MOLECULAR CLONING AND SEQUENCE ANALYSIS OF CHITINASE GENE *chiA* FROM LOCAL ISOLATE OF *Bacillus licheniformis* MS1

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Detection of chitin-degrading bacteria from natural sources such as rhizosphere soil is useful for the isolation of bacteria that produce antifungal or other novel compounds. A high correlation between chitinolysis and production of bioactive compounds has been reported (Chen *et al.,* 1991; Pisano *et al*., 1992; Hoster *et al.,* 2005). Microorganisms, which secret a complex of mycolytic enzymes, are considered to be used as biological control agents of plant diseases (Helisto *et al.,* 2001; Chang *et al.,* 2003; Hoster *et al.,* 2005). Chitin hydrolysis is performed by a major pathway composed of three separate enzymes to break down polymeric chitin to chitin oligosaccharides, diacetylchitobiose and N-actylglucosamine, separately or synergistically and consecutively in the degradation of chitin to free N-actylglucosamine. Endochitinase (E.C. 3.2.1.14) is a poly (1,4-(N-acetyl-β-D-glucosaminide))-glycanohydrolases, which produce multimers of β-N-acetylglucosamine by random hydrolysis of β-1-4-linkages in chitin and chitodextrins while Exochitinase (E.C. 3.2.1.52) β-1,4-N-acetylhexosaminidase, is catalyzing the sequential release of soluble dimers starting at the non-reducing end of the polymer. Chitobiases (E.C. 3.2.1.30) or β-N-acetylglucosaminidase, helps the hydrolysis of chitobiose into monomers of N-actylglucosamine (Souza *et al.,* 2003). It is though that chitin hydrolysis occurs either by a sequential or synergetic action of endochitinase, exochitinase, and chitobiases (Tanaka *et al.,* 2003). While exo- and endo-chitinases are able to depolymerize chitin alone. The presence of both activities significantly increases the efficiency of chitinolytic system (Howard *et al.,* 2003). Recently, the cloning and expression of the chitinase gene and its introduction into the biologically susceptible species or the construction of recombinant strains with new capacities have been recommended to be one of the interesting areas of chitinase studies and applications. Chitinase genes have been cloned and characterized from many microorganisms (Ueda *et al.,* 2003; Hobel *et al.,* 2005; Yano *et al.,* 2005; Yong *et al.,* 2006). Some of which were either transformed into plants and/or bacterial strains to increase their ability to control phytopathogens (Koby *et al.,* 1994; Punja,

2001) or were high level of expression in *Escherichia coli* cells to enhance the activity of *Bacillus thuringiensis* to control pests (Regev *et al*., 1996; Sampson and Gooday, 1998). Chitinase has received increased attention because of their potential application in the biological control of plants-pathogenic fungi and pests, as well as in the bioconversion of shellfish chitin wastes (Chang *et al.,* 2003; Hoster *et al.,* 2005). In our previous study, local isolate of *Bacillus licheniformis* MS1 isolated from agricultural fields, Giza, Egypt, was found to be one of the most producing a large amount of chitinase enzyme (Kamil *et al.,* 2007). For this reason this isolate was selected in the present study for cloning, sequencing and molecular analysis of its chitinase gene (*chiA*).

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions

The local isolate of *Bacillus lichneiformis* MS1 and MS3 were used in this study was originally isolated from agricultural fields, Giza, Egypt (Kamil *et al.,* 2007). *Escherichia coli* JM109 was used as the recipient strain for recombinant plasmid and it was grown in Luria-Bertani medium (Trypton 10.0 g -Yeast extract 5.0 g- NaCI 10.0 g- ddH_2O up to 1 liter). pGEM-T vector was used as a cloning vector.

Polymerase chain reaction (PCR)

The Polymerase Chain Reaction (PCR) is a procedure that allows rapid determination of the presence or absence of a target DNA sequence in any genetic material. In this study, the PCR was performed in a 50 µl reaction volume containing 1X PCR buffer (10 mM Tris-HCl pH 8.3; 50 mM KCl; 2 mM $MgCl_2$ and 0.01% (w/v) gelatine), 250 µM each of dGTP, dATP, dCTP and dTTP (dNTPs), 2.5 units of Taq DNA polymerases, 100 pmol of each primer and the DNA template. The DNA was extracted from the bacterial cells by boiling the bacterial growth in a water bath for 5 min to lyses the cells and then the tubes were spun briefly to collect the condensate (Carozzi *et al.,* 1991). PCR reaction conditions were: 94°C for 4 min; 35 cycles of denaturation at 94°C; annealing at 58°C; and extension at 72°C for 1 min each, followed by 7 min extension at 72°C.

Cloning of chitinas gene chiA

The complete coding region of chitinase gene *chiA* was amplified from MS1 isolate using sense primer (ChiF: 5`-ATGAAGAAAGCCGCTTCATCT-3`) and antisense primer (ChiR: 5`-GGAACGGGCTGTCACATAACGG-3`). The PCR product was ligated into pGEM-T vector in the presence of T4 DNA ligase for overnight at 4°C. The recombinant clones were amplified in *E. coli* JM109.

DNA sequence

DNA sequencing was carried out using the automated DNA sequencing method. The automated sequencing

reactions was performed with ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystem, USA) using the ABI PRISM (310 Genetic Analyzer). Both DNA strands were fully sequenced using primer walking method.

3D homology modeling software

Homology modeling was carried out using Swiss-Model, protein modeling server (Guex and Peitch 1997; Schwede *et al.*, 2003; Arnold *et al.*, 2006) accessible *via* the EXPASY (http://www.expasy.org/). Superimposition of *Bacillus licheniformis* MS1 *chiA* model was constructed by using RasMol (http://www.umass.edu/microbio/rasmol/) and Deep-View program (http://spdbv.vital-it.ch/). The functional domains were identified from the NCBI conserved domain database (CDD) (http://www.ncbi.nlm.nih.gov/betweenz/query.cgi?db=cdd)

RESULTS AND DISCUSSION

Amplification of full-length chiA gene from Bacillus Licheniformis MS1

The polymerase chain reaction (PCR) was used to amplify and isolate the full-length of *chiA* gene from the *Bacillus licheniformis* MS1 which exhibited the chitinase activity and antifungal properties. Amplification of 2116 bp fragment was performed using pair of primers ChiF and ChiR on total genomic DNA of MS1 and MS3 as shown in (Fig. 1). The expected amplified 2116 bp gene product belonging to the *chiA* gene was obtained from both local isolate of *B. licheniformis* MS1 and MS3.

Cloning and transformation of *chiA* gene from *B. liceniformis* MS1

The previous gene products of the putative *chiA* gene from MS1 were purified and ligated into pGEM-T Easy plasmid multiple cloning sites. Following ligation, plasmids were transformed into *E. coli* competent cells. The transformants were plated on LB- ampicillin plates containing X-gal and IPTG for identification of recombinants by the blue/white colony screen. One hundred white colonies designated G1 to G100 were picked and plated on a master plate, as well as being cultured in liquid media for DNA minipreps. Blue colonies do not contain the recombinant DNA molecule while the white colonies were the one may contain the target gene. The selected white colonies were subjected to different verification tests (PCR using specific PCR primers, plasmid miniprep and restriction digestion) to detect the recombinant clones harboring the specific gene encoding the chitinase gene from the local *Bacillus licheniformis* isolate MS1. The plasmid was isolated from the positive clone designated pChiA-BL1 and subjected to DNA sequence.

Nucleotide and amino acid sequence analysis of pChiA-1

The complete nucleotide sequence of the *chiA* gene from the local isolate of *Bacillus licheniformis* (MS1) was obtained. The nucleotide sequence and the

predicted amino acid sequence showed in Fig. (2). The sequence of the 5' and 3' ends were obtained using primer walking method. The open reading frame chiA-1 has A+T=54.3% and G+C=46.4%. The chiA-BL-1 gene contain one open reading frame (ORF) of 2082 bases starting at the ATG codon, ending with the stop codon TAA, and encodes 693 amino acids residues. The molecular mass of the protein calculated from the deduced amino acid sequence was 77613.49 Daltons and an isoelectric point of 5.380. Its amino acid composition can be divided into four categories: non-polar (Ala, Ile, Leu, Phe, Trp and Tyr), polar (Asn, Cys, Gln, Ser, Thr and Val), acidic (Asp and Glu) and basic (Lys and Arg). The ratio of the presence of each category is 29.43%, 26.98%, 13.41% and 9.95%, respectively. Comparing the deduced amino acid sequence of the local *Bacillus licheniformis* chiA gene with those registered in the NCBI's conserved domain databases (CDD) revealed that the mature protein was a modular enzyme composed of two conserved domains in the following order: glycosyl hydrolysis family 18 domain (Glyco_18 domain) reported by (Papanikolau *et al.*, 2001; Thamthiank *et. al.*, 2001; Rey *et al.*, 2004) a region of 170 amino acids from 532 to 691 found at the C-terminus of chitinase C found in members of glycoside hydrolase family 18. (http://www.ncbi.nlm.nih.gov/structure/cdd/wrpsb.cgi). Additionally, the signal peptide online tool (SignalP 3.0 Server, (http://www.cbs.dtu.dk/services/SignalP) was used to reveal the N-terminal cleavable signal peptide of MS1 *chiA*. The results showed that the deduced N-terminal sequence of 26 amino acids contained a typical signal peptide sequence. The signal cleavage site was between 26 Ala and 27 Glu. The nucleotide and deduced amino acid sequence of the cloned chitinase *chiA* from *Bacillus licheniformis* MS1 were compared with all published *chiA* sequences by BLAST searches and the results showed that the putative mature *chiA* exhibited a high sequence homology with *ChiA* from *Bacillus licheniformis* ATCC 14580 (sequence identity, 95%) and *Bacillus licheniformis* DSM 13 (95%), followed by chitinase from *Bacillus pumilus* (82%), and chitinase from *Herpetosiphon aurantiacus* ATCC 23779 (57%), *Streptomyces olivaceoviridis* (57%), and chitinase from *Streptomyces coelicolor* (55%), *Vibrio vulnificus* (54%) and a low sequence homology with chitinase from *Pyrococcus kodakaraensis* (26%). Figure 3, showed the protein alingment tree of MS1 Chitanse gene with the different bacterial chitinse genes available in Genbank data base as retrieved from (http://www.ncbi. nlm.nih.gov/structure/cdd/wrpsb.cgi). The results showed that the most related chitinge gene to MS1 is the chitinase A-BL1 (*Bacillus licheniformis*). Furthermore, based on structural alignment a theoretical 3D model for MS1 chitinse gene was obtained as showed in Fig. (4).

SUMMARY

The local isolate of *Bacillus licheniformis* MS1 that has been isolated from the rhizosphere of some plants col-

lected from different agriculture fields, Giza, Egypt, and screened for production of chitinase enzyme was used in this study. MS 1 was found to produce a large amount of this enzyme. Therefore, this isolate was selected for cloning, sequencing and molecular analysis of chitinase gene (*chiA*). The complete open reading (ORF) of *chiA* gene was amplified from *Bacillus licheniformis* MS1using chitinase specific primers. The coding region of *chiA* was cloned as a 2.116 kb DNA segment into pGEM-T vector. The complete nucleotide sequence of *chiA* gene from MS1 was determined. Nucleotide sequence of coding region revealed a single ORF with 693 amino acid coding capacity without upstream consensus promoter sequence. The molecular mass of protein calculated from deduced amino acids sequences was 77 and 613.49 KDa and had isoelectric point of 5.380. Alignment of the deduced *ChiA* amino acid sequence with other *ChiA* proteins revealed that, *ChiA* is most closely related to *ChiA* from *Bacillus licheniformis* ATTC 14580 (95%) and *Bacillus licheniformis* DSM13 (95%) followed by chitinase from *Bacillus pumilus* (82%). Analysis of the deduced amino acid sequence of *ChiA* from local isolate MS1 showed the mature chitinase after cleavage of signal peptide was composed of two conserved domains.

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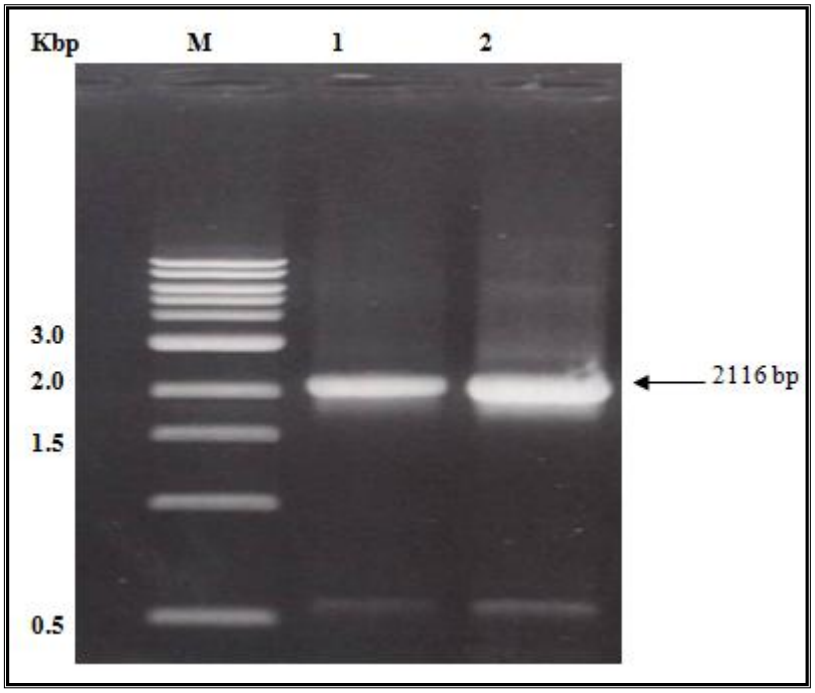

Fig. (1): PCR amplification of *chiA* gene using specific primers ChiF and ChiR. Arrow in lanes 1 and 2 referred to 2116 bp *chiA* gene that was detected in the *Bacillus licheniformis* MS1 and MS3, respectively. Lane M contained molecular mass standards 1 kb (numbers on the left are bp).

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   1 atgaagaaagccgcttcatctttttatcttgtatgctgctcctcgcgctttttatcccg   60
      M  K  K  A  A  S  S  F  L  S  C  M  L  L  L  A  L  F  I  P    20
  61 aaccggcagatttccgcagagacttcaagcgaaagaccggactgccgtccggaagggctt  120
      N  R  Q  I  S  A  E  T  S  S  E  R  P  D  C  R  P  E  G  L    40
 121 tgggactcaggcgttgaacatgtgccgtactgcgatgtatacgaccgggacggccgtgaa  180
      W  D  S  G  V  E  H  V  P  Y  C  D  V  Y  D  R  D  G  R  E    60
 181 aagcttgccaatcagttagaccggagaatcatcgggtactttacaagctggcgtactgga  240
      K  L  A  N  Q  L  D  R  R  I  I  G  Y  F  T  S  W  R  T  G    80
 241 aaaggcaatcaagatcgttatttagtaaacgatatcccctggaagtacttgagccacatt  300
      K  G  N  Q  D  R  Y  L  V  N  D  I  P  W  K  Y  L  S  H  I   100
 301 aattatgcgtttgcccacatcggggaagatcatcggatttcagtcggagatgagacagat  360
      N  Y  A  F  A  H  I  G  E  D  H  R  I  S  V  G  D  E  T  D   120
 361 gagaacaacccgtccatcgggatgacctggccggagcattccgatgtaaaaatggatcaa  420
      E  N  N  P  S  I  G  M  T  W  P  E  H  S  D  V  K  M  D  Q   140
 421 accttgccgtataaggggcattttaaccttatccatcaatataaagagaaatacccgaat  480
      T  L  P  Y  K  G  H  F  N  L  I  H  Q  Y  K  E  K  Y  P  N   160
 481 gtaaaagttttggcggctgccggcggttgggccgaaaccggcggttatgttgacagggac  540
      V  K  V  L  A  A  A  G  G  W  A  E  T  G  G  Y  V  D  R  D   180
 541 gggaagcggattccaagcggcggctttattcgatgacaacgaatggagacggatctgta  600
      G  K  R  I  P  S  G  G  F  Y  S  M  T  N  G  D  G  S  V     200
 601 aaccacaagggtattcacacctttgcggagtcggtcgttgcgtttctcagaaaatacgag  660
      N  H  K  G  I  H  T  F  A  E  S  V  V  A  F  L  R  K  Y  E   220
 661 atcgatggaatcgatattgactatgagtaccctacatcaatgcaggatgcaggaaatcct  720
      I  D  G  I  D  I  D  Y  E  Y  P  T  S  M  Q  D  A  G  N  P   240
 721 gcagattggaacatcgccaatccgcggcgcacgggatttaatacaagctttgaggcattg  780
      A  D  W  N  I  A  N  P  R  R  T  G  F  N  T  S  F  E  A  L   260
 781 atgaaaacattaagagaaaagcttgatcaagcgtccgctgaagatggaaagtactacatg  840
      M  K  T  L  R  E  K  L  D  Q  A  S  A  E  D  G  K  Y  Y  M   280
 841 ctgaccattgcagcaccatcctcggcatatttactgaggggatggaaacgtttaagccg  900
      L  T  I  A  A  P  S  S  A  Y  L  L  R  G  M  E  T  F  K  P   300
 901 cttcgatatgtcgattatgtccatatcatgtcctatgatcttcatggagcctggaatgag  960
      L  R  Y  V  D  Y  V  H  I  M  S  Y  D  L  H  G  A  W  N  E   320
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      F  V  G  P  N  A  S  L  F  D  N  G  E  D  A  E  L  K  H  S   340
1021 aacatatatactacgccggaatacgaagggatcggatatttaaatacggactgggcttac 1080
      N  I  Y  T  T  P  E  Y  E  G  I  G  Y  L  N  T  D  W  A  Y   360
1081 cattattttagaggagctatggaatcggggcgaatcaatatcggtgttccttattatacg 1140
      H  Y  F  R  G  A  M  E  S  G  R  I  N  I  G  V  P  Y  Y  T   380
1141 agaggctggaagaatgtgaacggcggagtaaacgggctgtggggaacatccaaaagcgca 1200
      R  G  W  K  N  V  N  G  G  V  N  G  L  W  G  T  S  K  S  A   400
1201 gattgtcctcagggactcaggcaatgcggtgacggagcaactggaatcgataatatttgg 1260
      D  C  P  Q  G  L  R  Q  C  G  D  G  A  T  G  I  D  N  I  W   420
1261 cacgataaagatgagcaggggaatgaaattggagcaggggcaaacccgatgtggcacgcc 1320
      H  D  K  D  E  Q  G  N  E  I  G  A  G  A  N  P  M  W  H  A   440
1321 aaaaaccttgaaaagggcattgccggttcttatctggaaaggtatggattgagcaaggct 1380
      K  N  L  E  K  G  I  A  G  S  Y  L  E  R  Y  G  L  S  K  A   460
1381 gatttgacaggcgcttataaaaggcattatgacgcgggggctggcagcgccttggctttgg 1440
      D  L  T  G  A  Y  K  R  H  Y  D  A  G  L  A  A  P  W  L  W   480
1441 aatccagaaaagaaagtgtttctatcgacagaggatgaggaatcgatcaaaacgaaggcg 1500
      N  P  E  K  K  V  F  L  S  T  E  D  E  E  S  I  K  T  K  A   500
1501 gattatgtcgttgataaaggtatcgggggtgtcatgttttgggatttgagcggtgattat 1560
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1561 gactggtatcccgaacgaaacggcaataaaggagaatacgatattggcaatactttaacg 1620
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1621 agcacgctgtatcacaaattctcggatacaacaccatacgataaccggctcagcacatcg 1680
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1681 ccttttcctgatgaatcattggatgtagacatagagctgacgaactttccattaggagat 1740
      P  F  P  D  E  S  L  D  V  D  I  E  L  T  N  F  P  L  G  D   580
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1801 ggaggttctgttatcgaatttgatgtgccgacatccacttcccctcggtttggaagctgg 1860
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      S  G  D  Q  V  E  V  I  A  K  G  H  T  G  P  N  I  G  G  L   640
1921 acgggagatttccaccgcatcggagtgactctatcaagctggaaaacgattaaacctggt 1980
      T  G  D  F  H  R  I  G  V  T  L  S  S  W  K  T  I  K  P  G   660
1981 gaggcggcggagtttcagcttgtttattatttgccgatcagcggcccttcaaatttcacg 2040
      E  A  A  E  F  Q  L  V  Y  Y  L  P  I  S  G  P  S  N  F  T   680
2041 atatcaatagatggaaaaaagtacaggcttaaagtgaagtaagtgtgcagccgttagatg 2100
      I  S  I  D  G  K  K  Y  R  L  K  V  K  - 693
2101 tgacagcccgttccaa 2116
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Fig. (2): Nucleotides sequence of chitinase A gene (*chi A*) and deduced amino acid sequence from the local isolate *Bacillus licheniformis* (MS1), the sequence extends, 2082 nucleotides in length that has one open reading frame. The translation products of the *chiA* gene are shown below the nucleotide sequence.

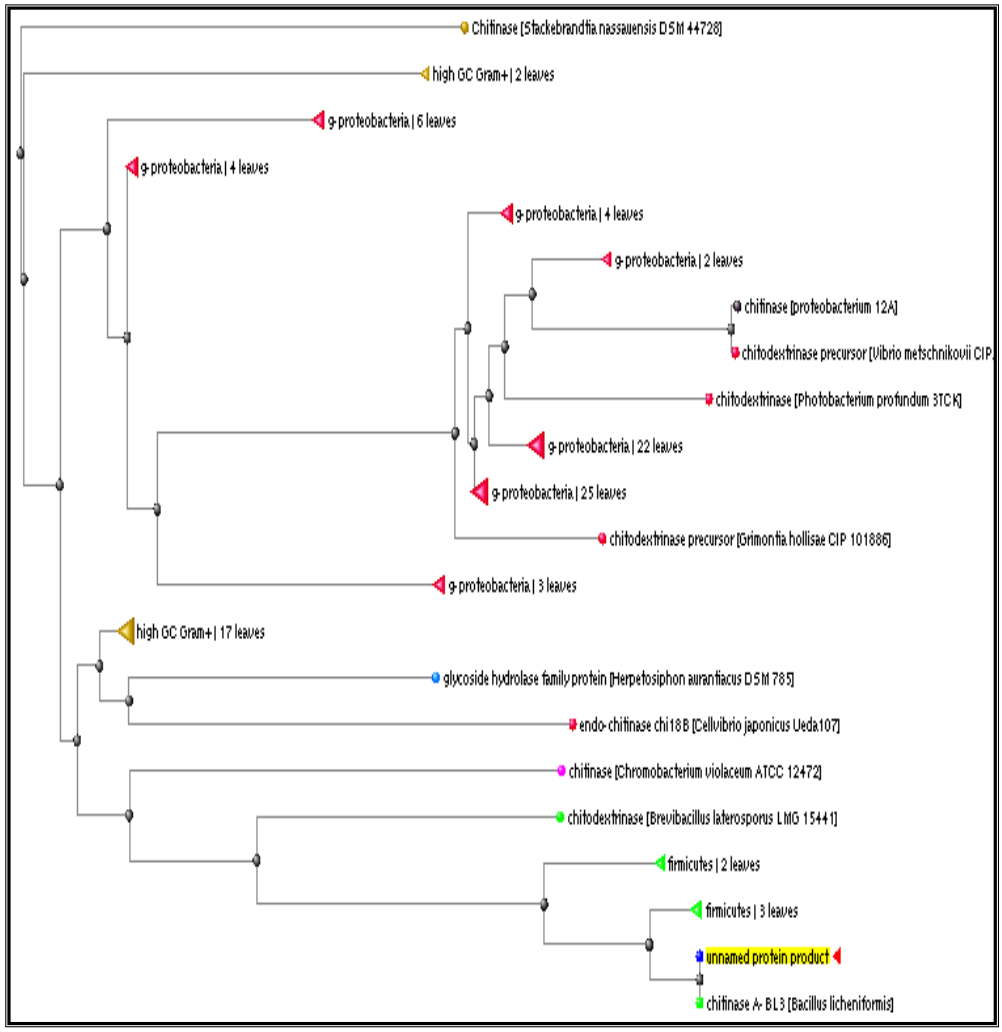

Fig. (3): Protein alignment tree of chitinse gene from MS1 with different bacterial chitinase genes avalible in GenBank. The MS1 chitinase is indicated on the figure by unnamed protein product.

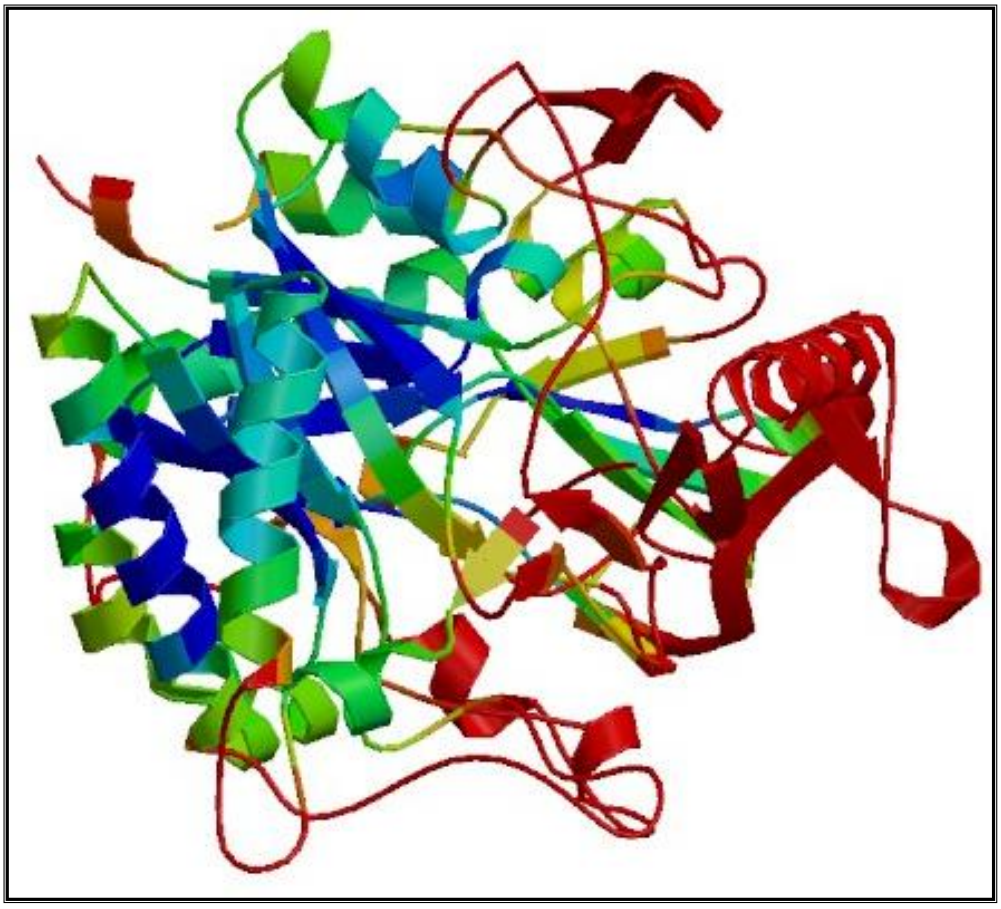

Fig. (4): *Bacillus licheniformis* MS1 *ChiA* 3D structure of homology modeling. Domain 1 is in trequaze, domain2 in green, domain3 in blue and domain 4 in orange.