Constitutive chitosanase from *Bacillus thuringiensis* B-387 and its potential for preparation of antimicrobial chitooligomers

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Abstract

The article proves the ability of the entomopathogenic strain *B. thuringiensis* var. *dendrolimus* B-387 to high the constitutive production (3–12.5 U/mL) of extracellular chitosanase, that was found for the first time. The enzyme was purified in 94-fold by ultrafiltration, affinity sorption and cation-exchange chromatography and characterized biochemically. The molecular mass of the chitosanase determined using SDS-PAGE is 40 kDa. Temperature and pH-optima of the enzyme are 55 °C and pH 6.5, respectively; the chitosanase was stable under 50–60 °C and pH 4–10.5. Purified chitosanase most rapidly (∼43 µM/mL × min, K_M ~ 0.22 mg/mL, k_cat ~ 4.79 × 10^4 s^{-1}) hydrolyzed soluble chitosan of the deacetylation degree (DD) 85% by endo-mode, and did not degrade colloidal chitin, CM-cellulose and some other glucans. The main reaction products of the chitosan enzymolysis included chitobiose, chitotriose and chitotetraose. In addition to small chitooligosaccharides (CHOs), the studied chitosanase also generated low-molecular weight chitosan (LMWC) with average M_w in range 14–46 kDa and recovery 14–35%, depending on the enzyme/substrate ratio and incubation temperature. In some cases, the chitosan (DD 85 and 50%) oligomers prepared using crude chitosanase from *B. thuringiensis* B-387 indicated higher antifungal and antibacterial activities in vitro in comparison with the initial polysaccharides. The data obtained indicate the good prospect of chitosanase B-387 for the production of bioactive CHOs.
Introduction

Microbial chitinases (EC 3.2.1.14) and chitosanases (EC 3.2.1.132) are considered as one of excellent "tools" for the enzymatic conversion of various chitin-containing wastes generated annually in enormous quantities during capture and processing of shellfish (Wang et al. 2011; Das et al. 2016; Pechsrichuang et al. 2018; Kaczmarek et al. 2019; Subramanian et al. 2020; Doan et al. 2020). According to the data of the Food and Agriculture Organization of the United Nations (FAO), only aquaculture production of crustaceans and mollusks reached more than 25 million tons in 2016 (The State of World Fisheries and Aquaculture 2018). Chitin as the major structural polymer of exoskeleton, inner coverings and cell walls is found in most invertebrate animals, as well as in filamentous and yeast fungi, some unicellular algae and protozoan (Durkin et al. 2009; Abo Elsoud et al. 2019; Yadav et al. 2019; Garcia-Rubio et al. 2020; Jones et al. 2020). Therefore, chitin as a unique structural analog of cellulose in animal and fungal life is also occurred ubiquitously, being a minimum second polysaccharide (after cellulose) in its world abundance with an annual production of more than $10^{10}$–$10^{11}$ tons only in aquatic ecosystems (Beier and Bertilsson 2013). Utilization of numerous chitin-containing sources, on the one hand, is intended to overcome the challenge caused by the global pollution of marine waters and coastal areas with shellfish processing wastes (Yadav et al. 2019). On the other hand, chitin is a valuable material that is highly promising in diverse fields of biotechnology, medicine, pharmacology, agriculture etc. (Khan et al. 2017). The main biotechnological role assigned to chitosanases in view of the purposes of the chitin wastes utilization is the partial destruction of chitosan, being deacetylated chitin derivative, for production of water-soluble and low-viscous bioactive oligomers (beginning DP 5-6) (Naqvi and Moerschbacher 2017). The products of extensively hydrolyzed chitosan including D-glucosamine (GlcN) and chitobiose (GlcN$_2$) forming under the action of exo-chitosanases (EC 3.2.1.165) and some endo-chitosanases, respectively, are also valuable compounds for medicine/pharmacology and they present a promising starting material for
the chemical-enzymatic synthesis of higher chitooligosaccharides (CHOs) and their substituted derivatives (Jung and Park 2014). Enzymatic depolymerization of chitosan has many advantages over the physico-chemical methods of its destruction making the controlled process under mild conditions of reaction mixture including temperature, pH and ionic strength parameters possible; in the absence of random modifications of functional groups and formation of undesired byproducts (Kaczmarek et al. 2019). As compared with other enzymes displaying chitosanolytic activity, chitosanases demonstrate the highest specific action and effectively degrade the polysaccharide under minimal enzyme–substrate ratios resulting in a sharp decrease of reaction products impurity with microbial proteins and metabolites (Pechsrichuang et al. 2018). This is a crucial factor for the preparation of CHOs with high-quality characteristics required especially for medicinal, pharmacological and food uses. At the same time, the widespread biotechnological application of chitosanases is still restricted due to their production expense; therefore, the search of several approaches for its cost reduction remains one of current trends in explorations of these enzymes. In view of the characterization of alternative microbial producers of chitosanase (including the enzymes with remarkable catalytic and physico-chemical properties), the capability of their cultivation in the absence of such expensive inductor substrate as chitosan acquire significant importance. This work is the first to report constitutively produced chitosanase in member of Bacillus thuringiensis, entomopathogenic species that has been practiced on a wide scale as biopesticide (insecticide) in crop production, horticulture and related fields (Ibrahim et al. 2010).

To date, chitosanases from B. thuringiensis (Bt) have been limitedly characterized in few studies if compared with chitinases (Cruz Camarillo et al. 2004; Lee et al. 2007; Koboyashi et al. 2011). Moreover, their role in bacterial pathogenesis to various insect pests, as well as their involvement in antagonism against phytopathogenic fungi (in terms of antagonistic strains Bt) remains unexplored and far from being fully understood. The potential of chitosanases from Bt for production of bioactive CHOs is still being studied rather fragmentarily and reported in just a few works, mainly, in view of chitosan conversion to oligomers with polymerization degree (DP) 3–7 (Olicón-Hernández et al. 2016; Kang et al. 2018; Santos-Moriano et al. 2018). This study is intended to purify and characterize the extracellular constitutive chitosanase from B. thuringiensis B-387, and to evaluate its potential for the production of bioactive CHOs.

Materials and methods

Reagents

The chitosan (Mw ~ 370 kDa, DD 85%) and partially N-acetylated chitosan (Mw ~ 200 kDa, DD ~ 50%) were obtained from the Laboratory of Biopolymer Engineering of the Institute of Bioengineering of Federal Research Center “Fundamentals of Biotechnology” of Russian Academy of Sciences (Moscow, Russia). Flake crab shell chitin was provided by BioProgress Co. (Shchelkovo, Russia) and grinded in laboratory mill. Colloidal chitin was prepared from powdered crab shell chitin by the modified method of Rodriguez-Kabana et al. (1983). Colloidal chitosan was prepared as previously reported (Helistö et al. 2001). N-acetyl-β-D-glucosamine, D-glucosamine, p-nitrophenyl-N-acetyl-β-D-glucosaminide (p-NP-GlcNAc), p-nitrophenyl-N,N’-diacetyl-β-D-chitobioside (p-NP-GlcNAc₂), p-nitrophenol, sodium salt of carboxymethylcellulose, azokazein, laminarin and xylan from birch were purchased from Sigma Chemical Co., USA. CM-Sepharose Fast Flow was purchased from GE Healthcare (USA). The nutritional components of culture media (tryptone, yeast extract etc.) were obtained from Panreac (Barcelona, Spain) and HiMedia Laboratories Pvt. Ltd. (Mumbai, India). All other reagents, including components for SDS-PAGE, TLC, buffer salts etc. used in the study were qualified as analytical and high grade.

Microorganism cultivation and enzyme production

The chitosanase-producing strain, B. thuringiensis var. dendrulimus B-387 was obtained from the All-Russian Collection of Microorganisms (VKM, Pushchino—Moscow, Russia) and it is also available in ATCC under number 19266 (Gordon et al. 1973). The strain is the base of the biopesticide “Dendrobacillin” effectively employed against Siberian silkworm larvae (Talalaev et al. 1971). The strain was maintained under 36 °C on LB agar and on basal medium containing (g/L): K₂HPO₄•3H₂O, 1; KH₂PO₄, 0.5; (NH₄)₂HPO₄, 0.5; MgSO₄•7H₂O, 0.4; CaCl₂, 0.2; bacteropeptone, 3; yeast extract, 3; corn steep liquor, 0.5; colloidal chitin (from crab shells), 5; agar, 16 (pH ~6.8 before autoclaving). In order to produce chitosanase the strain was previously grown in LB broth overnight on Innova 40R shaker (New Brunswick Scientific, USA) under 36 °C and 220 rpm. Grown bacterial cells were inoculated into LB broth (1:50, v/v) and cultivated in 250-mL Erlenmeyer flasks for 4 days at the same conditions. Thereafter, the liquid culture was centrifuged (Sigma 2-16PK (Germany), 5 °C, 6800 x g for 30 min) for cell biomass removal, and clarified supernatant was subjected to further purification.
Purification of chitosanase and SDS-PAGE

At the initial stage, the culture supernatant was concentrated by ultrafiltration on VivaFlow 200 (Sartorius, Germany) module with 10 kDa cut-off membrane. The enzyme concentrate obtained after this procedure was further subjected to affinity sorption on colloidal chitosan (DD 85%, 5 mg/mL) as previously reported (Aktuganov et al. 2019). Finally, the chitosanase was purified by cation-exchange chromatography on CM-Sepharose Fast Flow using Biologic LP chromatographic system (Bio-Rad, USA). The chitosan-adsorbed fraction of the enzyme was loaded onto the column (2.5 × 10 cm) packed with CM-Sepharose previously equilibrated by four volumes of 50 mM tris–HCl buffer (pH 8.5). Unadsorbed proteins were removed from the sorbent with 75 mM of equilibrating buffer; subsequently the sorbent was successively washed by 0–500 mM of NaCl linear gradient (75 mL) and 500 mM of NaCl (100 mL) in the same buffer under flow rate 1.5 mL/min. Active fractions were pooled, desalted and concentrated using VivaPore 10/20 concentrators (Sartorius, Germany). Purity degree and molecular mass of the chitosanase were estimated by denaturing electrophoresis (SDS-PAGE) in 12.5% polyacrylamide gel with 0.1% of sodium dodecyl sulfate according to Laemmli (1970). PageRuler Broad Range unstained protein ladder (Thermo Scientific, USA) containing a mixture of eleven recombinant proteins ranging from 5 to 250 kDa was used as a molecular mass standard. Separated proteins in gel were visualized using a colloidal solution of Coomassie Brilliant Blue G-250 in the mixture of phosphoric acid (85%)—ethanol—water (2:20:80 v/v) containing 8% (w/v) sulfate ammonium. In further the gel was washed with deionized water to remove background staining.

Measurement of chitosanase/chitinase activity and protein determination

Chitosanase and chitinase activities were determined by hydrolysis rate of soluble chitosan (Mw ~ 370 kDa, DD 85%) and colloidal chitin, respectively. For chitosanase assay, 0.5 mL of 0.5% (w/v) of chitosan solution in 100 mM sodium acetate (pH 6) was added to 1 mL of enzyme solution in the same buffer. The reaction mixture was incubated for 1 h under 50 °C and stopped by 5-min heating in boiling water bath and centrifuged (Eppendorf Mini-Spin Plus, 9700 × g for 5 min); reducing sugars concentration in 1 mL of supernatant was measured spectrophotometrically by the Schales’ method (Imoto and Yagishita 1971) using a calibrating curve with D-glucosamine (GlcN) as standard (in range 100–200 μg/mL). The release rate of 1 μM equivalent of GlcN per min in 1 mL of reaction mixture at the described conditions was expressed as 1 U of chitosanase activity. Chitinase was assayed by the same protocol and proportions, except that 0.5% (w/v) of colloidal chitin suspension and 50 mM phosphate citrate (pH 6) were used as a substrate and buffer solution, respectively. In that case, concentration of chitin degradation products was estimated on a calibration curve (in range 150–250 μg/mL) with N-acetyl-D-glucosamine (GlcNAc). The reaction mixture containing an equivalent volume of the enzyme solution incubated for 1 h (50 °C) without a substrate was used as a control.

Viscometric assay of soluble chitosans (DD 50 and 85%) hydrolysis by the chitosanase was evaluated using glass capillary viscometer of Ostwald type with a capillary diameter of 0.82 mm (Ecroskhim Co. Ltd., Russia) (Fig. S3).

Protein content in the enzyme samples was estimated by the Warburg and Cristian method according to the CHS protocol (2006).

Physico-chemical and kinetic characterization of chitosanase

Physico-chemical properties of the chitosanase including pH and temperature optima and stabilities, storage stability, effect of metal cations, salts, detergents and other additives were analyzed according to the description in a previously published study (Aktuganov et al. 2019). Substrate specificity of the purified enzyme was assayed against 0.5% colloidal chitosan (DD 85%), 0.5% soluble chitosan (DD 50%), 0.5% colloidal chitin, 0.5% carboxymethyl cellulose sodium salt (CMC-Na), 0.2% laminarin, 0.5% xylan and 1% azokasein. Conditions for the enzyme reaction with the mentioned substrates were analogous for standard chitosanase assay except for the incubation time while using laminarin (10 min), xylan (10 min) and azokasein (30 min). Protocols for determination of respective enzymatic activities were previously reported (Helistö et al. 2001). Relative activity of purified chitosanase toward the listed substrates was expressed in the percentage portion of its activity indicated in relation to the main substrate. The chitosanase was also tested for hydrolysis of chromogenic analogues of chitin dimer and trimer, p-nitrophenyl-N-acetyl-β-D-glucosaminide (p-NP-GlcNAC) and p-nitrophenyl-N,N’-diacetyl-β-D-chitobioside (p-NP-GlcNAC)₂, respectively (Aktuganov et al. 2003).

Kinetic constants (Kₘ and Vₘₐₓ) were calculated by the measurement and using linearization equation Vₘₐₓ = V₀ + [V₀/S₀] × Kₘ according to the Eisental and Cornish-Bowden method (1974). Kinetic of chitosan (DD 85%) destruction by purified chitosanase was analyzed for the incubation period 10–240 min at standard conditions.

Thin-layer chromatography

Low-molecular weight products of chitosan enzymatic cleavage were assayed using thin-layer chromatography on Silica gel 60 F₂₅₄ aluminum sheets (10×20 cm) (Merck, Springer
Germany). Purified chitosanase (44 μg – 0.42 U) was diluted tenfold by deionized water and incubated with the equivalent volume of 0.5% (w/v) soluble chitosan (DD 85%) for 10, 30, 60 and 240 min under 50 °C and pH 6. Samples of the reaction mixture taken at different time intervals were applied (1–2 μL) on TLC sheets. The solution including n-BuOH: NH₄OH(28%): MeOH: H₂O (5:4:2:1) was used as mobile phase in TLC chamber. After the migration of solvent front to upper edge of TLC sheet it was dried and treated with 0.25% (w/v) of ninhydrin solution in acetone for staining of mobile oligomers. A commercial mixture including chitosan oligomers with polymerization degree n = 2–6 (“Sigma”, USA) as well D-glucosamine (“Sigma”, USA) was used as a standard.

**HP-SEC of LMWC released under chitosan enzymatic hydrolysis**

The crude chitosanase complex was added to 2% (w/v) chitosan (DD 85%) solution in 50 mM sodium acetate buffer (pH 6) at volume ratios 1:600, 1:120 and 1:60 (enzyme–substrate ratios were 0.001, 0.005 and 0.012 U/mg, respectively) and incubated for 60 min under 50 °C (for ratios 1:600 and 1:60) or 70 °C (for 1:120). The residual substrate (LMWC) was precipitated by adding a 2 M solution of NaOH (1:1, v/v), centrifuged, washed by deionized water to neutral reaction and re-dissolved in 50 mM sodium acetate buffer (pH 6). The prepared solution was lyophilized; dry residue was weighted and again re-dissolved in deionized water for further analysis. Average molecular mass (M₆₅), number-average molecular mass (Mₙ), and polydispersity index (M₆₅ / Mₙ) of LMWC were assessed by high performance size-exclusion chromatography (HP-SEC) using liquid chromatograph S-2100 (Sykam, Germany) with the PhenylSep-GFC-P 4000 column (7.8 x 300 mm) (Phenomenex, USA) and precolumn (5.0 x 2 mm). The chromatography was performed under a flow rate 0.5 ml/min, temperature 30 °C and pressure 2 MPa. The mixture of 50 mM acetic acid and 150 mM ammonium acetate (pH 5.1) was used as eluent. The column was calibrated using dextran standards (Mw 1.08; 4.44; 9.89; 43.5; 66.7; 123.6 and 196.3 kDa) (Sigma, USA). Chromatogram analysis was carried out using software Chrom&Spec v. 1.6 (Ampersand Inc., Russia).

**Antibacterial and antifungal assays**

The bactericidal effect of chitosan oligomers was examined against the strains of opportunistic bacteria including *Bacillus cereus* VKM B-688, *Enterobacter cloaceae* IB-34-CPA, *Escherichia coli* XL1-Blue and *Pseudomonas aeruginosa* IB-39D from microbial collection of the Ufa Institute of Biology of UFRC RAS (*E. cloaceae* and *P. aeruginosa*), All-Russian Collection of Microorganisms (VKM) (*B. cereus*) and Evrogen company (Russia) (*E. coli*). Most of the tested fungal strains in antifungal assays were obtained from the VKM collection including plant pathogens such as *Alternaria alternata* (Fr.) Keis. F-3047, *Botrytis cinerea* Pers. F-894, *Fusarium culmorum* (W.G.Sm.) Sacc. F-844, *F. oxysporum* Schlecht. emend. Snyder & Hansen F-137 and *Rhizoctonia solani* J.G. Kühn F-895. The strain *Bipolaris sorokiniana* (Sacc.) Shoemaker IB-G12 was taken from the collection of the Ufa Institute of Biology of UFRC RAS. The chitosans DD 85% and 50% (1 % w/v) were previously hydrolyzed using the crude enzyme complex of *B. thuringiensis* B-387 during 60 min at 50 °C and enzyme–substrate ratio 0.002 U/mg. Total reaction mixtures were centrifuged, sterilized by filtration through bacterial syringe filters GVS (USA) with pore size 0.20 μm and lyophilized in sterile vial. Weighted quantities (25–1000 μg) of dried chitosan hydrolysates (CHOs) were dissolved in MQ-water immediately prior to the performance of the experiment. Antibacterial/antifungal assays were carried out in 96-well polystyrene cultural plates (Corning Inc., USA) with a test medium volume 200 μL per well. The antimicrobial activity of CHOs was determined as a growth inhibition (%) of the tested bacterial and fungal cultures, respectively, in LB and potato-dextrose broths. Optical density of grown microbial cultures was measured using the plate reader EnSpier (Perkin-Elmer, USA) at 600 nm after 24-h and 72-h incubation (28 °C) of bacteria and micromycetes, respectively. Bacterial cultures were pre-cultivated for overnight in LB broth under 30 °C and 220 rpm. Then grown bacteria were diluted by sterile LB broth to optical density value A₆₀₀ = 0.05 and 100 μL of prepared bacterial suspensions that were filled into wells. Suitable quantities of tested CHOs were added to the aliquots and adjusted to final volume (200 μL) with sterile LB broth. Fresh 6 days-old fungal cultures were taken for preparation of spore suspension (10⁵ spores per mL) in sterile potato-dextrose broth using counting chamber. Other manipulations for antifungal assay were analogous to these in the mentioned above antibacterial test. The growth inhibition (GI) degree (%) of test-microorganisms was calculated according to the formula: GI = [(A₆₀₀K–A₆₀₀E)/A₆₀₀K] • 100, in which A₆₀₀K and A₆₀₀E are the values of optical density for microbial cultures in control and experimental samples, respectively. CHOs doses were substituted with same volumes of sterile physiological solution in the control variants. Positive control samples included same concentrations of original polymeric chitosans (DD 85% and 50%). Minimal aliquots of CHOs causing no less than 50% bacterial / fungal growth reduction were taken as minimal inhibiting concentrations (MIC).
Statistical analysis

The experiments for the evaluation of fungicidal/bactericidal activities of CHOs as well as viscosimetric assay and enzymatic activities’ measurements were performed in triplicate; the data are presented as mean values with standard deviation (SD). The differences between the compared samplings were considered significant at p < 0.05.

Results

Culture growth and chitosanase production

Chitosanase production in LB broth reached the maximal level (> 3 U/mL) to 48 h of cultivation insignificantly increasing later. Bacterial growth demonstrated maximal value at 24 h (~ 6.6 × 10^8 CFU/mL) sharply declining by the 4th day of fermentation (Fig. 1). This behavior can probably be the result of autolysis of bacterial cells in course of sporulation that was earlier registered for the representatives of the species (Chen et al. 2018). During fermentation pH value of culture liquid increased from 6.5 to 9, however, it had no visible effect on chitosanase activity. Along with the chitosanase production, strain B-387 also demonstrated relatively slight basal chitinase activity (~ 0.1 U/mL) under cultivation in LB broth (Fig. 1). Its chitinase production increased in 3–fivefold in the medium supplemented with 0.5% (w/v) of colloidal chitin (data are not presented). Hence, it seems that chitinases of B-387 are inducible enzymes in contrast to chitosanases.

Purification of chitosanase

The chitosanase from culture supernatant of B. thuringiensis B-387 was reproducibly purified using three sequential procedures to an electrophoretically homogenous state. The main steps of the purification are summarized in Table 1. The ultrafiltration procedure commonly resulted in a tenfold increment of enzyme activity in concentrate volume (U/mL), while specific chitosanase activity increased approximately by three times. Affinity sorption demonstrated reversible binding of the enzyme by colloidal chitosan (5 mg/mL, DD ~ 85%) ranged from 70 to 75% of its total initial activity in the crude preparation (data are not presented). Maximal effectiveness of the chitosanase purification was reached by means of cation-exchange chromatography on CM-Sepharose. The sharp activity peak of the enzyme was revealed within minor protein peaks eluted by linear gradient of 0–500 mM M NaCl, while the non-adsorbed proteins of the major peaks did not indicate chitosanase activity (Fig. 2a). The final purification degree of the chitosanase was about 94-fold; molecular mass of the purified enzyme was approximately 40 kDa according to SDS-PAGE (Fig. 2b, line 3). The chitosanase fraction not adsorbed on colloidal chitosan after the affinity sorption step was also effectively purified on CM-Sepharose under the same conditions to an electrophoretically homogenic state and was revealed as protein band with same molecular mass (Fig. 2b, lines 4–5).

Table 1 Purification of the chitosanase from B. thuringiensis B-387

| Purification stage                              | Total protein, mg | Total chitosanase activity, U | Specific chitosanase activity, U/mg | Enzyme recovery, % | Purification degree (-fold) |
|-------------------------------------------------|-------------------|-------------------------------|-------------------------------------|--------------------|-----------------------------|
| Culture supernatant                              | 4722.30           | 1619.68                       | 0.34                                | 100                | 1                           |
| Ultrafiltration on VivaFlow 200 module, ≤ 10 kDa | 822.7             | 931.32                        | 1.13                                | 57.5               | 3.3                         |
| Affinity adsorption on colloidal chitosan (0.5%) | 204.79            | 730.48                        | 3.57                                | 45.1               | 10.4                        |
| Cation-exchange chromatography on CM-Sepharose   | 4.57              | 147.39                        | 32.25                               | 9.1                | 94                          |

Fig. 1 Growth rates of B. thuringiensis B-387, changes in its chitosanase/chitinase production and the culture pH dynamics during submerged cultivation in LB broth (36 °C, 220 rpm)
Physico-chemical properties of purified chitosanase

The influence of pH on the chitosanase activity and stability was assayed by solving chitosan (DD ~ 85%) under a limitedly wide range of values (pH 3–10.5). As expected, the purified enzyme functioned most actively in range of pH 5–7.5 with optimum at pH 6.5 (Fig. 3a). Furthermore, unlike with many bacterial chitosanases, the enzyme retained noticeable activity at pH 8–9, when chitosan partially settles out forming suspension systems. The enzyme demonstrated stability under pH 5–10.5 completely maintaining its original activity after 24 h pre-incubation (Fig. 3a). The temperature dependence of the enzyme activity is characterized by a pronounced optimum at 55 °C, however, the chitosanase was highly active also at 60–65 °C and retained more than 20% of its activity at 75 °C (Fig. 3b). The enzyme was moderately stable under 50–60 °C sustaining approximately 75 and 50% of the original activity, respectively, after 1 h incubation in the absence of substrate. The crude chitosanase demonstrated satisfactory stability during long-term storage. Common storage of the culture supernatant during 7 months in a non-sterile vessel at 5 °C differed little in its residual activity (93–100%) from other approaches including the use of sterilized containers, the addition of the preservative agent and the lyophilization procedure (Fig. S1).

The purified enzyme was assayed in the presence of a limited number of salts and chemicals (Table 2). None of the tested bivalent metal salts displayed an activating effect on the chitosanase including such potential co-factors and activators as Mg^{2+}, Ca^{2+}, Fe^{3+}, Mn^{2+} and Zn^{2+}. The strongest inhibitors among metal cations were Hg^{+} (at 10 mM and 1 mM), Cd^{2+} (10 and 1 mM), Zn^{2+} (10 mM) and Ag^{+} (10 and 1 mM). Among other verified chemicals only sodium lauryl sulfate (10 mM) expectedly exerted severe inhibition, whereas non-ionic surfactants, urea and polyglycols did not have visible effect except for tween-80 (10 mM) that solely displayed distinct activating action (Table 2). Sodium chloride distinctly (by 40–50%) suppressed the enzyme’s activity only under concentrations higher than 1 M (Fig. S2). This salt is capable to improve chitosan solubilization at some concentrations range thereby favoring to increase by times the activity of some hydrolases as, for instance, the exo-chitosanase from *Penicillium* sp. IB-37-2 (Aktuganov et al. 2019). However, such effect was not observed for the chitosanase of Bt B-387.

Kinetic characteristics and substrate specificity

The purified chitosanase most actively hydrolyzed soluble chitosan with DD 85%. Kinetic constants (K_M and V_max) calculated by measurement and using linearization equation V_max = V_0 + [V_0/S_0] × K_M according to the Eisental and Cornish-Bowden method (1974) amounted 0.22 mg/mL and approximately 43 µM/mL × min, respectively (Fig. 3, c–d). Empirical calculation from substrate concentration dependence curve resulted in a similar value of K_M but a lesser V_max ~ 34,987 µM/mL × min. Nearby value of specific V_max was obtained from the enzyme concentration dependence curve (Fig. 3c). According to this, catalytic constant k_cat was 4.792 × 10^4 s^{-1}, k_cat/K_M = 1.948 × 10^5 mg^{-1} × s^{-1}.

Among other substrates, colloidal chitosan (DD 80–85%) and partially N-acetylated soluble chitosan (DD 50%) were more slowly hydrolyzed by the chitosanase (V_max was 19.08 and 12.56 µM/mL × min; K_M was 0.98 and 3.19 mg/mL, respectively) (Table 3). Viscosity kinetic assay also indicated a lower depolymerization rate of chitosan with DD 50% by the enzyme (Fig. S3). The purified
The enzyme did not degrade colloidal chitin, chromogenic analogues of chitooligosaccharides p-NP-GlcNAc and p-NP-(GlcNAc)₂, Na-CMC, laminarin, xylan and azokazein (Table 3).

The main enzymatic reaction products of extensively hydrolyzed chitosan DD 85% (60–240 min) include chitobiose, chitotriose, chitotetraose and chitopentaose according to TLC assay (Fig. 4). Short incubation (10 min) resulted in rapid decrease of the substrate viscosity with formation of mixture containing longer chitooligomers (n = 4–7 etc.).

Thus, the chitosanase of Bt B387 demonstrates a typical pattern for the endo-mode of chitosan splitting.

**Chitosan conversion by the chitosanase into water-soluble chitooligomers**

Crude enzyme complex of Bt B-387 converted more than 95% of chitosan (DD 85%) into water-soluble oligomers within a 60-min incubation at enzyme–substrate ratios 0.01–0.04 U/mg. Preliminary HP-SEC analysis of LMWC...
preparing by 60-min enzymatic hydrolysis of 2% chitosan (at enzyme/substrate ratio 0.001 U/mg) indicated its average molecular weight about 46 kDa with polydispersity index (PDI) 2.03 (Fig. 5a). Ten-fold increase of enzyme–substrate ratio (to 0.012 U/mg) resulted in predominant generating of LMWC with the average molecular weight 16.7 kDa and PDI 1.55 (Fig. 5c). Interestingly, that increase of incubation temperature from 50 to 70 °C leaded to formation of LMWC with Mwa ~ 14.3 kDa (PDI 1.62) under midline enzyme–substrate ratio (0.005 U/mg) (Fig. 5b). Recovery of LMWC by dry weight under described conditions reached 29.2, 35 and 14.6%, respectively. The findings demonstrate the probability of the responsive approach in potential application of the Bt B-387 chitosanase for diverse challenges including preparation of LMWC with variable molecular weight or small chitooligosaccharides with DP = 2–5.

Fungicidal and antibacterial activity of chitooligosaccharides prepared using the chitosanase of Bt B-387

This work analyzed preliminary fungicidal activity of total CHO mixtures formed after 60-min hydrolysis of 1% chitosans with DD 85 and 50% by the crude chitosanase from Bt B-387 (~0.048 U/mg). It is worth to note, that antifungal effect of the chitosan breakdown products (including LMWC) deviated differently from the original polymer action depending on tested fungal species. On the one hand, inhibition degree of F. oxysporum and R. solani was definitely decreased in both chitosans enzymatic hydrolysates under the concentration range 25–150 µg/mL (Table 4). It is not surprising, taking into account relatively extensive hydrolysis of chitosan in this experiment. Nevertheless, increase of fungicidal effect of identical CHO mixtures against some other species was symptomatic. For example,
Fig. 5 HP-SEC analysis of LMWCs prepared from chitosan (DD 85%) 60-min hydrolysis by the crude chitosanase under enzyme–substrate ratios (U/mg) 0.001 (a), 0.005 (b) and 0.01 (c). The incubation temperature for a and c was 50 °C; for b—70 °C. PolySep-GFC-P 4000 column (7.8 x 300 mm). Injection volume was 20 µl.

(a) 84.6 mV
M_N: 22743.5
M_W: 46237
M_W/M_N: 2.03298 (PDI)
M_Z: 71680.4
M_Z+1: 91797.8
M_P: 52323.3 (Molecular weight for largest peak)

(b) 42.3 mV
M_N: 9161.33
M_W: 14808.6
M_W/M_N: 1.61643 (PDI)
M_Z: 22140.1
M_Z+1: 29294.7
M_P: 10989.4 (Molecular weight for largest peak)

(c) 42.3 mV
M_N: 10735
M_W: 16661.9
M_W/M_N: 1.55211 (PDI)
M_Z: 23907.2
M_Z+1: 30732.8
M_P: 14643.6 (Molecular weight for largest peak)
growth inhibition of blueberry blossom blight causative agent, *B. cinerea*, was registered under significantly lower concentrations of CHOs, than both initial chitosans (Table 4). Enzymatic cleavage products of chitosan DD 50% demonstrated increment of growth-inhibiting activity against *A. alternaria* and *B. sorokiniana* contrary to *F. culmorum* that was suppressed in greater degree by CHOs prepared by hydrolysis of chitosan DD 85%.

Comparison of antibacterial effects of enzymatic hydrolysates and original chitosans demonstrated evident activation of the CHOs inhibition degree against *E. cloaceae* and *E. coli* (Table 4). By contrast, *B. cereus* and *P. aeruginosa* displayed significant resistance both to chitosans and CHOs; and activities of the hydrolysates generally dropped relative to the initial polymers (Table 4).

### Discussion

The capability of *B. thuringiensis* B-387 to constitutive production of extracellular chitosanase was found for the first time in case of this species. Previously, such feature was demonstrated just in some strains of *Bacillus* spp. (Choi et al. 2004; Kim et al. 2004). Most of the reported chitosanase-producing strains of *Bacillus*, including *B. thuringiensis* belonging to *B. cereus* group, together with other known bacterial producers, require chitosan addition into culture medium (Koboyashi et al. 2011; Thadathil, Velappan 2014; Olicón-Hernández et al. 2016). Lee et al. (2007) characterizing expression of chitosanases family GH8 in the variety of *B. thuringiensis* strains found chitosanolytic activity among 12 from 29 serovars of this species at their growth on LB medium, supplemented with soluble chitosan. Although, the strain Bt B-387 demonstrated sustainable chitosanase secretion in the average range of 3–4 U/mL, its productivity can be increased to the enzyme yield 12–12.5 U/mL through simple empirical fit of cultivation conditions without application of specific substrate induction and chitin-containing carbon sources (unpublished data). It is highly likely that further statistical optimization of crucial growth parameters will allow to reach higher the chitosanase production by the Bt B-387 comparable or superior to efficiency reported for some other natural *Bacillus* strains (Choi et al. 2004; Chang et al. 2007; Gao et al. 2008). As distinct from the chitosanase, the chitinase of the Bt B-387 is inducible enzyme and it is found in minor quantities (≤ 0.1 U/mL) under the cultivation of this strain in LB broth. Colloidal chitin as the main carbon source induced the increase of chitinase production by the strain to approximately 0.3–0.7 U/mL depending on the substrate content (data are not presented). Differential expression of the chitosanase and chitinase in Bt B-387 is probably associated with a specific mechanism of its insect pathogenesis that should be clarified in further.

The enzyme purification based on ultrafiltration, affinity sorption and cation-exchange chromatography steps detected sole isoform of the chitosanase *M*ₘₜₙ 40 kDa according to SDS-PAGE. Interestingly, the chitosanase fractions differing in the colloidal chitosan (DD 85%) sorption abilities demonstrated identical molecular mass values after the final purification under same conditions (Fig. 2b). Most likely, this fact evidences on incomplete adsorption of the same chitosanase on the specific substrate, since it exerted similar physic-chemical and kinetic properties (data are not shown). Cation-exchange chromatography on CM-Sepharose was the

### Table 4

| Fungal strains               | MIC, µg/mL | Chitosan DD 85% | CHOds DD 85% | Chitosan DD 50% | CHOds DD 50% |
|-----------------------------|-----------|----------------|-------------|----------------|-------------|
| *Alternaria alternata* (Fr.) Keisl. F-3047 | 70 ± 9    | 70 ± 5         | 80 ± 3      | 70 ± 3         |
| *Bipolaris sorokiniana* (Sacc.) Shoemaker IB-G12 | 110 ± 8   | 100 ± 5        | 180 ± 5     | 120 ± 3        |
| *Botrytis cinerea* Pers. F-894       | 190 ± 12  | 70 ± 4         | 210 ± 13    | 140 ± 12       |
| *Fusarium culmorum* (W.G.Sm.) Sacc F-844 | 110 ± 7   | 80 ± 7         | 80 ± 2      | 90 ± 5         |
| *Fusarium oxysporum* Schlecht. emend. Snyder & Hansen F-137 | 110 ± 8   | 130 ± 14       | 100 ± 5     | 130 ± 10       |
| *Rhizoctonia solani* J.G. Kühn F-895 | 20 ± 5    | 30 ± 6         | 30 ± 7      | 40 ± 6         |

| Bacterial strains               | MIC, µg/mL | Chitosan DD 85% | CHOds DD 85% | Chitosan DD 50% | CHOds DD 50% |
|-----------------------------|-----------|----------------|-------------|----------------|-------------|
| *Bacillus cereus* B-688 | 1100 ± 100 | 1610 ± 150  | 550 ± 30    | 500 ± 45       |
| *Enterobacter cloaceae* IB-34-4CPA | 90 ± 7    | 60 ± 3       | ND²         | ND²           |
| *Escherichia coli* XL1-Blue | 120 ± 15 | 70 ± 8       | 300 ± 25    | 140 ± 20       |
| *Pseudomonas aeruginosa* IB-39D | 1260 ± 110 | > 2000       | 1390 ± 150  | 1170 ± 90      |

²The hydrolysates were prepared after 60-min treatment of original polysaccharides by the crude chitosanase from *B. thuringiensis* B-387

ND not determined
most effective and highly selective technique for the chitosanase purification to the electrophoretically homogenous state. Kobayashi et al. (2011) earlier purified the chitosanase from *B. thuringiensis* JAM-GG01 with analogous manner using cation-exchange chromatography on CM-Toyopearl under alkaline conditions of borate buffer (pH 9.5) eluting the enzyme by linear gradient of 0.15 M NaCl. However, the general purification scheme reported by these authors was more complex and included the steps of the enzyme rechromatography on the same resin at weakly acid equilibrating MOPS buffer (pH 6) and preliminary anion-exchange chromatography on SuperQ-Toyopearl. Molecular mass of the purified chitosanase (40 kDa) also indicated near value to the aforementioned chitosanase (43 kDa) from the *B. thuringiensis* JAM-GG01 (Kobayashi et al. 2011). Moreover, various previously characterized chitosanases from *Bacillus* species belonging to the glycosyl hydrolases (GH) family 8 demonstrated similar Mw values (40–46 kDa) (Mitsutomi et al. 1998; Kurakake et al. 2000; Choi et al. 2004; Gao et al. 2008; Liang et al. 2012). These are commonly bifunctional enzymes or chitosanases that are capable to hydrolyze CM-cellulose and other glucans. Interestingly, GH 46 chitosanases displaying a narrow substrate specificity commonly have the molecular masses in range 27–31 kDa (Kilani-Feki et al. 2013; Tomita et al. 2013; Luo et al. 2020).

GH8-chitosanases are not infrequently reported in *B. thuringiensis* and related species (Lee et al. 2007; Gao et al. 2008). However, the chitosanase of Bt B-387 manifested no hydrolyzing activity against chitin, CM-cellulose and some other β-1,4— and β-1,3-glucans (Table 3). The chitosanolytic activity of the purified enzyme decreased markedly against the less acetylated chitosan form (DD 50%) suggesting its preference affinity and specific recognition of GlcN residues. The GH8 chitosanase from *B. thuringiensis* JAM-GG01 also did not hydrolyze cellulose and other glucans except for lichenan (Kobayashi et al. 2011). Biochemical and catalytic characteristics of the purified chitosanase are not generally exclusive among the majority of reported bacterial chitosanases, nevertheless, it is characterized with one of the lowest $K_M$ and higher specific $V_{max}$ values. The temperature 70 °C is crucial for the purified chitosanase of Bt B-387 which lost >90% of its activity after 1 h of incubation. But, most likely, chitosan increases of the enzyme thermostability as it was reported for many bacterial chitosanases (Thadathil, Velappan 2014). Indirectly, this fact is supported by the obvious chitosan hydrolysis by the purified enzyme at 75–80 °C. Despite the proximity of the temperature optimum of the chitosanase from Bt B-387 to such of *B. thuringiensis* JAM-GG01 it differs markedly in its pH-optimum and demonstrates much higher thermostability (Kobayashi et al. 2011). The effect of the tested metal cations and surfactants on Bt B-387 chitosanase activity is roughly the same as it was previously reported for various bacterial chitosanases. Chitosan (DD 85%) hydrolysis by the chitosanase resulted in the rapid formation of CHO with DP ≥ 2–6 on the initial reaction step (5–10 min) in accordance with endo-mechanism. Longer-term incubation accumulated chitobiase, chitotriose and chitotetraose as the main final products. It was coherent with almost complete conversion (95%) of chitosan by the crude enzyme complex of Bt B-387 into small water-soluble chitooligosaccharides molecules for 60-min reaction. Lower enzyme–substrate ratios increased the yield of low-molecular weight chitosans with $M_w$ in range 14–46 kDa (Fig. 5). As it is known, LMWCs ($M_w$ ≥ 15 kDa) demonstrate higher antimicrobial activity compared to smaller oligomers and CHO with DP 2–6 (Park et al. 2008, 2015; Zhao 2019). Lin et al. (2009) using various statistical models illustrated significant reverse relationship between logarithm of $M_w$ and minimal inhibition concentration of LMWCs prepared from chitosans with DD 80 and 92% using chitinase, lysozyme and cellulase. Antimicrobial activity of tested LMWCs increased within $M_w$ ranges 8.5–13.5, 12–24.8, < 6–9.6 and 10.1–14.8 kDa (Lin et al. 2009). According to other data, antifungal activity of LMWCs with $M_w$ 15–40 kDa decreased sharply compared to chitoooligomers with $M_w$ 4–10 kDa (Stepnova et al. 2007). Antimicrobial tests of CHO mixtures resulted from extensively hydrolyzed chitosans (DD 85 and 50%) by crude chitosanase complex of Bt B-387 demonstrated ambiguous results manifesting both in decline and increase of the growth-inhibiting effect against several fungal and bacterial strains as compared to the initial polymers. This fact is obviously contingent on significant inter- and intraspecific variation of bacterial/fungal sensitivity to LMWC action determined by the specificity of the strain (Stepnova et al. 2007; Lin et al. 2009; Palma-Guerrero et al. 2010). Presumptive mechanisms for such specificity are usually attributed to the variety of interactions of LMWC with microbial plasma membrane lipids (Stepnova et al. 2007; Palma-Guerrero et al. 2010; Sanchez et al. 2017). Different sensitivity of bacterial and fungal cells to chitosan and CHO is probably also determined by significant variations in hydrophobicity of cell surface for several species (Polyudova et al. 2019). In gram-positive bacteria, such as *Staphylococcus aureus*, development of resistance to chitosan correlates with the acquired changes in its cell wall structure such as decrease of total negative charge of the cell surface, emergence of cross-resistance to some antimicrobial agents, transformations in cell tropism and metabolism, receptor histidine kinase repair etc. (Raafat et al. 2017).

Our findings acknowledged retaining of the acceptable antimicrobial activity for extensively hydrolyzed chitosan oligomers mixtures containing predominantly highly soluble small CHO molecules. Alongside this, the control of such parameters as enzyme–substrate ratio and reaction temperature allow to shift the balance between breakdown
products upwards for LMWC with $M_{w(A)}$ 14–16 kDa regulating thereby the final antimicrobial activity of the produced oligomers. Although enzymatic conversion of high molecular weight chitosan into LMCW with wide range of $M_{w(A)}$ is sometimes considered as a versatile problem realized only by the coordinated application of several hydrolases (including non-specific enzymes) (Lin et al. 2009), its implementation through controlled destruction by a single enzyme or an enzyme complex such as Bt B-387 chitosanase may by quite realistic. So, the main results of this study conclusively illustrate versatile potential of the chitosanase from Bt B-387 for enzymatic preparation of water-soluble LMWC and CHOs exerting antimicrobial activity. A more detail exploration of the various controlled parameters contribution in this process is supposed to be done in further.

Summarizing, the high basal level of the endochitosanase synthesis (3–3.5 U/mL) by B. thuringiensis, which does not require the addition of chitosan into nutrient medium, as well as the sizeable production potential of this culture allow to consider it as a promising microbial producer of chitosanase for various biotechnological processes. The chitinase of B. thuringiensis B-387, unlike chitosanase, is an inducible enzyme secreted notably only in the presence of colloidal chitin, therefore crude chitosanase preparation comprises just traces of chitinolytic activity. The purified enzyme differs in some of their biochemical characteristics from the previously reported Bt chitosanases. The chitosanase may be effectively employed for the polymer conversion into both short-chain CHOs and water-soluble LMWC. In the latter case, generation of bioactive LMWC with the desired molecular weight is well-controlled by such parameters as the enzyme/substrate ratio and incubation temperature. Enzymatic hydrolysates of chitosans with DD 85% and 50% demonstrated higher growth-inhibiting activity compared to the initial polymers against certain plant pathogenic fungi and opportunistic pathogenic bacteria. The findings confirm the perspective of this enzyme for managed preparation of bioactive chitosan oligomers.

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**Author contributions** The main conceptualization and supervision by GA and AM; GA and VS carried out researches, analyzed the data and prepared the paper. NG, EG, LK, AB and SL contributed to experiments, methodologies, and resources. GA, VS and NG were responsible for discussion and editing.

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**Data availability** All data generated or analyzed during this study are included in this published article and its supplementary information file.

**Declarations**

**Conflict of interest** All the authors declare that they have no conflict of interest.

**Ethical approval** This article does not contain any studies with human participants or animals performed by any of the authors.

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