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Biodegradation of chitinous substances and chitinase production by the soil actinomycete *Streptomyces rimosus*

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ABSTRACT

A chitinolytic actinomycete was isolated from agricultural soil in the centre of Poland. It was identified as *Streptomyces rimosus*. Identification was based on standard biochemical tests and on 16S rRNA gene sequence analysis. The degradation of chitinous substances (shrimp shell waste, crab chitin powder and chitosan) was examined on the basis of oxygen consumption by applying OxiTop (WTW). The enzyme catalyzed the hydrolysis of the disaccharide 4-MU-$(GlcNAc)_3$ most efficiently and was therefore classified as an endochitinase. The chitinase was purified from culture medium by fractionation with ammonium sulfate and affinity chromatography. The purified proteins were subjected to identification by mass spectrometry. Based on analysis of the resultant fragments of the amino acid sequence of chitinase produced by the examined bacteria, degenerate primers for PCR were designed. The results of our research prove that the substances which are metabolised by *S. rimosus* the most efficiently are chitosan and the shrimp shell waste. Chitinolytic activity examination showed that shrimp shell waste was the finest inducer of chitinase synthesis. The molecular mass of the purified enzyme was 63 kDa. The highest activity of chitinase was obtained at the temperature of 40°–45 °C. Optimal pH for chitinase activity was 7.0. The activity of the purified chitinase was stabilized by Mg^{2+} ions. The activity of the enzyme was inhibited by Hg^{2+} and Pb^{2+} ions. Chitinase inhibited the growth of the fungal phytopathogens *Fusarium solani* and *Alternaria alternata*. The nucleotide sequence of the amplified gene fragment proved to be similar to the sequence of chitinase-encoding genes of the glycoside hydrolases family 18.

1. Introduction

Apart from cellulose, chitin is the major source of natural organic compounds (Yang et al., 2009). This long biopolymer chain contains N-acetyl-D-glucosamine (GLcNAc) monomers forming covalent β-1,4 linkages (Kim et al., 2007; Xayphakatsaa et al., 2008). Chitin is widely dispersed in the structural components of numerous organisms including crustacean and mollusc shells, arthropod exoskeletons as well as fungal cell walls (Ikeda et al., 2009; Lee et al., 2009). Chitin is created from marine food production waste, e.g. shrimp and crab shells or krill (Xu et al., 2008). Approximately 75% of the total weight of shellfish such as shrimp, crab and krill is considered waste, and chitin comprises from 20% to 58% of the dry weight of the said waste (Wang et al., 1997). Both degradation and recycling of chitin constitute an important phase in maintaining the global circulation of carbon and nitrogen (Hoang et al., 2011). Generally, they consist of microbiological processes, because chitin may be used by a micropopulation as the only source of carbon and nitrogen (Nielsen and Sørensen, 1999). Chitinases play an important role in the decomposition of chitin and potentially in the utilization of chitin as a renewable resource (Zarei et al., 2010). The enzymatic hydrolysis of chitin to free N-acetylglucosamine proceeds with the participation of chitinolytic enzymes which catalyze the hydrolytic depolymerization of chitin (Gohel et al., 2006; Saks and Jankiewicz, 2010). These enzymes are classified into glycosyl hydrolase subfamilies 18 and 19 on the basis of amino acid similarities within the catalytic domain (Henrissat and Bairoch, 1993). Chitinases are commonly found in many soil bacteria and fungi which are able to degrade chitin, a component of the fungal cell wall and a constituent of the exoskeletons of worms and arthropods (Hoster et al., 2005; Meanwell and Shama, 2008; Yu et al., 2008). Among the chitinolytic bacteria, several *Actinobacteria* and *Streptomyces* species are thought to degrade the chitinous cell wall of plant fungal pathogens through the production of chitinases and antibiotics (Kawase et al., 2006; Yu et al., 2008).

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The objective of this research was to investigate the extent of degradation of chitinous substances through both the actinomycete strain isolated from the soil and the purification as well as characterization of chitinases.

2. Materials and methods

2.1. Isolation and screening of actinomycetes

Chitinolytic actinomycetes were isolated from agricultural soil from the centre of Poland. Detection of chitinolytic activity was based on clearing zones around bacterial colonies growing on Actinomycete Isolation Agar (Difco) medium with colloidal chitin (2%). Cultures were incubated for 7 days at 28 °C. Colonies with the biggest halos were isolated and used for further investigations Strain identification involved morphological and biochemical tests (Williams et al., 1994) (Table 1) and on 16S rRNA gene sequence analysis. Amplification of the 16S rRNA gene was carried out using the universal primers 27F and 1401R (Watanabe et al., 2001). The matrix in the reaction was constituted by the genomic DNA separated from cells of bacteria in the subsequent stage of logarithmic growth, applying the method described by Kutchma et al. (1998). After purification, the product of PCR was sequenced in the DNA Sequencing and Oligonucleotides Synthesis Laboratory at IBB (PAN – Polish Academy of Sciences, Poland). The obtained nucleotide sequences were compared with sequences deposited in available databases of GenBank/EMBL/DDBJ using the software BLAST. Based on analysis of the sequence of the gene encoding 16S rRNA, the strain was classified as *Streptomyces rimosus* (GenBank accession no. AB7252387).

2.2. Biodegradation of chitinous substances

Biodegradation of chitinous substances was determined by the respirometric method. The method is based on changes in pressure following oxygen consumption in a hermetically closed bottle containing the sample. This apparatus measures the oxygen consumption almost continuously over the incubation period. The measurement of BOD with OxiTop® Control was carried out according to the operating instruction provided by the supplier WTW (1998) and the author's own materials. The experiment focused on the degradation of chitinous substances –crab shell powder chitin (Sigma–Aldrich), shrimp shell waste and chitosan (Sigma–Aldrich) – by the actinomycete strain isolated from the soil which is characterized by high activity of chitinases and was identified as *S. rimosus*. To conduct the experiment 100 ml of inorganic medium, prepared according to the standardised procedure ISO 14853 (International Standardisation, 1999), were placed in a measuring vessel. Subsequently, chitinous substances were added (0.05 g). Growth medium was inoculated with 2 ml of *S. rimosus* suspension. Samples were incubated for 14 days at 26 °C. Samples without chitinous substances were the *control samples*. The samples were analyzed in triplicate. Biodegradation rate was calculated by comparing the BOD with the theoretical oxygen demand (ThOD) and expressed as a percentage (O'Malley, 2006). Shrimp waste 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. Protein and fat from shrimp shells were removed using trypsin and ether, respectively.

Table 1
Morphological, physiological and biochemical characteristics of *Streptomyces rimosus*.

Characteristic	Results	Characteristic	Results
Mycelial pigment red-orange	–	Growth at 45 °C	-
Diffusible pigment produced	–	37 °C	+
Diffusible pigment yellow-brown	–	Growth with (% w/v) :	+
Melanin production on:		NaCl (% 4)	+
Peptone yeast iron agar	–	NaCl (% 7)	+
Tyrosine agar	–	Sodium azide (0.01)	–
Antibiosis against to:		Phenol (0.1)	–
Bacillus subtilis	+	Utilization of nitrogen sources:	
Micrococcus luteus	+	L-Cysteine	–
Lecithinase activity	+	L-Valine	–
Lipolysis	+	L-Phenylalanine	–
Pectin hydrolysis	+	L-Histidine	+
Chitin hydrolysis	+	Utilization of carbon sources:	
Proteolytic activity	+	Sucrose	–
H_2S production	+	Mannitol	+
Nitrate reduction	–	Raffinose	–
Resistance to:		Dextran	–
Neomycin (50 mg/ml)	+		
Rifampicin (5 mg/ml)	–		
Penicillin G (10 IU)	+		

2.3. Chitinase production

Five hundred ml of liquid medium containing: 0.2% sodium caseinate, 0.01% asparagines, 0.05% K_2HPO_4, 0.01% $MgSO_4 \cdot 7H_2$ (pH 7.5) were inoculated with 10 ml of *S. rimosus* suspension (10^6 spores/ml). The substrate for microbial chitinase production was added into each medium: shrimp shell waste, crab shell powder chitin or chitosan (2%). The culture was performed at 28 °C for 10 days with shaking (100 rpm) and then cultures were centrifuged at 10,000 × g for 10 min at + 4 °C. All experiments were conducted in triplicate.

2.4. Determination of chitinolytic activity

The fluorogenic substrates 4-methylumbelliferyl-β-D-N,N′, N′-triacetylchitotriose [4-MU-$(GlcNAc)_3$], 4-methylumbelliferyl-β-D-N, N′-diacetylchitobioside [4-MU-$(GlcNAc)_2$] and 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide [4-MU-GlcNAc] were used to detect endochitinases, exochitinases and N-acetylhexosaminidases, respectively (McCreath and Gooday, 1992; Lindahl and Finlay, 2005). Substrates were supplied by Sigma–Aldrich. Stock solutions of fluorogenic substrates were prepared in 50% ethanol at a concentration of 0.8 mM and stored at −20 °C. The reaction mixture contained: 1 ml crude enzymes, 0.125 ml substrate 4MU- $(GlcNac)_{1-3}$ solution (the final concentration in a sample was 50 μM/L and 0.125 ml of phosphate buffer (50 mM, pH 7). The mixture was incubated for 1 h in the dark at 40 °C. The control, prior to addition of the substrate, was treated with 0.1 ml solution of $HgCl_2$ in order to deactivate the enzymes present in the sample (final concentration: 4 mM/l). The mixture was incubated for 1 h in the dark at 40 °C. After incubation, enzymatic reactions were stopped by adding $HgCl_2$. The released 4-methy lumbelliferone (MU) was measured fluorimetrically at 318 nm excitation and 445 nm emission using the Hitachi F 2500 spectrofluorometer. One unit of enzyme activity (U) was defined as the amount of enzyme required to release 1 nM MU/mg protein/h. Protein content was determined according to method of Bradford (1976) with bovine serum albumin as a standard.

2.5. Purification of chitinase

Purification of chitinases involved two steps: fractionation with ammonium sulfate and chitin affinity chromatography. All purification procedures were carried out at the temperature of 4 °C. Cultures of *S. rimosus* were carried out in the medium

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containing shrimp shell (2%). After 6 days of incubation at 26 °C the culture was centrifuged at 10,000 rpm at 4 °C. The culture medium (2 l) was precipitated using ammonium sulfate to 85% saturation. The protein deposit was obtained by centrifugation (16,000 × g, 30 min) and dissolved in 50 mM sodium phosphate buffer (pH 7.0) and dialyzed against the same buffer overnight. The second step was chitin affinity chromatography according to the modified method of Escott et al. (1998). The same volume of 1% (w/v) colloidal chitin in 50 mM sodium phosphate buffer (pH 7.0) was added to the desalted enzyme solution and incubated for 2 h at 4 °C. This solution was centrifuged (10,000 × g, 15 min), the supernatant was discarded and the deposit was washed twice with 50 mM sodium phosphate buffer (pH 7.0). The bound proteins were eluted with 50 mM acetate buffer, pH 4.0 and dialyzed overnight. After each purification phase the activity of chitinases and protein content were determined. The highly purified enzyme preparation (50 μg/ml) was sent to the Mass Spectrometry Laboratory at IBB PAN Poland. A protein sample previously digested using trypsin was separated on a nanoAcquity UPLC (Ultra Performance LC) system and analyzed with an Orbitrap-based mass spectrometer.

2.6. Cloning of part of the chitinase gene

Isolation of genomic *S. rimosus* DNA was carried out with the use of Genomic DNA Purification Kit (Fermentas). For cloning the following pairs of primers were used:

F: 5′ GTCCAGCCTTTCTATTCTC 3′

R: 5′AGGAGTAGAGGATCTTGATG 3′

The PCR product was ligated into vector pGEM®-T (Promega) and propagated in *Escherichia coli* JM109 cells. Plasmids were isolated from white bacterial colonies in which the presence of the gene coding for chitinase in the plasmid was additionally verified by PCR using the abovementioned primers. Plasmids containing a DNA insert in the form of *S. rimosus* chitinase gene were sent for sequencing to the Laboratory of DNA Sequencing and Oligonucleotide Synthesis (IBB PAN Poland).

2.7. Characteristic of purified chitinase

The molecular mass of the enzyme was determined. Electrophoresis under denaturing conditions (SDS PAGE) was performed in 12% polyacrylamide gel according to the procedure described by Laemmli (1970) in Tris-Glycine buffer, pH 8.3. The protein bands were visualized using Coomassie Brilliant Blue R-250. The molecular mass of the enzyme was estimated by SDS-PAGE. The following molecular weight standards were used: phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21 kDa), and lysozyme (14 kDa). The optimum temperature activity of chitinase was determined between 40 and 60 °C. The optimum pH was determined in a range of 4.0–8.0. The buffer systems were the following: 50 mM acetate buffer for the pH 4–5 and 50 mM sodium phosphate buffer for the pH range of 6–8. The effects of various chemicals on enzyme activity was determined following pre-incubation of the purified chitinase for 30 min at 4 °C in the presence of divalent metal ions (Mg, Ca, Hg, Zn, Mn, Cd, Pb) and SDS, urea. Final concentrations of the chemicals were 1 mM. Afterward the substrate was added and the residual activity was tested. Antifungal activity of chitinase was also estimated using growth inhibition assay described earlier by Wang et al. (2002). The chitinase was tested for inhibitory activity against the growth of phytopathogenic strains: *Alternaria alternata*, *Fusarium oxysporum*, *Fusarium solani*, *Fusarium culmorum*, *Botrytis cinerea*. The fungi came from the Bank of Plant Pathogens in Poznan.

3. Results and discussion

3.1. Biodegradation of chitinous substances and production of chitinolytic enzymes

Degradation of chitinous substances was determined on the basis of oxygen consumption by *S. rimosus*. The highest oxygen consumption was recorded in the presence of chitosan (980 mgO_2/g OC after 14 days) and shrimp shells (890 mgO_2/g OC after 14 days). The chitin powder was used the very least (Fig. 1). From the analysis of data it appears that the highest oxygen consumption by *S. rimosus* in the presence of all chitinous substances took place after 10 days of incubation. In this case the uptake of oxygen was not so intensive. Biodegradation rate was calculated by comparing the BOD with the theoretical oxygen demand (ThOD). The study revealed that chitosan was biodegradable to the greatest extent (42.5% after 14 days). *S. rimosus* was also effective in shrimp shell decomposition (38.2% after 14 days). The chitin powder was degraded to the smallest extent (19.7% after 14 days) (Table 3). Hoang et al. (2011) studied the degradation of shrimp shells by *Streptomyces* sp TH-11 using an electron microscope and atomic force microscopy. They observed a significant reduction in the weight of shrimp shells on the following days: 7, 12 and 16. The researchers reported that chitin powder was well degraded by *Streptomyces* sp TH-11. This substrate constituted a good inducer of chitinases. Many authors report that shrimp shells and chitin powder are excellent material for the production of chitinases (Wang et al., 2005; Chang et al., 2010; Ghasemi et al., 2010; Hoang et al., 2011). This proves that the aforementioned substances are efficiently used by microorganisms as a source of carbon and nitrogen. Chitinases of *S. rimosus* were strongly induced by shrimp shells. The activity of actinomycete chitinases was slightly lower when the chitin powder was present in the substrate, whereas chitosan turned out to be the weakest inducer of chitinases (Fig. 2.) Wang et al. (1997, 2002a,b) conducted numerous research on the use of shrimp waste by bacteria. Shrimp and crab shell powder was used for production of chitinase by *Pseudomonas aeruginosa Bacillus amyloliquefaciens* V656 and *Monascus purpureus* CCRC31499. Rattanakit et al. (2007) reported that shrimp waste was an appropriate substrate for the culture of *Aspergillus* sp. S1–13 on solid

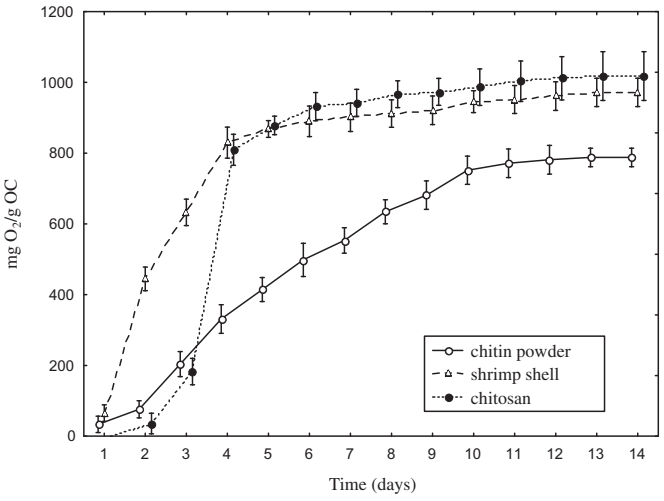

Fig. 1. Biological oxygen demand (BOD) obtained for tested chitinous substances.

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Table 2
Total organic carbon contents in the different samples.

Sample	TOC (%)
Chitin powder	45.7
Chitosan	40.2
Shrimp shell	26.9

nutrient medium. The organism synthesizes the same or even bigger amount of enzymes than a fungus grown on the nutrient medium with an addition of colloidal chitin. There is no doubt that shrimp shell waste was efficiently utilized by chitinases of microbiological origin. Nevertheless, many researchers claim that colloidal chitin is the best inducer of chitinases (Kim et al., 2007; Zarei et al., 2010). In all probability, for some microorganisms the absorption of colloidal chitin is easier in comparison with shrimp shells or chitin powder. Without any doubt, shrimp shell waste is efficiently utilized by chitinases of microbiological origin. Proper chitinous waste disposal is rare. However, if it is carried out effectively, it solves the environmental problems and has enormous economic value (Wang et al., 2002). According to our research, chitinous substances were intensively degraded during 10 days, after that period the degradation was less intensive. This may be caused by the appearance of different metabolites which can contribute to the inhibition of metabolism in the actinomycete.

Our studies show that *S. rimosus* produces different chitinolytic enzymes (Table 4). The use of fluorogenic substrates for determining the substrate specificity of chitinases with regard to the bond split is ambiguous. Based on the literature *N*- acetylglucosaminidases degrade 4-MU GlcNAc, exochitinases degrade 4-MU $(GlcNAc)_2$, and endochitinases 4-MU $(GlcNAc)_3$ and 4-MU $(GlcNAc)_4$ (McCreath and Gooday, 1992; Arora et al., 2003; Ruiz-Sànchez et al., 2005). According to Watanabe et al. (1990), exochitinases should not be able to release free 4 MU from 4-MU-$(GlcNAc)_3$. However, their studies indicate that the amount of free 4 MU released from 4- MU-$(GlcNAc)_3$ was 10 times higher than from 4-MU-$(GlcNAc)_2$. In turn, Lindahl and Finlay (2005) showed that 4MU-GlcNAc and 4MU-$(GlcNAc)_2$ were degraded by commercial endochitinases, but to a lesser degree than 4MU-$(GlcNAc)_3$. However, in samples where fluorescence with 4MU-$(GlcNAc)_3$ is low compared to that observed with 4MU-GlcNAc and 4MU-$(GlcNAc)_2$, fluorogenic substrates may be used as specific indicators of their respective target enzyme activities. In the samples used in our studies the highest enzyme activity was observed with the use of 4-MU-$(GlcNAc)_3$, which would point to the presence of an endochitinase. High activity of the enzyme was also observed with 4-MU-GlcNAc as the substrate. A review of the literature shows that could be a *N*-acetyl-β-glucosaminidase (exochitinase) (Eilenberg et al., 2006), i.e. a *N*-acetylhexosaminidase (exochitinase) (Harman et al., 2004; Lindahl and Finlay, 2005). Chitinolytic enzymes from *S. rimosus* showed highest activity after 6 days of incubation. Afterward, their activity decreased (Table 4). The lower activity of the enzymes after 6 days of incubation can be induced by depletion of growth-promoting substances and the presence of metabolites which not only inhibit the development of the actinomycete, but also inhibit the activity of the enzyme itself. Dahiya et al. (2005) reported maximum activity of chitinases from

Table 3
Biodegradation of the tested chitin substances (%).

No. of days	Chitosan	Chitin powder	Shrimp shell
6	21.2	5.2	19.3
10	34.3	15.4	30.9
14	42.5	19.7	38.2

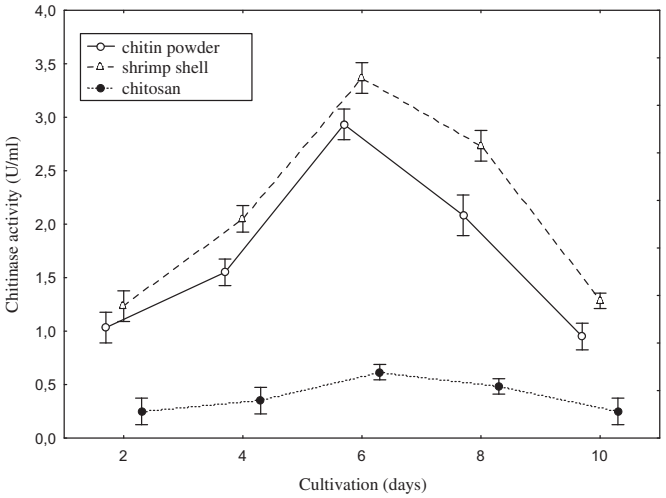

Fig. 2. Effect of time and chitinous substances on production of chitinase of *Streptomyces rimosus*.

Enterobacter sp. NRG4 obtained after 6 days of incubation. In some strains *Bacillus laterosporous* MML2270, *Bacillus* sp., *Streptomyces venezuelae* P_{10} and *Stenotrophomonas maltophilia*, maximum chitinase has already been observed after 4 days of incubation (Mukherjee and Sen 2006, Shanmugaiah et al., 2008; Xiao et al., 2009; ; Jankiewicz et al., 2012.

3.2. Purification of chitinases

Table 5 shows purification steps for chitinase from *S. rimosus*. Ammonium sulfate precipitation and chitin affinity adsorption resulted in 3.2-fold purified enzyme preparation with 24% recovery of activity. Purified chitinases were active at the level of 25 nmol/mg Narayana and Vijayalakshmi (2009) purified chitinase from *Streptomyces* ANU 6277 in a two-step process: ammonium sulfate precipitation and gel filtration by Sephadex G-100. The purification of the chitinase was increased to 5 fold with general yields at the level of 46.2%. Yano et al. (2008) applied a five-stage purification of chitinases from *Streptomyces cyaneus* Sp-27 and purified the enzyme preparation to 16 − fold with a general yield of 9.3%. However, Kavitha and Vijayalakshmi (2011) purified chitinases from *Streptomyces tendae* TK-VL 333 and obtained 3-fold purification of the enzyme preparation to with extremely high recovery of activity − 43%.Table 2.

3.3. Identification of purified enzymatic protein

The peptide sequence which was identified using mass spectrometry seemed to be similar to the sequences of chitinase C *Streptomyces albus* J1074, (Genbank accession: ZP_06593566). However, there were only 10% overlaps with chitinase C from *S. albus* J1074. Consequently, the additional cloning of one part of

Table 4
Chitinolytic activity of *Streptomyces rimosus* using different fluorescent substrates.

Substrate	Enzyme	Specific activity (U/mg protein)			
		2 Days	4 Days	6 Days	8 Days
4-MU GlcNAc	N-acetylhexaminidase	32.3	70.3	78.8	65.2
4-MU $(GlcNAc)_2$	Exo-chitinase	3.2	9.5	10.8	9.1
4-MU $(GlcNAc)_3$	Endo-chitinase	43.5	80.2	83.4	72.3

The substrate for microbial chitinolytic enzymes production was added into medium shrimp shell waste (2%). Cultivation was performed at 28 °C for 8 days.

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Table 5
Purification of chitinase from *Streptomyces rimosus*.

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification factor
Culture supernatant	230	1750	7.8	100	1
NH_4SO_4 (85%) dialysis	51	670	13.1	38	1.7
Affinity adsorption dialysis	17	425	25	24	3.2

Table 6
Characteristic of purified chitinase from *Streptomyces rimosus*.

Item	Characteristic
Molecular weight (kDa)	50
Optimal temperature (°C)	40
Optimal pH	8
Activator	Mg^{2+}, Ca^{2+}
Inhibitor	Hg^{2+}, Pb^{2+}
Antifungal activity	*Alternaria alternata*,*Fusarium solani*

the gene coding for chitinase strain *S. rimosus*. The obtained nucleotide sequence (900 bp), after being translated into the amino acid sequence, showed full identity with the sequence of *S. albus* J1074 chitinase (Fig. 3). The nucleotide sequence of the amplified gene fragment was similar to the sequence of genes encoding chitinases from the glycoside hydrolases family 18, type C.

3.4. Characteristic of purified chitinase

Table 6 shows the characteristics of purified chitinase. After native electrophoretic separation of the purified preparation, one protein band with molecular weight 63 kDa was observed (Fig. 4). The molecular weight of chitinases produced by actinobacteria and determined by SDS-PAGE is different: 35 kDa in *Microbispora* sp. V2 (Nawani et al. 2002), 43 kDa and 45 kDa in *Streptomyces albovinaceus* (El-Abyad et al. 1993), 49 kDa in *Streptomyces griseus* HUT 6037 (Tanabe et al. 2000), 66 kDa in *S. venezuelae* P10 (Mukherjee and Sen, 2006). The optimal temperature for purified chitinase *S. rimosus* activity was 40 °C. The optimum reaction temperatures of chitinases from *Streptomyces* were reported to be at the level of 40 °C (Gomes et al., 2001; Freeman et al., 2004). The optimum temperature of chitinase activity *Streptomyces* CU 36 was 40 °C and 45 °C (Kamel et al., 1993) and for *Streptomyces* sp. IK 30 °C (Margino et al., 2010).

Optimal pH for chitinase activity from *S. rimosus* was 7.0. According to Margino et al. (2010) chitinase from *Streptomyces* sp. IK was optimally active at pH 6.7. Han et al. (2009) reported that the chitinase from *Streptomyces* sp. DA11 had maximum activity at pH 8.0. As for other species of *Streptomyces*, the optimum pH for chitinase activity was from 3.3 to 7.5 (Gomes et al., 2001).

The activity of the purified chitinase was stabilized by Mg^{2+} ions, while Hg^{2+} and Pb^{2+} ions strongly inhibited the enzyme activity. Similar results were obtained by other researchers (Kim et al., 2007; Han et al., 2009). Antifungal activity of the purified chitinase against *F. solani* and *A. alternata* was observed. Many bacteria produce antifungal chitinase: *Enterobacter* sp. NRG4, *Pseudomonas* sp. TKU015, *Streptomyces hygroscopicus*, *Bacillus pumilus* SG2 (Dahiya et al., 2005; Wang et al., 2008; Prapagdee et al., 2008; Ghasemi et al., 2010). *S. rimosus* is recognized mainly because of the fact that it produces an antifungal antibiotic (Yu et al., 2008). Our research shows the potential of

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  1mrsahpahdg sgsrprplrr lrrrtlaalt glaipfagmv glaapsqaad apsasytrtq
                                     GLAIPFAGMV GLAAPSQAAD APSASYTRTQ
 61dwgtgfqarw snwtlewefp agtkvtlpwe aevsndgnrw taknrswngt tvqntgdtpl
   DWGTGFQARW SNWTLEWEFP AGTKVTLPWE AEVSNDGNRW TAKNRSWNGT TVQNTGDTPL
121 lapgasttfg fngtgdgapt gcklngvpce gaeqpedtpp tapgtptvsk vtrnaatlsw
    LAPGASTTFG FNGTGGGAPT GCKLNGVPCE GAEQPEDTPP TAPGTPAISK VTRNAATLSW
181 taatddkgvk dyevlrngtr vatvtgrtft dhdlapgtdy syavrardta kqtgpfsgaa
    TAATDDKGVK DYEVLRNGTR VATVTGRTFT DHGLAPGTDY SYTVRARDTA KQTGPFSGAA
241 kartkgkaap apkskitmgy ftewgiydkn yqvknlvtsg saekithiny afgdvrdgkc
    KARTKGKAAP APKSKITMGY FTEWGIYDKN YQVKNLVTSG SAEKITHINY AFGDVRDGKC
301 vagdteaayg kvftadeavd gvadrpdqpl hgnlnqlrkl kaefphikvl wsfggwswsg
    VAGDTEAAYG KVFTADEAVD GVADRPDQPL HG..........................
361 gfgeavkdap afarschelv edprwadvfd gidldweypn acgdtcddsp pealtdmmra
421 mraefgtdll aaaiaadase ggkldradya daaeyvdwyn vmtydyfgtw aaqgptaphs
481 pltaypgiqg ehntssatia klrgkgipak klllgigaygrgwtgvtqda pggtatgpaa
541gtyeagneey rvlaekcpat gtaggtayak cgddwwsydt petvtgkmaw akkqklggaf
601  lwefagdgak gdlframheg lr
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Fig. 3. Multiple-sequence alignment of chitinase from studied *Streptomyces rimosus*. Small letters : amino acid sequence of chitinase C from *Streptomyces albus* J1074 (Genbank accession number: ZP_06593566) BIG LETTERS : amino acid sequence translated from nucleotide sequence from obtained chitinase *S. rimosus* clone, Letters Underlined: LC–MS/MS analysis of chitinase *S. rimosus*.

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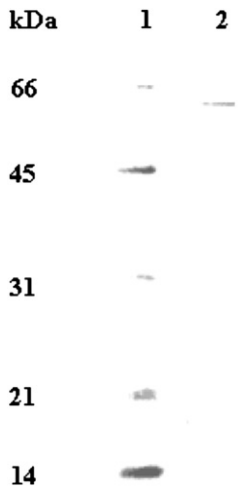

Fig. 4. SDS–PAGE analysis of purified chitinases from *Streptomyces rimosus* 1. Molecular weight markers. 2. Purified chitinase preparation.

chitinase produced by *S. rimosus*. Not only does the chitinase produced by this microorganism degrade chitinous substances, but it also inhibits the growth of phytopathogens.

4. Conclusion

The studies described investigate the extent of degradation of chitinous substances through both the actinomycete strain isolated from the soil and the purification as well as characterization of chitinases *S. rimosus* is recognized mainly because of the fact that it produces antimicrobial antibiotic. Our research shows the potential of chitinase produced by *S. rimosus*. The chitinase produced by this microorganism degrades the chitinous substances and also inhibits growth of phytopathogens. In this way they contribute to the circulation of carbon and nitrogen in nature and they take part in plant protection. Biological combating plant pathogens provides alternative methods of treating plant diseases without the adverse effects of chemical fungicides, which are usually expensive and can cause environmental pollution. Our research shows that *S. rimosus* produces different chitinolytic enzymes. The most active was endochitinase, which may be of great importance in the biocontrol.

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References

Arora, N., Ahmad, T., Rajagopal, R., Bhatnagar, R.K., 2003. A constitutively expressed 36 kDa exochitinase from *Bacillus thuringiensis* HD-1. Biochemical and Biophysical Research Communications 307, 620–625.

Bradford, M.M., 1976. Rapid and sensitive methods for the quantitation of microgram quantities of protein utilizing the principle of protein - dye binding. Analytical Biochemistry 72, 248–254.

Chang, W.T., Chen, M.L., Wang, S.L., 2010. An antifungal chitinase produced by *Bacillus subtilis* using chitin waste as a carbon source. World Journal of Microbiology and Biotechnology 26, 945–950.

Dahiya, N., Tewari, R., Tiwari, R.P., Hoondal, G.S., 2005. Production of an antifungal chitinase from *Enterobacter* sp. NRG4 and its application in protoplast production. World Journal of Microbiology and Biotechnology 21, 1611–1616.

Eilenberg, H., Pnini-Cohen, S., Schuster, S., Movtchan, A., Zilberstein, A., 2006. Isolation and characterization of chitinase genes from pitchers of the carnivorous plant Nepenthes khasiana. Journal of Experimental Botany 57, 2775–2784.

El-Abyad, M.S., El-Sayad, M.A., El-Shanshoury, A.R., El-Sabbagh, S.M., 1993. Towards the biological control of fungal and bacterial diseases of tomato using antagonistic *Streptomyces* spp. Plant Soil 149, 185–195.

Escott, G.M., Hearn, V.M., Adams, D.J., 1998. Inducible chitinolytic system of *Aspergillus fumigatus*. Microbiology 144, 1575–1581.

Freeman, S., Minzm, O., Kolesnik, I., Barbul, O., Zveibil, A., Maymon, M., 2004. Trichoderma biocontrol of *Colletotrichum acutatum* and *Botrytis cinerea* and survival in strawberry. European Journal of Plant Pathology 110, 361–370.

Ghasemi, S., Ahmadian, G., Jelodar, N.B., Rahimian, H., Ghandili, S., Dehestani, A., Shariati, P., 2010. Antifungal chitinases from Bacillus pumilus SG2: preliminary report. World Journal of Microbiology and Biotechnology 26, 1437–1443.

Gohel, V., Singh, A., Vimal, M., Ashwini, Ph., Chatpar, H.S., 2006. Bioprospecting and antifungal potential of chitynolitic microorganisms. African Journal of Biotechnology 5, 54–72.

Gomes, R.C., Semedo, L.T.A.S., Soares, R.M.A., Linhares, L.F., Ulhoa, C.J., Alviano, C.S., 2001. Purification of thermostable endochitinase from *Streptomyces* RC1071 isolated from a cerrado soil and its antagonism against phytopathogenic fungi. Journal of Applied Microbiology 90, 653–661.

Han, Y., Yang, B., Zhang, F., Miao, X., Li, Z., 2009. Characterization of antifungal chitinase from marine *Streptomyces* sp. DA11 associated with south China Sea Sponge Craniella Australiensis. Marine Biotechnology 11, 132–140.

Harman, G.E., Petzoldt, R., Comis, A., Chen, J., 2004. Interactions between *Trichoderma harzianum* strain T22 and maize inbred line Mo17 and effects of these interactions on diseases caused by *Pythium ultimum* and *Colletotrichum graminicola*. Phytopathology 94, 147–153.

Henrissat, B., Bairoch, A., 1993. New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. Biochemical Journal 293, 781–788.

Hoang, K.C., TH, Lai, Lin, C.S., Chen, Y.T., Liau, C.Y., 2011. The chitinolytic activities of Streptomyces sp. TH-11 International Journal of Molecular Sciences 12, 56–65.

Hoster, F., Schmitz, J.E., Daniel, R., 2005. Enrichment of chitinolytic microorganisms: isolation and characterization of a chitinase exhibiting antifungal activity against phytopathogenic fungi from a novel *Streptomyces* strain. Applied Microbiology and Biotechnology 66, 434–442.

Ikeda, M., Miyauchi, K., Mochizuki, A., Matsumiya, M., 2009. Purification and characterization of chitinase from the stomach of silver croaker *Pennahia argentatus*. Protein Expression and Purification 65, 214–222.

International Standardisation, ISO 14853, 1999. Determination of the Ultimate Aerobic Biodegradability of Plastic Materials in an Aqueous Medium Method by Measuring the Oxygen Demand in a Closed Respirometer.

Jankiewicz, U., Swiontek Brzezinska, M., Saks, E., 2012. Identification and characterization of a chitinase of *Stenotrophomonas maltophilia*, a bacterium that is antagonistic towards fungal phytopathogens. Journal of Bioscience and Bioengineering 113, 30–35.

Kamel, Z., Heikel, N., Fahmy, F., 1993. Extracellular chitinase from *Streptomyces* sp. and its antifungal activity. Acta Pharmaceutica Turcica 35, 135–143.

Kavitha, A., Vijayalakshmi, M., 2011. Partial purification and antifungal profile of chitinase produced by *Streptomyces tendae* TK-VL-333. Annals of Microbiology 61, 597–603.

Kawase, T., Yokokawa, S., Saito, A., Fujii, T., Nikaidou, N., Miyashita, K., Watanabe, T., 2006. Comparison of enzymatic and antifungal properties between family 18 and 19 chitinases from *S. coelicolor* A3(2). Bioscience Biotechnology and Biochemistry 70, 988–998.

Kim, H.S., Timmis, K.N., Golyshin, P.N., 2007. Characterization of a chitinolytic enzyme from *Serratia* sp. KCK isolated from kimchi juice. Applied Microbiology and Biotechnology 75, 1275–1283.

Kutchma, A.J., Roberts, M.A., Knaebel, D.B., Crawford, D.L., 1998. Small-scale isolation of genomic DNA from *Streptomyces* mycelia or spores. Biotechniques 24, 452–456.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–688.

Lee, Y.G., Chung, K.C., Wi, S.G., Lee, J.C., Bae, H.J., 2009. Purification and properties of a chitinase from *Penicillium* sp. LYG 0704. Protein Expression and Purification 65, 244–250.

Lindahl, B.D., Finlay, R.D., 2005. Activities of chitinolytic enzymes during primary and secondary colonization of wood by basidiomycetous fungi. New Phytologist 169, 389–397.

Margino, S., Nugroho, A.J., Asmara, W., 2010. Purification and characterization of *Streptomyces* sp. IK chitinase. Indian Journal of Biotechnology 15, 29–36.

McCreath, K.J., Gooday, G.W., 1992. A rapid and sensitive microassay for determination of chitinolytic activity. Journal of Microbiological Methods 14, 229–237.

Meanwell, R.J., Shama, G., 2008. Production of streptomycin from chitin using *Streptomyces griseus* in bioreactors of different configuration. Bioresource Technology 99, 5634–5639.

Mukherjee, G., Sen, S.K., 2006. Purification, characterization, and antifungal activity of chitinase from *Streptomyces venezuelae* P10. Current Microbiology 5, 265–269.

Narayana, K.J.P., Vijayalakshmi, M., 2009. Chitinase production by *Streptomyces* sp. Anu 6277. Brazilian Journal of Microbiology 40, 725–733.

Nawani, N.N., Kapadnis, B.P., Das, A.D., Rao, A.S., Mahajan, S.K., 2002. Purification and characterization of a thermophilic and acidophilic chitinase from *Microbispora* sp. V2. Journal of Applied Microbiology 93, 965–975.

Nielsen, M.N., Sørensen, J., 1999. Chitynolitic activity of *Pseudomonas fluorescens* isolates from barley and sugar beet rhizosphere. FEMS Microbiology Ecology 30, 217–227.

O'Malley, L.P., 2006. Evaluation and modification of the OECD 301F respiratory biodegradation test methods with redard to test substance concentration and inoculum. Water Air Soil Pollution 177, 251–265.

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Prapagdee, B., Kuekulvong, C., Mongkolsuk, S., 2008. Antifungal potential of extracellular metabolites produced by *Streptomyces hygroscopicus* against phytopathogenic fungi. International Journal of Biological Sciences, 330–337.

Rattanakit, N., Yano, Sh., Plikomol, A., Wakayama, M., Tachiki, T., 2007. Purification of Aspergillus sp. S1-13 chitinases and their role in saccharification of chitin in mash of solid-state culture with shellfish waste. Journal of Bioscience and Bioengineering 103, 535–541.

Ruiz-Sànchez, A., Cruz-Camarillo, R., Salcedo-Hernàndez, R., Barboza-Corona, J.E., 2005. Chitinases from *Serratia marcescens* Nima. Biotechnology Letters 27, 649–653.

Saks, E., Jankiewicz, U., 2010. Chitinolytic activity of bacteria (in polish). Progress Biochemistry 56, 427–434.

Shanmugaiah, V., Mathivanan, N., Balasubramanian, N., Manoharan, P.T., 2008. Optimization of cultural conditions for production of chitinase by *Bacillus laterosporous* MML2270 isolated from rice rhizosphere soil. African Journal of Biotechnology 7, 2562–2568.

Tanabe, T., Kawase, T., Watanabe, T., Uchida, Y., Mitsutomi, M., 2000. Purification and characterization of a 49 kDa chitinase from *Streptomyces griseus* HUT 6037. Journal of Bioscience and Bioengineering 89, 27–32.

Wang, S.L., Chiou, S.H., Chang, W.T., 1997. Production of chitinase from shellfish waste Pseudomonas aeruginosa K-187. Proceedings of the National Science Council, Republic of China 21, 71–78.

Wang, S.L., Yen, Y.H., Tsiao, W.J., Chang, W.T., Wang, C.L., 2002. Production of antimicrobial compounds by *Monascus purpureus* CCRC31499 using shrimp and crab shell powder as a carbon source. Enzyme and Microbial Technology 31, 337–344.

Wang, S.L., Hsiao, W.J., Chang, W.T., 2002a. Purification and characterization of an antimicrobial chitinase extracellularly produced by *Monascus purpureus* CCRC31499 in a shrimp and crab shell powder medium. Journal of Agricultural and Food Chemistry 50, 2249–2255.

Wang, S.L., Shih, I.L., Liang, T.W., Wang, C.H., 2002b. Purification and characterization of two antifungal chitinase extracellularly produced by *Bacillus amyloliquefaciens* V656 in a shrimp and crab shell powder medium. Journal of Agricultural and Food Chemistry 50, 2241–2248.

Wang, S.L., Yen, Y.H., Tzeng, G.C., Chienyan, Hsieh, C., 2005. Production of antifungal materials by bioconversion of shellfish chitin wastes fermented by *Pseudomonas fluorescens* K-188. Enzyme and Microbial Technology 36, 49–56.

Wang, S.L., Chena, S.J., Wang, C.L., 2008. Purification and characterization of chitinases and chitosanases from a new species strain *Pseudomonas* sp. TKU015 using shrimp shells as a substrate. Carbohydrate Research 343, 1171–1179.

Watanabe, T., Oyanagi, w., Suzuki, K., Tanaka, H., 1990. Chitinase system *of Bacillus circulans* WL-12 and importance of chitinase Al in chitin degradation. Journal of Bacteriology 172, 4017–4022.

Watanabe, K., Kodama, Y., Harayama, S., 2001. Design and evaluation of PCR primers to amplify bacterial 16S ribosomal DNA fragments used for community fingerprinting. Journal of Microbiological Methods 44, 253–262.

Williams, S.T., Sharpe, M.E., Holt, J.G., 1994. Bergey's Manual of Systematic Bacteriology, vol. 4. Williams and Wilkins, London.

WTW, 1998. Applikationsbericht BSB 997 230: Respirometrische BSB5-Bestimmung von häuslichem Abwasser mit dem OxiTop® Control – oder OxiTop®- Messsystem. Wissenschaftlich-technische Werkstätten (WTW), Weilheim.

Xayphakatsaa, K., Tsukiyamaa, T., Inouyeb, K., Okumotoa, Y., Nakazakia, T., Tanisaka, T., 2008. Gene cloning, expression, purification and characterization of rice (Oryza sativa L.) class II chitinase CHT11. Enzyme and Microbial Technology 43, 19–24.

Xiao, L., Xie, Ch., Cai, J., Lin, Z.J., Chen, Y.H., 2009. Identification and characterization of a chitinase-produced *Bacillus* showing significant antifungal activity. Current Microbiology 58, 528–533.

Xu, Y., Gallert, C., Winter, J., 2008. Chitin purification from shrimp wastes by microbial deproteination and decalcification. Applied Microbiology and Biotechnology 79, 687–697.

Yang, C.Y., Ho, Y.C., Pang, J.C., Huang, S.S., Tschen, J.S., 2009. Cloning and expression of an antifungal chitinase gene of a novel Bacillus subtilis isolate from Taiwan potato field. Bioresource Technology 100, 1454–1458.

Yano, S., Rattanakit, N., Honda, A., Noda, Y., Wakayama, M., Plikomol, A., Tachiki, T., 2008. Purification and characterization of chitinase A of *Streptomyces cyaneus* SP-27: an enzyme participates in protoplast formation from Schizophyllum commune Cymelia. Bioscience, Biotechnology, and Biochemistry 72, 54–61.

Yu, J., Liu, Q., Liu, Q., Liu, X., Sun, Q., Yan, J., Qi, X., Fan, S., 2008. Effect of liquid culture requirements on antifungal antibiotic production by *Streptomyces rimosus* MY02. Bioresource Technology 99, 2087–2091.

Zarei, M., Aminzadeh, S., Zolgharnein, H., Safahieh, A., Ghoroghi, A., Motallebi, A., Daliri, M., Lotfi, A.S., 2010. *Serratia marcescens* B4A chitinase product optimization using Taguchi approach. Iranian Journal of Biotechnology 8, 252–262.

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