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Original Research

Utilization of Shrimp-Shell Waste as a Substrate for the Activity of Chitinases Produced by Microorganisms

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Abstract

Chitinolytic microorganisms such as bacteria, fungi, and actinomycetes, were isolated from the water and bottom sediments of Lake Chełmżyńskie and the soil of its watershed. The impact of various forms of chitin on the chitinolytic activity of these microorganisms was investigated. Bacteria isolated from the analyzed environments exhibited the highest chitinolytic activity in the presence of colloidal chitin. High activity was also observed in a culture medium containing shrimp shells. The analysis of chitinolytic activity of actinomycetes demonstrated that strains isolated from the soil had considerably higher activity levels than those isolated from lacustrine water and bottom sediments. All soil actinomycetes exhibited the highest activity in a culture medium containing colloidal chitin and shrimp shells. Actinomycetes collected in water and bottom sediments were characterized by similar activity levels. Chitinolytic fungi exhibited the maximum activity also in a medium containing colloidal chitin. The presence of shrimp shells in a culture medium had the lowest impact on chitinolytic activity. Among the analyzed forms of chitin, the impact of chitin powder on the activity of chitinases was the least stimulating.

Keywords: chitinolytic microorganisms, chitinase, shrimp waste

Introduction

The function of chitinases varies between microorganisms. In bacteria, chitinases are important in feeding and parasitism. In fungi, protozoa, and invertebrates, they also play a part in morphogenesis. Chitinases are associated with defense mechanisms of plants and vertebrates [1]. There also arises a possibility of utilizing genetically modified microorganisms with high chitinolytic activity in biological pest (insects) control. Genetically modified *Bacillus thuringiensis* is an example of such an organism [2]. In agriculture, techniques using genetic manipulations of the chit42 gene from *Tichoderma harzianum* seem promising for improving plant resistance to fungal pathogens This gene encodes an active endochitinase, which has a very strong anti-fungal effect. It has been introduced into genomes of tobacco, apple tree, and potato, and when inserted with a constitutive promoter undergoes continuous expression. These transgenic plants are characterized by considerably higher resistance to fungal pathogens [3].

Chitinases can be also added to common fungicides and insecticides, not only to increase their strength and activity, but also to minimize the concentration of their chemically synthesized components, which pose serious threats to the environment and human health. Studies conducted by Bhushan and Hoondal [4] showed that chitinases from

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Bacillus sp. BG – 11 were highly compatible with common fungicides and insecticides. Chitinases also play a role in the utilization of crustacean waste [5]. Shrimp waste is considered an important source of chitin. A significant amount of shrimp waste is produced in Asia (primarily in Thailand – the main shrimp exporter – and India). Its utilization, however, is rarely carried out properly. Effective utilization would not only alleviate the environmental impact of shrimp waste, but also would provide considerable economic benefits [6]. Fungi can be potential biological control agents of insect, fungi and nematodes due to the production of fungial enzymes with an antagonistic action [7, 8].

The purpose of the present study was to investigate chitinolytic microorganisms from various habitats with high activity in order to determine whether chitinases produced by these organisms play a role in utilization of shrimp waste.

Materials and Methods

Object of the Study

Samples used in the study were obtained from water and bottom sediments of Lake Chełmżyńskie and from the soil of its watershed. The lake and its watershed are situated in the southern part of the Pojezierze Chełmińskie region within the Fryba River basin. The subglacial Chełmżyńska trough was formed as a result of drainage of ice-sheet meltwater, and presently lacustrine water fills the deepest section of the trough. Morphometric and trophic parameters of Lake Chełmżyńskie are included in Table 1.

Table 1. Morphometric and trophic characteristics of Lake Chełmżyńskie.

Characteristic	Value
Area (ha)[(1)]	271.1
Maximal deph (m)[(1)]	27.1
Mean deph (m)[(1)]	6.0
pH [(2)]	7.6-8.5
TP (mg dm^{-3}) [(2)]	0.04-0.05
TN (mg dm^{-3}) [(2)]	1.1-1.80
Electrolytic conductivity (μS cm^{-1}) [(2)]	601-703
Chlorophill a (μg dm^{-3})	26.4-56.9

[(1)] Data supplied by Provincial Inspectorate of Environmental Service in Bydgoszcz.
[(2)] Data supplied by Department of Environmental Microbiology and Biotechnology, Nicolaus Copernicus Universisty (data for 0.5 m depth, spring, summer, autumn 2007).

Isolation of Chitinolytic Microorganisms

Chitinolytic microorganisms were isolated from water, bottom sediments, and soil with Koch's plate technique by the surface inoculation on appropriate culture media. Analyzed samples were 10-times diluted, and 0.1 ml samples were placed on the medium surface. Inoculation was carried out in three parallel replicates. Chitinolytic bacteria were isolated using a medium containing (g/l): peptone (0.1), iron sulfate (0.1), ammonium sulfate (0.1), iron gluconate (0.1), colloidal chitin (7 g of dry mass), and agar (15). Nystatin (0.1 g/l) was added to the medium to inhibit the growth of fungi. After a 10-day incubation at 22ºC, areas around bacterial colonies were examined for lighter coloration, which indicated their capability to decompose chitin. These strains were isolated and placed in a culture medium with the above composition. The purity of isolated strains was checked with the Gram staining technique. Chitinolytic actinomycetes were isolated using the Hsu and Lockwood [9] medium containing (g/l): colloidal chitin (4 g of dry mass), magnesium sulfate (0.5), iron sulfate (0.01), zinc sulfate (0.001), manganese chloride (0.001), potassium phosphate, monobasic (0.7), and potassium phosphate, dibasic (0.3), and agar (20). Actinomycetes were incubated for 14 days at 28ºC, then areas around the cultures were examined for lighter coloration. These strains were transferred to test tubes with a fresh culture medium with the above composition. On classifying the colony into actinomyces, we considered the following characteristics [10]:

- the colony edges blurred because of radiating hyphae of substrate mycelium;
- the colony surface appearance: matt, chalk, down-like; sometimes aerial mycelium noticeable;
- the colony structure: tough, compact, leather-like;
- substrate-adherence degree: high;
- some actinomyces' colonies give off strong soil-like smell.

Microscopic fungi were isolated using the Czapek Dox medium containing (g/l): sodium nitrate (3), potassium dihydrogen phosphate (1), saccharose (20), potassium chloride (0.5), magnesium sulfate (0.5), iron sulfate (0.01), and agar (15). Streptomycin (30 mg/l) was added to the culture medium to inhibit the growth of bacteria. Following a 14-day incubation at 25ºC, 50-colony samples were inoculated to test tubes with the Czapek Dox medium, and used to determine the chitinolytic properties using the fluorometric method [11, 12]. The purity of fungi was examined with macroscopic and microscopic methods, staining the samples with methylene blue in lactophenol.

Culturing Chitinolytic Microorganisms

Eight-strain samples of bacteria, fungi, and actinomycetes isolated from water, bottom sediments, and soil were used to determine the chitinolytic activity. Chitinolytic microorganisms were cultured in 250 ml Erenmeyer flasks containing 100 ml of a culture medium. Bacteria were cultured in a medium containing (g/l): peptone 0.1; iron sulfate 0.1; ammonium sulfate 0.1; yeast

extract 0.1; and iron (II) sulfate 0.1. Fungi were cultured in a medium containing (g/l): sodium nitrate 3; potassium dihydrogen phosphate 1; saccharose 20; potassium chloride 0.5; magnesium sulfate 0.5; and iron sulfate 0.01. And finally, actinomycetes were cultured in medium containing (g/l): potassium phosphate dibasic 0.7; potassium phosphate monobasic 0.3; magnesium sulfate 0.5; zinc sulfate 0.001; and manganese chloride 0.001. Colloidal chitin (1.0 g), chitin powder (0.1 g), and ground shrimp shells (0.05 g) were added to each flask. The culture medium was inoculated with 1 ml samples of microorganism suspension obtained from a 72-hour incubation on agar slants. Each strain was analyzed in two replicates. Shrimp waste from the shrimp processing plant Krymar in Iłów was used in this study. Chitin powder was prepared from crab shells (Sigma-Aldrich, Poznan, Poland). Colloidal chitin was prepared after Lingappa and Lockwood [13].

Incubation was carried out for 4 days at 25°C, and then the cultures were centrifuged at 10,000 g/min for 10 min at + 4ºC. Acellular post-culturing liquid was used to determine the activity of chitinases, while the sediment was analyzed for protein content.

Determination of Chitinase Activity

The activity of chitinases produced by microorganisms was determined using the synthetic fluorogenic substrate 4-methylumbelliferyl N-acetyl-β-D-glucosaminide (4MU-GlcNac) (Sigma-Aldrich, Poznan, Poland). Methylcellosolve solvent (EGME, C3H8020) (Sigma-Aldrich, Poznan, Poland) was used to prepare a basic 1 mmol L^{-1} solution of 4MU-GlcNac, which was then stored at -20ºC. Prior to analysis, a 0.5 mmol L^{-1} working solution was obtained by diluting the basic solution twofold with spectrally pure water. For all assays, duplicates of 4.0 ml acellular post-culturing liquid, 0.5 ml substrate solution (final concentration was 50 µmol L^{-1}) and 0.5 ml of potassium phosphate buffer (final concentration 10 mmol L^{-1}; pH of the sample) were mixed and incubated for hours in darkness at in situ temperature. The control sample, prior to the addition of the substrate, was heated in boiling bath in order to deactivate the enzymes present in the sample. After incubation, enzymatic reactions were interrupted thermally. Fluorescence was additionally measured in all analyzed and control samples at the beginning and after incubation. The liberated methylumbelliferone was measured fluorimetrically at 318 nm excitation and 445 nm emission using a Hitachi F 2500 spectrofluorometer. The spectrofluorometer was calibrated with a solution of pure methylumbelliferone (Sigma-Aldrich, Poznan, Poland) [12]. One unit of chitinase activity was defined as 1 nmol 4-methylumbelliferone (MU) liberated per mg protein per hour.

Determination of Protein Content

The protein content in cultures of bacteria, fungi, and actinomycetes was determined with the Bradford [14] method.

Table 2. Effect of chitin sources on the activity of bacterial chitinase (nmol MU mg^{-1} protein h^{-1}), ± standard deviation (n=3).

No. strain	Origin of bacteria	Colloidal chitin	Chitin powder	Shrimp shells
1	Water	2.50±0.5	0.71±0.22	2.87±0.32
2		2.35±0.12	0.54±0.10	3.61±0.35
3		1.47±0.17	0.53±0.17	0.61±0.14
4		2.69±0.22	2.06±0.12	4.23±0.25
5		3.75±0.16	1.41±0.18	5.15±0.22
6		4.59±0.29	1.65±0.16	2.27±0.21
7		3.59±0.19	0.89±0.69	3.63±0.21
8		3.82±0.12	1.25±0.14	3.56±0.13
1	Bottom sediments	1.56±0.24	0.31±0.04	1.63±0.14
2		0.41±0.18	0.33±0.13	1.30±0.10
3		4.35±0.25	1.04±0.14	6.37±0.21
4		0.48±0.21	0.63±0.15	1.07±0.11
5		1.52±0.34	1.02±0.07	0.37±0.07
6		1.35±0.08	0.42±0.09	0.56±0.11
7		0.65±0.13	0.30±0.03	0.60±0.13
8		0.78±0.12	0.35±0.11	0.70±0.09
1	Soil	2.35±0.10	0.85±0.06	1.53±0.09
2		2.44±0.09	0.52±0.07	1.52±0.07
3		1.19±0.065	0.48±0.10	0.54±0.11
4		2.88±0.17	0.43±0.10	1.02±0.09
5		1.42±0.10	0.65±0.10	1.12±0.09
6		1.10±0.10	0.85±0.08	1.16±0.03
7		1.64±0.08	0.54±0.08	0.59±0.04
8		2.98±0.11	0.95±0.07	1.15±0.12

Results

Chitinolytic microorganisms were characterized by variable activity. In water, high chitinase activity was observed in bacteria and actinomycetes, while in bottom sediments, in fungi. In soil, actinomycetes exhibited noticeably higher chitinolytic activity than the other two groups of microorganisms. The highest chitinase activity of planktonic bacteria was observed in a medium containing colloidal chitin (1.47-4.59 nmol MU mg^{-1} protein h^{-1}) and shrimp shells (0.61-5.15 nmol MU mg^{-1} protein h^{-1}) (Fig. 1 and Table 2). Similarly in benthic bacteria, maximum activity was observed in the presence of colloidal chitin (0.41-4.35 MU nmol mg^{-1} protein h^{-1}) and shrimp shells (0.37-6.37 nmol MU mg^{-1} protein h^{-1}). Soil bacteria exhibited the highest activity in a culture medium containing colloidal chitin (1.10-2.88 nmol MU mg^{-1} protein h^{-1}).

Table 3. Effect of chitin sources on the activity of actinomycetales chitinase (nmol MU mg^{-1} protein h^{-1}), ± standard deviation (n=3).

No. strain	Origin of actinomycetes	Colloidal chitin	Chitin powder	Shrimp shells
1	Water	3.72±0.08	1.25±0.09	5.03±0.20
2		4.22±0.08	3.21±0.04	2.15±0.08
3		4.31±0.09	3.17±0.07	2.07±0.06
4		1.35±0.10	0.20±0.05	0.44±0.06
5		3.38±0.12	1.21±0.03	1.14±0.05
6		1.17±0.05	1.23±0.04	2.93±0.05
7		3.54±0.10	3.16±0.04	4.20±0.08
8		5.25±0.12	3.12±0.05	4.15±0.11
1	Bottom sediments	6.23±0.24	4.36±0.13	4.15±0.06
2		4.52±0.08	1.05±0.06	6.45±0.19
3		4.52±0.25	1.09±0.04	1.08±0.08
4		1.24±0.13	0.61±0.11	0.72±0.14
5		1.53±0.12	0.15±0.06	0.12±0.04
6		1.16±0.07	0.57±0.08	0.42±0.06
7		2.00±0.08	0.33±0.13	0.38±0.10
8		3.22±0.11	1.12±0.11	1.10±0.09
1	Soil	52.93±0.14	31.39±0.15	37.99±0.14
2		32.45±0.15	20.36±0.26	51.11±0.27
3		64.17±0.30	13.38±0.13	37.97±0.15
4		7.21±0.21	4.31±0.13	4.03±0.15
5		3.43±0.40	0.56±0.07	1.11±0.11
6		2.52±0.28	0.67±0.10	1.52±0.31
7		3.61±0.26	3.55±0.11	0.34±0.07
8		5.12±0.11	2.14±0.08	1.98±0.11

The analysis of chitinolytic activity of actinomycetes showed that strains isolated from the soil had considerably higher activity levels than those isolated from water or bottom sediments (Fig. 2 and Table 3). All soil actinomycetes had the highest activity in a culture medium containing colloidal chitin (2.52-64.17 nmol MU mg^{-1} protein h^{-1}). These organisms also showed high chitinase activity in the presence of shrimp shells (0.34-51.11 nmol MU mg^{-1} protein h^{-1}). In actinomycetes isolated from water and bottom sediments, chitinolytic activity levels were similar in the presence of various forms of chitin. Chitinolytic fungi reached the maximum activity in a culture medium containing colloidal chitin, and the highest activity was exhibited by the strains isolated from soil (2.16-11.31 nmol MU mg^{-1} protein h^{-1}). The presence of shrimp shells in a medium had the lowest impact on chitinolytic activity (Fig. 3 and Table 4). Chitin powder as a source of chitin in a culture medium had the least significant effect on the activity of chitinases produced by analyzed groups of microorganisms.

Discussion

The ability of bacteria, fungi, and actinomycetes to produce chitinolytic enzymes is common in nature. The great majority of these organisms are able to effectively break down chitin and use it as a source of carbon and energy and to synthesize chitinases during this process [15]. It is believed that chitinases are induced enzymes, which means that the expression of encoding genes is induced by a specific factor or factors. This expression is regulated by the repressor/inducer system. Chitin can function as an inducer, and glucose or other easy-to-assimilate source of carbon can serve as a repressor [16]. According to Frändberg and Schnürer [17], the production of chitinases can be induced

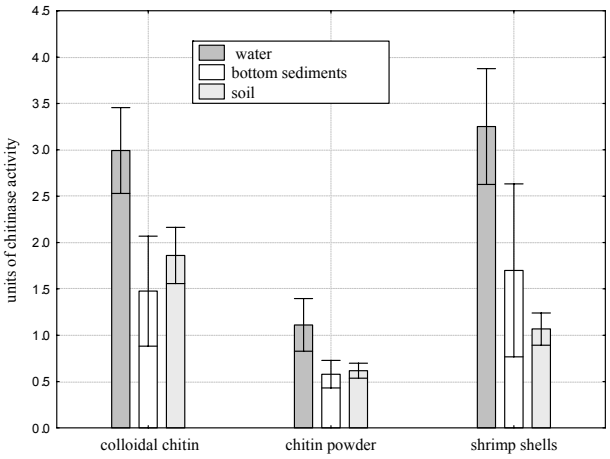

Fig. 1. Average chitinolytic activity of bacteria isolated from water, bottom sediments and soil.

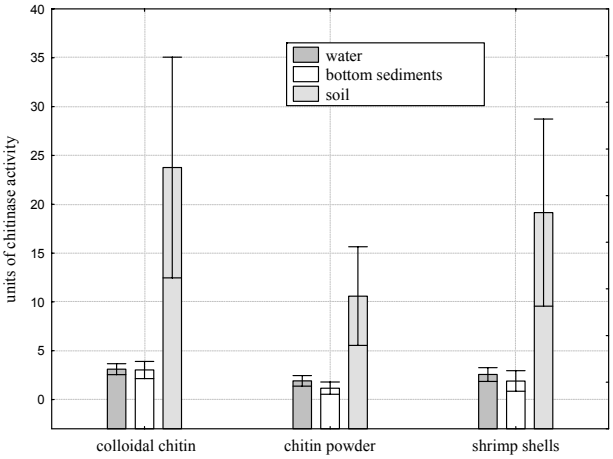

Fig. 2. Average chitinolytic activity of actinomycetes isolated from water, bottom sediments and soil.

Table 4. Effect of chitin sources on the activity of fungial chitinase (nmol MU mg^{-1} protein h^{-1}), ± standard deviation (n=3).

No. strain	Origin of fungi	Colloidal chitin	Chitin powder	Shrimp shells
1	Water	2.49±0.17	1.48±0.20	0.17±0.06
2		2.16±0.15	2.12±0.14	0.15±0.09
3		3.29±0.20	5.47±0.31	0.29±0.05
4		0.54±0.06	0.58±0.15	0.23±0.06
5		0.64±0.11	4.04±0.09	0.32±0.10
6		1.05±0.06	2.17±0.15	0.48±0.13
7		0.42±0.15	1.33±0.13	1.74±0.11
8		3.25±0.15	2.45±0.10	2.12±0.15
1	Bottom sediments	3.33±0.13	4.52±0.14	1.04±0.12
2		1.66±0.21	1.53±0.10	0.25±0.08
3		4.79±0.14	1.75±0.08	2.63±0.17
4		3.33±0.11	3.34±0.10	1.09±0.04
5		5.34±0.12	3.44±0.22	2.13±0.09
6		3.29±0.14	2.22±0.12	1.35±0.10
7		2.29±0.05	1.65±0.14	0.89±0.18
8		2.65±0.09	1.52±0.11	1.12±0.08
1	Soil	4.12±0.10	3.14±0.06	0.65±0.06
2		2.76±0.10	0.77±0.12	0.86±0.11
3		11.31±0.22	8.64±0.11	4.84±0.10
4		2.52±0.18	1.32±0.09	1.68±0.23
5		2.16±0.06	1.02±0.11	1.55±0.08
6		4.21±0.14	3.32±0.08	1.33±0.11
7		4.29±0.14	2.24±0.11	5.96±0.22
8		5.23±0.13	2.45±0.13	2.32±0.11

by the presence of chitin and chitooligosaccharides in a culture medium. In the absence of these substances, no extracellular secretion of chitinases was observed. The presence of N-acetylglucosamine (GlcNAc) did not induce chitinase secretion, which could be a result of catabolic repression. Monreal and Reese [18] reported that the production of chitinases by *Serratia marcescens* could even be inhibited by GlcNAc. In general, it was concluded that endochitinases were synthesized if chitin was the only source of carbon in a given environment, while the presence of N-acetylglucosamine induced the production of exochitinases [19, 20].

According to the present study, analyzed microorganisms exhibited the highest chitinolytic activity in the presence of colloidal chitin. Haran et al. [21] reported that adding chitin to a culture medium increased the activity of genes that encode these enzymes. Furthermore, according to Nampoothiro et al. [22], chitin, due to its colloidal character, is easier to assimilate and metabolize by microorganisms. Also, Nakaew's [23] study demonstrated that specific strains of fungi and bacteria showed high chitinolytic activity in media containing a 1% solution of colloidal chitin. In contrast, Wiwat et al. [3] reported that the activity of chitinases produced by *Bacillus circulans* No. 4.1 was high in the presence of colloidal chitin, but was the highest in the substrate containing glycol chitin. According to Nampoothiro et al. [22], the activity of chitinases produced by *Trichoderma harzianum* was most significantly induced by colloidal chitin. In contrast, in the presence of chitin powder and chitin flakes, this fungus showed low chitinolytic activity. El-Tarabily et al. [24] obtained similar results in the *Serratia marcescens, Streptomyces viridodiasticus,* and Micromonospora *carbonacea* strains – the highest activity was observed in culture media containing colloidal chitin. Admittedly, colloidal chitin is easier metabolized by microbes than other forms of chitin, but the present study demonstrated that shrimp shells also had a clearly stimulating impact on chitinase activity. Certain strains (primarily bacteria and actinomycetes) showed higher chitinolytic activity precisely in the presence of shrimp shells. The Rattanakit et al. [25] study demonstrated that in a culture medium containing shrimp waste, *Aspergillus* sp. synthesized the same or even larger amounts of chitinolytic enzymes than in a medium with colloidal chitin. Sabry [26], investigating microbiological decomposition of shrimp waste, reported that *Alcaligenes denitrificans* and *Bacillus subtilis* exhibited the highest chitinolytic activity in the presence of colloidal chitin, and *Bacillus amyloliquefaciens* and *Bacillus megaterium*, in a medium containing shrimp shells.

There is no doubt that the activity of chitinases depends on the type of a culture medium and individual characteristics of a given microbe. The present study demonstrated that shrimp shells could be used as a substrate in chitinase synthesis. Between 100 and 200 billion tons of "shell material" is produced annually, but from this total amount, only 5,000 tons of chitin is salvaged from North Sea crab shells and from Pacific and Greenland shrimp. The remaining

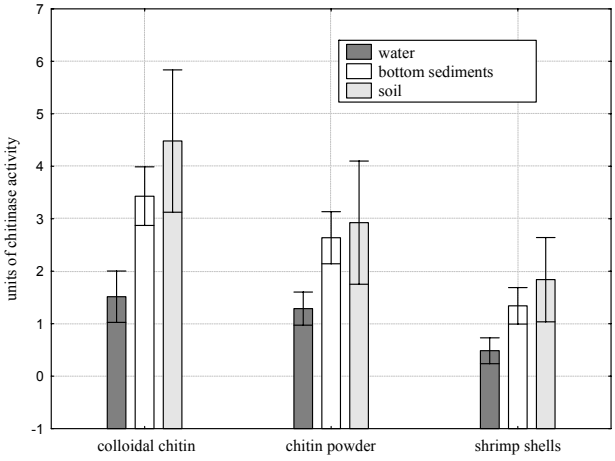

Fig. 3. Average chitinolytic activity of fungi isolated from water, bottom sediments and soil.

amount represents an enormous quantity of unutilized biomass; therefore, there is a growing interest and concern related to recycling waste containing chitin. Proper utilization of chitin waste is uncommon in spite of the fact that, if effective, recycling chitin waste would not only solve environmental problems, but would also provide significant economic benefits [27].

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