

Article

# Cloning and Expression of the Chitinase Encoded by *ChiKJ406136* from *Streptomyces Sampsonii* (Millard & Burr) Waksman KJ40 and Its Antifungal Effect

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**Abstract:** The present study demonstrated that the chitinase gene *ChiKJ406136* of *Streptomyces sampsonii* (Millard & Burr) Waksman KJ40 could be cloned using a PCR protocol and expressed in *Escherichia coli* (Migula) Castellani & Chalmers BL21 (DE3), and the recombinant protein had antifungal effect on four forest pathogens (*Cylindrocladium scoparium* Morgan, *Cryphonectria parasitica* (Murrill) Barr, *Neofusicoccum parvum* Crous, and *Fusarium oxysporum* Schl.) and also had the biological control effects on *Eucalyptus robusta* Smith leaf blight, *Castanea mollissima* BL. blight, *Juglans regia* L. blight and *J. regia* root rot. The results showed that *ChiKJ406136* was efficiently expressed and a 48 kilodalton (kDa) recombinant protein was obtained. No significant change in protein production was observed in the presence of different concentrations of IPTG (isopropyl- $\beta$ -D-thio-galactoside). The purified protein yield was greatest in the 150 mmol/L imidazole elution fraction, and the chitinase activities of the crude protein and purified protein solutions were 0.045 and 0.033 U/mL, respectively. The antifungal effects indicated that mycelial cells of the four fungi were disrupted, and the control effects of the chitinase on four forest diseases showed significant differences among the undiluted 10- and 20-fold dilutions and the control. The undiluted solution exhibited best effect. The results of this study provide a foundation for the use of *S. sampsonii* as a biocontrol agent and provides a new source for the chitinase gene, providing a theoretical basis for its application.

**Keywords:** biological control; chitinase gene; cloning; expression; *Streptomyces sampsonii*

## 1. Introduction

*Streptomyces* Waksman & Henrici, the largest genus of the phylum Actinobacteria, consists of a group of Gram-positive, aerobic, non-motile, catalase positive, and non-acid-fast bacteria with a filamentous form that resembles fungi [1–3]. The members of *Streptomyces* are well-known for their ability to produce a variety of bioactive compounds with different bioactivities such as antibacterial [4–9], antifungal [10], antiviral [11], immunosuppressive [12], anticancer, and antioxidant properties [13–15]. Thereinto, *Streptomyces sampsonii* is widely distributed in nature, having been isolated from *Maytenus aquifolia* Mart. [16], soil [17–22], marine sediment [23,24], and medicinal plants [25]. In particular, numerous isolates have been obtained from soil, such as garden soil [18], alpine soil from the Himalayan glacier region [19], fertile soil in Korea [26], and rhizosphere soil of healthy poplar trees [20]. *S. sampsonii* possesses antagonistic activity against fungal pathogens, including those of the genera *Alternaria* Nees and *Phomopsis* Sacc. & Roum. [19], and the species *Candida albicans* (C.P. Robin) Berkhout, *Aspergillus niger* van Tieghem, *Microsporium gypseum* (E. Bodin) Guiart

& Grigoraki, *Trichophyton* sp. (Castell.) Sabour. [18] and *Rhizoctonia violacea* (Tul.) Pat. [20]. Previous studies demonstrated that the bioactive compounds of *S. sampsonii* have important applications in various fields [3]. For example, crude extracts showed antitumor activity against glioblastoma multiforme (GBM) cells, inhibiting cell growth by 70.04% [25], and the supernatant of a *S. sampsonii* culture showed biological activity against the root-knot nematode [26]. In the purified components, soil isolates of *S. sampsonii* can produce heptaene polyene antibiotics [18,27]. In addition, *S. sampsonii* has been shown to produce hydrolytic enzymes, such as amylase, chitinase, protease, and lipase [19]. Studies at the molecular level have focused on strain identification and the phylogenesis of related species [28–35]. The complete genome sequence of *Streptomyces sampsonii* KJ40 was recently described by our lab [36], resulting in the discovery of a large number of gene encoding chitinases and enzymes involved in secondary metabolite production. However, little is known regarding the metabolic pathways and genetic regulation in this strain, limiting its practical application.

Chitin, a linear polymer of  $\beta$ -1,4-glucosidicosamine (GlcNAC), is the second most abundant polysaccharide in nature. Chitin can be degraded by chitinolytic enzymes, that is chitinase. Chitinases (EC 3.2.1.14) are widely present in a great variety of organisms, including insects, fungi, yeast, higher plants, vertebrates, arthropods and humans [37]. Due to the functions of degrading chitin, their antitumor activities, and antihypertensive activities, chitinases have been widely exploited in various fields, such as industrial, agricultural and medicinal applications [38,39]. As it is well-known, control of plant pests and diseases by application of biological environmentally friendly agents has received great attention. Chitinases, as an alternative to the use of chemical, have been holding great promise in control of fungal and insect pathogens of plants. The functions of chitinases in various organisms are diverse, as well as their mechanisms in biotechnological applications. In fungi, previous studies have demonstrated chitinases can inhibit the growth of fungi (such as *Verticillium dahliae* Kleb. [40], *Colletotrichum gloeosporioides* (Stoneman) Spauld. & H. Schrenk, *Fusarium graminearum* (Schwein.) Petch, *Fusarium oxysporum* (Schlecht.) Snyder & Hansen, *Alternaria alternata* (Fr.) Keissl. [41], and *Rhizoctonia solani* Kühn [42,43]) by impacting their synthesis of cell wall, apical growth and morphogenesis of fungal hyphae. In insects, it was reported by Gadelhak et al. [44] that the chitinases produced by *Actinoplanes philippinensis* Couch, *A. missouriensis* Couch, and *Streptomyces clavuligerus* Higgins & Kastner could inhibit the emergence of *Drosophila melanogaster* Meigen adults from pupae. In plants, Prasad et al. [45] showed that transgenic peanuts plants enhanced their sustained resistance to fungal diseases by over-expressing chitinase genes. Additional transgenic plants expressing chitinases have been successfully created including lemon tree [46], wheat [47] and carrots [48], and so on, this suggests the possibility that to control fungal or insect disease with chitinase transgenic plants or chitinase, and its potential applications is becoming the future.

*Streptomyces sampsonii* KJ40 was previously isolated from the rhizosphere soil of a poplar plantation in China. Field tests demonstrated that the fermentation filtrate of this bacterium can help to control poplar purple root rot and reduce tree morbidity [20]. Extracellular secondary metabolites or hydrolytic enzymes, including chitinase, can significantly inhibit fungal growth [19,23,26]. At present, the studies of chitinases from *S. sampsonii* primarily focus on the biological activity of the fermentation liquid. However, the chitinase gene from this strain has not been studied, and the activity of the protein is unknown. In this study, we identified the chitinase gene from the complete genome sequence of *S. sampsonii* KJ40, which was subsequently cloned into a prokaryotic expression vector, to express and purify the recombinant chitinase protein. In particular, we analyzed the antifungal effect of the purified chitinase on the mycelial morphology of four pathogenic fungi and its ability to control four plant diseases. The goal of study was to characterize the recombinant *S. sampsonii* KJ40 chitinase for future large-scale industrial production and further accelerated application in agriculture, industry and medicine.

## 2. Materials and Methods

### 2.1. Strains, Plasmids and Plant Samples

The strain *S. sampsonii* KJ40 (accession number: LORI00000000) was isolated from the rhizosphere soil of *Populus szechuanica* Schneid. and was provided by the Key Laboratory of Forest Protection in the Sichuan Province, which was preserved in the Chinese General Microbiological Culture Collection Center (CGMCC No.5996). The complete genome sequence length of *S. sampsonii* KJ40 was 7261502 bp, containing 6605 genes, 1260 tandem repeat sequences, 804 minisatellite DNAs, 67 microsatellite DNAs, 90 tRNAs, 9 rRNAs, and 19 sRNAs, the average GC content was 73.41% [36]. The strains were grown at 37 °C on Gause's No. 1 synthetic medium with ampicillin (Sigma-Aldrich, Llc., Shanghai, China) (100 µg/mL).

The pathogenic fungi (*Cylindrocladium scoparium* Morgan, *Cryphonectria parasitica* (Murrill) Barr, *Neofusicoccum parvum* Crous, and *Fusarium oxysporum* Schl.) were provided by the Key Laboratory of Forest Protection in Sichuan Province. The *Escherichia coli* strains Trans5α and BL21 (DE3) were purchased from Beijing TransGen Biotech Co., Ltd. (China). The plasmids pMD19-T vector and pET-32a vector were purchased from Dalian TaKaRa Bio Inc. (China).

For the plant samples, healthy, one-year-old *Eucalyptus robusta* Smith (twenty leaves per plant), *Castanea mollissima* BL. (five twigs per plant), and *Juglans regia* L. (five twigs per plant) seedlings were used. The plant samples were planted in a greenhouse (Temperature 25–28 °C, humidity 60–70%) as the Sichuan Agricultural University in Chengdu, Sichuan Province, China (elevation 503 m, 30°97'01.1" N, 103°81'46.1" E).

### 2.2. PCR Amplification of the Chitinase Gene

Based on an analysis of the complete genome sequence of *S. sampsonii* KJ40 and function prediction and a functional prediction analysis, one chitinase-encoding gene was identified and was named *ChiKJ406136*. Using Premier 5.0, the primers PL (5'-ATGCGTACCCGCTGATCG-3') and PR (5'-TCAGCAGCTGAGGTTGTCG-3') were designed to amplify the *ChiKJ406136* gene. Genomic DNA was prepared from strain KJ40 using a TIANamp Bacteria DNA Kit (Tiangen Biotech Co., Ltd., Beijing, China). All PCR reactions were performed in a total volume of 25 µL and contained 10 µL of ddH<sub>2</sub>O, 12.5 µL of 2× TransTaq High Fidelity (HiFi) PCR SuperMix I, 0.5 µL of Primer STAR HS DNA Polymerase, and 1 µL of each primer pair (10 µmol/L). The following PCR thermo-cycling conditions were used: 35 cycles of 94 °C for 3 min, 57.8 °C for 30 s, 72 °C for 1 min, and a final extension of 72 °C for 5 min. The PCR fragments were separated on a 1% agarose gel, purified using a TIANgel Midi Purification Kit (Tiangen Biotech Co., Ltd., Beijing, China) and sequenced by Invitrogen (ThermoFisher Scientific Co., Ltd., Shanghai, China).

### 2.3. Construction of a Cloning Vector Harboring the Chitinase Gene

The PCR fragments and the pMD 19-T vector were firstly digested for 1 h in a 10 µL reaction mixture that contained 1 µL of pMD 19-T vector, 2 µL of PCR fragment, 2 µL ddH<sub>2</sub>O and 5 µL of solution I. Next 10 µL of the ligation products was added to 100 µL of *E. coli* Trans5α competent cells, incubated without shaking and performed transformation following manufacturer's instruction. Then the transformed white bacterial colonies were isolated, pipetted, spread and cultured on a LB/X-Gal/Amp medium plate. The plate was cultured overnight at 37 °C. Subsequently, the positive recombinants were identified by PCR. PCR was performed with 1 µL of isolated bacterial cells, 1 µL each of the forward and reverse primers, 12.5 µL of 2× TransTaq High Fidelity (HiFi) PCR SuperMix I and 9.5 µL of ddH<sub>2</sub>O. The positive recombinants were then sequenced by Invitrogen (ThermoFisher Scientific Co., Ltd., Shanghai, China), of which the colony with correct insertions were cultured, and the plasmids were extracted using a Plasmid Midi Kit (Omega Bio-Tek Inc., Norcross, GA, USA). The cloning vector plasmids pMD19-T-*ChiKJ406136*-1 was double digested in a 50 µL reaction mixture

containing 16 µL of DNA, 1 µL of *Hind*III, 1 µL of *Bam*HI, 5 µL of 10× NEBuffer and 27 µL of ddH<sub>2</sub>O. Finally, the plasmids were stored at −20 °C, and the recombinant strains were stored at −80 °C.

#### 2.4. Gene Sequence and Chitinase Protein Identification

NCBI BLAST (Basic Local Alignment Search Tool) was used to generate a nucleotide sequence alignment, and the sequence and open reading frames (ORFs) were assembled using DNAMAN software (Lynnon Biosoft, Quebec, QC, Canada). Then, the amino acid sequences were determined using BLAST and the theoretical molecular weight and isoelectric point (pI) of the protein was calculated with the ExPasy Compute pI/Mw and ProtParam tool. Later, for the prediction of the protein's local hydrophobicity, transmembrane regions and signal peptide, ProtScale, TMHMM Server v. 2.0 and SignalP 4.1 Server (DTU Bioinformatics, Kemitovet, Lyngby, Denmark) were applied, respectively. At end, NPS@SOPMA and SWISS-MODEL tools were used to predict secondary structure and tertiary structure of the protein.

#### 2.5. Construction of the Chitinase Expression Vector

First, PCR with flanking restriction sites and without the signal peptide sequence from the cloning plasmid pMD19-T-*Chi*KJ406136-1 as template, was exploited to amplify chitinase gene *Chi*KJ406136. The forward primer PL (5'-CGCGGATCCGACACCCGCGCCGCCGCG-3') and reversed primer PR (5'-CCGCTCGAGTCAGCAGCTGAGGTTGTCG-3') were used in the amplification and the resulting plasmid was named as pMD19-T-*Chi*KJ406136-2. Later, the positive recombinants were mixed with the pMD19-T-*Chi*KJ406136-2 plasmid and *E. coli* Trans5α. After that, a second PCR was exploited with pMD19-T-*Chi*KJ406136-2 plasmid as a template. The resulting amplicon and the pET32a (+) plasmid were double digested with two restriction enzymes BamHI and XhoI, after which the products were purified with a TIANgel Midi Purification Kit (Tiangen Biotech Co., Ltd., Beijing, China). Next, a 20 µL recombination reaction system, consisting of 6 µL of chitinase gene fragments, 2 µL of pET32a (+) enzyme-digested products, 2 µL of 10× T4 DNA Ligase Buffer, 9 µL of ddH<sub>2</sub>O and 1 µL of T4 DNA ligase, was generated, mixed with 100 µL of *E. coli* Trans5α competent cells, incubated on ice for 30 min and heat shocked at 42 °C for 90 s. The purified product was sequenced by Invitrogen (ThermoFisher Scientific Co., Ltd., Shanghai, China). After adding 900 µL of Gause's No. 1 synthetic medium and incubating at 37 °C for 1 h, all transformed cells were inoculated onto Gause's No. 1 synthetic medium containing ampicillin (100 µg/mL) and incubated at 37 °C for 12 h. Finally, the plasmids were extracted from the culture liquid and detected by double digestion with restriction enzymes BamHI and XhoI.

#### 2.6. Induced Expression of the Recombinant Chitinase Gene *Chi*KJ406136

Recombinant expression plasmids were transformed into BL21 (DE3) competent cells, and a single colony was picked and inoculated into 10 mL of Gause's No. 1 synthetic medium containing 100 µg/mL ampicillin, which was cultured in a shaker set at 200 rpm and at 37 °C for 12 h. Next, 300 µL of the culture liquid was mixed with 30 mL of Gause's No. 1 synthetic medium containing 100 µg/mL ampicillin and cultured in a shaker set at 200 rpm and at 37 °C until the culture reached the logarithmic phase (OD 600 (optical density) = 0.6–0.8). Eighteen milliliters of the culture were then distributed into six sterile and dry cuvettes, after which isopropyl-b-D-thio-galactoside (IPTG, Merck) was added to the cultures at final concentrations of 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 mmol/L. The controls consisted of a strain harboring an empty vector that was induced or uninduced with IPTG (1 mmol/L). Approximately 1 mL of culture was withdrawn after 3 h of induction, which was then centrifuged at 12,000 rpm for 1 min, and the supernatant was discarded. The cell pellets were re-suspended in 1 mL of PBS (phosphate buffered saline) and centrifuged at 12,000 rpm for 1 min, followed by a second wash with PBS. Next, the cells were lysed, and the proteins were denatured in 20 µL of 5× denaturing buffer (60 mmol/L Tris-HCl, 25% glycerol, 2% sodium dodecyl sulfate (SDS), 0.1% bromophenol blue (Sigma-Aldrich Llc., Shanghai, China)) and 60 µL of PBS in a boiling water bath for 10 min. The samples were then centrifuged at 10,000 rpm for 10 min, and 4.5 µL of the

supernatant was loaded onto a 12.5% SDS–polyacrylamide gel (SDS–PAGE) run at a constant voltages of (90 and 180 V) through stacking and separating gels, respectively, using a Bio-Rad Mini-PROTEIN Tetra Electrophoresis system (Bio-Rad Co., Ltd., Beijing, China).

### 2.7. Dissolubility Determination and Purification of the Recombinant Chitinase

Approximately 1 mL of culture induced with IPTG (16 °C, overnight) was centrifuged at 12,000 rpm for 1 min. The cell pellet was gently re-suspended in 1 mL of PBS and then centrifuged at 12,000 rpm for 1 min, followed by a second wash with 60 µL of PBS. The final pellet was then lysed by 10 successive freeze-thaw cycles in liquid nitrogen and was subsequently centrifuged at 12,000 rpm for 5 min. The collected precipitates were re-suspended in 60 µL of 8 mol/L carbamide, and then was left to stand for 30 min. Next, the 60 µL treated precipitates and supernatant samples were mixed with 20 µL of 4× Protein SDS-PAGE loading buffer and incubated in a boiling water bath for 10 min. The samples were then centrifuged at 12,000 rpm for 10 min, and the supernatants were loaded onto a 12.5% SDS–polyacrylamide gel and run to determine the contents of expression products of the supernatants and precipitates, for confirming whether the expression protein existed in soluble form or in inclusion body form. The recombinant chitinase was purified using a One-Stop His-Tagged Protein Miniprep Pack (Tiandz, Inc., Beijing, China).

### 2.8. Determination of the Concentration and Activity of the Recombinant Chitinase

The concentrations of the crude extract and purified recombinant chitinase fractions were determined by using the Bradford Protein Quantitative Assay Kit (Solarbio, Inc., Beijing, China). Chitinase activities were quantitated by using 1% of colloidal chitin as substrate, the assay was processed as following steps: (1) add 6 g of chitin powder to 60 mL hydrochloric acid, stir for 24 h on a magnetic plate, then dilute with distilled water to a volume of 1 L; (2) centrifuge for 10 min and collect white colloidal chitin precipitate; (3) wash and suspend the pellet repeat times till pH around 6.5 with 100 mL of distilled water; (4) suspend Pipette 10 mL of 6% colloidal chitin mother liquor to 50 mL distilled water, then add 1.2 g of agar, mix, sterilize pour into culture dishes, and cool to room temperature. The colloidal chitin mediums were drilled with a 5 mm diameter perforator, and 50 µL of the crude and purified recombinant chitinase were injected into the hole, respectively, the supernatant of cell lysate from a strain with pET32a (+) was as control. The activity was checked after five days of incubation at 30 °C. The quantitative estimation of chitinase activity was determined by using the Chitinase Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

### 2.9. Effects of the Recombinant Chitinase on Pathogenic Fungi

Antifungal activity of recombinant chitinase was determined by a hyphal growth inhibition assay against four different pathogenic fungi (*C. scoparium*, *C. parasitica*, *N. parvum*, and *F. oxysporum*). The four fungi were cultured on potato dextrose agar (PDA) culture medium for several days until their mycelia covered the entire plate. Next, 10 µL of purified enzyme was aliquoted onto a glass slide, then a few mycelia were transferred from the plates with sterile tweezers and placed onto the enzyme solution for 20 h. The mycelial morphology was later observed every 2 h with an electron microscope.

### 2.10. Determination of Biological Control Effects in a Pot Experiment

Five mycelial sections (8 mm) of each assayed pathogenic fungus (*C. scoparium*, *C. parasitica*, *N. parvum*, *F. oxysporum*) were obtained by the punch method and added to each bottle of PDA liquid medium (potato 200 g, glucose 20 g, agar 15–20 g, distilled water 1000 mL, pH 7.0); pathogen spores were at a concentration of  $10^5$ /mL after 72 h.

Fifty healthy annual seedlings including *E. robusta*, *C. mollissima*, and one hundred annual *J. regia* seedlings, were treated as follows: (1) plants were inoculated by wound inoculation [49] with 10 mL of pathogenic fungal suspension, including the leaves of *E. robusta*, the twigs of *C. mollissima* and *J. regia*, and the roots of *J. regia*; (2) after 15 days, 10 mL of the undiluted recombinant chitinase solution and

the 10-, 20-fold dilutions were sprayed onto the plants [50], with sterile water serving as the control; and (3) after 20 days, the disease symptoms were evaluated. Each treatment was repeated ten times. Disease was scored as follows: (I) No disease observed on the plant; (II) withering twigs/leaves/roots less than 25%; (III) withering twigs/leaves/roots 25–50%; (IV) withering twigs/leaves/roots 51–75%; and (V) withering twigs/leaves/roots more than 75%.

Statistical analyses: All data were subjected to one-way analysis of variance to determine the significance of individual differences at the  $p < 0.05$  level. Significant means were compared using the least significant difference (LSD) test. All statistical analyses were conducted using the SPSS commercial statistical package (SPSS, Version 17.0 for Windows, SPSS Inc., Chicago, IL, USA).

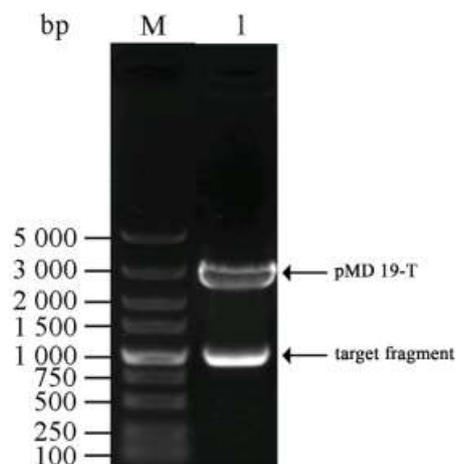
### 3. Results

#### 3.1. Cloning and Identification of the Chitinase Gene *ChiKJ406136*

The amplified chitinase gene PCR fragment was 864 bp and appeared as a single band (Figure 1). The cloning vector plasmids pMD19-T-*ChiKJ406136*-1 was double digested with the restriction enzymes BamHI and XhoI (Figure 2), resulting in the production of two clear bands of approximately 900 bp (target fragment: *ChiKJ406136*-1) and 2890 bp (vector plasmid pMD19-T) in size. This result demonstrated the successful cloning of the *ChiKJ406136*, which was submitted to NCBI under accession number MG323510.



**Figure 1.** The electrophoresis results of the PCR products of the *ChiKJ406136* gene PCR product M: DL2000 DNA Marker; 1: The PCR products of the *ChiKJ406136* gene products.



**Figure 2.** Confirmation the cloning vector by restriction enzyme digestion M: DL5000 DNA Marker; 1: The products of pMD19-T-*ChiKJ406136*-1 vector restriction enzyme digestion.

### 3.2. Analysis of the *ChiKJ406136* Gene Sequence and Protein Bioinformatics

The nucleotide sequence of the chitinase gene *ChiKJ406136* was 99% similar to that of the sequence of the 864 bp chitinase genes from *S. albus* SM254 and *Streptomyces* sp. FR-008. The fragment contained a 864-bp open reading frame (ORF) encoding a 287 amino acid protein (Figure 3), which contained a conserved region in the glycoside hydrolases family 19 domain. The amino acid sequence of *ChiKJ406136* was 100% identical to that of the chitinase genes from *Streptomyces* sp. SM8, *Streptomyces wadayamensis*, *Streptomyces* sp. FR-008, *S. griseus* subsp. *griseus*, *Streptomyces* sp. ScaeMP-6W, *Streptomyces* sp. IgraMP-1, and *Streptomyces* sp. BvitLS-983, as well as the chitinase A from *S. albus*.

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      10      20      30      40      50      60      70      80      90
1      ATGAGCCTGTGATCGCGCTGAGCGGTCGTGCGGGCTCGTCGTCTCCCCGCGCCACCGCACAGGCCGCGCCTGCCCAACGCG
1      M S L L I A L S A V V A A L V V L P A A T A Q A A A C A N A

      100     110     120     130     140     150     160     170     180
91     TGGAACGCTCCGCCGTACACCGCGGGCCAGAGCGCTCGTACAACGGGCGCAACTACCGCCAAGTGGTGGACGCAGAACGAGCGG
31     W N A S A V Y T G G Q S A S Y N G R N Y T A K W W T Q N E R

      190     200     210     220     230     240     250     260     270
181    CCCGGCAGCTCCGACGTCTGGGCCGACCAGGGTGCCTGTGTGGCGGGGGGGAGCAGCCAGCGCTCGTCGTGAGCGAGGCCAG
61     P G S S D V W A D Q G A C G G G G G E Q P S G F V V S E A Q

      280     290     300     310     320     330     340     350     360
271    TTCAACAGATGTTCCGAACCGAACTCTTCTACACCTACAGCGGGCTCACCGCGCCCTCAGCTCGTACCGCGCTCGCCAACCC
91     F N Q M F P N R N S F Y T Y S G L T A A L S S Y P A F A N T

      370     380     390     400     410     420     430     440     450
361    GGCAGCGACGAGGTCAAGAAGCGCGAGGCCCGCCCTTCTCGCCAACGTCAGCCACGAGACGGCGCGCCTCGTCCACATCAAGGAAGTC
121    G S D E V K K R E A A A F L A N V S H E T G G L V H I K E V

      460     470     480     490     500     510     520     530     540
451    AACGAGGCCAACTACCCCACTACTGCGACCGGAACCCAGCCCTACGGCTGCCGCGGGCCAGGCCGCTACTACCGCGCGGCCCATC
151    N E A N Y P H Y C D R N Q P Y G C P A G Q A A Y Y G R G P I

      550     560     570     580     590     600     610     620     630
541    CAGCTCAGCTGGAACCTCAACTACAAGGCCGCGGTGACGCCCTCGGCATCGACCTGCTGAACAACCCCTACCTGGTCGAGCAGAAGCC
181    Q L S W N F N Y K A A G D A L G I D L L N N P Y L V E Q N A

      640     650     660     670     680     690     700     710     720
631    TCCGTCGCTGGCGGACCGCCTGTGGTACTGGAACACCCAGTCCGGCCCGGCACGATGACCCCGCACAACGCCATCGTCAACAACCGG
211    S V A W R T G L W Y W N T Q S G P G T M T P H N A I V N N R

      730     740     750     760     770     780     790     800     810
721    GGCTTCGGCGAGACCATCCGCTCGATCAACGGCTCGATGAGTGAACGGCGGCAACCCGCGCCAGGTGCGAGCGCATCGACAAGTTC
241    G F G E T I R S I N G S I E C N G G N P A Q V Q S R I D K F

      820     830     840     850     860
811    ACCTCTTCGCGCAGATCCTCGGCACCCACCGGCTCCAACCTGTACTGTGA
271    T S F A Q I L G T T T G S N L Y C *

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**Figure 3.** The sequence of *ChiKJ406136* gene and its encoded amino acid sequence.

The protein encoded by *ChiKJ406136* was calculated to contain one signal peptide, with the cleavage site predicted to be located between amino acids 24 and 25, such that the mature peptide started at amino acid 25. In addition, the chitinase was predicted to contain five N-glycosylation sites (aa32, NASA; aa49, NYTA; aa136, NVSH; aa209, NASV; and aa250, NGSI). The secondary structure prediction of the protein revealed the following: the number of the alpha helices was 90 (31.36%); the number of extended chains was 66 (23%); the number of random coils was 100 (34.84%), the number of  $\beta$ -turns was 31 (10.80%). The results showed that the alpha helix and random coil features were dominant in the protein. The tertiary structure prediction results of the chitinase are shown in Figure 4.

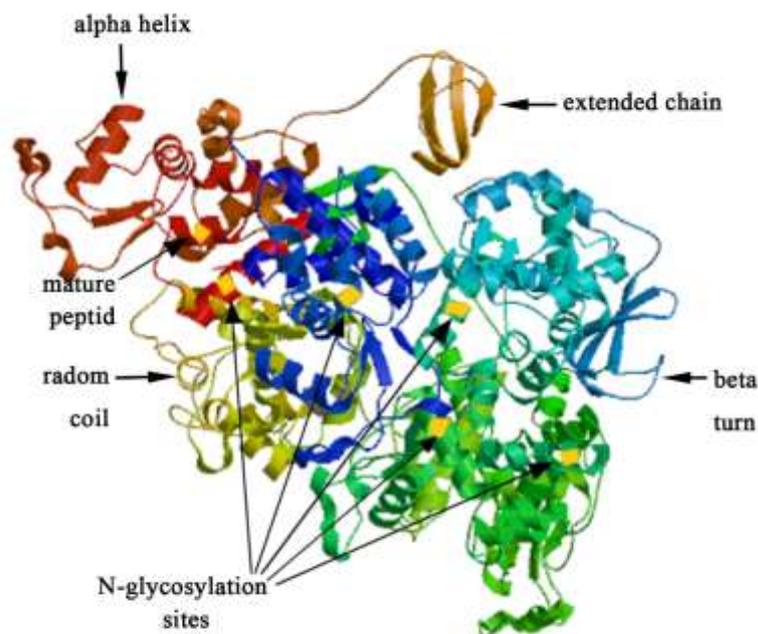


Figure 4. Prediction of protein tertiary structure.

### 3.3. Induction of Recombinant Chitinase Expression

The recombinant expression plasmid pMD19-T-*ChiKJ406136-2* was digested with restriction enzymes BamHI and XhoI (Figure 5), yielding one 800-bp band (target fragment: *ChiKJ406136-2*) that was identical to the predicted size of the SSKJ-6136 gene with the signal peptide removed, demonstrating the successful construction of the recombinant expression vector. SDS-PAGE analysis (Figure 6) showed that the expression of *ChiKJ406136* in *E. coli* BL21 (DE3) cells containing pET32a (+)-*ChiKJ406136-2* was robust and increased with the time of induction. In contrast, cells cultured in a similar manner and harboring the empty vector or pET32a (+)-*ChiKJ406136-2* without induction did not overexpress any particular protein, while cells harboring the empty vector that were induced with 1 mmol/L IPTG (isopropyl- $\beta$ -D-thio-galactoside) expressed a 20.4 kilodalton (kDa) protein. The induction of pET32a (+)-*ChiKJ406136* expression in the presence of all different concentrations of IPTG showed the presence of a 48 kDa protein band, where the molecular weight included the tagged protein and the removed signal peptide. This result also demonstrated that the concentration of IPTG had no effect on the molecular weight of the resulting recombinant protein.

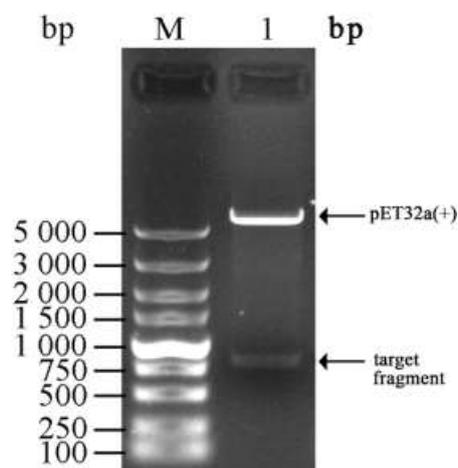
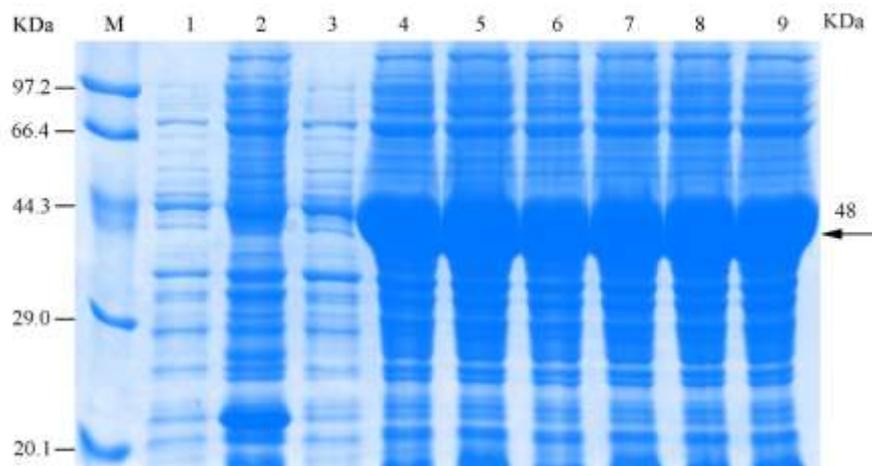


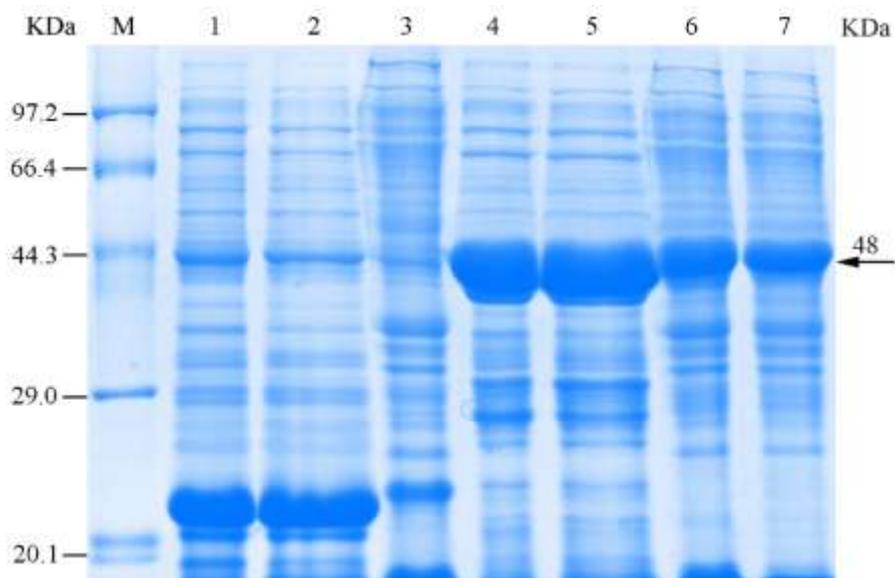
Figure 5. Confirmation of the expression vector by restriction enzyme digestion M: DL5000 DNA marker; 1: The products of the pET32a (+)-*ChiKJ406136* vector by restriction enzyme digestion.



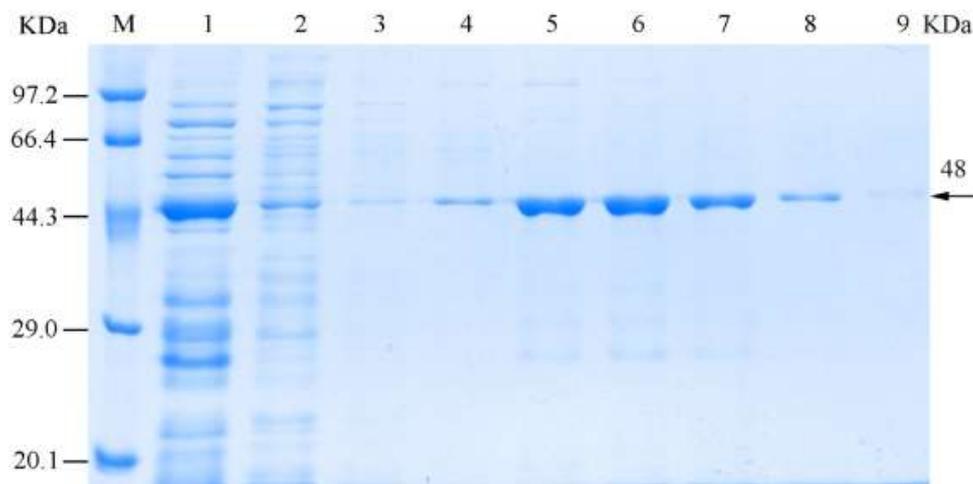
**Figure 6.** SDS (sodium dodecyl sulfate)-PAGE (polyacrylamide gel) analysis of pET32a (+)-*ChiKJ406136* expression induced by IPTG (isopropyl- $\beta$ -D-thio-galactoside) M: Protein marker (low molecular weight standard); 1: pET-32a (+) without induction; 2: pET-32a (+) induced by IPTG (1.0 mmol/L); 3-9: pET32a (+)-*ChiKJ406136* induced by IPTG, at concentrations of IPTG was 0, 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 mmol/L.

#### 3.4. Solubility and Purification of the Recombinant Chitinase

After an overnight induction at 16 °C, the recombinant protein was primarily insoluble, as the eluted fractions contained precipitated in the form of inclusion bodies (Figure 7). Imidazole concentrations of 10, 50, 100, 150, 200, 250, and 300 mmol/L were used to elute the recombinant protein from the column, resulting in protein being eluted at all concentrations, with the highest amount eluted using 150 mmol/L imidazole (Figure 8).



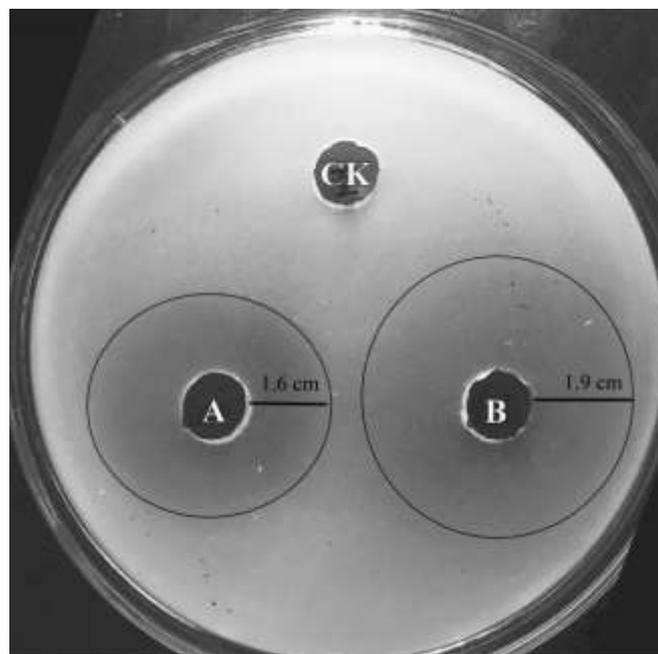
**Figure 7.** The detection of soluble recombinant proteins induced at 16 °C M: Protein marker (low molecular weight standard); 1, 2: Supernatant of cell lysate from a strain harboring pET32a (+); 3: Sediment of cell lysate from a strain harboring pET32a (+); 4, 5: Supernatant of cell lysate from a strain harboring the recombinant vector; 6: Sediment of cell lysate from a strain harboring the recombinant vector.



**Figure 8.** Purification and detection of recombinant protein M: Protein marker (low molecular weight standard); 1: Supernatant of cell lysate from the recombinant vector; 2: Effluent by gravity; 3–9: Fractions eluted with 10, 50, 100, 150, 200, 250 and 300 mmol/L imidazole.

### 3.5. Properties of the Recombinant Chitinase

Both the crude and purified proteins exhibited chitin hydrolysis activity (Figure 9), the diameters of chitin hydrolysis circles of crude and purified proteins were 1.6 cm and 1.8 cm, respectively. After being diluted 8-fold, the OD (optical density) values of the crude and purified recombinant proteins were 0.732 and 0.02, and had concentrations of 2.06 and 0.07 mg/mL, respectively (Table 1). In addition, the crude and purified proteins exhibited chitinase activities of 0.045 and 0.033 U/mL and the specific activities of 0.022 and 0.471 U/g, respectively. The recovery rates of the enzyme activities were 100% and 73.33% for the crude and purified recombinant chitinase fractions, respectively.



**Figure 9.** Detection of chitin hydrolysis by recombinant proteins on the colloidal chitin medium (1% chitin) CK: 50  $\mu$ L supernatant of cell lysate from a strain harboring pET32a (+); A: 50  $\mu$ L crude recombinant protein *ChiKJ406136*; B: 50  $\mu$ L purified recombinant protein *ChiKJ406136*. Culturing for 5 d, the diameters of chitin hydrolysis circles were measured. Black circles indicate the area of chitin hydrolysis.

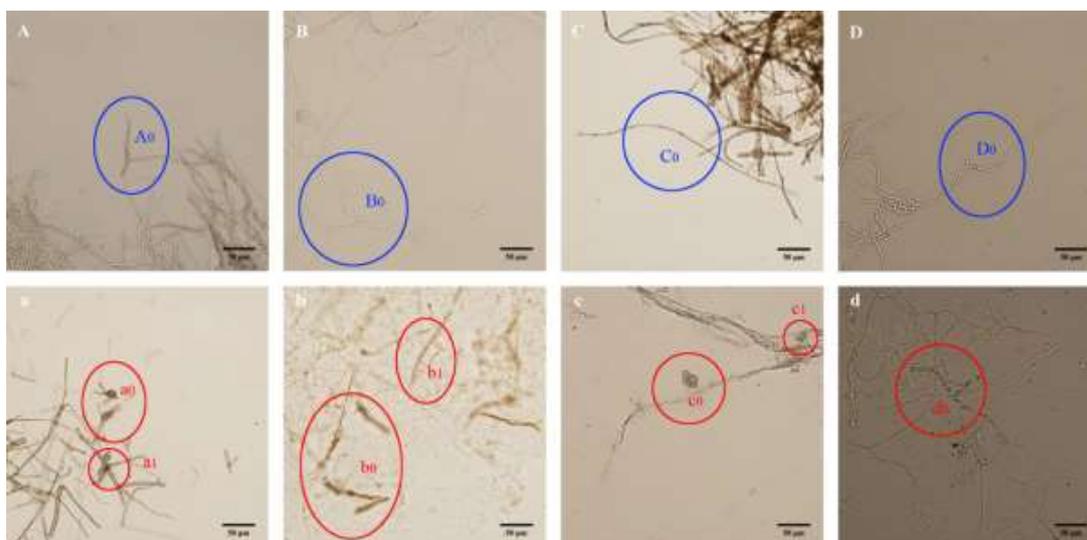
**Table 1.** Quantitative comparison between crude recombinant protein and purified recombinant protein <sup>a</sup>.

Purification Procedure	Concentration of Protein (mg/mL)	Chitinase Activity (U/mL)	Specific Activity (U/mg)	Purification Fold	Recovery Rate of Enzyme Activity (%)
<i>ChiKJ406136</i> -crude protein	2.06 ± 0.04 a	0.045 ± 0.010 a	0.022 ± 0.008 b	1 b	100 a
<i>ChiKJ406136</i> -purified protein	0.07 ± 0.01 b	0.033 ± 0.009 a	0.471 ± 0.050 a	21.41 a	73.33 b

<sup>a</sup> Data are presented as the means ± SD (standard deviation) ( $n = 3$ ). Lowercase letters after the same column indicate a significant difference between crude protein and purified protein at  $p < 0.05$  by the LSD (least significant difference) test.

### 3.6. Effect of the Recombinant Chitinase on Pathogenic Fungi

After being treated with the recombinant chitinase for 2 h, the mycelial morphology of four pathogenic fungi (*C. scoparium*, *C. parasitica*, *N. parvum* and *F. oxysporum*) that are known to be affected by *S. sampsonii* KJ40 was observed. Interestingly, the mycelial morphology of all four fungi was altered by the chitinase treatment (Figure 10), as the mycelia were mixed and partly broken down into chunks. Specifically, the merogenesis of the cells increased, inflating the mycelia of *N. parvum* (Figure 10c), and the mycelia of *F. oxysporum* were broken and distorted (Figure 10d).



**Figure 10.** Hyphal morphology of pathogenic fungi treated with *ChiKJ406136* A–D: The typical morphology of mycelia from *C. scoparium*, *C. parasitica*, *N. parvum*, and *F. oxysporum* (400×), A0, B0, C0, D0: normal hyphal; a–d: The morphology of mycelia from *C. scoparium*, *C. parasitica*, *N. parvum*, *F. oxysporum* treated with the recombinant chitinase (400×), a0, a1-mycelia were mixed, b0, b1-mycelia were broken down, c0, c1-mycelia were inflated, d0-mycelia were broken and distorted.

### 3.7. Biological Control Effects of Chitinase on Potted Plants

The biological control effects of the recombinant chitinase over the course of 20 days in pot experiments are shown in Table 2. The incidences of four diseases were as high as 85% in the control plants, while the recombinant chitinase solutions had different control effects at different dilutions. The biological control effects decreased at higher chitinase dilutions, with the undiluted solution exhibiting the best effect. The control effects of the 20-fold dilution was significantly reduced (below 55%).

**Table 2.** Biological control effect of the recombinant chitinase against *Eucalyptus robusta* Smith leaf blight, *Castanea mollissima* BL. blight, *Juglans regia* L. blight and *J. regia* root rot in pot experiment <sup>a</sup>.

Diluted Solution	Diseases on the Plant Samples											
	<i>E. Robusta</i> Leaf Blight			<i>C. Mollissima</i> Blight			<i>J. Regia</i> Blight			<i>J. Regia</i> Root Rot		
	Incidence (%) <sup>b</sup>	Disease Index <sup>c</sup>	Control Effect (%) <sup>d</sup>	Incidence (%)	Disease Index	Control Effect (%)	Incidence (%)	Disease Index	Control Effect (%)	Incidence (%)	Disease Index	Control Effect (%)
–	9.0 ± 1.0 d	6.8 ± 0.5 d	92.7 ± 1.5 a	12.0 ± 2.0 d	13.3 ± 1.1 d	86.1 ± 1.7 a	8.0 ± 1.5 d	6.4 ± 0.4 d	93.5 ± 3.1 a	10.0 ± 1.0 d	12.4 ± 0.9 d	90.4 ± 2.5 a
10-fold	21.0 ± 2.0 c	15.9 ± 1.1 c	79.0 ± 2.3 b	30.0 ± 2.0 c	20.2 ± 2.1 c	70.7 ± 2.0 b	19.0 ± 1.0 c	12.3 ± 1.5 c	80.6 ± 2.8 b	22.0 ± 1.0 c	19.0 ± 1.2 c	75.2 ± 2.2 b
20-fold	44.0 ± 3.0 b	33.6 ± 1.6 b	50.5 ± 2.5 c	55.0 ± 3.0 b	41.7 ± 2.4 b	42.6 ± 2.8 c	42.0 ± 2.0 b	31.8 ± 1.9 b	52.4 ± 2.0 c	46.5 ± 2.5 b	38.3 ± 2.5 b	46.1 ± 1.8 c
Control	92.0 ± 2.0 a	66.7 ± 2.1 a	–	90 ± 2.0 a	70.5 ± 2.3 a	–	85 ± 2.0 a	64.9 ± 2.4 a	–	90 ± 2.0 a	65.8 ± 2.2 a	–

<sup>a</sup> Data are presented as the means ± SD ( $n = 10$ ). Lowercase letters after the same column indicate a significant difference among bacterial concentrations at  $p < 0.05$  by the LSD (least significant difference) test; <sup>b</sup> Incidence (%) = (number of infected plants/total number of inoculated plants) × 100; <sup>c</sup> Disease index = ( $\Sigma$ (numerical value of each disease rating × number of twigs for each disease rating))/(total twigs × most serious disease rating) × 100; <sup>d</sup> Control effect (%) = ( $\Sigma$ (control disease index-treatment disease index)/control disease index) × 100.

#### 4. Discussion

Chitinases have been reported to improve the disease resistance and have been used as insect and biological fungicides and pesticides in agroforestry. Historically, studies have focused on using chitinase-producing microorganisms to directly control plant fungal diseases, which is based on the ability of these microorganisms to decompose the chitin that is present in the fungal cell walls [51]. Baek et al. [42] and Kern et al. [43] demonstrated that *Trichoderma virens* Pers. Gv29-8 and *Metarhizium anisopliae* (Metchnikoff) Sorokin can produce chitinase, respectively, and that they could inhibit the mycelial growth of *Rhizoctonia solani*, *Alternaria alternata*, *Fusarium oxysporum*, *Penicillium aculeatum* Peyronel, *Trichoderma harzianum* Rifai, *Rhizopus* sp. Ehrenb. and *Botrytis cinerea* Pers. Although chitinase have great potential for use in a number of applications, the activity observed for these enzymes has been low. For the commercial production of chitinase, previous studies focused that produced high levels of chitinase or on increasing the enzyme production by changing fermentation conditions [52–54]. Nevertheless, due to complex regulatory mechanisms, fermentation separation and immature extraction processes, the successful industrial production of chitinase is rare. The development of molecular biology techniques provides a new way to solve this problem, by cloning a chitinase gene into an appropriate vector for efficient expression to meet the needs of large-scale production and research and applications.

The protein encoded by the *S. sampsonii* *ChiKJ406136* gene has a domain belonging to the glycoside hydrolase family 19, and *Streptomyces* enzymes belongs to this chitinase family [55]. Family 19 chitinases were initially identified in higher plants, while only family 18 chitinases were observed in microorganisms. In a study by Ohno et al. [56], family 19 chitinase-encoding genes were identified from *Streptomyces griseus* (Krainsky) Waksman & Henrici HUT6037, outside higher plants, and family 19 members were also found in other actinomycetes [57–60]. Nevertheless, Family 19 chitinases are not frequently found in bacteria and never in *S. sampsonii*. The tertiary structure of chitinase family 19 is similar to that of lysozyme. The molecular weight of the chitinase from different biological sources varies greatly, from 20 to 90 kDa, with bacterial chitinases varying in size from 20 to 60 kDa, similar to plant chitinases (25–40 kDa) and smaller than insect chitinases (40 to 85 kDa) [61]. Similarly, the molecular weight of the *ChiKJ406136*-encoded protein expressed in *E. coli* was 30.6 kDa, and the weight of protein after removing the signal peptide was 28.3 kDa in our study. However, from the SDS-PAGE analysis of protein bands, the expressed protein bands were observed to be close to 48 kDa. The primary reason for this result is that the total molecular weight of the protein expressed in the pET-32a (+) vector is 20.4 kDa, which contains Trx-tags size of 12 kDa. Taking into account the His-tags (0.8 kDa) and S-tag (1.7 kDa), the remaining 5.1 kDa were derived from 54 amino acids between these tags and the termination codon.

The advantages of using an *E. coli* expression system are its well-characterized genetic background, fast reproduction rate, low cost, and high level of expression; in addition, there are numerous expression vectors, hosts and purification systems [62]. Exogenous proteins are easily degraded by the host proteases or form inclusion bodies when expressed at high levels. Currently, many studies have investigated protein re-naturation in vitro, but the process of protein re-naturation is often time-consuming. Therefore, exploring the soluble expression of exogenous proteins in *E. coli* has potentially, wide applications [63]. By lowering the culture temperature, the protein synthesis rate and the concentration of polymeric intermediates are reduced, avoiding the formation of inclusion bodies [64]. The *ChiKJ406136* gene was highly expressed at 16 °C from the pET-32a vector. The recombinant protein primarily existed in the supernatant in the soluble form. We easily cloned the chitinase-encoding gene into the histidine tag-containing pET expression vector and identified the expressed protein. In addition, the recombinant *ChiKJ406136*-encoded protein was eluted with different concentrations of an imidazole solution. The elution efficiency of the recombinant *ChiKJ406136*-encoded protein was high, and the recovery rate of enzyme activity was as high as 73.33%. The specific activity of the purified *ChiKJ406136* protein was 0.471 U/g, higher than the reported in a study by

García-Fraga et al. [65], where the activity of the purified recombinant *Ptchi19p*-encoded protein from *Pseudoalteromonas tunicata* Holmström was 0.228 U/g.

Similar to the previous studies of antifungal activity, for the four assayed fungi used in our study, we observed complete destruction of the mycelial morphology when all four pathogenic fungi were treated with the purified chitinase from *S. sampsonii* KJ40. Such a breakdown is likely due to the hydrolysis of chitin that exists in the fungal cell walls. *Chi18H8*, which encodes a chitinase from *Bacillus thuringiensis* Berliner BUPM255, was cloned and expressed by Hjort et al. [41]. The purified enzyme could inhibit the mycelial growth of *Colletotrichum gloeosporioides* (Stoneman) Spauld. & H. Schrenk, *F. graminearum*, *F. oxysporum* and *A. alternata*. The recombinant protein encoded by *Ptchi19p* from *P. tunicata* could also inhibit the mycelial growth of *F. oxysporum* and *Aspergillus niger* [65]. Through in vitro testing, Reyes-Ramírez et al. [66] demonstrated that a *Bacillus thuringiensis* chitinase had biocontrol potential against *Sclerotium rolfsii* (Curzi) C.C. Tu & Kimbr., *Aspergillus terreus* Thom., *Aspergillus Flavus* Link, *Nigrospora* sp. Mason with 25–82% inhibitory effects. Kirubakaran and Sakthivel [67] reported that a 35 kDa chitinase had significant effects on *B. cinerea* and *F. oxysporum* using a concentration of 1.2 U/g. Lee et al. [68] showed that a *Bacillus licheniformis* Carl. chitinase could inhibit 50% of the hyphal growth of *A. terreus*. In addition, some studies demonstrated the antifungal effect in pot experiments in greenhouses. For example, Viterbo et al. [69] reported that a 36 kDa endochitinase from *T. harrzianum* had some efficacy, but it did not reach significance for the disease caused by *B. cinerea* and *R. solani*. An antifungal chitinase of approximately 30kDa was isolated from *Sorghum bicolor* (L.) Moench using chromatographic techniques and showed a broad-spectrum antifungal activity toward devastating fungal pathogens that attack rice, tobacco, tea and clover at concentration of 18–36 µg/mL [70]. Those studies have been seen that the chitinases from either plants or bacteria display antifungal activity, which might make them a great alternative to the use of chemical products for the biological control of pests [59,61,71]. But as each enzyme may present its own characteristics that make it useful for a specific function, the search for new chitinases from different organisms is of interest. Hence, compared to the above results, the results of our study revealed an excellent ability to control some local plant diseases using lower concentrations of the purified chitinase. Interestingly, the effects of purified chitinases were better than *S. sampsonii* KJ40 using in disease control caused by *F. oxysporum* with the incidence, disease index and control effect of 60.67%, 52.88%, 21.25%, respectively [72]. Specifically, the ability of the chitinase to control plant diseases, including *E. robusta* leaf blight, *C. mollissima* blight, *J. regia* blight and *J. regia* root rot, were first reported by us.

## 5. Conclusions

In conclusion, we showed that a chitinase from *S. sampsonii* can be expressed and purified in high amounts with ease. The purified enzyme exhibits antifungal activity against plant pathogenic fungi and inhibits the diseases these fungi cause. As studies of the chitinase and the encoding gene from *S. sampsonii* were previously lacking, our results can improve and supply a biocontrol protein, providing a new resource for chitinase genes. Furthermore, our results may have potential application for the biocontrol of other phytopathogenic fungi and transgenic resistance breeding in plants, which may be used for commercial purposes. However, the N-terminal amino acid sequence and the mechanism of *S. sampsonii* chitinase action require further investigation.

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