NATIONAL TECHNICAL UNIVERSITY OF ATHENS

School of Chemical Engineering

Department IV Department of Synthesis and Development of Industrial Processes

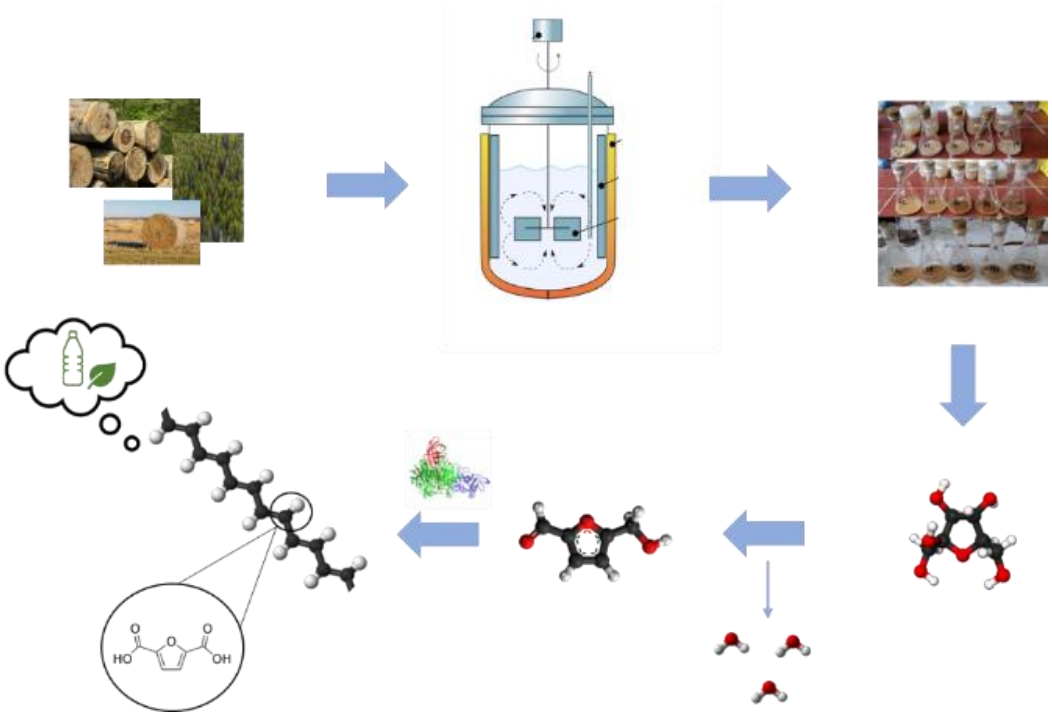
Biotechnology Laboratory

DOCTORAL THESIS

Valorization of lignocellulosic biomass for the production of fructose and furan monomer derivatives

Grigorios Dedes

Chemical Engineer NTUA



Supervisor: Evangelos Topakas

Athens 2023

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Η έγκριση της διδακτορικής διατριβής από την Ανώτατη Σχολή Χημικών Μηχανικών του Εθνικού Μετσόβιου Πολυτεχνείου δεν υποδηλώνει αποδοχή των γνωμών του συγγραφέα (Ν. 5343/1932, Άρθρο 202).

Η υλοποίηση της διδακτορικής διατριβής συγχρηματοδοτήθηκε από την Ελλάδα και την Ευρωπαϊκή Ένωση (Ευρωπαϊκό Κοινωνικό Ταμείο) μέσω του Επιχειρησιακού Προγράμματος «Ανάπτυξη Ανθρώπινου Δυναμικού, Εκπαίδευση και Δια Βίου Μάθηση», 2014-2020, στο πλαίσιο της Πράξης «Ενίσχυση του ανθρώπινου δυναμικού μέσω της υλοποίησης διδακτορικής έρευνας- Υποδράση 2: Πρόγραμμα χορήγησης υποτροφιών ΙΚΥ σε υποψηφίους διδάκτορες των ΑΕΙ της Ελλάδας»

Στην οικογένειά μου,

Βασίλη, Βασιλική, Μιχάλη, Δημήτρη και Τσίκο

“We are what we overcome”

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LIST OF PUBLICATIONS

1. Dedes, G., Karnaouri, A., Marianou, A. A., Lappas, A. A., Topakas, E. (2023). Evaluation of OxiOrganosolv pretreated hardwood and softwood lignocellulose as substrates for the chemoenzymatic production of 5-hydroxymethylfurfural (HMF). *Under preparation*

2. **Dedes, G.**, Karnaouri, A., Marianou, A. A., Kalogiannis, K. G., Michailof, C. M., Lappas, A. A., & Topakas, E. (2021). Conversion of organosolv pretreated hardwood biomass into 5-hydroxymethylfurfural (HMF) by combining enzymatic hydrolysis and isomerization with homogeneous catalysis. Biotechnology for Biofuels, 14(1), 1–11. https://doi.org/10.1186/s13068-021-02022-9

3. **Dedes, G.**, Karnaouri, A., & Topakas, E. (2020). Novel routes in transformation of lignocellulosic biomass to furan platform chemicals: From pretreatment to enzyme catalysis. Catalysts, 10(7), 1–25. https://doi.org/10.3390/catal10070743

4. Katsimpouras, C., **Dedes, G.**, Bistis, P., Kekos, D., Kalogiannis, K. G., & Topakas, E. (2018). Acetone/water oxidation of corn stover for the production of bioethanol and prebiotic oligosaccharides. Bioresource Technology, 270(July), 208–215. https://doi.org/10.1016/j.biortech.2018.09.018

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*Publications 1, 2, 3 and 4 resulted from the work presented in this thesis

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2. **Dedes G.**, Karnaouri A., Marianou A. A., Champesi P., Kalogiannis K. G., Michailof C. M., Topakas E. Production of 5-hydroxymethylfurfural (HMF) from organosolv pretreated beechwood by combining enzymatic hydrolysis and isomerization with homogeneous catalysts. Poster presentation, Society for Industrial Microbiology and Biotechnology, 71st Annual Meeting and Exhibition, 8-11 August, 2021, Austin, Texas, USA.

3. **Dedes G.,** Karnaouri A., Marianou A. A., Champesi P., Kalogiannis K. G., Michailof C. M., Topakas E. Integration of biocatalysis and chemical processes for the valorization of lignocellulosic biomass towards the production of 5-hydrxymethylfurfural. Poster presentation, 9th Conference of Mikrobiokosmos, 16-18 December 2021, Athens, Greece.

4. **Dedes G.**, Karnaouri A., Triantafillidis K. S., Topakas E. Development of novel chemocatalytic processes for the production of furans from lignocellulosic biomass. Poster presentation, 7th International Conference on Sustainable Solid Waste Managements, 26-29 June, Herakleion, Crete, Greece.

5. Katsimpouras C., **Dedes G.,** Bistis P., Kalogiannis K. G., Topakas E., Production of cellulosic ethanol from acetone/water oxidized corn stover at high solids content. Flash presentation, Cost Action FP1306, 4th Workshop, 12-14 March 2018, Thessaloniki, Greece.

ΕΥΧΑΡΙΣΤΙΕΣ

Η παρούσα διατριβή εκπονήθηκε στο Εργαστήριο Βιοτεχνολογίας της Σχολής Χημικών Μηχανικών του Εθνικού Μετσόβιου Πολυτεχνείου (Ε.Μ.Π.). Χρηματοδοτήθηκε εν μέρει από το Πρόγραμμα Ενίσχυσης Βασικής Έρευνας ΠΕΒΕ 2020 του Εθνικού Μετσόβιου Πολυτεχνείου καθώς και από το Επιχειρησιακό Πρόγραμμα «Ανάπτυξη Ανθρώπινου Δυναμικού, Εκπαίδευση και Δια Βίου Μάθηση», 2014-2020, στο πλαίσιο της Πράξης «Ενίσχυση του ανθρώπινου δυναμικού μέσω της υλοποίησης διδακτορικής έρευνας- Υποδράση 2: Πρόγραμμα χορήγησης υποτροφιών ΙΚΥ σε υποψηφίους διδάκτορες των ΑΕΙ της Ελλάδας» του Ιδρύματος Κρατικών Υποτροφιών (ΙΚΥ).

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Γρηγόρης Δέδες,

Αθήνα, Οκτώβριος 2023

LIST OF ABBREVIATIONS

AAO	Aryl Alcohol Oxidase
ACO	Acetone
AFEX	Ammonia Fiber Expansion
APS	Ammonium Persulfate
AWO	Acetone Water Oxidation
BGL	β-1,4-glucosidase
BHMF	2,5-nis(hydroxymethyl)furan
BVMOs	Bayer-Villinger Monooxygenases
CBB	Coomassie Brilliant Blue
CBH	Cellobiohydrolase
CBM	Carbohydrate Binding Module
CROs	Copper Radical Oxidases
DA	Diers-alder
DFF	Diformylfuran
DHMF	5,5’-dihydroxymethyl furoin
DM	Dry Matter
DMSO	Dimethyl Sulfoxide
DNS	3,5-dinitrosalicylic acid
EG	Endo-1,4-glucanase
EtOH	Ethanol
FA	Furfural
FAR	Furfural Reductase

FCA	Furancarboxylic Acid
FDCA	2,5-furandicarboxylic acid
FoGalOx	Fusarium Oxysporum Galactose oxidase
FOL	Furfuryl Alcohol
GAO	Galactose oxidase
GlO	Glyoxal Oxidase
GMC	Glucose-Methanol-Choline
HMFCA	2,5-hydroxymethylfuran carboxylic acid
HMFO	5-hydroxymethylfurfural oxidase
HPLC	High Performance Liquid Chromatography
HRP	Horseradish Peroxidase
iBuOH	Isobutanol
ICP-OES	Inductively coupled plasma atomic emission spectroscopy
IL	Ionic Liquid
IMAC	Immobilized Metal Affinity Chromatography
LPMO	Lytic Polysaccharide Monooxygenase
MW	Molecular Weight
OD	Optical Density
PAMO	Phenylacetone Monooxygenase
Pao	Periplasmic aldehyde oxidase
PCR	Polymerase Chain Reaction
PEF	Poluethylene furanoate
PET	Polyethylene terephthalate
PHAs	Polyhydroxyalkanoates

PLA	Polylactic acid
POM	Polyoxometalate
PUFAs	Polyunsaturated Fatty acids
SDS-PAGE	Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis
SSF	Simultaneous Saccharification and Fermentation
TEMED	Tetramethylethylenediamine
TEMPO	2,2,6,6-tetramethylpiperidine-1-oxyl
THF	Tetrahydrofuran
UPO	Unspecified Peroxygenase

ΠΕΡΙΛΗΨΗ

Τα τελευταία χρόνια, το ενδιαφέρον για βιοδιϋλιστήρια λιγνινοκυτταρινούχου βιομάζας έχει μεταφερθεί από την παραγωγή βιοκαυσίμων στην ανάπτυξη διεργασιών για τη σύνθεση χημικών υψηλής προστιθέμενης αξίας. Η παρούσα διατριβή αποσκοπεί στην ανάπτυξη διεργασιών για την αξιοποίηση υποστρωμάτων λιγνινοκυτταρινούχου βιομάζας, όπως οξιάς, πεύκου και στελεχών αραβοσίτου, με σκοπό την παραγωγή φρουκτόζης και 5-υδροξυμέθυλφουρφουράλης (HMF). Επιπλέον, εξετάζει την ανακάλυψη και παραγωγή μιας οξειδάσης της γαλακτόζης από το νηματοειδή μύκητα *Fusarium oxysporum* καθώς και την ικανότητά του να καταλύει αντιδράσεις οξείδωσης του HMF για την παραγωγή μονομερών του φουρανίου.

Στο πρώτο στάδιο της διατριβής μελετήθηκε ως κύρια πηγή λιγνινοκυτταρινούχου βιομάζας το ξύλο οξιάς για την παραγωγή HMF. Αρχικά, τα δείγματα αυτά υπέστησαν μια ήπια προκατεργασία οξείδωσης (OxiOrganosolv) με χρήση υδατικών διαλυμάτων αιθανόλης (EtOH), ακετόνης (ACO), και τετραϋδροφουρανίου (THF) ως διαλύτη. Ακολούθησαν πειράματα ενζυμικής υδρόλυσης και ισομερείωσης στις πλούσιες σε κυτταρίνη πούλπες που προέκυψαν από την προκατεργασία με στόχο την αξιολόγηση αυτών ως πρώτη ύλη για την παραγωγή φρουκτόζης. Τα βέλτιστα αποτελέσματα παρατηρήθηκαν για το δείγμα που προκατεργάστηκε στους 175 ^{o}C για 120 min, οδηγώντας στην παραγωγή 55.2 g φρουκτόζης/ 100 g προκατεργασμένης βιομάζας. Έτσι, το δείγμα αυτό χρησιμοποιήθηκε για ένα νέο πείραμα υδρόλυσης και ισομερείωσης μεγαλύτερης κλίμακας, το αποτέλεσμα του οποίου ήταν η παραλαβή ενός υδρολύματος με συγκέντρωση φρουκτόζης 104.5 g/L και γλυκόζης 25.0 g/L. Το υδρόλυμα αυτό χρησιμοποιήθηκε στη συνέχεια για να μελετηθεί η χημική αφυδάτωση των σακχάρων σε HMF με όξινους διαλύτες Brønsted. Από όλους τους διαλύτες που δοκιμάστηκαν, ο καλύτερος βρέθηκε να είναι το φορμικό οξύ (3.5% v/v) οδηγώντας στην παραγωγή HMF με εκλεκτικότητα 55.8%.

Στο επόμενο στάδιο της διατριβής, δείγματα οξιάς και πεύκου προκατεργάστηκαν μέσω της διεργασίας OxiOrganosolv με την προσθήκη πολυοξομεταλλικών καταλυτών στο διαλύτη της προκατεργασίας, με στόχο να μελετηθεί τόσο η επίδραση του τύπου της λιγνινοκυτταρινούχου βιομάζας ως αρχική τροφοδοσία, όσο και η προσθήκη του καταλύτη σε διάφορα συστήματα οργανικών διαλυτών. Συγκεκριμένα, οι οργανικοί διαλύτες που χρησιμοποιήθηκαν ήταν οι EtOH, ACO, THF καθώς και η ισοβουτανόλη (iBuOH), ενώ οι πολυοξομεταλλικοί καταλύτες ήταν το εμπορικά διαθέσιμο φωσφομολυβδικό οξύ $H_3PMo_{12}O_{40}$ x H2O (HPMo), καθώς και οι τροποποιημένοι με προσθήκη μετάλλων καταλύτες FePMo and CuPMo. Το καλύτερο δείγμα οξιάς οδήγησε στην παραγωγή 54.9 g φρουκτόζης/ 100 g προκατεργασμένης βιομάζας, ενώ το καλύτερο δείγμα πεύκου σε 53.4 g φρουκτόζης/ 100 g προκατεργασμένης βιομάζας, αντίστοιχα, και τα δύο για προκατεργασία με iBuOH στους 175 ^{o}C για 120 min απουσία καταλύτη. Στη συνέχεια, τα καλύτερα δείγματα υπέστησαν χημειοκαταλυτική αφυδάτωση με φορμικό οξύ (3.5% v/v) οδηγώντας στην παραγωγή HMF με εκλεκτικότητα 49.9%.

Στο τρίτο στάδιο της διδακτορικής διατριβής, στελέχη αραβοσίτου προκατεργάστηκαν με χρήση μικροκυμάτων σαν πηγή θέρμανσης με σκοπό να συγκριθεί αυτός ο μηχανισμός θέρμανσης με τον παραδοσιακό μηχανισμό θέρμανσης με αντιστάσεις. Τα πειράματα αυτά έλαβαν χώρα στους 150 ^{o}C με ένα μείγμα νερού και ACO, προσθέτοντας οξικό οξύ ως καταλύτη. Δέκα διαφορετικές δοκιμές έλαβαν χώρα με σκοπό να εξεταστεί η επίδραση της συγκέντρωσης οξικού οξέος και χρόνου προκατεργασίας στα δείγματα, ενώ η αξιολόγηση της επίδρασης της προκατεργασίας ως προς την ικανότητα σακχαροποίησης του στερεού κλάσματος που προέκυψε, πραγματοποιήθηκε με πειράματα ενζυμικής υδρόλυσης. Τα αποτελέσματα έδειξαν πως το καλύτερο υλικό ήταν αυτό που προκατεργάστηκε με συγκέντρωση οξικού οξέος 0.5% v/v για 120 min. Προς χάριν σύγκρισης, ακολούθησε προκατεργασία του ίδιου υλικού σε αυτόκαυστο στις ίδιες συνθήκες. Τα δείγματα που προήλθαν από προκατεργασία με μικροκύματα και σε αυτόκαυστο, εν τέλει υπέστησαν ενζυμική υδρόλυση και ισομερείωση παράγοντας 28.4 και 28.7 g φρουκτόζης/ 100 g προκατεργασμένης βιομάζας, αντίστοιχα. Ωστόσο, οι χρόνοι προκατεργασίας ανάμεσα στις δύο μεθόδους έδειξαν διαφορά 1 h σε διάρκεια, αναδεικνύοντας τη δυνατότητα των μικροκυμάτων να πετύχουν ίδια αποτελέσματα με την παραδοσιακή προκατεργασία σε λιγότερο χρόνο.

Τέλος, με στόχο την ανακάλυψη νέων βιοκαταλυτών για τη βιομετατροπή των ενώσεων φουρανίων προς μονομερείς ενώσεις υψηλής προστιθέμενης αξίας, μελετήθηκε μια πρωτεΐνη με ενεργότητα οξειδάσης της γαλακτόζης από το μύκητα *F. Oxysporum.* Το εν λόγω ένζυμο επιλέχθηκε αναζητώντας οξειδωτικά ένζυμα με ενεργότητα σε φουράνια που ανήκουν στην οικογένεια AA5_2 της βάσης δεδομένων της CAZy. Το γονίδιο που κωδικοποιεί την πρωτεΐνη απομονώθηκε από το DNA του μικροοργανισμού με χρήση μοριακών τεχνικών και παράχθηκε μέσω ετερόλογης έκφρασης σε κύτταρα *Pichia pastoris* X33. Κατόπιν προσδιορισμού των ιδανικών συνθηκών λειτουργίας του, αποδείχθηκε πως το ένζυμο ήταν ικανό να πραγματοποιήσει αποτελεσματικά την οξείδωση του HMF καθώς και των οξειδωμένων παραγώγων αυτού, αποδεικνύοντας πως μια ολοκληρωμένη χημειο-ενζυμική διεργασία για την παραγωγή οξειδωμένων μονομερών φουρανίων από τη λιγνινοκυτταρινούχο βιομάζα είναι εφικτή.

ABSTRACT

Over the past few years, the interest in lignocellulose biomass biorefineries has shifted from the production of biofuels to the development of processes for high-added value chemical synthesis. The present work attempts to develop processes for the valorization of lignocellulosic substrates, namely beechwood, pine and corn stover, with the aim to produce fructose and 5-hydroxymethylyfurfural (HMF). Additionally, this thesis examines the discovery and production of a galactose oxidase from the filamentous fungus *Fusarium oxysporum* as well as its ability to catalyze oxidation reactions on HMF for the production of furan monomers.

In the first stage of the thesis, beechwood biomass was studied as the main feedstock for the production of HMF. Initially, these samples were subjected to an oxidative pretreatment (OxiOrganosolv) with the use of aqueous solutions of ethanol (EtOH), acetone (ACO) and tetrahydrofuran (THF) as the solvent. Subsequently, enzymatic saccharification and fermentation experiments were carried out on the cellulose-rich pulps that derived from the pretreatment with the aim of evaluating them as the raw material for fructose production. The best results were observed for the sample pretreated at 175 ^{o}C for 120 min, leading to the production of 55.2 g fructose/ 100 g pretreated biomass. As a result, this sample was used for a scale-up saccharification and isomerization experiment that led to the production of a hydrolysate with a concentration of 104.5 g/L fructose and 25.0 g/L glucose. This hydrolysate was then used to study the chemical dehydration of sugars to HMF using Brønsted acid solvents. Out of the all the solvents studied, formic acid (3.5% w/w) proved to be the best performing one leading to the production of HMF with a 55.8% selectivity.

In the next stage of the thesis, beechwood and pine biomass were subjected to OxiOrganosolv pretreatment with the addition of polyoxometalate catalysts in the solvent, with the aim to examine both the effect of the nature of the biomass used as a feedstock, and the addition of the catalysts in a variety of organic solvents. In specific, the organic solvents in these experiments were EtOH, ACO, THF, as well as isobutanol (iBuOH), while the polyoxometalate catalysts were the commercially available phosphomolybdic acid $H_3PMo_{12}O_{40}$ x H2O (HPMo), as well as the custom- made metal substituted catalysts FePMo and CuPMo. The best performing beechwood sample led to the production of 54.9 g fructose/ 100 g pretreated biomass, while the best performing pine sample 53.4 g fructose/ 100 g pretreated biomass, respectively, both for pretreatment with iBuOH at 175 ^{o}C for 120 min in the absence of catalyst. Subsequently, the best performing samples were subjected to chemical dehydration experiments with formic acid (3.5% v/v) leading to the production of HMF with 49.9% selectivity.

The third stage of the thesis included the pretreatment of corn stover biomass using microwave heating in order to compare this heating mechanism with the resistance mediated heating of the

traditional autoclave pretreatment. These experiments took place at 150 ^{o}C with a mixture of water: ACO as the solvent with the addition of acetic acid as the catalyst. Ten different runs were carried out in order to discover the effect of acetic acid concentration and pretreatment duration on the biomass, while the evaluation of the samples was performed by the results of enzymatic saccharification experiments. The results showed that the best performing material was the one pretreated with an acetic acid concentration of 0.5% v/v for 120 min. For the sake of comparison, the same material was subjected to autoclave pretreatment under the same conditions. The samples of the microwave and autoclave pretreatment were also subjected to saccharification and isomerization experiments yielding 28.4 and 28.7 g fructose/ 100 g pretreated biomass, respectively. However, the total pretreatment times of the two processes exhibited a discrepancy of 1 h highlighting the potential of microwaves in achieving the same results in shorter amounts of time.

Finally, with the aim of discovering novel biocatalysts for the bioconversion of furans into high added value monomers, the last stage of the thesis included the examination of a protein with galactose oxidase activity from *F. oxysporum*. This enzyme was selected after searching for oxidative enzymes with activity on furans that belong to the AA5_2 family of the CAZy database. The enzyme encoding gene was isolated from the DNA of the microorganism using molecular techniques and produced by means of heterologous expression in *Pichia pastoris* X33 cells. After the determination of its optimal conditions, the enzyme was able to efficiently oxidize HMF as well as its oxidative derivatives, proving that an integrated chemoenzymatic process for the production of oxidative furan monomers from lignocellulose is possible.

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INTRODUCTION

1. Introduction

1.1 **Lignocellulosic biomass**

Lignocellulosic biomass refers to the plant biomass derived mainly from agricultural and forest residues and is valorized in many sectors towards the production of valuable products, such as animal feed, biofuels, chemical synthesis and materials. To date, $200x10^9$ tons of lignocellulosic biomass are produced annually, while the US Department of Energy estimates that 1,3 billion tons of lignocellulosic biomass are produced annually in the United States, of which, 933 million derive from agriculture and 368 million from forests (De Bhowmick et al., 2018). Lignocellulosic biomass, up until recently, had been utilized to produce second generation biofuels (mainly bioethanol and biodiesel). However, its low energy cost compared to other sources of raw materials together with its high productivity, has led to the expansion of its valorization to fields other than the energy department, such as the production of high added value materials (De Bhowmick et al., 2018). In any case, the key to lignocellulose utilization lies in the valorization of the monomeric sugars present in its composition. In fact, just as lignocellulose constituted an alternative in the replacement of first-generation biofuels with second generation ones (food versus fuel conflict), now it also provides a candidate to produce high added value chemicals from residues that would otherwise be discarded (Sousa et al., 2015).

Lignocellulose has traditionally been linked to the term ‘biofuel’. Biofuel is the fuel produced mainly from biobased materials such as bacteria, algae and terrestrial plants. As a result, biofuels can be categorized into two groups depending on their origin. Primary biofuels are produced by direct burning of woods, animal wastes, forest and crop residues (Rodionova et al., 2017). Depending on their source of biomass, benefits, limitations and production process, secondary biofuels can be further categorized into first, second, third and fourth generation biofuels (Ale et al., 2019). First generation biofuels also known as conventional biofuels are produced from crops containing starch, sugar, and vegetable oil. The sources of first-generation biofuels are among others corn, wheat, sugarcane for the production of bioethanol, as well as soybean and rapeseed for biodiesel production (Ale et al., 2019). The crops for the first-generation biofuel are also sources for human and animal feed, causing the competition between food and fuel production and eventually resulting in the increase in the food prices. Second-generation biofuels are mainly produced from the lignocellulosic biomass of non-food crops and agricultural residues. Wood, organic waste, corn stover, rice or wheat straw and some fast-growing trees as poplar serve as a feedstock for second generation biofuels. While this generation of biofuels did not originate directly from food sources, the demand for arable lands with food crops and the requirement for forest clearance, caused another food versus fuel debate. Third-generation biofuels are generally produced from algae and seaweed and they typically refer to biodiesel (Johnson et al., 2016). The main advantages of this source of biomass are their rapid growth rate, high photosynthetic efficiency and the low cultivation cost as microalgae can be cultivated in sewage and wastewater

(Wang et al., 2016). However, their growth and lipid content strongly depend on the cultivation conditions and factors such as temperature, light intensity, CO_2 concentration, pH value, and the nutrient composition of the culture medium. Finally, fourth-generation biofuels are produced from genetically engineered microorganisms such as microalgae and cyanobacteria. The microorganisms are genetically modified to increase their CO_2 intake during photosynthesis creating an artificial "carbon sink" and enhancing biofuel production (Halfmann et al., 2014; Johnson et al., 2016). CO_2 assimilation, wastewater treatment, and reduction of greenhouse gases emission can be considered as the environmental advantages of fourth-generation biofuels; however, the impact of gene modification process on the environment still requires more research. In fact, even though first- and second-generation biofuels have been the subject of extensive research throughout the year, third- and fourth-generation biofuels are still actively developing. While all these sources have traditionally been linked to the term biofuels as far as their exploitation is concerned, the saturation of research on biofuels has led to the emergence of different biorefineries with the aim to produce other high added value compounds such as chemicals, nutraceuticals and biobased polymers (Dedes et al., 2021; Karnaouri et al., 2021).

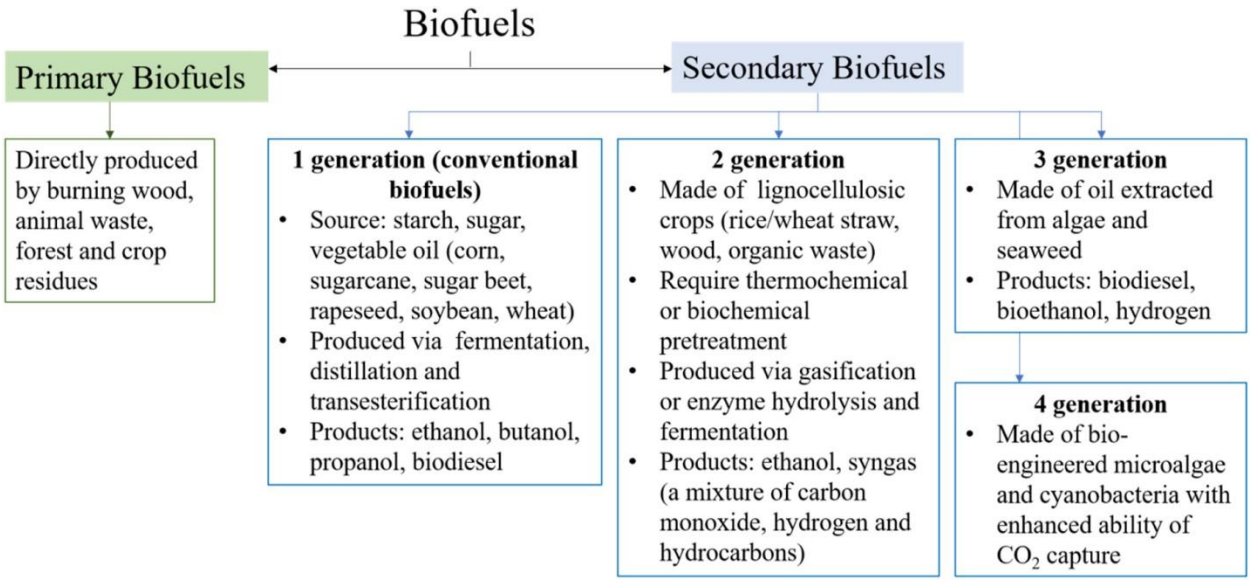


Figure 1.1: The categories and generations of biofuels (Rodionova et al., 2017)

Lignocellulose has been traditionally used to produce second-generation biofuels due to the rising concern over depleting fossil fuels and greenhouse gas emissions. As a result, there was a shift in interest towards alternative and non-conventional fuel, mainly bioethanol, originating from bio-renewable sources including sugars, starches and lignocellulosic materials (Demain et al., 2005; Hill et al., 2006). In the US, bioethanol is primarily produced from corn starch feedstocks while in

Brazil mainly produced from sugarcane juice and molasses. Together, these countries account for 89% of the current global bioethanol production (Renewable Fuels Association, 2010). In addition, while European countries account for a much smaller amount of the worldwide bioethanol production, biodiesel produced in Europe primarily in France and Germany remains by far more substantial and accounts for approximately 56% of the global production mainly because of the rising importance of diesel engines and feedstock opportunity costs according to the Official Journal of the European Union (2009). However, although corn-based and sugar-based ethanol are promising substitutes to gasoline production mainly in the transportation sector, they are not sufficient to replace a considerable portion of fossil fuel presently consumed worldwide each year (Bell & Attfield, 2009). Furthermore, ethical concerns about the use of food as fuel raw materials have encouraged efforts to be more focused on the potential of inedible feedstock alternatives (food versus fuel conflict) (Sun & Cheng, 2002). Lignocellulosic biomass materials constitute a substantial renewable substrate for bioethanol production that does not compete with food production and animal feed, while simultaneously contributing to environmental sustainability. Additionally, lignocellulosic biomass can be supplied on a large-scale basis from different low-cost raw materials such as municipal and industrial wastes, wood and agricultural residues (Cardona & Sánchez, 2007). Forestland materials typically include woody biomass, namely, hardwoods and softwoods, followed by sawdust, pruning and bark thinning residues. Softwoods typically include evergreen tree species such as pine, cedar, spruce, cypress, fir, hemlock and redwood, while hardwoods include trees such as poplar, willow, oak, cottonwood and aspen (Limayem & Ricke, 2012). Agricultural residues are mostly comprised of agricultural wastes such as corn stover, corn stalks, rice and wheat straws as well as sugarcane bagasse. The use of municipal and industrial wastes as raw materials for biorefineries is associated with the limitation of environmental problems such as the disposal of garbage household, processing papers, food processing, by-products, black liquors and pulps (Limayem & Ricke, 2012). The productivity and constitution of lignocellulose feedstocks can be summarized in the following tables.

Table 1.1: Productivity of lignocellulosic feedstocks according to the US Department of Energy

Biomass	**Million dry tons/year**
Agricultural residues	428
Forest residues	370
Energy corps	377
Grains and corn	87
Municipal and industrial wastes	58

Table 1.2: Constitution of different feedstocks of lignocellulosic biomass

Feedstock	Cellulose (%)	Hemicellulose (%)	Lignin (%)	References
Forest Residues				
Beechwood	48.5	30.8	27.3	(Kalogiannis et al., 2015)
Pine	46.7	20.1	29.0	(Zhu et al., 2010)
Oak	43.8	14.8	29.1	(Kumar & Wyman, 2009a)
Agricultural residues				
Corn Stover	31.7	20.0	12.6	(Liu et al., 2014)
Rice Stalks	41.5	18.0	17.7	(Sun & Tao, 2013)
Cotton Stalks	30.0	13.0	31.0	(Binod et al., 2012)
Wheat	35.2	22.2	22.1	(Toquero & Bolado, 2014)
Energy Crops				
Switchgrass	30.3	33.2	16.4	(Istrate et al., 2021)
Sugarcane bagasse	39.8	34.5	4.0	(Matsakas & Christakopoulos, 2013)
Municipal residues				
Food waste	18.3	7.6	2.2	(Matsakas et al., 2014)
Newspapers	49.3	12.2	19.2	(Guerfali et al., 2015)

The main characteristic of lignocellulosic biomass is its rigid and recalcitrant structure, which has led to the development of specific methods to make it amenable to processes for the valorization of its components. One such typical process involves, firstly, a *pretreatment* step for the effective fractionation of the lignocellulose components, mainly cellulose, hemicellulose and lignin. Pretreatment is then followed by *enzymatic hydrolysis* where cellulose and hemicellulose are decomposed to their monomeric sugars (Kumar et al., 2009). These sugars can be used as a carbon source in *fermentation* processes for biofuel production, such as ethanol and methane (Alvira et al., 2013; Hu et al., 2018) or for a wide range of high added value materials, such as lactic acid, succinic acid and omega-3 fatty acids (Abdel-Rahman et al., 2011; Karnaouri et al., 2020a). In addition, lignocellulosic monosaccharides can also function as raw materials for chemical or enzymatic synthesis processes for advanced chemicals with a plethora of uses in polymers,

pharmaceuticals and nutraceuticals (Putro et al., 2016). Amongst these fields, the currently most interesting one is the synthesis of platform chemicals for the production of plastics and polymers. This field has recently gained increased attention due to it being a green alternative to the traditional petrochemical synthesis of polymers, effectively transforming substances that would otherwise be discarded, to high added value products (Huang et al., 2010).

1.2 Valorization of lignocellulosic feedstocks

Lignocellulosic biomass, as mentioned above, is the most abundant plant material on the planet whose sources encompass, among others, herbaceous plant species, and woody crops as well as agricultural and forest residues (Cherubini, 2010). However, the deconstruction of the plant cell wall poses a bottleneck on the industrialization of lignocellulose valorization, due to its recalcitrant nature. The term 'biomass recalcitrance' refers to the rigid structure of lignocellulosic biomass rendering it immune to enzymatic digestibility. Overcoming this recalcitrance barrier and rendering lignocellulose amenable to subsequent chemical and enzymatic procedures, calls for a process known as pretreatment. Pretreatment is the initial step of the biomass conversion process to monosaccharides, aiming to alter the rigid structure and chemical composition of biomass in order to facilitate the access of enzymes on cellulose and hemicellulose, which are the main sources of utilizable sugars (De Bhowmick et al., 2018). The second underlying role of pretreatment is the effective fractionation of the three biomass constituents, namely lignin, cellulose and hemicellulose leading to pure streams of each fraction, thus facilitating each stream's separate valorization (Matsakas et al., 2018). However, different types of pretreatments lead to different solid and liquid streams. As a result, the type of pretreatment can vary depending on the need of the application. The appropriate pretreatment process can be designed based on the interest of each fraction of lignocellulose. For example, the need for high glucose content demands a solid pulp rich in cellulose but, on the other hand, a process designed on studying lignin can benefit from a liquid fraction with diluted lignin that can be easily extracted and isolated in its pure form. Understanding, how the chemical composition and physical structure of biomass contribute to its recalcitrance, as well as their effect on enzymatic hydrolysis of lignocellulose, would greatly help to improve the current pretreatment technologies, and probably promote the development of novel pretreatment processes (Axelsson et al., 2012).

Lignocellulose biorefineries are integrated systems that are set up with regard to possible products deriving from biomass. Traditionally, in the past lignocellulose biorefineries had an energy-driven objective and as a result, were set up to produce biofuels. However, the wide variety of compounds present in lignocellulose along with the advancement of science on a multitude of different fields, gave rise to another type of biorefineries: the material driven biorefineries. While the target of energy driven biorefineries was the generation of expendable biofuels as replacements to fossil fuels, the target of material driven biorefineries was the development of processes that produce

high added value chemicals or biomaterials from a fraction of biomass that would otherwise be discarded. The wide variety of material driven biorefineries include compounds such as fragrancies, high-value nutraceuticals, aromatic compounds, succinic acid, polyethylene, polylactic acid, methylcellulose, nanocellulose. In addition, another field gaining momentum as far as lignocellulose valorization is concerned in the generation of furans, either as a by-product of biomass pretreatment or as a target product of cellulose and hemicellulose treatment for the production of monomers with the aim to produce bio-based monomers. This is the objective of the present study.

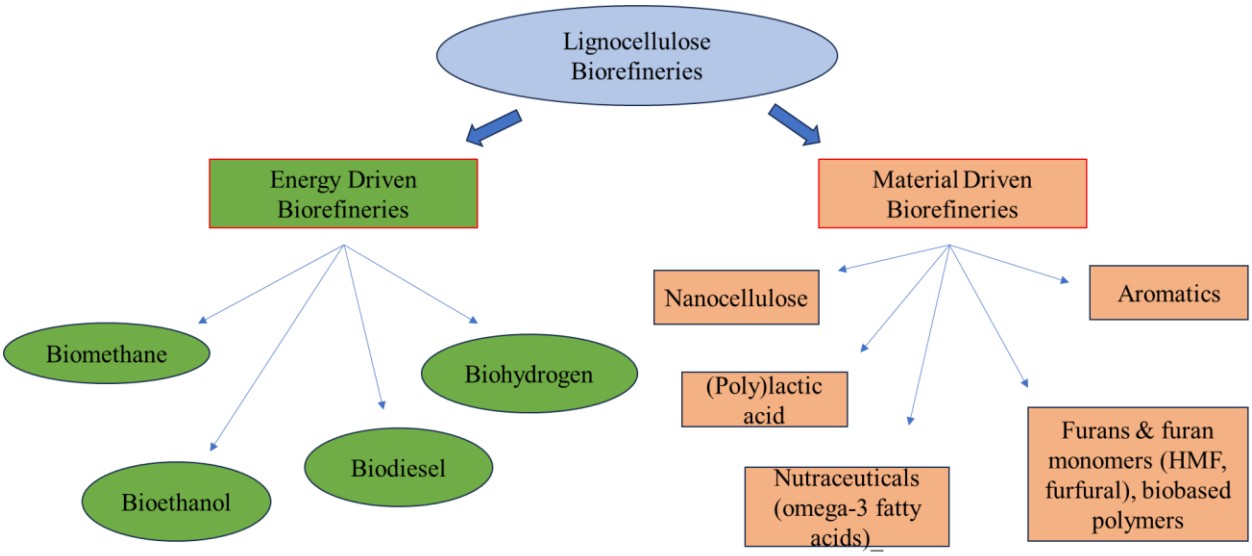


Figure 1.2: The wide variety of lignocellulose biorefineries

1.3 Plant cell wall composition

Lignocellulose is mainly comprised of three compounds; cellulose, hemicellulose (polymeric carbohydrates) and lignin (an aromatic polymer) that are intertwined together in a complex structure. In addition, lignocellulose also contains pectin, proteins, extractives and inorganic compounds in smaller amounts (Brandt et al., 2013; Taherzadeh & Karimi, 2008). The exact proportions of each fraction can vary depending on the type of the plant cell wall, as different types of feedstocks can carry different amounts of each compound. Pectin, proteins, extractives and inorganic compounds are also present in lignocellulose in smaller quantities. The composition of each feedstock on these ingredients is different: softwood, which represents the most abundant type of wood in forest residues consists approximately of 33-42% cellulose, 22-40% hemicellulose and 27-32% lignin (Nhuchhen et al., 2014). Hardwood, respectively, contains 38-51% cellulose, 17-38% hemicellulose and 21-31% lignin (Menon & Rao, 2012). Finally grasses contain 25-95%

cellulose, 20-50% hemicellulose and 0-40% lignin (Arsène et al., 2013; Smit & Huijgen, 2017). The complex matrix formed by these compounds functions as a shield against microorganisms and enzymes targeting the structural sugars of lignocellulose, effectively preventing its degradation. The key to the valorization of lignocellulosic biomass for the production of furans, lies in the valorization of the streams for cellulose and hemicellulose and specifically the pentoses and the hexoses that constitute them. Each type of sugar has its own catalytic pathway as hexoses lead to the production of 5-hydroxymethylfurfural (HMF), while pentoses lead to the production of furfural (2-furaldehyde, FA).

1.3.1 Cellulose

Cellulose, found in lignocellulose, is the primary and most plentiful component in plant cell walls, making up 35-50% of the biomass depending on its type. It is made up of D-glucose units linked together in a linear fashion through 1-4-β glucosidic bonds. This structure allows for a stretched chain formation, with hydrogen bonds connecting the chains into flat sheets. Moreover, when multiple cellulose strands pack together, crystalline fibrils are formed (O'Sullivan, 1997). The crystalline nature of cellulose refers to its organized and repetitive arrangement of glucose monomers. This arrangement allows cellulose to form strong, rigid structures in plant cell walls, providing mechanical strength to plant tissues. The hydrogen bonds between the glucose units in cellulose contribute to its high degree of polymerization, meaning that it consists of a large number of glucose units linked together. The high degree of polymerization makes cellulose less flexible and contributes to its insolubility in water and most solvents. The long, linear chains of cellulose molecules form a rigid structure that is resistant to being broken down by enzymes. However, it is important to note that while cellulose as a whole is insoluble, glucose and its oligomers (short chains of glucose) can be soluble in water and some other solvents. This is because the shorter chains do not exhibit the same degree of intermolecular interactions and can have some flexibility, allowing them to dissolve (Nhuchhen et al., 2014). Cellulose can be broken down into glucose molecules by enzymes called cellulases. There are different types of cellulases, including endoglucanases, cellobiohydrolases, and β-glucosidases, each playing a specific role in the breakdown process (Vermaas et al., 2015; Volynets et al., 2017). One of the significant applications of cellulose is in the production of bioethanol, a renewable fuel source. Glucose derived from cellulose can be fermented by microorganisms like yeast to produce ethanol. This offers an alternative to fossil fuels and helps reduce greenhouse gas emissions. Cellulose also shows potential in other areas, such as the production of cellooligosaccharides. These short chains of glucose units have prebiotic properties and can be used as functional food ingredients to promote gut health and enhance beneficial gut bacteria growth (Karnaouri et al., 2019).

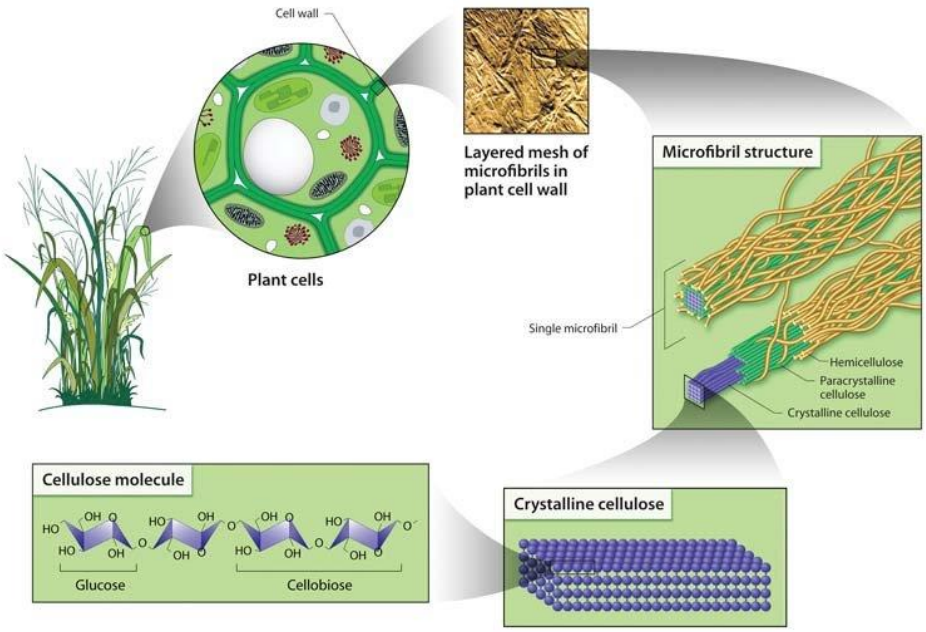


Figure 1.3: Schematic representation of cellulose structure within lignocellulose (Goyal et al., 2008)

1.3.2 Hemicellulose

Hemicellulose is the second most abundant category of polysaccharides in lignocellulosic biomass. It primarily consists of pentose sugars, such as xylose and arabinose, with a polymerization degree ranging from 100 to 200. Additionally, smaller quantities of hexose sugars like glucose, mannose, and galactose are also found (Nhuchhen et al., 2014). The linear connection of the xylose units forms the xylan backbone of hemicellulose. The arrangement of the xylan backbone can be modified with the addition of acetyl and methyl groups, as well as cinnamic, ferulic, glucuronic, and galacturonic acids that attach to the sugars of the backbone as decorations. These modifications can vary depending on the source of the lignocellulose and play a role in connecting lignin and hemicellulose (Timell, 1967). Alternatively, another type of hemicellulose exists in the form of mannan where xylose is substituted by mannose as the sugar monomer. Hemicelluloses typically make up 15-35% of plant biomass depending on its source. Of this percentage, xylans are the main hemicellulose components constituting about 20-30% of the biomass of hardwoods and herbaceous plants, whereas in some tissues of grasses and cereals xylans can account up to 50%. Xylans are usually available in large quantities as by-products of forest, agriculture, agro-industries, wood and pulp and paper industries. Mannan-type hemicelluloses like glucomannans and galactoglucomannans are the major hemicellulosic components of the secondary wall of softwoods whereas in hardwood they occur in minor quantities (Ebringerová et al., 2005).

Table 1.3: Different types of hemicellulose along with their respective source, amount and composition (Gírio et al., 2010)

Polysaccharide type	Biomass origin	Amount (% biomass)	Backbone	Side chains
Arabinogalactan	Softwoods	1-35	β-D-Gal	β-D-Gal
				α-L-Ara
				β-L-Ara
Xyloglucan	Hardwoods, XG grasses	2-25	β-D-Glc	β-D-Xyl
			β-D-Xyl	β-D-Gal
				α-L-Ara
				α-L-fuc
				Acetyl
Galacturomannan	Softwoods	10-25	β-D-Man	β-D-Gal
			β-D-Glc	Acetyl
Glucomannan	Softwoods, Hardwoods	2-5	β-D-Man	
			β-D-Glc	
Glucuronoxylan	Hardwoods	15-30	β-D-Xyl	4-O-Me-α-D-GlcA
				Acetyl
Arabinoglucuronoxylan	Grasses, cereals, softwoods	5-10	β-D-Xyl	4-O-Me-α-D-GlcA
				α-L-Ara
Arabinoxylans	Cereals	0.15-30	β-D-Xyl	α-L-AraFeruloyl
Glucuronoarabinoxylans	Grasses and cereals	15-30	β-D-Xyl	α-L-Ara
				4-O-Me-α-D-GlcA
				Acetyl
Homoxylans	Algae		β-D-Xyl	

Hemicellulose has been found to bind to cellulose, creating a complex matrix that is highly resistant to enzyme breakdown and provides structural strength to plant tissue. Unlike cellulose, hemicellulose is more easily broken down due to its non-crystalline nature. This characteristic is utilized in various deconstruction methods, which may involve pretreatment with solvents and high temperature and pressure, enzymatic treatment with hemicellulose-degrading enzymes, or a combination of both (Nhuchhen et al., 2014). The complexity of hemicellulose as an enzyme substrate requires the use of an array of multiple types of enzymes, which typically involve xylanases (EC 3.2.1.8), β-mannanases (EC 3.2.1.78), α-L-arabinofuranosidases (EC 3.2.1.55), α-D-glucuronidases, β-xylosidases (EC 3.2.1.37) and esterases, such as acetyl xylan esterases (EC 3.1.1.72) and feruloyl esterases (EC 3.1.1.73). Each type of these enzymes has a specific activity on the polymer of hemicellulose and can be used either as part of an enzymatic cocktail for the removal of hemicellulose to produce a cellulose rich fraction of biomass for cellulose valorization, or as a highly selective means to produce high added value compounds such prebiotics (Katsimpouras et al., 2018). Research has shown that removing hemicellulose increases the conversion efficiency of cellulose (Katsimpouras et al., 2018; Yang & Wyman, 2004). Finally, a recent trend with increased popularity involves the exploitation of the pentose fraction removed through pretreatment for the production of furans by means of chemically catalyzed dehydration, while simultaneously incorporating the furans that are formed through pretreatment as inhibitors (Luo et al., 2019).

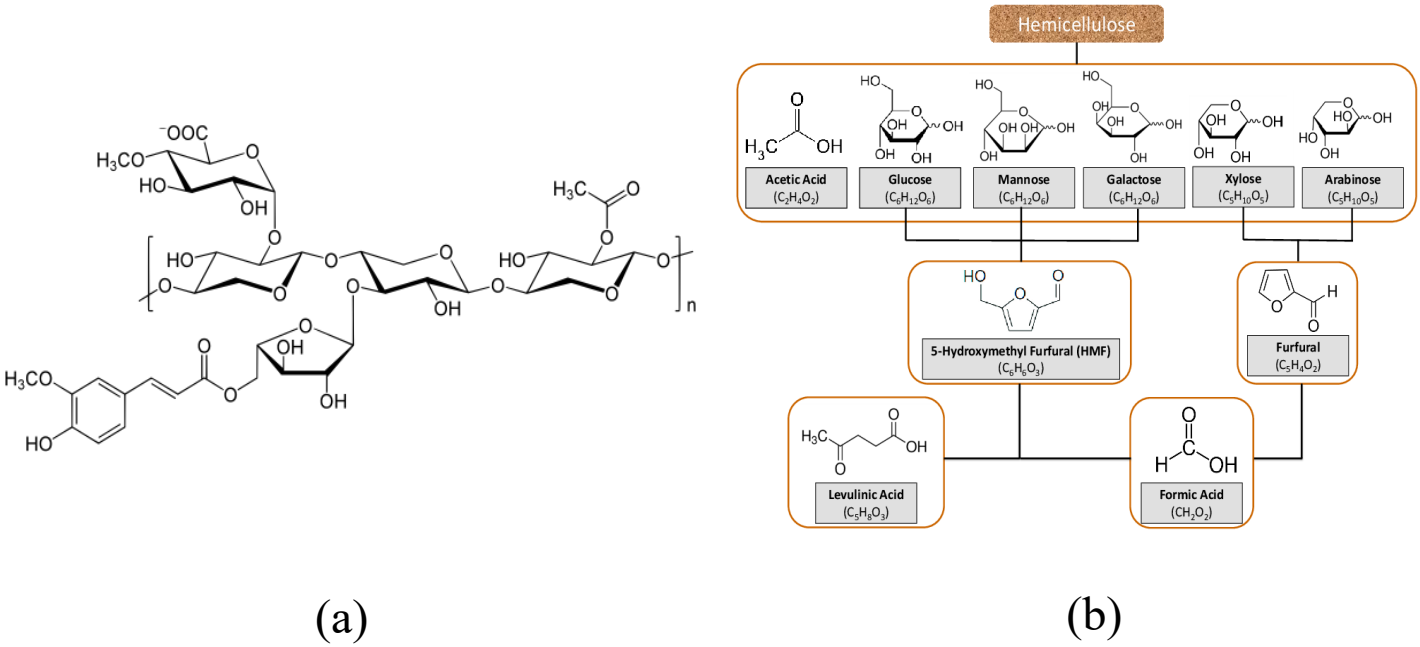


Figure 1.4: (a) Structure of hemicellulose, (b) Valorization of hemicellulose constituents in the context of a furan based biorefinery (Dwivedi et al., 2023)

1.3.3 Lignin

Lignin, a naturally occurring substance, is the most abundant substance made up of aromatic components in nature (Calvo-Flores & Dobado, 2010). It is a complex, amorphous, three dimensional long-chain and heterogeneous high molecular weight aromatic polymer of phenylpropanes of 3C attached with 6C atom rings, methoxy groups and non-carbohydratic polyphenolic substances connected by ether linkages (Anwar et al., 2014). It consists of three different primary phenylpropanes monomer units, namely *p*-coumaryl (abbreviated as 'H' unit, *p*-hydroxy phenyl propanol or 4-hydroxycinnamyl), coniferyl alcohol (abbreviated as 'G' unit, guaiacyl propanol or 3-methoxy4-hydroxycinnamyl) and sinapyl alcohol (abbreviated as 'S' unit, syringyl alcohol or 3,5-dimethoxy 4-hydroxycinnamyl), which vary in ratio depending on the plant it comes from (Nhuchhen et al., 2014). Lignin is present in plant biomass in percentages of 12-25%, although its content in herbaceous plants like grasses is between 10-19%, in softwoods between 25-35% and in hardwoods between 18-25%. It also serves as a reinforcement and protection for plant tissue, defending it against pathogens and insects as well as oxidative stress (Sticklen, 2008). Moreover, lignin also holds a key role in the resistance of the plant cell wall to enzymatic degradation. In the plant cell wall, lignin is connected to hemicellulose, thus forming a matrix that surrounds cellulose. This physical barrier not only prevents hydrolytic enzymes from accessing cellulose and hemicellulose but also absorbs cellulases irreversibly, hindering them from reaching cellulose (Palonen et al., 2004; Sewalt et al., 1997; Yang & Wyman, 2006). Consequently, effective enzymatic treatment requires the removal of lignin through a process known as delignification, typically achieved through pretreatment. Lignin is only soluble in organic solvents and not in water, making organosolv or alkali-catalyzed organosolv processes the most effective pretreatment methods for lignin removal (Kienberger, 2019; Zhao et al., 2009). However, enhancing enzymatic digestibility is not solely reliant on lignin removal. Dilute acid pretreatment, for instance, removes only a portion of lignin but still improves the digestibility of cellulose due to the removal of hemicellulose and changes in lignin distribution and structure (Axelsson et al., 2012).

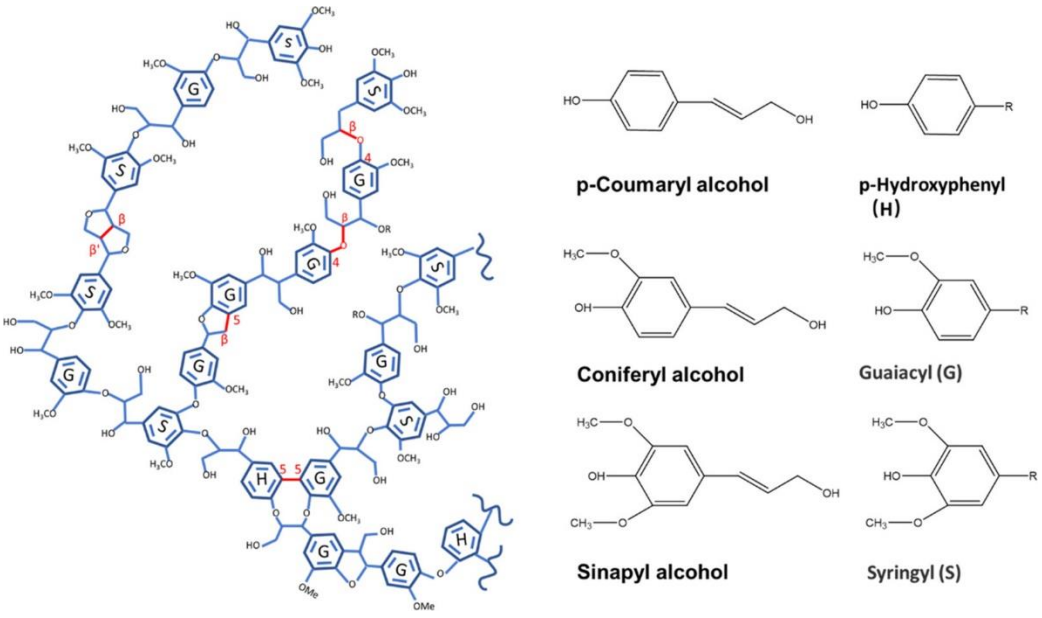


Figure 1.5: The structure of lignin as an aromatic polymer comprised of the three phenylpropane units (H,G,S) as adapted from Zhang & Naebe (2021)

The enzymatic deconstruction of lignin is typically performed by an array of oxidative enzymes, whose main representatives are laccases (EC 1.10.3.2) and lignin peroxygenases (EC 1.11.1.14) (Kumar & Chandra, 2020). Laccases are copper-containing enzymes that consist of monomeric, dimeric and tetrameric glycoproteins that catalyze the oxidation of the lignin phenolic compounds in two ways, direct and indirect oxidation. Direct oxidation contains the oxidation of the substrate to the similar radical as a result of direct contact that occurs with the copper cluster. Indirect oxidation accomplishes the same reaction, however through a mediator (Matera et al., 2008). Lignin peroxidases belong to family oxidoreductase, which degrades lignin and its derivatives in the presence of H_2O_2 due to the presence of a heme group containing iron ions. The overall lignin peroxygenase catalyzed mechanism is a two-step reaction involving the native enzyme of the ferric resting state: (1) the radical cation oxoferryl unstable intermediate compound I and (2) the impartial oxoferryl intermediate compound II (Castro et al., 2016). Other enzymes involved in lignin degradation are manganese peroxidases (EC 1.11.1.13), feruloyl esterases (EC 3.1.1.73), aryl alcohol oxidases (EC 1.1.3.7), quinone reductases (EC 3.1.1.73), lipases (EC 3.1.1.3), xylanases (EC 3.2.1.8) and catechol 2,3-dioxygenases (EC 1.13.11.2) (A. Kumar & Chandra, 2020).

In the context of furan-based valorization of lignocellulose, lignin remains as a byproduct that has potential uses in various applications (Kienberger, 2019). Among its uses, it can be utilized as an eco-friendly alternative to numerous petrochemically derived substances, rubber additives, thermoplastics and pharmaceuticals (Zakzeski et al., 2010). However, the lack of sufficient

processing techniques poses hurdles to the development of a full-fledged lignin based biorefinery. As a result, research on lignin is limited to biochemicals that fall into two categories, namely, macromolecules and aromatics. The category of macromolecules includes materials such as carbon fibers, benefiting from the high carbon content of lignin (Baker & Rials, 2013), polyurethane products (Strassberger et al., 2014) and thermoset, resin and adhesive macromolecules (Yan Zhao et al., 2016). Aromatic compounds typically involve BTX moieties (butane, toluene and xylene) (Jongerius, 2013), phenols (Liu et al., 2012) and vanillin (Fache et al., 2015). However, despite the potential of lignin as an aromatic polymer, the lack of knowledge on its utilization still renders it as an obstacle to lignocellulose degrading enzymes that needs to be removed.

1.4 Pretreatment of lignocellulose

The term '*pretreatment*' refers to the separation of each fraction of lignocellulosic biomass in order to receive its components to the highest possible purity. As mentioned above, pretreatment is the means to achieve an effective fractionation, and aims to alleviate the biomass of any constraints towards hydrolysis and increase the yield of the monomeric hexoses and pentoses from cellulose and hemicellulose, respectively (De Bhowmick et al., 2018). Typically, this is achieved through the disruption of the complex structure of lignocellulose, therefore facilitating the access of enzymes to the desired compound. Efficient fractionation plays a crucial role in the overall process of developing an effective pretreatment technology for furan-based biomass valorization. The main objective is to separate the biomass into two clean fractions: a solid pulp rich in cellulose and free from lignin, which can be hydrolyzed to glucose, and a liquor enriched with hemicellulose and lignin that can be easily separated and removed. In addition to this, other factors such as reducing the size of the biomass particles, improving cellulose accessibility, and minimizing the formation of inhibitory products that may have detrimental effects in the next step should also be taken into consideration. These products mainly involve carboxylic acids, furan derivatives and phenolic compounds, that are a result of sugar degradation and lignin deconstruction during pretreatment and are detrimental to the function of fermentative microorganisms in fermentation processes (Palmqvist & Hahn-Hägerdal, 2000; Varga et al., 2004). However, within the frame of furan-based biorefineries, HMF and FA produced in the pretreatment are the target byproduct and not an inhibitory one. As a result, the generation of furans in pretreatment can also be integrated in order to increase the overall yield. Nevertheless, it has been reported that furans can inhibit the action of enzymes such as isomerases, cellulases and/or hemicellulases diminishing sugar yields (Saritha et al., 2012). In addition, acidic compounds, such as formic or levulinic acid also might affect other reactions such as the dehydration reaction of sugars towards the production of furans. The method of pretreatment can change depending on the source of the feedstock or the downstream process and the fraction that is of particular interest. Some pretreatment techniques focus on breaking the connections between biomass components using high temperatures or

pressure, while others involve the use of chemical or biological catalysts. Pretreatment is an essential step in order to receive a material that is amenable to enzymatic processes with high yields. As a result, the meticulous customization of the pretreatment method in order to fit in all the parameters, such as feedstock, solvent, catalyst with the aim of achieving the desired end-product, is of utmost importance. Overall, there are four main categories of pretreatments: physical, chemical, physico-chemical, and biological pretreatment.

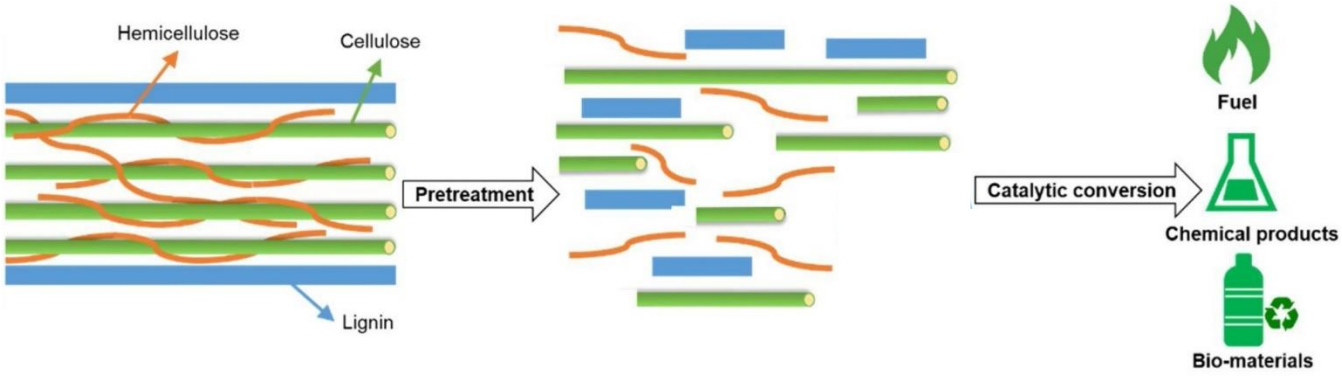


Figure 1.6: The role of pretreatment in lignocellulose biorefineries as adapted from Liao et al. (2020)

1.5 Fractionation technologies

1.5.1 Physical pretreatment

Physical pretreatment often involves mechanically treating lignocellulosic biomass to reduce particle size and enhance the accessibility of biomass to hydrolytic enzymes. Achieving a small particle size and utilizing effective stirring techniques are crucial in order to maximize the yield of subsequent hydrolysis due to securing better mass transfer conditions of the enzyme to the surface of the biomass (Katsimpouras et al., 2017).

The most common mechanical treatment for lignocellulose includes the use of different milling techniques for the reduction of the residue to the desired particle size along with other mechanical actions such as shearing, compressing, crushing, friction and stretching. Typically, all the above techniques are frequently combined with other pretreatment methods in order to process lignocellulose materials. Different milling technologies include disk mills, bead mills and ball mills that can be implemented to grind the biomass sample (Lomovskiy et al., 2020; Mayer-Laigle et al., 2018; Shen et al., 2020), or hammers, knife mills and jet mills that can be utilized to crush biomass particles (Victorin et al., 2020).

Extrusion is another type of physical pretreatment that units mechanical and thermal effects in a single machine. It is carried out in extruders, which consist of one or two axis with different screw

elements that can be adjusted to the desirable screw profile. The screws spin inside a tight barrel whose temperature can be regulated. The biomass is continuously fed to the machine, where it is transported and forced to pass through the narrow space between the screw and the barrel wall, generating high shearing forces that alter fiber structures mechanically. The target of this type of pretreatment is the particle size reduction and fibrillation. However, not all studies report adequate effectiveness on biomass samples and, as a result, it is often combined with chemicals in order to increase the overall yield (Gallego-García et al., 2023).

Ultrasonication is also another common physical technology for lignocellulose pretreatment that is based on the cavitation effect caused during irradiation with ultrasonic energy, an acoustic wave that oscillates at frequencies above 16 kHz. Acoustic cavitation is induced when the ultrasonic wave is propagated in a specific liquid medium, promoting compression (when the molecules of the solvent are pressed together) and rarefaction (when the molecules of the solvent are separated) cycles that form microbubbles containing gas. The collapse of such microbubbles triggers different physical and chemical effects, including heating, acoustic cavitation, acoustic streaming, nebulisation, and radical formation, thus altering biomass structure (Flores et al., 2021).

Finally, a recent type of physical pretreatment that has gathered a lot of interest is the pretreatment through microwave irradiation. This technology enhances hydrothermal biomass fractionation by promoting the interaction between substrate and reaction medium, although applying low pretreatment severities. Microwaves are non-ionizing radiations using wavelengths of 0.01-1m and 300-300,000 MHz. This unique heating mechanism benefits the pretreatment system through the rapid increase in temperature, thus greatly reducing heating time and offering a uniform and selective volumetric heating performance as well as increased energy transfer efficiency (Flores et al., 2021; Kostas et al., 2017).

1.5.2 Chemical pretreatment

An alternative to effective lignocellulose fractionation is the chemical pretreatment, which involves the use compounds such as alkali, acids, organic solvents and ionic liquids. Although chemical pretreatment has been utilized for many years, it also has undergone serious changes in order to be cost competitive and environmentally friendly. In this context, the latest trend in chemical pretreatment is the implementation of low chemical catalyst loading, low amount of liquid (solvent or water), as well as limited washing for catalyst recovery and conditioning.

The use of alkali is based on its ability to solubilize lignin and increase cellulose digestibility, while simultaneously exhibiting low cellulose/hemicellulose solubilization (Carvalheiro et al., 2008). In specific, NaOH has been reported to cause cellulose swelling as well as decrease its degree of polymerization and crystallinity, resulting in the dissolution of lignin structure (Taherzadeh & Karimi, 2007, 2008). Alkali pretreatment also involves the use of lime ($Ca(OH)_2$),

which has been reported to achieve hemicellulose deacetylation thus removing lignin from the pulp, while also providing the opportunity for its easy recovery through the use of CO_2 post pretreatment (Mosier et al., 2005a). Alkali compounds are frequently utilized in small amounts as additional catalysts for physico-chemical pretreatments. In general, alkaline pretreatment is applied to substrates such as agricultural residues or herbaceous crops with low lignin content.

In contrast, acid is used to break down and extract the hemicellulose component. Acid pretreatment can be undertaken using concentrated or diluted acid to dissolve the hemicellulose. However, utilizing concentrated acid involves challenges such as equipment corrosion, difficulties in recovering the acid, and high operational expenses, which make it impractical for large-scale commercial use. However, utilizing diluted acid not only helps hemicellulose dissolve, but also breaks it down into its individual components, generating sugars that can be fermented. A significant disadvantage of acid pretreatments is the production of furans, which can impede the growth of microorganisms involved in fermentation and the production of ethanol (Saha et al., 2005). Nevertheless, these substances can be effectively utilized to enhance the overall production of a furan-centered biorefinery concept. The widely employed acid is H_2SO_4, although hydrochloric, phosphoric, and nitric acid have also been utilized for this purpose (Mosier, Hendrickson, et al., 2005). Finally, the use of fumaric and maleic acid has been linked to lower amounts of FA produced when compared to H_2SO_4 (Kootstra et al., 2009). Acid pretreatment has been applied to many different lignocellulose substrates, such as corn stover, beechwood, rice and switch grass exhibiting promising results (Kumar et al., 2009; Saha et al., 2005; Zu et al., 2014).

The most promising work on chemical pretreatment lies in the use of organic solvents for lignocellulose fractionation. The principle of the use of organic solvents in the pretreatment lies in their ability to dissolve lignin from the biomass thus removing it to the liquid fraction. Consequently, once the solvent is evaporated from the liquor, the water insoluble lignin is then received in its pure form (Zhao et al., 2009). The result of organosolv pretreatment is the generation of three fractions: a fraction of pure dry lignin, a liquid fraction rich in hemicellulose and a solid fraction of relatively pure cellulose. Biorefineries that are mainly focused on the solid cellulose-rich fraction also benefit from organosolv pretreatment in other ways as in alkali pretreatment, organic solvents also lead to cellulose swelling increasing its degradability. To efficiently break down biomass into its components, organosolv pretreatment is combined with other types of pretreatment. Commonly used organic solvents or their aqueous solutions include methanol, ethanol, acetone, ethylene glycol, and tetrahydrofurfuryl alcohol. By using a mixture of water and organic solvent, it is possible to separate hemicellulose and lignin, as hemicellulose dissolves in water and lignin dissolves in organic solvents. However, in organosolv procedures, it is important to effectively remove the solvents through separation techniques, as they may hinder enzymatic hydrolysis or sugar fermentation (Sun & Cheng, 2002). Moreover, the higher expenses associated with using these solvents for industrial purposes result in a preference for solvents that are simple to separate and have low molecular weights and boiling points, like methanol and ethanol. The

organosolv method shows great potential in a furan-based biorefinery process because it enables the extraction of lignin-free components, specifically a solid fraction rich in cellulose and a liquid fraction abundant in hemicellulose, which can be utilized in the production of HMF and FA respectively. In addition, aside from the valorization of the cellulosic fraction, the ability of organosolv pretreatment to produce pure fractions of lignin and hemicellulose leads to the possibility of high added value chemical synthesis.

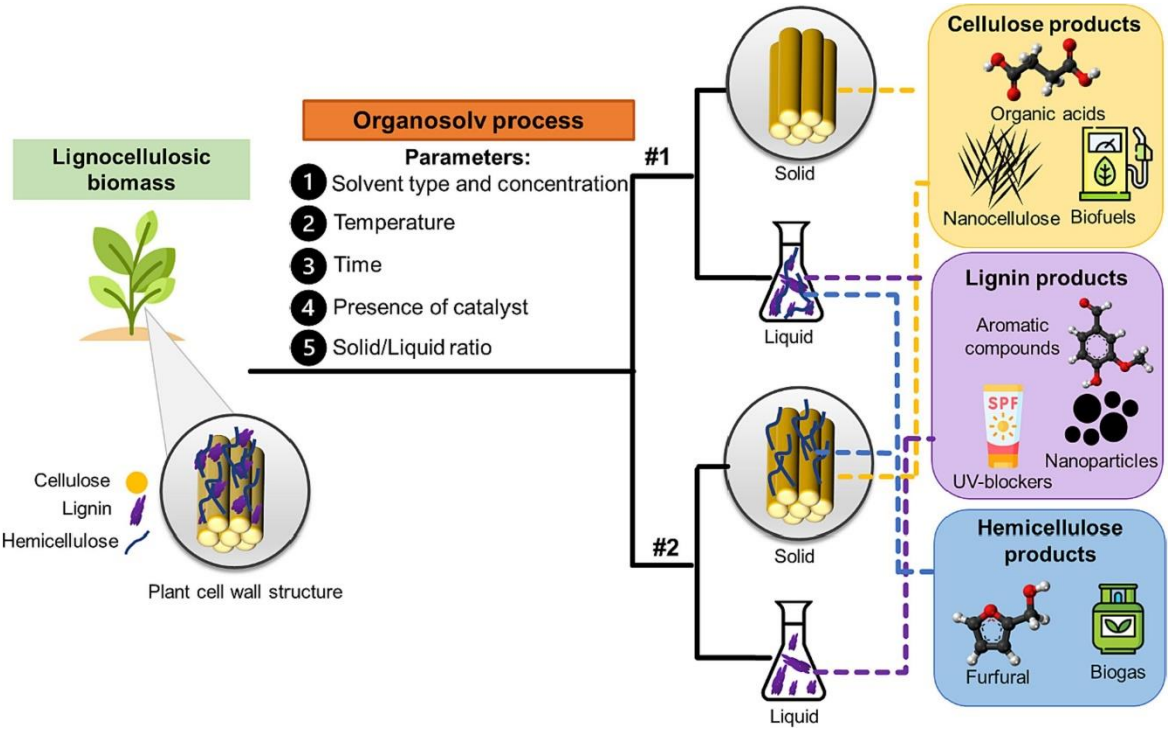


Figure 1.7: Different types of hemicellulose along with their respective source, amount and composition (Gírio et al., 2010)

Finally, over the past few years ionic liquids (ILs) have been introduced to the pretreatment process. ILs are salts with high organic cations and low inorganic anions that are liquid in relatively low temperatures and have low vapor pressure. Their low vapor pressure constitutes them a viable candidate for pretreatment as they can be easily recovered. Their mode of action in pretreatment lies in the rupture of the bonds between lignin, cellulose and hemicellulose and consequently, the rupture of the macromolecules without the formation of inhibitory substances. In that context, ILs have shown promising results both for the fortification of cellulose hydrolysis, as well as the production of HMF from lignocellulose (Li et al., 2009; Nguyen et al., 2016). The next step for IL pretreatment is to include ILs that are generated from lignocellulose itself, as result leading to a circular process that can be applied to future biorefineries.

1.5.3 Physicochemical pretreatment

This type of pretreatment involves the simultaneous use of high temperature and pressure conditions as well as the addition of chemicals as catalysts. This type of pretreatment combines the effects of chemical solvents on biomass structure, along with breaking bonds and separating components through extreme temperature and pressure conditions.

The most common representative for this type of pretreatment is steam explosion. This process involves subjecting biomass to pressurized steam for a short period of time, followed by a sudden decrease in pressure. This sudden pressure change causes the plant cell wall polymers to rupture, allowing the hemicellulose to be removed and separating the cellulose fibrils, making them easier to break down with enzymes. Additionally, the harsh mechanical conditions also affect the lignin fraction, temporarily changing its composition (Pielhop et al., 2016). Acetic acid is often produced during steam explosion pretreatment due to the exposure of hemicellulose acetyl groups to high temperatures. This is why some studies recommend using low concentrations of acid to facilitate hemicellulose degradation. Further degradation of acetic acid leads to the formation of furans like FA and HMF, as well as formic and levulinic acid (Mosier et al., 2005b). Overall, steam explosion pretreatment is considered as a safe process that does not require the use of hazardous materials and has been extensively studied in pilot units such as the one of NREL in Golden (Colorado, USA) and the one of SEKAB Örnsköldsvik (Sweden). In addition, it has also been implemented in second generation ethanol production units such as those of Iogen (Ottawa, Canada), Inbicon (Kalundborg, Denmark) and Abengoa (Salamanka, Spain).

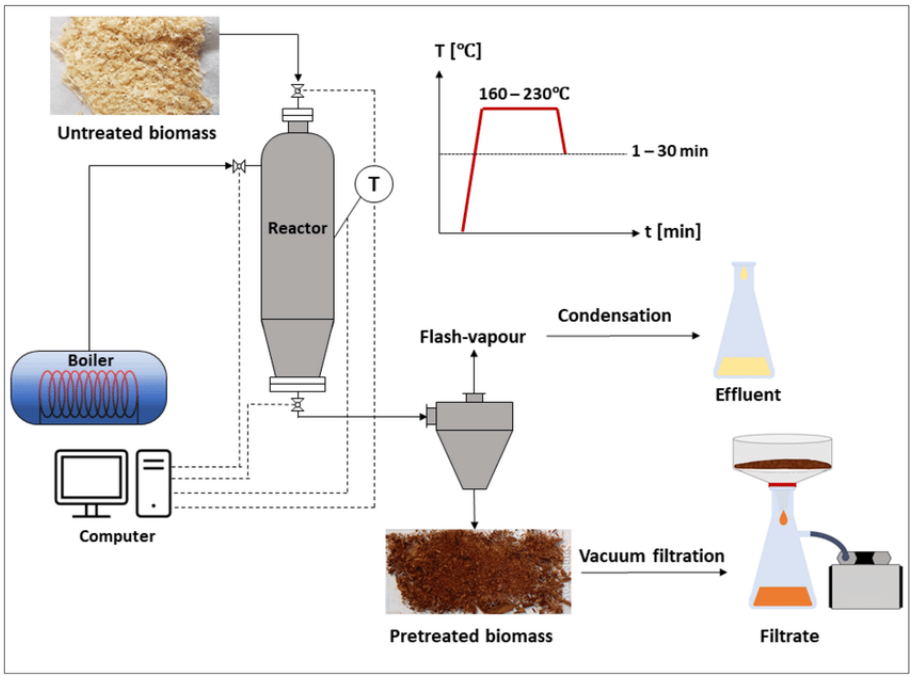


Figure 1.8: Steam explosion pretreatment Ghoreishi et al. (2022)

Another method of biomass pretreatment is liquid hot water pretreatment, which involves exposing biomass to high temperatures ranging from 160°C to 230°C, without the immediate decompression step. This process results in the solubilization and removal of hemicellulose, while slightly affecting the structure of lignin and cellulose (Palonen et al., 2004). The effective interaction of water and biomass can be achieved in three ways: (i) the co-current method, where the biomass is heated along with the water inside the reactor, where they remain for the total duration of the process, (ii) the counter current method, where the water and the biomass are mixed by moving in opposite directions and the (iii) the flowthrough method, where the water, as it is implied, flows through the biomass that is immobilized on a flat surface, dragging along the diluted fractions (Liu & Wyman, 2003; Yang & Wyman, 2004). The effectiveness of this process can be boosted by adding dilute acids, such as sulfuric or acetic acid in small quantities with the risk of generating inhibitory compounds. The addition of the acid leads to the disruption of the acetyl groups of the xylan backbone in hemicellulose, therefore increasing the efficiency of this method.

Ammonia Fiber Expansion (AFEX) follows the principle of the steam explosion pretreatment with the difference of implements anhydrous ammonia in low temperatures ranging from 60 ^{o}C to 100 ^{o}C followed by a sudden release in pressure. This results in the rapid expansion of ammonia, which creates a pretreated dry material where the cellulose fibers swell and lose their crystalline structure. Although this process only removes a small amount of lignin and hemicellulose, it has been found to break the linkages between lignin and carbohydrates, making the material more easily digestible by enzymes. This is likely due to the partial removal of lignin, which absorbs the enzymes responsible for cellulose degradation (Wyman et al., 2005). Another benefit of this pretreatment is the minimal production of byproducts that could interfere with subsequent biological processes, such as phenolic fragments of lignin, and the easy separation of ammonia despite its high volatility (Teymouri et al., 2005). On the other hand, wet oxidation is a pretreatment method that subjects lignocellulosic biomass to high temperature (between 170 ^{o}C and 200 ^{o}C) and pressure (from 10 to 12 bar O_2) conditions, using oxygen or air as a catalyst, for a short period of time (10 to 15 minutes). This process effectively breaks down and solubilizes the hemicellulose and lignin fractions of the biomass, with the aim of producing ethanol in a later fermentation step (Katsimpouras et al., 2017; Martín et al., 2008). However, the cost of oxygen and chemical catalysts required for wet oxidation can be major roadblocks to the industrialization and advancement of this technology.

Finally, physicochemical pretreatment also includes wet oxidation techniques for the disruption of lignocellulose. In this type of pretreatment, biomass and solvents are introduced in the reactor along with a constant feed of air/oxygen in high temperatures (<120 ^{o}C) for a specific duration. Its effectiveness lies in mainly three parameters: the pressure of the oxygen, the temperature and the duration. The basic principle of this pretreatment is that in high temperatures, water catalyzes the

hydrolytic reactions that cause hemicellulose to break down in smaller chains, while lignin is oxidized and therefore cannot reattach to cellulose and hemicellulose. In addition, the cellulosic fraction remains unchanged (Szijártó et al., 2009). The result of this process is a solid pulp with high delignification and high cellulose content. Over the past few years, there have been many attempts to customize this process to include an organic solvent in this process. The most promising representative in this case is acetone (Acetone/Water Oxidation, AWO), ethanol and tetrahydrofuran, with many studies exhibiting very promising results (Dedes et al., 2021; Gong et al., 2012; Jafari et al., 2016; Katsimpouras et al., 2017). The incorporation of an organic solvent in this process means that nearly all of the lignin present in the biomass can be isolated in the organic fraction of the solvent and its recovery can lead to high added value derivatives. A customization of this method was set up by Kalogiannis et al. (2020) and was implemented to pretreat lignocellulosic biomass samples used in this study.

1.5.4 Biological pretreatment

This type of pretreatment utilizes microorganisms for the degradation of the lignin and hemicellulose fractions, while the cellulose fraction remains intact. The main microorganisms involved in biological pretreatment are brown, white, and soft rot fungi. These fungi produce lignin degrading enzymes, such as laccases and peroxidases, that are capable of breaking down lignin efficiently (Kumar & Wyman, 2009b). Like all pretreatment processes, biological pretreatment can be combined with other treatment types for the production of a material with high cellulose content and susceptible to enzyme digestibility. For example, Itoh et al. successfully used a combination of biological and organosolv pretreatment to produce ethanol (Itoh et al., 2003). Although biological pretreatment offers advantages such as low capital cost, low energy demands, and a mild environmental impact, its use is restricted by slow hydrolysis rates and lengthy time requirements (Sun & Cheng, 2002). The following table presents studies utilizing microorganisms for the pretreatment of lignocellulose.

Table 1.4: Different biological pretreatment strategies involved in the pretreatment of lignocellulose and their advantages.

Microorganism	Biomass	Major effects	References
Punctualaria sp. TUFC20056	Bamboo culms	50% of lignin removal	(Suhara et al., 2012)
Irpex lacteus	Corn stalks	82% of hydrolysis yield	(Du et al., 2011)
Fungal consortium	Straw	7-fold increase in hydrolysis	(Taha et al., 2015)
Pleurotus. ostreatus/Pleurotus pulmonarius	Eucalyptus grandis saw dust	20-fold increase in hydrolysis	(Castoldi et al., 2014)
Phaneorchaete chrysosporium	Rice husk	–	(Potumarthi et al., 2013)
Fungal consortium	Corn stover	43.8% lignin removal/7-fold increase in hydrolysis	(Song et al., 2013)
Ceriporiopsis subvermispora	Wheat straw	Minimal cellulose loss	(Cianchetta et al., 2014)
Ceriporiopsis subvermispora	Corn stover	2–3-fold increase in reducing sugar yield	(Wan & Li, 2011)
Fungal consortium	Plant biomass	Complete elimination of use of hazardous chemicals	(Dhiman et al., 2015)

Overall, pretreatment is a process that aims to efficiently separate the polymeric constituents of lignocellulosic biomass. This result can be achieved upon the use of extreme mechanical, physical or chemical conditions that cause the links between lignin, hemicellulose and cellulose to disrupt. However, each type of pretreatment also presents a series of drawbacks that can either refer to high capital costs or the formation of by-products. In order for the pretreatment process to be cost-effective and competitive, there is a need for deep understanding of both the specifications of the raw materials as well as the targeted end-products. The following table summarizes the different types of pretreatment along with their respective advantages and disadvantages.

Table 1.5: Comparison of the different types of pretreatment.

Type of Pretreatment	**Advantages**	**Disadvantages**
Milling	Reduction of cellulose crystallinity	High energy demand
Alkali Pretreament	Hemicellulose and lignin removal	High residence times/ Formation of salts that bind to the biomass
Acid Pretreatment	Dilution of hemicellulose/ Change of lignin structure	Formation of inhibitors/ Solvent recovery demand
Organosolv	Dilution of hemicellulose and lignin/ reduction in cellulose crystallinity/ recovery of pure lignin	Demand for solvent recovery/ High solvent cost
Ionic Liquids	Reduction in cellulose crystallinity	ILs function as enzyme inhibitors/ High cost/ Demand for IL recovery
Steam explosion	Dilution of hemicellulose/ Change of lignin structure/ Low cost	Partial hemicellulose degradation/ Formation of toxic compounds
Hydrothermal	Release of hemicellulose oligomers/ Absence of chemical catalysts	High energy demands
Ammonia Fiber Expansion	Low inhibitor concentrations/ Biomass granulation not needed	Low effectiveness on high lignin biomass/ High cost due to ammonia
Wet Oxidation	High degree of hemicellulose and lignin solubilization/ Increased purity of lignin	High equipment and solvent cost
Biological	Low energy demand/ Delignification/ Cellulose Depolymerization/ Partial Hemicellulose hydrolysis/ Mild conditions	Very slow pretreatment/ Sugar consumption/ Low industrialization potential

1.6 Enzymatic hydrolysis of cellulose

As described in paragraph 1.3.1, cellulose is comprised of D-glucose units linked together with β-glycosidic bonds forming a structure with high crystallinity index, therefore exhibiting difficulties in its disruption. Breaking down cellulose to glucose can be achieved through the use of chemicals, such as acids or ionic liquids or enzymatically through the use of cellulases (Taherzadeh & Karimi, 2007). While the deconstruction of cellulose has traditionally been achieved through chemicals, the employment of enzymes offers disruption with high selectivity as well as mild environmental conditions. However, due to the recalcitrant nature of real lignocellulosic feedstocks, enzymatic hydrolysis of cellulose is a difficult task that requires the use of an arsenal of many different enzymes. These enzymes can be divided into three classes: exo-1,4-β-D-glucanases (cellobiohydrolases, CBHs, EC 3.2.1.176), endo-1,4-β-D-glucanases (EGs, EC 3.2.1.4), and β-glucosidases (BGLs, EC 3.2.1.21) that are all members if the glucoside hydrolase families (GH) in the Carbohydrate Active Enzymes (CAZy) database (http://www.cazy.org/).

The typical procedure of cellulose degradation typically involves the synergistic action of these three classes. In this context, EGs work arbitrarily on both soluble and insoluble parts of β-1,4-glucans. Their mode of action is focused on attaching to the amorphous regions of cellulose hydrolyzing the internal glycosidic bonds between glucose units, producing smaller chains of glucose with different polymerization degrees. These smaller chains then function as a substrate to CBHs, which bind to the ends of the glucose chains releasing units of cellooligosaccharides. The apparent synergy between these two classes of enzymes lies in the increase of free ends for CBHs, through the work of EGs. However, CBHs have a strong product inhibition and, as a result, their activity drops with cellooligosaccharide accumulation. Hence, BGLs are necessary in order to cleave glucose units from oligosaccharides (Eriksson et al., 2002; Väljamäe et al., 2003). In addition to these classes of enzymes, lytic polysaccharide monooxygenases (LPMOs) also contribute to the disruption of the cellulose polysaccharide by means of oxidation. LPMOs are auxiliary activity copper ion enzymes whose role is to reduce the molecular oxygen in the presence of an external electron donor. The reduced dioxygen then extracts a single hydrogen from the substrate leading to the cleavage of the β-1,4-glucosidic bond. LPMOs oxidize the glucose carbon in cellulose chains at either the C1 or the C4 position, resulting in the formation of reduced as well as oxidized monomers at the end of the hydrolysis (Singhania et al., 2021). As a result, LPMOs function synergistically with hydrolytic enzymes to boost the degradation of polysaccharidic substrates to increase sugar yield (Karnaouri et al., 2017). Currently there is a great repertoire of commercially available cellulolytic cocktails for efficient biomass hydrolysis to monosaccharides, mostly produced by thermophilic filamentous fungi (Rodrigues et al., 2015). In addition, numerous single enzymes have been cloned and expressed heterologously in eukaryotic hosts; this facilitates not only the study of mode of action of specific enzyme activities, but also the construction of tailor-made cocktails targeted for specific substrates (Karnaouri et al., 2013, 2018; Karnaouri et

al., 2014). Overall, cellulose hydrolysis despite being the result of a complex combination of different enzyme activities, has extensively been examined.

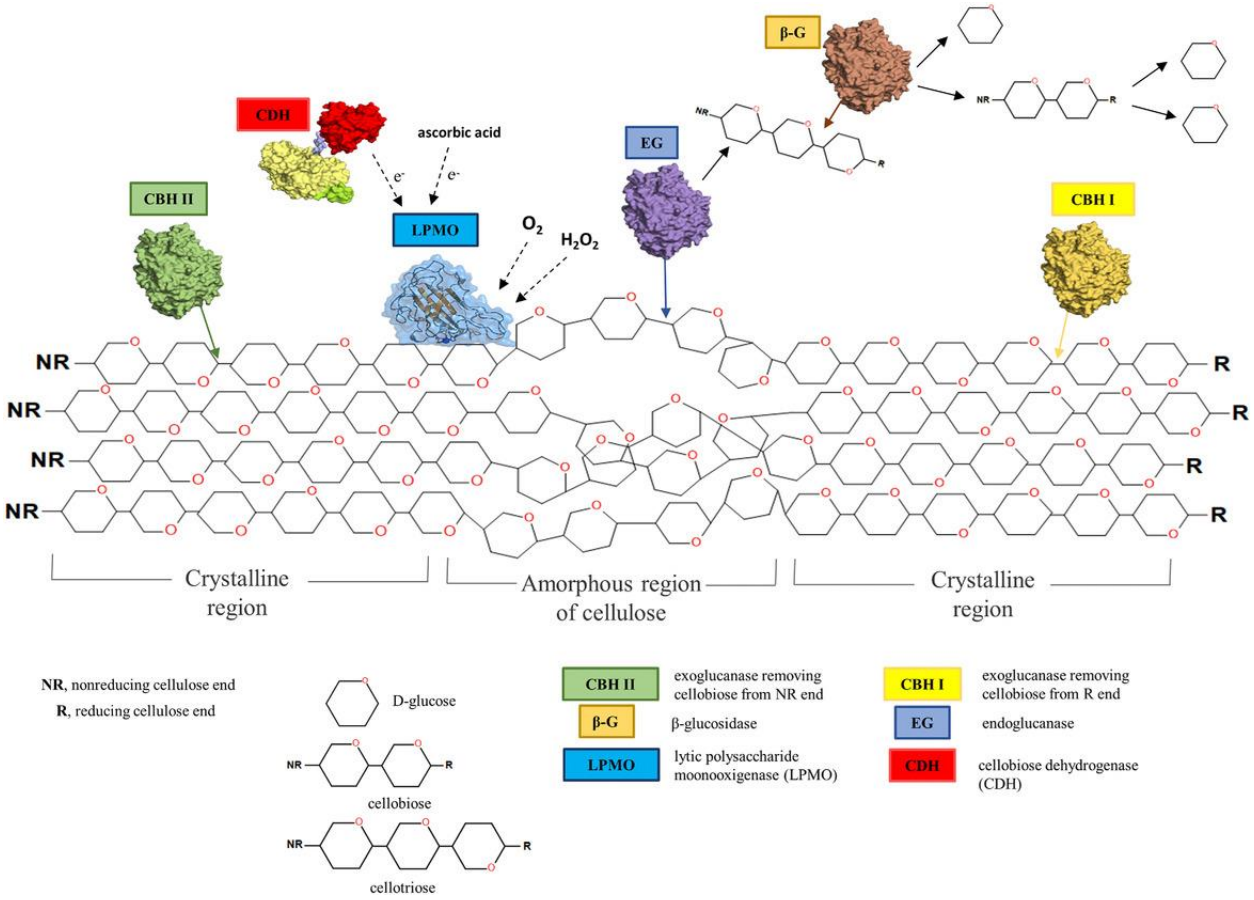


Figure 1.9: The complete schematic representation of enzymatic cellulose degradation for the production of glucose (Andlar et al., 2018)

1.7 Enzymatic hydrolysis of hemicellulose

The degradation of hemicellulose also requires the use of a wide variety of enzymes, even though hemicellulose is an amorphous polymer contrary to cellulose. The reason behind this is the wide variety of its compounds as described above. To begin with, the hydrolysis of xylan can be achieved through the use of different enzymes with various specificities and modes of action. Endo-1,4-β-xylanases (EC 3.2.1.8) are responsible for the breakdown of the xylan chain at random spots, while β-xylosidases (EC 3.2.1.37) release xylose units from the end of these chains. The separation of the side units is accomplished with α-L-arabinofuranosidases (EC 3.2.1.55), α-glucuronidases (EC 3.2.1.139) and the acetyl xylan esterases (EC.3.1.1.72). The degradation of mannan typically includes β-mannanases (EC 3.2.1.139), β-mannosidases (EC 3.2.1.25) and BGLs (EC 3.2.1.21). The endo action of β-mannanases catalyzes the random rupture of β-glucosidic bonds within the manna chain, releasing small length mannooligosaccharides. Then, β-

mannosidases release mannose from the non-reducing ends of mannooligosaccharides as well as mannobiose. Finally, the release of 1,4-glucopyranose units from oligosaccharides derived from glucomannan is accomplished by the action of BGLs. In addition, auxiliary enzymes such as α-galactosidases and acetyl xylan esterases are used for the separation of the D-galactopuranosidic and acetic acid side units, respectively. Feruloyl esterases (EC 3.1.1.73) belong to the group of carboxyl-esterases (EC 3.1.1.1) and target the ester bond between hydroxycinnamyl acids (ferulic or *p*-coumaric acid) or the dihydro-dimers of ferulic acid on the main polymer chains of xylan present in the plant cell wall. The result of this action is the rupture of the linkage between lignin and hemicellulose and, consequently, the disruption of the complex matrix of polymers that prevents access of cellulolytic enzymes to cellulose. As a result, most feruloyl esterases can be used synergistically with xylanases and cellulases for the deconstruction of the plant cell wall.

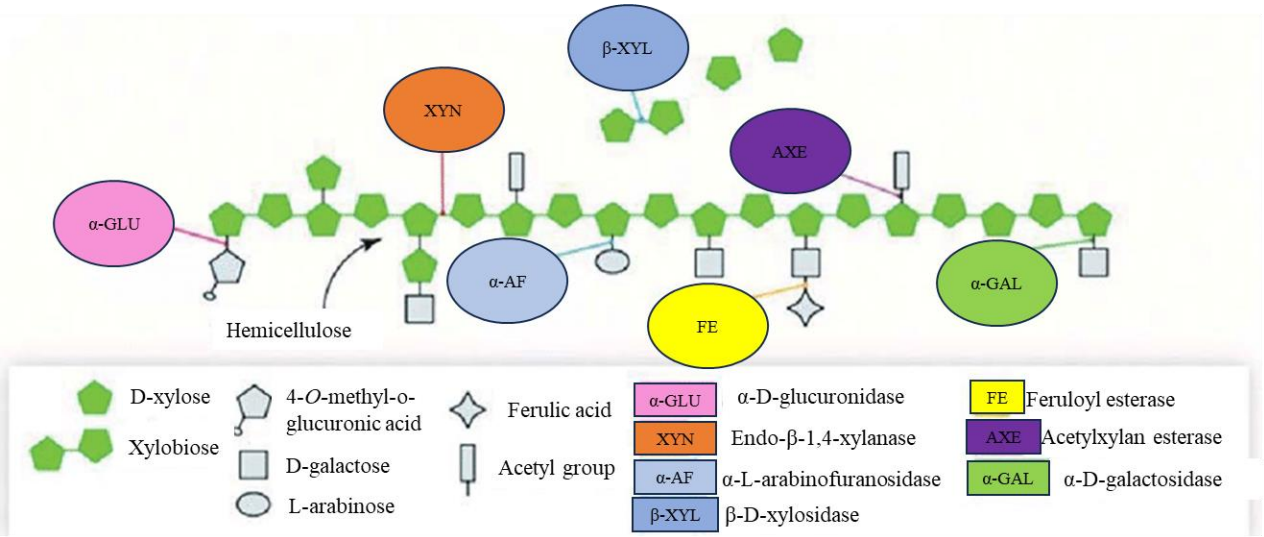


Figure 1.10: Schematic representation of the enzymatic deconstruction of hemicellulose (Madadi et al., 2017)

1.8 Inhibition of enzymatic hydrolysis

Breaking down cellulose is a complex process that can be hindered in various ways. The primary obstacle to enzymatic digestion is the intricate structure of the plant cell wall. The combination of lignin and hemicellulose polymers forms a matrix around the cellulose, making it difficult for cellulolytic enzymes to access and break down the cellulose. Therefore, pretreatment is crucial for maximizing the overall efficiency of the process, as it removes the main barriers of lignin and hemicellulose. However, the hydrolysis of cellulose still presents numerous challenges.

The first factor in the performance of enzymes is the substrate composition and the amount of lignin and hemicellulose present in it. It is known that cellulases and hemicellulases act by

attaching themselves on the cellulose fibers through one of their domains, namely the carbohydrate binding module (CBM), whose role is to stabilize the enzyme on the carbohydrate substrate and coordinate the hydrolysis (Várnai et al., 2014). As mentioned before, lignin has the ability to absorb enzymes non-reversibly consequently hampering their activity on cellulose and decreasing hydrolysis yields. As a result, it is pivotal to develop efficient pretreatment strategies in order to minimize lignin and hemicellulose content and eliminate the adverse effects of residual lignin (Matsakas et al., 2018).

Another key factor affecting hydrolysis yields studied in numerous reports is the effect of substrate concentration. High substrate concentration (above 12% solids loading) can pose serious drawbacks due to the increased viscosity of the reaction medium and the subsequent poor mass transfer conditions that it entails. This can be explained due to the fact that enzymes are soluble in the liquid fraction of the hydrolysis, however, their target is the surface of cellulose in the biomass. By increasing the amount of substrate, not only it is difficult to effectively secure enzyme mass transfer on cellulose, but also substrate inhibition phenomena are more prevalent, therefore diminishing the saccharification rates. In their work, Katsimpouras et al. (2007) portrayed the importance of the stirring technique to the yield of the hydrolysis, by comparing a traditional stirring system with flasks to an alternative free fall mixer. While traditional flasks accomplished stirring by moving in a horizontal axis, in the case of the free fall mixer, stirring ocurred through the spinning of fans that drifted the biomass vertically as well, then finally returning it the main reaction medium. As a result, biomass was much more easily liquified and the enzymes had easier access to their substrate increasing cellulose conversion, highlighting the advantages offered by effective mixing. These findings are extremely important because they shed light on the nature of hydrolysis as a process. By implementing an effective stirring technique, it is possible to increase substrate concentration and obtain a high glucose syrup, which is a prerequisite to high fructose concentrations after isomerization and, finally, high furan concentrations, within the frame of a furan-based valorization process.

On another note, hydrolysis can also be inhibited by degradation products produced in the pretreatment, such as phenolic compounds, furans, carboxylic acids), or by the end-product of the process. During the enzymatic deconstruction of cellulose, released glucose shows a typical product inhibition to BGLs (Decker et al., 2000), which leads to the accumulation of cellobiose in the hydrolysate. Cellobiose in turn, functions as an inhibitor to CBHs impeding their function (Holtzapple et al., 1990). These phenomena indicate that reaching the complete cellulose conversion to glucose is practically impossible due to the formation of inhibitors in the form of products. As a result, in order to overcome this obstacle, many reports examining sugar fermentation suggest a different strategy by incorporating saccharification and fermentation as one step in the same process (Simultaneous Saccharification and Fermentation, SSF). In this process, by incorporating the fermentative microorganism in the hydrolysis mixture, it is possible to both produce glucose through cellulose degradation and consume it through fermentation at the same

time. This way, the cellulolytic enzymes are alleviated off their inhibitors continuing cellulose deconstruction while the fermentative microorganism keeps consuming the produced glucose, increasing both the cellulose conversion and the end-product. It is also important to take into consideration that the use of different enzymes and microorganisms means difference in their pH and temperature optima. Furthermore, products such as formic acid can affect the reaction by altering its pH, although this can be solved by using buffer solutions (Panagiotou & Olsson, 2007). Nevertheless, it is evident that cellulose hydrolysis is a matter of extensive research on many different fronts and fields. Reaching high saccharification yields, means optimizing a number of different factors depending on the needs of the process.

1.9 Isomerization of hexoses and pentoses

Isomerization is a reaction where a compound is transformed to another with the same molecular weight, in other words an isomer. In this thesis, isomerization is mainly studied on the transformation of glucose to fructose, on the basis of valorizing the glucose produced by cellulose degradation. However, xylose present in hemicellulose can also be isomerized to xylulose.

Throughout literature, many reports describe different chemical routes for the transformation of glucose to HMF (Jadhav et al., 2012; Ståhlberg et al., 2010), and of pentoses to FA (Danon et al., 2014), by using metal catalysts, ionic liquids and other methods. However, the high cost of such materials, combined with the necessity for post-product purification raised the need for a development of a cost-effective, environmentally friendly process to convert glucose to HMF. To produce furan derivatives from sugars derived from lignocellulose in milder reactions, it is necessary to first convert glucose and xylose into fructose and xylulose, respectively. This is because ketoses, such as fructose and xylulose, can be dehydrated more easily than aldoses when Lewis acid catalysts are present. This dehydration process leads to the formation of furans, specifically HMF and FA (Choudhary et al., 2011; Román-Leshkov et al., 2006). Developing an efficient isomerization step is crucial to achieving high furan yields.

The enzymatic conversion of glucose to fructose can be performed through the use of a glucose isomerase. This enzyme is commonly used in the production of high fructose syrups from starches (Seyhan Tükel & Alagöz, 2008). The commercial enzyme preparation Sweetzyme IT from *Streptomyces murinus* has been successfully used on an industrial scale to produce fructose from glucose found in corn liquor (Dehkordi et al., 2009). However, the isomerization reaction faces a thermodynamic equilibrium that limits the conversion of glucose to fructose to around 50%. One method to shift this equilibrium in favor of fructose is by adding sodium tetraborate to the reaction. The borate ions react with glucose and fructose, forming ionized esters that attach to the second carbon of the ring. These complexes do not participate in the reaction because they are not recognized by the isomerase. Borate ions have a higher affinity for ketoses than aldoses in the equilibrium between borate and borate esters. This shift in equilibrium results in a higher

conversion of glucose to fructose, increasing it from the default 50% to as much as 80% (Takasaki, 1971; van den Berg et al., 1994). This approach allows for the production of HMF with high yields and selectivity, while also being environmentally friendly. A recent study by Wang et al. demonstrated that the addition of borate in a ratio of 0.5 to sugar significantly increased the isomerization of glucose and xylose-rich streams obtained from the pretreatment of corn stover, achieving approximately 80% glucose and 90% xylose conversion (Wang et al., 2019b).

In addition to cellulose, hemicellulose, particularly xylan, is a significant component of the plant cell wall. Considering this, the previously mentioned system for producing HMF through glucose isomerization could also be applied to convert xylose into FA. Similar to glucose, xylose can be transformed into FA using metal catalysts. However, by employing an isomerase, xylose can be converted into xylulose, which can then be dehydrated into FA more easily using acid and base catalysts (Takagaki et al., 2010; Yang et al., 2012). This suggests the potential for developing a system where both glucose and xylose, obtained from lignocellulose hydrolysis, can undergo an isomerization step followed by a dehydration step to produce HMF and FA, respectively, in a single process. This approach allows for the integration of xylose valorization alongside glucose in the biorefinery process, preventing the hemicellulose side stream to be discarded entirely.

1.10 Furans and furan derivatives

Furan (C_4H_4O) is a heterocyclic organic chemical compound of high volatility with a boiling point of 32 ^oC. It can be formed through various pathways from different precursors such as polyunsaturated fatty acids (PUFAs), amino acids, carbohydrates and ascorbic acid under thermal or oxidative conditions. To date, the four most important mechanisms featuring the major routes of furan formation are (1) the oxidation of polyunsaturated fatty acids, (2) the thermal degradation of amino acids under thermal conditions, (3) the thermal degradation or rearrangement of carbohydrates such as glucose, fructose and lactose and (4) the ascorbic acid decomposition including its derivatives as precursors (Batool et al., 2021). Furans have traditionally attracted a lot of attention as inhibitory substances in microorganism processes as well as the food industry due to hints on their carcinogenicity and frequent occurrence on thermally driven foods, such as different coffees, baby foods, cereals and meat products. In fact, the International Agency for Research on Cancer (IARC), US Department of Health and Human Services and National Toxicology Program (NTP) had classified furan as a possible carcinogen (Class 2B) (IARC 1995); (NTP 1993) (Batool et al., 2021). However, the main risks associated with furan consumption still remain unclear.

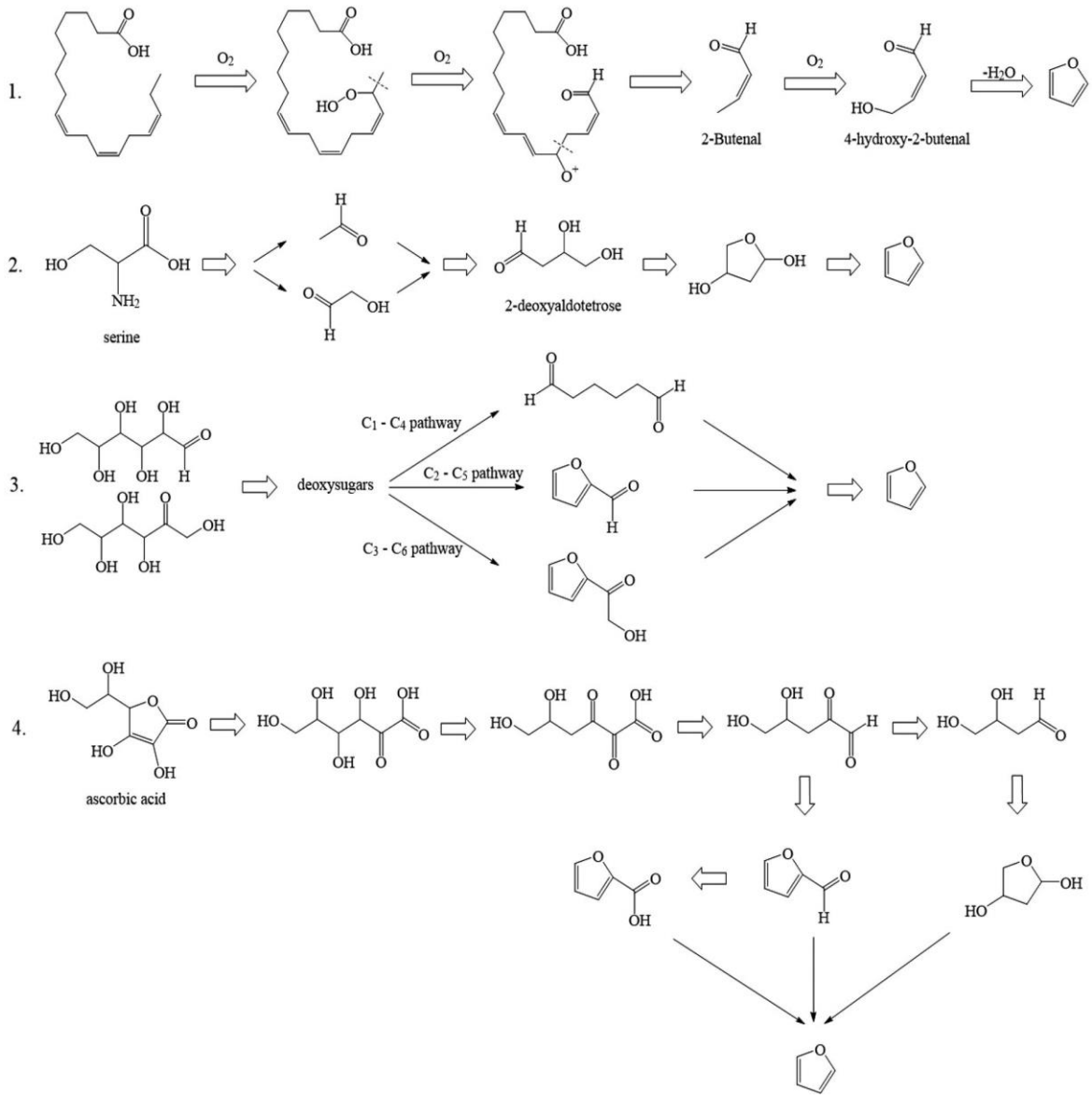


Figure 1.11: The four main mechanisms for the formation of furans (1) describes the pathway from polyunsaturated fatty acids (2) describes the pathway from serine amino acid (3) describes the pathway from hexose sugars (4) describes the pathway from ascorbic acid precursors (Batool et al., 2021)

On the other hand, furans have recently received increased attention in another field. Interest in polymers from renewable resources is growing vigorously as part of the general concern for sustainability, which also includes the more pressing problem of alternative energy sources. The chemistry involved in the synthesis of these biomass-based macromolecules is much more frequently associated with the modification of natural polymers (cellulose, starch, chitin, etc.) or oligomers (lignin, tannins, vegetable oils, etc.) rather than with the more classical fashion involving monomers and their polymerization. Within the latter strategy, the materials obtained

from monomers like terpenes, lactic acid and sugars bear intrinsically the structure imprint of their precursors and hence a specific set of related properties. The situation is qualitatively different with polymers prepared with furan monomers or through furan chemistry, because the approach resembles the petrochemical counterpart in the sense that it provides entry to a whole host of monomers and hence a variety of macromolecular structures with different properties. In this peculiar version of a biorefinery, two first- generation furan derivatives, FA and HMF, readily prepared from ubiquitous C5 and C6 carbohydrate resources, respectively, represent the precursors to that rich array of monomer structures, suitable for any type of polymerization process. Schemes 1–2 illustrate this point through a choice of monomers which have been synthesised and polymerized (Belgacem, 2008; Gandini, 2010). In addition to the straightforward research activities consisting of preparing these monomers, studying their behaviour in polymerization and copolymerisation systems and assessing the properties and possible applications of the ensuing materials, another domain of investigation calls upon some furan-specific chemical features, which enable original polymers to be prepared. These include end-functionalized macromolecules, block and graft copolymers and, more originally, the exploitation of the marked dienic character of the furan heterocycle through the application of the Diels–Alder (DA) reaction, to prepare thermos-reversible polymer architectures and hence, readily recyclable and mendable materials (Gandini & Belgacem, 2007).

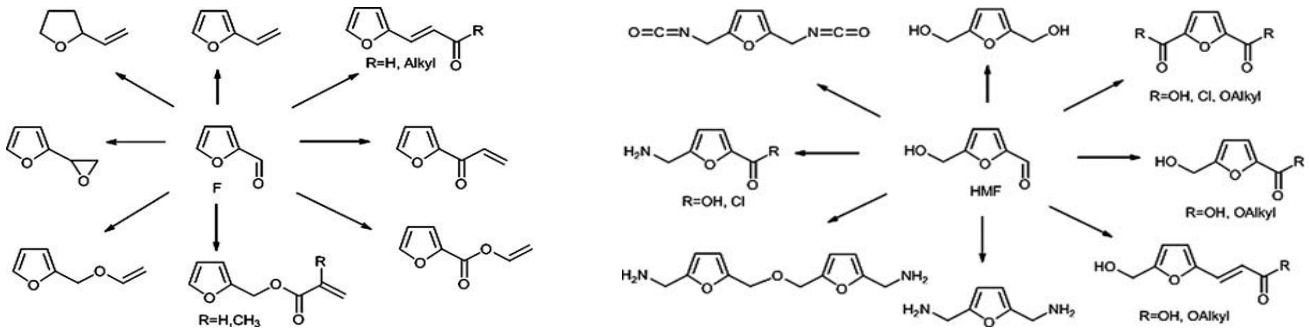


Figure 1.12: A selection of monomers derived from FA (Scheme 1) and HMF (Scheme 2) (Gandini, 2010)

Despite the well-established acid-based technologies which produce some 300 000 tons of FA yearly, mostly from the pentoses of corncobs and sugarcane bagasse, recent interest in novel systems associated with the chemical exploitation of biomass, has revived studies on its preparation. These include the use of zeolites, and of conventional Brønsted acids in an ionic liquid. The study of FA as a monomer or comonomer in the elaboration of often ill-defined ‘‘furan resins’’ has not been a particularly stimulating issue, and little contribution has enriched this topic. Within the same context, the recent surge of investigations related to the synthesis of HMF from

biomass-derived mono- and polysaccharides is one of the characteristic indicators of the growing impact of research on renewable resources. These investigations include mostly the use of *D*-fructose, but also inulin and glucose, as the precursors, and a broad selection of novel catalysts, reaction media, and/or process conditions. In the space of a few years this issue has thus been advanced so considerably that it brought HMF to the verge of becoming an industrial commodity. Given, however, that it is difficult and impractical to store HMF because of its proneness to degradation even under relatively mild surroundings, its in *situ* transformation into a stable derivative seems most appropriate. The chemistry of HMF is well documented and two obvious pathways are likely to be privileged in the search of that viable derivative, the oxidation to the corresponding dialdehyde or diacid. Indeed, together with the recent interest in preparing HMF through novel and more efficient procedures, studies examine its oxidation to 2,5-diformylfuran and 2,5-furandicarboxylic acid (FDCA) or its esters, which are all highly stable molecules and very valuable potential monomers functioning as feedstocks, as it can be concluded from their increasing number of publications. The furan ring possesses unique traits, like its dienic character, that make it susceptible to Diers-Alder reactions for the polymerization with other dienophiles. Moreover, furanic compounds like FDCA are considered viable green alternatives to benzyl derived monomers. According to the US Department of Energy, FDCA is placed among the top 12 high added value chemicals and is considered a promising platform chemical with a wide range of applications (Werpy & Petersen, 2004). Its structural similarity to terephthalic acid constitutes it as a candidate for synthesis of polymers such as polyethylene furanoate (PEF), a green alternative to poly(ethylene terephthalate) (PET). The annual market need of PET is estimated around 50 million tons consequently offering the opportunity to shift polymer synthesis from fossil fuels to biobased FDCA (Sousa et al., 2015). Nevertheless, the applications of FDCA extend to other polyesters like polyamines and polyurethanes (Moreau et al., 2004). Apart from FDCA, furancarboxylic acid (FCA), which is derived from FA and the valorization of pentoses, also shows great promise as it can create dimers that resemble bisphenol series through a condensation reaction with aldehydes and ketones, introducing new properties to polyesters (Delidovich et al., 2016).

To date, lignocellulose valorization is achieved through chemical catalysis, which includes the use of costly metal catalysts or environmentally detrimental solvents (Delidovich et al., 2016; Jadhav et al., 2012; Ståhlberg et al., 2010). Hence, over the past few years there is an attempt to alter the valorization process and to include biocatalysts, which naturally demand mild environmental conditions and low cost. In this context, FDCA and FCA have received attention as substrates for polymer synthesis from biomass. However, the use of highly selective catalysts infers a cascade of chemical reactions, with a wide range of substrates, each with their own properties and advantages towards the final product (Yuan et al., 2020a). The production of FDCA requires the use of enzymes such as oxidases and peroxygenases, while the use of transaminases leads to the

production of amines. Setting up such a process would offer a green and sustainable approach to the replacement of petrochemically produced polymers.

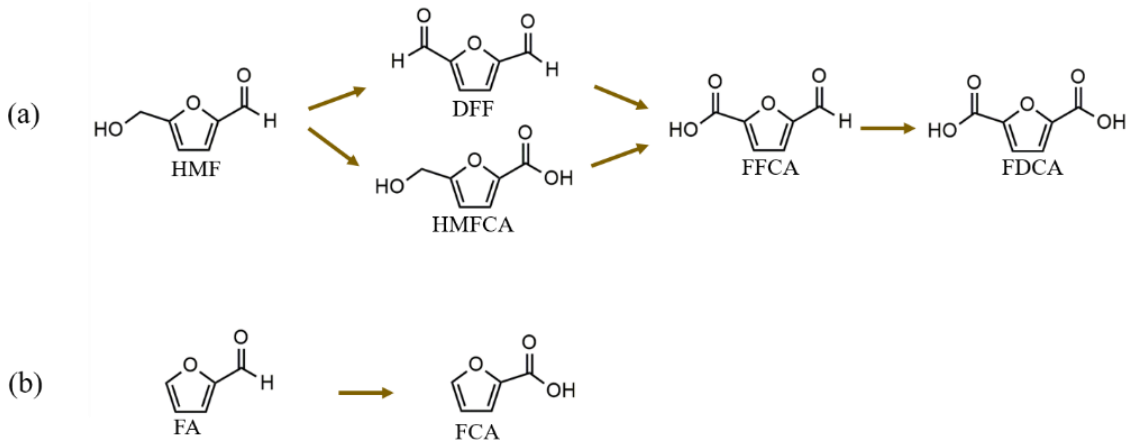


Figure 1.13: Schematic representation of oxidation of furan-derivatives that have been reported in the literature (a) HMF to FDCA, (b) FA to FCA. HMF: 5-hydroxymethylfurfural, DFF: 2,5-diformylfuran, HMFCA: 2,5-hydroxymethylfuran carboxylic acid, FFCA: 2,5-formylfurancarboxylic acid, FDCA: 2,5-furandicarboxylic acid, FA: furfural, 2-furaldehyde, FCA: 2-furancarboxylic acid or furoic acid.

1.11 Enzymatic conversion of furans to value-added monomers

Furans have been in the spotlight of biotechnological research either as inhibitors in processes such as microbial fermentation or, more recently, as starting materials in chemical synthesis of different valuable monomers. Throughout the years many studies report the use of microorganisms utilized to metabolize furans. However, the mechanisms of this metabolism are complex and due to the number of different enzymes secreted, the final product is not highly selective. Furthermore, when working with microbial cultures, there are additional challenges related to the organism's preferred pH and temperature conditions, as well as the downstream processes involved in obtaining the final product. Due to these factors, there was a demand for catalysts that could selectively convert furans, while being easily controlled and removed from the reaction. It was also desirable for these catalysts to operate under mild conditions. As a result, in recent years, significant efforts have been made to identify enzymes capable of catalyzing the conversion of furans. One of the most prominent fields of furan biotransformation is the oxidation. In this field, furans, and mainly HMF and FA are the substrates to a cascade of oxidative reactions, using the enzyme as a catalyst. These reactions, as their name suggest, require the use of oxidative enzymes, such as alcohol oxidases, galactose oxidases, glyoxal oxidases and peroxidases. Other types of catalytic pathways involve the use of transaminases for the reductive amination of furans and dehydrogenases to produce furan alcohols.

1.11.1 Oxidative reactions for HMF

The most interesting representative that can be derived from furans is FDCA, which is a substitute for the petrochemically synthesized terephthalic acid. That way, FDCA can be used to produce PEF as a substitute for PET. FDCA can be produced from HMF by following two routes. In the first route, HMF is converted to 5-hydroxymethyl-2-furancarboxylic acid (HMFCA), while in the second route HMF is converted to furan-2,5-dicarbaldehyde (DFF). Both routes transform each respective chemical to 2,5-formylfurancarboxylic acid (FFCA) which can, in turn, finally lead to FDCA. In order to perform the above reactions, there is a need for catalysts that can perform oxidation in alcohols and aldehydes. Due to the promiscuity in the mode of action of these oxidative enzymes, different activities have been found to participate in the cascade of furan oxidation, typically involving oxidases, such as alcohol oxidases, peroxygenases, laccases, lipases, galactose oxidases and a HMF oxidase as well as catalases and peroxygenases.

In 2013, Krystof et al. reported the use of a one pot system of 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO)-lipase for the conversion of HMF to FDCA. While this system includes a chemical catalyst (TEMPO) for the oxidation of HMF to DFF, it also includes the use of a lipase as a biocatalyst for further oxidation to FDCA. In 2015, Carro et al. tried to produce FDCA by means of a fully enzymatic cascade reaction. In their experiments they used an aryl-alcohol oxidase with the aim of performing all the oxidative steps required. However, while AAO successfully catalyzed the production of FFCA in high yields, further oxidation was inhibited from the hydrogen peroxide produced in the reaction (H_2O_2). For that reason, they performed the same reaction by adding an unidentified peroxygenase (UPO) after the effect of AAO in the reaction, eventually producing FDCA in a yield of 91%. Similarly, Serrano et al. (2019) also investigated the effect of an AAO from *Pleurotus eryngii* on HMF for FDCA production. While they proved that the AAO is able to efficiently oxidize HMF to FFCA, further oxidation to FDCA was inhibited. However, by using FFCA as an initial substrate to the AAO, the enzyme managed to transform the substrate to FDCA. Then, they also confirmed that H_2O_2 has an inhibitory effect on the second oxidation and for that reason they also added a catalase in the reaction, which led to a complete transformation of HMF to FDCA after 12 days.

All the above, indicate the ability of the aryl-alcohol enzymes from the AA3 CAZy family (EC 1.1.3.7) to perform the oxidation in HMF. However, due to the inability of the enzymes to completely produce FDCA, some reports examined the possibility of performing all the oxidation reactions with a single biocatalyst, instead of a cascade. Based on the oxidative ability of other enzymes of the AA3 family, such as glucose oxidases (EC 1.1.3.4), alcohol oxidases (EC 1.1.3.13) and pyranose oxidases (EC 1.1.3.10), Dijkman et al. (2014) characterized an HmfH (Koopman et al., 2010) on a genetic level. Following this, due to the fact that they never managed to obtain the enzyme by means of expression, they sought for homolog enzymes and were led to an HMF oxidase (HMFO) from *Methylovorus sp*, strain MP688. The HMFO again produced FFCA in high amounts, however the latter's low degree of hydration did not permit its further hydration to HMF.

For that reason, by solving the crystal structure and performing a double mutation, they managed to achieve a 1000-fold increase in the catalytic efficiency of the enzyme on FFCA, ultimately producing FDCA in higher yields. On the other hand, driven by this study Viña-Gonzalez et al. (2020) tried to follow a similar mutational pattern on the amino sequence of an AAO in order to render it able to perform all the three oxidation steps required for FDCA production. Indeed, while the wild type AAO initially strongly impeded the linkage of FFCA in the catalytic site of the enzyme, by introducing a Trp residue to a mutant carrying a H91N mutation that resembles the active site and structure of the HMFO, they managed to achieve a 6-fold increase in the production of FDCA from HMF. While the actual FDCA yield is relatively low (3%) the authors mention that this is the first time this enzyme achieved complete FDCA production, rending it a potential future biocatalyst for furan valorization.

1.11.2 The recently revisited AA5 family

The members of the broad AA5 family of CAZy database (http://www.cazy.org/AA5.html) have recently attracted significant interest regarding their potential in catalyzing oxidative modifications of furan derivatives. This group encompasses oxidases that require oxygen as a receptor (EC 1.1.3.-), galactose oxidases (GAOs) (EC 1.1.3.9), glyoxal oxidases (GlOs) (EC 1.2.3.15), alcohol oxidases (EC 1.1.3.13) and raffinose oxidases (EC 1.1.3.-). Among other oxidative enzymes, galactose oxidases and glyoxal oxidases have also been reported able to catalyze the conversion of furans similar to the aforementioned examples of the AAOs and HMFO. Over the last years, there is an increasing number of studies examining the potential of these enzymes in cascade processes on HMF conversion.

The first report of an AA5 enzyme acting on HMF was from the group of McKenna et al. (2015), who examined the ability of a custom developed variant of a GAO from *Fusarium graminearum* concerning FDCA synthesis. Again, GAO was able to convert HMF to DFF but not further oxidize it to FDCA. For that reason, they utilized an *E. coli* periplasmic aldehyde oxidase (PaoABC) that was found to be active on HMF, DFF and FFCA to see if the conversion to FDCA was possible. The advantage of using PaoABC in the process was that it does not require the hydrate form of FFCA, in contrast to the other catalysts used in the process. Indeed, by adding both GAO and PaoABC to HMF they observed such a conversion, although incomplete due to the fact that PaoABC also produced HMFCA from HMF, which is a poor substrate for GAO. Hence, on a following experiment they allowed GAO to completely oxidize HMF to DFF before adding PaoABC to the system, accomplishing to achieve complete transformation to FDCA. They also mention the addition of catalase in the system to relieve the oxidative enzymes of the H_2O_2 produced throughout the process and provide the O_2 needed. Following this study, they tried to design a one-pot process of the above reaction (McKenna et al., 2017). For that reason, they added a horseradish peroxidase (HRP) in the HMF-GAO-PaoABC-catalase system, that was found to

significantly boost the activity of GAO towards DFF production. This way, the HMFCA production was minimal and FDCA was produced in a 100% yield.

In 2015 Qin et al. (2015) examined the oxidation of HMF using an array of enzymes. Firstly, they used a xanthine oxidase from *E. coli* producing HMFCA at a yield of 94% after 7 days, without progressing in further oxidation steps. Secondly, they used three laccases to monitor the production of FFCA using TEMPO as a mediator. The best performing enzyme achieved an 82% yield, while small yields of 4% and 10% were also observed for DFF and FDCA respectively. Finally, they used an enzyme cascade of GAO, HRP and catalase to produce DFF from HMF. Then, they used ethyl acetate to extract the HMF and DFF from the system. The final step included the use of a lipase B (CAL-B) to convert DFF to FDCA, whose yield was 88%.

Recently, Karich et al. (2018) tried to design an enzyme cocktail to benefit from both the routes of the FDCA synthesis process, that included a UPO, a GAO and an AAO. In specific, they support that GAO's and AAO's role was to oxidize the HMF towards HMFCA and DFF respectively while producing H_2O_2 in the process and UPO's role was double: as oxidative factor to HMF and its oxidized derivatives and to relieve the H_2O_2 that was produced throughout the reaction. With this experiment they observed that the production of FFCA was rapid while the step from FFCA to FDCA was the most time consuming. However, after 24 h they observed an 80% FDCA yield in the reaction, while HMF was almost entirely converted to its oxidized derivatives.

Daou et al. (2019) examined the HMF oxidation of three GlOs from the *Pycnoporus cinnabarus* with high protein sequence identity. Even though none of these enzymes exhibited HMF conversion over 40%, they found out that for PciGlO2 and PciGlO3, the activity doubled with the addition of catalase. The main oxidized product of using exclusively GlOs on HMF was HMFCA. In order to bypass the inability to further oxidise HMFCA, they utilized an AAO in a series of reactions in combination with the GlOs. Eventually, after leaving AAO to oxidize HMF to FFCA, they added GlO to the reaction in presence of catalase, producing small amounts of FDCA.

Mathieu et al. (2020) recently discovered a radical AAO oxidase from *Colletotrichum graminicola* that is able to efficiently catalyze the oxidation of aryl alcohols but demonstrates weak activity towards carbohydrates. As such, they support the idea that this AAO (EC 1.1.3.7) is the first member of the AA5 family, whose activity is typically attributed to the flavin dependent glucose-methanol-choline (GMC) oxidoreductase family AA3. In their work, they also verified the ability of the AAO to oxidize HMF significantly better than the aforementioned biocatalysts. For that reason, they initially decided to characterize the biocatalyst as an HMF oxidase but decided to do otherwise due to its activity towards aryl alcohols. In addition, they found out that the enzyme can efficiently completely oxidize HMF towards DFF to which it cleanly stops, but can only partially convert HMFCA in FFCA, with a yield of 54%.

While most enzymes require additional factors in order to perform the oxidation reaction, such as (NADPH/$NADP^+$)-dependent oxidases of flavin-dependent enzymes, this does not seem to be the case for the AA5 family enzymes. Most members of this family are copper dependent, easy to produce and purify enzymes that can function under no other requirement to perform the oxidation (Dong et al., 2018). This is the reason why these enzymes can contribute significantly to the furan valorization procedure. However, in any case it is evident that a cascade of enzymes is required in order to either reach the final steps of the subsequent oxidations or achieve high yields in the respective step. In most cases, the use of catalysts, such as catalases or peroxygenases, is required in order to alleviate the system of the H_2O_2 produced from the oxidation, especially in the case of the AA5 family. Nevertheless, it is a promising observation that through protein engineering, proper mutants of a single enzyme can perform the entire oxidative pathway, even at small yields. As a result, further investigation can potentially transfer research from the current whole cell stage to a single enzyme stage, significantly boosting the valorization process.

1.11.3 Biocatalytic routes for furfural

Similarly to HMF, FA also displays a potential for oxidative conversion. The corresponding oxidative pathway is separated again into two routes. The first route leads to the production of 2-furoic acid, while the second route leads to the production of furfuryl alcohol. Nevertheless, while the products of the oxidative pathways for FDCA can be intertwined, this does not seem to be the same for FA, where each conversion seems to be more distinct. Early reports mention this process as a biodetoxification process in fermentation, therefore utilizing whole cell catalysis for the reduction of FA (Pérez et al., 2009; Ran et al., 2014; Viña-Gonzalez et al., 2020). However, further studies have been made to identify the specific enzymes involved in FA degradation.

The first report on the properties of an enzyme that catalyzes the conversion of FA was made from Gutiérrez et al. (2006), when they purified a FA reductase (FAR) from an *E. coli* strain, utilizing it to reduce FA to furfuryl alcohol. In 2011, Li et al. efficiently managed to extract and purify a FA reductase (FurX) from *Cupriavidus necator*, belonging in the Zn-dependent alcohol dehydrogenase family, producing furfuryl alcohol from FA. They also (Wang et al., 2014), sought to find homologs for the aforementioned reductase coming up with three alcohol dehydrogenases from yeast (YADH1), *E. coli* (EcADH) and *Pseudomonas aeruginosa* (PaADH). Testing them against the results from FurX, he verified the acitivity of alcohol dehydrogenases on FA to produce furfuryl alcohol. In their work, Krystof et al. (Krystof et al., 2013), mentioned that the TEMPO-lipase system they developed for HMF transformation is also able to effectively oxidize FA to furoic acid. Following this study, Kumar & Fraaije (2017) tested the ability of 15 Bayer-Villinger monoxygenases (BVMOs) to oxidize FA. While they verified that all 15 monooxygenases exhibited activity in FA, they decided to further examine a phenylacetone monooxygenase (PAMO). They then found that PAMO was able to convert FA in a 60% yield after 12 h with furoic

acid being the main product. They also mention the generation of a small amount of another byproduct that they identify as the formyl ester created from the BV oxidation reaction. They then support that this ester can be transformed into the corresponding alcohol and formic acid.

All the above verify the possibility of effectively valorizing FA towards the production of its oxidized derivatives as well. Formed from the hemicellulose sugars of lignocellulose, it is understandable that FA valorization is also a key towards lignocellulose biorefineries integration. For that reason, the promiscuity of utilizing oxidative enzymes on FA as well can potentially contribute towards that goal.

1.11.4 Other enzymatic activities and future perspectives

Another interesting group of compounds that can be produced from furans includes furfurylamines, that can be generated by transaminases. Transaminases (EC 2.6.1.18) are highly attractive versatile enzymes that catalyze the transformation of aldehydes and ketones towards the synthesis of chiral amines. These biocatalysts can provide a sustainable high yield, selective route to amines under mild aqueous conditions, by transfer of an amino group from a donor substrate to an acceptor compound. They have been used for the amination of FA and derivatives to access furfurylamines (Blume et al., 2015), while the high potential of the utilization of this enzyme family in industrial biotransformation processes is demonstrated by Merck's biosynthesis of the antidiabetic drug Sitagliptin (Neto et al., 2015). Reductive amination of furans is possible both for HMF and FA as starting materials. Both aldehyde groups of HMF or FA can be substituted with primary amines to generate amides with the use of transaminases. Transaminases have been reported to hold a primary role in one step biotransformation of furans towards the production of furfurylamines with multiple applications although research on this type of reaction is still limited.

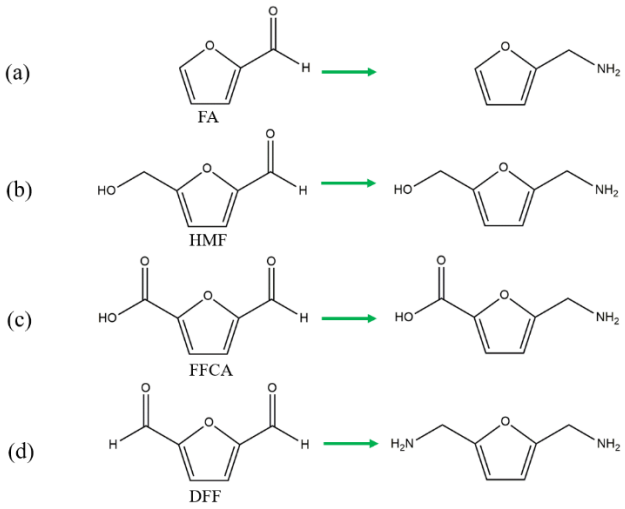


Figure 1.14: Schematic representation of furan-derivatives that have been reported in the literature to be transformed by aminotransferases to their corresponding amines. (a) HMF to HMFA, (b) FA to FFA, (c) FFCA and (d) DFF to monoamine and diamine respectively.

Furan alcohols, such as furfuryl alcohol (2-hydroxymethyl furan, FOL) and 2,5-bis(hydroxymethyl)furan (BHMF) that are originating for furans, namely FA and HMF respectively (Figure 1.14), are used as starting materials in synthesis of biopolymers (Oberleitner et al., 2014). BHMF is a versatile building block in the synthesis of polymers, fuels, and macrocycle polyethers; this molecule is converted to 5,5′-dihydroxymethyl furoin (DHMF) which undergoes through enzymatic polycodensation reactions catalyzed by lipase towards aliphatic–aromatic oligoesters (Kaswurm et al., 2013). The reduction of the keto-group of furans into an alcohol can be catalyzed through the use of alcohol dehydrogenases (ketoreductases, EC 1.1.1.1) (Domínguez de María & Guajardo, 2017; Yan et al., 2018) or whole cells and their array of oxidative enzymes (Gutiérrez et al., 2006; Jiang et al., 2016; Laadan et al., 2008; Li et al., 2011a; Li et al., 2017).

Various furan-based monomers discussed previously can be used as starting materials to create different compounds with unique chemical properties and diverse applications. These synthetic pathways involve multiple steps and incorporate both chemical catalysis and enzymatic reactions. Biocatalysts play a crucial role in these transformations, offering high selectivity under mild reaction conditions. For instance, lipases and proteases have been shown to produce a wide range of polyamides and polyesters, as explored in a review by (Lambert & Wagner, 2017), thereby enabling the utilization of furan through enzymes. Epoxides, which are versatile intermediate compounds, can undergo various reactions like nucleophilic substitution and hydrolysis, resulting in the production of a range of products (Gandini, 2011; Marotta et al., 2018). Several scientific

research groups have studied the chemistry of epoxidation reactions and emphasized the significance of the ring-opening process in synthetic reactions. Furanic compounds containing epoxide groups can be utilized in the bonding of polycarbonate via cationic photo-curing, and compounds with a phenyl ring can be used to prepare adhesives due to their rigid structure and hydrophobic nature. Furanic diglycidyl esters derived from FDCA offer a sustainable alternative to their petrochemical aromatic counterparts (Peterson et al., 2005). Enzymes have not been extensively studied for the addition of epoxide groups, although chemical methods have been reported. Furan ring epoxidation can result in rearrangement and ring opening, leading to activated monomers and new possibilities for synthesis. It has been observed that furan can be oxidized by cytochrome P450 enzymes to produce the activated monomer metabolite cis-2-butene-1,4-dial (Thibodeaux et al., 2012). P450 epoxidases contain a heme coenzyme that enables the addition of oxygen atoms to alkene double bonds, generating reactive electrophiles (Peter Guengerich, 2003; Peterson, 2013). The oxidation of furan by P450 can lead to the formation of either an epoxide-1 or a cis-enedione intermediate (Wang et al., 2017). Peroxygenases, a class of enzymes that catalyze oxyfunctionalization reactions, have also gained attention for their versatility and simplicity, although they exhibit lower selectivity compared to P450 monooxygenases (Alonso-Fagúndez et al., 2014). When enzymes such as peroxygenases or P450 cytochrome monooxygenases are added, furan derivatives can undergo epoxidation, followed by epoxide ring opening to form reactive epoxide metabolites. These metabolites can then undergo rearrangement to produce tricarbonyl metabolites, potentially leading to the synthesis of maleic acid or other compounds from furans. The synthesis of maleic acid through a FA epoxide monomer has previously been reported. To achieve the biotransformation and enrichment of targeted bio-bricks, as well as the functionalization of activated monomers, highly selective oxidative biocatalysts with high specific activities are necessary. By expanding the use of enzymes and discovering novel enzyme activities or designing catalysts with specific properties, innovative processes can be developed to produce bio-based monomers and reactive compounds.

Purpose of this thesis

This work focuses on the development of an integrated process to transform real lignocellulosic biomass samples to furan monomers in an effective and green way. To that end, the utilization of hardwood and softwood biomass has been the central core of the thesis. To begin with, beechwood residues have been pretreated through an oxidative organosolv pretreatment process using a multitude of different organic solvents, temperatures and residence times. The resulting pulps have been tested on their amenability to enzymatic saccharification and potential to produce fructose through isomerization. The setup of the optimal conditions of saccharification and isomerization experiments was performed on pure commercial cellulose. Following these experiments, the best performing hydrolysates were subjected to chemical hydrolysis to examine HMF production.

Additionally, a similar oxidative organosolv process was applied to beechwood and pine residues, however, with the addition of polyoxometalate catalysts (POMs). The three different POMs used were a commercial phosphomolybdic acid hydrate ($H_3PMo_{12}O_{40}$) and two custom-made alternates, where H_2 was replaced with Fe and Cu ions. Similarly, the best performing hydrolysates were also subjected to chemical dehydration for HMF production. Following these experiments, corn stover biomass was used to examine acetic acid catalyzed organosolv pretreatment using microwaves as a heating source. The input on the efficiency of the pretreatment was acetic acid concentration and residence time, while the output was enzymatic digestibility and fructose yield. Finally, the last part of the study was the discovery of a galactose oxidase from *Fusarium oxysporum* for the examination of its potential to oxidize HMF and its derivatives. The enzyme was produced by means of heterologous expression, purified and tested on galactose for the optimum conditions. Then, it was subjected to HMF oxidation reactions with the aim to produce FDCA as the result of the cascade of the reactions.

MATERIALS AND METHODS

2. Materials and Methods

2.1 Lignocellulosic biomass

The lignocellulosic biomass samples that were used in this thesis originated from corn stover and beechwood feedstocks. Corn stover biomass was provided by the Department of Agriculture, Crop Production and Rural Environment, University of Thessaly. Post collection, the biomass was subjected to milling using a laboratory scale mill so as to pass through a 0.5 mm screen and then stored at room temperature. Beechwood sawdust biomass samples were samples of the commercialy available Lignocel® (HBS 150-500) with particle size 150-500 μm d and were used as a representative of hardwood species. Southern yellow pine with the same particle size was used as representative of softwood species.

2.2 Microorganisms

The *Escherichia coli* TOP10 strain (Invitrogen, Carlsbad, CA, USA) was used for the multiplication and isolation of recombinant plasmid vectors.

The single cell methylotrophic yeast X33 *Pichia pastoris* strain (genotype: wildtype, phenotype: MutS) (Invitrogen, Carlsbad, CA, USA) was used to produce recombinant enzymes. *P. pastoris* constitutes an eukaryotic heterologous expression system with many advantages of higher expression systems such as post-translational modifications (formation of disulfide bonds, protein glycosylation). As a system it is easy to use, with high growth rate when cultivated at low-cost medium. Its ability to grow even in the presence of methanol as a sole carbon source is what differentiates it from other eukaryotes. In specific, the alcohol oxidase enzyme, expressed from two genes with high homology (97%), *AOX1* and *AOX2*, catabolizes methanol to formaldehyde in the presence of oxygen. Higher enzyme yields are achieved by *AOX1*, contrary to the *AOX2* that leads to lower yields. Strains in which both genes are expressed can grow in high methanol concentrations (phenotype MutS, methanol utilization plus), while strains where only *AOX2* is expressed can grow in low methanol concentrations (phenotype Mut^+, methanol utilization slow). Finally, this yeast facilitates the heterologous expression of proteins in high concentrations due to the low expression of endogenous proteins.

2.3 Enzymes and reagents

The commercialy available EasySelect™ *Pichia* Expression kit as well as the pPICZaC plasmid vector were supplied from Invitrogen (Waltham, MA, USA), while the restriction enzymes *Cla*I, *Xba*I, and *Sac*I from Takara (Kusatsu, Japan,). Pure cellulose Avicel was supplied from Macherey Nagel (Germany). The enzyme cocktails Cellic® Ctec 2 (cellulase) και Sweetzyme® (glucose

isomerase) were supplied from Novozymes (Bagsværd, Denmark). Cellic® Ctec 2 had a concentration of 140 mg/mL and an activity of 168 FPU/mL and the immobilized glucose isomerase Sweetzyme® had an activity of 150 U/g.

2.4 Laboratory equipment

The laboratory equipment that was used for the experiments of this thesis is listed below:

- Labo Autoclave (Sanyo, Osaka, Japan)
- ZHWY-211C Incubator (Zhicheng, Shanghai, China)
- Orbital uncubator S150 (Stuart, Scientific, Staffordshire, UK)
- Thermomixer Comfort with installed stirring (Eppendorf, Hamburg, Germany)
- Waterbaths
- Econo Gradient peristaltic pump (Bio-rad, Hercules, CA, USA)
- Waters SF-2120 fraction collector (Millipore, USA)
- Orbit LS stirring device (Labnet, Berkshire, UK)
- Vacuum Filtration Pump (PALL, Gelman laboratories, USA)
- Christ ALPHA 1-4 freeze drying device (B. Braun Biotech International, Melsungen, Germany)
- WTW 537 pH electrode and detection device, WTW (Germany)
- Amicon Stirred Cell Model 8400 ultrafiltration device (Millipore, USA)
- Ingenious Bio Imaging depiction device (Syngene, Cambridge, UK)
- Ultrapure water production devices Direct-Q (Merck Millipore, Billerica, MA, USA) and Labaqua (Biosan, Latvia)
- High Performance Liquid Chromatography (HPLC) System with Refractive Index Detector (Shimadzu, Japan)
- Beckman® Model TJ-6 Centrifuge (Brea, CA, USA)
- UV-Vis S-22 photometer (Boeco, Hamburg, Germany)
- Mini-PROTEAN™ Tetra SDS-PAGE system (Bio-rad, CA, USA)
- Consort E863 electrophoresis power supply (Consort bvba, Belgium)
- MicroPulser™ Elelctroporator (Bio-rad. CA, USA)

2.5 Sugar Detection

The concentration of total reducing sugars in solutions was detected using the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959). The detection is achieved spectrophotometrically through the oxidation of the aldehyde functional groups of reducing sugars to carboxylic acids accompanying the colored reaction with the DNS reagent. Glucose detection was accomplished using the commercial GOD/PAP method. This method is based on the oxidation of glucose in the presence

of a glucose oxidase that leads to the production of H_2O_2. The detection is achieved through a chromophore compound absorption at 510 nm that is the result of the reaction between a peroxidase, H_2O_2 and chloro-4-phenol.

2.6 Compositional analysis

The compositional analysis of the untreated as well as the pretreated lignocellulosic biomass samples to quantify the cellulose, hemicellulose and lignin fractions was carried out according to the National Renewable Energy Laboratory (NREL) protocol for Structural Carbohydrate and Lignin in Biomass (Sluiter et al., 2004). In summary, the composition is calculated by quantifying each fraction separately as a result of a two-stage acid hydrolysis. Lignin is separated into two subcategories; the acid insoluble Klason lignin that is detected spectrophotometrically in the ultraviolet spectrum, and the acid soluble lignin that is calculated by weight analysis. Hemicellulose and cellulose are broken down into their monomeric sugars that are in a soluble form in the hydrolysis mixture and can be detected by HPLC analysis.

2.7 High Performance Liquid Chromatography

The quantitative analysis of the compositional analysis sugars and the detection of the oxidative derivatives of HMF was performed through High Performance Liquid Chromatography (HPLC). The HPLC equipment includes a solvent dispersion system (Shimadzu, LC-20AD), a refractive index detector (Shimadzu RID 10A), a UV detector (Varian ProStar), an autosampler (Shimadzu, SIL-20A) and a data processing software (LC solution v1.24, SP1, Shimadzu). The column used for the detection of compositional analysis sugars was the Aminex HPX-87P (300x7.8 mm, particle size 9 μm, Bio-Rad, Hercules, CA) equipped with a CarbboPac and a De-Ashing Bio-Rad micro-guard column, while for the detection of furan derivatives the column was the Aminex HPX-87H (300x7.8 mm, particle size 9 μm, Bio-Rad, Hercules, CA) equipped with a Cation-H Bio-Rad micro-guard column. The running setup for sugar detection was the following: analysis time was set to 20 min, column temperature was set to 85 ^oC and the mobile phase was ultrapure water with a flow rate of 0.6 mL/min, while the detection was conducted on the RID detector. Respectively, for the detection of furan derivatives the analysis time was set to 40 min, the column temperature was set to 60 ^oC and the mobile phase was 10 mM H_2SO_4 with a flow rate of 0.8 mL/min, while the detection occurred in the UV detector and the wavelength was 264 nm. In any case, the samples were filtrated through a 0.45 μm syringe membrane filter to remove excess solids.

2.8 Protein Concentration Determination

The quantification of protein in the crude solution of cultures was achieved through the Bradford method (Bradford, 1976). This method is based on the attachment of the Coomassie Brilliant Blue G-250 pigment on proteins. The dye forms a strong, noncovalent complex with the protein's carboxyl group by van der Waals force and amino group through electrostatic interactions. During the formation of this complex, the red form of Coomassie dye first donates its free electron to the ionizable groups on the protein, which causes a disruption of the protein's native state, consequently exposing its hydrophobic pockets. These pockets in the protein's tertiary structure bind non-covalently to the non-polar region of the dye via the first bond interaction (van der Waals forces) which position the positive amine groups in proximity with the negative charge of the dye. The bond is further strengthened by the second bond interaction between the two, the ionic interaction. When the dye binds to the protein, it causes a shift from 465 nm to 595 nm, which is why the absorbance readings are taken at 595 nm. The calibration of the protein concentration was made with bovine serum albumin.

The protein concentration in pure samples following the isolation and purification step was performed spectrophotometrically at a 280 nm wavelength. This method is applicable to proteins containing tryptophan, tyrosine or disulfide bonds that absorb light at 280 nm. The concentration is calculated by using the Lambert-Beer equation:

$$Cp = \frac{A280\ x\ MW}{\varepsilon}$$

where C_p (mg/mL) is the protein concentration, A280 is the absorption, MW is the molecular weight of the protein and ε is extinction coefficient ($M^{-1}cm^{-1}$). The extinction coefficient for each protein was calculated through the ProtParam ExPASY tool.

2.9 Protein Sequence Analysis

The multiple sequence alignment was performed by Clustal Omega (Sievers et al., 2011). The signal peptide prediction was made by SignalP 4.0 (Petersen et al., 2011). Possible Glycosylation sites were predicted using the NetNGlyc (https://services.healthtech.dtu.dk/services/NetNGlyc-1.0/) and NetOGlyc (https://services.healthtech.dtu.dk/services/NetOGlyc-4.0/) online tools (R. Gupta & Brunak, 2002; Steentoft et al., 2013).

2.10 Electrophoresis Methods

2.10.1 Sodium dodecyl sulphate – polyacrylamide gel electrophoresis, SDS-PAGE

The sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE) is a method of separating protein molecules according to their size and is used to calculate the molecular weight of protein samples. Polyacrylamide is a neutral chemical compound, with a wide range of pH and temperature stability that is the result of the polymerization of acrylamide through the cross-linking of bis-acrylamide units. Polymerization is achieved by the presence of a chemical catalyst, namely ammonium persulfate (APS) as well as tetramethylethylenediamine (TEMED). The pore size of the gel is defined by the respective protein size.

The SDS causes the deconstruction of proteins after heating, as it disrupts secondary linkages and binds to the polypeptides. As a result, the polypeptide is neutrally charged and its mobility inside the gel is a result of it molecular weight exclusively.

The SDS-PAGE is a multi-buffer system between two gels stacked one over the other. The three buffers are the running buffer, the stacking gel buffer and the resolving gel buffer. The samples are loaded in small wells formed in the stacking gel and by applying voltage in the running buffer, the protein samples slowly pass through the resolving gel where they are later colored and depicted. Electrophoresis took place in a 12.5% w/w acrylamide gel, where 50 V were initially applied for 30 min followed by a voltage of 100 V for 1 h. Samples were prepared by mixing 20 μL of the protein sample with 4 μL loading buffer. Loading buffer recipe was Tris-HCl buffer 0.2 M, DTT 0.4 M, SDS 277 mM 8.0% (w/v), bromophenol blue 6 mM and glycerol 4.3 M.

Table 2.1: Composition of the stacking and the resolving gels for the SDS-PAGE

Component	Resolving Gel	Stacking Gel
dH_2O	1.2 mL	0.75 mL
0,75 mM Tris/SDS 0.2% (pH 8.8)	2.4 mL	-
0.25 mM Tris/SDS 0.2% (pH 6.8)	-	0.95 mL
40% bis-acrylamide	1.18 mL	0.19 mL
TEMED	5 μL	3.75 μL
10% APS	25 μL	12.5 μL

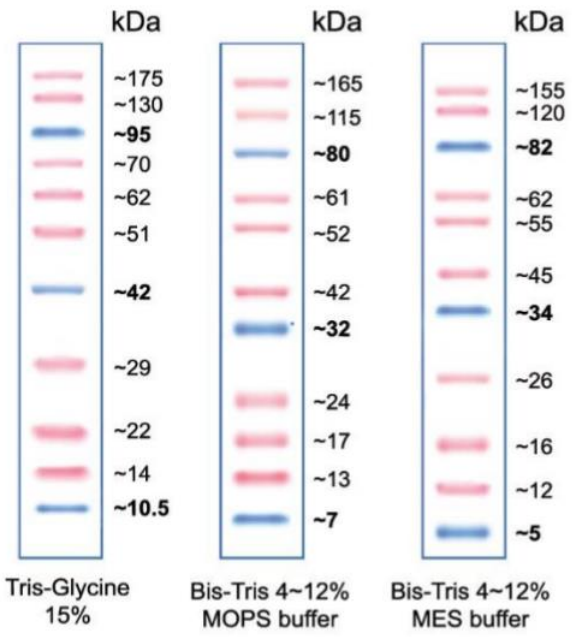


Figure 2.1: The molecular weights of the protein samples of the ladder used to calculate protein size.

2.10.2 Coloring Proteins in polyacrylamide gels

Protein samples in polyacrylamide gels were colored with the Coomassie Brilliant Blue G-250 (CBB) compound. CBB coloring is based on the interactions between the acidic and alkaline amino acids of protein and the organic units of the coloring compound. The acrylamide gel was submerged in a staining solution for 1 h and then transferred to a destaining solution for 2 h that was replaced with new one every 30 min. During the staining and destaining process the gel was constantly stirred at room temperature. The staining solution was a mixture of 40% (v/v) methanol, 10% (v/v) acetic acid, 50% (v/v) H_2O and 0.4% (v/v) CBB, while the destaining solution was a mixture of 20% (v/v) methanol, 10% (v/v) acetic acid, 70% (v/v) H_2O.

2.10.3 Agarose gel electrophoresis

Agarose gel electrophoresis is the most efficient method for nucleic acid separation. The DNA samples are loaded in specific wells formed in the gel and then are separated by applying voltage. The negative charge of DNA samples, due to their phosphate units, caused them to move towards the positive electrode inside the electric field. The progression of the DNA samples is dependent on a number of different parameters, namely: the size of the DNA, the concentration of agarose, the structure of the DNA, the voltage applied, the presence of ethidium bromide, the type of agarose and the electrophoresis buffer. After the electrophoresis, DNA samples are colored and depicted by applying UV light.

The electrophoresis gel was made by mixing 0.5 g agarose (AppliChem, Barcelona, Spain) in 50 mL TBE buffer (Tris/Borate/EDTA) at high temperatures to solubilize agarose. Electrophoresis took place at 50 V for 45 min. Afterwards 4 μL of Midori Green Advance nucleic acid stain was added in order to render the DNA samples visible to UV light. DNA samples were mixed with loading buffer before their introduction inside the gel to enable them to submerge inside the buffer due to higher density. The DNA FastGene DNA Ladder 1 kb (Nippon Genetics Europe, Dűren, Germany) was used to calculate DNA length and the DNA samples were depicted using the InGenius Bio Imaging device (Syngene, Cambridge, UK).

2.11 Lignocellulosic Biomass Pretreatment

2.11.1 Organosolv Microwave Pretreatment

The microwave assisted organosolv pretreatment of biomass took place in the Anton Paar Monowave 450 reactor (Anton Paar GmbH, Austria) of the Chemical Process Engineering Laboratory of the School of Chemical Engineering NTUA, under Professor G. Stefanidis. As depicted in figure 2.2, the instrument is comprised of the oven with a chamber for microwave emission (a) and a small glass bottle where the pretreatment takes place (b), equipped with a septum cap, for pressure measurement due to deformation, and a small glass chamber where an external thermometer is introduced. Temperature can also be measured by infrared detection, although less precisely. The whole arrangement is also equipped with an air supply in order to ensure fast cooling post pretreatment with a minimum 6 bar air pressure threshold in order to function.

Figure 2.2: The Anton Paar Monowave 450 instrument (a) the main setup with the microwave chamber (b) the glass bottle with the septum cap and thermometer chamber

Biomass pretreatment took place at 10% solid biomass loading while the solvent was a 50:50 v/v ratio mixture of water and acetone (H_2O/ACO) with the addition of acetic acid as a catalyst in concentrations of 0.5-2.5% (v/v). Biomass was introduced in the chamber either directly or after being soaked with the solvent for a set amount of time and the effect of residence time and acetic acid concentration was examined. The power profile to achieve a maximum temperature of 150 ^oC was set to 100 W for 90 sec, then 50 W for 60 sec followed by the duration of the pretreatment step (30 min to 2 h) and a colling step. Pretreatment took place at a solid to liquid ratio of 0.13 w/v. The minimum temperature of the cooling step was set to 60 ^oC, from where the chamber was opened and the biomass was collected by means of filtration, rinsed with extra water and left to dry in a 60 ^oC oven overnight. Afterwards, the solid pulp was used for compositional analysis, hydrolysis and isomerization experiments.

2.11.2 Oxidation pretreatment experiments (OxiOrganosolv)

The oxidation pretreatment experiments were conducted at the Chemical Process and Energy Resources Institute (Center for Research and Technology Hellas, Thessaloniki, Greece). The biomass feedstock for these experiments was the commercially available beechwood sawdust (Lignocel® HBS 150-500, JRS GmbH and Co KG, Germany) and bark-free pine trimming of *Pinus* sp. as a representative of softwood biomass. The biomass was pretreated with a mixture of 50:50 v/v of H_2O/organic solvent as a liquid phase at a solid to liquid ratio equal to 1:10. The reactor vessel was pressurized with 100% O_2 and heated up to the desirable temperature. Pressure values were set to 8 bar and 16 bar. The three temperature values examined were 150 ^oC, 160 ^oC and 175 ^oC. Reaction times varied between 60 and 120 min. Tetrahydrofuran (THF), acetone (ACO), ethanol (EtOH) and isobutanol (iBuOH) were used as the organic solvents. After cooling down and reactor depressurization, the solid pulp was obtained through vacuum filtration, sequentially washed with warm organic solvent and distilled water and air dried. The final moisture of the pulps was 5-8% w/w. The cellulose, hemicellulose and lignin content of the untreated and pretreated lignocellulosic biomass were determined on a dry feed basis (% w/w) following the protocols provided by the National Renewable Energy Laboratory (NREL).

POMs catalysts were also added to the pretreatment experiments described above. POMs added were either commercially available or custom made in-house. Commercial phosphomolybdic acid hydrate ($H_3PMo_{12}O_{40}$ x H_2O or HPMo) was supplied by Sigma-Aldrich (US). Copper and iron-exchanged phosphomolybdic acid salts ($M_3PMo_{12}O_{40}$ with M=Cu or Fe) were prepared as follows: 5 mmol $H_3PMo_{12}O_{40}$ x H_2O were dissolved in 50 mL of distilled water. The above solution was added dropwise to a 50 mL aqueous solution of 1 M $CuCl_2$ or $FeCl_2$ x 4 H_2O under vigorous stirring at room temperature. The mixture was stirred for about 30 min and then the temperature was raised to 50 °C and stirring was continued for 60 min, followed by drying at 80 °C. The final dried solid salts ($Cu_3PMo_{12}O_{40}$ or Cu-PMo and $Fe_3PMo_{12}O_{40}$ or Fe-PMo) were burned at 200 °C

for 4 h with a temperature increase rate of 5 °C/min using air. Chemical composition was determined by Inductive Coupled Plasma - Atomic Emission Spectroscopy (ICP-AES). These experiments were carried out at the Centre for Research and Technology Hellas (CERTH) and the catalysts were kindly offered by ms Stamatoula Karakoulia.

2.12 Enzymatic Hydrolysis

Enzymatic hydrolysis is one the most pivotal stages of the HMF production process, as the concentration of glucose produced constitutes the baseline for the following processes of isomerization and dehydration. As a result, high glucose concentration is demanded if high yields of fructose production are required. In addition, given that enzymatic hydrolysis is performed by the binding of cellulolytic enzymes to the surface of cellulose fibers, it is necessary to establish the parameters that will ensure the efficient mass transfer conditions of the enzymes on cellulose. Hence, a variety of values were examined on parameters such as stirring, substrate concentration and enzyme loading.

Firstly, the optimum hydrolysis conditions experiments were performed on conventional stirring systems (horizontal incubator bases) in 100 mL Erlenmeyer flasks. Hydrolysis was performed using the enzymatic cocktail Cellic® Ctec2 (Novozymes, Bagsværd, Denmark) at 50 ^{o}C and 180 rpm for 72 h. The ideal solids concentration and enzyme loading conditions were determined with enzymatic hydrolysis on pure Avicel cellulose (Avicel, Macherey Nagel, Germany) for a wide range for each parameter. Solids loading values ranged from 2% w/w to 20% w/w, with an enzyme loading of 15 mg enzyme/g dry matter, while enzyme loading ranged from 5 mg enzyme/ g DM to 30 g enzyme/g DM. Cellic® Ctec2 was used for all hydrolysis experiments and was diluted in 50 mM citrate phosphate buffer pH 5. The initial buffer concentration was 100 mM. However, it was observed that high buffer concentrations and subsequently high phosphate ion concentrations led to the formation of humins during the chemical dehydration step, decreasing the overall yield. As a result, 50 mM buffer concentration was established.

Following the conventional stirring hydrolysis experiment, a custom free fall mixer was utilized to examine hydrolysis in an enhanced stirring. The free fall mixer setup accomplishes mixing though the spinning of fans that transfer the material and returning it to the main volume after each turn. As a result, better mass transfer conditions are accomplished and cellulolytic enzymes present in the liquid part of the mixture are granted easier access to the cellulose fibers leading to better absorption. The free fall mixer experiment took place for 12 h at 50 ^{o}C and 25% w/w solids loading while enzyme loading was 15 mg enzyme/g DM. Spinning of the fans was set to 7 rpm, while the spinning trend was reversed every 2 min. All glucose concentrations in the hydrolysis experiments were determined with the use of the GOD/PAP method.

Figure 2.3: The enzymatic hydrolysis experiment. Top to bottom: lignocellulosic biomass in the flasks, the biomass-Cellic Ctec2® mixture at the start of the hydrolysis, the mixture after the hydrolysis

2.13 Isomerization reactions

Isomerization is the step following enzymatic hydrolysis for the transformation of glucose to fructose. Isomerization reactions were performed with the use of the commercially available Sweetzyme® (Novozymes A/S, Bagsværd, Denmark), an immobilized glucose isomerase. However, in order to achieve high yields, a preparation of the samples post hydrolysis is required. Firstly, hydrolysates were centrifuged for the separation of the liquid phase from the solid phase, followed by the addition of sodium tetraborate in the liquid phase at a mass ratio of 1:1 towards glucose. Next, reaching pH 7 was achieved with the addition of NaOH 1 M. Adding such small volumes of NaOH due to its high concentration results in minimum dilution of glucose. For that reason, glucose concentration was considered the same even after the pH adjustment. Glucose isomerase was then introduced to the prepared samples at a 5% w/v loading towards the total reaction volume. Samples were then incubated at 50 ^{o}C, 180 rpm for 24 h. After the end of

isomerization glucose was also measured with the GOD/PAP method. The reaction yield was calculated the reduction of glucose, as the molar ratio of glucose to fructose in the reaction is 1:1. As a result, yield was calculated with the following equation:

$$\%\ Fructose\ Yield = \frac{Glinitial - Glfinal.}{Glinitial}$$

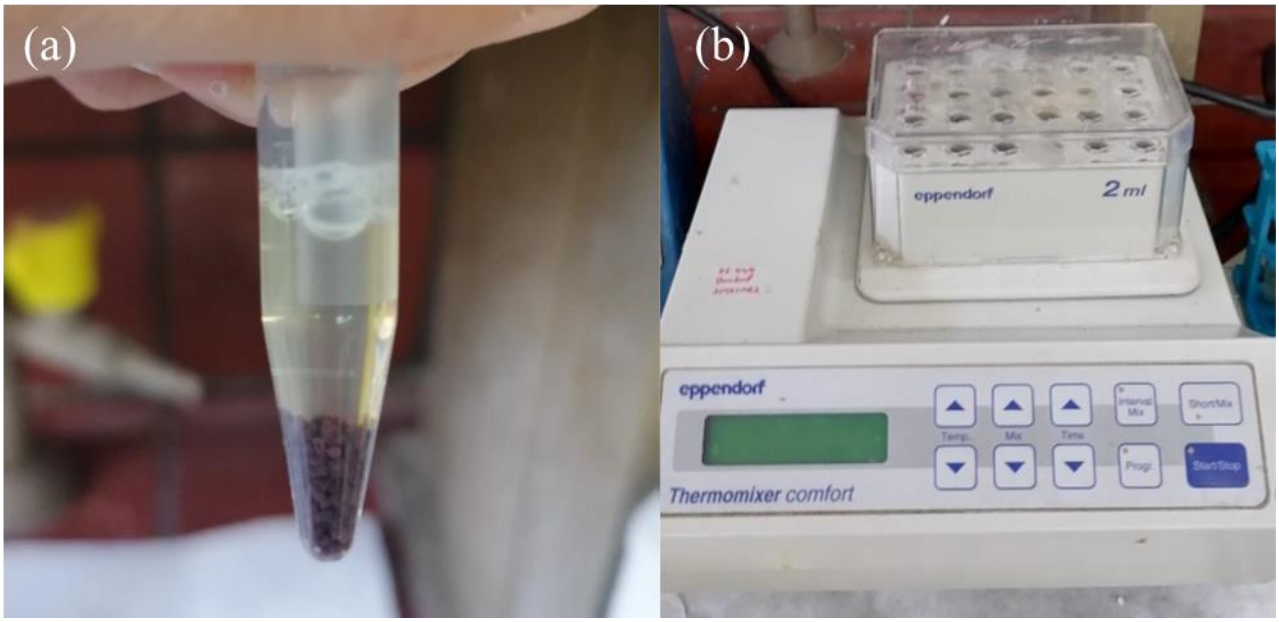


Figure 2.4: The isomerization experiment. (a) The mixture of the hydrolysate, sodium tetraborate and the immobilized isomerase, (b) The thermomixer for the incubation at 50 ^{o}C overnight

2.14 Chemical Dehydration to HMF

The dehydration reaction of sugars to HMF also took place at the Chemical Process and Energy Resources Institute (Center for Research and Technology Hellas, Thessaloniki, Greece). The sugar-rich liquor resulting from the enzymatic hydrolysis and isomerization of biomass was converted to furans over homogenous and/or heterogeneous catalysts. Thus, HCl 37% w/w, H_3PO_4 85% w/w, formic acid 99% w/w and maleic acid (solid form) used as homogenous catalysts, were purchased from Sigma Aldrich and used as received. Zeolite H-mordenite, employed as a heterogenous catalyst, was prepared via calcination of the NH_4-form zeolite CBV 21A (Si/Al = 10) (Zeolyst International) at 500 °C for 3 h in air. The experiments were carried out in a batch, stirred, autoclave reactor (C-276 Parr Inst., USA), under N_2 gas. The sugar-rich liquor was diluted with water, to suppress side reactions and increased formation of by-products (until a final sugar concentration of 2.5% w/w) and the corresponding catalyst was added. The mixture was charged into the reactor and heated to the desired temperature. The reaction was allowed to proceed for a

given time under continuous stirring. After completion, the reactor was cooled swiftly; the solution was filtered to remove solids (i.e. catalyst, by-products etc.) and analyzed by Ion Exchange Chromatography (ICS-5000, Dionex, USA). The quantification was based on external calibration, using standard solutions of sugars (glucose, mannose, xylose, fructose, galactose, arabinose and rhamnose), sugar alcohols (sorbitol and mannitol), HMF and organic acids (formic, acetic, glycolic, lactic, levulinic, propionic and butyric acid). The analysis of sugars was performed using a CarboPac PA1 (10 μm, 4 × 250 mm) column and guard column (10 μm, 4 × 30 mm) connected to a pulsed amperometric detector (PAD). The eluent was 20 mM NaOH at a 0.6 ml/min flow rate and the total analysis time was 75 min. The analysis of the organic acids was performed on an AS-15 (9 μm, 4 × 250 mm) column and pre-column (9 μm, 4 × 30 mm) connected to a conductivity detector (CD). The eluent was 8 mM NaOH at a 1 ml min−1 flow rate and the total analysis time was 75 min.

The conversion of sugars (glucose and fructose), the yields and the selectivity of the products (weight based) were calculated according to the following equations:

$$Sugars\ Conversion\ \% = 100\ x \frac{sugars\ reacted\ g}{sugars\ initial\ g}$$

$$Product\ Yield\ \% = 100\ x \frac{product\ produced\ g}{sugars\ initial\ g}$$

$$Product\ selectivity\ \% = 100\ x \frac{product\ yield\ \%}{sugars\ conversion\ \%}$$

2.15 Heterologous expression of the recombinant galactose oxidase

2.15.1 PCR amplification of the galactose oxidase gene

The gene encoding the galactose oxidase *Fo*GalOx (protein id: 15967, https://mycocosm.jgi.doe.gov/cgi-bin/dispGeneModel?db=Fusoxrap1&id=15967) was amplified by PCR from the genome of *Fusarium oxysporum* using the primers TtGO15967F and TtGO15967R (Table 2.2), which were designed based on the available sequence and contained the recognition sites of the restriction enzymes *Cla*I and *Xba*I on the corresponding 5'-ends. DNA amplification was performed by the KOD Hot Start polymerase for 35 cycles of denaturation (95 ^{o}C for 20 sec), annealing (57 ^{o}C for 30 sec) and extension (70 ^{o}C for 50 sec). Prior to these cycles an early stage of polymerase activation was carried out at 95 ^{o}C for 2 min. After these cycles there was an extension step at 70 ^{o}C for 2 min. The DNA sequence contained no introns, had a length of 2036 bp. Post DNA amplification, the sequence was separated by agarose gel electrophoresis and isolated with the PCR clean up kit (Macherey-Nagel, Dűren, Germany).

Table 2.2: Primer sequences used for the amplification of *Fo*GalOx from the *F. oxysporum genome*

Primer	**Sequence (5' → 3')**	**Length (bp)**
15967F	GCATCGATGGTCGCCATCTCCCAACCGG	28 bp
15967R	CGTCTAGATACTGAGTAACGAGAAGAGTACTCGCAAC	37 bp

2.15.2 Ligation to the PCR™-blunt plasmid vector

The isolated DNA sequence was introduced to the PCR™-blunt plasmid vector by means of ligation using a T4 DNA ligase (Takara, Japan). The reaction mixture ratios are depicted in table 2.3 After ligation the recombinant vectors were used to transform *Escherichia coli* TOP10 cells.

Table 2.3: PCR™-blunt ligation reaction protocol depicting the compound ratios

Compound	**Volume (µL)**
PCR™-blunt plasmid vector	1
PCR product	5
Ligation buffer	1
H_2O	2
Ligase	1

2.15.3 Escherichia coli cells transformation

The transformation procedure of a microorganism is typically accomplished by the attachment of the recombinant DNA sequence to the microorganism surface and then absorbed through the membranes. The laboratory transformation of cells requires that the cells be rendered competent through a series of specific techniques. The treatment of cell with $CaCl_2$ followed by a thermal heat-shock or electroporation are two of the most common techniques. The exact mechanism of

the interaction is yet to be discovered. However, the assumption is that the DNA molecules can be absorbed onto the cell surface due to the presence of Ca^{2+} ions and then pass through the membranes during the heat shock.

In order to create competent *E. coli* cells, LB medium precultures of 5 mL total volume were inoculated with *E. coli* TOP10 cells and incubated at 37 ºC for 16 h under continuous agitation (180 rpm). Then, 1 mL was collected from the precultures and transferred to 100 mL LB cultures followed be incubation at 37 ºC until a final OD of 0.4-0.6. After reaching the desired OD, cultures were cooled and centrifuged. The cells were collected from the precipitate and then resuspended in 12 mL of 0.1 M $MgCl_2$. This procedure was repeated and the cells were again resuspended in 4 mL $CaCl_2$ 0.1 M and the mixture was incubated at 4 ºC for 16 h. Finally, 1.9 mL of 50% v/v glycerol were added to the mixture and then aliquots of 0.1 mL were stored at -80 ºC.

The introduction of recombinant DNA vectors into the competent *E. coli* TOP10 cells was accomplished through heat-shock. The vector was added to the 0.1 mL aliquot of *E. coli* cells and then incubated at 4 ºC for 30 min. The heat-shock stage was then carried out by incubating the mixture at 42 ºC for 1.5 min. After the heat-shock stage, 0.2 mL of LS-LB (Low salt LB) was added to the mixture followed by an incubation at 37 ºC for 1 h. Finally, the cells were introduced to LS-LB petri dish cultures with the proper antibiotic and incubated at 37 ºC for 16 h.

The collection of plasmid vectors from transformed cells requires, firstly, an inoculation of 5 mL LS-LB cultures containing zeocin with the cells and an incubation at 37 ºC for 16 h with 180 rpm agitation. The collection was then carried out according to the instructions of the commercial NucleoSpin® Plasmid mini kit (Macherey-Nagel, Dϋren, Germany).

2.15.4 Restriction Enzyme Digestion

Restriction endonucleases have been classified into four main categories, namely type I, II, III, IV and almost every type requires the presence of a metal cofactor like Mg^{2+}. Type II restriction endonucleases make up the largest category of characterized enzymes, mainly due to their utility as recombinant DNA technology tools. Typically, restriction endonucleases' function is the rupture of the DNA sequence at specific spots depending on their recognition site.

Digestion reactions were carried out in order to isolate the *Fo*GalOx gene with the aim to use it to transform pPICZαC plasmid vectors. The reactions were performed according to the protocols of table 2.5 The recognition sites of each restriction enzyme used in this thesis are depicted in table 2.4.

Table 2.4: Recognition sites of restriction enzymes used in this thesis.

Restriction enzyme	Recognition Site	Origin
*Xba*I	5’ … T C T A G_A … 3’ 3’ … A G A T C T … 5’	*Xanthomonas badrii*
*Cla*I	5’ … A T C G_A T … 3’ 3’ … T A G C T A … 5’	*Caryophanon latum*
*Sac*I	5’ … G A G C T C … 3’ 3’ … C T C G A G … 5’	*Streptomyces achromogenes*
*Pme*I	5’ … G T T T A A A C … 3’ 3’ … C A A A T T T G … 5’	*Pseudomonas mendocina*

Table 2.5: Digestion reaction protocol depicting the compound ratios.

Compound	Volume (μL)
Recombinant plasmid vector	5
Restriction enzyme buffer	2
Restriction enzyme	1
H_2O	11

Results of the digestion enzymes were checked with agarose gel electrophoresis. The isolation and clean-up of the desired DNA fraction were accomplished using the NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel, Düren, Germany).

2.15.5 Ligation to pPICZαC plasmid vector

The ligation of a sticky end DNA sequence, as a result of digestion with restriction enzymes, with the plasmid vector pPICZαC was performed again using the T4 DNA ligase (Takara, Kusatsu, Japan). In addition to the DNA ligase the ratio of the rest of the reagents is depicted in table 2.6. Control reactions dismissing either the DNA sequence or the ligase were also made. Following the ligation to pPICZαC the recombinant vector was multiplied by the transformation of TOP10 *E.*

coli cells according to the protocol of paragraph 2.14.3 and then was isolated again according to the protocol of the NucleoSpin® Plasmid mini kit (Macherey-Nagel, Düren, Germany).

Table 2.6: pPICZαC ligation reaction protocol depicting the ratio of each compound.

	Amount in µL				
Reagent	**1:3**	**1:5**	**1:7**	**Ctrl 1**	**Ctrl 2**
pPICZαC vector	1	1	1	1	1
DNA sequence	3	5	7	-	-
T4 DNA ligase	1	1	1	1	-
Buffer	2	2	2	2	2
Ultrapure H_2O	13	11	9	16	17

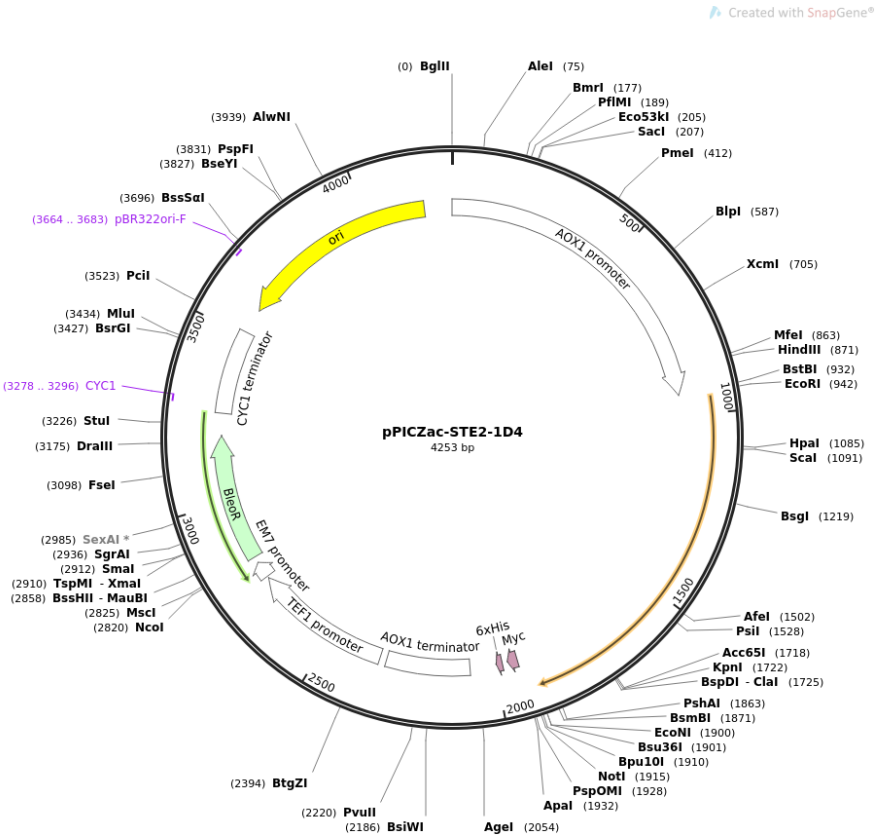


Figure 2.5: The sequence of pPICZαC containing the restriction sites for each restriction enzyme respectively.

2.15.6 Pichia pastoris cells transformation

The transformation of *P. pastoris* cells was accomplished by means of electroporation. The electroporation technique uses short high-voltage pulses in order to render the cell membrane temporarily permeable by DNA molecules. Linear DNA molecules with free ends are more likely to pass through the membrane and be incorporated to the chromosomes of the host, thus producing stable clones. For that reason, a procedure called DNA linearization with restriction enzymes is required prior to electroporation. *Fo*GalOx was linearized using the *Pme*I restriction enzyme and was used to transform *P. pastoris* X33 cells by electroporation.

The following paragraph presents the protocol for the preparation of *P. pastoris* X33 electrocompetent cells. YPD medium liquid cultures with a final volume of 50 mL were inoculated with *P. pastoris* cells and then incubated at 30 ºC for 16 h under 200 rpm agitation. Then, 10 mL were collected from the precultures and used to inoculate large scale YPD cultures with a final volume of 300 mL. Large scale cultures were also incubated at 30 ºC 200 rpm until a final OD_{600nm} of 1.3-1.5. The cultures were cooled and centrifuged and the cells collected from the precipitate and resuspended in 300 mL of ultrapure water. After further centrifugation cells were resuspended in 150 mL ultrapure water. Then two further centrifugation and resuspension steps followed where cells were resuspended in 12 mL and 1.5 mL of 1 M sorbitol, respectively.

The electroporation was carried out on 80 μL of electrocompetent *P. pastoris cells* with the linear DNA molecule. Both the cells and the DNA were mixed and after a 5 min incubation were transferred to the electroporation cuvette. An electric pulse of 2 kV for 4 ms was applied to the cuvette using the Gene Pulser device (Bio-Rad, Hercules, CA, USA). Then, 1 mL of 1 M sorbitol was immediately added to the cuvette and the mixture was incubated at 30 ºC for 1 h. After incubation the cell suspension was introduced to YPDS petri dishes in the presence of 100 μg/ mL zeocin. The recombinant colonies that were able to grow on the petri dish in the presence of the antibiotic were then collected and grown in cultures at 30 ºC for 3-10 d. The best performing colonies were scanned by enzyme activity reactions and then used to create glycerol stock aliquots stored at -80 ºC.

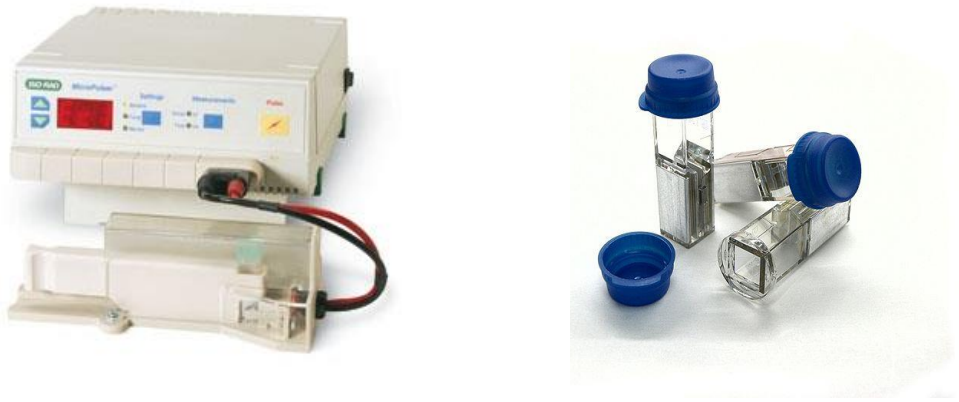


Figure 2.6: Electroporator and cuvettes used in the electroporation experiments.

2.15.7 Production of the recombinant biocatalyst from fungal cultures

(i) petri dish cultures and liquid precultures

Firstly, transformed X33 *P. pastoris* cells from glycerol stock storage were used to create YPD medium petri dish cultures. After incubation at 30 ºC for 48 h cells were collected and used to inoculate small scale precultures with BMGY medium and a total volume of 200 mL. After incubation at 30 ºC for 20 h samples were collected for OD_{600nm} measurement. The optical density of the cultures was used to calculate the culture volume needed to inoculate large scale cultures with a final OD of 1.

(ii) cultures in Erlenmeyer flasks

The optical density measurement was followed by the collection of the needed volume of the culture. The collected volume was centrifuged for 15 min at 3000 rpm. The BMGY medium supernatant was discarded and the cells in the precipitate were resuspended in BMMY medium. The mixture was then transferred to large 2 L Erlenmeyer flasks and incubated at 30 ºC for 5 d with a daily feed of 0.5% v/v methanol.

Table 2.7: List of reagents for the culture media preparation.

	BMGY (1 L)	**BMMY (1 L)**
Yeast Extract	10 g	10 g
Peptone	20 g	20 g
H_2O	700 mL	800 mL
Potassium Phosphate Buffer pH 6	100 mL	100 mL
YNB	3.4 g Yeast Nitrogen Base 10 g Ammonium Sulphate In 100 mL H_2O	3.4 Yeast Nitrogen Base 10 g Ammonium Sulphate In 100 mL H_2O
Biotin	2 mL	2 mL
Glycerol 10%	100 mL	-
Methanol 0.5%	-	1.25 mL

(iii) Filtration and ultrafiltration techniques

For the collection of the recombinant enzyme in a pure form the crude mixture of the culture after the 5 d incubation, firstly, a centrifuge step was required for the precipitation of the cells and the cell fragments. Following this, the crude was vacuum filtrated with filter papers of different pore sizes, namely 0.8 μm, 0.45 μm and 0.2 μm consecutively (Supor® 200, PALL Life Sciences). The medium was then introduced to the molecular ultrafiltration device Amicon Stirred Cell Model 8400, where it was condensed to using PM-10 membranes with a pore size of 10 kDa until a final volume of 50 mL was achieved.

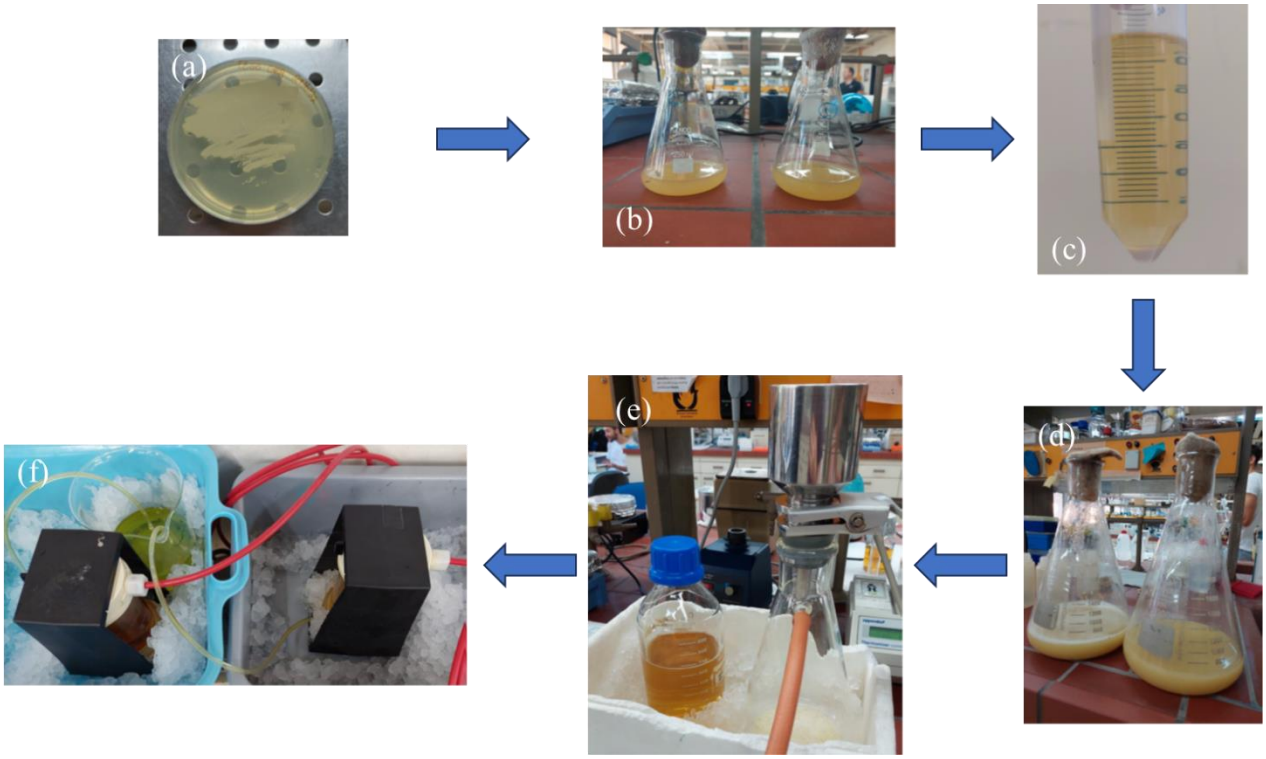


Figure 2.7: The complete scheme for the heterologous expression of recombinant proteins. (a) petri dish cultures, (b) growth in precultures, (c) centrifugation and collection of cells as a precipitate, (d) large scale cultures, (e) vacuum filtration, (f) molecular filtration

(iv) Isolation and purification of proteins with Immobilized Metal Affinity Chromatography (IMAC)

Immobilized Metal Affinity Chromatography (IMAC) was used as a technique for the isolation of recombinant proteins, due to its ability to bind the 6 histidine amino acids at the end of the protein sequence (His-tag) to the active metal resins (Ni^{2+}, Co^{2+}, Zn^{2-}, Cu^{2+}) taking advantage of the negative charge of amino acid residues. The metal ions in the resin used in this work we Co^{2+}.

The first step for the isolation of proteins was the equilibration of the culture medium to the chromatography resin buffer (20 mM Tris-HCl, 300 mM NaCl pH 8, TALON). The equilibration was achieved using the semi-permeable membrane dialysis technique (dialysis tubing cellulose membrane, flat width 25 mm, 12000 Da, Sigma-Aldrich, St. Louis, MO, USA). These membranes allow the passage of ions by osmosis on the internal and the external of the membrane until a final equilibrium is restored. Dialysis was carried out for 20 h at a temperature of 4 ^{o}C. Following this, the equilibrated culture medium was introduced to a Talon® Metal Affinity Resin chromatography column (Clontech Laboratories Inc., Kusatsu, Japan) where the following solvents and amounts were passed through the resin:

- 1 resin volume of distilled water for resin cleaning
- 10 resin volumes of TALON pH 8 for the equilibration of the resin with the culture medium
- Culture medium and collection of the flowthrough fraction
- Washing of the resin with TALON pH 8 and collection of the wash fraction
- TALON-imidazole solution with a range of concentrations between 5 and 100 mM for the elution of the protein
- Elution of the resin with TALON and water to restore it to the initial conditions
- Storage in a solution of EtOH 20% v/v

Following the protein purification experiment, the protein was transferred to a 20 mM Tris-HCl pH 8 buffer by means of dialysis in which it was stored at 4 ^{o}C for future use.

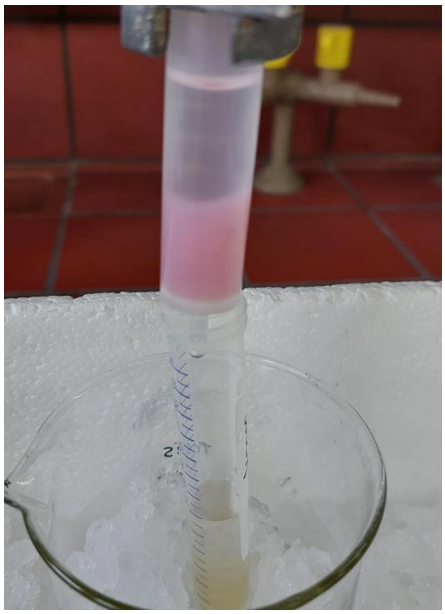


Figure 2.8: The IMAC chromatography column for the purification of recombinant proteins

2.16 Assay for enzyme activity

Enzymatic activity reactions were carried out with galactose as a substrate. The reaction mixture contained 50 mM galactose, 50 mM potassium phosphate buffer pH 6, galactose oxidase at a concentration of 0.0128 mg/mL and a mixture of 0.6 mg/mL HRP (horseradish peroxidase) and 1 mg/mL Phenol Red. The reaction mixture was incubated at 40 ^{o}C for 20 min and the reaction was stopped by adding NaOH 1 M. The reaction products were detected spectrophotometrically at 610 nm.

2.17 Determination of the temperature and pH optimum

The determination of the temperature optimum of enzymes was carried out using the assay for enzyme activity. Temperature values ranged from 20 ^{o}C to 70 ^{o}C and pH values ranged from 3.0 to 10.0. The different pH ranges were achieved by using the following buffers: Citrate phosphate buffer (pH 3-6), Potassium phosphate buffer (pH 6-8) and Bis-Tris (pH 8-10).

2.18 Furan substrate reactions

Furan substrate reactions for the examination of activity on furans took place at 40 ^{o}C, 1000 rpm for 24 h. The buffer used for the reactions was potassium phosphate buffer pH 6.0. Substrates of the reactions were HMF, DFF, HMFCA and FFCA in a concentration of 2 mM and *Fo*GalOx concentration in the reaction was 0.0182 mg/mL. Additionally, HRP and catalase were added to the reaction to alleviate H_2O_2 generation in a concentration of 0.26 mg/mL and 0.33 mg/mL respectively. HRP and catalase were commercially available and purchased from Sigma-Aldrich (USA). Reactions were stopped with the addition of HCl 1 M and the products were detected with HPLC analysis according to paragraph 2.7. Standards for the reactions were HMF, DFF, HMFCA, FFCA and FDCA in concentrations of 2 mM, 5 mM and 10 mM.

RESULTS AND DISCUSSION

Results and discussion

3. HMF production from organosolv pretreated hardwood biomass

Lignocellulosic biomass has been in the spotlight of the scientific community for many years due to its potential as a feedstock for the production of second-generation biofuels. However, over the past decade, due to the saturation of studies in this field, the interest of lignocellulose biorefineries has shifted towards high-added value chemical synthesis paving the way for the exploration of the potential of all the different compounds present in lignocellulose. However, in all the types of different biorefineries, one thing has remained a staple to effectively valorize lignocellulosic feedstocks, and that is biomass pretreatment. Biomass pretreatment is a process that is able to disrupt the recalcitrant structure of lignocellulose and achieve the fractionation and isolation of its constituents rendering them more amenable to chemical and enzymatic procedures, which is the essence of biomass valorization. In this context, this chapter examines the effect of an oxidative organosolv pretreatment on beechwood biomass samples and its ability to render them amenable to enzymatic saccharification. Furthermore, it also examines the mechanism of glucose isomerization with the aim to produce fructose in high yields and implements the optimal conditions to isomerization reactions on real biomass hydrolysates. Additionally, another reaction studied in this chapter is the dehydration of fructose to HMF in the presence of Brønsted acid solvents. All these processes were examined with the aim to create an integrated process, where real lignocellulosic substrates would be broken down in order to produce fructose and, ultimately, HMF in high yields.

3.1 Setting up the hydrolysis and isomerization experiments

In order to study the biomass hydrolysis and isomerization and optimize the fructose and HMF production, it was necessary to identify the ideal conditions for the hydrolysis parameters, namely solids loading, enzyme loading and sodium tetraborate loading. Hence, prior to hydrolysis of pretreated lignocellulosic samples, preliminary screening of different initial solids concentration and enzyme loading was carried out. Avicel® PH-101 (Sigma-Aldrich, US) was used as a model substrate due to it being pure cellulose. The results, depicted in figure 3.1, indicate that % cellulose conversion yield remained high (72.9%) up to 10% w/w solids loading, while for higher concentration it was decreasing, eventually reaching 49.8% for 16% w/w solids. As far as the enzyme loading is concerned, it is evident that the increase in cellulose conversion between the 10 mg enzyme/g dry matter (DM) and the 15 mg enzyme/g DM is much greater than the respective one between 15 and 20 mg enzyme/g DM, with the respective increase being 15.4% and 8.7%. Furthermore, for enzyme loading higher than 20 mg/g DM, the increase in cellulose conversion is not as significant. Taking into account that the enzyme cost comprises a significant bottleneck in

the overall economic integration of the process, the optimum enzyme concentration was set at 15 mg enzyme/g DM in all biomass experiments.

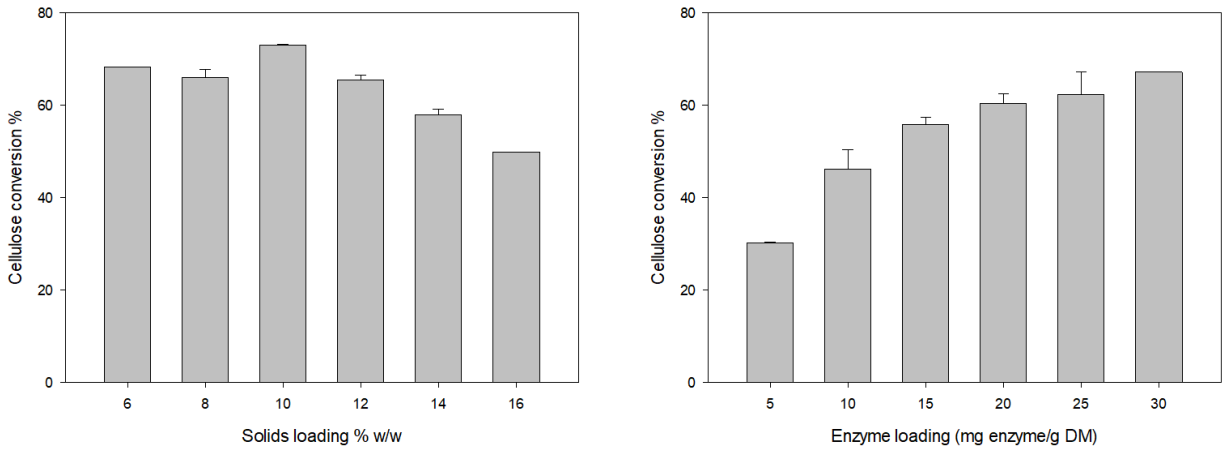


Figure 3.1: Study for the optimum solids and enzyme loading conditions.

Similarly, the optimum ratio of borate to glucose leading to the highest yield during isomerization reaction was identified with pure glucose as substrate. As presented in figure 3.2 the maximum yield was observed for a molar ratio sodium tetraborate to glucose equal to 0.28, so this condition was used in all isomerization reactions with biomass hydrolysates. Other studies report that the borate to sugar molar ratio that maximizes the conversion yields lies within the range of 0.33-0.5 (Wang et al., 2019a), or it is 0.5 (Huang et al., 2010). This may be attributed to differences in the molecular weight of the chemical depending on the origin. For example, sodium tetraborate decahydrate (our in-house chemical) can lead to a lower molar ratio needed for ideal isomerization compared to anhydrous. In any case, figuring out the ideal ratio of sodium tetraborate is a pivotal factor to take into consideration as far as the economic integration of the process is concerned.

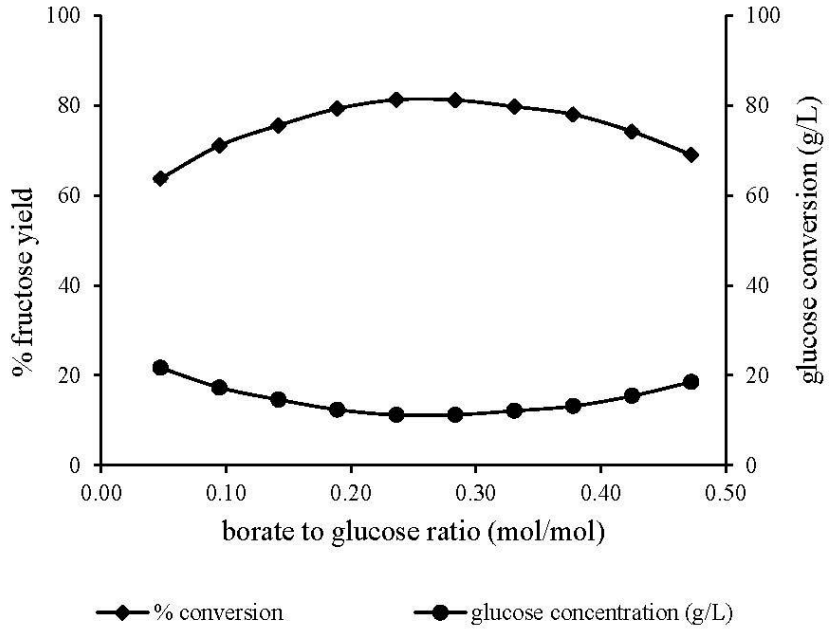


Figure 3.2: Study of the optimum borate loading for the isomerization reactions. Effect of different molar ratios of borate to glucose, on the reduction of glucose concentration and the yield of the isomerization reaction.

3.2 Screening of different biomass pretreatment processes for the production of fructose

Organosolv pretreatment processes have been shown to be extremely effective in an attempt to delignify lignocellulosic biomass especially with a small percentage of acid catalysts, leaving behind a sugar-rich solid fraction that can be used for the production of furan derivatives (Kalogiannis et al., 2020). By integrating an acid-free organosolv biomass fractionation process together with high-gravity enzymatic hydrolysis for the saccharification of sugar streams and efficient enzymatic isomerization, a concentrated fructose syrup is obtained. This chapter focuses on the application of a number of different homogeneous catalysts for the efficient dehydration of sugars to furans and their subsequent evaluation (Dedes et al., 2021).

After the optimal hydrolysis conditions were defined as described in paragraph 3.1, a number of different pretreated biomass samples were tested on both their susceptibility towards hydrolysis and the production of fructose. The different pretreatment conditions concerning the organic solvent employed (acetone-ACO, ethanol-EtOH, tetrahydrofuran-THF), the pretreatment conditions as well as the composition of all the pretreated lignocellulose biomasses are described

in table 3.1. Hydrolysis and isomerization yields are described in 3.2. It is profound that the cellulose-rich pulps that were obtained after organosolv fractionation were amenable to enzymatic saccharification, thus reaching cellulose to glucose conversion yields higher than 70% in most cases, compared to 25.9% conversion of the untreated feedstock. These results underline the efficiency of the OxiOrganosolv pretreatment process and verify the study previously reported by Kalogiannis et al. (2020). By employing organic solvents and mild oxidative conditions, OxiOrganosolv fractionation achieves efficient delignification and leads to solid pulps with high cellulose (even higher than 855 w/w) and low lignin content (even lower than 2% w/w), which can serve as starting materials for the production of value added products through biocatalysis and fermentation processes (Karnaouri et al., 2020b, 2021). Removal of lignin is of pivotal importance in the production of furans from biomass, since not only non-specific adsorption of enzymes onto the substrate during saccharification and isomerization is eliminated, but also the presence of phenolic compounds might interfere with the subsequent sugars dehydration step towards the production of HMF, thus causing adverse effects and lowering the overall process yield

Table 3.1: Pretreatment conditions and compositional analysis of each lignocellulosic biomass sample

Biomass No.	Pressure (bar)	Temperature (°C)	Reaction time (min)	Lignin (%)	Cellulose (%)	Hemicellulose (%)
			ACO/H_2O			
1	16	160	60	13.7	62.2	20.2
2	16	160	120	3.2	76.6	13.3
3	8	160	120	10.5	66.8	18.4
4	16	175	30	3.6	79.7	15.7
5	16	175	60	1.2	83.3	15.3
6	16	175	120	1.6	86.1	8.7
7	8	175	120	4.6	82.3	13.9
8	16	175	120	12.3	61.6	19.2
			EtOH/H_2O			
9	16	160	60	16.2	56.8	20.0
10	16	160	120	6.4	73.0	16.0
11	8	160	120	10.5	66.4	21.0
12	16	175	60	2.7	77.1	15.7
13	16	175	120	2.1	82.6	14.5
14	8	175	120	3.9	75.6	16.4
			THF/H_2O			
15	16	160	60	11.4	69.0	15.5
16	16	160	120	5.5	79.1	12.1
17	8	160	120	14.9	65.3	15.0
18	16	175	60	5.1	85.3	10.8
19	16	175	120	2.6	85.2	10.6
20	8	175	120	6.5	76.4	13.6

Table 3.2: Hydrolysis yields and glucose isomerization to fructose for different pretreated biomass samples. Numbers in parenthesis represent the standard error values. All experiments were run in duplicates.

Biomass No.	**Glucose (g/L)**	**Cellulose to glucose conversion (%)**	**Glucose to fructose conversion (%)**	**g fructose/ g cellulose**	**g fructose/100 g pretreated biomass**
			ACO/H_2O		
1	59.91 ± 0.49	77.98 ± 0.64	78.80 ± 0.14	0.61 ± 0.10	38.00 ± 1.66
2	87.78 ± 2.22	92.79 ± 2.34	79.87 ± 0.13	0.74 ± 0.10	56.79 ± 1.34
3	68.17 ± 2.15	82.71 ± 2.61	76.34 ± 0.16	0.63 ± 0.16	42.16 ± 2.36
4	80.16 ± 0.22	81.44 ± 0.22	77.23 ± 0.15	0.63 ± 0.13	50.15 ± 1.60
5	71.97 ± 0.99	69.99 ± 0.97	75.00 ± 0.17	0.51 ± 0.13	42.38 ± 1.54
6	71.58 ± 0.22	67.34 ± 0.21	73.68 ± 0.18	0.50 ± 0.12	42.72 ± 1.42
7	78.06 ± 0.99	76.83 ± 0.98	78.58 ± 0.14	0.60 ± 0.10	49.68 ± 1.23
8	42.78 ± 0.37	56.25 ± 0.48	68.12 ± 0.21	0.38 ± 0.12	23.61 ± 1.88
			EtOH/H_2O		
	33.73 ± 0.00	50.39 ± 0.00	71.53 ± 0.00	0.36 ± 0.00	19.55 ± 0.00
9	55.38 ± 0.25	79.06 ± 0.35	74.38 ± 0.17	0.59 ± 0.14	33.37 ± 2.45
10	80.26 ± 1.18	89.11 ± 1.31	76.30 ± 0.16	0.68 ± 0.13	49.61 ± 1.78
11	48.37 ± 1.63	59.02 ± 1.32	72.37 ± 0.68	0.43 ± 0.01	28.36 ± 1.84
12	84.59 ± 1.06	88.88 ± 1.11	75.94 ± 0.17	0.64 ± 0.16	49.36 ± 2.03
13	82.23 ± 2.65	80.67 ± 2.15	73.13 ± 0.44	0.59 ± 0.02	48.70 ± 2.41
14	86.08 ± 0.56	92.21 ± 0.45	79.05 ± 0.09	0.73 ± 0.00	55.15 ± 0.55
			THF/H_2O		
15	79.51 ± 0.12	93.36 ± 0.14	80.11 ± 0.13	0.75 ± 0.13	51.60 ± 1.83
16	78.70 ± 1.81	80.57 ± 1.85	78.19 ± 0.14	0.63 ± 0.10	49.85 ± 1.27
17	57.60 ± 0.32	71.49 ± 0.40	74.14 ± 0.17	0.53 ± 0.12	34.59 ± 1.84
18	83.27 ± 2.58	79.10 ± 2.45	79.12 ± 0.14	0.63 ± 0.09	53.37 ± 1.02
19	78.98 ± 0.84	75.09 ± 0.68	70.80 ± 0.23	0.53 ± 0.01	45.28 ± 0.74
20	79.47 ± 1.54	84.31 ± 1.24	78.97 ± 0.26	0.67 ± 0.01	50.81 ± 1.51
Untreated	12.86 ± 0.19	25.97 ± 0.15	70.89 ± 0.31	0.18 ± 0.00	7.38 ± 0.35
Avicel	76.05 ± 0.26	64.51 ± 0.21	80.40 ± 0.11	0.50 ± 0.08	48.21 ± 0.81

Correlating the effect of organic solvent in concert with the pretreatment temperature and the residence time with the saccharification yields of the cellulose-rich pulps, fractionation with ACO and THF favored a higher glucose release at 160 ºC, compared to 175 ºC. A shorter ACO pretreatment (30 min) was more efficient at higher temperature (175 ºC), reaching 80.16 g/L glucose (81.44% conversion), while 120 min were required at 160 ºC to reach 87.78 g/L glucose (92.79% conversion), respectively. A similar trend was observed with THF and, to a lesser extent, with EtOH. Regarding the isomerization step, glucose conversion to fructose was approximately 70-80% in all different trials, without showing any particular trend, indicating that there was little of no effect of the biomass characteristics on the activity of glucose isomerase, also considering that the reaction conditions were previously optimized. As shown in figure 3.3, when considering the overall process of pretreatment, hydrolysis and isomerization, it is easy to point out that all solvents perform better when pretreatment occurs at 160 ºC compared to 175 ºC, while lowering the pressure from 16 to 8 bar has varying effects depending also on the cooking temperature. The highest fructose yield obtained with all three solvents is approximately 27 g fructose/100 g of initial biomass; this is achieved when pretreatment occurs at 120 min, 160 ºC and 16 bar in case of ACO, 120 min, 175 ºC and 8 bar in case of EtOH and 60 min, 160 ºC and 16 bar in case of THF, respectively. Considering that direct saccharification and isomerization of untreated biomass results in 7.38 g fructose/100 g of untreated biomass, it is highlighted that acid organosolv pretreatment promotes a 4-fold increase of the overall fructose yield.

While there are numerous reports studying the conversion of lignocellulosic biomass-derived glucose to HMF through the use of metal catalysts or ionic liquids (Perez et al., 2018; Yuan et al., 2020b), there are scarce reports examining the isomerization pathway. An early study examining HMF production via fructose reported 90% glucose conversion to fructose in optimal borate loading and a following 63.3% HMF yield during the dehydration step (Huang et al., 2010). However, these results report the isomerization of pure glucose and not a hydrolysate obtained after the enzymatic saccharification of a real lignocellulosic substrate. Another work reports similar isomerization experiments on pure sugars in order to transfer these conditions to real dry matter (DMR) hydrolysates (Wang et al., 2019a). The isomerization experiments took place in 30 g/L pure glucose, 90 g/L pure glucose and 90 g/L glucose in DMR hydrolysate. The respective fructose yields were 83%, 77% and approximately 75% with the gradual decrease attributed either to the increase of the substrate or due to the fact that the hydrolysate contains various other substances that could potentially affect the isomerization reaction. The present work focuses mainly on the valorization of lignocellulosic biomasses and as a result examines the enzymatic saccharification. Nevertheless, the fructose yield managed to reach similar levels to those in literature, as the highest conversion is 80.11%, while simultaneously generating high glucose concentrations, which is also important in order to reach high fructose concentrations. This is probably the first work that examines a number of differently pretreated biomasses with regard to

their potential to produce fructose, in fact optimally transforming more than half of the pretreated biomass in fructose (56.79 g fructose/100 g pretreated biomass).

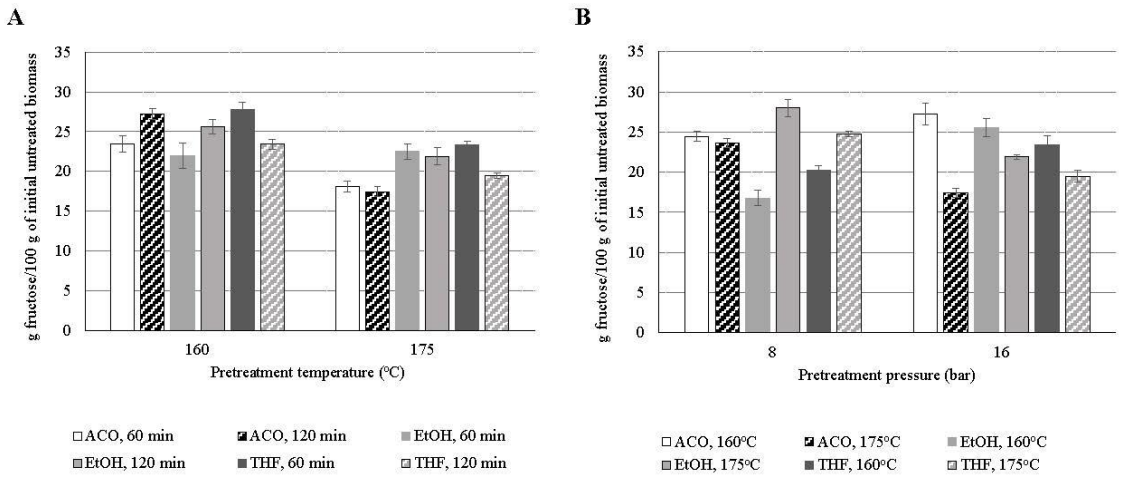


Figure 3.3: Effect of solvent, temperature and time when pretreatment is operated at O_2, pressure of 16 bar (A) and effect of pressure and temperature when pretreatment is operated for 120 min (B) on the overall fructose yield.

3.3 Scale up reaction and dehydration to furans

In order to obtain a concentrated fructose syrup that could serve as a starting material for the production of furans, a scale-up reaction was designed (figure 3.4). The employment of a high-gravity process, namely with a solid loading higher than 20% w/w DM is a prerequisite in order to obtain highly concentrated sugar syrups. However, it often suffers from poor mass transfer conditions; the adverse effects can be alleviated through a partial enzymatic hydrolysis step, which is referred to as liquefaction (van Putten et al., 2013). Liquefaction was performed in a two-step process, starting with incubation in a free-fall mixer and subsequent stage in an Erlenmeyer flask, which was proven to be an efficient strategy in order to obtain a liquor with 152.3 g/L reducing sugars.

Figure 3.4: Overall scheme of scale-up reaction towards the production of a fructose-rich syrup.

The sugar-rich hydrolysate obtained after isomerization of the scale-up reaction, containing 104.5 g/L fructose and 25 g/L glucose, as well as sodium tetraborate in a ratio to sugars equal to 0.28, was used as a substrate to produce HMF. Initially, a blank experiment was conducted in the absence of any additional catalyst, at 150 °C for 60 min, by diluting the substrate (10 g) in polar aprotic solvent DMSO (40 g) so as to obtain a DMSO/H_2O (4/1) mixture, according to a previous study by Marianou et al. (2018). Under the reaction conditions employed, HMF was not obtained as an end-product despite the almost complete conversion of sugars (99.0%) (table 3.3). In fact, the only products identified were organic acids glycolic, acetic and formic, with respective values of yield and selectivity below 4.6%, in all cases (table 3.3). Similar results were obtained in neat H_2O (i.e. omitting DMSO), as at sugars conversion of 98.9% the products detected were the same organic acids but at respective yield and selectivity below 3.2% (table 3.3). The only difference in this case, was that some traces of HMF were also detected, but with negligible yield (0.5%) and selectivity (0.5%). Thus, it was concluded that certain components of the liquid fraction, originating from hydrolysis and isomerization processes, act as catalysts causing conversion of sugars all the way to humins.

Table 3.3: Sugars (glucose and fructose, 2.5% w/w) conversion to HMF in the absence of any additional catalyst (150 ^{o}C, 60 min).

Solvent	Sugars Conversion (%)	HMF selectivity (%)	Yield				
			HMF	Glycolic Acid	Acetic Acid	Formic Acid	Levulinic Acid
H_2O	98.9	0.5	0.5	1.8	-	3.1	2.1
DMSO/H_2O (4/1)	99.0	-	-	1.4	4.6	3.2	-

Since the conversion of sugars is promoted by acidic ions, it was assumed that either the buffer citrate-phosphate from the hydrolysis step or the sodium tetraborate from the isomerization step might be the culprits causing the undesired conversion of the sugars to humins. Therefore, three aqueous standard solutions were prepared containing: (1) glucose/fructose/buffer citrate-phosphate, (2) glucose/fructose/sodium tetraborate, (3) glucose/fructose/buffer citrate-phosphate/sodium tetraborate, simulating the composition of the actual liquor. In each solution, the pH was adjusted to 7 with NaOH, simulating the actual process and afterwards it was subjected to heating at 150 ^{o}C for 60 min. According to the experimental results (table 3.4), sodium tetraborate alone proved highly active, as the conversion of sugars reached 99.6% while HMF yield (0.1%) and selectivity (0.1%) were negligible. On the other hand, citrate-phosphate buffer alone demonstrated mild catalytic activity, with sugars conversion and HMF selectivity reaching low values of 23.1% and 30.2% respectively. Finally, in the presence of both citrate-phosphate and sodium tetraborate, despite sugars conversion reaching 95.4%, the yield of HMF was barely 0.1%. Consequently, it may safely be concluded that the presence of sodium tetraborate in the reaction medium, and more specifically of boron species (tetraborate anion, boric acid) generated by its interaction with water, boosts the glucose conversion reactions towards the formation of by-products (humins), as also indicated by the dark brown color of the solution after the reaction (figure 3.5). According to previous studies (Istasse et al., 2020; Ståhlberg et al., 2011; Xu et al., 2015), high content of boron species can lead to the formation of a complex with the sugar bound to two borate molecules. These complexes of fructose are likely to be more stable than the mono-borate complexes of fructose, thus blocking the subsequent elimination of water in the first step of the conversion to HMF (proposed reaction mechanism by Ståhlberg et al. 2011) and simultaneously favoring fructose polymerization reactions (Akien et al., 2012; Zhao et al., 2021). The above hypothesis is also reinforced by the negligible production of formic (yield 2.3%) and levulinic (yield 1.5%), valuable organic acids derived from HMF (table 3.4).

Table 3.4: Effect of citrate-phosphate buffer and sodium tetraborate addition on sugars conversion to HMF (150 ^{o}C, 60 min). Experiments were run in duplicates and standard error deviation was 2.5%.

Solvent	Additive	Sugars conversion (%)	HMF Selectivity (%)	Yield (%)			
				HMF	Glycolic acid	Formic acid	Levulinic acid
dH_2O	Borax*, NaOH	99.6	0.1	0.1	0.9	2.2	1.5
Buffer citrate phosphate (5mM)	NaOH	23.1	30.2	7.0	0.2	0.2	0.1
Buffer citrate phosphate (5mM)	Borax*, NaOH	95.4	0.1	0.1	1.0	2.3	1.5

*sodium tetraborate decahydrate

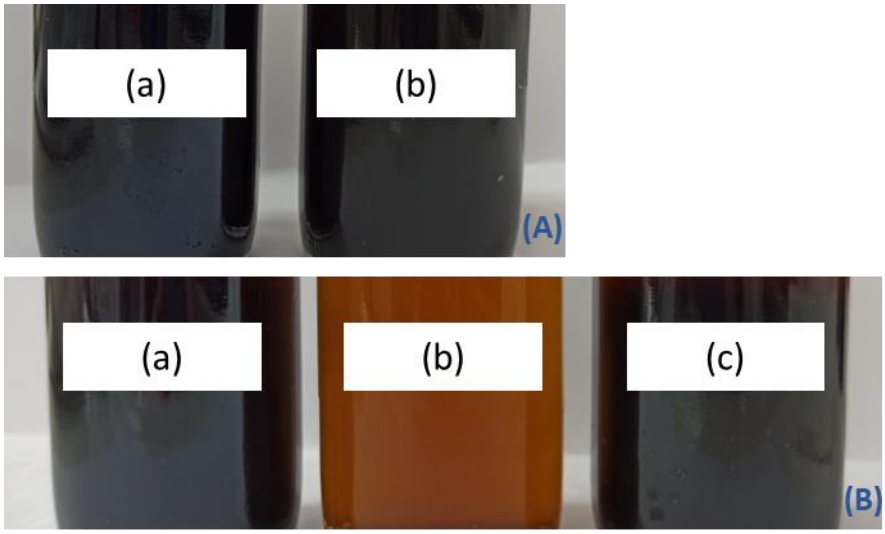


Figure 3.5: The color gained by the solutions after: **(A)** blank experiments in **(a)** H_2O and **(b)** DMSO/H_2O (4/1) mixture and **(B)** in pure aqueous solution of sugars in the presence of **(a)** Borax and NaOH, **(b)** buffer citrate phosphate (5mM) and NaOH and **(c)** Borax, buffer citrate phosphate (5mM) and NaOH.

Considering the necessity of both the presence of sodium tetraborate and citrate-phosphate buffer for the efficient enzymatic hydrolysis and isomerization of glucose to fructose, efforts were directed towards identifying the proper acid for decomposition of sodium tetraborate in order to minimize its effect on the production of HMF. Using this approach, Huang et al. (2010) reported

63% yield and 71.8% selectivity of HMF, with 88% conversion of sugars, performing the reaction in the presence of HCl, in water/MIBK diphasic system. In addition, using the same inorganic acid (HCl), but different biphasic system (Dioxane/H_2O (2/1)), Wang et al. (2018) reported even higher HMF yield (75%) and selectivity (88%), while the corresponding values using aqueous solution of H_2SO_4 (0.1 M) were 39% and 57%, based on the study of Rivas et al. (2019). Under strong acidity provided by HCl or H_2SO_4, sodium tetraborate is decomposed to boric acid and the corresponding sodium salt. Boric acid catalyzes the formation of HMF, however it also enables polymerization side reactions, as mentioned by (Istasse et al., 2020), especially in the presence of salts as demonstrated by Hansen et al. (2011). Apart from the boron species, another crucial factor for the selective synthesis of HMF is pH. It was demonstrated (Baugh & McCarty, 1988) that in acidic pH below 2.0 the formation of HMF is favored, contrary to higher pH values where sugars decomposition products such as organic acids and humins, are enhanced. Based on these and our results, as presented in table 3.4, the coupling of enzymatic and chemocatalytic processes requires the addition of an acid in order to convert the borate ions to boric ions and simultaneously act as a catalyst for the dehydration reaction. Thus, the effect of two inorganic and two organic acids was examined, as presented in table 3.5. Furthermore, the effect of a solid acid catalyst, namely H-Mordenite, was examined without prior acidification of the solution.

Table 3.5: Sugars (glucose and fructose) conversion to HMF over homogeneous and heterogeneous catalysts at 150 ^{o}C, 60 min. Experiments were run in duplicates.

Catalyst (final concentration % w/w)	**Sugars conversion (%)**	**HMF yield (%)**	**HMF selectivity (%)**
HCl (0.7%)	76.1	36.3	47.7
H_3PO_4 (3.0%)	83.1	43.7	52.6
formic acid (3.5%)	79.9	44.6	55.8
maleic acid (3.0%)	75.4	41.8	55.5
H-Mordenite (2.6%)	97.6	1.4	1.4

Thus, sugars-rich hydrolysate was acidified by addition of concentrated HCl, H_3PO_4 formic or maleic acid until pH 1–2, to ensure the dissociation of the borate species followed by dilution with

H2O at a ratio 1 to 4. Afterwards, the acidified and diluted hydrolysate was treated at 150 °C for 60 min. According to the experimental results (table 3.5), both the inorganic and the organic acids exhibited similar performance, thus catalyzing fructose conversion to HMF within a range of 75.4–83.1%, reaching yield between 36.3% to 44.6% and a selectivity between 47.7% to 55.8%. Compared to the results in the absence of any catalyst, these results validate the need for inactivating sodium tetraborate to boost the synthesis of HMF and suppress the subsequent decomposition reactions. Other products detected were organic acids formic and levulinic (table 3.5), but with respective yields below 3.8% and 2.6% in all cases, except the one, where formic acid was used as catalyst. In that case, the concentration of the produced formic acid could not be calculated, while the yield and selectivity of levulinic acid reached the highest values of 10.0% and 12.6%, respectively. In fact, among the homogeneous catalysts tested, formic acid presented the best results with HMF yield and selectivity reaching values of 44.6% and 55.8%, respectively, while sugars conversion was 79.9%. Furthermore, to evaluate the possibility of applying heterogeneous catalysis, H-mordenite was added to the sugars-rich hydrolysate, followed by treatment at 150 °C for 60 min. However, it did not have a discernible effect on HMF synthesis probably due to the presence of borate ions, as explained above. Since formic acid offered the highest HMF yield and selectivity, it was studied further regarding the effect of the reaction conditions on the synthesis of HMF from the sugar-rich hydrolysate. Thus, sugar-rich hydrolysate acidified by formic acid, was treated at three levels of temperature ranging from 120 to 175 °C and four different levels of reaction time ranging from 30 to 180 min. The results (figure 3.6) indicated that sugars (i.e. both glucose and fructose) conversion increased with temperature and time, while HMF selectivity and yield presented a maximum at 150 °C for 60 min (55.8% and 44.6%, respectively). At temperatures higher than 150 °C and longer reaction times, HMF selectivity and yield decrease, likely due to the formation of unidentified products (humins), as indicated form the dark brown color of the reaction solution. Consequently, the optimum reaction conditions for HMF selectivity and yield, are 150 °C for 60 min of reaction time. Overall, HMF can be produced from the sugar-rich hydrolysate at 45% yield using formic acid for alleviating the detrimental effect of borate species on the dehydration of sugars. The proposed process starting from actual lignocellulosic biomass results in the synthesis of HMF at satisfactory yields, while it is performed in aqueous solutions, without intermediate separation steps and omitting the use of extreme reaction conditions and inorganic acids, thus complying with the principles of green chemistry and being cost-effective.

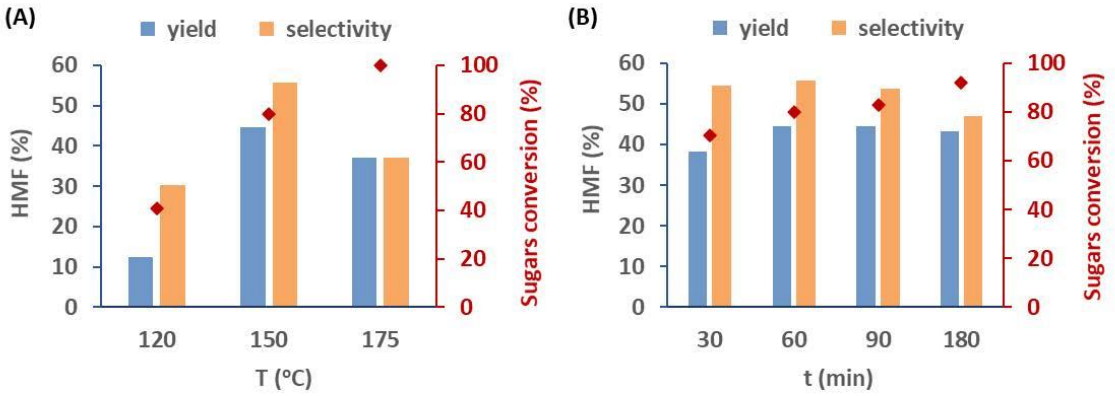


Figure 3.6: Effect of reaction (A) temperature (t=60 min) and (B) time (T=150 ^{o}C) on HMF synthesis from hexoses (glucose 0.5 %w/w and fructose 2.1% w/w) with formic acid catalysts, in the liquid product after isomerization (formic acid 3.5% w/w). (Primary y axis (bars): % HMF yield and selectivity, Secondary y axis (bullets): sugars conversion)

In the concept of lignocellulose valorization towards other high added value products, this work presents a combination of processes to effectively transform lignocellulosic biomass to HMF, including pretreatment, enzymatic saccharification and isomerization as well as chemical dehydration of fructose to HMF. Ultimately, the fact that more than half of lignocellulose was converted to fructose and the subsequent high yield and selectivity of HMF, support the feasibility of the entire process. Designing suitable enzymes that can effectively catalyze the oxidation of HMF can also boost the feasibility of a potential integrated process starting from lignocellulose and leading to biobased monomers.

4. HMF production from polyoxometalate assisted pretreatment of hardwood and softwood biomass.

As mentioned above, lignocellulosic biomass opens a wide range of fields regarding its valorization among which the synthesis of chemicals, food additives, pharmaceuticals and polymeric materials gather a lot of interest (Espro et al., 2021; Sugiarto et al., 2022). Polymeric materials in specific have showed a number of particularly interesting studies in the field of bio-based polymer synthesis including nanocellulose, polylactic acid (PLA), and polyhydroxyalkanoates (PHAs), such as such as poly(3-hydroxypropionic acid) and polyhydroxybutyrate, by combining microbial fermentation and enzyme-mediated processes with chemical catalysis (Chorozian et al., 2022; He et al., 2022; Jin et al., 2022). However, the crucial first step of biomass pretreatment is continuously expanding in order to customize the biomass sample to the needs of the final product. As a result, it is mandatory to also expand the range of pretreatment techniques and explore the respective products. In this context, while the previous chapter examines the oxidative organosolv pretreatment of beechwood in the absence of a catalyst, this chapter focuses on expanding the range of materials by testing not only oxidative organosolv pretreated softwood (pine) samples, but also solid beechwood pulps that are pretreated with isobutanol (iBuOH) as the organic phase of the liquid solvent. In addition, pulps from pretreatment using different POMs as catalysts were also included, in an attempt to lower the pretreatment temperature (Karnaouri et al., 2023), in order to examine the impact of those catalysts on saccharification, isomerization and HMF production. The hydrolysates of the best performing materials were then used in dehydration reactions and the HMF yields were examined. A step-by-step comparison of employing the solid pulp from different pretreatments will allow for evaluating the effect of solid pulps composition and/or the presence of compounds that may affect the production of HMF.

4.1 POM assisted pretreatment of beechwood and pine samples

Polyoxometalates (POMs) constitute a class of highly ordered metal oxide clustered polyanions with unique features and exceptional properties in selective binding/absorption and extraction of metal ions, finding numerous applications in wastewater treatment, pollutants degradation and desulfurization of fuels (Jelinek et al., 2021; Omwoma et al., 2015). Moreover, due to their redox properties and strong Brønsted/Lewis acidity, POMs hold a key role in the development of green chemical transformation pathways for the production of fine chemicals by replacing the mineral acids homogeneous catalysts often used in these processes, while they have been also applied to medicinal chemistry (Bijelic et al., 2019). Their reversible multistep reduction and electron storage properties render them suitable to be used in rechargeable batteries or microbial fuel cells, acting as electron transfer systems that generate electricity (Huang et al., 2020). Within the frame of

biomass valorization, POMs have been used in chemocatalytic conversion of lignocellulosic biomass into chemicals and fuels, including, among others, hydrolysis of polysaccharides, dehydration of monosaccharides to furans, oxidation and hydrogenation of carbohydrates to carboxylic acids or hexitols, respectively (Ponce et al., 2023; Zhong et al., 2021). POMs with different functionalization exhibit different selectivity and products, while their high stability and easy regeneration after utilization render them attractive catalysts in biomass biorefining technologies (Shatalov, 2016). While POMs have been used in chemocatalytic biomass valorization to valuable products, studies employing them in biomass pretreatment as delignification promoting catalysts are limited (Ding et al., 2017; Zhao et al., 2017). Novel structures have recently attracted the interest of the scientific community, e.g. Keggin and Wells–Dawson structures partially substituted with low-cost transition metals, regarding their application in processes with oxidation ability such as alkenes oxidation (Farsani et al., 2015) and other redox demanding processes, such as esterification reactions for biodiesel synthesis applied in liquid environment (Cai et al., 2018; Zhang et al., 2019). Specificity of lignin depolymerization process lies in the fact that the ideal catalyst should have redox properties in a sufficient degree, quite high to oxidize lignin but also low enough to reoxidize (and thus regenerate) it again due to the presence of the oxidizing agent. Based on this premise, POMs consisting of oxidizing metals such as Mo, Cu and Fe are considered promising candidates for depolymerizing lignin in an effective and controllable way (Karnaouri et al., 2023).

This chapter examines the effect of an oxidative organosolv pretreatment process for the production of HMF, implementing four different organic solvents (EtOH, ACO, THF and iBuOH), on lignocellulosic feedstocks of pine and beechwood. A total of 29 samples with different pretreatment conditions were tested regarding their susceptibility to enzymatic saccharification and fructose production, while the best conditions were selected to further HMF yield and selectivity. The exact pretreatment conditions for each sample as well as the compositional analysis of the solid pulps are presented in (table 4.1). By implementing enzymatic saccharification and isomerization, following the process already established in paragraph 3.1 (Dedes et al., 2021), it was possible to effectively produce fructose and HMF from real biomass samples. The evaluation of the pretreatment was performed by correlating the saccharification results for each sample with the three pretreatment parameters: temperature, residence time and type of solvent. In all cases, the initial pressure of pretreatment was 16 bar and oxidation was achieved with the constant flux of oxygen in the chamber. The results from the saccharification and isomerization reactions were organized into two groups depending on the nature of the biomass. The first group includes beechwood biomass samples (Lignocel®) which underwent isobutanol pretreatment at three different temperatures (150 ^{o}C, 160 ^{o}C and 175 ^{o}C) as well as Lignocel® samples in which the polyoxometalates were introduced to the pretreatment solvent at 150 ^{o}C, while the second group includes pine biomass samples which were pretreated with the organic solvents mentioned

above at 175 ^{o}C. Each group's fructose production in g fructose/ 100 g pretreated biomass was depicted in figures 4.1 and 4.2.

Table 4.1: Pretreatment conditions and compositions of pretreated beechwood and pine samples

Catalyst	Solvent	Temperature	Reaction Time	Biomass type	Lignin (%)	Cellulose (%)	Hemicellulose (%)
No Catalyst	iBuOH	150	60	Beechwood	19.8	55.5	25.0
	iBuOH	150	120	Beechwood	12.4	65.9	19.4
	iBuOH	160	60	Beechwood	13.4	68.2	21.6
	iBuOH	160	120	Beechwood	2.6	87.8	16.0
	iBuOH	175	60	Beechwood	1.8	87.4	13.1
	iBuOH	175	120	Beechwood	1.6	92.6	11.6
	ACO	150	120	Beechwood	13.5	62.7	21.0
	EtOH	150	120	Beechwood	15.0	56.7	20.7
	THF	150	120	Beechwood	7.8	73.1	13.3
HPMo	iBuOH	150	120	Beechwood	2.2	93.3	5.3
	ACO	150	120	Beechwood	2.7	89.1	7.9
	EtOH	150	120	Beechwood	4.6	86.6	12.2
	THF	150	120	Beechwood	4.5	90.4	7.7
FePMo	iBuOH	150	120	Beechwood	10.5	78.5	9.8
	ACO	150	120	Beechwood	3.9	82.0	7.0
	EtOH	150	120	Beechwood	4.7	77.7	9.9
CuPMo	iBuOH	150	120	Beechwood	6.1	86.6	8.2
	ACO	150	120	Beechwood	9.7	83.3	6.8
	EtOH	150	120	Beechwood	3.3	90.0	3.3
No Catalyst	iBuOH	175	60	pine	2.4	89.7	2.9
	iBuOH	175	120	pine	3.8	83.6	4.6
	ACO	175	60	pine	4.2	82.8	10.1
	ACO	175	120	pine	2.5	89.2	7.8
	EtoH	175	60	pine	7.2	77.6	13.2
	EtOH	175	120	pine	6.4	79.6	9.7
	THF	175	60	pine	26.8	60.8	6.7
	THF	175	120	pine	17.6	71.3	6.8

4.2 Enzymatic saccharification of pretreated beechwood pulps and production of fructose

The results of the saccharification and isomerization experiments of biomasses pretreated with different metal catalysts are depicted in table 4.2. Metal catalysts examined were a commercially available phosphomolybdic acid ($H_3PMo_{12}O_{40}$ x H_2O, HPMo), while the other two were custom made salts by replacing H_2 with metal ions from Fe ($Fe_3MO_{12}OP$) and Cu ($Cu_3MO_{12}OP$) (Karnaouri et al., 2023). The cellulose conversion of samples pretreated with iBuOH was 52.36% with the absence of catalyst, dropping to 48.79% for the HPMo catalyst, followed by an increase to 53.06% and 53.43% for Fe-PMo and Cu-PMo, respectively. The corresponding results for ACO were 57.69% (no catalyst), 55.53% (HPMo), 58.22% (Fe-PMo) and 41.04% (Cu-PMo), while for EtOH cellulose conversions results were 57.72% (no catalyst), 56.95% (HPMo), 56.90% (Fe-PMo) and 46.95% (Cu-PMo). Finally, THF as a solvent showed a very high cellulose conversion yield, reaching 79.02% in the absence of catalyst, however, a significant drop in the presence of HPMo at 49.11% discouraged further examination with the rest of the catalysts. These results indicate that the use of HPMo, as well as its metal substituted structures, does not necessarily contribute positively to enzymatic processes following pretreatment. In this work, the concept of implementing metal catalysts in the pretreatment process was conceived due to their redox potential and their ability to interact with lignin as electron donors in order to achieve high delignification and cellulose content in the residual pulps (Ponce et al., 2023; Zhong et al., 2021). As a result, the oxidation of lignin would lead to its removal in the pretreatment process, and indeed in all runs the addition of catalyst increased the cellulose content of the pulp according to table 4.2. Nevertheless, diluted POMs present in the pretreatment solvent may have been absorbed and incorporated in the solid pulp during pretreatment. As a result, these catalysts can play a role in the subsequent enzymatic processes due to the well-established interactions between POMs and enzymes from previous studies (Gil & Carbó, 2022; Gumerova & Rompel, 2021). In their review, Lentink et al. (2023) mention that these interactions are largely governed by factors such as the ionic strength of the medium, the pH, the temperature, the presence of other species such as surfactants and, in the case of metal-substituted POMs, the nature of the metal ion. In addition, the negative charge of POMs can also interact with the positively charged amino acids in the enzyme sequence resulting in the hydrolysis of peptide bonds or the oxidation of amino acids and, in turn, enzyme inhibition (Absillis & Parac-vogt, 2012). While this can be very useful in fields demanding high purity protein isolation such as crystallography or medicine (isolation of proteins from complex matrices such as human blood or tissue), in this case POMs can have a detrimental effect to the overall yield of the process. For example, cellulose conversion, which is the means for saccharification evaluation, seems to drop upon use of HPMo, with THF exhibiting a drop in cellulose conversion of nearly 20%. This also seems to be the case for Cu-PMo as cellulose conversion remains on the same levels only for iBuOH as a solvent. In addition, the same pattern is also passed down to the isomerization experiments. While glucose conversion to fructose usually

ranges between 70%-80%, the use of HPMo and CuPMo seems to inhibit isomerization, resulting in cellulose conversion values below 70%. This can possibly be attributed to the nature of the metal catalyst utilized. Keggin-type POMs, such as the one used in this work, have been observed demonstrating exceptional absorption affinity towards proteins in 0,05 M Tris-HCl buffer (Chen et al., 2015). As a result, small catalyst residues present in the samples may have absorbed the biocatalysts of saccharification and isomerization removing them from the reaction and, therefore, reducing their yield. In addition, the presence of copper ions in metal POMs, has been shown to produce reactive oxygen species in the vicinity of the protein binding site resulting in protein fragmentation (Abdelhameed et al., 2023). Hence, traces of Cu-PMo may induce cleavage of cellulases or the glucose isomerase lowering their activity. Nevertheless, Fe-PMo does not seem to affect enzymatic activity in these processes. In fact, in the case of iBuOH and ACO, cellulose conversion is slightly increased, a result that can be reinforced by studies reporting the upregulation of EG activities (Cano-Ramírez et al., 2016; Chinedu et al., 2011).

Table 4.2: Description of pretreatment conditions, enzymatic digestibility of obtained pulps after treatment with Cellic® CTec2 and glucose isomerization to fructose yields with Sweetzyme® of OxiOrganosolv pretreated beechwood samples. All experiments were run in duplicates and numbers represent the mean values.

Catalyst	Organic solvent	Pretreatment conditions	% Cellulose content in pulp	% Cellulose conversion to glucose	% Glucose conversion to fructose
No catalyst	iBuOH	150°C, 60 min	55.54	52.36 ± 3.09	73.06 ± 1.39
		150°C, 120 min	65.87	62.78 ± 0	73.6 ± 0.1
		160°C, 60 min	68.16	66.67 ± 3.14	70.7 ± 3.0
		160°C, 120 min	87.75	73.39 ± 0	79.5 ± 0.2
		175°C, 60 min	87.42	68.20 ± 1.47	79.8 ± 0.3
		175°C, 120 min	92.6	70.07 ± 0.56	84.6 ± 1.2
	ACO	150°C, 60 min	62.74	57.69 ± 2.39	78.04 ± 5.32
	EtOH	150°C, 60 min	56.72	57.72 ± 0.29	78.11 ± 2.11
	THF	150°C, 60 min	73.09	79.02 ± 1.70	74.17 ± 0.01
HPMo	iBuOH	150°C, 60 min	93.3	48.79 ± 2.44	64.71 ± 2.59
	ACO	150°C, 60 min	89.13	55.53 ± 1.27	60.41 ± 2.36
	EtOH	150°C, 60 min	86.63	56.95 ± 0.44	64.75 ± 0
	THF	150°C, 60 min	90.39	49.11 ± 2.37	61.46 ± 6.59
Fe-PMo	iBuOH	150°C, 60 min	78.48	53.06 ± 0.67	74.66 ± 1.8
	ACO	150°C, 60 min	81.96	58.22 ± 0.8	77.17 ± 1.15
	EtOH	150°C, 60 min	77.74	56.90 ± 1.86	77.9 ± 0.72
Cu-PMo	iBuOH	150°C, 60 min	86.58	53.43 ± 2.12	74.72 ± 1.07
	ACO	150°C, 60 min	83.3	41.04 ± 3.94	68.53 ± 2.34
	EtOH	150°C, 60 min	89.9	46.95 ± 1.46	68.98 ± 0.51

Table 4.3: Hydrolysis yields and glucose isomerization to fructose for different OxiOrganosolv pretreated beechwood samples. All experiments were run in duplicates and numbers represent the mean values.

Catalyst	**Organic solvent**	**Pretreatment conditions**	**Glucose after hydrolysis (g/L)**	**Fructose after isomerization (g/L)**	**g of fructose/100 g of pretreated biomass**	**g of fructose/100 g of untreated biomass**
No catalyst	iBuOH	150°C, 60 min	56.2 ± 0.6	41.3 ± 0.3	21.2 ± 0.2	20.5 ± 0.1
		150°C, 120 min	51.04 ± 0	37.6 ± 0.1	30.4 ± 0.1	18.6 ± 0
		160°C, 60 min	56.09 ± 2.64	39.6 ± 3.6	32.1 ± 2.9	20 ± 1.8
		160°C, 120 min	79.5 ± 0	63.2 ± 0.2	51.2 ± 0.1	24.2 ± 0.1
		175°C, 60 min	73.60 ± 1.59	58.7 ± 1.6	47.6 ± 1.3	22.6 ± 0.6
		175°C, 120 min	80.10 ± 0.63	67.8 ± 1.7	54.9 ± 1.4	23.8 ± 0.6
	ACO	150°C, 60 min	44.7 ± 0.2	34.9 ± 0.5	28.3 ± 0.4	26.3 ± 0.4
	EtOH	150°C, 60 min	40.4 ± 1.6	31.5 ± 0.9	25.5 ± 0.8	19.3 ± 0.6
	THF	150°C, 60 min	71.3 ± 1.5	52.9 ± 1.2	42.8 ± 1.0	24.5 ± 0.6
HPMo	iBuOH	150°C, 60 min	56.2 ± 2.8	36.4 ± 0.3	29.5 ± 0.2	12.9 ± 0.1
	ACO	150°C, 60 min	61.1 ± 1.40	36.9 ± 1.9	29.9 ± 1.6	13.6 ± 0.7
	EtOH	150°C, 60 min	60.9 ± 0.47	39.4 ± 0.3	31.9 ± 0.2	16.1 ± 0.1
	THF	150°C, 60 min	54.8 ± 2.65	33.7 ± 4.9	27.3 ± 3.9	13.1 ± 1.9
Fe-PMo	iBuOH	150°C, 60 min	51.4 ± 0.6	38.4 ± 1.9	31.1 ± 1.5	17.0 ± 0.8
	ACO	150°C, 60 min	58.9 ± 0.8	45.5 ± 0.3	36.8 ± 0.3	17.7 ± 0.1
	EtOH	150°C, 60 min	54.6 ± 1.8	42.5 ± 1.9	34.5 ± 1.5	17.4 ± 0.8
Cu-PMo	iBuOH	150°C, 60 min	57.1 ± 2.3	42.7 ± 2.5	34.6 ± 2.0	17.6 ± 1.0
	ACO	150°C, 60 min	42.2 ± 4.0	28.9 ± 3.9	23.4 ± 3.1	11.8 ± 1.6
	EtOH	150°C, 60 min	52.1 ± 1.6	35.9 ± 0.8	29.1 ± 0.7	13.3 ± 0.3

The results of both table 4.3 and figure 4.1 also offer insight on the dependance of beechwood hydrolysis and isomerization on pretreatment time and temperature for iBuOH. Firstly, it is apparent that, in any case, longer pretreatment times lead to higher cellulose percentages in the pulps and better cellulose conversions. In fact, harsher pretreatment conditions lead to higher cellulose content and, in the case of 175 ^{o}C and 120 min, cellulose content in the pulp reaches 92.6%. This exhibits a difference of more than 30% between the harshest and the mildest pretreatment conditions, as the run of 150 ^{o}C and 60 min leads to 55.54% cellulose in the pulp. In addition, cellulose conversion follows the same trend with the highest conversion being 73.39% (160 ^{o}C, 120 min) followed by the second highest 70.07% (175 ^{o}C, 120 min). However, if we take into consideration the isomerization results, where the 175 ^{o}C, 120 min run shows the highest glucose to fructose conversion of 84.6%, it is evident that this sample also produced the highest amount of fructose with 54.9 g fructose/ 100 g pretreated material. Moreover, as depicted in figure 4.1, there is a sudden jump in the production of fructose from 32.1 g fructose/ 100 g pretreated sample (160 ^{o}C, 60 min) to 51.2 (160 ^{o}C, 120 min), 47.6 (175 ^{o}C, 60 min) and 54.9 (175 ^{o}C, 120 min). This can partially be explained to the glucose concentration, which follows the same trend and is the substrate for fructose production, nevertheless glucose to fructose conversion is also higher in runs with harsher pretreatment conditions as opposed to milder ones. These results are comparable to the work of Saikia et al. (2020) who implemented a system for the production of fructose through the direct hydrolysis of inulin. In their study, the optimum fructose production was 65.7 g fructose/ g inulin, albeit through the hydrolysis of a pure fructose polymer. Furthermore, present work shows significantly higher fructose yields than studies implementing custom made chemical catalysts for the isomerization of fructose. For example, Zhang et al. (2020) were able to reach 32.58% fructose yield from pure glucose using a custom Al-hydrochar catalyst for 160 ^{o}C for 20 min, while Yang et al. (2022) implemented a similar Ca-hydrochar catalyst to reach 31% fructose yield at 120 ^{o}C for 45 min. Consequently, this study presents an effective way to produce fructose selectively from a complex system such as lignocellulose, while simultaneously achieving it in mild conditions, a prerequisite for enzymatic procedures.

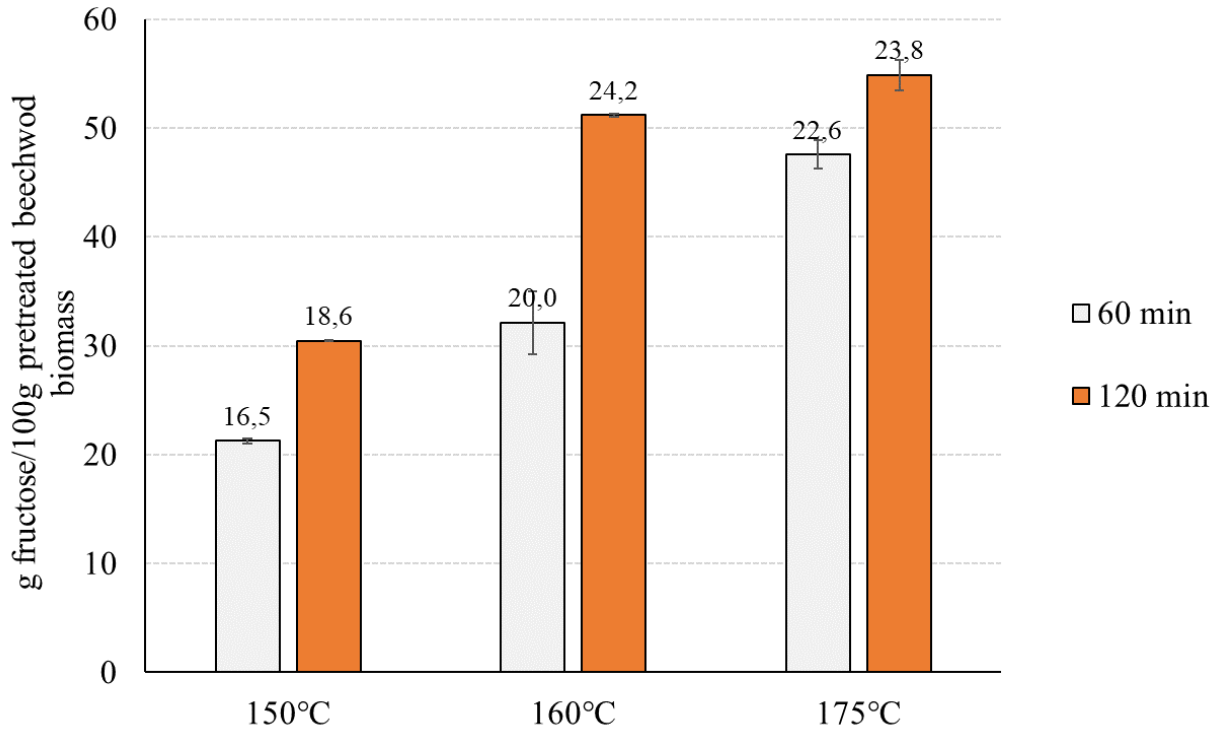


Figure 4.1: Production of fructose per 100 g of pretreated solid pulp after enzymatic hydrolysis with Cellic® CTec2and isomerization with Sweetzyme® of OxiOrganosolv pretreated beechwood samples with iBuOH at three different cooking temperatures (150, 160, 175ºC) and two residence times (60, 120 min). Labels indicate the production of fructose (g) per 100 g of initial untreated beechwood.

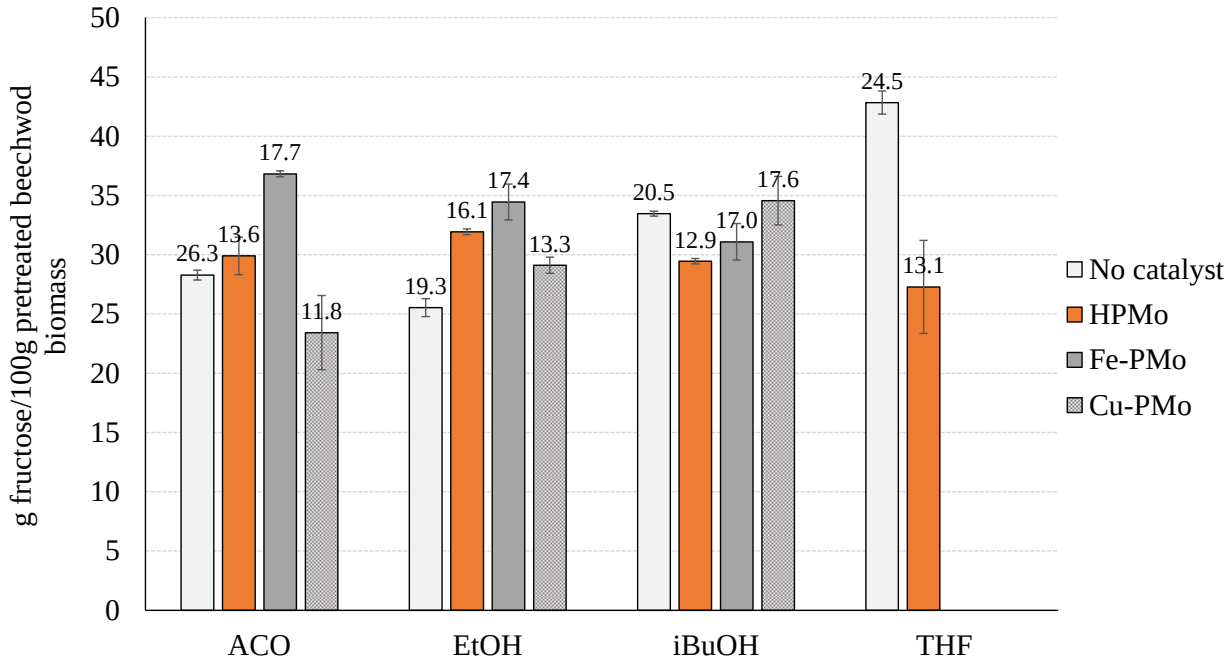


Figure 4.2: Production of fructose (g) per 100 g of pretreated solid pulp after enzymatic hydrolysis with Cellic® CTec2 and isomerization with Sweetzyme® of OxiOrganosolv pretreated beechwood samples (150 ºC, 60 min, O2 16 bar) with four different organic solvents and POMs catalysts at a loading of 5%. All experiments were run in duplicates and numbers represent the mean values. Labels indicate the production of fructose (g) per 100 g of initial untreated beechwood.

4.3 Enzymatic saccharification of pretreated pine pulps and production of fructose

The results of pine hydrolysis indicate that, while being susceptible to hydrolysis, pine as a feedstock leads to lower fructose production. Firstly, beechwood biomass, which was pretreated at milder conditions (150 ºC, 60 min), exhibited cellulose conversions above 50% for every solvent (69.12% for iBuOH, 57.69% for ACO, 57.72% for EtOH and 79.02% for THF). The respective cellulose conversion results for pine samples pretreated at 175 ºC were 62.83%, 46.79%, 67.02%, 31.71%) indicating that the use of EtOH at high temperatures is preferable, contrary to ACO and THF, which appear to perform better at low temperatures as it is also observed in previous work (Dedes et al., 2021). In addition, cellulose conversion of pine samples at higher pretreatment durations seems to be on par with the corresponding beechwood samples for iBuOH and EtOH, but significantly lower for ACO and THF (table 4.4). These low saccharification yields for THF can be attributed to the high lignin content of the pretreatment sample, that works as a binding layer for the hydrolytic enzymes of cellulose, inhibiting their function (Vermaas et al., 2015). In the case of ACO, the cellulose conversions might be the result of enzyme inhibitors produced in the pretreatment process. These inhibitors are essentially degradation products formed in different amounts depending on the nature of the pretreatment and have been found to negatively affect the

ensuing downstream biocatalytic processes (Jönsson & Martín, 2016). However, if the isomerization process is taken into consideration, it is evident that iBuOH as a pretreatment solvent not only assists the saccharification process, but also boosts the isomerase activity leading to higher fructose yields. In specific, while the pattern of glucose-to-fructose conversion fluctuating between the range of 70-80%, in the iBuOH pretreated pine samples it increases above 80%, effectively reaching 86.4% for 60 min residence time and 87.3% for 120 min residence time. This in turn translates to 19.3 and 20.3 g fructose/ 100 g untreated biomass, respectively, which shows nearly a twofold increase than most of the rest of the pine samples. In addition, while in most cases beechwood appears to be better as a feedstock, the 60 min iBuOH pretreated pine sample exhibits a 48.7 g fructose production/ 100 g of pretreated biomass, which is higher than the corresponding beechwood sample (47.6 g fructose/ 100 g pretreated biomass) as depicted in figure 4.3.

Table 4.4: Description of pretreatment conditions, enzymatic digestibility of obtained pulps after treatment with Cellic® CTec2 and glucose isomerization to fructose yields with Sweetzyme® of OxiOrganosolv pretreated pine samples. All experiments were run in duplicates and numbers represent the mean values.

Organic solvent	**Pretreatment conditions**	**% Cellulose content in pulp**	**% Cellulose conversion to glucose**	**% Glucose conversion to fructose**
iBuOH	175°C, 60 min	89.68	62.83 ± 0.49	86.4 ± 0.1
	175°C, 120 min	83.64	73.13 ± 1.02	87.3 ± 0.4
ACO	175°C, 60 min	82.75[a]	46.79 ± 1.31	73.2 ± 1.1
	175°C, 120 min	89.17[a]	36.97 ± 0.57	79.6 ± 0.1
EtOH	175°C, 60 min	77.6[a]	67.02 ± 0.82	74.5 ± 0.5
	175°C, 120 min	79.57[a]	56.60 ± 0.5	75.7 ± 1.7
THF	175°C, 60 min	60.8[a]	31.71 ± 0.30	73.9 ± 0.4
	175°C, 120 min	71.31[a]	38.17 ± 0.89	76.1 ± 0.7

Table 4.5: Hydrolysis yields and glucose isomerization to fructose for different OxiOrganosolv pretreated pine samples. All experiments were run in duplicates and numbers represent the mean values.

Organic solvent	Pretreatment conditions	Glucose after hydrolysis (g/L)	Fructose after isomerization (g/L)	g of fructose/100 g of pretreated biomass	g of fructose/100 g of untreated biomass
iBuOH	175°C, 60 min	69.6 ± 0.5	60.1 ± 0.5	48.7 ± 0.4	19.3 ± 0.2
	175°C, 120 min	75.5 ± 1.1	65.9 ± 1.3	53.4 ± 1	20.3 ± 0.4
ACO	175°C, 60 min	47.8 ± 1.3	35 ± 1.5	28.3 ± 1.2	12.7 ± 0.5
	175°C, 120 min	40.7 ± 0.6	32.4 ± 0.5	26.3 ± 0.4	10.9 ± 0.2
EtOH	175°C, 60 min	64.2 ± 0.8	47.8 ± 0.8	38.7 ± 0.7	19.7 ± 0.3
	175°C, 120 min	55.6 ± 4.9	42.1 ± 4.7	34.1 ± 3.8	16.2 ± 1.8
THF	175°C, 60 min	23.8 ± 0.2	17.6 ± 0.3	14.2 ± 0.2	7.9 ± 0.1
	175°C, 120 min	33.6 ± 0.8	25.6 ± 0.9	20.7 ± 0.7	10.5 ± 0.4

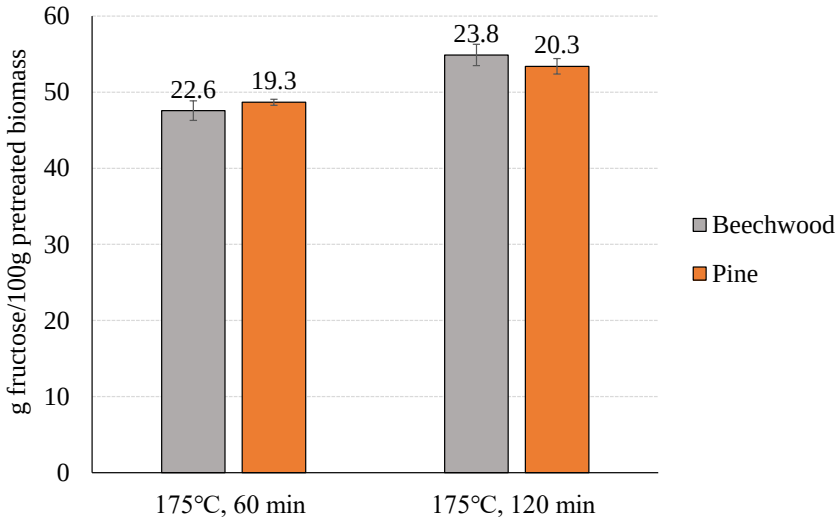


Figure 4.3: Comparison between beechwood and pine regarding the production of fructose (g) per 100g of pretreated solid pulp after enzymatic hydrolysis with Cellic® CTec2 and isomerization with Sweetzyme® with iBuOH at 175ºC for two different residence times (60, 120 min). All experiments were run in duplicates and numbers represent the mean values. Labels indicate the production of fructose (g) per 100 g of initial untreated beechwood.

4.4 Production of HMF

Optimum sugars-rich hydrolysates originated after enzymatic isomerization, were investigated as substrates for the production of HMF. Prior to reaction, the samples were acidified by adding concentrated formic acid until reaching pH of 1–2, since this particular acid was identified as the most appropriate among those tested for the dissociation of the borate species of sodium tetraborate in order to minimize its negative effect on the production of HMF (Dedes et al., 2021). Afterwards, the acidified hydrolysates containing mainly fructose and lower amounts of glucose were diluted with H_2O at a ratio 1 to 4 and treated at 150 °C for 60 min. Under the reaction conditions employed, sugars were effectively converted to HMF in all cases, with respective values of selectivity and yield ranging between 13 – 49 % (Table 4.6) and 12 - 40 % (Table 4.7). Other detected products (Table 4.7) were traces of mannose (yield below 4 % in any case), an isomer of glucose and fructose produced via epimerization, and organic acids levulinic and formic, formed through rehydration of HMF in a 1:1 molar ratio. The yield of levulinic acid remained below 15 % in all cases (Table 4.7). Regarding formic acid, its concentration could not be directly determined, due to its role as the reaction catalyst and only an assumption could be made based on the reaction stoichiometry (nominal values in Table 4.7). Apart from the liquid products, unidentified solid by-products of dark brown color (humins), were also formed, revealing that HMF degradation side-reactions also occurred. These solids were removed from the reaction mixture though filtration, washed with water and, after drying at 100 ^{o}C overnight, were weighed and subjected to carbon analysis. According to experimental results, almost all of them consisted of 60-65% of carbon, while their yield was below 7,5% (weight based) in all cases (Table 4.7). However, the sum of humins and products detected by IC analysis did not complete the mass balance, suggesting the presence of unidentified liquid products such as water-soluble oligomers (could not be measured) and water-soluble humins precursors as indicated by the dark brown color of the reaction mixtures (van Zandvoort et al., 2013).

The sugar-rich hydrolysates which were classified into two groups: beechwood hydrolysates and pine hydrolysates, depending on the biomass used as the initial feedstock for saccharification, were selected in order to infer conclusions regarding the impact of both the feed and the pretreatment method on HMF production. Starting from the first group, hydrolysates derived from beechwood pretreated with THF and iBuOH at 150 °C in the absence of a catalyst yielded similar HMF production, with respective values of 8.7 g and 8.3 g/ 100 g pretreated biomass. However, in the case of the No. 1 hydrolysate, there was a higher sugar conversion (82 %) and greater selectivity toward HMF (49% based on sugars) compared to the No. 4 hydrolysate (75% and 39% based on sugars), without a simultaneous significant increase in byproducts (Table 4.6). Considering the better performance of the No. 1 hydrolysate, the impact of the pretreatment temperature was further examined by using samples obtained from beechwood after pretreatment at three different temperatures: 150 °C (No. 1), 160 °C (No. 2), and 175 °C (No. 3). The results, presented in Table 4.6, indicate that with an increase in pretreatment temperature, there is a slight reduction in both

sugar conversion and HMF selectivity. However, the production of HMF from the pretreated feedstock doesn't exhibit the same trend as its values increase from 8 to 14 % w/w. The pretreatment temperature appears to exert some influence in the composition/structure of the initial feedstock, consequently affecting the behavior of the hydrolysates during subsequent HMF production.

The effect of POM pretreatment catalysts on subsequent process steps of the process was also evaluated by using optimal hydrolysates received after pretreatment of beechwood with HPMo (No 5 and 6), FePMo (No 7) and CuPMo (No. 8 and 9). Additionally, ICP-OES analysis was conducted to investigate the potential presence of metals that may have leached from the pretreatment catalysts and reached the hydrolysates during the prior process steps (Table 4.8). According to the ICP-OES analysis of the liquid samples, the Molybdenum (Mo) content in hydrolysates No. 1 and 2 was found to be negligible (Table 4.8), suggesting that the presence of this metal does not impact HMF production. Nonetheless, the presence of the catalyst during pretreatment appears to influence the feedstock, as the resulting hydrolysates exhibited lower HMF selectivity (32% and 33% respectively), despite achieving higher sugar conversion (81% and 84% respectively) when compared to those derived from feedstocks pretreated without the presence of a catalyst (Table 4.6). Furthermore, the production of HMF was 5.9 g and 5.8 g/100 g pretreated feedstock respectively, values lower when compared to the ones received from hydrolysates No. 1 and 3 (Table 4.6). This trend was also observed by the No. 7 hydrolysate, where sugars conversion reached its highest value of 93%, but with both the lowest selectivity (13%) and HMF yield based on the pretreated biomass (3.1 % w/w). In this case, the ICP-OES analysis revealed the presence of Mo and iron (Fe) in the reaction medium (Table 4.8), metals that promote side reactions leading to the formation of organic acids (levulinic and formic) and humins, as also indicated by the highest selectivity of these products (Table 4.7). Hydrolysates No. 8 and 9 appear to be more efficient in HMF production compared to the above-mentioned hydrolysates, especially the one with iBuOH, which exhibited high HMF selectivity (46%) and increased yield (8.4 g/ 100 g pretreated feedstock), both comparable to those achieved by No. 1 (49% and 8.3 g/100 g pretreated feedstock, respectively) without any pretreatment catalyst. From the perspective of metal content, both hydrolysates were found to contain nearly the same amount of copper (Cu) but varying amounts of Mo. Notably, the hydrolysate derived from feedstock pretreated with iBuOH exhibited a lower concentration of Mo, suggesting that iBuOH may enhance the catalyst's stability, resulting in negligible Mo leaching. It is likely that the presence of copper in low quantities contributes to the enhanced production of HMF, which contrasts with the roles of Fe and Mo, which further promote the reaction.

In the case of second group, which comprised hydrolysates derived from pine, the selected samples underwent pretreatment without the use of a catalyst at 175 ^{o}C for 120 min, with EtOH (No. 10), ACO (No. 11) and iBuOH (No. 13) respectively. The harsher reaction condition applied in pretreatment in order to increase the susceptibility of pine to saccharification. According to

experimental data (Table 4.6), hydrolysate No. 10 presented the highest HMF selectivity (50%) among the tested hydrolysates, converting sugars to HMF at 39%. Nevertheless, in this case, the overall yield of HMF from pretreated biomass was 9% w/w, slightly lower compared to 11% w/w obtained by hydrolysate No. 13. Regarding the iBuOH pretreated hydrolysates, the one received after pine pretreatment at the same reaction temperature (175 ^{o}C), but in lower reaction time (60 min), namely hydrolysate No. 12, was also examined for the production of HMF, presenting the exact same trend with the one observed in No. 13. This fact implies that pretreatment time with iBuOH in pine does not have a significant effect on the subsequent production of HMF. On the other hand, hydrolysate No. 11 demonstrated a different behavior compared to the other hydrolysates leading to a reduced conversion of sugars (60%) and the lowest overall yield of HMF from the pretreated biomass (5% w/w). In addition, the production of organic acids was enhanced, even though the selectivity of HMF reached almost 44%.

In summary, it was demonstrated that the production of HMF is favored by hydrolysates originated after the pretreatment of both feedstocks with iBuOH as organic solvent. In a comparison between the beechwood and pine, in the absence of pretreatment catalyst, beechwood proved more efficient for the production of HMF resulting in highest yield of 14 g / 100 g pretreated biomass. Regarding the hydrolysates obtained from pulps pretreated upon addition of POM catalysts, the highest HMF yield and selectivity were both observed by the hydrolysate obtained from beechwood pretreated with iBuOH upon addition of Cu-PMo catalyst, achieving 46% (selectivity) and yield of 8.4 g /100 g pretreated feedstock. The presence of Fe and Mo seems to suppress the production of HMF, most probably favoring the formation of degradation products, such as humins, organic acids and other soluble compounds. This is an interesting observation highlighting the effect of different catalysts on the products and subsequently the entirety of the valorization process. In the present study, hydrolysates obtained from beechwood biomass pretreated with Cu-PMo catalyst can serve as a starting material for HMF synthesis with relatively good yield and selectivity, while in another study on polyoxometalate pretreatment (Karnaouri et al., 2023), the presence of copper ions in the hydrolysates rendered this pulp cytotoxic and, thus not suitable for microbial fermentation. On the contrary, hydrolysates from beechwood biomass pretreated with Fe-PMo catalyst can be successfully used as carbon sources for microorganisms for the production of valuable products, however in this study it was shown that the formation of HMF is inhibited. These results suggest that the use of different POMs may affect not only the composition but also the quality of the pulp constituents and, consequently, determine the possible scenarios for its valorization.

Table 4.6: HMF synthesis from sugars in the liquid product after dehydration with formic acid (Reaction conditions: 150 °C, 60 min). All experiments were run in duplicates and numbers represent the mean values. Standard error deviation was ≤ 2.5% in all measurements.

Hydrolysate No.	Organic Solvent	Catalyst	Pretreatment conditions	Sugars conversion (%)	HMF Selectivity (%)	g HMF/ 100 g pretreated biomass
Lignocel						
1	iBuOH	no catalyst	150 ºC, 120 min	82.4	49.0	8.3
2	iBuOH	no catalyst	160 ºC, 120 min	75.9	47.8	13.0
3	iBuOH	no catalyst	175 ºC, 120 min	71.7	39.3	14.1
4	THF	no catalyst	150 ºC, 60 min	74.7	38.9	8.7
5	EtOH	HPMo	150 ºC, 60 min	81.2	32.4	5.9
6	iBuOH	HPMo	150 ºC, 60 min	84.1	33.3	5.8
7	ACO	Fe-PMo	150 ºC, 60 min	92.9	13.1	3.1
8	EtOH	Cu-PMo	150 ºC, 60 min	78.6	36.1	5.8
9	iBuOH	Cu-PMo	150 ºC, 60 min	75.3	45.9	8.4
Pine						
10	EtOH	no catalyst	175 ºC, 120 min	77.6	49.9	9.2
11	ACO	no catalyst	175 ºC, 120 min	60.3	43.5	5.0
12	iBuOH	no catalyst	175 ºC, 60 min	73.7	39.5	9.9
13	iBuOH	no catalyst	175 ºC, 120 min	74.1	40.8	11.3

Table 4.7: Sugars conversion to HMF and other side-products in the presence of formic acid as a catalyst (Reaction conditions: 150 °C, 60 min). All experiments were run in duplicates and numbers represent the mean values. Standard error deviation was ≤ 2.5% in all measurements.

Hydrolysate No.	Products (g/L)				Selectivity (%)			
	HMF	Mannose	Levulinic acid	Formic acid*	Mannose	Levulinic acid	Formic acid*	HMF
Lignocel								
1	8.4	0.4	2.1	4.3	2.4	12.6	25.2	40.3
2	5.6	0.3	1.3	2.6	2.3	11.1	22.2	36.3
3	5.6	0.3	1.2	2.4	2.4	8.6	17.2	28.2
4	4.1	0.1	1.3	2.6	0.8	12.2	24.4	29.1
5	6.8	1.1	2.1	4.2	5.0	9.9	19.9	26.3
6	3.8	0.3	1.6	3.1	2.8	13.7	27.5	28.0
7	3.2	0.5	4.0	8.0	2.0	16.6	33.2	12.2
8	3.3	0.3	1.2	2.4	2.8	13.2	26.4	28.4
9	9.0	0.8	2.1	4.3	3.9	10.9	21.8	34.5
Pine								
10	9.4	0.8	0.7	1.5	4.5	3.8	7.7	38.7
11	2.5	0.2	1.0	2.0	4.2	17.5	34.9	26.3
12	4.0	0.2	1.1	2.2	2.4	10.6	21.2	29.1
13	4.5	0.4	1.1	2.2	3.9	9.7	19.4	30.2

*Nominal values considering the stoichiometry of HMF rehydration to formic acid and levulinic acid
**Calculations based on weight

Table 4.8: Molybdenium (Mo), iron (Fe) and copper (Cu) originating from POM catalysts during pretreatment, dehydration and isomerization processes within the hydrolysates

Hydrolysate No.	ICP-OES (ppm)		
	Fe	Cu	Mo
5	-	-	0
6	-	-	4
7	77	-	405
8	-	38	59
9	-	44	8

5. Production of fructose from microwave pretreated corn stover

As it has already been established, the development of a proper pretreatment process is essential to achieve the desired end-product. Tampering with the parameters of temperature, solvent, catalyst and residence time can often be either extremely efficient, leading to a lignocellulosic pulp highly amenable to enzymatic saccharification, or detrimental, consequently inhibiting enzymatic activities and lowering the overall end-product yields. However, there are other secondary parameters that can potentially affect pretreatment, such as biomass granulation or the introduction of gas in the pretreatment chamber as well as the nature of the gas, that are usually overlooked. One such parameter is also the nature of the heating mechanism. Microwaves are a heating mechanism whose main advantage lies in their ability to increase temperature in much shorter amounts of time, depending on the power applied, as compared to the conventional resistance mediated heating. In addition, it has not yet been revealed whether the application of microwaves affects the structure of lignocellulose differently and what this means in terms of saccharification or isomerization. As a result, this chapter examines the application of microwaves for the organosolv pretreatment of corn stover samples, in an attempt to firstly, discover the efficacy of this process when compared with previous hydrothermal pretreatments and secondly, compare its effect on lignocellulose in contrast to resistance mediated pretreatment.

5.1 Compositional analysis of untreated corn stover biomass

The compositional analysis of all lignocellulosic biomass samples in this study was performed according to the protocols on NREL. Untreated corn stover biomass was found to contain 38.8±0.9 % cellulose, 23.5±1.1% hemicellulose, 20.2±0.7% lignin and 5.3±0.2% ash on a dry basis. These results are in accordance with corn stover compositions found in literature (Avci et al., 2013; Zu et al., 2014).

5.2 Screening of microwave organosolv pretreatment conditions for corn stover biomass

Pretreatment is the process that aims to separate and remove fractions of the plant cell wall, such as hemicellulose and lignin, in order to render the recalcitrant untreated biomass more amenable to enzymatic saccharification (Galbe & Zacchi, 2002). It is considered a stage of pivotal importance in lignocellulose valorization due to the inhibition of enzymatic activity by the aforementioned fractions. Organosolv pretreatment aims to remove parts of both lignin and hemicellulose by using mixtures of water (where hemicellulose is soluble) and organic solvents (where lignin is soluble), such as acetone, ethanol and isobutanol as the liquid fraction. The aim of this work is to examine the effect of organosolv pretreatment to an in-house lignocellulosic

biomass source as well as compare the results of an alternative heating mechanism to the traditional resistance mediated heating.

In this chapter, corn stover underwent said organosolv pretreatment with the aim to discover the ideal conditions for glucose release and subsequently fructose yield after isomerization. Pretreatment experiments took place in an Anton Paar Monowave 450 reactor where the heating was accomplished through microwave radiation. Pretreatment temperature was set to 150 ^{o}C, which is relatively low for pretreatment processes, in order to avoid high pressures in the chamber due to the low boiling point of the liquid fraction. This was imperative for the viability of the process as pressure was measured through the deformation of the septum cap and, as a result, the cap had no means of being reinforced in order to withstand high pressures. In addition, the pretreatment solvent was a mixture of 50:50 H_2O/ACO with a boiling point of 64.5 ^{o}C at atmospheric pressure. As a result, runs at temperatures above 150 ^{o}C formed high pressures that the septum could not withstand therefore exploding and releasing the mixture in the chamber. Hence, microwave pretreatment was evaluated on two other parameters: the residence time and the presence and ratio of acetic acid as a catalyst. Acetic acid concentration varied from 0% to 1.5% and for each acetic acid concentration two residence times of 60 min and 120 min were studied. Furthermore, glucose release for each pulp was studied by hydrolysis screening experiments using a solids loading of 3% w/w and an enzyme loading of 15 mg enzyme/ g DM for 24 h. The compositional analysis and the % solubilization of each pulp during the pretreatment are depicted in table 5.1.

Table 5.1: Screening of corn stover pretreatment conditions on the composition of the resulting pulps

Biomass No.	Acetic acid content (%)	Temperature (^{o}C)	Time (min)	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Solubilization (%)	Total mass closure (%)
1	0	150	60	34.9	19.0	18.5	23.9	72.5
2	0	150	120	35.0	18.5	20.2	28.1	73.7
3	0.5	150	60	31.8	17.0	20.9	25.1	69.8
4	0.5	150	120	36.0	16.7	18.4	28.5	71.1
5	1	150	60	36.6	18.6	18.0	25.9	73.1
6	1	150	120	37.3	16.5	17.6	40.5	71.4
7	1.5	150	60	38.1	19.0	15.5	26.2	72.7
8	1.5	150	120	37.0	15.6	18.6	33.2	71.2
Untreated	-	-	-	38.8	23.5	20.2	-	-

The results indicate that in most cases pretreatment was able to remove parts of both the hemicellulose and lignin fractions, especially in more severe pretreatment conditions where the acetic acid content was higher. However, in all cases the cellulose content of each pulp was also lower than that of the untreated biomass. This can be explained due to the high amounts of solubilization as more than 20% of the initial biomass was channeled to the liquid fraction of the pretreatment. It is evident that the addition of acetic acid as a replacement of O_2 as a catalyst resulted in inferior results concerning the delignification of biomass. Katsimpouras et al. (2018) examined the acetone/water oxidation of corn stover at 175 ^oC for three different pressure values of 8.5 atm, 20 atm and 40 atm at room temperature. In their work they mention that the high delignification of the biomass to two parameters: hemicellulose hydrolysis and the role of oxygen. The hydrolysis of hemicellulose leads to the disruption of its bonds with lignin and, as a result, lignin is depolymerized and solubilized in the acetone fraction. On the other hand, oxygen cleaves the carbon-carbon and ether linkages of the building blocks of lignin, which have been found to be very reactive under wet oxidation conditions (Gong et al., 2012). The combination of these factors led to further depolymerization of lignin resulting in pulps containing lignin at less than 10%. Furthermore, Kalogiannis et al. (2020) applied this technique to expand to other lignocellulosic substrates, such as beechwood (as representative of hardwood species) and yellow pine (as a representative of softwood species). They validated the efficacy of this pretreatment, achieving a 97% removal of lignin in a single stage for both substrates, while also producing close to zero degradation products. As a result, it is evident that acetone/water oxidation is a superior technique when it comes to removal of lignin and increase of cellulose in the final pulp.

According to the hydrolysis screening results of table 5.2, the best performing pulp after the 24-hour hydrolysis was sample no. 4. In specific, the introduction of acetic acid at a low concentration (0.5%) for 120 min led to the production of 10.0 g/L glucose. This indicated that sample No. 4 showed a two-fold increase in cellulose conversion in comparison to the untreated corn stover, and consequently the production of 333.5 mg glucose/ g biomass in comparison with 177.7 mg glucose/ g untreated biomass. Therefore, these pretreatment conditions were selected for a larger scale hydrolysis in order to produce fructose. In addition, these conditions were also implemented in an autoclave pretreatment run with the aim to compare conventional resistance mediated heating and microwave heating. As it is evident in table 5.3, the difference between the heating methods did affect the material in a significantly different way. Microwave pretreatment produced slightly better results than the conventional autoclave resulting in a 41.5 g/L, whereas autoclave pretreatment resulted in 39.9 g/L glucose release after a 72 h saccharification experiment with 10% solids loading and 15 mg Cellic® CTec2/ g DM. After the isomerization experiments 73.6% and 71.6% of glucose was transformed to fructose for microwave and autoclave pretreatment, while 28.4 g fructose were produced from 100 g microwave pretreated biomass and 28.7 g fructose from 100 g autoclave pretreated biomass respectively. However, while the saccharification results were similar for both heating mechanisms, one aspect of the pretreatment process is significantly

different. The energy given to the system through microwave radiation, depending on the power of the instrument, is more direct than that of conventional resistance mediated heating and, as a result temperature rises in a much faster pace. This, when combined with a colling source (e.g. feeding pressurized air to the chamber), can lead to extreme discrepancies in pretreatment time between conventional and microwave pretreatment. More specifically in our case, early experiments when handling the Monowave 450 showed that when implementing maximum power, the desired temperature was reached at less than 15 sec. However, the extreme rise in pressure resulted in the septum exploding. As a result, it was necessary to construct a custom profile where the final temperature would be reached in a milder way. This profile demanded a two-step process, where in the first step a power of 100 W was applied for 1 min followed by a second step where a power of 50 W was applied for 30 sec in order to reach the 150 ^{o}C threshold. In addition, the Monowave 450 was also equipped with a pressurized air feed to help with the cooling of the system, which was achieved in 20 min. On the other side, the conventional resistance mediated heating procedure of the pretreatment mixture needed nearly 45 min to reach the same temperature and due to the lack of cooling system, cooling was accomplished in nearly 90 min. As a result, there was a difference in overall pretreatment time of 1 h in order to complete a single run. Consequently, it is evident that while autoclave pretreatment offers advantages concerning pretreatment conditions such as high temperature and pressure, microwave pretreatment can contribute to the economic aspect of the pretreatment by diminishing pretreatment times and allowing more runs to be held.

Katsimpouras et al. (2016) also designed a model to optimize the microwave assisted hydrothermal pretreatment of corn stover with 0.3% v/v acetic acid as catalyst. The model uses temperature and time as the input in order to optimize glucose release as the output after an 8-hour enzymatic hydrolysis experiment with 3% w/w solids loading. After incorporating several pretreatment runs, the predicted optimal conditions to maximize glucose release were 231.2 ^{o}C for 15.8 min, while the predicted glucose was 7.7 g/L. De Bari et al. (2014) implemented an enzymatic saccharification and isomerization step of steam explosion pretreated corn stover to produce bioethanol from xylitol fermentation. In their work, they mention that the hydrolysate was partially isomerized in order to be fed to yeast for fermentation and it contained glucose at a concentration of 18.7 mmolC (mmoles of compound x number of C atoms). However, they do not provide data on glucose concentration after hydrolysis or fructose concentration after isomerization. Pang et al. (2013) implemented a combination of steam explosion and microwave pretreatment in order to hydrolyze corn stover. The best pulp was the result of a 5 min steam explosion process at 200 ^{o}C, followed by a 540 W irradiation for 3 min that gave a maximum glucose, xylose and total sugar yield 57.4, 17.8 and 75.2%, respectively (corresponding to 100% maximum total sugars in feedstock) after enzymatic hydrolysis. In addition, there are numerous reports that utilize corn stover for the production of chemicals other than bioethanol (Alipour & Omidvarborna, 2016; Chen et al., 2019; Giarola et al., 2016; Liu et al., 2022). However, all these studies report the use of metal catalysts for the direct

synthesis of the desired chemical as a one pot reaction from corn stover. The present study is the first work that aims to produce high added value chemicals from microwave pretreated corn stover, by implementing enzymes as catalysts post pretreatment with the aim of maintaining mild conditions in every step. Future work requires the use of the isomerized hydrolysate in dehydration reactions to produce HMF and the application of oxidative enzymes to produce bio-based furan monomers.

Table 5.2: Enzymatic saccharification results for the pretreated pulps. Saccharification conditions were: 3% w/w solids, 15 mg Cellic® Ctec2/g DM at 50 °C for 24 h

Biomass No.	**Glucose concentration (g/L)**	**Cellulose conversion (%)**	**mg glucose/ g biomass**
1	7.3	16.8	242.0
2	9.3	21.6	311.5
3	8.1	20.7	270.6
4	10.0	22.5	333.5
5	8.4	18.5	278.6
6	7.6	16.5	253.7
7	8.4	17.8	279.3
8	8.8	19.2	291.8
Untreated	5.3	11.1	177.7

Table 5.3: Comparison of glucose release (g/L), cellulose conversion (%) and fructose yield (g fructose/ 100 g pretreated material) between microwave and autoclave pretreated corn stover

Type of pretreatment	**Glucose concentration (g/L)**	**Cellulose conversion (%)**	**g fructose/ 100 g pretreated biomass**
Autoclave	39.9	89.9	28.7
Microwave	41.5	93.2	28.4

6. Bioconversion of HMF derivatives by a *F. oxysporum* galactose oxidase

Furans have traditionally been viewed in lignocellulose biorefineries as inhibitory compounds in fermentation and, as a result, the main focus in lignocellulose valorization studies was to eradicate their generation. However, over the past few years the shift in interest away from biofuels and towards high-added value chemicals, has led to the emergence of numerous studies that examine the production of HMF from lignocellulose as a versatile platform chemical. The interest in HMF lies in its ability to produce FDCA through a cascade of oxidative reactions. Throughout literature, there are many studies that examine accomplishing these reactions with the use of chemical and metal catalysts (Gupta et al., 2018; Sajid et al., 2018). However, there is also a significant number of studies that explore the application of biocatalysts to accomplish this result with the aim to introduce a greener perspective that leads to biobased monomers. This chapter falls into the latter category and constitutes a case study for the green functionalization of HMF. In this chapter, the expression of a galactose oxidase from *F. oxysporum* as well as its potential to catalyze HMF oxidation reactions is explored, in an attempt to introduce an eco-friendly process for the production of FDCA from lignocellulosic biomass samples.

6.1 Bioinformatic analysis

The oxidation of alcohols to carbonyl compounds is one of the most important reactions in synthetic chemistry and many oxidizing reagents exist for this purpose. However, most of these reagents are required in stoichiometric quantities, which usually is expensive and toxic. Thus, catalytic oxidation processes are deemed valuable, especially biocatalysis by alcohol oxidases, which require molecular oxygen as an oxidant and can be performed in aqueous solutions (Parikka et al., 2015). Galactose oxidases (EC 1.1.3.9) are single copper metalloenzymes that catalyze the oxidation of primary alcohols to corresponding aldehydes with strict regioselectivity for the galactose C6 primary hydroxyl group. In addition, certain non-sugar primary alcohols having a structure and configuration similar to the C4-C6 fragment of galactose have also been reported to be substrates of galactose oxidases (Siebum et al., 2006).

The history of galactose oxidases is characterized by the renaming and classification of their source organisms. Galactose oxidase was first detected in cultivation media of *Polyporus circinatu*s (Levasseur et al., 2013), which was later identified as *Dactylium dendroides* (NRRL 2903; ATCC 46032), a mycoparasite of *P. circinatus*. *D. dendroides* was later reclassified as *Fusarium graminearum* (Niessen and Vogel 1997). Recently, galactose oxidases have been classified as members of the newly established auxiliary activity (AA) family AA5 in the Carbohydrate-Active enZYmes database (http://www.cazy.org/). This family includes copper radical oxidases and two subfamilies, AA5_1 and AA5_2, containing presently glyoxal oxidases and galactose oxidases,

respectively, which share similar tertiary structures and virtually identical active sites despite their different catalytic specificities and low sequence similarities.

The crystallographic structure of the galactose oxidase was successfully determined in 1991 by X-ray diffraction with a 1.7 Å resolution. It revealed an interesting feature of the active site copper ion coordinated in a nearly square pyramidal geometry (Ito et al., 1991). The enzyme structure contained three different domains, composed predominantly by β-structure with short turns. The copper at the active site is coordinated by Tyr495 as an axial ligand, His496, His581, Tyr272 and a weakly coordinated water molecule (pH 7) as an equatorial ligand. The authors also concluded that there is a second organic cofactor post-translationally derived from the covalent bond between Tyr272 and Cys228. The thioether bond that links the two residues affects both the structure and reactivity of the protein (Whittaker, 2002). The Cu and the Tyr272 of the galactose oxidase active site can exist in the following three distinct oxidation states: the catalytically active one with Cu (II) and tyrosyl radical, the intermediate state with Cu (II) and tyrosine, and the lowest state with Cu (I) and tyrosine (Himo et al., 2000). The catalytic mechanism proposed by Whitaker considers as the first step the binding of the substrate to copper displacing the water ligand with a subsequent transfer of a proton from the alcohol to the axial Tyr495. Afterwards, a hydrogen atom is transferred from the substrate to the tyrosyl radical. The resulting substrate ketyl radical is then oxidized through electron transfer to the copper center, yielding Cu (I) and aldehyde. In the end, Cu (I) and tyrosine are re-oxidized by molecular oxygen, regenerating Cu (II) and tyrosyl while producing as subproduct H_2O_2 (Whittaker, 1993). In the active form of GAO, the copper atom is at oxidation state (II) and the tyrosine is in a radical form. Reduction of the tyrosine radical generates the inactive form of GAO, which can be rescued by treating the inactive form with mild oxidants, such as, hexacyanoferrate (III), iridium (IV) chloride, molybdic cyanide, sodium periodate, potassium dichromate, or copper sulfate. Peroxidases can also enhance the action of GAO by oxidizing the inactive form to the active radical form. When fully reduced, the copper atom is at oxidation state (I) and can react with molecular oxygen to generate hydrogen peroxide. Since high concentrations of hydrogen peroxide are reported to inactivate GAO, catalase can be added to the reaction mixture to degrade H_2O_2 and prolong GAO activity (Parikka et al., 2015).

This chapter examines the discovery and expression of a galactose oxidase from *Fusarium oxysporum* with the aim to catalyze the oxidation reaction of HMF to its derivatives. The goal of this project was to subject the enzyme to reactions with furans originating from the dehydration reactions of real biomass hydrolysates in order to create an integrated pathway for the production of biobased monomers from lignocellulose. The search for this particular galactose oxidase was aimed by looking for biocatalysts exhibiting furan activity. In specific, a galactose oxidase from *Fusarium gramirearum* (M. E. Cleveland et al., 2021) showed activity on conversion of HMF to other furans, therefore it was selected as model enzyme for the discovery of new proteins. The search for homologous sequences in filamentous fungi resulted in the selection of a *Fusarium oxysporum* galactose oxidase (EC 1.1.3.9) gene belonging to the AA5_2 subfamily for

heterologous expression in *P. pastoris* and application for the oxidation of furans. The gene encoding the *Fo*GalOx consists of 2046 bp encoding a 682 amino acid protein that possesses a 16 amino acid putative N-terminal signal peptide, as well as 3 *N*- and 16 *O*-glycosylation putative sites and a theoretical molecular weight of 81.5 kDa. The amino acid sequence presented in table 6.1 exhibited a 99% homology to the amino acid sequence of a characterized galactose oxidase from *F. oxysporum* (Ma et al., 2010) and a 82% homology to a respective GalOx sequence from *F. gramirearum* (Rogers et al., 2007). The three sequencies were compared using the Clustal Omega alignment tool and as depicted in figure 6.1 the alignment showed great similarities between them suggesting the existence of identical active site formed by the amino acids Cys230, Tyr255, Tyr497, His498 and His583 of the *Fo*GalOx sequence.

Table 6.1: Amino acid sequence of the *Fo*GalOx gene. Amino acids in red indicate the signal peptide.

Protein	Amino acid sequence
*Fo*GalOx	MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPFSNSTNNGLLFINTTIASIAAKEEGVSL EKREAEASMVAISQPAAKAETPEGSLQFLSLRASAPIGTAINRDKWRVTCDSQHEGDECSKAIDGDRDTFWHTAWAA GGTNDPKPPHTITIDMGSSQNVNGLSVLPRQDGSDHGWIGRHNVFLSTDGKNWGDAVATGTWFADNTEKYSNFET RPARYVRLVAVTEANDQPWTSIAEINVFKAASYTSPQPGLGRWGPTLDFPIVPVAAAVEPTSGKVLVWSSYRNDAFG GSPGGVTLTSTWDPSTGVISQRTVTVTKHDMFCPGISMDGNGQVVVTGGNDAQKTSLYDSSSDSWIPGPDMKVAR GYQSSATLSNGRVFTIGGSWSGGIFEKNGEVYDPSSKTWTSLPGALVKPMLTADQQGLYRSDNHGWLFGWKKGSVF QAGPSTAMNWYYTTGNGGVKSAGKRQSSRGTDPDAMCGNAVMYDAVKGKILTFGGSPSYQDSDATTNAHIITISEP GSTPKTVFASNGLYYPRTFHTSVVLPDGNVFITGGQQRGIPFADSTPQLTPELYVPNDDTFYKQQPNSIVRVYHSISLLL PDGRVFNGGGGLCGDCDTNHFDAQIYTPNNLYDSNGKLATRPKITKVSAKSVKVGGKITITADTSIKQASLIRYGTSTHT VNTDQRRIPLSLRRTGTGNSYSFQVPSDSGIALPGYWMLFVMNSAGVPSVASTLLVTQYLEQKLISEEDLNSAVDHHH HHH

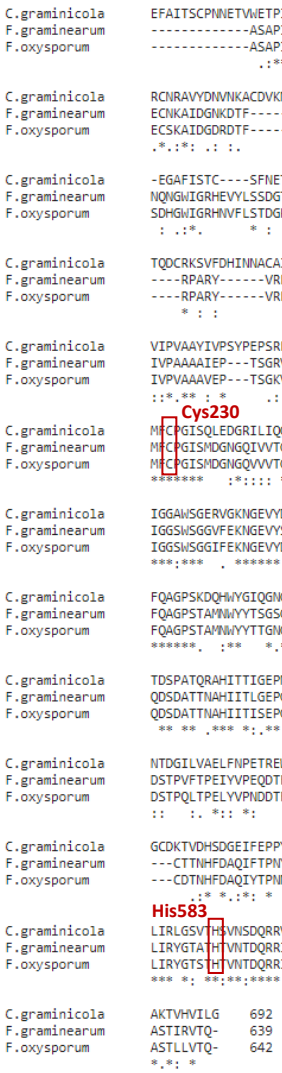


Figure 6.1: Multiple sequence alignment of *Fo*GalOx, a galactose oxidase from *F. oxysporum* (EXK84413.1) and a galactose oxidase from *F. graminearum* (2EIC_A). The amino acids pointed out are the ones that participate in the formation of the active site and are maintained in the *Fo*GalOx sequence.

In addition, the *Fo*GalOx sequence, along with other galactose oxidase sequences exhibiting activity on furans were also used as templates for the discovery of homologues in other fungal genomes, resulting in the retrieval of 10 novel uncharacterized sequencies with at least 45% homology. The Genbank accession numbers of these sequences are the following: XP_007876557 from *Pseudozyma flocculosa*, XP_029741679 from *Sporisorium graminicola*, XP_011390486 from *Ustilago maydis*, XP_041410401 from *Ustilago hordei*, XP_025587588 from *Fusarium venenatum*, XP_038762307 from *Botrytis sinoallii*, XP_035350800 from *Talaromyces rugulosus*, XP_041141830 from *Aspergillus flavus*, XP_043151733 from *Aspergillus udagawae*, XP_024684908 from *Aspergillus novofumigatus*. All the above sequencies represent potential proteins with putative furan activity and were used to construct a cladogram, depicted in figure

6.2, revealing that galactose oxidase sequences with high homology can be found in basidiomycetes and ascomycetes.

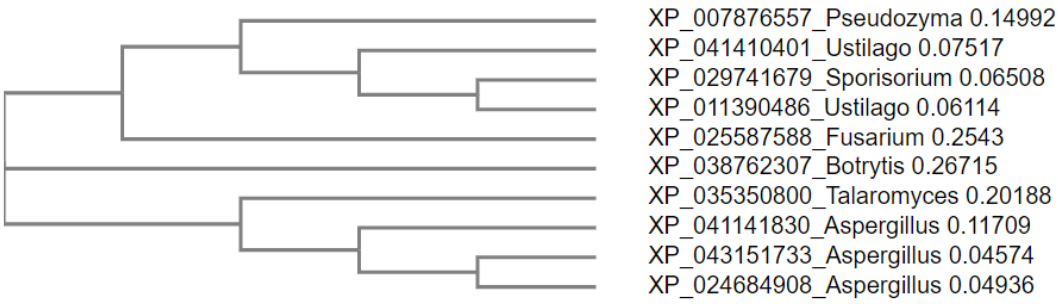


Figure 6.2: Cladogram of relationship of galactose oxidase genes in different basidiomycete and ascomycete strains. Branch lengths are arbitrary.

6.2 Heterologous expression and activity tests

As depicted in figure 6.3 the *Fo*GalOx gene was isolated from the *F. oxysporum* genome and was introduced to PCR blunt vectors. The multiplication and isolation of the gene took place in TOP10 *E. coli* cells. Afterwards the gene was isolated and through ligation, it was used to transform pPICZαC plasmid vectors, which were later linearized. After linearization these vectors were used to transform *P. pastoris* X33 cells and were cultivated in petri dish cultures with zeocin. The grown colonies were transferred to small scale cultures in order to examine the production of the enzyme. The best performing colony was used to create glycerol stocks and stored at -80 ^{o}C.

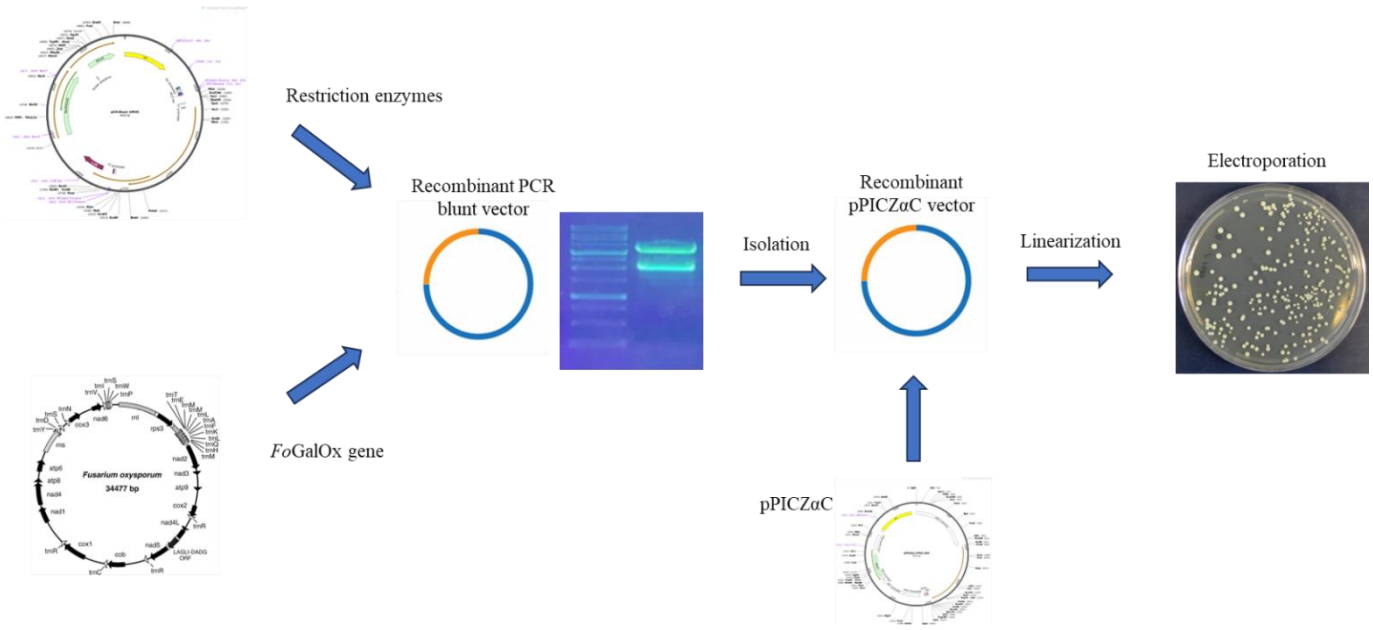


Figure 6.3: Transformation of P. pastoris cells, construction of recombinant pPICZαC plasmid vectors, restriction analysis, plasmid linearization and electroporation of X33 cells.

The heterologous expression of *Fo*GalOx was carried out as described in paragraph 2.15 and was isolated and purified by means of IMAC chromatography. After its purification the enzyme was subjected to SDS-PAGE in order to verify its molecular weight. As depicted in figure 6.4 the molecular weight of *Fo*GalOx was calculated at around 72 kDa, which was 9 kDa short of the predicted weight of 81 kDa. This might be attributed to the presence of the signal peptide during the calculation of the molecular weight. *Fo*GalOx concentration in the purified fraction was calculated at 0.182 mg/mL meaning that 182 mg of pure protein were produced from the *P. pastoris* cultures.

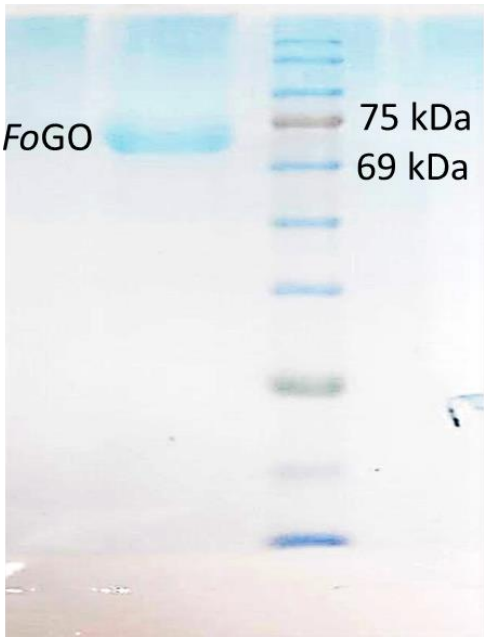


Figure 6.4: SDS-PAGE of purified *Fo*GalOx expressed in *P. pastoris* (left lane) and standard protein markers (right lane).

In addition, the enzyme was subjected to activity tests as described in paragraph 2.17 in order to discover the temperature and pH optima. A total of 7 temperature values was examined that ranged from 25 ^{o}C to 55 ^{o}C, examining the release of H_2O_2 from the oxidation of galactose. The temperature optimum of *Fo*GalOx was found to be 40 ^{o}C at pH 6.0 while the enzyme also retains 91% of its activity at 35 ^{o}C. In addition, the enzyme appears to retain its activity at temperatures above 40 ^{o}C, as even at 55 ^{o}C its relative activity is 64.3%. Furthermore, the results of the pH optimum test, show that the optimum pH for the action of *Fo*GalOx is 6.0. In specific, among the two different buffers used at pH 6.0 the enzyme appears to perform better at potassium phosphate buffer. Further increase in pH resulted in enzyme activity diminishing, eventually reaching 0% relative activity at pH 10.0. These results are in accordance with the work of (Paukner et al., 2014), who were the first to discover the enzyme of *Fo*GalOx and complete its biochemical characterization of galactose substrate. However, it was mandatory to test the optimal conditions of the enzyme in order to be able to utilize it in furan oxidation reactions, As a result, all furan reactions were held at these experimental conditions.

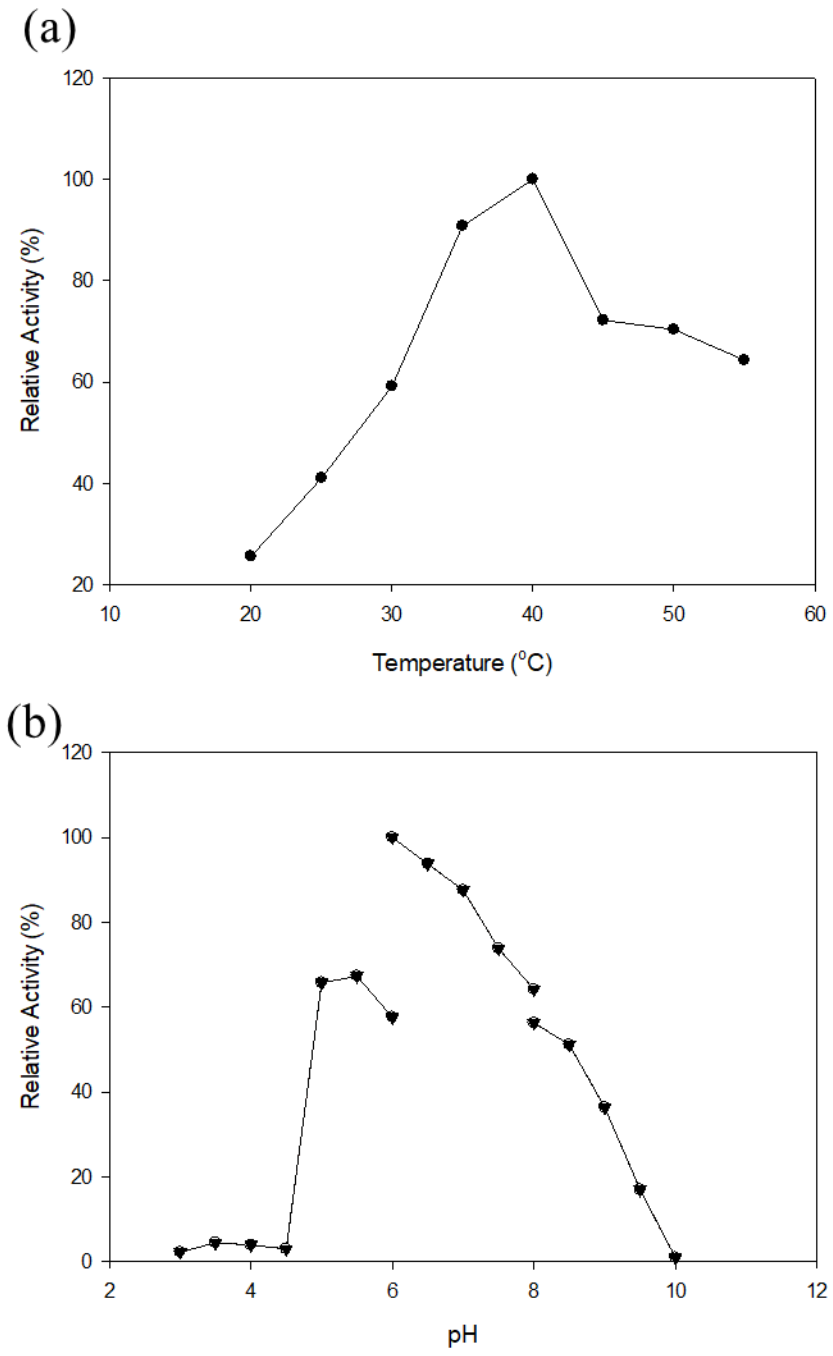


Figure 6.5: Effect of (a) temperature and (b) pH on the activity of *Fo*GalOx. The buffers used for each range of pH were: 3-6 Citrate-phosphate buffer, 6-8 potassium phosphate buffer, 8-10 Bis-Tris buffer. Experiments were run in duplicates.

6.3 Activity on furan substrates

The replacement of chemical oxidants with redox enzymes is a topic of considerable contemporary interest. In this context the utilization of member of the copper radical oxidases (CROs) constitutes an appealing alternative to co-factor dependent oxidases (Ribeaucourt et al., 2022). This chapter examines the application of the produced and purified *Fo*GalOx to test reactions on furan substrates on the optimum conditions discovered in the previous paragraph.

The furan substrates tested were HMF, DFF, HMFCA and FFCA. Initial reactions with HMF as a substrate showed relatively low to no activity of the enzyme. The interpretation of this phenomenon lied in the nature of the oxidative enzymes and their mode of action. As described above, the active site of galactose oxidases contains a tyrosine radical bound to a copper (II) ion complex, which is reduced to a nonradical tyrosine copper (I) complex during the oxidation of the substrate (Toftgaard Pedersen et al., 2015). This tyrosine radical can also undergo a reduction to a nonradical copper (II) complex, which is inactive. Therefore, the enzyme requires a single-electron oxidation in order to regenerate the active site (Whittaker, 2002). Although extensive studies have been made to find suitable activator molecules, such as potassium ferricyanide ($K_3[Fe(CN)_6]$), the most commonly used activator remains horseradish peroxidase (HRP) (H. C. Johnson et al., 2021). In addition, hydrogen peroxide (H_2O_2) is the by-product of galactose oxidase catalyzed oxidation, however, it has been shown to both inhibit and deactivate the enzyme (Kwiatkowski et al., 1981). Hydrogen peroxide is soluble to water and as a result, in order to alleviate the problem of its concentration, catalase was added to all the reactions to dissolve H_2O_2 into H_2O and O_2. Finally, reactions were also set up with the absence of HRP in order to examine the effect of only catalase in the oxidation reaction. Furan substrate concentration was 2 mM, *Fo*GalOx concentration was 0.0182 mg/mL and HRP and catalase concentrations were 0.26 mg/mL and 0.33 mg/mL, respectively.

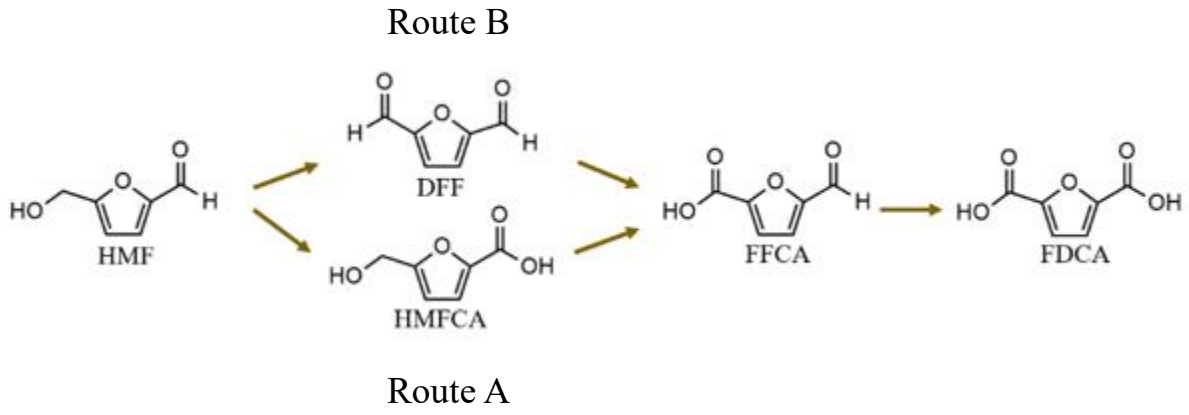


Figure 6.6: Schematic representation of oxidation of furan-derivatives that have been reported in the literature (a) HMF to FDCA, (b) FA to FCA.

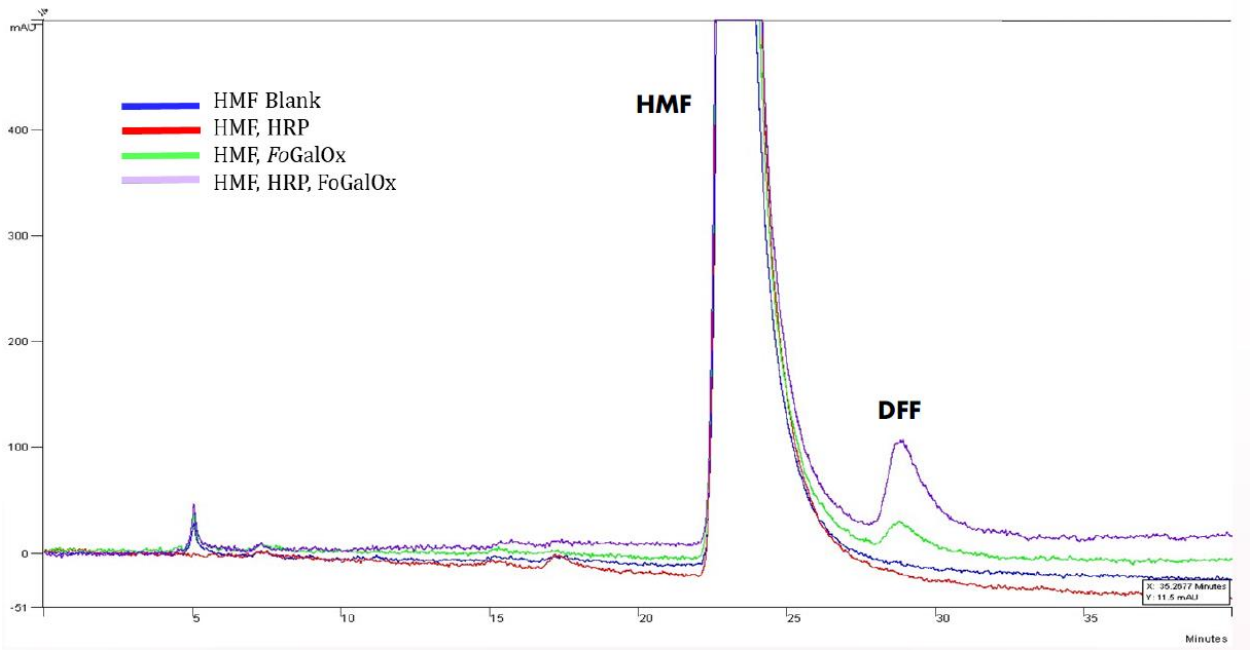


Figure 6.7: Reaction of *Fo*GalOx and HMF at 40 °C, pH 6.0 for 24 h

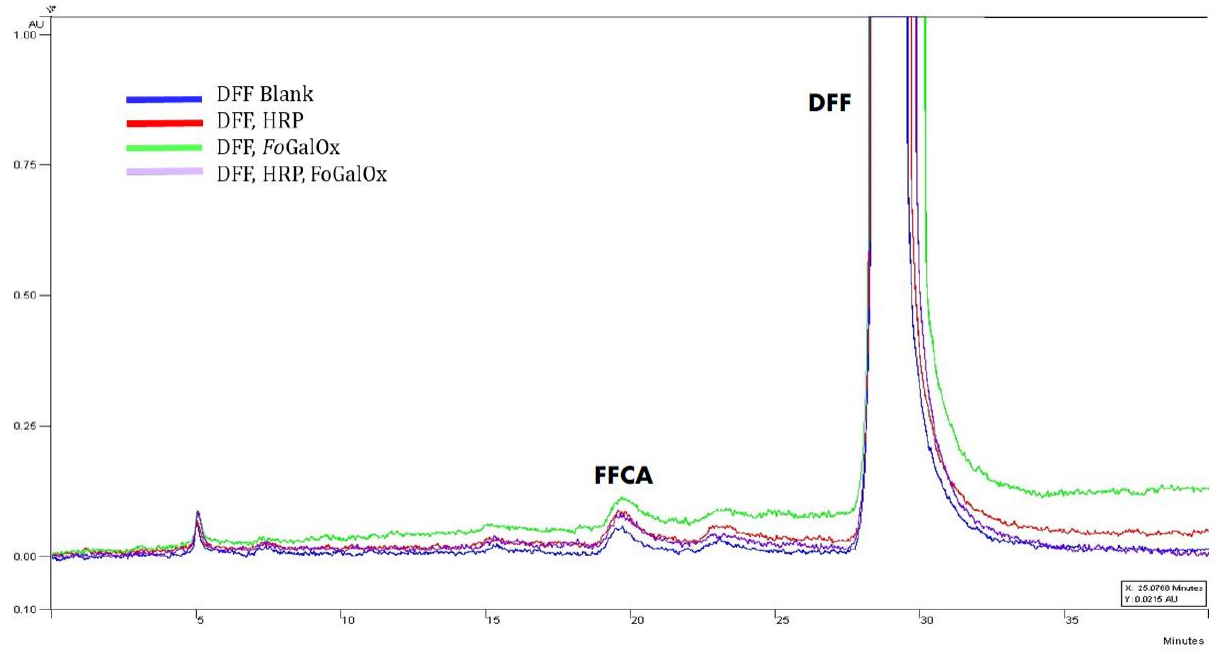


Figure 6.8: Reaction of *Fo*GalOx and DFF at 40 °C, pH 6.0 for 24 h

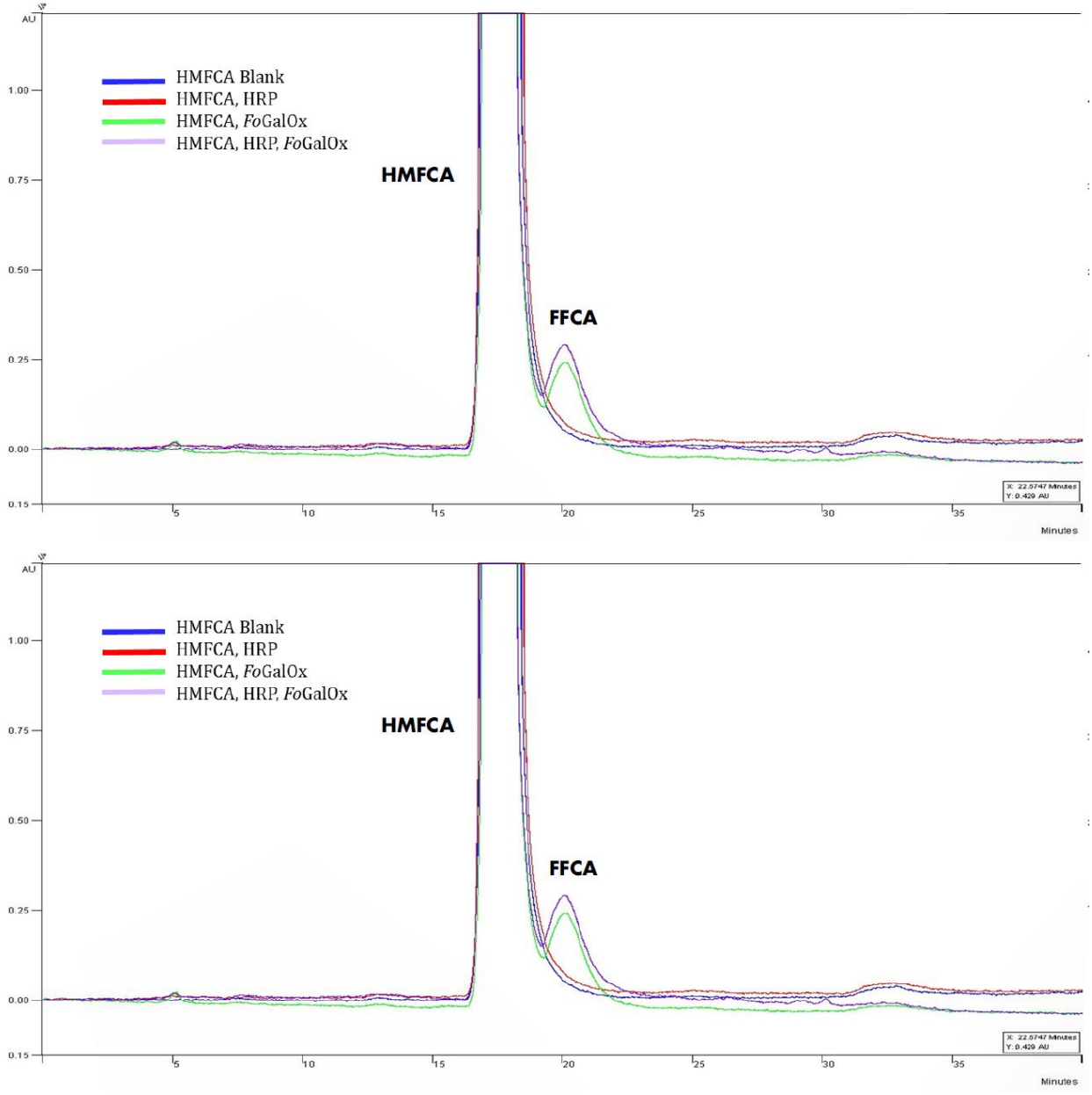


Figure 6.9: Reaction of *Fo*GalOx and HMFCA at 40 ^{o}C, pH 6.0 for 24 h

Figure 6.6 shows the oxidative cascade reaction of furans starting from HMF until the final stage of FDCA. Figures 6.7, 6.8, 6.9 show the results of 24 h reactions of each furan substrate with *Fo*GalOx as analyzed by HPLC chromatography. Although complete biochemical characterization of *Fo*GalOx on each furan substrate, which was the original goal, was not accomplished within this thesis, it was possible to validate the effectiveness of the biocatalyst in transforming furans. Starting with the activity on HMF, it is evident that *Fo*GalOx produces DFF and, in fact a substantial increase in DFF production was observed upon adding HRP to the reaction. This also

suggests that the enzyme follows route B as the pathway of the oxidation. Dijkman et al. (2014), published an extensive study, examining the activity of an FAD-dependent oxidase of the glucose-methanol-choline oxidoreductase (GMC) family on numerous substrates in order to discover the exact mechanism of the oxidation. In their work, they hypothesized that the enzyme, which later they would refer to as a HMF oxidase (HMFO), would be able to follow both routes due to the fact that it was active on both DFF and HMFCA. However, HMFO produced exclusively DFF when reacting with HMF. This can be explained by taking a look in the mechanism of the oxidation. In the first route the aldehyde group of HMF is oxidized to the carboxylic acid, thus yielding HMFCA. A second oxidation then yields FFCA and this will ultimately lead to the production of FDCA. In the second route, the alcohol group of HMF is oxidized to the corresponding aldehyde, thus yielding DFF followed by oxidations to FFCA and FDCA. Their findings showed that HMFO oxidizes the alcohol group of HMF rather than the aldehyde substituent suggesting that the enzyme was a true alcohol oxidase. However, several oxidases have been reported to perform oxidation reactions on aldehyde groups (Gesell et al., 2011; Van Hellemond et al., 2009). In all these cases, it turns out that the aldehyde itself is not oxidized, but the hydrated *gem*-diol form is converted by the enzyme. As a result, figure 6.6 can be refined to an alternative version as presented in figure 6.10.

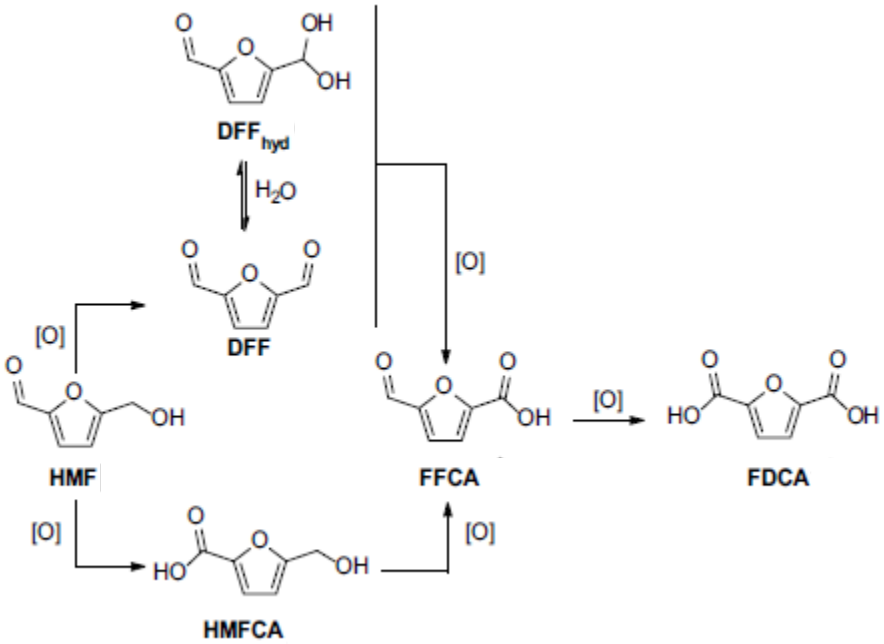
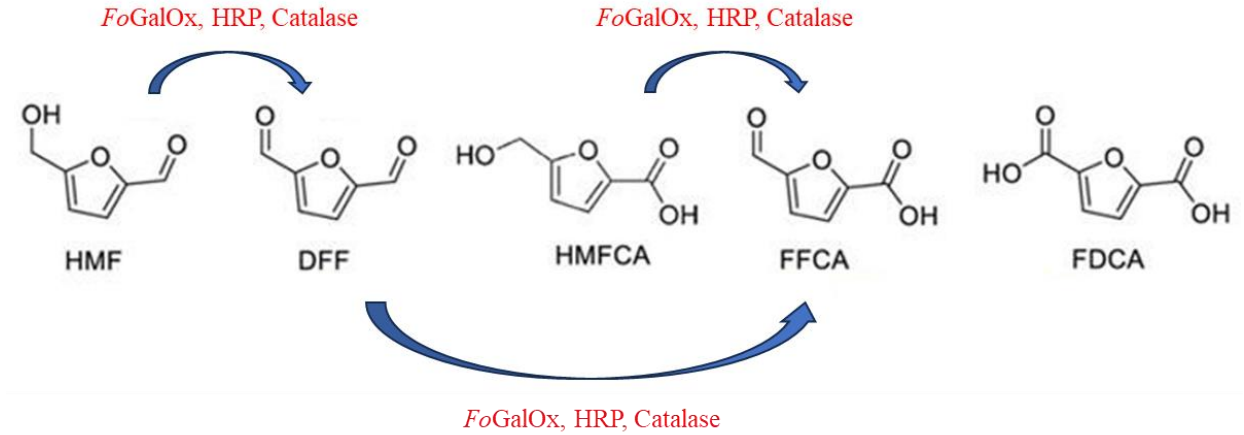


Figure 6.10: The refined expression of the oxidative pathway of furans (M. E. Cleveland et al., 2021)

As a result, by starting with HMF as a substrate the alcohol oxidation product of DFF will be



formed (route B). The aldehyde group of HMF cannot be oxidized because it is not present in its hydrated form. A significant fraction of DFF is hydrated, and therefore it is readily oxidized to FFCA. In addition, when the sole substrate is HMFCA, the enzyme is also able to perform oxidation of its alcohol group therefore producing FFCA as well. However, only a minor fraction of FFCA is in its hydrated form, which in turn explains the minor activity on FFCA, and becomes the compound accumulated during the reaction. This appears to be the case both on *Fo*GalOx as well as on the majority of AA5 oxidative enzymes as according to figures 6.8 and 6.9 *Fo*GalOx was active on both DFF and HMFCA with its activity boosted by the addition of HRP and catalase, despite exhibiting no activity on FFCA (data not shown).

Figure 6.11: Schematic representation of the activity of the *Fo*GalOx, HRP and catalase on furan substrates

Carro et al. (2015) in their study, also validated this hypothesis when examining the capacity of a *Pleurotus eryngii* aryl-alcohol oxidase to oxidize furans and produce FDCA. Even though initial reactions with AAO only reached the FFCA stage of the oxidation, they later implemented an *Agrocybe aegerita* unspecific peroxygenase (UPO) in succession to the AAO and managed to convert 91% of the initial HMF to FDCA. However, in their study they do not report activity of their enzyme to HMFCA. Cajnko et al. (2020) examined the activity of a *P. pastoris* alcohol oxidase and a *Dactylium dendroides* galactose oxidase on these furan derivatives either separately or in a multi-enzyme one pot reaction. The alcohol oxidase was able to oxidize HMF to DFF (25.6% yield) even producing a small amount of FFCA (3.1% yield) in the same reaction and was also able to produce FFCA (3.2% yield) from DFF substrate. However, it was not able to oxidize HMFCA. On the other hand, the galactose oxidase was active on all three substrates producing 5.1% DFF from HMF, 1.3% FFCA from DFF and 2.7% FFCA from HMFCA. When combining the two enzymes in a one pot reaction with catalase and HRP they were able to increase DFF yield

to 36.7% but no further oxidation was seen. Cleveland et al. (2021) reported the discovery of two AA5 radical copper oxidases from *Fusarium oxysporum* and *Fusarium graminearum* and examined their activity on a variety of substrates in order to classify them. Due to the fact that these enzymes shared a 60% pairwise identify versus the archetype of *Fusarium graminearum* galactose oxidase, they initially believed that these enzymes were galactose oxidases. However, increased activity on aryl alcohols as compared to galactose led to their classification as AAOs. *Fox*AAO, *Fgr*AAO and HMF reaction product analysis by H-NMR spectroscopy revealed a mixture of DFF (69% for *Fgr*AAO, 76% for *Fox*AAO) and FFCA (31% for *Fgr*AAO, 24% for *Fox*AAO) presumably via the aldehyde hydrate route. Furthermore, H-NMr analysis following the incubation of *Fgr*AAO and *Fox*AAO with HMF at 1.5 h and 3 h did not indicate the formation of HMFCA. However, reactions with HMFCA as a substrate resulted in 84% and 96% FFCA yield, respectively, indicating that the enzymes were able to perform the oxidation of the alcohol branch. Based on the results of Pedersen et al. (2015), Birmingham et al. (2021) tried to optimize a galactose oxidase enzyme by means of protein engineering in order to boost its oxidative potential to furans. They discovered that a double mutation at the F290 and S291 amino acids (mutant F290W/S291R) increased the $k_{cat,app}$ for HMF by 50%, whule the $K_{M,app}$ showed a 5-fold increase. Additionally, other strains that were the result of three rounds of mutations also showed high performance on HMF above 70% for 24 h reactions with the best strain exhibiting a 81% conversion. Cleveland et al. (2021) performed a survey of substrate specificity on 18 AA5 copper radical oxidases. Of them, 5 were found active on furans. An alcohol oxidase from *Colletorichum gloeosporioides* showed a 100% conversion of HMF to a 91:9 mixture of FFCA and FDCA, while being able to oxidize both HMF and HMFCA efficiently. An alcohol oxidase from *Puricularia oryzae* showed an incomplete conversion of 10 mM HMF producing predominantly DFF with 2% FFCA, while also showing full conversion of HMFCA and DFF to mostly FFCA. An alcohol oxidase from *Penicillium rubens* oxidized 100% of 10 mM HMF to DFF (including its hydrated form) and FFCA. An alcohol oxidase from *Aspergillus flavus* showed similar conversion percentages to *Pru*AlcOx for HMF, HMFCA and DFF with no conversion observed for FFCA. A galactose oxidase from *Fusarium oxysporum* displayed low percent conversion of HMF and had a similar product profile to *Pru*AlcOx. Of all these enzymes *Cgl*AlcOx and *Por*AlcOx also exhibited high conversion of FFCA to FDCA (24% and 49% yield, respectively). They mention that this may be caused by their active site architecture being able to accommodate the aldehyde from (or its hydrate) of FFCA, which results in higher (%) conversions to FDCA as compared to other CROs. However, in this case these enzymes should also be able to transform HMF to HMFCA, which was not observed in this study. Contrary to all the above studies, Mathieu et al. (2020) discovered an AAO from *Colletotrichum graminicola* that showed full HMF conversion to DFF, while also showing 46% conversion of HMFCA to FFCA. Nevertheless, the enzyme was unable to catalyze DFF oxidation which was intriguing due to the fact that the monohydrate form of DFF was present at a high proportion (50%). Finally, Milić et al. (2022) attempted FDCA synthesis in biphasic and monoaqueous conditions using a galactose oxidase and lipase B from *Candida*

antarctica. Initially, by testing the activity of galactose oxidase in biphasic solvents with the organic fraction being heptane, toluene, dodecane, ethyl acetate, ethyl hexanoate they reported that the enzyme retained most of its activity and even managed to produce DFF in biphasic EtOAc-H_2O systems. Eventually, after 72 h of reaction the lipase was introduced in the mixture and after 24 h of reaction, the two phases were incorporated into one with the formation of a white precipitation, namely FDCA.

It is evident that the mechanism of furan oxidation is a combination of many different parameters. Like flavin dependent oxidases, the ability of CROs to use molecular oxygen as a co-substrate is a valuable asset to their application in biocatalysis, avoiding the requirement for co-factor regeneration of NAD-dependent oxidases. This work studied the expression and application of a AA5_2 *Fusarium oxysporum* galactose oxidase for furan oxidation. It was shown that the biocatalyst follows the main principles of furan oxidation as the majority of the other members of the subfamily. Despite not being able to fully unravel the kinetic parameters on each furan derivative, early experimentation showed its ability to catalyze at least a single stage of furan oxidation. Future work, will focus on the optimization of enzyme concentration, enzyme combinations and reaction parameters in order to overcome the obstacle of the single stage oxidation with the aim of producing FDCA from HMF. Such an achievement will pave the way for the application of the biocatalyst in lignocellulose derived HMF streams integrating the project of furan monomer transformation from real biomass samples.

Table 6.2: Report of all oxidative enzymes on the oxidation of furan substrates. Each box depicts the product of the reaction with each substrate. (%) Yields are depicted in parentheses.

Type of enzyme	Microorganism	Substrate				Source
		HMF	DFF	HMFCA	FFCA	
Galactose oxidase	*Fusarium oxysporum*	DFF	FFCA	FFCA	no activity	Present work*
HMF oxidase	*Methylovorus* sp. strain MP688	FDCA (6.1)	FDCA (5.9)	FDCA (6.1)	FDCA (7.4)	(Dijkman et al., 2014a)
Aryl alcohol oxidase	*Pleurotus eryngii*	FFCA (94), FDCA (6)	FFCA (86), FDCA (14)	not tested	FDCA (11)	(Carro et al., 2015)
Alcohol oxidase	*Pichia pastoris*	DFF (25.6), FFCA (3.1)	FFCA (3.2)	no activity	FDCA (11.6)	(Cajnko et al., 2020)
Galactose oxidase	*Dactylium dendroides*	DFF (5.1)	FFCA (1.3)	FFCA (2.7), FDCA (0.2)	FDCA (0.6)	
Aryl alcohol oxidase	*Fusarium graminearum*	DFF (69), FFCA (31%)	FFCA (49)	FFCA (84)	no activity	(Cleveland et al., 2021)
Aryl alcohol oxidase	*Fusarium oxysporum*	DFF (76), FFCA (24)	FFCA (51)	FFCA (96)	no activity	
Galactose oxidase mutant	*Fusarium* NRLL 2903	DFF (81)	not reported	not reported	not reported	(Birmingham et al., 2021)
Alcohol oxidase	*Colletorichum gloeosporioides*	FFCA (91), FDCA (9)	FFCA (87), FDCA (13%)	FFCA (92), FDCA (8)	FDCA (24)	(Cleveland et al., 2021)
Alcohol oxidase	*Puricularia oryzae*	DFF (49), FFCA (2)	FFCA (72), FDCA (28)	FFCA (83), FDCA (17)	FDCA (49)	

*Qualitative data

Type of enzyme	Microorganism	Substrate				Source
		HMF	DFF	HMFCA	FFCA	
Alcohol oxidase	*Penicilium rubens*	DFF (52), FFCA (48)	FFCA (38)	FFCA (100)	FDCA (5)	
Alcohol oxidase	*Aspergilus flavus*	DFF (52), FFCA (48)	FFCA (40)	FFCA (100)	no activity	(Cleveland et al., 2021)
Galactose oxidase	*Fusarium oxysporum*	DFF (29), FFCA (4)	FFCA (12)	FFCA (17)	FDCA (7)	
Aryl alcohol oxidase	*Colletotrichum graminicola*	DFF (100)	no activity	FFCA (46)	not reported	(Mathieu et al., 2020)
Galactose oxidase	not reported	DFF (36.7), FDCA (not reported)	not reported	not reported	not reported	(Milić et al., 2022)

CONCLUSIONS

7. Conclusions

The worries over the constant depletion of fossil fuel reserves has led to a shift in the interest of the scientific community towards renewable energy. In addition, the continually increasing worldwide demand for energy and chemicals necessitates the emergence of utilizing sustainable carbon sources able to support the production of numerous value-added compounds that can be employed as starting materials, precursors or building blocks for, among others, biofuels, polymers and pharmaceuticals. In this regard, lignocellulosic biomass is a potential candidate since it is the most abundant renewable carbon source on the planet. In the past, lignocellulosic biomass has been reported as a promising feedstock to produce biofuels, such as bioethanol. However, since the biomass constituents can lead to the production of many other value-added products, along with the fact that large-scale bioethanol production faces some limitations, such as the amount of biomass needed for its sufficient production as well as the increased cost of its downstream processes, lignocellulose has started to expand as a raw material within the frames of the biorefinery concept. This study focused on the development of appropriate processes for the valorization of multiple lignocellulosic biomass samples to produce fructose as well as HMF and its oxidative derivatives.

The most studied feedstock of this work was beechwood biomass, which was the subject of several different pretreatment processes. The first part included the study of an oxidative organosolv pretreatment process (OxiOrganosolv) with the aim to produce HMF from beechwood biomass. This pretreatment examined the effect of four parameters, mainly choice of solvent (ACO, EtOH, THF), temperature (150 ^{o}C, 160 ^{o}C and 175 ^{o}C), pressure (8 bar and 16 bar) and reaction time (60 min and 120 min), on fructose production after the saccharification and isomerization step. The highest fructose yield obtained was 55.2 g fructose/ 100 g pretreated biomass for the sample pretreated with EtOH at a pressure of 8 bar, temperature of 175 ^{o}C for 120 min. This sample was the one used for scale-up hydrolysis using a custom-made free fall mixer to produce a high-sugars concentration hydrolysate that would be used as a substrate for chemical hydrolysis and HMF production. Eventually, after an 18 h-saccharification step followed by isomerization, the hydrolysate had a final concentration of 25.0 g/L glucose and 104.5 g/L fructose. Then, the development of the dehydration reactions involved the implementation of five different Brønsted acid solvents to perform the dehydration. Among HCl, H_3PO_4, formic acid, maleic acid and a commercial H-modernite, the best performing one was formic acid managing to reach a HMF yield of 44.6% with 55.8% selectivity after an incubation period of 60 min at 150 ^{o}C. As a result, this was the first work examining HMF production from lignocellulosic biomass samples in a green and integrated process. Up until then, there were few reports describing thoroughly the individual process steps of producing HMF from lignocellulosic biomass, especially by coupling enzymatic and chemo-catalysis. Fructose production was mainly studied from pure sugar solutions and HMF production was mainly accomplished through the use of expensive metal catalysts. Nevertheless,

this work was able to apply environmentally friendly biocatalysts under mild and homogeneous acid catalysts in low concentrations to design an integrated process that is able to break down lignocellulose and produce fructose and HMF in high yields.

The second part of the lignocellulose valorization comprised of the examination of the oxidative pretreatment with the addition of POMs as catalysts. This work used two different biomass sources, beechwood as a representative of hardwood biomass and pine as a representative of softwood biomass. Following the concept described above, beechwood was pretreated on a number of different pretreatment conditions, however, this time the four different parameters examined were: the solvent (iBuOH, ACO, EtOH, THF), the presence and type of POM catalyst (no catalyst, HPMo, Fe-PMo, Cu-PMo), the pretreatment temperature (150 ^{o}C, 160 ^{o}C and 175 ^{o}C) and the residence time (60 min and 120 min). The highest fructose yield in this process was observed for the pretreatment with iBuOH at 175 ^{o}C for 120 min, where 54.9 g fructose/ 100 g pretreated biomass were produced. Among the samples pretreated with POM catalysts, the highest fructose yield was observed for pretreatment with Fe-PMo and ACO pretreatment where the production was 36.8 g fructose/ 100 g pretreated biomass. Pine samples were pretreated with the same solvents, however, only at a temperature of 175 ^{o}C. The best performing sample was the one pretreated with iBuOH and was able to produce 53.4 g fructose/ 100 g pretreated biomass, which was very similar to the highest yield obtained in beechwood. The best performing hydrolysates were used to produce HMF with formic acid as a catalyst, following the same protocol as described above. The optimal HMF selectivity was 49.9% and was observed for the EtOH pretreated pine pulp in the absence of POMs, while the highest HMF selectivity for beechwood was 47.8% for iBuOH, 160 ^{o}C, 120 min and no catalyst. The highest HMF selectivity for the POM pretreated pulps was 45.9% for the iBuOH Cu-PMo pretreatment. The results of this work show that the effect of POMs in the pretreatment is dependent on the end product of the process. The concept of implementing metal catalysts in the pretreatment process was conceived due to their redox potential and their ability to interact with lignin as electron donors to achieve high delignification and cellulose content in the residual pulp. However, it was evident in many cases that the presence of metal cations can negatively affect each step, as enzymatic processes exhibited lower yields when Cu-PMo was incorporated in the pretreatment. Furthermore, HMF yields were correspondingly inhibited by the addition of Fe-PMo in the pretreatment, suggesting that Fe ions might lead to the production of humins during the dehydration step. As a result, this study indicates that the use of different POMs in the pretreatment is crucial to the final constitution of the pulp, therefore posing limits to the possible valorization pathways.

In order to further evaluate the effect of pretreatment on lignocellulosic biomass, microwave-assisted pretreatment was tested on corn stover biomass. The concept behind this work was firstly to introduce an effective organosolv pretreatment process that benefited from the rapid increase in temperature achieved by microwave heating in order to diminish the overall pretreatment duration and, secondly to compare the heating mechanism of microwaves to the traditional one of the

autoclave pretreatment. The type of pretreatment was an organosolv process with ACO, with the addition of acetic acid as a catalyst in concentrations lower than 2% v/v. The parameters examined in this type of pretreatment were among others, the compositions of the resulting pulps as well as their amenability to saccharification and isomerization. The results showed that this type of pretreatment exhibited lower degrees of delignification when compared to the previous pretreatments in this study as the lowest lignin content observed in the pulps was 15.5%, which was translated to a 4.7% decrease in the lignin content of the untreated material. Nevertheless, as far as saccharification is concerned, the best performing pulp was the one treated with 0.5% v/v acetic acid at 150 ^{o}C for 120 min, exhibiting a glucose release of 10.0 g/L after a 24 h-hydrolysis experiment, which was nearly double of the glucose release of the untreated material (5.3 g/L). In addition, cellulose conversion was 22.5% which corresponded to 333.5 mg glucose/ g biomass, which are higher in comparison with the untreated biomass where the respective values were 11.1% and 177.7 mg glucose/ g biomass. As a result, these reaction conditions were also tested on a pretreatment run performed in an autoclave equipment, in order to compare microwave and traditional resistance mediated heating. The ensuing saccharification and isomerization experiments, which were performed in optimal conditions resulted in similar glucose releases between the two types of pretreatments. Autoclave pretreatment led to a glucose release of 39.9 g/L with a cellulose conversion of 89.9%, while microwave pretreatment released 41.5 g/L glucose with a cellulose conversion of 93.2%. Eventually, fructose yield values were 28.7 g fructose/ 100 g pretreated biomass for autoclave and 28.4 g fructose/ 100 g pretreated biomass, suggesting that there is no significant difference between the two processes. However, microwave assisted pretreatment was able to achieve the same results in a much smaller amount of total pretreatment time as the duration exhibited a 1 h discrepancy between microwave and autoclave runs. In addition, when compared to other studies, this work shows that corn stover can still lead to respectable glucose yields even when treated at lower temperatures due to the addition of the organic solvent. As a result, this study proved that it is possible to produce fructose from microwave pretreated corn stover with the aim to produce high added value chemicals by implementing enzymes as catalysts post pretreatment with the aim of maintaining mild conditions in every step.

Finally, this work examined the heterologous expression and production of a galactose oxidase from *F. oxysporum* and its ability to perform oxidation on furan substrates. The idea behind this work was the development of a case study that would lead to the green functionalization of HMF from lignocellulose. The gene encoding this enzyme was found after gene mining on biocatalysts with potential activities on furans. Eventually, the gene of the galactose oxidase was found as a member of the CAZy AA5 subfamily 2, consisting of 2046 bp and 682 amino acids. The amino acid sequence exhibited a 99% homology to the amino acid sequence of a characterized galactose oxidase from *F. oxysporum* and a 82% homology to a respective galactose oxidase sequence from *F. gramirearum.* The enzyme was produced by means of heterologous expression from *P. pastoris*

cells following the isolation of the encoding gene from the genome of *F. oxysporum* and its incorporation in *P. pastoris* X33 cells. After purification and isolation the enzyme was tested on its natural substrate performing optimally at a temperature of 40 ^{o}C and a pH of 6.0, while its molecular weight was identified at around 72 kDa. After identifying the optimal conditions, the enzyme was subjected to reactions with furan substrates managing to effectively oxidize HMF to DFF, and DFF and HMFCA to FFCA, respectively. It was evident that the enzyme followed the pathway of oxidizing the alcohol group of HMF and producing the corresponding aldehyde (DFF), while the enzyme was also able to oxidize the alcohol group of HMFCA when it was used as a substrate. This was in accordance with all the oxidative enzymes used to oxidize furans in literature, further proving its classification as a AA5_2 member. In addition, the enzyme also exhibited low yields in every reaction, which was also the case for most galactose oxidases tested on furans, in contrast to alcohol and aryl alcohol oxidases, which seem to have significantly higher yields. Even though, the complete biochemical characterization of this galactose oxidase was not achieved within the frame of this thesis, it was possible to validate its capacity to oxidize furans paving the way for eventually producing FDCA from HMF.

8. Future perspectives

The variety of compounds present in lignocellulose results in infinite potential regarding the final product of lignocellulose biorefineries. The customization of the processes for the disruption and valorization of its recalcitrant nature is pivotal toward the end-product. The first part of this work examined the production of fructose and HMF from lignocellulose. While it was evident that HMF can be produced at high yields from lignocellulose, further optimizing the pretreatment processes to receive materials more amenable to saccharification, will pave the way for higher fructose yields and subsequently better HMF production. To that end, the OxiOrganosolv process is a very effective way of pretreating lignocellulosic biomass. However, it has mainly been examined with ACO as the organic part of the solvent and this work also provides data on iBuOH, EtOH and THF. The incorporation of more organic solvents in the process might shed some light on the mechanism of the pretreatment and lead to the production of more amenable pulps. In addition, this work showed the potential of polyoxometalates as catalysts for pretreatment. The substitution of metal ions in the catalyst will uncover more scenarios in which polyoxometalates can be used in order to reinforce enzymatic processes in biorefineries. While OxiOrganosolv has been tested on both forest and agricultural residues, it is important that more lignocellulosic substrates are examined as substrates such as oak, wheat, cotton residues and sugarcane bagasse that have been proven to be promising candidates for lignocellulose biorefineries. Furthermore, a potential substrate for such a pretreatment would be a blend of different lignocellulosic feedstocks as this type of feedstock is similar to real streams that are made available from industries. Finally, a future perspective for the OxiOrganosolv pretreatment lies in its scale up as the use of O_2 as a catalyst can present a number of problems and needs further investigation.

Regarding the nature of the pretreatment, future work can also focus on expanding the type of pretreatment. This work presented the possibility of implementing microwaves as a heating source in pretreatment. However, in this work pretreatment was carried out in a small scale, using a limited number of parameters such as the type of solvent, the temperature and the residence time. It is necessary to expand the range of the final pretreatment temperature, which was not feasible in this work due to the high pressures in the pretreatment chamber. By performing pretreatment runs in a number of different temperatures it will be possible to enlarge the collection of pulps that will pave the way for a potential scale up of the microwave pretreatment. In addition, the examination of the temperature profiles using infrared technology might provide crucial results on heat transfer through organic solvents that will lead to the construction of models predicting the ideal power that needs to be applied for optimal results. Consequently, a better estimation of the economic feasibility of microwaves will be possible.

Finally, future perspectives include the work on biocatalysts able to catalyze furan oxidation. While this work was able to validate the activity of a galactose oxidase on furans, further enzyme discovery will lead to the expansion of the arsenal of enzymes with furan activity. However, as far

as *Fo*GalOx is concerned, the low yields of the oxidation reactions observed in this work, necessitates the examination of the reaction parameters. Including more enzymes in the cascade of the reactions is necessary in order to boost the *Fo*GalOx activity and increase the yields of the products. This way, it might be possible to reach the final stage of the oxidation and produce FDCA from HMF. Furthermore, another future perspective is the determination of the kinetic parameters on HMF as well as the discovery of substances that could potentially inhibit the activity of the enzyme. After optimizing all the oxidation reaction parameters, it will be possible to move from using pure chemicals as substrates to HMF produced from hydrolysates. This will be the final step to create an integrated pathway that presents the complete valorization of a lignocellulosic substrate from the moment it was collected from nature to finally producing FDCA as a bio-based monomer.

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Appendix A – List of figures

Appendix B – List of tables