Supporting Information (SI)

Biocatalysis of Heterogenously-expressed Chitosanase for Preparation of Desirable Chitosan Oligosaccharides Applied against Phytopathogenic Fungi

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1 Materials and Methods

1.1 Materials

Chitosan [the degree of deacetylation (DDA) $\geq$ 95%] and colloidal chitin were purchased from Aladdin (China), while microcrystalline cellulose was purchased from Sigma (USA). COSs (purity $\geq$ 95%), including chitobiose: $(GlcN)_2$, chitotriose: $(GlcN)_3$, chitotetraose: $(GlcN)_4$, chitopentaose: $(GlcN)_5$ and chitohexaose: $(GlcN)_6$ were purchased from Long Dragon Bio. (Huizhou, China). Glycosidase PNGase F was purchased from NEB (Beijing, China). The strain *Pichia pastoris* GS115 and the vector pPIC9K were purchased from Invitrogen (USA). Phytopathogenic fungi, such as *Botrytis cinerea*, *Fusarium graminearum*, *Alternaria alternate*, *Magnaporthe grisea*, *Erysiphe cichoracearum*, and *Alternaria solani* were kindly provided by Prof. Jichuan Kang, College of Life Sciences, Guizhou University, Guiyang, China. All other chemicals were of analytical grade.

1.2 Methods

1.2.1 Prediction of the PI and molecular mass of the Csn75

The PI and molecular mass of the Csn75 were predicted by ExPASy ProtParam tool (https://web.expasy.org/protparam/#opennewwindow). Input the amino acid sequence of Csn75 into the frame on the website, and then click the "computer parameters", the result was shown in Figure S2.

1.2.2 Prediction of the potential N-glycosylation sites of the Csn75

Potential N-glycosylation sites of Csn75 were predicted by NetNGlyc 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc/#opennewwindow). Input the amino acid sequence of Csn75 into the frame on the web site, then click "Submit", the result was showed as shown in Figure S3.

1.2.3 Hydrolysis circle on the chitosan agar plate

To further confirm whether the gene of interest was correctly expressed, the hydrolysis of chitosan agar plate by fermentation supernatant was studied. Briefly, Oxford cups were placed on a agar plate containing 0.5% (w/v) chitosan, then, 200 µL/cup of the fermentation supernatant of GS115-pPIC9K-Csn75 was added into the Oxford cups and then the plate was incubated at 50 °C for 3 h, GS115-pPIC9K was used as control. Then, significant hydrolysis circles were observed around the Oxford cup filled with GS115-pPIC9K-Csn75 but there was no hydrolysis circle around the Oxford cup filled with the supernatant of GS115-pPIC9K, the result was shown in Figure S4.

1.2.4 Activity of determination of glycosylated and de-glycosylated Csn75

The activity of the glycosylated enzyme produced by GS115-pPIC9K-Csn75 was compared with the de-glycosylated enzyme produced in *Escherichia coli* in previous studies. In short, the enzyme activity of glycosylated and de-glycosylated enzyme solutions with the same protein concentration after purification was determined. The glycosylated enzyme activity measured under standard conditions was defined as 100%. The result was shown in Figure S4.

1.2.5 Kinetic analysis of purified Csn75

The kinetic parameters were analyzed using various concentrations of chitosan, [S] indicated the chitosan concentration, for ease of calculation, 1/[S] was ranging from 0.6 to 1.6 mL/mg with the interval of 0.2. The reactions were performed at 50 °C for 5 min in 0.2 M sodium acetate buffer at pH 5.5. The *Km* and *Vmax* values were calculated using the nonlinear regression analysis by program Graft. The result was shown in Figure S5.

1.2.6 Determination of different DP oligosaccharides contents.

HPLC was used to establish standard curve of content and peak area for chitobiose: $(GlcN)_2$, chitotriose: $(GlcN)_3$, chitotetraose: $(GlcN)_4$, chitopentaose: $(GlcN)_5$ and chitohexaose: $(GlcN)_6$, respectively. Briefly, the standard curves of $(GlcN)_2$, $(GlcN)_3$, $(GlcN)_4$, $(GlcN)_5$ and $(GlcN)_6$ were established by loading different amounts (2.5, 5, 10, 15, 20 μL) of the oligosaccharides on HPLC, respectively. Use the mass of the oligosaccharide as the ordinate and the corresponding peak area as the abscissa to make a standard curve, then, the regression equation of each oligosaccharide can be obtained by linear fitting. Finally, the content of the oligosaccharide in the sample can be calculated by the peak area of the corresponding retention time and the regression equation.

1.2.7 Protein concentration determination

Protein concentration was estimated by the Bradford assay method[1], using bovine serum albumin (Sigma) as the standard.

2 Figures and Tables of Supporting Information

2.1 Four steps for chitosanase purification and devices parameters of each step
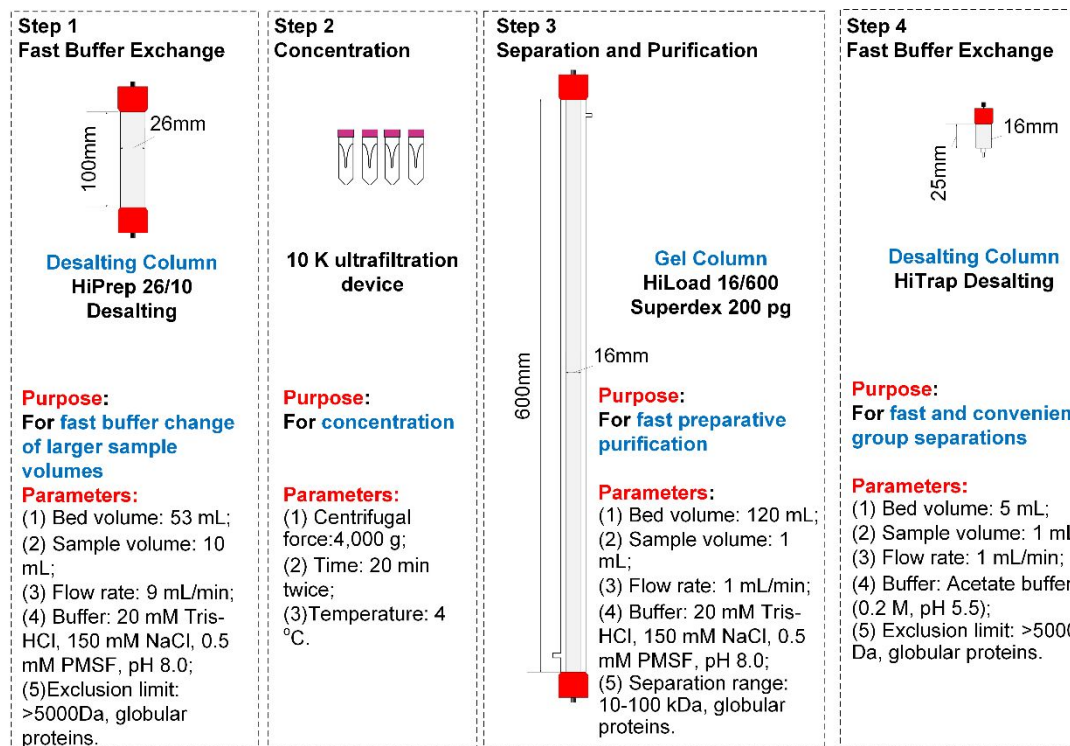
Figure S1 Four steps for chitosanase purification and devices parameters of each step

2.2 Molecular mass and PI of Csn75 predicted by ExPASy ProtParam tool

The PI of Csn75 was 6.05 and the molecular mass Csn75 was 23.5 kDa according to the predicted result as shown in Figure S2.

```
Number of amino acids: 221

Molecular weight: 23463.11

Theoretical pI: 6.05

Amino acid composition:    CSV format
Ala (A)   18        8.1%
Arg (R)    0        0.0%
Asn (N)   17        7.7%
Asp (D)   18        8.1%
Cys (C)    6        2.7%
Gln (Q)    6        2.7%
Glu (E)    7        3.2%
Gly (G)   26       11.8%
His (H)    8        3.6%
Ile (I)   12        5.4%
Leu (L)   12        5.4%
Lys (K)   21        9.5%
Met (M)    3        1.4%
Phe (F)   11        5.0%
Pro (P)   10        4.5%
Ser (S)   20        9.0%
Thr (T)    7        3.2%
Trp (W)    2        0.9%
Tyr (Y)    6        2.7%
Val (V)   11        5.0%
Pyl (O)    0        0.0%
Sec (U)    0        0.0%

 (B)      0         0.0%
 (Z)      0         0.0%
 (X)      0         0.0%


Total number of negatively charged residues (Asp + Glu): 25
Total number of positively charged residues (Arg + Lys): 21
```

Figure S2 Molecular mass and PI of Csn75 predicted by ExPASy ProtParam tool

2.3 Potential N-glycosylation sites of the Csn75 predicted by NetNGlyc 1.0 server

Three potential N-glycosylation sites of Csn75 are found at N23, N57, and N67 according to the result shown in Figure S3.

NetNGlyc 1.0 Server - prediction results
Technical University of Denmark

```
Asn-Xaa-Ser/Thr sequons in the sequence output below are highlighted in blue.
     Asparagines predicted to be N-glycosylated are highlighted in red.
```

Output for 'Sequence'

```
Name:  Sequence         Length:  313
MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPFSNSTNNGLLFINTTIASIAAKEEGV        80
SLEKREAEAYVEYNLPNNLEQIYDKHKGKCSKVLAKGFTNGDASQGKSFSYCGDIPGAIFISSSKGYTNMDINCDGANNS       160
AGKCANDPSGQGETAFKSDVKKFGISDLDANIHPYVVFGNEDHSPKFKPQSHGMQPLSVMAVVCNGQLHYGIWGDTNGGV       240
STGEASISLADLCFPNEHLDGNHGHDPNDVLFIGFTSKDAVPGATAKWKAKNAKEFEDSIKSIGDKLVAGLKA
......................N...............................N.........N..............       80
...............................................................................      160
...............................................................................      240
.........................................................................            320

(Threshold=0.5)
----------------------------------------------------------------------------------
SeqName        Position  Potential   Jury    N-Glyc
                                   agreement result
----------------------------------------------------------------------------------
Sequence        23 NTTT   0.6683    (9/9)    ++
Sequence        57 NSTN   0.6277    (8/9)    +
Sequence        67 NTTI   0.6466    (9/9)    ++
Sequence       158 NNSA   0.2327    (9/9)    ---
----------------------------------------------------------------------------------
```
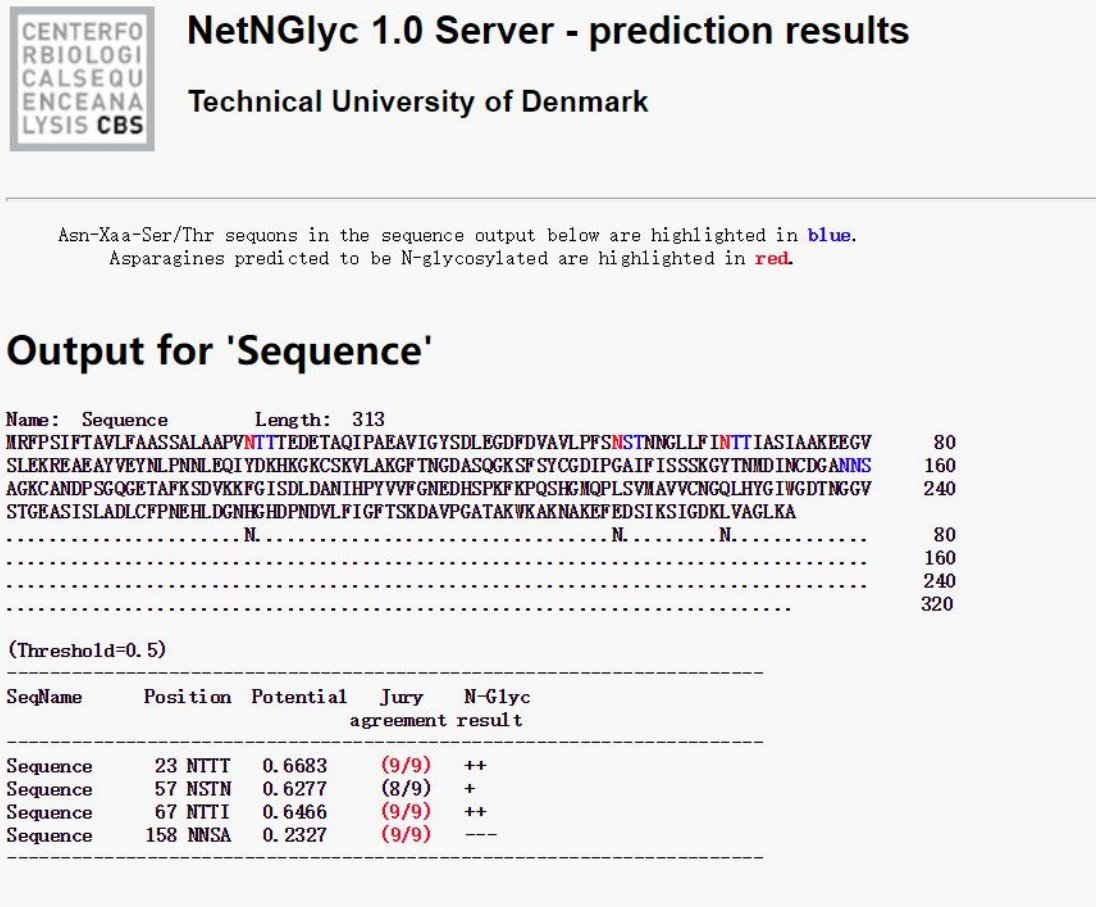
Figure S3 Potential N-glycosylation sites of the Csn75 predicted by NetNGlyc 1.0 server

2.4 Hydrolysis circle on the chitosan agar plate

Significant hydrolysis circles were observed around the Oxford cup filled with the supernatant of GS115-pPIC9K-Csn75 on the chitosan agar plate, but there was no hydrolysis circle around the Oxford cup filled with the supernatant of GS115-pPIC9K, as shown in Figure S4.
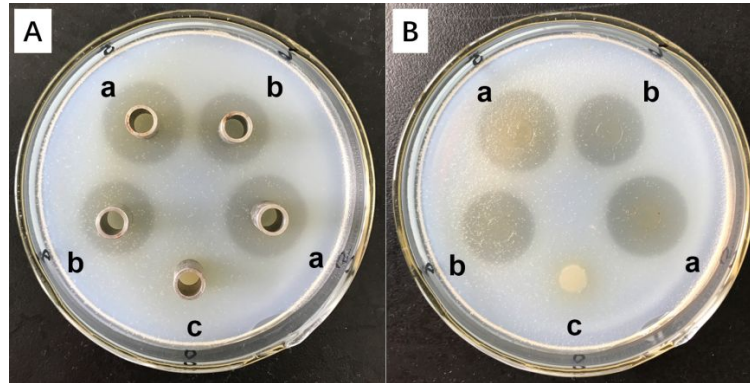

Figure S4 Hydrolysis circles of fermentation supernatant of GS115-pPIC9K-Csn75 (a, b) and GS115-pPIC9K (c) on chitosan agar plate with Oxford cups (A) and without Oxford cups (B). a and b respectively represent different positive transformants of GS115-pPIC9K-Csn75

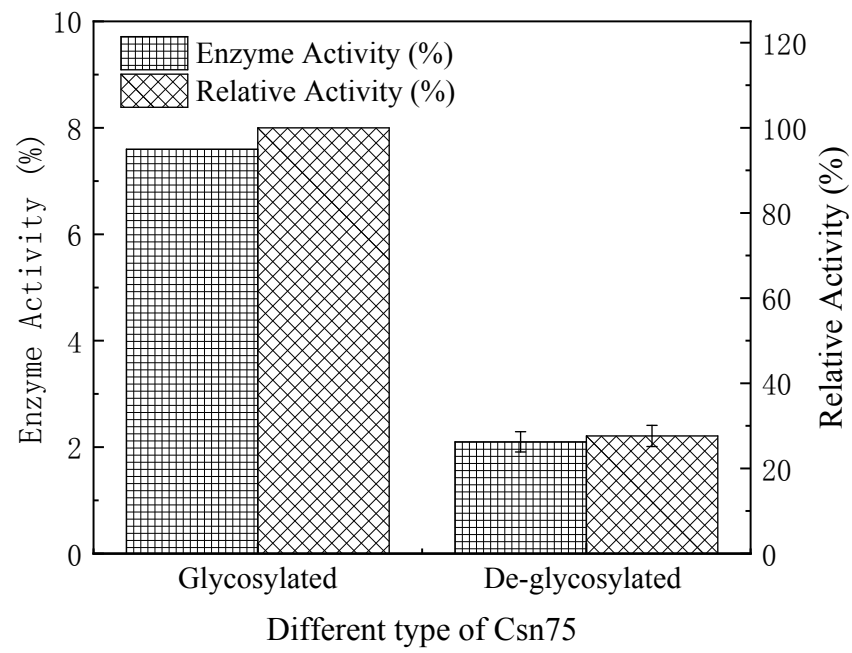2.5 Comparison of glycosylated and de-glycosylated enzyme activities

Figure S5 Comparison of glycosylated and de-glycosylated enzyme activities

2.6 The kinetic parameters analysis

The kinetic parameters were analyzed using various concentrations of chitosan, [S] indicated the chitosan concentration, for ease of calculation, 1/[S] was ranging from 0.6 to 1.6 mL/mg with the interval of 0.2. The linear fitting equation is y=0.0756x+0.1658, the $\frac{1}{V_m}$ equal to the intercept on axis y, and the $-\frac{1}{K_m}$ equal to the intercept on the axis x. The K_m and V_{max} values were calculated as follow:

$$\frac{1}{V_m} = 0.1658 \qquad (S1)$$

$$-\frac{1}{K_m} = -\frac{0.1658}{0.0756} \qquad (S2)$$

Therefore, V_m is 6.03 $\mu mol \cdot mL^{-1} \cdot min^{-1}$, and K_m is 0.46 mg/mL.
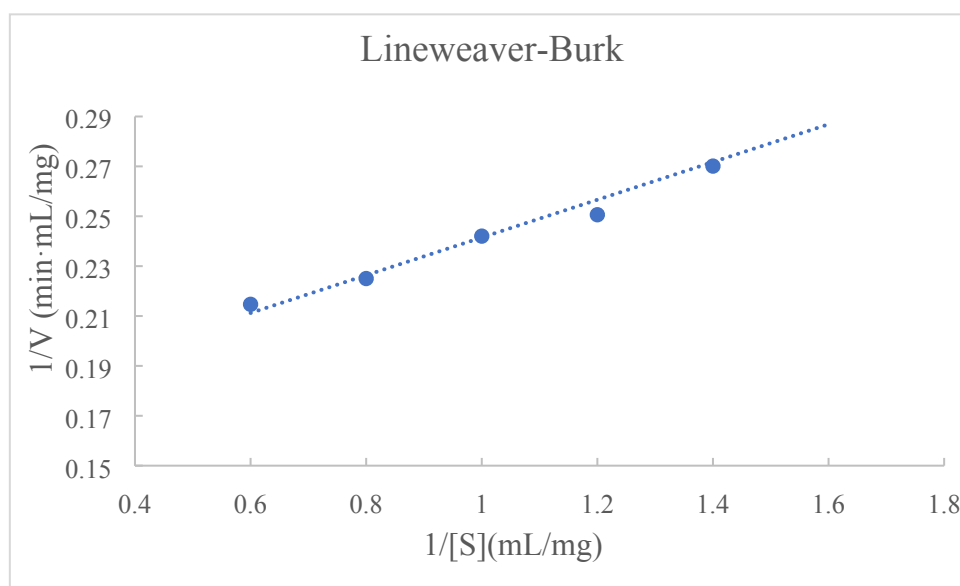

Figure S6 Lineweaver-Burk plot of the Csn75 activity

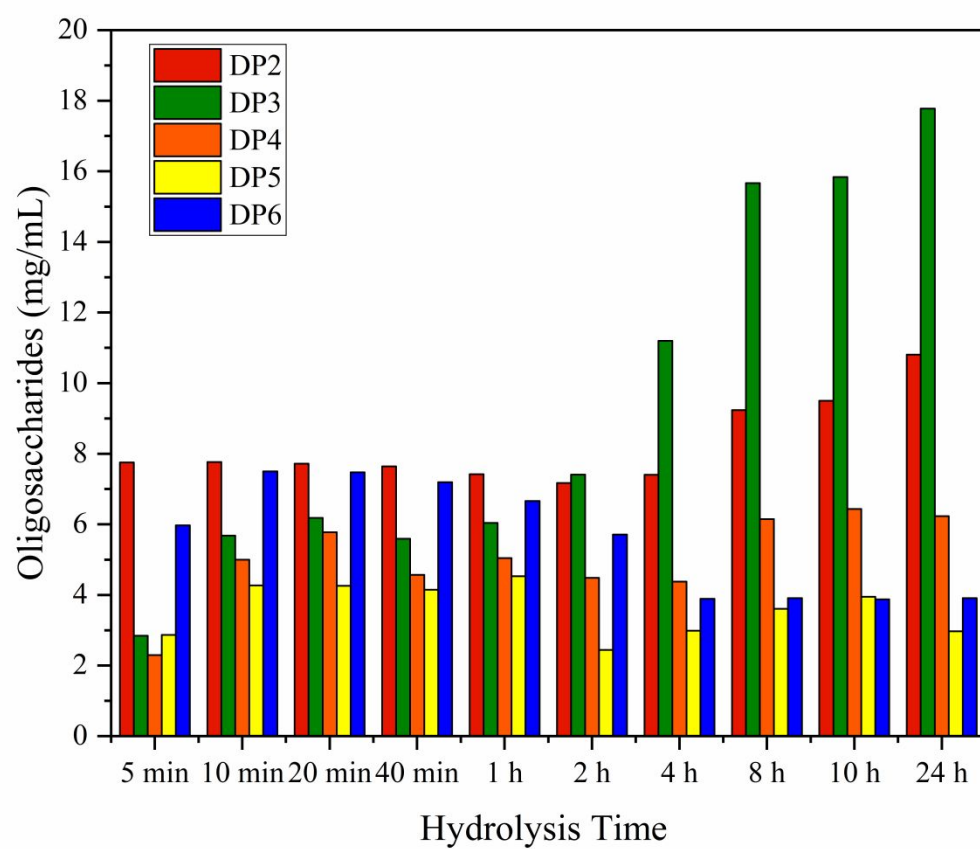## 2.7 Contents of different DP oligosaccharides at different hydrolysis time

Figure S7 Contents of different DP oligosaccharides at different hydrolysis time

2.8 Comparison of hydrolysis glycosidic bond of the endo and exo-chitosanases and classification of endo-chitosanases

Table S1 Comparison of hydrolysis glycosidic bond of the endo and exo-chitosanases and classification of endo-chitosanases based on the splitting specificity[2-3]

Name	Classification	Linkage				Beginning from
		D-D	D-A	A-D	A-A	
Endo-chitosanase	Subclass I	√	√	×	×	Reducing end
	Subclass II	√	×	×	×	
	Subclass III	√	×	√	×	
	Subclass IV	√	√	√	×	
Exo-chitosanase		√	√	×	×	Non reducing end

D: GlcN (D-glucosamine); A: GlcNAc (N-acetyl-D-glucosamine)

2.9 Comparison of properties of chitosanases

Table S2 Comparison of properties of chitosanases

GH family	Species	Protein name	Source	Mw (kDa)	Enzyme activity	pH	T (°C)	Metal-ion activators	Metal-ion inhibitors	K_m	V_m	Substrate specificity	EP	Ref.
GH75	*Aspergillus sp.*W2 (CGMCC7018)	csnw2	mutant	28	34 U/mg	6.0	55	Ca^{2+}, Mn^{2+}, Mg^{2+}	Fe^{2+}, Zn^{2+}, Ge^{2+} Ni^{2+}, Cu^{2+}	NM	NM	Chitosan (DDA: 92%)	DP 2-6 (DDA 92%)	[4]
GH75	*Aspergillus fumigatus* ATCC13073	NM	wild	24.5	8.8 U/mg	6.0	40	NM	NM	1.20-7.99 mM	3.10-4.52 μmol/min·mg	Chitosan (highly DDA)	DP 2-4	[5]
GH75	*Aspergillus sp. Y2K*	NM	wild	25	NM	6.5	65-70	Mn^{2+}, Zn^{2+},Ca^{2+}	Fe^{2+}, Cu^{2+},Ba^{2+}	NM	NM	Chitosan (highly DDA)	DP 3-5	[6]
GH46	*Janthinobacterium sp.* 4239	NM	wild	33	1500 U toward chitosan (80%DD,1000 kDa)	5-7	45	NM	NM	NM	NM	Chitosan (DDA: 55%, 80%)	DP 2-3 (DDA 80%)	[7]
GH46	*Bacillus subtilis* HD145	NM	mutant	31	9000 U/mg	5.5	50	NM	NM	NM	NM	Chitosan (DDA: 50%, 70%, 90%)	DP 2-4 (DDA 50%, 70%); DP 2-5 (DDA 90%)	[8]
GH46	*Bacillus subtilis 168*	NM	mutant	30	12000 U/mL	5-6	40-50	NM	NM	1.57mg/ mL	530 μmol/sec·mg	Chitosan (DDA: 75%-90%)	DP 2-4	[9]
NM	*Pseudoalteromonas sp. SY39*	CsnM	wild	28	393.2 U/mg	5.9	40	Mg^{2+}, Li^+, K^+, Ca^{2+}, Ba^{2+}	Al^{3+}, Cu^{2+}, Ni^{2+}, Fe^{2+}, Co^{2+}, Zn^{2+}	NM	NM	NM	DP 2-3	[10]
GH46	*Staphylococcus capitis*	Csn-CAP	mutant	35	89.2 U/mg	7	30	Mn^{2+},Zn^{2+}, Cu^{2+}	Ba^{2+}, Mg^{2+}, Ca^{2+}, Ni^{2+}	NM	NM	Chitosan (DDA:85%)	DP 2-3	[11]
GH46	*Gynuella sunshinyii YC6258*	GsCsn46A	mutant	29	260.87 U/mg	5.5	30	NM	NM	NM	NM	Chitosan (DDA≥95%)	DP 2-3	[12]
NM	*Microbacteriu*	ChiN	wild	30	9.6 U/mL	6.2	50	Mn^{2+}, Cu^{2+}	Hg^{2+}, Ag^+	1260U/	5 mg chitosan	Chitosan (DDA:	DP 2-4	[13]

	msp. OU01									mg protein	/mL	62%-100%)		
GH5	*Streptomyces griseus* HUT 6037	NM	mutant	34	86.2 U/mL	NM	NM	NM	NM	NM	NM	CMC, chitosan	DP 3-4	14
GH8	*Bacillus sp.* KCTC 0377BP	NM	wild	45	1700 U/mL	NM	NM	NM	NM	1.1mg chitosan /mL	NM	Carboxymethylated cellulose; Chitosan (DDA: 40%-100%)	DP 2-3	15

DP: Degree of Polymerization

DDA: Degree of Deacetylation

NM: Not Mentioned

EP: End Products (use DP of the Chitosan oligosaccharide to represent)

2.10 Determination of the chitosanase activity and protein content in the supernatant during fermentation

Table S3 Determination of the chitosanase activity and protein content in the supernatant during fermentation

Time (h)	Total Protein Content (mg/mL)	Enzyme Activity (U/mL)
0	1.383±0.0585 [a]	1.848±0.249 [a]
12	1.335±0.0751 [a]	1.862±0.365 [a]
24	1.811±0.0904 [b]	2.545±0.185 [a]
36	2.057±0.0663 [b]	3.335±0.387 [ab]
48	2.575±0.1021 [c]	4.626±0.541 [b]
60	3.688±0.0915 [d]	11.525±0.555 [c]
72	3.866±0.0738 [d]	13.115±0.245 [cd]
84	3.947±0.0759 [de]	13.535±0.504 [d]
96	4.201±0.0982 [e]	13.734±0.671 [d]

Data are presented as mean values ± standard deviation (n = 2). Different superscript letters in the same column indicate significantly different ($P < 0.05$).

2.11 Substrate specificity of the Csn75

Table S4 Substrate specificity of the Csn75

Substrate	Relative activity (%)
Chitosan (DDA≥95%)	100
Chitin	ND
Microcrystalline Cellulose	ND

"ND" means not detected.

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