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Journey of enzymes in entomopathogenic fungi

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ABSTRACT

Entomopathogenic fungi are well-known biological control agents of insects that have broadly replaced the chemicals used in biopesticides for agricultural purposes. The pathogenicity of entomopathogenic fungi depends on the ability of its enzymatic equipment, consisting of lipases, proteases and chitinases, which degrade the insect's integument. Additionally, the researchers studied the content of β-galactosidase, -glutaminase, and catalase within entomopathogenic fungi. With highly focused investigations on the use of enzymes for green technology, the group of entomopathogens are slowly gaining applications in these areas, even as phytopathogenic fungi (disease originator). This brief review will serve as a reference of the enzymes derived from entomopathogenic fungi and of their current and potential applications.

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1. Introduction

The footprint of enzyme-mediated processes has been observed even in ancient civilization. Today, of the approximately 4000 known enzymes, 200 enzymes are used commercially. Until the 1960s, total annual sales of enzymes were only several million dollars, but since then, the market has grown spectacularly [1,2]. Today, an increasing number of enzymes are produced affordably as a result of a better understanding of production biochemistry, bioprocess technologies, and protein engineering. Depending on environmental factors such as pH and temperature, one particular enzyme can catalyse different transformations, and as a result, the commercial use of enzymes has continued to increase. Twelve major producers along with 400 minor suppliers fulfil the industrial enzyme demand globally, but not many investigations have been proposed on the development of robust lipase bioreactor systems for commercial use [3]. Enzymes of fungal origin are more preferable due to the easy removal of cells during downstream processing. In recent years, microorganisms have been used as potential sources of industrially relevant enzymes, accelerating the interest in the study of extracellular enzymatic activity in several microorganisms [4]. Many investigations and studies have been focused on the discovery and characterization of novel and naturally occurring enzymes from sources that have been overlooked [5]. Searching for novel enzymes has renewed the interest in enzyme profiles of fungal strains following isolation from diverse and hitherto unexplored habitats. The entomopathogenic fungi are considerably vary in terms of their parasitic activity and virulence. The ability of a microorganism to invade into a host is called its degree of pathogenicity, and the ability to kill its host in controlled conditions is known as virulence. Evolution has the greatest impact on the host–pathogen relation. On the other hand, insects develop different strategies through the course of evolution for defending themselves from pathogens [5]. The fungi genera have showed evolutionary adaptations that allow them to invade through the immune system of insects to complete the infection process [6]. Recently new data have been revealed about genetic evolution that is responsible for the development of host resistance or pathogen virulence (shown in Fig. 1). Apparently, entomopathogenic fungi double their genes following replication; one copy of the replicated genes keeps the original properties, while the other copies get evolutionarily modified in respect to amino acids, stimulating a functional divergence. These important adaptations carry forward its evolution and persistence within the ecosystem, where the pathogen-host interaction occurs [7].

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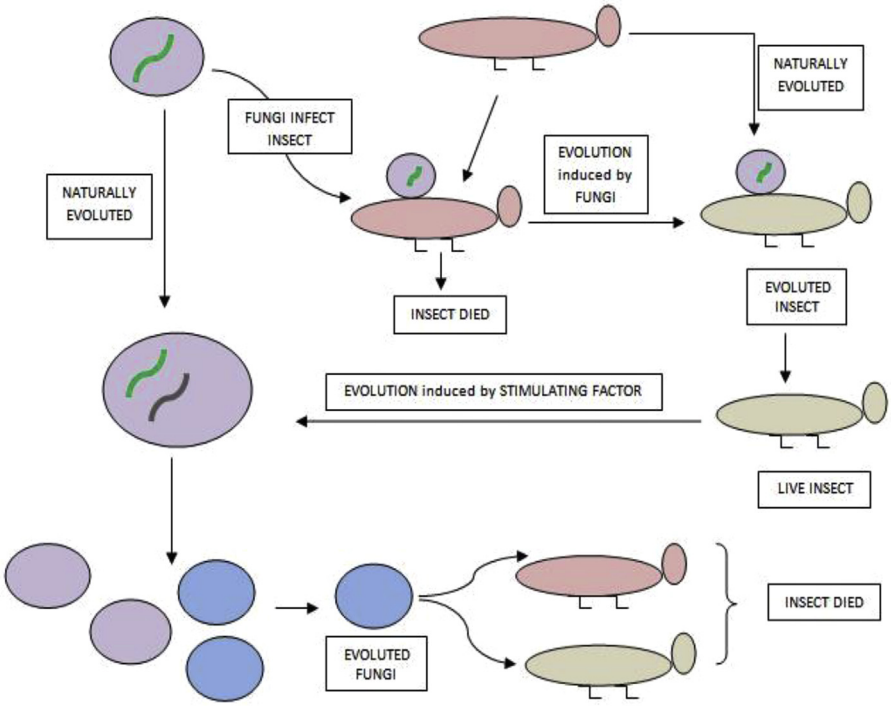

Fig. 1. Evolution of resistant fungi.

The present review discusses the evolutionary journey of many entomopathogenic microorganisms with variant enzymatic activity of different enzymes.

2. Occurrence and biology of entomopathogenic fungi

In broad terms, entomopathogenic fungi have life cycles that organise and adapt with insect-host stages and varying environmental conditions. Species and sometimes isolates within a species have been seen to behave differently depending on insect-host range, infection levels, germination rates and optimum temperature [8–10]. Members from the family of entomopathogenic fungi are generally considered to be opportunists that infect many species, including a range of insects, and host death is due to toxin production, which initiates defence responses [10,11]. Infection does not get in the way of the insect feeding system and movement, but conidia penetrate through a cavity in the abdomen of infected insects over a long period of time prior to death [8]. The families of Entomophthorales have biotrophic relationships with host insects following little or no saprophytism [11–14].

Entomopathogenic fungi are found amongst the families of Zygomycota and Ascomycota and in the class of Hyphomycetes in Deuteromycota [11], as well as in the families of Chytridiomycota and Oomycota. It is important to cite that fungal infections occur in other arthropods as well as insects and/or species that are not pests for cultivated crops. The asexually produced fungal spores or conidia that are largely responsible for infection are dispersed throughout the environment in which the insect hosts are present. When conidia get attached to the cuticle of a suitable host, they germinate following host recognition and activation of enzymatic reactions of the host as well as those of the fungal parasite [11]. The invasion of the insect body and circulatory system (hemolymph) starts once the fungus has penetrated the cuticle of the external insect skeleton. Structures and steps for the invasion of insect tissues include the formation of germ tubes, appresoria and penetration pegs [11]. The entomophthoralean fungi, with chitinuous walls resembling hyphal bodies, spread throughout the insect by penetration and utilization of host nutrients, which causes death of the host by physiological starvation 3–7 days after infection. Some entomophthoralean species initially form round-shaped protoplasts that either lack sugar residuesin the outer cell layers or veil their presence to evade recognition by insect haemocytes [8,11,15]. However, after the death of the insect host, the fungus has been found to appear from the dead host, and sporulation occurs outside the insect skeleton. Sporulation can occur internally when ambient humidity hinders external sporulation. *Metarhizium anisopliae* were seen to sporulate on the internal surfaces of the dried out hosts. The attachment structures signify that fungi continue to stay in the new hosts for subsequent transmission. Entomopathogenic fungi are found naturally; they can also be taken from the external environment and can be cultured in the laboratory. For example, the fungus *Beauveria bassiana* has been reported to exist naturally in more than 700 species of hosts [16]. However, it is quiet fortunate that *B. bassiana* is the most common natural host for almost all major insect taxa found in generally temperate regions. The fungal life cycle is defined by the infection of hosts, where the fungi build up a large population after producing huge numbers of conidia.

Entomopathogenic fungi play an important role in natural biological control agents for many insects and other arthropods and frequently behave as epizootics that significantly decrease host populations [17–20]. The significance of fungi in regulating the insect population was noticed early in recorded history by the ancient Chinese [10]. This was due to the existence of many natural epizootics and their distinct symptoms related to fungus-induced mortality [20,21]. Approximately 750 species of entomopathogenic fungi are known; among them, 85 genera have been found all through the classes of fungi [10,20,22,23]. The most important difference was observed in the method of infection; most entomopathogens infect hosts through their gut (shown in Fig. 2).

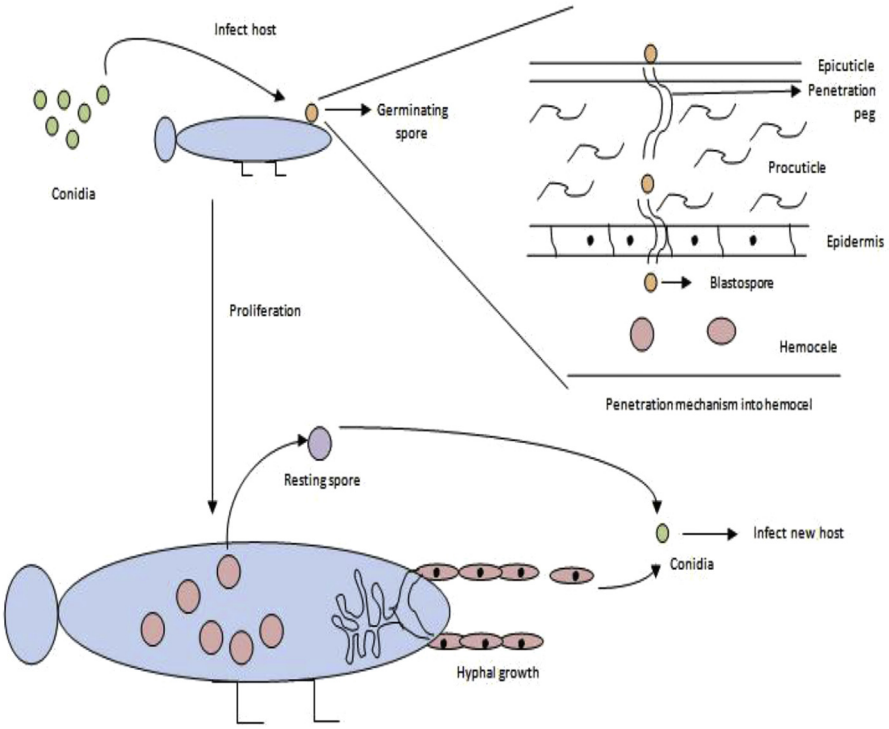

Fig. 2. Fungal pathogenicity against insects.

However, particular fungi belonging to the orders Hemiptera and Homoptera typically enter the insect cuticle and therefore are the most important pathogens identified to contaminate insects by means of sucking mouthparts [10].

The life cycle of entomopathogenic fungi on the insect cuticle begins with spore germination and penetration followed by vigorous proliferation of fungal hyphae, which ultimately desiccate the hosts, and continues with the production of infective spores that can insert themselves immediately into the cuticle to repeat the cycle, which requires a period of dormancy [24]. Adherence processes involve both physical and chemical interactions [25]. Epicuticular compounds such as fatty acids, amino acids, and glucosamines are assumed to play significant roles in regulating the specificity and pathogenicity of entomopathogenic fungi [26–29]. Moisture conditions favour spore germination, and possibly requires free water [30–33] in the absence of measureable precipitation [30,34]. The penetration of the cuticle by entomopathogenic fungi is accomplished by the development of the germ tube or by the formation of appressoria [10,26]. The process of vegetative reproduction in the insect haemocoel is common to most entomopathogenic fungi [10] and is usually described by distinct yeast-like structures or hyphal bodies. This type of growth allows the entomopathogen to invade and spread out rapidly followed by colonization of the host circulatory system and by increasing the surface area of typical filamentous fungal mycelium inside the nutrient medium. Several species from the order Entomophthorales produce vegetative protoplasts (cells without cell walls) within the haemocoel [35–37], which may help the pathogen detect the host immune responses. The length of the incubation period varies from species to species, and disease development during the vegetative propagation stage is typically temperature-dependent [18]. Fungi have been observed to elicit insect immune responses, but it is not known with gradually decreasing the release of mycoses [35,38,39]. According to the reported articles, both insects as well as entomopathogenic fungi were observed to manufacture a variety of proteases that degrade cuticle [40]. Smith and Grula, 1982 [28], also identified a wide range of trypsin, chymotrypsin, elastases, collagenase and chymoelastase as being responsible for cuticle degradation [35,39,41]. In this review we have described the reported enzymes responsible for fungal infections in insects.

2.1. Lipases

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are serine hydrolases that are widely studied for their physiological and industrial potential. These enzymes help to catalyse the hydrolysis of glycerol and long-chain fatty acids with synthesis of ester bonds. The activities of lipases are triggered only when adsorbed to an oil–water interface [42]. Since the 1980s, the charms of the biocatalyst properties of lipases have gained increasing demand in industry [43]. Lipases are also extremely versatile because they can catalyse numerous different reactions that are widely applied in multiple industries, such as in dairy and food manufacture, in the leather and detergent industries, in the production of cosmetics and pharmaceuticals and in organic synthesis reactions, especially reactions in non-aqueous media [44,45]. For example, in Brazil, there are large quantities of fat wastes from vegetable oil refineries that could be used as a carbon source in commercial lipase production.

Microbial lipases are the major type of commercially produced lipases. In recent years, studies on lipases, mainly of microbial origin, have increased because of the great potential of lipases in commercial fields. Secretion of fungal and bacterial lipases may be linked to ease of nutrient absorption from external nutrient sources, whereas pathogenic microbes secrete lipases in order to invade a new host. Fungi are one of the most important lipase sources for industrial applications because fungal enzymes are usually secreted extracellularly, simplifying the extraction from the fermentation media [46]. Various kinds of insect plagues have been controlled extensively by extracellular lipases produced by *M. anisopliae*, and

for this reason, it has become the most studied filamentous entomopathogen [47]. The type of lipase, along with the concentration of carbon and nitrogen sources and temperature play a substantial role in lipase production. In 2001, Sharma et al. described that different microorganisms pose different physiologies for lipase biosynthesis controlled by different regulating mechanisms [48]. Basically, lipase production in microorganisms is enhanced, and it varies not only by the lipid source but also by its concentration [49–51]. In experimental observations of Silva et al., the different lipid sources that best induced lipase production in *M. Anisopliae* were rice oil (4.46 ± 0.63 U/ml), soybean oil (4.26 ± 1.18 U/ml), olive oil (4.25 ± 0.69 U/ml), sunflower oil (4.23 ± 0.34 U/ml), sesame oil (3.51 ± 0.78 U/ml) and hydrogenated soybean fat (3.50 ± 1.00 U/ml) [52].

The epicuticle, the external layer of the insect's cuticle, is hydrophobic in nature and acts as the first barrier against microbial attack. A heterogeneous mix of lipids, long-chain alkenes, esters and fatty acids is the main constituent of insect cuticle. Lipases are responsible for the hydrolysis of ester bonds of lipoproteins, fats and waxes found at the interior part of the insect integument [53]. They significantly penetrate the cuticle and initiate nutrient release by breaking down the integument. The degradation of the epicuticle is followed by the production of fungal protease (Pr1), which degrades the proteinaceous material positioned in the procuticle [54]. A defence mechanism of insects has been identified and associated with the secretion of lactone B, which is responsible for the inhibition of lypolytic activity, which impedes subsequent entomopathogenic infection. Adhesion of the spores to the epicuticle with the help of lipase is a mandatory pre-step that initiates the degradation of fatty acids and alkenes in the cuticle waxy surface.

There are two types of lipases (Lipases I and II) that have been purified to homogeneity [55], using column chromatography on DEAE-Toyopearl. Lipase I consists of two polypeptide chains [chain A is small peptide in size, and it conjoins with a sugar moiety, whereas chain B is a large peptide chain of 34 kDa molecular weight]; Lipase II is a 30 kDa protein with a single polypeptide chain [55]. It was reported by Ohnishi et al., that an *Aspergillus oryzae* strain produced at least two kinds of extracellular lipolytic enzymes, L1 and L2. It was found that Lipase L1, a monomeric protein, has a molecular weight of 24 kDa, and it has the ability to cleave all ester bonds present in triolein [56].

Germination of conidia of *Nomuraearileyi* fungus was extensively enhanced when it was coupled with a lipase of 81.3 kDa secreted by *N. rileyi*. It improved the mortality of *Spodoptera litura* larvae [57]. Recently, cytochrome P450 monooxygenase (CYP52) has also been identified, which is important for the alkenes and insect epicuticle by breaking down the chain of hydrocarbons [58]. In *B. bassiana*, the *Bbcyp*52*x*1 gene encoded the lipase activity with an enzymatic complex known as CYP52X1. It has been proved that an additional combined activity of ω hydroxylase, which is capable of adding terminal hydroxyl groups in fatty acids and epoxides, is shown by CYP52X1 [59–61]. CYP52 fungal enzymes were found to be flexible; the presenting isoforms showed different activities and specificities in relation to the kind of alkanes and/or fatty acids [62], offering a great advantage to the entomopathogenic fungi to use these substrates as nutrients. Moreover, epicuticle degradation by the *Bbcyp*52*x*1 gene cluster of *B. bassiana* has been found during the initial stages of the infection. Nevertheless, after degradation of the cuticle, its role was no longer required. For this reason, the breakdown of lipid substrates by the entomopathogenic fungi occurs just at the time of cuticle penetration [59]. In *Metarhizium robertsii*, the MrCYP52 gene cluster from a cytochrome P450 subfamily has been recognised. However, being different from other entomopathogenic fungi, these fungi do not show fatty acids Ω-hydroxylation activity, and alkanes are mainly oxidized by the terminal methyl group, where the promoter region contains CREA sites, indicating that it is regulated by catabolite repression [63]. Dwayne and George showed that *B. bassiana* cultured *in vitro* produced lipase, but production was not seen until the early stationary phase because it was not induced until five days post-germination. In terms of an *in vivo* function, lipase may not be important for penetration of the integument, but it may be important for the next step of growth within the insect haemocoel, which has a lipid composition of 1.5–5.5% (w/v). *In vitro*, lipase production was induced by triglyceride substrates and was not inhibited by fatty acids present in the culture medium [64].

2.2. Proteases

Proteases build up a large group of hydrolytic enzymes that cleave the peptide bonds of proteins and break them into small peptides and amino acids. Since proteases play a role in almost cellular functions, they are found in plants and animals, as well as in microorganisms, including viruses. However, proteases are extensively present in nature, and microbes also serve as a preferred source of these enzymes [65]. If we take a brief view of the industrial enzymes, 75% are hydrolases and proteases from plant, animal and microbe sources, and they account for approximately 60% of total enzyme sales. Proteases are the enzymes that are considered as the most important for the infective process, and these characteristics and the potential industrial demand for these enzymes promote the production of these enzymes [66].

After the epicuticle has been broken down by lipases, the invading fungi produce great quantities of Pr1 (serine-protease), which degrades the proteinaceous material. On the other hand, further degradation of solubilised proteins in to amino acids by amino peptidases and exopeptidases is done to provide nutrients for entomopathogenic fungi [67]. The most frequently studied proteolytic enzymes are the subtilisin-like serine-protease Pr1 and trypsin-like protease Pr2. The Pr1 gene is related to eleven isoforms that have been identified and cloned, including a metallo protease [68]. The molecular structure of subtilisin-like protease Pr1 consists of five cysteines forming two disulphide bridges, and the residual cysteine was found near the catalytic triad made of Asp39, His69 and Ser224. The activities of Pr1 and Pr2 have been determined in *B. bassiana*, *M. anisopliae*, *Lecanicillium lecanni*, *Nomuraea rileyi* and *Metarhizium flavoviride* [69]. These proteases are secreted during the first cuticle degradation stage, and they stimulate the signal transduction mechanism by activating protein kinase A (PKA) mediated by AMPc [70]. It has been validated that the extracellular involvement of protease Pr1 in cuticle penetration is initialised by the infection of the cuticle [66,71]. Additionally, protease Pr1 was also found as a virulence indicator in entomopathogenic fungi [72]. High pathogenicity on *Heliothusarmigera* and cotton aphids has been observed by locally isolated entomopathogenic fungi grown on Sabouraud dextrose medium by laboratory bioassay methods. These experiments were designed to check similarities at the level of virulence-determining factors. VCP1 serine-like protease virulence factors, which are pathogenic toward both *Heliothus* and aphids, were grown in liquid medium at a large scale [73].

Recently, new strategies such as genetic engineering and many protease recombination have been employed to improve the virulence of entomopathogenic fungi. *M. anisopliae* has been transformed experimentally with the introduction of gene *aaIT*. A neurotoxin, regulated by the promoter of *mcl*1 and encoded by the *aaIT* gene of the *Androctonus australis* corpion, was expressed in its haemolymph [74]. Genetic modification of *Beauveria bssiana* has been done through the co-transformation of *Pr*1 with the aaIT gene

of *A. australis*. However, there was no synergistic effect observed because protease Pr1 was seen to degrade the AAIT protein [75]. For this reason, it was necessary to evaluate the interaction between the two kinds of proteins and the precise stage at which they were expressed. This meant, in order to degrade AAIT in the hymolymph, Pr1A should be expressed at the first stage of the infection. It was found that protease Pr1 could act in collaboration with chitinases for the cuticle degradation. So, an attempt was taken to construct a fused gene having both protease and chitinase activities. This transformation showed that an evident time reduction has been found in the insect's death, when the *cdep*1 gene encoded a *prl*A homologue co-transformed with the *Bbchit*1 gene in *B. bassiana* was compared to insect death due to non-protein breakdown [70]. In another case, cDNA of the protease gene CDEP was isolated from *B. bassiana* and fused with the *cry*1*Ac* gene of *Bacillus thuringiensis*, while the expression of the gene was regulated by the native promoter of the fungus. This experiment improves the insecticide activity over the *Helicoverpa armigera* larvae [76]. Of late, a Pr1 protease of 38 kDa that is highly homologous to serine protease (subtilisin type) secreted by *Lecanicillium* (*Verticillium*) *lecanii* was expressed following cloning into *Escherichia coli*; Pr1 protease showed an antifungal activity against diverse phytopathogenic fungi. This led to the exploiting entomopathogenic fungi to control plant diseases [77].

According to Bagga et al., for any fungus, this is the highest number of subtilisin that assures the cuticle degradation of insects of different orders for its nutrition [78]. The analysis of three entomopathogenic fungal genomes demonstrated that *B. bassiana, Cordyceps militaris* and *Metarhizium* spp. independently evolved the lineage. The genes of proteases and chitinases were expressed for pathogenesis in these three types of entomopathogenic fungi and showed similar evolutionary patterns [79,80]. *Lecanicillium* spp. overcame the cuticle barrier of insects during the penetration process with the help of Pr1 protease and its isoforms, which have an advantage on the basis of regulation and substrate specificity [81]. As a result, a relatively high number of isoforms of subtilisin in *M. anisopliae* are identified by evolutionary similarities [78]. The diversity in the isoforms offered entomopathogenic fungi an advantage of being capable of infecting insects of different orders.

In the process of enzymatic degradation, the type of chymoelastase serine protease Pr1 served as a major cuticle-degrading enzyme because 70% of the cuticle was composed of protein. Pr1 has been reported to show considerable ability to degrade cuticle [82], with a high concentration at the site of the penetration peg [83,84]. The pathogenicity of *V. Lecanii*, which was related to various characteristics of growth and enzymatic activities, was marked by the production of extra cellular chitinases as well as high germination and sporulation rates and was virulent against the aphid *Macrosiphoniella sanborni* [85]. *Verticillium lecanii* also produced and diffused toxins with insecticidal properties [86,87]. *M. anisopliae* was also reported to release several extracellular cuticle-degrading proteinases produced by PR1, a determinant of pathogenicity. Paterson et al. reported that the proteinaceous component(s) of insect cuticle was capable of inducing PR1 production [88].

It was reported by Shiv Shankar et al., that *Beauveria* sp. that was grown in glucose-yeast extract medium was supplemented with 2% mustard seed cake at 28 °C, 180 rpm for 3–4 days produced protease, and the crude enzyme broth was used for purification. The *Beauveria* sp. MTCC5184 secreted two alkaline proteases in the crude culture filtrate, and the major protease, BAP (protease from *Beauveria* sp. MTCC 5184), was purified to homogeneity. Specific activity was determined to be highest in 40–50%, 50–60%, and 60–70% fractions of the ammonium sulphate precipitation process. Unwanted proteins were removed by the precipitation of crude broth at 0–40% in large-scale process purification, and further precipitation of the supernatant at 40–70% was performed to obtain partially purified protease. The partially obtained protease was then subjected to carboxy methyl-cellulose column chromatography, where the protease was tightly bound. Approximately 56% of the protease was eluted in un-adsorbed fractions, as they are not bound to the matrix, while all the impurities bound to and remained in the matrix. Therefore, after a final purification step with a 10-fold purification, the specific activity of the protease increased to 60.39 U/mg from 6.025 U/mg. A total yield of 38.6% for purified protease was reported [65,89]. Similarly, protease from *B. bassiana* was purified by 5-fold purification with a recovery of 22% [90].

Production of the proteases have also been carried out in bioreactors. Rao et al. composed an optimum medium for protease production consisting of the following components (in%, w/v): 0.72 shrimp shell powder, 0.60 soy powder, 0.19 sucrose and 0.68 yeast extract. Theoretical protease production, with applied methodology, of 281.14 U/ml was observed in 7 days of cultivation. Further studies were carried out in a 5 l stirred-tank and 30 l airlift bioreactors for production. In a 5-L stirred-tank bioreactor, maximum protease activity of 238.77 U/ml was attained in 6 days of incubation when the aeration rate and agitation speed were controlled at 0.6 vvm and 150 rpm, respectively, whereas in a 30-L airlift bioreactor, the maximum protease activity was 283.84 U/ml with controlled an aeration rate of 0.9 vvm operated in bubble column mode. It was observed that the enzyme activity attained in the 30-L airlift bioreactor was close to that obtained from the shaker-flask cultivation study (280.72 U/ml) in 5 days of cultivation [91]. *Isaria fumosoroseus* isolate IF28.2 secretes extracellular proteases (Pr1 and Pr2), which were investigated as a function of carbon source (with effect on diamondback moth cuticle). The supernatant containing 1% glucose with 1% diamond back moth cuticle presents the highest level of Pr1 and Pr2 activity (18.83 ± 1.25 and 12.44 ± 1.36 U/mg/hr, respectively). When diamond back moth cuticle was supplied as a nutrient source to depressed mycelia, the maximum Pr1 production was observed in the supernatants. Additionally, the maximum Pr2 activity of depressed mycelia has been observed in the cultures containing chitin as the source of basic nutrients [92].

2.3. Chitinases

Chitin is a combined polymer of β-1,4 N-acetyl glucosamine and is one of the most abundant polymers in nature after cellulose [93]. It was considered the main structural component of fungal cellular walls and of exoskeletons of invertebrates [94]. Chitinases hydrolyse the β-1,4 bonds of chitin polymer, producing a predominant N, N′-diacetylchitobiose. This is carried out by the breakdown of N-acetyl glucosamine (GlcNAc) monomer by chitobiose. Chitinases are widely distributed in plants, bacteria, fungi, insects and vertebrates [95]. They collaborate with proteases to degrade the insect's cuticle [96] and are associated with different stages of the life cycle (germination, hyphal growth, morphogenesis, nutrition and defence against competitors) of entomopathogenic fungi [97]. The genome of filamentous fungi contains chitinases responsible for various physiological functions including: a) chitin degradation in the fungal cellular walls or in the exoskeletons of arthropods used as nutrient sources; b) remodelling of cell walls during hyphae growth, branching, hyphae fusion, autolysis and competence; c) also, protection from other fungi located in the same ecological niche [97,98].

The chitinases are classified into GH (glycoside hydrolase) families 18 and 19 [99,100]. GH (glycoside hydrolase) Families 18 and 19 have no difference in their three-dimensional structures and

resemble chitinase classes III and V produced by bacteria, fungi, virus, animals and plants [101]. The first molecular characterization was made for chitinase from *M. anisopliae* and was the *chit*1 gene [102]. A substantial homology has been found among *Chit*1, a 42 kDa protein, and chitinases of *Aspergillus album* and *Trichoderma harzianum*. Moreover, the *chit*2 gene of *M. anisopliae* codes for a 42 kDa chitinase protein that is controlled by catabolic repression [103]. The *chit*2 gene showed the same regulatory mechanism as proteases (signal transduction) [104]. The analysis of the *chit*2 gene expression profile indicated the presence of two different transcripts from RNA splicing; these transcripts encode two different proteins with different biological activities [105]. The expression of chitinase coded by the *chit*3 gene of *M. anisopliae* is dependent on the infection by *Rhipicephalus* (*Boophilus*) *microplus* tick [106]; it is considered to have specialised exochitinase and endochitinase enzymatic activities, both of which are involved in heat stress adaptation [107]. It was also proved that with protease Pr1, improved chitinase expression can be attempted through genetic engineering. For example, the *chit*1 gene of *M. anisopliae* was over expressed but with no increase in the virulence against *Manduca sexta* [108]. An alteration in the spores, hyphae and appressorium has been observed when a constitutive promoter is used for overexpression of the *chit2* gene of *M. anisopliae* [109]. Two types of chitinases, *chit*1 and *chit*2 genes, were isolated and characterised from *L. lecanii* [95]. A 40.9 kDa protein, coded by the *chit*1 gene, is composed of two functional domains for catalytic activities positioned at the extreme N-terminal end and a chitin binding domain (ChBD) found at the extreme C-terminal end. This gene was homologous to chitinases of *Hypocrea lixii* AAT37496, *Coniothyrium minitans* and *Aspergillus fumigatus*. With a prominent homology to the chitinases produced by *Aphanocladium album*, *Verticillium fungicola* and *B. bassiana,* this *chit*2 gene consisted of the same catalytic domain and ChBD sites. The *Bbchit*1 gene of *B. bassiana* coded for a protein with a molecular weight of 33 kDa, and it was also homologous to *T. harzianum* and *Streptomyces avermitilis* MA-4680 chitinases [110]. Nonetheless, it was not similar to chitinases produced by other entomopathogenic fungi, and they indicated that there were many differences among chitinases produced by these fungi. Meanwhile, the *Bbchit*1 gene was demonstrated to contain two ChBD binding sites; its chitinolytic activity increased by an evolution process directed by the construction of a series of variants. The variants SHU-1 and SHU-2 showed maximum enzymatic activity as result of the amino acid mutations outside of the catalytic and substrate binding regions [111]. The virulence of *B. bassiana* improved for silkworm mouth *Bombyx mori* with production from a recombinant *Bbchit*1 gene, constructed by fusing the *Bbchit*1 gene with the chitin binding domain (ChBD), under the regulation of the promoter with overexpression of chitinase and reducing the desiccation period of the infected insect [112]. Afterwards, a hybrid protein with the ability to increase the binding capability of protease to chitin if insect cuticle has been obtained by recombination of the ChBD fragment from *B. mori* with the *CDEP*-1 gene of *B. bassiana* was shown to have serine protease activity. This recombinant strain showed increased pathogenicity over *Myzus persicae* larvae due to the solubilization of protein components during insect cuticle degradation [113].

Chitinases accomplished critical functions in their process of growth and degradation of the fungal cell wall and insect cuticle, with chitin being a major component of both. Fungal virulence is often determined with the extracellular chitinases [114,115]. St Leger et al., reported the production of chitinolytic enzymes, *N*-acetyl-β-D-glucosaminidases and endochitinases, in *M. anisopliae*, *M. flavoviride*, and *B. bassiana* when growth media was supplemented with insect cuticle. *M. flavoviride* also secreted 1,4-β-chitobiosidases into the cuticle media. The chitinase from *M. anisopliae* comprised acidic proteins (pH 4.8) of 43.5 kDa and 45 kDa. Both bands and their respective N-terminal sequences were identified and found alike an endochitinase from *T. harzianum* [116]. Valadares-Inglis and Peberdy located significant induction of chitinolytic enzymes in enzymatically produced protoplasts, but no significant induction was observed from mycelia of *M. anisopliae*. Mainly, cell-bound chitinolytic activity was found in mycelia and protoplasts, but they show their activity in the membrane fraction [117]. cDNA of chitinase from *M. anisopliae* was grown in chitin-containing media as the sole carbon source [118]. The molecular mass was determined to be approximately 60 kDa, and the optimum pH was 5.0 [116]. The team investigated, cloned the chitinase gene (*chit*1) from *M. anisopliae* sf. *acridum* ARSEFstrain324 in *M. anisopliae* sf. *anisopliae* ARSEF strain 2575 *(Chit*1). Conversely, *M. anisopliae* sf. *anisopliae* ARSEF strain 2575 *(chit*1) produced chitinase earlier than the wildtype strain in chitin-containing medium. They proposed the necessity of the development of soluble chitin inducer following hydrolysis of chitin by *chit*1 for its production. However, the overexpression of chitinase did not expose any altered virulence to *M. sexta*, and this suggested that wild-type levels of chitinase were not limited for cuticle penetration [119]. In 2003, the expression and characterization of the chitinase was reported; it has a molecular weight of 42 kDa when produced by *M. anisopliae* in *E. coli* using a bacteriophage T7 (promoter based expression vector) [120]. Baratto et al. in their referred work, performed transcriptional analysis of the chitinase *chit*2 gene of *M. anisopliae* and showed that it has 1542 bp encoding 419 amino acids. The gene was interrupted by two introns and contained a signal peptide of 19 amino acids. It showed that CreA/Crel/Crr1-binding domains aided the up and down regulation of transcription of the *chit*2 gene by chitin and glucose, respectively [103]. It was found that the extracellular constitutive chitin deacetylase (CDA) produced by *M. anisopliae* converted chitin, a β-1,4-linked N-acetylglucosamine polymer, into its deacetylated form of chitosan (glucosamine polymer). Solubilised melanin generally cannot inhibit CDA [121]. The upstream regulatory sequences of 33 KDa of *Bbchit*1 was cloned based on the N-terminal amino acid sequence. The *Bbchit*1, which is present as a single copy in the *B. bassiana* genome, has no introns. CreA/Crel-binding elements are often found in the regulatory sequence of *Bbchit*, which controls carbon metabolism of fungi. A similarity was found among the amino acid sequences of *Bbchit*11 and those of the endochitinases of *S. avermitilis, Streptomyces coelicolor*, and *T. harzianum* (Chit36Y) [110].

2.3.1. Mechanisms of chitin degrading enzymes

Chitinolytic enzymes can be divided into *N*-acetylglucosaminidases and chitinases, which significantly differ in their breakdown patterns. *N*-acetylglucosaminidases (EC 3.2.1.52) catalyse the breakage of terminal, non-reducing *N*-acetylglucosamine (GlcNAc) residues from chitin. In general, the highest affinity of *N*-acetylglucosaminidases has been shown for N,N-diacetylchitobiosedimier $(GlcNAc)_2$, and they cleave it into two monomers [122]. According to the CAZy classification reported by Coutinho and Henrissat (Carbohydrate Active Enzymes database) [123], *N*-acetylglucosaminidases belong to glycoside hydrolase (GH) family 20. It is important that these enzymes not be referred to as exochitinases, which is has been incorectly reported in the literature. Chitinases (EC 3.2.1.14) are members of GH families 18 and 19 and catalyse the hydrolysis of the β-1,4 linkages of chitin and chitooligomers, resulting in the release of short-chain chitooligomers or monomers. GH families 18 and 19 do not share similar sequences; they have different three-dimensional structures [101,124–126] and different catalytic mechanisms. β-anomeric and α-anomers products were formed catalytically by GH 18 chitinases (retaining mechanism) [127]

and GH 19 chitinases (inverting mechanism) [128]. Furthermore, according to the cleavage patterns, chitinases can be grouped into endo- and exochitinases. Endochitinases degrade chitin from any point along the polymer chain, forming randomly sized chains of monomers, whereas exochitinases cleave from the end of the non-reducing chain and release $(GlcNAc)_2$ monomers. However, the enzymatic properties of chitinases were observed to be more complex but also more flexible than those reflected in the exochitinase andendochitinase classification. Another classification of chitinases by grouping them into processive and non-processive enzymes was done by *Serratia marcescens* [129–131]. Processive chitinases do not release from the enzyme-substrate complex after cleavage hydrolysis, but slide into the next cleavage site through the active site-tunnel. The presence of a carbohydrate binding domain enhanced its ability to move to the next cleavage site. Complete dissociation of enzyme from the enzyme-substrate complex after hydrolysis has been observed in the case of non-processive chitinases [129]. Chitinases were reported to contain various carbohydrate-binding modules (CBMs) that included different families of classically defined chitin- and cellulose-binding domains due to their preferred affinity for these carbohydrates [123]. Extensive data, classification and applications of these CBMs made them vital. In general, there are two mechanistic pathways for acid catalysed glycosyl hydrolysis that resulted in the following (schematic presentation Fig. 3):

1. Retention of the stereochemistry of the anomeric oxygenat C-1 position relative to the initial configuration or,
2. Inversion of the stereochemistry [132]. The mechanism proceeded with protonation of β-(1,4) glycosidic oxygen, and this lead to an oxocarbenium ion intermediate, which is also stabilised by an external carboxylate through covalent or electrostatic interactions. Water catalyses the nucleophilic reaction and generates hydrolysed products that retain the initial anomeric configuration. This is usually referred to as the double displacement mechanism. This resulted in the second most frequently discussed hydrolysis system (single-displacement reaction), with a water molecule that acts as a nucleophile. Hydrolysed products that were generated by Family 18 chitinases retained their anomeric configuration at the C-1 position. However, the X-ray crystal structure of two family 18 chitinases showed the absence of a second acidic residue in the active site that is capable of stabilizing the oxocarbeniumion. Thus, no consistency of the single- and the double-displacement mechanisms were observed with the structure and hydrolysed products. Additional experimental and theoretical evidence pointed to an oxazoline ion intermediate formed through a chimaera assisted by the neighbouring N-acetyl group [132].

2.3.2. Chitinase production and purification

Microbial chitinase has been produced by techniques of liquid batch fermentation, continuous fermentation, and fed-batch fermentation. Moreover, solid-state fermentation, along with biphasic cell systems, have also been used to produce chitinase. Extracellular chitinase production has been reported to be influenced by media components such as carbon sources, nitrogen sources, and agricultural residues such as rice bran, wheat bran, etc. [133,134]. The chitinase production was improved when glucose was used with chitin in the production medium. In contrast, Miyashita et al. reported that glucose has a suppressing effect on chitinase production [135]. Several other physical factors, such as pH and temperature, also affected chitinase production. Fungal chitinases were purified by different methods, such as ion exchange chromatography, fractional ammonium sulphate precipitation hydrophobic interaction chromatography, gel filtration chromatography, chitin-affinity chromatography and isoelectric focussing (IEF). From the value of fold purification of different fungal chitinases, the value of most purified chitinases was low [136–142]. This low value might be due to a synergistic action of the different isoforms of chitin degradation in crude supernatant and to the loss of chitinase activity during the purification progress [138]. Although many researches have been carried out on the production and purification of chitinases from fungi, the characterization of these enzymes has been limited to few fungi. In mycoparasites, purification was mainly focused on the chitinolytic system of *T. harzianum*. Several chitinases, including endochitinases, chitobiosidases and N-acetyl-glucosaminidases, from *T. harzianum* have been purified [136,137,143]. The purification of chitinases from other mycoparasites, such as *A. album* and *Gliocladium virens* [140], *Fusarium chlamydosporum* [144], *Trichothecium roseum* [141], *Stachybotry elegans* [145], and *Talaromyces flavus* [142] have also been reported. It has been observed that N-acetyl-D-glucosaminidase, endochitinase, and exochitinase are extensively produced by *B. bassiana* [110,146] and *M. anisopliae* [118,147] and those enzymes are subsequently purified and characterised for greater purposes. In contract to mycoparasitic fungi, among nematophagous fungi, only two chitinases from *Verticillium chlamydosporium and Verticillium suchlasporium* have been isolated with high purity and characterised [148]. Recently, chitinases of thermophilic fungi have been noticed for their thermostability, such as from *Thermomyces lanuginosus* [149]. Purification of chitinases from *Candidaalbicans*, *Sacchromyces cerevisiae* [150], and *Trichoderma asperellum* [151] was also reported to lead to the economical production of several useful products. Chitin and chitosan are the most abundantly present polymers of fungal cell walls. Biochemical analysis of chitin and chitosan provided specific information about their structures and cytochemical localisation, which determined the functional specialisation of these polymers. Benhamou and Asselin used wheat germ agglutinin–gold complex and chitinase–gold complex, as probes in order to detect GlcNAc residues in the fungal secondary cell walls [152]. Barley chitosanase was tagged with colloidal gold particles for the localization of chitosan in the spore and in hyphal cell walls of fungi. This technique was used to study the presence of chitosan in the cell walls of *Ophiostomaulmi* and *Aspergillus niger* [153]. Gold-labelled chitinase complexes have also been used for the immune cytochemical and cytochemical localisation of chitin and N-acetyl-D-glucosamine residues in a biotrophic mycoparasite.

2.4. β-galactosidase

To study whole-cell permeabilisation, the β-galactosidase activity in permeabilised blastospores was determined. Whole-cell permeabilisation resulted in the ability to extrapolate the *in vitro* enzyme concentrations to those found *in vivo* [154]. The method was selected on the basis of microorganisms and their compatibility with the permeabilisation system. The whole-cell permeabilisation for in situ assays has been achieved with organic solvents. The assay was performed by the chromogenic substrate method of MacPherson and Khachatourians with modified BS (blastospores) permeabilisation [155]. By using a solvent-based method, permeabilisation of fungi was developed first in yeast. A solution of toluene:ethanol was used as the permeabilising agent to study the levels of nitratereductase, glucose-6-phosphatase, alkaline/acid phosphatase, β-galactosidase, hexokinase and pyruvatekinase in *Saccharomyces cerevisiae* and *Candida albicans* [55,154,156–159]. Similarly, permeabilisation with a solution of

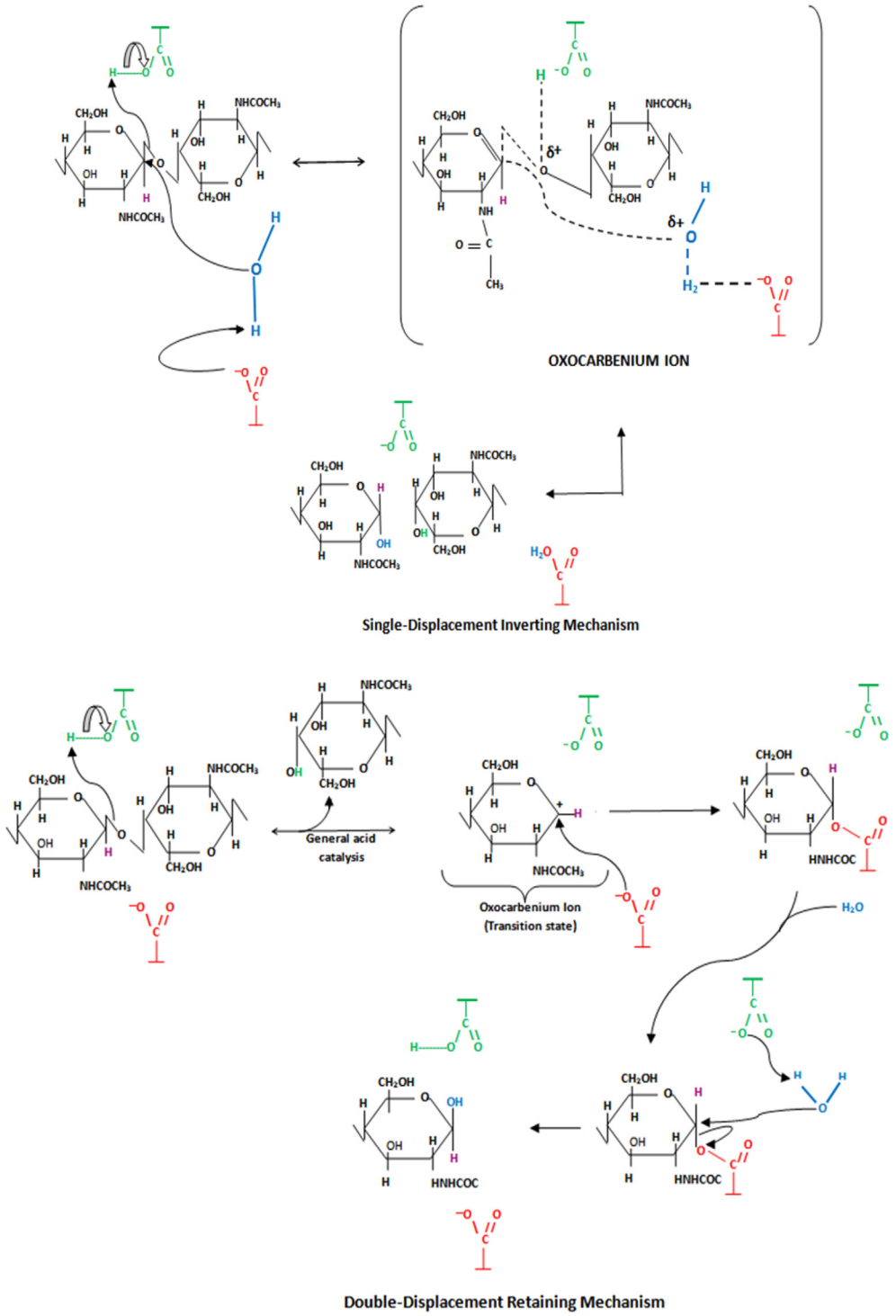

Fig. 3. The hydrolysis mechanism of displacement reaction proposed for the family 18 chitinase (Single displacement) and family 19 chitinase (Double displacement).

toluene:ethanol has proved advantageous for in situ enzyme assays of filamentous fungi [160]. Heating the cells in detergent solutions of 1–2% (v/v) has also been reported. However, the use of alcohols, antibiotics, detergents, ethylene diamine tetra acetic acid (EDTA) and methods such as osmotic shock, and freezing and thawing had also enabled in situ assays in yeast and filamentous fungi [158,161,162]. Comparatively, permeabilisation methods based on different cell treatments were recognized to be difficult because each method produced cells with different permeability alterations, each of which were suitable under different conditions. β-Galactosidase was partially purified from cell-free extracts of the entomopathogenic fungus *B. bassiana* by ammonium sulphate precipitation and CM-Sephadex chromatography followed by size fractionation of pooled β-galactosidase on Sephadex G-150. Enzyme activity was optimal at pH 6.6 to 7.0 at 50 °C, and rapidly decreased during incubation at 60 °C. Non-denaturing polyacrylamide gel electrophoresis indicated that a single polypeptide is responsible for β-galactosidase activity. The cations calcium or zinc were required for activity in addition to phosphate and potassium [163].

2.5. Catalase

The ability of entomopathogenic fungi to infect insects was found to be a complex process with variation in the expression of many genes. Most of the genes that have been associated with regulating host invasion and countering host defence have been identified from the analysis of *B. Bassiana*-expressed sequence tags [164,165]. Additionally, from EST (Express sequence tagging) analysis, cytochrome P450s have been identified as virulence-determinant genes that hydrolyse complex lipid in insect epicuticle [58,166]. However, proteases and chitinases have been the main competitors for overexpression of virulencein *B. bassiana*, whereas the contributions of catalases for the infection process in *B. bassiana* remain unknown. Thus, a PCR-based suppression hybridisation technique (PCR-SSH) was used to determine differential gene expression patterns during a 2-h infection period of *Spodoptera exigua* larvae with *B. bassiana* BCC2659. It was shown that death of the host required 3–8 days, which represented an initial contact period of those genes up-regulated during the first interaction with the host. The catalase gene (catE7) and ten other genes were found to be up-regulated within 2 h, and these results were confirmed by RT-PCR. After catalases were activated by stress and detoxification, the full-length catE7 gene of *B. bassiana* was cloned. The hyper-expression of BbcatE7 was examined by the insecticidal activity of the fungus. Under control of the constitutive G-protein domain promoter, BbcatE7 was transformed into wild-type *B. bassiana*. Transgenic *B. bassiana* strains over expressed catE7 and germinated faster than the wild-type, and insect bioassays revealed increased virulence. Meanwhile, the BbcatE7 over expressed strains were no more resistant than the wild-type to hydrogen peroxide stress. Among the set of up-regulated genes identified in this study, the catalase gene was considered to have a potential role in the pathogenic process and was chosen for further study [167].

Catalases are important for the virulence activity of plant pathogenic fungi. Catalases might respond to oxidative and other stresses, as well as participate in the remediation of toxic metabolites. Furthermore, bioinformatic analysis of BbcatE7 and its protein product suggests that it encodes a peroxisomal catalase enzyme. Such enzymes have been implicated as a virulence factors in *B. bassiana* cultivated in insect-like hydrocarbons, where *B. bassiana* showed increased catalase activity in peroxisomes, where lipids are degraded (β-oxidation) [61]. The experiments indicated that transgenic *B. bassiana* increased catalase (Bb::BbCatE7-2) activity relatively modestly by two fold; nevertheless, the transgenic *B. bassiana* displayed more toxicity for insects than the wild type. In addition, Bb::BbcatE7 strains germinated faster on PDA (Potato dextrose agar) media as well as on PDA containing H_2O_2 than the wildtype. The overall vegetative growth of the strains was similar in the presence and absence of H_2O_2 in media. The mechanism behind such an effect may be because increased catalase activity helped the conidia to overcome the hyper-oxidant stage faster than in the normal germination process [168]. It was assumed that catalase activity eliminated ROS (reactive oxygen species) and other host-derived toxins present on the cuticle when fungi invaded insect tissues and the haemoceol, and insects responded by production of hydrogen peroxide [169] and other reactive oxygen species when haemocytes phagocytosed the invaded fungi [170]. Catalase activity might also reduce insect defence capabilities, such as melanisation. Chantasingh et al. concluded that there was a relatively small increase in catalase activity (two-fold) that increased the efficacy of *B. bassiana* to target insect hosts. This reported result was used to screen for catalase in over expressed strains or for the more effective genetically engineered strains. Catalase activity was combined with other virulence determinants, including protease and chitinase activities, whose overexpression was known to increase the virulence of entomopathogenic fungi. Finally, additional catalase genes were found within *B. Bassiana*, and an investigation into their roles in fungal development and virulence was warranted [167].

2.6. L-Glutaminase

The use of nutritionally inert materials for solid state fermentation (SSF) makes it possible to accurately design media, to monitor process parameters and to develop scale-up strategies and various engineering aspects that ease the conventional SSF that uses organic solid substrates such as wheat bran [171]. This provides a homogenous aerobic condition to the medium throughout the fermenter and will not contribute impurities to the fermentation product [172]. It was reported earlier that polystyrene, a commercially available insulating and packaging material could be used as an inert solid media for the study of L-glutaminase activity in marine *Vibrio costicola* under SSF [173,174], while ion exchange resins and polyurethane foam have been used as inert carriers for SSF with fungi. In the present study, we evaluated the potential of *Beauveria* sp., an alkalophilic and salt-tolerant fungus that was isolated from marine sediment [175], for L-glutaminase, an exozyme product, using polystyrene as solid support under solid state fermentation. Hence, initially the various process parameters were optimized to deliver efficient L-glutaminase production by *Beauveria* sp. under solid state fermentation conditions. Enzyme production increased along with an increase in moisture content from 15.21 U/ml at 60% (v:w) to a maximum of 32.17 U/ml at 80% (v:w) [176]. Two optimal pH conditions were observed; at 27 °C, efficient L-glutaminase production that favoured high enzyme yield was observed at pH 6.0 (23.37 U/ml) and at pH 9.0 (23.96 U/ml). Most microbial extracellular enzymes were produced at high levels at a growth pH that is close to the optimal pH required for the maximal enzyme activity [177]. Incubation at 27 °C at pH 9.0, enhanced enzyme production (32.17 U/ml) compared to other temperatures considered. Nevertheless, a considerable level of enzyme production was obtained under other pH and temperature conditions. These factors are largely characteristic of the organism and vary for each species [178].

3. Molecular regulation of enzymes

Almost all lipases showed common structural architecture in a single protein domain. Exceptions were found in lipases from higher eukaryotes with complex functions, interaction molecular interactions and regulation. The genes encoding specific metabolic processes of lipases were found to be tightly regulated. The expression of lipolytic proteins was often seen to be induced and modulated by several parameters. The regulation of gene expression of lipase isoenzymes varied between enzymes isolated from different source organisms, and these lipases were encoded by a family of analogous genes. Usually, protein isoforms are closely related in sequence and biochemical features but are not identical, and differences can affect catalytic functions. Although there are variations in primary sequences, all lipases display the same structural architecture, the α/β hydrolase fold, with identical catalytic machineries. Lipases of known 3D structure are currently classified [179] into 7 families based on the elements of the basic fold: acetylcholinesterase-like, gastric lipase, lipase, fungal lipase, bacterial lipase, pancreatic lipase N-terminal domain, and cutinase-like. In α/β hydrolase, the active site has a catalytic triad that comprises a nucleophile, an acidic residue and a histidine [180]. The substrate hydrolysis follows a two-step mechanism. The active

serine acts as nucleophile enhanced by a proton transferred to the catalytic histidine with the formation of an ester bond. A tetrahedral intermediate was formed, and it carried a negative charge on the carbonyl oxygen atom and stabilised NH groups through hydrogen bonding. The proton on the histidine was transferred to the oxygen of the ester bond that cleaved and formed a covalent intermediate with the fatty acid from the substrate that was esterified to serine. The second step of the reaction was carried out by deacylation of the enzyme that hydrolysed the covalent intermediate. The proton was transferred from water to the active site serine and formed a hydroxide ion that attacked the carbonyl carbon atom in the covalently bonded substrate–enzyme intermediate. Further, the negatively charged tetrahedral intermediate became stabilized by hydrogen bonds to the oxyanion hole. Finally, histidine donated a proton to the oxygen atom of the active serine, and the acyl component was released [181].

All proteases catalyse the addition of water across amide (and ester) bonds, which are cleaved by a nucleophilic reaction on the carbonyl carbon of the covalent bond, which is easily broken by the enzyme. The exact mechanisms involved for cleavage and active site substituents vary widely for different protease subtypes. This provided the classification of proteases into serine proteases, cysteine proteases, metalloproteases, etc. [182]. Nevertheless, the substrate specificities for different proteases are diverse aspects of physiological activity; they all recognise internal peptide bonds or those of residues at the NH_2 or COOH terminus of the molecule, as well as residues at other amino- or carboxyltermi of the side chains of the bond to be cleaved. Proteases digested long protein chains into shorter fragments by breaking down the peptide bonds that link amino acid residues [183]. Proteases catalyse by one of two mechanisms: 1) Aspartic, glutamic and metallo proteases activate a water molecule, which hydrolyses the peptide bond with a nucleophilic reaction; 2) serine, threonine and cysteine proteases use the acatalytic triad. This residue covalently links protease to the substrate protein by a nucleophilic attack and releases the first half of the product. Furthermore, this covalent acyl-enzyme intermediate is hydrolysed to complete catalysis by releasing the second half of the product and the free enzyme [184].

Chitinase production by fungi is inducible in nature, and chitinase activities are generally detected when the fungi are grown in a chitin-containing medium [136,138,140,143,145]. It has been reported that chitinase gene expression in fungi is controlled by a repressor/inducer system, in which chitin or other products of degradation (such as *N*-acetyl-β-glucosamine) act as inducers, and glucose or easily metabolised carbon sources act as repressors. The regulation of chitinase genes *ech42* of *T. harzianum* (encoding 42 kDa endochitinase), *chit36* (encoding a 36 kDa endochitinase), *chit33* (encoding a 33 kDa endochitinase) and *nag1* (encoding *N*-acetyl-β- glucosaminidase) was studied in detail. The four genes were induced by fungal cell walls, colloidal chitin or in the absence of a carbon source [185,186], whereas the presence of high concentrations of glucose or glycerol inhibited their expression. Further studies showed that *ech42* gene transcription was also induced by physiological factors, such as low temperature and high osmotic pressure. Again, the mechanism for the regulation of *nag1* expression in *T. harzianum* was observed; it was induced by chitooligosaccharides of low molecular weight and by its own catabolic products (GlcNAc) [187].

The chit33 gene of *T. harzianum* was reported to be strongly repressed in the presence of glucose and expressed when absent [188]. In addition, the *N*-acetyl-glucosaminidase of *Trichoderma atroviride* was essential for the induction of *ech42* by chitin, but *chit42* was not induced by chitin in a mutant that did not express the gene *nag1* [189]. Glucose repression was studied as a common phenomenon in the regulation of fungal chitinase genes, and it is now better understood (Fig. 4).

4. Applications

The applications of enzymes from entomopathogenic fungi are wide-spread due their natural origin, their high specificity of action and their generally non-toxic nature. They work best under mild conditions of moderate temperature and near neutral pH and are also easily inactivated when the reaction has gone as far as desired. The following table (Table 1) shows a collection of different entomopathogenic fungi and the enzymes they produce.

Lipases are applied in organic chemical processing, in detergent processing, in the synthesis of biosurfactants, in the oleochemical industry, in the dairy industry, in the agrochemical industry, and in pharmaceutical processing. The required properties of lipases are already available, and continued efforts are still underway for commercializing biotransformation and synthesis [190]. Lipases used in detergents were found to be thermostable and remained active in the alkaline environment. The lipases were not often used

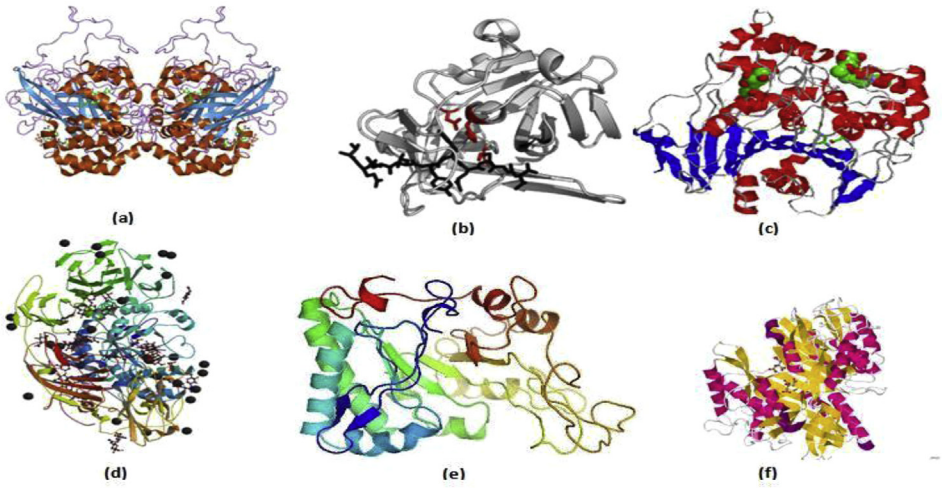

Fig. 4. Crystallography structure of (a) Catalase (EC 1.11.1.6); superfamily 7, (b) Protease (EC 3.4.22.44) with complexes of peptide substrate and active site triad residues, (c) Lipases (triacylglycerolacyl hydrolases, EC 3.1.1.3) built on an alpha/beta hydrolase fold and employed catalytic triad, (d) β-galactosidase (EC 3.2.1.23) each unit consists of 5 domains, (e) Computational structure prediction of *Beauveria bassiana* (AHA93892.1.), (f) L-Glutaminase (3.5.1.2) dimeric structure.

Table 1
List of entomopathogenic fungi with corresponding enzymes.

Enzymes	Entomopathogenic fungi
Lipase	*Fusarium oxysporum, Metarhizium anisopliae, Aspergillus flavus, Beauveria bassiana*
Protease	*Metarhizium anisopliae, Beauveria bassiana, Verticilium lecanii, Paecilomyces fumsoroseus, Isaria fumsoroseus, Tolypocladium niveum*
Chitinase	*Trichoderma atroviride, Trichoderma harzianum, Tichoderma virens, Metarhizium anisopliae, Beauveria bassiana, Nomurae rileyi, Aschersonia aleyrodis, Verticillium lecanii, Isaria fmosorosea*
β-galactosidase	*Aspergillus* spp., *Aspergillus foelidis, Beauveria bassiana, Aspergillus fonsecaeus, Aspergillus oryzae, Auerobasidium pullulans, Curvularia inequalis, Fusarium monilliforme, Metarhizium anisopliae, Metarhizium robertsii*
Catalase	*Lecanicillium muscarium, Fusarium oxysporum, Verticillum dahlia, Aspergillus phoenicis*
L-glutaminase	*Beauveria bassiana, Trichoderma koningii, Aspergillus flavus, Acremonium forcatum, Aspergillus wentii MTCC 1901, Trichoderma harzianum*

in oleochemical transformations [191] where γ-linolenic acid is processed, a polyunsaturated fatty acid (PUFA), or where others like astaxanthine (food colourant), methyl ketones (for flavoured molecules of blue cheese), and 4-hydroxydecanoic acid (fruit flavour) are processed [191]. Additionally, lipid esters were modified, including isopropyl myristate used in cosmetics, and monoglycerides were used as emulsifiers in food and pharmaceutical applications. Chiral and anti-inflammatory features in lipases have raised the importance and demand of these enzymes in efficient methods of biological activity [191,192].

Chitinases are mostly isolated from bacterial and fungal sources. They are generally suggested for use as agents for the biological protection of crops. Chitinolytic enzymes can be used as supplements for chemical fungicides from bacterial sources and as pesticides from fungal sources to increase their effectiveness against pathogens and to reduce the concentrations of these harmful chemicals [94,100]. Since chitinases are biodegradable and antifungal, they are also useful for environmental and agricultural uses, food technology and cosmetics. Chitin present in the solid waste from shellfish was converted to single-cell protein by chitinolytic enzymes. The breakdown of chitin by chitinase produced GlcNAc, chitooligosaccharides, and glucosamine, which has a very essential role in pharmaceuticals and acts against allergic inflammation [193]. NAG, the monomer obtained from the cleavage of chitin, is used in the manufacture of food products such as sweeteners, growth factors, chemicals, and pharmaceutical intermediates [194].

L-glutaminase (L-glutamine amidohydrolase EC3.5.1.2) has gained attention for its potential as an anticancer agent and as a flavour-enhancing agent in the food industry. It increased the glutamic acid content of the food, thereby imparting flavour [195]. Salt-tolerant and heat-stable L-glutaminase increased its efficiency and demand in the food industry [196]. The commercial importance of L-glutaminase stipulates the search for better-yielding viable source strains as well as economically viable bioprocesses for the large-scale production of the enzyme [173].

The selection of the appropriate protease complex by trial and error methods is necessary for specific applications. With the proper selection of enzymes, either limited proteolysis or complete hydrolysis of most proteins to amino acids could be obtained. Microbial proteolytic enzymes from different fungi and bacteria are available. With few exceptions, most fungal proteases tolerate and act effectively over a wide pH 4.0–8.0. Fungal protease has been used for centuries in the production of soy sauce and tamari sauce [197]. For industrial applications, microbial proteases are extracted from growth medium. One of the largest usages of fungal protease is in baking bread and crackers [198]. Cereal foods were treated with proteolytic enzymes, where proteins got modified and resulted in better processing and improved product handling and also increased their drying capacity. Proteolytic enzymes are basically used for tenderizing meats and animal casings for processed meats. Pharmaceutical and clinical applications of fungal proteases include their usage for digestive aids [199].

Catalases, essentially free of other enzymes, are obtained from fungal sources and are readily used for cold-sterilisation of milk for cheese processing [200]. Further, hydrogen peroxide is added to the milk to sterilise it, and catalase was used to remove the residual hydrogen peroxide before further processing the milk into cheese.

β-galactosidase is one of the significant enzymes used in food processing, offering nutritional, technological, and environmental applications [201]. Immobilizing enzyme allowed for enzyme reutilisation and resulted in increased activity in suitable microenvironments. The optimum pH range for the entomopathogenic fungal enzymes was determined to be 6.6–7.0 for processing of acid whey and its ultrafiltration permeate [155]. The immobilized enzyme showed up to 99% hydrolysis, and thus, it could be applied successfully for the hydrolysis of lactose in milk or whey. On the other side, thermostable enzymes have the unique ability to retain their activities at higher temperatures for consistent periods [163]. Thus, cold-active and thermostable enzymes have great potential and have drawn the attention of researchers.

5. Conclusions and perspective

The enzymes described in this review take part in a wide range of physiological processes, including morphogenesis, pathogenesis, parasitism, growth regulation, and immunity. The rapid advances in different techniques and interdisciplinary approaches will preferentially enable us in the next few years to further extend our knowledge about this group of enzymes and their versatile functions. The application of different molecular biology techniques has allowed us to identify and understand the functions of lipases, proteases, chitinases, β-galactosidases, catalases and L-glutaminases in the infective process of entomopathogenic fungi. Even though there are prolonged disagreements regarding the action sequence and importance of several enzymes, in the last few years, it has been shown that these enzymes regulate several infective processes and that their function as control agents are seldom considered. This fact has provided researchers in the field of biotechnology with an opportunity to implement bioprocess engineering for large-scale production of these enzymes along with strategies to establish applications for the enzymatic extracts, as well as possible applications as fungicides in the environment based on green technology.

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References

[1] T. Godfrey, S. West, Introduction to industrial enzymology, in: S.West T.Godfrey (Ed.), Industrial Enzymology, Stockton Press, New York, 1996, pp. 1–8.
[2] D. Wilke, Chemicals from biotechnology: molecular plant genetics will challenge the chemical and fermentation industry, Appl. Microbiol. Biotechnol. 52 (1999) 135–145.

[3] R. Sharma, Y. Chisti, U.C. Banerjee, Production, purification, characterization, and applications of lipases, Biotechnol. Adv. 19 (2001) 627–662.
[4] M.J.P. Goud, A. Suryam, V. Lakshmipathi, M.A. Singara Charya, Extracellular hydrolytic enzyme profiles of certain South Indian basidiomycetes, Afr. J. Biotechnol. 8 (2009) 354–360.
[5] I.M. Dubovskiy, M.M.A. Whitten, O.N. Yaroslavtseva, C. Greig, V.Y. Kryukov, E.V. Grizanova, K. Mukherjee, A. Vilcinskas, V.V. Glupov, T.M. Butt, Can insects develop resistance to insect pathogenic fungi? PLoS One 8 (2013) e60248.
[6] C.S. Wang, R.J.St. Leger, A collagenous protective coat enables *Metarhizium anisopliae* to evade insect immune responses, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 6647–6652.
[7] H. Graur, W.H. Li, Fundamentals of Molecular Evolution, second ed., Sinauer Associates, Sunderland, 2000.
[8] J.K. Pell, J. Eilenberg, A.E. Hajek, D.C. Steinkraus, Biology, ecology and pest management potential of Entomophthorales, in: T.M. Butt, C. Jackson, N. Magan (Eds.), Fungi as Biocontrol Agents: Progress, Problems and Potential, CAB International, Wallingford, 2001, pp. 71–153.
[9] K.E. Shaw, G. Davidson, S.J. Clark, B.V. Ball, J.K. Pell, D. Chandler, K.D. Sunderland, Laboratory bioassays to assess the pathogenicity of mitosporic fungi to Varroa destructor (Acari: Mesostigmata), an ectoparasitic mite of the honeybee, *Apis mellifera*, Biol. Control 24 (2002) 266–276.
[10] D.W. Roberts, R.A. Humber, Entomogenous fungi, in: G.T. Cole, B. Kendrick (Eds.), Biology of Conidial Fungi, Academic Press, New York, 1981, pp. 201–236.
[11] R.A. Samson, H.C. Evans, J.P. Latg, Atlas of Entomopathogenic Fungi, Springer, Berlin, Heidelberg, 1988.
[12] R.A. Humber, Evolution of entomopathogenicity in fungi, J. Invertebr. Pathol. 98 (2008) 262–266.
[13] H.C. Evans, Use of Clavicipitalean fungi for the biological control of arthropod pests, in: J.F. White, C.W. Bacon, N.L. Hywel-Jones, J.W. Spatafora (Eds.), Clavicipitalean Fungi, Marcel Dekker, New York, 2003, pp. 519–550.
[14] J.W. Spatafora, M. Blackwell, Molecular systematics of unitunicate perithecia ascomycetes: the Clavicipitales Hypocreales connection, Mycologia 85 (1993) 912–922.
[15] T.R. Glare, R.J. Milner, Ecology of entomopathogenic fungi, in: D.K. Arora, L. Ajello, K.G. Mukerji (Eds.), Handbook of Applied Mycology, Marcel Dekker, New York, 1991, pp. 547–612.
[16] G.D. Inglis, M.S. Goettel, T.M. Butt, H. Strasser, Use of hyphomycetous fungi for managing insect pests, in: T.M. Butt, C.W. Jackson, N. Magan (Eds.), Fungi as Biocontrol Agents: Progress, Problems and Potential, CABI International/AAFC, Wallingford, United Kingdom, 2001, pp. 23–69.
[17] H.D. Burges, Safety, safety testing and quality control of microbial pesticides, in: H.D.Burges,Microbial Control of Pests and Plant Diseases 1970-1980, Academic Press,London, 1981, pp. 737–767.
[18] R.I. Carruthers, T.L. Larkin, R.S. Soper, Simulation of insect disease dynamics: an application of SERB to a rangeland ecosystem, Simulation 51 (1988) 101–109.
[19] D.M. MacLead, Entomophthorales infections, in: E.A. Steinhaus (Ed.), Insect Pathology: An Advanced Treatise, Academic Press, New York, 1963, pp. 189–231.
[20] C.W. McCoy, R.A. Samson, D.G. Boucias, Entomogenous fungi, in: C.M. Ignoffo, N.B. Mandava (Eds.), Handbook of Natural Pesticides, Microbial Insecticides, Part A, Entomogenous Protozoa and Fungi, vol. 5, Mr ic Press, Boca, Raton, Fla, 1988.
[21] E.A. Steinhaus, Microbial diseases of insects, in: P.E. DeBach (Ed.), Biological Control of Insect Pests and Weeds, Chapman and Hall, London, 1964, pp. 515–547.
[22] A.T. Gillespie, E.R. Moorhouse, The use of fungi to control pest of agricultural and horticultural importance, in: J.M. Whipps, R.D. Lumsdon (Eds.), Biotechnology of Fungi for Improvement of Plant Growth, Cambridge University Press,London, 1989, pp. 55–84.
[23] J.V. Bell, Verticillium lecanii on the bean rust fungus, Uromyces appendiculatus, Trans. Br. Mycol. Soc. 79 (1974) 362–364.
[24] K. Al-Aidoors, D.W. Roberts, Mutants of *Metarhizium anisopliae* with increased virulence toward mosquito larvae,Can, J. Genet. Cytol. 11 (1978) 211–219.
[25] J. Fargues, Adhesion of the fungal spore to the insect cuticle in relation to pathogenicity, in: D.W. Roberts, J.R. Aist (Eds.), Infection Processes of Fungi, The Rockefeller Foundation, New York, 1984, pp. 90–110.
[26] D.G. Boucias, J.C. Pendland, Host recognition and specificity of entomopathogenic fungi, in: D.W. Roberts, J.R. Aist (Eds.), Infection Processes of Fungi, Rockefeller Foundation, New York, 1984, pp. 185–196.
[27] J.L. Kerwin, Fatty acid regulation of the germination of Erynia variabilis conidia on adults and puuparia of the lesser housefly *Fannia canicularis*, Can. J. Microbiol. 30 (1983) 158.
[28] R. Smith, E.A. Grula, Toxic components on the larvae surface of the corn earworm (*Heliothis zea*) and their effects on germination and growth of *Beauveria bassiana*, J. Invertebr. Pathol. 36 (1982) 15–22.
[29] S.P. Woods, E.A. Grula, Utilizable surface nutrients on *Heliothis zea* available for growth of *Beauveria bassiana*, J. Invertebr. Pathol. 43 (1984) 359–369.
[30] J.P. Kramer, The house-fly mycosis caused by entomophthora muscae: influence of relative humidity on infectivity and conidial germination, J. N. Y. Entomol. Soc. 88 (1980) 236–240.
[31] G.G. Newman, G.R. Carner, Environmental factors affecting conidial sporulation and germination of Entomophthora gammae, Environ. Entomol. 4 (1975) 615–618.
[32] D.W. Roberts, A.S. Campbell, Stability of entomopathogenic fungi, Misc. Publ. Entomol. Soc. Am. 10 (1977) 19–76.
[33] M. Shimazu, Factors affecting conidial germination of Entomophthora del phacis Hori, Appl. Entomol. Zool. 12 (1977) 260.
[34] B.A. Mullens, J.L. Rodriguez, J.A. Meyer, An epizootiological study of Entomophthora muscae in muscoid fly populations on southern California poultry facilities, with emphasis on *Musca domestica*, Hilgardia 55 (1987).
[35] T.M. Butt, C. Jackson, N. Magan, Fungi as Biocontrol Agents: Progress, Problems, and Potential, CABI Publishing, Wallingford, United Kingdom, 2001.
[36] J.P. Latge, L. Sampeoro, P. Brey, M. Diaquin, Aggressiveness of Conidiobolus obscurus against the pea aphid - influence of cuticular extracts on ballistospore germination of aggressive and nonaggressive strains, J. Gen. Microbiol. 133 (1987).
[37] R.A. Nolan, Protoplasts from Entomophthorales, in: J.F. Peberdy, L. Ferenczy (Eds.), Fungal Protoplasts:Applications in Biochemistry and Genetics, Marcel Dekker, New York, 1985, pp. 87–112.
[38] A.P. Gupta, Hemocytic and Humoral Immunity in Arthropods, John Wiley and Sons, New York, 1987.
[39] R.J.St. Leger, Studies on adaptation of *Metarhizium anisopliae* to life in the soil, J. Invertebr. Pathol. 98 (2008) 271–276.
[40] R.I. Samuels, I.C. Paterson, Cuticle-degrading proteinase from insect moulting fluid and culture filtrates of entomopathogenic fungi, Comp. Biochem. Physiol. B Biochem. Mol. Biol. 110 (1995) 661–669.
[41] M.J. Bidochka, G.G. Khachatourians, Regulation of extracellular protease in the entomopathogenic fungus Beauveria bassiana, Exp. Mycol. 12 (1988) 161–168.
[42] L. Alberghina, R.D. Schmid, R. Verger, Lipases: Structure, Mechanism and Genetic Engineering, Weinheim, Federal Republic of Germany, New York, NY, USA, 1991.
[43] J. Anguita, L.B. Rodriguez-Aparicio, G. Naharro, Purification, gene cloning, amino acid sequencing analysis and expression of an extracellular lipase from an *Aeromonas hydrophila* human isolate, Appl. Environ. Microbiol. 59 (1993) 2411–2417.
[44] C.C. Akoh, S.W. Chang, G.C. Lee, J.F. Shaw, Enzymatic approach to biodiesel production, J. Agric. Food Chem. 55 (2007) 8995–9005.
[45] J.A. Alford, D.A. Pierce, Lipolytic activity of microorganisms at low and intermediate temperatures. III. Activity of microbial lipases at temperatures below 0°C, J. Food Sci. 26 (1961) 518–524.
[46] A. Pandey, S. Benjamin, C.R. Soccol, P. Nigam, N. Krieger, V.T. Soccol, The realm of microbial lipases in biotechnology, Biotechnol. Appl. Biochem. 29 (1999) 119–131.
[47] H. Sierotzki, F. Camastral, P.A. Shah, M. Aebi, U. Tuor, Biological characteristics of selected Erynia neoaphidis isolates, Mycol. Res. 104 (2000) 213–219.
[48] S.K. Sharma, C.E. Olsen, V.S. Parmr, Lipase catalyzed synthesis of optically enriched a-haloamides, Bioorg. Med. Chem. 9 (2001) 1345–1348.
[49] M.M.D. Maia, A. Heasley, M.M. Camargo de Morais, E.H.M. Melo, M.A. Morais Jr., W.M. Ledingham, L.F. Jl, Effect of culture conditions on lipase production by *Fusarium solani* in batch fermentation, Bioresour. Technol. 76 (2001) 23–27.
[50] S.F. Lin, Production and stabilization of a solvent-tolerant alkaline lipase from *Pseudomonas pseudoalcaligenes* F-111, J. Ferment Bioeng. 82 (1996) 448–451.
[51] G. Corzo, S. Revah, Production and characteristics of the lipase from *Yarrowia lipolytica* 681, Bioresour. Technol. 70 (1999) 173–180.
[52] W. Silva, S. Mitidieri, A. Schrank, M.H. Vainstein, Production and extraction of an extracellular lipase from the entomopathogenic fungus Metarhizium anisopliae, Process Biochem. 40 (2005) 321–326.
[53] S. Ali, Z. Huang, S.X. Ren, Production and extraction of extracellular lipase from the entomopathogenic fungus *Isaria fumosoroseus*(Cordycipitaceae: Hypocreales), Biocontrol. Sci. Technol. 19 (2009) 81–89.
[54] W.O.B. Silva, L. Santi, A. Scharank, M.H. Vainstein, *Metarhizium anisopliae* lipolytic activity plays a pivotal role in *Rhipicepthalus (Boophilus) microplus* infection, Fungal Biol. 144 (2010) 10–15.
[55] K. Kohno, M. Wada, D. Schlessinger, M. D'urso, S. Tanabe, T. Oshiro, F. Imamoto, Stability of YACs containing ribosomal or RCP/GCP locus DNA in wild-type *S. cerevisiae* and RAD mutant strains, DNA Res. 1 (1994) 191–199.
[56] K. Ohnishi, Y. Yoshida, J. Toita, J. Sekiguchi, Purification and characterization of a novel lipolytic enzyme from *Aspergillus oryzae*, J. Ferment Bioeng. 78 (1994) 413–419.
[57] P. Supakdamrongkul, A. Bhumiratana, C. Wiwat, Characterization of an extracellular lipase from the biocontrol fungus, Nomuraea rileyi MJ, and its toxicity toward *Spodoptera litura*, J. Invertebr. Pathol. 105 (2010) 228–235.
[58] N. Pedrini, S. Zhang, M.P. Juárez, N.O. Keyhani, Molecular characterization and expression analysis of a suite of cytochrome P450 enzymes implicated in insect hydrocarbon degradation in the entomopathogenic fungus *Beauveria bassiana*, Microbiology 156 (2010) 2549–2557.
[59] S. Zhang, E. Wideman, G. Bernard, A. Lesot, E. Pinot, N. Pedrini, N.O. Keyhani, CYP52X1, representing new cytochrome P450 subfamily, displays fatty acid hydroxylase activity and contributes to virulence and growth on insect cuticular substrates in entomopathogenic fungus *Beauveria bassian*, J. Biol. Chem. 28 (2012) 13477–13486.

[60] N. Pedrini, R. Crespo, M.P. Juárez, Biochemistry of insect epicuticle degradation by entomopathogenic fungi, Comp. Biochem. Physiol. C Toxicol. Pharmacol. 146 (2007) 124–137.
[61] N. Pedrini, M.P. Juárez, R. Crespo, M.J.T. de Alaniz, Clues on the role of *Beauveria bassiana* catalases in alkane degradation events, Mycologia 98 (2006) 528–534.
[62] I.N. Van Bogaert, S. Groeneboer, K. Saerens, W. Soetaert, The role of cytochrome P450 monooxygenases in microbial fatty acid metabolism, FEBS J. 278 (2011) 206–221.
[63] L. Lin, W. Fang, X. Liao, F. Wang, D. Wei, R.J. St. Leger, The *MrCYP*52 cytochrome P450 monooxygenase gene of *Metarhizium robertsii* is important for utilizing insect epicuticular hydrocarbons, PLoS One. 6 (2011) e28984.
[64] D.H. Dwayne, G.G. Khachatourians, Production of an extracellular lipase by Beauveria bassiana, Biotechnol. Lett. i0 (1988) 637–642.
[65] J. Sharma, A. Singh, R. Kumar, A. Mittal, Partial purification of an alkaline protease from a new strain of *Aspergillus oryzae* AWT 20 and its enhanced stabilization in entrapped Ca-alginate beads, Internet J. Microbiol. 2 (2006) 1–14.
[66] U. Mustafa, G. Kaur, Extracellular enzyme production in *Metarhizium anisopliae* isolates, Folia Microbiol. 54 (2009) 499–504.
[67] C. Wang, M.A. Typas, T.M. Butt, Detection and characterization of *Pr*1 virulent gene deficiencies in the insect pathogenic fungus Metarhizium anisopliae, FEMS Microbiol. Lett. 213 (2002) 251–255.
[68] R.J.St. Leger, M.J. Bidochka, D.W. Roberts, Isoforms of the cuticle-degrading *Pr*1 proteinase and production of a metalloproteinase by Metarhizium anisopliae, Arch. Biochem. Biophys. 313 (1994) 1–7.
[69] S.Q. Liu, Z.H. Meng, J.K. Yang, Y.K. Fu, K.Q. Zhang, Characterizing structural features of cuticle-degrading proteases form fungi by molecular modeling, BMC Struct. Biol. 7 (2007) 33.
[70] W. Fang, J. Feng, Y. Fan, Y. Zhang, M.J. Bidochka, R.J. St. Leger, Y. Pei, Expressing a fusion protein with protease and chitinase activities increases the virulence of the insect pathogen Beauveria bassiana, J. Invertebr. Pathol. 102 (2009) 155–159.
[71] M.H. Sun, X.Z. Liu, Carbon Requirements of some entomopathogenic and mycoparasitic Hyphomycetes as fungal biocontrol agents, Mycopathology 161 (2006) 295–305.
[72] J. Castellanos-Moguel, M. González-Barajas, T. Mier, M.R. Reyes Montes, E. Aranda, C. Toriello, Virulence testing and extracellular subtilisin-like (*Pr*1) and tripsina-like (*Pr*2) activity during propagule production of *Paecilomyces fumosoroseus* isolates from whiteflies (Homoptera: Aeyrodidae), Rev. Iberoam. Micol. 24 (2007) 62–68.
[73] A.A. Shahid, A. Sattar, B. Chaudhry, S. Riazuddin, Determination of protein virulence factors and pathogenicity of entomopathogenic fungi, Pak. J. Biochem. Mol. Biol. 36 (2003) 100–107.
[74] C. Wang, R.J. St. Leger, A scorpion neurotoxin increases the potency of a fungal insecticide, Nat. Biotechnol 25 (2007) 1455–1456.
[75] D.M. Lu, M. Pava-Ripoli, Z. Li, C.Wang, Insecticidal evaluation of *Beauveria bassiana* engineered to express a scorpion neurotoxin and a cuticle degrading protease, Appl. Microbiol. Biot. 81 (2008) 515–522.
[76] L. Xia, Z. Zeng, X. Ding, F. Huang, The expression of a recombinant *Cry*1*Ac* gene with subtilisin-like protease *CDEP*2 gene in acrystalliferous *Bacillus thuringiensis* by Red/ET homologous recombination, Curr. Microbiol. 59 (2009) 386–392.
[77] G. Yu, J. Liu, L. Xie, X. Wang, S. Zhang, H. Pan, Characterization, cloning and heterologous expression of a subtilisin-like serine protease gene *VlPr*1 from Verticillium lecanii, J. Microbiol. 50 (2012) 939–946.
[78] S. Bagga, G. Hu, S.E. Screen, R.J. St. Leger, Reconstructing the diversification of subtilisins in the pathogenic fungus *Metarhizium anisopliae*, Gene 324 (2004) 159–169.
[79] Y. Xu, R. Orozco, E.M.K. Wijeratne, A.A.L. Gunatilaka, S.P. Stock, I. Molnár, Biosynthesis of the cyclooligomer Depsipeptide Beauvericin, a virulence factor of the entomopathogenic fungus *Beauveria bassiana*, Chem. Biol. 15 (2008) 898–907.
[80] G. Xiao, S.H. Ying, P. Zheng, Z.L. Wang, S. Zhang, X.Q. Xie, Y. Shang, R.J. St Leger, G.P. Zhao, C. Wang, M.G. Feng, Genomic perspectives on the evolution of fungal entomopathogenicity in Beauveria bassiana, Sci. Rep. 2 (2012) 483.
[81] N.J. Bye, A.K. Charnley, Regulation of cuticle-degrading subtilisin proteases from the entomopathogenic fungi, *Lecanicillium* spp: implications for host specificity, Arch. Microbiol. 189 (2008) 81–92.
[82] R.J.St. Leger, R.M. Cooper, A.K. Charnley, Distribution of chymoelastases and trypsin-like enzymes in five species of entomopathogenic Deuteromycetes, Arch. Biochem. Biophys. 258 (1987) 121–131.
[83] R.J.St. Leger, A.K. Charnley, R.M. Cooper, Characterization of cuticle degrading proteases produced by the entomopathogen Metarhizium anisopliae, Arch. Biochem. Biophys. 253 (1987) 221–232.
[84] M.S. Goettel, R.J. St. Leger, R.L. Rizzo, R.C. Staples, D.W. Roberts, Ultrastructural localization of a cuticle degrading protease produced by the entomopathogenic fungus *Metarhizium anisopliae* during penetration of host cuticle, J. Gen. Microbiol. 135 (1989) 2233–2239.
[85] M.A. Jackson, M.R. McGuire, L.A. Lacey, S.P. Wraight, Liquid culture production of desiccation tolerant blastospores of the bioinsecticidal fungus *Paecilomyces fumosoroseus*, Mycol. Res. 101 (1997) 35–41.
[86] N. Claydon, J.F. Grove, Insecticidal secondary metabolic products from the entomopathogenous fungus *Verticillium lecanii*, J. Invertebr. Pathol. 40 (1982) 413–418.
[87] G. Gindin, I. Barash, N. Harari, B. Raccah, Effect of endotoxic compounds isolated from Verticillium lecanii on the sweet potato whitefly *Bemisia tabaci*, Phytoparasitica 22 (1994) 189–196.
[88] I.C. Paterson, A.K. Charnley, R.M. Cooper, J.M. Clarkson, Partial characterization of specific inducers of a cuticle-degrading protease from the insect pathogenic fungus *Metarhizium anisopliae*, Microbiology 140 (1994) 3153–3159.
[89] S. Shankar, M. Rao, R. Seeta Laxman, Purification and characterization of an alkaline protease by a new strain of Beauveria sp, Process Biochem. 46 (2011) 579–585.
[90] A. Zibaee, A.R. Bandani, Purification and characterization of the cuticle-degrading protease produced by the entomopathogenic fungus, Beauveria bassiana in the presence of Sunn pest Eurygaster integriceps (Hemiptera: Scutelleridae) cuticle, Biocontrol Sci. Technol. 19 (2009) 797–808.
[91] Y.K. Rao, S.C. Lu, B.L. Liu, Y.M. Tzeng, Enhanced production of an extracellular protease from *Beauveria bassiana* by optimization of cultivation processes, Biochem. Eng. J. 28 (2006) 57–66.
[92] S. Ali, Z. Huang, S. Ren, Biocontrol Sci. Technol. (2009) 523–535.
[93] R.N. Tharanathan, F.S. Kittur, Chitin—the undisputed Biomolecule of great potential, Crit. Rev. Food Sci. Nutr. 43 (2003) 61–87.
[94] V. Seidl, Chitinases of filamentous fungi: a large group of diverse proteins with multiple physiological functions, Fungal Biol. Rev. 22 (2008) 36–42.
[95] Z.X. Lu, A. Laroche, H.C. Huang, Isolation and characterization of chitinases from *Verticillium lecanii*, Can. J. Microbiol. 51 (2005) 1045–1055.
[96] R.J.St. Leger, R.M. Cooper, A.K. Charnley, Characterization of chitinase and chitobiose produced by the entomopathogenic fungus Metarhizium anisopliae, J. Invertebr. Pathol. 58 (1991) 415–426.
[97] D.J. Adams, Fungal cell wall chitinases and glucanases, Microbiology 150 (2004) 2029–2035.
[98] J. Yang, B. Tian, L. Liang, K.Q. Zhang, Extracellular enzymes and the pathogenesis of nematophagous fungi, Appl. Microbiol. Biot. 75 (2007) 21–31.
[99] B. Henrissat, A. Bairoch, New families in the classification of glycosyl hydrolases based on amino acid sequence similarities, Biochemistry 293 (1993) 781–788.
[100] D.C. Li, Review of fungal chitinases, Mycopathologia. 161 (2006) 345–360.
[101] Y. Kezuka, M. Ohishi, Y. Itoh, J. Watanabe, M. Mitsutomi, T. Watanebe, T. Nonaka, Structural studies of a two-domain chitinase from *Streptomyces griseus* HUT6037, J. Mol. Biol. 358 (2006) 472–484.
[102] M.R. Bogo, C.A. Rota, H. Rota, H. Pinto Jr., M. Ocampos, C.T. Correa, M.H. Vainstein, A. Schrank, A chitinase encoding gene (*chit*1 gene) from the entomopathogen *Metarhizium anisopliae*: isolation and characterization of genomic and full- length cDNA, Curr. Microbiol. 37 (1998) 221–225.
[103] C.M. Baratto, V. Dutra, J. Tomazzoni, L. Barbosa, M. Henning, A. Schrank, Isolation, characterization, and transcriptional analysis of the chitinase *chit2* gene (DQ011663) from the biocontrol fungus *Metarhizium anisopliae* var. anisopliae, Curr. Microbiol. 53 (2006) 217–221.
[104] W. Fang, M. Pava-Ripoli, S. Wang, R.J. St. Leger, Protein kinase a regulates production of virulence determinants by the entomopathogenic fungus, *Metarhizium anisopliae*, Fungal Genet. Biol. 46 (2009) 277–285.
[105] J.T. Boldo, K.B. Amaral, A. Junges, P.M. Pinto, C.C. Staats, M.H. Vainstein, A. Schrank, Evidence of alternative splicing of the *chit2* chitinase gene from *Metarhizium anisopliae*, Gene 462 (2010) 1–7.
[106] M.V. da Silva, L. Santi, C.C. Staats, A.M. da Costa, E.M. Colodel, D. Driemeier, M.H. Vainstein, A. Schrank, Cuticle induced endo/exoacting chitinase CHIT30 from *Metarhizium anisopliae* is encoded by an ortholog of the *chit3* gene, Res. Microbiol. 156 (2005) 382–392.
[107] C.C. Staats, L. Kmetzsch, I. Lubeck, A. Junges, M.H. Vainstein, A. Schrank, *Metarhizium anisopliae* chitinase CHIT30 is involved in heat-shock stress and contributes to virulence against Dysdercus peruvians, Fungal Biol. 117 (2013) 137–144.
[108] S. Screen, G. Hu, R.J. St. Leger., Transformants of *Metarhizium anisopliae* sf. *anisopliae* overexpressing chitinase from *Metarhizium anisopliae* sf. *acridum* show early induction of native chitinase but are not altered in pathogenicity to *Manduca sexta*, J. Invertebr. Pathol. 78 (2002) 260–266.
[109] J.T. Boldo, A. Junges, K.B. Amaral, C.C. Staats, M.H. Vainstein, A. Schrank, Endochitinase CHI2 of the biocontrol fungus *Metarhizium anisopliae* affects its virulence toward the cotton stainer Bug *Dysdercus peruvians*, Curr. Genet. 55 (2009) 551–560.
[110] W. Fang, B. Leng, Y. Xiao, K. Jin, J. Ma, Y. Fan, J. Feng, X. Yang, Y. Zhang, Y. Pei, Cloning of *Beauveria bassiana* gene *Bbchit*1 and its application to improve fungal strain virulence, Appl. Environ. Microbiol. 71 (2005) 363–370.
[111] Y. Fan, W. Fang, Y. Xiao, X. Yang, Y. Zhang, M.J. Bidochka, Y. Pei, Directed evolution for increased chitinase activity, Appl. Microbiol. Biotechnol. 76 (2007) 135–139.
[112] Y. Fan, W. Fang, S. Guo, X. Pei, Y. Zhang, Y. Xiao, D. Li, K. Jin, M.J. Bidochka, Y. Pei, Increased insect virulence in *Beauveria bassiana* strains overexpressing an engineered chitinase, Appl. Environ. Microb. 73 (2007) 295–302.
[113] Y. Fan, X. Pei, S. Guo, Y. Zhang, Z. Luo, X. Liao, Y. Pei, Increased virulence using engineered protease- chitin binding domain hybrid expressed in the entomopathogenic fungus Beauveria bassiana, Microb. Pathog. 49 (2010) 376–380.
[114] M.J. Bidochka, G.G. Khachatourians, The implication of metabolic acids produced by *Beauveria bassiana* in pathogenesis of the migratory grasshopper, *Melanoplus sanguinipes*, J. Invertebr. Pathol. 58 (1991) 106–117.

[115] D.D. Hegedus, G.G. Khachatourians, Identification and differentiation of the entomopathogenic fungus *Beauveria bassiana* using polymerase chain reaction and single-strand conformation polymorphism analysis, J. Invertebr. Pathol. 67 (1996) 289–299.
[116] R.J.St. Leger, L. Joshi, M.J. Bidochka, N.W. Rizzo, D.W. Roberts, Characterization and ultrastructural localization of chitinase from Metarhizium anisopliae, M. flavoviride and *Beauveria bassiana* during fungal invasion of host (Manduca sexta) cuticle, Appl. Environ. Microbiol. 62 (1996) 907–912.
[117] M.C. Valadares-Inglis, P.W. Inglis, J.F. Peberdy, Sequence analysis of catalytic domain of a Metarhizium anisopliae chitinase, Braz. J. Genet. 20 (1997) 161–164.
[118] S.C. Kang, S. Park, D.G. Lee, Purification and characterization of a novel chitinase from the entomopathogenic fungus Metarhizium anisopliae, J. Invertebr. Pathol. 73 (1999) 276–281.
[119] S.E. Screen, G. Hu, R.T. St Leger, Transformants of Metarhizium anisopliae sf. anisopliae overexpressing chitinase from Metarhizium anisopliae sf. acridum show early induction of native chitinase but are not altered in pathogenicity to Manduca sexta, J. Invertebr. Pathol. 78 (2001) 260–266.
[120] C.M. Baratto, M.V. Da Silva, L. Santi, L. Passaglia, I.S. Schrank, M.H. Vainstein, A. Schrank, Expression and characterization of the 42 kDa chitinase of the biocontrol fungus *Metarhizium anisopliae* in Escherichia coli, Can. J. Microbiol. 49 (2003) 723–726.
[121] P. Nahar, V. Ghormade, M.V. Deshpande, The extracellular constitutive production of chitin deacetylase in *Metarhizium anisopliae*: possible edge to entomopathogenic fungi in the biological control of insect pests, J. Invertebr. Pathol. 85 (2004) 80–88.
[122] M. Horsch, C. Mayer, U. Sennhauser, D.M. Rast, b-N-acetylhexosaminidase: a target for the design of antifungal agents, Pharmacol. Ther. 76 (1997) 187–218.
[123] P.M. Coutinho, B. Henrissat, Carbohydrate-active enzymes: an integrated database approach, in: H.J. Gilbert, G. Davies, B. Henrissat, B. Svensson (Eds.), Recent Advances in Carbohydrate Bioengineering, The Royal Society of Chemistry, Cambridge, 1999, pp. 3–12.
[124] A. Perrakis, I. Tews, Z. Dauter, A.B. Oppenheim, I. Chet, K.S. Wilson, C.E. Vorgias, Crystal structure of a bacterial chitinase at 2.3, A resolution, Structure 2 (1994) 1169–1180.
[125] A.C. Terwisscha van Scheltinga, M. Hennig, B.W. Dijkstra, The 1.8 A resolution structure of hevamine, a plant chitinase/lysozyme, and analysis of the conserved sequence and structure motifs of glycosyl hydrolase family 18, J. Mol. Biol. 262 (1996) 243–257.
[126] D.M. van Aalten, B. Synstad, M.B. Brurberg, E. Hough, B.W. Riise, V.G. Eijsink, R.K. Wierenga, Structure of a two-domain chitotriosidase from *Serratia marcescens* at 1.9-A resolution, Proc. Natl. Acad. Sci. U.S.A. 97 (2000) 5842–5847.
[127] K.A. Brameld, W.D. Shrader, B. Imperiali, W.A. Goddard 3rd, Substrate assistance in the mechanism of family 18 chitinases: theoretical studies of potential intermediates and inhibitors, J. Mol. Biol. 280 (1998) 913–923.
[128] K.A. Brameld, W.A. Goddard 3rd, The role of enzyme distortion in the single displacement mechanism of family 19 chitinases, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 4276–4281.
[129] S.J. Horn, A. Sorbotten, B. Synstad, P. Sikorski, M. Sorlie, K.M. Varum, V.G. Eijsink, Endo/exo mechanism and processivity of family 18 chitinases produced by Serratia marcescens, FEBS J. 273 (2006) 491–503.
[130] A. Sorbotten, S.J. Horn, V.G. Eijsink, K.M. Varum, Degradation of chitosans with chitinase B from *Serratia marcescens*.Production of chito-oligosaccharides and insight into enzyme processivity, FEBS J. 272 (2005) 538–549.
[131] T. Uchiyama, F. Katouno, N. Nikaidou, T. Nonaka, J. Sugiyama, J. Watanabe, Roles of the exposed aromatic residues in crystalline chitin hydrolysis by chitinase A from Serratia marcescens 2170, J. Biol. Chem. 276 (2001) 41343–41349.
[132] I. Tews, A.C. Scheltinga, V. Terwisscha, A. Perrakis, K.S. Wilson, B.W. Dijkstra, Substrate assisted catalysis unifies two families of chitinolytic enzymes, J. Am. Chem. Soc. 119 (1997) 7954–7959.
[133] B. Bhushan, Isolation, Purification, Characterization and Scale up Production of a Thermostable Chitinase from an Alkalophilic Microorganism, Ph.D. thesis, Department of Microbiology, Panjab University, Chandigarh, 1998.
[134] N. Dahiya, R. Tewari, R.P. Tiwari, G.S. Hoondal, Chitinase production in solid state fermentation by Enterobacter sp. NRG4 using statistical experimental design, Curr. Microbiol. 51 (2005) 1–9.
[135] K. Miyashita, T. Fujii, Y. Sawada, Molecular cloning and characterization of chitinase gene from Streptomyces lividans, J. Microbiol. 137 (1991) 2065–2072.
[136] G.E. Harman, C.K. Hayes, M. Lorito, R.M. Broadway, A. Di Pietro, C. Peterbauer, A. Tronsmo, Chitinolytic enzymes of *Trichoderma harzianum*: purification of chitobiosidase and endochitinase, Phytopathology 83 (1993) 313–318.
[137] C.J. Ulhoa, J.F. Peberdy, Purification and some properties of the extracellular chitinase produced by Trichoderma harzianum, Enzyme Microb. Technol. 14 (1992) 236–241.
[138] J.De La Cruz, M. Rey, J.M. Lora, A. Hidalgo-Gallego, F. Dominguez, J.A. Pintor-Toro, A. Llobell, T. Benitez, Isolation and characterization of three chitinases from Trichoderma harzianum, Eur. J. Biochem. 206 (1992) 859–867.
[139] C. Kunz, O. Sellam, Y. Bertheau, Purification and characterization of a chitinase from the hyperparasitic fungus Aphanocladium album, Physiol. Mol. Plant Pathol. 40 (1992) 117–131.
[140] A.Di Pietro, M. Lorito, C.K. Hayes, R.M. Broadway, G.E. Harman, Endochitinase from Gliocladium virens: isolation, characterization, and synergistic antifungal activity in combination with gliotoxin, Phytopathology 83 (1993) 308–313.
[141] D.C. Li, S.H. Zhang, K.Q. Liu, J. Lu, Purification and characterization of a chitinase from the mycoparasitic fungus *Trichothecium roseum*, J. Gen. Appl. Microbiol. 50 (2004) 35–39.
[142] D.C. Li, S. Chen, J. Lu, Purification and partial characterization of two chitinase from the mycoparasitic fungus *Talaromyces flavus*, Mycopathologia 159 (2005) 223–229.
[143] S. Haran, H. Schickler, A. Oppenheim, I. Chet, New components of the chitinolytic system of Trichoderma harzianum, Mycol. Res. 99 (1995) 441–446.
[144] N. Mathivanan, V. Kabilan, K. Murugesan, Purification, characterization, and antifungal activity of chitinase from *Fusarium chlamydosporum*, a mycoparasite to groundnut rust, Puccinia arachidis, Can. J. Microbiol. 44 (1998) 646–651.
[145] G. Taylor, S. Jabaji-Hare, P.M. Charest, W. Khan, Purification and characterization of an extracellular exochitinase, b-N-acetylhexosaminidase, from fungal mycoparasite Stachybotrys elegans, Can. J. Microbiol. 48 (2002) 311–319.
[146] M.J. Bidochka, K.I. Tong, G.G. Khachatourians, Partial purification and characterization of two extracellular N-acetyl-D-glucosaminidases produced by the entomopathogenic fungus Beauveria bassiana, Can. J. Microbiol. 39 (1993) 41–45.
[147] D.S. Pinto, C.C. Barreto, A. Schrank, C.J. Ulhoa, M. Henning Vainstein, Purification and characterization of an extracellular chitinase from the entomopathogen Metarhizium anisopliae, Can. J. Microbiol. 43 (1997) 322–327.
[148] V.E. Tikhonov, L.V. Lopez-Llorca, J. Salinas, H.B. Jansson, Purification and characterization of chitinases from the nematophagous fungi Verticillium chamydosporium and V. suchlasporium, Fungal Genet. Biol. 35 (2002) 67–78.
[149] R.F. Guo, D.C. Li, R. Wang, Purification and properties of a thermostable chitinase from thermophilic fungus Thermomyces lanuginosus, Acta Microbiol. Sin. 45 (2005) 270–274 (in Chinese).
[150] K.J. Mellor, R.O. Nicholas, D.J. Adams, Purification and characterization of chitinase from Candida albicans, FEMS Microbiol. Lett. 119 (1994) 117–119.
[151] O. Ramot, A. Viterbo, D. Friesem, A. Oppenheim, I. Chet, Regulation of two homodimer hexosaminidases in the mycoparasitic fungus *Trichoderma asperellum* by glucosamine, Curr. Genet. 45 (2004) 205–213.
[152] N. Benhamou, A. Asselin, Attempted localization of a substrate for chitinase in plant cells reveals abundant N-acetyl D-glucosamine residues in secondary walls, Biol. Cell 67 (1989) 341–350.
[153] J, Grenier, N. Benhamou, A. Asselin, Colloidal gold complexed chitosanase: a new probe for ultrastructural localization of chitosan in fungi, J. Gen. Microbiol. 137 (1991) 2007–2015.
[154] R. Serrano, J.M. Gancedo, C. Gancedo, Assay of yeast enzymes in situ: a potential tool in regulation studies, Eur. J. Biochem. 34 (1973) 479–482.
[155] J.M. Mac Pherson, G.G. Khachatourians, Partial purification and characterization of b-galactosidase produced by *Beauveria bassiana*, Biotechnol. Appl. Biochem. 13 (1991) 217–230.
[156] V.P. Choudary, R. Rao, Regulatory properties of yeast nitrate reductase in situ, Can. J. Microbiol. 22 (1976) 35–42.
[157] S.P. Ram, P.A. Sullivan, M.G. Shepherd, The in situ assay of *Candida albicans* enzymes during yeast growth and germ– tube formation, J. Gen. Microbiol. 129 (1983) 2367–2378.
[158] J.E. Seip, R. Di Cosimo, Optimization of accessible catalase activity in polyacrylamide gel–immobilized *Saccharomyces cerevisiae*, Biotechnol. Bioeng. 40 (1992) 638–642.
[159] S. Shailasree, B. Nagajyothi, S.G. Bhat, S. Sekhar, N. Bhat, Preparation of detergent permeabilized bakers' yeast whole cell catalase, Process Biochem. 34 (1999) 349–354.
[160] M.R. Sorol, E. Pereyra, C. Mizyrycki, S. Silvia Rossi, S. Moreno, Protein kinase A activity in permeabilized cells as an approximation to *in vivo* activity, Exp. Cell Res. 271 (2001) 337–343.
[161] H. Felix, Permeabilized cells, Anal. Biochem. 120 (1982) 211–234.
[162] S. Sesták, V. Farkas, In situ assays of fungal enzymes in cells permeabilized by osmotic shock, Anal. Biochem. 292 (2001) 34–39.
[163] S.B. Martinez, R.A. Speckman, b-galactosidase treatment of frozen dairy product mixes containing whey, J. Dairy Sci. 71 (1988) 893–900.
[164] E.M. Cho, D. Boucias, N.O. Keyhani, EST analysis of cDNA libraries from the entomopathogenic fungus Beauveria (Cordyceps) bassiana. I. Evidence for stagespecific gene expression in aerial conidia, *in vitro* blastospores and submerged conidia, Microbiology 152 (2006) 2855–2864.
[165] E.M. Cho, L. Liu, W. Farmerie, N.O. Keyhani, EST analysis of cDNA libraries from the entomopathogenic fungus Beauveria (Cordyceps) bassiana. II. Fungal cells sporulating on chitin and producing oosporein, Microbiology 152 (2006) 2843–2854.
[166] J.G. Mantilla, N.F. Galeano, A.L. Gaitan, M.A. Cristancho, N.O. Keyhani, C.E. Gongora, Transcriptome analysis of the entomopathogenic fungus *Beauveria bassiana* grown on cuticular extracts of the coffee borer (Hypothenemus hampei), Microbiology 158 (2012) 1826–1842.
[167] C. Duriya, K. Supattra, O. Nemat, K. Keyhani, H. Boonyapakron, A. Thoetkiattikul, B. Kusol Pootanakit, A. Lily Eurwilaichitr, Identification of

catalase as an early up-regulated gene in *Beauveria bassiana* and its role in entomopathogenic fungal virulence, Biol. Control 67 (2013) 85–93.
[168] C.D. Michan, F. Lledias, W. Hansberg, W. Hansberg, Asexual development is increased in Neurospora crassa cat-3-null mutant strans, Eukaryot. Cell 2 (2003) 798–808.
[169] E. Vierstraete, P. Verleyen, F. Sas, G. Van den Bergh, A. De Loof, L. Arckens, L. Schoofs, The instantly released Drosophila immune proteome is infection-specific, Biochem. Biophys. Res. Commun. 317 (2004) 1052–1060.
[170] V.V. Glupov, M.F. Khvoshevskaya, Y.L. Lovinskaya, I.M. Dubovski, V.V. Martemyanov, J.Y. Sokolova, Application of the nitroblue tetra-zolium reduction method for studies on the production of reactive oxygen species in insect haemocytes, Cytobios 106 (2001) 165–178.
[171] Y. Zhu, J.O. Smits, W. Knol, J. Bol, A novel solid state fermentation system using polyurethane foam as inert carrier, Biotechnol. Lett. 16 (1994) 643–648.
[172] K.E. Aidoo, R. Hendry, B.J.B. Wood, Solid state fermentations, Adv. Appl. Microbiol. 28 (1982) 201–237.
[173] G.N. Prabhu, M. Chandrasekaran, Polystyrene — an inert carrier for glutaminase production by marine *Vibrio costicola* under solid state fermentation, World J. Microbiol. Biotechnol. 11 (1995) 683–684.
[174] G.N. Prabhu, M. Chandrasekaran, L-Glutaminase production by marine *Vibrio costicola* under solid-state fermentation using different substrates, J. Mar. Biotechnol. 4 (1996) 176–179.
[175] P.V. Suresh, M. Chandrasekaran, Utilization of prawn waste for chitinase production by the marine fungus *Beauveria bassiana* by solid state fermentation, World J. Microbiol. Biotechnol. 14 (1998) 655–660.
[176] G.N. Prabhu, M. Chandrasekaran, Impact of process parameters on L-glutaminase production by marine *Vibrio costicola* under solid state fermentation using polystyrene as inert support, Process Biochem. 32 (1997) 285–289.
[177] M.A. McTigue, C.T. Kelly, W.M. Fogarty, E.M. Doyle, Production studies on the alkalophilic amylase of three alkalophilic *Bacillus* sp. Biotechnol. Lett. 16 (1994) 569–574.
[178] M. Chandrasekaran, P. Lakshmanaperumalsamy, D. Chandramohan, Combined effect of environmental factors on spoilage bacteria, Fish Tech. (India). 28 (1991) 146–153.
[179] A.G. Murzin, S.E. Brenner, T. Hubbard, C. Chothia, SCOP: a structural classification of proteins database for the investigation of sequences and structures, J. Mol. Biol. 247 (1995) 536–540.
[180] D.L. Ollis, E. Cheah, M. Cygler, B. Dijkstra, F. Frolow, S.M. Franken, M. Harel, S.J. Remington, I. Silman, J. Schrag, J.L. Sussman, K.H.G. Verschueren, A. Goldman, The alpha/beta hydrolase fold, Protein Eng. 5 (1992) 197–211.
[181] C.A. Orengo, T.P. Flores, W.R. Taylor, J.M. Thornton, Identification and classification of protein fold families, Protein Eng. 6 (1993) 485–500.
[182] A. Barrett, J. Woessner, N. Rawlings, Handbook of Proteolytic Enzymes, second ed., Elsevier Academic Press, London,UK, 2004.
[183] N.M. Hooper, Proteases in Biology and Medicine, Portland Press, London, 2002.
[184] O. Kohei, New families of carboxyl peptidases: serine-carboxyl peptidases and glutamic peptidases, J. Biochem Tokyo 151 (2012) 13–25.
[185] N.M. De Las Mercedes Dana, M.C. Limon, R. Mejias, R.L. Mach, T. Benitez, J.A. Pintor-Toro, C. Kubicek, Regulation of chitinase 33 (chit33) gene expression in *Trichoderma harzianum*, Curr. Genet. 38 (2001) 335–342.
[186] A. Viterbo, M. Montero, O. Ramot, D. Friesem, E. Monte, A. Llodell, I. Chet, Expression regulation of the endochitinase vhit36 from *Trichoderma asperellum* (T. harzianumT-203), Curr. Genet. 42 (2002) 114–122.
[187] R.L. Mach, C.K. Peterbauer, K. Payer, S. Jaksits, S.L. Woo, S. Zeilinger, C.M. Kullnig, M. Lorito, C.P. Kubicek, Expression of two major chitinase genes of *Trichoderma atroviride* (T.harzianum P1) is triggered by different regulatory signals, Appl. Environ. Microbiol. 65 (1999) 1858–1863.
[188] M.C. Limon, E. Margolles-Clark, T. Benitez, M. Penttila, Addition of substrate-binding domains increases substrate-binding capacity and specific activity of a chitinase from Trichoderma harzianum, FEMS Microbiol. Lett. 198 (2001) 57–63.
[189] K. Brunner, M. Montero, R.L. Mach, C.K. Peterbauer, C.P. Kubicek, Expression of the ech42 (endochitinase)gene of Trichoderma atroviride under carbon starvation is antagonized via a BrlA-like cis-acting element, FEMS Microbiol. Lett. 218 (2003) 258–264.
[190] A. Liese, K. Seelbach, C. Wandrey (Eds.), Industrial Biotransformations, Wiley-VCH, Weinheim, 2000.
[191] D. Undurraga, A. .Markovits, S. Erazo, Cocoa butter equivalent through enzymic interesterification of palm oil midfraction, Process Biochem. 36 (2001) 933–939.
[192] J.Y. Xin, S.B. Li, Y. Xu, J.R. Chui, C.G. Xia, Dynamic enzymatic resolution of naproxen methyl ester in a membrane bioreactor, J. Chem. Technol. Biotechnol. 76 (2001) 579–585.
[193] J.H. Kuk, W.J. Jung, G.H. Jo, J.S. Ahn, Selective preparation of *N*-acetyl-D-glucosamine and *N,N*_-di acetyl chitobiose from chitin using a crude enzyme preparation from Aeromonassp, Biotechnol. Lett. 27 (2005) 7–11.
[194] P. Setthakaset, R. Pichyankura, A. Ajavakon, M. Sukwattanasinitt, Preparation of *N*-acetyl-D-glucosamine using enzyme from Aspergillus sp, J. Met. Mater. Min. 18 (2008) 53–57.
[195] J. Roberts, J.S. Holcenberg, W.C. Dolowy, Antineoplastic activity of highly purified bacterial glutaminase, Nature 227 (1970) 1136–1137.
[196] M. Moriguchi, K. Sakai, R. Tateyama, Y. Furuta, M. Wakayama, Isolation and characterization of salt tolerant glutaminase from marine *Micrococcus luteus* K-3, J. Ferment Bioeng. 77 (1994) 621–625.
[197] J.C. Hoogerheide, Microbial enzymes other than fungal amylases, in: L.A. Underkofler, R.J. Hickey (Eds.), Industrial Fermnlentations, Chemical Publishing Co., New York, 1954.
[198] J.A. Johnson, B.S. Miller, The Relationship between dough consistency and proteolytic activity, Cereal Chem. 30 (1953) 471–479.
[199] W.J. Ferracone, Enzymes-Their function and use in spotting, Neighborhood Dryclean. 5 (1951) 13–14.
[200] D. Herbert, P. Jane, Crystalline bacterial catalase, Biochem. J. 43 (1948) 193–202.
[201] T.P. Shukla, L.E. Wierzbicki, Beta-galactosidase technology: a solution to the lactose problem, Food Sci. Nutr. 25 (1975) 325–356.