Luleå University of Technology
Biochemical Process Engineering

National Technical University of Athens
School of Chemical Engineering

DIPLOMA THESIS

Food grade Oligosaccharides with high prebiotic value from forest biomass

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Αξιοποίηση βιομάζας δασικής προέλευσης για την παραγωγή ολιγοσακχαριτών με πρεβιοτική δράση

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Εκτεταμένη περίληψη

Τα πρεβιοτικά έχουν οριστεί ως «μη-πεπτόμενα συστατικά τροφίμων που ενισχύουν επιλεκτικά την ανάπτυξη και τη δραστηριότητα των ωφέλιμων βακτηρίων του παχέος εντέρου» και κατέχουν μεγάλο μερίδιο αγοράς στη βιομηχανία τροφίμων και στις φαρμακοβιομηχανίες. Ωστόσο, όλο και περισσότερη προσπάθεια καταβάλλεται για την ανάπτυξη καινοτόμων, βιώσιμων και ανταγωνιστικών συστατικών που μπορούν να εφαρμοστούν στην αγορά των λειτουργικών προϊόντων τροφίμων. Ένας συγκεκριμένος τύπος διατροφικών υδατανθράκων, οι άπεπτοι ολιγοσακχαρίτες (NDOs) έχουν κατεξοχήν ρόλο σε αυτή την κατηγορία συστατικών και θεωρούνται ιδανικό θρεπτικό υπόστρωμα για την ανάπτυξη και βελτίωση της ωφέλιμης εντερικής βακτηριακής χλωρίδας, δηλαδή των προβιοτικών βακτηρίων.

Η επωφελής σημασία των προβιοτικών έχει παρατηρηθεί εδώ και πολλά χρόνια με τη χρήση τους στη βακτηριακή ζύμωση του γάλακτος. Η εντερική βακτηριακή χλωρίδα και συγκεκριμένα του παχέος εντέρου αποτελεί περίπου το 95 % του συνολικού αριθμού κυττάρων στο ανθρώπινο σώμα και περιέχει πάνω από 500 διαφορετικά είδη βακτηρίων. Συνεπώς, παίζει πολύ σημαντικό ρόλο στη διατήρηση της υγείας του ανθρώπου, ενώ διαταραχές στη σύνθεσή της συνδέονται με την πρόκληση ορισμένων γαστρεντερικών ασθενειών, ακόμα και νόσων. Μερικές από αυτές είναι οι ιογενείς διάρροιες, το σύνδρομο του ευερέθιστου εντέρου, οι φλεγμονώδεις νόσοι (ασθένεια του Κρον) ακόμα και καρκίνος του παχέος εντέρου. Η συνδυασμένη δράση των προβιοτικών βακτηρίων με τα κατάλληλα πρεβιοτικά συστατικά, που εμπεριέχεται στον όρο «συμβιοτικά», παρέχει μια ενισχυμένη προσέγγιση στη διατήρηση μιας ισορροπημένης εντερικής βακτηριακής χλωρίδας.

Τα πιο γνωστά πρεβιοτικά συστατικά είναι οι φρουκτο-ολιγοσακχαρίτες (FOS), οι γαλακτο-ολιγοσακχαρίτες (GOS) και οι ξυλο-ολιγοσακχαρίτες (XOS) που ανήκουν στην κατηγορία των άπεπτων ολιγοσακχαριτών (NDOs). Οι άπεπτοι ολιγοσακχαρίτες είναι ολιγοσακχαρίτες με βαθμό πολυμερισμού 2-10 και η πρεβιοτική τους δράση ορίζεται από την ικανοποίηση ορισμένων χαρακτηριστικών, όπως είναι η ανθεκτικότητα στο γαστρικό υγρό και η αντίσταση στην υδρόλυση από τα ένζυμα του πεπτικού σωλήνα, ο μεταβολισμός τους από τα βακτήρια της εντερικής βακτηριακής χλωρίδας και τελικά η παραγωγή θρεπτικών συστατικών. Τελευταία έχει δοθεί προσοχή στους άπεπτους ολιγοσακχαρίτες με πρεβιοτική δράση που προέρχονται από την υδρόλυση της κυτταρίνης, τους λεγόμενους κελλο-ολιγοσακχαρίτες (COS). Αυτό που δίνει μεγάλη σημασία στους κελλο-ολιγοσακχαρίτες (COS) είναι ότι προέρχονται από την κυτταρίνη, που είναι η πιο διαδεδομένη οργανική ένωση στη Γη και αποτελεί το κύριο συστατικό του φυτικού κυτταρικού τοιχώματος. Επιπλέον, αποτελεί το βασικότερο συστατικό της λιγνινοκυτταρινούχας βιομάζας, η οποία αποτελείται επίσης από ημικυτταρίνη και

λιγνίνη και είναι μια ανεξάντλητη, ανανεώσιμη και εναλλακτική πρώτη ύλη. Συνεπώς, η αξιοποίησή της για την παραγωγή χρήσιμων και υψηλής διατροφικής σημασίας σακχάρων καθίσταται ιδιαίτερα ελκυστική.

Η σακχαροποίηση της λιγνινοκυτταρινούχας βιομάζας προκύπτει από μια σειρά διεργασιών που ξεκινάει με την προκατεργασία της βιομάζας που έχει ως αποτέλεσμα τη διάσπαση της άκαμπτης κρυσταλλικής δομής της και την αποδόμηση των βασικών συστατικών της, με στόχο την απομάκρυνση της ημικυτταρίνης και λιγνίνης, έτσι ώστε να διευκολυνθεί η μετέπειτα υδρόλυση της κυτταρίνης. Η υδρόλυση της κυτταρίνης με στόχο το σχηματισμό κελλοβιόζης επιτυγχάνεται με την δράση ειδικών ενζύμων, των κυτταρινασών, που μπορούν να παραχθούν από ορισμένους μικροοργανισμούς, κυρίως βακτήρια και μύκητες. Οι κυτταρινάσες αποτελούν ένα πολυσύνθετο ενζυμικό σύστημα όπου τα βασικά ένζυμα δρουν συνεργιστικά με στόχο την αποικοδόμηση της κυτταρίνης. Συγκεκριμένα, η β-1,4-ενδογλουκανάση (EG) αποικοδομεί με τυχαίο τρόπο τις άμορφες περιοχές της κυτταρίνης δημιουργώντας ελεύθερα άκρα στην αλυσίδα (αναγωγικά και μη αναγωγικά), ενώ οι β-1,4-εξωγλουκανάσες ή κελλοβιοϋδρολάσες (CBH) απελευθερώνουν μόρια κελλοβιόζης από τα άκρα. Η απόδοση της ενζυμικής υδρόλυσης αυξάνεται με την παρουσία ενός εξειδικευμένου ενζυμικού μίγματος, γι' αυτό και οι σύγχρονες μελέτες εστιάζουν στην ανεύρεση νέων συστατικών που θα συμμετέχουν στη δημιουργία ενισχυμένων ενζυμικών κοκτέιλ. Το ένζυμο λυτική μονοοξυγενάση των πολυσακχαριτών (Lytic polysaccharide monooxygenase, LPMO) με οξειδωτική ικανότητα έχει αποδειχθεί ότι δρα σε συνεργασία με τις κυτταρινάσες για την αποτελεσματική αποικοδόμηση της λιγνινοκυτταρινούχας βιομάζας οπότε αποτελεί βασικό ένζυμο για την παρούσα εργασία. Μια ειδική κατηγορία ενζύμων, οι «επεξεργαστικές» (processive) κυτταρινάσες, έχουν το πλεονέκτημα να προσδένονται ισχυρά στην κυτταρική αλυσίδα και να καταλύουν διαδοχικές υδρολύσεις προτού αποδεσμευτούν από το υπόστρωμα, αυξάνοντας την υδρολυτική απόδοση. Συνεπώς, η συμμετοχή τους στη δημιουργία ενζυμικών κοκτέιλ με ενισχυμένη δράση κρίνεται απαραίτητη.

Ο στόχος της παρούσας διπλωματικής εργασίας είναι να επεκτείνει την ήδη υπάρχουσα γνώση σχετικά με τους άπεπτους ολιγοσακχαρίτες (NDOs) με δυνητικά πρεβιοτική δράση αξιοποιώντας την άφθονη ανεκμετάλλευτη λιγνοκυτταρινούχα βιομάζα. Επομένως, η συγκεκριμένη μελέτη εστιάζει στην αποτελεσματική παραγωγή κελλο-ολιγοσακχαριτών (COS) μέσω της διεργασίας της ενζυμικής υδρόλυσης της δασικής βιομάζας.

Αρχικά, τα βασικά ένζυμα που χρησιμοποιήθηκαν για την αποικοδόμηση της λιγνοκυτταρινούχας βιομάζας παράχθηκαν ετερόλογα από τη μεθυλότροφη ζύμη *P. Pastoris* ενώ προήλθαν από το γονιδίωμα του μύκητα *T. thermophila*. Συγκεκριμένα, με

το συνδυασμό της ενδογλουκανάσης (*Tt*EG5) (75 kDa), της εξωγλουκανάσης *Tt*CBH7 (80 kDa) και του ενζύμου με οξειδωτική δράση *Tt*LPMO (60 kDa) παρατηρείται αυξημένη απόδοση της ενζυματικής υδρόλυσης. Στη συνέχεια, η συνδυασμένη δράση του παραπάνω ενζυμικού μίγματος βελτιώθηκε με την προσθήκη ενός «επεξεργαστικού ενζύμου» (processive), της κελλοβιοϋδρολάσης *Pa*Cbh6A, που επιλέχθηκε αφού έδειξε καλή λιγνοκυτταρινολυτική ικανότητα ανάμεσα στις 14 εμπορικές κυτταρινάσες που εξετάσθηκαν. Έπειτα, το συγκεκριμένο μίγμα ενζύμων βελτιστοποιήθηκε με τη χρήση στατιστικού μοντέλου ως προς τον καλύτερο συνδυασμό που θα μεγιστοποιήσει την απόδοση της υδρόλυσης των προκατεργασμένων λιγνοκυτταρινούχων υποστρωμάτων (σημύδα και έλατο).

Προκειμένου να παραχθούν σημαντικές ποσότητες κελλο-ολιγοσακχαριτών (COS) πραγματοποιήθηκαν υδρολύσεις μεγαλύτερης κλίμακας (6 % αρχική συγκέντρωση υποστρώματος) χρησιμοποιώντας τα δύο είδη δασικών υπολειμμάτων, όπου προέκυψε ότι η απόδοση της κελλοβιόζης ήταν διπλάσια με τη χρήση της σημύδας ως υπόστρωμα (19.2 %) συγκριτικά με τη χρήση του ελάτου (9.6 %). Τα προϊόντα της υδρόλυσης ανακτήθηκαν με διεργασίες υπό πίεση φιλτραρίσματος μέσω μεμβράνης προτού δοκιμαστεί η πρεβιοτική τους δράση. Τα προβιοτικά στελέχη που χρησιμοποιήθηκαν ανήκουν στην κατηγορία βακτηρίων της εντερικής χλωρίδας (*μπιφιδοβακτήρια* και *γαλακτοβάκιλλοι*) ενώ τελικά μόνο δύο στελέχη του είδους των *λακτοβακίλλων* (*L. gasseri* and *L. plantarum*) αποδείχθηκε ότι μπορούν να χρησιμοποιήσουν την καθαρή κελλοβιόζη ως πηγή άνθρακα, με το στέλεχος *L. Plantarum* να έχει το μεγαλύτερο ρυθμό ανάπτυξης (μ=0.407 h^{-1}).

Τέλος, η ανάπτυξη των επιλεγμένων προβιοτικών στελεχών του είδους των *λακτοβακίλλων* εμφάνισε διαφορετική συμπεριφορά ανάλογα με τη λιγνοκυτταρινούχα προέλευση των κελλο-ολιγοσακχαριτών (COS). Συγκεκριμένα, μόνο οι κελλο-ολιγοσακχαρίτες που προήλθαν με τη χρήση της σημύδας ως υπόστρωμα αποτέλεσαν καλή πηγή άνθρακα για την ανάπτυξη και των δύο στελεχών, ενώ εκείνοι που προήλθαν με τη χρήση ελάτου ως υπόστρωμα προώθησαν ελάχιστα την ανάπτυξη μόνο του στελέχους *L. plantarum* (μ=0.039 h^{-1}).

Η παρατήρηση αυτή συνδέεται με τη διαφορετική σύσταση των προκατεργασμένων δασικών υποστρωμάτων. Ειδικότερα, στην περίπτωση του προκατεργασμένου ελάτου παρατηρείται μεγαλύτερο ποσοστό εναπομένουσας λιγνίνης (14.9 %) συγκριτικά με την προκατεργασμένη σημύδα (7.1 %). Η λιγνίνη που έχει παραμείνει στο λιγνοκυτταρινούχο υπόστρωμα δρα παρεμποδιστικά όχι μόνο στη δράση των κυτταρινασών, μειώνοντας την απόδοση της ενζυμικής υδρόλυσης, αλλά και στη μετέπειτα ζύμωση των παραγόμενων σακχάρων από τα βακτηριακά στελέχη. Η αναστολή της βακτηριακής ανάπτυξης οφείλεται στην ύπαρξη των φαινολικών

ενώσεων που σχηματίζονται κατά τη διάρκεια της προκατεργασίας της πρώτης ύλης, αλλά και πιθανότατα κατά το στάδιο της σακχαροποίησης.

Η αξιοποίηση βιομάζας δασικής προέλευσης για την παραγωγή ολιγοσακχαριτών με πρεβιοτική δράση είναι μια βιοτεχνολογική εφαρμογή διεπιστημονικής φύσης και παραμένει ανοιχτή η δυνατότητα της περαιτέρω μελέτης της έτσι ώστε να αποκτηθεί μια ολοκληρωμένη άποψη.

Abstract

Prebiotics have been defined as "non-digestible food ingredients that selectively promote the growth and the activity of the beneficial bacteria that exist in the colon" and own a big market share in the food and pharmaceutical industries. However, more and more effort is dedicated in the development of novel, sustainable and cost-competitive ingredients that can be used in the functional food product market.

The purpose of this thesis is to expand the already existing knowledge regarding the non-digestible oligosaccharides (NDOs) with prebiotic potential by taking advantage of the unexploited high abundant lignocellulosic residues. Therefore, the main focus of this study is on the efficient production of cello-oligosaccharides (COS) through enzymatic hydrolysis process using forest biomass.

Firstly, the key enzymes that were used for the lignocellulose degradation were originally encoded by *T. thermophila* and expressed heterologously in the methylotrophic *P.pastoris* yeast. Particularly, when the endoglucanase *Tt*EG5 (75 kDa), the exoglucanase *Tt*CBH7 (80 kDa) and the oxidative enxyme *Tt*LPMO (60 kDa) were combined, an increase in the enzymatic hydrolysis yields was observed. The combined action of the above enzymatic mixture was then improved with the addition of a processive cellobiohydrolase, *Pa*Cbh6A, that was chosen after showing a good lignocellulolytic potential among the 14 commercial cellulases that were studied. The defined enzymatic mixture was afterwards optimized towards organosolv pretreated hardwood (birch) and softwood (spruce) substrates so as to maximize the cellobiose yields.

In order to produce significant amounts of COS larger scale reactions (6 % initial concentration of the substrate) using the two types of forest residues were carried out, where the cellobiose yield was 2-fold higher using birch as substrate (19.2 %) than using spruce (9.6 %). The hydrolysis products were recovered with pressure driven membrane processes prior to testing their prebiotic ability. Among the gut related bacteria (*bifidobacteria* and *lactobacilli*) that were used as probiotic strains, only two of the *lactobacillus* genus (*L. gasseri* and *L .plantarum*) proved to efficiently use pure cellobiose as a carbon source, with *L. plantarum* showing the highest growth rate (μ=0.407 h^{-1}).

Finally, the lignocellulose-derived COS had a different outcome on the growth of the two *lactobacilli* strains that was dependent on the biomass type that was used. Particularly, only the birch-derived COS proved to be an efficient carbohydrate source for the growth of both *lactobacilli* strains, while spruce-derived COS resulted in a minor growth of only the *L. plantarum* strain (μ=0.039 h^{-1}).

The evaluation of the prebiotic potential of lignocellulose-derived COS is a biotechnological application of interdisciplinary nature and remains to be further tested in order to obtain a holistic aspect.

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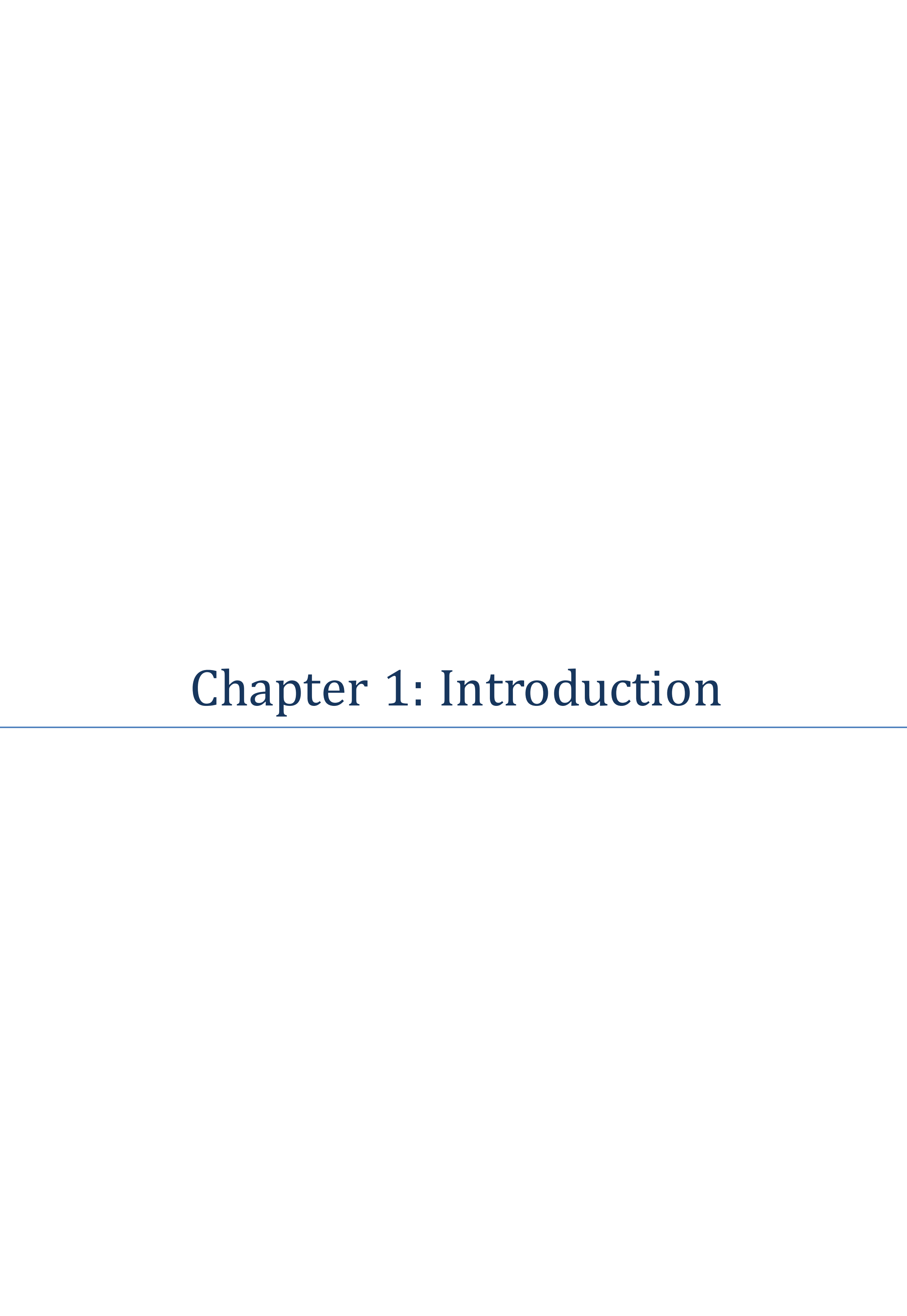


Chapter 1: Introduction

1 Introduction

1.1 Plant cell wall

The plant cell wall is a distinctive characteristic of plants and it has a macromolecular structure that surrounds the cell and is essential for its protection and survival (Caffall and Mohnen, 2009). Cell wall polysaccharides are currently separated into cellulose, hemicellulose and pectic polymers. The 90 % of the cell wall is consisted of carbohydrates (mostly hexoses and pentoses) and the remaining 10 % is consisted of proteins (Albersheim, 1975; Mccann and Knox, 2010). The plant cell wall structure includes three different layers, the primary cell wall, the middle lamella and the secondary cell wall as depicted in **Figure 1.1**. The secondary cell wall is further divided into three layers, the inner layer (S3), the middle layer (S2) and the outer layer (S1). Cellulose hemicellulose and lignin are distributed differently in these layers. It has been found that in wood fibers cellulose concentration increases from the middle lamella to the secondary wall. The layers S2 and S3 of the lamella have a have the highest cellulose concentration. Hemicellulose is mostly distributed in the secondary wall, while the lignin percentage decreases when the distance increases into the middle lamella and the cell corner has the highest lignin average concentration (Agarwal, 2006; McMillan, 1994; Yang, 2001). The primary cell wall and the middle lamella are formed during the initial growth and expansion of the cell and are more abundant in pectin compared to the secondary walls. Thus they play a major role in the wall's structural integrity and cell adhesion. The secondary cell wall is present in some cells as an additional layer when the growth of the primary wall ceases and becomes furthermore thickened and strengthened (Caffall and Mohnen, 2009; Cosgrove, 2005; Scheller and Ulvskov, 2010). Both primary and secondary cell walls are fibrous composites and have several polysaccharides in common. The composition of the primary plant cell wall is typically consisted of cellulose microfibrils (9-25%), hemicelluloses (25-50%), pectins (10-35%) and proteins (10%). The secondary plant cell wall is composed by cellulose (40-80%), hemicellulose (10-40%) and lignin (5-25%) (Salisbury and Ross, 1992; Goodwin and Mercer, 1983). As already mentioned the creation of the secondary cell wall is linked with the thickening of the primary wall with the inclusion of lignin into the cell wall matrix and is responsible for the generation of a woody tissue (Schuetz *et al.*, 2013; Theander and Aman, 1984). In both cell walls cellulose microfibrils form the fibrous component that creates the framework of the cell wall, although they have a more organized structure in the secondary cell wall, since the primary cell wall is subjected to changes that occur during the maturation of the cell (Hatfield, 1989). In the primary wall, xyloglucan is the main hemicellulose and provides the cross linking between non cellulosic and cellulosic polymers (Wolf *et al.*, 2012; McFarlane *et al.*, 2014). Pectins are a major component of the middle lamella and are defined as heterogeneous polysaccharides that have a galacturonic acid containing backbone and provide structural support to the primary cell wall by interacting with cellulose via hydrogen bonds (Zykwinska *et al.*, 2005; Wang *et al.*, 2012;

Atmodjo *et al*., 2013). Proteins may be minor components of the cell wall but they have either a structural (extensins) or an enzymatic function by taking part in the cell wall modification and the polysaccharide cross linking (Goodwin and Mercer, 1983; Albenne *et al*., 2013). At the secondary plant cell walls cellulose microfibrils are embedded in lignin, a phenolic compound and therefore this connection adds further rigidity and recalcitrance in the structure of the cell wall (Bidlack and Malone, 1992). The accumulated biomass in the secondary cell walls accounts for the majority of the plant biomass, which is the main source of biomass on Earth (Chen, 2014).

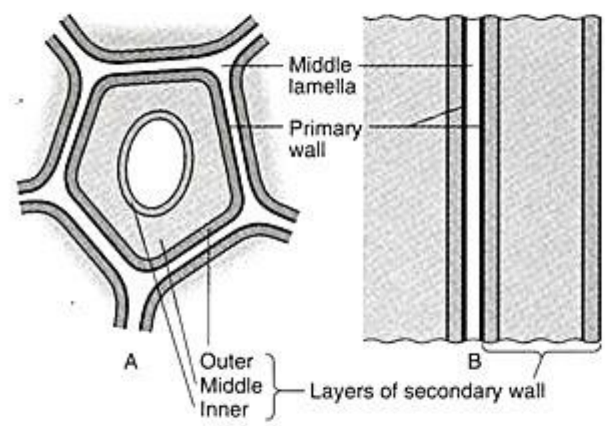


Figure 1.1. Plant cell wall structure. A) In cross section, B) In longitudinal section. (Sen *et al*., 2005)

1.1.1 Lignocellulose structure

Lignocellulose is the most abundant biomass produced from photosynthesis that is renewable and is produced in large amounts every year. Particularly, each year 200 billion metric tons of lignocellulosic biomass is produced worldwide (Ragauskas et al., 2006; Zhang et al., 2006a). This biomass includes waste from forestry, agricultural and agro-industrial practices, paper pulp and timber industries which when accumulating in such degree can cause environmental problems. Lignocellulose is composed by the plant cell wall materials, cellulose, hemicellulose and lignin, although the chemical composition of plants differs and is influenced by genetic and environmental factors (**Table 1.1**) (Betts *et al*., 1991; Deobald and Crawford, 1997). Since the chemical composition of lignocellulose is based on carbohydrates, it can be used as a substrate of enormous biotechnological value for producing several high value added products, such as ethanol, food additives, organic acids, enzymes and others (Mussatto and Teixeira, 2010).

Table 1.1. Chemical composition of lignocellulosic materials (Betts *et al.*, 1991)

Raw material	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Hardwoods	45-55	24-40	18-25
Softwoods	45-50	25-35	25-35
Grasses	25-40	25-50	10-30

The current models for the lignocellulose structure include cellulose microfibrils of a diameter about 10-25 nm (microfibrils are insoluble cable-like structures that are composed of 30 to 36 hydrogen bonded glucan chains) that form a network which consists the skeleton of the cell wall. Hemicellulose and lignin are wrapped around the microfibrils and this cross linking forms a three dimensional structure (**Figure 1.2**). Cellulose and hemicellulose are mainly linked though hydrogen bonds. In addition to the hydrogen bond, hemicellulose and lignin are linked with a chemical bonding, which usually refers to bonds between galactose or arabinose residues and carbohydrates (Chen, 2014; Chundawat et al., 2011; Yang, 2001).

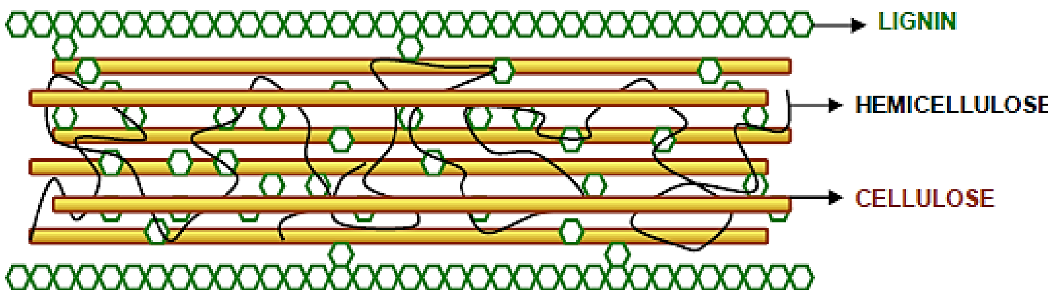


Figure 1.2. Representation of lignocellulosic structure (Mussatto and Teixeira, 2010).

1.1.1.1 Cellulose

Cellulose is the most widespread and abundant organic resource on Earth with a huge economic value of a total annual amount of several billion tons. It is found mostly in plants, although it is present in some animals (such as tunicates), bacteria and marine algae (Chen, 2014). It is a high molecular weight linear polysaccharide and is composed by D-glucose units that are linked by β-1,4-glycosidic bonds (also called glucan or glucopyranose chains). The chemical formula of cellulose is $(C_6H_{10}O_5)_n$, whereas n is the degree of polymerization (DP) which varies between 100 and 10,000 (Chen, 2014; Chundawat et al., 2011). The repeating unit of cellulose is called cellobiose and is consisted of two dehydrated repeating units of D-glucose as shown in **Figure 1.3** (Chen, 2014; Mussatto and Teixeira, 2010). In nature cellulose is synthesized by membrane proteins, namely terminal complexes (TCs) in chains that are known as protofibrils and consequently form microfibrils with 2-20 nm cross sections depending on the source of cellulose (Eyley and Thielemans, 2014). The adjacent cellulose molecules are linked together with hydrogen and Van der Walls bonds and as a result cellulose is packed into microfibrils. These bonds tend to arrange the cellulose chains in parallel alignment and form a crystalline structure (Ha et al., 1998; Zhang and Lynd, 2004). Therefore, cellulose exists in several crystalline forms. It can have highly crystalline regions (around 2/3 of the total cellulose) and amorphous regions. Since the molecules in the amorphous regions are less compact than the in crystalline regions, they are also soluble and more easily degradable (O'Sullivan, 1997; Zhang and Lynd, 2004). The cellulose crystallinity ranges between 30-80% refers to the percentage of all the cellulose that is occupied in the crystalline region (Yang, 2001). There are five types of crystal modification in solid cellulose and Type 1 is the crystal form of the natural cellulose and the most abundant which has a two chain monoclinic unit cell. Types II, III, IV and X are the crystal forms of the cellulose that has gone through artificial processing (Chen, 2014; Chundawat et al., 2010).

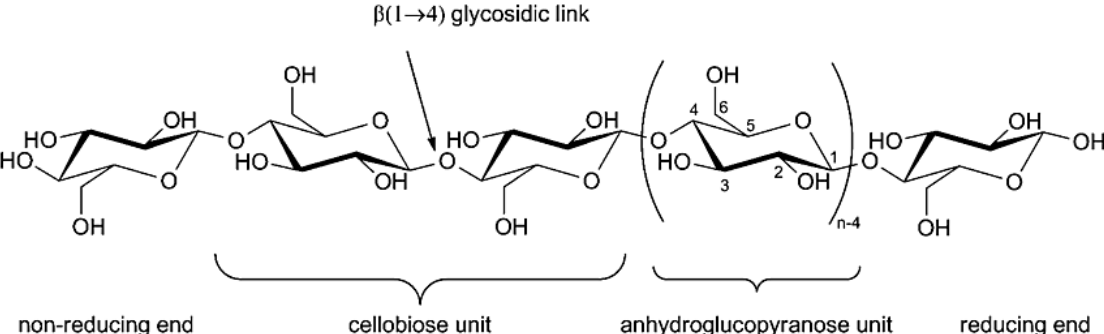


Figure 1.3. Representation of the cellulose chain showing the glucose repeating unit, together with the atom numbering, the glycosidic bond, the reducing and non-reducing ends respectively. (Eyley and Thielemans, 2014)

1.1.1.2 Hemicellulose

Hemicellulose was first identified by Schulze (1891) as the polysaccharide that can be extracted from plants by alkaline solutions (Zhang *et al.*, 2006c; Chundawat *et al.*, 2011). Hemicellulose is an heterogeneous group of polysaccharides that is composed of both linear and branched polymers of five different sugars, D-xylose, L-arabinose, D-mannose, D-glucose, D-galactose as well of other components such as acetic, glucuronic and ferulic acids (Eriksson *et al.*, 1990; Mussatto and Teixeira, 2010). It has an average degree of polymerization (DP) 70-200 and its composition differs depending on the cell tissue and the plant species (Chundawat *et al.*, 2011; Zhao *et al.*, 2012). The hemicellulose backbone is similar with that of cellulose and is made up of 1,4-β-D-linkages between its backbone sugars (Eriksson *et al.*, 1990; Cosgrove, 2005). According to the main sugar of the backbone, hemicellulose can have different classifications, for example xylans, mannans, glucans, glucuronoxylans, arabinoxylans, glucomannans, galactomannans, galactoglucomannans, β-glucans and xyloglucans (Mussatto and Teixeira, 2010). The two most abundant hemicelluloses are xyloglucan and arabinoxylan while the principal hemicelluloses in softwood are the galactoglucomannans while in hardwood the glucuronoxylans (Eriksson *et al.*, 1990; Cosgrove, 2005). Since hemicellulose has a lower DP and fewer crystalline structures than cellulose, it is more easily degraded and thus easier to be hydrolyzed (Chen, 2014; Fengel and Wegener, 1989).

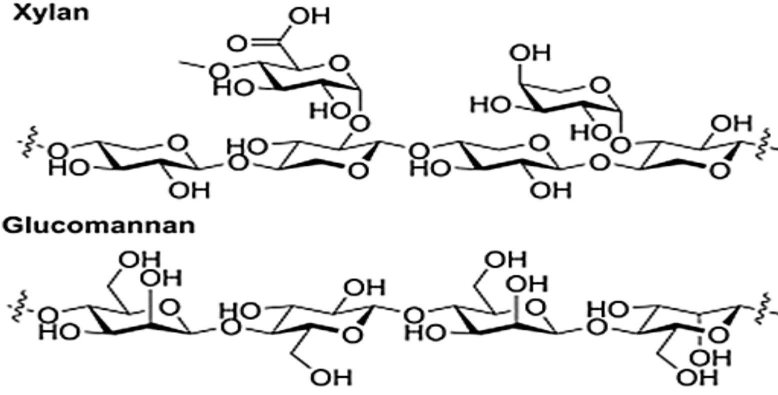


Figure 1.4. Representation of the structure of the main hemicelluloses that are found at hardwoods (xylan) and softwoods (glucomannan) (Shrotri *et al.*, 2017)

1.1.1.3 Lignin

After cellulose, lignin is one of the most abundant organic polymers in plants. Since lignin binds the cells, the fibers and vessels it has an important role for protecting the plants against pathogen invasion and insects (Sticklen, 2008). Lignin is a quite complex molecule and its structure varies a lot depending on the cell tissue type and the plant species. It is a polyphenolic polymer constituted by three basic monomeric phenylpropanoid units that are linked in a three dimensional structure, p-hydroxyphenyls (H), guaicyls (G), and syringyls (S) (Boerjan *et al.*, 2003; Grabber, 2005; Mussatto and Teixeira, 2010). The p-phenyl monomer (H type) is derived from coumaryl alcohol, guaiacyl monomer (G type) is derived from coniferyl alcohol and syringyl monomer (S type) from sinapyl alcohol. The basic structural monomers of lignin are depicted in **Figure 1.5**. At the hardwoods, the predominant lignins are the G and S monolignols with traces of H units, while at the softwoods lignins are composed of G units (Chundawat et al., 2011). Lignin is cross-linked with hemicellulose with at least two types of linkages: a) diferulic acid bridges and b) ester linkage between lignin and glucuronic acid attached to xylans (de Vries and Visser, 2001). Since there are no chains containing repeating units, lignin is extremely resistant to chemical and enzymatic degradation (Malherbe and Cloete, 2002).

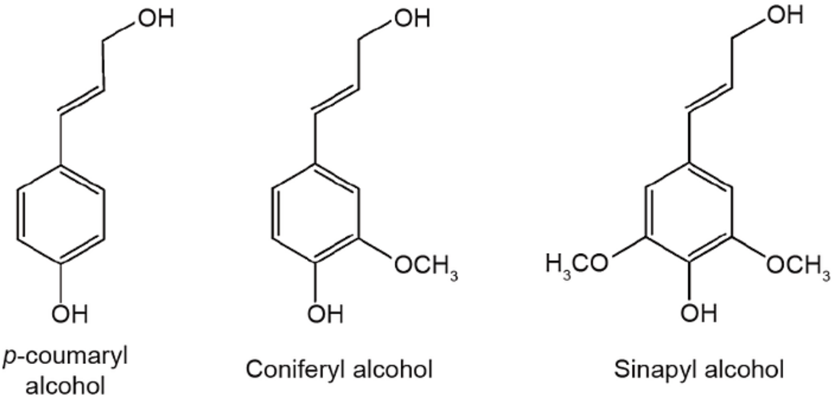


Figure 1.5. Lignin structural units (Achinas *et al.*, 2017).

1.1.2 Lignocellulose pretreatment

Lignocellulose is a very recalcitrant material and the primary objective of the lignocellulose pretreatment is the potential to access the cellulose and hemicellulose which are encrusted by lignin within the lignocellulose matrix (Malherbe and Cloete, 2002). The recalcitrance of the lignocellulosic biomass contributes to higher biomass conversion costs and is attributed to several natural factors such as a) the epidermal tissue of the plant body, b) the relative amount of the thick wall tissue, c) the degree of lignification, d) the structural heterogeneity and cell wall components complexity such as microfibrils and matrix polymers, e) the enzyme's challenge to act on an insoluble substrate, f) the inhibitors to subsequent fermentations that exist in cell walls or are generated during the conversion processes (Himmel et al., 2007). Therefore, the enzymatic hydrolysis of biomass is limited due to factors that can be classified in two groups, the direct factor, such as the accessible surface area and the indirect factors which include biomass structure relevant factors (pore size and volume, particle size and specific surface area), chemical compositions (lignin, hemicelluloses and acetyl group) and cellulose structure-relevant factors (cellulose crystallinity and degree of polymerization (DP)) (Zhao *et al*., 2012). However, the main factors causing the biomass recalcitrance are the low accessibility of crystalline cellulose fibers which prevent the enzymes to act efficiently and the presence of lignin and hemicellulose on the surface of cellulose preventing the enzymes to access the substrate (Zhang, 2008).

The biomass degradability differs according to the species of the lignocellulose, for instance, woody biomass is harder to be hydrolyzed than other types of lignocellulose and particularly softwood is more resistant to degradation than hardwood (Jönsson *et al*., 2013). Thus, the pretreatment process is necessary for disrupting the crystalline cellulose structure and breaking down the lignin so that the acids or the enzymes can easily access the cellulose and hydrolyze it into monomers (**Figure 1.6**) (Behera et al., 2014).

Figure 1.6. Schematic of the pretreatment effect at the biomass structure (Mosier et al., 2005)

Several pretreatment methods have been developed during the last decades. These methods can be divided into different categories, physical (milling and grinding), physicochemical (steam explosion, hydrothermolysis, wet oxidation, etc.), chemical (alkali, dilute acid, oxidizing agents and organic solvents), biological, electrical or a combination of them (Behera et al., 2014).

1.1.2.1 Physical pretreatment

Physical pretreatment is classified into two categories, mechanical and non-mechanical pretreatments and the main target for both is the increase of the accessible surface area and the pore size, as well as the decrease of the crystallinity and the degree of polymerization of the cellulose (Fan *et al*., 1982; Harmsen *et al*., 2010). Different types of physical processes such as milling, pyrolysis and irradiation are used. The physical pretreatment step is a necessary step for the further chemical or biochemical processing as the size reduction is one of the most effective methods for making cellulose more easily accessible to enzymatic treatment (Behera et al., 2014)

1.1.2.2 Chemical pretreatment

Chemical pretreatment is considered to be one of the most promising methods for enhancing the biodegradability of cellulose. It is has been extensively used for the delignification of the cellulosic materials in the paper-pulp industry (Zheng *et al*., 2009). This method takes advantage of some chemicals that have been reported to have a significant effect on the native

lignocellulose structure, do not produce toxic residues and the reactions are carried out at room temperature and pressure (Mtui, 2009). The chemicals that are used range from alkalis, acids, oxidizing agents, organic solvents and salts (Agbor et al., 2011; Behera et al., 2014). However, it should be considered that concentrated acids are not preferred since they are corrosive and thus should be recovered to make the pretreatment economically feasible (Agbor et al., 2011). According to the chemicals and the pretreatment conditions that are used there are differences on the pretreatment mechanisms. For instance, acids are used mainly for the hydrolysis of cellulose and the most common acids that are applied are the sulfuric acid, the HCl and the nitric acid, while the dilute acid hydrolysis is among the most commonly applied chemical pretreatment methods (Taherzadeh and Karimi, 2008). In the alkaline hydrolysis the most common alkalis or bases that are used are the NaOH, KOH, ammonia and lime. The alkaline hydrolysis causes the swelling of the biomass which results to an enhanced biomass degradability (Agbor et al., 2011; Taherzadeh and Karimi, 2008).

1.1.2.3 Biological pretreatment

Biological pretreatment is associated with the action of some micro-organisms that are able to degrade lignin, hemicellulose and polyphenols. The most common micro-organisms that are reported to take part in the biological pretreatment are several fungi such as brown-, white- and soft-rot fungi, with the last having a more effective action (Agbor et al., 2011; Taherzadeh and Karimi, 2008). These fungi are able to produce lignocellulolytic enzymes, which work synergistically to degrade the plant cell wall (Dashtban *et al.*, 2009; Behera *et al.*, 2014). Biological pretreatment is an environmentally friendly process since it requires low energy, mild conditions and no chemicals. However, the pretreatment rate is very slow and it requires careful control of growth conditions and large space to perform (Agbor et al., 2011).

Table 1.2 Summary of methods used for the pretreatment of lignocellulose (Andersen, 2007)

Physical[a]	Chemical[a]	Biological[b]
Ball-milling	Alkali	Fungi
Two-roll milling	Sodium hydroxide	Brown rots (attack cellulose)
Hammer milling	Ammonia	*Piptoprus betulinus*
Colloid milling	Ammonium sulfite	*Laetiporus sulphureus*
Vibro energy milling	Acid	*Trametes quercina*
High pressure steaming	Sulfuric acid	*Fomitopsis pinicola*
Extrusion	Hydrochloric acid	*Gloephyllum saepiarium*
Expansion	Phosphoric acid	White rots (attack both lignin and cellulose)
Pyrolysis	Gas	*Formes fomentarius*
High energy radiation	Chlorine dioxide	*Phellinus ignarius*
	Nitrogen dioxide	*Ganoderma appalanatum*
	Sulfur dioxide	*Amillaria mellea*
	Oxidizing agents	*Pleurotus ostreatus*
	Hydrogen peroxide	Red rot (attack both lignin and cellulose)
	Ozone	*Formitopsis annos*
	Cellulose solvents	Bacteria
	Cadoxen (ethylene diamine and water)	
	CMCS	
	Solvent extraction of lignin	
	Ethanol-water extraction	
	Benzene-ethanol extraction	
	Ethylene-glycol extraction	
	Butanol-water extraction	
	Swelling agents	

[a]: From Fan et al., 1982; [b]: From Schurz, 1978; CMCS: is composed of sodium tartarate, ferric chloride, sodium sulfite, and sodium hydroxide solution.

1.1.2.4 Physicochemical pretreatment

The pretreatments that combine physical and chemical processes are referred to as physicochemical processes. This category includes the majority of the pretreatment technologies such as steam explosion, liquid hot water, ammonia fiber/freeze explosion, wet oxidation, ammonia recycle percolation, aqueous ammonia, organosolv and CO_2 explosion (Agbor et al., 2011). The use of ionic liquids and cellulose solvent-based lignocellulose fractionation processes has recently been proposed (Zhu et al., 2009). These pretreatment forms depend mostly on the process conditions and solvents that are used and affect the physical and chemical properties of the biomass (Agbor et al., 2011; Behera et al., 2014).

- *Organosolv*

Organosolv (OS) pretreatment has attracted much attention and is considered one of the most promising pretreatment methods due to its big potential in the lignocellulosic biomass delignification and fractionation (Matsakas et al., 2018). In OS pretreatment method a large number of organic or aqueous-organic solvents can be used at elevated temperatures of 150-200°C with or without the addition of catalysts such inorganic acids HCl or H_2SO_4 or organic acids such as oxalic, salicylic and acetylsalicylic acid (Agbor et al., 2011; Taherzadeh and Karimi, 2008). The solvents that are used in the process are methanol, ethanol, acetone, ethylene glycol, triethylene glycol, and tetrahydrofurfuryl alcohol (Thring *et al.*, 1990). However the solvents that are applied should be separated and recovered with evaporation and

condensation and recycled to reduce the operational costs of the process. This step is very important since the solvents may have an inhibitory effect to the growth of microorganisms, enzymatic hydrolysis and fermentation or anaerobic digestion of the hydrolysate (Agbor et al., 2011; Kumar et al., 2009; Sun and Cheng, 2002). The procedure that is followed at the OS pretreatment is the one that is depicted at **Figure 1.7** and can be described as follows. First, the cellulose rich pretreated solids are separated from the pretreated liquor by filtration and then a recovery of the solvent is achieved through distillation leading to the precipitation of lignin and leaving behind the aqueous solution that contains the solubilized hemicellulose (Matsakas et al., 2019)

The OS is a very selective method that yields three different fractions: a solid fraction enriched in cellulose, a hemicellulose fraction that contains xylose and xylans and a lignin fraction (Duff and Murray, 1996; Raghavendran et al., 2018) dilutes in the organic solvent phase. The lignin that is produced by OS pretreatment has very low ash content, is sulfur free and maintains a structure close to the natural one by retaining the majority of β-ether bonds (Matsakas et al., 2019). Additionally, the OS is the only physicochemical pretreatment method that is very effective for the pretreatment of high lignin lignocellulose materials, such as softwoods (Agbor et al., 2011).

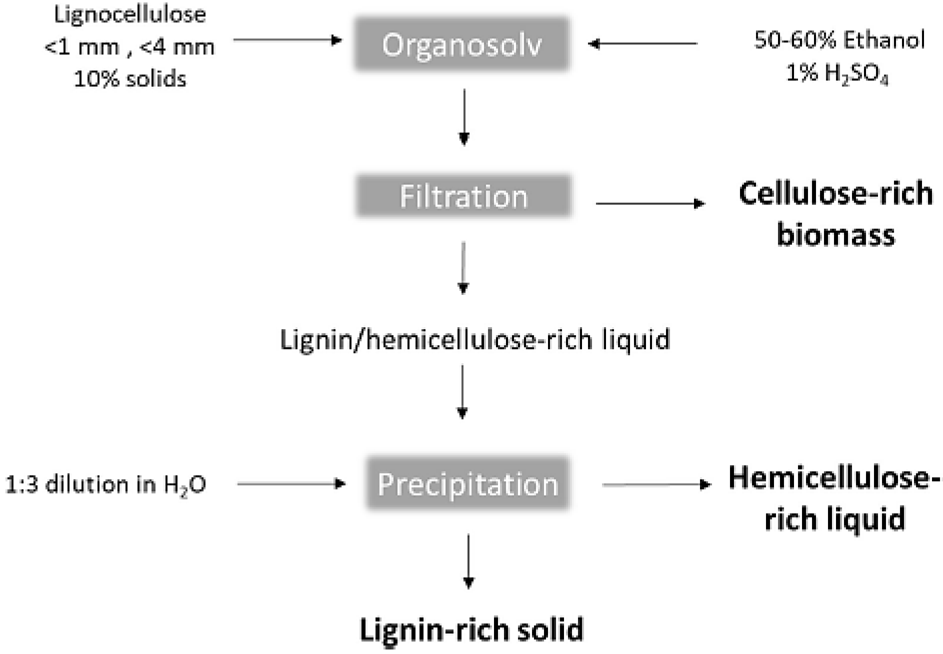


Figure 1.7. Representation of the organosolv pretreatment of birch and spruce biomass to obtain cellulose rich biomass (Raghavendran et al., 2018)

1.1.3 Lignocellulose biodegradation

As already mentioned the main components of lignocellulose are the cellulose, hemicellulose and lignin. There are some cellulose-degradable organisms that have the ability to use lignocellulose as feedstock and can be both microorganisms and animals (e.g. fungi, bacteria, actinomycetes). These lignocellulose degradable microorganisms can be divided into different categories according to the lignocellulose components that are able to degrade (Chen, 2014):

1. Cellulose degradable microorganisms, including fungi, actinomycetes, bacteria, protozoa etc. Several strains of these microorganisms are *Polyporus* spp., *Agaricales* spp., *Trichoderma* spp., and *Myrothecium* spp., *Sporocytophaga myxococcoides, Streptomyces antibioticus*, etc.

2. Hemicellulose degradable microorganisms. In this category fungi dominate at the early stage of hemicellulose degradation, while actinomycetes at the later stage. The number of fungi groups that degrade hemicellulose is greater than that for cellulose degradation.

3. Lignin degradable microorganisms, including Basidiomycota and *Aphyllophorales* spp. fungi, such as *Fomes* spp., *Polyporus* spp., *Polystictus* spp., etc.

The main species though for lignocellulose degradation are the fungi, which provide more reasonable cellulolytic enzyme systems and high level cellulase production (Chen, 2014; Sánchez, 2009). Among the most intensively studied microorganisms is the filamentous fungus *Trichoderma reesei* and is used for the commercial production of hemicellulases and cellulases. The fungal degradation occurs extracellularly, either in association with the outer cell layer or in the extracellular environment, because of the insolubility of lignin, cellulose and hemicellulose. The extracellular enzymatic systems of fungi can be divided into two types, the hydrolytic system, which produces hydrolases that are responsible for polysaccharide degradation, and a unique oxidative and extracellular ligninolytic system, which degrades lignin and opens phenyl rings (Sánchez, 2009). The fungi that is able to efficiently degrade lignin is the white rot fungi that belongs to the basidiomycetes, with *P. chrysosporium* being the most studied strain. This strain can simultaneously degrade cellulose, hemicellulose and lignin, whereas others such as *Ceriporiopsis subvermispora* tend to remove lignin in advance of cellulose and hemicellulose (Howard et al., 2003; Sánchez, 2009).

1.1.3.1 The fungus Thermothelomyces thermophila

Thermothelomyces thermophila (previously known as *Myceliophthora thermopila* or *Sporotrichum thermophile*) is a thermophilic filamentous fungus that is classified as an ascomycete and is very attractive for biotechnological applications and especially in the cellulose degradation process. This fungal type has the ability to synthesize a complete set of enzymes that are necessary for the cellulose breakdown (Karnaouri et al., 2014a).

The importance of this fungus lays in its thermophilic characteristic, which makes the enzymes that are produced tolerant in higher temperatures. The use of higher reaction temperatures in the hydrolysis process has several advantages such as the enhanced hydrolytic capability of the thermophilic enzymes compared to the mesophilic enzymes which is associated with higher hydrolysis rates and reduced incubation time (Berka et al., 2011; Singh, 2016). Additionally, the higher temperatures reduce the risk of contamination and improve the viscosity of the substrate and the mass transfer. Another important advantage is that the thermophilic enzymes can be stored at room temperature for prolonged periods and tolerate organic solvents. All these factors can help in reducing the production cost of lignocellulose bioconversion, with the use of improved and low cost biocatalysts (Turner *et al.*, 2007).

The genome of *T. thermophila* together with the one of another thermophilic fungus *Thielavia terrestris* were the first that were described for the thermophilic eukaryotes and the first complete telomere-to-telomere genomes for filamentous fungus. The genome analysis results for the *T. thermophila* revealed a genome size of 38.7 Mbp that is comprised by 9500 genes, organized in 7 chromosomes (Berka et al., 2011). This large genome size indicates its potential to encode a big variety of industrially important enzymes such as carbohydrate active enzymes (CAZy), proteases, oxidoreductases and lipases, while more than 200 sequences have been identified to encode exclusively plant cell wall degrading enzymes (Karnaouri et al., 2014a). Additionally, *T. thermophila* exhibits the highest number that can be found in fungi of accessory enzymes that belong to the auxiliary activity enzymes AA9 and family 1 carbohydrate binding modules (CBMs) but also a relatively high number of arabinoxylan degrading enzymes (Berka et al., 2011; Hinz et al., 2009). Particularly, eleven putative xylanases that belong to GH 10 and 11 families originate from the *T. thermophila*, whereas only five originate from *A. niger* and *T. reesei*. Moreover, fourteen arabinofuranosidases that belong to the GH families 43, 51 and 62 originate from *T. thermophila*, while fourteen from *A. niger* and only three from *T. reesei*. Thus, the *T. thermophila* is distinguished as a promising source of hemicellulolytic enzymes (Karnaouri et al., 2014a).

The optimal temperature of *T. thermophila* is between 40 and 50 °C, although it can be cultivated at temperatures between 25 and 55 °C (Morgenstern et al., 2012). The enzymes that are characterized from *T. thermophila* are found to act better on the range of 50 – 70 °C (Karnaouri et al., 2014a). Several enzymes from *T. thermophila* have already been studied, such as endoglucanases from GH families 5 and 7 (Karnaouri et al., 2014b, 2017), an ethanol tolerant

β-glucosidase (Karnaouri et al., 2013), the polysaccharide monooxygenase Cel61a and LPMO9 (Dimarogona et al., 2012b; Karnaouri et al., 2017), cellobiohydrolases from the families I and II (CBH6 and CBH7) (Karnaouri et al., 2018) and others, and have been expressed in *Pichia pastoris* yeast before characterization. For this thesis, the enzymes that were used were the following: one endoglucanase (EG5), one cellobiohydrolase (CBH7) and the lytic polysaccharide monooxygenase LPMO), all encoded by *T. thermophila* genes, heterologously expressed and produced in *P. pastoris* yeast.

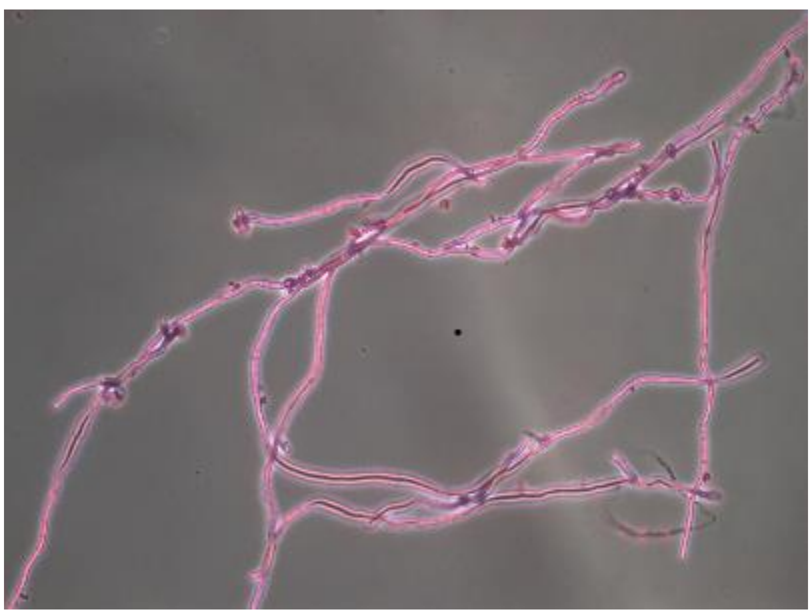


Figure 1.8. ***Thermothelomyces thermophila*** **ATCC 42464 (from https://www.diark.org)**

1.2 Cellulose in model substrates

Natural lignocellulosic substrates have complicated structure and therefore not well suited for fundamental and detailed characterization of enzymes. Thus, the performance of the enzymes against natural lignocellulosic materials is completely different from the one against model cellulosic substrates. The advantage of performing experiments with model substrates is that the purity of the substrate is constant. However, those substrates do not have exactly the same characteristics as the cellulose in the plant cell wall materials as depicted at **Table 1.3**.

Cellulose derivatives with long polymerization degree (DP) can be dissolved in water because of their chemical substitutions. Ionic substituted carboxymethyl cellulose (CMC) is a very common model substrate for the determination of cellulose activity. It is often used for the determination of endoglucanase activity, called CMCase, as endoglucanases are able to cleave intramolecular β-1,4-glucosidic bonds randomly, resulting in a huge decrease of DP (i.e., specific viscosity) of CMC. CMC is high polymer cellulose and has two very important physical parameters, the degree of substitution (DS) and the DP. CMC's solubility is closely associated with the DS, so that when the DS > 0.3-0.7, CMC is soluble in water. Commercial CMCs usually have a DS < 1.5 (Karlsson et al., 2002; Wood and Bhat, 1988; Zhang et al., 2006b).

Cellulose containing insoluble substrates for the determination of cellulase activity includes almost pure celluloses. The crystallinity index (CrI) of cellulose is not strongly associated with hydrolysis rates but it can be used for indicating the changes in cellulose characteristics for one material before and after treatment (Zhang et al., 2006b; Zhang and Lynd, 2004) .

Avicel is microcrystalline cellulose derived by acid hydrolysis of wood, and is commonly considered to be a crystalline substrate. However, microcrystalline cellulose still contains a significant fraction of amorphous cellulose (Zhang *et al*., 2006). Avicel is a good substrate for exoglucanase activity assay as it has a low DP and low accessibility and therefore it has been thought by some researchers that "avicelase" activity is equivalent to exoglucanase activity (Wood and Bhat, 1988; Zhang et al., 2006b). However, it has been reported that some endoglucanases can release a considerable amount of reducing sugars from avicel (Zhang and Lynd, 2004). Avicel® PH-101 is a white powder with the particle size of about 50 µm.

PASC (phosphoric-acid swollen cellulose) is considered to be a representative of amorphous cellulose, with the macromolecular structure of cellulose as a moiety of fibers (Zhang et al., 2006b) . It was prepared from Avicel by phosphoric acid treatment, usually by adding 85 % o-phosphoric acid (Wood, 1988). Opposed to the dry, solid, powder-like composition of Avicel, PASC is relatively viscous, unclear (cloudy) liquid. The properties of amorphous cellulose vary greatly depending on the cellulose origins, reaction temperature and time but also reagent types and concentrations. Thus, the comparison of hydrolysis rates on several types of

amorphous cellulose from different laboratories or even batches are impossible (Zhang et al., 2006b).

Table 1.3. Summary of main characteristics of cellulosic model substrates

Substrate	CrI	DP	SSA (m^2/g)	Reference
PASC	0-0.04	100-1000	240	a,b
Avicel	0.5-0.6	150-500	20	a,b
CMC	N.A.	100-2000	N.A	b

CrI: Crystallinity index, DP: Degree of polymerization, SSA: Specific surface area, a: (Zhang and Lynd, 2004)*; b:* (Zhang et al., 2006b) .

The accessibility of the cellulolytic enzymes to the cellulose fibers/chains is essential for efficient hydrolysis process. Thus, the accessibility to cellulose is related to the specific surface area and crystallinity of the substrate, but also the particle size, porosity and presence of associated compounds such as hemicelluloses and lignin as already mentioned previously. Zhang et al., 2006a investigated the influence of phosphoric acid treatment of Avicel on hydrolysis and observed dramatic differences in the hydrolysis rates of the cellulosic samples before (Avicel) and after phosphoric acid treatment (PASC). Those differences have been related to the differences in structure, and thus accessibility, of the two investigated substrates as it is shown in **Figure 1.9**. The crystal and fiber structure of Avicel is maintained intact, while the structure of PASC is evidently disordered, so that a larger surface area (larger SSA due to swelling) for binding is provided to the enzymes.

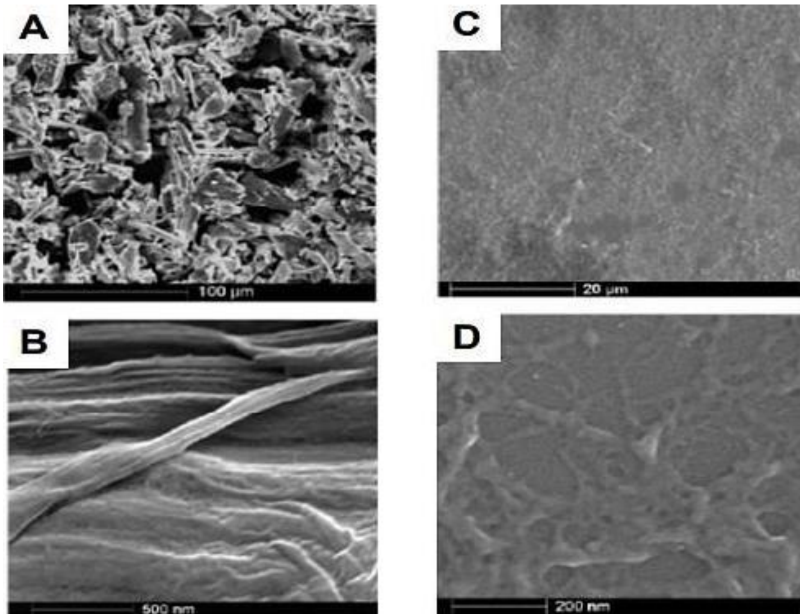


Figure 1.9. Scanning electron microscopy (SEM) images of intact Avicel (A,B) and PASC (C,D) with two different magnifications. (Zhang et al., 2006a)

1.3 Enzymatic hydrolysis process

The pretreatment methods for the lignocellulose bioconversion have a number of disadvantages, such as high energy consumption, low yields and environmental pollution. Therefore, there is a need of an economical, clean and efficient method for the conversion of renewable biomass into high value-added products. The enzymes, acting as catalysts have the key role for this sustainable biotransformation when acting together with the pretreatment technology.

The enzymatic hydrolysis process has attracted much attention as it has a number of advantages such as mild conditions, high specificity, less by-products, high sugar yield, simple equipment and no need for corrosion resistant materials and acid recovery device. However, there are some factors that restrict the efficient enzymatic hydrolysis of lignocellulose and result in low hydrolysis rates and higher prices for the enzymes leading in higher production costs. Therefore, there is a need to focus in an efficient saccharification of biomass that is conducted by the enzymatic hydrolysis method to improve the general yields of the lignocellulose utilization, reduce the production costs and create a more sustainable bio-based process. Several ways for achieving that are 1) an enhanced and appropriate pretreatment technology before the enzymatic hydrolysis, 2) the construction of a synergistic cellulase mixture that improves the enzymatic hydrolysis rate and eliminates product inhibition, and 3) strengthening of the enzymatic hydrolysis process by achieving a full utilization and circulation of the cellulase and developing new enzymatic hydrolysis techniques (Chen, 2014). This thesis is focused on the second aim, which is the use of novel enzymatic cocktails.

The enzymatic hydrolysis of cellulose is a reaction carried out by lignocellulolytic enzymes. Many enzymes are involved in the cell wall deconstruction but the main three categories that are considered necessary are the cellulases, hemicellulases and the accessory enzymes that include hemicellulose debranching, phenolic acid esterase and possibly lignin degrading and modifying enzymes (Himmel et al., 2007). When the hemicellulose post pretreatment barrier associated with the cell wall microfibrils has been weakened by the biomass pretreatment methods, cellulolytic enzymes can access and hydrolyze the crystalline cellulose core of the solid mass (Himmel et al., 2007).

1.4 Cellulases

1.4.1 Molecular structure of cellulases

Cellulases are modular enzymes that are composed of distinct units called Domains or Folds or Modules, which can fold independently. Typically, these enzymes are comprised by a C-terminal carbohydrate binding module (CBM) which can be adsorbed on the cellulose surface and is comprised by small fragments with a molecular weight of 10 kDa. The CBM is linked to an N-terminal catalytic domain (CD). The CD has a catalytic function and can only hydrolyze soluble cellulose (Chen, 2014; Soni *et al*., 2018). The CBM domain is found in the amino terminal or carboxy terminal of the cellulase and is connected to the CD domain through a relatively long highly glycosylated connection bridge (linker peptide). These linkers usually have a disordered arrangement and they are 5-100 residues long. They are composed mostly of proline, hydroxyl amino acid residues (serine and threonine), alanine and glycine. The carbohydrate binding module (CBM) is grouped into 71 families according to amino acid sequence and crystal structure and is thought to be the crucial accessory domain (Boraston *et al*., 2004; Soni *et al*., 2018). The function that makes the CBM so important is the facilitation of cellulose hydrolysis by bringing the catalytic (CD) domain closer to the cellulose network (cell surface), enhancing the hydrolytic activity toward the insoluble cellulose (Morana *et al*., 2011; Soni *et al*., 2018).

The majority of the fungal cellulases have a two domain structure with one catalytic domain (CD) and one cellulose binding domain (CBM), while there are some cellulases (mostly endoglucanases) that lack the cellulose binding domain. Additionally, there are some bacterial cellulases that possess multiple catalytic domains, CBMs and other domains (Morana *et al*., 2011; Ng and Cheung, 2011).

Cellulase *T. reesei* Cel7A is the best characterized cellulase and the major component of fungal enzyme cocktails. It consists of a small Family 1 CBM, an *O*-glycosylated linker and a large catalytic domain (CD) that contains 50 Å tunnel of threading cellulose chains and three sites for *N*-glycosylation (Divne et al., 1998). A schematic representation of the enzyme's processive mode of action on cellulose is depicted in **Figure 1.10**. The steps that are involved in this process include binding of the cellulase to the biomass, recognition of a free cellulose chain end, threading of the chain into the active site tunnel and decrystallization from the substrate, the hydrolysis reaction, product expulsion and reformation of the catalytically active complex (Chundawat et al., 2011).

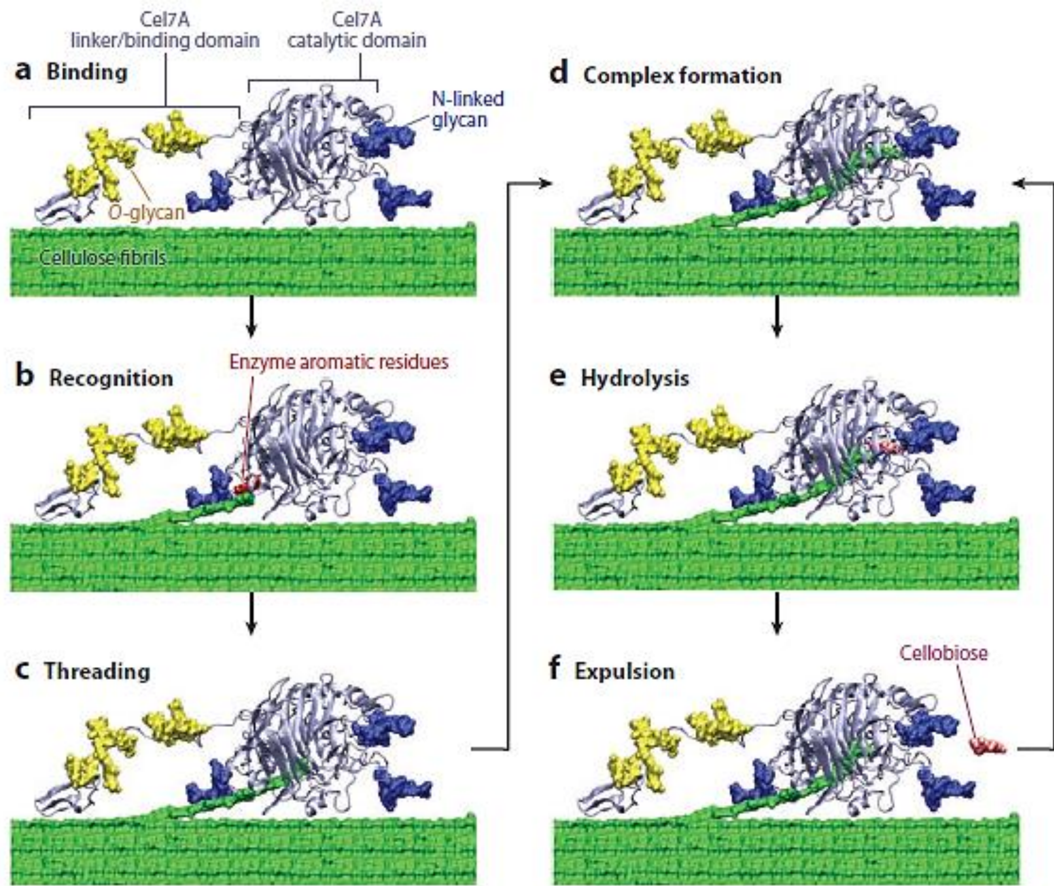


Figure 1.10. Steps involved in the mode of action of the *Trichoderma reesei* exocellulase (Cel7A) on crystalline cellulose. The yellow space-filling representation is *O*-glycosylation, the dark blue spacefill is *N*-glycosylation, the light blue schematic view is the Cel7A enzyme, and the green substrate is a cellulose microfibril. (*a*) Cel7A binding to cellulose, (*b*) recognition of a reducing end of a cellulose chain, (*c*) initial threading of the cellulose chain into the catalytic tunnel, (*d*) formation of a catalytically active complex, (*e*) hydrolysis (the product is shown in pink spacefill), and (*f*) product expulsion and threading of another cellobiosyl unit (Chundawat et al., 2011)

1.4.2 Reaction mechanism of cellulases

The lignocellulose hydrolysis is a heterogeneous reaction since the insoluble substrate (cellulose fibers) interact with the soluble cellulases (Chen, 2014; Soni *et al.*, 2018). The cellulose chains must be accessible to the cellulases in order to achieve an efficient hydrolysis, thus the cellulose microfibrils that are packed with hemicellulose and lignin must be loosened to make the surface area more accessible for the enzymes.

The process of the enzymatic action on cellulose is proposed by Mandels and Reese and is based on the C1-Cx model and suggests that natural cellulose must be synergistically decomposed by different enzymes (Mandels and Reese, 1999). The basic hydrolysis pattern can be described as follows:

$$\text{Crystal cellulose} \xrightarrow{C_1} \text{Amorphous cellulose} \xrightarrow{C_x} \text{Cellulose} \xrightarrow{\beta-\text{glucosidase}} \text{Glucose}$$

At the beginning, the disruption of the cellulose matrix is caused by an unknown component of enzymatic system that is referred to as swelling factor (C1) and makes the substrate accessible for the hydrolytic enzymes (Cx) during the initial stage of saccharification (Soni et al., 2018). Fiber swelling is observed and fragmentation of cellulose into short fibrils takes place, while no release of reducing sugars is observed during this step that is known as amorphogenesis (Esteghlalian et al., 2001). Then, the cellulose chains become available to the cellulolytic enzymes (Cx) and hydrolysis takes place together with the liberation of soluble sugars. The cellulolytic enzymes act on β-1,4-glycosidic bonds of the cellulosic network either by endocleavage or exocleavage. Among cellulases, exoglucanases preferably hydrolyze crystalline regions, whereas endoglucanases hydrolyze soluble and amorphous regions. Finally, β-1,4-glucosidase hydrolyzes cellobiose into glucose (Soni *et al.*, 2018). Thus, the cellulose hydrolysis is achieved by a phenomenon that is known as synergism between the cellulase system (including endoglucanases, exoglucanases and β-glucosidases).

1.4.3 Classification of cellulases

Cellulases belong to a class of enzymes that catalyze the hydrolysis of cellulose. Generally, cellulase is a complex multienzyme system composed of multiple components. According to the substrate specificity, cellulases may be divided into the following components: endoglucanases (EC 3.2.1.4), exoglucanases (EC 3.2.1.74), and β-glucosidases (EC 3.2.1.21) (Chen, 2014; Li et al., 2010).

The cellulases are grouped with many of the hemicellulases and other polysaccharides as *O*-glycoside hydrolases (EC 3.2.1.x). However, the lignin degrading enzymes are sometimes classified as cellulases, although this classification is usually considered inappropriate. Since the complete range of substrate specificity for individual enzymes is rarely determined, it has been suggested an alternative classification of glycoside hydrolases (GH) into families based on their amino acid sequence similarity (Morana et al., 2011). Since the number of identified glycoside hydrolases increases significantly, an integrated database (Carbohydrate-Active enZyme, CAZy) which is continuously updated has been created (http://www.cazy.org/) (Coutinho and Henrissat, 1999). Cellulases are found in different GH families (5, 6, 7, 8, 9, 12, 44, 45, 48, 51, 61, and 74) suggesting that enzymes with similar substrate specificities may belong to different families. For example, GH family 9 contains cellulases from bacteria, fungi, plants and animals, while GH family 7 and GH family 8 contain only fungal and bacterial hydrolases, respectively. Additionally, cellulases from the same microorganism can be found in

different families (e.g *Clostridium thermocellum* contains endoglucanases and exoglucanases from families 5, 8, 9, 44, and 48) (Shoham *et al.*, 1999).

In addition to the CAZy classification, Henrissat *et al.*, 1998 proposed another way to distinguish the enzymes in which the first three letters indicate the preferred substrate, while the number the glycoside hydrolase family and the following capital letter the order in which the enzymes were first reported. For example, the enzymes CBHI, CBHII, and EGI of *Trichoderma reesei* are designated Cel7A (CBHI), Cel6A (CBHII), and Cel6B (EGI) (Morana et al., 2011).

1.4.3.1 Endoglucanases

Endoglucanase (EG) or endocellulase is a 1,4-β-D-glucan-4-glucan hydrolase (EC 3.2.1.4). EGs are often called carboxymethyl cellulases because their enzyme activity is usually determined using the carboxymethylcellulose artificial substrate (CMC) (Lynd *et al.*, 1991; Zhou *et al.*, 2004; Zhang *et al.*, 2006b). EG hydrolyzes randomly internal β-1,4-glycosidic linkages in soluble (such as CMC) and amorphous regions of the cellulose. It generates oligosaccharides with different lengths and exposes new chain ends by cutting at the inner sites of the amorphous region of the polysaccharide chain. This action results in a rapid decrease of the polymer length and thus of the polymerization degree as well as in a gradual increase of reducing sugars concentration (Morana *et al.*, 2011; Chen, 2014; Soni *et al.*, 2018).

1.4.3.2 Exoglucanases

Exoglucanases are further subdivided in cellobiohydrolases (CBHs) (EC 3.2.1.91) and cellodextrinases (EC 3.2.1.74). The cellodextrinase mainly liberates cellobiose by hydrolyzing cellooligosaccharides. The CBH is the major component of the fungal cellulase system accounting for 40-70 % of the total cellulase proteins (Esterbauer et al., 1991). It is a β-1,4-glucan cellobiohydrolase that can hydrolyze cellulose chains by removing processively two units (cellobiose) either from the non-reducing or the reducing ends (e.g *T. reesei* CBHI and CBHII) (Zhang and Lynd, 2004). This action results in rapid release of reducing sugars but the polymer length doesn’t have significant changes. CBH prefers highly crystalline compounds like cellooligosaccharides and Avicel. It can be further categorized into two groups according to the main product that is obtained due to its reaction. Is the main product is glucose it is named Glucanohydrolase and if the main product is cellobiose it is named Cellobiohydrolase (Morana *et al.*, 2011; Chen, 2014; Soni *et al.*, 2018).

- *Processivity*

A crucial property of the cellobiohydrolases (CBHs) is their processivity. The enzyme processivity is the ability to remain attached to the substrate in between subsequent hydrolytic reactions. This mechanism is beneficial for the efficient degradation of crystalline substrates because the enzyme remains closely associated with the detached single polymer chain in between subsequent hydrolytic steps (Horn et al., 2012a). Due to the 180° rotation between consecutive sugar units, processive degradation of cellulose yields disaccharides (Davies and Henrissat, 1995; Rouvinen et al., 1990). All products generated by the processive enzymes are soluble, except for the first product. Thus, the products of the processive enzymes create much more reducing ends, than for example endoglucanases (EGs) which mainly generate insoluble reducing ends (Horn et al., 2012a).

Processive cellulases from several microorganisms have been studied such as *Trichoderma reesei*, *Humicola insolens*, *Thermobifida fusca*, *Clostridium cellulolyticum*, etc (Horn et al., 2012a). However, the molecular mechanism of processivity and the implication of processivity on the conversion of biomass are partly understood. This is due to the nonstandard kinetic complexities of the enzymatic reaction especially regarding the identification of the product "release" process. Further research needs to be done regarding the structural basis, the molecular mechanism and the biotechnological implications of cellulase processivity (Horn et al., 2012a).

1.4.3.3 β-Glucosidases

β-1,4-Glucosidase (BG) hydrolyzes soluble cellobiose and other cellodextrins with a DP up to 6 from the non-reducing ends and produces glucose. The hydrolysis rate of BG increases with decrease of the substrate's size (Zhang *et al.*, 2006; Morana *et al.*, 2011; Chen, 2014).

1.4.4 Auxiliary enzymes

The Lytic polysaccharide monooxygenase (LPMO) is a relatively novel cellulose degrading enzyme that was initially discovered for its activity on chitin degradation (Horn et al., 2012b; Vaaje-Kolstad et al., 2010) . The LPMO belongs to the auxiliary activities (AA) enzyme class and is currently categorized in auxiliary activity families 9-11 and 13 of the CAZy database (Levasseur et al., 2013).

It is a copper dependent monooxygenase (metalloenzyme) that uses molecular oxygen and an electron donor to break glycosidic bonds (Hemsworth et al., 2013; Horn et al., 2012b; Vaaje-Kolstad et al., 2010). The main electron donors are either derived from natural or exogenous sources and are small molecule reductants such as gallic and ascorbic acids, biomass derived

soluble compounds, phenols from fungi or lignin, etc. (Monclaro and Filho, 2017). LPMO is based on an oxidative mechanism that cleaves cellulose which is thought to be mediated through a divalent metal ion that is coordinated by a histidine-brace. The latter, has three nitrogen ligands that are provided by the amino group and side chain of two N- terminal His residues, respectively. The active site of the LPMO is planar and cleaves cellulose with a mechanism that involves the conserved carboxylic acid residues that are located within this site (Karnaouri et al., 2017). A generalized model mechanism of the action of these enzymes has not yet been identified; however the most common oxidation takes place at the C1 and/or C4 carbon in the glucose ring structure (Dimarogona et al., 2012a).

LPMOs have been associated with the depolymerization of recalcitrant polysaccharide chains in their crystalline regions based on the principle of oxidative disintegration in order to release oxidized oligosaccharides (Vaaje-Kolstad et al., 2010). The LPMO has a strong effect in the saccharification process as it enhances the soluble sugar yield from the lignocellulosic biomass and has been reported to work in association with the other cellulolytic enzymes (Hemsworth et al., 2013; Patel et al., 2016).

1.4.5 Synergism between the enzymes

For the complete saccharification of lignocellulosic biomass a repertoire of core and accessory hydrolytic enzymes that belong to different hydrolase families are required which act in a synergistic way.

Three cellulases represent the core enzymes: endoglucanases (EG), exo-glucanases (cellobiohydrolases (CBH)), and β-glucosidases (BG). These enzymes act in a different mode of action on the cellulose substrate. The endoglucanases randomly hydrolyze intramolecular β-1,4-glucosidic bonds on amorphous cellulose surfaces, generating new chain ends (reducing and non-reducing). The cellobiohydrolases cleave the cellulose chain in a processive manner from either the reducing or non- reducing ends, depending on the CBH type and producing cellobiose as product. Then, the released oligosaccharides are hydrolyzed to glucose by the β-glucosidases so that the system is relieved by end product inhibition (Davies and Henrissat, 1995; Himmel et al., 2007).

Synergism among the cellulases and the non-cellulolytic partners is based on variation in their substrate specificities, the endo-/exo-catalytic mechanisms and removal of the inhibitory products (Kim et al., 2014). Synergism of these enzymes to accessory hydrolytic and non-hydrolytic enzymes, e.g. esterases, lyases, and oxidases, attacking the hemicellulose and lignin-carbohydrate complex as well as various auxiliary enzymes and proteins enhances the catalytic efficiency on lignocelluloses (Bunterngsook et al., 2018). A schematic representation of the

synergistic actions of these enzymes in different polymers (lignin, hemicellulose, cellulose) is depicted in **Figure 1.11**.

The C1-Cx model as described previously (Section 1.4.2) is another way of explanation of the synergism among the enzymes. Among the C1 factors that have been suggested, the non-catalytic cellulose loosening expansin proteins and the lytic polysaccharide monooxygenase (LPMOs) oxidative enzymes are very promising for the enhanced lignocellulose bioconversion (Cosgrove, 2017; Monclaro and Filho, 2017). Particularly, the LPMOs have been associated with the promotion of the amorphogenesis of the substrate, possibly acting as the first component (C1) on the hydrolysis mechanism (Dimarogona et al., 2012a; Karnaouri et al., 2017). The LPMOs have gained much interest the last years for their huge potential in the enzymatic polysaccharide conversion, demonstrating that the oxidative attack together with the hydrolytic attack is very essential in the biomass degradation process (Dimarogona et al., 2012a; Karnaouri et al., 2017; Monclaro and Filho, 2017). As mentioned before, LPMOs require substances to act as electron donors and there are some indications suggesting that lignin could act as an electron donor (Monclaro and Filho, 2017). Some researchers indicated that the LPMOs can use non cellulosic biomass components even at low concentrations, such as soluble compounds, lignin and potentially hemicellulose (Hu et al., 2014). Therefore, lignin in biomass can play a key role for this new generation enzymatic cocktails including the LPMO. It has also been demonstrated that there is no antagonistic relation between the oxidoreductive cellulose degrading system and the hydrolytic cellulase system. The LPMOs tend to attack the highly crystalline cellulose regions, while the endoglucanases (EGs) attack the amorphous regions and the cellobiohydrolases (CBHs) require a chain end in order to act processively in the crystalline cellulose (Dimarogona *et al.*, 2012).

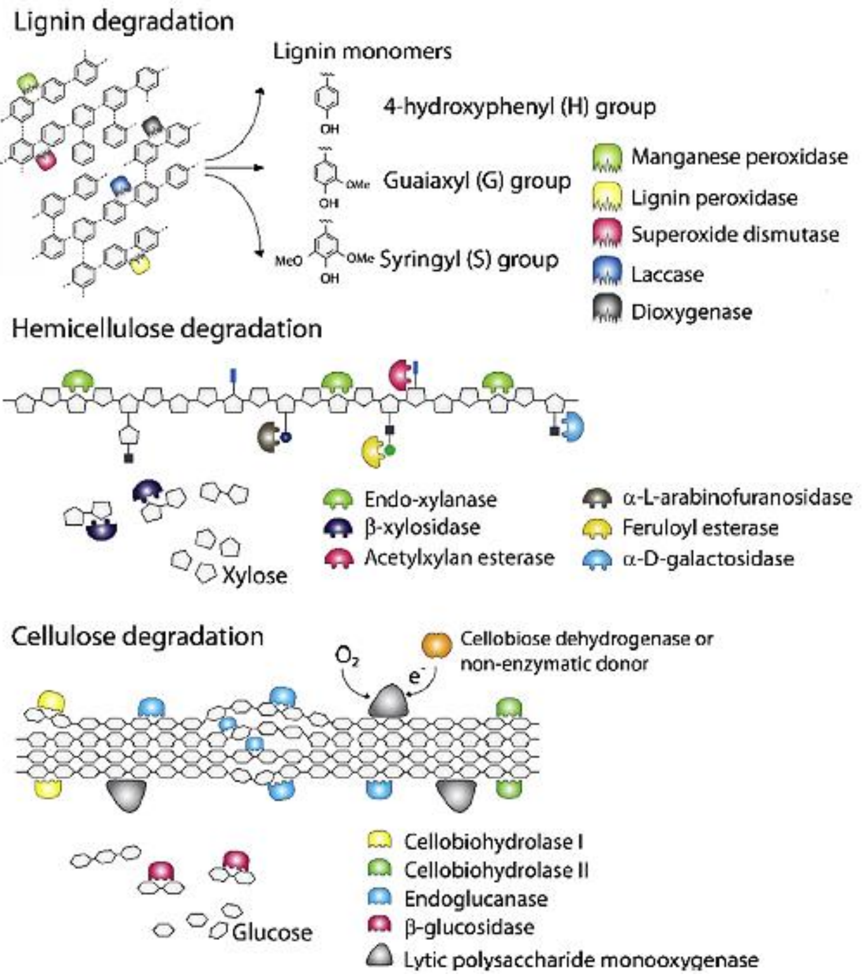


Figure 1.11. Representation of lignocellulose degradation (lignin, hemicellulose and cellulose polymers) by the synergistic actions of glycosyl hydrolases and auxiliary activity enzymes (Champreda et al., 2019)

1.5 Pichia pastoris yeast

Approximately 30 years ago the ability of certain yeasts to utilize methanol as a sole source of carbon and energy was discovered by Koichi Ogata (Ogata et al., 1969). *Pichia pastoris* is one of approximately 30 yeast species that represent two different genera (*Pichia* and *Candida*) capable of metabolizing methanol (Lee and Komagata, 1980).

Yeasts are used as expression systems for the production of eukaryotic heterologous proteins as they combine the molecular genetic manipulation and growth characteristics of prokaryotic organisms together with the subcellular machinery for performing post-translational protein modification of eukaryotes. The first yeast that was selected for this purpose was the *Saccharomyces cerevisiae*, although it did not prove ideal for as foreign gene expression host (Cregg *et al.*, 1993). The methylotrophic *P. pastoris* yeast proved advantageous compared to the *S. cerevisiae* as a potential expression host.

As a yeast, *P. pastoris* is a single celled microorganism that is easy for culture and manipulation. Furthermore, being an eukaryote is capable of many post translational modifications such as proteolytic processing, folding, disulfide bond formation and glycosylation. Thus, many proteins that end up inactive in bacterial systems are produces as active molecules in *P. pastoris* host. Additionally, the system of *P. pastoris* is generally faster, easier and less expensive to use than systems originated form higher eukaryotes and also gives higher expression levels (Higgins and Cregg, 1998). Therefore, several fermentation techniques were developed for maintaining the organism in large volume continuous culture and at cell densities in excess of 100 grams/liter dry cell weight. The growth medium for *P. pastoris* yeast is inexpensive and is consisted of a mixture of salts, trace elements, biotin and a carbon source (Cregg *et al.*, 1993). The successful expression of more than 200 different heterologous proteins in *P. pastoris* yeast has already been published and a website has been created and maintained by the Cregg lab which lists heterologous proteins expressed in *P. pastoris* (http://www.kgi.edu/html/noncore/program4.htm#jc) (Cregg et al., 2000).

The *P. pastoris* yeast is one the most suitable protein production host systems with the successful expression of many industrial enzymes as well as pharmaceutically relevant proteins, membrane proteins and small bioactive and antimicrobial peptides, with the need to find alternatives for induction to replace methanol for industrial scale fermentations (Ahmad et al., 2014).

1.5.1 Methanol metabolism

The methanol metabolic pathway seems to be the same in all yeasts and involves a unique set of pathway enzymes (Veenhuis *et al.*, 1983). The first step is the methanol oxidization to formaldehyde, generating hydrogen peroxide in the process by the enzyme alcohol oxidase (AOX). This first step in methanol metabolism takes place within a specialized organelle that is called the peroxisome for avoiding the hydrogen peroxide toxicity and at the same time to keep away toxic hydrogen peroxide from the rest of the cell (Higgins and Cregg, 1998). Catalase which is also found inside the peroxisome is responsible for the degradation of hydrogen peroxide to oxygen and water. A formaldehyde portion leaves the peroxisome and is further oxidized to formate and carbon dioxide by two cytoplasmic dehydrogenases, reactions that are an energy source for cells growing on methanol. The remaining formaldehyde forms cellular constituents by a reaction that is catalyzed by the third peroxisomal enzyme, the dihydroxyacetone synthase (DHAS). The subsequent reactions of methanol assimilation and dissimilation are localized in the cytosol (Cereghino and Cregg, 2000).

The AOX enzyme is a homo-octomer and each subunit contains one noncovalently bound FAD (flavin adenine dinucleotide) cofactor. It has a poor affinity for O2 and methylotrophic yeasts appear to compensate for this deficiency by synthesizing large enzyme amounts (Higgins and Cregg, 1998). In *P. pastoris* there are two genes that code for AOX, the AOX1 and the AOX2. However, only the AOX1 is responsible for the majority of alcohol oxidase activity in the cell (Cregg et al., 1989). The AOX1 gene expression is induced by methanol to high levels. The level of this gene expression is generally lower (~5% of total soluble protein) in methanol grown shake flask cultures than in fermentor cultures (≥30%) (Couderc and Baratti, 1980). The AOX and the DHAS enzymes are undetectable in cells cultured on carbon sources such as glucose, glycerol or ethanol, however the presence of methanol is essential for the production of the DHAS enzyme and the expression of the AOX1 gene which is controlled at the level of transcription (Cereghino and Cregg, 2000; Tschopp et al., 1987).

1.5.2 Secretion of heterologous proteins

Yeast expression systems are very important for their ability to secrete heterologous protein products. Since 2002 there has been a great increase in foreign protein production in *P. pastoris* yeast. The heterologous proteins in *P. pastoris* yeast can be expressed either intracellularly, or extracellularly with secretion into the medium. The production of large amounts of heterologous proteins in shake flask cultures is difficult since there are many limitations, such as the volume, the oxygen transfer, the substrate addition and the inability to

monitor these factors efficiently. Thus, the use of bioreactors is preferable since a simultaneous monitoring of all these parameters is possible (Macauley-Patrick et al., 2005).

The secreted heterologous protein comprises the majority of the total protein in the medium since *P. pastoris* secretion of endogenous proteins occurs in low levels and its culture medium contains no added proteins (Barr *et al*., 1992). The secretion step requires the presence of a signal sequence on the foreign protein in order to target it to the secretory pathway and it serves as a first step in purification, separating the foreign protein from the cellular proteins (Higgins and Cregg, 1998).

1.5.3 Post translational modifications

P. pastoris yeast has the ability to perform many of the posttranslational modifications that are generally associated with higher eukaryotes as previously mentioned. One of the most common and most complex is the O- and N-linked glycosylation of secreted proteins. Differences in the number and type of sugar units added by humans as compared with the ones added by *P. pastoris* yeast can be problematic for the use of yeast secreted proteins as therapeutic products (Cereghino et al., 2002; Cregg et al., 2000). Since the recombinant proteins are not compatible with the mammalian ones, once they are introduced into the bloodstream they are rapidly cleared from it. To overcome this challenge two strategies have been suggested; changing the amino acid sequence of a secreted protein to eliminate glycosylation sites; or to engineer *P. pastoris* strains with more human-type glycosylation properties (Cereghino et al., 2002). The most desirable option is the second, however it is the most complex. One group of researchers proved that is possible to engineer the glycosylation pathway in *P. pastoris* by humanizing glycoproteins produced in *P. pastoris* (Bobrowicz et al., 2004). Engineering *Pichia* cells is possible due to the increased knowledge about the optimal glycosylation patterns, although it is not possible to generalize these patterns since for each recombinant protein it should be considered the context of the system in which it is expressed and the terms of the purpose for which it will be used (Macauley-Patrick et al., 2005).

1.6 Probiotics

The word "probiotic" is derived from the Greek word that means "for life". The first generally accepted definition for probiotics was given by Fuller (1989) who defined probiotics as "live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance". However, the importance of the probiotics was pointed out many years ago by Metchnikoff who first regarded the use of probiotics in yoghurt fermentation process (Fuller, 1992). The most commonly found probiotics in humans are the lactic acid bacteria, *lactobacilli* and the *bifidobacteria* species. The probiotic products may contain either a single strain or a mixture of bacterial strains. Probiotics have been associated with beneficial effects in a wide variety of biomedical conditions such as; diarrhea, constipation, colitis, recolonization by pathogens, flatulence, gastroenteritis, gastric acidity, immunostimulation, hypercholesterolemia, carcinogenesis etc. Furthermore, they contribute to the synthesis of nutrients and improve bioavalaibility; some probiotics are known to exert anti-oxidative activity (Gibson and Roberfroid, 1995; Pandey et al., 2015).

The importance of the probiotics lays in the fact that the microflora in the human large intestine is thought to compromise about 95 % of the total cells in the body and consists up to 10^{12} bacteria for every gram of gut contents, therefore through the characteristics of the host's microflora it plays a major role in the host's nutrition and general well-being (Gibson and Roberfroid, 1995; Manning and Gibson, 2004). The gut's microflora composition is thus the main target that should be regulated through diet or through functional food products.

1.6.1 Lactic acid bacteria (LAB)

The LAB are classified as GRAS (generally recognized as safe) microorganisms (Adams 1999). One important characteristic of the LAB is their diverse capacities to utilize carbohydrates, which is reflected by the high number of genes encoding putative sugar transporters. The components that are responsible for the catalysis of sugar transport and phosphorylation are the predicted phosphoenolpyruvate-dependent sugar PTSs (Postma *et al.*, 1993). The LAB mainly employ homo- and hetero-fermentative fermentation. The homofermentative species only ferment hexoses and almost exclusively (85%) to lactic acid. The heterofermentative species metabolize pentoses and hexoses and produce lactic acid, ethanol (or acetic acid) and CO_2 (Hammes and Vogel, 1995).

The main components that are produced by the intestinal LAB during the fermentation of the NDOs are the short chain fatty acids (SCFA). The SCFA are volatile acids and the most abundant which represent the 90-95% of the SCFA that exist in the colon are the acetic acid

(C2), the propionic acid (C3) and the butyric acid (C4) (Ríos-Covián et al., 2016). Lactate, which is not a SCFA, is the main product of the LAB and is not accumulated in the colon due to the presence of some species that convert lactate into SCFA (Flint et al., 2014). The amount of SCFA is directly related with human health, as the diet affects the composition of the gut microbiome and therefore the production of different SCFA (Brüssow and Parkinson, 2014; Louis *et al*., 2014). It has been found that generally the SCFA are associated with the inhibition of pathogenic microorganisms and the nutrient absorption increase, while acetate is related with the inhibition of enteropathogens by the *Bifidobacterium* species (Fukuda et al., 2011; Macfarlane and Macfarlane, 2012). Additionally, the butyrate and the propionate have a higher effect on obesity prevention than acetate (Gao et al., 2009; Lin et al., 2012). Studies are also highlighting the effect of butyrate against the colorectal cancer development (Canani et al., 2011; Keku et al., 2015).

1.6.1.1 Lactobacilli

The *Lactobacillus* genus is the largest, most diverse group among the LAB, constituted by over 100 species (Zhang and Cai, 2014) . According to several studies, certain strains have probiotic characteristics as they show beneficial effects on gut functionality (Galdeano and Perdigón, 2006; Gueimonde et al., 2006; Kalliomäki et al., 2001). *Lactobacillus plantarum* species is found in the human's gastrointenstinal tract (GIT) (Siezen et al., 2010a) and is known to have the largest genome size between other LAB, encoding a significantly high number of PTS genes (Cai et al., 2009; Kleerebezem and Hugenholtz, 2003; Morita et al., 2009). This has been related with the strain's ability to ferment a wide range of carbohydrates (Bringel *et al*., 2001). It's a facultatively heterofermentative species (Salvetti *et al*., 2012).

Another predominant species of the *Lactobacillus* genera is *Lactobacillus gasseri*. It can be found in the GIT, as well as in the oral cavity and the vaginal system and it has homofermentative and thermophilic characteristics (Francl et al., 2010; Singroha et al., 2017). It has a considerable number of PTS genes (Zhang *et al*., 2013). *Lactobacillus reuteri* is also a homofermentative species (Salvetti *et al*., 2012). According to Zhang *et al*., 2013 this strain has a low number of PTS genes and thus exhibits limited capacity for carbohydrate utilization compared to other *lactobacilli* strains.

1.6.1.2 Bifidobacteria

The *Bifidobacterium* genus is another important group of the LAB, although they have different phylogenetic origins (Biavati, 2001). They are heterofermentantive microorganisms and one of the largest microbial populations in the human GIT, as they consist the 91% of the total gut microbiota (Harmsen et al., 2000). Studies that used species of this genus as probiotics showed a positive effect in the management of active ulcerative colitis (Kato et al., 2004). Additionally,

this LAB genus is known to synthesize vitamins that belong to B family, although this vitamin producing ability differs among the different *Bifidobacterium* species (Pompei et al., 2007).

Like the other LAB of the colon, the *Bifidobacteria* are able to metabolize in a strain dependent way different carbohydrates (de Vrese and Schrezenmeir, 2008). Generally, the polymeric carbohydrates are first degraded to low molecular weight oligosaccharides and subsequently to monosaccharides using carbohydrate degrading enzymes. These monosaccharides are then converted to intermediates of the hexose fermentation pathway (de Vries and Stouthamer, 1967). All the species that belong to this genus use a metabolic pathway (the 'bifid' shunt) for the degradation of hexoses which is different to that of the *Lactobacillus* genus (Felis and Dellaglio, 2007). The bifidobacterial genome has proved to include genes that encode certain carbohydrate modifying enzymes, such as glycosyl hydrolases, sugar ABC transporters, PEP-PTS (PEP—phosphoenolpyruvate; PTS—phosphotransferase system) components that are necessary for the degradation of plant- and host- derived carbohydrates (Pokusaeva *et al.*, 2011).

The *Bifidobacterium longum* is one of the most representative species that belong to this genus (Harmsen et al., 2000). Its genome contains a high number of various genes that are responsible for the fermentation of different carbohydrates (Lorca et al., 2007). *Bifidobacterium animalis* subsp. *lactis* has a significantly smaller genome than that of *B.longum* and thus smaller number of genes that are responsible for the carbohydrate fermentation (Barrangou et al., 2009). The *Bifidobacterium adolescentis* genes number is between that of the *B.animalis* subsp. *lactis* and the *B.longum* (Pokusaeva et al., 2011). Depending on the carbohydrate-degrading enzymes of the *Bifidobacteria* species there is accordingly a preference on the substrates which can subsequently lead to the discovery of new possible prebiotic candidates.

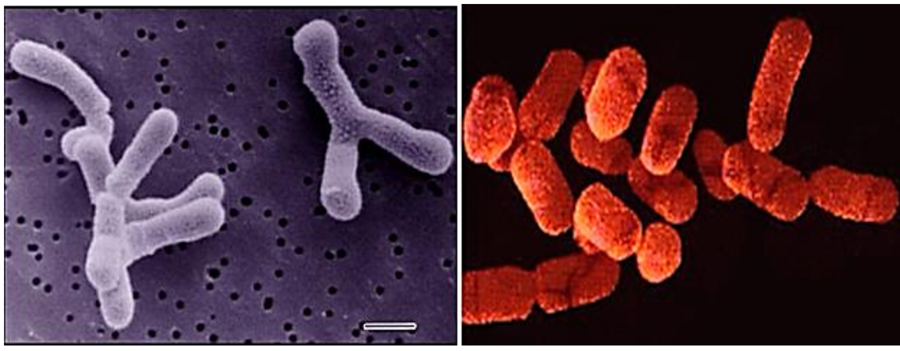


Figure 1.12. (left) *Bifidobacterium adolescentis* is a gram positive bacterium with a Y- or V-shape, while *Lactobacillus plantarum* (right) is a gram positive bacterium with a rod shaped structure with rounded ends (https://microbewiki.kenyon.edu).

1.7 Prebiotics

Prebiotics were first defined as "non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one, or a limited number of bacteria in the colon that can improve the host health."(Gibson and Roberfroid, 1995). As potential prebiotic is considered any food that contains carbohydrates, and in particular oligosaccharides, although it should satisfy certain characteristics, such as; 1) resistance to gastric acidity, to hydrolysis by mammalian enzymes and to gastrointestinal absorption, 2) fermentation by intestinal microbiota, 3) selective stimulation of the growth and/or activity of the intestinal bacteria that contribute to health and well-being. According to the first characteristic, resistance to digestion, the prebiotic does not need to be completely indigestible but significant amounts should be available in the intestine (especially the large bowel) in order to provide a fermentation substrate. The third characteristic is the most difficult to fulfill (Gibson and Roberfroid, 1995).

A wide variety of dietary carbohydrates are considered as potential substrates for bacterial fermentation, with resistant starch (RS) being the most quantitatively important. Additionally, non-starch polysaccharides (NSP) including plant derived substrates such as pectin, cellulose, hemicellulose and xylan have a large contribution, as well as sugars and oligosaccharides such as lactose, raffinose and fructo-oligosaccharides (FOS). Finally, proteins and peptides are also potential candidates. Prebiotic intake is also possible to be obtained through the diet through certain fruits and vegetables, however the levels of the natural prebiotics is too low, indicating the need for enhancing the levels of prebiotic intake (Manning and Gibson, 2004). Among the candidate prebiotics only bifidogenic, non-digestible oligosaccharides fulfill all the above criteria for being considered as functional prebiotics (Gibson and Roberfroid, 1995).

A combination of probiotics and prebiotics lead to the term of synbiotics. Synbiotics were defined as mixtures of probiotics and prebiotics that beneficially affect the host by improving the survival and Implantation of live microbial dietary supplements in the gastrointestinal tract of the host (Andersson et al., 2001). Synbiotics were developed to overcome possible survival difficulties for probiotics. Based on several observations the use of sunbiotics shows an improvement of survival of the probiotic bacteria during the passage through the upper GIT (Pandey *et al.*, 2015).

1.7.1 Non digestible oligosaccharides (NDOs)

The non-digestible oligosaccharides are carbohydrates with a low degree of polymerization (DP) and therefore low molecular weight that contain between 3 and 10 sugar units (Mussatto

and Mancilha, 2007). Some of them, such as inulin from chicory have a degree of polymerization up to 60 or down to 2 such as lactulose or cellobiose. Commercial NDOs are mixtures of oligosaccharides with variable DP (Swennen *et al.*, 2006). The concept of the NDOs first originated from the observation that the anomeric C atom (C1 or C2) of the monosaccharide units of some dietary oligosaccharides has a configuration that makes their osidic bounds non digestible to the hydrolytic activity of the human digestive enzymes (Roberfroid and Slavin, 2000). Particularly, the indigestibility of the NDOs results either from the configuration of the glycosidic bond between monomeric sugar units, or the substrate selectivity of gastrointestinal digestive enzymes. The inability of humans to digest NDOs is due to the fact the sugar units of the NDOs are linked by glycosidic bonds that have a β-configuration and thus cannot be degraded by human gastrointestinal enzymes since they are specific for α-configuration glycosidic bonds (Swennen *et al.*, 2006).

The main categories of non-digestible oligosaccharides include carbohydrates in which the monosaccharide unit is fructose, galactose, mannose, arabinose, glucose and/or xylose, such as fructooligosaccharides (FOS), galactooligosaccharides (GOS), mannooligosaccharides (MOS), arabinooligosaccharides (AOS), xylooligosaccharides (XOS) and cellooligosaccharides (COS) (Roberfroid and Slavin, 2000).

Various health benefits are associated with the NDOs, such as improved blood lipid metabolism, regulation of gastrointestinal function, prevention and treatment of constipation, increased vitamin synthesis and improved human immunity. Additionally, they can be used as protective agent when bacteria encounter changes of temperature, pH and other growth conditions (Chen *et al.* 2007). Since the NDOs beneficially affect the host, they are considered as functional foods, and their ability to be fermented by the beneficial bacterial species of the colon, contributes to their prebiotic characteristic.

Over 20 different types of NDOs are currently available in the market and they are either extracted from natural sources, obtained by enzyme processing, or produced chemically (Swennen *et al.*, 2006). The main way for NDOs production is the enzymatic process, since it offers mild reaction conditions and less chemically harsh by-products.

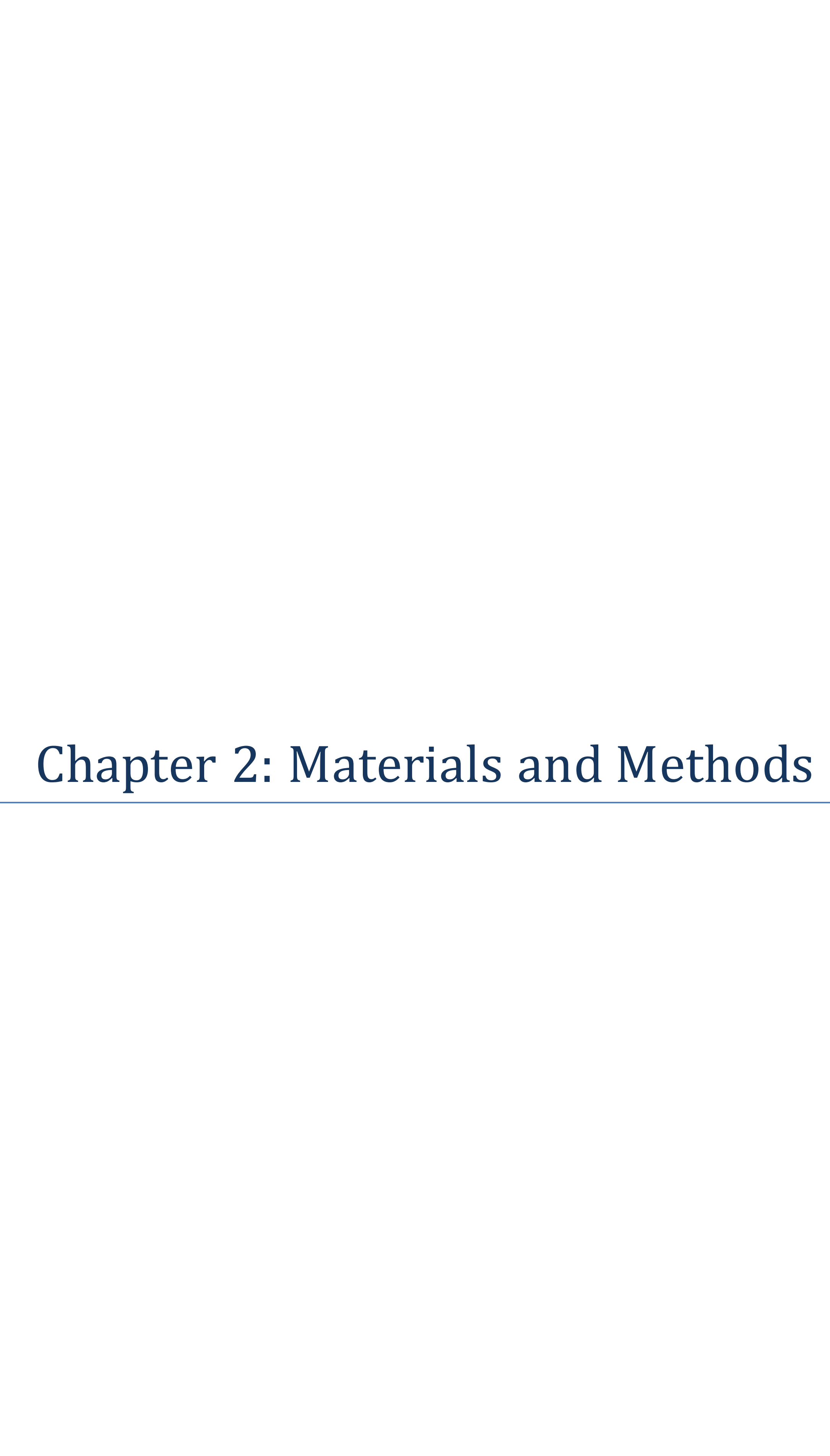
A new source of NDOs is plant cell wall polysaccharides. Such plant polysaccharides are often present in large amount in fiber rich by products and wastes (e.g. lignocellulosic biomass etc.). The availability of well-defined enzymes or enzyme combinations for the production of NDOs from these substrates is a prerequisite (Mussatto and Mancilha, 2007). The plant cell wall polysaccharides as a novel source for the production of prebiotic oligosaccharides has gained increasing attention, since it offers a sustainable and attractive utilization of the agricultural and industrial residues leading to the development of a bio-based economy. This thesis focuses on the conversion of lignocellulosic biomass as raw material for the production of NDOs as a high value added products for a potential prebiotic utilization. Particularly, these NDOs are cellooligosaccharides (COS).

1.7.2 Prebiotic potential of cellobiose

Cellooligosaccharides (COS) are linear oligosaccharides that are composed of β-1,4-linked glucopyranose units. The COS are important functional oligosaccharides that are significant in the feed and food industry with an increasing interest as potential prebiotic. Additionally, they can be derived from the most abundant carbon source on Earth; lignocellulose, therefore they are potentially the most abundant available NDOs. However, there is still limited information regarding the large scale production of COS. Two main strategies are being used for the production of COS; acid based and enzyme based hydrolysis of the cellulose. The latter, is considered as more attractive as previously mentioned for the use of milder reaction conditions and the less monomer production (Mussatto and Mancilha, 2007).

The bioavailability of cellobiose in humans has already been evaluated with cellobiose tolerance tests and breath hydrogen excretion (Nakamura *et al*., 2004). It has consequently been observed that after ingestion cellobiose can be fermented by the gut microflora and that it cannot be hydrolyzed by the digestive enzymes, therefore it reaches the colon undigested (Basholli-Salihu et al., 2013). Few studies have demonstrated the cellobiose effect on the growth of Bifidobacterium species and showed that it has a higher prebiotic potential than FOS (Pokusaeva et al., 2011b). Additional studies have been carried out with humans and rodents and suggested the beneficial effects of cellobiose on carbohydrate metabolism, diabetes and obesity (Satouchi *et al*., 1996; Watanabe, 1998).

The effect of cellobiose on the growth of gut microbiota (*Lactobacillus* and *Bifidobacteria* species) has yet to be determined. In this thesis, the cellobiose production from the abundant lignocellulose biomass source was investigated as well as the cellobiose prebiotic potential on lactic acid bacteria.



Chapter 2: Materials and Methods

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals-Equipment

The equipment that was used for this thesis and was available at Luleå Tekniska Universitet (LTU) is the following:

- Water thermostat bath WB-4MS (Biosan, Latvia)
- pH-meter pHenomenal® 1100 H (VWR U.S)
- Eppendorf ThermoMixer® C Incubator (Eppendorf, Germany)
- Q-POD® Ultrapure Water Remote Dispenser (Millipore U.S)
- Dionex™ ICS-5000+ Capillary HPIC™ System and Chromeleon™ Chromatography Data System (CDS) Software (ThermoFischer Scientific, U.S)
- Systec V-100 (Systec GmbH, Germany) vertical Top Loading Autoclave
- Spectra max M2 Microplate Reader (Molecular Devices, U.S)
- Spectrophotometer GENESYS™ 10S UV-Vis (ThermoFischer Scientific, U.S)
- Pellicon® XL Device and Labscale™ TFF System (Millipore U.S)
- ÄKTA Prime Plus liquid chromatography system, equipped with Prime View 5.31. software (GE Healthcare Life Sciences)
- Novex™ XCell *SureLock*™ Mini-Cell (Invitrogen , U.S) with pre-casted Nu-PAGE® Novex 4-12% Bis-Tris gels
- Eppendorf® Centrifuge 5804/5804R (Eppendorf, Germany) and Microcentrifuge Galaxy Mini (VWR U.S)
- HPLC 200 (Perkin Elmer U.S), equipped with an RID detector, oven and Aminex HPX-87N Column (Biorad U.S)
- Nanofiltration system (Sterlitech, U.S.), HP4750 High Pressure Stirred Cell
- Gel imaging and analysis system InGenius BioImaging, Syngene (U.K.) equipped with GeneSnap v6.05 and GeneTools v3.06 software.
-

2.1.2 Enzymes

To study the cellobiose production from biomass, commercially available processive enzymes were used. Four of them were purchased from Megazyme (U.S.A) and are the following:

- endo-1,4-b-D-glucanase (EG5) from *Talaromyces emersonii* (E-CELTE)
- endo-1,4-b- D-glucanase (EG7) from *Trichoderma longibrachiatum* (E-CELTR)
- cellobiohydrolase I (CBH7) from *Trichoderma longibrachiatum* (E-CBHI)
- cellobiohydrolase II (CBH6) of microbial origin. (E-CBHIIM)

A total amount of 14 processive cellulases were purchased from NZYTech Lda. (Portugal) and are summarized at the following table together with some details of interest:

Table 2.1. Processive cellulases purchased from NZYTech

Number	Enzyme name	Short name	Organism	Architecture	Temperature
1	Cellobiohydrolase 48A	CcCel48A	*Clostridium cellulolyticum*	GH48	37
2	Cellobiohydrolase 5A	CtCbh5A	*Clostridium thermocellum*	GH5	60
3	Cellulase 9A	CtCel9A	*Clostridium thermocellum*	GH9	60
4	Cellobiohydrolase 9A	CtCbh9A	*Clostridium thermocellum*	GH9	60
5	Cellulase 9B	CtCel9B	*Clostridium thermocellum*	GH9	60
6	Cellobiohydrolase 6A	PaCbh6A	*Podospora anserina*	GH6	50
7	Cellulase 9W	CcCel9W	*Clostridium cellulolyticum*	GH9	37
8	Cellulase 9M	CcCel9M	*Clostridium cellulolyticum*	GH9	37
9	Cellulase 9R	CcCel9R	*Clostridium cellulolyticum*	GH9	37
10	Cellulase 9A	CcCel9A	*Clostridium cellulovorans*	GH9	37
11	Reducing-end cellobiohydrolase 48A	CsCbh48A	*Clostridium stercorarium*	GH48	60
12	Cellulase 9J	CcCel9J	*Clostridium cellulolyticum*	GH9	37
13	Cellulase 9Q	CcCel9Q	*Clostridium cellulolyticum*	GH9	37
14	Cellulase 9A	RfCel9A	*Ruminococcus champanellensis*	GH9	50

Apart from the commercially available enzymes, three recombinant biocatalysts were used in the experiments. The enzymes, primarily encoded by the thermophilic fungus *Thermothelomyces thermophila* (previously known as *Sporotrichum thermophile* or *Myceliophthora thermophila*), were expressed in the heterologous host *Pichia pastoris* yeast. The enzymes included two endoglucanases of Glycoside Hydrolase family GH5 (*Tt*EG5) and GH7 (*Tt*EG7) (Karnaouri et al., 2014b; Karnaouri et al., 2017), a GH7 cellobiohydrolase (*Tt*CBH7) and a lytic polysaccharide monooxygenase (*Tt*LPMO) of Auxiliary Activity family AA9 (Karnaouri et al., 2017, 2018). The procedure that was followed will be analyzed in the Section 2.2.1 of this thesis.

2.1.3 Substrates

Organosolv-pretreated birch (B4: 200 ◦C for 30 min, 60% (v/v) EtOH, B1: 200◦C for 15 min, 60% (v/v) EtOH) and spruce (S3: 200 ◦C for 30 min, 52%(v/v) EtOH) were used as substrates (Matsakas et al., 2018, 2019). The compositional analysis of the materials is described in **Table 2.2**.

Carboxymethyl cellulose (CMC), microcrystalline cellulose Avicel PH-101 and cellooligosaccharides (DP2-6) were obtained from Sigma-Aldrich (USA). Celloheptaose (DP7) and celooctaose (DP8) were obtained from Elicityl - Oligotech® (France). Phosphoric acid swollen cellulose (PASC) was prepared from Avicel, following the protocol initially described by Wood, 1988.

Table 2.2. Compositional analysis for the pretreated materials used for hydrolysis.

Sample	Cellulose(%)	Hemicellulose(%)	Lignin(%)	References
Birch (B1)	66.3	22	7.8	Matsakas et al., 2018
Birch (B4)	67.1	21	7.1	Matsakas et al., 2018
Spruce(S3)	66	6	14.9	Matsakas et al., 2019

The composition is given on w/w dry mass.

2.1.4 Bacterial strains

Six bacterial strains that belong to the *lactobacilli* and *bifidobacteria* species (3 strains for each species respectively) were included in this study as they are representative species with probiotic properties. *Bifidobacterium adolescentis* DSM 20083, *Bifidobacterium longum* DSM 20219, Lactobacillus gasseri DSM 20077 and *Lactobacillus reuteri* DSM 20016 were purchased from DSMZ, Braunschweig, Germany). *Bifidobacterium animalis subsp.lactis* and *Lactobacillus plantarum* ATCC 8014 were generously offered by Essum AB company.

2.1.5 Buffer solutions and media

- ***Pichia pastoris* cultivation media and Buffers**

Potassium phosphate buffer 1 M: 150 mL of K2PO4 1 M and 400 mL of KH2PO4 1 M were mixed and the pH was adjusted to 6.0.

YNB 13.4 % (w/v) (yeast nitrogen base solution): 3.4 g YNB (without ammonium sulfate and amino acids) and 10g Ammonium sulfate were dissolved in 100mL dH2O.

Biotin 0.02 % (w/v): 20 mg biotin was dissolved in 100 mL dH2O.

Glycerol 10 % (w/v): 10 g glycerol was dissolved in 100 mL dH2O.

BMGY (Buffered Glycerol complex medium): 1% (w/v) yeast extract, 2% (w/v) peptone, 1M phosphate buffer pH 6.0, 1.34% (w/v) YNB, 4x10-5% (w/v) biotin, 1% (v/v) glycerol

BMMY (Buffered Methanol complex medium): 1% (w/v) yeast extract, 2% (w/v) peptone, 1M phosphate buffer pH 6.0, 1.34% (w/v) YNB, 4x10-5% (w/v) biotin, 0.5% (v/v) methanol. [For the BMMY that was used for the production of *Tt*CBh7 the YNB was 2.34% (w/v)]

TALON Buffer 10X: 3M NaCl, 500mM Tris, pH was adjusted to 8.0 with HCl.

TALON Buffer 1X with imidazole: 0.3M NaCl, 50mM Tris, 100mM imidazole, pH was adjusted to 8.0 with HCl

Tris Buffer 10X: 200mM Tris, pH was adjusted to 8.0 with HCl.

- **SDS Page Buffers**

Sample preparation buffer: 3.55 mL 0.25 M Tris Base pH 6.8, 1.8 mL glycerol 50% (v/v), 1.8 mL *β*–mercaptoethanol, 0.71 gr SDS, 2.85 mL bromophenol blue 0.1 % (w/v)

Running buffer: 3.03 g/l Tris-base, 14.4 g/l glycine, 1 g/l SDS, pH 8.3

Staining solution: 0.4% (w/v) Coomassie G-250, 400 mL methanol, 100 mL acetic acid, 500 mL mQH2O

Destaining solution: 450 mL methanol, 100 mL acetic acid, 450 mL mQH2O

Marker: Novex™ Sharp Pre-stained Protein Standard (Invitrogen, U.S.A)

- **Lowry Assay**

Lowry Solution: 49 mL 0.2 M NaOH, 49 mL 2% (w/v) Na2CO3, 1 mL 1% (w/v) CuSO4.5H2O, 1 mL 2% (w/v) NaK tartrate

Folin reagent: Folin & Ciocalteu's phenol reagent 2 N (Merck, U.S)

- **Other Buffers**

Phosphate-citrate Buffer 100mM pH 5.0: 100 mM citric acid, 200 mM sodium phosphate dibasic, 0.02% (w/v) NaN_3

Ammonium acetate Buffer 100mM pH 5.5: 100mM ammonium acetate, pH was adjusted to 5.5 with 0.1 M Acetic acid

- **Bacteria culture media and carbohydrate sources**

The growth medium for the *Lactobacillus* strains was MRS medium with cysteine (Medium 232 DSMZ, Germany) and for the *Bifidobacteria* strains the Bifidobacterium medium (Medium 58 DSMZ, Germany). For the cello-oligosaccharides (COS) experiments, both bacterial species were cultivated in MRS broth that contained

cellobiose instead of glucose as carbohydrate source. The pH of the medium was adjusted according to the medium's instructions prior autoclaving (121 °C for 20min).

The commercial carbohydrate sources that were used, D-glucose and D-cellobiose, were obtained from Sigma-Aldrich (U.S.A). Birch and spruce derived cello-oligosaccharides (COS) were produced by the enzymatic treatment of the biomass using an enzymatic cocktail with recombinant enzymes as will be described in Section 2.2.4 .

2.1.6 Nanofiltration

- **Chemicals and membranes**

For the nanofiltration trials the membranes that were used and their characteristics are depicted at **Table 2.4**. The membranes were purchased from Sterlitech (USA) as well as the nanofiltration vessel. For the experiments, D-glucose 180.156 g/mol and D-cellobiose 342.3 g/mol were purchased from Sigma-Aldrich (U.S.A) and were used for the model solutions. Their characteristics are depicted at **Table 2.3**.

Table 2.3. Chemical properties of glucose and cellobiose.

	Glucose	Cellobiose
Molecular formula	$C_6H_{12}O_6$	$C_{12}H_{22}O_{11}$
Molecular structure	HO, O, OH, HO, OH, OH	HO, O, OH, OH, OH, HO, O, O, OH, OH, OH
Molecular weight (g/mol)	180.16	342.3

Table 2.4.Nanofiltration membranes and their characteristics.

Manufacturer	Type	Pore size/MWCO*	Polymer	pH	Flux (GFD/psi)
Dow Filmtec™	NF270	~200-400 Da	Polyamide-TFC	2-11	72-98/130
GE Osmonics™	DL	~150-300 Da	Polyamide-TFC	2-10	28/220
Synder™	NFX	~150-300 Da	Polyamide-TFC	3-10.5	20-25/110
Synder™	NFW	~300-500 Da	Polyamide-TFC	4-10	45-50/110
TriSep™	TS40	~200 Da	Polypiperazine-amide-TFC	2-11	20/110

**MWCO: molecular weight cut-off.*

2.2 Methods

2.2.1 Production of heterologously expressed of *Tt*EG5, *Tt*LPMO, *Tt*CBH7

In this thesis, the heterologous expression of two cellulases, one Endo-1,4-β-d-glucanase that belongs to the glycoside hydrolase family 5 (EG5) and one Exo-1,4-β-d-glucanase, also called cellobiohydrolase that belongs to the glucoside hydrolase family 7 (CBH7) and of one Lytic polysaccharide monooxygenase (LPMO), all from the filamentous fungus *T. thermophila*, is described. The recombinant enzymes were produced from the methylotrophic yeast *Pichia pastoris*, secreted to the culture medium and purified.

2.2.1.1 Production of the recombinant enzymes

The expression of the recombinant protein genes was performed in methylotrophic *P. pastoris* yeast. Cultivation of *P. pastoris* liquid cultures was performed under continuous agitation (200 rpm) in BMGY first and then in BMMY media for the induction of the expression, at 30 °C in Erlenmeyer shake flasks. Firstly, the recombinant gene was inoculated in BMGY media for 20-24 h. The absorbance (OD_1) of the *P. pastoris* pre-culture at 600 nm was measured and the starting OD (OD_2) for the main culture in BMMY media is 1 (according to *Pichia pastoris* protocols, Invitrogen). The volume of the pre-culture that is needed and which contains the amount of cells that are necessary for the resuspension in the main culture is calculated according to the equation $OD_1V_1 = OD_2V_2$. Then, the necessary volume of the pre-culture was centrifuged (4 °C, 5000 rpm for 10min), the supernatant was discarded and the cells were collected and resuspended in BMMY medium. Methanol 0.5% (v/v) was added to induce the expression of the recombinant proteins. The cultures were incubated under continuous agitation (200 rpm), at 30 °C, while the required methanol was added daily. The cultivation lasted for 120 – 168 h. The recombinant enzyme was secreted extracellularly into the culture medium, so after the end of the cultivation, the culture was centrifuged (4 °C, 8000 rpm for 10min), the supernatant was collected and filtered under vacuum with membrane filters of pore size 0.45 μm and 0.2 μm sequentially.

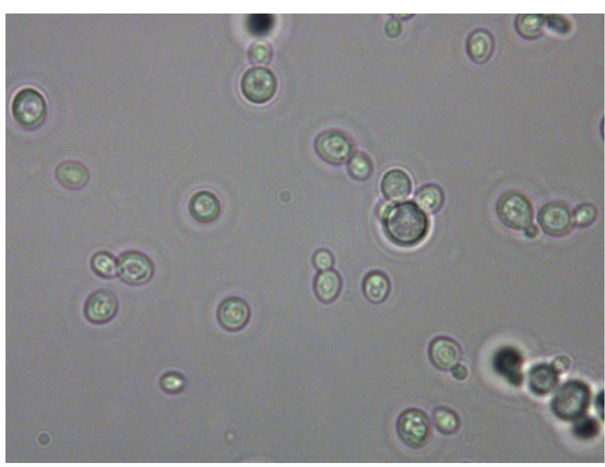


Figure 2.1. P. pastoris cells growing at shake flasks showing normal budding phenotype, as well as two buds attached to the mother cell (Mickey Mouse-like appearance) (Magnitude x100)

2.2.1.2 Purification of the recombinant enzymes

The culture supernatant containing the recombinant protein was concentrated until approximately 50 mL using a LabScale Tangential Flow Filtration system (TFF) (Millipore) with exclusion membrane size 10 kDa (Pellicon XL Ultrafiltration Module Biomax 10 kDa, Millipore). Afterwards, the pH of the concentrate was exchanged to 8.0 using dialysis tubing membranes with ten volumes of Talon Buffer 1X, for 12 h at 4°C under continuous agitation. After filtration with membrane filters (0.22 µm pore size), the concentrate was purified by single step immobilized metal ion affinity chromatography (IMAC) on an ÄKTA Prime Plus system (GE Healthcare Life Sciences) equipped with a cobalt-charged resin (Talon Metal Affinity Resin, Clontech, U.S.). The column was washed first with 100 mL mQ water and then with 100 mL Talon buffer 1X at a flow rate of 2 mL/min. Then, the sample containing the histidine-tagged protein was loaded onto the column at a flow rate 1 mL/min. The column was washed with 100 mL of Talon buffer 1X at a flow rate of 2 mL/min, or until the OD_{280nm} reached the baseline indicating that all non-histidine tagged proteins had been eluted. Then, a linear gradient from 0 to 100 mM imidazole Talon Buffer 1X (100 mL) was applied at a flow rate of 2 ml/min and the protein peak finally appears as in **Figure 2.2**. Finally, the purified protein was again subjected in buffer exchange with dialysis membranes against Tris Buffer 1X in order to remove imidazole and salts. The amount of the purified protein was determined using the Lowry assay (Smith et al., 1985) as will be described later on.

Fractions (2 mL) containing the protein of interest were taken after the TFF filtration procedure and IMAC chromatography for further examination on sodium dodecyl sulfate–polyacrylamide gel electrophoresis SDS-PAGE.

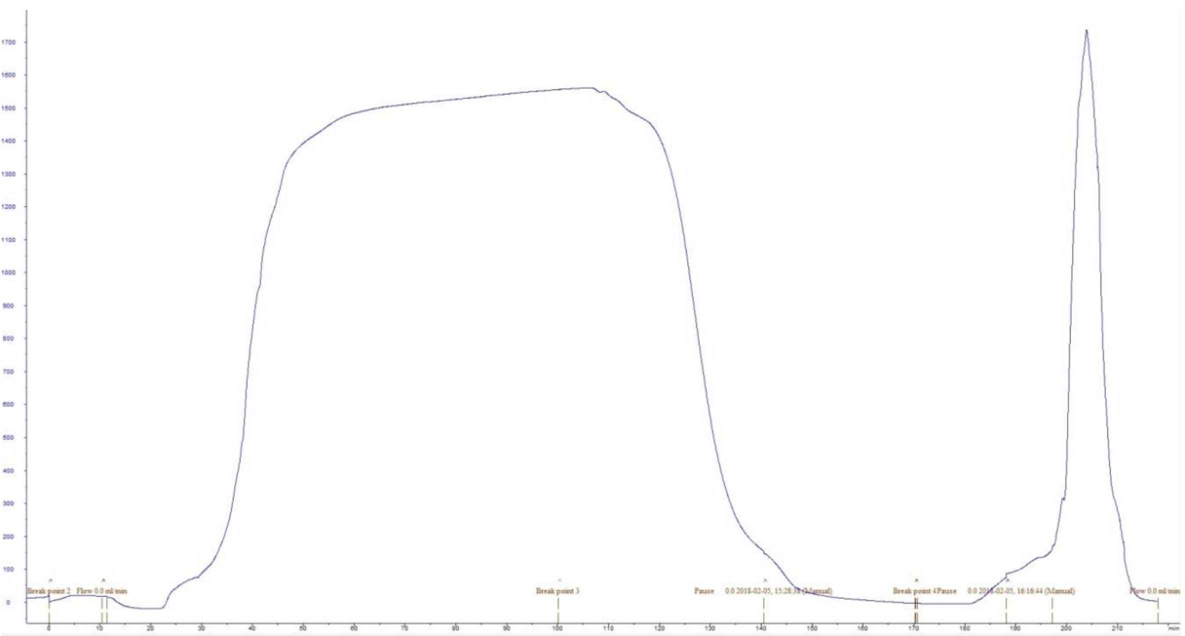


Figure 2.2 Protein peak of *Tt*CBH7 during IMAC chromatography.

2.2.1.3 Enzyme activity assay

The amount of protein was determined by the Lowry Protein Assay microplate procedure at 750 nm, using BSA (bovine serum albumin) as a standard (Smith et al., 1985). Results for the total amount of protein will be described later in Section 3.1.

*Tt*EG5 and *Tt*CBH7 activity was determined on PASC 0.5 % (w/v) and Avicel 5% (w/v) for 60 min, at 50 °C and 1200 rpm in 100 mM citrate–phosphate buffer pH 5.0 using an enzyme loading of 20 mg/g for the PASC substrate and 25 mg/g for the Avicel substrate (Karnaouri et al., 2017, 2018). The samples were then centrifuged and the concentration of total reducing sugars that were released by the activity of the enzymes at the supernatant was determined using the dinitrosalicylic acid reagent (DNS) and glucose for the standard curve (Miller, 1959). For the DNS method in 125μl sample were added 125μl of DNS reagent, then it was boiled in 100°C for 5 min, centrifuged and 1 mL of distilled water was added. In the end, the absorbance was measured at 540 nm. One unit (U) of enzyme activity was defined as the amount of enzyme that liberated 1 μmol of glucose equivalents per minute under assay conditions.

*Tt*LPMO activity was determined on PASC 0.5 % (w/v) after 24 h incubation at 50°C and 1000 rpm in 100m M citrate–phosphate buffer pH 5.0, using 1 mM ascorbic acid as reducing agent and an enzyme loading of 20 mg/g. The sample then was inactivated by boiling for 15 min prior to analysis using high-performance anion exchange chromatography (HPAEC). The analysis was conducted using an ICS 5000SP system (Dionex, Thermo Fisher Scientific Inc.) with a pulsed amperometric detector equipped with a disposable electrochemical gold electrode, using a CarboPac PA1 4 × 250 mm

analytical column and a CarboPac PA1 4 × 50 mm guard column, at 30 °C. 10 μL of samples were injected and the reaction products were eluted at 1 mL/min with initial conditions set to 0.1 M NaOH (100% eluent A). This step was succeeded by a linear gradient toward 10% eluent B (1 M NaOAc in 0.1 M NaOH) after 10 min and 30% B after 25 min; a 5 min exponential gradient (Dionex curve 6) to 100% B followed. Integration of chromatograms was performed using Chromeleon 7.0 software (Karnaouri et al., 2017).

2.2.2 Enzymatic Hydrolysis

Forest residues are by nature heterogeneous in composition, size, structure and properties. Thus, the degradability of these materials is different. To accomplish the efficient hydrolysis process of different types of lignocellulosic materials, novel lignocellulolytic enzyme mixtures have to be customized. Hydrolysis of organosolv pretreated natural substrates (birch and spruce as forest residues) was tested in the presence of two cellulases (EG5, CBH7) and one accessory enzyme that increases the efficiency of the cellulases (LPMO). Cooperative action between the three cellulolytic enzyme classes is essential for the efficient enzymatic hydrolysis.

2.2.2.1 Hydrolysis of lignocellulosic materials with the recombinant enzymes

Hydrolysis of lignocellulosic feedstocks (pretreated birch and spruce from forest biomass) was performed using different enzymatic cocktails consisted of the three recombinant enzymes (*Tt*EG5, *Tt*CBH7, *Tt*LPMO) (**Table 2.5**). Enzymatic reactions were performed in 2 mL microtubes, at 50∘C, under agitation of 1100 rpm, with 3% (w/v) initial dry matter content. The enzymes were loaded at 25 mg/g substrate. All reactions were performed in 100 mM phosphate citrate buffer pH 5.0 and contained 0.02 % (w/v) sodium azide to prevent microbial contamination. Samples were taken twice at 24 and 72 h. Each sample was put in a new microtube and was centrifuged so as to achieve separation of the soluble products from the insoluble substrate. Then the supernatant was transferred again to a clean microtube and was boiled for 5 min at 100°C for inactivating the enzyme. The sample was afterwards filtered (0.22 μM pore size) and then the released sugars of interest (cellobiose and glucose) were quantified with isocratic ion-exchange chromatography using an Aminex HPX-87N column with a micro-guard column at 85 °C (Bio-Rad Laboratories, Hercules, CA, USA) using 0.01 M Na2PO4 at a flow rate of 0.6 mL/min as the mobile phase.

Table 2.5. Preliminary combinations used for the hydrolysis tests with the recombinant enzymes.

Combination number	Enzyme proportions			Enzyme proportions (µg)		
	*Tt*EG5	*Tt*LPMO	*Tt*CBH7	*Tt*EG5	*Tt*LPMO	*Tt*CBH7
1	0	0	1	0	0	750
2	0	0,2	0,8	0	150	600
3	0	0,3	0,7	0	225	525
4	0,3	0,1	0,6	225	75	450
5	0,3	0,3	0,4	225	225	300
6	0,4	0,1	0,5	300	75	375
7	0,5	0	0,5	375	0	375
8	0,7	0,3	0	525	225	0
9	0,8	0,2	0	600	150	0
10	1	0	0	750	0	0

For testing the activity of the recombinant cellobiohydrolase (*Tt*CBH7) similar reactions were performed with different enzymatic cocktails using the commercial cellobiohydrolase (CBHI) from Megazyme (U.S.A) (**Table 2.6**).

Table 2.6. Preliminary combinations used for the hydrolysis tests with CBH7 from Megazyme.

Combination number	Enzyme proportions			Enzyme proportions (µg)		
	*Tt*EG5	*Tt*LPMO	CBHI	*Tt*EG5	*Tt*LPMO	CBHI
1	0	0	1	0	0	750
2	0	0,2	0,8	0	150	600
3	0	0,3	0,7	0	225	525
4	0,3	0,1	0,6	225	75	450
5	0,3	0,3	0,4	225	225	300
6	0,4	0,1	0,5	300	75	375
7	0,5	0	0,5	375	0	375

2.2.2.2 Hydrolysis tests with NZYtech enzymes

In this thesis, the mode of action of the NZYTech enzymes was tested with activity tests on cellulosic polysaccharides. Three different model substrates were used (PASC, Avicel and CMC) as well as soluble substrates cello-oligosaccharides with polymerization degree from 5 to 8. Additionally, the activity of the NZYTech enzymes was tested in pretreated natural substrates (birch and spruce as forest residues).

- ***Hydrolysis of model substrates***

Hydrolysis of PASC 3% (w/v) was performed using all the NZYtech enzymes. The enzymatic reaction was performed in safe lock 2 mL microtubes at 37, 50 and 60◦C

according to the enzyme optimal temperature of action and with 1100 rpm agitation. The initial substrate content was 0.5% (w/v) and the enzyme loading was 20 mg/g substrate. All reactions were performed in 100 mM phosphate citrate buffer pH 5.0 and contained 0.02 % (w/v) sodium azide to prevent microbial contamination. Samples were taken at 2, 8 and 24 h. Each sample was put in a new microtube and was centrifuged so as to achieve separation of the soluble products from the insoluble substrate. The supernatant was transferred again to a new microtube and was boiled for 5 min at 100◦C for inactivating the enzyme and was filtered (0.22 μM pore size). The presence of oligosaccharides was verified with HPAEC/PAD chromatography, as described previously. Same procedure was followed using Avicel 3 % (w/v) and CMC 1 % (w/v) as substrates that were prepared in 100 mM phosphate citrate buffer pH 5.0.

- ***Kinetics of hydrolysis on soluble oligosaccharides***

Hydrolysis of cello-oligosaccharides with DP 5 - 8 was performed using all the NZYtech enzymes. The reaction conditions were the same as for the hydrolysis of the insoluble substrates, as well as the procedure that was followed. For the determination of the hydrolysis kinetics samples were taken every 20 min for a total duration of 100 min. The released oligosaccharides were evaluated with HPAEC/PAD chromatography, as previously described.

For the identification and quantification of hydrolysis products, D-glucose and G2-G8 cello-oligosaccharides were used as carbohydrate standards (**Figure 2.3**) (Karnaouri et al., 2014b). Assuming that the condition $[Eo] \ll [So] \ll Km$ is satisfied (where [Eo] and [So] represent the concentrations of the enzyme and substrate, respectively), the enzymatic hydrolysis of cello-oligosaccharides was regarded as a first-order reaction. Since the integrated rate equation for the first-order kinetics can be written as $k \times t = \ln([So]/[St])$, where $k=(kcat/Km)[enzyme]$, whereas [So] and [St] represent substrate concentration prior to the start of the reaction and at a specified time during the reaction, respectively, the estimation of the catalytic efficiency of the enzymes against cello-oligosaccharides was made using the above equation (Matsui et al., 1991).

- ***Hydrolysis of lignocellulosic materials***

Hydrolysis of lignocellulosic feedstocks (birch, spruce) was performed using all the NZYtech enzymes. The enzymatic reaction was performed in safe lock 2 mL microtubes at 37, 50 and 60 ◦C according to the enzyme and with 1100 rpm agitation. The initial substrate content was 1.5 % (w/v) and the enzyme loading was 8 mg/g substrate. Samples were taken at 48 h and the released oligosaccharides were

evaluated with HPAEC/PAD chromatography, after the procedure that was previously described.

2.2.3 Experimental design

The cellobiose yields from organosolv pretreated birch and spruce with different enzyme mixtures was studied. For this experimental design, a mixture of five enzymes, including the most promising processive enzyme that was chosen after the study of the NZYtech enzymes, together with three commercial cellulases EG5, CBH7, EG7 (Megazyme, U.S.A) and the recombinant *Tt*LPMO were used. The experimental design was set up with the software Design Expert 7.0.0. (Stat-Ease Inc.). The algorithmically built *D-optimal* design was employed for the generation of 20 experimental combinations that are depicted at the **Table 2.7**. In all the combinations the summary of the enzymes was equal to 1 (100%) so that only the proportion of each enzyme to vary except from EG7 that remained constant and equal to 5 % for all the combinations. The evaluation of the results towards the maximum cellobiose yields was performed with the same software using the most appropriate model that fits the experimental data (Karnaouri et al., 2016, 2018). The reactions were performed at 50 ^oC under 1100 rpm agitation with 3% (w/v) initial dry matter and with an enzyme loading 25 mg/g substrate in 100 mM phosphate citrate buffer pH 5.0 with 0.02% sodium azide for the prevention of contamination, at a final volume of 1 mL. Samples were taken at 24 and 48 hours and each sample was put in a new microtube and was centrifuged so as to achieve separation of the soluble products from the insoluble substrate. The supernatant was transferred again to a new microtube and was boiled for 5 min at 100∘C for inactivating the enzyme and was filtered (0.22 μM pore size). Then, the released sugars (glucose and cellobiose) were quantified with isocratic ion-exchange chromatography using an Aminex HPX-87N column with a micro-guard column at 85 ^oC (Bio-Rad Laboratories, Hercules, CA, U.S.A) using 0.01 M Na2PO4 at a flow rate of 0.6 mL/min as the mobile phase.

Table 2.7. Experimental combinations used for the hydrolysis tests.

Combination number	Enzyme proportions				
	EG5	*CBH7*	*Tt*LPMO	*PaCbh6a*	EG7
1	0.190	0.570	0.095	0.095	0.050
2	0.190	0.570	0.095	0.095	0.050
3	0.285	0.285	0.285	0.095	0.050
4	0.475	0.285	0.095	0.095	0.050
5	0.285	0.285	0.190	0.190	0.050
6	0.380	0.285	0.190	0.095	0.050
7	0.190	0.285	0.285	0.190	0.050
8	0.190	0.285	0.285	0.190	0.050
9	0.190	0.475	0.095	0.190	0.050
10	0.475	0.285	0.095	0.095	0.050
11	0.190	0.380	0.285	0.095	0.050
12	0.190	0.380	0190	0.190	0.050
13	0.333	0.428	0.095	0.095	0.050
14	0.190	0.380	0.095	0.285	0.050
15	0.285	0.285	0.095	0.285	0.050
16	0.333	0.428	0.095	0.095	0.050
17	0.380	0.285	0.095	0.190	0.050
18	0.190	0.475	0.190	0.095	0.050
19	0.190	0.285	0.190	0.285	0.050
20	0.190	0.285	0.190	0.285	0.050

2.2.4 Scale up reaction

A scale up reaction with birch (B4) and spruce (S3) as substrates (composition as depicted at **Table 2.2** of Section 2.1.3) was necessary for a larger scale production of cellobiose for the probiotic tests. For the reactions, the initial substrate content was 6 % w/v OS pretreated birch (B4) and spruce (S3). The optimal combination of the enzymes that were used is the one that will be mentioned at Section 3.3 of this thesis. The enzyme loading was 25 mg/g substrate. The reaction was 100 mL in total and was performed in 1 L shake flask in 50 °C under continuous agitation 170 rpm. The reaction was prepared in 0.014 M Ammonium acetate buffer pH. 5.5. Change of the buffer compared to the one that was used at the previous hydrolysis tests and the decrease on its concentration was carried out in order to achieve the minimum salt content on the hydrolysis product. The enzymes were loaded in two steps sequentially in order to maximize hydrolysis and prevent end-product inhibition, by maintaining the same total enzyme loading. First, an enzyme loading of 15 mg/g was added and was incubated for 48 h. Then, the hydrolysate was filtrated, measured and boiled at 100 °C for 10 min for the inactivation of the enzymes. The biomass that remained was washed

with the same buffer and the washed hydrolysate was also measured and boiled. The washed biomass was again placed in the shake flask and the rest 10 mg/g enzyme loading was added together with the proper amount of buffer for another 48 h. Then, the same procedure for washing the biomass was followed. All the hydrolysates (original and washed) were filtrated with 0.22 μm pore size filter and then samples were taken for HPLC analysis for identifying and quantifying the cellobiose and glucose content.

For the removal of the protein content all the hydrolysates were mixed together, measured and filtrated with a LabScale Tangential Flow Filtration system (TFF) (Millipore) with exclusion membrane size 5 kDa (Pellicon XL Ultrafiltration Module Biomax 5 kDa, Millipore).

Then, the permeate was filtrated with nanofiltration and the retentate that occured was placed for freeze drying. Samples were taken from all the steps for HPLC analysis. At the end, 10 mg of the freeze dried product were diluted in 1 mL mQ water and the sample was analyzed with isocratic ion-exchange chromatography using an Aminex HPX-87H column with a micro-guard column at 65 °C (Bio-Rad Laboratories, Hercules, CA, U.S.A) using 0.1 M H_2SO_4 at a flow rate of 0.6 mL/min as the mobile phase and HPAEC/PAD chromatography for determining the cellobiose and glucose final content.

2.2.5 Chromatographic techniques

The chromatographic techniques that were used in this thesis for the detection of the hydrolysis products were HPLC chromatography with an RID detector and HPAEC chromatography.

2.2.5.1 HPAEC/PAD

Analysis by high performance anion exchange chromatography (HPAEC) coupled with pulsed amperometric detection (PAD) provides high detection sensitivity of mono-, di- and oligosaccharides.

The analysis was conducted using an ICS 5000SP system (Dionex, Thermo Fisher Scientific Inc.) with a pulsed amperometric detector equipped with a disposable electrochemical gold electrode, using a CarboPac PA1 4 × 250 mm analytical column and a CarboPac PA1 4 × 50 mm guard column, at 30 °C. 10 μL of samples were injected and the reaction products were eluted at 1 mL/min with initial conditions set to 0.1 M NaOH (100% eluent A). This step was succeeded by a linear gradient toward 10% eluent B (1 M NaOAc in 0.1 M NaOH) after 10 min and 30% B after 25 min; a 5 min

exponential gradient (Dionex curve 6) to 100% B followed. Integration of chromatograms was performed using Chromeleon 7.0 software (Karnaouri et al., 2017).

The eluents were prepared using purified water from milli Q system. 5.279 mL of Sodium hydroxide were used for 1 L of eluent B, and then the eluent was spurged for 10 min with nitrogen gas. For 1 L of eluent C, 5.279 mL of Sodium hydroxide and 82.03 g of Na-acetate are needed. For removing the salts eluent C is filtrated with 0.22 μm pore size filter before spurging with nitrogen.

The separation mechanism is based on the weakly acidic properties of sugar molecules in basic solutions (pH above 12), while the detection employs the ability of the gold electrode surface to catalyze the oxidation of polar compounds in alkaline media.

Standard mixtures of neutral sugars (G1-G8) (**Figure 2.3**) whose concentrations were similar to those of typical sample hydrolysates were analyzed so that based on the retention times and peak areas of the standard sugars, peaks of the real hydrolysate samples can be identified and quantified.

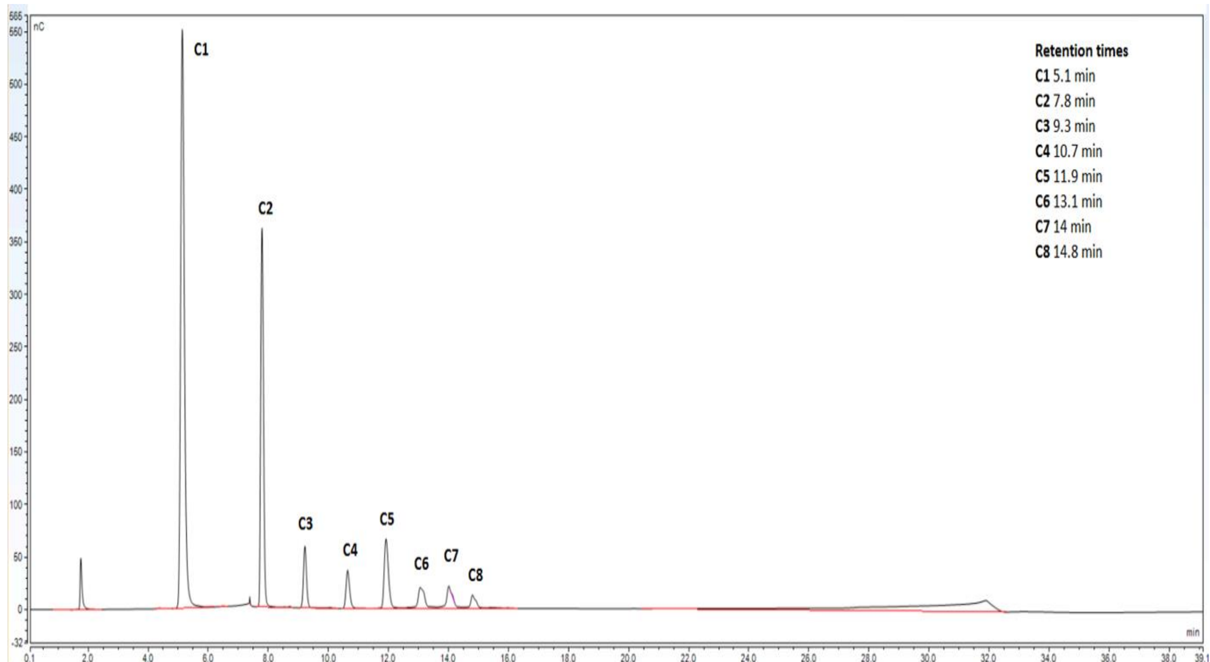


Figure 2.3 Chromatograph of a neutral sugar standard solution containing glucose (C1), cellobiose (C2), cellotriose (C3), cellotetraose (C4), cellopentaose (C5), and cellohexaose (C6), celloeptaose (C7), cellooctaose (C8)

2.2.5.2 HPLC

High performance Liquid Chromatography (HPLC) is one of the most popular and mature analytical techniques and by far the most widely used separation technique.

HPLC has gained its popularity mainly due to its reliability (use of pressure driven liquid support) and versatility (possibility of adjusting the composition of both mobile and stationary phases). The chromatographic mode or separation mechanism depends on the overall interactive relationships between the stationary phase, the mobile phase and the analyte.

A schematic of an HPLC system is shown in **Figure 2.4**, with emphasis on the flow path of the solvent (solid arrows), as it proceeds from the solvent reservoir to the detector. The solvent is usually referred to as the mobile phase or eluent and for this thesis the eluent that was used was sodium phosphate dibasic, Na_2HPO_4, 10 mM for the analysis with Aminex HPX-87N column and H_2SO_4 100 mM for the analysis with Aminex HPX-87H column. After the injection of the sample, which requires a minimal preparation, usually filtration through 0.22 μm hydrophilic filter, a separation takes place within the column, and separated sample components leave (are eluted or washed from) the column, with detection by RID (refractive index detector). The fundamental nature of the separation is determined mainly by the choice of column, which in this thesis was the N and H columns (Aminex, Biorad U.S.A) that are packed with a polymer based matrix (polystyrene divinylbenzene). The Aminex N column has a sodium ionic form, while the Aminex H column has a hydrogen ionic form and is optimized for the analysis of organic acids and particularly for the separation of organic acids alone or in combination with carbohydrates, alcohols, fatty acids or neutral compounds.

The Aminex HPLC columns separate compounds using the ion-moderated partition chromatography technique. As carbohydrate analysis columns they separate compounds using a combination of size exclusion and ligand exchange mechanisms:

- In oligosaccharide separations, size exclusion is the primary mechanism. Low crosslinked resins allow carbohydrates to penetrate, and oligosaccharides separate by size
- For monosaccharide separations, ligand exchange is the primary mechanism and involves the binding of hydroxyl groups of the sugars with the fixed counterion of the resin. Ligand exchange is affected by the nature of the counterion (Pb2+, Ca2+, etc.) and by the spatial orientation of the carbohydrate's hydroxyl groups

The analysis is taking place for 30 min, at a flow rate of 0.6 ml/min at 85 °C or 65 °C for N and H column respectively and the separation of glucose and cellobiose is noticeable due to the difference in their retention times.

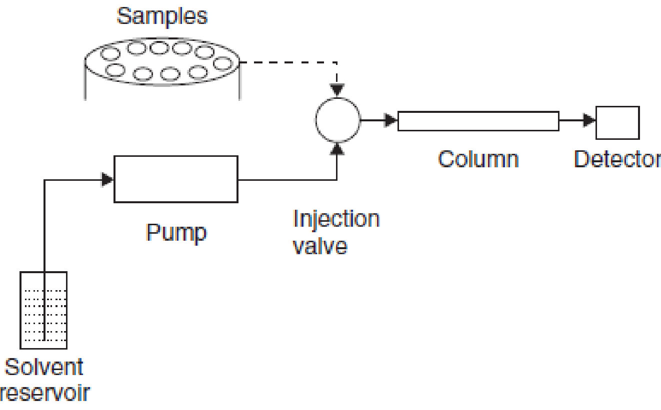


Figure 2.4 HPLC system diagram (Snyder et al., 2010)

2.2.6 Product recovery

In bioconversion processes of cellulosic biomass the product recovery step is of major importance and is mainly accomplished by pressure driven membrane separation processes for separation, purification and concentration of desired products because of its high recovery or removal efficiency, the low energy input, the moderate operating conditions and the easy integrating with other operating units (Strathmann, 2001; Wiesner and Aptel, 1996) Additionally, an advantage of the membrane separation techniques is that they are easy to scale up for commercial production. Two types of pressure driven membrane processes are ultrafiltration (UF) and nanofiltration (NF).

2.2.6.1 Tangential flow filtration (TFF)

The UF membranes can retain macromolecules with molecular weight ranging from 1000 to 100,000 g/mol and are especially used for protein separation or concentration, while the membrane pore size is 1-100 nm. The membrane's molecular weight cut off (MWCO) is usually defined as the equivalent molecular weight of the smallest protein that will exhibit above 90 % rejection and consists the basis for the choice of a membrane (Saxena et al., 2009). The ultrafiltration method that was used for the purposes of this thesis is a tangential flow filtration (TFF) system as depicted in **Figure 2.5**. The modules that were used for the filtration were the flat sheet cassettes, which provide a physical separation of the retentate and filtrate streams, high membrane packing densities, easy access for cleaning and replacement and good mass transfer characteristics (Saxena et al., 2009). For reducing the filtration time, three cassettes with membranes of the same molecular cut off were placed at the ultrafiltration system and participated at the filtration process simultaneously.

If the purpose of the filtration is the protein concentration then the fraction that is collected is the retentate, whereas if a protein separation is needed the fraction that is collected is the permeate. The separation of proteins by membranes is based on the differential transmission of molecules through the membrane. The molecules with molecular weight bigger than the membrane's molecular cut off are retained by the membrane while the molecules with molecular weight smaller than the membrane's are passing at the permeate. The transmission of molecules is characterized by the sieving coefficient parameter that is given by the following equation:

$$S_j = \frac{C_p}{C_F} \quad [1]$$

Whereas C_p and C_F are the concentrations of the protein in the permeate and in the feed respectively (Christy et al., 2002).

The special characteristic of the tangential flow filtration system is that the filtration is based on the control of the filtrate flux (and thus the transmembrane pressure) and fluid mechanics for minimizing the fouling effect and taking advantage of the concentration polarization effect. Fouling of the membrane occurs when the concentration of the solute increases in a way that blocks the walls of the membranes and reduces the effective pore size. Hence, the sieving effect of the solute decreases (Van Reis et al., 1999). At TFF system, since the feed pressure can be controlled, an optimum flux can be achieved.

Additionally, the feed solution flows parallel to the membrane surface as it is depicted in **Figure 2.6(b)** instead of in a dead-end way (**Figure 2.6(a)**), minimizing the membrane wall blocking from the high solute concentration as the molecules are able to circulate along the length of the membrane and not necessarily through it, reducing at the same time the concentration polarization effect.

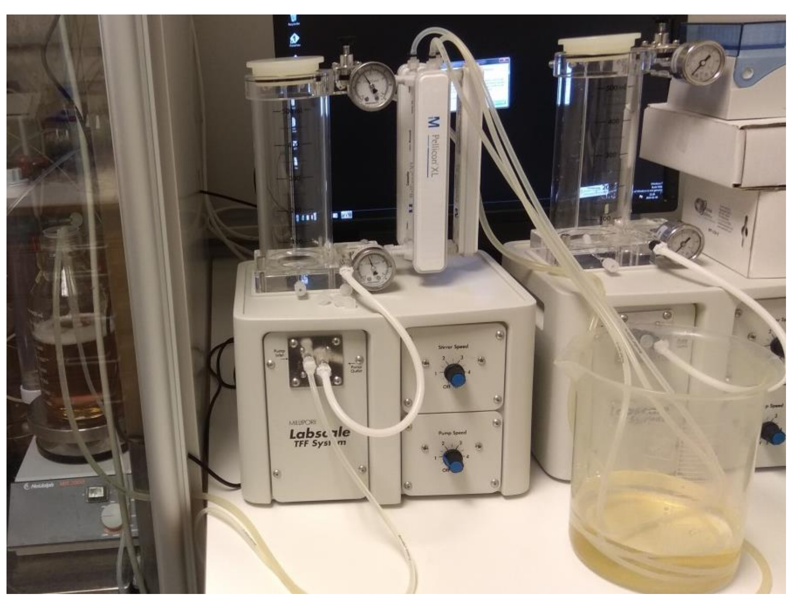


Figure 2.5. Ultrafiltration system that was used.

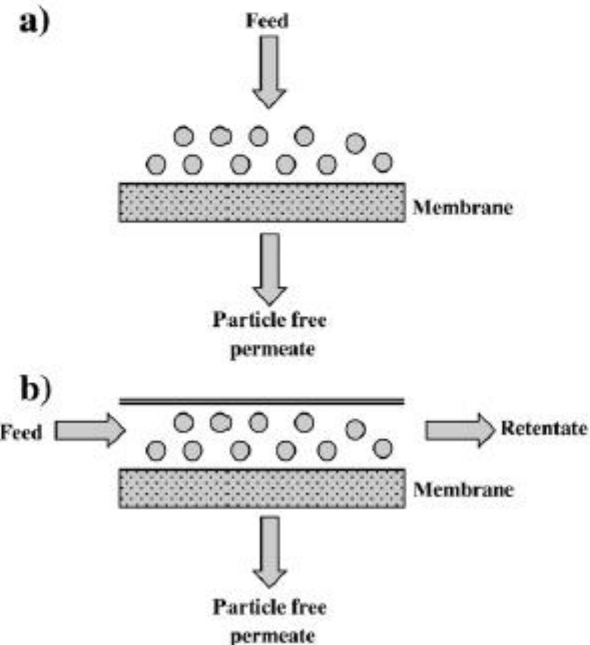


Figure 2.6. Graphic explanation of (a) dead-end and (b) tangential flow filtration. (Saxena et al., 2009)

2.2.6.2 Nanofiltration

The aim of the nanofiltration at the product purification step is the separation of cellobiose and glucose for obtaining a solution potentially pure of cellobiose. This step is especially important for enzymatically produced biofunctional oligosaccharides. Nanofiltration is an easy maintenance and cost competitive alternative that can offer

promising results. However, cellobiose and glucose molar masses differ only by a factor of 1.9 which inevitably makes the separation process extremely difficult.

Nanofiltration is defined as a process that separates solvent, monovalent salts and small organics from divalent ions and larger species with molecular weight in the range of 200 – 1000 g/mol and a membrane pore size of 20 - 70 nm (Vandezande et al., 2008). In **Figure 2.7** is depicted a schematic explanation of the nanofiltration method. Particularly, the set-up is consisted by a dead end filtration vessel in which a continuous stirring is on the membrane surface is applied for avoiding the concentration polarization effect. The feed solution of oligosaccharides is supplied at the nanofiltration vessel and eventually during the filtration the retentate becomes richer on oligosaccharides with DP higher than 1, in this case cellobiose, while the permeate's composition is the smaller oligosaccharides (glucose), other molecules of small molecular weight and salts.

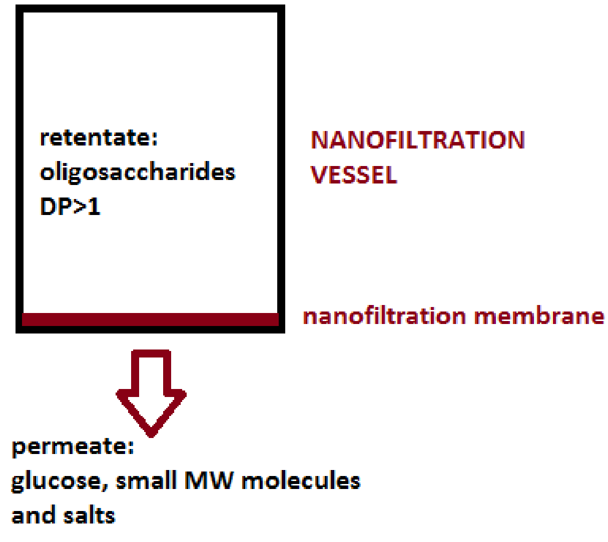


Figure 2.7. Graphic explanation of nanofiltration method.

- ***Experimental set-up and procedure***

The nanofiltration vessel that is depicted at **Figure 2.8** is a magnetically stirred dead-end stainless steel cell (Sterlitech, USA) with a working volume of 300 mL and effective membrane area of 14.6 cm^2 and 500 rpm continuous agitation. A constant pressure of 5, 10, 15 or 20 bar was provided by filling nitrogen gas into the cell, while the permeate was collected in a beaker placed on an electronic scale in order to calculate the permeate flux. When the tests were carried out at higher than room temperatures (40, 50 or 60 °C), heat was supplied by hot water that was placed in a stainless steel bowl in which the nanofiltration vessel was placed.

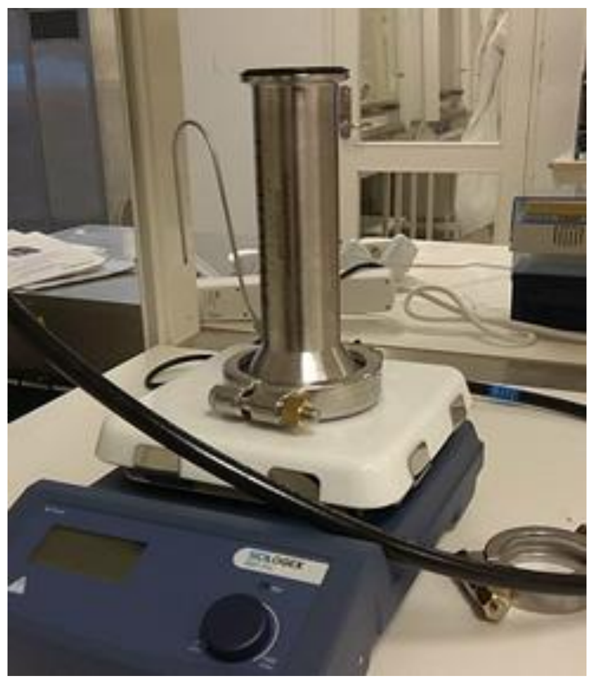


Figure 2.8 Nanofiltration vessel (Sterlitech) that was used for the experiments.

- ***Water permeability measurements***

The membranes were stored in 1 % solution of sodium metabisulfite (SMBS) and prior to the permeability tests they were rinsed with dH2O and placed in the nanofiltration dead-end cell. Then, the cell was filled with 50 mL dH2O and a pressure of 10 bar was applied. Measurements of the permeate's weight were taken every 5 min for 20 min in total. The water permeability of every membrane was determined every time before and after the use of the membrane as an indication of the membranes efficiency and whenever a change was detected the membrane was discarded and replaced by a new one. The tests for the water permeability were always determined at room temperature.

- ***Nanofiltration of pure sugars - model solutions***

The model solutions that were used for the experiments were prepared with commercial glucose and cellobiose in dH2O. The total feed concentration that was tested was 5 and 20 mg/mL. The filtration was performed at ratio of cellobiose to glucose 9: 1, at different pressures (5, 10, 15 and 20 bar), while the pH was constant at 5.0 and the experiments were carried out at room temperature. The membranes that were more suitable for the experimental purposes were further tested at different temperatures (40, 50 and 60 °C). The feed volume was 50 mL. During the filtrations the feed solutions were concentrated by a factor of 2 and samples of the permeate were taken every 15 min on average depending on the flow rate of the permeate. In the end of the filtration sample was taken also from the inside of the vessel (retentate). All samples were then filtrated with 0.22 μm pore size filters and were analysed with HPLC chromatography.

- ***Nanofiltration of enzymatic reaction***

For the enzymatic reaction a commercial mixture of enzymes (Celluclast) was used for the hydrolysis of birch (B4) biomass. The hydrolysis solution was produced by a 50 mL reaction with 3% (w/v) initial DM, 10 mg/g substrate enzyme loading and 100 mM phosphate-citrate buffer pH 5.0 after 48 hours of incubation. The hydrolysate was filtrated with 0.22 μm pore size filter and was then used for the nanofiltration trials. The trials were conducted at a pressure of 10 bar and room temperature for every membrane, and for the more efficient ones at higher temperatures (40, 50 and 60 °C). Again, the feed volume was 50 mL and the solution was concentrated by a factor of 2. Samples from the permeate were taken every 10 min and from the retentate in the end of the filtration. All samples were filtrated with 0.22 μm pore size filters and analyzed with HPLC chromatography. In the end of the filtration and after all the samples were taken, the permeate and the retentate were again mixed together, a new sample was taken and analyzed so that it can be used as the initial feed for the next membrane trial.

- ***Nanofiltration parameters***

The permeate's flux is calculated as follows:

$$J = \frac{V}{A * t} \quad [2]$$

Whereas J is the permeate flux (L / m^2 h), V is the permeate volume (L), A is the membrane area (m^2) and t is the permeation time (h) (Dalwani et al., 2010).

The water permeability of the membrane (L_p) is given by the following equation:

$$L_p = \frac{J_w}{TMP} \quad [3]$$

Whereas J_w is the water flux (L/h) and TMP the transmembrane pressure (m^2 * bar).

The retention of a solute j (R_j) is obtained by the equation [4]:

$$R_j = \left(1 - \frac{C_{p,j} * V_p}{C_{F,j} * V_F}\right) * 100\% \quad [4]$$

Whereas $C_{p,j}$ is the concentration of the solute j in the permeate and $C_{F,j}$ the concentration of the solute j in the feed. V_p is the volume of the permeate and V_F is the volume of the feed. For the calculations the average original feed and final retentate concentration were used, as the calculation based on the time dependent mass balance during the trials was not easy to be determined.

The cellobiose separation factor is given by the equation [5] in which the observed retentions of cellobiose (R_{cell}) and glucose (R_{gl}) are used. A separation factor greater than one corresponds to cellobiose enrichment in the retentate as compared to the feed solution (Morthensen et al., 2015).

$$X_{cell} = \frac{R_{cell}}{R_{gl}} \quad [5]$$

2.2.7 Evaluation of COS prebiotic potential

2.2.7.1 Culture procedure and media preparation

For the experimental purposes, both bacterial species (*lactobacilli* and *bifidobacteria*) were cultivated anaerobically at the optimal growth temperature of 36 °C from stock cultures. The stock cultures were maintained at -80 °C in the appropriate medium supplemented with 50 % (v/v) glycerol as a cryoprotectant. Cells from the culture broth of each species were subcultured (5% v/v) into fresh media and incubated 24 h anaerobically at 36 °C. The OD of the preculture was measured at 600 nm and the volume of the inoculum was then calculated so that the starting OD of the main culture is 0.3. The cells were collected after centrifugation at 4000 rpm for 10 min and resuspended in MRS medium containing cellobiose. The incubation for all the cultures was achieved in a Vinyl anaerobic chamber (COY Laboratory products, U.S.A) under strict anaerobic atmosphere using a regulated gas mixture.

The plant hydrolysates from birch and spruce biomass using an enzymatic cocktail with the recombinant enzymes were recovered with the procedure that is described in Section 2.2.4 of this thesis, prior to use as a carbohydrate source for the probiotic tests. A 2% (w/v) cellobiose content media was prepared from the final products of the scale up reactions, where the sugars where dissolved directly in MRS broth. The final product from spruce proved difficult to dissolve and required heating to 60 °C. The obtained media was then sterilized by membrane filtration using 0.22 μm pore size filters (Sartorius). Additionally, a 2% (w/v) carbohydrate content media was prepared with the commercial cellobiose as a positive control and was autoclaved normally (121 °C for 20min).

2.2.7.2 Evaluation of bacterial growth

- ***cellobiose as carbon source***

Firstly, for testing the effectiveness of cellobiose as prebiotic candidate, the growth of all strains was tested on commercial cellobiose. The growth cultures (50 mL) with glucose as carbohydrate source were prepared as described above. The cells from the preculture were inoculated in 50 mL MRS media containing 2 % (w/v) cellobiose and in MRS media with 2 % (w/v) glucose that was used as a positive control. The cultures were incubated under anaerobic conditions, as previously mentioned, for a maximum of 190 h. The absorbance was measured at the optical density of 600 nm (OD_{600}) using a spectrophotometer. All the cultures were grown in duplicates.

- ***Plant derived cello-oligosaccharides as carbon source***

The selected strains (*Lactobacilli* strains) that were able to grow on commercial cellobiose were tested on the birch and spruce derived cello-oligosaccharide (COS). The growth cultures (50 mL) with glucose as carbohydrate source were prepared as described above. The cells from the preculture were inoculated in 10 mL MRS media containing 2 % (w/v) birch and spruce COS and in MRS media with 2 % (w/v) commercial cellobiose that was used as a positive control. The cultures were incubated under anaerobic conditions, as previously mentioned, for a maximum of 100 h. The absorbance was measured at the optical density of 600 nm (OD_{600}) using Microplate spectrophotometer (Spectra max M2 Microplate Reader, Molecular Devices, U.S.A). All the cultures except from the positive control were grown in duplicates.

The carbohydrate consumption of the *Lactobacilli* and *Bifidobacteria* strains and the production of short chain fatty acids (SCFA) was analyzed using HPLC chromatography (HPLC 200, Perkin Elmer U.S, equipped with an RID detector, oven and Aminex HPX-87H Column (Biorad U.S)).

Chapter 3: Results

3 Results

3.1 Heterologous expression of *Tt*EG5, *Tt*LPMO, *Tt*CBH7

The recombinant proteins of interest were purified (Section 2.2.1.2) and further examined with SDS-page for the determination of their molecular weight. As indicated in **Figure 3.1**, the molecular weight of the purified *Tt*LPMO enzyme was estimated to be 60 kDa, whereas the molecular weight of *Tt*EG5 and *Tt*CBH7 was 75 kDa and 80 kDa, respectively.

The amount of protein that was secreted was estimated by the Lowry protein assay (Smith et al., 1985) and reached 67.5 mg of pure *Tt*EG5, 44 mg of pure *Tt*LPMO and 35 mg of pure *Tt*CBH7 per L of cultivation (**Table 3.1**).

The purified *Tt*EG5 and *Tt*CBH7 were assayed towards different substrates for their activity. *Tt*EG5 showed a specific activity of 1.06 U/mg on PASC 0.5 % w/v and 0.16 U/mg on Avicel 5 % w/v. *Tt*CBH7 exhibits a specific activity of 0.55 U/mg on PASC 0.5 % w/v and very low activity on Avicel 5 % w/v (0.02 U/mg) (**Table 3.2**).

As for the oxidative enzyme *Tt*LPMO, its activity was determined towards PASC that resulted in the generation of C1 and C4-oxidized products as shown in chromatograph of **Figure 3.2**. *Tt*LPMO is an enzyme with dual oxidative regioselectivity producing C1/C4 double oxidized products. C1 oxidized oligosaccharides elute at 13-19 min, while C4-oxidized oligosaccharides elute at 19-30 min retention time in HPAEC/PAD. The *Tt*LPMO activity towards PASC occurred as expected from other studies (Frommhagen et al., 2015; Karnaouri et al., 2017).

Table 3.1. Total amount of protein

Protein amount (mg)		
***Tt*EG5**	***Tt*LPMO**	***Tt*CBH7**
67.5	44	35

The results were calculated per lt of cultivation

Table 3.2. Specific activities of purified enzymes

	***Tt*EG5**	***Tt*CBH7**
PASC	1.06	0.55
AVICEL	0.16	0.02

The specific activities are expressed in Units per mg enzyme.

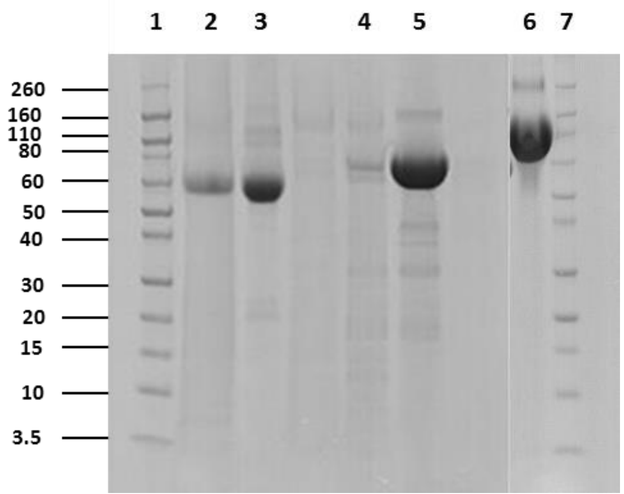


Figure 3.1. SDS Page of enzymes : 1. Novex® sharp pre-stained protein marker, 2.*Tt*LMPO after TFF, 3.purified *Tt*LMPO, 4.*Tt*EG5 after TFF, 5.purified *Tt*EG5, 6. Purified *Tt*CBH7, 7. Novex® sharp pre-stained protein marker

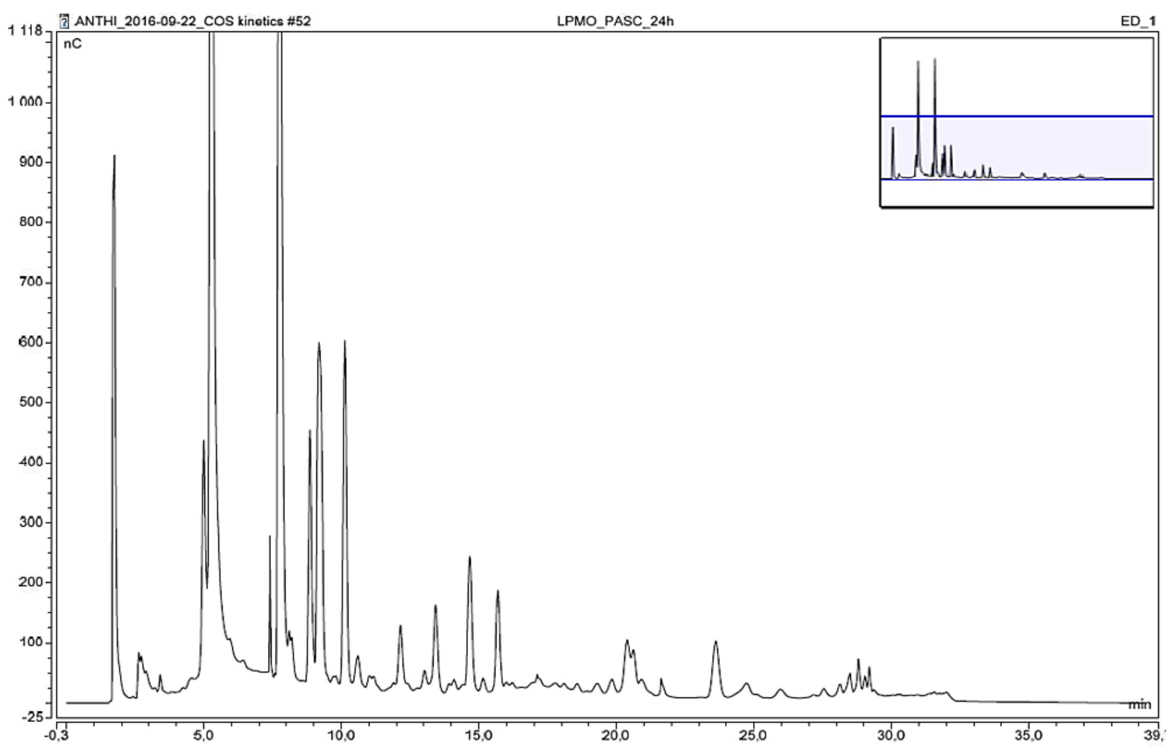


Figure 3.2. HPAEC/PAD chromatograph showing the activity of the recombinant *Tt*LPMO towards PASC with the production of oxidative products.

3.2 Enzymatic hydrolysis

3.2.1 Hydrolysis of lignocellulosic materials with the recombinant enzymes

The three recombinant enzymes (*Tt*EG5, *Tt*LPMO, *Tt*CBH7) were used for the conduction of the preliminary experiments that will determine the relative abundances of the enzymes that will maximize the cellobiose yield. The combinations of the three enzymes are depicted on **Table 2.5** and the equation that describes the cellobiose yield is given as follows:

$$Cellobiose\ yield\ (\%) = \frac{cellobiose\ concentration\ \left(\frac{mg}{mL}\right) * 1.05\ * 100}{\frac{mg\ substrate}{mL\ reaction} * cellulose\ percentage\ * 1.1} \quad [6]$$

Whereas, the cellulose percentage for birch is 66.3 % for B1 and 67.1 % for B4 and 66 % for spruce (S3) (**Table 2.2** of Section 2.1.3).

The results, as depicted in **Table 3.3** showed that for all the lignocellulosic substrates the optimum combination of the enzymes that maximizes the cellobiose yield is the same and particularly the combination no.5, which corresponds to 30 % *Tt*EG5, 30 % *Tt*LPMO and 40 % *Tt*CBH7 (**Table 2.5**). This observation confirms that a relative abundance of all the three enzymes is required for the efficient degradation of lignocellulosic biomass, with *Tt*CBH7 participating in a higher degree than the other two enzymes in the enzymatic mixture. Thus, the construction of an enzymatic cocktail is necessary for achieving higher cellobiose yields.

The cellobiose yield that is achieved by this enzyme combination for birch is 9.7 % and 12.1 % for B1 and B4, respectively. For spruce the maximum cellobiose yield that is achieved is 7.9 %. It can be therefore noticed that OS pretreated birch B4 yields higher cellobiose amounts among the other lignocellulosic substrates reaching 90.4 mg of cellobiose from 1 g of biomass, following to substrate B1 with 71.5 mg CB for 1 g of biomass and as expected lastly spruce (S3) with 58 mg CB for 1 g of biomass. It is observed that the hydrolysis yields for the OS pretreated substrates decrease as the lignin content increases (B4: 7.1 %, B1: 7.8 %, S3: 14.9 %). The higher lignin content of spruce is an obstacle for the efficient hydrolysis since the carbohydrate-acting enzymes and particularly cellulases have a CBM module that can be adsorbed onto the lignin compounds of the substrate in a way that hampers their activity and leads to lower production yields (Karnaouri et al., 2018).

It should also be mentioned that the cellobiose to glucose ratio is quite low for birch (4.7 for B4 and 6.2 for B1) while for spruce is quite high (18). According to these results, the limits of the relative abundances of the enzymes for the following experimental design for birch and spruce substrates were chosen as depicted at **Table 3.4**.

Table 3.3. Preliminary experiments with various combinations of the recombinant enzymes.

Combi-nation number	Birch (B1)			Birch (B4)			Spruce (S3)		
	CB yield (%)	mg CB/g biomass	CB/GL ratio	CB yield (%)	mg CB/g biomass	CB/GL ratio	CB yield (%)	mg CB/g biomass	CB/GL ratio
1	5.6	41.6	5.4	7.3	54.7	2.9	3.9	28.7	13.6
2	8.5	62.9	4.6	9.4	70.1	3.5	4.2	30.9	12.6
3	7.6	56.0	5.6	7.1	53.4	3.5	3.3	24.1	12.6
4	7.0	51.5	4.9	11.2	83.4	4.3	4.5	32.8	14.1
5	**9.7**	**71.5**	**6.2**	**12.1**	**90.4**	**4.7**	**7.9**	**58.0**	**18.0**
6	9.3	68.9	6.9	10.4	77.5	4.2	5.3	39.0	16.6
7	8.2	60.3	5.3	9.4	70.3	3.9	4.6	33.8	16.4
8	5.3	39.1	14.3	5.7	42.6	4.7	6.0	43.7	16.7
9	6.0	44.1	11.7	5.6	41.6	5.0	5.1	37.3	17.4
10	5.6	41.4	17.9	4.4	32.6	4.8	5.4	39.4	19.4

The reaction conditions are 3 % w/v DM, 25 mg/g enzyme load for 72 h in 50 °C. The results are expressed as a percentage of the conversion of the total cellulose content of the substrates. The bold values indicate the enzyme combination giving the maximum hydrolysis yields.

Table 3.4. Upper and lower limits for all variables used for the experimental design.

	Model variable	Upper limit	Lower limit
EG5	A	0.2	0.5
CBH7	B	0.3	0.6
***Tt*LPMO**	C	0.1	0.3
***Pa*Cbh6a**	D	0.1	0.3

The same experiment was carried out with only one birch substrate (B4) and spruce (S3) with the replacement of the recombinant *Tt*CBH7 with the commercial cellobiohydrolase CBHI so that the activity of the recombinant enzyme is compared with that of the commercial one. As shown in **Table 3.5**, the best combination is again no.5 which corresponds to 30 % *Tt*EG5, 30 % *Tt*LPMO and 40 % CBHI as previously mentioned. The results show once again that birch substrate gives higher hydrolysis yields (11.7 %) as verified by the high cellobiose amounts (87.1 mg CB for 1 g of biomass) than spruce with hydrolysis yield 5.3 % and cellobiose yield reaching 39 mg CB for 1 g of biomass, indicating again that birch is more suitable substrate for the purposes of this study. However, the cellobiose yields are generally lower after the replacement of the recombinant *Tt*CBH7 with the commercial CBHI, proving the high activity of the *Tt*CBH7 and its importance at the synergism together with the recombinant *Tt*EG5 and *Tt*LPMO.

Table 3.5. Preliminary experiments with various combinations of the recombinant enzymes (*Tt*EG5, *Tt*LPMO) and commercial CBH7.

Combination number	Birch (B4)			Spruce (S3)		
	Cellobiose yield (%)	mg CB/g biomass	Cell/gl ratio	Cellobiose yield (%)	mg CB/g biomass	Cell/gl ratio
1	7.8	57.8	5.3	3.4	24.6	2.6
2	9.3	69.5	6.1	2.6	19.3	2.5
3	6.7	50.0	4.0	3.0	21.9	2.6
4	9.9	73.7	5.7	2.7	20.1	1.4
5	**11.7**	**87.1**	**6.7**	**5.3**	**39.0**	**4.7**
6	11.4	85.2	6.3	3.8	27.9	3.5
7	10.5	78.5	5.2	4.8	35.4	5.8

3.2.2 Hydrolysis tests with the NZYtech enzymes

A study of 14 processive enzymes from bacterial and fungal origin (**Table 2.1** of Section 2.1.2) was carried out for illustrating the mode of action of each enzyme and point out their potential use as lignocellulose degrading cellulases. The main characteristics for the best candidate cellulase that will participate in the construction of an enzymatic mixture together with the recombinant enzymes are the high enzymatic activity together with a high cellobiose yield and a low glucose production.

The majority of the enzymes come from the bacteria *Clostridium cellulolyticum* and *Clostridium thermocellum*. The mesophilic bacterium *C.cellulolyticum* belongs to the group of bacteria that use multienzyme complexes called cellulosomes to digest cellulose (Bélaich et al., 1997). The most abundant family among the already sequenced cellulases from this organism is the glycoside hydrolase (GH) family 9 (Ding et al., 1999), therefore five out the six cellulases from *C.cellulolyticum* that were used belong to the GH family 9, while one of them belongs to the GH famlily 48. *C.thermocellum* is a strictly anaerobic, thermophilic bacterium that also possesses a cellulosomal enzyme system and is one of the most efficient systems discovered to date (Bayer et al., 1983). The three out of four enzymes from *C.thermocellum* that were used belong to the GH family 9, while one belongs to the GH family 5. Only one cellulase from the NZYtech enzymes was from the bacterium *Ruminococcus champanellensis* and belongs also to the GH family 9. *R. champanellensis* is anaerobic, cellulolytic, gram positive bacterium that is the only to date human colonic bacterium that is capable of degrading crystalline cellulose (Moraïs et al., 2016). Two more cellulases belonging to the GH9 and GH48 families come from the bacteria *Clostridium cellulovorans* and *Clostridium stercorarium,* respectively. Lastly, one cellulase belongs to the family GH6 and is the only one that comes from an eukaryotic organism, particularly the ascomycete *Podospora anserina* which is a fungus that is

thought to degrade specifically the recalcitrant parts of lignocellulose and encodes a large diversity of CAZymes.

The exo-acting cellulases that belong to the GH family 48 are known to hydrolyze the cellulose chains from the reducing ends, while GH9 and GH6 enzymes act processively from the non-reducing ends. There are as well certain processive endo-acting enzymes that belong to the GH5 and GH9 families (Leis et al., 2017; Wilson and Kostylev, 2012).

- ***Hydrolysis of model substrates***

For the determination of the cellulase activity and for testing the substrate specificity of the enzymes, hydrolysis was carried out on three different cellulosic polysaccharides (PASC, Avicel and CMC). Together with the 14 processive cellulases, the recombinant *Tt*EG5 was tested for comparison. The released sugars from the hydrolysis of the cellulosic polysaccharides were detected with HPAEC/PAD chromatography as mentioned in Section 2.2.5.1. The evaluation of the results was based on the chromatograph peaks of the oligosaccharides with DP 1-5 (**Figure 2.3**). The product profile as well as the total cellulose conversion into oligosaccharides was calculated with the following equation (eq.7):

$$Cellulose\ conversion\ (\%) = \frac{(C1 + C2 * 1.05 + C3 * 1.07 + C4 * 1.08 + C5 * 1.09) * 100}{Csubstrate * 1.1} \quad [7]$$

Where the concentration of glucose, oligosaccharides and initial substrate are calculated in mg/mL of reaction volume and 1.05, 1.07, 1.08 and 1.09 are the conversion rates of cellobiose, C3, C4, and C5 to glucose respectively. The cellulose percentage for PASC, Avicel and CMC was considered as 100%.

The results of the **Table 3.6** show the percentage of the products that are released during the hydrolysis with PASC 3 % w/v as a substrate. The no.15 recombinant enzyme, *Tt*EG5, has the highest catalytic activity (7.53 %) together with the highest cellobiose yield (84.2 %) while it produces 8.1 % glucose. The enzyme no.6, *PaCbh6a*, a cellobiohydrolase 6A from the fungal organism *Podospora Anserina* is also very active (6.33 %) with cellobiose yield 82.1 % and very low glucose percentage (2.2 %). The enzyme no.8, *CcCel9M*, exhibits the next higher activity (6.10 %) but it has very low cellobiose yields (30 %) and quite high glucose percentage (11.4 %).

With Avicel 3 % w/v as substrate same observations can be made (**Table 3.7**), although all the enzyme activities are significantly lower than with PASC substrate. The no.15, recombinant enzyme, *Tt*EG5, has an activity of 2.52 %, the highest among the other 14 enzymes and 93.2 % cellobiose yield while 6.8 % glucose yield. Next, is again the enzyme no.6, *PaCbh6a*, with activity 1.06 %, cellobiose yield 92.5 % and only 2.5 %

glucose perecentage. The activity of the enzyme no.8, *CcCel9M*, is 0.71 % and the cellobiose yield is again quite low (36 %) and the glucose percentage relatively high (12.5 %).

The activity results with CMC 1 % w/v as the cellulosic substrate are quite different than with PASC and Avicel substrates (**Table 3.8**). On CMC the enzymes with the highest activity are the enzyme no.2, *CtCbh5A*, with activity of 1.51 % and cellobiose yield 78.3 % and enzyme no.4, *CtCbh9A*, with 1.37 % enzymatic activity but very low cellobiose yield (44.8 %). The recombinant enzyme, no.15 *Tt*EG5, has again high cellobiose yield (93.3 %), although it does not exhibit the highest activity among the other enzymes (1.25 %). The enzyme no.6, *PaCbh6a*, shows lower percentages than the previous activity tests both for the cellobiose yield (76.4 %) and the enzymatic activity (0.68 %).

Table 3.6. Activity tests results of NZYtech enzymes on PASC substrate.

PASC			Products (%)					
	Enzyme name	Temperature (°C)	C1	C2	C3	C4	C5	Activity (%)
1	*CcCel48A*	37	1.0	57.2	21.5	20.4	0.0	2.26
2	*CtCbh5A*	60	2.1	71.1	26.2	0.7	0.0	4.41
3	*CtCel9A*	60	11.7	47.7	14.2	26.4	0.0	0.17
4	*CtCbh9A*	60	44.2	0.0	55.8	0.0	0.0	0.04
5	*CtCel9B*	60	0.0	75.4	19.8	4.8	0.0	0.82
6	***PaCbh6A***	**50**	**2.2**	**82.1**	**13.1**	**1.1**	**1.6**	**6.33**
7	*CcCel9W*	37	5.9	40.2	36.1	13.0	4.9	3.02
8	*CcCel9M*	37	11.4	30.0	23.6	35.0	0.0	6.10
9	*CcCel9R*	37	1.0	16.0	25.4	41.2	16.4	1.28
10	*CcCel9A*	37	14.4	80.7	3.7	1.2	0.0	4.78
11	*CsCbh48A*	60	2.1	70.2	19.7	6.1	1.8	3.30
12	*CcCel9J*	37	0.0	0.0	0.0	0.0	0.0	0.02
13	*CcCel9Q*	37	6.7	60.1	26.8	3.4	3.0	3.87
14	*RfCel9A*	50	1.9	35.5	19.1	32.0	11.5	2.61
15	***TtEG5***	**50**	**8.1**	**84.2**	**2.9**	**1.8**	**3.1**	**7.53**

For the activity tests the reaction conditions are 0.5% w/v DM, 20 mg/g enzyme load for 24 h. The bold values indicate the enzymes with the highest activity yields.

Table 3.7. Activity tests results of NZYtech enzymes on Avicel substrate.

Avicel			Products (%)					
	Enzyme name	**Temperature (°C)**	**C1**	**C2**	**C3**	**C4**	**C5**	**Activity (%)**
1	*CcCel48A*	37	1.3	61.6	29.8	7.3	0.0	0.39
2	*CtCbh5A*	60	4.1	79.0	16.8	0.0	0.0	0.43
3	*CtCel9A*	60	0.0	52.2	24.1	23.6	0.0	0.06
4	*CtCbh9A*	60	0.0	0.0	100.0	0.0	0.0	0.02
5	*CtCel9B*	60	0.0	70.1	29.9	0.0	0.0	0.13
6	***PaCbh6A***	**50**	**2.5**	**92.5**	**5.0**	**0.0**	**0.0**	**1.06**
7	*CcCel9W*	37	7.6	50.7	41.6	0.0	0.0	0.22
8	*CcCel9M*	37	12.5	36.0	50.1	1.4	0.0	0.71
9	*CcCel9R*	37	5.2	30.0	42.6	22.3	0.0	0.10
10	*CcCel9A*	37	3.5	91.5	5.0	0.0	0.0	0.41
11	*CsCbh48A*	60	1.8	75.8	22.5	0.0	0.0	0.61
12	*CcCel9J*	37	0.0	0.0	0.0	0.0	0.0	0.00
13	*CcCel9Q*	37	1.4	74.0	24.7	0.0	0.0	0.20
14	*RfCel9A*	50	2.2	56.9	18.4	22.5	0.0	0.10
15	***TtEG5***	**50**	**6.8**	**93.2**	**0.0**	**0.0**	**0.0**	**2.52**

Table 3.8. Activity tests results of NZYtech enzymes on CMC substrate.

	CMC		Products (%)					
	Enzyme name	**Temperature (°C)**	**C1**	**C2**	**C3**	**C4**	**C5**	**Activity (%)**
1	*CcCel48A*	37	0.0	49.4	25.7	24.9	0.0	0.24
2	***CtCbh5A***	**60**	**3.4**	**78.3**	**12.4**	**5.9**	**0.0**	**1.51**
3	*CtCel9A*	60	0.0	33.7	18.5	31.5	16.3	0.51
4	***CtCbh9A***	**60**	**8.4**	**44.8**	**36.8**	**7.6**	**2.5**	**1.37**
5	*CtCel9B*	60	0.0	70.8	27.4	1.8	0.0	0.97
6	*PaCbh6A*	50	3.0	76.4	20.6	0.0	0.0	0.68
7	*CcCel9W*	37	7.7	53.6	34.5	4.2	0.0	1.07
8	*CcCel9M*	37	5.8	25.2	18.9	24.7	25.4	0.42
9	*CcCel9R*	37	1.3	14.8	28.9	35.8	19.2	1.08
10	*CcCel9A*	37	11.2	81.4	7.4	0.0	0.0	1.03
11	*CsCbh48A*	60	5.2	59.5	21.0	5.9	8.4	1.08
12	*CcCel9J*	37	0.0	0.0	0.0	0.0	0.0	0.00
13	*CcCel9Q*	37	6.7	59.0	22.8	0.0	11.6	1.21
14	*RfCel9A*	50	1.8	44.2	20.1	24.9	9.0	1.13
15	***TtEG5***	**50**	**6.7**	**93.3**	**0.0**	**0.0**	**0.0**	**1.25**

Table 3.6-Table 3.8 show the results from PASC, Avicel and CMC hydrolysis describing the percentage of the products that are released, as well as the catalytic activity of each enzyme. The recombinant endoglucanase *Tt*EG5 produced the highest cellobiose (G2) amounts from all the model substrates that were used (84.2 - 93.3 %) (lowest and highest detected percentages), and glucose (6.7 – 8.1 %) to lesser extent while it produced traces of G3 - G5 products. *Tt*EG5 demonstrated higher activity towards PASC (7.53 %) followed by Avicel (2.52 %) and lastly by CMC (1.25 %) as indicated also by Karnaouri et al., 2017.

Among the NZYtech enzymes the one that observed to be the most active is the cellulase from the fungal organism *P.anserina* which showed similar substrate specificity with *Tt*EG5, demonstrating higher activity towards PASC (6.33 %) and Avicel (1.06 %) and very low ability to hydrolyze CMC (0.68 %). These results are in accordance with a study that indicated higher specific activity of this cellulase towards Avicel, than CMC (PASC was not tested as a substrate) (Poidevin et al., 2013). In this study it was observed that CMC might not be a good substrate for this *P.anserina* cellulase, whereas it is more active towards crystalline cellulose. In the same study the processivity of the cellulase is verified with the observation of its active site tunnel topology, giving another explanation to the lower activity on CMC, as the substituted glucose units have more difficulty entering the narrow active site. These indications suggest that *PaCbh6A* can be considered as an exo-acting cellobiohydrolase. Additionally, the main hydrolysis products from these model cellulose substrates for the *P.anserina* cellulase was cellobiose (G2) (76.4 - 92.5 %) and to lesser extent cellotriose (G3) (5 - 20.6 %) and glucose (G1) (2.2 - 3 %) once more being in accordance with the results from the previously mentioned study.

The substrate preference among the NZYtech cellulosomal cellulases and the released products vary substantially. For example, the *C.thermocellum* cellulases *CtCel9A, CtCbh9A, CtCel9B* demonstrate almost no activity towards PASC and Avicel, while their activity is higher when tested towards CMC substrate. Therefore, due to their ability to hydrolyze CMC these enzymes can be considered as endo-processive cellobiohydrolases (Leis et al., 2017). However, it is already known that the distinction between endo- and exo-glucanases is hard to establish experimentally (Poidevin et al., 2013). The cellulase from *C.cellulolyticum, CcCel9J* showed no activity at all towards any of the substrates. In contrast, the cellulases *CcCel9W, CcCel9R, CcCel9A, CcCel9Q* from *C.cellulolyticum*, *CsCbh48A* from *C.cellulovorans*, *RfCel9A* from R.*champanellensis* and *CtCbh5A* from *C.thermocellum* appear active in both PASC and CMC. The enzymes CcCel48A and CcCel9M from *C.cellulolyticum* appear to be most active in PASC than in CMC.

- ***Kinetics of hydrolysis on oligosaccharides***

The hydrolysis pattern of the NZYtech cellulases was further studied on soluble cello-oligosaccharides with DP from 5- 8 with a kinetics study with which the constant k=kcat/km is calculated. The results of the kinetic study are described on **Table 3.9**, where is shown the catalytic efficiency (kcat/km) of each cellulase for every substrate. As expected from the previous screening on model substrates, the enzymes with the highest catalytic efficiency are the recombinant *Tt*EG5 and the cellulase from *P.anserina* with values on the range of 10^5 and 10^4, respectively. Another observation that can be made is that while the DP of the substrate changes the behavior of each cellulase varies. For example, the recombinant *Tt*EG5 follows a G6 > G5 > G4 > G3 pattern showing higher kcat/km values towards G6 which is in accordance with the results of another study (Karnaouri et al., 2017). The same pattern is observed for the PaCbh6A cellulase. Other enzymes that show an increasing activity together with the increase of the DP of the oligosaccharides are the cellulases: no.1, no.4, no.7, no.11, no.13, no.14, while other enzymes show a decrease on their activity (no.3, no.9). On the other hand, there are also some enzymes that their activity remains the same despite the increase on the DP (no.2, no.5, no.8, no.10).

The product profile of the cellulases on soluble oligosaccharides is depicted at **Table 3.10**. Each cellulase has a distinctive mode of action on each substrate, however some similarities can be noticed. Particularly, the mode of action of *CtCel9A, PaCbh6A, CcCel9W* are the same, as well as the mode of action of the recombinant *Tt*EG5 with the *CcCel9Q* and *CtCbh9A* with *CcCel9M*.

Table 3.9. Catalytic efficiency of the NZYtech enzymes on cello-oligosaccharides with DP 5-8.

	Enzyme name	Temperature (°C)	kcat/km			
			C5	**C6**	**C7**	**C8**
1	*CcCel48A*	37	1.37 x 10^3	2.89 x 10^3	4.35 x 10^3	4.41 x 10^3
2	*CtCbh5A*	60	8.18 x 10^3	8.15 x 10^3	8.3 x 10^3	9.01 x 10^3
3	*CtCel9A*	60	6.45 x 10^2	6.76 x 10^2	2.55 x 10^2	9.3 x 10^1
4	*CtCbh9A*	60	7.46 x 10^2	7.61 x 10^2	2.01 x 10^2	2.36 x 10^2
5	*CtCel9B*	60	1.71 x 10^3	1.56 x 10^3	1.59 x 10^3	1.88 x 10^3
6	***PaCbh6A***	**50**	**5.13 x 10^4**	**3.46 x 10^4**	**8.87 x 10^4**	**8.11 x 10^4**
7	*CcCel9W*	37	1.13 x 10^3	8.74 x 10^3	2.18 x 10^4	2.86 x 10^4
8	*CcCel9M*	37	2.99 x 10^4	2.52 x 10^4	3.17 x 10^4	2.78 x 10^4
9	*CcCel9R*	37	-	2.04 x 10^3	1.23 x 10^3	8.51 x 10^2
10	*CcCel9A*	37	1.37 x 10^3	1.34 x 10^3	1.34 x 10^3	1.79 x 10^3
11	*CsCbh48A*	60	1.00 x 10^3	1.56 x 10^3	2.97 x 10^3	3.70 x 10^3
12	*CcCel9J*	37	-	-	-	-
13	*CcCel9Q*	37	3.26 x 10^3	3.81 x 10^3	5.04 x 10^3	6.51 x 10^3
14	*RfCel9A*	50	1.12 x 10^2	1.94 x 10^3	5.77 x 10^3	5.93 x 10^3
15	***TtEG5***	**50**	**5.18 x 10^4**	**6.21 x 10^4**	**1.51 x 10^5**	**1.98 x 10^5**

For the kinetics study the reaction conditions are 0.5% w/v DM and 20 mg/g enzyme load. Samples were taken every 20 min for a total duration of 100 min. The bold values indicate the enzymes with the highest catalytic efficiency.

Table 3.10. Mode of action of the NZYtech enzymes for each cello-oligossacharide with DP from 5-8.

	Enzyme name	Temperature (°C)	Mode of action			
			C5	**C6**	**C7**	**C8**
1	*CcCel48A*	37	G3+G2	G3+G4	G4 to G1	G5 to G2
2	*CtCbh5A*	60	G3+G2	G4 to G2	G4 to G2	G6 to G1
3	*CtCel9A*	60	G3+G2	G4 to G2	G4 to G2	G5 to G2
4	*CtCbh9A*	60	G4 to G1	G4 to G2	G4 to G2	G5 to G2
5	*CtCel9B*	60	G3+G2	G4 to G2	G5 to G2	G6 to G2
6	***PaCbh6A***	**50**	**G3+G2**	**G4 to G2**	**G4 to G2**	**G5 to G2**
7	*CcCel9W*	37	G3+G2	G4 to G2	G4 to G2	G5 to G2
8	*CcCel9M*	37	G4+G1	G4+G2	G4 to G2	G5 to G2
9	*CcCel9R*	37	-	G3	G4+G3	G5 to G3
10	*CcCel9A*	37	G4 to G1	G4 to G2	G5 to G2	G4 to G2
11	*CsCbh48A*	60	G3+G2	G4 to G2	G6 to G1	G6 to G1
12	*CcCel9J*	37	-	-	-	-
13	*CcCel9Q*	37	G3+G2	G4 to G2	G4 to G2	G6 to G2
14	*RfCel9A*	50	-	G4 to G2	G5 to G2	G6 to G2
15	***TtEG5***	**50**	**G+G2**	**G4 to G2**	**G4 to G2**	**G6 to G2**

- ***Hydrolysis of lignocellulosic materials***

The screening of the mode of action of the NZYtech enzymes in addition with the recombinant *Tt*EG5, *Tt*CBH7 and commercial CBH6 and CBH7 cellulases was carried out on three different OS pretreated forest materials; two birch substrates (B1, B4) and one spruce (S3) (pretreatment conditions and composition mentioned at Section 2.1.3) for testing their lignocellulolytic degrading potential.

The activity test results on OS pretreated birch (B1) are shown at **Table 3.11**. The commercial CBH7 enzyme, no.17 has the highest activity yield (5.49 %) while the cellobiose and glucose yields are 77.2 % and 11.8 %, respectively. The next most active enzyme is the recombinant enzyme *Tt*EG5 with 2.17 % activity yield, cellobiose and glucose yields 85.3 % and 11.3 %. No.2 enzyme, *CtCbh5A*, appears to be quite active on birch (B1) substrate with 1.90 % activity yield. However, it produces relatively low cellobiose (77.6 %) and high glucose percentage (16.4 %). The enzyme no.6, *PaCbh6a*, shows quite low activity (0.64 %) but the cellobiose yield is very high 94.9 %, while it produces zero amounts of glucose.

The results of the activity tests of the enzymes on OS pretreated birch (B4) are shown at **Table 3.12**. The enzyme no.17, commercial CBH7 has an activity yield of 6.46 %, cellobiose and glucose percentages of 83.3 % and 13.7 %, respectively. Next, the recombinant enzyme no.15, *Tt*EG5, has an activity yield of 4.19 % and cellobiose percentage 80.5 %, while the glucose percentage is quite high 17.9 %. The enzyme no.2 *CtCbh5A* exhibits an activity of 4.94 %, cellobiose yield 78.5 % but high glucose percentage 19.9 %. The enzyme no.6, *PaCbh6a*, even though is not among the most active ones (1.20 % activity yield) it produces high cellobiose amounts (92.3 %) and low glucose percentages (6.9 %) as previously mentioned.

Table 3.13 shows the results of the activity tests on OS pretreated spruce (S3). The most active enzymes are again enzyme no.17, commercial CBH7 with activity yield of 9.85 %, enzyme no.15, recombinant *Tt*EG5 (5.81 %) and no.2, *CtCbh5A* with a yield of 4.26 %. However, the glucose percentage of enzyme no.2 is relatively high (16.2 %). The enzyme no.6, *PaCbh6a* has an activity yield of 2.32 % and may not be among the most active ones, but it produces high cellobiose amounts (92.1 %) and zero amounts of glucose.

Table 3.11. Activity tests of Nzytech enzymes on Birch (B1) substrate.

	Birch (B1)		Products (%)				
	Enzyme name	**Temperature (°C)**	**C1**	**C2**	**C3**	**C4**	**Activity (%)**
1	*CcCel48A*	37	0.0	90.9	9.1	0.0	0.23
2	***CtCbh5A***	**60**	**16.4**	**77.6**	**4.2**	**1.7**	**1.90**
3	*CtCel9A*	60	0.0	0.0	0.0	0.0	0.00
4	*CtCbh9A*	60	0.0	100.0	0.0	0.0	0.04
5	*CtCel9B*	60	0.0	76.6	23.4	0.0	0.04
6	*PaCbh6A*	50	0.0	94.9	5.1	0.0	0.64
7	*CcCel9W*	37	0.0	67.2	16.6	16.2	0.90
8	*CcCel9M*	37	3.5	41.1	23.8	1.7	1.28
9	*CcCel9R*	37	89.5	0.0	4.9	5.6	0.28
10	*CcCel9A*	37	10.6	80.2	5.6	3.5	1.68
11	*CsCbh48A*	60	0.0	94.9	5.1	0.0	0.59
12	*CcCel9J*	37	0.0	0.0	0.0	0.0	0.00
13	*CcCel9Q*	37	2.3	63.8	16.4	17.5	0.94
14	*RfCel9A*	50	0.0	0.0	0.0	0.0	0.00
15	***TtEG5***	**50**	**11.3**	**85.3**	**2.2**	**1.2**	**2.17**
16	*TtCBH7*	60	11.8	75.8	12.4	0.0	0.32
17	***CBHI Megazyme***	**50**	**11.8**	**77.2**	**4.9**	**6.1**	**5.49**
18	*CBHII Megazyme*	50	0.0	100.0	0.0	0.0	0.04

For the activity tests the reaction conditions are 1.5% w/v DM, 8 mg/g enzyme load for 48 h. The bold values indicate the enzymes with the highest activity yields.

Table 3.12. Activity tests of NZYtech enzymes on Birch (B4) substrate.

	Birch (B4)		Products (%)				
	Enzyme name	**Temperature (°C)**	**C1**	**C2**	**C3**	**C4**	**Activity (%)**
1	*CcCel48A*	37	0.0	85.0	6.0	9.1	0.32
2	***CtCbh5A***	**60**	**19.9**	**78.5**	**0.8**	**0.7**	**4.94**
3	*CtCel9A*	60	0.0	0.0	0.0	0.0	0.00
4	*CtCbh9A*	60	0.0	98.5	1.5	0.0	0.05
5	*CtCel9B*	60	0.0	98.8	1.2	0.0	0.04
6	*PaCbh6A*	50	6.9	92.3	0.8	0.0	1.20
7	*CcCel9W*	37	10.3	79.6	1.0	9.0	1.48
8	*CcCel9M*	37	34.6	61.4	1.0	3.1	2.35
9	*CcCel9R*	37	89.2	0.0	0.7	10.1	0.17
10	*CcCel9A*	37	13.2	84.6	0.8	1.3	2.55
11	*CsCbh48A*	60	8.9	87.8	0.8	2.4	0.75
12	*CcCel9J*	37	0.0	0.0	0.0	0.0	0.00
13	*CcCel9Q*	37	13.6	73.8	0.9	11.7	0.95
14	*RfCel9A*	50	0.0	0.0	0.0	0.0	0.00
15	***TtEG5***	**50**	**17.9**	**80.7**	**0.8**	**0.7**	**4.19**
16	*TtCBH7*	60	23.8	69.1	0.9	6.2	0.26
17	***CBHI Megazyme***	**50**	**13.7**	**83.3**	**0.8**	**2.2**	**6.46**
18	*CBHII Megazyme*	50	0.0	99.2	0.8	0.0	0.07

Table 3.13. Activity tests of NZYtech enzymes on Spruce (s3) substrate.

	Spruce (S3)		Products (%)				
	Enzyme name	Temperature (°C)	C1	C2	C3	C4	Activity (%)
1	*CcCel48A*	37	0.0	73.6	15.6	10.7	1.06
2	***CtCbh5A***	**60**	**16.2**	**81.0**	**2.8**	**0.0**	**4.26**
3	*CtCel9A*	60	0.0	58.0	31.0	11.0	0.06
4	*CtCbh9A*	60	0.0	0.0	100.0	0.0	0.02
5	*CtCel9B*	60	0.0	59.6	29.1	11.3	0.14
6	*PaCbh6A*	50	0.0	92.1	7.9	0.0	2.32
7	*CcCel9W*	37	0.0	63.4	27.7	8.9	0.72
8	*CcCel9M*	37	33.3	42.2	22.9	1.5	2.53
9	*CcCel9R*	37	0.0	26.6	25.6	47.8	0.13
10	*CcCel9A*	37	10.7	86.8	2.5	0.0	2.71
11	*CsCbh48A*	60	5.0	86.2	8.7	0.0	2.12
12	*CcCel9J*	37	0.0	0.0	0.0	0.0	0.00
13	*CcCel9Q*	37	12.2	77.2	10.6	0.0	1.10
14	*RfCel9A*	50	0.0	53,9	15.5	30.6	0.17
15	***TtEG5***	**50**	**10.1**	**88.8**	**1.0**	**0,0**	**5.81**
16	*TtCBH7*	60	10.1	76.3	11.6	1.9	0.65
17	***CBHI Megazyme***	**50**	**15.3**	**84.5**	**0.2**	**0.0**	**9.85**
18	*CBHII Megazyme*	50	0.0	82.8	17.2	0.0	0.26

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The **Table 3.11-Table 3.13** show the hydrolysis results on the two birch substrates (B1, B4) and on spruce (S3) describing the activity yields and the released products of each cellulase. It is observed that the substrate preferences and the mode of action of the released products are very similar to all the substrates. That is, the enzymes that seemed to be most active had the same behavior for all the lignocellulosic substrates. Particularly, for all the substrates the cellulase with the highest activity was the commercial cellobiohydrolase CBHI, followed by the recombinant *Tt*EG5 and then the bacterial cellulase from the bacterial organism *C.thermocellum*, *CtCbh5A*.

The high activity of the endoglucanase (EG5) and the cellobiohydrolase (CBH) on the lignocellulosic substrates was expected, as these enzymes are of high importance on the lignocellulose degradation process. In addition, these cellulases also have the ability to release cellobiose as a major product which is verified by the results of this test with cellobiose percentages for both CBHI and *Tt*EG5 between 77.2 and 88.8 % (lowest and highest detected percentages). These enzymes produce also low glucose amounts (10.1 – 17.9 %) and traces of cellotriose (G3) and cellotetraose (G4).

The cellulase *CtCbh5A* from the NZYtech enzymes that appeared to have the highest activity towards lignocellulosic substrates (1.90 – 4.94 %) releases as well cellobiose as the main product (77.6- 81 %), however it releases significant glucose amounts (16.2- 19.9 %) and also traces of cellotriose (G3) and cellotetraose (G4). It is worth mentioned that the eukaryotic cellulase from *P.anserina,* PaCbh6A, even though it does not show very high activity yields (0.64- 2.32 %) on lignocellulosic substrates, it produces very high cellobiose amounts (92.1- 94.9 %) together with minor glucose percentages (0- 6.9 %) and traces of cellotriose (G3).

The mode of action of the cellulases regarding the released products is very similar with the one mentioned from the hydrolysis tests with the model cellulosic substrates. However, the activity yields of the NZYtech cellulases demonstrated huge differences among the model substrates and the lignocellulosic ones. Particularly, the cellulase from the fungal organism *P.anserina, PaCbh6A* that seemed to be most active towards the model substrates had a significant decrease on the activity when tested to the "real" cellulosic substrates, where the most active cellulase proved to be the *CtCbh5A* from the bacterium *C.thermocellum*. Nevertheless, the ability of the fungal cellulase *PaCbh6A* to produce very high cellobiose amounts together with almost zero amounts of glucose render it a good candidate for the participation in the cellulolytic enzyme mixtures for the high production of cellobiose as a main product.

3.4 Experimental design

The activity tests that were performed for the NZYtech enzymes led to the conclusion that the enzyme no.6, the Cellobiohydrolase 6A, *PaCbh6A* from the fungal organism *P.anserina* will participate to the experimental design for birch (B4) and spruce (S3) biomass in order to determine the optimal enzyme combination for the maximum cellobiose yield. The preliminary results with the recombinant enzymes helped to choose the appropriate limits of the relative abundances of the enzymes for the experimental design (**Table 3.4**). The enzymes that were used for this design were the following three commercial cellulases (EG5, CBH7, EG7), together with the recombinant *Tt*LPMO and the processive enzyme that was chosen from the NZYtech enzymes, *PaCbh6a*. The maximum cellobiose yield that was released from the 48 h hydrolysis of birch was achieved with an enzyme combination that was consisted of 26 % EG5, 29 % CBH7, 12 % *Tt*LPMO, 29 % *Pa*Cbh6a and 5 % EG7. For the case of spruce, the optimal combination for the maximum cellobiose production was consisted of 34 % EG5, 42 % CBH7, 10% *Tt*LPMO, 10 % *Pa*Cbh6a and 5% EG7. After identifying the optimal enzyme mixtures that maximize the cellobiose yield, the commercial cellulases CBH7 and EG7 were replaced with the recombinant ones (*Tt*EG7 production was not a part of this thesis). These optimal enzyme combinations were used later on for the scale-up reactions.

3.5 Scale up reaction

A larger scale reaction of 100 mL was carried out using the optimized enzymatic combinations that occurred for each substrate (birch, spruce). The main target is the maximum production of cellobiose so that it can be tested later on as a carbohydrate source for several probiotic strains. The cellobiose yield was calculated by the equation [6], whereas the cellulose content for the OS pretreated birch (B4) is 67.1 % and for spruce (S3) 66% (**Table 2.2**). As shown at **Table 3.14** birch (B4) gives higher cellobiose yields (19.2 %) than spruce (S3) (9.6 %) as expected from the preliminary results (Section 3.2.1). The cellobiose to glucose ratio is quite low both for birch (6) and for spruce (5.1).

Table 3.14. Final products results from scale up reactions.

Biomass	Cellobiose yield (%)	mg CB/g biomass	mg GL/g biomass	CB/GL ratio
Birch	19.2	133.8	22	6
Spruce	9.6	84	16.5	5.1

The reactions were performed with 6 % w/v initial DM, 25 mg/g enzyme load for 96 h. The cellobiose yield occurs from the HPLC chromatography results in the end of each reaction, while the other values occur from the HPAEC/PAD chromatography results of the final freeze dried product.

3.6 Nanofiltration

For the experimental purposes of this thesis a screening of five different nanofiltration membranes was performed. The effect of different parameters was tested for evaluating the performance of each membrane regarding the best separation of cellobiose/glucose.

3.6.1 Effect of feed concentration

As depicted at **Table 3.15**, the model solution with commercial cellobiose and glucose was tested at different total sugar concentrations (5 and 20 mg/mL) with a constant ratio cellobiose/glucose of 9: 1. It should be mentioned that the cellobiose separation factors for all the membranes are quite low, with values not higher than 1.5, indicating that the cellobiose/glucose system is difficult to separate. Increasing the total feed concentration resulted in increased osmotic pressure of the solution and therefore in lower effective pressure (driving force) which reduced the permeate flux (data not shown) (Morthensen et al., 2015). As shown, an increase at the feed concentration didn't have a significant change on the cellobiose separation factor for all the pressures that were applied. It can only be noticed a very small decrease on the separation factor (on the range of 0.01 - 0.07) for most of the membranes which can be attributed to the increase on the total concentration of monosaccharide as it has been mentioned by Mah et al., 2014 even though their study was based on a different system (xylose/glucose).

The different feed concentration didn't have a significant effect also on the observed retention of both cellobiose and glucose (**Table 3.16**). The only observation that can be made is a minor increase on the retention percentages with the increase of the feed concentration for some membranes, at the range of maximum 2.4 % for cellobiose (DL membrane) and 6.6 % for glucose (NF270 membrane). For membrane NFX the trials were carried out only at 5 mg/mL feed concentration as the permeate flux was very high (data not shown) and the separation factor was the lowest (1.00) therefore it was not suitable for furthermore study.

Table 3.15. Separation factors for all the nanofiltration membranes at different feed concentrations and pressures with the model solution.

Membranes	Feed concentration (mg/mL)	Separation factor			
		5 bar	10 bar	15 bar	20 bar
NF270	5	-	**1.12**	-	1.03
	20	-	1.05	-	1.02
DL	5	1.11	**1.14**	1.11	1.03
	20	-	1.10	1.07	1.07
NFX	5	-	1.00	-	-
NFW	5	1.01	-	-	-
	20	0.96	-	-	-
TS40	5	-	1.03	1.07	-
	20	-	**1.11**	1.04	-

The cellobiose separation factor is given by the equation 5. Bold values indicate the membranes with high separation factors.

Table 3.16. Observed retention for cellobiose and glucose for all the membranes at different feed concentrations with the model solution at 10 bar*.

Membranes	Feed concentration (mg/mL)	Retention (%)	
		cellobiose	glucose
NF270	5	**96.3**	**86.4**
	20	**97.4**	**93.0**
DL	5	86.6	76.0
	20	89.0	81.3
NFX	5	98.8	98.5
NFW	5	64.0	63.2
	20	58.1	60.4
TS40	5	**93.0**	**90.2**
	20	**93.6**	**84.4**

**The data show the results for the trials that were carried out at room temperature and 10 bar except from the membrane NFW that the trial pressure was 5 bar. The retention values are given by the equation 4. Bold values indicate the membranes with the best retention combination.*

3.6.2 Effect of pressure

It was furthermore investigated whether an increase of the separation factor could be achieved by increasing the pressure. The overall conclusion comparing the separation factor values of **Table 3.15** for all the membranes is that while increasing the pressure the separation factor decreases. Therefore, the optimal pressure for the maximum separation factor was chosen to be 10 bar. The membranes that appeared to have the higher separation factors at 10 bar were the membrane DL (1.14), NF270 (1.12) and TS40 (1.11). For evaluating the membranes' efficiency at the enhanced separation of cellobiose/glucose system a comparison of the observed retentions of cellobiose and

glucose is necessary. **Table 3.16** depicts the observed retention for cellobiose and glucose for all the membranes at the optimized pressure of 10 bar. An efficient separation is achieved by high retention percentages of cellobiose and low retention percentages for glucose. Thus, the membranes that best satisfy this demand are the membrane NF270 (96.3 % for cellobiose and 86.4 % for glucose) and the membrane TS40 (93.6 % for cellobiose and 84.4 % for glucose).

3.6.3 Effect of temperature

It has been mentioned by previous studies that an increase in the temperature affects the separation process by having an impact on the feed solution properties but also on the pore structure of the membrane (Sharma et al., 2003). By the increase of the temperature the viscosity of the feed solution is decreased and the permeate flux increases (data not shown). The latter can be explained by the pore swelling of the membrane (Morthensen et al., 2015). The influence of temperature at the membranes that were chosen (NF270, TS40) as the more efficient regarding the separation of cellobiose/glucose system was investigated at 10 and 20 bar in order to understand if the increase at the pressure has an impact at the membrane's performance.

As shown at **Table 3.17**, the increase of the temperature seems to be better combined with the feed concentration of 20 mg/mL, since the separation factor is increased more significantly than with the 5 mg/mL feed concentration. For membrane NF270 and 20 mg/mL feed concentration the separation factor increases at higher temperatures, although the increase is mostly significant from room temperature, 25 °C (1.05) to 40 °C (1.21) while it increases at a smaller extent at higher temperatures. For the membrane TS40 on the other hand and feed concentration 20 mg/mL the increase of the separation factor is significant from 40 °C (1.21) to 50 °C (1.39). It is again noticed that higher pressure doesn't help at the separation process, as tested with membrane NF270 at 20 bar since the increase at the separation factor was less significant than the one at 10 bar.

However, from the results at **Table 3.18**, it is observed that generally as the temperature increases the retention for both cellobiose and glucose decreases. Consequently, the best working temperature depends on the purpose of the filtration and also at the membrane. If higher cellobiose retention is needed, without losing important cellobiose amounts the suitable temperature is room temperature (25 °C). On the other hand, if the purpose is the largest possible separation of the cellobiose/glucose system, despite the cellobiose loss amounts, then an increase at the temperature is suggested. For membrane NF270 the optimal temperature is 40 °C and for TS40 50 °C for the largest separation of the cellobiose/glucose system.

Table 3.17. Separation factors for the best nanofiltration membranes at different feed concentrations, pressures and temperatures with the model solution.

Membranes	Feed concentration (mg/mL)	Separation factor					
		10 bar			20 bar		
		40 °C	50 °C	60 °C	40 °C	50 °C	60 °C
NF270	5	1.32	1.23	1.31	1.02	1.09	1.15
	20	1.21	1.26	1.36	1.10	1.08	1.29
TS40	5	1.00	1.16	-	-	-	-
	20	1.21	1.39	-	-	-	-

The cellobiose separation factor is given by the equation 5.

Table 3.18. Observed retention for cellobiose and glucose at different feed concentrations and temperatures with the model solution at 10 bar.

Membranes	Feed concentration (mg/mL)	Retention (%)							
		RT		40 °C		50 °C		60 °C	
		CB	GL	CB	GL	CB	GL	CB	GL
NF270	5	96.3	86.4	91.4	69.2	89.1	72.6	87.6	66.8
	20	97.4	93.0	95.5	79.1	91.2	72.5	94.9	69.9
TS40	5	93.0	90.2	99.3	99.2	98.6	84.6	-	-
	20	93.6	84.4	91.3	75.5	89.3	64.3	-	-

The retention values are given by the equation 4. CB stands for cellobiose, GL for glucose and RT for room temperature.

3.6.4 Nanofiltration with the enzymatic hydrolysate

A screening of the membranes was performed with the real hydrolysate solution as described in Section 2.2.6.2, for comparison with the results with the model solution. The trials were carried out at the optimal pressure of 10 bar and at room temperature for all the membranes except the membrane NFW, which showed from the trials with the model solution very low performance. For the membranes that proved to be more efficient (NF270 and TS40) the trials were also carried out at higher temperatures (40, 50 and 60 °C). As shown at **Table 3.19** the results were in accordance with the ones with the model solution. Particularly, the membranes with the highest separation factor were membrane NF270 (1.58 at 40 °C) and TS40 (1.35 at 50 °C). From the results at **Table 3.20** it can be observed that the retention for both cellobiose and glucose reduced with the increase of the temperature for membrane NF270, while for TS40 the cellobiose retention increased from 40 °C (91.2 %) to 50 °C (94.6 %). For the real hydrolysate solution again the same conclusions are made regarding the most efficient separation of cellobiose/glucose. If higher cellobiose retention is needed the optimal temperature is 25 °C, while for the largest separation of cellociose/glucose for membrane NF270 the optimal temperature is 40 °C and for membrane TS40 50 °C.

Table 3.19. Separation factors for different membranes and temperatures with the enzymatic hydrolysate at 10 bar.

Membranes	Separation factor			
	RT	40 °C	50 °C	60 °C
NF270	1.06	**1.58**	1.26	1.32
DL	1.04	-	-	-
NFX	1.01	-	-	-
TS40	-	1.13	**1.35**	-

The separation factor is given by the equation 5. Bold values indicate the membranes with high separation factors

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Table 3.20. Observed retention for cellobiose and glucosecfor different membranes and temperatures with the enzymatic hydrolysate at 10 bar.

Membranes	Retention (%)							
	RT		40 °C		50 °C		60 °C	
	cellobiose	glucose	cellobiose	glucose	cellobiose	glucose	cellobiose	glucose
NF270	98.3	92.8	**88.3**	**55.9**	88.9	70.5	83.9	63.5
DL	88.2	84.6	-	-	-	-	-	-
NFX	99.8	98.4	-	-	-	-	-	-
TS40	-	-	91.2	80.9	**94.6**	**69.9**	-	-

The retention values are given by the equation 4. Bold values indicate the membranes with the best retention combination.

3.6.5 Water permeabilities

The average water permeabilities (L_p) of all the membranes were calculated with the equation 3 and are depicted at **Table 3.21**. The loss of initial water permeability during the nanofiltration trials was found to be 1 unit for all the membranes (data not shown). Hence, membrane fouling didn't seem significant in any of the cases.

Table 3.21. Average water permeability for all the membranes.

Membranes	Water permeability (L_p)
NF270	8
DL	7
NFX	6
NFW	13
TS40	8

The water permeability trials were carried out at 10 bar and at room temperature. The values were calculated with the equation 3.

3.7 Evaluation of COS prebiotic potential

3.7.1 Growth potential of *Bidibobacteria* and *Lactobacilli* strains on cellobiose

The growth potential of different bacterial probiotic strains was studied in the presence of pure cellobiose using glucose as a positive control. The evaluation of the results is described by both the increase of the optical density (OD_{600}) and the carbohydrate utilization as summarized in **Figure 3.3**. As depicted in **Table 3.22**, only one of the *Bifidobacterium* strains, *B. adolescentis*, showed a minor growth on the culture media that was supplemented with 2% w/v cellobiose as a carbon source. Particularly, the growth rate of *B. adolescentis* on cellobiose was very low (μ= 0.014 h^{-1}) compared to that when grown on glucose (μ=0.107 h^{-1}) which can be also observed by the low final optical density value (final OD_{600} = 1.06 ± 0.07) and the big incubation times that were required for the growth on cellobiose (190 h) as shown in **Figure 3.3a**. Thus, this strain was not chosen for the further experimental studies for the evaluation of the prebiotic potential of the plant-derived COS. The *Lactobacillus* strains showed generally higher growth potential, as two of them finally were able to efficiently use cellobiose as a carbon source (**Table 3.22**). The *L. gasseri* strain showed a good growth on cellobiose with a growth rate value similar to that of glucose (μ=0.212 h^{-1}) and quite high final optical density value (final OD_{600} = 1.56 ± 0.03) (**Figure 3.3b**). The *L. plantarum* strain appeared to be the most promising, as it shows the highest growth rate on cellobiose (μ=0.407 h^{-1}) as verified by the high final optical density value (final OD_{600} = 7.35 ± 0.04) while it is also the only strain that consumes completely the carbohydrate content at the first 25 h of fermentation as shown in **Figure 3.3c**. The growth rates of all the strains were lower when cultured in cellobiose than when cultured in glucose. The only metabolite that is produced by both *lactobacilli* strains when grown on pure cellobiose is the lactic acid, as depicted in **Table 3.23 and Table 3.24**. Production of short chain fatty acids (acetic, propionic, butyric acid) was not detected.

Table 3.22. Cellobiose utilization by *Bifidobacterium* and *Lactobacillus* strains.

Bifidobacterium strains	Cellobiose growth	Lactobacillus strains	Cellobiose growth
B. adolescentis DSM 20083	+	*L. gasseri DSM 20077*	++
B. longum DSM 20219	-	*L.plantarum ATCC 8014*	++++
B.animalis subsp. lactis	-	*L. reuteri DSM 20016*	-

A minus sign (-) indicates that final OD_{600} < 0.6, + indicates final OD_{600} = 0.6 - 1, ++ indicates final OD_{600} = 1 - 2, +++ indicates final OD_{600} = 2 - 5, ++++ indicates final OD_{600} > 5.

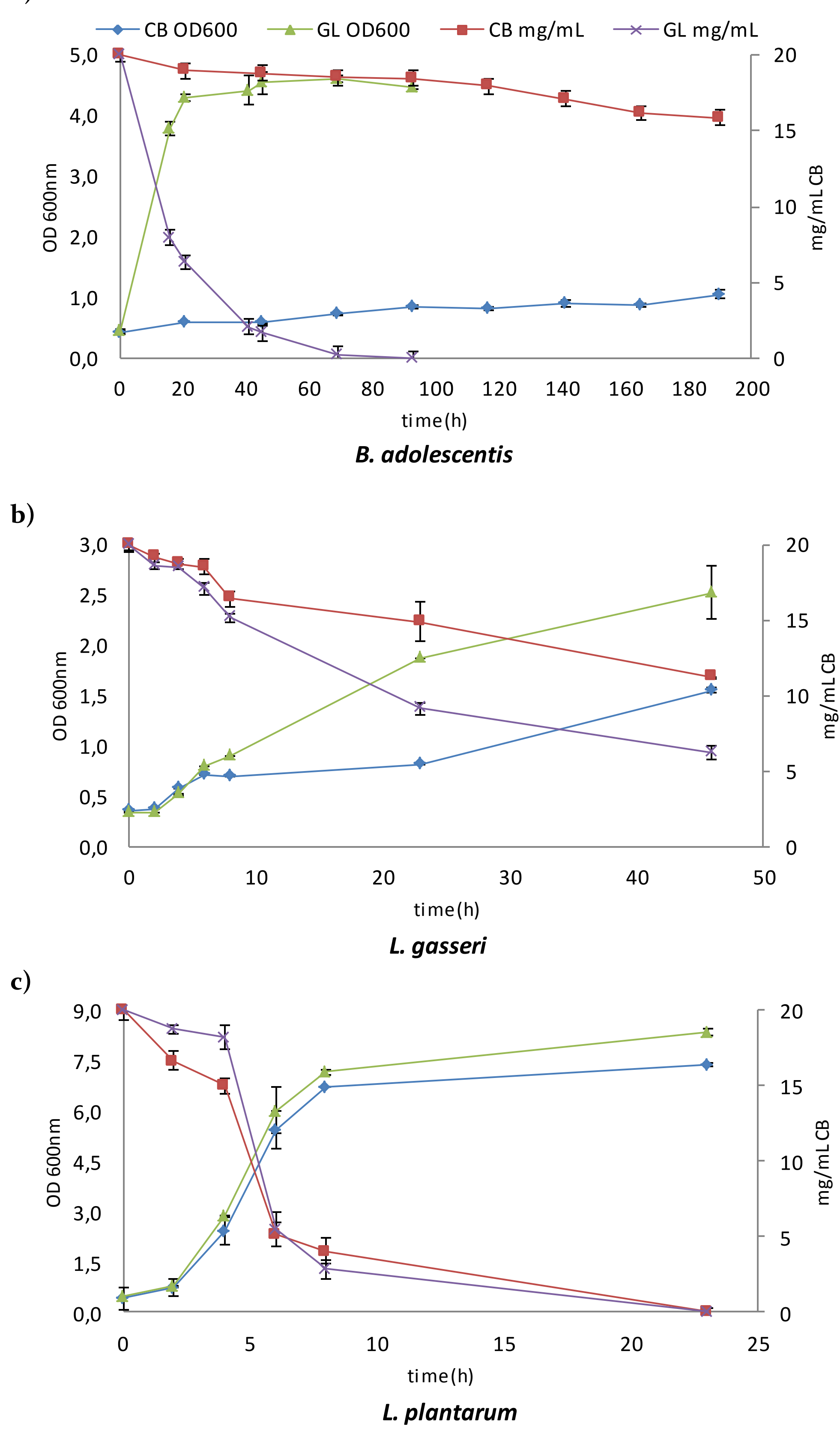


Figure 3.3. Growth curve and carbohydrate consumption of (a) *Bidibobacterium adolescentis*, (b) *Lactobacillus gasseri* and (c) *Lactobacillus plantarum* grown on culture media supplemented with 2% (w/v) commercial cellobiose and 2% (w/v) glucose used as positive control. All the cultures were grown in duplicates.

Table 3.23. Fermentation metabolites of *L. gasseri* upon growth on pure cellobiose, birch and spruce COS-rich hydrolysates.

	L. gasseri					
mg/mL	**cellobiose**		**birch**		**spruce**	
	0h	75h	0h	75h	0h	75h
cellobiose	20.0 ± 1.06	0.9 ± 0.12	20.0 ± 2.04	5.2 ± 1.88	20.0 ± 0.06	19.9 ± 1.32
lactic acid	0.16 ± 0.06	21.4 ± 0.91	0.2 ± 0.03	20.0 ± 0.05	0.2 ± 0.09	0.4 ± 0.03
acetic acid	4.0 ± 0.21	4.05 ± 0.05	7.9 ± 1.04	8.4 ± 1.07	6.6 ± 0.19	8.44 ± 1.39
propionic acid	0.9 ± 0.03	1.21 ± 0.12	0.8 ± 0.10	1.4 ± 0.17	0.7 ± 0.15	1.16 ± 0.18

No significant amounts of formic and butyric acid were detected.

Table 3.24. Fermentation metabolites of *L.plantarum* upon growth on pure cellobiose, birch and spruce COS-rich hydrolysates.

	L. plantarum					
mg/mL	**cellobiose**		**birch**		**spruce**	
	0h	26h	0h	26h	0h	75h
cellobiose	20.0 ± 1.51	0.0 ± 0.00	20.0 ± 1.69	0.0 ± 0.00	20.0 ± 0.76	12.3 ± 1.25
lactic acid	0.4 ± 0.01	21.8 ± 2.85	0.3 ± 0.05	28.4 ± 2.67	0.3 ± 0.00	21.2 ± 1.67
acetic acid	3.4 ± 0.15	3.9 ± 0.71	7.9 ± 0.92	8.4 ± 0.91	6.9 ± 0.25	8.8 ± 1.61
propionic acid	0.9 ± 0.01	0.9 ± 0.03	0.8 ± 0.09	1.0 ± 0.04	0.6 ± 0.02	1.2 ± 0.15

No significant amounts of formic and butyric acid were detected.

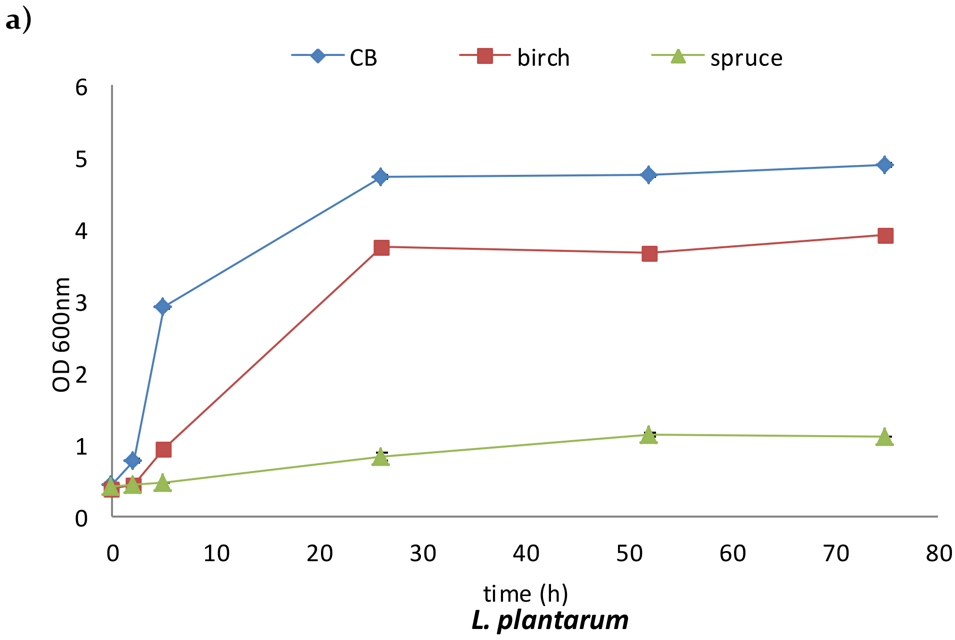
3.7.2 Growth potential of *Lactobacilli* strains on plant-derived COS

The *Lactobacillus* strains (*L.gasseri* and *L.plantarum*) that were able to utilize the pure commercial cellobiose were used for testing the prebiotic effect of the plant-derived cello-oligosaccharides (COS). Both strains were able to utilize the birch-derived COS from the larger scale hydrolysis (Section 2.2.4) as demonstrated by the increase of the optical density value and the consumption of the cellobiose content (**Figure 3.4, Figure** 3.5). The most effective strain was again the *L. plantarum* with a growth rate μ=0.161 h^{-1} as verified also by the high optical density value (final OD_{600} = 3.92 ± 0.01). In addition, this strain is able to degrade completely the cellobiose at the first 26 h of fermentation (**Figure 3.5a**), when compared to the 75 hours that are required by the *L. gasseri* strain (**Figure 3.5b**). The test of the prebiotic effect of the spruce-derived COS from the larger scale hydrolysis did not prove very promising, as the *L.plantarum* strain exhibited very low growth rate (μ=0.039 h^{-1}), while *L.gasseri* no growth at all (**Figure 3.5b**).

From the analysis of the fermentation products (**Table 3.23 and Table** 3.24) it is observed that again no significant amounts of short chain fatty acids were produced, while it should be mentioned that the acetic acid that was detected at the beginning of the fermentation originates from the cultivation media but also from the buffer that was used in the hydrolysis. As previously mentioned, the main metabolite that is

produced during the fermentation by both *lactobacilli* strains is the lactic acid. However, it should be noted that the concentration of lactic acid is much higher than the cellobiose that is consumed, which might be explained from the fact that there was some residual glucose in the biomass hydrolysates and that they might also contain other sugars that can be consumed by the bacteria and were not detected by the HPLC chromatography.

a)



b)

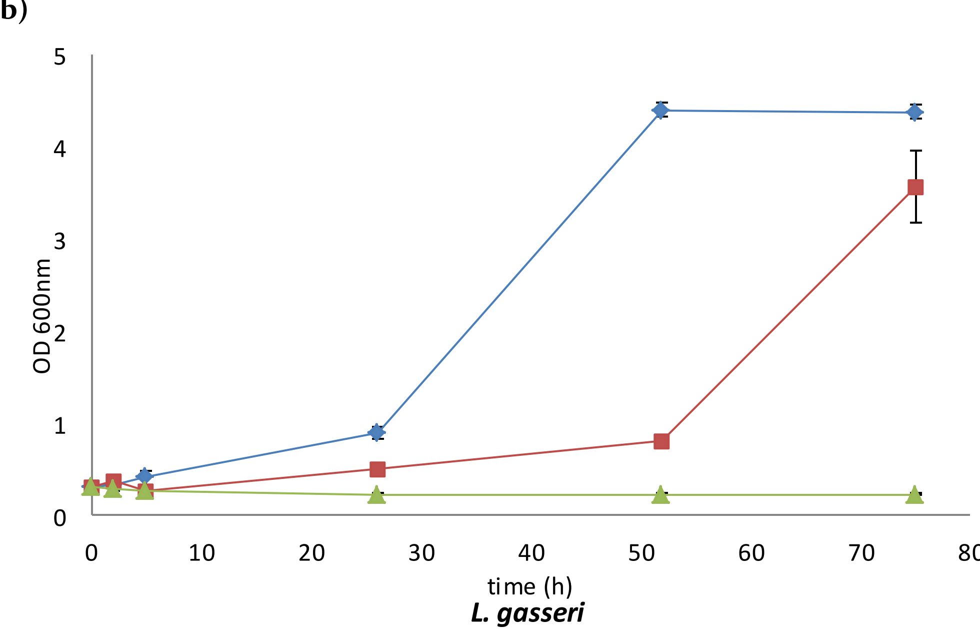
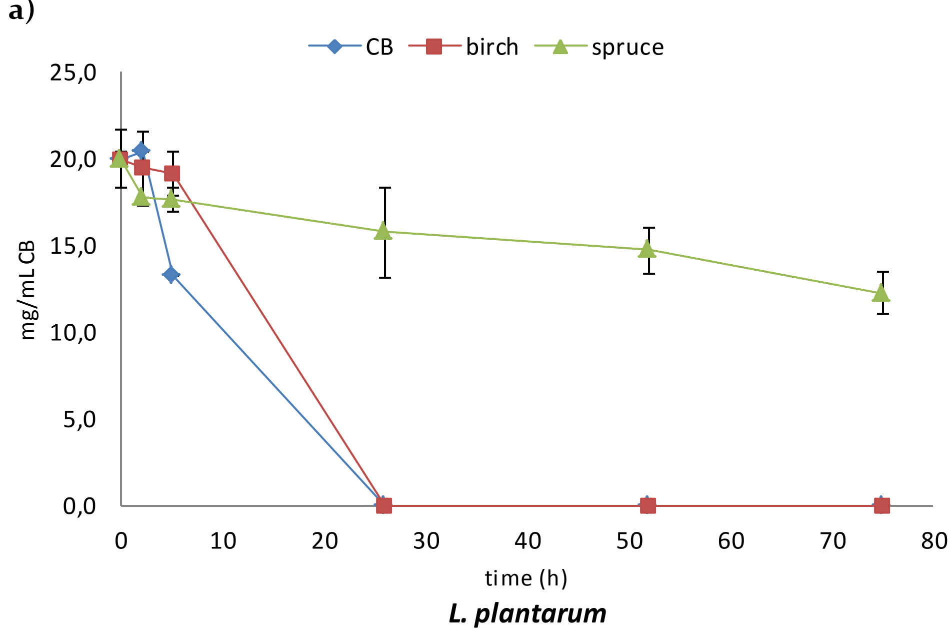


Figure 3.4. Growth curve of (a) *Lactobacillus plantarum* and (b) *Lactobacillus gasseri* cultured MRS media supplemented with 2% commercial cellobiose (control), 2% (w/v) birch and spruce-derived COS by the hydrolysis with the recombinant enzymes.

a)



b)

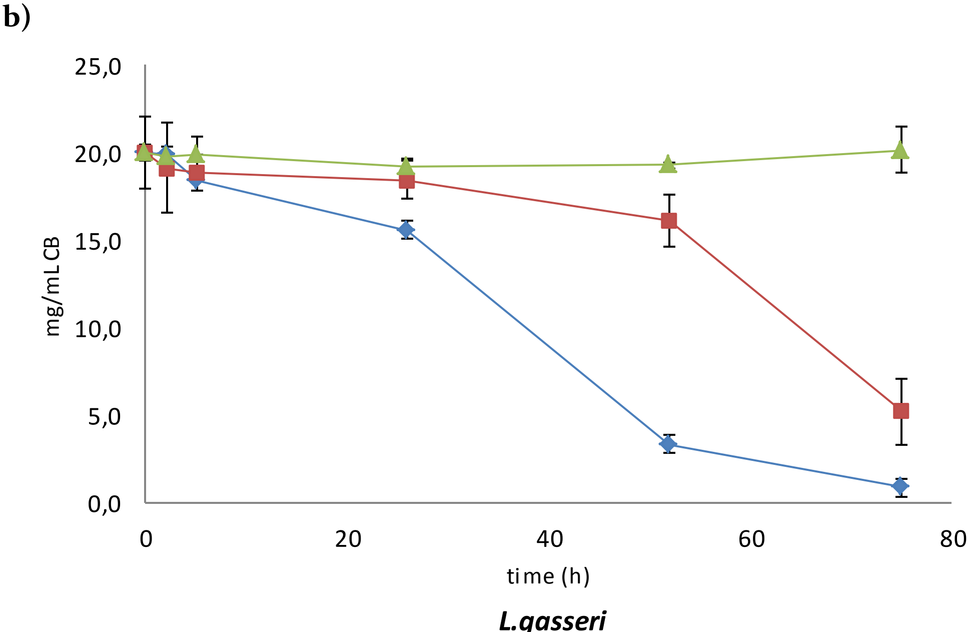


Figure 3.5. Consumption of cellobiose present in the (a) *Lactobacillus plantarum* and (b) *Lactobacillus gasseri* cultures with 2% (w/v) commercial cellobiose and 2% (w/v) birch and spruce-derived COS by the hydrolysis with the recombinant enzymes. Growth experiments were done in duplicates except control with cellobiose that was done only a single time.

Chapter 4: Discussion

4 Discussion

4.1 Heterologous expression of enzymes

The experimental purposes of this thesis required the production of three recombinant enzymes; one endoglucanase (EG5), one exoglucanase or cellobiohydrolase (CBH7) and one accessory enzyme, the lytic polysaccharide monooxygenase (LPMO). The genes of these enzymes were from the filamentous fungus *Thermothelomyces thermophila* and were expressed heterologously in the methylotrophic *Pichia Pastoris* yeast.

T. thermophila is a thermophilic fungus that owns a complete enzymatic repertoire for the bioconversion of complex carbohydrates, in this case for depolymerization of lignocellulosic material. Most of these enzymes exhibit high thermostability. *T. thermophila*'s cellulases have been shown to remain stable for temperatures up to 60°C (Morgenstern et al., 2012). Particularly, the studied enzymes have been shown to work efficiently in 50 °C for *Tt*EG5 and *Tt*LPMO (Karnaouri et al., 2017) and 60°C for *Tt*CBH7 (Karnaouri et al., 2018). Enzyme thermostability is essential during the saccharification process, which converts lignocellulosic biomass to reducing sugars, because of the harsh process conditions that are usually used as a pretreatment step in order to make the biomass more suitable for enzymatic hydrolysis. Another advantage is the higher resistance in contamination as well as the increased catalytic rate at higher temperatures that potentially reduces processing times, saving energy and improving fermentation yields.

The effectiveness of the methylorophic *P.pastoris* yeast as a heterologous host has already been studied and chosen for its various advantages, such as its capability to perform several post translational modifications as the eukaryotic organisms and the relatively easy culture techniques (Higgins and Cregg, 1998). The recombinant genes were grown and harvested as described in Section 2.2.1.1 (Topakas et al., 2012), the cultures were kept in shaking incubator at 30 °C (200 rpm) for maximum 7 days with the daily addition of 0.5 % v/v methanol, for the protein induction. Finally, after the completion of the induction, the proteins were purified (Section 2.2.1.2) and their molecular weight, concentration and hydrolytic activity towards PASC and Avicel substrates was determined. The molecular weight as shown in **Figure 3.1** was estimated 75 kDa for *Tt*EG5 and 80 kDa for *Tt*CBH7 which are in accordance with the results from similar studies (Karnaouri et al., 2017, 2018). Regarding the molecular weight of *Tt*LPMO, it was estimated to be 60 kDa, which is almost the double than the

one mentioned in other studies (Frommhagen et al., 2015, 2016). The *Tt*EG5 enzyme showed the highest specific activity towards PASC (1.06 U/mg), although the value is quite low when compared to another similar study where the activity of *Tt*EG5 on PASC reached 3.4 U/mg (Karnaouri et al., 2017). The *Tt*CBH7 enzyme showed as well highest specific activity towards PASC (0.55 U/mg), although it was also too low compared to a study were the activity value was 3.21 U/mg (Karnaouri et al., 2018).

4.2 Enzymatic hydrolysis of lignocellulosic forest materials

Lignocellulosic biomass is a renewable source that contains energy rich polysaccharides that when subjected to enzymatic hydrolysis they break down to sugars which can be further fermented to produce a variety of high added-value products. However, lignocellulose degradation is a challenging process due a number of factors linked with the recalcitrance and complex nature of this material. The saccharification of the lignocellulosic biomass is hence limited due to these factors. Particularly, as major requirements are considered the hemicellulose and lignin removal, the decrease of the degree of polymerization of the cellulose chain, the decrease of the cellulose crystallinity index (CrI) which is also accompanied by the decrease of the particle size and thus the increase of the accessible surface area (Mansfield et al., 1999; Zhao et al., 2012). Therefore, since lignocellulosic saccharification is necessary for the production of food grade fermentable sugars (this study is particularly focused on cellobiose) there is a necessity to overcome the difficulty and complexity of the depolymerization of this substrate. The pretreatment of the forest biomass is a prerequisite for achieving high hydrolysis yields and the organosolv method has proven a promising solution (Raghavendran et al., 2018).

It should also be mentioned the importance to create multicomponent cellulase mixtures that act synergistically upon the hydrolysis process and eventually increasing significantly the hydrolysis rates and overall yield. The beneficial effects of the synergism between endo- and exo- glucanases (EGs and CBHs) have already been reported (Andersen et al., 2008; Kleman-Leyer et al., 1996) as well as of the significant role of the "accessory" oxidative enzyme (LPMO) in the enzymatic cocktail (Karnaouri et al., 2017). Additionally, the endoglucanase EG5 and the cellobiohydrolase CBH7 are enzymes of major importance for the purposes of this thesis since the major product from the action of these enzymes on lignocellulosic substrates appeared to be the release of cellobiose. Particularly, EG5 has the advantage that is not as susceptible to cellobiose inhibition as CBH7, indicating their potential use in the large scale

production of food grade cellooligosaccharides (Karnaouri et al., 2017). For the current study these three key enzymes were produced heterologously as previously mentioned. Additionally, improving the enzymes that are already used in the lignocellulose bioconversion is of high importance. Therefore, during this thesis the screening of 14 processive bacterial and fungal cellulases was carried out for finally choosing the one that demonstrates higher activity and can be used to create novel enzymatic cocktails. As the most promising candidate it was chosen the eukariotic cellulase from the fungal organism *P. anserina*, *Pa*Cbh6A. An optimization design using the OS pretreated birch and spruce substrates was carried out in order to determine the optimal enzyme mixtures that maximize the cellobiose yield.

It has been reported that the bioconversion of lignocellulosic biomass is profitable when the enzymatic hydrolysis is performed at high solids (substrate) concentrations (≥15 %). The advantages that are linked with the increased substrate concentration are the increased product concentrations together with the decreased energy and water demands and thus the reduced operational costs (Kristensen et al., 2009; Roche et al., 2009). In the current study the enzymatic hydrolysis was performed at low solid concentration (<10 %), particularly, at 6 % w/v substrate loading using shake flasks.

The scale-up reactions were carried out using OS pretreated birch (B4) and spruce (S3) as lignocellulosic substrates and the optimized cellulase mixtures that occurred from the previously mentioned experimental design. The composition and structure of the lignocelullosic materials differs according to the type of wood that is used. The main hemicellulolytic component of hardwoods (birch) is xylan, while for softwoods (spruce) is glucomannan. Consequently, the response of the different materials to the pretreatment method that is being used is also distinctive. It has been reported that after the organosolv pretreatment of both birch and spruce biomass the lignin removal is more significant for birch than for spruce and therefore the enzymatic digestibility showed higher improvement in the case of birch than in the case of spruce (Nitsos et al., 2016; Raghavendran et al., 2018), as it can also be observed by the composition of the OS pretreated substrates that were used for this study (**Table 2.2** of Section 2.1.3).

The hydrolysis results showed a 2-fold higher cellobiose yield in the case of birch (19.2 %) compared to spruce, producing 133.8 mg cellobiose per g of biomass. As mentioned previously the higher lignin content for spruce (14.9 %) is one explanation for the lower hydrolysis yields. However, hydrolysis yields for both substrates were lower than expected. It has been mentioned that increasing the biomass concentration is linked as well with certain disadvantages that can offset the advantage of the increased product concentration (Kristensen et al., 2009). For example, high solids loading can cause insufficient mixing resulting in the increased viscosity of the slurries which was

observed mostly in the case of spruce, therefore becoming an obstacle for the mass transfer mechanisms throughout hydrolysis and thus blocking the efficient interaction between the cellulases and the substrate (Felby et al., 2008). Another factor that has been associated with the reduced hydrolysis is the inhibition of the cellulases that is caused by the sugars that are released and especially cellobiose. Additionally, the remaining hemicellulose derived products on the substrate, like xylan, also impact the hydrolysis rates of cellulases (Qing et al., 2010). Even though generally the inhibition is more apparent at high-solid concentrations it is also significant at low-solid concentrations (Modenbach and Nokes, 2013) and therefore can be applicable for the current study.

4.3 Cellobiose as a prebiotic candidate

Nowadays food and pharmaceutical industries show a growing interest on the development of functional food products. That is, products that demonstrate various beneficial effects for the consumer such as the improvement of the bioavailability of a particular component and eventually the reduction of the risk of certain diseases and the general amelioration of the person’s well-being. The main target for these high-added value food ingredients are the non- digestible oligossacharides (NDOs), such as cellobiose. Their importance is given by the fact that these oligosaccharides are able to be fermented by the human intestinal flora and therefore promote the growth of the beneficial gut bacteria such as *Bifidobacteria* and *Lactobacilli*.

The abundance of the lignocellulosic biomass together with its ability to generate high-added value oligosaccharides, such as cello-oligosaccharides (COS) through enzymatic treatment as previously mentioned, make it a sustainable source for the potential larger scale production of these novel food-grade ingredients. Therefore, the focus of this study is to elucidate the prebiotic potential of cellobiose not only as a pure component but as the main product from lignocellulose hydrolysis. In total six *bifidobacteria* and *lactobacilli* probiotic strains were tested for their ability to consume cellobiose as a sole carbon source.

The summarized results regarding the strains that could efficiently grow on cellobiose are depicted at **Table 3.22**. The *bifidobacteria* strains that were tested were unable to efficiently consume pure cellobiose, with only one strain (*B. adolescentis*) showing very low growing ability (μ= 0.014 h^{-1}) and therefore was not further tested upon lignocellulose-derived COS. This observation is in accordance with the results from another study (Pokusaeva et al., 2011b) where the growth of 36 different *bifidobacteria*

strains (including *B. adolsecentis*, *B. longum* and *B. animalis* subsp. *lactis*) was tested on cellobiose and finally none of these three particular strains was able to show a significant growth. However, it should be noted that the bacterial ability to metabolize particular carbohydrates is species and strain-dependent and is also connected with the number of genes that each strain possesses for encoding the enzymes that are able to metabolize plant-derived oligo- and poly-saccharides (Hopkins et al., 1998; Pokusaeva et al., 2011a). Therefore, further research needs to be carried out with different *bifidobacteria* species or strains to better understand their substrate preferences.

On the other hand, the *lactobacilli* species showed higher preference on pure cellobiose, with two (*L.gasseri* and *L.plantarum*) out of the three strains that were tested to be able to efficiently grow. Particularly, *L.plantarum* was the most promising strain demonstrating significantly higher growth rate (μ=0.407 h^{-1}) and fast cellobiose degradation (23 h) (**Figure 3.3c**). The *L.plantarum* strain is a very important and versatile species of the lactic acid bacteria (LAB) as it can be isolated from a variety of food and non-food related environmental niches such as fermented vegetables and the human gastrointestinal tract (GIT) (Siezen et al., 2010b). In addition, as explained in Section 1.6.1.1, it is known to have the largest genome size compared to other bacterial species and therefore it is able to encode higher number of transporter genes, particularly 25 PTS (phosphotransferase system) which is translated in its ability to ferment a wider range of carbohydrates (Kleerebezem et al., 2003).

Both *lactobacilli* strains were able to grow on the birch-derived COS that were generated by the larger scale hydrolysis using the constructed enzymatic cocktail as previously described. As expected, the *L.plantarum* proved to be again the most promising probiotic strain, having a growth rate of μ=0.161 h^{-1} and degrading cellobiose at the first 26 h of fermentation (**Figure 3.4.a**). Regarding the growth of the *lactobacilli* strains on spruce-derived COS that were generated using the same hydrolysis conditions as with birch, both strains showed a poor growing ability. Concluding, birch-derived COS provided an efficient carbohydrate substrate for the *lactobacilli* strains, in contrast with the spruce-derived COS, indicating that the prebiotic potential of COS is strongly linked with the lignocellulose material that is used as a substrate for the hydrolysis. A possible explanation can be again attributed to the different substrate composition. Particularly, the higher lignin content of spruce compared to that of birch indicates the presence of higher content of phenolic compounds that occur from the partial lignin breakdown during pretreatment and can also be formed during carbohydrate degradation. Phenolic compounds have been already associated with inhibitory effects in the fermentation of lignocellulosic hydrolysates, and particularly the low molecular weight ones are considered as the most toxic (Palmqvist and Hahn-

Hägerdal, 2000). Therefore, for the improvement of the fermentation process of the lignocellulosic hydrolysates care should be taken regarding the optimization of the substrate pretreatment and hydrolysis conditions as well as the detailed analysis of the hydrolysates, in order to perform specific detoxification processes. Particularly, it has been reported that the most efficient detoxification method is the treatment with activated charcoal as well as the enzymatic treatment with laccase, a phenol-oxidizing enzyme, however the choice (or the combination) of detoxification methods depends on the hydrolysate composition as well as on the fermenting microorganism (Guo et al., 2013). In this sense, future studies should pay bigger attention on the effect and mechanism of inhibition by phenolic compounds on cell growth.

During the fermentation of the cello-oligosaccharides (COS) the bacteria produce certain metabolites as depicted at **Table 3.23, Table 3.24**. However, these *lactobacilli* strains did not seem to favor the production of short chain fatty acids (SCFA) and the acetic acid that is present originates from the culture media and the buffer that was used in the biomass hydrolysis. As expected, lactic acid was the main metabolite, since *L.gasseri* and *L.plantarum* are known to be homofermentative and facultatively heterofermentative species, respectively, which are known to produce almost exclusively lactic acid as explained in Section 1.6.1.1.

4.4 Conclusions and future trends

Undoubtedly, nowadays there is a turn to a more organic and sustainable lifestyle in general but also regarding the support of functional food products. Increased consumer awareness for the improvement of digestive health and for the benefits that are offered by prebiotics raise the demands for a higher but at the same time cost-competitive production of these health related food ingredients. Prebiotics have been already extensively studied and explored commercially and a great variety of oligosaccharides with prebiotic properties is already in use. However, the demand for novel and sustainable ingredients with prebiotic potential leads to the cellulose-derived oligosaccharides, cello-oligosaccharides (COS) which can be produced by an enzyme based hydrolysis of insoluble cellulose. The importance of COS is given by the fact that cellulose is the most important and common polymer that is found on Earth, with wood and annual plants as the main natural and renewable resources. Nevertheless, since the high production demands of COS would be limited using only land resources, there is a need for the use of novel non-conventional cellulosic feedstocks. Although cellulose can also be produced from the cell walls of certain algae

and bacteria, tunicates, which live in the oceans, are the only known animal source for cellulose and might contain up to 60 % cellulose (Zhao and Li, 2014). In addition, the tunicate cellulose is observed that is chemically identical with plant cellulose. Particularly, the species *Ciona Intestinalis* can be farmed in high densities, therefore allowing the large scale cellulose production which make it an excellent non-conventional marine source candidate for the COS production. In this sense, the prebiotic potential of marine algae is increasingly being studied with main focus on the search and development of methods to improve the extraction of the functional poly- and oligosaccharides. In this regard, marine prebiotics, either from algae or from the tunicate species remain to be more extensively studied in order to establish a new non-conventional resource, other than the lignocellulosic biomass, for the production of novel biobased products suitable for human consumption.

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