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Dietary fibre production from oat hulls

Schmitz, Eva

2022

Document Version:
Publisher's PDF, also known as Version of record

Link to publication

Citation for published version (APA):
Schmitz, E. (2022). *Dietary fibre production from oat hulls*. Division of Biotechnology, Lund University.

Total number of authors:
1

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Dietary fibre production from oat hulls

EVA SCHMITZ | DIVISION OF BIOTECHNOLOGY | LUND UNIVERSITY

Printed by Media-Tryck, Lund 2021 NORDIC SWAN ECOLABEL 3041 0903

Is waste the solution?

Humankind has been exploiting its habitat for too long. We are well aware of the consequences that await us, if we do not start living more sustainably. Can we accomplish that without losing our modern standard of living? One approach promising exactly this is the bioeconomy. In a bioeconomy all products are made from renewable biomass instead of fossil resources. Agricultural waste, such as oat hulls, is an excellent raw material for this purpose as it is already available in large quantities at low costs and does not compete with the food industry. Besides establishing appropriate raw materials, many production lines have to be redesigned or reinvented. This requires the effort of many researchers and developers, each providing one small solution contributing a little part to achieving the big challenge of sustainable living. This thesis describes one of these small solutions.

LUND UNIVERSITY

ISBN: 978-91-7422-854-0

Faculty of Engineering
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Dietary fibre production from oat hulls

By Eva Schmitz

LUND
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DOCTORAL DISSERTATION

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To be defended at Kemicentrum lecture hall A the 3rd of February 2022 at 9.15.

Faculty opponent
Professor Henk Schols
Wageningen University & Research

Organization LUND UNIVERSITY Division of Biotechnology P.O. Box 124 SE 22100 Lund, Sweden	**Document name** Doctoral thesis **Date of issue** 2022-01-03
Author Eva Schmitz	**Sponsoring organizations**: BioInnovation (Vinnova, Formas, Swedish Energy Agency), Lantmännen Research Foundation

Title and subtitle
Dietary fibre production from oat hulls

Abstract

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In this work, oat hulls were found to be an excellent source of dietary fibres as they are rich in lignocellulose (up to 83 % of its dry weight) and the hemicellulose arabinoxylan (35 %). Arabinoxylan has been identified as a promising dietary fibre with anti-inflammatory properties. The hull's outstanding composition, however, is threatened by the presence of extreme weather events such as unusual heat and low precipitation during its growth phase leading to a loss of 25 % of its lignocellulose and 31 % of its hemicellulose content. Simultaneously, the separation of grain and hull is impeded leading to lower industrial grain yields and higher starch contamination of the hull fraction.

Despite its excellent chemical composition for the production of dietary fibres, its optical properties are rather unfavourable in the eyes of consumers due to its dark coloration which challenges its seamless inclusion in a variety of products. In this work, a mild and effective bleaching method was developed using alkaline hydrogen peroxide. This method is suitable for the production of a white insoluble dietary fibre product with a CIE L* (lightness parameter) value of above 85, while retaining the hull's lignocellulosic content. The method is robust yielding similar bleaching results despite large raw material batch variation caused by different weather conditions during the oat's growth phase.

For oat hulls to be used as source for soluble dietary fibres with potential prebiotic effects, their recalcitrance needs to be overcome. In this work, an ultrasonication assisted alkali pre-treatment method was developed that specifically targeted the solubilisation of the hemicellulose fraction. A 10 min ultrasonication stage in water followed by a 9 h sodium hydroxide (5 M) stage at 80 ºC resulted in the solubilisation of 75 % of the present hemicellulose.

For soluble dietary fibres to have a prebiotic effect, shorter chain lengths are favourable. The production of (arabino-)xylooligosaccharides up to a degree of polymerisation of 6 was achieved in high yields (57 %) by combining a ferulic acid esterase (FAE) with GH11 and GH5 xylanases. The FAE provides more access of the hemicellulose backbone to xylanases and supports further solubilisation, while the two xylanases hydrolyse the backbone in different parts of the xylan chain.

Overall, oat hulls were found to have a great potential as source for the production of both soluble and insoluble dietary fibres. In preliminary product evaluation and upscaling trials, the developed methods for the production of both fibre types were promising for application on industrial scale.

Key words: oat hulls, dietary fibre, chemical composition, bleaching, pre-treatment, enzyme synergy

Classification system and/or index terms (if any)

Supplementary bibliographical information		**Language** English, Swedish, German
ISSN and key title		**ISBN** 978-91-7422-854-0 (print) 978-91-7422-855-7 (pdf)
Recipient's notes	**Number of pages** 80	Price
	Security classification	

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Dietary fibre production from oat hulls

By Eva Schmitz

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Faculty of Engineering
Department of Chemistry
Division of Biotechnology

ISBN 978-91-7422-854-0 (print)
ISBN 978-91-7422-855-7 (pdf)

Printed in Sweden by Media-Tryck, Lund University
Lund 2021

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"It's a dangerous business, going out of your door.
You step onto the road, and if you don't keep your feet,
there's no knowing where you might be swept off to."

- J. R. R. Tolkien

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Abstract

The current threats of climate change connected to the unsustainable consumption of fossil resources paired with an increasing world population demands the exploration of alternative raw materials for the production of food, fuel and specialty chemicals. Agricultural waste streams present suitable sources due to their availability in large quantities at low costs without competing with current food production. Among them are oat hulls, the outer protective layer of the oat grain, which are currently mostly disposed via incineration.

In this work, oat hulls were found to be an excellent source of dietary fibres as they are rich in lignocellulose (up to 83 % of its dry weight) and the hemicellulose arabinoxylan (35 %). Arabinoxylan has been identified as a promising dietary fibre with anti-inflammatory properties. The hull's outstanding composition, however, is threatened by the presence of extreme weather events such as unusual heat and low precipitation during its growth phase leading to a loss of 25 % of its lignocellulose and 31 % of its hemicellulose content. Simultaneously, the separation of grain and hull is impeded leading to lower industrial grain yields and higher starch contamination of the hull fraction.

Despite its excellent chemical composition for the production of dietary fibres, its optical properties are rather unfavourable in the eyes of consumers due to its dark coloration which challenges its seamless inclusion in a variety of products. In this work, a mild and effective bleaching method was developed using alkaline hydrogen peroxide. This method is suitable for the production of a white insoluble dietary fibre product with a CIE L* (lightness parameter) value of above 85, while retaining the hull's lignocellulosic content. The method is robust yielding similar bleaching results despite large raw material batch variation caused by different weather conditions during the oat's growth phase.

For oat hulls to be used as source for soluble dietary fibres with potential prebiotic effects, their recalcitrance needs to be overcome. In this work, an ultrasonication assisted alkali pre-treatment method was developed that specifically targeted the solubilisation of the hemicellulose fraction. A 10 min ultrasonication stage in water

followed by a 9 h sodium hydroxide (5 M) stage at 80 °C resulted in the solubilisation of 75 % of the present hemicellulose.

For soluble dietary fibres to have a prebiotic effect, shorter chain lengths are favourable. The production of (arabino-)xylooligosaccharides up to a degree of polymerization of 6 was achieved in high yields (57 %) by combining a ferulic acid esterase (FAE) with GH11 and GH5 xylanases. The FAE provides more access of the hemicellulose backbone to xylanases and supports further solubilisation, while the two xylanases hydrolyse the backbone in different parts of the xylan chain.

Overall, oat hulls were found to have a great potential as source for the production of both soluble and insoluble dietary fibres. In preliminary product evaluation and upscaling trials, the developed methods for the production of both fibre types were promising for application on industrial scale.

Popular science summary

Climate change caused by an unsustainable use of fossil resources as well as a growing world population with increasing health problems are two leading challenges we are facing in the present time. Both of these challenges require us to identify and develop new ways of using sustainable resources for the production of everyday goods as well as healthy food products.

The agricultural industry generates many waste streams that are presently underutilised. Many of these waste streams are composed of the parts of the plants that are not edible. One example of a waste stream produced in large amounts at low costs is the oat hull. The hull is the outer non-edible shell of the oat grain. Its function is to protect the grain from wind, weather and diseases. Therefore, it is a very tough and hard material, which is difficult to process.

In my doctoral studies, I analysed the oat hull's chemical composition in order to understand, if we can use it as a sustainable resource for the production of other materials. I found that the hull is rich in dietary fibres. Dietary fibres are an important part of our diet, which we generally consume too little of. Various health benefits are associated with the consumption of dietary fibres including lower levels of inflammation and a strengthened immune system. Oat hulls are particularly rich in a specific dietary fibre called arabinoxylan, which makes them rather unique compared to other agricultural side streams. Unfortunately, this valuable arabinoxylan content is threatened by extreme weather events such as unusual heat and little precipitation. In the very hot and dry summer of 2018, the oat hull lost 31 % of its arabinoxylan content. This does not only show that our plants are becoming less nutritious, but also that the processing methods we are developing today need to be adaptable to the yearly variation of the plants.

Dietary fibre products can be separated into two categories: insoluble and soluble fibres. Naturally, the oat hull is very rich in insoluble fibres, which are rather unaccepted by consumers due to their dark brown colour. This colour makes it difficult to include them in other food products such as pasta and bread without changing the visual appearance of the pasta or bread. Therefore, I developed a suitable and robust bleaching method for the food industry in this thesis. The resulting insoluble dietary fibres are

off-white in colour and have a neutral smell, which increases their consumer acceptance. The developed method is robust as it gives similar results on oat hull batches that were grown in different years and therefore contain different amounts of arabinoxylan.

Soluble dietary fibres have recently received a lot of attention due to their potential of being prebiotic. Prebiotics are substances that specifically stimulate the growth and fermentation of beneficial gut bacteria, the so called probiotics, which in turn exert various health benefits on their host. Naturally, oat hulls do not contain any soluble dietary fibres due to their chemical structure. The individual parts are heavily interconnected, which hinders their solubilisation. In order to produce soluble dietary fibres, these linkages between the different parts must be broken. Processes that can achieve this are called pre-treatments. In this thesis, I developed a pre-treatment method, which is capable of solubilising 75 % of the arabinoxylan present in the oat hull. The method involves two stages: ultrasonication (a physical method) and alkali treatment (a chemical method). The ultrasound waves are weakening the structure, introducing more space for the alkali chemical, which then breaks the connecting bonds.

In order for soluble dietary fibres to have prebiotic effects, they need to be further reduced in size. Specific proteins called enzymes are capable of facilitating these reactions. The great advantage of using enzymes instead of chemicals for these reactions is their specificity, which enables us to tailor very specific potentially prebiotic molecules. Even though the pre-treated soluble fibres have lost some of their complexity, their structure still needs to be attacked by more than one type of enzyme in order to produce significant yields of short dietary fibres. In this thesis, I studied the interactions of various enzymes with different functions on the solubilised oat hull material and found a combination of three enzymes that results in a high yield of short potentially prebiotic dietary fibres from oat hulls.

Overall, I discovered that oat hulls have great potential as new source for the production of both soluble and insoluble dietary fibres. In preliminary product evaluation and upscaling trials, the developed methods were promising for use on industrial scale.

Populärvetenskaplig sammanfattning

Klimatförändringar orsakade av en ohållbar användning av fossila resurser såväl som en växande världsbefolkning med ökande hälsoproblem är två ledande utmaningar vi står inför just nu. Båda dessa utmaningar kräver att vi identifierar och utvecklar nya sätt att använda hållbara resurser för produktionen av vardagsvaror samt hälsosamma livsmedelsprodukter.

Jordbruksindustrin genererar många avfallsflöden som för närvarande är underutnyttjade. Många av dessa avfallsströmmar består av de delar av växterna som inte är ätbara. Ett exempel på en avfallsström som produceras i stora mängder till låga kostnader är havreskalet. Skalet är det yttre, inte ätbara skiktet av havrekornet. Dess funktion är att skydda havrekärnan från vind, väder och sjukdomar. Därför är det ett mycket tufft och hårt material, vilket är svårt att bearbeta.

I mina doktorandstudier analyserade jag havreskalets kemiska sammansättning för att förstå om vi kan använda den som en hållbar resurs för produktion av olika material. Jag fann att skalet är rikt på kostfibrer. Kostfiber är en viktig del av vår kost, som vi i allmänhet konsumerar för lite av. Olika hälsofördelar förknippade med intag av kostfibrer är till exempel lägre nivåer av inflammation och ett förstärkt immunförsvar. Havreskalen är särskilt rika på en specifik kostfiber som kallas arabinoxylan, vilket gör dem ganska unika jämfört med andra jordbruks sidoströmmar. Tyvärr hotas detta värdefulla arabinoxylaninnehåll av extrema väderhändelser som ovanlig värme och för lite nederbörd. Under den mycket varma och torra sommaren 2018 tappade havreskalet 31 % av sitt arabinoxylaninnehåll. Detta visar inte bara att våra växter blir mindre näringsrika, utan också att de bearbetningsmetoder vi utvecklar idag måste kunna anpassas till växternas årliga variation.

Kostfiberprodukter kan delas in i två kategorier: olösliga och lösliga fibrer. Havskalet är naturligt mycket rikt på olösliga fibrer, som inte är så tilltalande för konsumenterna på grund av deras mörkbruna färg. Denna färg gör det svårt att inkludera dem i andra livsmedelsprodukter som pasta och bröd utan att ändra pastans eller brödets utseende. Därför utvecklade jag en lämplig och robust blekningsmetod för livsmedelsindustrin i denna avhandling. De resulterande olösliga kostfibrerna är benvita och har en neutral lukt, vilket ökar deras konsumentacceptans. Den utvecklade metoden är robust

eftersom den ger liknande resultat på havreskal som odlats under olika år och därför innehåller olika mängder arabinoxylan.

Lösliga kostfibrer har nyligen fått stor uppmärksamhet på grund av deras potential att vara prebiotiska. Prebiotika är ämnen som specifikt stimulerar tillväxt och jäsning av fördelaktiga tarmbakterier, de så kallade probiotika, som i sin tur ger olika hälsofördelar för sin värd. Havreskalen innehåller naturligt inga lösliga kostfibrer på grund av deras kemiska struktur. De enskilda delarna är starkt sammankopplade, vilket hindrar deras löslighet. För att producera lösliga kostfibrer måste dessa kopplingar mellan de olika delarna brytas. Processer som kan uppnå detta kallas förbehandlingar. I denna avhandling utvecklade jag en förbehandlingsmetod som kan lösa upp 75 % av det arabinoxylan som finns i havreskalen. Metoden innefattar två steg: ultraljudsbehandling (en fysisk metod) och alkalibehandling (en kemisk metod). Ultraljudsvågorna försvagar strukturen och introducerar mer utrymme för alkalikemikalien, som sedan bryter bindningarna.

För att lösliga kostfibrer ska ha prebiotiska effekter måste de minskas ytterligare i storlek. Specifika proteiner som kallas enzymer kan underlätta dessa reaktioner. Den stora fördelen med att använda enzymer istället för kemikalier för dessa reaktioner är deras specificitet, vilket gör att vi kan skräddarsy potentiellt prebiotiska molekyler. Även om de förbehandlade lösliga fibrerna har tappat en del av sin komplexitet, behöver strukturen fortfarande attackeras av mer än en typ av enzym för att ge betydande utbyten av korta kostfibrer. I denna avhandling studerade jag interaktionerna mellan olika enzymer med olika funktioner på det lösliga havreskalmaterialet och hittade en kombination av tre enzymer som resulterade i ett högt utbyte av korta potentiellt prebiotiska kostfibrer från havreskal.

Sammantaget upptäckte jag att havreskalen har en stor potential som ny källa för produktion av både lösliga och olösliga kostfibrer. I preliminära produktutvärdering och uppskalningsförsök var de utvecklade metoderna lovande för användning i industriell skala.

Populärwissenschaftliche Zusammenfassung

Der durch eine nicht nachhaltige Nutzung fossiler Ressourcen verursachte Klimawandel sowie eine wachsende Weltbevölkerung mit zunehmenden Gesundheitsproblemen sind zwei zentrale Herausforderungen der Gegenwart, denen wir uns stellen müssen. Beide Herausforderungen verlangen von uns, neue Wege der Nutzung nachhaltiger Ressourcen für die Produktion von Gütern des täglichen Bedarfs sowie von gesunden Lebensmitteln zu identifizieren und zu entwickeln.

Die Agrarindustrie erzeugt viele Abfallströme, die derzeit nicht ausreichend genutzt werden. Viele dieser Abfallströme bestehen aus nicht essbaren Pflanzenteilen, die in großen Mengen zu geringen Kosten produziert werden. Ein Beispiel ist die Haferhülse. Diese Hülse ist die äußere, nicht essbare Hülle des Haferkorns. Ihre Funktion besteht darin, den Samen vor Wind, Wetter und Krankheiten zu schützen. Daher besteht sie aus einem sehr zähen und harten Material, das schwer zu verarbeiten ist.

In dieser Dissertation habe ich die chemische Zusammensetzung der Haferhülse analysiert, um zu verstehen, ob wir sie als nachhaltige Ressource für die Herstellung anderer Materialien nutzen können. Meine Ergebnisse zeigen, dass die Hülse außergewöhnlich reich an Ballaststoffen ist. Ballaststoffe sind ein wichtiger Bestandteil unserer Ernährung, von dem wir in der Regel zu wenig konsumieren. Mit dem Verzehr von Ballaststoffen sind verschiedene gesundheitliche Vorteile verbunden, darunter der Rückgang von Entzündungen und ein gestärktes Immunsystem. Haferhülsen sind besonders reich an dem spezifischen Ballaststoff Arabinoxylan, was sie im Vergleich zu anderen landwirtschaftlichen Abfallströmen einzigartig macht. Bedauerlicherweise ist dieser wertvolle Arabinoxylangehalt durch extreme Wetterereignisse wie starke Hitze und geringe Niederschläge bedroht. Im sehr heißen und trockenen Sommer 2018 verlor die Haferhülse 31 % ihres Arabinoxylangehalts. Dies zeigt nicht nur, dass unsere Pflanzen weniger nahrhaft werden, sondern auch, dass Verarbeitungsmethoden an die jährliche Variation der Pflanzen angepasst werden müssen.

Ballaststoffprodukte können in zwei Kategorien unterteilt werden: unlösliche und lösliche Ballaststoffe. Die Haferhülse ist von Natur aus sehr reich an unlöslichen

Ballaststoffen, wird aber aufgrund ihrer dunkelbraunen Farbe von Verbrauchern wenig akzeptiert. Denn die dunkle Farbe erschwert es, die Hülse ohne optische Veränderungen in andere Lebensmittel wie Nudeln oder Brot zu integrieren. Daher habe ich in dieser Arbeit eine geeignete und robuste Bleichmethode für die Lebensmittelindustrie entwickelt. Die resultierenden unlöslichen Ballaststoffe sind cremefarben und haben einen neutralen Geruch, was ihre Verbraucherakzeptanz erhöht. Die entwickelte Methode ist robust, da sie bei Haferhülsenchargen, die in verschiedenen Jahren angebaut wurden und daher unterschiedliche Mengen an Arabinoxylan enthalten, ähnliche Ergebnisse liefert.

Lösliche Ballaststoffe haben in letzter Zeit aufgrund ihres präbiotischen Potenzials viel Aufmerksamkeit erhalten. Präbiotika sind Substanzen, die gezielt das Wachstum und die Fermentation von nützlichen Darmbakterien, den sogenannten Probiotika, stimulieren, die im Gegenzug verschiedene gesundheitliche Vorteile für ihren Wirt auslösen. Von Natur aus enthalten Haferhülsen aufgrund ihrer chemischen Struktur keine löslichen Ballaststoffe. Deren einzelne Bestandteile sind vielschichtig miteinander verbunden, was ihre Löslichkeit behindert. Um lösliche Ballaststoffe herzustellen, müssen diese Verbindungen zwischen den verschiedenen Teilen aufgebrochen werden. Verfahren, die dies erreichen können, werden Vorbehandlungen genannt. In dieser Arbeit habe ich eine Vorbehandlungsmethode entwickelt, die in der Lage ist, 75 % des in der Haferhülse vorhandenen Arabinoxylans zu lösen. Das Verfahren umfasst zwei Stufen: Ultraschall- (eine physikalische Methode) und Alkalibehandlung (eine chemische Methode). Die Ultraschallwellen schwächen die Struktur und schaffen mehr Platz für die Alkalichemikalie, die im Anschluss die Verbindungen aufbricht.

Damit lösliche Ballaststoffe präbiotisch wirken können, müssen sie weiter verkleinert werden. Bestimmte Proteine, sogenannte Enzyme, sind in der Lage, diese Reaktionen zu beschleunigen. Der große Vorteil der Verwendung von Enzymen anstelle von Chemikalien für diese Reaktionen ist ihre Spezifität, die es uns ermöglicht, sehr bestimmte potenziell präbiotische Moleküle zuzuschneiden. Auch wenn die vorbehandelten löslichen Ballaststoffe etwas an Komplexität verloren haben, muss deren Struktur noch von mehr als einer Enzymart angegriffen werden, um signifikante Ausbeuten an potentiellen Präbiotika zu erzielen. In dieser Arbeit habe ich die Interaktionen verschiedener Enzyme mit unterschiedlichen Funktionen auf dem löslichen Haferhülsenmaterial untersucht und eine Kombination von drei Enzymen gefunden, die zu einer hohen Ausbeute an kurzen potenziell präbiotischen Ballaststoffen führt.

Insgesamt habe ich festgestellt, dass Haferhülsen ein großes Potenzial als neue Quelle für die Produktion von löslichen und unlöslichen Ballaststoffen haben. In vorläufigen Produktbewertungs- und Hochskalierungsversuchen waren die entwickelten Verfahren vielversprechend für den Einsatz in der Industrie.

List of papers

This thesis is based on the following papers, referred to by their Roman numerals and provided at the end of the thesis. All papers are reprinted with permission of their respective publishers.

I. **Warming weather changes the chemical composition of oat hulls**

E. Schmitz, E. Nordberg Karlsson and P. Adlercreutz

2020, Plant Biology 22(6): 1086-1091

II. **Chemical and biochemical bleaching of oat hulls: The effect of hydrogen peroxide, laccase, xylanase and sonication on optical properties and chemical composition**

E. Schmitz, J. Francis, K. Gutke, E. Nordberg Karlsson, P. Adlercreutz and M. Paulsson

2021, Biotechnology Reports 30: e00624

III. **Ultrasound assisted alkaline pre-treatment efficiently solubilises hemicellulose from oat hulls**

E. Schmitz, E. Nordberg Karlsson, P. Adlercreutz

2021, Waste and Biomass Valorization 12: 5371-5381

IV. **Lignocellulose degradation for the bioeconomy: the potential of enzyme synergies between xylanases, ferulic acid esterase and laccase for the production of arabinoxylo-oligosaccharides**

E. Schmitz, S. Leontakianakou, S. Norlander, E. Nordberg Karlsson and P. Adlercreutz

2022, Bioresource Technology 343: 126114

My contribution to the papers

All work described in this thesis was performed under the supervision of Prof. Patrick Adlercreutz and Prof. Eva Nordberg Karlsson at the Division of Biotechnology.

I. I planned the study, performed all experiments, evaluated the data, wrote the manuscript and submitted it as corresponding author.

II. Together with J. Francis, I planned, performed and evaluated the biochemical bleaching experiments. K. Gutke and M. Paulsson carried out the chemical bleaching experiments and I took part in their evaluation by performing the chemical characterisation of all materials. I wrote the majority of the manuscript with the help of M. Paulsson and submitted it as corresponding author.

III. I planned the study, performed all experiments, evaluated the data, wrote the manuscript and submitted it as corresponding author.

IV. I planned the study, performed all experiments on oat hulls as well as the enzyme stability experiments on all substrates and evaluated the corresponding data. I discussed the data produced by my co-authors S. Leontakianakou and S. Norlander with them, wrote the manuscript and submitted it as corresponding author.

Papers not included in the thesis

The following papers were also written as part of my PhD education, but are not included in this thesis.

i. **Endo-xylanases as tools for production of substituted xylooligosaccharides with prebiotic properties**

E. Nordberg Karlsson, E. Schmitz, J. A. Linares-Pastén, P. Adlercreutz

2018, Applied Microbiology and Biotechnology 102: 9081-9088

ii. **Altering the water holding capacity of potato pulp via structural modifications of the pectic polysaccharides**

E. Schmitz, E. Nordberg Karlsson, P. Adlercreutz

2021, Carbohydrate Polymer Technologies and Applications 2: 100153

1 Introduction

Modern economies largely depend on finite and environment polluting fossil resources. Recent decades have shown that their vast consumption is not sustainable, posing manifold threats in the form of climate change and resource scarcity. While it is not possible to reliably predict the approximate time point of their exhaustion, the depletion rates of the world's largest fossil oil fields are increasing at an alarming rate suggesting the almost complete consumption of the world's reserves by 2100 [1]. A potential alternative economic strategy, which addresses and relieves the negative effects of fossil resource consumption, is the bioeconomy [2, 3]. In a bioeconomy, fossil resources are substituted with renewable biological feedstocks from which essential materials, chemicals and energy are produced [3-5]. No single widely accepted bioeconomy definition exists and the manifold available definitions differ greatly depending on the national and international strategies they originate from. However, all definitions mention the requirement of new scientific and technological developments based on advanced biological and chemical conversion technologies for a successful establishment [4, 5]. Assuming that those are developed in a cost competitive manner, the positive impacts of a sustainable bioeconomy on environmental challenges are outstanding and an important step towards fulfilling the United Nations' sustainable development goals [4].

For the successful establishment of a sustainable bioeconomy, it is important that all components are sustainable, beginning with the utilised raw material. Ideally, these biomass feedstocks should be available in sufficient quantities year-round and at low costs. They must not add further to environmental pollution and be accepted as suitable raw materials by society. Their holistic use avoiding the production of waste streams is favoured [2]. Lignocellulosic waste streams from the agricultural and forestry industries present an excellent group of biomaterials fulfilling many of these criteria: they are available at low cost and in large quantities, they do not require the cultivation of more land than currently utilised and do not compete with the production of biomass for food [3].

One potential agricultural waste stream suitable as starting material in a bioeconomy is oat hulls. Oat hulls are the outer, protective layer of the oat grain. They are typically disposed during oat production for food purposes by incineration or land filling, posing

further risks for environmental pollution [6]. This is unfortunate as the hull's lignocellulosic structure consists of building blocks having great potential for products in many industries, such as dietary fibre supplements in the food industry, biobased films for the packaging industry [7] and composites for building materials in the construction industry [8]. The challenge to accessing its potential as raw material lies in breaking its recalcitrant structure, i.e. overcoming its resistance to biological and chemical decomposition. Therefore, the most important technologies to be developed in the framework of enabling the establishment of bioeconomies include suitable conversion technologies or pre-treatments, which make the raw material accessible for further modification. However, subsequent processing techniques should not be disregarded, as they need to be more specific and highly adapted to the industry developing the end products.

1.1 Aim and scope of the thesis

The present thesis is aiming at supporting the establishment of a bioeconomy in Sweden by contributing scientific knowledge in three areas: a potential new raw material, a suitable pre-treatment method to access its building blocks and other processing techniques for the development of end products, in particular dietary fibres.

The suitability of oat hulls as potential biomass feedstock for the bioeconomy and source of dietary fibres is assessed by characterisation of its chemical composition in **Paper I**. Interesting fractions are identified and climate effects discussed. A potential application as insoluble dietary fibre supplement requiring minimal processing suitable for the food industry is identified and described in **Paper II**. To enable further applications in various industries, an efficient pre-treatment method aiming at solubilising its hemicellulose fraction was developed. The method itself as well as the insight it is giving on the oat hull's lignocellulosic structure is discussed in **Paper III**. For the production of a variety of different soluble compounds the pre-treated material needs to be further hydrolysed. An exploration of enzyme synergies to produce potentially prebiotic fibres in high yields was conducted and evaluated in **Paper IV**. For a more complete understanding of the enzyme interactions, two additional lignocellulosic substrates besides oat hulls were utilised in the study.

2 Oat hulls

The oat plant is an annual grass belonging to the taxonomic family *Gramineae* or *Poaceae* together with other small grained cereals such as wheat, rye and barley [9]. Oats are ranked under the genus *Avena*, of which 27 species or sub-species are currently known. The agriculturally most dominating species is *Avena sativa*, commonly known as cultivated oats, which occupies 75 % of the world's oat production [9, 10]. The edible part of the plant, the groat, is often covered by a resilient hull or husk, contributing between 25 and 35 % of the grains weight [11]. In literature, contradicting terminologies are often used to describe the parts of the oat plant. In this thesis, the terminology explained in Figure 2.1 is adopted.

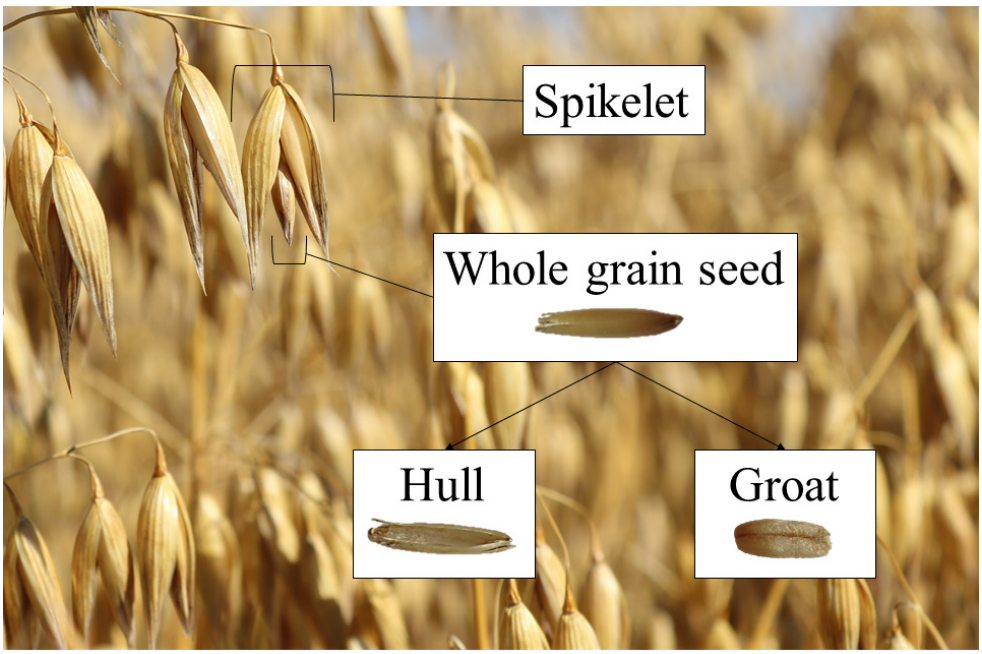


Figure 2.1: Terminology of the oat plant parts used in this thesis.
Various amounts of whole grain seeds can be found in one spikelet.

Oat as domestic crop appears much later than other small grained cereals in human history. Its place of origin is still unknown, but is suspected to be in the Mediterranean basin or the Middle East. During most of its cultivation history, its nutritional value was vastly underestimated. First records mention its presence as undesirable weed in wheat and barley fields until its cultivation began during the Roman conquest of Europe. In these times, it was almost exclusively fed to horses [12]. Introduction of oat fields in new areas went along with the expansion of the Roman cavalry. Once Great Britain was reached, oat cultivation flourished and became an essential part of European crop production due to the simultaneous onset of a change in climate. Increasingly moist weather conditions and a reduction of soil pH gave rise to lower barley and wheat yields, the most commonly grown cereals at that time. Oat had a particularly great success in Scotland, Ireland and Wales, where it became the most popular grain in the 18th century and a fundamental ingredient in people's diets [12, 13]. Increasing urbanisation in the late 19th century led to a decrease in the need for horses and hence, oat. Concurrently, oat was replaced as a major protein source in animal feed with higher protein yielding crops, such as soybean [9, 12]. In the northern latitudes of both Canada and Europe, oat production did not suffer as great of a reduction as fewer alternative crop choices are competitive in the local climate conditions [12]. Worldwide, however, oat production was more than halved from 1971 (54.5 million tonnes) to 1999 (24.1 million tonnes). Production quantities after 1999 remained rather constant with both positive and negative fluctuations of maximal 5 million tonnes [14].

Recent trends in food consumption as well as discoveries of the outstanding health benefits of oat consumption are forecasting a demand for an increased oat production in the coming years, especially in Europe and North America. Oat for human consumption is gaining particular attention as vegan alternative for dairy and meat products [9, 15]. Historically, no great breeding efforts have been undertaken which increased the content of a particular fraction. Hence, the present day *Avena sativa* contains many fractions in considerable quantities, whose health benefits are currently being discovered. These fractions include soluble fibres, protein, oil, and specific vitamins and minerals such as iron, magnesium, phosphorus, zinc, copper, manganese and selenium. Compared to other cereals, the amino and fatty acid profiles in the protein and oil fractions in oat are superior due to their greater content in all the essential amino acids and more favourable ratio of unsaturated to saturated fatty acids [9, 10, 12, 15]. Particular attention, however, has been given to the soluble fibre β-glucan. A large variety of positive health benefits has been associated with its consumption such as reduced blood glucose and serum cholesterol levels, reduced risks for cardiovascular diseases, gastrointestinal disorders, type II diabetes as well as central adiposity and obesity. In most cases these effects are attributed to changes in the gut

microbiota composition upon consumption of β-glucan [15]. Considerably fewer studies have investigated the effects of insoluble fibres such as arabinoxylan. Those are also present in the oat kernel, but to a much larger extent in the hull (**Paper I**). While too few human studies have been conducted to draw reasonable conclusions, the ones carried out so far are pointing towards positive effects on inflammation, improved lung performance and reduced mortality rates in respiratory-related diseases [10].

As this literature study reveals, almost all attention has so far been given to the oat groat. Scientific literature on the oat hull is scarce. Many studies go as far as claiming that the groat contains almost all the nutritive value, while the hull is considered of little value or even a negative component in feed and impedes processing. Modern breeding efforts have therefore been aiming at reducing the hull content of the grain [12]. As the hull's main function in the oat plant is to protect the groat from diseases and environmental stresses, those reductions in hull content are likely to result in further problems. Studies comparing hulled *Avena sativa* with a hulless variant (falsely classified as a different species, *Avena nuda*, in the past) show that the hulless variant experiences greater reductions in grain yields, especially under severe water stresses [9]. As those could occur more frequently in the future due to climate change, it is safe to assume that the hull is here to stay. The outlook of increasing production quantities of hulled oats demands further processing of the hull to prevent it from being wasted, ideally in a bioeconomy context. To assess its potential for various applications and design the most value-adding processes, a detailed understanding of its chemical composition is required.

2.1 Chemical composition

As oat hulls have not received much attention in the biochemical research community so far, only superficial chemical characterisations can be found in scientific literature. Those are solely reporting the presence and quantities of the biggest fractions, but do not characterise them in more detail [16-18]. **Paper I** provides the first detailed chemical characterisation of oat hulls produced in large quantities by the agricultural industry. There is an abundant amount of oat varieties belonging to the species *Avena sativa*, which all differ slightly in their chemical composition. In this work, an industrially available mixture of the varieties Kerstin and Galant was analysed and processed. These varieties are grown in large quantities in Sweden and therefore represent a suitable fraction for use as raw material in a bioeconomy process. As a mixture is used for characterisation as well as all process experimentation, it is likely that the results are not variety specific and can be transferred to other varieties.

Oat hulls resemble in their overall chemical composition other cereal by-products such as hulls and straws in that they are mostly composed of lignocellulose. Lignocellulose is a material containing three main fractions: cellulose, hemicellulose and lignin. Cellulose is a linear homopolymer composed of β-1,4-linked glucose units, which assemble in microcrystalline structures. Hemicellulose is a branched heteropolymer composed of various material specific units of hexoses, pentoses and uronic acids. Lignin is a highly irregular organic polymer formed by non-repeating phenyl-propane units with varying degrees of methoxylation [19]. Commonly in cereal by-products, cellulose makes up the largest amount of the three fractions, followed by hemicellulose and lignin [20]. Oat hulls stand in contrast to this trend being mostly composed of hemicellulose with lignin being the second largest fraction and cellulose the third (**Paper I**). This unique chemical composition becomes evident in Table 2.1. Barley hull is the closest to oat hull regarding chemical composition, also exhibiting an exceptionally large hemicellulose content. However, this fraction seems to be more susceptible to changes based on environmental conditions and species varieties as a larger range of hemicellulose content could be found in literature as for oat hull. Compared to the oat groat, the chemical composition of the oat hull is very different, only containing small amounts of the nutritionally dominating fractions starch, protein and lipids. This explains why literature so far claims that the oat's nutritive value is entirely in the groat [12]. However, recent advances in the understanding of the role of the gut microbiota on health underlines the importance of consuming appropriate dietary fibres [10]. While the oat groat is generally considered a good source for dietary fibres, only 1-2 % of its weight are composed of dietary fibres. In contrast, the hull contains up to 84 % of lignocellulose, the fraction in which dietary fibres can be found. This indicates that the nutritive value of oat hulls for human consumption is potentially still vastly underestimated.

Table 2.1: Chemical composition of oat hulls as well as other parts of the oat plant and similar cereal by-products. The numbers represent percentage ranges based on dry weight. The fractions for which no data is available are labelled ND.

Component	Oat groat	Oat hull	Oat straw	Barley hull	Wheat straw
Cellulose	ND	16-26	31-38	34-39	34-39
Hemicellulose	1-2	24-35	20-28	12-36	23-30
Lignin	2-6	13-25	4-15	14-22	12-20
Starch	67-74	3-16	ND	10-11	ND
Protein	12-17	1-7	4	4-5	2-4
Lipid	5-9	1-2	ND	2-4	2
Ash	1	5-6	3	5	3-6
Literature source	[21-24]	**Paper I, III**	[20, 25, 26]	[20, 27, 28]	[20, 29-32]

Further interesting fractions in the oat hull are the phenolics and the minerals. Plant phenolics are a ubiquitous group of phytochemicals composed of a benzene ring equipped with one or more hydroxyl groups. In the plant, their main function is to serve as a protecting agent against environmental stresses and provide structural stability. Due to their ability to act as strong antioxidants, they are utilised by various industries in products intended for human consumption [33]. The most well studied phenolic acid in lignocellulosic materials is ferulic acid, a derivative of cinnamic acid. Structurally, it acts as a linker molecule in lignocellulose covalently cross-linking hemicellulose polymers via ferulic acid dimers or connecting hemicellulose with lignin forming an ester bond with hemicellulose side chains and an ether bond with lignin moieties [34, 35]. However, it is most known for its capability of acting as strong antioxidant, anti-inflammatory, anti-microbial, anti-allergic, anti-carcinogenic and immunomodulating agent [10, 36]. All these effects are the result of its ability to scavenge free radicals by donating a hydrogen atom from its phenolic hydroxyl group upon absorption of ultra-violet light. Oat hulls are rich in ferulic acid, containing up to 2840 µg/g (**Paper I, III**). This content is similar to the one in barley hull (2807 µg/g) [37], but considerably higher than that in oat bran (1689 µg/g, **Paper IV**). While the high amounts of phenolics present in oat hulls have been known for many years, the food industry still ignores the hull, claiming that the bran contains the highest amount [10]. This provides possibilities for other industries, such as the chemical and cosmetic industry, to utilise the ferulic acid in oat hulls as source for UV absorbers in sun screens or pre-cursors for the growing vanillin production. One drawback of using ferulic acid from biomass is the difficulty of obtaining adequate extraction yields, underlining the need for the development of efficient processing technologies [36].

Another minor fraction in oat hulls that deserves attention are the minerals. Oat groats are classified by the US Food and Drug Administration (FDA) as well as the European Food Safety Authority (EFSA) as good sources for iron, magnesium, phosphorus, copper and manganese for human consumption [10]. Table 2.2 provides an overview of the content of these minerals in the groat and the hull. While the hull contains fewer amounts of copper and manganese as the groat, it is richer in iron and phosphorus and comparable in its magnesium content. Hence, it should also be considered as a potential source of minerals for human consumption.

Table 2.2: Mineral composition of the oat groat and hull. All numbers are given in µg/g based on dry weight.

Mineral	Oat Groat	Oat Hull
Iron	39-63	11-130
Magnesium	800-1540	480-1500
Phosphorus	2630-3930	310-4400
Copper	3-300	1-5
Manganese	30-106	27-52
Literature source	[38-40]	**Paper I**

2.2 Hemicellulose content

Hemicellulose is an amorphous polysaccharide, whose composition varies widely across materials. It is commonly classified based on its predominant monosaccharide unit. In grasses and annuals, to which the cereals belong, the corresponding monosaccharide is xylose. Therefore, the hemicellulose is classified as xylan. Xylans can be further categorised into various subclasses depending on the occurrence of the second most predominant monosaccharide units [41]. In oat hulls, the second most common hemicellulose monosaccharide unit is arabinose (**Paper I**), hence, its hemicellulose can be classified as arabinoxylan. Arabinoxylans are composed of a backbone of β-1,4-linked xylopyranosyl units (Xyl*p*), which can be substituted with arabinofuranosyl (Ara*f*) residues at positions O-2 and/or O-3. Further backbone substituents include other monosaccharide units such as glucose or galactose, uronic acid units such as glucuronic acid, acetyl groups and phenolic acid units such as ferulic or coumaric acid. Phenolic acids are typically esterified at position 5 of the Ara*f* side groups [42]. A graphical representation of the arabinoxylan structure with the most common units present in oat hulls is displayed in Figure 2.2. Arabinoxylan belongs to the low hydration polysaccharides, whose main functions are to stabilise the plant's cell walls and support wall elongation. Therefore, it is predominantly found on the surface of cellulose microfibrils in the secondary cell wall [41].

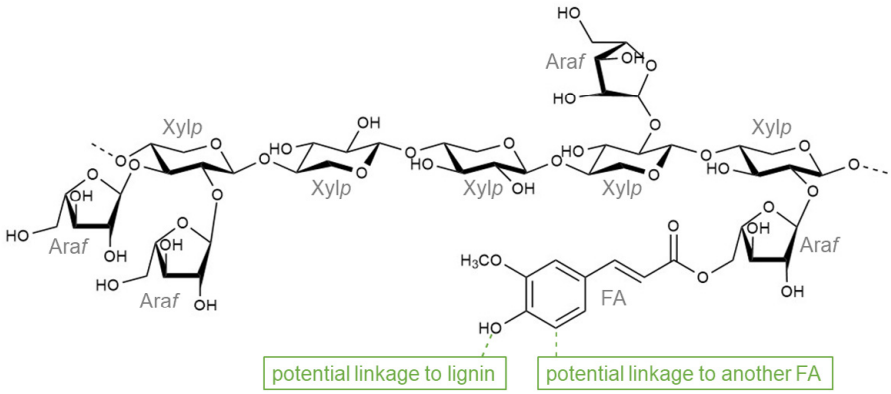


Figure 2.2: Schematic representation of the chemical structure of arabinoxylan.
Only the most common substituents in oat hull hemicellulose are displayed. The amount of arabinofuranosyl (Ara*f*) to xylopyranosyl (Xyl*p*) units is overestimated for oat hull arabinoxylan in order to show the various linkage possibilities. The abbreviation FA stands for ferulic acid unit.

A summary of the hemicellulose composition in oat hulls is given in Table 2.3. Besides Ara*f* and Xyl*p* only minor amounts of other substituents are present. A common way of reporting the degree of substitutions is the arabinose-to-xylose (A/X) ratio. The higher the achieved number the more arabinose substituents can be found on the xylan backbone, which gives an approximation of the amount of branches in the polymer. The A/X ratio of oat hulls is with 0.1 to 0.2 rather low. Much higher ratios of 0.5 to 1.0 have been reported for the oat groat [34]. More branched polymers are often more water soluble, simplifying their industrial processing. The low number of substituents on the xylan backbone in oat hulls, therefore, suggests the need for harsher treatment methods in order to obtain solubility. On the other hand, more branched polymers are less accessible to enzymatic attack, which could counteract the advantages of their water solubility.

Table 2.3: Hemicellulose composition of oat hulls. The numbers represent percentage ranges based on anhydrous hemicellulose content.

Component	Quantity in oat hulls
Arabinose	10-15
Galactose	4-6
Glucose	0
Mannose	0-2
Xylose	74-83
Galacturonic acid	1-3
Glucuronic acid	2-3
A/X ratio	0.1-0.2
Literature source	**Paper I, III**

In general, hemicelluloses are described as having a lower stability than cellulose. Together with its features of being biodegradable and biocompatible, many industries have expressed their interest in the polymer. From a food perspective, the polymer is interesting as dietary fibre component, emulsifier and edible food coating. The packaging industry could modify it via oxidation, reduction, esterification and etherification to produce packaging films that are less permeable to oxygen, but have a high aroma permeability and light transmittance. In the healthcare and pharmaceutical industry, cereal arabinoxylans have potential as prebiotics, antimicrobial films and encapsulation materials for probiotics as well as active pharmaceutical ingredients in drug delivery formulations. From a materials chemistry perspective, the possibilities seem infinite. Xylose and arabinose can be converted to furfural, which serves as a precursor for the production of many important products, such as transportation fuels, lubricants, rubbers, cements and adhesives. Xylose can also be converted to 2,3-butanediol, butanol, succinic acid, ethanol, ethylene derivatives, lactic acid and glycerol. All of those are precursors for further important materials, which are nicely summarised by Qaseem and colleagues [42]. This exemplifies the growing demand for sustainable sources of hemicelluloses and hence, the need to find new appropriate biomaterials rich in hemicellulose. Based on its chemical composition, the oat hull is very competitive.

2.3 Lignocellulose structure

One of the main obstacles of utilising lignocellulosic materials for the production of value-added goods is their recalcitrance, i.e. their resistance to degradation. The severity of biomass recalcitrance is directly dependent on its structural complexity being affected by a variety of structural factors based on chemical composition and physical interactions [42, 43]. Among the chemical species, the abundance of the following is contributing greatly to the material's recalcitrance: lignin, hemicellulose, acetyl groups, ferulate cross-linking and moisture. Among the physical interactions, the following play a significant role: polymer crystallinity, degree of polymerisation (DP), accessible surface area and porosity [42-46]. The importance of each of these factors varies between different lignocellulosic materials. An insightful simulation inspired schematic representation of the complex lignocellulosic structure can be found in the perspective article by Petridis and Smith [47].

Due to the large variability of lignocellulose originating from different sources, their tertiary structure can also differ significantly. However, certain features have been identified for all materials so far. Cellulose is characterised by long, linear chains of glucose units with DPs between 4,000 and 14,000 [6, 43]. These chains expose a vast amount of hydroxyl groups on their surface leading to the formation of a network of well-ordered hydrogen bonds, which allows a compact packing in crystalline microfibrils [48]. This hydrogen bonding extends to the surrounding hemicellulose, which has been described as coating to the cellulose microfibrils, acting as a physical barrier to cellulose degradation [6, 43, 45, 46].

Hemicellulose exhibits a more amorphous structure than cellulose due to the presence of heterosaccharide branches and acetyl groups. The DP of hemicellulose is with numbers between 50 and 300 significantly lower than that of cellulose [43, 48]. In cereals, the major hemicellulose is arabinoxylan, which consists of a backbone of xylopyranosyl units (Xyl*p*) decorated with arabinofuranosyl (Ara*f*) side groups. These Ara*f* units can be further esterified at their C-5 hydroxyl group to hydroxycinnamic acids, mainly ferulic and *p*-coumaric acid [49, 50]. Their abundance is one of the factors determining the recalcitrance of a material due to their ability to form cross-links between different hemicellulose chains via dimerization as well as to form ether bonds with lignin moieties. This type of ester-ether linkage is the primary covalent connection between hemicellulose and lignin [19, 48, 50, 51].

Lignin is often described as the 'glue' of lignocellulose keeping the other fractions together and thereby providing mechanical strength and significantly increasing the recalcitrance [43, 45]. While its covalent linkages to hemicellulose via hydroxycinnamic

acid bridges have been confirmed, it is still unclear, if its interaction with cellulose also occurs covalently or rather non-covalently as in the case of the hemicellulose-cellulose interaction [48]. The randomness and variety of the phenylpropane units and their interunit linkages in lignin make this the most challenging fraction to characterise and degrade. It is this intricate composition and the nature of the phenolic acid cross-linking rather than its abundance that regulate its recalcitrance [46].

As oat hulls have not been in focus of many research efforts so far, very little is known about its lignocellulosic structure. The composition analysis in **Paper I** (see Table 2.1) suggests a different structure to that of other common agricultural side streams, due to a rather large hemicellulose fraction followed by an equal distribution of both cellulose and lignin [20]. The lack of hemicellulose side chain decorations (see Table 2.3) suggests a less amorphous and more crystalline structure, different to the hemicellulose found in many other sources. Fewer decorations allow the hemicellulose polymers to form more hydrogen bonds leading to a better assembly and increased recalcitrance [52]. This structural possibility together with the large amount of present ferulate cross-linking could explain its recalcitrance to not only enzymatic attack, which are often mainly hindered by the presence of manifold side chain substituents [52], but also chemical attack as found in **Papers II, III** and **IV**. Alkaline hydrogen peroxide treatment should easily cleave the alkali labile ester bonds [48], however, this was not the case in **Paper II**. The increased hemicellulose crystallinity could impose mass transfer limitations that prevent the chemicals from entering the tight network [44]. This observation could be confirmed in **Paper III**, where ultrasonication was used to successfully enable an alkaline treatment to cleave these ester bonds. Ultrasonication most likely caused a disruption of the tight network and reduced hemicellulose crystallinity. Even after alkali solubilisation of the hemicellulose fraction in **Paper III**, certain parts of the hemicellulose remain insoluble in water, but could be further solubilised by combined hydrolysis with a GH11 xylanase and a ferulic acid esterase in **Paper IV**. In this combination, these enzymes are known to act well on insoluble regions of arabinoxylan. The lack of a synergistic action of a GH10 xylanase with a ferulic acid esterase proposes the lack of more substituted, solubilised regions as this enzyme combination generally releases more products from these regions. The hypothesis of a partially crystalline arabinoxylan structure is further supported by previous findings on arabinoxylan structure of whole oat grains including the hulls [53]. In this study, oat arabinoxylan was extracted in sequential steps with increasing alkalinity. The last extraction step utilised incubation in 6 M NaOH and yielded sparsely substituted arabinoxylan with an A/X ratio of 0.11. This fraction had a tendency to aggregate, solubilise poorly and be more resistant to enzymatic breakdown compared to more easily extracted fractions. The similarity in structure and physical

properties to the oat hull arabinoxylan assessed in this thesis suggests that this fraction originates from the hull.

2.4 Changing climate considerations

The change in climate Europe experienced 1500 years ago enabled oat cultivation to flourish [13]. The change in climate we are currently experiencing might have the opposite outcome. In Sweden, oat is a popular crop grown in large quantities (see Figure 2.3 for an example). In recent normal weather years, such as 2016, 2017 and 2019, yields of 4.4 to 4.8 tonnes per hectare were achieved. In the extreme weather year of 2018, which was characterised by very high average temperatures (2-3 °C higher) and little precipitation (33-50 % less), this yield was drastically reduced to only 2.5 t/ha [14, 54]. Additionally, not only the yield, but also the nutrient composition of food crops is known to be influenced by many factors including environmental conditions [10]. The oat crop is no exception to this. Previous studies have shown that the β-glucan content and structure in oats is also very susceptible to different environmental conditions during plant growth including environmental temperature and precipitation quantities [15, 55].

Figure 2.3: Oat fields located on the Swedish island Öland (A) and at the edge of the town Lund in Southern Sweden (B).
The images were taken in June and August 2021, respectively.

Oat hulls are a very tough and recalcitrant material designed to protect the groat from environmental stresses. However, the stresses of high temperatures and low precipitation cause a drastic change in their chemical composition suggesting a weakened ability to protect the groat (**Paper I**). In 2018, the lignocellulosic content of oat hulls grown in Sweden was reduced by approximately 25 % compared to regular weather years. Especially the hemicellulose fraction in the lignocellulose suffered great losses with a reduction of about 21 %. Interestingly, not only the total amount of hemicellulose, but also its composition changed. Overall, less backbone Xyl*p* and more side chain units, especially Ara*f*, were present in the 2018 grown hulls. While Ara*f* and Xyl*p* units made up 10.0 and 82.3 % of the hemicellulose in 2017, respectively, the Ara*f* content was increased to 15.4 % and the Xyl*p* content decreased to 74.2 % in 2018. This shift in composition is also visible in the A/X ratio, which increased from 0.1 to 0.2. The hull hemicellulose produced by the oat plant in warmer and drier weathers is therefore more branched, suggesting a higher rigidity, potentially as protection mechanism (higher defence capacity) in extreme environments. According to this hypothesis, the phenolic acid content should be upregulated to enable stronger cross-linking in the lignocellulose. This behaviour has been observed in many plants,

when they are exposed to environmental stresses including high temperatures and drought [56]. However, the opposite was observed in oat hulls. The phenolic acid content was decreased by up to 63 %. While this would be detrimental for an industrial process requiring the phenolic content of oat hulls as raw material, it could also have implications for the industries utilising the other fractions. Phenolic acids play an important role in the plant's protection mechanism against fungal and microbial attack [57]. Hence, warmer and drier years could lead to the production of more microbially infected hulls, which could be unsuitable for their intended use or in the least reduce the quality of their products. The other smaller quantity fractions protein, starch, lipids and minerals were also found in different amounts. Both the protein and starch content was increased in the hull grown in the extreme weather, suggesting developmental complications leading to immaturity at the end of the growth phase. It is known that the protein content in immature hulls is higher than in mature hulls [58]. **Paper I** furthermore shows that the increased starch content originates from the groat, which was bound more tightly to the hull in 2018 resulting in less optimal industrial peeling. The lipid content as well as the minerals iron, potassium, magnesium, phosphorus and sulphur were also increased, however, only slightly, which should not significantly impact industrial processes.

Despite these detrimental changes in the chemical composition of oat hulls, their ability to protect the groat was maintained during the growth season of 2018 in Sweden. Analysis of the chemical composition of oat groats grown in Sweden in the seasons of 2017 to 2020, did not show a considerable difference in β-glucan content in 2018. Across all years a generally rather large fluctuation of 6 % points was found. In contrast, the total dietary fibre as well as arabinoxylan content in these oat batches was very stable even in 2018 with fluctuations of less than 3 % points [59]. These results demonstrate the preeminent resilience of the oat plant and the plant's requirement to grow a hull. Hulless variants will most likely not provide us with these benefits in the future.

Short seasons of cool and wet climates with long periods of daylight are optimal growth conditions for oat. Together with the rye plant, oats are the most versatile among the cereals concerning soil adaptation. They even thrive in both acidic soils of pH 4.5 and alkaline soils of pH 9.0. Furthermore, different varieties have been bred that display a high tolerance to salinity. However, all varieties are very sensitive to heat and especially drought [9]. Even rather novel cultivars, which were expected to be better adapted to the changing climate conditions, did not thrive well in warmer and drier conditions [60]. This is most likely due to their inherently poor water management. Oats require a deeper root system than other cereals to supply themselves with adequate water amounts and grow best in soils with high water holding capacity. On average, they

require about 600 mL of water for each gram of plant dry matter produced. In comparison, other small grain cereals require about 300 mL [9, 61].

As more extreme weather events are expected to become more frequent in the future, all industrial processes developed for the conversion of oat hulls need to be robust and adaptable to rather large seasonal variabilities in their chemical composition. Both the food industry as well as the pulp and paper industry have a long history of working with constantly changing raw materials. These industries can therefore provide valuable insights for the successful establishment of novel bioconversion processes.

3 Bleaching for an insoluble fibre product

The dietary fibre fraction of lignocellulosic materials can be further subdivided into water-soluble and water-insoluble fibre fractions. The water-insoluble fraction is typically composed of lignin and non-starch polysaccharides with higher degrees of polymerisation (DP) originating from the cellulose and hemicellulose fractions that cannot be degraded by gastrointestinal enzymes. (For a discussion of the water-soluble fraction please refer to Chapter 4.) Up to date, these fractions are largely used as supplementation in animal feed. However, interest in the use of these fibres for human consumption has been increasing since the late 1980s, resulting in a variety of available supplemented food products including baked goods, beverages and ice cream [62, 63].

This interest was initially sparked by the found health benefits associated with the consumption of insoluble dietary fibres (IDFs). IDFs have been shown to exhibit a high water holding capacity which results in an increased faecal bulk volume and transit time as well as a reduced absorption of nutrients [55, 64, 65]. In mice and pig studies, these properties were correlated to a decreased nutrient digestion and absorption including the lowered fermentation by the gastrointestinal flora. While these effects can be seen negatively for animal farming intended for human consumption, direct human consumption of the IDFs can be very beneficial, especially for diabetic patients and individuals preferring a high-fat Western style diet [64, 66]. Due to the reduced energy digestibility in the presence of IDFs, studies on humans and mice reported a moderate weight and fat loss or lesser weight gain compared to control diets together with an increased feeling of satiety. Inflammatory markers were significantly reduced and insulin sensitivity improved. In mice, the effects were as substantial as preventing the obese phenotype when fed the same diet as the control group but supplemented with IDFs [66].

IDF for human consumption are often sourced from the by-products of milling cereal grains [62]. Oat bran is a rather popular source containing 6-12 % of IDF [65], being claimed the oat fraction richest in dietary fibre. However, IDF was found to amount

to 80-87 % of the oat hull (unpublished data connected to **Paper I**, [65]) making it a far more valuable material for sourcing IDFs.

A large disadvantage of IDFs and products rich in these, such as the oat hull, is their rough texture, dark appearance and commonly perceived bad flavour [67]. As these features often defer consumers, efforts are being undertaken to modify the IDFs in a way to make them more attractive [67, 68]. One promising way is by altering their particle size, which has effects on their surface area potentially leading to improvements in consumer acceptance. Another commonly performed alteration method in the food industry to increase consumer favourability is bleaching. Besides improving optical properties, various bleaching methods are also employed to enhance product quality via the removal of unwanted tastes and smells as well as the alteration of functional properties, such as elevated fibre swelling capacities [69, 70].

3.1 Bleaching methods

Bleaching methods can be grouped into three different categories depending on the mechanism of the bleaching reaction: oxidising methods, reducing methods and biochemical methods. These methods eliminate the visible colour by chemically modifying or removing the chromophore, i.e. the part of the molecule absorbing light at a specific wavelength in the visible spectrum. After the bleaching reaction, the bleached substance is either lacking a chromophore completely or contains a shorter chromophore which can only absorb radiation of wavelengths shorter than those in the visible spectrum. As the names suggest oxidising methods oxidise the double bonds in the chromophore, while reducing methods reduce them into single bonds. Biochemical methods involve the action of a single or a combination of a variety of different enzymes [70].

The history of bleaching is long; bleaching being mentioned as an ordinary activity in human lives already in biblical times. Through most of history reducing methods were employed, combining the reducing power of sunlight with chemicals such as lemon juice [70, 71]. A big revolution in the bleaching industry was triggered by the discovery of chlorine gas in 1774. This potent oxidising bleaching agent reduced the bleaching time from months to hours enabling the establishment of large pulp mills with an unforeseen high through-put of bleached pulp [71, 72]. Despite the great successes and developments in chlorine bleaching, the chlorine era was accompanied with various health and environmental problems. The 1970s mark a turn in the bleaching industry. The first elemental chlorine free and totally chlorine free oxidising bleaching methods became commercial giving rise to the current postchlorine era. While the introduction

of oxidising agents such as chlorine dioxide, ozone and peroxides reduced the environmental burden of the bleaching industry, the threats are not eliminated. A promising alternative is provided by the biochemical bleaching methods. First mentioned in scientific literature in 1986 [73], these methods are still in their infancy compared to their chemical counterparts. However, the paper industry in traditional bleaching countries such as Sweden, Finland, Canada and the USA has partly been implementing them in their bleaching repertoire since as early as 1999 [74].

For bleaching methods to be applicable in the food industry, they do not only have to be effective, selective towards the coloured species leaving the remaining components intact, inexpensive, safe for the workers and cause no pollution to the environment [72], but also leave no harmful traces affecting the health of the consumer. Hence, the list of possible agents is rather short. One widely approved agent is hydrogen peroxide, an oxidant in the bleaching process. It is known to effectively remove colours in a variety of materials, while having little to no effect on the chemical composition and nutritional value of the food product [70]. Its functional properties can also be positively altered by the treatment leading to an increased water and oil absorption capacity [69, 75]. Additionally, alkaline hydrogen peroxide can also act as an antimicrobial agent combining two treatment steps in one. The only by-products generated during the bleaching reaction are oxygen and water, making it an ideal candidate. On the other side, residual hydrogen peroxide left after the reaction needs to be removed by catalases as it can cause off-flavours and corrode the equipment [70]. These oxidised off-flavours after bleaching have been reported for certain food products, such as whey [70], while more lignocellulosic materials with a minimal protein and fat content do not seem to be affected [69].

The negative aspects of alkaline hydrogen peroxide bleaching can be reduced or eliminated by the additional application of biochemical bleaching methods. Especially off-flavours and bitterness can be avoided and removed during enzyme treatment [70, 76]. Currently, a variety of different enzymes are tested for their bleaching suitability. Popular candidates belong to the lipoxygenases, peroxidases, laccases and xylanases [70, 74, 76]. While the first three enzymes catalyse oxidation reactions that could lead to direct brightening effects, xylanases are typically referred to as bleaching aids, rather than bleaching agents, due to their indirect action. The hydrolysis of the xylan present in the material enables other bleaching agents to better access the chromophores, leading to reduced amounts of bleaching chemicals required to achieve the same level of brightness [74]. While the combination of biochemical bleaching methods with alkaline hydrogen peroxide is tested in the pulp and paper industries [71, 74], the food industry has not widely explored this option so far.

3.2 Bleaching of oat hulls

Despite having an excellent chemical composition for the use as insoluble dietary fibre supplement (see Chapter 2 and **Paper I**), oat hulls have not been utilised as a food product so far. One challenge with introducing food products containing oat hull fibres to the market, is the fact that the oat hull supplementation will darken the food product considerably, negatively affecting consumer acceptance. Hence, a bleaching method, which is suitable for the food industry, is required to utilise this exceptional fibre source. The colour in lignocellulosic materials mostly originates from their lignin and phenolic components. As displayed in Table 2.1 and **Paper I**, oat hulls are rich in both lignin as well as phenolic acids. Due to their tight incorporation in the lignocellulosic structure, a suitable bleaching agent must be able to penetrate this network. The pulp and paper bleaching industry is very experienced in effectively treating such materials, raising the question if there is a possibility to adapt their methods, such as a combination of oxidative chemical and biochemical treatments, to the food industry.

3.2.1 Chemical bleaching

Alkaline hydrogen peroxide treatment for the modification of oat hulls has previously been investigated in a number of studies [75, 77-79]. These studies, however, aimed at optimising the functional properties of the oat hull fibres rather than improving their optical appearance. Furthermore, rather harsh reaction conditions were applied at temperatures as high as 110 °C. For the application of the hulls in the food industry, it is important that the chemical composition and especially the fibre content is altered as little as possible. Therefore, milder conditions are desirable. The possibility of applying those, potentially in combination with biochemical methods to reduce the required chemical load, was investigated in **Paper II**.

For the biochemical bleaching stage, the enzyme laccase was chosen. Laccases belong to the multicopper oxidases, being able to oxidize phenols and in combination with a mediator, such as 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), even non-phenolic lignin units. During the reaction a phenoxy radical is formed, which shortens the chromophore by depolymerising lignin leading to a reduction or shift in colour. A significant advantage of using laccases is that they are not very substrate specific, hence, any method developed with them is most likely applicable on other similar substrates as well. The drawback of this unspecificity is that they also catalyse the repolymerisation reaction, which could darken the material instead by forming chromophoric conjugated carbonyl structures [80]. Hence, it is crucial to perform the bleaching reactions under suitable, well-defined conditions. One possibility of

addressing this problem is by using the laccases in combination with alkaline hydrogen peroxide bleaching, which effectively removes the potentially formed carbonyl structures [80]. Additionally, the application of xylanases could prove beneficial. Positive effects of using those as bleaching aids on xylan rich materials for the reduction of required bleaching agents have been reported repeatedly [74, 81-83]. A third method successfully reducing the amount of necessary bleaching chemicals was found to be ultrasonication [84]. Similar to the xylanases, ultrasonication is suspected to open up the lignocellulosic structure, making the chromophores more accessible to the bleaching agents. The effectiveness of these methods on the bleachability of oat hulls was tested in **Paper II**. The optical properties in the study are reported as brightness (in percent) and lightness (L*; the grey scale axis in the CIE L*, a*, b* colour space).

The optimal chemical charges yielding the highest lightness and brightness values were found to be 150 kg hydrogen peroxide (H_2O_2) and 10 kg sodium hydroxide (NaOH) per barrel dry ton (bdt). An overview of the developed chemical bleaching method is displayed in Figure 3.1. Under these process conditions, the brightness of unmilled Swe17 oat hulls (grown in Sweden during 2017) increased from 22.4 % to 42.0 % and the lightness was improved by 21.6 units from 64.6 to 86.2. These differences are clearly visible to the naked eye (see Figure 3.1). Unfortunately, there is no standard sample preparation procedure to measure the optical properties of materials such as oat hulls, making comparison of these results with those from other studies difficult. Previous studies on alkaline hydrogen peroxide treatment of oat hulls reported an increase in the L* value from 44.8 to 51.4 [77] and from 46.8 to 53.5 [78]. While the absolute values are not comparable due to a different sample preparation for optical analysis, the increase in units is. Compared to the 21.6 units higher L* value after bleaching in the present study, the previous studies reported improvements of only 6.6 and 6.7 units, respectively. Hence, the alkaline hydrogen peroxide treatment method in **Paper II** is superior to those previously reported for the purpose of oat hull bleaching.

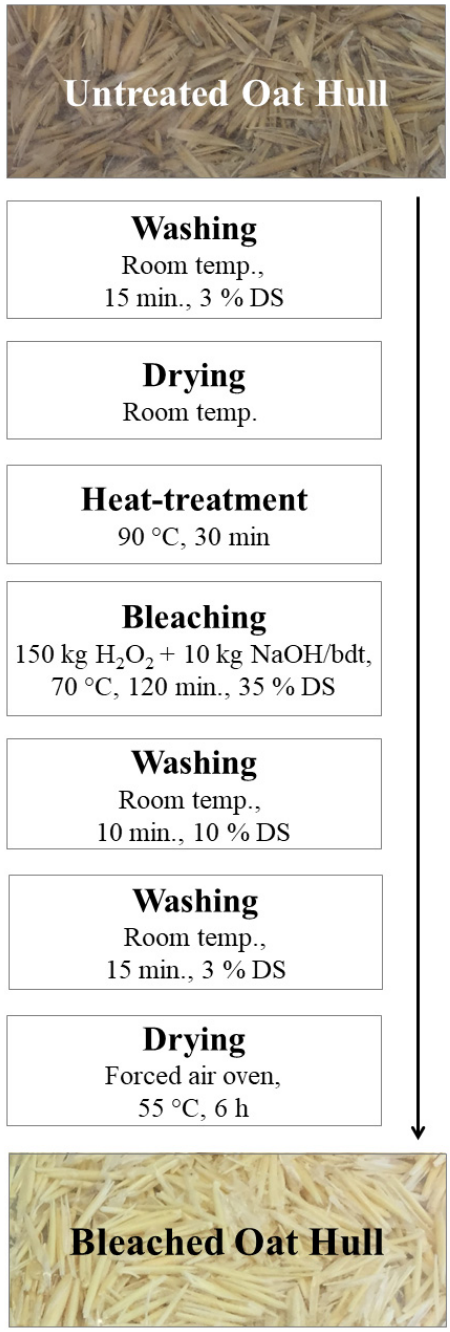


Figure 3.1: Schematic illustration of the developed bleaching process for oat hulls.
The abbreviations DS and bdt stand for dry solids content and barrel dry ton, respectively.

As the chemical composition of oat hulls is significantly altered by different environmental conditions during its growth phase, the concentration of its chromophores and hence, its colour, is also prone to change from year to year (see Figure 3.2). A suitable bleaching method must therefore be robust enough to cope with these changes or be easily adaptable to the present material. While the four oat hull batches analysed in **Paper II** exhibit rather different brightness and lightness values before bleaching (see Figure 3.2), they appear rather similar after bleaching with a brightness of above 40 % and a lightness of above 85. Hence, the developed method displayed in Figure 3.1 is robust enough to cope with the yearly variations in oat hull composition and does not need to be adapted.

	Swe16	Swe17	Swe18	Swe19
Lignin content [%]	74.5	82.7	62.3	71.7
Ferulic acid content [µg/g]	2596	2319	1339	3327
Brightness [%]	22.4	22.4	27.0	25.9
L* value	66.3	64.6	70.7	68.2

Figure 3.2: Optical appearance and properties of oat hulls grown in Sweden in different years including chemical content information of potentially chromophore containing species.
The number in the name of the different batches stands for the growth year. L* describes the grey scale axis in the three-dimensional colour system CIE L*, a*, b*.

As previously explained, it is important that the bleaching method does not significantly affect the nutrient composition. The suitability of the developed method regarding this parameter could also be confirmed in **Paper II**. The treatment mainly removed the starch fraction (most likely in the washing stages), while only minimally affecting the remaining lignocellulosic fractions (see Figure 3.3). None of the hemicellulose was removed and its composition regarding arabinoxylan content was not altered either. Surprisingly, even though the oat hulls lost their colour, neither the lignin nor the phenolic acid content was reduced to a considerable amount. Therefore, neither of those fractions seem to contain the chromophores. Alkaline hydrogen peroxide is known to remove coniferaldehydes in certain pulps, suggesting that oat hull colour may originate from these structures. However, a colorimetric assay revealed that

only a small fraction of these are removed by the treatment (see Figure 3.3). This difference compared to the literature results most likely originates from the fact that alkaline hydrogen peroxide bleaching is applied to pre-treated materials in literature, such as thermomechanical pulp, which are known to soften irreversibly at high temperatures [85, 86]. The coniferaldehydes in the non pre-treated oat hulls in this study might be too interconnected with the lignocellulosic network. It remains unclear which chemical species in the hulls is responsible for its colour. Quinones could be other potential structures to explore.

	Optical Appearance	Cellulose [%]	Hemicellulose [%]	Lignin [%]	Starch [%]	Ferulic Acid [µg/g]	Coniferaldehyde Presence
Untreated		24.8	27.6	19.3	4.7	3327	
Bleached		26.4	32.2	17.2	2.3	3320	

Figure 3.3: Differences in optical appearance and chemical composition before and after bleaching with 150 kg/bdt H_2O_2 and 10 kg/bdt NaOH of oat hulls.
The pink colour in the last column indicates the presence of coniferaldehyde structures.

3.2.2 Biochemical bleaching

In combination with the described chemical bleaching method, biochemical bleaching utilising a laccase, a xylanase and ultrasonication was tested. While significant positive effects on bleaching and the reduction of bleaching chemicals have been reported on lignocellulosic materials such as rice and wheat straw [81, 82], only minor changes were found for oat hulls. The use of a mediator is known to improve the action of the employed laccase by enabling the oxidation of molecules, which are too large to fit into the enzyme's active site [87]. However, the commonly used ABTS stained the hulls dark blue and could not be removed after several cycles of washing with water. Hence, it is unsuitable for bleaching oat hulls for food purposes. A special feature of the chemical bleaching method developed in this study is that the hulls are not treated in any way prior to bleaching. They are neither milled nor destarched. This keeps the processing steps to a minimum and should allow easy implementation in industry.

Therefore, the small savings in bleaching chemicals that could be obtained by adding a biochemical bleaching stage do not justify the additional investments required for implementing a biochemical bleaching stage. This stage would require additional processing steps such as milling and dewatering. The two straws that were successfully bleached with biochemical methods were also pre-treated to a kraft pulp [81, 82]. These pulps are less recalcitrant than the oat hulls as their lignin fraction is dissolved and extensively chemically modified, explaining the more effective action by the enzymes. While proving the effectiveness of biochemical methods is important to trigger further developments in this field, a timely implementation of oat hull processing for the production of an insoluble dietary fibre product is more likely to be achieved with the developed alkaline hydrogen peroxide bleaching method.

3.2.3 Sensory product evaluation

A common challenge limiting the applicability of alkaline hydrogen peroxide bleaching of cereals in the food industry is the occurrence of unpleasant smells upon storage. Those smells are the result of lipid oxidation, generally referred to as rancidification [88]. As food products with rancid smells are detrimental to consumer acceptance, a small smell study with a panel of seven participants was conducted (unpublished data). The panellists received unbleached as well as alkaline hydrogen peroxide bleached oat hulls that were treated with various hydrogen peroxide concentrations and had been stored at room temperature for nearly a year. The panellists reported the presence of a cereal smell that was reduced upon increasing concentrations of applied bleaching chemicals resulting in smells that were described as “neutral”, “weak”, “vanilla” and “baking powder”. No unpleasant smells were reported by any of the panellists suggesting that the lipid fraction in oat hulls is too small to result in significant amounts of lipid oxidation products. Therefore, consumer acceptance of an alkaline hydrogen peroxide bleached oat hull product seems promising regarding colour appearance and smell.

4 Pre-treatment for a soluble fibre product

The other fraction of dietary fibres present in lignocellulosic materials is water-soluble fibres. In cereals, those consist of non-starch polysaccharides being part of the hemicellulose fraction, including β-glucan and arabinoxylan [55]. Due to their ability to absorb substantial amounts of water, they exhibit a high viscosity in the intestine, which increases intestinal transit time [55, 64, 66]. There are diverting assessments on whether this is a benefit for human health. The increased transit time allows the digestive system to degrade the consumed material more completely leading to improved energy digestibility and potential weight gain [64, 66]. On the other hand, reduced glucose and cholesterol adsorption in the intestine and serum levels have repeatedly been associated with the consumption of soluble dietary fibre rich diets [55, 66]. One positive aspect of consuming soluble dietary fibres reported by all studies is the increase of beneficial bacterial species in the large intestine, which ferment the fibres into a variety of short chain fatty acids (SCFAs) [64, 66]. Manifold beneficial attributes on human and animal health have been correlated by an increased SCFA production including maintenance of the gut barrier function, reduction of insulin resistance and appetite as well as protection and retardation of carcinoma cell lines [65, 89].

Many of these beneficial effects have been confirmed for the consumption of soluble oat bran fibre. Additionally, oat bran fibre triggered the activity reduction of several digestive enzymes, such as amylase, lipase and chymotrypsin, weakening the digestion of starch, lipids and proteins. The reduced enzyme activities were attributed to the increased viscosity in the intestine caused by both the swelling soluble fibres themselves as well as an upregulated mucin production. The onset of an increased mucin production, which was triggered by the consumption of the soluble oat fibres, lasted even after soluble oat fibre consumption was terminated [65]. Hence, oat provides a fantastic source of dietary fibres.

In comparison to oat bran, where about half of the dietary fibres are soluble and the other half insoluble [65], the naturally occurring soluble fibre fraction in oat hulls is negligible (0.38 %; unpublished data connected to **Paper I**). This feature is owed to the hull's outstanding recalcitrance (see Chapter 2.3). However, as the oat hull is very rich in hemicellulose and the health benefits of soluble oat fibres have met considerable

consumer interest, fibre modifications resulting in greater solubility could be another option to turn this underutilised material into a value-added product. Solubility issues are often reported as a major problem in the processing of biomaterials in the framework of the bioeconomy [5]. Hence, developing novel methods and adapting existing ones to new materials is of utmost importance for the success of the bioeconomy. A suitable solubilisation method for hemicellulose in oat hull could therefore lead to the development of many more products besides soluble dietary fibres. Methods addressing solubility improvements among other features are often grouped under the umbrella term pre-treatments.

4.1 Pre-treatment methods

Pre-treatment methods are the initial processing steps of lignocellulosic biomass aiming at reducing its recalcitrance to allow fractionation, solubilisation and hydrolysis of its constituents [6, 42, 43, 90]. For a pre-treatment method to be applicable on industrial scale, it needs to be efficient, minimise associated costs and energy demands, preserve the fractions of interest, limit the formation of toxic inhibitors, allow for a recycling of used chemicals and avoid the formation of environmentally unfriendly effluent streams [42, 43, 90]. During the last four decades, many research efforts in the area led to the development of a wide array of pre-treatment methods. However, none of these were capable of meeting all of the mentioned requirements. Commonly, they are classified into one of four categories depending on their mode of action: physical, chemical, physicochemical or biological methods [6, 43].

With the aim of solubilising the hemicellulose fraction in oat hulls, a selection of promising pre-treatment methods from each of these categories except for the biological ones were tested in **Paper III**. A summary of these methods, associated benefits and challenges on employing them in industrial scale and effectiveness on solubilising hemicellulose in oat hulls is given in Table 4.1. The reported percentages of solubilised material were not further characterised, so it has to be noted that those can also contain other fractions besides hemicellulose. The biological methods are included in the table to allow the comparison of benefits and challenges of all categories. As they exclusively contain degrading techniques, which are not aim of this study, they were excluded from testing on oat hulls. For the same reason, the commonly applied acid pre-treatment was omitted. The chemical pre-treatment method of utilising alkaline hydrogen peroxide, which was introduced in Chapter 3, has also received significant attention for the solubilisation of hemicellulose [91]. However, as it did neither affect the chemical composition nor the quantity of the insoluble fraction of oat hulls in **Paper II**, it was not tested further.

Table 4.1: Overview of pre-treatment methods suitable for reducing the recalcitrance of lignocellulosic biomass. A summary of the associated benefits and challenges of each method as well as the amount of solubilised material in oat hulls is given based on dry weight.

Pre-treatment Method	Benefits	Challenges	Fraction of solubilised material in oat hulls [%]	Literature Source
		Physical Methods		
Mechanical (Milling)	No production of toxic side streams	Energy intensive	0	[6, 45, 90]
Irradiation (Microwave)	High-energy efficiency Short processing time Small space requirements Uniform treatment Excellent control	Increases degradation of polysaccharides High installation cost	4.4	[6, 48, 90]
Ultrasonication	Short processing time Low temperature Little required solvent Green technology Suitable for continuous flow through process	Difficult control of reaction temperature Upscaling difficulties	0.1	[90, 92]
		Chemical Methods		
Alkali	Low cost Solubilises lignin and hemicellulose specifically	Time consuming process High pollution High chemical recovery cost	23.8	[48, 90]
Deep eutectic solvents	Mild reaction conditions Simple preparation Green solvents Reusability of chemicals	Challenging separation of solvents and products after treatment High viscosity	1.0	[93, 94]
		Physicochemical Methods		
Autoclaving	High efficiency Low reactor cost No intermediate inhibition Do not use chemicals besides water Low formation of toxic compounds Beneficial life cycle assessment Direct utilisation of solubilised material	Energy intensive Highly diluted product	3.5	[6, 45, 48, 90]
		Biological Methods		
Microorganisms	Low energy demand Eco-friendly process Mild conditions No generation of toxic compounds Very selective	Time consuming process Large space requirements Limited process control Consumption of products by microorganisms	Not tested	[6, 45, 48, 90]
Enzymes	Low energy demand Mild conditions Environmental benefits Very selective	Time consuming process	Not tested	[6, 45, 48, 90]

Among the physical pre-treatment methods, the mechanical ones including milling are certainly the most commonly applied. Their main action is to reduce biomass size, introducing a greater surface area and reduced crystallinity [6]. These effects do not induce hemicellulose solubility on their own, but have a positive influence on the efficiency of subsequent pre-treatment techniques [45]. Hence, milling is always applied in combination with other treatments and was used as a first step in all treatments tested in **Paper III**.

Irradiation of lignocellulosic biomass has been studied using gamma rays, electron beams and microwaves [90]. As microwaves are readily accessible, this irradiation technique was analysed for hemicellulose solubilisation in **Paper III**. Microwaves are capable of uniformly penetrating the biomass, where they produce thermal energy and induce vibration of polar molecules causing disruptions in the lignocellulosic network [6, 90]. As the effectiveness of microwaves is dependent on the dielectric properties of the irradiated material, solubilisation yields can vary significantly. While only 4.4 % of the oat hull material could be solubilised under harsh microwave conditions, nearly 51 % of wheat bran including 48 % of its arabinoxylan fraction and 57 % of brewer's spent grain were solubilised [95, 96]. Therefore, microwave irradiation was deemed unsuitable for hemicellulose solubilisation in oat hulls.

Ultrasonication was the third physical pre-treatment tested on oat hulls. Ultrasound waves interact with the lignocellulosic material in two ways, via sonochemical and mechanoacoustic effects. The sonochemical effects lead to the generation of oxidising radicals, while mechanoacoustic effects cause cavitation events that alter the material's surface structure and fracture the cross linking in between polymers [6, 90]. The severity of the cavitation events is highly dependent on the frequency of the applied ultrasound waves. When applied adequately local hot spots are created with temperatures of up to 5000 °C and pressures of 500 atm. Fortunately, these hot spots are limited to a duration of a few microseconds, whose collapse can cause the generation of short-life-time radicals from either the biomass or the solvent [90, 92]. Solubilisation successes of polysaccharides after ultrasonication treatment have been reported for corn bran (14.7 %) [97] and sugarcane bagasse (5.1 %) [84]. However, on oat hulls and many other materials the solubilisation yield after only ultrasonication treatment is minute. Only 0.1 % of the oat hull material could be solubilised. The great improvements in solubilisation yield on these materials are often only observed when ultrasonication is combined with other pre-treatment techniques [90].

Chemical pre-treatment methods were among the first ones to be developed and remain an important category today due to their great effectiveness. Especially alkaline methods have been extensively studied on a wide variety of materials due to their ability to specifically target lignin and hemicellulose for solubilisation and degradation. Upon

alkaline treatment, the lignocellulosic matrix is altered in two ways. At lower temperatures, the cellulose fraction swells, which reduces its crystallinity, generating a more porous structure with larger internal surfaces. At higher temperatures, the ester linkages connecting hemicellulose with lignin or different strands of hemicellulose polymers to each other are hydrolysed. With increasing temperatures, even ether bonds are broken. The cleavage of both types of bonds causes a decrease in the degree of polymerisation and leads to hemicellulose as well as lignin solubilisation [45, 48, 90]. One of the most commonly used alkaline reagents is sodium hydroxide. When used for treatment of sugarcane bagasse, 32 % of its insoluble components could be solubilised [98]. Similarly high results were found in a pre-treatment screening on oat hulls. Nearly 24 % of the oat hull material was solubilised, which constitutes about half of the combined hemicellulose and lignin content in the material.

As classical chemical pre-treatment methods such as alkali generally have the disadvantage of using chemicals causing pollution of effluent streams, a rather recently introduced category of green solvents was also tested for their ability to solubilise hemicellulose from oat hulls. This group of solvents is known under the name of deep eutectic solvents (DES). It consists of a variety of eutectic mixtures composed of a hydrogen bond donor and a hydrogen bond acceptor, which build strong hydrogen bonding interactions resulting in lower melting points of the mixture compared to the melting points of the individual components. Very often they are liquid at room temperature. Both components can be of natural origin increasing their acceptability in industries considering only green and sustainable methods. Due to their composition, DES are capable of disrupting intermolecular hydrogen bonding in lignocellulose, which supports the solubilisation of its polymers [93, 94]. Their dissolution power has been proven on *E. globulus* wood, where it dissolved 92 % of its xylan fraction [93]. However, the lignocellulosic structure of oat hulls seems to be too recalcitrant for hydrogen bond cleavage to have an effect on hemicellulose solubility. Only 1 % of the hull material could be solubilised with a eutectic mixture of choline chloride and glycerol.

Due to their extensive list of benefits, physicochemical pre-treatment methods are an attractive group to study. They operate by executing thermal and/or pressure stresses on the treated material. As cellulose, hemicellulose and lignin have different thermal stabilities, fractionation can be achieved. Hemicellulose exhibits the lowest thermal stability and should therefore be the easiest to target for solubilisation [48]. Hydrothermal pre-treatments are named in various ways including autoclaving and liquid hot water treatment, but describe very similar processes [90]. On beech wood, the thermal stresses imposed by the treatment with liquid hot water was shown to increase the pore volume of the material and hence, its specific surface area [48]. On

rye bran those effects led to the solubilisation of 54 % of the material, which constituted 45 % of its arabinoxylan fraction [99]. However, those effects could not be replicated on oat hulls, where only 3.5 % was solubilised.

4.2 Ultrasonication combined with alkaline treatment

The pre-treatment screening results on oat hulls showed that methods targeting the cleavage of hydrogen bonds in the material, such as autoclaving or treatment with deep eutectic solvents, failed at solubilising considerable amounts of hemicellulose. This demonstrates that the recalcitrance of oat hull lignocellulose is also caused by its extensive network of covalent linkages. Pre-treatment methods capable of disrupting covalent bonds are microwaving, ultrasonication and alkaline treatment. While both microwaving and ultrasonicating the material did not cause the material to solubilise, the treatment with alkaline solvents is very promising. A possible explanation for this behaviour is the ability of alkaline solvents to specifically target the cleavage of ester and ether bonds in the material. Those are the bonds linking the different hemicellulose chains to one another and lignin species. Additionally, alkaline solvents can increase the porosity of the biomass by swelling of the cellulose fraction enabling itself access to more protected regions. However, the results of alkaline hydrogen peroxide treatment in **Paper II** show that rather harsh alkaline conditions need to be applied for solubilisation of significant amounts. At similarly low concentration of sodium hydroxide larger amounts of hemicellulose were solubilised from both corn bran and sugarcane bagasse [84, 97].

One alternative to increasing the harshness of pre-treatment methods for the improvement of solubilisation yields is the combination of several different methods. As most pre-treatment processes are limited to acting on certain aspects of the material, a combined application could lead to synergistic effects. These effects have been observed for the combination of alkaline reagents with several other pre-treatment methods [48]. One attractive method is ultrasonication. Due to its ability to form oxidising radicals, it has been shown to act very effectively in combination with other oxidising methods including alkaline treatment [90]. Besides this, it is also capable of mechanically disrupting the biomass creating more space for the penetration of the alkaline reagent [84].

Initial trials of combining these two methods for the treatment of oat hulls in **Paper III** revealed that the solvent used during the ultrasonication stage has an exceptional impact on the effectiveness of the solubilisation in the alkali stage (see Figure 4.1). Ultrasonication itself did not lead to the solubilisation of any considerable amounts

when carried out in either water or 2 M sodium hydroxide (NaOH). Subsequent treatment stages in water after ultrasonication in water or in NaOH after ultrasonication in NaOH did not result in yields different to those when the material was only subjected to the water or NaOH incubation stages without ultrasonication. However, when ultrasonication was performed in water followed by a 2 M NaOH incubation, the solubilisation yield was nearly tripled compared to the NaOH incubation without ultrasonication. This behaviour has so far not been observed on other lignocellulosic materials such as wheat straw and corn bran. In those cases, ultrasonication in an alkaline medium resulted in increased solubilisation yields [97, 98]. A combination of two circumstances is most likely responsible for this behaviour. The first circumstance deals with the outstanding recalcitrance of oat hulls. Compared to corn bran, oat hulls contain 22 times more lignin (**Paper I** and [100]). Additionally, both corn bran and sugarcane bagasse contain more heavily substituted xylan than oat hulls, which has been shown to be solubilised more readily (**Paper I** and [84, 97, 100]). The second circumstance concerns the fact that the effectiveness of ultrasonication depends on the quantity and severity of the formed cavitation events. Those can be heavily influenced by a variety of factors including the viscosity of the solvent. Alkaline solutions have a higher viscosity than water, which is increasing with increasing concentration. In higher viscosity environments, fewer cavitation events occur resulting in a reduced production of radicals as well as less extreme high local temperatures and pressures [92].

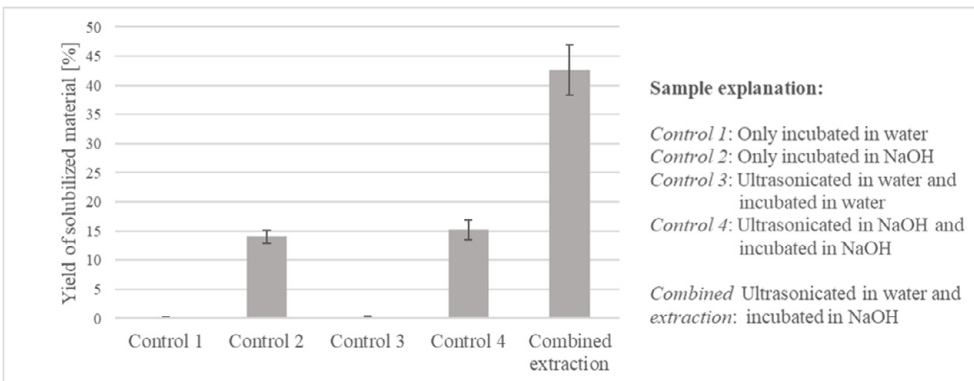


Figure 4.1: Solubilisation results of the combined ultrasonication and alkali pre-treatment trial as well as four controls showing the amount of solubilised material as a fraction of the destarched and milled oat hulls in percent (n = 2).
A short description of the treatment of all samples is given to the right of the graph.

After the general procedure of the ultrasound assisted alkaline treatment was established, the method was optimised towards increasing hemicellulose solubilisation yield in **Paper III**. When performing optimisation studies it is always advantageous to not only look at individual factor influences on the system, but also their interactions in a factorial design. The single-variable approach of optimisation introduces experimental biases often only leading to the discovery of quasi-optima. The determination of the true optimum of a system requires a more mathematical design generally referred to as factorial design or design of experiments (DOE) [101]. According to general practices in this field, two DOEs were designed and executed in **Paper III** evaluating the four factors ultrasonication length, NaOH concentration, NaOH incubation length and NaOH incubation temperature on oat hull hemicellulose solubility. A second DOE was required as the design space of the first DOE was too narrow. The verified optimal conditions resulting from these experiments were a 10 min long ultrasonication stage followed by incubation in 5 M NaOH at 80 ºC for 9 hours. Under these conditions, 75 % of the hemicellulose fraction in oat hulls was solubilised. This constitutes a significantly higher yield than was found on less recalcitrant materials with the same combined pre-treatment method (wheat straw: 65 %, corn bran: 32 %) [97, 98]. In both of these studies, merely the effect of certain parameters on the solubilisation yield was investigated rather than conducting a parameter optimisation considering factor interactions. This underlines the importance of including factor interaction effects in optimisation studies and highlights the benefit of utilising DOE in the development of successful bioconversion technologies.

The fraction of the major constituents solubilised with this method from the original oat hulls is displayed in Figure 4.2. As expected, mostly the hemicellulose and lignin fractions were attacked and solubilised by the treatment, while the cellulose fraction remained completely insoluble [90]. The dissolution of nearly the entire starch fraction is also understandable due to the naturally great solubility of starch at high temperatures in water, which is even further increased upon ultrasonication [102]. The 20 times reduction of ferulic acid content in the pre-treated material compared to the untreated hulls furthermore shows that the phenolic acid linkages between hemicellulose molecules and towards lignin were effectively removed by the developed method explaining the success of this developed method.

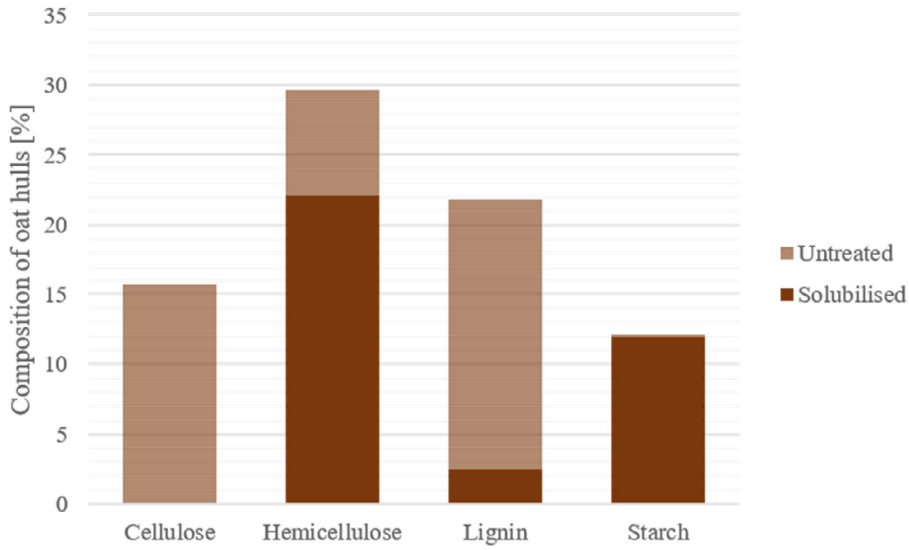


Figure 4.2: Composition of the untreated oat hulls including labelling of fractions that were solubilised during the developed ultrasonication assisted alkaline pre-treatment.
The pre-treatment was therefore able to solubilise 0 % of the cellulose fraction, 75 % of the hemicellulose fraction, 12 % of the lignin fraction and 98 % of the starch fraction.

4.3 Upscaling trials

For the developed method to be applicable in industry it needs to be scalable. Alkaline hydrogen peroxide treatment on industrial scale is widely established in the pulp and paper industries. However, there are fewer examples of industrial scale ultrasonication treatments [92]. A currently available equipment comprises an ultrasonication probe rather than a bath as in the method developed in **Paper III**. Both ultrasonication types are used for the degradation of biomass, but differ in their power output. Ultrasonication with a probe is generally more disrupting to the biomass [92]. While this feature seems advantageous to oat hull treatment, especially on larger scale, the probe intensity output needs to be adjusted. Therefore, the optimum conditions for hemicellulose solubilisation from oat hulls were investigated in a design of experiment study similar to that described in **Paper III** (unpublished study). A laboratory scale ultrasonication probe with a capacity of up to 4 L by an industrial scale equipment manufacturer was used, which is intended for translating all parameters from laboratory to industrial scale. The varied factors in the study were ultrasonication length, ultrasonication amplitude strength as well as sodium hydroxide treatment length. An even higher optimum yield compared to **Paper III** was found. After 30 seconds of

ultrasonication at an amplitude of 68 % followed by a 4 hours long sodium hydroxide stage, 92.6 % of the hemicellulose was solubilised at a 2 g scale. Besides the increased yield, this method has the advantage of being shorter in overall treatment time (4 hours compared to 9.5 hours), which simplifies its application in industry. Additionally, the NaOH treatment temperature could be reduced from 80 °C to 60 °C. At these conditions a slightly larger laboratory scale of 300 g was tested, resulting in a solubilisation yield of 82.5 %. While this yield is lower compared to the 2 g scale, it is still larger than the yield of 75 % found in **Paper III**. Therefore, this adapted method for an ultrasonication probe is promising for testing on pilot scale.

An initial evaluation of the solubilised material showed that not only the yield is affected by the different ultrasonication methods, but also the size of the solubilised material. A size exclusion chromatography analysis revealed that the solubilised material after ultrasonication in a bath is longer compared to the material treated with the ultrasonciation probe, resulting in approximate degrees of polymerisation of 405 and 34, respectively (unpublished results). These differences need to be further evaluated and taken into account when assessing subsequent treatment steps as a shorter size could be beneficial for the functionality of a prebiotic product (see Chapter 5). As this shorter material was not available at the time the enzymatic hydrolysis in Chapter 5 was conducted, only the material solubilised in the ultrasonication bath is being discussed in the remainder of this thesis.

5 Enzymatic hydrolysis for a soluble fibre product

Despite the outstanding solubilisation of oat hull hemicellulose achieved with the pre-treatment method developed in **Paper III**, this treatment is likely not sufficient to produce a soluble dietary fibre product stimulating the growth of and fermentation by a variety of beneficial gut microbiota. For soluble fibres to be termed prebiotics, they need to be used by microorganisms that exert a health benefit to their host [103]. This typically limits soluble fibres to sizes with degrees of polymerisation (DP) of less than 60. Different studies suggest opposing results on whether fibres with DPs of more or less than 10 are more effective prebiotics [104-106]. In either case, the pre-treated soluble oat hull fibres need to be shortened. A reduction in size can selectively be obtained via enzymatic hydrolysis. Direct enzyme treatment without the application of a pre-treatment for the production of prebiotics from oat hulls would be ideal. However, enzyme hydrolysis trials have shown that no enzyme was active on non-pre-treated oat hulls (unpublished data). This observation is rather expected as the outer layers of cereal seeds and other lignocellulosic materials are known to be resistant to enzymatic degradation [46, 99, 107]. Adequate pre-treatments open the inter- and intramolecular network of the material by removing decorations on the main chain hemicellulose as well as reducing the amount of present lignin [46, 90, 107, 108]. This generates a higher number of enzymatic binding sites which significantly improves the biomass conversion [19]. The presence of lignin has been shown to not only impose physical barriers, but also directly inhibit the action of various hydrolysing enzymes via adsorption of the enzymes onto the lignin. The ultrasound assisted alkaline pre-treatment method developed in **Paper III** removes most of the lignin from the hemicellulose fraction and might modify the remaining one in a way that is not inhibiting to enzymatic hydrolysis anymore [46]. Therefore, a pre-treatment step is indispensable despite it significantly increasing the cost of the process [109].

One challenge of applying a pre-treatment step prior to enzymatic hydrolysis is their compatibility. Pre-treatments have been shown to produce by-products that inhibit further enzymatic degradation by inducing conformational changes in the enzymes [109, 110]. The identity and quantity of these by-products is highly influenced by the

utilised plant biomass as well as the type and severity of the pre-treatment. Different enzymes have been shown to cope differently with their presence, some being more greatly inhibited than others [110, 111]. Hence, the successful production of prebiotics from oat hulls is dependent on the integration of both the pre-treatment and enzymatic hydrolysis steps. Neutralisation after alkaline treatments of biomass have been shown to effectively remove inhibitors including salts, phenolic acids, aldehydes and furfural [90]. In **Paper IV**, the effectiveness of neutralisation was confirmed as the selected enzymes were more active on the pre-treated oat hulls, when a neutralisation step was added at the end of the pre-treatment method.

Nature has produced a vast variety of enzymes capable of acting on complex lignocellulosic substrates. These are mostly produced by microorganisms, including bacteria, fungi and yeasts. As these microorganisms tend to live in communities involving various species, the degrading enzymes produced by only one organism are usually not sufficient for complete biomass degradation [19, 112]. Modern biotechnology succeeded at identifying and industrially producing a large variety of interesting enzymes sourced from manifold environments. In order to act on the hetero-xylan present in the hemicellulose fraction of oat hulls, a consortium of different carbohydrate active enzymes is most likely required [112]. It is noteworthy to consider different representatives of the same group or class of enzymes as those have been shown to exhibit varying activities on different lignocellulosic substrates, including oat hulls [111, 113].

5.1 Suitable enzyme classes

Nature has developed an immense amount of different enzyme classes for the degradation of lignocellulose. As the purpose of this thesis is to develop methods for biomass conversion in industry, only those that are commercially available will be covered. For the production of short (branched) oligosaccharides that could potentially act as prebiotics from ultrasound-assisted alkali pre-treated oat hulls, three groups are of particular interest: xylan main chain cleaving enzymes, lignin removing enzymes and those acting on the connections between hemicellulose and lignin. The enzyme class characteristics that make them interesting for hydrolysis of the pre-treated oat hulls are presented in the following sub-sections. A graphical indication on the points of cleavage these enzymes are catalysing is given in Figure 5.1. As the solubilised material does not contain any cellulose, cellulases are omitted. Further excluded enzyme classes are those acting on the xylan side chains, as branched xylooligosaccharides present an interesting group of products for the selective stimulation of probiotics.

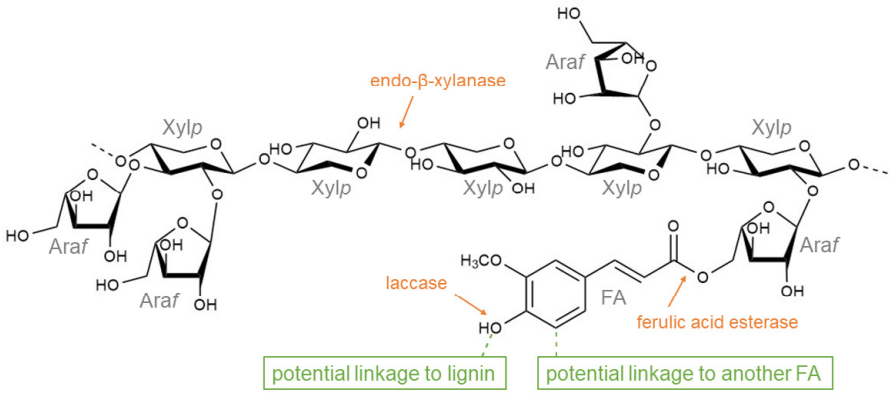


Figure 5.1: Schematic indication of the enyzmatic cleavage sites in arabinoxylan.
The displayed arabinoxylan structure only contains the most common substituents found in oat hulls: xylopyranosyl (Xyl*p*), arabinofuranosyl (Ara*f*) and ferulic acid (FA) units. Only the enzyme classes studied in more detail in this thesis are shown. As the exact point of cleavage of the various endo-β-xylanases depends on the presence of the surrounding substituents, it is not further classified into families.

5.1.1 Glycoside hydrolase family 10 (GH10)

Members of GH10 are xylanases hydrolysing the β-1,4-linked glycosidic bonds in the inner part of xylan polymers, i.e. they are endo-acting. This class is one of the most intensively studied classes for xylan degradation and has been claimed to be the most preferred class due to their more accommodating substrate specificity [99, 108, 114]. GH10 xylanases are characterised by a rather small, but open active site, which enables them to accommodate substituents close to the point of cleavage in sites -3, -2, +1 and +2. Different members allow substituents in varying positions of these subsites, but many of them can accommodate a substitution in -2 [46, 99, 108, 114]. Allowed substituents include single C-3 linked arabinofuranosyl (Ara*f*) units as well as Ara*f* units that are further connected to feruloyl substituents [99, 107]. The glycone subsites -3 to -1 exhibit a strong binding affinity towards the substrate, while the aglycone subsites are characterised by weaker binding affinities. For a catalytic cleavage to occur, the bound substrate must contain at least two consecutive unsubstituted xylopyranosyl (Xyl*p*) units [114-116]. GH10 xylanases are in many cases multi-domain enzymes increasing their size to about 40 kDa. This larger size inhibits them from penetrating into a tight and insoluble lignocellulosic network. Therefore, they are more active on shorter and soluble substrates including xylooligosaccharides down to DPs of 3 [108, 114]. The resulting products are typically rather small and can be linear or branched. Due to the structure of the catalytic site, branches occur at the non-reducing end of the products, while two unsubstituted Xyl*p* units are present at the reducing end [115]. Their activity on pre-treated agricultural side streams has been shown for various

materials including wheat and rye bran [99, 108]. A screening of different members of this class is generally recommended as the allowance of substituents in different parts of the active site generates different hydrolysis products [115].

5.1.2 Glycoside hydrolase family 11 (GH11)

Members of GH11 are similarly to GH10 also widely studied endo-xylanases acting on the same β-1,4-glycosidic linkages between backbone Xyl*p* units [114]. Their active site, however, differs significantly to that of GH10 members; being very narrow and less accommodating to side chain substituents in the substrates, GH11 members are more active on unsubstituted, linear materials [46, 112, 114]. Ara*f* substituents have been reported to be accommodated in subsites -3, -2, +2 and +3 by certain members, however, none allow the presence of any side chain substituents in subsites -1 and +1 [108, 114, 115]. Generally, GH11 members prefer longer substrates (DP > 3) with increasing affinity for substrates exhibiting longer DPs due to unfavourable binding affinities in the aglycone subsites. Their catalytic activity requires the presence of at least three consecutive unsubstituted Xyl*p* units in the active site [107, 114, 116]. Nevertheless, branched products, including those with feruloyl substituents, can still be generated by GH11 members. If substituents are present on the main chain, the hydrolysis of the glycosidic bonds occurs one linkage prior to the substituted Xyl*p*, towards the reducing end. Resulting products often have two unsubstituted Xyl*p* units at the reducing end [107, 108, 115]. Overall, GH11 catalysed products tend to be larger than those catalysed by members of GH10 [114]. Due to their smaller overall size of < 30 kDa, generally being single domain, GH11 xylanases are capable of penetrating insoluble lignocellulosic networks, making them interesting candidates for the hydrolysis of rather recalcitrant material [99, 108, 114].

5.1.3 Glycoside hydrolase family 5 (GH5)

The enzymes belonging to GH5 catalyse the hydrolysis of a large variety of β-linked polymers [115]. For this thesis, members of subfamily 34 (GH5_34) are of particular interest as they act on the β-1,4-glycosidic bonds in the inner part of xylan polymers. However, unlike members of GH10 and GH11, they require an α-1,3-Ara*f* substitution in the -1 position of their active site for the catalytic hydrolysis to occur leading to their characterisation as arabinoxylanases [107, 112, 115]. Their open catalytic cleft allows Ara*f* substitutions in all subsites from -2 to +2 including double substitutions, hence, they are ideal candidates for the production of arabinose substituted products [115, 117]. These products are often branched at their reducing

end [112]. Despite being of great interest for the production of arabinoxylo-oligosaccharides, not many members have been studied in detail so far. The most well studied GH5_34 arabinoxylanase *Ct*Xyl5A is a multi-domain protein containing several carbohydrate binding modules, which are suspected to aid in the hydrolysis of complex heteroxylans. Whether their larger size imposes restrictions for the penetration into more cross-linked materials has not been studied so far, however, a study on model rye and wheat arabinoxylan suggests promising activities [117].

5.1.4 Ferulic acid esterase (FAE)

Hydrolysis of the xylan backbone by (arabino-)xylanases can be hindered by cross-links between different hemicellulose chains as well as lignin. Tightly interconnected networks reduce the spaces required by the enzymes to penetrate the material and find suitable binding sites. The most common cross-link in lignocellulosic materials is an ester-ether bridge involving a hydroxycinnamic acid. In cereals, this acid is ferulic acid [37, 118]. The hydrolysis of the ester bond bound to Ara*f* substituents on the xylan main chain can effectively be catalysed by enzymes generally referred to as ferulic acid esterases. Rather few representatives have been characterised in detail so far. Despite their name, they have been shown to also act on ester linkages between arabinoxylan and other hydroxycinnamates including the second most common phenolic acid *p*-coumaric acid [52, 112, 119]. A positive effect of employing FAEs together with main chain cleaving xylanases for the degradation of lignocellulosic materials has been shown in various examples [46, 49, 119]. Both the release of saccharides as well as phenolic acids was enhanced when these different classes were applied simultaneously. Interestingly, a combination of FAE and GH11 mainly enhanced the release of monomeric acids, while the combined action of FAE and GH10 released dimers more efficiently [119]. However, not only the xylanase combination partner, also the identity of the FAE representative has a large effect on the efficiency of ester bond hydrolysis on different substrates underlining once more the importance of identifying the most appropriate enzyme candidate for the present lignocellulosic material [113].

5.1.5 Laccase

Another type of substance potentially inhibiting the activity of (arabino-)xylanases present in lignocellulosic materials including the pre-treated oat hulls is lignin. One type of lignin modifying enzymes that has been studied for a wide variety of industrial applications is laccase [120]. Laccases are multicopper oxidases catalysing oxidation reactions of phenolic compounds including lignin via the formation of oxygen radicals. The reaction is driven by the reduction of molecular oxygen to water [19, 119, 121].

While positive effects of their catalysis on the activity of hemicellulolytic enzymes have been shown, their combined mode of action is not well understood [112, 119]. One observation reports that laccases support the action of hemicellulases by removing inhibiting phenolic compounds, which have been freed by accessory enzymes, via repolymerisation reactions [122]. The previously reported successes in increasing the hydrolysis degree of complex lignocellulosic materials paired with their ability to act on a broad range of substrates make them interesting candidates for the further degradation of the pre-treated oat hulls.

5.2 Enzyme synergy

Industrial bioconversion processes are inherently complex due to the versatility and heterogeneity of the incoming raw material. These challenges can only be overcome by the application of various cooperating enzymes. This cooperation, where the enzymes' combined activities are greater than the theoretical sum of their single activities, is termed synergy [50, 111, 112]. A valuable means of quantifying synergy is the degree of synergy (DS). The term describes the mathematical relationship between individual and combined enzyme activities. In the calculation, the reaction rate, conversion yield or product yield when the enzymes are applied together is divided by the sum of the enzyme activities or conversion yields of the individual enzymes employed in the reaction [46, 111, 123]. DS values can be grouped into three categories: those that are less than 1, those that are equal to 1 and those that are greater than 1. Enzyme combinations resulting in DS values less or equal to 1 indicate that the enzymes are not acting synergistically and degrade the substrate independently. If the values are far less than 1, it furthermore suggests that the enzymes are inhibiting each other, for example by competing for the same substrate binding sites. This type of inhibition is sometimes referred to as anti-synergy. DS values larger than 1, however, are a strong indication for synergy [46, 111].

Analysis of enzyme synergy in such a systematic manner generates a greater understanding of key enzymes and cooperation mechanisms and provides information about the substrate structure, which is crucial for the development of effective enzyme cocktails that reduce enzyme load and reaction time of industrial processes [46, 50, 111]. One challenge in performing valuable experimental studies to achieve this goal, is the determination of specific enzyme variants that are adequate for the generation of the desired products. Inspiration for suitable classes and combinations can be found in microorganisms in nature. Plant biomass degrading specialists tend to express a large variety of glycoside hydrolases, lignin modifying enzymes as well as further accessory

enzymes. Interestingly, some microorganisms also produce several variants of the same enzyme class in order to be able to degrade a larger substrate variety; a phenomenon referred to as multiplicity [52, 111]. This suggests that not only combining members of various classes is of value, but also the combination of several representatives of the same class.

Based on the mode of action of the involved enzymes, synergy can be further classified into two types: homeosynergy and heterosynergy. Homeosynergy describes the synergistic behaviour of two or more enzymes catalysing the cleavage of the same bonds, such as in the main chain, while heterosynergy involves enzymes with different targets, such as debranching and main-chain cleaving enzymes [46, 112]. Heterosynergy typically occurs in one of two ways. Side-chain active enzymes remove the branches of heteroxylans, which results in more binding sites for main-chain acting enzymes. This cooperation typically results in the production of many short xylooligosaccharides and has been shown to occur both when the enzymes are applied simultaneously or sequentially. In another case, synergy occurs when main-chain acting enzymes liberate short decorated oligosaccharides, which are the preferred target for side-chain cleaving enzymes. This type of cooperation often significantly increases the amount of free side chain substituents [112]. Irrespective of which mode of synergy is the dominant one, degradation of the xylan main-chain does not occur in a linear fashion and is dependent on a variety of factors including enzyme ratios, specific characteristics of both the enzymes and the substrate as well as the enzyme formulation. Hence, predictions of optimal enzyme combinations are rarely confirmed by biochemical assays [46, 112].

5.2.1 Oat hulls as substrate

Due to the challenge of accurately predicting enzymatic activities as well as the presence of synergies on lignocellulosic materials, the effect of a variety of 14 different commercial enzyme mixtures was examined in **Paper IV**. These enzymes included representatives of amylases, cellulases, GH10 and GH11 xylanases, GH5 arabinoxylanases, GH30 glucuronoarabinoxylanases, ferulic acid esterases and laccases. Other common accessory enzymes such as acetyl xylan esterases, which support the action of main chain cleaving enzymes, were omitted as alkaline pre-treatment is known to remove all acetyl decorations [112]. Previous studies on the development of treatment processes of lignocellulose involving both a pre-treatment as well as an enzymatic hydrolysis step, stressed the importance of ensuring that both stages are compatible with one another [6, 90]. An important parameter to consider when employing alkaline pre-treatment methods is neutralisation. Neutralisation can lead to the removal of potential enzyme inhibitors that might negatively impact the subsequent

enzyme hydrolysis stage [90]. This effect could also be observed on oat hulls pre-treated with the method developed in **Paper III** at lab scale. When the 14 different enzymes were applied on non-neutralised pre-treated oat hulls, an 81 % lower arabinoxylan conversion was achieved compared to the conversion of the neutralised arabinoxylan (see **Paper IV**). Hence, only neutralised pre-treated oat hull material was used in all enzymatic synergy reactions.

An initial screening study according to a DOE experimental set-up allowing factor interactions, found that six out of the 14 enzyme mixtures had a significant effect on the hydrolysis of arabinoxylan. Those were the arabinoxylanase *Ct*XynA from NZYTech (GH5_*Ct*), the GH10 xylanase E-XYNBS from Megazyme (GH10_X), the GH11 xylanases Pentopan Mono BG (GH11_P) and Feed from Novozymes (GH11_F), the ferulic acid esterase E-FAERU from Megazyme (FAE_R) and the laccase NS51003 from Novozymes (Lac_N). An overview of their activities on oat hulls expressed as degree of synergy or conversion yield is given in Figure 5.2. With nearly 40 % of all tested enzyme combinations, rather many resulted in synergy. However, only one homeosynergy was found, that between the enzymes GH11_F and GH5_*Ct*. This observation led to the conclusion that oat hull hemicellulose is composed of stretches with a higher density of Ara*f* substitutions and stretches that are rather undecorated as discussed in section 2.3. The observed synergy could result from two potential mechanisms. The ability of GH11_F to act on the water insoluble parts of the hemicellulose (all material is alkali soluble, but not water soluble) might solubilise some of the originally insoluble, more substituted parts that can then be accessed by the bulkier GH5_*Ct*, which was not able to reach them without prior solubilisation by GH11_F. Alternatively (or simultaneously), GH5_*Ct* could produce longer oligosaccharides from the substituted regions which then only carry few substitutions allowing GH11_F to degrade them further into smaller oligosaccharides. Homeosynergies involving xylanases from different glycoside hydrolase families have in the past been shown to be beneficial for the production of XOS due to their ability of generating higher yields and a larger diversity of xylooligosaccharides [112]. This behaviour could also be observed on oat hulls (see Figure 5.3).

	Enzyme Combination	DS	Yield [%]
Individual enzymes	GH11_P	N/A	32.1
	GH11_F	N/A	36.2
	GH10_X	N/A	42.4
	GH5_*Ct*	N/A	1.2
	FAE_R	N/A	0.1
	Lac_N	N/A	0.1
Xylanases	GH11_P + GH11_F	0.50	34.0
	GH11_P + GH10_X	0.59	43.9
	GH11_F + GH10_X	0.61	48.2
	GH11_P + GH5_*Ct*	0.72	23.8
	GH11_F + GH5_*Ct*	1.52	56.7
	GH10_X + GH5_*Ct*	0.86	37.6
	GH11_P + GH11_F + GH10_X	0.39	43.5
	GH11_P + GH11_F + GH5_*Ct*	0.45	31.2
	GH11_P + GH10_X + GH5_*Ct*	0.48	36.5
	GH11_F + GH10_X + GH5_*Ct*	0.60	48.0
	GH11_P + GH11_F + GH10_X + GH5_*Ct*	0.38	42.5
Ferulic acid esterase Xylanases	FAE_R + GH11_P	1.02	32.8
	FAE_R + GH11_F	1.27	46.0
	FAE_R + GH10_X	0.79	33.8
	FAE_R + GH5_*Ct*	4.78	6.2
	FAE_R + GH11_F + GH10_X	0.55	43.5
	FAE_R + GH11_F + GH5_*Ct*	1.52	57.2
	FAE_R + GH10_X + GH5_*Ct*	0.77	33.6
	FAE_R + GH11_P + GH10_X + GH5_*Ct*	0.46	35.2
	FAE_R + GH11_F + GH10_X + GH5_*Ct*	0.55	44.3
Laccase Ferulic acid esterase Xylanases	Lac_N + GH11_P	1.13	36.4
	Lac_N + GH11_F	1.29	46.8
	Lac_N + GH10_X	0.92	39.1
	Lac_N + GH5_*Ct*	10.92	14.0
	Lac_N + FAE_R	87.98	16.6
	Lac_N + GH11_P + GH10_X	0.48	35.9
	Lac_N + GH11_F + GH10_X	0.50	39.4
	Lac_N + GH11_F + GH5_*Ct*	1.50	56.1
	Lac_N + GH10_X + GH5_*Ct*	0.79	34.5
	Lac_N + FAE_R + GH11_P	1.17	37.8
	Lac_N + FAE_R + GH11_F	1.43	52.1
	Lac_N + FAE_R + GH10_X	0.77	32.7
	Lac_N + FAE_R + GH5_*Ct*	8.66	12.0
	Lac_N + GH11_F + GH10_X + GH5_*Ct*	0.60	45.4
	Lac_N + FAE_R + GH11_F + GH10_X	0.48	37.8
	Lac_N + FAE_R + GH11_F + GH5_*Ct*	1.37	51.6
	Lac_N + FAE_R + GH11_F + GH10_X + GH5_*Ct*	0.52	41.5

Figure 5.2: Degree of synergies (DS) and conversion yields of arabinoxylan in percent of various enzyme combinations on ultrasound assisted alkali pre-treated oat hull.
DS results of larger than 1 denote synergy and are marked in green. DS results of less than 1 suggest enzyme inhibition and are marked in orange. The conversion yield describes the fraction of arabinoxylan that was converted into oligosaccharides up to a degree of polymerisation of 6 and that could be identified with the available standards. The results of the enzyme combinations producing the highest yields are marked in green. Those producing the lowest yields are marked in orange.

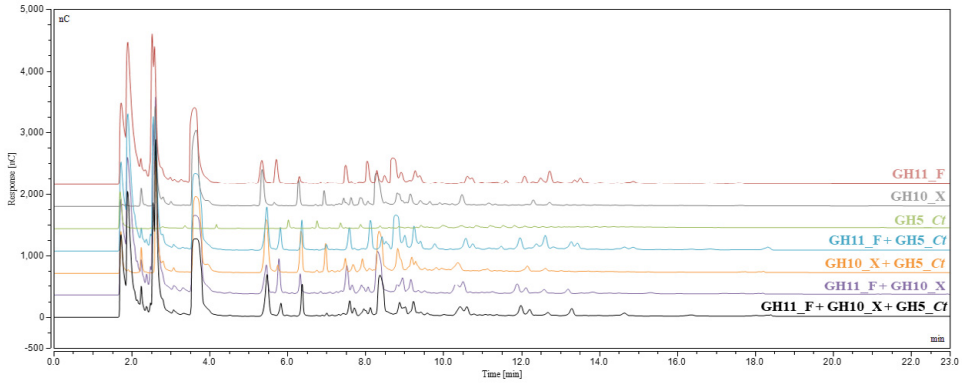


Figure 5.3: HPAEC chromatograms depicting the product profiles resulting from reactions of GH11_F, GH10_X and GH5_*Ct* (arabino-)xylanases alone or in combination on ultrasound-assisted alkali pre-treated oat hulls. The combination of enzymes do not only change the quantities of certain products, but also generate novel products that could not be produced by any of the xylanases alone.

In contrast, all heterosynergies involving FAE_R resulted in synergy except for those also containing GH10_X. This result is rather surprising as previous studies on enzyme synergy involving FAEs and xylanases reported the highest synergies when FAEs were combined with GH10 xylanases [46]. However, not many studies combining xylanases with accessory enzymes have been performed so far. Previous research focus was lying mostly on the combination of various xylanases or xylanases with cellulases [124]. One exploratory study investigating the synergy of FAEs and representatives of GH10 and GH11 found that these synergies are very substrate dependent with different results on wheat and barley [52]. Hence, this opposing trend observed on oat hulls might be specific for this substrate.

The same pattern of enzyme combinations resulting in synergy was also found for those involving Lac_N. All combinations involving GH10_X resulted in DS values far less than 1, while all other combinations resulted in synergy including the three highest DS values of all experiments. The ability of Lac_N to improve the hydrolytic activity of the other enzymes is most likely caused by its ability to repolymerise free phenolic acids. Those are present in the pre-treated oat hulls as result of the alkaline treatment, which is known to cleave ester bonds [48], and are further released by the action of FAE_R (see **Paper IV**). The repolymerisation activity of Lac_N was confirmed via size exclusion chromatography as well as free phenolic acid analysis. After reactions with Lac_N no free phenolic acids, including ferulic acid, could be detected, while they were present in those reactions not involving Lac_N. Furthermore, the lignin species exhibited a larger size after reaction with Lac_N, which suggests that the free phenolic acids were incorporated into the present lignin. Phenolic compounds can inhibit and even

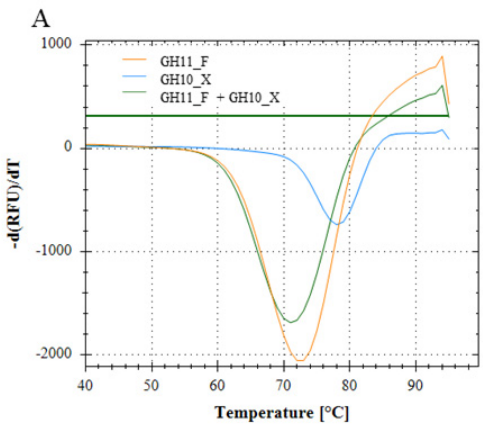
deactivate xylanases by triggering changes in their confirmation. While the exact mechanism of how this is achieved has not been understood, yet, the ability of laccase to scavenge phenolic compounds to support xylanase activity is well known. In a study on sugarcane bagasse, the presence of laccase was able to prevent loss of xylanase activity by 60-70 % [122].

Besides the identity of the enzymes involved, their order of application can have a large impact on the resulting DS. Again, no trend shared by all lignocellulosic materials exists [111]. However, it seems that arabinoxylan conversion benefits from the simultaneous addition of the enzymes as opposed to their sequential addition [46, 49]. For the arabinoxylan conversion of pre-treated oat hulls, this behaviour could be confirmed. While all in **Paper IV** tested enzyme combinations, regardless of the order of application, resulted in synergy (with the exception of one), their DS values differed greatly. In all cases but one, the simultaneous addition yielded higher values, which is why only simultaneous reactions were further analysed in this study. For the enzyme combination FAE_R + GH11_F, the mode of application did not impact the resulting DS, which suggests an interesting reaction mechanism. As the same DS of 1.3 was achieved regardless of the order of enzyme addition, the GH11 xylanase must be capable of acting on ferulic acid esterified xylan, producing (A)XOS linked to a ferulic acid unit. Only after the cleavage of the ester bonds by FAE_R, the products can be detected as (A)XOS.

The degree of synergy is a powerful means to describe enzyme interactions, however, it can be misleading when reaching high conversion yields is the main objective. As previously described [46, 123], the reactions on oat hull confirm that the highest yields are not necessarily achieved for those enzyme combinations resulting in the highest DS (see Figure 5.2). The highest DS (88) on oat hull was reached with the enzyme combination of FAE_R and Lac_N. However, this combination resulted in a conversion yield of only 17 %, which is far from the 57 % reached by the combination of FAE_R, GH11_F and GH5_*Ct*. The reason for this is the very small yields achieved by the single enzymes. Neither of the enzymes is active on the main chain and should therefore only indirectly contribute to its hydrolysis by supporting the activity of main chain cleaving enzymes. However, side reactions can always occur, which increased the yield of arabinoxylan conversion slightly when FAE_R and Lac_N were applied simultaneously. As DS is a ratio, small improvements on small starting yields have an over proportionately large impact. Therefore, it should not be used as a tool for determination of the optimal enzyme combination for industrial processes aiming at optimising yields.

Besides promoting greater accessibility to the substrate, enzyme synergy can also result from direct interactions between the enzymes. These interactions could lead to both an

increase or decrease in physical stability of the enzyme. Analyses of the melting temperatures of the utilised enzymes on oat hulls in combination without presence of the substrate showed that the enzymes associated with one another as only one melting curve peak was detected opposed to the two expected ones (one for each enzyme). The melting temperature of this combined peak was often below the temperatures when they were analysed alone (see Figure 5.4A). This shows that both enzymes become destabilised when they are simultaneously present in the reaction mixture. Unfortunately, Lac_N could not be included in these analyses due to interfering compounds in the formulation.



B

Enzyme Combination	Only Enzymes	Oat Hull
GH11_P	53.7	53.3
GH11_F	72.7	72.3
GH10_X	78	78
GH5_*Ct* Domain 1	55	52.5
GH5_*Ct* Domain 2	72	69.7
GH11_F + GH10_X	71.7	71.7
GH11_F + GH5_*Ct*	70	72.3
GH11_P + GH10_X + GH5_*Ct*	55	54.7
FAE_R	59.3	59.5
FAE_R + GH11_F	72	71.7
FAE_R + GH11_F + GH5_*Ct*	71.3	72.7

Stabilizing | No Change | Destabilizing

Figure 5.4: Melt curves for selected enzymes showing a destabilising effect when present in combination (A) as well as average enzyme melting temperatures (n = 3) of all tested combinations in only buffer or in presence of the pre-treated oat hull substrate (B).
An increase in the melting temperatures indicates a stabilising effect, while a decrease suggests a destabilising effect.

These results alone cannot explain the synergy effects described in Figure 5.2. Hence, the oat hull substrate was added to the subsequent melt curve analyses. Interestingly, the enzyme combinations resulting in DS < 1 did not exhibit a change in melting temperature compared to the melting temperatures of the enzymes in absence of the substrate (see Figure 5.4B). The enzyme combinations with DS > 1, however, also exhibited an increase in melting temperature suggesting that an increased enzyme stability is contributing to their synergistic effects. One exception to this trend was found. The combination of GH11_F and FAE_R exhibits a clearly synergistic DS of 1.56, but no change in their melting temperature was observed. Enzyme stability is therefore only one of the factors contributing to enzyme synergy.

5.2.2 Other lignocellulosic substrates

Besides oat hulls, enzyme synergy was also investigated on three other lignocellulosic substrates in **Paper IV** in order to elucidate the importance of the substrate for the observed enzymatic synergies. These lignocellulosic materials were insoluble, destarched oat bran as well as destarched and ultrasound-assisted alkaline pre-treated, soluble oat and corn bran. No enzyme combination was found that behaved equally on all substrates, suggesting a stronger dependency of enzyme activity on the present substrate as opposed to other present enzymes. A similar observation has recently been made with cellulose-active enzymes and different cellulosic substrates [125]. Among the pre-treated materials, an inverse relationship between substrate recalcitrance and amount of enzyme combinations resulting in synergy was found. The more recalcitrant the substrate, the more enzyme combinations resulted in DS > 1. While lignocellulose recalcitrance depends on many factors, mainly the lignin and ferulic acid content were used here to establish the order of recalcitrance; oat hulls being the most recalcitrant followed by corn and soluble oat bran. The same trend, however, could not be observed for the insoluble oat bran. Based on its lignin and ferulic acid content, its recalcitrance should be in between those of oat hull and corn bran, however fewer enzymes combinations resulted in synergy compared to corn bran. This can most likely be explained by its insolubility limiting the access of the enzymes to the substrate, complicating a direct comparison of these substrates.

Due to this strong substrate dependency, the establishment of a universal enzymatic process is unattainable. However, heterosynergies seem to be more widespread applicable compared to homeosynergies. Synergistic enzyme combinations involving FAE_R and a xylanase were found on all substrates, indicating that FAEs can be powerful tools to enable greater accessibility of the main chains in lignocellulosic materials. The type of xylanase acting in synergy differed among the materials, most likely due to their different backbone substitutions. Interestingly, the combination with GH10_X resulted in synergy on the insoluble oat bran, while the combination with GH11_F resulted in synergy on the soluble oat bran. According to the size and penetration abilities of these enzyme classes, the opposite trend was expected. However, the pre-treated soluble oat bran contains most likely less main chain decoration compared to the non-pre-treated insoluble oat bran, which might explain the higher activity of GH11_F on this material.

Heterosynergies involving Lac_N were only found for the two substrates containing the highest amounts of lignin and ferulic acid: oat hulls and insoluble oat bran. Lac_N was found to also repolymerise the free phenolic acids present in the insoluble oat bran reactions. Therefore, it is likely that the observed synergy arises from the same mechanism of removing inhibitory compounds to the xylanases as previously described

for the oat hull. Based on the lack of laccase synergy on corn bran and soluble oat bran, their content of phenolic compounds seems to be too low to be inhibitory to the xylanases.

Homeosynergies were only found on corn bran besides the oat hull indicating the presence of differently substituted regions in the materials. In both cases, those combinations resulted in high yields, showing that even though they are less common, they can be rather important for industrial conversion processes. The mismatch between enzyme combinations resulting in high DS values and yields previously reported for the oat hulls, were also found for all the other materials.

The hypothesis of changes in enzyme stability being responsible for the synergistic effects was, similarly to the observations on the oat hull, only partly confirmed. While the relationship of higher melting points meaning greater enzyme stability and higher DS values was confirmed in most enzyme combinations, substrate dependent exceptions were found as well. The combination of GH11_F and GH10_X resulted in DS < 1 for both corn bran and soluble oat bran. While the melting temperature of the enzymes when applied on oat bran was also decreased as expected, it was increased on corn bran suggesting a greater enzyme stability. Hence, the enzyme stability experiments confirm the overall observation that substrate dependency is the most dominating factor for enzyme synergies.

6 Concluding remarks

Based on the research findings in this thesis, oat hulls are suggested as new sustainable resource to be considered for the industrial production of dietary fibres. Their suitability was shown via an analysis of their chemical composition (**Paper I**) and the development of production methods of both insoluble (**Paper II**) and soluble dietary fibres (**Papers III** and **IV**), which are feasible on industrial scale.

Dry oat hulls are composed of 84 % lignocellulose, the largest component being hemicellulose with 35 %. This large amount of hemicellulose in the form of arabinoxylan is rather unique among cereal waste streams and hence makes them ideal candidates for the production of dietary fibres.

The dietary fibres present in oat hulls are naturally insoluble suggesting their use as insoluble dietary fibre supplement. Due to their dark colour consumer acceptance of food products supplemented with the dark fibre is rather low. Therefore, an effective and robust bleaching method was developed resulting in an off-white insoluble dietary fibre product that showed promising consumer acceptance in initial tests.

Due to the increasing consumer interest in soluble dietary fibre products, another processing method was developed to solubilise the dietary fibres present in oat hulls. Their natural recalcitrance was overcome by an ultrasonication assisted alkali pre-treatment method, which showed similar solubilisation yields in initial upscaling trials. For these soluble fibres to become prebiotic a further reduction in chain length is beneficial. This was achieved in acceptable yields by the synergistic action of a ferulic acid esterase, a GH11 xylanase and a GH5 xylanase.

The results of the synergy experiments further supported our understanding of enzyme interactions and highlighted that enzyme synergy is very substrate specific. Therefore, biomass processing methods cannot simply be transferred to similar materials, but need to be adapted for every specific raw material.

6.1 Future perspectives

Based on oat hull availability and the lack of competing high-value applications, their use as dietary fibre source for various food products is highly promising. While initial upscaling trials were successful, trials on industrial scale still need to be carried out. The value of the products can be further increased significantly, if health claims could be used on their packaging. This requires more detailed evaluation of the products in various trials. For the soluble product to be attributed a prebiotic functionality, fermentation studies with probiotic strains should be carried out as a first step.

Utilising oat hulls as dietary fibre source is only one option in supporting the establishment of a successful bioeconomy. The bioeconomy requires the production of manifold everyday goods, besides food, from biomaterials. Oat hulls should be further assessed in this regard. Their rather unsubstituted xylan fraction could also be the basis for the production of biobased packaging materials or composites for construction material. Furthermore, applications for the lignin and cellulose fractions that remain insoluble after pre-treatment should be explored. The pre-treatment method developed in this thesis would ideally be only one part in a biorefinery making use of every fraction of the oat hull.

Acknowledgements

Modern day science is never the work of a single person and a PhD journey encompasses substantially more than what is written on the pages of this book. It requires the efforts of a team. I have been so lucky to have an incredible team by my side, whom I would like to thank here from the bottom of my smiling heart.

The first thank you goes to my supervisors, *Patrick* and *Eva*, for trusting me to succeed on this journey. Thank you for giving me the freedom to go my own way, allowing me to experiment not only with enzymes and biomass, but also with topics and fields.

For connecting this experimentation with the real world, I am grateful to my industrial partners at Lantmännen and Nouryon. *CG*, *Christian* and *Annelie*, thank you for your continued support throughout this journey. A special thanks goes to *Magnus* for the detailed questions, countless corrections and constant availability, even after our collaboration had officially ended.

A lot of experimentation would not have taken off without the support of my scientific family, the Bioorganic group. Thank you *Ariana*, *Siri*, *Nikolina*, *Cecilia*, *Savvina*, *Eimantas*, *Cristina*, *Ruby* and *Carl*, for welcoming me into your circle, supporting me with failed experiments and sharing the burden of fixing equipment. *Nikolina*, thank you for the wonderful atmosphere in the office where no topic was left undiscussed.

Similarly, I would like to thank the members of the Fermentation group for adopting me half-way through my studies: *Roya*, *Andrius*, *Zehui*, *Leila A*, *Leila K*, *Emmanuel*, *Anna*, *Madeleine*, *Israt*, *Zubaida* and *Fang*. Thank you for giving me a place to share my laughter and frustration and to discuss my thoughts on carbohydrate biochemistry. A special thank you goes to *Roya* for introducing me to nearly every equipment I touched and every method I used on this journey. Without you, we would all be lost. For introducing me to the world of teaching, a big credit goes to *Anna*. Thank you for taking away my nervousness with your calm nature and structured working approach.

Some of my greatest teachers started out as my students in degree projects or the enzyme technology course, first and foremost *Juanita*. Thank you for keeping me on my toes and asking questions I would have never thought of myself. You contributed immensely to my professional growth and helped me build self-confidence.

A PhD journey goes far beyond a regular work experience. Odd working hours and lab problems that occupy one's thoughts even during 'free times' are a frequent companion. Thank you, *Sara*, for putting those times into perspective and teaching me about a healthy work-life balance in many highly cherished fika breaks. You are an inspiration.

In similar terms, I also owe the greatest of thanks to *Zehui* and *Savvina*. Thank you for always being there, be it in the lab fixing analytical equipment or after work baking and enjoying home cooked meals. Being in a foreign country during a pandemic is a horrifying experience, especially when you start feeling that itchiness in your throat. Thank you for cycling into town in the rain to pick up Covid tests, keeping my spirits high in those scary times and simply being there, becoming my family.

This journey would have never started without the countless pep-talks and encouraging words over brunch with my Malmö girls, *Madhuri* and *Samantha*. Thank you for being there for me in my times of uncertainty and pushing me to click that apply button.

There are a few people in my life that never doubted that I would embark on this journey, even long before I started considering it myself. They are the silent supporters, invisible at work, but nonetheless important, and a major reason for my successes. *Claudio*, thank you for your tragi-comedic career advice and pushing me beyond my comfort zone when necessary. *Iris*, *Makieu*, *Carmen*, *Papul* and *Nancy*, my Jacobs girls, thank you for always cheering in my corner, no matter how far apart we have moved from one another. Thank you for the surprise calls and cards, the check-ins. Thank you for reminding me who I am, when I start to forget. *Tanja*, dieser Dank geht auch an dich. Du bist immer für mich da, und das schon seit der Mittelstufe. Damals hast du mich bei der Lateinnachhilfe gelehrt, dass es nichts gibt, dass ich nicht lernen kann. Manchmal muss ich nur die Lernmethode wechseln. Dieser Rat hat mich während meiner gesamten Promotion begleitet und ohne dich wäre dieses Buch wahrlich nicht so gut geworden.

Embarking on a PhD journey is not a lonely adventure. It involves the entire family. *Helen*, vielen Dank für deine unermüdliche Unterstützung, deine Positivität und deine emotionalen Pep-talks jedes Mal wenn ich an mir selbst zweifle. Danke, dass du meine mentalen Wände immer wieder einreißt. *Mama* und *Papa*, dieses Buch ist genauso euer Verdienst wie meiner. Nur wegen eurer Förderung meiner Neugier und den Lehren das Wesentliche im Auge zu behalten und nicht aufzugeben, bin ich überhaupt so weit gekommen. Vielen Dank für euer Vertrauen und euren Glauben an mich.

E infine, *Tiago*, tu sai meglio di chiunque altro cosa significhi davvero questa tesi. Non ci sono parole per esprimere la gratitudine che ho nei tuoi confronti. Il nostro viaggio è iniziato qui e solo per questo sarei disposta a rivivere da capo tutti i problem legati al dottorato. Sei la mia montagna, la mia roccia. Grazie.

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Paper I

plant biology

Plant Biology ISSN 1435-8603

RESEARCH PAPER

Warming weather changes the chemical composition of oat hulls

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Keywords
biorefinery; chemical composition; climate effects; lignocellulosic biomass; oat hull.

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Editor
R. Leegood

Received: 30 January 2020; Accepted: August 9 2020

doi:10.1111/plb.13171

ABSTRACT

- The current threats of climate change are driving attention away from the petrochemical industry towards more sustainable and bio-based production processes for fuels and speciality chemicals. These processes require suitable low-cost starting material. One potential material assessed here is the oat hull. Its overall chemical composition has so far not been fully characterized. Furthermore, it is not known how it is affected by extreme weather events.
- Oat hulls (Kerstin and Galant varieties) grown during 'normal' weather years (2016 and 2017) are compared to the harvest of the warmer and drier year (2018). Standard methods for determination of plant chemical composition, with focus on carbohydrate composition, are utilized.
- Oat hulls grown in 'normal' weather conditions (2017) are rich in lignocellulose (84%), consisting of 35% hemicellulose, 25% lignin and 23% cellulose. Arabinoxylan was found to be the major biopolymer (32%). However, this composition is greatly influenced by weather variations during the oat growth phase. A lignocellulose reduction of 25% was recorded in the warmer and drier 2018 harvest. Additionally, a 6.6-fold increase in starch content, a four-fold increase in protein content and a 60% decrease in phenolic content was noted.
- Due to its high lignocellulose composition, with an exceptionally large hemicellulose fraction, the chemical composition of oat hulls is unique among agricultural by-products. However, this characteristic is significantly reduced when grown in warmer and drier weather, which could compromise its suitability for use in a successful biorefinery.

INTRODUCTION

Oat (*Avena sativa* L.) is a crop grown worldwide and is now attracting more attention due to the discovery of its many health benefits as well as an increased interest in plant-based diets. Its increasing popularity is reflected in global production quantities, which have risen by nearly 30%, from 19 million tons in 2010 to 26 million tons in 2017, and is expected to increase further (FAOSTAT Database, 2019). Hence, the handling of low value side streams will also become more important. One large quantity by-product of oat production is the grain's outer shell, *i.e.* the hull (or husk), which makes up 25% to 35% of the entire grain (Redaelli & Berardo 2007). Currently, its value is rather low as it is seen as a waste product, which is often burned for energy production. Due to its lignocellulosic composition (*i.e.* cellulose, hemicellulose and lignin), however, it has great potential as starting material in a biorefinery process, which aims to produce conventional and novel fuels as well as specialty chemicals from biomass. Potential products include biobased films for packaging (Sousa *et al.* 2016) and composites for construction and building material (Vo & Navard 2016). Compared to traditional petroleum-based refineries, biorefineries are attractive alternatives as they are based on the conversion of renewable material and emit zero net CO_2 into the atmosphere (Amoah *et al.* 2019). In order to assess the oat hull's suitability to act as a biorefinery starting material, as well as to design the most value-adding process, an in-depth understanding of its complex chemical composition is crucial. All characterizations performed so far have only assessed the quantity of its main components and have not further analysed its lignocellulose composition (Welch *et al.* 1983; Crosbie *et al.* 1985; Thompson *et al.* 2000).

Particularly large quantities of oats (up to 2.6 million tons per year) are grown in the Nordic countries due to the ability of oats to thrive in the short seasons of cool and wet climates with long periods of daylight (Buerstmayr *et al.* 2007; FAOSTAT Database, 2019). Previous studies have shown that oat growth is greatly influenced by changes in climate conditions. Elevated temperatures (especially during the early growth season) as well as reduced precipitation significantly impact agricultural yield of many oat varieties at diverse growth locations (Buerstmayr *et al.* 2007; Peltonen-Sainio *et al.* 2011; Klink *et al.* 2014). Even more recently bred oat cultivars, which were expected to be better adapted to current weather conditions, have suffered from significant yield losses in warmer and drier years (Klink *et al.* 2014). The oat hull chemical content has been shown to remain the same at elevated temperatures, indicating that it is similarly affected as the grain (Peltonen-Sainio *et al.* 2011). As oat growth is so sensitive to altered weather conditions, it is likely that not only macroscopic factors such as grain yield, plant height and lodging severity are influenced, but also its chemical composition. This must be taken into consideration when constructing a biorefinery process to produce speciality chemicals from oat hulls. Adaptive process

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engineering and construction flexibility will become increasingly more important to allow successful production during years with different weather profiles.

The objective of this study was to analyse the chemical composition of oat hulls and assess their suitability as starting material in a biorefinery. Furthermore, changes in the composition due to extreme weather conditions at comparable locations were analysed and evaluated in the context of the productivity of a new oat hull-based biorefinery. As years with altered weather conditions are expected to occur more frequently in the future, this information is essential to consider during biorefinery process design.

MATERIAL AND METHODS

Raw material and chemicals

Four different oat hull batches were supplied by Lantmännen ek. för (Stockholm, Sweden). The hulls in the three batches from Sweden (one batch per investigated year) were pooled from several fields in the region of Mälardalen in central Sweden. A minority of hulls also came from the regions Östergötland and Västergötland. One additional batch, grown in Denmark in 2018, was also investigated. More detailed information about batch characteristics are summarized in Table 1. All hulls were separated from the grains utilizing a Bühler BSSA stratopact HKE50HP-Ex peeler (Höflinger Millingsystems, Neustadt an der Weinstraße, Germany).

All chemicals were purchased from Merck (Sweden) unless otherwise specified.

Moisture and ash content analysis

The moisture and ash content were determined by drying at 105 °C overnight followed by incineration at 575 °C for 24 h, respectively.

Total starch analysis

Analysis of total starch content was performed using the Total Starch kit from Megazyme. Protocol K-TSTA 09/14 method a was followed.

For visual confirmation of the presence of starch, whole hulls from batches Swe17 and Swe18 were taken. A drop of undiluted Lugol reagent (Merck) was added to either the interior or exterior side of the hull for 3 min. Imaging was performed using a Nikon OPTIPHOT-2 microscope equipped with a Plan 20/0.50 DIC 160/0.17 objective and a Nikon Digital Sight DS-2 Mv camera. The software used for image analysis was NIS-Elements D 3.1 (Nikon, Tokyo, Japan).

Protein content analysis

The protein content was measured according to the Dumas technique by applying 25 mg of sample to a FlashEA 1112 series N/Protein Analyzer (Thermo Scientific, Waltham, MA, USA) using aspartic acid as the standard. For nitrogen to protein content conversion, a factor of 5.83 was used.

Lipid content analysis

Total lipid content was analysed by lipid extraction in a 2:1 chloroform:methanol (v/v) solution based on the methods of Folch *et al.* (1957) and Lee *et al.* (1995). To aid extraction, the samples were homogenized for 60 s using an Ultra Turrax T 25 digital (IKA, Staufen im Breisgau, Germany) blender at 12,000 rpm. The organic phases were separated by mixing with 0.5% sodium chloride solution at a 2:5 ratio, followed by centrifugation for 5 min at 3,893x*g*. Subsequently, the bottom chloroform layer was isolated and the lipids retrieved and weighed after evaporation of the solvent.

Structural carbohydrates and lignin content analysis

Determination and characterization of lignin, cellulose and hemicellulose content was performed according to the NREL Laboratory Analytical Procedure (NREL/TP-510-42618, 2012). Extracted monosaccharides and uronic acids were identified and quantified using an HPAEC-PAD (ICS-5000, Thermo Scientific, Sunnyvale, CA, USA) equipped with a CarboPac PA20 analytical column (150 mm × 3 mm; 6 µm) as well as a guard column (30 mm × 3 mm), as previously described in Falck *et al.* (2014). For the separation of arabinose and rhamnose, the mobile phase concentration was increased to 10 mM sodium hydroxide. Uronic acids were analysed according to the same method, with a mobile phase consisting of 90 mM sodium hydroxide and 150 mM sodium acetate.

Phenolic acid content analysis

Phenolic acids were extracted and quantified according to the method described in Sajib *et al.* (2018). As not all phenolics

Table 1. Description of oat hull batches used in this study. Weather data were retrieved from the archives of the Swedish Meteorological and Hydrological Institute (SMHI Database, 2019) and the Danish Meteorological Institute (DMI Database, 2019). Summer is defined as the months of June, July and August.

Batch	Oat variety (Seed origin)	Harvest year	Growth location	Average temperature summer [°C]	Average precipitation summer [mm]
Swe16	Kerstin and Galant (SW-Seed, Sweden)	2016	Sweden	15.0–16.5	49–130
Swe17	Kerstin and Galant (SW-Seed, Sweden)	2017	Sweden	13.5–15.0	49–98
Swe18	Kerstin and Galant (SW-Seed, Sweden)	2018	Sweden	16.5–18.5	33–65
Den18	Symphony and Poseidon (Saaten Union, Germany)	2018	Denmark	17.7	47.2

were identified, the total phenolics content of the different batches is evaluated by comparing the total area beneath all peaks.

Mineral content analysis

For the detection and quantification of minerals, the oat hulls were first dissolved in nitric acid with wet combustion in a microwave system. Subsequently, the samples were analysed *via* inductively coupled plasma optical emission spectroscopy using an iCap 7400 ICP-OES (Thermo Scientific). The report limits for the individual minerals in $\mu g\ g^{-1}$ were as follows: Al: 1.0; Ba: 0.2; Ca: 20; Cr: 0.1; Cu: 0.1; Fe: 0.5; K: 10; Mg: 20; Mn: 0.1; Na: 5.0; P: 10; S: 20.

RESULTS AND DISCUSSION

Chemical composition

The chemical composition of the four different oat hull batches grown in similar locations, but under different weather conditions (see Table 1), was analysed. Batch Swe17 was chosen as model batch to describe the general composition of oat hulls as it contains a mixture of two common oat varieties grown in Sweden (*i.e.* Kerstin and Galant) under 'normal' weather conditions. The quantity of its main components is summarized in Table 2. The composition analysis shows that oat hulls are a highly lignocellulosic material, comprising 83.9% of the hull dry weight, which is about 8% more than commonly found in oat straw; this places hulls among the most lignocellulose rich agricultural wastes, comparable to wheat straw (Isikgor & Becer 2015). The largest fraction of the hull lignocellulose is hemicellulose (35.1%), followed by lignin with 25.4%. The majority of the lignin is acid insoluble, corresponding to 91.7% of the lignin fraction. The cellulose content at 23.4% is slightly lower than the lignin content. This composition is unique to oat hulls and differentiates them from many other agricultural waste products, as these are typically richest in cellulose and poorest in lignin (Isikgor & Becer 2015). After lignocellulose, the most abundant component is ash, i.*e.* inorganic materials content, with 5.2%. Minor components include starch (2.5%), proteins (1.4%) and lipids (0.9%). The low lipid content, which is in line with previous findings (Bryngelsson *et al.* 2002), most likely describes the waxy coating on the outer surface of the hull and does not penetrate into the deeper layers.

Table 2. Chemical composition of four oat hull batches grown under different weather conditions (see Table 1 for description). All numbers represent triplicate percentages based on dry weight.

Component	Swe16	Swe17	Swe18	Den18
Lignin	25.4 ± 1.7	25.4 ± 2.3	19.7 ± 0.2	12.9 ± 0.7
Acid insoluble	*23.1 ± 1.7*	*23.3 ± 2.3*	*17.2 ± 0.2*	*10.5 ± 0.5*
Acid soluble	*2.3 ± 0.2*	*2.1 ± 0.1*	*2.5 ± 0.2*	*2.4 ± 0.2*
Cellulose	17.2 ± 1.6	23.4 ± 2.6	16.0 ± 0.5	25.7 ± 0.2
Hemicellulose	33.1 ± 0.9	35.1 ± 0.1	27.9 ± 0.3	24.0 ± 0.1
Starch	8.5 ± 0.4	2.5 ± 0.6	16.3 ± 1.9	12.9 ± 0.4
Ash	6.0 ± 0.3	5.2 ± 0.1	5.7 ± 0.1	6.3 ± 0.1
Protein	1.7 ± 0.4	1.4 ± 0.2	5.4 ± 0.5	7.4 ± 0.4
Lipid	0.5 ± 0.2	0.9 ± 0.4	1.5 ± 0.1	1.4 ± 0.3

When comparing the composition of all batches analysed in this study, large variability becomes evident (see Table 2). This also holds true for the two batches grown under 'normal' weather conditions, *i.e.* Swe16 and Swe17. A lower cellulose composition was found in batch Swe16, although the starch content was increased. This shows that the chemical composition of oat hulls is influenced by more than just climate changes. However, the remaining components remain very similar among batches. Hence, the general composition of batch Swe17 described above will be used as representative of a 'normal' growth year for further comparison with the batches grown in the climatically irregular year 2018. These latter two batches showed very large differences compared to Swe16 and Swe17. The largest variability was found in the starch composition, which substantially increased in both 2018 batches by factors of 6.6 (Swe18) and 5.2 (Den18) in comparison with Swe17. The starch content measured in this study is likely not part of the hull itself but represents a minor fraction of the grain that remains attached to the hull during the dehulling process (observation at the industrial dehulling site; CG Pettersson, Lantmännen ek. för., personal communication). The hulls were removed industrially with a Bühler peeler, which is not as thorough as peeling by hand. Therefore, the presence of minor contaminants from the grain in the hull fraction is likely. To confirm this hypothesis, un-milled hulls from batches Swe17 and Swe18 were stained on either the interior or exterior side of the hull with Lugol reagent, which selectively colours starch. The results clearly show that starch is only present on the interior side of the hull (see Fig. 1). The starch content results for batches Swe18 and Den18 indicate that the hull is more tightly bound to the grain when grown in warmer seasons with lower precipitation. This development would greatly impact a biorefinery process. Generally, it is challenging to process starch-containing materials due to the starch gelling nature. Processes that do not account for this might become clogged and unable to process the material; therefore, starch might have to be degraded first when it exceeds a certain process-dependent limit.

The lignocellulosic content of the hulls grown in 2018 decreased to 63.6% and 62.6%, respectively, of values in 2017. This represents a loss of approximately 25%, which would also have significant implications for a biorefinery process. Mostly the hemicellulose and lignin fractions of the lignocellulose were reduced. However, in the lignin fraction it is only the acid-insoluble part that showed a significant reduction. The acid-soluble part remains the same. The cellulose content differed greatly among all batches, so that no trend related to weather variations could be determined. On the other hand, both protein and lipid content are increased for the 2018 growth season. This increase might also be partially caused by a larger grain content in the hull fraction, as described above for starch. However, an increase in both components when the crops are grown in warmer temperatures has also been observed for soybean seeds (Wolf *et al.* 1982) and oat grains (only protein measured; Peltonen-Sainio *et al.* 2011), suggesting an additional higher production of proteins and lipids by the plant under warmer conditions. Immature hulls are known to contain more protein than mature hulls (Pomeranz *et al.*, 1976). This is an indication that the extreme weather conditions inhibited

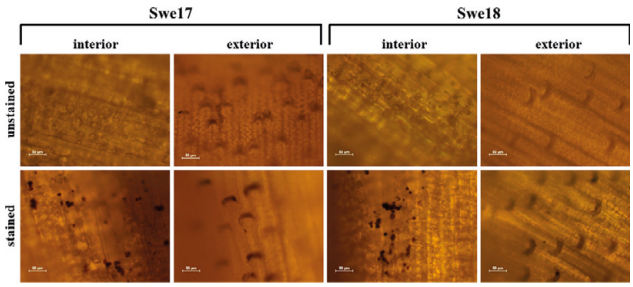

Fig. 1. Microscope images depicting the Swe17 and Swe18 oat hull surfaces from the interior and exterior sides with and without staining (Lugol reagent). The stained starch is visible as dark spots.

ripening. Regarding lipid content, an increase in the thickness of the waxy layer on the surface of the hull is most likely the result of a protective mechanism in the plant to prevent evaporation of water from the grain in warmer weather. The only constant content between years was for ash, which is unlikely to be influenced by differences in weather.

Hemicellulose composition

Xylose, at 28.9%, was the most abundant monosaccharide in the hemicellulose of oat hulls, followed by arabinose (3.5%) (see batch Swe17 in Table 3). This indicates that arabinoxylan is the most abundant biopolymer, with an arabinose to xylose (A/X) ratio of 0.1. Compared to the oat grain, in which A/X ratios are 0.5 to 1.0, depending on the extraction method have been measured, this is a rather low ratio indicating the presence of few chemical branches (Virkki *et al.* 2005). Further minor monosaccharides present are galactose (1.4%), mannose (0.1%) and rhamnose, whose concentration was too low to be quantified. Additionally, minor galacturonic (0.5%) and glucuronic (0.8%) acid substitutions were found. The composition of batch Swe16 was very similar, the only difference being the absence of mannose and rhamnose, which indicates that the hemicellulose composition is rather stable in 'normal' years.

However, there were variations in the monosaccharide composition of hemicellulose in the hotter and drier years. These mainly influence the xylose content, which is greatly decreased (24% for Swe18 and 38% for Den18). The arabinose, galactose and uronic acid content remained similar, leading to a doubling of the A/X ratio for Den18 (0.2 compared to 0.1). The content of the minor monosaccharides rhamnose and mannose differed between Swe18 and Den18, indicating that also growth location influences chemical composition. While neither of the monosaccharides could be detected in Den18, both increased in Swe18 (NQ rhamnose and 0.2% mannose). Overall, this analysis shows that not only the hemicellulose amount, but also its composition is altered during differences in weather conditions. This becomes important when a biorefinery aims to produce speciality chemicals based on xylose, or when xylose fermenting microorganisms are used for the production of biofuels.

Table 3. Hemicellulose composition of four oat hull batches grown under different weather conditions (see Table 1 for description). ND, not detected; NQ, not quantifiable; A/X ratio, arabinose to xylose ratio. All numbers represent triplicate percentages based on dry weight, except for the A/X ratio, which is not a percentage.

Component	Swe16	Swe17	Swe18	Den18
Xylose	27.4 ± 0.9	28.9 ± 0.3	21.9 ± 0.2	17.8 ± 0.1
Arabinose	3.3 ± 0.0	3.5 ± 0.0	3.2 ± 0.6	3.7 ± 0.0
Galactose	1.2 ± 0.0	1.4 ± 0.2	1.3 ± 0.1	1.4 ± 0.0
Rhamnose	ND	NQ	NQ	ND
Mannose	ND	0.1 ± 0.1	0.2 ± 0.0	ND
Galacturonic acid	0.5 ± 0.0	0.5 ± 0.0	0.6 ± 0.0	0.6 ± 0.0
Glucuronic acid	0.8 ± 0.0	0.8 ± 0.1	0.7 ± 0.0	0.5 ± 0.0
A/X ratio	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0

Phenolic composition

Oat hulls have previously been of interest in the food industry to help prevent lipid oxidation due to their high antioxidant activity originating from their phenolic content (Xing & White, 1997). Several studies have reported the presence of a variety of different phenolic compounds (Xing & White 1997; Emmons & Peterson 1999; Bryngelsson *et al.* 2002; Varga *et al.* 2018), which were confirmed in this study. However, the detected quantities are only comparable to the study by Varga *et al.* (2018). Among previous studies, the largest total phenolic amount reported was 560 $\mu g\ g^{-1}$ (Xing & White 1997), which is more than 16 times less than the amount found in this study for the oat hull batches grown in a 'normal' weather year (Swe16 and Swe17; see Table 4). These large variations are not caused by differences in oat varieties and growth locations, but rather a difference in the effectiveness of the extraction method. While the previous studies which reported lower content of phenolics only extracted the soluble compounds, Varga *et al.* (2018), as well as this study, utilized a method suitable for the extraction and analysis of bound phenolic compounds. The most abundant phenolic acid found in this study was a mixture of *p*-coumaric acid and vanillin (6920 and 6557 $\mu g\ g^{-1}$, respectively), which were not separable in the analysis method established by Sajib *et al.* (2018). The remaining identified phenolic acids, found in decreasing abundance, were ferulic acid (2596 and 2319 $\mu g\ g^{-1}$) and *p*-hydroxybenzaldehyde (215 and 200 $\mu g\ g^{-1}$). Ferulic acid is known to form cross-links between arabinoxylan chains as well as binding hemicellulose to lignin *via* ester bonds (Virkki *et al.* 2005; Amoah *et al.* 2019). As oat hulls are very rich in arabinoxylan, hemicellulose and lignin (see Tables 2 and 3), the high ferulic acid content is acceptable.

Table 4. Total and identified phenolic composition of four oat hull batches grown under different weather conditions (see Table 1 for description). All identified phenolic compounds represent triplicate measurements based on dry weight in μg g^{-1}. The total phenolics composition is given as percentage of batch Swe16.

Phenolic compound	Swe16	Swe17	Swe18	Den18
p-hydroxybenzaldehyde	214.5 ± 4.3	200.3 ± 11.1	68.7 ± 1.8	60.9 ± 3.3
p-coumaric acid/vanillin	6919.6 ± 224.8	6557.2 ± 304.8	2521.3 ± 86.4	3008.6 ± 66.8
Ferulic acid	2596.2 ± 40.4	2318.5 ± 111.3	1339.2 ± 41.0	1435.6 ± 43.4
Total	100	99.8	43.1	37.2

A large decrease in all phenolics was found in the two batches grown in the hotter and drier year, accumulating to a total decrease of about 57% and 63% for batches Swe18 and Den18, respectively, compared to both Swe16 and Swe17. This finding supports previous results of Menga *et al.* (2010), who found that environmental factors affect the production of phenolic compounds more than genetic differences in oat variety. Many plants up-regulate phenolics expression under stress, including high temperatures and drought, to increase the plant's defence capacity (Akula & Ravishankar, 2011). However, this trend could not yet be confirmed for oats. Peterson *et al.* (2005) found no significant differences in the avenanthramides (phenolic alkaloids unique to oats) content of oats grown in the same location under both dry and irrigated conditions. Instead, differences in phenolics content depending on the maturity stage have been found, with higher content in immature seeds (Alfieri & Redaelli, 2015). This is in contrast to the observation found for the protein content and questions whether maturation is inhibited in warmer weather.

The drastic decrease in phenolic content would not only have implications for biorefinery use, aiming to utilize the antioxidant activity of oat hulls, but could affect any process utilizing oat hulls, as the cinnamic acids among the phenolic acids, *i.e. p*-coumaric and ferulic acid, have been shown to be important in antifungal and antimicrobial protection mechanisms of the plant rather than in plant antioxidant activity (Bryngelsson *et al.* 2002). Therefore, more microbially infected hulls might be harvested in warmer and drier years, which could influence the quality of any product produced from the hulls.

Mineral composition

A mineral composition analysis revealed that with 3100 μg g^{-1}, K is by far the most abundant mineral (see Table 5), followed by Ca (840 μg g^{-1}), Mg (480 μg g^{-1}), P (310 μg g^{-1}) and S (250 μg g^{-1}). All remaining minerals were found in minor concentrations of 31 μg g^{-1} and below in the Swe17 batch, representing a 'normal' growth year. The mineral composition of the second batch grown under 'normal' weather conditions (Swe16) is very similar. With only higher amounts of Na, P and S, which corresponds to the slightly higher ash content displayed in Table 2.

In contrast, there were large differences in the two batches grown in the climatically more stressful year of 2018. In general, all minerals found had higher concentrations, this trend being more pronounced for batch Den18, and supported by the higher measured ash content (see Table 2). The largest increases were seen for the minerals Fe, K, Mg, P and S. The higher Fe content might be explained by the harsher milling process that was applied on both Swe18 and Den18 and could therefore be contamination introduced by the milling. The largest difference between Swe18 and Den18 is the Ca content, which increased significantly for Swe18, while remaining at approximately the same level as the 'normal' year batches for Den18.

Table 5. Mineral composition of four oat hull batches grown under different weather conditions (see Table 1 for description). All numbers represent duplicate measurements of one hydrolysed sample based on dry weight in μg g^{-1}.

Minerals	Swe16	Swe17	Swe18	Den18
Aluminium	1.4	2.9	13	10
Barium	1.3	1.7	2.7	4
Calcium	820	840	1400	880
Chromium	<0.1	<0.1	0.1	0.5
Copper	1.3	1.4	3.3	4.7
Iron	11	16	76	130
Potassium	2700	3100	5000	6400
Magnesium	560	480	950	1500
Manganese	27	31	52	49
Sodium	89	15	59	14
Phosphorus	550	310	2000	4400
Sulphur	380	250	810	1300

Suitability of oat hulls for biorefineries

Overall, oat hulls are an attractive starting material for lignocellulose-utilizing biorefineries. However, its lignocellulose composition (determined for Kerstin and Galant varieties grown in Sweden during the 'normal' weather year 2017) differed from other lignocellulosic agricultural by-products, containing an unexpectedly high amount of hemicellulose (35.1%) and lignin (25.4%), but relatively little cellulose (23.4%). The hemicellulose consists exclusively of arabinoxylan with very little galactose, rhamnose, mannose and uronic acid substitutions. Oat hull arabinose to xylose (A/X) ratio at 0.1 is also very low, making it attractive for applications requiring unsubstituted xylan. In addition, a very high content of phenolic compounds was detected, which could be valuable for the food industry due to their antioxidant activity. The remaining components are only present in minor amounts, facilitating its processing. However, this chemical composition is greatly influenced by different weather conditions during the oat growth phase. A loss of about 25% of lignocellulose content was observed for the two batches grown in an average 2 °C to 5 °C warmer summer with up to 75% less precipitation compared to a 'normal' year. This loss is mostly due to a reduction in the hemicellulose and (acid

insoluble) lignin fractions. The cellulose content fluctuates independent of weather trends. Even the hemicellulose monosaccharide composition changed, as characterised by a decrease in the xylose fraction of up to 8%. Furthermore, the starch content increased by a factor of 6.6, which is most likely due to the higher grain content in the hull fraction resulting from difficulties in separating the hull from the grain. Finally, a decrease in the phenolic content of around 60% was noted, which hints at higher susceptibility of the plant to microbial attack. These are very large variations that need to be taken into consideration when designing a new oat hull-based biorefinery.

CONCLUSION

Oat hulls have high potential to serve as starting material for a biorefinery due to their extensive lignocellulose content (84%). This lignocellulose is exceptionally rich in hemicellulose (35%), which consists exclusively of arabinoxylan with only a few arabinose substitutions (A/X ratio of 0.1), making it an attractive source for unsubstituted xylan. However, this chemical composition is greatly influenced by weather conditions (25% loss of lignocellulose content in the warmer and drier year). This needs to be considered when designing a new oat hull-based biorefinery as the trend for warmer and drier seasons in the Nordic regions is increasing.

ACKNOWLEDGEMENTS

We would like to thank Katarina Gutke and Magnus Paulsson at Nouryon for performing the mineral content analysis and making helpful suggestions. We are furthermore grateful to CG Pettersson at Lantmännen for providing the raw material and for fruitful discussions. This work was supported by the strategic innovation program BioInnovation funded by Vinnova, Formas and the Swedish Energy Agency.

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Paper II

Biotechnology Reports 30 (2021) e00624

Contents lists available at ScienceDirect

Biotechnology Reports

journal homepage: www.elsevier.com/locate/btre

Chemical and biochemical bleaching of oat hulls: The effect of hydrogen peroxide, laccase, xylanase and sonication on optical properties and chemical composition

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ARTICLE INFO

Article history:
Received 27 October 2020
Received in revised form 26 April 2021
Accepted 27 April 2021

Keywords:
Oat hulls
Chemical bleaching
Hydrogen peroxide
Biobleaching
Enzymes
Dietary fibre

ABSTRACT

Oat hulls are an excellent dietary fibre source for food supplements due to their rich lignocellulose composition as well as their great abundance as low-value agricultural side stream. For the production of white fibre supplements, a mild, but effective bleaching of the hulls is required. Chemical bleaching with hydrogen peroxide and sodium hydroxide was here found to be a suitable method increasing the CIE L^* value (corresponds to a lightness value) above 85. The developed method is mild, retaining the hull's chemical composition. Only a minor decrease in coniferaldehyde structures upon bleaching was detected. Colour and chemical variabilities of oat hulls from different growth seasons did not influence the required bleaching conditions to achieve the desired optical properties. The inclusion of biochemical bleaching steps utilizing the xylanase Pentopan Mono BG, the laccase NS51003 and sonication was industrially not feasible as they could not reduce the required amount of subsequently applied bleaching chemicals significantly.
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1. Introduction

In recent years, the consumer interest for healthier food products has increased, including fibre-enriched products. Oat hulls are an excellent source for additional dietary fibre due to their rich lignocellulose composition as well as their great abundance as low-value byproduct from oat production. Their lignocellulose content can reach up to 84 % and the hemicellulose fraction up to 35 % depending on the growth conditions [1]. This makes the oat hull composition superior to wheat straw, which is currently commonly used as fibre supplement. Wheat straw contains a similarly large lignocellulose fraction, however only 23–30 % is hemicellulose [2]. The hemicellulose from cereals is of particular interest as a fibre supplement as it can be either added as insoluble fibre to reduce hunger feelings for an increased amount of time after consumption [3] or broken down into smaller soluble fragments with potentially prebiotic effects in the gut [4]. However, there is an important drawback of utilizing oat hulls as fibre supplements. Oat hulls are naturally dark in colour, which alters the optical appearance of the end products, such as pasta or bread. White fibres are generally preferred as they can easily be incorporated in food formulations without altering the optical properties. Therefore, bleaching of oat hulls is a required processing step for their use in food products.

Bleaching can be performed either with chemical or biochemical methods or a combination thereof. Traditional chemical bleaching typically employed elemental chlorine, which made the process very effective and economical, but environmentally unfriendly. To counteract this negative aspect, modern chemical bleaching follows either totally chlorine-free methods or elemental chlorine free methods, which use chlorine chemicals such as chlorine dioxide or hypochlorite. Both methods utilize oxygen based bleaching agents, such as elemental oxygen, hydrogen peroxide (H_2O_2) or ozone [5]. The inclusion of biochemical methods could also lower the negative impact of bleaching on the environment by reducing the consumption of the required bleaching chemicals to achieve similar or better optical properties. These methods could, therefore, contribute to the achievement of the sustainable development goal 12 discussing responsible consumption and production [6]. A very promising candidate is

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https://doi.org/10.1016/j.btre.2021.e00624
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Table 1
Description of oat hull batches used in this study. The data were taken from our previous study [1] and the methods described below. The copper, iron and manganese content before and after milling (in brackets) is given.

Batch	Harvest year	Lignocellulose content [%][1]	Starch content [%][1]	Ferulic acid content [μg/g][1]	Cu [μg/g]	Fe [μg/g]	Mn [μg/g]
Swe16	2016	74.5 ± 1.0	8.5 ± 0.4	2596 ± 40	1.3	11	27
Swe17	2017	82.7 ± 0.4	2.5 ± 0.6	2319 ± 111	1.7 (1.4)	18 (16)	36 (31)
Swe18	2018	62.3 ± 0.5	16.3 ± 1.9	1339 ± 41	1.4 (3.3)[2]	17 (76)[3]	27 (52)[2]
Swe19	2019	71.7 ± 0.4	12.1 ± 0.5	3327 ± 222	2.4 (2.4)	24 (45)[3]	40 (42)

[1] Analysis performed on milled oat hulls.
[2] The higher content in the milled oat hulls indicates that the milled and unmilled hulls are from different batches as this increase is not seen for Swe17 and Swe19.
[3] Batches Swe18 and Swe19 were milled with industrial milling equipment, while Swe17 was milled in a lab mill. The higher Fe content in batches Swe18 and Swe19 is likely due to contamination from the industrial milling equipment since the Fe content of the different unmilled oat hull batches was in the range 11–24 μg/g.

Table 2
Description of the optical properties of the unmilled oat hull batches used in this study. The optical properties are described using the three-dimensional colour system CIE L^*, a^*, b^*, which consists of a grey scale axis (L^*), a green-red axis (a^*), and a blue-yellow axis (b^*).

Batch	Harvest year	Brightness [%]	L^*	a^*	b^*
Swe16	2016	22.4	66.3	3.0	21.2
Swe17	2017	22.4	64.6	2.4	18.1
Swe18	2018	27.0	70.7	2.9	21.0
Swe19	2019	25.9	68.2	2.3	18.4

Table 3
Factor settings for the design of experiment study (Swe19 hulls) showing the minimum and maximum values for each factor, which enclose the design space.

Factor	Unit	Minimum	Maximum
Sonication length	min	10	60
Laccase concentration	U/g	6	18
Xylanase concentration	U/g	5	15

the enzyme laccase. Laccases are multicopper oxidases, which are capable of oxidizing phenols and even non-phenolic lignin units, when combined with an oxidation mediator. During the laccase reaction with its substrate a phenoxy radical is formed, which can depolymerise lignin and hence increase the brightness of the material. However, the action of the phenoxy radical could also result in counterproductive lignin repolymerization as well as their conversion into chromophoric conjugated carbonyl structures, such as quinones, which darken the material instead. These chromophoric structures can be removed by alkaline or oxidative chemical bleaching stages with a lower chemical loading than if only chemical bleaching had been performed [5]. Therefore, a combination of laccase and chemical bleaching is very attractive.

Previous studies on both wood kraft pulp and lignocellulosic material from agricultural sources have shown that the use of xylanase alone or in combination with laccase can reduce the need for bleaching chemicals [7–9]. Xylanases are capable of separating the lignin carbohydrate complexes, which makes the material more accessible to the bleaching chemicals. In one case, the combined treatment of xylanase and laccase on kraft pulp made a reduction of the bleaching chemical sodium hypochlorite of up to 42 % possible [9]. A similar loosening of the lignin carbohydrate complexes was observed by Sun and colleagues [10], when extracting hemicellulose from sugarcane bagasse via ultrasonication. Therefore, sonication could be another potential processing step to reduce the required amount of both chemical and biochemical agents for bleaching.

Only chemical bleaching has so far been tested on oat hulls utilizing either alkaline (NaOH) hydrogen peroxide extrusion [11–13] or peracetic acid [14,15]. Alkaline hydrogen peroxide extrusion caused an increase of the oat hull's L^* value, which measures the grey-scale, of 15 % (from 44.8–51.4) [11] and 23 % (from 46.8–53.5) [12]. However, neither of the studies aimed at increasing the optical properties of the oat hulls, but rather their modification for fibre functionalization or isolation of nanofibrillated cellulose. Therefore, the bleaching methods were not optimized towards this parameter. The alkaline hydrogen peroxide bleached hulls were, however, also tested for their suitability as fibre supplement in a food product, i.e. cookies, regarding their physical and sensory characteristics [16]. A very high acceptance level of 91 % among consumers was reached, indicating the suitability of utilizing alkaline hydrogen peroxide as bleaching agent.

The present study assesses the chemical and biochemical bleachability, including sonication, of oat hulls and evaluates the changes imposed by the developed process on their fibre composition. The main objective is to find a suitable bleaching method yielding good optical properties for use in the food industry for the production of white fibre supplements.

2. Materials and methods

2.1. Raw materials, enzymes and chemicals

Four different oat hull batches were supplied by Lantmännen ek. för. All batches were a mixture of Kerstin and Galant varieties grown in central Sweden (Mälaren Valley, with minorities coming from Östergötland (English exonym East Gothland) and Västergötland (English exonym West Gothland)) in different years. All seeds were obtained from SW-Seed (Sweden). As the chemical composition of oat hulls can vary considerably depending on the environmental conditions during the growth season [1], the darkness of the batches differed visibly. A more detailed

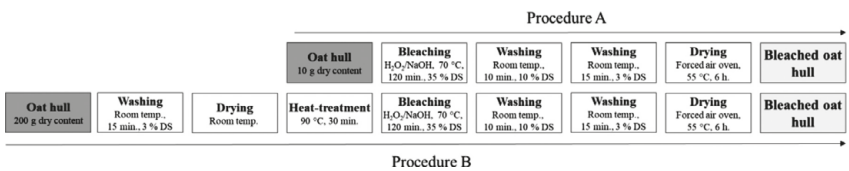

Fig. 1. Schematic illustration of the bleaching procedures used in this work. The abbreviation DS stands for dry solids content.

description of the batches is given in Tables 1 and 2. The hulls were industrially separated from the grains utilizing a Bühler BSSA stratopact HKE50HP-Ex peeler (Höflinger Millingsystems) and milled for the biochemical bleaching study using an industrial size hammer mill with a milling capacity of 1 t/h located at the Lantmännen factory in Järna.

The enzyme α-amylase from *Bacillus licheniformis* (type XII-A) was obtained from Sigma-Aldrich for starch removal. For biochemical bleaching, the laccase NS51003 and the glycoside hydrolase family 11 xylanase Pentopan Mono BG from Novozymes were used. For the chemical bleaching, hydrogen peroxide (Eka HP C59, 59 %, Nouryon) and sodium hydroxide (NaOH, 1 M, Scharlau) were used.

2.2. Chemical bleaching

Two different bleaching procedures have been used in this study (see Fig. 1). For the determination of the optimal amount of sodium hydroxide required to reach the highest brightness and L^* values at each H_2O_2 loading (Swe16–Swe19), for production of a larger amount of bleached material to be milled (Swe17), as well as for the chemical bleaching stage following biochemical bleaching (Swe18, Swe19), the following procedure was used (Procedure A): Hydrogen peroxide (50–150 kg/bone dry ton (bdt)), sodium hydroxide (5–15 kg/bdt) and deionized water were mixed into an oat hull sample (10 g dry weight) by kneading where after the sample (35 % dry solids content) was transferred into a plastic bag that was immersed into a water bath (70 °C) and incubated for 120 min. The chemical loadings and bleaching conditions were chosen based on previous experience (bleaching of wheat bran, unpublished results). The bleaching was stopped by diluting the oat hull sample to a dry solids content of 10 % with deionized water. After 10 min (room temperature), the suspension was dewatered on a Büchner funnel (containing a supporting wire, Monodur, 112 μm) generating a solid fraction and a filtrate (Filtrate I). The bleached oat hull was then washed (3 % dry solids content, 15 min, room temperature) with deionized water, the suspension was dewatered on a Büchner funnel (containing a supporting wire, Monodur, 112 μm) and the solid fraction dried in a forced air oven (Husqvarna Type QW100D) at 55 °C for six hours. The pH and

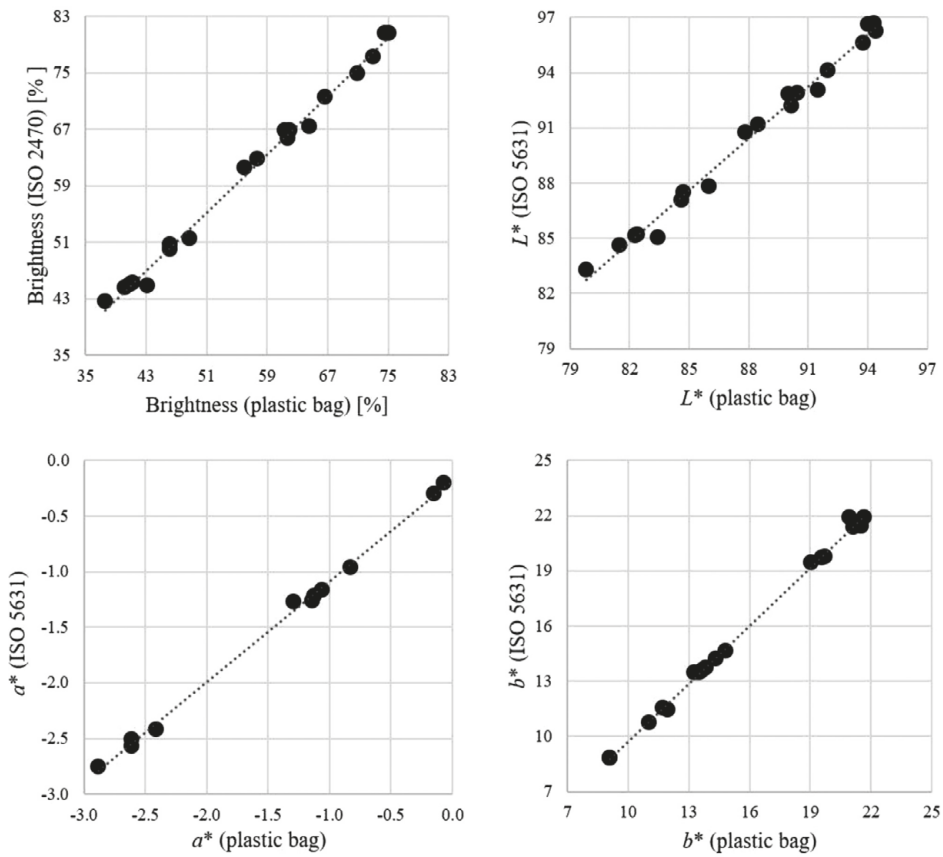

Fig. 2. Optical properties (brightness, L^*, a^* and b^*) of wood pulp pad samples produced from unbleached and bleached softwood thermomechanical and kraft pulps measured according to standard procedures (ISO 2470, ISO 5631) and for the corresponding pulp pad samples placed in a plastic bag (the method used in this work).

residual H_2O_2 content (iodometric titration using 0.05 M sodium thiosulphate from Merck) were determined by analysing Filtrate I. For Swe19, the oat hulls were heat treated (90 °C, 30 min) prior to bleaching to denature enzymes that caused hydrogen peroxide decomposition.

A larger amount of bleached oat hull (Swe19) was prepared in a modified procedure where the bleached oat hull was used for studying the chemical changes that occur during bleaching as well as for evaluating the effect of milling (Procedure B). The following modifications were made to the procedure described above. An unbleached and unmilled oat hull sample (200 g dry weight) was washed in deionized water (3 % dry solids content, 15 min, room temperature) whereafter the suspension was dewatered on a Büchner funnel (containing a supporting wire, Monodur, 112 μm) and the solid fraction dried in room temperature for two days. The dried oat hull was then subjected to a heat treatment (90 °C, 30 min) after which the bleaching procedure was continued as described above. The modifications were done to have a more robust process for industrial implementation, e.g. to be able to handle oat hulls that have been stored for various periods of time and to have a more uniform starting material with low or limited enzymatic activity.

2.3. Biochemical bleaching

The bleaching potential of laccase (NS51003), xylanase (Pentopan Mono BG) and sonication on oat hulls was assessed. To establish a suitable protocol for the experiments involving the laccase, the influence of mediator use, oxygen sparging and drying were investigated in a pre-study. All pre-study experiments were carried out on the milled and destarched Swe18 batch. The starch was removed with α-amylase according to the protocol by Sajib and colleagues [17]. Milling before treatment was a necessary process step in order to increase the surface area available for enzymatic attack.

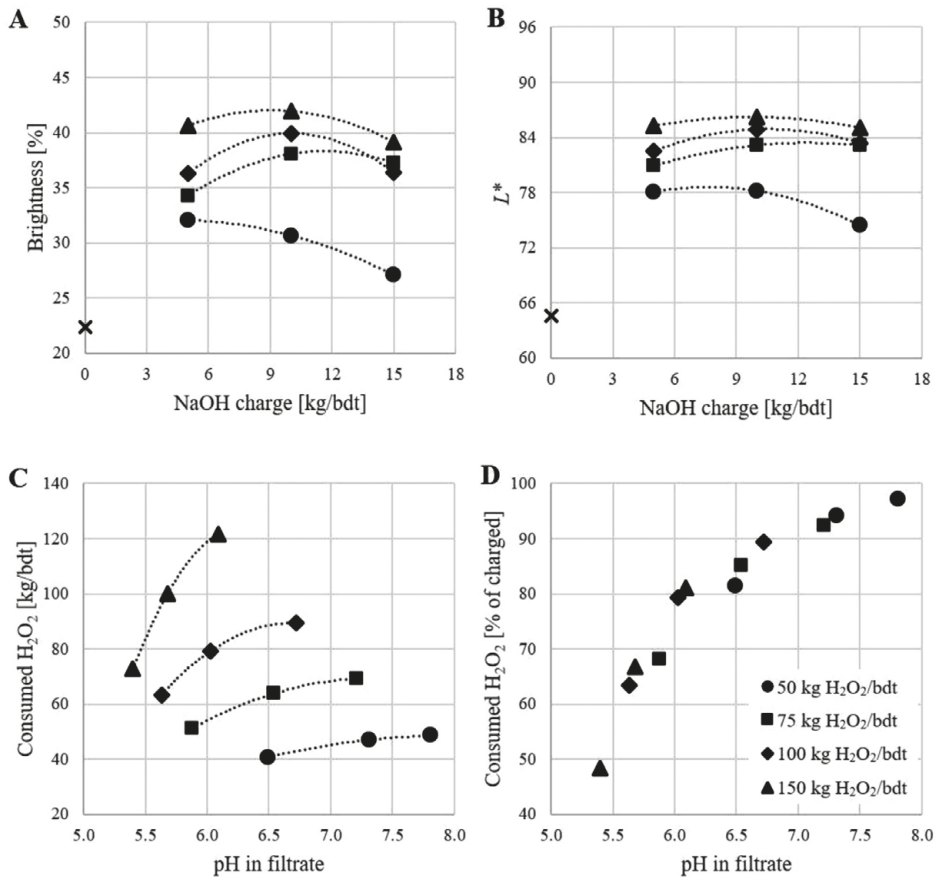

Fig. 3. Optimization of alkali loading. The optical properties brightness (A) and L^* (B) for unbleached (symbol x) and hydrogen peroxide bleached (filled symbols) unmilled oat hulls (Swe17) as a function of alkali loading. The amount of consumed hydrogen peroxide during bleaching of the hulls is reported as a function of the end pH in the filtrate (C and D).

To assess the influence of a mediator on oat hull bleachability, 615 U laccase/g oat hulls (dry weight) were incubated with 0.5, 1 and 3 mM 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS; Sigma-Aldrich) at 45 °C for 24 h under constant shaking in a shaking incubator. The reaction was carried out in 0.1 M sodium acetate buffer at pH 4.5 with a 10 % (w/v) solid loading. Subsequently, the samples were centrifuged at 3893 *g* for 20 min and the supernatants discarded. Two cycles of washing with MilliQ water and subsequent centrifuge separation were carried out.

To assess the influence of increased oxygen pressure on oat hull bleachability with laccase, 12 U laccase/g oat hulls (dry weight) were incubated according to the same procedure as above with the exception that air was sparged into the reaction medium for 10 min at the start of the reaction and 4 h later. The washed hulls were dried overnight in a 60 °C oven or freeze dried in a LyphLock 12 lyophilizer (Labconco). Subsequently, the hulls were bleached chemically according to Procedure A described above utilizing chemical loadings of 145 kg/bdt H_2O_2 and 10 kg/bdt NaOH.

In order to determine the best conditions for bleaching oat hulls with laccase, xylanase and sonication, a design of experiment study was carried out. The study was planned and statistically analysed utilizing the software MODDE 12.1 (Sartorius Stedim Data Analytics AB) based on an orthogonal (balanced) full factorial design. A detailed description of the variables used is displayed in Table 3. The hulls used for this study were industrially milled Swe19 hulls. In contrast to the oat hulls in the pre-study, the Swe19 hulls were not destarched as their starch content was lower compared to Swe18 hulls (see Table 1) and should therefore not inhibit the enzymatic reactions. The bleaching reactions were carried out successively starting with sonication in Milli-Q water, followed by xylanase treatment in 20 mM sodium phosphate buffer at pH 6.9 and laccase treatment in 0.1 M sodium acetate buffer at pH 4.5, all with a 10 % (w/v) solid loading. For sonication, a

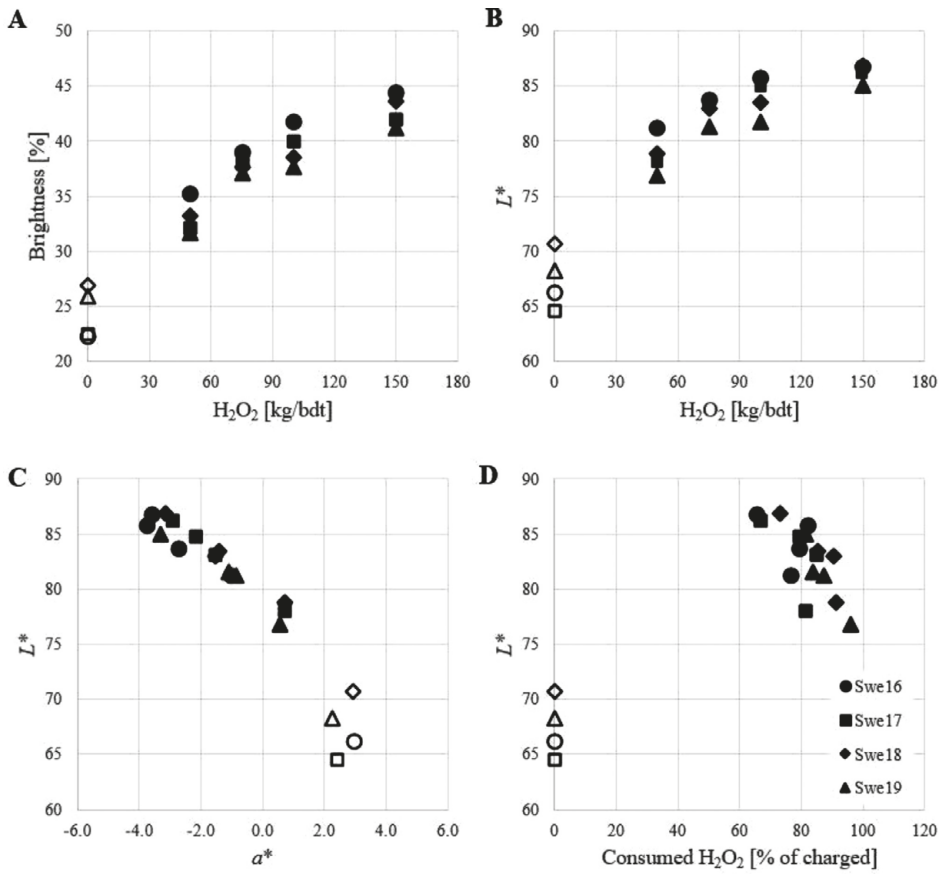

Fig. 4. Bleaching of oat hulls from different growth seasons. The optical properties brightness (A) and L^* (B) for unbleached (unfilled symbols) and hydrogen peroxide bleached (filled symbols) unmilled oat hulls (Swe16-Swe19) as a function of hydrogen peroxide loading at optimal alkali loading. The L^* values are further correlated to the respective a^* values (C) as well as the consumed hydrogen peroxide (D). The legend for all four graphs is given in D.

Labassco Sonorex RK100H with a frequency of 35 kHz (Bandelin) was used. Both enzymatic reactions were carried out for 24 h at 45 °C under constant shaking. In between treatments the hulls were washed twice with MilliQ water using centrifugation. The final pellets were freeze dried in a LyphLock 12 lyophilizer (Labconco). To assess how the treatment influenced the requirements for subsequently applied bleaching chemicals, the hulls were bleached chemically according to Procedure A described above utilizing 10 kg/bdt NaOH and either 50, 100 or 150 kg/bdt H_2O_2.

2.4. Optical properties analyses

The optical properties (C, d/0°) of the unbleached as well as bleached oat hulls were analysed using a Technidyne Color Touch X™ spectrophotometer. The dried oat hull samples were disintegrated in a coffee grinder (Krups Type 203B) for 20 s whereafter the gently mechanically treated (still coarse) oat hulls were placed into a plastic bag before determining the optical properties in the spectrophotometer. This method is not according to the standard ISO procedures (ISO 2470; ISO 5631) but was developed to be able to determine the optical properties of particulate matter. In order to determine the correlation between the used method and the standard ISO methods, optical properties of wood pulp pad samples produced from unbleached and bleached softwood thermomechanical and kraft pulps were analysed, using both standard and the modified procedures. The obtained brightness and CIE L^* (grey scale axis), a^* (green-red axis), and b^* (blue-yellow axis) values can be found in Fig. 2. The brightness and L^* values were lower when determined using the developed method compared to the ISO method whereas the a^* and b^* values were less affected. The decrease is about five units for brightness and two to three units for L^* when determining the optical properties of samples placed in a plastic bag, i.e. the optical

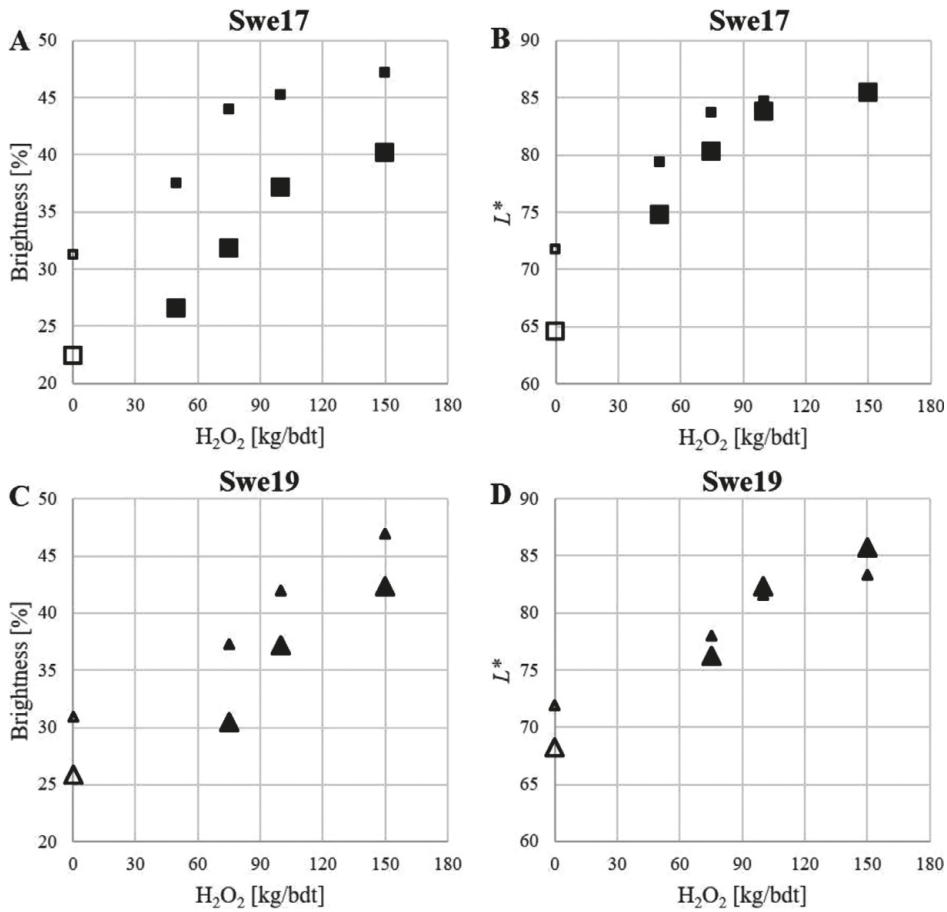

Fig. 5. Effects of milling on optical properties. The optical properties brightness (A and C) and L^* (B and D) of unbleached (unfilled symbols) and hydrogen peroxide bleached (filled symbols) Swe17 (A and B) and Swe19 (C and D) oat hulls before (large symbols) and after (small symbols) laboratory scale milling in a Perten 3100 mill equipped with a 0.8 mm sieve (Perkin Elmer).

properties of both unbleached and bleached oat hull samples were underestimated in this work.

2.5. Structural carbohydrates and lignin content analyses

The lignin, cellulose, hemicellulose and starch content of the 200 g scale unbleached and bleached samples was analysed. Before analysis, the whole hulls were milled in a coffee grinder for one cycle at grind size 8 (Model: BCG820BSSUK; Sage). The lignocellulose fraction was characterized according to the NREL Laboratory Analytical Procedure (NREL/TP-510-42618, 2012). The extracted monosaccharides were identified and quantified utilizing HPAEC-PAD (ICS-5000, Dionex, Thermo Scientific) equipped with a CarboPac PA20 analytical column (150 mm × 3 mm, 6 μm) as well as a respective guard column (30 mm × 3 mm) as previously described by Falck and colleagues [18]. The starch content was determined utilizing the "Total Starch" kit from Megazyme (https://www.megazyme.com/total-starch-assay-kit); method "a" of protocol K-TSTA 09/14 was followed.

2.6. Phenolic acid content analysis

The phenolic acids of the 200 g scale unbleached and bleached samples were extracted, separated and quantified according to the HPLC method described by Sajib and colleagues [17]. Before extraction, the whole hulls were milled in a coffee grinder for one cycle at grind size 8 (Model: BCG820BSSUK; Sage). The total phenolic acid content is reported as percentage based on the total phenolic acid content measured in an unbleached reference oat hull sample of the same batch.

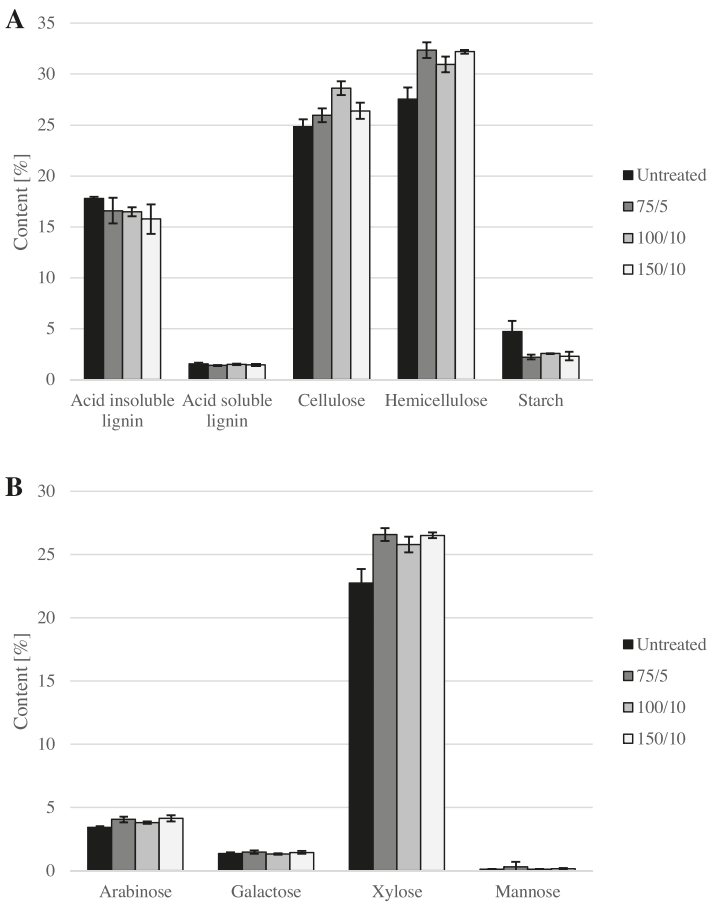

Fig. 6. Carbohydrate composition of untreated and bleached Swe19 oat hulls (A) and monosaccharide composition in hemicellulose fraction (B). Bleaching conditions were 75 kg H_2O_2/bdt and 5 kg NaOH/bdt (denoted as 75/5 in the graphs), 100 kg H_2O_2/bdt and 10 kg NaOH/bdt (100/10) and 150 kg H_2O_2/bdt and 10 kg NaOH/bdt (150/10). All analyses were performed in triplicates.

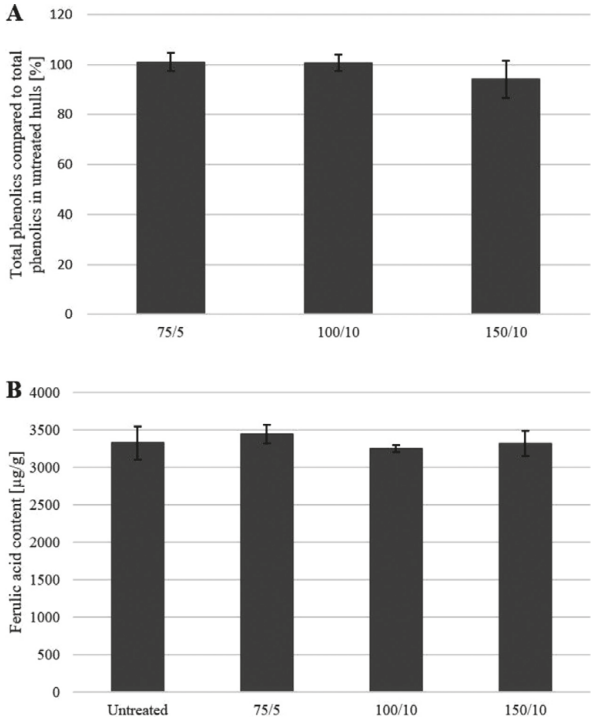

Fig. 7. Total phenolics in bleached samples (Swe19) compared to untreated hulls (A) and total ferulic acid content in untreated and bleached hulls (B). The total phenolics content is reported as percentage based on the total phenolics content measured in an unbleached reference oat hull sample of the same batch. Bleaching conditions were 75 kg H_2O_2/bdt and 5 kg NaOH/bdt (denoted as 75/5 in the graphs), 100 kg H_2O_2/bdt and 10 kg NaOH/bdt (100/10) and 150 kg H_2O_2/bdt and 10 kg NaOH/bdt (150/10). All analyses were performed in triplicates.

2.7. Wiesner test

The presence of coniferaldehyde structures in the untreated as well as bleached oat hulls was determined with the Wiesner test [19]. The Wiesner reagent was prepared by dissolving 200 mg phloroglucinol in 8 mL of 20 % ethanol. Subsequently, 2 mL of concentrated hydrochloric acid were added. An oat hull aliquot was immersed in the phloroglucinol–HCl stain for 1 min, after which it was air dried at room temperature overnight. Imaging was performed utilizing a Canon SLR camera.

2.8. Metal content analysis

For the detection and quantification of copper, iron and manganese, the oat hulls were first dissolved in nitric acid under wet combustion using a microwave system. Subsequently, the samples were analysed (in duplicate) via inductively coupled plasma optical emission spectroscopy using an iCap 7400 ICP-OES (Thermo Scientific). The report limits for the individual metals in µg/g were as follows: Cu: 0.1; Fe: 0.5; Mn: 0.1.

3. Results and discussion

3.1. Chemical bleaching

3.1.1. Optimization of alkali to hydrogen peroxide ratio

The chemical bleaching method investigated in this study was based on the combined action of hydrogen peroxide (H_2O_2) and sodium hydroxide (NaOH). One stage alkaline hydrogen peroxide bleaching was chosen due to its simplicity and previous experience from bleaching of wheat bran (unpublished results). For each hydrogen peroxide loading there is an optimal alkali loading that gives the highest brightening effect and this loading is influenced by the material to be bleached (e.g. transition metal ion content, amount of acidic groups) and the bleaching conditions employed (time, temperature, dry solids content). Further, the bleaching in this part of the study was performed on unmilled, not destarched oat hulls (Swe16–Swe19) since this makes such a procedure easier to implement in industrial scale (less process steps, ease of dewatering). The prerequisite is that the oat hulls are homogenously bleached, since the dried and bleached hulls are to be milled

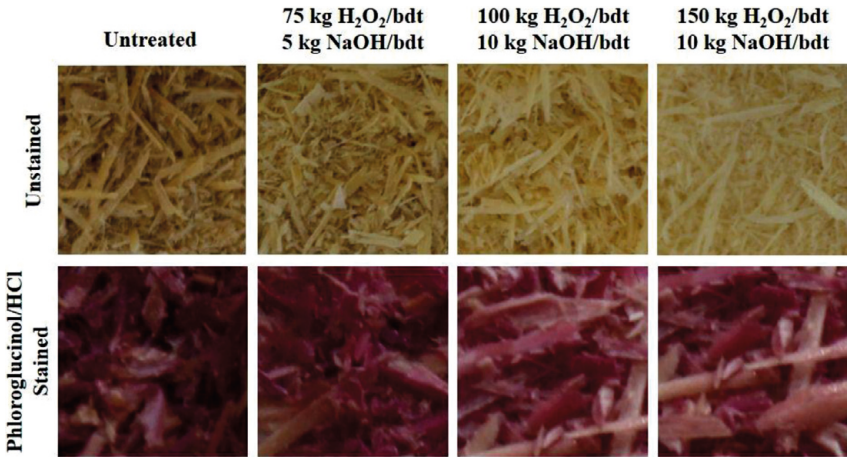

Fig. 8. Visual appearance of the untreated and bleached oat hulls at different concentrations of bleaching chemicals before and after staining with phloroglucinol–HCl solution. The pink colour indicates the presence of coniferaldehyde structures.

before mixing into the end products. Two of the oat hull batches (Swe17, Swe19) were therefore milled to a flour after bleaching and the optical properties were determined both before and after milling (see Section 3.1.3).

The bleachability of Swe17 is used to exemplify the importance of optimizing the alkali loading when bleaching with H_2O_2. Fig. 3 shows the brightness and L^* values for Swe17 at four different H_2O_2 loadings (50, 75, 100 and 150 kg/bdt) as a function of the alkali loading. The optimal NaOH loading was found to be 5 kg/bdt for 50 kg H_2O_2/bdt and 10 kg NaOH/bdt for the higher hydrogen peroxide loadings. About 65–85 % of the loaded H_2O_2 was consumed (higher consumption at the lower H_2O_2 loadings) and an increase of up to about 20 and 22 units for brightness and L^*, respectively, was possible to achieve. The optical properties were underestimated with the procedure employed in this work (see Materials and methods); the brightness should be about five units higher and the L^* value 2–3 units higher than measured. The total yield loss during the bleaching and washing stages was found to be less than 10 %.

3.1.2. Effect of annual variations of oat hulls on bleaching conditions

Fig. 4 shows the optical properties for the four unmilled oat hull batches with different growth years (2016–2019) at the optimal alkali loading when bleached with 50–150 kg H_2O_2/bdt. The optimal NaOH loading was 5 kg/bdt when charging 50 kg H_2O_2/bdt for Swe16-Swe19 and 5–15 kg/bdt for the rest of the H_2O_2 loadings (depending on the oat hull batch). Despite the rather large variation in unbleached optical properties (see Table 2) and chemical composition (see Table 1 and reference [1]), the bleaching response was rather similar with a possibility to reach a L^* value of above 85 before milling, which corresponds to an 23–33 % increase. Besides increasing the brightness and L^* value (lightness), H_2O_2 bleaching decreased the a^* value (greener) and increased the b^* value (more yellow). A previous study on micronized oat hulls reported a similar behaviour for the colour coordinates upon alkaline hydrogen peroxide extrusion [12]. However, the analysis was performed on sheets containing only 10 % oat hulls. Therefore, these results are not directly comparable. The hydrogen peroxide consumption in the present study was higher for Swe18 and Swe19 (75-95 % of loading at optimal alkali loading) compared to Swe16 and Swe17 (65–85 %) which points to a higher H_2O_2 decomposition (less H_2O_2 available for bleaching) in the former case. The optical properties (especially the L^* value) were also lower for Swe18 and Swe19. Hydrogen peroxide decomposition is catalysed by transition metals (especially manganese) but can also be caused by the presence of enzymes (catalase) [20]. The manganese content was, however, similar for Swe16-Swe19 whereas the iron content was higher for Swe19

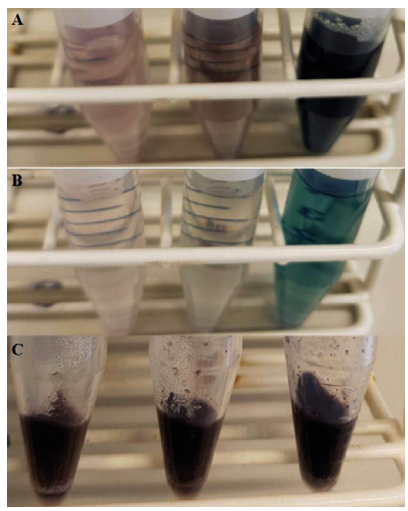

Fig. 9. Colour of oat hulls (Swe18) treated with laccase and 0.5 mM, 1 mM and 3 mM ABTS (from left to right) at different wash stages: supernatant after the first water wash cycle (A), supernatant after the second wash cycle (B) and laccase-ABTS treated oat hulls after the second wash cycle (C).

Table 4
The optical properties colour coordinates (L^*, a^*, b^*) and brightness of untreated, laccase treated and laccase treated with air sparging, destarched and milled oat hulls (Swe18) followed by chemical bleaching and freeze drying. The conditions for chemical bleaching were 145 kg/bdt H_2O_2 and 10 kg/bdt NaOH at 70 °C, 120 min and a dry solids content of 35 %.

Sample	L^*	a^*	b^*	Brightness [%]
Untreated + H_2O_2 bleached	84.8	−3.2	29.8	38.6
Laccase treated + H_2O_2 bleached	83.9	−3.2	29.7	37.3
Laccase treated with air sparging + H_2O_2 bleached	84.3	−3.2	29.5	38.0

Table 5
The optical properties colour coordinates (L^*, a^*, b^*) and brightness of untreated, oven dried as well as freeze dried destarched and milled oat hulls (Swe18). All samples were chemically bleached with the following conditions: 145 kg/bdt H_2O_2 and 10 kg/bdt NaOH at 70 °C, 120 min and a dry solids content of 35 %.

Sample	L^*	a^*	b^*	Brightness [%]
Untreated + H_2O_2 bleached	85.9	−2.5	31.3	38.9
Oven dried + H_2O_2 bleached	62.2	2.4	19.9	19.3
Freeze dried + H_2O_2 bleached	83.9	1.6	27.3	39.1

(Table 1). A heat treatment to deactivate/inhibit enzymatic activity prior to H_2O_2 bleaching could improve the robustness of the proposed bleaching process. Removing transition metals using chelating agents (e.g. EDTA) could also be considered even though previous experience with similar materials (wheat bran, unpublished results) and transition metal contents have shown a limited effect of such a treatment.

3.1.3. Effect of milling on optical properties

The H_2O_2 bleaching described above was performed on unmilled oat hulls. Milling releases new surfaces, hence a reduction of the optical properties would be expected, if the hulls were only bleached on the surface. However, the opposite was observed (see Fig. 5), which implies that the entire material was bleached. The improvement of the optical properties compared to the unmilled hulls is likely to be an effect of an increased light scattering ability. There was, however, a difference between Swe17 and Swe19 oat hull batches where the brightness and L^* values increased less for Swe19. It is likely that the two bleaching procedures used could explain this; the mixing of bleaching chemicals into Swe19 (200 g batch) was less effective and it is likely that the bleaching was not as homogeneous as for Swe17 (multiple 10 g batches). Further, the laboratory milling introduced iron (from 18 to 99 μg/g for Swe17 and from 24 to 210 μg/g for Swe19) which could influence the optical properties negatively if coloured iron complexes (with e.g. phenolic compounds) are formed. The increase in brightness was 7–12 and 5–7 units for Swe17 and Swe19, respectively. The increase in the L^* value varied but was highest for unbleached and low bleached hulls (Swe17).

3.1.4. Fibre content and composition

As the bleached material is intended to be used as a fibre supplement in food products, it is important to understand how hydrogen peroxide bleaching affects the fibre content and composition. Therefore, three larger batches of Swe19 hulls were bleached with varying chemical loadings (75 kg H_2O_2/bdt and 5 kg NaOH/bdt; 100 kg H_2O_2/bdt and 10 kg NaOH/bdt; 150 kg H_2O_2/bdt and 10 kg NaOH/bdt; Procedure B). As shown in Fig. 6A, no significant differences in the carbohydrate content were found for untreated and bleached samples except for the starch content. Furthermore, the hemicellulose composition, which is mainly arabinoxylan (polymer consisting of mainly arabinose and xylose monomers), is not substantially affected either (see Fig. 6B).

Additionally, the amount of fibres found in the filtrate after bleaching (Filtrate I, see section 2.2) was analysed. Only very small amounts of fibres were found (0.02 % of treated hull fraction), supporting the finding that only minor amounts of fibres are lost during bleaching.

3.1.5. Phenolics content

Phenolics as well as lignin have been considered to significantly contribute to colour formation in biomass [5,21]. As the lignin composition was not affected to any large extent during bleaching and there is an exceptionally high presence of phenolic acids in oat hulls [1,22], the observed colour change might be due to a change in the phenolic acid composition. Therefore, the total phenolics content in the untreated and bleached Swe19 samples was analysed. As shown in Fig. 7, no difference in the phenolic acid content or composition (exemplified with ferulic acid content) was observed, proving that the low molecular weight phenolics are not affected by hydrogen peroxide bleaching. As neither the lignin is removed nor the phenolics composition changed, bleaching must

Table 6
Factor settings and L^* results for different H_2O_2 loadings of the design of experiment study using milled oat hulls (Swe19). The NaOH loading was 10 kg/bdt for all H_2O_2 loadings.

Sample	Sonication length [min]	Laccase concentration [U/g]	Xylanase concentration [U/g]	L^* at H_2O_2, 50 [kg/bdt]	L^* at H_2O_2, 100 [kg/bdt]	L^* at H_2O_2, 150 [kg/bdt]
Ref[1]	–	–	–	78.1	82.5	86.9
1	10	6	5	79.3	83.1	84.0
2	10	18	5	79.5	82.8	83.8
3	10	6	15	79.3	82.0	84.1
4	10	18	15	78.3	82.1	83.9
5	60	6	5	79.9	82.6	83.8
6	60	18	5	79.3	83.1	83.6
7	60	6	15	77.3	82.7	83.9
8	60	18	15	77.5	82.0	83.9
9	35	12	10	79.3	82.6	83.8
10	35	12	10	77.8	83.1	83.8
11	35	12	10	78.6	82.5	84.3

[1] Milled oat hulls (Swe19) that were heat treated (90 °C, 30 min) prior to H_2O_2 bleaching.

occur via a lignin-retaining mechanism. Under such conditions, phenolic structures, especially the present hydroxyphenyl type phenolics, have been shown to be very stable [23–25].

Other strongly coloured species common in wood lignin are coniferaldehyde structures. Those are bound to the ends of the lignin polymers and hence, their side chains can easily be removed by an oxidative cleavage with bleaching chemicals resulting in less coloured α-carbonyl and hydroquinone structures. Pan and colleagues [24] have demonstrated that the bleaching effect of alkaline hydrogen peroxide on spruce thermomechanical pulp is mainly attributed to the removal of these structures. In order to analyse if these structures are also present in oat hull lignin and removed upon alkaline hydrogen peroxide bleaching, the Wiesner test was performed on unbleached and bleached oat hull samples. The Wiesner test involves a specific colour reaction of a phloroglucinol–HCl solution with coniferaldehyde structures. The dark pink colour of the unbleached oat hulls confirms the presence of coniferaldehydes (see Fig. 8). Alkaline hydrogen peroxide bleaching does lead to a reduction in the intensity of the pink colour indicating that some coniferaldehyde structures are removed. However, the strongest hydrogen peroxide bleached hulls are still stained by the Wiesner reagent showing that not all structures are removed. This is most likely due to the inaccessibility of those groups within the hull's structure. Pan and colleagues [24] have shown that for a complete removal of all coniferaldehydes in spruce thermomechanical pulp, prior isolation of the lignin is required. Fig. 8 furthermore shows that the overall bleaching of the oat hulls seems to be more profound than the removal of coniferaldehyde structures. This suggests that other coloured species, potentially quinones, are removed upon bleaching.

These data show that chemical bleaching with alkaline hydrogen peroxide is a very suitable method for bleaching oat hulls intended for the use as fibre supplement in food products as a good brightness and lightness is reached, while the fibre composition is not substantially affected.

3.2. Biochemical/chemical bleaching of milled oat hulls

There are many reports in the literature suggesting the use of enzymes alone or in combination with chemicals for biomass bleaching [5,7,8]. Of particular interest for oat hull bleaching are laccases and xylanases in combination with sonication, as those have been shown to increase brightness in combination with chemical bleaching of wheat and rice straw, which have a similar chemical content compared to oat hulls [7]. In Ziaie-Shirkolaee et al. [7], an increase of up to 6.5 brightness points was obtained, which led to a possible reduction of the used bleaching chemical chlorine dioxide of up to 25 %. In order to successfully analyse the influence and interaction of the laccase, xylanase and sonication on the bleachability of oat hulls in a design of experiment study, a pre-study with laccase only was performed which aimed at determining the best framework conditions for the reaction.

3.2.1. Laccase including treatments

Laccases have been shown to bleach better in presence of a mediator [5,8]. A commonly used one is 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS). However, the reaction of laccase and ABTS stained the hulls blue and it was not possible to wash (H_2O) away the colour, even after two cycles (see Fig. 9). Therefore, ABTS is unsuitable for the purpose of bleaching oat hulls and no mediator was used in the following trials.

Another variable that could limit laccase activity is the availability of oxygen in the reaction medium. In order to assess the influence of this variable, the laccase reaction was performed with and without air sparging. The results show no difference in optical properties after a subsequent H_2O_2 bleaching stage (see Table 4). Hence, oxygen presence does not seem to be a limiting factor.

While assessing the importance of air sparging, another important factor influencing hull colour was noted. When the hulls were oven dried (60 °C, overnight) after the enzymatic treatment, they became darker than the untreated ones. The formation of darker, insoluble compounds when oat hulls are subjected to higher temperatures has been observed before by Cardoso et al. [11], who suspected Maillard reactions and caramelization to be the cause. This darkening could not be reversed by subsequent chemical bleaching using H_2O_2/NaOH in the present study (see Table 5). Therefore, alternative freeze drying was tested. This drying method did not influence the hull colour and was therefore used for the trials described in Section 3.2.2.

3.2.2. Factorial design for enzymatic treatment and sonication

Based on the results of the pre-study, a design of experiment study was executed with varying concentrations of laccase and xylanase as well as sonication length. All treatments had a similar bleaching effect modifying the L^* value slightly by -0.8 to 1.8 or -0.5 to 0.6 units for 50 or 100 kg H_2O_2/bdt, respectively (see Table 6). The best result was achieved for sample 5 as it shows the highest L^* value at the lowest hydrogen peroxide loading. This sample has a long sonication time and low enzyme concentrations, indicating that sonication might be more important than enzymatic treatment. However, this difference is insignificant compared to only chemical bleaching. Furthermore, the achieved savings in bleaching chemical consumption are rather small. Considering that several extra processing steps are required for the enzymatic treatment including a milling and dewatering stage, this process would not be feasible on industrial scale. Therefore, it can be concluded that, using the enzymes selected here, the enzymatic treatments previously shown to be effective on other lignocellulosic materials, did not have the capability to bleach oat hulls. This is most likely due to the difference in recalcitrance of the different materials. The chemical composition of oat hulls suggests many ferulic acid ester linkages cross-linking the hemicellulose polymers to each other as well as to lignin [1]. This tight packing makes the material rather inaccessible to enzymatic attack. The lignocellulosic material previously bleached by biochemical methods was kraft pulp [7,8] and has hence been pre-treated with sodium sulphide and sodium hydroxide at high temperatures. This process leads to a dissolution and degradation of lignin making the hemicellulose more accessible to enzymatic attack [20].

4. Conclusion

Chemical bleaching with hydrogen peroxide and sodium hydroxide is a suitable and robust method for bleaching of oat hulls intended to be used as dietary fibre supplements. Oat hull batch variability does not influence the required conditions for the desired optical properties very much despite variations in unbleached optical properties and chemical composition. Unmilled oat hulls were successfully bleached on the inside as well as outside, making it possible to simplify industrial processing by adding a milling step after the bleaching stage. Additionally, this type of chemical bleaching seems to be rather mild as the overall chemical composition was not substantially affected. Alkaline hydrogen peroxide is therefore an ideal bleaching option for retaining the oat hull composition that makes the bleached oat hull suitable as food ingredient.

In contrast, biochemical bleaching utilizing a xylanase, a laccase and sonication only had a minor positive effect on the L^* value. A possible reduction of bleaching chemicals in a subsequent

chemical bleaching step is, based on the current results, judged to be too small to be industrially feasible considering the extra processing steps required for biochemical bleaching. To implement this methodology in industry, more enzyme candidates should be tested. Elucidation of exactly which chemical structures are removed or modified in the chemical bleaching process is an interesting topic for future research. This could also give hints on which enzymes might give bleaching effects on oat hulls and similar materials.

Author contributions

Contributor Role	Authors
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Data Curation	/
Formal Analysis	Eva S, Juanita, Katarina, Magnus
Funding Acquisition	Eva NK, Patrick, Magnus
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Methodology	Eva S, Juanita, Katharina, Magnus
Project Administration	Eva NK, Patrick, Magnus
Resources	/
Software	/
Supervision	Eva NK, Patrick, Magnus
Validation	Eva S, Juanita, Katharina, Magnus
Visualization	Eva S, Magnus
Roles/Writing – Original Draft	Eva S, Magnus
Writing – Review & Editing	All

Role of funding source

This work was supported by the Swedish Governmental Agency for Innovation Systems (VINNOVA) [grant number 2017-02713] within the strategic innovation program BioInnovation, jointly organised by VINNOVA, The Swedish Research Council for Enviroment, Agricultural Sciences and Spatial Planning (Formas) and the Swedish Energy Agency. The award was received by ENK and PA. The funders had no role in study design, data collection, analysis and interpretation, decision to publish, or preparation of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Authors Magnus Paulsson and Katarina Gutke were employed by the company Nouryon. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Acknowledgements

The authors thank Patrik Johansson, Nouryon, for his skilful technical assistance in hydrogen peroxide bleaching of untreated and pre-treated oat hulls. The authors are furthermore grateful to CG Pettersson, Lantmännen, for providing the raw material and milling the bleached oat hulls.

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Paper III

Waste and Biomass Valorization
https://doi.org/10.1007/s12649-021-01406-0

ORIGINAL PAPER

Ultrasound Assisted Alkaline Pre-treatment Efficiently Solubilises Hemicellulose from Oat Hulls

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Received: 15 January 2021 / Accepted: 19 February 2021
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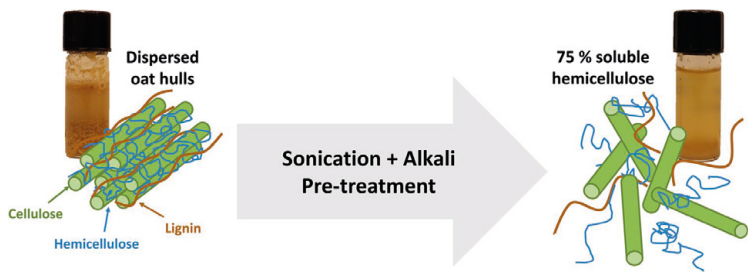
Abstract
The establishment of sustainable bioeconomies requires the utilization of new renewable biomaterials. One such material currently seen as a waste product is oat hulls. Oat hulls exhibit a great potential for the production of dietary fibres due to their exceptionally large hemicellulose content (35%). Their recalcitrant structure however requires a suitable pre-treatment method to access and process the hemicellulose. After a screening of various physical, chemical and physico-chemical pre-treatment methods, including autoclaving, ultrasonication, microwave-, deep eutectic solvents-, as well as alkaline treatments, a combined ultrasonication and alkali pre-treatment method was here found to be the most suitable. A factorial design resulted in optimized conditions of 10 min ultrasonication in water, followed by an incubation in 5 M NaOH at 80 ºC for 9 h yielding solubilisation of 72% of all hemicellulose in the hulls. The method was shown to efficiently break the ester bonds between ferulic acid and the hemicellulose main chain, contributing to its solubilisation.

Graphic Abstract



Keywords Oat hulls · Ultrasonication · Alkali pre-treatment · Hemicellulose solubilisation

Statement of Novelty

The successful transition of modern economies to sustainable bioeconomies requires the development of suitable methods for the utilization of renewable biomaterials. Oat hulls are currently an underutilized agricultural side stream with interesting chemical properties. Their exceptionally large hemicellulose content makes them great candidates for the production of health promoting food ingredients, such as dietary fibres. Due to the heavy crosslinking of its lignocellulose components, a suitable pre-treatment method is required. The in this study developed method yields a very high amount of soluble hemicellulose; higher than previously reported for oat hulls or other lignocellulosic material.

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Published online: 12 March 2021

Introduction

With a growing world population, an increase in food production quantities is required. Alongside the production of food, larger amounts of agricultural side streams are generated. To date, those are mainly underutilized and often regarded as waste leading to improper handling increasing the risk for serious environmental pollution [1]. Additionally, the trend towards establishing bioeconomies around the world is demanding smarter treatment solutions. One such agricultural side stream produced in large quantities is oat hulls, i.e. the shell protecting the edible oat grain. In 2018, 23 million tons of oat were produced worldwide [2]. As the hull makes up 25–35% of the entire grain [3], considerable amounts of oat hull waste have to be treated.

Oat hulls like many other agricultural side streams are mainly composed of lignocellulose [4]. Lignocellulose is a complex, inter-connected network of cellulose, hemicellulose and lignin. Cellulose is a linear polymer composed of β-1,4-linked D-glucose units arranged in microfibrils. Hemicellulose is heterogeneous and includes branched polymers arranged in random and amorphous structures. In oat hulls, hemicellulose is mainly xylan, which is composed of a xylose backbone with limited substituents of arabinose, galactose, uronic and phenolic acids [4]. Lignin is an amorphous organic polymer composed of phenylpropane units differing in methoxyl group substitutions on the aromatic rings [1, 5]. The exact composition of lignocellulose varies greatly depending on the biomass as well as growth conditions [4]. Oat hulls consist of approximately 23% cellulose, 35% hemicellulose and 25% lignin under regular growth conditions. The hemicellulose is mostly arabinoxylan with few side chain substitutions [4]. The three main components in lignocellulose are strongly interconnected via various types of linkages. Cellulose is mostly connected to hemicellulose via hydrogen bonds, while lignin is covalently attached to hemicellulose via phenolic acid ester-ether bridges [5]. This complex structural organization alongside with its heterogeneous chemical content makes lignocellulose very resistant to chemical and biological decomposition, i.e. recalcitrant [1, 6]. Hence, efficient processing methods that are tailor-made for the respective biomass are required to overcome its recalcitrance and enable further use. These processing methods are typically referred to as pre-treatments.

Traditionally, pre-treatments were mostly explored with the aim of increasing the accessibility of cellulose for the production of biofuels [1, 5, 7]. Hence, they were evaluated on their efficacy to remove and break down hemicellulose and lignin. The societal pressure to move towards a stronger bioeconomy manufacturing various products from biomass as well as recent food trends demanding fibre-enriched food products require the valorization of not only the cellulose, but also the hemicellulose and lignin fractions. This requires a reassessment of the previously evaluated pre-treatment techniques. Oat hulls are exceptionally rich in hemicellulose (35% of total dry weight), which makes them an excellent starting material for the production of dietary fibres. In order to exert beneficial prebiotic effects in the gut, they need to be solubilized [8]. Therefore, the suitability of a variety of pre-treatment techniques for the solubilisation of hemicellulose was evaluated in this study.

The different pre-treatment types are often classified according to their mode of action. The main groups are physical, chemical, physico-chemical and biological techniques [1, 5, 7]. The physical pre-treatments assessed in this study are milling, microwaving and ultrasonication. Milling is commonly used in combination with other pre-treatments as it increases the surface area by reducing particle size, which allows greater accessibility for further modification [1]. The irradiation of lignocellulosic biomass with microwaves weakens its recalcitrance by generating heat as well as vibrations of polar molecules leading to substantial collisions in a uniform way throughout the entire sample [7]. Ultrasonication causes a cavitational destabilization of the lignocellulose matrix by both generating oxidizing radicals (sono-chemical effect) and altering the surface structure (mechano-acoustic effect) [7]. As chemical pre-treatment methods, the effect of alkali and deep eutectic solvents (DES) was investigated. When biomass is incubated in alkali reagents the cellulose tends to swell, breaking its crystalline structure, which generates a greater porosity and exposes a larger surface area. Additionally, ester linkages, and to a lower degree ether linkages, between hemicellulose and lignin are broken under alkaline conditions, which significantly increases their solubility and leads to a decrease in the degree of polymerization [5, 7]. In contrast, DES have also been shown to efficiently solubilize hemicellulose (xylan) by disrupting intermolecular hydrogen bonds between the polysaccharides, which leads to an overall structural destabilization [9]. They are eutectic mixtures of a hydrogen bond donor and a hydrogen bond acceptor with strong hydrogen bond interactions. Due to their structural resemblance to the more established ionic liquids (ILs), they are often classified as a new group of ILs. The physico-chemical pre-treatment techniques are often based on stability differences of the individual lignocellulose components when exposed to thermal or pressure stresses. In this study, autoclaving was used to induce hydrothermal stress. Hemicelluloses are known to have a lower thermal stability than lignin and cellulose. They can therefore be solubilized at certain elevated temperatures, while cellulose and lignin remain intact [5]. Biological pre-treatments are still in their infancy, however positive results have been achieved with a laccase, which

oxidizes the lignin and hence makes the other components more readily accessible [1]. No biological pre-treatments were assessed in this study as the focus was placed on non-degrading techniques aiming at only solubilizing the material. For the same reason, no acid pre-treatment was tested as chemical pre-treatment.

The objective of the present study was to find a suitable pre-treatment method and optimize its conditions for maximizing the solubilisation of hemicellulose in oat hulls. This processing step is crucial for the production of hemicellulose fibres with prebiotic potential. Implementing such an application in industry would support the shift towards establishing a bioeconomy.

Materials and Methods

Raw Materials and Chemicals

Two different oat hull batches supplied by Lantmännen ek. för. were used in this study. Both batches were a mixture of Kerstin and Galant varieties grown in central Sweden (Mälaren Valley, with minorities coming from Östergötland (English exonym East Gothland) and Västergötland (English exonym West Gothland)), but in different years: 2017 (Swe17) and 2019 (Swe19). All seeds were obtained from SW-Seed (Sweden). Due to the different environmental conditions during the growth phase, the hemicellulose content in the batches differed, being 35.1% in Swe17 and 29.6% in Swe19 [4, 10]. In both batches, the hulls were industrially separated from the grains utilizing a Bühler BSSA stratopact HKE50HP-Ex peeler (Höflinger Millingsystems) and milled with an industrial size hammer mill (milling capacity of 1 t/h) located at the Lantmännen facilities in Järna.

For starch removal, the enzyme α-amylase from *Bacillus licheniformis* (type XII-A) was obtained from Sigma-Aldrich. All chemicals were purchased from Merck (Sweden) unless otherwise specified.

Autoclave Pre-treatment

The suitability of using an autoclave pre-treatment for the solubilisation of oat hull hemicellulose was assessed on destarched and milled Swe17 oat hulls. Destarching was performed according to the protocol by Sajib and colleagues [11]. Subsequently, the hulls were dispersed in Milli-Q water at a ratio of 1:10 (w/v) and autoclaved at 121 ºC for 15 h (Inspecta, Thermo Scientific). After treatment, the liquid phase containing the solubilized material was separated from the solid phase via centrifugation at 3893×*g* for 20 min. The experiment was performed in six replicates.

Microwave Pre-treatment

For testing the microwave pre-treatment, 20 g of destarched and milled Swe17 oat hulls were dispersed in 160 mL Milli-Q water in a microwave beaker. The beaker was tightly closed and fixed in the sample holder of an Ethos PLUS 2 microwave (Milestone). The microwave was set to heat to 180 ºC in 10 min and hold the treatment temperature for 30 min. After microwaving, the liquid phase was collected by centrifugation at 3800×*g* for 30 min. The treatment was run in duplicate.

Ultrasonication Pre-treatment

Ultrasonication was tested for its suitability of solubilizing oat hull hemicellulose. Destarched and milled Swe19 oat hulls were dispersed in Milli-Q water at a ratio of 1:5 (w/v) and placed in a Labassco Sonorex RK100H ultrasonic bath (Bandelin) with a frequency of 35 kHz for 30 min. Subsequently, the liquid fraction was separated via centrifugation at 3893×*g* for 10 min. The experiment was performed in duplicate.

Deep Eutectic Solvents Pre-treatment

Two different deep eutectic solvents (DES) were tested for their capability in solubilizing hemicellulose in oat hulls: choline chloride:glycerol ([Chol]Cl:Gly) and choline chloride:ethylene glycol ([Chol]Cl:Ethyl) (molar ratio 1:2). The solvents were prepared by continuously stirring the reagents at 80 ºC in an oil bath until a homogenous clear liquid was formed. Destarched and milled Swe17 oat hulls were mixed with either of the DES at a ratio of 1:16 (w/v) and incubated at 115 ºC for 3 h under constant shaking. After treatment, the liquid and solid fractions were separated via centrifugation at 3893×*g* for 20 min. Both treatments were performed in triplicate.

Alkali Pre-treatment

The effect of alkali incubation on the solubilisation of hemicellulose in oat hulls was tested with both sodium hydroxide (NaOH) and potassium hydroxide (KOH). Destarched and milled Swe17 oat hulls were dispersed in 2 M of either base at a ratio of 1:5 (w/v) and incubated at 40 ºC for 16 h with continuous shaking. Subsequently, the liquid phase was separated from the solid phase via centrifugation at 3893×*g* for 20 min. The supernatant was neutralized with 37% hydrogen chloride to a final pH of 5–6. Both treatments were run in duplicate.

Combined Ultrasonication and Alkali Pre-treatment

The effect of combining ultrasonication and alkali pre-treatments was tested on destarched and milled Swe19 oat hulls. Five different types of treatments were performed in duplicate with a total treatment time of 2 h. In each treatment the oat hull to liquid ratio was 1:5 (w/v). In two treatments, the oat hulls were incubated at 40 ºC for 2 h under constant shaking in either Milli-Q water or 2 M NaOH (control 1 and 2). In another two treatments, the oat hulls were first sonicated in a Labassco Sonorex RK100H ultrasonic bath (Bandelin) with a frequency of 35 kHz for 30 min and then incubated at 40 ºC for 1.5 h while being immersed in either Milli-Q water or 2 M NaOH (control 3 and 4). In the final treatment, the oat hulls were first sonicated for 30 min in Milli-Q water, then the water was separated by centrifugation at 3893×*g* for 10 min and discarded. The remaining pellet was immersed in 2 M NaOH and incubated at 40 ºC for 1.5 h. The final liquid fraction after all treatments was isolated using centrifugation at 3893×*g* for 10 min.

Design of Experiment Studies

In order to determine the best conditions for extraction and solubilisation of hemicellulose from oat hulls, two design of experiment studies (DOEs) were carried out on milled Swe19 oat hulls. The studies were planned and statistically analysed with the software MODDE 12.1 (Sartorius Stedim Data Analytics AB) based on a reduced central composite face design (DOE 1) and a central composite orthogonal design (DOE 2). The individual experiments were carried out following the procedure for the final treatment type described in section 'Combined Ultrasonication and Alkali Pre-treatment', but the factors ultrasonication length, temperature, NaOH concentration and NaOH extraction time were varied. A summary of the factor settings used in both studies is given in Table 1.

Table 1 Factor settings for the design of experiment studies showing the minimum and maximum values for each factor, which enclose the design space. In DOE 2, the factors temperature and NaOH extraction time were kept constant at 80 ºC and 9 h, respectively

Factor	Unit	Minimum	Maximum
DOE 1			
Ultrasonication length	min	10	60
Temperature	ºC	20	80
NaOH concentration	M	0.5	5
NaOH extraction time	h	0.5	15
DOE 2			
Ultrasonication length	min	1	10
NaOH concentration	M	4	10

Soluble Hemicellulose Analysis

The solubilized fibres in the final liquid fractions of all pre-treatment types were precipitated by addition of four volumes of 99.5% ethanol and incubation at 4 ºC overnight. After precipitation, the ethanol was removed by centrifugation at 3893×*g* for 5 min and evaporation. The dry fibres were weighed for yield determination.

The extracted fibres of the DOE studies as well as the optimized treatment were further characterized for their hemicellulose content following the NREL Laboratory Analytical Procedure (NREL/TP-510-42618, 2012). Extracted monosaccharides were identified and quantified utilizing HPAEC-PAD (ICS-5000, Dionex, Thermo Scientific) equipped with a CarboPac PA20 analytical column (150 mm × 3 mm, 6 µm) as well as a respective guard column (30 mm × 3 mm) as previously described by Falck et al. [12]. The starch content of the extracted fibre samples was determined using the "Total Starch" kit from Megazyme. Protocol K TSTA 09/14 method a was followed.

The phenolic acid content of both the untreated Swe19 batch as well as the extracted fibres from the same batch were analysed according to the method described by Sajib et al. [11].

Results and Discussion

Screening of Pre-treatment Methods

For the extraction and solubilisation of hemicellulose from the oat hull lignocellulosic matrix, a variety of common physical, chemical and physico-chemical pre-treatment methods were tested. Milling was performed on all oat hull material prior to processing with the other pre-treatment types. To assess the potential and allow comparability of the very different methods, rather harsh conditions were chosen for all of them. Several reaction conditions for the microwave and deep eutectic solvents treatments were tested, however, only the best results are presented here. The amounts of extracted and solubilized material based on oat hull dry weight are shown in Fig. 1. It has to be noted that this extracted material was not further characterized regarding its chemical composition, meaning that it most likely contains a mixture of cellulose, hemicellulose and lignin. Under the investigated conditions, both types of alkali treatments by far solubilized the most oat hull material (29% by KOH and 24% by NaOH). All other pre-treatments resulted in yields lower than 5%. This demonstrates the great recalcitrance of oat hulls as much higher results could be achieved with other lignocellulosic materials using the same methods (see Table 2).

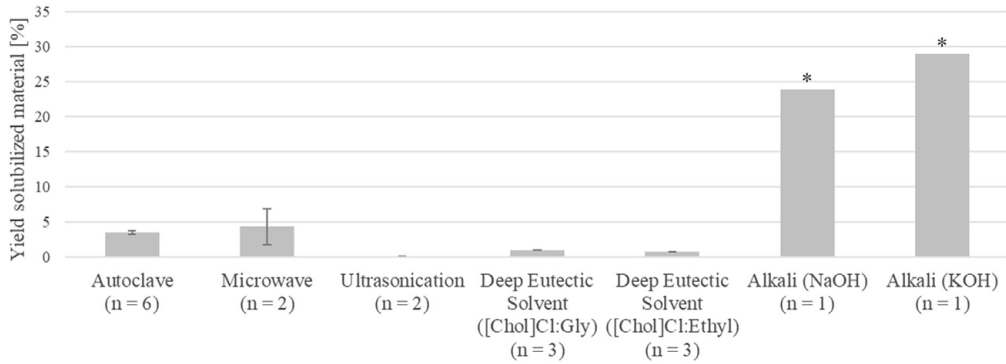

Fig. 1 Extraction results of various pre-treatments showing the amount of solubilized material as a fraction of the destarched and milled oat hulls in percent (dry weight solubilized material of total dry weight). The replicate numbers are reported as n. The two treatments, which were only performed once, are marked with an asterisk (*)

Table 2 Comparison of extraction results of the tested pre-treatment methods showing the amount of solubilized material (or fractions if further specified) from the destarched and milled oat hulls in this study and similar lignocellulosic materials in other studies. All numbers are given as percent

Pre-treatment method	Yield of solubilized material from oat hulls [%]	Yield of solubilized material from other lignocellulosic materials [%]	References
Autoclave	3.5	5.7 (brewer's spent grain) *17.2 of arabinoxylan fraction*	[11]
		54 (rye bran) *45 of arabinoxylan fraction*	[12]
Microwave	4.4	50.8 (wheat bran) *48 of arabinoxylan fraction*	[13]
		57 (brewer's spent grain) *of hemicellulose*	[14]
Ultrasonication	0.1	14.7 (corn bran) *of polysaccharides*	[15]
		5.1 (sugarcane bagasse)	[16]
Deep eutectic solvents	1.0 ([Chol]Cl:Gly) 0.8 ([Chol]Cl:Ethyl)	92 (E. globulus wood) *of xylan*	[9]
Alkali	23.8 (NaOH) 29.0 (KOH)	32.3 (sugarcane bagasse)	[17]
		67.2 (rice straw) *of hemicellulose* *88.5 with* H_2O_2	[18]

Autoclaving was not a successful approach to solubilise hemicellulose from the lignocellulose matrix, and was limited to conditions that did not degrade the hemicellulose which is the least thermostable polymer in the lignocellulose complex. Targeting of the hydrogen bond interactions by DES, did also not result in solubilisation. Thus, it seems that the breakage of covalent bonds binding hemicellulose into the oat hull matrix is crucial for hemicellulose solubilisation. To achieve this, three methods were included in the screening: ultrasonication, microwave treatment and alkaline treatment. Neither ultrasonication, nor microwave treatment led to significant solubilisation. The oxidizing radicals generated during ultrasonication and the collisions of polar molecules caused by microwave irradiation seem to not be sufficient enough to break these covalent bonds. Alkaline treatment was more successful, and has the advantage of both breaking ester and ether bonds connecting the hemicellulose to lignin as well as increasing porosity by cellulose swelling, which allows the reagents to penetrate the entire material. However, the alkaline conditions need to be rather harsh as a previously established alkaline hydrogen peroxide treatment method, utilized for other types of biomass materials [5], did not solubilize much material or alter the oat hull's lignocellulosic composition [10].

Combined Pre-treatments Using Ultrasonication and Alkali

The effectiveness of alkali pre-treatment has been reported to be significantly increased when combined with other types of pre-treatment [5]. One successful combination is the sequential application of ultrasonication followed by alkali treatment. On sugarcane bagasse, this combined treatment increased the yield as ultrasonication led to a mechanical disruption of the cell wall resulting in an increased accessibility of the material to alkali attack [16]. Therefore, the effect of combining ultrasonication with alkali pre-treatment for the solubilisation of oat hull hemicellulose was tested. Even though slightly higher values were obtained for the KOH treatment in the screening trial (Fig. 1), all further tests were performed with NaOH due to its easy availability for industrial scale treatments.

The results of this combined extraction are visualized in Fig. 2. Alongside the preferred sequential ultrasonication in water followed by incubation in NaOH, several alternatives (controls) were included in the study, involving varying the solvent during the ultrasonication or incubation step. It can be seen that incubation in water only (during 2 h) (control 1) as well as ultrasonication in water with subsequent incubation in water during a corresponding period of time (control 3) were not capable of solubilizing the oat hull hemicellulose. Incubation of the oat hulls in NaOH (control 2), on the other hand, did dissolve a notable fraction, despite use of a shorter incubation period (2 h) than in the screening (16 h, Fig. 1). However, this yield could not be increased substantially when the oat hulls were first sonicated in NaOH, followed by incubation in the same solvent (control 4). This stands in contrast to previous studies on wheat straw and corn bran, which showed positive effects on the hemicellulose extraction yield when sonicated in an alkali medium [15, 17]. The present study however demonstrates that the yield of extracted material was nearly tripled (combined extraction in Fig. 2) when the material was first ultrasonicated in water, followed by replacement of the water by alkali and incubation at the same temperature and time as used in control 4.

The cavitation events triggered by ultrasonication that lead to the degradation of biomass are strongly influenced by the solvent the material is dispersed in. One important solvent parameter influencing the number of triggered cavitation events is viscosity [19]. NaOH solutions have a higher viscosity than water, becoming more pronounced with increasing NaOH concentration. Ultrasonication in control 4 in the present study was performed in a more concentrated NaOH solution than in the study showing good hemicellulose extraction from corn bran [15]. Therefore, fewer cavitation events might have been generated in this study producing fewer radicals and less extreme high local temperature and pressure effects. It furthermore hints at a more recalcitrant lignocellulose structure in oat hulls compared to corn bran. Ultrasonication in water alone has been shown to mainly solubilize heavily substituted xylan from sugarcane bagasse due to the lower amount of possible interactions with cellulose [16]. As oat hull hemicellulose contains very few side chain substitutions, its solubilisation must require harsher conditions explaining why ultrasonication alone failed to solubilize much material. However, the results in Fig. 2 show that it must destabilize the material in a way that enables easier solubilisation by a subsequent alkali treatment.

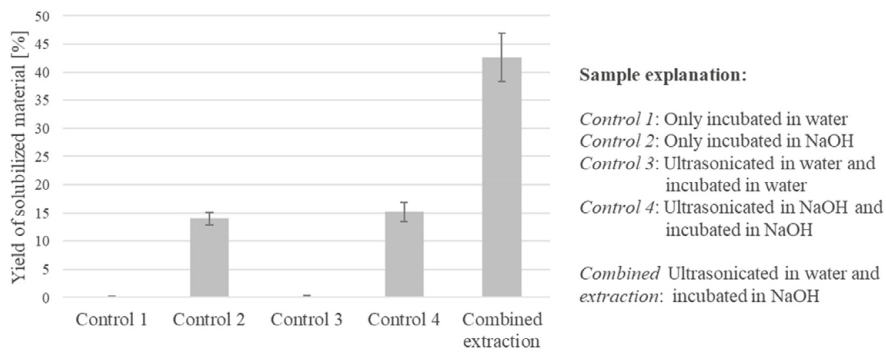

Fig. 2 Extraction results of the combined ultrasonication and alkali pre-treatment trial as well as four controls showing the amount of solubilized material as a fraction of the destarched and milled oat hulls in percent (n = 2). A short description of the treatment of all samples is given to the right of the graph

Pre-treatment Method Optimization

First Design of Experiment Study

Based on the promising results of the combined ultrasonication and alkali extraction screening study, a design of experiment study (DOE) was carried out to determine the optimal conditions for hemicellulose solubilisation. Contrary to the analysis in the screening studies, the extracted material was analysed regarding its lignocellulose composition and the yield numbers reported here only include the extracted hemicellulose fraction with respect to the whole oat hull sample. Additionally, the destarching step prior to extraction (used in the screening trials above) was omitted as the starch content in the sample was experimentally found to be low enough (12%) to not influence the extraction. Based on the screening trials, the factors selected to evaluate the influence on the extraction yield were ultrasonication length, NaOH concentration, NaOH incubation time and NaOH incubation temperature. A reduced central composite face design was determined to be the most suitable for analysis as it allows the analysis of second order terms (quadratic) for four factors while minimizing the necessary number of experiments. Following the suggestion by the modelling software MODDE, 23 extractions varying the factor values were carried out. The yield results from those extractions generated a model with high summary of fit parameters ($R2 = 0.87$; $Q2 = 0.76$; Model validity = 0.78; Reproducibility = 0.89), which indicate that the created model fits well to the experimental data. With the factor settings applied in the DOE 1 design space (Table 1), the model suggests that the ultrasonication length does not have an effect on hemicellulose solubilisation, indicating that the minimum of 10 min of ultrasonication might be sufficient for the effect seen in the screening test (see Fig. 3A). Both NaOH concentration and incubation temperature are very important, however the optima are lying outside the design space at the higher ends, suggesting that even higher concentrations and higher temperatures might yield better results. NaOH incubation time is also a very important factor and the optimum was found within the design space at 8.96 h. The optimizer function of the software suggested a maximum yield of 21.9% solubilized hemicellulose relative to the entire oat hull under the following conditions: 55 min of ultrasonication, 5 M NaOH concentration, 80 °C incubation temperature and 9 h incubation time. To verify the validity of the model, a triplicate experiment at exactly these conditions was carried out. The result was a yield of $21.3 \pm 1.3\%$, which is very close to the predicted value. Therefore, the model seems to be corresponding to reality very well.

As not only hemicellulose is solubilized during the treatment, but also lignin and cellulose, the DOE factors were also assessed for their influence on hemicellulose purity in the extracted material. As shown in Fig. 3B, the purity in general is rather low yielding a maximum of 0.22, i.e. 22%, at the highest NaOH concentration, lowest incubation temperature and shortest incubation time. At those conditions, the hemicellulose yield (4.8%) is very low. This suggests the presence of a small fraction of easily accessible, potentially more substituted, hemicellulose that can be solubilized at milder conditions. The major fraction, however, seems to be very strongly anchored to the lignocellulosic matrix requiring some degradation and solubilisation of lignin and cellulose for its liberation. As the main aim of the study is to maximize the amount of soluble hemicellulose, the following optimizations only regarded hemicellulose yield and not its purity.

Second Design of Experiment Study

In order to increase the design space and evaluate extractability of oat hull hemicellulose at shorter ultrasonication times and higher NaOH concentrations, a second DOE study was designed. Based on the results of the first DOE, the treatment time was fixed at the optimal 9 h of incubation and the incubation temperature at 80 °C as higher temperatures require specialized equipment and might not be suitable for large scale production. Ultrasonication times of 1 to 10 min and NaOH concentrations of 4–10 M were investigated in a central composite orthogonal design allowing the analysis of second order terms (quadratic) for the two factors in 11 experiments. The generated model fits well to the experimental data as shown by the high summary of fit parameters: $R2 = 0.96$; $Q2 = 0.86$; Model validity = 0.75; Reproducibility = 0.97. In this design space (DOE 2, Table 1), the extraction yield was very strongly dependent on ultrasonication time, which also had a significant interaction term with NaOH concentration. The model suggests that the ultrasonication time should be kept low at lower NaOH concentrations and high at higher NaOH concentrations (see Fig. 4). However, all results, even the optimum (19.1%), in this design space are below the optimum in the previous study. Therefore, it can be concluded that shorter ultrasonication times and higher NaOH concentrations do not increase the yield. The higher NaOH concentrations might also lead to a degradation of the polysaccharides instead of further solubilisation.

Final Method Adjustments and Analyses

The DOE studies suggest that the optimal conditions for hemicellulose solubilisation are incubation at 80 ºC with 5 M NaOH for 9 h. However, it remains unclear, if a shorter ultrasonication time of 10 min yields as much as the predicted best setting at 55 min. Therefore, a final study was performed in which only the ultrasonication length was

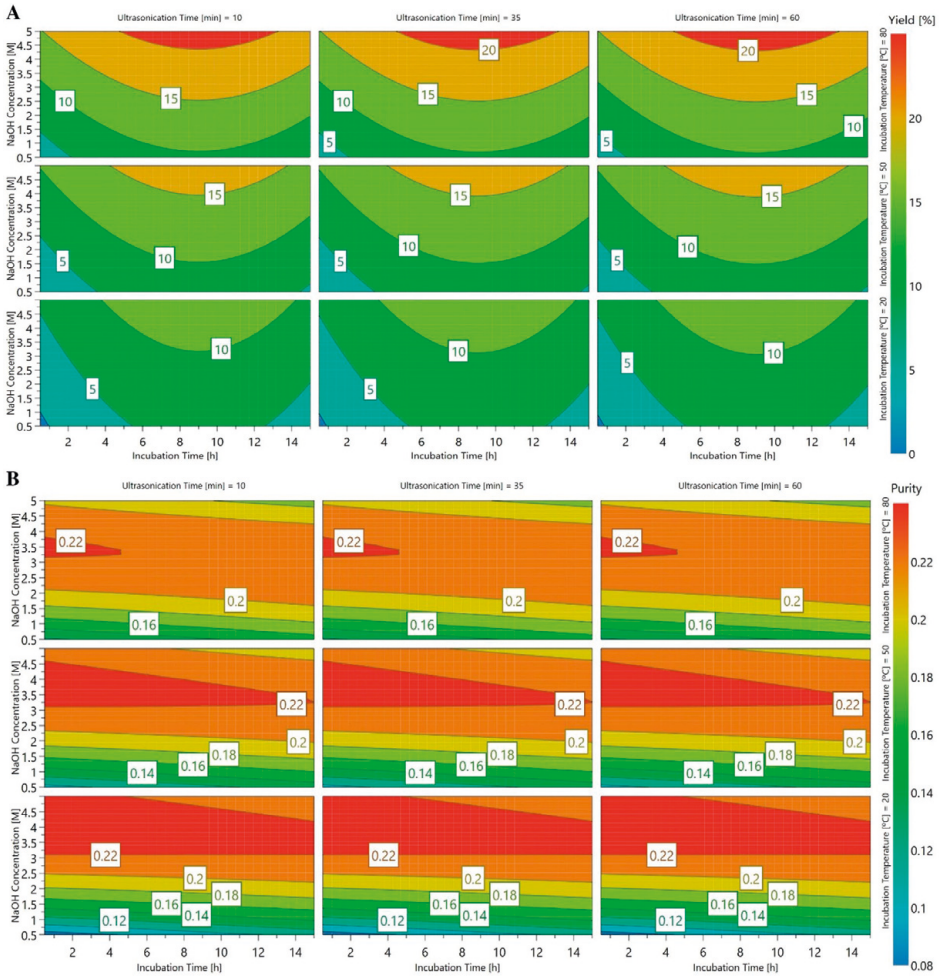

Fig. 3 Response contour plot showing the influence of ultrasonication time, NaOH concentration, incubation time and incubation temperature on the hemicellulose solubilisation yield (**A**) and hemicellulose purity in the extracted material (**B**) in the first DOE study

varied followed by the same optimal conditions of all other parameters. The results displayed in Fig. 5 show that there are no large differences between the yields of solubilized hemicellulose among all the sonicated samples. This shows that a 10 min long ultrasonication step is sufficient.

For further chemical characterization of the soluble material obtained with this final optimized extraction method, a larger batch starting with 25 g of the milled oat hulls was produced. The resulting soluble material is a heterogeneous mixture of many components (see Table 3). Of the initial oat hull starting material 24.6% of the lignocellulose components were solubilized. The largest fraction was hemicellulose (22.1% of total dry weight) followed by starch (11.9%) and lignin (2.5%). This corresponds to a solubilisation of 74.7% of all available hemicellulose in the oat hull. This yield is significantly higher than results from previous similar extraction protocols combining ultrasonication and alkali treatments on wheat straw (65% yield of hemicellulose) [17] and corn bran (32% yield of hemicellulose) [15] for hemicellulose solubilisation, exemplifying

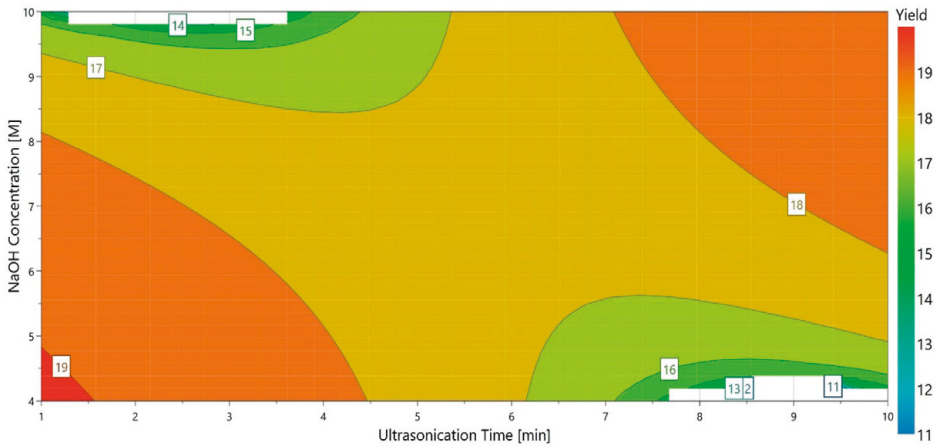

Fig. 4 Response contour plot showing the influence of ultrasonication time and NaOH concentration on the hemicellulose solubilisation yield in the second DOE study

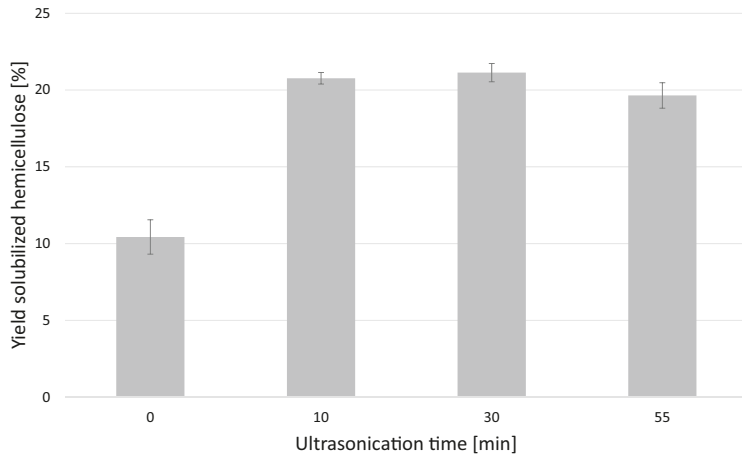

Fig. 5 Extraction results of ultrasonication length trial showing the amount of solubilised hemicellulose as a fraction of the oat hull sample. All tests were performed in duplicate

the importance of optimizing reaction parameters as well as choosing the best pre-treatment method for the raw material under investigation.

Additionally, the phenolic acid content before and after extraction was analysed. Of special interest is ferulic acid as it acts as a linker between the individual hemicellulose strands as well as between hemicellulose and lignin, significantly contributing to the recalcitrance and insolubility of the material [5]. The extracted material contains 20 times less ferulic acid than the untreated oat hulls (see Table 4), showing that the developed pre-treatment method is very efficient in removing these structures. The greater solubility of the pre-treated material is therefore (at least partly) due to the breakage of intermolecular connections between individual hemicellulose molecules and hemicellulose and lignin molecules. Similarly, a 15 times reduction of all phenolic acids was observed after the pre-treatment suggesting that the developed method is effective in breaking ester bonds.

For successful application of this extraction and solubilisation process in industry, the method needs to be up-scalable. The greatest challenge here is to find appropriate equipment. The alkali treatment stage can easily be implemented

Table 3 Quantities of the major components in the untreated Swe19 oat hulls, in the solubilized material after pre-treatment (combined ultrasonication and alkali extraction) in percentage of the original material as well as the solubilized amount related to the original amount present in the untreated oat hulls

Component	Untreated [%]	Solubilized material [%]	Solubilized fraction of original material [%]
Cellulose	15.7 ± 3.0	0	0
Hemicellulose	29.6 ± 2.0	22.1 ± 3.4	74.7
Arabinose	*4.2 ± 0.2*	*3.4 ± 0.6*	*81.0*
Galactose	*1.6 ± 0.2*	*1.0 ± 0.2*	*62.5*
Xylose	*23.3 ± 1.2*	*17.5 ± 2.7*	*75.1*
Mannose	*0.5 ± 0.4*	*0.2 ± 0.0*	*40.0*
Lignin	21.8 ± 0.1	2.5 ± 1.8	11.5
Starch	12.1 ± 0.5	11.9 ± 0.5	98.3

The two DOE labels in the graph were displayed as italics as means of visual separation from the columns. They represent two breaks in the table that do not follow the column structure

Table 4 Phenolic acid content in Swe19 oat hull batch before and after combined pre-treatment of ultrasonication and alkali extraction. The total phenolics content is reported as area underneath all peaks in the HPLC chromatograms due to lacking standards for all phenolic acids

Swe19 batch	Ferulic acid content [µg/g]	Total phenolics [mAU*min]
Before Pre-treatment	2840 ± 447	152 ± 20
After Pre-treatment	141 ± 137	10 ± 7

as large scale alkali processing is commonly employed in pulp mills. For this reason it has previously been judged to be the most competitive pre-treatment technique [20]. Large scale ultrasonication applications are not as widespread, however, many efforts to establish it have been made in recent years [19] providing a promising outlook for the application of ultrasonication-assisted alkaline extraction on large scale.

Conclusion

Ultrasonication-assisted alkaline extraction for the solubilisation of hemicellulose in oat hulls was shown to be a very suitable pre-treatment method. The optimal treatment conditions are 10 min of ultrasonication in water, followed by a 9 h long incubation in 5 M NaOH at 80 °C. At these conditions, 74.7% of the hemicellulose present in the raw material was solubilized.

Acknowledgements The authors are grateful to Juanita Francis for the deep eutectic solvent pre-treatment method analysis and CG Pettersson, Lantmännen, for providing the raw material.

Author contributions All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Eva Schmitz. The first draft of the manuscript was written by Eva Schmitz and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Funding Open access funding provided by Lund University. This work was supported by the strategic innovation program BioInnovation with funding from Vinnova, Formas, the Swedish Energy Agency, Lantmännen Research Foundation, Lyckeby Starch and Nouryon Pulp and Performance Chemicals.

Declaration

Conflict of interest The authors have no conflicts of interest to declare that are relevant to the content of this article.

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Paper IV

Bioresource Technology 343 (2022) 126114

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Lignocellulose degradation for the bioeconomy: The potential of enzyme synergies between xylanases, ferulic acid esterase and laccase for the production of arabinoxylo-oligosaccharides

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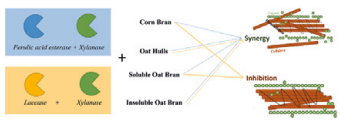
HIGHLIGHTS

- Enzyme synergy is highly substrate dependent.
- High degrees of synergy do not necessarily lead to high yields.
- Homeosynergies between xylanases increase yields the most, up to 57%.
- Heterosynergies of ferulic acid esterase and xylanases were found on all substrates.
- Synergies of laccase and xylanases exist only for high lignin containing substrates.

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Enzymatic synergy
Lignocellulose degradation
Recalcitrant biomass
Carbohydrate active enzymes
(arabino)xylooligosaccharides

ABSTRACT

The success of establishing bioeconomies replacing current economies based on fossil resources largely depends on our ability to degrade recalcitrant lignocellulosic biomass. This study explores the potential of employing various enzymes acting synergistically on previously pretreated agricultural side streams (corn bran, oat hull, soluble and insoluble oat bran). Degrees of synergy (oligosaccharide yield obtained with the enzyme combination divided by the sum of yields obtained with individual enzymes) of up to 88 were obtained. Combinations of a ferulic acid esterase and xylanases resulted in synergy on all substrates, while a laccase and xylanases only acted synergistically on the more recalcitrant substrates. Synergy between different xylanases (glycoside hydrolase (GH) families 5 and 11) was observed particularly on oat hulls, producing a yield of 57%. The synergistic ability of the enzymes was found to be partly due to the increased enzyme stability when in combination with the substrates.

1. Introduction

The polluting effects due to the use of fossil resources and especially the climate effects of the carbon dioxide emissions are forcing the world's large economies to consider more sustainable raw materials for the production of essential goods, such as plastics and fuel. A potential

Abbreviations: (A)XOS, (Arabino)xylooligosaccharides; A/X, Arabinose to xylose ratio; AX, Arabinoxylan; CE, Carbohydrate Esterase; CB, Corn Bran; DS, Degree of Synergy; DOE, Design of Experiment; FAE, Ferulic acid esterase; GH, Glycoside hydrolase; IOB, Insoluble oat bran; Lac, Laccase; OB, Oat bran; OH, Oat hull.

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https://doi.org/10.1016/j.biortech.2021.126114
Received 31 August 2021; Received in revised form 6 October 2021; Accepted 7 October 2021
Available online 11 October 2021
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alternative strategy, the bioeconomy, employs advanced biology and chemical conversion technologies to produce fundamental building blocks of materials, chemicals and energy from various renewable biomass feedstocks (de Besi & McCormick, 2015; Zilberman et al., 2013). Central to this strategy is the development of sustainable methods, which address and relieve the effects of climate change and scarcity of natural resources (de Besi & McCormick, 2015). Hence, biological raw materials from various sources need to be evaluated for their suitability. One attractive source is lignocellulosic waste streams from the agricultural and forestry industries as they are available at low cost and in large quantities. Additionally, they do not compete with the land requirement and biomass production for food purposes (Ubando et al., 2020).

Lignocellulose consists of a complex network of cellulose, hemicellulose and lignin with varying ratios depending on the type of biomass as well as growth conditions (Schmitz et al., 2020). Cellulose consists of β-1,4-linked D-glucose units forming linear polymers, which assemble in a microfibril structure. Hemicellulose is a heterogeneous polymer with manifold branches arranged in random structures. Lignin is composed of phenyl-propane units with varying methoxyl group substitutions on the aromatic rings assembled in an irregular organic polymer (Ravindran & Jaiswal, 2016). These components are closely linked to one another via hydrogen bonds and ester-ether bridges, making the material very recalcitrant, i.e. resistant to biological and chemical decomposition (Himmel et al., 2007). In order to create valuable products from these components, suitable environmentally friendly conversion technologies are required for the successful implementation in a bioeconomy strategy (Sun et al., 2016).

Ideally, such a conversion technology degrades recalcitrant biomasses from various sources, into smaller units from which the desired products can be built. Biomass from different sources have different chemical composition and consequently different conversion technologies are needed to make efficient use of them (Tursi, 2019). Ideally, the biomass degradation should not be complete, but only to a degree that enables efficient and simple production of the desired materials. Breaking down complex substrates into very specific products is efficiently done by enzymes (Malherbe & Cloete, 2002). To date, many industrial processes are already employing enzymes and their use has been suggested to promote the establishment of bioeconomies (Ubando et al., 2020). However, yields are often too low to be economically feasible on large scale (Singh et al., 2019). There are many reasons for this, such as enzyme inhibition by toxic degradation products, limited access to the substrate and time constraints (Malherbe & Cloete, 2002). Protein engineering has proven to be very useful for improving the activity and stability of enzymes under process conditions. This has made it possible to enhance the efficiency of many bioprocesses, including bioethanol production from lignocelluloses (Lugani et al., 2020).

In nature, lignocellulose degrading organisms circumvent the limitations of single enzyme usage by employing enzymes from various classes simultaneously. The combined activity of these enzymes leads to a more efficient substrate hydrolysis, generally referred to as enzymatic synergy (Malgas et al., 2017). The synergistic behaviour of the enzymes improves the performance of the individual ones by removing hindrances that impede their activity. These combined efforts can, furthermore, reduce the reaction time required for hydrolysis and hence, the processing time in an industrial setting. Due to this increased enzymatic activity as well as the reduced processing time, an industrial conversion technology based on enzyme synergy could be more economical than a comparable one based on the action of only one enzyme.

So far, much effort has been placed on establishing optimal ratios of two (Beaugrand et al., 2004) or three enzymes (Sanhueza et al., 2018; Suwannarangsee et al., 2012). However, these studies focused mainly on optimizing yields rather than understanding the underlying mechanisms for the observed behaviour. Knowledge of the effects of protein–protein interactions between synergistic enzymes and their behaviour towards intermediate lignocellulose degradation products is crucial for the development of successful bioconversion processes and has previously been identified as a major knowledge gap (Malgas et al., 2017).

The purpose of this study is to explore and understand how a large variety of relevant enzymes acting on lignocellulose operate together. Similarities and differences for four different lignocellulosic substrates (corn bran, oat hull, soluble and insoluble oat bran) are discussed. The corn bran, oat hull and soluble oat bran were ultrasound assisted alkali pretreated, while the insoluble oat bran was only destarched. The biodegradation of the hemicellulose fraction, arabinoxylan, into oligosaccharide units ((A)XOS) is in focus since these products have a potential as health promoting food ingredients. A better understanding of the synergistic activities of carbohydrate active enzymes enables the development of robust industrial methods for the degradation of biomass in high yields. The development of these methods is crucial to compete with the fossil industry and will pave the way for the establishment of successful bioeconomies.

2. Materials and methods

2.1. Substrates

Four different lignocellulosic substrates were used in this study: corn bran, soluble and insoluble oat bran fiber and oat hull. The corn bran was provided by Bunge Limited (USA), while the oat bran fiber and oat hull were supplied by Lantmännen ek. för. (Sweden). The oat bran fiber is an insoluble side stream during industrial extraction of proteins from oat bran. In the remainder of this article, it will be referred to as only oat bran. Before use as substrates for the enzyme experiments, the brans were destarched and the hulls were milled with an industrial size hammer mill (milling capacity of 1 t/h) located at the Lantmännen facilities in Järna. Subsequently, the brans as well as the hulls were pretreated by ultrasound assisted alkali according to our previously developed method (Schmitz et al., 2021). Additionally, destarched but not alkali pretreated oat bran was used as a substrate in the enzyme reactions. As this material is insoluble in water, it is denoted as insoluble oat bran in the remainder of this article. More detailed information about the substrates and their chemical composition are provided in Table 1. All chemical analyses were carried out according to the methods previously described in (Schmitz et al., 2020).

2.2. Enzymes

A variety of 14 different commercially available carbohydrate-active enzymes was employed in this study. Table 2 lists these enzymes, provides further information about their identity and use in this study, and describes their catalytic reaction.

2.3. Substrate pretreatment

Both of the brans were destarched prior to further modification. For this, 100 μg of each sample were suspended in sodium phosphate buffer (100 mM, pH 6) at a ratio of 1:10 (w/v) for corn bran and 1:20 (w/v) for oat bran, and boiled at 70 °C for 5 min to allow the starch to gelatinize. The samples were cooled to 40 °C and incubated for 2 h after addition of 10 μL of both α–amylase from *Bacillus licheniformis* (type XII-A; ≥ 500 U/mg, Sigma-Aldrich) and amyloglucosidase from *Aspergillus niger* (300 U/mL, Megazymes). The solubilized glucose molecules were separated by centrifugation at 1,500 × g for 10 min and the pellet was further washed with Milli-Q water via cloth filtration three times. The remaining pellet was freeze dried in a LyphLock 12 lyophilizer (Labconco).

After destarching, the brans as well as the unprocessed hulls were pretreated according to the ultrasound assisted alkali method described in (Schmitz et al., 2021). In brief, the substrates were dispersed in Milli-Q water at a ratio of 1:5 (w/v) and sonicated in a Labassco Sonorex RK100H ultrasonic bath (Bandelin) with a frequency of 35 kHz for 10

Table 1
Description of the substrates used for the enzyme experiments after pretreatment in this study.

Substrate	Variety (Seed origin)	Harvest year	Growth location	Arabinoxylan content [%]	Lignin content [%]	Ferulic acid content [μg/g]
Corn bran	Unknown	2019	USA	29.1 ± 3.7	1.2 ± 0.0	1139 ± 7
Oat bran	Kerstin and Galant (SW-Seed, Sweden)	2019	Sweden	5.4 ± 0.1	0.2 ± 0.0	6 ± 1
Insoluble oat bran	Kerstin and Galant (SW-Seed, Sweden)	2019	Sweden	14.2 ± 0.5	16.2 ± 1.5	1689 ± 13
Oat hull	Kerstin and Galant (SW-Seed, Sweden)	2019	Sweden	20.9 ± 3.3	20.3 ± 2.7	141 ± 137

min. Subsequently, the liquid and solid fractions were separated by centrifugation at 3,893 × g for 10 min. The solid fraction was then mixed with 5 M sodium hydroxide and incubated at 63 °C for 9 h under constant shaking. The liquid fraction was isolated via centrifugation at 3,893 × g for 10 min and either used directly or neutralized first with 37% hydrochloric acid to a pH between 5 and 6. The arabinoxylan in this fraction was precipitated via addition of four volumes of 99.5% ethanol and incubation at 4 °C overnight. Subsequently, the ethanol was removed by centrifugation at 3,893 × g for 5 min and evaporation. After complete evaporation, the material was freeze dried. In the case of the oat hull extract, the material was resuspended in Milli-Q water first to allow for better solubilisation in the following treatments. This lyophilized material was used as substrate in all enzymatic experiments.

In order to understand the effect of the salts present in the substrates after pretreatment, the pretreated substrates were desalted for selected experiments. Desalting was carried out by resuspending 50 mg/mL of substrate in Milli-Q to a final volume of 20 mL and placed in a dialysis membrane 12,000 Dalton MWCO. The samples were desalted overnight in room temperature in 2 L beakers filled with Milli-Q, and subsequently freeze- dried.

2.4. Enzymatic synergy reactions

All enzymatic synergy reactions were carried out in 100 mM sodium phosphate buffer (pH 6). For each reaction, 50 mg of one of the substrates (see Table 1) was dissolved in 1 mL of buffer. The enzymes were added simultaneously at room temperature at a concentration of 100 U/g of oat hull or 50 U/g of corn or oat bran as described in Table 2. The reactions were carried out at 40 °C for 24 h under constant shaking. To terminate the reactions, the samples were boiled at 110 °C for 5 min.

Deviations from this general procedure were made in the screening study as well as the sequential addition analysis. The screening study was based on a design of experiment generated by the software MODDE 12.1 (Sartorius Stedim Data Analytics AB). In 35 independent experiments, the enzyme concentrations were investigated as factors in a fractional factorial resolution IV design in the range of 10 to 100 U/g. The centre point at enzyme concentrations of 55 U/g was investigated in triplicate. All of these experiments were performed in duplicate resulting in a total of 70 samples. Only the substrate oat hull was used in this screening study.

The enzyme reactions in the sequential addition analysis differed from the general protocol by adding only one enzyme at a time. The reaction with the first enzyme was carried out for 24 h according to the method described above, terminated and the second enzyme was added for which the procedure was repeated.

2.5. Analysis of enzyme reaction products

The conversion yield of all enzymatic reactions was determined by quantification of the present mono- and oligosaccharides in the terminated reaction mixtures. Quantification was carried out by HPAEC–PAD (ICS-5000, Dionex, Thermo Scientific) equipped with a CarboPac PA200 analytical column (250 mm × 3 mm, 5.5 μm) as well as a respective guard column (50 mm × 3 mm). The mobile phase was composed of 100 mM sodium hydroxide throughout the run and a gradient of sodium acetate at 0–10 min of 0–100 mM after which it was kept constant at 100 mM until the end of the run at 23 min. The standards used for quantification were arabinose (Sigma–Aldrich), xylose (Sigma-Aldrich), xylooligosaccharides X_2 to X_6 (Megazyme), and the following arabinoxylooligosaccharides (Megazyme): A^2XX, A^3X, XA^2XX, XA^3XX and

Table 2
List of all enzymes employed in this study including their suppliers' information, amounts used in experiments on oat hull and description of their catalytic reaction.

Name in this study[1,2]	Enzyme Type[2]	Name given by supplier	Supplier	E.C. Numbers	Amount used in experiments [μL][3]	Catalytic Reaction
GH11(P)	Xylanase (GH11)	Pentopan Mono BG	Novozymes	3.2.1.8	19 (10 g/L stock)	
GH11(F)	Xylanase (GH11)	Feed Xylanase	Novozymes	3.2.1.8	19	Hydrolysis of glycosidic bonds in xylan backbone. No substituents (Ara*f*, MeGlcA) allowed in −1 and + 1 subsites.[1]
—	Xylanase (GH11)	Grindamyl H460	Danisco	3.2.1.8	18 (10 g/L stock)	
—	Xylanase (GH11)	Powerbake 900	Danisco	3.2.1.8	17 (10 g/L stock)	
—	Xylanase (GH11)	E-XYLNP	Megazyme	3.2.1.8	1	
GH10	Xylanase (GH10)	E-XYNBS	Megazyme	3.2.1.8	5	Hydrolysis of glycosidic bonds in xylan backbone.
—	Xylanase (GH10)	E-XYNBCM	Megazyme	3.2.1.8	7	Substituents (Ara*f*, MeGlcA) allowed in + 1 subsite, but not −1 subsite.[1]
GH5	Arabino-xylanase (GH5)	*Ct*Xyn5A (GH5-CBM6)	NZYTech	3.2.1.-	10 (100 μg/mL stock)	Hydrolysis of glycosidic bonds in xylan backbone. Ara*f* substituent required in −1 subsite and allowed in + 1 subsite.[1]
—	Glucurono-*arabino*-xylanase (GH30)		Novozymes	3.2.1.136	20	Hydrolysis of glycosidic bonds in xylan backbone. MeGlcA and GlcA substituents required in −2 subsite.[4]
—	Cellulase	Celluclast 1.5 L	Novozymes	3.2.1.4	20 (10 g/L stock)	Hydrolysis of glycosidic bonds in cellulose.
—	Gluco-amylase	Saczyme	Novozymes	3.2.1.3	20 (10 g/L stock)	Hydrolysis of glycosidic bonds in starch. Exo-acting.
—	α-amylase	Termamyl 120	Novozymes	3.2.1.1	20 (10 g/L stock)	Hydrolysis of α glycosidic bonds in starch. Endo-acting.
FAE	Ferulic acid esterase (CE1)	E-FAERU	Megazyme	3.1.1.73	13	Hydrolysis of ester bonds between ferulic acid and hemicellulose.
Lac	Laccase	NS51003	Novozymes	1.10.3.2	5	Oxidation of phenolic species.

[1] Enzymes studied with respect to synergistic effects are given the names listed in the first column.
[2] The abbreviation GH stands for glycoside hydrolase family and the abbreviation CE stands for carbohydrate esterase family.
[3] The amounts used for the experiments on oat hull correspond to an enzyme activity of approximately 100 U/g of substrate. For the experiments on corn and oat bran, half of the amounts in the table corresponding to 50 U/g were used to obtain similar conversion levels due to their lower recalcitrance.
[4] The allowed interactions in the −1 and + 1 subsites of the catalytic domain are substituent dependent.

$A^{2+3}XX$.

Selected enzyme reaction products after the hydrolysis with laccase and/or ferulic acid esterase were further analysed by size exclusion chromatography according to the method by Salas-Veizaga and colleagues (Salas-Veizaga et al., 2017). Instead of the refractive index detector, a UV detector at a wavelength of 320 nm was used. Additionally, the amount of ferulic acid released during the reactions was quantified utilizing the HPLC method developed by Sajib and colleagues (Sajib et al., 2018).

2.6. Degree of synergy calculation

The degree of synergy for all enzymatic reactions was calculated based on the conversion yield (see section 2.5.) according to the formula described by Van Dyk and Pletschke (Van Dyk & Pletschke, 2012), where the conversion yield of the combined enzyme mixture is divided by the theoretical sum of the enzymes' individual conversion yields.

2.7. Enzyme stability analysis

To assess changes in the enzyme stability in presence of the various substrates and other enzymes, melting curves were generated and analysed according to the Thermofluor assay as described by Boivin and colleagues (Boivin et al., 2013). In brief, the respective enzyme and substrate solutions were mixed in an unskirted 96-well plate with buffer and SYPRO Orange dye (BioRad) on ice in the following concentrations and order: 11 µL Milli-Q water, 5 µL 5 × 100 mM sodium phosphate buffer (pH 6.0), 5 µL 5 × substrate solution (1.25 mg/mL), 2 µL 20 µM enzyme solution, 2 µL SYPRO Orange dye (62 × stock). Subsequently, the plate was centrifuged at 4 °C and 2,500 × g for 30 s to ensure adequate mixing. The melting curve analysis was then performed in a CFX96 Real-Time System C1000 Touch Thermal Cycler (Bio-Rad) according to the Bio-Rad Protein Thermal Shift Assay Bulletin 7180.

3. Results and discussion

Enzymes in nature often act synergistically for the complete hydrolysis of complex and recalcitrant lignocellulosic materials, meaning that their combined activity is greater than the sum of their individual activities on the given substrate. This synergy can be further differentiated into homeosynergy, where all enzymes are cleaving the main chain, and heterosynergy, where main chain cleaving and debranching enzymes are combined (Van Dyk & Pletschke, 2012). Due to the complexity of the substrate and large variety of catalysed reactions when considering hemicellulose degradation by various enzymes, adequate synergy quantification is a major obstacle, especially when comparing different substrates (Van Dyk & Pletschke, 2012). To cope with this challenge, a combination of two different evaluation methods is employed in this study: substrate conversion yield and enzyme stability.

3.1. Initial considerations

The capability of enzymes to act synergistically can be influenced by a variety of external factors. Substrate pretreatment has been shown to both increase and decrease the required enzyme loading (Van Dyk & Pletschke, 2012), stressing the need for an assessment of the integration of the pretreatment and enzyme hydrolysis steps. In this study, most substrates were alkali pretreated as it is a widely used method (Xu & Sun, 2016) and was shown to be the optimal one for the solubilisation of oat hull hemicellulose (Schmitz et al., 2021). Alkali pretreatment of lignocellulosic substrates as well as drying, which is an essential part of the pretreatment method, have previously been shown to impair enzyme hydrolysis. While it is out of the scope of this study to minimize the negative effects of these factors, the effect of neutralization of the alkali phase after extraction on the capability of enzyme synergy was assessed (see Fig. 1). The results show that a neutralized substrate allows for a greater hydrolysis yield by a mixture of 14 enzymes. Therefore, all enzyme experiments in this study were performed on neutralized alkali pretreated substrates.

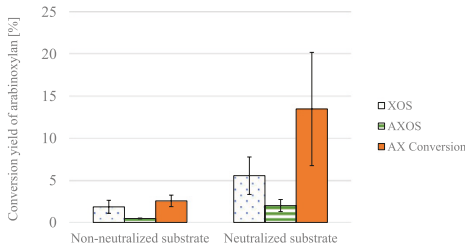

Fig. 1. Conversion yield of arabinoxylan (AX) after enzymatic hydrolysis of ultrasound assisted alkaline pretreated oat hull substrate, whose soluble fibres in the alkali phase were either directly precipitated or neutralized first with hydrochloric acid to pH 5–6 (n = 2). A mixture of 14 different enzymes was used for the hydrolysis. The quantified hydrolysis products are xylooligosaccharides (XOS) and arabinoxylooligosaccharides (AXOS). The AX conversion is defined as the sum of XOS, AXOS, arabinose and xylose monosaccharides.

Another important consideration when evaluating enzyme synergies is the use of pure versus technical grade enzymes. A better understanding of the specific enzyme reactions can often be more easily deduced from pure enzymes as additional components in the technical grade enzyme mixtures have been shown to impact reaction results (Van Dyk & Pletschke, 2012). However, this increased understanding will not support the establishment of bioconversion processes if the pure enzymes cannot be used in an industrial setting. As the purpose of this study is to provide more information for the implementation of these processes in industry, only commercially available enzyme preparations were considered.

3.2. Enzyme synergy based on conversion yield

A total of 14 different commercial enzyme preparations including amylases, cellulases, xylanases of glycoside hydrolase (GH) families 10 and 11, an arabinoxylanase of family GH5, a glucuronoarabinoxylanase of family GH30, a laccase as well as a ferulic acid esterase were screened for their synergistic activity on the pretreated oat hull substrate in a design of experiment (DOE) study. We have previously shown that oat hulls are more recalcitrant than many other lignocellulosic materials (Schmitz et al., 2021). Therefore, the screening was only performed on this substrate. The less recalcitrant corn and oat bran are expected to be hydrolysed by those enzymes acting on oat hull. The final DOE model suggested that only the following enzymes led to a significant conversion of the substrate: Arabinoxylanase 5A (GH5), E-XYNBS (GH10), Pentopan Mono BG (GH11(P)), Feed xylanase (GH11(F)), E-FAERU (FAE) and NS51003 (Lac). The chosen enzymes have a specific mode of action on the substrate backbone, and other recalcitrant elements present there, such as lignin. In contrast, the others probably only attacked bonds in the substrate that were not crucial for increasing substrate conversion. Only the chosen enzymes were considered in further explorations of their synergy. During the first hydrolysis trials on corn and oat bran, it became apparent that their starch content was too high for the enzymes other than the amylases to have an effect. Hence, those substrates were destarched prior to all enzyme hydrolysis experiments in this study.

An established way of comparing enzyme synergies on cellulose based on the conversion yield is by calculating the degree of synergy (DS) (Van Dyk & Pletschke, 2012). The substrate conversion yield of the enzyme mixture is divided by the sum of all substrate conversion yields of the individual enzymes present in the mixture (see section 2.6). For

more complex substrates, such as lignocellulose, the quantification of conversion yields is more challenging as a variety of different bonds are being hydrolysed. In this study, the conversion of arabinoxylan present in a complex lignocellulosic matrix is of main interest. Therefore, all conversion yields are based on the arabinoxylan fraction. As a result, the action of those enzymes, not acting directly on the arabinoxylan main chain, is not directly elucidated. This limitation of quantifying enzyme synergy is addressed by further analysis methods discussed in section 3.3. Another constraint of the conversion yields is that they are solely based on the quantification of those products for which standards are commercially available, hence, stressing the need for better conversion analysis methods for complex substrates.

The DS of two to five enzyme combinations on the pretreated substrates corn bran, oat hull and oat bran as well as the non-pretreated insoluble oat bran are displayed in Fig. 2A. The data demonstrates that enzyme synergy and inhibition are highly substrate dependent. Many enzyme combinations were found that only resulted in synergy on one of the substrates, suggesting that enzyme activity and stability are more influenced by the present substrate rather than the other enzymes. A similar observation has recently been made on cellulose (Tokin et al., 2020). For the pretreated substrates, a general trend of an inverse relationship between substrate recalcitrance and the number of enzyme combinations acting synergistically can be seen. Substrate recalcitrance depends on many factors of which lignin content and ferulate cross-linking are of great importance (Van Dyk & Pletschke, 2012). The pretreated oat hulls contain by far the most lignin (20.3 ± 2.7 %, see Table 1) and also resulted in the highest number of synergistic enzyme combinations. The pretreated oat bran contains hardly any lignin (0.2 ± 0.0 %), explaining why this substrate resulted in the fewest synergistic combinations. This also correlates to the sparse presence of ferulate cross-linking. Oat bran only contains 6 ± 1 μg/g ferulic acid, while there are 1139 ± 7 μg/g in corn bran. The few cross-links in oat bran probably makes the material rather accessible for the enzymes, explaining why more enzyme combinations yield in synergy on corn bran.

The insoluble oat bran, on the other hand, does not follow this trend. According to its lignin and ferulic acid composition, the number of enzyme combinations resulting in synergy should be between those of oat hull and corn bran. As this oat bran material is insoluble in the reaction buffer, the enzymes' access to the substrate is very different as opposed to the soluble pretreated materials. Hence, a direct comparison is not possible. The potential inhibiting effect of the salts present in the substrates after pretreatment was ruled out as being a reason for the observed differences in enzyme synergy on pretreated and insoluble oat bran after an experiment on desalted pretreated oat bran and oat hull. The synergy of FAE and GH10 was tested on those materials and found to be very similar to the results on non-desalted pretreated materials (DS of 0.92 for oat bran and 1.00 for oat hull).

No enzyme combination was found, where the DS was larger than 1 on all substrates. The combination resulting most universally in synergy is FAE + a xylanase, a heterosynergy, which has previously been shown to result in synergy on complex carbohydrates (Mkabayi et al., 2020). In the case of corn bran it is the xylanase GH5, while pretreated oat bran requires the xylanase GH11(F) and insoluble oat bran the xylanase GH10. Oat hulls result in synergy with either as well as both GH11(F) and GH5 xylanases. GH11(F), GH10 and GH5 are xylanases specifically hydrolysing the glycosidic bond between two xylose monomers. While GH11(F) does not allow any substitutions of the xylose backbone in the −1 and + 1 subsites in its active site, GH10 can accommodate arabinofuranosyl (Ara*f*) and 4-O-methyl-α-D-glucopyranosyl (MeGlcA) substituents in the + 1 subsite, but not the −1 subsite. GH5 catalyses the very specific hydrolysis of a glycosidic bond between two xylose monomers of which one needs to be substituted with an arabinose (subsite −1) (Bhattacharya et al., 2020). Its + 1 subsite can accommodate further Ara*f* substituents. Consequently, these enzymes are capable of acting on slightly different substrates. Oat hulls seem to contain parts with arabinose substitutions and free xylose backbone, while this observation suggests that the majority of pretreated oat bran consists of unsubstituted xylose backbone and the majority of the xylose backbone in corn bran seems to be arabinose substituted. These indications could however not be confirmed by the arabinose to xylose (A/X) ratios of the two materials. The A/X ratios are with 0.52 for corn bran and 0.59 for pretreated oat bran rather similar. This suggests that the structure of oat bran must consist of regions with very highly substituted arabinose side chains on which GH5 cannot act and regions of rather unsubstituted xylose backbone. Corn bran on the other hand most likely has a more even distribution of arabinose on its backbone. In both cases, the xylanases require the arabinoxylan backbone to be freely accessible. FAE is able to provide such conditions (Mkabayi et al., 2020). It catalyses the hydrolysis of ferulic acid ester bonds linking the arabinoxylan strings to one another or lignin (Wang et al., 2020). These ferulic acid bridges contribute significantly to the recalcitrance of the material and have been shown to inhibit hemicellulose access to various enzymes including xylanases and laccases (de Oliveira et al., 2015). Their removal, therefore, provides more space for the remaining enzymes to act on the xylan backbone.

Heterosynergies involving xylanases in combination with a laccase were only found for the two most recalcitrant materials: oat hull and insoluble oat bran. Laccases are multicopper oxidases, catalysing the oxidation of phenolic and lignin units via the formation of a phenoxyradical (Widsten & Kandelbauer, 2008). The presence of lignin and other phenolic species has been shown to inhibit the activity of xylanases (Boukari et al., 2011; Mathibe et al., 2020). The fact that only those substrates containing the largest amounts of lignin and ferulic acid (see Table 1) result in synergies involving laccase, suggests that the laccase is capable of converting some of the inhibiting species into less inhibitory polymers. The potential of laccase to increase xylanase activity in this manner has been observed before (Ladeira Ázar et al., 2018). This hypothesis is supported by the fact that also some of the highest conversion yields were found for laccase/xylanase combinations on oat hull and insoluble oat bran. Further investigations on the action of laccase in combination with xylanases are described in section 3.4.

Only isolated homeosynergies on corn bran and oat hull were found. Those, however, seem to be rather important combinations as they coincide with high yields (see Fig. 2B). On these substrates, there is a clear need for various types of xylanases to cleave the main chain glycosidic bonds, which are in proximity to other substituents. In the case of oat hull, the combination of GH11(F) and GH5 gave rise to synergy as well as a high yield. This underlines the previous finding that oat hull arabinoxylan must be composed of areas with arabinose substitutions and unsubstituted xylan main chain. Corn bran, on the other hand, requires the use of GH11(F), GH10 and GH5 to achieve a high yield and synergy. In addition to the described structural implications on oat hull, the necessity of GH10 indicates that there are regions in corn bran with substituents other than arabinose. These substituents are mostly glucose, galactose and glucuronic acid. Their presence could make the material more resistant to xylanase-catalysed hydrolysis, which would explain the overall very low yields achieved for this substrate. Preliminary studies on corn bran degradation have also shown that higher yields can be achieved with these enzymes when the corn bran was subjected to a different pretreatment method, underlining once more the importance of adapting the pretreatment and enzyme hydrolysis stage to each material (unpublished data).

Counterintuitively, an increased enzyme synergy does not necessarily lead to a greater conversion yield (Andersen et al., 2008; Van Dyk & Pletschke, 2012). This phenomenon could be confirmed in this study (see Fig. 2B). While the combination of different xylanases as well as FAE and a xylanase both give rise to the highest synergies and conversion yields, the combinations are not exactly the same. The combination with the highest synergy was found on oat hull (Lac + FAE; DS = 88) and resulted in a rather low conversion yield (17%). This can be explained by the fact that the DS is more influenced by small changes when the single enzyme yields are also small as in the case of FAE. When larger single

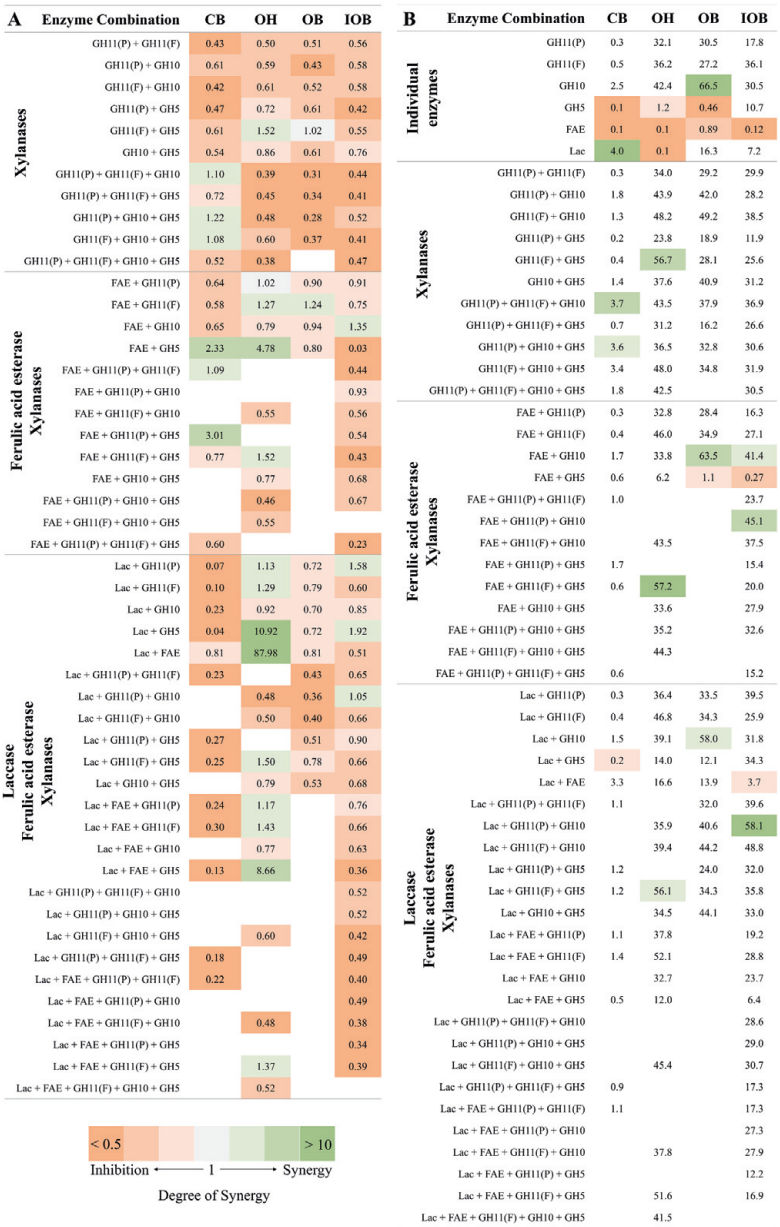

Fig. 2. A) Degree of synergy (DS) of various enzyme combinations on the substrates corn bran (CB), oat hull (OH), oat bran (OB) and insoluble oat bran (IOB). A DS greater than 1 denotes synergy, while a DS < 1 denotes enzyme inhibition. The enzymes are not influencing each other's activity when DS $= 1$. B) Total conversion yields in percent of arabinoxylan present in the substrates. The reactions yielding in the highest conversions are marked in green and those resulting in the lowest conversions are marked in red for each substrate. All data is based on duplicate analyses. Details on the enzymes used are given in Table 2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

enzyme yields are present, small improvements in the yield will give rise to DS values only slightly above 1 as in the case of combining only xylanases. Consequently, small improvements on smaller yields are over proportionately weighed in DS calculations. While DS is a good means of analysing the influence of different enzymes on each other, it should not be utilized for determining the optimal enzyme combination in an industrial bioconversion process. Yields are more relevant in this case. An interesting example underlining this can be seen on the substrate oat bran. The overall highest yield (67%) is found on the pretreated bran with only one enzyme (GH10). The non-pretreated insoluble oat bran, however, needs a combination of three enzymes (Lac + GH11(P) + GH10) to achieve its highest yield (58%). Hence, enzyme synergies are only beneficial to achieve high yields in some cases.

The products generated in the highest yielding reactions described in Fig. 2B furthermore differ greatly among the different biomasses. Overall, a large fraction of the arabinoxylan was converted into oligosaccharides. XOS products represented 34, 60, 48 and 40% of the AX conversion from corn bran, oat hulls, pretreated oat bran and non-pretreated insoluble oat bran, respectively. The AXOS fractions were slightly lower with 19, 29, 42 and 33%.

Enzyme synergies have been reported for both simultaneous and sequential addition of the enzymes. While it remains challenging to predict, which mode of application yields the highest synergies on various substrates, most often better results are obtained for simultaneous addition when examining arabinoxylan substrates (de Vries et al., 2000; Van Dyk & Pletschke, 2012). This holds especially true when the overall aim is only a partial instead of a complete hydrolysis. The present study could confirm this general trend for the substrate oat hull. As shown in Fig. 3, all sequential additions yield lower DSs than the simultaneous additions regardless of the order, except for the combination FAE + GH11(F). In this combination, every application mode resulted in approximately the same DS, suggesting that the GH11(F) xylanase is capable of producing (A)XOS that are still connected to either lignin or other AX products via ferulic acid linkages after reaction. Only once these linkages are cleaved by FAE, these products can be detected by HPAEC-PAD. As these linkages remained intact after harsh alkali pretreatment, they are most likely not cleaved by the alkaline mobile phase during HPAEC analysis, as usually observed. No sequential combination resulted in higher DS than simultaneous application, therefore only simultaneous addition was further assessed in the subsequent parts of this study. It is noteworthy that despite the differences in degree of synergy, all enzyme additions regardless of the application mode resulted in synergy with the exception of the sequential addition of Lac + GH5.

3.3. Enzyme synergy based on enzyme stability

The second method for enzyme synergy detection and characterization we employed was stability measurements via a Thermofluor assay. This assay allows the detection of the melting temperatures of the enzymes. Therefore, enzyme stability in this study describes the physical stability of the proteins rather than the duration for which enzymatic reactions can be carried out. Unfortunately, impurities in the commercial laccase mixture made analysis with this method impossible. Interesting combinations excluding laccase from Fig. 2 were chosen and the results are displayed in Fig. 4. The single enzymes were found to have higher melting temperatures than when they were present in combination with others (see column “only enzymes” in Fig. 4A) indicating that they have a destabilizing effect on one another. Unexpectedly, the enzyme combinations always gave rise to only one peak instead of two. In the case of FAE + GH11(F) the enzyme with the lower melting temperature when alone (FAE) was stabilized in the combination, while the melting temperature of GH11(F) was not affected to a great extent. Another unexpected observation was made for the melting curve of GH5. The application of the single enzyme gave rise to two peaks. Those are most likely belonging to the two different domains of the enzyme. The lower melting temperature domain seems to become very stable when in combination with other enzymes as this peak disappears completely. All these observations indicate that the selected enzymes associate with one another in solution, which can have both positive and negative effects on their stability.

The different substrates exhibit a significant effect on the stability of the enzymes resulting in both more and less stable enzymes. The stability effects displayed in Fig. 4A are in correspondence with the synergy data from Fig. 2 for the following enzyme combinations: GH11(F) + GH5, GH11(P) + GH10 + GH5 and FAE + GH11(F) + GH5. The combination GH11(F) + GH10 results in a decreased stability and inhibitory DS on oat bran, hence, the methods are confirming each other. On corn bran, however, the results are pointing in opposite directions. While the enzyme stability is increased, the inhibitory DS value (see Fig. 2A) is even lower than on oat bran. While enzyme stability seems to contribute to their synergistic action in most cases, stability alone is not an appropriate indication for the resulting DS in every case. This is also underlined by the observation that the melting temperatures of many enzyme combinations were not affected by the different substrates, while the conversion yield DS in Fig. 2 shows very large differences. It also has to be noted that most melting temperatures found are much higher than the temperature utilized in the enzymatic reaction experiments (40 °C) indicating that all enzymes are rather stable under reaction conditions. Therefore, more unidentified factors seem to play a role in establishing synergy. While it remains important to screen various enzymes for their activity on specific substrates and no universally

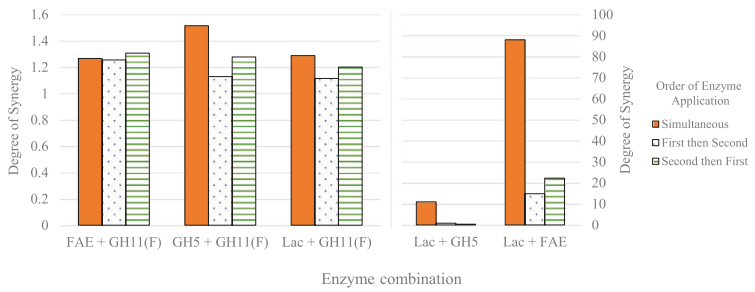

Fig. 3. Degree of synergy of two enzyme combinations applied simultaneously or in sequence on oat hull substrate. Due to the large difference in absolute value of degree of synergy the last two enzyme combinations are plotted on another axis on the right. Details on the enzymes used, FAE, GH11(F), GH5 and Lac, are given in Table 2.

A

Enzyme Combination	Only Enzymes	CB	OH	OB
Xylanases				
GH11(P)	53.7	53.0	53.3	54
GH11(F)	72.7	72.7	72.3	69.7
GH10	78	78	78	78
GH5 Domain 1	55	49	52.5	49.3
GH5 Domain 2	72	71.7	69.7	72
GH11(F) + GH10	71.7	74.3	71.7	69.7
GH11(F) + GH5	70	70.7	72.3	69.7
GH11(P) + GH10 + GH5	55	55	54.7	53
Ferulic acid esterase + Xylanases				
FAE	59.3	57.3	59.5	57
FAE + GH11(F)	72	71.5	71.7	71.7
FAE + GH11(F) + GH5	71.3	72.7	72.7	70.7

Stabilizing | No Change | Destabilizing

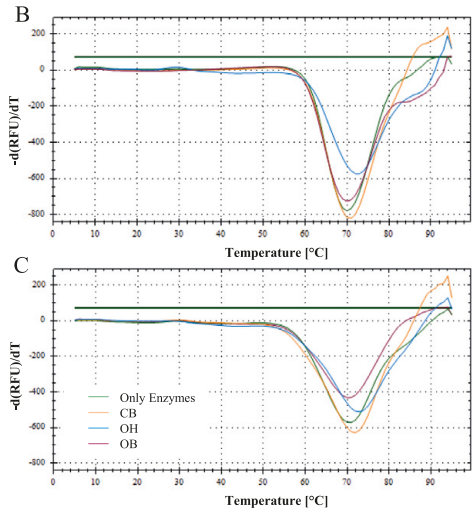

Fig. 4. Average enzyme melting temperatures (n = 3) in only buffer or presence of the substrates corn bran (CB), oat hull (OH) and oat bran (OB) (A). An increase in the melting temperature marks a stabilizing effect, while a decrease demonstrates a destabilizing effect. The melt curves for the enzyme combination of GH11(F) + GH5 and FAE + GH11(F) + GH5 are shown as examples in B and C, respectively. The y-axis in both B and C describes the derivative of relative fluorescent units (RFU) over temperature in °C. The curve legend in C also applies to B. Details on the enzymes used are given in Table 2.

collaborating enzyme combinations could be found, heterosynergies should especially be further explored in the future. Additional aspects influencing synergy that were not subject of this study are enzyme loadings, substrate concentrations, reaction times and side activities. As those can have a large influence on the yield, it is important to further evaluate those interactions.

3.4. A closer look at heterosynergy

Drawing universal conclusions on enzyme synergies is challenging as they seem to be mostly substrate dependent. However, more often heterosynergies compared to homeosynergies were found in Fig. 2. In most cases, the addition of FAE to the xylanases leads to a greater synergy than combining only xylanases. This is most likely attributable to its action in making the substrate more accessible to xylanases. In contrast, the enzyme laccase seems to act very specifically on only the high lignin substrates oat hull and insoluble oat bran. Laccase is an interesting enzyme receiving attention by a large variety of industries (Mate & Alcalde, 2017). Even though there is a broad interest in the enzyme, its action and effect on hemicellulose structure is only poorly understood. This is due to the difficulty of monitoring its reaction, which involves the generation of phenoxy radicals from phenolic groups which can either depolymerise or repolymerise lignin (Widsten & Kandelbauer, 2008). To elucidate its action on complex lignocellulose in combination with other enzymes, selected reactions on oat hull were analysed by size exclusion chromatography. The substrate only gave rise to one distinct peak, indicating the presence of one specific lignin species. Due to the lack of appropriate standards, its size could not be determined. After treatment with either FAE or Lac, the peak's retention time shifted to a slightly earlier elution and its height was reduced by 19 and 49%, respectively. There are two potential reasons for this behaviour: the size of the lignin could either be increased to a small extent after enzyme treatment while its quantity was reduced or the double bond conjugation in the lignin was altered in a way that its absorbance at 320 nm was lowered. This observation was confirmed by total soluble lignin content analyses after enzyme reaction (substrate: 0.41%, FAE: 0.37%, Lac: 0.38%). Interestingly, the combined addition of Lac and FAE did not only reduce the peak height and lignin content the most, but also resulted in a larger shift of the peak's retention time to an earlier elution. This indicates an overall larger size of the lignin species as result of repolymerization. A similarly large shift was observed for the enzyme combination Lac + GH5, indicating the presence of enzyme side activities whose further characterization could be beneficial for understanding the reaction outcomes and designing optimal bioconversion processes.

These data could be confirmed with the analysis of free ferulic acid present after enzymatic reaction. Ferulic acid is a hydroxycinnamic acid, which can be covalently bound to lignin via an ether bond. While its biosynthetic pathway is still not entirely understood, its synthesis is tightly interconnected to that of lignin suggesting the possibility of repolymerization of ferulic acid with lignin (de Oliveira et al., 2015). All pretreated substrates before enzymatic reaction (control) contained free ferulic acid which dissolved in the reaction buffer (see Fig. 5). As expected, this amount increased after reaction with FAE. One drawback of the applied analysis method is that it only quantified the amount of free ferulic acid. Those species that are still attached to lignin as well as those present as free diferulic acid, which connected two hemicellulose strands before enzymatic cleavage, are not detected. Therefore, the increase of free ferulic acid compared to control samples in all substrates is not as high as one might expect. After the reaction with laccase, however, nearly all free phenolic acids are removed (only data for ferulic acid is shown in Fig. 5). This occurs most likely via repolymerization to lignin and explains the presence of larger lignin species after reaction with laccase. The same observation was made for the reaction with both FAE and Lac. Laccase is capable of also repolymerizing the additional ferulic acid freed after reaction with FAE correlating with the previous finding of giving rise to the largest lignin molecules.

4. Conclusions

Among the 14 different enzymes screened in this study, both homeo-

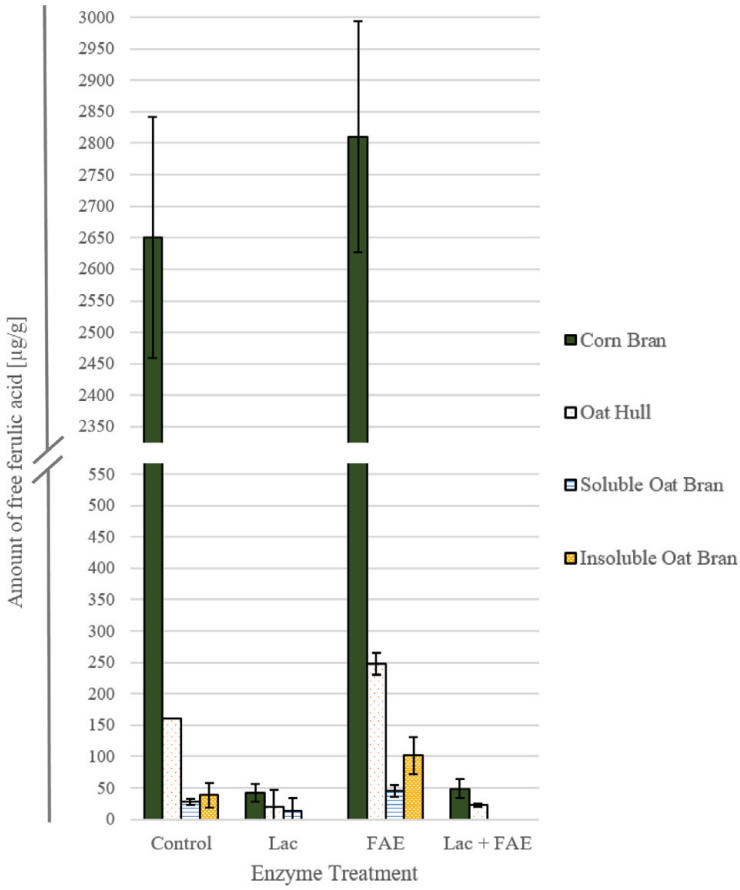

Fig. 5. Amount of free ferulic acid in µg/g in the substrates corn bran, oat hull, soluble and insoluble oat bran before (control) and after reaction with laccase (Lac), ferulic acid esterase (FAE) and Lac + FAE (n = 2).

and heterosynergies were found. Heterosynergies involving FAE were most universal, while combinations with laccase resulted in synergies only on the more recalcitrant substrates. Simultaneous addition of the enzymes gave higher DS than sequential addition. High DS did not necessarily lead to high yield. Synergies between different xylanases were particularly efficient in producing a high yield from the normally recalcitrant oat hulls. Melt curve analysis indicated that the combined enzymes associate with one another, which has both positive and negative effects on their stability, at a higher temperature.

CRediT authorship contribution statement

Eva Schmitz: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Visualization. **Savvina Leontakianakou:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing - review & editing. **Siri Norlander:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing - review & editing. **Eva Nordberg Karlsson:** Writing - review & editing, Supervision, Funding acquisition. **Patrick Adlercreutz:** Writing - review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by the Swedish Foundation for Strategic Research (the Industrial Research Center ScanOats, IRC15-0068) and the strategic innovation programs Swelife and Medtech4Health, joint ventures by Swedeńs Innovation Agency (Vinnova), Formas and the Swedish Energy Agency (Dnr 2019-05428).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2021.126114.

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Supplementary Data

Lignocellulose degradation for the bioeconomy: the potential of enzyme synergies between xylanases, ferulic acid esterase and laccase for the production of arabinoxylo-oligosaccharides

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Appendix to Section 3.2. Enzyme Synergy Based on Conversion Yield

A

	Enzyme Combination	CB	OH	OB	IOB
Xylanases	GH11(P) + GH11(F)	0.14	0.02	0.05	0.14
	GH11(P) + GH10	0.09	0.01	0.03	0.46
	GH11(F) + GH10	0.15	0.01	0.08	0.01
	GH11(P) + GH5	0.09	0.00	0.01	0.10
	GH11(F) + GH5	0.14	0.05	0.20	0.10
	GH10 + GH5	0.03	0.07	0.07	0.50
	GH11(P) + GH11(F) + GH10	0.14	0.02	0.09	0.09
	GH11(P) + GH11(F) + GH5	0.02	0.02	0.07	0.01
	GH11(P) + GH10 + GH5	0.16	0.03	0.16	0.46
	GH11(F) + GH10 + GH5	0.08	0.01	0.15	0.21
	GH11(P) + GH11(F) + GH10 + GH5	0.12	0.01	N/A	0.18
Ferulic acid esterase Xylanases	FAE + GH11(P)	0.14	0.03	0.05	0.60
	FAE + GH11(F)	0.24	0.04	0.41	0.41
	FAE + GH10	0.34	0.04	0.16	0.34
	FAE + GH5	0.09	0.86	0.59	0.10
	FAE + GH11(P) + GH11(F)	0.01	N/A	N/A	0.05
	FAE + GH11(P) + GH10	N/A	N/A	N/A	0.26
	FAE + GH11(F) + GH10	N/A	0.00	N/A	0.09
	FAE + GH11(P) + GH5	0.07	N/A	N/A	0.04
	FAE + GH11(F) + GH5	0.20	0.08	N/A	0.07
	FAE + GH10 + GH5	N/A	0.02	N/A	0.62
	FAE + GH11(P) + GH10 + GH5	N/A	0.00	N/A	0.47
	FAE + GH11(F) + GH10 + GH5	N/A	0.00	N/A	N/A
	FAE + GH11(P) + GH11(F) + GH5	0.02	N/A	N/A	0.08
Laccase Ferulic acid esterase Xylanases	Lac + GH11(P)	0.03	0.01	0.02	0.41
	Lac + GH11(F)	0.01	0.08	0.12	0.06
	Lac + GH10	0.03	0.04	0.06	0.40
	Lac + GH5	0.02	0.16	0.24	0.68
	Lac + FAE	0.08	2.15	0.28	0.28
	Lac + GH11(P) + GH11(F)	0.02	N/A	0.09	0.18
	Lac + GH11(P) + GH10	N/A	0.00	0.02	0.06
	Lac + GH11(F) + GH10	N/A	0.03	0.02	0.06
	Lac + GH11(P) + GH5	0.07	N/A	0.01	0.12
	Lac + GH11(F) + GH5	0.04	0.00	0.08	0.05
	Lac + GH10 + GH5	N/A	0.01	0.01	0.54
	Lac + FAE + GH11(P)	0.00	0.02	N/A	0.13
	Lac + FAE + GH11(F)	0.02	0.00	N/A	0.05
	Lac + FAE + GH10	N/A	0.03	N/A	0.45
	Lac + FAE + GH5	0.02	0.31	N/A	0.05
	Lac + GH11(P) + GH11(F) + GH10	N/A	N/A	N/A	0.37
	Lac + GH11(P) + GH10 + GH5	N/A	N/A	N/A	0.41
	Lac + GH11(F) + GH10 + GH5	N/A	0.06	N/A	0.28
	Lac + GH11(P) + GH11(F) + GH5	0.08	N/A	N/A	0.25
	Lac + FAE + GH11(P) + GH11(F)	0.00	N/A	N/A	0.14
	Lac + FAE + GH11(P) + GH10	N/A	N/A	N/A	0.38
	Lac + FAE + GH11(F) + GH10	N/A	0.02	N/A	0.24
	Lac + FAE + GH11(P) + GH5	N/A	N/A	N/A	0.20
	Lac + FAE + GH11(F) + GH5	N/A	0.01	N/A	0.14
	Lac + FAE + GH11(F) + GH10 + GH5	N/A	0.00	N/A	N/A

B

	Enzyme Combination	CB	OH	OB	IOB
Individual enzymes	GH11(P)	0.06	0.02	2.1	2.5
	GH11(F)	0.08	0.88	6.1	9.0
	GH10	0.51	0.30	4.7	21.6
	GH5	0.07	1.36	0.21	8.9
	FAE	0.08	0.14	0.17	0.10
	Lac	0.41	0.02	5.9	5.4
Xylanases	GH11(P) + GH11(F)	0.12	1.24	7.0	13.7
	GH11(P) + GH10	0.01	0.48	0.37	2.6
	GH11(F) + GH10	0.20	0.82	1.6	17.1
	GH11(P) + GH5	0.10	0.05	1.6	2.5
	GH11(F) + GH5	0.16	1.81	0.70	6.0
	GH10 + GH5	0.18	2.95	7.2	13.8
	GH11(P) + GH11(F) + GH10	0.00	1.91	15.2	8.1
	GH11(P) + GH11(F) + GH5	0.08	1.32	1.8	8.9
	GH11(P) + GH10 + GH5	0.08	2.04	17.5	1.5
	GH11(F) + GH10 + GH5	0.32	0.81	17.9	4.2
	GH11(P) + GH11(F) + GH10 + GH5	0.05	0.76	N/A	1.0
Ferulic acid esterase Xylanases	FAE + GH11(P)	0.08	0.93	0.40	12.9
	FAE + GH11(F)	0.19	1.48	3.1	20.7
	FAE + GH10	0.78	1.58	6.1	24.3
	FAE + GH5	0.10	1.12	0.77	0.12
	FAE + GH11(P) + GH11(F)	0.15	N/A	N/A	7.4
	FAE + GH11(P) + GH10	N/A	N/A	N/A	13.2
	FAE + GH11(F) + GH10	N/A	0.33	N/A	12.7
	FAE + GH11(P) + GH5	1.07	N/A	N/A	7.2
	FAE + GH11(F) + GH5	0.39	3.09	N/A	5.0
	FAE + GH10 + GH5	N/A	0.74	N/A	9.2
	FAE + GH11(P) + GH10 + GH5	N/A	0.01	N/A	0.69
	FAE + GH11(F) + GH10 + GH5	N/A	0.26	N/A	N/A
	FAE + GH11(P) + GH11(F) + GH5	0.04	N/A	N/A	0.41
Laccase Ferulic acid esterase Xylanases	Lac + GH11(P)	0.11	0.28	6.8	21.7
	Lac + GH11(F)	0.11	2.85	4.7	11.0
	Lac + GH10	0.21	1.84	12.7	15.4
	Lac + GH5	0.02	0.20	0.67	31.9
	Lac + FAE	0.16	0.41	9.2	3.7
	Lac + GH11(P) + GH11(F)	0.20	N/A	0.01	21.1
	Lac + GH11(P) + GH10	N/A	0.05	2.7	28.5
	Lac + GH11(F) + GH10	N/A	2.61	9.1	20.6
	Lac + GH11(P) + GH5	0.28	N/A	4.5	18.3
	Lac + GH11(F) + GH5	0.07	0.03	12.4	17.9
	Lac + GH10 + GH5	N/A	0.24	6.7	12.9
	Lac + FAE + GH11(P)	0.05	0.66	N/A	9.1
	Lac + FAE + GH11(F)	0.02	0.15	N/A	11.5
	Lac + FAE + GH10	N/A	1.22	N/A	8.7
	Lac + FAE + GH5	0.01	0.43	N/A	4.8
	Lac + GH11(P) + GH11(F) + GH10	N/A	N/A	N/A	0.40
	Lac + GH11(P) + GH10 + GH5	N/A	N/A	N/A	0.73
	Lac + GH11(F) + GH10 + GH5	N/A	4.64	N/A	0.49
	Lac + GH11(P) + GH11(F) + GH5	0.08	N/A	N/A	1.3
	Lac + FAE + GH11(P) + GH11(F)	0.07	N/A	N/A	0.51
	Lac + FAE + GH11(P) + GH10	N/A	N/A	N/A	0.50
	Lac + FAE + GH11(F) + GH10	N/A	1.54	N/A	0.28
	Lac + FAE + GH11(P) + GH5	N/A	N/A	N/A	0.11
	Lac + FAE + GH11(F) + GH5	N/A	0.50	N/A	0.04
	Lac + FAE + GH11(F) + GH10 + GH5	N/A	0.17	N/A	N/A

Figure S1. Standard deviations of the degree of synergy (DS) of various enzyme combinations on the substrates corn bran (CB), oat hull (OH), oat bran (OB) and insoluble oat bran (IOB) reported in Figure 2 (A), as well as of the total conversion yields in percent of arabinoxylan present in the substrates (B). All data is based on duplicate analyses.

Appendix to Section 3.4. A Closer Look at Heterosynergy

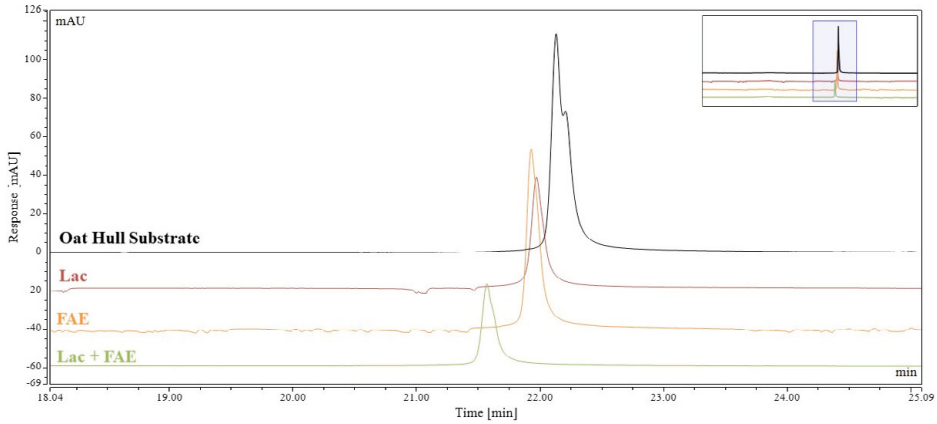

Figure S2. Size exclusion chromatograms showing UV absorbance at 320 nm for various enzyme combinations on the substrate oat hull in full size. The displayed chromatogram is zoomed in on the time axis to enhance the visibility of the peaks. The full chromatogram is visible in the upper right corner. Sample order from top to bottom: only oat hull substrate, laccase (Lac), ferulic acid esterase (FAE), Lac+FAE. The discussion on size changes of the lignin fraction in the oat hull material after enzymatic treatment with laccase and ferulic acid esterase is based on these data.