Review Article

Production, purification and properties of fungal chitinases—A review

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After cellulose, chitin is the second most abundant organic and renewable polysaccharide in nature. This polymer is degraded by enzymes called chitinases which are a part of the glycoside hydrolase family. Chitinases have many important biophysiological functions and immense potential applications especially in control of phytopathogens, production of chito-oligosaccharides with numerous uses and in treatment and degradation of chitinous biowaste. At present many microbial sources are being explored and tapped for chitinase production which includes potential fungal cultures. With advancement in molecular biology and gene cloning techniques, research on fungal chitinases have made fast progress. The present review focuses on recent advances in fungal chitinases, containing a short introduction to types of chitinases, their fermentative production, purification and characterization and molecular cloning and expression.

Keywords: Chitin, Chitinase, Fungal sources, Gene cloning, Purification and Characterization

Introduction

Chitin, a poly-β-1,4-*N*-acetylglucosamine (NAG), is the most abundant polysaccharide found in nature after cellulose, and is biocompatible, biodegradable and bioabsorbable[1]. Henri Braconnot, a French professor of natural history, discovered chitin in 1811 after the discovery of a "material particularly resistant to usual chemicals" by A. Hachett, an English scientist in 1799. The presence of nitrogen in chitin was demonstrated by Lassaigne in 1843[2]. In nature chitin exists in two conformations as α chitin, where the arrangement of individual polymeric chains is in antiparallel fashion or as β chitin, where it is in a parallel fashion. Chitin is the main component of arthropod exoskeletons and connective tissues, fungal cell walls and is also found to a certain extent in other marine organisms. Among the best characterized sources of chitin are shellfish (including shrimp, crab, lobster, and krill), oyster, and squid. About 29.9, 1.4, and 0.7 million tons of chitin is harvested from shellfish, oyster and squid respectively per year. The chitin contents in crustaceans usually range from 2-12% of the whole body mass. Up to 75% of waste generated from processing of shellfish consists of chitin. The chitin content from processing waste on the basis of dry weight is 13-26% for crabs, 14-42% for shrimps and 34-49% in case of krill[3,4]. In the natural state chitin is found to be complexed with proteins, lipids, pigments and minerals such as calcium carbonate[4]. Hence demineralisation and deproteinisation of the chitin containing biomass is necessary to obtain chitin in the pure form. Traditionally this is carried out using concentrated acids or alkalis, which poses corrosion, disposal and safety related environmental problems. The alternative to this is processing of the chitin wastes using low-cost chitin degrading enzymes. The production of chitinolytic enzymes from various microbial sources and use of these enzymes for various applications including the production of NAG and its oligomers have been reported[4,19].

Chitinases are chitin hydrolysing enzymes which have gained interest in different biotechnological applications. This is due to their ability to degrade chitin in the fungal cell wall and insect exoskeleton, due to which they are used as antimicrobial or insecticidal agents useful in biocontrol of plant pathogens. Another useful application of chitinase is for bioconversion of chitin, into pharmacological active products, namely *N*-acetylglucosamine and chito-oligosaccharides. These are useful as antimicrobials, immunoenhancers which activate the host defence system, drug delivery carriers, antioxidants, in haemostasis and wound healing, blood cholesterol control and food preservation[1,3,4]. Other interesting applications include the preparation of protoplasts from filamentous fungi and the production of single cell protein[3]. Cloning, expression and characterization of

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chitinases from various microorganisms, including bacteria and fungi have been reported[3,16].

Sources of chitinase—Chitinases are synthesized in a wide range of organisms including viruses[3], bacteria[5], fungi[6], insects[7], higher plants[8] and mammals[9]. Most organisms (bacteria, plants and insects) have large families of chitinases with distinct functions, including digestion, defence against pathogens, cuticle turnover and cell differentiation.

Plant chitinases—In plants, chitinases are produced as pathogenesis-related proteins which are induced by the attack of phytopathogens and confer the plant with self defence ability against such pathogens. Chitinases can also be induced by application of chitooligosaccharides and plant growth regulators like ethylene which forms the basis of pathogen biocontrol in plants[8]. Chitinases inhibit the growth of fungi synergistic with other enzymes like β-1,3-glucanase in different plant varieties like rice, wheat, potatoes, tobacco, citrus fruits, beans, tomatoes, corn, yam and peas[10]. Chitinases have been utilized for biological control of insect pests on transgenic plants either alone or in combination with other insecticidal proteins[6,10]. These chitinases have also been shown to have a role during embryogenesis, seed germination and growth of seedlings[8]. The chitinases of plants are generally endochitinases of smaller molecular weight than the chitinases present in insects. Their size ranges from 25–40 kDa[5].

Insect chitinases—Chitinases or chitinase-like proteins have been found in all insect species belonging to different orders including dipterans, lepidopterans, coleopterans, hemipterans and hymnopterans. The chitinases present in the insects have been described from *Tribolium castaneum, Bombyx mori, Manduca sexta, Culex pipiens, Apis mellifera* etc[6,7]. The production of enzymes in insects is regulated by hormones during the transformation of the larvae. These enzymes play important roles as degradative enzymes during ecdysis, where the cuticle is degraded to chitooligosaccharides by endochtinases, which is further hydrolised by exoenzymes to N-acetyl-glucosamine. This is then salvaged to synthesize a new cuticle. Insect chitinases also have a defensive role against their own parasites[7,11]. The molecular weight of these chitinases ranges from 40–85 kDa. Chitinases are also found in crustaceans like shrimps, krills and prawns[6].

Mammalian chitinases—All known mammalian chitinases belong to the GH18 family and are further subdivided as true chitinases with chitinolytic activity and chitinase like proteins which only have chitin binding ability without any enzymatic activity[12]. Chitotriosidase was the first human chitinase to be identified which was produced by macrophages in Gaucher patients[9]. Since this enzyme showed antifungal properties, it was proposed that this enzyme may be involved in defence against chitin-containing pathogens. After the discovery of chitotriosidase, a second chitinase named acidic mammalian chitinase (AMCase) was isolated by Boot *et al.*[13] and shown to be expressed primarily in the gastrointestinal tract and lung of both mouse and human. Based on its expression profile, it was proposed to have a dual function in innate immunity and chitin digestion[12]. Recent studies performed on several bat species suggest that AMCase may actually be involved in chitin digestion in insectivorous mammals[14].

Microbial chitinases—Chitinases are widely distributed in bacteria such as *Serratia, Chromobacterium, Klebsiella, Bacillus, Pseudomonas, Clostridium, Vibrio, Arthrobacter, Beneckea* and *Aeromonas*[6]. Among *Actinomycetes, Streptomyces* species are good sources of bioactive compounds including antibiotics, enzymes etc. Production of chitinolytic enzymes has been identified in various *Streptomyces* sp., including *S. antibioticus, S. griseus, S. plicatus, S. lividans, S. aureofaciens* and *S. halstedii*[15]. Yeasts and fungi with yeast-like growth forms have low numbers of chitinases. Chitinase genes have been identified in *Saccharomyces cerevisiae, Candida albicans, Kluyveromyces lactis* and the dimorphic fungi-yeast *Paracoccidioides brasiliensis*[16,17]. Chitinases are found in filamentous fungi including *Trichoderma, Oenicillium, Penicillium, Lecanicillium, Neurospora, Mucor, Beauveria, Lycoperdon, Aspergillus, Myrothecium, Conidiobolus, Metharhizium, Stachybotrys* and *Agaricus*[5,6,16,17].

The possible reasons why fungi produce chitinases have been proposed and discussed[16–18]. The chitin polymer is insoluble in aqueous media and is complex having high molecular weight. Hence uptake of chitin as such and its degradation within the cell by microorganisms is not possible. For this purpose they secrete enzymes with different specificity so as to transform or hydrolyse chitin from different exogenous chitin sources and use the degradation products as source of nutrients. The fungal cell wall is predominantly composed of chitin and hence

chitinases play an important role in cell wall remodelling during the fungal life cycle, hyphal growth and extension, branching, hyphal fusion and autolysis. Chitinases are also produced as competition and defence mechanism against other fungi or to colonise other arthropods including insects (entomopathogenic fungi) or nematodes (nematode-trapping fungi).

Classification of chitinases

The classification of chitinases is based on the mode of action as proposed by International Union of Biochemistry and Molecular Biology (IUBMB, 1992) and CAZy (http://www.cazy.org/) into two broad categories. First one is endochitinase (E.C. 3.2.1.14), which randomly hydrolyses the chitin polymer to generate soluble low molecular weight polymers. The other one is exochitinase, which consists of two categories, chitobiase (E.C. 3.2.1.29), which catalyze the progressive release of di-acetylchitobiose starting at the non-reducing end of the chitin and β-N-acetylglucoaminidases (E.C. 3.2.1.52), which successively remove NAG units from the non-reducing end of the products generated by endochitinases and chitobiases with higher affinity to the latter[6,19,20]. Based on amino acid sequence similarity they can be grouped into glycosyl hydrolase families (GH) 18, 19 and 20 which are structurally unrelated[21]. Fungal chitinases predominantly belong GH family 18 which also consists of chitinases from bacteria[6,20]. Family 19 chitinases have mostly been identified in plants. Family 20 includes the β-N-acetylhexosaminidases from bacteria like *Vibrio harveyi, Streptomycetes*, and humans[3]. The catalytic mechanism of chitinases family 18 involves substrate-assisted catalysis, which retains the anomeric configuration of the product. Family 18 chitinases are mainly inhibited by allosaminidin[6,8].

Fungal chitinases can be divided into three major subgroups, namely, A, B and C, based on the amino acid sequences of their GH 18 modules. These subgroups differ in the architectures of their substrate-binding cleft and, thus, their catalytic activities (exo *vs.* endo) and also contain different carbohydrate-binding modules (CBM)[3,17]. Subgroup A chitinases contain a catalytic domain, but no CBMs, and have an average molecular mass of 40–50 kDa. Subgroup B chitinases have variable sizes and domain structures with molecular mass ranging from 30–90 kDa. The smaller proteins (30–45 kDa) generally contain CBMs while the larger proteins (~90 kDa) are bound to the plasma membrane. Subgroup C is a novel subgroup of fungal chitinases which typically are of 140–170 kDa range and have a chitin-binding domain (CBM 18). The presence of CBMs in the enzymes enables them to bind more tightly to the insoluble chitin substrate which enhances their processivity[20].

Production of fungal chitinases

Fungal chitinase has been produced predominantly by submerged fermentation (SmF) process. In addition to this, solid-state fermentation (SSF) has also been carried out for the production of chitinase. SmF has advantages in process control and easy recovery of extracellular enzymes but the products are dilute as compared to those obtained by SSF. Use of cheap raw material and the relative easiness of process operations are advantages of SSF, although problems are encountered with substrate sterilization, temperature and *p*H control, maintenance of culture purity and length of the process. Higher titres of chitinase enzyme were produced by SSF method using *Verticillium lecanii* as compared to SmF[22].

Generally, chitinase produced from microorganisms is inducible in nature. It is generally observed that presence of chitin in the production medium enhances the chitinase yield to a great extent. Colloidal chitin is the best inducer of chitinase enzyme in comparison to other sources of chitin[23,24]. Chitin at a concentration of 1-1.5% led to the maximal production of chitinase[24,25]. The production of extracellular chitinase is influenced by various physical parameters including pH, incubation temperature, aeration and media components like carbon sources, nitrogen sources and other micronutrients. In SSF the substrate type like agricultural residues including wheat bran, rice bran, chitin flakes, waste products obtained from different marine organisms like crabs, shrimps and prawns etc.[23,26,27,28] influence the chitinase production.

Addition of carbon sources other than chitin was found to have a mixed effect. The addition of glucose and maltose showed an increase in the chitinase production as reported by Sandhya *et al.*[25] for *Trichoderma harzianum*.

The time of incubation has a profound effect on the production of chitinase, in that the production levels increases to a certain maximum level over a period of time after which it decreases with further incubation. The main reason for decrease in production may be the depletion of nutrients in the fermentation medium. It may also be due to production of inhibitory

products in the medium resulting in the inactivation of enzyme secretory system or degradation of the enzyme itself. As reported by Ali and Ibrahim[29] for *Chaetomium thermophilum*, Sandhya *et al.*[25] for *Trichoderma harzianum* and Wasli *et al.*[30] for *Trichoderma virens*, maximum chitinase production was observed within 96 h of incubation. But in case of *Alternaria alternata*, chitinase production peaked at 7th day[24], while the best chitinase production was attained after 8 days for *Thermomyces lanuginosus*[29]. In case of SSF, 96 h was required for maximum chitinase production by *Trichoderma harzianum*[23], while 120 h of incubation was required for *Verticillium lecanii*[22]. A strain of *Trichoderma harzianum* gave maximum chitinase activity for rice bran after incubation for 6 days[28]. But in case of *Penicillium aculeatum*, chitinase production peaked at 72 h[27].

Nitrogen sources play a crucial role in the synthesis of enzymes involved in various primary as well as secondary metabolic pathways. Supplementation of different nitrogen sources in the chitinase production medium and their effects on the enzyme production has been elucidated. Yeast extract has been reported to be the most favourable organic nitrogen source for chitinase production by *Trichoderma harzanium*[23]. Both peptone and tryptone remarkably increased the chitinase production by *Trichoderma harzanium*[25]. In case of *Trichoderma harzianum*[28] and *Basidiobolus ranarum*[31] a combination of peptone and malt extract resulted in highest chitinase production. Corn Steep Liquor was used as the only nitrogen source for chitinase production by *Penicillium janthinellum* by Fenice *et al*[32]. The inorganic nitrogen source sodium nitrate caused the highest chitinase production as reported by Sharaf[24] for *Alternaria alternata* and Binod *et al.*[27] for *Penicillium aculeatum*, while addition of ammonium sulphate produced the highest chitinase by *Trichoderma virens*[30].

Inoculum type also influences the chitinase production period and level. Mycelial inoculum resulted in faster and higher chitinase production as compared to spore inoculum for *Trichoderma harzanium*[25] and *Verticillium lecanii*[22]. But in general spore suspension was used as inoculum for production of chitinase[24,27,28].

The initial *p*H and the incubation temperature play an important role in the production of chitinase. It is generally observed that fungal chitinase production takes place at a more acidic *p*H (less than 6)[30,33,34]. However exceptions were observed in *Basidiobolus ranarum*[31] and *Beauveria bassiana*[26] where the optimal *p*H was 9.0 and 9.5 respectively. In case of SSF, the generally used substrates being agro-residues which possess very good buffering capacity, adjustment of the medium *p*H is not necessary[23]. *p*H optimisation of the substrate was carried out by Sudhakar and Nagarajan[28] and by Suresh and Chandrasekaran[26]. For both SSF and SmF, chitinase production is generally highest in the mesophilic temperature range of 25-35 °C (Table 1). An exception to this was *Beauveria bassiana*[33], where optimum temperature for chitinase production was reported to be 40 °C.

The particle size of the substrate also plays an important role in microbial growth and product yield during SSF. An optimal particle size has to be determined to balance between the surface area and the inter-particular space of the substrate for efficient growth as well as mass transfer and gaseous exchange. A particle size of 425-600 μm was optimised by Suresh and Chandrasekaran[26] for maximum chitinase production. Similarly the initial moisture content also influences the chitinase production by SSF. Moisture content of 60-75% was found to be optimum for most of the fungal chitinases[22]. Bioprocess optimisation using statistical methods has also been applied for achieving highest possible chitinase enzyme production. The optimisation of medium was carried out by Sudhakar and Nagarajan[28] for maximum chitinase production by *Trichoderma harzianum* using combination of Plackett Burman Design (PBD) and Central Composite Design (CCD) while Ghanem *et al.*[35] achieved a 1.81 fold increase in chitinase production by *Aspergillus terreus* using PBD and Box Behnken Design. Similar bioprocess optimisation using CCD was done and 1.81 fold and 7.71 fold increase was observed in the chitinase production by *Trichoderma virens*[30] and *Basidiobolus ranarum*[31] respectively.

Whole cell immobilisation methods have been applied for production of chitinases. Conidiospores of the fungus *Penicillium janthinellum* P9 were reported to be immobilised on polyurethane sponge and chemically-modified macroporous cellulose carrier by Fenice *et al.*[36] and cultivated for the production of chitinolytic enzymes in repeated-batch process and continuous process. There was negligible decrease in enzyme activity after repeated use of the immobilised carrier. Also, increased chitinase yield was obtained for the continuous process in fluidised bed reactor system.

Table 1—Fungal Chitinase production

Microorganism	Method of Production	Initial *pH* of medium	Incubation temperature (°C)	Substrate	Yield	Application	Reference
Alternaria alternata	SmF	5.0	30	Colloidal chitin	6.41 U/mL	Biocontrol against fruitfly larvae	24
Aspergillus fumigatus	SmF	5.0	32	Flake chitin	N.S.	N.S.	37
Aspergillus fumigatus	SmF	7.0	37	Colloidal chitosan	5.86 U/mL	N.S.	38
Aspergillus terreus	SmF	6.0	30	Fish scale waste	4.31 U/mL	N.S.	35
Basidiobolus ranarum	SmF	9.0	25	Colloidal chitin	3.47 U/mL	Biocontrol (antifungal) agent	31
Beauveria bassiana	SSF	9.5	27	Prawn waste	248.0 U/gds	N.S.	26
Beauveria bassiana	SSF	5.0	40	Wheat bran supplemented with silkworm chrysalis	126 U/gds	N.S.	33
Colletotrichum gloeosporioides	SmF	N.S.	28	Colloidal chitin	14.93 U/mL	N.S.	39
Fusarium Oxysporum	SSF	6.0	30	Wheat bran supplemented with chitin flakes	23.6 U/gds	N.S.	40
Gliocladium catenulatum	SmF	4.5	30	Sclerotia powder	230.84 U/mL	Biocontrol (antifungal) agent	34
Monascus purpureus	SmF	7.0	25	Shrimp and crab shell powder	191 U/mL	Biocontrol (antifungal) agent	41
Penicillium aculeatum	SSF	5.0	30	Wheat bran supplemented with chitin flakes	12.53 U/gds	Production of NAG	27
Penicillium janthinellum	SmF	4.0	24	Colloidal chitin	0.69 U/mL	N.S.	32
Rhizopus oryzae	SmF	N.S.	30	Starch	3.56 U/mL	N.S.	42
Talaromyces flavus	SmF	6.0	28	Colloidal chitin	6.2 U/mL	Biocontrol (antifungal) agent	43
Trichoderma aureoviride	SmF	4.7	28	Colloidal chitin	0.036 U/mL	N.S.	44
Trichoderma harzianum	SmF	5.5	30	Colloidal chitin	14.7 U/mL	N.S.	25
Trichoderma harzianum	SSF	4.5	30	Wheat bran supplemented with colloidal chitin	3.18 U/gds	Biocontrol (antifungal) agent	23
Trichoderma harzianum		6.0	35	Rice bran	66.5 U/mL	N.S.	45
Trichoderma virens	SmF	4.0	30	Colloidal chitin	0.147 U/mL	N.S.	30
Trichothecium roseum	SmF	6.0	28	Crab shell chitin	0.78 U/mL	Biocontrol (antifungal) agent	46
Verticillium Chlamydosporium	SmF	N.S.	24	Colloidal chitin	0.095 U/mL	Biocontrol (Plant parasitic nematode) agent	47
Verticillium suchlasporium	SmF	N.S.	24	Colloidal chitin	0.0975 U/mL	Biocontrol (Plant parasitic nematode) agent	47
Verticillium lecanii	SmF/SSSF	6.0	25	Shrimp waste silage (SMF), Sugarcane bagasse supplemented with shrimp waste silage (SSF)	966 U/gds (SmF), 1674 U/gds (SSF)	N.S.	22

N.S. = Not specified

Expression and production of chitinases using molecular cloning methods

Many naturally occurring organisms have proved to be major sources of chitinolytic enzymes, but for being an industrially potent strain or effective pathogen resistance, increased levels of enzyme production are always desirable. Molecular cloning methods are being effectively exploited to achieve

overproduction of chitinases by improving the organism through introduction of stable genetic modifications.

A *gpd-Bbchit1* construct, in which the chitinase encoding *Bbchit1* gene driven by the *Aspergiullus nidulans* constitutive promoter, was transformed into the genome of *B. bassiana*, and three transformants that overproduced Bbchit1 were obtained by Fang et al[48]. Insect bioassays revealed that overproduction of Bbchit1 enhanced the virulence of *B. bassiana* for aphids increasing the mortality level of the latter.

Many expression systems were developed for chitinase production, by cloning the chitinase genes isolated from other microorganisms[49]. The chitinase cDNA was isolated from a mycoparasitic fungus *Tricothecium roseum* by Pan et al[50] by PCR amplification and the chitinase was expressed as a fusion protein in *Escherichia coli*. The recombinant *E. coli* efficiently yielded a high amount of active chitinase with high antifungal activity. Similar overexpression of chitinase gene from *Trichoderma atroviride* in *E. coli* without its signal peptide and with an N-terminal His-tag was produced as inclusion bodies, from which the active chitinase was recovered using a simple refolding procedure[51]. Kopparapu et al[52] cloned the chitinase gene from the thermophilic fungus *Paecilomyces thermophila* in *E. coli* which expressed it as an intracellular soluble protein. To obtain enough active chitinase for performing *in vitro* functional analysis, *Bbchit1* gene from *Beauvaria bassiana* was expressed in *E. coli* and *P. pastoris* by Fan et al[53]. The yield and the specific activity of the extracellular chitinase (Bbchit1) produced by *P. pastoris* were significantly higher than that of the refolded Bbchit1 from *E. coli* produced as inclusion bodies which indicated *P. pastoris* was a convenient expression system for the efficient production of Bbchit1. Similarly an endochitinase gene *ech42* from *Trichoderma atroviride* was cloned and expressed in *Pichia pastoris* using a constitutive expression system and the recombinant protein was secreted into the culture medium as glycoprotein[54], while two thermostable chitinase genes were cloned from two thermophilic fungi, *Thermoascus aurantiacus var. levisporus* and *Chaetomium thermophilum* and expressed in *P. pastoris*[55]. The chitinase gene from the thermophilic fungus, *Thermomyces lanuginosus* was cloned and overexpressed in *Saccharomyces cerevisiae* and the recombinant chitinase was produced as soluble secreted protein[56]. Similarly a gene coding for chitinase

from *Trichoderma asperellum* was cloned and expressed as a fusion protein in *Saccharomyces cerevisiae* and extracellular chitinase production occurred after 12 h of induction with 2% galactose[57].

The presence of a chitin-binding domain (ChBD), plays an important role in the biocontrol activity of the fungal chitinase activity against plant pathogens. With this in view, Limon et al[58] engineered hybrid chitinases with stronger chitin-binding capacity by fusing a ChBD from *Nicotiana tabacum* and a cellulose-binding domain from *Trichoderma reesei*. The chimeric chitinases had higher hydrolytic activity than the native chitinase on high molecular mass insoluble substrates such as ground chitin or chitin-rich fungal cell walls. They were more effective at inhibiting the growth of phytopathogens like *Rhizoctonia solani*, *Botrytis cinerea* and *Phytophthora citrophthora* than the wild type[59]. Similarly several *Beauvaria bassiana* hybrid chitinases were constructed by Fan et al[60]. Of these the one with a ChBD derived from the *Bombyx mori* chitinase fused to the *B. bassiana* chitinase showed the greatest ability to bind to chitin. Constitutive expression of this hybrid chitinase gene by *B. bassiana* reduced time to death of insect hosts by 23% in comparison to the wild-type fungus.

Disease resistance in transgenic plants has been improved, by the insertion of a chitinase gene from a biocontrol fungus. The gene encoding a strongly antifungal endochitinase was transferred to tobacco and potato from the mycoparasitic fungus *Trichoderma harzianum* and high expression levels of the fungal gene were obtained. The selected transgenic lines were highly tolerant or completely resistant to the foliar pathogens including *Alternaria alternata*, *Alternaria solani*, *Botrytis cinerea*, and the soil borne pathogen *Rhizoctonia solani*[61]. Overexpression of endochitinase genes of *Trichoderma harzianum* in transgenic tobacco, conferred broad tolerance to biotic and abiotic stress including resistance to fungal and bacterial pathogens, salinity and heavy metals[62]. Transgenic expression of chitinase genes from *Trichoderma virens* in cotton plants resulted in an elevated defensive state and increased resistance to the pathogen *Rhizoctonia solani*[63,64].

Purification and characterisation of fungal chitinases

In mycoparasitic fungi, the purification and characterisation of the chitinolytic system of *Trichoderma* species has widely been reported

including endochitinases, chitobiosidases and N-acetyl-glucosaminidases[65-69]. The purification of chitinases from other mycoparasitic fungi has also been reported, such as *Fusarium chlamydosporum*[70], *Stachybotrys elegans*[71], *Talaromyces flavus*[43], *Trichothecium roseum*[46], *Colletotrichum gloeosporioides*[39], *Rhizopus oryzae*[42], *Aspergillus fumigatus*[37,38] and *Penicillium janthinellum*[72]. In entomopathogenic fungi, N-acetyl-D-glucosaminidase, endochitinase, and exochitinase have been purified and characterized from *Beauveria bassiana*[48] and *Metarhizium anisopliae*[73]. Only two chitinases from the nematophagous fungi *Verticillium chlamydosporium* and *Verticillium Suchlasporium* have been purified and characterized[47].

The purification of fungal chitinases to homogeneity is carried out by different methods including fractional ammonium sulphate precipitation, ion exchange chromatography, hydrophobic interaction chromatography, gel filtration chromatography, chitin-affinity chromatography, and isoelectric focusing (IEF).

Most chitinolytic fungi have been found to produce more than one kind of chitinase. Many studies report *Trichoderma harzianum* to produce several individual chitinases including N-acetyl-glucosaminidases (102 and 73 kDa), endochitinases (52, 42, 33 and 31 kDa) and chitobiosidase (40 kDa)[16]. Similarly at least six different chitinases have been reported to be produced by the entomopathogenic fungus *Metarhizium anisopliae*[16]. *Talaromyces flavus* produces at least two kinds of chitinases[43]. Two exochitinases and an endochitinase are produced by the mycoparasite *Stachybotrys elegans*[71]. By gel filtration chromatography Binod *et al.*[74] showed four chitinase fractions of different molecular masses ranging from 27 to 82 kDa. Similarly two chitinases of *Penicillium janthinellum* were purified by preparative isolectric focussing having molecular weights of 45 and 35.4 kDa[72]. Four chitinase isozymes were reported from *Moniliophthora perniciosa* a phytopathogen of *Theobroma cacao*[75]. These multiple chitinases act in a mutually synergistic way and hence have a complementary effect between them.

Most purified fungal chitinases have been characterized. The characteristics mainly contain molecular weight, isoelectric *p*H, optimal *p*H, optimal temperature, thermostability, *p*H stability, effect of different metal ions and inhibitors, and antifungal activity.

Molecular masses of fungal chitinases generally range from 30-50 kDa (Table 2) with few of them having higher masses like that of 81 kDa for *Monascus purpureus*[41] and 104 kDa for *Aspergillus fumigatus*[38]. The isoelectric *p*H of the fungal chitinases ranges within 3–8[16].

Most of the purified fungal chitinases have a similar optimal *p*H and show moderate to high activity within the *p*H range of 4–8 (Table 2). Similarly the *p*H stability of the fungal chitinases varies within the range of *p*H 4–8. However as reported by Jung *et al.*[38], an *Aspergillus fumigatus* chitinase showed a comparatively broader range of *p*H stability from *p*H 2 to 10 while a chitinase of *Penicillium janthinellum* showed a *p*H stability in an acidic range of *p*H 2 to 4.5[72]. The optimum temperature for most fungal chitinases are within 40-50 °C and show moderate activity within the range of 30-70 °C (Table 2). Also the thermal stability is seen generally upto 50 °C. However, chitinases from thermophilic fungi including *Thermomyces lanuginosus*[56,76], *Rhizopus oryzae*[42], strains of *Trichoderma*[67,69] and *Gliocladium catenulatum*[34] exhibit high optimum temperature at 55-60 °C and thermostability of up to 50 °C.

Metal ions play an important role in biological catalysis by forming complexes with the enzymes and maintaining or disrupting the 3 dimensional structure and configuration[77]. Ca^{2+} was observed to enhance chitinase activity in many reports[34,42,66,76,78]. Mg^{2+} enhanced activity of chitinase produced by *Penicillium* sp.[78], *Anaeromyces* sp. and *Orpinomyces* sp.[79], and *Paecilomyces thermophila*[52]. Wang *et al.*[41] reported that Fe^{2+} enhanced the activity of the chitinase produced by *Monascus purpureus*. Activity of chitinase obtained from *Penicillium* sp.[78] was also enhanced by Mn^{2+} while that from *Trichoderma harzianum* was enhanced by both Mn^{2+} and Cu^{2+} [80]. Ba^{2+} was reported to enhance activity of chitinases produced by *Thermomyces lanuginosus*[76] and *Rhizopus oryzae*[42]. The major inhibitors of chitinase was Hg^{2+} [38,39,52,66,76,78,79] since it reacts with -SH groups found in cysteine residues in the protein chain and disrupts the tertiary structure. The other inhibitory metal ions include Ag^{2+} [34,37,66,76], Cu^{2+} [34,38,42,78], Zn^{2+} [34,37,41,78], Fe^{2+} [66,70,76,78] and Co^{2+} [39,67]. Similarly detergents like sodium dodecyl sulfate and solvents like acetone alter the three dimensional structure and cause denaturation of protein and thus loss in activity of the chitinase. SDS strongly inhibited enzyme activity as observed for chitinases from *Rhizopus oryzae*[42], *Anaeromyces* sp. and *Orpinomyces* sp.[79], and

Table 2—Properties of fungal chitinases

Organism	pH		Temperature (°C)		Molecular Mass (kDa)	Sp. Activity (U/mg)	Reference
	Optimum	Range	Optimum	Range			
Anaeromyces mucronatus	6.5	2.5 to 8	40	20 to 50	N.S.	50.2 pkat/mg	79
Aspergillus fumigatus	5	4 to 8	60	45 to 60	46	3.93	37
Aspergillus fumigatus	6	3 to 6	50-60	35 to 70	104	9.19	38
Chaetomium thermophilum	5.5	5 to 6	60	40 to 70	47	6.09	55
Colletotrichum gloeosporioides	7	4 to 8	50	30 to 55	43	413 U/ug	39
Fusarium chlamydosporum	5	4 to 7	40	30 to 50	40	200	70
Gliocladium catenulatum	5.0-6.0	4 to 10	60	20 to 70	51	12.17	34
Metarhizium anisopliae	5	3.5 to 7	37	N.S.	60	5.13	73
Monascus purpureus	7	5 to 8	40	30 to 50	81	1.02	41
Orpinomyces sp.	6.5	2.5 to 8	50	40 to 60	N.S.	95.6 pkat/mg	79
Paecilomyces thermophila	4.5	4 to 6	50	35 to 55	43.7	N.S.	52
Penicillium sp. LYG 0704	5	3 to 8	40	30 to 60	47	12.3	78
Penicillium aculeatum	5.5	4.5 to 6	50	40 to 65	82.6, 33.9 and 29.1	8	74
Rhizopus oryzae	5.5-6.0	5 to 8.5	60	50 to 70	50	65.2	42
Talaromyces flavus	5	4 to 8	40	20 to 40	41	5.6	43
	6	4 to 9	40	20 to 40	32	4.3	
Thermoascus aurantiacus	8.0	6 to 10	50	40 to 60	48	26.19	55
Thermomyces lanuginosus	4.5	3 to 9	55	50 to 60	48	35.51	76
Thermomyces lanuginosus	6.5	5 to 7	60	30 to 70	42	N.S.	56
Trichoderma atroviride	4	3 to 7	40	10 to 60	42	N.S.	54
Trichoderma harzianum	4	3 to 6	60	30 to 60	36	N.S.	67
Trichoderma reesei	5	6 to 8	55	30 to 55	46	71.5	69
Trichoderma viride	3.5	3 to 6	50-55	30 to 60	28	1.7	66
Trichothecium roseum	5	4 to 8	40	20 to 40	39	64.1	46

Paecilomyces thermophila[52]. Ethanol, methanol and acetone reduced the chitinase enzyme activity of *Monascus purpureus*[41]. EDTA which is a chelating agent capable of binding metal ions in solution has been reported to reduce the activity of chitinases produced by *Colletotrichum gloeosporioides*[39], *Trichoderma viride*[66], *Monascus purpureus*[41] and *Thermomyces lanuginosus*[76]. But it had negligible effect on chitinases produced by *Aspergillus fumigatus*[37,38] and an activity enhancing effect was seen on the chitinase produced by a *Penicillium* sp.[78]

Many reports prove the efficacy of fungal chitinases in degrading fungal cell walls and inhibit fungal growth *in vitro*. Two chitinases purified from *Talaromyces flavus* had activity against cell wall of *Verticillium dahliae, Sclerotinia sclerotiorum* and *Rhizoctonia solani*, and inhibited spore germination and germ tube elongation of *Alternaria alternata, Fusarium moniliforme,* and *Magnaporthe grisea*[43]. In another study, the chitinase produced by *Gliocladium catenulatum* was found to inhibit the conidial germination, hyphal growth and sclerotial germination of various plant pathogenic fungi including *Sclerotinia scleotiorum*, *Rhizoctonia solani*, and *Botrytis cinerea*[34]

while chitinase produced by *Trichoderma harzianum* was found to inhibit the growth of *Sclerotium rolfsii*[80]. Similarly chitinases have been purified from other fungi including *Monascus purpureus*[41], *Tricothecium roseum*[46] and *Fusarium chlamydosporum*[70] and shown to have biocidal activity against a large number of fungal pathogens. In addition, chitinases from thermophilic fungi are also promising for biocontrol and enzymatic conversion of chitin because of their thermostability[34].

Conclusions

Chitin is one of the underutilised bioresources in the world even though it is available on large scale especially from the marine waste. With novel advances in scientific research, the production of chitin polymer degrading/modifying chitinases which produce chitooligosacharides and NAG which are in turn applied in various fields of chemistry, biomedical, biotechnology, agriculture and environment protection is increasing. The production of fungal chitinases using the SSF method though practiced is still limited. A wide scope for extensive research to achieve industrial scale production of the enzyme by SSF still exists. Utilisation

of chitinous wastes as substrate in SSF is conducive both economically and environmentally. Research in expression and production of fungal chitinase in plants using molecular cloning methods is a useful tool in their protection from phytopathogens. The aim of further research should be in the discovery of novel chitinases, especially from fungi growing in extreme environments, determining biological functions of chitinase in plant pathogen interactions, regulation mechanism between different chitinases in the same organism, and engineering of chitinases with specific properties.

Acknowledgement

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