Journal of the Taiwan Institute of Chemical Engineers 000 (2017) 1–6

Contents lists available at ScienceDirect

Journal of the Taiwan Institute of Chemical Engineers

journal homepage: www.elsevier.com/locate/jtice

Enhancement of chitosanase secretion by *Bacillus subtilis* for production of chitosan oligosaccharides

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ARTICLE INFO

Article history:
Received 22 September 2016
Revised 1 December 2016
Accepted 24 December 2016
Available online xxx

Keywords:
Bacillus subtilis
Chitin
Chitosan
Chitosanase
Protein sorting signals
Glycoside hydrolases

ABSTRACT

Chitosan oligosaccharides (COSs) are produced by hydrolysis of chitosan (the deacetylation product of chitin) by chitosanases. COSs are widely used in food, textile, pharmaceutical, and medical applications. The chitosanase (Csn) encoded by the *Bacillus subtilis* 168 *csn* gene, which belongs to the glycosyl hydrolase family 46, was recombinantly expressed in *B. subtilis* PT5. Csn fused with various signal peptides was secreted into culture supernatants and its production evaluated. The highest specific activity achieved was 156 ± 4.68 U/ml using the *aprE* signal peptide rather than the original *csn* signal peptide. The optimal temperature and pH for enzyme activity were determined as 47 °C and 5.4, respectively, using central composite design (CCD). Recombinant Csn could efficiently hydrolyze both α and β type chitosan to dimer, trimer, and tetramer COSs. Further, the optimal medium for chitosanase production was predicted and successfully determined using Box–Behnken experimental designs. The maximum Csn protein production level that could be achieved using the optimal fermentation medium (0.76% lactose, 1.63% yeast extract, and 2.31% glutamate) was 208.23 ± 5.19 U/ml in a 5-l fermenter. The present study demonstrates that recombinant *B. subtilis* Csn has potential for use as a biocatalyst to develop industrial-scale production of COSs.

1. Introduction

Chitin and its protein-bound forms are widespread in nature; after cellulose, chitin is the second most abundant natural polymer [1]. Chitosan, the deacetylation product of chitin, is an unbranched, straight-chain cationic polymer derived from a combination of *N*-acetyl-D-glucosamine (GlcNAc) and D-glucosamine (GlcN). In general, the proportion of GlcN in chitosan is >80%; however, GlcN and GlcNAc are randomly distributed within the overall structure of the molecule [2]. Chitosan has applications in various fields, including food, textile, pharmaceutical, and cosmetic production, among others. Chitosan oligosaccharides (COSs) are small molecules comprising 2–20 monomers derived from hydrolysis of chitosan by chemical or enzymatic methods [3,4]. The properties of COSs include high solubility, high biological activity, and easy absorption by organisms. The length and deacetylation of COSs are important factors determining their biological functions [5–7]. COSs have various biological activities, including enhancing the immune system, lowering cholesterol, increasing the amount of probiotic species (*e.g.*, *Bifidobacterium bifidum* and *Lactobacillus* spp.), and improving the absorption of calcium to strengthen bone [6,7]. COSs can also be used to regulate blood sugar levels in patients with diabetes [7,8]. Enzymatic production of COSs is achieved by hydrolysis of the 1,4-glycosidic bond of chitosan, catalyzed by chitosanases, enzymes produced by many organisms [9–21]. The use of chitosanases for the production of COSs has a number advantages over chemical methods, including mild reaction conditions, environmental sustainability, ability to produce COSs containing higher proportions of specific products, and lack of monosaccharide production [4]. Therefore, the concept of large-scale production of chitosanase has attracted attention in chitosan-related industries. *Bacillus subtilis* 168 chitosanase (Csn) is an extracellular enzyme, has a high potential for use in industrial applications, and has been expressed or secreted using *Escherichia coli* [4,7]. Although *E. coli* can express or secrete *B. subtilis* chitosanase, concerns that the enzyme produced in this way may stimulate immune responses to endotoxins have limited its potential for use in some applications [22]. *B. subtilis* is a 'generally recognized as safe' (GRAS) bacterial strain, and is one of the most well-known prokaryotes, with an important position in scientific research. *B. subtilis* is used for generation of products such as enzymes, amino acids, and chemicals in the food, beverage, detergents industries [23,24]. *B. subtilis* can also be used as a platform for the production

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http://dx.doi.org/10.1016/j.jtice.2016.12.040
1876-1070/

Table 1
Strains and plasmids used in this study.

	Relevant characteristics	Source
Strain		
E. coli		
DH5α	*deoR endA1 gyrA96 hsdR17 supE44 thi1 recA1 lacZM15*	Lab. collection
B. subtilis		
PT5	DB428, △*wpr::T7gene 1*	
PT5(MT-Csn)	PT5, △*mpr*:: P_{T7} /*csn*-erm	This study
PT5(MT01-Csn)	PT5, △*mpr*:: P_{T7} /*aprN* SP-M*csn*-erm	This study
PT5(MT02-Csn)	PT5, △*mpr*:: P_{T7} /*phoD* SP-M*csn*-erm	This study
PT5(MT04-Csn)	PT5, △*mpr*:: P_{T7} /*ywbN* SP-M*csn*-erm	This study
PT5(MT05-Csn)	PT5, △*mpr*:: P_{T7} /*epr* SP-M*csn*-erm	This study
PT5(MT06-Csn)	PT5, △*mpr*:: P_{T7} /*oppA* SP-M*csn*-erm	This study
PT5(MT07-Csn)	PT5, △*mpr*:: P_{T7} /*xynD* SP-M*csn*-erm	This study
PT5(MT08-Csn)	PT5, △*mpr*:: P_{T7} /*bglS* SP-M*csn*-erm	This study
PT5(MT09-Csn)	PT5, △*mpr*:: P_{T7} /*yncM* SP-M*csn*-erm	This study
PT5(MT10-Csn)	PT5, △*mpr*:: P_{T7} /*yfhK* SP-M*csn*-erm	This study
Plasmid		
pMT-Csn	pMT with the complete sequence of Csn	This study
pMT1-Csn	*aprN* SP instead of *csn* SP in pMT-Csn	This study
pMT2-Csn	*phoD* SP instead of *csn* SP in pMT-Csn	This study
pMT4-Csn	*ywbN* SP instead of *csn* SP in pMT-Csn	This study
pMT5-Csn	*epr* SP instead of *csn* SP in pMT-Csn	This study
pMT6-Csn	*oppA* SP instead of *csn* SP in pMT-Csn	This study
pMT7-Csn	*xynD*SP instead of *csn* SP in pMT-Csn	This study
pMT8-Csn	*bglS* SP instead of *csn* SP in pMT-Csn	This study
pMT9-Csn	*yncM* SP instead of *csn* SP in pMT-Csn	This study
pMT10-Csn	*yfhk* SP instead of *csn* SP in pMT-Csn	This study

Abbreviations: erm, erythromycin resistant determinant; *csn*, full-length of *B. subtilis* chitosanase Csn; M*csn*, mature form of *B. Subtilis* chitosanase Csn; P_{T7}, T7 promoter; SP, signal peptide.

of recombinant proteins [25–27] and chemicals [28–30]. In this study, Csn, was recombinantly expressed fused to various signal peptides, using an efficient *B. subtilis* system, secreted into the culture supernatant, and its production evaluated. Optimal reaction conditions were assessed according to the central composite design (CCD) method [31]. The results indicated that recombinant Csn expressed from the *B. subtilis* system has high potential for development for use in the industrial-scale production of COSs. Furthermore, the fermentation medium for chitosanase production was optimized by response surface methodology using Box–Behnken design [32,33]. Finally, large-scale production of chitosanase by *B. subtilis* was achieved. The supernatant containing chitosanase can be used directly to convert chitosan into chitosan oligosaccharides.

2. Materials and methods

2.1. Bacterial strains, culture conditions and chemicals

E. coli strain DH5α was used to create expression vectors and *B. subtilis* strain PT5 was used to evaluate the recombinant secretion of Csn [34]. Recombinant strains were grown in shake flasks with medium (as indicated), and culture growth determined by measuring the optical density at 600 nm (OD_{600}) using a U-1500 spectrophotometer (Hitachi). Cultures were inoculated with sufficient inoculum to yield an OD_{600} of 0.1 after overnight cultivation, and grown at 37 °C with agitation at 200 rpm. Ampicillin (100 μg/ml) and erythromycin (10 μg/ml) were used as selection markers to screen for *E. coli* harboring plasmids and *B. subtilis* integrants, respectively. The α-form and β-form commercial chitosan were purchased from Charming & Beauty Co., Ltd. Lactose, yeast extract, and glutamate were purchased from Wellwiz Enterprise Co., Ltd, USB Co. Ltd, and Ve Wong Corporation, respectively.

2.2. Construction of chitosanase expression vectors

The strains and vectors, and primers used in this study are listed in Tables 1, and 2, respectively. PCR was carried out using a 2720 Thermal Cycler (Applied Biosystems), with high-fidelity Phusion DNA polymerase (New England BioLabs). The PCR conditions were as follows: denaturation at 98 °C for 30 s; followed by 28 cycles of denaturation at 98 °C for 10 s, annealing at 60 °C for 10 s, and extension at 72 °C for 2 kb/min; and a final extension step at 72 °C for 5 min. Integration vector pMT1 DNA was used as template to amplify a 4.422 kb (MT0102) PCR fragment, using primers MT01 and MT02, and *B. subtilis* PT5 (a derivative of *B. subtilis* 168) chromosomal DNA was used as a template to amplify a 0.849Kb (Csn) amplicon, using the primers Csn01 and Csn02 [34]. Both PCR products were digested with *Nru*I/*Bam*HI and ligated to one another to

Table 2
Primers used in this study.

	Relevant characteristics	Source
Primer		
MT01	CTCGAGGGATCCCTGCAGGCA (*Bam*HI)	
MT02	ATCGGATCGCGACCCTCTCCTTTTAAAAAATCTAG (*Nru*I)	
Csn01	GGAAGTTCGCGAATGAAAATCAGTATGCAAAAAGC (*Nru*I)	*csn* 5'
Csn02	TTTCCTGGATCCTTATTTGATTACAAAATTACCGTAC (*Bam*HI)	*csn* 3'
Csn03	AACGGTCTCGAGGGCGGGACTGAATAAAGATCAA (*Xho*I)	M*csn* 5'
aprN01	AGAGGGTCGCGAGTGAGAAGCAAAAAATTGTGG (*Nru*I)	aprN SP
aprN02	GATCGACTCGAGGCAGCCTGCGCAGACATGTTG (*Xho*I)	aprN SP
phoD01	GGGGATCTCGCGAATGGCATACGACAGTCGTT (*Nru*I)	*phoD* SP
phoD02	TGTTTCCTCGAGGCAGCATTTACTTCAAAGGCCCCA (*Xho*I)	*phoD* SP
ywbN01	GTAACTCGCGAATGAGCGATGAACAGAAAAAG (*Nru*I)	*ywbN* SP
ywbN02	TGTTTCCTCGAGGCGCAACGGCTGCCCCCGCCA (*Xho*I)	*ywbN* SP
epr01	CATAGTTCGCGAATGAAAAACATGTCTTGCAAAC (*Nru*I)	*epr* SP
epr02	TGTTTCCTCGAGGCGGCGCTAGCAACAAGCGGA (*Xho*I)	*epr* SP
oppA01	ATGCATTCGCGAATGAAAAAACGTTGGTCGATTG (*Nru*I)	*oppA* SP
oppA02	TAGCTACTCGAGGCCGCGCTCAGCACGAGAGTG (*Xho*I)	*oppA* SP
xynD01	ATGCATTCGCGAATGAGGAAAAAGTGTAGCGT (*Nru*I)	*xynD* SP
xynD02	TAGCTACTCGAGGCAGCATACGCAGACTTCCCAG (*Xho*I)	*xynD* SP
bglS01	ATGCATTCGCGAATGCCTTATCTGAAACGAGTG (*Nru*I)	*bglS* SP
bglS02	TAGCTACTCGAGGCAGCTGAGGCAGTAGCAGTGA (*Xho*I)	*bglS* SP
yncM01	ATGCATTCGCGAATGGCGAAACCACTATCAAAAG (*Nru*I)	*yncM* SP
yncM02	TAGCTACTCGAGGCTGCTTTTGCGACTTGAGCGT (*Xho*I)	*yncM* SP
yfhK01	ATGCATTCGCGAATGAAAAAGAAACAAGTAATGCTC (*Nru*I)	*yfhK* SP
yfhK02	TAGCTACTCGAGGCAGCTTTTGCTGCGGGAGCGGA (*Xho*I)	*yfhK* SP

Please cite this article as: P.-C. Su et al., Enhancement of chitosanase secretion by Bacillus subtilis for production of chitosan oligosaccharides, Journal of the Taiwan Institute of Chemical Engineers (2017), http://dx.doi.org/10.1016/j.jtice.2016.12.040