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Isolation of putative chitinase II gene fragment from *Serratia plymuthica* strain UBCR_12

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ABSTRACT

Chitinase is mostly investigated in Serratia marcescens, but not in S. plymuthica. In order to detail investigated chitinase from S. plymuthica, a putative chitinase II fragment has been successfully isolated from S. plymuthica strain UBCR_12. Isolation was performed by PCR-based cloning strategy by applying a pair of primer, specifically designed to amplify chitinase II fragment. BLAST result showed that the cloned fragment has 93% similarity to chitinase of S. plymuthica strain 3Re4-18 (CP012097) and others. Cluster analysis showed that the putative chitinase sequence has very close related to chitinase A (ChiA). Furthermore, domain analysis showed that the putative chitinase has only one domain, GH18_chitinase, as catalytic domain resembles high similarity to the ChiA domains. Based on its 3D-modeling structure analysis we assumed that the cloned chitinase fragment belongs to the subfamily of Chi indicating the similar function to SpChiD.

Keywords: *Chitinase, Serratia Plymuthica, CDD Analysis, PCR-Based Cloning, GH18 Domain.*

INTRODUCTION

Chitinases have attracted many attentions due to their role in biocontrol of fungal phytopathogens [1]. Their ability to degrade chitin directly into low molecular weight chitooligomers [2], could be used to degrade fungal cell wall which is mainly characterized by a complex structure composed of chitin 1,3-β and 1,6-β-glucan, mannan, and protein [3]. Chitin is a linear polymer of β-1,4-N-acetylglucosamine (GlcNAC) [4] and is essential for cell viability for many types of fungi [5]. The degradation of chitin in fungi cell wall by chitinase activity will be one of the effective mechanisms to inhibit the growth of the fungal pathogen in a plant. In *Serratia marcescens*, chitinase was extensively studied and divided into five groups, chitinase A (*Chi*A), chitinase B (*Chi*B), chitinase C (*Chi*C), *Chi*C2, and CBP21[6-10]. Three of them (*Chi*A, *Chi*B, and *Chi*C) have been extensively detailed studied for their function, mechanism, and structure. While *Chi*C2 which belongs to subfamily *Chi*C is responsible to the catalytic domain of *Chi*C. Meanwhile, CBP21 is a chitin-binding protein having 21 kDa in weight and its production is coregulated with three other chitinases, although the role of this protein is not known [10].

Species of *S. plymuthica* has also been known for its chitinase activity and therefore is used to inhibit several phytopathogenic fungi [11-18]. The species was identified to contain at least one type of chitinase, for instance, *Chi*A [19]. So far, no *Chi*B and *Chi*C are isolated from *S. plymuthica*. The previous study indicated that our novel isolate *S. plymuthica* strain UBCR_12 showed a complex chitinase activity. The species showed obvious ability to inhibit *Colletotrichum gloeosporioides* [18], a phytopathogenic fungi in many horticultural crops. Homology analysis based on its 16S rRNA gene showed a significant hit with *S. plymuthica* AS12 (CP002774). Detailed annotation of the species revealed six chitinases Open Reading Frame (ORFs). Based on this data, we designed specific primer combination in order to isolate all those six chitinases related genes.

Here we report the isolation and further analysis of a putative 1.281 bp-chitinase gene fragment. The isolation of 1.281 bp-putative chitinase has been previously reported in *Coccidiodes immitis* [20], but has not been reported so far from *S. plymuthica* as well as from *S. marcescens*. PCR-based cloning as an effective and efficient method to isolate a target gene was chosen and used in this study. This method using Polymerase Chain Reaction (PCR) technique to isolate and amplify the target gene by in vitro. The advantage of this method is that it yields only the target gene in a numerous of DNA fragment concentration for easily and quickly. Generally, to construct a recombinant DNA technology, the desired DNA fragment is prepared in large quantity using PCR and the amplicon (the PCR-amplified DNA) thus obtained is cloned into the vector of choice [21].

MATERIALS AND METHODS

The research was performed in Lab of Biotechnology, Faculty of Agriculture, Andalas University from December of 2015 to July of 2016. PCR-based cloning approach has been performed, started by designing of specific primer combination, in vitro

amplification, heat shock transformation using *Escherichia coli* DH5α [22], recombinant analysis, and finally nucleotide sequence analysis.

Designing of putative 1.281 bp chitinase specific primer

Six ORFs of 1.281 bp putative chitinase were selected from six accessions of *S. plymuthica* deposited in GenBank (https://www.ncbi.nlm.nih.gov/nuccore/333488955/?report=genebank) (CP002773, CP002774, CP002775, CP007439, CP006566, and CP006250) and used for designing the specific primer combination. The multi-alignment analysis was performed in offline software ClustalX2. The multi-alignment result further processed using Bio edit software to define the consensus sequence. The consensus sequence was finally used as a template for primer designing in the online software of primer3plus (http://www.bioinformatics.nl/cgibin/primer-3plus/primer3plus.cgi). One pair of specific primer combination was designated with Chi-1281bp-F as forward primer (5-TAA-CATGGCATTACCCCGTA-3'). The primer sequence covered four nucleotides added in the upstream of the start codon (ATG). The reverse primer was designated with Chi-1281bp-R (5'-ATCGCCTGTNGGTTTAGCG-3'). The primer position is 20 bases located in downstream the stop codon. The expected PCR product was estimated of 1.299 bp.

In vitro amplification and cloning of putative 1.281 bp chitinase fragment

Chromosomal DNA of *S. plymuthica* strain UBCR_12 was isolated as described Syafriani [18]. PCR reaction was performed in a volume of 25 µL, containing 5 ng/µL of DNA, 1.5 µL each primer (10 pmol/µL), and filled until 25 µL with PCR-grade H_2O. PCR reaction was run using 35 cycles composing of 94 ºC; 60 s, 55 ºC; 60 s, and 72 ºC; 90 s for each cycle. Initial denaturation was done at 94 ºC for three minutes and followed by a final extension at 72 ºC for five minutes. The amplicon was expected as a single fragment of 1.299 bp in size, ligated to pGem-T easy vector. The ligation subsequently transformed into *E. coli* DH5α by heat shock method. Enrichment of transformant was done by incubating in 250 µL Luria Bertani (LB) medium for 20 minutes under shaking condition at 37 ºC for 150 rpm. Transformation suspension was grown on LB selective medium, completed with 100 mm IPTG, 3% X-Gal, and 100 mg/mL Ampicilin prior incubation at 37 ºC, for overnight.

The selected recombinant plasmid DNA was further purified using Wizard Plus SV Minipreps DNA Purification System per Promega's protocol (Promega, USA). The amplicon was sent to 1st BASE for sequencing from both T7 and SP6 termini. Nucleotide sequences were compared for their homology using BLASTn tool available at NCBI website (http://blast.ncbi.nlm.nih.gov). Furthermore, the target sequence was analyzed in Mega6 software [23] to build the phylogenetic tree. Finally, 3D-modeling of protein structure analysis was also performed for our chitinase gene by using Phyre2, an online web portal [24].

RESULTS AND DISCUSSION

Amplification of the DNA genome of *S. plymuthica* strain UBCR_12 with the specific primer produced a single fragment as expected in size of 1.299 bp (Figure 1). The PCR product was then cloned into a pGem-T easy vector and was further sequenced.

The sequence data of putative chitinase was further analysed with BLASTn [25], phylogenetic tree (MEGA6) [23], ORF Finder [26], Conserved Domain Database (CDD) [27, 28], and 3D-modeling protein structure [24].

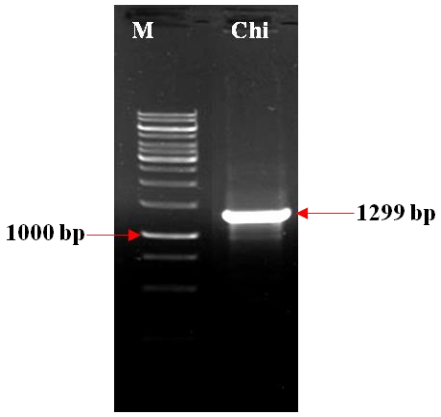

Figure-1: Amplification result of chitinase gene target produced a single 1.299 bp fragment

Homology and cluster analysis

Trimming and editing of sequence data verified successfully a 1.299 bp in length. Homology comparison showed 93% similarity to the chitinase gene of some *S. plymuthica* deposited in the gene bank, for instance, CP012097, CP007439, CP015613, CP006566, and CP002775. Comparison with other different *Serratia* species exhibited only 85% similarity for instance with *S. proteamaculans* 568 (CP000826) and 84% with *S. liquefaciens* strain HUMV-21 (CP011303). Based on this result, we designated our sequence as putative chitinase *S. plymuthica* strain UBCR_12. The complete sequence was deposited into NCBI database with accession number: KX863673. Phylogenetic tree analysis showed that the sequence in this study mostly related to chitinase A (*Chi*A) (Figure 2). *Chi*A is major chitinase which retards fungal growth and known as an effective agent in biocontrol of fungal pathogens of several economic crops [29]. It also regarded as the most abundant in nature [30]. The nucleotide size of this gene is around 1.692 bp in *S. marcescens* and most of *S. plymuthica*, where its molecular weight is about 58-kDa [7, 19]. *Chi*A is also classified as one of GH18 chitinase among the four others, such as *Chi*B (54 kDa), *Chi*C (52 kDa), *Chi*C (35 kDa), and CBP21 (21 kDa) [6].

Cluster analysis revealed four different groups of chitinase GH18 from gram-negative bacteria (Figure 2), where chitinase A (group I) showed a high evolutionary relatedness to chitinase B (group II) and also to chitinase C (group III). Based on amino acid sequence similarity, the family 18 of chitinases can be classified into three subfamilies namely A, B, and C [31]. The chitinase B belongs to subfamily A [30], therefore the evolutionary relatedness of chitinase A to chitinase B is higher than chitinase A to chitinase C. On the other hand, chitinase C belongs to the subfamily of chitinase B [10], thus chitinase B showed a quite relatedness to chitinase C. Moreover, putative chitinase (group IV) surprisingly showed the higher evolutionary relatedness to chitinase A group. Although the putative chitinase in this study (KX863673) showed a high relatedness mostly to putative chitinase from S. plymuthica, but it was also showed quite a high relatedness to *S. marcescens* and *S. proteamaculans*. Since our

sequence shows a high relatedness to chitinase A, this result lead us to an assumption that our putative chitinase sequence has similar domain structure and mechanism in degrading chitin substrate.

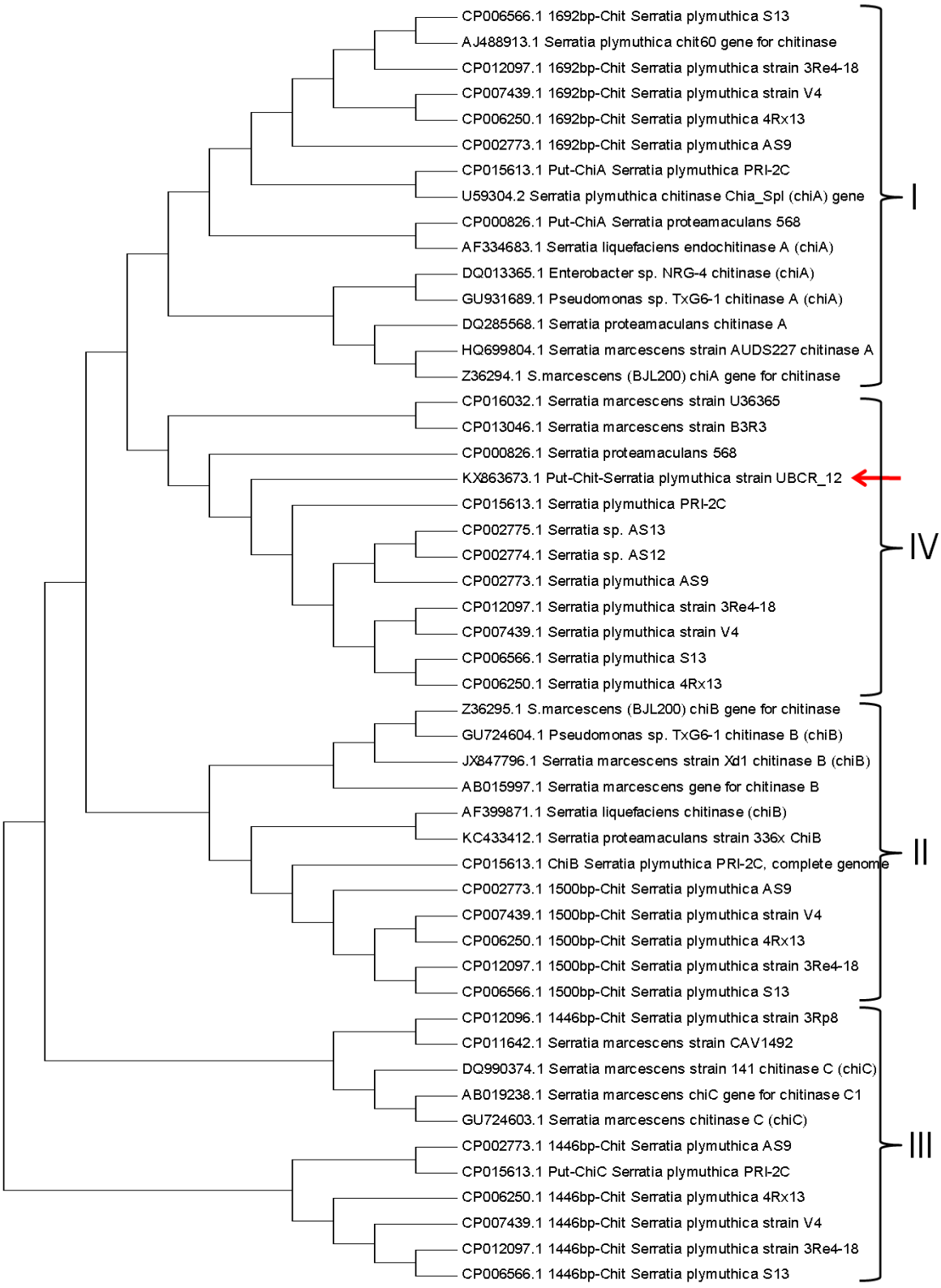

Figure-2: Phylogenetic tree of putative chitinase gene sequence of *S. plymuthica* strain UBCR_12 with 49 closely related selected accessions. The tree was designed by Kimura 2-model parameter method embedded in Mega6 using bootstrap value with 1000 replication.

Open reading frame (ORF) finder and conserved domain database (CDD) analysis

ORF finder analysis was performed on NCBI (http://www.ncbi.nlm.gov/orffinder/). The aim of this analysis is to reveal the number of ORF spanning along the 1.299 bp-putative chitinase gene sequence. Besides, this analysis is purposed to predict functionality domain of the sequence. ORF analysis successfully identified six putative ORFs ranging from 102 bp to 1.281 bp. The longest ORF containing 426 amino acids started with ATG and ended with TAA as the stop codon.

CDD analysis was used to get the domain structure of the putative chitinase fragment. The result showed that the 1.281 bp sequence of putative chitinase has only one domain classified as GH18_chitinase domain (Figure 3). This domain is well known as a catalytic domain. The CDD analysis result also showed that the catalytic domain of putative chitinase gene in this study has similarity to some other of catalytic domain from some species. Furthermore, as we can see in Fig. 3, the domain structure of our putative chitinase was also similar to catalytic domain of GH18 chitolectin chitotriosidase, GH18 IDGF, GH18 chitinase-like, GH18 plant chitinase class V, GH18 zymocin alpha, GH18 narbonin, GH18 chitobiase, GH18 304 chitinase, GH18 EndoS-like, GH18 trifunctional, GH18 CFLE spore hydrolase, GH18 SI-CLP, and GH18 chitinase-like superfamily.

GH18 is an ancient family of chitinases, widely distributed in archaea, bacterial, and eukaryotes, including human and fungi, whose members catalyze the hydrolysis of chitin, a structural component of the cell wall of fungi and the exo-skeleton of arthropods [32]. Thus, the CDD analysis in this study showed that our chitinase has similarity to other species. Besides, the GHs can be classified as either exo or endo depending on whether the enzyme cleaves the substrate from a terminus or randomly at internal sites along the polymer, respectively. The endochitinases are non-processive and have shallow substrate-binding clefts, while exochitinases are often processive with deep substrate-binding clefts [9, 33]. The exochitinase can be divided into two subcategories: chitobiosidases (catalyze the progressive release of diacetylchitobiose units starting at the non-reducing end of chitin microfibrils) and N-acetyl-β-(1,4)-D-glucosaminidases (cleave the oligomeric products of endochitinases and chitobiosidases, generating monomers of GlcNAc [34]. In general, *Chi*A and *Chi*B are known as exochitinase, while *Chi*C is known as endochitinase [35]. Since our CDD analysis showed that the chitinase in this study has homologous protein to GH18 chitobiase and also *Chi*A, we assumed that our chitinase is an exochitinase enzyme. Chitobiase is an enzyme that cleaves off N-acetylglucosamine from the non-reducing end of chitobiose and higher chitooligosaccharides [36].

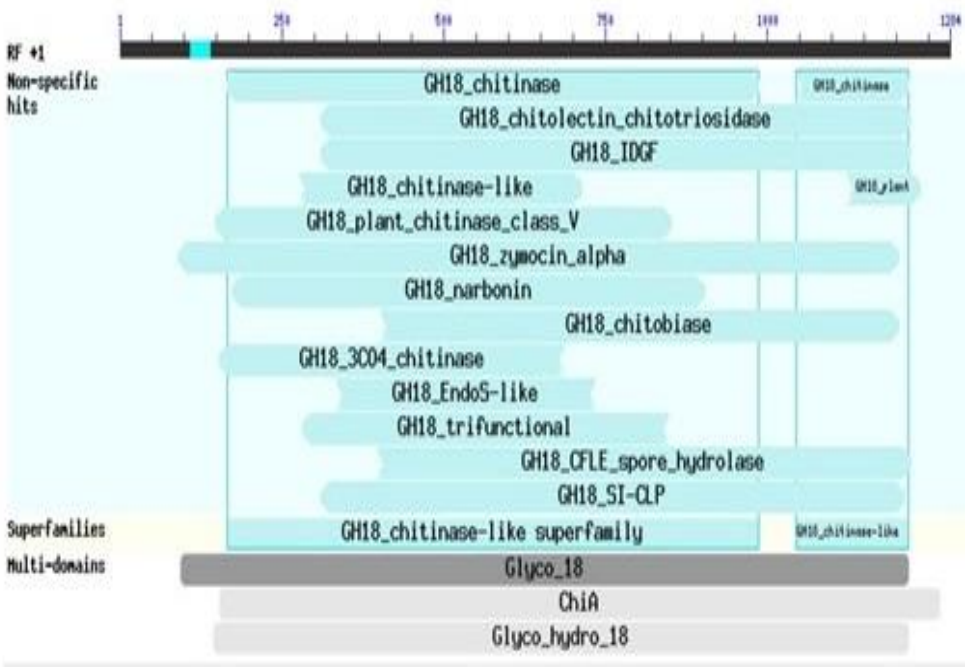

Figure-3: CDD analysis of 1.281 bp-putative chitinase gene sequence to other chitinases from some species

Figure 3 showed that the structure of ChiA domain is a part of Glyco 18 (GH18) and have strong similar domain structure to our 1.281 bp-putative chitinase. It proved our previous assumption that our sequence has similar domain structure to *Chi*A. Since the 1.281 bp-putative chitinase in this study has only one domain, we assumed that the catalytic domain of our chitinase is a subfamily of *Chi*A. Eventually, besides some other species have similar domain structure to putative chitinase in this study, a 1.281 bp-chitinase sequence has also been described from *Coccidiodes immitis* which encode a protein of 427 amino acids and having a predicted size of 47 kDa [20]. *C. immitis* is a eukaryotic organism and identified as pathogenic fungi to human. Multi-alignment of the sequence with accession number U33265 against the 1.281 bp-putative chitinase showed a low identity of 46.35%. It indicated that both sequences are completely different although they have similar size (1.281 bp). Based on this reason, performed 3D-modeling.

3D-modeling of putative chitinase structure

The 3D-modeling of putative chitinase structure showed that our 1.281 bp-putative chitinase has similarity to the crystal structure of putative chitinase II from *Klebsiella pneumoniae*. However, only 394 amino acid residues (92%) that could be modeled with 100% confidence by the single highest scoring template. Based on this result, we revealed that our sequence is a putative chitinase II. Chitinase II is a chitinase class that only found in plant, fungi, and bacteria [34]. This result has supported the

previous CDD analysis result showing specific domain structure similar with the chitinase from plant and others. Class II chitinase has exo active site, lack of the cysteine-rich N-terminal domain, but has sequence similarity with class I chitinase [37]. This result confirmed our presumption that the isolated chitinase has exochitinase activity.

Figure-4: Modeling of 1.281 bp-putative chitinase

Species of *Serratia* sp. is known as one of family *Enterobacteriaceae* [38]. That is why it is not surprising that our sequence has similarity with the putative chitinase II from *K. pneumonia* and the *Chi*D of *Serratia proteamaculans* (*SpChi*D) about 79%. Detailed domain structure determination showed that *SpChi*D folds into a single catalytic domain containing a $(\beta/\alpha)_8$ TIM barrel conformation. In comparing between the putative chitinase II in this study, the *SpChi*D consists of 406 amino acid residues, which is a bit shorter than our chitinase (426 amino acids), but they have 93.6% similarity. Madhuprakash *et al*. [39] described that *SpChi*D (PDB: 4LGX) has multiple active sites, hydrolytic and/or chitobiase activities. Based on its exonuclease specific domain, we suggested that our chitinase has similar function with *SpChi*D.

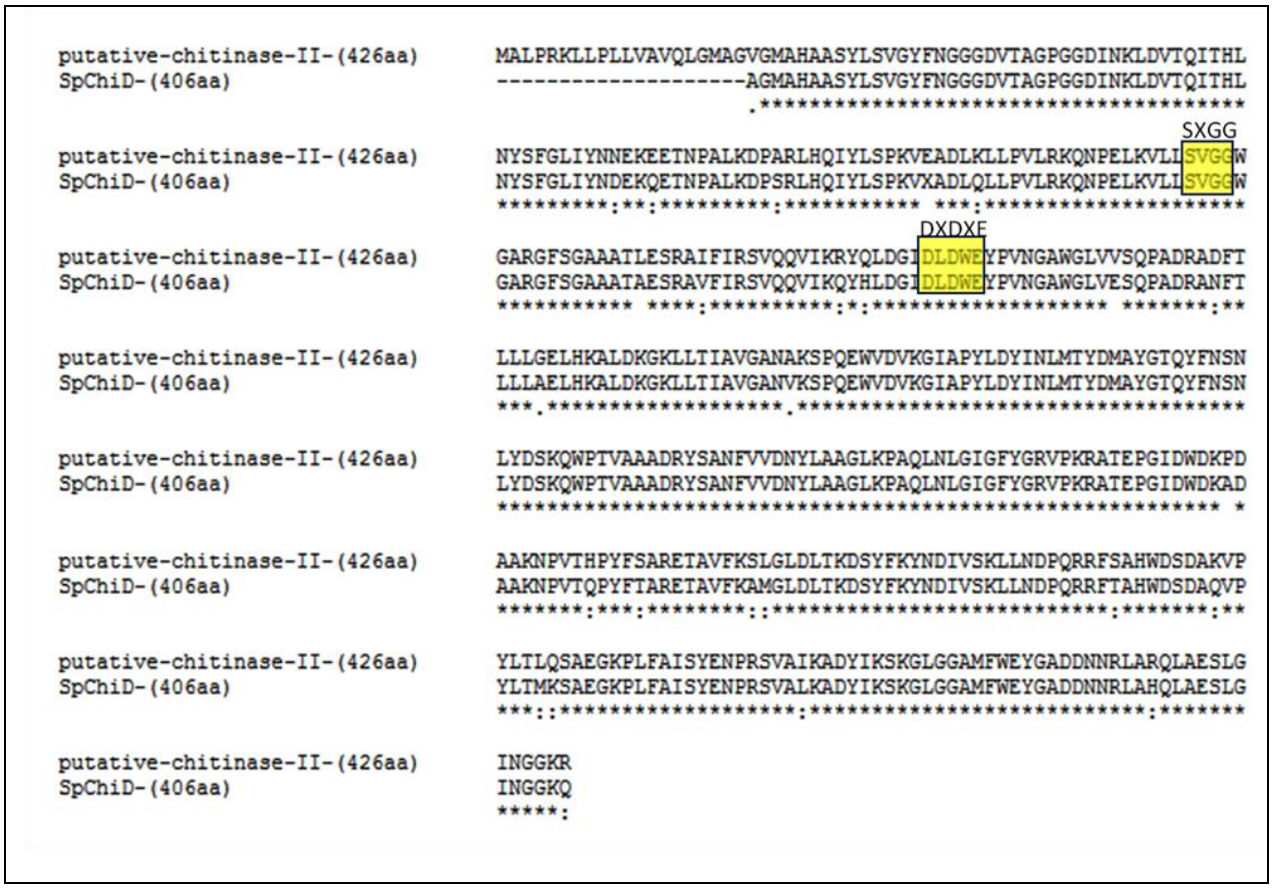

Figure-5: Alignment of putative chitinase II from *S. plymuthica* strain UBCR_12 with *SpChi*D

Identification of catalytic motif of SXGG and DXDXE residue[33], we found that both motifs are also present in our sequence (Fig.5 showed by yellow block). TIM barrel of GH family 18 enzymes contains the active site a catalytic motif of DXDXE residue that includes a glutamic acid which protonates the oxygen in scissile glycosidic bonds [40]. In our sequence, it is represented by DLDWE. Such conserved motif was also found in Chi3023 from *Bacillus thuringiensis* serovar morrisini, characterized by Asp^{207}-Leu^{208}-Asp^{209}-Trp^{210}-Glu^{211} [41]. Moreover, the motif DXDXE contains the so-called chitinase insertion domain (CID), located between amino acid residues 310 and 379, identified by the conserved residues YxR. Chitin has been suggested to insert at this site to reach the active site, and it consists of a small α+β domain which is the main structural feature of subfamily A of family 18 chitinases [30].

Another highly conserved residue is a serine that is part of a less well known but equally diagnostic SXGG motif [42]. Serine was though to help in the stabilization of a temporary surplus of negative charge that develops on the first aspartate of the catalytic sequence motif during catalysis, while glutamate in the active site acts as the catalytic acid [43, 44]. The SXGG motif

corresponds to substrate binding site[45]. This motif that is followed by a Trp in processive chitinases is vital for processivity and the ability to efficiently degrade insoluble chitin[46]. In our case, the SXGG motif is represented by SVGG. The SVGG motif has also been found in chitinase A1 (PDB: 1ITX) from *Bacillus circulans* [47], thus this data is supporting our above preassumption.

CONCLUSION

This study has successfully isolated a 1.281 bp-putative chitinase from *S. plymuthica* strain UBCR_12. Based on CDD analysis, it could be concluded that the isolated sequence is classified as putative chitinase II which has high similarity to *Chi*A sequence. Domain analysis successfully identified only one domain (catalytic domain). The 3D-modeling structure analysis showed similar structure and function to *SpChi*D from *S. proteamaculans*. However, further expression assay has to be performed in order to prove our claim.

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