THE ROLE OF CHITINOLYTIC BACTERIA AND FUNGI IN BIODEGRADATION OF CRUSTACEAN REMAINS IN LACUSTRINE HABITATS

ABSTRACT: This study presents results of research on occurrence of chitinolytic bacteria and fungi in water, bottom sediments, and watershed soil of an eutrophic lake and on their ability to use the crustacean skeletons (shrimp waste) as a respiration substrate. It was found that the respiration rate of bacteria and fungi during decomposition of chitin varied in different environments. The participation of chitinolytic microorganisms in water (13%) and soil (18%) was greater than in bottom sediments (5%). The respiration activity in the presence of all parts of shrimp waste and shrimp exoskeletons observed in chitinolytic bacteria was higher than that of fungi. But fungi demonstrated the highest metabolic activity in the presence of the shrimp head sections. The highest respiration activity was observed in planktonic and soil bacteria, while the lowest, in benthic strains. The chitinolytic bacteria used well all examined respiration substrates (all parts of shrimp waste – 671 mg O₂ g⁻¹ protein in 5 days, the shrimp head sections – 851 mg O₂ g⁻¹ protein in 5 days and shrimp exoskeletons – 490 mg O₂ g⁻¹ protein in 5 days). No significant differences in respiration activity were observed in chitinolytic fungi isolated from water, bottom sediments and soil. All of fungal strains demonstrated the highest metabolic activity in the presence of the shrimp head sections (average 1083 mg O₂ g⁻¹ protein in 5 days). Shrimp exoskeletons were oxidized the least efficiently (average 160 mg O₂ g⁻¹ protein in 5 days). Certain strains were not using them at all.

KEY WORDS: chitinolytic bacteria, chitinolytic fungi, respiratory activity

Chitin is a (1→4)-β-linked, insoluble, and unbranched homopolymer of N-acetylglucosamine (De Boer et al. 1999, Cottrell et al. 1999). It is second the most common polymer on Earth after cellulose (Sheng et al. 2001). Microbiological degradation of chitin is an essential process in the global recycling of nitrogen and carbon (Nielsen and Sørensen 1999) and chitinolytic microorganisms play an important role in the natural environment. They are used in neutralization of chitin waste and production of biological fungicides that control the growth of fungal pathogens of plants (Gohel et al. 2006). Chitin present in waste from marine crustacea is used in production of biological fungicides (Wang et al. 2002, Chang et al. 2007). Large quantities of shell waste, which consists of heads and exoskeletons, could be used as source of livestock feed. However, its usefulness as animal feed is primarily limited by a high content of chitin (17.6%), which cannot be effectively digested by organisms (Sachindra and Mahendrakar 2005). Only certain microorganisms are capable of decomposing this polymer. Shrimp waste is
often treated as common rubbish and is processed to obtain livestock feed or is used as an inexpensive natural nitrogen fertilizer. Pollutants containing chitin are carried from a watershed area to stagnant waters where they are degraded by various groups of microorganisms. The purpose of this study was to investigate the role of bacteria and microscopic fungi in biodegradation of chitin, to isolate the chitinolytic strains, and to determine the rate at which these strains use shrimp waste and shrimp exoskeletons, which constitute the primary source of chitin.

For the analyses, bacteria and fungi isolated from the water and bottom sediments of the lake and from the soil of its watershed were used. The lake (Lake Chełmżyńskie) in central Poland is a temperate, eutrophic, deep lake. Morphometric and trophic parameters are presented in Table 1.

### Table 1. Morphometric and trophic characteristics of Lake Chełmżyńskie

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Area (ha)(1)</td>
<td>271.1</td>
</tr>
<tr>
<td>Maximal depth (m)(1)</td>
<td>27.1</td>
</tr>
<tr>
<td>Mean depth (m)(2)</td>
<td>6.0</td>
</tr>
<tr>
<td>pH (2)</td>
<td>7.6 – 8.5</td>
</tr>
<tr>
<td>TP (mg dm⁻³) (2)</td>
<td>0.04 – 0.05</td>
</tr>
<tr>
<td>TN (mg dm⁻³) (2)</td>
<td>1.1 – 1.80</td>
</tr>
<tr>
<td>Electrolytic conductivity (μS cm⁻¹) (2)</td>
<td>601 – 703</td>
</tr>
<tr>
<td>Chlorophyll a (μg dm⁻³)</td>
<td>26.4 – 56.9</td>
</tr>
</tbody>
</table>

(1) Data supplied by Provincial Inspectorate of Environmental Service in Bydgoszcz
(2) Data supplied by Department of Environmental Microbiology and Biotechnology, Nicolaus Copernicus University (data for 0.5 m depth, spring, summer, autumn 2006).

The water samples were collected at depths of up to 5 cm with an automatic pipettor PipetBoy (De Wille Biotechnology), while the bottom sediment samples (up to 5 cm), with an O5 cm × 0.75 m tube scoop directly to sterile twist jars. All samples were aseptically transferred to sterile glass jars and placed on ice in an insulated container at ± 7°C. They were analyzed immediately after they arrived in the laboratory. The time between sample collection and microbiological analyses did not exceed 5 hours. The samples were collected seasonally in the spring (April 3, 2006), summer (August 21, 2006), and autumn (October 20, 2006).

Heterotrophic bacteria in water, bottom sediments, and soil were isolated with a plate technique applying a surface inoculation on nutrient agar. After 7-day incubation at 20°C, 50 colonies were inoculated to each test tube with nutrient agar and then were used to determine the chitinolytic properties with the fluorometric method (Hoppe 1993; Martinez et al. 1996).

Fungi in water, bottom sediments, and soil were isolated with a plate technique applying a surface inoculation on Czapek Dox agar. After 14-day incubation at 25°C, 50 colonies were inoculated to each test tube containing Czapek Dox agar and used to determine the chitinolytic properties with the fluorometric method (Hoppe 1993; Martinez et al. 1996).

The activity of chitinases produced by bacteria and fungi was determined with an organic Sigma-made substrate: 4-methylumbelliferyl N-acetyl-β-D-glucosaminide (4MU-GlcNac) marked with the fluorophore MUF (methylumbelliferyl). Methylcellulose solvent (EGME, C3H8O20) (Sigma) was used to prepare a basic 1 mM solution of 4MU-GlcNac. The solution was stored at −20°C. Prior to analysis, the working 0.5 mM solution was obtained by diluting basic solution twofold with spectrally pure water. Cell – free post-culture liquid was poured into 4.5 cm³ disposable polyester containers – three analyzed samples and one control. The final concentration of the substrate equaled 50 μM. 0.5 cm² of the working solution was added to three containers with samples, while the control, prior to addition of the substrate, was treated with 0.1 cm² saturated solution of HgCl₂, in order to deactivate the enzymes present in the sample (final concentration: 4 mM). Enzymatic reactions were conducted...
for 5 hours. Next, the enzymatic reactions in analyzed samples were interrupted by adding 0.1 cm$^3$ HgCl$_2$. Fluorescence was additionally measured in all analyzed and control samples at the beginning and after incubation. The increase in fluorescence caused by enzymatic cleavage of fluorogenic substrate was measured with a Hitachi F 2500 spectrofluorometer model T-2500 in 3 cm$^3$ quartz cuvettes. The length of the excitation/emission wavelengths was 318/445 nm. Before each experiment the procedure was calibrated by fluorescence reading of MUF standard solutions (20nM – 1 mM).

The respiration activity of chitinolytic bacteria and fungi in the presence of shrimp waste was determined with the measurement system OxiTop Control 12. This apparatus measures oxygen consumption almost continuously over the incubation period. The measurement of BOD with OxiTop$^\text{a}$ Control was carried out according to the operating instruction provided by the supplier (WTW 1998). For the respirometric analyses, the authors used samples of 10 strains of chitinolytic bacteria and fungi isolated from water, bottom sediments, and soil, which were characterized by the highest activity of chitinases. Whole shrimp waste, head sections, and exoskeletons were used as respiration substrates. Shrimp wastes originated from a processing facility. The company has been shelling shrimp (Pandalus borealis) from the North Sea since 1991. Shrimp waste was dried at 105°C, ground, and sterilized in an autoclave for 20 min. at 117°C.

The isolated chitinolytic bacteria and fungi were cultured on agar slants for 72 hours at 25°C. Afterwards, the cultures were removed from the slants with sterile distilled water and used for respirometric analyses. Each measuring vessel contained a magnetic stirrer and 50 cm$^3$ of mineral medium (ISO 14851 1999). Medium was inoculated with 2 cm$^3$ of the prepared suspension. Shrimp waste (0.04g) was added after the temperature in the containers had stabilized; next, a rubber carrier containing absorbent CO$_2$ (0.4 g NaOH) was placed in the neck of a vessel. The OxiTop measuring heads were tightly screwed on, and the vessels were placed on a mixing platform in a thermostatic cabinet. The incubation was carried out for 5 days at 25°C. The measured values were recorded in the OC 110 control system in the “Special BOD” mode. Samples without respiration substrate additions were used as a control (endogenous respiration). All samples were analyzed in three replicates. The respiration activity was expressed in mg of O$_2$ g$^{-1}$ of protein in 5 days. The protein content was determined according to the Bradford (1976) method.

Statistical analyses were done using STATISTICA 6’2003 for Windows. The analysis of variance ANOVA was the main analytical method. Percentage of strains of chitinolytic bacteria and fungi from different environments were compared using t – test.

It was found that the participation of bacteria and fungi in decomposition of chitin varied in different environments (Fig. 1).

![Fig. 1. The contribution (%) of chitinolytic bacteria (B) and fungi (F) in water, bottom sediment and soil. * – significance level $P<0.05$.](image-url)
The participation of chitinolytic microorganisms in water and soil was greater than in bottom sediments. In water, chitinolytic bacteria on average constituted 13%, in soil – 18%, and in bottom sediments – only 5% of the total numbers of heterotrophic bacteria. The share of fungi in decomposition of chitin was greater. In the water, they constituted no less than 50%, in soil – 47%, and in bottom sediments – 42% of all fungi.
The respiration rates of chitinolytic bacteria and fungi in the presence of shrimp waste demonstrated significant differences. The respiration activity of chitinolytic bacteria was higher than that of fungi. But fungi demonstrated the highest metabolic activity in the presence of the shrimp head sections (Fig. 2). It was observed that strains of planktonic bacteria and soil bacteria had the highest respiration activity in the presence of all kinds of shrimp waste, while benthic bacteria – the lowest (R < 0.00 and O < 0.00) (Fig. 3). The analyzed strains demonstrated the most intense respiration activity in the presence of the shrimp heads. Planktonic strains used on average 950 mg of O₂ g⁻¹ of protein in 5 days, benthic bacteria – 701 mg of O₂ g⁻¹ of protein in 5 days, and soil bacteria 903 mg of O₂ g⁻¹ of protein in 5 days. All investigated bacterial strains used shrimp exoskeletons the least effectively.

No differences in respiration activity were observed in chitinolytic fungi isolated from water, bottom sediments and soil (R < 0.63 and O < 0.00) (Fig. 4) All of fungal strains demonstrated the highest metabolic activity in the presence of the shrimp head sections. The amount of oxygen used by fungi was much greater than the amount used by bacteria. Fungi isolated from water used on average 1156 mg of O₂ g⁻¹ of protein in 5 days, benthic – 1101 mg of O₂ g⁻¹ of protein in 5 days and soil – 991 mg of O₂ g⁻¹ of protein in 5 days. Shrimp exoskeletons were oxidized the least efficiently by chitinolytic fungi. Some strains were not using them at all (Fig. 4).

Chitin, in spite of its complex structure, constitutes an important source of carbon and nitrogen for heterotrophic microorganisms. Its breakdown is catalyzed by chitinases synthesized primarily by bacteria, fungi, and actinomycetes. The fraction of microorganisms that hydrolyze chitin varies. However, there is not enough information as to which group of organisms plays the most important role in that process. Our study demonstrated that fungi participation in decomposing chitin is much more substantial than that of bacteria. The kind of environment also has a great impact on occurrence of chitinolytic microorganisms. The percentage of chitinolytic microorganisms in water and soil was much greater than in bottom sediments. This phenomenon is common in water bodies. In lacustrine bottom sediments and also in eutrophic lakes rich in organic matter, the fraction of chitinolytic

![Fig. 4. Oxygen uptake by chitinolytic fungi isolated from different environments. Vertical bars represent ± SD.](image)
bacteria is much smaller than in the water. The presence of organic matter may hinder the usage of chitin by microorganisms. According to Swiontek Brzezińska (2004), on average 5–11% of heterotrophic bacteria present in water of eutrophic lake were capable of breaking down chitin, while in oligo-mesotrophic lake, this value ranged from as much as 10 to 19%. In bottom sediments of eutrophic lake, the author observed only 3–4% of chitinolytic bacteria and in oligo-mesotrophic lake – 5–7%. Mudryk (1991) reports that 11% of bacteria found in the surface waters of estuarine lake break down chitin, while in bottom sediments, only 5%. Podgór ska (2002) found only 0–6% of chitinolytic bacteria in Baltic sea water and beach sand, while Skórczewski (2003) observed 7% of chitinolytic bacteria in estuarine lake.

Soil is a heterogeneous environment and a high fraction of chitinolytic bacteria and fungi might be associated with high concentration of chitin and, in general, higher abundance of microorganisms. One gram of ploughed soil contains up to 10^6 organisms capable of decomposing chitin (Schlegel 2003). According to Marszewska-Żemielęcka et al. (1974) chitin is decomposed the most efficiently by actinomycetes. Paul and Clark (2000) suggest that as much as 90% of soil actinomycetes are capable of breaking down this polysaccharide. Active actinomycetes participate in chitin remineralization; however, it is certain that bacteria and fungi also significantly contribute to decomposition of this polysaccharide in soil. Even though, the participation of fungi in decomposition of chitin is greater than that of bacteria, their activity is lower. This study suggests that the activity of chitinases produced by bacteria isolated from shrimp waste was much higher than the activity of fungal chitinases (Swiontek Brzezińska et al. 2006). It could be concluded that bacteria are more effective in biodegradation of chitin.

In addition to enzymatic activity, the respiration activity of microorganisms is an essential indicator describing the intensity of transformation of organic matter in water bodies (Gossens et al. 1984). The respiration activity could be measured with respirometric, enzymatic, and radiologic methods. Currently, the respirometric method with a Clark electrode is widely used. This method enables a fast measurement of activity, but various substrates are oxidized with different intensity; therefore, the authors of this study used the respirometric measurement system OxiTop Control 12, which enables recording results even at 24-hour intervals. This is particularly important when using substrates that are difficult to assimilate, such as chitin. Until recently, the manometric method is a respirometric method based on pressure change following oxygen consumption in a hermetically closed bottle containing the sample (Hufschmid et al. 2003). New generation of devices used for determining respiration activity are equipped with an electronic system that measures pressure and memory where the values recorded in successive days are stored (WTW 1998). Vähäoja et al. (2005), when investigating biodegradation of different types of oil in underground water, found that OxiTop Control device used to determine the consumption of oxygen produced more accurate results in comparison to traditional methods. Hufschmid et al. (2003) arrived at similar conclusions when examining pollution in industrial sewage.

This study demonstrated that chitinolytic bacteria and fungi oxidized shrimp waste with different intensities. The majority of isolated strains of bacteria metabolized whole shrimp waste most actively, head sections were metabolized slightly less actively, and exoskeletons were metabolized the least efficiently. In contrast, chitinolytic fungi used head sections much more efficiently, and some of their strains did not use exoskeletons at all. The fact that bacteria and fungi used exoskeletons less efficiently may suggest that this substrate requires more time to initiate oxidation. Because of its complex structure, chitin is considered a difficult-to-assimilate compound, whose oxidation requires long period of time, even if it is conducted by chitinolytic microorganisms. In contrast, more active utilization of head sections could be associated with oxidation of protein and lipids (predominant compounds in this section). Strzelczyk et al. (1988), Mudryk (1997) and Grover and Chrzanowski (2000) maintain that cellulose is not
a respiration substrate that is actively used. Likewise chitin, this compound is hard to assimilate and few microorganisms are capable of remineralizing this polymer. Mudryk and Podgórska (2005), who examined the respiration activity of bacteria isolated from marine beach sand, found that the analyzed strains used glucose and casein hydrolysate most actively and cellobiose and sodium pyruvate – least actively. Massardier-Nageotte et al. (2006) investigated the aerobic biodegradability of four different polymers by microbial inoculums extracted from the soil using OxiTop respirometer. Among tested polymers that is degraded the most is MB – blend starch + polycaprolactone and polyactic acid is almost not degraded.

This study demonstrated that chitinolytic bacteria oxidized shrimp waste more effectively than fungi. Many fungi strains did not use shrimp exoskeletons at all. It was also observed that shrimp exoskeletons hindered the growth of fungi, which is in accordance with findings of Joo (2005) and Wang et al. (2002). Bacillus cereus QQ308 produce antifungal hydrolytic enzymes, comprising chitinase, chitosanase and protease, when grown in a medium containing shrimp and crab shell powder produced from marine waste (Chang et al. 2007). Therefore, it could be assumed that bacteria play more important role in degradation of chitin compounds than fungi.

The metabolic activity of microflora certainly depends on the environment from which the strains were isolated. This study demonstrated that chitinolytic bacteria and fungi isolated from water metabolized shrimp waste with higher intensity. Mudryk and Podgórska (2005) obtained similar results. That author reports that consumption of oxygen in the presence of analyzed substrates was higher for planktonic strains. Strzelczyk and Mielczarek (1971) observed that epiphytic bacteria were characterized by the highest activity, planktonic bacteria – medium, and bacteria isolated from bottom sediments – the lowest activity. The higher metabolic activity of planktonic bacteria in comparison to that of benthic bacteria was also observed by Donderski and Strzelczyk (1992).

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