

## Chitinolytic activity of bacteria and fungi isolated from shrimp exoskeletons

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### Abstract

Microbiological analysis of shrimp exoskeletons demonstrated considerable differences in abundance of heterotrophic bacteria and fungi. The number of heterotrophic bacteria was greater by two orders of magnitude than that of fungi. The survey, however, did not reveal significant differences in abundances of bacteria and fungi in samples collected during different months of the survey. The percent contribution of chitinolytic fungi in shrimp exoskeletons was greater than that of bacteria that hydrolyze chitin. The activity of chitinase bacteria was always higher than fungi. Chitinases produced by bacteria demonstrated the highest level of activity at 40°C and pH = 8. In contrast, fungal chitinases showed the highest activity at 50°C and pH = 5.

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## INTRODUCTION

Chitin is a long-chain, structural polysaccharide; it is constructed from N-acetylglucosamines linked together by  $\beta$ -1,4 glycosidic bonds (Requera and Leschine 2001). It is the second largest of the polysaccharides (after cellulose), which are abundant in nature. Chitin is present in the exoskeleton of arthropods, coelenterates, flatworms, protozoa, molluscs, and crustaceans (Suzuki et al. 1998). Furthermore, the cell wall of fungi is constructed of chitin and chitosan (Schlegel 2003). Chitin is broken down by endo- and exo- enzymes known as chitinases (EC3.2.1.14), as well as  $\beta$ -N – acetylhexaminidase (EC3.2.1.52). These enzymes are produced by bacteria, fungi, and actinomycetes, among others.

Chitin and its derivatives are popular in the national and international medical community due to their wide range of practical applications, for example, modern dressing materials, new pharmaceuticals, and preparations used to treat local changes of an oral cavity (Struszczyk 2002). Chito-oligosaccharides used in biotechnology often originate from waste created during seafood processing. Products made of exoskeletons of crabs and shrimp accelerate wound healing processes, stimulate the immunological system, improve antitumor properties, and are even used in waste water treatment. In Taiwan, where seafood is processed on an industrial scale, enormous amounts of waste containing chitin are produced. Utilization of this waste has brought about two benefits: decreased pollution and the production of chito-oligosaccharides, which are useful in biotechnology (Huang et al. 1996). In Poland, shrimp waste is treated as common rubbish and is processed to obtain livestock feed or used as an inexpensive natural nitrogen fertilizer. The demand for seafood products is increasing and so is the amount of waste produced during its processing. Chitin waste is active organic matter. Various biochemical processes take place in the shrimp waste before it is processed. These processes are conducted by a variety of physiologically diverse organisms. The fungal species growing in large numbers in shrimp waste not only decompose shrimp exoskeletons but also cause allergies. Microorganisms with high chitinolytic activity can be used as vaccines that protect soil from excessive deposits of chitin. There is a lot of information in the literature concerning chitinase activity of single strains of bacteria and fungi, but we still do not know which group of microorganisms is the most effective in decomposing this polymer. Finding strains characterized by high activity of chitinases may be essential for biotechnology. Thus, the aim of this study was to determine the intensity with which chitinolytic bacteria and fungi degrade shrimp waste depending on certain physical and chemical factors and whether strains of these microorganisms are particularly active.

## MATERIALS AND METHODS

Samples for this study were collected from shrimp waste, which originated from the processing factory PPHU KRYMAR in Ilów. The factory shells shrimp and imports approx. 17 tons of shrimp 2 to 3 times per month. The facility processes *Pandalus borealis*, which is caught in the polar circle region near Greenland. Approximately 360 tons of waste are produced yearly as a result of the facility's activity.

Samples for microbiological analyses were collected from fresh shrimp waste, which constituted the source of chitin. Samples were collected directly into sterile jars, which were then placed on ice in an insulated container and transported to the laboratory. The time prior to beginning the microbiological analyses did not exceed 5 hours. The study material was collected on 16 December 2005, 15 February 2006, and 6 May 2006.

The number of heterotrophic bacteria in shrimp waste was determined by the plate count method. Following this protocol, 90 cm<sup>3</sup> of sterile water was added to 10 g of waste. The mixture was homogenized for 2 minutes at 4000 rpm. The homogenized liquid was diluted with sterile water ten times. This diluted mixture was inoculated on a surface containing nutrient agar. After a 7-day incubation at 20°C, the colonies that grew on this medium were counted and results were expressed per 1 g of fresh waste. Subsequently, 50 colonies were isolated from each sample and used for determination of chitinolytic properties.

The number of fungi in shrimp waste was estimated by the plate count method. The diluted mixture was cultured on a surface containing Czapek Dox agar. After a 14-day incubation at 25°C, the colonies that grew on this medium were counted and the results were expressed per 1 g of shrimp waste. 50 colonies of fungi were isolated from each sample and were inoculated into test tubes containing Czapek Dox agar. These strains were used to determine the chitinolytic activity.

Chitinolytic properties of bacteria isolated from the waste were determined using a solid base containing both the nutrient agar and additional colloidal chitin (7 g dm<sup>-3</sup>). Strains of bacteria were spot-inoculated into culture medium and incubated at 20°C for 14 days. After the incubation, the zone around the colonies was checked for lighter areas, which indicate that the bacteria were capable of breaking down chitin. These strains were used in further analyses. Colloidal chitin was prepared according to Lingappa and Lockwood (1962). Due to the fact that fungi do not produce a positive result, i.e. a lighter zone on the nutrient agar in the presence of additional colloidal chitin, this method cannot be used for preliminary determination of chitinolytic properties of these microorganisms.

To determine chitinolytic activity, 150 strains of both bacteria and fungi isolated from shrimp waste were used. Bacteria were cultured in Erlenmeyer flasks, which contained 50 cm<sup>3</sup> of liquid culture medium composed of: peptone – 0.1 g, FeSO<sub>4</sub> · 7 H<sub>2</sub>O – 0.1 g, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> – 0.1 g, yeast extract – 0.1 g, iron gluconate – 0.1 g, colloidal chitin – 2.0 g dry mass, and tap water – 1.0 dm<sup>3</sup>. The culture medium was inoculated with 1 cm<sup>3</sup> of bacterial suspension obtained from the 72-hour bacterial incubation on agar slants. Bacteria were incubated for 7 days at 20°C and then were centrifuged for 10 min at 10 000 g min<sup>-1</sup>, at a temperature of ±4°C. Cell-free post-culture liquid, which contained chitinolytic enzymes, was used to determine chitinolytic activity, while the sediment was used to analyze bacterial protein. Fungi were cultured in Erlenmeyer flasks, which contained 100 cm<sup>3</sup> of liquid culture medium composed of: yeast extract – 4 g, malt extract – 10 g, glucose – 4 g, colloidal chitin – 2 g. dry mass, and distilled water – 1 dm<sup>3</sup>. The culture medium was inoculated with 1 ml of fungi suspension obtained from the 7-day incubation on Czapek Dox agar slants. Fungi were incubated for 7 days at 25°C. Subsequently, the cultures were centrifuged for 10 min at a temperature of ±4°C, and the activity of chitinolytic enzymes was tested in clear post-culture liquid. Mycelium was used to analyze protein. Three replicate flasks of each treatment combination were used. Protein was determined using Bradford's method (1976).

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) (Hoppe 1983, 1993). Methylcellosolve 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 cm<sup>3</sup> disposable polyester containers - three analyzed samples and one control. The final concentration of the substrate equaled 50 μM. 0.5 cm<sup>3</sup> 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 cm<sup>3</sup> saturated solution of HgCl<sub>2</sub> 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<sup>3</sup> HgCl<sub>2</sub> (Mudryk and Skórczewski 2004). 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<sup>3</sup> 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 (20 nM – 1 mM) (Martinez et al. 1996). The quantity  $\text{nM MUF} \times \text{mg}^{-1} \text{ protein} \times \text{h}^{-1}$  was adopted as a measure of the activity of chitinases produced by bacteria and fungi.

The activity of chitinases produced by bacteria and fungi was analyzed at temperatures of 10, 20, 30, 40, and 50°C. The effect of different pH values on the activities was also studied. The buffers were: 50 mM citrate-phosphate buffer (pH ranging from 5.0 to 7.0), 50 mM Tris-HCl buffer (pH 8.0 and 9.0). In order to determine the activity of chitinases in environments with different pH, we calibrated a method for different values of pH. First, we prepared a MUF solution with concentrations in accordance with the analytical curve, and then we measured the fluorescence of this solution for different values of pH.

## RESULTS

Microbiological analysis of shrimp waste demonstrated significant differences in abundance of heterotrophic bacteria and fungi (Table 1). The number of heterotrophic bacteria was greater by two orders of magnitude than that of fungi. The heterotrophic bacteria on shrimp exoskeletons was, on average,  $15 \times 10^4$  per g shrimp waste and fungi average  $12 \times 10^2$  per g shrimp waste. The survey, however, did not reveal significant differences in abundance

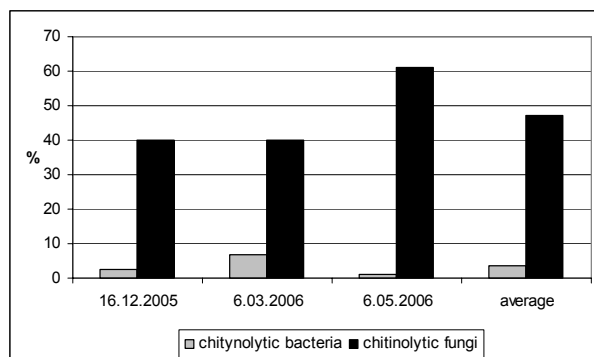
**Table 1**

The number of heterotrophic bacteria and fungi inhabiting shrimp exoskeletons

Date of collection shrimp waste	Number of heterotrophic bacteria in 1 g shrimp waste	Number of fungi in 1 g shrimp waste
16.12.2005	$11 \times 10^4$	$17 \times 10^2$
15.02.2006	$19 \times 10^4$	$15 \times 10^2$
6.05.2006	$15 \times 10^4$	$4 \times 10^2$
Average	$15 \times 10^4$	$12 \times 10^2$

of bacteria and fungi in samples collected in different months of the survey. The percent contribution of chitinolytic fungi in shrimp exoskeletons was greater (average of 47%) than that of bacteria that hydrolyze chitin (average of 3.5%) (Fig. 1).

The activity of chitinase of bacteria and fungi isolated from shrimp exoskeleton was proportional to the incubation time. The highest levels of activity were observed after 5 hours and the values were higher for bacteria than fungi (mean per strains of bacteria  $3.5 \text{ nM MUF} \times \text{mg}^{-1} \text{ protein}$  and mean per strains of fungi  $1.57 \text{ nM MUF} \times \text{mg}^{-1} \text{ protein}$ ) (Table 2). Chitinases produced by bacteria demonstrated maximum activity at 40°C (mean per strains



**Fig. 1.** Proportion of chitinolytic bacteria and fungi in breaking shrimp exoskeletons.

**Table 2**

The effect of incubation time on activity of chitinase produced by bacteria and fungi

Incubation time [h]	Chitinolytic bacteria			Chitinolytic fungi		
	range	mean	± SD	range	mean	± SD
1	0.22–9.80	1.37	± 2.01	0.19–1.22	0.55	± 0.39
2	0.50–18.40	1.86	± 3.64	0.20–1.60	0.79	± 0.51
3	0.58–24.78	2.53	± 4.87	0.24–2.54	1.03	± 0.69
4	0.77–25.49	2.90	± 5.04	0.24–2.81	1.42	± 0.89
5	0.76–25.49	3.50	± 5.37	0.24–3.40	1.57	± 1.00

of  $4.47 \text{ nM MUF} \times \text{mg}^{-1} \text{ protein} \times \text{h}^{-1}$ ) and at  $\text{pH} = 8$  (mean per strains of  $1.73 \text{ nM MUF} \times \text{mg}^{-1} \text{ protein} \times \text{h}^{-1}$ ) (Table 3, 4), whereas fungal chitinases demonstrated maximum activity at  $50^\circ\text{C}$  (mean per strains of  $1.93 \text{ nM MUF} \times \text{mg}^{-1} \text{ protein} \times \text{h}^{-1}$ ) and at  $\text{pH} = 5$  (mean per strains of  $0.51 \text{ nM MUF} \times \text{mg}^{-1} \text{ protein} \times \text{h}^{-1}$ ).

## DISCUSSION

Chitin is a structural component of numerous invertebrates and fungi. In water bodies, its main sources are exoskeletons of crustaceans, insects, and diatoms (Schlegel 2003, Suzuki et al. 1998). Biological decomposition of chitin is carried out by enzymes, called chitinases (Schlegel 2003). Chitinases are produced by bacteria (Huang et al. 1996), actinomycetes (Mahadevan and

**Table 3**

The effect of temperature on activity of chitinase produced by bacteria and fungi

Temperature [°C]	Chitinolytic bacteria			Chitinolytic fungi		
	range	mean	SD	range	mean	SD
10	0.0–8.15	1.04	± 1.90	0.0–2.30	0.54	± 0.74
20	0.10–16.21	1.57	± 3.26	0.20–3.90	0.87	± 1.12
30	0.16–25.81	3.22	± 6.29	0.17–4.70	1.11	± 1.43
40	0.08–25.81	4.46	± 7.79	0.09–5.50	1.41	± 1.69
50	0.0–16.63	1.47	± 3.71	0.0–5.80	1.43	± 1.94

**Table 4**

The effect of pH on activity of chitinase produced by bacteria and fungi

pH	Chitinolytic bacteria			Chitinolytic fungi		
	range	mean	± SD	range	mean	± SD
5	0.10 - 4.05	0.75	± 0.99	0.14 - 1.78	0.68	± 0.51
6	0.15 - 8.15	1.40	± 2.06	0.10 - 1.32	0.51	± 0.44
7	0.11 - 16.2	1.50	± 3.28	0.1 - 1.15	0.36	± 0.35
8	0.17 - 9.77	1.75	± 1.98	0.04 - 1.07	0.32	± 0.34
9	0.0 - 4.02	0.77	± 1.02	0.0 - 0.57	0.15	± 0.14

Crawford 1997), fungi (Pinto et al. 1997), and by yeast and plants (Bhushan and Hoondal 1998).

The number of heterotrophic bacteria on shrimp exoskeletons was greater by two orders of magnitude than that of fungi. Ampe and Thiéry (1998) observed a similar phenomenon. They found more aerobic and anaerobic bacteria than fungi in the digestive track of the fairy shrimp *Branchinella spinosa*. The number of bacteria and fungi increased in the direction from the foregut to the hindgut. The number of fungi in different environments is often smaller than that of bacteria. Swiontek Brzezinska et al. (2006 unpub.) found that the number of heterotrophic bacteria was much higher than that of fungi in soil and water.

Our study demonstrated that the percent contribution of chitinolytic fungi in shrimp exoskeletons was greater than chitinolytic bacteria. Swiontek Brzezinska et al. (2006 unpub.) obtained similar results in the soil of the Chełmżyński Lake watershed. In soil samples, the authors found that the proportion of fungi breaking down chitin is much higher than that of bacteria. Environments rich in easily accessible organic matter may constitute a barrier for utilization of hard-to-decompose compounds, e.g. chitin. In support of this thesis is the observation

of a low proportion of chitinolytic bacteria in eutrophic lakes, bottom sediments, etc. ZoBell and Rittenberg (1938) while surveying marine bottom sediments, observed that only 0.1-1% of heterotrophic bacteria were capable of breaking down chitin. Mudryk (1991) found that in the surface waters of estuarine Lake Gardno 10.6% of bacteria break down chitin, while in bottom sediments, only 5%. Podgórska and Mudryk (2003) found that in a Baltic Sea sand beach only 4.3% of chitinolytic bacteria were able to break down chitin. According to Donderski and Swiontek Brzezinska (2001), in waters of oligomesotrophic Lake Jasne, which is devoid of organic matter, as much as 6.7-38.1% of heterotrophic bacteria were capable of breaking down chitin; whereas, in eutrophic Lake Jeziorak – only 4.4-23.3% of bacteria were able to break it down. Rheinheimer (1987) found several thousand bacteria hydrolyzing chitin in 1 cm<sup>3</sup> of water collected in the western Baltic and its fjords. In oxygenated sediments there were several dozen thousand such bacteria per gram of dry matter. These bacteria primarily inhabited exoskeletons of dead crayfish. However, such rich sources of chitin as crayfish, crab, or shrimp exoskeletons, can't always stimulate the growth of chitinolytic bacteria. Our research supports this idea. Zdanowski and Vosjan (1998) observed a similar phenomenon, also finding an insignificant percent of chitinolytic bacteria in krill.

Although our research points towards a greater percentage participation of fungi in breaking down chitin, the activity of chitinolytic enzymes is much greater in heterotrophic bacteria. Therefore, it can be assumed that bacteria break down chitin more effectively than fungi.

Our study demonstrated that chitinolytic activities of bacteria were always higher than that of fungi. Swiontek Brzezinska et al. (2006 unpub.) obtained similar results among bacteria and fungi isolated from the soil of the Chełmżyński Lake watershed and the water of the Chełmżyński Lake. Chitin is present in the cell wall of many fungi, and it has been used to improve the biological control of photogenic fungi. The abundance of chitin may inhibit fungal growth and activity.

Temperature is certainly an important factor that affects the enzymatic activity. According to numerous studies, chitinases are active at temperatures ranging from 20 to 50°C (Frändberg and Schnürer 1994, Huang et al. 1996, Bhushan and Hoondal 1998, Wiwat et al. 1999, Bendt et al. 2001). Our data is in accordance with data found in the literature. Chitinolytic enzymes produced by bacteria and fungi displayed the highest activity at 40-50°C. Bendt et al. (2001) observed high chitinolytic activity in the psychrotolerant bacteria *Vibrio* sp. at temperatures ranging from 30 to 45°C. Huang et al. (1996), researching chitinolytic activity in *Aeromonas* sp., observed that the optimal temperature ranged from 20 to 50°C. Thermostable chitinases produced by *Bacillus* sp. BG - 11 were the most active between 40 and 60°C. At these temperatures,

chitinolytic activity remained stable (Bhushan and Hoondal 1998). Wiwat et al. (1999) found that the bacteria *Bacillus circulans* Nr 4.1. displayed the highest chitinolytic activity at 40°C; at 30 and 50°C, activity was slightly lower. Tominaga and Tsujisaka (1976), when surveying activity of chitinases in the actinomycete *Streptomyces orientalis*, observed the highest activity between 30 and 60°C. Full inactivation of the enzyme occurred at 75°C. Fenice et al. (1998), researching chitinolytic activity in *Penicillium janthinellum* P9 in a Bench-Top Bioreactor, observed that the optimal temperature ranged from 22 to 26°C

Numerous studies have demonstrated that chitinases are characterized by activity across a wide range of pH values = 4.0 – 8.5 (Morrissey et al. 1976, Wiwat et al. 1999, Bendt et al. 2001). The results of our study demonstrated that bacterial chitinases displayed the highest activity at pH = 8.0. However, chitinases produced by fungi reached maximum activity at pH = 5. Enzymatic activity of microorganisms may be related to their habitat. Bacteria prefer a neutral or slightly alkaline environment (the pH of most waters or soils), while fungi prefer acidic conditions. Donderski (1984) found that the maximum chitinase activity of planktonic and benthic bacteria occurred at pH=5.9 or pH=6.0. Chitinases produced by *Arthrobacter* sp. displayed the highest activity at pH = 5.0, and the lowest at pH = 3.5 and 7.0 (Morrissey et al. 1976). Monreal and Reese (1969) observed that optimal pH for chitinase activity in *Serratia marcescens* was pH = 6.4. Wiwat et al. (1999) found that the activity of chitinases in *Bacillus circulans* No. 4.1 occurred at a wide range of pH values, from 3.0 to 12. However, the highest level of chitinolytic activity occurred at a pH of 7 – 8. Other researchers obtained similar results. According to Bhushan and Hoondal (1998), optimum activity of chitinases in *Bacillus* sp. BG – 11 occurred at a pH of 8.5, while Frändberg and Schnürer (1994) observed the highest chitinolytic activity at pH = 8.0 in *Bacillus pabuli* K1. It is evident from the Bendt et al. (2001) study that chitinases produced by the psychrotolerant bacteria *Vibrio* sp. were the most active at pHs of 7 and 8. According to Hiraga et al. (1997, after Bhushan and Hoondal 1998) *Aeromonas hydrophila* displayed the highest level of chitinase activity at pH values ranging from 5.0 to 8.0. Fenice et al. (1998) researching chitinolytic activity in *Penicillium janthinellum* P9 in Bench-Top Bioreactor, observed that the optimal pH ranged from 4.0 to 4.5

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