Characterization of the Chitinolytic System During the Mycoparasitic Interaction Between *Trichoderma aggressivum* f. *aggressivum* and Different Host Strains of *Agaricus bisporus*

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Abstract

Green mould is a serious disease of commercially grown mushrooms, the causal agent being attributed to the filamentous soil fungus *Trichoderma aggressivum* f. *aggressivum* and *T. aggressivum* f. *europaeum*. Found worldwide, and capable of devastating crops, this disease has caused millions of dollars in lost revenue within the mushroom industry.

One mechanism used by *Trichoderma* spp. in the antagonism of other fungi, is the secretion of lytic enzymes such as chitinases, which actively degrade a host's cell wall. Therefore, the intent of this study was to examine the production of chitinase enzymes during the host-parasite interaction of *Agaricus bisporus* (commercial mushroom) and *Trichoderma aggressivum*, focusing specifically on chitinase involvement in the differential resistance of white, off-white, and brown commercial mushroom strains.

Chitinases isolated from cultures of *A. bisporus* and *T. aggressivum* grown together and separately, were identified following native PAGE, and analysis of fluorescence based on specific enzymatic cleavage of 4-methylumbelliferyl glucoside substrates. Results indicate that the interaction between *T. aggressivum* and *A. bisporus* involves a complex enzyme battle. It was determined that *T. aggressivum* produces a number of chitinases that appear to correlate to those isolated in previous studies using biocontrol strains of *T. harzianum*. A 122 kDa *N*-acetylglucosaminidase of *T. aggressivum* revealed the highest and most variable activity, and is therefore believed to be an important predictor of antifungal activity. Furthermore, results indicate that brown strain resistance of mushrooms may be related to high levels of a 96 kDa *N*-acetylglucosaminidase, which showed elevated activity in both solitary and dual cultures with *T. aggressivum*.

Overall, each host-parasite combination produced unique enzyme profiles, with the majority of the differences seen between day 0 and day 6 for the extracellular chitinases. Therefore, it was concluded that the antagonistic behaviour of *T. aggressivum* does not involve a typical response, always producing the same types and levels of enzymes, but that mycoparasitism, specifically in the form of chitinase production, may be induced and regulated based on the host presented.

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Introduction

Agaricus bisporus commonly known as the 'white' or 'button' mushroom is responsible for more than 90% of all mushroom crops in North America (Rinker & Chalmers, 1998; USDA, 2001). Since its crude beginning in Paris caves, advances in technology have greatly improved production techniques of this economically important crop (Miles & Chang, 1997). However, the drive for increased yields in combination with flawed sterile techniques has at times resulted in large-scale losses due to contamination (Seaby, 1987; Staunton, 1987). Although there are a number of fungal diseases known to plague mushroom growers, *Trichoderma* infection is often the most problematic.

Trichoderma spp. are filamentous fungi commonly found in soil where they lead active lives as saprotrophs. However, given the opportunity they can also become extremely effective mycoparasites capable of killing a number of other fungi for utilization of their nutrients (Deacon, 1997). As a result, *Trichoderma* species are often exploited for their parasitic abilities, and are used as biocontrol agents against a number of plant pathogens (see reviews Papavizas 1985; Chet 1987, 1993). The antifungal properties of *Trichoderma harzianum* have been studied extensively, and research has given rise to a number of strains that can be used against a host of soilborne fungi. Consequently these same parasitic activities have caused extensive damage to commercial mushroom crops, since *A. bisporus* is easily parasitized in a phenomenon commonly known as green mould disease.

Green mould is characterized by dense white mycelial growth, followed by extensive green sporulation, and has been known to destroy 30-100% of mushroom crops (Wuest *et*

al., 1996). Outbreaks of this disease have been experienced in both the United Kingdom and North America, causing a loss of revenue in the tens of millions of dollars for North America alone (Castle *et al.*, 1998).

It was initially thought that green mould disease was caused by two aggressive biotypes of *T. harzianum*. It has since been proposed that these aggressive biotypes are independent species, distinct from *T. harzianum* the commonly used biocontrol agent. The causal agent of green mould in North America is now referred to as *Trichoderma aggressivum* f. *aggressivum*, while its European counterpart has been named *T. aggressivum* f. *europaeum* (Samuels *et al.*, 2002).

Trichoderma spp. use a number of mechanisms in its antagonism of other fungi, yet it is the secretion of lytic enzymes such as proteases, glucanases, and chitinases that have received the most attention by researchers. Recent studies have focused on the chitinolytic system of *Trichoderma*, and at least seven distinct enzymes have been discovered (Haran *et al.*, 1996).

The intent of this study was to examine the contribution of chitinase enzymes during the host-parasite interaction of *A. bisporus* and *T. aggressivum*, focusing specifically on chitinase involvement in the differential resistance of commercial mushroom strains.

Literature Review

Agaricus bisporus

There are over 2,500 mushroom varieties grown in the world today, ranging from the common button mushroom to Asia's exotic Shiitake. Although specialty mushrooms have become increasingly popular, the commercial button mushroom, *Agaricus bisporus* is still responsible for 32% of the world's total cultivated mushrooms (Chang, 1999). In North America *Agaricus* production continues to dominate, accounting for greater than 90% of all mushroom crops (USDA, 2001; Rinker & Chalmers, 1998).

The commercial mushroom is a fungus belonging to the large worldwide order of Agaricales. Found within this group are many other edible mushrooms, such as the crimini, oyster and enoki mushrooms, as well as a number of poisonous and hallucinogenic species (Moore-Landecker, 1996; Miles & Chang, 1997).

Although there are more than 5,000 species contained within this order, they share the common characteristic of a fleshy moist basidioma bearing a hymenium that is exposed at maturity (Moore-Landecker, 1996). The common button mushroom has all the general traits of this order, however it is set apart by its production of only two basidiospores on each basidium, instead of the usual four, hence the name 'bisporus' (Miles & Chang, 1997).

Mushroom Production

Early cultivation of button mushrooms began in France during the reign of Louis XIV, where mushrooms were grown in special caves near Paris. From France, mushroom production spread to England where it was found to be a very easy crop to grow, requiring minimal materials and labour (The Mushroom Council, 2002). In the late 19th

century mushroom production made its way to North America, where today commercial cultivation of mushrooms accounts for $274 million dollars of Canada's agricultural produce. As the leader in mushroom production, Ontario growers harvest more than 50% of the 190 million pounds of mushrooms produced in Canada each year (Statistics Canada, 2002).

As mushrooms have become an economically important crop, the methods of production have improved with new technologies. However, there are still six basic steps that more or less follow the natural course of mushroom growth. It is during these procedures that many of the disease causing agents (pathogens) of mushrooms are first introduced. Thus, to fully understand the interaction between host and pathogen, knowledge of the production process is greatly beneficial.

The process of mushroom production described by The Mushroom Council (2002) takes about four months and begins with the preparation of compost. The most widely used components are horse manure and straw, however synthetic compost, consisting of such materials as hay and corncobs, may also be used. Both types of compost require the addition of a nitrogen source, and the conditioning agent gypsum. This first step, referred to as phase I takes place outdoors, where long piles of composting materials are thoroughly watered, and turned every few days. The temperature within the piles eventually rises as a result of the heat generated from bacterial and fungal activity. Decomposition of the organic matter by these microbes provides the nutrients necessary to support mushroom growth. After 7-14 days, phase II composting begins. This step takes place indoors in a much more controlled environment. Compost is packed into trays or long shelves, and the air temperature is raised using steam to at least 60°C, which

is maintained for several hours. These conditions will kill microbial and insect parasites, while allowing thermophilic fungi and bacteria to continue to be active, converting ammonia into protein and reducing the pH (Moore-Landecker, 1996).

The compost, which has been cooled, is transferred to the growing area for the spawning step. Mushroom spawn, which has been prepared under sterile laboratory conditions, is used to inoculate the compost. The term spawn refers to vegetative mushroom mycelium that have been allowed to grow into cereal grains. In Canada and the U.S., commercial mushroom farmers have any number of companies to purchase spawn from. Growers have a choice of four major mushroom cultivars: (i) smooth white, (ii) off-white, (iii) cream and (iv) brown, however consumer preference generally leads farmers to produce mainly white and off-white varieties (A. Castle, pers. comm.).

After mycelia have colonized the compost for a period of 14 to 21 days, the process of casing begins. Compost is covered with a casing layer 3-5 cm thick consisting of soil or a mixture such as sphagnum and chalk. The casing layer is watered with a fine spray 2 to 4 times weekly (Moore-Landecker, 1996). Air temperature is dropped and room is purged of carbon dioxide to induce fruit body production. Soon, small, white, pin-like protrusions appear through the casing layer. This step known as pinning, may be carefully controlled to allow for maximum mushroom production.

The final step of cropping occurs when mushroom caps reach 2-8 cm in size. At 6 to 12 day intervals, new crops or "breaks" of mushrooms will appear (Figure 1), which are generally hand picked, and once harvested are cooled quickly.

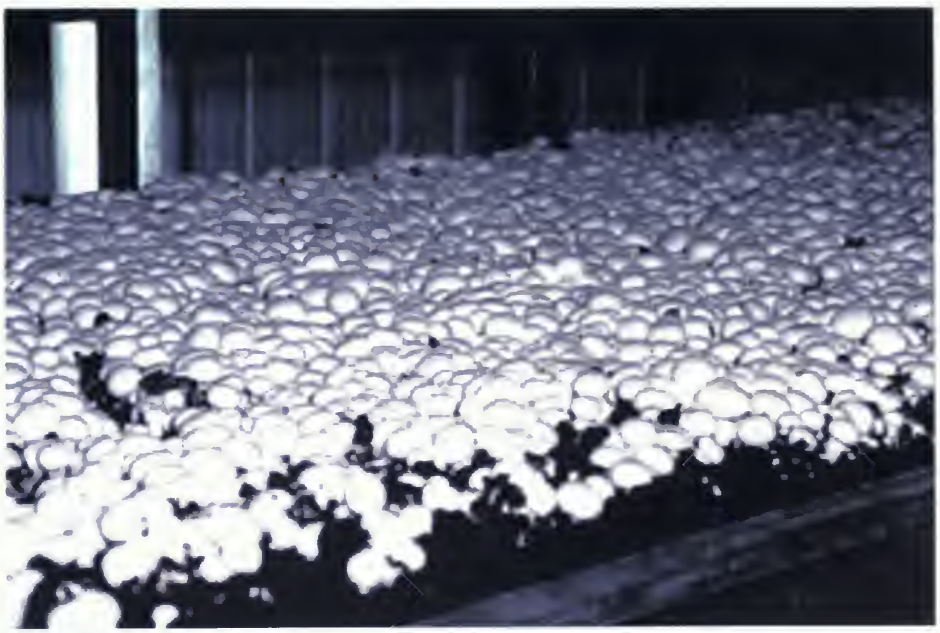

Figure 1 – Commercial mushroom production of *A. bisporus*.
Picture from: <http://www.plant.uoguelph.ca/faculty/drinker/>

Mushroom Pests and Diseases

Although each step of mushroom production is carefully controlled by way of humidity, temperature and CO_2 levels, the occurrence of unwanted pathogens is more difficult to control. Mushroom cultivation is a form of monoculture, an approach in which only a few strains are grown. This practice may lead to genetic instability within a crop and often increases its susceptibility to disease (Horgen & Castle, 2002). *Agaricus* is vulnerable to attack by viruses, bacteria, microscopic fungi, nematodes and a number of insects (Fletcher *et al.*, 1986). Furthermore, fungal disease management programs are made more difficult due to the fact that the commercial mushroom itself is a fungus. Although there are wide variety of insect pests and diseases that may affect a mushroom crop, there are a few that are particularly prevalent and pose the greatest risk to crop yields and product marketability.

Insect Pests

Problematic to all farmers, including mushroom growers, are invertebrate pests. The warm humid environment of the mushroom house allows for year-round survival of a variety of insects. Often these pests colonize a crop, whereby larvae may feed on the growing fungal mycelium, or burrow tunnels into the mushroom stem and caps (Fletcher *et al.*, 1986). The tiny cecid, phorid, and sciarid flies are common associated with *Agaricus* crops. Along with a variety of mites these small flies move easily throughout a farm, and can severely damage crops (Fletcher *et al.*, 1986). Most control measures rely heavily on insecticides, however these chemicals are both costly and hazardous to human health and the environment. New programs have begun using integrated pest

management, combining chemical and biological control in which natural predators are introduced to eliminate these pests (Fleischer, 2002).

The insects themselves may cause little or no damage to crops, however once an incidence of parasitism has occurred, it often opens the door for other more serious pathogens to flourish (Fletcher *et al.*, 1986).

Fungal Diseases

The fact that the mushroom itself is a fungus does not limit the wide range of fungal pathogens that actively parasitize it. The most common and often most problematic of the mushroom mycoparasites are *Verticillium fungicola*, *Mycogone perniciosa*, and *Hypomyces rosellus* as well as a number of aggressive moulds of the *Trichoderma* species (Fletcher *et al.*, 1986). Symptoms of *Verticillium* problems, commonly referred to as dry bubble disease, include cap spotting, distortion and splitting of the mushroom. *Mycogone* causes a soft decay of the cap and stem, while *Hypomyces* often distorts mushroom caps, and prevents gill formation (Fletcher *et al.*, 1986). Although fungicides such as benomyl and thiabendazole can often control these diseases, the over use of such chemicals can lead to tolerance in these pathogens.

More aggressive parasites such as *Trichoderma aggressivum*, known to cause green mould disease, are often more problematic. The production of large numbers of resilient spores, paired with aggressive colonization of mushroom crops creates a unique problem that is more difficult to manage. Hence, most mushroom farmers consider *Trichoderma* to be one of the most serious of all fungal diseases (Fletcher *et al.*, 1986).

Classification of Trichoderma

Classification of the filamentous soil fungus *Trichoderma*, has undergone many revisions over the years. This genus was originally comprised of four species, *T. aureum, T. nigrescens, T. roseum*, and *T. viride*, which were separated by their differently coloured conidia. It is now known that these species are unrelated, and the name *Trichoderma* is associated only with members displaying green conidiospores (Bissett, 1991) (Figure 2). Although many *Trichoderma* isolates can be identified by macroscopic characteristics, recent attempts to delineate the *Trichoderma* tree have used a number of molecular techniques including randomly amplified polymorphic DNA (RAPDs) and restriction fragment length polymorphisms (RFLPs) (Castle *et al.*, 1998; Muthumeenakshi and Mills, 1995). A large number of *Trichoderma* species are commonly found on mushroom farms including *T. harzianum, T. virens, T. viride, T. koningii, T. pseudokoningii, T. longibrachiatum* and *T. atroviride* (Rinker *et al.*, 1995). Most of these species do not pose a serious threat to crops, and are often regarded as indicators of underlying problems. However, one species in particular, *T. harzianum*, has proven to be an unwelcome and remarkably hostile invader of mushroom farms. Initially four biotypes of this species were identified, referred to as Th1, Th2, Th3, and Th4. Of these biotypes, Th1 is generally regarded as the least problematic as a non-aggressive inhabitant of mushroom houses (Gams & Meyer, 1998; Samuels *et al.*, 2002). Furthermore, Th3 is no longer considered a *T. harzianum* isolate, and has been reidentified as *T. atroviride* (Castle *et al.*, 1998; Gams & Meyer, 1998; Ospina-Giraldo *et al.*, 1998).

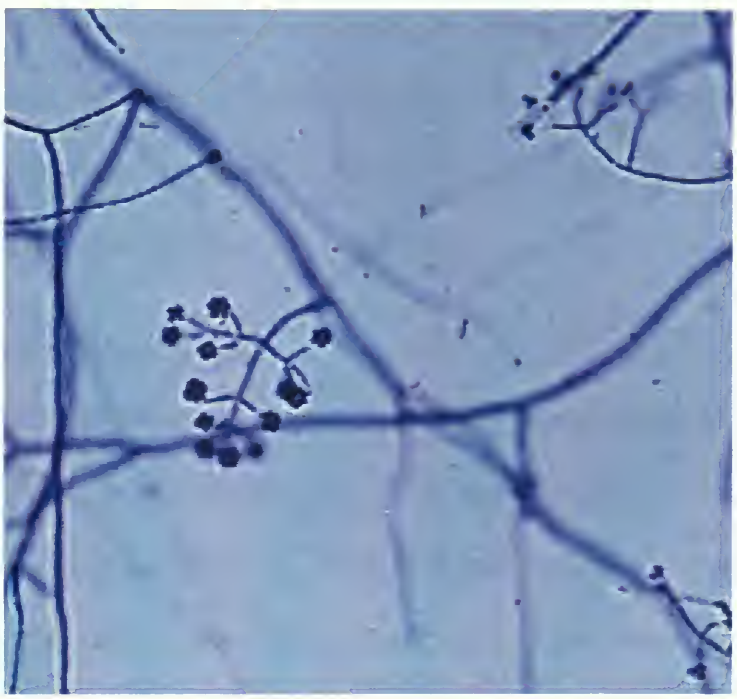

Figure 2 – Microscopic view of *Trichoderma* conidial heads and filaments. Conidia are one-celled ovoid spores produced from the tips of phailides which are often found as short whorls branching from conidiophores. Picture from:
<http://soils1.cses.vt.edu/ch/biol_4684/Microbes/trichoderma.html>

It is Th2 and Th4 which pose the most risk, and have been deemed responsible for outbreaks of green mould disease. The biotype Th2 is associated with outbreaks of green mould in Ireland and the U.K., while Th4 has caused epidemics in North America.

Recently, the phylogenetic relationship of these biotypes has been re-evaluated, and although long suspected, it has now been proven that Th1 is a separate species from Th2 and Th4. As a result, the biotype Th4 has been described as a novel species, and renamed *Trichoderma aggressivum* f. *aggressivum*, while Th2 is now referred to as *T. aggressivum* f. *europaeum* (Samuels *et al.*, 2002).

Trichoderma as a Mycoparasite

Trichoderma spp. are commonly described as filamentous soil fungi found in a wide range of environments. Within the soil, these fungi lead an active life as saprotrophs. However, given the opportunity they can also become extremely effective mycoparasites capable of killing a number of other fungi for utilization of their nutrients (Deacon, 1997). As a result, *Trichoderma* species are often exploited for their parasitic abilities, and are used as biocontrol agents against a number of plant pathogens (see reviews Papavizas 1985; Chet 1987, 1993). The antifungal properties of *Trichoderma* have been studied extensively, and research has given rise to a number of strains which can be used against a host of soilborne fungi. The most common plant pathogenic fungi against which this fungus is used include *Rhizoctonia solani*, *Sclerotium rolfsii* and *Botrytis cinerea* (Chet, 1987).

Highly effective strains that have the ability to persist in the hosts' natural environment, which are inexpensive, and easy to store, as well as having a mode of application allowing for its full expression, make the ideal biocontrol agent (Harman,

2002). Harman and associates at Cornell University created such an agent in the 1980's. *T. harzianum* strain 1295-22, also known as T-22 has demonstrated the ability to colonize and protect the entire root system of a crop against a number of fungal pathogens (Harman, 2002). Although the mechanisms of this biocontrol are not well understood, a considerable amount of research is being conducted to gain further insight and allow for strict control of these types agents.

Green Mould Disease

Although the antagonistic properties of *Trichoderma* have proven beneficial in the biocontrol of many plant pathogens, these same parasitic activities have caused extensive damage to commercial mushroom crops. The problems caused by *Trichoderma* include green mould disease, and cap spotting. Green mould disease, characterized by white mycelial growth followed by extensive green sporulation, has been known to destroy 30-100% of mushroom crops (Wuest *et al.*, 1996). Decreased yields have been experienced world-wide, causing a loss of revenue in the tens of millions of dollars for North America alone (Castle *et al.*, 1998).

The first outbreak of green mould disease is known to have occurred in Ireland in 1986, and shortly after was also discovered in England (Seaby, 1987). Canada saw its first incidence of green mould in British Columbia in 1990, and by 1992 Ontario farmers also reported infection of mushroom crops. Since Ontario produces more than 50% of Canada's mushrooms (OASCC, 1998), and green mould has been identified on at least two-thirds of Ontario mushroom farms (Rinker *et al.*, 1997) there is a great potential for devastation of crops resulting in large scale losses within Canada's mushroom industry. To decrease the possibility of future green mould epidemics research is currently being

conducted to look at all aspects of the parasitic interaction between *Trichoderma aggressivum* and the commercial button mushroom *Agaricus bisporus*.

Signs and Symptoms of Green Mould

The complete lack of mushroom production is the most obvious sign of a *Trichoderma* infestation (Seaby, 1989). Since its mycelia are white, as is the mycelium of *Agaricus*, and may grow rapidly over the compost or casing layer it is often difficult to detect. Infection may occur at any stage of mushroom production, however early introduction of this mycoparasite is often more detrimental to crop yields (Anderson *et al.*, 2001). Following complete colonization by *Trichoderma* mycelia, masses of green spores are often observed on the casing surface (Figure 3). A late stage infection is less of a concern, and may cause only limited green patches on casing material or cap spotting on mushrooms.

Although non-productive areas on the casing surface are generally the final and most serious sign of green mould, there are a number of other early indicators of its presence within a mushroom house. The most prominent of which is a characteristic cinnamon colour on the mushroom caps, caused by the presence of red pepper mites (*Pygmephorus* spp.) which feed on green mould (Staunton, 1987). Although the mites are not directly harmful, feeding on the green mould damages the mushrooms making them unmarketable, as well as providing a carrier for spread of the disease (Staunton, 1987; Seaby, 1989; Muthumeenakshi & Mills, 1996).

Figure 3 – The bag on the left shows casing with healthy spawn growth, while the bag on the right shows the effects of green mould caused by *Trichoderma aggressivum*. Picture from: <http://mushgrowinfo.cas.psu.edu/Trichoderma%20Green%20Mold.html>

Sources and Methods of Control

The difficulty in controlling green mould disease can largely be attributed to the diversity of vectors responsible for the spread of *Trichoderma* spores. The microscopic spores may be dispersed by wind, dust particles, insects, rodents as well as workers and farm tools (Seaby 1989, 1996). Furthermore, infestation may be difficult to detect and, therefore, control as apparently normal spawn runs can give way to large areas of green sporulation (Seaby, 1996).

Contamination by this mycoparasite can most often be attributed to personnel and equipment (Seaby, 1996). *Trichoderma* spores appear to gain entry into growing areas through attachment to workers hands and clothing. Similarly, trays and cleaning equipment, which have not been sufficiently steamed, may continue fungal spread. In a study conducted by Rinker and coworkers (1997) *Trichoderma* spores were detected in virtually all aspects of the production process. However, initiation of the disease is often blamed on poorly composted substrate, produced as a consequence of bulk pasteurization (Staunton, 1987). Thus, the drive for increased yields in combination with flawed sterilization techniques during cultivation may result in contamination of mushroom crops by green mould.

Presently, the best means to localize and control this disease is to prevent the spread of *Trichoderma* spores throughout entire farms. This is accomplished using strict sanitation programs whereby workers are required to have clean hands and clothes before working in the production area. Zones highly susceptible to contamination may be protected by physical barriers, and by limiting the number of workers in these areas. Furthermore, the

disinfection of all trays, shelving, and mushroom house structures is essential to the control of green mould (Seaby 1987, 1989; Staunton 1987; Rinker *et al.*, 1997).

In addition to good sanitary practices, fungicides may also be used. The most common in the control of green mould disease is the systemic fungicide benomyl. Benomyl and its primary metabolite carbendazim bind to microtubules interfering with cell functions such as cell division and intracellular transportation (Deacon, 1997). Although there are conflicting reports, this chemical has been associated with a number of health problems ranging from mild skin irritations, to cancer and serious birth defects (Pesticides News, 1997). In 2001, DuPont the leading supplier of benomyl, voluntarily discontinued manufacturing of this product, citing mainly financial reasons. However, in September of 2002 the U.S. Environmental Protection Agency cancelled registration of all benomyl-containing products (U.S. EPA, 2002). Currently farmers are continuing to use existing stocks, but they will soon be looking for alternatives in the face of possible green mould outbreaks.

Resistance of A. bisporus to Green Mould Disease

Resistance to infection occurs when the natural ability of a host prevents or restricts the parasitic fungus from successful invasion. A variety of mechanical or biochemical factors, normally present, or produced in response to fungal infection may be involved in host resistance (Moore-Landecker, 1996).

Resistance to green mould disease was first reported in 1996 by Fletcher, as well as Rinker & Alm, where outbreaks on mushroom farms revealed brown strains that were much less affected than their white counterparts. Further investigation by Anderson and coworkers (2001) gave credit to these findings with the observation that mushroom yields

from brown strains infected with *T. aggressivum* were significantly higher than those of white and off-white hybrid strains. The mechanisms behind this resistance are not well understood, and current research on this topic is scarce. However, resistance to *Trichoderma* spp. exhibited by Shiitake mushrooms (*Lentinula edodes*) may provide insight to the possible mechanisms involved.

The means by which resistance occurs in the Shiitake mushroom has been attributed to the formation of melanins, produced by the oxidation of phenolic compounds within the host cell wall, following attack by *Trichoderma* (Tokimoto & Komatsu, 1995). Melanins are well known as pigments, however they may also protect fungi against enzymatic and invasive attack by mycoparasites (Jacobson & Tinnell, 1993). At this time it is unknown if the presence of melanins, the production of antibiotics or the secretion of a host's own hydrolytic enzymes contribute to brown strain resistance of *A. bisporus*. Consequently, a number of studies have begun to examine each of these components with respect to their involvement in green mould disease.

Modes of Antagonism

The antagonistic properties of *Trichoderma harzianum* have long been known, and its ability to parasitize other fungi, including the commercial button mushroom, have made it a subject of great interest to researchers.

It has been determined that *Trichoderma* spp. employ a number of mechanisms in the parasitism of other fungi, including competition for space and nutrients, antibiosis, and the release of hydrolytic enzymes. The first method of parasitic involvement is based on the ease at which *Trichoderma* can outgrow *A. bisporus*. Consequently, compost fully colonized by *Agaricus* mycelia exhibit increased resistance to infection by *Trichoderma*

(Seaby, 1987). The release of antibiotics is another important factor in the parasitic abilities of *T. harzianum*, and has been the focus of a number of studies. It has been found that *Trichoderma* produces both volatile and non-volatile antibiotics, which act in various ways to interfere with the growth and development of nearby fungal species (Deacon, 1997: Dennis & Webster, 1971a,b). In addition to antibiosis, *T. harzianum* is also known to excrete lytic enzymes such as proteases, glucanases, and chitinases into its surroundings, which effectively degrade the cell wall of host fungi as well as aid in utilization of the hosts' nutrients (Haran *et al.*, 1996).

Although all three modes of antagonism are important in the parasitic activities of members of this genus, it is the release of hydrolytic enzymes that has gained the most attention by scientists.

Fungal Cell Walls

Familiarity with the structural components of fungal cell walls is key to understanding the importance of hydrolytic enzymes and their involvement during host-parasite interactions. The cell walls are extremely important for survival, providing support and protection for the fungus. Degradation of this wall is potentially harmful, allowing for exposure of internal components and a vulnerability to physical and chemical attacks, which may ultimately lead the organisms' death (Deacon, 1997).

The cell walls of fungi are composed of various polysaccharides including cellulose, glucans, and chitin, the amount of each dependent on the division, phylum, and species of fungus. The rigid structure of the fungal cell wall can be attributed to the chitin and glucan microfibrils that are embedded in a gel-like matrix. Small polysaccharides,

proteins, lipids, salts and pigments are the general components of this matrix (Moore-Landecker, 1996).

The cell walls of the commercial button mushroom *A. bisporus*, have been found to contain approximately 43% (w/w) chitin, 41% glucan, 16% protein and 1.5% lipid (Michalenko *et al.*, 1976). These components are organized into an inner and outer layer, with a layer of mucilage often present on the outer surface (Figure 4).

As one of the main structural components of the fungal cell wall, glucans are polysaccharides composed of glucose units held together by glycosidic linkages. These bonds may exist in a number of orientations, most commonly β-1,3 and β-1,6 (Moore, 1998). Chitin is the second most abundant polymer in nature, and is a structural component of a variety of organisms. Produced in the cytosol by chitin synthase, this β-1,4-linked polymer of *N*-acetyl-D-glucosamine (GlcNAc) is easily integrated into newly forming cell walls (Moore, 1998). The cross-linking of these microfibrils produces a lattice difficult to break, such that a parasite must secrete a variety of enzymes to degrade the host's cell wall. Enzymes with this ability are collectively known as hydrolytic enzymes, and are often found in plants and fungi. These enzymes are largely recognized for their role in mycoparasitic activities, yet they may also be found in plants to combat or resist attack by fungal pathogens (Sahai & Manocha, 1993). The most widely studied in host-parasite interactions are proteases, glucanases and chitinases, as they have the ability to degrade cell wall components.

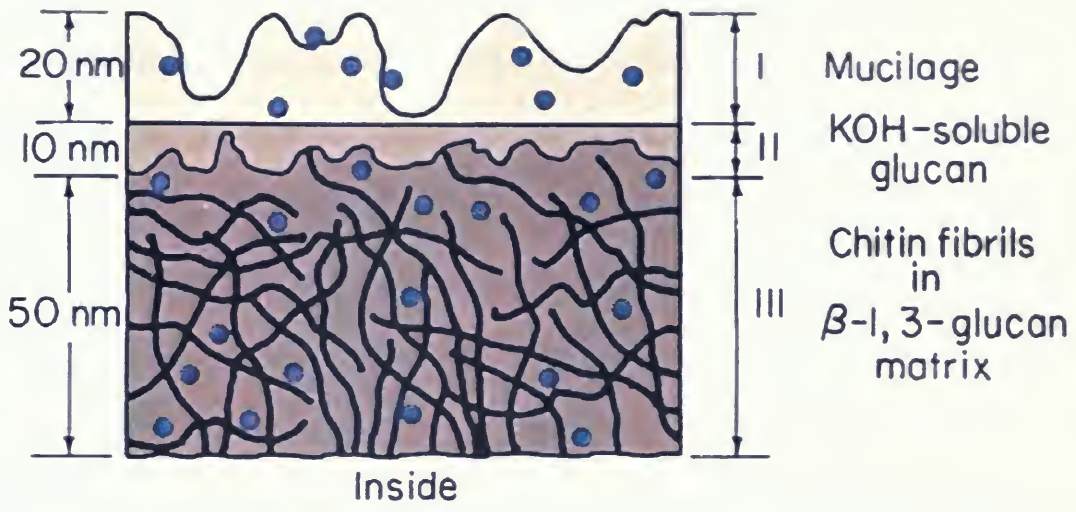

Figure 4 – Diagrammatic model of the cell wall of *Agaricus bisporus* adapted from Michalenko *et al.* (1976). The wall is composed of three layers: (I) an outer proteinaceous mucilage layer, (II) an alkaline soluble layer of α–glucans, and (III) an inner-most layer containing a matrix of chitin and β–glucans (● protein; ⌒ chitin fibrils)

Proteases

The structural integrity of the fungal cell wall is enhanced by the presence of a variety of proteins and glycoproteins (Moore, 1998). Thus, proteases may play a significant role in the mycoparasitic abilities of *Trichoderma* spp.

Proteases are considered to be important in host cell wall degradation, as well as efficient utilization of subsequently released nutrients. Increased susceptibility to lysis by both glucanase and chitinase has been demonstrated following proteolytic digestion of host cell walls (Sivan & Chet, 1989). More recently, research has focused on a 31 kDa basic proteinase isolated from *T. harzianum*. The gene encoding this enzyme (*prb1*) has been cloned and characterized, and support for its involvement in parasitic activities has mounted. Geremia and coworkers (1993) first determined that *prb1* was induced by autoclaved mycelia, fungal cell wall preparations, as well as chitin containing media. It has since been found that *prb1* induction occurs rapidly during direct confrontation assays with *Rhizoctonia solani*. Additionally, transgenic strains of *Trichoderma* carrying multiple copies of *prb1* have demonstrated an increased level of control of the plant disease caused by *R. solani* (Flores *et al.*, 1997).

Studies of protease involvement in the parasitic activities of *T. harzianum* may give rise to improved biocontrol strains of this organism. However, increased knowledge of all modes of antagonism contributes greatly to the understanding of green mould disease.

Glucanases

Fungi are capable of synthesizing a number of β–glucans, each of which may have diverse roles depending on their size, structure, physical and chemical properties, as well as their location (Haran *et al.*, 1996). Found largely within the cell wall, β–glucans

function as structural support, and occasionally as a source of nutrition. The degradation of cell wall glucans by lytic enzymes known as glucanases, may severely limit a host's ability to survive.

The two main groups of glucanases are categorized based on the hydrolysis products they produce. Glucanases acting in an exo- type fashion release glucose residues by the sequential cleavage of the β–glucan chain from the non-reducing end. Internal cleavage of the glucan may also be seen in which an endoglucanase cleaves β-linkages at random sites (Haran *et al.*, 1996). Since β-1,3-glucans are a large component of many phytopathogen cell walls (Bartnicki-Garcia, 1968), β-1,3-glucanases produced by *T. harzianum* have been studied intensely to determine their involvement in mycoparasitism, and for exploitation in biological control.

A number of β-1,3-glucanases have been isolated from *T. harzianum* strains commonly used as biocontrol agents. The complex glucanase system consists of a number of β-1,3-endoglucanases with molecular masses ranging from 29 to 76 kDa (De La Cruz *et al.*, 1993; Noronha & Ulhoa, 1996, 2000, Noronha *et al.*, 2000), and at least two β–1,3-exoglucanases (31 & 110 kDa) (Kitamoto *et al.*, 1987; Cohen-Kupiec *et al.*, 1999). The production of these enzymes has been demonstrated in response to numerous inducers including chitin, laminarin and purified fungal cell walls.

Without the right arsenal of enzymes, the intertwining glucan and chitin fibrils, hidden beneath a mucilage layer, may be impossible for a mycoparasite to degrade. Thus, successful digestion of a host's cell wall may require the cooperative efforts of multiple enzymes. Since the mucilage covering is largely composed of glucan, it is possible that glucanase activity may precede the hydrolytic activity of chitinase (Haran *et al.*, 1996).

Chitinases

Classification

Chitin is a natural polymer found not only within fungal cell walls, but also the exoskeletons of insects and crustaceans (Cohen-Kupiec & Chet, 1998). As such, a large number of organisms possess enzymes collectively known as chitinases, which are capable of degrading chitin fibrils. The function of these enzymes may vary widely depending on their source. Amino acid sequence similarities, which are usually indicative of folding similarities, have been used to group chitinases into five classes, all of which belong to either family 18 or 19 glycosyl hydrolases. Classes I, II, and IV make up the family 19 glycosyl hydrolases and are comprised of chitinases of plant origin (Cohen-Kupiec & Chet, 1998). Researchers have found that plant chitinases are anti-fungal in nature, and are thought to play an integral role in protecting the host from invasion by fungal pathogens (Sahai & Manocha, 1993). Some specific examples of this are transgenic tobacco plants that constitutively express a bean chitinase revealing an enhanced resistance to *Rhizoctonia solani* (Broglie *et al.*, 1991), similarily, cucumber resistance to *Colletotrichum lagenarium* has also been correlated with chitinase levels (Irving & Kuc, 1990).

Class I chitinases have an N-terminal cysteine-rich chitin-binding domain separated from the catalytic domain by a proline- and glycine-rich spacer region, variable in both size and composition. Most class I chitinases are synthesized as propeptides, and are eventually directed to the vacuole by a short carboxy-terminal signal sequence. Deletion of this signal peptide redirects a class I chitinase to the apoplast while retaining the enzymatic activity (Grover *et al.*, 2001). Found mainly in dicotyledons class II chitinases are similar to class I except they do not have the N-terminal and spacer region.

Furthermore, the lack of the carboxy-terminal vacoular targeting signal indicates that these chitinases do not bind chitin, and are generally secreted to the apoplast. Class IV chitinases, again found mainly in dicotyledons, are a group of extracellular chitinases that share 41-47% sequence identity with class I chitinases in the catalytic domain, however, they have shorter chain lengths due to four internal deletions (Cohen-Kupiec & Chet, 1998).

Class III chitinases are unique in having a structure unrelated to any other class of plant chitinases. These enzymes, derived mainly from plants and fungi belong to family 18 of the glycosyl hydrolases, and appear to be more closely related to the bacterial chitinases. Members of the class III chitinases generally have lysozyme activity, including the bifunctional lysozyme/chitinase enzyme of *Havea brasiliensis* (Cohen-Kupiec & Chet, 1998). These enzymes may have a variety of functions depending on the organism they are associated with. In fungi multiple functions have been assigned for the chitinases they produce, including nutrition, development, morphogenesis and mycoparasitism (Sahai & Manocha, 1993).

Class V, also family 18 glycosyl hydrolases, represent mostly bacterial chitinases. Largely to meet nutritional needs bacteria may produce a variety of chitinases. Class V enzymes show no homology to classes I, II and IV, and are characterized by the duplication of the N-terminal chitin-binding domain. Two proteins from tobacco represent this class, and are unrelated to other plant chitinases but share sequence similarity to bacterial exochitinases (Cohen-Kupiec & Chet, 1998; Robert *et al.*, 2002).

Within each of these classes, enzymes may be divided into three principle types: (i) β-1,4-*N*-acetylglucasaminidases, which function as exochitinases, are responsible for

splitting the chitin polymer into GlcNAc monomers, (ii) endochitinases which randomly cleave internal sites within the chitin chain; and (iii) chitobiosidases which catalyze the progressive release of diacetylchitobiose such that no monosaccharides or oligosaccharides are formed (Harman *et al.*, 1993; Sahai & Manocha, 1993). These activities are summarized in Figure 5.

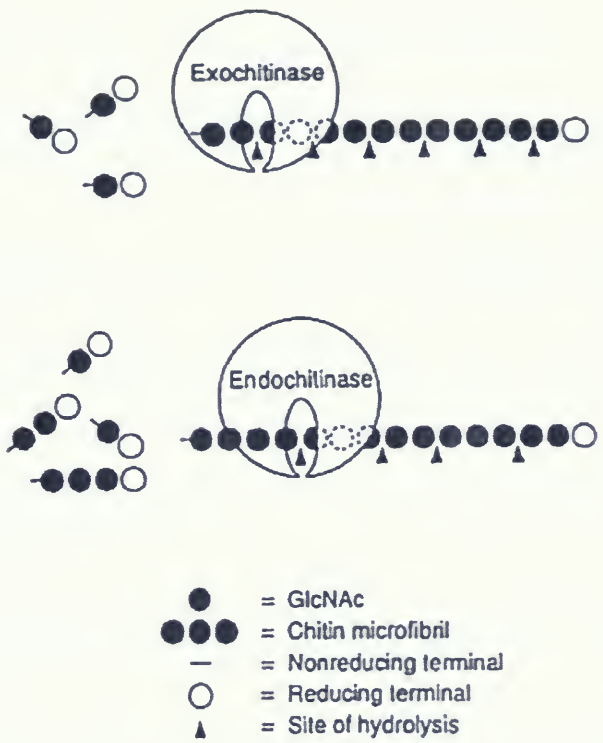

Figure 5 – Mechanism of exochitinase and endochitinases action (Sahai & Manocha, 1993).

Mycoparasitic Involvement

Initial speculation of lytic enzyme involvement in the antifungal activities of *Trichoderma* spp., began with observation of host-parasite interactions using scanning (SEM) and transmission (TEM) electron microscopy. Penetration holes and lysed sites have been observed following the removal of *T. harzianum* coiled around host hyphae, indicating the presence of cell-wall degrading enzymes (Elad *et al.*, 1983). Furthermore, a study by Cherif and Benhamou (1990) revealed that contact was not necessary for parasitism to occur, and the detection of *N*-acetylglucosamine residues suggested that chitinase enzymes might be involved. More recently research has focused on isolating and purifying chitinases in attempts to resolve the chitinolytic system of *T. harzianum.*

Numerous chitinases have been detected as secreted enzymes of various *Trichoderma* spp. Induction of such enzymes may occur in the presence of chitin or fungal cell walls (Carsolio *et al.*, 1994; Garcia *et al.*, 1994; Limón *et al.*, 1995; Peterbauer *et al.*, 1996), and may also be produced as a result of starvation (Limón *et al.*, 1995; Mach *et al.*, 1999). Consequently, as inducible enzymes chitinases are also subject to catabolite repression, in which glucose has been deemed responsible (Limón *et al.*, 1995; Carsolio *et al.*, 1994; Peterbauer *et al.*, 1996). To date researchers have determined that there are at least seven chitinases which make up the complex chitinolytic system of *T. harzianum.* Included are exochitinases (CHIT 102 & CHIT 73), endochitinases (CHIT 52, CHIT 42, CHIT 33 & CHIT 31), and the chitobiosidase CHIT 40 (Haran *et al.*, 1996). A summary of these enzymes can be seen in Table 1. Although *Trichoderma* spp. may secrete these enzymes during growth on various carbon sources, it is unknown if all are essential to parasitic activity (Haran *et al.*, 1996).

Table 1 – Summary of *T. harzianum* chitinolytic activities induced by chitin (Haran *et al.*, 1996).

Designation	Apparent Mass (kDa)	Activity	Strain	Reference
CHIT 102	102-118	*N*-acetylglucosaminidase	TM	Haran *et al.* (1995)
			39.1	Ulhoa & Peberdy (1991)
CHIT 73	73	*N*-acetylglucosaminidase	TM	Haran *et al.* (1995)
			P1	Lorito *et al.* (1994a)
CHIT 52	52	Endochitinase	TM	Haran *et al.* (1995)
CHIT 42	40-42	Endochitinase	CECT 2413	De La Cruz *et al.* (1992)
			39.1	Ulhoa & Peberdy (1991)
			P1	Harman *et al.* (1993)
CHIT 40	40	Exochitinase (chitobiosidase)	P1	Harman *et al.* (1993)
CHIT 33	33-37	Endochitinase	CECT 2413	De La Cruz *et al.* (1992)
			TM	Haran *et al.* (1995)
CHIT 31	31-33	Endochitinase	CECT 2413	De La Cruz *et al.* (1992)
			TM	Haran *et al.* (1995)

Regulation of chitinases is not well understood, and attempts to elucidate the chitinolytic system have been made more difficult by their isolation from various independent strains of *T. harzianum* in which differential expression of these enzymes has been observed (Inbar & Chet, 1995; Haran *et al.*, 1996). It has yet to be determined if chitinase induction is a consequence of, or a prerequisite, for mycoparasitism. However, recent studies in which a number of chitinases have been cloned are helping to clarify this issue. Three genes, *ech33*, *ech42*, and *nag1* have been cloned and characterized as genes encoding CHIT 33, CHIT 42 (both endochitinases), and the *N*-acetylglucosaminidase CHIT 73, respectively (Carsolio *et al.*, 1994; Garcia *et al.*, 1994; Hayes *et al.*, 1994; Limón *et al.*, 1995; Peterbauer *et al.*, 1996). The detection of *ech42* prior to contact of *T. harzianum* with its host is indicative of an antagonistic role (Woo *et al.*, 1998; Zeilinger *et al.*, 1999). While the specific inducers of chitinase expression remain unknown, it has been well established that basal levels of CHIT 102 are produced by *T. harzianum*. More recent studies have determined that CHIT 42 is also produced at low levels, suggesting that induction may be triggered by chitin oligomers produced by constitutive activity of one or both of these enzymes (Inbar & Chet, 1995; Schickler *et al.*, 1998; Zeilinger *et al.* 1999).

In summary, it appears that *Trichoderma* spp. are capable of forming a wide array of both exo- and endochitinases, with increasing evidence of their involvement in the mycoparasitic activities of *T. harzianum*. Research of the chitinolytic system has focused mainly on the creation of a more effective biocontrol agent, while the specific role of chitinases in green mould disease has been largely untouched. Furthermore, few studies

have focused on the mechanisms of fungal resistance to *Trichoderma* spp., and the contribution of hydrolytic enzymes to this resistance is unknown at this stage.

Experimental Focus

The lytic enzymes involved in mycoparasitism, specifically the chitinolytic system is of particular interest to researchers, and it is on this system that my research focused. The intent of this study was to examine the production of chitinase enzymes during the host-parasite interaction of *A. bisporus* and *T. aggressivum.*

Based on reviewed literature the question posed was - Do different host strains of *A. bisporus*, i.e. brown strains which appear to be more resistant than white strains, have an effect on the types or levels of chitinases produced by *T. aggressivum*? To answer this, four strains of *A. bisporus*, representing white, off-white and brown mushrooms were used. Intra- and extracellular proteins were isolated from *T. aggressivum* and *A. bisporus* grown independently, as well as from dual cultures grown for a period of 14 days. Following separation by polyacrylamide gel electrophoresis, detection of specific chitinases was accomplished using activity gel analysis as described by Tronsmo and Harman (1993).

Materials and Methods

Strains and Media

Agaricus bisporus commercial strains Amycel 2400 large brown, Horst U1 off-white hybrid, Sylvan SB65 large brown, and Sylvan 130 mid-range (white) hybrid, were maintained on *Schizophyllum* complete media (SCM) that consisted (l^{-1}) of: 0.5 g $MgSO_4 \cdot 7H_2O$, 0.46 g KH_2PO_4, 1.0 g K_2HPO_4, 2.0 g peptone, 2.0 g yeast extract, 20.0 g glucose, (agar 20 g) (Snider & Raper, 1958). Cultures were initiated by excising a ~10 mm x 5 mm section from a mature culture, and placing the section upside down onto the centre of a new SCM plate. All strains were subcultured monthly. *Trichoderma aggressivum* isolate T586 was maintained on malt extract agar (MEA) that consisted per liter of: 20.0 g malt extract, (20.0 g agar). Subculturing of *T. aggressivum* was performed every two weeks by taking a loopful of conidia from a mature plate and touching the loop to a new MEA plate.

Enzyme Production

Solitary Cultures

Proteins were isolated from both *A. bisporus* and *T. aggressivum* solitary cultures for enzyme identification. *Agaricus* cultures were grown by transferring ~10 mm x 5 mm pieces of mature culture to plates containing SCM broth. These cultures were allowed to grow for three weeks, at which time they were harvested for enzyme isolation. Similarly, *T. aggressivum* spores (10^7 - 10^8/ml) were suspended in MEA, and grown for a period of five days before intra- and extracellular proteins were extracted.

Dual Cultures

Agaricus cultures were grown in the same manner as solitary culturing described above. Once these cultures had grown for three weeks they were inoculated with 1 ml of *Trichoderma* spore suspension 10^7 - 10^8 conidia/ml. Dual cultures were harvested at one-day intervals beginning at day 0 through to day 14.

Enzyme Isolation

At the appropriate time intervals cultures were harvested for enzyme isolation using a modified procedure by Haran *et al.* (1995). Harvesting included filtering 3 plates of dual cultures through Whatman No. 4 filter paper to separate mycelia (intracellular protein) from culture liquid (extracellular protein). Mycelia were then ground using a mortar and pestle, following which were suspended in 30 ml of protease inhibitor extraction buffer (1 mM EDTA, 10 mM Tris-HCl, pH 7.4, 1 µM PMSF). This suspension was then homogenized using a Sorvall Omni-Mixer for 3 min at 4°C, sonicated for 3 min at 4°C, and homogenized again at 4°C for 3 min. Homogenate was centrifuged at 12000 *g* for 20 minutes at 4°C and supernatant collected. Proteins were precipitated from the supernatant by the addition of ammonium sulphate (75% saturation). Once the ammonium sulphate had dissolved, the solution was kept at 4°C for 1 h., following which proteins were collected by centrifuging at 12000 *g* for 20 min. The pellet was resuspended and liquid enzyme suspensions were further purified by dialyzing (MWCO 12000-14000) overnight against protease inhibitor extraction buffer at 4°C. Samples in dialysis tubes were placed on a dry bed of polyethylene glycol and concentrated to an approximate volume of 1 ml. Proteins were stored at – 20°C until needed. Protein

concentration was determined according to Bradford (1976) using the Bio-Rad protein-assay dye reagent and bovine serum albumin (BSA) as a standard.

Enzyme Identification

For detection of specific chitinolytic activity, proteins were separated using both sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and Native-PAGE, whereby gels and buffers contained no SDS, which acts to denature proteins. Samples containing the standard amount of 5 μg of protein were prepared in sample buffer containing 125 mM Tris-Cl (pH 6.8), 20% glycerol (v/v), [4% SDS (w/v)], and 0.2% bromophenol blue, and only samples separated by SDS-PAGE were heated for 3 min. at 37°C. The samples were loaded into 1.0 mm gels with a 4% acrylamide/bis-acrylamide stacking gel, and separated along an 11% acrylamide/bis-acrylamide resolving gel in a Bio-Rad Mini-Protean II cell. Gels were run in the presence of run buffer (25 mM Tris, 250 mM glycine, [0.1% SDS]) for 3 hours at 70 volts for the best resolution of bands. Following electrophoresis SDS gels were washed using 25% isopropanol to remove SDS and allow for renaturation of the enzymes. The wash buffer was changed at 5 minute intervals for 15 minutes. Gels were then washed with dH_2O for 5 minutes. An agarose substrate solution was prepared by heating 20 ml of 100 mM sodium acetate (pH 4.8) containing 1% agarose in a heating oven at 50°C. For detection of specific chitinolytic activity, one of three substrates was added to the agarose solution to achieve a final concentration of 0.025 mg/ml. The specific substrates 4-methylumbelliferyl N-acetyl-β-D-glucosaminide [4-MU(GlcNAc)], 4-methylumbelliferyl N-acetyl-β-D-N,N'-diacetylchitobioside [4-MU$(GlcNAc)_2$], and 4-methylumbelliferyl N-acetyl-β-D-N,N',N''-triacetylchitotriose [4-MU$(GlcNAc)_3$] (Sigma) produce a

fluorescent product following enzymatic hydrolysis. These substrates allow for detection of the three different chitinase types by acting as dimeric, trimeric, and tetrameric substrates respectively (Figure 6). *N*-acetylglucosaminidases act in an exo type fashion splitting the polymer into GlcNAc monomers, and will be detected using all substrates. Endochitinases cleave randomly at internal sites along the chitin chain, producing low-molecular-mass multimers, requiring at least the trimer for activity. Chitobiosidases catalyze the progressive release of diacetylchitobiose in a step-wise fashion, such that no monosaccharides or oligosaccharides are formed, and therefore will only be detected in the presence of the trimeric substrate (Haran *et al.*, 1995).

For native gels the procedure was essentially the same, however since there was no SDS present the isopropanol step was not required and gels were simply washed in dH_2O for 5 minutes before being placed in the agarose-substrate solution. Once gels were placed in the agarose-substrate solution they were rotated slowly for 10 minutes prior to detection under ultraviolet light.

Determination of Molecular Mass

To determine the molecular weight of proteins separated by SDS-PAGE, a fluorescent molecular weight marker was used (Sigma). Following electrophoresis, marker bands were visualized on activity gels, and the migration distance was measured to create a standard curve. Determination of molecular mass using non-denaturing PAGE was much more involved, since both mass and charge contribute to the migration of a native protein. Using Sigma's Nondenatured Protein Molecular Weight Marker Kit, four proteins of known molecular mass were run on native polyacrylamide gels of varying concentrations (6%, 8%, 10%, and 12%).

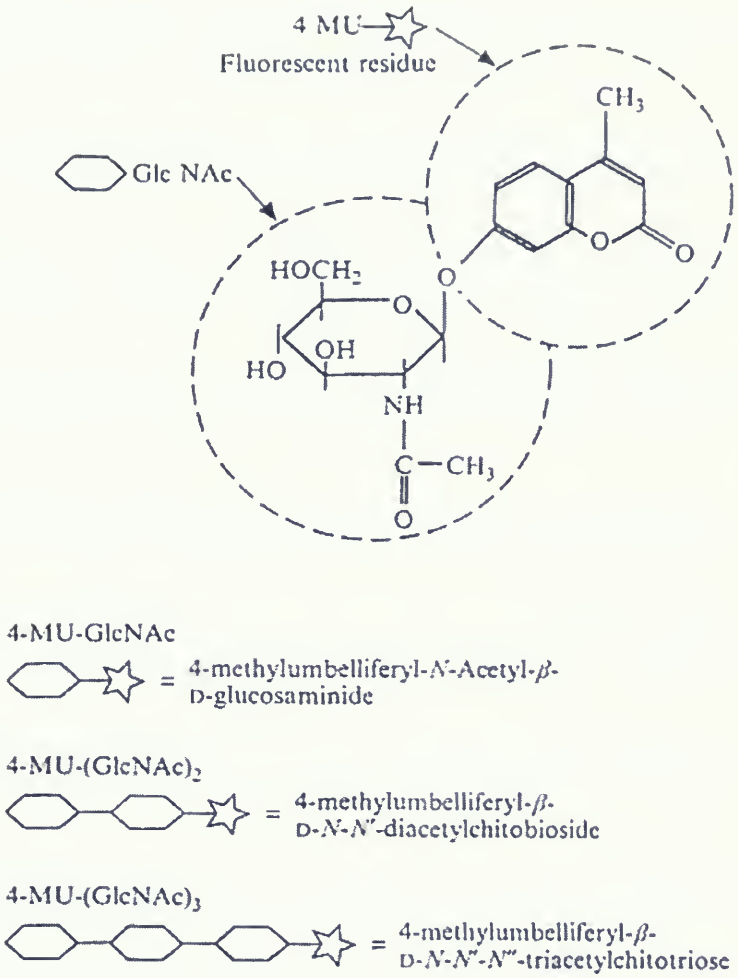

Figure 6 – Schematic presentation of 4-methylumbelliferyl (4-MU) glucosides of *N*-acetylglucosamine oligosaccharides as substrates for the detection of chitinolytic enzymes (Haran *et al.*, 1995).

Marker proteins were visualized by Coomassie staining, in which gels were placed on a shaker at slow speed in a solution of 0.25% Coomassie Brilliant Blue R-250, 40% methanol, and 10% acetic acid. Excess dye was removed by washing with destaining solution consisting of 40% methanol, and 10% acetic acid until the background was sufficiently clear. Migration distances of proteins and dye front were measured to calculate R_f values, and create a standard curve as outlined in the Sigma Technical Bulletin No. MKR-137. Proteins of unknown molecular weight were run on 6%, 8%, 10%, and 12% native polyacrylamide gels for calculation of R_f values, which were used to determine molecular mass from the standard curve.

At the time of molecular weight determination each sample had been run many times and similarities in banding patterns had been observed. Therefore, specific samples were chosen to represent those believed to have the same molecular weight, based on previously run gels. Large charts were created containing all R_f values, and the corresponding log values (see Appendix for calculations) for each representative, which included 68 samples from the 1st and 2nd replicates. Based on migration patterns across the varying gel concentrations, as seen in the log values, similar patterns were grouped together. The values from a minimum of five bands were averaged and the standard deviation calculated. Molecular weight was determined using the standard curve which had been created.

Quantification

Chitinolytic activity was detected by the released fluorescent product, 4-methylumbelliferone (4-MU). Fluorescent signals, seen as activity gel bands, were viewed with the Bio-Rad Gel Doc 2000 system, and intensity measurements were

performed with Fuji's Image Gauge version 3.46 software. Known concentrations of 4-MU were used to create a standard curve that allowed for conversion of intensity readings of samples to enzyme activity. One unit (U) of enzyme activity was defined as the amount of enzyme that catalyzed the release of 1 ng of 4-methylumbelliferone min^{-1}. Specific activity was defined as units (U) of enzyme activity per microgram of crude protein extract.

The average from six intensity readings for each known sample was used to create a standard for quantification of fluorescent bands. For unknowns, two bands per replica for each sample were used to calculate specific activity. The intensity measurements were averaged and standard deviation determined.

Results

Culture Conditions

Cultures used for protein isolation were grown in petri dishes containing liquid medium. This small-scale culturing method provided the best means of reducing contamination, and minimizing cultures lost as a result. Although a wide range of unwanted fungi and bacteria were observed at times, liquid cultures of *A. bisporus* were most commonly infested with a bacterial contaminant, as identified by Dr. A.J. Castle. The addition of ampicillin at a concentration of 50µg/ml, acted to significantly reduce the number of cultures lost due to bacteria.

Macroscopic observations during co-culturing allowed for visualization of the parasitism of *A. bisporus* by *T. aggressivum*. Initial detection of *Trichoderma* hyphae occurred within twenty-four hours of dual culture initiation. This was seen as a slight cloudiness of the medium accompanied by small amounts of white hyphal growth. By day 3, a thin film of hyphae was observed, and by day 5 it had thickened, and white filamentous growth could be seen on the film. Co-culturing was completed after day 14, by which time the entire plate was grown over with thick white mycelia, the culture liquid had darkened significantly, and small amounts of greenish yellow spores could be seen (Figure 7). No discernible differences at the macroscopic level were observed between cultures containing different host strains of *A. bisporus* and *T. aggressivum*.

Figure 7 – Macroscopic examination of co-cultures of *Agaricus bisporus* strain Sylvan SB65 and *Trichoderma aggressivum*. Photograph (**A**) shows a mature culture of *A. bisporus* grown in liquid culture, newly inoculated with *Trichoderma* spores, and representing T = 0. Photograph (**B**) is a higher magnification of *A. bisporus* mycelia at T = 0, and (**C**) represents day 1, with the beginnings of *Trichoderma* hyphae slightly visible amongst the larger *Agaricus* colonies. (**D**) shows the thick layer of *Trichoderma* mycelia present at day 5, while day 14 is represented in photograph (**E**).

Protein Isolation and Analysis

Quantification

Presently there are no known procedures for quantification of bands that fluoresce as a result of the release of 4-methylumbelliferone (4-MU). Since this is an uncharged molecule, standards for image analysis could not be achieved using gel electrophoresis. Therefore, a new method was created in which varying concentrations of 4-MU were pipetted into gel comb indents of a polyacrylamide gel to form band like patterns that could be analyzed using image analysis software. Intensity measurements were performed using Fuji's Image Gauge software, which allowed for standard band areas and background subtraction. A sample picture, and standard curve are represented in Figure 8.

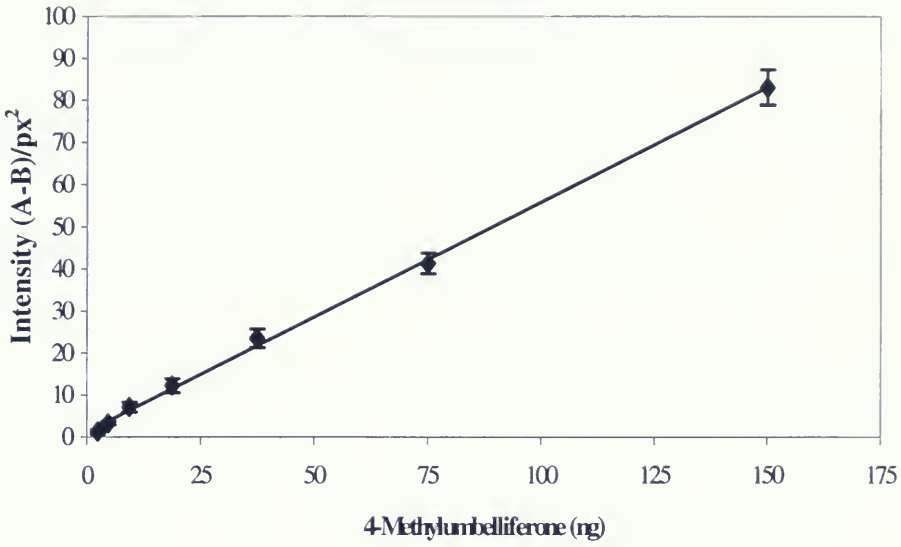

Figure 8 – Gel picture shows the fluorescence of 4-MU visible under ultraviolet light with the quantity in ng of 4-methylumbelliferone listed below. The resulting standard curve represents the average intensity readings for six replicates of each concentration.

Molecular Weight Estimation

To determine the molecular weight of proteins separated by native-PAGE, Sigma's Nondenatured Protein Molecular Weight Marker Kit was used. Four proteins of known molecular mass were run on native polyacrylamide gels of varying concentrations (6%, 8%, 10%, and 12%), and marker proteins were visualized by Coomassie staining (Figure 9). Migration distance of proteins and dye front were measured to calculate R_f values, which were plotted against gel concentration (Figure 10), and used to create a standard curve as outlined in the Sigma Technical Bulletin No. MKR-137. Proteins of unknown molecular weight were also run on 6%, 8%, 10%, and 12% native polyacrylamide gels for calculation of R_f values, which were used to determine molecular mass from the standard curve (Figure 11). Based on this method, estimations of apparent molecular masses of chitinase enzymes isolated during this study are summarized in Table 2. The molecular weight was averaged from a minimum of 5 bands, and the resulting standard error calculated.

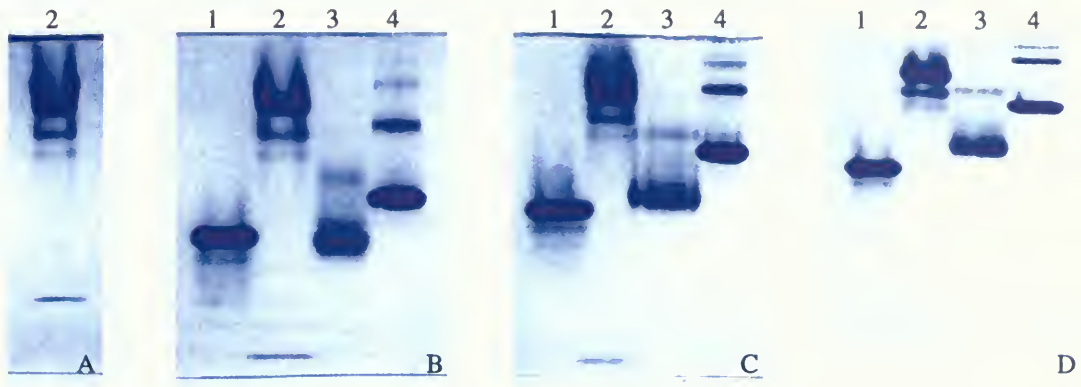

Figure 9 – Electrophoretic banding patterns of standard proteins separated by (**A**) 6%, (**B**) 8%, (**C**) 10%, and (**D**) 12% native polyacrylamide gels. Standard 1 represents α–Lactalbumin (14.2 kDa), standard 2 represents Carbonic anhydrase (29 kDa), standard 3 represents Chicken egg albumin (45 kDa), and standard 4 represents a monomer (66 kDa) and dimer (132 kDa) of Bovine serum albumin.

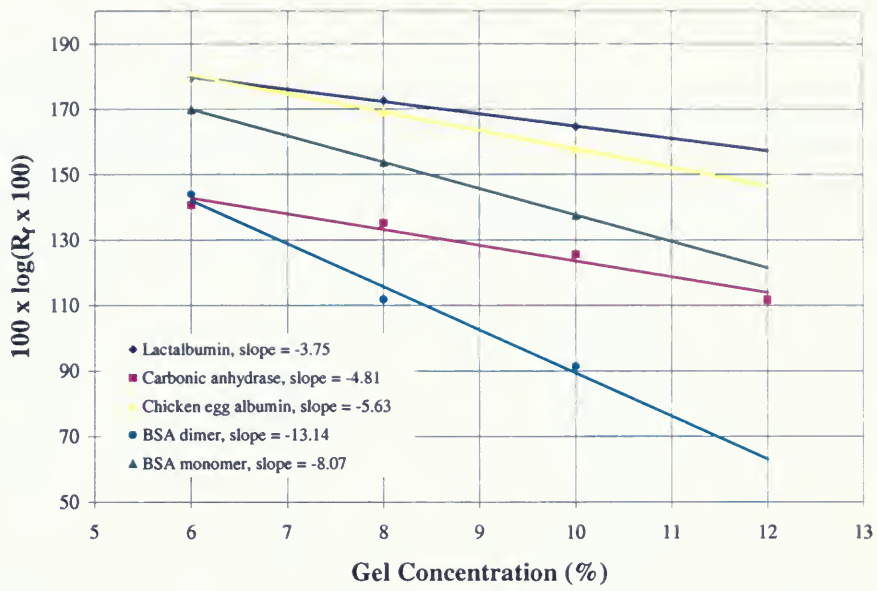

Figure 10 – Determination of standard slopes based on the calculated relative mobility of each standard protein.

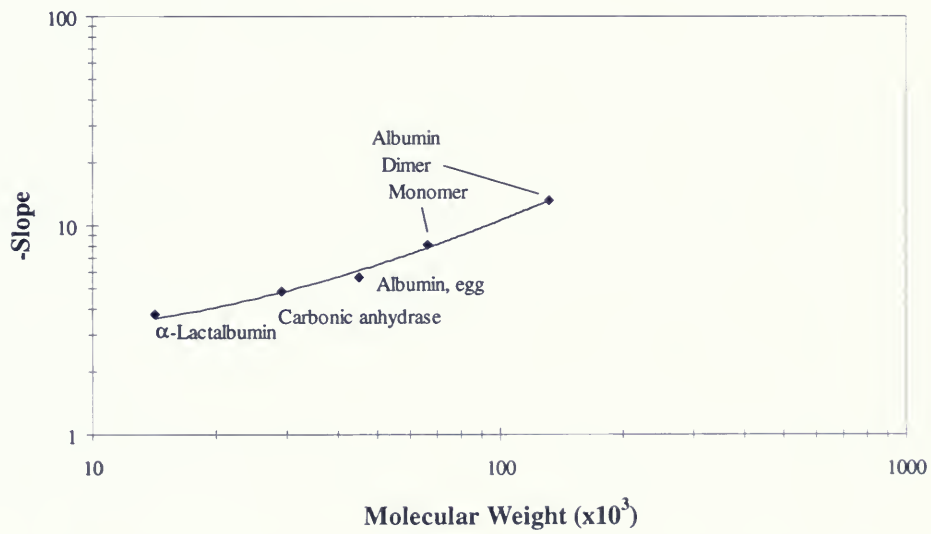

Figure 11 – Plot of negative slopes versus molecular weights used for the determination of unknown molecular masses.

Table 2 – Summary of apparent molecular weights of chitinase enzymes isolated under both solitary and dual culture conditions.

Chitinase	Mol. Mass (kDa ± SE)
1	131 ± 3
2	125 ± 2
3	122 ± 4
4	111 ± 3
5	105 ± 2
6	96 ± 2
7	40 ± 2
8	36 ± 1

Enzyme Profiling

Proteins were initially separated under the denaturing conditions of SDS-PAGE. However, enzyme analysis of gels revealed only *N*-acetylglucosaminidases. In attempts to determine if other enzyme types were present but not detectable, a number of variables were altered, including: (i) temperature of agarose-substrate solution, (ii) use of potassium phosphate buffer pH 6.7, which had a higher pH than the sodium acetate buffer, and (iii) electrophoresis using native gels (without SDS). The first two modifications failed to resolve any new enzyme types, however under non-denaturing conditions a number of additional chitinases were now detected (Figure 12).

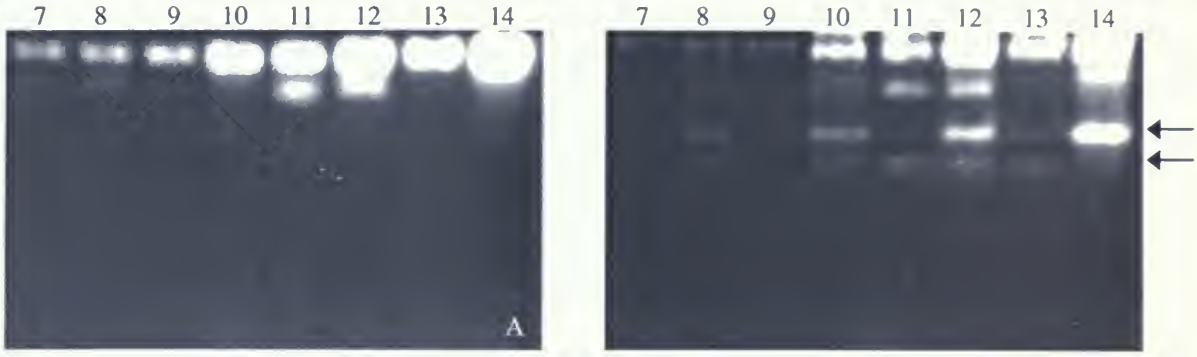

Figure 12 – Comparison of activity gels representing day 7 through 14 dual cultures of *T. aggressivum* with *A. bisporus* strain SB65. Both gels were visualized using 4-MU-$(GlcNAc)_2$. **(A)** Samples were separated under denaturing conditions using SDS-PAGE **(B)** Protein samples were separated under non-denaturing conditions. Arrows indicate enzymes that had been previously undetected.

Chitinolytic Activity of Solitary Cultures

Intracellular and extracellular proteins were isolated in duplicate from solitary cultures of all four *Agaricus* strains as well as *T. aggressivum*. Samples were analyzed on polyacrylamide gels for characterization of chitinases prior to co-cultivation studies.

In solitary culture it was observed that *Trichoderma* produced a number of chitinases, including an *N*-acetylglucosaminidase detected intracellularly, with an apparent molecular mass of 122 kDa. Additionally, a 36 kDa endochitinase was isolated from mycelial samples, while filtrate samples revealed a 40 kDa chitobiosidase, determined to be such as it was seen only in the presence of the trimeric substrate 4-MU-$(GlcNAc)_2$.

Higher chitinase activity was observed for brown strains, Amycel 2400, and Sylvan SB65, for both mycelial (3.78 U/µg and 1.82 U/µg crude protein extract, respectively) and filtrate samples (3.80 U/µg, 1.92 U/µg and 1.50 U/µg, respectively). Specific activity calculations for all four *Agaricus* strains are presented in Table 4. Several chitinases, all *N*-acetylglucosamindases, of apparent molecular weights of 111, 105 and 96 kDa were detected (Figure 13).

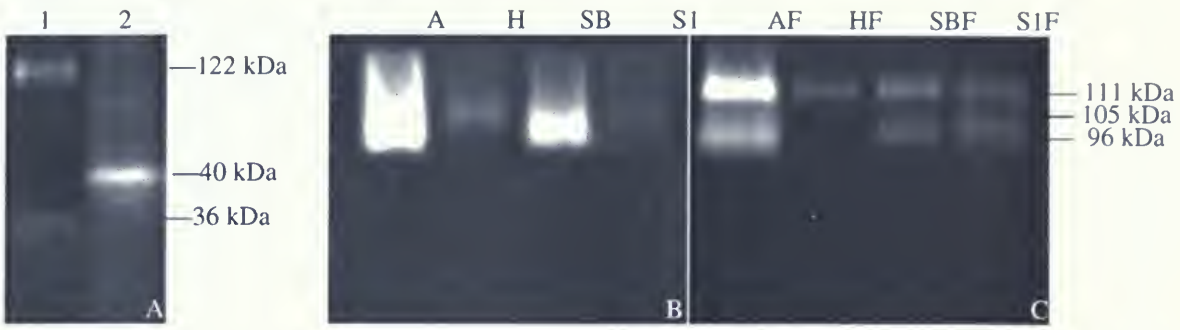

Figure 13 – Detection of chitinolytic activity of proteins produced from (**A**) *T. aggressivum* solitary cultures with lane 1 representing intracellular proteins, and lane 2 representing extracellular proteins; (**B**) intracellular proteins isolated from *A. bisporus* strains A-Amycel 2400, H-Horst U1, SB-Sylvan SB65, and S1-Sylvan 130, and (**C**) extracellular proteins collected from the filtrate of the same four *A. bisporus* strains. Activity was visualized using 4-MU-$(GlcNAc)_2$ for *T. aggressivum* and 4-MU-(GlcNAc) for *A. bisporus* strains.

Table 3 - Chitinolytic activity of solitary *Trichoderma* cultures, representing both intracellular and extracellular isolations. Enzyme type NAG refers to *N*-acetylglucosaminidases, Endo represents endochitinases, and Chito represents chitobiosidases.

Sample	Mol. mass (kDa)	Specific activity (U/μg crude protein)	4-MU-(GlcNAc)	4-MU-$(GlcNAc)_2$	4-MU-$(GlcNAc)_3$	Chitinase Type
Intra-	122	0.83	+	+	+	NAG
Intra-	36	0.65	-	+	+	Endo
Extra-	40	2.92	-	+	-	Chito

Table 4 - Chitinolytic activity of solitary *Agaricus bisporus* cultures (Amycel 2400, Horst U1, Sylvan SB65, Sylvan 130), representing both intracellular and extracellular isolations. The abbreviation NAG refers to the enzyme *N*-acetylglucosaminidases.

Sample	Mol. mass (kDa)	Specific activity (U/μg crude protein)	4-MU-(GlcNAc)	4-MU-$(GlcNAc)_2$	4-MU-$(GlcNAc)_3$	Chitinase Type
Intracellular						
Amycel	96	3.78	+	+	+	NAG
Horst	105	0.85	+	+	+	NAG
SB65	96	1.82	+	+	+	NAG
S130	105	0.43	+	+	+	NAG
Extracellular						
Amycel	111	3.80	+	+	+	NAG
	96	3.80	+	+	+	NAG
Horst	111	1.06	+	+	+	NAG
SB65	111	1.92	+	+	+	NAG
	96	1.50	+	+	+	NAG
S130	111	1.19	+	+	+	NAG
	96	1.21	+	+	+	NAG

Chitinolytic Activity of Dual Cultures

Dual cultures of each *Trichoderma – Agaricus* combination were grown in triplicate for isolation of intracellular and extracellular proteins. Each trial period (day 0-14) was collected on two separate occasions, resulting in a minimum of 240 samples. All samples were run on native polyacrylamide gels three times, and each gel was incubated with one of the three 4-MU glucoside substrates for determination of enzyme type. Activity gels of intracellular enzymes revealed two *N*-acetylglucosaminidases (122 kDa and 96 kDa), a 36 kDa endochitinase and a 40 kDa chitobiosidase. An example of dual culture activity gels can be seen below in Figure 14. The day at which these enzymes were first detected varied slightly between dual culture combinations, and can be seen in Tables 5 - 8.

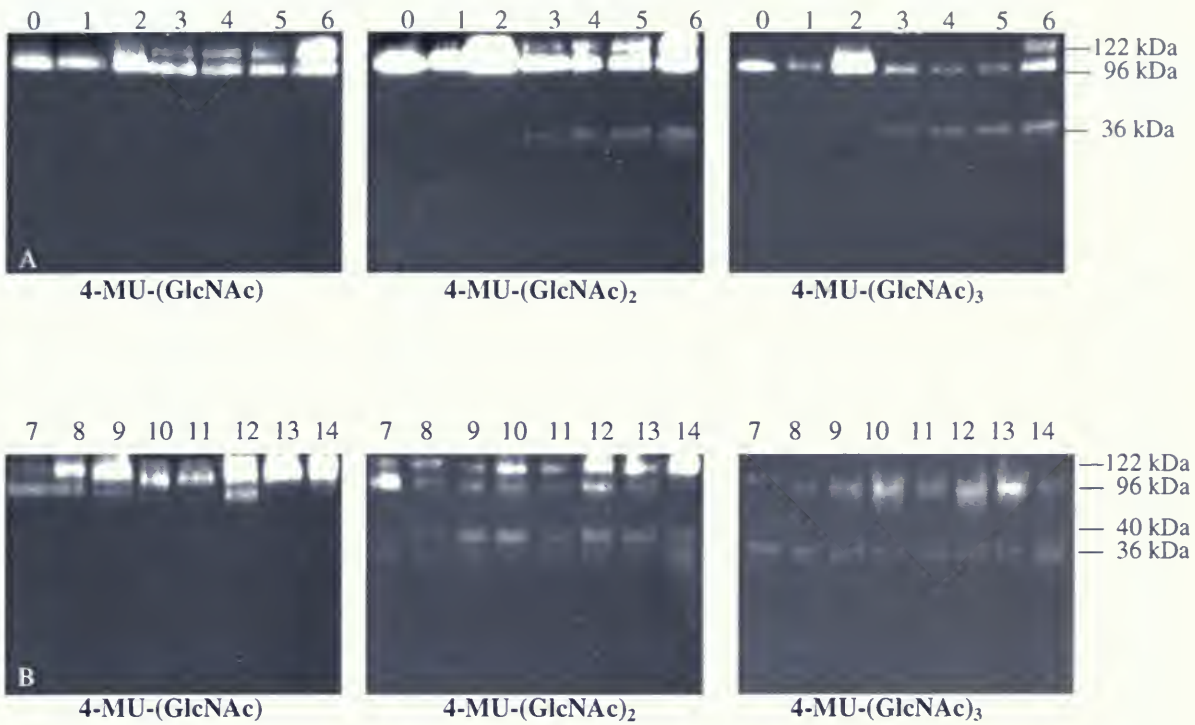

Figure 14 – Detection of intracellular chitinolytic activity of proteins isolated from dual cultures of *T. aggressivum* and *A. bisporus* strain Sylvan SB65 A (day 0 –6) and B (day 7-14). Activity was visualized following incubation with one of three specific substrates 4-MU-(GlcNAc), 4-MU-$(GlcNAc)_2$, or 4-MU-$(GlcNAc)_3$.

Table 5 - Chitinolytic activity of intracellular enzymes isolated from dual cultures of *T. aggressivum* and *A. bisporus* strain Amycel 2400. Enzyme type NAG refers to *N*-acetylglucosaminidases, Endo represents endochitinases, and Chito represents chitobiosidases.

Day	Mol. mass (kDa)	Specific activity (U/μg crude protein)	4-MU-(GlcNAc)	4-MU-$(GlcNAc)_2$	4-MU-$(GlcNAc)_3$	Chitinase Type
0	96	3.50	+	+	+	NAG
1	96	3.50	+	+	+	NAG
2	122	0.99	+	+	+	NAG
	96	3.50	+	+	+	NAG
3	122	1.21	+	+	+	NAG
	96	3.50	+	+	+	NAG
	36	0.21	-	+	+	Endo
4	122	1.21	+	+	+	NAG
	96	3.50	+	+	+	NAG
	36	0.42	-	+	+	Endo
5	122	2.37	+	+	+	NAG
	96	3.50	+	+	+	NAG
	36	0.61	-	+	+	Endo
6	122	3.96	+	+	+	NAG
	96	3.50	+	+	+	NAG
	36	0.85	-	+	+	Endo
7	122	4.67	+	+	+	NAG
	96	3.50	+	+	+	NAG
	40	0.85	-	+	-	Chito
	36	0.57	-	+	+	Endo
8	122	4.44	+	+	+	NAG
	96	3.50	+	+	+	NAG
	40	0.99	-	+	-	Chito
	36	0.50	-	+	+	Endo

Day	Mol. mass (kDa)	Specific activity (U/μg crude protein)	4-MU-(GlcNAc)	4-MU-$(GlcNAc)_2$	4-MU-$(GlcNAc)_3$	Chitinase Type
9	122	3.73	+	+	+	NAG
	96	0.63	+	+	+	NAG
	40	1.00	-	+	-	Chito
	36	0.38	-	+	+	Endo
10	122	4.45	+	+	+	NAG
	96	2.12	+	+	+	NAG
	40	0.98	-	+	-	Chito
	36	0.35	-	+	+	Endo
11	122	1.64	+	+	+	NAG
	96	1.29	+	+	+	NAG
	40	1.00	-	+	-	Chito
	36	0.51	-	+	+	Endo
12	122	4.68	+	+	+	NAG
	96	1.41	+	+	+	NAG
	40	1.15	-	+	-	Chito
	36	0.59	-	+	+	Endo
13	122	4.63	+	+	+	NAG
	96	1.40	+	+	+	NAG
	40	1.14	-	+	-	Chito
	36	0.78	-	+	+	Endo
14	122	4.68	+	+	+	NAG
	96	1.29	+	+	+	NAG
	40	1.10	-	+	-	Chito
	36	0.77	-	+	+	Endo

Table 6 - Chitinolytic activity of intracellular enzymes isolated from dual cultures of *T. aggressivum* and *A. bisporus* strain Horst U1. Enzyme type NAG refers to *N*-acetylglucosaminidases, Endo represents endochitinases, and Chito represents chitobiosidases.

Day	Mol. mass (kDa)	Specific activity (U/μg crude protein)	4-MU-(GlcNAc)	4-MU-$(GlcNAc)_2$	4-MU-$(GlcNAc)_3$	Chitinase Type
0	96	0.71	+	+	+	NAG
1	96	0.77	+	+	+	NAG
2	122	0.50	+	+	+	NAG
	96	0.70	+	+	+	NAG
	36	0.29	-	+	+	Endo
3	122	0.34	+	+	+	NAG
	96	0.11	+	+	+	NAG
	36	0.38	-	+	+	Endo
4	122	1.89	+	+	+	NAG
	96	0.25	+	+	+	NAG
	36	0.45	-	+	+	Endo
5	122	2.36	+	+	+	NAG
	96	0.34	+	+	+	NAG
	36	0.44	-	+	+	Endo
6	122	3.33	+	+	+	NAG
	96	1.94	+	+	+	NAG
	36	0.46	-	+	+	Endo
7	122	4.28	+	+	+	NAG
	96	0.33	+	+	+	NAG
	36	0.43	-	+	+	Endo
8	122	4.32	+	+	+	NAG
	96	1.07	+	+	+	NAG
	40	0.78	-	+	-	Chito
	36	0.70	-	+	+	Endo

Day	Mol. mass (kDa)	Specific activity (U/μg crude protein)	4-MU-(GlcNAc)	4-MU-$(GlcNAc)_2$	4-MU-$(GlcNAc)_3$	Chitinase Type
9	122	4.32	+	+	+	NAG
	96	1.25	+	+	+	NAG
	40	0.83	-	+	-	Chito
	36	0.61	-	+	+	Endo
10	122	4.32	+	+	+	NAG
	96	0.95	+	+	+	NAG
	40	1.18	-	+	-	Chito
	36	0.57	-	+	+	Endo
11	122	4.32	+	+	+	NAG
	96	0.85	+	+	+	NAG
	40	1.06	-	+	-	Chito
	36	0.73	-	+	+	Endo
12	122	3.62	+	+	+	NAG
	96	0.56	+	+	+	NAG
	40	0.82	-	+	-	Chito
	36	0.47	-	+	+	Endo
13	122	4.32	+	+	+	NAG
	96	0.71	+	+	+	NAG
	40	0.99	-	+	-	Chito
	36	0.83	-	+	+	Endo
14	122	4.32	+	+	+	NAG
	96	0.50	+	+	+	NAG
	40	0.79	-	+	-	Chito
	36	0.42	-	+	+	Endo

Table 7 - Chitinolytic activity of intracellular enzymes isolated from dual cultures of *T. aggressivum* and *A. bisporus* strain Sylvan SB65. Enzyme type NAG refers to *N*-acetylglucosaminidases, Endo represents endochitinases, and Chito represents chitobiosidases.

Day	Mol. mass (kDa)	Specific activity (U/μg crude protein)	4-MU-(GlcNAc)	4-MU-$(GlcNAc)_2$	4-MU-$(GlcNAc)_3$	Chitinase Type
0	96	1.92	+	+	+	NAG
1	96	1.92	+	+	+	NAG
2	122	0.34	+	+	+	NAG
	96	1.92	+	+	+	NAG
3	122	0.81	+	+	+	NAG
	96	1.92	+	+	+	NAG
	36	0.43	-	+	+	Endo
4	122	1.85	+	+	+	NAG
	96	1.92	+	+	+	NAG
	36	0.90	-	+	+	Endo
5	122	1.77	+	+	+	NAG
	96	1.92	+	+	+	NAG
	36	0.51	-	+	+	Endo
6	122	3.52	+	+	+	NAG
	96	1.92	+	+	+	NAG
	36	0.53	-	+	+	Endo
7	122	2.75	+	+	+	NAG
	96	1.22	+	+	+	NAG
	40	0.16	-	+	-	Chito
	36	0.48	-	+	+	Endo
8	122	3.06	+	+	+	NAG
	96	0.74	+	+	+	NAG
	40	0.43	-	+	-	Chito
	36	0.46	-	+	+	Endo

Day	Mol. mass (kDa)	Specific activity (U/μg crude protein)	4-MU-(GlcNAc)	4-MU-$(GlcNAc)_2$	4-MU-$(GlcNAc)_3$	Chitinase Type
9	122	3.86	+	+	+	NAG
	96	1.02	+	+	+	NAG
	40	1.43	-	+	-	Chito
	36	0.40	-	+	+	Endo
10	122	2.56	+	+	+	NAG
	96	1.99	+	+	+	NAG
	40	1.72	-	+	-	Chito
	36	0.32	-	+	+	Endo
11	122	2.02	+	+	+	NAG
	96	1.27	+	+	+	NAG
	40	0.65	-	+	-	Chito
	36	0.36	-	+	+	Endo
12	122	4.09	+	+	+	NAG
	96	1.46	+	+	+	NAG
	40	1.53	-	+	-	Chito
	36	0.38	-	+	+	Endo
13	122	4.07	+	+	+	NAG
	96	1.42	+	+	+	NAG
	40	1.08	-	+	-	Chito
	36	0.41	-	+	+	Endo
14	122	4.09	+	+	+	NAG
	96	1.42	+	+	+	NAG
	40	0.99	-	+	-	Chito
	36	0.53	-	+	+	Endo

Table 8 - Chitinolytic activity of intracellular enzymes isolated from dual cultures of *T. aggressivum* and *A. bisporus* strain Sylvan 130. Enzyme type NAG refers to *N*-acetylglucosaminidases, Endo represents endochitinases, and Chito represents chitobiosidases.

Day	Mol. mass (kDa)	Specific activity (U/μg crude protein)	4-MU-(GlcNAc)	4-MU-$(GlcNAc)_2$	4-MU-$(GlcNAc)_3$	Chitinase Type
0	96	0.3	+	+	+	NAG
1	96	0.68	+	+	+	NAG
2	122	0.75	+	+	+	NAG
	96	0.32	+	+	+	NAG
	36	0.41	-	+	+	Endo
3	122	1.53	+	+	+	NAG
	96	0.29	+	+	+	NAG
	36	0.47	-	+	+	Endo
4	122	1.85	+	+	+	NAG
	96	0.30	+	+	+	NAG
	36	0.50	-	+	+	Endo
5	122	3.88	+	+	+	NAG
	96	1.66	+	+	+	NAG
	36	0.53	-	+	+	Endo
6	122	3.72	+	+	+	NAG
	96	0.27	+	+	+	NAG
	36	0.54	-	+	+	Endo
7	122	3.83	+	+	+	NAG
	96	0.30	+	+	+	NAG
	40	0.25	-	+	-	Chito
	36	0.58	-	+	+	Endo
8	122	3.95	+	+	+	NAG
	96	1.53	+	+	+	NAG
	40	0.27	-	+	-	Chito
	36	0.60	-	+	+	Endo

Day	Mol. mass (kDa)	Specific activity (U/μg crude protein)	4-MU-(GlcNAc)	4-MU-$(GlcNAc)_2$	4-MU-$(GlcNAc)_3$	Chitinase Type
9	122	3.96	+	+	+	NAG
	96	1.23	+	+	+	NAG
	40	0.56	-	+	-	Chito
	36	0.74	-	+	+	Endo
10	122	4.33	+	+	+	NAG
	96	0.89	+	+	+	NAG
	40	0.21	-	+	-	Chito
	36	0.73	-	+	+	Endo
11	122	4.00	+	+	+	NAG
	96	0.92	+	+	+	NAG
	40	0.45	-	+	-	Chito
	36	0.55	-	+	+	Endo
12	122	3.92	+	+	+	NAG
	96	0.67	+	+	+	NAG
	40	0.11	-	+	-	Chito
	36	0.74	-	+	+	Endo
13	122	4.02	+	+	+	NAG
	96	1.27	+	+	+	NAG
	40	0.52	-	+	-	Chito
	36	0.93	-	+	+	Endo
14	122	3.91	+	+	+	NAG
	96	0.53	+	+	+	NAG
	40	0.47	-	+	-	Chito
	36	0.56	-	+	+	Endo

Extracellular enzymes collected from the filtrate of dual cultures revealed six different enzyme types. Only *N*-acetylglucosaminidases were observed in the early days of dual culturing, with estimated molecular weights ranging from 96 to 131 kDa. Additionally, a 40 kDa chitobiosidase was seen initially on day 8 of all four *Agaricus – Trichoderma* combinations. It was observed that the most variation of chitinolytic activity occurred between day 0 and day 6 as seen in the activity gels represented in Figure 15. Following the flurry of enzyme production, the types of chitinases produced remained constant after day 6, although there were differences in the specific activities of each enzyme type between dual culture combinations (Figure 16). All four combinations reveal differences in the types and levels of production of chitinase enzymes (Tables 9 – 12).

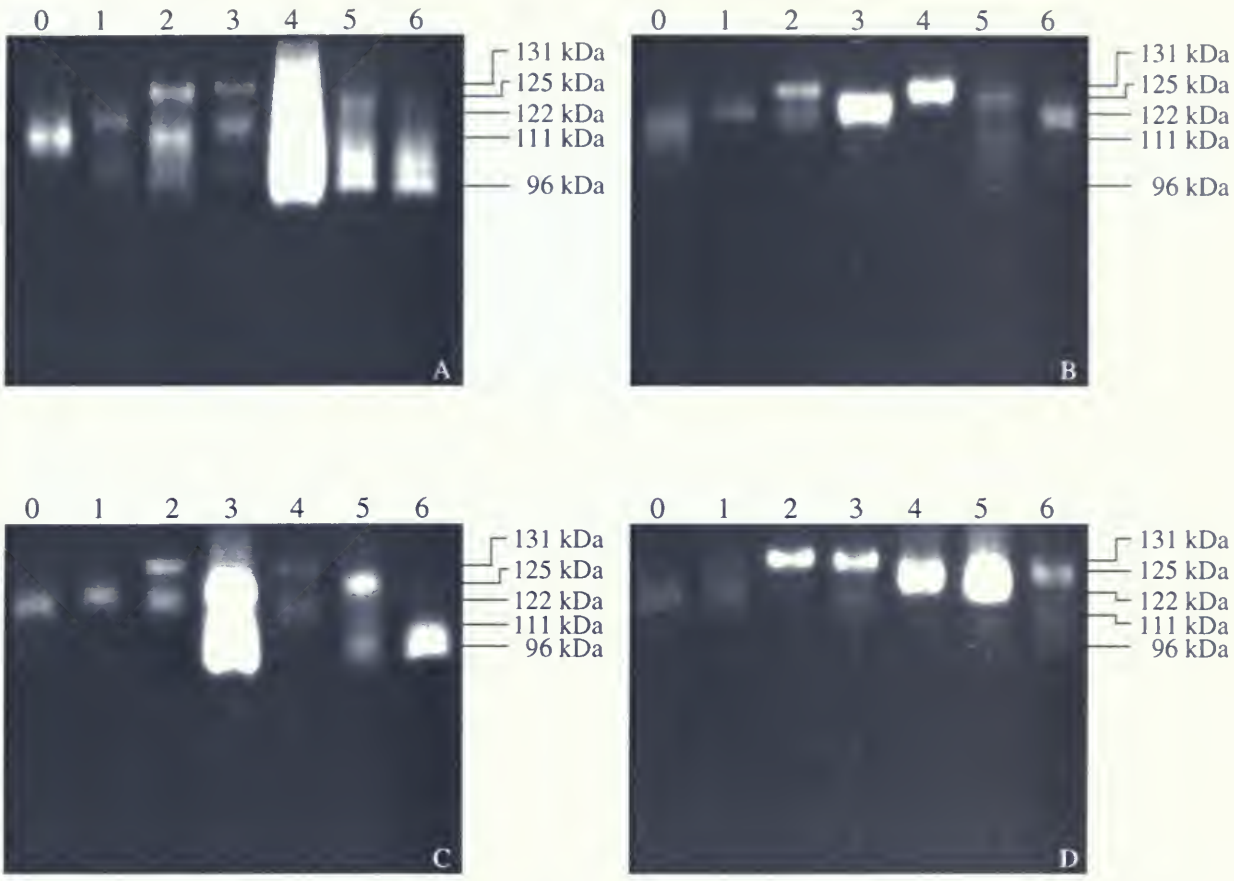

Figure 15 – Detection of extracellular chitinolytic activity of proteins isolated between day 0 through day 6, from dual cultures of *T. aggressivum* and *A. bisporus* strains: (**A**) Amycel 2400 (**B**) Horst U1 (**C**) Sylvan SB65 (**D**) Sylvan 130. Activity was visualized following incubation using the dimeric substrate 4-MU-(GlcNAc).

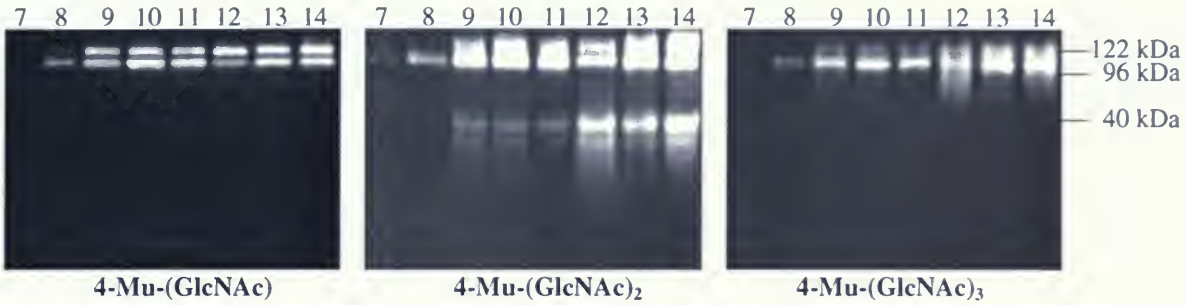

Figure 16 – Detection of extracellular chitinolytic activity of proteins isolated from dual cultures of *T. aggressivum* and *A. bisporus* strain Amycel 2400 (day 7 –14). Activity was visualized following incubation with one of three specific substrates 4-MU-(GlcNAc), 4-MU-$(GlcNAc)_2$, or 4-MU-$(GlcNAc)_3$.

Table 9 - Chitinolytic activity of extracellular enzymes isolated from dual cultures of *T. aggressivum* and *A. bisporus* strain Amycel 2400. Enzyme type NAG refers to *N*-acetylglucosaminidases, and Chito represents chitobiosidases.

Day	Mol. mass (kDa)	Specific activity (U/μg crude protein)	4-MU-(GlcNAc)	4-MU-$(GlcNAc)_2$	4-MU-$(GlcNAc)_3$	Chitinase Type
0	111	3.15	+	+	+	NAG
1	122	1.32	+	+	+	NAG
	96	1.28	+	+	+	NAG
2	131	2.01	+	+	+	NAG
	111	2.63	+	+	+	NAG
	96	1.35	+	+	+	NAG
3	131	1.45	+	+	+	NAG
	111	1.64	+	+	+	NAG
	96	1.20	+	+	+	NAG
4	96	3.59	+	+	+	NAG
5	125	1.31	+	+	+	NAG
	96	3.57	+	+	+	NAG
6	122	0.85	+	+	+	NAG
	96	3.57	+	+	+	NAG
7	122	1.43	+	+	+	NAG
	96	2.54	+	+	+	NAG
8	122	2.82	+	+	+	NAG
	96	1.77	+	+	+	NAG
	40	1.23	-	+	-	Chito
9	122	3.17	+	+	+	NAG
	96	3.51	+	+	+	NAG
	40	1.68	-	+	-	Chito
10	122	3.13	+	+	+	NAG
	96	3.48	+	+	+	NAG
	40	1.66	-	+	-	Chito

Day	Mol. mass (kDa)	Specific activity (U/μg crude protein)	4-MU-(GlcNAc)	4-MU-$(GlcNAc)_2$	4-MU-$(GlcNAc)_3$	Chitinase Type
11	122	3.15	+	+	+	NAG
	96	3.48	+	+	+	NAG
	40	1.67	-	+	-	Chito
12	122	2.91	+	+	+	NAG
	96	2.94	+	+	+	NAG
	40	2.40	-	+	-	Chito
13	122	3.65	+	+	+	NAG
	96	3.29	+	+	+	NAG
	40	2.26	-	+	-	Chito
14	122	3.67	+	+	+	NAG
	96	3.29	+	+	+	NAG
	40	2.47	-	+	-	Chito

Table 10 - Chitinolytic activity of extracellular enzymes isolated from dual cultures of *T. aggressivum* and *A. bisporus* strain Horst U1. Enzyme type NAG refers to *N*-acetylglucosaminidases, and Chito represents chitobiosidases.

Day	Mol. mass (kDa)	Specific activity (U/μg crude protein)	4-MU-(GlcNAc)	4-MU-$(GlcNAc)_2$	4-MU-$(GlcNAc)_3$	Chitinase Type
0	111	1.21	+	+	+	NAG
1	122	1.13	+	+	+	NAG
2	131	2.48	+	+	+	NAG
	111	1.26	+	+	+	NAG
3	122	3.37	+	+	+	NAG
4	131	3.28	+	+	+	NAG
5	125	1.20	+	+	+	NAG
	96	0.80	+	+	+	NAG
6	122	1.86	+	+	+	NAG
	96	0.99	+	+	+	NAG
7	122	3.77	+	+	+	NAG
	96	1.40	+	+	+	NAG
8	122	3.18	+	+	+	NAG
	96	0.70	+	+	+	NAG
	40	0.93	-	+	+	Chito
9	122	3.17	+	+	+	NAG
	96	0.94	+	+	+	NAG
	40	0.91	-	+	+	Chito
10	122	3.85	+	+	+	NAG
	96	1.21	+	+	+	NAG
	40	1.64	-	+	+	Chito
11	122	3.81	+	+	+	NAG
	96	1.04	+	+	+	NAG
	40	1.81	-	+	+	Chito

Day	Mol. mass (kDa)	Specific activity (U/μg crude protein)	4-MU-(GlcNAc)	4-MU-$(GlcNAc)_2$	4-MU-$(GlcNAc)_3$	Chitinase Type
12	122	3.44	+	+	+	NAG
	96	1.05	+	+	+	NAG
	40	1.93	-	+	+	Chito
13	122	4.32	+	+	+	NAG
	96	1.07	+	+	+	NAG
	40	1.86	-	+	+	Chito
14	122	4.42	+	+	+	NAG
	96	0.93	+	+	+	NAG
	40	1.98	-	+	+	Chito

Table 11 - Chitinolytic activity of extracellular enzymes isolated from dual cultures of *T. aggressivum* and *A. bisporus* strain Sylvan SB65. Enzyme type NAG refers to *N*-acetylglucosaminidases, and Chito represents chitobiosidases.

Day	Mol. mass (kDa)	Specific activity (U/μg crude protein)	4-MU-(GlcNAc)	4-MU-$(GlcNAc)_2$	4-MU-$(GlcNAc)_3$	Chitinase Type
0	111	1.65	+	+	+	NAG
1	122	1.83	+	+	+	NAG
2	131	2.21	+	+	+	NAG
	111	1.87	+	+	+	NAG
3	122	3.66	+	+	+	NAG
	96	3.66	+	+	+	NAG
4	131	1.08	+	+	+	NAG
	111	1.15	+	+	+	NAG
	96	0.44	+	+	+	NAG
5	125	2.90	+	+	+	NAG
	96	1.85	+	+	+	NAG
6	122	0.79	+	+	+	NAG
	96	2.98	+	+	+	NAG
7	122	1.74	+	+	+	NAG
	96	2.55	+	+	+	NAG
8	122	1.60	+	+	+	NAG
	96	3.01	+	+	+	NAG
	40	1.21	-	+	+	Chito
9	122	3.15	+	+	+	NAG
	96	2.63	+	+	+	NAG
	40	1.37	-	+	+	Chito
10	122	3.58	+	+	+	NAG
	96	2.60	+	+	+	NAG
	40	1.98	-	+	+	Chito

Day	Mol. mass (kDa)	Specific activity (U/μg crude protein)	4-MU-(GlcNAc)	4-MU-$(GlcNAc)_2$	4-MU-$(GlcNAc)_3$	Chitinase Type
11	122	3.45	+	+	+	NAG
	96	2.61	+	+	+	NAG
	40	1.20	-	+	+	Chito
12	122	3.45	+	+	+	NAG
	96	2.63	+	+	+	NAG
	40	2.19	-	+	+	Chito
13	122	3.28	+	+	+	NAG
	96	2.49	+	+	+	NAG
	40	2.03	-	+	+	Chito
14	122	3.65	+	+	+	NAG
	96	2.65	+	+	+	NAG
	40	2.26	-	+	+	Chito

Table 12 - Chitinolytic activity of extracellular enzymes isolated from dual cultures of *T. aggressivum* and *A. bisporus* strain Sylvan 130. Enzyme type NAG refers to *N*-acetylglucosaminidases, and Chito represents chitobiosidases.

Day	Mol. mass (kDa)	Specific activity (U/μg crude protein)	4-MU-(GlcNAc)	4-MU-$(GlcNAc)_2$	4-MU-$(GlcNAc)_3$	Chitinase Type
0	111	1.09	+	+	+	NAG
1	122	0.98	+	+	+	NAG
2	131	3.14	+	+	+	NAG
3	131	3.14	+	+	+	NAG
	111	0.83	+	+	+	NAG
4	122	3.65	+	+	+	NAG
5	122	3.66	+	+	+	NAG
	96	0.61	+	+	+	NAG
6	125	1.29	+	+	+	NAG
	96	0.54	+	+	+	NAG
7	122	3.15	+	+	+	NAG
	96	0.89	+	+	+	NAG
8	122	3.09	+	+	+	NAG
	96	1.23	+	+	+	NAG
	40	0.27	-	+	+	Chito
9	122	3.45	+	+	+	NAG
	96	1.26	+	+	+	NAG
	40	0.35	-	+	+	Chito
10	122	3.13	+	+	+	NAG
	96	1.44	+	+	+	NAG
	40	0.30	-	+	+	Chito
11	122	3.86	+	+	+	NAG
	96	0.61	+	+	+	NAG
	40	1.42	-	+	+	Chito

Day	Mol. mass (kDa)	Specific activity (U/μg crude protein)	4-MU-(GlcNAc)	4-MU-$(GlcNAc)_2$	4-MU-$(GlcNAc)_3$	Chitinase Type
12	122	3.18	+	+	+	NAG
	96	1.73	+	+	+	NAG
	40	0.35	-	+	+	Chito
13	122	4.12	+	+	+	NAG
	96	1.66	+	+	+	NAG
	40	2.58	-	+	+	Chito
14	122	4.24	+	+	+	NAG
	96	1.59	+	+	+	NAG
	40	2.56	-	+	+	Chito

Quantification of the fluorescent product 4-methylmubelliferone (4-MU), allowed for determination of specific activity of enzymes separated by polyacrylamide gel electrophoresis. Intensity measurements were performed with Fuji's Image Gauge version 3.46 software, and a standard curve previously seen in Figure 8 was used to convert intensity readings of samples to enzyme activity. One unit (U) of enzyme activity was defined as the amount of enzyme which catalyzed the release of 1 ng of 4-methylumbelliferone min^{-1}, and specific activity was calculated as units of enzyme activity per microgram of crude protein extract. A standard (5 µg) of protein extract was loaded into the wells of polyacrylamide gels, however there were a few instances in which protein concentration was low and a higher amount of protein was added. In these cases specific activity was calculated using the differential amount that had been used. Each *Agaricus – Trichoderma* combination revealed differences in specific activity for individual enzyme types, although a number of parallels could be seen.

Similarities were generally observed between the dual cultures containing brown *Agaricus* strains Amycel 2400, and Sylvan SB65, as well as between the off-white strain Horst U1, and white strain Sylvan 130. In Figure 17 it can be seen that the 122 kDa *N*-acetylglucosaminidase of *T. aggressivum* increased for all four dual culture combinations. The 96 kDa *N*-acetylglucosaminidase of *A. bisporus* maintained a steady level of activity for the brown strain Amycel 2400 until day 8, following which a sharp decrease could be seen. A similar trend was observed for Sylvan SB65, however, the drop in activity occurred after only 6 days and was not as severe. By comparison of Horst U1 and Sylvan 130 both had lower activities of the 96 kDa *N*-acetylglucosamindase, which they maintained for the full time course. Although the overall trend was an increase in the 40 kDa chitobiosidase, which appeared later than the other *T. aggressivum* enzymes, activity levels for this enzyme were somewhat lower than the other two enzyme types, and were

similar for all host varieties. All four combinations demonstrated a slow increase in the 36 kDa endochitinase, with only small changes from one day to the next. This enzyme appeared early, concomitantly with the 122 kDa N-acetylglucosaminidase, however its activity was much lower and there was no significant difference seen between the different host varieties.

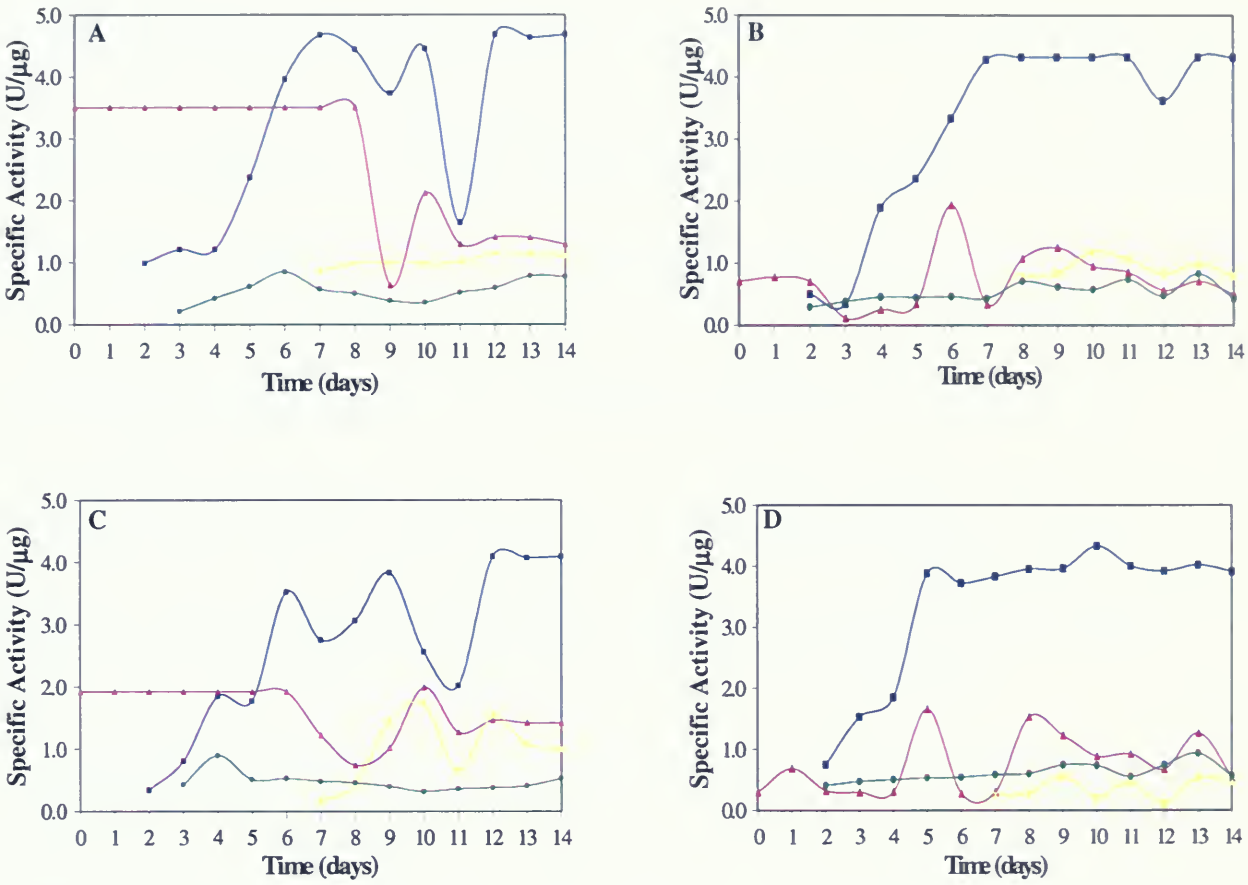

Figure 17 – Specific activity of *N*-acetylglucosaminidase ▪ (122 kDa), ▲ (96 kDa), chitobiosidase (40 kDa), and endochitinase ● (36 kDa) produced intracellularly during dual culturing of *T. aggressivum* and *A. bisporus* strains: **(A)** Amycel 2400, **(B)** Horst U1, **(C)** Sylvan SB65, and **(D)** Sylvan 130. All values are means of two bands per replica with standard deviations of less than 10%.

The extracellular protein samples collected over a period of fifteen days, revealed the presence of a number of different enzymes, some of which had been observed in solitary cultures, as well as in intracellular protein extracts from dual cultures. During the early stages of co-cultivation, daily changes in types and levels of chitinases were observed, as seen previously in Tables 9 - 12. After day 6, the types of chitinases detected remained constant with only three chitinases being detected, including 122 kDa and 96 kDa *N*-acetylglucosaminidases, and a 40 kDa chitobiosidase (Figure 18). The general trend in activity levels for all four combinations showed an increase in the 122 kDa protein, and after an increase in activity of the 96 kDa *N*-acetylglucosaminidase its activity appeared to plateau. However there was a considerable difference in the amount of activity despite the general trends. It can be seen in Figure 18 (A) and (C) that the 96 kDa *N*-acetylglucosaminidase had a level of activity comparable to that of the 122 kDa enzyme, both ranging around 3.0 U/µg after day 6. However in the dual cultures containing the white and off-white mushroom strains, as represented in Figure 18 (B) and (D), the level of activity of the 122 kDa enzyme was around 3.5 U/µg, while the 96 kDa *N*-acetylglucosaminidase was detected at a much lower level, which was approximately 1.0 U/µg. The specific activity of the 40 kDa chitobiosidase appeared to be different for each *Trichoderma – Agaricus* combination, however each showed an overall increase in this enzyme from the time it was first detected until day 14.

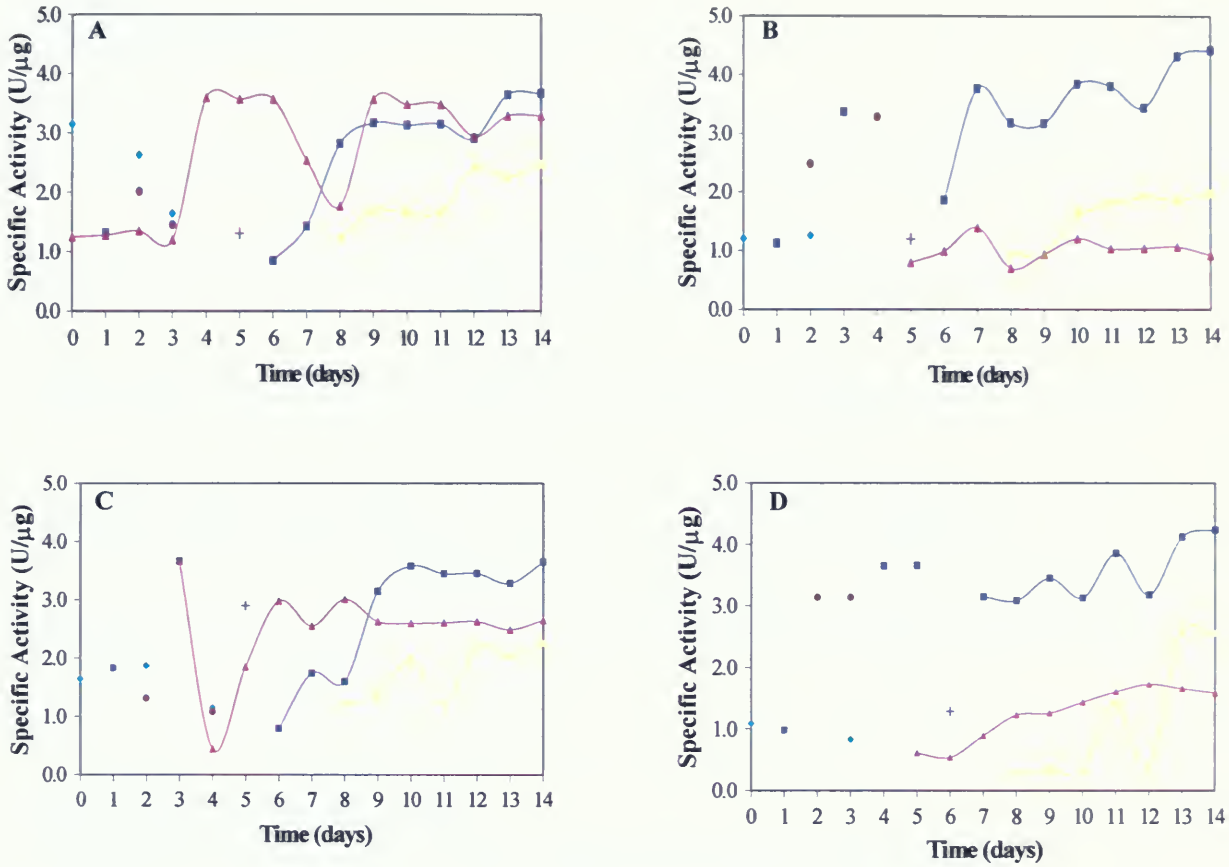

Figure 18 – Specific activity of *N*-acetylglucosaminidase • (131 kDa), + (125 kDa), ◆ (111 kDa), ■ (122 kDa), ▲ (96 kDa), and chitobiosidase (40 kDa) produced extracellularly during dual culturing of *T. aggressivum* and *A. bisporus* strains: **(A)** Amycel 2400, **(B)** Horst U1, **(C)** Sylvan SB65, and **(D)** Sylvan 130. All values are means of two bands per replica with standard deviations of less than 10%.

Discussion

Culture Conditions

Observation of dual cultures allowed for visualization, at the macroscopic level, of interactions between *Agaricus bisporus* and *Trichoderma aggressivum*. Although this study was focused at the molecular level of interaction, comparison of chitinase activity with the timing of growth was helpful. One of the key features of *Trichoderma* spp. that make it such an aggressive mycoparasite is its ability for rapid growth (Deacon, 1997), allowing it to quickly colonize a host. This was confirmed by the fact that *Trichoderma* hyphae were observed within twenty-four hours of inoculation, and by the third day had completely covered the plate. However, differences at this level between different host-parasite combinations were not detected.

Recovering Chitinolytic Activity

In this study, chitinase enzymes were separated using both SDS and native polyacrylamide gel electrophoresis. The anionic detergent SDS (sodium dodecyl sulfate) actively denatures protein structure by binding with the hydrophobic residues of polypeptides (Voet & Voet, 1995). Therefore, to recover enzymatic activity of proteins, denaturants such as SDS must be removed following electrophoresis. This was accomplished using a 25% (v/v) isopropanol wash, which effectively removed SDS from the gel, and allowed for refolding of proteins. However, during the course of this study it was determined that not all enzyme types, specifically endochitinases and chitobiosidases, were observed using this method. In attempts to determine if these enzymes were present but not detected, a number of variables were altered including

separating proteins by native polyacrylamide gel electrophoresis. Since the proteins were in their native form for this process, isopropanol was not required to restore activity.

The majority of researchers have reported chitinase activity using SDS-PAGE (Haran *et al.*, 1995; Ulhoa & Peberdy, 1991,1992; Lorito *et al.*, 1994a; De La Cruz *et al.*, 1992; Harman *et al.*, 1993), and in our own lab comparison of SDS and native gels had been tested with no differences observed (Corfe, 2000). However, it is possible that the samples compared by Corfe did not contain endochitinases, and chitobiosidases, as there were a number of days in which only *N*-acetylglucosaminidases were present. A sensitivity to repeated alcohol washes, is one potential explanation for non-detectable enzymes. It is also possible that the SDS may have permanently denatured certain chitinases such that subsequent removal was insufficient for restoration of activity. Although the reason for the discovery of enzymes which had previously been undetected, is unknown, all possible chitinase types were detectable using native-PAGE, and therefore this technique was adopted for use in the present study.

Chitinolytic Activity of Solitary Cultures

A characterization of the enzyme profiles of solitary cultures allowed tentative assignments of the origins of chitinase enzymes detected during co-culturing of *A. bisporus* and *T. aggressivum*. This experimental approach also allowed for the determination of chitinase types produced in the absence of inducers, which is important in determining specific enzyme involvement during parasitic interactions.

With regards to enzyme production, a number of studies have been undertaken to determine the types of glucanases produced by *A. bisporus* (Galán *et al.*, 1999; Manning & Wood, 1983; Raguz *et al.*, 1992). However, there has been no investigation into the

chitinases that may be produced by this fungus, and consequently, there was no basis for comparison for chitinases isolated in this study. Given that all four mushroom strains were the same species of *Agaricus*, it was not surprising to find that they produced similar enzyme profiles. Analysis of chitinolytic activity revealed a number of *N*-acetylglucosaminidases produced intra- and extracellularly prior to inoculation with *T. aggressivum* spores. Within the mycelia, two *N*-acetylglucosaminidases with estimated weights of 105 kDa and 96 kDa, were found. It is interesting to note that both brown strains Amycel 2400 and Sylvan SB65 produced only a 96 kDa *N*-acetylglucosaminidase intracellularly, while the off-white Horst U1, and white strain Sylvan 130, produced a 105 kDa *N*-acetylglucosaminidase. In examining the production of secreted chitinases by *A. bisporus* it was observed that all four strains produced a 111 kDa and a 96 kDa *N*-acetylglucosaminidase, with the exception of Horst U1, which appeared to produce only the 111 kDa enzyme. Furthermore, in looking at the specific activities of these enzymes, as previously reported in Table 4, there are again similarities between the two brown strains, and between the off-white, and white strains. Overall, higher chitinase activity was observed for brown strains Amycel 2400, and Sylvan SB65 relative to the off-white and white strains. Although it is unknown at this point why they exhibit higher chitinase activity levels, it is suspected that this may be a part of increased resistance observed in brown strains, and should be examined further.

A number of studies have looked at the chitinases produced by *Trichoderma* spp. prior to interaction with a host. It has been found that *T. harzianum* constitutively produces a 102 kDa *N*-acetylglucosaminidase (Haran *et al.*, 1995; Inbar & Chet, 1995; Schickler *et al.*, 1998), and more recently it has been determined that a 42 kDa endochitinase may

also be produced at low levels (Zeilinger *et al.* 1999). In the present study solitary cultures of *T. aggressivum* were found to produce three chitinases, including an *N*-acetylglucosaminidase with an apparent molecular mass of 122 kDa, which was found intracellularly. Additionally, an endochitinase with an estimated weight of 36 kDa, and a 40 kDa chitobiosidase were also detected. Similar to previous studies involving CHIT 102, the 122 kDa *N*-acetylglucosaminidase was observed at low levels within the mycelia. Although CHIT 102, as indicated by its name, has been reported to have a molecular weight of 102 kDa, enzymes thought to be the same have been reported to have masses up to 118 kDa (Ulhoa & Peberdy, 1991; Haran *et al.*, 1995). Based on this research it is uncertain, but quite possible that the 122 kDa *N*-acetylglucosaminidase isolated from *T. aggressivum* is very similar to the CHIT 102 enzyme previously discovered in *T. harzianum*.

Chitinolytic Activity of Dual Cultures

Intracellular and extracellular chitinases were collected and analyzed for all four host-parasite combinations. In examining the activity of mycelial (intracellular) samples, it was determined that there were four distinct enzymes present. Based on the results of solitary culturing it was established that a 122 kDa *N*-acetylglucosaminidase, 40 kDa chitobiosidase, and 36 kDa endochitinase were most likely a product of *T. aggressivum*. Additionally, a 96 kDa *N*-acetylglucosaminidase was thought to belong to *A. bisporus*. Although activity gels did not reveal the 96 kDa *N*-acetylglucosaminidase within the mycelia of *A. bisporus* strains Horst U1, and Sylvan 130, these enzymes or an inactive zymogen precursor may have been present at levels not detectable using these methods, as they were later detected in dual culturing. Evidence for membrane-bound fungal

chitinase zymogens has previously been established by McMurrough and Bartnicki-Garcia (1973), as well as Molano and co-workers (1979).

A number of trends in the data could be seen among the four possible host-pathogen arrangements, although each *Agaricus* – *Trichoderma* combination revealed differences in specific activity for the intracellular enzymes detected. Based on the observed rapid growth of *Trichoderma*, it was expected that an increase in enzyme activity might also be seen. In all cases, it was observed that the 122 kDa *N*-acetylglucosaminidase from *Trichoderma* increased over the two-week period, while the 40 kDa and 36 kDa enzymes remained at relatively low levels intracellularly. Therefore, it appears that the 122 kDa *N*-acetylglucosaminidase may be the best indicator of *T. aggressivum*'s involvement in the interaction. This is based on the assumption that the 122 kDa *N*-acetylglucosaminidase of *T. aggressivum* is analogous to CHIT 102 of *T. harzianum*, both of which appear in solitary culture, and are the first enzymes detected and upregulated during anti-fungal activities.

Activity gels for dual cultures of extracellular samples were considerably more complex, and during the first 6 days of co-culturing a number of different enzymes were detected, including *N*-acetylglucosaminidases of estimated molecular weights 131, 125, 122, 111, and 96 kDa. Some of these enzymes had previously been discovered in solitary cultures, as well as in intracellular protein extracts from dual cultures. Changes in the types and levels of enzyme production were observed on a daily basis during the early days of the fungal interaction. However, after day 6, each *Trichoderma* – *Agaricus* combination appeared to maintain relatively constant levels of enzyme activity. All four combinations revealed an increase in activity of the 122 kDa *N*-acetylglucosaminidase,

and after an increase in activity of the 96 kDa *N*-acetylglucosaminidase its activity leveled off. Yet despite similar trends, the overall amount of activity measured was considerably different. Common to both brown strains Amycel 2400, and Sylvan SB65 was the high level of 96 kDa *N*-acetylglucosaminidase. In contrast, cultures containing the off-white strain Horst U1, and the white strain Sylvan 130, produced the 96 kDa enzyme with consistently lower activity levels.

These results lead to several points of observation. First of all, examining intracellular enzyme profiles, both brown strains began with reasonably high levels of 96 kDa *N*-acetylglucosaminidase, and maintained this level fairly consistently until around day 6-8, following which intracellular activity of this enzyme dropped while extracellular production rose. Given the assumption that *Agaricus* produced this enzyme, it could be predicted that it contributes to brown strain resistance, whereas high levels of the 96 kDa *N*-acetylglucosaminidase may be key to warding off *Trichoderma*. This is further supported by the observation that intracellular extracts of off-white and white strains did not contain detectable levels of this enzyme. Thus, despite the high levels of the *Trichoderma* 122 kDa *N*-acetylglucosaminidase activity, brown strains are able to maintain, and possibly fight back with the early and high activity of the 96 kDa *N*-acetylglucosaminidase.

At day 8, additional extracellular enzymes were detected, and the first of the two enzymes was determined to be a 40 kDa chitobiosidase. Initially, it was thought that the second enzyme migrating faster than the 40 kDa enzyme (Figure 16) might be the 36 kDa endochitinase previously detected. However, closer examination lead to the classification of the second enzyme as an isoenzyme of the 40 kDa chitobiosidase. This conclusion

was based on comparing activity gels containing the suspected isoenzyme, to those established to have both the 36 and 40 kDa enzymes. It was observed that the second band was only seen in the presence of the trimeric substrate 4-MU-$(GlcNAc)_2$, and not the tetrameric substrate, as the 36 kDa endochitinase did. Therefore this enzyme would be a chitobiosidase, rather than an endochitinase. Furthermore, the activities observed for the isoenzyme were relative to those of the 40 kDa chitobiosidase, and were never seen in higher levels or present in the absence of the 40 kDa enzyme. This is in contrast to activity gels containing both the 36 and 40 kDa enzymes, which often had variable activities, neither enzyme appearing to be dependent on the other. Examining the enzyme pattern it can be seen that the second enzyme activity was poorly resolved. This often arises when there are a number of enzymes of similar molecular weight. And finally, during molecular weight determination, equal spacing between the 40 kDa enzyme, and the isoenzyme was maintained across the different gel percentages. It appeared that the two bands were moving together, which was not seen on any other gels where different enzymes moved completely separate from one another, with spacing between bands changing with the gel percentage.

In terms of activity of the 40 kDa chitobiosidase, it appeared to be different for each *Trichoderma – Agaricus* combination. However, each showed an overall increase in the activity of this enzyme from the time it was first detected until day 14. This enzyme may also be involved in the parasitic interaction, but its low level of production and lack of a predictable trend, make it difficult to determine how it may be involved.

Correlation with Previous Research

Previous investigations into the chitinolytic system of *Trichoderma* spp. have been done almost exclusively with strains of *T. harzianum* commonly used as biocontrol agents. Recently, it has been established that *T. harzianum* is not the same species as those responsible for green mould disease, formerly referred to as Th2 and Th4. Therefore, *Trichoderma* species known to cause disease outbreaks have been renamed, and the North American type is now referred to as *T. aggressivum* f. *aggressivum*, while its European counterpart has been named *T. aggressivum* f. *europaeum* (Samuels *et al.*, 2002). It was the North American species *T. aggressivum* that was used in the present study.

Researchers have discovered seven distinct chitinase enzymes isolated from *T. harzianum*, produced in response to numerous inducers including chitin, *N*-acetylglucosamine, and purified fungal cell walls. Included are *N*-acetylglucosaminidases (CHIT 102 & CHIT 73), endochitinases (CHIT 52, CHIT 42, CHIT 33 & CHIT 31), and the chitobiosidase CHIT 40 (Haran *et al.*, 1996). During the course of this study, a number of chitinases were isolated, and believed to be analogous to those found in *T. harzianum*. However, it appears that *T. aggressivum*, although having some similar enzymes, lacks the same activity as *T. harzianum*. And therefore lends support to the findings that *Trichoderma harzianum*, and *Trichoderma aggressivum*, are indeed different species of this filamentous fungus.

Considering the production of enzymes by *A. bisporus*, it is interesting to note that there were chitinases detected for the entire duration of the experiment. Upon observation of dual cultures macroscopically (Figure 7), it seems that *Trichoderma* had completely overgrown the plate by day 14, and that it was unlikely that *A. bisporus* was

alive. Yet, throughout the two-week period, the abundance of chitinases produced by *Agaricus* appeared to cycle, in that enzyme production failed to cease, possibly as a resistance mechanism against attack by *T. aggressivum*. Since *Agaricus* enzyme production was not completely lost during the time period studied, and in fact was able to cycle in abundance, it appears that *Agaricus* remained viable. This finding is in agreement with another study (Droganes, 1998), in which *A. bisporus* and *T. aggressivum* were co-cultured for a prolonged period of time, and had become completely overgrown with *Trichoderma*. Pieces of this culture were removed and were placed on fresh plates containing benomyl (a fungicide used to eliminate *Trichoderma* spp.). Surprisingly, *Agaricus* was able to re-grow, and thus it appears that at some point *Agaricus* may be able to inhibit *Trichoderma* from causing further damage, although it is unclear how this would be accomplished. Looking at green mould disease outbreaks, this may be the case when only partial damage of mushroom crops is seen, where production yields are severely decreased, but not completely.

At the organismal level, *A. bisporus* has been seen to produce compounds that stimulate *Trichoderma aggressivum* f. *europaeum* growth in culture. It seems that simultaneous growth of both fungal species is allowed due to tolerance to the other's antibiotic production (Mumpuni *et al.*, 1998). This may also be the case for hydrolytic enzymes, where each are producing chitinases, and are able to compete during their interaction. However, some strains of *A. bisporus* (ie. off-white/white) may not be able to compete as well, possibly due to their low levels of 96 kDa *N*-acetylglucosaminidase activity.

It is often seen that enzymes having similar functions are regulated together, and although this study did not look specifically at the regulation of chitinases, in carefully comparing the activities of each enzyme it appears that these enzymes are not co-regulated. This corresponds with previous studies in which genes encoding for chitinases, such as *nag1* and *ech42*, found their expression to be regulated independently (Mach *et al.*, 1999). Similarly, Limón and colleagues (1995) reported differences in gene expressions of CHIT42 and CHIT33, which suggested that each chitinase was regulated independently. While it appears that chitinase expression may occur in a specific order it has been determined that the mechanism of regulation is different for each enzyme.

Origins of Chitinase Expression

One of the difficulties within this experiment was that it was unclear which organism had produced specific enzymes. Previous research has indicated that up-regulated chitinases are produced by *Trichoderma*, and in this study a number of the enzymes detected closely match the type and size of those previously isolated from *Trichoderma harzianum* (Haran *et al.*, 1996). Based on solitary culturing it was determined that the 122 kDa *N*-acetylglucosaminidase, as well as the 40 kDa chitobiosidase, and the 36 kDa endochitinase detected during dual culturing most likely are produced by *Trichoderma aggressivum*. It can be further concluded that the 96, 105, and 111 kDa *N*-acetylglucosaminidases originate from *Agaricus bisporus* as they were detected during its solitary culturing. However, it cannot be conclusively proven which organism produced the 131 kDa and 125 kDa *N*-acetylglucosaminidases, using the results of this study.

One way of determining which organism produced the enzymes, would be to use a permeable membrane to physically separate host from parasite. Several studies have

used this approach with reasonable success. However, there have been large discrepancies in results obtained using this type of experiment, believed to stem from the different types of membranes used (Cortés *et al.*, 1998; Zeilinger *et al.*, 1999; Kullnig *et al.*, 2000). Using a permeable membrane relies on the assumption that induction of the parasitic response occurs by low molecular mass diffusible factors, and that contact between the host and parasite is not required.

Another way to determine the origin of enzyme production is to introduce one of the organisms after it has been killed. Studies of this nature have been done by introducing various fungal cell walls, which have been collected and sterilized, to established *Trichoderma* cultures. Researchers have used *Rhizoctonia solani* and *Sclerotium rolfsii* most often, and *A. bisporus* has been used within our own lab. It has consistently been shown that *Trichoderma* spp. produce a variety of chitinases in response to fungal cell walls (Garcia *et al.*, 1994; Limón *et al.*, 1995; Peterbauer *et al.*, 1996; Mach *et al.*, 1999).

Although these studies are important in determining the types of chitinases that it is possible to produce, and that the mere presence of a host cell wall can induce their production, this is not a completely accurate representation of what actually occurs during the parasitism commonly referred to as green mould disease. Consequently, this study was undertaken to look closely at the enzyme profiles resulting from two living organisms, actively engaged in a parasitic or competitive relationship. Although it was determined that there is a high probability of many of the enzymes originating from *Trichoderma*, it is unknown if all of them did. Especially within the early stages of the interaction, it is suspected that *Agaricus bisporus* may have produced some of the chitinases seen, perhaps as a defense mechanism. Therefore, there are a number of

directions in which this research could take, some of which involve clarifying the origin the various chitinases.

Future Research Directions

To date, researchers have primarily been concerned with the chitinases produced by *Trichoderma* spp., and have not taken an in depth look at enzymes produced by the hosts for a full understanding of the interaction of the two organisms during parasitism. Therefore, it is proposed that to further clarify which organism produces specific chitinases, a reverse of the standard experiment be performed. This means, that mycelia of *Trichoderma* would be collected and sterilized, and introduced to growing cultures of *Agaricus* for observation chitinases produced. At the same time molecular techniques could also be used to resolve the issue of chitinase origin. One such method uses isolated RNA, and a variety of primers for reverse transcription PCR (RT-PCR). However this technique requires prior knowledge of the estimated size of the polypeptide, as well as nucleic acid or protein sequence information for primer design.

Once it has been determined which chitinases each organism produced, the next logical step would be to test their involvement in parasitism. It has been well established that chitinases contribute to the antagonistic activities of *Trichoderma* spp., demonstrated early on by studies using purified chitinases to observe their effects on different fungal species (De la Cruz *et al.*, 1992, 1993; Lorito *et al.*, 1993, 1994a, 1994b; Schirmböck *et al.*, 1994). More recently, researchers have begun to characterize the genes responsible for these enzymes, and to date three chitinase genes (*ech33*, *ech42*, and *nag1*) have been cloned with research focusing on *ech42* (Carsolio *et al.*, 1994, 1999; Garcia *et al.*, 1994; Hayes *et al.*, 1994; Limón *et al.*, 1995; Peterbauer *et al.*, 1996). Preliminary results of

observed gene expression, confirm an antagonistic role for these enzymes. Therefore, it would seem reasonable that the future course of research, based on the findings of this study also proceed in the direction of molecular genetics. Based on high levels of the 122 kDa *N*-acetylglucosaminidase produced by *T. aggressivum*, it seems that this enzyme may be the best predictor of antifungal activity. Furthermore, the differential activity of the 96 kDa *N*-acetylglucosaminidase attributed to *A. bisporus*, may be an indicator of brown strain resistance, and thus warrants further investigation. The role of these specific enzymes may be clarified by creating strains of *T. aggressivum* and *A. bisporus*, in which the genes encoding these chitinases are removed, or subjected to altered regulation.

Although chitinases appear to play an important role during host-parasite interactions, it is unlikely that they act alone. It has been found that hydrolytic enzymes such as chitinases, glucanases and proteases act synergistically to parasitize a host (Lorito *et al.*, 1993, 1994a). Therefore, future research should also look to broaden the scope of enzymes examined for a complete picture of the parasitic interaction.

To develop sustainable green mould management strategies, a full understanding of the relationship between *A. bisporus* and *T. aggressivum* is required. The eventual outcome of this research is to apply knowledge gained to the control of green mould disease. Once researchers have determined the mechanism(s) underlying resistance, it may be possible to create strains of *A. bisporus* resistant to infection by *T. aggressivum*. Furthermore, researchers focusing on the mechanisms underpinning the induction of antagonistic behaviour by *Trichoderma* spp., may be able to disrupt the signaling process and therefore prevent disease. Regardless of the specific area of research, further

knowledge of the interaction between *A. bisporus* and *T. aggressivum* should hopefully improve current strategies in the prevention and control of green mould disease.

Summary

Green mould is a serious problem, which has caused world-wide losses for mushroom growers. Presently the best means of controlling this disease is through strict hygiene, and by the use of chemical fungicides such as benomyl. However, the long-term goal is to develop safer and more effective control measures. This project was designed to look at the production of chitinases by *Trichoderma aggressivum* to determine if there were observable differences in the enzyme profiles during parasitic interactions with different host strains of *A. bisporus*. It was determined that *T. aggressivum* produces a number of chitinases that appear to correlate to those isolated in previous studies using biocontrol strains of *T. harzianum*. Recently, Samuels and co-workers (2002) established that *T. aggressivum*, formerly referred to as Th4, is not the same species as *T. harzianum* (Th1). Results of this study lend support to the division of species as *T. aggressivum* lacked the same activities described in *T. harzianum*.

It appears that the parasitic interaction between *T. aggressivum* and *A. bisporus* involves a complex enzyme battle, yet there were a number of clear trends. The 122 kDa *N*-acetylglucosaminidase of *T. aggressivum* revealed the highest and most variable activity, and is therefore believed to be an important predictor of antifungal activity. Furthermore, high levels of the 96 kDa *N*-acetylglucosaminidase observed in brown strains of *A. bisporus*, may be significant in conferring resistance.

Overall, each host-parasite combination produced different enzyme profiles. Therefore, it can be concluded that the antagonistic behaviour of *T. aggressivum* does not involve a typical response, but that mycoparasitism, specifically in the form of chitinases, may be induced and regulated based on the host presented.

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Appendix

Bradford Assay

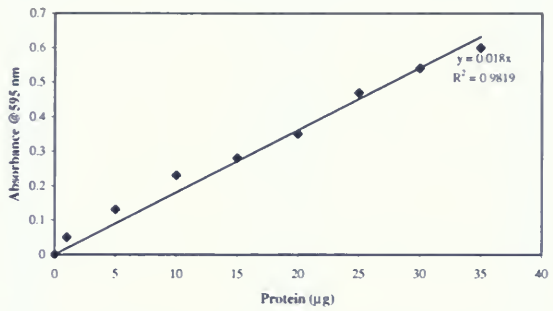

Figure A1 – Bradford assay standard curve of absorbance versus BSA protein concentration.

Table A1 – Absorbance readings at 595 nm of solitary *Trichoderma* samples for determination of protein concentration based on standard curve (T = intracellular; TF = extracellular).

Sample	1st Replica		2nd Replica	
	O.D. at 595nm	Protein (µg)	O.D. at 595nm	Protein (µg)
T	0.25	13.51	0.32	17.14
TF	0.11	6.25	0.23	12.47

Table A2 - Absorbance readings at 595 nm of solitary *Agaricus* samples for determination of protein concentration based on standard curve (intracellular samples: A-Amycel 2400, H-Horst U1, SB-Sylvan SB65, and S1-Sylvan 130; extracellular samples = F).

Sample	1st Replica		2nd Replica	
	O.D. at 595nm	Protein (µg)	O.D. at 595nm	Protein (µg)
A	0.24	13.33	0.32	17.77
H	0.15	8.33	0.65	36.11
SB	0.15	8.33	0.37	20.56
S1	0.08	4.44	0.42	23.33
AF	0.25	13.89	0.21	11.67
HF	0.24	13.33	0.19	10.56
SBF	0.22	12.22	0.24	13.33
S1F	0.13	7.22	0.19	10.56

Table A3 - Absorbance readings at 595 nm of intracellular samples isolated from dual cultures of *T. aggressivum* and *A. bisporus* strain Amycel 2400 for determination of protein concentration based on standard curve.

Sample (day)	1st Replica		2nd Replica	
	O.D. at 595nm	Protein (µg)	O.D. at 595nm	Protein (µg)
0	0.29	15.40	0.28	27.85
1	0.12	5.34	0.37	22.82
2	0.23	11.85	0.50	30.99
3	0.29	15.40	0.20	12.15
4	0.52	29.02	0.25	15.29
5	0.60	33.76	0.28	17.17
6	0.31	16.59	0.05	2.73
7	0.09	3.56	0.08	4.61
8	0.28	14.81	0.40	24.71
9	0.40	21.92	0.40	24.71
10	0.21	10.67	0.58	36.01
11	0.20	10.08	0.42	25.96
12	0.45	24.88	0.26	15.92
13	0.31	16.59	0.10	5.87
14	0.62	34.94	0.44	27.22

Table A4 - Absorbance readings at 595 nm of intracellular samples isolated from dual cultures of *T. aggressivum* and *A. bisporus* strain Horst U1 for determination of protein concentration based on standard curve.

Sample (day)	1st Replica		2nd Replica	
	O.D. at 595nm	Protein (µg)	O.D. at 595nm	Protein (µg)
0	0.18	8.89	0.08	5.12
1	0.27	14.22	0.11	7.04
2	0.37	20.14	0.13	8.33
3	0.18	8.89	0.35	22.41
4	0.11	4.75	0.33	21.13
5	0.35	18.96	0.41	26.25
6	0.19	9.48	0.19	12.17
7	0.47	26.06	0.52	33.29
8	0.36	19.55	0.48	30.74
9	0.20	10.07	0.22	14.09
10	0.15	7.12	0.49	31.38
11	0.30	15.99	0.54	34.58
12	0.17	8.30	0.44	28.18
13	0.16	7.01	0.5	32.02
14	0.24	12.44	0.29	18.57

Table A5 - Absorbance readings at 595 nm of intracellular samples isolated from dual cultures of *T. aggressivum* and *A. bisporus* strain Sylvan SB65 for determination of protein concentration based on standard curve.

Sample (day)	1st Replica		2nd Replica	
	O.D. at 595nm	Protein (µg)	O.D. at 595nm	Protein (µg)
0	0.07	2.38	0.12	7.68
1	0.30	15.99	0.05	3.21
2	0.32	17.18	0.20	12.80
3	0.10	4.15	0.36	23.05
4	0.22	11.25	0.40	25.61
5	0.44	24.28	0.40	25.61
6	0.05	1.19	0.09	5.77
7	0.39	21.32	0.50	32.02
8	0.54	30.20	0.45	28.16
9	0.21	10.66	0.47	30.09
10	0.27	14.21	0.56	35.85
11	0.12	5.33	0.43	27.53
12	0.16	7.70	0.38	24.33
13	0.19	9.48	0.54	34.58
14	0.08	2.97	0.25	16.00

Table A6 - Absorbance readings at 595 nm of intracellular samples isolated from dual cultures of *T. aggressivum* and *A. bisporus* strain Sylvan 130 for determination of protein concentration based on standard curve.

Sample (day)	1st Replica		2nd Replica	
	O.D. at 595nm	Protein (µg)	O.D. at 595nm	Protein (µg)
0	0.05	2.73	0.25	16.10
1	0.08	4.64	0.30	19.12
2	0.43	26.90	0.10	6.41
3	0.23	14.18	0.40	25.61
4	0.60	37.71	0.45	28.82
5	0.62	38.98	0.19	12.17
6	0.05	2.74	0.09	5.76
7	0.60	37.71	0.45	28.80
8	0.16	9.73	0.43	27.53
9	0.22	13.54	0.28	17.93
10	0.10	5.92	0.31	19.85
11	0.16	9.73	0.33	21.13
12	0.15	9.09	0.39	24.97
13	0.38	23.72	0.22	14.09
14	0.21	12.91	0.25	16.01

Table A7 - Absorbance readings at 595 nm of extracellular samples isolated from dual cultures of *T. aggressivum* and *A. bisporus* strain Amycel 2400 for determination of protein concentration based on standard curve.

Sample (day)	1st Replica		2nd Replica	
	O.D. at 595nm	Protein (μg)	O.D. at 595nm	Protein (μg)
0	0.19	11.64	0.11	5.64
1	0.12	7.12	0.24	12.83
2	0.14	8.46	0.11	5.64
3	0.16	9.73	0.35	18.92
4	0.12	7.18	0.25	13.11
5	0.16	9.73	0.14	7.30
6	0.15	9.09	0.19	10.06
7	0.17	10.36	0.47	25.56
8	0.15	9.09	0.24	12.83
9	0.14	8.45	0.32	17.26
10	0.12	7.18	0.27	14.49
11	0.12	7.18	0.19	9.79
12	0.18	11.00	0.47	25.56
13	0.11	6.55	0.20	10.62
14	0.16	9.73	0.15	7.85

Table A8 - Absorbance readings at 595 nm of extracellular samples isolated from dual cultures of *T. aggressivum* and *A. bisporus* strain Horst U1 for determination of protein concentration based on standard curve.

Sample (day)	1st Replica		2nd Replica	
	O.D. at 595nm	Protein (μg)	O.D. at 595nm	Protein (μg)
0	0.18	11.00	0.35	18.92
1	0.13	7.82	0.30	16.16
2	0.11	6.55	0.29	15.60
3	0.14	8.46	0.18	9.51
4	0.15	9.09	0.10	5.08
5	0.06	3.37	0.37	20.03
6	0.13	7.82	0.08	3.70
7	0.18	11.00	0.15	7.85
8	0.15	9.09	0.31	16.70
9	0.41	25.62	0.13	6.74
10	0.18	11.00	0.16	8.41
11	0.22	13.54	0.36	19.47
12	0.28	17.36	0.16	8.13
13	0.12	7.19	0.23	12.00
14	0.20	12.27	0.23	12.00

Table A9 - Absorbance readings at 595 nm of extracellular samples isolated from dual cultures of *T. aggressivum* and *A. bisporus* strain Sylvan SB65 for determination of protein concentration based on standard curve.

Sample (day)	1st Replica		2nd Replica	
	O.D. at 595nm	Protein (μg)	O.D. at 595nm	Protein (μg)
0	0.07	5.05	0.45	24.45
1	0.19	12.26	0.10	5.08
2	0.24	15.26	0.40	21.68
3	0.20	12.86	0.43	23.34
4	0.14	9.25	0.20	10.62
5	0.18	11.66	0.45	13.11
6	0.08	5.65	0.08	3.98
7	0.09	6.25	0.58	31.65
8	0.05	3.85	0.15	7.85
9	0.16	10.45	0.19	10.06
10	0.15	9.85	0.25	13.38
11	0.05	3.85	0.33	17.82
12	0.18	11.66	0.32	17.26
13	0.13	8.65	0.25	13.38
14	0.15	9.85	0.39	21.14

Table A10 - Absorbance readings at 595 nm of extracellular samples isolated from dual cultures of *T. aggressivum* and *A. bisporus* strain Sylvan 130 for determination of protein concentration based on standard curve.

Sample (day)	1st Replica		2nd Replica	
	O.D. at 595nm	Protein (μg)	O.D. at 595nm	Protein (μg)
0	0.11	7.46	0.32	17.26
1	0.26	16.45	0.13	6.47
2	0.16	10.45	0.04	1.76
3	0.20	12.85	0.26	13.94
4	0.14	9.25	0.17	8.96
5	0.21	13.46	0.22	11.73
6	0.12	8.06	0.25	13.38
7	0.14	9.26	0.25	13.38
8	0.20	12.86	0.20	10.62
9	0.06	4.45	0.11	5.64
10	0.11	7.45	0.36	19.47
11	0.25	15.87	0.41	22.24
12	0.16	10.45	0.23	12.28
13	0.24	15.27	0.20	10.62
14	0.11	7.45	0.22	11.73

Quantification

Standards

Table A11 – Average of six intensity readings used to create standard for quantification of fluorescent bands (A – arbitrary units; B – background; px^2 – pixel area (px^2 = 473)). Integration = 0.5 sec.

4-MU (ng)	Intensity (A-B)/px^2							
	1	2	3	4	5	6	Avg.	Std. Dev.
150.0	70.52	88.25	89.41	82.51	88.75	79.13	83.09	4.55
75.0	31.71	41.79	38.98	41.39	41.78	44.73	40.06	2.04
37.5	15.50	24.41	23.76	28.31	24.67	21.13	22.96	2.57
18.8	8.75	15.58	13.16	12.58	12.11	10.75	12.15	1.77
9.4	8.35	8.82	7.52	6.34	6.71	5.24	7.16	1.33
4.7	2.00	3.69	3.32	3.02	3.42	3.40	3.14	0.24
2.3	0.89	2.44	1.23	1.00	1.08	1.44	1.34	0.58

Table A12 – Average of six intensity readings used to create standard for quantification of fluorescent bands (A – arbitrary units; B – background; px^2 – pixel area (px^2 = 473)). Integration = 0.8 sec.

4-MU (ng)	Intensity (A-B)/px^2							
	1	2	3	4	5	6	Avg.	Std. Dev.
150.0	113.8	123.6	127.3	125.6	123.8	122.8	122.81	1.81
75.0	54.32	64.58	71.07	75.27	80.08	74.55	69.978	5.74
37.5	26.54	39.41	41.73	43.98	38.16	37.72	37.923	2.62
18.8	14.53	21.14	18.21	22.92	19.84	16.4	18.84	2.52
9.4	13.74	12.99	10.04	12.85	9.57	8.46	11.27	2.03
4.7	2.75	5.97	4.72	6.64	5.72	3.30	4.85	1.29
2.3	1.79	2.7	0.94	2.1	3.07	0.45	1.84	1.12

Table A13 – Average of six intensity readings used to create standard for quantification of fluorescent bands (A – arbitrary units; B – background; px^2 – pixel area (px^2 = 473)). Integration = 1.2 sec.

4-MU (ng)	Intensity (A-B)/px^2							
	1	2	3	4	5	6	Avg.	Std. Dev.
150.0	132.9	134.1	130.5	136.8	134.1	133.7	133.68	2.24
75.0	88.71	96.22	94.55	91.24	97.62	102.6	95.15	4.18
37.5	45.98	58.48	56.56	66.93	60.79	62.76	58.58	4.00
18.8	25.78	34.69	30.72	27.71	27.38	33.44	29.95	3.29
9.4	14.87	15.91	17.7	14.49	15.64	19.31	16.32	1.89
4.7	6.21	7.75	7.65	6.77	7.60	11.21	7.865	1.72
2.3	4.09	6.13	2.86	2.67	3.03	5.16	3.99	1.57

Specific Activity

Intensity measurements were obtained using Fuji's Image Gauge software, and averages from a minimum of two bands per replica for each sample was used to calculate specific activity.

Sample Calculation:

Given an integration of 0.5 sec. the equation from the standard curve is $y = 0.5455x + 1.3119$, where y = measured intensity, and x = 4-methylumbelliferone (ng). Thus for a measured intensity of 12.74:

$$12.74 = 0.5455x + 1.3119$$
$$x = (12.74 - 1.3119)/0.5445$$
$$x = 20.95 \text{ ng}$$

Since one unit (U) of enzyme activity was defined as the amount of enzyme which catalyzed the release of 1 ng of 4-methylumbelliferone min^{-1}, and specific activity was calculated as units of enzyme activity per microgram of crude protein extract:

Specific activity = (x/min)/μg of crude protein extract
= (20.95 ng/10 min)/5 μg of crude protein extract
= 0.42 ng/min/μg of crude protein extract

Table A14 – Specific activity calculated using intensity measurements taken from activity gels of chitinases isolated from solitary cultures of *T. aggressivum* (T = intracellular, TF = extracellular) and *A. bisporus* (intracellular samples: A-Amycel 2400, H-Horst U1, SB-Sylvan SB65, and S1-Sylvan 130; extracellular samples = F).

Sample	Size (kDa)	Spec. Activity 1st	Spec. Activity 2nd	Avg.	Std. Dev.
T	122	0.78	0.88	0.83	0.07
T	36	0.62	0.67	0.65	0.04
TF	40	2.99	2.85	2.92	0.10

Sample	Size (kDa)	Spec. Activity 1st	Spec. Activity 2nd	Avg.	Std. Dev.
A	96	3.90	3.65	3.78	0.17
H	105	0.81	0.89	0.85	0.06
SB	96	1.71	1.93	1.82	0.16
S1	105	0.40	0.46	0.43	0.04
AF	111	3.60	4.00	3.80	0.28
	96	3.60	4.00	3.80	0.28
HF	111	1.11	1.00	1.06	0.08
SBF	111	1.83	2.01	1.92	0.13
	96	1.42	1.57	1.50	0.11
S1F	111	1.27	1.11	1.19	0.11
	96	1.26	1.16	1.21	0.07

Table A15 – Specific activity calculated using intensity measurements taken from intracellular activity gels of 122 kDa and 96 kDa *N*-acetylglucosaminidases isolated from dual cultures of *T. aggressivum* and *A. bisporus* strain Amycel 2400.

Sample (day)	Size (kDa)	Spec. Activity 1st	Spec. Activity 2nd	Avg.	Std. Dev.
0	122	-	-	-	-
1	122	-	-	-	-
2	122	1.05	0.93	0.99	0.08
3	122	1.13	1.29	1.21	0.11
4	122	1.16	1.26	1.21	0.07
5	122	2.48	2.26	2.37	0.16
6	122	4.07	3.84	3.96	0.17
7	122	4.36	4.98	4.67	0.44
8	122	4.40	4.48	4.44	0.06
9	122	3.52	3.93	3.73	0.28
10	122	4.51	4.39	4.45	0.08
11	122	1.69	1.59	1.64	0.07
12	122	4.84	4.51	4.68	0.23
13	122	4.36	4.90	4.63	0.38
14	122	4.88	4.47	4.68	0.28

Size (kDa)	Spec. Activity 1st	Spec. Activity 2nd	Avg.	Std. Dev.
96	3.40	3.60	3.50	0.14
96	3.40	3.60	3.50	0.14
96	3.40	3.60	3.50	0.14
96	3.40	3.60	3.50	0.14
96	3.40	3.60	3.50	0.14
96	3.40	3.60	3.50	0.14
96	3.40	3.60	3.50	0.14
96	3.40	3.60	3.50	0.14
96	3.40	3.60	3.50	0.14
96	0.67	0.59	0.63	0.06
96	2.25	1.98	2.12	0.20
96	1.20	1.38	1.29	0.13
96	1.34	1.47	1.41	0.10
96	1.31	1.49	1.40	0.13
96	1.22	1.35	1.29	0.08

Table A16 – Specific activity calculated using intensity measurements taken from intracellular activity gels of 40 kDa and 36 kDa chitinases isolated from dual cultures of *T. aggressivum* and *A. bisporus* strain Amycel 2400.

Sample (day)	Size (kDa)	Spec. Activity 1st	Spec. Activity 2nd	Avg.	Std. Dev.
0	40	-	-	-	-
1	40	-	-	-	-
2	40	-	-	-	-
3	40	-	-	-	-
4	40	-	-	-	-
5	40	-	-	-	-
6	40	-	-	-	-
7	40	0.79	0.91	0.85	0.08
8	40	1.03	0.94	0.99	0.07
9	40	0.98	1.02	1.00	0.03
10	40	1.03	0.94	0.98	0.07
11	40	0.96	1.04	1.00	0.06
12	40	1.07	1.23	1.15	0.11
13	40	1.11	1.18	1.14	0.06
14	40	1.03	1.16	1.10	0.10

Size (kDa)	Spec. Activity 1st	Spec. Activity 2nd	Avg.	Std. Dev.
36	-	-	-	-
36	-	-	-	-
36	-	-	-	-
36	0.22	0.20	0.21	0.01
36	0.40	0.44	0.42	0.03
36	0.64	0.57	0.61	0.06
36	0.81	0.89	0.85	0.06
36	0.60	0.53	0.57	0.04
36	0.47	0.53	0.50	0.04
36	0.36	0.40	0.38	0.03
36	0.35	0.35	0.35	0.00
36	0.54	0.48	0.51	0.04
36	0.54	0.63	0.59	0.06
36	0.83	0.73	0.78	0.07
36	0.79	0.74	0.77	0.03

Table A17 – Specific activity calculated using intensity measurements taken from intracellular activity gels of 122 kDa and 96 kDa *N*-acetylglucosaminidases isolated from dual cultures of *T. aggressivum* and *A. bisporus* strain Horst U1.

Sample (day)	Size (kDa)	Spec. Activity 1st	Spec. Activity 2nd	Avg.	Std. Dev.
0	122	-	-	-	-
1	122	-	-	-	-
2	122	0.53	0.47	0.50	
3	122	0.31	0.36	0.34	
4	122	1.95	1.83	1.89	
5	122	2.45	2.27	2.36	
6	122	3.12	3.54	3.33	
7	122	4.51	4.06	4.28	
8	122	4.56	4.08	4.32	
9	122	4.56	4.08	4.32	
10	122	4.56	4.08	4.32	
11	122	4.56	4.08	4.32	
12	122	3.42	3.81	3.62	
13	122	4.56	4.08	4.32	
14	122	4.56	4.08	4.32	

Size (kDa)	Spec. Activity 1st	Spec. Activity 2nd	Avg.	Std. Dev.
96	0.69	0.73	0.71	0.03
96	0.77	0.76	0.77	0.01
96	0.74	0.66	0.70	0.06
96	0.11	0.11	0.11	0.00
96	0.24	0.25	0.25	0.01
96	0.36	0.32	0.34	0.03
96	1.84	2.03	1.94	0.13
96	0.31	0.35	0.33	0.03
96	1.04	1.10	1.07	0.04
96	1.32	1.17	1.25	0.11
96	0.99	0.91	0.95	0.06
96	0.87	0.82	0.85	0.03
96	0.53	0.59	0.56	0.04
96	0.75	0.66	0.71	0.07
96	0.53	0.47	0.50	0.04

Table A18 – Specific activity calculated using intensity measurements taken from intracellular activity gels of 40 kDa and 36 kDa chitinases isolated from dual cultures of *T. aggressivum* and *A. bisporus* strain Horst U1.

Sample (day)	Size (kDa)	Spec. Activity 1st	Spec. Activity 2nd	Avg.	Std. Dev.
0	40	-	-	-	-
1	40	-	-	-	-
2	40	-	-	-	-
3	40	-	-	-	-
4	40	-	-	-	-
5	40	-	-	-	-
6	40	-	-	-	-
7	40	-	-	-	-
8	40	0.79	0.77	0.78	0.01
9	40	0.86	0.79	0.83	0.06
10	40	1.15	1.21	1.18	0.04
11	40	1.01	1.12	1.06	0.07
12	40	0.78	0.86	0.82	0.06
13	40	1.05	0.92	0.99	0.08
14	40	0.74	0.84	0.79	0.07

Size (kDa)	Spec. Activity 1st	Spec. Activity 2nd	Avg.	Std. Dev.
36	-	-	-	-
36	-	-	-	-
36	0.28	0.30	0.29	0.01
36	0.36	0.40	0.38	0.03
36	0.47	0.42	0.45	0.04
36	0.43	0.45	0.44	0.01
36	0.48	0.43	0.46	0.03
36	0.40	0.46	0.43	0.04
36	0.74	0.65	0.70	0.06
36	0.62	0.60	0.61	0.01
36	0.58	0.55	0.57	0.03
36	0.69	0.77	0.73	0.06
36	0.43	0.50	0.47	0.04
36	0.85	0.80	0.83	0.04
36	0.41	0.43	0.42	0.01

Table A19 – Specific activity calculated using intensity measurements taken from intracellular activity gels of 122 kDa and 96 kDa *N*-acetylglucosaminidases isolated from dual cultures of *T. aggressivum* and *A. bisporus* strain Sylvan SB65.

Sample (day)	Size (kDa)	Spec. Activity 1st	Spec. Activity 2nd	Avg.	Std. Dev.	Size (kDa)	Spec. Activity 1st	Spec. Activity 2nd	Avg.	Std. Dev.
0	122	-	-	-	-	96	1.84	2.00	1.92	0.11
1	122	-	-	-	-	96	1.84	2.00	1.92	0.11
2	122	0.35	0.32	0.34	0.03	96	1.84	2.00	1.92	0.11
3	122	0.79	0.83	0.81	0.03	96	1.84	2.00	1.92	0.11
4	122	1.75	1.95	1.85	0.14	96	1.84	2.00	1.92	0.11
5	122	1.81	1.73	1.77	0.06	96	1.84	2.00	1.92	0.11
6	122	3.43	3.60	3.52	0.13	96	1.84	2.00	1.92	0.11
7	122	2.94	2.56	2.75	0.27	96	1.29	1.14	1.22	0.10
8	122	3.24	2.88	3.06	0.25	96	0.76	0.72	0.74	0.03
9	122	3.75	3.90	3.83	0.10	96	1.09	0.95	1.02	0.10
10	122	2.47	2.65	2.56	0.13	96	2.13	1.84	1.99	0.20
11	122	1.97	2.06	2.02	0.06	96	1.28	1.26	1.27	0.01
12	122	4.16	4.02	4.09	0.10	96	1.36	1.55	1.46	0.13
13	122	4.17	3.96	4.07	0.14	96	1.51	1.33	1.42	0.13
14	122	4.16	4.02	4.09	0.10	96	1.51	1.33	1.42	0.13

Table A20 – Specific activity calculated using intensity measurements taken from intracellular activity gels of 40 kDa and 36 kDa chitinases isolated from dual cultures of *T. aggressivum* and *A. bisporus* strain Sylvan SB65.

Sample (day)	Size (kDa)	Spec. Activity 1st	Spec. Activity 2nd	Avg.	Std. Dev.	Size (kDa)	Spec. Activity 1st	Spec. Activity 2nd	Avg.	Std. Dev.
0	40	-	-	-	-	36	-	-	-	-
1	40	-	-	-	-	36	-	-	-	-
2	40	-	-	-	-	36	-	-	-	-
3	40	-	-	-	-	36	0.44	0.42	0.43	0.01
4	40	-	-	-	-	36	0.94	0.85	0.90	0.07
5	40	-	-	-	-	36	0.48	0.54	0.51	0.04
6	40	-	-	-	-	36	0.51	0.55	0.53	0.03
7	40	0.15	0.17	0.16	0.01	36	0.51	0.44	0.48	0.04
8	40	0.41	0.45	0.43	0.03	36	0.49	0.43	0.46	0.04
9	40	1.47	1.38	1.43	0.07	36	0.38	0.41	0.40	0.03
10	40	1.63	1.81	1.72	0.13	36	0.31	0.33	0.32	0.01
11	40	0.69	0.60	0.65	0.06	36	0.33	0.38	0.36	0.03
12	40	1.44	1.62	1.53	0.13	36	0.40	0.36	0.38	0.03
13	40	1.01	1.14	1.08	0.10	36	0.37	0.44	0.41	0.04
14	40	0.91	1.06	0.99	0.10	36	0.56	0.50	0.53	0.04

Table A21 – Specific activity calculated using intensity measurements taken from intracellular activity gels of 122 kDa and 96 kDa *N*-acetylglucosaminidases isolated from dual cultures of *T. aggressivum* and *A. bisporus* strain Sylvan 130.

Sample (day)	Size (kDa)	Spec. Activity 1st	Spec. Activity 2nd	Avg.	Std. Dev.
0	122	-	-	-	-
1	122	-	-	-	-
2	122	0.78	0.72	0.75	
3	122	1.46	1.60	1.53	
4	122	1.75	1.94	1.85	
5	122	3.95	3.81	3.88	
6	122	3.89	3.55	3.72	
7	122	3.75	3.90	3.83	
8	122	3.79	4.11	3.95	
9	122	4.01	3.90	3.96	
10	122	4.15	4.51	4.33	
11	122	4.07	3.92	4.00	
12	122	3.86	3.98	3.92	
13	122	3.91	4.12	4.02	
14	122	3.84	3.97	3.91	

Size (kDa)	Spec. Activity 1st	Spec. Activity 2nd	Avg.	Std. Dev.
96	0.31	0.29	0.30	0.01
96	0.63	0.72	0.68	0.06
96	0.32	0.32	0.32	0.00
96	0.30	0.28	0.29	0.01
96	0.32	0.27	0.30	0.03
96	1.58	1.73	1.66	0.11
96	0.26	0.28	0.27	0.01
96	0.28	0.31	0.30	0.01
96	1.46	1.60	1.53	0.10
96	1.14	1.31	1.23	0.11
96	0.86	0.92	0.89	0.04
96	0.98	0.85	0.92	0.08
96	0.66	0.68	0.67	0.01
96	1.27	1.26	1.27	0.01
96	0.51	0.55	0.53	0.03

Table A22 – Specific activity calculated using intensity measurements taken from intracellular activity gels of 40 kDa and 36 kDa chitinases isolated from dual cultures of *T. aggressivum* and *A. bisporus* strain Sylvan 130.

Sample (day)	Size (kDa)	Spec. Activity 1st	Spec. Activity 2nd	Avg.	Std. Dev.
0	40	-	-	-	-
1	40	-	-	-	-
2	40	-	-	-	-
3	40	-	-	-	-
4	40	-	-	-	-
5	40	-	-	-	-
6	40	-	-	-	-
7	40	0.24	0.26	0.25	0.01
8	40	0.28	0.25	0.27	0.03
9	40	0.59	0.53	0.56	0.04
10	40	0.20	0.22	0.21	0.01
11	40	0.42	0.47	0.45	0.04
12	40	0.12	0.10	0.11	0.01
13	40	0.53	0.50	0.52	0.03
14	40	0.50	0.43	0.47	0.04

Size (kDa)	Spec. Activity 1st	Spec. Activity 2nd	Avg.	Std. Dev.
36	-	-	-	-
36	-	-	-	-
36	0.39	0.42	0.41	0.03
36	0.44	0.50	0.47	0.04
36	0.52	0.47	0.50	0.04
36	0.51	0.55	0.53	0.03
36	0.51	0.56	0.54	0.04
36	0.54	0.62	0.58	0.06
36	0.62	0.57	0.60	0.04
36	0.79	0.69	0.74	0.07
36	0.75	0.70	0.73	0.04
36	0.52	0.57	0.55	0.04
36	0.68	0.79	0.74	0.07
36	0.99	0.88	0.93	0.08
36	0.59	0.52	0.56	0.04

Table A23 – Specific activity calculated using intensity measurements taken from extracellular activity gels of chitinases isolated from dual cultures of *T. aggressivum* and *A. bisporus* strain Amycel 2400 (left), and Horst U1 (right).

Sample (day)	Size (kDa)	Spec. Activity 1st	Spec. Activity 2nd	Avg.	Std. Dev.
0	111	3.02	3.28	3.15	0.18
1	122	1.40	1.23	1.32	0.13
	96	1.25	1.31	1.28	0.04
2	131	1.95	2.07	2.01	0.08
	111	2.73	2.52	2.63	0.14
	96	1.41	1.29	1.35	0.08
3	131	1.55	1.34	1.45	0.14
	111	1.72	1.56	1.64	0.11
	96	1.25	1.14	1.20	0.08
4	96	3.43	3.75	3.59	0.23
5	125	1.25	1.36	1.31	0.07
	96	3.69	3.45	3.57	0.17
6	122	0.79	0.90	0.85	0.08
	96	3.43	3.71	3.57	0.20
7	122	1.42	1.43	1.43	0.01
	96	2.36	2.72	2.54	0.25
8	122	2.77	2.86	2.82	0.06
	96	1.75	1.79	1.77	0.03
	40	1.14	1.31	1.23	0.13
9	122	3.25	3.09	3.17	0.11
	96	3.57	3.44	3.51	0.10
	40	1.55	1.80	1.68	0.17
10	122	3.04	3.22	3.13	0.13
	96	3.58	3.37	3.48	0.16
	40	1.57	1.75	1.66	0.13
11	122	3.26	3.03	3.15	0.16
	96	3.43	3.53	3.48	0.07
	40	1.61	1.72	1.67	0.08
12	122	2.87	2.94	2.91	0.04
	96	3.06	2.82	2.94	0.17
	40	2.51	2.24	2.40	0.23
13	122	3.60	3.70	3.65	0.07
	96	3.18	3.39	3.29	0.14
	40	2.35	2.16	2.26	0.13
14	122	3.76	3.57	3.67	0.13
	96	3.13	3.44	3.29	0.21
	40	2.49	2.45	2.47	0.03

Sample (day)	Size (kDa)	Spec. Activity 1st	Spec. Activity 2nd	Avg.	Std. Dev.
0	111	1.18	1.23	1.21	0.03
1	122	1.20	1.06	1.13	0.10
2	131	2.63	2.32	2.48	0.21
	111	1.21	1.31	1.26	0.07
3	122	3.14	3.60	3.37	0.33
4	131	3.13	3.42	3.28	0.21
5	125	1.24	1.15	1.20	0.07
	96	0.76	0.83	0.80	0.04
6	122	1.95	1.77	1.86	0.13
	96	1.01	0.96	0.99	0.03
7	122	3.65	3.89	3.77	0.17
	96	1.32	1.47	1.40	0.11
8	122	3.15	3.21	3.18	0.04
	96	0.74	0.65	0.70	0.07
	40	0.97	0.88	0.93	0.07
9	122	3.16	3.18	3.17	0.01
	96	0.99	0.88	0.94	0.07
	40	0.85	0.96	0.91	0.08
10	122	4.00	3.70	3.85	0.21
	96	1.12	1.29	1.21	0.11
	40	1.53	1.75	1.64	0.16
11	122	3.95	3.66	3.81	0.20
	96	1.01	1.07	1.04	0.04
	40	1.92	1.69	1.81	0.17
12	122	3.52	3.36	3.44	0.11
	96	1.05	1.04	1.05	0.01
	40	2.02	1.84	1.93	0.13
13	122	4.30	4.33	4.32	0.03
	96	1.08	1.06	1.07	0.01
	40	2.00	1.71	1.86	0.20
14	122	4.33	4.50	4.42	0.13
	96	0.96	0.90	0.93	0.04
	40	1.84	2.11	1.98	0.18

Table A24 – Specific activity calculated using intensity measurements taken from extracellular activity gels of chitinases isolated from dual cultures of *T. aggressivum* and *A. bisporus* strain Sylvan SB65 (left), and Sylvan 130 (right).

Sample (day)	Size (kDa)	Spec. Activity		Avg.	Std. Dev.
		1st	2nd		
0	111	1.68	1.62	1.65	0.04
1	122	1.96	1.69	1.83	0.20
2	131	2.14	2.25	2.21	0.10
	111	1.85	1.89	1.87	0.03
3	122	3.64	3.67	3.66	0.03
	96	3.63	3.68	3.66	0.03
4	131	1.15	1.01	1.08	0.10
	111	1.16	1.13	1.15	0.03
	96	0.41	0.46	0.44	0.04
5	125	2.85	2.95	2.90	0.07
	96	1.96	1.73	1.85	0.16
6	122	3.00	2.96	0.79	0.03
	96	0.83	0.74	2.98	0.07
7	122	1.64	1.83	1.74	0.13
	96	2.56	2.54	2.55	0.01
8	122	1.57	1.62	1.60	0.04
	96	3.05	2.97	3.01	0.06
	40	1.14	1.27	1.21	0.08
9	122	3.14	3.16	3.15	0.01
	96	2.63	2.63	2.63	0.00
	40	1.27	1.47	1.37	0.14
10	122	3.56	3.59	3.58	0.01
	96	2.55	2.64	2.60	0.07
	40	1.94	2.01	1.98	0.06
11	122	3.46	3.44	3.45	0.01
	96	2.80	2.42	2.61	0.27
	40	1.28	1.11	1.20	0.11
12	122	3.55	3.34	3.45	0.16
	96	2.54	2.72	2.63	0.13
	40	2.20	2.17	2.19	0.01
13	122	3.51	3.04	3.28	0.33
	96	2.36	2.61	2.49	0.17
	40	1.95	2.11	2.03	0.11
14	122	3.55	3.74	3.65	0.13
	96	2.47	2.82	2.65	0.25
	40	2.31	2.20	2.26	0.08

Sample (day)	Size (kDa)	Spec. Activity		Avg.	Std. Dev.
		1st	2nd		
0	111	1.01	1.16	1.09	0.10
1	122	1.00	0.96	0.98	0.03
2	131	3.10	3.18	3.14	0.06
3	131	3.10	3.18	3.14	0.06
	111	0.85	0.80	0.83	0.04
4	122	3.53	3.76	3.65	0.16
5	122	3.73	3.58	3.66	0.10
	96	0.62	0.60	0.61	0.01
6	125	1.21	1.36	1.29	0.11
	96	0.53	0.55	0.54	0.01
7	122	3.25	3.04	3.15	0.16
	96	0.93	0.85	0.89	0.06
8	122	3.14	3.03	3.09	0.08
	96	1.19	1.27	1.23	0.06
	40	0.25	0.28	0.27	0.03
9	122	3.63	3.27	3.45	0.25
	96	1.31	1.20	1.26	0.08
	40	0.33	0.37	0.35	0.03
10	122	3.22	3.03	3.13	0.14
	96	1.54	1.33	1.44	0.14
	40	0.32	0.27	0.30	0.03
11	122	3.75	3.97	3.86	0.16
	96	0.57	0.64	0.61	0.04
	40	1.37	1.46	1.42	0.06
12	122	3.07	3.29	3.18	0.16
	96	1.82	1.63	1.73	0.13
	40	0.37	0.34	0.35	0.03
13	122	4.26	3.98	4.12	0.20
	96	1.67	1.65	1.66	0.01
	40	2.69	2.47	2.58	0.16
14	122	4.43	4.04	4.24	0.28
	96	1.65	1.53	1.59	0.08
	40	2.44	2.67	2.56	0.16

Determination of Molecular Weight

At the time of molecular weight determination each sample had been run many times, and it was seen that there were a number of similarities in banding patterns. Therefore, a number of samples were chosen to represent those believed to have the same molecular weight, based on these previously run gels.

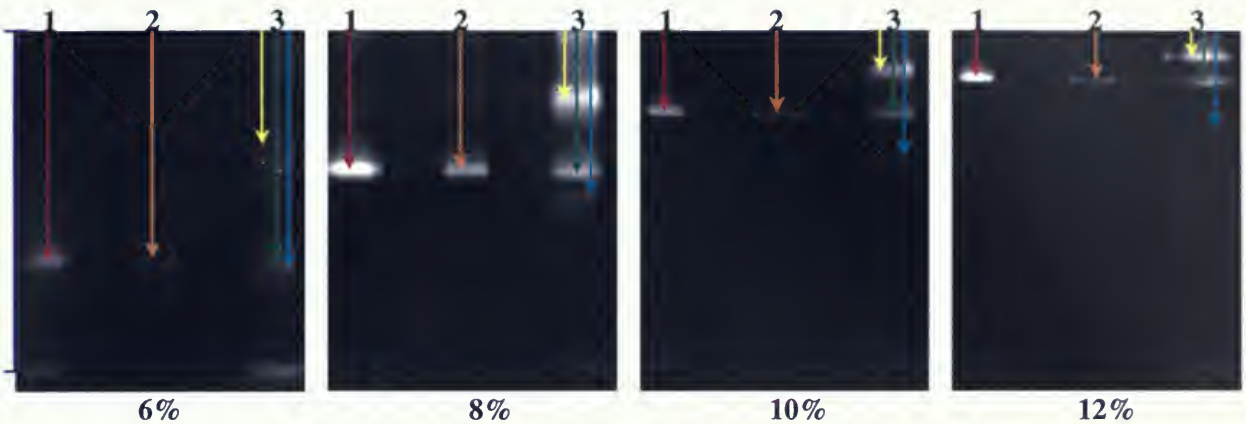

Figure A2 – Example of gels run using various concentrations for molecular weight determination. Migration distance indicated for each sample.

Sample Calculation:

① For protein in lane 1 (6%): migration distance (protein) = 33, and dye front = 52

$$\therefore R_f = \text{p./d.f.}$$
$$= 33/52$$
$$= 0.63$$

② R_f values are converted to plot against gel concentration:
100 [Log (R_f x 100)]

Table A25 – Example of chart used to record migration distances and R_f calculated values.

	Gel Concentration			
Migration distance	**6%**	**8%**	**10%**	**12%**
Protein	33	20	12.5	7.5
Dye Front	52	52	50	50
R_f	0.63	0.38	0.25	0.15
Log Value	179.9	157.9	139.8	117.6

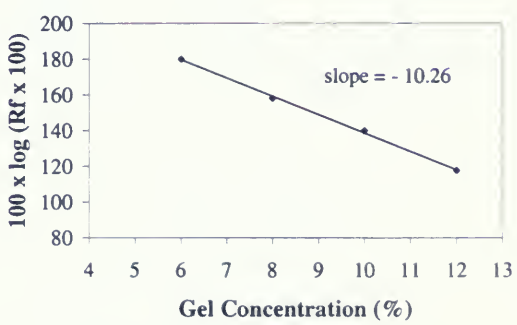

Figure A3- Determination of slope from raw data.

③ To determine molecular weight the equation from the standard curve is

$y = 0.0811x + 2.4356$, where y = -slope, and x = molecular weight (kDa).

Thus for a -slope of 10.26:

$$10.26 = 0.0811x + 2.4356$$
$$x = (10.26 - 2.4356)/0.0811$$
$$x = 96.5 \text{ kDa}$$

Large charts were created containing all R_f values, and the corresponding log values for each representative, which included 68 samples from the 1st and 2nd replicates. Based on migration patterns across the varying gel concentrations, as seen in the log values, similar patterns were grouped together. The values from a minimum of five bands were averaged and the slope calculated. Molecular weight was determined using the previously described calculation.

Table A26 – Molecular weight determination for representative enzymes isolated from dual and solitary cultures of *Trichoderma* and *Agaricus*. Log values averaged from a minimum of five bands for each enzyme type.

Enzyme	Avg. Log Values				-Slope	Mol. Weight	Std. Dev.
	6%	8%	10%	12%		(kDa)	
1	162.32	123.04	106.07	81.29	13.00	130.3	3.1
2	159.33	136.55	111.39	83.82	12.58	125.1	2.0
3	159.22	137.66	113.67	85.13	12.31	121.8	3.8
4	170.68	148.87	131.38	100.40	11.42	110.7	3.2
5	166.93	150.78	127.88	101.47	10.96	105.2	1.9
6	179.92	159.19	139.94	118.06	10.24	96.3	2.0
7	169.23	157.40	147.03	134.63	5.71	40.3	2.2
8	177.74	169.81	157.75	146.09	5.35	35.9	1.4