PRODUCTION OF HUMAN FUCOSYLTRANSFERASES IN *TRICHODERMA REESEI*

Anna Gryshyna

Department of Chemistry and Biomolecular Sciences,
Macquarie University, Sydney, Australia

Supervisors
Prof. Helena Nevalainen
Prof. Nicolle Packer
Dr. Liisa Kautto

MACQUARIE University
SYDNEY·AUSTRALIA

A thesis submitted in fulfilment of the requirements for the degree of
Doctor of Philosophy
May 2017

“Why do you go away? So that you can come back. So that you can see the place you came from with new eyes and extra colours. And the people there see you differently, too. Coming back to where you started is not the same as never leaving.”

― Terry Pratchett

TABLE OF CONTENTS

Abstract

Glycosylation is the most complex posttranslational modification specific to eukaryotes and also some bacteria. . One of the bottlenecks limiting progress in the glycobiology research is the non-availability and/or high price of glycan-modifying enzymes, such as fucosyltransferases, due to the imperfections of existing production systems.

The aim of this project was to evaluate the potential of a filamentous fungus *Trichoderma reesei* as an expression host for recombinant human α-1,3/4 fucosyltransferases (FUT3 and FUT4). The principles discovered and approaches used can be applied to other mammalian glycoproteins of biomedical interest.

In the current work, cDNAs encoding the C-terminal catalytic domains of the human α-1,3/4 fucosyltransferases were synthesised according to the *T. reesei* codon usage and assembled into expression vectors under the strong cellobiohydrolase 1 (*cbh1*) promoter.

FUT3 and FUT4 were produced as a fusion to a Strep-tag, mCherry fluorescent protein and the core-linker fragment of the endogenous CBH1 protein to facilitate protein purification and secretion. Due to the better performance of the transformants producing the recombinant FUT3 protein, most of the work was carried out with the transformants producing FUT3, while the FUT4 protein was not studied in detail.

Screening of *T. reesei* transformants demonstrated not only secretion of a fusion protein but also its degradation, most probably by the extracellular proteases of *T. reesei*. Protease inhibition studies suggested that the main protease damaging the recombinant FUT3 was a serine protease active at acidic pH. The recombinant protein was purified using a Strep-Tactin column, and the estimated yield of FUT3 was about 70 mg/l. N-terminal sequencing of purified proteins revealed that some of them were N-terminally blocked; one protease cleavage site was identified at the N-terminus of the mCherry protein.

Cultivation of the *T. reesei* transformants at a neutral pH and with addition of a serine protease inhibitor PMSF demonstrated that degradation of the recombinant FUT3 in the culture supernatants could be suppressed.

The activity of the purified recombinant FUT3 was assayed and preliminary results indicated that recombinant FUT3 has certain enzymatic activity although further studies are necessary to confirm these data.

In conclusion, it was established that a recombinant human fucosyltransferase FUT3 could be produced in *T. reesei*. Further studies into optimisation of the cultivation conditions and strain development to limit the proteolytic degradation are required to achieve higher production levels.

STATEMENT OF DECLARATION

I certify that the work presented in this thesis has not been submitted as a part of the requirement for a degree or course to any institution or university other than Macquarie University.

I also certify that this thesis is an original piece of research conducted by me between March 2013 and May 2017 and it contains no material previously published or written by any other person except where due reference is made in the text.

I hereby give my consent to allow a copy of my thesis to be deposited in the University Library for consultation, loan and photocopying forthwith.

Anna Gryshyna (43119328)

May 2017

ACKNOWLEDGEMENTS

My PhD journey was an exiting and challenging experience, with lots of ups and downs and I would like to thank to everyone who helped me to get through the hard times, sometimes even without realising it.

I would like to sincerely thank my supervisors, Professor Helena Nevalainen, Professor Nicki Packer and Dr. Liisa Kautto for their commitment, guidance, and patience throughout my study and for giving me a great opportunity in life.

Many thanks to my fellow postgraduate students, officemates, group members and members of the faculty: Zhiping, Mafruha, Wisam, Aneesh, Karthik, Jashan, Arun, Shaz, Jenny, Rain, Jodie, Ian, Elsa, Catherine and many many others. All of you contributed to my wonderful PhD experience. A special thank you to Dr. Angela Sun for her help and patience and for being a great example of a young and competent scientist.

A very special thank you goes to my friend Phil for his willingness to talk to me and listen to my complaints and also for sending me food from another continent. Our unconventional friendship has helped me to get through the worst months of my PhD. Many thanks to my awesome housemates Jenny, Mathilda and Emily for educating me in the Aussie culture and for being the first people to call me Dr. Anna.

Most importantly, I would like to express my endless gratitude to my family for their love and encouragement; they kept believing in me even when I did not believe in myself. Without their support I would not have been able to achieve this.

PRESENTATIONS AND AWARDS

(1) Poster presentation at the Australian Society for Microbiology conference, Canberra (2015).

(2) Poster presentation at the 13th European conference of Fungal Genetics, Paris (2016).

(3) Invited book chapter: Gryshyna, A., Kautto, L., Peterson, R., & Nevalainen, H. (2016). On the Safety of Filamentous Fungi with Special Emphasis on *Trichoderma reesei* and Products Made by Recombinant Means. In M. Schmoll & C. Dattenböck (Eds.), Gene Expression Systems in Fungi: Advancements and Applications (pp. 441–457). Springer International Publishing. http://doi.org/10.1007/978-3-319-27951-0_20.

(4) Macquarie University Postgraduate Research Fund (PGRF) travel grant (AUD 5000) (2016).

LIST OF ABBREVIATIONS

Abbreviations frequently used in the text are:

a.a.	Amino acids
bp	Nucleotide base pairs
BSA	Bovine serum albumine
CBH1	Cellobiohydrolase 1
CLS	Cellobiose/lactose/soybean flour medium
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine-tetra-acetic acid
ER	Endoplasmic reticulum
FUT3	Human fucosyltransferase 3
FUT4	Human fucosyltransferase 4
g	Gravity force
kb	Kilobase pairs
kDa	Kilodalton
LB	Luria Broth
mRNA	Messenger ribonucleic acid
M	Moles per litre
min	Minute
PAGE	Polyacrylamide gel electrophoresis

PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
RNA	Ribonucleic acid
SB	Sodium borate buffer
SDS	Sodium dodecyl sulphate
Sec	Second
TBS	Tris buffered saline
UV	Ultraviolet
V	Volt
v/v	Volume per volume
v/w	Volume per weight
w/w	Weight per weight

1

INTRODUCTION

1.1 Glycosyltransferases

Carbohydrate structures, or glycans, play diverse roles in a wide range of biological processes, such as protein folding and degradation, cell adhesion and trafficking, cell signalling, fertilisation and embryogenesis (Varki & Lowe, 2009). They are also involved in pathogen recognition and immune responses, either on their own as cell surface receptors, or by modifying the function of the protein they are attached to (Varki, 2017). Glycosylation is the most complex posttranslational (and also co-translational) modification that requires consecutive interactions of dozens of enzymes and substrates. Three types of glycosylation have been described so far: *N*-glycosylation (glycans attached to an Asn of an Asn-X-Ser/Thr consensus sequence, where X is any amino acid), *O*-glycosylation (glycans attached to a Ser or Thr), and *C*-glycosylation (glycans attached to a tryptophan). Approximately 50% of human proteins are glycosylated (Walsh & Jefferis, 2006), with 1-2% of the human genome dedicated to encoding glycan-modifying enzymes (Coutinho et al., 2003).

N-linked glycosylation is the most studied type of glycosylation, and it is an important factor to consider when planning for recombinant protein production. Glycosylation usually takes place in the endoplasmic reticulum (ER) and Golgi apparatus of eukaryotic cells, although *N*-glycosylated proteins produced by bacteria have been described (Benz & Schmidt, 2002). Enzymes involved in the process of glycosylation can be classified by the type of action into glycosyltransferases that catalyse the transfer of a sugar molecule to the glycan chain, and glycosidases that catalyse the hydrolysis of glycosidic linkages. Additionally, both glycosyltransferases and glycosidases are classified by the type of sugar they act on (fucose, mannose, galactose, glucose, N-acetylglucosamine).

1.2 Fucosyltransferases

Fucosyltransferases are the enzymes that transfer an L-fucose sugar from a GDP-fucose (guanosine diphosphate-fucose) donor substrate to an acceptor substrate. The acceptor substrate can be another sugar as in the case of *N*-linked glycosylation, or a protein, as in the case of *O*-linked glycosylation. So far, 13 carbohydrate specific fucosyltransferases have been found in humans (Becker & Lowe, 2003). The vast majority of fucosyltransferases are located in the Golgi apparatus; however, *O*-fucosyltransferases have been shown to localise in the endoplasmic reticulum (ER). Fucosyltransferases demonstrate different, although sometimes overlapping enzymatic activity profiles (Table 1.1).

Table 1.1. Human *N*-fucosyltransferases and their involvement in the synthesis of the human glycans.

Linkage	**Fucosyltransferase**	**Reference**	**Comments**
α-1,2	FUT1 and FUT2	Kelly et al., 1995; Larsen et al., 1990	ABO blood group antigen synthesis
α-1,3	FUT3-FUT7 and FUT9	Kaneko et al., 1999; Natsuka et al., 1994	Lewis antigen generation
α-1,4	FUT3 and FUT5		
α-1,6	FUT8	Miyoshi et al., 1999	Core fucosylation
α-1,3	FUT10 and FUT11	Mollicone et al., 2009	Introduce fucose to the core GlcNAc of the N-glycan on conalbumin glycopeptides and biantennary N-glycan acceptors but not to the short lactosaminyl acceptor substrates.

Fucosyltransferases have a complex tissue- and cell type-specific expression pattern, which changes constantly depending on the developmental stage and pathological process. For example, fucosyltransferase 6 (FUT6) is abundantly expressed in epithelial cells and in the liver, kidneys, and gastrointestinal tissues, and minimally expressed in spleen, lungs, and cervix. FUT6 is not found in the brain, adrenal cortex or peripheral blood leukocytes. The

tissue distribution of fucosyltransferase 3 (FUT3) is similar to that of FUT6, except that FUT3 is not expressed in liver and is less abundant in kidneys. Fucosyltransferase 5 (FUT5) is only minimally expressed in spleen, liver, colon, and testes (de Vries et al., 2001; Cameron et al., 1995; Kaneko et al., 1999). Fucosyltransferase 4 (FUT4) is expressed in a wide variety of cells and tissues (Gersten et al., 1995). High levels of fucosyltransferase 7 (FUT7) are detected in hematopoietic cells, such as leukocytes, and in endothelial cells of the venule (Natsuka et al., 1994; Sasaki et al., 1994). FUT4 and FUT7 are involved in the synthesis of fucosylated cell surface epitopes that are recognised by selectins (Niemelä et al., 1998). Fucosyltransferase 9 (FUT9) is abundantly found in the brain, stomach, spleen, and peripheral blood cells (Kaneko et al., 1999). Interestingly, FUT9 is among the more conserved fucosyltransferases, as the difference between human, rat, and mouse FUT9 amino acid sequence is only 1% (Kaneko et al., 1999). FUT4 and FUT9 are produced early in human embryogenesis, whereas FUT3 and FUT6 transcripts appear only after the 10^{th} week of development (Cailleau-Thomas et al., 2000).

Out of all identified fucosyltransferases, three (FUT3, 5 and 6) have a high degree of sequence similarity (~ 90%) and are only found in humans and chimpanzees (Costache et al., 1997). FUT3, 5 and 6 have evolved relatively recently and are not present in functional forms in all humans. While FUT3, 5 and 6 not appear to have an essential biological role, FUT4 and FUT7 are necessary for normal leukocyte trafficking and function (Weninger et al., 2000).

All fucosyltransferases are type II transmembrane proteins and consist of an N-terminal cytoplasmic tail, membrane-spanning region (signal-anchor domain), stem region and a globular C-terminal catalytic domain (Breton et al., 1998) (Figure 1.1). Signal-anchor domain determines the location of a fucosyltransferase within the Golgi apparatus. Stem regions of some glycosyltransferases are reported to have cleavage sites for endogenous proteases which

release transferases from the Golgi membrane in response to inflammation (Paulson & Colley, 1989).

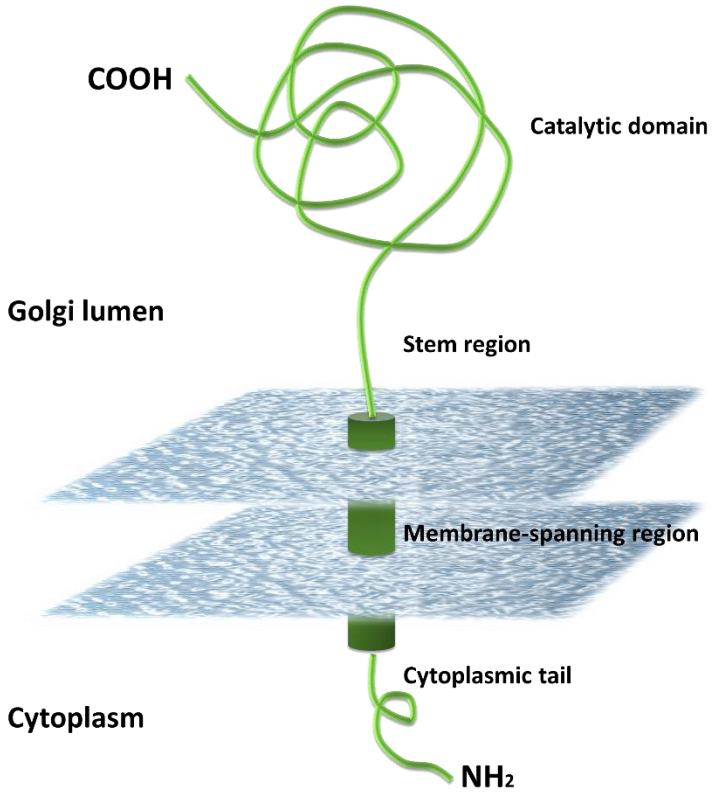


Figure 1.1. Schematic structure of fucosyltransferases featuring N-terminal cytoplasmic tail, membrane-spanning domain, stem region and C-terminal catalytic domain facing the Golgi lumen. Modified from Paulson and Colley (1989).

Crystal structures of some mammalian fucosyltransferases have been determined, revealing conserved folds present in these enzymes, their substrate binding sites and substrate specificities (Baruah et al., 2016; Ihara et al., 2007). Human fucosyltransferases identified thus far have between two to five potential glycosylation sites in the C-terminal region (de Vries at al., 2001). The use of site-directed mutagenesis to eliminate glycosylation sites from some human fucosyltransferases, such as FUT3, FUT5 and FUT6 resulted in the loss of enzymatic activity (Christensen et al., 2000). It has been demonstrated that a significant portion of the N-terminal region can be deleted without affecting catalytic activity, whereas removing only a few amino acids from the C-terminus of the FUT5 enzyme resulted in a complete loss of

enzyme activity (Xu et al., 1996).

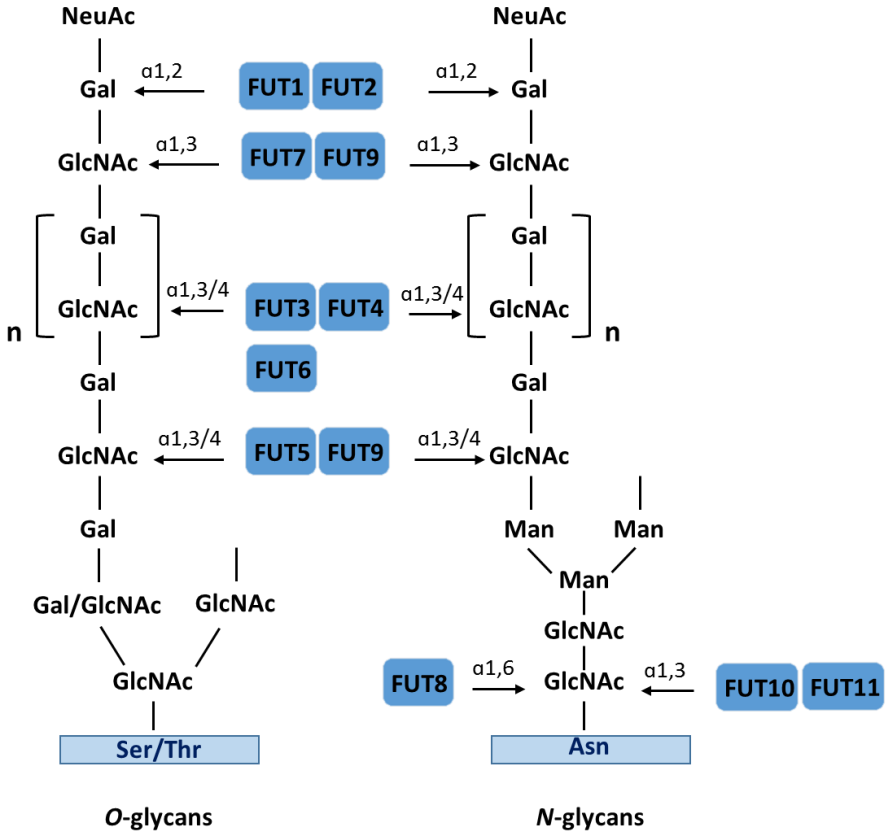


Figure 1.2. A simplified representation of the specific activities of the human *N*-fucosyltransferases. Out of all fucosyltransferases shown, only FUT3 and FUT5 have two enzymatic activities (α-1,3 and α-1,4). NeuAc – sialic acid, Gal – galactose, GlcNAc - N-acetylglucosamine, Man – mannose. Modified from Tu et al. (2013).

Two *O*-fucosyltransferases have been identified so far. Of these, OFUT1 adds fucose directly to polypeptide chains (Wang et al., 2001; Stahl et al., 2008). A putative OFUT2 has been identified in the human genome by comparison with fucosyltransferase encoding sequences in the *Drosophila melanogaster* genome (Roos et al., 2002).

Two N-fucosyltransferases expressed in this study are described in more detail in the following sections. FUT4 has been chosen for its essential role in the biological functions and FUT3 for having two enzymatic activities (α-1,3 and α-1,4) which could be useful for *in vitro* applications.

1.2.1 Fucosyltransferase 3

Fucosyltransferase 3 (FUT3) or galactoside 3(4)-L-fucosyltransferase is an enzyme encoded by the *FUT3* gene in humans, located on chromosome 9. FUT3, also known as Lewis blood group fucosyltransferase, has both α-1,3 and α-1,4 fucosyltransferase activities. The enzyme catalyses the transfer of fucose sugar from the GDP-fucose donor substrate to N-acetylglucosamine (GlcNAc) in α-1,3 and α-1,4 linkages in Gal-GlcNAc glycan structures (Figure 1.2). FUT3 is involved in the synthesis of VIM-2, Lewis^{a}, Lewis^{b}, sialyl Lewis^{x} and non-sialylated Lewis^{x} antigens (Oriol, 1995). Not all humans have an active FUT3 enzyme; individuals who are missing an active FUT3 are Lewis blood group negative (Le^{-}), while those who express an active FUT3 are Lewis-positive (Le^{+}).

Human FUT3 has two C-terminal glycosylation sites (Asn154 and Asn185) (Holmes et al., 2000). The role of glycosylation in the activity of FUT3 has been investigated (Christensen et al., 2000). Overall inhibition of glycosylation with core glycosylation inhibitor tunicamycin and castanospermine, which inhibits trimming of glucose residues, resulted in the loss of enzymatic activity and the activity reduced to 40%, respectively. Inhibition studies also demonstrated that *N*-linked glycosylations of both glycosylation sites and trimming of terminal glucose residues are necessary for the full activity of the FUT3 enzyme. Site-directed mutagenesis of *N*-glycosylation sites resulted in a series of mutants each with lower enzymatic activity than the wild type enzyme. Removal of an Asn154 glycosylation site or both glycosylation sites resulted in complete loss of enzymatic activity while removal of Asn185 resulted in a drop in activity to 27% of the wild-type enzyme. FUT3 is commercially produced in a mouse myeloma cell line by R&D Systems, USA (https://www.rndsystems.com).

1.2.2 Fucosyltransferase 4

Fucosyltransferase 4 (FUT4), also known as a myeloid-specific α-1,3 fucosyltransferase, is an enzyme that transfers fucose to N-acetylglucosamine. It catalyses the synthesis of the non-sialylated Lewis^{x} (CD15) antigen and VIM-2 antigen (Nakayama et al., 2001). Human *FUT4* gene is located on chromosome 11. Orthologues of the *FUT4* gene have been found across a wide range of higher eukaryotes such as armadillo and chimpanzee (http://www.ensembl.org). Overexpression of *FUT4* in breast cancer tissues and serums as compared to the normal tissues is associated with the proliferation and metastasis of breast cancer (Yan et al., 2015). FUT4 could thus serve as a novel biomarker for the diagnosis and prognosis of breast cancer. The human FUT4 enzyme has two potential glycosylation sites in the C-terminal region (Lowe et al., 1991). A study conducted on rat FUT4 (Baboval et al., 2000) demonstrated that the protein required glycosylation for activity but that intracellular localisation of the protein was not affected by changes in glycosylation. Decreased activity occurred at the levels of 64%, 5%, and 1% of the wild-type enzyme by mutations at Asn117, Asn218 N-glycosylation sites or both, respectively (Baboval et al., 2000). Overall, human FUT4 and its functions have not been studied in detail, and the enzyme is not commercially available at the present time.

1.3 Role of fucosylation in biological processes

Functional properties of oligosaccharides are often defined by specific terminal glycan modifications, including fucosylation. For example, fucosylation of glycoproteins affects the biological activities of adhesion molecules and growth factor receptors (Miyoshi et al., 2008). A number of pathological conditions, such as cancer and inflammation, are accompanied by increased levels of fucosylation of specific proteins. Abnormally fucosylated glycans/glycoproteins could provide novel tumour markers or targets for cancer therapy. Some fucosylated glycoproteins such as AFP-L3 (α-fetoprotein), and antibodies, which recognise

fucosylated oligosaccharides such as sialyl Lewis$^{a/x}$, have been used as tumour markers (Miyoshi et al., 2008). Upregulated fucosylation was observed in some cases of pancreatic cancer (Singh et al., 2015) and potential biomarkers are being developed. Upregulated fucosylated cytokines are also observed in rheumatoid arthritis (Isozaki et al., 2014).

The ABO blood group antigens are among the most well-known fucosylated glycans (Lowe, 1993). Lewis antigens are another example of fucosylated blood group antigens. Among other functions, these structures play a role in host-microbe interactions. For example, the gastric pathogen *Helicobacter pylori* attaches to the Lewis^{b} antigens expressed on the surface of the gastric epithelium. Individuals expressing other forms of the Lewis antigen are characterised by decreased occurrence of peptic ulcer disease (Hooper & Gordon, 2001). In many tumours, increase in Lewis^{y} and antigen H is observed, which correlates with poor clinical prognosis (Tan et al., 2015; Madjid et al., 2005). CD15 antigen (Lewis^{x}) has potential to be used as a biomarker of serious bacterial infections in infants admitted to hospital (Markic et al., 2012).

Fucose is an abundant component of gut surface glycans. Gut fucosylation is closely connected with bacterial colonisation, which could be a part of an increased innate immune response. For example, germ-free mice do not maintain ileal fucosylation after weaning, but colonisation with bacteria from conventionally housed mice restores it (reviewed in Pickard & Chervonsky, 2015). Feeding with exogenous fucosylated glycans was shown to improve the survival rate of mice, susceptible to gut inflammation (Pham et al., 2014). Evidence suggests that gut microbiota metabolise host fucose, so in some cases bacteria trigger host fucosylation in order to use fucose as a food source (Hooper et al., 1999). Fucose, especially when introduced during severe sickness of the host, supports the beneficial activity of resident bacteria while suppressing their potential virulence (Pickard et al., 2014). In addition to their potential use as a carbon and energy source, fucosylated glycans can serve as adhesion sites or receptors for pathogens, including *S. typhimurium*, *H. pylori*, enterotoxigenic *E. coli*, and norovirus

(reviewed in Pickard & Chervonsky, 2015).

1.4 Need of recombinant glycosyltransferases

Pure and defined glycan structures are essential research tools in glycobiology. Unlike proteins and nucleic acids, which are synthesised according to a pre-determined template and can be obtained in homogeneous forms using biological methods such as recombinant expression and polymerase chain reaction, glycans produced in biological systems are heterogeneous. Furthermore, the yield that can be obtained from biological systems is rather low.

Glycans can be synthesised *in vitro* both chemically and enzymatically. Chemical synthesis allows to obtain homogenous glycans in larger quantities but requires harsh conditions and preparation of specialised precursor compounds; also, chemical synthesis is not selective, so protection of reactive groups is necessary. Another drawback of chemical synthesis of glycans is difficulty in achieving the required stereochemistry and regio-selectivity.

Enzymatic synthesis of glycans *in vitro* is an attractive alternative, allowing synthesis of an amazing diversity of glycan structures with defined anomeric configurations and linkages. The use of glycan-modifying enzymes does not require harsh conditions or protection of reactive groups, and the enzymes have distinct specificities for their donor and acceptor substrates. In many cases of *in vitro* glycan synthesis chemical and enzymatic tools are combined; typically, the core structure is chemically synthesised, and then specific glycosyltransferases are used for precise modifications (Li et al., 2015).

One of the bottlenecks of the *in vitro* enzymatic glycan synthesis is the availability of the glycan-modifying enzymes. While some enzymes, for example, galactosyltransferase from bovine milk and bacterial sialyltransferases expressed in *E. coli* (Sigma-Aldrich, USA), are readily available from commercial sources, others have to be discovered, expressed and

purified in order to perform a synthesis of a particular glycan structure. Availability of various glycan-modifying enzymes would allow synthesis and modifications of glycans *in vitro* for a wide variety of applications, ranging from the basic studies of their functional roles in various biological processes to the discovery of new diagnostic biomarkers and therapeutic reagents.

Eukaryotic glycosyltransferases exhibit narrow substrate specificity towards glycan acceptors, which makes them a preferred choice to use in targeted and specific glycosylation reactions (Li et al., 2015). Also, nucleotide sugar donor substrates for mammalian glycosyltransferases are usually commercially available. Differently to this, substrates for bacterial glycan modifying enzymes may be difficult to obtain as they have to be chemically or enzymatically synthesised or extracted from bacteria. Glycosyltransferases of human origin are particularly suited for the synthesis of mammalian oligosaccharides, such as sialylated and branched glycans, because these reactions are difficult to carry out with either chemical methods or bacterial enzymes (Hidari et al., 2005).

Abnormal glycans and changes in glycosylation profiles are often associated with various diseases such as cancer and atherosclerosis and can be used as biomarkers. Therefore, unravelling the structures and functions of glycans is essential to understanding their role in pathological processes, and for developing diagnostic tools and treatments for human diseases. Structurally defined *N*-glycans (especially isomers) provide standards and probes for MS-based *N*-glycan analysis.

1.4.1 Glycan arrays

Synthetic glycans are widely used in glycan arrays composed of various oligosaccharides immobilised on a solid surface in a spatially-defined arrangement, which allow to study interactions of various carbohydrate structures with other proteins or glycoproteins to identify the glycan binding partners in biological samples and analyse their specificity. They are also

used as probes for binding of viruses and bacteria to glycans and studies into mechanisms of infection (Amonsen et al., 2007; Paulson & de Vries, 2013). Further on, glycan arrays find uses in profiling the substrate specificity of glycan-modifying enzymes (Willis et al., 2009; Laurent et al., 2008), in searching for glycan binding antibodies and lectins, and identifying ligands that could be used to modulate their activity (Shivatare et al., 2016; Amin et al., 2013).

1.4.2 Therapeutic glycoproteins

Recombinant glycoprotein therapeutics are the fastest growing class of pharmaceutical reagents, which comprises more than one-third of approved biotechnology-based medicines. Glycoproteins such as clotting factors, hormones, cytokines, antisera, enzymes, enzyme inhibitors, Ig-Fc-fusion proteins, and monoclonal antibodies are used in the treatment of cancer, autoimmune diseases, and various enzyme and hormone deficiencies (Kreitman, 2003; Sola & Griebenow, 2010; Ghaderi et al., 2012). Biopharmaceuticals with functions potentially dependent on glycosylation include erythropoietin (EPO), antibodies, blood factors, some interferons (IFNs) and some hormones (Walsh & Jefferis, 2006). The glycan structures attached to the proteins can dramatically affect protein stability, yield, pharmacokinetic properties, bioactivity and immunogenicity and therefore impact the cost and success of the treatment (Arnold et al., 2007; Durocher & Butler, 2009; Sola & Griebenow, 2009).

For example, removal of any of the *N*-glycosylation sites on the recombinant EPO had no significant effect on the *in vitro* activity but substantially reduced its *in vivo* activity (Delorme et al., 1992). When additional *N*-linked glycosylation sites were introduced into the recombinant EPO, improved efficacy and catabolic half-life were reported (Egrie & Browne, 2001).

Recombinant IFN-β is commercially produced in a glycosylated form in CHO (Chinese Hamster Ovary) cells (Avonex; Biogen-Idec, USA) and as a non-glycosylated product in *E.*

coli (Betaseron; Chiron, USA). Both proteins have passed regulatory requirements for quality, safety and efficacy. However, the non-glycosylated IFN-β demonstrated reduced *in vitro* activity, increased protein aggregation and increased sensitivity to thermal denaturation (Runkel et al., 1998). Similar observations were made for glycosylated and non-glycosylated IFN-γ produced in mammalian cells and in *E. coli* (Sareneva et al., 1994 and 1996).

Most common production systems for recombinant proteins include bacteria, yeast, insect and mammalian cells. Bacterial expression systems for recombinant proteins are widely used, but limited as most bacteria lack certain post-translational modification (PTM) mechanisms including glycosylation, which makes the production of human proteins cumbersome (Skretas et al., 2009). The glycosylation pattern of a recombinant protein can vary significantly depending on the production host: (1) bacterial systems mostly lack a glycosylation machinery, (2) yeast, plants and insect cell systems generate immunogenic glycan types absent in humans, and (3) mammalian systems provide human-like complex glycans. Although therapeutic glycoproteins produced in mammalian cell lines, such as Chinese Hamster Ovary (CHO), murine myeloma (NS0) or Baby Hamster Kidney (BHK), are mostly decorated with human-like glycans, they still can differ from human glycans (Ghaderi et al., 2012). Such non-human glycan structures trigger an immune response, therefore increasing the immunogenicity of therapeutical recombinant glycoproteins.

Glycoproteins are naturally heterogeneous and usually exist in a number of glycoforms which differ both in glycosylation site occupancy (macroheterogeneity) and glycan structures (microheterogeneity) (Sola & Griebenow, 2010; Khmelnitsky, 2004; Walsh & Jefferis, 2006). Differently glycosylated proteins vary greatly both in physicochemical and pharmacological properties, which affects therapeutic efficacy of the protein medicines. *In vitro* glycoengineering is one of the developing approaches, which allows production of homogenously glycosylated proteins with defined properties and increased efficacy

(Challener, 2016). One example of such approach is *in vitro* glycoengineering of IgG1 (Thomann et al., 2015) using a combination of chemical and enzymatic methods. Monoclonal IgG1 expressed in CHO cells was treated with glycan-modifying enzymes to change the content of the terminal galactose and/or sialic acid of Fc glycans, and increase the binding of hyper-galactosylated IgG1 to the substrate.

1.5 Heterologous expression of glycosyltransferases

Many human glycosyltransferases are glycosylated (Breen, 2002; de Vries et al., 2001), which makes their expression in the most widely used expression host for recombinant proteins, *E. coli*, rather complicated due to the limited posttranslational modifications in this organism. Even so, successful expression of recombinant glycosyltransferases in *E. coli* has been reported (Lauber et al., 2015; Skretas et al., 2009). However, extensive genetic manipulations of the production strain were required in order to ensure proper folding and glycosylation. Human sialyltransferase ST6GalNAc I (ST6) expressed in an engineered *E. coli* strain carrying mutations that provided an increased oxidative cytoplasmic environment (Skretas et al., 2009) was the first reported human glycosyltransferase produced in an active form in a bacterial host. Later, human GalNAcT2 glycosyltransferase was expressed in *E. coli* together with redox folding helpers (Lauber et al., 2015). A number of human glycosyltransferases have been produced in yeast *Pichia pastoris* and *Saccharomyces cerevisiae* (Malissard et al., 2000; Shimma et al., 2006; Salo et al., 2005). However, even soluble forms of recombinant glycosyltransferases produced in *S. cerevisiae* were not secreted, and in some cases hyperglycosylation may have occurred (Malissard et al., 1999). Several other expression systems such as mammalian cells, insect cells and plant tissues have been used to express recombinant glycosyltransferases successfully (White et al., 1995; Palacpac et al., 1999; Kato et al., 2017). Some examples of recombinant glycosyltransferases are provided in Table 1.2.

Table 1.2. Examples of functional recombinant eukaryotic glycosyltransferases.

Glycosyltransferase origin	Expression host	Yield	Activity	Reference
α-1,3-fucosyltransferases (*A. thaliana* and *D. melanogaster*)	*Pichia pastoris*	Not determined	1.0 U/l (*A. thaliana*) 0.0015 – 0.00165 U/l (*D. melanogaster*)	Bencurova et al., 2003
α-1,4-fucosyltransferase (tomato)	*Pichia pastoris*	Not determined	0.8 – 1.0 U/l	
α-1,3/4-fucosyltransferase (human)	*Pichia pastoris*	About 30 mg/l	2 cpm*10^{-6}/h*mg prot (cpm – concentration of fucose radioisotope (counts per minute)	Gallet et al., 1998
β-1,4-galactosyltransferase I (human)	*Pichia pastoris*	Not determined	0.6 U/l	Malissard et al., 2000
α-1,3-fucosyltransferase VI (human)	*Pichia pastoris*	Not determined	3 U/l	
α-2,6-sialyltransferase ST6Gal I (human)	*Pichia pastoris*	Not determined	0.3 U/l	
UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferase 2 (GalNAcT2) (human)	*E. coli*	0.32 mg per gram of cell pellet	139.6 ± 27.9 pmol/min/μg	Lauber et al., 2015
α-2,6(N)-sialyltransferase (human)	*Saccharomyces cerevisiae*	Not determined	0.31 U/l	Borsig et al., 1995
FUT 1,3,6,7,8,9 (human)	*Saccharomyces cerevisiae*	Not determined	>100 U (1 U = 1 pmol of product/5 OD_{600} unit of cell wall/24 h) Precise analysis of each enzyme was not performed.	Shimma et al., 2006
β-1,3-N-acetylglucosaminyltransferase 3 (β3GnT3) (human)	*Saccharomyces cerevisiae*	Not determined		
GalNAc alpha-2,6-sialyltransferase 2 (ST6GalNAc2) (human)	COS-7 cells *S. cerevisiae*	Not determined Not determined	11.4 pmol/h/ μg >100 U	Samyn-Petit et al., 2000; Shimma et al., 2006

All existing expression systems have certain drawbacks that make their use for the production of recombinant human glycosyltransferases only partially successful. Posttranslational modifications are difficult to achieve in *E. coli* and while yeast cells are capable of posttranslational modifications, they often hyperglycosylate the proteins which could affect

the activity of the recombinant products. Mammalian and insect cells are a more attractive choice in terms of bioactivity of human enzymes produced in these systems, but they are expensive and difficult to handle; also, obtained yields are usually low which increases the cost of a recombinant product and therefore limits its market availability. An overview of various expression systems is provided in Table 1.3.

Table 1.3. Characteristics of various expression systems for recombinant protein production. Adapted from Nevalainen et al. (2005).

Characteristics	***Escherichia coli***	**Filamentous fungi**	**Yeast**	**Insect cells**	**Mammalian cells**
Cell growth	Hours to days	About one week	Days to one week	Days to one week	Weeks
Cost of growth medium	Low to medium	Low to medium	Low to medium	High	High
Expression level	Low to high	Low to high	Low to high	Low to high	Low to high
Secretion capability	Secretion to periplasm	Secretion to medium	Secretion to medium	Secretion to medium	Secretion to medium
Post-translational modifications					
Protein folding	Refolding usually required	Refolding might be required	Refolding might be required	Proper folding	Proper folding
***N*-linked glycosylation**	None	Mammalian-type core, no sialic acid, non-human sugars added	High mannose, no sialic acid, non-human sugars added	Complex, no sialic acid, non-human sugars added	Complex, non-human sugars added
***O*-linked glycosylation, phosphorylation, acetylation, acylation**	No	Yes	Yes	Yes	Yes

Filamentous fungi represent an attractive alternative to expression systems described above,

as they combine eukaryotic posttranslational machinery with relatively high yield and less complex cultivation techniques.

1.6 Filamentous fungus *Trichoderma reesei*

Members of the genus *Trichoderma* degrade wood and plant biomass into sugars by secreting a large variety of hydrolytic enzymes (Kubicek et al., 2008). One of them, *Trichoderma reesei,* has demonstrated a great potential for secretion of both native and recombinant proteins and has been successfully used as an industrial microorganism (Peterson & Nevalainen, 2012; Bishof et al., 2016). Some mutant strains of *T. reesei* are known to secrete more than 100 g/l of fungal proteins into the cultivation medium (Cherry & Fidantsef, 2003). Such secretion capability makes *T. reesei* an attractive expression host for recombinant proteins, industrial enzymes, and secondary metabolites (Nevalainen et al., 1994; Olempska-Beer et al., 2006; Jeoh et al., 2008). One of the most widely used mutant strains is the *T. reesei* Rut-C30 (Montenecourt & Eveleigh, 1979), obtained by modification of the wild type strain QM6a by a range of mutagenic agents such as UV light and chemical treatment with nitrosoguanidine (NTG) (Montenecourt & Eveleigh, 1977a; Montenecourt & Eveleigh, 1977b; Montenecourt & Eveleigh, 1979; Nevalainen et al., 1980). It is a hypercellulolytic strain, low in protease (Sheir-Neiss & Montenecourt, 1984) with reported protein secretion level of up to 40 g/l (Durand et al., 1988) and with cellulase production levels 15-20 times higher than that of QM6a (reviewed by Peterson & Nevalainen, 2012). *T. reesei* is a producer of many industrially relevant enzymes used in the food and animal feed industries, such as α-and β-amylases, glucoamylases, cellulases, β-glucanases, xylanases, pectinases, proteases and lipases (Jeenes et al., 1991), both native and heterologous. Some examples of enzymes produced in *T. reesei* and commercialised by the members of the Association of manufacturers and formulators of enzyme products (AMFEP; http://www.amfep.org) and their industrial applications are

provided in Table 1.4.

Table 1.4. Examples of enzymes expressed in *T. reesei* and commercialised by AMFEP members. Adapted from Gryshyna et al. (2016).

Enzyme activity	**Donor organism**	**IUBMB number***	**Application**
Amylase (alpha)	*Aspergillus* sp.	3.2.1.1	Food/technical
Catalase	*Aspergillus* sp.	1.11.1.6	Technical
Cellulase	*Staphylotrichum* sp.	3.2.1.4	Technical
Glucanase (endo-1,3(4)-beta)	*Trichoderma* sp.	3.2.1.6	Food/feed/technical
Glucoamylase or Amyloglucosidase	*Trichoderma* sp.	3.2.1.3	Food/technical
Glucosidase (alpha)	*Aspergillus* sp.	3.2.1.20	Food
Glucosidase (beta)	*Trichoderma* sp.	3.2.1.21	Technical
Laccase	*Thielavia* sp.	1.10.3.2	Technical
Mannanase (endo-1.4-beta)	*Trichoderma* sp.	3.2.1.78	Feed/technical
Pectin methylesterase or Pectinesterase	*Aspergillus* sp.	3.1.1.11	Food/feed/technical
Phospholipase A2	*Aspergillus* sp.	3.1.1.4	Food/feed/technical
Phospholipase B	*Aspergillus* sp.	3.1.1.5	Food/feed
Polygalacturonase or Pectinase	*Aspergillus* sp.	3.2.1.15	Food/feed/technical
Xylanase	*Actinomadura* sp.	3.2.1.8	Feed
Xylanase	*Aspergillus* sp.	3.2.1.8	Food

*IUBMB - refers to the enzymes nomenclature of the International Union of Biochemistry and Molecular Biology (http://www.chem.qmul.ac.uk/iubmb/enzyme/)

Reports on the expression of non-fungal proteins in *T. reesei* are less frequent than those describing expression of fungal proteins. It is reflective of the more challenging nature of expression of proteins originating from higher organisms in *T. reesei*. Early examples of mammalian protein expression include the production of active calf chymosin and an engineered Fab antibody fragment in *T. reesei* (Harkki et al., 1989; Nyyssönen & Keränen, 1995). List of mammalian proteins successfully produced in *T. reesei* at the time is provided

in Table 1.6. Although *T. reesei* has a potential as an expression host for mammalian proteins, the reported yields are substantially lower than those of fungal proteins. Constantly developing genetic engineering techniques and a better understanding of the expression mechanisms of *T. reesei* allow to expect improvements in the production of recombinant mammalian proteins in *T. reesei.*

1.7 Transformation techniques

Genetic instability is a major bottleneck in *T. reesei* transformation. It usually is a consequence of a lack of integration of the incoming DNA into the genome or due to the tandem integration of multiple gene copies, which may be excised through a loop-out event (Aw & Polizzi, 2013; Jørgensen et al., 2014).

Delivery of DNA into the fungal cell is an essential step in creating strains for the production of heterologous proteins. Existing methods differ in transformation efficiency and stability of the transformants. These methods include protoplast transformation (Penttilä et al., 1987), electroporation, lithium acetate treatment, biolistic bombardment (Lorito et al., 1993, Te'o et al., 2002) and *Agrobacterium tumefaciens* - mediated transformation (Zeilinger, 2004). Two of these, also used in the current work, are discussed in detail below.

1.7.1 Protoplast transformation

PEG-mediated protoplast transformation was the first method described for filamentous fungi. This method includes treatment of fungal cells with cell wall degrading enzymes in order to make cells permeable, osmotic stabilisation of protoplasts, most commonly with sorbitol, and the DNA intake facilitated by PEG and $CaCl_2$. Protoplast transformation has been reported to result in a high transformation frequency of up to 500 transformants per µg of DNA in *T. reesei* (Gruber et al., 1990). It is, however, laborious and time-consuming. The efficiency of

protoplast formation could differ from batch to batch due to the inconsistent activity of the lytic enzymes (Ruiz-Diez, 2001). Another drawback of protoplast transformation is that it has been particularly difficult with the generally used Rut-C30 strain, seemingly due to a reduced ability of the strain to regenerate the cell wall (Nevalainen et al., 1995; Bergquist et al., 2002).

1.7.2 Biolistic bombardment

The biolistic bombardment was initially introduced in 1987 for plant transformation (Klein et al., 1987). In this method, tungsten or gold particles coated with DNA are accelerated to a high speed to bombard the target structure such as chloroplasts, plant cells, selected tissues or conidia. This method is less laborious than the traditional protoplast transformation and can be performed on unicellular fungal conidia. The biolistic transformation has been applied in several filamentous fungi, such as *A. nidulans*, *N. crassa*, *Magnaporthe grisea*, *T. harzianum* (reviewed in Ruiz-Diez, 2002) and *T. reesei* (Hazell et al., 2000; Te'o et al., 2002). Biolistic bombardment efficiencies for different cell types are provided in Table 1.5.

Table 1.5. Efficiency of biolistic bombardment for different cell types (continued onto the next page).

Cell type	**Transformation frequency**	**Reference**
E. coli JA221	1.25×10^4/μg of DNA	Smith et al., 1992
Erwinia srewartii DC283	12.5/μg of DNA	
A. nidulans	81/μg of DNA	Fungaro et al., 1995
Paecilomyces fumosoroseus (fungus)	33 – 153/μg of DNA	Chaves Barreto et al., 1997
T. harzianum	600 – 800/μg of DNA	Lorito et al., 1993
T. reesei	36.6/μg of DNA	Te'o et al., 2002
Physcomitrella patens (moss)	20 – 40/μg of DNA	Šmídková et al., 2010
Chicken embryos	Transformation efficiency 100%, survival rate 25%, the number of expression units in the embryo body cells ranged from 100 to	Ribeiro et al., 2001

	1,000.	
Soybean Bean Cotton	9% 2.7% 0.55% Transformation efficiency calculated as the total number of fertile transgenic plants divided by the total number of bombarded embryonic axes.	Rech et al., 2008

Similar to protoplast transformation, optimising the bombardment parameters by varying the chamber vacuum, size and density of the particles used for the bombardment, and the driving power source is required to obtain high yields of transformants.

1.8 Expression of recombinant proteins in *T. reesei*

Efficient strategies for the production of recombinant proteins in filamentous fungi are constantly developed, as the market of recombinant proteins keeps expanding. Higher production efficiencies and consequently, lower costs of the final product are requirements for obtaining a commercial viability.

The production of heterologous proteins by filamentous fungi is often limited whereas the production of homologous proteins can be high (Iwashita, 2002). Many fungi can secrete gram per liter amounts of endogenous and heterologous fungal gene products in the culture medium but attempts to produce high levels of proteins of bacterial, plant, and mammalian origin have been less successful (Nevalainen & Peterson, 2014a). The factors limiting the amount of heterologous products produced in filamentous fungi have been codon usage, protein folding, glycosylation and proteolytic degradation (Nevalainen & Peterson, 2014a). The strategies used for the overproduction of recombinant proteins in filamentous fungi have included introduction of multiple copies of the gene of interest, use of the promoters of highly expressed genes, expression from a locus of a highly expressed gene, minimising the proteolytic

degradation and development of improved cultivation media (Verdoes et al., 1995). Additionally, expression of heterologous non-fungal proteins in filamentous fungi has been improved by fusing the corresponding gene to the 3' end of a homologous gene or a fragment thereof (Gouka et al., 1997; Paloheimo et al., 2003).

1.8.1 Choice of the promoter for recombinant expression in *T. reesei*

The choice of promoter is one of the essential steps in planning the production of a recombinant protein. The native promoter of a gene of interest generally is replaced by a promoter of a highly expressed gene homologous to the host to improve transcription levels of the target gene (Ward et al., 1990). Depending on their mode of activation, promoters are classified into the constitutive and inducible promoters. Constitutive promoters are constantly active at a consistent rate regardless of environmental factors and are isolated from the "housekeeping" genes that have a continuous role in the cellular maintenance. Unlike constitutive promoters, inducible promoters are switched on in response to specific inducing factors, such as a certain carbon source, the availability or absence of specific metabolites, temperature, light and growth phase.

1.8.1.1 The cbh1 promoter

The cellobiohydrolase 1 (CBH1) enzyme constitutes approximately 60% of the total secreted protein produced by *T. reesei* (Nevalainen, 1985; Harkki et al., 1989). Therefore, the *cbh1* gene promoter is considered to be the most powerful promoter in *T. reesei* (Harkki et al., 1991) and is widely used in heterologous gene expression (Nevalainen & Peterson, 2014b). Some examples of recombinant proteins expressed under the control of the *cbh1* promoter are provided in Table 1.6.

The *cbh1* promoter is strongly induced by sophorose and cellulose, and also by some soluble carbon sources, such as lactose (Ilmén et al., 1997), and repressed in the presence of glucose.

A few independent mechanisms of activation and repression of the *cbh1* promoter have been identified so far, including transcription enhancers ACEI (Saloheimo et al., 2000), ACEII (Aro et al., 2001) and Xyr1 (Stricker et al., 2006), and repressors Rce1 (Cao et al., 2017) and CREI.

The carbon catabolite repressor CREI binds to the *cbh1* promoter at three binding sites and reduces its strength (Strauss et al., 1995; Ilmén et al., 1996a). Mutation of a single CREI binding site in the *cbh1* promoter resulted in disabling of glucose repression mechanism (Ilmén et al., 1996b). The Rut-C30 strain contains a truncated carbon catabolite repressor gene *cre1* and therefore is catabolite derepressed and able to produce cellulases on glucose-containing medium (Ilmén et al., 1996a).

When these repressor binding sites were deleted, and multi-copy activator binding sites were introduced in the *cbh1* promoter, the glucose repression effect was eliminated, increasing production levels of the glucuronidase (*gus*) reporter gene in *T. reesei* Rut-C30 M3 (Liu et al., 2008). The promoter designed by Liu et al. (2008) was later used to express the native *T. reesei* β-glucosidase with a 3.7-fold increase in activity compared to the parental strain Rut-C30 (Zhang et al., 2010).

Zou et al. (2012) tested the hypothesis that replacement of the negative regulator binding sites of the *cbh1* promoter with positive regulator binding sites such as transcription activator ACEII and the HAP2/3/5 complex may further improve the expression level of heterologous genes. Results showed that replacement of all three *cre1* binding sites significantly increased the expression of a green fluorescent protein reporter in both inducing and repressing conditions.

Among other developments of the *cbh1* promoter is the first application of the blue light-mediated regulation of DNA transcription to *T. reesei* by Wang et al. (2014). Blue light exposure has significant advantages over chemical inducers; it is cheap, easy to apply, could

be instantly removed from the system and therefore is useful for precise control of the gene expression. The modified *cbh1* promoter was successfully used to express the heterologous DsRed protein on glucose when induced by exposure to the blue light.

Table 1.6. Examples of proteins which have been expressed in *T. reesei* under the *cbh1* promoter and in fusion with the *cbh1* signal sequence (ss) and the CBH1 carrier protein. Adapted from Nevalainen & Peterson (2014b) and Curach (2004) (continued onto the next page).

Protein/gene source	**Yield**	**Comments**	**Reference**
Fungal			
Hydrophobin I Hydrophobin II (*T. reesei*)	0.5 g/l 240 mg/l	Coding sequence directly under *cbh1* promoter	Bailey et al., 2002
Lipase (*Aspergillus niger*)	0.3 g/l	Fusion to *cbh1* ss RNAi silencing of the native *cbh1* gene	Qin et al., 2012
Thermophilic xylanase II (*Humicola grisea* var. *Thermoidea*)	0.5 g/l	Fusion to *cbh1* ss	de Faria et al., 2000
Bacterial			
Xylanase (*Actinomadura flexuosa*)	N/A	Fusion to mannanase I core and linker region	Paloheimo et al., 1998
Xylanase (*Nonomuraea flexuosa*)	0.82 g/l	Fusion to *cbh2* core and duplicated linker	Paloheimo et al., 2003
Thermophilic xylanase B (*Dictyoglomus thermophilum*)	100 mg/l 0.5 – 1g/l	Fusion to *cbh1* ss	Te'o et al., 2000 Bergquist et al., 2002
Mammalian			
Calf chymosin (bovine)	40 mg/l 100 mg/l	Fusion to *cbh1* ss Fusion to *cbh1* core-linker	Harkki et al., 1989 Nykänen et al., 2002
Interleukin-6 (human)	5 mg/l	Fusion to *cbh1* core-linker	Demolder et al., 1994
Fab antibody fragments (mouse)	1 mg/l 40 mg/l 150 mg/l	40 mg/l and 150 mg/l yields from fusion to *cbh1* core-linker	Nyyssönen et al., 1993

Erythropoietin (human)	97 mg/l	Fusion to *cbh1* ss *cbh1* promoter was optimised (Liu et al., 2008)	Zhong et al., 2011
α-galactosidase A (human)	20 mg/l	Fusion to *cbh1* carrier	Smith et al., 2014
	636 mg/l	Intracellular production using a gamma zein peptide from maize (ZERA)	
Obestatin (human)	5.5×10^{-6} mg/l	Fusion to *cbh1* core-linker	Sun et al., 2016
Interferon alpha-2b	4,5 g/l	Fusion to *cbh1* carrier Protease deficient strain, cultured with addition of protease inhibitors	Landowski et al., 2016
Plant			
Endopeptidase B (barley)	0.05 g/l 0.5 g/l	Coding sequence directly under *cbh1* promoter Higher yield achieved in a low protease mutant strain	Saarelainen et al., 1997

Other inducible cellulase promoters such as *cbh2* (cellobiohydrolase 2), *xyn2* (xylanase 2) and *egl2* (endoglucanase 2) have also been used for heterologous gene expression (Miyauchi et al., 2013 and 2014) but their full potential has not been explored.

1.8.1.2 Non-cellulase promoters

The use of the inducible cellulase promoters leads to the production of the high amount of endogenous cellulases, which is not always desirable as it exhausts the protein secretion pathway. The use of promoters active in the presence of glucose allows to produce not only heterologous proteins but also specific native hydrolases free of most other hydrolytic enzymes. Some promoters, active in the carbon catabolite repressing conditions are reviewed in Keränen and Penttilä (1995).

Li et al. (2012) identified two constitutive promoters active under high glucose conditions, *eno* (enolase) and *pdc* (pyruvate decarboxylase), and tested them for expression of a homologous xylanase II. The results demonstrated the highest levels of expression of the recombinant xylanase II up to date (82% and 83% respectively). Another constitutive promoter *rp2*

(ribosomal protein) was applied to express a β-glucosidase (*bgla)* from *Aspergillus niger* with 3 folds higher yield than secretion of the native β-glucosidase by the wild type *T. reesei* strain (He et al., 2013).

The *pki1* promoter isolated from the gene encoding pyruvate kinase of *T. reesei* (Schindler et al., 1993) is commonly used as a part of expression cassettes to drive the expression of the hygromycin phosphotransferase (*hph*) selection marker gene in *T. reesei* (Te'o et al., 2000; Bergquist et al., 2002; Sun et al., 2016). The *pki1* promoter has also been applied to express native *T. reesei* xylanase 1 and xylanase 2 enzymes under glucose catabolite repression conditions at 76 and 145 U/mg of protein respectively in contrast to 26 U/mg produced by the non-transformant strain (Kurzatkowski et al., 1996).

1.8.2 Signal sequence and protein secretion

Efficient expression of heterologous proteins in fungal hosts requires successful transport of the recombinant protein through the secretory pathway where they undergo posttranslational modifications including protein folding and glycosylation (Shusta et al., 1998; Nuttall et al., 2002; Conesa et al., 2001).

Signal peptides, or signal sequences, are short peptides attached to the N-terminus of the majority of secreted proteins, which direct them towards the secretory pathway. Within the endoplasmic reticulum (ER), the signal sequences are cleaved off by signal peptidases, and the proteins undergo primary glycosylation, folding and proteolytic processing (Nelson & Cox, 2000). After that, the proteins are packed and transported in vesicles along the secretory pathway to the Golgi complex or a structure equivalent to Golgi (Shusta et al., 1998; Nuttall et al., 2002), where post-translational modifications, such as further glycosylation and trimming, occur. Unfolded proteins are sorted in the ER and targeted to either proteasomes or to vacuoles for degradation (Shoji et al., 2008).

Fusion of the protein of interest to a signal sequence of a highly secreted homologous protein such as that of the CBH1 has been found to promote the secretion of heterologous proteins (van den Hondel, 1991; reviewed in Su et al., 2012). Some examples of heterologous proteins produced in fusion with the CBH1 signal sequence under control of the *cbh1* promoter are provided in Table 1.6.

1.8.3 Fusion to a carrier protein

Making the protein to be produced as a fusion to a highly secreted native protein is a widely used strategy to enhance the production of heterologous proteins by filamentous fungi. It is believed that the homologous carrier protein promotes efficient expression and secretion of recombinant protein by stabilising the mRNA, improving the translocation of the protein to the ER, facilitating proper folding, and protecting the protein from the proteolytic degradation (Simonen et al., 1994; Samuelsson & Uhlén, 1996; Archer & Peberdy, 1997).

Glucoamylase (*glaA*) of *A. niger* is widely used as a fusion protein for heterologous expression by *Aspergillus* species and found to significantly increase the yield and stability of heterologous proteins. Human interleukin-6 was successfully produced in fusion with a glucoamylase by *A. niger* (Broekhuijsen et al., 1993) and *A. nidulans* (Contreras et al., 1991) as well as humanised antibodies produced by *A. niger* (Ward et al., 2004). It was demonstrated that such fusion not just protected heterologous proteins during the passage through a secretion pathway but in some cases also stabilised mRNA levels (Jeenes et al., 1993; Gouka et al., 1997).

Fusion with core and linker domains of the native cellobiohydrolase 1 (CBH1) of *T. reesei* was found to increase expression of an antibody fragment-CBH1 fusion protein by over 150 fold (Nyyssönen & Keränen, 1995) and calf chymosin by 5 fold (Nykänen, 2002), but the fusion strategy was not beneficial in the expression of a bacterial thermophilic xylanase (Te'o

et al., 2000). Fusion with CBH1 not only improved secretion but also increased the levels of mRNA encoding the fusion protein, possibly by enhancing transcription or by stabilising the mRNA. Fusion with other homologous proteins such as cellobiohydrolase 2 and mannanase 1 was also used (Paloheimo et al., 2003). Some examples of fusion with native protein carriers are provided in Table 1.6. Linkers, often containing protease cleavage sites for the removal of the carrier proteins, are usually introduced between the target protein and its fusion partner to increase flexibility of the construct.

1.8.4 Optimising the codon usage of the incoming genes

The level of gene expression of heterologous genes introduced into fungal cells depends on various factors such as mRNA stability and processing, availability of transfer RNAs (tRNAs) and translational efficiency, which are, to some degree, dependent on the sequence of the gene to be expressed. Different organisms are often biased towards one of the several codons that encode the same amino acid over the others. The codon usage preferences are reflected in the abundance of tRNAs specific to each codon which subsequently affects the rate of transcription and translation of a particular coding sequence. Differences in codon usage incur difficulties in the expression of heterologous genes, especially from distance species. Codon optimisation, or adjusting codons of a heterologous gene to match the codon usage preferences of the expression host is a common method in heterologous protein expression (reviewed in Su et al., 2012).

Analysis of cellulase genes from *T. reesei* showed an overall A/T content of less than 40% and a strong bias against codons ending with A/T. An AT-rich (61%) xylanase (*xynB*) gene from a bacterium *Dictyoglomus thermophilum,* that prefers A or T at the third codon position, was successfully expressed in *T. reesei* after the codons were changed according to the host codon usage, while the original gene failed to express (Te'o et al., 2000).

Codon optimised *Cas9* gene of *Streptococcus pyogenes* was successfully expressed in *T. reesei* (Liu et al., 2015), which was the first application of CRISPR/Cas9 genome-editing system in filamentous fungi. Codon optimisation also made it possible to use a FLP/FRT recombination system from the yeast *S. cerevisiae* for marker recycling in filamentous fungi (Kopke et al., 2010; Yamada et al., 2014).

1.8.5 Optimisation of the production host

One of the main obstacles for the successful production of higher eukaryotic proteins is the high amount of secreted proteases produced by *T. reesei*. Approaches used to overcome this problem are, for example, control of the cultivation conditions (pH, nitrogen source) to inhibit specific groups of proteases and the use of low protease strains, obtained by either traditional mutagenesis or by targeted deletion of the protease genes. Random mutagenesis has resulted in the isolation of low protease strains, such as Rut-C30 (Montenecourt & Eveleigh, 1979) and ALKO2221 (Saarelainen et al., 1997). However, random mutagenesis is not a targeted approach, and could introduce undesired (and unpredicted) changes into the fungal genome.

The total number of proteases estimated by analysis of the *in silico* predicted secretome of *T. reesei* amounts to 383 (Druzhinina et al., 2012). Thirty-nine proteases were identified by proteomic studies of the *T. reesei* QM6a culture supernatant, and their expression was described to be pH dependent (Adav et al., 2011).

The ensemble of proteases secreted by *T. reesei* is active across a wide range of pH. Acid aspartic protease was found to be the dominant protease at pH 5 and lower (Haab et al., 1990), while at pH 7 an alkaline serine protease constituted 55% of the total protease activity (Zhang et al., 2014). Some proteases identified in *T. reesei* are listed in Table 1.7.

Table 1.7. Protease genes identified in *T. reesei* and their accession numbers in GenBank (https://www.ncbi.nlm.nih.gov/genbank/) and Joint Genome Institute (JGI, http://jgi.doe.gov) databases.

Protease	**GenBank/JGI accession number**	**Reference**
Trypsin-like alkaline serine protease	CB895877	Dienes et al., 2007
Alkaline serine protease	EGR49466.1	Zhang et al., 2014
Aspartic proteases	EGR52488.1 EGR52940.1	
Aspartic proteases	tre74156 tre53961 tre121133 tre77579 tre81004 tre122076 tre79807 tre121306 tre119876	Landowski et al., 2015 and 2016
Glutamic proteases	tre69555 tre106661	
Subtilisin-like proteases	tre123244 tre51365 tre58698 tre123865 tre58698	
Trypsin-like serine protease	tre73897	
Sedolisin protease	tre123865	
Metalloproteases	tre122703 tre60581 tre81070 tre108592	

Due to the high number of proteases and their wide activity range, deletion of all of them would be impossible with the technology currently in hand. Instead, a way to go would be

identification and sequential removal of the most problematic proteases for a particular heterologous gene product, as demonstrated by Landowski et al. (2015 and 2016). After deletion of nine protease genes, the obtained strain had only 4% of total protease activity of the parental strain and demonstrated improved stability and production levels of interferon alpha-2b. This type of approach is very labour and resource intensive. However, discovery of novel genetic manipulation tools such as marker recycling, CRISPR/Cas9 genome editing system and creation of strains with enhanced frequency of homologous recombination allows to expect more developments in the area.

1.8.6 Culture-based strategies

Another approach towards lowering protease activity and improving the yields of the heterologous proteins would be by controlling the cultivation conditions as production efficiency is not solely determined by genetic characteristics of the production strain but is also influenced by environmental factors. Maximal protein production can be achieved through optimisation of the culture and growth parameters (Ahamed & Vermette, 2008). The levels of the protein production are known to be affected by the type, concentration, and quality of the carbon source, aeration, temperature, pH, and growth rate (Mandels, 1975; Bailey & Tähtiharju, 2003; Haki & Rakshit, 2003). For example, it has been shown that different *T. reesei* mutant strains can be induced to different degrees by various carbon sources. The cellulase activity in Rut-C30 and QM6a strains was most strongly induced by Avicel PH-101 (microcrystalline cellulose manufactured by Sigma-Aldrich), but in QM9414 the highest cellulase activities were achieved by using the microcrystalline cellulose manufactured by J.T. Baker (Dashtban et al., 2011). The concentration of the carbon source also has an effect: it was reported that the highest cellulase induction for QM6a, QM9414, and Rut-C30 was achieved when 1%, 1.25%, and 1.5% of their most desirable carbon source was

present in the culture medium respectively (Dashtban et al., 2011). These findings suggest that it is necessary to evaluate the recombinant protein production under different concentrations of the carbon source. Jampala et al. (2015) demonstrated that out of seven carbon sources and eleven nitrogen sources evaluated for induction of cellulase genes expressed under native promoters, maltose and ammonium hydrogen carbonate showed a significant effect on cellulase activity and maltose and ammonium sulphate showed a significant effect on xylanase activity. Use of an organic nitrogen source had no influence on cellulase production by *T. reesei* (Rodriguez-Gomez & Hobley, 2013), while increased levels of proteases have been reported (Kredics et al., 2005; Haab et al., 1990). Both micro- and macromorphology of fungal cultures influence the rate of growth and product formation. Morphological forms, which also contribute to productivity, are, in turn, influenced by pH, the agitation rate and aeration levels, especially in bioreactor cultures (Ilias & Hoq, 1998; Gibbs et al., 2000).

1.8.6.1 Dissolved oxygen

Trichoderma reesei is an obligatory aerobe. Dissolved oxygen (DO) is one of the key factors in biomass formation and cellulase production. Oxygen deficiency negatively affects cell growth and cellulase production and could also cause sporulation. Low DO (less than 30%) causes the formation of pellets, which decreases cellulase production (Cui et al. 1997; Domingues et al. 2000). The effect of DO on fungal morphology has been reported in the literature. Cui et al. (1997 and 1998) found that pellets were denser when DO was close to the saturation level while in the case of very low DO levels, pellets were rather weak and fluffy. The biomass per wet pellet volume and the porosity of the pellets were all functions of DO and the pellet size. Smaller pellets formed under higher DO levels had a higher intrinsic strength. Wongwicharn et al. (1999) reported that two distinct morphologies were observed when varying the oxygen enrichment in the gas supply. Under oxygen limiting conditions,

long, sparsely branched hyphae with a low percentage of "active" length were formed. Under higher oxygen enrichment levels (30-50%), shorter hyphae with more branching and a higher percentage of "active" length were formed. The production of both native and heterologous enzymes positively correlated with the higher active length and tip numbers (Wang et al., 2005).

1.8.6.2 pH

Low pH (2-3) is beneficial for the filamentous growth while higher pH values (5–6) lead to pellet formation (Wainwright et al., 1993). Pellet size decreases with increasing pH. In bioreactor cultivations, pellets are favoured over mycelial growth as they ensure better oxygen transfer. Smaller pellets are preferred over larger pellets due to a higher ratio between active mycelium (on the surface of the pellet) and inactive/dead mycelium (in the centre of the pellet) (Punt et al., 2002).

Different pH values of the culture medium may be optimal for maximum yield of cellulases by *Trichoderma* strains growing on different carbon sources. According to Ryu and Mandels (1980), pH range of 3.0 to 4.0 was optimal for pure cellulose as a carbon source, but a higher initial pH was beneficial for lignocelluloses. In other studies, the maximum yield of cellulases was obtained in pH range of 3.0–5.0 (Mukhopadhyay & Nandi, 1999; Chen &Wayman, 1993; Kadam & Keutzer, 1995).

1.9 Protein purification strategies

Choosing an appropriate purification tag is an important step in designing a recombinant protein production, especially in the case of proteins with potential biomedical applications, where purity is crucial. Purification tag should not be degraded in the *T. reesei* medium also containing secreted proteases, sustain the growth conditions used and not interfere with the

activity and folding of recombinant protein; for these reasons, short tags are preferred. Purification process should occur under mild conditions, which do not damage the recombinant protein. The cost of the purification process also plays a role in the selection of the method of purification, especially if large-scale production is envisioned. Purification methods that have been used in *T. reesei* system are reviewed below.

1.9.1 Carbohydrate-binding modules

Carbohydrate-binding modules (CBMs) are functional modules of enzymes involved in plant biomass degradation; they are used as affinity purification tags in various systems (Wang & Hong, 2014; Rodriguez et al., 2004, Hong et al., 2008). Substrates of specific CBM proteins are widely available and cheap; this approach also allows large-scale purification, high product purity and mild treatment which retains bioactivity (reviewed in van der Kaaij et al., 2010). However, binding of CBMs to cellulose containing matrices is not always reversible which limits the use of CBMs as purification tags. Also, filamentous fungi produce many native cellulose-binding proteins which makes selective binding of the recombinant proteins impossible. It has been demonstrated that glycosylation of CBMs by eukaryotic hosts could affect their binding properties (Boraston et al., 2001).

1.9.2 Polyhistidine tag

Polyhistidine tag (His-tag) is one of the most commonly used affinity tags in the purification of recombinant proteins. It consists of six or more histidine residues attached to either N- or C-terminus of the target protein. His-tag purification system requires buffer exchange which complicates the purification process and may result in loss of the target protein. The first use of polyhistidine tag in *T. reesei* demonstrated that fusion with His-tag lead to increased protease degradation of the native Cel61A protein (Karlsson et al., 2001). Among other reported problems are degradation of His-tag (Kontkanen et al., 2009) and failure of His-

tagged recombinant swollenin from *Trichoderma pseudokoningii* S38 to bind to the column probably because of proteolysis or masking of the tag (Yao et al., 2008). Other, more successful applications of His-tag in *T. reesei* expression system are the purification of recombinant swollenin from *Penicillium oxalicum* (Kang et al., 2013) and native swollenin (Andberg et al., 2015). Such unreliable performance calls for meticulous experimental design if His-tag is to be used in the *T. reesei* system.

1.9.3 Short peptide tags

FLAG-tag and HA-tag are short (8 a.a. and 9 a.a. respectively) peptide tags. Short peptide tags are biochemically inert and therefore do not affect protein folding and do not interfere with the protein functions. Removal of such tags is usually not necessary unless the target protein is to be used *in vivo*. Both tags have been proven to work in *T. reesei* expression system (Salles et al., 2007; Yao et al., 2008) and found not to interfere with the bioactivity or the biodegradation of the recombinant protein. Purification of FLAG-tagged and HA-tagged proteins requires the use of specific antibodies, which makes these tags not feasible for large-scale purification.

The Strep-tag II is a short synthetic peptide of 8 amino acids (WSHPQFEK), which has an affinity to Strep-Tactin, a specifically engineered streptavidin (Schmidt & Skerra, 2007). Unlike FLAG-tag and HA-tag, Strep-tag purification does not require the use of antibodies, which reduces the cost of the process. Strep-tag II has been successfully used in *T. reesei* by Pakula et al. (2016) and Smith et al. (2014) in fusion with lipase from *Dipodascus capitatus* and human α-galactosidase A respectively. Strep-tag II is well suited for the analysis of functional proteins because the purification procedure can be performed under physiological conditions. However, it was observed that human α-galactosidase A became unstable during the Step-tactin binding process when the pH of an acidic culture was increased to the neutral

pH range of Strep-Tactin binding conditions (Smith et al., 2014), therefore, compatibility of the recombinant protein with the conditions of the purification process should also be taken into consideration. Besides purification, peptide tags can also be used for the detection of recombinant proteins via Western blotting if antibodies for the target protein are not available.

1.10 Fluorescent reporter proteins

The use of fluorescent reporter proteins started with the discovery of the green fluorescent protein from the jellyfish *Aequorea victoria* and the cloning of the gene encoding it (Prasher et al., 1992). Since then, other fluorescent proteins and their mutants have been found and developed and the number of their applications have been steadily increasing. Fluorescent reporters are now widely employed in labelling of protein biosensors and in studies into gene transcription and protein localisation (González-Vera & Morris, 2015). GFP has been used as an indicator of the production phase of heterologous protein produced by *E. coli* on an industrial scale (Albano et al., 1998; Baker et al., 2002) and for various studies in fungal biology (summarised in Jensen et al., 2004). Fluorescent reporters can also be targeted to specific organelles by fusion with the appropriate localisation signal (de Giorgi et al., 1996).

The mCherry fluorescent protein was derived from a red fluorescent protein isolated from *Discosoma* sp. It is a monomeric protein which is resistant to photobleaching and has higher photostability compared to other monomeric fluorophores (Shaner et al., 2004). Among other applications, the mCherry protein has been used for quantification of secreted proteins (Duellman et al., 2015). The mCherry was used in this study for the preliminary screening of *T. reesei* transformants secreting recombinant FUT3 and FUT4 enzymes. It has been demonstrated previously that the mCherry gene did not require codon optimisation for expression in *T. reesei* and the protein was relatively stable in the culture supernatants of *T. reesei* (Sun, 2008).

1.11 AIMS OF THE PROJECT

The overall aim of this project was to evaluate the potential of a filamentous fungus *Trichoderma reesei* as a production host for human fucosyltransferases FUT3 and FUT4. As discussed above, currently used production systems do not fully satisfy the increasing need for human glycan-modifying enzymes for various research applications. Successful experiments in the past have demonstrated that *T. reesei* is a suitable host for expression of mammalian proteins; however, this was the first attempt to express functional human fucosyltransferases 3 and 4 in *T. reesei*.

Specific aims, refined over the course of the project, included:

1. Expression of the FUT3 and FUT4 in *T. reesei* in fusion with the main cellobiohydrolase 1 carrier protein and with and without the mCherry tag for transformant screening.

2. Assessing the performance of selected transformants in shake flask cultivations, and analysing degradation of the heterologous proteins in the culture supernatant.

3. Purification of the recombinant FUT3 protein, evaluation of its activity and determination of protease cleavage sites for *T. reesei* proteases within the protein.

4. Optimisation of the yield and stability of the recombinant FUT3 by identifying a group of proteases playing a role in the proteolytic degradation of the FUT3 protein and limiting their activities by adjusting the pH of the culture medium, and cultivation with the addition of protease inhibitors.

5. Cultivation of the selected transformant CMF3-44 producing recombinant FUT3 in fusion with the mCherry protein in a laboratory fermenter under continuous pH control for minimising the effect of acidic proteases on the heterologous protein.

2

MATERIALS AND METHODS

2.1 Common reagents and solutions

All chemicals used were purchased from Sigma-Aldrich (Australia) unless otherwise specified. All reagents, buffers and media were prepared using Millipore Milli-Q filtered H_2O. Sterilisation of most liquids was carried out by autoclaving at 121°C for 20 min. Frequently used solutions are listed in Table 2.1. All percent quantities are in w/v unless otherwise specified. Methods related to particular experiments are described in detail in each appropriate chapter.

Table 2.1 Common chemicals and solutions.

Solution	**Components**
SB (20x)	0.008% NaOH, 0.045% H_3BO_3, pH 8.0
TE (10x)	100 mM Tris-HCl, 10 mM EDTA, pH 8.0
LB	1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.0
LA	15 g/l agarose in LB medium
PBS	137 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4 \times 2H_2O$, 2 mM KH_2PO_4, pH 7.4
TBS	50 mM Tris-HCl, pH 7.4, 140 mM NaCl
Conidia collection solution	0.9% NaCl, 0.01% (v/v) Tween 80
Revco storage solution	42 mM K_2HPO_4, 22 mM KH_2PO_4, 1.7 mM sodium citrate, 0.4 mM $MgSO_4$, 30% (v/v) glycerol

2.2 Microbial strains and cultivation procedures

2.2.1. *E. coli* strain and cultivation

E. coli strain DH5α (New England Biolabs, US) was used for plasmid propagation and cloning work. Luria-Bertani broth (LB) and Luria-Bertani agar (LA) (Table 2.1) were used for propagation of *E. coli* and its recombinants. Selection medium containing 100 μg/ml ampicillin was used for selection and maintaining of recombinants. *E. coli* cultures were incubated at 37°C, and liquid cultures were agitated at 250 rpm. Stock cultures were prepared using Revco storage solution (Table 2.1) and stored at -80ºC.

2.2.2. *E. coli* transformation

The preparation of competent DH5α cells and transformation of *E. coli* were performed according to Inoue et al. (1990). For each transformation, 200 µl of competent cells were incubated with 1-5 µl of plasmid DNA on ice for 30 min, then heat-pulsed at 42°C for 45 sec and incubated on ice for another 2 min. The cells were agitated at 37°C in 0.8 ml LB (Table 2.1) for 1 h before plating on LB agar containing 100 µg/ml ampicillin. Colonies were counted after an overnight incubation at 37°C.

2.2.3. *T. reesei* strain and preparation of conidial suspensions

The hypercellulolytic strain Rut-C30 (Montenecourt & Eveleigh, 1979) was used as a host strain for recombinant protein production. *T. reesei* strains were maintained on Potato Dextrose Agar (PDA) plates (Difco, USA). The plates were incubated at 28ºC in the dark for three days and then moved to the room temperature and natural light to enhance conidiation. Conidia were collected by adding 5 ml of conidia collection solution (Table 2.1) and then gently scraping the surface of the PDA plates with a spreader. The conidial suspension was filtered through sterile cotton wool-stuffed micropipette tips to remove hyphae, and the number of conidia in the solution was counted using a haemocytometer. Conidial suspensions were stored either at 4ºC temporarily or at -80ºC in Revco storage solution (Table 2.1) for extended periods.

2.2.4. Cultivation of *T. reesei* in liquid medium

The liquid medium used in this study was CLS (cellobiose/lactose/soybean flour extract), described in Lim et al. (2001). To make 1 liter, 15 g KH_2PO_4, 5 g $(NH_4)_2SO_4$, 10 ml 100× trace elements (50 mg $FeSO_4 \times 7H_2O$, 16 mg $MnSO_4 \times H_2O$, 14 mg $ZnSO_4 \times 7H_2O$, and 20 mg CoCl in 100 ml H_2O) and 10 g of D-(+)-cellobiose were added to ~ 750 ml H_2O and the pH

was adjusted to 6.5 with KOH. Prepared solution was sterilised by autoclaving before adding 2.4 ml 1 M $MgSO_4$ and 5.4 ml 1 M $CaCl_2$, 50 ml 20% lactose and 200 ml soybean flour extract, which were sterilised separately. The soybean flour extract was prepared by autoclaving 15% soybean flour in water followed by centrifugation for 20 min at 9000× g to remove insoluble particles.

Cultivation in liquid medium was conducted in either 24-well plates containing 3 ml of medium in each well or in 250 ml flat bottom conical flasks containing 50 ml of medium. Shake flasks were inoculated with 1×10^8 of freshly harvested conidia per 50 ml of medium. 24-well plates, used for preliminary screening of a large number of transformants, were inoculated with small pieces of mycelium from the PDA plates. Liquid cultures were incubated at 28 °C with shaking at 250 rpm in the dark to prevent conidiation.

2.3 General molecular cloning techniques

The methods for general DNA manipulations were as described in Sambrook & Russell (2001). Propagation of plasmids was carried out in *E. coli* DH5α strain. Plasmid DNA extraction was performed with the QIAprep spin miniprep kit (Qiagen, Germany). PCR products and DNA digested with restriction endonucleases were cleaned up with the QIAquick PCR purification kit (Qiagen, Germany). DNA fragments isolated from agarose gels were purified with the QIAquick gel extraction kit (Qiagen, Germany). DNA concentration was measured using NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, USA).

All restriction enzymes were obtained from Fermentas (USA) and New England Biolabs (USA) unless otherwise specified. Restriction reactions were carried out according to the manufacturer's instructions. DNA ligation was performed with T4 DNA ligase (Fermentas, USA). Ligation reactions were set up according to the manufacturer's instructions and

incubated at room temperature for one hour.

2.3.1 DNA sequencing

DNA sequencing was performed by Macrogen (South Korea). DNA samples and primers were prepared according to the Macrogen user's guide. For one sequencing reaction, 5 µl of plasmid DNA (100 – 200 ng/µl) or 5 µl of purified PCR product (10 – 60 ng/µl) were submitted together with 5 µl of a suitable primer (10 pmol/µl).

2.3.2 Polymerase Chain Reaction

AmpliTaq Gold polymerase (Applied Biosystems, USA) was used for routine PCR reactions. Q5® High-Fidelity DNA (New England Biolabs, USA) was used for reactions with difficult amplicons, such as gDNA and amplicons with high GC content. PCR reactions were carried out according to the manufacturers' protocols. All primers for PCR were synthesised by Macrogen (South Korea) and by Integrated DNA Technologies (USA). Primers used in this project are listed in Table 2.2. Standard reaction conditions were: denaturation at 95ºC for 10 min in the first cycle and 30 sec for the rest of the reaction cycles; annealing at 56-60ºC for a period of 30 sec, depending on the primers used; extension at 72ºC for 30 sec to 1 min depending on the length of the amplified fragment and up to 2 min for the final cycle. The amplification steps were repeated for up to 40 cycles.

Table 2.2. List of primers used in this project (continued onto the next page).

Primer	**5' to 3' sequence**	**Purposes**
P1mCherryFwd	GGCGGCAAGCTTATACGTAATGGTGAGCAAGGGCGAGG	Amplification of mCherry gene from the pmCherry plasmid. Confirmation of insertion of the mCherry DNA fragment into the pCBH1corlin plasmid by colony PCR. Detection of the mCherry gene in gDNA extracted from *T. reesei* transformants.
P1mCherryRev	GCGGCGGGATCCCTCGAGCTTGTCGTCGTCGTCCTTGTACAGCTCGTCCATGC	
P2FUT3Fwd	TACTTCAACCTCACCATGTC	Confirmation of insertion of the FUT3 DNA fragment into the pCBH1corlin and pCM plasmids by colony PCR.

		Detection of FUT3 gene in gDNA extracted from *T. reesei* transformants.
P2FUT3Rev	CTTCTCGGTGATGTAGTCG	
P2FUT4Fwd	CCTCCGCTTCAACATCAG	Confirmation of insertion of the FUT4 DNA fragment into the pCBH1corlin and pCM plasmids by colony PCR. Detection of FUT4 gene in gDNA extracted from *T. reesei* transformants.
P2FUT4Rev	CTCTCGAAGTTCATCCAGAC	
corlinSeqFwd	TCAAGCAGCTGACTGAGATG	Sequencing of the pCF3, pCF4, pCMF3 and pCMF4 plasmids.
hph2.fwd	CTCTCCCTCGTCGAAGCCGC	Detection of the Hygromycin resistance gene in gDNA extracted from *T. reesei* transformants.
hygroSpeIRev	CGGGGATCCACTAGTCATGCATCTATTC	
fut3_corr.fwd	GATCCGCTAGCTCAGGTGAA	Amplification of the FUT3 DNA fragment from the pUC57-FUT3 plasmid.
fut3_corr.rev	AAGCTTACTCGAGATGGAGCCACC	
fut4_corr.fwd	ATATCGGATCCGCTAGCTCA	Amplification of the FUT4 DNA fragment from the pUC57-FUT4 plasmid.
fut4_corr.rev	AAGCTTACTCGAGATGGAGCCACC	

Colony PCR was performed to select positive *E. coli* transformants after the transformation with ligation mix as a preliminary confirmation of insertion of the gene of interest into the plasmid. Single colonies from transformation plates were picked with a sterile pipette tip and added to the PCR reaction mix instead of a template DNA.

2.3.3 Agarose gel electrophoresis

DNA analyses and PCR product confirmations were carried out in 1% agarose gels (Amresco, USA) prepared in 1× SB buffer (Table 2.1). The GelRed stain (Biotium, USA) was used for visualisation of nucleic acids as instructed by the manufacturer. DNA was mixed with 6× gel loading dye (30% (v/v) glycerol, 0.25% bromophenol blue in dH2O) before electrophoresis was performed at 80 V for 50 min in 1× SB buffer. The 1 Kb plus DNA ladder (Fermentas, USA) was used in all DNA gel electrophoreses.

2.4. *T. reesei* expression vector

The pCBH1corlin vector (Te'o et al., 2000) was used in this study as a backbone for construction of the expression cassettes for the FUT3 and FUT4 genes. The pCBH1corlin

vector comprises of the CBH1corlin expression platform (Te'o & Nevalainen, 2010) cloned into the pUC19 vector for propagation in *E. coli*. Main features of the CBH1corlin expression cassette are shown in Figure 2.1.

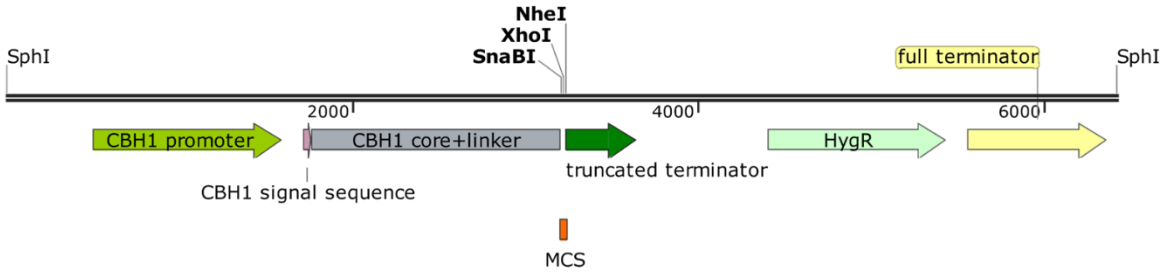


Figure 2.1. CBH1corlin expression cassette (6421 bp) containing a promoter, signal sequence, full and truncated terminators of cellobiohydrolase 1 gene, Hygromycin resistance gene (*HygR*), DNA fragment encoding the core and linker domains of the cellobiohydrolase 1 and multiple cloning site (MCS). Restriction sites of the restriction enzymes used in this study are shown. Image created using SnapGene software (from GSL Biotech; available at snapgene.com).

The pCBH1corlin vector (Figure 2.2) comprises the hygromycin resistance gene under the control of *T. reesei* pyruvate kinase promoter (*pki*) which allows selection of the *T. reesei* transformants carrying the expression cassette, promoter, signal sequence and terminator of the cellobiohydrolase 1 (CBH1) enzyme, multiple cloning site and DNA sequence encoding the core and linker domains of the CBH1 protein.

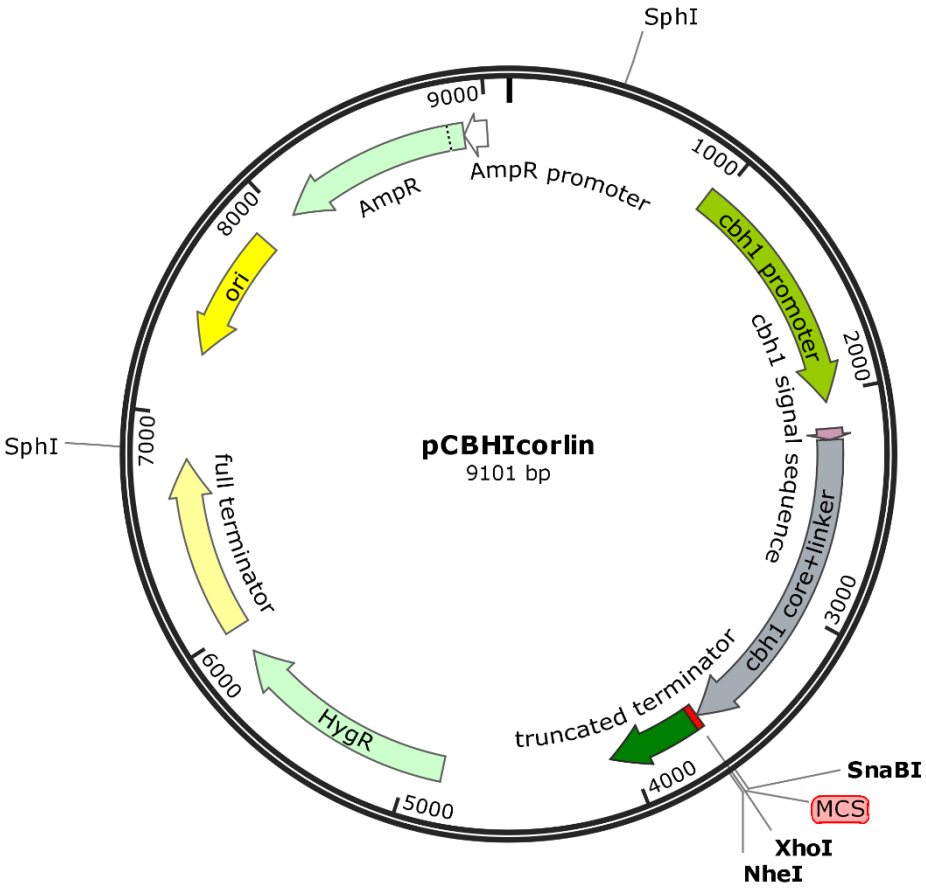


Figure 2.2. Map of the pCBH1corlin vector: CBH1corlin expression cassette (Figure 2.1), cloned into the *Sph*I restriction site of the pUC19 plasmid. Origin of replication (ori) and ampicillin resistance gene (AmpR) of the pUC19 plasmid are shown. Image created using SnapGene software (from GSL Biotech; available at snapgene.com).

2.5 Transformation of *T. reesei*

2.5.1 Biolistic bombardment

The biolistic bombardment using the Bio-Rad PDS-1000/He biolistic particle delivery system was performed as described by Te'o et al. (2002).

Preparation of DNA-coated gold microparticles. Approximately 50 mg of gold particles (Inbio Gold, Australia) were resuspended in 1 ml H_2O. For each bombardment procedure, 10 µl of the gold particle solution was mixed with 500 ng of DNA, 50 µl 2.5 M $CaCl_2$, and 20 µl

0.1 M spermidine. Prepared mixture was vortexed for 2-3 min and incubated for 10 min on ice. The supernatant was removed by quick centrifugation, and the DNA-coated gold particles were resuspended in 10 µl absolute ethanol and applied onto the macrocarrier (Bio-Rad, USA).

Preparation of conidia. On the day of bombardment, approximately 10^7 fresh conidia were spread onto the centre of each PDA plate. The plates were dried and incubated at 28ºC for 1 hour before bombardment.

***Bombardment procedure*.** The shooting chamber and all other components of the gene gun were UV-irradiated for 30 min and subsequently wiped with 70% (v/v) ethanol before the procedure. Helium at 800 *psi* pressure was applied to create 25 mm Hg vacuum. The bombardment was performed with 650 *psi* rupture disks (Bio-Rad, USA) and an optimal target distance of approximately 3 cm was used.

After the bombardment, PDA plates were incubated at 28ºC for a minimum of 2 hours before overlaying with PDA containing 80 U/ml of Hygromycin B (AG Scientific, USA). Plates were incubated at 28°C for another two to three days until colonies become visible. Single colonies were transferred onto fresh PDA-Hygromycin B plates for the second round of selection.

2.5.2 Protoplast transformation

Protoplast transformation was performed as described by Penttilä et al. (1987).

Preparation of the protoplasts. One day before the transformation, freshly harvested fungal conidia were spread onto sterile cellophane discs placed on the PDA plates and incubated overnight at 28°C. On the day of the procedure, cellophane discs were removed from the PDA plates and incubated with lysing enzymes from *Trichoderma harzianum* (40 mg/ml solution prepared in 1.2 M $MgSO_4$ – 10mM sodium phosphate buffer, pH 5.8) to digest the cell walls. The formation of the protoplasts was monitored under the microscope until enough protoplasts

detached from the mycelium (Figure 2.3).

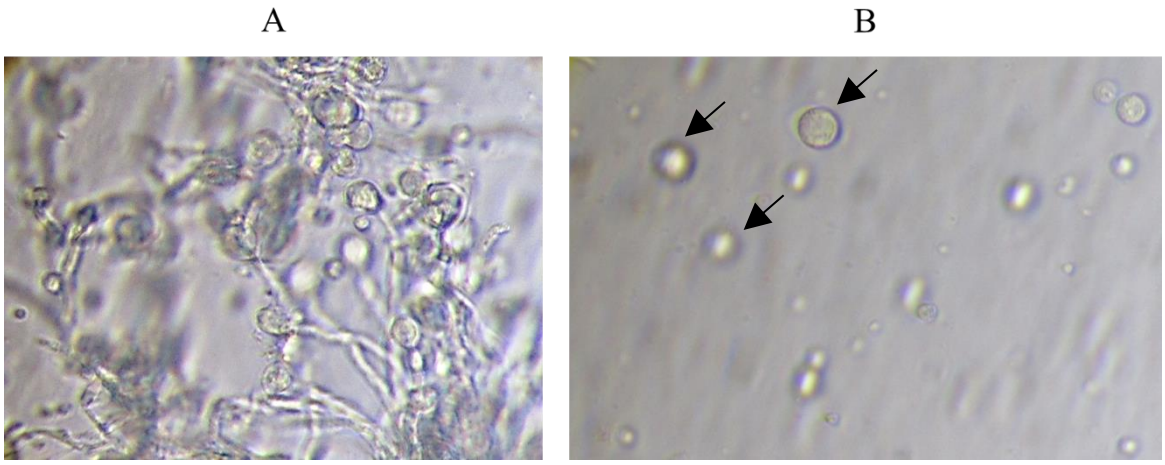


Figure 2.3. Rut-C30 sample before the enzyme treatment (**A**) and detached protoplasts (indicated with arrows) (**B**).

The protoplast solution was filtered through a glass filter (#1 or #2) with 0.1 M MgSO4 – sodium phosphate solution as a washing buffer. Obtained suspension was overlaid with an equal volume of 0.6 M sorbitol – 0.1 M Tris-HCl pH 7.0 buffer and centrifuged for 15 min at 4000× g. Protoplasts were removed from the interphase or the bottom of the tube, washed with an equal volume of 1.2 M sorbitol – 10 mM Tris-HCl pH 7.5 solution and centrifuged for 5 min at 4000× g. This step was repeated three times. After the final washing step protoplasts were resuspended in 400 - 800 µl of 1.2 M sorbitol – 10 mM Tris-HCl – 10 mM $CaCl_2$ solution, pH 7.5 (200 µl/transformation).

Transformation of the protoplasts. For each transformation, 5 µg DNA was added to 200 µl of protoplasts followed by addition of 50 µl of PEG solution (40% PEG6000 – 50 mM $CaCl_2$ – 10 mM Tris-HCl, pH 7.5). After incubation on ice for 20 min, 2 ml of 40% PEG solution was added, and protoplast solution was incubated for 5 min at room temperature. Next, 4 ml of 1.2 M sorbitol – 10 mM Tris-HCl – 10mM $CaCl_2$, pH 7.5 were added.

For regeneration of transformed protoplasts, 100-200 µl of protoplast solution were plated onto selective medium (1.5% KH_2PO_4, 0.5% $(NH_4)_2SO_4$, 2% glucose, 1 M sorbitol, 1.8% agar,

60 U/ml Hygromycin B, 2.4 mM $MgSO_4$, 5.4 mM $CaCl_2$, pH 5.5) in 10 ml of top agar containing Hygromycin B (60 - 80 U/ml). Plates were incubated at 28°C for 3 - 5 days and emerging colonies were transferred onto fresh PDA-Hygromycin B plates (80 U/ml) for the second round of selection.

2.6 Extraction of fungal genomic DNA

Fungal strains were grown for seven days (until conidiation) on sterile cellophane discs placed on PDA plates. The mycelia were harvested and ground into a fine powder in liquid nitrogen. To 50-100 mg of ground mycelium, 750 µl of lysis buffer (50 mM Tris-HCl, 50 mM EDTA, 3% SDS, 1% (v/v) 2-mercaptoethanol) was added and mixed thoroughly by vortexing. After an hour of incubation at 65ºC, 700 µl of chloroform: phenol (1:1) was added and mixed by inverting. The mixture was then centrifuged at 13, 000× g for 15 min.

A volume of 600-650 µl of the aqueous (top) phase was removed to a new tube, avoiding any cellular debris at the interface. Further, 700 µl of SEVAG (chloroform: isoamyl alcohol, 24:1) was added to the aqueous phase, mixed by inverting and centrifuged at 13,000× g for 5 min. The aqueous phase was then removed to a new tube. Subsequently, 20 µl of 3 M sodium acetate and 1 ml of isopropanol were added into the tube and mixed gently by inverting until ropes of DNA appeared. The tube was centrifuged at 13,000× g for 30 s to pellet the DNA. The pelleted DNA was washed twice with 70% (v/v) ethanol. The tube was centrifuged and the ethanol removed. DNA was briefly dried in a vacuum centrifuge and resuspended in fresh sterile Milli-Q water. Extracted gDNA was used in PCR reactions to confirm the integration of the expression cassette into the *T. reesei* genome.

2.7. Measurement of secreted mCherry fluorescence

The mCherry fluorescence was measured in the culture supernatants as a preliminary indication of recombinant protein production by the *T. reesei* transformants. *T. reesei* culture supernatants were centrifuged at 13, 000× g for 10 min to remove any cellular debris. About 100 µl of the culture supernatants were pipetted to 96 well microtiter plate (Greiner, USA). Fluorescence readings were taken using FluoStar Galaxy plate reader (BMG Lab technologies, Australia) at an excitation/emission wavelength of 560/630 nm.

2.8 Protein gel electrophoresis

Precast NuPAGE Bis-Tris Mini gels (Invitrogen, USA) with a 4-12% gradient were used for protein separation and visualisation as well as self-prepared SDS-PAGE gels (Table 2.3). Pre-stained protein standard Novex Sharp (Invitrogen) and Strep-tag protein ladder (iba, Germany) were used for molecular weight determination. Protein samples mixed with 4× loading buffer (Invitrogen) were heated at 70°C for 10 min before loading on the gel. Loaded gels were run at 200 V for 40 min in MOPS buffer (Invitrogen, US).

Table 2.3. Components of the self-prepared SDS-PAGE gels.

Protein gel	**Components**
Separating gel	12.5% acrylamide:bis (Bio-Rad, USA) 3.4 mM Tris HCl pH 8.8 0.07% (v/v) TEMED (Tetramethylethylenediamine) 0.05% ammonium persulfate 0.4% SDS
Stacking gel	4% acrylamide:bis 0.74 mM Tris-HCl pH 6.8 0.07% (v/v) TEMED 0.05% ammonium persulfate 0.4% SDS

Gels were fixed in 7% (v/v) acetic acid/10% (v/v) methanol before staining in Coomassie blue G-250 for 1 h - overnight, followed by destaining in 1% (v/v) acetic acid before visualisation

on a light box.

2.9 Western blot analysis

Western blotting was performed to identify the recombinant proteins in the *T. reesei* culture supernatants and also to analyse the Strep-tag purified proteins. Specific antibodies (Table 2.4) and Strep-Tactin AP conjugate were used for identification; alkaline phosphatase (AP)-labelled secondary antibodies and BCIP/NBT substrate (Invitrogen, USA) were used for visualisation. Proteins on an SDS-PAGE gel (section 2.8) were transferred onto PVDF or nitrocellulose membrane for 15 min at 1.3 A and 25 V in Bio-Rad Trans-Blot Turbo buffer using a Bio-Rad Trans-Blot Turbo system (Bio-Rad, USA). For Western blotting with specific antibodies, the membrane was blocked for 1 h at room temperature using TBS buffer (Table 2.1) with 3% skim milk and 0.05% (v/v) Tween 20 (BDH Chemicals Ltd., England) with gentle shaking. The membrane was incubated with a suitable primary antibody (Table 2.4) diluted in 3% skim milk in TBS buffer overnight at 4ºC with gentle shaking. The membrane was then washed three times for 10 min each with TBS-Tween 20 buffer at room temperature and incubated for an hour with the secondary antibody (Table 2.4) in TBS buffer with gentle shaking. After incubation with the secondary antibody, the membrane was washed three times for 5 min each in TBS-Tween 20 buffer at room temperature to remove the excess antibody. The washed membrane was then treated with BCIP/NBT alkaline phosphatase detection solution.

Table 2.4. Antibodies and solutions used in Western blotting (continued onto the next page).

Primary antibody/conjugate	**Secondary antibody**	**Detection method**
anti-mCherry (polyclonal) Abcam, #ab183628	Goat anti-rabbit IgG (AP) Abcam, #ab6722	BCIP/NBT alkaline phosphatase detection kit (Invitrogen, #00-2209)
anti-FUT3 (polyclonal) Abcam, #ab102844		

anti-FUT3 (polyclonal) Santa Cruz Biotechnology, #sc-14874	Donkey anti-goat IgG (AP) Santa Cruz Biotechnology, #sc-2022	
anti-FUT4 (monoclonal) Abcam, #ab181461	Goat anti-mouse IgG (AP) Abcam, #ab98724	
anti-CBH1 (polyclonal) courtesy of Dr. Junior Te'o		
Strep-Tactin AP conjugate iba, #2-1503-001	N/A	

For identification of the Strep-tagged recombinant proteins, the membrane was blocked using 20 ml TBS buffer with 3% bovine serum albumin (BSA) and 0.1% (v/v) Tween 20 overnight at 4ºC. After blocking, the membrane was washed three times using TBS buffer with 0.1% (v/v) Tween 20 for 5 min each at room temperature. After the last wash step, 2.5 µl of Strep-Tactin AP conjugate in 10 ml of TBS buffer with 0.1% (v/v) Tween 20 was added to the membrane and incubated for 60 min at room temperature with gentle shaking. The membrane was washed twice for one min each with TBS-Tween 20 buffer at room temperature and then twice with TBS buffer. The membrane was then incubated in BCIP/NBT solution for colour development. The chromogenic reaction was continued until the desired intensity of colour was achieved.

2.10 Purification of Strep-tagged proteins

Purification of recombinant Strep-tagged proteins was performed according to the Strep-tag purification manual available at https://www.iba-lifesciences.com. Buffers used in the purification procedure are listed in Table 2.5.

Table 2.5. Buffers used for purification of Strep-tagged proteins.

Buffer	**Components**
Buffer W	100 mM Tris-HCl pH 8.0,150 mM NaCl, 1 mM EDTA
Buffer E	100 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 2.5 mM desthiobiotin
Buffer R	100 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM HABA (hydroxy-azophenyl-benzoic acid), pH 8.0

T. reesei transformants were cultured for 48 h in 50 ml of CLS medium as described in section 2.2.4. Cultures were centrifuged at 4000× g for 30 min; supernatants were collected and used immediately. If long-term storage was required, supernatants were stored at -80°C and Mini EDTA-free protease inhibitor Cocktail tablets (Roche) were added (one tablet per 50 ml of the supernatant). Gravity flow columns filled with 5 ml of Strep-Tactin Sepharose resin (iba, Germany) were equilibrated with 10 ml of buffer W. Supernatants were applied on the column using WET FRED flow regulation system from iba (Germany) keeping the flow speed at approximately 1 ml/min.

The columns were washed five times with 5 ml of buffer W. Recombinant proteins were eluted from the column with buffer E in 6 2.5 ml fractions. After elution, the columns were washed three times with 25 ml of buffer R. The colour change from yellow to red indicated the regeneration process. Regenerated columns were overlaid with 2 ml of buffer R and stored at 4°C. Elution fractions were dialysed against distilled water overnight at 4°C and used in Western blotting, N-terminal sequencing and protease inhibition studies.

2.11 Sample preparation for N-terminal sequencing

At least 2 picomoles of the protein to be sequenced were run on the SDS-PAGE gel and transferred onto the PVDF membrane as described in sections 2.8 and 2.9. After the transfer, the membrane was stained with freshly prepared 0.1% Coomassie Blue R250 in 40% (v/v) methanol for 30 seconds. Stained membrane was destained with 50% (v/v) methanol until bands were visible and the background was clear, rinsed with water and dried.

3

EXPRESSION OF THE RECOMBINANT FUT3 AND FUT4 IN *TRICHODERMA REESEI*

3.1 Introduction

The filamentous fungus *Trichoderma reesei* has a long history of use as a producer of non-recombinant and recombinant hydrolytic enzymes for food and feed industries, agriculture, textile and paper treatment (Jeoh et al., 2008). However, expression of mammalian gene products in *T. reesei* remains a challenge. The first mammalian protein successfully produced in *T. reesei* was calf chymosin at 40 mg/l, expressed as a fusion to the highly secreted endogenous CBH1 protein (Harkki et al., 1989). Among other accomplishments are a murine Fab fragment expressed at 150 mg/l (Nyyssönen et al., 1993) and a recently reported interferon alpha-2b at the level of 4.5 g/l produced in a multiple protease-deficient host strain (Landowski et al. 2016).

In this project, hypercellulolytic low in protease *T. reesei* strain Rut-C30 (Montenecourt and Eveleigh, 1979) was used as an expression host for two recombinant human fucosyltransferases, FUT3 and FUT4. Recombinant human FUT3 and FUT4 were expressed from the pCBH1corlin vector (Te'o et al., 2000) described in detail in section 2.4. Briefly, the pCBH1corlin vector features the cellobiohydrolase I (*cbh1*) gene promoter, contained in a 2.2 kb long nucleotide sequence, protein secretion signal, and a 247 bp truncated transcription terminator of the *cbh1* gene. The *cbh1* promoter is strongly induced by cellulose and repressed in the presence of glucose (Ilmén et al., 1996a). The pCBH1corlin also contains DNA sequences encoding the core and linker fragments of the CBH1 protein to assist in expression and secretion of the recombinant proteins (Nyyssönen et al., 1993; Smith et al., 2014). Transformation selection marker is the hygromycin B resistance gene expressed under the *T. reesei pki* promoter.

Two expression constructs were created for each fucosyltransferase; in both constructs, the FUT3 or FUT4 protein was produced in fusion with Strep-tag II to aid in protein detection and purification, and the core and linker domains of the CBH1 enzyme to boost secretion (section 1.8.3). Additional components included into one of the constructs were DNA encoding the fluorescent protein mCherry and the enterokinase cleavage site. The mCherry protein was initially introduced into the fusion protein to track its production and secretion in the fungal hyphae; it was also used for initial screening of transformants. The 15 bp (GACGACGACGACAAG) enterokinase cleavage site was introduced into the construct in order to remove the fusion partners (CBH1 and mCherry) during protein purification.

In this study, Western blotting using the Strep-Tactin AP conjugate against Strep-tag II was the main method applied for the screening of transformants in order to identify the best producers of recombinant fucosyltransferases.

3.2 Materials and Methods

3.2.1 Optimisation of the codon usage and construction of the FUT3 and FUT4 expression cassettes

Structures of the FUT3 and FUT4 enzymes and their gene sequences are described in detail in section 1.2 and in Appendices 1 and 3. Gene sequences encoding only the truncated C-terminal catalytic domains of the FUT3 and FUT4 were codon-optimised according to the *T. reesei* codon usage preference (Table 3.1). The N-terminal cytoplasmic tail and membrane-spanning region (see Figure 1.1) were not included in the construct. Such truncation allows secretion of the recombinant fucosyltransferases outside of a cell but does not affect enzymatic activity (de Vries et al., 2001).

Table 3.1. Codon usage in *Trichoderma reesei* and humans. Higher percentage indicates a higher priority in codon usage (adapted from Te'o et al., 2000 and Castro-Chavez, 2011).

Amino acid	Codon	Amino acid codon usage in *T. reesei* (%)	Most used codons in humans per amino acid (%)	Amino acid	Codon	Amino acid codon usage in *T. reesei* (%)	Most used codons in humans per amino acid (%)
Gly	GGG	15		**Ser**	AGT	5	
	GGA	14			AGC	25	24
	GGT	18			TCG	22	
	GGC	60	34		TCA	8	
Glu	GAG	82	58		TCT	16	
	GAA	18			TCC	25	
Asp	GAT	31		**Asn**	AAT	19	
	GAC	69	54		AAC	81	53
Val	GTG	23	46	**Met**	ATG	100	100
	GTA	4		**Ile**	ATA	4	
	GTT	18			ATT	33	
	GTC	56			ATC	64	47
Ala	GCG	17		**Thr**	ACG	29	
	GCA	12			ACA	10	
	GCT	22			ACT	20	
	GCC	49	40		ACC	41	36
Arg	AGG	13		**STOP**	TGA	19	47
	AGA	7	21		TAG	44	
	CGG	12			TAA	37	
	CGA	16		**Cys**	TGT	24	
	CGT	17			TGC	76	54
	CGC	35					
Leu	TTG	10		**Gln**	CAG	81	73
	TTA	1			CAA	19	
	CTG	36	40	**His**	CAT	17	
	CTA	2			CAC	83	58
	CTT	14		**Lys**	AAG	92	57
	CTC	38			AAA	8	
Pro	CCG	22		**Phe**	TTT	38	
	CCA	12			TTC	62	54
	CCT	25		**Tyr**	TAT	25	
	CCC	40	32		TAC	75	56
Trp	TGG	100	100				

Alignment of the original and codon-optimised FUT3 and FUT4 cDNA sequences and their respective amino acid translations can be found in Appendices 2 and 4.

Truncated and codon optimised cDNAs were synthesised by GenScript (USA) (http://www.genscript.com) together with the 24 bp N-terminal Strep-tag II and a short 12 bp linker. Restriction sites for *Xho*I and *Nhe*I restriction enzymes were introduced into the N- and

C-termini of the construct. The *Cla*I restriction site was introduced between the Strep-tag II and the linker to allow the replacement of the purification tag, if found necessary (Figure 3.1).

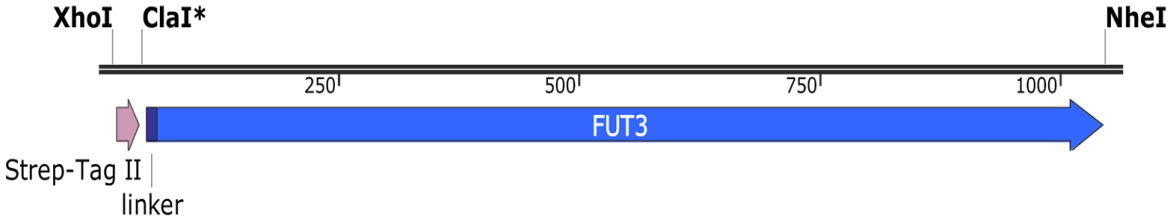


Figure 3.1. FUT3 cDNA in fusion with N-terminal Strep-tag II and linker (1063 bp). The *Xho*I and *Nhe*I restriction sites were used in the construction of the expression cassette. Image created using SnapGene software (from GSL Biotech; available at snapgene.com).

Synthesised sequences were supplied in pUC57 plasmids for propagation in *E. coli* (Figure 3.2).

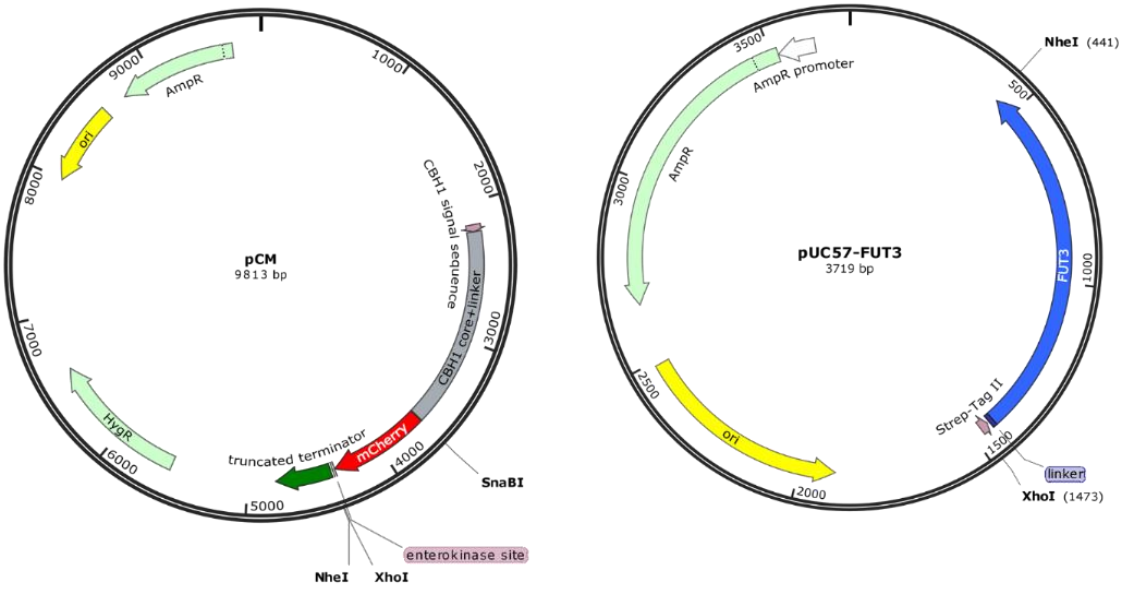


Figure 3.2. Maps of the pCM (pCBH1corlin plasmid containing the mCherry gene) and pUC57-FUT3 plasmids; only major features are shown. Image created using SnapGene software (from GSL Biotech; available at snapgene.com).

The mCherry gene was PCR amplified from the pmCherry plasmid (http://www.clontech.com) with P1mCherryRev and P1mCherryFwd primers (see Table 2.2). Restriction sites and a C-terminal enterokinase cleavage site were added during amplification.

The amplified fragment was digested with *SnaB*I and *Xho*I restriction enzymes and cloned into the pCBH1corlin plasmid. The obtained plasmid was called pCM (**C**BH1-**m**Cherry) (Figure 3.2).

Plasmids pUC57-FUT3 and pUC57-FUT4 were digested with *Xho*I and *Nhe*I restriction enzymes. The resulting fragments were purified from the gel and cloned into the corresponding sites of the pCM plasmid (pCMF3 and pCMF4). Assembly of the pCMF3 vector is illustrated in Figure 3.3.

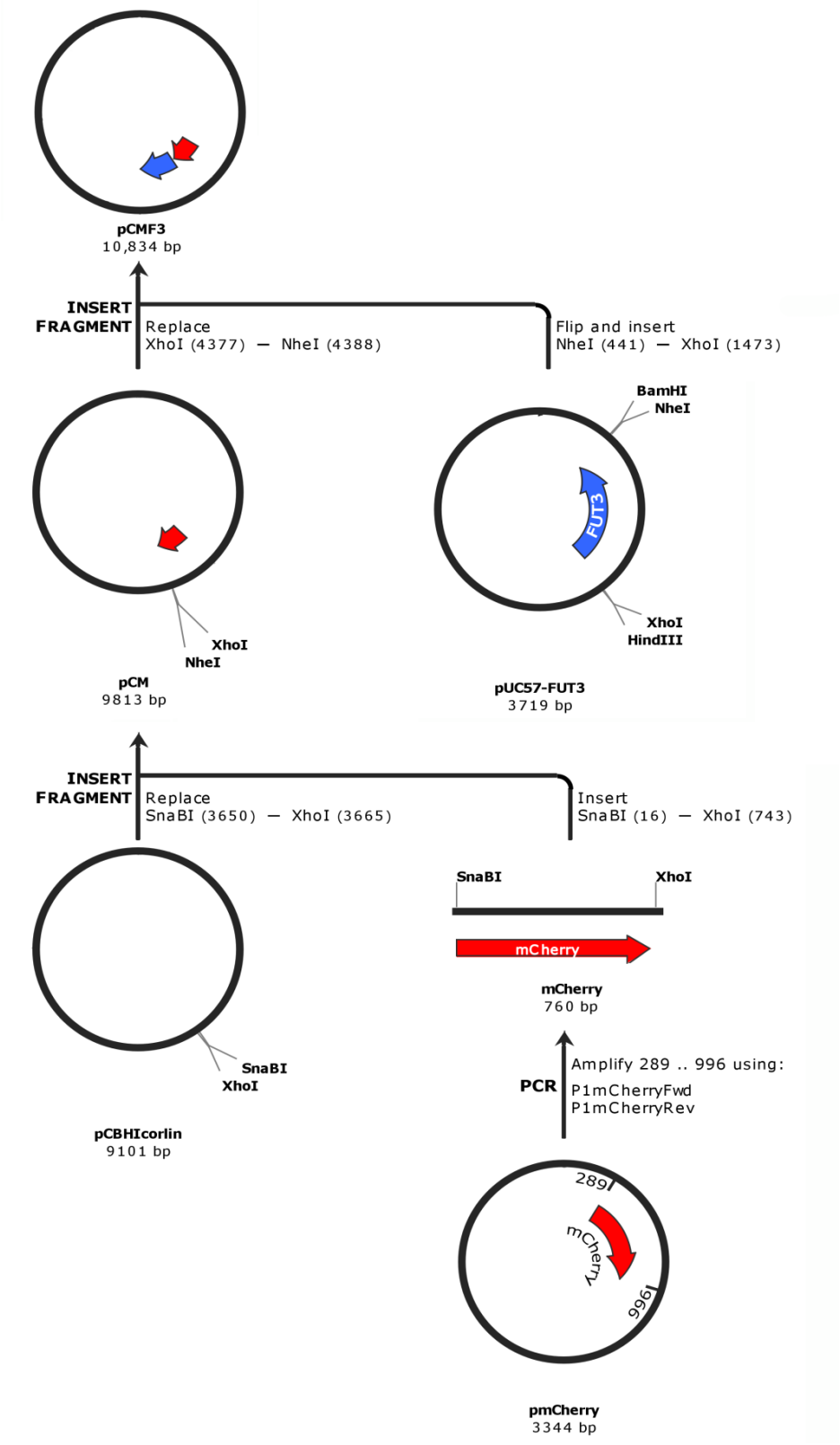


Figure 3.3. Assembly of the pCMF3 vector. Only major features of the expression cassette are shown. The mCherry gene (red arrow) was amplified from the pmCherry plasmid and cloned into the pCBH1corlin plasmid (pCM). Next, FUT3 DNA fragment (blue arrow) was cut out from the pUC57-FUT3 plasmid and cloned into the pCM plasmid (pCMF3). Image created using SnapGene software (from GSL Biotech; available at snapgene.com).

To construct expression cassettes without the mCherry gene, FUT3 and FUT4 fragments were amplified from the pUC57-FUT3 and pUC57-FUT4 plasmids using fut3_corr.rev, fut3_corr.fwd, fut4_corr.rev and fut4_corr.fwd primers (see section 2.3.2 for detailed information). Amplified fragments were digested with *Xho*I and *Nhe*I restriction enzymes and cloned into the pCBH1corlin plasmid (pCF3 and pCF4 plasmids). Assembly of the pCF3 plasmid is illustrated in Figure 3.4.

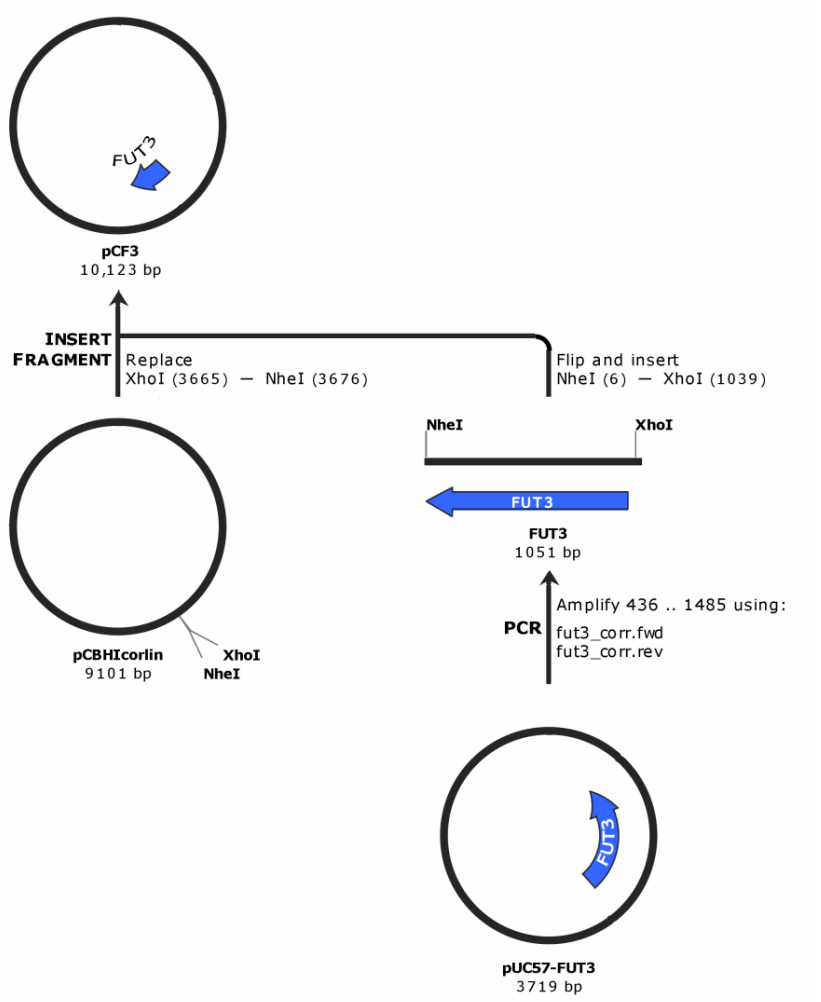


Figure 3.4. Assembly of the pCMF3 vector. Only major features of the expression cassette are shown. The FUT3 DNA fragment (blue arrow) was amplified from the pUC57-FUT3 plasmid and cloned into the pCBH1corlin plasmid (pCF3). Image created using SnapGene software (from GSL Biotech; available at snapgene.com).

At each step of plasmid construction, integration of the fragments into the expression cassette was confirmed by PCR with specific primers (Table 2.2) and by Sanger sequencing. As the

final step, all constructed plasmids were sequenced using the corlinSeqFwd primer. The sequencing primer was designed to bind upstream of the multiple cloning site of the pCBH1corlin plasmid (Figure 3.5), which allows to obtain good sequencing data of the DNA fragments cloned into the plasmid. Sequencing results can be found in Appendices 5 and 6.

Figure 3.5. The binding site of corlinSeqFwd primer (224 bp upstream of the multiple cloning site), used for sequencing of the pCMF and pCF plasmids. Image created using SnapGene software (from GSL Biotech; available at snapgene.com).

Up to scale maps of pCMF3 and pCF3 plasmids, the binding site of a sequencing primer and location of the restriction sites of the enzymes used in this study are highlighted in Figure 3.6.

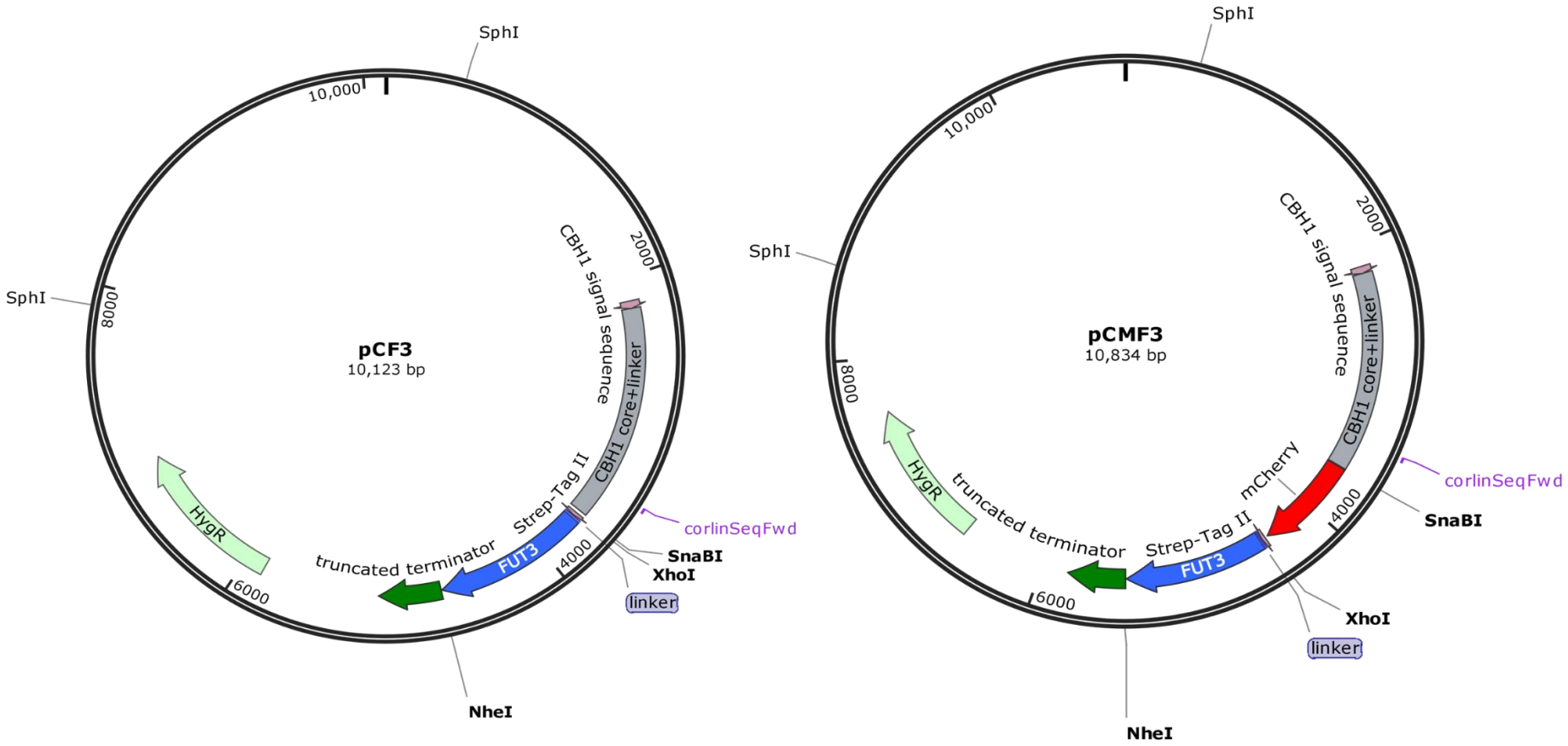


Figure 3.6. Maps of the pCF3 and pCMF3 plasmids. Major features of the expression cassettes are displayed: mCherry (red), FUT3 (blue), CBH1 core and linker (grey). Image created using SnapGene software (from GSL Biotech; available at snapgene.com).

Expression cassettes were excised from the plasmid backbone by digestion with *Sph*I, gel-purified and used to transform *T. reesei*.

3.2.2 Transformation of *T. reesei*

T. reesei strain Rut-C30 was transformed with linear FUT3 and FUT4 expression cassettes using both biolistic bombardment and protoplast transformation (transformation methods are described in section 2.5). Obtained transformants were subjected to two rounds of selection with Hygromycin B (final concentration 80 U/ml). Protoplast transformation was not successful, and all initial transformants obtained with this method did not survive the second round of selection with Hygromycin B. Transformants obtained by biolistic bombardment were grown on PDA plates for seven days before collecting the conidia into a spore collection solution for the cultivation studies.

3.2.3 Screening of transformants based on mCherry fluorescence

T. reesei transformants containing the pCMF3 or pCMF4 construct were cultivated in 3 ml CLS medium at 28°C for three days. Fluorescence of the mCherry protein in the culture supernatants was measured as described in section 2.7.

3.2.4 Western blotting

Western blot analysis was performed as described in section 2.9 with some variations in the protein electrophoresis and blotting conditions. Both precast gels (Invitrogen, USA) and freshly prepared self-cast gels were used for protein electrophoresis. Nitrocellulose and PVDF membranes were interchangeably used in Western blotting. BSA was used as a blocking agent in Western blots with the Strep-Tactin AP conjugate and skim milk was used in Western blots with antibodies. Western blotting was repeated 2-3 times for each set of samples. Strep-tag ladder (iba, cat#2-1011-100) served as a positive control in all Western blots with the Strep-

Tactin AP conjugate.

3.3 Results and Discussion

3.3.1 Screening of the transformants by mCherry fluorescence and Western blotting

Three rounds of both protoplast transformation and biolistic bombardment were performed in order to obtain the sufficient number of transformants, selected on the basis of their resistance to Hygromycin B. The results are summarised in Table 3.2.

Table 3.2. Summary of the *T. reesei* transformation with CMF (containing mCherry) and CF (no mCherry) expression cassettes.

Transformation method	Number of transformants	Comments
Protoplast transformation	N/A	Transformation plates were contaminated.
Protoplast transformation	8 CMF3 and 8 CMF4 transformants	Transformants did not survive the second round of selection with Hygromycin B. Confluent growth on transformation plates did not allow to select individual transformants; lower concentration of protoplasts was used in the next round of transformation.
Protoplast transformation	60 CMF4 and 50 CMF3 transformants	Transformants did not survive the second round of selection with Hygromycin B.
Biolistic bombardment	10 CMF3 transformants	All transformants survived the second round of selection with Hygromycin B.
Biolistic bombardment	19 CMF3 transformants and 21 CMF4 transformant	Four CMF3 transformants were lost due to contamination.
Biolistic bombardment	8 CF3 and 10 CF4 transformants	All transformants survived the second round of selection with Hygromycin B.

Protoplast transformation resulted in a high number of initial transformants, however, transformants obtained with this method did not survive the second round of selection with Hygromycin B. Instability of transformants usually results from the lack of integration of a DNA cassette into the genome and has been previously described to be a major bottleneck for *T. reesei* transformation (Jørgensen et al. 2014). Another possible reason of such instability is

the tandem integration of multiple copies of a DNA cassette, which could be excised through a loop-out event (Aw & Polizzi, 2013; Jørgensen et al., 2014). It has been demonstrated that protoplast transformation has been particularly difficult with Rut-C30, seemingly due to a reduced ability of the strain to regenerate the cell wall (Nevalainen et al., 1995; Bergquist et al., 2002). This aspect was not studied further as stable transformants were obtained via biolistic bombardment.

After two rounds of selection with Hygromycin B, genomic DNA was extracted from some transformants (8 CMF3 and 7 CMF4 transformants from the first and second rounds of biolistic bombardment) (section 2.6). The presence of the *hph* gene conferring resistance to hygromycin, the mCherry gene and the FUT3 and FUT4 encoding genes in the genomic DNA was confirmed by PCR amplification with specific primers (see Table 2.2). All PCR reactions were positive, confirming integration of the expression cassettes into the genome (data not shown). While most of the transformants had a similar phenotype to the expression host Rut-C30 (dark green spores), a few transformants had unusually light, almost white spores (Figure 3.7, A); however, no differences in growth speed and viability of the transformants were observed.

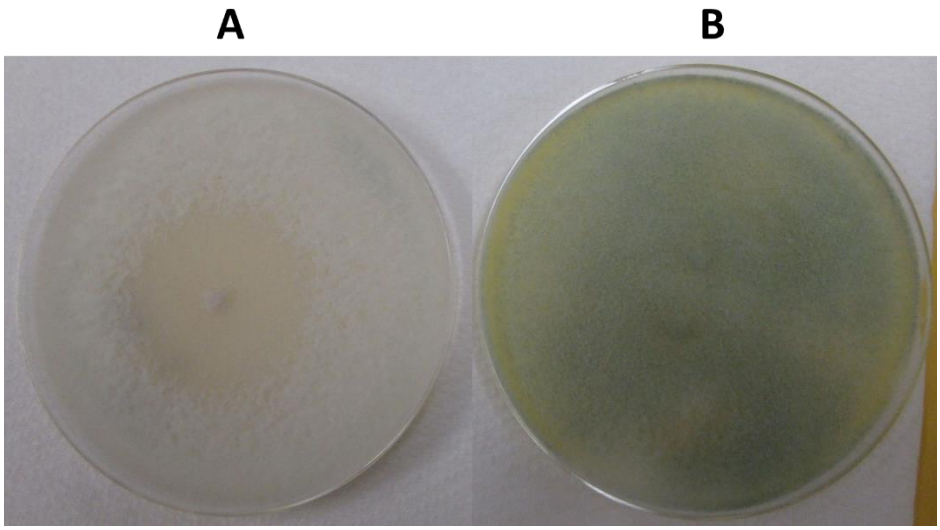


Figure 3.7. Colony appearance of the CMF3-1 transformant obtained by biolistic bombardment (**A**) and the expression host Rut-C30 (**B**).

After a stable integration of the expression cassettes into the *T. reesei* genome was confirmed, the next step was to screen the transformants for the production of the recombinant proteins. Fluorescent tags are often used as a rapid and high-throughput method of screening, especially when a large number of transformants needs to be processed (Bird et al., 2015; Jia & Jeon, 2016; Wong & Truong, 2010). However, there is a recognised possibility of the fluorescent tag becoming cleaved off from the fusion protein (Huang et al., 2014). In case this should happen, high levels of mCherry fluorescence might not correlate with high amounts of recombinant fucosyltransferases as the recombinant enzyme might become cleaved from the mCherry protein and degraded. Even so, when a large number of transformants needs to be screened, the strategy is worth pursuing to indicate that the fusion protein is being expressed and secreted.

Fluorescence of the mCherry protein was measured in the culture supernatants of 10 CMF3 transformants obtained in the first round of biolistic bombardment as the first indication of production and secretion of recombinant fusion proteins. Selected transformants with a high or low level of fluorescence measured in the culture supernatant were further analysed for the

presence of Strep-tagged recombinant proteins to establish a potential correlation. Results of the first round of such screening of the CMF3 transformants are shown below in Figure 3.8.

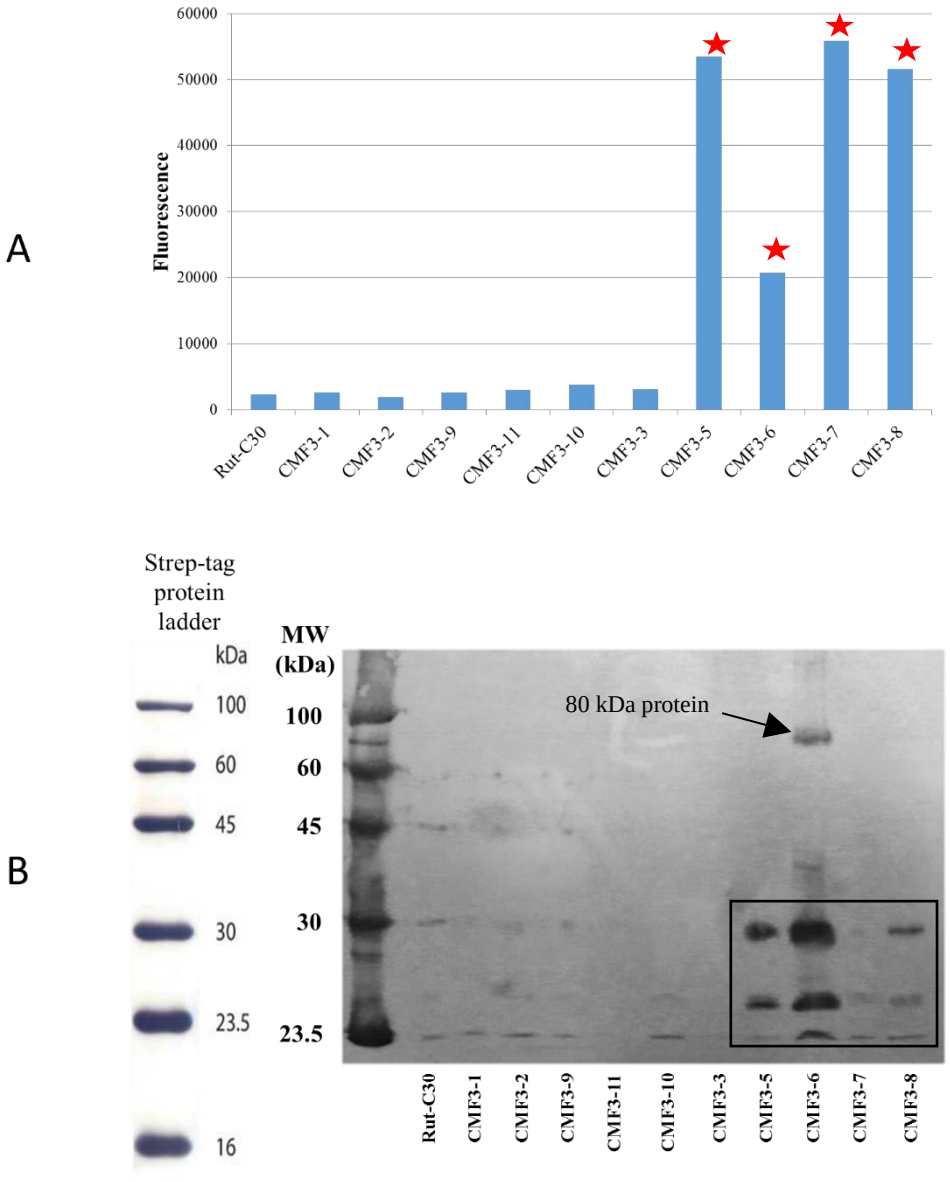


Figure 3.8. Fluorescence of the mCherry protein (**A**) and detection of Strep-tagged proteins (**B**) in the three day culture supernatants of CMF3 transformants. Transformants with high levels of the mCherry fluorescence that also secreted recombinant Strep-tagged proteins are labelled with red stars. Four transformants characterised by high levels of mCherry fluorescence secreted low MW Strep-tagged proteins (indicated by a rectangle) and only one of them secreted an 80 kDa protein, indicating possible truncation of the heterologous FUT3 protein.

Four CMF3 transformants (CMF3-5, CMF3-6, CMF3-7 and CMF3-8) exhibited high levels of mCherry fluorescence. Western blot analysis demonstrated that the culture supernatant of these transformants also contained Strep-tagged recombinant proteins of about 30 and 25 kDa

(indicated by a rectangle in Figure 3.8); transformant CMF3-6 additionally secreted an 80 kDa Strep-tagged protein. No full-sized recombinant proteins (115.4 kDa) were detected in any of the analysed CMF3 transformants. The occurrence of multiple protein bands of various molecular weights recognised with the Strep-Tactin AP conjugate indicates cleavage of the recombinant fusion protein (discussed in detail in Chapter 5). Transformants with low levels of fluorescence detected in their culture supernatants seemed not to produce recombinant proteins carrying a Strep-tag II (Figure 3.8).

Transformants obtained in the second round of *T. reesei* transformation via biolistic bombardment (both CMF3 and CMF4) were analysed in a similar way (Figure 3.9).

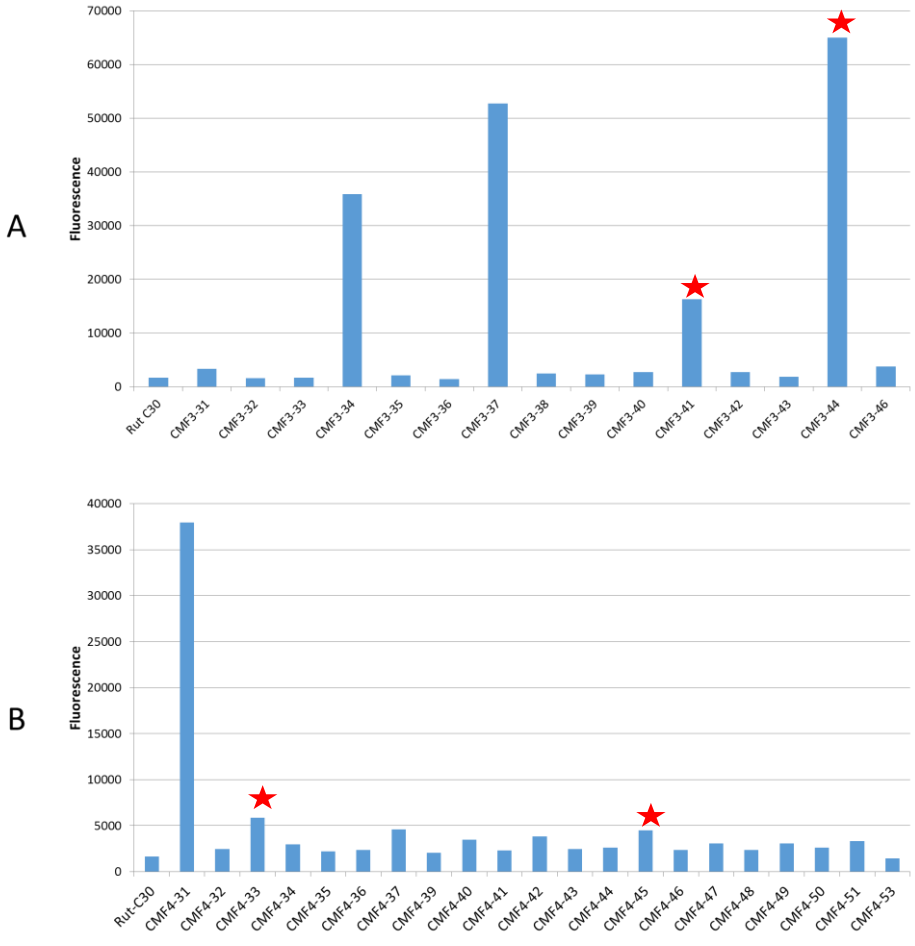


Figure 3.9. Fluorescence of the mCherry protein in the culture supernatants of CMF3 (**A**) and CMF4 (**B**) transformants obtained in the second round of biolistic bombardment. Transformants that demonstrated secretion of the recombinant Strep-tagged proteins are labelled with red stars.

Despite the substantial number of transformants (19 CMF3 and 21 CMF4 transformants) obtained in the second round of transformation, only five of them, CMF3-34, 37, 41 and 44, and CMF4-31 exhibited high levels of mCherry fluorescence. When analysed by Western blotting, not all of these transformants characterised by high levels of mCherry fluorescence demonstrated the occurrence of Strep-tagged proteins in their culture supernatants (Figure 3.10).

Interestingly, transformants CMF4-33 and CMF4-45 with relatively low levels of mCherry fluorescence demonstrated secretion of an about 100 kDa protein which is approximately 20 kDa smaller than predicted size of the CMF4 protein (118.4 kDa); the 100 kDa protein may be a product of C- or N-terminal cleavage; this was different to the outcome of the first round of transformant screening, where a correlation between the secreted mCherry fluorescence and the presence of Strep-tagged recombinant proteins in the culture supernatants was observed. As mentioned earlier, the mCherry protein may be cleaved off from the FUT protein and exist independently in the culture supernatants. As the analysis was carried out on three-day cultures, there is a possibility that at that stage of cultivation either mCherry or the FUT4 part may have become degraded resulting in a discrepancy in the measurements of mCherry fluorescence and Western blot analysis. However, after these experiments, a reliable relationship between high mCherry fluorescence and the level of the production of the FUT3 or FUT 4 fusion protein could not be established.

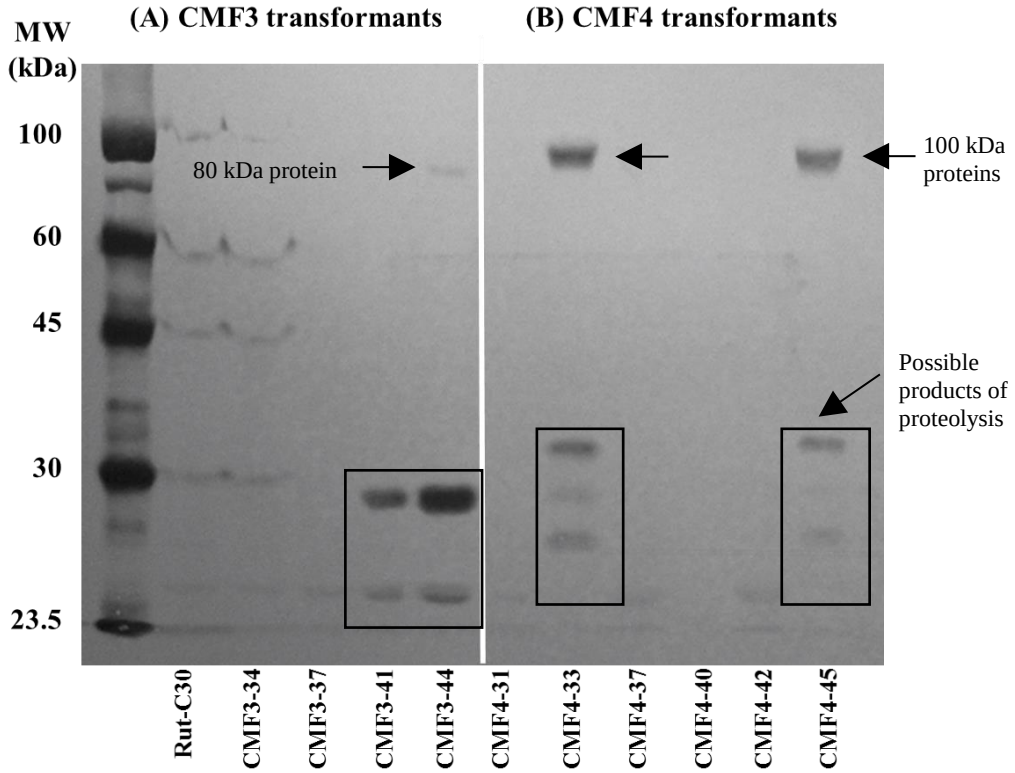


Figure 3.10. Western blot detection of Strep-tagged proteins in equal amounts of the culture supernatants of CMF3 (**A**) and CMF4 (**B**) transformants obtained in the second round of biolistic bombardment.

Transformants obtained in the second round of biolistic bombardment also secreted low MW recombinant proteins (25 – 35 kDa) (indicated by rectangles in Figure 3.10) as well as recombinant proteins of ~ 100 kDa (indicated by arrows in Figure 3.10). It should be noted that full-sized recombinant proteins (115.4 kDa for CMF3 and 118.2 kDa for CMF4) were not detected in the culture supernatants of any of the CMF3 and CMF4 transformants. Instead, other high molecular weight recombinant proteins (approximately 80 kDa and 100 kDa) were observed in Western blots together with low molecular weight recombinant proteins. The nature of these proteins and possible reasons for the absence of full-sized proteins will be further discussed in the following chapters. Interestingly, Strep-tagged proteins detected in the culture supernatants of the CMF3 and CMF4 transformants were different in size for the two groups of transformants. Both CMF3 and CMF4 constructs included the CBH1 core and linker protein, the mCherry protein and either the FUT3 or FUT4 protein so the different sizes of the

degradation products detected in the culture supernatants of the transformants could potentially be explained by different protease cleavage sites in the FUT3 and FUT4 proteins.

Transformants (CMF3-5, 6, 7, 8, 34, 37, 41, 44, CMF4-33 and CMF4-45) that demonstrated positive results in Western blots with the Strep-Tactin AP conjugate were further analysed for the presence of recombinant FUT3 and FUT4 proteins in the culture supernatants. None of the CMF4 transformants showed positive results in the Western blot analysis with a commercial anti-FUT4 antibody (Abcam, #ab181461) raised against the C-terminus of human fucosyltransferase 4 (data not shown). This could be a result of low antibody affinity or concentration of the FUT4 below the detection level. It is also possible that the recombinant CMF4 protein was C-terminally cleaved resulting in deletion of the antibody recognition site.

Only the CMF3-44 transformant, also characterised by high level of mCherry fluorescence (Figure 3.9) demonstrated a positive reaction in the Western blotting with a commercial anti-FUT3 antibody (Abcam, #ab102844) (Figure 3.11): two major bands of about 80 kDa and 45 kDa were detected. While the 45 kDa band was not observed in the Western blots with the Strep-Tactin AP conjugate, the 80 kDa band can be seen as a faint band in Figure 3.10.

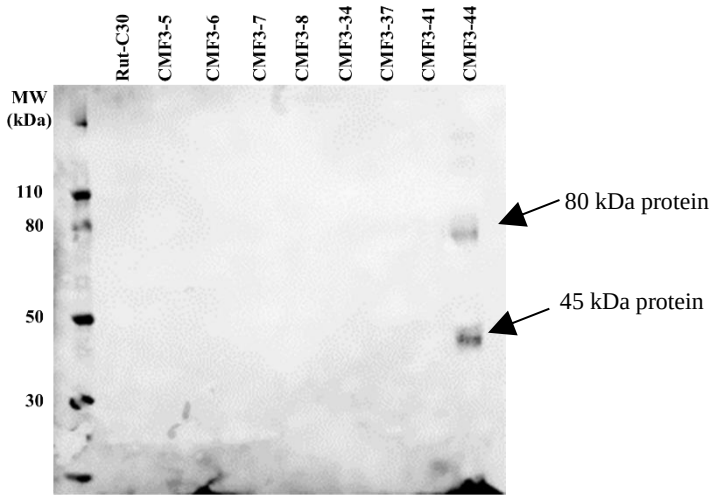


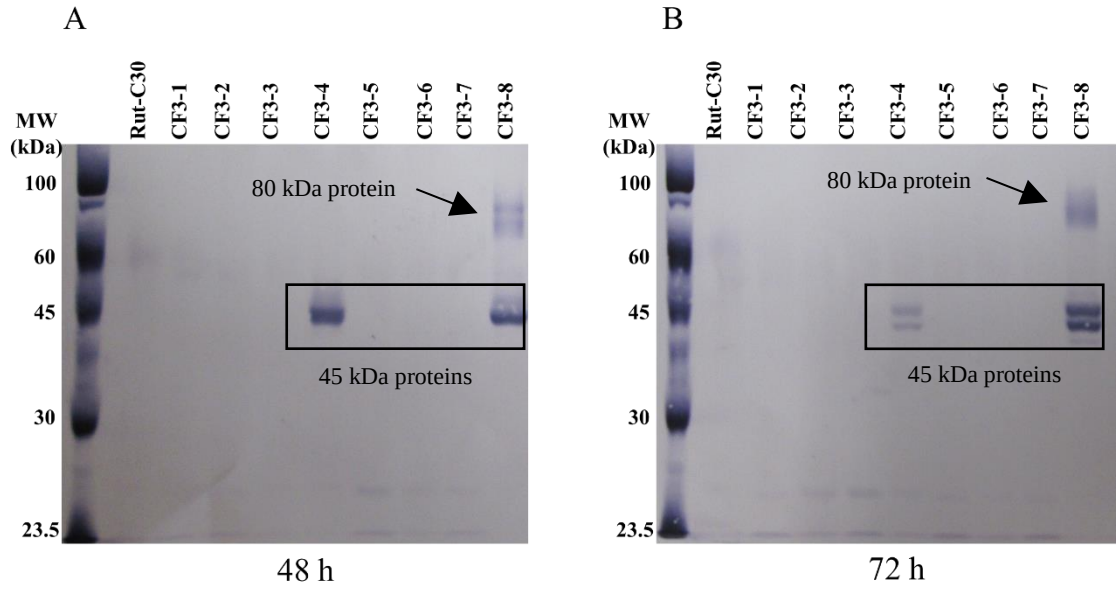
Figure 3.11. Western-blot detection of the FUT3 proteins in equal volumetric amounts of the culture supernatants of CMF3 transformants using a commercial anti-FUT-3 antibody.

The anti-FUT3 antibody used in this study targets the C-terminus of the human fucosyltransferase 3, which has two glycosylation sites (Christensen et al., 2000). It is possible that due to potential differences in glycosylation between humans and the fungus (Deshpande et al., 2008), FUT3 produced in *T. reesei* had low affinity to the antibody.

Positive controls (commercial FUT3 and FUT4 enzyme proteins) were not available at the time of the experiment which makes these results inconclusive. Proteins of approximately the same size (80 kDa) were detected in the culture supernatant of the CMF3-44 transformant with both anti-FUT3 antibody and Strep-Tactin AP conjugate which was an indication for that the Strep-tag II and the FUT3 protein remained together. This suggested that the presence of a recombinant FUT3 in the culture supernatant can be detected by the Strep-Tactin AP conjugate. Therefore, it was decided to use Western blotting with the Strep-Tactin AP conjugate as a primary detection method, as the use of commercial anti-FUT3 and anti-FUT4 antibodies for routine detection was not an option because of the high cost and the issues discussed above.

3.3.2. Screening of non-mCherry transformants by Western blotting

Culture supernatants of eight CF3 and 10 CF4 transformants carrying expression cassettes without mCherry were analysed by Western blotting now taking into consideration the possibility that the FUT part may become degraded by the third day of incubation. Thus the samples taken on day 2 (48 h) and day 3 (72 h) were analysed. Similarly to the CMF4 transformants with mCherry, CF4 transformants without mCherry did not show any signs of recombinant protein expression using an anti-FUT4 antibody (Abcam, #ab181461) and also no Strep-tagged proteins were detected (data not shown). Out of eight CF3 transformants, two (CF3-4 and CF3-8) demonstrated the presence of Strep-tagged proteins in Western blots with the Strep-Tactin AP conjugate in both 48 h and 72 h culture supernatants (Figure 3.12). None of the eight CF3 transformants tested demonstrated a positive reaction in the Western blotting with a commercial anti-FUT3 antibody (Abcam, #ab102844) (data not shown).



.

Figure 3.12. Western blot detection of Strep-tagged proteins in the culture supernatants of CF3 transformants after 48 (**A**) and 72 (**B**) hours of cultivation. The 45 kDa Strep-tagged proteins secreted by both transformants are indicated by rectangles.

Unlike the mCherry-containing CMF3 transformants, the transformant CF3-8 produced a recombinant protein of the expected size (about 80 kDa) (indicated by an arrow in Figure 3.12) as well as a smaller recombinant protein of about 45 kDa. Transformant CF3-4 only showed the presence of a 45 kDa recombinant protein. A 45 kDa Strep-tagged protein detected in both transformants could be a result of proteolytic cleavage of the fusion protein. The absence of the 80 kDa band in the culture supernatant of the CF3-4 transformant could be a result of different protease profiles between the two transformants due to, for example, disruption of some protease genes by random integration of the expression cassette into the *T. reesei* genome. Another explanation could be slower growth and/or slower protein secretion of the recombinant protein by the CF3-4 transformant which resulted in a concentration of the 80 kDa protein below detection levels.

3.4 Summative discussion

Expression cassettes for FUT3 and FUT4 were successfully constructed and used to transform *T. reesei* Rut-C30. Two transformation techniques were used from which protoplast transformation produced high numbers of unstable transformants that lost resistance to Hygromycin B during the second round selection. Biolistic bombardment, on the other hand, resulted in the stable integration of the expression cassette into the *T. reesei* genome. Obtained transformants were screened for the production of recombinant proteins first by measuring the fluorescence of the mCherry protein in the culture supernatants and then by detecting recombinant proteins by Western blotting using an the Strep-Tactin AP conjugate and a commercial antibody raised against human FUT3 or FUT4. A positive correlation between the mCherry fluorescence detected in the culture medium and secretion of the recombinant human glycosyltransferases FUT3 and FUT4 could not be established within the realms of the experiments carried out here. The transformant CMF3-44 came closest in fulfilling the

requirements in expressing strong fluorescence and producing FUT3-positive and Strep-tag positive bands of the same size in Western blots (Figure 3.10 and Figure 3.11), suggesting that the FUT3 protein and the Strep-tag II were not cleaved off each other, and that detection of the Strep-tag II could be used as an indication of the presence of the recombinant FUT3.

Full-sized recombinant proteins were not observed in any of the CMF transformants expressing the recombinant FUT3 or FUT4 in fusion with the mCherry protein. This could be a result of proteolytic degradation of the recombinant products by *T. reesei* proteases during secretion or in the culture supernatant. This aspect will be studied in more detail in the following chapter. Another less likely reason could be an error during transcription or translation.

One of the non-mCherry CF3 transformants (CF3-8, Figure 3.12) produced a recombinant protein close in size to the predicted full-sized protein on day 2 and day 3 of cultivation. The transformant also produced a truncated form of the recombinant protein which may suggest that the FUT3 proteins without mCherry may be more stable, perhaps due to the smaller size of the recombinant protein or because of a closer proximity of the FUT3 to the CBH1 carrier protein, which could protect the recombinant protein from degradation by native proteases (Nyyssönen & Keränen, 1995). While Strep-tag II could be cleaved from the FUT3 protein, the size (80 kDa) of the proteins produced by transfromant CF3-8 observed in Western blots (Figure 3.12) suggests that these two fusion partners were intact and existed as one protein.

Due to apparent insufficient activity/specificity of the commercial anti-FUT3 and anti-FUT4 antibodies raised against human enzymes and the absence of commercial FUT3 and FUT4 enzymes to be used as the positive controls, it was decided to continue experiments using detection of the Strep-tag II as the main detection method for the recombinant proteins until a better detection system was made available.

The Strep-tag II is small in size and resistant to proteolytic degradation, and therefore could be detected in even the smallest degradation products which allows to observe various degradation products at different time points and growth conditions and use this information to hypothesise about possible cleavage sites in the fusion protein.

Despite the substantial number of transformants obtained via biolistic bombardment (29 CMF3, 21 CMF4, 10 CF4 and 8 CF3 transformants), only some of them expressed recombinant Strep-tagged proteins. Transformant CMF3-44 secreted an 80 kDa protein recognised by both the Strep-Tactin AP conjugate and anti-FUT3 antibody, and transformant CF3-8 produced a recombinant protein of the expected size (80 kDa) which was also recognised by the Strep-Tactin AP conjugate. Therefore, it was decided to focus on these two transformants in the studies to follow. Some other CMF3 transformants and a few CMF4 transformants were used in a handful of experiments discussed in the following chapter for comparison purposes, but they were not investigated further.

4

OPTIMISATION OF CULTURE CONDITIONS

4.1 Introduction

The work carried out above suggested that recombinant proteins were produced but then degraded at some point during production, secretion or cultivation. This could be a result of incomplete transcription/translation or caused by proteolysis during or after secretion. The proteolytic system of *T. reesei* is a major factor affecting the production of heterologous proteins (Ward, 2012; Dienes et al., 2007; Zou et al., 2012). Even though the *T. reesei* Rut-C30 strain used in this study is characterised by lower protease activity in comparison with the wild-type strain QM6a, it still has hundreds of proteases, mostly unidentified or predicted on the gene level (Kredics et al., 2005; Adav et al., 2011; Martinez et al., 2008). There is not enough knowledge on the differences in protease profiles of *T. reesei* strains. Secretome analyses of Rut-C30 and another high-secreting *T. reesei* strain CL847 grown on a medium promoting production of cellulases and hemicellulases demonstrated the absence of trypsin in the Rut-C30 cultures (Herpoël-Gimbert et al., 2008).

A number of methods to decrease or inhibit harmful effects of protease activity exists. For example, modifications of the protease profile can be achieved via traditional mutagenesis and screening for low protease strains or by targeted gene manipulations such as RNA silencing and protease gene knock-outs (Mäntylä et al., 1998; Saloheimo & Pakula, 2012; Landowski et al., 2015 and 2016) as well as deletion of protease regulatory factors (Landowski et al., 2017). Bioprocessing methods such as early harvesting of the product, pH control and use of protease inhibitors (Smith et al., 2014; Zhang et al., 2014; van den Homberg et al., 1997) do not introduce changes into the genome of a production host but are directed towards lowering protease production and inhibition of already expressed proteases. As an example of such approach, cultivation at a lower temperature and higher pH decreased proteolytic degradation of the homologous Cel61A in *T. reesei* (Karlsson et al., 2001).

Trichoderma tends to grow at an acidic pH and produce acidic proteases that have been shown to harm heterologous proteins (van den Hombergh et al., 1997). The pH control strategy is a simple approach to inhibit acidic protease activity and increase the yield of heterologous proteins. One possible drawback of this strategy is that at the neutral pH, heterologous proteins could be exposed to neutral and alkaline proteases that may be harmful as well. An example of the pH control approach combined with a knock-out of a major alkaline protease is described by Zhang et al. (2014); enhanced production and stability of the heterologous alkaline endoglucanase EGV from *Humicola insolens* was observed using the *T. reesei* Rut-C30 Δspw protease deficient strain and adjusting the pH to 7.0 during cultivation as compared to the parental strain.

In this study, the optimal time of cultivation was identified in testing a series of transformants and a pH control strategy was applied in order to stabilise recombinant proteins in the culture supernatants. This allowed to extend further the cultivation time for obtaining higher yields of the recombinant FUT3 protein.

4.2 Materials and Methods

4.2.1 Time-dependent protein degradation

To study the influence of the cultivation time on the stability of the recombinant FUT3 and FUT4 in the culture supernatants, a series of CMF3, CF3 and CMF4 transformants that were selected for their ability to secrete recombinant Strep-tagged proteins in Chapter 3, were grown in 250 ml shake flasks in 50 ml of CLS medium. Samples were taken every 24 hours during three days and analysed by Western blotting with the Strep-Tactin AP conjugate as described in section 2.9.

4.2.2 pH-dependent degradation

Transformants CF3-4 and CF3-8 without mCherry and CMF3-44 and CMF4-33 containing mCherry as a fusion partner were grown in shake flask cultures as described earlier (section 2.2.4), divided in the control and experimental groups. The pH in the experimental flasks was periodically measured and manually adjusted to 6.5 – 7 with 5M KOH while the pH in the control flasks was not adjusted. Samples from both groups were analysed in Western blots with the Strep-Tactin AP conjugate as described in section 2.9. Western blotting was repeated 2-3 times.

4.2.3 Extraction of soluble intracellular proteins

Intracellular proteins were extracted from the CMF3-44 transformant and analysed for the presence of recombinant proteins inside the hyphae. The CMF3-44 transformant was grown in 50 ml CLS in 250 ml shake flasks over four days. Every day one flask was harvested: mycelia from liquid cultures were collected by centrifugation (5000× g, 10 min). The pellet was washed in 0.9% NaCl and then frozen in liquid nitrogen. The frozen mycelial pellet was ground to a fine powder under liquid nitrogen, and approximately 0.5 g was added to 5 ml of citrate buffer (50 mM citric acid, 50 mM sodium citrate, pH 5.0) containing 1 mM PMSF and 0.1% protease inhibitor cocktail (Roche, Germany). The sample was vortexed and incubated at 4°C for approximately 1 h. Next, the soluble fraction was separated from the insoluble material by centrifugation at 12 000× g for 20 min. The soluble fraction was stored at 4°C and used within two days to detect recombinant proteins by measuring the mCherry fluorescence and by Western blotting with the Strep-Tactin AP conjugate.

4.3 Results and Discussion

4.3.1 Time-dependent degradation of recombinant proteins produced by CMF3 and CMF4 transformants

Five CMF3 transformants and three CMF4 transformants, producing recombinant FUT3 and FUT4 proteins respectively, were selected based on their ability to produce secreted Strep-tagged proteins (section 3.3.1) and were further analysed to identify the optimal time of cultivation with a view of the balance between the maximal yield and degradation of the recombinant proteins. Culture supernatants taken after 24, 48 and 72 h of cultivation were analysed by Western blotting with the Strep-Tactin AP conjugate. Results of Western blotting are shown in Figure 4.1.

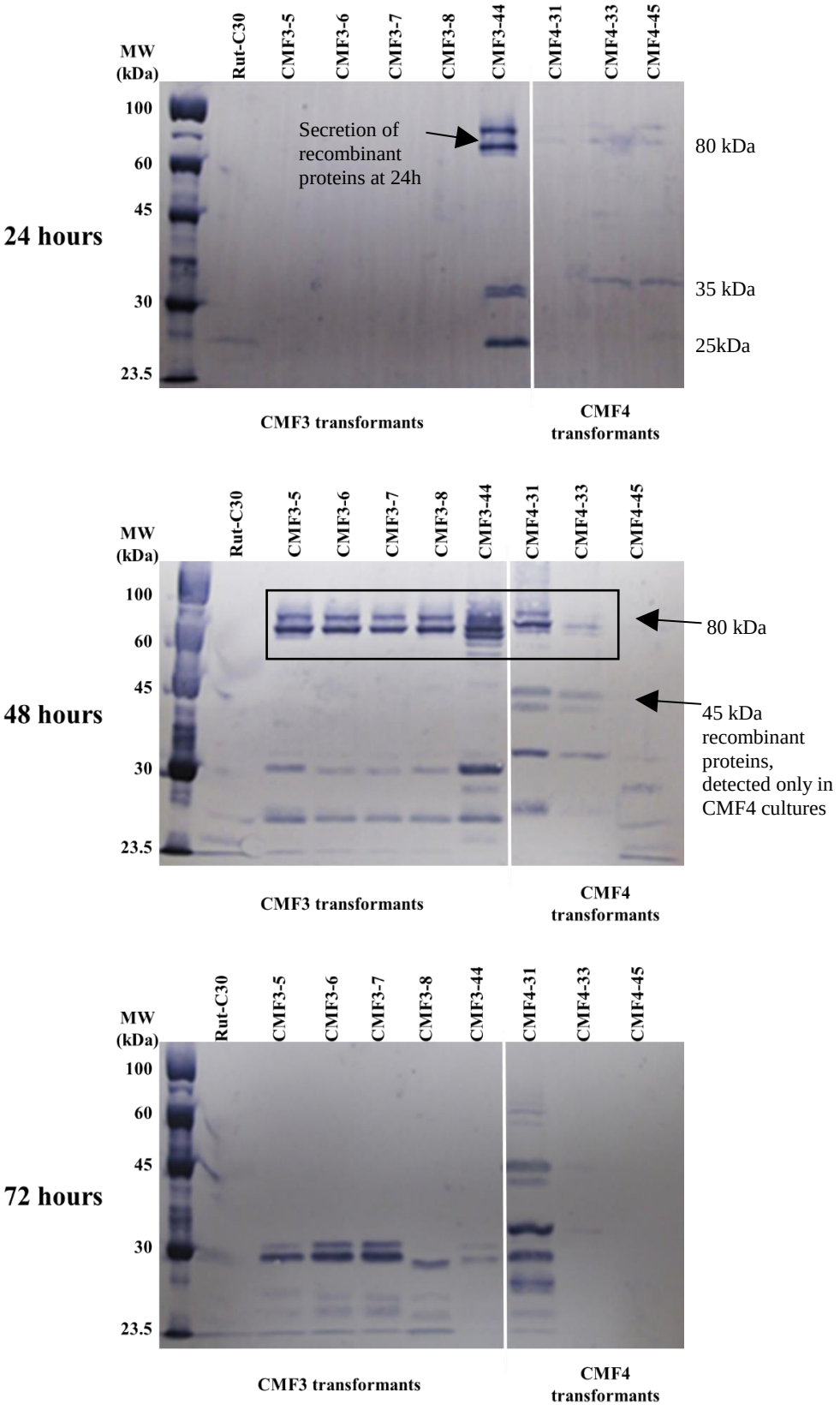


Figure 4.1. Western blot detection of Strep-tagged proteins in the culture supernatants of selected CMF3 and CMF4 transformants after 24, 48 and 72 hours of cultivation.

Out of eight transformants, three (CMF3-44, CMF4-33 and CMF4-45) expressed recombinant fusion proteins in the first 24 h of cultivation. From these, CMF3-44, which also exhibited high mCherry fluorescence (Figure 3.9) looked especially strong.

On the second day into cultivation (48 h), low MW (25 - 30 kDa) and high MW (around 80 kDa) recombinant proteins were detected in the culture supernatants of all transformants tested. Proteins in the culture supernatants of the CMF4 series of transformants already demonstrated advanced signs of degradation. By 72 hours into cultivation, high molecular weight products were completely degraded with the exception of transformant CMF4-31, which still showed a faint band of a high molecular weight protein. Full-sized CMF3 or CMF4 proteins (115.4 kDa and 118.4 kDa) were not observed in any of the transformants even at the earliest stages of cultivation, which may indicate that an initial cleavage of the recombinant FUT3 and FUT4 proteins occurs inside the cell or during secretion while further degradation happens outside the cell. This aspect was further explored in section 4.3.4.

Degradation patterns were similar within each series of transformants (CMF3 and CMF4) but different between the series. CMF3 transformants produced recombinant proteins of about 80, 35 and 25 kDa; CMF4 transformants also secreted an 80 kDa recombinant protein but lower molecular weight recombinant proteins detected in the supernatants of CMF4 transformants were slightly larger than those of the CMF3 transformants and additional protein bands of ~ 45 kDa were also observed (Figure 4.1). Similar degradation patterns between different transformants within each group suggested that the degradation was not random but rather caused by specific proteases targeting specific cleavage sites. This aspect was further explored in Chapters 5 and 6, where specific protease activities and cleavage sites were analysed. Obtained results suggest that the suitable time of cultivation is two days and also explain why high MW products were not observed in most transformants during the initial screening, performed after three days of cultivation (section 3.3.1). Double bands of the similar molecular

weight detected in the culture supernatants of all screened transformants (Figure 4.1) could be a result of the proteolysis at the closely located protease cleavage sites; alternatively, the bands with the slightly defferent molecular weight could be the different glycoforms of the same protein

4.3.2 pH-dependent degradation of recombinant proteins produced by CMF3 and CMF4 transformants

Transformant CMF3-44, selected in section 4.3.1 as a good producer of recombinant protein, was grown in three parallel cultures with and without pH adjustment, as described in section 4.2.2. Culture supernatant samples were taken after 48, 60, 72 and 84 h of cultivation and analysed by Western blotting with Strep-Tactin AP conjugate. Results of the Western blotting are shown in Figure 4.2 and Figure 4.3. As demonstrated previously (section 4.3.1), expression of the recombinant products begins around 48 h of cultivation, therefore, samples taken at the earlier time points were not analysed.

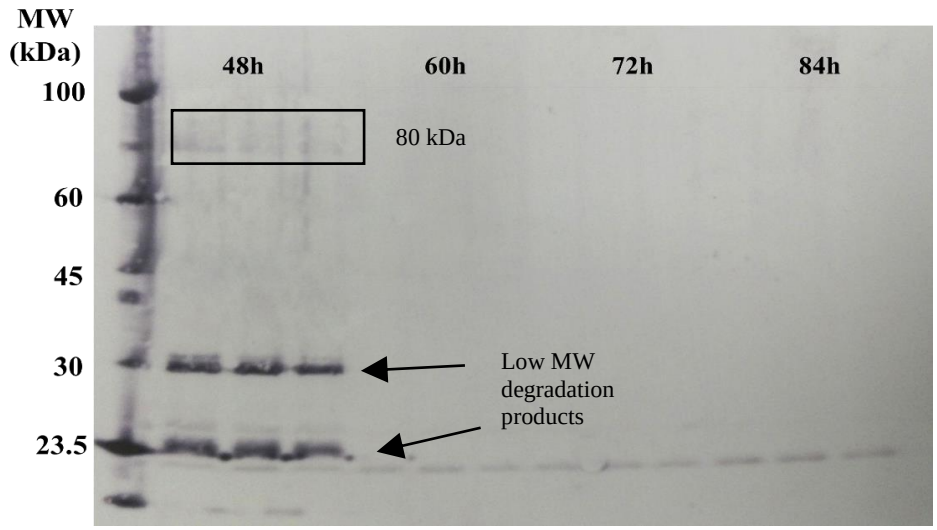


Figure 4.2. Western blot detection of Strep-tagged proteins in culture supernatants of the CMF3-44 transformant without pH adjustment. An 80 kDa recombinant protein (rectangular box) was only observed at 48 h of cultivation. Number of low MW recombinant proteins (indicated by arrows) also decreased after 48 h of cultivation.

An 80 kDa recombinant protein was observed at 48 h in all biological replicates, and at 60 h,

the recombinant protein was completely degraded, similarly to the previous experiment (Figure 4.1) where 80 kDa recombinant proteins produced by the CMF3-44 transformant were degraded and low MW proteins also exhibited signs of degradation after 72 h of cultivation.

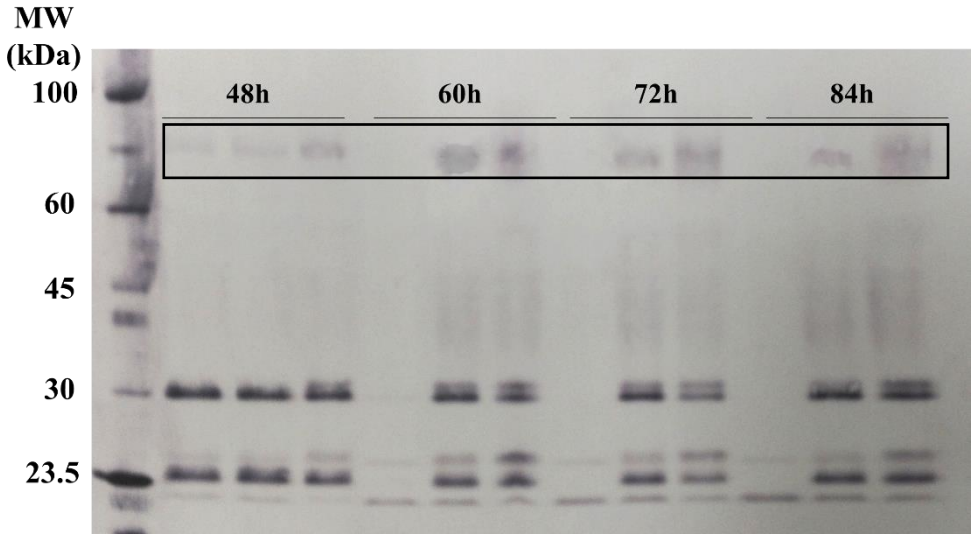


Figure 4.3. Western blot detection of Strep-tagged proteins in culture supernatants of the CMF3-44 transformant in cultures with pH adjustment. An 80 kDa recombinant proteins observed throughout the cultivation, are indicated by a rectangle.

For the cultures with pH control (Figure 4.3), the results correlated among the biological replicates with the exception of one culture that was accidentally adjusted to pH 12 at 48 h, which may have caused growth inhibition or increase in production of alkaline proteases. High (80 kDa) recombinant proteins were detected in the culture supernatants up to day 4 of cultivation (84 h). Low MW (30 and 25 kDa) recombinant proteins were also observed in pH controlled samples indicating that some degradation did occur despite pH adjustment. The pH curves of the CMF3-44 cultures with and without pH adjustment are provided in Figure 4.4 (B).

To further support the findings and compare the effect of the pH on the stability of the recombinant CMF3 and CMF4 proteins, transformant CMF4-31 was grown with and without pH adjustment as described in section 4.2.2. In the control cultures, pH gradually decreased from 6.5 (pH of the culture medium before inoculation) to below 5 (Figure 4.4, A).

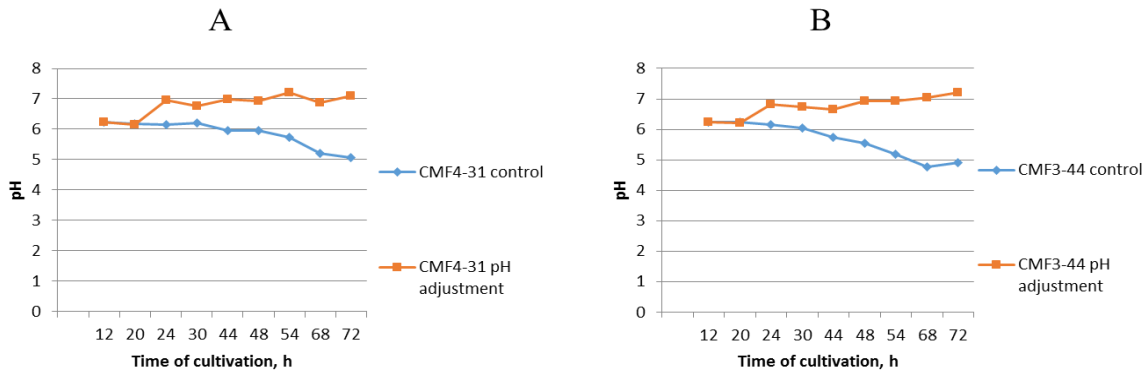


Figure 4.4. pH profiles of CMF4-31 (**A**) and CMF3-44 (**B**) cultures grown with and without pH adjustment (control).

Equal volumetric amounts of the culture supernatants were analysed in Western blots with Strep-Tactin AP conjugate (Figure 4.5). Transformant CMF4-31, which expressed recombinant FUT4 protein in fusion with the mCherry protein and CBH1 carrier, exerting high mCherry fluorescence, behaved similarly to transformant CMF3-44 when grown at the neutral pH (Figure 4.3). Secretion of the recombinant proteins in the culture without pH control started around 48 h of cultivation while in a culture with pH adjustment secretion was delayed or, possibly, the concentration of the recombinant proteins was below the detection level.

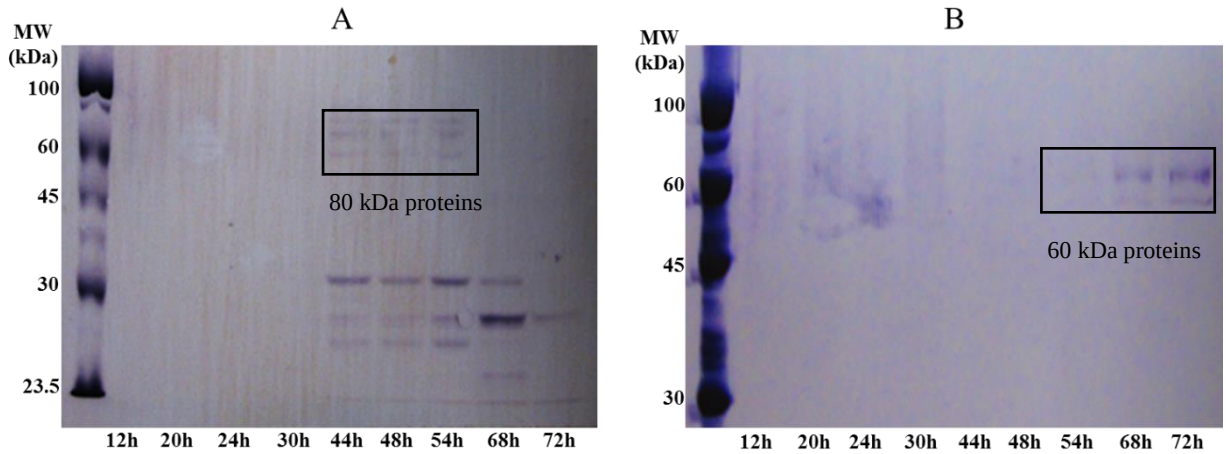


Figure 4.5. Western blot detection of Strep-tagged proteins in culture supernatants of the CMF4-31 transformant. (**A)** control culture without pH adjustment; (**B)** pH adjusted to 7.0. High MW proteins detected in both cultures at different time points are indicated by rectangles.

Overall, recombinant proteins were more stable at pH 7.0 and, similarly to the previous experiment, were detected in the culture supernatant even after 72 h of cultivation, although the size of the proteins was different (bands of about 80 kDa in a culture without pH adjustment and 60 kDa bands in a culture without pH adjustment). Also, low MW recombinant proteins were not observed in the culture with pH adjustment. It is possible that the FUT4 protein, unlike FUT3, was susceptible to the degradation by the neutral or alkaline proteases, which were induced by the neutral pH of the culture.

4.3.3 pH- and time-dependent degradation of recombinant proteins produced by the non-mCherry CF3 transformants

Transformants CF3-4 and CF3-8, which expressed the FUT3 protein without mCherry, were analysed to identify the optimal time of cultivation for the production of recombinant fusion FUT3 and also to study the effect of pH of the growing culture on the stability of the recombinant proteins. Without pH adjustment, the pH in both cultures gradually decreased from 6.5 (pH of the culture medium before inoculation) to 5 (Figure 4.6). The pH in the experimental cultures was kept between 6.5 and 7.0 as described in section 4.2.2.

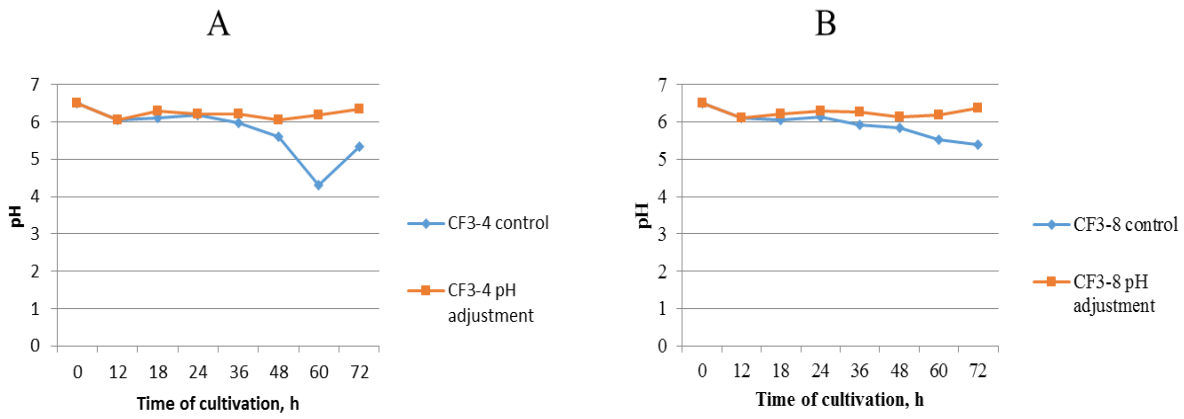


Figure 4.6. pH profiles of the CF3-4 (**A**) and CF3-8 (**B**) cultures grown without pH adjustment (control) and with pH adjustment.

Samples were taken every 6-12 hours and equal volumetric amounts of the culture supernatants were analysed by Western blotting with the Strep-Tactin AP conjugate. Results of the experiments for the transformant CF3-4 are shown in Figure 4.7. As described in section 3.3.2 (Figure 3.12), CF3-4 secreted a truncated recombinant protein (about 45 kDa). Recombinant proteins of the same size were observed in both the control and pH adjusted cultures. Recombinant products in both cultures were detected in the culture supernatants at around 36 hours of cultivation. In the control culture without pH adjustment, degradation of the recombinant products occurred at some point between 48 and 60 hours of cultivation, which corresponds to the results obtained for CMF3 and CMF4 transformants in section 4.3.2. When the pH was adjusted to 7.0, recombinant proteins remained stable up to 72 hours of cultivation.

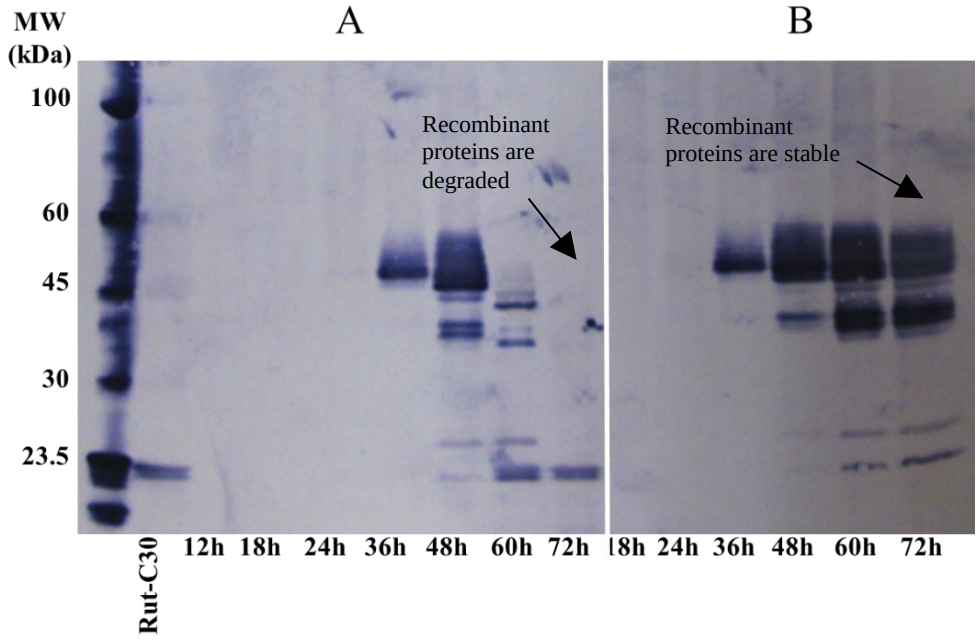


Figure 4.7. Western blot detection of Strep-tagged proteins in the culture supernatants of the CF3-4 transformant. (**A)** control culture without pH adjustment; (**B)** pH adjusted to 7.0.

Slightly different results were obtained for the transformant CF3-8. Unlike CF3-4, this transformant secreted both full-sized (about 80 kDa) and truncated (45 kDa) recombinant proteins. Similarly to CF3-4, these protein were detected in the culture supernatants at 36 hours

of cultivation and signs of degradation were visible after 60 hours of cultivation (Figure 4.8).

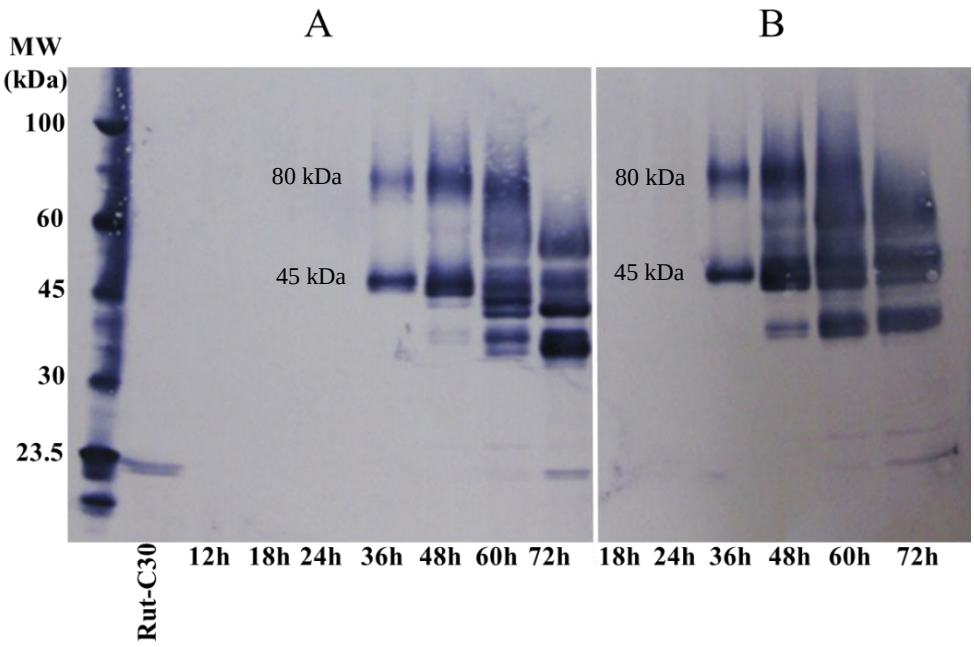


Figure 4.8. Western blot detection of Strep-tagged proteins in culture supernatants of the CF3-8 transformant. (**A)** control culture without pH adjustment; (**B)** pH adjusted to 7.0.

The effect of pH shift was less prominent for the CF3-8 than for transformants CMF3-44 and CMF4-31, which implies that stability of a shorter fusion FUT3 protein without the mCherry subunit was less affected by acidic conditions. Also, recombinant proteins produced by the CF3-8 strain seem to be slightly more stable: although proteolytic degradation took place after 48 hours into cultivation, recombinant proteins close to the size of 80 kDa were still detected even after 60 hours of cultivation (Figure 4.8, B).

4.3.4 Analysis of the intracellular and secreted proteins of the CMF3-44 transformant

An assumption was made in Chapter 3 that initial cleavage of the fusion proteins occurred inside the cell or during the secretion. To further investigate this hypothesis, extracellular and intracellular proteins of the CMF3-44 transformant were analysed by Western-blotting with Strep-Tactin AP conjugate and the fluorescence of the mCherry protein was measured (Figure 4.9).

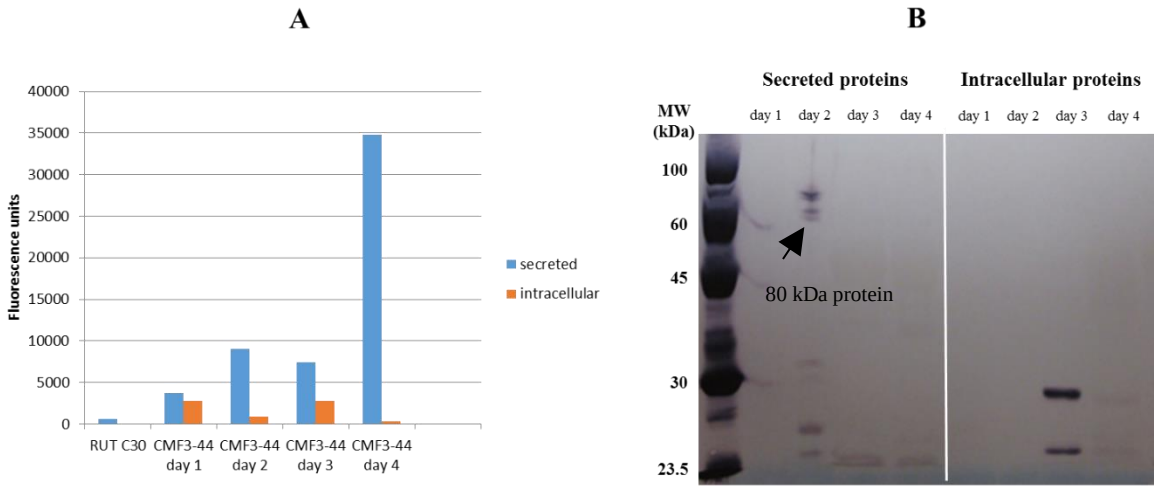


Figure 4.9. mCherry fluorescence (**A**) and Strep-tagged proteins (**B**) detected by Western blotting in the culture supernatants and in the soluble intracellular protein fraction of the CMF3-44 transformant over the course of four days.

The intensity of the mCherry fluorescence in the culture supernatants of the CMF3-44 cultures gradually increased throughout four days of cultivation (Figure 4.9, A), as a result of secretion and accumulation of the mCherry protein in the culture medium. Fluorescence measured in the soluble intracellular protein fraction was low/undetectable, and no pattern was observed. This might indicate that there was no accumulation of the fusion protein in the secretory pathway. Strep-tagged proteins, including an 80 kDa protein, were detected in the culture supernatants on day 2 (Figure 4.9, B), similarly to the previous experiments (section 4.3.2). No high MW recombinant proteins were detected in the intracellular protein fraction, and 30 kDa and 25 kDa proteins were detected on day 3 and 4 (Figure 4.9, B). The presence of the 30 kDa and 25 kDa recombinant proteins in the intracellular protein fraction could indicate that the initial cleavage occurs inside the cell and later on secreted proteins are further degraded by extracellular proteases. Co-localisation of the mCherry protein and the ER was investigated by confocal microscopy (Figure 4.10).

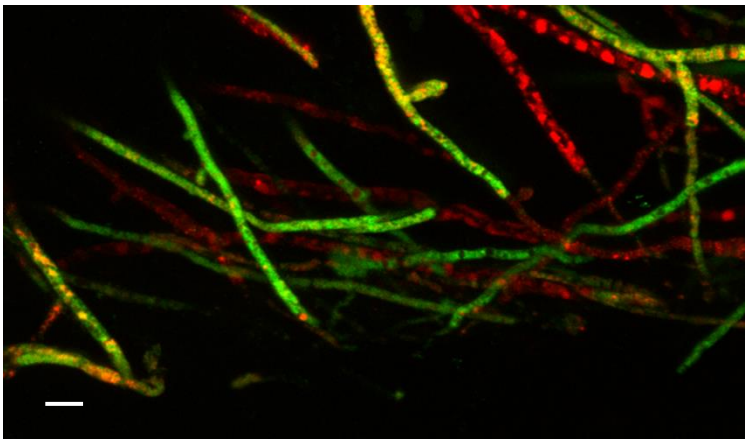


Figure 4.10. Confocal microscopy image of hyphae of the CMF3-44 transformant cultivated for 48 h. The green colour indicates the endoplasmic reticulum (ER) stained with ER-Tracker™ Green BODIPY® FL Glibenclamide (Life Technologies, Australia). The red colour indicates mCherry fluorescence visualised in the hyphae. Co-localisation of the mCherry protein and the ER is shown in yellow. The staining and microscopy were performed at Macquarie University Microscopy Facility. Scale bar is 10 μm.

Confocal microscopy demonstrated co-localisation of the mCherry fluorescent tag and the ER at 48 h of cultivation. Accummulation of the mCherry protein outside of the ER (bright red spots) may indicate that some recombinant proteins were not directed into the secretory pathway. The presence of low MW Strep-tagged proteins (which were identified as products of the cleavage of the mCherry protein in Chapter 5) suggests that this particular cleavage occurs inside the cell.

4.4 Summative discussion

Cultivation experiments revealed similarities and also some differences between the *T. reesei* transformants producing smaller (CF3) and larger (CMF3 and CMF4) fusion proteins.

It was demonstrated that the optimal time of cultivation for all tested transformants was around 48 hours with a view pf obtaining a good yield of recombinant protein. While Western blotting does not allow to precisely compare the yield of recombinant proteins produced by different strains or in different conditions, it seemed that the CF3 transformants secreted higher amounts of recombinant proteins and that secretion began at an earlier stage of cultivation, 36 hours compared to 48 hours for the CMF3 and CMF4 transformants. Recombinant proteins produced

by CF3 transformants were also slightly less prone to time-dependent degradation, as an about 80 kDa protein, corresponding to the full-sized fusion FUT3 protein, was detected in the culture supernatants even after 60 h of cultivation (Figure 4.8).

Recombinant proteins produced by the CMF3-44, CMF4-31, CF3-4 and CF3-8 selected for screening were less degraded when transformants were grown at a neutral pH compared to the non-adjusted conditions which could indicate that extracellular degradation of the recombinant proteins was at least partially caused by acidic proteases.

Transformants CMF4-31 and CMF3-44 behaved similarly in the pH control experiments. Both types of transformants produced fusion proteins containing mCherry, which seemed to be more prone to time-dependent degradation; in cultures without pH control, little or no recombinant proteins were detected after 60 h of cultivation (Figure 4.3 and 4.5). The effect of pH control was also more prominent for proteins produced by CMF3 and CMF4 transformants containing mCherry than for the smaller CF3 proteins. It has been hypothesised that closer proximity of the CBH1 core and linker carrier protein to a heterologous protein can protect it from degradation (Nyyssönen & Keränen, 1995). Also, there might be additional protease cleavage sites in mCherry and linker regions. Although the mCherry protein is stable in *T. reesei* supernatants (Sun, 2008) it is reported to have a cleavage site at the N-terminus (Huang et al., 2014), which does not affect its fluorescent properties but could result in proteolysis of the fusion protein.

CF3 transformants without mCherry exhibited similar results to CMF3 transformants in terms of the pH shift: recombinant proteins produced by the CF3 transformants were more stable in the cultures with neutral pH, thus supporting the hypothesis of acidic proteases playing a role in the degradation of the recombinant FUT3.

Considering the low number of CMF4 transformants, the absence of positive CF4

transformants and also unavailability of a commercial FUT4 enzyme to be used as a positive control in activity assays and Western blots, it was decided to focus on the transformants CMF3-44 and CF3-8 expressing the recombinant FUT3 protein in further experiments.

5

PURIFICATION AND ANALYSES OF PURIFIED PROTEINS

5.1 Introduction

Choosing an appropriate purification tag is an important step in designing a recombinant protein production, especially in the case of proteins with potential biomedical applications where purity is crucial. The cost of the purification process also plays a role in the selection of the method of purification. Purification process should occur under mild conditions, and a tag must be compatible with *T. reesei* medium and growth conditions.

Strep-tag II, used in this study, has been used successfully used in *T. reesei* in earlier research for the purification of lipase from *Dipodascus capitatus* and human α-galactosidase A (Pakula et al., 2016, Smith et al., 2014). It is stable in *T. reesei* and due to the small size (8 a.a) does not interfere with the folding and activity of the protein it is attached to, so removal of the tag after purification may not be necessary, depending on the intended application. Strep-tag II is well suited for the analysis of functional proteins because the purification procedure can be carried out under physiological conditions.

Observations made in the previous chapters pointed to the possibility that the recombinant FUT3 protein was degraded by acidic proteases of *T. reesei* and that such degradation was not random but rather occurred at specific cleavage sites (section 4.3.1). Identification of such proteases and their cleavage sites is a necessary step in order to improve the yields of the recombinant FUT3 and would be beneficial not only for this project but also for the production of other heterologous proteins in *T. reesei*. The information obtained could be used to design an appropriate protease deficient production strain or to modify expression cassettes for the expression of heterologous proteins in order to remove known cleavage sites.

In this chapter, recombinant Strep-tagged FUT3 proteins were purified from the culture supernatants of the CMF3-44 and CF3-8 transformants. After purification, components of the fusion protein (mCherry protein, CBH1 core and linker protein, Strep-tag II and FUT3 protein)

were identified by Western blotting with respective antibodies. N-terminal sequencing of the recombinant proteins purified from the culture supernatant of the CMF3-44 transformant was carried out in order to identify specific protease cleavage sites. Purified proteins were also used in protease inhibition studies (section 6.3.1), and enzymatic activity of the recombinant FUT3 was analysed.

5.2 Materials and Methods

5.2.1 Purification of Strep-tagged recombinant proteins

The transformants CMF3-44 and CF3-8 were grown for 48 h in shake flasks containing 50 ml CLS to induce the *cbh1* promoter (4 flasks per each strain). Cultures were centrifuged two times for 20 min at 5000× g to remove fine particles that can block purification columns. Obtained supernatants (approximately 40 ml from 50 ml culture) were loaded on a gravity flow column containing 5 ml Strep-Tactin Sepharose or Strep-Tactin Superflow HC as described in section 2.10.

5.2.2 N-terminal sequencing of the CMF3-44 proteins

Previous experiments (section 4.3.1) suggested that degradation of the recombinant proteins was not random but rather occurred at certain cleavage sites. In order to identify specific protease cleavage sites, recombinant proteins purified from the culture supernatant of the CMF3-44 transformant were subjected to N-terminal sequencing. Purified proteins were run on an SDS-PAGE gel, transferred onto PVDF membrane and stained with Coomassie R-250 (section 2.11). Four major bands of the size about 80 kDa (double band), 35 kDa and 25 kDa were cut out from the membrane and sent for sequencing to Australian Proteome Analysis Facility (APAF) (www.proteome.org.au).

5.2.3 Activity assays

Glycosyltransferase activity kit (R&D Systems, cat# EA001) was used to determine the activity of the recombinant proteins, purified from the culture supernatants of the CMF3-44 and CF3-8 transformants. The principle of the activity assay is shown in Figure 5.1.

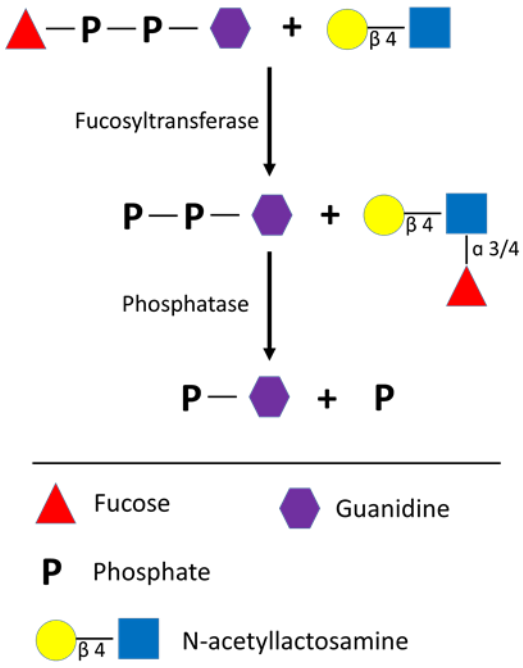


Figure 5.1. Principle of the fucosyltransferase activity assay. After the fucose molecule is transferred from GDP-fucose to the acceptor substrate, a phosphatase cleaves one inorganic phosphate from the guanidine-diphosphate. The amount of phosphate is then detected in a colorimetric reaction with Malachite Green Reagents. Modified from www.rndsystems.com

Activity assay was performed as described in the product datasheet (https://resources.rndsystems.com/pdfs/datasheets/ea001.pdf). Briefly, working solutions of donor and acceptor substrates (10 µl each) were mixed with coupling phosphatase 1 (5 µl). The reaction was initiated by addition of a recombinant protein or a commercial fucosyltransferase 3 (25 µl). Assay buffer was used in the negative controls in place of the fucosyltransferase enzyme. After incubation at 37°C, Malachite Green Reagents A and B (30 µl each) were added to the reaction. Next, after incubation at room temperature for 20 min, OD 620 was measured and adjusted by substracting the reading of the negative control.

Coupling phosphatase 1, assay buffer, phosphate standard and Malachite Green Reagents were included in the kit. Positive control and donor and acceptor substrates were purchased separately. Recombinant human fucosyltransferase 3 (R&D Systems, cat# 4950-GT) expressed in a mouse myeloma cell line, was used as a positive control. Five mM human IgG and 2.4 mM lactosamine were used as the acceptor substrates. GDP-Fucose (240 uM – 2.5 mM) was used as a donor substrate. Activity assays were carried out at 37°C for 20 h (with IgG as a substrate) and for 2 h (with lactosamine). All samples were analysed in duplicates, as per manufacturer's recommendations.

5.3 Results and Discussion

Samples taken before and during purification of recombinant proteins from the CMF3-44 and CF3-8 cultures were analysed by Western blotting with Strep-Tactin AP conjugate (Figure 5.2). In both cases, flow-through and wash fractions contained high amounts of recombinant proteins, which suggests that the amount of recombinant Strep-tagged proteins in the sample was higher than the binding capacity of the purification column. The binding capacity of the Strep-Tactin Sepharose resin is 50 to 100 nmol of recombinant Strep-tagged protein per 1 ml of resin (up to 8 mg in case of an 80 kDa protein). The high amount of recombinant FUT3 in the flow through fraction indicated that not all the protein was bound to the column and that the binding capacity was indeed, too low. The choice of the column was based on the existing information about the yields of heterologous mammalian proteins in *T. reesei* which were usually below 100 mg/l (see Table 1.6). It is also possible that the conditions of the purification process such as pH of the buffers and the flow speed, required optimisation. However, this aspect was not studied further as the obtained amounts of the recombinant proteins were sufficient for their analyses, and optimisation of the purification process did not fit into the time frame of the project.

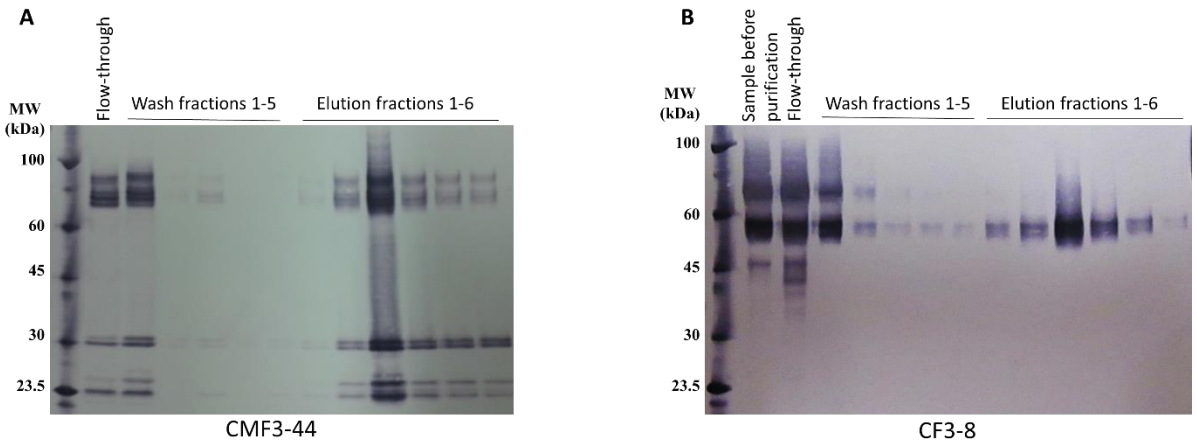


Figure 5.2. Western blot analysis of samples taken during purification of the recombinant proteins produced by the CMF3-44 (**A**) and CF3-8 (**B**) transformants. The presence of unbound Strep-tagged proteins in the flow-through and wash fractions indicates that the amount of Strep-tagged proteins in the 40 ml of culture supernatant applied on the column was higher than the binding capacity of the column.

Elution fractions were pooled together, dialysed against water overnight at 4°C and concentrated in Speedvac. The concentration of the purified proteins was measured, and the total amount of the recombinant Strep-tagged protein purified from 200 ml of the CMF3-44 culture supernatant was calculated to be 7 mg (45 mg per 1 litre of the culture). Taking into account the amount of unbound proteins in the flow-through and wash fractions, the overall yield of recombinant FUT3 was estimated to be close to 70 mg/l level. The yield of recombinant proteins from the CF3-8 culture was lower (approximately 40 mg/l). Purified and concentrated proteins analysed by Western blotting with the Strep-Tactin AP conjugate are shown in Figure 5.3:

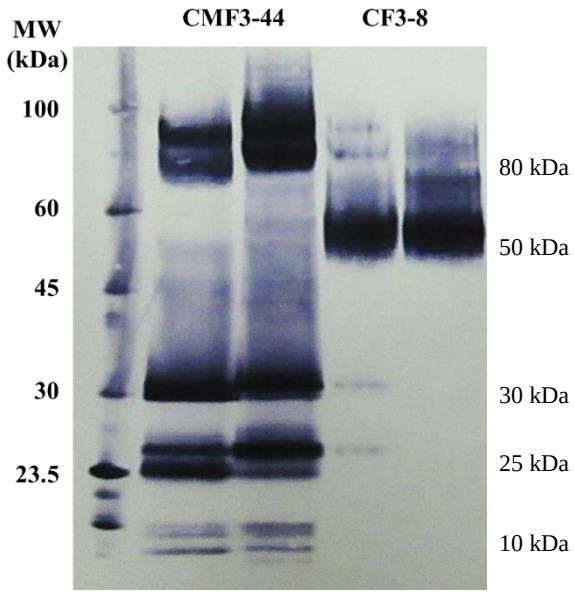


Figure 5.3. Concentrated recombinant proteins (purified from the culture supernatants of the CMF3-44 and CF3-8 transformants) obtained in two rounds of purification. Approximately 0.2 µg of purified protein was loaded in each well; detection was performed by Western blotting with the Strep-Tactin AP conjugate.

After purification of the Strep-tagged proteins from the culture supernatant of the CMF3-44 transformant, five major bands (double bands of 80 and 25 kDa and a 30 kDa band) were detected by Western blotting (Figure 5.3) together with fainter bands of approximately 10 kDa. In the fraction of proteins purified from the CF3-8 transformant, a 50 kDa band dominated, and less intense bands of about 80 kDa were observed, similarly to the bands detected in the culture supernatants of the respective transformants (Figure 4.1 and 4.8). Even though recombinant Strep-tagged proteins were shown to exist as multiple truncated products of different size, the fraction of Strep-tagged proteins, purified from the culture supernatants, will be referred to as "CMF3-44 protein" and "CF3-8 protein" in the following chapters.

5.3.1 Identification of the components of the recombinant protein in Western blots

Identification of the FUT3 protein in culture supernatants in the earlier analyses using an antibody raised against human fucosyltransferase 3 (Abcam, #ab102844) was not completely successful and did not give reliable and reproducible results perhaps due to a low affinity of

the antibody to recombinant FUT3 or low concentration of the FUT3 in the culture supernatant. Here, a new anti-FUT3 antibody (Santa Cruz Biotechnology cat# sc-14874) was used to confirm the presence of the FUT3 component in the proteins obtained by the purification using the Strep-Tactin Sepharose column and provide support to the earlier assumption that the larger Strep-tagged recombinant proteins also contained FUT3 (Chapter 3). Other components of the fusion FUT3 protein were also identified to shed some light on the compositions of the degradation products observed throughout the study.

The components of the recombinant protein produced by the CMF3-44 transformant: Strep-tag II (1.1 kDa), CBH1 carrier protein (47.8 kDa), mCherry (26.7 kDa) and FUT3 (38.2 kDa) were detected in Western blots with respective antibodies (section 2.9). The two bands of around 80 kDa were seen in all Western blots including the Western with a specific anti-FUT3 antibody (Santa Cruz Biotechnology cat# sc-14874) raised against a peptide mapping near the C-terminus of human fucosyltransferase 3. This further confirms the earlier hypothesis (Chapter 3) that Western blotting with the Strep-Tactin AP conjugate did detect the recombinant FUT3 protein and that the tag had not been removed from some forms of the fusion protein.

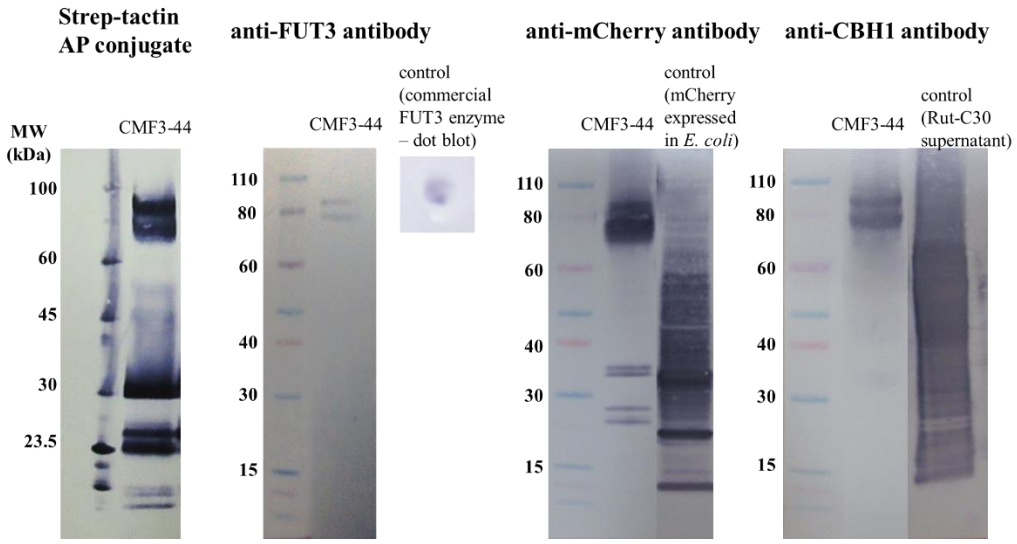


Figure 5.4. Detection of the different components of the Strep-tagged fusion proteins purified from the CMF3-44 culture supernatants by Western blotting. Approximately 0.2 µg of the purified protein was analysed in each blot. Positive controls used were: Strep-tag ladder, commercial FUT3 enzyme (0.5 µg), cell lysate of *E. coli* expressing the mCherry protein and Rut-C30 (expression host) culture supernatant.

The 35 and 25 kDa bands were detected only in Western blots with Strep-Tactin AP conjugate and anti-mCherry antibody (Figure 5.4), which allows to hypothesise about the location of possible cleavage sites. It also showed that previous analysis of the culture supernatants with the Srep-tactin AP conjugate detected degraded proteins that did not contain FUT3.

The structure of the CMF3 protein and theoretical molecular weights of its components are shown in Figure 5.5. The anti-FUT3 antibody targets the C-terminus of human FUT3 protein so it can be assumed that the 80 kDa bands contained intact FUT3. The anti-CBH1 antibody is polyclonal and therefore can detect even short/truncated fragments of the CBH1 core and linker protein which allows to assume with a certain level of caution that the fusion protein was N-terminally cleaved somewhere in the CBH1 core/linker region, leaving enough of CBH1 to be detected. Nyyssönen and Keränen (1995) described production of the heavy Fd chain of the Fab antibody in fusion with the CBH1 core-linker protein by *T. reesei* and reported that the CBH1 carrier was intracellularly cleaved from its fusion partner by an unidentified protease, which could also happen to the FUT3 protein produced in fusion with the CBH1

core-linker protein.

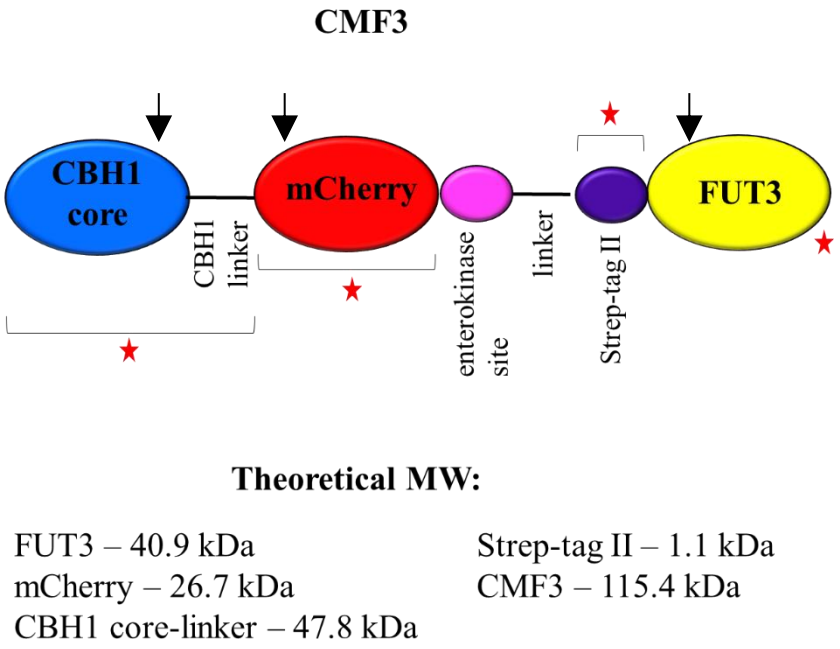


Figure 5.5. Schematic structure of the CMF3 protein and molecular weights of its components. Arrows indicate possible protease cleavage sites. Antibody binding sites are indicated by stars.

The low molecular weight proteins bands of about 35 kDa and 25 kDa obtained from CMF3-44 transformant contain the Strep-tag II and the mCherry protein; therefore, by knowing that mCherry is 26.7 kDa, and the Strep-tag II, linker and the enterokinase cleavage site are 20 a.a. in total and do not add much to the size of a protein, we can hypothesise that there might be protease cleavage sites located at the N-terminal region of the FUT3 or N-terminal region of the mCherry (Figure 5.5).

Western blot analyses of the recombinant proteins purified from the culture supernatant of the transformant CF3-8 demonstrated that two protein bands above and below 80 kDa contained the Strep-tag II, FUT3 and the CBH1 carrier (Figure 5.6). A band of approximately 50 kDa only contained the Strep-tag II and the CBH1 carrier.

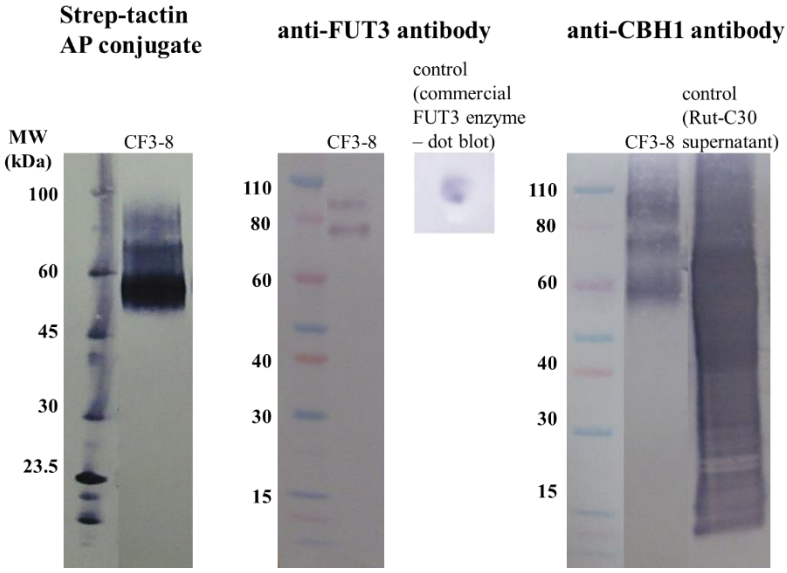
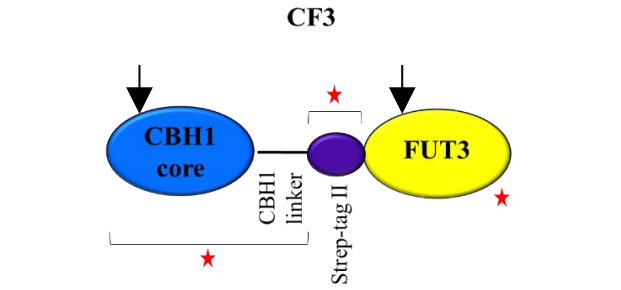


Figure 5.6. Detection of the different components of the recombinant fusion protein produced by the CF3-8 transformant by Western blotting. Approximately 0.2 µg of the purified protein was analysed in each blot. Positive controls used were: Strep-tag ladder, commercial FUT3 enzyme (0.5 µg), and Rut-C30 culture supernatant.

As discussed above, possible proteolytic cleavage sites could be located within the CBH1 core-linker protein and at the N-terminus of the FUT3 protein; therefore, two bands of approximately 80 kDa containing the Strep-tag II, CBH1 and FUT3 could be products of the cleavage within the CBH1 protein. A 50 kDa band only containing the Strep-tag II and CBH1 could be a result of the cleavage at the N-terminus of FUT3, as shown in Figure 5.7.



Theoretical MW:

FUT3 – 40.9 kDa
mCherry – 26.7 kDa
CBH1 core-linker – 47.8 kDa
Strep-tag II – 1.1 kDa
CF3 – 88.7 kDa

Figure 5.7. Schematic structure of the CF3 protein and molecular weights of the components. Arrows indicate possible protease cleavage sites. Antibody binding sites are indicated with stars.

It appears that the recombinant proteins produced by transformants CMF3-44 and CF3-8 are cleaved at different cleavage sites within the CBH1 carrier protein. While the recombinant fusion protein produced by the CMF3-44 strain (full size 115.4 kDa) was hypothesised to be cleaved at the C-terminus of the CBH1 core-linker resulting in 80 kDa proteins, the CF3-8 made protein (full size 88.7 kDa) was possibly cleaved at the N-terminus of the CBH1 core-linker, and such cleavage produced proteins of approximately 80 kDa. Such differences could be a result of differences in the folding of the CMF3 and CF3 recombinant proteins as the cleavage sites could be exposed to proteases or protected from the proteolysis by domains of the fusion protein.

5.3.2 N-terminal sequencing of the purified recombinant proteins produced by the CMF3-44 transformant

Four major protein bands purified from the culture supernatant of the CMF3-44 transformant (section 5.3) were subjected to N-terminal sequencing in order to specify the position where the protease cleavage occured (Figure 5.5). N-terminal sequencing requires at least 2 picomoles of protein (0.16 µg in the case of an 80 kDa protein and 0.06 µg in the case of a 30 kDa protein). At least five major bands were observed in the fraction of Strep-tagged proteins purified from the CMF3-44 transformant (Figure 5.8). To ensure that each protein to be sequenced was present in sufficient concentration, a total of 5.6 µg of the purified recombinant protein was used to prepare samples for the N-terminal sequencing.

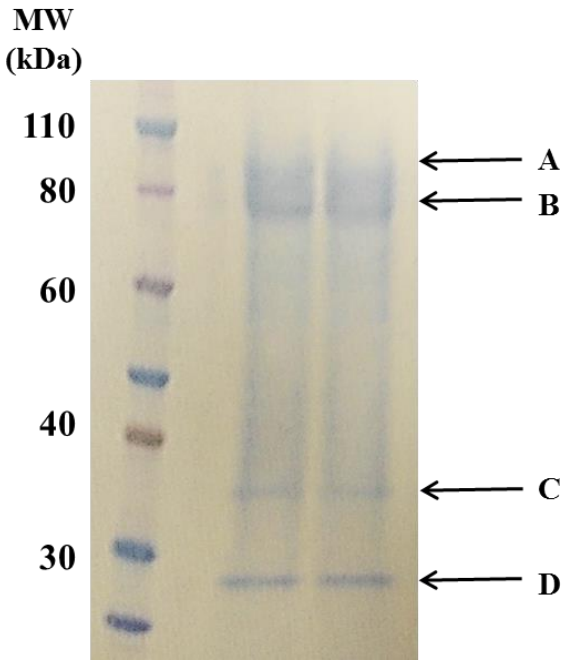


Figure 5.8. Recombinant proteins purified from the culture supernatant of the CMF3-44 transformant, blotted onto a PVDF membrane and stained with Coomassie R-250 prior to N-terminal sequencing. Protein bands to be sequenced are indicated with letters from A-D and pointed by arrows.

Out of the four analysed proteins, two 80 kDa forms (Figure 5.8, A and B), were N-terminally blocked which made the N-terminal sequencing impossible at this point of experimental work. Protein with the lowest molecular weight (~25 kDa, Figure 5.8, D) gave a very low signal possibly due to an insufficient sample concentration, and no reliable sequence was recorded. Sequencing of the protein band C (Figure 5.8) with MW of ~ 35 kDa was successful and the amino acid sequence GEEDN was obtained. This sequence was found at the N-terminus of the mCherry protein (Figure 5.9).

MVSKGEEDNMAIIKEFMRFKVHMEGSVNGHEFEIEGEGEGRPYEGTQTAKLKV
TKGGPLPFAWDILSPQFMYGSKAYVKHPADIPDYLKLSFPEGFKWERVMNFEDG
GVVTVTQDSSLQDGEFIYKVKLRGTNFPSDGPVMQKKTMGWEASSERMYPED
GALKGEIKQRLKLKDGGHYDAEVKTTYKAKKPVQLPAYNVNIKLDITSHNEDYTIV
EQYERAEGRHSTGGMDELYK

Figure 5.9. Amino acid sequence of the mCherry fluorescent protein. Amino acids identified by N-terminal sequencing are highlighted in green.

Huang et al. (2014) studied the degradation of the mCherry fluorescent tag by the acidic

proteases in lysosomes of mammalian cell lines. Among other findings, they demonstrated that removal of the 11 N-terminal residues of mCherry (MVSKGEEDNMA) generated a cleavage-resistant fluorescent protein. N-terminal sequencing of the proteins purified from the CMF3-44 transformant demonstrated cleavage of the mCherry protein in the same region, so it is possible that the cleavage was caused by the same class of proteases targeting the same amino acid sequence. Results of Western blotting (Figure 5.4) and N-terminal sequencing are summarised in Table 5.1:

Table 5.1. Summary of analyses of the recombinant proteins purified from the CMF3-44 transformant.

Protein size	**WB identification**				**Theoretical cleavage sites**	**N-terminal sequencing results**
	Strep-tag II	**CBH1**	**FUT3**	**mCherry**		
~ 80 kDa (double band)	+	+	+	+	CBH1 core/linker region	N-terminally blocked
~ 35 kDa	+	-	-	+	N-terminal region of FUT3 or N-terminal region of mCherry	Cleavage site identified at the N-terminus of mCherry protein
~ 25 kDa	+	-	-	+	N-teminal region of FUT3 or N-terminal region of mCherry	Low signal

Two cleavage sites (AQ and RQ) for Kex2-like peptidases of *T. reesei* have been described (de Faria et al., 2002; Miyauchi et al., 2013). One RQ site was found at the N-terminus of the FUT3 protein (Figure 5.10).

RVSRDDATGSPRAPSGSSRQDTTPTRPTLLILLRTWPFHIPVALSRCSEMVPGTAD
CHITADRKVYPQADMVIVHHWDIMSNPKSRLPPSPRPQGQRWIWFNLEPPPN
CQHLEALDRYFNLTMSYRSDSDIFTPYGWLEPWSGQPAHPPLNLSAKTELVAWA
VSNWKPDSARVRYYQSLQAHLKVDVYGRSHKPLPKGTMMETLSRYKFYLAFEN
SLHPDYITEKLWRNALEAWAVPVVLGPSRSNYERFLPPDAFIHVDDFQSPKDLAR
YLQELDKDHARYLSYFRWRETLRPRSFSWALDFCKACWKLQQESRYQTVRSIAA
WFT

Figure 5.10. Amino acid sequence of the FUT3 protein; Kex2-like peptidase cleavage site is highlighted in red.

A protein of approximately 35 kDa produced by the CMF3-44 transformant (Figure 5.8, band

C) could be a product of the cleavage by a Kex2-like protease and by an unknown protease that cleaves at the N-terminus of the mCherry protein as shown in Figure 5.11. Theoretical MW of the product of such cleavage is 30.7 kDa.

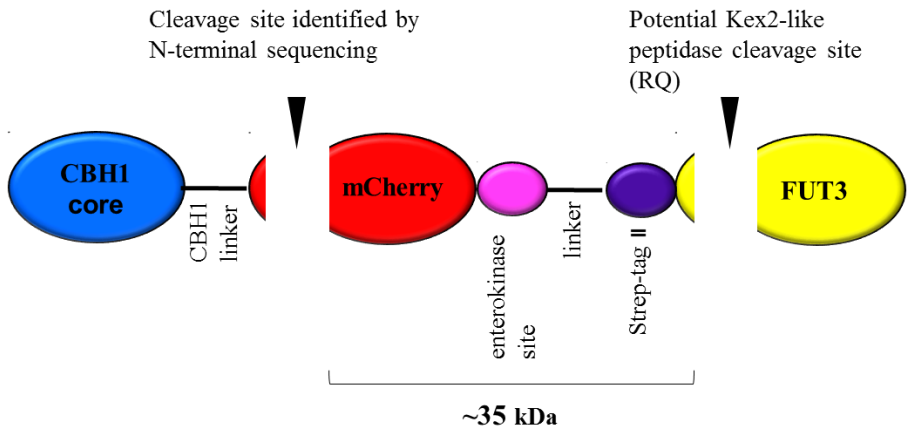


Figure 5.11. Schematic structure of the CMF3 fusion protein. Sites of protease cleavage that produces a protein of approximately 35 kDa are shown.

Obtained information corresponds with the results of Western blotting (section 5.3.1) where only the mCherry protein and Strep-tag II were detected in the ~35 kDa protein and assumptions were made about the position of the cleavage sites in the protein produced by the CMF3-44 transformant (Figure 5.5).

Three AQ cleavage sites (de Faria et al., 2002) were found in the CBH1 core-linker protein (Figure 5.12).

MYRKLAVISAFLATARAQSACTLQSETHPPLTWQKCSSGGTCTQQTGSVVIDAN
WRWTHATNSSTNCYDGNTWSSTLCPDNETCAKNCCLDGAAYASTYGVTTSGN
SLSIGFVTQSAQKNVGARLYLMASDTTYQEFTLLGNEFSFDVDVSQLPCGLNGA
LYFVSMDADGGVSKYPTNTAGAKYGTGYCDSQCPRDLKFINGQANVEGWEPS
SNNANTGIGGHGSCCSEMDIWEANSISEALTPHPCTTVGQEICEGDGCGGTYS
DNRYGGTCDPDGCDWNPYRLGNTSFYGPGSSFTLDTTKKLTVVTQFETSGAIN
RYYVQNGVTFQQPNAELGSYSGNELNDDYCTAEEAEFGGSSFSDKGGLTQFKK
ATSGGMVLVMSLWDDYYANMLWLDSTYPTNETSSTPGAVRGSCSTSSGVPAQ
VESQSPNAKVTFSNIKFGPIGSTGNPS

Figure 5.12. Amino acid sequence of the CBH1 core-linker protein; Kex2-like peptidase cleavage sites are highlighted in red.

It is possible that the proteins of about 80 kDa produced by the CMF3-44 transformant, which were hypothesised to be cleaved at the C-terminus of the CBH1 carrier protein, are a result of the cleavage by a Kex2-like peptidase, as shown in Figure 5.13.

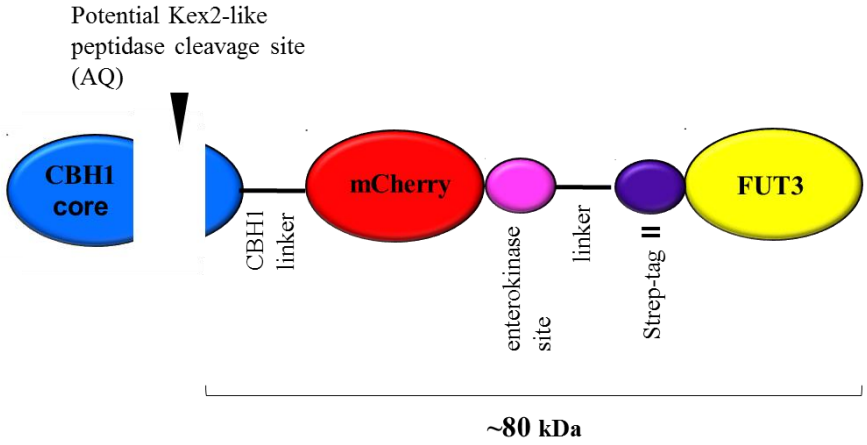


Figure 5.13. Schematic structure of the CMF3 fusion protein. A site of protease cleavage that produces a protein of approximately 80 kDa is shown.

It is estimated that approximately half of all proteins cannot be directly sequenced by Edman degradation because they have a blocked N-terminal residue. Natural causes of the N-terminal block include an N-acetyl amino acid, a glycosylated amino acid, or a pyrrolidone carboxylate group. Three *N*-glycosylation sites have been identified in the catalytic domain of the CBH1 and one of them (Asn384) is located at the C-terminus of the protein (Qi et al., 2014). *O*-glycosylation sites have been found in the linker region of the CBH1 protein (Klarskov et al., 1997; Maras et al., 1997; Harrison et al., 1998). As it was hypothesised in section 5.3.1 that two recombinant proteins of ~ 80 kDa were cleaved somewhere in the C-terminus of the CBH1 core/linker protein, glycosylation could explain the N-terminal blockage of those proteins.

The N-terminal block could also occur during protein purification as a result of sample manipulations, but given the fact that all samples were processed simultaneously and at identical conditions and only two out of four samples were found to be N-terminally blocked, it is more likely that the blockage was natural. Some N-terminal blocking groups can be removed but such procedure requires a significant amount of the protein and does not always

work because the type of blockage is mostly unknown. Attempts to remove the N-terminal blockage would require purification of large amounts of recombinant protein, which was not feasible due to the time allocated for this research.

5.3.3 Activity assays

Enzymatic activity of the recombinant proteins purified from the CMF3-44 and CF3-8 culture supernatants was analysed using a colorimetric assay (section 5.2.3). The fucosyltransferase 3 enzyme can transfer fucose in α-1,3 and α-1,4 linkages to the N-acetylglucosamine (GlcNAc) of the β-D-Gal-(1→4)-D-GlcNAc substrate (lactosamine). Two different substrates, lactosamine and human IgG, which has lactosamine structures among its glycans, were used in the activity assays. Lactosamine is a more suitable substrate for assaying the fucosyltransferase activity, but it was not available at the beginning of activity assay experiments, thus IgG was used as a substitute to start with. The assay used in this study measures total fucosyltransferase activity and does not allow to differentiate between α-1,3 and α-1,4 activities.

The assay is based on the detection of free GDP released after the transfer of fucose from GDP-Fucose to the acceptor substrate. Specific phosphatase releases inorganic phosphate from the GDP and the amount of the phosphate is detected by Malachite Green phosphate detecting reagents A and B. Enzyme activity is calculated as the amount of phosphate (pmol/min) released by one µg of the enzyme. First, the relationship between the absorbance and amount of phosphate in the reaction was established by creating a phosphate standard curve using dilutions of the phosphate standard (provided in the assay kit) (Figure 5.14).

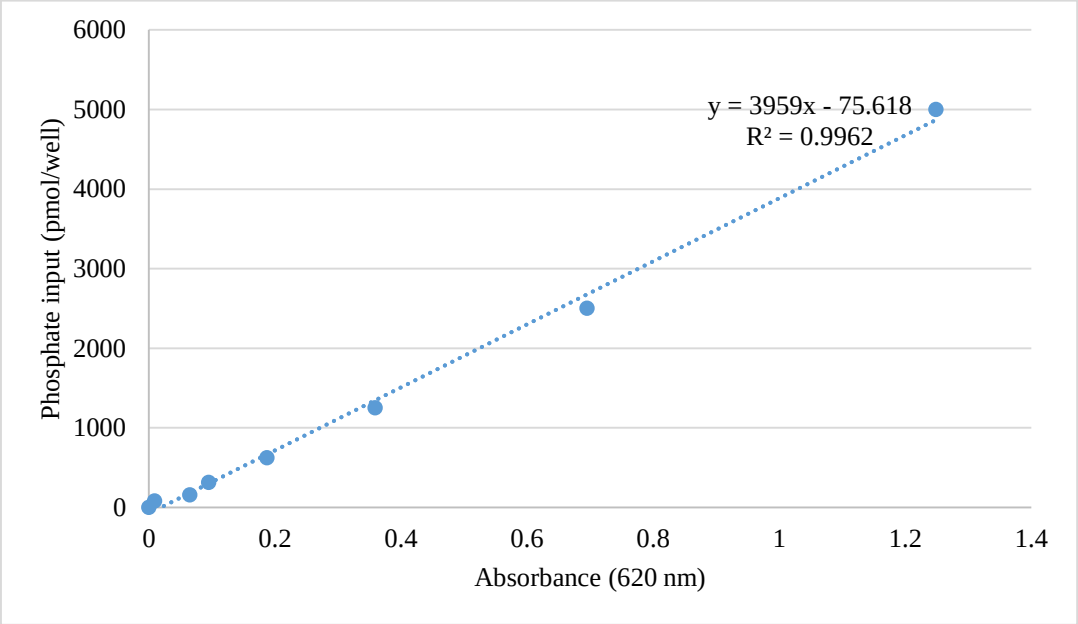


Figure 5.14. A phosphate standard curve. The slope of the linear regression line, 3959 pmol/OD, represents the amount of phosphate corresponding to a unit of absorbance at 620 nm. It is referred to as the phosphate conversion factor (CF) in subsequent calculations. Assay was performed in duplicate and average values were used to create the standard curve.

Next, the activity of the commercial FUT3 was measured according to the protocol provided by the manufacturer. The manufacturer guarantees specific activity of >25 pmol/min/µg under the described conditions (240 µM GDP-Fucose, 2.4 mM lactosamine, time of incubation – 20 min). Specific activity was calculated by the following formula: **Specific Activity** = **S** (OD/µg) x **CF** (pmol/OD)/**Time** (minutes), where S is the slope of the linear regression line (Figure 5.15) and CF is a conversion factor obtained from the phosphate standard curve (Figure 5.14). Specific activity of the commercial FUT3 enzyme was calculated to be 117 pmol/min/µg.

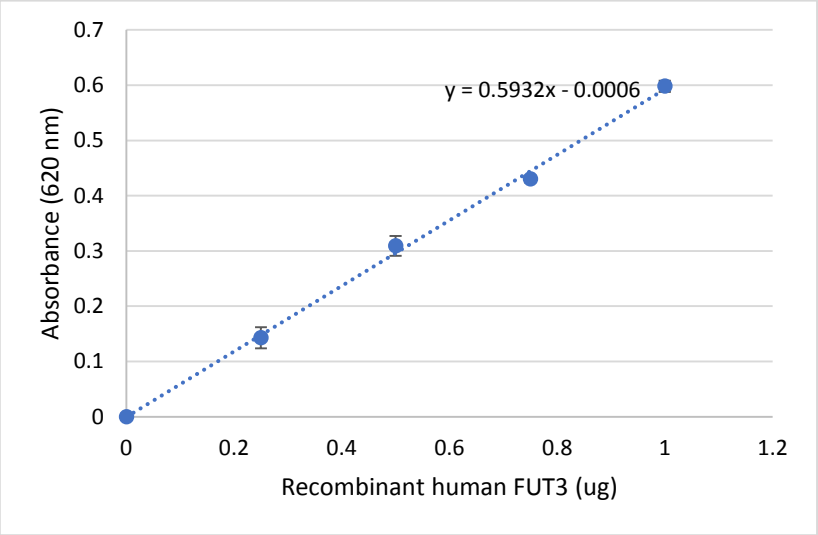


Figure 5.15. Recombinant human FUT3 (R&D Systems, cat#4950-GT) assay. Using the conversion factor of 3959 pmol/OD, the specific activity was calculated to be 117 pmol/min/µg ((0.5932 x 3959) ÷ 20). Assay was performed in duplicate and average values were used to create the curve. Error bars represent minimal and maximal values.

Activities of the CMF3-44 and CF3-8 recombinant proteins purified from the culture supernatants of the respective transformants were first assayed using IgG as a substrate. Specific activities were calculated to be 1.56 pmol/min/µg for the FUT3 from CMF3-44 and 4.15 pmol/min/µg for FUT3 from CF3-8 using the formula described above (Figure 5.16 and 5.17).

IgG has various glycan structures, only a few of them being able to accept terminal fucose; IgG was used at the highest recommended concentration (5 mM), and reactions were carried out for 20 h (maximal recommended time). However, measured activities were still very low.

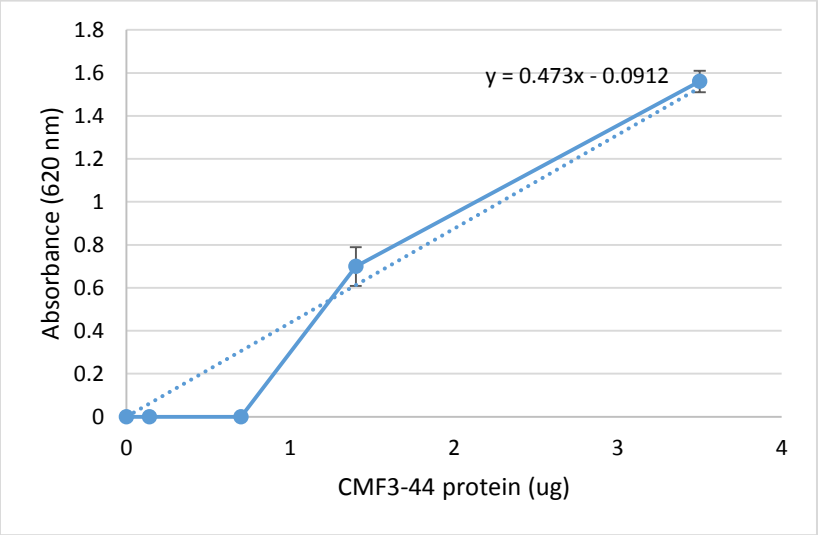


Figure 5.16. Activity assay for the CMF3-44 produced recombinant protein with IgG as a substrate. Activity: 1.56 pmol/min/µg. Assay was performed in duplicate and average values were used to create the curve. Error bars represent minimal and maximal values.

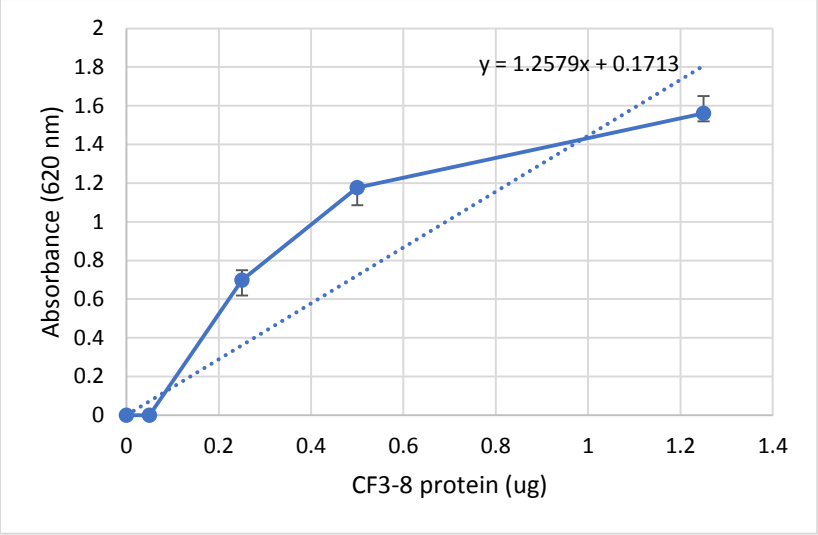


Figure 5.17. Activity assay for the CF3-8 produced recombinant protein with IgG as a substrate. Activity: 4.15 pmol/min/µg. Assay was performed in duplicate and average values were used to create the standard curve. Error bars represent minimal and maximal values.

The assay was repeated with a more suitable substrate (lactosamine) in a concentration recommended by the manufacturer (2.4 mM). Activities measured were approximately ten times higher than in assay with IgG which could be a result of higher number of acceptor structures in lactosamine. Specific activities were calculated to be 11 pmol/min/µg for the CMF3-44 and 47 pmol/min/µg for the CF3-8 protein (Figure 5.18 and 5.19).

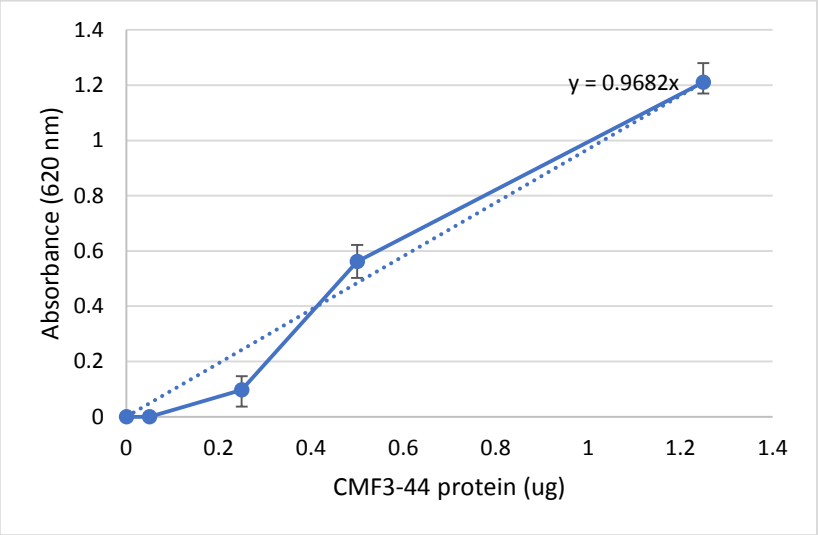


Figure 5.18 Activity assay for the CMF3-44 produced recombinant protein with lactosamine as a substrate. Activity: 11 pmol/min/µg. Assay was performed in duplicate and average values were used to create the standard curve. Error bars represent minimal and maximal values.

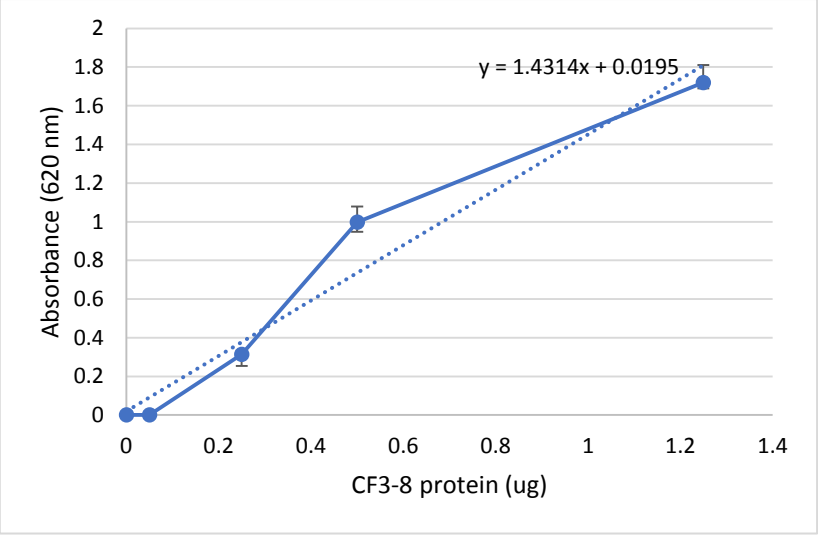


Figure 5.19. Activity assay for the CF3-8 produced recombinant protein with lactosamine as a substrate. Activity: 47 pmol/min/µg. Assay was performed in duplicate and average values were used to create the standard curve. Error bars represent minimal and maximal values.

In both assays, with IgG and lactosamine as a substrate, the CF3-8 protein demonstrated approximately four times higher activity than the CMF3-44 protein. It could be at least partially explained by different compositions of the two proteins and, consequently, different protease cleavage sites and degradation patterns. At least seven protein bands were detected in the fraction of Strep-tagged proteins purified from the CMF3-44 transformant (Figure 5.3), and only two of them were detected by the anti-FUT3 antibody (Figure 5.4). Only three major

bands were observed in the CF3-8 Strep-tag II protein fraction (Figure 5.3) and two of them were demonstrated to contain the FUT3 protein (Figure 5.6), so while equal amounts of each protein were used in activity assays, amount of the proteins containing FUT3 in the CMF3-44 samples could have been lower than in CF3-8 samples.

5.4 Summative discussion

Recombinant Strep-tagged proteins were successfully purified from the culture supernatants of the CMF3-44 and CF3-8 transformants. It turned out that not all Strep-tagged proteins present in the culture supernatants could bind to the purification resin, therefore, further optimisation of the purification technique will be required either by increasing the amount of the purification resin, using a resin with a higher binding capacity, decreasing the amount of the culture supernatant applied on the purification column or by optimising the conditions of the purification process.

Analysis of the purified proteins by Western blotting with specific antibodies targeting components of the fusion FUT3 protein demonstrated that two recombinant proteins of about 80 kDa purified from the culture supernatant of the CMF3-44 transformant contained all subunits of the fusion protein, including the FUT3 protein. The low molecular weight bands only contained the Strep-tag II and the mCherry protein. Out of the three major proteins purified from the culture supernatant of the CF3-8 transformant, two proteins of approximately 80 kDa contained all subunits of a fusion protein (CBH1 carrier, Strep-tag II and FUT3) and a protein of about 50 kDa only contained the CBH1 carrier and a Strep-tag II.

Results obtained in Western blotting with different antibodies allowed to hypothesise about the position of the protease cleavage sites; to confirm the hypothesis, four proteins purified from the CMF3-44 transformant were subjected to the N-terminal sequencing. Out of the four analysed samples, two 80 kDa proteins that contained the CBH1 core and linker, mCherry,

Strep-tag II and the FUT3 protein were N-terminally blocked which made the N-terminal sequencing impossible and one gave a very low signal due to an insufficient amount of the sample. Sequencing of the ~ 35 kDa protein was successful, and a cleavage site located at the N-terminus of the mCherry protein was identified. Enzymatic activity of the CMF3-44 and CF3-8 made recombinant proteins was measured to be 11 pmol/min/μg for the FUT3 from CMF3-44 and 47 pmol/min/μg for the FUT3 from CF3-8, which were lower than the activity of the commercial FUT3 enzyme expressed in mammalian cells (117 pmol/min/μg). This could be a result of differences in glycosylation between fucosyltransferases expressed in fungal and mammalian hosts (Deshpande et al., 2008) or a possible C-terminal cleavage of the FUT3 protein which affected the active center of the enzyme. Human fucosyltransferase 3 has two N-glycosylation sites at the C-terminus of the enzyme; while it has been demonstrated that glycosylation is necessary for the FUT3 activity (Christensen et al., 2000), specific glycan structures of the FUT3 and their possible effect on the activity of the enzyme have not been studied. Also, recombinant proteins secreted by the CMF3-44 and CF3-8 transformants seem to be degraded by extracellular proteases, and only a small fraction of purified Strep-tagged proteins contained the FUT3 protein (Figure 5.4 and 5.6), so the actual activity of the recombinant FUT3 produced in *T. reesei* could be higher. For example, recombinant Strep-tagged proteins purified from the culture supernatant of the transformant CF3-8 consist of two faint 80 kDa bands which contain the FUT3 protein and one intense 50 kDa band which does not contain FUT3 (Figure 5.3 and 5.6), and have enzymatic activity of 47 pmol/min/μg. Assuming that FUT3-containing proteins represent 50% of the total Strep-tagged proteins produced by the CF3-8 transformant, the actual enzymatic activity would be 94 pmol/min/μg which is close to the activity of the commercial FUT3 enzyme (117 pmol/min/μg). To further investigate this assumption, separation of the FUT3-containing proteins from the other Strep-tagged proteins is required, either by size-exclusion chromatography or by purification with

specific anti-FUT3 antibodies. Results of the FUT3 activity assays are preliminary and require further optimisation, however, they demonstrate that recombinant FUT3 expressed in *T. reesei* is functional.

6

PROTEASE INHIBITION STUDIES

6.1 Introduction

Observations made in Chapter 4 indicated that keeping the pH of the culture medium at 7.0 during cultivation stabilised secreted recombinant proteins and prevented or slowed down their further degradation; this suggests that such degradation was caused by proteases active at an acidic pH. To achieve better control over the production process, identification of specific proteases or classes of proteases responsible for the degradation of specific recombinant products is necessary. There are four main types of proteases: aspartic, serine, cysteine and metalloproteases according to the nature of their catalytic active site. Based on the pH optimal for their functioning, proteolytic enzymes can be characterised as alkaline, neutral or acidic proteases. At pH 5 and lower, the major protease of *T. reesei* has been shown to be an acid aspartic protease (Haab et al., 1990). When pH was kept at 6.0, Dienes et al. (2007) purified a trypsin-like alkaline serine protease from the *T. reesei* QM9414 culture supernatant.

Protease activity can be eliminated or reduced by adjustment of culture conditions (e.g. pH or carbon source), targeted deletion of protease genes or by the use of protease inhibitors. For example, in their work Zhang et al. (2014) combined the pH control strategy with disruption of a major alkaline serine protease (SWP) gene and demonstrated an increase in production of a heterologous alkaline endoglucanase EGV from *Humicola insolens* by *T. reesei*. SWP protease was found to be a subtilisin-like serine protease. Protease inhibition strategies are described in detail in sections 1.8.5 and 1.8.6.

The first step in choosing the right protease inhibition strategy is to explore cultivation conditions and identify specific proteases or classes of proteases responsible for the degradation of a particular heterologous protein. In this chapter, differences in protease profiles between the CMF3-44 cultures grown with and without pH adjustment were studied. Protease inhibitors for the major groups of proteases, aspartic and serine, were used to identify

specific groups of proteases in the *T. reesei* culture supernatant responsible for the degradation of the recombinant fusion FUT3 proteins.

6.2 Materials and Methods

6.2.1 Total protease activity assay

Total extracellular protease activity in the culture supernatants of the CMF3-44 strain grown with and without pH adjustment (section 4.3.2) and in the culture supernatant of the parental strain Rut-C30 was determined as described in Benitez et al. (2001). Briefly, 100 µl of azocasein (5 mg/ml) in 100 mM Tris-HCl buffer (pH 6.5 and 5.0) was incubated with 100 µl of culture supernatants (at an appropriate dilution) for 12 h at 28°C with continuous shaking. The reaction was stopped by the addition of 400 µl of 10% (v/v) trichloroacetic acid. The tubes were centrifuged, and the supernatant was removed to a new Eppendorf tube. An equal volume of 0.5 M NaOH was added into the tube, and the absorbance was measured at 440 nm. The assay was carried out in triplicate. The pH of the assay buffer was chosen based on the pH of the analysed sample: samples of the CMF3-44 culture without pH adjustment and the Rut-C30 sample were analysed at pH 5.0 and samples of the CMF3-44 culture grown with pH adjustment were analysed at pH 6.5 since the pH was adjusted to stay between 6 and 7.

6.2.2 Protease assays with class-specific substrates

Specific activities of aspartic, chymotrypsin-like and subtilisin-like proteases were measured in the culture supernatants of the CMF3-44 strain grown with and without pH adjustment (section 4.3.2) and in the culture supernatant of a parental strain Rut-C30. All samples for the aspartic and subtilisin-like protease assays included 50 µl of culture supernatant (at an appropriate dilution), 140 µl of suitable buffer and 10 µl of a fluorescent substrate (Table 6.1). 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 on a fluorometer (FLUOstar Galaxy, BMG Lab Technologies, Germany) with the excitation and emission wavelengths of 360 nm and 460 nm respectively. In the negative controls, 50 µl of buffer was used instead of the culture supernatant. Positive controls were as above with the culture supernatant replaced with 50 µl of 0.1 mg/ml purified enzyme (Table 6.1).

To assay the chymotrypsin-like protease activity, 50 µl of culture supernatant was mixed with 200 µl of suitable buffer and 10 µl of substrate (Table 6.1). The samples were incubated at 37˚C for 10 min in the dark, and the reaction was 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 onto a transparent microtitre plate and absorbance measured at 410 nm.

Table 6.1. Specific substrates, buffers and positive controls used to assay the activitiy of aspartic, chymotrypsin-like and subtilisin-like proteases. The concentration of all substrates was 0.05 mM.

Protease class	Specific substrate	Positive control	Buffer	Reference
Aspartic	Boc- Leu- Ser- Thr- Arg- amino-4-methylcoumarine	Pepsin from porcine gastric mucosa (40 units/mg)	10 mM Na-acetate buffer, pH 3.5	Takeuchi et al., 1988
Chymotrypsin	N- Benzoyl- L-Tyrosine- P-nitroanilide	Bovine pancreas alpha-chymotrypsin (10-30 units/mg)	0.1 mM Tris-HCl buffer pH 8, 5 mM CaCl2	Novillo et al., 1997
Subtilisin	N- Suc- Ala- Ala- Ala 7- amino-4-methylcoumarin	Subtilisin A from *Bacillus sp* (7-15 units/mg)	0.1 mM Tris-HCl buffer pH 8, 5 mM CaCl2	Markaryan et al., 1996

6.2.3 Identification of protease classes with protease inhibitors

Degradation of the Strep-tagged recombinant proteins purified from the culture supernatants of the CMF3-44 and CF3-8 transformants (section 5.3) was studied with and without different protease inhibitors in the culture supernatant of the expression host, Rut-C30. The method was modified from Landowski et al. (2015). Rut-C30 was grown for six days in 300 ml of CLS

medium. Total protein concentration in the supernatant was 2 mg/ml. pH of the CLS medium at the beginning of the cultivation was 6.5, and after six days of cultivation it was measured to be 5.15. Protease inhibitors targeting major groups of proteases identified in the Rut-C30 strain and in the CMF3-44 transformant were used: pepstatin (aspartic proteases), chymostatin (chymotrypsin-like serine proteases) and PMSF (serine proteases).

Fifty µl of a purified protein sample (0.05 mg/ml) (section 5.3) were added to the 50 µl of the Rut-C30 culture supernatant with and without PMSF (10 mM), chymostatin (100 µM) or pepstatin (50 µM) and incubated at 37°C. Reactions were stopped after 1 h and 18 h of incubation and equal volumes were analysed by Western blotting with the Strep-Tactin AP conjugate as described earlier (section 2.9). In the negative control samples, the Rut-C30 supernatant was replaced with water.

6.2.4 Cultivation with protease inhibitors

Transformant CMF3-44 was cultured with addition of the serine protease inhibitor PMSF to establish whether it was possible to inhibit proteolytic degradation of the recombinant FUT3 during cultivation. Such approach would allow to extend the time of cultivation and therefore increase the yield of the recombinant proteins.

CMF3-44 was grown in shake flask cultures in 50 ml of CLS medium. Test cultures were divided into two groups: in group one, PMSF was added before inoculation (0 hours of cultivation) and then every 12 hours, and in another group, PMSF was added after 36 hours cultivation and then every 12 hours. Equal volumes of the culture supernatants were analysed by Western blotting with Strep-Tactin AP conjugate as described earlier. Ten mM PMSF used in the protease inhibition assays was found to be toxic for *T. reesei* so lower concentrations (2.5 mM and 5 mM) were used.

6.3 Results and Discussion

6.3.1 Total extracellular protease activity

Total protease activity in the culture supernatants of the CMF3-44 cultures grown with and without pH adjustment was measured using azocasein as a substrate. Results are shown in Figure 6.1. Total protease activity in the CMF3-44 cultures with and without pH adjustment was similar at all the timepoints analysed. In both cultures, the total protease activity slightly increased from 48 h to 60 h of cultivation, and then dropped by 72 h of cultivation. Similar total protease activity despite differences in the pH profiles of the two cultures, could be a result of induction and inhibition of proteases, active at acidic or neutral pH.

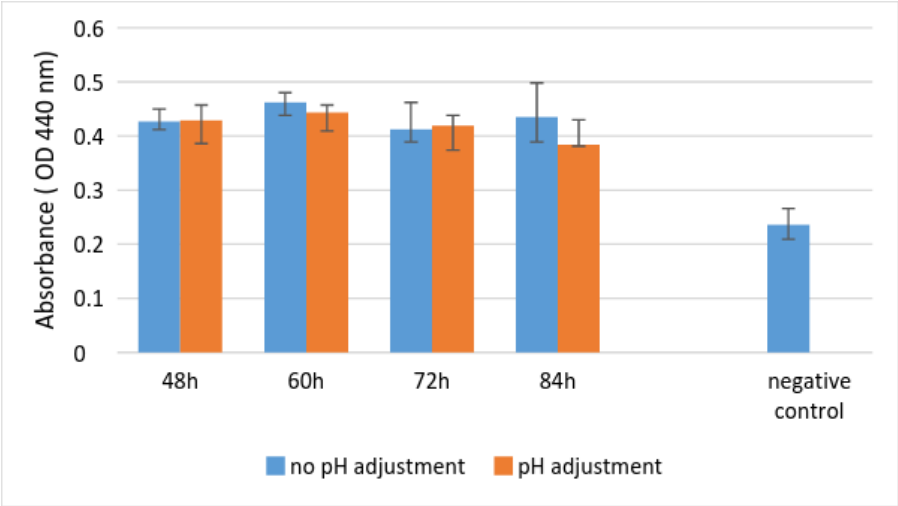


Figure 6.1. Total extracellular protease activity in the culture supernatants of CMF3-44 strain grown with and without pH adjustment. Assays were carried out in triplicate. Error bars represent minimal and maximal activities measured among three biological replicates. In a negative control reaction, the culture supernatant was replaced with assay buffer. Equal amounts of protein (2.5 µg) were used in each reaction.

6.3.2 Characterisation of specific protease activities

Protease assays with specific substrates were performed to measure protease activities in the CMF3-44 cultures grown with and without pH adjustment (section 4.3.2). In a previous study Bali (2012) described protease profiles for *T. reesei* Rut-C30 and its mutant CVt expressing a

heterologous Venus protein and reported the presence of aspartic, chymotrypsin-like and subtilisin-like proteases in culture supernatants of both strains; therefore it was decided to perform activity assays for these major groups of proteases.

The extracellular aspartic protease activity was measured at pH 3.5 (Figure 6.2). In the CMF3-44 culture grown without pH adjustment, the aspartic protease activity slowly increased from 48 h to 84 h of cultivation. In the CMF3-44 culture grown at the neutral pH (6.5 - 7), aspartic protease activity stayed at approximately the same level from 48 to 84 h of cultivation, with a slight drop at 72 h, and was lower than in the culture without pH control, especially at 72 and 84 h of cultivation.

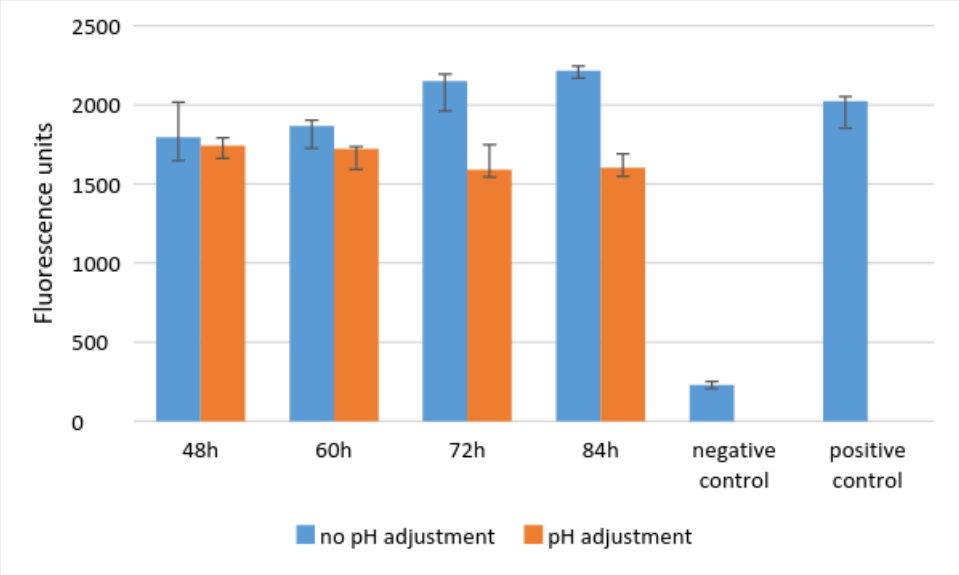


Figure 6.2. Aspartic protease activity in the culture supernatants of CMF3-44 strain grown with and without pH adjustment. Pepsin from porcine gastric mucosa served as a positive control (> 40 units/mg). In a negative control reaction, the culture supernatant was replaced with assay buffer. Assays were carried out in triplicate. Error bars represent minimal and maximal activities measured among three biological replicates. Equal amounts of protein (2.5 µg) were used in each reaction.

Differences in the aspartic protease activity in the cultures with and without pH adjustment reflect the pH of the culture medium (Figure 4.4, B). The pH in the CMF3-44 culture without pH adjustment decreased from 6.5 to below 5, creating favourable environment for the secretion of aspartic proteases (Gente et al., 2001), and pH in the culture with pH kept close

to neutral range (6.5 - 7) was at least partially inhibitory towards production of aspartic proteases.

Chymotrypsin-like proteases are active in the pH range from 6.0 to 8.0 (Rick, 1974, Aguirre et al., 2009), which correlates with the protease activity measured in the CMF3-44 cultures. Chymotrypsin-like protease activity in the CMF3-44 culture grown at the neutral pH gradually increased from 48 h to 84 h of cultivation. The CMF3-44 culture without pH adjustment was characterised by overall lower chymotrypsin-like protease activity; the lowest protease activity was detected at 72 h, which corresponds to the drop of the pH on day 3 of cultivation (Figure 6.3).

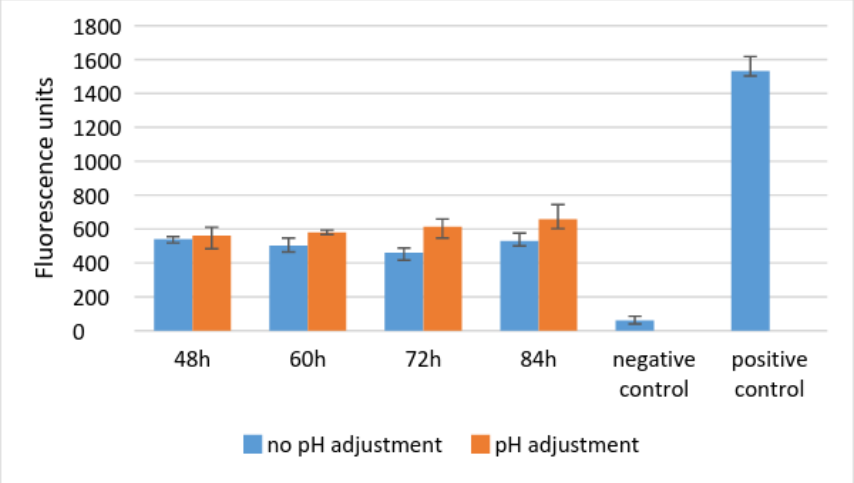


Figure 6.3. Chymotrypsin-like protease activity in the culture supernatants of CMF3-44 strain grown with and without pH adjustment. Bovine pancreas alpha-chymotrypsin served as a positive control (10 - 30 units/mg). In a negative control reaction, the culture supernatant was replaced with assay buffer. Assays were carried out in triplicate. Error bars represent minimal and maximal activities measured among three biological replicates. Equal amounts of protein (2.5 µg) were used in each reaction.

Subtilisin-like protease activity in the CMF3-44 cultures with and without pH adjustment increased gradually from 48 h to 84 h of cultivation (Figure 6.4), however, subtilisin-like protease activity in the culture with pH adjustment was notably higher. Subtilisin-like proteases are active in a broad pH range (6.0 – 8.0) (Pannkuk et al., 2015, Ekici et al., 2008). It is possible that the acidic pH in the CMF3-44 culture without pH adjustment was less

favourable for the secretion of subtilisin-like proteases.

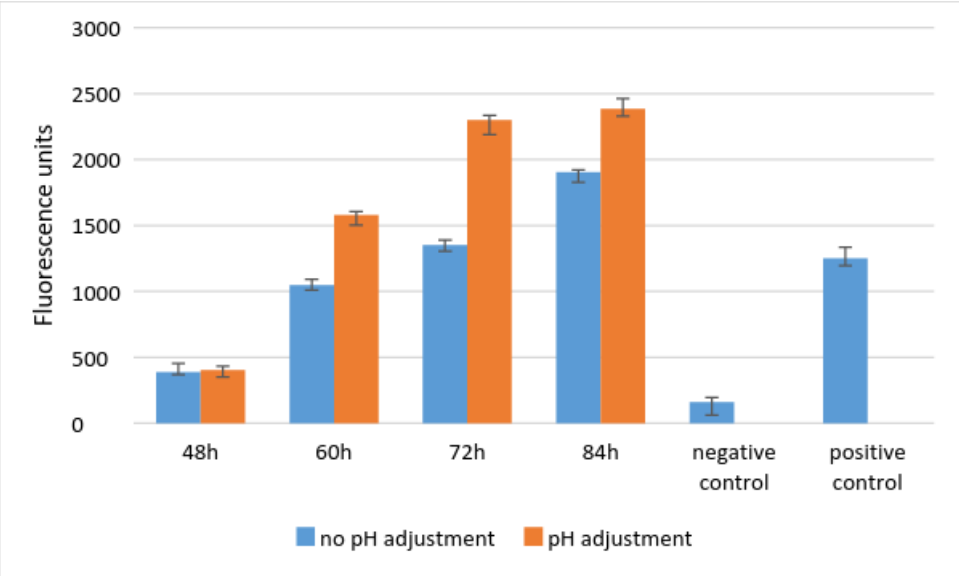


Figure 6.4. Subtilisin-like protease activity in the culture supernatants of CMF3-44 strain grown with and without pH adjustment. Subtilisin A from *Bacillius* sp. served as a positive control. In a negative control reaction, the culture supernatant was replaced with assay buffer. Assays were carried out in triplicate. Error bars represent minimal and maximal activities measured among three biological replicates. Equal amounts of protein (2.5 µg) were used in each reaction.

6.3.3 Identification of extracellular protease classes taking part in degradation of the recombinant fusion FUT3 protein

As shown above, aspartic, chymotrypsin-like and subtilisin-like protease activities were detected at different ratios in the culture supernatants of the CMF3-44 transformant both with and without pH adjustment. However, the experimental set-up did not allow to establish which particular protease class was responsible for the extracellular proteolysis of the recombinant FUT3 protein. This aspect was assessed using the recombinant proteins, purified from the culture supernatants of the CMF3-44 and CF3-8 transformants (Chapter 5). Purified CMF3-44 and CF3-8 proteins were incubated in the culture supernatant of the expression host Rut-C30 with the addition of specific protease inhibitors. Similarly to the supernatants of the CMF3-44 transformant, the Rut-C30 culture supernatant contained aspartic, chymotrypsin-like and subtilisin-like protease activities (Figure 6.5) which made it suitable to be used as a

protease background.

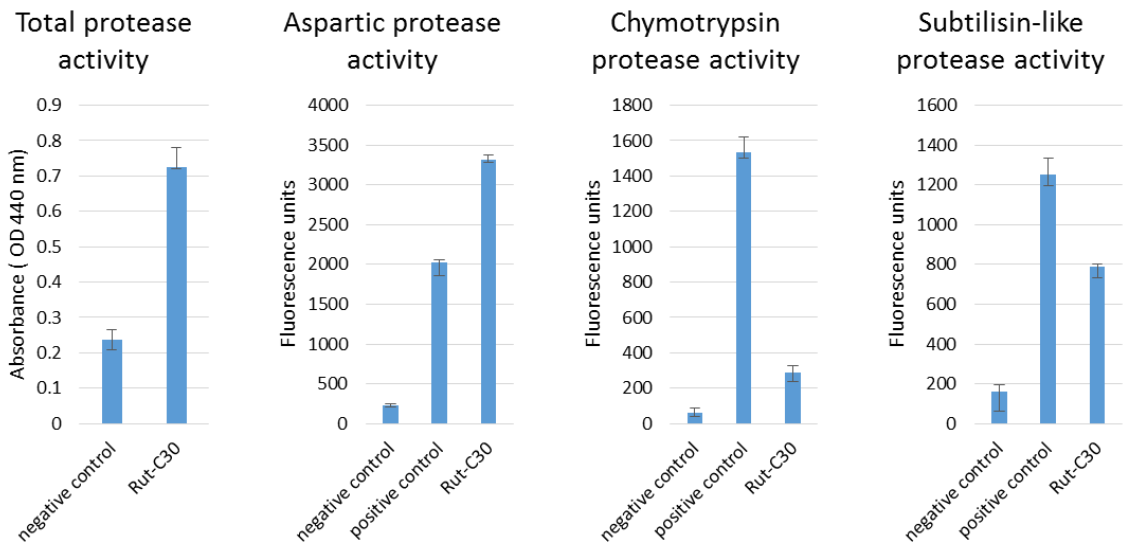


Figure 6.5. Total protease activity and specific aspartic, chymotrypsin-like and subtilisin-like protease activities in the six day Rut-C30 culture supernatant. In a negative control reaction, the culture supernatant was replaced with assay buffer. Positive controls are listed in Table 6.1. Assays were carried out in triplicate. Error bars represent minimal and maximal activities measured among three technical replicates. Equal amounts of protein (2.5 µg) were used in each reaction.

Accordingly, protease inhibitors targeting major protease classes found to be present in the culture supernatant of Rut-C30 strain and in the culture supernatants of the CMF3-44 transformant (section 6.3.2) were used; these were pepstatin (aspartic proteases), chymostatin (chymotrypsin-like serine proteases) and PMSF (serine proteases). Samples were analysed by Western blotting with the Strep-Tactin AP conjugate. Results of identification of protease classes for the proteins purified from the culture supernatant of the CMF3-44 transformant are shown in Figure 6.6.

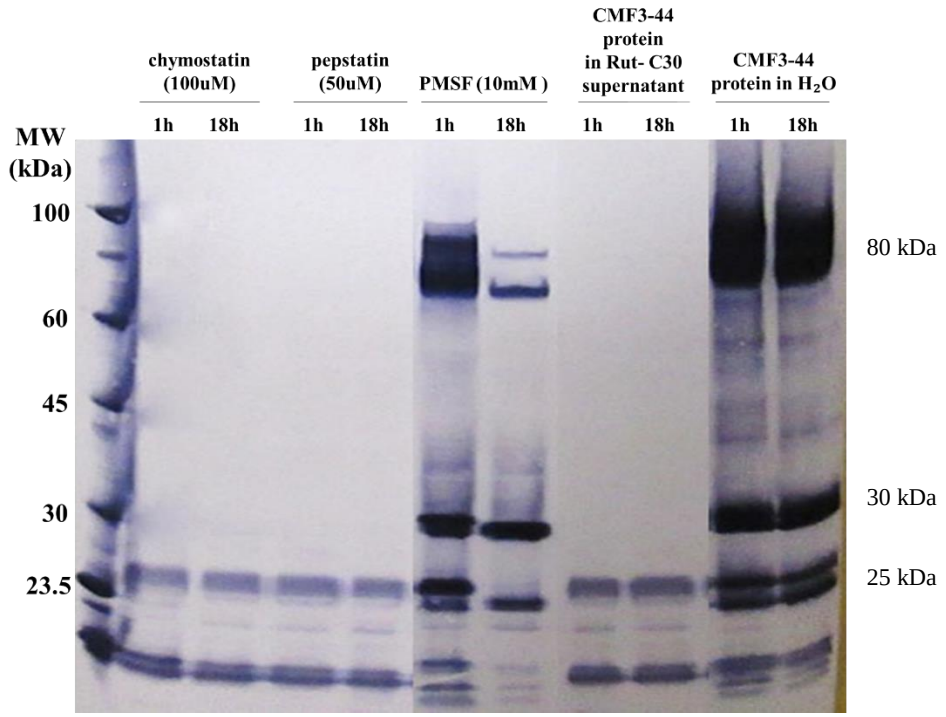


Figure 6.6. Stability of recombinant proteins produced by the CMF3-44 transformant in the Rut-C30 culture supernatant. Addition of a serine protease inhibitor (PMSF) postponed degradation of the recombinant protein by Rut-C30 proteases while chymostatin and pepstatin (inhibitors of chymostatin-like and aspartic proteases) did not affect protein stability.

Although aspartic proteases are known to dominate in *T. reesei* cultures grown at acidic pH (Haab et al., 1990), the addition of pepstatin did not affect the stability of recombinant protein produced by CMF3-44 as it was degraded after one hour of incubation in the Rut-C30 supernatant (Figure 6.6). It is possible that despite the high amount of aspartic proteases secreted by *T. reesei*, proteolytic degradation of the CMF3-44 protein was caused by another class of proteases, also active at acidic pH. Results of the aspartic protease activity assay (section 6.3.2) and pH control experiments (section 4.3.2) support this hypothesis: while the stability of the recombinant proteins was improved by maintaining the pH of the culture within 6.5 – 7.0 range (Figure 4.3), indicating that proteolysis was caused by proteases active at an acidic pH, substantial levels of acidic protease activity were detected in culture supernatants of the CMF3-44 transformant grown at the neutral pH.

Incubation of the CMF3-44 protein in the Rut-C30 culture supernatant with two serine

protease inhibitors, chymostatin and PMSF, demonstrated that addition of PMSF improved stability of the recombinant FUT3 and inhibited its proteolytic degradation for up to 18 hours while chymostatin had no such effect. After one hour into cultivation with PMSF, the amount of the 80 kDa protein was similar to the control sample (recombinant protein incubated in water), and after 18 h of incubation the amount of recombinant proteins reduced substantially, probably as a result of degradation of PMSF (Figure 6.6). Chymostatin has selectivity for chymotrypsin-like serine proteases (Umezawa et al., 1970) while PMSF is a nonspecific inhibitor of serine proteases (Ritchie, 2013). Considering the results obtained in the protease inhibition assays and observations made in the pH control experiments (section 4.3.2) one can assume that the proteolysis of the recombinant CMF3-44 protein in the culture supernatant of the Rut-C30 expression host was at least partially caused by serine proteases sensitive to PMSF and active at acidic pH.

Similar results to CMF3-44 were observed in protease identification assay with the proteins purified from the CF3-8 culture (Figure 6.7). Addition of PMSF partially inhibited proteolytic degradation of the recombinant FUT3 in the culture supernatant of Rut-C30 while addition of pepstatin and chymostatin had no effect on the protein stability. After one hour of incubation with PMSF, protein bands of 80 kDa and 45 kDa were observed, although the bands were fainter than in the control samples, indicating that some degradation did occur, and after 18 h of incubation only the 45 kDa band was observed (Figure 6.7).

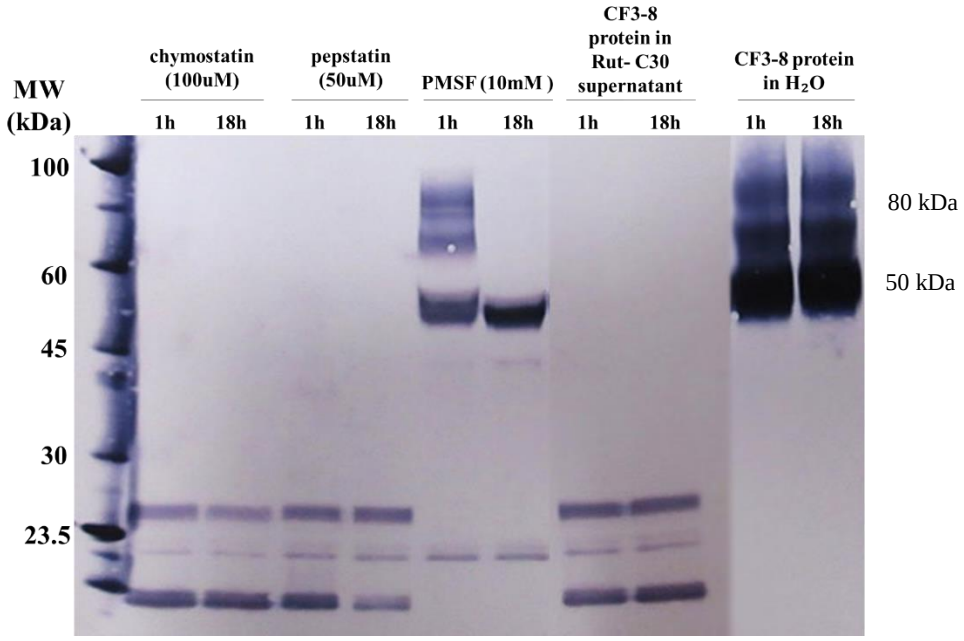


Figure 6.7. Stability of the recombinant proteins produced by the CF3-8 transformant in the Rut-C30 culture supernatant. Addition of a serine protease inhibitor (PMSF) partially inhibited the degradation of the recombinant proteins by Rut-C30 proteases while addition of chymostatin and pepstatin did not improve protein stability.

To sum up, based on the protease inhibition studies and pH control experiments (section 4.3.2), one can assume that serine proteases sensitive to PMSF and active at acidic pH are at least partially responsible for the extracellular degradation of recombinant proteins produced by CMF3-44 and CF3-8 transformants. One such protease, active at low pH but not inactivated by pepstatin, was first found in *A. fumigatus* (Reichard et al., 2005) and classified as a sedolisin serine protease. Landowski et al. (2016) demonstrated great improvement in IFNα-2b production by *T. reesei* via deletion of the subtilisin-related serine protease SLP7 gene.

CMF3-44 cultures grown at a neutral pH were characterised by higher stability of the recombinant proteins (section 4.3.2) and also by increased activity of serine proteases (section 6.3.2). Activity assays for serine proteases (chymotrypsin-like and subtilisin-like) were performed at pH 8.0, as the majority of serine proteases have a pH optimum in the neutral to slightly alkaline range (Cuervo et al., 2008, Ekici et al., 2008), which did not allow to analyse the differences in acidic serine proteases activities between the cultures.

6.3.4 Cultivation of the CMF3-44 transformant with addition of a protease inhibitor

As demonstrated above, degradation of purified recombinant proteins could be inhibited by addition of PMSF to the Rut-C30 culture supernatant. To investigate the possibility of cultivation of *T. reesei* with the addition of protease inhibitors, various concentrations of PMSF were added to the growing culture. Transformant CMF3-44 was chosen for this experiment as the one with better results in protease inhibition studies (section 6.3.1). Culture supernatant samples were taken at 36 h, 48 h and 67 h of cultivation and analysed by Western blotting with the Strep-Tactin AP conjugate. Results of Western blotting are shown in Figure 6.8.

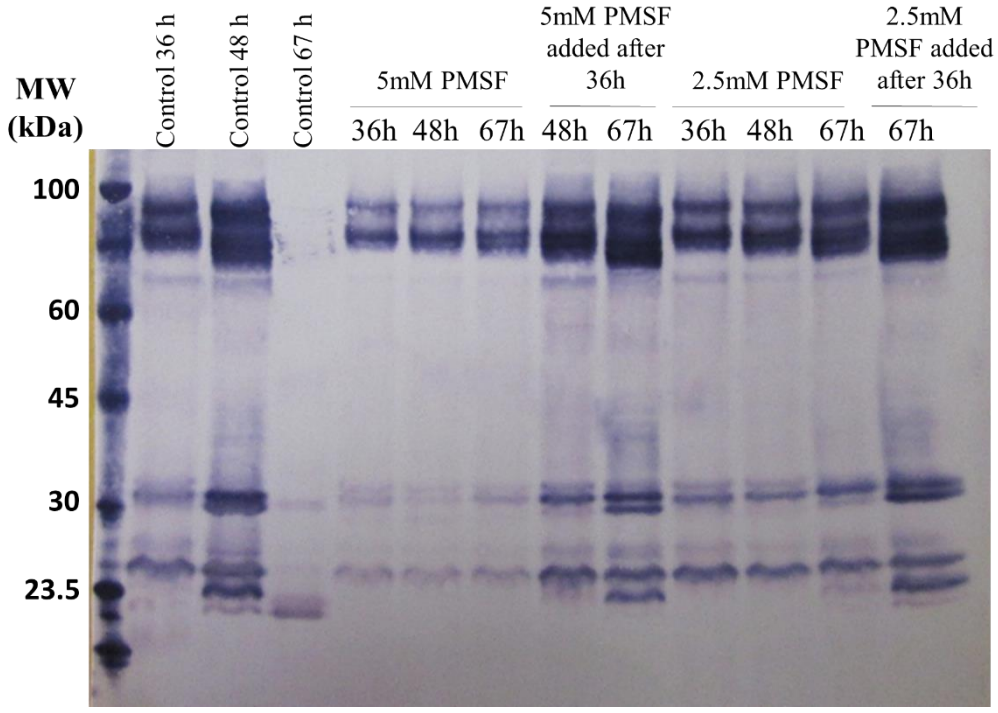


Figure 6.8. CMF3-44 cultivation with the addition of varying amounts of PMSF to the culture. Equal volumetric amounts were analysed by Western blotting with the Strep-Tactin AP conjugate. The 80 kDa protein (double bands) was detected in all cultures grown with the addition of PMSF on day 3 of cultivation (67 h) while recombinant proteins in the control culture were degraded after 48 h of cultivation.

The addition of PMSF even at a low concentration (2.5 mM) successfully inhibited degradation of the recombinant FUT3 in a growing culture up to day 3 (67 h) of cultivation

while in the control samples without PMSF, recombinant proteins became degraded after day 2 (48 h) of cultivation. Obtained results show that addition of protease inhibitors during cultivation makes it possible to increase the time of cultivation, for example, in continuous and fed-batch fermentation processes, combined with pH adjustment.

PMSF is unstable at ambient temperatures and in water-based solutions. According to the results of protease inhibition assays, inhibitory properties of PMSF began to decrease after 18 hours of incubation. Therefore, PMSF needs to be periodically added to the culture to keep the concentration at a satisfactory level. PMSF is only soluble in ethanol or methanol, so when used at high concentrations, either PMSF itself or the solvent can be harmful to *T. reesei*. When PMSF was added to the cultures at the beginning of cultivation, the amount of recombinant proteins was lower as compared to the control culture without PMSF addition or to the cultures to which PMSF was added after 36 hours of cultivation. It is possible that *T. reesei* needs a certain amount of time to grow and develop to the stage where it can overcome the toxic effect of PMSF, for example by remodelling of the cell wall in response to stress (Ene et al., 2015; Heilmann et al., 2013). Light microscopy of the cultures with addition of PMSF revealed an increased number of conidia compared to the control cultures without PMSF addition (data not shown), which could also be a result of the toxicity of PMSF. As demonstrated in the previous experiments (section 4.3.1), proteolytic degradation of recombinant FUT3 begins sometime around 48 hours of cultivation, so the addition of PMSF after 36 hours appears a good time point.

The addition of protease inhibitors is not feasible in large-scale industrial processes and is mostly used in small scale experiments as a proof of concept, or in sensitive mammalian cell cultures (Diaz-Nido, 1991; Clincke et al., 2011). However, it demonstrates that targeted inhibition of specific protease activities in the growing cultures makes it possible to protect recombinant proteins during cultivation and increase the time of cultivation if necessary.

Smith et al. (2014) applied this approach to the production of a human galactosidase in fusion with the CBH1 carrier in *T. reesei* Rut-C30 mus 53Δ strain with enhanced homologous recombination frequency, reporting an increase in the yield of the recombinant galactosidase by the addition of a strong cocktail of protease inhibitors to the culture medium.

6.4 Summative discussion

Experiments described in this chapter were aimed at identification of specific classes of proteases responsible for the degradation of the recombinant fusion FUT3 protein. Protease activity assays revealed that while the total protease activity in the CMF3-44 cultures grown with and without pH adjustment was similar, the culture grown at a neutral pH was characterised by a decrease of the aspartic protease activity and increase in the activities of alkaline chymotrypsin-like and subtilisin-like serine proteases (section 6.3.2). The Rut-C30 culture supernatant, used in the protease identification assays, was characterised by a higher total protease activity and aspartic protease activity, whereas chymotrypsin-like and subtilisin-like protease activities in the culture supernatant of a Rut-C30 culture were lower than corresponding protease activities in the CMF3-44 cultures (Figure 6.5). This could be at least partially accounted for by the acidic pH of the Rut-C30 culture (5.15) and longer time of cultivation (6 days); it is also possible that differences in protease profiles between the expression host and the transformant are caused by production of the heterologous protein (Bali, 2012).

Incubation of the purified CMF3-44 and CF3-8 proteins in the culture supernatant of the expression host Rut-C30 with and without the addition of protease inhibitors demonstrated that acidic serine proteases are at least partially responsible for the extracellular proteolysis of the recombinant FUT3 proteins. Inhibition of proteolytic degradation by the addition of protease inhibitors was more prominent for the proteins produced by the CMF3-44

transformant than for the CF3-8 produced proteins. Effect of the PMSF treatment on the recombinant FUT3 protein without mCherry (CF3-8) was less obvious even after 1 hour after addition of PMSF. It is possible that the CMF3 construct has additional protease cleavage sites for the acidic serine proteases within the mCherry fusion protein, such as the cleavage site identified by N-terminal sequencing in Chapter 5, and therefore benefits more from the PMSF addition.

Cultivation of the CMF3-44 transformant with the addition of varying concentrations of PMSF at different time points demonstrated that periodical addition of PMSF to the growing culture had a positive effect on the stability of the recombinant proteins. While this approach is not practical for the large scale production, it could be used in small-scale cultivations to increase the yield of the recombinant proteins for further analyses.

7

BIOREACTOR CULTIVATIONS OF THE CMF3-44 TRANSFORMANT

7.1 Introduction

Bioreactor cultivations provide not only containment but also make it possible to control environmental parameters at predetermined values. Among the conditions that can be controlled are dissolved oxygen (DO), pH, temperature, agitation rate, redox potential, dissolved carbon dioxide, cell concentration, cell growth, substrate concentration, inlet gas flow and composition, volume, pressure, and fluid dynamics (Palomares et al., 2004).

Macro- and micromorphology of fungi depend on culture conditions and, conversely, affect protein production (Palomares et al., 2004, Thomas & Zhang, 1998). Oxygenation is one of the critical parameters in fungal fermentation; maintaining oxygenation at the desired level could be problematic, especially at the later stages when the viscosity of the culture increases, as intense sparging and agitation cause shearing of mycelium. Increased oxygenation leads to the formation of pellets; such changes in macromorphology are often accompanied by a decrease in protein production. Influence of fermentation parameters on fungal morphology and yield of proteins of interest are reviewed in detail in section 1.8.6.

Experiments described in section 4.3.2 demonstrated that stability of the recombinant FUT3 protein in the growing cultures was improved when pH was adjusted to neutral. However, the experimental design (shake flask cultures with manual pH control) did not allow precise control and recording of physical parameters, such as temperature, dissolved oxygen, agitation rate, gas flow and pH, and also posed a high risk of contamination.

The CMF3-44 transformant was selected for the fermentation studies as it demonstrated good performance in the pH control experiments (section 4.3.2) and protease inhibition studies (section 6.3.3). Time of fermentation was limited to four days. As it was previously shown that proteolytic degradation takes place between day 2 and day 3 of cultivation (section 4.3.1), one the goals of the experiment here was to demonstrate that the time of cultivation could be

extended past that critical point. While longer cultivation is possible and would be beneficial with regard to the yield of the heterologous product, it would require control of substrate depletion and periodical feeding of the culture, which did not fit into the time frame of the current research. Inoculation with both fungal conidia and a pre-culture are commonly used in fungal fermentations. For this experiment, inoculation with conidia was chosen as it allowed to observe morphological changes and changes in physical parameters (pH, aeration and agitation) at the early stages of growth. Also, inoculation with spores mimics the conditions in the shake flask cultivations performed earlier (section 4.3.2), which is important for comparison of the results of the experiments.

7.2 Materials and Methods

The CMF3-44 strain was grown in a 3 litre New Brunswick BioFlo/CelliGen 115 bioreactor (working volume 1.5 L) in CLS medium at 28°C with pH maintained at 7.0 by the addition of sterile 5 M KOH. Agitation was achieved with one pitched-blade impeller. Foaming was prevented by addition of the non-silicone antifoam 204 (Sigma). Dissolved oxygen (DO) was set at above 30% using a combination of agitation (50 – 400 rpm) and sparging with air (0.1 – 1 Lpm). Fresh conidia (30 x 10^8) were used as inoculum to mimic conditions of the shake-flask cultures. Samples were taken at 24, 36, 48, 60, 72, 84 and 96 hours (1-4 days) of cultivation and analysed by Western blotting with the Strep-Tactin AP conjugate. Additionally, samples were stained with Trypan Blue, which allows to differentiate between dead and live cells, and analysed by light microscopy using Olympus BH2 microscope at 40× magnification.

7.3 Results and Discussion

7.3.1 Cultivation parameters and fungal morphology

In order to confirm the influence of a neutral pH on the stability of the recombinant FUT3 (section 4.3.2), transformant CMF3-44 was grown in a bioreactor with and without pH control. Critical fermentation parameters (pH, agitation, aeration and dissolved oxygen) were recorded and can be found in Appendices 7 - 11.

Starting pH of the culture medium was 6.5. One of the bioreactor cultures was grown with the pH set at 7.0 (Appendix 7) while in another culture pH naturally dropped to below 5 and slightly increased on day 4 of cultivation (Appendix 8). Similar pH curves were observed when pH was manually controlled in the shake flask cultures (section 4.3.2).

Dissolved oxygen was maintained at above 30% by varying agitation and aeration. Data recorded during cultivation with pH control are provided in Appendices 9 - 11. CMF3-44 bioreactor culture grown without pH control showed similar patterns (data not shown). The agitation speed and aeration rate rapidly increased to the set maximum (400 rpm, 1.0 Lpm) after approximately 24 hours of cultivation (Appendices 10 and 11) in order to maintain dissolved oxygen at the required level, indicating an increase of the viscosity of the culture due to the accumulation of biomass. After 24 h, dissolved oxygen, agitation and aeration remained stable up to day four of fermentation.

Fungal morphology at various growth stages was inspected using light microscopy. Samples were mixed with Trypan Blue to identify dead cells. Both cultures, with and without pH control, were morphologically similar. During the first two days of cultivation, only the primary form of mycelium was observed, characterised by long filaments and little or no branches (Velkovska et al., 1997) (Figure 7.1).

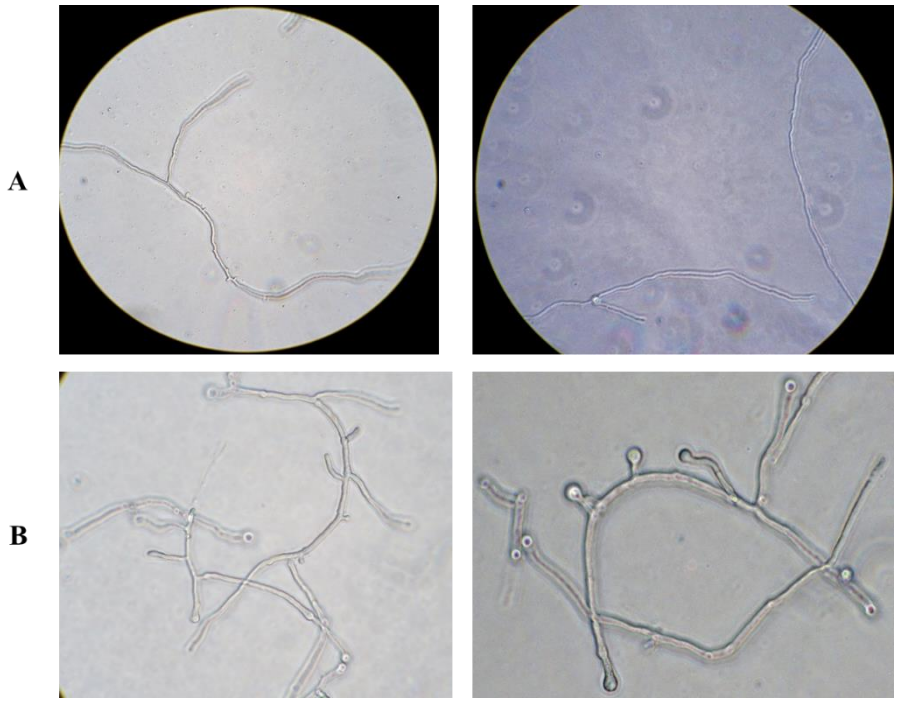


Figure 7.1. Primary mycelium of the CMF3-44 transformant at 24 h (**A**) and 48 h (**B**) observed at 40× magnification. No obvious morphological differences between cultures grown at pH 7 and without pH adjustment were detected. A total of 30 views were screened.

Primary mycelium is common for the initial stage of growth, with fast accumulation of biomass and low protein secretion. This stage could be shortened by inoculation with a pre-culture, which allows to reduce the time of fermentation. Inoculation with conidia was chosen for this experiment as it allowed to observe morphological changes and changes in physical parameters, such as pH, at the early stages of growth.

Velkovska et al. (1997) reported that only the secondary form of mycelium, characterised by a large number of branches, contributed to cellulase production by *T. reesei*. Highly branched mycelium has a larger surface for interaction with the growth substrate, which may increase promoter activity relevant for the use of the substrate and subsequently improve the protein production levels. Protein secretion occurs at the apical or subapical hyphal regions, therefore secondary mycelium has higher secretion capacity (Conesa et al., 2001). Secondary mycelium was observed in the CMF3-44 cultures after two days of cultivation (Figure 7.2).

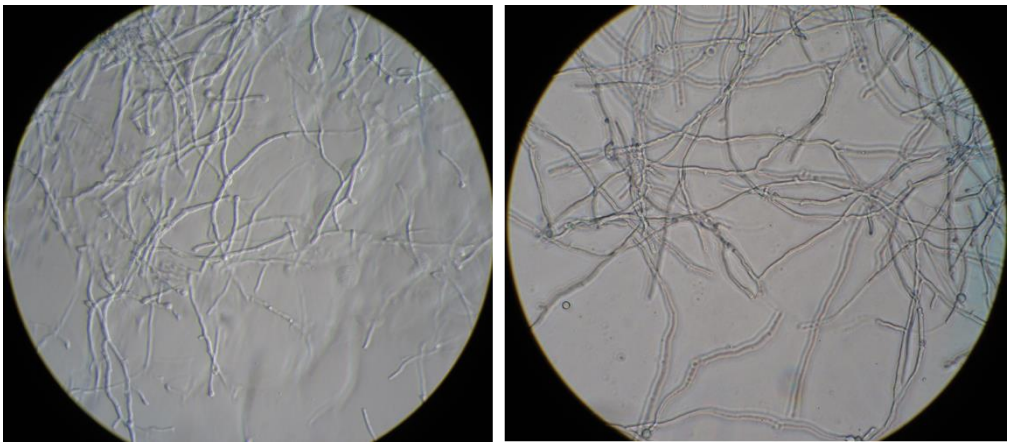


Figure 7.2. Secondary mycelium of the CMF3-44 transformant after 60 hours of cultivation observed at 40× magnification. No morphological differences between cultures grown at pH 7 and without pH adjustment were detected. A total of 30 views were screened.

No dead mycelia (which would be stained blue by the Trypan blue stain) or conidiation or shearing of the mycelium were observed throughout the four days of fermentation, which indicates that no substrate exhaustion occurred and physical parameters of fermentation were not harmful to the fungus (Palomares et al., 2004).

A factor that has a significant effect on fungal morphology is pH. Experiments conducted by Li et al. (2013) demonstrated that when pH of the *T. reesei* 30s-3-13 (a mutant of Rut-C30) cultures was maintained at 4.0, only primary mycelium was observed throughout a three day cultivation. In cultures grown at pH 5.0 and 6.0, secondary mycelium appeared at 60 h, and a higher rate of mycelial autolysis and conidia formation was observed at the higher pH. In the current study, there were no significant differences in the morphology between a culture with the pH set at 7.0 and a culture where pH was allowed to drop naturally to below 5.0 under the cultivation conditions used.

7.3.2 Western blot analysis of recombinant proteins in bioreactor cultures

Samples taken during fermentation were analysed by Western blotting with the Strep-Tactin AP conjugate. In the culture without pH control (Figure 7.3, B), expression of the recombinant fusion protein started at around 36 hours of cultivation; at 60 hours of cultivation, high

molecular weight recombinant proteins were mostly degraded, and at the later stages of cultivation, only low molecular weight proteins were observed.

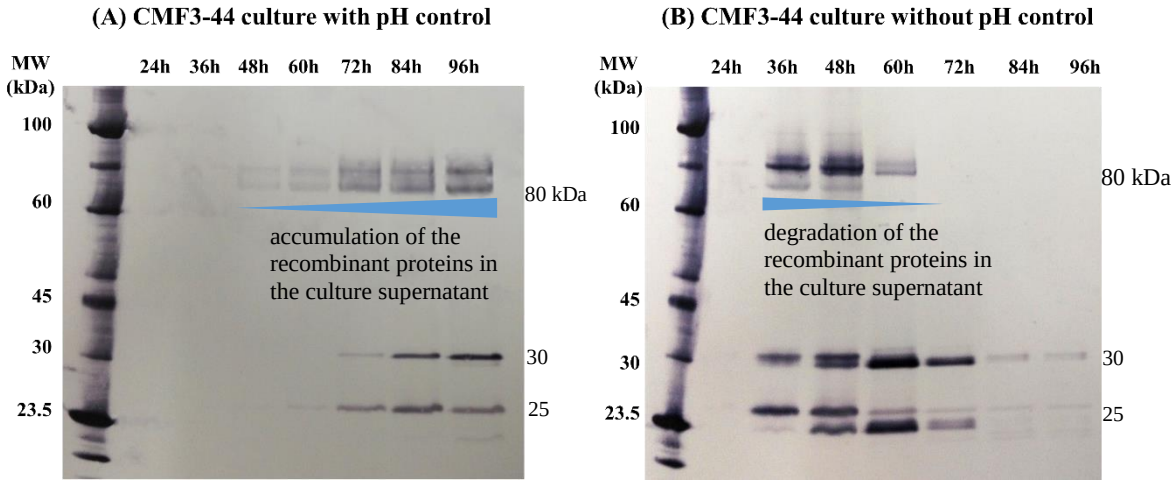


Figure 7.3. Strep-tagged proteins detected in the CMF3-44 culture supernatant throughout 96 h of cultivation in a fermentor with pH control (**A**) and without pH control (**B).**

Analysis of the recombinant Strep-tagged proteins produced in the culture with pH control (Figure 7.3, A) demonstrated that secretion started at around 48 hours of cultivation and the amount of recombinant proteins gradually increased between 48 and 96 hours of cultivation.

As it was demonstrated before (section 4.3.2), secretion of the recombinant proteins in the cultures with pH adjusted to neutral was delayed compared to the cultures without pH adjustment. A similar delay was observed in the bioreactor cultures. Such delay in the production of the recombinant proteins by a culture grown at pH 7.0 could be a result of decreased activity of the *cbh1* promoter at neutral pH (Mukhopadhyay & Malik, 1980). According to Li et al. (2013), pH 5.0 was optimal for the production of exoglucanases (including CBH1). In the current research, the amount of the recombinant proteins detected by Western blotting in the pH controlled culture gradually increased with time (Figure 7.3, A), which may indicate that protease degradation was inhibited, allowing accumulation of the heterologous products. Low molecular weight bands (~30 and ~25 kDa), which were previously demonstrated to be products of the cleavage within the mCherry protein (sections

5.3.1 and 5.3.2), were observed at smaller amounts in a culture with the pH control compared to a culture without pH control, which also could be a result of inhibition of a specific protease responsible for a particular cleavage.

7.4 Summative discussion

Experiments conducted and described in this chapter supported preliminary results obtained in section 4.3.2. The positive effect of the neutral pH on the production of recombinant fusion FUT3 proteins was demonstrated. These results, in turn, support the conclusion made in Chapter 6 that acidic proteases are responsible for the extracellular degradation of the recombinant proteins. Production of the recombinant FUT3 in a culture with pH control was delayed in time compared to the culture without pH control, but on the positive side, the protein was not degraded throughout four days of cultivation. No differences in conidia formation and mycelial morphology were observed throughout four days of fermentation, as cultures with and without pH control developed in a similar way. Physical parameters of fermentation (agitation between 50 and 400 rpm and aeration between 0.1 and 1.0 Lpm) were sufficient to keep DO at above 30% but did not cause shearing of the mycelium or formation of pellets and clumps.

To sum up, a positive effect of neutral pH on the stability of the recombinant FUT3 protein in the culture supernatant of a growing culture was confirmed; it was demonstrated that cultivation time could be extended past two days (optimal cultivation time for the cultures without pH adjustment) in order to increase the yield of recombinant proteins, which is necessary for the additional studies of the recombinant FUT3. Sufficient physical parameters of cultivation were established and accumulation of the recombinant products with time was also demonstrated (Figure 7.3).

8

SUMMARY, CONCLUSIONS AND FUTURE WORK

8.1 Summary

The overall goal of this study was to express functional human fucosyltransferases FUT3 and FUT4 in the filamentous fungus *Trichoderma reesei*. Due to the more promising results obtained with the recombinant FUT3, experimental work was mainly focused on the FUT3-producing transformants. Successful expression of the FUT3 enzyme was achieved, and factors influencing the yield and stability of the recombinant fucosyltransferases were analysed as follows:

(1) Two expression cassettes for the production of the human FUT3 and FUT4 in *T. reesei* were constructed and successfully introduced into the *T. reesei* Rut-C30 via biolistic bombardment (Chapter 3). Preliminary screening of obtained transformants demonstrated that high levels of mCherry fluorescence in the culture supernatants did not correlate with the presence of the recombinant proteins in the culture supernatants. Further experiments demonstrated that mCherry was partially cleaved from the FUT3 protein.

(2) Proteolytic degradation of the recombinant FUT3 and FUT4 proteins was observed, and culture-based strategies were implemented to improve the yield and stability of the FUT3. A specific class of proteases taking part in the degradation of the recombinant fusion FUT3 protein was identified, and one protease cleavage site within the mCherry fusion partner was described (Chapter 4, 5, 6 and 7).

(3) Purification tag (Strep-tag II) was not cleaved from some forms of the the FUT3 fusion protein, and was successfully used for purification of the recombinant FUT3. Preliminary activity assays demonstrated certain enzymatic activity of the purified FUT3 (Chapter 5).

8.1.1 Composition of the expression cassettes and transformation of *T. reesei* (1)

Two expression cassettes for the production of the human FUT3 and FUT4 in *T. reesei* under control of the *cbh1* promoter were constructed. One of the expression cassettes was designed to produce fucosyltransferases in fusion with the mCherry fluorescent protein and core and linker domains of the native CBH1 protein (CMF3 and CMF4 proteins) while another cassette encoded a shorter fusion protein featuring FUT3 or FUT4 and the CBH1 carrier (CF3 and CF4 proteins). Two transformation methods, protoplast transformation and biolistic bombardment, were used to introduce the expression cassettes into the *T. reesei* genome. Protoplast transformation produced more transformants than the biolistic bombardment, but none of them survived the second round of selection with 80 U/ml of Hygromycin B. Instead, biolistic bombardment resulted in the formation of stable transformants (Chapter 3). Further analysis of the proteins produced by the different types of transformants demonstrated that the recombinant protein produced in CMF3 transformants was more susceptible to proteolysis compared to recombinant proteins made in CF3 transformants and had additional cleavage site within the mCherry fusion partner (Chapter 5). Considering these findings, the use of mCherry fluorescence as an indication of the production and secretion of a recombinant FUT3 fusion protein was not reliable in the *T. reesei* expression system.

8.1.2 Analysis of proteolytic degradation of the FUT3 protein (2)

It was observed that only one out of 38 screened transformants (CF3-8) secreted recombinant FUT3 of the expected size while other transformants secreted smaller recombinant proteins of different sizes (Chapter 3). It was assumed that the observed proteins were a result of degradation of the recombinant proteins by extracellular proteases of *T. reesei*. Conducted experiments demonstrated that production of recombinant FUT3 and FUT4 started on day 2 of cultivation and by day 3 most of the recombinant proteins were undetectable (Chapter 4).

Cultivation of transformants at a neutral pH supported prolonged stability of the recombinant proteins in the culture supernatant (up to 4 days of cultivation) compared to the non-adjusted conditions (Chapter 4 and 7). The most harmful class of proteases was identified by incubation of the purified recombinant proteins produced by CMF3-44 and CF3-8 in the culture supernatant of the expression host with inhibitors for the major classes of proteases detected in the *T. reesei* supernatant. PMSF (broad spectrum inhibitor of serine proteases) had a positive effect on the stability of the recombinant FUT3: the protein was detected even after 18 h of incubation while in the control sample without addition of PMSF, the recombinant protein was degraded after 1 h of incubation (Chapter 6). Combining these results with the observations made in the pH control experiments, it was assumed that serine proteases active at an acidic pH play a role in the proteolysis of the recombinant FUT3. The addition of PMSF to the growing culture also had a positive effect on the stability of the recombinant FUT3, but also a toxic effect of high concentrations of PMSF was observed. The addition of protease inhibitors to the growing culture is not feasible for the large-scale cultivations, however, it could be used in small-scale experimental cultures to improve the yields of heterologous proteins and as a proof of concept to demonstrate the effect of inhibition of specific classes of proteases on the stability of heterologous proteins.

8.1.3 Purification and assaying the activity of the recombinant FUT3 (3)

Western blot analysis of the culture supernatants with an anti-FUT3 antibody and with the Strep-Tactin AP conjugate suggested that Strep-tag II was not cleaved from the FUT3 protein and therefore could be used for purification of the recombinant FUT3 (Chapter 3). After purification, the yield of the recombinant protein produced by the CMF3-44 transformant was estimated to be around 70 mg/l while the yield from the CF3-8 transformant was approximately 40 mg/l. Western-blot analysis of the purified recombinant proteins with

specific antibodies targeting the components of the fusion protein revealed that high molecular weight proteins (~80 kDa) purified from the CMF3-44 and CF3-8 transformants contained all components of the fusion construct: FUT3, mCherry, CBH1core-linker and the Strep-tag II for CMF3-44 and FUT3, CBH1core-linker and Strep-tag II for CF3-8. Preliminary activity assays demonstrated that FUT3 recombinant fusion proteins produced by the CMF3-44 and CF3-8 transformants exhibited certain enzymatic activity; however, the shorter recombinant protein without mCherry purified from the culture supernatant of the transformant CF3-8 was more active, possibly because of the smaller number of degradation products which did not contain the FUT3 protein and therefore contained higher amount of the FUT3 protein in the sample (Figure 5.19). N-terminal sequencing of the recombinant proteins purified from the culture supernatant of the CMF3-44 transformant demonstrated that two proteins of about 80 kDa recognised by both the Strep-Tactin AP conjugate and anti-FUT3 specific antibody were N-terminally blocked. A cleavage site at the N-terminus of the mCherry protein was identified by N-terminal sequencing of a ~35 kDa protein produced by the CMF3-44 transformant.

Overall, production and purification of the functional recombinant human fucosyltransferase 3 (FUT3) in *T. reesei* were accomplished. While the main aim of the study has been achieved, the project could benefit from additional experiments, discussed below.

8.2 Future work

8.2.1 Optimisation of the purification method and further analyses of the recombinant proteins

It was observed in Chapter 5 that the high amount of the recombinant proteins was present in the flow-through fraction, indicating that the binding capacity of the Strep-Tactin purification columns used in this study was not sufficient to purify the required amounts of the recombinant protein for further studies, therefore, optimisation of the purification technique will be required

for further studies. It could include the use of bigger purification columns and Strep-Tactin resins with higher binding capacity or optimisation of the purification parameters, such as pH. Purification of larger quantities of the recombinant protein would allow to attempt removal of the N-terminal blockage (Chapter 5) and identification of the protease cleavage sites in the proteins produced by the CMF3-44 and CF3-8 transformants. Another issue to be addressed is determining the activity of the recombinant FUT3. Culture supernatants of all analysed transformants displayed multiple truncated proteins (degradation products of the fusion FUT3 protein) containing the Strep-tag II, and only a few of these products contained the FUT3 protein. Therefore, it is necessary to develop a purification method using HPLC or specific antibodies for separation of the FUT3 containing protein from the proteolysis products.

The activity assay used in this study measured the total fucosyltransferase activity; differentiation between α-1,3 and α-1,4 activities would require additional experiments, for example, MS analysis of the acceptor substrates used in the fucosyltransferase reactions.

8.2.2 Optimisation of the expression host

While certain inhibition of the proteolytic degradation of the recombinant FUT3 fusion protein was achieved by maintaining the neutral pH of the culture medium and by cultivation with protease inhibitors, multiple degradation products still were observed and, in the case of the CMF3-44 transformants, full-sized fusion proteins were not detected. Expressing the FUT3 protein in a genetically engineered protease-deficient strain could improve the yield and stability of the recombinant FUT3 as has been demonstrated for other heterologous proteins of human origin (Landowski et al., 2016; Zhang et al., 2014).

Targeted integration of the expression cassette into the *cbh1* locus could also improve the yield of the recombinant FUT3, as knocking-out the *cbh1* gene encoding the major secreted *T. reesei* protein would reduce the amount of the protein in the secretory pathway and thus reduce

secretion stress (Miettinen-Oinonen, 2004). As the first step towards improvement of targeted integration, work on a *T. reesei* strain with enhanced homologous recombination frequency has been started. When ready, such strain would also make it easier to create various protease deficient strains tailored to the expression of specific heterologous proteins.

8.2.3 Glycan analysis

As demonstrated in the previous studies (Christensen et al., 2000), glycosylation of the FUT3 is necessary for its activity. Functional human fucosyltransferase 3 has been produced in expression hosts with different glycosylation machineries, such as mammalian cell lines (BHK-21) as well as yeasts *S. cerevisiae* and *P. pastoris* (Costa et al., 1997; Shimma et al., 2006; Gallet et al., 1998) which indicates that the relative size of the glycan structures is more important for the fucosyltransferase activity than the glycan composition. Unlike highly branched and hypermannosylated yeast glycans, glycosylation in *T. reesei* is more human-like, with moderate number of mannose residues; however, the glycans still lack the terminal sialic acid and fucose residues (Deshpande et al., 2008). Considering the fact that specific glycan structures of the human FUT3 have not been described in the literature, it would be interesting to compare the glycan structures of the commercial FUT3 and of the FUT3 expressed in *T. reesei* and establish a relationship between the glycosylation and FUT3 enzyme activity.

8.3 Conclusion

Recombinant human fucosyltransferase 3 (FUT3) has been successfully expressed in *T. reesei* at an estimated level of 70 mg/l. This compares well with other recombinant proteins produced in *T. reesei* (Table 1.6) and other fungi (Lubertozzi & Keasling, 2009) but does not reach the yields of recombinant human interferon alpha-2b (4.5 g/l) produced in *T. reesei* strain carrying

multiple deletions of genes encoding harmful proteases (Landowski et al., 2016). Improvement of the yield and stability of the recombinant FUT3 is necessary for further studies and potential large-scale production. Nevertheless, findings of this research provide a proof of concept for recombinant expression of a human fucosyltransferase in *T. reesei* and contribute to the knowledge on the production of heterologous human proteins in this industrially-exploited fungal host.

References

Adav, S. S., Ravindran, A., Chao, L. T., Tan, L., Singh, S., & Sze, S. K. (2011). Proteomic analysis of pH and strains dependent protein secretion of *Trichoderma reesei*. *Journal of Proteome Research*, *10*(10), 4579–4596. http://doi.org/10.1021/pr200416t

Aguirre, C., Castro-Guillén, J. L., Contreras, L., Mendiola-Olaya, E., de la Vara, L. G., & Blanco-Labra, A. (2009). Partial characterization of a chymotrypsin-like protease in the larger grain borer (*Prostephanus truncatus* (Horn)) in relation to activity of *Hyptis suaveolens* (L.) trypsin inhibitor. *Journal of Stored Products Research*, *45*(2), 133–138. http://doi.org/http://doi.org/10.1016/j.jspr.2008.11.001

Ahamed, A., & Vermette, P. (2008). Culture-based strategies to enhance cellulase enzyme production from Trichoderma reesei RUT-C30 in bioreactor culture conditions. *Biochemical Engineering Journal*, *40*(3), 399–407. http://doi.org/10.1016/j.bej.2007.11.030

Association of Manufacturers and Formulators of Enzyme Products (AMFEP). http://www.amfep.org

Albano, C. R., Randers-Eichhorn, L., Bentley, W. E., & Rao, G. (1998). Green Fluorescent Protein as a Real Time Quantitative Reporter of Heterologous Protein Production. *Biotechnology Progress*, *14*(2), 351–354. http://doi.org/10.1021/bp970121b

Aleksenko, A., & Clutterbuck, A. J. (1997). Autonomous Plasmid Replication in *Aspergillus nidulans*: AMA1 and MATE Elements. *Fungal Genetics and Biology*, *21*(3), 373–387. http://doi.org/10.1006/fgbi.1997.0980

Amin M. N., McLellan J. S., Huang W., Orwenyo J., Burton D. R., Koff W. C., … Wang L-X. (2013). Synthetic glycopeptides reveal the glycan specificity of HIV-neutralizing antibodies. *Nature Chemical Biology*, *9*, 521–526. doi:10.1038/nchembio.1288

Amonsen, M., Smith, D. F., Cummings, R. D., & Air, G. M. (2007). Human Parainfluenza Viruses hPIV1 and hPIV3 Bind Oligosaccharides with α2-3-Linked Sialic Acids That Are Distinct from Those Bound by H5 Avian Influenza Virus Hemagglutinin. *Journal of Virology*, *81*(15), 8341–8345. http://doi.org/10.1128/JVI.00718-07

Andberg, M., Penttilä, M., & Saloheimo, M. (2015). Swollenin from *Trichoderma reesei* exhibits hydrolytic activity against cellulosic substrates with features of both endoglucanases and cellobiohydrolases. *Bioresource Technology*, *181*, 105–113. http://doi.org/10.1016/j.biortech.2015.01.024

Archer, D. B., & Peberdy, J. F. (1997). The molecular biology of secreted enzyme production by fungi. *Critical Reviews in Biotechnology*, *17*(4), 273-306. http://dx.doi.org/10.3109/07388559709146616

Arnold, J., Wormald, M., Sim, R., Rudd, P., & Dwek, R. (2007). The impact of glycosylation on the biological function and structure of human immunoglobulins. *Annual Review of Immunology, 25*(1), 21-50. doi:10.1146/annurev.immunol.25.022106.141702

Aro, N., Saloheimo, A., Ilmén, M., & Penttilä, M. (2001). ACEII, a novel transcriptional activator involved in regulation of cellulase and xylanase genes of *Trichoderma reesei*. *The Journal of Biological Chemistry*, *276*(26), 24309–24314. http://doi.org/10.1074/jbc.M003624200

Aw, R., & Polizzi, K. M. (2013). Can too many copies spoil the broth? *Microbial Cell Factories*, *12*, 128. http://doi.org/10.1186/1475-2859-12-128

Baboval, T., Koul, O., & Smith, F.I. (2000). N-glycosylation site occupancy of rat α-1, 3-fucosyltransferase IV and the effect of glycosylation on enzymatic activity. *Biochimica et*

Biophysica Acta (BBA) - General Subjects, 1475(3), 383–389. http://doi.org/10.1016/S0304-4165(00)00094-5

Bailey, M. J., Askolin, S., Horhammer, N., Tenkanen, M., Linder, M., Penttilä, M., & Nakari-Setälä, T. (2002). Process technological effects of deletion and amplification of hydrophobins I and II in transformants of *Trichoderma reesei*. *Applied Microbiology and Biotechnology*, *58*(5), 721-727. doi:10.1007/s00253-002-0966-z

Bailey, M. J., & Tähtiharju, J. (2003). Efficient cellulase production by *Trichoderma reesei* in continuous cultivation on lactose medium with a computer-controlled feeding strategy. *Applied Microbiology and Biotechnology, 62*(2), 156-162. doi:10.1007/s00253-003-1276-9

Baker, K. N., Rendall, M. H., Patel, A., Boyd, P., Hoare, M., Freedman, R. B., & James, D. C. (2017). Rapid monitoring of recombinant protein products: a comparison of current technologies. *Trends in Biotechnology, 20*(4), 149–156. http://doi.org/10.1016/S0167-7799(01)01914-X

Bali, H. (2012). Detection and identification of extracellular proteases in *Trichoderma reesei* (Masters thesis, Macquarie University, Sydney).

Becker, D. J., & Lowe, J. B. (2003). Fucose: biosynthesis and biological function in mammals. *Glycobiology, 13*(7), 41R-53R. doi: 10.1093/glycob/cwg054

Bencúrová, M., Rendić, D., Fabini, G., Kopecky, E-M., Altmann, F., & Wilson, I. B. H. (2003). Expression of eukaryotic glycosyltransferases in the yeast *Pichia pastoris*. *Biochimie, 85*(3-4), 413-422. http://doi.org/10.1016/S0300-9084(03)00072-5

Benitez, J. A., Silva, A. J., & Finkelstein, R. A. (2001). Environmental Signals Controlling Production of Hemagglutinin/Protease in *Vibrio cholerae*. *Infection and Immunity, 69*(10), 6549–6553. http://doi.org/10.1128/IAI.69.10.6549-6553.2001

Benz, I., & Schmidt, M. A. (2002). Never say never again: protein glycosylation in pathogenic bacteria. *Molecular Microbiology, 45*(2), 267–276. doi:10.1046/j.1365-2958.2002.03030.x

Bergquist, P., Te'o, V., Gibbs, M., Cziferszky, A., de Faria, F. P., Azevedo, M., & Nevalainen, H. (2002). Expression of xylanase enzymes from thermophilic microorganisms in fungal hosts. *Extremophiles*, *6*(3), 177–184. http://doi.org/10.1007/s00792-001-0252-5

Bird, L. E., Rada, H., Verma, A., Gasper, R., Birch, J., Jennions, M., … Owens, R. J. (2015). Green Fluorescent Protein-based Expression Screening of Membrane Proteins in *Escherichia coli*. *Journal of Visualized Experiments*, (95), 52357. http://doi.org/10.3791/52357

Bischof, R., & Seiboth, B. (2014). Chapter 12 – Molecular Tools for Strain Improvement of *Trichoderma spp*. In Biotechnology and Biology of *Trichoderma*. Elsevier. http://doi.org/10.1016/B978-0-444-59576-8.00012-6

Bischof, R. H., Ramoni, J., & Seiboth, B. (2016). Cellulases and beyond: the first 70 years of the enzyme producer *Trichoderma reesei*. *Microbial Cell Factories*, *15*(1), 106. http://doi.org/10.1186/s12934-016-0507-6

Boruah, B. M., Kadirvalraj, R., Wang, S., Ramiah, A., Wood, Z. A., & Moremen, K. W. (2016). Crystal structure and mutagenesis studies of mammalian fucosyltransferase-9 (FUT9) reveals the catalytic core and substrate specificity. *The FASEB Journal*, *30*(1), lb119. Retrieved from http://www.fasebj.org/content/30/1_Supplement/lb119.abstract

Broekhuijsen, M. P., Mattern, I. E., Contreras, R., Kinghorn, J. R., & van den Hondel, C. A. (1993). Secretion of heterologous proteins by *Aspergillus niger*: production of active human interleukin-6 in a protease-deficient mutant by KEX2-like processing of a glucoamylase-hIL6 fusion protein. *Journal of biotechnology*, *31*(2), 135-145. https://doi.org/10.1016/0168-1656(93)90156-H

Boraston, A. B., Warren, R. A., & Kilburn, D. G. (2001). Glycosylation by *Pichia pastoris* decreases the affinity of a family 2a carbohydrate-binding module from *Cellulomonas fimi*: a functional and mutational analysis. *Biochemical Journal*, *358*(Pt 2), 423–430. doi: 10.1042/bj3580423

Borsig, L., Ianov, S.X., Herrmann, G.F., Kragl, U., WanUdrey, C. & Berger, E.G. (1995). Scaled-up expression of human α-2,6(N)sialyltransferase in *Saccharomyces cerevisiae*. *Biochemical and Biophysical Research Communications, 210*(1), 14-20. https://doi.org/10.1006/bbrc.1995.1621

Breen, K. C. (2002). The role of protein glycosylation in the control of cellular N-sialyltransferase activity. *FEBS Letters, 517*(1-3), 215-218. http://doi.org/10.1016/S0014-5793(02)02626-1

Breton, C., Oriol, R., & Imberty, A. (1998). Conserved structural features in eukaryotic and prokaryotic fucosyltransferases. *Glycobiology*, *8*(1), 87–94. https://doi.org/10.1093/glycob/8.1.87

Brown, J. S., Aufauvre-Brown, A., & Holden, D. W. (1998). Insertional mutagenesis of *Aspergillus fumigatus*. *Molecular and General Genetics, 259*(3), 327–335. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/9749676

Bundock, P., den Dulk-Ras, A., Beijersbergen, A., & Hooykaas, P. J. (1995). Trans-kingdom T-DNA transfer from *Agrobacterium tumefaciens* to *Saccharomyces cerevisiae*. *The EMBO Journal*, *14*(13), 3206–3214. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC394382/

Cailleau-Thomas, A., Coullin, P., Candelier, J. J., Balanzino, L., Mennesson, B., Oriol, R., & Mollicone, R. (2000). FUT4 and FUT9 genes are expressed early in human embryogenesis. *Glycobiology*, *10*(8), 789–802. https://doi.org/10.1093/glycob/10.8.789

Cao, Y., Zheng, F., Wang, L., Zhao, G., Chen, G., Zhang, W. & Liu, W. (2017). Rce1, a novel transcriptional repressor, regulates cellulase gene expression by antagonizing the transactivator Xyr1 in *Trichoderma reesei*. *Molecular Microbiology*. Accepted Author Manuscript. doi:10.1111/mmi.13685

Cameron, H. S., Szczepaniak, D., & Weston, B.W. (1995). Expression of human chromosome 19p α(1,3)-fucosyltransferase genes in normal tissues. Alternative splicing, polyadenylation, and isoforms. *Journal of Biological Chemistry*, *270*(34), 20112–20122. doi: 10.1074/jbc.270.34.20112

Castro-Chavez, F. (2011). Most Used Codons per Amino Acid and per Genome in the Code of Man Compared to Other Organisms According to the Rotating Circular Genetic Code. *NeuroQuantology : An Interdisciplinary Journal of Neuroscience and Quantum Physics*, *9*(4), 500. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3445418/

Chakraborty, B. N., Patterson, N. A., & Kapoor, M. (1991). An electroporation-based system for high-efficiency transformation of germinated conidia of filamentous fungi. *Canadian Journal of Microbiology*, *37*(11), 858–863. doi:10.1139/m91-147

Challener, C. A. (2016). Controlling Glycosylation for Improved Product Quality. *BioPharm International*, *29*(6), 32-34. Retrieved from http://www.biopharminternational.com/controlling-glycosylation-improved-product-quality

Cherry, J. R., & Fidantsef, A. L. (2003). Directed evolution of industrial enzymes: an update. *Current opinion in biotechnology*, *14*(4), 438-443. http://doi.org/10.1016/S0958-1669(03)00099-5

Chaves Barreto, C., Cardoso Alves, L., Lima Aragão, F. J., Rech, E., Schrank, A., & Henning Vainstein, M. (1997). High frequency gene transfer by microprojectile bombardment of intact conidia from the entomopathogenic fungus *Paecilomyces fumosoroseus*. *FEMS Microbiology Letters*, *156*(1), 95–99. http://doi.org/10.1111/j.1574-6968.1997.tb12711.x

Chen, S., & Wayman, M. (1993). Use of sorbose to enhance cellobiase activity in a *Trichoderma reesei* cellulase system produced on wheat hydrolysate. *Biotechnology Techniques*, *7*(5), 345–350. http://doi.org/10.1007/BF00152539

Christensen, L. L., Jensen, U. B., Bross, P., & Orntoft, T. F. (2000). The C-terminal N-glycosylation sites of the human α1,3/4-fucosyltransferase III, -V, and -VI (hFucTIII, - V, and -VI) are necessary for the expression of full enzyme activity. *Glycobiology*, *10*(9), 931–939. https://doi.org/10.1093/glycob/10.9.931

Clincke, M.-F., Guedon, E., Yen, F. T., Ogier, V., & Goergen, J.-L. (2011). Characterization of metalloprotease and serine protease activities in batch CHO cell cultures: control of human recombinant IFN-γ proteolysis by addition of iron citrate. *BMC Proceedings*, *5*(Suppl 8), P115. http://doi.org/10.1186/1753-6561-5-S8-P115

Conesa, A., Punt, P. J., van Luijk, N., & van den Hondel, C. A. M. J. J. (2001). The secretion pathway in filamentous fungi: a biotechnological view. *Fungal Genetics and Biology*, *33*(3), 155–171. http://doi.org/10.1006/fgbi.2001.1276

Contreras, R., Carrez, D., Kinghorn, J. R., van den Hondel, C. A. M. J. J., & Fiers, W. (1991). Efficient KEX2-like processing of a glucoamylase-interleukin-6 fusion protein by *Aspergillus*

nidulans and secretion of mature interleukin-6. *Biotechnology (NY), 9*(4), 378–381. doi: 10.1038/nbt0491-378.

Costa, J., Grabenhorst, E., Nimtz, M., & Conradt, H. S. (1997). Stable Expression of the Golgi Form and Secretory Variants of Human Fucosyltransferase III from BHK-21 Cells: PURIFICATION AND CHARACTERIZATION OF AN ENGINEERED TRUNCATED FORM FROM THE CULTURE MEDIUM. *Journal of Biological Chemistry, 272*(17), 11613–11621. http://doi.org/10.1074/jbc.272.17.11613

Costache, M., Apoil, P. A., Cailleau, A., Elmgren, A., Larson, G., Henry, S., … Mollicone, R. (1997). Evolution of fucosyltransferase genes in vertebrates. *Journal of Biological Chemistry, 272*(47), 29721–29728. doi: 10.1074/jbc.272.47.29721

Coutinho, P. M., Deleury, E., Davies, G. J., & Henrissat, B. (2003). An Evolving Hierarchical Family Classification for Glycosyltransferases. *Journal of Molecular Biology, 328*(2), 307-317. http://doi.org/10.1016/S0022-2836(03)00307-3.

Cuervo, P., Mesquita-Rodrigues, C., d'Avila Levy, C. M., Britto, C., Pires, F. A., Gredilha, R., … Jesus, J. B. (2008). Serine protease activities in *Oxysarcodexia thornax* (Walker) (Diptera: Sarcophagidae) first instar larva. *Memórias do Instituto Oswaldo Cruz, 103*(5), 504-506. https://dx.doi.org/10.1590/S0074-02762008000500018

Cui, Y. Q., Van der Lans, R. G. J. M., & Luyben, K. C. A. M. (1997). Effect of agitation intensities on fungal morphology of submerged fermentation. *Biotechnology and Bioengineering, 55*(5), 715–726. doi: 10.1002/(SICI)1097-0290(19970905)55:5<715::AID-BIT2>3.0.CO;2-E

Cui, Y. Q., Van der Lans, R. G. J. M., & Luyben, K. C. A. M. (1998). Effects of dissolved oxygen tension and mechanical forces on fungal morphology in submerged fermentation.

Biotechnology and Bioengineering, 57(4), 409–419. doi: 10.1002/(SICI)1097-0290(19980220)57:4<409::AID-BIT4>3.0.CO;2-Q

Curach, N. C. (2005). An investigation into the *hex1* gene and gene promoter for the enhancement of protein production in *Trichoderma reesei*. (PhD Thesis, Macquarie University, Sydney).

Dashtban, M., Buchkowski, R., & Qin, W. (2011). Effect of different carbon sources on cellulase production by *Hypocrea jecorina* (*Trichoderma reesei)* strains. *International journal of biochemistry and molecular biology, 2*(3), 274-286. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3193291/

de Faria, F. P., Te'o, V. S., Bergquist, P. L., Azevedo, M. O., & Nevalainen, K. M. (2002). Expression and processing of a major xylanase (XYN2) from the thermophilic fungus *Humicola grisea var. thermoidea* in *Trichoderma reesei*. *Letters in applied microbiology, 34*(2), 119-123. 10.1046/j.1472-765x.2002.01057.x

De Giorgi, F. De, Brini, M., Bastianutto, C., Marsault, R., Montero, M., Pizzo, P., … Rizzuto, R. (1996). Targeting aequorin and green fluorescent protein to intracellular organelles. *Gene, 173*(1), 113–117. http://doi.org/https://doi.org/10.1016/0378-1119(95)00687-7

de Groot, M. J., Bundock, P., Hooykaas, P. J., & Beijersbergen, A. G. (1998). *Agrobacterium tumefaciens*-mediated transformation of filamentous fungi. *Nature Biotechnology, 16*(9), 839–42. doi:10.1038/nbt0998-839

Delorme, E., Lorenzini, T., Giffin, J., Martin, F., Jacobsen, F., Boone, T., & Elliott, S. (1992). Role of glycosylation on the secretion and biological activity of erythropoietin. *Biochemistry, 31*(41), 9871–9876. doi: 10.1021/bi00156a003

Demolder, J., Saelens, X., Penttilä, M., Fiers, W. & Contreras, R. (1994). KEX2-like processing of glucoamylase-interleukin 6 and cellobiohydrolase-interleukin 6 fusion proteins by *Trichoderma reesei*. 2nd European Conference on Fungal Genetics, Lunteren. Abstract B38.

Deshpande, N., Wilkins, M. R., Packer, N., & Nevalainen, H. (2008). Protein glycosylation pathways in filamentous fungi. *Glycobiology*, *18*(8), 626–637. http://doi.org/10.1093/glycob/cwn044

de Vries, T., Knegtel, R. M., Holmes, E. H., & Macher, B. A. (2001). Fucosyltransferases: structure/function studies. *Glycobiology*, *11*(10), 119R–128R. http://doi.org/10.1093/glycob/11.10.119R

Diaz-Nido, J., Armas-Portela, R., & Avila, J. (1991). Addition of protease inhibitors to culture medium of neuroblastoma cells induces both neurite outgrowth and phosphorylation of microtubule-associated protein MAP-1B. *Journal of Cell Science*, *98*(3), 409–414. Retrieved from http://jcs.biologists.org/content/98/3/409

Dienes, D., Börjesson, J., Hägglund, P., Tjerneld, F., Lidén, G., Réczey, K., & Stålbrand, H. (2007). Identification of a trypsin-like serine protease from *Trichoderma reesei* QM9414. *Enzyme and Microbial Technology*, *40*(5), 1087–1094. http://doi.org/10.1016/j.enzmictec.2006.08.013

Dodd, S. L., Lieckfeldt, E., & Samuels, G. J. (2003). *Hypocrea atroviridis sp. nov.*, the teleomorph of *Trichoderma atroviride*. *Mycologia, 95*(1), 27-40. doi: 10.2307/3761959

Domingues, F. C., Queiroz, J. A., Cabral, J. M., & Fonseca, L. P. (2000). The influence of culture conditions on mycelial structure and cellulase production by *Trichoderma reesei* Rut

C30. *Enzyme and Microbial Technology*, *26*(5-6), 394-401. http://doi.org/10.1016/S0141-0229(99)00166-0

Druzhinina, I. S., Shelest, E., & Kubicek, C. P. (2012). Novel traits of *Trichoderma* predicted through the analysis of its secretome. *FEMS Microbiology Letters*, *337*(1), 1–9. http://doi.org/10.1111/j.1574-6968.2012.02665.x

Duellman, T., Burnett, J., & Yang, J. (2015). Quantitation of secreted proteins using mCherry fusion constructs and a fluorescent microplate reader. *Analytical Biochemistry*, *473*, 34–40. http://doi.org/10.1016/j.ab.2014.12.010

Durand, H., Clanet, M., & Tiraby, G. (1988). Genetic improvement of *Trichoderma reesei* for large scale cellulase production. *Enzyme and Microbial Technology 10*(6), 341-346. https://doi.org/10.1016/0141-0229(88)90012-9

Durocher, Y., & Butler, M. (2009). Expression systems for therapeutic glycoprotein production. *Current Opinion in Biotechnology*, *20*(6), 700-707. http://doi.org/10.1016/j.copbio.2009.10.008

Ensembl (http://www.ensembl.org)

Egrie, J. C., & Browne, J. K. (2001). Development and characterization of novel erythropoiesis stimulating protein (NESP). *British Journal of Cancer*, *84*(Suppl 1), 3–10. http://doi.org/10.1054/bjoc.2001.1746

Ekici, Ö. D., Paetzel, M., & Dalbey, R. E. (2008). Unconventional serine proteases: Variations on the catalytic Ser/His/Asp triad configuration. *Protein Science : A Publication of the Protein Society*, *17*(12), 2023–2037. http://doi.org/10.1110/ps.035436.108

Ene, I. V., Walker, L. A., Schiavone, M., Lee, K. K., Martin-Yken, H., Dague, E., … Brown, A. J. P. (2015). Cell Wall Remodeling Enzymes Modulate Fungal Cell Wall Elasticity and Osmotic Stress Resistance. *mBio*, *6*(4), e00986–15. http://doi.org/10.1128/mBio.00986-15

Fungaro, M. H. P., Rech, E., Muhlen, G. S., Vainstein, M. H., Pascon, R. C., de Queiroz, M. V, … de Azevedo, J. L. (1995). Transformation of *Aspergillus nidulans* by microprojectile bombardment on intact conidia. *FEMS Microbiology Letters*, *125*(2), 293–297. http://doi.org/http://dx.doi.org/10.1016/0378-1097(94)00512-P

Gallet, F., Vaujour, H., Petit, J. M., Maftah, A., Oulmouden, A., Oriol, R., … Julien, R. (1998). Heterologous expression of an engineered truncated form of human Lewis fucosyltransferase (Fuc-TIII) by the methylotropic yeast *Pichia pastoris*. *Glycobiology*, *8*(9), 919-925. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/9675225

GenBank (https://www.ncbi.nlm.nih.gov/genbank/)

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*(6), 323-328. http://dx.doi.org/10.1007/s002940000166

Gems, D., Johnstone, I. L., & Clutterbuck, A. J. (1991). An autonomously replicating plasmid transforms *Aspergillus nidulans* at high frequency. *Gene*, *98*(1), 61–67. http://doi.org/10.1016/0378-1119(91)90104-J

Gersten, K. M., Natsuka, S., Trinchera, M., Petryniak, B., Kelly, R. J., Hiraiwa, N., …. Lowe, J. B. (1995). Molecular cloning, expression, chromosomal assignment, and tissue-specific expression of a murine a-(1,3)-fucosyltransferase locus corresponding to the human ELAM-1 ligand fucosyltransferase. *Journal of Biological Chemistry*, *270*(42), 25047–25056. doi:10.1074/jbc.270.42.25047

Ghaderi, D., Zhang, M., Hurtado-Ziola, N. & Varki, A. (2012). Production platforms for biotherapeutic glycoproteins. Occurrence, impact, and challenges of non-human sialylation. *Biotechnology and Genetic Engineering Reviews, 28*, 147–175. doi:10.5661/bger-28-147

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*(1), 17–48. http://dx.doi.org/10.1080/07388550091144177

Gomes-Barcellos, F., Pelegrinelli-Fungaro, M. H., Furlaneto, M. C., Lejeune, B., Pizzirani-Kleiner, A. A., & Azevedo, J. L. (1998). Genetic analysis of *Aspergillus nidulans* unstable transformants obtained by the biolistic process. *Canadian Journal of Microbiology, 44*(12), 1137-1141. doi:10.1139/w98-213

González-Vera, J. A., & Morris, M. C. (2015). Fluorescent Reporters and Biosensors for Probing the Dynamic Behavior of Protein Kinases. *Proteomes, 3*(4), 369–410. http://doi.org/10.3390/proteomes3040369

Gouka, R. J., Punt, P. J., & van den Hondel, C. A. M. J. J. (1997). Efficient production of secreted proteins by *Aspergillus*: progress, limitations and prospects. *Applied Microbiology and Biotechnology, 47*(1), 1-11. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/9035405

Gruber, F., Visser, J., Kubicek, C. P., & Graaff, L. H. (1990). Cloning of the *Trichoderma reesei pyr*G gene and its use as a homologous marker for a high-frequency transformation system. *Current genetics, 18*(5), 447-451. doi:10.1007/BF00309915

Gryshyna, A., Kautto, L., Peterson, R., & Nevalainen, H. (2016). On the Safety of Filamentous Fungi with Special Emphasis on *Trichoderma reesei* and Products Made by Recombinant Means. In M. Schmoll & C. Dattenböck (Eds.), *Gene Expression Systems in Fungi:*

Advancements and Applications (pp. 441–457). Cham: Springer International Publishing. http://doi.org/10.1007/978-3-319-27951-0_20

Guangtao, Z., Hartl, L., Schuster, A., Polak, S., Schmoll, M., Wang, T., … Seiboth, B. (2009). Gene targeting in a nonhomologous end joining deficient *Hypocrea jecorina*. *Journal of Biotechnology, 139*(2), 146–151. http://doi.org/10.1016/j.jbiotec.2008.10.007

Haab, D., Hagspiel, K., Szakmary, K., & Kubicek, C.P. (1990). Formation of the extracellular proteases from *Trichoderma reesei* QM 9414 involved in cellulase degradation. *Journal of Biotechnology, 16*(3-4), 187–198. https://doi.org/10.1016/0168-1656(90)90035-A

Haki, G. D., & Rakshit, S. K. (2003). Developments in industrially important thermostable enzymes: a review. *Bioresource technology, 89*(1), 17-34. http://doi.org/10.1016/S0960-8524(03)00033-6

Harkki, A., Mäntylä, A., Penttilä, M., Muttilainen, S., Bühler, R., Suominen, P., … Nevalainen, H. (1991). Genetic engineering of *Trichoderma* to produce strains with novel cellulase profiles. *Enzyme and Microbial Technology, 13*(3), 227-233. https://doi.org/10.1016/0141-0229(91)90133-U

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*(6), 596-603. doi:10.1038/nbt0689-596

Harrison, M. J., Nouwens, A. S., Jardine, D. R., Zachara, N. E., Gooley, A. A., Nevalainen, H., & Packer, N. H. (1998). Modified glycosylation of cellobiohydrolase I from a high cellulase-producing mutant strain of *Trichoderma reesei*. *European Journal of Biochemistry, 256*(1), 119–127. doi: 10.1046/j.1432-1327.1998.2560119.x

Hartl, L., & Seiboth, B. (2005). Sequential gene deletions in *Hypocrea jecorina* using a single blaster cassette. *Current Genetics*, *48*(3), 204–211. http://doi.org/10.1007/s00294-005-0011-8

Hazell, B. W., Te'o, V.S., Bradner, J. R., Bergquist, P. L., & Nevalainen, K. M. (2000). Rapid transformation of high cellulase-producing mutant strains of *Trichoderma reesei* by microprojectile bombardment. *Letters in Applied Microbiology, 30*(4), 282-286. doi:10.1046/j.1472-765x.2000.00715.x

He, R., Zhang, C., Guo, W., Wang, L., Zhang, D., & Chen, S. (2013). Construction of a plasmid for heterologous protein expression with a constitutive promoter in *Trichoderma reesei*. *Plasmid*, *70*(3), 425–429. http://doi.org/10.1016/j.plasmid.2013.09.004

Heilmann, C. J., Sorgo, A. G., Mohammadi, S., Sosinska, G. J., de Koster, C. G., Brul, S., … Klis, F. M. (2013). Surface Stress Induces a Conserved Cell Wall Stress Response in the Pathogenic Fungus *Candida albicans*. *Eukaryotic Cell*, *12*(2), 254–264. http://doi.org/10.1128/EC.00278-12

Herpoël-Gimbert, I., Margeot, A., Dolla, A., Jan, G., Molle, D., Lignon, S., … Asther, M. (2008). Comparative secretome analyses of two *Trichoderma reesei* RUT-C30 and CL847 hypersecretory strains. *Biotechnol Biofuels*, *1*(1), 18. http://doi.org/1754-6834-1-18 [pii]\r10.1186/1754-6834-1-18

Hidari, K. I., Horie, N., Murata, T., Miyamoto, D., Suzuki, T., Usui, T., & Suzuki, Y. (2005). Purification and characterization of a soluble recombinant human ST6Gal I functionally expressed in *Escherichia coli*. *Glycoconjugate Journal, 22*(1–2), 1–11. doi: 10.1007/s10719-005-0845-9

Holmes, E. H., Yen, T. Y., Thomas, S., Joshi, R., Nguyen, A., Long, T., … Macher, B. A. (2000). Human α1,3/4 fucosyltransferases: characterization of highly conserved cysteine residues and N- linked glycosylation sites. *Journal of Biological Chemistry*, *275*(32), 24237–24245. doi: 10.1074/jbc.M000888200

Hong, J., Ye, X., Wang, Y., & Zhang, Y. H. (2008). Bioseparation of recombinant cellulose-binding module proteins by affinity adsorption on an ultra-high-capacity cellulosic adsorbent. *Analytica Chimica Acta*, *621*(2), 193–9. doi:10.1016/j.aca.2008.05.041

Hooper, L. V., Xu, J., Falk, P. G., Midtvedt, T., & Gordon, J. I. (1999). A molecular sensor that allows a gut commensal to control its nutrient foundation in a competitive ecosystem. *Proceedings of the National Academy of Sciences of the United States of America*, *96*(17), 9833–9838. doi: 10.1073/pnas.96.17.9833

Hooper, L.V., & Gordon, J. I. (2001). Glycans as legislators of host-microbial interactions: spanning the spectrum from symbiosis to pathogenicity. *Glycobiology*, *11*(2), 1R–10R. https://doi.org/10.1093/glycob/11.2.1R

Huang, L., Pike, D., Sleat, D. E., Nanda, V., & Lobel, P. (2014). Potential Pitfalls and Solutions for Use of Fluorescent Fusion Proteins to Study the Lysosome. *PLoS ONE*, *9*(2), e88893. https://doi.org/10.1371/journal.pone.0088893

Ihara, H., Ikeda, Y., Toma, S., Wang, X., Suzuki, T., Gu, J., … Taniguchi, N. (2007). Crystal structure of mammalian α1,6-fucosyltransferase, FUT8. Glycobiology, 17(5), 455-466. doi:10.1093/glycob/cwl079

Ilias, M. & Hoq, M. (1998). Short Communication: Effect of agitation rate on the growth and production of xylanase free of cellulase by *Thermomyces lanuginosus* MH4 in bioreactor.

World Journal of Microbiology and Biotechnology, 14(5), 765-767. doi:10.1023/A:1008837410130

Ilmén, M., Onnela, M. L., Klemsdal, S., Keränen, S., & Penttilä, M. (1996a). Functional analysis of the cellobiohydrolase I promoter of the filamentous fungus *Trichoderma reesei*. *Molecular and General Genetics, 253*(3), 303-314. doi:10.1007/PL00008597

Ilmén, M., Thrane, C., & Penttilä, M. (1996b). The glucose repressor gene *cre1* of *Trichoderma*: Isolation and expression of a full-length and a truncated mutant form. *Molecular and General Genetics, 251*(4), 451-460. doi:10.1007/BF02172374

Ilmén, M., Saloheimo, A., Onnela, M. L., & Penttilä, M. E. (1997). Regulation of cellulase gene expression in the filamentous fungus *Trichoderma reesei*. *Applied and Environmental Microbiology, 63*(4), 1298-1306. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC168424/

Inoue, H., Nojima, H., & Okayama, H. (1990). High efficiency transformation of *Escherichia coli* with plasmids. *Gene, 96*(1), 23-28. http://doi.org/10.1016/0378-1119(90)90336-P

Isozaki, T., Ruth, J. H., Amin, M. A., Campbell, P. L., Tsou, P.-S., Ha, C. M., … Koch, A. E. (2014). Fucosyltransferase 1 mediates angiogenesis, cell adhesion and rheumatoid arthritis synovial tissue fibroblast proliferation. *Arthritis Research & Therapy, 16*(1), R28. http://doi.org/10.1186/ar4456

International Union of Biochemistry and Molecular Biology (http://www.chem.qmul.ac.uk/iubmb/enzyme/)

Iwashita, K. (2002). Recent studies of protein secretion by filamentous fungi. *Journal of Bioscience and Bioengineering, 94*(6), 530–5. https://doi.org/10.1016/S1389-1723(02)80191-8

Iwakuma, H., Koyama, Y., Miyachi, A., Nasukawa, M., Matsumoto, H., Yano, S., … Kasumi, T. (2015). Generation of a glucose de-repressed mutant of *Trichoderma reesei* using disparity mutagenesis. *Bioscience, Biotechnology and Biochemistry, 8451*, 1–7. http://doi.org/10.1080/09168451.2015.1104236

Jampala, P., Murugan, P., Ramanujam, S., & Uppuluri, K. B. (2015). Investigation on the Effect of Carbon and Nitrogen Sources for the Production of Cellulosome by *Trichoderma Reesei* NCIM 1186 Using Saturated Placket Burman Design. *Biosciences Biotechnology Research Asia, 12*(2), 1577-1586. doi: 10.13005/bbra/1819

Jeenes, D. J., Mackenzie, D. A., Roberts, I. N., & Archer, D. B. (1991). Heterologous protein production by filamentous fungi. *Biotechnology & genetic engineering reviews, 9*, 327-367. http://dx.doi.org/10.1080/02648725.1991.10750006

Jeenes, D. J., Marczinke, B., MacKenzie, D. A., & Archer, D. B. (1993). A truncated glucoamylase gene fusion for heterologous protein secretion from *Aspergillus niger*. *FEMS Microbiology Letters, 107*(2-3), 267-271. doi: 10.1111/j.1574-6968.1993.tb06041.x

Jensen, D. F., & Ashulz, A. (2004). Exploitation of GFP-technology with filamentous fungi. In: Handbook of Fungal Biotechnology. vol. 20. (Eds.) D. K. Arora. Marcel and Dekker. New York: 441-451.

Jeoh, T., Michener, W., Himmel, M. E., Decker, S. R., & Adney, W. S. (2008). Implications of cellobiohydrolase glycosylation for use in biomass conversion. *Biotechnology for biofuels, 1*(1), 10. doi: 10.1186/1754-6834-1-10

Jia, B., & Jeon, C. O. (2016). High-throughput recombinant protein expression in *Escherichia coli*: current status and future perspectives. *Open Biology*, *6*(8), 160196. http://doi.org/10.1098/rsob.160196

Joint Genome Institute (http://jgi.doe.gov)

Jørgensen, M. S., Skovlund, D. A., Johannesen, P. F., & Mortensen, U. H. (2014) A novel platform for heterologous gene expression in *Trichoderma reesei* (teleomorph *Hypocrea jecorina*). *Microbial Cell Factories, 13,* 33. doi:10.1186/1475-2859-13-33

Kadam, K. L., & Keutzer, W. J. (1995). Enhancement in cellulase production by *Trichoderma reesei* Rut-C30 due to citric acid. *Biotechnology Letters, 17*(10), 1111–1114. http://doi.org/10.1007/BF00143112

Kaneko, M., Kudo, T., Iwasaki, H., Ikehara, Y., Nishihara, S., Nakagawa, S., … Narimatsu, H. (1999). α1,3-Fucosyltransferase IX (Fuc-TIX) is very highly conserved between human and mouse; molecular cloning, characterization and tissue distribution of human Fuc-TIX. *FEBS Letters, 452*(3), 237–242. http://doi.org/10.1016/S0014-5793(99)00640-7

Kang, K., Wang, S., Lai, G., Liu, G., & Xing, M. (2013). Characterization of a novel swollenin from *Penicillium oxalicum* in facilitating enzymatic saccharification of cellulose. *BMC Biotechnology, 13,* 42. http://doi.org/10.1186/1472-6750-13-42

Karlsson, J., Saloheimo, M., Siika-aho, M., Tenkanen, M., & Penttila, M. (2001). Homologous expression and characterization of Cel61A (EG IV) of *Trichoderma reesei. European Journal of Biochemistry, 268*(4), 6498–6507. doi: 10.1046/j.0014-2956.2001.02605.x

Kato, T., Kako, N., Kikuta, K., Miyazaki, T., Kondo, S., Yagi, H., … Park, E. Y. (2017). N-Glycan Modification of a Recombinant Protein via Coexpression of Human Glycosyltransferases in Silkworm Pupae. *Scientific Reports, 7*(1), 1409. http://doi.org/10.1038/s41598-017-01630-6

Kelly, R. J., Rouquier, S., Giorgi, D., Lennon, G. G., & Lowe, J. B. (1995). Sequence and expression of a candidate for the human secretor blood group a(1,2)fucosyltransferase gene

(FUT2). Homozygosity for an enzyme-inactivating nonsense mutation commonly correlates with the non-secretor phenotype. *Journal of Biological Chemistry*, *270*(9), 4640-4649. doi: 10.1074/jbc.270.9.4640

Keränen, S., & Penttilä, M. (1995). Production of recombinant proteins in the filamentous fungus *Trichoderma reesei*. *Current Opinion in Biotechnology*, *6*(5), 534–537. http://doi.org/10.1016/0958-1669(95)80088-3

Khmelnitsky, Y. L. (2004). Current strategies for in vitro protein glycosylation. *Journal of Molecular Catalysis B: Enzymatic*, *31*(4–6), 73-81. http://doi.org/10.1016/j.molcatb.2004.07.002.

Klarskov, K., Piens, K., Stahlberg, J., Høj, P. B., Van Beeumen, J. & Claeyssens, M. (1997). Cellobiohydrolase I from *Trichoderma reesei*: identification of an active site nucleophile and additional information on sequence including the glycosylation pattern of the core protein. *Carbohydrate Research, 304*(2), 143–154. https://doi.org/10.1016/S0008-6215(97)00215-2

Klein, T. M., Wolf, E. D., Wu, R., & Sanford, J.C. (1987). High velocity mecroprojectiles for delivering nucleic acids into living cells. *Nature, 327*, 70-73. doi:10.1038/327070a0

Kontkanen, H., Westerholm-Parvinen, A., Saloheimo, M., Bailey, M., Rättö, M., Mattila, I., … Buchert, J. (2009). Novel *Coprinopsis cinerea* polyesterase that hydrolyzes cutin and suberin. *Applied and Environmental Microbiology*, *75*(7), 2148–57. doi: 10.1128/AEM.02103-08

Kopke, K., Hoff, B., & Kuck, U. (2010). Application of the *Saccharomyces cerevisiae* FLP/FRT recombination system in filamentous fungi for marker recycling and construction of knockout strains devoid of heterologous genes. *Applied and Environmental Microbiology*, *76*(14), 4664–4674. http://doi.org/10.1128/AEM.00670-10

Krappmann, S. (2007). Gene targeting in filamentous fungi: the benefits of impaired repair. *Fungal Biology Reviews, 21*(1), 25–29. http://doi.org/10.1016/j.fbr.2007.02.004

Kredics, L., Antal, Z., Szekeres, A., Hatvani, L., Manczinger, L., Vágvölgyi, C., & Nagy, E. (2005). Extracellular Proteases of *Trichoderma* Species. *Acta Microbiologica et Immunologica Hungarica, 52*(2), 169–184. http://doi.org/10.1556/AMicr.52.2005.2.3

Kreitman, R. J. (2003). Recombinant toxins for the treatment of cancer. *Current opinion in molecular therapeutics, 5*(1), 44–51. http://dx.doi.org/10.1517/14712598.2.8.785

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*(10), 753-763. doi: 10.1631/jzus.B0860015

Kubodera, T., Yamashita, N., & Nishimura, A. (2002). Transformation of *Aspergillus sp*. and *Trichoderma reesei* Using the Pyrithiamine Resistance Gene (*ptrA*) of *Aspergillus oryzae*. *Bioscience, Biotechnology, and Biochemistry, 66*(2), 404–406. http://doi.org/10.1271/bbb.66.404

Kück, U., & Hoff, B. (2010). New tools for the genetic manipulation of filamentous fungi. *Applied Microbiology and Biotechnology, 86*(1), 51–62. http://doi.org/10.1007/s00253-009-2416-7

Kurzatkowski, W., Torronen, A., Filipek, J., Mach, R. L., Herzog, P., Sowka, S., & Kubicek, C. P. (1996). Glucose-induced secretion of *Trichoderma reesei* xylanases. *Applied and environmental microbiology, 62*(8), 2859-2865. Retrieved from http://aem.asm.org/content/62/8/2859.long

Landowski, C. P., Huuskonen, A., Wahl, R., Westerholm-Parvinen, A., Kanerva, A., Hänninen, A. L., … Saloheimo, M. (2015). Enabling low cost biopharmaceuticals: A

systematic approach to delete proteases from a well-known protein production host *Trichoderma reesei*. *PLoS ONE*, *10*(8), 1–28. http://doi.org/10.1371/journal.pone.0134723

Landowski, C. P., Mustalahti, E., Wahl, R., Croute, L., Sivasiddarthan, D., Parvinen, A. W., … Saarinen, J. (2016). Enabling low cost biopharmaceuticals : high level interferon alpha - 2b production in *Trichoderma reesei*. *Microbial Cell Factories*, *15*(1), 104. http://doi.org/10.1186/s12934-016-0508-5

Landowski, C., Westerholm-Parvinen, A., & Saloheimo, M. (2017). Regulatory protein deficient *Trichoderma* cells and methods of use thereof. Google Patents. Retrieved from http://www.google.com/patents/WO2017025586A1?cl=en

Larsen, R. D., Ernst, L. K., Nair, R. P., & Lowe, J. B. (1990). Molecular cloning, sequence, and expression of a human GDP-L-fucose:beta-D-galactoside 2-alpha-L-fucosyltransferase cDNA that can form the H blood group antigen. *Proceedings of the National Academy of Sciences of the United States of America*, *87*(17), 6674–6678. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC54599/

Lauber, J., Handrick, R., Leptihn, S., Dürre, P., & Gaisser, S. (2015). Expression of the functional recombinant human glycosyltransferase GalNAcT2 in *Escherichia coli*. *Microbial Cell Factories*, *14*, 3. http://doi.org/10.1186/s12934-014-0186-0

Li, J., Wang, J., Wang, S., Xing, M., Yu, S., & Liu, G. (2012). Achieving efficient protein expression in *Trichoderma reesei* by using strong constitutive promoters. *Microbial Cell Factories*, *11*(1), 84. http://doi.org/10.1186/1475-2859-11-84

Li, C., Yang, Z., He Can Zhang, R., Zhang, D., Chen, S., & Ma, L. (2013). Effect of pH on cellulase production and morphology of Trichoderma reesei and the application in cellulosic

material hydrolysis. *Journal of Biotechnology*, *168*(4), 470–477. http://doi.org/10.1016/j.jbiotec.2013.10.003

Li, L., Liu, Y., Ma, C., Qu, J., Calderon, A. D., Wu, B., … Wang, P. G. (2015). Efficient Chemoenzymatic Synthesis of an N-glycan Isomer Library. *Chemical Science (Royal Society of Chemistry : 2010)*, *6*(10), 5652–5661. http://doi.org/10.1039/C5SC02025E

Lim, D., Hains, P., Walsh, B., Bergquist, P., & Nevalainen, H. (2001). Proteins associated with the cell envelope of *Trichoderma reesei*: a proteomic approach. *Proteomics*, *1*(7), 899–909. http://doi.org/10.1002/1615-9861(200107)1:7<899::AID-PROT899>3.0.CO;2-#

Liu, T., Wang, T., Li, X., & Liu, X. (2008). Improved heterologous gene expression in *Trichoderma reesei* by cellobiohydrolase I gene (*cbh1*) promoter optimization. *Acta Biochimica et Biophysica Sinica*, *40*(2), 158–165. http://doi.org/10.1111/j.1745-7270.2008.00388.x

Liu, R., Chen, L., Jiang, Y., Zhou, Z., & Zou, G. (2015). Efficient genome editing in filamentous fungus *Trichoderma reesei* using the CRISPR/Cas9 system. *Cell Discovery*, *1*, 15007. http://doi.org/10.1038/celldisc.2015.7

Lorito, M., Hayes, C. K., Di Pietro, A., & Harman, G. E. (1993) Biolistic transformation of *Trichoderma harzianum* and *Gliocladium virens* using plasmid and genomic DNA. *Current Genetics*, *24*(4), 329–356. doi:10.1007/BF00336788

Lowe, J. B., Kukowska-Latallo, J. F., Nair, R. P., Larsen, R. D., Marks, R. M., Macher, B.A., … Ernst, L. K. (1991). Molecular cloning of a human fucosyltransferase gene that determines expression of the Lewis X and VIM-2 epitopes but not ELAM-1 dependent cell adhesion. *Journal of Biological Chemistry*, *266*(6), 17467–17477. Retrieved from http://www.jbc.org/content/266/26/17467.long

Lowe, J. B. (1993). The blood group-specific human glycosyltransferases. *Bailliere's Clinical Haematology*, *6*(2), 465–492. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/8043935

Lubertozzi, D., & Keasling, J. D. (2009). Developing *Aspergillus* as a host for heterologous expression. *Biotechnology Advances*, *27*(1), 53–75. http://doi.org/10.1016/j.biotechadv.2008.09.001

Madjd, Z., Parsons, T., Watson, N. F., Spendlove, I., Ellis, I., & Durrant, L. G. (2005). High expression of Lewisy/b antigens is associated with decreased survival in lymph node negative breast carcinomas. *Breast Cancer Research*, *7*(5), R780–R787. http://doi.org/10.1186/bcr1305

Malissard, M., Zeng, S. & Berger, E. G. (1999). The yeast expression system for recombinant glycosyltransferases. *Glycoconjugate Journal*, *16*(2), 125-39. doi:10.1023/A:1007055525789

Malissard, M., Zeng, S., & Berger, E.G. (2000). Expression of functional soluble forms of human beta-1,4-galactosyltransferase I, alpha-2,6-sialyltransferase, and alpha-1,3-fucosyltransferase VI in the methylotrophic yeast *Pichia pastoris*. *Biochemical and Biophysical Research Communications*, *267*(1), 169-173. https://doi.org/10.1006/bbrc.1999.1946

Mandels, M. (1975). Microbial sources of cellulase. *Biotechnology and bioengineering symposium*, *5*, 81-105.

Mandels, M., Parrish, F. W., & Reese, E. T. (1962). Sophorose as an inducer of cellulase in *Trichoderma viride*. *Journal of bacteriology*, *83*(2), 400-408.

Mäntylä, A., Paloheimo, M. & Suominen, P. (1998). Industrial mutants and recombinant strains of *Trichoderma reesei*. In G. E. Harman & C. Kubicek (Eds), *Trichoderma and Gliogladium*, vol. 2 (pp. 291–304). London: Taylor and Francis.

Maras, M., De Bruyn, A., Schraml, J., Herdewijn, P., Claeyssens, M., Fiers, W. & Contreras, R. (1997). Structural characterization of N-linked oligosaccharides from cellobiohydrolase I secreted by the filamentous fungus *Trichoderma reesei* RUTC 30. *European Journal of Biochemistry*, *245*(3), 617–625. doi: 10.1111/j.1432-1033.1997.00617.x

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*(8), 2211–2215. doi: 10.1128/jb.178.8.2211-2215.1996

Markic, J., Jeroncic, A., Polancec, D., Bosnjak, N., Markotic, A., Mestrovic, J., & Culic, V. C. (2013). CD15s is a potential biomarker of serious bacterial infection in infants admitted to hospital. *European Journal of Pediatrics*, *172*(10), 1363–9. doi:10.1007/s00431-013-2047-y

Martinez, D., Berka, R. M., Henrissat, B., Saloheimo, M., Arvas, M., Baker, S. E., … Brettin, T. S. (2008). Genome sequencing and analysis of the biomass-degrading fungus *Trichoderma reesei* (syn. *Hypocrea jecorina*). *Nature Biotechnology*, *26*(5), 553–560. http://doi.org/10.1038/nbt1403

Meyer, V., Mueller, D., Strowig, T., & Stahl, U. (2003). Comparison of different transformation methods for *Aspergillus giganteus*. *Current Genetics*, *43*(5), 371–377. http://doi.org/10.1007/s00294-003-0406-3

Meyer, V. (2008). Genetic engineering of filamentous fungi - Progress, obstacles and future trends. *Biotechnology Advances*, *26*(2), 177–185. http://doi.org/10.1016/j.biotechadv.2007.12.001

Miettinen-Oinonen, A. (2004). *Trichoderma reesei* strains for production of cellulases for the textile industry. VTT Publications. Retrieved from http://www.vtt.fi/inf/pdf/publications/2004/P550.pdf

Miyauchi, S., Te'o, V. S., Bergquist, P. L., & Nevalainen, K. M. H. (2013). Expression of a bacterial xylanase in *Trichoderma reesei* under the *egl2* and *cbh2* glycosyl hydrolase gene promoters. *New Biotechnology, 30*(5), 523–530. http://doi.org/10.1016/j.nbt.2013.02.005

Miyauchi, S., Te'o, V. S. J., Nevalainen, K. M. H., & Bergquist, P. L. (2014). Simultaneous expression of the bacterial *Dictyoglomus thermophilum xyn*B gene under three different *Trichoderma reesei* promoters. *New Biotechnology, 31*(1), 98-103. http://doi.org/10.1016/j.nbt.2013.08.002

Miyoshi, E., Moriwaki, K., & Nakagawa, T. (2008). Biological Function of Fucosylation in Cancer Biology. *The Journal of Biochemistry, 143*(6), 725-9. http://doi.org/10.1093/jb/mvn011

Miyoshi, E., Noda, K., Yamaguchi, Y., Inoue, S., Ikeda, Y., Wang, W., … Taniguchi, N. (1999). The α1-6-fucosyltransferase gene and its biological significance, *Biochimica et Biophysica Acta (BBA) - General Subjects, 1473*(1), 9-20. http://doi.org/10.1016/S0304-4165(99)00166-X.

Mollicone, R., Moore, S. E., Bovin, N., Garcia-Rosasco, M., Candelier, J-J., Martinez-Duncker, I., & Oriol R. (2009). Activity, splice variants, conserved peptide motifs, and phylogeny of two new α1, 3-fucosyltransferase families (FUT10 and FUT11). *Journal of Biological Chemistry, 284*(7), 4723–4738. doi: 10.1074/jbc.M809312200

Montenecourt, B. S., & Eveleigh, D. E. (1977a). Preparation of mutants of *Trichoderma reesei* with enhanced cellulase production. *Applied and environmental microbiology*, *34*(6), 777-782. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC242747/

Montenecourt, B. S., & Eveleigh, D. E. (1977). Semiquantitative Plate Assay for Determination of Cellulase Production by *Trichoderma viride*. *Applied and Environmental Microbiology, 33*(1), 178–183. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC170619/

Montenecourt, B. S., & Eveleigh, D. E. (1979). *Selective Screening Methods for the Isolation of High Yielding Cellulase Mutants of Trichoderma reesei*. In Hydrolysis of Cellulose: Mechanisms of Enzymatic and Acid Catalysis (pp. 289-301). doi: 10.1021/ba-1979-0181.ch014

Mukhopadhyay, S. N., & Malik, R. K. (1980). Increased production of cellulase of *Trichoderma sp*. by pH cycling and temperature profiling. *Biotechnology and Bioengineering*, *22*, 2237-2250. doi: 10.1002/bit.260221104

Mukhopadhyay, S., & Nandi, B. (1999). Optimization of Cellulase Production by *Trichoderma reesei* ATCC 26921 Using a Simplified Medium on Water Hyacinth Biomass. *Journal of Scientific and Industrial Research, 58*(2), 107–111.

Nakayama, F., Nishihara, S., Iwasaki, H., Kudo, T., Okubo, R., Kaneko, M., … Narimatsu, H. (2001). CD15 Expression in Mature Granulocytes Is Determined by α1,3-Fucosyltransferase IX, but in Promyelocytes and Monocytes by α1,3-Fucosyltransferase IV. *Journal of Biological Chemistry, 276*(19), 16100-16106. doi: 10.1074/jbc.M007272200

Natsuka, S., Gersten, K. M., Zenita, K., Kannagi, R., & Lowe, J. B. (1994). Molecular cloning of a cDNA encoding a novel human leukocyte α1,3-fucosyltransferase capable of synthesizing

the sialyl Lewis X determinant. *Journal of Biological Chemistry*, *269*(24), 16789–16794. Retrieved from http://www.jbc.org/content/269/24/16789.long

Nevalainen, H. (1985). Genetic improvement of enzyme production in industrially important fungal strains. VTT, Technical Research Centre of Finland, publications 26, Espoo Finland.

Nevalainen, H., Lavygina, I., Neethling, D. & Packer, N. (1995). The biochemical nature of the cell envelope of a high cellulase-secreting mutant differs from that of the *Trichoderma reesei* wild type. *Journal of Biotechnology, 42*(1), 53–59. https://doi.org/10.1016/0168-1656(95)00064-W

Nevalainen, K. M. H., Palva, E. T., & Bailey, M. J. (1980). A high cellulase-producing mutant strain of *Trichoderma reesei*. *Enzyme and Microbial Technology, 2*(1), 59-60. https://doi.org/10.1016/0141-0229(80)90009-5

Nevalainen, H., Paloheimo, M., Miettinen-Oinonen, A., Torkkeli, T. K., Turunen, M., Fagerstrom, R., … Rambosek, J. (1994). Production of phytate degrading enzymes in *Trichoderma*. Google Patents. Retrieved from http://www.google.com/patents/EP0659215A1?cl=en

Nevalainen, H., Te'o, V. S., & Bergquist, P. L. (2005). Heterologous protein expression in filamentous fungi. *Trends in biotechnology, 23*(9), 468-474. http://dx.doi.org/10.1016/j.tibtech.2005.06.002

Nevalainen, H., & Peterson, R. (2014a). Making recombinant proteins in filamentous fungi-are we expecting too much? *Frontiers in Microbiology, 5*, 75. http://doi.org/10.3389/fmicb.2014.00075

Nevalainen, H., & Peterson, R. (2014b). *Heterologous Expression of Proteins in Trichoderma*. *Biotechnology and Biology of Trichoderma*. Amsterdam: Elsevier. http://doi.org/10.1016/B978-0-444-59576-8.00007-2

Nelson, D., & Cox, M. (2000). *Lehninger Principles of Biochemistry*. New York: Worth Publishers.

Nielsen, M. L., De Jongh, W. A., Meijer, S. L., Nielsen, J., & Mortensen, U. H. (2007). Transient marker system for iterative gene targeting of a prototrophic fungus. Applied and Environmental Microbiology, 73(22), 7240–7245. http://doi.org/10.1128/AEM.01839-07

Nielsen, J. B., Nielsen, M. L., & Mortensen, U. H. (2008). Transient disruption of non-homologous end-joining facilitates targeted genome manipulations in the filamentous fungus *Aspergillus nidulans*. *Fungal Genetics and Biology, 45*(3), 165–170. http://doi.org/10.1016/j.fgb.2007.07.003

Niemelä, R., Natunen, J., Majuri, M. L., Maaheimo, H., Helin, J., Lowe, J. B., … Renkonen, R. (1998). Complementary acceptor and site specificities of Fuc-TIV and Fuc-TVII allow effective biosynthesis of sialyl-TriLex and related polylactosamines present on counterreceptors of selectins. *Journal of Biological Chemistry, 273*(7), 4021–4026. doi: 10.1074/jbc.273.7.4021

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*(3), 247-254. https://doi.org/10.1016/S0965-1748(96)00092-6

Nuttall, J., Vine, N., Hadlington, J. L., Drake, P., Frigerio, L. & Ma, J. K.-C. (2002). ER–resident chaperone interactions with recombinant antibodies in transgenic plants. *European Journal of Biochemistry, 269*, 6042–6051. doi: 10.1046/j.1432-1033.2002.03302.x

Nykänen, M. J. (2002). Protein secretion in *Trichoderma reesei*. Expression, secretion and maturation of cellobiohydrolase I, barley cysteine proteinase and calf chymosin in Rut-C30. (PhD thesis, University of Jyväskylä, Jyväskylä).

Nyyssönen, E., Penttilä, M., Harkki, A., Saloheimo, A., Knowles, J.K.C., & Keränen, S. (1993). Efficient production of antibody fragments by the filamentous fungus *Trichoderma reesei*. *Nature Biotechnology, 11*(5), 591-595. doi:10.1038/nbt0593-591

Nyyssönen, E., & Keränen, S. (1995). Multiple roles of the cellulase CBHI in enhancing production of fusion antibodies by the filamentous fungus *Trichoderma reesei*. *Current genetics, 28*(1), 71-79.

Ouedraogo, J. P., Arentshorst, M., Nikolaev, I., Barends, S., & Ram, A. F. J. (2015). I-SceI-mediated double-strand DNA breaks stimulate efficient gene targeting in the industrial fungus *Trichoderma reesei*. *Applied Microbiology and Biotechnology, 99*(23), 10083–10095. http://doi.org/10.1007/s00253-015-6829-1

Ouedraogo, J. P., Arentshorst, M., Nikolaev, I., Barends, S., & Ram, A. F. J. (2016). I-SceI enzyme mediated integration (SEMI) for fast and efficient gene targeting in *Trichoderma reesei*. *Journal of Biotechnology*, *222*, 25–28. http://doi.org/10.1016/j.jbiotec.2016.02.012

Olempska-Beer, Z. S., Merker, R. I., Ditto, M. D., & DiNovi, M. J. (2006). Food-processing enzymes from recombinant microorganisms--a review. *Regulatory toxicology and pharmacology, 45*(2), 144-158. http://doi.org/10.1016/j.yrtph.2006.05.001

Oriol, R. (1995). ABO, Hh, Lewis and secretion. Serology, genetics and tissue distribution. In J. P. Cartron and P. Rouger (Eds.), *Molecular basis of major blood group antigens* (pp. 37–73). London: Plenum Press.

Ozeki, K., Kyoya, F., Hizume, K., Kanda, A., Hamachi, M., & Nunokawa, Y. (1994). Transformation of intact *Aspergillus niger* by electroporation. *Bioscience, Biotechnology, and Biochemistry, 58*(12), 2224–2227. http://doi.org/10.1271/bbb.58.2224

Pakula, T. M., Nygren, H., Barth, D., Heinonen, M., Castillo, S., & Penttilä, M. (2016). Genome wide analysis of protein production load in *Trichoderma reesei*. *Biotechnology for Biofuels, 9*, 132. http://doi.org/10.1186/s13068-016-0547-5

Palacpac, N. Q., Yoshida, S., Sakai, H., Kimura, Y., Fujiyama, K., Yoshida, T., & Seki, T. (1999). Stable expression of human β1,4-galactosyltransferase in plant cells modifies N-linked glycosylation patterns. *Proceedings of the National Academy of Sciences, 96*(8), 4692–4697. http://doi.org/10.1073/pnas.96.8.4692

Paloheimo, M., Mäntylä, A., Vehmaanperä, J., Hakola, S., Lantto, R., Lahtinen, T., … Suominen, P. (1998). Thermostable xylananses produced by recombinant *Trichoderma reesei* for pulp bleaching. In M. Claeyssens, W. Nerinckx & K. Piens (Eds.), *Carbohydrates from Trichoderma reesei and other microorganisms, Structures, Biochemistry, genetics and applications* (pp. 255-264). Cambridge: The Royal Society of Chemistry.

Paloheimo, M., Mäntylä, A., Kallio, J., & Suominen, P. (2003). High-Yield Production of a Bacterial Xylanase in the Filamentous Fungus *Trichoderma reesei* Requires a Carrier Polypeptide with an Intact Domain Structure. *Applied and Environmental Microbiology, 69*(12), 7073–7082. http://doi.org/10.1128/AEM.69.12.7073-7082.2003

Palomares, L. A., Estrada-Moncada, S., & Ramírez, O. T. (2004). Production of Recombinant Proteins. In P. Balbás & A. Lorence (Eds.), Recombinant Gene Expression: Reviews and Protocols (pp. 15–51). Totowa, NJ: Humana Press. http://doi.org/10.1385/1-59259-774-2:015

Pannkuk, E. L., Risch, T. S., & Savary, B. J. (2015). Isolation and Identification of an Extracellular Subtilisin-Like Serine Protease Secreted by the Bat Pathogen *Pseudogymnoascus destructans*. *PLoS ONE 10*(3): e0120508. https://doi.org/10.1371/journal.pone.0120508

Paulson, J. C., & Colley, K. J. (1989). Glycosyltransferases. Structure, localization, and control of cell type-specific glycosylation. *Journal of Biological Chemistry, 264*(30), 17615–17618. Retrieved from http://www.jbc.org/content/264/30/17615.long

Paulson, J. C., & de Vries, R. P. (2013). H5N1 receptor specificity as a factor in pandemic risk. *Virus Research*, *178*(1), http://doi.org/10.1016/j.virusres.2013.02.015

Pham, T. A. N., Clare, S., Goulding, D., Arasteh, J. M., Stares, M. D., Browne, H. P., … Lawley, T. D. (2014). Epithelial IL-22RA1-Mediated Fucosylation Promotes Intestinal Colonization Resistance to an Opportunistic Pathogen. *Cell Host & Microbe*, *16*(4), 504–516. http://doi.org/10.1016/j.chom.2014.08.017

Penttilä, M., Nevalainen, H., Ratto, M., Salminen, E., & Knowles, J. (1987). A versatile transformation system for the cellulolytic filamentous fungus *Trichoderma reesei*. Gene, *61*(2), 155-164. http://doi.org/10.1016/0378-1119(87)90110-7

Peterson, R., & Nevalainen, H. (2012). *Trichoderma reesei* RUT-C30--thirty years of strain improvement. Microbiology, *158*(1), 58-68. doi: 10.1099/mic.0.054031-0

Pickard, J. M., Maurice, C. F., Kinnebrew, M. A., Abt, M. C., Schenten, D., Golovkina, T., … Chervonsky, A. V. (2014). Rapid fucosylation of intestinal epithelium sustains host-

commensal symbiosis in sickness. *Nature*, *514*(7524), 638–641. http://doi.org/10.1038/nature13823

Pickard, J. M., & Chervonsky, A. V. (2015). Intestinal fucose as a mediator of host-microbe symbiosis. *Journal of Immunology (Baltimore, Md. : 1950)*, *194*(12), 5588–5593. http://doi.org/10.4049/jimmunol.1500395

Prasher, D. C., Eckenrode, V. K., Ward, W. W., Prendergast, F. G., & Cormier, M. J. (1992). Primary structure of the *Aequorea victoria* green-fluorescent protein. *Gene*, *111*(2), 229–233. http://doi.org/https://doi.org/10.1016/0378-1119(92)90691-H

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*(5), 200–206. http://dx.doi.org/10.1016/S0167-7799(02)01933-9

Qi, F., Zhang, W., Zhang, F., Chen, G., & Liu, W. (2014). Deciphering the Effect of the Different N-Glycosylation Sites on the Secretion, Activity, and Stability of Cellobiohydrolase I from *Trichoderma reesei*. *Applied and Environmental Microbiology*, *80*(13), 3962–3971. http://doi.org/10.1128/AEM.00261-14

Qin, L., Cai, F., Dong, X., Huang, Z., Tao, Y., & Huang, J. (2012). Improved production of heterologous lipase in *Trichoderma reesei* by RNAi mediated gene silencing of an endogenic highly expressed gene. *Bioresource Technology*, *109*, 116–122. http://doi.org/10.1016/j.biortech.2012.01.013

R&D Systems (https://www.rndsystems.com)

Rech, E. L., Vianna, G. R., & Aragao, F. J. L. (2008). High-efficiency transformation by biolistics of soybean, common bean and cotton transgenic plants. *Nature Protocols*, *3*(3), 410–418. http://dx.doi.org/10.1038/nprot.2008.9

Reichard, U., Léchenne, B., Asif, A. R., Streit, F., Grouzmann, E., Jousson, O., & Monod, M. (2006). Sedolisins, a new class of secreted proteases from *Aspergillus fumigatus* with endoprotease or tripeptidyl-peptidase activity at acidic pHs. *Applied and Environmental Microbiology, 72*(3), 1739–1748. http://doi.org/10.1128/AEM.72.3.1739-1748.2006

Ribeiro, L.A., Mariani, P.D.S.C., Azevedo, J.L., Rech, E.L., Schmidt, G.S., & Coutinho, L.L.. (2001). A biolistic process for in vitro gene transfer into chicken embryos. *Brazilian Journal of Medical and Biological Research, 34*(9), 1115-1124. https://dx.doi.org/10.1590/S0100-879X2001000900003

Rick, W. (1974). In *Methods of Enzymatic Analysis*, 2nd ed., Volume II, (pp 1009-1012). New York, NY: Academic Press Inc.

Ritchie, C. (2013). Protease inhibitors. *Materials and methods, 3*, 169. DOI//dx.doi.org/10.13070/mm.en.3.169

Rodriguez, B., Kavoosi, M., Koska, J., Creagh, A. L., Kilburn, D. G., & Haynes, C. A. Inexpensive and generic affinity purification of recombinant proteins using a family 2a CBM fusion tag. *Biotechnology Progress, 20*(5), 1479–89. doi:10.1021/bp0341904

Rodriguez-Gomez, D., & Hobley, T. J. (2013). Is an organic nitrogen source needed for cellulase production by *Trichoderma reesei* Rut-C30? *World Journal of Microbiology and Biotechnology, 29*(11), 2157–2165. doi:10.1007/s11274-013-1381-6

Roos, C., Kolmer, M., Mattila, P., & Renkonen, R. (2002). Composition of *Drosophila melanogaster* proteome involved in fucosylated glycan metabolism. *Journal of Biological Chemistry, 277*(5), 3168-3175. doi: 10.1074/jbc.M107927200

Ruiz-Díez, B. (2002). A Review: Strategies for the transformation of filamentous fungi. *Journal of Applied Microbiology*, *92*(2), 189–195. http://doi.org/10.1046/j.1365-2672.2002.01516.x

Runkel, L., Meier, W., Pepinsky, R. B., Karpusas, M., Whitty, A., Kimball, K., … Goelz, S. E. (1998). Structural and functional differences between glycosylated and non-glycosylated forms of human interferon-*β* (IFN-*β*). *Pharmaceutical Research*, *15*(4), 641–649. doi: 10.1023/a:1011974512425.

Ryu, D. D. Y., & Mandels, M. (1980). Cellulases: Biosynthesis and applications. *Enzyme and Microbial Technology*, *2*(2), 91–102. http://doi.org/http://dx.doi.org/10.1016/0141-0229(80)90063-0

Saarelainen, R., Mantyla, A., Nevalainen, H., & Suominen, P. (1997). Expression of Barley Endopeptidase B in *Trichoderma reesei*. *Applied and Environmental Microbiology, 63*(12), 4938–4940. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1389312/

Salles, B. C., Te'o, V. S., Gibbs, M. D., Bergquist, P. L., Filho, E. X., Ximenes, E. A., & Nevalainen, K. M. (2007). Identification of two novel xylanase-encoding genes (*xyn5* and *xyn6*) from *Acrophialophora nainiana* and heterologous expression of *xyn6* in *Trichoderma reesei*. *Biotechnology letters, 29*(8), 1195-1201. doi:10.1007/s10529-007-9380-z

Salo, H., Sievi, E., Suntio, T., Mecklin, M., Mattila, P., Renkonen, R., & Makarow, M. (2005). Co-expression of two mammalian glycosyltransferases in the yeast cell wall allows synthesis of sLex. *FEMS Yeast Research, 5*(4-5), 341-350. doi: 10.1016/j.femsyr.2004.11.007

Saloheimo, A., Aro, N., Ilmén, M., & Penttilä, M. (2000). Isolation of the *ace1* Gene Encoding a Cys2-His2 Transcription Factor Involved in Regulation of Activity of the Cellulase Promoter

cbh1 of *Trichoderma reesei*. *Journal of Biological Chemistry*, *275*(8), 5817–5825. http://doi.org/10.1074/jbc.275.8.5817

Saloheimo, M., & Pakula, T. M. (2012). The cargo and the transport system: Secreted proteins and protein secretion in *Trichoderma reesei* (*Hypocrea jecorina*). *Microbiology*, *158*(1), 46–57. http://doi.org/10.1099/mic.0.053132-0

Sambrook, J., & Russell, D. (2001). Molecular Cloning: A Laboratory Manual, 3rd edn. NY: Cold Spring Harbor.

Samuelsson, E., & Uhlen, M. (1996). Chaperone-like effect during in vitro refolding of insulin-like growth factor I using a solubilizing fusion partner. *Annals of the New York Academy of Sciences, 782*, 486-494. doi: 10.1111/j.1749-6632.1996.tb40586.x

Samyn-Petit, B., Krzewinski-Recchi, M. A., Steelant, W. F., Delannoy, P. & Harduin-Lepers, A. (2000). Molecular cloning and functional expression of human ST6GalNAc II. Molecular expression in various human cultured cells. *Biochimica et Biophysica Acta (BBA) - Bioenergetics, 1474*(2), 201-211. http://doi.org/10.1016/S0304-4165(00)00020-9

Sanchez, O., & Aguirre, J. (1996). Efficient transformation of *Aspergillus nidulans* by electroporation of germinated conidia. *Fungal Genetics Newsletter, 43*(21), 48–51. https://dx.doi.org/10.4148/1941-4765.1317

Sareneva, T., Pirhonen, J., Cantell, K., Kalkkinen, N., & Julkunen, I. (1994). Role of N-glycosylation in the synthesis, dimerization and secretion of human interferon-gamma. *Biochemical Journal, 303*(Pt 3), 831–840.

Sareneva, T., Mortz, E., Tolo, H., Roepstorff, P. & Julkunen, I. (1996). Biosynthesis and N-glycosylation of human interferon-γ. *European Journal of Biochemistry, 242*(2), 191–200. doi: 10.1111/j.1432-1033.1996.0191r.x

Sasaki, K., Kurata, K., Funayama, K., Nagata, M., Watanabe, E., Ohta, S., … Nishi, T. (1994). Expression cloning of a novel α1, 3-fucosyltransferase that is involved in biosynthesis of the sialyl Lewis X carbohydrate determinants in leukocytes. *Journal of Biological Chemistry, 269*, 14730–14737.

Schindler, M., Mach, R. L., Vollenhofer, S. K., Hodits, R., Gruber, F., Visser, J., … Kubicek, C. P. (1993). Characterization of the pyruvate kinase-encoding gene (*pki1*) of *Trichoderma reesei*. *Gene, 130*(2), 271-275.

Schmidt, T. G. M., & Skerra, A. (2007). The Strep-tag system for one-step purification and high-affinity detection or capturing of proteins. *Nature Protocols, 2*(6), 1528–1535. http://dx.doi.org/10.1038/nprot.2007.209

Schuster, A., Bruno, K. S., Collett, J. R., Baker, S. E., Seiboth, B., Kubicek, C. P., & Schmoll, M. (2012). A versatile toolkit for high throughput functional genomics with *Trichoderma reesei*. *Biotechnology for Biofuels*, *5*(1), 1. http://doi.org/10.1186/1754-6834-5-1

Shaner, N. C., Campbell, R. E., Steinbach, P. A., Giepmans, B. N. G., Palmer, A. E., & Tsien, R. Y. (2004). Improved monomeric red, orange and yellow fluorescent proteins derived from *Discosoma sp*. red fluorescent protein. *Nature Biotechnology, 22*(12), 1567–1572. http://dx.doi.org/10.1038/nbt1037

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.

Shivatare, S. S., Chang, S.-H., Tsai, T.-I., Tseng, S. Y., Shivatare, V. S., Lin, Y.-S., … Wong, C.-H. (2016). Modular synthesis of *N*-glycans and arrays for the hetero-ligand binding analysis of HIV antibodies. *Nature Chemistry*, *8*(4), 338–346. http://doi.org/10.1038/nchem.2463

Shimma, Y., Saito, F., Oosawa, F., & Jigami, Y. (2006). Construction of a Library of Human Glycosyltransferases Immobilized in the Cell Wall of *Saccharomyces cerevisiae*. *Applied and Environmental Microbiology*, *72*(11), 7003–7012. http://doi.org/10.1128/AEM.01378-06

Shoji, J., Arioka, M., & Kitamoto, K. (2008). Dissecting cellular components of the secretory pathway in filamentous fungi: insights into their application for protein production. *Biotechnology Letters, 30*(1), 7–14. http://doi.org/10.1007/s10529-007-9516-1

Shusta, E. V, Raines, R. T., Pluckthun, A., & Wittrup, K. D. (1998). Increasing the secretory capacity of *Saccharomyces cerevisiae* for production of single-chain antibody fragments. *Nature Biotechnology, 16*(8), 773–777. http://dx.doi.org/10.1038/nbt0898-773

Simmons, E. G. (1977). Classification of some cellulase-producing *Trichoderma* species. Paper presented at Second International Mycological Congress (University of South Florida, FL).

Simonen, M., Jamsa, E., & Makarow, M. (1994). The role of the carrier protein and disulfide formation in the folding of beta-lactamase fusion proteins in the endoplasmic reticulum of yeast. *The Journal of biological chemistry, 269*(19), 13887-13892. Retrieved from http://www.jbc.org/content/269/19/13887.long

Singh, S., Pal, K., Yadav, J., Tang, H., Partyka, K., Kletter, D., … Haab, B. B. (2015). Upregulation of Glycans Containing 3’ Fucose in a Subset of Pancreatic Cancers Uncovered Using Fusion-Tagged Lectins. *Journal of Proteome Research, 14*(6), 2594–2605. http://doi.org/10.1021/acs.jproteome.5b00142

Skretas, G., Carroll, S., DeFrees, S., Schwartz, M. F., Johnson, K. F., & Georgiou, G. (2009). Expression of active human sialyltransferase ST6GalNAcI in *Escherichia coli*. *Microbial Cell Factories, 8,* 50. http://doi.org/10.1186/1475-2859-8-50

Šmídková, M., Holá, M., & Angelis, K. J. (2010). Efficient biolistic transformation of the moss *Physcomitrella patens*. *Biologia Plantarum*, *54*(4), 777–780. http://doi.org/10.1007/s10535-010-0141-9

Smith, F. D., Harpending, P. R., Sanford, J. C. (1992). Biolistic transformation of prokaryotes: factors that affect biolistic transformation of very small cells. Microbiology, 138, 239-248. doi: 10.1099/00221287-138-1-239

Smith, W., Jäntti, J., Oja, M., & Saloheimo, M. (2014). Comparison of intracellular and secretion-based strategies for production of human α-galactosidase A in the filamentous fungus *Trichoderma reesei*. *BMC Biotechnology*, *14*, 91. http://doi.org/10.1186/s12896-014-0091-y

Snapgene (www.snapgene.com)

Solá, R. J., & Griebenow, K. (2009). Effects of Glycosylation on the Stability of Protein Pharmaceuticals. *Journal of Pharmaceutical Sciences*, *98*(4), 1223–1245. http://doi.org/10.1002/jps.21504

Solá, R. J., & Griebenow, K. (2010). Glycosylation of Therapeutic Proteins: An Effective Strategy to Optimize Efficacy. *BioDrugs : Clinical Immunotherapeutics, Biopharmaceuticals and Gene Therapy*, *24*(1), 9–21. http://doi.org/10.2165/11530550-000000000-00000

Stahl, M., Uemura, K., Ge, C., Shi, S., Tashima, Y. & Stanley, P. (2008). Roles of Pofut1 and O-fucose in mammalian Notch signalling. *The Journal of biological chemistry*, *283*, 13638-13651. doi:10.1074/jbc.M802027200

Steiger, M. G., Vitikainen, M., Uskonen, P., Brunner, K., Adam, G., Pakula, T., … Mach-Aigner, A. R. (2011). Transformation system for *Hypocrea jecorina* (*Trichoderma reesei*) that favors homologous integration and employs reusable bidirectionally selectable markers.

Applied and Environmental Microbiology, *77*(1), 114–121. http://doi.org/10.1128/AEM.02100-10

Strauss, J., Mach, R. L., Zeilinger, S., Hartler, G., Stöffler, G., Wolschek, M., & Kubicek, C. P. (1995). Cre1, the carbon catabolite repressor protein from *Trichoderma reesei*. *FEBS Letters*, *376*(1-2), 103–107. doi: 10.1016/0014-5793(95)01255-5

Stricker, A. R., Grosstessner-Hain, K., Würleitner, E., & Mach, R. L. (2006). Xyr1 (Xylanase Regulator 1) Regulates both the Hydrolytic Enzyme System and d-Xylose Metabolism in *Hypocrea jecorina*. *Eukaryotic Cell*, *5*(12), 2128–2137. http://doi.org/10.1128/EC.00211-06

Su, X., Schmitz, G., Zhang, M., Mackie, R. I., & Cann, I. K. O. (2012). *Heterologous Gene Expression in Filamentous Fungi*. *Advances in Applied Microbiology* (Vol. 81). Elsevier. http://doi.org/10.1016/B978-0-12-394382-8.00001-0

Sugui, J. A., Chang, Y. C., & Kwon-Chung, K. J. (2005). *Agrobacterium tumefaciens*-Mediated Transformation of *Aspergillus fumigatus*: an Efficient Tool for Insertional Mutagenesis and Targeted Gene Disruption. *Applied and Environmental Microbiology*, *71*(4), 1798–1802. http://doi.org/10.1128/AEM.71.4.1798-1802.2005

Sun, A. (2008). Fluorescent tagging of the endoplasmic reticulum in *Trichoderma reesei*. (Honours thesis, Macquarie University, Sydney).

Sun, A., Peterson, R., Te'o, J., & Nevalainen, H. (2016). Expression of the mammalian peptide hormone obestatin in *Trichoderma reesei*. *New Biotechnology*, *33*(1), 99–106. http://doi.org/10.1016/j.nbt.2015.08.004

Takeuchi, M., Ueno, Y., & Ichishima, E., (1988). Fluorogenic substrate of *Aspergillus* aspartic proteinase. *Agricultural and Biological Chemistry, 52,* 1279-1280.

Tan, M., Zhu, L., Zhuang, H., Hao, Y., Gao, S., Liu, S., … Lin, B. (2015). Lewis Y antigen modified CD47 is an independent risk factor for poor prognosis and promotes early ovarian cancer metastasis. *American Journal of Cancer Research*, *5*(9), 2777–2787.

Tani, S., Tsuji, A., Kunitake, E., Sumitani, J.-I., & Kawaguchi, T. (2013). Reversible impairment of the ku80 gene by a recyclable marker in *Aspergillus aculeatus*. *AMB Express*, *3*(1), 4. http://doi.org/10.1186/2191-0855-3-4

Thomann, M., Schlothauer, T., Dashivets, T., Malik, S., Avenal, C., Bulau, P., … Reusch, D. (2015). *In Vitro* Glycoengineering of IgG1 and Its Effect on Fc Receptor Binding and ADCC Activity. *PLoS ONE*, *10*(8), e0134949. http://doi.org/10.1371/journal.pone.0134949

Thomas, C. R., & Zhang, Z. (1998). The Effect of Hydrodynamics on Biological Materials. In E. Galindo & O. T. Ramírez (Eds.), *Advances in Bioprocess Engineering: Volume II* (pp. 137–170). Dordrecht: Springer Netherlands. http://doi.org/10.1007/978-94-017-0643-8_9

Te'o, V.J.S., Cziferszky, A.E., Bergquist, P.L. & Nevalainen, K.M.H. (2000). Codon optimisation of xylanase gene *xynB* from the thermophilic bacterium *Dictyoglomus thermophilum* for expression in the filamentous fungus *Trichoderma reesei*. *FEMS Microbiology Letters, 190*(1), 13-19. http://doi.org/10.1016/S0378-1097(00)00308-6

Te'o, V. S. J., Bergquist, P. L., & Nevalainen, K. M. H. (2002). Biolistic transformation of *Trichoderma reesei* using the Bio-Rad seven barrels Hepta Adaptor system. *Journal of Microbiological Methods*, *51*(3), 393–399. http://doi.org/10.1016/S0167-7012(02)00126-4

Te'o, V. S. J., & Nevalainen, K. M. H. (2010). Multiple promoter platform for protein production. Google Patents. Retrieved from http://www.google.com/patents/EP2245164A1?cl=en

Tu, Z., Lin, Y.-N., & Lin, C.-H. (2013). Development of fucosyltransferase and fucosidase inhibitors. *Chemical Society Reviews*, *42*(10), 4459–4475. http://doi.org/10.1039/C3CS60056D

Umezawa, H., Aoyagi, T., Morishima, H., Kunimoto, S., & Matsuzaki, M. (1970). Chymostatin, a new chymotrypsin inhibitor produced by actinomycetes. *Journal of Antibiotics (Tokyo), 23*(8), 425-7.

van den Hombergh, J. P. T. W., van de 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*(7), 256–263. http://dx.doi.org/10.1016/S0167-7799(97)01020-2

van den Hondel, C. A. M. J. J., Punt, P. J., & van Gorcom, R. F. M. (1991). Heterologous gene expression in filamentous fungi. In J. W. Bennett and L. L. Lasure (Eds.), *More Gene Manipulations in Fungi*, (pp. 396 – 428). New York: Academic Press.

van der Kaaij, R. M, Ram, A. F. J., Schaap, P., & Punt, P. J. (2011). Genomic Approaches for Identification of the Biopolymer Degrading Enzyme Network of *Aspergillus niger*. In The Mycota. Industrial applications (2nd edition). doi:10.1007/978-3-642-11458-8_20

Varki, A. (2017). Biological roles of glycans. *Glycobiology*, *27*(1), 3–49. http://doi.org/10.1093/glycob/cww086

Varki, A., & Lowe, J. B. (2009). Biological Roles of Glycans. In A. Varki, R. D. Cummings, J. D. Esko, H. H. Freeze, P. Stanley, C. R. Bertozzi, G. W. Hart, & M. E. Etzler (Eds.), *Essentials of Glycobiology*, *2nd edition*. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press.

Velkovska, S., Marten, M. R., & Ollis, D. F. (1997). Kinetic model for batch cellulase production by *Trichoderma reesei* RUT C30. *Journal of Biotechnology, 54*(2), 83–94. http://doi.org/10.1016/S0168-1656(97)01669-6

Verdoes, J. C., Punt, P. J., & Hondel, C. A. M. J. J. (1995). Molecular genetic strain improvement for the overproduction of fungal proteins by filamentous fungi. *Applied Microbiology and Biotechnology, 43,* 195-205. doi:10.1007/BF00172812

Wainwright, M. P., Trinci, A. P. J., & Moore, D. (1993). Aggregation of spores and biomass of *Phanerochaete chrysosporium* in liquid culture and the effect of anionic polymers on this process. *Mycology Research,97*, 801–6.

Walsh, G. & Jefferis, R. (2006). Post-translational modifications in the context of therapeutic proteins. *Nature Biotechnology, 24*(10), 1241–1252. http://doi.org/10.1038/nbt1252

Wang, D., & Hong, J. (2014). Purification of a Recombinant Protein with Cellulose-Binding Module 3 as the Affinity Tag. In R. J. Giannone & A. B. Dykstra (Eds.), *Protein Affinity Tags: Methods and Protocols* (pp. 35–45). New York, NY: Springer New York. http://doi.org/10.1007/978-1-4939-1034-2_3

Wang, Y., Shao, L., Shi, S., Harris, R. J., Spellman, M. W., Stanley, P., & Haltiwanger, S. (2001). Modification of epidermal growth factor-like repeats with O-fucose. Molecular cloning and expression of a novel GDP-fucose protein O-fucosyltransferase. *Journal of Biological Chemistry, 276*(43), 40338-40345. doi: 10.1074/jbc.M107849200

Wang, W., Shi, X.-Y., & Wei, D.-Z. (2014). Light-mediated control of gene expression in filamentous fungus *Trichoderma reesei*. *Journal of Microbiological Methods*, *103*, 37–9. http://doi.org/10.1016/j.mimet.2014.05.017

Wang, L., Ridgway, D., Gu, T., & Moo-Young, M. (2005). Bioprocessing strategies to improve heterologous protein production in filamentous fungal fermentations. *Biotechnology Advances*, *23*(2), 115–129. http://doi.org/10.1016/j.biotechadv.2004.11.001

Ward, O. P. (2012). Production of recombinant proteins by filamentous fungi. *Biotechnology Advances*, *30*(5), 1119–1139. http://doi.org/10.1016/j.biotechadv.2011.09.012

Ward, M., Wilson, L. J., Kodama, K. H., Rey, M. W., & Berka, R. M. (1990). Improved production of chymosin in *Aspergillus* by expression as a glucoamylase-chymosin fusion. Biotechnology (NY) *8*, 435-440.

Ward, M., Lin, C., Victoria, D. C., Fox, B. P., Fox, J. A., Wong, D. L., … Wang, H. (2004). Characterization of Humanized Antibodies Secreted by *Aspergillus niger*. *Applied and Environmental Microbiology*, *70*(5), 2567–2576. http://doi.org/10.1128/AEM.70.5.2567-2576.2004

Weninger, W., Ulfman, L. H., Cheng, G., Souchkova, N., Quackenbush, E. J., Lowe, J. B., & von Andrian, U. H. (2000). Specialized contributions by FucT-IV and FucT-VII during leukocyte rolling in dermal microvessels. *Immunity*, *12*, 665–676. http://dx.doi.org/10.1016/S1074-7613(00)80217-4

White, T., Bennett, E. P., Takio, K., Sorensen, T., Bonding, N., & Clausen, H. (1995). Purification and cDNA cloning of a human UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase. *Journal of Biological Chemistry*, *270*, 24156–24165. doi: 10.1074/jbc.270.41.24156

Willis, L. M., Zhang, R., Reid, A., Withers, S. G., & Wakarchuk, W. W. (2009). Mechanistic investigation of the endo-α-*N*-acetylgalactosaminidase from *Streptococcus pneumoniae R6*. *Biochemistry*, *48*(43), 10334–10341. http://doi.org/10.1021/bi9013825

Wongwicharn, A., McNeil, B., Harvey, L. M. (1999). Effect of oxygen enrichment on morphology, growth, and heterologous protein production in chemostat cultures of *Aspergillus niger* B1-D. *Biotechnology and Bioengineering*, *65*, 416–424. doi: 10.1002/(SICI)1097-0290(19991120)65:4<416::AID-BIT6>3.0.CO;2-Z

Wong, S. S. C., & Truong, K. (2010). Fluorescent Protein-Based Methods for On-Plate Screening of Gene Insertion. *PLoS ONE*, *5*(12), e14274. http://doi.org/10.1371/journal.pone.0014274

Xu, Z., Vo, L., & Macher, B. A. (1996). Structure-function analysis of human α1, 3-fucosyltransferase. Amino acids involved in acceptor substrate specificity. *Journal of Biological Chemistry*, *271*, 8818–8823. doi: 10.1074/jbc.271.15.8818

Yamada, Y., Maeda, M., Alshahni, M. M., Monod, M., Staib, P., & Yamada, T. (2014). Flippase (FLP) recombinase-mediated marker recycling in the dermatophyte *Arthroderma vanbreuseghemii. Microbiology, 160(Pt 10)*, 2122–2135. http://doi.org/10.1099/mic.0.076562-0

Yan, X., Lin, Y., Liu, S., Aziz, F., & Yan, Q. (2015). Fucosyltransferase IV (FUT4) as an effective biomarker for the diagnosis of breast cancer. *Biomedicine & Pharmacotherapy*, *70*, 299–304. doi: 10.1016/j.biopha.2014.12.048

Yao, Q., Sun, T. T., Liu, W. F., & Chen, G. J. (2008). Gene cloning and heterologous expression of a novel endoglucanase, swollenin, from *Trichoderma pseudokoningii* S38. *Bioscience, Biotechnology, and Biochemistry*, *72*(11), 2799-2805. http://doi.org/10.1271/bbb.80124

Zeilinger, S. (2004). Gene disruption in *Trichoderma atroviride* via *Agrobacterium*-mediated transformation. *Current Genetics, 45*, 54–60. doi:10.1007/s00294-003-0454-8

Zhang, J., Zhong, Y., Zhao, X., & Wang, T. (2010). Development of the cellulolytic fungus *Trichoderma reesei* strain with enhanced b-glucosidase and filter paper activity using strong artifical cellobiohydrolase 1 promoter. *Bioresource Technology*, *101*(24), 9815–9818. http://doi.org/10.1016/j.biortech.2010.07.078

Zhang, G., Zhu, Y., Wei, D., & Wang, W. (2014). Enhanced production of heterologous proteins by the filamentous fungus *Trichoderma reesei* via disruption of the alkaline serine protease SPW combined with a pH control strategy. *Plasmid*, *71*(1), 16–22. http://doi.org/10.1016/j.plasmid.2014.01.001

Zhong, Y., Liu, X., Xiao, P., Wei, S., & Wang, T. (2011). Expression and Secretion of the Human Erythropoietin Using an Optimized *cbh1* Promoter and the Native CBH I Signal Sequence in the Industrial Fungus *Trichoderma reesei*, *165*, 1169–1177. http://doi.org/10.1007/s12010-011-9334-8

Zou, G., Shi, S., Jiang, Y., van den Brink, J., de Vries, R. P., Chen, L., … Zhou, Z. (2012). Construction of a cellulase hyper-expression system in *Trichoderma reesei* by promoter and enzyme engineering. *Microbial Cell Factories*, *11*(1), 21. http://doi.org/10.1186/1475-2859-11-21

Appendices

1) FUT3 aminoacid sequence (NCBI Reference Sequence: NP_000140.1)

MDPLGAAKPQWPWRRCLAALLFQLLVAVCFFSYLRVSRDDATGSPRAPSGSSRQDT
TPTRPTLLILLWTWPFHIPVALSRCSEMVPGTADCHITADRKVYPQADTVIVHHWDI
MSNPKSRLPPSPRPQGQRWIWFNLEPPPNCQHLEALDRYFNLTMSYRSDSDIFTPYG
WLEPWSGQPAHPPLNLSAKTELVAWAVSNWKPDSARVRYYQSLQAHLKVDVYGR
SHKPLPKGTMMETLSRYKFYLAFENSLHPDYITEKLWRNALEAWAVPVVLGPSRSN
YERFLPPDAFIHVDDFQSPKDLARYLQELDKDHARYLSYFRWRETLRPRSFSWALDF
CKACWKLQQESRYQTVRSIAAWFT

N-terminal cytoplasmic tail is highlighted in yellow, membrane-spanning region in highlighted in green and C-terminal catalytic domain is highlighted in blue.

2) Alignment of original and codon-optimised FUT3 DNA sequences

```
   1 R  V  S  R  D  D  A  T  G  S  P  R  A  P  S  G  S  S  R  Q

   1 cgtgtgtcccgagacgatgccactggatcccctagggctcccagtgggtcctcccgacag

   1 R  V  S  R  D  D  A  T  G  S  P  R  A  P  S  G  S  S  R  Q

   1 cgcgtctcccgcgacgacgccaccggctccccccgcgccccagcggctcctcccgccag

   1          10        20        30        40        50

  21 D  T  T  P  T  R  P  T  L  L  I  L  L  R  T  W  P  F  H  I

  61 gacaccactcccacccgccccaccctcctgatcctgctacggacatggcctttccacatc

  21 D  T  T  P  T  R  P  T  L  L  I  L  L  R  T  W  P  F  H  I

  61 gacaccacccccacccgccccaccctcctgatcctgctccgcacctggcccttccacatc

  61          70        80        90        100       110

  41 P  V  A  L  S  R  C  S  E  M  V  P  G  T  A  D  C  H  I  T

 121 cctgtggctctgtcccgctgttcagagatggtgcccggcacagccgactgccacatcact

  41 P  V  A  L  S  R  C  S  E  M  V  P  G  T  A  D  C  H  I  T
```

```
121 cccgtcgccctgtcccgctgcagcgagatggtccccggcaccgccgactgccacatcacc

121          130       140       150       160       170

 61 A  D  R  K  V  Y  P  Q  A  D  M  V  I  V  H  H  W  D  I  M

181 gccgaccgcaaggtgtacccacaggcagacatggtcatcgtgcaccactgggatatcatg

 61 A  D  R  K  V  Y  P  Q  A  D  M  V  I  V  H  H  W  D  I  M

181 gccgaccgcaaggtctacccccaggccgacatggtcatcgtccaccactgggacatcatg

181          190       200       210       220       230

 81 S  N  P  K  S  R  L  P  P  S  P  R  P  Q  G  Q  R  W  I  W

241 tccaaccctaagtcacgcctcccaccttccccgaggccgcaggggcagcgctggatctgg

 81 S  N  P  K  S  R  L  P  P  S  P  R  P  Q  G  Q  R  W  I  W

241 tccaaccccaagagccgcctcccccctcccccgccccagggccagcgctggatctgg

241          250       260       270       280       290

101 F  N  L  E  P  P  P  N  C  Q  H  L  E  A  L  D  R  Y  F  N

301 ttcaacttggagccacccccctaactgccagcacctggaagccctggacagatacttcaat

101 F  N  L  E  P  P  P  N  C  Q  H  L  E  A  L  D  R  Y  F  N

301 ttcaacctggagcccccccccaactgccagcacctggaggccctggaccgctacttcaac

301          310       320       330       340       350

121 L  T  M  S  Y  R  S  D  S  D  I  F  T  P  Y  G  W  L  E  P

361 ctcaccatgtcctaccgcagcgactccgacatcttcacgccctacggctggctggagccg

121 L  T  M  S  Y  R  S  D  S  D  I  F  T  P  Y  G  W  L  E  P
```

361 ctcaccatgtcctaccgcagcgactccgacatcttcaccccctacggctggctggagccc

361 370 380 390 400 410

141 W S G Q P A H P P L N L S A K T E L V A

421 tggtccggccagcctgcccacccaccgctcaacctctcggccaagaccgagctggtggcc

141 W S G Q P A H P P L N L S A K T E L V A

421 tggagcggccagcccgcccacccccccctcaacctcagcgccaagaccgagctggtcgcc

421 430 440 450 460 470

161 W A V S N W K P D S A R V R Y Y Q S L Q

481 tgggcggtgtccaactggaagccggactcagccagggtgcgctactaccagagcctgcag

161 W A V S N W K P D S A R V R Y Y Q S L Q

481 tgggccgtcagcaactggaagcccgacagcgcccgcgtccgctactaccagagcctgcag

481 490 500 510 520 530

181 A H L K V D V Y G R S H K P L P K G T M

541 gctcatctcaaggtggacgtgtacggacgctcccacaagcccctgcccaaggggaccatg

181 A H L K V D V Y G R S H K P L P K G T M

541 gcccacctcaaggtcgacgtctacggccgctcccacaagcccctgcccaagggcaccatg

541 550 560 570 580 590

201 M E T L S R Y K F Y L A F E N S L H P D

601 atggagacgctgtcccggtacaagttctacctggccttcgagaactccttgcaccccgac

201 M E T L S R Y K F Y L A F E N S L H P D

601 atggagacctgagccgctacaagttctacctggccttcgagaactccctgcaccccgac

601 610 620 630 640 650

221 Y I T E K L W R N A L E A W A V P V V L

661 tacatcaccgagaagctgtggaggaacgccctggaggcctgggccgtgcccgtggtgctg

221 Y I T E K L W R N A L E A W A V P V V L

661 tacatcaccgagaagctgtggcgcaacgccctggaggcctgggccgtccccgtcgtcctg

661 670 680 690 700 710

241 G P S R S N Y E R F L P P D A F I H V D

721 ggccccagcagaagcaactacgagaggttcctgccacccgacgccttcatccacgtggac

241 G P S R S N Y E R F L P P D A F I H V D

721 ggccccagccgcagcaactacgagcgcttcctgccccccgacgccttcatccacgtcgac

721 730 740 750 760 770

261 D F Q S P K D L A R Y L Q E L D K D H A

781 gacttccagagccccaaggacctggcccggtacctgcaggagctggacaaggaccacgcc

261 D F Q S P K D L A R Y L Q E L D K D H A

781 gacttccagagccccaaggacctggcccgctacctgcaggagctggacaaggaccacgcc

781 790 800 810 820 830

281 R Y L S Y F R W R E T L R P R S F S W A

841 cgctacctgagctactttcgctggcgggagacgctgcggcctcgctccttcagctgggca

281 R Y L S Y F R W R E T L R P R S F S W A

841 cgctacctgagctacttccgctggcgcgagacctgcgccccgctccttcagctgggcc

841 850 860 870 880 890

301 L D F C K A C W K L Q Q E S R Y Q T V R

901 ctggatttctgcaaggcctgctggaaactgcagcaggaatccaggtaccagacggtgcgc

301 L D F C K A C W K L Q Q E S R Y Q T V R

901 ctggacttctgcaaggcctgctggaagctgcagcaggagtcccgctaccagaccgtccgc

901 910 920 930 940 950

321 S I A A W F T *

961 agcatagcggcttggttcacctga

321 S I A A W F T *

961 agcatcgccgcctggttcacctga

961 970 980

Original sequence is highlighted in green. Modified bases in codon-optimised sequence are highlighted in red.

3) FUT4 aminoacid sequence (NCBI Reference Sequence: NP_002024.1)

MRRLWGAARKPSGAGWEKEWAEAPQEAPGAWSGRLGPGRSGRKGRAVPGWASW

PAHLALAARPARHLGGAGQGPRPLHSGTAPFHSRASGERQRRLEPQLQHESRCRSST

PADAWRAEAALPVRAMGAPWGSPTAAAGGRRGWRRGRGLPWTVCVLAAAGLTC

TALITYACWGQLPPLPWASPTPSRPVGVLLWWEPFGGRDSAPRPPPDCRLRFNISGC

RLLTDRASYGEAQAVLFHHRDLVKGPPDWPPPWGIQAHTAEEVDLRVLDYEEAAA

AAEALATSSPRPPGQRWVWMNFESPSHSPGLRSLASNLFNWTLSYRADSDVFVPYG

YLYPRSHPGDPPSGLAPPLSRKQGLVAWVVSHWDERQARVRYYHQLSQHVTVDVF

GRGGPGQPVPEIGLLHTVARYKFYLAFENSQHLDYITEKLWRNALLAGAVPVVLGP

DRANYERFVPRGAFIHVDDFPSASSLASYLLFLDRNPAVYRRYFHWRRSYAVHITSF

WDEPWCRVCQAVQRAGDRPKSIRNLASWFER

N-terminal cytoplasmic tail is highlighted in yellow, membrane-spanning region is highlighted in green and C-terminal catalytic domain is highlighted in blue.

4) Alignment of original and codon-optimised FUT4 DNA sequences

```
   1 G   Q   L   P   P   L   P   W   A   S   P   T   P   S   R   P   V   G   V   L

   1 gggcagctgccgccgctgccctgggcgtcgccaaccccgtcgcgaccggtgggcgtgctg

   1 G   Q   L   P   P   L   P   W   A   S   P   T   P   S   R   P   V   G   V   L

   1 ggccagctccccccctccctgggccagccccaccccagccgccccgtcggcgtcctc

   1           10          20          30          40          50


  21 L   W   W   E   P   F   G   G   R   D   S   A   P   R   P   P   P   D   C   R

  61 ctgtggtgggagcccttcgggggcgcgatagcgccccgaggccgccccctgactgccgg

  21 L   W   W   E   P   F   G   G   R   D   S   A   P   R   P   P   P   D   C   R

  61 ctctggtgggagcccttcggcggccgcgacagcgccccccgccccccccccgactgccgc

  61           70          80          90          100         110


  41 L   R   F   N   I   S   G   C   R   L   L   T   D   R   A   S   Y   G   E   A

 121 ctgcgcttcaacatcagcggctgccgcctgctcaccgaccgcgcgtcctacggagaggct

  41 L   R   F   N   I   S   G   C   R   L   L   T   D   R   A   S   Y   G   E   A

 121 ctccgcttcaacatcagcggctgccgcctcctcaccgaccgcgccagctacggcgaggcc

 121           130         140         150         160         170
```

```
 61 Q  A  V  L  F  H  H  R  D  L  V  K  G  P  P  D  W  P  P  P
181 caggccgtgcttttccaccaccgcgacctcgtgaaggggccccccgactggcccccgccc
 61 Q  A  V  L  F  H  H  R  D  L  V  K  G  P  P  D  W  P  P  P
181 caggccgtcctcttccaccaccgcgacctcgtcaagggccccccccgactggcccccccccc
181          190       200       210       220       230

 81 W  G  I  Q  A  H  T  A  E  E  V  D  L  R  V  L  D  Y  E  E
241 tggggcatccaggcgcacactgccgaggaggtggatctgcgcgtgttggactacgaggag
 81 W  G  I  Q  A  H  T  A  E  E  V  D  L  R  V  L  D  Y  E  E
241 tggggcatccaggcccacaccgccgaggaggtcgacctccgcgtcctcgactacgaggag
241          250       260       270       280       290

101 A  A  A  A  A  E  A  L  A  T  S  S  P  R  P  P  G  Q  R  W
301 gcagcggcggcggcagaagccctggcgacctccagccccaggcccccgggccagcgctgg
101 A  A  A  A  A  E  A  L  A  T  S  S  P  R  P  P  G  Q  R  W
301 gccgccgccgccgccgaggccctcgccaccagcagcccccgccccccggccagcgctgg
301          310       320       330       340       350

121 V  W  M  N  F  E  S  P  S  H  S  P  G  L  R  S  L  A  S  N
361 gtttggatgaacttcgagtcgccctcgcactccccggggctgcgaagcctggcaagtaac
121 V  W  M  N  F  E  S  P  S  H  S  P  G  L  R  S  L  A  S  N
361 gtctggatgaacttcgagagccccagccacagccccggcctccgcagcctcgccagcaac
361          370       380       390       400       410
```

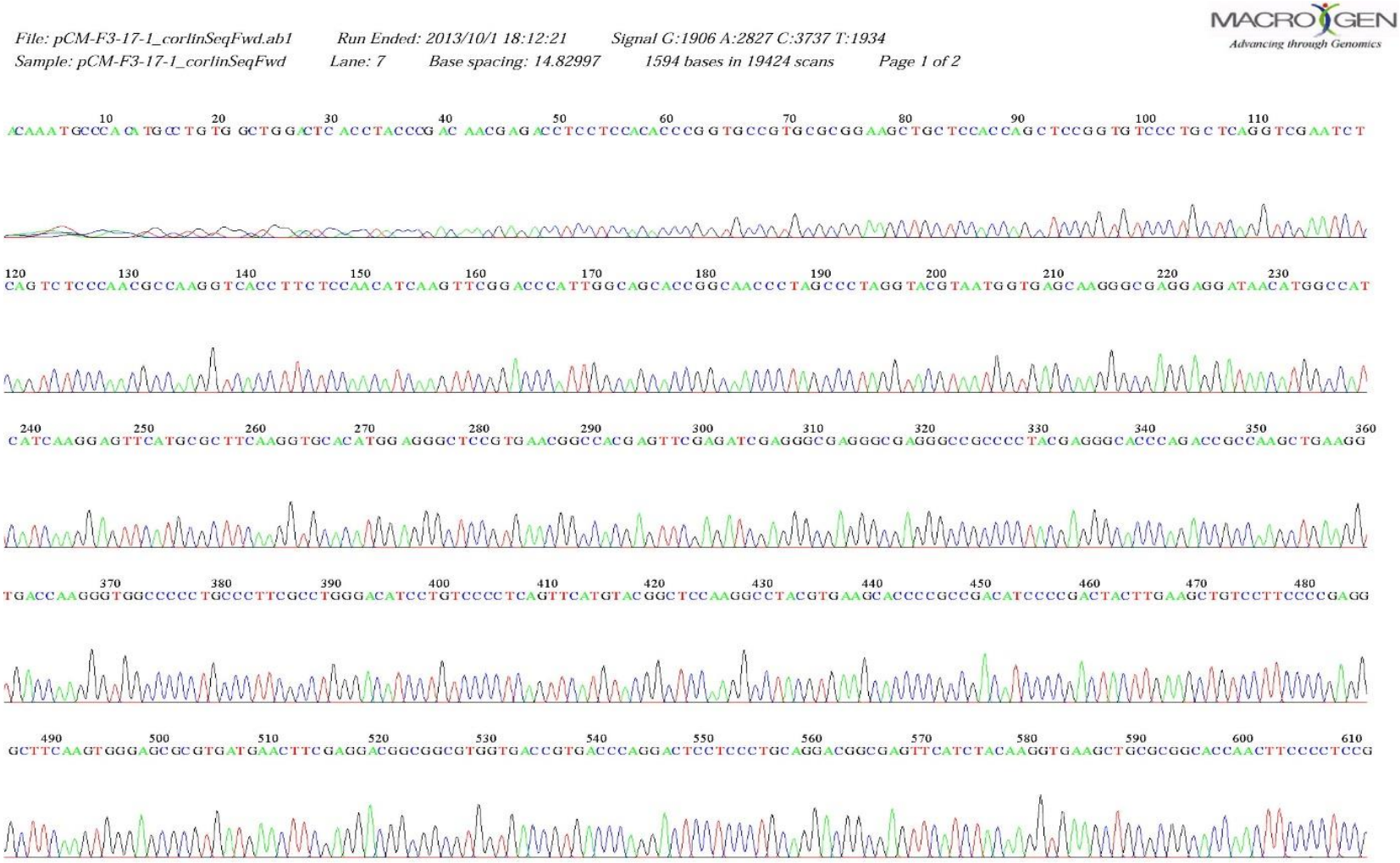
```
141 L  F  N  W  T  L  S  Y  R  A  D  S  D  V  F  V  P  Y  G  Y

421 ctcttcaactggacgctctcctaccgggcggactcggacgtctttgtgccttatggctac

141 L  F  N  W  T  L  S  Y  R  A  D  S  D  V  F  V  P  Y  G  Y

421 ctcttcaactggacctcagctaccgcgccgacagcgacgtcttcgtccctacggctac

421          430       440       450       460       470


161 L  Y  P  R  S  H  P  G  D  P  P  S  G  L  A  P  P  L  S  R

481 ctctaccccagaagccacccggcgacccgccctcaggcctggccccgccactgtccagg

161 L  Y  P  R  S  H  P  G  D  P  P  S  G  L  A  P  P  L  S  R

481 ctctaccccgcagccaccccggcgacccccccagcggcctcgcccccccctcagccgc

481          490       500       510       520       530


181 K  Q  G  L  V  A  W  V  V  S  H  W  D  E  R  Q  A  R  V  R

541 aaacaggggctggtggcatgggtggtgagccactgggacgagcgccaggcccgggtccgc

181 K  Q  G  L  V  A  W  V  V  S  H  W  D  E  R  Q  A  R  V  R

541 aagcagggcctcgtcgcctgggtcgtcagccactgggacgagcgccaggcccgcgtccgc

541          550       560       570       580       590


201 Y  Y  H  Q  L  S  Q  H  V  T  V  D  V  F  G  R  G  G  P  G

601 tactaccaccaactgagccaacatgtgaccgtggacgtgttcggccggggcgggccgggg

201 Y  Y  H  Q  L  S  Q  H  V  T  V  D  V  F  G  R  G  G  P  G

601 tactaccaccagctcagccagcacgtcaccgtcgacgtcttcggccgcggcggccccggc

601          610       620       630       640       650
```

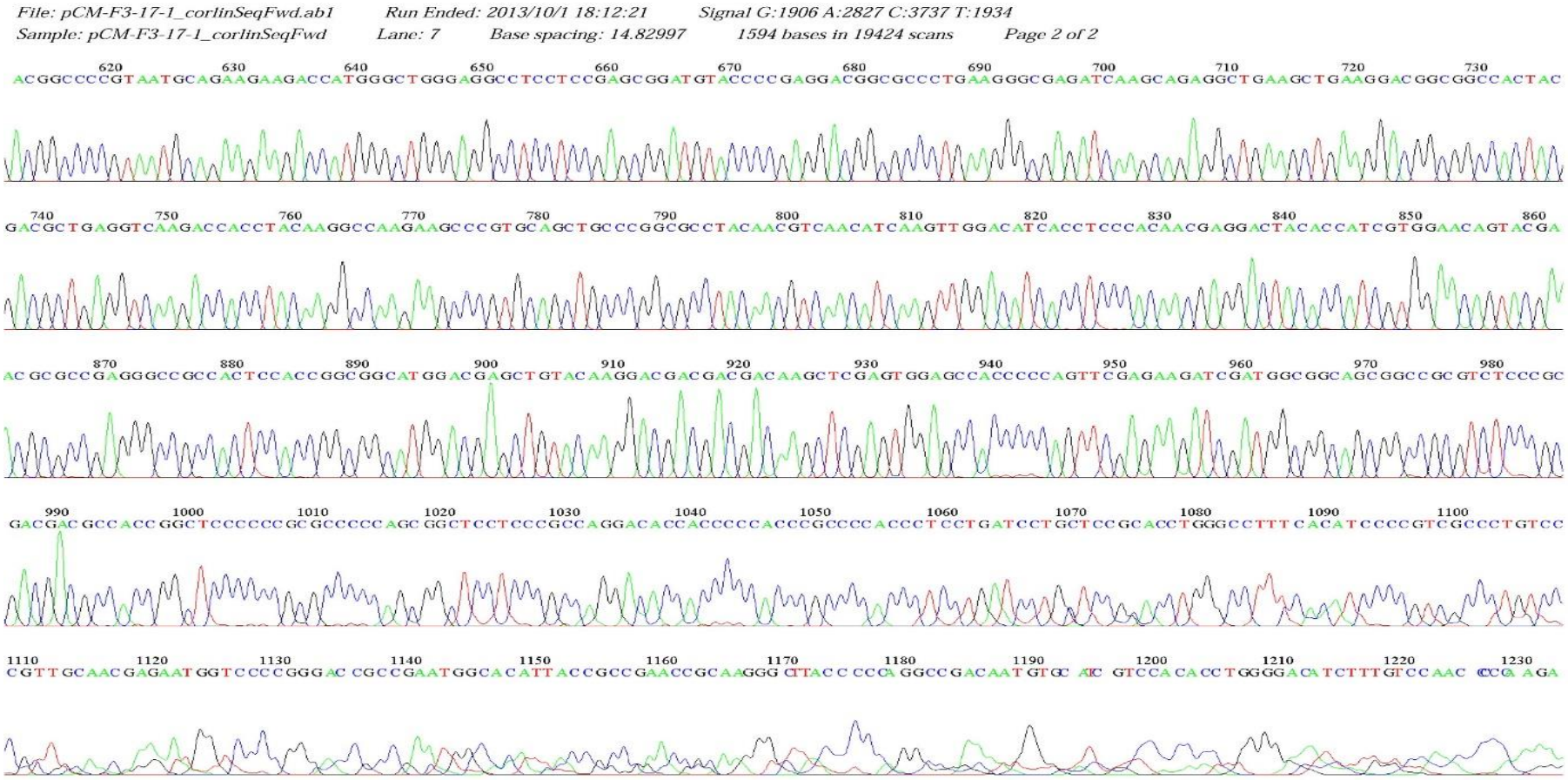
```
221 Q  P  V  P  E  I  G  L  L  H  T  V  A  R  Y  K  F  Y  L  A
661 cagccggtgcccgaaattgggctcctgcacacagtggcccgctacaagttctacctggct
221 Q  P  V  P  E  I  G  L  L  H  T  V  A  R  Y  K  F  Y  L  A
661 cagcccgtccccgagatcggcctcctccacaccgtcgcccgctacaagttctacctcgcc
661          670       680       690       700       710

241 F  E  N  S  Q  H  L  D  Y  I  T  E  K  L  W  R  N  A  L  L
721 ttcgagaactcgcagcacctggattatatcaccgagaagctctggcgcaacgcgttgctc
241 F  E  N  S  Q  H  L  D  Y  I  T  E  K  L  W  R  N  A  L  L
721 ttcgagaacagccagcacctcgactacatcaccgagaagctctggcgcaacgcctcctc
721          730       740       750       760       770

261 A  G  A  V  P  V  V  L  G  P  D  R  A  N  Y  E  R  F  V  P
781 gctggggcggtgccggtggtgctgggcccagaccgtgccaactacgagcgctttgtgccc
261 A  G  A  V  P  V  V  L  G  P  D  R  A  N  Y  E  R  F  V  P
781 gccggcgccgtccccgtcgtcctcggccccgaccgcgccaactacgagcgcttcgtcccc
781          790       800       810       820       830

281 R  G  A  F  I  H  V  D  D  F  P  S  A  S  S  L  A  S  Y  L
841 cgcggcgccttcatccacgtggacgacttcccaagtgcctcctccctggcctcgtacctg
281 R  G  A  F  I  H  V  D  D  F  P  S  A  S  S  L  A  S  Y  L
841 cgcggcgccttcatccacgtcgacgacttccccagcgccagcagcctcgccagctacctc
841          850       860       870       880       890
```

```
 301 L  F  L  D  R  N  P  A  V  Y  R  R  Y  F  H  W  R  R  S  Y
 901 cttttcctcgaccgcaaccccgcggtctatcgccgctacttccactggcgccggagctac
 301 L  F  L  D  R  N  P  A  V  Y  R  R  Y  F  H  W  R  R  S  Y
 901 ctcttcctcgaccgcaaccccgccgtctaccgccgctacttccactggcgccgcagctac
 901          910       920       930       940       950

 321 A  V  H  I  T  S  F  W  D  E  P  W  C  R  V  C  Q  A  V  Q
 961 gctgtccacatcacctccttctgggacgagccttggtgccgggtgtgccaggctgtacag
 321 A  V  H  I  T  S  F  W  D  E  P  W  C  R  V  C  Q  A  V  Q
 961 gccgtccacatcaccagcttctgggacgagccctggtgccgcgtctgccaggccgtccag
 961          970       980       990       1000      1010

 341 R  A  G  D  R  P  K  S  I  R  N  L  A  S  W  F  E  R  *
1021 agggctggggaccggcccaagagcatacggaacttggccagctggttcgagcggtga
 341 R  A  G  D  R  P  K  S  I  R  N  L  A  S  W  F  E  R  *
1021 cgcgccggcgaccgcccaagagcatccgcaacctcgccagctggttcgagcgctga
1021          1030      1040      1050      1060      1070
```

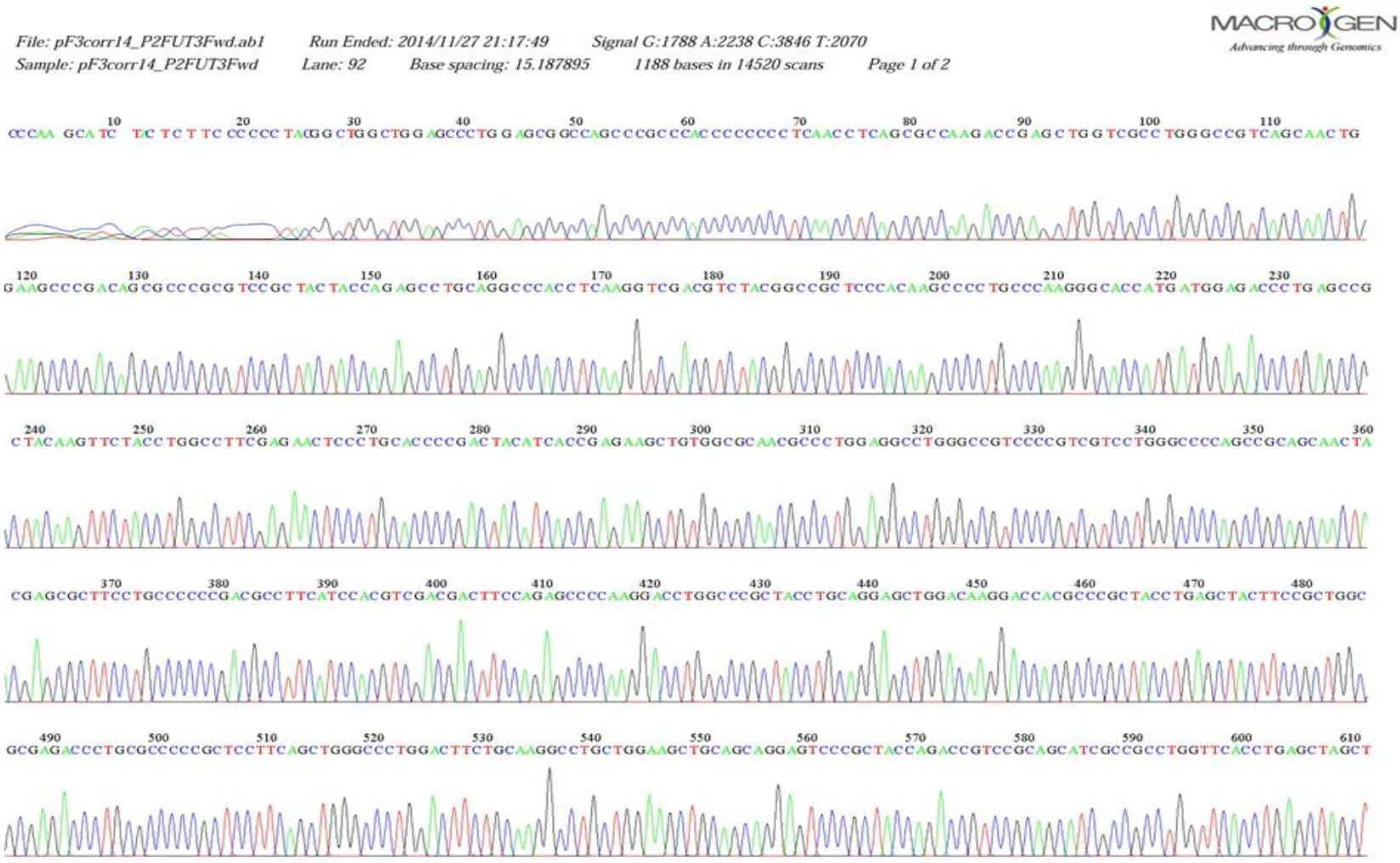
Original sequence is highlighted in green. Modified bases in codon-optimised sequence are highlighted in red.

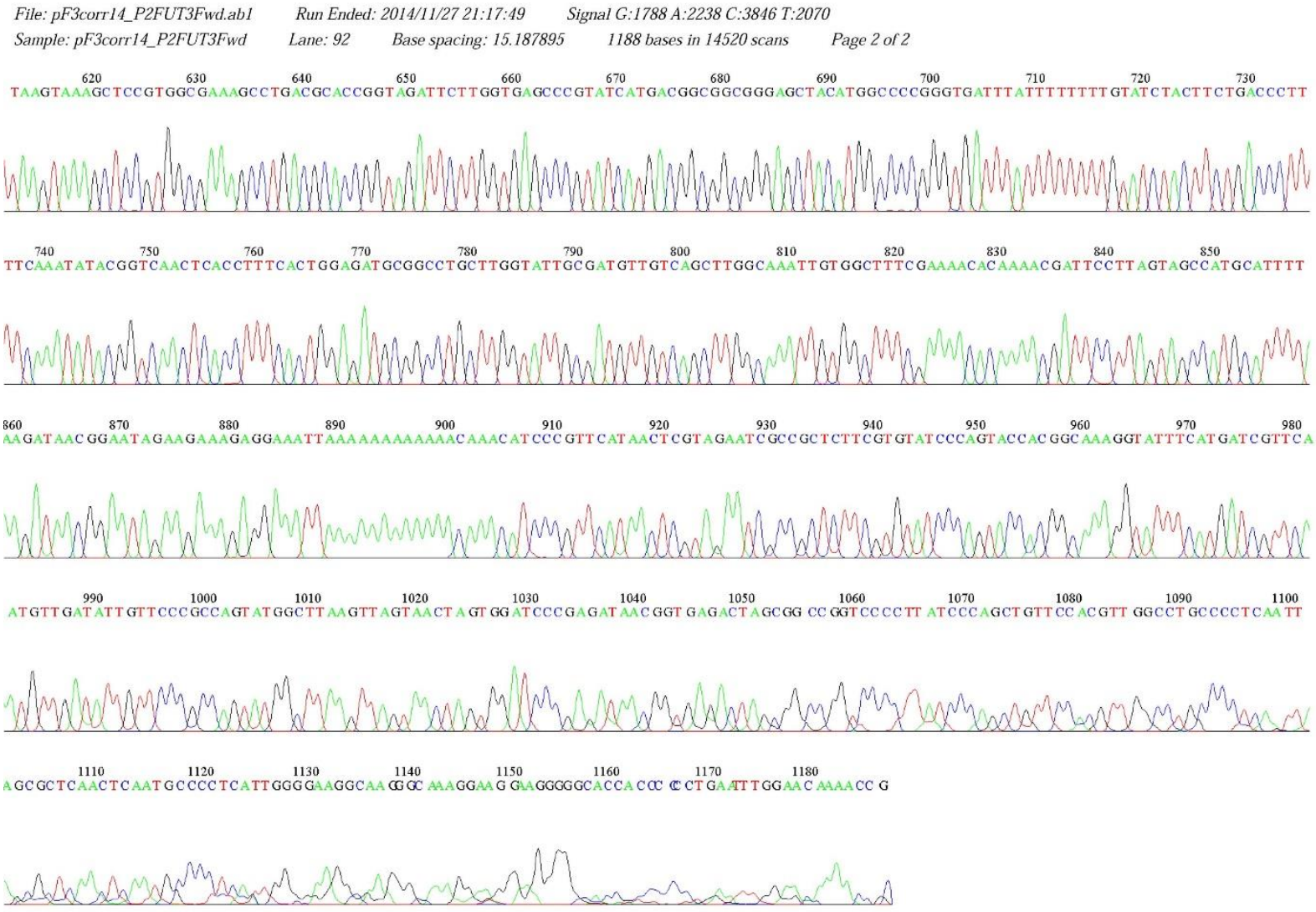
5) Sequencing of the pCMF3 plasmid



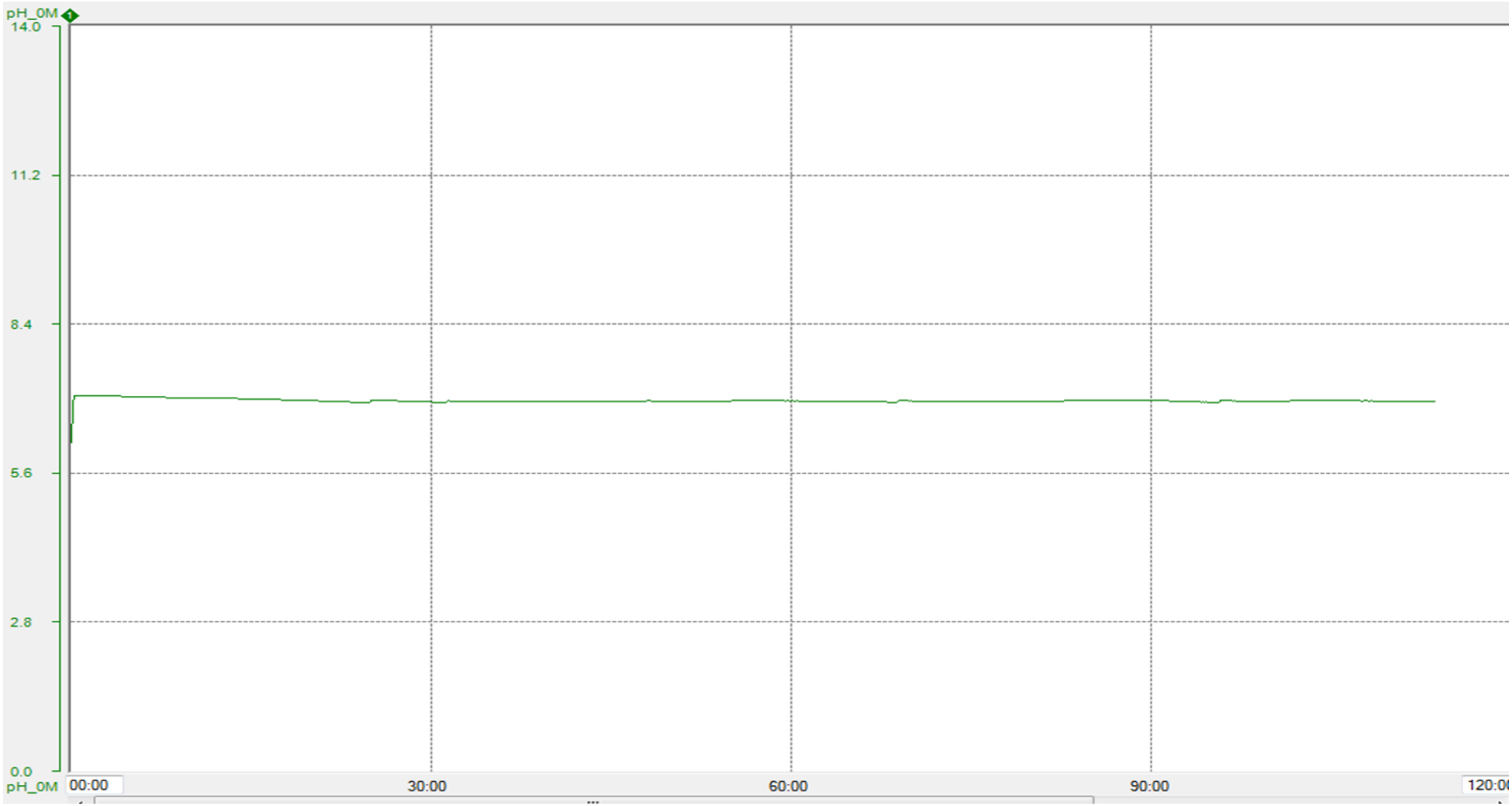

File: pCM-F3-17-1_corlinSeqFwd.ab1 Run Ended: 2013/10/1 18:12:21 Signal G:1906 A:2827 C:3737 T:1934
Sample: pCM-F3-17-1_corlinSeqFwd Lane: 7 Base spacing: 14.82997 1594 bases in 19424 scans Page 2 of 2

6) Sequencing of the pCF3 plasmid

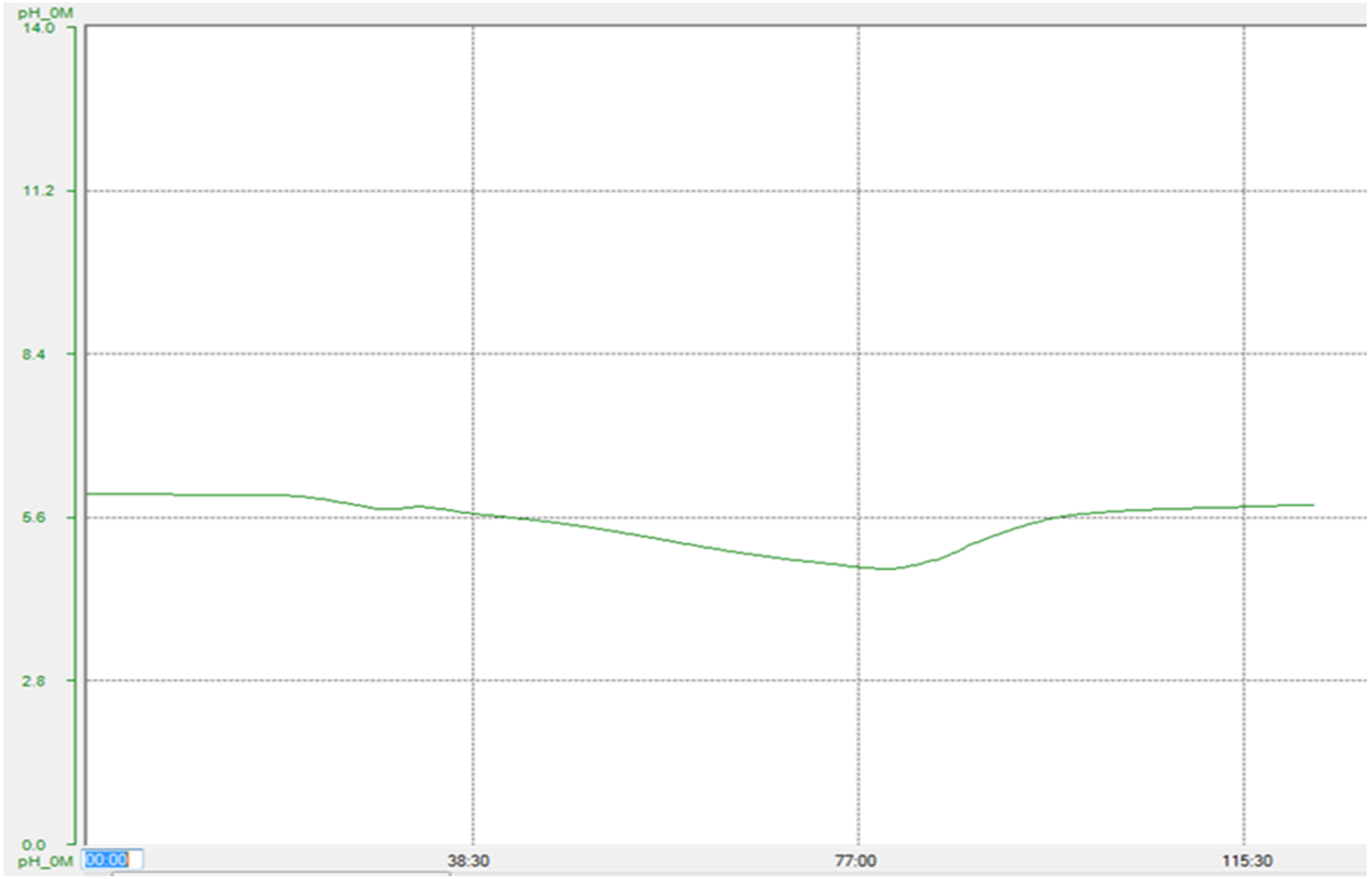



File: pF3corr14_P2FUT3Fwd.ab1 Run Ended: 2014/11/27 21:17:49 Signal G:1788 A:2238 C:3846 T:2070
Sample: pF3corr14_P2FUT3Fwd Lane: 92 Base spacing: 15.187895 1188 bases in 14520 scans Page 2 of 2
620 630 640 650 660 670 680 690 700 710 720 730
TAAGTAAAGCTCCGTGGCGAAAGCCTGACGCACCGGTAGATTCTTGGTGAGCCCGTATCATGACGGCGGCGGGAGCTACATGGCCCCGGGTGATTTATTTTTTTTGTATCTACTTCTGACCCTT
740 750 760 770 780 790 800 810 820 830 840 850
TTCAAATATACGGTCAACTCACCTTTCACTGGAGATGCGGCCTGCTTGGTATTGCGATGTTGTCAGCTTGGCAAATTGTGGCTTTCGAAAACACAAAACGATTCCTTAGTAGCCATGCATTTT
860 870 880 890 900 910 920 930 940 950 960 970 980
AAGATAACGGAATAGAAGAAAGAGGAAATTAAAAAAAAAAAAACAAACATCCCGTTCATAACTCGTAGAATCGCCGCTCTTCGTGTATCCCAGTACCACGGCAAAGGTATTTCATGATCGTTCA
990 1000 1010 1020 1030 1040 1050 1060 1070 1080 1090 1100
ATGTTGATATTGTTCCCGCCAGTATGGCTTAAGTTAGTAACTAGTGGATCCCGAGATAACGGTGAGACTAGCGGCCGGTCCCCTTATCCCAGCTGTTCCACGTTGGCCTGCCCCTCAATT
1110 1120 1130 1140 1150 1160 1170 1180
AGCGCTCAACTCAATGCCCCTCATTGGGGAAGGCAAGGCAAAGGAAGGAGGGGGCACCACCCCCTGAATTTGGAACAAAACCG

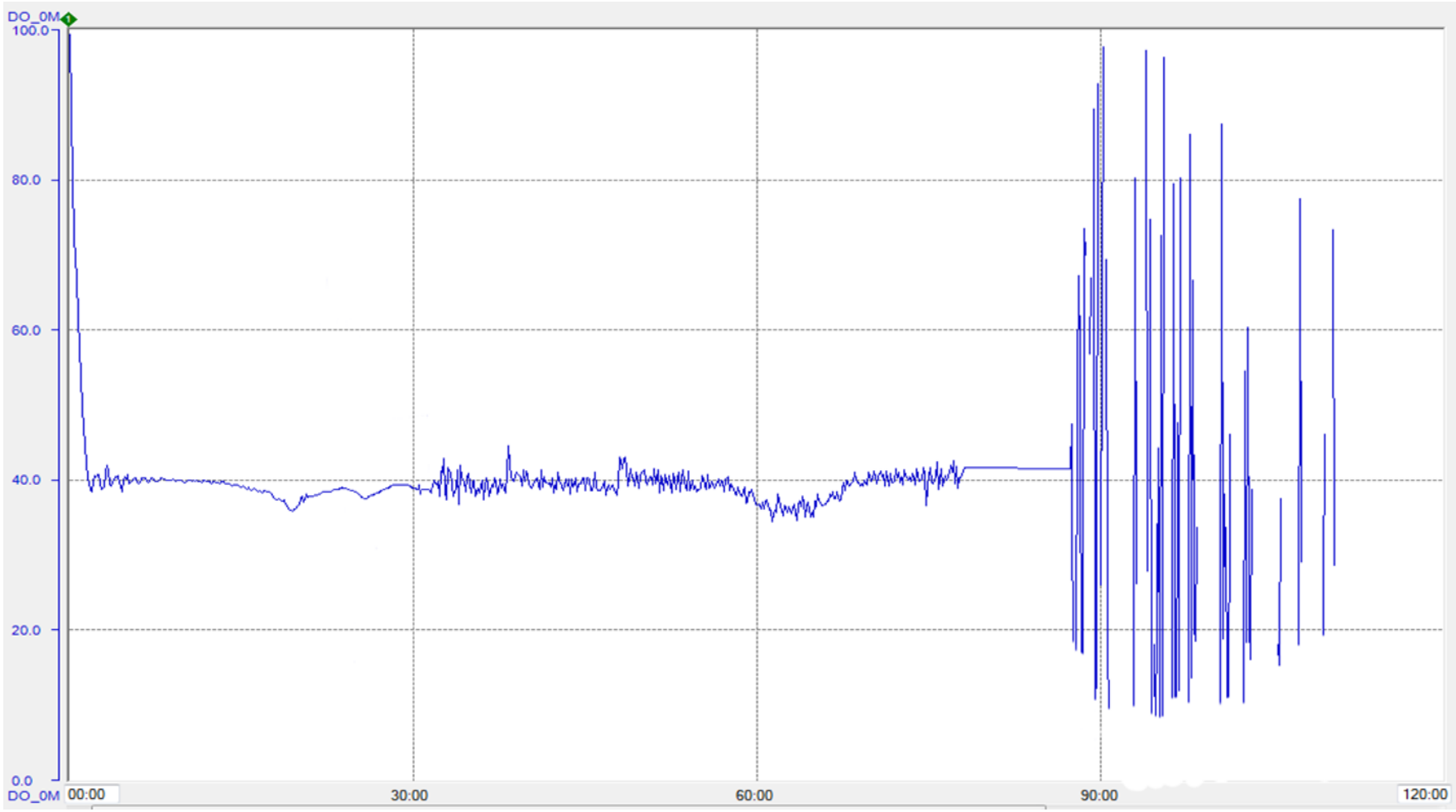
7) pH curve of the CMF3-44 bioreactor culture with pH control



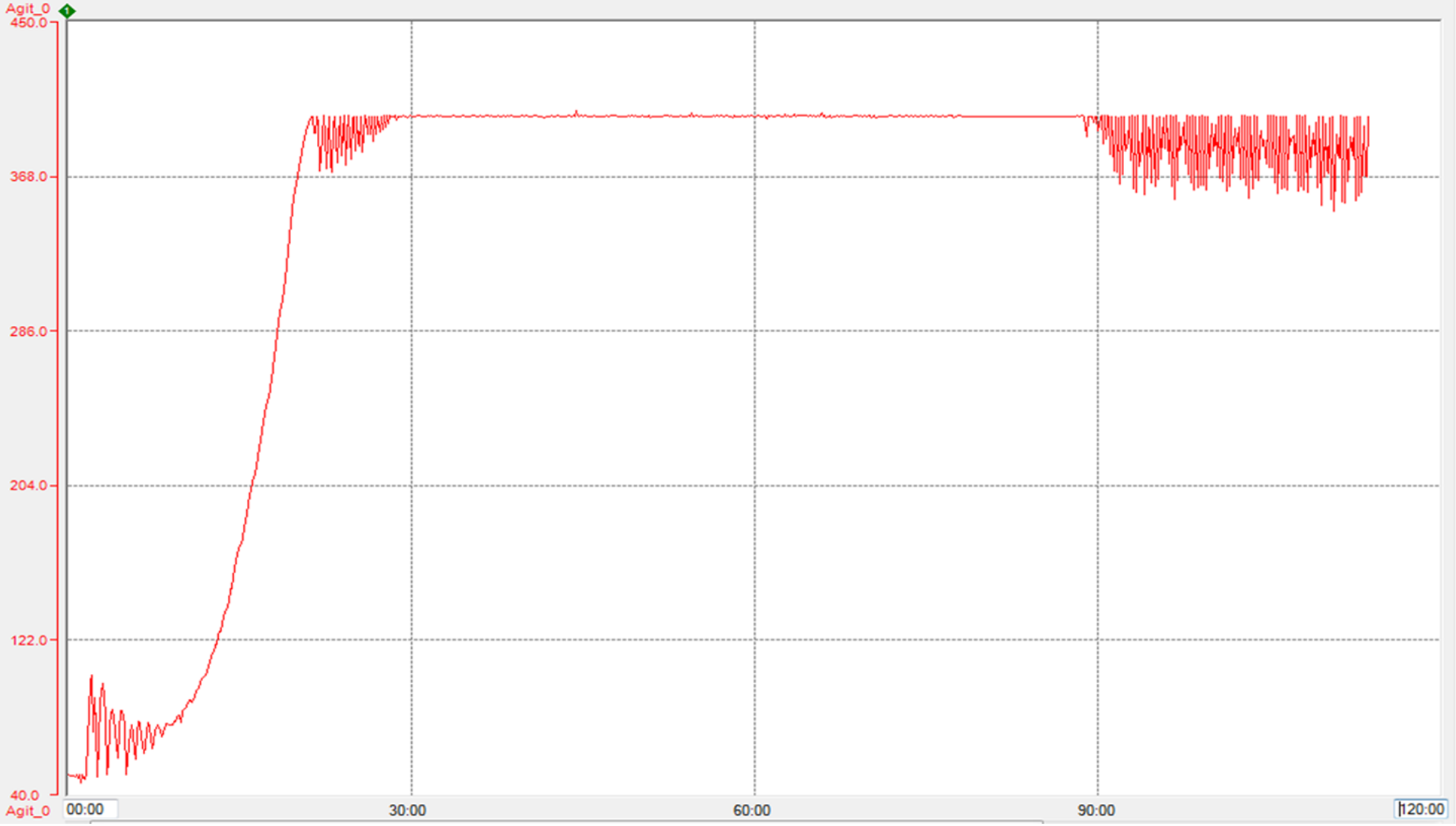
8) pH curve of the CMF3-44 bioreactor culture without pH control



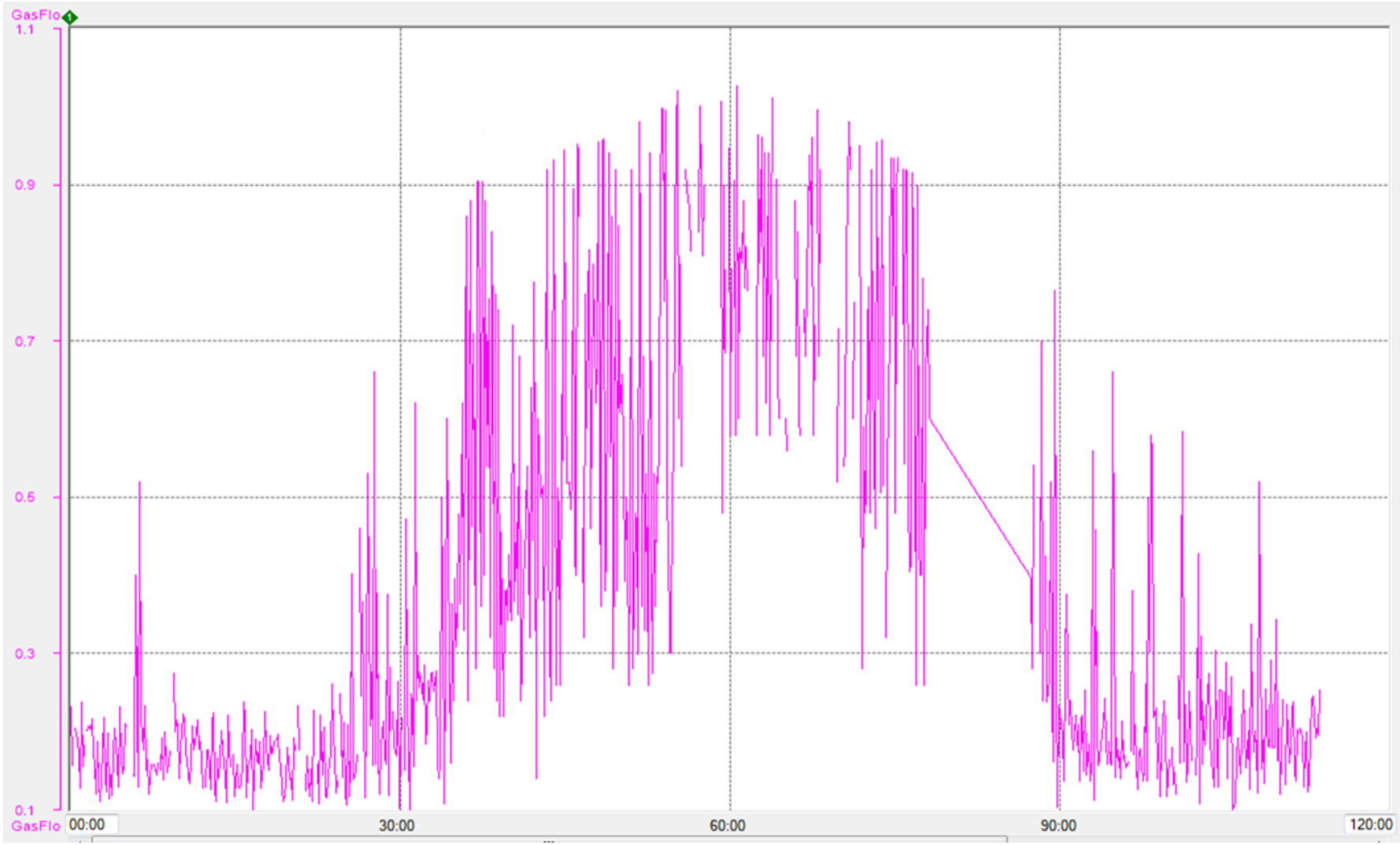
9) Dissolved oxygen concentration in the CMF3-44 bioreactor culture with pH control



10) Agitation recorded for the CMF3-44 bioreactor culture with pH control



11) Aeration recorded for the CMF3-44 bioreactor culture with pH control



12) Biosafety approval letter

From: Bio Safety <biosafety@mq.edu.au>
Sent: 22 May 2015 12:28
To: Helena Nevalainen
Subject: Approval for Exempt Dealing "Expression of bacterial, fungal, plant and animal genes encoding harmless products in Trichoderma reesei (GRAS: Generally recognized As Safe)" (5201500378)

Re: Exempt Dealing "Expression of bacterial, fungal, plant and animal genes encoding harmless products in *Trichoderma reesei* (GRAS: Generally recognized As Safe)" (5201500378)

Thank you for your response to concerns raised by the Institutional Biosafety Committee (IBC) regarding the above application. The Chair has reviewed your response and approval of the above Exempt Dealing has been granted, effective 20 May 2015.

Approval has been granted subject to your compliance with the Office of the Gene Technology Regulator's standard conditions for exempt work listed below:

1. The project must be conducted in accordance with the OGTR Guidance Notes for the Containment of Exempt Dealings (http://www.ogtr.gov.au/internet/ogtr/publishing.nsf/Content/ExemptDealGuideSept11_2-htm
2. The Guidance Notes are only applicable to exempt dealings conducted under the *Gene Technology Act 2000*. They do not provide guidance for laboratory safety, good laboratory practice or work health and safety issues. For these purposes, refer to AS/NZS 2243.3:2010.
3. You must inform the Institutional Biosafety Committee if you complete or abandon the exempt dealings with GMOs.

Please note the following standard requirements of approval:
1. Approval will be for a period of 5 years *subject to the provision of annual reports* (http://www.research.mq.edu.au/current_research_staff gene_technology_and_biosafety/submitting_a_new_application). If, at the end of this period the project has been completed, abandoned, discontinued or not commenced for any reason, you are required to submit a Final Report. If you complete the work earlier than you had planned you must submit a Final Report as soon as the work is completed. Please contact the Committee Secretary at biosafety@mq.edu.au in order to obtain a report.

A Progress/Final Report for this project will be due on: 20 May 2016.

2. If you will be applying for or have applied for internal or external funding for the above project it is your responsibility to provide the Macquarie University's Research Grants Management Assistant with a copy of this email as soon as possible. Internal and External funding agencies will not be informed that you have final approval for your project and funds will not be released until the Research Grants Management Assistant has received a copy of this email.

If you need to provide a hard copy letter of Final Approval to an external organisation as evidence that you have Final Approval, please do not hesitate to contact the Committee Secretary at biosafety@mq.edu.au or by phone 9850 4063.

Please retain a copy of this email as this is your formal notification of final Biosafety approval.

Yours Sincerely

Biosafety Secretariat
Research Office
Level 3, Research Hub, Building C5C East
Macquarie University
NSW 2109 Australia