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Extremophilic fungal Chitinases: Properties and perspectives

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ABSTRACT

Extremophiles survive and grow in extreme environments formerly considered hostile for life. These fungi have developed strategies to withstand harsh conditions. Due to their extreme stability, the proteins or extremozymes from these organisms offer new opportunities for biocatalysis. The wide spectrum of applications of chitin derivatives in various fields ranging from medicine to cosmetics makes the exploration of extremophilic chitinases necessary. This review summarizes the recent developments on the characterization of extremophilic chitinases and the success in cloning and expression of their genes.

Keywords : Chitinase, fungi, GH family 18, extremozymes, thermophiles, chitooligomers

INTRODUCTION

Chitin, a Greek word for 'envelop', was discovered in 1811 as a substance occurring in mushrooms. It is the most widespread aminopolysaccharide in nature and is estimated annually to be produced almost as much as cellulose. It is a cationic aminopolysaccharide, composed of β (1-4) linked N-acetyl-D-glucosamine (NAG) residues. It is present in the exoskeleton of invertebrates (crustaceans, molluscs), insects and in marine diatoms and fungi among microorganisms. Derivatives of chitin oligomers have also been implicated as morphogenic factors in the communication between leguminous plants and *Rhizobium* and even in vertebrates, where they may be important during early stages of embryogenesis (Bakkers *et al*., 1999). Chitin is widely distributed in fungi, occurring in *Basidiomycetes*, *Ascomycetes* and *Phycomycetes*, where it is a component of the cell walls and structural membranes of mycelia, stalks, and spores with amounts varying between traces and up to 45% of the organic fraction.

Chitin polymers tend to form microfibrils (also referred to as rods or crystallites) that are stabilized by hydrogen bonds formed between the amine and carbonyl groups. X-ray diffraction analysis suggested that chitin occurs in two crystalline allomorphs, a more abundant α- and less common β- form. In both forms chitin chains are arranged in sheets, the chains in any one sheet have the same direction. Fungal and yeast cell walls contain α-chitin (Saito *et al.,* 2000). In α-chitin, adjacent sheets have the chains that are antiparallel and tightly held by a number of inter and intra-sheet H-bonds (Aranaz *et al*., 2009). In addition, non-crystalline, transient states have also been reported in fungi (Vermeulen and Wessels, 1986).

Recently, the commercial value of chitin has increased because of the beneficial properties of its soluble derivatives, which are suitable in chemistry, biotechnology, agriculture, food processing, cosmetics, veteniary, medicine, dentistry, environment protection and paper or textile production (Synowiecki and Al-Khateeb, 2000; Tharanathan and Kittur, 2003). Chitin monomer finds application in the treatment of joint damage, treatment of inflammatory bowel disease, production of cosmetics, sialic acid production, waste water treatment, drug delivery vehicles and others. Applications of chitooligosaccharides (COSs) include drugs against asthma, antibacterial agents, ingredients in wound dressings, anticancer therapy, nerve regeneration and vectors in gene therapy. Several other potential effects of COS have been described, including immune modulatory effects, antifungal activities and lowering effect on serum glucose levels in diabetics (Chen *et al*., 2010; Tharanathan and Kittur, 2003). These derivatives also include chitosan, a partially deacetylated and soluble form of chitin, which has a broader range of applications (Ravi Kumar, 2000). Chemical hydrolysis and deacetylation of chitin require large amounts of HCl, NaOH and water (Ravi Kumar, 2000). The development of efficient enzyme-based methods for chitin processing might be more ecologically compatible and reduce requirements for energy and resources of this process (Hartl *et al*., 2012).

ENZYMES ACTING ON CHITIN AND CHITOSAN

Chitinases catalyze the hydrolysis of chitin to its oligosaccharides and can be classified into two major categories (Graham and Sticklen, 1994). Endochitinases (EC 3.2.1.14) that cleave chitin randomly at internal sites, generating soluble, low molecular mass multimers of NAG such as chitotetraose, chitotriose and diacetylchitobiose, and exochitinases that are further divided into two subcategories: chitobiosidases and β-(1,4) N-acetyl glucosaminidases. Chitobiosidases (EC 3.2.1.29) catalyze the progressive release of diacetylchitobiose starting at the non-reducing end of chitin microfibril. β-(1,4) N-acetyl glucosaminidases (GlcNAcase, EC 3.2.1.30) or chitobiases catalyze the release of terminal, non-reducing N-acetylglucosamine residues in an exo-type fashion from chitin and oligomers of chitin. In general they have the highest affinity for the dimer N,N'-diacetylchitobiose $(GlcNAc)_2$ and convert it into two monomers (Horsch *et al*., 1997).

According to the glycosyl hydrolase classification system, based on the amino acid sequence similarity, chitinases

are placed in families 18, 19, and 20 (Henrissat and Bairoch, 1993). Family 18 and 19 chitinases are distinguished mainly on the basis of occurrence, catalytic mechanism and 3D structure. All fungal chitinases belong to family 18. Within the family 18, two distinct classes of fungal chitinase may be identified based on the similarity of enzymes to family 18 chitinases from plants or bacteria (Pishko *et al*., 1995; Takaya *et al*., 1998). Therefore, fungal chitinases are divided into fungal/bacterial (corresponding to Class V) chitinases, similar to chitinases found in bacteria, and fungal/plant (corresponding to Class III) chitinases, which are similar to chitinases from plants (Adams, 2004; Takaya *et al*., 1998; Jaques *et al*., 2003) [**Fig. 1**]. Recently, according to phylogenetic analysis, it has been suggested that fungal chitinases of family 18 can be divided into three groups: group A and group B (corresponding to class V and III of chitinases, respectively), whereas a novel group C comprises high molecular weight chitinases which have a domain structure similar to *Kluyveromyces lactis* killer toxins (Seidl *et al*., 2005). Class III or group B chitinases have shallow and open active site architecture. In contrast, class V/group A chitinases, have a deep and tunnel shaped active site (Hartl *et al*., 2012). According to domain structure and size, chitinases from extremophilic fungi studied to date appear to belong to group A or fungal/bacterial class of chitinases (Li *et al*., 2010; Ramli *et al*., 2011; Kopparapu *et al*., 2012; Palanivelu and Vaishna, 2013).

The family 20 includes the β-N-acetylhexosaminidases from bacteria, fungi, and mammals. As chitin in most of the organisms occur as a heteropolymer of acetylated and deacetylated glucosamine residues, chitin deacetylases and chitosanases too form a part of the repertoire of chitin hydrolyzing enzymes. Chitin/chitooligosaccharide deacetylases cleaves off the acetyl group from NAG residues in chitin/chitooligosaccharide and chitosanases hydrolyze the β-glycosidic bond between deacetylated chitin residues. Chitin deacetylases are included in the carbohydrate esterase family 4 of glycoside hydrolases (Caufrier *et al*., 2003). Chitosanases have been classified into family 5, 8, 46, 75 and 80 of glycoside hydrolases (Cheng *et al*., 2006).

CHITINASES FROM EXTREMOPHILES

The use of enzymes or microorganisms as biocatalysts for the formation of various products has many advantages such as specificity, mild operating conditions among others. Due to harsh industrial process conditions, there are demands for biocatalysts that function in process conditions. Mesophilic organisms have been the source of majority of industrial enzymes, their application is restricted due to their limited stability at the extremes of temperature, pH and ionic strength (Gomes and Steiner, 2004). On the other hand, extremophiles are a potent source of extremozymes, which show stability in extreme conditions. There is growing interest in exploiting the microorganisms that are capable of growth in extreme environments (Gomes and Steiner, 2004).

Various groups of the extremophiles possess unique features, which can be harnessed to provide enzymes with a wide range of application possibilities. Extreme environments include those with either high (55 to 121 °C) or low (–2 to 20 °C) temperatures, high salinity (2–5 M NaCl) sugar concentration, and high alkalinity (pH>8) or high acidity (pH<4). The extremophilic microbes can also tolerate other extreme conditions like high pressure, high levels of radiation or toxic compounds, or conditions that we consider unusual, such as living in rocks deep below the surface of the earth or living in extremely dry areas with very low water and nutrient availability (Gomes and Steiner, 2004). In addition, extremophiles may be found in environments with a combination of extreme conditions such as high temperature and high acidity, high temperature and high alkalinity or high pressure and low temperature. Most of the extremophiles which have been identified to date belong to the domain of the archaea, although many extremophiles from the eubacterial and eukaryotic kingdoms have also been identified and characterized recently (Vieille and Zeikus, 2001; Ramli *et al*., 2011). In this review, an attempt has been made to discuss about chitinases from thermophilic fungi.

THERMOPHILIC FUNGAL CHITINASES

Of all extremozymes, thermostable enzymes have attracted most attention and thermophilic microorganisms are amongst the most studied of the extremophiles. Such enzymes are of great industrial and biotechnological interest due to the fact that the enzymes are thermostable and better suited for industrial processes operating at high temperatures. There are many advantages of conducting industrial processes at high temperature such as faster

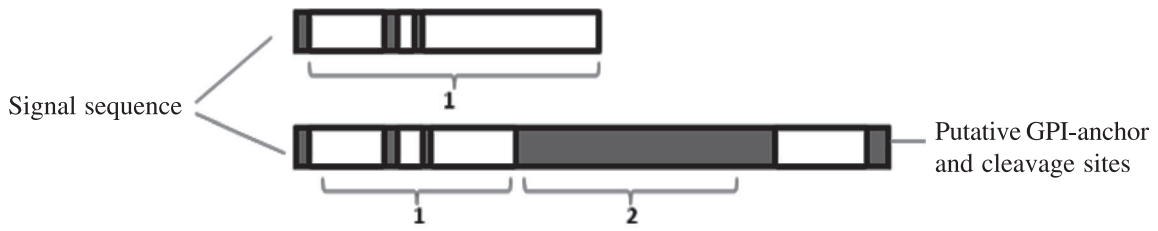

Fig. 1. Comparison of domains of the ChiB1 fungal/bacterial chitinase (433 aa) and ChiA1 fungal/plant chitinase (825 aa) of *Aspergillus fumigatus*. 1, Catalytic domain; 2, Serine/Threonine rich domain (Adams, 2004).

reaction rates, higher process yields due to increased solubility of substrates and products, and the decreased risk of contamination (Vieille and Zeikus, 2001).

Thermophilic organisms are particularly important in biotechnology as the source of thermostable enzymes. Although thermostable enzymes have been reported from mesophiles, the productivity is high in thermophiles and the frequency of finding more thermostable and more chemoresistant enzymes is higher in thermophiles than in mesophiles (Maheshwari *et al*., 2000; Vieille and Zeikus, 2001). The exploration of chitinases from thermophilic bacteria and fungi is also interesting from the point of view of understanding the mechanism of thermotolerance of such enzymes.

Thermophily in fungi is not as extreme as in eubacteria or archaea. However, 0.06% of the recorded fungal species are able to breach the upper temperature limit of eukaryotes and can thrive at temperatures between 45 and 55 °C (Cooney and Emerson, 1964; Maheshwari *et al*., 2000). Thermophilic fungi have been reported from decomposing organic materials like wood chips, animal dung, cereal straw, municipal refuge and several others in which the activity of mesophiles result in thermogenic conditions or man-made habitats such as cooling towers, effluent of nuclear power reactors and ducts employed for thermal insulation (Satyanarayana and Johri,1999).

Chitinolytic enzymes have been described from a few thermophilic fungi: *Mucor miehei, Talaromyces emersonii, T. leycettanus* and *Thermomyces lanuginosus* (Jensen and Olsen, 1999). Thermophilic fungal chitinases exhibit high temperature optimum and high thermostability. *Talaromyces emersonii* produces an inducible chitinolytic system consisting of a chitinase and an N-acetylglusamidase with optimal activities in the range of 65 to 75 °C (Hendy *et al*., 1990; McCormack *et al*., 1991). A 48 kD chitinase is reported from *Thermomyces lanuginosus* which is stable for 20 min at 70 °C and for 25 min at 65 °C (Guo *et al*., 2005). Likewise two chitinases from *Thermoascus aurantiacus* var. *levisporus* and *Chaetomium thermophilum* possess high thermostability (Li *et al*., 2010).

CHITINASES OF OTHER EXTEMOPHILES

Only a few studies have been done on chitinases of fungal extremophiles other than thermophiles. Notable among these are the chitinases of psychrophilic fungi (Fenice *et al*., 1998; Ramli *et al*., 2011). These are important for biocontrol of microbial spoilage of refrigerated foods, biocontrol of phytopathogens in cold environments and/ or treatment of chitin-rich wastes at low temperature. In industrial applications, psychrophilic chitinases are becoming attractive partly because of ongoing efforts to decrease energy consumption. At low temperatures, the kinetic energy of reacting molecules is too low to allow reactions to occur. Psychrophilic or cold-adapted enzymes compensate in this situation by having a highly flexible protein structure and conformation (Ramli *et al*., 2012).

PRODUCTION OF EXTREME FUNGAL CHITINASES

The most common method employed for production of chitinases is submerged fermentation (SmF) process and owing to complex nutritional requirements of extreme fungi, complex medium like YPD medium containing colloidal chitin as inducer has been generally used for wild type cultivation and expression of chitinase (Ramli *et al*., 2012). When expressed in heterologous hosts, expression medium e.g. buffered methanol complex (BMMY) medium has been used (Li *et al*., 2010).

PURIFICATION AND CHARACTERIZATION

Purified extremophilic fungal chitinases have been characterized in terms of their molecular weight, optimal pH, optimal temperature, thermostability, and glycosylation. Usually, extremophilic fungal chitinases are monomeric proteins (Guo *et al*., 2008; Kopparapu *et al*., 2012). The molecular weight of extremophilic fungal chitinases spans a very narrow range (43-50 kDa) [**Table 1**]. Optimal pH and temperature are almost in the same range for the majority of the purified chitinases from thermophilic fungi. Thermophilic fungal chitinases are active in the pH range between 4.0 and 8.0 and have a high temperature maximum for activity around 50–60 °C (**Table 1**). In addition, they exhibit remarkable thermal stability and are stable at 45-55 °C with longer half-lives at 60 °C and 70 °C than those from other fungi (Guo *et al*., 2005). The lower temperature limit of psychrophilic fungal chitinases is 5 °C (Fenice *et al*., 1998; Ramli *et al*., 2011).

MECHANISM OF ACTION

The catalytic mechanism of glycosyl hydrolases involves two catalytic residues and proceeds through a geometrically

Table. 1. Characterization of chitinases from extrememophilic fungi

Organism	Opt. pH/ range	MW kD	Opt. temp.	Reference
Talaromyces emersonii	5-5.5	-	65	McCormack *et al*., 1991
Thermomyces lanuginosus	4.5	48	55	Guo *et al*., 2005; Guo *et al*., 2008
Thermoascus aurantiacus	8	48.4	50	Li *et al*., 2010
Chaetomium thermophilum	5.5	47.3	60	Li *et al*., 2010
Paecilomyces thermophila	4.5	43.7	50	Kopparapu *et al*., 2012
Verticillium lecanii A3	4	45	40	Fenice *et al*., 1998
Glaciozyma antarctica PI12	4	50	15	Ramli *et al*., 2011

distorted oxocarbonium intermediate (**Fig. 2**). The β-1,4 glycosidic bond between sugars is broken involving the protonation of the leaving group alcohol leading to an oxocarbonium ion intermediate, which is then stabilized by a second carboxylate (either through covalent or electrostatic interactions). A nucleophilic attack by water which replaces the leaving sugar group yields the hydrolysis products. Family 18 chitinases retains the initial anomeric configuration and the reaction is commonly referred to as the double displacement mechanism of hydrolysis. Whereas family 19 chitinases follow a single displacement mechanism and the hydrolysis products show inversion of anomeric configuration.

It is proposed that two conserved carboxylic acids are important in retaining the substrate assisted catalytic mechanism of family 18 hydrolases (Perrakis *et al*., 1994). One has been proposed to act as a general acid to protonate the leaving group and glutamate serves this role in a wide range of glycohydrolases. A second carboxylic acid generally aspartate in chitinases either assists the catalytic glutamate or acts as base to stabilize the transition state (Bortone *et al*., 2002).

STRUCTURE OF FUNGAL CHITINASE GENES

All fungal chitinases belong to family 18 of the glycosylhydrolase superfamily (Henrissat, 1999). A characteristic of the family 18 chitinases is their multi-domain structure. Typically, the basic structures of fungal chitinase of family 18 are composed of five domains or regions: N-terminal signal peptide region, catalytic domain, serine/theronine-rich region, chitin-binding domain, and C-terminal extension region. Most fungal chitinases, however, lack the last three domains, which do not seem to be necessary for chitinase activity because naturally occurring chitinases that lack these regions are still enzymatically active. The extremophilic chitinases are, in general, simple and compact proteins consisting of a single catalytic domain characteristic of family 18 chitinases (Guo and Li, 2006). Alignments of the extremophilic fungal chitinase protein sequences show that these enzymes are highly similar (**Fig. 3**). Comparison with the related fungal chitinase sequences from GenBank database identified by BLASTp searches showed that chitinases from thermophilic fungi, *Thermoascus aurantiacus* (TaCHIT1), *Chaetomium thermophilum* (CtCHIT1) and *Paecilomyces thermophilum* (PtChiA) are composed of a single catalytic domain without any other domains (chitinbinding domain and Ser/Thr rich domain), similar to 33 kDa chitinase from *Trichoderma harzianum* and 48 kDa chitinase from thermophilic *Thermomyces lanuginosus* (Limon *et al*., 1995; Guo and Li, 2006).

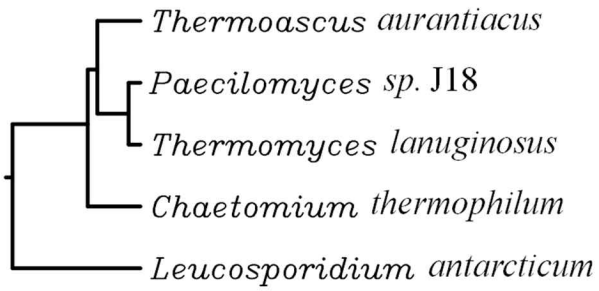

Fig. 3. Dendogram showing phylogeny among extremophilic fungal chitinases.

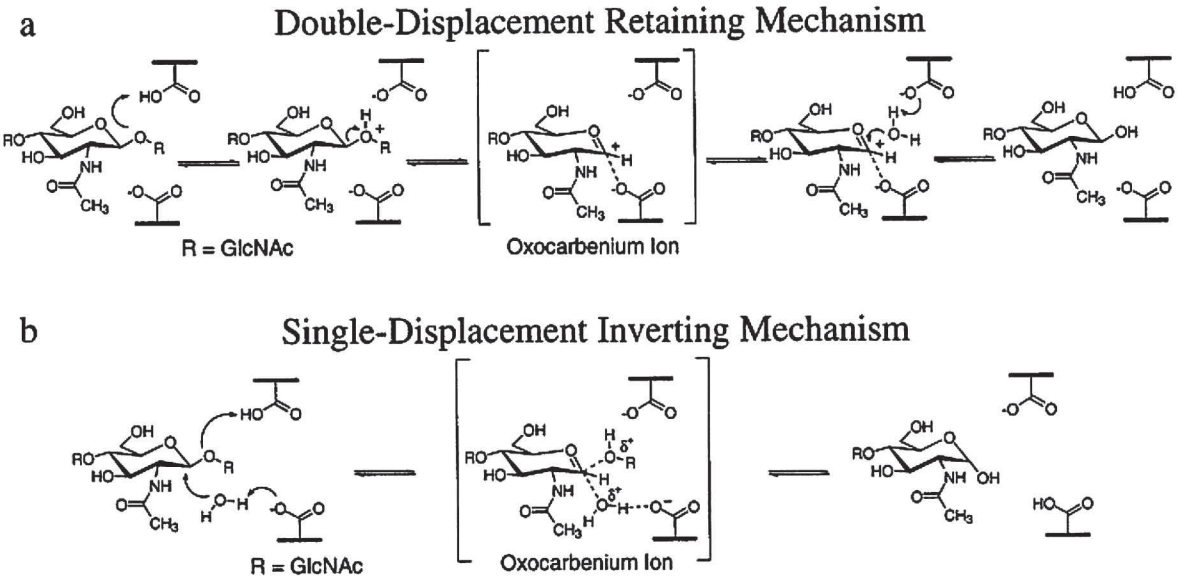

Fig. 2. The hydrolysis mechanism of chitinases (a) Double-displacement mechanism proposed for family 18 chitinases and (b) Single-displacement mechanism proposed for family 19 chitinases.

The catalytic domain of fungal chitinases is responsible for the hydrolysis of the substrate. Sequence alignments reveal two highly conserved regions within the catalytic domain. The two consensus regions or motifs, SxGG and DxxDxDxE, correspond to a substrate-binding site and a catalytic domain, respectively, in family 18 chitinases (Henrissat, 1991). Catalytic domain and consensus regions for chitinases from thermophilic fungi have also been analyzed (Li *et al*., 2010; Guo and Li, 2006). The catalytic domains of chitinases from *Thermoascus aurantiacus* var. *levisporus* and *Chaetomium thermophilum* are similar to those of other fungal chitinases in family 18 of glycosyl hydrolases (Li *et al.,* 2010). Alignment of genes encoding these two proteins and the most similar other fungal chitinases revealed that these chitinases share the LSIGGWT and DXXDXDXE motifs that are considered to be substrate-binding site and a chitin-catalyzing domain, respectively (Watanabe *et al*., 1993; Hollis *et al*., 2000; Li, 2006; Li *et al*., 2010). No other domains like chitin-binding domain, Ser/Thr rich domain or signal peptide region is present in the extreme chitinases reported so far. The only exception is the cold adapted chitinase from *Glaciozyma antarctica* PI12 which contains a putative N- terminal signal peptide of 19 amino acids suggesting its secretory nature (Ramli *et al*., 2011). The chitinase gene from another thermophilic fungus, *Paecilomyces thermophila* (PtChiA) does not have a potential signal peptide but consists of a potential N-glycosylation site, N-X-S/T (Kopparapu *et al*., 2012).

CLONING EXPRESSION AND REGULATION OF CHITINASE GENES

Production of fungal chitinase is commonly induced in the presence of chitin and is controlled by a repressor/inducer system (McCormack *et al*., 1991). In this system, chitin or other oligosaccharide products of chitin degradation (such as N-acetyl-β-D-glucosamine and glucasamine) act as inducers while glucose or other easily metabolized carbon sources act as repressors. The molecular basis of glucose repression to fungal chitinase gene expression has been studied in mesophilic fungi. It is demonstrated that the URS (upstream regulatory sequence) in the fungal chitinase promoters plays a key role in the regulation of glucose repression. The protein product of the regulatory gene creA/cre1 is a negatively acting transcription factor that binds to DNA sequence motif with the consensus sequence SYGGRG in the URS (Chuan, 2006). In the presence of glucose, the activated CreA/Cre1 protein binds to the consensus motif, and represses transcription of chitinase genes. Though the upstream regulatory elements and the repertoire of transcription factors influencing chitinase gene expression has not been described in thermophilic fungi to date, thermophilic fungal chitinases are also expected to possess a repressor/inducer system. The chitinase gene from the thermophilic fungus *Thermomyces lanuginosus* overexpressed in *Saccharomyces cerevisae* was strongly repressed by glucose even when the growth was three fold higher than the chitin induced culture (Prasad and Palanivelu, 2012). It is, therefore, likely that chitinase gene regulation in thermophilic fungi may share certain similarities with other fungal family 18 chitinases.

Most chitinolytic microorganisms have been found to produce more than one kind of chitinase. *Trichoderma harzianum* produce seven individual chitinases (Haran *et al*., 1995; Harman *et al*., 1993; De La Cruz *et al*., 1992). In a similar fashion multiple forms of chitinases are also produced in thermophilic fungi. *Talaromyces emersonii* produces at least two kinds of chitinases (McCormark *et al*., 1991). These multiple chitinases may have a mutually synergistic and complementary effect between them (Chuan, 2006).

Only a few genes encoding extremophilic fungal chitinases have been sequenced, expressed and purified. Most cloned chitinase genes of extremophilic fungi are expressed well in host organisms, such as *E. coli* and yeast. Expression of some extremophilic fungal chitinase

Table 2. The properties of extremophilic fungal chitinases expressed in heterologous hosts.

Fungus	Amino acid	Expression vector /Host/GenBank Accession No.	Optimal pH andTemp.	Thermal Stability	MW (kDa)	Reference
Thermomyces lanuginosus -RMB	390	pXcmkn12 *E. coli* DH5α JQ801444	nd	nd	nd	Palanivelu and Vaishna, 2013
Paecilomyces thermophila J18	400	pMD-18T *E. coli* JN129126	pH 4.5 50 °C	up to 40 °C	43.7	Kopparapu *et al*., 2012
Thermomyces lanuginosus ATCC 44008	——	pLC9 *Saccharomyces cerevisiae* SEY 2101 JQ801444	pH 6.560 °C	> 60% activity after 6 h at 50 °C	42	Prasad and Palanivelu, 2012
Thermoascus aurantiacus var. *levisporus*	399	pPIC9 K *Pichia pastoris* EF608144	pH 8.0 50 °C	95.3% activity after 60 min at 50 °C	48.4	Li *et al*., 2010
Chaetomium--thermophilum	402	pPIC9 K *Pichia pastoris* EU697741	pH 5.560 °C	96.7% activity after 60 min at 60 °C	47.3	Li *et al*., 2010
Glaciozyma antarctica PI12	404	pPICZaA *Pichia pastoris* GS115JF901326	pH 4.0 15 °C	Retained activity from 5 to 25 °C	50	Ramli *et al*., 2011

genes in heterologous hosts is summarized in **table 2**. In recent years, genome sequencing of some model extremophilic fungi has been completed, such as *Myceliophthora thermophila* and *Thielavia terrestris* (Berka *et al*., 2011). By genome analysis, new and more chitinase genes have been found. Genome analysis of *M. thermophila* ATCC 42464 and *T. terrestris* NRRL 8126 reveals the presence of at least 18 and 26 sequences encoding glycosyl hydrolase family 18 proteins.

THREE-DIMENSIONAL (3D) STRUCTURE

Structural information of extremophilic chitinase is scarce. However, structure of a novel cold adapted chitinase, CHI II, from psychrophilic *Glaciozyma antarctica* PI12 has been modelled based on a predictive method known as comparative modelling or homology modelling. Homology modelling develops a useful 3D model for a protein's structure in the absence of an experimentally determined structure. Due to low sequence identity to other experimentally determined structures, the structure of CHI II was determined by threading which involves the comparison of an amino acid sequence to a library of known folds (Ramli *et al*., 2012).

The structural prediction of CHI II is based on the catalytic domain of chitinase A1 from *Bacillus circulans* WL-12 (1ITX). The secondary structural alignment between CHI II and 1ITX showed a good alignment at 8 α-helices and 9 β-strands. The CHI II 3D model has features similar to other family 18 chitinases like two signature sequences (XXGG and DXXDXDXE) corresponding to residues 90–96 and 125–132 in CHI II lying along the barrel strands 3 and 4 (S3 and S4) that help to form the active site cleft on the carboxyl end of the β-barrel (**Fig 4a**). The proposed catalytic residues for the modelled CHI II structure are Asp125–Glu132. Site-directed mutagenesis studies have shown that the glutamate in the DXXDXDXE signature motif in family 18 chitinases acts as the catalytic acid while all aspartates in the DXXDXDXE motif contribute to catalysis (Synstad *et al*., 2004). Another common feature is the conserved $(\beta/\alpha)_8$ fold or TIM barrel (an alternating pattern of α-helices and β-strands within a single domain) in the catalytic domain of CHI II. The TIM-barrel structure is known as a general folding motif of family 18 chitinases.

Two small β-domains, attached at the top of the α/β–barrel domain, which provides a deep cleft for substrate binding, are commonly present in microbial chitinase enzymes such as those from *B. circulans* (Watanabe *et al*., 2003) and *Serratia marcescens* (Perrakis *et al*., 1994). CHI II chitinase lacks β -domain 1 and was found to have only β -domain 2 in the predicted structure. β-domain 2 includes the chitinase insertion domain (CID) region. CID is also known as an (α + β) insertion in the TIM-barrel catalytic domain. The CID in CHI II is formed by an insertion between β -strand 7 (S7) and α -helix 7 (H7) comprised of residues 255–357. It contains six β-strands and one helix and forms a deep substrate-binding cleft that is assumed to be important in processive hydrolysis of the chitin chain (Watanabe *et al*., 2003). The TIM-barrel domain plus the CID domain can bind long chain substrates by providing a deep substrate binding cleft, which may not be the case for enzymes containing only the TIM-barrel domain (Li and Greene, 2010).

The CHI II 3D model also revealed a few conserved and exposed aromatic residues, including Trp92, Trp204, Trp210 and Tyr362 (**Fig. 4b**). Based on the comparison of the CHI II chitinase with the catalytic domain of from *Bacillus circulans* WL-12, it was suggested that these surface-exposed aromatic residues are important for crystalline chitin hydrolysis and for guiding the chitin chain to the catalytic cleft so that effective catalysis can take place (Ramli *et al*., 2012).

Though the overall structure of the extremophilic enzymes resemble their mesophilic counterparts, several structural

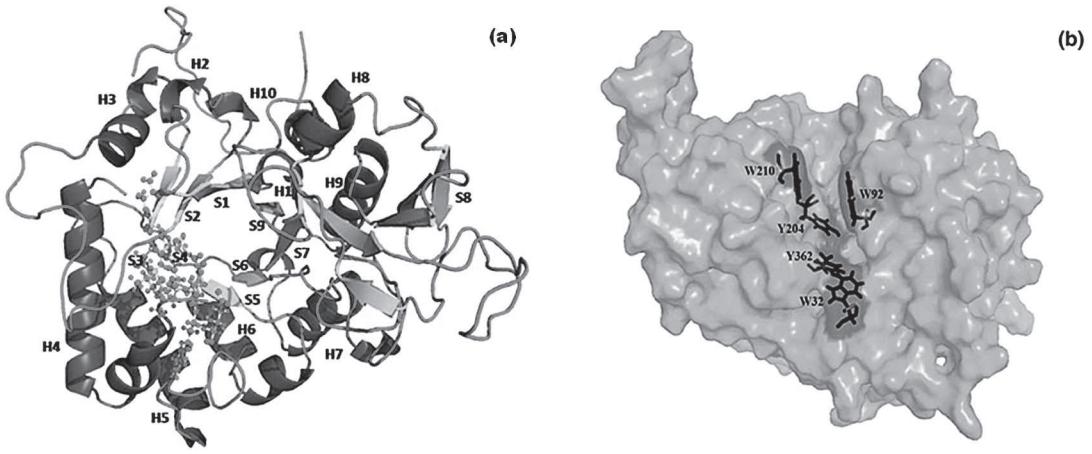

Fig. 4. (a) 3D model of CHI II showing β strands (S1-S9) and α helices (H1-H10). The two signature sequences are represented in balls and sticks and (b) Substrate binding cleft of CHI II showing the positions of aromatic amino acids (Ramli *et al*., 2012).

alterations like substitutions in the amino acid sequence or change in the number of protein-protein or protein-solvent interactions, contribute towards flexibility and stability of protein at lower and higher temperature, respectively. Psychrophilic enzymes possess higher structural flexibility compared to their mesophilic and thermophilic counterparts (Russell *et al.,* 1998). The reduced aromatic residue content and a longer hydrophilic loop (at residue 261–284) in the CID of CHI II contribute to its adaptation to low temperatures (Ramli *et al.,* 2012).

A multiple alignment of mesophilic, thermophilic and psychrophilic chitinases revealed that in cold adapted CHI II, proline at many places is substituted by hydrophobic amino acids and glycine and alanine replace different amino acids. In addition, a comparative structural analysis of CHI II with mesophilic chitinase from *Coccidioides immitis* revealed a reduction in the number of salt bridges, fewer hydrogen bonds and an increase in the exposure of the hydrophobic side chains to the solvent in CHI II (Hollis *et al.,* 2000). Together these adaptations in CHI II results in increased flexibility of the enzyme, allowing for an adaptation to low temperatures (Ramli *et al*., 2012).

CONCLUSIONS

Exploration of chitinases from extremophilic fungi is limited to those that can withstand temperature extremes. A systematic characterization of chitinases from thermophilic fungi is called for understanding their thermostability and evolutionary relationships to their mesophilic counterparts. As compared to extremozymes from extremophilic bacteria and archaea, the understanding of the nature and mechanisms of extremophily of proteins from fungi is very limited. The exploration and characterization of chitinases from extrememophilic fungi and understanding of structural features related to their extreme stability is necessary for developing better and more versatile chitinases for biotechnological applications.

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