



Isolation, Characterization, and Insecticidal Activity of Chitinase Gene (*chiA*) From *Aspergillus niger* (EM77)

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ABSTRACT

Chitin, which is found in the structure of insect cuticles and peritrophic membranes, is degraded by the chitinase enzymes. The degradation decreases the feeding and defenses of the insect and weakens it. Therefore, chitinases are important enzymes that have potential for being used as biological control agents against harmful insects. In the present study, genes encoding exo-chitinases (e.g. *chiA*) have been isolated from *A. niger*. The partial sequence includes 1484 bp open reading frame encodes for 493 amino acid. No intron was found in the *chiA* of *A. niger* (EM77). The deduced amino acid sequence of *chiA* of *A. niger* (EM77) clearly indicated the presence of three conserved domains: ChtBD1 superfamily, chitin recognition protein, and Glyco-18 domain. Chitinase enzyme was partially-purified from the culture filtrate of *A. niger* (EM77) and was fractionated using ethanol concentrations (25-75%). Thus, the molecular mass of the semi-purified chitinase enzyme (~ 30 kD) was observed after purification compared with unpurified total protein. So, the molecular weight of the protein seemed to be unusual from other fungal chitinases which are around 50-70 kD. The partially-purified chitinase was evaluated as the highest activity (42.39 Units/ml) 0.5 g/l of the chitin substrate concentration at 30°C for six days. Insecticidal activity of partially-purified *A. niger* EM77 chitinase has been estimated against *S. littoralis* after 96 hours and the mortality rate of neonate larvae was 38.9%. That was because of the ability of chitinase to disrupting the peritrophic membrane of insect cells. So, chitinase producing ability of *A. niger* (EM77) could be serving as an effective biopesticide to control harmful pests which destroy importantly economic crops instead of hazardous chemical pesticide.

INTRODUCTION

Chitin, an insoluble homopolymer of β -(1, 4)-linked N-acetyl-D-glucosamine (GlcNAc), is an important component of exoskeletons (insects), crustacean shells (marine living) and fungal cell (Brine and Austin, 1981). After cellulose, chitin is the second most abundant component of biomass in nature (Zainol Abidin *et al.*, 2020). Due to the presence of chitinolytic enzymes (chitinases), chitin does not accumulate in the environment and can be degraded to GlcNAc short monomers (Abo Elsoud & El Kady, 2019). Chitinases play significant physiological and ecological roles in chitin metabolism (Souza *et al.*, 2011). Also, it has an important role in the bio-control of pathogenic fungi and insect control as a pesticide (Hamid *et al.*, 2013). Chitinases can be found in a wide range of organisms, including bacteria, fungi, plants, insects and vertebrates (Hamid *et al.*, 2013). Many microorganisms produce chitinase to feed on chitin as a carbon and energy source (Rathore & Gupta, 2015). Some bacterial strains (e.g. *Serratia* and *Bacillus*) are known to produce four different types of chitinases. However, most of the filamentous fungi have been recorded to produce about 20 different chitinases with higher activity levels than those of plants and bacteria (Hartl *et al.*, 2012). Many fungi produce chitinase enzyme to degrade the substrate as a defense mechanism, as well as to gather nutrients for their survival under adverse environmental conditions (da Silva *et al.*, 2005). *Aspergillus* species is a dominant and most potent species for chitinase production. Amongst, *Aspergillus terreus* and *A. niger* (LOCK62) were found to be the efficient source of chitinase production having various applications (Brzezinska & Jankiewicz, 2012). Mainly, fungi have several chitinase genes encoding enzymes with diverse chitinase activities (Ramaiah *et al.*, 2000). Chitinase genes from several fungi species have been characterized (Danışmazoğlu *et al.*, 2015). Genes encoding chitinase enzymes have been cloned from a wide

range of yeasts and filamentous fungi (Jaques *et al.*, 2003). All cloned chitinase enzymes belong to the glycohydrolase family18 based on the percentage of similarity to family18 (Zhong *et al.*, 2015); two distinct classes can be identified chitinases from plants or bacteria. Chitinase genes have been cloned and characterized from many microorganisms, including *Trichoderma artroviridae* (Takaya *et al.*, 1998), *Aspergillus fumigatus* (Xia *et al.*, 2001), *Paecilomyces* sp (Dong *et al.*, 2007), and *Aspergillus nidulans* (Shin *et al.*, 2009),

Spodoptera littoralis are serious pests of forest or agricultural crops throughout the world. Chemical insecticides that have a variety of side effects on the environment have been used for several years against these pests. Because of their hazardous effects, these chemicals are no longer recommended for agricultural pest management (Abdel-Aziz *et al.*, 2013). Chitinase is able to effectively break down glycosidic bonds of chitin polymer in the content of Insects' peritrophic membranes (PM) and allow the passage of all kinds of components found in the midgut through this barrier. In this way, some bacteria and their toxic components reach the endothelial cells of the midgut and become a threat to insect life (Bahar *et al.*, 2012). Consequently, any defect in PM reduces feeding and protection against microbial attack (Langer *et al.*, 2000).

In the current study, an assessment of chitinase producing ability of *A. niger* (EM77) was performed, gene and amino acid sequences were analyzed against other reported chitinase sequences deposited into the NCBI. To the best of our knowledge, this research is the first to isolate chitinase from *A. niger*. Furthermore, an insecticidal assay of *A. niger* chitinase against *S. littoralis* larvae was measured.

MATERIALS AND METHODS

Fungal Source and Growth Conditions:

A previously isolated and identified *Aspergillus niger* (EM77) (Accession no. KF774181) was maintained on potato dextrose agar (PDA) medium at 30°C for six

days in a rotary shaker (200 rpm) with a daily cycle consisting of 15 h of light and 9 h of darkness and it was preserved at -80°C in 50% (v/v) glycerol.

Preparation of Fungal Expressed Chitinase:

Fungal mycelia of *A. niger* (EM77) were grown on chitinase inducing medium containing ground chitin (0.5 g/l) in basal salts medium following the procedure of Wang *et al.* (2013).

RNA Isolation:

Grown mycelia were harvested flash-frozen in liquid nitrogen then ground to a fine powder in a porcelain mortar. Total RNA was isolated from homogenized fungal mycelia using the RNeasy® Mini Kit (Qiagen, Netherlands). Concentrations of RNA were determined using the Nanodrop ND-1000 (Nanodrop). First-strand cDNA was prepared using 1 μg of RNA, and following the manufacturer's instructions of SuperScript® II system (Invitrogen) including the reverse transcriptase and the oligo-dT primer.

Specific primers *chiF*, 5'-ATGTCAGTCATTTTCGGCATTTC-3', and *chiR*, 5'-TCATGGTACCATCATCCAGA-3' were designed to amplify the open reading frame of the 5' termini of chitinase gene. The primers were designed according to the data derived by the automatic computational analysis of the annotated genomic sequence of *A. niger* chitinase that was accessioned as XM-001396587.

PCR was conducted using the Go Taq DNA polymerase (Promega) in a thermocycler machine following the stepwise condition for; 30 cycles, as denaturation step for 60 sec. at 94°C , annealing step for 90 sec. at 55°C , extension step for 60 sec. at 72°C , and a final extension step for 7 min of 70°C . The amplified fragment was separated by electrophoresis on 1.0% agarose gel stained with ethidium bromide (0.5 $\mu\text{g}/\text{ml}$) and was visualized on a UV gel documentation system (BioRad, USA). The amplicon was excised from the agarose gel then purified from unincorporated PCR primers and

dNTPs using the QIAquick PCR purification kit following the manufacturer's instructions (Qiagen, Netherlands). The sequence of *A. niger* chitinase fragment was sent for sequencing to LGC Genomics GmbH on a 3730xl DNA Analyzer (Applied Biosystems™/Thermo Fisher Scientific). Then it was BLAST searched on the National Center for Biotechnology Information (NCBI) database and compared with the obtained sequence on the Sequence® Software. Sequencing runs were performed at the Chitinase gene (*chiA*) of *A. niger* (EM77) was submitted to the NCBI database (accession No. MH878942).

Sequence Alignments and Phylogenetic Analysis:

Sequence of chitinase gene (*chiA*) which obtained for our isolate *A. niger* (EM77) was compared to other sequences of other *Aspergillus* species deposited to the NCBI GenBank using the "BLASTN" and "BLASTX" tools at the National Center for Biotechnology Information website (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Alignment of nucleotide and deduced amino acid sequences with selected Chitinase genes from other *Aspergillus* species were done using T-Coffee Multiple Sequence Alignment Tools (<http://www.tcoffee.org/Projects/tcoffee/index.html#DOWNLOAD>). Box shading of conserved regions was applied using the sequence manipulation suite (http://www.bioinformatics.org/sms2/color_align_cons.html). The phylogenetic tree was constructed based on the amino acid sequences using the neighbor-joining method of MEGA X software Kumar *et al.* (2016); www.megasoftware.net). The conserved domains within the amino acid or the coding nucleotide sequence were detected using the website of <https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>.

Exochitinase Crude Enzyme Production:

A culture of *A. niger* (EM77) was grown on a basal medium broth (BM) supplemented with chitin as the following (g/l): chitin 0.5; K_2HPO_4 , 3.0; yeast extract,

3.5; peptone, 2.0; KCL, 3.0; NaNO₃, 3.0, MgSO₄, 0.5; FeSO₄ 0.01 (pH 6.2) at 30°C for six days with a daily cycle consisting of 15 h of light and 9 h of darkness. Then 50 ml of the BM broth were inoculated with one ml of spore suspension approximately equal (1×10^6 CFU/ml). The inoculated flasks were incubated at 30 °C for six days in a rotary shaker (200 rpm). At the end of the incubation period, the cultures were centrifuged for 15 min. using a cooling centrifuge. The culture filtrate was used as the crude enzyme.

Exochitinase Partial Purification:

Exochitinase of *A.niger* (EM77) was fractionated using ethanol concentrations (25-75%). Each fraction has produced a precipitate that was obtained by centrifugation at 10000 xg, for 15 min. at 4°C then assayed for enzymatic activity.

Exochitinase Activity Assay:

Exochitinase activity was determined following the method of Matsumoto et al (2004) using the chromogenic substrate p-nitrophenyl-β-D-N-acetylglucosaminide (PNP-β-GlcNAc) as a substrate. One unit of the enzyme activity was defined as the amount of enzyme releasing 1 μmol of p-nitrophenol per minute under the specified assay conditions.

Polyacrylamide Gel Electrophoresis:

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) technique (Laemmli 1970) was performed at a 12% concentration to determine the molecular mass of the exochitinase enzyme that was fractionated with the highest purification fold. The standard protein marker contained a mixture of 11

known proteins that were resolved specific bands with known molecular weight ranged between 10 and 175 kDa.

Insecticidal Activity:

The insecticidal effect using surface contamination bioassay of partially- purified chitinase was applied to the first-instar larvae of the Egyptian cotton leafworm (*Spodoptera littoralis*). Briefly, castor bean leaves (*Ricinus communis*) were soaked with partially purified exo-chitinase as 0.5 and 1.0 ml of partially -purified enzyme (42.39 Units/ml). The assay was performed in cups of artificial diet. For the control treatment, water has substituted the exo-chitinase doses in the artificial diet. Three replicates were done for each treatment, where each replicate has contained 30 larvae. The cups were incubated at 26 ± 1 °C, 60 % humidity, and a 12 h light/dark photoperiod (El-Defrawi et al., 1964 and El-barky *et al.*, 2008). Mortality was monitored daily for seven days after treatment by counting the number of dead larvae.

RESULTS

Characterization of Exo-Chitinase cDNA and Sequence Analysis:

A fragment at the size of 1484 bp was amplified from the cDNA synthesized from the RNA of the *A. niger* (EM77) (Fig. 1). The obtained fragment was sequenced and identified as a partial sequence of the chitinase gene using the BLASTx and BLASTn tools of the NCBI GenBank database. The exo-chitinase fragment could not be produced when chitin was not supplemented in the growing medium of *A. niger* (EM77).

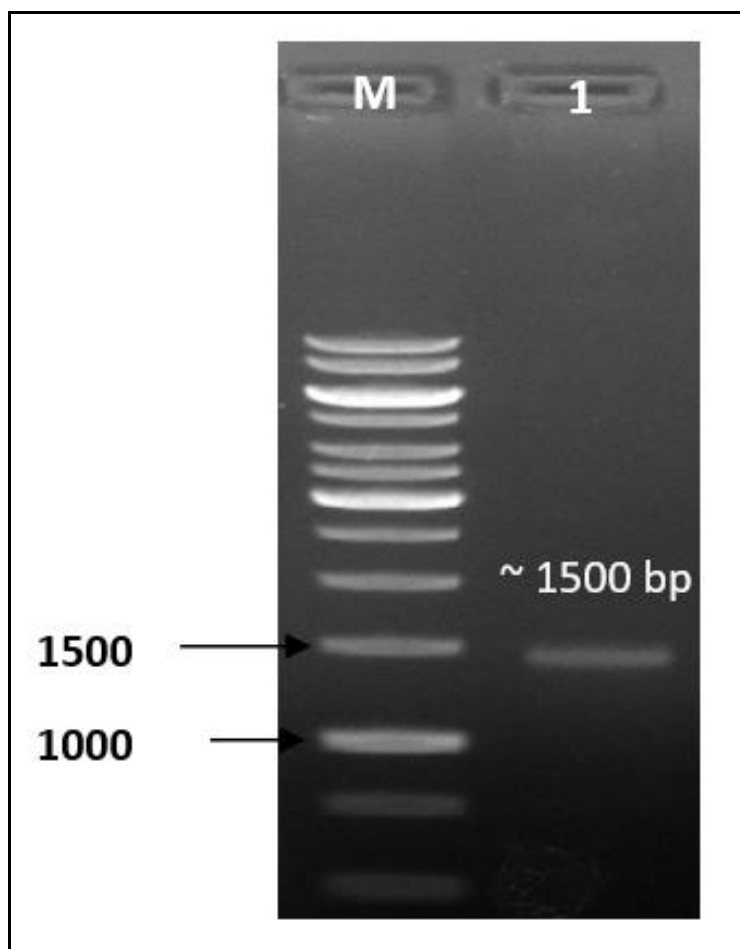


Fig. 1: An ethidium bromide stained gel of PCR amplification of *chiA* gene from *A. niger* (EM77). Lane M 1 Kb marker, lane 1 ~ 1484 bp amplified fragment of *chiA* gene.

The nucleotide sequence of the obtained fragment has deduced an ORF of 1484 bp and 493 of amino acid as shown in Fig. (2). No introns were detected in the nucleotide sequence of *chiA* of *A. niger* (EM77). Further analysis of the deduced amino acid sequence of *chiA* of *A. niger* (EM77) has clearly indicated three conserved domains. The first domain was allocated between the amino acids numbered 53 and 94 (42 – aa), which corresponds to ChtBD1

super-family with e-value of 6.67e-09. The second domain was allocated between the amino acids numbered 112 and 134 (20 aa), which corresponds to the chitin recognition protein with e-value 1.17e-09. The third domain was allocated between the amino acids numbered 150 and 491 (440-aa), which corresponds to the Glyco-18 domain (Fig. 2). The third domain is proof that the *chiA* of *A. niger* (EM77) belongs glycosyl hydrolase family18 of chitinases.

| | |
|------|---|
| 1 | atgtcagtcatttcggcattttctgctctgcctgttcgggtggcaactgacttcacagcgt |
| | <u>M S V I S A F L L C L F G W Q L T S S A</u> |
| 61 | gtcttcgcgcaccaatctcgtctggggagctcttcocgtccaatctcgaccctgtctatcct |
| | <u>V F A H N L A W G A L P S N L D P V Y P</u> |
| 121 | ttccaatttctttacttcttctgctagtgatgggtacacctgtgccaagaag |
| | <u>F Q F L S T S F A S D D ¹G Y T C A K D K</u> |
| 181 | ggatgtaagataggatgctgcgaccattgactccactggctcgggttattgtggtttt |
| | <u>G C K I G C C G P I D S T G S G Y C G F</u> |
| 241 | ggccccgagttctgcggaataattgcacctcgaactcgcgatcgcaagagcagtggtgac |
| | <u>G P E C G K N C T S N C D R K S E C D</u> |
| 301 | cctggctgggggaagaatggctcaagcatccactgttcocgtaaatgtttgtgtgac |
| | <u>P G W G K E W S Q A S ²T C P L N V C C S</u> |
| 361 | aagttcggcttctgcggaaccaccaaggacttctgtggcgatgccaccgtcgtgtctccc |
| | <u>K F G E C G T T K D C G D A T V V S P</u> |
| 421 | caatgcagcggcaccagtgccaaagggcggacagtcggatactacgaaggggtggaatttg |
| | <u>Q C S G T S A K G ³R T V G Y Y E G W N L</u> |
| 481 | gaacgtccatgtggaataatgggtccacctgatatccccctcggctactatacacacatc |
| | <u>E R P C G K M G P P D I P L G Y Y T H I</u> |
| 541 | aaactacggttcgcttccatogaccctcagacattcccgcatcgcctcccattggacaatctc |
| | <u>N Y A F A S I D P Q T F R I A P M D N L</u> |
| 601 | acagcgtcattgtaccaagggcgaacagcactcaagcgccaacagagtcattctcgaggtg |
| | <u>T A S L Y Q G V T A L K R Q Q S H L E V</u> |
| 661 | tggattgccattgggggatgggcattcaatgaccgggtccgaccggaaccatttctct |
| | <u>W I A I G G W A F N D P G P T R T T F S</u> |
| 721 | gacctggcgttccgaggctgctcaggatacattcttcggatcccttgattagctttatg |
| | <u>D L A A S E A A Q D T F F G S L I S F M</u> |
| 781 | cagaagaatcggtttgggttagatattgactgggaataacctgtgaccgcaagcagc |
| | <u>Q K N R F D G V D I D W E Y P V T D E R</u> |
| 841 | ggtgggagaccgagattatgtcaattttgtgactttgggtgaaaagactcagagaacag |
| | <u>G G R P E D Y V N F V T L V K R L R E Q</u> |
| 901 | ttgaatcagacggggagaagtatgggatctccatcactttgocgacatcatacgggtac |
| | <u>L N Q T G R K Y G I S I T L P T S Y W Y</u> |
| 961 | ctgagaggattcgcacattgtcaatctcagcgcgatgtcgatttccttaacgtcatgtct |
| | <u>L R G F D I V N L E P H V D F L N V M S</u> |
| 1021 | tatgatattcagggcacttgggactctacgaacaagcagctcggaccgtacgccttcgca |
| | <u>Y D I H G T W D S T N K Q L G P Y A F A</u> |
| 1081 | cataccaatctaacggagattaacagcgcctctagaacttctctggcgcaataacatcaat |
| | <u>H T N L T E I N S A L E L L W R N N I N</u> |
| 1141 | ccagagagagttaaccttggactaggtttctacgggtcagtttcacaaatgaagaatccc |
| | <u>P E R V N L G L G F Y G R S F T M K N P</u> |
| 1201 | gggtgcaagaagcctggctgtgagttctctggaggggcacgggcaggtgaatgtaccggc |
| | <u>G C K K P G C E F S G G A R A G E C T G</u> |
| 1261 | acaccggcgtgttggctacgtatgaggtcaacaagatcattcagaagggcgccgacag |
| | <u>T P G V L A T Y E V N K I I Q K G G T E</u> |
| 1321 | atgacctggcaaaagcgcagcagttaccatcgtaacctgggacaaggatcaatgggtt |
| | <u>M T L D K D A A V T I V T W D K D Q W V</u> |
| 1441 | tccattgataaccaagagacactacggatgaagatggactatgccataagcggctcct |
| | <u>S I D N Q E T L R M K M D Y A N K R C L</u> |
| 1501 | gggggaactatgggtctgggcaatagatctggatgatggtaccat |
| | <u>G G T M V W A I D L D D G T</u> |

Fig. 2: Nucleotide sequence of chitinase A gene (*Chi A*), its translation from an ATG startcodon (GenBank accession number MH878942). The deduced amino acid sequence is shown in one-letter code under the ORF. The three conserved domains of fungal chitinase are underlined. Domain no. 1 is ChtBD1 super family (from 53 – 94), domain no. 2 is Chitin recognition protein (from 112 - 134) and domain no. 3 is Glyco_18 domain (from 150 – 491). carbohydrate binding site (chemical binding) is shown in bold and highlighted amino acid letters.

Phylogenetic Analysis:

Phylogenetic analysis of *A. niger* (EM77) chitinase (*chiA*) partial protein sequence with respect to chitinase genes sequences of other *Aspergillus* species (*A. niger* XP_001396624, *A. awamori* GCB27978, *A. neoniger*, XP_025483904, *A. piperis* XP_025521389, *A. kawachii*

GAA86292, *A. homomorphus* XP_025553913, *A. candidus* XP_024670775 and *A. campestris* XP_024688746) was shown in (Fig.3). At the amino acid level, the *A. niger* (EM77) chitinase (*chiA*) sequence has produced a 100 % similarity sequence to *A. niger* (Acc. No. XP_001396624), 95% to that of *A. awamori*, *A. neoniger* and *A.*

piperis (Acc. No. GCB27978, (XP_025483904), *A. piperis* (XP_025521389) and *A. kawachii* (GAA86292) were closer to each other and clustered together in a different clade. Chitinase genes from *A. candidus* (XP_024670775) and *A. campestris* (XP_024688746) were closer to each other than other chitinases and clustered together in a third clade (Fig.3).

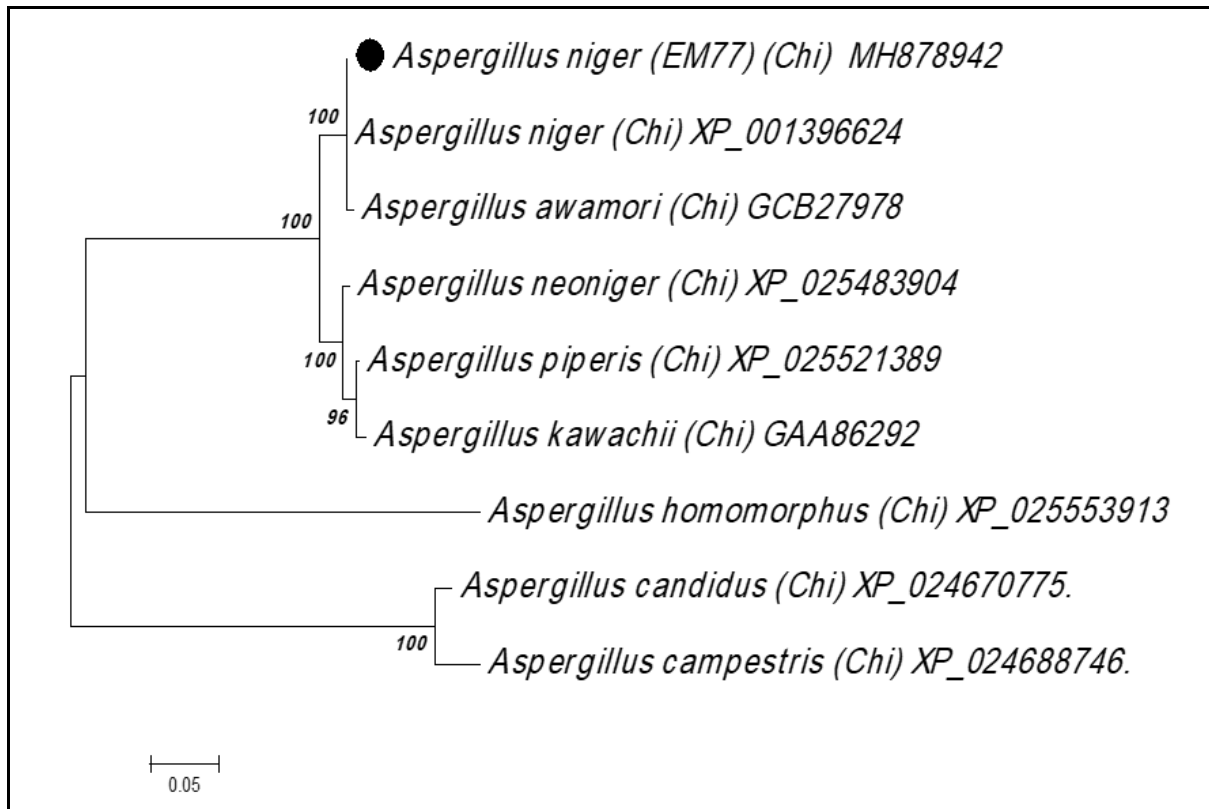


Fig. 3: Phylogenetic analysis of *A. niger* (EM77) chitinase (*chi*) partial protein sequence with *chi* genes from selected *Aspergillus* species. Tree was constructed with Neighbor-joining method using MEGA X software. The analysis involved 493 amino acid sequences. All positions containing gaps and missing data were eliminated. Alignment of the sequences was done with CLUSTALW, bootstrap values (in percent) are calculated from 1000 resampling.

Exo-Chitinase Activity and Molecular Weight:

The chitinase activity was evaluated based on the ethanol fractions methods of Matsumoto *et al.* (2004). Fractions obtained at 50 and 75% of ethanol have scored the heights purification folds 2.9 and 3.4-fold

respectively, and were collected together. Also, the maximum activity was observed by using the basal media supplemented with 0.5 g/l chitin then incubates at 30°C for six days. The protein concentration was 42.39 units/ml.

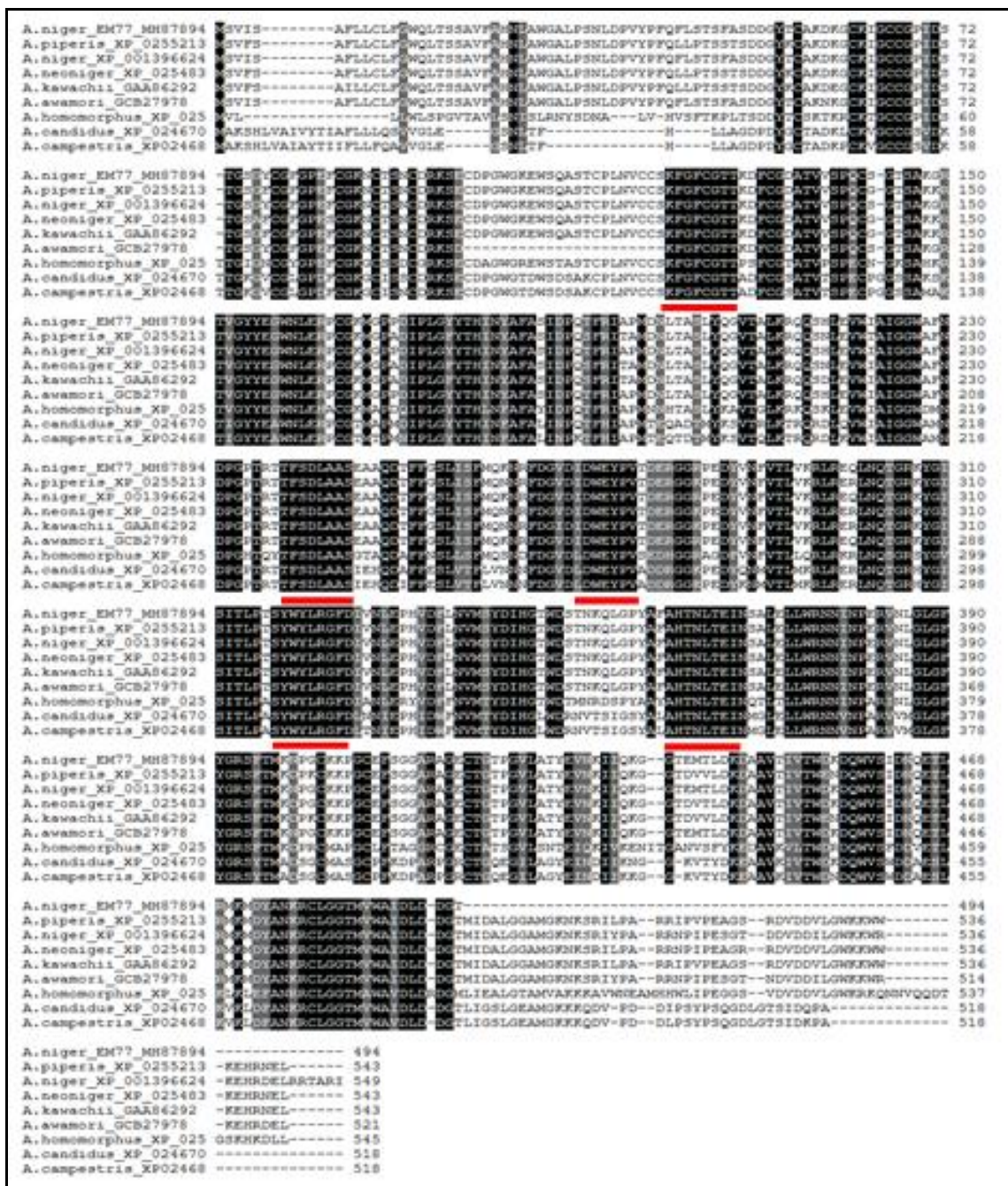


Fig. 4: Multiple amino acid sequence alignment of *A. niger* (EM77) chitinase (*chi*) with some selected chitinase genes from other *Aspergillus* species showing the highly conserved signature motifs (red underlined). Alignment was done with T-Coffee Multiple Sequence Alignment Tools: <http://www.tcoffee.org/Projects/tcoffee/index.html#DOWNLOAD>. Box shading of conserved regions done using Sequence Manipulation Suite: http://www.bioinformatics.org/sms2/color_align_cons.html.

Protein Electrophoresis Analysis:

From the SDS-PAGE analysis, the purified protein of chitinase enzyme was

observed at the molecular weight of ~ 30 kD in comparison to the with crud and purified protein (Fig. 5).

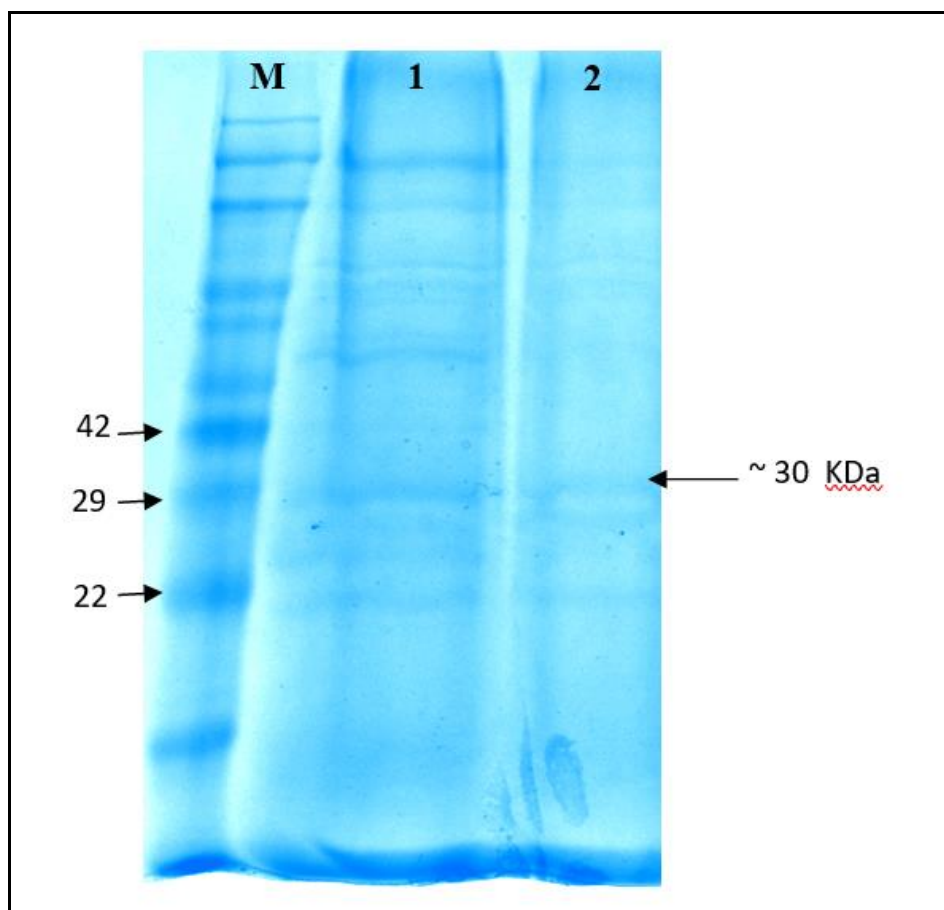


Fig. 5: SDS-PAGE analysis of the crud and semi-purified protein. Lane M: pre-stained protein marker; Lane 1: total protein of *A. niger* (EM77) cells; Lane 2: semi-purified chitinase protein (~30 KDa).

Bioassay of Insecticidal Activity:

Different concentrations of partially-purified *A. niger* EM77 chitinase were tested for its efficiency on first instar *Spodoptera littoralis* larvae. Death of *S. littoralis* larvae has begun after 48 h. using only 1.0 ml of (42.39 Units/ml) partially -purified chitinase.

The highest mortality rate was after 96 hours (hrs). The mortality rates were increased from 48 to 96 hrs post-treatment. Noticeable mortality rates were 6.7 %, 23.3 % and 38.9% at 48, 72, and 96 hrs respectively (Table 1, and Fig.6).

Table 1: The mortality % of semi-purified *A. niger* EM77 chitinase treated larvae of *S. littoralis*

| Mortality Time (hrs.) | Treatments | | | |
|--------------------------|--------------------|-------------------------------------|-------------------------------------|-----------|
| | Water (Control) | 0.5 ml (42.39 U/ml chitinase) | 1.0 ml (42.39 U/ml chitinase) | P value |
| 48 | 0 ± 0.0 | 2.0 ± 1.15 | 13.30 ± 1.20 | 0.004 ** |
| 72 | 0.33 ± 0.3 | 14.0 ± 2.08 | 23.33 ± 2.96 | 0.007 ** |
| 96 | 0.67 ± 0.3 | 19.0 ± 0.58 | 33.33 ± 2.02 | <0.001 ** |

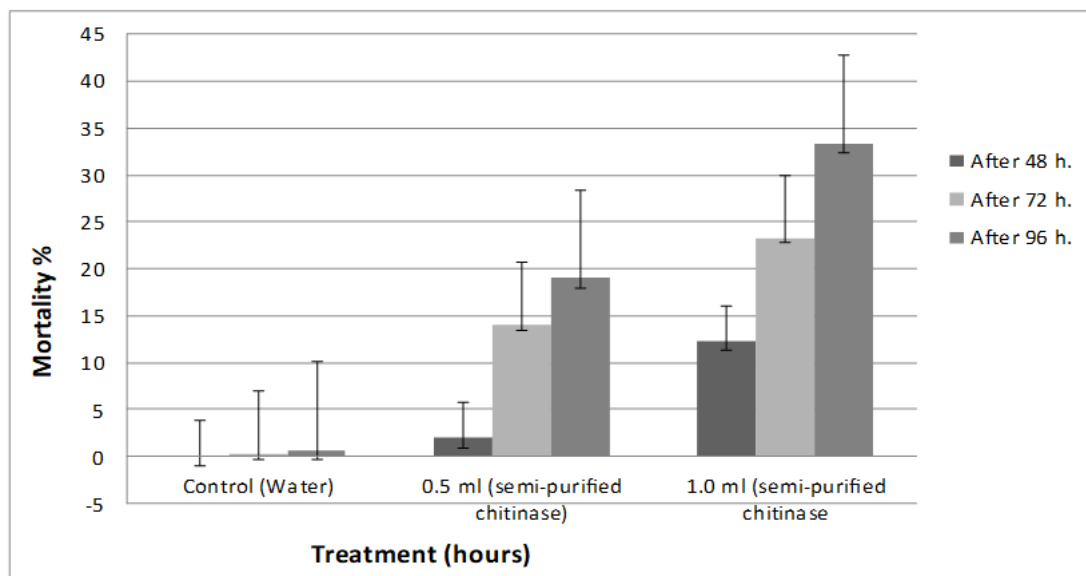


Fig.6: The mortalities rates of *S. littoralis* at different time post *A. niger* EM77 chitinase treatments.

DISCUSSION

Chitin oligosaccharide exists naturally in many organisms including exteriors of insects and fungi. Upon degradation of chitin by a number of organisms, severe damage and even death may occur in pathogens and pests whose external surfaces contain this polymer. Currently, chemical fungicides and insecticides are the major means of controlling these disease-causing agents (Veliz *et al.*, 2017). However, due to the potential harm that these chemicals cause to the environment and to human and animal health, new strategies are being developed to replace or reduce the use of fungal- and pest-killing compounds in agriculture. Thus, microbes producing chitinases have attained great attention for the last few decades. Microorganisms producing chitinolytic enzymes were considered as bio-control agents (Farag *et al.*, 2016), amongst were the *Aspergillus* species (Ni *et al.*, 2015 and Stoykov *et al.*, 2015), that more specifically have included the *A. niger* that produces the chitinase with the highest activity (Awad *et al.*, 2017).

In the current study, an ORF (1484 bp) of *chiA* gene was isolated and encodes as a

chitinase enzyme of fungal class *A. niger* (EM77). The ORF was submitted to the NCBI database (accession No. MH878942) and analyzed using bioinformatics tools, which showed no previous record with a sequence of *chiA* gene from *A. niger*. All published sequences of chitinase genes from some *Aspergillus* species that occur in NCBI database were derived by automatic computational analysis using the gene prediction method for genomic sequences. This suggests that the ORF sequence is the first record of *chiA* gene from *A. niger*.

The deduced amino acid sequence of *chiA* for *A. niger* (EM77) was found to have 100 % identity with the deduced sequence of *chiA*, a fungal/plant chitinase from *A. niger* (XP_001396624) (Pel *et al.*, 2007) and 95 % identity with the deduced sequence for the *A. awamori* (GCB27978) (Kusuya *et al.*, 2018), *A. neoniger* (XP_025483904) (Vesth *et al.*, 2018) and *A. piperis* (XP_025521389) fungal/plant chitinase (Mondo *et al.*, 2018).

Based on the similarity percentages of amino acid sequences, most chitinase genes that have been characterized to date are classified into two families of glycosyl hydrolases (family18 and 19) (Meng *et al.*, 2015). Thus, the deduced amino acid

sequence of chitinase (*chiA*) of the isolate EM77 has included the characteristic substrate-binding site and the catalytic motifs cited by Vocadlo and Davies (2008). Therefore, it can be denoted as a member of the family18 of glycosyl hydrolases. The characterized chitin-binding domain (ChtBD1) was found important to specifically bind chitinases to insoluble chitin (Limón, *et al.*, 2004). Despite the fact that some fungal chitinases may lack the presence of ChtBD domain (Duo-Chuan, 2006), however, the chitinase in the current study has included the ChtBD.

Chitinase enzyme produced by *A. niger* (EM77) was partially purified using ethanol fraction due to the insolubility of precipitated proteins. Coagulation of chitinase proteins with ethanol fractions agreed with Tandjaoui *et al.* (2015) who reported that precipitation of enzymes by ethanol and acetone was not stable in most enzymes and insoluble in water and buffer solutions that in turn would hinder the purification processes. On the other side, other workers preferred precipitation of chitinase with ammonium sulphate, for instance; *Bacillus cereus*, *Streptomyces griseorubens* and *A. terreus* (Liang *et al.*, 2014; Karthik *et al.*, 2015; and Rashad *et al.*, 2017). The chitinase was exhibited maximum activity (42.39 Units/ml). These results were in agreement with those other chitinase preparation obtained from *B. thuringiensis* (25.11 U/ml) (Gomaa and El-Mahdy, 2018).

Chitinases enzyme is glycosyl hydrolases with sizes ranging from 20 kDa to about 90 kDa (Bhattacharya *et al.*, 2007). Great varieties of chitinase can be found among fungal species, and each species can produce different chitinase isomers, which have different catalytic properties (Homthong *et al.*, 2016). In the present study, a single band at ~ 30 kDa was determined as the molecular weight of the partially purified, which is approximately close to that of the chitinase produced from *Aspergillus flavus* (30 kDa); *Monacrosporium thaumasium* (30kDa),

Bacillus cereus IO8 (30 kDa), *Lecanicillium lecanii* 43H (33 kDa), *Bacillus licheniformis* strain JS (22 kDa) and *Streptomyces* sp.(40 kDa), *A. nidulans* (44 kDa) (Beltagy *et al.*, 2018, Karthik *et al.*, 2015; Nguyen *et al.*, 2015; Soares *et al.*, 2015; Hammami *et al.*, 2013; Waghmare and Ghosh, 2010; Yamazaki *et al.*, 2007). Our finding does not in agreement with the results of identified purified 45 kDa chitinase from *A. fumigatus* (Jaques *et al.*, 2003).

The potential use of chitinase for controlling insect pests has been demonstrated in several studies. In our study, partially-purified exo-chitinase of *A. niger* EM77 had insecticidal activity against *S. littoralis* larvae after 96 hours (38.9%). That was because of the ability of chitinase to disrupting peritrophic membrane of insect cells (Zhong *et al.*, 2015). As well as chitinase of family18 and 19 were known for its catalytic activity similar to lysozyme and chitosanase in their activities (Sahai and Manocha, 1993), which could be serving as an effective biopesticide to control harmful pests which destroy importantly economic crops instead of hazardous chemical pesticide.

Conclusion

Several reports have extensively highlighted the importance of chitinases for various biotechnological and environmental applications. So, the increasing number of databases describing and chitinase genes and protein structure and function can help to streamline the engineering of chitinases in a more rational manner to yield chitinases with improved activity and stability specifically for the various biotechnological applications. In our study, gene encoding exo-chitinases (*chiA*) were characterized by the isolate EM77 as the first to be published in *Aspergillus niger*. Characterization of the new chitinase has included molecular, biochemical, and bioassay techniques. Results indicated that the *A. niger* EM77 is a fungal strain that can produce amounts of chitinase (42.39 Units/ml) within six days. In addition, the partial-purified chitinases enzyme has shown a promising insecticidal

activity (~39 % mortality in four days) against *S. littoralis* larvae.

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