

**Detection and identification of  
extracellular proteases in  
*Trichoderma reesei***

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## Abstract

A major factor affecting the yield of heterologous protein production from filamentous fungi is the degradation of the target protein by proteases produced by the host organism. One approach to reduce this degradation by proteases is the identification of the proteases produced from the fungi followed by inactivation of the gene(s) encoding the harmful proteases. This project involved characterisation of the protease profile of three strains of the filamentous fungus *Trichoderma reesei*: QM6a (wild type), Rut-C30 (transformation host) and CVt (producing a heterologous Venus protein). Firstly, zymography was used to allow comparison of extracellular proteases produced from these three strains. Then, liquid assays using specific fluorescent substrates, and mass spectrometric analysis were used to identify the extracellular protease types secreted by the Rut-C30 and CVt strains. Both strains of *T. reesei* examined were found to produce extracellular proteases that were active across neutral to acidic pH range. Liquid protease activity assays using specific substrates and inhibitors for each protease type showed that there was a resemblance in the protease types which were secreted by *T. reesei* (Rut-C30 and CVt) strains. Aspartic proteases, chymotrypsin-like proteases and subtilisin-like proteases activities were detected by this method. However, there was higher aspartic protease activity in Rut-C30 supernatants whereas there was higher chymotrypsin-like protease and subtilisin-like protease activities in CVt supernatants. In addition, mass spectrometric analysis (MALDI TOF/TOF MS/MS and LC ESI MS/MS) showed both similarities and differences in the types of proteases between the two strains. Aspartic protease, chymotrypsin-like protease and subtilisin-like protease were identified from both strains. However, three proteases (carboxypeptidase, metallopeptidases and serine protease) were detected from the CVt strain only. This project paves the way for molecular engineering of protease deficient production hosts.

## **Declaration**

I certify that the research presented in this thesis is original work carried out by the author. The work has not been presented for a higher degree to any university or institution other than Macquarie University, and contains no material previously published or written by any other person except where due reference is made in the text.

Hana Faisal Bali

## Abbreviations

CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
DTT	dithiothreitol
ESI	electrospray ionisation
FU	fluorescence units
IEF	isoelectric focusing
IPG	immobilised pH gradient
kDa	kilodaltons
LC	liquid chromatography
MALDI	matrix assisted laser desorption ionisation
MCA	7-amino-4-methylcoumarin
MS	mass spectrometry
MS/MS	tandem mass spectrometry
NCBI	National Center for Biotechnology Information
PAGE	polyacrylamide gel electrophoresis
PDA	potato dextrose agar
PMF	peptide mass fingerprinting
Q-TOF	quadrupole time-of-flight
SDS	sodium dodecyl sulphate
TFA	trifluoroacetic acid
v/v	volume/volume
w/v	weight/volume

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## Chapter 1: Introduction

*Trichoderma reesei* is a filamentous fungus currently exploited for the large-scale production of proteins (mainly enzymes) applicable to industrial processes. The number of recombinant proteins of interest is increasing and developing to areas such as biopharmaceuticals. As a consequence, the *T. reesei* expression system requires further optimisation and diversification to cater for the specific requirements of each commercially-relevant protein. In particular, there is a need to identify the proteases that cause the degradation of heterologous proteins. This chapter provides the background on *T. reesei* as a production host and an outline of the different kinds of proteases produced by this fungus.

### 1.1 The potential of filamentous fungi for industrial applications

Filamentous fungi belong to a diverse group of lower eukaryotic microorganisms. They have a number of properties that make them important both scientifically and economically. The economic importance can be illustrated by the various products that are secreted by filamentous fungi, such as enzymes, organic acids, polysaccharides, alkaloids, pigments, mycotoxins, and antibiotics, as reviewed by Nevalainen (1985). Filamentous fungi produce a broad spectrum of extracellular enzymes accounting for around 40 % of enzymes used in industry (Lowe, 1992; Penttilä *et al.*, 2004), in a global market worth over \$ 2 billion dollars (Nevalainen and Te'o, 2003). Fungal enzymes are used in the production of detergents, textiles, starch, baking goods, animal feed, beverages, brewing and dairy products (Saxena *et al.*, 2004).

The fact that fungi can secrete a large amount of proteins (mainly hydrolytic enzymes) into the culture medium has an important advantage for industry; the secretion makes the harvesting of desired products easier since there is no need to break the cells and to eliminate the intracellular proteins, a process that is normally tedious and expensive. Also, filamentous fungi perform post-translational modification (glycosylation, folding, proteolytic cleavage) of gene products in a way analogous to higher eukaryotes, which is very important if mammalian proteins are to be expressed. Moreover, fungi can be grown on a cheap and undefined medium which makes large-scale protein production economically feasible. The secreted levels of native proteins are high in optimal production environments; for example, *Aspergillus niger* can produce more than 20 grams of glucoamylase per litre (Berka *et al.*, 1990; Xin *et al.*, 2010) and *T. reesei* has been shown to produce hydrolases at up to 100 grams per litre (Cherry and Fidantsef, 2003). Filamentous fungi have also been used as host organisms for the production of heterologous gene products (Nevalainen *et al.*, 2005). For example, calf chymosin has been produced in *A. awamori* and *T. reesei* (Harkki *et al.*, 1989; Uusitalo *et al.*, 1991; Schuster and Schmoll, 2010) and mouse antibody (Fab) fragments in *T. reesei* (Nyyssönen *et al.*, 1993; Schuster and Schmoll 2010). Several heterologous enzymes have been successfully expressed in some strains of filamentous fungi, but the secreted yields of these enzymes rarely reach grams per liter. Stable recombinants can be isolated from filamentous fungi thus enabling controlled strain improvement. Therefore, fungi continue to be investigated for development as protein expression systems. Table 1-1 contains a number of industrially important products made by filamentous fungi (Gibbs *et al.*, 2000).

**Table 1-1** Some industrial products made by fermentations with filamentous fungi. Adapted from Gibbs *et al.*, 2000 with some modifications.

Product	Microorganism	Reference
<b><u>Antibiotics</u></b>		
Penicillin G and V	<i>Penicillium chrysogenum</i>	van den Berg <i>et al.</i> , 2008
Cephalosporin C	<i>Cephalosporium acremonium</i>	van de Kamp <i>et al.</i> , 1999 Elander <i>et al.</i> , 2003
Griseofulvin	<i>Penicillium patulum</i>	Gibbs <i>et al.</i> , 2000
<b><u>Enzymes</u></b>		
Glucose oxidase, pectinase and phytase	<i>Aspergillus niger</i>	Wiebe, 2003
Xylanase and invertase	<i>Aspergillus awamori</i>	Hölker <i>et al.</i> , 2004
α-amylase and glucoamylase	<i>Aspergillus oryzae</i>	te Biesebeke <i>et al.</i> , 2005
Cellulase, xylanase and endoglucanase	<i>Trichoderma sp.</i>	Polizeli <i>et al.</i> , 2005 Dutta <i>et al.</i> , 2008
<b><u>Mycotoxins</u></b>		
Aflatoxins, citrinin and ochratoxin	<i>Aspergillus sp.</i>	Torelli <i>et al.</i> , 2006
Trichothecenes and zearalanone	<i>Fusarium sp.</i>	Gibbs <i>et al.</i> , 2000
Citrinin, ochratoxin	<i>Penicillium sp.</i>	Greenhill <i>et al.</i> , 2008

**Table 1-1** (continued)

<b><u>Other native fungal products</u></b>		
Riboflavin	<i>Ashbya gossypii</i>	Lim <i>et al.</i> , 2001 Park <i>et al.</i> , 2006
Citric and gluconic acid	<i>Aspergillus niger</i>	Schuster <i>et al.</i> , 2002 Magnuson and Lasure, 2004 Ramachandran <i>et al.</i> , 2006
Kojic acid and biotin	<i>Aspergillus oryzae</i>	Krishna, 2005
<b><u>Recombinant heterologous proteins</u></b>		
Fab antibody fragments	<i>Trichoderma reesei</i>	Nyyssönen <i>et al.</i> , 1993
Human interleukin-6	<i>Aspergillus niger</i>	Punt <i>et al.</i> , 2002 Grimm <i>et al.</i> , 2005
Tissue plasminogen activator (tPA)	<i>Aspergillus niger</i>	Nevalainen <i>et al.</i> , 2005
Thermophilic xylanase	<i>Trichoderma reesei</i>	Mäntylä <i>et al.</i> , 2007

## 1.2 *Trichoderma* species

*Trichoderma* species are filamentous, soft – rot fungi. They are ubiquitous colonisers of cellulosic materials and are thus abundant on decaying plant material and wood (Kubicek *et al.*, 2008; Jaklitsch 2009). They also can occur in the rhizosphere of plants where they can induce systemic resistance against pathogens (Harman 2000). *Trichoderma* species are characterised by rapid growth, typically bright green conidia (occasionally yellow) and a highly branched conidiophore structure (Gams and Bissett 1998; Schuster and Schmoll, 2010). They are able to grow in different natural

environments such as the rich and diversified habitat of a tropical rainforest as well as in a fermentor in a laboratory. This is because *Trichoderma* species respond to their environment by regulation of growth, conidiation and enzyme production, and hence adjust their lifestyle to prevailing conditions. Most of this genus is saprophytic but some species are capable of mycoparasitism (Klein and Eveleigh, 1998; Schuster and Schmoll, 2010).

Some *Trichoderma* species (such as *T. harzianum*, *T. virens* and *T. asperellum*) are suitable as biocontrol agents against fungal and bacterial diseases on agricultural crops (Benitez *et al.*, 2004). Some species have been shown to be opportunistic plant symbionts, enhancing systemic resistance of plants (Yedidia, 1999; Ruocco *et al.*, 2009).

*Trichoderma* species have been used for production of food additives and related products (Blumenthal, 2004). Various *Trichoderma* enzymes are applied to improve the brewing process ( $\beta$ -glucanases), as macerating enzymes in fruit juice production (pectinases, cellulases and hemicellulases), as feed additive in livestock farming (xylanases) and for the production of pet food (Schuster and Schmoll, 2010).

### **1.3 *Trichoderma reesei***

*Trichoderma reesei* was first identified as the culprit organism that caused a rapid degradation of US military canvas tents and cotton clothing in Bougainville during World War II (Linko, 1993). The parent strain of practically all industrial *T. reesei* production strains is QM6a (Mandels and Reese, 1957). From the time of its discovery until 1977, this isolate was classified as *T. viride*, but it has since been characterised as a distinct species and renamed *T. reesei* (Simmons *et al.*, 1977).

*T. reesei* is used for large scale production of proteins due to its secretion capacity and cheap and easy cultivation. Also, molecular tools and methods are available to produce recombinant strains of *T. reesei* for industrial protein production (Seidl *et al.*, 2006). *T. reesei* can be regarded as a safe host organism and many enzyme preparations produced by recombinant strains have been assigned approved status by appropriate national or international authorities (reviewed by Nevalainen *et al.*, 1994).

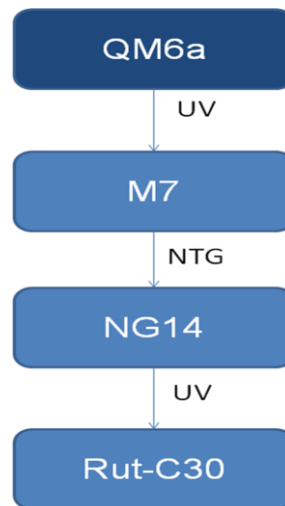
*T. reesei* degrades plant material by secreting numerous enzymes (such as cellbiohydrolases, endoglucanases,  $\beta$ -glucosidases, and xylanases), which act synergistically in the hydrolysis of cellulose and hemicellulose to oligosaccharides and monomeric sugars. Some of the current research with *T. reesei* is focused on improvement of the efficiency of cellulase production in order to decrease the cost of the production of bioethanol (biofuel) from cellulosic waste material (Kumar *et al.*, 2008). *T. reesei* strains that are used in industry to produce cellulases are genetically engineered by targeted genetic modifications such as efficient use of inducible promoters, increased gene copy numbers, or removal of genes encoding undesired secreted proteins (Nevalainen *et al.*, 1994; Jun *et al.*, 2009).

Recently, the potential of *T. reesei* has been investigated for the heterologous production of antibodies (Joosten *et al.*, 2003) with a view of incorporating the antibodies into a whole range of everyday products such as shampoos and skin care creams for targeting the active ingredients to the specific surface.

*T. reesei* Rut-C30 was produced as a result of a random mutagenesis and screening program for high cellulase production (Montenecourt and Eveleigh, 1979). The strain

Rut-C30 produces around 35 grams of extracellular protein per liter which is approximately four times more than the parent QM6a strain (Durand and Clanet, 1988; Margolles-Clark *et al.*, 1997; Jun *et al.*, 2009). The extracellular cellulolytic system of *T. reesei* is composed of 60-80 % cellobiohydrolases (exoglucanases), 20-36 % endoglucanases and 1 %  $\beta$ -glucosidases, all of which act synergistically in the conversion of cellulose into glucose (Zaldívar *et al.*, 2001; Muthuvelayudham and Viruthagiri, 2006).

*T. reesei* Rut-C30 is the best characterised publicly available *T. reesei* strain. It was generated by three mutagenesis steps (Figure 1.1). The first step was an ultra- violet light (UV) mutagenesis of QM6a leading to isolation of the M7 strain, which was mutagenised further with N-nitrosoguanidine (NTG) to produce the NG14 strain (Montenecourt and Eveleigh, 1979). UV mutagenesis was further applied on NG14 and Rut-C30, a catabolite repression deficient mutant, was isolated. This strain has been reported to contain a truncated carbon catabolite repressor gene *cre I* (deletion of ~ 2.5 kb in the *cre I* gene mediating glucose repression) and a frame-shift mutation in the  $\beta$ -glucosidase II encoding gene. The genome lacks an 85 kb DNA fragment in the scaffold 15, and as a result misses 29 genes in comparison with the wild type QM6a (Seidl *et al.*, 2008). The strain is known as a low protease producer (Sheir-Neiss and Montenecourt, 1984) and exhibits 11 % of the total protease activity of the wild-type QM6a (Peterson *et al.*, 2011). However, there has been no published characterization of the types of proteases *T. reesei* Rut-C30 or the wild-type QM6a secretes.



**Figure 1.1** Pedigree of *T. reesei* Rut-C30 and its relationship to the wild type isolate QM6a (Montenecourt and Eveleigh, 1979). Mutagens used are marked as UV (ultra-violet light) and NTG (N-nitrosoguanidine).

*T. reesei* mutant strains are frequently used as production hosts for recombinant proteins (Nevalainen *et al.*, 2005). For example, *T. reesei* Rut-C30 has been used as surrogate host for production of an endochitinase from *T. harzianum*. The endochitinase gene *P1* was isolated and over expressed in Rut-C30 under the promoter of the major cellulase gene (*cbh I*). The endochitinase activity in the recombinant strain represented about 20 fold increase over the endochitinase activity produced by *T. harzianum* (Margolles-Clark *et al.*, 1996a).

In previous work carried out in the EDGE (Enzyme Development and Gene Expression) laboratory, *T. reesei* Rut-C30 was used as a transformation host to generate a recombinant strain producing a cellobiohydrolase I – Venus fusion protein where Venus represents a protein heterologous to *Trichoderma* (Kautto, 2009). Venus protein is a genetic mutant of green fluorescent protein, derived from *Aequorea victoria*. Its molecular weight is 27 kDa, its excitation peak is 514 nm and its emission



peak is 527 nm (Rekas *et al.*, 2002). The resulting *T. reesei* CVt transformant strain was used in the current work to explore the production of specific proteases by a recombinant strain expressing a heterologous protein. These results were compared to the proteases produced from a non-transformant *T. reesei* Rut-C30 strain.

To optimise *T. reesei* as host for the production of heterologous proteins, it is necessary to have an understanding of the basic fungal biology of protein secretion.

#### **1.4 The secretory pathway of *T. reesei***

Efficient secretion is one of the most important features of filamentous fungi that make them attractive as hosts for protein production (Nevalainen *et al.*, 2005). To date, the secretory pathway of *T. reesei* has not been described in detail. A general model for secretion in eukaryotic microorganisms has been developed through experimental studies of *Saccharomyces cerevisiae* (Diener *et al.*, 2004). Briefly, after the DNA transcription, which occurs in the nucleus, the mRNA is translated to protein after leaving the nucleus. The newly made protein interacts with chaperone molecules allowing folding on the site of the endoplasmic reticulum (ER). The protein leaves the ER in transport vesicles and moves to the Golgi, where the protein is modified further by glycosylation and packed into secretory vesicles. These secretory vesicles fuse with the cytoplasmic membrane, and open out releasing their entire contents into the extracellular space (Nevalainen *et al.*, 2005; Conesa *et al.*, 2001).

#### **1.5 The effects of proteases on protein production**

One of the major causes of lowering the yields of a foreign protein produced in a heterologous system is its degradation by proteases. Proteolysis can occur within the secretory pathway or following secretion (van den Hombergh *et al.*, 1997; O'Donnell

*et al.*, 2001). Modifications to the protease profile can be achieved via traditional mutagenesis and screening, or targeted gene manipulation (Mäntylä *et al.*, 1998; Saloheimo and Pakul, 2012). Isolation of particular protease encoding genes allows for specific manipulation and inactivation of these loci; for example deletion of the gene encoding the aspartic protease trichodermapepsin from *T. reesei* resulted in a 94% decrease in acid protease activity (Mäntylä *et al.*, 1998). Notably, deletion of the *pepA* gene (aspartic protease gene) increased bovine prochymosin production in *T. reesei* by more than 66% (Berka *et al.*, 1990; Wang *et al.*, 2008) and reduced the degradation of over expressed thaumatin in *A. niger* (Moralejo *et al.*, 2000).

The protease profile of *A. niger* has been partially characterized (Van den Hombergh *et al.*, 1997). These proteases include aspartic, serine, and carboxy proteases, which are similar to proteases that have been identified from *T. reesei* supernatants (Bradford, 2000). Some studies have been carried out with *Aspergillus* addressing the effect of proteases on the yield of recombinant proteins. For example, the recombinant *A. niger* B1-D was genetically modified to secrete hen egg white lysozyme (HEWL) and the effects of bioprocess parameters on extracellular proteases of this strain were studied. A significant reduction in HEWL production occurred as a result of increased activity of extracellular proteases in the cultures, correlating to rising temperature (thermal stress) in early exponential phase. The proteases were predominantly acid proteases (aspartic), in addition to serine-, cysteine- and metallo- proteases (Li *et al.*, 2007). However, no studies assessing the impact of extracellular proteases from *Trichoderma* on recombinant protein production have been published. Although heterologous fungal proteins can be expressed in *T. reesei*, the proteases still seem to cause degradation which reduces

the protein yield (Nevalainen *et al.*, 2005). The reduction of protease activity therefore seems important in achieving higher production yields of heterologous proteins. In addition to protease deficient strains generated via random mutagenesis or by genetic engineering, bio-processing techniques, such as downstream processing at low temperatures, the early separation of product from proteases, pH control and the use of protease inhibitors, have been used to reduce the proteolysis. The spectrum of proteases in individual species can be very diverse and species-specific. Therefore, the analysis of the extracellular proteases to reveal the dominant protease type is very important when working towards enhanced yields of heterologous proteins.

## **1.6 Types of proteases**

Proteases are secreted naturally by filamentous fungi to hydrolyse proteins. They play various roles in cellular metabolism, nutrition and morphogenesis (Archer and Peberdy, 1997; Delgado-Jarana *et al.*, 2002). Proteases are classified as exopeptidases, which cleave one or a few amino acids from the N- or C- terminus, or endopeptidases, which cleave internally in polypeptide chains (also called proteinases). They are four main types of proteases: aspartyl, serine, cysteine and metalloproteases according to the nature of their catalytic active site (Table 1-2).

**Table 1-2** The range of pHs at which particular classes of proteases are active (Uhlir, 1990), and their specific inhibitors.

<b>Class</b>	<b>Catalytic residue in active site</b>	<b>pH range</b>	<b>Specific inhibitor*</b>
<b>Aspartyl</b>	Aspartic acid	Acidic	<b>Pepstatin A</b> <b><math>\alpha</math>2-Macroglobulin</b>
<b>Serine</b> • Chymotrypsin • Trypsin • Elastase • Subtilisin	Serine	Neutral-alkaline	<b>AEBSF</b> (4-2-aminoethyl-benzenesulfonyl fluoride hydrochloride) <b>EACA</b> (6-Aminohexanoic acid) <b>Aprotinin (BPTI)</b> basic pancreatic trypsin inhibitor <b>Phosphoramidon disodium salt</b> <b>Bestatin hydrochloride</b>
<b>Cysteine</b>	Cysteine	Acidic-neutral	<b>E-64</b> (2S,3S -trans-epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester) <b>N-Ethylmaleimide</b>
<b>Metallo-protease</b>	Metal ion - $Zn^{2+}$	Acidic-neutral	<b>EDTA-<math>Na_2</math></b> (disodium ethylenediaminetetra-acetate) <b>1,10-phenanthroline</b>

\* Some examples of specific inhibitors for each protease class.

## **1.7 Strategies to identify extracellular proteases in culture supernatants**

Strategies that can be employed to identify extracellular proteases in culture supernatants include zymography, protease assays, and mass spectrometry.

### **1.7.1 One and two -dimensional gel electrophoresis and zymography**

The extracellular proteases in the culture supernatant can be separated by one-dimensional (1D) and two-dimensional (2D) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). In 1D electrophoresis proteins are separated according to molecular weight only, while in 2D electrophoresis proteins are separated according to molecular weight and isoelectric point (pI). These are common and efficient methods of separating the proteins according to their electrophoretic mobility (Choi *et al.*, 2001; Walker, 2005).

Zymography is a technique based on electrophoretic separation on an SDS-PAGE gel that contains a substrate for the enzyme of interest (eg. gelatin, casein, or fibrin for proteases) copolymerised with the polyacrylamide gel. Zymography has been used to detect the activity of a variety of enzymes such as xylanases, lipases, chitinases and proteases (Lantz and Ciborowski, 1994; Sharma *et al.*, 2008; Soni *et al.*, 2008; Peterson *et al.*, 2011a). The substrate is degraded by the enzymes that are renatured after the electrophoretic separation. In the case of protease zymograms, this results in white bands or spots (1D or 2D zymogram respectively) against dark background when stained with Coomassie Blue dye.

### 1.7.2 Protease assays

Assays using specific protease substrates and inhibitors (Table 1-2) can be applied to determine the different protease types in a culture supernatant (Table 1-3). These substrates are peptides combined with 7-amino-4-methylcoumarin (MCA), so when a protease cleaves the specific peptide chain, the MCA will fluoresce at a peak excitation wavelength 380 nm and emit at a peak of 460 nm. An absorbance assay for chymotrypsin activity is used where the absorbance is measured at 410 nm (Goller *et al.*, 1998; Weder *et al.*, 1993). Fluorescence and absorbance based assays have been used previously to identify extracellular proteases in *T. reesei* (Goller *et al.*, 1998; Bradford, 2000). Information gained by this approach can then be used in the future to identify the genes encoding particular proteases to eliminate them in order to enhance the yields of heterologous proteins produced in fungi.

**Table 1-3** Protease types and commercially available specific substrates (Sigma-Aldrich, Australia). Commercially available enzymes that can serve as positive controls for each protease type are also shown.

Protease type	Specific substrate	Positive control enzyme
<b>Aspartyl</b>	Boc- Leu- Ser- Thr- Arg- amino-4-methylcoumarin	Pepsin from porcine gastric mucosa
<b>Cysteine</b>	Z- Arg- Arg 7-amino-4-methylcoumarin	Papain from <i>Papaya latex</i>
<b>Metallo-proteases</b>		
▪ <b>Collagenase</b>	N-Suc-Gly-Pro-Leu-Gly-Pro 7-amino-4-methylcoumarin	Collagenase from <i>Clostridium histolyticum</i>
▪ <b>Non-collagenase</b>	N- Suc- Ala-Ala-Phe 7-amino-4- methylcoumarin	Metallo-enzyme type IX from <i>Bacillus polymyxa</i>
<b>Serine</b>		
▪ <b>Trypsin</b>	NA-CBZ-L-Arg 7-amino-4-methylcoumarin	Trypsin from bovine pancreas
▪ <b>Chymotrypsin</b>	N- Benzoyl- L- Tyrosine- P- nitroanilide	Bovine pancreas alpha-chymotrypsin
▪ <b>Elastase</b>	N-Suc-Ala-Ala-Pro- Phe 7-amino-4-methylcoumarin	Elastase type 1 from porcine pancreas
▪ <b>Subtilisin</b>	N- Suc- Ala- Ala- Ala 7-amino-4-methylcoumarin	Subtilisin A from <i>Bacillus</i> sp

### **1.7.3 Protein identification by mass spectrometry**

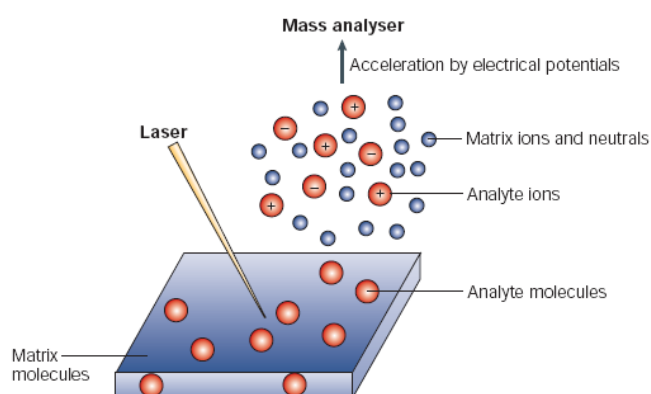
Mass spectrometry is a high throughput method for identifying proteins. It is a very accurate method that weighs peptides after digestion with a suitable enzyme (trypsin) and the masses of these peptides can then be matched against the theoretical peptide masses of known proteins for identification. The two types of mass spectrometry used in the current study were matrix-assisted laser desorption/ionisation time-of-flight tandem mass spectrometry (MALDI-TOF/TOF MS/MS) and liquid chromatography electrospray ionisation tandem mass spectrometry (LC ESI MS/MS). MALDI-TOF/TOF MS/MS mass spectrometers are hybrid mass spectrometers that combine a MALDI ion source with a TOF analyzer, a CID fragmentation cell and another TOF analyser to produce MS/MS fragmentation spectra (Gogichaeva *et al.*, 2007). LC ESI MS/MS involves separation of peptides according to their hydrophobicity prior to electrospray ionisation and fragmentation (Chernushevich *et al.*, 2001). These two methods have been used widely to identify proteins in *Trichoderma* spp., for example in the identification of a novel aspartic protease secreted by *T. harzianum* (Suárez *et al.*, 2005), and the identification of subunits of the *T. reesei* 20S proteasome (Grinyer *et al.*, 2006; Kim *et al.*, 2007).

#### **1.7.3.1 MALDI-TOF/TOF MS/MS for identification of proteins separated by SDS-PAGE**

MALDI-TOF/TOF MS/MS can be used for identification of protein bands from an electrophoresis gel. Protein bands are excised and digested with a specific protease (trypsin), then mixed with a large excess of ultraviolet absorbing matrix which normally contains a low-molecular weight aromatic acid. The excess matrix molecules transfer the embedded non-volatile analyte molecules into gas phase



when the analyte is irradiated with a focused laser beam of appropriate wavelength (Figure 1.2). Then singly protonated analyte ions are formed which are accelerated by electric potentials into a tandem mass spectrometer. The spectra are used to search databases to identify the proteins (Blackstock and Weir, 1999; Yates, 2000; Steen and Mann, 2004).

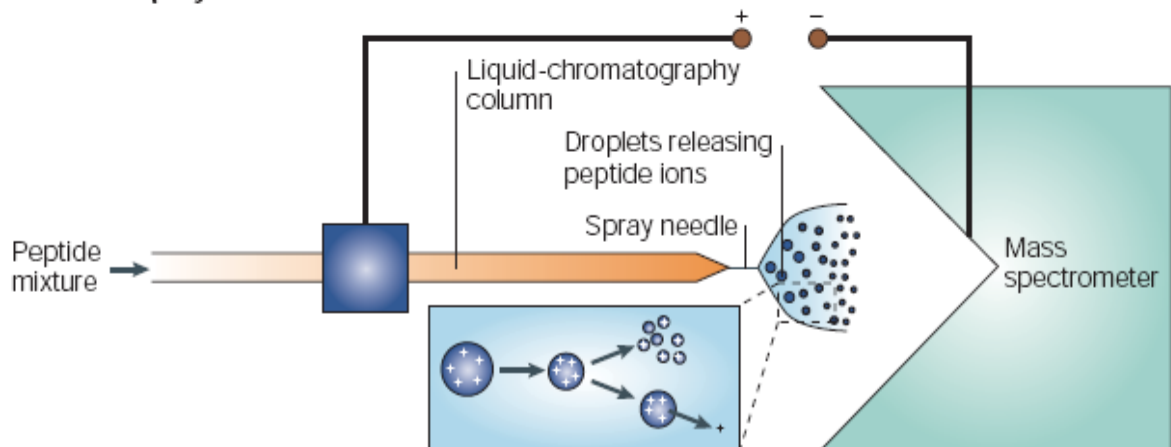


**Figure 1.2** Ionisation in matrix-assisted laser desorption/ionisation time-of-flight tandem mass spectrometry (MALDI- TOF/TOF MS/MS). Steen and Mann (2004).

#### 1.7.3.2 LC ESI MS/MS for identification of proteins separated by SDS-PAGE

The protein bands are excised from an electrophoresis gel and digested with a specific protease (trypsin) to convert the protein to peptides. The peptides are injected onto a micro-scale capillary high- performance liquid chromatography (LC) column (50-150  $\mu\text{m}$  inner diameters). The peptides are eluted according to their hydrophobicity by using a solvent gradient. When the peptides reach the end of the column they flow through a needle (Figure 1.3). At the end of the needle the liquid vaporises and the peptides subsequently become ionised by the action of a strong electrical potential in a process called electrospray ionisation (ESI; Steen and Mann,

2004). Then the ionised peptides are analysed by tandem mass spectrometry (MS/MS).



**Figure 1.3** Ionisation in liquid chromatography electrospray ionisation tandem mass spectrometry (LC ESI MS/MS). Steen and Mann (2004).

### 1.7.3.3 Database searching

The mass spectra obtained from MALDI-TOF/TOF and LC ESI MS/MS can be analysed using the MASCOT search engine (Matrix Science Ltd, London UK; Perkins *et al.*, 1999). The peptide fragmentation pattern is compared to theoretical fragmentation patterns calculated from known proteins in NCBItr ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) or SwissPort ([www.expasy.org/sport/](http://www.expasy.org/sport/)) databases.

## 1.8 Aims of this study

*Trichoderma reesei* shows promise as an expression host for the production of heterologous proteins. However, proteases pose a major problem affecting the yields of foreign gene products thus limiting the use of *T. reesei* as an expression host.

The overall aim of the project was to profile and identify proteases secreted by *Trichoderma reesei* as the first step towards knowledge-based modification of protease profiles.

The specific aims were to:

- 1- Profile secreted proteases of two strains of *T. reesei*, Rut-C30 (transformation host) and CVt (producing a heterologous Venus protein), to allow comparison of extracellular proteases produced by these two strains. This was carried out with zymography.
- 2- Identify the extracellular protease types in the Rut-C30 and CVt strains using specific fluorescent substrates in a liquid assay, and mass spectrometric analysis to identify protein bands excised from protein gels.

## Chapter 2: Materials and Methods

### 2.1 Frequently used reagents, buffers and gels

All chemicals used were of analytical grade and were obtained from Sigma-Aldrich (Australia) unless otherwise stated. Agarose was obtained from Amresco (USA) and Avicel cellulose from Fluka (Germany). The reagents, buffers, and culture medium used throughout this work were prepared using water filtered by Millipore Milli-Q Academic filtration system (MQ water) and sterilised by autoclaving at 121°C for 20 min. Some of the commonly used buffers and solutions are listed in Table 2-1, and commonly used protein gels are listed in Table 2-2.

**Table 2-1** Buffers and solutions used throughout experimental work.

Buffers and solutions	Reagents
<b>Conidia harvesting solution</b>	0.9 % (w/v) NaCl 0.05 % (w/v) Tween 80
<b>Protein gel loading solution / sample buffer (5X)</b>	0.05 M Tris HCl pH 6.8 50 % (w/v) glycerol 12 % (w/v) SDS 0.1 % (w/v) bromophenol blue
<b>Running buffer (10X) for gel electrophoresis</b>	10 mM (w/v) Tris 14 mM (w/v) glycine 1 % (w/v) SDS
<b>Zymogram renaturing buffer</b>	2.5% (v/v) Triton-X
<b>Zymogram developing buffer</b>	15 mM Tris HCl 0.2 M NaCl 5 mM CaCl 0.02 % (v/v) Brij 35
<b>Fixing buffer for protein gels</b>	7 % (v/v) acetic acid 10 % (v/v) methanol
<b>Equilibration solution for two dimensional gels</b>	50 mM (w/v) Tris-HCl pH 8.8 6 M (w/v) urea 30 % (w/v) glycerol 2 % (w/v) SDS Traces of bromophenol blue

**Table 2-2** Protein gels commonly used throughout experimental work.

Protein gel	Contents
<b>12.5 % SDS PAGE gel</b>	12.5 % (w/v) acrylamide:bis (Bio-Rad, Australia) 3.4 mM (w/v) Tris HCl pH 8.8 0.07 % (v/v) TEMED* 0.05 % (w/v) ammonium persulfate 0.4 % (w/v) SDS
<b>Gelatin zymogram</b>	12.5 % (w/v) acrylamide:bis (Bio-Rad, Australia) 0.1 % (w/v) gelatin 3.4 mM (w/v) Tris HCl pH8.8 0.07 % (v/v) TEMED* 0.05 % (w/v) ammonium persulfate 0.4 % (w/v) SDS
<b>Casein zymogram</b>	12.5 % (w/v) acrylamide:bis (Bio-Rad, Australia) 0.1 % (w/v) casein 3.4 mM (w/v) Tris HCl pH8.8 0.07 % (v/v) TEMED* 0.05 % (w/v) ammonium persulfate 0.4 % (w/v) SDS

Contents refer to separating gel only. Stacking gel was 4 % (w/v) acrylamide, 0.74 mM (w/v) Tris-HCl pH 6.8, 0.4 % (w/v) SDS (Laemmli, 1970).

\* Tetramethylethylenediamine.

## 2.2 Growth medium

The *Trichoderma* strains were grown in a medium containing Avicel cellulose, lactose and soybean flour (ALS medium). The medium contained 15 g/L  $\text{KH}_2\text{PO}_4$ , 5 g/L  $(\text{NH}_4)_2\text{SO}_4$ , 10 mL (per liter) 100 x trace elements (100 mg  $\text{FeSO}_4 \times 7\text{H}_2\text{O}$ , 20 mg  $\text{MnSO}_4 \times \text{H}_2\text{O}$ , 20 mg  $\text{ZnSO}_4 \times 7\text{H}_2\text{O}$ , 40 mg  $\text{CoSO}_4 \times 7\text{H}_2\text{O}$  in 200 ml MQ  $\text{H}_2\text{O}$ ; Penttilä *et al.*, 1987), 0.2 % (v/v) Tween 80 (ICI Americas USA), 2 % Avicel (Fluka, Germany) and 1.5 % soy bean flour, with the pH adjusted to 6.5 with 5 M KOH. The solution was autoclaved at 121°C for 20 min before adding (per liter) 50 mL 20 % (w/v) lactose, 2.4 mL 1 M  $\text{MgSO}_4$  and 5.4 mL 1 M  $\text{CaCl}_2$ , which had been pre-sterilised separately.

## **2.3 Fungal strains and culture conditions**

### **2.3.1 *T. reesei* strains**

The strains used were the wild-type *Trichoderma reesei* QM6a (Mandels and Reese, 1957), the low protease strain of *Trichoderma reesei*, Rut-C30 (Montenecourt and Eveleigh 1979) and the recombinant *Trichoderma reesei* CVt expressing the heterologous Venus protein, provided by Dr Liisa Kautto (Kautto, 2009).

### **2.3.2 Cultivation on a solid medium**

Cultures were maintained on potato dextrose agar (PDA) plates that were incubated at 28°C in the dark for 6-7 days. Conidia were collected by flooding the plate with 8 mL of conidia harvesting solution (Table 2-1) and gently abrading with a glass rod spreader. The conidial suspension was filtered through non-absorbent cotton wool to remove any hyphae and other debris. The concentration of conidia within the suspension was calculated using a haemocytometer. Conidial suspensions were used immediately or frozen at -20°C until required.

### **2.3.3 Cultivation in a liquid medium**

Fungi were cultivated in 50 mL of the ALS medium in 250 mL conical flasks. Each 50 mL culture was inoculated with  $1 \times 10^8$  conidia, unless otherwise stated. Cultures were grown (in duplicate) at 28°C on a shaker at 250 rpm for 7 days. On each day, a 2 mL sample from each culture was collected in an Eppendorf tube and centrifuged at  $10,000 \times g$  for 5 min. The supernatants were tested for pH and used for the enzyme assays and zymograms.

## **2.4 Protein assay**

Protein concentration of the sample was measured using the Bio-Rad DC protein assay kit according to the manufacturer's instructions (Lowry *et al.*, 1951). Incubation time was 15 min at room temperature in the dark.

## **2.5 Protein separation gels**

### **2.5.1 One-dimensional electrophoresis (1DE)**

Samples of *T. reesei* Rut-C30 and CVt culture supernatants (as described in Section 2.3.3) were mixed with an equal volume of the electrophoresis sample buffer (Table 2-1) before electrophoresis on 12.5 % (w/v) acrylamide SDS-PAGE gels (Table 2-2), (Laemmli, 1970). The gels were run using 1 x running buffer (Table 2-1) under standard conditions (100 V for 30 min, then 130 V for 50min). Thereafter, the gels were incubated in a fixing buffer for 40 min (Table 2-1). The gels were stained with Coomassie Brilliant Blue G (Bio-Rad) for 4 h and destained with acetic acid (1 % v/v).

### **2.5.2 Two-dimensional electrophoresis (2DE)**

#### **2.5.2.1 2DE Sample preparation**

Samples for 2DE were prepared using *T. reesei* Rut-C30 culture supernatants only. The supernatants (5 mL) were incubated in Trichloroacetic acid (20 % v/v TCA) on ice for 30 min. The precipitate was then collected by centrifugation at 2500 rpm for 15 min at 4°C and precipitated protein washed with acetone and stored in ice for 30 min. After incubation on ice the precipitate was collected once more by centrifugation at 2500 rpm for 15 min at 4°C and dried for 10 min at room temperature to remove the acetone. The precipitated protein pellets were resolubilised by adding 2.5 ml of sample solubilisation buffer (8 M urea, 2 % CHAPS, 1 % w/v DTT) and leaving for 2 h at room temperature. The insoluble material was separated by centrifugation at 2500 rpm for 15 min and the protein supernatant was collected for isoelectric focusing (IEF).

#### **2.5.2.2 The first dimension (iso-electric focusing, IEF)**

Dry strips (immobilised pH gradient IPG strips) were re-hydrated by adding 50 µl of the protein sample (Section 2.5.2.1), 150 µl sample solubilisation buffer and 4 µl

tracking dye to each strip. The strips were left for 6 h at room temperature before subjecting to isoelectric focusing. Prior to the second dimension on SDS-PAGE, the IPG strips were equilibrated in 5 ml equilibration solution (Table 2-1) with DTT (0.1 g) for 15 min. Another equilibration step for 15 min was carried out by adding iodoacetamide (0.25 g) instead of DTT.

### **2.5.2.3 The second dimension (SDS-PAGE)**

The IPG strips were placed horizontally on the SDS-PAGE gels and electrophoresis was carried out under a constant current of 5 mA/gel for 20 min followed by 12 mA/gel for 2 h. The SDS-PAGE gel was run using 1 x Tris-Glycine running buffer (Table 2-1). The gels were stained with Coomassie blue for 6 h and destained with 1 % (v/v) acetic acid (Nandakumar *et al.*, 2006).

## **2.6 Zymogram activity gels**

Zymography was used to profile the protease activity in the *T. reesei* culture supernatants using different protease substrates (gelatin and casein).

### **2.6.1 One-dimensional gelatin zymogram gels**

Protease activity of the *T. reesei* QM6a, Rut-C30 and CVt culture supernatants was first profiled using gelatin zymogram gels (Table 2-2). The gels were run using the running buffer (Table 2-1) at standard running conditions (100 V for 30 min, then 130 V for 50 min). Thereafter, the gel was incubated in the zymogram renaturing buffer for 40 min. The gels were rinsed three times with MQ water followed by equilibration overnight in the zymogram-developing buffer (Table 2-1) at different pHs (3.5, 4.5, 5.5 and 6.5). After incubation, the gels were stained with Coomassie Brilliant Blue G (Bio-Rad) for 4 h and destained with 1 % (v/v) acetic acid (Choi *et al.*, 2001).



In addition, the gelatin zymogram gels were produced under reducing conditions by adding  $\beta$ -mercaptoethanol to the sample buffer before electrophoresis (Table 2-1).

Also, the gelatin zymogram gels were produced using culture supernatant from cultures inoculated with  $3 \times 10^7$  spores per ml in ALS medium under the same conditions described previously in Section 2.3.3.

### **2.6.2 One-dimensional casein zymogram gels**

Protease activity of the culture supernatant was also determined using casein zymogram gels (Table 2-2). The gels were run using the running buffer (Table 2-1) at standard running conditions (100 V for 30 min, then 130 V for 50 min). Thereafter, the gels were incubated in renaturing zymogram buffer for 40 min (Table 2-1). The gels were rinsed three times with MQ water followed by equilibration overnight in zymogram developing buffer at different pHs (3.5 and 6.5). After incubation the gels were stained with Coomassie Brilliant Blue G (Bio-Rad) for 4 h and destained with (1 % v/v) acetic acid (Choi *et al.*, 2001).

### **2.6.3 Two-dimensional gelatin zymogram gels (2D)**

Samples were prepared using *T. reesei* Rut-C30 culture supernatants. The IEF (first dimension) and the electrophoresis were carried out as previously described (Sections 2.5.2.1, 2.5.2.2 and 2.5.2.3). However, in the 2D gelatin zymogram, the SDS-PAGE gels contained 0.1 % (w/v) gelatin (Table 2-2).

After electrophoresis the gels were incubated in renaturing zymogram buffer for 4 h (Table 2-1). The gels were rinsed three times with MQ water followed by equilibration overnight in zymogram developing buffer at pH 6.3 (Table 2-2). After incubation, the gels were stained with Coomassie Brilliant Blue G (Bio-Rad) for 4 h and destained with 1 % (v/v) acetic acid (Nandakumar *et al.*, 2006).

## **2.7 Enzyme activity assays**

Enzyme assays were carried out using serial dilutions of *T. reesei* Rut-C30 and CVt culture supernatants and specific substrates to detect and characterise the types of extracellular protease activity. Assays were carried out using the substrates listed in Table 2-3.

### **2.7.1 Fluorescence assays with specific substrates**

All samples for the fluorescent assays included 50 µl of culture supernatant (at an appropriate dilution) 140 µl of suitable buffer and 10 µl of a fluorescent substrate (Table 2-3). The samples were pipetted into a 96 well black microtitre plate (Greiner Bio One, Germany) and the plate was incubated at 37°C for 10 min in the dark. The fluorescence was determined by a fluorometer (FLUOstar Galaxy, BMG Lab Technologies, Germany) with the excitation and emission wavelengths of 360 nm and 460 nm respectively. Negative controls included all ingredients of the sample mixture except that 50 µl of buffer was used instead of the culture supernatants. Positive controls were as above with sample replaced with 0.1 mg/ml of purified enzyme (Table 2-3).

### **2.7.2 Assays for chymotrypsin activity**

To assay for chymotrypsin activity, the samples contained 50 µl of culture supernatant, 200 µl of suitable buffer and 10 µl of substrate (Table 2-3). The samples were incubated at 37°C for 10 min in the dark and the reaction stopped by the addition of 500 µL of 30 % (v/v) acetic acid. The samples were centrifuged at 16,000 × *g* for 5 min. 200 µl of the supernatant was transferred to a transparent microtitre plate and absorbance measured at 410 nm (Table 2-3).

**Table 2-3** Specific protease substrates, commercially available pure enzymes (positive controls) and the buffers used in this study.

<b>Protease class</b>	<b>Specific substrate<sup>1</sup></b>	<b>Positive control</b>	<b>Buffer</b>	<b>Reference</b>
<b>Aspartic</b>	Boc- Leu- Ser- Thr- Arg- amino- 4- methylcoumarine	Pepsin from porcine gastric mucosa (40 units /mg)	1 M phosphate buffer pH 3	Takeuchi <i>et al.</i> , 1988
<b>Chymotrypsin</b>	N- Benzoyl- L- Tyrosine- P- nitroanilide	Bovine pancreas alpha- chymotrypsin (10-30 units /mg)	0.1mM Tris- HCl buffer pH 8 contain 5 mM CaCl <sub>2</sub>	Novillo <i>et al.</i> , 1997
<b>Cysteine</b>	Z- Arg- Arg 7- amino-4- methylcoumarin	Papain from <i>Papaya latex</i> (10 units /mg)	0.1 M phosphate buffer pH 6.5 containing 20 mM EDTA and 50 mM L- cysteine	Powers <i>et al.</i> , 2000
<b>Subtilisin</b>	N- Suc- Ala- Ala- Ala 7- amino-4- methylcoumarin	Subtilisin A from <i>Bacillus sp</i> (7-15 units /mg)	0.1mM Tris- HCl buffer pH 8 contain 5 mM CaCl <sub>2</sub>	Markaryan <i>et al.</i> , 1996

All substrates and positive controls were obtained from Sigma Aldrich, Australia.

<sup>1</sup>Concentration of all substrates was 0.05 mM.

The fluorescence and chymotrypsin assays were repeated using specific inhibitors for each type of protease (Table 2-4). The inhibitor was added prior to the addition of the substrate.

**Table 2-4** Specific protease inhibitors and final concentrations used in these studies.

<b>Protease class</b>	<b>Inhibitor<sup>1</sup></b>	<b>Concentration</b>
Serine	AEBSF, 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride	0.1 mM
Cysteine	E-64, (2S,3S)-trans-epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester	14 $\mu$ M
Aspartic	Pepstatin	1 $\mu$ M

<sup>1</sup>All inhibitors were obtained from Sigma Aldrich, Australia.

## 2.8 Proteomic analysis of extracellular proteases

Proteomic analysis was performed with the assistance of the Australian Proteome Analysis Facility (APAF Ltd) at Macquarie University.

### 2.8.1 MALDI TOF/TOF MS/MS

The extracellular proteases were subjected to MALDI TOF/TOF MS/MS using protein bands excised from the 1D gelatin zymogram and SDS-PAGE gels.

#### 2.8.1.1 Sample preparation for MALDI TOF/TOF MS/MS

Protein bands displaying activity on the 1D protease zymogram gels were excised. In addition, bands were excised from the 1D SDS-PAGE gels from areas estimated to contain protein that aligned to the bands on the 1D zymogram gels. The gel pieces were destained with acetonitrile/ammonium bicarbonate and incubated at 37°C for 15 min. Destaining was repeated three times and the residual liquid was discarded. Each sample was dried by adding 20  $\mu$ l of 25 mM CAN (ceric ammonium nitrate) and

incubated for 10 min at room temperature. The acetonitrile was removed and the sample incubated at 37 °C until the gel pieces were completely dry. The samples were digested using 25 µl trypsin (15 ng/ul trypsin in 25 Mm (NH<sub>4</sub>)HCO<sub>3</sub>) for 1 h at 4°C, then the excess trypsin solution was removed. The resulting peptides were re-acidified, desalted and concentrated by zip tip (PerfectPure C18, Eppendorf).

#### **2.8.1.2 Data acquisition by MALDI TOF/TOF MS/MS**

Samples were eluted directly onto the sample plate with 1 µl of matrix (α-cyano-4-hydroxy cinnamic acid, 4 mg/ml in 70 % (v/v) acetonitrile, 0.06 % (w/v) trifluoroacetic acid TFA) and allowed to air-dry. The samples were analysed by MALDI TOF/TOF MS/MS on an Applied Biosystems (USA) 4800 Proteomics Analyser. Spectra were acquired in a reflector mode in the mass range of 700 to 3500 Da. The peptides were characterised further by the MS/MS (TOF/TOF) mode for isolation and fragmentation then re-accelerated to measure their masses and intensities. MS and MS/MS spectra from the same sample were merged in a single mascot generic file (mgf) prior to submission for database searching.

#### **2.8.1.3 Data processing following MALDI TOF/TOF MS/MS**

The mgf file generated by MALDI TOF/TOF MS/MS was analysed using the database searching program, Mascot (Matrix Science Ltd, London UK). Peaklists were searched against fungal proteins in the Swiss-Prot (<http://www.ebi.ac.uk/uniprot>) and NCBI nr (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) databases.

## **2.8.2 LC ESI MS/MS**

The extracellular proteases were analysed using LC ESI MS/MS of excised protein bands from 1D SDS-PAGE gels.

### **2.8.2.1 Sample preparation for LC ESI MS/MS**

Protein bands were excised from the 1D SDS-PAGE gels from areas estimated to include protein that aligned to the bands on the 1D zymogram and were cut into 1 mm pieces with a scalpel. Samples were washed and destained with 250 µl of 50 % acetonitrile/100 mM ammonium bicarbonate and incubated with slow vortexing at 37°C for 15 min. For alkylation 50 µl of freshly prepared reducing agent, (25 mM dithiothreitol in 1 mL of 25 mM (NH<sub>4</sub>)HCO<sub>3</sub>, was added and the sample incubated for 30 min at 56°C. Then, 50 µl of reducing reagent (55 mM iodoacetamide in 25 mM (NH<sub>4</sub>)HCO<sub>3</sub>, was added and the sample incubated for 30 min at room temperature in the dark. The gel pieces were washed with 250 µL of MQ water for 15 min, and then washed twice with 250 µl of washing solution (50 % acetonitrile/100 mM ammonium bicarbonate) and incubated at 37°C for 15 min. This step was repeated three times and the residual liquid was discarded. Each sample was dried by adding 20 µl of acetonitrile and incubated for 10 min at room temperature. The acetonitrile was removed and the sample incubated at 37°C until gel pieces were completely dry. The samples were digested by 25 µl of trypsin (15 ng/µL trypsin in 25 mM (NH<sub>4</sub>)HCO<sub>3</sub>, for 1 h at 4°C, then the excess trypsin solution was removed. Ammonium bicarbonate (25 mM (NH<sub>4</sub>)HCO<sub>3</sub>) was added to cover the gel pieces and the sample was incubated for 16 h at 37°C.

### **2.8.2.2 Data acquisition by LC ESI MS/MS**

The sample (40 µl) was injected onto a peptide trap (Michrome peptide Captrap) for pre-concentration and desalted with 0.1 % (v/v) formic acid, 2 % (v/v) acetonitrile at 8 µl/min. The peptide trap was then switched into line with the analytical column (SGE ProteCol C18, 300 Å, 3 µm, 150 µm x 10 cm). Peptides were eluted from the column using a linear solvent gradient with steps from H<sub>2</sub>O: CH<sub>3</sub>CN (100:0; + 0.1 % (v/v) formic acid) at 500 nl/min over a 50 min period (Agilent 1100 nano-LC system). The LC eluent was subjected to positive ion nano-flow electrospray MS analysis on a QSTAR quadrupole time-of-flight mass spectrometer (Applied Biosystems, USA) which was operated in an information-dependent acquisition mode (IDA).

In IDA mode a TOF MS survey scan was acquired (m/z 400-1600, 0.5 second), with the three largest multiply charged ions (counts > 25) in the survey scan sequentially subjected to MS/MS analysis. MS/MS spectra were accumulated for 2 seconds (m/z 100-1600). MS and MS/MS spectra from the same sample were merged in a single mgf file prior to submission for database searching.

### **2.8.2.3 Data processing following LC ESI MS/MS**

The mgf files were analysed using the database searching program, Mascot (Matrix Science Ltd, London UK). Peaklists were searched against Fungi in the NCBI nr database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Currently, the NCBI nr database does not include the *T. reesei* genome or protein sequences derived from it. Therefore, the mass spectra from the LC ESI MS/MS was also searched against a database containing *T. reesei* protein sequences, derived from the *T. reesei* genome, available from the JGI database (<http://genome.jgi-psf.org/Trire2/Trire2.download.ftp.html>).

*T. reesei* protein sequences were uploaded from JGI onto the global proteome machine organisation database (GPM; <http://www.thegpm.org>). Peaklists derived from the mass spectra were searched against the *T. reesei* protein sequences and identifications were made where significant matches were achieved ( $p < 0.05$ ). ExPASy tools “pI/Mw” ([http://expasy.org/tools/pi\\_tool.html](http://expasy.org/tools/pi_tool.html)) were used to determine the pI and MW of identified proteins, “Signal P” (<http://www.cbs.dtu.dk/services/SignalP/>) was used to predict the presence and location of signal peptide cleavage sites in amino acid sequences and PSORT database (<http://www.psort.org/>) was used to determine the sub-cellular localisation of identified proteins.

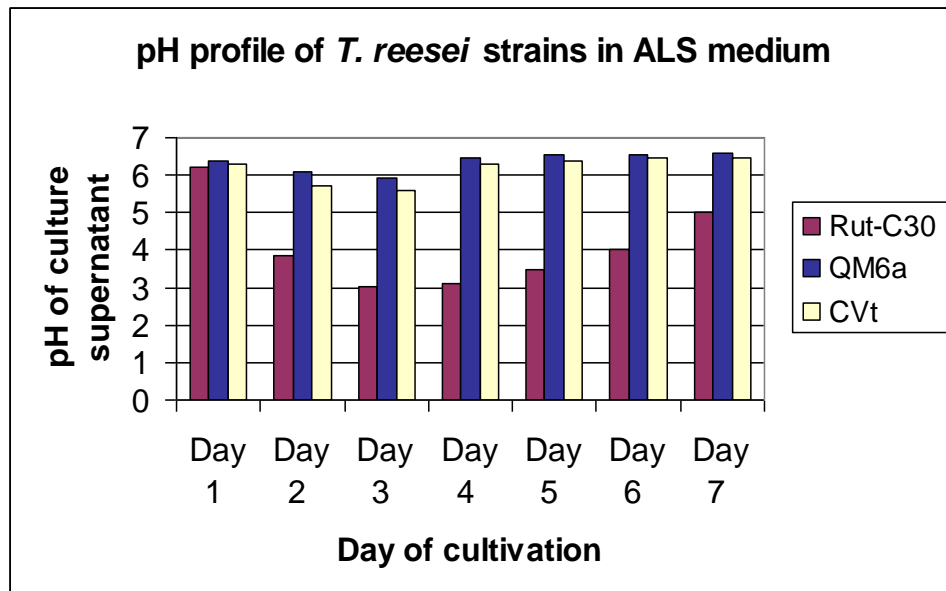


## Chapter 3: Results and Discussion

Extracellular proteases of selected strains of *T. reesei* were studied to reveal and identify the dominant extracellular protease(s) potentially responsible for the degradation of expressed heterologous proteins. Firstly, the pH profiles of the liquid cultures of *T. reesei* QM6a, Rut-C30 and transformant CVt producing the heterologous Venus protein were determined (Section 3.1), and the proteases from the culture supernatants were profiled according to molecular mass using zymogram gels (Section 3.2). Enzyme activity assays with specific substrates were used to determine the types of proteases in the culture supernatants of Rut-C30 and CVt (Section 3.3), and protease identifications were made by mass spectrometry (Section 3.4).

### 3.1 pH profiles of fungal cultures

The pH profiles of the *T. reesei* QM6a, Rut-C30 and CVt cultures were determined across the seven day incubation period (Figure 3.1) to investigate the effect of the fluctuating culture pH (a result of fungal growth) on protease activity. In the culture of Rut-C30, acidic substances were secreted that lowered the pH of the medium (Eneyskaya *et al.*, 1999). The pH dropped rapidly in the early growth phase, reaching its lowest (pH 3) on Day 3. From Day 4, the pH of the Rut-C30 culture medium began to rise again to reach pH 5 on Day 7. In contrast, the pH of the wild type QM6a and CVt cultures dropped only slightly in the early growth phase (Day 3, pH 5.91 and pH 5.61, respectively), and returned to approximate pH 6.5 by Day 7 (Figure 3.1).



**Figure 3.1** The pH profiles of the cultures of *T. reesei* Rut-C30, QM6a and CVt grown in ALS medium at 28 °C for seven days.

The dissimilar pH profiles of the Rut-C30 and QM6a cultures indicated a difference in secretion between the two strains. The variation could reflect the genetic changes in the hypercellulolytic (low protease) *T. reesei* strain Rut-C30 compared to the wild-type QM6a strain (Peterson and Nevalainen, 2012). It was intriguing that the pH profile of CVt, a transformant of Rut-C30 carrying a gene for the fluorescent Venus protein, was also different from the pH profile of its parent strain Rut-C30. The Rut-C30 culture dropped to an acidic pH 3 during the cultivation period, whereas the CVt culture did not drop below pH 5.5, resulting in a similar pH profile to that of the wild-type QM6a. However, the CVt strain was established in this laboratory and the pH profile and growth rate was consistent with that previously observed (Kautto, 2009). Furthermore, concurrent work in the laboratory confirmed the production of the Venus protein by the strain by microscopy.

The discrepancy between the pH profiles of Rut-C30 and CVt could have been a consequence of the slightly retarded growth of the CVt strain compared to Rut-C30. On solid medium (PDA), CVt required 14-15 days to produce a well conidiating culture plate compared to 7 days for Rut-C30. CVt also grew more slowly than Rut-C30 in liquid medium and a smaller mycelial mass was observed. There may have been insufficient time for CVt to secrete a similar amount and type of the acidic substances into the liquid culture medium due to the slower fungal growth pattern. Rapid growth of Rut-C30 and the corresponding drop in pH of the culture supernatant is regularly reported in the laboratory; a slower growth rate and a correspondingly smaller drop in the pH of the supernatants of transformant strains when grown in liquid media has also frequently been observed (eg. Kautto, 2009; Sun, 2012).

The pH profiles of CVt and Rut-C30 have been documented previously using a comparable, cellulase-inducing culture medium (CLS medium, containing minimal salts, trace elements, 1 % (w/v) cellobiose, 1 % (w/v) lactose and 1 % (w/v) soybean flour, pH 6.5; Kautto 2009) with results consistent with those recorded here. It seems apparent that Rut-C30 and CVt strains have different secretion characteristics.

The main protease produced by *T. reesei* is an acidic protease '*Trichoderma*-pepsin' (Eneyskaya, *et al.*, 1999), which has been associated with degradation of heterologous proteins previously (Margolles-Clark *et al.*, 1996b). Because acidic proteases are more likely to be secreted in response to an acidic growth medium (Gente *et al.*, 2001), the drop in pH recorded in the Rut-C30 culture in this study, and the maintenance of a close to neutral pH in the QM6a and CVt cultures, were considered features of particular interest. Whether the variation in the pH profiles between the strains had impacted on the amount and type of protease activity was to be determined in of the next phase of the work.

## **3.2 Profiles of protease activity by zymography**

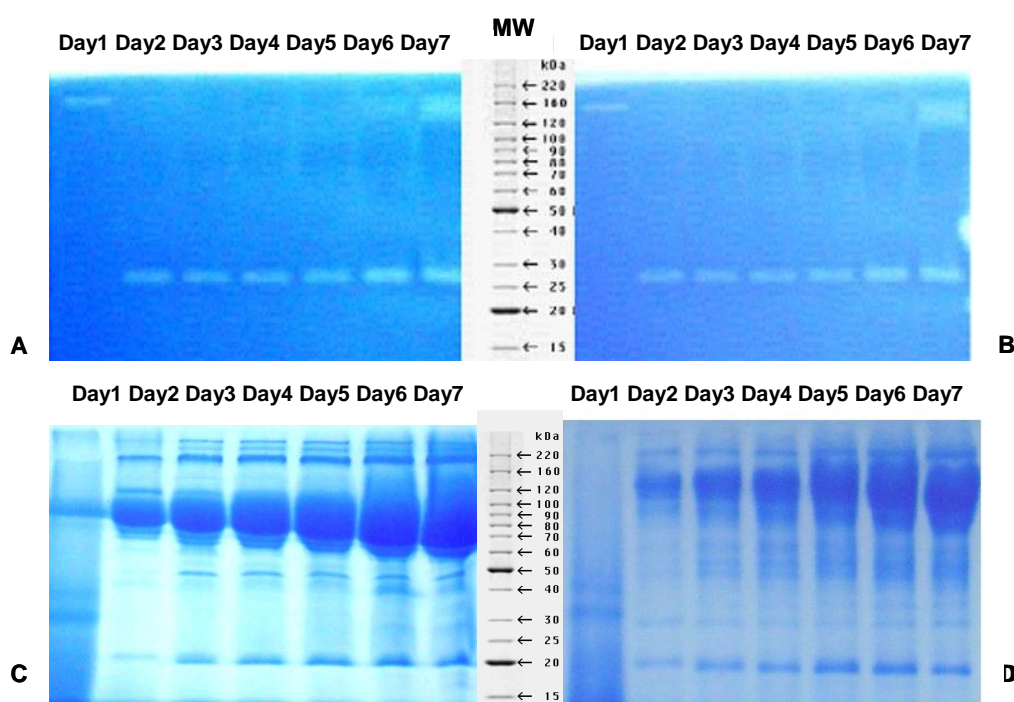
Zymography was employed to profile the extracellular proteases secreted by the *T. reesei* strains under the given growth conditions. Preliminary work was carried out using the Rut-C30 strain only (Section 3.2.1). The purpose of the preliminary work was to develop and fine-tune the zymogram technique, and to gain a base-line understanding of the hypercellulolytic strain Rut-C30 in terms of protease activity in the ALS culture medium (Section 2.2). A suitable sample preparation technique (Section 3.2.1.1), protein substrate (Section 3.2.1.2) and developing buffer (Section 3.2.1.3) were defined, and attempts were made to produce a 2D zymogram from the Rut-C30 culture supernatant (Section 3.2.1.4). After the refinement of an efficient zymogram technique, the number of conidia in the culture inoculum was varied to determine any impact on protease activity in the Rut-C30 culture, as displayed on the zymograms (Section 3.2.2). Finally, the refined techniques were used to compare the extracellular proteases of the *T. reesei* strains Rut-C30, QM6a and CVt under the specific growth conditions (Section 3.2.3).

### **3.2.1 Refinement of the zymogram technique**

#### **3.2.1.1 Sample preparation**

It was considered that using the reducing agent  $\beta$ -mercaptoethanol in the gel loading buffer (Section 2.6.1) could enhance protein separation during SDS-PAGE of the fungal supernatants, which in turn could result in more distinct proteolytic bands on the zymograms (Rojas *et al.*, 2009). In order to investigate this concept, and to determine whether the reducing agent impacted on the overall protease activity displayed on the zymogram, Rut-C30 supernatant was prepared with and without adding  $\beta$ -mercaptoethanol to the loading buffer prior to electrophoresis (Section 2.5.1).

As shown in Figure 3.2. the protease activity profile displayed on zymograms produced from the *T. reesei* Rut-C30 culture supernatant was similar in the presence or absence of the reducing agent. Zymogram bands indicating protease activity were evident from proteins of approximately 160 kDa in the supernatant from the Day 1, Day 6 and Day 7 samples and from 26 kDa proteins from Day 2 to 7. Further discussion of the protease profile of Rut-C30 is contained in subsequent sections of the thesis.

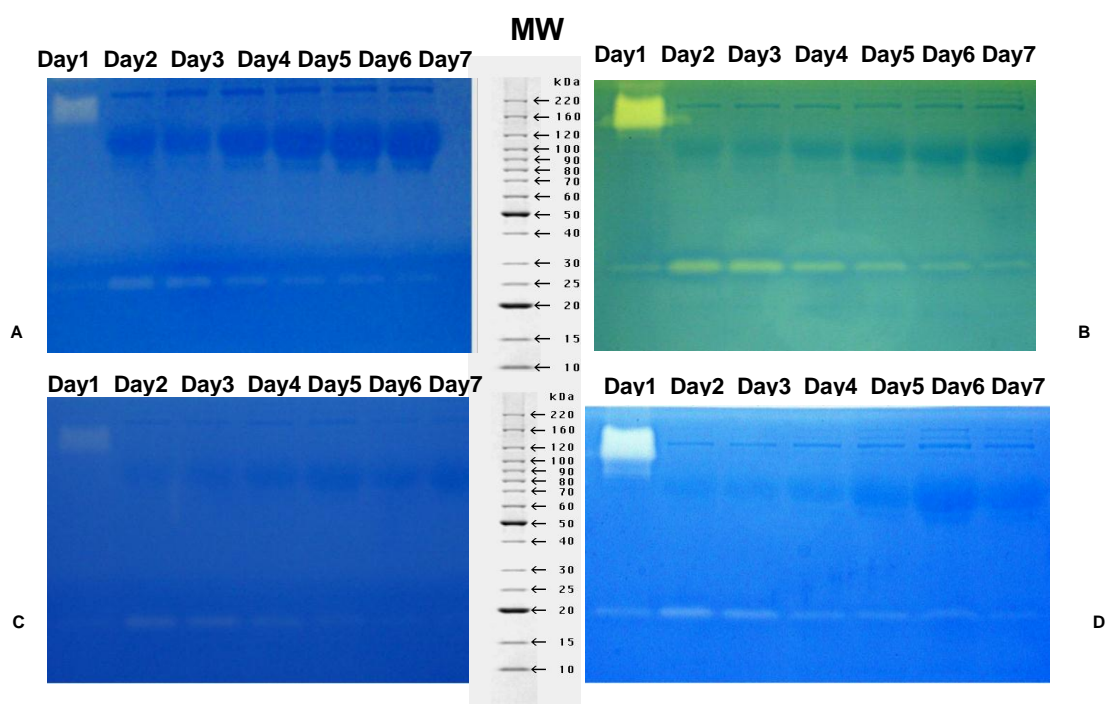


**Figure 3.2** 1D protease zymograms and SDS-PAGE gels of culture supernatants (20  $\mu$ l) of *T. reesei* Rut-C30 grown in ALS medium at 28 °C for seven days. The zymogram gels contained 0.1 % (w/v) gelatin and were incubated at pH 6. (A) 1D – zymogram and (C) 1D- SDS-PAGE gel using culture supernatant prepared in a loading buffer without reducing agent. (B) 1D – zymogram and (D) 1D- SDS-PAGE gel using culture supernatant prepared in loading buffer with reducing agent.

The addition of  $\beta$ -mercaptoethanol did not change the visualisation of the proteolytic bands on the zymogram used to profile the protease activity in the *T. reesei* Rut-C30 culture supernatant. Therefore, from this point onwards, supernatant samples were prepared in the absence of reducing agent for zymograms in this study.

### 3.2.1.2 Protease substrates for zymography

It is advisable to try several substrates when working with enzymes to determine the most suitable (sensitive) substrate for the enzyme detection. Casein or gelatin substrates (0.1 % w/v) were added to the separating gels in the 1D- zymogram gels (Table 2.2). At this stage, two different pHs of developing buffer (3.5 and 6.5) were used. The effect of the pH of the developing buffer was explored further in work described in Section 3.2.1.3.



**Figure 3.3** 1D-protease zymograms of culture supernatants (40  $\mu$ l) of *T. reesei* (Rut-C30) grown in ALS medium at 28  $^{\circ}$ C for seven days. Gels were stained with Coomassie Blue G-250. Gels **A** and **C** contained 0.1 % (w/v) casein. The gels were incubated at pH 6.5 and 3.5 respectively. Gels **B** and **D** contained 0.1 % (w/v) gelatin were incubated at pH 6.5 and 3.5 respectively.

In general, the gelatin substrate is widely used for zymography because it is readily available and economical. Gelatin is a result of partial hydrolysis of collagen (Ward and Courts, 1977), whereas casein protein is a phosphor-protein from mammalian milk (Kunz and Lönnerdal, 1990). Gelatin zymogram gels have shown a higher enzymatic sensitivity than casein zymogram gels in previous studies (Choi *et al.*,

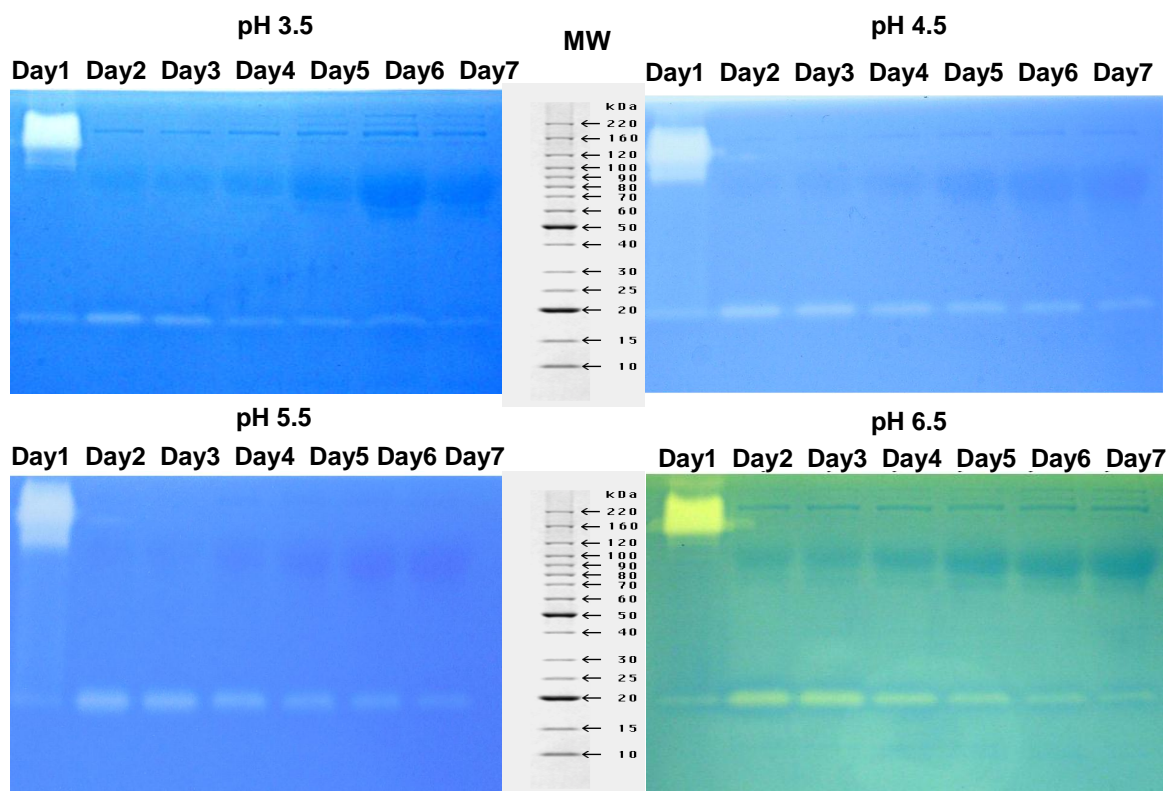
2001). Similarly, the gelatin zymogram gels in this work (Figure 3.3 B and D) showed very clear proteolytic bands at the zone of gelatin digestion against the dark blue stained background of undigested gelatin. However, the gelatin zymogram in Figures 3.2 A and 3.3 B and D show a slight difference in proteolytic bands, with bands that appear at a high molecular weight at Day 6 and 7. These differences could be due to the slight variability in both the zymogram development and the culture growth, in addition to the amount of the loaded culture supernatants (40  $\mu$ l in Figure 3.2 A and 20  $\mu$ l in Figure 3.3 B and D). However, the high molecular weight proteolytic band at Day 6 and 7 shown in Figure 3.2 did not reappear in the future zymogram gels.

The bands on the gelatin zymogram gels (Figure 3.3 B and D) were clearer than those on the casein zymogram gels (Figure 3.3 A and C). Also, the widths of the proteolytic band from the Day 1 sample in the gelatin zymogram gels spanned across 160 - 220 kDa, whereas the corresponding bands were narrower in the casein zymogram gels, from approximately 170 - 210 kDa. The lower bands (26 kDa) in the gelatin zymogram gels were also more distinct than the corresponding bands in the casein zymogram gels. The casein appeared to be less sensitive than gelatin, possibly owing to the more complex structure of casein, which could have limited the proteolysis (Swaisgood, 1993). However, the number and molecular weights of the zymogram bands was the same on the gelatin and casein gels. Considering that the gelatin zymograms appeared to be more sensitive than the casein zymograms in detecting the extracellular protease activity, the gelatin substrate was used in future experiments.

### 3.2.1.3 Zymogram developing buffer

A series of zymograms was produced using developing buffers (Table 2.1) at various pHs to determine the effect of pH on the activity of proteases in the gels (Figure 3.4). After electrophoresis and renaturing (Section 2.5.1), the gels were incubated in developing buffer at pH 3.5, 4.5, 5.5 or 6.5. The developing buffer was the second buffer after the renaturing buffer and its function was to allow the protease/s to digest the zymogram substrate (gelatin in this experiment).

The change in the pH of the developing buffer did not affect the extracellular protease activities on the zymogram gels (Figure 3.4), and all four zymograms of Rut-C30 supernatant showed a similar pattern of bands with approximate molecular weights of 26 kDa and 160 - 220 kDa. The 160 - 220 kDa band covered a wide area and was evident in Day 1 only, whereas the 26 kDa (lower band) looked thinner and was evident from Day 2 to Day 7.





**Figure 3.4** 1D-zymography of culture supernatants (40 µl) of *T. reesei* Rut-C30 grown in ALS medium at 28 °C for seven days. The gels contained 0.1 % (w/v) gelatin as a substrate, stained with Coomassie Blue G-250. The pHs of the developing buffers are written on the top of each gel.

The pH of the developing buffer did not influence the activity of the extracellular protease/s in the zymogram gels. This suggested the proteases in the culture supernatants could maintain activity across acidic and neutral pH ranges. At this point, at least some of the active proteases in the culture supernatants were postulated to be aspartic proteases, which are active in both acidic and neutral pH ranges (Uhlir, 1990). As mentioned previously, the main protease produced by *T. reesei* is known to be an acidic protease 'Trichoderma-pepsin' (Eneyskaya *et al.*, 1999). However, the protease/s in Day 1 (Figure 3.4) were secreted when the culture medium was close to neutral pH (Figure 3.1). This may not have been as conducive to the secretion of acidic proteases, and thus the 160 – 220 kDa band shown in Day 1 was considered less likely to represent an acidic protease. Nevertheless, the activity of this 160 – 220 kDa band on the zymogram gels was also not affected by the variation in the pH of the developing buffer. Therefore, it appeared that the proteases secreted into the culture medium on Day 1 were also active across a wide pH range (3.5 – 6.5).

The low number of proteolytic bands shown on the zymogram gels of Rut-C30 supernatant reflected the low number of proteases secreted by the Rut-C30 strain, possibly resulting from the random mutagenesis process used to produce the hypercellulolytic strain previously reported to have low protease activity (Sheir-Neiss and Montenecourt, 1984; Peterson *et al.*, 2011). All bands were produced using developing buffers across a broad pH range. As all the Rut-C30 extracellular

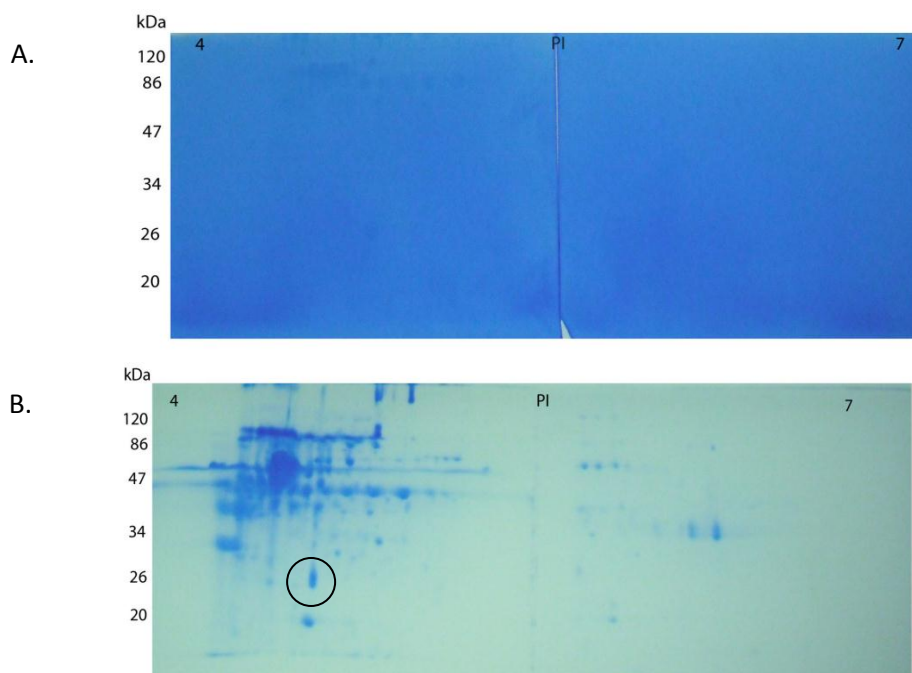
proteases seemed to be active (and therefore detectable) at pH 6, from this point forward pH 6 was chosen for the developing buffer for all zymograms.

The next step towards better separation of the proteins in the culture supernatants and the detection of individual proteins displaying protease activity was to apply 2D-gel electrophoresis and zymography.

#### **3.2.1.4 Two dimensional electrophoresis and zymography**

Two dimensional zymography (Section 2.5.2) was attempted to detect the protease activity in the *T. reesei* Rut-C30 supernatant from Day 7, which should have appeared as clearing spots in the 2D gelatin zymogram gels. 2D-SDS-PAGE gels without gelatin substrate (Section 2.5.2) were run in parallel with the intention of aligning the clearing spots on the 2D zymogram with the protein spots on the 2D SDS-PAGE gel; protein in the matching spots could then be identified by mass spectrometry. The protein separation in 2D electrophoresis depends on two dimensions, first on the pI of the proteins, then on the molecular weight of the proteins. The majority of proteins secreted by *T. reesei* Rut-C30 have a pI between 4 and 7 (Herpoël-Gimbert *et al.*, 2008) so this pI range was chosen as appropriate for the work carried out here.

Unfortunately, the 2D zymogram did not show any spots that would have indicated degradation of the gelatin substrate (Figure 3.5A). This result could have been caused by the addition of DTT in the 2D sample preparation step (Section 2.5.2.1), which is considered to be the reason for inactivation of proteases in some previous studies (Rossano *et al.*, 2011). However, when the 2D sample preparation was carried out without the addition of the DTT, the zymogram gel still did not show any protease activity.



**Figure 3.5 (A)** 2D gelatin zymogram and **(B)** 2D SDS-PAGE gel of *T. reesei* Rut-C30 culture supernatant (Day 7). The zymogram gels contained 0.1 % (w/v) gelatin and were incubated in the developing buffer pH 6, and stained with Coomassie Blue G-250. The circled spot on the 2D SDS-PAGE gel at 26 kDa could be the protease that formed the proteolytic band in 1D zymogram gel (Figure 3.2A, Day 7).

The lack of activity in the 2D zymogram (Figure 3.5A) could also have been due to the presence of trichloroacetic acid in the 2D sample mixture, which could have irreversibly denatured the proteases (Saitoh *et al.*, 2007). The iodoacetamide in the 2D sample preparation mixture may also have inhibited the protease activity owing to the irreversible alkylating effect on cysteine proteases (if the culture supernatant contained cysteine proteases). As a result blue gels without any proteolytic spots were produced (Figure 3.5A).

The 2D zymogram technique was not effective for the visualisation of protease activity in the culture supernatant of *T. reesei* Rut-C30. However, 2D SDS-PAGE carried out on gels without substrate resulted in the successful separation of proteins across two dimensions, resulting in hundreds of spots from pI 4-7 (Figure 3.5B). Most of the protein spots were distributed across pI 4 - 5. The majority of these proteins

are known to be cellulases, predominantly CBHI and CBHII, which usually appear as train of spots (Herpoël-Gimbert *et al.*, 2008). The circled spot on the 2D SDS-PAGE gel at molecular mass 26 kDa (Figure 3.5B) was considered to be a potential candidate for the protease that formed the proteolytic band in the 1D zymogram gels (Figure 3.2A). On the other hand, it is also possible that a protein of a concentration below that detectable by Coomassie Blue (ie. without a visible blue “spot” on the 2D gel) could have been the 26 kDa protease of interest. This method has been used by Choi (2003) and it revealed three extracellular proteases from the culture supernatants. However, in that study a bacterial strain was used and the types of the proteases that were detected on the 2D gelatin zymogram gels and identified by MALDI TOF, were ATP-dependant proteases and extracellular metallo-proteases (Choi *et al.*, 2003). It is possible that *T. reesei* Rut-C30 culture supernatant did not have these types of proteases.

#### **3.2.1.5 Summary of the refined zymogram technique**

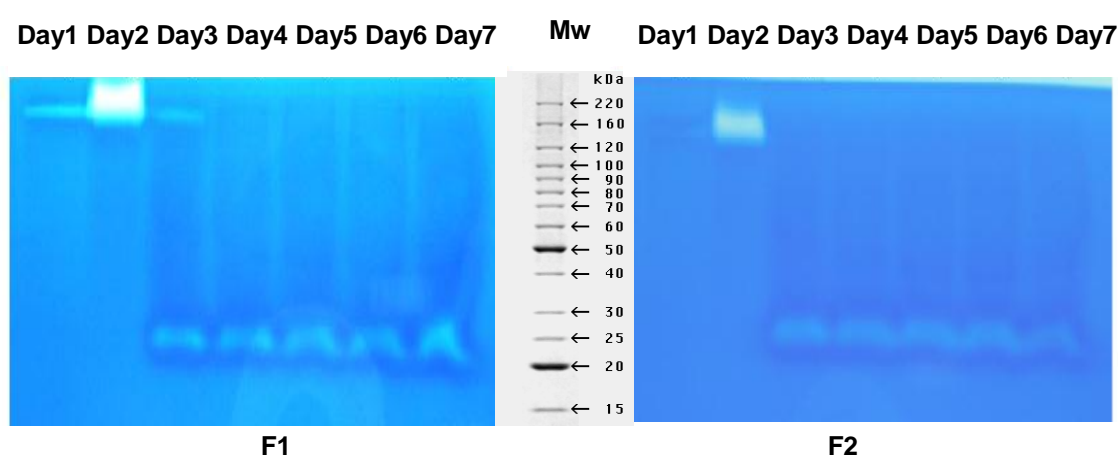
Due to the difficulties encountered detecting the *T. reesei* Rut-C30 proteases by 2D zymography, 1D zymography was considered the best choice for further experiments in which the protease activity of Rut-C30 was to be compared with that of the wild-type *T. reesei* QM6a and CVt, a transformant derived from Rut-C30, producing the heterologous Venus protein (Section 3.2.3). The preliminary work (Sections 3.2.1.1 – 3.2.1.4) indicated that culture supernatants were best prepared for zymography without adding the reducing agent to the SDS-PAGE loading buffer (Section 3.2.1.1), gelatin was the superior substrate for the zymogram gels (Section 3.2.1.2) and pH 6 was an appropriate pH for the zymogram developing buffer (Section 3.2.1.3).

Before using the refined technique to compare the extracellular proteases of the three *T. reesei* strains (Section 3.2.3), concurrent work in the laboratory prompted the

investigation of a potential influencing factor on protease activity, namely the number of conidia used in the initial culture inoculum.

### 3.2.2 The effect of inoculum size on protease activity

Concurrent work in the laboratory (Te'o, unpublished material) had indicated the final yield of secreted protein from *T. reesei* Rut-C30 grown in liquid media could be influenced by the quantity of conidia used for the initial culture inoculation (Section 2.3.3); however, the cause of this effect had not been fully determined. One possible cause was the variable secretion of proteases as a function of the initial concentration of conidia in the culture medium. To explore this concept, two 50 ml of cultures of Rut-C30 were prepared (Section 2.3.3), the first (F1) inoculated with  $1 \times 10^8$  conidia and the second (F2) inoculated with  $3 \times 10^7$  conidia. On each day a 2 ml sample from each culture was collected and the protease activity in the supernatants was analysed by 1D zymography on gels containing gelatin as substrate. The experiment was carried out twice. Notably, the pH profiles across the seven day cultivation period were very similar in the F1 and F2 cultures of Rut-C30 to that previously observed (Figure 3.1).



**Figure 3.6** 1D-zymograms of culture supernatants (40 µl) of *T. reesei* (Rut-C30) grown in ALS medium at 28 °C for seven days. Gelatin (0.1 % w/v) was used as a substrate in the gels, which were incubated at pH 6, and stained with Coomassie Blue. F1: supernatant from culture inoculated with  $1 \times 10^8$  conidia per ml of medium. F2: supernatant from culture inoculated with  $3 \times 10^7$  conidia per ml of medium.

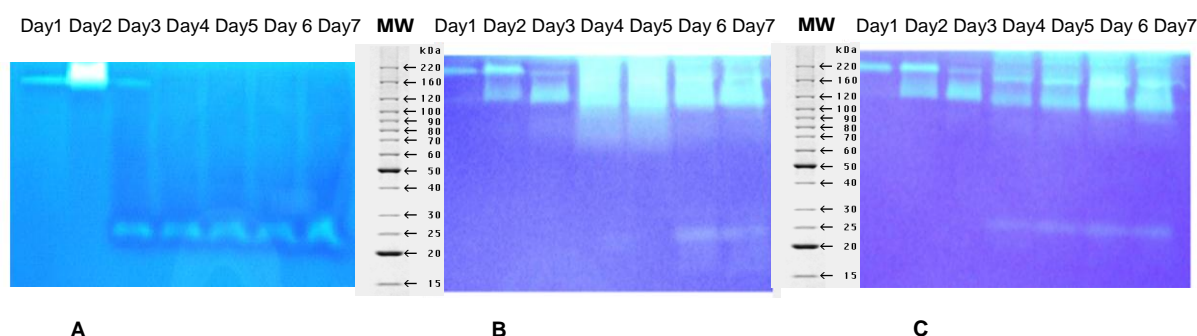
Changing the number of conidia in the inoculant did not greatly affect the proteolytic banding pattern on the zymograms (Figure 3.6) but more subtle differences could be detected. The clarity of all of the bands was greater in the zymogram produced from the supernatant of F1 (inoculated with  $1 \times 10^8$  conidia), indicating slightly higher protease activity than seen from the supernatant of F2 (inoculated with  $3 \times 10^7$  conidia). Furthermore, there was evidence of activity from protease(s) of approximately 160 kDa in the F1 supernatant from Day 3 that was not detected in the F2 supernatant on Day 3 (Figure 3.6). This finding indicated that inoculating the culture medium with  $1 \times 10^8$  conidia resulted in slightly higher protease activity.

The usual aim of an experiment of this type would be to determine the quantity of conidia in the culture inoculant that would result in the highest yield of a target protein, ideally coupled with reduced protease activity. However, the focus of the work described here was to profile the secreted proteases of the *T. reesei* strains under the defined growth conditions (Section 2.2 – 2.3). To do this, slightly higher protease activity was beneficial (provided the profile of activity was similar) because it could potentially increase the probability of detecting and identifying the proteases. Considering these results, the conidial concentration used to inoculate the 50 ml culture medium in further work carried out in this study was  $1 \times 10^8$  conidia.

Following the development and fine-tuning of the zymogram technique, and the establishment of a baseline understanding of the Rut-C30 protease profile under the defined growth conditions, the protease activity of Rut-C30 could be compared with that of the wild-type *T. reesei* QM6a and CVt, a transformant derived from Rut-C30, producing the heterologous Venus protein.

### 3.2.3 Profiles of the extracellular proteases of *T. reesei* Rut-C30, QM6a and CVt by 1D-zymography

The protease activity in the culture supernatants of *T. reesei* Rut-C30, QM6a and CVt (grown in ALS medium, Section 2.2) was visualised and compared using 1D gelatin zymogram gels prepared according to the refined technique (Section 3.2.1.5).



**Figure 3.7** 1D-zymograms of culture supernatants (40  $\mu$ l) of *T. reesei* strains (**A**- Rut-C30, **B**- QM6a and **C**- CVt) grown in ALS medium at 28 °C for seven days. The gels contained 0.1 % (w/v) gelatin, stained with Coomassie Blue G-250.

The zymogram gel of *T. reesei* Rut-C30 culture supernatants (Figure 3.7A) indicated the secretion of a high molecular weight protease (about 160 kDa) from Day 1 – 3 of cultivation, as seen in the preliminary work (Section 3.2.1). The zymogram band from the Day 2 supernatant was smeary and extended from 160-220 kDa, indicating a higher level of protease activity from one or several proteases of a high molecular weight. The corresponding band from Day 3 supernatant was narrower, indicating weaker protease activity from high molecular weight protease(s). In addition, the Rut-C30 zymogram revealed a 26 kDa protease in the supernatant from Day 3 - 7. Similar proteolytic bands have been detected in Rut-C30 culture supernatants using a gelatin zymogram in past research (Peterson *et al.*, 2011).

At this stage, the 160-220 kDa bands seen from the Rut-C30 supernatant on Day 1-2 (Figure 3.7A) were predicted to be serine, cysteine or metallo-proteases (Table 1.2), which are more likely to be secreted when the culture medium is at an approximately neutral pH (Table 3.1). The pH of Rut-C30 supernatant dropped to 3 on Day 3, followed by the disappearance of the 160-220 kDa bands, supporting the hypothesis that these high molecular weight bands represented serine, cysteine or metallo-proteases according to their pH range of activity. The smeariness of the zymogram bands from the Rut-C30 supernatant from Day 2 (160- 220 kDa) may have been due to the lack of separation of the sample through the zymogram gel or due to over-loading (40 µl). Also, the protease/s themselves could have been responsible for the smeary band by hydrolysing the substrate (gelatin) whilst travelling through the gel.

The zymogram bands at approximately 26 kDa from the Rut-C30 supernatant on Day 3, 4, 5, 6 and 7 (Figure 3.7A) were predicted to be aspartic proteases for two reasons: first, it was known that the main protease produced by *T. reesei* is an acidic protease (Eneyskaya *et al.*, 1999; second, the pH of the Rut-C30 supernatant from Day 3 to Day 7 was in the acidic pH range (Figure 3.1).

Overall, samples taken from the Rut-C30 culture medium across the seven days of the cultivation period produced relatively few proteolytic bands on the zymogram in comparison to the other two strains QM6a (Figure 3.7B) and CVt (Figure 3.7C), which reflected the low protease activity that has been previously reported from the Rut-C30 strain (Sheir-Neiss and Montenecourt, 1984; Peterson *et al.*, 2011). The low protease activity of the Rut-C30 strain is one of the advantages of Rut-C30 as an expression host compared to other high-secreting strains such as CL 847 (Herpoël-Gimbert *et al.*, 2008). The high protein secretion and high cellulolytic activity are the



main characteristics of the Rut-C30 strain (Montenecourt and Eveleigh, 1979; Bisaria and Ghose, 1981; Peterson and Nevalainen, 2012).

The zymogram produced from the QM6a culture supernatants (Figure 3.7B) indicated the secretion of a high molecular weight protease (about 220 kDa) from Day 1. Two zymogram bands were evident from the Day 2 supernatant indicating proteases of 120 kDa and 220 kDa. The 220 kDa protease appeared to be weaker in activity in the Day 3 supernatant and the 120 kDa protease exhibited higher activity, visualised by a stronger and clearer band. Supernatant from the QM6a culture on Day 4, 5, 6 and 7 exhibited a high amount of protease activity apparently from a number of high molecular weight proteases at the top part of the zymogram. However, less activity from the high molecular weight proteases (100 - 220 kDa) was evident on Day 6 and Day 7. Furthermore, a lower molecular weight band of 26 kDa appeared on these last two days, possibly indicating the eventual secretion of the aspartic protease that was postulated to be secreted in the Rut-C30 supernatant from Day 3.

The differences between the QM6a and Rut-C30 zymogram probably were partly a result of the genetic changes that have been found in the Rut-C30 strain compared to the wild-type QM6a (Peterson and Nevalainen, 2012). Around 100 kb of genomic DNA is missing in the genome of Rut-C30 compared to the wild type QM6a, including the truncation of the *creI* gene (Ilmén *et al.*, 1996). Also, the mutation affected 25 additional genes in Rut-C30 (Le Crom *et al.*, 2009). It has been shown that the Rut-C30 strain has a lower protease activity than the wild-type QM6a (Peterson *et al.*, 2011). Therefore, it was expected that there would be more proteolytic bands in the QM6a zymogram than in the Rut-C30 zymogram under the same conditions. However, the exact reasons for the low protease production in Rut-C30 remain

unclear. The genetic difference between the two strains could also contribute to the differences in the pH profiles of the strains (Figure 3.1), and thus could indirectly effect the protease secretion during the cultivation period.

The zymogram of the CVt culture supernatant (Figure 3.7C) more closely resembled that of QM6a than Rut-C30. The secretion of a high molecular weight (220 kDa) protease was evident from Day 1. There was evidence of further protease activity from a number of proteases of 120 - 210 kDa on Day 2, and on Day 3 there was less activity from the 220 kDa protease and more from the 120 – 210 kDa proteases. From Days 4 – 7 the protease activity of the high molecular weight proteases steadily increased, resulting in an almost continuous area of protease activity from 94 – 210 kDa. Faint bands from a protease of approximately 26 kDa were also evident in the CVt supernatants from Day 4 – 7. Again, it was considered that the 26 kDa protease could have been the same postulated aspartic protease as seen on the Rut-C30 zymogram gel (Figure 3.7A). However, the 26 kDa band appeared from Day 2 in the Rut-C30 supernatant whereas in the CVt supernatant it appeared from Day 4.

The zymograms of Rut-C30 and CVt (Figure 3.7A and C) were shown to be different, which was unexpected because CVt strain was derived from the Rut-C30 strain and the only designed alteration in the CVt strain compared to Rut-C30 was the addition of the Venus protein at the *cbh1* locus (Kautto, 2009). However, CVt also had a slower growth rate than Rut-C30, which could reflect a change in metabolism and correspondingly impact on the protease secretion (Kautto, 2009). Furthermore, the different protease activity profile in the zymogram between CVt and Rut-C30 culture supernatants could be related to the differences in their pH profiles (Figure 3.1). Similarly to QM6a, the CVt culture maintained a pH range close to neutral throughout the cultivation period, which may have resulted in the secretion of a range of

proteases active in the neutral pH range (serine, cysteine and metallo-proteases). In contrast, the Rut-C30 culture medium dropped to an acidic pH which may have been primarily conducive to the production of acidic proteases.

Profiling the protease activity of *T. reesei* Rut-C30 on the 1D zymogram and comparing it to the wild-type QM6a gave an indication of the effect of the random mutagenesis process, used to create the hypercellulolytic Rut-C30, on its protease activity. Comparison between the Rut-C30 strain (surrogate host) and a transformant derived from it (CVt strain, producing Venus protein) revealed further differences in the protease profiles between these strains. One of the main focuses of this study was to investigate how the secretion of heterologous proteins impacts on protease activity, so the following work focused on the transformant CVt and its parent strain Rut-C30 only. The next step was to determine the types of proteases secreted by the two *T. reesei* strains using liquid assays (Section 3.3).

### **3.3 Liquid assays – Determining the types of proteases secreted by Rut-C30 and CVt**

Liquid assays were carried out to compare the types of proteases in the culture supernatants of *T. reesei* Rut-C30 and CVt, and their activity. Firstly, the protein concentrations of the culture supernatants were determined across the cultivation period (Section 3.3.1). Fluorescent and colorimetric protease assays using substrates specific to various protease types were conducted (Section 3.3.2 – 3.3.3), and assays were repeated using specific protease inhibitors to confirm the results (Section 3.3.4).

### 3.3.1 Protein concentration of Rut-C30 and CVt culture supernatants

Before proceeding to the liquid assays, the protein concentrations of the Rut-C30 and CVt culture supernatants were determined across the seven day cultivation period (Table 3.2).

**Table 3.2** The protein concentration of Rut-C30 and CVt culture supernatants measured using Bio-Rad DC protein assay (mg/ ml).

Day	Rut-C30	CVt
Day 1	4.4	3.9
Day 2	4.3	3.88
Day 3	5.2	4.91
Day 4	5	4.59
Day 5	4.2	3.91
Day 6	4.51	4.13
Day 7	5.3	4.65

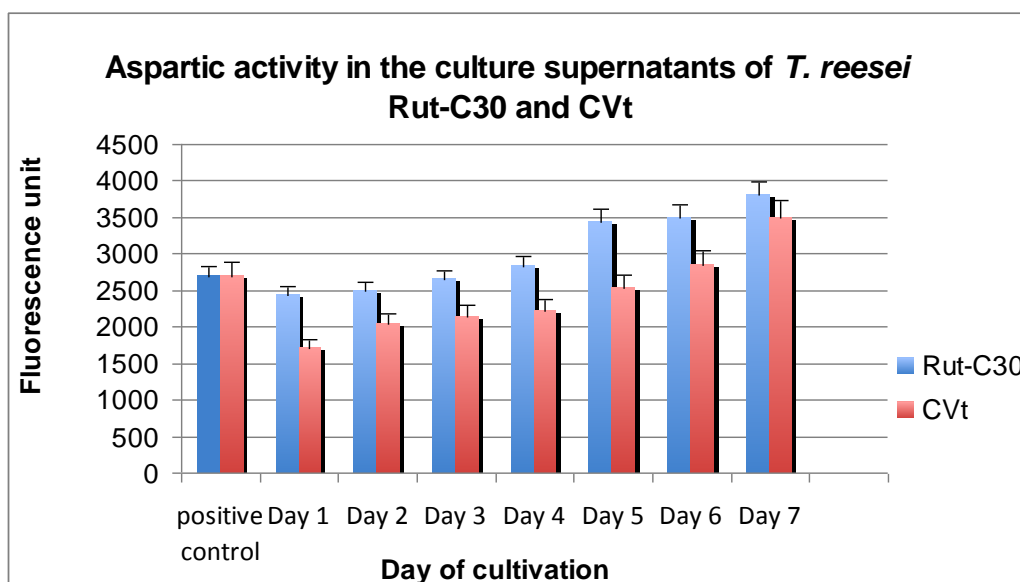
The protein concentration of the Rut-C30 culture supernatant was slightly higher than the protein concentration of the CVt culture supernatant throughout the entire cultivation period (Table 3.2) and a similar fluctuation pattern was recorded in both strains. It was surprising that the observed slower growth rate of CVt in comparison to Rut-C30 (Section 3.1) did not impact greatly on the total protein secretion; however the slower growth rate and other features of the CVt strain may have caused changes to the types of proteins, and particularly the types of proteases, that were secreted.

### 3.3.2 Characterisation of protease activity by fluorescent assays

Protease assays were performed using specific fluorescent substrates to ascertain the types of proteases that were secreted by Rut-C30 and CVt into the culture

medium. Of the four fluorescent substrates used (Table 2.3), substrates specific to aspartic, cysteine and subtilisin proteases resulted in detection of fluorescence above the background level, indicating that they were being cleaved by specific proteases in the samples. Commercial purified specific proteases were used as positive controls (Table 2.3).

Substrate for the aspartic protease type (Boc- Leu- Ser- Thr- Arg- amino-4-methylcoumarin, Table 2.3) was cleaved by extracellular proteases of *T. reesei* Rut-C30 at pH 3, and the activity increased gradually from Day 1 to Day 7 (Figure 3.8).



**Figure 3.8** Aspartic protease activity in the culture supernatants of *T. reesei* RUT-C30 and CVt. Assays were carried out at 37 °C with an incubation time of 10 min. Fluorescence was measured using excitation at 360 nm and emission at 460 nm. Pepsin from porcine gastric mucosa served as a positive control (> 40 units / mg). Assays were carried out in triplicate.

The extracellular aspartic protease activity of *T. reesei* CVt at pH 3 (Figure 3.8) was detected from Day 1 to Day 4, then significantly increased on Days 5, 6 and 7. The aspartic protease activity in the CVt supernatants was lower than the aspartic protease activity in Rut-C30 supernatants (Figure 3.8). The pH of the culture medium could at least partly account for these results. The pH of the CVt culture medium

across the seven cultivation days (Figure 3.1) remained in the close to neutral range, whereas the Rut-C30 culture medium dropped into the acidic pH range. Aspartic proteases are more likely to be secreted and are more active in an acidic medium (Gente *et al.*, 2001).

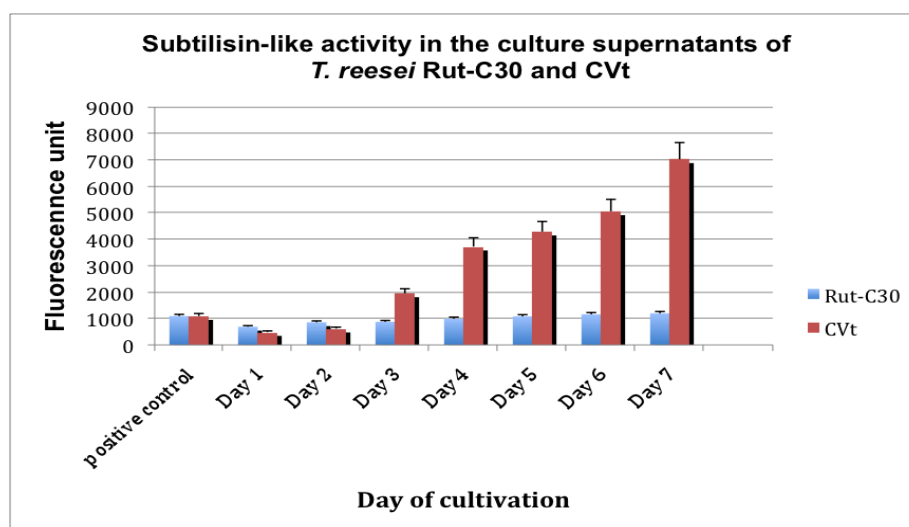
A higher overall level of protease activity was seen in the zymogram of CVt culture supernatants compared to Rut-C30 (Figure 3.7C and 3.7A respectively). However, the high proteolytic activity was mainly from high molecular weight protein/s not seen on the Rut-C30 zymograms. It was hypothesised that the high molecular weight bands were not aspartic proteases (Section 3.2.1.3) as their secretion occurred early in the growth period when the pH of the medium was still in the close to neutral range (Figure 3.1). The assay results provide further evidence for this hypothesis. If the high molecular weight proteases were aspartic then one would expect substantially higher protease activity from the CVt strain, however this was not the case.

The 26 kDa bands on the CVt zymogram, postulated to be caused by an aspartic protease, were observed from Day 5 (Figure 3.7C) whereas the 26 kDa bands on the Rut-C30 zymogram were observed from Day 2 (Figure 3.7A). On Day 5, the aspartic protease activity in the CVt supernatant reached similar levels as Rut-C30 at Day 2. The level of activity on Day 2 in Rut-C30 supernatants and Day 5 in CVt supernatants appeared to be the lowest level that could be detected in the zymograms; although aspartic protease activity was detected by assay on Day 1 in Rut-C30 culture supernatants and at Day 1, 2, 3 and 4 in CVt culture supernatants, it was not detected on the zymograms (Figure 3.7).

The cysteine-type protease substrate (Z- Arg- Arg 7-amino-4-methylcoumarin) was not cleaved by extracellular proteases in the *T. reesei* Rut-C30 or CVt culture supernatants. However, a cysteine protease has been found to be secreted from

*Trichoderma* species in the presence of heavy (toxic) metals in the growth medium (Kredics *et al.*, 2005; Raspanti *et al.*, 2009). The results could imply that Rut-C30 did not secrete a cysteine protease in this work because the growth medium (ALS medium) did not contain heavy metal. Alternatively the gene for the cysteine protease may have been inactivated during the mutagenesis that resulted in the high cellulolytic Rut-C30 strain of reported low protease activity (Sheir-Neiss and Montenecourt, 1984; Peterson *et al.*, 2011). Another possible reason why no cysteine protease activity was identified by the fluorescent substrate could be that a cysteine protease may have been cleaved by other proteases.

The subtilisin type substrate (N- Suc- Ala- Ala- Ala 7- amino-4-methylcoumarin, Table 2.3) was cleaved by extracellular proteases of *T. reesei* Rut-C30 at pH 8 (Figure 3.9). Activity was detected from Day 1 and increased gradually to Day 7. The subtilisin-like protease activity of CVt was lower than that of Rut-C30 on the first two days but then increased to higher than Rut-C30 on Day 3. Throughout the rest of the cultivation period the subtilisin-like activity of CVt supernatant continued to increase dramatically until it was almost seven times that of Rut-C30 on Day 7 (Figure 3.9).



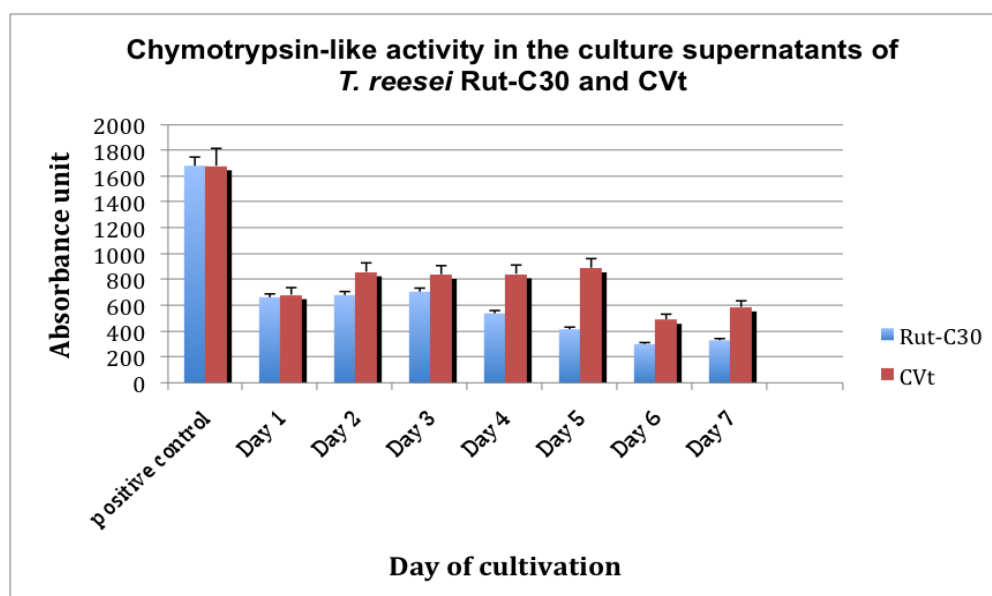
**Figure 3.9** Subtilisin-like activity in the culture supernatants of *T. reesei* Rut-C30 and CVt. Assays were carried out at 37 °C with an incubation time of 10 min. Fluorescence was measured using excitation 360 nm and emission 460 nm. Subtilisin A from *Bacillus* sp served as a positive control.

The lower subtilisin-like activity levels in the CVt supernatant in comparison to Rut-C30 in the first two days of cultivation (Figure 3.9) could be partly explained by the slower growth rate of the CVt strain (Section 3.1 and Kautto, 2009). The differences in the pH profiles of the two strains throughout the entire seven days of cultivation (Figure 3.1) could also have affected the amount of subtilisin-like proteases secreted. Whereas the acidic nature of the Rut-C30 culture medium may have encouraged the secretion of aspartic proteases, the maintenance of a closer to neutral pH in the CVt culture may have been more favorable for the secretion and activity of subtilisin-like proteases. The higher subtilisin-like protease activity in the CVt supernatants in the last five days (Figure 3.9) correlated well with the appearance of the high molecular weight (160 – 220 kDa) bands in the zymogram gel of CVt on the same days (Figure 3.7C). The subtilisin-like protease activity in Rut-C30 supernatants also increased from Day 1 to Day 7 (Figure 3.9) but it was substantially lower than the subtilisin-like activity of CVt supernatants, and apparently not high enough to be detected in the zymogram gel of Rut-C30 (Figure 3.7A). It appeared that the subtilisin-like protease activity may have been induced by expression of the heterologous Venus protein.

### **3.3.3 Characterisation of protease activity by colorimetric assays**

Colorimetric assays were carried out using the specific substrate N- Benzoyl- L- Tyrosine- P- nitroanilide (Table 2.3), which allowed the detection of the chymotrypsin-like protease activity through measuring the absorbance at 410 nm. The chymotrypsin substrate > 98 % specific to chymotrypsin protease (Sigma Aldrich [www.sigmaaldrich.com](http://www.sigmaaldrich.com) ).





**Figure 3.10** Chymotrypsin-like activity in the culture supernatants of *T. reesei* Rut-C30 and CVt. Assays were carried out at 37 °C with an incubation time of 10 min. Absorbance was measured at 410 nm. Bovine pancreas alpha-chymotrypsin served as a positive control (10 - 30 units / mg).

The chymotrypsin type substrate was cleaved by extracellular proteases of *T. reesei* Rut-C30 at pH 8 (Figure 3.10). Activity was detected on all seven days. However, the chymotrypsin-like activity in the Rut-C30 supernatant declined from Day 3 to Day 6 then increased slightly on Day 7 (Figure 3.10). Again, this could be related to the pH profile of the Rut-C30 culture because lower chymotrypsin-like activity was detected when the culture medium was in an acidic pH range (Figure 3.10, Figure 3.1). The chymotrypsin-like activity was at its highest in the Rut-C30 supernatant on the first three days (Figure 3.10). The protease activity observed on the Rut-C30 zymogram during that time was from proteins of 160-220 kDa (Figure 3.7A), suggesting these high molecular weight proteins could have included chymotrypsin-like proteases. It is notable that the chymotrypsin-like protease activity was detected in all seven days in the liquid assays whereas the high molecular weight band only showed on the zymogram on the first three days. This may have been because the zymogram technique was not as sensitive to the lower protease activity as the fluorescent assay.

Chymotrypsin-like activity was present from Day 1 in the CVt supernatant and increased in Day 2. The chymotrypsin-like activities were similar on Days 2 - 5, decreased on Day 6, and increased slightly on Day 7. The chymotrypsin-like protease activity in the CVt supernatants detected by liquid assay correlated well with the appearance of the high molecular weight (160 - 220 kDa) bands in the zymogram gel of CVt (Figure 3.7C) from Day 1 to Day 7; similar bands were only visible in the Rut-C30 supernatant on Day 1 - 2 (Figure 3.7A). This strongly suggested that the protease activity detected in the CVt supernatant was at least partly due to a high molecular weight chymotrypsin-like protease. However, the liquid assay revealed a decrease in the chymotrypsin-like protease activity of the CVt supernatant on Day 6 and 7 whilst more proteolytic bands appeared at 94 - 220 kDa on the CVt zymogram on these days. These results indicated that another type of high molecular weight protease, other than the chymotrypsin-like protease, could have been secreted by CVt on the last two days of cultivation.

The following tables provide a summary of the specific protease activities detected in the culture supernatants of *T. reesei* Rut-C30 and CVt (Section 3.3), and the molecular weights of protein bands displaying protease activity on the zymograms produced from the supernatants of the two strains (Section 3.2.3).

**Table 3.4** Summary of the protease activity detected in the Rut-C30 culture supernatants. FU, AU: the fluorescence units and absorbance units (respectively) produced from the liquid assays using Rut-C30 culture supernatant and specific substrates for each protease type (Sections 3.3.2 and 3.3.3). MW: the approximate molecular weight (kDa) of the proteolytic band/s that were visualised on the Rut-C30 zymogram (Section 3.2.3, Figure 3.7A).

Protease activity	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Aspartic (FU)	2433	2493	2643	2832	3435	3493	3804
Subtilisin (FU)	687	857	873	991	1080	1159	1197
Chymotrypsin (AU)	663	680	591	538	415	299	329
Zymogram bands (MW, kDa)	160	160-220	26, 160	26	26	26	26

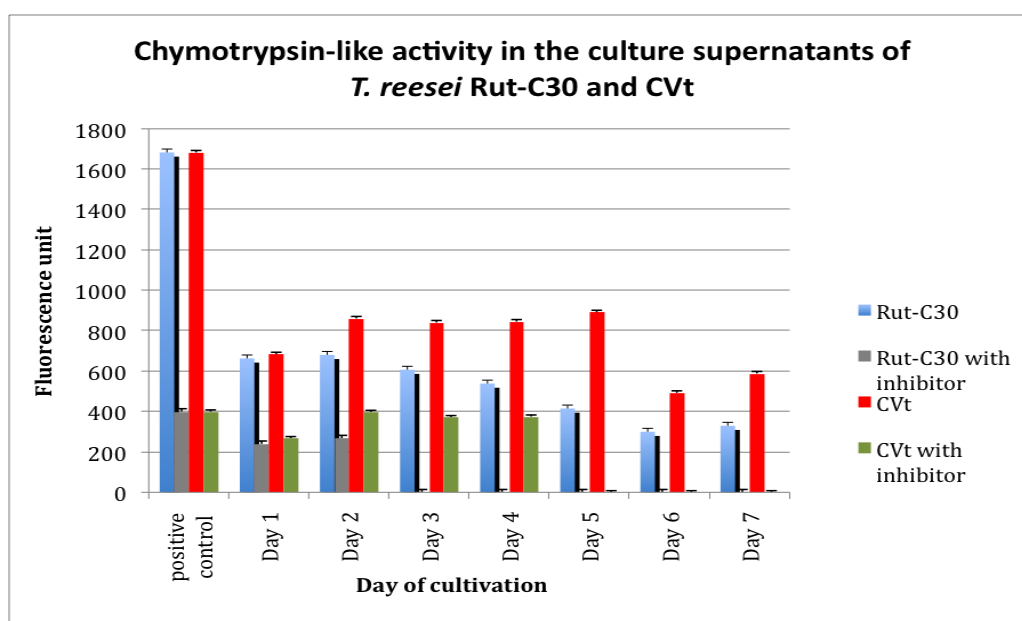
**Table 3.5** Summary of the extracellular protease activity of the CVt culture supernatants. FU, AU: the fluorescence units and absorbance units (respectively) produced from the liquid assays using Rut-C30 culture supernatant and specific substrates for each protease type (Sections 3.3.2 and 3.3.3). MW: the approximate molecular weight (kDa) of the proteolytic band/s that were visualised on the CVt zymogram (Section 3.2.3, Figure 3.7C).

<b>Protease activity</b>	<b>Day 1</b>	<b>Day 2</b>	<b>Day 3</b>	<b>Day 4</b>	<b>Day 5</b>	<b>Day 6</b>	<b>Day 7</b>
<b>Aspartic (FU)</b>	1715	2039	2141	2228	2530	2854	3493
<b>Subtilisin (FU)</b>	486	617	1952	3724	4292	5061	7033
<b>Chymotrypsin (AU)</b>	683	861	841	845	891	492	588
<b>Zymogram bands (MW, kDa)</b>	220	100-220	100-220	26, 94-220	26, 94-220	26, 94-220	26, 94-220

### **3.3.4 Confirmation of results by inhibition of specific protease activities**

Protease assays with the specific fluorescent substrates (Section 3.3.2) were repeated a further three times using specific inhibitors (Section 2.7.2, Table 2.4). The appropriate inhibitors completely prevented the protease activity of the positive controls and the secreted proteases in each case, thus confirming the existence of aspartic and subtilisin-like proteases in the culture supernatants of Rut-C30 and CVt.

When the colorimetric assays of chymotrypsin-like activity were carried out using a specific chymotrypsin-like protease inhibitor (Section 2.7.2, Table 2.4) the chymotrypsin-like activities of the positive control and supernatant samples were substantially reduced but not completely inhibited (Figure 3.11). The addition of the chymotrypsin-like protease inhibitor (AEBSF) to the Rut-C30 culture supernatants from the Day 1 and 2 still resulted in detectable activity. Likewise, the CVt culture supernatants from Day 1 – 4 still showed some chymotrypsin-like protease activity after the addition of AEBSF. These results indicated that an insufficient concentration of AEBSF inhibitor was being used ie. the concentrations of the positive control protease and the chymotrypsin-like protease in certain samples (culture supernatants of Rut-C30 on Days 1 and 2, and CVt on Days 1 - 4) were too high to be fully inhibited by the AEBSF concentration. However, as a substantial reduction in chymotrypsin-like activity was achieved in the presence of the inhibitor in each case, the existence of chymotrypsin-like proteases in the Rut-C30 and CVt culture supernatants could be confirmed.



**Figure 3.11** Chymotrypsin-like activity in the culture supernatants of *T.reesei* Rut-C30 and CVt. Assays were carried out at 37 °C with an incubation time of 10 min. Absorbance was measured at 410 nm. The same procedure was repeated with adding chymotrypsin-like protease inhibitor (0.1 mM AEBSF, 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride). Bovine pancreas alpha-chymotrypsin served as a positive control (10 - 30 units / mg). Assays were carried out in triplicate.

The results from the liquid assays of protease using specific substrates and inhibitors for each protease type showed that there was a certain resemblance in the protease types secreted by *T. reesei* Rut-C30 and CVt strains. The assays showed aspartic-like protease activity in both the *T. reesei* Rut-C30 and the CVt supernatants and this was confirmed by application of aspartic-specific inhibitors which successfully inhibited activity. Chymotrypsin and subtilisin protease assays carried out with and without specific inhibitors confirmed chymotrypsin-like and subtilisin-like protease activity in the culture supernatants of both Rut-C30 and CVt. However, according to the cysteine protease liquid assays, neither the *T. reesei* Rut-C30 nor the CVt supernatant had cysteine-like protease activity.

Although the liquid assays revealed that Rut-C30 and CVt strains secreted a similar suite of protease types when grown under the same culture conditions, there were

marked differences in the level of activity of each protease type in the culture supernatants of the two strains. Across the entire cultivation period higher levels of aspartic protease activity were detected in the Rut-C30 culture than the CVt culture, although on Day 6 and Day 7 the aspartic-like protease activity of CVt was close to that of Rut-C30 (Figure 3.8). In contrast, there were higher levels of subtilisin-like protease activity in the Rut-C30 culture than the CVt culture on the first two days of cultivation. However, from Day 3 the subtilisin-like protease activity of Rut-C30 remained almost constant, whereas the CVt culture exhibited a dramatic increase in subtilisin-like protease activity, surpassing that of Rut-C30 on Day 3 and reaching almost seven times that of its parent strain by Day 7 (Figure 3.9). The chymotrypsin-like protease activities of Rut-C30 and CVt were similar on the first day of cultivation, but there was a decrease in the chymotrypsin-like protease activity of the Rut-C30 culture from Day 3, resulting in higher chymotrypsin-like protease activity in the CVt cultures from Day 2 to Day 7 (Figure 3.10 and 3.11).

The results from the liquid assays complemented those achieved from the zymography, leading to an enhanced understanding of the protease profile of the Rut-C30 and CVt strains under the given culture conditions. The next step was to identify the proteases secreted by the two strains by MALDI TOF/TOF MS/MS and LC ESI MS/MS.

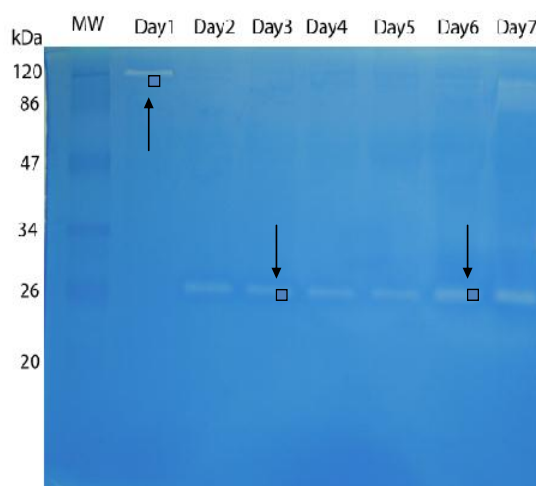
### **3.4 Proteomic analysis of extracellular proteases**

In the final stages of the study, mass spectrometry was used to identify the extracellular proteases secreted by *T. reesei* Rut-C30 and the transformant CVt, producing the heterologous Venus protein, under the culture conditions (Section 2.2 and 2.3). Initial experiments were carried out using Rut-C30 culture supernatants only, with the aim of finding the most efficient mass spectrometric technique for protease identification. Attempts were made to identify Rut-C30 extracellular proteases from zymograms and SDS-PAGE gels by MALDI TOF/TOF MS/MS (Section 3.4.1) and LC ESI MS/MS (Section 3.4.2). The LC ESI MS/MS method was then used to identify and compare the secreted proteases of CVt and Rut-C30 (Section 3.4.3).

#### **3.4.1 MALDI TOF/TOF MS/MS of *T. reesei* Rut-C30 culture supernatant**

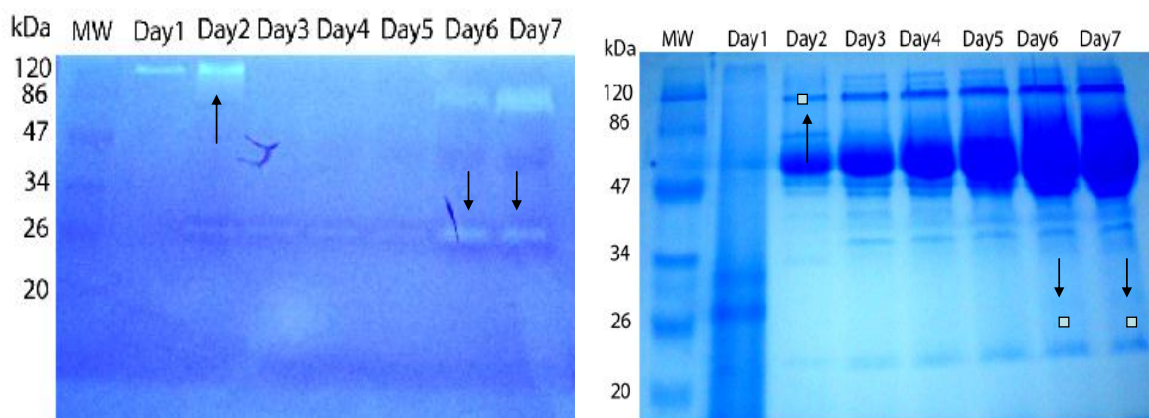
*T. reesei* Rut-C30 was grown for seven days in ALS medium as previously described (Section 2.2 - 2.3). Samples were taken from the medium each day and a 1-D zymogram was produced from the supernatants (Section 2.6.1) using the refined technique described in Section 3.2.1.5. Bands from the zymogram gel were excised as shown in Figure 3.12 and subjected to MALDI TOF/TOF MS/MS analysis (Section 2.8.1).





**Figure 3.12** 1D zymogram produced from culture supernatants (40  $\mu$ l) of *T. reesei* Rut-C30 grown in ALS medium at 28 °C for seven days. The gel contained 0.1 % (w/v) gelatin, was incubated in a developing buffer at pH 6 and stained with Coomassie Blue G-250. Arrows and boxes indicate areas excised from the gel.

Unfortunately, the MALDI TOF/TOF MS/MS did not reveal the identity of the *T. reesei* proteases in the zymogram bands. The only protein that could be identified from the bands was gelatin, the substrate used for the zymogram gel. The gelatin dominated the mass spectra, masking the other proteins that may have existed in the bands. Because of the substrate interference, an alternative strategy was used. Rut-C30 supernatant was subjected to SDS-PAGE on two gels run in parallel. The first gel contained gelatin and was used to produce a protease zymogram as just described. The second gel, without substrate (the SDS-PAGE gel), was processed and stained using the standard SDS-PAGE procedure (Section 2.5.1). Protein bands were excised for identification by MALDI TOF/TOF MS/MS from an area on the SDS-PAGE gel estimated to contain protein that aligned to the band on the zymogram gel (indicated by arrows in Figure 3.13).



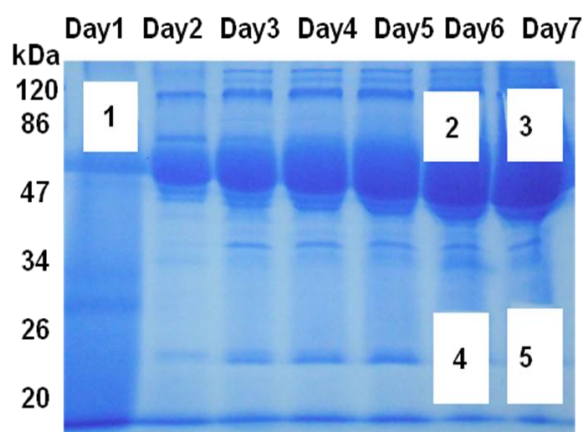
**Figure 3.13** 1D zymogram (left) and SDS-PAGE gel (right) of culture supernatants (40  $\mu$ l) of *T. reesei* Rut-C30 grown in ALS medium at 28 °C for seven days. The zymogram contained 0.1 % (w/v) gelatin, and was incubated in a developing buffer at pH 6, and stained with Coomassie Blue G-250. Arrows on the left indicate the zymogram bands that were aligned with protein bands from SDS-PAGE gel (right), according to molecular weight. Squares on the SDS-PAGE gel (right) indicate areas excised from the gel for MALDI TOF/TOF MS/MS.

Two proteins were identified by MALDI TOF/TOF MS/MS of the protein bands of approximately 26 kDa excised from the SDS-PAGE gel (Day 6 and Day 7 supernatant, Figure 3.13). However, the identified proteins were xylanases and no proteases were detected (data not shown). *T. reesei* produces xylanases of around 20-21 kDa (Tenkanen *et al.*, 1992). The xylanases are secreted in far greater quantities than the Rut-C30 proteases (Herpoël-Gimbert *et al.*, 2008) so it seemed likely that the xylanases from the protein band could have monopolised the mass spectra, masking the presence of any proteases of a similar molecular weight and relatively low abundance. In addition, it was possible that the excised protein band from the SDS-PAGE gel may not have, in fact, corresponded to the protein that was responsible for the band on the zymogram, despite the intention to align these two bands. The protein substrate (gelatin) in the zymogram gel retarded the movement of the samples during electrophoresis, so discerning the corresponding band on the SDS-PAGE gel was difficult (Peterson *et al.*, 2009).

In response to these results it was decided that a modification to the technique was needed. Firstly, it was evident that it could be beneficial to excise larger bands from the SDS-PAGE gel in order to increase the probability of including the protease responsible for the zymogram bands of interest. Secondly, LC ESI MS/MS (Section 2.8.2) was used instead of MALDI TOF/TOF MS/MS for protein identification. During the liquid chromatography (LC) phase of LC ESI MS/MS, protein mixtures are separated according to their hydrophobicity before being subjected to mass spectrometric analysis (Section 1.7.3.2). As a consequence, less abundant proteins can enter the fragmentation phase of ESI MS/MS at a different time than the highly abundant proteins, thus increasing the likelihood of their detection and identification.

### 3.4.2 LC ESI MS/MS of *T. reesei* Rut-C30 culture supernatant

*T. reesei* Rut-C30 culture supernatants were subjected to SDS-PAGE as described above (Section 3.4.1). Protein bands were excised from the SDS-PAGE gel (Figure 3.14) from a large area estimated to contain a protein that aligned to the bands on previous zymogram gels (eg. Figure 3.12).



**Figure 3.14** SDS-PAGE gel of culture supernatants (40  $\mu$ l) of *T. reesei* Rut-C30 grown in ALS medium at 28 °C for seven days. Numbers 1, 2, 3, 4 and 5 indicate the excised areas that were analysed by LC ESI MS/MS.

The excised protein bands (Figure 3.14, areas 1, 2, 3, 4 and 5) were analysed by LC ESI MS/MS and protein identifications are shown in Table 3.3. Protein identifications were assigned when the Mowse scores were significant ( $p < 0.05$ ) for spectra searched against the *T. reesei* database (<http://genome.jgi-psf.org/Trire2>) using the Mascot search engine (Perkins *et al.*, 1999; Section 2.8.2.3). For spectra processed through the Global Proteome Machine (GPM; [www.thegpm.org](http://www.thegpm.org)), identifications were assigned when the  $\log_e$  score was lower than the statistically significant cut-off values assigned by the GPM algorithm (Fenyő *et al.*, 2010; Section 2.8.2.3).

A subtilisin-type protease (predicted MW 93.3 kDa) was identified from each of the excised bands containing proteins of 70 – 120 kDa from the supernatant of Rut-C30 on Days 1, 6 and 7 (Figure 3.14, areas 1, 2 and 3; Table 3.3). Two aspartic proteases (predicted MW 26.5 and 24.3 kDa) were identified from each of the excised bands containing proteins of 20 – 28 kDa from supernatant of Rut-C30 on Day 6 and 7 (Figure 3.14, areas 4 and 5; Table 3.3); however, one of the aspartic proteases was identified as an intracellular protein. The presence of the intracellular aspartic protease in the culture supernatant could have been a result of cell lysis during the late stages of growth, a topic that is also addressed in the following sections.

**Table 3.3** Proteases identified<sup>a</sup> by LC ESI MS/MS from the SDS-PAGE gel (Figure 3.14) produced from the culture supernatants of *T. reesei* Rut-C30 grown in ALS medium.

Excised area <sup>b</sup>	Accession number	Description <sup>c</sup>	pI <sup>d</sup>	MW <sup>d</sup> (kDa)	Sub-cellular localisation <sup>e</sup>	Strain
1,2,3	jgi 51365	Peptidase S8, subtilisin	5.12	93.3	Extracellular	Rut-C30
4,5	jgi 69555	Peptidase A4, aspartic-type endopeptidase activity	4.53	26.5	Extracellular	Rut-C30
4,5	jgi 106661	Peptidase A4, aspartic-type endopeptidase activity	4.28	24.3	Intracellular	Rut-C30

<sup>a</sup> Protein identifications were assigned when the Mowse scores were significant ( $p < 0.05$ ) using the Mascot search engine, and/or when the  $\log_e$  score was lower than the statistically significant cutoff values assigned by the GPM algorithm (GPM search engine).

<sup>b</sup> The excised area refers to the area from the SDS-PAGE gel excised for LC ESI MS/MS, as shown in Figure 3.14.

<sup>c</sup> Accession number and descriptions were as assigned in the jgi database for *T. reesei* (<http://genome.jgi-psf.org/Trire2>).

<sup>d</sup> The pI and Mw were calculated using ExPASy (<http://expasy.org/tools/>).

<sup>e</sup> The sub-cellular localisation was as predicted by PSORT (<http://www.psорт.org/>)

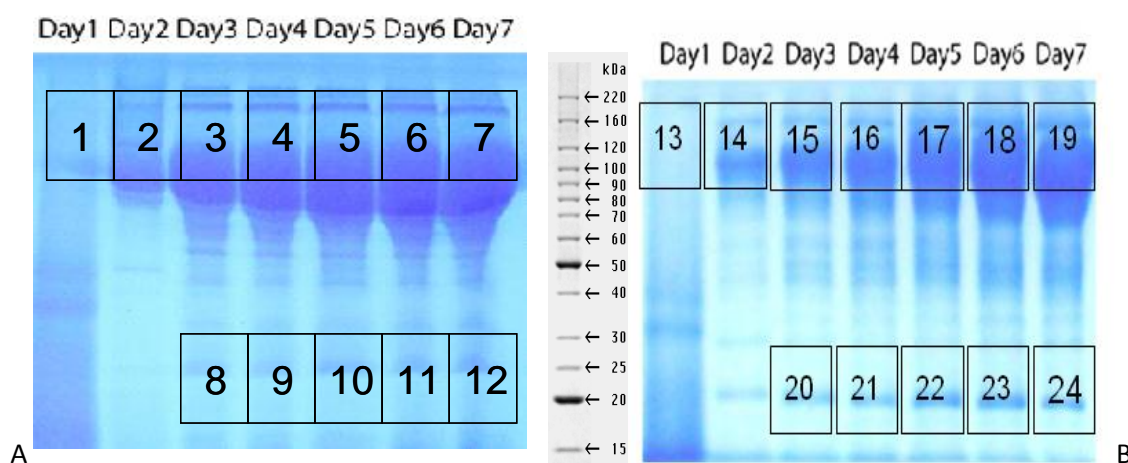
The protein sequences of the two identified aspartic proteases (Table 3.3) were aligned against the protein sequence of Pep 1, *Trichoderma reesei* aspartic protease pro- precursor (Appendix 1) to determine if one of the identified aspartic proteases could be Pep 1. There was a 27.3% sequence similarity between Pep 1 and the identified 26.5 kDa aspartic protease with accession number jgi|69555, and 25% sequence similarity between Pep 1 and the identified 24.3 kDa intracellular aspartic protease with accession number jgi|106661 (Table 3.3). Therefore, neither of the two identified aspartic proteases appeared to be the Pep 1 enzyme.

Three proteases were identified from the Rut-C30 supernatant using LC ESI MS/MS of proteins excised from five large areas on the SDS-PAGE gel (Figure 3.14). This was a better result than achieved by MALDI TOF/TOF MS/MS analysis of small excision areas of the gel (Section 3.4.1). Therefore, these results confirmed that excising a large area of the gels encompassing protein around the molecular weight of interest, and analysing the protein content by LC ESI MS/MS was an improved strategy for identifying the extracellular proteases. The next step was to apply these techniques to identify and compare the extracellular proteases produced by Rut-C30 with that of the Rut-C30-derived transformant CVt, producing the heterologous Venus protein.

### **3.4.3 LC ESI MS/MS of CVt and Rut-C30 culture supernatant**

*T. reesei* Rut-C30 and transformant CVt were grown in ALS medium (Section 2.3). Samples were collected every day and the supernatants were used to produce SDS-PAGE gels (Figure 3.15) as described above (Section 3.4.1). Bands were excised from large areas of the SDS-PAGE gels (Figure 3.15) encompassing proteins of a molecular weight corresponding to bands seen on previous zymograms produced

from the supernatants of the two strains (Figure 3.7B and 3.7C). The protein bands were subjected to LC ESI MS/MS for protein identification and a total of seven proteases were identified (Table 3.4).



**Figure 3.15** 1D SDS-PAGE gels of culture supernatants (40  $\mu$ l) of *T. reesei* (A) Rut-C30 and (B) CVt grown in ALS medium at 28  $^{\circ}$ C for seven days. The rectangles show the excised areas from each gel.

Four proteases (an aspartic protease, a chymotrypsin-like protease and two subtilisin-like proteases) were identified in the culture supernatants of both Rut-C30 and CVt (Table 3.4). The aspartic protease and the chymotrypsin-like protease were detected from each of the areas excised from the gels encompassing proteins of 16 – 30 kDa from the Rut-C30 and CVt supernatants from Day 3 – Day 7 (areas 8 – 12 and 20 – 24, Figure 3.15). The subtilisin-like proteases were detected from the high molecular weight (100 – 220 kDa) proteins in the supernatants of both strains from Day 1 to Day 7 (areas 1 – 7 and areas 13 – 19, Figure 3.15). However one of the identified subtilisin-like proteases was predicted to be an intracellular protein by PSORT (<http://psort.hgc.jp/>). It is possible that an intracellular protein was present in the supernatant due to cell lysis during cultivation or centrifugation.

**Table 3.4** Proteases identified by LC ESI MS/MS from the SDS-PAGE gels (Figure 3.15) produced from the culture supernatants of *T. reesei* Rut-C30 and CVt grown in ALS medium.

Excised area	Accession number	Description	pI	MW <sub>(kDa)</sub>	Sub-cellular localisation	Strain/s
1-7 13-19	jgi 81517	Peptidase S8, subtilisin	7.54	153.8	Intracellular	Rut-C30 and CVt
1-7 13-19	jgi 51365	Peptidase S8, subtilisin	5.12	93.3	Extracellular	Rut-C30 and CVt
8-12 20-24	jgi 73897	Peptidase S1, chymotrypsin	5.83	26.4	Extracellular	Rut-C30 and CVt
8-12 20-24	jgi 69555	Peptidase A4, aspartic-type endopeptidase	4.53	26.5	Extracellular	Rut-C30 and CVt
13-19	jgi 58282	Peptidase M14, carboxypeptidase A,	5.68	102.6	Intracellular	CVt
13-19	jgi 75159	Peptidase M1, neutral zinc metallopeptidase	5.34	98.8	Intracellular	CVt
13-19	jgi 66608	serine-type peptidase S9	5.34	101.2	Intracellular	CVt

<sup>a</sup> Protein identifications were assigned when the Mowse scores were significant ( $p < 0.05$ ) using the Mascot search engine, and/or when the  $\log_e$  score was lower than the statistically significant cutoff values assigned by the GPM algorithm (GPM search engine).

<sup>b</sup> The excised area refers to the area from the SDS-PAGE gel excised for LC ESI MS/MS, as shown in Figure 3.15.

<sup>c</sup> Accession number and descriptions were as assigned in the jgi database for *T. reesei* (<http://genome.jgi-psf.org/Trire2>).

<sup>d</sup> The pI and Mw were calculated using ExPASy (<http://expasy.org/tools/>).

<sup>e</sup> The sub-cellular localisation was as predicted by PSORT (<http://www.psорт.org/>)



Three proteases were identified from high molecular weight proteins (areas 13 – 19 Figure 3.15) in the supernatant of CVt from Day 1 to Day 7; none of these proteases were identified from the supernatant of Rut-C30 (Table 3.4). The proteases specific to the CVt supernatant were a carboxypeptidase A, a neutral zinc metallo-peptidase and a serine protease, all of which were predicted to be intracellular proteins by PSORT. Intracellular proteins may have been present in the culture supernatant of CVt due to cell lysis during cultivation and/or centrifugation. If due to cell lysis, the presence of the intracellular proteins in the culture supernatant of CVt and not Rut-C30 suggests that the CVt cell membrane may have been more susceptible to breakage than that of Rut-C30. Perhaps this was due to increased cellular stress as a result of transformation and heterologous protein production. It is possible that the cell membrane of CVt was more susceptible to breakage than that of the parent strain Rut-C30, and the presence of more intracellular proteases in the CVt supernatant than the Rut-C30 supernatant suggests this may have been the case. It is also possible that the production of intracellular protease was higher in CVt than in Rut-C30. Proteomic analysis of the mycelia of both strains would be necessary to investigate this but was not attempted in this study. However, the detection of intracellular proteases in the culture supernatants was not an unusual result and has been reported previously (eg. Grinyer *et al.*, 2006).

The identification of aspartic, chymotrypsin-like and subtilisin-like proteases in the supernatant of Rut-C30 and CVt correlated well with the results of the liquid assays which had revealed these specific protease activities (Section 3.3). Cysteine proteases were not identified and correspondingly no cysteine protease activity was detected in the liquid assays. LC ESI MS/MS is not a quantitative mass spectrometric technique (Bantscheff *et al.*, 2007) so it could not provide any more

information than the assays about the amount of each protease type in the supernatants on different days. Quantification of individual target proteases could be achieved in future work by techniques such as iTRAQ (isobaric Tag for Relative and Absolute Quantitation; Ross *et al*, 2004).

It was previously proposed that the approximately 26 kDa protease present in the supernatant of the *T. reesei* strains was an aspartic protease following the comparison of the results of the liquid assays and zymograms (Section 3.3.2). Mass spectrometry had now confirmed the identity of the aspartic protease. Likewise, it was postulated that a protease of between 160 and 220 kDa could be responsible for the subtilisin-like activity, which was seven times higher in the CVt culture than in the Rut-C30 culture by the end of the cultivation period (Section 3.3.2). Correspondingly, a subtilisin-like protease was identified in the culture supernatants of Rut-C30 and CVt from protein of approximately 100 - 220 kDa.

The comparison of zymogram activity to the results of the chymotrypsin-like protease assay had led to the proposition that the detected chymotrypsin-like activity emanated from a high molecular weight protein (Section 3.3.2). There was a reservation in this proposition, however, due to a discrepancy in which the chymotrypsin-like activity of CVt had decreased in the last days of cultivation whereas the high molecular weight bands on the protease zymogram band had widened and increased in number (Section 3.3.2). The identification of a chymotrypsin-like protease of approximately 26 kDa (Table 3.4) provided an explanation not previously considered, i.e. the chymotrypsin-like protease was of a similar molecular weight as the aspartic protease and thus may have been represented in that 26 kDa band alongside the aspartic protease.

The identification of three additional proteases at around 100 kDa (carboxypeptidase, metallopeptidase, serine-type peptidase, Table 3.4) in the supernatant of CVt, but not in the supernatant of Rut-C30, could explain the presence of the additional high molecular weight bands on the zymogram of CVt supernatant (Figure 3.7C), particularly evident in Days 6 and 7. The additional proteases in the supernatant of CVt, a strain representative of a transformant producing a heterologous protein, is a concern for heterologous protein production because the yield of the target protein could be considerably reduced due to protease degradation. Whether the additional proteases in the culture medium were the result of cell lysis or abnormal secretion as a result of genomic changes caused by the transformation process was beyond the scope of this study. Genome sequencing of the CVt strain, such as 454 sequencing (Margulies *et al.*, 2005), could reveal specific DNA mutations that could be linked to the observed results.

## Chapter 4: Summary and future prospects

*Trichoderma reesei* has a natural high capacity for protein secretion and is currently used for industrial enzyme production. However, a major impediment for the use of *T. reesei* as an expression host for heterologous gene products is their degradation by extracellular proteases, which can substantially reduce final product yields (Nevalainen and Te'o, 2003; Peterson and Nevalainen, 2012). The aim of this project was to profile, identify and compare the extracellular proteases produced by several strains of *T. reesei*: the wild-type QM6a, the high protein secreting mutant Rut-C30 (transformation host) and CVt (a transformant derived from Rut-C30, producing a heterologous Venus protein). The comparison between the Rut-C30 and CVt strains was undertaken to investigate how the expression of heterologous proteins impacts on extracellular protease activity. The study provides a valuable contribution towards understanding the proteolytic degradation of heterologous proteins. The work was carried out in several stages, as summarised below.

### 4.1 Refinement of the zymogram technique

The first stage of the work involved the refinement of the zymogram technique for efficient profiling of the extracellular proteases in the culture supernatants. It was found that a good protease profile on the zymogram gels could be achieved without the addition of a reducing agent ( $\beta$ -mercaptoethanol) to the gel loading buffer (Section 3.2.1.1), and gelatin was chosen as the most sensitive substrate to be used in the zymogram gels (Section 3.2.1.2). The banding pattern and intensity remained the same when the zymogram gels were incubated in developing buffers of pH ranging from 3.5 to 6.5, so pH 6 was chosen as an appropriate pH for the developing buffer in future work (Section 3.2.1.3). Attempts at 2D zymography were not

successful (Section 3.2.1.4), so 1D zymography under the refined conditions was deemed the most efficient method for profiling the extracellular protease of the *T. reesei* strains in this work. Changing the size of the culture inoculant from  $1 \times 10^8$  conidia to  $3 \times 10^7$  conidia caused a very slight decrease in protease activity evidenced on the zymogram; however, the banding pattern was very similar. Therefore it was decided that inoculation with  $1 \times 10^8$  conidia was most appropriate for detection and identification of the extracellular proteases in this work (Section 3.2.2).

#### **4.2 Comparison of the protease profiles of *T. reesei* Rut-C30, QM6a and CVt by zymography**

Zymography was used to compare the proteases in the supernatants of *T. reesei* Rut-C30 (transformation host), QM6a (wild-type) and CVt (producing a heterologous Venus protein). The zymograms revealed differences in protease secretion between the three strains grown under the same conditions (Section 3.2.3). The zymogram produced from Rut-C30 supernatant showed a lower number of proteolytic bands compared to the zymogram produced from the QM6a (wild-type) supernatant, reflecting the previously documented characteristic of Rut-C30 as a low protease mutant strain (Sheir-Neiss and Montenecourt, 1984). More surprisingly, a large number of proteolytic bands were evident on the zymogram of CVt, indicating substantially higher protease activity in the CVt supernatant than in Rut-C30, the parent strain of CVt.

#### **4.3 Characterisation of the proteases in the supernatants of *T. reesei* Rut-C30 and CVt by liquid assays**

Liquid assays were carried out using specific fluorescently labelled and coloremtric substrates (Table 2.3) to characterise the types of protease activity in the *T. reesei*

Rut-C30 and CVt supernatants. Aspartic proteases were detected in the supernatants of both strains; however, there was higher aspartic protease activity in Rut-C30 supernatant than in the CVt supernatant (Section 3.3.2). The increase in aspartic activity of Rut-C30 occurred concurrently with the drop in pH of the Rut-C30 culture medium (Section 3.1). Conversely, the culture medium of CVt remained at a near neutral pH though the cultivation period (possibly due to a slower growth rate) and a higher level of subtilisin-like and chymotrypsin-like activity was recorded (Section 3.3.3). No cysteine protease activity was detected in the supernatants of either strain. From these results, it seemed apparent that Rut-C30 and CVt secreted the same types of proteases under the same culture conditions but there were differences in the levels of activity of each of the protease types between the two strains. In particular, it appeared that production of the heterologous Venus protein had resulted in a massive increase in subtilisin-like protease activity.

#### **4.4 Identification of the proteases in the *T. reesei* Rut-C30 and CVt supernatants by mass spectrometry**

Two well-established mass spectrometric techniques, MALDI TOF/TOF MS/MS and LC ESI MS/MS were trialed to identify the proteases in the Rut-C30 culture supernatant (Section 3.4.1 and 3.4.2). LC ESI MS/MS was found to be the most effective technique, allowing the identification of Rut-C30 proteases from large excised areas of an SDS-PAGE gel encompassing proteins of a molecular weight matching that of bands on a corresponding zymogram (Section 3.4.2). The LC ESI MS/MS technique was then used to identify proteins excised from SDS PAGE gels of *T. reesei* Rut-C30 and CVt supernatants, again by choosing the excised areas to match bands on zymograms of Rut-C30 and CVt supernatants (Section 3.4.3).

Four proteases were identified as common to the supernatants of both Rut-C30 and CVt (Table 3.4, Section 3.4.3), an aspartic protease, a chymotrypsin-like protease and two subtilisin-like proteases. One of the subtilisin-like proteases was predicted to be an intracellular protein. The identification of the aspartic, chymotrypsin-like and subtilisin-like proteases were in line with the detection of their corresponding activities in the liquid assays (Section 3.3). In addition, an aspartic protease has been previously identified as the main secreted protease of *T. reesei* (Margolles-Clark *et al.*, 1996b). Three additional proteases were identified in the supernatant of CVt only, a carboxypeptidase A, a neutral zinc metallo-peptidase and a serine protease, all of which were predicted to be intracellular proteins. The presence of proteases in the supernatant that were not expected to be secreted, according to gene sequence, could be at least partly explained by an increased amount of cell lysis in the CVt strain, possibly as a result of cellular stress due to transformation and heterologous protein production.

#### **4.5 Conclusions and future work**

Distinct profiles of extracellular protease activity were revealed from a high protein secreting mutant *T. reesei* Rut-C30 and a transformant CVt producing a heterologous protein, cultivated under the same defined growth conditions. Rut-C30 and CVt were found to secrete the same types of proteases but there were marked differences in the activity levels of each protease type in the culture medium. The Rut-C30 strain predominantly secreted an aspartic (acidic) protease, in response to the drop in pH of the culture medium of Rut-C30 during the cultivation period. The transformant CVt, on the other hand, experienced a slower growth rate and maintained a relatively even and near neutral pH of the culture medium during the growth period; as a result, there was less secretion and activity of the aspartic

protease, and increased secretion and activity of a subtilisin-like (neutral – alkaline) protease. Further investigation of the effect of culture pH on protease production could be carried out by controlling the pH in a small laboratory fermenter, and examining the protease profiles of the supernatants of CVT and Rut-C30 under these conditions. Adding to the protease profile of CVt was the presence of intracellular proteases, most likely due to cell lysis late in the cultivation period. Increased cell lysis in the transformant strain may have indicated cellular stress due to heterologous protein production. The presence of intracellular proteins in the culture medium, possibly due to increased cellular stress associated with the production of heterologous proteins, is an issue that needs to be acknowledged and addressed in the future.

Moreover, this study has revealed that the process of heterologous protein degradation by proteases is complex. Knowledge of the proteases secreted by a transformant host is only a starting point because a different suite of proteases and/or different levels of activity of each protease type can be experienced by a transformant strain. The study points towards the need for specific profiling of the proteases secreted by individual transformants of interest to gain insight into specific targets for further genetic manipulation to increase product yield. Further analysis could include the separation and purification of the various proteases secreted by the fungi. Also, genetic engineering could be conducted, such as a knock-out of the gene encoding the protease that causes the greatest degradation of the particular foreign protein of interest, for example the aspartic and subtilisin-like proteases identified in this study.



## References

- Archer, D.B., Peberdy, J.F., 1997. The molecular biology of secreted enzyme production by fungi. *Critical Reviews in Biotechnology*. 17: 273-306.
- Bantscheff, M., Schirle, M., Sweetman, G., Rick, J., Kuster, B., 2007. Quantitative mass spectrometry in proteomics: a critical review. *Analytical and Bioanalytical Chemistry*. 389: 1017-1031.
- Benitez, T., Rincon, A.M., Limon, M.C., Codon, A.C., 2004. Biocontrol mechanisms of *Trichoderma* strains. *International Microbiology*. 7: 249-260.
- Berka, R.M., Ward, M., Wilson, L.J., Hayenga, K.J., Kodama, K.H., Carloma, L.P., Thompson, S.A., 1990. Molecular cloning and deletion of the gene encoding aspergillopepsin A from *Aspergillus awamori*. *Gene*. 86: 153-162.
- Bisaria, V.S., Ghose, T.K., 1981. Biodegradation of cellulosic materials: substrates, microorganisms, enzymes and products. *Enzyme and Microbial Technology*. 3: 90-104.
- Blackstock, W.P., Weir, M.P., 1999. Proteomics: quantitative and physical mapping of cellular proteins. *Trends in Biotechnology*. 17: 121-127.
- Blumenthal, C., 2004. Production of toxic metabolites in *Aspergillus niger*, *Aspergillus oryzae*, and *Trichoderma reesei*: justification of mycotoxin testing in food grade enzyme preparations derived from the three fungi. *Regulatory Toxicology and Pharmacology*. 39: 214-228.
- Bradford, S., 2000. Characterisation of the protease profile of two mutant strains of *Trichoderma reesei*. Honours thesis. Macquarie University, Sydney, Australia.
- Chernushevich, I.V., Loboda, A.V., Thomson, B.A., 2001. An introduction to quadrupole time-of-flight mass spectrometry. *Journal of Mass Spectrometry*. 36: 849-865.
- Cherry, J.R., Fidantsef, A.L., 2003. Directed evolution of industrial enzymes: an update. *Current Opinion in Biotechnology*. 14: 438-443.
- Choi, N.S., Yoon, K.S., Lee, J.Y., Han, K.Y., Kim, S.H., 2001. Comparison of three substrates (casein, fibrin, and gelatin) in zymographic gel. *Journal of Biochemistry and Molecular Biology*. 34: 531-536.
- Conesa, A., Punt, P.J., van Luijk, N., van den Hondel, C.A., 2001. The secretion pathway in filamentous fungi: a biotechnological view. *Fungal Genetics and Biology*. 33: 155-171.
- Delgado-Jarana, J., Rincón, A.M., Benítez, T., 2002. Aspartyl protease from *Trichoderma harzianum* CECT 2413: cloning and characterization. *Microbiology*. 148: 1305-1315.

- Diener, S.E., Dunn-Coleman N., Foreman P., Houfek, T.D., Teunissen, P.J.M., van Solingen, P., Dankmeyer, L., Mitchell, T.K., Ward, M., Dean, R.A., 2004. Characterization of the protein processing and secretion pathways in a comprehensive set of expressed sequence tags from *Trichoderma reesei*. FEMS Microbiology Letters. 230: 275-282.
- Durand, H., Clanet, M., 1988. Genetic improvement of *Trichoderma reesei* for large scale cellulase production. Enzyme and Microbial Technology. 10: 5710-5721.
- Dutta, T., Sahoo, R., Sengupta, R., Ray, S.S., Bhattacharjee, A., Ghosh, S., 2008. Novel cellulases from an extremophilic filamentous fungi *Penicillium citrinum*: production and characterization. Journal of Industrial Microbiology and Biotechnology. 35: 275-82.
- Elander, R.P., 2003. Industrial production of beta-lactam antibiotics. Applied Microbiology and Biotechnology. 61: 385-392.
- Eneyskaya, E.V., Kulminskaya, A.A., Savel'ev, A.N., Savel'eva, N.V., Shabalin, K. A., Neustroev, K.N., 1999. Acid protease from *Trichoderma reesei*: limited proteolysis of fungal carbohydrases. Applied Microbiology and Biotechnology. 52: 226-231.
- Fenyő, D., Eriksson, J., Beavis, R., 2010. Mass spectrometric protein identification using the global proteome machine. Methods in Molecular Biology. 673: 189-202.
- Gams, W., Bissett, J., 1998. Morphology and identification of *Trichoderma*. In: Harmann, G.E., Kubicek, C.P. (eds) *Trichoderma and Gliocladium*, Vol. 1, Basic Biology, Taxonomy and Genetics, Taylor and Francis, London, pp. 3-34.
- Gente, S., Billon-Grand, G., Poussereau, N., Fèvre, M. 2001. Ambient alkaline pH prevents maturation but not synthesis of ASPA, the aspartyl protease from *Penicillium roqueforti*. Current Genetics. 38: 323-328.
- Ghosh, A., Al-Rabiai, S., Ghosh, B.K., 1982. Increased endoplasmic reticulum content of a mutant of *Trichoderma reesei* (RUT-C30) in relation to cellulase synthesis. Enzyme and Microbial Technology. 4: 110-113.
- Gibbs, P.A., Seviour, R.J., Schmid, F., 2000. Growth of filamentous fungi in submerged culture: problems and possible solutions. Critical Reviews in Biotechnology. 20:17-48.
- Gogichaeva, N.V., Williams, T., Alterman, M.A., 2007. MALDI TOF/TOF tandem mass spectrometry as a new tool for amino acid analysis. Journal of the American Society for Mass Spectrometry. 18:279-284.
- Goller, S.P., Schoisswohl, D., Baron, M., Parriche, M., Kubicek, C.P., 1998. Role of endoproteolytic dibasic proprotein processing in maturation of secretory proteins in *Trichoderma reesei*. Applied and Environmental Microbiology 64: 3202-3208.

- Greenhill, A.R., Blaney, B.J., Shipton, W.A., Frisvad, J.C., Pue, A., Warner, J.M., 2008. Mycotoxins and toxigenic fungi in sago starch from Papua New Guinea. *Letters in Applied Microbiology*. 47: 342-347.
- Grimm, L.H., Kelly, S., Krull, R., Hempel, D.C., 2005. Morphology and productivity of filamentous fungi. *Applied Microbiology and Biotechnology*. 69: 375-384.
- Grinyer, J., Kautto, L., Traini, M., Willows, R.D., Te'o, J., Bergquist, P., Nevalainen, H., 2006. Proteome mapping of the *Trichoderma reesei* 20S proteasome. *Current Genetics*. 51: 79-88.
- Harkki, A., Uusitalo, J., Bailey, M., Penttilä, M., Knowles, J.K.C., 1989. A novel fungal expression system: secretion of active calf chymosin from the filamentous fungus *Trichoderma reesei*. *Nature Biotechnology*. 7: 596-603.
- Harman, G.E. 2000. Myths and dogmas of biocontrol. Changes in perceptions derived from research on *Trichoderma harzianum* T22. *Plant Disease*. 84: 377-393.
- Herpoël-Gimbert, I., Margeot, A., Dolla, A., Jan, G., Mollé, D., Lignon, S., Mathis, H., Sigoillot, J.C., Monot, F., Asther, M., 2008. Comparative secretome analyses of two *Trichoderma reesei* RUT-C30 and CL847 hypersecretory strains. *Biotechnology for Biofuels*. 1: 18.
- Hölker, U., Höfer, M., Lenz, J., 2004. Biotechnological advantages of laboratory-scale solid-state fermentation with fungi. *Applied Microbiology and Biotechnology*. 64: 175-186.
- Ilmén, M., Onnela, M.L., Klemsdal, S., Keränen, S., Penttilä, M., 1996. Functional analysis of the cellobiohydrolase I promoter of the filamentous fungus *Trichoderma reesei*. *Molecular and General Genetics*. 253: 303-314.
- Jaklitsch, W.M., 2009. European species of *Hypocrea*. Part I. The green-spored species. *Studies in Mycology*. 63: 1-91.
- Joosten, V., Lokman, C., van den Hondel, C.A.M.J.J., Punt, P.J., 2003. The production of antibody fragments and antibody fusion proteins by yeast and filamentous fungi. *Microbial Cell Factories* 2: 1.
- Jun, H., Bing, Y., Keying, Z., Xuemei, D., Daiwen, C., 2009. Strain improvement of *Trichoderma reesei* Rut C-30 for increased cellulase production. *Indian Journal of Microbiology*. 49: 188-195.
- Kautto, L.K., 2009. *Trichoderma reesei* proteasome and genome-wide effects of the expression of mutant cellobiohydrolase I. PhD thesis, Department of Chemistry and Biomolecular Sciences, Macquarie University, Sydney, Australia. <http://minerva.mq.edu.au:8080/vital/access/manager/Repository/mq:9273>.

- Kim, Y., Nandakumar, M.P., Marten, M.R., 2007. Proteomics of filamentous fungi. *Trends in Biotechnology*. 25: 395-400.
- Klein, D., Eveleigh, D.E., 1998. Ecology of *Trichoderma*. In: Harmann, G.E., Kubicek, C.P. (eds) *Trichoderma and Gliocladium*, Vol. 1, Basic Biology, Taxonomy and Genetics. Taylor and Francis, London. pp. 57-74.
- Kredics, L., Antal, Z., Szekeres, A., Hatvani, L., Manczinger, L., Vágvölgyi, C., Nagy, E., 2005. Extracellular proteases of *Trichoderma* species. A review. *Acta Microbiologica et Immunologica Hungarica*. 52: 169-184.
- Krishna, C., 2005. Solid-state fermentation systems - an overview. *Critical Reviews in Biotechnology*. 25: 1-30.
- Kubicek, C.P., Komon-Zelazowska, M., Druzhinina, I. S., 2008. Fungal genus *Hypocrea/Trichoderma*: from barcodes to biodiversity. *Journal of Zhejiang University, Science B*. 9: 753-763.
- Kumar, R., Singh, S., Singh, O.V., 2008. Bioconversion of lignocellulosic biomass: biochemical and molecular perspectives. *Journal of Industrial Microbiology and Biotechnology*. 35: 377-391.
- Kunz, C., Lönnerdal, B., 1990. Human-milk proteins: analysis of casein and casein subunits by anion-exchange chromatography, gel electrophoresis, and specific staining methods. *The American Journal of Clinical Nutrition*. 51: 37-46.
- Laemmli, U. K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. 227: 680-685.
- Lantz, M.S., Ciborowski, P., 1994. Zymographic techniques for detection and characterization of microbial proteases. *Methods in Enzymology*. 235: 563-94
- Le Crom, S., Schackwitz, W., Pennacchio, L., Magnuson, J.K., Culley, D.E., Collett, J.R., Martin, J., Druzhinina, I.S., Mathis, H., Monot, F.D.R., Seiboth, B., Cherry, B., Rey, M., Berka, R., Kubicek, C.P., Baker, S.E., Margeot, A., 2009. Tracking the roots of cellulase hyperproduction by the fungus *Trichoderma reesei* using massively parallel DNA sequencing. *Proceedings of the National Academy of Sciences*. 106: 16151-16156.
- Li, Q., Harvey, L.M., McNeil, B., 2008. The effects of bioprocess parameters on extracellular proteases in a recombinant *Aspergillus niger* B1-D. *Applied Microbiology and Biotechnology*. 78: 333-341.
- Lim, S.H., Jong Soo Choi, J.S., Park, E.Y., 2001. Microbial production of riboflavin using riboflavin overproducers, *Ashbya gossypii*, *Bacillus subtilis*, and *Candida famate*: an Overview. *Biotechnology and Bioprocess Engineering*. 6: 75-88.
- Linko, M., 1993. Opening address. The 2<sup>nd</sup> TRICEL symposium on *Trichoderma reesei* cellulases and other hydrolases, Espoo. Foundation of Biotechnical and Industrial Fermentation Research 9.

- Lowe, D.A., 1992. Fungal enzymes. In: Arora, D.K., Elander, R.P., Mukerji, K.G. (eds), Handbook of Applied Mycology. Fungal Biotechnology. Marcel Dekker Inc., New York. pp. 681-706.
- Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. The Journal of Biological Chemistry. 193: 265-75.
- Magnuson, J.K., Lasure, L.L., 2004. Organic acid production by filamentous fungi. In: Lange, J., Lange, L. (eds), Advances in Fungal Biotechnology for Industry, Agriculture, and Medicine. Kluwer Academic/Plenum Publishers, New York. pp. 307-340.
- Mandels, M., Weber, J., Parizek, R., 1971. Enhanced cellulase production by a mutant of *Trichoderma viride*. Applied Microbiology. 21: 152-154.
- Mandels, M., Reese, E.T., 1957. Induction of cellulase in *Trichoderma viride* as influenced by carbon sources and metals. Journal of Bacteriology. 73: 269-278.
- Mäntylä, A., Paloheimo, M., Suominen, P., 1998. Industrial mutants and recombinant strains of *Trichoderma reesei*. In: Harmann, G.E., Kubicek, C.P. (eds) *Trichoderma and Gliocladium*, Vol. 2, Enzymes, biological control and commercial applications. Taylor and Francis, London. pp. 291-309.
- Mäntylä, A., Paloheimo, M., Hakola, S., Lindberg, E., Leskinen, S., Kallio, J., Vehmaanperä, J., Lantto, R., Suominen, P., 2007. Production in *Trichoderma reesei* of three xylanases from *Chaetomium thermophilum*: a recombinant thermoxylanase for biobleaching of kraft pulp. Applied Microbiology and Biotechnology. 76: 377-386.
- Margolles-Clark, E., Hayes, C.K., Harman, G.E., Penttilä, M., 1996a. Improved production of *Trichoderma harzianum* endochitinase by expression in *Trichoderma reesei*. Applied and Environmental Microbiology. 62: 2145-2151.
- Margolles-Clark, E., Tenkanen, M., Nakari-Setälä, T., Penttilä, M., 1996b. Cloning of genes encoding alpha-L-arabinofuranosidase and beta-xylosidase from *Trichoderma reesei* by expression in *Saccharomyces cerevisiae*. Applied and Environmental Microbiology. 62: 103840-3846.
- Margolles-Clark, E., Ilmén, M., Penttilä, M., 1997. Expression patterns of ten hemicellulase genes of the filamentous fungus *Trichoderma reesei* on various carbon sources. Journal of Biotechnology. 57: 167-179.
- Margulies, M., Egholm, M., Altman, W.E., Attiya, S., Bader, J.S., Bemben, L.A., Berka, J., Braverman, M.S., Chen, Y.J., Chen, Z., Dewell, S.B., Du, L., Fierro, J.M., Gomes, X.V., Godwin, B.C., He, W., Helgesen, S., Ho, C.H., Irzyk, G.P., Jando, S.C., Alenquer, M.L.I., Jarvie, T.P., Jirage, K.B., Kim, J.B., Knight, J.R., Lanza, J.R., Leamon, J.H., Lefkowitz, S.M., Lei, M., Li, J., Lohman, K.L., Lu,

- H., Makhijani, V.B., McDade, K.E., McKenna, M.P., Myers, E.W., Nickerson, E., Nobile, J.R., Plant, R., Puc, B.P., Ronan, M.T., Roth, G.T., Sarkis, G.J., Simons, J.F., Simpson, J.W., Srinivasan, M., Tartaro, K.R., Tomasz, A., Vogt, K.A., Volkmer, G.A., Wang, S.H., Wang, Y., Weiner, M.P., Yu, P., Begley, R.F., Rothberg, J.M., 2005. Genome sequencing in microfabricated high-density picolitre reactors. *Nature* 437: 376-380.
- Markaryan, A., Lee, J.D., Sirakova, T.D., Kolattukudy, P.E., 1996. Specific inhibition of mature fungal serine proteinases and metalloproteinases by their propeptides. *Journal of Bacteriology*. 178: 2211-2215.
- Martínez, T.F., Alarcón, F.J., Díaz-López, M., Moyano, F.J., 2000. Improved detection of amylase activity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with copolymerized starch. *Electrophoresis*. 21: 2940-2943.
- Montenecourt, B.S., Eveleigh, D.E., 1979. Selective screening methods for the isolation of high yielding cellulase mutants of *Trichoderma reesei*. In: Brown, R., Jurasek, L. (eds), *Hydrolysis of Cellulose: Mechanisms of Enzymatic and Acid Catalysis*, *Advances in Chemistry Series* 181. American Chemical Society, Washington, D.C. pp. 289-301.
- Moralejo, F.J., Cardoza, R.E., Gutierrez, S., Sisniega, H., Faus, I., Martín, J.F., 2000. Overexpression and lack of degradation of thaumatin in an aspergillopepsin A defective mutant of *Aspergillus awamori* containing an insertion in the pepA gene. *Applied Microbiology and Biotechnology*. 54: 772-777.
- Muthuvelayudham, R., Viruthagiri, T., 2006. Fermentative production and kinetics of cellulase protein on *Trichoderma reesei* using sugarcaneb and rice straw. *African Journal of Biotechnology*. 5: 1873-1881.
- Nandakumar, M.P., Cheung, A., Marten, M.R., 2006. Proteomic analysis of extracellular proteins from *Escherichia coli* W3110. *Journal of Proteome Research*. 5: 1155-1161.
- Nevalainen, H., 1985. Genetic Improvement of Enzyme Production in Industrially Important Fungal Strains. Publication 26. VTT Technical Research Center of Finland, Espoo.
- Nevalainen, H., Suominen, P., Taimisto, K., 1994. On the safety of *Trichoderma reesei*. *Journal of Biotechnology*. 37: 193-200.
- Nevalainen, H., Te'o, V.S., Bergquist, P.L., 2005. Heterologous protein expression in filamentous fungi. *Trends in Biotechnology*. 23: 468-474.
- Nevalainen, H., Te'o, V.S., 2003. Enzyme production in industrial fungi- molecular genetic strategies for integrated strain improvement. *Applied Mycology and Biotechnology*. 3: 241-259.
- Novillo, C., Castañera, P., Ortego, F., 1997. Inhibition of digestive trypsin like proteases from larvae of several lepidopteran species by the diagnostic

- cysteine protease inhibitor E64. *Insect Biochemistry and Molecular Biology*. 27: 247-254.
- Nyyssönen, E., Penttilä, M., Harkki, A., Saloheimo, A., Knowles, J.K., Keränen, S., 1993. Efficient production of antibody fragments by the filamentous fungus *Trichoderma reesei*. *Bio/technology*. 11: 591-595.
- O'Donnell, D., Wang, L., Xu, J., Ridgway, D., Gu, T., Moo-Young, M., 2001. Enhanced heterologous protein production in *Aspergillus niger* through pH control of extracellular protease activity. *Biochemical Engineering Journal*. 8: 187-193.
- Penttilä, M.E., Nevalainen, H., Salminen, E., Rättö, M., Knowles, J. 1987. Versatile transformation system for the cellulolytic filamentous fungus *Trichoderma reesei*. *Gene* 61:155-164.
- Penttilä, M., Limon, C., Nevalainen, H., 2004. Biotechnology of *Trichoderma*. In: Arora, D.K., Bridge, P.D., Bhatnagar, D. (eds), *Handbook of Fungal Biotechnology (Mycology Series 20)*. Marcel Dekker, New York. pp. 413-427.
- Park, E.Y., Zhang, J.H., Tajima, S., Dwiarti, L., 2007. Isolation of *Ashbya gossypii* mutant for an improved riboflavin production targeting for biorefinery technology. *Journal of Applied Microbiology*. 103: 468-476.
- Perkins, D.N., Pappin, D.J.C., Creasy, D.M., Cottrell, J.S., 1999. Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis*. 20: 3551.
- Peterson, R., Nevalainen, H., 2012. *Trichoderma reesei* RUT-C30 – thirty years of strain improvement. *Microbiology*. 158: 58-68.
- Peterson, R., Grinyer, J., Joss, J., Khan, A., Nevalainen, H., 2009. Fungal proteins with mannanase activity identified directly from a Congo Red stained zymogram by mass spectrometry. *Journal of Microbiological Methods*. 79: 374-377.
- Peterson, R., Grinyer, J., Nevalainen, H., 2011. Extracellular hydrolase profiles of fungi isolated from koala faeces invite biotechnological interest. *Mycological Progress*. 10: 207-218.
- Polizeli, M.L., Rizzatti, A.C., Monti, R., Terenzi, H.F., Jorge, J.A., Amorim, D.S., 2005. Xylanases from fungi: properties and industrial applications. *Applied Microbiology and Biotechnology*. 67: 577-591.
- Powers, J.C., Kam, C.M., Ricketts, K.M., Casillas, R.P., 2000. Cutaneous protease activity in the mouse ear vesicant model. *Journal of Applied Toxicology*. 20: S177-S182.
- Punt, P.J., van Biezen, N., Conesa, A., Albers, A., Mangnus, J., van den Hondel, C., 2002. Filamentous fungi as cell factories for heterologous protein production. *Trends in Biotechnology*. 20: 200-206.

- Ramachandran, S., Fontanille, P., Pandey, A., Larroche, C., 2006. Gluconic acid: properties, applications and microbial production. *Food Technology and Biotechnology*. 44: 185-195.
- Raspanti, E., Cacciola, S.O., Gotor, C., Romero, L.C., Garcia, I., 2009. Implications of cysteine metabolism in the heavy metal response in *Trichoderma harzianum* and in three *Fusarium* species. *Chemosphere*. 76: 48-54.
- Rekas, A., Alattia, J.R., Nagai, T., Miyawaki, A., Ikura, M., 2002. Crystal structure of venus, a yellow fluorescent protein with improved maturation and reduced environmental sensitivity. *The Journal of Biological Chemistry*. 277:50573-50578.
- Rojas, J.A., Cruz, C., Mikán, J.F., Villalba, L.S., Cepero de García, M.C., Restrepo, S., 2009. Isoenzyme characterization of proteases and amylases and partial purification of proteases from filamentous fungi causing biodeterioration of industrial paper. *International Biodeterioration & Biodegradation*. 63: 169-175.
- Ross, P.L., Huang, Y.N., Marchese, J.N., Williamson, B., Parker, K., Hattan, S., Khainovski, N., Pillai, S., Dey, S., Daniels, S., Purkayastha, S., Juhasz, P., Martin, S., Bartlet-Jones, M., He, F., Jacobson, A., Pappin, D.J., 2004. Multiplexed protein quantitation in *Saccharomyces cerevisiae* using amine-reactive isobaric tagging reagents. *Molecular & Cellular Proteomics*. 3: 1154-69.
- Rossano, R., Larocca, M., Riccio, P., 2011. Digestive enzymes of the crustaceans *Munida* and their application in cheese manufacturing: a review. *Marine Drugs*. 9: 1220-1231.
- Ruocco, M., Lanzuise, S., Vinale, F., Marra, R., Turra, D., Woo, S. L., Lorito, M., 2009 Identification of a new biocontrol gene in *Trichoderma atroviride*: the role of an ABC transporter membrane pump in the interaction with different plant-pathogenic fungi. *Molecular Plant-Microbe Interactions*. 22: 291-301.
- Saitoh, E., Yamamoto, S., Okamoto, E., Hayakawa, Y., Hoshino, T., Sato, R., Isemura, S., Ohtsubo, S., Taniguchi, M., 2007. Identification of cysteine proteases and screening of cysteine protease inhibitors in biological samples by a two-dimensional gel system of zymography and reverse zymography. *Analytical Chemistry Insights*. 2: 51-59.
- Saloheimo, M., Pakula, T.M., 2012. The cargo and the transport system: secreted proteins and protein secretion in *Trichoderma reesei* (*Hypocrea jecorina*). *Microbiology*. 158: 46-57.
- Saxena, R.K., Malhotra, B., Batra, A., 2004. Commercial importance of some fungal enzymes. In: D. K. Arora (ed), *Handbook of Fungal Biotechnology (Mycology Series 20)*. Marcel Dekker, New York. pp. 287-298.



- Schuster, E., Dunn-Coleman, N., Frisvad, J., van Dijck P., 2002. On the safety of *Aspergillus niger* – a review. *Applied Microbiology and Biotechnology*. 59: 426-35
- Schuster, A., Schmoll, M., 2010. Biology and biotechnology of *Trichoderma*. *Applied Microbiology and Biotechnology*. 87: 787-799.
- Seidl, V., Schmoll, M., Scherm, B., Balmas, V., Seiboth, B., Migheli, Q., Kubicek, C. P., 2006. Antagonism of *Pythium* blight of zucchini by *Hypocrea jecorina* does not require cellulase gene expression but is improved by carbon catabolite derepression. *FEMS Microbiology Letters*. 257: 145-151.
- Seidl, V., Gamauf, C., Druzhinina, I.S., Seiboth, B., Lukas, H., Christian, P.K., 2008. The *Hypocrea jecorina* (*Trichoderma reesei*) hypercellulolytic mutant RUT C30 lacks a 85 kb (29 gene-encoding) region of the wild-type. *BMC Genomics*. 9: 327-342.
- Sharma, M., Chadha, B.S., Kaur, M., Ghatora, S.K., Saini, H.S., 2008. Molecular characterization of multiple xylanase producing thermophilic/thermotolerant fungi isolated from composting materials. *Letters in Applied Microbiology*. 46: 526-535.
- Sheir-Neiss, G., Montenecourt, B.S., 1984. Characterization of the secreted cellulases of *Trichoderma reesei* wild type and mutants during controlled fermentations. *Applied Microbiology and Biotechnology*. 20: 46-53.
- Simmons, E.G., 1977. Classification of some cellulase-producing *Trichoderma* species. In: Bigelow, H.E., Simmons, E.G. (eds), *Biology of Trichoderma* symposium, 2nd International Mycological Congress Abstracts. University of South Florida, Tampa, FL. pp. 618.
- Soni, R., Nazir, A., Chadha, B.S., Saini, H.S., 2008. Novel sources of fungal cellulases for efficient deinking of composite paper waste. *BioResearch*. 3: 234-246.
- Steen, H., Mann, M., 2004. The abc's (and xyz's) of peptide sequencing. *Nature Reviews Molecular Cell Biology*. 5: 699-711.
- Suárez, M.B., Sanz, L., Chamorro, M.I., Rey, M., González, F.J., Llobell, A., Monte, E., 2005. Proteomic analysis of secreted proteins from *Trichoderma harzianum* Identification of a fungal cell wall-induced aspartic protease. *Fungal Genetics and Biology*. 42: 924-934.
- Sun, A. 2012. Heterologous expression of the mammalian peptide hormone obestatin in *Trichoderma reesei*. PhD thesis, Department of Chemistry and Biomolecular Sciences, Macquarie University, Sydney, Australia.
- Swaigood, H.E., 1993. Genetic perspectives on milk proteins: comparative stud1 ES and nomenclature. *Journal of Dairy Science*. 76: 3054-3061.

- Takeuchi, M., Ueno, Y., Ichishima, E., 1988. Fluorogenic substrate of *Aspergillus* aspartic proteinase. *Agricultural and Biological Chemistry*. 52: 1279-1280.
- te Biesebeke, R., Record, E., van Biezen, N., Heerikhuisen, M., Franken, A., Punt, P. J., van den Hondel, C.A., 2005. Branching mutants of *Aspergillus oryzae* with improved amylase and protease production on solid substrates. *Applied Microbiology and Biotechnology*. 69: 44-50.
- Tenkanen, M., Puls, J., Poutanen, K., 1992. Two major xylanases of *Trichoderma reesei*. *Enzyme and Microbial Technology*. 14: 566-574.
- Torelli, E., Firrao, G., Locci, R., Gobbi, E., 2006. Ochratoxin A-producing strains of *Penicillium* spp. isolated from grapes used for the production of "passito" wines. *International Journal of Food Microbiology*. 106: 307-312.
- Uhlig, H., 1990. Survey of industrial enzymes. In: Gerhartz, W. (ed), *Enzymes in Industry: Production and Application*. VCH, Weinheim. pp. 77-91.
- Uusitalo J. M., Nevalainen H., Harkki A. M., Knowles J. K., Penttilä M. E., 1991. Enzyme production by recombinant *Trichoderma reesei* strains. *Journal of Biotechnology*. 17: 35-49.
- van de Kamp, M., Driessen, A.J.M., Konings, W., 1999. Compartmentalization and transport in  $\beta$ -lactam antibiotic biosynthesis by filamentous fungi. *Antonie Van Leeuwenhoek*. 75: 41-78.
- van den Berg, M.A., Albang, R., Albermann, K., Badger, J.H., Daran, J.-M., Driessen, A.J.M., Garcia-Estrada, C., Fedorova, N.D., Harris, D.M., Heijne, W.H.M., Joardar, V.S., Kiel, J.A.K.W., Kovalchuk, A., Martin, J.F., Nierman, W.C., Nijland, J.G., Pronk, J.T., Roubos, J.A., van der Klei, I.J., van Peij, N.N.M.E., Veenhuis, M., von Doehren, H., Wagner, C., Wortman, J.R., Bovenberg, R.A.L., 2008. Genome sequencing and analysis of the filamentous fungus *Penicillium chrysogenum*. *Nature Biotechnology*. 26: 1161-1168.
- van den Hombergh, J.P., Van den Vondervoort, P.J., Fraissinet-Tachet, L., Visser, J., 1997. *Aspergillus* as a host for heterologous protein production: the problem of proteases. *Trends in Biotechnology*. 15: 256-263.
- Walker, J.M., 2005. Protein structure, purification, characterisation and function analysis. In: Wilson, K., Walker, J.M. (eds), *Principles and Techniques of Biochemistry and Molecular Biology*. Cambridge University Press, New York. pp. 349-404.
- Wang, Y., Xue, W., Sims, A.H., Zhao, C., Wang, A., Tang, G., Qin, J., Wang, H., 2008. Isolation of four pepsin-like protease genes from *Aspergillus niger* and analysis of the effect of disruptions on heterologous laccase expression. *Fungal Genetics and Biology*. 45: 17-27.
- Ward, A.G., Courts, A., 1977. *The Science and Technology of Gelatin*. Academic Press, London.

- Weder, J.K., Haussner, K., Bokor, M.V., 1993. Use of fluorogenic substrates to visualize trypsin and chymotrypsin inhibitors after electrophoresis. *Electrophoresis*. 14: 220-226.
- Wiebe, M., 2003. Stable production of recombinant proteins in filamentous fungi – problems and improvements. *Mycologist*. 17: 140-144.
- Xin, L., Jibin, S., Manfred, N., Josef, W., An-Ping, Z., Ursula, R., 2010. The intra- and extracellular proteome of *Aspergillus niger* growing on defined medium with xylose or maltose as carbon substrate. *Microbial Cell Factories*. 9: 23.
- Yates, J.R., 2000. Mass spectrometry - from genomics to proteomics. *Trends in Genetics*. 16: 5-8.
- Yedidia, B.N.C., 1999. Induction of defense responses in cucumber plants (*Cucumis sativus* L.) by the biocontrol agent *Trichoderma harzianum*. *Applied and Environmental Microbiology*. 65: 1061-1070.
- Zaldívar, M., Velásquez, J.C., Contreras, I., María Pérez, L., 2001. *Trichoderma aureoviride* 7-121, a mutant with enhanced production of lytic enzymes: its potential use in waste cellulose degradation and/or biocontrol. *Electronic Journal of Biotechnology*. 4: 3.
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