Gene cloning, functional expression and characterization of a novel GH46 chitosanase from *Streptomyces avermitilis* (SaCsn46A)

Jing Guo (✉ guojing113@cczu.edu.cn)

Changzhou University <https://orcid.org/0000-0002-7622-6810>

Yi Wang

Changzhou University

Wenjun Gao

Changzhou University

Xinrou Wang

Changzhou University

Xin Gao

Changzhou University

Zaiwei Man

Changzhou University

Zhiqiang Cai

Changzhou University

Qing Qing

Changzhou University

Research Article

Keywords: *Streptomyces avermitilis*, Chitosanase, Glucosamine, Chitooligosaccharides, Glycoside hydrolase (GH) family 46, Enzyme properties

Posted Date: August 11th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-785979/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Version of Record: A version of this preprint was published at Applied Biochemistry and Biotechnology on September 20th, 2021. See the published version at <https://doi.org/10.1007/s12010-021-03687-6>.

Abstract

A novel glycoside hydrolase (GH) family 46 chitosanase (SaCsn46A) from *Streptomyces avermitilis* was cloned and functionally expressed in *Escherichia coli* Rosetta (DE3) strains. SaCsn46A consists of 271 amino acids, which includes a 34-amino acids signal peptide. The protein sequence of SaCsn46A shows maximum identity (83.5%) to chitosanase from *Streptomyces sp.* SirexAA-E. Then the mature enzyme was purified to homogeneity through Ni-chelating affinity chromatography with a recovery yield of 78% and the molecular mass of purified enzyme was estimated to be 29 kDa by SDS-PAGE. The recombinant enzyme possessed a temperature optimum of 45 °C and a pH optimum of 6.2, and it was stable at pH ranging from 4.0 to 9.0 and below 30 °C. The K_m and V_{max} values of this enzyme were $1.32 \text{ mg} \cdot \text{mL}^{-1}$, $526.32 \text{ } \mu\text{M} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$, respectively (chitosan as substrate). The enzyme activity can be enhanced by Mg^{2+} and especially Mn^{2+} , which could enhance the activity about 3.62-fold at a 3 mM concentration. The enzyme can hydrolyze a variety of polysaccharides which linked by β -1,4-glycosidic bonds such as chitin, xylan and cellulose, but it could not hydrolyze polysaccharides linked by α -1,4-glycosidic bonds. The results of thin layer chromatography and HPLC showed that the enzyme exhibited an endo-type cleavage pattern and could hydrolyze chitosan to glucosamine (GlcN) and $(\text{GlcN})_2$. This study demonstrated that SaCsn46A is a promising enzyme to produce glucosamine and chitooligosaccharides (COS) from chitosan.

Introduction

Chitosan is a kind of linear polymer which was composed of β -1, 4-linked D-glucosamine (GlcN) units. Chitosan is a group of biomacromolecules that is commonly prepared by the partial chemical deacetylation of chitin(34, 4). As the second most abundant polymer in nature, chitin occurs in the exoskeletons of fungi, crustaceans, insects and algae. (14).

Chitooligosaccharides (COS) are the hydrolyze products of chitosan with degrees of polymerization (DP) 2–10(26). COS have many biological activities such as anti-tumor(21), immune enhancing(19), anti-oxidizing(13), free radical scavenging(15) and antimicrobial activity(33). GlcN has gained its popularity as nutraceutical having application in weight loss and in treatment of osteoarthritis, knee pain and back pain(9). Consequently, it is a popular compound in medicine, agriculture and rises interest in converting chitosan into COS and GlcN(24). COS and GlcN can be prepared from chitosan by a variety of methods, such as chemical, physical and enzymatic(10). Compare with other methods, enzymatic conversion to produce COS and GlcN produced has attracted extensive attention as an environmentally friendly, high efficiency and controllable and alternative to chemical and physical production. Many enzymes have been reported to hydrolyze chitosan to produce chitooligosaccharides (COS) such as chitosanase, chitinase(22), protease(20), cellulase, lipase(28). Among these enzymes, chitosanase (EC. 3.2.1.132) is the most attractive because of its high activity and specificity.

Chitosanase is a kind of enzyme that hydrolyzes chitosan degradation into COS or GlcN. Various chitosanases have been identified from many species(17). Due to the sequences in the Carbohydrate

Active Enzymes database (www.cazy.org)(16), chitosanases are categorized into glycoside hydrolases (GH) families 46(24, 26, 29), 75(34), 80(32) and 8(35). GH46, GH75, and GH80 currently contain exclusively chitosanases, while the GH8 family contains a few other glycoside hydrolases, such as cellulase, lichenase, and xylanase(35). It is remarkable that chitosanases from fungi are mainly distributed among the GH75 family, but chitosanases from bacteria are mainly belonging to GH46 family, a few belong to GH80(17).

S. avermitilis is a kind of aerobic bacteria and were mostly reported to produce avermectins(1) and ivermectin(3). Heggset reported a chitosanase from the GH75 family produced by *S. avermitilis*, which mainly produce long oligomers ($DP \geq 2$)(5). However, to our knowledge, no report is available regarding on gene cloning and characterization of GH46 family chitosanase from *S. avermitilis*. Accordingly, in this study, a novel chitosanase designated as SaCsn46A was cloned by PCR and functionally expressed in *E. coli* Rosetta (DE3). The characterization of its biochemical properties suggests that SaCsn46A has high activity and pH stability and could efficiently convert colloidal chitosan into GlcN and $(GlcN)_2$, which was rarely reported by other chitosanases.

Materials And Methods

Culture medium, chemicals, strains, and plasmids

Escherichia coli JM 109, *E. coli* DH5 α and *E. coli* Rosetta (DE3) used in this study was grown at 37 °C with shaking at 160 r.p.m. in Luria-Bertani medium which contained ($g \cdot L^{-1}$) peptone 10, yeast extract 5, NaCl 10. Gene cloning was conducted through the plasmids pMD18-T and *E. coli* JM109 (Takara). Gene expression was conducted through the plasmids pET-28a (Novagen, Germany) and *E. coli* Rosetta (Takara). The genetic manipulation reagents (Ex Taq™, T4 DNA ligase, restriction endonuclease, buffers and dNTP) were purchased from Takara (Dalian, China). The genomic DNA extraction, plasmid isolation kits, ampicillin, kanamycin, isopropyl β -D-1-thiogalactopyranoside (IPTG), protein purification and protein electrophoresis reagents were purchased from Sangon (Shanghai, China). COSs with 2–3 DPs were purchased from Qingdao BZ Oligo Biotech Co., Ltd. (Qingdao, China).

DNA cloning and sequence analysis

Two primers (sacsnF, 5'-3': CGGGATCCGCACCCGTCGGCCTGGACGAC; sacsnR, 5'-3': CCCAAGCTTTCAGCCGATGTGGTAGCTGTC) were designed and used to amplify the chitosanase gene, and the signal peptide was eliminated. The PCR products were cloned into pMD18-T and then digested by the designated restrictive endonuclease and cloned into the corresponding sites (BamHI-HindIII) of the expression vector pET-28a to generate pET-SaCsn46A, the recombinant enzyme carry the His-tag and T7-tag belonging to the pET-28a plasmid at the N-terminal. The signal peptide was predicted using Signal P 4.0 (<http://www.cbs.dtu.dk/services/SignalP/>). Homology searches in GenBank were performed using the BLAST program. The molecular mass of the protein was determined using the software DNAMAN (<http://www.lynnon.com/>). Multiple alignments of SaCsn46A sequence were performed with Clustal W

(<https://www.genome.jp/tools-bin/clustalw>) and ESprint 3.0 (<http://esprint.ibcp.fr/ESPrint/ESPrint/>). Phylogenetic trees were constructed using neighbor-joining algorithm in MEGA 5.0 (<http://www.megasoftware.net/>). Three-dimensional structure homology model of SaCsn46A was constructed by Swiss-Model (<http://swissmodel.expasy.org>)

Expression and purification of SaCsn46A

E. coli Rosetta (DE3) cells harboring the designated plasmid were incubated in Luria-Bertani medium containing 50 µg/mL kanamycin and grown at 37 °C to OD₆₀₀ of 0.6. The incubation of the target protein production was performed by adding IPTG to a final concentration of 1 mM. The cultures were grown for an additional 12 h at 16 °C, and protein expression in the cells and culture supernatant were analyzed by SDS-PAGE.

All the following purification steps were carried out at 4 °C. To purify recombinant chitosanase, the induced culture was centrifuged for 10 min at 10000 g. The cell pellet was suspended in buffer I (0.02 M Tris-HCl, pH 8.0, 0.5 M NaCl and 10 % glycerol) and washed twice with the same buffer. The washed cell pellet was suspended in buffer I, disrupted by sonication on an ice bath, and then centrifuged for 30 min at 10000 g. The resulting supernatant was applied on a Ni-Sepharose column previously charged with Ni (II). After the column was washed with buffer II (0.02 M Tris-HCl, pH 8.0, 0.5 M NaCl, 0.02 M imidazole and 10 % glycerol), elution was performed with a 0.08 M imidazole (0.02 M Tris-HCl, pH 8.0, 0.5 M NaCl, 0.08 M imidazole and 10 % glycerol). Elution contains chitosanase activity was collected, imidazole was removed by dialysis, and the enzyme was stored at -20°C.

Properties of purified SaCsn46A

Chitosanase activity was determined by 3, 5-dinitrosalicylic acid (DNS) method, as described previously(4). The content of reducing sugar was determined by measuring optical density (OD) at 520 nm using D-(+)-glucosamine as standard. The reaction mixture was incubated at 45 °C for 10 min. All reactions were done in triplicate, and their mean and standard deviation values were used for analysis. One unit (U) of chitosanase activity is defined as the amount of enzyme liberating 1 µmol of D-(+)-glucosamine-equivalent reducing sugars per minute under the above conditions.

Chitosanase profiles assay for pH were determined at pH ranging from 3.0 to 8.0 at 40 °C in the following buffers: 0.05 M acetate buffer (pH 4.5-6.0) and 0.05 M phosphate buffer (pH 6.0–8.0). pH stability of chitosanase was determined by measuring the residual activities under optimal conditions (pH 6.2, 10 min) after pre-incubation at 0 °C and pH 4.0–9.0. Buffers used were 0.05 M acetate buffer (pH 4.0–6.0), 0.05 M phosphate buffer (pH 6.0–8.0) and 0.05M Tris-HCl buffer (pH 9.0). Chitosanase profiles assay for temperature were determined at temperatures ranging from 20 to 90 °C at 5 intervals at optimal pH. Thermostability was conducted by measuring the residual activities under optimal conditions (pH 6.2, 45 °C) after pre-incubation at 0–60 °C and pH 7.5 for 2 h. pH stability was conducted by measuring the residual activities under optimal conditions after pre-incubation at 0 °C and pH 3.0–9.0 for 2 h. A control sample with no pretreatment was taken as 100 %.

The substrate specificity of SaCsn46A was determined by DNS method. The reaction mixtures, containing 0.05 M phosphate buffer (pH 6.2), the tested polysaccharide substrates (1%) and appropriate amount of SaCsn46A, were incubated at 45 for 20 min. Control samples were treated in the same way in the absence of SaCsn46A. The amount of reducing sugar in the supernatant was determined by measuring the OD520. One unit (U) of enzyme activity was defined as the amount of enzyme that liberated 1 μmol of reducing sugar per min under the above assay conditions.

The effects of metal ions on chitosanase activity were determined by assaying the enzyme activity at pH 6.2 and 45 °C for 10 min in the presence of various ions at different concentration.

The catalytic constant was determined by Lineweaver-Burk plot method using chitosan in ranging of 0.1–10 mg/mL at optimal pH 6.2 and 45 °C for 10 min in the presence of 3 mM Mn^{2+} .

Hydrolytic properties of purified SaCsn46A

The analysis of the hydrolytic products of SaCsn46A was performed by analyzing the products of colloidal chitosan (1%, w/v; 90% DDA) using thin layer chromatography (TLC) method. The purified enzyme was incubated with colloidal chitosan at 28 °C in phosphate buffer (pH 6.2). Samples withdrawn at different times were immediately boiled for 20 min, then centrifuged for 10 min at 10000 g. The hydrolysis products were spotted onto a silica gel plate, which was developed in a solvent system which contains ethyl acetate-methanol-water-25% ammonia (5:9:1:1.5, v/v/v/v). The plate was sprayed with 0.3% ninhydrin (dissolved in 95% ethanol, w/v), followed by heating at 120 °C for 10 min. The hydrolysis products were analyzed by HPLC system (Waters 1525, Waters, USA) equipped with evaporative light scattering detector (ELSD). The GlcN and $(\text{GlcN})_2$ were separated on high performance sugar column (Shodex Asahipak NH_2 P504E, Shodex, Kyoto, Japan), eluted by acetonitrile and distilled water (70/30, w/w) mixture with a flow rate of $1.0 \text{ mL} \cdot \text{min}^{-1}$ at 30 °C.

Results And Discussion

Sequence analysis of the chitosanase

Based on Genbank database and amino acid sequence analysis, the chitosanase gene from *S. avermitilis* (GenBank: KUN55720.1) was selected for analysis. The gene contained 816 bp (base pair), which encoding 271 amino acids. The SaCsn46A has a 'leader peptide' in the secretion of this enzyme and a cleavage site at the N-terminus (Ala34-Ala35) which was determined by the Signal P 4.0 program. The protein sequence of SaCsn46A shows maximum identity to chitosanase from *Streptomyces* sp. (Genbank accession No. WP_028800608.1).

The protein sequences of SaCsn46A and other chitosanases belonging to four different GH families were selected from the GenBank database to constructed a phylogenetic tree (see Fig. 1(a)). SaCsn46A is clearly a member of GH46 family chitosanase. As can be seen from the multiple sequence alignment of GH 46 family members in Fig. 1(b), the SaCsn46A contained two key active site residues (E22 and D40),

that are conserved in all GH46 family members. The residues E22 and D40 were replaced by A22 and A40, respectively and the mutants were mostly inactive (data not shown).

Homology model of SaCsn46A was built by the Swiss-Model server with the chitosanase from *Streptomyces* sp. SirexAA-E as template (PDB ID: 4ily.1A), which shares 83.5% identity to SaCsn46A. Two amino acid residues in the catalytic active center (E22 and D40) were illustrated with sticks in the 3D-structure of SaCsn46A. These results suggest that SaCsn46A is a novel member of family GH46.

Heterologous expression and purification of the chitosanase

The SaCsn46A gene was cloned from *S. avermitilis* and expressed in *E. coli* Rosetta (DE3) successfully with a N-terminal His-tag and without signal peptide. The recombinant enzyme were purified to electrophoretic homogeneity by a Ni-NTA affinity column (result was shown in Fig. S1). The molecular weight of purified recombinant enzyme was estimated to be 29 kDa by SDS-PAGE which was about 4 kDa larger than the wild mature enzyme, corresponding to the size of T7-tag and His-tag at N-terminal of the recombinant enzyme (3.7 kDa). The molecular weight of the most reported chitosanases were ranging from 20 to 60 kDa(31, 23, 7, 6, 24), but Kim reported a chitosanase produced by *Aspergillus fumigatus* was 108 kDa(8) and Chen obtained a chitosanase produced by *Aspergillus* sp. CJ22-326 was 109 kDa(2). The molecular weight of SaCsn46A is close to a chitosanase from *Streptomyces albolongus* (29.6 kDa)(4).

Chitosanase activity and substrate specificity

The specific activity of SaCsn46A was determined after purification step with Ni-NTA column was increased from $121.2 \text{ U} \cdot \text{mg}^{-1}$ to $424.2 \text{ U} \cdot \text{mg}^{-1}$, obtaining a purification fold of 3.5 and recovery yield of 78% (Results are listed in Table 1).

Table 1
Effects of different substrates on SaCsn46A

Substrates	Relative activity (%)
Colloidal chitosan	100 ± 1.67
Power chitosan	1.77 ± 0.12
Colloidal chitin	1.77 ± 0.22
Power chitin	1.48 ± 0.17
Cellulose	0.46 ± 0.09
Carboxymethyl cellulose	2.39 ± 0.15
Xylan	2.23 ± 0.17
Soluble starch	N.D.
Dextrin	N.D.
N.D., the enzyme activity was not detected.	

In order to determine the substrate specificity of SaCsn46A, we tested its ability to hydrolyze different polysaccharides, and the results are listed in Table 2. We found that SaCsn46A can hydrolyze a variety of polysaccharides which were linked by β -1,4-glycosidic bonds, but it could not hydrolyze polysaccharides linked by α -1,4-glycosidic bonds such as soluble starch and dextrin. The enzyme showed a high hydrolysis activity toward colloidal chitosan, and displayed low hydrolysis activity toward powder chitosan (7.51 ± 0.72 U/mg), colloidal chitin (7.51 ± 0.93 U/mg), powder chitin (6.28 ± 0.93 U/mg), cellulose (1.95 ± 0.93 U/mg), carboxymethyl cellulose (10.14 ± 0.64 U/mg) and xylan (9.46 ± 0.93 U/mg), shows a broad of substrate spectrum. It can be seen from the experimental results that although SaCsn46A can hydrolyze a variety of polysaccharides, its catalytic capacity for cellulose is the lowest, which may be related to the solubility of cellulose and the space size of constituent monomers. The broad spectrum properties of substrate are different from the reported chitosanase belonging to family GH46, such as Csn21c from *S. albolongus*(4), Csn-BAC from *Bacillus* sp. MD-5(29), Csn-CAP from *Staphylococcus capitis*(24) and BaCsn46A from *Bacillus amyloliquefaciens*(18). These enzymes strictly hydrolyze colloidal chitosan and have no catalytic effect on powder chitosan and other polysaccharides.

Table 2
Effects of different ions on SaCsn46A

Ions	Relative activity (%)	
	1 mM	2 mM
Control	100 ± 1.89	100 ± 2.18
Cu ²⁺	95.32 ± 2.45	46.76 ± 2.34
Fe ²⁺	103.20 ± 2.04	107.25 ± 1.51
K ⁺	100.67 ± 1.88	79.92 ± 2.52
Mg ²⁺	103.73 ± 1.98	127.85 ± 1.74
Ba ²⁺	92.79 ± 1.94	76.94 ± 2.43
Ca ²⁺	87.85 ± 1.59	63.47 ± 1.89
Zn ²⁺	89.05 ± 2.62	48.70 ± 2.09
EDTA	34.56 ± 3.28	21.61 ± 2.79

Biochemical characterization of the purified chitosanase

The optimal temperature for SaCsn46A was determined in different temperature ranges (20–90 °C). The maximum relative chitosanase activity was observed at 45 °C (Fig. 3a), similar optimal temperature (45 °C) has been reported from *Paenibacillus dendritiformis* chitosanase(25), while was higher than that of *Gynuella sunshinyii* chitosanase (30 °C)(17), *Bacillus* sp. BY01 chitosanase (35 °C)(30), *Bacillus* sp. MD-5 chitosanase (40 °C)(29) and *S. capitatus* chitosanase (40 °C)(24), and lower than *Bacillus* sp. chitosanase (60 °C)(13), *B. amyloliquefaciens* chitosanase (55 °C)(12) and *S. albolongus* chitosanase (50 °C)(4). Most reported microbial chitosanases have an optimum reaction pH in the range 4 to 8 (27). Optimal chitosanase activity was observed at pH 6.2 in 50 mM phosphate buffer (Fig. 3b), while SaCsn46A was pH-sensitive (Fig. 3b), less than 30% relative activity was observed at pH value below 6 or above 7, however more than 80% relative activity was observed at pH6-7 in 50 mM phosphate buffer.

Thermostability illustrated that the SaCsn46A was stable below 30 °C (Fig. 3c). SaCsn46A has good pH stability at 4.0–9.0 (Fig. 1d), retained more than 80% of its maximal activity at pH 5.0–9.0 after incubation for 2 h, the stability characteristic of SaCsn46A was similar to *Bacillus* sp. chitosanase (stable at pH 3.6–9.8 below 30 °C)(13).

The effects of metal ions on the activity of SaCsn46A are presented in Table 3. The enzyme activity was inhibited by Cu²⁺, Ba²⁺, Ca²⁺ and Zn²⁺ at both 1 mM and 2 mM concentration. The enzyme activity was inhibited by K⁺ at 2 mM (79.92%). K⁺ and Mg²⁺ of 1mM has no effect on enzyme activity. Fe²⁺ at both 1 mM and 2 mM displayed slight promoting effect on enzyme activity. Notably, the enzyme activity can be

increased to 3.62-fold by Mn^{2+} at 3 mM concentration, and stimulatory effect was observed when the concentration of Mn^{2+} was 9 mM (Fig. 4). Most chitosanases can be enhanced activity by Mn^{2+} , for example, Csn21c from *S. albolongus*(4), Csn-BAC from *Bacillus* sp. MD-5(29) and CsnB from *Bacillus* sp. BY01(30), that were enhanced to 2.0-fold, 1.79-fold and 2.57-fold, respectively, after the addition of Mn^{2+} , obviously Mn^{2+} shows more stimulatory effect on SaCsn46A than other chitosanases. EDTA showed a significant inhibitory effect on SaCsn46A, because EDTA is a chelator of divalent cations, suggesting that this chitosanase is a metalloenzyme.

The K_m value (colloidal chitosan as substrate) of SaCsn46A was $1.32 \text{ mg}\cdot\text{mL}^{-1}$, which was lower than that of Csn21c from *S. albolongus* ($7.4 \text{ mg}\cdot\text{mL}^{-1}$)(4), BaCsn46A from *B. amyloliquefaciens* ($2.8 \text{ mg}\cdot\text{mL}^{-1}$)(18), GsCsn46A from *G. sunshinyii* ($1.97 \text{ mg}\cdot\text{mL}^{-1}$)(17). Notably, this value is very low, indicating a high affinity with the substrate. The $V_{\max}$ value of SaCsn46A was determined that was $526.32 \text{ }\mu\text{M}\cdot\text{mg}^{-1}\cdot\text{min}^{-1}$, which was higher than that of Csn21c from *S. albolongus* ($263.1 \text{ }\mu\text{M}\cdot\text{mg}^{-1}\cdot\text{min}^{-1}$) and GsCsn46A from *G. sunshinyii* ($358.65 \text{ }\mu\text{M}\cdot\text{mg}^{-1}\cdot\text{min}^{-1}$)(17), while was much lower than BaCsn46A from *B. amyloliquefaciens* ($7142.9 \text{ }\mu\text{M}\cdot\text{mg}^{-1}\cdot\text{min}^{-1}$)(18).

Hydrolytic properties of chitosanase

The hydrolysis products of colloidal chitosan (1%, w/v, 90%DDA) were detected by TLC, results are listed in Fig. 5. After 7 h incubation, chitosan was complete degraded by SaCsn46A, two clear spots could be detected on the TLC plate. The mobility ratio of these spots was in good agreement with GlcN and $(\text{GlcN})_2$ markers, indicated that this enzyme can hydrolyze chitosan into GlcN and $(\text{GlcN})_2$. This conclusion was followed verified by high performance liquid chromatography (HPLC) analysis (Fig. 4). There is one product peak with an appearance time of 6.192 min in Fig. 4 (c), which is consistent with the appearance time of GlcN Fig. 4 (a), and the appearance time of the other product peak is 7.245 min, which is consistent with the peak time of $(\text{GlcN})_2$ Fig. 4 (b). The ratio of the two hydrolytic products is 1:2.8 ($\text{GlcN}:(\text{GlcN})_2$). As shown in TLC analysis (Fig. 4), CHOS with different DPs appeared after degraded for 5 min. After 30 min incubation, $(\text{GlcN})_2$ and $(\text{GlcN})_3$ became main products. As the reaction continued, only two visible spots were left on the TLC plate after complete degradation. Recently, most microorganisms produced chitosanases were reported to produce DP2-7 CHOS(11–13, 16–18, 24, 25, 29, 30), only Csn21c from *S. albolongus* was reported that can hydrolyze chitosan into GlcN and $(\text{GlcN})_2$ (4), however, the hydrolysis behavior of SaCsn46A is completely different from that of Csn21c. When Csn21c hydrolyzes chitosan, GlcN was existed at the initial stage of the hydrolysis process, however, SaCsn46A hydrolyzes chitosan, higher DP products appear first, then gradually degrade into GlcN and $(\text{GlcN})_2$, that indicate SaCsn46A is an endo-type enzyme catalyzing the cleavage of β -1,4-glycosidic linkage. Although this hydrolysis characteristic of SaCsn46A is similar to other reported GH46 family chitosanases, hydrolysates of SaCsn46A have lower degree of polymerization.

Conclusion

A novel chitosanase gene was cloned from *S. avermitilis* and expressed in *E. coli* Rosetta. The recombinant enzyme has its optimal enzyme activity under condition of 45 °C in 50 mM pH 6.2 phosphate buffer, and was very stable at pH range from 4.0 to 9.0 and below 30 °C. The enzyme activity can be strongly enhanced about 3.5-fold by Mn^{2+} at a 3 mM concentration. The enzyme can hydrolyze a variety of β -1,4-glycosidic bonds-linked polysaccharides which shows a good application prospect. The hydrolysis products were mainly GlcN and (GlcN)₂ which demonstrated that SaCsn46A was a good potential candidate for the industrial production of GlcN and (GlcN)₂ in mild conditions.

Declarations

Author Contribution Conceptualization: J. Guo, Z.W. Man. Methodology: J. Guo, W.J. Gao. Data analysis: J. Guo, Y. Wang. Software: J. Guo. Writing original manuscript: J. Guo. Review and revising manuscript: Y. Wang, W.J. Gao., X.R. Wang, X. Gao, Z.W. Man, Z.Q. Cai, Q. Qing. Funding acquisition: J. Guo, Z.W. Man, Z.Q. Cai, Q. Qing. All authors reviewed and approved the final manuscript.

Funding This work was supported by the National Natural Science Foundation of China (31700075), the Natural Science Foundation of the Jiangsu Higher Education Institution of China (19KJB180001), the Key Research and Development Program of Shandong Province, China (2019JZZY020605), the Initial Research Funding of Changzhou University (ZMF17020115), the Extracurricular Innovation and Entrepreneurship Fund for College Students of Changzhou University (ZMF19020280), the Natural Science Foundation of Jiangsu Province (BK20181465).

Data Availability All data generated or analyzed during this study are included in this published article and its supplementary information files.

Ethics Approval and Consent to Participate Not applicable.

Consent for Publication Not applicable.

Conflict of Interest The authors declare no competing interests.

References

1. Chen, J., Liu, M., Liu, X., Miao, J., Fu, C., Gao, H. ... Zhang, L. (2016). Interrogation of *Streptomyces avermitilis* for efficient production of avermectins. *Synthetic and Systems Biotechnology*, 1, 7–16
2. Chen, X., Xia, W., & Yu, X. (2005). Purification and characterization of two types of chitosanase from *Aspergillus* sp. CJ22-326. *Food Research International*, 38, 315–322
3. Deng, Q., Xiao, L., Liu, Y., Zhang, L., Deng, Z., & Zhao, C. (2019). *Streptomyces avermitilis* industrial strain as cell factory for Ivermectin B1a production. *Synthetic and Systems Biotechnology*, 4, 34–39
4. Guo, N., Sun, J., Wang, W., Gao, L., Liu, J., Liu, Z. ... Mao, X. (2019). Cloning, expression and characterization of a novel chitosanase from *Streptomyces albolongus* ATCC 27414. *Food*

5. Heggset, E. B., Tuveng, T. R., Hoell, I. A., Liu, Z., Eijsink, V. G. H., & Vårum, K. M. (2012). Mode of Action of a Family 75 Chitosanase from *Streptomyces avermitilis*. *Biomacromolecules*, 13, 1733–1741
6. Hirano, K., Arayaveerasid, S., Seki, K., Adams, D. J., & Mitsutomi, M. (2014). Characterization of a Chitosanase from *Aspergillus fumigatus* ATCC13073. *Bioscience, Biotechnology, and Biochemistry*, 76, 1523–1528.
7. Kim, P. I., Kang, T. H., Chung, K. J., Kim, I. S., & Chung, K. (2004). Purification of a constitutive chitosanase produced by *Bacillus* sp. MET 1299 with cloning and expression of the gene. *FEMS Microbiology Letters*, 240, 31–39
8. Kim, S. Y. S., & Lee, D. H., Ke Ho. (1998). Purification and Characteristics of Two Types of Chitosanases from *Aspergillus fumigatus* KH-94. *Journal of Microbiology and Biotechnology*, 8, 568–574
9. Kumar, M., Dangayach, P., & Pareek, N. (2020). Enhanced glucosamine production through synergistic action of *Aspergillus terreus* chitozymes. *Journal of Cleaner Production*, 262, 121363
10. Lin, S., Qin, Z., Chen, Q., Fan, L., Zhou, J., & Zhao, L. (2019). Efficient Immobilization of Bacterial GH Family 46 Chitosanase by Carbohydrate-Binding Module Fusion for the Controllable Preparation of Chitooligosaccharides. *Journal of Agricultural and Food Chemistry*, 67, 6847–6855
11. Liu, G. L., Li, Y., Zhou, H. X., Chi, Z. M., & Madzak, C. (2012). Over-expression of a bacterial chitosanase gene in *Yarrowia lipolytica* and chitosan hydrolysis by the recombinant chitosanase. *Journal of Molecular Catalysis B: Enzymatic*, 83, 100–107
12. Luo, S., Qin, Z., Chen, Q., Fan, L., Jiang, L., & Zhao, L. (2020). High level production of a *Bacillus amyloliquefaciens* chitosanase in *Pichia pastoris* suitable for chitooligosaccharides preparation. *International Journal of Biological Macromolecules*, 149, 1034–1041
13. Ma, C., Li, X., Yang, K., & Li, S. (2020). Characterization of a New Chitosanase from a Marine *Bacillus* sp. and the Anti-Oxidant Activity of Its Hydrolysate. *Marine Drugs*, 18.
14. Mao, X., Guo, N., Sun, J., & Xue, C. (2017). Comprehensive utilization of shrimp waste based on biotechnological methods: A review. *Journal of Cleaner Production*, 143, 814–823
15. Park, P. J., Je, J. Y., & Kim, S. K. (2003). Free Radical Scavenging Activity of Chitooligosaccharides by Electron Spin Resonance Spectrometry. *Journal of Agricultural and Food Chemistry*, 51, 4624–4627
16. Pechsrichuang, P., Lorentzen, S. B., Aam, B. B., Tuveng, T. R., Hamre, A. G., Eijsink, V. G. H., & Yamabhai, M. (2018). Bioconversion of chitosan into chito-oligosaccharides (CHOS) using family 46 chitosanase from *Bacillus subtilis* (BsCsn46A). *Carbohydrate Polymers*, 186, 420–428
17. Qin, Z., Chen, Q., Lin, S., Luo, S., Qiu, Y., & Zhao, L. (2018). Expression and characterization of a novel cold-adapted chitosanase suitable for chitooligosaccharides controllable preparation. *Food Chemistry*, 253, 139–147
18. Qin, Z., Luo, S., Li, Y., Chen, Q., Qiu, Y., Zhao, L. ... Zhou, J. (2018). *Biochemical properties of a novel chitosanase from Bacillus amyloliquefaciens and its use in membrane reactor* (97 vol., pp. 9–16). LWT

19. Rahimnejad, S., Yuan, X., Wang, L., Lu, K., Song, K., & Zhang, C. (2018). Chitooligosaccharide supplementation in low-fish meal diets for Pacific white shrimp (*Litopenaeus vannamei*): Effects on growth, innate immunity, gut histology, and immune-related genes expression. *Fish & Shellfish Immunology*, 80, 405–415
20. Ribas Fonseca, L., Porto Santos, T., Czaikoski, A., & Cunha, L., R. (2020). Modulating properties of polysaccharides nanocomplexes from enzymatic hydrolysis of chitosan. *Food Research International*, 137, 109642
21. Salah, R., Michaud, P., Mati, F., Harrat, Z., Lounici, H., Abdi, N. ... Mameri, N. (2013). Anticancer activity of chemically prepared shrimp low molecular weight chitin evaluation with the human monocyte leukaemia cell line, THP-1. *International Journal of Biological Macromolecules*, 52, 333–339
22. Shehata, A. N., Aty, A. E., Darwish, A. A., Wahab, D. A. A., & Mostafa, F. A., W. A. and. (2018). Purification, physicochemical and thermodynamic studies of antifungal chitinase with production of bioactive chitosan-oligosaccharide from newly isolated *Aspergillus griseoaurantiacus* KX010988. *International Journal of Biological Macromolecules*, 107, 990–999
23. Su, C., Wang, D., Yao, L., & Yu, Z. (2006). Purification, Characterization, and Gene Cloning of a Chitosanase from *Bacillus* Species Strain S65. *Journal of Agricultural and Food Chemistry*, 54, 4208–4214
24. Sun, H., Cao, R., Li, L., Zhao, L., & Liu, Q. (2018). Cloning, purification and characterization of a novel GH46 family chitosanase, Csn-CAP, from *Staphylococcus capitis*. *Process Biochemistry*, 75, 146–151
25. Sun, H., Mao, X., Guo, N., Zhao, L., Cao, R., & Liu, Q. (2018). Discovery and Characterization of a Novel Chitosanase from *Paenibacillus dendritiformis* by Phylogeny-Based Enzymatic Product Specificity Prediction. *Journal of Agricultural and Food Chemistry*, 66, 4645–4651
26. Sun, H., Yang, G., Cao, R., Mao, X., & Liu, Q. (2020). Expression and characterization of a novel glycoside hydrolase family 46 chitosanase identified from marine mud metagenome. *International Journal of Biological Macromolecules*, 159, 904–910
27. Thadathil, N., & Velappan, S. P. (2014). Recent developments in chitosanase research and its biotechnological applications: A review. *Food Chemistry*, 150, 392–399
28. Xia, W., Liu, P., & Liu, J. (2008). Advance in chitosan hydrolysis by non-specific cellulases. *Bioresource Technology*, 99, 6751–6762
29. Yang, G., Sun, H., Cao, R., Liu, Q., & Mao, X. (2020). Characterization of a novel glycoside hydrolase family 46 chitosanase, Csn-BAC, from *Bacillus* sp. MD-5. *International Journal of Biological Macromolecules*, 146, 518–523
30. Yang, Y., Zheng, Z., Xiao, Y., Zhang, J., Zhou, Y., Li, X. ... Yu, H. (2019). Cloning and Characterization of a Cold-adapted Chitosanase from Marine Bacterium *Bacillus* sp. BY01. *Molecules*, **24**.
31. Yoon, H. G., Kim, H. Y., Lim, Y. H., Kim, H. K., Shin, D. H., Hong, B. S., & Cho, H. Y. (2000). Thermostable Chitosanase from *Bacillus* sp. Strain CK4: Cloning and Expression of the Gene and Characterization of the Enzyme. *Applied and Environmental Microbiology*, 66, 3727

32. Yorinaga, Y., Kumasaka, T., Yamamoto, M., Hamada, K., & Kawamukai, M. (2017). Crystal structure of a family 80 chitosanase from *Mitsuaria chitosanitabida*. *FEBS Letters*, 591, 540–547
33. Yue, L., Li, J., Chen, W., Liu, X., Jiang, Q., & Xia, W. (2017). Geraniol grafted chitosan oligosaccharide as a potential antibacterial agent. *Carbohydrate Polymers*, 176, 356–364
34. Zhang, J., Cao, H., Li, S., Zhao, Y., Wang, W., Xu, Q. ... Yin, H. (2015). Characterization of a new family 75 chitosanase from *Aspergillus* sp. W-2. *International Journal of Biological Macromolecules*, 81, 362–369
35. Zhou, Z., Zhao, S., Liu, Y., Chang, Z., Ma, Y., Li, J., & Song, J. (2016). A highly Conserved Aspartic Acid Residue of the Chitosanase from *Bacillus* Sp. TS Is Involved in the Substrate Binding. *Applied Biochemistry and Biotechnology*, 180, 1167–1179

Figures

Figure 1

Bioinformatics analysis of SaCsn46A. (a) Neighbor-joining phylogenetic tree. Phylogenetic analysis was carried out using MEGA 5.0 software. Chitosanases in different families are framed in red; (b) multiple amino acid sequence alignment of SaCsn46A and other chitosanases belonging to GH46 family (signal peptide were removed from these protein sequences). The typical catalytic residues (E and D) are indicated by red underlines. The listed sequence included the chitosanases from *Streptomyces* sp. SirexAA-E (4ILY), *Streptomyces* sp. N174 (1CHK), *Microbacterium* sp. OU01 (ABM91442.1), *Bacillus amyloliquefaciens* (QJW64146.1) and *Bacillus* sp. MD-5 (ASB61783.1); (c) Homology modeling structure of SaCsn46A.

Figure 2

Enzymatic properties of SaCsn46A. (a) Effects of temperature on SaCsn46A; (b) Effects of pH on SaCsn46A; (c) enzyme inactivation at different temperatures; (d) pH stability. The 100% relative activity of SaCsn46A was 424.2 U/mg.

Figure 3

Effects of different Mn²⁺ concentration on SaCsn46A

Figure 4

Thin layer chromatography (TLC) analysis of hydrolysis products of colloidal chitosan and HPLC analysis of hydrolysis products of colloidal chitosan by SaCsn46A. (a) GlcN standard; (b) (GlcN)₂ standard; (c) hydrolysis products of colloidal chitosan by SaCsn46A; Std, COS standards.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementalResult.docx](#)