood 1975). After 14-day incubation at 25°C, the zone around the colonies was examined for lighter areas, which indicated capability of actinomycetes to decompose chitin. Colonies without a clear zone were counted among microorganisms that do not decompose chitin. All chitinolytic colonies were transferred to Hsu and Lockwood (1975) agar slants and used in further tests.

A total of 47 strains (13 from water, 14 from bottom sediments, and 20 from soil) were used to determine chitinolytic activity. Actinomycetes were cultured in Erlenmeyer flasks, which contained 50 cm<sup>3</sup> of liquid culturing medium, composed of: potassium phosphate, dibasic – 0.7 g, potassium phosphate, monobasic – 0.3 g, magnesium sulfate – 0.5 g, zinc sulfate – 0.001 g, magnesium chloride – 0.001 g, colloidal chitin – 2 g dry mass, and distilled water – 1000 mL. Culture medium was inoculated with 1 mL of actinomycete suspension obtained from 72-hour bacterial incubation on agar slants. Actinomycetes were incubated for 4 days at 25°C, and then centrifuged for 10 min at 10 000 g min<sup>-1</sup> and 4°C. A cellular post-culturing liquid containing chitinolytic enzymes was used to determine chitinolytic activity, while the sediment was analyzed for protein content.

The activity of chitinases produced by actinomycetes was determined using an organic substrate (Sigma) 4-methylumbelliferyl N-acetyl-β-D-glucosaminide (4MU-GlcNac) marked with fluorescent particles MUF (methylumbelliferyl). Methylcellosolve solvent (EGME, C3H8O2) (Sigma) was used to prepare basic 1 mmol L<sup>-1</sup> solution of 4MU-GlcNac, which was then stored at -20°C (Hoppe 1993). Prior to analysis, working solution of 0.5 mmol L<sup>-1</sup> was obtained by diluting basic solution twofold with spectrally pure water. Acellular post-culturing liquid was poured into 4.5 mL disposable polyester containers – three analyzed samples and one control. The final concentration of the substrate in a sample equaled 50 μmol L<sup>-1</sup>. An

0.5 mL measure of the working solution was added to each of the three containers with samples, while the control sample, prior to addition of the substrate, was placed in boiling bath in order to deactivate the enzymes present in the sample. The enzymatic reactions were carried out for an hour, and then, were interrupted thermally, the same as the control sample. An increase in fluorescence caused by fission of the fluorogenic substrate and release of the MUF molecule was measured in a Hitachi F 2500 spectrofluorometer. The length of the inducing/emission wave was set to 318/445 nm. The spectrofluorometer was calibrated with solutions of pure fluorescent molecules MUF (Sigma). For this purpose, a basic 10 mmol L<sup>-1</sup> solution of MUF was prepared and then diluted with spectrally pure water (Fluka) (Martinez *et al.* 1996). The quantity of nmol MUF mg<sup>-1</sup> protein h<sup>-1</sup> was adopted as a measure of chitinase activity. Colloidal chitin was prepared according to Lingappa and Lockwood (1962). The protein content was determined according to the Bradford (1976) method.

Activity of chitinases produced by actinomycetes was examined in relation to the temperature (10–50°C) and different chitinous substances, such as, colloidal chitin, chitin powder, and shrimp shells. When investigating the impact of temperature and various chitin substances on activity of chitinases produced by actinomycetales surveyed factor was respected without division of the strains depending on the reaction of the environments of their origin.

The results were analyzed in STATISTICA 6.0. Variance analysis (ANOVA) was the primary statistical method used in calculations. This method facilitated comparison of the following independent factors: habitat's pH and season.

### 3. RESULTS

The actinomycetes were the most abundant in soil (average of  $18 \times 10^3$  CFU g<sup>-1</sup> in farmland soil and  $9 \times 10^3$  CFU g<sup>-1</sup> in sandy soil), and the least abundant in water (average of  $2.7 \times 10^1$  CFU mL<sup>-1</sup> in water with neutral pH and  $0.6 \times 10^1$  CFU mL<sup>-1</sup> in water with the alkaline pH) (Table 2). The 2-way ANOVA analysis demonstrated significant dif-

Table 2. The number of actinomycetes in the lake water, bottom sediment and in soil of drainage basin, ( $\pm$  standard deviation  $n = 3$ ), estimated with the method of plate technique and expressed as CFU – Colony Forming Units.

Season	Reaction of environment	Environment		
		Water CFU $\times 10^1 \text{ mL}^{-1}$	Bottom sediments CFU $\times 10^2 \text{ g}^{-1}$	Soil CFU $\times 10^3 \text{ g}^{-1}$
spring	neutral	3 $\pm$ 1.0	162 $\pm$ 10.6	24 $\pm$ 1.0
	alkaline	0.7 $\pm$ 0.6	93 $\pm$ 10.0	12 $\pm$ 2.0
summer	neutral	1 $\pm$ 1.0	15 $\pm$ 6.8	17 $\pm$ 2.0
	alkaline	0.3 $\pm$ 0.6	8 $\pm$ 1.0	8 $\pm$ 1.0
autumn	neutral	3 $\pm$ 1.0	27 $\pm$ 10.0	13 $\pm$ 2.5
	alkaline	0.7 $\pm$ 0.6	16 $\pm$ 6.4	7 $\pm$ 1.5
average	neutral	2.7 $\pm$ 1.0	68 $\pm$ 9.1	18 $\pm$ 1.8
	alkaline	0.6 $\pm$ 0.6	39 $\pm$ 5.8	9 $\pm$ 1.5

Table 3. 2-way ANOVA test comparing the influence of the season (1), pH of habitat (2) on the number of actinomycetes.

Factor	Water			Bottom sediments			Soil		
	df	F	P	df	F	P	df	F	P
Season	2	3.5	0.057	2	79.3	0.000	2	23.4	0.000
pH of habitat	1	18.5	0.000	1	12.3	0.003	1	79.3	0.000

Explanations: df – number of independent variables, F- among-groups variance to the within – group variance, P – significance level

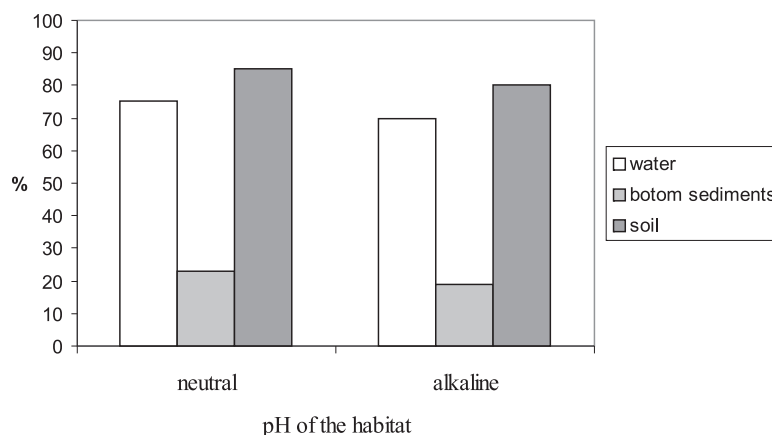


Fig. 1. The contribution (%) of chitinolytic actinomycetes in their total numbers in lake water, bottom sediment and in the soil.

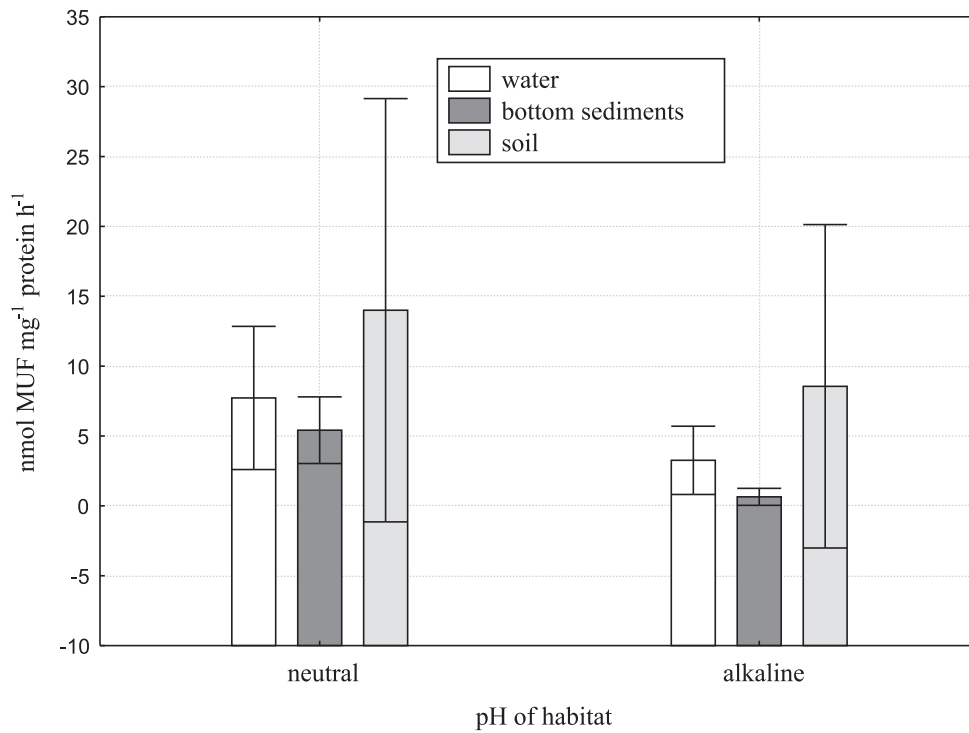


Fig. 2. Chitinolytic activity of actinomycetes isolated from lake water, botom sediments and the soil (vertical bars represent standard deviation) estimated with the fluorometric method and expressed as MUF (methylumbelliferyl) mg<sup>-1</sup> protein h<sup>-1</sup>.

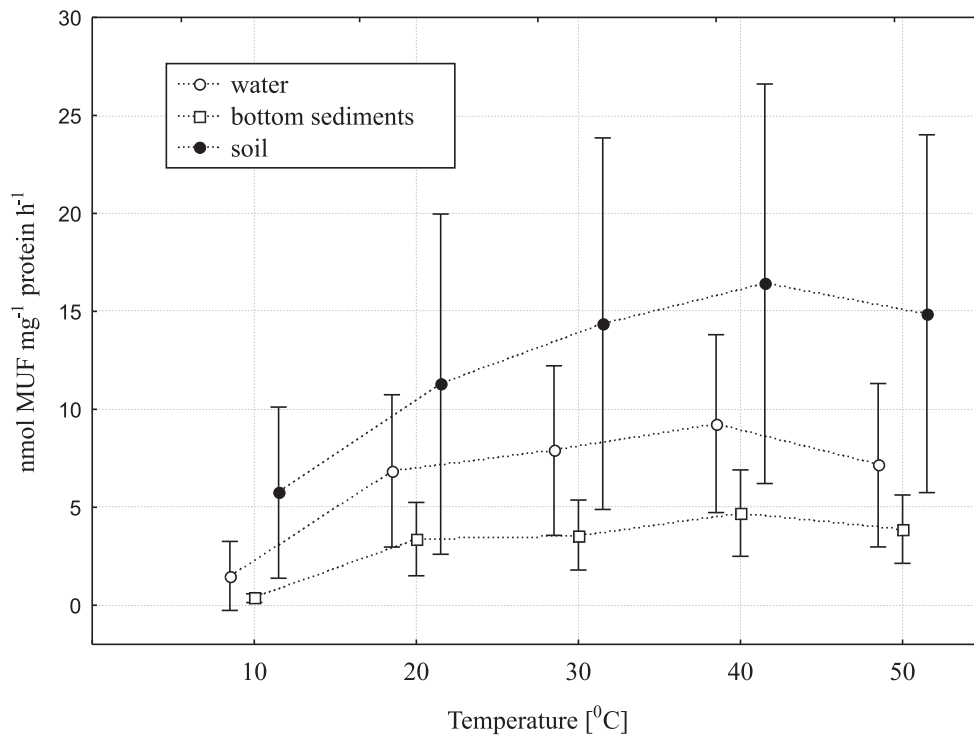


Fig. 3. Influence of temperature on chitinolytic activity of actinomycetes isolated from lake water, botom sediments and the soil (vertical bars represent standard deviation) estimated with the fluorometric method and expressed as MUF (methylumbelliferyl) mg<sup>-1</sup> protein h<sup>-1</sup>.

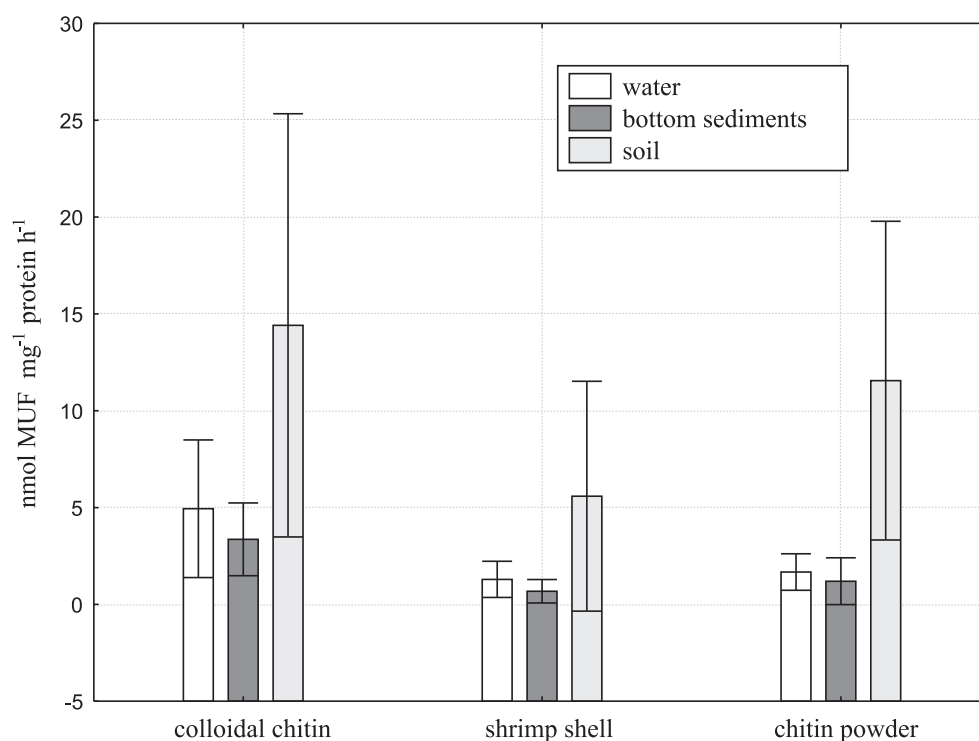


Fig. 4. Influence of chitinous substance on chitinolytic activity of actinomycetes isolated from water, bottom sediments and the soil (vertical bars represent standard deviation) estimated with the fluorometric method and expressed as MUF (methylumbelliferyl)  $\text{mg}^{-1}$  protein  $\text{h}^{-1}$ .

ferences in actinomycete abundances across seasons and values of habitat's pH and season (Table 3). The highest abundances of actinomycetes were recorded in spring in habitats with neutral pH.

The highest percentage of chitinolytic actinomycetes in their total numbers was observed in soil (average of 80% in sandy soil and 85% in farmland soil) (Fig. 1). Chitinolytic actinomycetes were also abundant in water (average of 73%). In silt and sandy sediments, percentages of chitinolytic actinomycetes equaled 23 and 15%, respectively (Fig. 1).

Chitinolytic activities (Fig. 2) of actinomycetes isolated from water, bottom sediments, and soil were different. Strains isolated from neutral pH habitats had higher activity of chitinases than those from alkaline habitats. Actinomycetes obtained from soil were characterized by the highest activity (average of 14  $\text{nmol MUF mg}^{-1}$  of protein  $\text{h}^{-1}$  in strains from farmland soils and 8.5  $\text{nmol MUF mg}^{-1}$  of protein  $\text{h}^{-1}$  in sandy soil), while the lowest activity was observed among benthic actinomycetes (average of 5.4  $\text{nmol MUF mg}^{-1}$  of

protein  $\text{h}^{-1}$  in strains isolated from silt and 0.65  $\text{nmol MUF mg}^{-1}$  of protein  $\text{h}^{-1}$  in sandy sediments) (Fig. 2).

Examination of the impact of temperature and the type of chitinous substrate on the activity of chitinases produced by actinomycetes demonstrated that their activity peaked at 40° (Fig. 3) and in the presence of colloidal chitin (Fig. 4).

#### 4. DISCUSSION

According to this study, actinomycetes were the most abundant in soil environment, and the least, in water. Soil is inhabited not only by relatively higher numbers of actinomycetes, but also by larger populations of bacteria and fungi (Swiontek Brzezinska *et al.* 2008b). The highest abundances of microorganisms are observed in the upper 30 cm soil layer; below that depth, their numbers rapidly decrease. Soil draining and calcium treatment create favorable conditions for growth of actinomycetes. In alkaline peat, these microbes are very active. Their numbers in the farmland soil oscillate around

one million per gram of soil (Marszevska-Ziemięcka *et al.* 1974). According to a study conducted by Barabasz and Smyk (1997), abundances of bacteria and actinomycetes in natural soils are very high and considerably exceed that of fungi. That study demonstrated that in heavily managed soil, the abundances of bacteria and actinomycetes decrease substantially, which is accompanied by excessive growth of fungi pathogenic to plants (Barabasz and Smyk 1997). Actinomycetes not only inhabit soils, but are also present in aquatic environment. Jensen *et al.* (1991) and Burns *et al.* (2003) found actinomycetes in seawater, and Terkina *et al.* (2002) and Klausen *et al.* (2004b) in fresh water. Actinomycetes were found in association with cat-tail (*Typha latifolia*) and bulrush (*Scirpus acutus*) roots in constructed wetland (Hatano *et al.* 1994) and in sewage treatment scum (Jenkins *et al.* 1993).

Ability to decompose chitin is a characteristic property of actinomycetes. Paul and Clark (2000) found that 90% of all actinomycetes obtained from soil belonged to *Streptomyces* genus and nearly all were able to decompose chitin, however, El-Fiky *et al.* (2003) reported that only 7% actinomycetes from the rhizosphere of tomato and green pepper were capable of decomposing chitin. According to this study, chitinolytic actinomycetes found in water constituted 70–75% of the total number of actinomycetes, while those isolated from soil, 80–85%. The lowest numbers of actinomycetes capable of degrading chitin were found in bottom sediments (19–23%). Lower percentage of chitinolytic actinomycetes in a habitat could be related to a considerable accumulation of organic matter. Numerous studies reported that chitinolytic microorganisms were more abundant in environments that are poor in nutrients. The presence of organic matter may hinder the usage of chitin by microorganisms. Swiontek Brzezinska (2004), when analyzing abundances of chitinolytic bacteria in water and bottom sediments in lakes with various nutrient content, observed that the percentage of chitinolytic bacteria was higher in meso-oligotrophic lake (10–19% in water and 5–7% in bottom sediments) than in eutrophic lake (5–11% in water and 3–4% in bottom sediments). Similar results were ob-

tained by Mudryk (1991) in estuarine lake, where 10.6% of bacteria from surface water were capable of degrading chitin, and only 5% of those from bottom sediments.

Measurements of enzymatic activity of microorganisms provide a reliable indicator of intensity of decomposition and transformation of organic matter in a natural environment. In recent years, measuring enzymatic activity with a spectrofluorometric method (Brown and Goulder 1996, Martinez *et al.* 1996) has become increasingly common. This method utilizes a complex composed of an organic substrate bound with a fluorogenic molecule MUF/MCA (Lamy *et al.* 1999). In order to evaluate the activity of endochitinolytic (chitinases) and exochitinolytic (N-acetylglucosaminidase) enzymes, specific substrates are used, for example, chito-oligomers, 4-MU-oligomers, or CMC-RBV (Patil *et al.* 2000). The most accurate methods use  $^3\text{H}$ -chitin and fluorogenic substrates (Yang and Hamaguchi 1980, Cabib 1988). Application of these fluorogenic substrates permits conducting simple, effective, and very accurate in situ measurements of extracellular enzymatic activity (Lamy *et al.* 1999). This study demonstrated that actinomycetes isolated from soil were characterized by a wide range of activity, with the highest values among all analyzed strains. Chitinases produced by actinomycetes collected in bottom sediments were the least active. Unfortunately, the available information on chitinolytic activity of actinomycetes inhabiting water and bottom sediments is still quite limited. High activity of chitinases produced by soil microorganisms could be associated with easier access to organic matter (including chitin) than in an aquatic environment. Podgórska (2002) reported that bacteria inhabiting beach sand of sea coast synthesized wide spectrum of hydrolytic enzymes more actively than bacteria collected from water. Small percentage of chitinolytic actinomycetes occurring in bottom sediments could be a result of the fact that chitin undergoes decomposition in water, and only small quantities of this substance reach sediments.

In natural environment, the activity of enzymes produced by microorganisms, including chitinases, in general takes place at

temperatures not exceeding 30°C. For experimental purposes, in order to estimate potential activity of investigated enzymes at higher temperatures, there have been undertaken researches within wider temperature scope.

Activity of chitinases depends on temperature and the type of chitinous substrate, among other things. This study showed that chitinases produced by actinomycetes had the highest activity at 40°C. Tominga and Tsujisaka (1976) observed that *Streptomyces orientalis* strain showed high activity between 30 and 60°C. Gomes *et al.* (2000), who analyzed activity of 5 chitinolytic strains of actinomycetes isolated from soil, observed that activity levels peaked at 40–50°C. A strain described as 218 was active even at 70°C. Chitinases produced by microorganisms are active across a wide range of temperature. Swiontek Brzezinska *et al.* (2007) reported that bacterial and fungal chitinases were the most active between 40 and 50°C. Wiwat *et al.* (1999) found that bacteria *Bacillus circulans* No. 4.1. showed the highest chitinolytic activity at 40°C, with slightly lower values observed at 30 and 50°C. Bendt *et al.* (2001) observed high chitinolytic activity in psychrotolerant bacteria *Vibrio sp.* at temperatures ranging from 30 to 45°C.

Chitinase activity largely depends on the type of chitinous substrate. Some chitinases are constitutive, while others are induced and require a provision of an appropriate chitinous substrate. According to this study, chitinases produced by actinomycetes from all examined habitats showed the highest activity in the presence of colloidal chitin. Nampoothiri *et al.* (2004) obtained similar results when investigating chitinase activity of *Trichoderma harzianum*. Furthermore, Joo (2005) reported that chitinolytic activity of *Streptomyces halstedii* was high in the presence of colloidal chitin; however, the highest activity levels were observed in medium containing a mixture of chitobiose, chitotriose, chitotetrose, and chitopentose. Ability to use different chitinous substrates could be the characteristic and specific for each strain. Colloidal chitin, due to its colloidal character, is relatively easy to assimilate and metabolize. Nakaew *et al.* (2000) demonstrated that specific strains of fungi and bacteria show very high chitinolytic ac-

tivity in mediums containing 1% solution of colloidal chitin. Rattanakit *et al.* (2002) reported that shrimp waste was also a suitable substrate for culturing *Aspergillus sp.* S1-13. This fungus synthesized equal or even larger amount of enzymes than a fungus cultured on medium with addition of colloidal chitin.

Observed differences in actinomycetales number and activity in the lake and the soil may be explained by higher accumulation of chitin substances in the soil. Attempts at estimating the amount of chitin in the biosphere have not so far brought satisfactory results, for there is no data concerning some ecosystems or they are fragmentary. Only for the hydrosphere such an assessment has been accomplished so far (totally  $1361 \times 10^6$  T y<sup>-1</sup>) (Grzybowska 2003). In the soil environment, the fungi continually synthesize the chitin. They produce large amounts of that substance (within the limits of 2.6 and 26.2 of the mycelium dry matter), depending on the soil characteristics, such as reaction and temperature, and on the fungus age. The chitin can also be found in annelids and selected fungi. It also builds the diatoms cell wall (Marszewska-Zięmięcka *et al.* 1974). This polymer allows microorganisms to continually synthesize chitinolytic enzymes and take active part in that compound decomposition. Similar phenomenon is observed in lakes. Bottom sediments contain much more highly specialized bacteria, for instance in fibre decomposition, than water. Easy accessible substrate undoubtedly stimulates enzymes activity and induces expression of genes, which encode the chitinase. Those genes activity increases if the culture is run on the medium with the chitin content (Haran *et al.* 1995).

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*Received after revising November 2008*