Ciência Rural, Santa Maria, v.53:4, e20210663, 2023 http://doi.org/10.1590/0103-8478cr20210663
ISSNe 1678-4596
BIOLOGY

Cloning, expression and antifungal effect of the recombinant chitinase from *Streptomyces sampsonii* KJ40

Shiwei Wang[1] Xinmei Fang[1] Kaige Liang[1] Shujiang Li[1] Shan Han[1] Tianhui Zhu[1*]

[1]College of Forestry, Sichuan Agricultural University, 611130, Chengdu, Sichuan Province, China. E-mail: zhuth1227@126.com. [*]Corresponding author.

ABSTRACT: *Streptomyces sampsonii is a kind of biocontrol bacterium with antifungal effects, and chitinase is one of the main antifungal substances. To improve and further study the structure and function of the chitinase gene of S. sampsonii, we amplified the target fragment by PCR, ligated the fragment to the expression vector pET-32a, introduced the resulting plasmid into Escherichia coli BL21 (DE3) and induced expression of the chitinase. Then, the recombinant chitinase was purified by is-labelled protein micro purification kit. A chitinase gene, Sschi61, was cloned from the genome and expressed in a prokaryote. The antifungal effect of the recombinant protein was also studied. Finally, the chitinase gene Sschi61 with a length of 1755 bp was obtained, and the expression of the 82 kDa recombinant chitinase was induced in E. coli by IPTG. The recombinant chitinase could inhibit the black spot pathogen of Eucommia ulmoides (Pestalotiopsis trachicarpicola). After the hyphae of the pathogen of black spot of Eucommia ulmoides (Pestalotiopsis trachicarpicola) were soaked with recombinant chitinase, the hyphae cells expanded, broke, and dissolved.*
Key words: *biological control, Streptomyces sampsonii, chitinase gene, cloning, prokaryotic expression.*

Clonagem, expressão e efeito antifúngico da quitinase recombinante de *Streptomyces sampsonii* KJ40

RESUMO: *Streptomyces sampsonii é uma espécie de bactéria de biocontrole com efeitos antifúngicos, sendo a quitinase uma das principais substâncias desse tipo. Para melhorar e estudar mais a estrutura e função do gene da quitinase de S. sampsonii, amplificamos o fragmento alvo por PCR, ligamos o fragmento ao vetor de expressão pET-32a, introduzimos o plasmídeo resultante em Escherichia coli BL21 (DE3) e induzimos expressão da quitinase. Em seguida, a quitinase recombinante foi purificada pelo kit de micropurificação de proteína marcada. Um gene da quitinase, Sschi61, foi clonado do genoma e expresso em um procarioto. O efeito antifúngico da proteína recombinante também foi estudado. Finalmente, o gene da quitinase Sschi61 foi obtido, contando comprimento de 1755 pb, e a expressão da quitinase recombinante de 82 kDa foi induzida em E. coli por IPTG. A quitinase recombinante pode inibir o patógeno da mancha preta de Eucommia ulmoides (Pestalotiopsis trachicarpicola). Após as hifas do patógeno da mancha preta de Eucommia ulmoides (Pestalotiopsis trachicarpicola) serem embebidas com quitinase recombinante, as células das hifas se expandiram, quebraram e se dissolveram.*
Palavras-chave: *controle biológico, Streptomyces sampsonii, gene quitinase, clonagem, expressão procariótica.*

INTRODUCTION

Streptomyces belongs to the family *Streptomyces* of actinomycetes, which are prokaryotes. It is a gram-positive filamentous bacterium whose morphology is similar to fungi (FLÄRDH et al., 2009; HASANI et al., 2014). *Streptomyces* can produce macrolides, amino glycosides, polypeptides and other antibiotics. Among them, the macrocyclic ester antibiotic FK506 can inhibit the replication of vaccinia virus in bsc-40 cells (HIGGINBOTHAM et al., 2010; REIS et al., 2006). Aminoglycoside antibiotics have a strong inhibitory effect on methicillin-resistant *Staphylococcus aureus*. *Streptomyces* can also produce many kinds of enzymes that are widely used in medicine, food and agriculture. PLD (ε-polylysine degrading enzyme) was isolated from the cell membranes of *S. albus* and *S. diastocologenes*. *S. mobaraensis* can produce transglutaminase, which can promote protein intramolecular or intermolecular cross connection, the interaction between protein and amino acid and protein intramolecular glutamine hydrolysis to change the structure and functional properties of proteins (KITO et al., 2002; YOKOYAMA et al., 2004). *S. albolongus* can produce the chitinase SaChiA4, which has potential application in chitin waste treatment. *Streptomyces* can also produce

Received 09.11.21 Approved 04.13.22 Returned by the author 07.13.22
CR-2021-0663.R1
Editors: Leandro Souza da Silva Fábio Nascimento

amylase, protease, lipase, and other enzymes (GAO et al., 2018). In addition, *Streptomyces* can secrete the growth hormone IAA to promote plant growth, increasing the dry weight of corn, cucumber, tomato, and other plants (IGARASHI et al., 2002; KHAMNA et al., 2009).

Chitin is a high molecular weight polymer that is connected by N-acetyl-2-amino-2-deoxy-D-glucose with a β-1,4 glycoside bond. According to the different crystal forms, chitin can be divided into α-chitin, β-chitin and γ-chitin (KHOUSHAB et al., 2010). Chitin has strong chemical stability due to its hydrogen bonds in and between molecules, making it difficult to study its application. Chitinase can hydrolyse β-1,4 glycoside bonds in chitin, and this reaction plays an important role in saving energy, reducing pollution and decomposing chitin produced by organisms (HAMMAMI et al., 2013; HARTL et al., 2012; KUMAR et al., 2000; XIAO et al., 2003). Studies have shown that chitinase can inhibit a variety of plant pathogenic fungi. Chitinase can also decompose chitin components in the cuticle of nematodes and reduce the economic losses of billions of dollars caused by plant parasitic nematodes to global agriculture and forestry (ABAD et al., 2008; MERZENDORFER et al., 2003). Therefore, chitinase has broad prospects in the field of biological control. At present, a wide range of studies on chitinases have been performed throughout the world, mainly including the biological control of *Botrytis cinerea*, plant soil-borne disease pathogenic fungi, and *Bipolaris sorokiniana* (INBAR et al., 1991; MAGNIN-ROBERT et al., 2007; ZHANG et al., 2000). Several chitinase genes in fungi, actinomycetes, and bacteria have been cloned and expressed; the Chit36 gene from *Trichoderma harzianum* was cloned and expressed. The soluble protein chi6769 from a new rare actinomycete (*Saccharothrix yanglingensis* HS. 015) was cloned and expressed, and the chitinase genes stmchia and stmchib from *Stenotrophomonas maltophilia* were cloned and expressed. These chitinases have antifungal activity and can combine with nanomaterials to increase their catalytic stability (LU et al., 2018; MOHAMMADZADEH et al., 2017; SUMA et al., 2013). Chitin oligosaccharide, amino oligosaccharide and other substances produced by chitinase-catalysed chitin hydrolysis increased the resistance to fungi and bacteria by increasing the secretion of plant-resistant enzymes.

With the huge losses of agricultural and forestry production due to plant fungal diseases, traditional chemical control has the risk of high pollution and high residue, while biological control has gradually become the main means of disease control because of its high efficiency, lack of pollution and long-lasting effect. Worldwide, an increasing number of microorganisms and their metabolites inhibited the survival and reproduction of pathogenic fungi. Streptomyces sampsonii has obvious antibacterial activity, which can inhibit the growth of various fungi and bacteria, potential insecticidal and antitumour activities, which have a certain lethal effect on root knot nematodes and tumor cells, the ability to promote growth and improve soil conditions; and the potential as a biological pesticide to improve low-cost organic land (KIM et al., 2011). S. sampsonii KJ40 is an actinomycete with multiple functions of disease prevention and growth promotion. The strain isolated from the rhizosphere of Poplar can prevent the occurrence of purple root rot and significantly improve the growth ability of trees. *P. trachicarpicola* can cause a variety of plant diseases, mostly foliar diseases, and is a pathogen of tea gray blight, brown leaf spot of *Polygonatum sibiricum*, leaf spots on mango, and twig blight of *Pinus bungeana* (Pinaceae: Pinoideae) in addition to black spot disease of *Eucommia ulmoides* (ZHANG et al., 2021; LIU et al., 2019; SHU et al., 2020; QI et al., 2020). In this study, the chitinase gene of S. sampsonii KJ40, Sschi61, was cloned and expressed in E. coli. The basic properties of chitinase and its effect on *P. trachicarpicola* were explored, laying a foundation for the research and development of a chitinase gene from S. sampsonii KJ40 for biological control.

MATERIALS AND METHODS

Strains, plasmids, plant samples and media

Strains: *S. sampsonii* KJ40 (accession number: LORI00000000) was provided by the forest protection and Forest Pathology Laboratory of Sichuan Agricultural University. The strain is preserved by the Chinese General Microbiological Culture Collection Center (CGMCC No. 5996). The key laboratory of forest protection in Sichuan Province provided the pathogenic fungus (*P. trachicarpicola*), which causes black spot of *Eucommia ulmoides*. It was isolated in the incidence area of black spot of *Eucommia ulmoides* disease in Dayi County, Chengdu, Sichuan Province.

Expression host strains: *Escherichia coli* BL21 (DE3) chemically competent cells were purchased from TransGen Biotech (China).

Plasmids: The plasmid pET-32a vector was purchased from TransGen Biotech (China).

Medium: (1) Nutrient broth medium (NB) consisted of the following: peptone 10 g, beef extract 5 g, sodium chloride 5 g, distilled water 1 L, pH 7.2-7.4. (2) LB medium consisted of the following: tryptone 10 g, yeast extract 5 g, sodium chloride 10 g, distilled water 1 L, pH 7.0. For solid medium, 18 g of agar was added to each litre of liquid medium.

Sequence analysis and protein bioinformatics prediction of the chitinase gene Sschi61

Based on the analysis of the whole genome sequence of *S. sampsonii* KJ40 and its functional prediction, a chitinase-coding gene named *Sschi61* (accession number: MW682322) was screened. The nucleotide sequence was compared by the BLAST program in the NCBI database, and the ORF and amino acid sequence were inferred by DNAMAN software. The amino acid fragment with high homology was selected for multiple sequence alignment by using Clustalx1.83 software. The protparam (http://web.expasy.org/protparam/) tool in the online ExPASY system was used to predict the amino acid composition, total number of positively and negatively charged residues, molecular formula, instability coefficient, fat coefficient and hydrophobicity. The tool for computing pI/Mw (http://web.expasy.org/computeupi/) was used to predict the theoretical molecular weight and theoretical isoelectric point of protein, the ProtScale (http://web.expasy.org/protscale/) tool was used to predict protein hydrophobicity, the TMHMM server v. 2.0 (http://www.cbs.dtu.dk/services/tmhmm-2.0/) tool was used to predict the transmembrane region, the Targetp 1.1 server (http://www.cbs.dtu.dk/services/targetp/) tool was used for subcellular localization analysis, the online software signalp 4.1 server (http://www.cbs.dtu.dk/services/signalp/) was used to predict protein signal peptide, the online software netnglyc 1.0 server (http://www.cbs.dtu.dk/services/netnglyc/) was used to predict the N-glycosylation sites, the online software net Phos 3.1 server (http://www.cbs.dtu.dk/services/netphos/) was used to predict phosphorylation sites, Scanprosite (http://prosite.expasy.org/scanprosite) analysed the functional sites and domains of its encoded proteins, NPs @ sopma was used to predict the secondary structure of the protein, and the online software SWISS-MODEL (http://swissmodel.expasy.org/interactive) was used to predict the tertiary structure of the protein.

PCR amplification of the chitinase gene Sschi61

Genomic DNA was prepared from *S. sampsonii* KJ40 by using a DNA extraction kit (made by Tiangen Biotech Co., Ltd., Beijing) for rhizobacteria. Primer Premier 5.0 software was used to design primers PF (5'-GCCATGGCTGATATCGGATCC ATGGTCGGCCTCGCCGCCCCCA-3') and PR (5'-GTGGTGGTGGTGGTGCTCGAG TCAGCGCAGCCCCTCGTG-3') to amplify the *Sschi61* gene. The PCR system contained ddH_2O 19 µl, 2 × TransTaq high fidelity (hifi) PCR Supermix 25 µl (buy from TransGen Biotech Co., Ltd.), genomic DNA 2 µl, 10 µmol/L upstream primer 2 µl, and 10 µmol/L downstream primer 2 µl. The following PCR thermal cycle conditions were used: 98 °C predenaturation for 2 min 98 °C denaturation for 15 seconds, 55 °C annealing for 30 seconds, 72°C extension for 20 seconds, 72 °C final extension for 7 min, 4 °C storage, and the step 2 to 4 was repeated for 35 cycles. PCR fragments were detected and separated on a 1.5% agarose gel, and the target fragments were recovered by the centrifugal column type DNA purification and recovery kit of Tiangen Biochemical Technology (Beijing) Co., Ltd.; the DNA fragments were used for the construction of subsequent expression vectors.

Construction of the expression vector with the chitinase gene Sschi61

The restriction endonucleases *BamH*I and *Xho* Ⅰ were used to cut the pET32a (+) vector at 37°C for 2h. The enzyme cutting reaction system included pET32a (+) vector 23 µl, *BamH*I and *Xho*I 2 µL each and 10 × NEBuffer 3 µL. After recovery by DNA purification and the Recovery Kit from Tiangen Biotech (Beijing) Co., Ltd., the products from the reaction were ligated to the expression vector to construct the recombinant expression vector. After purification, the target gene (3 µl), the vector enzyme product (2 µl) and sosoo mix (SoSoo Cloning Kit from TsingKe Biological Technology Co., Ltd., Beijing) (5 µl) were used. The reaction was incubated at 50°C for 15 min. At the end of the incubation period, 10 µl of the ligation reaction solution was added to 50 µl of competent *Escherichia coli* trans 5α sensitive cells, mixed evenly and placed in an ice bath for 30 min. After 90 seconds in a 42 °C water bath, the centrifuge tube containing the cells was placed in an ice bath for 5 min. Then, 500 µl of LB liquid medium was added. The mixture was cultured at 37°C and 200 rpm for 1 h. The mixture was used to coat LB plates (containing ampicillin) and incubated at 37 °C for 12-20 h. A single colony was placed in LB liquid medium for PCR amplification. The system and reaction procedure were consistent with those in "*PCR amplification of the chitinase gene Sschi61*". Colony

PCR products were examined by electrophoresis with a 1.5% agarose gel to detect whether there were any target bands. The positive bacterial solution identified by PCR was sent to Beijing Qingke Biotechnology Co., Ltd. for DNA sequencing. The plasmid with the correct sequence was extracted from the medium and stored at -20 °C.

Induced expression of the recombinant chitin gene Sschi61

The recombinant expression vector was transferred into BL21 (DE3) expression-sensitive cells. A single colony was cultured in 10 ml of LB at 37°C and 220 rpm overnight and inoculated in 10 ml of LB medium at a ratio of 1:100. When the OD600 reached 0.6, IPTG at a final concentration of 0.5 mM was added to induce expression, and the culture was incubated at 220 rpm and 37 °C overnight; no IPTG inducer was added as a negative control. After centrifugation at 4000 rpm for 10 min, the supernatant was discarded, and the bacteria were collected. SDS-PAGE was used to separate the protein, and Coomassie brilliant blue R-250 was added to stain the protein.

Purification and detection of recombinant Sschi61

Bacteria cells were broken by 400W ultrasound, 2s each time, 8s interval, as a cycle, 20min in total. After that, centrifuged at 4 °C and 12000 rpm for 20 min, the supernatant was collected, and affinity chromatography with Ni-IDA from Sangon Biotech (Shanghai) Co., Ltd. was performed. After the collected components were detected by SDS-PAGE, fractions were dialyzed overnight at 4 °C in buffer containing 50 mM Tris, 300 mM NaCl, 2 mM DTT, pH 8.0 and filtered with a 0.45 μM filter membrane; 1 ml of the solution was placed in a tube, which was frozen at -80 °C.

SDS-PAGE and Western blot were used to detect the purified protein. SDS-PAGE was performed on a 10 ml 8% separation gel and a 4 ml 5% concentrated gel. Then, the samples were loaded onto the gel, and electrophoresis, staining and destaining were carried out to obtain clear protein bands. The polyacrylamide gel detected by Western blot was made from 5% concentrated gel and 8% separation gel. SDS-PAGE protein buffer at the appropriate concentration was added to the collected protein sample, and then the denatured protein was heated. Then, the membrane was transformed and sealed. Antibody I had the label of His-tag, it recognized the His-tag fused to targeted proteins; the antibody company was SANGON biotechnology, No.: d110002. Antibody II was goat anti-rabbit; the antibody company was SANGON biotechnology, No.: d110058. Finally, a TMB kit was used to develop colour.

Concentration and activity determination of recombinant chitinase

The protein concentration was determined by a sk3071 non-interference protein quantitative kit, the assay kit company was SANGON biotechnology. The chitinase activity of purified protein was determined by a chitinase kit from Nanjing Jiancheng Bioengineering Institute. Enzyme activity unit definited that under the condition of 37 ℃, the amount of enzyme that 1mg protein decomposes chitin to produce 1mg of N-acetylglucosamine is 1 unit of enzyme activity in 1 h.

Effect of recombinant protein on mycelium and spore morphology of pathogenic fungi

P. trachicarpicola was cultured on a PDA plate for several days until the whole plate was covered with hyphae and spores were produced. A small number of pathogenic hyphae and spores were picked up by sterilized tweezers under very clean conditions and placed into purified recombinant protein solution with a concentration of 0.40 mg/ml for 24 h. A drop of protein solution containing pathogenic hyphae and spores was placed on a glass slide, and the morphology of hyphae and spores was observed by electron microscopy. Normal hyphae and spores that were not treated with recombinant protein solution were used as controls.

RESULTS

Sequence analysis and protein bioinformatics prediction

NCBI comparison results showed that the chitinase gene was more than 95% homologous to the *Sschi61* gene, and the ORF size was 1755 bp, encoding 584 amino acids (Figure 1). SMART domain prediction revealed three domains: CBD, FN3 and glyco. Among them, glycoprotein was the conserved region from the 217th amino acid residue to the 569th amino acid residue of the *Sschi61* gene, the carbohydrate domain of CBD was from the 14th amino acid residue to the 111th amino acid residue, and the type III domain of FN3 fibronectin was from the 122nd amino acid residue to the 196th amino acid residue. The phylogenetic tree showed that the

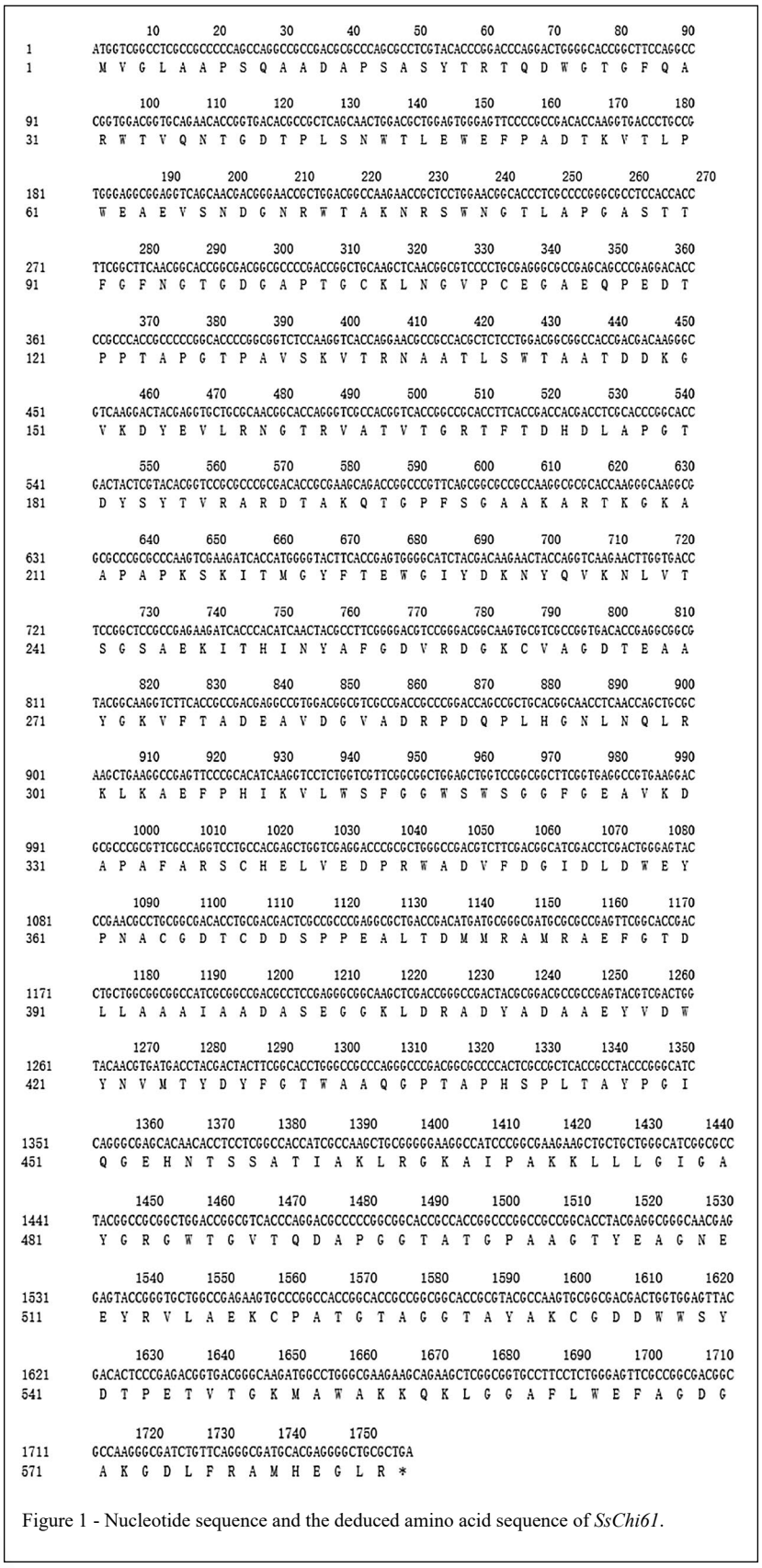

Figure 1 - Nucleotide sequence and the deduced amino acid sequence of *SsChi61*.

Sschi61 gene was closely related to *Streptomyces sanguis* (WP 071330519.1), and the glycohydrolase 18 family region was conserved. The amino acid sequence and physicochemical property analysis by software Protparam showed that the relative molecular weight of chitinase was approximately 62.3 kDa, the isoelectric point was 5.05, and the instability index was 23.78, indicating that the chitinase protein was a stable protein; the fat coefficient was 56.16, indicating that chitinase had general heat resistance. The total number of positively charged residues (Arg + Lys) was 57, the total number of negatively charged residues (ASP + Glu) was 74, and the formula was $C_{2760}H_{4164}N_{758}O_{868}S_{16}$. The total average hydrophilic coefficient was -0.510, indicating that the protein was hydrophilic. The chitinase protein was composed of 20 kinds of amino acids. Ala (a) was the most abundant amino acid, accounting for 14.2% of the total amino acids. Met (m), Cys (c) and His (H) were the least abundant amino acids, accounting for 1.4% of the total amino acids (Table 1). The TMHMM server v. 2.0 results showed that the chitinase protein had no transmembrane region and was a non-transmembrane protein. Subcellular localization of targetp 1.1 server software showed that the chitinase protein was intracellular protein. The netnglyc 1.0 server analysis showed that there were six N-glycosylation sites in the chitinase protein. The netphos 3.1 server tool was used to analyse the potential phosphorylation sites of amino acid sequences, and the results showed that the chitinase protein sequence had potential phosphorylation sites located on serine (SER), threonine (THR) and tyrosine (Tyr) amino acids; threonine had the most potential phosphorylation sites, with 35 sites. Serine had the second most potential phosphorylation sites, with 18 sites, and the least was tyrosine, with 11 sites. The software signalp 4.1 server predicted that a chitinase signal peptide site was present at amino acids 29 and 30 with a probability of 0.6938. The prediction of protein secondary structure showed that irregular curl accounted for 55.99%, elongation chain accounted for 17.47%, α-helix accounted for 21.75%, and β-angle accounted for 4.79% of the protein. It was speculated that the irregular curl structure may play a major role in protein secondary structure. The predicted results of the tertiary structure of the chitinase protein are shown in figure 2, the template PDB id used to model was 4W5U.

Amplification of the chitinase gene and construction of the expression vector

A single band with a fragment size of 1755 bp was obtained. The purified target gene fragment was ligated between the *BamH*I and *Xho*I enzyme sites of the vector pET32a (+) and transformed into *Escherichia coli* trans 5α cells. Sequencing of the positive clones revealed that there were 3 base mutations in the sequence compared with the target gene sequence, including 2 synonymous mutations and 1 missense mutation.

Induced expression and purification of recombinant chitinase

The expression of the pET32a (+)-*Sschi61* recombinant plasmid in BL21 (DE3) competent

Table 1 - The aminoacidic composition of the protein.

Amino acids	Number of amino acids	Percentage	Amino acids	Number of amino acids	Percentage
Ala (A)	83	14.2%	Leu (L)	31	5.3%
Arg (R)	24	4.1%	Lys (K)	33	5.7%
Asn (N)	19	3.3%	Met (M)	8	1.4%
Asp (D)	45	7.7%	Phe (F)	19	3.3%
Cys (C)	8	1.4%	Pro (P)	34	5.8%
Gln (Q)	13	2.2%	Ser (S)	24	4.1%
Glu (E)	29	5.0%	Thr (T)	58	9.9%
Gly (G)	67	11.5%	Trp (W)	21	3.6%
His (H)	8	1.4%	Tyr (Y)	21	3.6%
Ile (I)	11	1.9%	Val (V)	28	4.8%

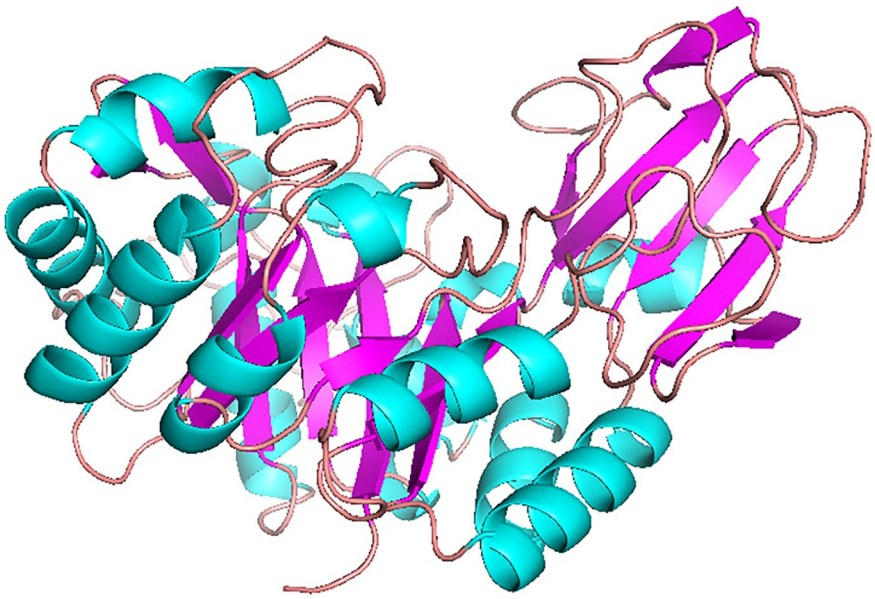

Figure 2 - Tertiary structure prediction.

Note: blue: α-helix; purple: β-turn; pink: random coil same as for figure 1.

cells was induced. The result of SDS-PAGE gel identification of the protein induced by IPTG were shown in figure 3. The protein was approximately 82 kDa in size and existed in the precipitate. Under the condition of 37 °C, the protein expression was highest. The results of SDS-PAGE after Ni agarose affinity chromatography are also shown in figure 3. The results showed that there was a target protein in the effluent, there was a small amount of heteroprotein in the eluate, there was more heteroprotein in eluate 3 than in eluate 4, there was more heteroprotein in eluate 4 than in eluate 5, and the eluate had a protein band with the same size as the target band. The SDS-PAGE results of the purified recombinant protein are shown in figure 4, and there were obvious bands at the corresponding positions, indicating that the purification of the recombinant protein was successful. The results of Western blot analysis are shown in figure 5, and there were obvious bands at the corresponding positions, one lane is a fusion protein.

Concentration and activity of recombinant chitinase

The concentration of chitinase recombinant protein was 1.07 mg/ml, which was determined by using a sk3071 non-interfering protein quantitative kit (by measure the absorbance value of different concentrations of protein, then draw the standard curve, and the activity of chitinase was 0.104 U/g, which was determined by the Nanjing Jiancheng Institute of Bioengineering by using a chitinase kit purified.

Effect of recombinant protein on the black spot pathogen Eucommia ulmoides

The results showed that after the hyphae of the black spot pathogen *Eucommia ulmoides* were soaked with recombinant protein, the hyphal cells expanded, broke and dissolved (ulcerated) (Figure 6); after the spores of the black spot pathogen Eucommia were soaked with recombinant protein, no obvious morphological changes were found, since the conidia may be resistant to enzymatic degradation in general.

DISCUSSION

In 1991, TOYODA et al found that chitinase produced by *Streptomyces griseus* could completely digest the haustorium of *Erysiphe*

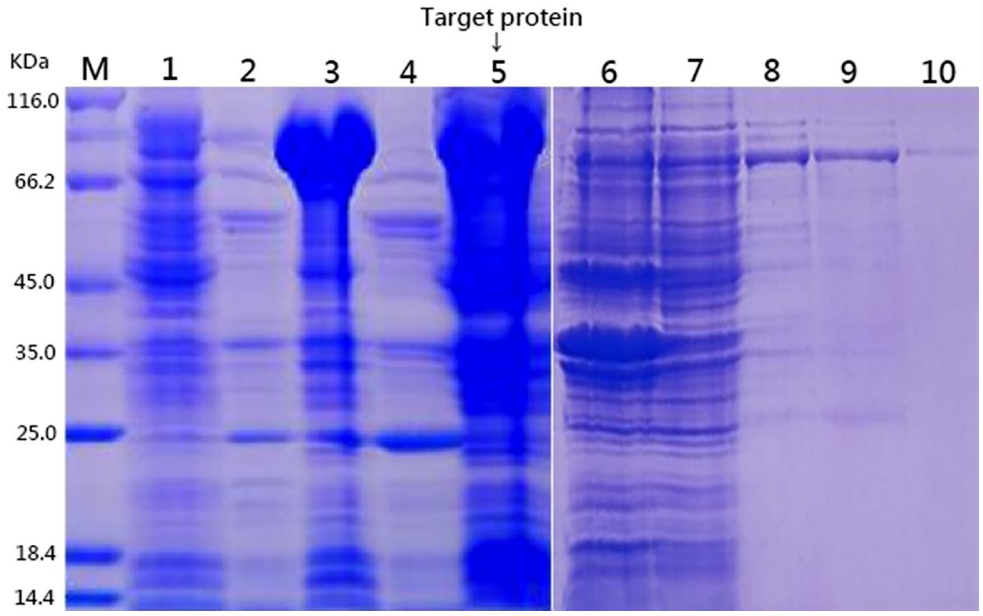

Figure 3 - SDS-PAGE pattern of the samples.

Note: M: Protein Marker; 1: the total protein before induction; 2: 20 ℃ supernatant; 3: 20 ℃ precipitation; 4: 37 ℃ supernatant; 5: 37 ℃ precipitation; 6: loading; 7: efflux; 8: 20 mM Imidazole elution fraction; 9: 50 mM Imidazole elution fraction; 10: 500 mM Imidazole elution fraction.

graminis f. sp. hordei after purification (TOYODA et al., 1991). The chitinase-producing microorganism and its chitinase can inhibit the growth of fungi in vitro. *Streptomyces* is considered to be the main source of chitinase, mainly *Streptomyces griseus*, *Streptomyces cyaneus*, *Streptomyces fradiae*, *Streptomyces venezuelae*, *Streptomyces coelicolor*, *Streptomyces erythraeus* and *purple Streptomyces violaceus* niger. At present, there are few studies on *S. sampsonii* regulators, and even fewer cloned and expressed complete chitinase genes. Only LI et al. cloned and expressed the chitinase gene chikj406136 with an ORF region of 864 bp, encoding 287 amino acids and containing the conserved region of the glycoside hydrolase family 19 (LI et al., 2018).

According to the similarity of amino acid sequences, chitinases from different organisms can be divided into five types, Ⅰ, Ⅱ, Ⅲ, Ⅳ and Ⅴ, which can be classified into two families of glycoside hydrolases. The amino acid sequence, structure and catalytic mechanism of these two families are different. Among them, type I, type II and type IV belong to the glycoside hydrolase 19 family and are mainly found in plants (KASPRZEWSKA, 2003). Type Ⅰ chitinase contains a C-segment signal peptide and a cysteine-rich region, which can make type I chitinase bind to the vacuole of target plant cells. Type Ⅱ and type Ⅳ chitinases are mainly found in dicotyledons. Type III and type V chitinases belong to 18 family, which is mainly found in bacteria and fungi. The catalytic domains of the chitinases 18 family have (α/β) s barrel structures; the «bacterial type" chitinases of this family was *exo*-chitinases and can produce chitin from the non-reducing end of chitin (ANDERSEN et al., 2005).

The conserved domain of the amino acid encoded by the *Sschi61* gene of *S. sampsonii* belongs to the glycoside hydrolase 18 family. However, the chitinases of the glycoside hydrolase 18 family have been mainly studied in insects. In *Bombyx mori*, the studies of chitinases of the glycohydrolase 18 (gh18) family have mainly involved purification, activity detection and gene cloning and expression (ABDEL-BANAT et al., 2002; PAN et al., 2010;

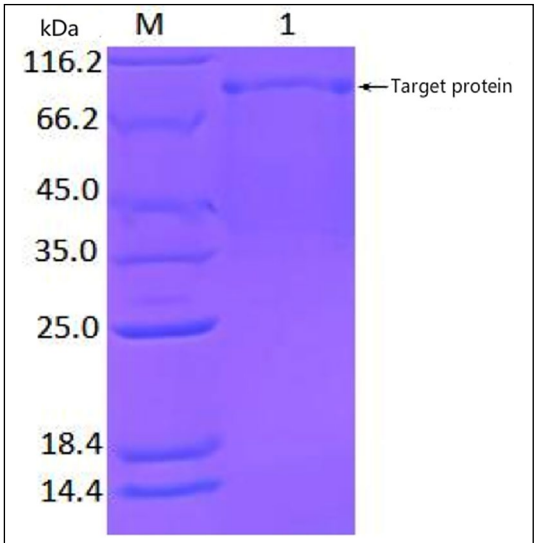

Figure 4 - SDS-PAGE pattern of purified chitinase.

Note: M: Protein marker; 1: Target protein.

TAKAHASHI et al., 2002). Insect chitinases belong to the glycoside hydrolase 18 family. The normal regulation of chitinase can destroy the metabolism of chitin in control pests; therefore, chitinases can be used as a new biological control strategy with great development potential (ARAKANE et al., 2009; NAKABACHI et al., 2010). Chitinases in plants belong to the glycoside hydrolase 19 family, the members of which contain a signal peptide and a highly homologous catalytic region. Under normal conditions, the expression of chitinase in plants is very low or non-existent. When infected by pathogenic bacteria, chitinase activity will increase rapidly. For example, when plants such as rape, bean, Arabidopsis and tobacco are infected by fungi, chitinase will be largely expressed in vivo, and enzyme activity will be greatly increased (FUKUDA et al., 1991; RASMUSSEN et al., 1992; ROBY et al., 1990; SAMAC et al., 1990). At the same time, transgenic research on rape and tobacco showed that the resistance of individuals that successfully expressed heterochitinase in the presence of pathogenic fungi was significantly higher than that of the control plants (BROGUE et al., 1991) This result suggested that the chitinase gene is an important defence gene in plants that plays an important role in plant growth and development, as well as in the stress resistance response.

The ORF of *Sschi61* is 1755 bp, encoding 584 amino acids, and the length of the gene is more than twice that of the chikj406136 gene. The glycohydrolase 18 region was conserved. The glycohydrolase 18 family contains a complete sequence of chitinase. Therefore, the analysis of the *S. sampsonii* chitinase gene *Sschi61* in this study improved and supplemented the knowledge of *S. sampsonii* chitinase biological control, laying a theoretical foundation for the research and application of the chitinase gene family in *S. sampsonii* biological control bacteria. This is a functional study of the chitinase system in *S. sampsonii* for biocontrol to study the biological control function of the chitinase family in *S. sampsonii* against pathogenic fungi.

Most of the protein function research requires a large amount of purified and soluble protein, and this is also important in this study. In order to achieve soluble expression, there are two common ideas: periplasmic space expression and intracytoplasmic expression (ZHANG et al., 2016). The oxidative environment of the periplasmic space can promote the formation of correct disulfide bonds, and at the same time, the internal protease content is low, avoiding the degradation of recombinant proteins (LEICHERT et al., 2004). OELSCHLAEGER et al. (2003) linked a signal peptide PelB at the N-terminal of scFv, s when expressing scFv, s single-chain

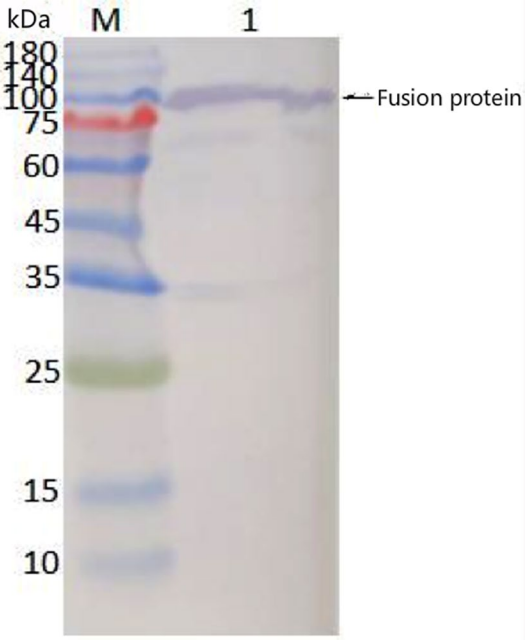

Figure 5 - Western Blot pattern of recombinant protein.

Note: M: Protein marker; 1: Recombinant protein.

antibody fragments in *E. coli*. The insertion of the signal peptide successfully guided the expression of exogenous proteins in the periplasmic space. To overcome the reductive environment in the cytoplasm, a mutant strain of cytoplasmic reductase can be used as an engineered strain to reduce the reducibility in the cytoplasm, thereby helping the recombinant protein expressed in the intracytoplasm to form correct disulfide bonds. The purpose of increasing the expression of soluble protein can be achieved by selecting an appropriate vector and host (KHODABAKHSH et al., 2013). In addition, the use of fusion tags to express and replace amino acids to increase the expression of soluble proteins is also a feasible method. KIM et al. used the method of amino acid substitution to successfully increase the expression level and activity of the soluble protein of α-1,3 fucosyltransferase mutants (KIM et al., 2017).

The chitinase gene in *S. sampsonii* has high homology with the chitinase genes of *Streptomyces albidoflavus*. In the study of *Streptomyces albidoflavus*, it was found that the fermentation broth of *Streptomyces albidoflavus* had a strong inhibitory effect on *Fusarium culmorum* (SKINNER, 1953). *Streptomyces albidoflavus* can produce the antifungal antibiotic antimycin A18, which can effectively inhibit the growth of a variety of pathogenic fungi and has the characteristics of high efficiency and low toxicity (YAN et al., 2010).

CONCLUSION

In this study, the recombinant chitinase from *Sschi61* was found to be toxic to the pathogen of black spot of *Eucommia ulmoides* (*Pestalotiopsis trachicarpicola*) and it could inhibit the black spot pathogen, after experiments. This experiment analysed the cloning and expression of the *S. sampsonii* chitinase gene *Sschi61*, and the chitinase gene family members of the *S. sampsonii* biocontrol gene were improved and supplemented, laying a theoretical foundation for the comprehensive analysis of the *S. sampsonii* chitinase gene family, supplemented the study of the chitinase gene of *S. sampsonii* KJ40. At the same time, a new idea of green prevention and treatment of black spot of *E. ulmoides* was also developed.

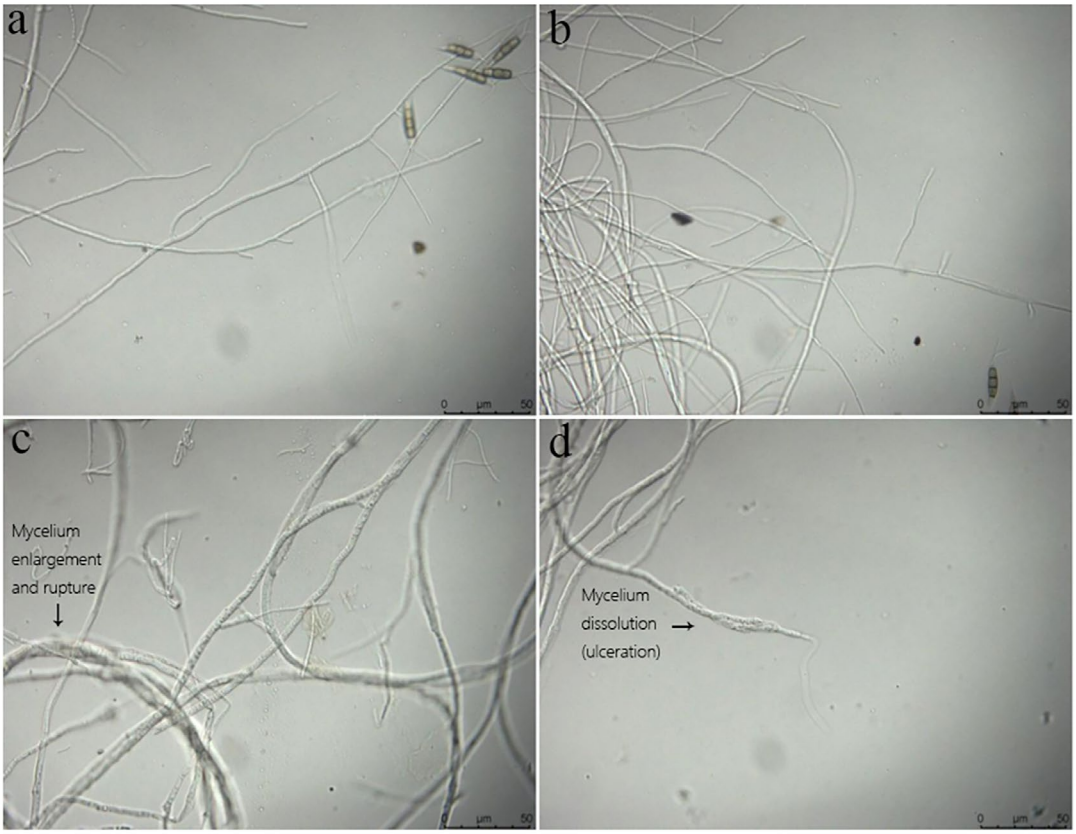

Figure 6 - Mycelium morphology.

Note: a and b: Normal mycelium; c: Mycelium enlargement and rupture; d: Mycelium dissolution (ulceration) same as figure 1.

ACKNOWLEDGEMENTS

This work was supported by the Foundation of Chinese National Science and Technology Platform Infrastructure (Grant No. 2005DK21207-13).

DECLARATION OF CONFLICT OF INTEREST

We have no conflict of interest to declare.

AUTHORS' CONTRIBUTIONS

Conceptualization: SWW, XMF and KGL. Data acquisition: SWW and KGL. Design of methodology and data analysis: SWW, XMF and KGL. SWW, XMF, SJL, SH and THZ prepared the draft of the manuscript. All authors critically revised the manuscript and approved of the final version.

REFERENCES

ABAD, P. et al. Genome sequence of the metazoan plant-parasitic nematode Meloidogyne incognita. **Nature Biotechnology**, v.26, p.909-915, 2008. Available from: <http://doi.org/10.1038/nbt.1482>. Accessed: Jul. 27, 2008. doi: 10.1038/nbt.1482.

ABDEL-BANAT, B. M. A. et al. Analysis of hydrolytic activity of a 65-kDa chitinase from the silkworm, Bombyx mori. **Biosci Biotechnol Biochem**, v.66, p.1119-1122, 2002. Available from: <https://www.tandfonline.com/doi/abs/10.1271/bbb.66.1119>. Accessed: May, 22, 2014. doi: 10.1271/bbb.66.1119.

ANDERSEN, O. A. et al. Natural product family 18 chitinase inhibitor. **Nat. Prod. Rep.**, v.22, p.563-579, 2005. Available from: <http://doi.org/10.1039/b416660b>. Accessed: Sept. 07, 2005. doi: 10.1039/b416660b.

ARAKANE, Y. et al. Insect chitinase and chitinase-like proteins. **Cell Mol. Life Sci.**, v.67, p.201-216, 2009. Available from: <https://link.springer.com/article/10.1007/s00018-009-0161-9>. Accessed: Oct. 09, 2009. doi: 10.1007/s00018-009-0161-9.

BROGUE, K. et al. Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani*. **Science**, v.254, p.1194-1197, 1991. Available from: <https://www.science.org/doi/abs/10.1126/science.254.5035.1194>. Accessed: Nov. 22, 1991. doi: 10.1126/science.254.5035.1194.

FLÄRDH, K. et al. *Streptomyces* morphogenetics: dissecting differentiation in a filamentous bacterium. **Nat. Rev. Microbiol**, v.7, p.36-49, 2009. Available from:<https://www.nature.com/articles/nrmicro1968>. Accessed: Jan., 2009. doi: 10.1038/nrmicro1968.

FUKUDA, Y. et al. Gene structure and expression of a tobacco endochitinase gene in suspension-cultured tobacco cells. **Plant Molecular Biology**, v.16, p.1-10, 1991. Available from: <https://link.springer.com/article/10.1007/BF00017912>. Accessed: Jan. 18, 1991. doi: 10.1007/BF00017912.

GAO, L. et al. Cloning, characterization and substrate degradation mode of a novel chitinase from *Streptomyces albolongus* ATCC 27414. **Food Chemistry**, v.261, p.329-336, 2018. Available from: <https://www.sciencedirect.com/science/article/pii/S0308814618306940>. Accessed: Sept. 30, 2018. doi: 10.1016/j.foodchem.2018.04.068.

HAMMAMI, I. et al. Partial purification and characterization of chi IO8, a novel antifungal chitinase produced by *Bacillus cereus* IO8. **Journal of Applied Microbiology**, v.115, p.358-366, 2013. Available from: <https://sfamjournals.onlinelibrary.wiley.com/doi/full/10.1111/jam.12242>. Accessed: May, 06, 2013. doi: 10.1111/jam.12242.

HARTL, L. et al. Fungal chitinases: diversity, mechanistic properties and biotechnological potential. **Applied Microbiology & Biotechnology**, v.93, p.533-543, 2012. Available from: <https://link.springer.com/article/10.1007/s00253-011-3723-3>. Accessed: Dec. 02, 2011. doi: 10.1007/s00253-011-3723-3.

HASANI, A. et al. *Streptomycetes*: characteristics and their antimicrobial activities. **Int. J. Adv. Biol. Biomed. Res.**, v.2, p.63-75, 2014. Available from: <https://studfile.net/preview/5194696>. Accessed: Dec. 02, 2016.

HIGGINBOTHAM, S. J. et al. Identification and characterisation of a *Streptomyces* sp. isolate exhibiting activity against methicillin-resistant *Staphylococcus* aureus. **Microbiological Research**, v.165, p.82-86, 2010. Available from: <https://www.sciencedirect.com/science/article/pii/S0944501309000020>. Accessed: Jan. 29, 2010. doi: 10.1016/j.micres.2008.12.004.

IGARASHI, Y. et al. Pteridic acids A and B, novel plant growth promoters with auxin-like activity from *Streptomyces hygroscopicus* TP-A0451. **The Journal of Antibiotic**, v.55, p.764-767, 2002. Available from: <https://www.jstage.jst.go.jp/article/antibiotics1968/55/8/55_8_764/_article/-char/ja/>. Accessed: May, 10, 2002. doi: 10.7164/antibiotics.55.764.

INBAR, J. et al. Evidence that chitinase produced by *Aeromonas caviae* is involved in the biological control of soil-borne plant pathogens by this bacterium. **Soil Biol. Biochem.**, v.23, p.973-978, 1991. Available from: <https://www.sciencedirect.com/science/article/abs/pii/003807179190178M>. Accessed: Mar. 20, 1991. doi: 10.1016/0038-0717(91)90178-m.

KASPRZEWSKA, A. Plant chitinases-regulation and function. **Cellular & molecular biology letters**, v.8, p.809-824, 2003. Available from: <https://pubmed.ncbi.nlm.nih.gov/12949620>. Accessed: Aug. 03, 2003.

KHAMNA, S. et al. Actinomycetes isolated from medicinal plant rhizosphere soils: diversity and screening of antifungal compounds, indole-3-acetic acid and siderophore production. **World Journal of Microbiology & Biotechnology**, v.25, p.649-655, 2009. Available from:<https://link.springer.com/article/10.1007/s11274-008-9933-x>. Accessed: Dec. 16, 2008. doi: 10.1007/s11274-008-9933-x.

KHODABAKHSH, F. et al. Cloning and Expression of Functional Reteplase in Escherichia coli TOP10. **Avicenna Journal of Medical Biotechnology**, v.5, p.168-175, 2013. Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3732866>. Accessed: Jan. 03, 2013.

KHOUSHAB, F. et al. Chitin research revisited. **Marine Drugs**, v.8, p.1988-2012, 2010. Available from: <https://www.mdpi.com/1660-3397/8/7/1988/htm>. Accessed: Jan. 28, 2010. doi: 10.3390/md8071988.

KIM, B. G. et al. **Helicobacter pylori*α*-1,3 fucosyltransferase gene and protein with improved soluble protein expression and activity, and thereof application for synthesis of α-1, 3 fucosyloligosaccharide**, 2017, Available from: <https://www.freepatentsonline.com/10336990>. Accessed: Jul. 02, 2019. Patent No.: 10336990 B2.

KIM, S. S. et al. Biological control of Root-knot nematode by *Streptomyces smpsonii* KK1024. **Korean Journal of Soil Science and Fertilizer**, v.44, p.1150-1157, 2011. Available from: <https://www.koreascience.or.kr/article/JAKO201120842650866.page>. Accessed: Dec. 31, 2011. doi: 10.7745/KJSSF.2011.44.6.1150.

KITO, M. et al. Purification and characterization of an ε-poly-L-lysine-degrading enzyme from an ε-poly-L-lysine-producing strain of *Streptomyces albulus*. **Archives of Microbiology**, v.178, p.325-330, 2002. Available from: <https://link.springer.com/article/10.1007/s00203-002-0459-6>. Accessed: Nov. 18, 2002. doi: 10.1007/s00203-002-0459-6.

LEICHERT, L. I. et al. Protein thiol modifications visualized *in vivo*. **PLOS Biology**, v.2. e333, 2004. Available from: <https://journals.plos.org/plosbiology/article?id=10.1371/journal.pbio.0020333>. Accessed: Oct. 05, 2004. doi: 10.1371/journal.pbio.0020333.

LI, S. J. et al. Cloning and Expression of the Chitinase Encoded by *ChiKJ406136* from *Streptomyces Sampsonii* (Millard & Burr) Waksman KJ40 and Its Antifungal Effect. **Forests**, v.9, p.699, 2018. Available from: <https://www.mdpi.com/1999-4907/9/11/699/htm>. Accessed: Nov. 11, 2018. doi: 10.3390/f9110699.

LIU, S. R. et al. Biological characteristics of pathogen causing *Polygonatum sibiricum* brown leaf spot. **Mycosystema**, v.38, p.768-777, 2019. Available from: <https://manu40.magtech.com.cn/Jwxb/CN/10.13346/j.mycosystema.180333>. Accessed: Nov. 07, 2019. doi: 10.13346/j.mycosystema.180333.

LU, Y. et al. Expression and characterization of a novel chitinase with antifungal activity from a rare actinomycete, *Saccharothrix yanglingensis* Hhs. 015. **Protein Expression & Purification**, v.143, p.45-51, 2018. Available from: <https://www.sciencedirect.com/science/article/pii/S1046592817303923>. Accessed: Nov. 05, 2017. doi: 10.1016/j.pep.2017.10.013.

MAGNIN-ROBERT, M. et al. Biological control of *Botrytis cinerea* by selected grapevine-associated bacteria and stimulation of chitinase and *β*-1,3 glucanase activities under field conditions.

Eur. J. Plant Pathol., v.118, p.43-57, 2007. Available from: <https://link.springer.com/article/10.1007/s10658-007-9111-2>. Accessed: Feb. 21, 2007. doi: 10.1007/s10658-007-9111-2.

MERZENDORFER, H. et al. Chitin metabolism in insects: structure, function and regulation of chitin synthases and chitinases. **Journal of Experimental Biology**, v.206, p.4393-4412, 2003. Available from: <https://journals.biologists.com/jeb/article/206/24/4393/34291/Chitin-metabolism-in-insects-structure-function>. Accessed: Dec. 15, 2003. doi: 10.1242/jeb.00709.

MOHAMMADZADEH, R. et al. Expression of chitinase gene in BL21 pET system and investigating the biocatalystic performance of chitinase-loaded AlgSep nanocomposite beads. **International Journal of Biological Macromolecules**, v.104, p.1664-1671, 2017. Available from: <https://www.sciencedirect.com/science/article/pii/S0141813016326745>. Accessed: Mar. 24, 2017. doi: 10.1016/j.ijbiomac.2017.03.119.

NAKABACHI, A. et al. Chitinase-like proteins encoded in the genome of the pea aphid, *Acyrthosiphon pisum*. **Insect Molecular Biology**, v.19, p.175-185, 2010. Available from:<https://resjournals.onlinelibrary.wiley.com/doi/full/10.1111/j.1365-2583.2009.00985.x>. Accessed: Feb. 23, 2010. doi: 10.1111/j.1365-2583.2009.00985.x.

OELSCHLAEGER, P. et al. Identification of factors impeding the production of a single-chain antibody fragment in Escherichia coli by comparing in vivo and in vitro expression. **Applied Microbiology Biotechnology**, v.61, p.123-132, 2003. Available from:<https://link.springer.com/article/10.1007/s00253-002-1190-6>. Accessed: Jan. 28, 2003. doi: 10.1007/s00253-002-1190-6.

PAN, Y. et al. Molecular cloning, Expression and Characterization of *BmIDGF* Gene from *Bombyx mori*. **Zeitschrift fur Naturforschung C**, v.65, p.277-283, 2010. Available from: <https://www.degruyter.com/document/doi/10.1515/znc-2010-3-417/html>. Accessed: Jan. 02, 2014. doi: 10.1515/znc-2010-3-417.

QI, M. et al. *Pestalotiopsis trachicarpicola*, a novel pathogen causes twig blight of *Pinus bungeana* (Pinaceae: Pinoideae) in China. **Antonie van Leeuwenhoek**, 2020. Available from: <https://link.springer.com/article/10.1007/s10482-020-01500-8>. Accessed: Nov. 27, 2020. doi: 10.1007/s10482-020-01500-8.

RASMUSSEN, U. et al. Cloning and character- ization of a pathogen-induced chitinase in *Brasica napus*. **Plant Molecular Biology**, v.20, p.277-287, 1992. Available from: <https://link.springer.com/article/10.1007/BF00014495>. Accessed: Oct., 1992. doi: 10.1007/bf00014495.

REIS, S. A. et al. FK506, a secondary metabolite produced by *Streptomyces*, presents a novel antiviral activity against Orthopoxvirus infection in cell culture. **Journal of Applied Microbiology**, v.100, p.1373-1380, 2006. Available from: <https://sfamjournals.onlinelibrary.wiley.com/doi/full/10.1111/j.1365-2672.2006.02855.x>. Accessed: Apr. 21, 2006. doi: 10.1111/j.1365-2672.2006.02855.x.

ROBY, D. et al. Activation of a bean chitinase promoter in transgenic tobaco plants by phytopathogenic fungi. **The Plant Cell**, v.2, p.999-1007, 1990. Available from:<https://doi.org/10.1105/tpc.2.10.999>. Accessed: Oct. 1, 1990. doi: 10.2307/3869239.

SAMAC, D. A. et al. Isolation and characterization of the genes encoding basis and acidic chiti- nase in *Arabidopsis thaliana*. **Plant Physiology**, v.93, p.907-914, 1990. Available from: <https://doi.org/10.1105/tpc.2.10.999>. Accessed: Oct. 01, 1990. doi: 10.1104/pp.93.3.907.

SHU, J. et al. Identification and characterization of pestalotioid fungi causing leaf spots on mango in southern China. **Plant disease**, v.104, p.1207-1213, 2020. Available from: <https://apsjournals.apsnet.org/doi/full/10.1094/PDIS-03-19-0438-RE>. Accessed: Feb. 15, 2020. doi: 10.1094/PDIS-03-19-0438-RE.

SKINNER, F. A. Inhibition of *Fusarium culmorum* by S*treptomyces albidoflavus*. **Nature**, v.172, p.1191, 1953. Available from:<https://www.nature.com/articles/1721191a0>. Accessed: Dec. 26, 1953. doi: 10.1038/1721191a0.

SUMA, K. et al. Chitinase A from *Stenotrophomonas maltophilia* shows transglycosylation and antifungal activities. **Bioresource Technology**, v.133, p.213-220, 2013. Available from: <https://www.sciencedirect.com/science/article/pii/S0960852413001260>. Accessed: Jan. 29, 2013. doi: 10.1016/j.biortech.2013.01.103.

TAKAHASHI, M. et al. A new chitinase-related gene, *BmChiR1*, is induced in the *Bombyx mori* anterior silk gland at molt and metamorphosis by ecdysteroid. **Insect Biochem. Mol. Biol.**, v.32, p.147-151, 2002. Available from: <https://www.sciencedirect.com/science/article/abs/pii/S0965174801001023>. Accessed: Dec. 17, 2001. doi: 10.1016/s0965-1748(01)00102-3.

TOYODA, H. et al. Suppression of the powdery mildew pathogen by chitinase microinjected into barley coleoptile epidermal cells. **Plant Cell Reportsi**, v.10, p.217-220, 1991. Available from: <https://link.springer.com/article/10.1007/BF00232561>. Accessed: Aug. 18, 1991. doi: 10.1007/BF00232561.

XIAO, C. et al. Synthesis and properties of degradable poly (vinyl alcohol) hydrogel. **Polymer Degradation & Stability**, v.81, p.297-301, 2003. Available from: <https://www.sciencedirect.com/science/article/pii/S0141391003001009>. Accessed: Jun. 11, 2003. doi: 10.1016/S0141-3910(03)00100-9.

YAN, L. L. et al. Antimycin A_{18} produced by an endophytic *Streptomyces albidoflavus* isolated from a mangrove plant. **The Journal of Antibiotics**, v.63, p.259-261, 2010. Available from: <https://www.nature.com/articles/ja201021>. Accessed: Mar. 19, 2010. doi: 10.1038/ja.2010.21.

YOKOYAMA, K. et al. Properties and application of microbial transglutaminase. **Applied Microbiology & Biotechnology**, v.64, p.447-454, 2004. Available from: <https://link.springer.com/article/10.1007/s00253-003-1539-5>. Accessed: Jan. 22, 2004. doi: 10.1007/s00253-003-1539-5.

ZHANG, X. et al. Biological characteristics of *Pestalotiopsis trachicarpicola*, a pathogen of tea gray blight. **Guizhou Agricultural Sciences**, v.49, p.38-43, 2021. Available from: <http://doi.org/10.3969/j.issn.1001-3601.2021.01.007>. Accessed: Jan. 14, 2021. doi: 10.3969/j.issn.1001-3601.2021.01.007.

ZHANG, Y.M. et al. Advances in Promoting Soluble Expression of Recombinant Protein in *Escherichia coli*. **China Biotechnology**, v.36, p.118-124, 2016. Available from: <https://www.cnki.net/kcms/doi/10.13523/j.cb.20160517.html>. Accessed: Dec. 22, 2015. doi: 10.13523/j.cb.20160517.

ZHANG, Z. et al. The Role of Chitinase Production by *Stenotrophomonas maltophilia* Strain C3 in Biological Control of *Bipolaris sorokiniana*. **Phytopathology**, v.90, p.384-389, 2000. Available from: <https://apsjournals.apsnet.org/doi/abs/10.1094/PHYTO.2000.90.4.384>. Accessed: Feb. 22, 2007. doi: 10.1094/PHYTO.2000.90.4.384.