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ORIGINAL PAPER

Chitooligosaccharides enzymatic production by *Metarhizium anisopliae*

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Abstract The products of chitosan hydrolysis are chitooligosaccharides and are used mainly for medical applications due to their specific biological activities. The objective of this study was to detect and identify the products of enzymatic hydrolysis of chitosan (dimers to hexamers) using a crude extract of chitosanolytic enzymes produced by the fungus *Metarhizium anisopliae*. These fungus was able to produce, during 48 h cultivation in a medium containing chitosan, chitooligosaccharides ranging from dimers, trimers, tetramers and pentamers at concentrations 0.2, 0.19, 0.06, 0.04 mg/mL, respectively, and the enzymatic activity was 2.5 U/L. Using the crude enzyme extract for chitosan hydrolysis, we detected the presence of dimers to hexamers at hydrolysis times of 10, 20, 30, 40, 50 and 60 min of enzymatic reaction, but the yields were higher at 10 min (54%). The hexamers was obtained only with 30 min of reaction with concentration of 0.004 mg/mL.

Keywords Palavras Chaves: *Metarhizium anisopliae* · Chitosan · Chitooligosaccharides · Hydrolysis of chitosan

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Introduction

Chitin is one of the most abundant polysaccharides in nature. It is one of the main components of the cell wall of fungi, the exoskeleton of arthropods, such as crustaceans (crab, lobster, and shrimp), and insects (ants, beetles, and butterflies); and is present in the radula of mollusks and in the beaks of cephalopods (e.g. squid and octopus) [1].

Chitosan is a deacetylated derivative of chitin which is a linear polysaccharide consisting of β-1,4-*N*-acetyl-glucosamine [2]. Chitosan is insoluble in water, but dissolves in aqueous solutions of organic acids, such as acetic, formic, and citric acids, and in inorganic acids, such as diluted hydrochloric acid. The solubility of chitosan is related to the amount of protonated amino groups ($-NH_3+$) in the polymer chain. More these groups are, the greater the electrostatic repulsion between the chains and the greater the solvation in water. Therefore, the degree of acetylation of chitosan has a marked effect on its solubility [3].

Chitosan demonstrates antimicrobial, antiviral, and antifungal properties that make it a favorable option for biomedical applications [4]. In the food industry, chitosan has been used as a stabilizer and thickener. It can also be used as a preservative, cleaning agent, and health food additive [5, 6]. In cosmetics, chitosan forms a protective and hydrating elastic coat on the surface of the skin that has the ability to bind the other ingredients that act on the skin. In the environmental field, chitosan has been used as adsorbent of heavy metal ions and organic compounds [7, 8].

Chitosan is hydrolyzed to chitooligosaccharides by chitinolytic enzymes, which are produced by microorganisms and some plants [9]. Chitosanases (EC 3.2.1.132) are glycosyl hydrolases that catalyze the β-1, 4 glycosidic bond hydrolysis of chitosan to produce glucosamine

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oligosaccharides. These enzymes are gaining increasing importance because of their products, low-molecular weight chitosans and chitosan oligomers, which are obtained by partial enzymatic hydrolysis and have applications in the medical and pharmaceutical fields [10, 11]. Chitosanases are found in microorganisms, bacteria, and fungi [12–14].

The entomopathogenic fungus *Metarhizium anisopliae* produces a chitinolytic system, which is believed to have a role in the digestion of insect cuticles during the development of diseases [15]. This microorganism is a filamentous fungus, Deuteromycetes, Moniliales order and Moniliaceae family [16]. A chitinolytic system was analyzed in terms of secretion and also in terms of regulation, demonstrating high variation among the isolates [17, 18].

Studies on chitosan have increased interest in its conversion to chitooligosaccharides, because these compounds are soluble in water and have potential use in various biomedical applications [19]. COS are known to exhibit many biological activities, such as antifungal [20], antibacterial [21–23], and antitumor [24, 25] activities; immune cell proliferation effects [26]; and protection against infection [21, 22].

The objective of this study was to produce and identify the products of enzymatic hydrolysis of chitosan (COS) using a crude extract of chitosanolytic enzymes produced by the fungus *M. anisopliae*.

Materials and methods

Reagents

Chitosan (85% deacetylated, molecular weight (MW): 90–190 KDa, acquired from Sigma–Aldrich (MO, USA)) was prepared using the modified method of Yabuki et al. [27]. The patterns of chitosan oligomers (dimers, trimers, tetramers, pentamers, hexamers) were acquired from Seikagaku Co. (Japan). All other reagents used were the highest quality available.

Microorganism

The microorganism, *M. anisopliae,* strain (CG374), used in this study was kindly provided by EMBRAPA Genetic Resources and Biotechnology (Brasilia/DF-Brazil). The strain was maintained on a potato dextrose agar (PDA) containing 1% of yeast extract at 4 °C.

Fermentation conditions

Spore suspension (10 mL) (10^7 spores/mL) from a 5-day-old culture in PDA medium was transferred using 2 mL of sterile water. Next, this spore suspension was transferred to a 250 mL Erlenmeyer containing 90 mL of culture medium consisting of: 0.2% chitosan, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.5% KCl, 0.3% yeast extract, 0.5% peptone, 0.2% $NaNO_3$, and 0.001% $FeSO_4$ (pH 5.5) [28]. The growth was carried out in a rotation incubator for 2 days at 25 °C and 110 rpm. From this suspension, 10 mL was transferred to 90 mL of the same medium. Samples of this culture were taken every 12 h, the broth was centrifuged at 13,400*g* for 15 min, and the supernatant was used to determine the enzyme activity assay and the reducing sugars.

Chitosanolytic activity

Enzymatic activity was assessed by determining the reducing sugars generated by chitosan hydrolysis. In this case, 500 µL of the fermented broth was mixed with 500 µL of chitosan solution solubilized in hydrochloric acid (0.1 N). The reaction was carried out for 30 min at 55 °C. To terminate the reaction, 2.5 mL of dinitrosalicylic acid was added and then cooled in an ice bath, and quantification of the reducing sugars was performed using a spectrophotometer (Thermo Spectronic) at 600 nm [29] and a standard curve with D-glucosamine. One unit (U) of chitosanase was defined as the amount of enzyme that is capable of releasing 1 µmol of reduced sugar equivalent to chitosan D-glucosamine/min.

Chitooligosaccharide production

The production of chitosan oligomers was accomplished through enzymatic hydrolysis (using the crude extract of chitosanolytic enzymes of the fungus *M. anisopliae*). The reactions were performed in a mixture containing a total volume of 1.0 mL (0.5 mL chitosan solution and 0.5 mL of crude enzyme extract). The reactions were incubated at 55 °C for 10, 20, 30, 40, 50, and 60 min. The reaction was stopped by immersing the tube in boiling water for 10 min [30]. For the time of 48 h, the hydrolysates were analyzed in HPLC.

Analysis and quantification of chitosan oligosaccharides

Detection of chitosan oligosaccharides was conducted using high-performance liquid chromatography (HPLC) and a Shim-Pack CLC-NH_2 column (Shimadzu Co., Japan). Oligomer analysis was carried out using acetonitrile (60%) as a mobile phase at a flow rate of 0.8 mL/min and a refractive index detector. The $(GlcN)_{n=2-6}$ chitooligomer peaks were quantified using a standard curve (1–10 mg/mL) in accordance with Liang et al. [31].

Results and discussion

Production of chitosanolytic enzymes

In this work, we chosen *M. anisopliae* for study because it is a entomopathogenic fungus and produce chitinolytic enzymes, which are believed to have a role in the digestion of insect cuticles, then it will possible produce inducible chitosanolytic enzymes when in the presence of chitosan as carbon and energy source. According to ours results, the fungus *M. anisopliae*, when grown in a medium containing chitosan as its sole source of carbon, is able to induce enzyme production with chitosanolytic activities. Figure 1a and b show chitosanolytic enzymatic activity and the concentration of reducing sugars, respectively, during cultivation of the fungus.

Observed in Fig. 1a are two peaks showing chitosanolytic activity (48 and 170 h) with the most activity, 2.5 U/L, occurring after 48 h of cultivation. A chitosanolytic activity of 1.5 U/L was obtained using the *Fusarium solani* fungus [32], which is the same order of magnitude as that obtained at 170 cultivation hours using *M. anisopliae*. Regarding the time at which the highest peak of chitosanolytic activity was obtained, the results are similar to that found by Valadares-Iglis and Peberdy [15], who studied production of chitinolytic enzymes by the *M. anisopliae* fungus in a medium containing chitin. *M. anisopliae*, when grown in a medium containing chitin, is capable of producing enzymes with chitinolytic activity. Some authors found that the chitinolytic enzymes of the *M. anisopliae* fungus also seem to be dependent on the induction system used, development stage, germination, or exponential growth phase [15, 33]. Entopathogenic fungi, such as *M. anisopliae*, when growing in a liquid culture containing insect cuticles as a carbon source, produce a variety of chitinolytic enzymes [17]. With respect to the peak obtained at 170 h of cultivation, a hypothesis for its existence is that during the microorganism's growth phase, it secretes enzymes extracellularly to allow growth, most of which are endochitosanases and during prolonged cultivation times it begins to secrete predominantly exochitosanases [15]. The amount of reducing sugars increased significantly, mainly due to the presence of chitosanolytic enzymes in the culture medium, and reached the maximum in 48 h, the length of time in which chitosanolytic activity is maximized. Thus, the amount of sugars formed by enzymatic hydrolysis is higher than that consumed by the microorganism. There is a slight reduction in reducing sugars after 48 cultivation hours (Fig. 1b). This fact can be explained by the use of reducing sugars for the metabolism of the fungus itself.

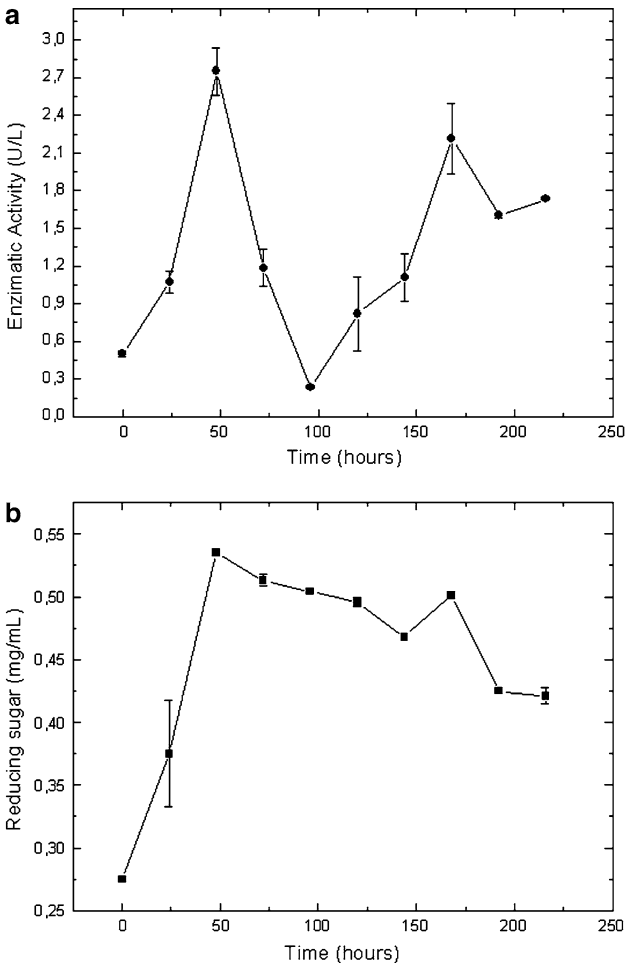

Fig. 1 **a** Production of chitosanase enzyme by *M. anisopliae* in medium containing chitosan (0.2%), at 25 °C, 110 rpm and **b** reducing sugar produced during cultivar. Values are means of triplicate replicase with standard deviations <10%

Detection and identification of oligomers produced during 48 h of cultivation

During cultivation of the *M. anisopliae* microorganism, the composition of the broth containing chitosanolytic enzymes was analyzed using HPLC, as shown in the chromatograms in Fig. 2a, b.

It is important to note that these oligomers were produced during 48 h of cultivation. These results show that during cultivation of the *M. anisopliae* fungus, the production of dimers to pentamers occurs. In Fig. 1a, we can observe that the concentration of reducing sugars with 48 h of cultivation was 0.55 mg/mL. According to Fig. 3, the concentration of total reducing sugars (dimers to pentamers) was 0.5 mg/mL, showing that the amount of reducing sugars produced during 48 h of cultivation consisted of chitooligomers from dimers to pentamers. The other growing periods were not analyzed because our objective was to determine the cultivation time in which we would see the highest enzymatic activity and assess whether

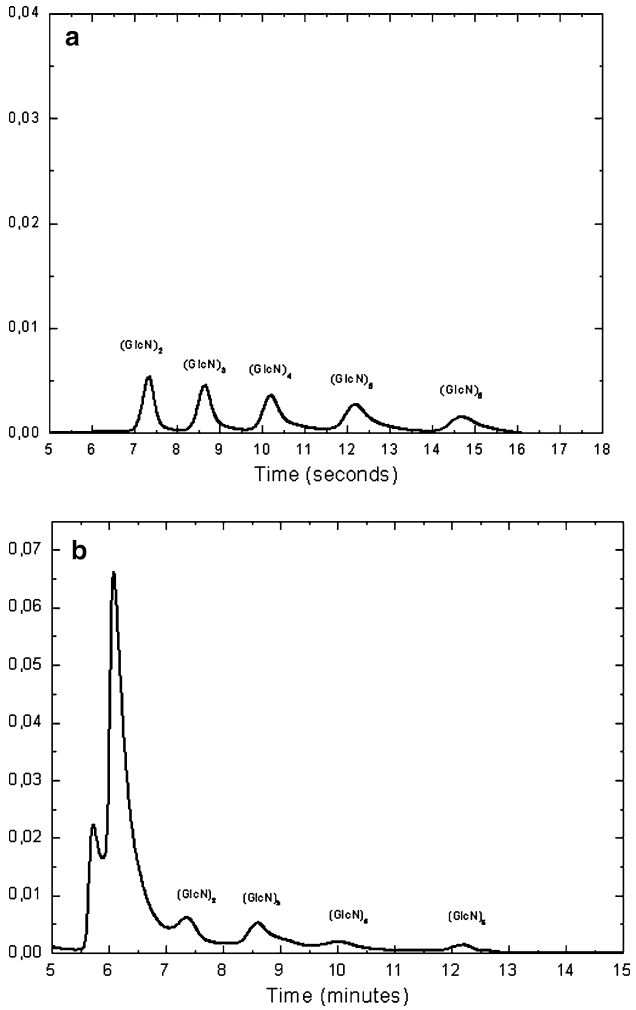

Fig. 2 Chromatograms of the (GlcN)n, 2-6 patterns (**a**) and of the production of COS formed over 48 h of cultivation of *M. anisopliae* (**b**). Chromatograms were performed through HPLC of Shim-Pack CLC-NH_2 column. Oligomer analysis was carried out using acetonitrile (60%) as a mobile phase at a flow rate of 0.8 mL/min and a refractive index (RI) detector

oligomer production occurred during this period. Later, the crude enzyme extract was used for chitosan hydrolysis.

Oligomer production using the crude enzyme extract produced by the fungus

The crude extract (cell free) containing chitosanolytic enzymes produced by the *M. anisopliae* fungus was used to hydrolyze chitosan (10 mg/mL) and the chitosanolytic activity of this extract was 2.3 U/mL during hydrolysis (data not shown).The chromatograms obtained from the enzymatic hydrolysis at different hydrolysis times (10, 20, 30, 40, 50 and 60 min) are shown in Fig. 4a–f, respectively. Table 1 shows the yields of chitosan oligomers obtained at different hydrolysis times.

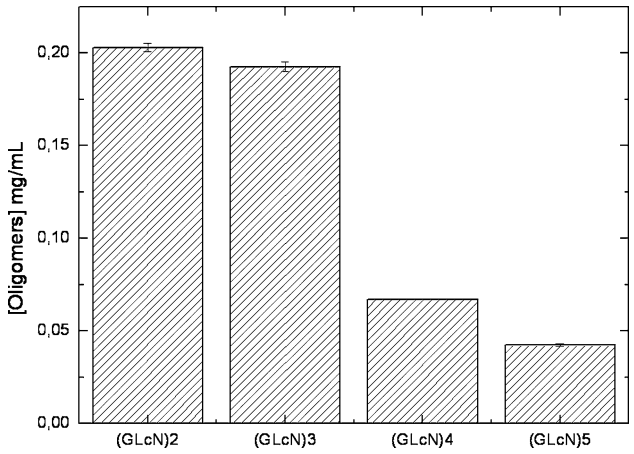

Fig. 3 Concentration of oligosaccharides (dimers to pentamers) during 48 h of fungus cultivation in medium containing 0.2% chitosan, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.5% KCl, 0.3% yeast extract, 0.5% peptone, 0.2% $NaNO_3$, and 0.001% $FeSO_4$ (pH 5.5). The growth was carried out in a rotation incubator for 2 days at 25 °C and 110 rpm

The data verify that the best yields were obtained within 10 min of hydrolysis (54%). Taking into consideration the time and productivity of oligomers using the crude enzyme extract of the *M. anisopliae* fungus, we can see that the enzymatic system is very efficient. Choi et al. [34] obtained a total yield of 40% oligomers over 60 reaction hours using *Bacillus* sp. KCTC 0377BP chitosanase; and Roncal et al. [35], using pure enzyme obtained from Sigma–Aldrich, obtained a yield of 46.3% COS.

According to Fig. 5, it is observed that the highest concentration of oligomers was obtained at 10 min of hydrolysis, and as hydrolysis occurs, the concentration of chitosan oligomers (dimers to pentamers) decreases. This decrease in the concentration of COS may suggest that there is an increase in the production of the chitosan monomer (GlcN) through a combined action mechanism of the complex of chitosanolytic enzymes, a result of both endo- and exo-enzymes [36].

The biological properties of COS depend on the degree of polymerization. Chitosan oligomers show higher biological activity than chitosan, and the best functional characteristics are obtained with oligomers with a degree of polymerization between pentamer and heptamer compared to oligomers with a relatively low degree of polymerization [37].

According to Fig. 5, the maximum concentration of pentamers (0.8 mg/mL) occurred with 10 min of hydrolysis. The hexamers were only detected after 30 min of hydrolysis at low concentrations (0.004 mg/mL).

The oligomer production system used in this study aimed at verifying the possibility that enzymes produced by the *M. anisopliae* fungus would hydrolyze chitosan into

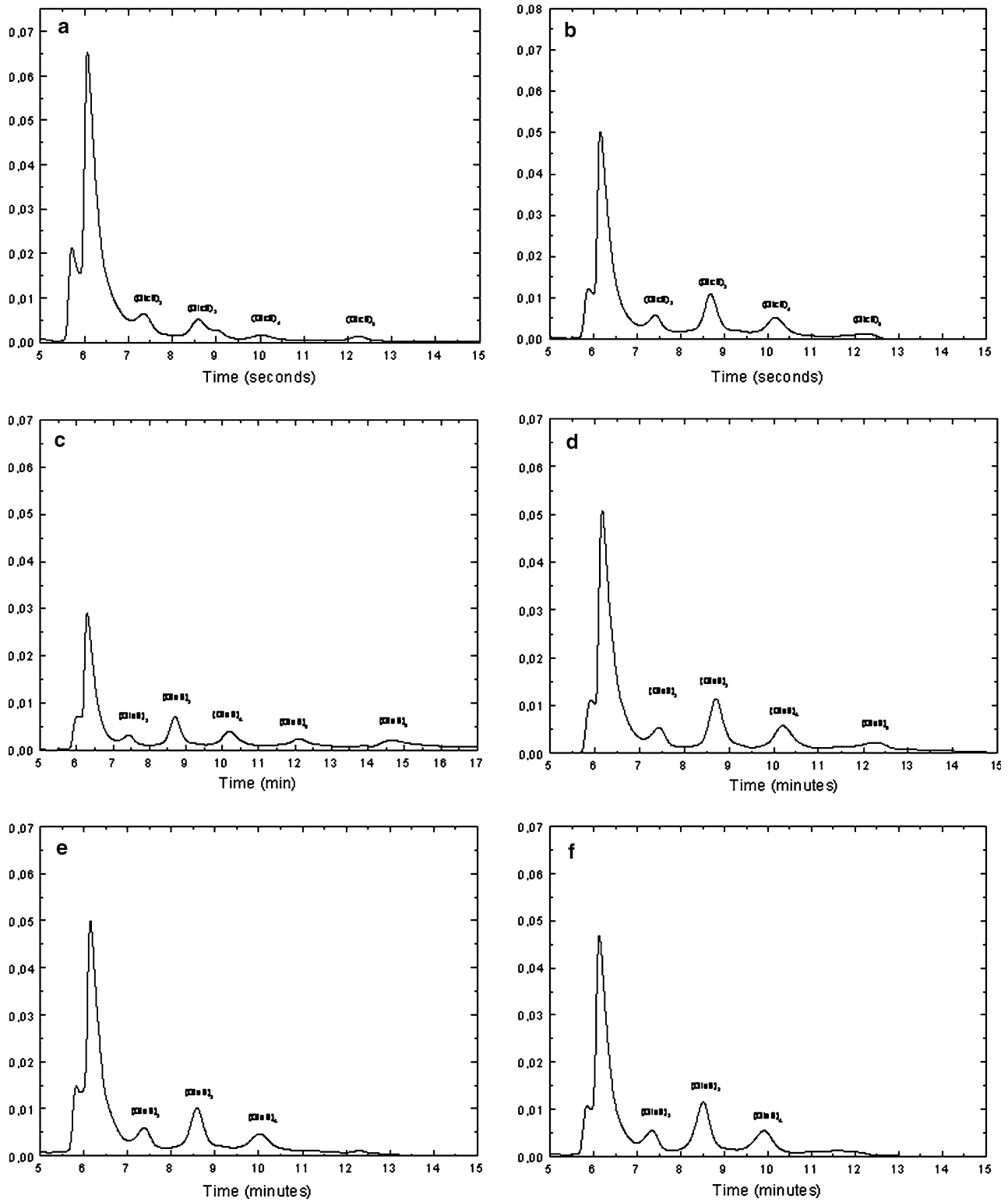

Fig. 4 Chromatograms of hydrolyzed chitosan formed by the incubation of chitosan with the crude enzyme extract produced by the *Metarhizium anisopliae* fungus. **a** 10, **b** 20, **c** 30, **d** 40, **e** 50, **f** 60 min. Chromatograms were performed through HPLC of Shim-Pack CLC-NH_2 column. Oligomer analysis was carried out using acetonitrile (60%) as a mobile phase at a flow rate of 0.8 mL/min and a refractive index (RI) detector

Table 1 $(GlcN)_{2-6}$ oligomers production by different times of hydrolysis measured in HPLC

Hydrolysis time (min)	Total yields (mg/mL) $(GlcN)_{2-6}$	Relative production[a] (10 mg/mL de chitosan) (%)
10	5.43	54.3
20	2.53	25.3
30	1.87	18.7
40	0.76	7.6
50	1.06	10.6
60	0.42	4.2

[a] Relative production was measured by the total $(GlcN)_{2-6}$ yield/chitosan concentration × 100

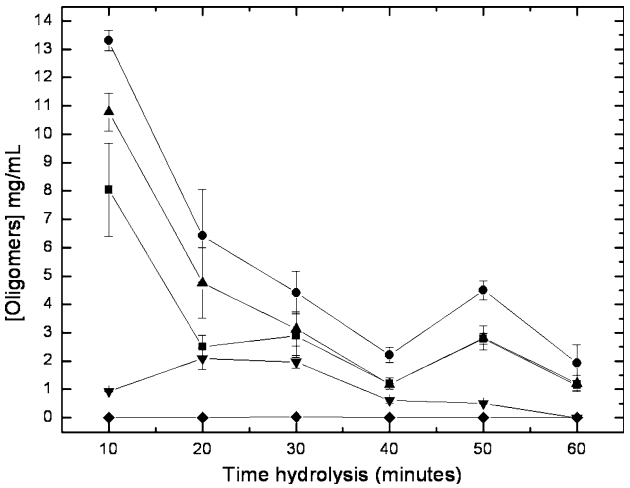

Fig. 5 Concentration of chitosan COS by the crude enzyme extract produced by *Metarhizium anisopliae*. Dimers (*filled square*), trimers (*filled triangle*), tetramers (*filled triangle*), pentamers (*inverted triangle*) and hexamers (*filled diamond*). The results represent the ±SD mean of three experiments in triplicate

oligomers of commercial interest. Most studies on oligomer production with a degree of polymerization between pentamers and hexamers were performed using a continuous system with immobilized enzymes [21, 22, 38–41]. However, in the present study a batch of satisfactory results were obtained.

Conclusion

COS have drawn considerable attention for their application, mainly in medicine. Several authors have studied the production of COS using immobilized enzyme systems with the goal of increasing the production of products of interest, mainly pentamers and hexamers. This process is more expensive because it usually works with the purified enzyme in a continuous production system. This study verified the ability of the *M. anisopliae* fungus to produce a crude enzyme extract in an induction medium using chitosan as a main source of carbon. Later, these chitosanolytic enzymes were used in chitosan hydrolysis. In addition, the oligomers formed during 48 h of microorganism cultivation were analyzed and the presence of dimers, trimers, tetramers and pentamers was found, but in small concentrations. To increase the concentration of oligomers chitosan hydrolysis was carried out using the crude enzyme extract produced by the fungus. Higher concentrations of oligomers were obtained during 10 min of hydrolysis. It was observed that the crude extract containing chitosanolytic enzymes produced by *M. anisopliae* is effective in the production of COS, facilitating its potential in industrial application.

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