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Overexpression of Chitinase A Gene from *Serratia marcescens* in *Bacillus subtilis* and Characterization of Enhanced Chitinolytic Activity

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HIGHLIGHTS

- *chiA* from *S. marcescens* was successfully overexpressed in *Bacillus subtilis* 168.
- Chitinase activity was increased 2.15 folds in recombinant *B. subtilis* after 2 h of IPTG induction.
- Optimum temperature and pH for the chitinase activity were 60 ^{o}C and pH 9.0, respectively.
- Zn^{2+} and SDS increased the chitinase activity 3.2 and 2.9 folds, respectively.

Abstract: Chitinase enzymes possess various usages in agriculture, biotechnology and medicine due to their chitin degrading property. Thus, efficient production of chitinase enzymes with desired properties has importance for its use. In this study, chitinase A (*chiA*) gene from *Serratia marcescens* Bn10 was cloned and heterologously overexpressed using pHT43 vector in *Bacillus subtilis* 168. The recombinant chitinase was characterized in terms of temperature, pH, and various effectors. The extracellular chitinase activity in recombinant *B. subtilis* was found 2.15-fold higher than the parental strain after 2 h of IPTG induction. Optimum temperature and pH for the extracellular chitinase activity in the recombinant *B. subtilis* were determined as 60 ^{o}C and pH 9.0, respectively. NaCl, Ca^{2+}, Mn^{2+}, Cu^{2+}, Zn^{2+}, sodium dodecyl sulfate (SDS), Tween-20, and ethanol increased the chitinase activity whereas Mg^{2+} caused an inhibition. The most notable increment on the chitinase activity was provided by Zn^{2+} (3.2 folds) and then by SDS (2.9 folds). The chitinase, overproduced by the recombinant *B. subtilis* 168 heterologously expressing *chiA*, was determined to have optimum activity at high temperature and alkaline conditions as well as various effectors increase its activity. The extracellular chitinase of recombinant *B. subtilis* might be a promising source for agricultural, biotechnological and medical applications.

Keywords: *Bacillus subtilis*; *Serratia marcescens*; chitinase; heterologous expression; enzyme activity.

INTRODUCTION

Chitin is a water-insoluble linear homopolymer of *N*-acetylglucosamine (GlcNAc) monomers linked with β-1,4 glycosidic bonds, and the second most abundant polysaccharide in nature, after cellulose, found mostly in the exoskeleton and midgut epithelium of insects, eggshell of nematodes, shells of crustaceans, and cell

walls of some fungi [1,2]. Water-soluble, degraded form of chitin is valuable both for biomass conversion and biotechnological applications [3].

Chitinases (EC 3.2.1.14) belong to the family of glycosyl hydrolases grouped into family 18 or 19 according to their amino acids similarity [4]. Endochitinases degrade chitin randomly at internal sites whereas exochitinases are responsible for removal of monomers or dimers of GlcNAc from the non-reducing end of chitin chains [1]. Chitinases possess a wide range of usage area such as biocontrol of fungal pathogens or insect pests, and production of single-cell proteins or ophthalmic preparations [5]. Therefore, chitinase enzymes from various organisms such as *Agave tequilana* [6], *Eisenia fetida* [7], *Ostrinia furnacalis* [8], and *Avena chinensis* [9] were studied. Positive role of chitinase under abiotic stress conditions in plants, such as low temperature and osmotic stress, has also been shown [10]. Low levels of enzyme activity and resulting amount of chitooligosaccharides limit the chitinase applications [4]. Thus, there are increasing efforts towards the increasing chitinase levels in organisms. For instance, increased production of a chitinase in cucumber provided resistance against *Fusarium oxysporum* [11].

Bacillus subtilis is one of the generally recognized as safe (GRAS) organisms making them useful for applications friendly to environment, such as utilization as a biocontrol agent [12,13]. Production and characterization of native or recombinant chitinase in *B. subtilis* have been investigated. Chitinases of *B. subtilis* TV-125 isolated from plants were characterized and their antifungal activity against *F. culmorum* was reported [14]. Biocontrol capacity of *B. subtilis* ATCC 11774 against the *Rhizoctonia solani* was shown by Saber and coauthors [15]. There are some studies on the production of recombinant chitinase as well. Rostami and coauthors [12] expressed chitinase gene from *B*. *pumilus* on the spore surface of *B. subtilis*, and showed its growth inhibitory activity on *R. solani* and *Trichoderma harzianum*. Chitinase gene from *B. subtilis* was also expressed in *E. coli* [13,16] or *Burkholderia vietnamiensis* [17].

Serratia marcescens is one of the chitinase over-producer bacteria [2,18,19]. There are some studies to ameliorate the characteristics of chitinase from *S. marcescens*. For instance, Emruzi and coauthors [20] applied site directed mutagenesis to increase the stability of chitinase from *Serratia marcescens* B4A. Chitinase A from *S. marcescens* (*Sm*ChiA) is highly powerful in the hydrolysis of crystalline chitin [21], and active in the extracellular environment. There are two domains of *Sm*ChiA. Both the catalytic and chitin binding domains have aromatic residues playing crucial roles in substrate binding and hydrolytic activity of the *Sm*ChiA [22].

In the present study, *chiA* gene from *S. marcescens* encoding chitinase A was cloned to an expression vector and introduced to *B. subtilis* 168. Increased chitinase activity in *B. subtilis* 168, and effects of temperature, pH, metals and some inhibitors on chitinase activity were shown.

MATERIAL AND METHODS

Bacteria and culture conditions

Serratia marcescens Bn10 was previously isolated from hazelnut beetle (*Balaninus nucum*) in Turkey [18,23]. *Bacillus subtilis* 168 was obtained from the Bacillus Genetic Stock Center [24]. *Escherichia coli* DH5α was used as cloning host. The bacterial strains were grown in Luria Broth (LB) at 30 ^{o}C for *B. subtilis* 168 and 37 ^{o}C for *S. marcescens* Bn10 and *E. coli* DH5α.

Cloning of *chiA* gene in *B. subtilis*

Genomic DNA of *S. marcescens* Bn10 was isolated using GeneJET Genomic DNA Purification Kit (Thermo Scientific) following the manufacturer's recommendations. The primers used for cloning of chitinase A (*chiA*) gene were SmChiF: 5´-ggatccatgcgcaaatttaataaaccgctg-3´ and SmChiR: 5´-tctagattattgaacgccggcgctgtt-3´. PCR mixture contained 1X *Taq* buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTP mix, 1 U *Taq* DNA polymerase, all from Thermo Scientific, 0.2 µM of each primers, 1 ng template DNA, and the reaction was performed at 94 ^{o}C for 3 min, 35 cycles of 94 ^{o}C for 1 min, 58 ^{o}C for 30 s, 72 ^{o}C for 2 min, and a final extension at 72 ^{o}C for 10 min. The PCR product consisted of the *chiA* open reading frame (ORF) was visualized on 0.8% agarose gel, and cloned into pGEM-T Easy Vector (Promega). The *chiA* gene was recovered from pGEM-T Easy and cloned into pHT43 (MoBiTech, Germany) using *Bam*HI, *Xba*I restriction enzymes (Figure 1). The *chiA* gene was cloned under P_{grac}01 promoter region including the promoter of *gro*E gene and the *lac*O operator as well as the Shine-Dalgarno sequence of the *gsiB* gene. Signal sequence of α-amylase (*amyQ*) was present on the plasmid so that the product was secreted to extracellular environment. The recombinant pHT43-*chiA* vector was obtained in *E. coli* DH5α.

The competent cells of *B. subtilis* 168 were prepared and the pHT43-chiA plasmid was introduced into *B. subtilis* according to the method of Klein and coauthors [25]. Recombinant bacteria were selected in the presence of 5 µg/ml chloramphenicol. Presence of pHT43-*chiA* in *B. subtilis* was verified by plasmid isolation and restriction enzyme digestion.

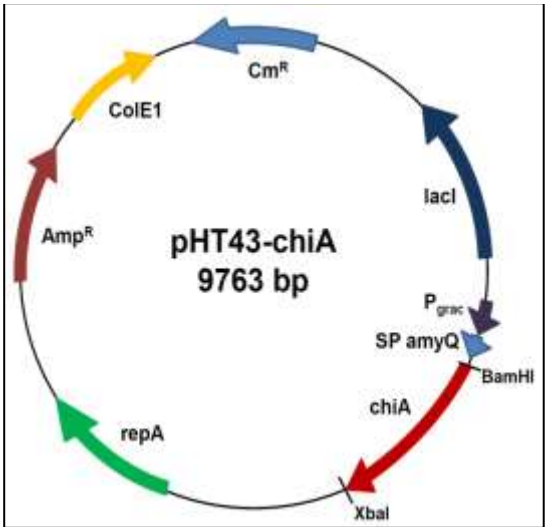

Figure 1. Map of pHT43-*chiA* vector.

Production of recombinant chitinase

Colloidal chitin was prepared according to Roberts and Selitrennikoff [26]. *B. subtilis* 168 carrying pHT43-*chiA* was cultured in 1.6% (w/v) Nutrient Broth (Merck) containing 0.2% (w/v) colloidal chitin and 5 µg/ml chloramphenicol at 30 ºC until the optical density at 600 nm (OD_{600}) was reached at 0.7. Then, the culture was supplemented with 1 mM final concentration of IPTG and incubated at 30 ºC for 2 and 4 h more [27]. The bacterial culture was centrifuged at 12000 rpm for 10 min and the supernatant were used as the source of crude enzyme. Same procedure was applied to *B. subtilis* 168 excluding chloramphenicol in the medium.

Determination of chitinase activity

The chitinase activity was measured via reducing sugar method of Miller [28]. The chitinase assay was performed as reported previously [19]. Standard assay conditions: 1 ml of crude enzyme source and 1 ml of substrate (1% colloidal chitin in 20 mM phosphate buffer, pH 7.5) were mixed and incubated at 50 °C for 60 min. Then, 2 ml of 1% 3,5-dinitrosalicylic acid (DNS, Sigma) was added and the mixture was boiled for 15 min. After centrifugation at 5000 rpm for 5 min, the absorbance was measured at 530 nm. One unit (U) of enzyme activity was defined as the amount of enzyme that releases 1 µmol *N*-acetylglucosamine per minute.

Characterization of chitinase activity

Effect of temperature on chitinase activity was determined under the standard assay conditions except incubation temperature at 30, 40, 50, 60, 70, 80 and 90 ºC. The effect of pH on chitinase activity was investigated under the standard assay conditions changing the pH values of the phosphate buffer as 3, 4, 5, 6, 7, 7.5, 8, 9 and 10. The effects of NaCl, $CaCl_2$, $MgCl_2$, $MnSO_4$, $ZnSO_4$, $CuSO_4$, and sodium dodecyl sulfate (SDS) in 1 mM concentrations as well as 1% (v/v) Tween-20 and ethanol were determined under standard assay conditions adding these materials to the assay mixture.

RESULTS AND DISCUSSION

Cloning and overexpression of *chiA* gene in *B. subtilis*

Enzymes have importance in many industrial and biotechnological processes, used as biocatalysts working under mild conditions requiring less energy and generating reduced waste [29,30]. Bioconversion of chitin to *N*-acetyl-D-glucosamine (GlcNAc) by highly efficient chitinases has economic value, and *B. subtilis* is a potent microorganism for this purpose [13]. Wang and coauthors [13] was able to produce 1.63 g of GlcNAc from 10 g of pretreated chitin using *B. subtilis* chitinase with a yield of 60% and 95% purity. Pan and coauthors [4] increased the chitinase activity of *B. subtilis* via molecular engineering strategies such as

optimization of ribosome binding site and addition of signal peptide. Moreover, *B. subtilis* chitinase expressed in *E. coli* was able to show nematicidal activity on *Meloidogyne javanica* [16]. In this study, we aimed to overproduce chitinase enzyme from *S. marcescens* in *B. subtilis* to increase the extracellular chitinolytic activity. The 1.7 kb chitinase A (*chiA*) gene was successfully amplified from genomic DNA of *S. marcescens* Bn10 (Figure 2A), cloned into pGEM-T Easy and pHT43 vectors in *E. coli*, and pHT43-*chiA* was introduced to *B. subtilis* 168 (Figure 2B).

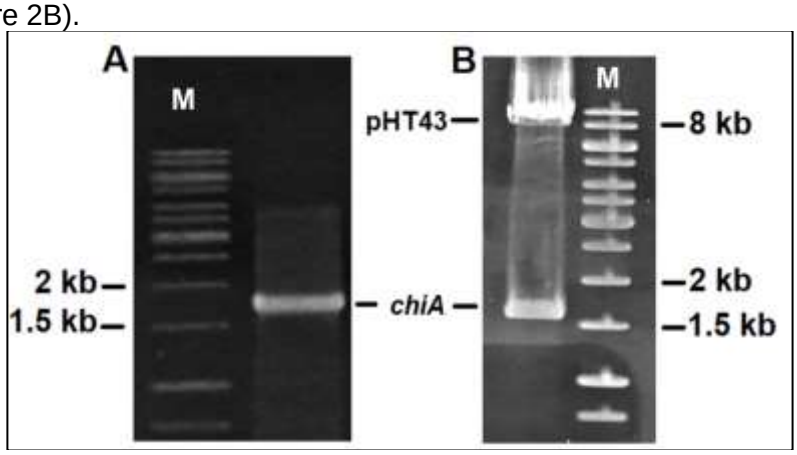

Figure 2. Cloning of *chiA* gene. (A) PCR product for *chiA* gene. (B) Digested *chiA* from pHT43-*chiA* from *B. subtilis* 168 with *Bam*HI and *Xba*I. M: 1 kb DNA marker.

The recombinant *B. subtilis* carrying *chiA* was cultured in the presence of chitin, and the *chiA* expression was induced by IPTG for 2 h and 4 h. Parental *B. subtilis* 168 was cultured in parallel under the same conditions. The culture supernatants were used as crude enzyme source and chitinase activities were compared. After 2 h of IPTG induction, chitinase activities of parental and recombinant *B. subtilis* 168 were 13.4 U/ml and 28.8 U/ml, respectively, corresponding to 2.15-fold increase in enzyme activity. 4 h of IPTG induction resulted in chitinase activities as 13.6 U/ml and 25.5 U/ml for parental and recombinant *B. subtilis* 168, respectively, corresponding to 1.88-fold induction in enzyme activity (Figure 3). Heterologous overexpression of *chiA* gene from *S. marcescens* Bn10 elevated the chitinase activity in *B. subtilis* 168 approximately 2 folds.

Heterologous expression of chitinase genes has been reported for various bacteria. Chitinase genes from *S. marcescens* were expressed heterologously in *B. thuringiensis* [18,31], *Enterobacter cloacae*, *Klebsiella oxytoca* [32], *Lactococcus lactis* and *Lactobacillus plantarum* [33]. Also, chitinase genes from *B. subtilis* were expressed in *Burkholderia vietnamiensis* [17] and *E. coli* [13,16]. Additionally, chitinase gene of *B. pumilus* was expressed on the spore coat of *B. subtilis* [12]. Previous studies and our results showed that heterologous expression of chitinase genes elevated the levels of enzyme activity in the host organism.

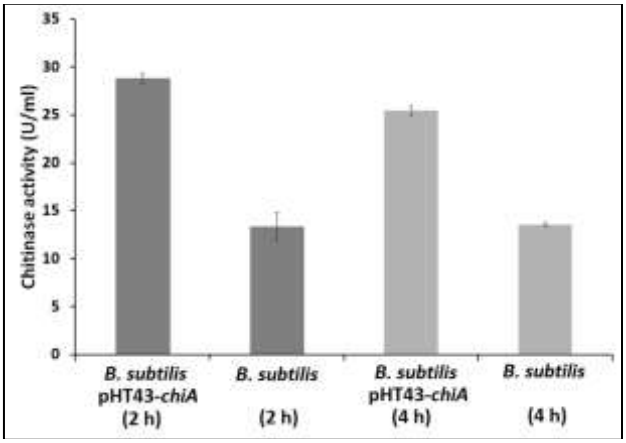

Figure 3. Comparison of chitinase production by recombinant *B. subtilis* 168 overproducing ChiA and parental strain upon 2 h and 4 h induction of IPTG.

Effects of temperature and pH on the chitinase activity

Since chitinase overproducer *B. subtilis* has potential for industrial use, the influences of various factors on the chitinolytic activity were investigated. Effect of temperature was investigated on the extracellular chitinase activity of *B. subtilis* 168 overproducing chitinase A (ChiA) from *S. marcescens* at the range of 30-90 ºC. The chitinase activity was elevated by increasing temperatures from 30 ºC to 60 ºC and the enzyme activity was decreased at the temperatures higher than 60 ºC (Figure 4). Therefore, the optimum temperature for chitinase activity in *B. subtilis* 168 carrying *chiA* from *S. marcescens* was determined as 60 ºC. The optimum temperature for chitinase activity in *S. marcescens* was reported as 45 ºC [18] and 50 ºC [19,20]. The chitinase activity in different strains of *B. subtilis* showed highest activity at 30 ºC [15], 50 ºC [14,34] or 60 ºC [4]. Optimum temperature for chitinase from *B. subtilis* expressed in *E. coli* was reported as 40 ºC [13]. Increased activity at high temperatures for chitinase produced by recombinant *B. subtilis* obtained in this study might be advantageous for biotechnological applications.

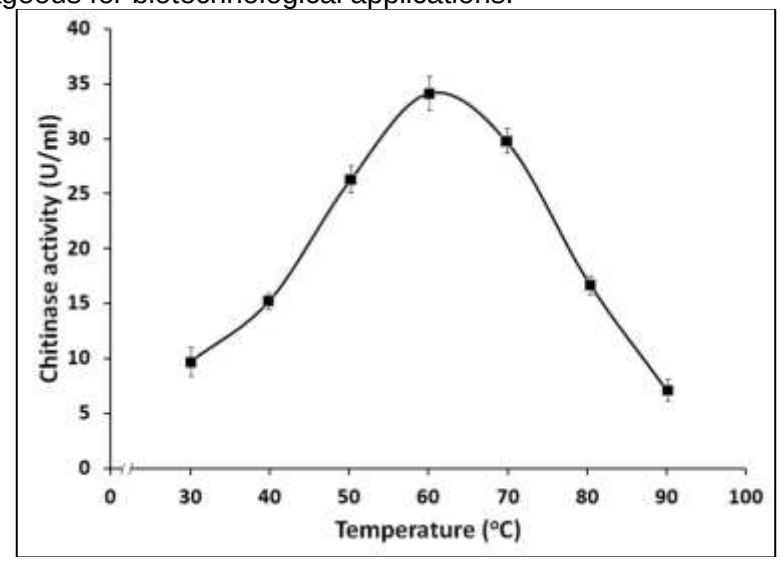

Figure 4. Effect of temperature on the extracellular chitinase activity of *B. subtilis* 168 overproducing ChiA.

Effect of pH on the extracellular chitinase activity of recombinant *B. subtilis* 168 was evaluated at the range of pH 3.0-10.0. Optimum activity for chitinase in *B. subtilis* was determined at pH 9.0 (Figure 5). A bimodal action was observed for chitinase activity with a shoulder at pH 5.0 and a peak at pH 9.0. This type of bimodal activity was reported for chitinase enzymes previously [18]. Highest chitinase activity for *S. marcescens* was found as pH 5.0, 6.0 [20], 7.0 [19], and 9.0 [18]. Optimum pH for *B. subtilis* chitinase was reported as 4.0 [14], 5.0 [4], 8.0 [15] and 9.0 [34]. Effect of pH was found different for varying strains.

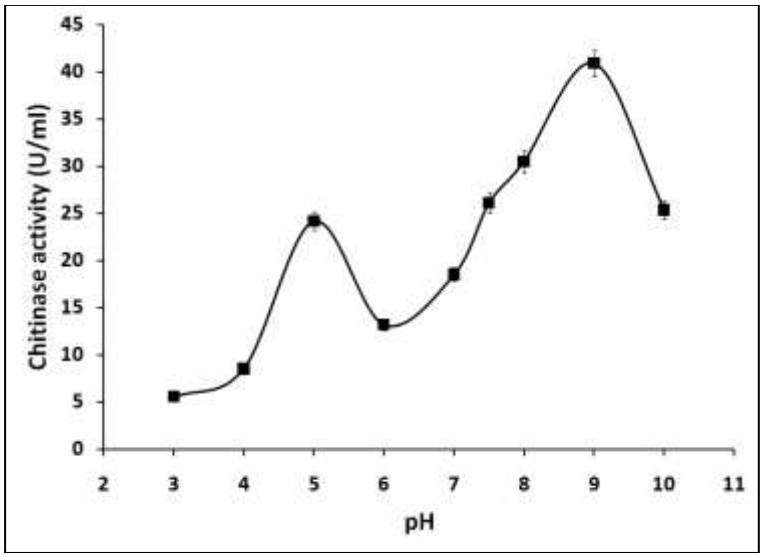

Figure 5. Effect of pH on the extracellular chitinase activity of *B. subtilis* 168 overproducing ChiA.

Influence of effectors on the chitinase activity

Influence of various effectors, Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+}, NaCl, SDS, Tween-20 and ethanol were investigated on the extracellular chitinase activity of *B. subtilis* 168 overexpressing *chiA* (Figure 6).

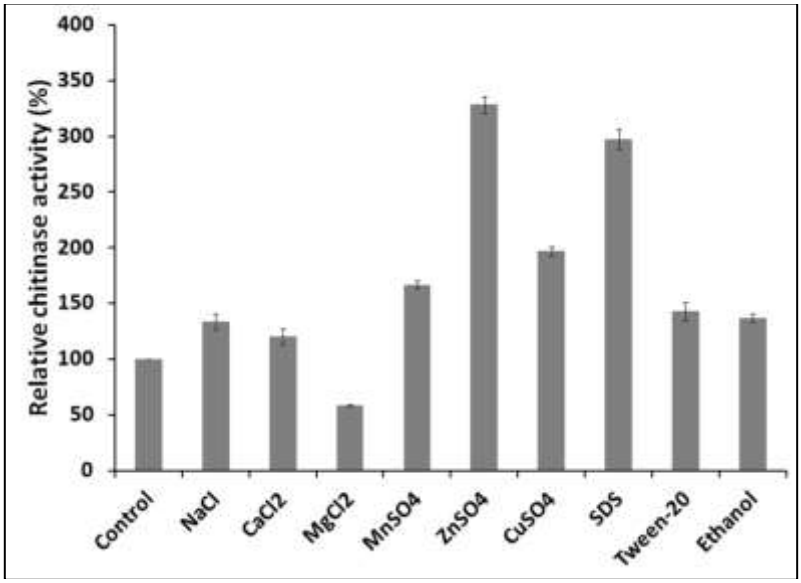

Figure 6. Influence of various effectors on the extracellular chitinase activity of *B. subtilis* 168 overproducing ChiA.

Although Mg^{2+} caused a negative effect, NaCl, Ca^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+}, SDS, Tween-20 and ethanol increased the chitinase activity 20% to 3.2 fold. The most remarkable enhancement on the chitinase activity was provided by Zn^{2+} (3.2 folds) and then by SDS (2.9 folds). Although SDS, a detergent, acts as an inhibitor on the activity of most enzymes [34,35,36], it may also increase the enzyme activity [37]. Inhibitory effect of Mg^{2+} was also observed for the chitinase activity of *B. subtilis* JN032305 [34] and *B. subtilis* WB600 at 15 mM concentration [4]. Interestingly, chitinase activity of *B. subtilis* WB600 was reported to be inhibited entirely by Zn^{2+} and strongly by Cu^{2+} [4] whereas the inhibitory effect of Zn^{2+} on the chitinase activity of *B. subtilis* JN032305 [31] and *S. marcescens* Bn10 [18] was not significant. Additionally, Mn^{2+} increased the chitinase activity of *B. subtilis* WB600 [4] but inhibited the chitinase of *B. subtilis* JN032305 [34] and *S. marcescens* Bn10 [18]. Influence of effectors on the chitinase activity alters in different studies.

CONCLUSION

Chitinase A (*chiA*) gene from *S. marcescens* Bn10 was successfully cloned and heterologously overexpressed using pHT43 vector in *B. subtilis* 168, in this study. The extracellular chitinase activity in recombinant *B. subtilis* was measured 2.15-fold higher than the parental strain. Optimum temperature and pH for the extracellular chitinase activity in recombinant *B. subtilis* were determined as 60 ^{o}C and pH 9.0, respectively. NaCl, Ca^{2+}, Mn^{2+}, Cu^{2+}, Tween-20, ethanol, especially Zn^{2+} and SDS increased the chitinase activity whereas Mg^{2+} caused a negative effect. Extracellular chitinase, produced by recombinant *B. subtilis* 168 overexpressing *chiA*, was found to be active at high temperatures and at alkali conditions as well as more active in the presence of various effectors, which makes it a promising source for agricultural and biotechnological applications.

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Conflicts of Interest: The authors declare no conflict of interest.

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