Universidade do Minho
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Maria Goreti Castro Sepúlveda

Production of recombinant carbohydrate-binding modules in *Pichia pastoris* and their application in the modification of paper fibers

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Dissertação de Mestrado
Mestrado em Bioengenharia

Trabalho efetuado sob a orientação da
Doutora Carla Cristina Marques de Oliveira
e co-orientação da
Professora Doutora Lucília Maria Alves Ribeiro Domingues

outubro de 2014

“Our greatest weakness lies in giving up. The most certain way to succeed is always to try just one more time”

Thomas A. Edison

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Abstract

Enzymes have been widely applied in the paper industry for the modification of pulp and paper properties. Although they have the potential to replace hazardous chemicals, their use in the hydrolysis of polysaccharides reduces both fiber strength and mass. To overcome these negative effects, the potential use of recombinant carbohydrate-binding modules (CBMs) in papermaking, has been receiving growing attention. A CBM is defined as a contiguous amino-acid sequence within a carbohydrate-active enzyme, presenting an independent fold and function, and carbohydrate-binding activity. In addition, glycosylation in fungal CBMs has been suggested to be important for the modification of pulp and paper properties

In order to improve cellulose fibers from the paper industry using an enzyme-free process, consisting of recombinant CBMs, we aimed to: (1) clone and produce in *Pichia pastoris* recombinant CBM1 from *Trichoderma reesei* (fungus) and CBM3 from *Clostridium thermocellum* (bacteria), (2) optimize the production and purification of the recombinant CBMs by immobilized metal ion affinity chromatography (IMAC) and (3) characterize the cellulose-binding capacity of the recombinant CBMs and study their effects in the properties of pulp (drainability) and paper fibers (wetability, bursting and tensile strengths, lengthening, tearing and air permeability).

Recombinant CBM1 fused to enhanced green fluorescent protein (eGFP), directly or by the native glycosylated CBM linker, was produced at high levels but mainly as intracellular protein. Their purification from *P. pastoris* cell-free extracts by IMAC resulted in low amounts, which compromised their characterization and application. Nevertheless, the two purified recombinant CBM-fusions were able to bind to cellulose. On the other hand, both recombinant versions of CBM3, glycosylated and non-glycosylated, were produced as secreted proteins with the non-glycosylated CBM3 exhibiting higher cellulose-binding affinity than the glycosylated version. Regardless of the lack of effect on pulp drainability, both CBM3 versions improved significantly the mechanical properties of *Eucaliptus globulus* papersheets, namely the burst and tensile strength indexes, up to 12% and 10% (in papersheets made of *E. globulus* pulp treated with non-glycosylated CBM3), respectively. In addition, non-glycosylated CBM3 decreased significantly the wetability and air permeability of papersheets. However, both recombinant CBM3 versions did not have any effect on papersheets made of a mixture of *E. globulus* and *Pinus sylvestris* (30:70) pulp. The modification of paper fibers by recombinant CBM3 was dependent on its concentration but not on its glycosylation. These results indicate that recombinant CBM3 play an important role in the improvement of surface/interface properties of cellulose fibers, contributing to paper products with higher quality.

Resumo

As enzimas têm sido amplamente aplicadas na indústria de papel para a modificação das propriedades de pasta e papel. Embora tenham o potencial para substituir produtos químicos perigosos, a sua utilização na hidrólise de polissacarídeos reduz a massa e a resistência das fibras. De forma a ultrapassar estes efeitos negativos, tem-se dado cada vez mais atenção à potencial utilização de módulos recombinantes de ligação a carbohidratos (CBMs) no fabrico de papel. Um CBM é definido como uma sequência de aminoácidos de uma enzima, que apresenta uma estrutura e função independentes, bem como atividade de ligação a carbohidratos. Além disso, a glicosilação dos CBMs de origem fúngica tem sido sugerida como importante para a modificação das propriedades da pasta e do papel.

De forma a melhorar as fibras de celulose da indústria de papel, utilizando um processo sem enzimas, e que consiste na utilização de CBMs recombinantes, propusemo-nos a: (1) clonar e produzir em *Pichia pastoris* o CBM1 recombinante de *Trichoderma reesei* (fungo) e o CBM3 de *Clostridium thermocellum* (bactéria), (2) optimizar a produção e purificação dos CBMs recombinantes por cromatografia de afinidade com iões metálicos imobilizados (IMAC) e (3) caraterizar a capacidade de ligação à celulose dos CBMs recombinantes e estudar os seus efeitos nas propriedades de pasta (drenabilidade) e fibras de papel (molhabilidade, rutura, resistência à tração, alongamento, rasgamento e permeabilidade ao ar).

O CBM1 recombinante fundido com a proteína verde fluorescente (eGFP), diretamente ou através do linker glicosilado nativo do CBM, foi produzido em níveis elevados, mas sobretudo de forma intracelular. A sua purificação a partir de extratos celulares de *P. pastoris* por IMAC resultou em baixas quantidades, o que comprometeu a sua caraterização e aplicação. No entanto, as proteínas de fusão eGFP-CBM purificadas apresentaram capacidade de ligação à celulose. Por outro lado, ambas as versões recombinantes do CBM3 foram produzidas em grande quantidade no meio de cultura, tendo a versão não glicosilada apresentado uma maior afinidade de ligação à celulose. Independentemente de não terem alterado a drenabilidade de pastas de papel, ambas as versões recombinantes do CBM3 melhoraram significativamente as propriedades mecânicas das folhas de papel de *Eucaliptus globulus*, nomeadamente, os índices de rutura e de resistência à tração, até 12% e 10% (em folhas de papel feitas a partir de pasta *E. globulus* tratada com a versão não glicosilada do CBM3), respetivamente. Além disso, a versão não glicosilada do CBM3 diminuiu significativamente a molhabilidade e permeabilidade ao ar das folhas de papel. Contudo, ambas as versões recombinantes do CBM3 não provocaram qualquer efeito em folhas de papel feitas a partir de pasta *E. globulus* e *Pinus sylvestris* (30:70). A modificação das fibras de papel pelo CBM3 recombinante foi dependente da sua concentração, mas não da sua glicosilação. Estes resultados indicam que o CBM3 recombinante desempenha um papel importante na melhoria das propriedades superficiais/interfaciais de fibras de celulose, contribuindo para produtos de papel com maior qualidade.

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Abbreviations

°C	degree Celsius
µg	microgram
µL	microliter
µM	micromolar
Aa	amino-acid(s)
Avicel	commercial name of microcrystalline cellulose
BMG	Buffered minimal medium containing Glycerol
BMM	Buffered minimal medium containing Methanol
bp	base pairs
BSA	Bovine Serum Albumin
CBD	Cellulose-binding domain
CBM	Carbohydrate-binding module
CD	Catalytic domain
Ct	*Clostridium thermocellum*
Da	Dalton
dH_2O	distilled water
DNA	deoxyribonucleic acid
DNS	3,5-dinitrosalicylic acid
dNTP	deoxyribonucleotide
EDTA	Ethylenediamine tetraacetic acid
eGFP	enhanced Green fluorescent protein
FITC	Fluorescein isothiocyanate
g	gram
g	relative centrifugal force
His-tag	Polyhistidine-tag
h	Hour
IMAC	Immobilized Metal ion Affinity Chromatography

L	Liter
LB	Luria-Bertani medium
LK	Linker sequence
M	molar concentration
min	minute
ms	millisecond
ng	nanogram
NL	Native Linker
nm	Nanometer
o/n	Overnight
O.d.	Oven dry
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PEG	Polyethilenoglycol
PES	Polyethersulfone
PMSF	Phenylmethanesulphonylfluoride
RAC	Regenerated amorphous cellulose
rpm	revolutions per minute
s	second
SAP	Shrimp Alkaline Phosphatase
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SOC	Super Optimal broth with Catabolite repression
°SR	Schopper-Riegler degree
TAE	Tris-Acetate-EDTA
TEMED	N,N,N',N'-Tetramethylethylenediamine
TEV	Tobacco etch virus
Tr	*Trichoderma reesei*
Tris	tris(hydroxymethyl)aminomethane
YPD	Yeast extract Peptone Dextrose medium

Aims

Since the mid 1980s, enzymes have been widely applied in the paper industry, namely, in the bleaching boosting with xylanases and fiber modification with cellulases. Despite of the benefits of this utilization, the hydrolytic activity is responsible for an elevated mass loss of the treated products. To avoid these negative effects, the use of carbohydrate-binding modules (CBMs) in papermaking has been investigated. It was demonstrated that the treatment of cellulose materials with CBMs improves significantly the interfacial properties of the fibers (Pala et al., 2001; Pinto et al., 2006). Additionally, glycosylation has been suggested to play a key role in the beneficial effects of CBMs in cellulose fibers, namely pulp drainability. According to the literature, the glycans attached to the CBMs have high water affinity, improving the hydration and stabilization of the fibers, thereby reducing the inter-fiber interactions and contributing to a better water drainage (Machado et al., 2009). Nevertheless, the methodology to obtain native CBMs results in contaminant residual catalytic activity. Therefore, the development of industrially attractive strategies to obtain pure glycosylated CBMs is desired. This goal may be attained through their production and purification in recombinant organisms capable of performing glycosylation, such as the methylotrophic yeast *P. pastoris*.

Thus, the main objective of this work is the improvement of cellulose fibers from the paper industry using recombinant glycosylated CBMs produced in *P. pastoris*, namely the family 1 CBM from *Trichoderma reesei* (fungus) Cel7A (cellobiohydrolase I) (*Tr*CBM1$_{Cel7A}$) and the family 3 CBM from *Clostridium thermocellum* (bacterium) CipA scaffold protein (*Ct*CBM3$_{CipA}$).

In summary, the specific aims of this study are:

1. Cloning and expression of *Ct*CBM3$_{CipA}$ (glycosylated and non-glycosylated versions) and *Tr*CBM1$_{Cel7A}$ (fused to eGFP, directly or by the native glycosylated CBM linker) in *P. pastoris*;
2. Optimization of the production conditions and purification of the recombinant CBMs;
3. Characterization of the recombinant CBMs in terms of cellulose-binding capacity and study of their effects in the properties of pulp (drainability) and paper fibers (wetability, bursting and tensile strengths, lengthening, tearing and air permeability).

CHAPTER 1

Introduction

1.1. Brief description of the papermaking process

The activity of pulp and paper sectors contributes strongly to the growth of the Portuguese economy. The Portucel Soporcel group, a Portuguese industry of paper and pulp, is one of the country's three leading exporters, and possibly the exporter generating the higher national value added, once their products are manufactured from national raw materials and resources. Thereby, 75% of eucalyptus wood and 73% of pine wood for this industry is sourced in Portugal (http://www.celpa.pt). Additionally, this group is leader in the production of high quality paper, such as the Navigator, considered to be the best office paper in the world (Opticom Internatioal Research, 2004). All of these factors help to place Portugal in the forefront of pulp and paper production.

Pulp and paper are manufactured from raw materials containing cellulose fibers, generally wood, recycled paper, and agricultural residues. Wood is mainly composed of cellulose, hemicellulose and lignin. Cellulose is the most abundant of the components, generally representing 40-45% of the wood dry weight (Sjostrom, 1993). Depending on the wood species, about 20-30% of wood dry weight is hemicellulose and about 15-20% is lignin.

The main steps in pulp and paper manufacturing are: raw material preparation and handling, pulp manufacturing, pulp washing and screening, chemical recovery, bleaching, stock preparation, and papermaking (Bajpai, 2012). Paper is made by separating the cellulosic fibers in wood and then removing the lignin that binds the fibers. Cellulosic pulp is manufactured from the raw materials, using chemical, chemimechanical and mechanical (including thermomechanical) methods. Nowadays, chemical pulping is used on most papers produced commercially in the world. The main aim in chemical pulping is to separate the wood fibers from each other in order to render them suitable for further industrial processing. The delignification of woody material can be carried out by cooking (digesting) the raw materials, using the kraft

(sulfate) or sulfite processes (Sjostrom, 1993). Today, the predominant pulping method is the Kraft process. In Kraft pulping, about 90% of wood lignin is solubilized during the treatment process (Sjostrom, 1993). The remaining 10% of lignin is mainly responsible for the brown colour of the Kraft pulp and unbleached paper. The main goal of bleaching is to remove the residual lignin from the pulp, without degrading the pulp carbohydrates, especially cellulose.

Paper is a network of cellulose fibers. Cellulose is the most abundant polymer in nature (Kimura and Itoh, 2004; Tamai et al., 2004) with convenient properties for quite different applications and industries, such as varied morphology, excellent mechanical properties, availability in large amounts and low cost (Belgacem et al., 1995). Although cellulose is a homopolymer of repeated units of cellobiose, the β -1,4-glycosidic linkages make the structural organization highly ordered and tightly packed (crystallinity), with few amorphous regions (Phitsuwan et al., 2013; Srisodsuk, 1994). The glucose residues are twisted by 180° in the polymeric chain (Figure 1.1), taking different conformations. The degree of crystallinity of cellulose depends very much on origin and type of pretreatment. Crystalline cellulose is highly resistant to microbial attack and enzymatic hydrolysis, whereas amorphous cellulose is degraded at a much faster rate (Eriksson et al., 1990).

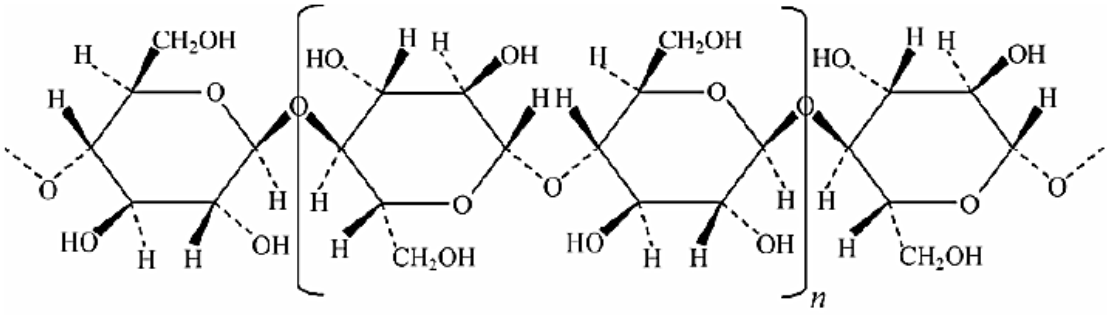

Figure 1.1 Detail of a cellulose chain, highlighting the repetitive unit cellobiose (n).

The increasing competition on the world paper market inspires producers to continually develop and improve their products. One way to meet this competition is to improve paper properties. However, understanding the mechanical properties of paper in fundamental terms is a difficult task, and it is an area of intense study. The mechanical properties of paper reflect some parameters, such as morphology, intrinsic chemistry, and structure of the individual fibers as well as the network structure of the paper. The paper properties evaluated in this work are briefly described below.

During stock preparation, and particularly at the refining stage, the properties of fibers are greatly changed due to breaking of intra-fiber hydrogen bonds, fibrillation of the exposed secondary wall, creation of inter-fiber or fibril bonds, fiber shortening and production of fiber debris.

The mechanical and physical properties of paper more studied are tear resistance, burst and tensile strengths (Figure 1.2). Additionally, in this work, the properties lengthening and air permeability were analyzed in the papersheets. It is a common practice to report most paper strength properties in the form of an index, i.e. normalized with respect to the grammage, in order to eliminate the effect of grammage variations.

Bursting strength is perhaps the most commonly measured strength property of paper and it is defined as the pressure required to cause bursting of a paper. In order to define this parameter, a sheet of paper is placed in a bursting strength tester and subjected to an increasing pressure – generated by a membrane – until its rupture. The force required to burst the paper is called burst resistance and measured in kiloPascal (kPa). Fundamentally, bursting strength is actually a combination of different properties, largely tear and tensile (Zhao et al., 2005). Tensile strength is other key parameter in paper production. It is determined by mechanically placing a strip of paper into a tensile tester that strains the paper and registers the force needed to break it (measured in newton, N) (Zhao et al., 2005). Resistance to tear is another property that has long been used to assess a paper's ability to stand up to the stresses imposed upon it, particularly in the production of printing, writing and packaging papers (Biermann, 1996). Internal tear strength is a property which suffers as a result of the increase in burst and tensile achieved with refining a papermaking stock (Seth et al., 1979). Although tear resistance is improved by fibrillation resulting from the action of the refiner in the early stages, the later reduction of fiber length creates a net reduction in tear strength. Other factors, such as increased homogeneity resulting from the refining action, are also said to reduce tear but to a lesser extent. Hence, the refining energy must give sufficient tensile strength without excessive loss of tearing resistance. The physical property, air permeability, is the ability to let air pass through the sheet. Air permeability is measured as the volume of air that flows in unit time through unit area under unit pressure difference. This property characterizes the degree of the sheet openness and is determined by the condition of the fiber mat structure (Rudman and Patterson, 1998).

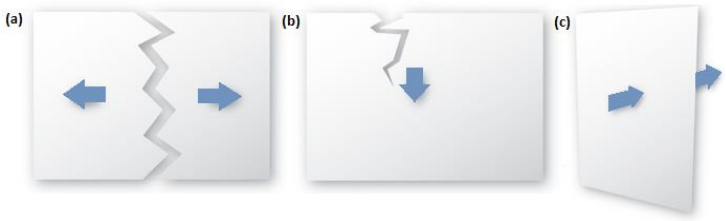

Figure 1.2 Schematic representation of the tensile strength (a), tear resistance (b) and burst strength (c).

However, it should be taken into account that these fiber properties are interdependent. For example, increasing bonding (to promote strength and stiffness) causes fibrillation and a larger surface area resulting in lower permeability. Hence, the developed characteristics in a paper are usually a compromise (Biermann, 1996).

1.2. Carbohydrate-binding modules

CBMs were previously defined as cellulose-binding domains (CBDs) based on the initial discovery of several modules that bound to cellulose (Gilkes et al., 1988; Tomme et al., 1988). The first CBD was described in 1986 by Tilbeurgh and coworkers (1986) when they treated a cellulase from *Trichoderma reesei*, with a protease. These authors obtained two peptides: the higher molecular weight peptide retained the cellulolytic activity, while the smaller one exhibited cellulose affinity, therefore being designated a cellulose-binding domain (CBD). However, other CBMs with different binding specificities were described, and that recognize chitin, β-glucans, starch, glycogen, inulin, pullulan, xylan, and many other different polysaccharides (arabinofuranose, mannan, fucose, lactose, galactose, polygalacturonic acid, β-D-galactosyl-1,4-β-D-N-acetylglucosamine, lipopolysaccharides, etc) (Guillén et al., 2010). CBMs are relatively small proteins (30-180 amino-acids), usually separated from the catalytic module by a flexible linker, which is often glycosylated to protect against proteolysis. The linker sequences are rich in hydroxyl amino-acids residues, such as serines, prolines and threonines (Shen et al. 1991; Tomme et al., 1995), and often feature metal ion coordination (Boraston et al. 2004). The most likely explanation for the role of linkers is that they provide to the catalytic module the conformational freedom it requires to work efficiently while it is anchored to its substrate by the CBM (Black et al., 1997). CBMs may be found in any domain of life in several carbohydrate-active proteins and their location within the parental protein can be either C or N terminal or,

more rarely, centrally positioned within the polypeptide chain (Wang et al., 2002; Zverlov et al., 2001).

1.2.1. Structure and classification

Three-dimensional structures of CBMs have been determined by X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy (Hashimoto, 2006). For determination of binding specificity of CBMs, several methods have been developed, and the most commons are affinity gels (Tomme et al., 2000) and macroarray assays (McCartney et al., 2004). For the detailed analysis of the kinetics of binding, ITC (isothermal titration calorimetry), fluorescence analysis, UV spectroscopy analysis and quantitative binding assays are commonly used (Kallas, 2006). The increasing number of three-dimensional structures of different CBMs in complex with their ligands provides an invaluable tool for the understanding of the binding specificities (Pires et al., 2004). The topography of the binding site determines the binding specificity of CBMs to substrates. Currently, almost 30 000 CBMs are described in the CAZy database, and based on evolutionary relationships, polypeptide folds and substrate preferences, grouped in 71 families, but the number of CBM families is still growing (CAZY server, http://www.cazy.org/Carbohydrate-Binding-Modules.html). Because the fold of proteins is better conserved than CBM sequences, some of the CBM families can be grouped into superfamilies or clans. However, this classification includes CBMs that display great variety in binding specificity. Thus, CBM families were classified into seven structural family folds (β-sandwich (1), β-trefoil (2), Cystein knot (3), Unique (4), OB fold (5), Hevein fold (6) and Hevein-like fold (7) (Boraston et al., 2004). The most common fold among CBMs is the β-sandwich fold, which can be divided into two fold sub-families: β-jelly roll and immunoglobulin folds (Hashimoto, 2006).

A classification based on substrate binding has also been proposed, where CBMs may also be classified into three types, which reflect the macromolecular structure of the target ligand (Boraston et al., 2004; Boraston et al., 2007). Generally, these three types are known as ‘surface-binding’ CBMs (type A), ‘glycan chain-binding’ CBMs (type B) and ‘small sugar-binding’ CBMs (type C) (Hashimoto, 2006). Type A CBMs, with affinity for insoluble and crystalline polysaccharides such as cellulose or chitin, have a flat or platform-like hydrophobic surface

composed of aromatic residues. This planar architecture of the binding site interacts with the glucosyl-pyranose ring of the substrate (Bolam et al., 1998; Boraston et al., 2004; Boraston et al., 2007; Guillén et al., 2010; Shoseyov et al., 2006). Nagy and coworkers (1998) concluded that Type A CBMs show little or no affinity for soluble carbohydrate. On the other hand, Type B CBMs bind to soluble and less-ordered plant structural polysaccharide chains, such as amorphous cellulose, mannan, or xylan (Boraston et al., 2007). The Type C or lectin-like CBMs that only bind to small sugars, for instance mono-, di-, or tri-saccharides due to steric restriction in the binding site, and are usually found in xylanases (Boraston et al., 2004; Guillén et al., 2010; Shoseyov et al., 2006).

1.2.2. Functions

The main function of CBMs is to potentiate the rate of catalysis by bringing their cognate enzymes into intimate contact with the target substrate, reducing the accessibility problem (Boraston et al., 2003; Hall et al., 1995; Montanier et al., 2009; Tomme et al., 1988; Tunnicliffe et al., 2005;). Additionally, they are thought to have four primary functions: proximity and avidity effects, substrate targeting and microcrystallite disruption.

The proximity effect is responsible for increasing the effective concentration of enzyme on the surface of the substrate, thereby improving the catalytic efficiency (Figure 1.3). This effect has been shown by genetically removing the CBM domain from the catalytic module of the wild-type enzyme and is observed mostly in enzymes that act on insoluble substrates and in cellulosomes (Bolam et al., 1998; Boraston et al., 2004; Moreira and Gama, 2009).

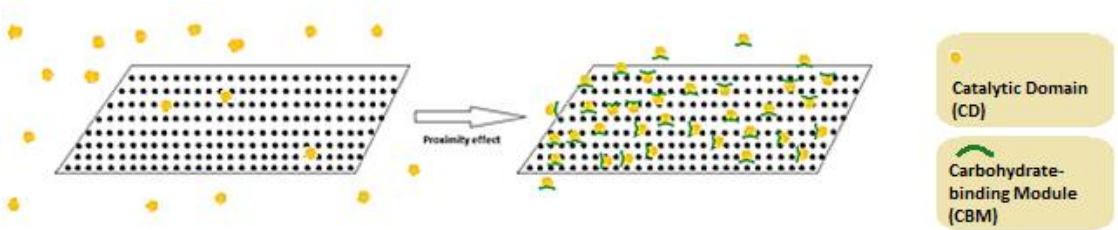

Figure 1.3 Schematic representation of the CBM mediated proximity effect.

Perhaps most interesting from the perspective of potential technical applications is the targeting function, directing the hydrolytic enzyme to its specific substrate, or specific regions of the polysaccharide (Figure 1.4). This function has been shown in several studies, serving as tool for the elucidation of protein-carbohydrate interaction mechanisms and as molecular probes for polysaccharide localization in situ to identify different polysaccharides in plant cell-walls (McCartney et al., 2004). This feature was related with the selective substrate affinity, distinguishing different crystalline, amorphous, soluble and non-soluble polysaccharides (Boraston et al., 2001; Carrard et al., 2000; McCartney et al., 2004; Notenboom et al., 2001; Tomme et al., 1995; Wang et al., 2002).

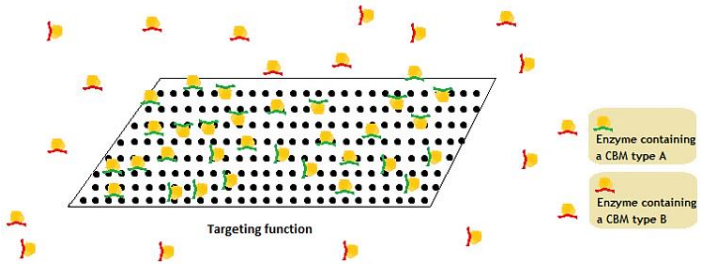

Figure 1.4 Schematic representation of the targeting effect of CBMs.

Some CBMs also display a disruptive function, making the substrate more susceptible to enzymatic hydrolysis (Figure 1.5). This function was demonstrated for the first time in 1991, by Din and coworkers (1991), when they isolated a non-catalytic cellulose-binding domain, from endoglucanase A (*Cellulomonas fimi*), which was able to disrupt the cellulose fibers. Binding of CBMs to a crystalline substrate leads to polysaccharide chains disorganization and enhancement of substrate availability (Din et al., 1991; Guillén et al., 2010; Moreira and Gama, 2009).

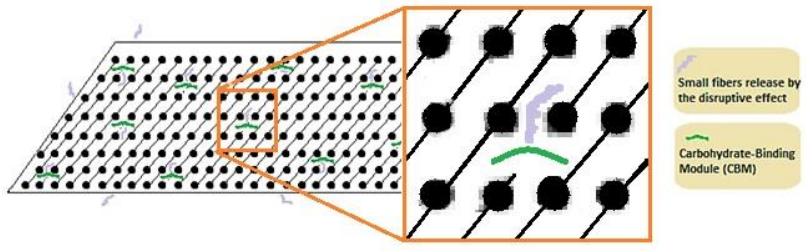

Figure 1.5 Schematic representation of the disruptive effect of the CBMs on polysaccharide fibers.

The concept of avidity effect was related to the presence of CBMs in multiples copies within the enzymes architecture (Figure 1.6). The multiple modules act cooperatively in the binding process and this multimodularity increases the avidity of the CAZyme for the substrate. Additionally, it has

been speculated that the multimodularity increases the avidity of the CAZyme for the substrate and heterogeneous multimodularity allows the enzyme to bind heterogeneous substrates simultaneously. This potential feature has been exploited for the creation of high affinity and multivalent CBMs (Abbott et al., 2008; Gregg et al., 2008).

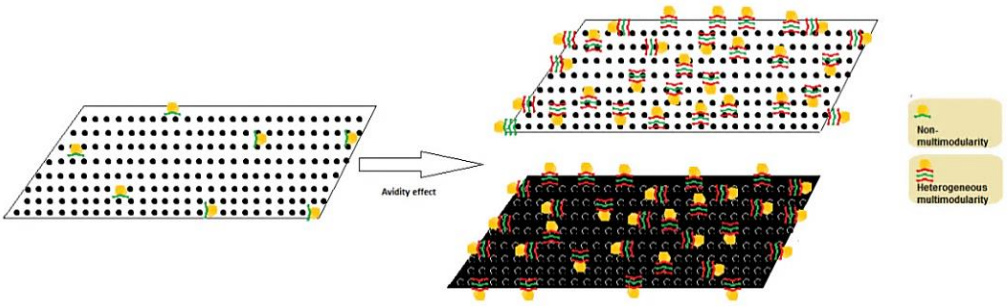

Figure 1.6 Schematic representation of the avidity effect of the enzyme containing multiple copies of CBMs. The multimodularity increases the avidity of the enzyme for the substrate, and heterogeneous multimodularity allows the enzyme to bind heterogeneous substrates.

1.2.3. Production and general applications of CBMs

CBMs were initially obtained from enzymes through proteolysis (Lemos et al., 2000; Pinto, 2006; Tilbeurgh et al., 1986), but are now increasingly produced by recombinant DNA technology (Moreira and Gama, 2009), which allows increased purity levels and the possibility of fusion to other bioactive molecules. However, the strategies for production of recombinant CBMs are not yet optimized. The main expression systems that have been used for their production are *Escherichia coli* and *Pichia pastoris*. Nevertheless, the choice of the expression system depends on the process goals and on the properties of the protein to be produced. The least expensive, easiest and quickest expression of proteins can be carried out in *E. coli*, but for expression of proteins that require post-translational modifications, *E. coli* is not the system of choice (Jahic, 2003). On the other hand, recombinant proteins produced in the methylotrophic yeast *P. pastoris* have several advantages over other eukaryotic and prokaryotic expression systems (section 1.3).

CBMs provide a wide range of tools since they present three basic properties: (i) CBMs are usually independently folding units and, therefore, can function autonomously in chimeric proteins; (ii) the attachment matrices are abundant and inexpensive and also have excellent chemical and physical properties; and (iii) the binding specificities can be controlled, and

therefore the right solution can be adapted to an existing problem (Shoseyov et al. 2006). These features have been extensively studied and have a great potential application in different fields of biotechnology (Figure 1.7).

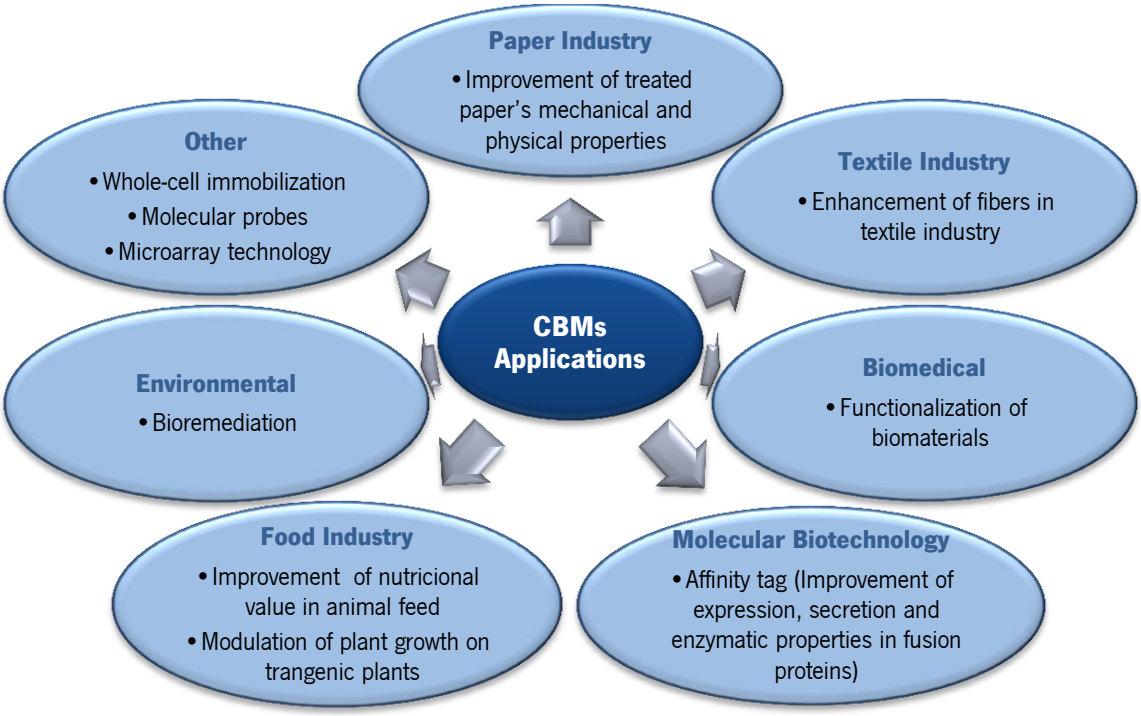

Figure 1.7 CBM applications and related areas.

CBMs have been successfully used as fusion tags for protein purification (Boraston et al., 2001; Guerreiro et al., 2008; Kavoosi et al., 2004; Ramos et al., 2010; Ramos et al., 2013; Rodriguez et al., 2004; Sugimoto et al., 2012; Wan et al., 2011), enzyme or cell immobilization (Hwang et al., 2004; Kauffmann et al., 2000; Nam et al., 2002) and for the functionalization of polysaccharide-based materials (Andrade et al., 2009; Carvalho et al., 2008; Moreira et al., 2008; Pértile et al., 2012). Another example is their application for targeting of hydrolytic enzymes to the desired substrate that has been exploited in the textile industry, resulting in a decrease in the quantity of enzyme necessary and consequently reduced costs (Cavaco-Paulo, 1998). Due to their substrate specificity, CBMs are valuable as analytical tools for the detection of specific polysaccharides in plant tissues as molecular probes (Ding et al., 2006; Hildén et al.,2003; McCartney et al., 2004; McCartney et al., 2006; von Schantz et al., 2009), and as part of carbohydrate microarrays (Moller et al. 2007). Finally, CBMs can be used in the paper industry (section 1.2.3.1).

1.2.3.1. The potential application of CBMs in the paper industry

Due to their independent fold and function, CBMs have many biotechnological applications, namely in the paper industry. It has been demonstrated that CBMs are capable of improving both pulp and paper properties. The improvement of pulp properties was related to the reduction of water and energy consumption, important variables to take into account in the final cost of the process. It has been shown that CBMs obtained by proteolysis, improve the mechanical properties of papersheets, enhance the substrate-binding capacity and cellulolytic activity of the native enzyme and reduce the acid character of the fibers (Levy et al., 2002; Pinto et al., 2004; Suurnakki et al., 1999; Thongekkaew et al., 2013). Additionally, family 1 CBM from *T. reesei* cellulases improved the air permeability and tensile strength of papersheets (Pala et al., 2001; Yokota et al., 2009). On the other hand, the CBMs may also improve the pulp drainability (Pala et al., 2001). Pulp drainability is the capacity of the pulp for water drainage and is measured by the Schopper-Riegler degree (°SR). The increase in drainage rate could lead to energy savings, and improve the runnability of recycled papermaking (Pinto, 2006).

Later, the production of CBMs by proteolysis, for example with papain, was replaced by recombinant DNA technology. This last method allows overcoming the purity limitations of the proteolysis approach, which can originate invalid results, due to enzymatic contamination. Levy and colleagues (2002) produced in *E. coli* a recombinant bifunctional cellulose-binding protein with two CBDs from *Clostridium cellulovorans* fused to form a cellulose crosslinking protein (CCP), able to mimic the chemistry of cellulose cross-linking. The purified recombinant bifunctional cellulose-binding protein was applied to Whatman filter paper and found to enhance its mechanical properties, such as tensile strength, brittleness, Young's modulus and energy to break. Additionally, CCP treatment could transform the paper into a water-repellent material. Moreover, the same authors verified a synergistic effect between CCP and cationic starch that resulted in higher mechanical performance of paper (Levy et al., 2003). Afterwards, Levy and coworkers (2004) constructed a bifunctional cross-linking molecule composed of starch and cellulose-binding modules CSCP (starch-cellulose cross-linking protein). This CSCP was able to bind soluble and insoluble starch to cellulose and to improve the mechanical properties of paper composed of cellulose fibers and starch. More recently, Shi and colleagues (2014) produced in *E. coli* four engineered double CBMs (family 1 or/and 3) and studied their performance on paper

properties improvement. The authors concluded that the constructs improved the folding endurance and tensile strength and significantly reduced the wetability of treated papers. On the other side, single CBMs have also been applied for fiber modification. Thereby, Cadena and colleagues (2010) concluded that a single CBM3b from the modular enzyme endoglucanase Cel9B of *Paenibacillus barcinonensis* affects fiber properties but is less active on pulp refining. Additionally, it was suggested that the glycosylation of fungal CBMs may be relevant for the modification of the fibers surface properties (Machado et al., 2009). The authors conjugated the CBM3 from *C. thermocellum*, recombinantly produced in *E. coli*, with activated polyethilenoglycol (PEG), to mimetize the glycosidic fraction present in fungal CBMs, and verified that the adsorption of this CBM to the fibers is reversible while it improved the drainability of *Eucalyptus globulus* and *Pinus sylvestris* pulps without affecting negatively the paper physical properties. It was stated that the conjugation CBM-PEG improves the hydration and stabilisation of the fibers, thereby reducing the inter-fiber interactions, suggesting that glycosylation is essential for this event.

All of these previous results suggest that CBM-based technology may have valuable applications in papermaking, namely in the improvement of mechanical properties of paper and in energy saving.

1.2.4. The CBM1 from *T. reesei* Cel7A (*Tr*CBM1$_{Cel7A}$)

The Family 7 cellobiohydrolase (Cel7A) is the most secreted protein under cellulase-induced conditions from *T. reesei* (Nogawa et al., 2001). This multidomain enzyme (Figure 1.8) consists of a carbohydrate-binding module (CBM) and a catalytic domain (CD) with N-glycosylation connected by a linker with substantial O-glycosylation (Divne et al., 1994; Divne et al., 1998; Kraulis et al., 1989).

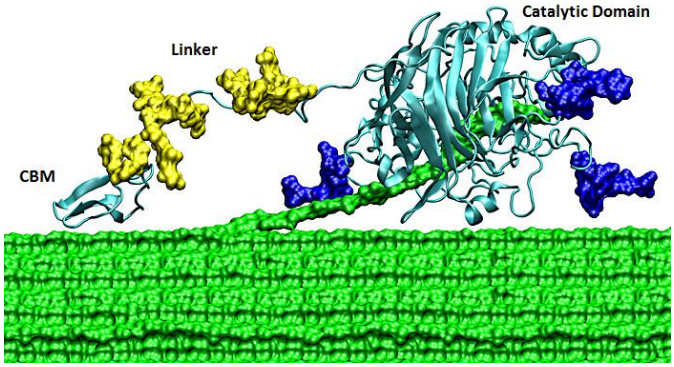

Figure 1.8 Cel7A from *T. reesei* on cellulose. The enzyme is shown in light blue, the O-glycosylation on the linker in yellow, the N-glycan on the CD in dark blue and cellulose in green. The pictures were prepared with visual molecular dynamics (VMD) (Humphrey et al., 1996).

In the process of cellulose hydrolysis by multimodular cellulases, the initial binding event is likely mediated by the CBM (Beckham et al., 2010). The *Tr*CBM1$_{Cel7A}$ from the Cel7A share with other members of this family the common feature of small molecular weight (approximately 3 kDa, less than 40 amino-acid residues), with two disulfide bonds. Moreover, it contains three hydrophobic amino-acids, such as tyrosine or tryptophan, which form a flat hydrophobic surface that interacts with the hydrophobic surface of crystalline cellulose (Dagel et al., 2011; Ding et al., 2006; Lethio et al., 2003; Nimlos et al., 2012; Sugimoto et al., 2012). The reversibility of binding of fungal CBMs to cellulose has been controversial, with some authors indicating that the binding is reversible (Carrard et al., 1999; Linder et al., 1996; Lymar et al., 1995; Otter et al., 1989; Tomme et al., 1995), while others defend its irreversibility (Beldman et al., 1987; Lethio et al., 2001; Linder et al., 1998; Reinikainen et al., 1997). *Tr*CBM1$_{Cel7A}$ was found to bind reversibly to cellulose (Shi et al., 2014).

The Cel7A linker, however, has received less attention than the CBM and CD, and its role in catalysis is not completely understood. Nevertheless, cellulase linkers have been hypothesized to serve several functions, such as a torsional leash (Srisodsuk et al., 1993), as a hinge or spring between the CBM and the CD (Srisodsuk et al., 1993; von Ossowski et al., 2005) and as a driver of the relative orientations of the CBM and CD. Additionally, glycosylation has been hypothesized to prevent proteolysis (Langsford et al., 1987). The Cel7A linker region contains O-glycosylation, as shown in Figure 1.8 and Figure 1.9. The glycosylation pattern of the linker sequence (Figure 1.9) was suggested by Harrison et al. (1998), in a work where they quantified the extent and heterogeneity of mannose residues (between 1 and 3 on each S and T residues).

Figure 1.9 Cel7A linker domain sequence, with the O-glycan shown in orange. Adapted from Harrison et al. (1998).

1.2.5. The CBM3 from *C. thermocellum* CipA (*Ct*CBM3$_{CipA}$)

The strictly anaerobic, thermophilic bacterium *C. thermocellum* produces one of the most efficient enzymatic cellulose degradation systems (Lynd et al., 2002). This feature is at least in part due to the formation of a huge enzyme complex, a cellulosome. In *C. thermocellum*, cellulosomes were shown to mediate cell binding to cellulose through the CBM3 borne by the cellulosomal scaffolding protein, CipA (Bayer et al., 1983; Bayer et al., 1998; Lamed et al., 1987). The scaffoldin protein CipA contains nine cohesin modules to which enzymes and other protein components specifically dock by virtue of dockerin modules (Figure 1.10).

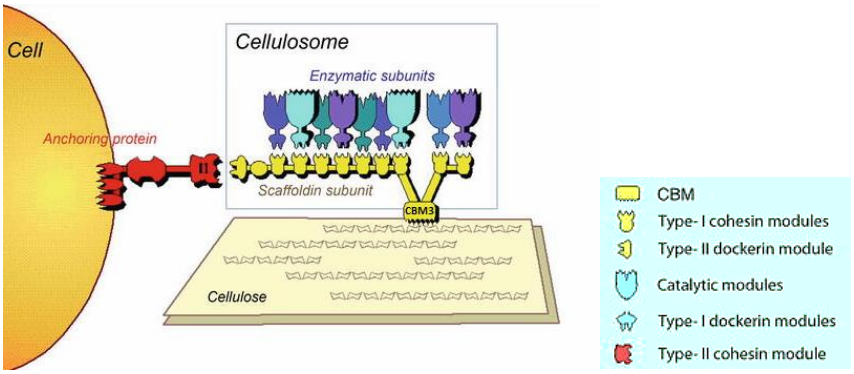

Figure 1.10 The cellulosome of *C. thermocellum*, showing the location of the CBM3, used in this thesis. Adapted from Bayer et al., 2013.

The function of the *Ct*CBM3$_{CipA}$ is well characterized and studied (Tormo et al., 1996; Tomme et al., 1998) and its three-dimensional structure was elucidated (Tormo et al., 1996). Adsorption of the bacterial *Ct*CBM3$_{CipA}$ is similar to the *Tr*CBM1$_{Cel7A}$ binding; both have in common a hydrophobic flat surface that interact with the hydrophobic surface of cellulose (Lethio et al., 2003; Ding et al., 2006; Dagel et al., 2011). Tormo and coworkers (1996) proposed that a flat conserved surface on the bottom of *Ct*CBM3$_{CipA}$, interact with crystalline cellulose, including the amino-acid residues which form the planar strip (W118, R112, D56, H57, Y67). The *Ct*CBM3$_{CipA}$ has a slightly tendency of to bind close to the chain ends (Sugimoto et al., 2012). Shoseyov and Warren (1997) suggested that the adsorption of CBM3 is quite stable, however, it was demonstrated that the adsorption may not be considered irreversible. More recently, Machado

and coworkers (2009) showed that CBM3 exhibited inter-fiber mobility, suggesting that the adsorption of CBM3 to the fibers is reversible.

1.3. The *P. pastoris* expression system

The introduction of the DNA recombinant technology in the seventies, allowed the production of proteins in several host organisms resulting in a faster and easier process compared to their natural sources (Demain and Vaishnav, 2009).

The methylotrophic yeast *P. pastori*s is a robust microorganism for the industrial production of a wide variety of recombinant proteins, including CBMs (Table 1.1). *P. pastoris* is a single-cell microorganism that is easy to manipulate and culture. Therefore, the powerful genetic techniques available for this organism make *P. pastoris* a system of choice for heterologous protein production. Nowadays, *P. pastoris* is the most frequently used yeast system for heterologous protein production (Gasser et al., 2013).

The development of strategies for cloning and production of heterologous proteins in *P. pastoris* should take into account some aspects, such as, the choice of the proper expression vector for intracellular or secreted production, selection of secretion signals, promoter–terminator combinations and suitable selection markers (Figure 1.11). Ahmad and coworkers (2014) affirmed that the choice of the proper expression vector and complementary host strain are the most important prerequisite for successful recombinant protein production. A variety of *P. pastoris* expression vectors and host strains with a wide range of genotypes are available. The choice of a specific strain is mainly determined by the required application (Daly and Hearn, 2005). The standard vector systems for intracellular and secretory expression include constitutive (P_{GAP}) and inducible promoters triggered by methanol or methylamine (P_{AOX1}, $P_{AOX2,}$ P_{FLD}) (Ahmad et al., 2014).

Table 1.1 Reported applications for recombinant CBMs produced in *P. pastoris*.

CBM/Origin	Application	Reference
CBM2a *Cellulomonas fimi*	**Functional and mutational analysis**	Boraston et al., 2001 (b)
CBM2a *Cellulomonas fimi*		Boraston et al., 2003
CBM21 *Rhizopus oryzae*	**Recombinant protein engineering and purification**	Lin et al., 2009
CBM1 *Trichoderma reesei* and *Phanerochaete chrysosporium*		Sugimoto et al., 2012
CBM3 *Clostridium thermocellum*		Wan et al., 2011
CBM2a *Cellulomonas fimi*		Boraston et al., 2001 (a)
CBD *Neocallimastix patriciarum* and *Candida antarctica*		Gustavsson et al., 2001
CBD *Neocallimastix patriciarum*		Rotticci-Mulder et al., 2001
CBD *Neocallimastix patriciarum* and *Candida antarctica*		Jahic et al., 2003
CBM1 *Trichoderma reesei*		Tang et al., 2013
CBM22-2 *Clostridium thermocellum*		Vuong and Master (2014)

The expression vectors are integrated into the *P. pastoris* genome to maximize the stability of expression strains (Cereghino and Cregg, 2000). Gene copy number has been identified as a 'rate-limiting' step in the production of recombinant proteins from *P. pastoris* (Clare et al., 1991). Increasing the number of copies of the expression cassette generally has the effect of increasing the amount of protein produced (Clare et al., 1991; Romanos, 1995; Vassileva et al., 2001).

P. pastoris has several advantages over other eukaryotic and prokaryotic expression systems: (1) rapid growth rate, coupled with ease of high cell-density fermentation; (2) high levels of productivity in an almost protein-free medium; (3) elimination of endotoxin and bacteriophage contamination; (4) ease of genetic manipulation of well-characterized yeast expression vectors; (5) the ability to engineer secreted proteins that can be purified from growth medium without harvesting the yeast cells themselves and (6) diverse posttranslational modifications that include polypeptide folding, glycosylation, methylation, acylation, proteolytic adjustment, and targeting to subcellular compartments (Li et al., 2007). Moreover, *P. pastoris* can grow within a wide pH range (pH 3-7) without any major changes in specific growth (Cos et al., 2006).

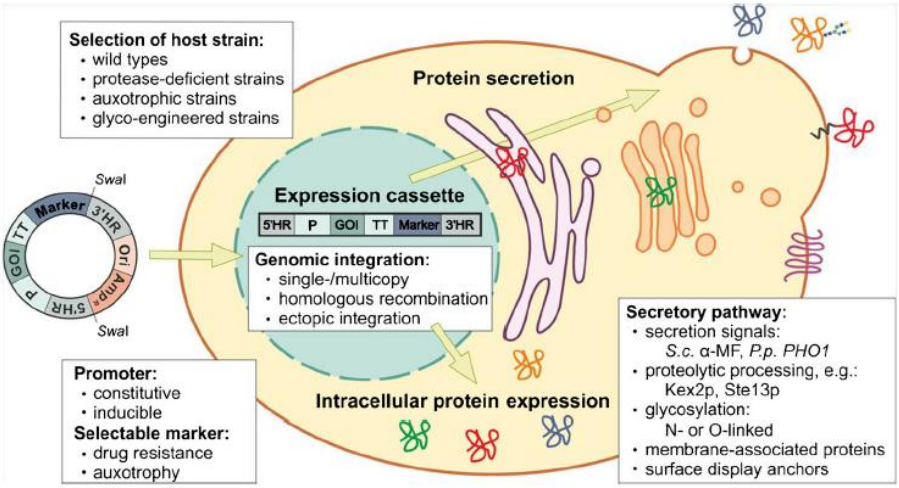

Figure 1.11 Some points that should be considered for the heterologous gene expression in *P. pastoris*. Reproduced from Ahmad et al., 2014.

Although *P. pastoris* expression systems are more expensive and time-consuming to develop than their *E. coli* counterparts, once they are established the cost of recombinant protein production is comparable (Potvin et al., 2012). The specific productivity of *P. pastoris* is generally low, but is compensated by the high cell densities achieved (Potvin et al., 2012).

1.3.1. Regulation of the methylotrophic metabolism

P. pastoris is a methylotrophic yeast, capable of metabolizing methanol as its sole carbon and energy source. Most of the promoters isolated for controlling recombinant protein production with *P. pastoris* are associated with methanol metabolism (Figure 1.12). The first step in the metabolism of methanol is the oxidation of methanol to formaldehyde and hydrogen peroxide using molecular oxygen by the enzyme alcohol oxidase. To avoid hydrogen peroxide toxicity, methanol metabolism takes place within a specialized cell organelle, the peroxisome, which sequesters toxic byproducts away from the rest of the cell. The enzyme alcohol oxidase has a poor affinity for O_2, and *P. pastoris* compensates by generating large amounts of the enzyme.

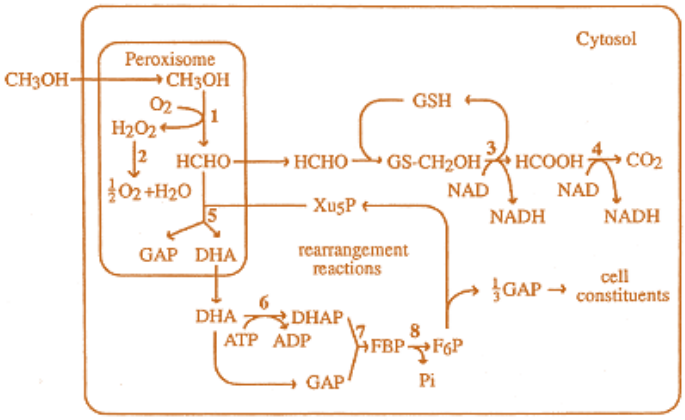

Figure 1.12 The methanol pathway in the methylotrophic yeast *P. pastoris*. 1, alcohol oxidase; 2, catalase ; 3, formaldehyde dehydrogenase; 4, formate dehydrogenase, 5, dihydroxyacetone synthase; 6, dihydroxyacetone kinase; 7, fructose 1,6-biphosphate aldolase; 8, fructose 1,6-bisphosphatase.

The promoter regulating the production of alcohol oxidase, *AOX*1, is one of the most used to drive heterologous protein expression in *Pichia*. This promoter is highly repressed in cells grown on glucose or glycerol but is strongly induced by methanol (Cereghino et al., 2002). There are three phenotypes of *P. pastoris* with regard to methanol utilization (Macauley-Patrick et al., 2005; Potvin et al., 2010) (Table 1.2). Currently, the majority of researchers use the Mut$^+$ phenotype (Macauley-Patrick et al., 2005).

Table 1.2 Phenotypes of *P. pastoris* according to the methanol utilization.

Phenotype	Genes	Description	Strains
Mut$^+$ "Methanol utilization plus"	✓ *AOX1* ✓ *AOX2*	Grows on methanol at wild-type rate and therefore requires a high dose of methanol in large-scale cultivations (Cereghino and Cregg 2000).	X-33 GS115
Mut^S "Methanol utilization slow"	**X** *AOX1* ✓ *AOX2*	*AOX*1 gene is deleted and therefore the methanol-uptake rate is slowed down due to weaker *AOX*2. In some cases, Mut^s strains have high productivities compared to the wild-type strains (Pla et al., 2006).	KM71 KM71H
Mut$^-$ "Methanol-utilizing minus"	**X** *AOX1* **X** *AOX2*	Strains with both *AOX* genes deleted are unable to grow on methanol and thus with low growth rates (Macauley-Patrick et al., 2005).	MC100-3

1.3.2. Protein glycosylation

During secretion, proteins are translocated into the Endoplasmic Reticulum (ER) lumen, where folding, formation of disulfide bonds and post-translational modifications such as glycosylation take place (Daly and Hearn, 2005; Gasser et al., 2013). Glycosylation is the covalent addition of polysaccharides to protein side chains, and is one of the most common and critical post-translational modification of proteins secreted by eukaryotic cells. This is classified accordingly to the ligation site of the carbohydrate chain to the protein. In Asn-Xxx-Ser/Thr sequence motifs, the glycosylation may occur at the asparagine residue (N-linked) or at serine and threonine residues (O-linked). *P. pastoris* is capable of adding both O- and N-linked carbohydrate moieties to secreted proteins (Cregg et al., 2000). Glycosylation of proteins in *P. pastoris* could modify the functions and characteristics of the recombinant proteins produced, that could become an issue for some applications (Eckart and Bussineau 1996). The yeast *P. pastoris* has been shown to N-glycosylate in a similar manner to *Saccharomyces cerevisiae* with a decreased tendency to hyperglycosylate (Grinna et al., 1989; Miele et al., 1997). The average chain length of glycoproteins expressed by *P. pastoris* is only 8–14 mannose residues, whereas that by *S. cerevisiae* is 40~150 residues (Li et al., 2007).

1.3.3. Secretion

Secretion of the recombinant protein is highly advantageous from a biotechnology point of view since it simplifies purification of the desired protein from other cellular matter. *P. pastoris* can produce heterologous proteins either intracellularly or extracellularly. Nevertheless, in order to avoid the usual first steps of purification, such as cell lysis, extracellular production of foreign proteins is more desirable. Moreover, *P. pastoris* cells secrete low amounts of endogenous proteins which, in combination with culture in simple mineral salts media, may result in a culture supernatant where the secreted heterologous protein comprises the vast majority of the total protein (Macauley-Patrick et al., 2005, Jahic et al., 2007). Secretion requires the presence of a signal sequence on the foreign protein to target it to the secretory pathway. Secretion signals can be attached to the protein of interest and the most commonly utilized are those of the heterologous *S. cerevisiae* alfa-mating factor (α-MF) and of the endogenous *P. pastoris* acid

phosphatase (PHO1) signal (Cereghino et al., 2000). The most successful secretion signal used with the *P. pastoris* expression system is the α-MF, however, variability in the number of N-terminal amino-acids is commonly reported with this secretion, reflecting incomplete signal sequence processing (Macauley-Patrick et al., 2005).

As the recombinant protein progresses through the secretory pathway, the α-MF signal is sequentially removed (Ahmad et al., 2014). Firstly, a pre-sequence with 19 amino-acid residues directs the protein to the endoplasmic reticle (ER) where it is subsequently removed by signal peptidase. Then, a pro-sequence with 66 amino-acid residues is removed in the late Golgi by the Kex2 endopeptidase. Finally there is a Glu-Ala repeat which is removed by the Ste13 exopeptidase exposing the N-terminal of the recombinant protein (Ahmad et al., 2014). One of the common problems encountered while using the α-MF secretion signal is non-homogeneity of the N-termini of the recombinant proteins due to incomplete STE13 processing. The efficiency of this process can be affected by the surrounding amino-acid sequence. For instance, the cleavage efficiencies of both Kex2 and Ste13 proteins can be influenced by the close proximity of proline residues and the tertiary structure formed by a foreign protein may protect cleavage sites from their respective proteases (Cereghino and Cregg, 2000). Recently, Yang and coworkers (2013) reported enhanced secretory protein production by optimizing the amino-acid residues at the Kex2 P1' site.

CHAPTER 2

Materials and methods

2.1. Sterilization procedure

All materials and culture media used in this work were sterilized by autoclaving at 121 °C during 20 min at a pressure of 1 bar. Thermolabile solutions were sterilized by filtration with 0.20 µm sterile Polyethersulfone (PES) filter units and thermolabile materials (such as the electroporation cuvettes) were sterilized by ultraviolet radiation exposing at a wave-length of 254 nm during 20 min.

2.2. Strains and plasmids

NZY5α competent cells of NZYTech (fhuA2Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17) was used as the bacterial cloning host for DNA manipulations and *P. pastoris* KM71H (arg4 aox1::ARG4) was used for recombinant protein production. Two plasmids, pPICZαA+eGFP-*Tr*CBM1$_{Cel7A}$ and pPICZαA+eGFP-NL-*Tr*CBM1$_{Cel7A}$ were constructed in this work. These plasmids contain the strong, highly-inducible P_{AOX1} promoter, the *S. cerevisiae* α-mating factor (α-MF) signal sequence and the *Sh ble* gene (*Streptoalloteichus hindustanus ble gene*) that confers resistance to Zeocin™. The strains used in this work are listed in Table 2.1.

Table 2.1 – *P. patoris* strains used in this work.

Strain/Plasmid	Description	Source
KM71H_eGFP-NL-*Tr*CBM1$_{Cel7A}$	Strain with Mut^s phenotype containing eGFP-NL-*Tr*CBM1$_{Cel7A}$ with native linker (NL)	This work
KM71H_eGFP-*Tr*CBM1$_{Cel7A}$	Strain with Mut^s phenotype containing eGFP-*Tr*CBM1$_{Cel7A}$ without NL	This work
KM71H_*Ct*CBM3$_{CipA}$-wt	Strain with Mut^s phenotype containing CBM3 wild type from *C. thermocellum*	Previously constructed by our research group
KM71H_*Ct*CBM3$_{CipA}$-mut	Strain with Mut^s phenotype containing CBM3 lacking the three potential N-glycosylation sites from *C. thermocellum*	Previously constructed by our research group

2.3. Bacteria and yeast storage

For bacteria and yeast cultures storage, the microorganisms were regularly spread in the appropriate selective medium and inverted agar plates sealed with parafilm were maintained at 4 °C. For permanent storage, stocks of each selected culture were prepared. Each strain was grown overnight in appropriate selective liquid medium in favorable conditions. Then, 1 mL of this culture was introduced in 9 mL of fresh medium and grown for more 5-6 h. Thereafter, 0.3 mL of sterile glycerol were added to 1 mL of the culture, mixed by vortexing and incubated on ice for 10 min. The tubes were stored at -80 °C.

2.4. Culture media

E. coli cells were cultured in Luria-Bertani (LB) medium (1% (w/v) tryptone, 0.5% (w/v) yeast extract and 1% (w/v) sodium chloride (NaCl), at pH 7.5), or low-salt LB medium (same composition as LB medium but with half of NaCl concentration) when using Zeocin™ (25 µg/mL) as the selection antibiotic. All strains were also grown in the corresponding solid media, obtained by the addition of 2% (w/v) agar. *P. pastoris* KM71H were grown in Yeast Extract Peptone Dextrose medium (YPD). Recombinant *P. pastoris* KM71H strains were grown in YPD supplemented with Zeocin™ (100 µg/mL) or Buffered Minimal medium containing Glycerol (BMG). Buffered Minimal medium containing Methanol (BMM) was used for recombinant protein production. The different media used for *P. pastoris* cultivation are listed in Table 2.2.

Table 2.2 Media for *P. pastoris* cultivation

Media	Composition
BMG	100 mM potassium phosphate pH 6.0, 1.34% yeast nitrogen base (YNB) with ammonium sulfate without amino-acids, 4 x 10^{-5}% biotin and 1% glycerol
BMM	100 mM potassium phosphate pH 6.0, 1.34% yeast nitrogen base (YNB) with ammonium sulfate without amino-acids, 4 x 10^{-5}% biotin and 0.5% methanol
YPD	2% (w/v) glucose, 2% (w/v) peptone, 1 % (w/v) yeast extract and 2% (w/v) agar

2.5. Construction of the expression plasmids pPICZαA+eGFP-*Tr*CBM1$_{Cel7A}$ and pPICZαA+eGFP-NL-*Tr*CBM1$_{Cel7A}$

2.5.1. Cloning strategy

The gene sequence of CBM1 from the *T. reesei* Cel7A (Cellobiohydrolase I) with (E1) and without (E2) its native linker (NL) was obtained by two PCR (polymerase chain reaction) strategies (Figure 2.1). The CBM1 coding sequence was fused in frame to eGFP ("enhanced green fluorescent protein") from pCG (Wan et al., 2011), together with a Tobacco Etch Virus (TEV) protease encoding sequence (to obtain free-eGFP CBMs when necessary). The eGFP-*Tr*CBM1$_{Cel7A}$ (E1) and eGFP-NL-*Tr*CBM1$_{Cel7A}$ (E2) were amplified by multiple-step primer extension PCR using codon optimized primers (Table 2.3), based on the codon usage of *P. pastoris*. The two constructs were inserted into the *EcoR*I and *Kpn*I restriction sites of pPICZαA and fused with the *S. cerevisiae* α-mating factor (α-MF) at its C terminus in the resulting plasmid. CBMs sequences were also His-tagged at the C-terminal for IMAC purification.

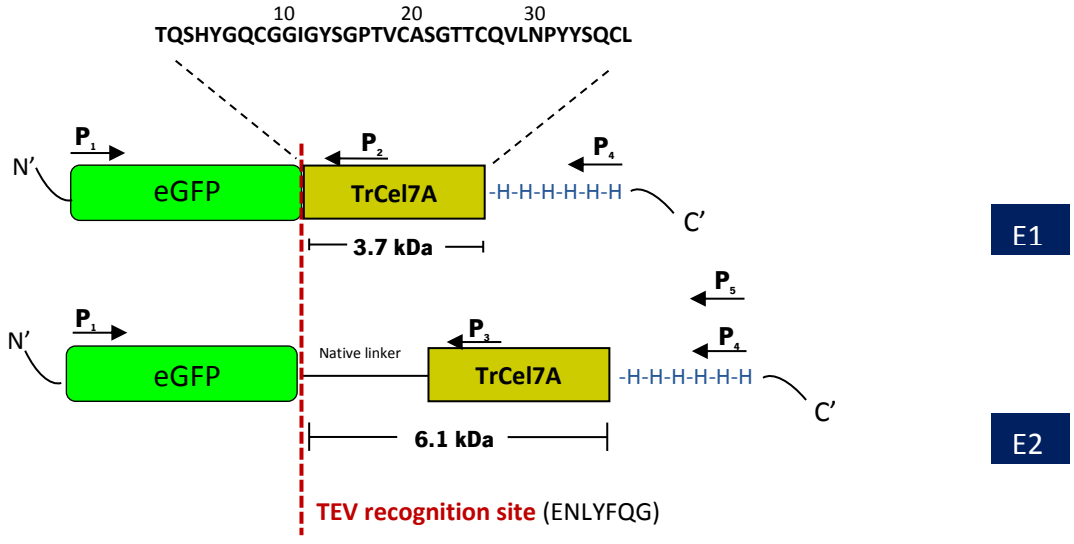

Figure 2.1 Strategies for cloning *Tr*CBM1$_{Cel7A}$ from *T. reesei* in *P. pastoris*

2.5.2. Primers

The primers used in this work to amplify the *Tr*CBM1$_{Cel7A}$ constructs are listed in Table 2.3

Table 2.3 Primers used in this work. The recognition sites of the restriction enzymes used in the cloning procedures are underlined (*EcoR*I: gaattc and *Kpn*I: ggtacc). The melting temperature of each primer is indicated (Tm). Lower case and upper case refer to the annealing sequence of primer with eGFP and CBM coding region sequence, respectively. In bold is represented the native linker coding region sequence.

	Primer	Sequence (5` →3`)	Tm (°C)
P$_1$	**TrCel7A_FW**	cggaattcatggtgagcaagggcgag	57,9
P$_2$	**TrCel7A_RV1.1**	CCACCACATTGACCGTAATGAGATTGAGTACCTTGAAAGTACAAGTTTTCcttgta cagctcgtccatgc	56
P$_3$	**TrCel7A_RV1.2**	CCACCACATTGACCGTAATGAGATTGAGT**TGGACCTGGAGAAGAACCAGT AGTAGTAGCTGGTCTTCTAGTAGTAGTAGTACCTCTGTTACCACCT GGTGG**ACCTTGAAAGTACAAGTTTTCcttgtacagctcgtccatgc	56
P$_4$	**TrCel7A_RV2**	ggggtaccTCAATGATGATGATGATGATGCAAACATTGAGAGTAGTATGGGTTCA AAACTTGACAAGTAGTACCAGAAGCACAAACAGTTGGACCAGAGTAACCAATA ccaccacattgaccgtaatgaga	56,9
P$_5$	**TrCel7A_RV3**	ggggtaccTCAATGATGATGATGATGATGC	51,0

2.5.3. Polymerase Chain Reaction (PCR)

2.5.3.1. Amplification of eGFP-*Tr*CBM1$_{Cel7A}$ and eGFP-NL-*Tr*CBM1$_{Cel7A}$

The eGFP-*Tr*CBM1$_{Cel7A}$ and eGFP-NL-*Tr*CBM1$_{Cel7A}$ was amplified by two-step or three-step primer extension PCR, respectively (Table 2.4). All reactions were carried out with 2 min of denaturation at 95 °C, followed by 30 cycles of denaturation for 2 min at 95 °C, 45 s of annealing at appropriate primer temperature (Table 2.4) and 1 min per kb of the fragment to be amplified at 72 °C for extension. A final extension step was conducted at 72 °C for 10 min. Vent DNA polymerase (Stratagene) was used.

Table 2.4 Primers and DNA template used for the amplification of eGFP-*Tr*CBM1$_{Cel7A}$ and eGFP-NL-*Tr*CBM1$_{Cel7A}$.

		Primers	Template	T (annealing) (°C)
eGFP-*Tr*CBM1$_{Cel7A}$	PCR1	P1 P2	pCG	55
	PCR2	P1 P4	PCR1 product	53
eGFP-NL-*Tr*CBM1$_{Cel7A}$	PCR3	P1 P3	pCG	50
	PCR4	P1 P4	PCR3 product	53
	PCR5	P1 P5	PCR4 product	53

2.5.3.2. Colony PCR

Colony PCR is a convenient high-throughput method to confirm correct plasmid constructions in transformants of *E. coli* and *P. pastoris*. For that, a small amount of each colony was collected with a sterile toothpick and directly added to the bottom of a PCR tube. The cells were microwaved for 2 cycles of 45 s at 900 W and immediately placed on ice. Thus, a PCR reaction mix with all components except the template was prepared and added to each PCR tube. NZYTaq DNA polymerase (NZYTech)) was used.

2.5.4. DNA electrophoresis

2.5.4.1. Agarose gel electrophoresis

Horizontal DNA electrophoresis, in agarose gels, is the standard procedure for separating DNA by size (e.g. length in base pairs). Gels of agarose were prepared with a concentration of 1% (w/v), in 1x TAE Buffer (Table 2.5) and for nucleic acid staining 0.006% (v/v) Green safe premium (NZYTech) was incorporated. Each well of the gel was loaded with DNA sample, previously mixed with 1x loading dye (Table 2.5). The running of the gel was made with 1x TAE Buffer at 60-100 V, until the tracking dye reached 2/3 of the gel length. The gel was visualized and photographed in a Molecular Imager ChemiDoc™ XRS + Imaging System (Bio-Rad) and analyzed using the Image Lab 4.0 software.

Table 2.5 Composition of TAE buffer and Loading dye stock solutions.

50x TAE buffer	2 M Tris-base 50 mM EDTA pH 8.0
6x Loading dye	25% (w/v) glycerol 20 mM EDTA 0.25% (w/v) Bromophenol blue

2.5.4.2. DNA molecular weight marker

The DNA molecular weight marker used in agarose gels for easy quantification (for PCR purpose) and size determination was NZYDNA Ladder III (NZYTech), a pattern of 14 bands, ranging from 200 to 10000 bp (Table 2.6).

Table 2.6 Molecular sizes of the bands from NZYDNA Ladder III

Band	Size (bp)	Band (ng)
1	10000	100
2	7500	75
3	6000	60
4	5000	50
5	4000	40
6	3000	30
7	2500	25
8	2000	20
9	1400	14
10	1000	100
11	800	80
12	600	60
13	400	40
14	200	20

2.5.5. DNA purification methods

2.5.5.1. DNA purification from PCR products

PCR products were purified with the QIAquick® PCR purification Kit (QIAGEN) according to the manufacturer's protocol.

2.5.5.2. DNA purification from agarose gel

DNA was recovered from agarose gels using the QIAquick® Extraction Gel Kit (QIAGEN). The protocol was performed according to the manufacturer's instructions.

2.5.6. DNA quantification and storage

DNA concentration was determined in a NanoDrop 1000 Spectrophotometer (Thermo Scientific) by loading 2 µL of sample. The apparatus measures the absorbance at 260 nm and converts it to DNA concentration (ng/µL).

DNA samples were stored at -20 °C or 4 °C in TE (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA), EB buffer (10 mM Tris-HCl, pH 8.5) or in sterile UP H_2O.

2.5.7. Enzymatic modification of DNA

2.5.7.1. Digestion with restriction enzymes

Double digestion reactions with *EcoR*I-HF and *Kpn*I-HF (New England Biolabs) were performed overnight at 37 °C, in the CutSmart™ Buffer. The final volume of reaction (10-20 µL) was adjusted with UP H_2O.

2.5.7.2. Plasmid DNA dephosphorylation

To prevent re-circularization of digested plasmid DNA during the ligation reaction, it was dephosphorylated. Thus, Shrimp Alkaline Phosphatase (SAP) (Fermentas™) was added to the digestion mixture of the pPICZαA. Afterwards, the linearized vector was purified from agarose gel, as described above (section 2.5.5.2).

2.5.7.3. Ligation vector-insert

Ligation of digested DNA fragments to digested pPICZαA was performed overnight at 4 °C with T4 DNA Ligase (Promega). Per ligation reaction, the vector quantity used was 100 ng and the insert amount was calculated by the next formula:

$$\text{insert (ng)} = \frac{\text{vector (ng)} \times \text{size of insert (Kb)}}{\text{size of vector (Kb)}} \times \text{molar ratio of } \left(\frac{insert}{vector}\right)$$

The molar ratio of insert/vector used was 3:1. To the ligation reaction mix 1 µL of 10x concentrated Ligase Buffer (Promega) and 1 U of T4 DNA Ligase (Promega) was added. The final volume of reaction (10 µL) was adjusted with sterile UP H_2O.

2.5.8. Transformation of *E. coli* cells by heat-shock method

Competent *E. coli* cells were used as the bacterial cloning host for DNA manipulation and were transformed with the ligation reactions by the heat-shock method. The DNA from the ligation reactions was gently mixed with 100 µL of competent cells and incubated on ice for 30 min. Then, the cells were heat-shocked for 40 s in a 42 °C water bath and incubated on ice for 2 min. Thereafter, 900 µL of room temperature SOC medium (2% (w/v) tryptone, 0.5% (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgSO_4.7H_2O$, 10 mM $MgCl_2.6H_2O$ and 20 mM glucose) was added, followed by incubation at 225 rpm and 37 °C for 1 h. The cell suspension was spread on low-salt LB agar plates with 25 µg/mL Zeocin™, in appropriate dilutions, and incubated overnight at 37 °C. Then, some Zeocin™-resistant transformants were picked and spread on low-salt LB plates with 25 µg/mL Zeocin™.

2.5.9. Plasmid DNA preparation from *E. coli*

Two methods for the extraction of plasmid DNA were performed: a rapid plasmid DNA extraction and a commercial kit method.

2.5.9.1. Rapid plasmid DNA extraction

Each *E. coli* colony was spread in a new selective low-salt LB with Zeocin™ agar plate and grown overnight at 37 °C. Then, the cells were collected and resuspended in 200 µL of UP H_2O and mixed in the vortex. Cellular lysis was performed by the addition of 200 µL of Solution I (1% (w/v) SDS and 0.2 M NaOH) followed by 4 times inversion to mix. To neutralize and precipitate cell extracts and other contaminants, 200 µL of solution II (3 M potassium acetate and 11.5% (v/v) acetic acid) were added, the tube gently inverted 4 times and then incubated on ice for 5 min. This solution was centrifuged for 2 min at 13200 rpm and the supernatant transferred to a new tube. An equal volume of isopropanol was added to the tube and mixed by inverting the tube 5 times. This suspension was centrifuged for 2 min at 13200 rpm. The supernatant was carefully removed and the pellet was air dried for 10 min and resuspended in 30 µL of UP H_2O.

2.5.9.2. Commercial kit method

To obtain higher quantities and purity of plasmid DNA, the GenElute™ Plasmid Miniprep Kit (Sigma) was used, and the procedure was performed according to the manufacturer's protocol.

2.6. Transformation of *P. pastoris* by electroporation and screening of multicopy transformants

The expression plasmids were transformed into *P. pastoris* KM71H by electroporation method using the EasySelect™ *Pichia* Expression Kit (Invitrogen) according to the manufacturer's protocol.

2.6.1. DNA transforming preparation

Before the transformation, the expression plasmids, approximately 5 µg, were linearized by *Sac*I digestion overnight at 37 °C. Then, the enzyme was inactivated by heating for 20 min at 65 °C, and DNA was purified (section 2.5.5.1).

2.6.2. Cells preparation

Firstly, *P. pastoris* KM71H cells were grown overnight in 5 mL of YPD medium in a 50 mL conical tube at 30 °C and 200 rpm. Subsequently, 200 mL of fresh YPD medium in a 1-L flask was inoculated with 250 µL of the overnight culture. The culture was grown overnight again to an OD_{600} = 1.3-1.5. The cells were centrifuge at 4,000xg for 5 min at 4 °C, and the pellet was resuspended with 200 mL of ice-cold, sterile UP H_2O. The cells were once more centrifuged and the pellet was resuspended with 100 mL of ice-cold, sterile UP H_2O. Thereafter, cells was centrifuged as described above, and ressuspended in 8 mL of ice-cold 1 M sorbitol. Finally, the cells were once more centrifuged and then ressuspended in 0.4 mL of ice-cold 1 M sorbitol to a final volume of approximately 1 mL.

2.6.3. Electroporation

Transformation by electroporation was performed in a Gene Pulser Xcell™ Electroporation System (Bio-Rad), using the manufacturer's instructions for *S. cerevisiae* (a voltage of 1500 V, a capacitance of 25 µF and a resistance of 200 Ω). Initially, a volume of 80 µL of cells was transferred to an ice-cold 0.2 cm electroporation cuvette, avoiding the formation of air bubbles and ensuring that the cell suspension was deposited at the bottom of the cuvette, was gently mixed with approximately 5 µg of linearized DNA (in 10 µL sterile water) and incubated on ice for 5 min. Then, the cuvette was carefully dried and inserted into the electroporation chamber, and the electric pulse was applied. Immediately after the pulse, 1 mL of ice-cold 1 M sorbitol was added to the cuvette. The time constant, with optimal values between 4 and 5 ms, was verified. The cuvette content was transferred to a sterile 1.5-mL tube and incubated at 30 °C without shaking for 2 h. The cell suspension was subsequently spread in solid plates of YPD medium with 1M sorbitol (YPDS) containing 100 µg/mL Zeocin™, in appropriate dilutions. Finally, the plates were incubated from 3-5 days at 30 °C until colonies were formed.

2.6.4. Screening of multi-copy transformants

For high-level production of recombinant proteins, it can be convenient to select a clone with a higher gene dosage of the desired gene. A convenient method to obtain these clones is making use of antibiotic-resistance genes, on the expression plasmid. It has been shown that transformants which are able to grown on enhanced concentrations of the selective drug are likely to harbor a higher copy number of expression plasmids (Gasser et al., 2013).

For selection of putative multi-copy recombinants, transformed cells were spread on increasing concentrations of Zeocin™. Thus, resulting colonies were transferred to YPD plates containing 500 and 1000 µg/mL Zeocin™ and incubated for 24 h at 30 °C. The integration of the plasmid into the yeast genome was confirmed by colony PCR (section 2.5.3.3).

2.7. Production and purification of recombinant CBMs

Briefly, for the production of recombinant proteins, the selected colonies were cultivated (shaking at 200 rpm) in BMG medium in 1-L Erlenmeyer flasks at 30 °C for 24 h, approximately. After centrifugation at room temperature (8500 rpm, 10 min) and removal of BMG, cell pellets were resuspended in BMM medium in 500-mL Erlenmeyer flasks to induce expression. To ensure sufficient aeration during induction, baffled Erlenmeyer flasks covered by two sheets of gaze were used. The induction media were then incubated at 30 °C and 200 rpm. Methanol was added every 24 h to maintain induction. In order to enhance the production of the recombinant proteins, some parameters, such as duration of the induction phase, methanol concentration and the concentration of the producing cells in the induction medium, were optimized.

2.7.1. Concentration of recombinant proteins from culture supernatants

After production under optimized conditions, yeast cells were removed from the fermentation broth by centrifugation. Thereafter, salts were precipitated by increasing the pH with 10 N NaOH to 7.5-8.0 and removed by centrifugation (10 min, 10 000 rpm at 4 °C). Concentration of recombinant proteins from culture supernatants was performed using a Vivaspin® 20 (Sartorius Stedim Biotech) with a 10,000 MWCO PES membrane and a capacity of 20 mL. First, the

Vivaspin® was washed with filtered distilled water by centrifuging for 10 min at 4000 x g and 4 °C. After that, the culture supernatant was applied to the Vivaspin® (20 mL of each time) and centrifuged in the same conditions. Then, Phosphate Buffer Saline (PBS) (137 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na_2HPO_4 and 2 mmol/L KH_2PO_4, pH 7.4) was applied by adding approximately 45 mL to the Vivaspin® (15 mL of each time). At final a protein concentrate was obtained and was stored at -20°C until use.

2.7.2. Cellular disruption of *P. pastoris*

P. pastoris proteic extracts were obtain by mechanical cellular disruption with glass beads (Φ 0.40-0.60 mm). Lysis occurred during 10 repetitions of 1 min vortexing and 1 min chilling on ice. After 30 min of centrifugation at 4 °C and 16,000g, the supernatants containing the soluble cytosolic proteins were removed and the pellets containing the membrane-associated protein fractions were further treated with solubilization buffer (disintegration buffer with 2% SDS), and centrifuged at 2,300g and 4 °C for 5 min. The resulting supernatants contained the membrane-associated proteins. The proteic extracts were purified by IMAC.

Membrane-associated proteins were separated from cytosolic proteins according to Hohenblum et al. (2004). Briefly, the cell pellet (present in 50 mL of the production medium) was thawed on ice and washed with 5 mL of ice cold PBS. Then, after 10 min of centrifugation at 4 °C and 13,200rpm, the washed cell pellet was ressuspended in 500 µL of disintegration buffer (PBS, 1 mM PMSF and 0.1 mM EDTA).

2.7.3. IMAC protein purification

Immobilized metal affinity chromatography (IMAC) is widely used for the purification of recombinant proteins (Cheung et al., 2012). The recombinant proteins were purified by IMAC, using nickel columns (suitable for the purification of His-tagged proteins), and then quantified for protein purification yield. This purification technique is based on the interaction of proteins with histidine residues (or Trp and Cys) on their surface with divalent metal ions (e.g., Ni^{2+}, Cu^{2+}, Zn^{2+},

Co^{2+}) immobilized via a chelating ligand. Histidine-tagged proteins have an extra high affinity in IMAC because of the multiple (6 to 10) histidine residues.

Initially, in order to enhance the efficiency of purification, the imidazole concentration of supernatant samples were adjusted to 40 mM imidazole. Then, the sample was filtered through 0.45 µm filter units and pH was adjusted to 7.4. After cleaning the pump and column purification with filtered distilled water, the column was loaded with nickel solution. Then, in order to remove the excess of nickel, approximately 30 mL of filtered distilled water were introduced into the column. IMAC purification begins with equilibration of the column with a binding buffer containing a low concentration of imidazole (40 mM). The sample was slowly loaded on the column, and for enhanced the purity of recombinant proteins, the column was washed using the binding buffer. Elution of bound proteins was performed with 300 mM of imidazole. Finally, the cleaning solution was loaded on the column. Aliquots of flow-through, washing, eluted and cleaning samples were prepared and analyzed by SDS-PAGE. The composition of the solutions used in the purification procedure is present in Table 2.7. Before being loaded onto the column purification, the solutions were filtered and the pH was adjusted to 7.4 in the day of purification. The column was cleaned and stored according to the manufacturer's instructions.

Table 2.7 Composition of IMAC solutions.

Solution	Composition
Nickel solution	0.1 M $NiSO_4$. $6H_2O$
Binding solution	40 mM Imidazol 20 mM phosphate buffer
Elution buffer	300 mM Imidazol 20 mM phosphate buffer
Cleaning solution	0,5 M NaCl 50 mM EDTA 20 mM phosphate buffer

The eluted protein fractions were dialyzed against PBS buffer (overnight at 4°C) to remove salts and imidazole.

2.7.4. Protein quantification

To estimate total protein from fermentation supernatant samples a NanoDrop 1000 Spectrophotometer (Thermo Scientific) was used. The protein concentration was estimated by molar extinction coefficient of 1.898 $M^{-1}cm^{-1}$ and 1.904 $M^{-1}cm^{-1}$ for *Ct*CBM3$_{CipA}$-wt and *Ct*CBM3$_{CipA}$-mut, respectively and 0.912 $M^{-1}cm^{-1}$ and 0.852 $M^{-1}cm^{-1}$ for eGFP-*Tr*CBM1$_{Cel7A}$ and eGFP-NL-*Tr*CBM1$_{Cel7A}$, respectively.

Protein concentrations were determined by the Bradford method with bovine serum albumin (BSA) as the standard, using the Bio-Rad Protein assay kit. The amount 160 µL of the protein sample were mixed with 40 µL of dye reagent concentrate in each well of a microtiter plate. After incubating the plate for 5 min at room temperature, the absorbance was measured at 595 nm. A blank was made with Sodium Acetate buffer (50 mM, pH 5.0) instead of protein sample. To convert absorbance to protein concentration a calibration curve was made with absorbance *versus* correspondent concentrations of BSA.

2.8. Analysis of proteins by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a technique for separating proteins based on their ability to move within an electrical current, which is a function of the length of their polypeptide chains or of their molecular weight. The most widely used gel system for separating of proteins by SDS-PAGE is the Laemmli system (1970). This classic system uses tris-glycine gels comprised of a stacking gel component and the resolving gel where varying acrylamide gel percentages are used to separate the proteins based on their mass weight.

2.8.1. Gel and sample preparation

The stacking and resolving gel composition used for proteins separation are presented in Table 2.8. The acrylamide composition of the resolving gel varied according to the molecular weight of protein to be separated.

Table 2.8 Gel composition used for SDS-PAGE analyzes.

	Stacking Gel	Resolving Gel	
	4%	**12%**	**15%**
Acrylamide (v/v)	3.84%	11.68%	14.48%
Bis-Acrylamide (v/v)	0.10%	0.32%	0.52%
Tris-HCl, pH 6.8	0.12 M	-	-
Tris-HCl, pH 8.8	-	0.375 M	0.375 M
SDS (w/v)	0.1%	0.1%	0.1%
APS (w/v)	0.05%	0.05%	0.05%
TEMED (v/v)	0.005%	0.005%	0.005%

The SDS-PAGE samples were prepared by mixing 16 µL of protein sample with 4 µL of 5x Sample Buffer (Table 2.9) and incubating 5 min at 100 °C. Each well of the gel was loaded with 15 µL of the mixture.

Table 2.9 Composition of the 5x Sample Buffer used for SDS-PAGE analysis.

5x Sample Buffer	0.313 M Tris-HCl, pH 6.8 50% (v/v) glycerol 10% (w/v) SDS 10% (v/v) β-mercaptoethanol 0.05% (w/v) Bromophenol Blue

The running of the gel was made with 500 mL of 1x Running Buffer (Table 2.10) at 15 mA, until the tracking dye reached the base of the gel.

Table 2.10 Composition of the 1x Running Buffer used for SDS-PAGE analysis.

1x Running Buffer	25 mM Tris base 192 mM Glycine (Sigma) 0.1% (w/v) SDS

2.8.2. Protein molecular weight marker

The protein molecular weight marker used was Thermo Scientific PageRuler Unstained Broad Range Protein Ladder, a mixture of 11 proteins (5 to 250 kDa) for use as size standards in

protein electrophoresis (SDS-PAGE). The sizes of the bands are presented in Table 2.11. The volume of 5 µL of molecular weight marker, per gel, was used.

Table 2.11 Molecular sizes of the bands from Thermo Scientific PageRuller Unstained Broad Range Protein Ladder. The 100, 50 and 20kDa protein bands are more intense for easy identification.

Band	Molecular weight (kDa)
1	250
2	150
3	**100**
4	70
5	**50**
6	40
7	30
8	**20**
9	15
10	10
11	5

2.8.3. Coomassie Brilliant Blue staining

Coomassie™ Brilliant Blue dyes (R-250 and G-250) form strong but non-covalent complexes with proteins, most probably based on a combination of van der Waals forces and electrostatic interactions. Coomassie R-250, the more commonly and sensitivity used of the two, can detect as little as 0.1 µg of protein. The Coomassie Brilliant Blue solution was prepared by dissolving 80 mg of Coomassie Brilliant Blue R-250 in 1 L of distilled water, for 2 - 4 h. After that, 3 mL of 37% HCl was added. The solution was stored at room temperature and protected from light.

To stain SDS-PAGE gels with Coomassie™ Brilliant Blue method, the gel was first rinsed in distilled water, heated in a microwave oven at 700 W for 30 seconds and then incubated for 3 min on a rotary shaker with gentle mixing. This operation was repeated for 3 times. After that, the gel was immersed in the Coomassie™ Brilliant Blue solution, heated in a microwave oven at 700 W for 30 seconds, and then incubated for 30 min, on a rotary shaker with gentle mixing. Gels were visualized and photographed in a Molecular Imager ChemiDoc™ XRS + Imaging System (Bio-Rad) and analyzed using the Image Lab 4.0 software.

2.9. Adsorption capacity of eGFP-*Tr*CBM1$_{Cel7A}$, eGFP-NL-*Tr*CBM1$_{Cel7A}$, *Ct*CBM3$_{CipA}$-wt and *Ct*CBM3$_{CipA}$-mut on cellulose

2.9.1. Qualitative adsorption to Avicel

The ability of the recombinant CBMs (*Tr*CBM1$_{Cel7A}$ and *Ct*CBM3$_{CipA}$) to bind to cellulose was evaluated in Avicel-binding assays. These affinity assays were done by mixing 0.4 mg of purified protein with 25 mg of Avicel. After 2 h of incubation at room temperature mixed by rotation, the mixture was centrifuged (13200g, 10 min, at room temperature) and the supernatant was collected (flow fraction). Then, the cellulose fibers were washed for five times with PBS buffer, to remove unbound protein. The elution of the bound protein was made by the addition of 40 µL 1x Sample Buffer directly to the fibers and boiling for 10 min. The flow fraction, washes and elution samples were analyzed by 15% (w/v) SDS-PAGE acrylamide gel stained with Coomassie Brilliant Blue (section 2.8.3).

2.9.2. CBM adsorption isotherm

The binding of both recombinant versions of *Ct*CBM3$_{CipA}$ to Avicel was assayed in eppendorf tubes containing different CBM concentrations (CBM$_{Initial}$, µmol/mL) and Avicel with a concentration of 10 mg/mL in 1 mL of buffer (50 mM sodium acetate, pH 5.0). Duplicate samples were incubated at 4 °C with constant shaking in a rotating shaker. After 2 h, Avicel was removed by centrifugation (12000xg, 10 min, 4 °C), and the free protein concentration left in the supernatant (CBM$_{Free}$, µmol/mL) was measured in a HORIBA Scientific spectrofluorimeter, operated at an emission and excitation wavelengths of 344 and 275 nm, respectively. Previously, the apparatus was calibrated using CBM solutions with known concentrations, previously determined for protein quantification (section 2.11). The bound CBM (µmoL/g) was calculated by subtracting free CBM concentrations from total CBM concentrations. The equilibrium association constants and target binding capacity were determined by nonlinear regression of bound versus free protein concentrations to Langmuir isotherm based on the equation as follows:

$$CBM_{Bound} = \frac{(CBM_{Inicial}) - (CBM_{Free})}{m_{Avicel}} \cdot V_R$$

where V_R (mL) correspond to the total adsorption volume and m_{Avicel} (g) the fibers mass. Non-linear regression analysis was used to calculate the parameters of the Langmuir adsorption isotherm:

$$CBM_{Bound} = \frac{CBM_{Max} \cdot K_a \cdot [CBM]_{Free}}{1 + K_a \cdot [CBM]_{Free}}$$

where CBM_{Bound} is the molar amount of protein adsorbed, per unit weight of cellulose, $[CBM]_{Free}$ is the molar protein concentration in the liquid phase at the adsorption equilibrium, CBM_{Max} and K_a are the maximum molar amount of protein adsorbed, per unit weight of cellulose, and the adsorption equilibrium constant, respectively.

2.9.3. Fluorescence adsorption studies

To analyze the adsorption of *Ct*CBM3$_{CipA}$ on the cellulose surface, conjugates of CBM with a fluorescence probe, fluorescein isothiocyanate (FITC), were produced. Fluorescein isothiocyanate (FITC) is a fluorescent probe widely used to attach a fluorescent label to proteins, by reacting with amine groups. The isothiocyanate group reacts with amino terminal and primary amines in proteins. The *Ct*CBM3$_{CipA}$ conjugation with FITC (Isomer I) was made using a ratio of 1:3 (protein: fluorochrome). The fluorochrome was dissolved in DMSO, and allowed to react with the protein overnight, in the dark, at room temperature, with magnetic stirring. The *Ct*CBM3$_{CipA}$-FITC conjugates were purified in a PD-10 desalting column (GE Healthcare), previously equilibrated with an appropriate buffer (PBS, pH 7.4). The FITC/Protein (F/P) molar ratio is defined as the ratio of moles of FITC to moles of protein in the conjugate. The absorbance of the conjugate sample at 280 nm and 495 nm was determined (Jasco V-560 spectrophotometer), and the F/P molar ratio was calculated by the next formula:

$$Molar\ F/P = \frac{A_{495} \times C}{A_{280} - [(0.35 \times A_{495})]}$$

Where, $C = \frac{MW \times E_{280}^{0.1\%}}{389 \times 195}$ is a constant value given for a protein, MW is the molecular weight of the protein, 389 is the molecular weight of FITC, 195 is the absorption $E^{0.1\%}$ of bound FITC at 490

nm at pH 13.0, (0.35 X A_{495}) is the correction factor due to the absorbance of FITC at 280 nm, $E^{0.1\%}$ is the absorption at 280 nm of a protein at 1.0 mg/mL.

The conjugated CBMs were allowed to adsorb on *E. globulus* fibers (4 mg/g o.d. pulp), for 30 min, at room temperature. The fibers were centrifuged (5 min, 13,200 rpm) and washed with buffer to remove the non-adsorbed CBM-FITC. Fluorescence microscopy observations were performed in an Olympus BX51 microscope, equipped with an Olympus DP72 attached, and using the cell^B software (all equipments are from Olympus Corporation, Tokyo, Japan).

2.10. Studies of the *Ct*CBM3$_{CipA}$-wt and *Ct*CBM3$_{CipA}$-mut effects on fibers

The following methods were performed with the aim of studying the effect of recombinant CBMs in pulp and paper properties. The pulps used in this work were: unbleached Kraft pulp (30% *E. globulus* and 70 % *Pinus sylvestris*), kindly supplied by the paper company Europac Kraft Viana, and bleached *E. globulus* pulp, gently provided by the Paper Science and Technology Department, Universidade da Beira Interior (UBI). The determination of the Schopper-Riegler index and mechanical properties (Figure 2.2) was performed in the Europac Kraft Viana.

Consistency or, more properly, "concentration" is the term used within the pulping and papermaking industries to describe the dry solid content of pulp slurry in water. This property was determined in two stages of this work: before each treatment of pulp, for the estimation of the wet pulp amount to process and before each drainability or mechanical test determination for the estimation of the volume of diluted pulp to test. In the first case, after ensure that the pulp is a homogeneous mixture, a representative sample (approximately 10 g) of the pulp suspension was collected and heated o/n at 105 °C to certify the complete removal of water. Afterwards, the wet pulp weight and oven dried (o. d.) pulp was correlated and the amount of wet pulp to be processed for each treatment was determined. In the second case, 500 mL of the diluted pulp was used to make a papersheet that was dried at 105 °C for approximately 10 min. The weight of paper sheet was determined and the volume of pulp for each test was calculated based on the amount of pulp which is required, 2 g or 1.2 g/papersheet for drainability and mechanical properties determination, respectively.

2.10.1. Water absorption time (WAT)

The water absorption time (WAT) is an important parameter in the manufacture of pulp and paper. It is an indicator of the ability of a sample of wood fiber or pulp mass to retain water. The effect of the glycosidic fraction on the surface properties of Whatman paper was analyzed by contact angle measurements. For determination of WRV, the absorption time of a water drop in the treated papers was determined. Thus, rectangular pieces of "home-made" bacterial cellulose (3,0 x 1,0 cm) were immersed in 20 mM Tris base, pH 7.0, containing increasing concentrations of CBM, for 2 h. Afterwards, the samples were then recovered and dried for 24 h at room temperature. A drop of distilled water (3 µL) was pipetted onto the CBMs treated paper surface and the changes in drop shape over time were recorded with time lapses of 20 ms using an optical Contact Angle System OCA (Dataphysics), and then analyzed frame by frame with SCA20 software.

2.10.2. Schopper-Riegler index (ºSR)

The pulp drainability is the capacity of the pulp for water drainage and is measured by the Schopper-Riegler degree (°SR). For determination of this property, 6 g o.d. (oven dry) of pulp were processed. The pulp suspensions were prepared by disintegrating the samples in sodium acetate buffer 50 mM, pH 5.0, for 5 min. This mechanical treatment allows fiber separation, without significantly altering their structural properties. After the disintegration step, a certain amount of recombinant CBM (2 or 4 mg/ g o.d. fiber) was added to the mixture. The reaction with the pulp occurred for more 25 min, at room temperature, with continuous vigorous mixing. To finish, the mixture was filtered and pulp samples were stored at 4 °C o/n. In order to estimate the CBM action conveniently, control assays (in the absence of recombinant CBMs) were made in parallel. Thereafter, the treated pulp was disintegrated in 2 L of distilled water to 10 000 revolutions. Firstly, to verify the accuracy of the tester the °SR was determined with distilled water (°SR must be equal to 4). Once the calibration of the Shopper-Riegler tester was verified, the tests for the pulp suspension was carry out immediately. After agitating vigorously the pulp suspension with a plastic 500 mL beaker, the volume corresponding to 2 g o.d. was measured to the °SR beaker and distilled water was precisely added to 1000 mL. With the lid closed, the suspension was poured into the Shopper-Riegler tester as soon as 1000 mL was reached. The °SR beaker was placed to the side orifice and after 5 sec the valve was open to release the suspension. When dripping stops, the °SR of water received from side orifice was

checked. Between measurements, the chamber and funnel were rinsed with distilled water. Each experimental condition (CBM assay and control) was tested 2 times and a good reproducibility was found between the results.

2.10.3. Mechanical properties

Fibers were treated with recombinant proteins (2 or 4 mg per gram of o.d. pulp) as described in the previous section. Thereafter, the treated pulp was disintegrated in 2 L of distilled water to 10 000 rotations. Then, the pulp was diluted in 3L of distilled water, and the consistency of the pulp was determined (section 2.15). Handsheets of 63 ± 1 g/m^2 grammage were prepared (each one with 1.2 g o.d. pulp) and pressed for 7 min at 50 Kg/cm^2. Determination of mechanical properties was achieved according to the usual standard procedures: permeability (ISO 5636/3 1992 F); tensile strength (ISO 1924/2 1985 F); tearing (ISO 1947 F) and bursting strength (ISO 2758 1983 F).

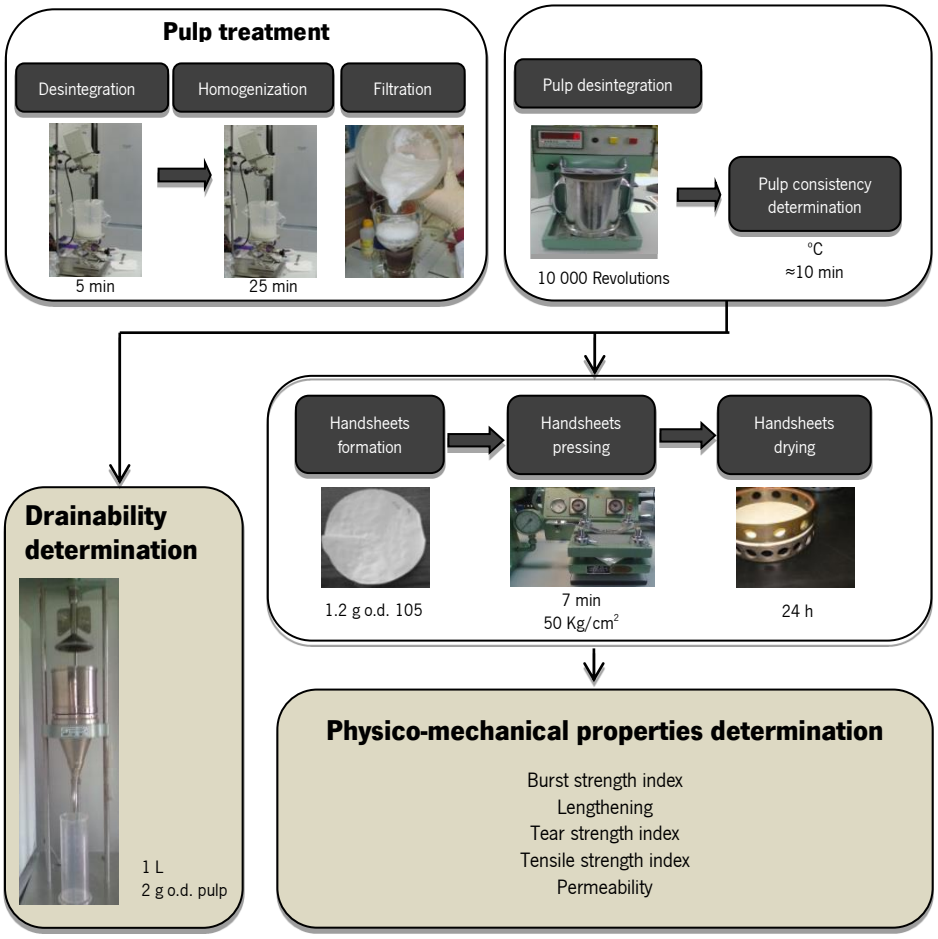

Figure 2.2 Main experimental steps for determination of the drainability of paper pulps and mechanical properties of papersheets.

2.11. Statistical analysis

One-way ANOVA followed by Tukey multiple comparisons test ($p = 0.05$) was performed using GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla California USA.

CHAPTER 3

Results and Discussion

3.1. Construction of the expression plasmids pPICZαA+eGFP-*Tr*CBM1$_{Cel7A}$ and pPICZαA+eGFP-NL-*Tr*CBM1$_{Cel7A}$

The DNA coding sequence of eGFP-*Tr*CBM1$_{Cel7A}$ (coding for eGFP fused to the *Tr*CBM1$_{Cel7A}$ without linker) and eGFP-NL-*Tr*CBM1$_{Cel7A}$ (coding for eGFP fused to *Tr*CBM1$_{Cel7A}$ with its native linker) was amplified (section 2.5.3) by three sequential PCRs (Table 2.4), using codon optimized primers (Table 2.3), resulting in final PCR products of 883 and 955 bp, respectively (Figure 3.1).

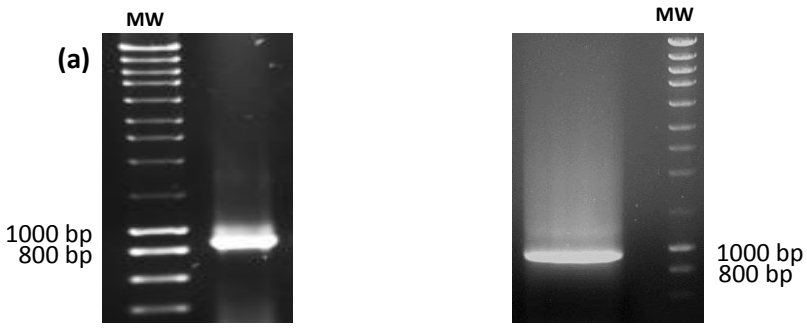

Figure 3.1 Amplification of the DNA coding sequences of eGFP-*Tr*CBM1$_{Cel7A}$ and eGFP-NL-*Tr*CBM1$_{Cel7A}$. (a) PCR product of eGFP-*Tr*CBM1$_{Cel7A}$, (b) PCR product of eGFP-NL-*Tr*CBM1$_{Cel7A}$. MW, molecular weight standards.

Each amplified fragment was digested with *EcoR*I-HF and *Kpn*I-HF (section 2.5.7.1) and ligated with T4 DNA ligase to the pPICZαA plasmid previously digested with the same enzymes (section 2.5.7.3) to generate pPICZαA_eGFP-*Tr*CBM1$_{Cel7A}$ and pPICZαA_eGFP-NL-*Tr*CBM1$_{Cel7A}$.

The resulting ligation reactions were used to transform competent *E. coli* cells (section 2.5.8). Afterwards, the transformant colonies obtained were analyzed for the presence of the insert. Thus, some colonies were randomly chosen for rapid plasmid DNA extraction (section 2.5.9.1) (Figure 3.2) and analyzed in agarose gel (section 2.5.4). This screening resulted in the selection of two clones, 4 and 8, potentially carrying the pPICZαA_eGFP-*Tr*CBM1$_{Cel7A}$ and pPICZαA_eGFP-NL-*Tr*CBM1$_{Cel7A}$ constructs, respectively, because they presented a MW higher than the other clones.

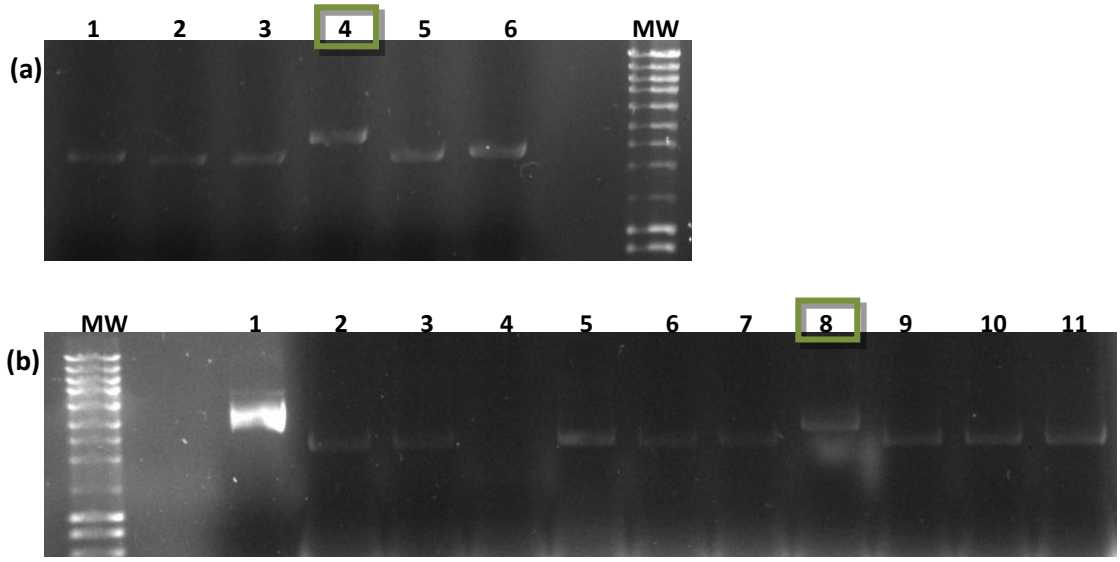

Figure 3.2 Screening of *E. coli* NZY5α transformants carrying pPICZαA+eGFP-*Tr*CBM1$_{Cel7A}$ (a) and pPICZαA+eGFP-NL-*Tr*CBM1$_{Cel7A}$ (b) by electrophoresis of plasmid DNA extracted through the rapid method. The boxes represent selected colonies for restriction analyses. Clones 4 (a) and 8 (b) appeared in the agarose gel with higher molecular weight than the other clones. Lane 1(b,), empty pPICZαA extracted with the commercial kit; MW, molecular weight standards.

Pure plasmid DNA was isolated from the selected clones (section 2.5.9.2) and then double digested with *EcoR*I-HF and *Kpn*I-HF (section 2.5.7.1) to confirm the presence of the insert (Figure 3.3).

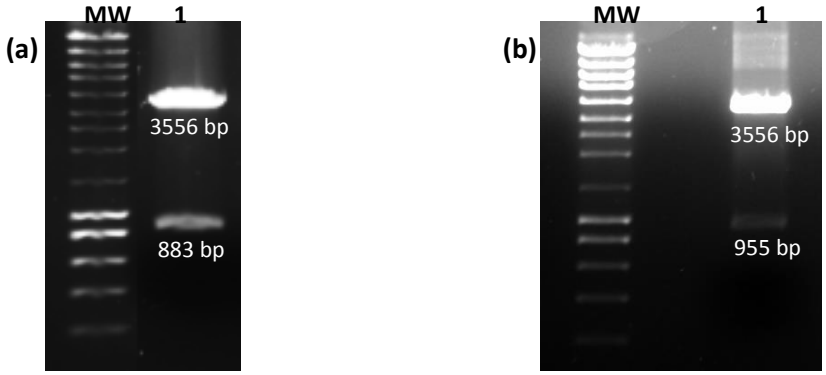

Figure 3.3 Confirmation of insertion of eGFP-*Tr*CBM1$_{Cel7A}$ (a) and eGFP-NL-*Tr*CBM1$_{Cel7A}$ (b) genes into pPICZαA plasmid by restriction analysis. (a) pPICZαA+eGFP-*Tr*CBM1$_{Cel7A}$ from clone 4 and (b) pPICZαA+eGFP-NL-*Tr*CBM1$_{Cel7A}$ from clone 8 digested with the cloning restriction enzymes, *EcoR*I and *Kpn*I. MW, molecular weight standards.

The correct sequence and directionality of the inserts was verified by sequencing with 5´ *AOX1* and the 3´ *AOX1* primers (located in *AOX*1 promoter and terminator, respectively).

The constructed expression plasmids (pPICZαA_eGFP-*Tr*CBM1$_{Cel7A}$ and pPICZαA_eGFP-NL-*Tr*CBM1$_{Cel7A}$) were linearized using *Sac* I (section 2.6.1) at the *AOX*1 locus for efficient integration into the *Pichia* genome, and transformed into *P. pastoris* KM71H by electroporation (section 2.6.3), with transformation efficiencies of 100 to 150 colonies per μg of DNA. The selection of transformants was made with Zeocin™ at a final concentration of 100 μg/mL. Multicopy integrated strains were selected in YPDS plates with increasing concentrations of Zeocin™ (500 and 1000 μg/mL) (section 2.6.4). The integration of plasmids into the yeast genome was confirmed by colony PCR using primers P1 and P5 (section 2.5.3.3; Figure 3.4).

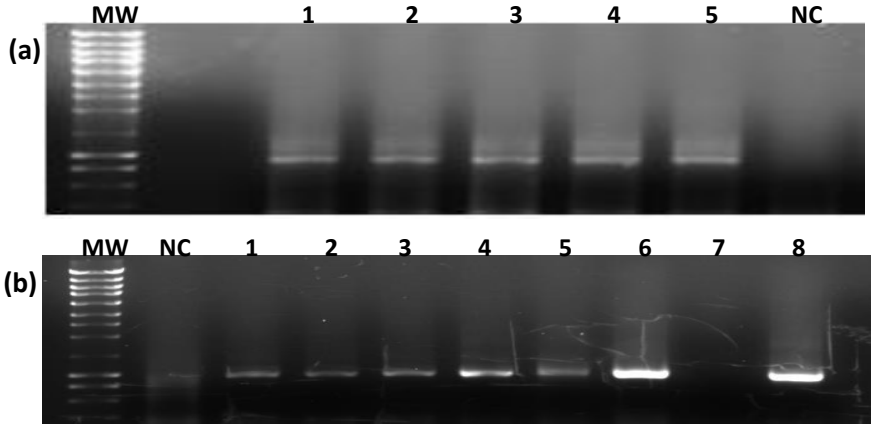

Figure 3.4 Confirmation of the insertion of the expression vectors eGFP-*Tr*CBM1$_{Cel7A}$ (a) and eGFP-NL-*Tr*CBM1$_{Cel7A}$ (b) into the genome of *P. pastoris* by colony PCR. NC, negative control; MW, molecular weight standards.

Some positive *P. pastoris* clones (clones 1, 2 and 3 (a) and 4, 6 and 8 (b) from Figure 3.4) were selected for the production of the recombinant fusion proteins, based on their ability to grown in YPD medium supplemented with the higher Zeocin™ concentrations used (500 or 1000 μg/mL).

3.2. Production of eGFP-*Tr*CBM1$_{Cel7A}$ and eGFP-NL-*Tr*CBM1$_{Cel7A}$ in *P. pastoris*

The *S. cerevisiae* α-factor signal sequence, included in the pPICZaA expression vector, was used for the secretion of the recombinant eGFP-*Tr*CBM1$_{Cel7A}$ and eGFP-NL-*Tr*CBM1$_{Cel7A}$ fusion proteins. Different induction conditions were tested to obtain extracellular production of both eGFP-*Tr*CBM1$_{Cel7A}$ and eGFP-NL-*Tr*CBM1$_{Cel7A}$, such as different induction temperatures (15 ºC and 30 ºC), final methanol concentrations (0.5% and 1% v/v) and induction times (4 and 5 days). However, regardless of the production conditions tested, intense bands corresponding to the

predicted molecular weights of these fusion proteins (approximately 33 kDa for eGFP-*Tr*CBM1$_{Cel7A}$ and 35 kDa for eGFP-NL-*Tr*CBM1$_{Cel7A}$) were not detected by SDS-PAGE analysis of the concentrated supernatants of recombinant *P. pastoris* cultures after 4 days of induction (Figure 3.5-a). Moreover, intense bands of higher molecular weight that might correspond to glycosylated versions of these proteins were also not observed. The concentrated supernatants of recombinant *P. pastoris* expressing eGFP-*Tr*CBM1$_{Cel7A}$ and eGFP-NL-*Tr*CBM1$_{Cel7A}$ were compared with the concentrated supernatants of the same strains that were submitted to the same induction period, but without supplementation with methanol (Figure 3.5-b). Similar results were obtained after 5 days of induction, thus indicating that these recombinant proteins (eGFP-*Tr*CBM1$_{Cel7A}$ and eGFP-NL-*Tr*CBM1$_{Cel7A}$) were not successfully expressed as secreted proteins in any of the production conditions tested.

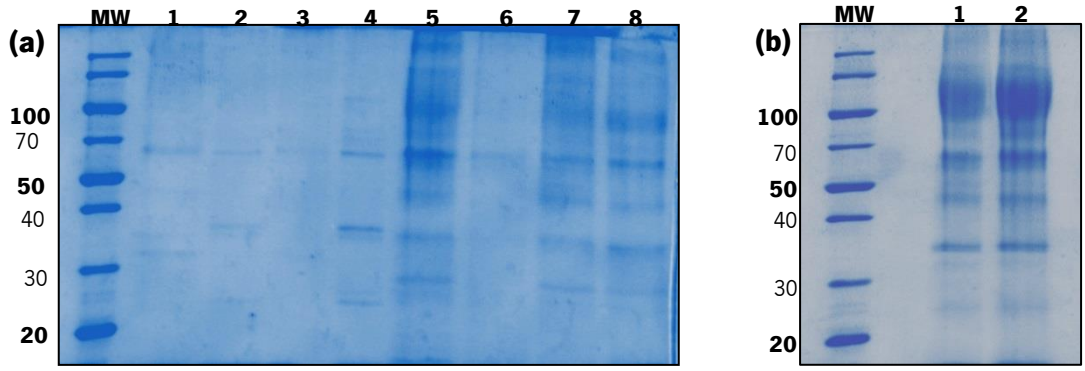

Figure 3.5 SDS–PAGE analysis (Coomassie Blue stained gel) of (a) 25X concentrated supernatants of *P. pastoris* expressing eGFP-*Tr*CBM1$_{Cel7A}$ (Lane 1-4) and eGFP-NL-*Tr*CBM1$_{Cel7A}$ (Lane 5-8), collected after 4 days of induction and (b) 25X concentrated supernatants of *P. pastoris* expressing eGFP-*Tr*CBM1$_{Cel7A}$ (Lane 1) and eGFP-NL-*Tr*CBM1$_{Cel7A}$ (Lane 2), without methanol supplementation in the induction period (used as control). (a) The temperature of induction and methanol supplementation rate were subjected to optimization (Lanes 1 and 5: 15 ºC, 0.5% methanol/day; Lanes 2 and 6: 15 ºC, 1% methanol/day; Lanes 3 and 7: 30 ºC, 0.5% methanol/day and Lanes 4 and 8: 30 ºC, 1% methanol/day. The predicted molecular weights for eGFP-*Tr*CBM1$_{Cel7A}$ and eGFP-NL-*Tr*CBM1$_{Cel7A}$ are approximately 33 and 35 kDa, respectively. (b) The temperature of induction was 30 ºC. MW, molecular weight standards.

For bioprocess development, GFP has been used as a reporter in both prokaryotic and eukaryotic cells (Albano et al., 1998; Cha et al., 2000; Chae et al., 2000; Chalfie et al., 1994; Cheng et al., 1996; DeLisa et al., 1999; Jones et al., 2004). However, production of soluble, secreted GFP or GFP-protein fusions in *P. pastoris* is not straightforward.

Since the recombinant proteins eGFP-*Tr*CBM1$_{Cel7A}$ and eGFP-NL-*Tr*CBM1$_{Cel7A}$ were not detected in the culture medium, the cells of the recombinant *P. pastoris* strains were observed by fluorescent

microscopy (Figure 3.6). Fluorescence microscopy images of the cells showed that both eGFP-*Tr*CBM1$_{Cel7A}$ and eGFP-NL-*Tr*CBM1$_{Cel7A}$ were expressed but as cell-associated proteins. Additionally, under the same culture conditions a higher accumulation of eGFP-NL-*Tr*CBM1$_{Cel7A}$ than of eGFP-*Tr*CBM1$_{Cel7A}$ was observed in the recombinant *P. pastoris* cells. These results point out to a possible bottleneck in the fusion protein folding and secretion pathway, resulting in intracellular accumulation. Similar results have been reported by Sjoblom et al., (2012), who suggested that the high-level expression of fluorescent GFP-fused proteins in *P. pastoris* leads to the intracellular accumulation of GFP into subcellular organelles, namely in the Golgi system.

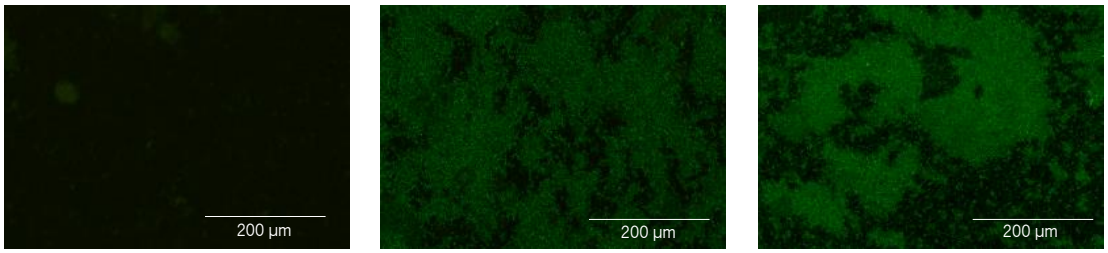

Figure 3.6 Fluorescence microscopy of KM71H *P. pastoris* cells transformed with (a) empty pPICZαA, (b) pPICZαA_eGFP-*Tr*CBM1$_{Cel7A}$ and (c) pPICZαA_eGFP-NL-*Tr*CBM1$_{Cel7A}$ vectors. The images (a) and (b) were acquired with an exposure time of 100 ms and (c) with an exposure time of 50 ms.

The secretion levels of this yeast are highly variable and, in several cases, intracellular accumulation of the recombinant protein destined for secretion has been observed (Hohenblum et al., 2004; Resina et al., 2005; Zupan et al., 2004). Additionally, decrease of the target protein production levels when fused to GFP is case dependent (Surribas et al., 2006). For instance, when eGFP was cloned in the C-terminal of recombinant *Ct*CBM3$_{CipA}$, the fused CBM was successfully secreted (Wan et al., 2011). However, in this study, the cloning of eGFP in the N-terminal of *Tr*CBM1$_{Cel7A}$ could have affected its secretion. To improve secretion systems it is important to have an understanding of the limiting steps within the secretory pathway. Various strategies can be employed in the future in order to improve secretion of recombinant *Tr*CBM1$_{Cel7A}$ in *P. pastoris*, like testing a different fusion order in the construct (i.e. *Tr*CBM1$_{Cel7A}$-eGFP), or replace the fusion partner. Alternatively, *P. pastoris* transformants resistant only to a lower Zeocin™ concentration (i.e. 100 µg/mL) can be selected. Eventually, a transformant with a

lower copy number of the expression cassette integrated in the genome would be able to secrete the recombinant fusion protein eGFP-CBM.

Since by fluorescence microscopy recombinant eGFP-*Tr*CBM1$_{Cel7A}$ and eGFP-NL-*Tr*CBM1$_{Cel7A}$ were observed to remain unsecreted, with the intended to investigate in which fraction of the cell were they retained, after mechanical cell lysis of *P. pastoris* cells, membrane-associated proteins were separated from cytosolic proteins (section 2.7.2) and immediately purified by IMAC, to avoid proteolytic degradation. The purifications of eGFP-*Tr*CBM1$_{Cel7A}$ and eGFP-NL-*Tr*CBM1$_{Cel7A}$ were analyzed by SDS-PAGE and these recombinant proteins were only found in the cytosolic extracts (Figure 3.7). However, both eGFP-*Tr*CBM1$_{Cel7A}$ and eGFP-NL-*Tr*CBM1$_{Cel7A}$ could not be successfully purified, once the binding of several host proteins to the IMAC column was detected.

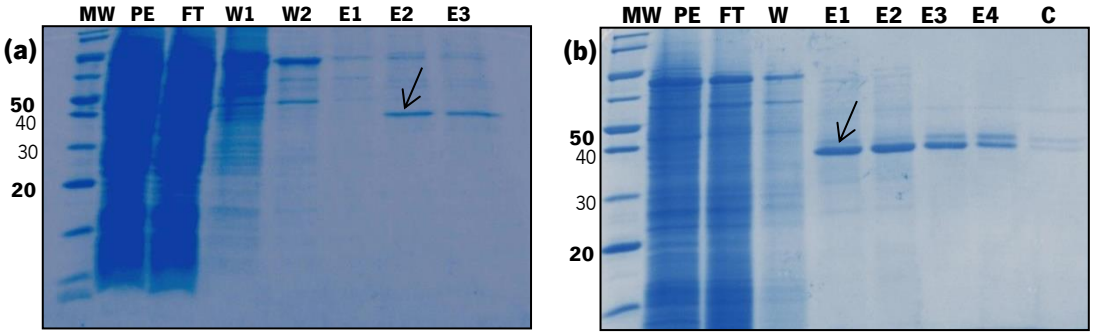

Figure 3.7 SDS-PAGE analyses (Coomassie Blue stained gel) of the IMAC purification of *P. pastoris* cytosolic extracts of the (a) KM71H_eGFP-*Tr*CBM1$_{Cel7A}$ and (b) KM71H_eGFP-NL-*Tr*CBM1$_{Cel7A}$. (MW) molecular marker, (PE) cytosolic extract sample loaded onto the column, (FT) flow-through sample, (W) washing sample, (E) eluted sample and (C) column cleaning sample. The arrows in the figure indicate the band corresponding to the purified fractions of *Tr*CBM1$_{Cel7A}$ and eGFP-NL-*Tr*CBM1$_{Cel7A}$).

Nevertheless, the adsorption of the eluted proteins to microcrystalline cellulose was subsequently confirmed by qualitative adsorption to Avicel (section 3.5.1).

3.3. Production of *Ct*CBM3$_{CipA}$-wt and *Ct*CBM3$_{CipA}$-mut in *P. pastoris*

Previously, two versions of the *C. thermocellum* CipA CBM3 coding region, both containing codons in preference in *P. pastoris*, were cloned into the *P. pastoris* KM71H genome by our research group using the pPICZαA plasmid; one coding for the native *Ct*CBM3$_{CipA}$ with three potential N-glycosylation sites (N14; N68; N124) – *Ct*CBM3$_{CipA}$-wt, and other coding for a mutated

*Ct*CBM3$_{CipA}$ with no potential N-glycosylation sites (amino-acid substitutions: N14Q; N68Q; N124Q) – *Ct*CBM3$_{CipA}$-mut, to be used as control in further studies.

Preliminary SDS-PAGE analysis of recombinant *P. pastoris* culture supernatnans (Figure 3.8) showed that both native (*Ct*CBM3$_{CipA}$-wt) and mutated (*Ct*CBM3$_{CipA}$-mut) *Ct*CBM3$_{CipA}$ were produced as secreted proteins, as expected. SDS-PAGE analysis also revealed that, as expected, only *Ct*CBM3$_{CipA}$-wt was produced highly N-glycosylated (as indicated by the smeared band in Figure 3.8, Lane 1). Additionally, as a result of low amounts of endogenous proteins secreted by *P. pastoris*, both recombinant versions of *Ct*CBM3$_{CipA}$ were observed to comprise the vast majority of the total protein in the concentrated culture supernatants.

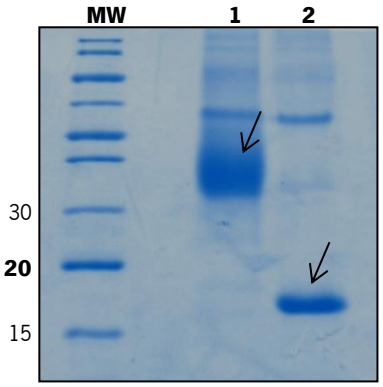

Figure 3.8 SDS–PAGE analysis (Coomassie Blue stained gel) of 25X concentrated supernatants of *P. pastoris* expressing *Ct*CBM3$_{CipA}$-wt (Lane 1) and *Ct*CBM3$_{CipA}$-mut (Lane 2). MW, molecular weight standards. The arrows in the figure indicate the bands corresponding to the *Ct*CBM3$_{CipA}$-wt and *Ct*CBM3$_{CipA}$-mut.

The recombinant *Ct*CBM3$_{CipA}$-mut version of the *Ct*CBM3$_{CipA}$ presented a band with its calculated molecular weight (approximately 18 kDa), while the putative glycosylated version, *Ct*CBM3$_{CipA}$-wt, presented a band with a higher apparent molecular weight (approximately 35 kDa) than predicted. This result can be explained by the fact that glycosylated proteins may suffer a significant delay in SDS-PAGE gels. Indeed, N-glycosylation of *Ct*CBM3$_{CipA}$-wt was previously confirmed in our research group by digestion with Endoglycosidase H, which caused the reduction of that high molecular weight to 18 kDa, which is the *Ct*CBM3$_{CipA}$-wt calculated molecular weight (Figure 3.9).

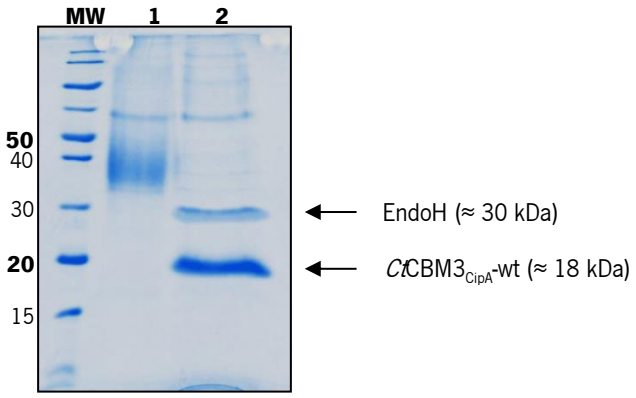

Figure 3.9 SDS-PAGE analysis (Coomassie Blue stained gel) of the *Ct*CBM3$_{CipA}$-wt before (Lane 1) and after (Lane 2) digestion with Endoglycosidase H (EndoH). MW, molecular weight standards.

To study the effects of these recombinant versions of *Ct*CBM3$_{CipA}$ on the surface/interface properties of cellulose fibers and access the importance of glycosylation for their effect, high amounts of these proteins were required. Therefore, in order to enhance the recombinant versions of *Ct*CBM3$_{CipA}$ production, the induction time and the concentration of the producing cells in the induction medium were submitted to optimization. SDS-PAGE analysis (Figure 3.10) showed that the amount of both recombinant proteins after 4 days of induction was higher than after 3 days of induction.

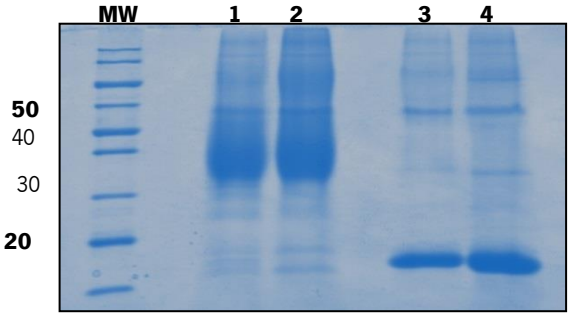

Figure 3.10 Effect of the induction period in the *Ct*CBM3$_{CipA}$-wt (Lanes 1 and 2) and *Ct*CBM3$_{CipA}$-mut (Lanes 3 and 4) expression. 25X concentrated supernatant of recombinant *P. pastoris* after 3 (Lanes 1 and 3) and 4 days (Lanes 2 and 4) of induction. MW, molecular weight standards.

After this optimization step, the concentration of the producing cells in the induction medium was duplicated and the production levels of recombinant versions of *Ct*CBM3$_{CipA}$ in the culture medium of *P. pastoris* could be increased for values ranging from 20 to 50 mg/L.

3.4. Purification of *Ct*CBM3$_{CipA}$-wt and *Ct*CBM3$_{CipA}$-mut from culture supernatants

Recombinant versions of *Ct*CBM3$_{CipA}$ were purified from *P. pastoris* cultures using the IMAC method (section 2.10), since they were cloned with a 6xhis-tag sequence at their C-terminal. The purified *Ct*CBM3$_{CipA}$-mut and *Ct*CBM3$_{CipA}$-wt were then analyzed by SDS-PAGE (Figure 3.11). Purification efficiencies, estimated by the ratio between the protein amount in eluted samples and the protein amount loaded onto the purification column, were approximately 0.43 and 0.58 for *Ct*CBM3$_{CipA}$-wt and *Ct*CBM3$_{CipA}$-mut, respectively. As observed in Figure 3.11, identical results were obtained for the two recombinant proteins, in respect to their high affinity for the nickel resin. This strong interaction translated into a high protein amount in the eluted samples. The elution fractions of *Ct*CBM3$_{CipA}$-mut (Figure 3.11-a, E1, E2, E3 and E4) consisted of a predominant protein of approximately 18 kDa. The elution fractions of *Ct*CBM3$_{CipA}$-wt (Figure 3.11-b, E1, E2 and E3) consisted of a predominant protein of approximately 35 kDa. These elution fractions also presented additional proteins with lower molecular weights, which may correspond to non-glycosylated and less glycosylated forms of recombinant *Ct*CBM3$_{CipA}$-wt.

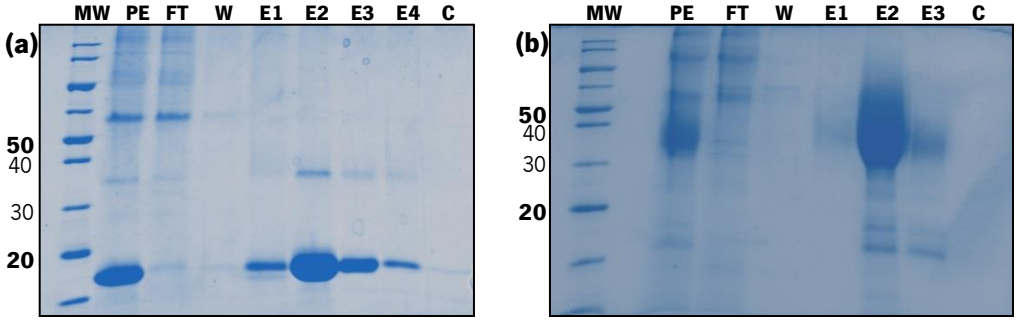

Figure 3.11 SDS-PAGE analyses (Coomassie Blue stained gel) of the IMAC purification of (a) *Ct*CBM3$_{CipA}$-mut and (b) *Ct*CBM3$_{CipA}$-wt produced by recombinant *P. pastoris*. (MW) molecular marker, (CS) 25X concentrated supernatant sample loaded onto the column, (FT) flow-through sample, (W) washing sample, (E) eluted sample and (C) column cleaning sample.

3.5. Adsorption of recombinant CBMs on cellulose fibers

3.5.1. Qualitative adsorption to Avicel

*Ct*CBM3$_{CipA}$ is a very well characterized and studied CBM (Tomme et al., 1995; Tormo et al., 1996). It is a Type A CBM ("surface-binding"), which means that it possesses a planar surface that interacts tightly with crystalline cellulose. The ability of the recombinant versions of *Ct*CBM3$_{CipA}$ to bind to cellulose was evaluated in Avicel-binding assays (Figure 3.12), confirming that both recombinant *Ct*CBM3$_{CipA}$-wt and *Ct*CBM3$_{CipA}$-mut showed affinity to Avicel, as revealed by their minor quantity in the unbound fraction (Figure 3.12, Lane 2) and their association with the carbohydrate fraction (Figure 3.12, Lane 5). The small unbound amount of *Ct*CBM3$_{CipA}$-wt and *Ct*CBM3$_{CipA}$-mut (Figure 3.12, Lanes 2), may be attributed to a relative excess of CBM in relation to Avicel.

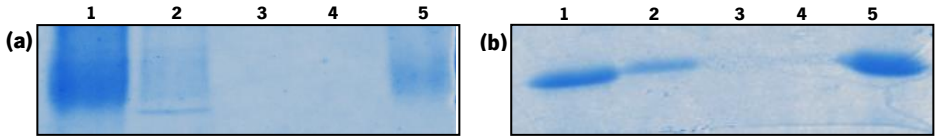

Figure 3.12 SDS-PAGE analyses (Coomassie Blue stained gel) for the binding of *Ct*CBM3$_{CipA}$-wt (a) and *Ct*CBM3$_{CipA}$-mut (b) to Avicel. Lanes: 1, *Ct*CBM3$_{CipA}$ added; 2, unbound; 3, first wash; 4, last wash and 5, *Ct*CBM3$_{CipA}$ desorption.

The binding ability of recombinant eGFP-*Tr*CBM1$_{Cel7A}$ and eGFP-NL-*Tr*CBM1$_{Cel7A}$ to cellulose was also analyzed by qualitative adsorption to Avicel (Figure 3.13). It has been suggested that *Tr*CBM$_{Cel7A}$ binds preferentially to the crystalline regions of cellulose and also demonstrates modest binding affinities to amorphous regions of cellulose and some cello-oligosaccharides (Nieves et al., 1991). SDS-PAGE analyses (Figure 3.13) confirmed that both recombinant eGFP-*Tr*CBM1$_{Cel7A}$ and eGFP-NL-*Tr*CBM1$_{Cel7A}$ exhibited capacity to bind to microcrystalline cellulose.

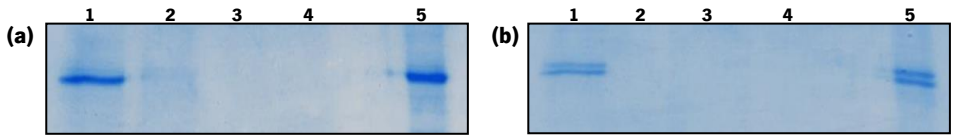

Figure 3.13 SDS-PAGE analyses (Coomassie Blue stained gel) for the binding of eGFP-T*r*CBM1$_{Cel7A}$ (a) and eGFP-NL-T*r*CBM1$_{Cel7A}$ (b) to Avicel. Lanes: 1, Purified fusion protein added; 2, unbound; 3, first wash; 4, last wash and 5, fusion protein desorption.

In addition, the two major proteins obtained in the purification of recombinant eGFP-NL-*Tr*CBM1$_{Cel7A}$ bound to Avicel, suggesting that the higher band in the SDS-PAGE gel (Figure 3.13-b)

possibly corresponds to an O-glycosylated fraction of this protein. This is consistent with the fact that in the native *Tr*CBM1$_{Cel7A}$ linker 9 potential sites exist for O-glycosylation (Harrison et al. 1998), and this linker is present in eGFP-NL-*Tr*CBM1$_{Cel7A}$ but not in eGFP-*Tr*CBM1$_{Cel7A}$.

3.5.2. Adsorption isotherms of *Ct*CBM3$_{CipA}$-wt and *Ct*CBM3$_{CipA}$-mut for Avicel

As previously mentioned, *Ct*CBM3$_{CipA}$ is characterized by a planar hydrophobic binding surface, comprised of conserved aromatic amino-acids, which could selectively interact with crystalline polysaccharides (Linder et al., 1995). The capacity of the recombinant *Ct*CBM3$_{CipA}$ versions (glycosylated and non-glycosylated) produced in this study to bind to Avicel, an insoluble form of cellulose, was evaluated. Microcrystalline cellulose (Avicel) is a typical model cellulosic substrate for the determination of the adsorption isotherms of CBMs (Boraston et al., 2001; Guo et al., 2013; Hong et al., 2008; Najmudin et al., 2005; Shi et al., 2014).

The ascending binding isotherms of *Ct*CBM3$_{CipA}$-mut (non-glycosylated) and *Ct*CBM3$_{CipA}$-wt (glycosylated) for Avicel (section 2.14.2) was determined (Figure 3.14) and in both cases, the experimental data fitted well to Langmuir model kinetics, with satisfactorily high corelation coefficients (R^2 = 0.9909 and R^2 = 0.9800 for *Ct*CBM3$_{CipA}$-mut and *Ct*CBM3$_{CipA}$-wt, respectively).

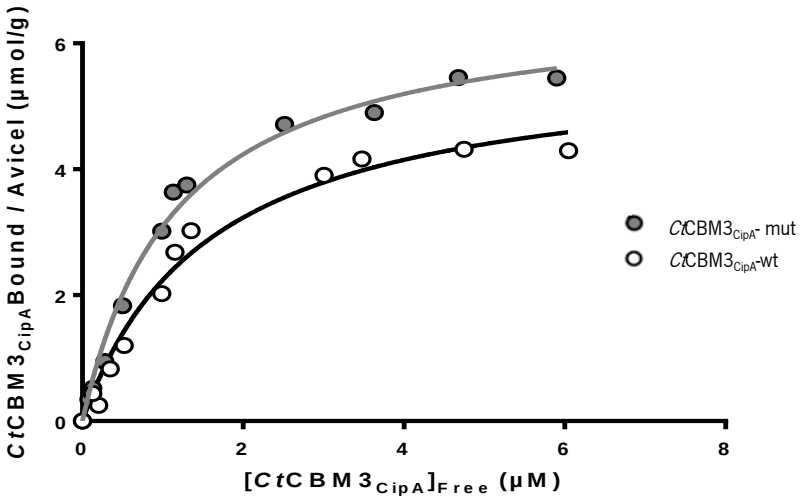

Figure 3.14 Adsorption isotherms of *Ct*CBM3$_{CipA}$-mut and *Ct*CBM3$_{CipA}$-wt for Avicel. The lines were obtained by non-linear regression of the Langmuir isotherm. The values represent the average of two independent replicates.

The adsorption equilibrium constant (K_a), and the maximum molar amount of protein adsorbed per unit weight of cellulose (CBM_{Max}) were determined by non-linear regression of bound *versus* free protein concentrations expressed as a Langmuir isotherm constant (Table 3.1).

Table 3.1 Binding parameters of *Ct*CBM3$_{CipA}$ versions to Avicel.

	CBM_{Max} (µmol/g)	K_a (µM)
***Ct*CBM3$_{CipA}$-mut**	6.72 ± 0.25	0.855 ± 0.127
***Ct*CBM3$_{CipA}$-wt**	5.79 ± 0.37	0.632 ± 0.260

It has been postulated that Avicel has a low binding capacity, with most of its binding surface located internally (Guo et al., 2013; Hong et al., 2007; Hong et al., 2008; Morag et al., 1995). Additionally, it was suggested that the K_a for the adsorption of *Ct*CBM3$_{CipA}$ to cellulose decreases with the decreased crystalline contents in cellulose substrates (Hong et al., 2008). This observation may suggest that the lower affinity of both *Ct*CBM3$_{CipA}$-mut and *Ct*CBM3$_{CipA}$-wt for Avicel, comparing to the reported affinity of *Ct*CBM3$_{CipA}$ for regenerate amorphous cellulose (RAC) (Wan et al., 2011), is probably related to the presence of amorphous portions on its structure. This is consistent with the nature of family 3 CBMs, which preferably bind to crystalline cellulose (Lethio et al., 2003; Ding et al., 2006; Dagel et al., 2011).

Moreover, the recombinant *Ct*CBM3$_{CipA}$-mut and *Ct*CBM3$_{CipA}$-wt exhibited differences in their adsorption to cellulose. The binding parameters of *Ct*CBM3$_{CipA}$-mut are higher than the binding parameters of *Ct*CBM3$_{CipA}$-wt, showing that *Ct*CBM3$_{CipA}$-mut has a higher affinity for cellulose. These different values are probably due to the N-glycosylation of *Ct*CBM3$_{CipA}$-wt, and/or the differences in their amino-acid composition; three asparagine residues (N14; N68; N124) were substituted by three glutamine residues in the *Ct*CBM3$_{CipA}$-mut amino-acid sequence. Nevertheless, according to the three-dimensional structure of CBM3 reported by Tormo et al. (1996), the three potential N-glycosylation sites (N14; N68; N124) were not located in the binding site of this CBM. In spite of this, the glycosylation can affect protein folding and consequently decrease the accessibility of the *Ct*CBM3$_{CipA}$ to the cellulose surface. Indeed, Wan and coworkers (2011) found that the adsorption ability of glycosylated *Ct*CBM3$_{CipA}$-eGFP

(produced in *P. pastoris*) to RAC was (6%) lower than that reported for non-glycosylated *Ct*CBM3$_{CipA}$-eGFP produced in *E. coli*.

3.5.3. Adsorption studies of *Ct*CBM3$_{CipA}$-FITC conjugates on *E. globulus* fibers

The microscopic observation of *E. globulus* fibers treated with FITC-labelled CBMs (Figure 3.15) allowed the characterization of the surface distribution. At the ratio of 4 mg CBM/g o.d. pulp, the *E. globulus* fibers appear to be completely covered by the CBM. In this assay, the incubation conditions and the CBM/g o.d. pulp ratio were the same as those used in the treatment of identical fibers for paper properties determination (section 3.6.3). A control assay was carried out using only FITC, in order to detect non-specific adsorption to cellulose. As it can be seen in Figure 3.15-a, no fluorescent emission was detected in the control fibers.

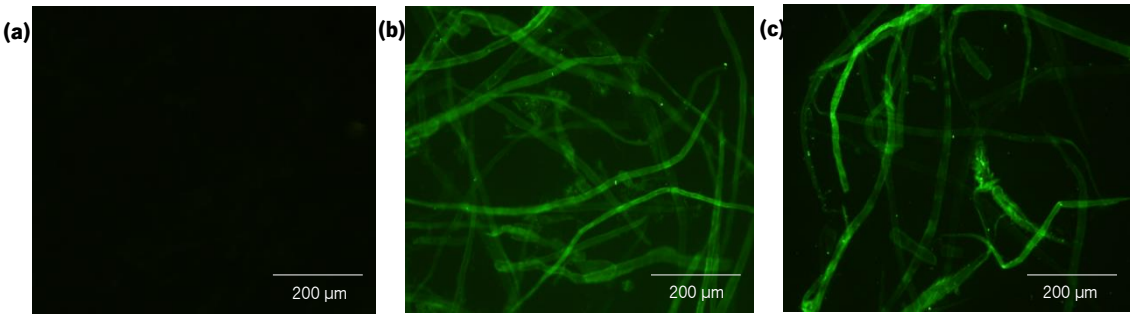

Figure 3.15 *E. globulus* fibers treated with free FITC (a) *Ct*CBM3$_{CipA}$-mut-FITC (b) and *Ct*CBM3$_{CipA}$-wt-FITC (c). The images were acquired with an exposure time of 200 ms.

The adsorption of *Ct*CBM3$_{CipA}$ versions on *E. globulus* fibers was heterogeneous: the CBMs were preferentially concentrated around the fiber's extremities and in some middle regions (Figure 3.16). These regions, that accumulated higher quantities of *Ct*CBM3$_{CipA}$, are characterized by a disordered packing of the cellulose microfibrils, resulting in a higher surface area available for CBM adsorption (Hildén et al., 2003; Pinto et al., 2006). This would explain why these regions in *E. globulus* fibers present a brighter fluorescence. Accordingly, other authors have shown that CBMs have a differential affinity for cellulose fibers with different crystalline properties and/or surface areas (Linder et al. 1996; Bothwell et al. 1997; Pinto et al., 2006). Namely, Pinto and

collegues (2006) also reported that the distribution of glycosylated fungal CBMs (labeled with FITC) on CF11 fibers was not uniform, but concentrated instead around the fiber's extremities.

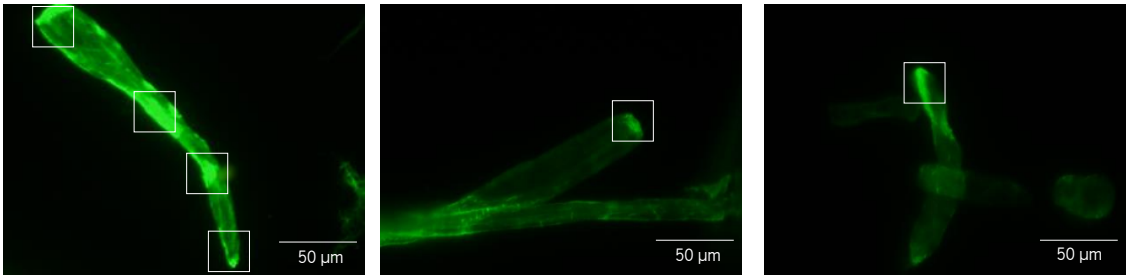

Figure 3.16 The adsorption of *Ct*CBM3$_{CipA}$-FITC on *E. globulus* fibers is not uniform, being higher on the fiber extremities. The images were acquired with an exposure time of 200 ms.

3.6. Effect of the recombinant CBMs (*Ct*CBM3$_{CipA}$-wt and *Ct*CBM3$_{CipA}$-mut) in paper fibers properties

The main goal of this part of the study was to analyze the effect of recombinant versions of *Ct*CBM3$_{CipA}$ on the pulp and paper properties.

3.6.1. Effect of *Ct*CBM3$_{CipA}$-wt and *Ct*CBM3$_{CipA}$-mut on the water absorption time (WAT) of treated bacterial cellulose fibers

The effect of recombinant *Ct*CBM$_{CipA}$ on the surface properties of "home-made" thin papers of commercial bacterial cellulose was analyzed by contact angles measurements. The results for WAT in the *Ct*CBM$_{CipA}$-treated papers are presented in Figure 3.17. Since the surface of the treated papers is very irregular and the WAT was too long (≈ 10-15 min), the measurements had high variation.

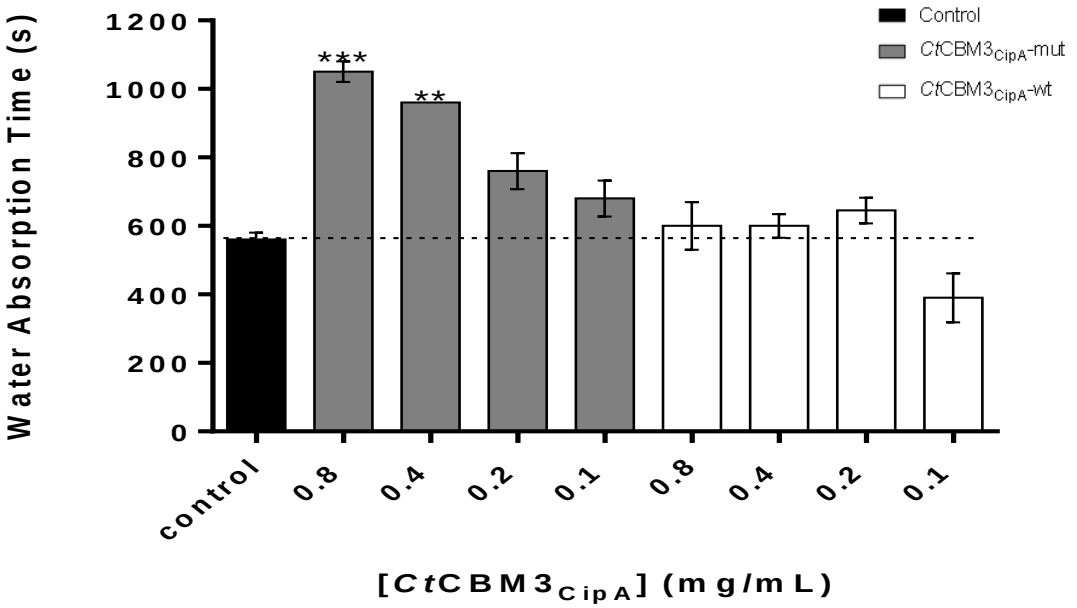

Figure 3.17 Water absorption time of "home-made" thin papers of commercial bacterial cellulose treated with *Ct*CBM3$_{CipA}$-wt and *Ct*CBM3$_{CipA}$-mut at different concentrations (measured by contact angles). The control refers to paper treated with buffer. The bars represent the mean ± SEM, n = 3. The statistical significance of the results in comparison with the control was determined by one-way ANOVA (Tukey's multiple comparisons test). **P<0.01; ***P<0.001.

As it can be seen in Figure 3.17, when recombinant *Ct*CBM3$_{CipA}$-mut was applied at concentrations of 0.8 and 0.4 mg/ml, the WAT increased significantly comparing with untreated papers. At the highest concentration used, *Ct*CBM3$_{CipA}$-mut increased 87.5% the WAT. In other words, the wetability of *Ct*CBM3$_{CipA}$-mut treated papers was significantly reduced compared to control paper (Figure 3.18). This decrease in wetability of treated paper could be related to an increase in the surface hydrophobicity of cellulose occupied by CBM (Levy et al., 2002). These results are consistent with the previous work reported by Levy and coworkers (2002), which demonstrated that treatments of Whatman cellulose filter paper with a CBM from *C. cellulovorans* and a cellulose cross-linking protein (CCP, double fusion of this CBM) resulted in a moderate and high surface hydrophobicity increase, respectively. Similarly, Shi et al., (2014) concluded that CBM fusions of CBM3 from *C. thermocellum* YS CipB with CBM1 from *Volvariella volvacea* significantly reduced the wetability of treated papers.

Additionally, it was verified that the WAT of *Ct*CBM3$_{CipA}$-mut treated papers increased with increasing protein concentrations (statistical differences between 0.8 mg/mL and 0.1 mg/mL

were found, with P<0.01). Similarly, Levy and coworkers (2002) verified that WAT of CBM- and CCP-treated papers also increased with increasing protein concentrations (Levy et al., 2002).

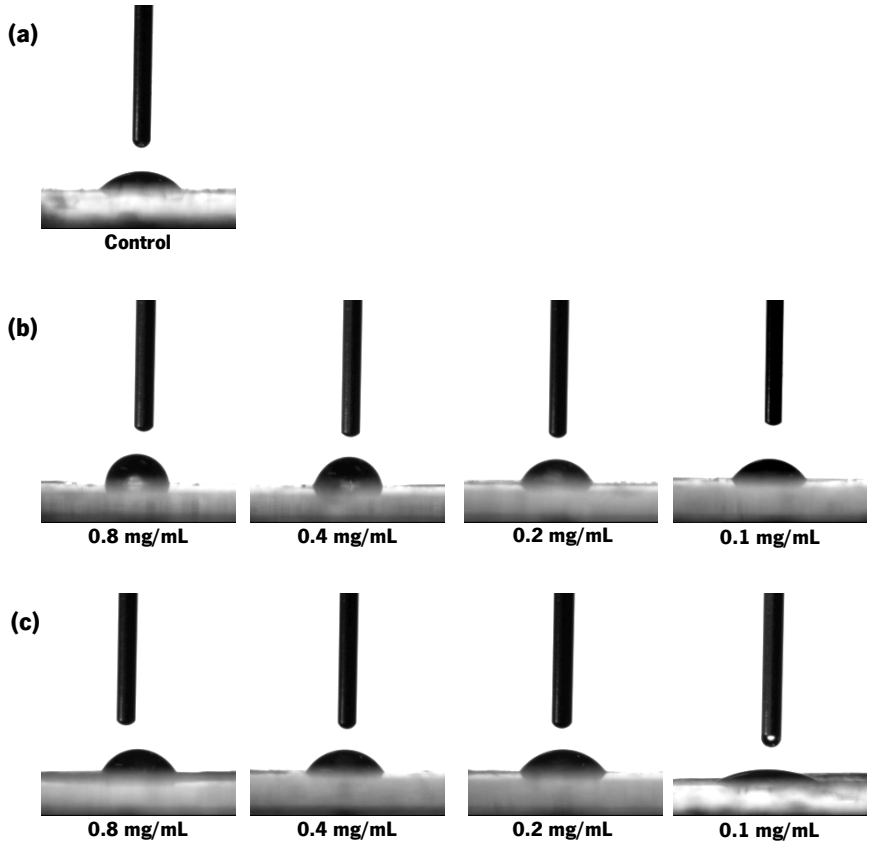

Figure 3.18 Interfacial contact angle of nontreated (a), *Ct*CBM3$_{CipA}$-mut (b) and *Ct*CBM3$_{CipA}$-wt (c) treated "home-made" thin papers of commercial bacterial cellulose. These pictures were taken immediately after the water droplets (3 µL) came in contact with the paper.

On the other hand, no statistical differences between C*t*CBM3$_{CipA}$-wt and the control were found, indicating that C*t*CBM3$_{CipA}$-wt does not change the WAT, and thus the wetability, of "home-made" thin papers of commercial bacterial cellulose for the concentrations tested (Figure 3.17; Figure 3.18). Additionally, it was verified that WAT of *Ct*CBM3$_{CipA}$-wt treated papers is slightly influenced by protein concentration (statistical differences between 0.2 mg/mL and 0.1 mg/mL were obtained, with P<0.05). Interestingly, at the lower concentration used (0.1 mg/mL), *Ct*CBM3$_{CipA}$-wt decreased 30.4% the WAT of treated papers. While the CBM appears to make the fiber more hydrophobic, demonstrating water-repellent properties, the glycosylation seems to revert that effect: i.e. it increases the fiber hydration, as suggested by Machado and colleagues (2009). The results show that C*t*CBM3$_{CipA}$-mut and C*t*CBM3$_{CipA}$-wt, for the same concentrations, modify

differently the WAT of treated papers (statistical differences at 0.8, 0.4 and 0.1 mg/mL, with $P<0.05$). Thus, glycosylation may be important for the effects of CBM3 on the surface properties of treated papers. However, the distinct effect of *Ct*CBM3$_{CipA}$-mut and *Ct*CBM3$_{CipA}$-wt on WAT of treated papers could also be related to the differences in their adsorption capacity to cellulose, as determined in section 3.5.2.

3.6.2. Effect of *Ct*CBM3$_{CipA}$-wt and *Ct*CBM3$_{CipA}$-mut on pulp drainability

The drainability is the measurable index of the refining degree of pulps, which is one of the most important stages in the paper production process. The Shopper-Riegler (°SR) number is a measurement of the drainability of a suspension of pulp in water. Pulp drainability decreases drastically with the refining process (°SR increases), because the fiber becomes more hydrated and fibrillated (Maximino et al., 2013; Pinto, 2006). The improvement of drainability may be significant for papermaking, allowing relevant energy savings. It is known that enzymes improve the pulps drainability (Bajpai, 2010; Bhardwaj et al., 1995; Jackson et al., 1993; Maximino et al., 2011; Pommier et al., 1989; Sarkar et al, 1995; Stork et al., 1995). Additionally, it has been demonstrated that CBMs, obtained by proteolytic digestion of the CBM-containing enzymes, are capable of significantly improving the drainability of paper pulps (Pala, et al., 2001; Pinto et al., 2004). However, the increased drainage may be also attributed to the residual hydrolytic activity present in the used CBM formulations.

In the present work, the effect of recombinant versions of *Ct*CBM3$_{CipA}$ on the drainability (Shopper-Riegler index, °SR) of bleached pulp of *E. globulus* and unbleached pulp of *E. globulus* and *P. sylvestris* (30:70) with different degrees of refining was analyzed (Table 3.2). Regardless of the variables studied, the recombinant CBMs did not have any effect on the pulps' drainability (Table 3.2).

Table 3.2 Shopper-Riegler index of the bleached *E. globulus* and unbleached *E. globulus* and *P. sylvestris* (30:70) fibers treated with *Ct*CBM3$_{CipA}$-mut and *Ct*CBM3$_{CipA}$-wt, and untreated fibers (control). The values represent the average ± SEM of two independent replicates.

Ratio (mg CBM/ g o.d. pulp)	Pulp	Control	*Ct*CBM3$_{CipA}$-mut	*Ct*CBM3$_{CipA}$-wt
2:1	*E. globulus* and *P. sylvestris* (30:70)	53.4 ± 0.6	53.3 ± 0.4	52.7 ± 0.7
	E. globulus	29.0 ± 0.0	28.5 ± 0.3	28.3 ± 0.3
4:1	*E. globulus*	22.8 ± 0.3	23.3 ± 0.3	23.0 ± 0.0

In a previous work, *Ct*CBM3$_{CipA}$ produced recombinantly from *E. coli* and used at a lower dosage than here (1 mg/g o.d. pulp) was also unable to modify *E. globulus* pulp drainability (Machado et al., 2009). However, when it was PEGylated, it led to a significant reduction of 15.7% in the °SR index for *E. globulus* pulp, thus increasing its drainability (Machado et al., 2009). The authors modify the CBM3, by pegylation, which as glycosylation is likely to be highly hydrated. Here, even after increasing the ratio of CBM3 in the pulp treatment (2 mg/g o.d. pulp and 4 mg/g o.d. pulp), the °SR index still did not change, showing that none of the recombinant versions of *Ct*CBM3$_{CipA}$ tested affected the drainability of the studied pulps, for the refining degrees used. It was surprising that the CBM3-mut did not change pulp drainability since it reduced significantly paper wetability (section 3.6.1). It is thought that water drainage is closely related with fibers hydration; a lower hydration, caused by CBM hidrophobicity, would lead to a negative effect on pulp drainage (Cadena et al., 2010; Machado et al., 2009). Additionally, no differences were detected between the pulps treated with *Ct*CBM3$_{CipA}$-mut and *Ct*CBM$_{CipA}$-wt, suggesting that glycosylation of CBM3 does not contribute to an improvement in pulp drainability.

3.6.3. Physico-mechanical properties of papersheets treated with *Ct*CBM3$_{CipA}$-wt and *Ct*CBM3$_{CipA}$-mut

The goal of this part of the study was to analyze the effect of *Ct*CBM3$_{CipA}$ on paper properties, namely bursting and tensile strengths, lengthening, tearing and air permeability. Previously, it was demonstrated that when *E. globulus* and *P. sylvestris* pulps were treated with 1 mg of *Ct*CBM3$_{CipA}$ per gram of fiber (o.d.), no effect on pulp and paper properties was observed (Machado et al., 2009). Thus, in this work the double amount of CBM was used (2 mg CBM/g

o.d.) in the treatment of two diferent pulps: a mixture of unbleached *E. globulus* and *P. sylvestris* pulp (30:70) and bleached *E. globulus* pulp. The properties of the resulting handsheets are shown in Figure 3.19.

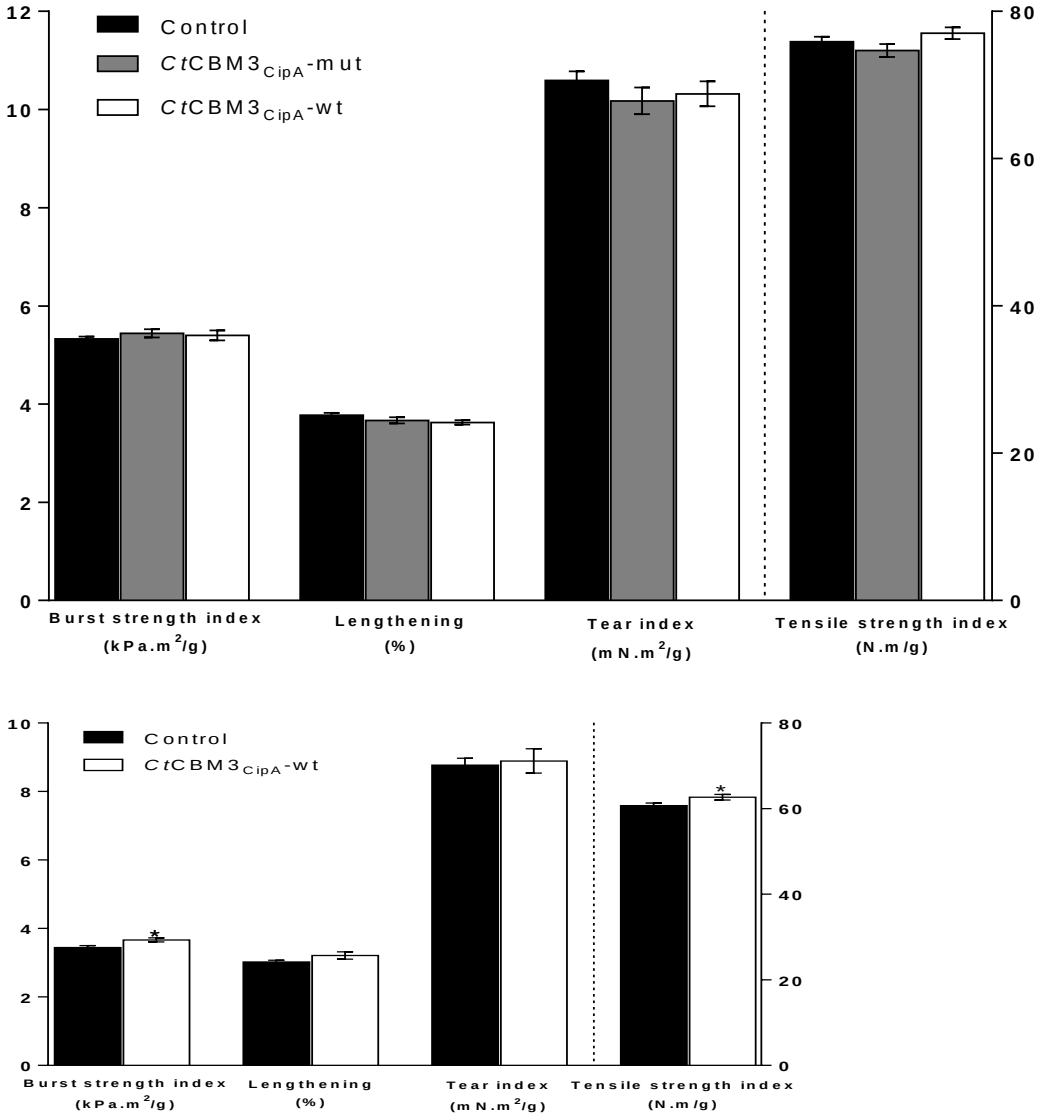

Figure 3.19 Physico-mechanical properties of untreated (control) and CtCBM3CipA-mut and CtCBM3CipA-wt treated (2 mg of CBM/g o.d) *E. globulus* and *P. sylvestris* (30:70) fibers (on top), and *E. globulus* fibers (below) (mean ± SEM). The statistical significance of the results in comparison with the control was determined by one-way ANOVA (Tukey's multiple comparisons test). *P<0.05.

The burst and tensile strength indexes of papersheets, obtained from *E. globulus* pulp treated with *Ct*CBM3$_{CipA}$-wt (2 mg/g o.d. pulp) were 6% and 3% higher than those of the control, respectively. CBMs did not have any effect on papersheets made of a mixture of *E. globulus* and *P. sylvestris* (30:70) probably due to its composition. The *P. sylvestris* fibers are predominant in

the mixture and the effect of the CBMs only on these fibers was not studied in this work. In addition, the mixture of pulps has some contaminants (including lignin), since it was not bleached, which can affect CBMs action.

Thereby, in order to enhance the effects on *E. globulus*, the amounts of *Ct*CBM3$_{CipA}$-mut and *Ct*CBM3$_{CipA}$-wt were duplicated again (4 mg/g o.d. pulp). The results obtained are presented in Figure 3.20.

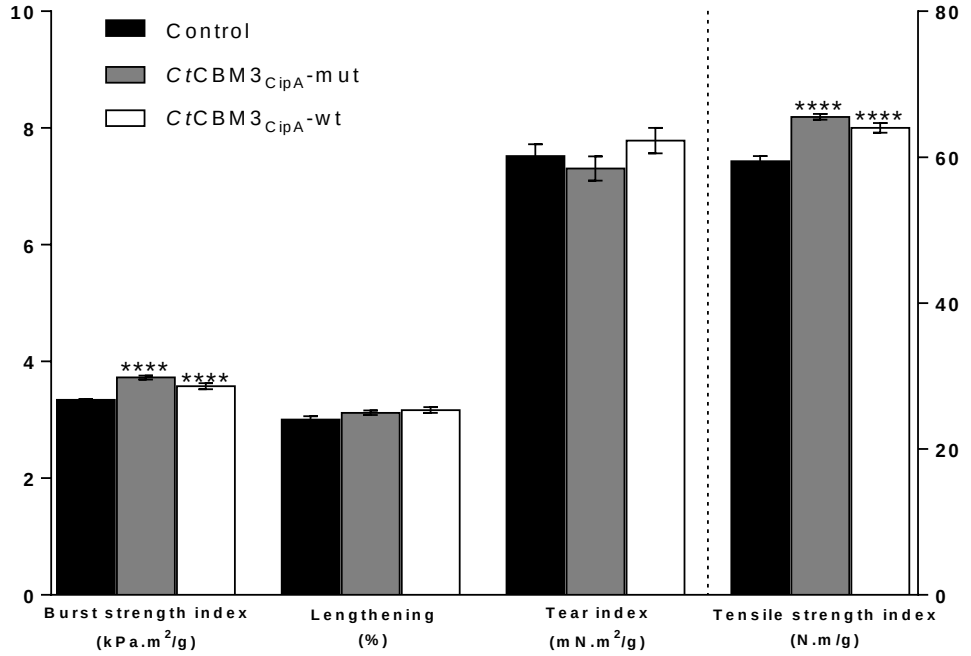

Figure 3.20 Physico-mechanical properties of untreated (control) and *Ct*CBM3$_{CipA}$-mut and *Ct*CBM3$_{CipA}$-wt treated (4 mg of CBM/g o.d) *E. globulus* fibers (mean ± SEM). The statistical significance of the results in comparison with the control was determined by one-way ANOVA (Tukey's multiple comparisons test). **** P<0.0001.

At this ratio, both recombinant versions of *Ct*CBM3$_{CipA}$ highly improved the burst and tensile strength indexes of papersheets made of *E. globulus* pulp (Figure 3.20), suggesting that the modification of these properties by recombinant versions of *Ct*CBM3$_{CipA}$ is dependent on its concentration. The higher ratio of *Ct*CBM3$_{CipA}$ (4 mg/g o.d. pulp) significantly improved the mechanical properties of papersheets, whereas a lower ratio (2 mg/g o.d. pulp) produced only a small effect. On the other hand, lengthening and tear index was not significantly (P>0.05) affected by the recombinant versions of *Ct*CBM3$_{CipA}$ treatment.

The results observed for burst and tensile strength indexes after *Ct*CBM3$_{CipA}$-mut treatment were similar to those of the *Ct*CBM3$_{CipA}$-wt. However, the effect of *Ct*CBM3$_{CipA}$-mut treatment was more evident. The burst strength of the papersheets made of *E. globulus* pulp treated with *Ct*CBM3$_{CipA}$-mut was approximately 12% greater than the papersheets made of untreated pulp, whereas the effect of *Ct*CBM3$_{CipA}$-wt was significantly smaller ($P<0.05$), resulting in only a 7% increase. The tensile strength of paper is highly influenced by its intrinsic fiber strength, as well as by the strength and amount of the fiber-fiber bonds (Cadena et al., 2010; Roberts et al., 1996). Accordingly, it was established that most of the fiber strength is obtained by the bonding that exists between the individual fibers (Spence 1987; Xu and Yang 1999). This intrafiber bonding improves the stress transfer between the fibers and it is considered to be one of the most important factors affecting the stress development in the fiber web under tensile deformation (Askling et al. 1998; Gassan and Bledzki 1998). Papersheets made of *E. globulus* pulp treated with *Ct*CBM3$_{CipA}$-mut and *Ct*CBM3$_{CipA}$-wt significantly increased their tensile strength over the papersheets made of untreated pulp, in about 10% and 8%, respectively. These results suggest that *Ct*CBM3$_{CipA}$ can crosslink between *E. globulus* fibers in the cellulose web. Paper tensile strength enhancement has also been reported in other works using other CBM-based approaches (Cadena et al., 2010; Kitaoka et al., 2001; Levy et al., 2002; Levy et al., 2004; Shi et al., 2014).

Papersheets made of *E. globulus* pulp treated with *Ct*CBM3$_{CipA}$ demonstrated a decrease in their permeability (Table 3.3), thereby substantially reducing their air resistance.

Table 3.3 Permeability of the papersheets from bleached *E. globulus* and unbleached mixture of *E. globulus* and *P. sylvestris* (30:70) fibers treated with *Ct*CBM3$_{CipA}$-mut and *Ct*CBM3$_{CipA}$-wt, and untreated fibers (control). The values represent the average ± SEM of two independent assays.

Ratio (mg CBM/ g o.d. pulp)	Refining (°SR)	Pulp	Permeability (mL/min)		
			Control	CtCBM3$_{CipA}$-mut	CtCBM3$_{CipA}$-wt
2:1	53.4 ± 0.6	*E. globulus* and *P. sylvestris* (30:70)	122.1 ± 1.0	123.6 ± 4.0	118.3 ± 4.1
	29.0 ± 0.0	*E. globulus*	1265.0 ± 15.5^{a}	N.A.	1221.3 ± 11.4^{b}
4:1	22.8 ± 0.3	*E. globulus*	1903.8 ± 12.1^{a}	1748.8 ± 14.6^{c}	1802.5 ± 30.5^{d}

Values in a row followed by a different letter superscript differed significantly with $P<0.05$ (a,b), $P<0.01$ (a,d) and $P<0.0001$ (a,c). N.A: not assayed.

The permeability of papersheets made of *E. globulus* pulp treated with *Ct*CBM3$_{CipA}$-mut and *Ct*CBM3$_{CipA}$-wt differed from the untreated pulp (control) by 8% and 5%, respectively. This result was expected since both recombinant *Ct*CBM3$_{CipA}$ versions reduced significantly the strength of papersheets. It is known that increasing bonding (to promote strength and stiffness) causes fibrillation and a larger surface area, and reduces air permeability (Biermann, 1996).

The air permeability of papersheets is an important parameter for paper industry, since it is related to the ability to remove water during the paper drying step, and consequently contribute to a more economical drying process. Thereby, these results suggest that the application of *Ct*CBM3$_{CipA}$ versions to *E. globulus* pulp can negatively affect the process, as lower permeability implies higher drying times and heat consumption, a slower production rate, and therefore higher production costs. On the other hand, more resistant papers can be generated by pulp treatment with recombinant versions of *Ct*CBM3$_{CipA}$.

CHAPTER 4

Conclusions and Future Remarks

This work consisted in the production of recombinant CBMs from *Trichoderma reesei* and *Clostridium thermocellum* in *Pichia pastoris*, and their application in the modification of paper fibers. The results obtained lead to the following conclusions:

- Recombinant family 1 CBM from *T. reesei* Cel7A (cellobiohydrolase I), fused to eGFP, without (eGFP-*Tr*CBM1$_{Cel7A}$) and with its native linker (eGFP-NL-*Tr*CBM1$_{Cel7A}$) were functionally produced in *P. pastoris*, but intracellularly accumulated;
- Recombinant family 3 CBM from *C. thermocellum* CipA scaffold protein, glycosylated (*Ct*CBM3$_{CipA}$-wt) and non-glycosylated (*Ct*CBM3$_{CipA}$-mut) versions, were produced in *P. pastoris* as secreted proteins;
- Glycosylation modified the *Ct*CBM3$_{CipA}$ affinity to cellulose. Recombinant *Ct*CBM3$_{CipA}$-wt bound to Avicel, nevertheless, with less affinity than the *Ct*CBM3$_{CipA}$-mut;
- The adsorption of both recombinant versions of *Ct*CBM3$_{CipA}$ on *E. globulus* fibers was heterogeneous. Fluorescence microscopy images showed that these CBMs were preferentially concentrated around the fiber extremities and in some middle regions;
- Recombinant non-glycosylated *Ct*CBM3$_{CipA}$ modified the wetability of cellulose, while the glycosylated version had no significant effect on this property. Treatment of "home-made" bacterial cellulose thin papers with *Ct*CBM3$_{CipA}$-mut resulted in a significant surface hydrophobicity increase;
- Both recombinant versions of *Ct*CBM3$_{CipA}$ were unable to modify the drainability of paper pulps (unbleached *Eucaliptus globulus* and *Pinus sylvestris* (30:70) pulp and bleached *E. globulus* pulp), for the conditions tested;
- Treatment of *E. globulus* pulp with recombinant *Ct*CBM3$_{CipA}$ modified the physico-mechanical properties of papersheets. The burst and tensile strength indexes of papersheets made of *E. globulus* pulp treated with both recombinant versions of

*Ct*CBM3$_{CipA}$ were significantly increased. A significant decrease in air permeability of papersheets after *Ct*CBM3$_{CipA}$ pulp treatment was also observed;

- The modification of the surface/interface properties of cellulose by recombinant *Ct*CBM3$_{CipA}$ is dependent on its concentration. The wetability, burst and tensile strength indexes were improved when higher concentrations were applied;
- Glycosylation was not important for the effect of *Ct*CBM3$_{CipA}$ on paper properties. Its presence did not enhance the effect of recombinant *Ct*CBM3$_{CipA}$.

In summary, the *Ct*CBM3$_{CipA}$ adsorption leads to a change on the surface properties of cellulose fibers, as revealed by the change in wettability, burst and tensile strengths and air permeability of papersheets. Thus, this CBM has potential to be used in the paper industry for the enhancement of paper properties. Nevertheless, a better understanding of the effects of CBMs on the surface and structural properties of cellulose fibers is required.

As future work it is proposed:

- Replacement of the fusion partner of *Tr*CBM1$_{Cel7A}$ or test a different fusion order, i.e. *Tr*CBM1$_{Cel7A}$-eGFP (eGFP placed at the C-terminal of the CBM instead at the N-terminal), for the efficient extracellular production of *Tr*CBM1$_{Cel7A}$ in *P. pastoris*;
- Application of other recombinant glycosylated CBMs (namely, recombinant *Tr*CBM1$_{Cel7A}$) in fiber modification. Recombinant glycosylated-engineered CBMs with different glycosylation levels can also be developed;
- Perform an economic study to address the feasibility of application of the recombinant CBMs obtained in this work in the paper industry;
- Use the recombinant DNA technology to fuse the *Ct*CBM3$_{CipA}$ with bioactive peptides, for the introduction of bioactive properties on paper (development of bioactive papers).

References

Abbott, D. W., Eirín-López, J. M., Boraston, A. B. (2008). Insight into ligand diversity and novel biological roles for family 32 carbohydrate-binding modules. Mol Biol Evol, 25: 155-167.

Ahmad, M., Hirz, M., Pichler, H., Schwab, H. (2014). Protein expression in *Pichia pastoris*: recent achievements and perspectives for heterologous protein production. Appl Microbiol Biotechnol, 98: 5301-5317.

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. Biotechnol. Prog., 14: 350–354.

Andrade, F. K., Moreira, S. M., Domingues, L., Gama, F. M. (2009). Improving the affinity of fibroblasts for bacterial cellulose using carbohydrate-binding modules fused to RGD. J. Biomed. Mater. Res. A.

Askling, C., Wagberg, L., Rigdahl, M. (1998). The effect of additives on the mechanical properties of dry-formed fiber networks. J. Mater. Sci., 33: 1997–2003.

Bajpai, P. (2010). Solving the problems of recycled fiber processing with enzymes. BioResources, 5(2): 1311-1325.

Bajpai, P. (2012). Biotechnology for Pulp and Paper Processing. Brief Description of the Pulp and Paper Making Process. Chapter 2: 7-14.

Bayer, E. A., Kenig, R., Lamed, R. (1983). Adherence of *Clostridium thermocellum* to cellulose. J. Bacteriol., 156: 818-827.

Bayer, E. A., Chanzy, H., Lamed, R., Shoham. Y. (1998). Cellulose, cellulases and cellulosomes. Current Opinion in Structural Biology, 8: 548-557.

Bayer, E. A., Shoham. Y., Lamed, R. (2013). Lignocellulose-Decomposing Bacteria and Their Enzyme Systems. The Procaryotes, 215-266.

Bhardwaj, N. K., Bajpai, P., Bajpai, K. (1995). Use of enzymes to improve drainability of secondary fibers. Appita, J., 48(5): 378-380.

Beckham, G. T., Matthews, J. F., Bomble, Y. J., Bu, L., Adney, W. S., Himmel, M. E., Nimlos, M. R., Crowley, M. F. (2010). Identification of amino acids responsible for processivity in a Family 1 carbohydrate-binding module from a fungal cellulase. J. Phys. Chem. B, 114: 1447–1453.

Beldman, G., Voragen, A.G., Rombouts, F. M., Searle-van Leeuwen, M. F., Pilnik, W. (1987). Adsorption and kinetic behavior of purified endoglucanases and exoglucanases from *Trichoderma viride*, Biotechnol. Bioeng., 30: 251–257.

Belgacem, M. N., Czeremuszkin, G., Sapieha, S., Gandini, A. (1995). Surface characterization of cellulose fibres by XPS and inverse gas chromatography. Cellulose, 2: 145-157.

Biermann, C. J. (1996). Wood and fiber fundamentals. In: Handbook of pulping and papermaking. Academic Press, San Diego, pp 754.

Black, G. W., Rixon, J. E., Clarke, J. H., Hazlewood, G. P., Ferreira, A., Bolam, D. N., Gilbert, H. J. (1997). Cellulose binding domains and linker sequences potentiate the activity of hemicellulases against complex substrates. Journal of Biotechnology, 57: 59-69.

Bolam, D. N., Ciruela, A., McQueen-Mason, S., Simpson, P., Williamson, M. P., Rixon, J.E., Boraston, A., Hazlewood, G. P., Gilbert, H. J. (1998). *Pseudomonas* cellulose-binding domains mediate their effects by increasing enzyme substrate proximity. Biochem. J. 331:775–781.

Boraston, A. B., McLean, B. W., Guarna, M. M., Amandaron-Akow, E., Kilburn, D. G. (2001a). A family 2a carbohydrate-binding module suitable as an affinity tag for proteins produced in *Pichia pastoris*. Protein Expr Purif 21: 417-423.

Boraston, A. B., Warren, R. A. J.., Kilburn, D. G. (2001b). Glycosylation by *Pichia pastoris* decreases the affinity of a family 2a carbohydrate-binding module from *Cellulomonas fimi*: a functional and mutational analysis. Biochem. J., 358: 423-430.

Boraston, A. B., Kwan, E., Chiu, P., Warren, R. A., Kilburn, D. G. (2003). Recognition and hydrolysis of noncrystalline cellulose. J Biol Chem 278: 6120-6127.

Boraston, A. B., Bolam, D. N., Gilbert, H. J., Davies, G. J. (2004). Carbohydrate-binding modules: fine-tuning polysaccharide recognition. Biochem. J. 382: 769-781.

Boraston A. B., Ficko-Blean E., Healey M. (2007). Carbohydrate recognition by a large sialidase toxin from *Clostridium perfringens*. Biochemistry 46: 11352-11360.

Bothwell, M. K., Daughhetee, S. D., Chaua, G. Y., Wilson, D. B., Walker, L. P. (1997). Binding capacities for *Thermomonospora fusca* E3, E4 and E5, the E3 binding domain, and *Thichoderma reesei* CBHI on avicel and bacterial microcrystalline cellulose. Bioresource Technol., 60: 169-178.

Cadena, E. M., Chriac, A. I., Pastor, F.I., Diaz, P., Vidal, T., Torres, A. L. (2010). Use of cellulases and recombinant cellulose binding domains for refining TCF kraft pulp. Biotechnol Prog., 26: 960-967.

Carrard, G. and Linder, M. (1999). Widely different off rates of two closely related cellulose-binding domains from *Trichoderma reesei*, Eur. J. Biochem., 262: 637–643.

Carrard, G., Koivula, A., Soderlund, H., Béguin, P. (2000). Cellulose-binding domains promote hydrolysis of different sites on crystalline cellulose. Proc. Natl. Acad. Sci., 97: 10342-10347.

Carvalho, V., Domingues, L., Gama, M. (2008). The Inhibitory Effect of an RGD-Human Chitin-Binding Domain Fusion Protein on the Adhesion of Fibroblasts to Reacetylated Chitosan Films. Mol Biotechnol., 40: 269-279.

Cavaco-Paulo, A. (1998). Mechanism of cellulase action in textile processes. Carbohydr. Polymers, 37: 273-277.

Cereghino, J. L. and Cregg, J. M. (2000). Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. FEMS Microbiol. Rev., 24: 45-66.

Cereghino, G. P., Cereghino, J. L., Ilgen, C., Cregg, J. M. (2002). Production of recombinant proteins in fermenter cultures of the yeast *Pichia pastoris*. Current Opinion in Biotechnology, 13: 329-332.

Cha, H. J., Wu, C. F., Valdes, J. J., Rao, G., Bentley, W. E. (2000). Observations of green fluorescent protein as a fusion partner in genetically engineered Escherichia coli: monitoring protein expression and solubility. Biotechnol. Bioeng., 67(5): 565–574.

Chae, H. J., DeLisa, M. P., Cha, H. Y., Weingand, W. A., Rao, G., Bentley, W. E. (2000). Framework for on-line optimisation of recombinant protein expression in high cell density *Escherichia coli* cultures using GFP-fusion monitoring. Biotechnol. Bioeng, 69(3): 275–285.

Chalfie, L., Tu, Y., Euskirchen, G., Ward, W. W., Prasher, D. C. (1994). Green fluorescent protein as a marker for gene expression. Science 263, 802–805.

Cheng, L., Fu, J., Tsukamoto, A., Hawley, R. G. (1996). Use of green fluorescent protein variants to monitor gene transfer and expression in mammalian cells. Nature Biotechnol, 14: 351–354.

Cheung, R. C., Wong, J. H., Ng, T., B. (2012). Immobilized metal ion affinity chromatography: a review on its applications. Appl Microbiol Biotechnol, 96:1411–1420.

Clare, J. J., Rayment, F. B., Ballantyne, S. P., Sreerkrishna, K., Romanos, M. A. (1991). High-level expression of tetanus toxin fragment C in *Pichia pastoris* strains containing multiple tandem integrations of the gene. BioTechnology, 9: 455–460.

Cos, O., Ramón, R., Montesinos, J., Valero, F. (2006). Operational strategies, monitoring and control of heterologous protein production in the methylotrophic yeast *Pichia pastoris* under different promoters: A review. Microbial Cell Factories, 5: 17.

Cregg, J. M. (2000). Expression in the Methylotrophic yeast *Pichia pastoris*. In Gene Expression Systems: Using Nature for the Art of Expression (Fernandez, J. M. and Hoeffler, J. P., eds.), Academic Press, San Diego, CA, pp. 157–191.

Dagel, D. J., Liu, Y. S., Zhong, L., Luo, Y., Himmel, M. E., Xu, Q., Zeng, Y., Ding, S. Y., Smith, S. (2011). In situ imaging of single carbohydrate-binding modules on cellulose microfibrils. J Phys Chem B, 115: 635–641.

Daly, R. and Hearn, M. (2005). Expression of heterologous proteins in *Pichia pastoris*: a useful experimental tool in protein engineering and production. J. Mol. Recognit., 18: 119-138.

DeLisa, M. P., Li, J., Rao, G., Weingand, W. A., Bentley, W. E. (1999). Monitoring GFP-fusion protein expression during high cell density cultivation of *Escherichia coli* using an on-line optical sensor. Biotechnol. Bioeng., 65(1): 54–64.

Demain, A. L. and Vaishnav, P. (2009). Production of recombinant proteins by microbes and higher organisms. Biotechnology Advances, 27: 297-306.

Din, N., Gilkes, N. R., Tekant, B., Miller Jr, R. C., Warren, R. A. J., Kilburn, D. G. (1991). Non-Hydrolytic Disruption of Cellulose Fibres by the Binding Domain of a Bacterial Cellulase. Bio/Technology, 9: 1096-1099.

Ding, S.-Y., Xu, Q., Ali, M. K., Baker, J. O., Bayer, E. A., Barak, Y., Lamed, R., Sugiyama, J., Rumbles, G., Himmel, M. E. (2006). Versatile derivatives of carbohydrate-binding modules for imaging of complex carbohydrates approaching the molecular level of resolution. BioTechniques, 41: 435-443.

Divne, C., Ståhlberg, J., Reinikainen, T., Ruohonen, L., Pettersson, G., Knowles, J. K., Teeri, T. T., Jones, T. A. (1994). The three-dimensional crystal structure of the catalytic core of cellobiohydrolase I from *Trichoderma reesei*. Science, 265: 524–528.

Divne, C., Ståhlberg, J., Teeri, T. T., Jones, T. A. (1998). High-resolution crystal structures reveal how a cellulose chain is bound in the 50-Å long tunnel of cellobiohydrolase I from *Trichoderma reesei*. J. Mol. Biol., 275: 309–325.

Eckart, M. R., Bussineau, C. M. (1996). Quality and authenticity of heterologous proteins synthesized in yeast. Curr Opin Biotechnol, 7: 525–530.

Eriksson, K. E. L., Blanchette, R. A., Ander, P. (1990). Microbial and enzymatic degradation of wood and wood components, Springer, Berlin Heidelberg New York.

Gassan, J. and Bledzki, A. K. (1998). Alkali treatment of lute fibers: relationship between structure and mechanical properties. J. Appl. Polym. Sci., 71: 623–629.

Gasser, B., Prielhofer, R., Marx, H., Maurer, M., Nocon, J., Steiger, M., Puxbaum, V., Sauer, M., Mattanovich, D. (2013). *Pichia pastoris*: protein production host and model organism for biomedical research. Future Microbiol, 8(2): 191-208.

Gilkes, N., Warren, R. A., Miller, R. J., Kilburn, D. G. (1988). Precise excision of the cellulose binding domains from two *Cellulomonas fimi* cellulases by a homologous protease and the effect on catalysis. J. Biol. Chem. 263:10401–10407.

Gregg, K. J., Finn, R., Abbott, D. W., Boraston, A. B. (2008). Divergent modes of glycan recognition by a new family of carbohydrat-binding modules. J Biol Chem, 283: 12604-12613.

Grinna, L. S., Tschopp, J. F. (1989). Size distribution and general structural features of N-linked oligosaccharides from the methylotrophic yeast, *Pichia pastoris*. Yeast, 5: 107–115.

Guerreiro, C. I., Fontes, C. M., Gama, F. M., Domingues, L. (2008). *Escherichia coli* expression and purification of four antimicrobial peptides fused to a family 3 carbohydrate-binding module (CBM) from *Clostridium thermocellum*. Protein Expression and Purification, 59: 161-168.

Guillén, D., Sánchez, S., Rodríguez-Sanoja, R. (2010). Carbohydrate-binding domains: multiplicity of biological roles. Appl Microbiol Biotechnol, 85:1241–1249.

Guo, J., Catchmark, J. (2013). Binding Specificity and Thermodynamics of Cellulose-Binding Modules from Trichoderma reesei Cel7A and Cel6A. Biomacromolecules, 14: 1268-1277.

Gustavsson, M., Lehtio, J., Denman, S., Teeri, T., Hult, K., Martinelle, M. (2001). Stable linker peptides for a cellulose-binding domain–lipase fusion protein expressed in *Pichia pastoris*. Protein Eng., 14: 711-7115.

Hall, J., G. W. Black, L. M. Ferreira, S. J. Millward-Sadler, B. R. Ali, G. P. Hazlewood, H. J. Gilbert. (1995). The non-catalytic cellulose-binding domain of a novel cellulase from *Pseudomonas fluorescens* subsp. Cellulose is important for the efficient hydrolysis of Avicel. Biochem. J., 309: 749–756.

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*. Eur. J. Biochem., 256: 119-127.

Hashimoto, H. (2006). Recent structural studies of carbohydrate-binding modules. Cell Mol Life Sc, 63: 2954–2967.

Hildén, L., Daniel, G., Johansson, G. (2003). Use of a fluorescence labelled, carbohydrate- binding module from *Phanerochaete chrysosporium* Cel7D for studying wood cell wall ultrastructure. Biotechnol. Lett., 25: 553-558.

Hohenblum, H., Gasser, B., Maurer, M., Borth, N., Mattanovich, D. (2004). Effects of gene dosage, promoters, and substrates on unfolded protein stress of recombinant Pichia pastoris. Biotechnology and Bioengineering, 85: 367-375.

Hong, J., Ye, X., Zhang, P. (2007). Quantitative Determination of Cellulose Accessibility to Cellulase Based on Adsorption of a Nonhydrolytic Fusion Protein Containing CBM and GFP with Its Applications. Langmuir, 23: 12535-12540.

Hong, J., Yea, X., Wanga, Y., Zhang, P. (2008). Bioseparation of recombinant cellulose-binding module-proteins by affinity adsorption on an ultra-high-capacity cellulosic adsorbent. Analytica Chimica Acta, 621: 193–199.

Humphrey, W., Dalke, A., Schulten, K. (1996). VMD, visual molecular dynamics. J. Mol. Graph., 14: 33–8, 27–8.

Hwang, S., Ahn, J., Lee, S., Lee, T., Haam, S., Lee, K., Ahn, I., Jung, J. (2004). Evaluation of cellulose-binding domain fused to a lipase for the lipase immobilization. Biotechnol. Lett., 26: 603-605.

Jackson, L. S., Heitmann, J. A., Joyce, T. W. (1993). Enzymatic modification of secondary fiber. Tappi J., 76(3): 147-154.

Jahic, M. (2003). Process techniques for production of recombinant proteins with *Pichia pastoris*. Royal Institute of Technology. Stockholm, Sweden.

Jahic, M., Veide, A., Charoenrat, T., Teeri, T., Enfors, S. (2007). Process technology for production and recovery of heterologous proteins with *Pichia pastoris.* Biotechnology Progress, 23(2): 516.

Jones, J. J., Bridges, A. M., Fosberry, A. P., Gardner, S., Lowers, R. R., Newby, R. R., James, P. J., Hall, R. M., Jenkins, O. (2004). Potential of real-time measurement of GFP-fusion proteins. J Biotechnol, 109(1-2): 201-211.

Kallas, A. (2006). Heterologous expression, characterization and applications of carbohydrate active enzymes and binding modules. Royal Institute of Technology, Stockholm (Sweden).

Kauffmann, C., Shoseyov, O., Shpigel, E., Bayer, E. A., Lamed, R., Shoham, Y., Mandelbaum, R. T. (2000). A novel methodology for enzymatic removal of atrazine from water by CBD-fusion protein immobilized on cellulose. Environ. Sci. Technol., 34: 1292-1296.

Kavoosi, M., Meijer, J., Kwan, E., Creagh, A., Kilburn, D., Haynes, C. (2004). Inexpensive one-step purification of polypeptides expressed in *Escherichia coli* as fusions with the family 9 carbohydrate-binding module of xylanase 10A from *T. maritima*. J. Chromatogr. B, 807: 87-94.

Kimura, S., Itoh, T. (2004). Cellulose synthesizing terminal complexes in the ascidians. Cellulose, 11: 377-383.

Kraulis, J., Clore, G. M., Nilges, M., Jones, T. A., Pettersson, G., Knowles, J., Gronenborn, A. M. (1989). Determination of the three-dimensional solution structure of the C-terminal domain of cellobiohydrolase I from *Trichoderma reesei*. A study using nuclear magnetic resonance and hybrid distance geometry-dynamical simulated annealing. Biochemistry, 28: 7241–7257.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227:680.

Lamed, R., Naimark, J., Morgenstern, E., Bayer, E. A. (1987). Specialized cell surface structures in cellulolytic bacteria. J. Bacterial., 169: 3792-3800.

Langsford, M. L., Gilkes, Singh, B., Moser, B., Miller, R. C., Warren, R. A., Kilburn, D. G. (1987). Glycosylation of bacterial cellulases prevents proteolytic cleavage between functional domains. FEBS Lett., 225: 163–167.

Lethio, J., Wernerus, H., Samuelson, P., Teeri, T., Stahl, S. (2001). Directed immobilization of recombinant staphylococci on cotton fibers by functional display of a fungal cellulose-binding domain. FEMS Microbiol. Lett., 195: 197-204.

Lethio, J., Sugiyama, J., Gustavsson, M., Fransson, L., Linder, M., Teeri, T. (2003). The binding specificity and affinity determinants of family 1 and family 3 cellulose binding modules. Proc. Natl. Acad. Sci., 100: 484-489.

Lemos M. A., Teixeira J. A., Mota M., Gama F. M. (2000). A simple method to separate cellulose-binding domains of fungal cellulases after digestion by a protease. Biotech Lett, 22: 70-707.

Levy, I., Nussinovitch, A., Shpigel, E., Shoseyov, O. (2002). Recombinant cellulose crosslinking protein: a novel papermodification biomaterial. Cellulose, 9: 91-98.

Levy, I., Paldi, T., Siegel, D., Shoseyov, O. (2003). Cellulose binding domain from *Clostridium cellulovorans* as a paper modification reagent. Nordic Pulp Paper Res. J., 18: 421–428.

Levy, I., Paldi, T., Shoseyov. O. (2004). Engineering a bifunctional protein that cross-bridges between starch and cellulose. Biomaterials, 25: 1841–1849.

Li, P., Anumanthan, A., Gao, X., Iangovan, K., Suzara, V., Düzgüne, N., Renugopalakrishnan, V. (2007). Expression of Recombinant Proteins in *Pichia Pastoris*. Appl Biochem Biotechnol, 142: 105-124.

Lin, S., Lin, I., Chou, W., Hsieh, C., Liu, S., Huang, R., Sheu, C., Chang, M. (2009). CBM21 starch-binding domain: A new purification tag for recombinant protein engineering. Protein Expression and Purification, 65: 261-266.

Linder, M., Lindeberg, G., Reinikainen, T., Teeri, T. T., Pettersson, G. (1995). The difference in affinity between two fungal cellulose-binding domains is dominated by a single amino acid substitution. FEBS Lett., 372: 96–98.

Linder, M., Salovuori, I., Ruohonen, L., Teeri, T. (2006). Characterization of a Double Cellulose-binding Domain. Synergistic high affinity binding to crystalline cellulose. The journal of Biological Chemistry, 271(35): 21268–21272.

Linder, M., Teeri, T. T. (1997). The roles and function of cellulose-binding domains. Journal of Biotechnology, 57: 15-28.

Linder, M., Nevanen, T., Soderholm, L., Bengs, O., Teeri, T. (1998). Improved immobilization of fusion proteins via cellulose-binding domains, Biotechnol. Bioeng., 60: 642–647.

Lymar, E., Li, B., Renganathan, V. (1995). Purification and characterization of a cellulose-binding beta-glucosidase from cellulose-degrading cultures of *Phanerochaete chrysosporium*, Appl. Environ. Microbiol., 61: 2976–2980.

Lynd, L., Weimer, P., van Zyl, W., Pretorius, I. (2002). Microbial Cellulose Utilization: Fundamentals and Biotechnology. Microbiol. Mol. Biol. Rev., 66(3): 506.

Macauley-Patrick, S., Fazenda, M., McNeil, B., Harvey, L. (2005). Heterologous protein production using the *Pichia pastoris* expression system. Yeast, 22: 249–270.

Machado, J., Araújo, A., Pinto, R., Gama, F. M. (2009). Studies on the interaction of the Carbohydrate Binding Module 3 from the *Clostridium thermocellum* CipA scaffolding protein with cellulose and paper fibres. Cellulose, 16: 817-824.

Maximino, M. G., Taleb, M. C., Adell, A. M., Formento, J. C. (2011). Application of hydrolytic enzymes and refining on recycled fibers. Cell. Chem. Tech., 45(5-6): 397-403.

Maximino, M. G., Taleb, M. C., Adell, A. M. (2013). Influence of the enzyme addition point on recycled industrial pulp properties. BioResources, 8(1): 1089-1099.

McCartney, L., Gilbert, H. J., Bolam, D. N., Boraston, A. B., Knox, J. P. (2004). Glycoside hydrolase carbohydrate-binding modules as molecular probes for the analysis of plant cell wall polymers. Anal. Biochem., 326: 49-54.

McCartney, L., Blake, A., Flint, J., Bolam, D., Boraston, A. B., Gilbert, H., Knox, J. (2006). Differential recognition of plant cell walls by microbial xylan-specific carbohydrate-binding modules. PNAS, 103: 4765–4770.

Miele, R. G., Nilsen, S. L., Brito, T., Bretthauer, R. K., Castellino, F. J. (1997). Glycosylation properties of the *Pichia pastoris*-expressed recombinant kringle 2 domain of tissue-type plasminogen activator. Biotechnol. Appl. Biochem, 25: 151–157.

Moller, I., Sørensen, I., Bernal, A. J., Blaukopf, C., Lee, K., Øbro, J., Pettolino, F., Roberts, A., Mikkelsen, J., Knox, J. P. (2007). High-throughput mapping of cell wall polymers within and between plants using novel microarrays. Plant J, 50: 1118-1128.

Montanier, C., van Bueren, A. L., Dumon, C., Flint, J. E., Correia, M. A., Prates, J. A., Firbank, S. J., Lewis, R. J., Grondin, G. G., Ghinet, M. G., Gloster, T. M., Herve, C., Knox, J. P., Talbot, B. G., Turkenburg, J. P., Kerovuo, J., Brzezinski, R., Fontes, C. M., Davies, G. J., Boraston, A. B., Gilbert, H. J. (2009). Evidence that family 35 carbohydrate binding modules display conserved specificity but divergent function. Proc Natl Acad Sci, 106: 3065-3070.

Morag, E., Lapidot, A., Govorko, D., Lamed, R., Wilchek, M., Bayer, E., Shoham, Y. (1995). Expression, Purification, and Characterization of the Cellulose-Binding Domain of the Scaffoldin Subunit from the Cellulosome of *Clostridium thermocellum*. Applied and Environmental Microbiology, 61(5): 1980–1986.

Moreira, S. M., Andrade, F. K., Domingues, L., Gama, M. (2008). Development of a strategy to functionalize a dextrin-based hydrogel for animal cell cultures using a starch-binding module fused to RGD sequence, BMC Biotechnol., 8:78.

Moreira, S. M., Gama, M. (2009). Carbohydrate Binding Modules: Functions and Applications. Biotechnology in Agriculture, Industry and Medicine Series. Nova Science publishers, Inc. New York. Chapter 4: 11-15.

Nagy, T., Simpson, P., Williamson, M. P., Hazlewood, G. P., Gilbert, H. J., Orosz, L. (1998). All three surface tryptophans in Type IIa cellulose binding domains play a pivotal role in binding both soluble and insoluble ligands. FEBS Lett, 429: 312– 6.

Najmudin, S., Guerreiro, C., Ferreira, L., Romão, M., Fontes, C., Prates, J. (2005). Overexpression, purification and crystallization of the two C-terminal domains of the bifunctional cellulase ctCel9D-Cel44A from *Clostridium thermocellum*. Acta Crystallographica, F61: 1043-1045.

Nam, J., Fujita, Y., Arai, T., Kondo, A., Morikaw, Y., Okada, H., Ueda, M., Tanaka, A. (2002). Construction of engineered yeast with the ability of binding to cellulose. J. Mol. Catalys., 17: 197-202.

Nimlos, M., Beckham, G., Matthews, J., Bu, L., Himmel, M., Crowley, M. (2012). Binding Preferences, Surface Attachment, Diffusivity, and Orientation of a Family 1 Carbohydrate-binding Module on Cellulose. J. Biol. Chem., 287: 20603-20612.

Nogawa, M., Goto, M., Okada, H., Morikawa, Y. (2001). L-Sorbose induces cellulase gene transcription in the cellulolytic fungus *Trichoderma reesei*. Curr Genet, 38: 329-334.

Notenboom, V., Boraston, A. B., Kilburn, D. G., Rose, D. R. (2001). Crystal structures of the family 9 carbohydrate-binding module from *Thermotoga maritima* xylanase 10A in native and ligand-bound forms. Biochemistry, 40: 6248-6256.

Otter, D. E., Munro, P. A., Scott, G. K., Geddes, R. (1989). Desorption of *Trichoderma reesei* cellulase from cellulose by a range of desorbents, Biotechnol. Bioeng., 34: 291–298.

Pala, H., Lemos, M. A., Mota, M., Gama, F. M. (2001). Enzymatic upgrade of old paperboard containers. Enzyme Microb Technol, 29: 274–279.

Pértile, R., Moreira, S., Andrade, F., Domingues, L., Gama, M. (2012). Bacterial Cellulose Modified Using Recombinant Proteins to Improve Neuronal and Mesenchymal Cell Adhesion. Biotechnol. Prog., 28: 526-532.

Phitsuwan, P., Laohakunjit, N., Kerdchoechuen, O., Kyu, K., Ratanakhanokchai, K. (2013). Present and potential applications of cellulases in agriculture, biotechnology, and bioenergy. Folia Microbiol, 58: 163–176.

Pinto, R., Moreira, S., Mota, M., Gama, M. (2004). Studies on the Cellulose-Binding Domains Adsorption to Cellulose. Langmuir, 20: 1409-1413.

Pinto, R., Carvalho, J., Mota, M., Gama, M. (2006). Large-scale production of cellulose-binding domains. Adsorption studies using CBM-FITC conjugates. Cellulose, 13: 557-569.

Pinto, J. (2006). Production of Cellulose-Binding Domains by Proteolysis; Studies on the Adsorption and Modification of Cellulose Fibres. Tese de Doutoramento em Engenharia Quimica e Biológica. Escola de Engenharia. Universidade do Minho.

Pires, V. M., Henshaw, J. L., Prates, J. A., Bolam, D. N., Ferreira, L. M., Fontes, C. M., Henrissat, B., Planas, A., Gilbert, H. J. and Czjzek, M. (2004). The crystal structure of the family 6 carbohydrate binding module from *Cellvibrio mixtus* endoglucanase 5a in complex with oligosaccharides reveals two distinct binding sites with different ligand specificities. J. Biol. Chem., 279: 21560–21568.

Pla, I. A., Damasceno, L. M., Vannelli, T., Ritter, G., Batt, C. A., Shuler, M. L. (2006). Evaluation of Mut^{+} and Mut^{S} *Pichia pastoris* phenotypes for high level extracellular scFv expression under feedback control of the methanol concentration. Biotechnol Prog, 22: 881–888.

Pommier, J. C., Fuentes, J. L., Goma, G. (1989). Using enzymes to improve the process and product quality in the recycled paper industry. Tappi J., 71(6): 187-191.

Potvin, G., Ahmad, A., Zhang, Z. (2010). Bioprocess engineering aspects of heterologous protein production in *Pichia pastoris*: A review. Biochem Eng J Corrected Proof. In Press.

Potvin, G., Ahmad, A., Zhang, Z. (2012). Bioprocess engineering aspects of heterologous protein production in *Pichia pastoris*: A review. Biochemical Engineering Journal, 64: 91– 105.

Ramos, R., Domingues, L., Gama, M. (2010). *Escherichia coli* expression and purification of LL37 fused to a family III carbohydrate-binding module from *Clostridium thermocellum*. Protein Expression and Purification, 71: 1-7.

Ramos, R., Moreira, S., Rodrigues, A., Gama, M., Domingues, L. (2013). Recombinant Expression and Purification of the Antimicrobial Peptide Magainin-2. Biotechnol. Prog., 29: 17-22.

Reinikainen, T., Takkinen, K., Teeri, T. (1997). Comparison of the adsorption properties of a single-chain antibody fragment fused to a fungal or bacterial cellulose-binding domain, Enzyme Microb. Technol., 20: 143–149.

Resina, D., Cos, O., Ferrer, P., Valero, F. (2005). De-veloping high cell density fed-batch cultivation strategies for heterologous protein production in *Pichia pastoris* us- ing the nitrogen source-regulated FLD1 promoter. Biotechnology and Bioengineering, 91: 760-767.

Roberts, J. C. (1996). The Chemistry of Paper. The Royal Society of Chemistry, Letchworth, UK.

Rodriguez, B., Kavoosi, M., Koska, J., Creagh, A. L., Kilburn, D. G., Haynes, C. A. (2004). Inexpensive and generic affinity purification of recombinant proteins using a family 2a CBM fusion tag. Biotechnol Prog, 20: 1479-1489.

Romanos, M. (1995). Advances in the use of *Pichia pastoris* for high-level gene expression. Curr Opin Biotechnol, 6: 527–533.

Rotticci-Mulder, J. C., Gustavssonm, M., Holmquist, M., Hult, K., Martinelle, M. (2001). Expression in *Pichia pastoris* of *Candida antarctica* lipase B and lipase B fused to a cellulose-binding domain. Protein Expr Purif, 21:386–392.

Rudman, I. and Patterson, T. F. (1998). Water and air permeability of wet sheets. Institute of paper science and technology. Atlanta, Georgia.

Sarkar, J. M., Cospe, D. R., Harting, E. J. (1995). Applying enzymes and polymers to enhance the freeness of recycled fiber. Tappi J., 78(2): 89-95.

Seth, R. S. (1979). Measurement of fracture resistance of paper. TAPPI, 62(7).

Shen, H., Schmuck, M., Pilz, I., Gilkes, N.R., Kilburn, D.G., Miller, R.C.J., Warren, R.A. (1991). Deletion of the linker connecting the catalytic and cellulose-binding domains of endoglucanase A (CenA) of *Cellulomonas fimi* alters its conformation and catalytic activity. J Biol Chem, 266: 11335-11340.

Shi, X., Zheng, F., Pan, R., Wang, J., Ding, S. (2014). Engineering and comparative characteristics of double carbohydrate binding modules as a strength additive for papermaking applications. BioResources, 9(2): 3117-3131.

Shoseyov, O. and Warren, R. A. J. (1997). Cellulose binding domains – a novel fusion technology for efficient, low cost purification and immobilization of recombinant proteins, innovations, No. 7.

Shoseyov, O., Shani, Z., Levy, I. (2006). Carbohydrate binding modules: biochemical properties and novel applications. Microbiol Mol Biol Rev, 70:283–295.

Sjoblom, M., Lindberg, L., Holgersson, J., Rova, U. (2012). Secretion and expression dynamics of a GFP-tagged mucin-type fusion protein in high cell density Pichia pastoris bioreactor cultivations. Advances in Bioscience and Biotechnology, 3: 238-248.

Sjostrom, E. (1993). Wood Chemistry, Fundamentals and Application, 2 ed, Academic Press Inc, San Diego, CA.

Spence, E. E. (1987). Encyclopedia of Polymer Science and Technology. 2nd ed. Vol. 10. Wily-Interscience, New York, NY, 761–786.

Srisodsuk, M., Reinikainen, T., Pentilla, M., Teeri, T. (1993). Role of the interdomain linker peptide of *Trichoderma reesei* cellobiohydrolase I in its interaction with crystalline cellulose. J. Biol. Chem., 268: 20756–20761.

Srisodsuk, M. (1994). Mode of action of *Trichoderma reesei* cellobiohydrolase I on crystalline cellulose. VTT Publications, 188: 107.

Stork, G., Pereira, H., Wood, T. M., Dusterhoft, E. M. Toft, A., Puls, J. (1995). Upgrading recycled pulps using enzymatic treatment. Tappi J., 78(2): 79-88.

Sugimoto, N., Igarashi, K., Samejima, M. (2012). Cellulose affinity purification of fusion proteins tagged with fungal family 1 cellulose-binding domain. Protein Expression and Purification, 82: 290–296.

Suurnakki, A., Tenkanen, M., Siika-aho, M., Niku-Paavola, M. L., Viikari, L., Buchert, J. (1999). *Trichoderma reesei* cellulases and their core domains in the hydrolysis and modification of chemical pulp. Cellulose, 7: 189-209.

Surribas, A., Resina, D., Ferrer, P., Valero, F. (2006). Limitations using GFP as a protein expression reporter in *Pichia pastoris*. Microbial Cell Factories, 5(1): 56.

Tamai, N., Tatsumi, D., Matsumoto, T. (2004). Rheological properties and molecular structure of tunicate cellulose in LiCl/1,3-dimethyl-2-imidazolidinone. Biomacromolecules, 5: 422-432.

Tang, C-D., Li, J-F., Wei, X-H., Min, R., Gao, S-J., Wang, J., Yin, X., Wu, M. (2013). Fusing a Carbohydrate-Binding Module into the *Aspergillus usamii* ß-Mannanase to Improve Its Thermostability and Cellulose-Binding Capacity by *In Silico* Design. PLoS ONE 8(5): e64766.

Thongekkaew, J., Ikeda, H., Masaki, K., Iefujgi, H. (2013). Fusion of cellulose binding domain from *Trichoderma reesei* CBHI to *Cryptococcus* sp. S-2 cellulase enhances its binding affinity and its cellulolytic activity to insoluble cellulosic substrates. Enzyme and Microbial Technology, 52: 241-246.

Tilbeurgh, H. V., Tomme, P., Claeyssens, M., Bhikhabhai, R., Pettersson, G. (1986). Limited proteolysis of the cellobiohydrolas I from *Trichoderma reesei*. Separation of functional domains. FEBS Letters, 204: 223-227.

Tomme, P., H. Van Tilbeurgh, G. Pettersson, J. Van Damme, J. Vandekerckhove, J. Knowles, and M. Teeri Claeyssens. (1988). Studies of the cellulolytic system of *Trichoderma reesei* QM 9414. Analysis of domain function in two cellobiohydrolases by limited proteolysis. Eur. J. Biochem., 170:575–581.

Tomme, P., Driver, D. P., Amandoron, E. A., Miller Jr, R. C., Warren, R., Kilburn, D. (1995). Comparison of a fungal (family I) and bacterial (family II) cellulose- binding domain. J Bacteriol, 177: 4356-4363.

Tomme, P., Boraston, A., McLean, B., Kormos, J., Creagh, A., Sturch, K., Gilkes, N., Haynes, C., Warren, R., Kilburn, D. (1998). Characterization and affinity applications of cellulose-binding domains. Jornal of Chromatography B, 715: 283-296.

Tomme, P., Boraston, A., Kormos, J., Warren, R., Kilburn, D. (2000). Affinity electrophoresis for the identification and characterization of soluble sugar binding by carbohydrate-binding modules. Enzyme Microb. Tech., 27: 453-458.

Tormo, J., Lamed, R., Chirino, A. J., Morag, E., Bayer, E. A., Shoham, Y., Steitz, T. A. (1996). Crystal structure of a bacterial family-III cellulosebinding domain: a general mechanism for attachment to cellulose. EMBO J, 15(21): 5739–5751.

Tunnicliffe, R.B., Bolam, D.N., Pell, G., Gilbert, H.J., Williamson, M.P. (2005). Structure of a mannan-specific family 35 carbohydrate-binding module: evidence for significant conformational changes upon ligand binding. J Mol Biol., 347(2): 287-96.

Vassileva, A., Chugh, D. A., Swaminathan, S., Khanna, N. (2001). Expression of hepatitis B surface antigen in the methylotrophic yeast *Pichia pastoris* using the GAP promoter. J Biotechnol, 88: 21–35.

von Ossowski, I., Eaton, J. T., Receveur-Bréchot, V. (2005). Protein disorder: conformational distribution of the flexible linker in a chimeric double cellulase. Biophys. J., 88(4): 2823–2832.

von Schantz, L., Gullfot, F., Scheer, S., Filonova, L., Gunnarsson, L., Daniel, G., Karlsson, E., Brumer, H., Ohlin, M. (2009). Affinity maturation generates greatly improved xyloglucan-specific carbohydrate binding modules. BMC Biotechnol., 9: 92.

Vuong, T. V., Master, E. R. (2014). Fusion of a Xylan-Binding Module to Gluco-Oligosaccharide Oxidase Increases Activity and Promotes Stable Immobilization. PLoS ONE, 9(4): e95170.

Wan, W., Wang, D., Gao, X., Hong, J. (2011). Expression of family 3 cellulose-binding module (CBM3) as an affinity tag for recombinant proteins in yeast. Appl Microbiol Biotechnol, 91: 789-798.

Wang, A. A., Mulchandani, A., Chen, W. (2002). Specific adhesion to cellulose and hydrolysis of organophosphate nerve agents by a genetically engineered *Escherichia coli* strain with a surface-expressed cellulose-binding domain and organophosphorus hydrolase. Appl. Environ. Microbiol., 68: 1684–1689.

Xu G. G. and Yang C. Q. (1999). Comparison of the kraft paper crosslinked by polymeric carboxylic acids of large and small molecular sizes: dry and wet performance. J. Appl. Polym. Sci., 74: 907–912.

Yang, S., Kuang, Y., Li, H., Liu, Y., Hui, X., Li, P., Jiang, Z., Zhou, Y., Wang, Y., Xu, A., Li, S., Liu, P., Wu, D. (2013). Enhanced production of recombinant secretory proteins in *Pichia pastoris* by optimizing Kex2 P1' site. PLoS ONE, 8:e75347.

Yokota, S., Matsuo, K., Kitaoka, T., Wariishi, H. (2009). Retention and paper-strength characteristics of anionic polyacrylamides conjugated with carbohydrate-binding modules. BioResources, 4: 234-244.

Zhao, Y., L., Hu, Z., S., Ragauskas, A. (2005). Improvement of paper propertie using starch-modified precipitated calcium carbonate filler. TAPPI Journal, 4(2): 3-7.

Zverlov, V. V., Volkov, I. Y., Velikodvorskaya, G. A. and Schwarz, W. H. (2001). The binding pattern of two carbohydrate-binding modules of laminarinase Lam16A from *Thermotoga neapolitana*: differences in glucan binding within family CBM4. Microbiology, 147: 621–629.

Zupan, A. L., Trobec, S., Gaberc-Porekar, V., Menart, V. (2004). High expression of green fluorescent protein in *Pichia pastoris* leads to formation of fluorescent particles. J Biotechnol, 109: 115-122.