1. Introduction

1.1 Filamentous fungi as sources for enzymes and metabolites

A considerable part of the industrial attraction of filamentous fungi is their ability to secrete large amounts of proteins into the growth medium. Filamentous fungi have been widely used as sources of metabolites and enzymes for centuries and still play very important roles in our everyday life. Genera such as *Mucor* and *Aspergillus* are used for making cheese and soy sauce and several other oriental food ingredients, and in the production of beverages. Enzymes such as α-amylases, glucoamylases, cellulases and proteases secreted by filamentous fungi are applied in many industries (Punt et al., 2002). However, only fungal proteinases and amylases will be discussed in the following sections.

In the year 2000, the global market for industrial enzymes was around $2 billion and the amount increases annually (Nevalainen and Te'o, 2003). The biggest enzyme user is the detergent industry (up to 34 %) followed by textiles (11 %), starch (12 %), baking (5 %), animal feed (7 %), beverages and brewing (7 %) and dairy processes (14 %) (Saxena et al., 2004). Extracellular enzyme yields as high as 100 g/l have been reported for *Trichoderma reesei* (Cherry and Fidantsef, 2003). The production levels of glucoamylase by industrial *Aspergillus* strains are up to 30 g/l (Finkelstein et al., 1989). In addition to enzymes, fungi also produce important metabolites such as antibiotics, organic acids, pigments and various food additives (Punt et al., 2002).

Filamentous fungi are also being developed and applied as cell factories for the expression of homologous and heterologous recombinant proteins for industrial purposes. The advantages of fungal expression systems over other available expression systems have been fully reviewed by Nevalainen et al. (2005). Examples of fungal expression systems include those based on the use of *T. reesei*, *A. niger* and *A. oryzae*. Recombinant gene products expressed in filamentous fungi feature a wide range of biocatalysts already on the market and some therapeutic proteins that, however, are not yet in the market (Adrio and Demain, 2003; Bergquist et al., 2002; Gerngross, 2004; Maras et al., 1999; Nevalainen et al., 2005; Punt et al., 2002). The best yields of recombinant products have been obtained from expressing a fungal gene in a fungal host. For example, secretion of *Rhizomucor*

miehei aspartic proteinase was up to 3 g/l in *A. oryzae* in a controlled fermentation (Christensen et al., 1988). However, the yields of non-fungal heterologous proteins have remained low. Several strategies (discussed in section 1.4) have been successfully applied to improve the secretion of foreign proteins in filamentous fungi.

1.1.1 Fungal proteinases

Fungi produce a large amount of proteinases which belong to several different families. Proteinases are generally classified into two major groups, exopeptidases and endopeptidases. In the exopeptidase group, there are two sub-groups, aminopeptidases and carboxypeptidases, characterized by their sites of action at the N- or C- termini of the target proteins. Several aminopeptidases have been studied from *Aspergillus* spp. including leucine aminopeptidase and lysine peptidase (Basten et al., 2001; Chien et al., 2002; Nampoothiri et al., 2005). Carboxypeptidases which can release a single amino acid or a dipeptide have been identified from several fungi such as *Aspergillus* spp., *Penicillium* spp. and *Metarhizium* spp. (Chiba et al., 1995; Degan et al., 1992; Joshi and St. Leger, 1999; Rao et al., 1998).

Fungal endopeptidases, which are divided into four sub-families based on their catalytic mechanism, have been well studied and widely applied in industry. The four sub-families are serine proteinases, aspartic proteinases, cysteine proteinases and metalloproteinases. Serine proteinases are named from the presence of a serine residue in the active site. They have been isolated from many fungi including *A. niger*, *A. nidulans*, *Paecilomyces lilacinus*, *Conidiobolus coronatus* and *Ophiostoma floccosum* (Abraham and Breuil, 1996; Fredericket al., 1993; Khan et al., 2003; Phadatare et al., 1997; vanKuyk et al., 2000). Generally, serine proteinases have a molecular weight in the range of 18 kDa to 35 kDa. However, higher molecular weight serine proteinases have also been reported from *A. niger* and *A. nidulans* (Rao et al., 1998). Aspartic proteinases are also known as acid proteinases; these proteinases depend on aspartic acid residues for activity. The aspartic acid residue in the active site is located within the motif Asp-Xaa-Gly, in which Xaa can be Ser or Thr. In general, aspartic proteinases secreted by fungi can be divided into two groups, pepsin-like aspartic proteinases produced by *Aspergillus*, *Penicillium*, *Rhizopus*,

Trichoderma and *Neurospora*, and rennin-like aspartic proteinases secreted by *Endothia* and *Mucor* (Delgado-Jarana et al., 2000; Haab et al., 1990; Rao et al., 1998; Yamashita et al., 1987). Cysteine proteinases are also called thiol proteinases because their activity is inhibited by thiol reagents. Generally, the activity of cysteine proteinases is enhanced by reducing reagents such as HCN or cysteine. So far, only a limited number of cysteine proteinases secreted by fungi have been reported (Rao et al., 1998). Metalloproteinases include diverse catalytic types which require a divalent metal ion for activity. Most metalloproteinases are inhibited by chelating agents such as EDTA, but not by sulfhydryl agents or diisopropylfluorophosphate. There are some reports of metalloproteinases found in fungi including *P. roqueforti*, *A. oryzae*, *A. sojae*, *A. fumigatus* (Markaryan et al., 1996; Rao et al., 1998).

1.1.1.1 Applications of proteinases in industry

Proteinases have been extensively applied in many industries, particularly in the detergent and food industry. In the detergent industry, proteinases are the key component in all kinds of detergents and the use of proteinases in laundry detergents accounts for approximately 25 % of the total worldwide sales of enzymes. Serine proteinases produced by *Bacillus* are used in most detergents. An alkaline proteinase from *Condidiobolus coronatus* has been applied to detergents in India (Rao et al., 1998). Fungal proteinases are also used in food industry mainly for cheese-making, baking, preparation of soya hydrolysate and meat tenderization. Furthermore, fungal proteinases have been used in leather processing and pharmaceutical industries and for basic research. In leather industry, enzymes, which not only improve the leather quality but also reduce environmental pollution, have become an alternative option to the use of chemicals. *Aspergillus* proteinases, combined with some other proteinases, have been applied to hydrolyze noncollagenous constituents of the skin and to remove nonfibrillar proteins (North, 1982; Rao et al., 1998).

1.1.1.2 Trifunctional probe for capturing the secreted proteinases

Activity-based chemical probes which can covalently modify the active sites of enzymes in complex proteomes have been developed to profile proteins on the basis of

their function (Adam et al., 2002; Cravatt and Sorensen, 2000). They have been reported to label the active residues, such as serine, cysteine or lysine, typically seen in proteinases and transferases. The active residue of a protein attacks the reactive group of the probe, resulting in formation of a covalent linkage between the active residue of the protein and the probe. In addition to the reactive group that serves as an activity tag, activity probes typically have three additional components: a linker region, a fluorescent reporter, and an affinity tag for purification of low abundance proteins (Scheme 1 in publication II). The fluorescent reporter group allows for the physical identification of the tagged protein on gels. The affinity tag facilitates affinity purification of the protein if required. The linker physically separates the different functionalities so that they will not interfere with one another's role. This activity-based approach has been successfully used to identify key enzyme activities in biological systems of the animal kingdom (Jessani and Cravatt, 2004). For example, Greenbaum and coworkers (Greenbaum et al., 2000) used this strategy to elucidate the important role of cysteine proteinases in the host cell invasion process of human malaria parasite *Plasmodium falciparum*. The *P. falciparum* proteome was subjected to cysteine protease probes, which identified four important protease activities from whole-cell lysates.

1.1.2 Fungal amylases

Amylases are hydrolases that cleave starch or starch breakdown products. The main amylolytic activities produced by fungi include α-amylase, β-amylase and glucoamylase (GA) (Pandey et al., 2000). Alpha amylase randomly cleaves amylose which contains only α-1,4- linked glucose subunits, and the cleavage products feature dextrin, maltose and glucose. Beta amylase catalyzes hydrolysis of α-1,4 glycosidic bonds from the non-reducing end of the polysaccharide chain and releases maltose molecules. Glucoamylase hydrolyzes α-1,4 and α-1,6 glycosidic linkages from the non-reducing end of the polysaccharide chain (Ventura et al., 1995); the end products of such reaction are single glucose units.

1.1.2.1 Applications of amylases in industry

Amylases are essential for our everyday life due to their broad applications in food, beverage, fermentation, textile, pulp and paper, animal feed, baking, detergent, fuel ethanol and brewing industries (www.novozyme.com and www.genencor.com). Most industrial amylases are sourced from microbes such as bacteria, fungi and yeasts, although amylases are also found in plants and animals. Glucoamylase and α-amylase are the most frequently isolated amylases from micro-organisms (Eksteen et al., 2003; Nunberg et al., 1984; Orlando et al., 1983; Ventura et al., 1995). Beta-amylase is generally obtained from plants, but the enzyme is also available from microbial sources (Ray, 2004; Yoshigi et al., 1995). Currently, α-amylases from *Aspergillus* spp. and *Bacillus* spp. are commonly used by industry, especially α-amylases from *B. amyloliquefaciens* and *B. licheniformis*. An acid stable α-amylase from *A. kawachii* is employed in starch hydrolysis for fermenting *Shochu* mash to prepare *Shochu*, an indigenous Japanese spirit (Kaneko et al., 1996). Glucoamylases from filamentous fungi constitute the major part of microbial glucoamylases for commercial applications. *Aspergillus* spp., particularly *A. niger* and *A. awamori*, have been used frequently for the production of glucoamylase (Wang and Webb, 1995). Although mesophilic amylases from various sources have been isolated, characterized and applied in industry, there is a further need for novel amylases that can perform at extreme conditions such as a high or low temperature, acidic or alkaline pH, high salt concentration and high pressure (Deutch, 2002; Hyun and Zeikus, 1985).

1.1.2.2 The structure of fungal α-amylase and glucoamylase enzymes

Generally, an α-amylase enzyme contains multiple domains, which constitute a catalytic domain and starch binding domain that are connected by a linker region (Boel et al., 1990; Kaneko, et al., 1996). Boel et al. (1990) investigated the α-amylase from *A. niger* and *A. oryzae* by X-ray diffraction. The results showed that there were two distinct domains, domain A and a smaller domain B, which were separated by a linker region. Furthermore, there was an essential Ca^{2+} binding site between the main body of the β barrel structure (domain A) and the smaller domain B. The Ca^{2+} ion preserved the structural integrity of the active site (Boel et al., 1990). An acid stable α-amylase (asAA) from *A. kawachii* characterized by Kaneko et al. (1996) showed 97 % homology to the α-amylase

from *A. niger*. Putative regions rich in threonine and serine (TS regions) were identified at the C-terminus of both asAA of *A. kawachii* and the α-amylase of *A. niger*. The TS region enables α-amylase to digest raw starch. Further studies have confirmed that the α-amylases from *Aspergillus* spp. which do not have the TS region could not degrade raw starch (Matsubara et al., 2004). Therefore, not all fungal α-amylases have the capability to degrade raw starch (Boel et al., 1990; Kaneko et al., 1996).

Considerably more attention has been paid to fungal glucoamylases which are well characterized due to their applications in industry, particularly glucoamylases from *Aspergillus* spp. (Nunberg et al., 1984; Stoffer et al., 1993; Ventura et al., 1995). Similarly to α-amylases, most fungal glucoamylases generally possess a catalytic domain at the N-terminus and a starch binding domain (SBD) at the C-terminus of the protein, and the two domains are connected by a linker region (Boel et al., 1984; Hayashida et al., 1989; Stone et al., 1993). However, there are also some variations to the structure: a glucoamylase from *Rhizopus oryzae* contains a catalytic domain at the C-terminus and SBD at the N-terminus (Ashikari et al., 1985). Some studies have indicated that the two forms of glucoamylase (GA I and GA II) are derived from a single glucoamylase gene locus in *A. niger* and *A. awamori* (Boel et al., 1984; Nunberg et al., 1984). GA I contains a catalytic domain, SBD and linker region, but GA II does not have the SBD. The SBD amino acid sequences of glucoamylases from *Aspergillus* spp. are highly conserved (Coutinho and Reilly, 1997). The glucoamylase from *A. niger* (GA I), which has been well investigated and characterized, is preferred as a model in the structure-function studies of SBDs in starch hydrolysis (Cornett et al., 2003; Juge et al., 2002; Southall et al., 1999). It was shown that digestion of insoluble starch by the GA I from *A. niger* was 80 times faster than that of GA II which lacks the SBD (Southall et al., 1999). Therefore, a functional SBD can bind to raw starch and enhance the capability of glucoamylase to digest raw starch. SBD also allows the catalytic domain of the glucoamylase to access large areas of the starch granule surface by disrupting the surface to enhance hydrolysis (Juge et al., 2002).

1.1.2.3 Regulation of the expression of *Aspergillus* α-amylase and glucoamylase

Alpha amylase and glucoamylase are enzymes subjected to carbon catabolite repression (Nagata et al., 1993; Ventura et al., 1995). Expression of the two enzymes is

repressed by different carbon sources. For example, production and secretion of α-amylase from *A. oryzae* was induced by starch, maltose and maltodextrins and repressed by glucose (Yabuki et al., 1977; Carlsen et al, 1996; Erratt et al., 1984; Mørkeberg et al., 1995). Production of α-amylase from *A. awamori* was also repressed by glucose (Bhella and Altosaar, 1987). In *A. niger* and *A. oryzae*, the expression of glucoamylase was induced by starch and maltose, but repressed by xylose (Fowler et al., 1990; Hata et al., 1992). According to some reports, glucose can also act as an inducer for the expression of glucoamylase from *A. niger* (Fowler et al., 1990). In *A. awamori*, glucoamylase was induced by glucose, starch and a variety of other hexose sugars or hexose sugar polymers and repressed by glycerol and xylose (Nunberg et al., 1984). Starch and maltose acted as inducers and glucose and xylose as repressors in the expression of glucoamylase from *A. terreus* (Ventura et al., 1995).

An increasing number of studies indicate that production of amylases in solid state fermentation (SSF) has considerable potential to minimize carbon catabolite repression (de Souza and Peralta, 2001; Nandakumar et al., 1999; Sudo et al., 1994). A full review of studies for the production of industrial enzymes including amylases by SSF is available at http://www.ias.ac.in/currsci/jul10/articles23.htm. Generally, a lower moisture content in the cultures plus decreased carbon catabolite repression in SFF resulted in the production of higher amount of amylases when compared to submerged fermentation. Nandakumar et al., (1999) also reported that production of α-amylase and amyloglucosidase by *A. niger* using SSF minimized carbon catabolite repression. This was considered to be due to a lower water content and the absence of medium agitation.

1.2 The secretory pathway of filamentous fungi

Many recent studies have focused on the protein secretion pathway of filamentous fungi with a view to finding reasons for the low yields of heterologous proteins obtained in these systems. The protein secretion pathway has been well studied and characterized in yeast and higher eukaryotes, which provides an excellent starting point for investigations into the secretion pathway of filamentous fungi. However, there are two significant differences between filamentous fungi, and yeast and higher eukaryotes: these are the polar

extension of the hyphal tips of fungal mycelia and the ability of hyphae to secrete large amounts of proteins into the culture medium (Conesa et al., 2001). Secretion pathways of *Aspergillus* and *Trichoderma*, which are commercially important organisms, have been widely explored at the molecular level (Conesa et al., 2001; Gordon et al., 2000a,b; Khalaj et al., 2001; Saloheimo et al., 1999). A general model for the protein secretion pathway in filamentous fungi is shown in Fig. 1.

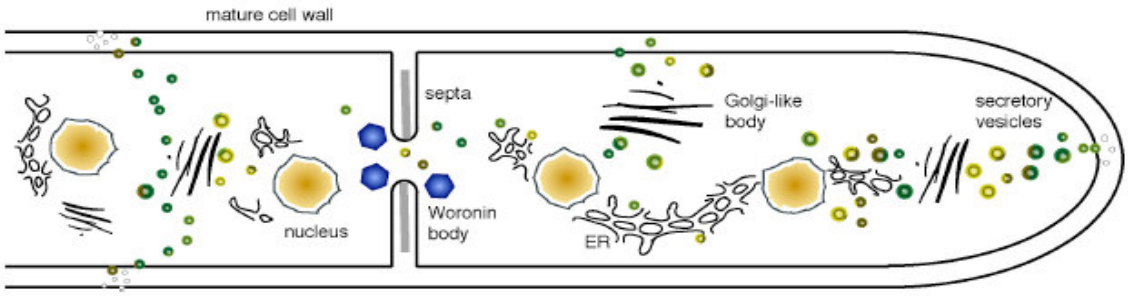

Fig. 1. Schematic diagram of the secretion pathway of filamentous fungi (Valkonen, 2003).

Proteins that will be secreted into the growth medium begin their journey by entering the endoplasmic reticulum (ER). Within the ER, the nascent proteins are folded and undergo subunit assembly and post-translational modifications such as glycosylation, disulfide bridge formation, and phosphorylation. Subsequently, proteins are packaged into vesicles and transported to the Golgi apparatus where they undergo further folding and modifications. Finally, proteins are packaged in secretory vesicles and directed to the plasma membrane from where they are secreted into the culture medium. Many studies have indicated that secretion of proteins occurs mostly at the apical cells or subapical regions of hyphae (Conesa et al., 2001; Gordon et al., 2000a, b). Gordon et al. (2000a,b) monitored protein secretion in *A. niger* using the green fluorescent protein fused to glucoamylase. The results revealed that the fluorescence was most intense at hyphal apices and some fluorescence was also localized in the hyphal cell walls and septa.

1.2.1 Protein quality control

ER, the largest subcellular organelle of the protein secretion pathway, is the place where protein translation, folding, assembly and covalent modification occur. However, these processes are not perfect and can produce misfolded or unfolded proteins that must be properly disposed of. One of the essential functions of ER is to ensure that only correctly folded proteins are allowed to proceed in the secretory pathway. Inhibition of the secretion of aberrant proteins is regarded as protein quality control (Sitia and Braakman, 2003). This process is mediated by the unfolded protein response (UPR) and ER-associated degradation (ERAD) which are tightly coordinated (Ellgaard and Helenius, 2001; Travers et al., 2000).

1.2.1.1 Unfolded protein response (UPR)

UPR, which involves a complex signal transduction pathway, can be induced by imposing stress to the ER. Conditions that disturb the ER function include accumulation of unfolded or misfolded proteins, overexpression of particular proteins and production of heterologous proteins (Umebayashi et al., 1999; Saloheimo et al., 1999). Subsequently, the expression level of chaperones and foldases in the ER is upregulated. The IRE1 protein, which possesses endoribonuclease activity and is the most upstream element of the UPR, acts as a sensor in sensing unfolded proteins in the ER and transfers the signal to the downstream portion of the UPR pathway (Valkonen et al., 2004). In yeast, IRE1 cleaves the borders of a 252-bp intron at the 3' end of the encoding region of the *hac1* mRNA during the induction of the UPR, which creates a mature HAC1 of 238 amino acids instead of the non-spliced form HAC1 of 230 amino acids (Cox and Walter, 1996). The *ire1* gene of *T. reesei* was isolated and characterized and it has been reported that it encodes a functional homologue of the yeast IRE1 protein (Valkonen et al., 2004). The transcription factor, HACA/HAC1, which plays an essential role in the UPR-pathway has been isolated and characterized from the filamentous fungi *T. reesei* and *A. nidulans* (Saloheimo et al., 2003). Similarly to the expression of yeast *hac1* gene, expression of *hac1/A* genes of *T. reesei* and *A. nidulans* is also activated by a non-spliceosomal intron-splicing event. In addition, truncation of the HACA/HAC1 mRNA at the 5' flanking region was observed upon the UPR induction. Therefore, the induction of UPR in filamentous fungi has different

features to that observed in yeast, and the mechanism seems to be more complicated (Saloheimo et al., 2003).

Several chaperones and foldases have been identified in the ER of filamentous fungi including a binding protein (BiP) that is a member of the HSP70 protein family, protein disulphide isomerase (PDI), and calnexin. BiP, which is one of the most abundant ER chaperones, can recognize unfolded polypeptides, facilitate their folding and prevent abnormal aggregation (Gething, 1999). BiP also binds to misfolded proteins to preclude them from continuing through the secretory pathway (Hijarrubia et al., 1997). A gene encoding BiP has been isolated and characterized from *A. awamori*. The deduced protein sequence showed very high similarity to BiP protein found in the lumen of ER in yeast, mammalian cells and plant cells (Hijarrubia et al., 1997). It has been observed that *bip1* of *T. reesei* was strongly induced immediately after the activation of *hac1* during the UPR induction (Pakula et al., 2003). PDI is one of the major foldases and catalyses the oxidization, reduction and isomerization of protein disulfide bonds during protein maturation in the ER. PDI also serves as a chaperone that binds to proteins and promotes protein folding when disulfide bonds are abscent (Conesa et al., 2001). Genes encoding PDI have been isolated from *A. niger* and *T. reesei* (Ngiam et al., 1997; 2000; Saloheimo et al., 1999). In *A. niger*, the *pdiA* gene encodes a protein containing 515 amino acids which include an ER-translocation signal sequence (20 aa) and a 495-aa mature protein. The predicted protein also has two thiol oxidoreductase active sites with a -CGHC- motif and a typical C-terminal-HDEL ER-retention signal (Ngiam et al., 1997). Functional analysis indicated that PDI was induced by the accumulation of unfolded proteins in the ER and overexpression of heterologous proteins. The study also showed the importance of PDI in the refolding of heterologous proteins and consequently, it may play an important role in improving the yield of heterologous proteins in filamentous fungi (Ngiam et al., 2000). The predicted protein sequence of PDI1 of *T. reesei* also contains a 20-aa N-terminal signal sequence and the putative C-terminal ER retention signal HDEL. Furthermore, it shows 60 % similarity to the PDI of *A. niger*. Detailed investigation into *T. reesei* suggested that expression of the *pdi1* gene was induced by ER stress and regulated by the carbon source. The level of *pdi1* mRNA was about 10 times higher when cellulose-containing medium was used instead of glucose medium (Saloheimo et al., 1999). Calnexin is a lectin-like

chaperone that transiently binds to monoglucosylated N-linked glycoproteins in the ER. In *A. niger*, production of a heterologous fungal manganese peroxidase was increased four to five times by overexpression of *clxA*, a gene encoding calnexin (Conesa et al., 2002).

1.2.1.2 ER-associated degradation (ERAD)

ERAD involves selective recognition of aberrant proteins, and export or retrotranslocation of these proteins from the ER to the cytoplasm for their subsequent degradation by the proteasome (Bonifacino and Weissman, 1998). This process is connected to UPR. Accumulation of unfolded proteins or misfolded proteins induces UPR that enhances the expression of genes encoding ER-resident chaperones and foldases in an attempt to fold proteins correctly (Sitia and Braakman, 2003). Terminally misfolded proteins are delivered to the cytoplasm, tagged with ubiquitin and degraded by the 26S proteasome. While the UPR has been intensively studied at a molecular level, the mechanism of ERAD is less well known. Only the *prs12* gene of *T. reesei* that is homologous to the mouse regulatory subunit 12 of the 26S proteasome has been characterized (Goller et al., 1998a). However, similar genes encoding the proteasome subunits can be identified from *N. crassa*, *A. nidulans* and *T. reesei* genome sequence databases. Recently, a master map of the 20S proteasome subunits from *T. reesei* has been generated (Grinyer et al., 2007). Thirteen out of the 14 20S proteasome subunits and several related proteins that co-purified with the 20S proteasome were identified. These data will be valuable for future studies addressing the role of the proteasome in fungal protein quality control.

1.3 The filamentous fungus *Ophiostoma*

Ophiostoma spp. have been found and isolated from a range of geographical areas such as Australasia, North American, Europe, Asia and South Africa (de Beer et al., 2003; Held et al., 2003; Hoffman and Breuil, 2004a; Jacobs and Kirisits, 2003). They are the most frequently isolated species from stained wood. Blue stain, which is grey, black or bluish discoloration of sapwood, is often caused by the presence of pigmented fungal hyphae. Blue staining fungi are typically early colonizers of freshly sawn lumber of many tree

species important for the timber industry. Generally, there are three groups of fungi that cause blue stain of wood: black yeast, dark moulds and species of *Ceratocystis*, *Ophiostoma* and *Ceratocystiopsis* (Seifert, 1993). *Ophiostoma* spp. belong to the *Ophiostomataceae* family, a large group of ascomycetes grouped together because of their evolutionary closeness and phenotypic and ecological similarities (Kim et al., 1999; Upadhyay, 1993). Most *Ophiostoma* species do not compromise the strength properties of wood, but do reduce the aesthetic quality of timber and decrease the economic value of lumber (White-McDougall et al., 1998). The most affected property of wood is the toughness which was reduced up to 30 % in heavily stained pine (Seifert, 1993). *Ophiostomataceae* family also contain a number of pathogens, such as *O. ulmi* and *O. novo-ulmi*, which cause Dutch elm disease (Brasier, 1991; Dorion et al., 1994). Dutch elm disease causes blockage of the water-conducting tissue within the tree and tree will die eventually.

1.3.1 Dimorphism of *Ophiostoma* spp.

Fungal dimorphism refers to an environmentally controlled reversible interconversion of yeast and mycelial morphologies. Both forms (Fig. 2) have been observed in *Ophiostoma* spp. (Kulkarni and Nickerson, 1981). The dimorphism of *O. ulmi sensu lato*, which is the causative agent of Dutch Elm disease, has been extensively studied (Gadd and Brunton, 1992; Kulkarni and Nickerson, 1981; Muthukumar and Nickerson, 1984; 1985). Campana (1978) suggested that the dimorphism of *O. ulmi sensu lato* was essential for the spreading of Dutch Elm disease. Budding yeasts were the major form during passive transportation of the fungus in the individual xylem vessels and the yeast-form cells were converted to the hyphal form to enable penetration from one vessel to another.

A. B.

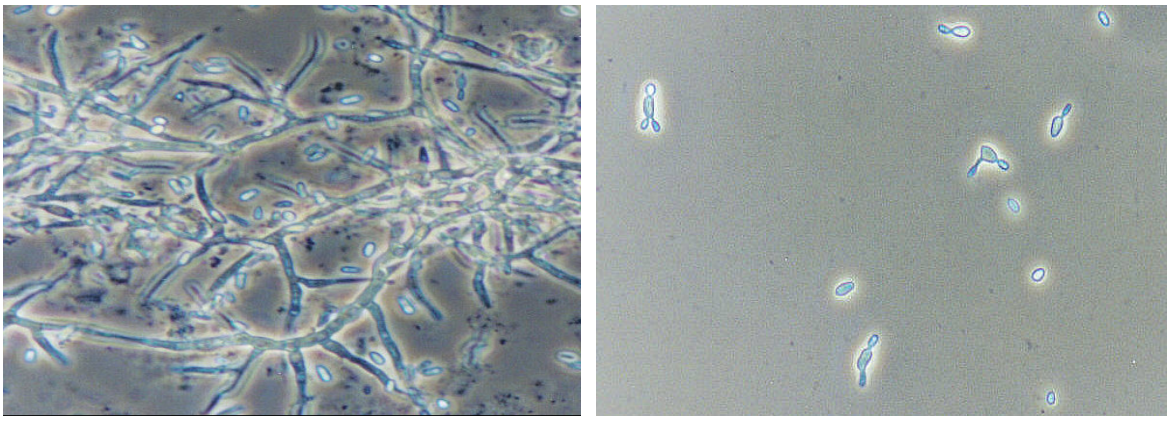

Fig. 2. Dimorphism of *Ophiostoma floccosum*. A. mycelial form, B. yeast like cells. 40x magnification.

Previous studies into factors regulating the yeast-mycelial form in other fungi have revealed that temperature, pH and phosphate transition metal ions can be essential for the conversion from one form to another in the liquid culture. However, Kulkarni and Nickerson (1981) reported that none of the above factors had a reproducible effect on the dimorphism of *O. ulmi sensu lato*. A particular nutritional parameter that controlled the *O. ulmi sensu lato* morphology was the nitrogen source present in a defined liquid medium. Formation of yeast cells was induced using proline as a nitrogen source whereas formation of the mycelial form was induced in a liquid medium containing ammonium ions, arginine or asparagine as a nitrogen source. Studies into the role of calcium in the dimorphism of *O. ulmi sensu lato* provided evidence that it was necessary for the formation of mycelia (Gadd and Brunton, 1992; Muthukumar and Nickerson, 1984; 1985), and that calcium and known calmodulin antagonists were able to reverse the morphology of cells (Muthukumar and Nickerson, 1985). Growth of *O. ulmi* was exclusively yeast-like in the absence of calcium from the culture medium, whereas the mycelial form of *O. ulmi* was detected when calcium was supplied up to a final concentration of 5 mM in a liquid growth medium. Approximately 22 % of the population turned into mycelial form after 28 h with 1 mM Ca^{2+}, which was the maximal concentration for the formation of a mycelial state (Gadd and Brunton, 1992).

1.3.2 Growth of *Ophiostoma* in wood

Colonization and the growth path of *Ophiostoma* spp. in wood have been well characterized. Dispersal of *Ophiostoma* is mediated by bark beetles and other animal vectors, rain splash water flow and air currents (Upadhyay, 1993). Blue staining fungi generally colonize wood during the seasoning or transportation of freshly sawn lumber. There are several factors that influence colonization such as the available nutrients, moisture and oxygen content of wood and the ambient temperature. It has been reported that the minimum moisture percentage for fungal growth in wood is 20 % and the optimum water content for maximal blue stain development is 60-80 % (Seifert, 1993). However, fully saturated wood will not be affected due to the very low concentration of oxygen. The optimal growth temperature for the blue staining fungi is 22 to 30 °C and the growth rate will be reduced if an unsuitable temperature is experienced. Nevertheless, serious blue staining can occur on wood which is stored at a low temperature in the range of 3-8 °C (Seifert, 1993).

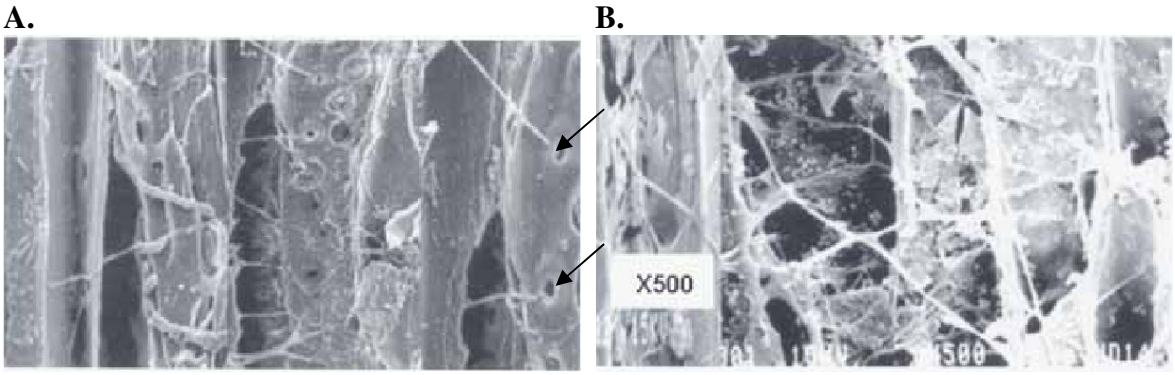

Fig. 3. Growth of *O. piliferum* in conifer wood chips (Croan, 2004). A. The mycelium of *O. piliferum* passes from one cell to the next through bordered pits and tracheids, the arrows point to the border pits. B. Heavy mycelial growth in radial tracheid cells.

A detailed investigation of the process of host invasion by blue staining *Ophiostoma* spp. has been conducted by Liese and Schmid (1961). *O. piliferum* and *O. piceae* were inoculated onto surface-sterilized spruce and blocks of fresh pine wood. Subsequent light microscope studies indicated that tracheids, rays and resin canals were all colonized. However, ray parenchyma, a tissue involved in food storage and radial translocation of sugars and water, was the most favourable path of growth. The growth of *Ophiostoma*

occured via bordered pits or through direct penetration of the wall from tracheid to tracheid (Fig. 3). Penetration was usually through the torus of the pit membrane and involvement of enzymes was not detected in the process. The growth of *Ophiostoma* from cell to cell in the medullary rays was mostly via the pits with some involvement of enzymes to destroy the pit membrane (Croan, 2004; Gibbs, 1993). Ballard and Walsh (1982) studied the colonization and growth of blue staining fungi, dispersed by bark beetles, in the xylem of lodgepole pine using a light microscope. Initially, the fungi were located in the beetle frass of the egg gallery from where they soon spread into the sapwood. Numerous uniserate rays of the sapwood and phloem were the essential paths for the radial fungal development. Fungi grew extensively in the rays and fungal hyphae moved into tracheids once they were established in the xylem rays. Fungal hyphae also attacked the resin canals (Fig. 4, A and B). Even though the growth path and dispersal vectors of *Ophiostoma* spp. in wood have been extensively studied, the interaction among dispersal vectors, *Ophiostoma* and the trees is very complicated and further exploration is needed to understand the biology of these interactions.

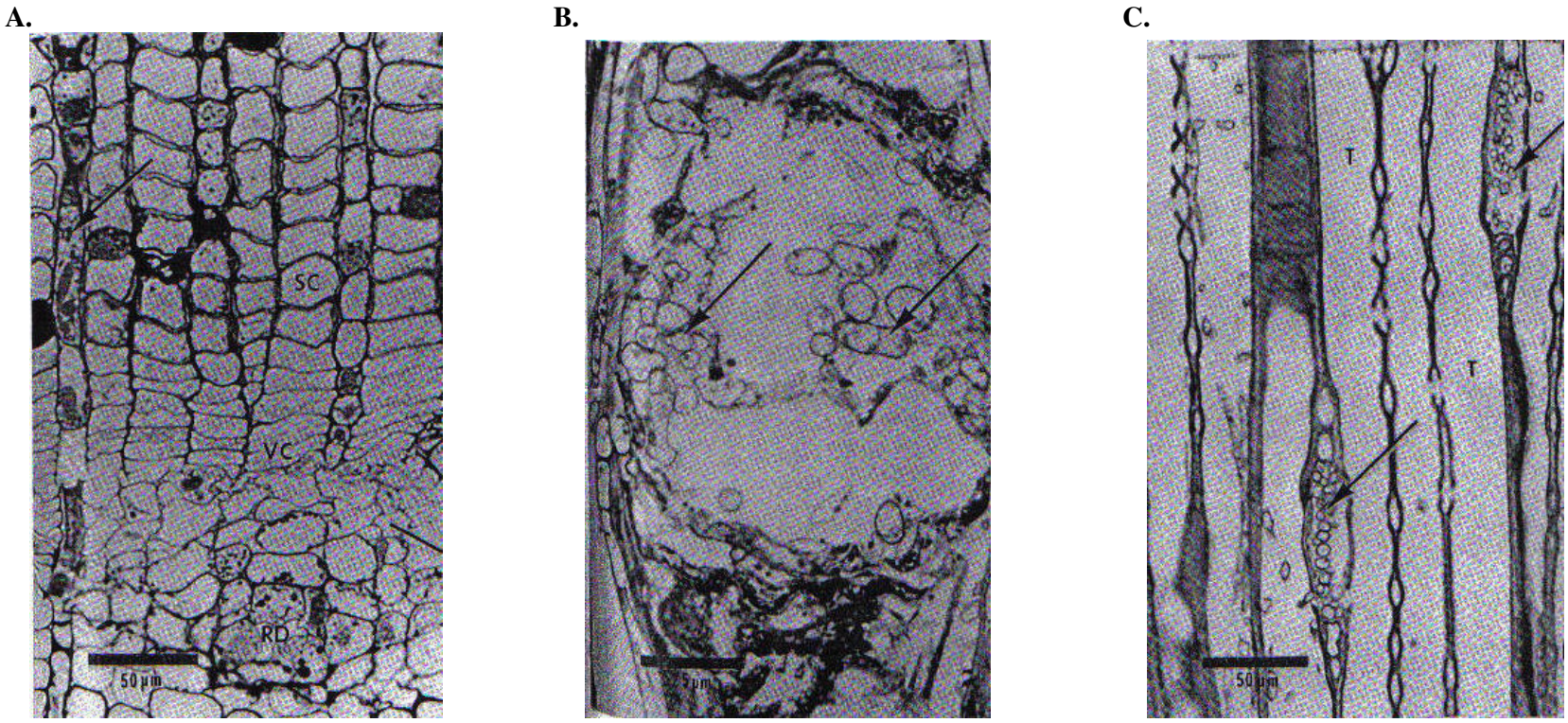

Fig. 4. Growth of blue stain fungi in xylem of lodgepole pine (Ballard and Walsh, 1982). In picture A, "RD" indicates the axial resin ducts. Picture B, tangential view of phloem ray possessing horizontal resin ducts. In picture C, the arrows point to fungal hyphal development through living ray parenchyma cells and "T" indicates axial tracheids.

1.3.3 Enzymes secreted by *Ophiostoma*

In general, *Ophiostoma* spp. do not degrade cellulose and lignin, and it has been shown that starch and soluble sugars in wood are the main carbon sources for their growth (Seifert, 1993). Thus, enzymes such as amylases are important for wood colonization. We have recently isolated and characterized a gene (*amy1*) from *O. floccosum* that encodes an α-amylase (Wu et al., 2006). It has been reported that plant pathogenic *Ophiostoma* spp. can secrete laccase and cellulolytic enzymes to facilitate their establishment and spreading in the wood (Binz and Canevascini, 1997; Przybył et al., 2006).

Trees store most of their nitrogen in proteins and amino acids. The content of easily assimilated nitrogen sources like ammonia is very low in trees and not sufficient to support fungal growth. Therefore, proteinases are essential for the growth of *Ophiostoma* in wood (Abraham and Breuil, 1996; Hoffman and Breuil, 2004a). The first proteinase isolated and characterized from *O. floccosum* was a subtilisin-like proteinase that belongs to the serine proteinase family (Abraham and Breuil, 1996). The purified subtilisin-like proteinase has a molecular mass of 33 kDa and a pI value of 5.6. Its activity was completely inhibited by PMSF and partially inhibited by some other serine proteinase inhibitors including DCI, DFP and AEBSF. Subsequently, the ability of this proteinase to degrade proteins from different origins was investigated. The results indicated it was capable of degrading a wide range of animal and plant proteins as well as proteins isolated from wood (Abraham et al., 1998). Isolation and analysis of genes encoding subtilisin-like proteinases in nine blue staining fungi including *O. ulmi*, *O. novo-ulmi* and *O. floccosum*, revealed the presence of three distinct groups of genes encoding subtilisin-like proteinases: Ofloc1 group, Opic group and Cr group. One gene copy representing each group was isolated from the genome of *O. floccosum* 387N (Hoffman and Breuil, 2002). Two gene copies of the Opic group and one gene copy of the Ofloc1 and Cr groups were found in the genome of *O. floccosum* isolate 55-1 (Hoffman and Breuil, 2004a). Expression analysis of the three genes encoding subtilisin-like proteinases in *O. piliferum* and *O. piceae* indicated that the expression of *opil1* gene (Ofloc1 group) was induced by BSA, but not affected by nitrogen or carbon catabolite repression. The expression of the *opic* gene (Opic group) was also induced by BSA and regulated by nitrogen or carbon catabolite repression. Production of the two subtilisin-like proteinases was regulated by pH. The latter proteinases may play an

important role in nutrient acquisition from wood as their expression responded to exogenous nutrient sources. However, the expression of *opil2* (Cr group) was not affected by the nutrient sources and pH tested. Sequence analysis of *opil2* showed high similarity to the *pepC* gene in *A. niger*, which encodes a vacuolar proteinase. These results suggested that *opil2* may play a housekeeping role (Hoffman and Breuil, 2004a).

Lipase is yet another key enzyme secreted by *Ophiostoma* during the colonization of resin ducts. This enzyme can catalyze hydrolysis of triacylglycerides into free fatty acids and glycerol. It has been shown that these hydrolytic products are important for the synthesis of melanin, a dark pigment produced by *Ophiostoma*. Extracellular lipases secreted by *O. piliferum* and *O. floccosum* have been purified and characterized (Brush et al., 1999; Gao and Breuil, 1998). Lipase purified from *O. floccosum* has a molecular mass of 35 kDa as determined by SDS-PAGE and 37 kDa when measured by size exclusion chromatography. The lipase was stable between pH 4 to 8 at a temperature below 40 °C, and had a pH optimum of approximately 5 and a temperature optimum of 30 °C (Gao and Breuil, 1998).

1.3.4 Applications of *Ophiostoma* in industry

Discoloration caused by blue staining fungi including *Ophiostoma* reduces the economic value of wood. The largest forestry company in New Zealand has estimated that the loss of value of wood caused by blue stain is at least $100 M per year (Vanneste, et al., 2002). Discoloration is due to dark pigmented mycelia that colonize wood and the dark colour within the fungal mycelia is caused by melanins. Chemical treatment has been applied to prevent blue stain during wood processing, storage and transportation. However, only five fungicides have been registered for commercial use: TCMTB (2-thiocyanomethylthiobenzothiazole), borax (sodium borate), copper-8-quinolinolate, 3-iodo-2-propynyl-butyl-carbamate and quaternary ammonium compounds (Seifert, 1993). Unfortunately, none of them is completely effective in preventing blue stain of the wood. Furthermore, problems originating from the use of these chemicals, particularly damage to the environment, have been of concern in many countries (Gao and Breuil, 1998).

The albino strain of *O. piliferum*, commercially named Cartapip™, was the first fungal product that was applied successfully in the pulp and paper industry to reduce pitch in the wood (Farrell et al., 1993). Pitch or wood extractives, which contain fats,

waxes, fatty acids, resins, phenolics, free and esterified sterols and terpenes accumulate during pulping processes, increase the toxicity of effluents and decrease the quality of the final products (George et al., 1999; White-McDougall et al., 1998). Treatment of non-sterilized wood chips after chipping with Cartapip^{TM} decreased the triglyceride content in the wood by 60-80 %. This operation extensively improved the quality of paper and the pulping efficiency, and reduced the effluent toxicity (Farrell et al., 1993; Zimmerman et al., 1995).

Subsequently, an environmentally sourced new approach using a biological control agent to prevent the blue stain instead of chemicals, has been conducted successfully (Behrendt et al., 1995a; b; Blanchette et al., 1992; White-McDougall et al., 1998). This treatment involved application of albino strains of blue staining fungi to freshly cut logs to colonize the sapwood, utilizing the available nutrients and therefore, inhibiting subsequent colonization by other economically harmful blue staining fungi. A range of colourless isolates of *Ophiostoma* spp. featuring *O. floccosum*, *O. piceae* and *O. ips* has been produced and applied as biological control agents to suit various areas and tree species. Some strains including *O. floccosum* significantly reduced the dark sapstain and also extensively degraded wood extractives (Behrendt et al., 1995a; 1995b; Held et al., 2003; Vanneste et al., 2002; White-McDougall et al., 1998).

1.3.5 *Ophiostoma* as a potential expression host

Ophiostoma spp. may provide some advantages over other reported fungal expression hosts. First, *Ophiostoma* can be used in applications requiring the presence of a whole organism. Examples include biological control (as discussed above) and modification of wood from the inside since *Ophiostoma* species are capable of invading wood via ray parenchyma cells (Ballard et al., 1982; Farrell et al., 1993; Gao and Breuil, 1998). Secondly, *Ophiostoma* spp. produce a relatively small number of highly secreted dominant proteins into the liquid culture, which can facilitate the downstream purification of any recombinant gene product made in the system. Dominant enzymes secreted by *Ophiostoma* include proteinases, lipases and amylases (Brush et al., 1999; Gao and Breuil, 1998; Wu et al., 2006). Genes encoding these efficiently secreted proteins provide a potential source for strong promoters for high level gene expression which is an essential requirement for an efficient expression system. Furthermore,

Ophiostoma can grow on a cheap medium, thus reducing the cost for large-scale production of commercially important proteins.

1.4 Requirements for the development of a novel expression system

The unique property of filamentous fungi to secrete copious amounts of proteins outside the cell and the ability to grow on cheap cultivation media make them very attractive for use as expression hosts for a range of valuable proteins (Nevalainen and Te'o, 2003). Currently, the filamentous fungi *T. reesei* and *A. niger* var. *awamori* are amongst the most favoured secretors used in industrial fermentations. There are several new filamentous fungal expression systems under development featuring *Chrysosporium lucknowense*, *Fusarium graminearum*, *A. sojae* and *A. japonicus*. Many attempts to develop new fungal systems have not been discussed in the peer-reviewed literature (Nevalainen et al., 2005).

There are four basic requirements for the development of a novel fungal expression system: i) a high protein-secreting strain; ii) a strong promoter for high level gene expression; iii) a suitable transformation selection marker and iv) an efficient transformation method to introduce exogenous DNA into the fungal genome.

1.4.1 Strategies for strain and yield improvement in filamentous fungi

1.4.1.1 UV mutagenesis to improve the secretion ability of the expression host

Even though improvement of the production of particular proteins is currently carried out by molecular means, the traditional random physical and chemical mutagenesis is still an effective procedure to obtain mutants with increased general secretion ability. Improvement of secretion of extracellular enzymes by random mutagenesis and screening has been successfully carried out with different filamentous fungi (Bailey et al., 1981; Mattern et al., 1992). Many of the high-secreting mutants isolated are currently used for industrial production of fungal enzymes and applied as expression hosts for recombinant gene products (Mäntylä et al., 1998). UV-irradiation is frequently used as the mutagenic agent because of its efficiency and the relative absence of danger to the user compared to some other mutagenic agents such as nitrosoguanidine and gamma irradiation. Bernier and Hubbes (1990) successfully

produced benomyl-tolerant mutants by exposing yeast-like *Ophiostoma* cells to UV light and N-methyl-N'-nitro-N-nitrosoguanidine (Bernier and Hubbes, 1990; 1994).

1.4.1.2 Molecular approaches to improve yields of recombinant proteins in filamentous fungi

The yields of recombinant proteins produced by filamentous fungi have continued to increase with the development of molecular biology and optimization of recombinant technologies. Molecular strategies that have been successfully applied to yield improvement are shown in Table 1. In addition, codon optimisation and overexpression of regulatory factors have also been proven to be useful to improve the production of recombinant proteins in filamentous fungi (Te'o et al., 2000; Van Peij et al., 1998). Among these, strong promoters, gene fusions and protease-deficient host strains have been most efficiently and widely applied. For example, recombinant production of an antibody fragment was improved more than 150-fold by fusing the gene encoding the antibody to the cellobiohydrolase I (*cbh1*) gene and the *cbh1* gene promoter of *T. reesei* (Nyyssönen and Keränen, 1995). Secretion of porcine pancreatic prophospholipase A_2, which was initially undetectable in a proteinase-producing *A. nige*r strain, was increased to around 10 mg/l when the expression cassette was transformed into a proteinase-deficient strain (Roberts et al., 1992). In addition, overproduction of regulatory activators, foldases and/or chaperones has also increased the yields of foreign proteins several fold in particular cases (Table 1; Conesa et al., 2002; Gouka et al., 1977a).

Table 1. Strategies applied to improve the yield of recombinant proteins in filamentous fungi.

Protein	**Fungal host and promoter**	**Strategy**	**Yield**	**Improvement factor**	**Reference**
Fab antibody fragment	*T. reesei*, *cbh1*	Fusion to sequences encoding an	150 mg/l	150 x	Nyyssönen and Keränen, 1995
Human interleukin-6	*A. nidulans*, *alcA*	endogenous protein	>100 mg/l	100 x	Hintz et al., 1995
Endoglucanase	*T. reesei*, *cbh1*	Introduction of multiple copies of the	N/R	3 x	Miettinen-Oinonen and Suominen, 2002
Thaumatin	*A. awamori*, *gdhA*	gene of interest	14 mg/l	N/R	Moralejo et al., 1999
Human interleukin-6	*A. niger*, *gpdA*	Proteinase-deficient host strain	15 mg/l	> 1000 x	Broekhuijsen et al., 1993
Thermophilic xylanase II	*T. reesei*, *cbh1*	Strong expression promoter	0.5 g/l	N/R	de Faria et al., 2002
Hen egg-white lysozyme	*A. niger*, *glaA*		1 g/l	20 x	Jeenes et al., 1993
Feruloyl esterase	*A. niger*, *gpdA*		1 g/l		Record et al., 2003
Bovine prochymosin	*A. awamori*, *glaA*	Application of UV mutagenesis to the transformant	1 g/l	100 x	Dunn-Coleman et al., 1991
Bovine prochymosin	*A. niger* var. *awamori*, *glaA*	Overproduction of	N/R	2.8 x	Valkonen et al., 2003
Laccase	*A. niger* var. *awamori*, *glaA*	chaperones and/or	N/R	7 x	Valkonen et al., 2003
Manganese peroxidase	*A. niger*, *gpdA*	foldases	>80 mg/l	5 x	Conesa et al., 2002
Hen egg-white lysozyme	*A. niger*, *glaA*	Medium optimization	209 mg/l	N/R	Gheshlaghi et al., 2005

Note: N/R indicates that the data was not reported in the respective reference.

1.4.2 Considerations in the construction of expression vectors

An expression vector serves as a vehicle for introducing a specific gene into the target cell for its expression. Basic constituents of an expression cassette are regulatory DNA sequences representing a gene promoter, the driving force for gene expression, and a transcription terminator sequence. In addition, an expression vector contains a transformation selection marker that may, for example, confer resistance to an antibiotic or encode an enzyme to allow usage of a specific substrate such as acetamide as the sole nitrogen source. A multiple cloning site is needed for insertion of the gene to be expressed in a particular system. A secretion signal may be included for secretion of the gene product into the culture medium. Many studies have indicated that foreign proteins are easily degraded intra- and extracellularly by fungal proteinases, decreasing the yield of foreign proteins considerably (Goller et al., 1998b; Gouka et al., 1996; 1997a; Nakajima et al., 2006; Nykänen et al., 2002; Spencer et al., 1998). Therefore, strategies featuring production of the foreign gene product as a fusion to an endogenous, well-secreted protein have been adopted in an attempt to decrease the degradation of the recombinant products by fungal proteinases.

1.4.2.1 Fungal promoters

Overall, promoters are classified into two groups: inducible promoters whose activity is triggered by either chemical or physical factors, and constitutive promoters which allow for continuous transcription of a gene. The inducing factor for promoter activity can be a carbon source, temperature, pH, or a particular metabolite. Currently, the commonly used gene promoters for gene expression in filamentous fungi are the inducible *glaA* gene promoter of glucoamylase A from *A. awamori*, the inducible *cbh1* gene promoter of cellobiohydrolase I from *T. reesei* and the constitutive *gpdA* gene promoter of glyceraldehyde-3-phosphate dehydrogenase from *A. nidulans* (Gouka et al., 1997a; Keränen and Penttilä, 1995; Nevalainen and Penttilä, 2004; Punt et al., 1990). The inducible Taka amylase gene promoter (*taaG2*) from *A. niger*, the inducible acetamidase *amdS* gene promoter from *A. nidulans*, the constitutive pyruvate kinase *pki1* gene promoter and the alcohol dehydrogenase *alcA* gene promoter also have been studied widely (Hynes

and Davis, 2005; Nagata et al., 1993; Schindler et al., 1993; Waring et al., 1989). Recently, the constitutive *hex1* gene promoter isolated from *T. reesei* has been investigated for protein production (Curach et al., 2004). Only the *glaA*, *taaG2*, *gpd* and *amdS* gene promoters will be discussed in the following sections.

1.4.2.1.1 The inducible *glaA* gene promoter from *A. niger* var. *awamori*

The best studied glucoamylase (GA) enzymes are produced by *A. niger* and *A. awamori* (Cornett et al., 2003). Regulation of GA expression occurs at the level of transcription: the synthesis of GA is strongly induced by starch and repressed by xylose (Fowler et al., 1990; Nunberg et al., 1984). The transcription start sites (TSS) of the *glaA* gene are located in the region between -73 and -52 from the start codon and only a further 140 bp is sufficient for transcription initiation. However, sequences required for high-level expression of GA are located in the *glaA* promoter region between -800 and -318 (Fowler et al., 1990; Verdoes et al., 1994). A protein-binding CCAAT motif which enhances transcription of the gene was identified within region I (from positions -464 to – 428) in a DNAase I footprinting experiment (Qiu et al., 2002). Consequently, Liu et al. (2003) demonstrated enhancement of the expression of a heterologous protein in *A. niger* by introducing multiple copies of region I containing the CCAAT-motif. Zhu et al. (2004) confirmed that transcription of the *glaA* gene in *A. niger* was regulated by CCAAT-binding proteins. These proteins have been purified, characterized and named as AngCP1 and AngCP2. These proteins bind to the regions -489 to -414 and -390 to -345, respectively. The *glaA* gene promoter has been applied broadly to drive expression of a variety gene products (Table 2) including enzymes used in industry and gene products of pharmaceutical interest (Schuster et al., 2002).

Table 2. Production of recombinant proteins in *Aspergillus* spp. using the *glaA* promoter.

Protein	**Origin**	**Expression host**	**Yield**	**Reference**
Glucoamylase	*A. niger*	*A. niger*	30 g/l[a]	Finkelstein et al., 1989
α-L-arabinofuranosidase	*A. niger*	*A. niger*	2.48 U/ml[b]	Flipphi et al., 1993
Aspartic protease	*Mucor miehei*	*A. awamori*	1.97 g/l[a]	Ward et al., 1993
Glucose oxidase	*A. niger*	*A. niger*	13.8 U/mg[c]	Whittington et al., 1990
Phytase	*A. niger* var. *ficuum*	*A. niger*	280 U/mg[c]	Van Gorcom et al., 1990
Xylanase	*A. awamori*	*A. niger*	72 kU/ml[b]	Van Gorcom et al., 1991
Prochymosin	Bovine	*A. awamori*	1 g/l[a]	Dunn-Coleman et al., 1991
Hen egg-white lysozyme	Hen	*A. niger*	1 g/l[a]	Jeenes et al., 1993
Human interleukin-6	Human	*A. nidulans*	5 mg/l[a]	Contreras et al., 1991
Human lactoferrin	Human	*A. awamori*	2 g/l[a]	Ward et al., 1995
Prophospholipase A2	Pig	*A. niger*	10 mg/l[a]	Roberts et al., 1992

Note: a, the amount of protein per liter of culture supernatant; b, specific enzyme activity (U) per millilitre of culture supernatant; c, specific enzyme activity (U) per milligram dry weight of mycelia.

1.4.2.1.2 Taka-amylase A (*taaG2*) gene promoter from *A. oryzae*

Taka-amylase A (TAA) produced by *A. oryzae* is an industrially exploited α-amylase. Production of TAA is strongly induced by starch and maltose and repressed by glucose. The *taaG2* gene is the major transcribed gene in *A. oryzae* and regulation of the *taaG2* gene promoter has been studied using *A. nidulans* which has no detectable amylase activity as an expression host (Nagata et al., 1993). Four putative CreA binding sites were located within approximately 400 bp upstream of the transcription start site of the *taaG2* gene and these sites contained sequences related to the CreA binding consensus sequence. The CreA protein which is involved in glucose repression of the *taaG2* gene, bound to three of the CreA binding sites identified in the promoter with high affinity, and one site with low affinity, as indicated by DNase I footprinting (Kato et al., 1996). A CCAAT-binding factor AnCP which is analogous to the HAP complex of *Saccharomyces cerevisiae* (Forsburg and Guarente, 1989; McNabb et al., 1995) acts as an activator in high-level expression of the *taaG2* gene in *A. nidulans* (Kato et al., 1998; Steidl et al., 1999). Another two factors have been investigated, SREB and amyR, that are involved in the induction of the expression of TAA in *A. nidulans*. SREB is referred to as a starch responsive element which binds to two sites located in the *taaG2* promoter regions, -204 to -189 and -182 to -168. A conserved nucleotide sequence, CGGNNATT, has been found at the two binding sites and also in region IIIa of the α-glucosidase *agdA* gene promoter from *A. oryzae* (Tani et al., 2000). AmyR, which is a transcriptional activator for amylase expression, binds to two types of sequences located in a number of promoters of *Aspergillus* genes encoding enzymes degrading starch. One of the sites contains the sequence CGG-N8-CGG in which the two CGG triplets are separated by eight nucleotides and the other site features one CGG triplet followed by the sequence AAATTTAA (Petersen et al., 1999).

1.4.2.1.3 The *gpdA* and *amdS* gene promoters of *Aspergillus* spp.

The constitutive *gpdA* gene promoter of *A. nidulans* (Punt et al., 1990) has been used widely for a range of purposes such as expression of the transcriptional factor ALCR, a fluorescent protein (GFP) to detect fungal colonisation of wood and a number of industrially-useful enzymes (de Vries et al., 1997; Lee et al., 2002; Mathieu et al., 1994;

Record et al., 2003; Rose et al., 2002). The acetamidase *amdS* gene promoter of *A. nidulans* is one of the best characterized gene promoters from filamentous fungi and has been used as a model for understanding fungal promoter regulation (Hynes and Davis, 2005).

1.4.2.2 Selection markers

The most commonly used selection markers for fungal transformation include auxotrophic markers and genes conferring antibiotic resistance (Table 3). Genes such as *argB* and *pyr4* are widely used to complement corresponding auxotrophic mutants that are deficient in the metabolism of arginine or uracil, respectively. A drawback of this type of approach is the need for auxotrophic mutants (Ruiz-Díez et al., 2002). The *amdS* gene which encodes an acetamidase enzyme has been isolated from *A. nidulans* (Hynes et al., 1983) and extensively applied as a selection marker for fungal transformations, including several *Aspergillus* spp., *Penicillium chrysogenum* and *T. reesei* (Debets et al., 1990; Kolar et al., 1988; Michielse et al., 2004; Saarelainen et al., 1997; Tilburn et al., 1983). The acetamidase enzyme allows the fungi to grow on acetamide or acrylamide by using them as a sole carbon and/or nitrogen source. The *E. coli* gene encoding β-glucuronidase (*gus*) (Jefferson et al., 1986) which hydrolyzes colourless glucuronide to produce a coloured product has also been used to transform fungi (Toda et al, 2001). Dominant drug resistance genes are preferred as selection markers for a wide range of fungi and are especially suitable for industrially-applied strains and pathogenic fungi. The *hph* gene from *E. coli* (Gritz and Davies, 1983) encoding a hygromycin B phosphotransferase has been proven to be of great value in the transformation of filamentous fungi including *A. niger*, *T. reesei*, *O. piceae* and *O. ulmi* (Hoffman and Breuil, 2004b; Mach et al., 1994; Punt et al., 1987; Royer et al., 1991; Tanguay and Breuil, 2003). The *kan* gene, which encodes resistance to the antibiotic G418, has also been applied to several fungi such as *N. crassa*, *Cephalosporium acremonium* and *Phycomyces blakesleanus* (Bull and Wootton, 1984; Penalva et al., 1985; Revuelta and Jayaram, 1986). Genes conferring antibiotic resistance have been expressed under a variety of constitutive fungal promoters and the expression cassettes have also included terminator regions to ensure proper transcription termination.

Table 3. Examples of selection markers available for fungal transformation.

Selection marker	**Function**
argB, *pyr4*	Complementation of auxotrophic mutation
amdS	Ability to use a novel energy source
gus	Colour production: β-glucuronidase hydrolyzes colourless glucuronides to coloured products
hph, *kan*, *benr*	Drug resistance

1.4.2.3 Function of the signal sequence and effects of proteolysis on foreign protein yields

The signal sequence, which will direct the protein to the secretory pathway (Bzymek et al., 2004; Jalving et al., 2000), is a short peptide which usually contains around three to 60 amino acids. The signal sequence can be found either at the N-terminus or the C-terminus of a protein and it is cleaved in the ER during secretion (Bzymek et al., 2004; Díez et al., 2002; Jalving et al., 2000).

In eukaryotes, enzymes involved in proteolytic processing are often calcium-dependent serine proteinases that belong to subtilisin and kexin families. A Kex2-like peptidase activity which cleaves a dibasic peptide sequence Lys-Arg or Arg-Arg has been characterized in many fungi including *T. reesei*, *A. niger* and *N. crassa* (Goller et al., 1998b; Nykänen et al., 2002; Rasmussen-Wilson et al., 1997; Svetina et al., 2000). Dibasic amino acid doublets, the Kex2 target sites, have been shown to predominate as cleavage target sequences in the secretory proteins from filamentous fungi (Calmels et al., 1991).

Although production of recombinant fungal proteins using fungi as cell factories is quite efficient, expression of heterologous proteins of non-fungal origin often results in a yield of only a few milligrams per liter (Fowler and Berka., 1991; Gouka et al., 1996; 1997b). Therefore, many approaches have been taken to improve the production of heterologous proteins in fungal hosts. These include introducing multiple copies of a particular gene, using proteinase-deficient host strains, fusing a DNA sequence encoding a well-secreted endogenous protein to the N-terminal end of a heterologous protein and application of strong promoters (Goller et al., 1998b; Gouka et al., 1996; 1997b; Spencer et al., 1998). It has been established that construction of a fusion is often a successful strategy

to increase the yields of foreign proteins. Routinely, a Kex2 cleavage site will be introduced between the carrier protein and the heterologous protein so that the target protein can be released from the fusion partner (Gouka et al., 1997b; Nakajima et al., 2006). The most common carrier proteins are *A. niger* glucoamylase (GA I) and *T. reesei* cellobiohydrolase I (CBHI) (Broekhuijsen et al., 1993; Goller et al., 1998b; Gouka et al., 1996; 1997b; Nyyssönen and Keränen, 1995). Production of human interleukin-6 (hIL-6) in *A. niger* was significantly improved when a partial GA encoding sequence was fused to the N-terminal end of hIL-6 (Broekhuijsen et al., 1993). Similarly, production of plant α-galactosidase (AGL) as a fusion protein in *A. awamori* was increased by 25-fold compared to the non-fusion approach (Gouka et al., 1997b). These examples amongst a number of other studies have indicated that the gene fusion approach is widely applicable for high level expression of recombinant gene products in filamentous fungi (Gouka et al., 1997b).

1.4.3 Transformation of filamentous fungi

Gene transformation systems are important for the molecular genetic manipulation and investigation of various organisms, including filamentous fungi. Briefly, molecular transformation involves introduction of exogenous DNA into recipient cells. The DNA introduced will be either integrated into the genome of the recipient cells or maintained as an autonomously replicating plasmid. As a general rule, the incoming DNA will integrate into the genome of filamentous fungi. Integration of exogenous DNA into the fungal genome is often ectopic rather than a result homologous recombination.

Current methods for introducing DNA into filamentous fungi include PEG-mediated protoplast transformation, chemical treatment, electroporation, biolistic bombardment and *Agrobacterium tumefaciens*-mediated transformation, reviewed in Olmedo-Monfil et al. (2004).

1.4.3.1 Methods of transformation

Protoplast transformation. A protoplast is a cell from which the wall has been removed either mechanically or enzymatically. Fungal protoplasts can be prepared from intact

conidia, germinating conidia, young hyphae or yeast-like cells. The choice of cell type depends on the particular fungus of interest (Buxton and Radford, 1983; Rossier et al., 1985; Ruiz-Díez, 2002; Tanguay and Breuil, 2003). A major factor for the successful preparation of protoplasts is the choice of a suitable lysing enzyme to degrade the cell walls. Several commercially available enzyme cocktails have been applied to produce protoplasts; these include a mixture of helicase and glusulase from the snail gut, Zymoylase T100 (Seikagaku Corp., Japan) produced by *Arthrobacter luteus* and Novozyme 234 (Novozymes, Denmark) that contains 1,3-glucanases and chitinases (Olmedo-Monfil et al., 2004; Ruiz-Díez, 2002). Among all these products, Novozyme 234 produced by the fungus *Trichoderma viride* is the most common lysing enzyme used. An osmotic stabilizer is needed to protect the protoplasts due to the absence of cell walls. Sorbitol, magnesium sulphate, mannitol and sodium chloride are the most common osmotic stabilizers, especially sorbitol with a concentration between 0.8 to 1.2 M. Another key factor in protoplast transformation is the calcium-PEG-DNA treatment. It has been reported that calcium ions facilitate the internalization of exogenous DNA and PEG can help with the uptake of DNA (Olmedo-Monfil et al., 2004). Among several PEG varieties, PEG 4000 (40 % v/v polyethylene glycol) has been used frequently in protoplast transformation. The commonly-used concentrations of calcium are 10 mM and 50 mM.

Chemical treatment. An alternative method to avoid protoplast formation is to use a chemical treatment combined with PEG to induce cell permeability. For example, lithium acetate at a concentration of 0.1 M is commonly used. PEG will cause the chemically treated cells to agglomerate which will make the DNA uptake easier. This approach has been successfully performed to transform *N. crassa*, *Coprinus lagopus*, *Humicola grisea* var. *thermoidea* and *Ustilago violacea* (Bej and Perlin, 1989; Binninger et al., 1987; Dantas-Barbosa, et al., 1998; Dhawale et al., 1984).

Electroporation. Electroporation relies on short duration, high amplitude electric fields to induce transient cell permeability. Foreign DNA is taken up by the cells during the change of permeability of the cell membrane induced by an electric pulse. Both intact conidia and protoplasts can be used for electroporation (Olmedo-Monfil et al., 2004; Ruiz-Díez, 2002).

Electroporation of germinated conidia of *A. nidulans* has also been studied (Sánchez and Aguirre, 1996). Ward et al. (1989) used protoplasts of *A. awamori* and *A. niger* for electroporation and the transformation efficiency was similar to that of protoplast transformation. Electroporation combined with PEG was used to transform the osmotically-sensitive cells of *T. harzianum* of whose cell wall had been partially digested (Goldman et al., 1990). The transformation frequency was quite high, up to 400 transformants per µg DNA. Electroporation has been successfully performed to transform *N. crassa*, *P. urticae*, *A. oryzae*, *A. niger* and *T. harzianum* (Chakraborty et al., 1991; Goldman et al., 1990; Ozeki et al., 1994; Sánchez and Aguirre, 1996).

Biolistic transformation. Biolistic transformation was originally developed for the transformation of plant tissues (Sanford, 1990). Consequently, it has been widely adopted to transform bacteria, yeasts, filamentous fungi and animal cells (Olmedo-Monfil et al., 2004). Biolistic transformation involves bombarding target cells with microparticles coated with the transforming DNA. This approach is particularly suitable for fungi for which protoplasts are difficult to obtain or which may show difficulties in the regeneration of protoplasts. Gold or tungsten microparticles are commonly used as a vehicle for introducing DNA into fungal cells. Te'o et al. (2002) transformed *T. reesei* using the Bio-Rad™ seven barrels hepta adaptor system. Several key factors that affected the transformation efficiency were investigated. The results indicated that the vacuum pressure in the bombardment chamber and the distance traveled by the microparticles before hitting the cells played a significant role in relation to transformation efficiency. Transformation efficiencies obtained were about the same with both circular and linear DNA. With the optimization of this technique, biolistic transformation has been conducted to transform a number of filamentous fungi such as *Cryptococcus neoformans*, *T. atroviride* and *A. nidulans* (Gomes-Barcellos et al., 1998; Kingsbury et al., 2004; Rocha-Ramírez et al., 2002).

Agrobacterium-mediated transformation. *Agrobacterium tumefaciens* is a Gram-negative bacterium which has been applied extensively to transfer genes to a variety of plants (Abuodeh et al., 2000). The principle of this method is that the bacterium can transfer a part

of Ti (tumor-inducing) plasmid, T-DNA, which is flanked by 25 bp direct repeats, into plant cells. Subsequently, T-DNA is integrated into the genome of the target cells by a conjugation-like process and the genes in the T-DNA will be expressed. The gene products of a native T-DNA will induce tumor formation in plant cells. A series of *vir* genes in the Ti plasmid play a major role in the process of T-DNA transfer. Expression of the *vir* genes is induced by phenolic acetosyringone. In principle, any genes located in the T-DNA region can be transferred (Li et al., 2000; Olmedo-Monfil et al., 2004). Recently, *Agrobacterium*-mediated transformation has been applied successfully to transform fungi (Abuodeh et al., 2000; Groot et al., 1998; Li et al., 2000; Mullins et al., 2000; Olmedo-Monfil et al., 2004). The great advantage of *Agrobacterium-mediated* transformation is versatility that allows the use of protoplasts, hyphae or spores as the target material for transformation (Mullins et al., 2000). Another important characteristic of this method is that it results in random integration of a single copy of the T-DNA into the host genome in most cases (Olmedo-Monfil et al., 2004).

Restriction enzyme-mediated integration (REMI). REMI is a method to introduce linearized DNA into cells in the presence of a restriction enzyme. The restriction enzyme is capable of generating compatible cohesive ends in the genome and thereby stimulate integration of the foreign DNA into cognate restriction sites in the chromosomes (Casas-Flores et al., 2004; Kahmann and Basse, 1999). REMI appears to increase percentage of single-copy integrations as well as overall transformation frequency. REMI has been widely applied for fungal transformation, expecially for transforming pathogens (Kahmann and Basse, 1999; Maier and Schafer, 1999; Mullins and Kang, 2001; Xu et al., 2005).

1.4.3.2 Transformation approaches applied to *Ophiostoma*

The first transformation method applied to *Ophiostoma* spp. was based on the use of protoplasts of *O. ulmi*, the causal agent of Dutch elm disease, (Royer et al., 1991). The protoplasts were prepared from yeast-like cells which were digested with Novozyme 234 in the presence of 2-mercaptoethanol. For the transformation, 50 mM $CaCl_2$ and 66 % PEG 4000 (w/v) were used and 0.6 M sucrose was added to the plates as an osmotic stabilizer. Both circular and linear DNA were used and the results showed that the transformation

efficiency was two- to four-fold higher when the DNA was linearized. The ratio of stable to unstable transformants was also higher with linear DNA. The most likely explanation for a higher transformation efficiency using linear DNA was increase of integration of the DNA into the fungal genome rather than improvement of the DNA uptake (Royer et al., 1991). *Ophiostoma piceae* and *O. quercus* were transformed by protoplast transformation and an even higher transformation efficiency, around 4.8 $x10^5$ transformants per µg DNA, was reported (Wang et al., 1999). *Agrobacterium tumefaciens*-mediated transformation has also been applied to transform *Ophiostoma* spp. *Agrobacterium tumefaciens* strains EHA105 and AGL-1 were used as carriers and four binary Ti vectors, which contained the *hph* gene under various fungal promoters and with or without the *A. nidulans trpC* terminator, were investigated (Tanguay and Breuil, 2003). This study indicated that the methodology was applicable for introducing foreign DNA into the genome of *Ophiostoma* spp. Another observation in the study was that a single copy of the incoming DNA was integrated in the genome in most transformants, which is a characteristic feature of *Agrobacterium*-mediated transformation (Tanguay and Breuil, 2003). *Agrobacterium tumefaciens*-mediated transformation was successfully conducted to disrupt the subtilase gene (*albin1*) in the genome of *O. piliferum*. Disruption of *albin1* gene resulted in alteration of the phenotype of transformants which decreased the staining of wood. Furthermore, production of extracellular proteinases was impaired and the ability of the transformants to grow on wood was also severely affected (Hoffman and Breuil, 2004b).

1.4.4 Reporter genes

Reporter genes encode proteins commonly used to investigate gene expression, expression enhancers and the efficiency of gene delivery systems. In addition, reporters can be used to trace proteins in the secretion pathway and define protein localization. Reporter genes can be directly expressed under the promoter sequences of interest or fused to the gene encoding a protein of interest. The most commonly used reporter genes are shown in Table 4.

Table 4. The commonly-used reporter genes.

Reporter gene	**Protein encoded**
lacZ (*E. coli*)	β-galactosidase
gfp (*Aequorea victoria*)	Green fluorescent protein
gus (*E. coli*)	β-glucuronidase
luc (*Photinus pyralis*)	Luciferase
DsRed (*Discosoma* spp.)	DsRed protein variants

The *lacZ* gene is one of the three structural genes of the *lac* operon of *E. coli* (Kalnins et al., 1983). Selection is based on the detection of the colored product that is produced by hydrolysis with β-galactosidase encoded by the *lacZ* gene. GFP is a fluorescent protein which turns green under blue light. It was found and characterized from the luminescent jellyfish *Aequorea victoria* by Shimomura in 1960 and the gene encoding GFP was isolated by Prasher in 1992 (Prasher, 1992). Shortly after this, Chalfie et al. (1994) used it as a reporter to study the gene promoter of *Caenorhabditis elegans* (Chalfie et al., 1994). Since then, GFP has been widely applied to study gene promoters, gene expression and protein secretion in a wide range of organisms (Ross-MacDonald et al., 1997; Santerre Henriksen et al., 1999). The gene encoding GUS was isolated from *E. coli* and has been used as a gene-fusion marker, especially in plant molecular biology (Jefferson et al., 1986; Toda et al., 2001). Lee et al. (2002) used the green fluorescent protein as a biomarker to monitor *Ophiostoma* fungi in wood and the results indicated that *gfp* could be successfully expressed in *Ophiostoma* spp. The β-glucuronidase enzyme hydrolyzes colourless glucuronides to coloured products. Luciferase is an enzyme that catalyzes oxidation of luciferin which results in production of light. Photon emission can be detected by a light sensitive apparatus such as a luminometer (Mankin et al., 1997). Among the reporter systems discussed above, GFP is particularly common in fungal biology (Bell et al., 1999; Du et al., 1999; Maor et al., 1998; Valdivia and Falkow, 1996). The unique quality of GFP is that it absorbs light at 238-395 nm and emits light at a maximum of 508 nm. Therefore, detection of GFP only requires UV light and oxygen, whereas the other reporter systems mentioned above rely on cofactors or substrates for activity (Lorang et al., 2001). Recently, a red fluorescent protein, DsRed discussed below in more detail, has increased its versatility as a fluorescent reporter (Garcia-Parajo et al., 2001.).

1.4.4.1 The fluorescent protein DsRed

The gene encoding DsRed was isolated and characterized from the Indo Pacific reef coral *Discosoma* spp. by Matz et al. (1999). It shows 26-30 % identity to *Aequorea* GFP. However, the excitation and emission maxima of the DsRed protein are at 558 nm and 583 nm, respectively. The emission of the DsRed protein is amongst the longest wavelength emissions reported so far for a wild-type fluorescent protein (Baird et al., 2000; Garcia-Parajo et al., 2001). The emergence of DsRed has contributed to many studies, particularly fluorescence resonance energy transfer (FRET) (Garcia-Parajo et al., 2001). Many studies have confirmed that DsRed is stable under harsh pH conditions and extremely resistant to photobleaching (Baird et al., 2000; Garcia-Parajo et al., 2001). These unique properties make DsRed a versatile reporter in multiple labelling experiments, for example, a partner for GFP in FRET experiments and protein localization (Garcia-Parajo et al., 2001; Mizuno et al., 2001). However, there are two drawbacks in the use of DsRed: a slow and potentially incomplete maturation of the protein to emit red fluorescence and its tendency to aggregate (Bevis and Glick, 2002; Mikkelsen et al., 2003). To overcome these deficiencies, different DsRed mutants that contain various amino acid substitutions have been developed. These substitutions have improved protein maturation and reduced the tendency of DsRed to aggregate (Mikkelsen et al., 2003). The DsRed1-E5 mutant commercialised as fluorescent timer (BD Biosciences Clontech, USA) had two substitutions, V105A and S197T, which increase its fluorescence intensity and provide a distinct spectral property. The S197T substitution is responsible for a spectral shift in the maturing fluorophore, so that the emitted fluorescence changes from green (excitation at 483 nm, emission at 500 nm) to red (excitation at 558 nm, emission at 583 nm) over time (Terskikh *et al.*, 2002). These unique characteristics of DsRed-E5 make it attractive to be used as a fluorescent clock to study promoter activity and gene expression. In this study, the mutant DsRed1-E5 fluorescent timer was applied to monitor promoter activity.

1.4.4.2 Thermostable xylanase B

Xylanases are glycanase enzymes which hydrolyze the linear polysaccharide beta-1,4-xylan into xylose. Xylanases have been isolated from many micro-organisms including bacteria, filamentous fungi and yeasts (Amoresano et al., 2000; Heck et al., 2002;

Rezende et al., 2002). A gene encoding the thermostable xylanase B has been isolated from *Dictyoglomus thermophilum* (Morris et al., 1998). The enzyme has temperature and pH optima of 85 °C and pH 6.5, respectively. A synthetic *xynB* gene coding for the catalytic domain of the *D. thermophilum* xylanase B has been constructed by modifying the codon usage to facilitate its expression in the filamentous fungus *T. reesei* (Te'o et al., 2000). Thermostable xylanase activity was detected in several transformants which indicated the potential to successfully produce the thermostable xylanase in a fungal host (Te'o et al., 2000).

1.5 Aims

The broad aim of this study was to develop *Ophiostoma floccosum* as a novel fungal expression system. There are two main reasons for choosing *O. floccosum* as model organism: i) *O. floccosum* is a relatively well studied non-pathogenic fungus compared to other sapstaining *Ophiostoma* spp. (Abraham et al., 1993; 1998; Abraham and Breuil, 1996; Hoffman and Breui, 2004; Wang et al., 1999) and ii) it has showen potential for industrial application as a biological control agent (Held et al., 2003). There were four major tasks to this project: i) improvement of the secretion ability of *Ophiostoma*; ii) identification and isolation of strong promoters for high level gene expression; iii) identification of suitable selection marker (s) for fungal transformation; and iv) development of efficient transformation protocol (s). Within this framework, the following procedures were also performed: profiling of proteinase activities in the culture supernatants of the parental strain and several selected mutants, construction of expression vectors for introducing the gene of interest into the fungal genome and testing the novel expression system using a thermostable xylanase B as a reporter.

2. Materials and Methods

The detailed materials and methods have been outlined in the appropriate publications (Table 5). The basic materials and methods used in this study are described in this chapter. All the chemicals used in this work were form Sigma (USA), unless otherwise specified.

Table 5. Materials and methods described in publications I –III.

Material and Method	**Publication**
Fungal strain	I, II, III
Induction and isolation of mutant strains by UV mutagenesis	I
Protein assay	I, II, III
Amylase assay	I
Lipase assay	I
General proteinase assay	I
SDS-PAGE	I, II, III
N-terminal sequencing	I, II
DNA sequencing	I
Southern blotting	III
Northern blotting	I, III
Extraction of fungal genomic DNA	I, III
Genomic walking PCR	I
Isolation of fungal total RNA	I, III
Amplification of the cDNA of α-amylase by RNA ligase mediated rapid amplification of cDNA ends (RLM-RACE) PCR	I
Construction of expression vectors pOAMY20 and pOAMYss	III
Protoplast transformation	III
Zymogram activity gel	III
Synthesis of a trifunctional (TriPS) probe	III
Stability of the TriPS probe under denaturing conditions	III
Sample preparation and assay for the detection of active serine proteinase by TriPS probe	III
Sample labeling and detection using TriPS probe	III

2.1 Selection of culture media

In addition to the general liquid cultivation medium described in publications I-III, several other carbon sources combined with soybean flour as a nitrogen source with minimal salts at pH 5.5 were studied. These were 2 % (w/v) Avicel cellulose, 1 % (v/v) lactose and 1.5 % (w/v) soy bean flour (ALS); 2 % Avicel cellulose and 1.5 % soy bean

flour (AS); 1 % lactose and 1.5 % soy bean flour (LS); 2 % (w/v) cellobiose, 1 % lactose and 1.5 % soy bean flour (CLS); 2 % cellobiose and 1.5 % soy bean flour (CS) and 3 % soluble starch and 1.5 % soy bean flour (SS). Cultivation using different pHs were carried out using SS medium with the pH adjusted to 5.5, 6.5 and 8 by addition of KOH (10 M).

2.2 Proteinase assays using fluorescent substrates

Specific proteinase activities were assayed by using substrates and reference materials listed in Table 6. Chymotrypsin-, elastase-, subtilisin- and trypsin-like proteinase activities, cysteine proteinase, metallo proteinase and aspartic proteinase activities were measured, with some modifications, as described (Novillo et al., 1997; Bieth et al., 1974; Markaryan et al, 1996; Leytus et al., 1983; Powers et al., 2000; Santana et al., 1997; Takeuchi et al., 1988) respectively.

Chymotrypsin-, elastase- and subtilisin-like proteinase activities were measured in 0.1 mM Tris-HCl buffer pH 8.0 containing 5 mM $CaCl_2$. Trypsin-like, cysteine, metallo and aspartic proteinase activities were determined in 67 mM sodium phosphate buffer (pH 7.6); 0.1 M phosphate buffer (pH 6.5) containing 20 mM EDTA and 50 mM L-cysteine; 50 mM tricine buffer (pH 7.5) with 10 mM $CaCl_2$ and 400 mM NaCl; and 1 M phosphate buffer pH 8.0, respectively. All assays were incubated at 37 ºC for 10 min and conducted in duplicate. All reaction mixtures except that for the chymotrypsin-like proteinase included 0.05 mM of substrate, 50 µl of sample and 140 µl of reaction buffer. The fluorescence was determined by a fluorimeter (FLUOstar Galaxy, BMG LabTechnologies, Germany) with the excitation and emission wavelengths of 360 nm and 460 nm, respectively. To assay for chymotrypsin-like proteinase activity, the reaction mixture contained 50 µl of culture supernatant, 200 µl of reaction buffer and 0.05 mM of substrate. The reaction was stopped by the addition of 500 µl of 30 % (v/v) acetic acid. The reactions were centrifuged at 16,000 *g* for 5 min and the absorbance measured at 410 nm.

Table 6. Specific proteinase substrates and commercial pure reference enzymes used in this study.

Substrate	**Specificity**	**Positive control**
	Serine type:	
N-Benzoyl – L – Tyrosine –p – nitroanilide	Chymotrypsin	Bovine pancreas alpha-chymotrypsin
N-Suc-Ala-Ala-Ala 7-amino-4-methylcoumarin	Elastase	Elastase Type 1 from porcine pancreas
N-Suc-Ala-Ala-Pro-Phe 7-amino-4-methylcoumarin	Subtilisin	Subtilisin A from *Bacillus* sp.
NA-CBZ-L-arg 7-amino-4-methylcoumarin	Trypsin	Trypsin from bovine pancreas
Na-CBZ-Arg-Arg 7-amino-4-methylcoumarin	Cysteine	Papain from *Papaya latex*
N-Suc-Gly-Pro-Leu-Gly-Pro-7-amino-4-methylcoumarin	Metallo	Collagenase from *Clostridium histolyticum*
Boc-Leu-Ser-Thr-Arg -amino-4-methylcoumarin (Pettide Institute, Japan)	Aspartic	Pepsin from porcine gastric mucosa

2.3 Choice of suitable selection markers

A series of selection markers used for fungal transformation in previously published studies was tested for *Ophiostoma* transformation. These included *amdS* encoding acetamidase, *kan* encoding G418 resistance and *hph* encoding hygromycin B resistance. Sensitivity of *Ophiostoma* to these and several other antibiotics was tested.

2.3.1 Sensitivity of parental strain J2026 and mutant MQ.5.1 to different antibiotics

Sensitivities of *O. floccosum* J2026 and MQ.5.1 to different antibiotics were screened. Spores (10^7, 7-day old) prepared as in publication I were spread onto PDA plates containing each appropriate antibiotic (Table 7). Test plates prepared in triplicate were incubated at 28°C for over two weeks.

Table 7. Different antibiotics and their concentrations used to screen the sensitivity of J2026 and MQ.5.1.

Strain / Antibiotic	J2026	MQ.5.1
G418 (μg/ml)	10	N/A
	30	N/A
	50	N/A
	80	N/A
	90	90
	100	100
	120	120
	150	N/A
Hygromycin B (unit/ml)	50	N/A
	100	N/A
	120	120
	150	150
	160	160
	180	N/A
	200	N/A
	250	N/A
Puromycin (μg/ml)	50	N/A
	100	N/A
Phleomycin (μg/ml)	50	N/A
	100	N/A

Note: "N/A" indicates not applied.

2.3.2 Capability of *Ophiostoma* to utilize acetamide as a sole nitrogen source

About 10^7 conidia from J2026, MQ 1.2 and MQ 5.1 were spread on minimal plates containing acetamide as the sole nitrogen source. The plates contained (per liter): 15 g KH_2PO_4, 10 mM acetamide, 12.5 CsCl, 12 g Agar (Difico), 10 ml 100 x Trace elements, 2.4 ml 1 M $MgSO_4$, 5.4 ml 1M $CaCl_2$ and 2 % (w/v) Glucose. All cultures were incubated at 28 °C for up to two weeks.

2.4 Construction of the expression vectors

All expression vectors constructed in this study contained the α-amylase gene promoter, signal sequence and transcription terminator sequence. The first expression vector, pOAMY20, was constructed by Dr. Junior Te'o (III). All other vectors described in this work and shownin Fig. 5 were constructed based on pOAMY20.

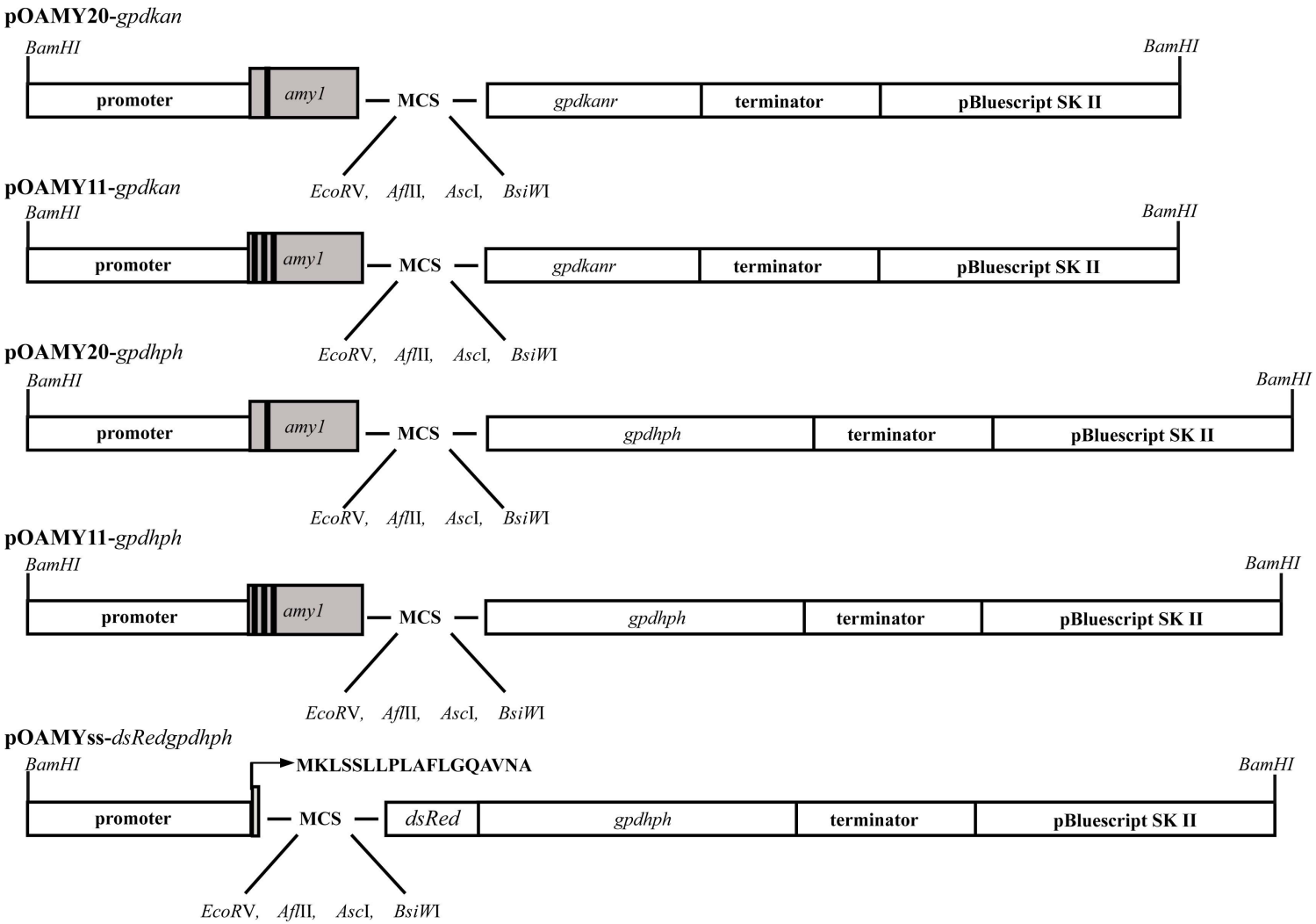

Fig. 5. Schematic diagram of expression vectors constructed in this study. "*amy1*", the gene encoding α-amylase of *O. floccosum*; "MCS", multiple cloning site; "*gpd*", glyceraldehyde-3-phophate dehydrogenase gene promoter from *Aspergillus nidulans*; "*kan*", the gene encoding aminoglycoside-3-phophotransferase, "*hph*", the gene encoding hygromycin phosphotransferase. Black boxes present the introns located in the *amy1* encoding region.

2.4.1 Construction of plasmids containing the G418 resistance gene (*kan*) under the *gpdA* promoter

The *gpd* promoter was amplified from plasmid pAN7-1 (Punt et al., 1987; Wang et al., 1999) using primers gpdBsiWI.for and gpdfusion.rev (Table 8). The gene encoding G418 resistance was amplified from plasmid pCWK1 (Walsh et al., 1998) and fused to the *gpdA* gene promoter using overlapping PCR. First, equal amount of the two gene fragments (100 ng of each), 25 µM dNTPs, 1 x buffer, 1 unit of Triple Master polymerase and H_2O to 25 µl were mixed and subjected to the following PCR conditions: 1 x (94°C, 2 min) and 15 x (94°C, 30 s; 60°C, 30 s; 72°C, 1 min). The DNA mixture was purified using the Qiagen PCR purification kit. The final PCR step was carried out using 2 µl of the above mixture as template, together with 100 ng of primers, 25 µM dNTP mix, 1 x buffer, 1 unit Triplemaster polymerase (Eppendorf, Australia) and H_2O to 50 µl. The reaction conditions were 1 x (94°C, 2 min) and 35 x (94°C, 30 s; 70°C, 20 s; 72°C, 2 min) and 1 x (72°C, 7 min). The fused gene fragment was inserted into the PCR 2.1 TA cloning vector (Invitrogen, USA) and amplified in *E. coli* DH5α. The plasmids PCR 2.1-*gpdkan* and pOAMY20 were digested with *BsiWI* and *PmeI* and ligated together, generating the recombinant vector pOAMY20-*gpdkan*. The expression vector pOAMY11-*gpdkan* was constructed based on pOAMY20 and contains the α-amylase gene promoter, partial α-amylase with all three introns and the α-amylase transcription terminator region (Fig. 5).

2.4.2 Construction of plasmids containing the hygromycin B resistance gene (*hph*) under the *gpdA* promoter

DNA fragment that contained the *gpdA* gene promoter and *hph* gene (*gpdhph*) was amplified from plasmid pAN7-1 and purified using the Qiagen PCR purification kit. The purified *gpdhph* PCR products were digested with restriction enzymes *BsiWI* and *PmeI*. The digested *gpdhph* DNA was inserted in such a way as to replace the *gpdkan* fragments in vectors pOAMY20-*gpdkan* and pOAMY11-*gpdkan*. The newly constructed expression vectors were named as pOAMY20-*gpdhph* and pOAMY11-*gpdhph*, respectively (Fig. 5).

2.4.2.1 Amplification of gene encoding DsRed and screening the functionality of the DsRed protein

The gene encoding DsRed was amplified from plasmid pHEN54RQ-*dsRed* (kindly provided by Dr. Curach) which was originally cloned from the pTimer plasmid (BD Biosciences Clontech, USA). The functionality of DsRed was tested in *E. coli* DH5α before inserting the gene into the *amy1* promoter-driven expression vectors. The *dsRed* gene was amplified by PCR, and digested with *BamHI* and *HindIII* and inserted into the pUC19 plasmid and transformed into *E. coli* DH5α. Transformants were inspected under a fluorescence microscope. The green and red fluorescence visualized indicated expression of a functional fluorescent protein.

2.4.2.2 Insertion of the gene encoding DsRed into expression vectors pOAMY20-*gpdhph* and pOAMY11-*gpdhph*

The gene coding for the *dsRed* fragment was excised from pUC19 and inserted into expression vectors pOAMY20-*gpdhph* and pOAMY11-*gpdhph* to generate recombinant plasmids pOAMY20-*dsRedgpdhph* and pOAMY11-*dsRedgpdhph*. Recombinant plasmid pOAMYss-*dsRedgpdhph* was obtained by replacing the partial α-amylase gene and its promoter region in pOAMY11-*dsRedgpdhph* with a gene fragment containing the α-amylase signal sequence and the α-amylase promoter region.

Table 8. Primers used to construct recombinant plasmids.

Primers	**5'-3'**	**Application**
gpdBsWiI.for	ACCATGCGTACGAATTCCCTTGTATCTC	amplify *gpd* promoter
gpdfusion.rev	TATGGCTCATGGTGATGTCTGCTCAAGCGG	amplify *gpd* promoter
kanfusion.for	AGACATCACC ATGAGCCATATTCAACGGG	amplify *Kan* gene
kanMssI.rev	CCAATTAGTTTAAACTTATTAGAAAAACTCATCGAG CAT	amplify *Kan* gene
hphMssI.rev	GTCGGCGTTTAAACTATTCC TTTGCCCTCGGAC	amplify *hph* gene
dsRedXbaI.for	GCTCTAGAGCACGTGATGGTGCGCTCCTCCAAGAAC	amplify *dsRed* gene
dsRedEcoI.rev	CGGAATTCCGGGCCCTACAGGAACAGGTGGTGGCG	amplify *dsRed* gene
XynBHindIII.for	GCCTGGAAGCTTGGATATCCAGACGTCTATCACCCT AACC	amplify *XynB* gene
XynBBamHI.rev	GAGGCCGGATCCTTAAGCTATTAGAAGGTGTTCTGG GTG	amplify *XynB* gene

2.5 Fungal transformation

2.5.1 Biolistic transformation

Biolistic transformation was carried out as described previously in Te'o et al. (2002) with some modifications. Freshly harvested conidia (7- to 21-day old, $5x10^7$) were spread on 20 ml PDA plates for hygromycin B or G418 selection or minimal plates containing acetamide and allowed to dry. A summary of the different DNAs used for biolistic transformation are shown in Table 9. Around 50 mg of M-10 tungsten or gold particles (Bio-Rad, USA) was washed three times with 1 ml absolute ethanol and twice with Milli Q H_2O. The particles were resuspended in 1 ml MilliQ H_2O. For each transformation, 10 µl of particles was coated with 1-15 µg of DNA.

Table 9. Plasmids used to transform *Ophiostoma floccosum*.

Plasmid	**Selection marker**	**Ref.**
pHEN11	*amdS*	Te'o et al., 2000
pAN7-1	*hph*	Wang et al., 1999
$pRLM_{EX}30$	*hph*	Mach et al., 1994
PCR 2.1-*gpdkan*	*kan*	This work
pOAMY11-*gpdkan*	*kan*	This work
pOAMY20-*gpdkan*	*kan*	This work
pOAMY11-*gpdhph*	*hph*	This work
pOAMY20-*gpdhph*	*hph*	This work
pOAMY11-*dsRedgpdhph*	*hph*	This work
pOAMY20-*dsRedgpdhph*	*hph*	This work
pOAMYss-*dsRedgpdhph*	*hph*	This work
pOAMY11-*xynBgpdhph*	*hph*	This work
pOAMYss-*xynBgpdhph*	*hph*	This work

Note: *amdS*, gene encoding acetamidase; *kan*, gene encoding G418 resistance and *hph*, gene encoding hygromycin B resistance.

The Bio-Rad gene gun system was sprayed with 70 % ethanol prior to use. The macro-carrier discs were dipped in absolute ethanol and air dried. The rupture discs at 650 psi, 900 psi, 1100 psi and 1350 psi were washed in absolute ethanol before use.

Subsequently, bombarded plates were incubated at 28 °C for 4-6 h and overlaid with 10 ml of molten PDA each containing different antibiotic (450 units/ml of hygromycin B or 300 µg/ml of G418). The final antibiotic concentrations were 150 units/ml for hygromycin B and 100 µg/ml for G418, respectively. Plates were incubated at 28 °C for up to 2-3 weeks. Potential transformants obtained from transformation with plasmids containing the *hph* gene were picked and transfered onto PDA plates

containing 200, 400, 600 and 800 units/ml of hygromycin B. The transformants which grew on the selection plates containing over 400 units/ml of hygromycin B were selected and their genomic DNAs were isolated and used for PCR analysis. In transformations with the plasmid containing the *amdS* gene, the plates were incubated at 28 °C after bombardment for up to two weeks.

2.6 Detection of the presence of DsRed using fluorescence microscopy

Potential transformants obtained by transforming with a circular vector pOAMY20-*dsRedgpdhph* or pOAMYss-*dsRedgpdhph* were incubated in liquid starch-soybean flour medium and samples were taken after 3, 4, 5 and 6-day incubation. The samples were fixed by adding paraformaldehyde to 4 % (v/v) and left in the fume hood for 1 h at room temperature. The cells were pelleted by centrifugation at 13,000 rpm for one min, washed three times using 1x PBS and resuspended in PBS. Wells were created on microscope slides with a silicon pen, coated with 0.1 % polyethyleneimine and air dried. Cells were added into the wells on the slides and left to settle for 10 min in the dark. The excess liquid was removed with filter paper and the slides were mounted by adding a small drop of the aqueous mountant containing an anti-fade agent. The slides were covered with cover slips and kept in the dark. Samples were visualized using fluorescence microscopy (BH2-RFCA, Olympus, Japan).

3. Results and Discussion

3.1 Improvement of the secretion ability of *O. floccosum* strain J2026 (I)

3.1.1 Selection of culture medium

One of the major aims of this project was to improve the secretion ability of *O. floccosum*. It has been established in a previous research with other fungi that screening for increased total protein concentration under optimized culture conditions provides a good guide for increased secretion capability of the organism (Finkelstein et al., 1989; Nevalainen, 1981). In a lead up to this study, several culture media and conditions were investigated to obtain high yields of secreted protein from *Ophiostoma* (unpublished data). Secreted proteins in the culture supernatants were separated and visualized on 12.5 % (w/v) SDS-PAGE and the protein amount and profiles were compared. The highest extracellular total protein secretion was achieved at pH 6.5 with soluble starch as a carbon source and soybean flour as nitrogen source. Starch is the key nutrient for sapstaining *Ophiostoma* spp. to support their growth in ray parenchyma cells, the most favourable path for *Ophiostoma* growth in the wood. Thus, a medium containing 3 % (w/v) soluble starch and 1.5 % (w/v) soybean flour was used for growing *Ophiostoma* in liquid culture.

3.1.2 Comparison of secretion between *T. reesei* wild type QM6a and *O. floccosum* J2026

To initially assess the potential of *Ophiostoma* to be developed as an efficient protein secretor, the wild type *O. floccosum* J2026 and a wild type *T. reesei* QM6a, which is the initial parental strain of a series of high protein-secreting *T. reesei* mutants, (Bailey and Nevalainen, 1981) were cultured in a liquid medium containing 3 % soluble starch and 1.5 % soybean flour. Proteins secreted into the culture medium from day 1 to day 5 were separated and profiles compared by 12.5 % SDS-PAGE (Fig. 6). Protein secretion by both strains was clearly increasing from day 2 to day 5 (Fig. 6, A, lane 2 to 5 and B, lane 3 to 6). A smear was observed in lane 1 (Fig. 6, A) and 2 (Fig. 6, B) which was due to the presence of soybean flour in the culture medium. Soybean flour contains a large amount of proteins which were visualized by SDS-PAGE (Fig. 6, B, lane 1).

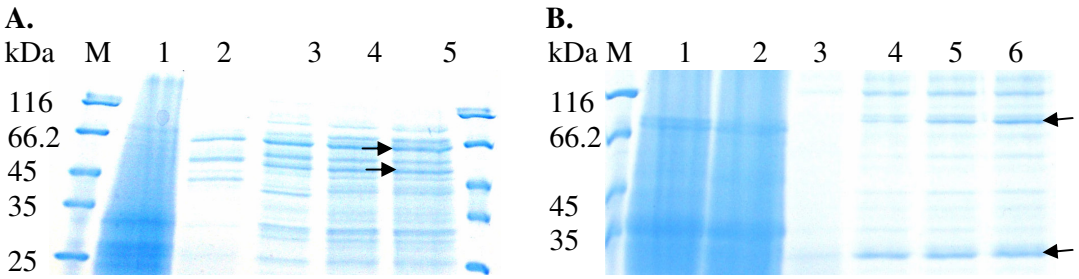

Fig. 6. Analysis of secreted proteins from *T. r*eesei QM6a and *O. floccosum* J2026 by 12.5 % SDS-PAGE. Each lane contained 60 µl of culture supernatant. A. QM6a. M, molecular weight marker; lane 1 to lane 5, culture supernatants from day 1 to day 5. B. J2026, M, molecular weight marker; lane 1 to lane 5, culture supernatants from day 1 to day 5. Arrows point to abundant secreted proteins.

Total protein concentration in the culture medium after incubation for five days was measured using a Bio-Rad DC kit (Bio-Rad) and was found to be 2.08 and 1.88 mg/ml for QM6a and J2026, respectively. Overall, the results showed that non-mutagenized *O. floccosum* J2026 and *T. reesei* QM6a secreted approximately similar amounts of proteins. Furthermore, the amount of dominant proteins in the culture supernatants of QM6a and J2026 seemed similar (indicated by arrows in Fig. 6). Considering the fact that mutants derived from *T. reesei* QM6a can secrete up to 40 g/l of protein into the culture medium (Durand et al., 1988), it seemed likely that high secretion of proteins from *Ophiostoma* could be achieved by a mutagenesis program.

3.1.3 UV mutagenesis to increase protein secretion in *O. floccosum* J2026

Traditional mutagenesis and screening program similar to that successfully applied to improve protein secretion in *T. reesei* (Bailey and Nevalainen, 1981) was conducted in this study. UV irradiation was chosen as the mutagen due to its relative safety to the user. The procedure is shown in Fig. 7. Briefly, spores of the parental strain were irradiated under UV light (254 nm) and samples were taken after the exposure of 1.5, 2, 3, 4 and 5 minutes. Mutagenized spores were then cultured on PDA plates for two to three days to allow colony formation. The survival rate at each time point compared to the non-mutagenized control was calculated and colonies with a survival rate of 2 - 20 % were transferred onto selection plates containing 2 % (w/v) insoluble starch. A clearing halo appeared around each colony due to starch degradation. Enhanced production of enzymes degrading starch (amylases) was indicated by an increase in the size of hydrolysis halos around the growing colonies compared to the non-mutagenized parental strain. Six to eight mutants with improved amylolytic activity

were chosen from each round of UV mutagenesis, and incubated in a minimal liquid culture medium containing 3 % soluble starch and 1.5 % soybean flour. Amylase, proteinase and lipase activity and total protein concentration in the culture medium were measured and the data used to select the parental strain for the next round of UV mutagenesis. Key characteristics for selection were an increase in amylase activity and total protein concentration, and a decrease in proteinase activity. In addition, lipase activity in the culture supernatant was also measured due to its importance for *Ophiostoma* growth in wood and potential for industrial applications (Farrell et al., 1993; Gao and Breuil, 1998).

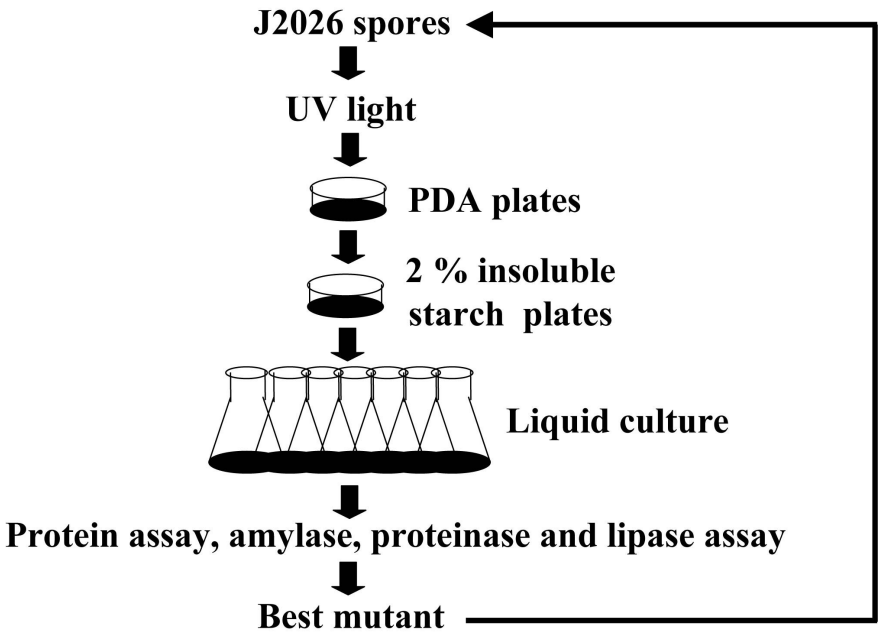

Fig. 7. Schematic diagram of the UV mutagenesis and screening procedure.

There were two reasons for using amylase activity as an indicator for enhanced protein secretion during repeated UV mutagenesis. First, starch and other soluble sugars are the major nutrients in ray parenchyma cells, therefore, amylases are important for the fungi to degrade nutrients available in their natural environment. An analogous strategy using increased cellulase secretion has been successfully applied to *T. reesei* (Bailey and Nevalainen, 1981; Montenecourt, 1983). Secondly, genes encoding amylolytic enzymes have strong promoters such as the *glaA* gene promoter of *A. niger* and *taaG2* gene promoter of *A. oryzae* which have been extensively characterized and applied to produce recombinant gene products (Cornett et al., 2003; Nagata et al., 1993).

A strong *Ophiostoma* gene promoter would be required for the construction of expression cassettes.

Degradation of foreign proteins by secreted fungal proteinases is one of the major reasons for low yields of recombinant gene products made in filamentous fungal hosts (Goller et al., 1998; Gouka et al., 1996; 1997a; Nakajima et al., 2006; Nykänen et al., 2002; Spencer et al., 1998). Therefore, obtaining proteinase-deficient mutants or mutants with decreased proteinase activity was a secondary goal in the screening procedure. In this study, five rounds of UV mutagenesis were performed and the results are shown and discussed in the following sections.

3.1.3.1 Analysis of selected mutants from the first round of UV mutagenesis

UV irradiation was performed to improve protein secretion of the parental strain J2026 using amylase activity as an indicator. From the first round of UV mutagenesis, 1025 colonies were patched onto plates containing 2 % (w/v) insoluble starch to screen for increased amylase activity. Eight candidates exhibiting the largest hydrolysis halos were chosen for further analysis. These mutants were cultured in a liquid medium and cultures were harvested after 5-days incubation on a shaker at 28 °C at 250 rpm in the dark. Amylase, proteinase and lipase activities and total secreted protein in the culture supernatants were measured as described in publication I. The results are presented in Table 10. In the first round, the best mutant named MQ.1.1 showed more than a three-fold increase in amylase activity compared to the parental strain J2026, and its extracellular proteinase activity was decreased more than 17-fold. Total protein concentration in the culture supernatant of the best mutant MQ.1.1 was also increased over three-fold. The ability of PNPC (C10) hydrolysis by lipase enzymes secreted by the mutant MQ.1.1 was increased 1.5-fold compared to the parental strain J2026. Differently to mutant MQ.1.1, there were three high-proteinase producing mutants, MQ.1.2, MQ.1.3 and MQ.1.7, in which the proteinase activity was increased more than three times compared to the parental strain after the first round UV mutagenesis. High-proteinase producing mutants were produced due to the random nature of mutations generated by UV mutagenesis (Table 10). However, according to the selection criteria outlined in section 3.1.3, a low proteinase-producing mutant, which was also a high-amylase producing strain, was picked for further mutagenesis.

Table 10. Amylase, lipase and proteinase activity and total protein concentration in the culture supernatants of the first round UV mutants of *O. floccosum* J2026. Strain MQ.1.1 has been highlighted.

Strain	Amylase activity (nkat)	Proteinase activity Azocasein solubilised (mg/ml)	Lipase activity PNP (µmol/min)		Total protein concentration (mg/ml)
			C10	C12	
J2026	10.3	4.43	8.77	2.23	0.05
MQ.1.1	**36.8**	**0.25**	**14.1**	**6.27**	**0.166**
MQ.1.2	18.4	13.25	5.55	0.76	0.177
MQ.1.3	18.2	13.03	4.26	0.16	0.164
MQ.1.4	13.3	4.97	9.91	1.55	0.094
MQ.1.5	11.9	7.12	4.01	3.96	0.099
MQ.1.6	10.5	0.48	8.44	6.42	0.085
MQ.1.7	10.0	13.41	5.09	0.48	0.076
MQ.1.8	9.5	5.81	7.2	1.23	0.106

In the early stages of this work, total protein concentration was measured using the Bio-Rad DC kit which is based on the Lowry method (Lowry et al., 1951)**.** Total protein concentration in the culture supernatant of the parental strain J2026 was around 2 mg/ml after 5 d of incubation (section 3.1.2). However, it appeared that there was a lesser amount of proteins (estimated by eye) in the culture supernatant of J2026, visualized by SDS-PAGE and Coomassie blue staining, than indicated by the assay using the DC kit. At the same time, a parallel protein assays using the Bradford method, which is based on Coomassie brilliant blue (Peterson, 1979), seemed to agree with the SDS-PAGE gels. In order to clarify the situation, known amounts of commercially available BSA (Bovine serum albumin; Bio-Rad) were loaded onto SDS-PAGE along with the culture supernatant of J2026 and stained with Coomassie blue. By comparing the staining of proteins with the 'BSA standard' and recording the intensity of the bands by a densitometer (not shown), it transpired that the actual total concentration of the proteins in the culture supernatant was about 10-20 times lower than indicated by the Lowry-based assay. Consequently, it was decided to use the Bradford method in the following experiments.

SDS-PAGE (12.5 %) was performed to visualize the protein profiles in the culture supernatants of the parental strain and eight selected mutants (Fig. 8). The analysis revealed different protein profiles. In the mutants, secretion of several proteins discussed in publication I seemed to have been improved by several-fold (indicated by arrows in Fig. 8), however, the profiles were variable due to the random nature of

mutations by UV irradiation. Overall, these results indicated that UV mutagenesis was efficient in producing mutants with improved general protein-secretion ability and that secretion of a particular protein may also be improved. Secretion of three proteins, each with a molecular weight of around 33 kDa, was extensively increased (Fig. 8, indicated by arrows in lanes MQ.1.2, MQ.1.3 and MQ.1.7), when the protein profiles of the culture supernatants of the three mutants were compared with the parental strain J2026. Correspondingly, proteinase activity in the culture medium of the three mutants was substantially increased when compared to the parental strain. This increase seemed rather specific since the amount of total secreted protein was either about the same or less than that in MQ.1.1 (Table 10). In a similar study, Szekeres et al. (2004) showed that improved secretion of a specific proteinase occurred after UV-mutagenesis (2004). Among several *T. harzianum* mutants which overproduced proteinase activity, there were strains in which the trypsin-like and chymotrypsin-like proteinase activities were 12.5 and 16 times higher compared to the wild-type strain. The protein with *Mr* of around 33 kDa was identified by N-terminal sequencing and its sequence showed high similarity to a subtilisin-like serine proteinase from *Ophiostoma* (Abraham and Breuil, 1996). According to the screening criteria, increased amylase and decreased proteinase activity plus increased total protein concentration, mutant strain MQ.1.1 was chosen as the parental strain for the second round of mutagenesis.

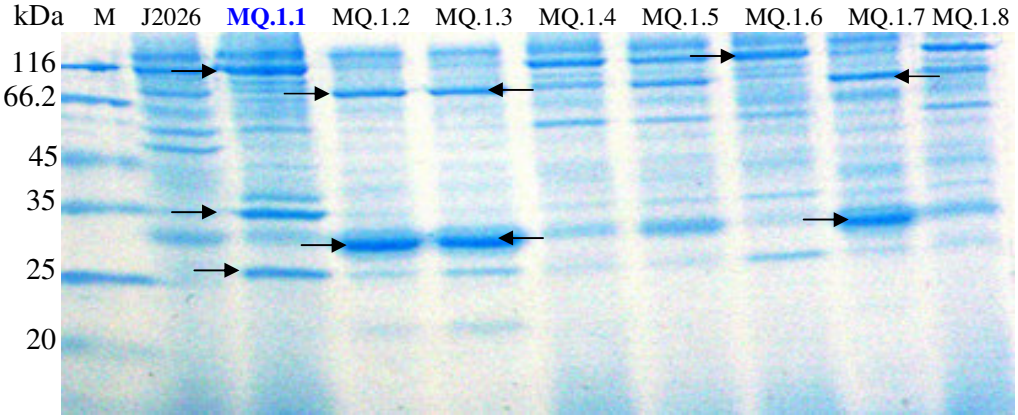

Fig. 8. SDS-PAGE analysis of proteins secreted into the culture medium of the first round UV mutants from *O. floccosum* J2026. Each lane contained 60 µl of culture supernatant. M, molecular weight marker. MQ.1.1-1.8, selected mutants. Arrows indicate proteins secreted at a higher level than in the parental strain J2026.

3.1.3.2 Analysis of selected mutants from the second round of UV mutagenesis

Second round UV mutagenesis was performed using MQ.1.1 as the parental strain. In this round, 606 colonies were patched onto starch plates to screen for improved amylase activity. Six candidates were chosen for further analysis in liquid culture. Amylase, proteinase and lipase activities and total protein concentration were measured as above and the results are presented in Table 11. In the second round, the best mutant, designated as MQ.2.1, exhibited over a 47-fold increase in amylase activity compared to the parental strain MQ.1.1. Extracellular proteinase activity of MQ.2.1 was decreased by 5-fold and total protein concentration in the culture supernatant of MQ.2.1 was increased more than 2.5-fold. Hydrolysis of PNPL (C12) was increased 1.5-fold compared to the parental strain MQ.1.1. After the second round, there seemed to be a continuing increase in the amylase activity, lipase activity and total protein concentration measured in the culture supernatants of the mutants. Therefore, it was confirmed that it was possible to improve the secretion ability of *Ophiostoma* by UV mutagenesis. Particularly, the amylase activity of mutant strain MQ.2.1 was increased dramatically compared to the parental strain. Furthermore, proteinase activity in most culture supernatants of the mutant strains was decreasing, which presented an additional benefit for the purpose of this study. Based on all the data, MQ.2.1 was chosen as the parental strain for the third round of UV mutagenesis.

Table 11. Amylase, lipase and proteinase activity and total protein concentration in the culture supernatants of the second round UV mutants from MQ.1.1. Strain MQ.2.1 has been highlighted.

Strain	**Amylase activity (nkat)**	**Proteinase activity azocasein solubilised (mg/ml)**	**Lipase activity PNP (µmol/min)**		**Total protein Concentration (mg/ml)**
			C10	**C12**	
MQ.1.1	15.0	0.362	17.4	9.6	0.103
MQ.2.1	**720.9**	**0.066**	**16.7**	**14.5**	**0.253**
MQ.2.2	34.2	6.54	12.6	5.1	0.076
MQ.2.3	14.6	0.119	15.4	7.8	0.126
MQ.2.4	12.0	0.06	9.7	3.4	0.122
MQ.2.5	25.2	0.27	17.8	10.3	0.163
MQ.2.6	18.1	0.103	15.7	9.8	0.108

Secreted proteins were visualized by 12.5 % SDS-PAGE to compare the extracellular protein profiles (Fig. 9). The protein profiles in the culture supernatant of

MQ.2.3, MQ.2.4, MQ.2.5 and MQ. 2.6 were similar to the parental strain. The profiles were well in line with amylase activity and total protein concentration in the culture medium of the parental strain and the mutants (Table 11). The protein profile in the culture supernatant of mutant MQ.2.1 was different to the parental strain and other mutants. Clearly, secretion of three high molecular weight proteins (indicated by arrows in Fig. 9) in the mutant MQ.2.1 had increased dramatically. Amylase activity in the culture supernatant of mutant MQ.2.1 was also increased compared to the parental strain MQ.1.1 and other mutants from this round. Therefore, it seemed likely that the three high molecular weight proteins could be enzymes involved in starch degradation. This assumption was confirmed by a zymogram gel assay (Fig. 15 in section 3.3) and N-terminal sequencing. The protein with a molecular mass of around 116 kDa was a starch-degrading enzyme as shown by zymogram gel analysis (unpublished data). Proteins with molecular masses of around 70 and 80 kDa were an α-amylase and a glucoamylase, respectively. It was expected that the high molecular mass proteins were enzymes involved in the degradation of starch since the screening was based on the increasing size of the hydrolysis halo around mutant colonies growing on starch. Generally, the molecular masses of known fungal α-amylases and glucoamylases are around 60 kDa and over 80 kDa, respectively (Pandey et al., 2000).

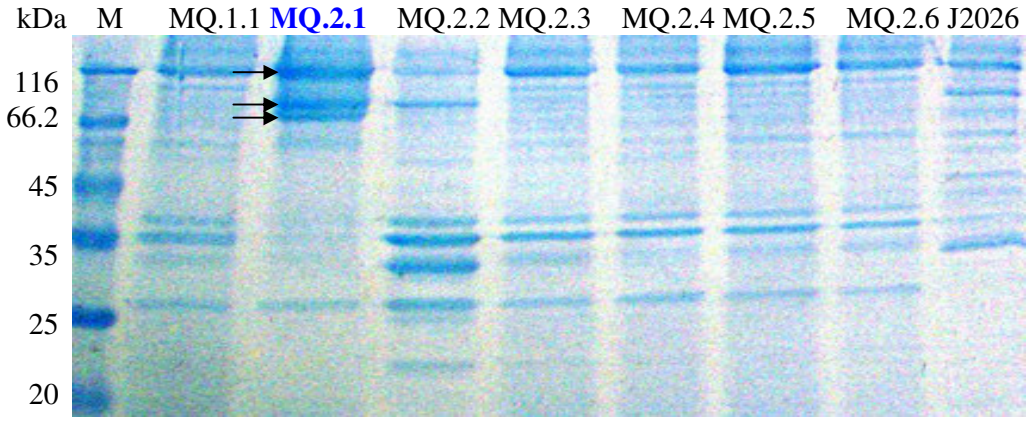

Fig. 9. SDS-PAGE analysis of proteins secreted into the culture medium of the second round UV mutants from MQ.1.1. Each lane contained 60 µl of culture supernatant. M, molecular weight marker. MQ.2.1-2.6, selected mutants. Arrows indicate proteins secreted at a higher level when compared to the parental strain MQ.1.1.

3.1.3.3 Analysis of selected mutants from the third round of UV mutagenesis

Third round UV mutagenesis was carried out using MQ.2.1 as the parental strain, and over 600 colonies were patched onto plates containing 2 % (w/v) insoluble

starch for screening of mutants with improved amylase activity. Six candidates were chosen for further analysis based on formation of a large halo around the colonies. Amylase, proteinase and lipase activities and total protein concentration in the culture supernatants of the parental strain MQ.2.1 and the six selected mutants were measured (Table 12). SDS-PAGE separation of proteins secreted into the culture medium from the third round UV mutants is shown in Fig. 10. The best mutant was found to be MQ.3.1 which showed a 1.2-fold increase in amylase activity when compared to the parental strain. In addition, its extracellular proteinase activity was decreased 1.5 times. The hydrolysis of PNPC (C10) by MQ.3.1 and most of mutant strains was increased slightly and remained high, but the ability of PNPL (C12) hydrolysis was decreased compared to the parental strain MQ.2.1. In this round, protein profiles in the culture supernatants of the parental strain MQ.2.1 and selected mutants were similar. Three dominant proteins (indicated by arrows in Fig. 10) which were shown to be enzymes degrading starch were secreted by all the strains screened. The protein profiles were consistent with the enzyme activity: relatively high amylase activity was detected in the culture supernatants of the parental strain MQ.2.1 and all mutants when compared with the mutants derived from the previous rounds. Even though the total protein concentration of the mutant MQ.3.3 was higher than in MQ.3.1, the amylase activity in the culture supernatant of MQ.3.3 was lower and proteinase activity much higher than in MQ.3.1. The nature of the series of proteins around 40-50 kDa (Lane MQ.3.3, Fig. 10) is not known. These may be proteinases as evidenced by the molecular weight (Rao et al., 1998) and increased proteinase activity in this mutant (Table 12). Therefore, strain MQ.3.1 was chosen as the parental strain for the fourth round of mutagenesis.

Table 12. Amylase, lipase and proteinase activity and total protein concentration in the culture supernatants of the third round UV mutants from MQ.2.1. Strain MQ.3.1 has been highlighted.

Strains	Amylase activity (nkat)	Proteinase activity azocasein solubilised (mg/ml)	Lipase activity PNP (µmol/min) C10	C12	Total protein concentration (mg/ml)
MQ.2.1	505.6	0.0666	17.6	12.9	0.209
MQ.3.1	**600.1**	**0.0427**	**19.4**	**10.8**	**0.203**
MQ.3.2	592	0.1009	16.2	12.7	0.194
MQ.3.3	516	0.4088	19.9	12.3	0.231
MQ.3.4	388	0.0515	19.2	10.9	0.191
MQ.3.5	301.4	0.0543	19.9	12.3	0.066
MQ.3.6	297	0.0543	19.7	9.7	0.093

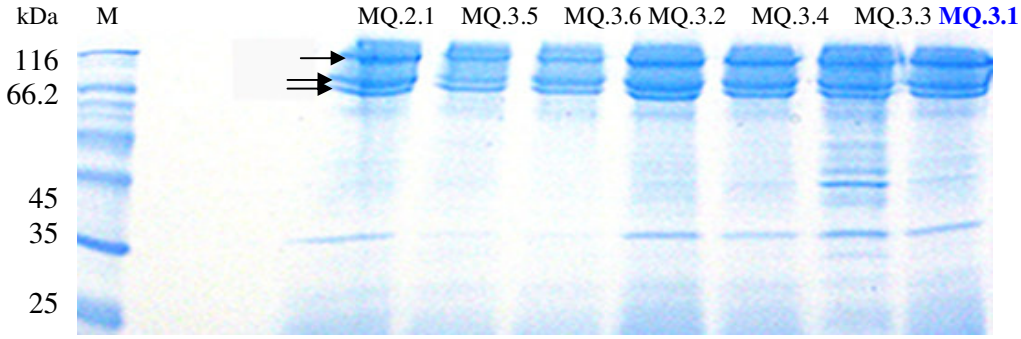

Fig. 10. SDS-PAGE analysis of proteins secreted into the culture medium of the third round UV mutants from MQ.2.1. Each lane contained 60 µl of culture supernatant. M, molecular weight marker. MQ.3.1-3.6, selected mutants. MQ.3.1 has been highlighted. Arrows pointed out the dominant secreted proteins.

3.1.3.4 Analysis of selected mutants from the fourth round of UV mutagenesis

Fourth round UV mutagenesis was performed using MQ.3.1 as the parental strain. From this round, 702 colonies were patched onto starch plates and screened for further improvement in amylase activity. Six candidates were chosen and grown in liquid medium and their culture supernatants were assayed for amylase, proteinase and lipase activity as well as total protein concentration. The results are presented in Table 13. Separation of proteins by 12.5 % SDS-PAGE was also conducted to compare the extracellular protein profiles (Fig. 11). Amylase activity of the mutant MQ.4.2 was the highest in this round of mutagenesis and was increased by only 1.1 times when compared to the parental strain MQ.3.1. Protein profiles in the culture supernatants of the parental strain MQ.3.1 and selected mutants were similar. The three dominant proteins (indicated by arrows in Fig. 11) were present in the culture medium of all strains screened. Based on the intensity of protein bands, it seemed that the amount of

the three dominant proteins secreted were similar between the parental strain MQ.3.1 and all mutants. This result was consistent with high amylase activity in the culture medium of the parental strain and mutants. Therefore, it seemed that amylase secretion had reached a plateau. In this round, PNPC hydrolysis by the mutant MQ.4.5 was relatively high compared to the parental strain and all mutants obtained so far (Table 13). It has been shown that the lipase produced by *O. piceae* has a molecular mass of around 35 kDa (Gao and Breuil, 1998). However, there was no notable difference in production of a protein in that molecular range from protein profiles visualized by SDS-PAGE (Fig. 11). Nevertheless, this mutant could be further explored and developed as an efficient lipase provider to decrease the amount of extractives (fats, resin acids, etc.) in wood processing.

Amylase activity in the culture supernatant of MQ.4.1 was slightly increased compared to the parental strain MQ.3.1. MQ.4.1 was chosen as the parental strain for the final round of UV mutagenesis, despite the fact that amylase activity in the culture medium of the mutant MQ.4.2 was higher. The total protein concentration in the culture medium of the mutant MQ.4.1 was the highest and the proteinase activity was the lowest among the mutants screened (Table 13).

Table 13. Amylase, lipase and proteinase activity and total protein concentration in the culture supernatants of the fourth round UV mutants from MQ.3.1. Strain MQ.4.1 has been highlighted.

Strains	**Amylase activity (nkat)**	**Proteinase activity azocasein solubilised (mg/ml)**	**Lipase activity PNP (µmol/min)**		**Total protein concentration (mg/ml)**
			C10	**C12**	
MQ.3.1	762.7	0.0787	18.4	5.8	0.264
MQ.4.1	**786.5**	**0.054**	**13.7**	**3.2**	**0.343**
MQ.4.2	870.8	0.0835	15.4	14.4	0.237
MQ.4.3	796.1	0.07	12.2	7.3	0.256
MQ.4.4	781.2	0.976	12.4	4.5	0.258
MQ.4.5	749.1	0.082	24.7	0.03	0.235
MQ.4.6	713.3	0.1075	10.8	0.01	0.282

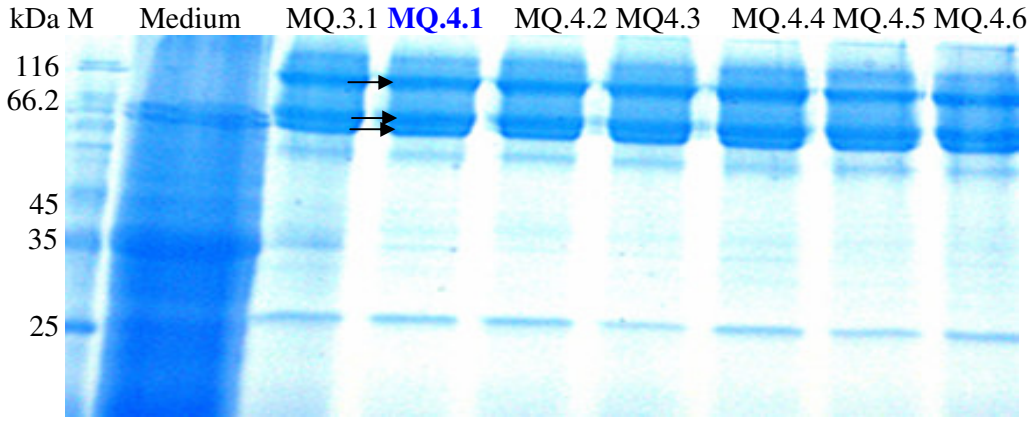

Fig. 11. SDS-PAGE analysis of proteins secreted into the culture medium of the fourth round UV mutants from MQ.3.1. Each lane contained 60 µl of culture supernatants. M, molecular weight marker. MQ.4.1-4.6, selected mutants. MQ.4.1 has been highlighted.Arrows pointed to dominant secreted proteins.

3.1.3.5 Analysis of selected mutants from the fifth round of UV mutagenesis

The fifth and final round of UV mutagenesis was performed using the mutant MQ.4.1 as the parental strain. Six mutants were chosen for culturing in liquid medium and total protein concentration, amylase activity and proteinase activity were measured (Table 14). In this round, amylase activity in the culture supernatant of the best mutant MQ.5.1 was increased 1.7 times, but the total protein concentration was decreased compared to the parental mutant. SDS-PAGE was conducted to compare the extracellular protein profiles (Fig. 12), which appeared similar between the parental strain MQ.4.1 and the selected mutants except for the mutant MQ.5.5 (Fig. 12). One of the dominant proteins of around 80 kDa present in the culture supernatants of the parental strain and other mutants was absent in the culture supernatant of mutant MQ.5.5 (indicated by a circle in Fig. 12). It is therefore possible that the gene encoding the corresponding protein had become inactivated during UV mutagenesis. At the time, amylase activity in the culture supernatant of mutant MQ.5.5 was relatively lower than that in other mutants, which could be expected as the protein with a molecular weight around 80 kDa had been identified as a glucoamylase (Fig.9). UV irradiation absorbs directly to the DNA bases and typically promotes the covalent attachment of adjacent pyrimidines to each other (Fincham et al., 1979) which typically destroys function of a gene. Even though the probability of hitting a particular gene in a fungal genome by random mutagenesis is of the order of 10^{-6} (Fincham et al., 1979), there are studies which show the loss of particular enzyme activity such as cellulase and proteinase (Mattern et al., 1992; Nevalainen and Palva, 1978).

Table 14. Amylase, lipase and proteinase activity and total protein concentration in the culture supernatants of the fifth round UV mutants from MQ.4.1. Strain MQ.5.1 has been highlighted.

Strains	Amylase activity (nkat)	Proteinase activity azocasein solubilised (mg/ml)	Lipase activity PNP (µmol/min)		Total protein concentration (mg/ml)
			C10	C12	
MQ.4.1	842.08	0.1046	19.9	10.4	0.3442
MQ.5.1	**1450.23**	**0.0876**	**23**	**6.2**	**0.2977**
MQ.5.2	876.85	0.1022	21.4	10.9	0.2818
MQ.5.3	875.47	0.084	21.9	10.8	0.3112
MQ.5.4	790.62	0.1094	19.6	9.2	0.2316
MQ.5.5	643.50	0.1686	17	8.7	0.209
MQ.5.6	738.08	0.1083	19.8	9.7	0.252

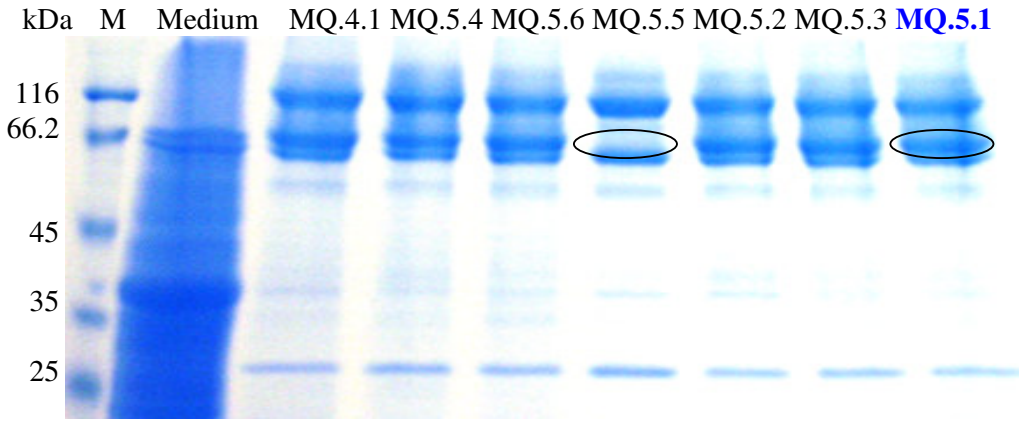

Fig. 12. SDS-PAGE analysis of proteins secreted into the culture medium of the fifth round UV mutants from MQ.4.1. Each lane contained 60 µl of culture supernatant. M, molecular weight marker. MQ.5.1-5.6, selected mutants. MQ.5.1 has been highlighted. The circled protein in lane MQ.5.1 was absent in the culture supernatant of mutant MQ.5.5 (the position is indicated by the circle in lane MQ.5.5).

In the fifth round, the amylase activity of the best mutant was increased, but the total protein concentration decreased. Therefore, amylase activity and concentration of the total secreted protein in the culture supernatant of the parental strain and the key mutant from each round of UV mutagenesis were plotted in order to identify any trends in relation to improvement (Fig. 13). It transpired that the amylase activity had increased about 72-fold over the parental strain after round two. From round two to round four, increase in the amylase enzyme activity appeared to plateau. However, the situation seemed to improve again from the fourth to the fifth (point 5 and point 6 in Fig. 13) round, by which the amylase activity had increased around 240 times compared to the parental strain J2026. At the same time, the change in the concentration of total secreted protein was steadily increasing from round one to round

four (point 2 and point 5 in Fig. 13). There was no increase in the total secreted protein from round four to round five (points 5 and 6 in Fig. 13). In the first and the second round of UV mutagenesis, total protein concentration in the culture medium of key mutants was increased along with improvement in amylase activity. In the third round and fourth round (points 4 and 5 in Fig 13), the total protein concentration was slightly enhanced with a steady change of amylase activity. However, total protein concentration in the culture medium of the key mutant MQ.5.1 decreased with an increase in amylase activity in the fifth round. It was therefore possible that improvement in the concentration of total secreted protein had also reached saturation. Therefore, UV mutagenesis was discontinued.

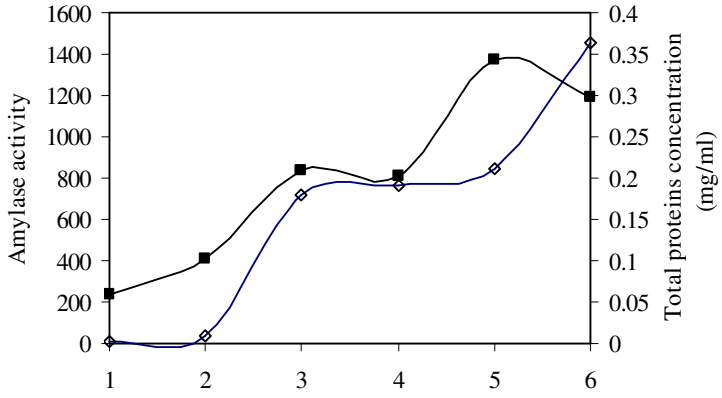

Fig. 13. Change of the amylase activity and total secreted protein in the culture supernatants of the parental strain and key mutants of each round of UV mutagenesis. The numbers 1 to 6 of x axis represent the nonmutagenized parental strain J2026, and mutants MQ.1.1, MQ.2.1, MQ.3.1, MQ.4.1 and MQ.5.1, respectively. "■" and "◊" indicate the concentration of total secreted protein and amylase activity of the parental strain and key mutants from each round of UV mutagenesis, respectively. Amylase activity assays were conducted in duplicate and total protein concentration was measured in triplicate.

3.1.4 Summary of the mutagenesis program

Five rounds of UV mutagenesis were carried out and around 3500 mutant colonies were screened on plates containing 2 % insoluble starch. A wide range of enzyme activities and secreted protein profiles were obtained from the 33 strains selected for further testing in liquid culture. After five rounds of UV mutagenesis, amylase activity in the culture supernatant had been improved dramatically. The amylase activity of the best mutant (MQ.5.1) was increased by 240 times over the

parental strain J2026. MQ.5.1 also displayed high lipase activity on both the 10-carbon (PNPC) and 12-carbon (PNPL) substrates. The amount of total protein in the culture supernatant of mutant MQ.5.1 was improved by 6-fold and the general proteinase enzyme activity was decreased by more than 40-fold making it a low proteinase-secreting mutant. Protein profiles in the culture supernatants of the parental strain J2026 and 33 selected mutants displayed notable differences, further exhibiting the effect of the mutagenization program. The amounts of several proteins in the culture supernatants of the four selected key mutants was increased by about 8- to 10-fold compared to the parental strain (indicated by arrows in Fig.1, publication I). Considering these improvements, a series of high secreting mutants derived from *O. floccosum* J2026 has been developed and representatives of which can be explored as expression hosts to produce recombinant gene products. The protein profiles in the culture supernatants of these mutants were variable and the production of several proteins was evidently improved. Efficiently expressed proteins provide a means for isolating strong promoters to drive the expression of recombinant gene products. The high-secreting mutant MQ.5.1 was chosen as the host strain of the *Ophiostoma* expression system developed in this study.

3.2 Proteinase profiles in the culture supernatants of the wild type strain J2026 and several key mutants

In the procedure of screening for increased protein secretion using α-amylase activity as an indicator, various mutant strains tested also exhibited a change in proteinase production. Characterization and identification of the proteinases of an expression host is important when attempting matching of the host with a particular recombinant protein to minimize yield loss by proteinases. Toward this aim, specific proteinase activities in the culture supernatants of the parental strain J2026 and series of key mutants were assayed using fluorescent labeled substrates which were specific for each group of proteinase. The proteinase profiles are shown in Table 15. All proteinase assays were conducted under both acidic (pH 5) and alkaline pH (pH 7.5 or 8) conditions.

Metalloproteinase activity was detected in the culture supernatants of the parental strain and all selected mutant strains under both pH conditions. Several

metalloproteinases isolated from fungi have been reported in the literature (Rao et al., 1998). Surprisingly, aspartic proteinase activity was detected in the culture supernatants of all strains assayed at pH 7.5. Aspartic proteinase is an acidic proteinase generally found to be active at acidic pH. It belongs to pepsin-like and rennin-like proteinases. It was possible that the aspartic proteinase acitivity detected in the culture supernatants of the parental strain and mutant strains in this study was a rennin-like proteinase that was also functional at a neutral pH. The recommended assay pH for rennin extracted from the fungus *Mucor miehei* was pH 7.5 (Martin et al., 1980). Cysteine proteinase activity was only detected in the culture supernatants of MQ.1.1 at pH 7.5 and in MQ.2.1, MQ.3.1, MQ.4.1 and MQ.5.1 under both pH conditions. There was no cysteine proteinase activity in the culture supernatants of the parental strain and mutant MQ.1.2 under the conditions applied in this study. There may be two possible explanations for this observation: i) expression of the gene encoding cysteine proteinase was induced or its expression facilitated by inactiviation of gene encoding the subtilisin-like proteinase in the parental strain J2026 during the first round of UV irradiation, or ii) a repression factor which suppresses expression of the gene encoding cysteine proteinase was inactivated. The possibilities can be explained further provided that genetic regulation of proteinase expression in *Ophiostoma* will become better known.

Subtilisin-like proteinase activity was detected only in the culture supernatants of the parental strain J2026 and MQ.1.2 under both pH conditions, but not in the culture supernatants of other selected key mutants. A subtilisin-like proteinase, one of the dominant proteins in the culture supernatants of J2026 and MQ.1.2 (Fig.1 and Table 3 in I), was identified by N-terminal sequencing in this study and has been reported previously (Abraham and Breuil, 1996). So far, it has been indicated that there are three subtilisin-encoding genes in *Ophiostoma floccosum* (Hoffman and Breuil, 2004a). Secretion of the subtilisin-like proteinase into the culture supernatant of MQ.1.2 was increased by several-fold when compared with the parental strain (Fig. 1, publication I). The absence of subtilisin-like proteinase activity in the culture supernatants of MQ.1.1, MQ.2.1, MQ.3.1, MQ.4.1 and MQ.5.1 may be explained by gene inactivation during the UV mutagenesis. It seemed the potential inactivation occurred in the first round of UV mutagenesis as MQ.1.1 was the parental strain for the following round of UV mutagenesis since no subtilising-like proteinase was detected in the culture supernatant of MQ.1.1.

In summary, production of proteinase decreased or increased by UV mutagenesis in a random manner. All results indicated that the proteinase profiles in the culture supernatants of the parental strain J2026 and selected key mutants were different and therefore, the different strains would provide an excellent portfolio of hosts for foreign gene expression. In addition, the results obtained will facilitate further tailoring of the proteinase profiles in an attempt to control the problem of proteolysis of heterologous gene products expressed in the *Ophiostoma* system. Addition of inhibitors to the culture medium to inactivate a particular proteinase or knock-out the gene encoding a specific proteinase that may cause harm to the foreign gene products of interest are examples of further developments.

Table 15. Specific proteinase activities in the culture supernatants of the parental strain *O. floccosum* J2026 and selected mutants using group-specific fluorescent labelled substrates.

Strain / Proteinase	J2026		MQ.1.1		MQ.1.2		MQ.2.1		MQ.3.1		MQ.4.1		MQ.5.1	
	pH 5	**pH 8**	**pH 5**	**pH 8**	**pH 5**	**pH 8**	**pH 5**	**pH 8**	**pH 5**	**pH 8**	**pH 5**	**pH 8**	**pH 5**	**pH 8**
Serine														
Chymotrypsin-like	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Elastase-like	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Subtilisin-like	+	+	-	-	+	+	-	-	-	-	-	-	-	-
pH	5	7.6	5	7.6	5	7.6	5	7.6	5	7.6	5	7.6	5	7.6
Trypsin	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Aspartic	-	+	-	+	-	+	-	+	-	+	-	+	-	+
Metallo	+	+	+	+	+	+	+	+	+	+	+	+	+	+
pH	5	7.5	5	7.5	5	7.5	5	7.5	5	7.5	5	7.5	5	7.5
Cysteine	-	-	-	+	-	-	+	+	+	+	+	+	+	+

Note: "+" indicates that the specific proteinase activity was detected and "-"indicates that there was no activity.

3.2.1 Activity-based identification of secreted serine proteinases of the filamentous fungus *Ophiostoma* (II)

In order to visualize and identify the secreted proteinases, a new approach was developed using activity-based chemical probes. A trifunctional probe (Fig. 14), which can label the active residue, such as serine, typically seen in serine proteinases, was used to identify active serine proteinases in the culture supernatant of *O. floccosum* MQ.1.2 which secretes a large amount of a subtilisin-like serine proteinase (Table 15). The active residue of an enzyme or protein attacks the reactive group of the probe, resulting in formation of a covalent linkage between the active residue of the protein and the probe (Fig. 14). In addition to the reactive group that serves as an activity tag, activity probes typically have three additional components: a linker region, a fluorescent reporter, and an affinity tag for purification of low abundance proteins (Fig. 14). The fluorescent reporter group faciliates identification of the tagged protein separated by SDS-PAGE. The affinity tag allows further purification of the protein by affinity when necessary. The linker physically separates out the different functionalities so they will not interfere with each other's function.

The reason for choosing serine proteinase as a test case were three-fold: i) the activity-based chemical probe to detect serine proteinases has already been developed and widely applied in many areas (Berger et al., 2004; Jeffery and Bogyo, 2003), ii) *Ophiostoma* serine proteinases have been identified previously (Abraham and Breuil, 1996) and iii) serine proteinase activity has been detected in this study (Table 15).

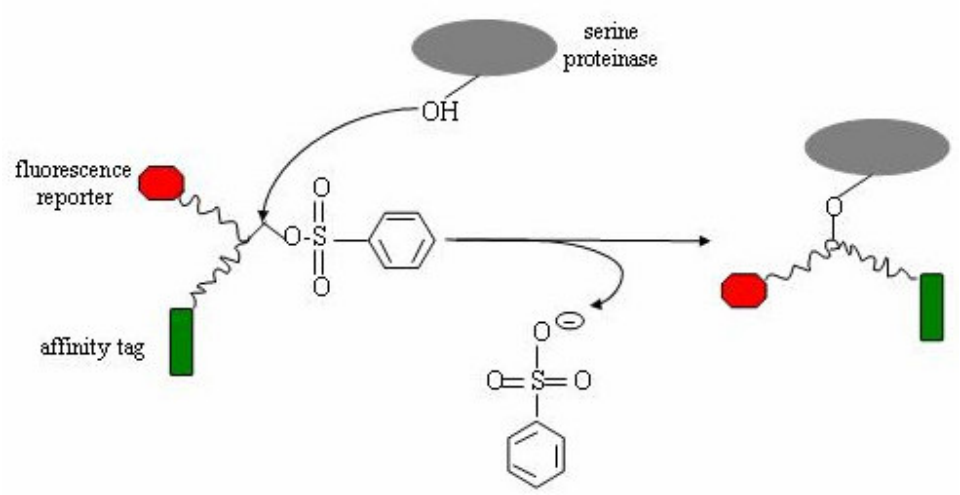

Fig 14. Composition of an activity probe and fluorescent labeling of the active serine residue of a proteinase. Modified from publication II.

Four proteins in the culture supernatant of MQ.1.2 were detected using the activity-based probe (Fig. 6, publication II) and were subsequently N-terminally sequenced for their identification (Table 16). Of the four proteins detected, two proteins were matched to *Ophiostoma* subtilisin-like serine proteinases which have been previously identified (Abraham and Breuil, 1996; Hoffman and Breuil, 2002). One protein did not match to the sequence of any proteins available in the NCBI data base and protein 3 (P3) was similar to the sequence of the probable endopeptidase K from *Neurospora*, a member of the same subtilisin-like proteinase family but located in a different sub-family that requires thiol for activation. All these data proved that the activity-based probes are expedient and applicable to capture and subsequently identify active proteinases in the culture supernatants of fungi. The probe strategy is more rapid and efficient and can directly discover enzymes in their active form compared to traditional approaches including liquid enzyme assay and zymogram gel assay.

Table 16. The N-terminal sequences of proteins in the culture supernatant of *O. floccosum* J2026 MQ.1.2 detected by the activity probe. Modified from publication II.

Protein	Sequence	Identification	Accession number	Matched fungal genus
P1	TFPKASVTVA	unknown	unknown	unknown
P2	AYTTQTGAPW	subtilisin-like proteinase	AAL08502.1	*Ophiostoma*
P3	VYDSQAGAGS	probable endopeptidase K	CAD71122	*Neurospora*
P4	ALTTQSGGTT	subtilisin-like proteinase	AAL08508	*Ophiostoma*

3.2.2 Summary

This is the first time that an activity-based probe has been used to identify secreted proteinases of fungal origin. The results derived from this part of the study were consistent with the proteinase activity assay using fluorescent substrates (Table 15). Considerable subtilisin-like proteinase activity was detected in the culture supernatant of MQ.2.1 by liquid enzyme assay. Importantly, the active enzymes captured can be visualized with certain equipment such as a laser-induced fluorescent scanner. Therefore, this new approach will be useful to rapidly screen proteinase profiles of particular fungi of interest provided that a suitable probe is available. A series of probes is available for identification of active proteinases including a probe for capturing cysteine proteinases (Greenbaum et al., 2000) and the selection is expanding continuously (Cravatt and Sorensen, 2000). For the purpose of this study, a known proteinase profile would facilitate the design of optimal cultivation conditions for a recombinant protein and selection of a suitable expression host deficient in proteinases that may degrade the protein of interest.

3.3 Isolation and characterization of the α-amylase gene (*amy1*) from *O. floccosum* MQ.5.1

The promoter is one of the key elements of an expression vector. Efficiently expressed proteins have strong promoters (Nevalainen et al., 2003), therefore, promoters of abundantly secreted proteins that also provide secretion signals were of particular interest in this study. Proteins in the culture supernatants of *O. floccosum* J2026 and the four selected mutants were visualized by 12.5 % SDS-PAGE (Fig.1, publication I) and five dominant protein bands were excised and N-terminally sequenced for their

identification. The N-terminal sequences of these proteins (P1-P5, Fig. 1, publication I) are shown in publication I, Table 2. One of the most efficiently secreted proteins was identified as an α-amylase with a molecular mass around 70 kDa.

A DNA fragment of approximately 930 bp in length was amplified from the genomic DNA of *O. floccosum* MQ.5.1 using degenerate primers designed against fungal genes similar to the *A. niger taaG2* gene sequence. The complete *amy1*-coding sequence and flanking regions were isolated by GWPCR as discussed in publication I. A 6.5 kb DNA fragment featuring the gene (2.1 kb) encoding the α-amylase enzyme, the 5' promoter region of α-amylase (2.6 kb) and the 3' terminator region (1.8 kb) were isolated and characterized. The *amy1* gene sequence isolated in this study is available from Genbank under accession number DQ526426. The detailed features and the *amy1* gene sequence are shown in Fig. 2 in publication I. Genomic DNA of MQ.5.1 was digested by restriction enzymes *HindIII*, *StuI*, *SmaI* and *PstI* and Southern blotting was carried out in order to determine the copy number of *amy1* gene in the genome of the mutant strain. A DIG-labeled DNA fragment encoding a portion of the catalytic domain of AMY1 was used as a probe. A single band which was detected in all digests indicated that there is only one copy of *amy1* gene in the genome of the mutant MQ.5.1 (Fig. 15).

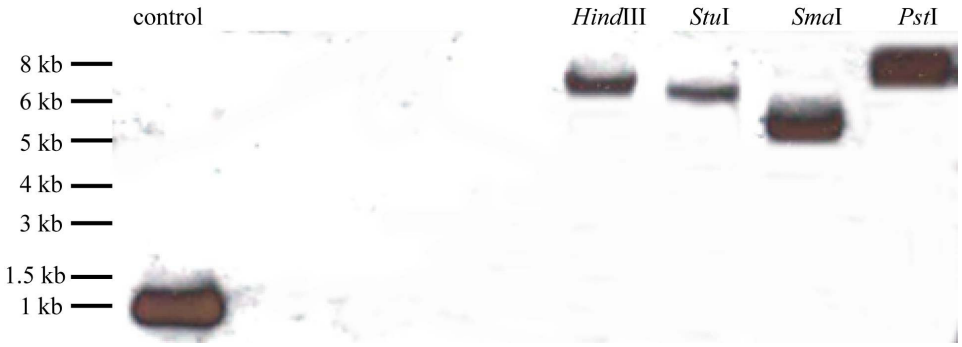

Fig. 15. Southern blotting to detect the *amy1* gene copy number in the genome of the mutant strain MQ.5.1. Control indicates DNA fragment containing part of gene encoding α-amylase (around 1 kb) was amplified from vector pOAMYss-*gpdhph* (pubilication III) by PCR.

Northern blotting analysis targeting the *O. floccosum* α-amylase mRNA showed multiple signals (Fig. 4, publication I). RLM-RACE PCR was performed to confirm the presence of multiple *amy1* transcripts, and determine the transcriptional start site (TSS) and polyadenylation site (s) of the transcripts. The results indicated that there were three

amy1 cDNAs generated from the single *amy1* gene locus. All three mRNAs were transcribed from a single starting site located at position 2635. Polyadenylation sites were located at positions 4951, 4952 and 4963, respectively (Fig. 3, publication I). The formation of multiple *amy1* transcripts was due to the differential splicing of introns located at various positions at the end of 3' end of *amy1*-coding gene (publication I). The event that multiple messages are derived from same gene locus is not unusual and has been reported previously (Boel et al., 1984; Curach et al., 2004; Nunberg et al., 1984). Nunberg and his coworkers found that two messages of *A. awamori* glucoamylase were formed from the same gene locus due to a differential mRNA splicing event. This resulted in two isoforms of glucoamylase, GAI and GAII. From these, GAI contained a full length glucoamylase enzyme including the catalytic domain, linker region and starch binding domain. However, GAII did not contain the starch binding domain. In our case, the full length of α-amylase contained all three domains whereas the other two short forms of α-amylase obtained did not possess the starch binding domain (discussed in the following text).

The gene corresponding to transcript I, which was the dominant message, encodes a protein of 630 amino acids with a predicted M_r of 67 kDa. Its amino acid sequence showed 72 % similarity to the putative α-amylase from *Aspergillus niger* (Boel et al., 1990). Analysis showed it to have a putative N-terminal signal sequence in addition to the catalytic domain, linker region and starch binding domain. The predicted N-terminal sequence and *Mr* of protein I corresponded to P3 that had been previously identified by N-terminal sequencing (Table 2, publication I). A zymogram assay was performed and the results indicated that it was an active enzyme in the culture medium (Fig. 16). Transcript II encodes a protein of 509 amino acids with a predicted *Mr* of 54 kDa and transcript III encodes a protein of 466 amino acids with a predicted *Mr* of 51 kDa. The two smaller isoforms of AMY1 protein contained the catalytic domain and part of the α-amylase linker region. Alignment of the three isoforms of AMY1 proteins is shown in Fig. 17. All three predicted proteins contained a putative N-terminal sequence and catalytic domain. In addition, protein II contained most of the linker region and protein III contained a small portion of linker region and additional 37 amino acids that did not match to the rest of the linker region or the starch binding domain. Particular function of the two isoforms of AMY1 would still remain to be explored.

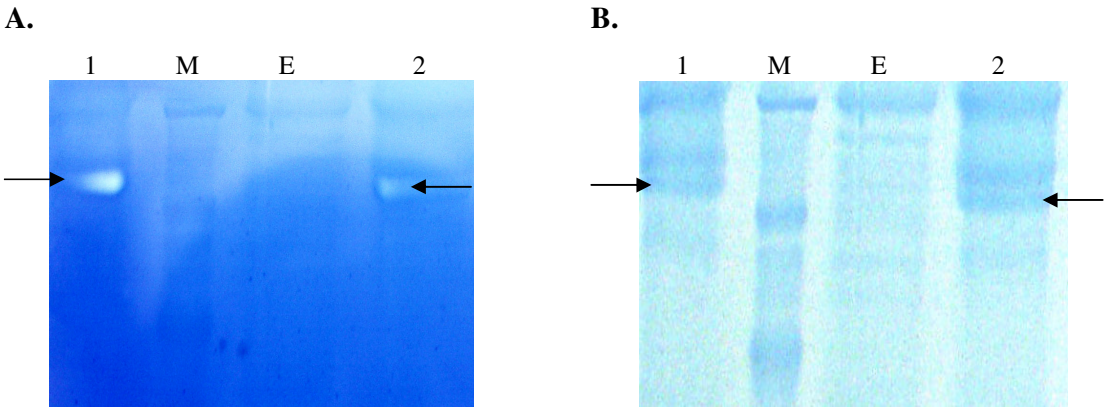

Fig. 16. Zymogram assay of starch hydrolyzing activity on 12.5 % SDS-PAGE with 0.25 % soluble starch. A. Gel was stained with Coomassie Blue. B. Gel was stained with Coomassie Blue and destained with water. Lane 1 and 2 in A and B contained 60 µl of culture supernatant of MQ.2.1. M indicates the molecular weight marker. E indicates empty lane. Arrows in picture A indicate the enzymes, which hydrolyzed the substrate visualized by staining with Coomassie Blue. Arrows in picture B indicate the corresponding proteins in picture A which stained with Coomassie Blue.

3.3.1 Summary

In summary, a 6.5-kb DNA fragment was isolated from the genomic DNA of a high –protein-secreting mutant MQ.5.1 by GWPCR. The fragment contained the *amy1* gene promoter, α-amylase gene and the transcription termination sequence. There was only one copy of the *amy1* gene in the genome of MQ.5.1. Three *amy1* transcripts were generated from a single *amy1* gene locus. They encode three forms of α-amylase, one of which features a complete enzyme consisting of a catalytic domain, linker region and a starch binding domain. The *amy1* gene promoter, gene encoding *amy1* and its transcriptional terminator provide tools for the construction of expression vectors for developing *Ophiostoma* as an expression system. Construction of such vector is described in section 3.4.

```
                1                                                  50
  Protein I     MKLSSLLPLA FLGQAVNALS PAEWRKQSIY FLLTDRFGRT DNSTSATCNT
  Protein II    MKLSSLLPLA FLGQAVNALS PAEWRKQSIY FLLTDRFGRT DNSTSATCNT
  Protein III   MKLSSLLPLA FLGQAVNALS PAEWRKQSIY FLLTDRFGRT DNSTSATCNT
                51                                                 100
  Protein I     GDRAYCGGSW QGVINHLDYI QGMGFTAIWI TPVTGQFYES TGDGTSYHGY
  Protein II    GDRAYCGGSW QGVINHLDYI QGMGFTAIWI TPVTGQFYES TGDGTSYHGY
  Protein III   GDRAYCGGSW QGVINHLDYI QGMGFTAIWI TPVTGQFYES TGDGTSYHGY
                101                                                150
  Protein I     WQQDIYSLNS HLGDQNDLKA LSAALHARGM YLMVDVVANH MGYDGAGSNV
  Protein II    WQQDIYSLNS HLGDQNDLKA LSAALHARGM YLMVDVVANH MGYDGAGSNV
  Protein III   WQQDIYSLNS HLGDQNDLKA LSAALHARGM YLMVDVVANH MGYDGAGSNV
                151                                                200
  Protein I     DYSVFDAFPS SSYFHSYCEI SNYDDQSNVE DCWLGDTTVS LPDLNTELTS
  Protein II    DYSVFDAFPS SSYFHSYCEI SNYDDQSNVE DCWLGDTTVS LPDLNTELTS
  Protein III   DYSVFDAFPS SSYFHSYCEI SNYDDQSNVE DCWLGDTTVS LPDLNTELTS
                201                                                250
  Protein I     VRSIWNSWVA GLVANYSIDG LRIDTVKHVE TSFWPGYNDA AGVYCVGEVF
  Protein II    VRSIWNSWVA GLVANYSIDG LRIDTVKHVE TSFWPGYNDA AGVYCVGEVF
  Protein III   VRSIWNSWVA GLVANYSIDG LRIDTVKHVE TSFWPGYNDA AGVYCVGEVF
                251                                                300
  Protein I     DGDPAYTCAY QNYMDGVLNY PIYYQLLSAF ESTSGSISNL YNMIKSVASD
  Protein II    DGDPAYTCAY QNYMDGVLNY PIYYQLLSAF ESTSGSISNL YNMIKSVASD
  Protein III   DGDPAYTCAY QNYMDGVLNY PIYYQLLSAF ESTSGSISNL YNMIKSVASD
                301                                                350
  Protein I     CADPTLLGNF IENHDNPRFA SYTSDYSLAQ NAISFLFFSD GIPIVYSGQE
  Protein II    CADPTLLGNF IENHDNPRFA SYTSDYSLAQ NAISFLFFSD GIPIVYSGQE
  Protein III   CADPTLLGNF IENHDNPRFA SYTSDYSLAQ NAISFLFFSD GIPIVYSGQE
                351                                                400
  Protein I     QHYSGGADPA NREATWLSGY STTATLYKHI KTTNQIRSLI IGKDSSWATS
  Protein II    QHYSGGADPA NREATWLSGY STTATLYKHI KTTNQIRSLI IGKDSSWATS
  Protein III   QHYSGGADPA NREATWLSGY STTATLYKHI KTTNQIRSLI IGKDSSWATS
                401                                                450
  Protein I     ANSPFYQDSN TIAMLKGSAS GSKVLTVLsn kgaSgssytl slgstgyssg
  Protein II    ANSPFYQDSN TIAMLKGSAS GSKVLTVLsn kgaSgssytl slgstgyssg
  Protein III   ANSPFYQDSN TIAMLKGSAS GSKVLTVLcl gerSqp.... ..........
                451                                                500
  Protein I     aslvelysct tvtvdssgnv pvPmasglpr vlVpsswVsG sglcgtavtt
  Protein II    aslvelysct tvtvdssgnv pvPmasglpr vlVpsswVsG sglcgtavtt
  Protein III   .......... ..llhcpdgl lwPfgnsvgh leVivfkVeG a*........
                501                                                550
  Protein I     gtatatgtst kattatatta tsctaatavs vvfnelattt ygenvyiigs
  Protein II    gtatatag*. .......... .......... .......... ..........
  Protein III   .......... .......... .......... .......... ..........
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Fig. 17. Alignment of predicted isoforms of AMYI proteins from *Ophiostoma floccosum* MQ.5.1. Residues highlighted by shadowing represent identical residues. Red colour -labeled regions are the signal sequences; black colour-labeled regions in capital font are the catalytic domain; pink colour-labeled regions are the linker regions and the blue colour-labeled region is starch binding domain.

3.4 Construction of *amy1* expression vectors (I, III)

An expression vector is a vehicle for the introduction and expression of a particular gene in a chosen host organism. Two of the crucial elements for such a vector are the expression cassette featuring a strong promoter and transcription termination sequences, and the transformation selection marker. The α-amylase gene promoter that drives expression of one of the most efficiently secreted *Ophiostoma* proteins, as shown in Fig. 1 (pubilication I), was chosen for the vector construction. Several selection markers were assessed for inclusion in the expression vector.

3.4.1 Screening of suitable selection markers for *O. floccosum* transformation

The preferred option was a gene encoding antibiotic resistance. The sensitivity of the parental strain *O. floccosum* J2026 and the mutant MQ.5.1 to several antibiotics was tested and the results are presented in Table 17. Both J2026 and MQ.5.1 were unable to grow on PDA plates containing 100 μg/ml of G418 or 150 units/ml of hygromycin B. Apart from antibiotic resistance genes, another potential dominant selective marker was the *amdS* gene, since we have shown that either the parental strain J2026 or MQ.5.1 could not use acetamide as a sole nitrogen source (unpublished data). Thus, the gene conferring G418 resistance (*kan*), the gene encoding hygromycin B resistance (*hph*) and the *amdS* gene were all considered as selection markers for *Ophiostoma* transformation. The constitutive *gpdA* promoter from the *Aspergillus nidulans* gene encoding glyceraldehyde-3-phosphate was used to drive the *hph* gene as described previously (Punt et al., 1987; Wang et al., 1999).

Table 17. Sensitivity of *Ophiostoma floccosum* J2026 and the mutant MQ.5.1 to different antibiotics.

Strain / Antibiotics	J2026		MQ.5.1	
	Concentration	**Growth**	**Concentration**	**Growth**
G418 (μg/ml)	10	Y	N/A	N/A
	30	Y	N/A	N/A
	50	Y	N/A	N/A
	80	Y	N/A	N/A
	90	Y	90	Y
	100	N	100	N
	120	N	120	N
	150	N	N/A	N/A
Hygromycin B (unit/ml)	50	Y	N/A	N/A
	100	Y	N/A	N/A
	120	Y	120	Y
	150	N	150	N
	160	N	160	N
	180	N	N/A	N/A
	200	N	N/A	N/A
	250	N	N/A	N/A
Puromycin (μg/ml)	50	Y	N/A	N/A
	100	Y	N/A	N/A
Phleomycin (μg/ml)	50	Y	N/A	N/A
	100	Y	N/A	N/A

Note: "Y" indicates growth; "N" indicates no growth and "N/A" indicates not applied.

3.4.2 Transformation of plasmids containing *kan*, *amdS* and *hph* into *O. floccosum*

To establish a suitable selection marker for *Ophiostoma* transformation, plasmids containing appropriate genes (listed below) were introduced into the parental strain J2026 and the mutant MQ.5.1 by biolistic bombardment and protoplast transformation.

3.4.2.1 Biolistic transformation of J2026 and MQ.5.1

Plasmids pHEN11 (*amdS*), $pRLM_{EX}30$ (*hph*), PCR2.1-*gpdkan* (*kan*) and pAN7-1 (*hph*) were transformed into the parental strain J2026 and the mutant MQ.5.1 by biolistic bombardment. Approximately two week-old spores of J2026 and MQ.5.1 were bombarded with tungsten particles (M-10) coated with 1 μg of plasmid DNA, using the seven barrel system of the Bio-Rad™ gene gun. The vacuum pressure was set at 1350 psi and the traveling distance of the particles to the spores was 3 cm. Spores bombarded with plasmid pHEN11 were plated onto minimal medium agar plates (pH

6.5) containing acetamide as the sole nitrogen source. PDA selection plates for transformation with plasmids pAN7-1, pRLM$_{EX}$30 and PCR2.1-*gpdkan* were subsequently overlaid with an appropriate concentration of hygromycin B or G418. All plates were bombarded with tungsten particles coated with the appropriate plasmid DNA, and one plate bombarded with tungsten particles without DNA was included as a negative control. The bombardment was repeated five times. Transformation with plasmids pHEN11 (*amdS*) and PCR2.1-*gpdkan* (*kan*) did not produce colonies growing on the appropriate selection plates after two weeks' incubation. The background growth was high in transformations with plasmids pAN7-1 and pRLMex30, which both contained the *hph* gene conferring resistance to hygromycin B. Some colonies even appeared on the selection plates bombarded with particles only (negative control). It seemed that *Ophiostoma* became more resistant to hygromycin B after transformation, a phenomenon which has also been observed in *T. reesei* (Markku Saloheimo, VTT Biotechnology, personal communication). Therefore, potential transformants were transferred onto PDA plates containing a higher concentration of hygromycin B (200 units/ml) to detect true transformants. Over 1000 individual colonies were screened, but there was no growth of colonies on the plates with 200 units/ml of hygromycin B.

In order to assess the overall success of biolistic transformation of filamentous fungi, previously published studies were reviewed. Te'o et al. (2000) have shown that two factors in biolistic bombardment were essential for transformation efficiency: the helium pressure as the driving force and the distance traveled by the microparticles before hitting the cells. Therefore, different pressures and various bombardment distances were tested to transform MQ.5.1. Different combinations of rupture pressures (650, 900, 1100 and 1350 psi) and bombardment distances (3 cm and 6 cm) were tested in bombarding plasmids pHEN11, pAN7-1 and pRLM$_{EX}$30 into *O. floccosum* J2026 and MQ.5.1 (Table 18). There was no growth of colonies on the selection plates from transformation with the plasmid pHEN11. Around 300 colonies grew on the selection plates from transformations with the plasmids pAN7-1 and pRLM$_{EX}$30 but some colonies also grew on the selection plates bombarded with tungsten only as a negative control. All potential transformants were further screened on PDA plates containing 200 units/ml of hygromycin B. However, no colonies grew on these plates.

Table 18. Various parameters trialed to introduce plasmids pHEN11, pAN7-1 and $pRLM_{EX}30$ into *O. floccosum* J2026 and MQ.5.1 by biolistic transformation. J206 and MQ.5.1 spores (14-d old) were transformed with plasmids pHEN11, pAN7-1 and $pRLM_{EX}30$ under different pressures combined with different travel distances of the particles.

Strain	Pressure (psi)	Plasmid	Distance (cm) 3	6
J2026			+	+
	650	pHEN11	+	+
		pAN7-1	+	+
		$pRLM_{EX}30$	+	+
	900	pHEN11	+	+
		pAN7-1	+	+
		$pRLM_{EX}30$	+	+
	1100	pHEN11	+	+
		pAN7-1	+	+
		$pRLM_{EX}30$	+	+
	1350	pHEN11	+	+
		pAN7-1	+	+
			+	+
MQ.5.1			+	+
	650	pHEN11	+	+
		pAN7-1	+	+
		$pRLM_{EX}30$	+	+
	900	pHEN11	+	+
		pAN7-1	+	+
		$pRLM_{EX}30$	+	+
	1100	pHEN11	+	+
		pAN7-1	+	+
		$pRLM_{EX}30$	+	+
	1350	pHEN11	+	+
		pAN7-1	+	+
		$pRLM_{EX}30$	+	+

Note: "+" indicates applied.

The next step was to investigate the effect of the spore age on the transformation efficiency. Consequently, MQ.5.1 spores of 3, 5, 10, 14 and 21 days were bombarded with the three plasmids using different shooting distance and bombardment presure (not

shown). No transformants were obtained from these attempts either. It appeared that biolistic transformation may be not suitable for transforming *O. floccosum*.

There are three possible reasons for the inability to obtain transformants: i) the promoter driving the expression of the selection marker was not functional; ii) integration of the transforming DNA into the fungal genome did not occur, or iii) biolistic bombardment was not suitable to for transforming *Ophiostoma*.

To contemplate the first possibility, plasmids containing the *amdS* gene under its own promoter have been successfully applied to transform a series of fungi, including ascomycetous *Aspergillus* spp. and *T. reesei* (Hazell et al., 2000; Kelly and Hynes., 1985; Wernars et al., 1985). In principle, the *amdS* gene promoter should also function in *Ophiostoma* spp., an ascomycete. However, there are no published studies on the use of the *amdS* gene as a selection marker to transform *Ophiostoma* to support this assumption. It has also been reported that the transformation efficiency is low, in general, using the *amdS* gene (Gomi et al., 1992). Based on these considerations, no further experiments were conducted to investigate the suitability of the *amdS* gene to transform *Ophiostoma*.

Plasmid $pRLM_{EX}30$ contains the *hph* gene under the control of the pyruvate kinase (*pki1*) gene promoter of *T. reesei* (Schindler *et al.*, 1993). The *pki* gene promoter is considered to be a constitutive gene promoter and has been used widely in *T. reesei* transformations with high transformation efficiency (de Faria et al., 2002; Te'o et al., 2000; 2002). In principle, there is no reason for this promoter not to be functional in *Ophiostoma*, provided the plasmid $pRLM_{EX}30$ was integrated into the *Ophiostoma* genome. However, there are no previous publications on the use of this plasmid for *Ophiostoma* transformation. In the plasmid pAN7-1, the *hph* selection marker gene is expressed under the constitutive *A. nidulans gpdA* promoter. Wang et al. (1999) have successfully transformed *Ophiostoma* spp. including *Ophiostoma floccosum* using the plasmid pAN7-1. Therefore, the *gpdA* promoter should be active if the DNA was integrated into the *Ophiostoma* genome. Thus, successful transformation should be obtained, at least, using the pAN7-1 plasmid.

To confirm the above assumption and to keep the otherwise handy hygromycin selection option on the table, ten colonies from transformations with plasmids pAN7-1 and $pRLM_{EX}30$ were randomly chosen and their genomic DNA was isolated. PCR was

performed to screen for integration of the *hph* gene into the *Ophiostoma* genome. No PCR products were obtained from any of the candidates screened. Therefore, it appeared that the reasons for not obtaining any transformants possibly were due to lack of integration of the plasmid DNA into the fungal genome and/or the ineffective transformation method. Olmedo-Monfil et al. (2004) have divided the integrative events into three main classes: homologous recombination, heterologous recombination and double homologous recombination. Heterologous recombination between the foreign gene and host genome DNA is the most common way of DNA integration in filamentous fungi (Ruiz-Díez, 2002), no apparent homology between the incoming DNA and that integration site in the recipient genome is needed. However, it has been shown that the homology between the incomimg DNA and the integration site in the genome of hosts will facilitate homologous integration events (Olmedo-Monfil et al., 2004; Ruiz-Díez, 2002). Therefore, further efforts into developing an efficient transformation protocol for *Ophiostoma* were focused on facilitating the integration of foreign DNA into the *Ophiostoma* genome by constructing expression vectors containing DNA encoding an endogenous *Ophiostoma* enzyme α-amylase and sequences flanking *amy1* gene locus (discussed below). In addition, protoplast transformation was trialed for the introduction of recombinant DNA into *Ophiostoma* (section 3.4.2.3).

3.4.2.2 Biolistic transformation of *amy1* expression vectors into *O. floccosum* MQ.5.1

To facilitate the integration of DNA into the *Ophiostoma* genome, four *amy1* expression vectors were constructed: pOAMY11-*gpdkan* (*kan*), pOAMY20-*gpdkan* (*kan*), pOAMY11-*gpdhph* (*hph*) and pOAMY20-*gpdhph* (*hph*). These expression cassettes that contain DNA homologous to the chromosomal DNA of *O. floccosum* were transformed into MQ.5.1 by biolistic bombardment under a pressure of 1350 psi and particle traveling distance of 3 cm. After transformation with pOAMY11-*gpdkan* and pOAMY20-*gpdkan*, there was no growth of colonies on selection plates containing 100 μg/ml of G418 after two weeks incubation. No transformants were obtained even though the concentration of G418 in the selection plates was reduced to 80 or 50 μg/ml to increase the chance of obtaining some transformants. After increasing the concentration of hygromycin B in the selection plates up to 200 units/ml, there were still

some colonies on plates which had been bombarded with microparticles without DNA. As noted earlier (3.4.2.1), it seemed that *Ophiostoma* became more resistant to hygromycin B after bombardment. Biolistic transformation requires high pressure for DNA-coated missiles to penetrate the cell wall. Colonies surviving this procedure may develop stronger cell walls or be otherwise more viable which may contribute to the increased hygromycin resistance.

3.4.2.3 Protoplast transformation of MQ.5.1 with plasmids pAN7-1 and $pRLM_{EX}30$

An alternative approach, protoplast transformation was carried out with the MQ.5.1 strain to overcome the problems experienced with biolistic transformation. The reason for attempting protoplast transformation was three-fold: i) protoplast transformation has been applied extensively to filamentous fungi including *Ophiostoma* spp. (Wang et al., 1999), ii) the general transformation efficiency is relatively high compared to other methods (Olmedo-Monfil et al., 2004), and iii) PEG solution used in protoplast transformation could markedly improve the uptake of DNA into the cells. In a previous study, Timberlake and Marshall (1989) showed that DNA molecules were internalized during the PEG induced protoplast transformation, whereas no transformation occurred when PEG was omitted.

The plasmids chosen for the experiments were pAN7-1, which has been successfully used to transform *Ophiostoma* by Wang et al. (1999) and $pRLM_{EX}30$ with a view of comparing the performance of the transformation marker (*hph*) under the *gpdA* and *pki1* gene promoters, respectively. Approximately 200 colonies from about 1000 colonies growing on the antibiotic selection plates after transformation were selected and transferred onto PDA plates containing either 200 units/ml or 400 units/ml of hygromycin B. All potential transformants grew on the PDA plates containing 200 units/ml of hygromycin B plates. However, the non-tranformed MQ.5.1 also grew on the PDA plates with 200 units/ml of hygromycin B as before. Eighty-four out of 200 potential transformants from the transformation with the plasmid pAN7-1 and 40 out of 200 potential transformants from the transformation with the plasmid $pRLM_{EX}30$ were able to grow on PDA plates containing 400 units/ml of hygromycin B. The

non-transformed MQ.5.1 did not grow on the selection plates incubated under the same conditions. Subsequently, 17 transformants representing each plasmid, that were able to grow on PDA plates containing 400 units/ml of hygromycin B were randomly chosen and transferred onto PDA plates containing 600 and 800 units/ml (Fig. 18) of hygromycin B. Thirteen (pAN7-1) and 15 ($pRLM_{EX}30$) transformants also grew on plates containing 600 and 800 units/ml of hygromycin B. Different growth rates among the potential transformants on the selection plates may have been because of different copy numbers of the *hph* gene integrated into the genome of the transformants (Fig. 18). However, this possibility was not explored further.

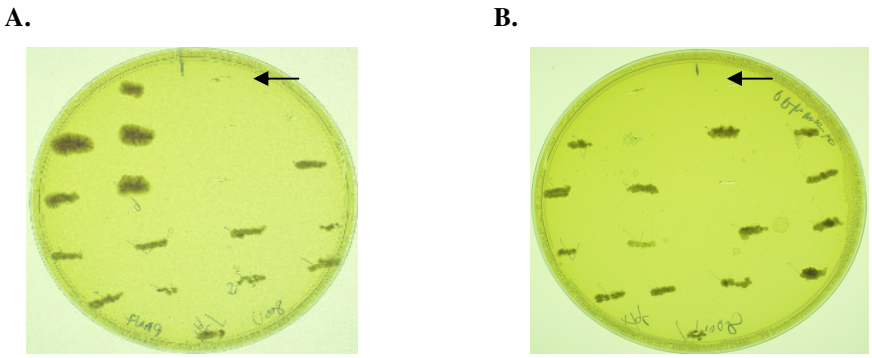

Fig. 18.Growth of transformants obtained with the plasmids pAN7-1 (A) and plasmid $pRLM_{EX}30$ (B). PDA plates contained 800 units /ml of hygromycin B. Arrows point to the non-transformed MQ.5.1 which was used as a negative control.

Six candidates representing each vector, which grew on plates with 800 units/ml of hygromycin B, were then randomly selected and their genomic DNAs were isolated for further analysis. PCR was performed to confirm integration of the *hph* gene into the *Ophiostoma* genome and the results indicated the presence of the *hph* gene in the genome of the 12 selected transformants. The transformation efficiency was calculated based on the numbers of transformants growing on the PDA plates containing 400 units/ml of hygromycin B. Transformation efficiency with the plasmids pAN7-1 and pRLMx-30 was 32.2 /µg DNA and 18.2 /µg DNA, respectively. The background growth on the control plates was still high using hygromycin B selection in combination with protoplast transformation.

Polyethylene glycol, which has been shown to help with the uptake of DNA into protoplasts, is a key factor for protoplast transformation (Olmedo-Monfil et al., 2004). Therefore, transformation with the plasmid pAN7-1 combined with PEG 4000 (60 %) or PEG 6000 (25 %) was investigated to study the influence of PEG on the

transformation efficiency. Fifty one (PEG 4000) and 47 (PEG 6000) out of 96 transformants screened were able to grow on PDA plates containing 400 units/ml of hygromycin B. Transformation efficiencies were about the same with PEG 4000 (9.6 /μg DNA) and PEG 6000 (8.2 /μg DNA). It appeared that the type of PEG solution did not affect transformation efficiency.

Regenration controls were prepared in each round of protoplast transformation to assess the viability of protoplasts. The average regenration frequency was around 75 %. The high concentration of hygromycin was required to obtain *Ophiostoma* transformants. This was not due to photodegradation of the antibiotic since the plates were always kept in the dark. Every hygromycin batch used for *Ophiostoma* studies was also used to successfully transform *T. reseei*. Overall, it appeared that the low efficiency for *Ophiostoma* transformation was not due to the low quality of protoplasts, photodegrtadation or old batch of the antibiotics. Even though these transformation efficiencies were lower than that mentioned above, they are within natural fluctuation recorded for individual transformations.

3.4.3 Summary

A selection marker plays an essential role in the transformation procedure. From the marker genes tested *hph*, *amdS* and *kan*, the *hph* gene was the only selection marker that worked in *Ophiostoma* transformation. Previously published studies also indicated that the *hph* gene is applicable for transformation of *Ophiostoma* protoplast and *Agrobacterium*-mediated transformation (Royer et al., 1991; Tanguay and Breuil, 2003; Wang et al., 1999). Therefore, the *hph* gene under the constitutive *gpdA* promoter was used as selection in further studies. Based on the results obtained from *Ophiostoma* transformation by biolistic bombardment and the protoplast method, the latter seemed more suitable for further optimization. Even though the transformation effeciency in protoplast transformation remained low and the background growth was still high on the selection plates using *hph* gene as seletion marker, true transformants as confirmed by PCR were obtained.

3.5 Testing the expression system using the fluorescent protein DsRed as a reporter

The mutant strain MQ.5.1 was transformed with expression vectors containing the gene encoding red fluorescent protein DsRed under the *amy1* gene promoter in order to test *Ophiostoma* as an expression host for production of a foreign protein. Functionality of the *dsRed* gene was first tested in *E. coli* DH5α before inserting it into the *amy1* based expression vectors pOAMY11-*gpdhph* and pOAMY20-*gpdhph*. Fluorescence microscopy confirmed that a functional DsRed protein was expressed in *E. coli* DH5α (Fig. 19). Subsequently, the *dsRed* gene was inserted into the two *amy1* based expression vectors and the recombinant vectors were named pOAMY20-*dsRedgpdhph* and pOAMY11-*dsRedgpdhph*. A third expression plasmid was constructed based on pOAMY11-*dsRedgpdhph* and was named pOAMYss-*dsRedgpdhph* (Fig. 5 in section 2.4).

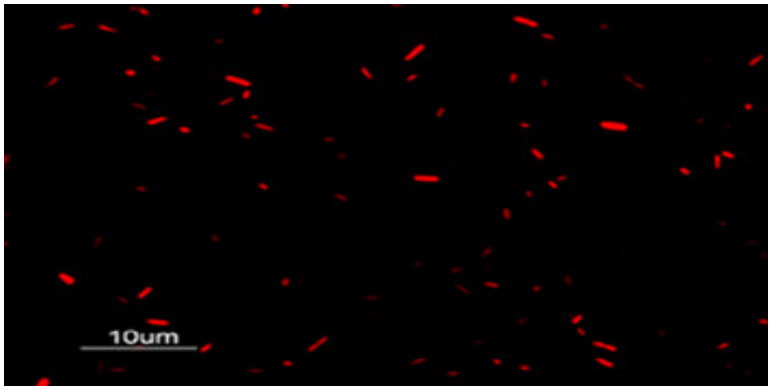

Fig. 19. Production of the DsRed protein in *E. coli* DH5α. A smear of *E. coli* cells transformed with pUC19-*dsRed* was exposed to green light (488 nm) emitting red fluorescence (100x magnification).

All three expression plasmids containing the *dsRed* gene were introduced into MQ.5.1 by protoplast transformation. Various modifications were carried out to the protocol including the use of different PEGs and transforming with different amounts of linearized and circular DNA to facilitate the integration of the gene cassette into the genome of MQ.5.1. From over 3000 potential transformants screened on PDA plates containing 400 units/ml of hygromycin B, a small number of transformants were obtained that contained the pOAMY20-*dsRedgpdhph* or the pOAMYss-*dsRedgpdhph* plasmid. PCR results, which amplified the DNA fragment containing part of gene

encoding *amy1* and full length of *dsRed* gene, indicated that integration of the expression cassettes into the genome of MQ.5.1 transformants had occurred. Subsequently, all transformants were grown in a liquid medium with 3 % soluble starch to induce the *amy1* gene promoter and cultures were screened for the presence of fluorescence. However, no fluorescence was detected from any of the transformants under conditions used in this study.

There could be two reasons for the contradictory results: i) the PCR was not functioning properly or ii) the DsRed protein was not functional or the *dsRed* gene was not integrated into the genome of *O. floccosum* MQ.5.1. Southern blot analysis was conducted to confirm the PCR results indicating integration of the *dsRed* gene into the genome of the transformants. The analysis was repeated three times but the prescence of the *dsRed* gene was not detected in the six transformants tested. At the same time, a DNA fragment containing *dsRed* gene used as positive control was detected by a 0.7-kb DIG-labeled DsRed-E5 probe, which indicated that the conditions used in Southern analysis was suitable. Therefore, it appeared that the *dsRed* gene had not integrated into the fungal genome and the results obtained from PCR and Southern blotting were contradictory. It was possible that the signal obtained from the PCR experiments was due to unspecific amplification which typically produced products with wrong sizes (Wang et al., 1999). However, this may not be the case in this study because the sizes of the PCR products were as expected whereas no products with corresponding sizes were obtained when using the genomic DNA of non-transformed MQ.5.1 as control. Therefore, it seemed that the PCR results were reliable. Compared to Southern blot analysis, PCR is more sensitive allowing amplification from 0.1 pg of DNA and visualization of the products in agarose gel. Thus, it was possible that the absence of signals from Southern blot analysis was because it was less sensitive (Ryan et al., 1997).

Northern blot analysis was then carried out to detect DsRed-specific mRNA. Total RNA was isolated from the mycelia collected at 72 and 120 h of growth, however, no DsRed message was detected. Based on all the data, it seemed that the *dsRed* gene was integrated into the genome of *Ophiostoma*. The absence of the red fluorescence in the culture supernatant of the transformants was due to non-transcription of *dsRed* gene as no signal was detected by Northern analysis. Thus, no further investigation was conducted to analyze these transformants.

3.5.1 Summary

It can be concluded that *Ophiostoma floccosum* strain J2026 and its mutant MQ.5.1 were very difficult to transform, based on the above results and the data presented in section 3.4. Even though transformants could be obtained using the *hph* gene as a selection marker, the background growth on the selection plates was high. An additional round of screening of the potential transformants on PDA plates containing a higher concentration of hygromycin B successfully decreased the number of potential transformants for further analysis by PCR and liquid culture. It is possible that some true transformants were overlooked in the screening procedure. In any case, the screening strategy in the above approach was found to be tedious, costly and time consuming with all the experimental phases carried out in order to screen for the true transformants (including selection on higher hygrymycin B plates, isolation genomic DNA, PCR, Southern and Northern analysis).

3.6 Expression of the xylanase B gene (*xynB*) in *O. floccosum* MQ.5.1 (III)

An alternative strategy was developed and applied to test the expression system due to the problems discussed above. The basic idea was to use expression of the thermostable Xylanase B protein as a reporter and the procedure is shown in Fig. 20. Briefly, vectors containing the *Dictyoglomus thermophilum xynB* gene under the *Ophiostoma amy1* promoter were introduced into MQ.5.1 by protoplast transformation using the *hph* gene as a selection marker. Subsequently, all colonies that grew on the selection plates containing 150 units/ml of hygromycin B were transferred onto *amy1* induction plates containing 2 % insoluble starch and incubated at 28 °C for 2- to 3- days. The plates were then overlaid with 0.5 % birchwood xylan and 1 % agarose and incubated at 70 °C over night to inactivate endogenous enzymes and develop thermostable xylanase activity. A clear halo would appear around colonies which produced an active xylanase after flooding the plates with 1 % Congo red.

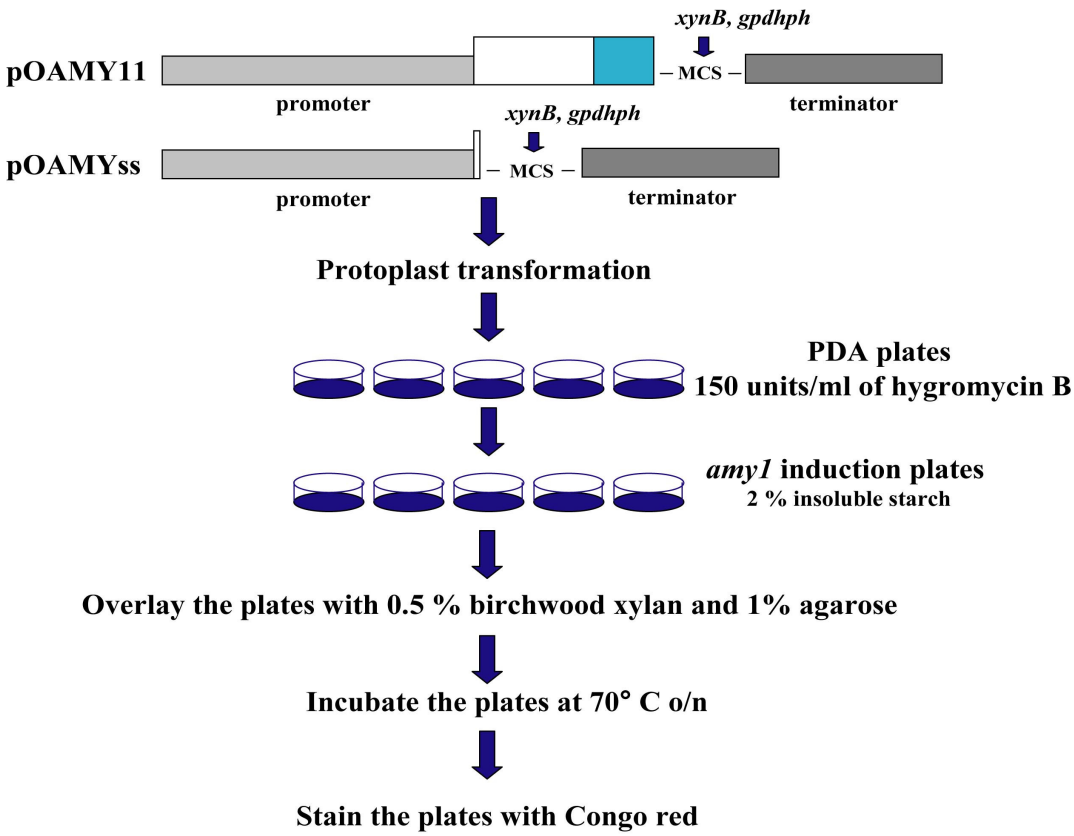

Fig. 20. Schematic diagram of the procedure for an alternative screening approach for *Ophiostoma* transformants. "*xynB*", gene encoding xylanase B; "*gpdhph*", *gpdA* gene promoter and gene conferring hygromycin B resistance; "MCS", mutiple cloning site.

Functionality of the *xynB* gene amplified by PCR from plasmid pHEN54-*xynB* was tested by transforming the recombinant plasmid into *E. coli* DH5α and screening for xylanase activity at 70 °C (Te'o et al., 2000). Around 100 *E. coli* recombinants were screened and all exhibited a clear halo around the colony indicating expression of an active xylanase. Subsequently, the *xynB* gene was inserted into the expression vectors pOAMY11-*gpdhph* containing a partial α-amylase gene sequence and pOAMYss-*dsRedgpdhph* containing the α-amylase secretion signal in place of the *dsRed* gene. The vectors were transformed into MQ.5.1 by two rounds of protoplast transformation and around 400 candidate transformants representing each expression cassette were screened. All 400 colonies were transferred from hygromycin B selection plates onto plates containing insoluble starch to induce the *amy1* promoter under which the *xynB* gene will be expressed. At this point, a clear halo appeared around each colony

after 2 to 3 d incubation due to degradation of starch by endogenous amylolytic enzymes.

The plates were then overlaid with 0.5 % (w/v) birchwood xylan and 1 % (w/v) agarose, incubated at 70ºC overnight and flooded with Congo Red. A clear halo around the colony against the red background should now indicate expression of an active thermotolerant xylanase under the *amy1* promoter. However, because of the background of underlying haloes originating from starch degradation (see above), it was difficult to distinguish whether a halo around each colony after the xylan overlay was a result of degradation of the xylan substrate or background only (Fig. 21). For example, there was a halo around the nontransformed strain MQ.5.1 derived from the degrading of starch by amylases. Even though the screening results were not conclusive, it was established that 30 potential transformants obtained from the transformation with pOAMY11-*xynBgpdhph* and 41 from the transformation with pOAMYss-*xynBgpdhph* exhibited haloes that were considerably larger than those around non-transformant colonies plated as a control. Consequently, ten colonies which exhibited the largest haloes from transformation with each vector were selected for cultivation in a liquid medium to confirm xylanase production and therefore successful transformation of *O. floccosum* MQ.5.1. Xylanase activity in the culture supernatant of the twenty strains was measured and one transformant (SS41), which showed up to 24 times higher xylanase activity than any other strain tested (data not shown) was chosen for further analysis.

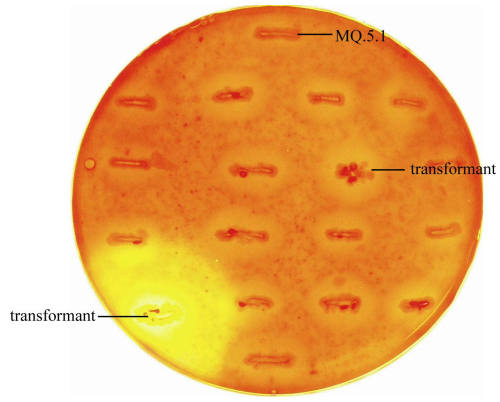

Fig. 21 Screening for thermostable xylanase activity on the starch induction plates. MQ.5.1, non-transformed strain.

Southern blot analysis (Fig. 2, publication III) revealed that at least three copies of the *xynB* gene had been integrated into the genome of transformant SS41, also partly explaining the considerable xylanase activity in the culture supernatant. The effect of soluble and insoluble starch and different pH conditions (pH 5.5, 6.0 and 6.5) were then studied on xylanase production by SS41. Production of xylanase B by *Ophiostoma* was not much different at pH 5.5, 6.0 and 6.5. However, insoluble starch seemed to support production of the recombinant xylanase: xylanase acitivity in the culture supernatant of SS41 in ISSM (2 % insoulble starch with 1.5 % soy bean flour) was more than 1.5 times higher than in SSM (3 % soluble starch with 1.5 % soybean flour). Growth on insoluble substrates is characteristic for *Ophiostoma* spp. in the nature. *Ophiostoma* spp. can grow in the resin ducts, move from cells to cells and utilize the available nutrients nearby (Abraham and Breuil, 1996; Behrendt et al., 1995 a,b).

Expression of the *xynB* gene in SS41 was also investigated at the transcriptional level and two signals were detected by Northern blot analysis. Generation of the two *xynB* transcripts similar in length could be due to different polyadenylation events. Three *amy1* transcripts were generated from the single *amy1* gene locus and their polyadenylation sites located at various positions (publication I). It was possible that transcription of the *xynB* gene under the regulation of *amy1* gene promoter was terminated at different positions and then polyadenylation occurred. Therefore, different sizes of the *xynB* transcript were generated.

Xylanase activity was also visualized by a zymogram activity assay (Fig. 4, publication III) and two proteins with *Mr* 30 and 27 kDa were detected which exhibited xylanase activity. These proteins were larger than the predicted molecular weight based on the gene sequence. Their occurence can be explained, if the AMYI signal peptide had not been cleaved from the xylanase and/or if the xylanase enzyme was post-translationally modified e.g. by glycosylation. There are two possible *N*-linked glycosylation sites at the N-terminus of the recombinant xylanase B (Dr. Te'o, personal communication). Potential glycosylation was not explored further in this study but glycosylated proteins could be visualized in gel using fluorescent probes (Ge et al., 2004). Despite the presence of multiple forms, the modified Xylanase B proteins remained active.

3.6.1 Summary

All results indicated that the *O. floccosum* mutant MQ.5.1 is capable of producing an active recombinant protein encoded by the *xynB* gene originating from the thermophilic bacterium *D. thermophilum* and that this activity can be applied as an indicator for successful transformation of *Ophiostoma*. Importantly, almost all potential transformants growing on the hygromycin B selection plates could be screened further for a conclusive result. Therefore, the chance of capturing true transformants was higher than using the previous strategy using hygromycin B selection only discussed in section 3.5. More importantly, the candidates screened by this approach were not only transformants which expressed the heterologous gene product, but could also secrete an active recombinant protein into the culture medium. In conclusion, an alternative strategy for detecting *Ophiostoma* transformants, based on a plate-assay for the expression of a thermostacble xylanase has been developed. This approach is quick, efficient and straightforward because the screening can be carried out on plates without any need to resort to PCR, Southern, or Northern analysis. Other genes may be introduced into *Ophiostoma* by cotransformation with the vector contaning the *xynB* gene. Meanwhile, this is the first report describing successful production of a thermotolerant foreign enzyme in *Ophiostoma* spp.