Universidade do Minho
Escola de Ciências

Mário Jorge Faria Barroca

Enhancement of the Biotechnological Value of a Commercial Cold-Adapted Xylanase

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Trabalho efetuado sob a orientação do
Doutor Tony Collins
e do
Professor Doutor Björn Johansson

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STATEMENT OF INTEGRITY

I hereby declare to have conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

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Resumo

As xilanases são O-glicosídeo hidrolases que clivam as ligações internas β-1,4-D-xilosídicas do xilano, um dos principais polissacarídeos presente nas plantas. Elas são importantes biocatalizadores industriais com diversas aplicações. O presente projeto foi centrado numa xilanase adaptada ao frio e altamente ativa, com o principal objetivo de desenvolver a sua potencial exploração. Neste projeto consta tanto uma parte de investigação fundamental, como aplicada, o que permite o aumento do conhecimento e exploração desta xilanase e consequentemente a sua expansão em novos processos e produtos. Concretamente, desenvolvemos o uso desta xilanase na produção de produtos de valor acrescentado a partir de uma fonte marinha sustentável e caracterizámos os fatores que determinam a sua performance em relação ao pH.

O xilano e os xilo-oligossacarídeos são polímeros de xilose e oligómeros com uma crescente variedade de aplicações. A maioria do xilano e xilo-oligossacarídeos estudados até à data são compostos por ligações β-1,4, e provém de plantas lignocelulósicas por processos caros, com múltiplas etapas, e prejudiciais ao ambiente. Ao tirar vantagem da ausência de lignina, do alto teor de xilano e de uma frágil parede celular da macroalga *Palmaria palmata*, conseguimos identificar e otimizar um eficiente sistema de biorrefinaria em cascata baseado em tecnologia "verde" de alto rendimento, para a produção sustentável de novos xilo-oligossacarídeos e xilano com ligações mistas β-1,3/β-1,4.

Um pré-requisito necessário para o desenvolvimento de qualquer enzima aplicada a processos comerciais é compreender a sua atividade e estabilidade nas condições de processamento. Os nossos estudos aprofundados sobre a atividade, estabilidade e solubilidade desta xilanase, em função do pH, demonstraram que a precipitação proteica é o principal factor limitante, dominando praticamente todo o espectro de pH ácido e a pH alcalino próximo do seu pI. Na verdade, foram observados processos reversíveis na actividade a pHs ligeiramente acídicos, no entanto são rapidamente mascarados pela precipitação, enquanto que a pHs alcalinos, a precipitação é acompanhada por alterações da conformidade da enzima, seguido da completa desnaturação a pH alcalinos extremos. Estes conceitos demonstram a complexidade de factores que determinam a dependência das proteínas em relação ao pH e serão determinantes para melhorar a aplicabilidade desta enzima na indústria.

PALAVRAS-CHAVE: Xilanase, Xilo-oligosacarídeos, Valorisação da macroalga

Abstract

Xylanases (EC 3.2.1.8) are O-glycoside hydrolases which cleave the internal β-1,4-D-xylosidic linkages of the plant heteropolysaccharide xylan and are important industrial biocatalysts with widespread application. The present study was centred around a highly active cold-adapted glycoside hydrolase family 8 xylanase with the overall aim of developing its exploitation potential. The study encompassed both applied and fundamental research to enable an improved understanding and exploitation of the xylanase and thereby permit its expansion into new processes and products. Specifically, the use of the enzyme in the production of value-added products from a sustainable marine resource was developed and the factors determining its pH related performance characterised.

Xylan and xylo-oligosaccharides are xylose polymers and oligomers with a growing variety of applications in diverse areas. Almost all xylan and xylo-oligosacharides studied to date have a β-1,4-linked backbone and have been isolated from land sourced lignocellulosic feedstocks via costly, multi-step, environmentally unfriendly processes. Taking advantage of the high xylan content, lignin free nature, weakly linked cell wall matrix and sustainability of the macroalgae *Palmaria palmata*, in combination with the high activity of the cold adapted xylanase, we identified and optimised an efficient, high yielding, scalable, green chemistry based cascading biorefinery system for the sustainable production of novel mixed linkage β-1,3/β-1,4 xylan and xylo-oligosaccharides.

A prerequisite to the development of any enzyme for any commercial process is an understanding of its activity and stability under process conditions. Interestingly, little is known of the performance of the cold-adapted xylanase with respect to pH, a critical process parameter. Our in-depth study investigating the pH dependence of activity, stability and solubility in this xylanase identified protein precipitation as playing a major role, dominating over almost the entire acidic pH range and at alkaline pHs close to the pI. Reversible processes affecting activity are indeed initially observed at slightly acidic pHs, but are quickly overwhelmed by precipitation, while at alkaline pHs this is accompanied by conformational alterations, with complete protein unfolding being evident at alkaline pH extremes. Such insights highlight the complexity of factors determining the pH dependence of proteins and will be determinant in an improved application of this xylanase in industry as well as in its tailoring to specific process conditions.

KEYWORDS: Xylanase, Xylo-oligosaccharides, Macroalgae valorisation

LIST OF PUBLICATIONS

The work performed during this PhD resulted in the following publications:

M. Barroca, G. Santos, C. Gerday, T. Collins, Biotechnological Aspects of Cold-Active Enzymes, in: R. Margesin (Ed.), Psychrophiles: From Biodiversity to Biotechnology, Springer International Publishing, Cham, 2017, pp. 461-475. DOI: 10.1007/978-3-319-57057-0_19.

D. Silva, G. Santos, **M. Barroca**, T. Collins, Inverse PCR for Point Mutation Introduction, Methods Mol Biol 1620 (2017) 87-100. DOI: 10.1007/978-1-4939-7060-5_5.

M. Barroca, G. Santos, B. Johansson, F. Gillotin, G. Feller, T. Collins, Deciphering the factors defining the pH-dependence of a commercial glycoside hydrolase family 8 enzyme, Enzyme and Microbial Technology 96 (2017) 163-169. DOI: 10.1016/j.enzmictec.2016.10.011.

National patent submission:

Collins, J. A., **Barroca, M.**, Process for the production of xylan and xylan products from marine biomass and their uses, Applicant: Universidade do Minho. Filing Date: August 27, 2020. **Portuguese Provisional Patent Application.** Application Number: 116669.

Publications not included in this thesis:

B. Monteiro, P. Ferraz, **M. Barroca**, S.H. da Cruz, T. Collins, C. Lucas, Conditions promoting effective very high gravity sugarcane juice fermentation, Biotechnology for Biofuels 11(1) (2018) 251. DOI: 10.1186/s13068-018-1239-0.

M. Barroca, P. Rodrigues, R. Sobral, M.M.R. Costa, S.R. Chaves, R. Machado, M. Casal, T. Collins, Antibiotic free selection for the high level biosynthesis of a silk-elastin-like protein, Scientific Reports 6(1) (2016) 39329. DOI: 10.1038/srep39329.

T. Collins, **M. Barroca**, F. Branca, J. Padrão, R. Machado, M. Casal, High Level Biosynthesis of a Silk-Elastin-like Protein in *E. coli*, Biomacromolecules 15(7) (2014) 2701-2708. DOI: 10.1021/bm5005564

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ACRONYMS

AACC	American Association of Cereal Chemists
Abs.	Absorbance
AIR	Alcohol Insoluble Residue
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
avDP	Average degree of polymerisation
BSA	Bovine serum albumin
CALB	*Candida antarctica* lipase B
CAZy	Carbohydrate-Active enZYmes database
CCD	Central composite design
CMC	Carboximetilcelulose
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNS	DiNitroSalicylic acid
dNTP	Deoxyribonucleotide triphosphate
DP	Degree of polymerisation
DTT	Dithiothreitol
EC	Enzyme Commission
EDTA	Ethylenediaminetetraacetic acid
GH	Glycoside hydrolase family
HPLC	High-performance liquid chromatography
IMTA	Integrated Multi-Trophic Aquaculture
iPCR	Inverse PCR
IUBMB	International Union of Biochemistry and Molecular Biology
IUPAC	International Union of Pure and Applied Chemistry
LB	Luria Bertani Broth
MW	Molecular weight
NS	Nelson-Somogyi

PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PES	Polyethersulfone
PNK	Polynucleotide 5'-hydroxyl-kinase
PVA	Polyvinyl alcohol
R&D	Research and development
RBB	Remazol Brilliant Blue
RIU	Refractive index units
RNA	Ribonucleic acid
RSM	Response surface methodology
RT-PCR	Real-time Polymerase chain reaction
SDM	Site-directed mutagenesis
SEC-HPLC	Size-exclusion chromatography
SNP	Single nucleotide polymorphisms
SOB	Super Optimal Broth
TAE	Tris-Acetate-EDTA
TCA	Trichloroacetic acid
UV	Ultraviolet

PREFACE

This thesis is centred around a cold-adapted xylanase isolated from an Antarctic bacterium. This enzyme has been previously studied at the physicochemical, functional and structural levels and has been successfully commercialised worldwide for use in baking applications due to its unique properties. The present work continues the study of this enzyme via both applied and fundamental studies aiming to further develop its biotechnological potential while also gaining a better understanding of its physicochemical properties. Specifically, the use of the enzyme for the efficient production of novel xylo-oligosaccharides is herein developed and optimised, and its pH-dependence investigated.

This manuscript is divided into three chapters: Introduction (Chapter I), Experimental procedures, results and discussions (Chapter II) and General conclusions and future perspectives (Chapter III). Each chapter is presented as a compilation of scientific publications which have either already been published, are to be submitted for publication, or are taken from a preliminary patent application.

Chapter I: Introduction

The introduction is made up of two review papers focused on the main subject areas of the thesis.

I.I. Xylan, Xylanases and Xylanase Families: Fundamentals and Applied Aspects

Taken from: M. Mendonça, M. Barroca, T. Collins, Endo-β-1,4 xylanase containing families: characteristics, singularities and similarities. **Review paper to be submitted.**

This section presents and discusses the current knowledge on the polysaccharide xylan, its structural makeup and applications, its degradation by enzymes, namely xylanases, and the variable characteristics and applications of these enzymes. In particular, an in-depth systematic review of the classification of these enzymes and their distribution in different families and related properties is presented so as to clear up the current ambiguity and confusion in the literature in relation to this.

I.II. Biotechnological Aspects of Cold-Active Enzymes

M. Barroca, G. Santos, C. Gerday, T. Collins, Biotechnological Aspects of Cold-Active Enzymes, in: R. Margesin (Ed.), Psychrophiles: From Biodiversity to Biotechnology, Springer International Publishing, Cham, 2017, pp. 461-475. **Published review.**

This section presents an overview of enzyme cold-adaptation with a focus on the biotechnological aspects of cold-adapted enzymes and their application in industry.

Chapter II: Experimental Procedures, Results and Discussions

This is divided into four sections, each being further divided into introduction, experimental procedures, results and discussion, and conclusions.

II.I. Macroalgae Xylan: Aqueous Extraction and Application as an Enzyme Assay Substrate

Taken from: Collins, J. A., Barroca, M., Process for the production of xylan and xylan products from marine biomass and their uses, Applicant: Universidade do Minho. Filing Date: August 27, 2020. **Portuguese Provisional Patent Application**. Application Number: 116669.

The aim of the applied studies of this project was to develop the use of the cold-adapted xylanase for the eco-friendly production of xylo-oligosaccharides from marine biomass. Section II.I focuses on the first step of this process, specifically, the development and optimisation of a high yielding environmentally friendly process for xylan extraction from a red macroalgae.

II.II. Xylanases for Xylo-oligosaccharides from Macroalgae Xylan: Process Development and Optimisation

Taken from: Collins, J. A., Barroca, M., Process for the production of xylan and xylan products from marine biomass and their uses, Applicant: Universidade do Minho. Filing Date: August 27, 2020. **Portuguese Provisional Patent Application**. Application Number: 116669.

This section describes the development, optimisation and characterisation of xylo-oligosaccharides production processes involving xylanase hydrolysis of macroalgae xylan prepared according to methods described in the previous section.

II.III. Deciphering the Factors Defining the pH-Dependence of a Commercial Glycoside Hydrolase Family 8 Enzyme

M. Barroca, G. Santos, B. Johansson, F. Gillotin, G. Feller, T. Collins, Deciphering the factors defining the pH-dependence of a commercial glycoside hydrolase family 8 enzyme, Enzyme and Microbial Technology 96 (2017) 163-169. **Published Paper.**

The cold-adapted xylanase has been intensively studied, in particular in relation to temperature, but little is known of its pH dependence. pH is also a critical industrial process parameter and an understanding of the pH dependence of enzymes performance is essential for successful industrial application while also enhancing fundamental knowledge on protein structure-function-stability-solubility relationships. This section presents a comprehensive fundamental study investigating the pH dependence of activity, stability and solubility in the cold-adapted enzyme.

II.IV. Inverse PCR for Point Mutation Introduction

D. Silva, G. Santos, M. Barroca, T. Collins, Inverse PCR for Point Mutation Introduction, in: L. Domingues (Ed.), PCR: Methods and Protocols, Springer New York, New York, NY, 2017, pp. 87-100. **Published Book Chapter.**

The results of the previous section will have important implications in the tailoring of the cold-adapted xylanase for a specific pH and process application. Site directed mutagenesis techniques are essential, widely used tools for protein tailoring and the study of protein structure and function. This subchapter presents a technical paper detailing an in-house optimised protocol for the inverse PCR technique for protein engineering.

Chapter III: General Conclusions and Future Perspectives

This last chapter discusses the general results of the study, highlighting the principal results and their potential implications as well as indicating future directions of study.

Chapter I

Chapter I.I

Xylan, Xylanases and Xylanase Families: Fundamentals and Applied Aspects

1. Xylan

Xylan is one of the most abundant polysaccharides in Nature, it is the second most abundant polysaccharide in terrestrial ecosystems and is a major constituent of plant cell walls, constituting the main component of plant hemicellulose [1, 2]. Plants represent around 80% (w/w, carbon content) of the total biomass present on earth, being trailed by bacteria, fungi, archaea, protists, animals and, lastly, with only a minute proportion, viruses. Most plant biomass, or phytomass, is concentrated in terrestrial environments, with less than 0.25% being found as marine biomass in the form of algae and seagrass [3]. Approximately 18% of land phytomass is currently consumed by land animals, but human consumption has been increasing steadily in recent years and projections indicate consumption of up to 50% of all plants cultivated each year in the coming decades [4]. As a result, xylan is a major natural constituent of human and animal diets wherein its concentration, composition, interactions and structure in plants impact the manufacturing, processing, quality, organoleptic properties and health aspects of various foods, feeds and beverages. Similarly, it impacts diverse plant-based technical products and production processes, such as in the pulp and paper and textile industries, and the recent shift in societal preferences away from petroleum based products towards biobased products will develop its utilisation in various further diverse areas such as in the biofuels, chemicals, materials, pharmaceuticals, health care and cosmetics industries [5, 6].

Xylan is found in a wide variety of plants where it functions in mechanical support, cell wall integrity and plant defence, and is also believed to play a role in plant growth and development. Its levels in plants ranges from 19-35% (w/w dry mass) in angiosperms, 7-14% (w/w dry mass) in gymnosperms and is present at highest levels in algae, ranging from 20-60% (w/w dry mass), and is even found in some bryophytes. In particular it is found at high levels in the wheat endosperms (70% of dry weight). It is localised in the secondary cell wall of dicots, and lignified tissues of monocots (20-35% w/w) and conifers (5-15% w/w), with residual amounts in the primary cell wall (2-5% w/w), and in both the primary and secondary cell walls of graminaceae (20-40% w/w in the primary and 40-50% w/w in the secondary cell wall). Most commonly, in these structures it is found at the interface between lignin and cellulose, being bound by covalent and/or hydrogen bonds, but in some plants, such as algae, it replaces cellulose and even lignin in the cell wall matrix [1, 2, 7-9].

1.1. Xylan Structure and Variability

The xylan structure is highly variable. It is composed of a backbone chain of 50 to 10000 D-xylopyranosyl units which are most commonly β-1,4-linked, but β-1,3 and mixed-linkage β-1,3/β-14, have also been reported [2, 10, 11] (Fig. 1). This backbone chain may or may not be branched to varying degrees with various side chain groups such as glucuronyl, methyl-D-glucuronyl, α-L-arabinosyl, galacturonyl, xylosyl, rhamnosyl, galactosyl, glucosyl, acetyl, feruloyl dehydrodiferuloyl, sinapyl and/or p-coumaroyl groups [12, 13]. These potential alternatives lead to a large variety of structural types with various different properties and functionalities, and highly variable xylan structures have been reported depending on the plant species and indeed also the plant tissues and plant age [7].

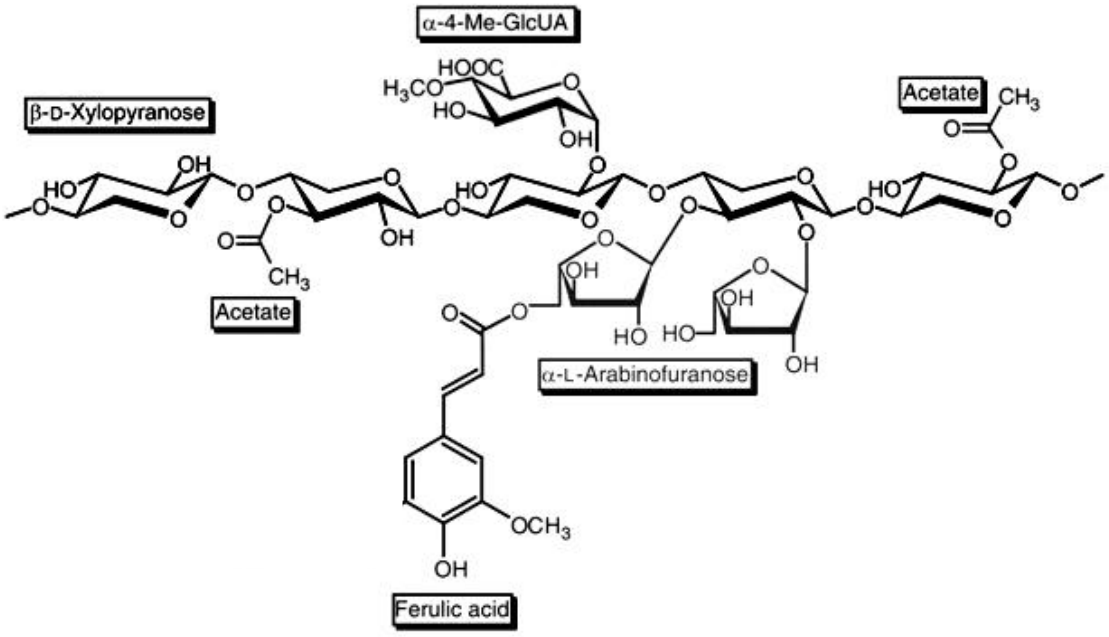

Fig. 1. Representation of the xylan structure and the various side-chain substitutions that can be observed in different plant sources. Adapted from Dodd, D. and Cann, I. 2009 [14].

The most abundant types of xylans currently known are homoxylans, arabinoxylans, glucuronoxylans and arabinoglucuronoxylans [15]. Homoxylans are composed of a linear xylose backbone chain which may or may not be substituted with xylose side chain branching and are predominantly present in algae and seagrass, but can also be found in tobacco stalk and esparto grass [2]. In acetyl-arabinoxylans, found in cereal grains and important in bread making and animal fodder, the partially acetylated β-1,4-linked xylose backbone is substituted with α-linked L-

arabinofuranose side chain residues at the carbon 2 (C-2) and/or carbon 3 (C-3) position of the xylose units, with some of these arabinofuranose residues being esterified with ferulic and/or coumaric acid [16]. In *O*-acetyl-4-*O*-(methyl)glucuronoxylan, found in hardwoods, ≈10% of the backbone xylose units are D-glucuronic acid or 4-O-methyl-D-glucuronic acid substituted at C-2, and ≈60% are mono or di-acetylated at C-2 and/or C-3[17]. Glucuronoxylans are typically very difficult to extract, usually requiring harsh chemical and physical treatments for extraction and resulting in a deacetylated insoluble xylan product [18, 19]. Acetyl-arabino-4-*O*-(methyl)glucuronoxylans are typically found in wheat bran, wheat straw, various corn and sunflower residues, bagasse, grasses and other annual plants. These are highly substituted xylans, with the mono- and di-acetylated backbones being side chain substituted every 5-12 xylose units at C-3, or both C-2 and C-3, with partially esterified arabinofuranose; and every 5-6 xylose units at C-2 with (methyl)-D-glucuronic acid side chain residues [20, 21]. Interestingly, corn fiber acetyl-arabino-4-*O*-methylglucuronoxylan is probably one of the most highly decorated and hydrolytic enzyme resistant xylans currently known. Over 70% of its partially mono- and di-acetylated xylose backbone residues have one or more arabinose, 4-*O*-methylglucuronic acid, or other side chain substitution, and the arabinose is often both esterified with ferulic acid and substituted with various mono, di and trisaccharides composed of α and β-linked xylose and α-linked D- and L-galactose residues [22]. Finally, as for acetyl-arabino-4-*O*-methylglucuronoxylans, softwood xylans are also both ≈10% L-arabinofuranose and ≈20% 4-*O*-methyl-D-glucuronic acid substituted, but these xylans are absent of acetylation, and substitutions occur at C-3 of the backbone xyloses.

The most common source of xylan in industry today is terrestrial lignocellulosic plants and their agricultural and industrial wastes which give rise to various complex, substituted, β-1,4-linked xylans as described above. These are produced via complex, costly, multistep processes with hazardous, corrosive chemicals (e.g., acids, alkali, solvents, ammonia, alkaline peroxide) and physical treatments (e.g., high temperature and/or pressures), with/without enzyme treatment. This can result in production of undesired side products and a high environmental footprint. In fact, these procedures frequently result in high amounts of inhibitor compounds such as acetic acid, furfural, hydroxymethylfurfural and lignin degradation products that can interfere with posterior xylan conversion processes and hence further downstream processing is required for their removal. In contrast, non-lignocellulosic marine biomass such as macroalgae have potential for extraction with more environmentally friendly and economically viable processes and offer prospective as

novel sources of novel xylan structures. Indeed, macroalgae xylan is a major focus of the present thesis and thus will now be overviewed here.

2. Macroalgae Xylan

Macroalgae (colloquially known as seaweeds) are an abundant and carbon neutral renewable marine resource which offer a rich, yet underexploited, source of numerous compounds including various polysaccharides, proteins, lipids, antioxidants, vitamins and minerals. They are under-exploited 'crops' which do not compete with traditional food crops for space or resources (arable land and freshwater) and in fact have several benefits over traditional land based crops in terms of productivity, nutritional value and environmental impact. In addition, from a technical point of view, the lack of lignin in many seaweeds implies that the harsh pre-treatment required for release of components from lignocellulosic biomass may not be required. Macroalgae are fast growing, macroscopic, marine autotrophs divided into three main taxonomic groups based on their pigmentation: green (Chlorophyta), red (Rhodophyta), and brown (Phaeophyta - related to Chromista) [2]. Various xylan structures, distinct to 'common xylans' of land plants, have been identified in both green and red macroalgae [23], but none, as yet, has been identified in brown macroalgae [24].

In green macroalgae, xylan has been mainly studied in the *Chlorophyta* and *Charophyta/Streptophyta*. *Chlorophyta*, whose morphology can vary from unicellular to more complex structures such as colonial, thalloid or siphonous forms, can be found in both marine and freshwater environments [25]. In these algae, and contrary to land plants, the cell wall is composed of either β-1,3-D-xylans or β-1,4-D-mannans, with cellulose being completely absent in most of the genera studied. The majority of genera contain non-substituted β-1,3 homoxylans, although in some cases these can change from β-1,3-D-xylans to β-1,4-D-mannans, or vice versa, in different stages of growth. Furthermore, and similar to the β-1,3-D-glucans of land plants, the β-1,3-D-xylans of this algae take on a triple helix structure, each helix being composed of six xylose residues per turn in a pitch of approximately 18 Å [26-28].

Charophytes are found in both soil and freshwaters and, due to highly similar cell wall structures, are believed to be the closest extant relatives of land plants with whom they are classified with in the same phylogenetic clade (the streptophytes) [29]. Indeed, recent studies focused on the evolution of land plants suggest that the β-1,4-D-xylans present in this algae were among the key cell wall innovations that led to the terrestrialisation and emergence of polysaccharide-based plant

cell walls. Charophytes are composed of a variety of different poylsaccharides in the cell wall, including xylan, cellulose, pectic polymers, arabinogalactan, and heteromannans. Most commonly, the xylan is in the form of β-1,4-D-heteroxylan in which the xylose backbone can be substituted, principally with α-L-arabinofuranose residues, at C-2 and/or C-3 [30, 31]. More rarely, lower amounts of β-1,3-D-homoxylans can also be found in some species, such as in *K. flaccidum*, this being similar in structure to that observed in Chlorophyte algae [32].

Rhodophyta, from Greek 'rose plant', includes all red algae found on earth. The majority of red algae are found in marine environments, with only a small percentage being found in freshwater. Color is not the only distinction in these algae as their cell wall and intracellular matrix also differs significantly from other plants. Most red seaweeds synthesise sulfated galactans such as agar, agarose and carrageenans which have been extensively used as powerful gelling and stabilizing agents with a vast array of applications [33, 34]. Other polysaccharides found in red algae are cellulose and xylan, with three types of xylan having been reported: the β-1,3-D-homoxylans that occur as microfibrils in the cell walls of *Porphyra umbilicalis*, similar to those found in green algae; small amounts of β-1,4-D-xylans, found in the walls of *Palmaria palmata* and *Scinaia hatei*; and the mixed linkage β-1,3/β-1,4-D-homoxylans found in Nemaliales and Palmariales, where xylan is the main polysaccharide present. Studies have indicated that the structure of these mixed-linkage xylans consists of an irregular distribution of solitary β-1,3 linkages within β-1,4-linked sequences of variable length in a random coil conformation [35]. Similar to other algae, the microfibrils of β-1,3-D-xylans in *Rhodophyta* occur as triple helices and, in some cases, can be replaced by cellulose, or vice versa, in different growth stages of the plant [35-37].

The red seaweed *Palmaria palmata* (also known as dulse, dillisk or dilsk) is probably one of the most xylan-rich macroalgae known. This is commonly found in the nothern Atlantic and Pacific waters and is one of the few species that has been commonly used for human consumption, being consumed in Europe and Asia for centuries [38]. It is characterised by a high xylan (20-60%) and protein (10-35%) content, low amounts of other components (lipids, phenolics, galactose, glucose etc.), the absence of lignin, and only minute quantities of cellulose [39, 40]. While a minor amount of β-1,4-linked D-xylan has been reported, the principal xylan present is a water-soluble homoxylan composed of β-1,3- and β-1,4-linked D-xylose units, with a proportion of 60-70% β-1,4 and 30-40% β-1,3, which are irregularly distributed and vary with season and the method of extraction. This xylan was shown in some studies to be partly acidic, likely due to the presence of sulfate, phosphate

and proteins, but this was not confirmed in other studies [36]. Interestingly also, this xylan has been found to be only weakly held in the cell wall matrix by hydrogen bond interactions [41]. Nevertheless, notwithstanding its structural makeup favouring xylan extraction, namely the absence of lignin, low cellulose levels, weakly held nature of xylan, and its high xylan content, *Palmaria palmata* has apparently been overlooked as a feedstock for xylan production.

3. Biotechnological Applications of Xylan

Three main routes are being developed for xylan application: as dietary fibre in food and feed applications; in materials, mainly in biocomposites production; and as a feedstock for breakdown to xylo-oligosaccharides and xylose for conversion to high-value products. In addition, its direct use in cosmetics, in skin and hair care, and as a substrate for enzyme activity assays have also been reported. In the former, it is suggested for use as a skin moisturiser, anti-wrinkle and anti-aging cream, and for hair and skin regeneration [42, 43]. In the latter, it is commonly used in R&D as a substrate for detection and measurement of activity of xylan hydrolytic enzymes, namely xylanases which degrade the xylan backbone [44].

Xylan has found use in food and feed applications where they can be integrated into food/feed and beverages to enhance health effects, or even consumed directly, as dietary fibre with health benefits such as the lowering of blood cholesterol and decrease in postprandial glucose and insulin responses [45].

Interest in its use in the production of bio-based products of industrial value has grown considerably in recent years due to the acknowledgment of this polysaccharide as a sustainable, abundant, renewable resource with potential for replacement of many petroleum based products. Nevertheless, a current limitation in its more widespread use is associated with a difficulty in obtaining high purity xylan and xylan hydrolysis products in large quantities at low cost. Furthermore, in relation to its use as a feedstock for hydrolysis to value-added products, difficulties have been encountered in the conversion of its monomeric units, specifically the pentose sugars xylose and arabinose, to value-added products, but recent advances in chemical conversion processes and, more importantly, in metabolic engineering, have been successful in making major advances to overcoming this limitation.

Xylan is currently being developed for use in a variety of materials and biocomposite preparations such as in films for packaging and hydrogels for water remediation, drug delivery and tissue regeneration [46]. Its biocompatibility and non-toxic characteristics as well as the ability to chemically modify its properties favour its use in such applications. Difficulties in film formation and solubility related issues has interfered with a more widespread use of xylan in packaging applications but recent studies have shown promising results, namely an increased biofilm hydrophobicity and decreased solubility, upon combination of xylan with compounds such as polyvinyl alcohol (PVA), nanofibrillated cellulose, sodium monochloroacetate, or/and glycerol [47-51]. Presently, xylan based films have been developed for packaging of oils, fats and fruits [52]. In relation to their use in hydrogels, synthetic hydrogels have been massively replaced by polysaccharide-based hydrogels due to the latter's structural variety, sustainability, and more simplified production. Such hydrogels are able to retain water up to a thousand times their dry mass, a characteristic that is required in many biomedical applications such as in drug delivery [53, 54]. Xylan hydrogels have also been shown to have potential use in tissue regeneration, where the addition of xylan to chitosan hydrogels showed improved rates of recovery for bone tissue regeneration when injected in rats [55, 56]. Importantly also, xylan is an ideal carrier for colon drug delivery as it is resistant to the human digestive system and is only processed in the human colon, thereby improving targeted delivery [57]. Finally, xylan based hydrogels have also found application in water remediation for desalination and heavy metal purification as they can be easily regenerated and the metals recovered for further use.

The principal valorisation route for xylan in the future is likely to be as a feedstock for the production of xylo-oligosaccharides and monomer sugars. Xylo-oligosaccharides are low molecular weight breakdown products of xylan with degrees of polymerisation of between 2-10 backbone xylose units [59, 60]. They can occur naturally at very low concentrations in some foods, such as fruits, honey and vegetables [58] but currently most xylo-oligosaccharides are produced by hydrolysis of xylan to xylo-oligosaccharides by high temperature, acid, alkali and/or enzyme treatment [58, 59]. Of these, enzymatic hydrolysis, with endo-β-1,4-xylanases (also known as endoxylanases), has gained much interest as it is the most specific and eco-friendly approach for xylo-oligosaccharide production and can avoid byproduct and xylose production [60, 61]. At present, it appears that almost all xylo-oligosaccharides are produced from land plant xylan and thus, depending on the xylan source and their production process, they may be substituted to varying degrees with various side-chain groups. Such difference in structures leads to differences in properties and

functionalities of the xylo-oligosaccharides [62]. Xylo-oligosaccharides have been shown to have a number of potential applications, with a principal focus being as prebiotics with a market value of ≈3.5 billion Euros (in 2017) this being spurred on by recent customer trends towards preventative health and healthier foods. Xylo-oligosaccharides are non-digestible oligosaccharides which are resistant to digestion in the gastrointestinal tract and can stimulate the growth and/or activity of beneficial intestinal bacteria [61]. They can stimulate growth of *Bifidobacteria*, *Bacteroides* and *Lactobacilli* and augment production of short chain fatty acid (acetate, propionate, butyrate, etc.) and lactate with positive health effects [63, 64]. Interestingly, they have been shown to be more resistant to both acidic pHs and high temperatures and more effective than other commonly used prebiotics, such as fructo-oligosaccharides and inulin [65, 66] and thereby should enable a more ready market acceptance. In addition to use as prebiotics, a number of other health related applications have also been reported for xylo-oligosaccharides, these include as anti-cancer agents, anti-oxidants and anti-microbials as well as immunostimulatory and anti-inflammatory agents and in diabetes treatment/prevention [67, 68]. Furthermore, due to a low-calorie value and sweet taste, e.g. xylobiose has a relative sweetness of 0.34 as compared to sucrose [69], they are suited to use as alternative low calorie sweeteners, especially in anti-obesity diets and thereby responding to the global obesity crisis and the introduction of a 'sugar tax' by various governments. Further potential food/feed applications include as emulsifying, stabilizing and fat replacer agents, while their application as anti-oxidants and moisturisers in cosmetics has also been described [66, 70-73].

In relation to the production of monomer sugars from xylan, complete xylan hydrolysis by enzymatic, physical or/and chemical treatment can lead to production of various monomers including xylose, arabinose, galactose, mannose, glucuronic acid and glucose. These can serve as platform intermediates for conversion to various high value products, yet the abundant xylan pentose monomers xylose and arabinose are currently underutilised due to difficulties associated with their conversion and their unavailability as highly pure commodity chemicals. Nevertheless, as previously mentioned, modern science is currently making advances in overcoming these limitations and these pentoses have already been shown to be efficient platform intermediates for the manufacture of various value-added industrial products with application in such diverse areas as platform chemicals, biofuels, health care, food and feed, ingredients, pharmaceuticals, solvents, and materials. Example products include xylitol for use in the food, dental and pharmaceutical industries [74-76]; xylonic acid for application in the food industry and construction, and as precursor to products such as polyamides and 1,2,4-butanetriol [77, 78]; bioethanol for biofuels

and conversion to further added-value products [79]; isopropanol for use as industrial solvent and precursor to propylene; lactic acid for use in packaging, textile and pharmaceutical industries [80-82]; and, furfural for use as a solvent or as an additive in products such as fungicides, inks and flavouring agents, as well as to produce other chemicals of interest such as polyurethane, furan, butanol, furoic acid, among others [83-86]

4. Xylan Degradation

The heterogeneity and complexity of xylan, as well as its variable accessibility in plant cell walls makes it highly recalcitrant to complete degradation. Indeed, micro-organisms have been required to develop strategic methodologies so as to enable its utilisation as a carbon source [12, 87]. Many micro-organisms have developed complex hydrolytic systems composed of multiple different, specialised enzymes cooperatively-acting towards a complete and efficient degradation of xylan into fermentable sugars for metabolism, and thus also contributing to the equilibrated recycling of hemicellulose in nature [88-90]. This xylanolytic system (Fig. 2) includes endo-β-1,3/1,4-D-xylanases (EC 3.2.1.8/EC 3.2.1.32) acting on the xylan backbone, and β-D-xylosidases (EC 3.2.1.37) releasing xylose from the non-reducing end, for the degradation of the xylan main chain, and acetylxylan esterases (EC 3.1.1.72), α-L-arabinofuranosidases (EC 3.2.1.55), α-D-glucuronidase (3.2.1.139) and ferulic acid esterases (EC 3.7.1.73) for the release of the side-groups attached to the main chain (Fig. 2) [12, 91, 92]. Many micro-organisms possess all these xylanolytic activities so as to enable for a more complete substrate hydrolysis, and frequently also possess multiple variants of the same enzymes, with different specificities and modes of action, to enable for a more efficient and greater extent of hydrolysis [12]. Typical examples of micro-organisms which have multiple xylanolytic activities include, *Spirochaeta thermophila* with xylanase, xylosidase, and arabinofuranosidase enzyme activities; *Bifidobacterium adolescentis*, *B. infantis* and *B. bifidum* with xylosidase and arabinosidase activities; *Anaerocellum thermophilum* with xylanase and xylosidase activities and diverse filamentous fungi with multiple xylanases, e.g., *Aspergillus ochraceus*, with three independent xylanases; *Trichoderma reesei*, with six independent xylanases and *Talaromyces versatilis,* with four independent xylanases [93-97].

Of these xylanolytic enzymes, the xylan backbone degrading endoxylanases (EC 3.2.1.8/EC 3.2.1.32) are the most critical and indeed also the most thoroughly studied and most used in

industry today. They are a focus of the present thesis and their classification and potential for application will be now presented.

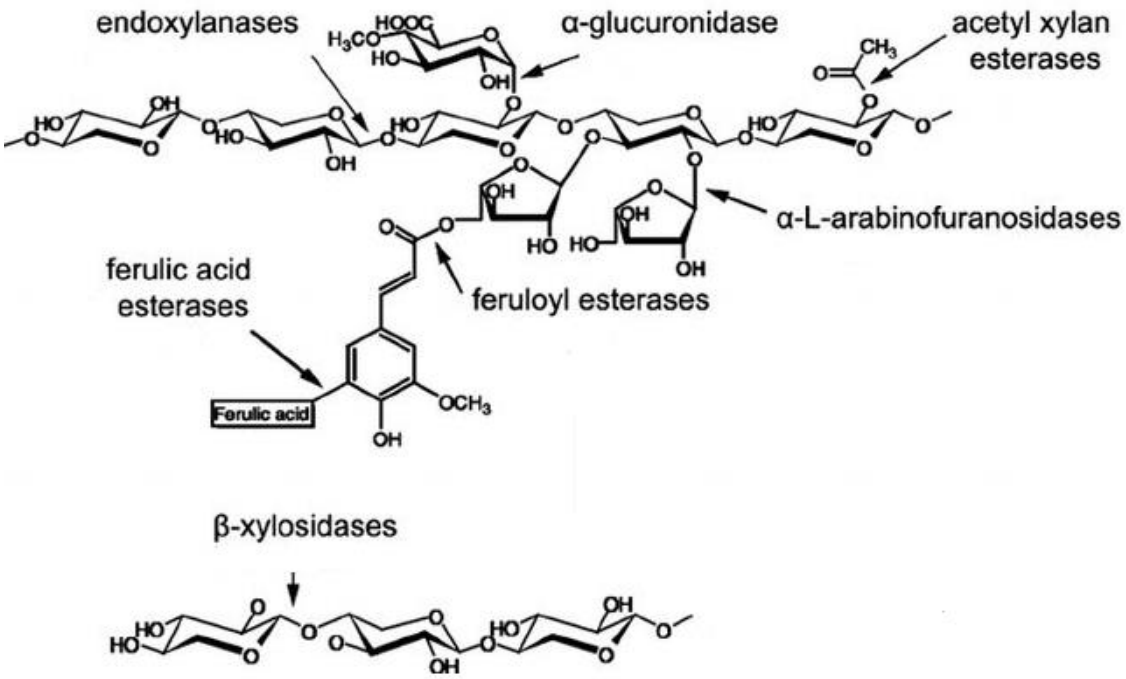

Fig. 2. Representation of the structure of xylan and the sites of its attack by various xylanolytic enzymes. Adapted from Sun, J. et al 2012 [98].

5. Classification of Xylanases

Enzymes acting on carbohydrates have been principally classified with two different classification systems. The Enzyme Commission (EC) number system, proposed by the International Union of Biochemistry and Molecular Biology (IUBMB), is based on the biochemical properties of enzymes, namely the reaction catalysed, substrate specificity and in some cases even the molecular mechanism. Of the 6 classes of enzymes currently recognised by this system, the hydrolases (EC number 3) are the most widespread used. Within this group, xylanases, or endoxylanases, are classified as glycosidases (EC 3.2.1.) that catalyse the hydrolysis of β-1,4- or β-1,3-D glycosidic bonds between internal xylose units in xylans (EC 3.2.1.8 and EC 3.2.1.32, respectively). In 1991, Henrissat suggested another classification system that divides enzymes into glycoside hydrolase families (GH) based on the primary sequence of the catalytic domain [99]. This created a much more robust classification scheme, where enzymes with analogous structures, specificity and mechanism of action were grouped together into families. It takes into account the evolutionary

relationships of enzymes and provides a powerful means for functional annotation, structure-function analysis, protein engineering and enzyme development for application. Based on this classification system, in September 1998 the Carbohydrate-Active enZYmes database (CAZy: http://www.cazy.org/) was proposed and adopted worldwide for a more complete classification of not only xylanases, but glycoside hydrolases in general and indeed of all carbohydrate-active enzymes that degrade, modify, or create glycosidic bonds. Currently, the CAZy database contains 168 glycoside hydrolase (GH) families and is the accepted classification system for glycoside hydrolases.

Most of the xylanases studied to date are found in GH-families 10 and 11, and were for quite some time believed to be solely confined to these 2 families. Indeed, xylanases present in these families comprise almost 90% (around 600) of all characterised xylanases in the CAZy database. Nevertheless, in 2005, Collins et al. documented 4 additional families containing xylanases and, ever since, the number of xylanase containing families and characterised members has continuously increased [12]. The presence of xylanases in GH families other than GH10 and 11 is now firmly acknowledged and in fact the numbers and investigations of these is growing rapidly as the exhibition of often novel, unique characteristics and/or substrate specificities by these heralds an important industrial potential.

Unfortunately, some ambiguity still exists in relation to the classification of xylanases within CAZy, in particular as to the actual number of GH families harbouring catalytic domains with true endoxylanase activity, and quite divergent numbers of xylanase containing families have been reported by different authors (Chakdar et al. 2016; Nguyen et al. 2018; Nordberg Karlsson et al. 2018). Our systematic study indicates that, presently, 12 different CAZy GH families containing enzymes with a demonstrated endoxylanase activity have been identified: GH5, GH7, GH8, GH10, GH11, GH12, GH26, GH30, GH43, GH51, GH98 and GH141. Xylanases are also listed in CAZy for GH16 and GH48, but a closer inspection shows these to be erroneously classified multifunctional proteins with multiple domains wherein the whole protein, including the xylanase catalytic domain, has been classified together in the families of each of its individual domains, in these cases, the GH16 or GH48 domains [100, 101]. Here we review the 12 different xylanase containing CAZy GH families identified and their xylanase members, with a focus on the diverse characteristics and substrate and linkage specificities of the diverse families. Taxonomic aspects and the application potential of the various specific families are also discussed.

5.1. Glycoside Hydrolase Family 5

GH5 sequences have been identified in a wide variety of organisms, predominantly bacteria and fungi, but have also been reported in archaea, plants, protists and metazoa, the latter being restricted to nematodes, molluscs, annelids and arthropods. GH5 is one of the largest and most diverse glycoside hydrolase families, with more than 17069 sequences of which 564 have been characterised, and with 27 different enzyme activities listed. In addition, many GH5 members are characterised by a broad substrate specificity, displaying activity on multiple substrates.

GH5 enzymes adapt a $(\beta/\alpha)_8$-barrel fold structure composed of eight parallel β-strands surrounded by eight α-helices and connected by long flexile loops at the larger top surface of the molecule and by simple α/β turns at the smaller surface on the bottom of the barrel [102-104]. Additional helices on the top and/or bottom of the barrel have also been observed in various proteins. This is the most common protein fold known, about 10% of all known enzymes display this structure, and it is observed in 24 different glycoside hydrolase families of CAZy. Among GH5 enzymes, only 7 amino acids are strictly conserved and catalysis occurs with retention of the anomeric configuration [104-106].

Initially, GH5 was composed solely of cellulases [107], however, the increasing number of submitted sequences and observed specificities and activities over the years has demanded for a number of revisions and reappraisals of the classification of this family. As a result, various enzymes, including endo-xylanases, were reclassified to GH30, and GH5 was sub-divided into 56 subfamilies, with enzymes listed in CAZy as having endo-xylanase activities being currently found in subfamilies GH5_4, GH5 21, GH5_25 and GH5_34 [108, 109].

Subfamily GH5_4 members are extracellular polyspecific enzymes that have been predominantly isolated from gastrointestinal and rumen bacteria and fungi. A large number or enzymes in this subfamily display activity on xylan, and Glasgow et al. even detected activity on beechwood xylan in 86% of the members examined [110]. Nevertheless, this activity is a side or subsidiary activity of much reduced intensity relative to the preferred substrates, which, depending on the enzyme, may be CMC, lichenan, mannan, or xyloglucan.

GH5_25 members also display a high catalytic promiscuity yet with a potential preference for mannan substrates. This subfamily is represented by both extracellular and intracellular enzymes and is dominated by thermophiles yet with recent identification of cold-adapted and mesophilic members [111, 112]. The presence of enzymes with endoxylanase activity in the subfamily is controversial. Hydrolysis activity was reported for two enzymes on beechwood xylan yet the same enzymes were not found to display activity on birchwood xylan when tested by other groups [110, 113]. On the other hand, a phylogenetically distinct member (ABN52701.1, from *Hungateiclostridium thermocellum* ATCC 27405) is listed in CAZy as displaying endoxylanase activity and indeed relatively high activities on beechwood, birchwood and arabino- xylans were reported [104]. Indeed suggestions have been made for classification of this enzyme as a separate phylogenetic group, or subfamily, and raises the questions as to whether a relatively high xylanase activity in GH5_25 is restricted to this group. Further studies are called for to better understand this.

In relation to the applied potential of subfamilies GH5_4 and GH5_25 members, their broad substrate specificity could be of advantage in biomass degradation applications, such as in the production of biofuels and various chemicals, by enabling an enhanced accessibility to and hydrolysis of cellulose, or even a more efficient co-degradation of multiple biomass polysaccharides for the production of fermentable hexose and pentose monosaccharides.

In contrast to the GH5 subfamilies described above which are polyspecific, initial studies indicate subfamilies GH5_21 and GH5_34 enzymes as being mono-specific and solely active on arabinoxylan. Only 36 members of GH5_21 and 9 of GH5_34 have been identified and only a few of these have been only incompletely characterised.

GH5_34 enzymes are mostly bacterial, extracellular, multi-domain proteins, with the exception of a single domain aquatic fungal enzyme (KXS18720.1). These enzymes have been shown to be highly specific for arabinoxylan degradation, with no activity on arabinose unsubstituted xylan, or on various glucan, mannan, arabinan, pectin or galactan substrates. Thus, GH5_34 enzymes can be classified as appendage specific xylanases, or more specifically, arabinoxylan-specific xylanases [114, 115]. Studies have shown that they specifically hydrolyse the backbone β-1,4-D-xylosidic linkage immediately subsequent to (i.e., towards the reducing end of) α-1,3-arabinose substituted xylose and thereby release products with a reducing end arabinose decorated xylose.

GH5_21 is composed of extracellular, possibly cell bound, bacterial enzymes from members of the *Bacteroidetes* phylum. Unfortunately, only a few studies have been reported on enzymes belonging to this subfamily, yet these suggest a potential specificity for arabinoxylan, similar to GH5_34 members [105, 116]. Regrettably, studies carried out on this subfamily were incomplete, and further studies to determine the true substrate and linkage specificity, product profiles and mechanisms of action of this sub-family are clearly required.

Finally, and importantly, enzymes from both these subfamilies GH5_21 and GH5_34 have potential as novel tools in the breakdown and valorisation of various arabinosylated substrates as well as in the design of specific xylo-oligosaccharides. In particular they should prove valuable in the degradation of highly substituted substrates such as rye and corn xylan which are recalcitrant to the currently employed GH10 and GH11 xylanases.

5.2. Glycoside Hydrolase Family 8

Of the 6332 members (79 characterised) belonging to GH8, only 8 have been shown to be xylanases. Interestingly, among other glycoside hydrolase activities, a special type of xylosidase/xylanase called a reducing-end-xylose releasing exo-oligoxylanase (EC 3.2.1.156) can also be found in this family. The majority of GH8 members are bacterial extracellular single domain enzymes, but a small number or multi-domain variants and eukaryotic derived enzymes have also been described. Five of the 8 xylanases are cold-adapted enzymes, characterised by high activities at low to moderate temperatures and reduced stabilities [117-121]. The remaining three are a thermolabile enzyme from a thermophilic host [122], and enzymes from human [123] and insect [124] gut microbiota.

GH8 xylanases are classified as “true” endoxylanases due to their high specificity for the xylan substrate, with no activity having been reported on any other substrate. They are active on a variety of xylan structures (i.e., birchwood, beechwood, rye, wheat, oat spelt and mixed linkage β-1,3/ β-1,4 xylans), exhibiting highest activity on high molecular weight, low substituted substrates. Indeed, a significant decrease in activity is observed for highly substituted xylans and for short-chain xylo-oligosaccharides, being mainly inactive on xylo-oligosaccharides with degrees of polymerisation ≤ 5 [117, 123, 124] and with studies suggesting the presence of up to 7 subsites [125].

The architectural design of GH8 members is an $(\alpha/\alpha)_6$ barrel, this is not shared by any other xylanase family, and they catalyse hydrolysis with inversion of the anomeric configuration. Within the catalytic site of GH8 enzymes, a glutamic acid residue has generally been accepted to function as the general acid while the general base residue has been found to vary. In fact, further division of GH8 members into 3 subfamilies based on the identity of the proton acceptor has been suggested, and xylanases are all found in subfamily A with an aspartic acid residue serving as the proton acceptor [126].

The specific characteristics of the GH8 endo-xylanases, in particular their tendency for preference of high molecular weight unsubstituted substrates and production of substituted high molecular weight products, lend them to various applications in which incomplete xylan hydrolysis is desired. For example, they can be employed in the production of high molecular weight highly substituted arabinoxylans for use as soluble dietary fiber in the food industry [127]. In addition, they can be employed in bread making to enhance dough stability, bread volume and crumb structure by solubilising insoluble arabinoxylan and producing high molecular weight solubilised arabinoxylans. Here, avoidance of over-hydrolysis of soluble and insoluble arabinoxylan is critical as this leads to undesirable sticky doughs and hence is suited to GH8 endoxylanases [128, 129].

5.3. Glycoside Hydrolase Family 10

GH10 contains the highest number of characterised xylanases among all GH families, presently 347 xylanases have been characterised of the total 4454 GH10 sequences identified. GH10 enzymes are mostly found in bacteria from various environments (the digestive tract, terrestrial soils, sedimentary environments, etc.), but are also reported in fungi and some plants [130-133].

The grand majority of xylanases allocated to this family have been described as monospecific, with different preferred xylan substrates depending on the enzyme. Nevertheless, a number of polyspecific enzymes have also been described, with low activities relative to their activity on xylan being also observed on low molecular mass cellulose substrates, xyloglucan, and/or glucomannan. In addition, GH10 enzymes displaying feruloyl esterase (3.1.1.73), cellobiohydrolase (3.2.1.-), alpha-arabinosidase and/or xylan endotransglycosylase (2.4.2.-) activities have also been reported [134-136]. GH10 enzymes are active on short chain xylo-oligosaccharides, are believed to have an active site composed of 4 to 5 subsites, and typically release xylobiose as the principal final

hydrolysis product with lower amounts of xylose, xylotriose and xylotetraose [134-137]. They can hydrolyse decorated substrates but require at least two unsubstituted xylose residues between branched residues. It has been shown that they can cleave the xylosidic linkage on the nonreducing end of a substituted residue but can only hydrolyse the third xylosidic linkage after a substituted xylose, thus giving rise to non-reducing end substituted xylo-trimers as the smallest potential decorated product [138].

As for GH5 enzymes, GH10 xylanases display a $(\beta/\alpha)_8$-barrel fold structure, also known as a TIM fold architecture, in which the two glutamic acid catalytic residues are held in β-strands 4 and 7, and catalysis occurs through the double displacement mechanism that conserves the anomeric configuration [139, 140]. Several members of this family can deviate from this architecture, displaying amino acid substitutions that form for example wider clefts that gives the enzyme the ability to degrade more branched xylans [141-143].

The xylanases in this family were among the first to be discovered and a number of family 10 enzymes have already found application, in particular in: improving the digestibility of animal feed stocks, leading to better nutrient utilisation and growth performance; in the textile industry, enhancing the bleaching and finishing stages via enhanced accessibility of chemicals; in the production of xylo-oligosaccharides and/or xyloses; and in the bioconversion of lignocellulosic wastes into useful economical products such as ethanol [72, 144-148].

5.4. Glycoside Hydrolase Family 11

GH11 is the GH family with the second highest number of characterised xylanases (268 of 1857 members). Similar to GH10, the majority of GH11 xylanases are found in bacteria and fungi but are also found in plants and insects [149-151].

GH11 xylanases are often called 'true' xylanases because they specifically target xylans and have no activity on any other substrate [152, 153]. A few exceptions to this rule have been reported, with activity on substrates other than xylan, including laminarin, lichenan, starch and pectin, as well as acetyl group removal from xylan (3.1.1.72), being demonstrated, yet these activities were very reduced or even negligible [149, 154]. GH11 xylanases are active on a wide variety of xylan types but the substrate of choice of most enzymes is believed to be glucuronoxylan, with slightly enhanced activity being observed for this [149, 154]. The predominant products of hydrolysis are

xylobiose and xylotriose, usually without release of xylose, and accompanied by larger xylo-oligosaccharides when acting on branched xylans [155, 156]. GH11 xylanases are less active on short chain xylo-oligosaccharides than GH10 enzymes and more hindered by side chain groups, thereby resulting in larger products which can serve as substrates for GH10 xylanases. Like GH10, they require at least two unsubstituted xylose residues between arabinosyl branched residues, but, in contrast, require at least three in glucuronyl and 4-*O*-methyl-glucuronyl substituted substrates. In further contrast to GH10, they can only cleave the second xylosidic linkage on the nonreducing end of a substituted residue, but can hydrolyse the second or third xylosidic linkage after, respectively, a arabinofuranosyl or 4-*O*-(methyl)-glucuronyl substituted xylose [155, 157].

GH 11 members are low molecular weight enzymes which catalyse hydrolysis via the retaining mechanism. They display a β-jelly roll fold structure consisting of two packed antiparallel β-sheets and an α-helix in which the two β-sheets are twisted and create a deep, narrow and long cleft which harbours the two glutamic acid catalytic residues [157, 158].

Together with GH10 enzymes, GH11 enzymes were among the first xylanases studied. A large variety of these are now commercially available from a variety of manufacturers and are commonly used in such areas as the pulp and paper industry and baking where their low molecular weight, believed to enable enhanced penetration into the pulp and paper macrosubstrates, and substrates specificity are advantageous [128, 159].

5.5. Glycoside Hydrolase Family 26

GH26 contains a total of 2444 members, including 76 biochemically characterised enzymes of which 6 are confirmed endo-1,3-β-D-xylanases (EC 3.2.1.32). This family contains most of the characterised endo-β-1,3-xylanases in the CAZy database but these have only been scantly studied. GH26 xylanases are only found in bacteria, mainly in proteobacteria that inhabit marine environments, which might be expected as β-1,3-xylan is believed to be only found in marine algae [160-162]. These enzymes specifically hydrolyse the β-1,3 glycosidic bonds between xylose residues in a xylan backbone and no activity has been detected on β-1,4 linked xylan. Hydrolyses products for these enzymes are mainly low molecular weight xylo-oligosaccharides, or even xylose following longer reaction periods [161-163]. To date, no structure of any GH26 xylanase has been reported, but similar to other members of this family these enzymes would be expected to possess

a $(\beta/\alpha)_8$ barrel structure and catalyse hydrolysis by a retaining double-displacement mechanism orchestrated principally by two glutamic acid catalytic residues [160, 161, 164].

5.6. Glycoside Hydrolase Family 30

Currently, GH30 is composed by 3023 members with only 39 having being characterised. Almost half of the characterised enzymes are endo-β-1,4-xylanases, some of which were former GH5 members that were reclassified into GH30 due to their closer evolutionary relationship. GH30 is divided into 9 subfamilies, based on the secondary structure arrangement of the side β-structure, and xylanases are allocated in subfamilies 7 and 8 [165-167].

Subfamily GH30_7 includes exclusively fungal xylanases from the Ascomycota phylum which prioritise glucuronoxylan hydrolyses, although they have also been shown to be active on other xylan substrates such as wheat arabinoxylan, oat spelt xylan and even carboxy-methylcellulose [168-170]. On the other hand, subfamily GH30_8 consists of bacterial enzymes, except for a xylanase identified in a nematode, which, contrary to subfamily 7, were shown to be specific for (methyl)glucuronoxylans and with the majority being appendage specific glucuronoxylanases [165, 171, 172].

The appendage specific glucuronoxylanases of GH30_8 are selective enzymes in which the presence of (methyl)glucuronic acid sidechains is essential for activity [173]. These enzymes recognise the glucuronic acid sidechain and cleave at the second xylosidic bond following the branch towards the reducing end. The -2 subsite of the active site of these enzymes accommodates a uronic acid decorated xylopyranose unit in which the uronic acid is stabilised by both ionic interactions and hydrogen bonds with specific active site amino acids. Thus, the xylo-oligosaccharides produced are characterised by the presence of a single glucuronic acid appendage. These enzymes generally do not have detectable activity on arabinoxylans or linear unsubstituted xylo-oligosaccharides [167, 168, 172].

All enzymes within the GH30 family contain a $(\beta/\alpha)_8$ catalytic domain closely associated to a nine-stranded side beta-structure ($(\beta/\alpha)_8$ + β fold) [166]. The side structure was initially believed to form together with the TIM barrel the catalytic centre and was later confirmed to play a crucial role in enzyme activity [166, 174]. Similar to GH5, GH10 and GH26 enzymes, with a similar structure of the catalytic domain, enzymes of GH30 catalyse hydrolysis via a double displacement

mechanism with retention of the anomeric configuration in which two glutamic residues play the role of the essential catalytic residues [166, 175].

GH30 enzymes could be important in product design, specifically in the design of various substituted xylo-oligosaccharides with diverse structures, properties and potential applications.

5.7. Glycoside Hydrolase Families 7, 12, 43, 51, 98 and 141

In addition to the GH families discussed above, xylanases have also been classified in GH families 7, 12, 43, 51, 98 and 141. Unfortunately only one xylanase has been studied in each of these families.

The only GH7 enzyme with xylanase activity studied to date was isolated from the fungus *Trichoderma reesei* [176, 177]. It was found to be an endoglucanase with slightly lower activity on both beechwood and grass xylan but as much as a 10-fold lower activity on short chain xylo-oligosaccharides than cello-oligosaccharides . Similar to GH10 enzymes, it has a small active site constituted by around 4 subsites, but similar to GH11 enzymes it releases a uronic acid substituted xylotetramer (aldopentauronic acid) as smallest product from glucuronoxylan. While the structure of this xylanase was not determined, similar to the other GH7 enzymes it is expected to display a β-jelly roll fold structure and catalyse hydrolysis with retention of the anomeric configuration [176-178]

GH12 houses a single archaeal non-specific xylanase. This thermoacidophilic xylanase (SSO1354), isolated from *Saccharolobus solfataricus*, is a single domain enzyme capable of hydrolysing, in decreasing order, beechwood xylan, oat spelt xylan and birchwood xylan [179, 180], with xylobiose and xylotriose as the main enzymatic products followed by medium sized xylo-oligosaccharides [179]. GH12 enzymes are characterised by a beta-jelly roll fold structure and catalyse hydrolysis with retention of the anomeric configuration [181-183].

As much as 18353 sequnces are classified in GH43, of which 183 have been characterised, but only one member has been reported to have endoxylanase activity. This is a 64 kDa enzyme, XYND, from *Paenibacillus polymyxa* which was reported to have both xylanase and α-L-arabinofuranosidase activities [132]. No other studies were carried out on this enzyme and further investigations are required to examine this enzyme and family further, and to verify the existence and importance of xylanase activity in this GH family.

GH51 contains 4146 sequences but only 83 have been characterised, including a non-specific xylanase from the gram-positive bacterium *Alicyclobacillus acidocaldarius*. Similar to the GH12 xylanase, this is a thermoacidophilic enzyme. More specifically, it is an endoglucanase that operates at temperatures up to 90 °C and pHs as low as pH 2-6, but also hydrolyses oat spelt xylan, carboxymethyl cellulose (CMC) and non-crystalline cellulose [12, 184]. This enzyme presumably cleaves its substrates via the retaining mechanism and the main reaction products are xylobiose and xylotetraose [184]. The catalytic structure of family GH51 enzymes is a $(\beta/\alpha)_8$ structure that is linked to a beta-sandwich domain consisting of 12 strands [185, 186].

For over 10 years, GH98 was solely composed of blood-group-substance endo- β-1,4-galactosidases (EC 3.2.1.102), until a novel specific glucuronoarabinoxylan endo-1,4-β-xylanase, isolated from *Bacteroides ovatus*, a human gut inhabiting bacterium, was attributed to this family [116, 187]. This enzyme displays no activity on glucuronoxylan nor arabinoxylan, but is extremely specific for corn glucuronoarabinoxylan and releases large sized xylo-oligosaccharide products. Studies revealed that substrate recognition depends strongly on side-chains containing both 3-*O*-β-xylose and 2-*O*-α-arabinose substitutions on a backbone xylose unit [116]. These double substitutions are thought to be relatively uncommon within the corn xylan and hence the observed large xylo-oligosaccharide products [116]. GH98 members display a $(\beta/\alpha)_8$ fold structure linked with a 11-stranded beta-sandwich structure and cleave substrates with inversion of the anomeric configuration [188, 189].

Lastly, GH141, with 434 sequences, has only two characterised members to date, an alpha-fucosidase and a xylanase. The xylanase (Xyn141E) was isolated from the thermophilic bacteria *Clostridium thermocellum* and catalyses hydrolysis of several types of xylans (glucuronoxylans and arabinoxylans), as well as other substrates such as CMC, barley beta-glucan and mannan at lower levels [135, 136, 190]. Hydrolyses produces xylo-oligosaccharides with degrees of polymerisation between 2 and 5 and several short decorated oligosaccharides [190]. The 3D structure of the xylanase has not been determined but investigation of the structure of the alpha-fucosidase of this family shows this to consist of a beta-helix catalytic domain linked to a beta-sandwich, this being a distinct catalytic fold to all other xylanase containing families (Ndeh et al. 2017).

6. Industrial Applications of Xylanases

Xylan is ubiquitous in plants and can have a major impact on the quality, mechanical, physical, chemical and organoleptic properties of plants, their extract and their products, be they for the food, feed or technical industries, as well as having an impact on the processing and/or manufacturing processes for these. Indeed properties such as the xylan concentration, composition, interactions and structure strongly impact numerous products and processes and the potential for selective modification and/or transformation of these by endo-xylanases highlights the enormous potential of these enzymes in diverse industries. The careful selection of specific xylanases with the desired substrate specificities and product profiles as well as the requisite specific activity and stability can enable for the careful specific modification of xylan to enhance various plant based processes and products. Indeed the value of these enzymes has been understood for quite some time and they have already found application in a wide range of applications, including in the food, animal feed, beverages, paper and biofuels industries [6, 191].

In the pulp and paper industry xylanases have been successfully used in the prebleaching of paper pulps, reduction of the beating times of virgin pulps, restoration of bonding and increased freeness in recycled fibers, as well as in the selective removal of xylans from dissolving pulps [12, 192, 193]. They are also essential in the animal feeds industry where they are used for pretreatment of feeds to hydrolyse the anti-nutritive factor xylan and thereby improve digestibility, reduce intestinal viscosity and hence improve both the weight gain and feed conversion efficiency of the animals [194-196]. In the bakery industry they are used to solubilise insoluble arabinoxylan and produce high molecular weight solubilised arabinoxylans which increase the quality of dough and bread, this improves dough flexibility, machinability and stability, enabling larger loaf volume as well as an improved crumb structure [6, 128]. In combination with cellulases and pectinases, xylanases have also been applied in the clarification and aromatisation of musts, wines and fruit juices, as well as for liquefying fruit and vegetables, and mainly to improve the maceration and extraction processes and lead to products of reduced viscosity and improved clarity [6, 12, 196, 197]. Furthermore, xylanases are classified as promising candidates for the treatment of wastes from agricultural and food industries. Here, the degradation of xylan into xylo-oligomers and xylose facilitates waste recycling and enables the production of numerous value-added biobased products as discussed above. [196]. Indeed xylanases can be used in biomass valorisation in replacing or reducing the use of the hazardous chemical and physical processes currently used in xylo-oligosaccharide and xylose production processes wherein they offer a more specific hydrolysis process avoiding byproduct formation and reducing the environmental impact.

7. The Cold-Adapted GH8 Xylanase

A cold-adapted GH8 xylanase has been isolated and studied by Collins et al. and its valorisation and in-depth characterisation was the focus of this PhD thesis. This xylanase, referred to as pXyl (psychrophilic xylanase), was isolated from the Antarctic bacterium *Pseudoalteromonas haloplanktis* TAH3a and has been extensively studied [117], in particular its relationship with temperature. pXyl was shown to have the typical cold-adapted characteristics of a high activity at low to moderate temperatures, with a maximum activity at 35 °C and retaining 90 % and 60% of its activity at 20 °C and 5 °C, respectively, as well as a low stability with an apparent melting temperature of 53.5 °C. Its tertiary structure was determined and substrate specificity studies indicated it to be highly specific for xylan, functioning as an endo-1,4-β-D-xylanase via an inverting mechanism. The two catalytic residues for this process were identified: glutamic acid 78 functioning as the proton donor and aspartic acid 281 as the proton acceptor. Furthermore, tertiary structure analysis showed this enzyme to consist of 13 α-helices and 13 β-strands where the α-helices form an $(\alpha/\alpha)_6$ barrel with six pairs of inner and outer helices surrounding the central axis (Fig. 3) [117, 198]. Importantly also, this enzyme is specific for xylan, is most active on long chain xylo-oligosaccharides, with 7 subsites having being suggested in its active site, and is insensitive to all known natural xylanase inhibitors [117, 199].

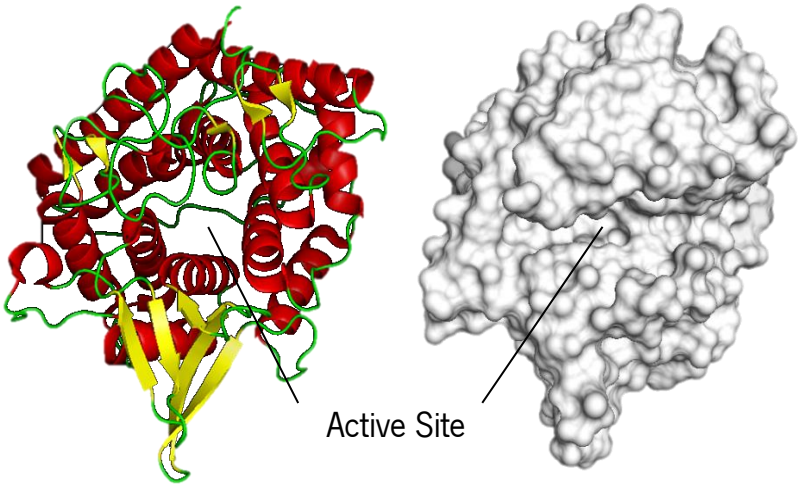

Fig. 3. Image showing a front view of the $(\alpha/\alpha)_6$-fold structure of the cold-adapted xylanase (pXyl). Image prepared with protein database structure 1H13.pdb using Pymol.

The high rate of catalytic activity, insensitivity to inhibitors and specificity of action of pXyl point to a high potential of this for application. In fact, it has already been shown to have a beneficial effect in baking, leading to an increase in loaf size and improvement of bread texture and is now being marketed worldwide for baking applications by Puratos N.V [128, 129].

Notwithstanding the in-depth knowledge available for this enzyme and its commercial success, its full biotechnological value remains to be exploited as it has not yet been tested in applications other than in baking, and the dependence of its performance on other critical industrial process parameters, such as pH, have not been studied in detail. The current thesis aims to address some of these shortcomings.

8. References

[1] A. Ebringerová, T. Heinze, Xylan and xylan derivatives – biopolymers with valuable properties, 1. Naturally occurring xylans structures, isolation procedures and properties, Macromolecular Rapid Communications 21(9) (2000) 542-556.
[2] Y.S.Y. Hsieh, P.J. Harris, Xylans of red and green algae: what is known about their structures and how they are synthesised?, Polymers 11(2) (2019) 354.
[3] Y.M. Bar-On, R. Phillips, R. Milo, The biomass distribution on Earth, Proceedings of the National Academy of Sciences 115(25) (2018) 6506.
[4] P. Alexander, C. Brown, A. Arneth, J. Finnigan, M.D.A. Rounsevell, Human appropriation of land for food: The role of diet, Global Environmental Change 41 (2016) 88-98.
[5] M. Pauly, K. Keegstra, Plant cell wall polymers as precursors for biofuels, Current Opinion in Plant Biology 13(3) (2010) 304-311.
[6] S. Subramaniyan, P. Prema, Biotechnology of microbial xylanases: enzymology, molecular biology, and application, Crit Rev Biotechnol 22(1) (2002) 33-64.
[7] H.V. Scheller, P. Ulvskov, Hemicelluloses, Annual Review of Plant Biology 61(1) (2010) 263-289.
[8] R. Zhong, D. Cui, Z.H. Ye, Secondary cell wall biosynthesis, New Phytol 221(4) (2019) 1703-1723.
[9] S. Ratnayake, C.T. Beahan, D.L. Callahan, A. Bacic, The reducing end sequence of wheat endosperm cell wall arabinoxylans, Carbohydrate Research 386 (2014) 23-32.
[10] A. Teleman, M. Tenkanen, A. Jacobs, O. Dahlman, Characterization of O-acetyl-(4-O-methylglucurono)xylan isolated from birch and beech, Carbohydrate Research 337(4) (2002) 373-377.
[11] W. Mackie, D.B. Sellen, Degree of polymerization and polydispersity of xylan from the cell wall of the green seaweed *Penicillus dumetosus*, Biopolymers 10(1) (1971) 1-9.
[12] T. Collins, C. Gerday, G. Feller, Xylanases, xylanase families and extremophilic xylanases, FEMS Microbiology Reviews 29(1) (2005) 3-23.
[13] J.V. Madeira, F.J. Contesini, F. Calzado, M.V. Rubio, M.P. Zubieta, D.B. Lopes, R.R. de Melo, Chapter 18 - Agro-industrial residues and microbial enzymes: an overview on the eco-friendly bioconversion into high value-added products, in: G. Brahmachari (Ed.), Biotechnology of Microbial Enzymes, Academic Press 2017, pp. 475-511.
[14] D. Dodd, I.K.O. Cann, Enzymatic deconstruction of xylan for biofuel production, Global change biology. Bioenergy 1(1) (2009) 2-17.
[15] A. Ebringerová, Structural diversity and application potential of hemicelluloses, Macromolecular Symposia 232(1) (2005) 1-12.
[16] Z. Zhang, C. Smith, W. Li, Extraction and modification technology of arabinoxylans from cereal by-products: A critical review, Food Research International 65 (2014) 423-436.
[17] S.-L. Chong, L. Virkki, H. Maaheimo, M. Juvonen, M. Derba-Maceluch, S. Koutaniemi, M. Roach, B. Sundberg, P. Tuomainen, E.J. Mellerowicz, M. Tenkanen, O-Acetylation of glucuronoxylan in *Arabidopsis thaliana* wild type and its change in xylan biosynthesis mutants, Glycobiology 24(6) (2014) 494-506.
[18] G. Hilpmann, N. Becher, F.A. Pahner, B. Kusema, P. Mäki-Arvela, R. Lange, D.Y. Murzin, T. Salmi, Acid hydrolysis of xylan, Catalysis Today 259 (2016) 376-380.
[19] J.S. Van Dyk, B.I. Pletschke, A review of lignocellulose bioconversion using enzymatic hydrolysis and synergistic cooperation between enzymes—Factors affecting enzymes, conversion and synergy, Biotechnology Advances 30(6) (2012) 1458-1480.

[20] N. Sorek, T.H. Yeats, H. Szemenyei, H. Youngs, C.R. Somerville, The implications of lignocellulosic biomass chemical composition for the production of advanced biofuels, BioScience 64(3) (2014) 192-201.
[21] T. Yamasaki, A. Enomoto, A. Kato, T. Ishii, M. Kameyama, H. Anzai, K. Shimizu, Enzymatically derived aldouronic acids from *Cryptomeria japonica* arabinoglucuronoxylan, Carbohydrate Polymers 87(2) (2012) 1425-1432.
[22] P. Biely, S. Singh, V. Puchart, Towards enzymatic breakdown of complex plant xylan structures: State of the art, Biotechnology Advances 34(7) (2016) 1260-1274.
[23] C. Jackson, S. Clayden, A. Reyes-Prieto, The Glaucophyta: The blue-green plants in a nutshell, Acta Societatis Botanicorum Poloniae 84 (2015) 149-165.
[24] E. Deniaud-Bouët, K. Hardouin, P. Potin, B. Kloareg, C. Hervé, A review about brown algal cell walls and fucose-containing sulfated polysaccharides: Cell wall context, biomedical properties and key research challenges, Carbohydr Polym 175 (2017) 395-408.
[25] H. Verbruggen, M. Ashworth, S.T. LoDuca, C. Vlaeminck, E. Cocquyt, T. Sauvage, F.W. Zechman, D.S. Littler, M.M. Littler, F. Leliaert, O. De Clerck, A multi-locus time-calibrated phylogeny of the siphonous green algae, Mol Phylogenet Evol 50(3) (2009) 642-53.
[26] E. Frei, R.D. Preston, Non-cellulosic structural polysaccharides in algal cell walls I. Xylan in siphoneous green algae, Proceedings of the Royal Society of London. Series B. Biological Sciences 160(980) (1964) 293-313.
[27] E.D.T. Atkins, K.D. Parker, R.D. Preston, The helical structure of the β-1, 3-linked xylan in some siphoneous green algae, Proceedings of the Royal Society of London. Series B. Biological Sciences 173(1031) (1969) 209-221.
[28] M. Ciancia, J. Alberghina, P.X. Arata, H. Benavides, F. Leliaert, H. Verbruggen, J.M. Estevez, Characterization of cell wall polysaccharides of the coencocytic green seaweed bryopsis plumosa (bryopsidaceae, chlorophyta) from the argentine coast1, Journal of Phycology 48(2) (2012) 326-335.
[29] F. Leliaert, D.R. Smith, H. Moreau, M.D. Herron, H. Verbruggen, C.F. Delwiche, O. De Clerck, Phylogeny and molecular evolution of the green algae, Critical Reviews in Plant Sciences 31(1) (2012) 1-46.
[30] I. Sørensen, J.K.C. Rose, J.J. Doyle, D.S. Domozych, W.G.T. Willats, The Charophycean green algae as model systems to study plant cell walls and other evolutionary adaptations that gave rise to land plants, Plant signaling & behavior 7(1) (2012) 1-3.
[31] I. Sørensen, F.A. Pettolino, A. Bacic, J. Ralph, F. Lu, M.A. O'Neill, Z. Fei, J.K.C. Rose, D.S. Domozych, W.G.T. Willats, The charophycean green algae provide insights into the early origins of plant cell walls, The Plant Journal 68(2) (2011) 201-211.
[32] J.K. Jensen, M. Busse-Wicher, C.P. Poulsen, J.U. Fangel, P.J. Smith, J.-Y. Yang, M.-J. Peña, M.H. Dinesen, H.J. Martens, M. Melkonian, G.K.-S. Wong, K.W. Moremen, C.G. Wilkerson, H.V. Scheller, P. Dupree, P. Ulvskov, B.R. Urbanowicz, J. Harholt, Identification of an algal xylan synthase indicates that there is functional orthology between algal and plant cell wall biosynthesis, New Phytologist 218(3) (2018) 1049-1060.
[33] J.R. Turvey, E.L. Williams, The structures of some xylans from red algae, Phytochemistry 9(11) (1970) 2383-2388.
[34] S.H. Brawley, N.A. Blouin, E. Ficko-Blean, G.L. Wheeler, M. Lohr, H.V. Goodson, J.W. Jenkins, C.E. Blaby-Haas, K.E. Helliwell, C.X. Chan, T.N. Marriage, D. Bhattacharya, A.S. Klein, Y. Badis, J. Brodie, Y. Cao, J. Collén, S.M. Dittami, C.M.M. Gachon, B.R. Green, S.J. Karpowicz, J.W. Kim, U.J. Kudahl, S. Lin, G. Michel, M. Mittag, B.J.S.C. Olson, J.L. Pangilinan, Y. Peng, H. Qiu, S. Shu, J.T. Singer, A.G. Smith, B.N. Sprecher, V. Wagner, W. Wang, Z.-Y. Wang, J. Yan, C. Yarish, S. Zäuner-Riek, Y. Zhuang, Y. Zou, E.A. Lindquist, J. Grimwood, K.W. Barry, D.S. Rokhsar, J. Schmutz, J.W.

Stiller, A.R. Grossman, S.E. Prochnik, Insights into the red algae and eukaryotic evolution from the genome of *Porphyra umbilicalis* (Bangiophyceae, Rhodophyta), Proceedings of the National Academy of Sciences (2017) 201703088.
[35] A.G. Viana, M.D. Noseda, A.G. Gonçalves, M.E.R. Duarte, N. Yokoya, M.C. Matulewicz, A.S. Cerezo, β-D-(1→4), β-D-(1→3) 'mixed linkage' xylans from red seaweeds of the order Nemaliales and Palmariales, Carbohydrate Research 346(8) (2011) 1023-1028.
[36] E. Deniaud, B. Quemener, J. Fleurence, M. Lahaye, Structural studies of the mix-linked β-(1->3)/β-(1->4)-D-xylans from the cell wall of *Palmaria palmata* (Rhodophyta), Int J Biol Macromol 33(1-3) (2003) 9-18.
[37] A.I. Usov, Chapter 4 - Polysaccharides of the red algae, in: D. Horton (Ed.), Advances in Carbohydrate Chemistry and Biochemistry, Academic Press 2011, pp. 115-217.
[38] M. Garcia-Vaquero, M. Hayes, Red and green macroalgae for fish and animal feed and human functional food development, Food Reviews International 32(1) (2016) 15-45.
[39] C.M. Keith, L.C.W. Jeffrey, F.J. Simpson, Review of chemical constituents of the red alga *Palmaria palmata* (dulse), Economic Botany 34(1) (1980) 27-50.
[40] Y. Peng, J. Hu, B. Yang, X.-P. Lin, X.-F. Zhou, X.-W. Yang, Y. Liu, Chapter 5 - Chemical composition of seaweeds, in: B.K. Tiwari, D.J. Troy (Eds.), Seaweed Sustainability, Academic Press, San Diego, 2015, pp. 79-124.
[41] E. Deniaud, J. Fleurence, M. Lahaye, Interactions of the mix-linked β-(1,3)/β-(1,4)-D-xylans in the cell walls of *Palmaria palmata* (Rhodophyta), Journal of Phycology 39(1) (2003) 74-82.
[42] C. Dal Farra, N. Domloge, D. Peyronel, Use of purified, water-soluble xylans extracted from *Palmaria*, Google Patents, 2003.
[43] M. Kanlayavattanakul, N. Lourith, Biopolysaccharides for skin hydrating cosmetics, (2015) 1867-1892.
[44] F. Meddeb-Mouelhi, J.K. Moisan, M. Beauregard, A comparison of plate assay methods for detecting extracellular cellulase and xylanase activity, Enzyme and Microbial Technology 66 (2014) 16-19.
[45] A. Ebringerová, Z. Hromádková, Xylans of industrial and biomedical importance, Biotechnology and Genetic Engineering Reviews 16(1) (1999) 325-346.
[46] G.-G. Chen, X.-M. Qi, Y. Guan, F. Peng, C.-L. Yao, R.-C. Sun, High strength hemicellulose-based nanocomposite film for food packaging applications, ACS Sustainable Chemistry & Engineering 4(4) (2016) 1985-1993.
[47] A. Abdulkhani, A. Najd Mazhar, S. Hedjazi, Y. Hamzeh, Preparation of xylan bio-composite films reinforced with oxidized carboxymethyl cellulose and nanocellulose, Polymer Bulletin (2019).
[48] D.P. Kamdem, Z. Shen, O. Nabinejad, Z. Shu, Development of biodegradable composite chitosan-based films incorporated with xylan and carvacrol for food packaging application, Food Packaging and Shelf Life 21 (2019) 100344.
[49] X.-f. Chen, J.-l. Ren, L. Meng, Influence of ammonium zirconium carbonate on properties of poly (vinyl alcohol)/xylan composite films, Journal of Nanomaterials 2015 (2015).
[50] J.S. Stevanic, C. Joly, K.S. Mikkonen, K. Pirkkalainen, R. Serimaa, C. Rémond, G. Toriz, P. Gatenholm, M. Tenkanen, L. Salmén, Bacterial nanocellulose-reinforced arabinoxylan films, Journal of Applied Polymer Science 122(2) (2011) 1030-1039.
[51] M. Alekhina, K.S. Mikkonen, R. Alén, M. Tenkanen, H. Sixta, Carboxymethylation of alkali extracted xylan for preparation of bio-based packaging films, Carbohydrate Polymers 100 (2014) 89-96.
[52] K.S. Mikkonen, M. Tenkanen, Sustainable food-packaging materials based on future biorefinery products: Xylans and mannans, Trends in Food Science & Technology 28(2) (2012) 90-102.

[53] C. Gao, J. Ren, C. Zhao, W. Kong, Q. Dai, Q. Chen, C. Liu, R. Sun, Xylan-based temperature/pH sensitive hydrogels for drug controlled release, Carbohydrate Polymers 151 (2016) 189-197.
[54] M. Chang, X. Liu, L. Meng, X. Wang, J. Ren, Xylan-based hydrogels as a potential carrier for drug delivery: effect of pore-forming agents, Pharmaceutics 10(4) (2018) 261.
[55] X.-W. Peng, L.-X. Zhong, J.-L. Ren, R.-C. Sun, Highly effective adsorption of heavy metal ions from aqueous solutions by macroporous xylan-rich hemicelluloses-based hydrogel, Journal of Agricultural and Food Chemistry 60(15) (2012) 3909-3916.
[56] J.R. Bush, H. Liang, M. Dickinson, E.A. Botchwey, Xylan hemicellulose improves chitosan hydrogel for bone tissue regeneration, Polymers for Advanced Technologies 27(8) (2016) 1050-1055.
[57] X.-F. Sun, H.-h. Wang, Z.-x. Jing, R. Mohanathas, Hemicellulose-based pH-sensitive and biodegradable hydrogel for controlled drug delivery, Carbohydrate Polymers 92(2) (2013) 1357-1366.
[58] A.K. Samanta, N. Jayapal, C. Jayaram, S. Roy, A.P. Kolte, S. Senani, M. Sridhar, Xylooligosaccharides as prebiotics from agricultural by-products: Production and applications, Bioactive Carbohydrates and Dietary Fibre 5(1) (2015) 62-71.
[59] M.J. Vázquez, J.L. Alonso, H. Domínguez, J.C. Parajó, Xylooligosaccharides: manufacture and applications, Trends in Food Science & Technology 11(11) (2000) 387-393.
[60] D.S. Naidu, S.P. Hlangothi, M.J. John, Bio-based products from xylan: a review, Carbohydrate Polymers 179 (2018) 28-41.
[61] C. Amorim, S.C. Silvério, K.L.J. Prather, L.R. Rodrigues, From lignocellulosic residues to market: Production and commercial potential of xylooligosaccharides, Biotechnology Advances 37(7) (2019) 107397.
[62] M.A. Kabel, L. Kortenoeven, H.A. Schols, A.G.J. Voragen, In vitro fermentability of differently substituted xylo-oligosaccharides, Journal of Agricultural and Food Chemistry 50(21) (2002) 6205-6210.
[63] C.K. Hsu, J.W. Liao, Y.C. Chung, C.P. Hsieh, Y.C. Chan, Xylooligosaccharides and fructooligosaccharides affect the intestinal microbiota and precancerous colonic lesion development in rats, J Nutr 134(6) (2004) 1523-8.
[64] C. Amorim, S.C. Silvério, B.B. Cardoso, J.I. Alves, M.A. Pereira, L.R. Rodrigues, In vitro assessment of prebiotic properties of xylooligosaccharides produced by *Bacillus subtilis* 3610, Carbohydrate Polymers 229 (2020) 115460.
[65] W.F. Broekaert, C.M. Courtin, K. Verbeke, T. Van de Wiele, W. Verstraete, J.A. Delcour, Prebiotic and other health-related effects of cereal-derived arabinoxylans, arabinoxylan-oligosaccharides, and xylooligosaccharides, Crit Rev Food Sci Nutr 51(2) (2011) 178-94.
[66] C.M. Courtin, K. Swennen, P. Verjans, J.A. Delcour, Heat and pH stability of prebiotic arabinoxylooligosaccharides, xylooligosaccharides and fructooligosaccharides, Food Chemistry 112(4) (2009) 831-837.
[67] A.A. Aachary, D. Gobinath, K. Srinivasan, S.G. Prapulla, Protective effect of xylooligosaccharides from corncob on 1,2-dimethylhydrazine induced colon cancer in rats, Bioactive Carbohydrates and Dietary Fibre 5(2) (2015) 146-152.
[68] R. Maeda, T. Ida, H. Ihara, T. Sakamoto, Induction of apoptosis in MCF-7 cells by β-1,3-xylooligosaccharides prepared from *Caulerpa lentillifera*, Biosci Biotechnol Biochem 76(5) (2012) 1032-4.
[69] H.-W. Park, M.-J. Kim, S. Seo, S. Yoo, J.-H. Hong, Relative sweetness and sweetness quality of xylobiose, Food Science and Biotechnology 26(3) (2017) 689-696.

[70] C. Valls, F.I.J. Pastor, T. Vidal, M.B. Roncero, P. Díaz, J. Martínez, S.V. Valenzuela, Antioxidant activity of xylooligosaccharides produced from glucuronoxylan by Xyn10A and Xyn30D xylanases and eucalyptus autohydrolysates, Carbohydrate Polymers 194 (2018) 43-50.
[71] J. Yang, P.H. Summanen, S.M. Henning, M. Hsu, H. Lam, J. Huang, C.-H. Tseng, S.E. Dowd, S.M. Finegold, D. Heber, Z. Li, Xylooligosaccharide supplementation alters gut bacteria in both healthy and prediabetic adults: a pilot study, Frontiers in physiology 6 (2015) 216-216.
[72] A. Moure, P. Gullón, H. Domínguez, J.C. Parajó, Advances in the manufacture, purification and applications of xylo-oligosaccharides as food additives and nutraceuticals, Process Biochemistry 41(9) (2006) 1913-1923.
[73] M.C.R. Mano, I.A. Neri-Numa, J.B. da Silva, B.N. Paulino, M.G. Pessoa, G.M. Pastore, Oligosaccharide biotechnology: an approach of prebiotic revolution on the industry, Applied Microbiology and Biotechnology 102(1) (2018) 17-37.
[74] L. Venkateswar Rao, J.K. Goli, J. Gentela, S. Koti, Bioconversion of lignocellulosic biomass to xylitol: an overview, Bioresource Technology 213 (2016) 299-310.
[75] T.L. de Albuquerque, I.J. da Silva Jr, G.R. de Macedo, M.V.P. Rocha, Biotechnological production of xylitol from lignocellulosic wastes: a review, Process Biochemistry 49(11) (2014) 1779-1789.
[76] N.L. Mohamad, S.M. Mustapa Kamal, M.N. Mokhtar, Xylitol biological production: a review of recent studies, Food Reviews International 31(1) (2015) 74-89.
[77] J. Zhu, Y. Rong, J. Yang, X. Zhou, Y. Xu, L. Zhang, J. Chen, Q. Yong, S. Yu, Integrated production of xylonic acid and bioethanol from acid-catalyzed steam-exploded corn stover, Appl Biochem Biotechnol 176(5) (2015) 1370-81.
[78] M. Bueno, F. Zamora, I. Molina, H.A. Orgueira, O. Varela, J.A. Galbis, Synthesis and characterization of optically active polyamides derived from carbohydrate-based monomers, Journal of Polymer Science Part A: Polymer Chemistry 35(17) (1997) 3645-3653.
[79] O. Sánchez, S. Montoya, Production of bioethanol from biomass: an overview, 2013, pp. 397-441.
[80] F.A. Castillo Martinez, E.M. Balciunas, J.M. Salgado, J.M. Domínguez González, A. Converti, R.P.d.S. Oliveira, Lactic acid properties, applications and production: a review, Trends in Food Science & Technology 30(1) (2013) 70-83.
[81] C. Gao, C. Ma, P. Xu, Biotechnological routes based on lactic acid production from biomass, Biotechnology Advances 29(6) (2011) 930-939.
[82] M. Singhvi, D. Joshi, M. Adsul, A. Varma, D. Gokhale, D-(−)-Lactic acid production from cellobiose and cellulose by *Lactobacillus lactis* mutant RM2-24, Green Chemistry 12(6) (2010) 1106-1109.
[83] G. Machado, S. Leon, F. Santos, R. Lourega, J. Dullius, M. Mollmann, P. Eichler, Literature review on furfural production from lignocellulosic biomass, Natural Resources 07 (2016) 115-129.
[84] A.S. Mamman, J.-M. Lee, Y.-C. Kim, I.T. Hwang, N.-J. Park, Y.K. Hwang, J.-S. Chang, J.-S. Hwang, Furfural: hemicellulose/xylosederived biochemical, Biofuels, Bioproducts and Biorefining 2(5) (2008) 438-454.
[85] A. Eseyin, A. Eseyin, Philip, H. Steele, An overview of the applications of furfural and its derivatives, International Journal of Advanced Chemistry 3 (2015) 42-47.
[86] A.K. Mathew, A. Abraham, K.K. Mallapureddy, R.K. Sukumaran, Chapter 9 - Lignocellulosic biorefinery wastes, or resources?, in: T. Bhaskar, A. Pandey, S.V. Mohan, D.-J. Lee, S.K. Khanal (Eds.), Waste Biorefinery, Elsevier 2018, pp. 267-297.
[87] A. Belancic, J. Scarpa, A. Peirano, R. Díaz, J. Steiner, J. Eyzaguirre, *Penicillium purpurogenum* produces several xylanases: Purification and properties of two of the enzymes, Journal of Biotechnology 41(1) (1995) 71-79.

[88] H. Wang, Z. Li, H. Liu, S. Li, H. Qiu, K. Li, X. Luo, Y. Song, N. Wang, H. He, H. Zhou, W. Ma, T. Zhang, Heterologous expression in *Pichia pastoris* and characterization of a novel GH11 xylanase from saline-alkali soil with excellent tolerance to high pH, high salt concentrations and ethanol, Protein Expression and Purification 139 (2017) 71-77.
[89] B. Saha, Hemicellulose bioconversion, Journal of industrial microbiology & biotechnology 30 (2003) 279-91.
[90] T.W. Jeffries, Utilization of xylose by bacteria, yeasts, and fungi, Pentoses and Lignin, Springer Berlin Heidelberg, Berlin, Heidelberg, 1983, pp. 1-32.
[91] S. Singh, A.M. Madlala, B.A. Prior, *Thermomyces lanuginosus*: properties of strains and their hemicellulases, FEMS Microbiology Reviews 27(1) (2003) 3-16.
[92] S. Yegin, Single-step purification and characterization of an extreme halophilic, ethanol tolerant and acidophilic xylanase from *Aureobasidium pullulans* NRRL Y-2311-1 with application potential in the food industry, Food Chemistry 221 (2017) 67-75.
[93] R.B. Hespell, Xylanolytic activities of *Spirochaeta thermophila*, Current Microbiology 29(6) (1994) 343-347.
[94] M. Michelin, T.M. Silva, J.A. Jorge, T. Maria de Lourdes, Purification and biochemical properties of multiple xylanases from *Aspergillus ochraceus* tolerant to Hg 2+ ion and a wide range of pH, Applied Biochemistry and Biotechnology 174(1) (2014) 206-220.
[95] P. Biely, V. Puchart, M.A. Stringer, K.B. Mørkeberg Krogh, *Trichoderma reesei* XYN VI–a novel appendage-dependent eukaryotic glucuronoxylan hydrolase, The FEBS journal 281(17) (2014) 3894-3903.
[96] M. Lafond, O. Guais, M. Maestracci, E. Bonnin, T. Giardina, Four GH11 xylanases from the xylanolytic fungus *Talaromyces versatilis* act differently on (arabino) xylans, Applied microbiology and biotechnology 98(14) (2014) 6339-6352.
[97] H. Zeng, Y. Xue, T. Peng, W. Shao, Properties of xylanolytic enzyme system in bifidobacteria and their effects on the utilization of xylooligosaccharides, Food Chemistry 101(3) (2007) 1172-1177.
[98] J. Sun, C. Tian, S. Diamond, N.L. Glass, Deciphering transcriptional regulatory mechanisms associated with hemicellulose degradation in *Neurospora crassa*, Eukaryotic Cell 11(4) (2012) 482-493.
[99] B. Henrissat, A classification of glycosyl hydrolases based on amino acid sequence similarities, Biochem J 280 (Pt 2)(Pt 2) (1991) 309-16.
[100] Y. Chu, Z. Hao, K. Wang, T. Tu, H. Huang, Y. Wang, Y.G. Bai, Y. Wang, H. Luo, B. Yao, X. Su, The GH10 and GH48 dual-functional catalytic domains from a multimodular glycoside hydrolase synergize in hydrolyzing both cellulose and xylan, Biotechnology for Biofuels 12(1) (2019) 279.
[101] H.J. Flint, J. Martin, C.A. McPherson, A.S. Daniel, J.X. Zhang, A bifunctional enzyme, with separate xylanase and beta(1,3-1,4)-glucanase domains, encoded by the xynD gene of Ruminococcus flavefaciens, Journal of Bacteriology 175(10) (1993) 2943-2951.
[102] C.M. Bianchetti, P. Brumm, R.W. Smith, K. Dyer, G.L. Hura, T.J. Rutkoski, G.N. Phillips, Jr., Structure, dynamics, and specificity of endoglucanase D from *Clostridium cellulovorans*, Journal of molecular biology 425(22) (2013) 4267-4285.
[103] N. McGregor, M. Morar, T.H. Fenger, P. Stogios, N. Lenfant, V. Yin, X. Xu, E. Evdokimova, H. Cui, B. Henrissat, A. Savchenko, H. Brumer, Structure-function analysis of a mixed-linkage β-glucanase/xyloglucanase from the key ruminal bacteroidetes *Prevotella bryantii* $B_1$4, The Journal of biological chemistry 291(3) (2016) 1175-1197.
[104] S.-F. Yuan, T.-H. Wu, H.-L. Lee, H.-Y. Hsieh, W.-L. Lin, B. Yang, C.-K. Chang, Q. Li, J. Gao, C.-H. Huang, M.-C. Ho, R.-T. Guo, P.-H. Liang, Biochemical characterization and structural analysis

of a bifunctional cellulase/xylanase from *Clostridium thermocellum*, The Journal of biological chemistry 290(9) (2015) 5739-5748.
[105] D. Dodd, Y.H. Moon, K. Swaminathan, R.I. Mackie, I.K. Cann, Transcriptomic analyses of xylan degradation by *Prevotella bryantii* and insights into energy acquisition by xylanolytic bacteroidetes, The Journal of biological chemistry 285(39) (2010) 30261-73.
[106] F. Barras, I. Bortoli-German, M. Bauzan, J. Rouvier, C. Gey, A. Heyraud, B. Henrissat, Stereochemistry of the hydrolysis reaction catalyzed by endoglucanase Z from *Erwinia chrysanthemi*, FEBS Letters 300(2) (1992) 145-148.
[107] B. Henrissat, M. Claeyssens, P. Tomme, L. Lemesle, J.P. Mornon, Cellulase families revealed by hydrophobic cluster analysis, Gene 81(1) (1989) 83-95.
[108] F.J. St. John, J.F. Preston, E. Pozharski, Novel structural features of xylanase A1 from *Paenibacillus* sp. JDR-2, Journal of Structural Biology 180(2) (2012) 303-311.
[109] H. Aspeborg, P. Coutinho, Y. Wang, H. Brumer, B. Henrissat, Evolution, substrate specificity and subfamily classification of glycoside hydrolase family 5 (GH5). BMC Evol Biol 12:186, BMC evolutionary biology 12 (2012) 186.
[110] E.M. Glasgow, K.A. Vander Meulen, T.E. Takasuka, C.M. Bianchetti, L.F. Bergeman, S. Deutsch, B.G. Fox, Extent and origins of functional diversity in a subfamily of glycoside hydrolases, Journal of molecular biology 431(6) (2019) 1217-1233.
[111] K. Ash, T. Brown, T. Watford, L.E. Scott, C. Stephens, B. Ely, A comparison of the *Caulobacter* NA1000 and K31 genomes reveals extensive genome rearrangements and differences in metabolic potential, Open biology 4(10) (2014) 140128.
[112] M. Martin, S. Biver, S. Steels, T. Barbeyron, M. Jam, D. Portetelle, G. Michel, M. Vandenbol, Identification and characterization of a halotolerant, cold-cctive marine endo-β-1,4-glucanase by using functional metagenomics of seaweed-associated microbiota, Applied and Environmental Microbiology 80(16) (2014) 4958-4967.
[113] S.R. Chhabra, K.R. Shockley, D.E. Ward, R.M. Kelly, Regulation of endo-acting glycosyl hydrolases in the hyperthermophilic bacterium *Thermotoga maritima* grown on glucan- and mannan-based polysaccharides, Applied and Environmental Microbiology 68(2) (2002) 545-554.
[114] A. Labourel, L.I. Crouch, J.L.A. Brás, A. Jackson, A. Rogowski, J. Gray, M.P. Yadav, B. Henrissat, C.M.G.A. Fontes, H.J. Gilbert, S. Najmudin, A. Baslé, F. Cuskin, The mechanism by which arabinoxylanases can recognize highly decorated xylans, The Journal of biological chemistry 291(42) (2016) 22149-22159.
[115] M.A.S. Correia, K. Mazumder, J.L.A. Brás, S.J. Firbank, Y. Zhu, R.J. Lewis, W.S. York, C.M.G.A. Fontes, H.J. Gilbert, Structure and function of an arabinoxylan-specific xylanase, The Journal of biological chemistry 286(25) (2011) 22510-22520.
[116] A. Rogowski, J.A. Briggs, J.C. Mortimer, T. Tryfona, N. Terrapon, E.C. Lowe, A. Baslé, C. Morland, A.M. Day, H. Zheng, T.E. Rogers, P. Thompson, A.R. Hawkins, M.P. Yadav, B. Henrissat, E.C. Martens, P. Dupree, H.J. Gilbert, D.N. Bolam, Glycan complexity dictates microbial resource allocation in the large intestine, Nature Communications 6(1) (2015) 7481.
[117] T. Collins, M.-A. Meuwis, I. Stals, M. Claeyssens, G. Feller, C. Gerday, A Novel Family 8 Xylanase, Functional and Physicochemical Characterization, The Journal of biological chemistry 277 (2002) 35133-9.
[118] C.C. Lee, R.E. Kibblewhite-Accinelli, K. Wagschal, G.H. Robertson, D.W. Wong, Cloning and characterization of a cold-active xylanase enzyme from an environmental DNA library, Extremophiles 10(4) (2006) 295-300.
[119] S. Elleuche, H. Piascheck, G. Antranikian, Fusion of the OsmC domain from esterase EstO confers thermolability to the cold-active xylanase Xyn8 from *Pseudoalteromonas arctica*, Extremophiles 15(2) (2011) 311-317.

[120] B. Guo, P.-Y. Li, Y.-S. Yue, H.-L. Zhao, S. Dong, X.-Y. Song, S. Caiyun, W.-X. Zhang, X.-L. Chen, X.-Y. Zhang, Z. Cheng, Y.-Z. Zhang, Gene cloning, expression and characterization of a novel xylanase from the marine bacterium, *Glaciecola mesophila* KMM241, Marine drugs 11 (2013) 1173-87.
[121] C.A. Fowler, G.R. Hemsworth, F. Cuskin, S. Hart, J. Turkenburg, H.J. Gilbert, P.H. Walton, G.J. Davies, Structure and function of a glycoside hydrolase family 8 endoxylanase from *Teredinibacter turnerae*, Acta crystallographica. Section D, Structural biology 74(Pt 10) (2018) 946-955.
[122] K.-H. Yoon, H. Yun, K. Jung, Molecular cloning of a *Bacillus* sp. KK-1 xylanase gene and characterization of the gene product, Biochemistry and molecular biology international 45 (1998) 337-47.
[123] P.-Y. Hong, M. Iakiviak, D. Dodd, M. Zhang, R.I. Mackie, I. Cann, Two new xylanases with different substrate specificities from the human gut bacterium *Bacteroides intestinalis* DSM 17393, Applied and Environmental Microbiology 80(7) (2014) 2084-2093.
[124] Y. Brennan, W.N. Callen, L. Christoffersen, P. Dupree, F. Goubet, S. Healey, M. Hernández, M. Keller, K. Li, N. Palackal, A. Sittenfeld, G. Tamayo, S. Wells, G.P. Hazlewood, E.J. Mathur, J.M. Short, D.E. Robertson, B.A. Steer, Unusual microbial xylanases from insect guts, Applied and Environmental Microbiology 70(6) (2004) 3609-3617.
[125] A. Pollet, J. Schoepe, E. Dornez, S.V. Strelkov, J.A. Delcour, C.M. Courtin, Functional analysis of glycoside hydrolase family 8 xylanases shows narrow but distinct substrate specificities and biotechnological potential, Applied Microbiology and Biotechnology 87(6) (2010) 2125-35.
[126] W. Adachi, Y. Sakihama, S. Shimizu, T. Sunami, T. Fukazawa, M. Suzuki, R. Yatsunami, S. Nakamura, A. Takénaka, Crystal structure of family GH-8 chitosanase with subclass II specificity from *Bacillus* sp. K17, Journal of molecular biology 343(3) (2004) 785-795.
[127] A. Pollet, J.A. Delcour, C.M. Courtin, Structural determinants of the substrate specificities of xylanases from different glycoside hydrolase families, Crit Rev Biotechnol 30(3) (2010) 176-91.
[128] T. Collins, A. Hoyoux, A. Dutron, J. Georis, B. Genot, T. Dauvrin, F. Arnaut, C. Gerday, G. Feller, Use of glycoside hydrolase family 8 xylanases in baking, Journal of Cereal Science 43 (2006) 79-84.
[129] A. Dutron, J. Georis, B. Genot, T. Dauvrin, T. Collins, A. Hoyoux, G. Feller, Use of family 8 enzymes with xylanolytic activity in baking, Google Patents, 2012.
[130] M. Lafond, A. Tauzin, V. Desseaux, E. Bonnin, E.-H. Ajandouz, T. Giardina, GH10 xylanase D from *Penicillium funiculosum*: biochemical studies and xylooligosaccharide production, Microbial Cell Factories 10(1) (2011) 20.
[131] A.P. MacCabe, M.T. Fernández-Espinar, L.H. de Graaff, J. Visser, D. Ramón, Identification, isolation and sequence of the *Aspergillus nidulans* xlnC gene encoding the 34-kDa xylanase, Gene 175(1) (1996) 29-33.
[132] F.Y. Bih, S.S.H. Wu, C. Ratnayake, L.L. Walling, E.A. Nothnagel, A.H.C. Huang, The predominant protein on the surface of maize pollen Is an endoxylanase synthesized by a Tapetum mRNA with a long 5′ leader, Journal of Biological Chemistry 274(32) (1999) 22884-22894.
[133] G. Mamo, O. Delgado, A. Martinez, B. Mattiasson, R. Hatti-Kaul, Cloning, sequence analysis, and expression of a gene encoding an endoxylanase from *Bacillus halodurans* S7, Molecular Biotechnology 33(2) (2006) 149-159.
[134] L. Fredriksen, R. Stokke, M.S. Jensen, B. Westereng, J.K. Jameson, I.H. Steen, V.G.H. Eijsink, Discovery of a thermostable GH10 xylanase with broad substrate specificity from the arctic mid-ocean ridge vent system, Applied and Environmental Microbiology 85(6) (2019).
[135] K. Wang, R. Cao, M. Wang, Q. Lin, R. Zhan, H. Xu, S. Wang, A novel thermostable GH10 xylanase with activities on a wide variety of cellulosic substrates from a xylanolytic *Bacillus* strain

exhibiting significant synergy with commercial Celluclast 1.5 L in pretreated corn stover hydrolysis, Biotechnology for Biofuels 12(1) (2019) 48.
[136] X. Xue, R. Wang, T. Tu, P. Shi, R. Ma, H. Luo, B. Yao, X. Su, The N-terminal GH10 domain of a multimodular protein from *Caldicellulosiruptor bescii* is a versatile xylanase/β-glucanase that can degrade crystalline cellulose, Applied and Environmental Microbiology 81(11) (2015) 3823-33.
[137] S.C. Teo, K.J. Liew, M.S. Shamsir, C.S. Chong, N.C. Bruce, K.-G. Chan, K.M. Goh, Characterizing a halo-tolerant GH10 xylanase from *Roseithermus sacchariphilus* strain RA and its CBM-truncated variant, International journal of molecular sciences 20(9) (2019) 2284.
[138] G. Pell, E.J. Taylor, T.M. Gloster, J.P. Turkenburg, C.M. Fontes, L.M. Ferreira, T. Nagy, S.J. Clark, G.J. Davies, H.J. Gilbert, The mechanisms by which family 10 glycoside hydrolases bind decorated substrates, The Journal of biological chemistry 279(10) (2004) 9597-605.
[139] J. Gebler, N.R. Gilkes, M. Claeyssens, D.B. Wilson, P. Béguin, W.W. Wakarchuk, D. Kilburn, R.C. Miller, R.A.J. Warren, S. Withers, Stereoselective hydrolysis catalyzed by related beta-1,4-glucanases and beta-1,4-xylanases, Journal of Biological Chemistry 267 (1992) 12559-12561.
[140] G.W. Harris, J.A. Jenkins, I. Connerton, R.W. Pickersgill, Refined crystal structure of the catalytic domain of xylanase A from *Pseudomonas fluorescens* at 1.8 A resolution, Acta Crystallographica Section D 52(2) (1996) 393-401.
[141] S.R. Andrews, S.J. Charnock, J.H. Lakey, G.J. Davies, M. Claeyssens, W. Nerinckx, M. Underwood, M.L. Sinnott, R.A. Warren, H.J. Gilbert, Substrate specificity in glycoside hydrolase family 10. Tyrosine 87 and leucine 314 play a pivotal role in discriminating between glucose and xylose binding in the proximal active site of *Pseudomonas cellulosa* xylanase 10A, The Journal of biological chemistry 275(30) (2000) 23027-33.
[142] O. Gallardo, P. Diaz, F.I. Pastor, Characterization of a *Paenibacillus* cell-associated xylanase with high activity on aryl-xylosides: a new subclass of family 10 xylanases, Applied Microbiology and Biotechnology 61(3) (2003) 226-33.
[143] V. Solomon, A. Teplitsky, S. Shulami, G. Zolotnitsky, Y. Shoham, G. Shoham, Structure-specificity relationships of an intracellular xylanase from *Geobacillus stearothermophilus*, Acta Crystallogr D Biol Crystallogr 63(Pt 8) (2007) 845-59.
[144] C. Valls, M.B. Roncero, Using both xylanase and laccase enzymes for pulp bleaching, Bioresource Technology 100(6) (2009) 2032-9.
[145] P.H. Selle, V. Ravindran, G.G. Partridge, Beneficial effects of xylanase and/or phytase inclusions on ileal amino acid digestibility, energy utilisation, mineral retention and growth performance in wheat-based broiler diets, Animal Feed Science and Technology 153(3) (2009) 303-313.
[146] S. Dhiman, J. Sharma, B. Battan, Pretreatment processing of fabrics by alkalothermophilic xylanase from *Bacillus stearothermophilus* SDX, Enzyme and Microbial Technology 43 (2008) 262-269.
[147] M. Butt, M. Tahir-Nadeem, Z. Ahmad, M. Sultan, Xylanases and their applications in baking industry, Food Technology and Biotechnology 46 (2008).
[148] A. Kallioinen, T. Puranen, M. Siika-aho, Mixtures of thermostable enzymes show high performance in biomass saccharification, Applied Biochemistry and Biotechnology 173 (2014).
[149] C.C. Lee, R.E. Kibblewhite-Accinelli, M.R. Smith, K. Wagschal, W.J. Orts, D.W. Wong, Cloning of *Bacillus licheniformis* xylanase gene and characterization of recombinant enzyme, Current Microbiology 57(4) (2008) 301-5.
[150] J.A. Pérez-Gonzalez, L.H. De Graaff, J. Visser, D. Ramón, Molecular cloning and expression in *Saccharomyces cerevisiae* of two *Aspergillus nidulans* xylanase genes, Applied and Environmental Microbiology 62(6) (1996) 2179-2182.

[151] C. Girard, L. Jouanin, Molecular cloning of cDNAs encoding a range of digestive enzymes from a phytophagous beetle, *Phaedon cochleariae*, Insect Biochem Mol Biol 29(12) (1999) 1129-42.
[152] L. Mao, P. Meng, C. Zhou, L. Ma, G. Zhang, Y. Ma, Molecular cloning and heterologous expression of an acid stable xylanase gene from *Alternaria* sp. HB186, World Journal of Microbiology and Biotechnology 28(3) (2012) 777-784.
[153] C.U. Baek, S.G. Lee, Y.R. Chung, I. Cho, J.H. Kim, Cloning of a Family 11 Xylanase Gene from *Bacillus amyloliquefaciens* CH51 Isolated from Cheonggukjang, Indian journal of microbiology 52(4) (2012) 695-700.
[154] Ó. Gallardo, P. Diaz, F.I.J. Pastor, Cloning and characterization of xylanase A from the strain *Bacillus* sp. BP-7: comparison with alkaline pI-low molecular weight xylanases of family 11, Current Microbiology 48(4) (2004) 276-279.
[155] W. Qu, W. Shao, Cloning, expression and characterization of glycoside hydrolase family 11 endoxylanase from *Bacillus pumilus* ARA, Biotechnology Letters 33(7) (2011) 1407-1416.
[156] S. Nakamura, K. Wakabayashi, R. Nakai, R. Aono, K. Horikoshi, Purification and some properties of an alkaline xylanase from alkaliphilic *Bacillus* sp. strain 41M-1, Applied and Environmental Microbiology 59(7) (1993) 2311-2316.
[157] A. Törrönen, A. Harkki, J. Rouvinen, Three-dimensional structure of endo-1,4-beta-xylanase II from *Trichoderma reesei*: two conformational states in the active site, The EMBO journal 13(11) (1994) 2493-2501.
[158] W.W. Wakarchuk, R.L. Campbell, W.L. Sung, J. Davoodi, M. Yaguchi, Mutational and crystallographic analyses of the active site residues of the *Bacillus circulans* xylanase, Protein science : a publication of the Protein Society 3(3) (1994) 467-475.
[159] G. Paës, J.G. Berrin, J. Beaugrand, GH11 xylanases: structure/function/properties relationships and applications, Biotechnology Advances 30(3) (2012) 564-92.
[160] M. Kiyohara, K. Sakaguchi, K. Yamaguchi, T. Araki, T. Nakamura, M. Ito, Molecular cloning and characterization of a novel beta-1,3-xylanase possessing two putative carbohydrate-binding modules from a marine bacterium *Vibrio* sp. strain AX-4, Biochem J 388(Pt 3) (2005) 949-957.
[161] F. Okazaki, N. Nakashima, C. Ogino, Y. Tamaru, A. Kondo, Biochemical characterization of a thermostable β-1,3-xylanase from the hyperthermophilic eubacterium, *Thermotoga neapolitana* strain DSM 4359, Applied Microbiology and Biotechnology 97(15) (2013) 6749-57.
[162] Y. Aoki, Y. Kamei, Preparation of recombinant polysaccharide-degrading enzymes from the marine bacterium, *Pseudomonas* sp. ND137 for the production of protoplasts of *Porphyra yezoensis*, European Journal of Phycology 41(3) (2006) 321-328.
[163] W.-S. Liang, T.-Y. Fang, H.-T. Lin, T.C. Liu, W.-J. Lu, W.-S. Tzou, S.-J. Tang, F.-P. Lin, S.-M. Liu, C.-L. Pan, Cloning, expression, and characterization of *Pseudomonas vesicularis* MA103 β-1,3-xylanase in *Escherichia coli* ClearColi BL21(DE3), Fisheries Science 81(6) (2015) 1135-1143.
[164] P. von Freiesleben, O.V. Moroz, E. Blagova, M. Wiemann, N. Spodsberg, J.W. Agger, G.J. Davies, K.S. Wilson, H. Stålbrand, A.S. Meyer, K.B.R.M. Krogh, Crystal structure and substrate interactions of an unusual fungal non-CBM carrying GH26 endo-β-mannanase from *Yunnania penicillata*, Scientific Reports 9(1) (2019) 2266.
[165] O. Gallardo, F.I.J. Pastor, J. Polaina, P. Diaz, R. Łysek, P. Vogel, P. Isorna, B. González, J. Sanz-Aparicio, Structural insights into the specificity of Xyn10B from *Paenibacillus barcinonensis* and its improved stability by forced protein evolution, The Journal of biological chemistry 285(4) (2010) 2721-2733.
[166] F.J. St John, J.M. González, E. Pozharski, Consolidation of glycosyl hydrolase family 30: A dual domain 4/7 hydrolase family consisting of two structurally distinct groups, FEBS Letters 584(21) (2010) 4435-4441.

[167] M. Vrsanská, K. Kolenová, V. Puchart, P. Biely, Mode of action of glycoside hydrolase family 5 glucuronoxylan xylanohydrolase from *Erwinia chrysanthemi*, FEBS Journal 274(7) (2007) 1666-77.
[168] Y. Nakamichi, T. Fouquet, S. Ito, M. Watanabe, A. Matsushika, H. Inoue, Structural and functional characterization of a bifunctional GH30-7 xylanase B from the filamentous fungus *Talaromyces cellulolyticus*, Journal of Biological Chemistry (2019) jbc.RA118.007207.
[169] M. Tenkanen, M. Vršanská, M. Siika-aho, D.W. Wong, V. Puchart, M. Penttilä, M. Saloheimo, P. Biely, Xylanase XYN IV from *Trichoderma reesei* showing exo- and endo-xylanase activity, FEBS Journal 280(1) (2013) 285-301.
[170] H. Luo, J. Yang, J. Li, P. Shi, H. Huang, Y. Bai, Y. Fan, B. Yao, Molecular cloning and characterization of the novel acidic xylanase XYLD from *Bispora* sp. MEY-1 that is homologous to family 30 glycosyl hydrolases, Applied Microbiology and Biotechnology 86(6) (2010) 1829-39.
[171] F.J. St John, D. Dietrich, C. Crooks, P. Balogun, V. de Serrano, E. Pozharski, J.K. Smith, E. Bales, J. Hurlbert, A plasmid borne, functionally novel glycoside hydrolase family 30 subfamily 8 endoxylanase from solventogenic *Clostridium*, Biochem J 475(9) (2018) 1533-1551.
[172] I.Q. Padilha, S.V. Valenzuela, T.C. Grisi, P. Díaz, D.A. de Araújo, F.I. Pastor, A glucuronoxylan-specific xylanase from a new *Paenibacillus favisporus* strain isolated from tropical soil of Brazil, Int Microbiol 17(3) (2014) 175-84.
[173] K. Nishitani, D. Nevins, Glucuronoxylan xylanohydrolase: A unique xylanase with the requirement for appendant glucuronosyl units, The Journal of biological chemistry 266 (1991) 6539-43.
[174] S.V. Valenzuela, P. Diaz, F.I.J. Pastor, Modular glucuronoxylan-specific xylanase with a family CBM35 carbohydrate-binding module, Applied and Environmental Microbiology 78(11) (2012) 3923-3931.
[175] C. Köhler, L. Hennig, C. Spillane, S. Pien, W. Gruissem, U. Grossniklaus, The Polycomb-group protein MEDEA regulates seed development by controlling expression of the MADS-box gene PHERES1, Genes Dev 17(12) (2003) 1540-53.
[176] P. Biely, M. Vrsanská, M. Claeyssens, The endo-1,4-beta-glucanase I from *Trichoderma reesei*: Action on beta-1,4-oligomers and polymers derived from D-glucose and D-xylose, Eur J Biochem 200(1) (1991) 157-63.
[177] G.J. Kleywegt, J.-Y. Zou, C. Divne, G.J. Davies, I. Sinning, J. Ståhlberg, T. Reinikainen, M. Srisodsuk, T.T. Teeri, T.A. Jones, The crystal structure of the catalytic core domain of endoglucanase I from *Trichoderma reesei* at 3.6 Å resolution, and a comparison with related enzymes11Edited by K.Nagai, Journal of molecular biology 272(3) (1997) 383-397.
[178] M. Penttilä, P. Lehtovaara, H. Nevalainen, R. Bhikhabhai, J. Knowles, Homology between cellulase genes of *Trichoderma reesei*: complete nucleotide sequence of the endoglucanase I gene, Gene 45(3) (1986) 253-63.
[179] R. Cannio, N. Di Prizito, M. Rossi, A. Morana, A xylan-degrading strain of *Sulfolobus solfataricus*: isolation and characterization of the xylanase activity, Extremophiles 8(2) (2004) 117-24.
[180] H.D. Sakai, N. Kurosawa, *Saccharolobus caldissimus* gen. nov., sp. nov., a facultatively anaerobic iron-reducing hyperthermophilic archaeon isolated from an acidic terrestrial hot spring, and reclassification of *Sulfolobus solfataricus* as *Saccharolobus solfataricus* comb. nov. and *Sulfolobus shibatae* as *Saccharolobus shibatae* comb. nov, International Journal of Systematic and Evolutionary Microbiology 68(4) (2018) 1271-1278.
[181] H.-W. Kim, M. Kataoka, K. Ishikawa, Atomic resolution of the crystal structure of the hyperthermophilic family 12 endocellulase and stabilizing role of the DxDxDG calcium-binding motif in *Pyrococcus furiosus*, FEBS Letters 586(7) (2012) 1009-1013.

[182] T. Gloster, F. Ibatullin, K. Macauley, J. Eklöf, S. Roberts, J. Turkenburg, M. Bjørnvad, P. Jørgensen, S. Danielsen, K. Johansen, T. Borchert, K. Wilson, H. Brumer, G. Davies, Characterization and three-dimensional structures of two distinct bacterial xyloglucanases from families GH5 and GH12, The Journal of biological chemistry 282 (2007) 19177-89.
[183] S. Khademi, D. Zhang, S. Swanson, A. Wartenberg, K. Witte, E. Meyer, Determination of the structure of an endoglucanase from *Aspergillus niger* and its mode of inhibition by palladium chloride, Acta Crystallogr D Biol Crystallogr 58 (2002) 660-7.
[184] K. Eckert, E. Schneider, A thermoacidophilic endoglucanase (CelB) from *Alicyclobacillus acidocaldarius* displays high sequence similarity to arabinofuranosidases belonging to family 51 of glycoside hydrolases, European Journal of Biochemistry 270(17) (2003) 3593-3602.
[185] C.R. dos Santos, P.O. de Giuseppe, F.H.M. de Souza, L.M. Zanphorlin, M.N. Domingues, R.A.S. Pirolla, R.V. Honorato, C.C.C. Tonoli, M.A.B. de Morais, V.P. de Matos Martins, L.M. Fonseca, F. Büchli, P.S.L. de Oliveira, F.C. Gozzo, M.T. Murakami, The mechanism by which a distinguishing arabinofuranosidase can cope with internal di-substitutions in arabinoxylans, Biotechnology for Biofuels 11(1) (2018) 223.
[186] T.A.C.B. Souza, C.R. Santos, A.R. Souza, D.P. Oldiges, R. Ruller, R.A. Prade, F.M. Squina, M.T. Murakami, Structure of a novel thermostable GH51 α-L-arabinofuranosidase from *Thermotoga petrophila* RKU-1, Protein science : a publication of the Protein Society 20(9) (2011) 1632-1637.
[187] K.M. Anderson, H. Ashida, K. Maskos, A. Dell, S.C. Li, Y.T. Li, A clostridial endo-beta-galactosidase that cleaves both blood group A and B glycotopes: the first member of a new glycoside hydrolase family, GH98, The Journal of biological chemistry 280(9) (2005) 7720-8.
[188] M.A. Higgins, G.E. Whitworth, N. El Warry, M. Randriantsoa, E. Samain, R.D. Burke, D.J. Vocadlo, A.B. Boraston, Differential recognition and hydrolysis of host carbohydrate antigens by *Streptococcus pneumoniae* family 98 glycoside hydrolases, The Journal of biological chemistry 284(38) (2009) 26161-26173.
[189] D.J. Rigden, Analysis of glycoside hydrolase family 98: Catalytic machinery, mechanism and a novel putative carbohydrate binding module, FEBS Letters 579(25) (2005) 5466-5472.
[190] S. Heinze, M. Mechelke, P. Kornberger, W. Liebl, W.H. Schwarz, V.V. Zverlov, Identification of endoxylanase XynE from *Clostridium thermocellum* as the first xylanase of glycoside hydrolase family GH141, Scientific Reports 7(1) (2017) 11178.
[191] R.A. Prade, Xylanases: from biology to biotechnology, Biotechnology and Genetic Engineering Reviews 13(1) (1996) 101-132.
[192] P. Bajpai, Biological bleaching of chemical pulps, Critical Reviews in Biotechnology 24(1) (2004) 1-58.
[193] L. Viikari, A. Kantelinen, J. Sundquist, M. Linko, Xylanases in bleaching: From an idea to the industry, FEMS Microbiology Reviews 13(2) (1994) 335-350.
[194] L.N. Twomey, J.R. Pluske, J.B. Rowe, M. Choct, W. Brown, M.F. McConnell, D.W. Pethick, The effects of increasing levels of soluble non-starch polysaccharides and inclusion of feed enzymes in dog diets on faecal quality and digestibility, Animal Feed Science and Technology 108(1) (2003) 71-82.
[195] N. Mathlouthi, J.P. Lallès, P. Lepercq, C. Juste, M. Larbier, Xylanase and beta-glucanase supplementation improve conjugated bile acid fraction in intestinal contents and increase villus size of small intestine wall in broiler chickens fed a rye-based diet, J Anim Sci 80(11) (2002) 2773-9.
[196] Q. Beg, M. Kapoor, L. Mahajan, G. Hoondal, Microbial xylanases and their industrial applications: a review, Applied microbiology and biotechnology 56(3) (2001) 326-338.

[197] O. Colagrande, A. Silva, M.D. Fumi, Recent applications of biotechnology in wine production, Biotechnology Progress 10(1) (1994) 2-18.
[198] T. Collins, D. De Vos, A. Hoyoux, S.N. Savvides, C. Gerday, J. Van Beeumen, G. Feller, Study of the active site residues of a glycoside hydrolase family 8 xylanase, Journal of molecular biology 354(2) (2005) 425-35.
[199] T. Collins, M.-A. Meuwis, C. Gerday, G. Feller, Activity, stability and flexibility in glycosidases adapted to extreme thermal environments, Journal of molecular biology 328(2) (2003) 419-428.

Chapter I.II

Biotechnological Aspects of Cold-Active Enzymes

Biotechnological Aspects of Cold-Active Enzymes

Mário Barroca, Gustavo Santos, Charles Gerday, and Tony Collins

Abstract Cold-adapted enzymes produced by organisms inhabiting permanently low temperature environments are typically characterized by a high activity at low to moderate temperatures and a poor thermal stability. Such characteristics make these enzymes highly attractive for various applications where they can enable more efficient, cost-effective, and environmentally friendlier processes than higher temperature-adapted enzymes. In this chapter, the biotechnological aspects of cold-adapted enzymes and their application in industry are reviewed and discussed with a focus on cleaning/detergents, food and beverages, molecular biology, biomedicine, pharmaceuticals, cosmetics, textiles, biofuels, and materials applications.

Contents

19.1 Introduction

Enzymes are highly specific biological catalysts that accelerate the rate of chemical reactions in the cells of living organisms. These natural catalysts are biodegradable, fast, efficient, and selective, and produce low amounts of by-products while also

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being less demanding with respect to process energy, raw materials and toxic components than many traditional chemical catalysts. Their exquisite catalytic power, specificity of action and reduced environmental footprint makes them seemingly ideal tools for numerous biotechnological applications. Indeed, they have already found application in many diverse industries including food, beverages, pharmaceuticals, detergents, textiles, leather, chemical, biofuels, animal feed, personal care, pulp and paper, diagnostics, and therapy, and are continuously developing into new areas. They are employed to enable improved and/or more economic and eco-friendly end-products and bioprocesses and can even facilitate the development of novel products and processes. The current market value of industrial enzymes is estimated at almost USD 5 billion (BCC Research 2017), up from USD 3.3 billion in 2010 (Blamey et al. 2017), and is expected to reach almost USD 6.3 billion by 2021 (BCC Research 2017).

The International Union of Biochemistry and Molecular Biology (IUBMB) and the International Union of Pure and Applied Chemistry (IUPAC) have classified enzymes into six main classes (Enzyme Commission, EC, numbers 1–6) based on the types of reactions catalyzed. Enzymes belonging to all six classes have found application, but the hydrolases (EC number 3) are the most widespread used, with lipases, proteases, glycosidases, and other hydrolytic enzymes accounting for almost 90% of the total industrial enzymes market share (Blamey et al. 2017). These are mainly used as high volume, commodity, or bulk enzymes in the manufacture of food, beverage, cleaning agents, textiles, biofuels, and pulp and paper. On the other hand, specialty enzymes, for use in high value, low volume products such as in diagnostics, research and development, and as biocatalysts, currently have a small market share but have shown strong growth in recent years. Improved health care and the adaption of a "personalized" medicine approach drive this growth in diagnostics. Similarly, the development of "Green chemistry," making use of the high substrate-, regio-, and enantio- selectivity of enzymes for the sustainable production, modification, and/or functionalization of pharmaceuticals, fine chemicals, flavors, fragrances, etc., drives growth in biocatalysis. Indeed, the current societal shift towards greener technologies and a more sustainable low carbon resource-efficient economy as well as the expansion of biotechnology into new fields previously dominated by petroleum-based chemicals will continue to drive growth of enzymes in the future. In fact, currently only about 11% of all chemicals are made from renewable raw materials, with the remaining being obtained from crude oil, natural gas, and coal (Blamey et al. 2017), and it is believed that enzymes will play a major role in the shift towards a greater utilization of renewables in the future and in the development of efficient, improved, environmentally friendly, and sustainable bio-based processes.

For successful integration into a particular application, the ideal enzyme needs to appropriately catalyze the desired reaction with the desired specificity under the conditions used. That is to say, the enzyme needs to have the required specificity and selectivity as well as a high activity and stability in the process environment. Unfortunately, and due mainly to the harsh conditions frequently used in industrial processes, few naturally occurring enzymes fulfill all of these requirements

(Bommarius and Paye 2013; Sarmiento et al. 2015). High temperatures, as used, for example, in pulp and paper manufacture and in bioconversion, are often required in processes to enable a better breakdown and improved solubility of substrates and products as well as reduced viscosity, higher mass transfer rates, easier separation of volatile products, reduced contamination, and a shift in the equilibrium of endothermic reactions towards products (Siddiqui 2015). Conversely, low temperatures are more suited for processes involving heat-sensitive or/and volatile components or/and where undesirable chemical side-reactions occurring at high temperatures and contamination problems are to be avoided, such as in the manufacture of many foods, beverages, fine chemicals, and pharmaceuticals. Furthermore, in addition to extremes of temperature, many industrial processes are also carried out under extremes of pH, pressure, salinity, and/or in the presence of detergents, non-aqueous solvents, etc. (Bommarius and Paye 2013; Sarmiento et al. 2015; Barroca et al. 2017). Hence, standard, naturally occurring enzymes, which are typically stable and active over a narrow range near moderate physical and chemical conditions, are unsuited for use in numerous industrial processes in which "unnatural" conditions are used. In an attempt to overcome this, scientists have turned to extremophilic enzymes (Liszka et al. 2012; Elleuche et al. 2014; Littlechild 2015; Siddiqui 2015) and protein engineering (Liszka et al. 2012; Bommarius and Paye 2013). The use of rational design or directed evolution to fine-tune or engineer the properties of a protein for a particular application has achieved some success, but the use of extremophilic enzymes already adapted to extreme conditions offers a more direct route. Indeed, extremophilic enzymes, produced by organisms inhabiting and adapted to extreme environments, have already been shown to be valuable tools for processes in which extreme conditions prevail. A large number of extremophilic enzymes are already commonly used in various diverse applications with the vast majority being thermophiles, these being active and stable at high temperatures and frequently also in the presence of harsh chemicals and detergents. In contrast, use of enzymes from other extreme environments appears much less developed. As an example, a search of patenting databases for patents on enzymes with the keywords "cold-active," "cold-adapted," "cold-resistant," or "psychrophilic" in the title or abstract identified 53 patents whereas a similar search using the keywords "thermostable," "heat-stable," "heat-resistant," and "thermophilic" gave over 60-times more hits. Interestingly, while currently being much less employed than thermophilic enzymes, cold-adapted enzymes have an enormous potential as highly valuable tools for various biotechnological applications, and this review will focus on these enzymes and their biotechnological aspects.

19.2 Cold-Adapted Enzymes

As discussed in Chap. 10 of this book and in numerous previous review papers on the subject (Santiago et al. 2016; Fields et al. 2015; Siddiqui 2015; Gerday 2013, 2014; Collins et al. 2002a, 2007, 2008; Huston 2008; D'Amico et al. 2006),

cold-adapted or psychrophilic enzymes, produced by organisms inhabiting permanently low temperature environments, are typically characterized by a high activity at low temperatures and a reduced stability as compared to their mesophilic and thermophilic homologs. They are believed to have overcome the low temperature challenge and maintained high activities at low temperatures by increasing the flexibility of specific regions of their molecular edifice. This increased flexibility enables a continued mobility of those regions important for enzyme activity, even in the low energy environment characteristic of low temperatures, and is achieved via a reduction in the number and/or strength of stabilizing interactions in the protein structure which in turn leads to the observed reduced structural stability of cold-adapted enzymes. Importantly, this balancing of flexibility, activity, and stability is believed to be key in enzyme adaptation to temperature, with thermophilic enzymes, in contrast to those from psychrophiles, being generally characterized by a high stability, low flexibility, and reduced low temperature activity.

The intrinsic attributes of a high activity at low temperatures and reduced stability of cold-adapted enzymes offer many advantages for use in a variety of commercial applications. Low temperature processes are common in the food and beverages industries and cold-adapted enzymes, highly active under these conditions, offer obvious benefits for such processes. Also, it is important to note that cold-adapted enzymes are not only more highly active than their mesophilic and thermophilic homologs at low temperatures, but frequently also show a higher activity at moderate temperatures. Hence, processes can be carried out at ambient temperatures without the need for energy input (for heating or cooling) and with lower quantities of cold-adapted enzyme being required as compared to enzymes adapted to higher temperatures. That is to say that these enzymes can be instrumental in developing processes with an improved economic and environmental impact. Furthermore, the thermolability of these enzymes offers solutions for those processes where a greater control is required and where a simple selective inactivation of the enzyme can be achieved by mild heat treatment (Margesin et al. 2003). Such a characteristic may prove beneficial for preserving product quality in the food and beverages industry and in biocatalysis or for sequential multi-step processes such as those used in molecular biology (Huston 2008). In fact, cold-adapted enzymes have already found application in these industries and their now commonplace application in cleaning/detergents applications as well as development into new markets underscores the potential and market value of these enzymes. In effect, market research by the Freedonia Group (The Freedonia Group 2016) has indicated that, in the mature commodity enzymes markets where competition among enzyme makers is intense, novel enzymes presenting efficient performance at lower temperatures will play an important role in allowing for market expansion in the future. In addition, while little explored at present, it is believed that in the future, psychrophilic enzymes will offer valuable tools as specialty enzymes in the preparation of temperature-sensitive pharmaceutical ingredients, fine-chemicals, flavors, and fragrances. In the following sections, the application of cold-adapted enzymes in various industrial enzymes markets will be discussed.

19.3 Cleaning/Detergents

This is probably the best developed market for cold-adapted enzymes with a large number of different hydrolytic enzymes (EC 3) being commercialized for this application by various companies throughout the world. See Sarmiento et al. (2015) for an in-depth review.

Enzymes are used as cleaning agents in household and industrial scale laundry and dishwashing, as well as for cleaning-in-place in the food, dairy, and brewing industries and even in the cleaning of buildings, carpets, contact lenses, etc. (Cavicchioli et al. 2002; Damhus et al. 2013; Sarmiento et al. 2015; Siddiqui 2015). This market currently accounts for approximately 20–25% of total industrial enzymes sales (BCC Research 2017). Enzymes break down stains, soiling, and deposits into more soluble products, thereby allowing for improved cleaning performance in, for example, laundry and dishwashing as well as for the deblocking and cleaning of filters and equipment in the food and beverages industries. Lipases hydrolyze lipids as found, for example, in grease, butter, oil, sauces, tears, molds, and biofilms; proteases break down proteins common in grass, blood, egg, milk, cheese, yoghurt, sweat, tears, molds, etc., and amylases are used for breakdown of starch soiling from cereals, pasta, potatoes, molds, biofilms, etc. Cellulases have also been used and act on oat products, such as cereals and snack bars. Furthermore, in laundry detergents, cellulases have an added benefit of contributing to fabric care; they degrade accessible broken cotton fibers (known as fuzz or pills) and thereby remove any captured dirt but at the same time reduce fuzz build up and hence also increase the softness and color brightness of cotton fabrics. Recently, attention has also been turned to mannanases and pectinases for use in cleaning detergents for removal of difficult stains due to gum, fruit products, juices, mayonnaise, tomato sauce, salad dressing, body lotions, personal care products, etc. Pullulanases have also been shown to have potential for biofilm removal (Antranikian et al. 2004). The action of enzymes allows for improved cleaning performance and in turn this improved effectiveness enables a reduction in the use of other more hazardous components, e.g., detergents, surfactants, polymers, alkaline builders (in laundry), phosphates (in dishwashing), and organic solvents (for cleaning-in-place), as well as reduced water consumption (Damhus et al. 2013; Sarmiento et al. 2015). Indeed, up to 25–50% reduction in a laundry detergent surfactant system has even been demonstrated upon use of enzymes (Damhus et al. 2013; Siddiqui 2015). Such modifications obviously lead to environmentally friendlier wash water wastes and more sustainable wash processes.

The major benefit of using cold-adapted enzymes in cleaning/detergents is that the process temperature and hence energy input can be reduced and thus enables an improved economic and environmental impact. It has been estimated that a reduction in wash temperature from 40 to 30 °C allows for a 30% saving in energy, corresponding to 100–300 g of CO_2 per wash (Cavicchioli et al. 2011; Siddiqui 2015), and already over 50% of laundry machine washes are carried out at low temperatures (Sarmiento et al. 2015). Furthermore, in laundry washing, low

temperatures also extend garment life by being less aggressive, reducing garment degradation, and lessening shrinkage and/or dye bleeding. Currently, the focus is on further reducing temperatures to approx. 20 °C and leading to further savings, and a continued growth in the use of cold-adapted enzymes in this application is thus forecast. In cleaning in-place applications in the food and beverages industries, the use of cold-adapted enzymes would avoid the need for warm cleaning washes and the poor stability of these enzymes would give a greater assurance of complete enzyme inactivation following heat treatment, a desirable characteristic for the cleaning of food, dairy, and brewing industry equipment. Additionally, cold-adapted enzymes could offer advantages in the cleaning of large immovable objects where heating is not viable, and indeed glucose oxidases, proteases, amylases, and lipases have already been shown to be effective in mold and biofilm removal from building surfaces (Webster and May 2006; Valentini et al. 2010). These were shown to enable effective building cleaning and conservation while also reducing the use of more aggressive cleaning agents. Similarly, enzymes have been shown to be effective in contact lens cleaning, and a thermolabile fish waste isolate protease has been shown to have potential as an efficient, non-hazardous cleaning agent for the removal of tear films and proteinaceous deposits on contact lens (Pawar et al. 2009).

19.4 Food and Beverages

Industrial enzymes for use in the food and beverages industries represent a relatively well developed market with current sales of nearly USD 1.5 billion (BCC Research 2017). They are used as food additives and processing aids in such diverse areas as the more traditional processes of cheese manufacturing, wine making, brewing, and bread making to the more recent applications in the preparation of functional foods and nutraceuticals (Chandrasekaran 2015). Enzymes are used in the manufacture, processing, preparation, and treatment of foods and beverages. They can enable improved process efficiency, reduced processing costs, and reduced environmental impact and, frequently also, enhance the flavor, nutritional value, appeal, digestibility, texture, and/or shelf life of the final product. Growing consumer preference for more natural, healthier, and flavorsome foods as well as an improved awareness of environmental issues and food safety is driving continued growth in the use of enzymes in this sector, and a compound annual growth rate of 4.7% through 2021 has been forecasted (BCC Research 2017).

Cold-adapted enzymes are particularly attractive in food and beverages preparation due to their high catalytic activity at temperatures that minimize spoilage, alterations in taste, and loss of nutritional value as well as their ease of inactivation (Huston 2008). They have found application in the dairy, baking, beverages, meat, and fish processing industries and in the production of functional foods.

In the dairy industry, a number of cold-adapted β-galactosidases, or lactases, have been developed for the production of lactose free milk and treatment of the

waste by-product whey. Approximately 65% of the human population has a reduced ability to digest lactose after infancy, with Asian and African populations being the most affected. β-Galactosidases hydrolyze lactose to glucose and galactose and hence can be used to remove lactose from dairy products and improve product digestibility while also enhancing sweetness. A variety of β-galactosidases are currently being marketed, but cold-adapted β-galactosidases offer the advantage of efficient hydrolysis at refrigeration temperatures which minimize problems associated with contamination and alteration of product organoleptic properties (Hoyoux et al. 2001; Ghosh et al. 2012; Stougaard and Schmidt 2012; Pawlak-Szukalska et al. 2014). In the valorization of whey, a by-product of cheese production and a waste disposal problem, the glucose and galactose produced by β-galactosidase treatment can be used as sweeteners in soft drinks and confectionary products, in the production of functional foods/nutraceuticals (Van de Voorde et al. 2014), and in biofuel production (Huston 2008). A recent study showed the potential of using a cold-adapted β-galactosidase for the initial steps of preparation of tagatose, a novel, low-calorie sweetener (Van de Voorde et al. 2014). Similarly, cold-adapted β-galactosidases have also been shown to be useful in the preparation of other low calorie sweeteners, namely, galactooligosaccharides (Karasova-Lipovova et al. 2003; Nakagawa et al. 2007; Schmidt and Stougaard 2010; Pawlak-Szukalska et al. 2014). In effect, in addition to hydrolysis, many β-galactosidases also display transglycosylation activities where monosaccharides are transferred to oligosaccharides with the production of di-, tri-, tetra-, and pentasaccharides. These can be produced in the milk or from whey, and in addition to functioning as low calorie sweeteners, they have also been found to be effective prebiotics, selectively stimulating the growth of beneficial intestinal microorganisms (Pawlak-Szukalska et al. 2014). Proteases are another family of enzymes which play an important role in the dairy industry and in particular during clotting and ripening in cheese making. Lipases and phospholipases are also used and due to the low temperatures employed, cold-adapted variants have been suggested for this (Huston 2008).

Amylases, xylanases, oxidases, asparaginases, and lipases are all commonly used in baking applications so as to improve product quality (dough machinability, bread texture, and shelf life), reduce the use of chemical additives (e.g., potassium bromate, emulsifiers, etc.), and reduce the production of acrylamide (in baked or fried products including biscuits, crisps, crackers, etc.). As yet, the majority of enzymes used appear to be of mesophilic or thermophilic origin whereas dough preparation and proofing is typically carried out at moderate temperatures at which cold-adapted enzymes offer considerable cost and efficiency advantages. Nevertheless, it appears that currently only one cold-adapted enzyme has found application in baking, namely, a cold-adapted xylanase (Collins et al. 2002b, 2006, 2012; Dornez et al. 2011; Dutron et al. 2012). Xylanases improve dough machinability, giving rise to a more flexible, easier-to-handle dough, larger loaf size, and improved crumb structure, and the cold-adapted xylanase was shown to be more effective than the other commercial xylanases studied (Collins et al. 2006, 2012; Dutron et al. 2012).

In the brewing, wine, and fruit and vegetable processing industries, pectinases (polygalacturonases, pectin lyases, and pectin methylesterase) and hemicellulases such as xylanases are used to increase extraction yield, improve clarification, reduce viscosity, and enhance color and flavor (Tu et al. 2013; Adapa et al. 2014). Rhamnogalacturonases, galactanases, and arabinanases have also been recently developed for these applications. Apparently no cold-adapted enzymes have been commercialized in this sector as yet but low temperature active enzymes are available, e.g., Lallzyme EX (Lallemand) is active between 5 and 20 °C (Sarmiento et al. 2015). Pectin esterases can also be used in the manufacture of fruit preparations composed of intact fruit pieces, and a cold-active pectin methylesterase was found to increase gelling and enhance fruit integrity during processing (Pan et al. 2014). Finally, pectinases, in addition to other glycoside hydrolases (EC 3.2.1), lipases, and proteases, can likewise be used for the treatment of food and beverage industry wastes with cold-adapted enzymes allowing for a more effective ambient temperature waste management (Margesin et al. 2005; Naga Padma et al. 2011; Tsuji et al. 2013).

In meat and fish processing, cold-active proteases can be used for tenderization and taste enhancement as well as improving the nutritional and functional properties of refrigerated products (He et al. 2004; Bjarnason and Benediktsson 2010; Venugopal 2016). They can be employed in the preparation of soluble protein hydrolysates for use as flavor enhancers, meat extracts, emulsifiers, and foaming agents and which have also been shown to exert health benefits such as antihypertensive, antioxidant, and immunoregulatory activity (Cazarin et al. 2015). A study by He et al. (2004) showed how a cold-adapted protease released more taste amino acids and essential amino acids from meat than a mesophilic protease during cold storage. Similarly, a marine protease was shown to be effective in the preparation of protein hydrolysates for use as flavor enhancers in foods for human consumption and animal feed (Bjarnason and Benediktsson 2010). Finally, the use of cold-adapted enzymes (proteases, lipases, chitinase etc.) in seafood processing (fish descaling, skin removal and degreasing, waste treatment, oil extraction, etc.) has also been discussed (Shahidi and Janak Kamil 2001; Junpei et al. 2016; Venugopal 2016).

19.5 Molecular Biology

Cold-adapted alkaline phosphatases (Kobori et al. 1984; Sullivan et al. 1988; Rina et al. 2000; Nilsen et al. 2008; Muller-Greven et al. 2012), both single and double stranded nucleases (Awazu et al. 2011) and uracil-DNA *N*-glycosylases (Lanes et al. 2002), are currently being commercialized as molecular biology tools by various companies (New England Biolabs Inc., ArcticZymes, Takara-Clontech, Affymetrix, Inc.). Alkaline phosphatases are most commonly used in the dephosphorylation of the 5′ end of DNA or RNA during cloning and end-labeling procedures. Nucleases, depending on their specificity, degrade single and/or double stranded DNA and/or RNA and are used, e.g., in removing contaminating

DNA/RNA from RNA preparations, PCR master mixes, and protein solutions. Uracil-DNA *N*-glycosylases are used in PCR, RT-PCR, site-directed mutagenesis, and SNP genotyping procedures to release free uracil from uracil-containing DNA (Sarmiento et al. 2015). In all these cases, in addition to a high activity at low temperatures being beneficial, the instability of cold-adapted enzymes is a determining factor in their successful application. This latter characteristic enables for simplified enzyme inactivation by moderate heat treatment as opposed to the time consuming chemical treatments or column purifications required with mesophilic or thermophilic variants which often also lead to sample loss and downstream contamination problems.

Recently, a cold-adapted polymerase has been commercialized by Arcticzymes for use in gene sequencing, molecular diagnostics, and other markets. Furthermore, cold-adapted ligases, recombinases, and proteinase k have been called for (Huston 2008; Sarmiento et al. 2015).

19.6 Biomedicine, Pharmaceuticals, and Cosmetics

Many pharmaceuticals, active pharmaceutical ingredients, fine chemicals, flavors, and fragrances are heat sensitive or/and volatile and hence must be synthesized at low temperatures at which cold-adapted enzymes are most active. In addition, it has been proposed that as a result of their proposed high structural flexibility, cold-adapted enzymes can operate at low water activity, such as in the aqueous/organic and non-aqueous solvent systems frequently used during organic synthesis of complex molecules (Huston 2008; Karan et al. 2012). In this market sector, hydrolases, oxidoreductases, lyases, transferases, reductases, carboxylases, etc. are becoming more commonly used but only a few cold-adapted enzymes have been investigated and below an overview of these is given.

The most widely used cold-adapted enzymes in this sector are lipases and esterases for the synthesis of optically pure intermediate compounds of synthetic value. In fact, lipases (mainly CALB) from *Candida antarctica* are among the most extensively and diversely used enzymes in organic synthesis. They are used in a broad range of surprisingly diverse applications, including the modification of sugars and sugar-related compounds, desymmetrization of complex prochiral drug intermediates, and resolution of racemic alcohols and amines (Huston 2008; Kirk and Christensen 2002; Suen et al. 2004) during the synthesis of various pharmaceuticals (e.g., calcium antagonists as antihypertensive drugs, NK1/NK2 antagonist for asthma treatment), cosmetics (e.g., iso-propyl myristate, a skin emollient), flavors, and fragrance esters.

As discussed above (Sect. 19.4), cold-adapted ß-galactosidases are suited to the production of tagatose (an antihyperglycemic agent) and galactooligosaccharides (prebiotics). Moreover, these have also been shown to catalyze the synthesis of heterooligosaccharides such as lactulose (for treatment of constipation and hepatic encephalopathy, use as a prebiotic, and use in diagnostics), galactosyl-xylose (use

in diagnostics), and alkyl glycosides (foaming agents) as well as glycosylated salicin (antiinflammatory agent) from lactose (Pawlak-Szukalska et al. 2014). Also, as discussed previously, cold-adapted proteases can be used for the preparation of bioactive peptides for use as antihypertensive, antioxidant, and immunoregulatory agents (Cazarin et al. 2015).

Cold-adapted proteases are currently being marketed as therapeutic agents against bacterial (biofilm breakdown) and viral (virus infectivity reduction) infections and in oral health care (plaque removal) and cosmetics (frown line reduction and dead or dried skin removal) (Fornbacke and Clarsund 2013).

Other cold-adapted enzymes with potential in biomedical applications include a marine α-galactosidases which was shown to be capable of converting B red blood cells into the universal blood type O cells for use in transfusion therapy (Balabanova et al. 2010) and a cold-active nitroreductase as a cancer prodrug activating enzyme using low temperature therapy for activation (Çelik and Yetiş 2012).

19.7 Other Applications

In the textiles industry, cold-adapted amylases, cellulases, and laccases have been developed for the rapid desizing, or starch removal, of woven fabrics, bio-finishing of cellulosic fabrics, and less abrasive enzymatic stonewashing and bleaching of denim (Sarmiento et al. 2015).

Cold-adapted enzymes, namely, cell wall degrading enzymes, amylases, laccases, lipases, and phospholipases, have been suggested for improving the energy efficiency and costs of biofuel (bioethanol, biodiesel, and biogas) production processes. In particular, they should find application in cold-cook or no-cook processes, simultaneous saccharification and fermentation (Festersen et al. 2005; Huston 2008; Gohel and Duan 2012; Ji et al. 2014; Wen et al. 2015), and low temperature biogas production (Akila and Chandra 2010).

Marine silicatein enzymes have been shown to be central in biomineralization and in the synthesis of biosilicates found in marine diatoms, radiolaria, and sponges (Shimizu et al. 1998; Wang et al. 2012). This has important implications in materials science and indicates the potential of cold-adapted enzymes for the synthesis of a variety of nanostructured mineral/organic composite materials under low temperature and mild chemical conditions. Examples of such materials include silica and siloxane polymers, bimetallic alloy nanoparticles, bimetallic perovskite-like materials, zirconia nanoparticles, spinel gallium oxide, etc. See Huston (2008) for a review.

19.8 Conclusions

The initial development of cold-adapted enzymes for use in industry was somewhat delayed as compared to mesophilic and thermophilic enzymes. Nowadays, however, they have found application in almost all sectors of the industrial enzymes

markets. A continued growth in their use is envisaged in the future as novel cold-adapted enzymes with unique properties are isolated from the vast and varied cold environments in the world and as techniques for their isolation (Cavicchioli et al. 2011), efficient production (Cavicchioli et al. 2011), purification, engineering (Liszka et al. 2012; Bommarius and Paye 2013), stabilization, and immobilization (Mateo et al. 2007) are further developed. In addition, the current shift towards a more environmentally friendly and sustainable economy and the development of enzymes for biocatalysis will enable expansion into new application areas and drive growth further.

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References

Adapa V, Ramya LN, Pulicherla KK, Sambasiva Rao KRS (2014) Cold active pectinases: advancing the food industry to the next generation. Appl Biochem Biotechnol 172 (5):2324–2337. doi:10.1007/s12010-013-0685-1

Akila G, Chandra TS (2010) Stimulation of biomethanation by *Clostridium* sp. PXYL1 in coculture with a *Methanosarcina* strain PMET1 at psychrophilic temperatures. J Appl Microbiol 108(1):204–213. doi:10.1111/j.1365-2672.2009.04412.x

Antranikian G, Breves R, Janßen F, Qoura FM (2004) Pullulanases from psychrophilic organisms. Patent Application WO2006032477A1, DE102004046116A1

Awazu N, Shodai T, Takakura H, Kitagawa M, Mukai H, Kato I (2011) Microorganism-derived psychrophilic endonuclease. Granted Patent US8034597B2

Balabanova LA, Bakunina IY, Nedashkovskaya OI, Makarenkova ID, Zaporozhets TS, Besednova NN, Zvyagintseva TN, Rasskazov VA (2010) Molecular characterization and therapeutic potential of a marine bacterium *Pseudoalteromonas* sp. KMM 701 α-galactosidase. Mar Biotechnol 12(1):111–120. doi:10.1007/s10126-009-9205-2

Barroca M, Santos G, Johansson B, Gillotin F, Feller G, Collins T (2017) Deciphering the factors defining the pH-dependence of a commercial glycoside hydrolase family 8 enzyme. Enzym Microb Technol 96:163–169. doi:10.1016/j.enzmictec.2016.10.011

BCC Research (2017) BCC research report. Global markets for enymes in industrial applications. BIO030J, Jan 2017. BCC Research LLC, USA

Bjarnason JB, Benediktsson B (2010) Protein hydrolysates produced with the use of marine proteases. Granted Patents CA2421058C, DE60007655D1/T2, EP1227736B1, US7070953B1

Blamey JM, Fischer F, Meyer H-P, Sarmiento F, Zinn M (2017) Enzymatic biocatalysis in chemical transformations: a promising and emerging field in green chemistry practice. In: Brahmachari G, Demain AL, Adrio JL (eds) Biotechnology of microbial enzymes, production, biocatalysis and industrial applications. Academic, New York, pp 347–403. doi:10.1016/B978-0-12-803725-6.00014-5

Bommarius AS, Paye MF (2013) Stabilizing biocatalysts. Chem Soc Rev 42:6534–6565. doi:10.1039/c3cs60137d

Cavicchioli R, Charlton T, Ertan H, Mohd Omar S, Siddiqui KS, Williams TJ (2011) Biotechnological uses of enzymes from psychrophiles. Microb Biotechnol 4(4):449–460. doi:10.1111/j.1751-7915.2011.00258.x

Cavicchioli R, Siddiqui KS, Andrews D, Sowers KR (2002) Low-temperature extremophiles and their applications. Curr Opin Biotechnol 13(3):253–261

Cazarin C, Lima G, da Silva J, Maróstica M (2015) Enzymes in meat processing. In: Chandrasekaran M (ed) Enzymes in food and beverage processing. CRC, Boca Raton, pp 337–351. doi:10.1201/b19408-17

Çelik A, Yetiş G (2012) An unusually cold active nitroreductase for prodrug activations. Bioorg Med Chem 20(11):3540-3550. doi:10.1016/j.bmc.2012.04.004

Chandrasekaran M (2015) Enzymes in food and beverage processing. CRC, Boca Raton

Collins T, Claverie P, D'Amico S, Georlette D, Gratia E, Hoyoux A, Meuwis MA, Poncin J, Sonan G, Feller G, Gerday C (2002a) Life in the cold: psychrophilic enzymes. In: Pandalai SG (ed) Recent research developments in proteins, vol 1. Transworld Research Network, Trivandrum, pp 13–26

Collins T, Meuwis MA, Stals I, Claeyssens M, Feller G, Gerday C (2002b) A novel family 8 xylanase, functional and physicochemical characterization. J Biol Chem 277 (38):35133–35139. doi:10.1074/jbc.M204517200

Collins T, Hoyoux A, Dutron A, Georis J, Genot B, Dauvrin T, Arnaut F, Gerday C, Feller G (2006) Use of glycoside hydrolase family 8 xylanases in baking. JCS 43:79–84

Collins T, D'Amico S, Marx J, Feller G, Gerday C (2007) Cold-adapted enzymes. In: Gerday C, Glansdorff N (eds) Physiology and biochemistry of extremophiles. ASM Press, Washington, DC, pp 165–179. doi:10.1128/9781555815813.ch13

Collins T, Feller G, Gerday C, Meuwis MA (2012) Family 8 enzymes with xylanolytic activity. Granted Patent US8309336B2

Collins T, Roulling F, Florence P, Marx JC, Feller G, Gerday C, D'Amico S (2008) Fundamentals of cold-adapted enzymes. In: Margesin R, Schinner F, Marx JC, Gerday C (eds) Psychrophiles: from biodiversity to biotechnology. Springer, Berlin, pp 211–227

D'Amico S, Collins T, Marx JC, Feller G, Gerday C (2006) Psychrophilic microorganisms: challenges for life. EMBO Rep 7(4):385–389

Damhus T, Kaasgaard S, Olsen HS (2013) Enzymes at work, 4th edn. Novozymes A/S, Denmark

Dornez E, Verjans P, Arnaut F, Delcour JA, Courtin CM (2011) Use of psychrophilic xylanases provides insight into the xylanase functionality in bread making. J Agric Food Chem 59 (17):9553–9562. doi:10.1021/jf201752g

Dutron A, Georis J, Genot B, Dauvrin T, Collins T, Hoyoux A, Feller G (2012) Use of family 8 enzymes with xylanolytic activity in baking. Granted Patents US8192772 (2012), EP1549147B1 (2011), CN1681392B (2010), DE60336153 D1 (2011), CA 2498014C (2011), ES2360942 (2011), DE60336153D1 (2011)

Elleuche S, Schroder C, Sahm K, Antranikian G (2014) Extremozymes—biocatalysts with unique properties from extremophilic microorganisms. Curr Opin Biotechnol 29:116–123. doi:10.1016/j.copbio.2014.04.003

Festersen RM, Olsen HS, Pedersen S (2005) Alcohol product processes. Granted Patents DE04718914T1, EP1604019B1, CN1788083B, US8772001B2

Fields PA, Dong Y, Meng X, Somero GN (2015) Adaptations of protein structure and function to temperature: there is more than one way to 'skin a cat'. J Exp Biol 218(12):1801–1811. doi:10.1242/jeb.114298

Fornbacke M, Clarsund M (2013) Cold-adapted proteases as an emerging class of therapeutics. Infect Dis Ther 2(1):15–26. doi:10.1007/s40121-013-0002-x

Gerday C (2013) Psychrophily and catalysis. Biology 2(2):719–741. doi:10.3390/biology2020719

Gerday C (2014) Fundamentals of cold-active enzymes. In: Buzzini P, Margesin R (eds) Cold-adapted yeasts: biodiversity, adaptation strategies and biotechnological significance. Springer, Berlin, pp 325–350. doi:10.1007/978-3-642-39681-6_15

Ghosh M, Pulicherla KK, Rekha VP, Raja PK, Sambasiva Rao KR (2012) Cold active beta-galactosidase from *Thalassospira* sp. 3SC-21 to use in milk lactose hydrolysis: a novel source from deep waters of Bay-of-Bengal. World J Microbiol Biotechnol 28(9):2859–2869. doi:10.1007/s11274-012-1097-z

Gohel V, Duan G (2012) No-cook process for ethanol production using indian broken rice and pearl millet. Int J Microbiol 2012:680232. doi:10.1155/2012/680232

He H, Chen X, Li J, Zhang Y, Gao P (2004) Taste improvement of refrigerated meat treated with cold-adapted protease. Food Chem 84(2):307–311. doi:10.1016/S0308-8146(03)00242-5

Hoyoux A, Jennes I, Dubois P, Genicot S, Dubail F, Francois JM, Baise E, Feller G, Gerday C (2001) Cold-adapted beta-galactosidase from the Antarctic psychrophile *Pseudoalteromonas haloplanktis*. Appl Environ Microbiol 67(4):1529–1535

Huston AL (2008) Biotechnological aspects of cold-adapted enzymes. In: Margesin R, Schinner F, Marx JC, Gerday C (eds) Psychrophiles: from biodiversity to biotechnology. Springer, Berlin, pp 347–364

Ji L, Yang J, Fan H, Yang Y, Li B, Yu X, Zhu N, Yuan H (2014) Synergy of crude enzyme cocktail from cold-adapted *Cladosporium cladosporioides* Ch2-2 with commercial xylanase achieving high sugars yield at low cost. Biotechnol Biofuels 7(1):130. doi:10.1186/s13068-014-0130-x

Junpei Z, Rui Z, Zunxi H, Zhifeng S, Xianghua T, Junjun L, Qian W (2016) Low-temperature salt-tolerant product-inhibition-resistant beta-N-acetyl glucosamine enzyme JB10NagA. Patent Application CN105483101A

Karan R, Capes MD, Dassarma S (2012) Function and biotechnology of extremophilic enzymes in low water activity. Aquat Biol 8(1):4. doi:10.1186/2046-9063-8-4

Karasova-Lipovova P, Strnad H, Spiwok V, Mala S, Kralova B, Russell NJ (2003) The cloning, purification and characterisation of a cold-active [beta]-galactosidase from the psychrotolerant Antarctic bacterium *Arthrobacter* sp. C2-2. Enzym Microb Technol 33(6):836–844

Kirk O, Christensen MW (2002) Lipases from *Candida antarctica*: unique biocatalysts from a unique origin. Org Process Res Dev 6(4):446–451. doi:10.1021/op0200165

Kobori H, Sullivan CW, Shizuya H (1984) Heat-labile alkaline phosphatase from Antarctic bacteria: rapid 5' end labelling of nucleic acids. Proc Natl Acad Sci USA 81:6691–6695

Lanes O, Leiros I, Smalas AO, Willassen NP (2002) Identification, cloning, and expression of uracil-DNA glycosylase from Atlantic cod (*Gadus morhua*): characterization and homology modeling of the cold-active catalytic domain. Extremophiles 6(1):73–86

Liszka MJ, Clark ME, Schneider E, Clark DS (2012) Nature versus nurture: developing enzymes that function under extreme conditions. Annu Rev Chem Biomol Eng 3:77–102. doi:10.1146/annurev-chembioeng-061010-114239

Littlechild JA (2015) Enzymes from extreme environments and their industrial applications. Front Bioeng Biotechnol 3:161. doi:10.3389/fbioe.2015.00161

Margesin R, Fauster V, Fonteyne PA (2005) Characterization of cold-active pectate lyases from psychrophilic *Mrakia frigida*. Lett Appl Microbiol 40(6):453–459

Margesin R, Feller G, Gerday C, Russell NJ (2003) Cold-adapted microorganisms: adaptation strategies and biotechnological potential. In: Britton G (ed) Encyclopedia of environmental microbiology. Wiley, New York. doi:10.1002/0471263397.env150

Mateo C, Palomo JM, Fernandez-Lorente G, Guisan JM, Fernandez-Lafuente R (2007) Improvement of enzyme activity, stability and selectivity via immobilization techniques. Enzyme Microb Technol 40(6):1451–1463. doi:10.1016/j.enzmictec.2007.01.018

Muller-Greven JC, Post MA, Kubu CJ (2012) Recombinant *Colwellia psychrerythraea* alkaline phosphatase and uses thereof. Granted Patents US8486665B2, US8129168B2

Naga Padma P, Anuradha K, Reddy G (2011) Pectinolytic yeast isolates for cold-active polygalacturonase production. Innov Food Sci Emerg Technol. 12(2):178–181. doi:10.1016/j.ifset.2011.02.001

Nakagawa T, Ikehata R, Myoda T, Miyaji T, Tomizuka N (2007) Overexpression and functional analysis of cold-active β-galactosidase from *Arthrobacter psychrolactophilus* strain F2. Protein Expr Purif 54(2):295–299. doi:10.1016/j.pep.2007.03.010

Nilsen I, Overbö K, Lanes O (2008) Shrimp alkaline phosphatase. Granted Patents US 7323325B2, DE60130567D1, DE60130567T2, EP1326890B1

Pan X, Tu T, Wang L, Luo H, Ma R, Shi P, Meng K, Yao B (2014) A novel low-temperature-active pectin methylesterase from *Penicillium chrysogenum* F46 with high efficiency in fruit firming. Food Chem 162:229–234. doi:10.1016/j.foodchem.2014.04.069

Pawar R, Vasudeo Z, Siddhivinayak B, Govind P (2009) Application of protease isolated from Bacillus sp. 158 in enzymatic cleansing of contact lenses. Biotechnology 8(2):276–280. doi:10.3923/biotech.2009.276.280

Pawlak-Szukalska A, Wanarska M, Popinigis AT, Kur J (2014) A novel cold-active β-d-galactosidase with transglycosylation activity from the Antarctic *Arthrobacter* sp. 32cB—Gene cloning, purification and characterization. Process Biochem 49(12):2122–2133. doi:10.1016/j.procbio.2014.09.018

Rina M, Pozidis C, Mavromatis K, Tzanodaskalaki M, Kokkinidis M, Bouriotis V (2000) Alkaline phosphatase from the Antarctic strain TAB5. Properties and psychrophilic adaptations. Eur J Biochem 267(4):1230–1238

Santiago M, Ramírez-Sarmiento CA, Zamora RA, Parra LP (2016) Discovery, molecular mechanisms, and industrial applications of cold-active enzymes. Front Microbiol 7:1408. doi:10.3389/fmicb.2016.01408

Sarmiento F, Peralta R, Blamey JM (2015) Cold and hot extremozymes: industrial relevance and current trends. Front Bioeng Biotechnol 3:148. doi:10.3389/fbioe.2015.00148

Schmidt M, Stougaard P (2010) Identification, cloning and expression of a cold-active beta-galactosidase from a novel Arctic bacterium, *Alkalilactibacillus ikkense*. Environ Technol 31 (10):1107–1114. doi:10.1080/09593331003677872

Shahidi F, Janak Kamil YVA (2001) Enzymes from fish and aquatic invertebrates and their application in the food industry. Trends Food Sci Tech 12(12):435–464. doi:10.1016/S0924-2244(02)00021-3

Shimizu K, Cha J, Stucky GD, Morse DE (1998) Silicatein α: cathepsin L-like protein in sponge biosilica. Proc Natl Acad Sci USA 95(11):6234–6238

Siddiqui KS (2015) Some like it hot, some like it cold: Temperature dependent biotechnological applications and improvements in extremophilic enzymes. Biotechnol Adv 33(8):1912–1922. doi:10.1016/j.biotechadv.2015.11.001

Stougaard P, Schmidt M (2012) Cold-active beta-galactosidase, a method of producing same and use of such enzyme. Granted Patents US8288143, EP2396403B1, EP2396403B8, CN102361974B

Suen W-C, Zhang N, Xiao L, Madison V, Zaks A (2004) Improved activity and thermostability of *Candida antarctica* lipase B by DNA family shuffling. Protein Eng Des Sel 17(2):133–140. doi:10.1093/protein/gzh017

Sullivan CW, Shizuya H, Kobori H (1988) Heat sensitive bacterial alkaline phosphatase. Granted Patent US4720458

The Freedonia Group (2016) World enzymes industry study with forecasts for 2020 & 2025, Study #3417. https://www.freedoniagroup.com/brochure/34xx/3417smwe.pdf

Tsuji M, Yokota Y, Shimohara K, Kudoh S, Hoshino T (2013) An application of wastewater treatment in a cold environment and stable lipase production of Antarctic basidiomycetous yeast *Mrakia blollopis*. PLoS One 8(3):e59376. doi:10.1371/journal.pone.0059376

Tu T, Meng K, Bai Y, Shi P, Luo H, Wang Y, Yang P, Zhang Y, Zhang W, Yao B (2013) High-yield production of a low-temperature-active polygalacturonase for papaya juice clarification. Food Chem 141(3):2974–2981. doi:10.1016/j.foodchem.2013.05.132

Valentini F, Diamanti A, Palleschi G (2010) New bio-cleaning strategies on porous building materials affected by biodeterioration event. Appl Surf Sci 256(22):6550–6563. doi:10.1016/j.apsusc.2010.04.046

Van de Voorde I, Goiris K, Syryn E, Van den Bussche C, Aerts G (2014) Evaluation of the cold-active *Pseudoalteromonas haloplanktis* β-galactosidase enzyme for lactose hydrolysis in whey permeate as primary step of D-tagatose production. Process Biochem 49(12):2134–2140. doi:10.1016/j.procbio.2014.09.010

Venugopal V (2016) Enzymes from seafood processing waste and their applications in seafood processing. In: Se-Kwon K, Fidel T (eds) Advances in food and nutrition research, vol 78. Academic, New York, pp 47–69. doi:10.1016/bs.afnr.2016.06.004

Wang X, Schloßmacher U, Wiens M, Batel R, Schröder HC, Müller WEG (2012) Silicateins, silicatein interactors and cellular interplay in sponge skeletogenesis: formation of glass fiber-like spicules. FEBS J 279(10):1721–1736. doi:10.1111/j.1742-4658.2012.08533.x

Webster A, May E (2006) Bioremediation of weathered-building stone surfaces. Trends Biotechnol 24(6):255–260. doi:10.1016/j.tibtech.2006.04.005

Wen J, Ren C, Huang N, Liu Y, Zeng R (2015) Draft genome of bagasse-degrading bacteria *Bacillus aryabhattai* GZ03 from deep sea water. Mar Genomics 19:13–14. doi:10.1016/j.margen.2014.11.004

Chapter II

Chapter II.I

Macroalgae Xylan: Aqueous Extraction and Application as an Enzyme Assay Substrate

Abstract

The idea to shift from a petro-based to a bio-based society has gained momentum in recent years. Xylan, as a major biomass component, has a potentially important role to play in this bio-based society yet limitations associated with lack of a cost effective, environmentally friendly production process for large quantities of highly pure xylan impede its widespread utilisation. Currently, xylan is principally obtained from land sourced lignocellulosic feedstocks via costly multi-step processes employing various hazardous chemicals, harsh physical treatments and/or enzyme(s) hydrolysis. Taking advantage of the high xylan content, lignin free nature, weakly linked cell wall matrix and sustainability of the red macroalga *Palmaria palmata*, we have developed an efficient, eco-friendly aqueous based process for the fractionation of the macroalgae into a soluble xylan rich fraction and an insoluble protein enriched fraction. The extraction process involves a proprietary macroalgae pretreatment step employing commonly used macroalgae preservation techniques, followed by aqueous extraction, and soluble-insoluble fraction separation. Response surface methodology was used in process optimisation and the high yielding optimised process developed was found to enable extraction of 70% of the total xylan present in the macroalgae for a product consisting of 73% (dry weight) xylan. The xylan product produced herein was demonstrated to be effective as a soluble substrate in assays for xylanase activity measurements and, following chemical coupling with a suitable dye, to function as a chromogenic substrate for plate based screening of xylanase activity. The extraction process developed in this study offers a simpler, more effective and environmentally friendly xylan production process than those currently employed and thereby should enhance the market potential of this polysaccharide. Furthermore, this study promotes the multi-valorisation of the red macroalga studied, enhancing the value of this marine biomass, and promoting growth in aquaculture and the seaweed industry.

1. Introduction

Plants represent ≈80 % (w/w, carbon content) of the total biomass on Earth [1] and xylan is one of the principal components of plants cell walls, constituting ≈10-30% of hardwoods, ≈7-10% of softwoods and <30% of annual plants [2]. It is the principal component of hemicellulose and is principally found at the interface between lignin and cellulose in plant cell walls where it is believed to be important in fibre cohesion and cell wall integrity. Hemicellulose is a complex heteropolysaccharide composed of a linear backbone chain of D-xylopyranosyl units linked via β-1-4 or/and β-1-3 xylosidic bonds. The structure can be of variable lengths and be substituted to varying degrees by side chains of arabinosyl, glucuronosyl, methylglucuronosyl, acetyl, feruloyl and/or p-coumaroyl residues [2-5] depending on the plant species, plant tissue and even the developmental stage. Such a heterogeneity of structures leads to various diverse xylan properties, namely diverse molecular weights, solubilities, stabilities, strengths, thermal behaviours, biological activities and applications.

The application potential of xylan is vast and growing, emerging as a component of biocomposites in for example new functionalised polymeric materials for drug delivery, water remediation and even edible films [6], and with a promising potential for substitution of petrol-based polymers in environmentally friendly products [3, 4, 7]. Furthermore, it has been shown to have application in food and feed (as dietary fibre) [8], in skin and hair care (moisturiser) [9], and in R&D as a substrate for detection and measurement of xylanase activity [10-12]. Importantly also, xylan can be hydrolysed to various high value products such as various xylo-oligosaccharides and monosaccharides. The former have already been shown to be effective safe and stable prebiotics [13, 14], being beneficial for gastrointestinal health, but also have potential in the food industry, health care and cosmetics [15-17] The latter, which are majoritarily composed of the pentose sugar xylose, are precursors for the synthesis of numerous high value platform chemicals, biomaterials, biofuels, food and non-food products including xylitol, furfural, xylonic acid, polyhydroxyalkanoate, 1,4 butanediol, isoproponal, etc. [3, 18].

While the application potential of xylan and its breakdown products is acknowledged, the absence of cost effective, environmentally friendly methods for production of large quantities of highly pure xylan is a major challenge presently severely limiting its use. Currently, xylan

is almost entirely isolated from terrestrial plants, mostly from lignocellulosic materials, even though the high lignin content and extensive covalent and non-covalent interactions within the xylan-cellulose-lignin complexes in these plants impedes xylan extraction. As a result, multi-step processes involving use of hazardous corrosive chemicals (e.g. acids, alkali, ammonia, alkaline peroxide, sodium hypochlorite, solvents) and/or harsh physical treatments (e.g. temperatures up to ≈200 °C, pressures up to ≈10 bar, high pressure steam blasting), with or without enzyme treatment are required [19-22]. These processes present various economic and environmental challenges, necessitate specialised equipment, often with high energy inputs, frequently produce undesired side products, pollutants and/or inhibitors and present costly downstream purification and chemical disposal requirements as well as a high environmental footprint [6, 23]. Hence they do not respond to the challenge for a low cost, environmentally friendly, high-purity xylan production process.

Recently, attention has been turned towards marine macroalgae as a reliable alternative xylan source. Macroalgae are a fast growing, abundant and carbon neutral, renewable marine resource which can be cultivated under controlled aquaculture conditions and can reduce strains on the worlds resources (e.g. on food crops, arable land and freshwater), while also reducing pesticides and fertiliser use as compared to terrestrial plants. *Palmaria palmata* ('dulse') is a xylan-rich red macroalgae commonly found on the northern coasts of the Atlantic and Pacific Oceans. It is predominantly constituted by a water soluble, non-substituted, mixed linkage β-1,3/β-1,4 xylan (20-60%) in addition to also high concentrations of proteins (10-35%) and reduced amounts of other components (galactose, floridoside, lipids, demosterol etc.) [24, 25]. Interestingly also, it lacks lignin and cellulose and has a weakly linked cell wall matrix in which it is believed that the xylan is only weakly maintained by H-bonds [26]. Such a structural make up could facilitate component extraction and reduce the need for harsh processing steps. In this study, we exploit these favourable properties of *Palmaria palmata* for development of a simple, environmentally friendly xylan extraction process. The developed process was optimised and characterised, the different extracts characterised and the potential of the xylan-rich extract as an R&D substrate in xylanase activity analysis demonstrated.

2. Materials and Methods

2.1. Macroalgae Biomass

Four different *Palmaria palmata* samples cultivated and pretreated using various different confidential proprietary procedures were supplied by ALGAplus, Ílhavo, Portugal. Both wild harvested macroalgae, collected in northern Portugal and the south-west of Ireland, and cultivated macroalgae produced by ALGAplus using in-house developed Integrated Multi-Trophic Aquaculture (IMTA) procedures were investigated. Upon harvesting, samples were immediately processed using ALGAplus proprietary preservation procedures involving diverse combinations of various different washing, drying and milling procedures.

2.2. Carbohydrate Quantification

Carbohydrate content was determined by HPLC analysis of samples following hydrolysis to monomers by 4% H_2SO_4 treatment at 121 °C, 1 bar pressure for 20 minutes. Hydrolysed samples were centrifuged at 9000 rpm for 30 minutes, filtered through a 0.22 µm polyethersulfone (PES) filter (Merck) and separated and quantified by HPLC on a ROA-organic acid H^+(8%) column (Phenomenex) at 60 °C. An Elite LaChrom (VWR Hitachi) chromatography system with an Elite LaChrom L-2490 RI detector (VWR Hitachi) at 40 °C was used for all experiments. Mobile phase was 2.5 mM H_2SO_4 at a flow rate of 0.7 mL/min for the first 7 minutes followed by 0.1 mL/min for a total period of 30 minutes. The EZChrom Elite 3.3.2 SP2 software was employed for data collection and analysis. Sugar monomer concentrations were calculated from respective standard curves of each monomer standard investigated (xylose, glucuronic acid, glucose and arabinose). For soluble samples and extracts, H_2SO_4 hydrolysis was carried out by direct addition of H_2SO_4 to a final concentration of 4% and treatment as described above. In the case of *P. palmata*, 10 g of dried alga was suspended in a final volume of 100 mL of 4% H_2SO_4 and four consecutive hydrolyses/extraction treatments completed and pooled before analysis by HPLC as described above.

2.3. Protein Concentration

Soluble protein content was determined using the Lowry method as described by Waterborg et al. 1984 [27]. One hundred µL of 2 M NaOH was added to an equal volume sample before addition of 1 mL of freshly mixed complex-forming reagent (100:1:1 by volume of 2% (w/v) Na_2CO_3: 1% (w/v) $CuSO_4 \cdot 5H_2O$: 2% (w/v) sodium potassium tartrate, respectively) and incubated at room temperature for 10 minutes. One hundred µL of 1 N Folin reagent (Themo Fisher Scientific) was then added and vigorous mixed and incubated for a further 30 minutes before absorbance measurement at 750 nm and protein concentration determination from a BSA standard curve.

2.4. Nitrogen/Protein Concentration

The Kjeldahl method was used in determination of the nitrogen/protein content of insoluble samples. 5 g samples were incubated with 10 mL of 96% H_2SO_4 and a 3.5 g Kjeldahl catalyst tablet (Kjeldahl SE (K_2SO_4+Se), Panreac) at 550 °C for 30 minutes in a FOSS Labtec DT 208 Digestor. After digestion, samples were cooled to room temperature before distillation in the Foss Kjeltectm 8400 Analyser Unit using the standard protocol suggested by the manufacture. Protein content was determined using a conversion factor of 6.25, which is commonly used in conversion of nitrogen-to-protein content in studies on seaweeds [28].

2.5. Lipid Content

The lipid content was determined as described by Bligh & Dyer, 1959 [29]. Dried samples were mixed with 0.8 volumes of water, 1 volume of chloroform and 2 volumes of methanol. After thoroughly vortexing, 1 volume of chloroform was added to the mixture followed by another mixing step. Thereafter, 1 volume of distilled water was added and the suspension stirred for 10 minutes. The resulting suspension consists of a chloroform/methanol/water mixture with a volumetric ratio of 2:2:1.8 (v/v/v). The mixture was filtered through a grade 1 paper filter (Whatman), transferred to a graduated cylinder and let stand until complete phase separation and clarification (20 minutes). The volume of the lower chloroform layer containing the purified lipids was recorded and the upper layer removed by aspiration. Five 1 mL samples of the chloroform layer were then evaporated at room temperature overnight

in a fume hood in pre-weighed tubes. After evaporation, samples were weighed and lipid content calculated as follow:

$$Total\ Lipid\ Content = \frac{Weight\ of\ lipid\ in\ sample\ x\ Volume\ of\ chloroform\ layer}{Volume\ of\ aliquot} \quad \textbf{(1)}$$

2.6. Ash Content

Ash content was analysed according to AACC Method 08-01.01 and Laurens et al, 2012 [30]. 10 g of dried samples were incubated at 550 °C for 16 hours in a muffle furnace (Nabertherm LVT 15/11) and then re-weighed.

2.7. Moisture Content

Moisture content was measured as described by Ahn et al, 2014 [31]. 10 g samples were dried in ceramic crucibles in a drying oven (80 °C) until constant weight was recorded.

2.8. Phenolic Compounds Content

Phenolic compounds were first extracted from 10 g samples by four consecutive extractions with 100 mL of distilled water at 98 °C, 180 rpm for 5 hours, and then pooled and mixed, before quantification as described by Machu et al, 2015 [32]. For this, 20 µL of extracted samples containing phenolic compounds was mixed with 1580 µL distilled water and 100 µL 2 N Folin reagent (Thermo Fisher Scientific) and incubated for 5 minutes at room temperature in the absence of light. 300 µL of freshly prepared 2 M $NaCO_3$ was then added and incubated for an additional 60 minutes at room temperature in the absence of light. Absorbance at 750 nm was measured and the phenolic content calculated from a galic acid standard curve.

2.9. Extraction of Palmaria palmata *Xylan*

2.9.1. Alcohol Insoluble Residue (AIR)

The alcohol insoluble residue was prepared as described by Deniaud et al, 2003 [26]. Briefly, 10 g of dried *P. palmata* were immersed in 100 mL of 70% boiling ethanol. The suspension was boiled for 20 minutes with vigorous mixing and recovered by filtration (grade 1 paper filter (Whatman)). The algal material was then repeatedly washed at room temperature with 70% ethanol, 96% ethanol, acetone, and chloroform:methanol (3:2 vol:vol) until each filtrate was colourless. The final residue was referred to as the alcohol insoluble residue (AIR) and was dried overnight at room temperature.

2.9.2. Alkaline Extraction

Two different concentrations of NaOH were tested for xylan extraction from *P. palmata*. 10 g of dried *P. palmata* were incubated with 100 mL of 0.5 and 1 M NaOH at 50 °C and room temperature, respectively, with vigorous agitation for 30 minutes. After incubation, the supernatant was separated from the insoluble components by centrifugation at 9000 rpm for 15 minutes, then neutralised using 37% HCl and finally filtered through a 0.22 µm PES filter (Merk) before quantification by HPLC.

2.9.3. AIR + Alkaline Extraction

A combination of both procedures described on 2.9.1 and 2.9.2 was performed. Ten g of *P. palmata* were first pre-treated using the AIR procedure followed by alkaline xylan extraction as described in 2.9.2.

2.9.4. Aqueous Extraction

Two aqueous extraction procedures were tested for xylan extraction of *P. palmata*. 10 g of dried *P. palmata* was incubated in 100 mL deionised water with vigorous agitation at room temperature for 30 minutes or 100 °C for 2 hours. Following incubation, the supernatant

was separated from the insoluble components by centrifugation at 9000 rpm for 15 minutes and filtered through a 0.22 µm PES filter (Merck).

2.9.5. AIR + Aqueous Extraction

Here, a combination of both procedures described on 2.9.1 and 2.9.4 was performed. 10 g of *P. palmata* were first treated with the AIR procedure before aqueous extraction at 100 °C.

2.10. Xylan: HPLC Analysis

The molecular weight range of the xylan produced was analysed with use of two SEC-HPLC columns; a PolySep-SEC GFC-P Linear (Phenomenex) HPLC column and a BioBasic™ SEC 60 (Thermo Scientific) HPLC column with fractionation ranges (pullulans) of, respectively, 1 KDa to 10 MDa and 300 Da to 6 KDa. In both cases, isocratic elution with water as the mobile phase was used with a constant flow rate of 0.5 mL/minute (BioBasic™ SEC 60 column) or 0.6 mL/minute (PolySep-SEC column) for a total period of 30 minutes. Standard curves were prepared with various molecular weight standards of 1 g/L dextran (Sigma) (4000, 40000 and 200000 Da) and polyethylene glycol (Sigma) (200, 300, 400, 1500, 3350, 4000 and 8000 Da) as well as 1 g/L xylose, xylobiose, xylotriose, xylotetraose, xylopentaose and xylohexaose standards (Megazyme). HPLC equipment and software employed were the same as reported for carbohydrate analysis in section 2.2. Prior to HPLC analysis all samples were treated with trichloroacetic acid (TCA) at a final concentration of 10% for at least 2 hours at room termperature, centrifuged at 14000 rpm for 15 minutes and filtered through a 0.22 µm PES filter (Merk) for sample deproteinisation.

A Rezex RSO-Oligosaccharide Ag^{+} (4)% (Phenomenex) HPLC column, which is asserted to enable identification of xylose oligosaccharides with degrees of polymerisation up to 14, was used in confirming the absence of low molecular weight components (xylooligosaccharides/xylose) in the soluble xylan rich fraction produced in this study. Isocratic elution was used with water as mobile phase at a constant flow rate of 0.15 mL/min for a total period of 90 min. 1 g/L xylose, xylobiose, xylotriose, xylotetraose, xylopentaose and xylohexaose standards were also analysed. HPLC equipment and software

employed were the same as reported for carbohydrate analysis (section 2.2). Sample preparation was the same as described above for the SEC-HPLC analysis (section 2.10).

2.11. Activity Assays

The DiNitroSalicylic acid (DNS) and Nelson-Somogyi (NS) assays were utilised in calculating the carbohydrate reducing ends content of samples for determination of the average degree of polymerisation and the xylanase activity on the various xylan substrates investigated, namely the *Palmaria palmata* xylan of the present study, beechwood xylan (Carl Roth), birchwood xylan (Sigma) and oat spelt xylan (Carl Roth). The DNS assay was performed as previously reported by [10, 11] and the NS assay was performed as described by McCleary et al, 2015 [11]. Xylanases representing four different CAZy glycoside hydrolase families were investigated: two glycoside hydrolase family 8 endoxylanases: pXyl and Nzy8A (Genebank accession number: CAD20872.1; code: CpXyn8A, respectively); two glycoside hydrolase family 11 endoxylanases: Mega11A and Nzy11A (Genebank accession number: ABW04217.1 and CAA84537.1, respectively); a glycoside hydrolase family 10 endoxylanase: Mega10A (Genebank accession number: ACE84499.1); and a glycoside hydrolase family 26 endoxylanase: GH26 xylanase (Genebank accession number: OHX64342.1). Assays were carried out at the optimal pH for activity in the appropriate buffer recommended for each enzyme:

- pXyl: Mops/NaCl (20 mM MOPS, 100 mM NaCl), pH 7.5
- Nzy8A: sodium phosphate (50 mM), pH 6.0
- Mega11A: sodium phosphate buffer (100 mM), pH 6.0
- Nzy11A: sodium phosphate (50 mM), pH 6.5
- Mega10A: sodium acetate buffer (100 mM), pH 5.0
- GH26 xylanase: 20 mM phosphate buffer, 1.5 M NaCl, pH 7.5

The soluble xylan-rich fraction extracted from *Palmaria palmata* was used directly in the assays. Oat spelt xylan, birchwood xylan and beechwood xylan were utilised directly as supplied. Soluble beechwood xylan was also used, this was prepared as follows: 100 g of beechwood xylan powder was incubated in 500 mL of deionised water overnight at 4 °C with

vigorous agitation. The extracted soluble xylan was then separated from the insoluble xylan by centrifugation at 50000g for 15 minutes, freeze dried, and stored at room temperature.

pXyl was produced and purified as described in Barroca et al. 2017 [33]. All other enzymes were used as supplied by the suppliers. The gene sequence encoding the GH26 OHX64342.1 was synthesised at NZYTech and inserted in the expression vector pET22b in *E. coli* BL21. The enzyme was produced and purified as described by Cai et al. 2018 [34].

2.12. Average Degree of Polymerisation (avDP)

For calculation of the average degree of polymerisation (avDP) of carbohydrate samples, the DNS and NS assays were performed as described for measurement of xylanase activity [10, 11] with the exception that the samples were directly used without the addition of enzyme. The avDP of the sample was calculated as follow:

$$avDP = \frac{Total\ \mu M\ of\ xylose\ in\ sample}{Total\ \mu M\ xylose\ equivalents\ measured\ by\ DNS \vee NS} \quad \textbf{(2)}$$

Total µM of xylose was calculated from the measured substrate weight and a D-xylopyranosyl molecular weight of 150 g/L.

2.13. Remazol Brilliant Blue Coupling to Xylan and Use in Plate Screening

Remazol Brillant Blue (RBB) was coupled to soluble xylan from *P. palmata* to give RBB-xylan for use as a novel chromogenic screening substrate. The coupling procedure was performed as follows [35]:

Two and a half g RBB dye (Sigma) was mixed with 2.5 g xylan in 60 mL deionised water before dropwise addition over 5 minutes of 20 mL of a 0.4 M sodium acetate solution (Merck), pH 11, with continuous stirring at room temperature. 20 mL of a 1.875 M NaOH solution, pH 13 was then added to initiate the coupling reaction and incubated for 90 minutes at room temperature with continuous mixing. 200 mL of 96% ethanol was then added to precipitate the RBB-Xylan. The solution was let stand at -20 °C for 15 minutes before filtering through a filter paper (Macherey Nagel) under vacuum. The precipitate was washed sequentially with 1 L of wash solution (16 mM sodium acetate solubilized in ethanol

70%) until the filtrate was colourless. Finally, two additional washes with 100 mL 70% ethanol and 50 mL acetone were carried out before filtering and then drying overnight at room temperature.

2.13.1. RBB to Xylose Ratio Determination

The prepared RBB-xylan was characterised in relation to the number of RBB molecules per molecule of xylose. 2 mg of the RBB-Xylan powder was dissolved in 10 mL of deionised water and centrifuged to remove any particles in suspension. After centrifugation, the supernatant was diluted to final RBB-xylan concentrations of 100 μg/mL and 50 μg/mL. The absorbance of both was measured at 595 nm and converted into a Remazol Brilliant Blue molar concentration using the Beer-Lambert Law with a RBB Molar Extinction Coefficient at 595 nm ($\varepsilon_{595\,nm}$) of 9.25 X 103 M^{-1} cm^{-1} as follows:

$$A(Absorbance) = c(MolarConcentration)xl(Pathlength)x\varepsilon(MolarExtinctionCoefficient) \quad \textbf{(3)}$$

The ratio of the number of moles or RBB per moles of xylose was then calculated using the known concentration of xylan used and a xylose residue molecular mass of 133.12 g/mol.

2.13.2. RBB Xylan Plates Preparation

RBB xylan plates were prepared using conventional LB media (1% Bacto-tryptone, 0.5% yeast extract, 1% NaCl, 2% agar, pH 7.5) supplemented with 0.1% (w/v) of RBB xylan.

2.14. Process Optimisation RSM-CCD

Response surface methodology (RSM) is a powerful methodology combining statistical and mathematical techniques to improve method development and optimisation processes. Its use in statistical experimental design results in reduced process variability combined with reduced resource requirements. The software program Design-Expert version 7.0.0 (Stat-Ease Inc) was employed for the central composite design (CCD) experimental design, model fitting, diagnosis and interpretation as well as for data plotting and navigation, and

identification of optimal conditions. The parameter description, ranges used and the model utilised are described in the results section of this study.

2.14.1. Model Fitting and Validation

Analyses of variance (ANOVA) was used for the determination of the statistical significance of the model used. Here, the Fisher's statistical test (F-test) was performed to determine the effect of each variable on each response in the study. The significance and the magnitude of the effects estimates of each variable and all their possible linear and quadratic interactions on the responses were determined. A confidence level of 95% significance that is, a *p*-value higher than 0.05, was implemented.

The selection of the model takes into account the significance of the model, evaluated by considering either the F-values or the *p*-values of the model and of the lack of fit. The lack-of-fit test compares the residual error of the selected model with the pure error of the data, calculated though the analyses of the variance between the 5 central points performed. If residual error significantly exceeds pure error, the model will show significant lack of fit, and another model may be more appropriate. In addition the adjusted R^2, which analyses the distance of the predicted value of the model from the actual data values, was also used for model selection. An adjusted R^2 higher than 85% was considered optimal for this study.

For the RSM-CCD analyses, a final total volume of 20 mL was used for each experimental run and the 3 following responses were measured: total xylan content (measured as total grams of xylose present in the supernatant), total protein content (measured as total grams of protein present in the supernatant), total ash content (measured as total grams of ash present in the supernatant). Follow completion of incubation, samples were centrifuged at 9000 rpm for 15 minutes and the total grams of each component present in the supernatant was analysed.

3. Results and Discussion

3.1. Palmaria palmata *Composition*

Palmaria palmata samples were prepared by ALGAplus using four different confidential proprietary production procedures and the carbohydrate, protein, lipid, phenolic compounds and ash content of these were analysed. From Fig. 1 it can be seen that sample 1 differentiates significantly from all other samples for all components except phenolics. It has higher xylan and protein content and lower levels of ash and lipids and thus constitutes an interesting sample for further analysis. Sample 2 was also found to a have higher content of xylan than samples 3 and 4, while sample 4 is characterised by an increased lipid content. Such variations reflect the different cultivation and pretreatment processes used.

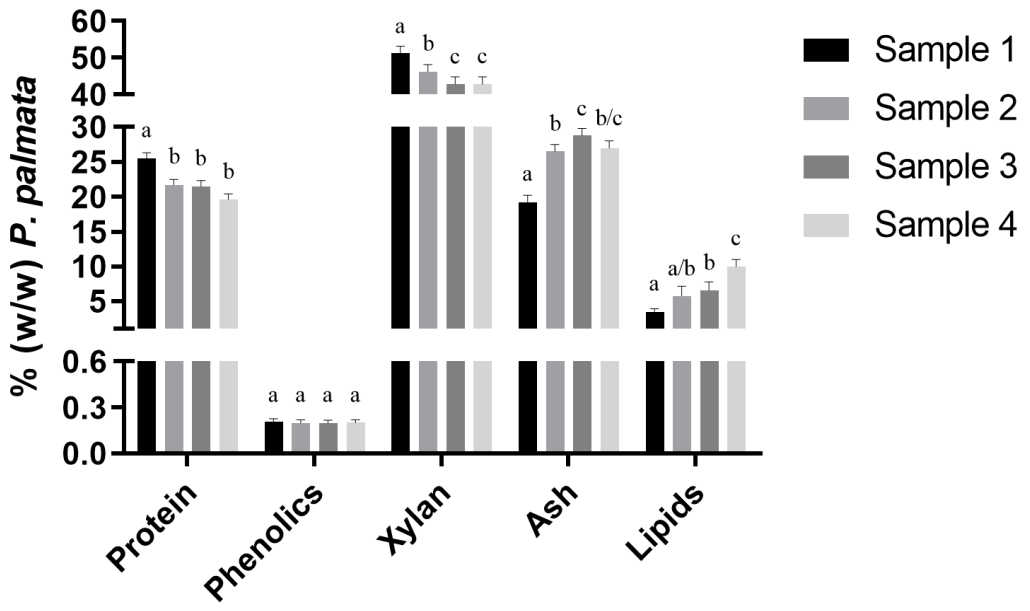


Fig. 1. Chemical composition of the four *Palmaria palmata* samples supplied by ALGAPlus. Values are expressed as % (w/w): g of component per 100 g of *Palmaria palmata*. Lower-case letter represent the results of the analysis of variance [two-way ANOVA] for the various samples examined. The same lower-case letter for different samples for a particular component indicates no significant differences in concentrations at a *p*-value ≤0.01, a different letter indicates a significant difference at a *p*-value ≤0.01.

The overall results for the compositional analysis are within the ranges of component concentrations reported for wild-harvested *Palmaria palmata* by other authors, with the xylan content (40-50%) being at the high end of this range (20-60%) [24, 25]. Indeed, the xylan concentration of this macroalgae is among the highest of any plant reported to date and

signals the high potential of this as a xylan source. Protein concentrations ranged from 20 to 25%, which is within the reported range (10-35%) and in fact is higher than most conventional protein sources used for human consumption (e.g. 13% in eggs, 21% in beef) [36]. Furthermore, this protein has been reported to have a high content (25-50%) of a variety of essential amino acids, such as lysine, methionine, leucine, phenylalanine, threonine, valine, histidine and isoleucine [37]. Thus, it is an interesting potential source of protein for human and animal consumption and represents an excellent alternative for vegetarians, an eating habit which is increasing significantly in popularity in recent years. Nevertheless, within the macroalgae matrix this protein is thought to be poorly digestible, which is believed to be mainly due to an anti-nutritive effect and high viscosity of the xylan present [38]. Indeed, a number of studies are focusing on enhancing the solubility and digestibility of this protein by protein extraction, xylan removal, use of solubilising agents and/or protease hydrolysis, and thereby should enhance the use of this macroalgae as a protein source in the future [38, 39]. In our study, ash concentrations between 20-30% were observed, which are similar to the levels observed by other authors (17–37%) [25] and mainly influenced by the washing process used, i.e. use of fresh water or sea water, but also the presence of shellfish contaminants. The ash content is relatively high as compared to most common vegetables (e.g. 2% for cabbage, 5% for soybean), but it consists mainly of NaCl and minerals that are essential in the human diet, such as iodine, magnesium, calcium and iron [40, 41]. Furthermore, NaCl can be beneficial for prolonging algal shelf-life and may positively affect taste. The lipid content of all samples tested was quite high, ranging from 3-10%, when compared with reported values of between 0.2-4% [42]. These variations may be due to the different washing and pre-treatment processes used by different manufactures, the presence of fish and/or shellfish contaminants, as well as the fact that most literature values are mainly for direct analysis of fresh algae as opposed to pre-treated dried algae as used here. The same reasons may be proposed for the higher residual concentrations of phenolic compounds (≈0.2%) observed in all algae samples analysed when compared to values reported in literature ranging from 1-3%.

It is important to note that wild harvested *Palmaria palmata* composition varies considerably with the cultivation and processing conditions used and namely with the cultivation season, available nutrients and temperature, and results in the broad ranges in component concentrations reported by various authors [24, 25]. Nevertheless, use of aquaculture

techniques in combination with defined pretreatment processes can overcome this by enabling a reproducible, tightly controlled, sustainable macroalgae production. This would reduce compositional variation while also preserving wild algal populations and enabling controlled, economically stable production and increased product safety. Importantly also, and as used in the present study, *Palmaria palmata* can be produced under the Integrated Multi-Trophic Aquaculture (IMTA) concept [43] which co-cultivates algae with other species, namely fish, and offers the advantage of being a more environmentally friendly, organic certified process, avoiding use of chemicals and also allowing for bioremediation through removal of fish wastes.

3.2. Xylan Extraction

A variety of protocols for extraction of xylan from biomass have been reported and we investigated 3 different methods (alkaline extraction, AIR + alkaline extraction, and aqueous extraction), and variants thereof, as previously reported for polysaccharide extraction from macroalgae, including *Palmaria palmata* [3, 26, 44]. Use of sulphuric acid at high temperatures was also investigated but was found to degrade the xylan to xylose monomers and other breakdown products, possibly furfural, and thus is not discussed here. Fig. 2 shows the results of the xylan and protein extraction yields in the soluble extracts for samples 1 and 4 only as samples 2 and 3 were found to give highly similar results to sample 4. It can be seen that sample 1 can again be differentiated from all other samples tested and is characterised by a significantly higher yield of xylan in all final extracts. Lowest xylan yields were obtained with direct alkaline extraction and with water extraction with a short incubation period (Fig. 2A), but this was enhanced almost two-fold upon combining alkaline extraction with a prior preparation of an alcohol insoluble residue (AIR). Surprisingly, direct aqueous extraction with prolonged incubation or/and boiling yielded similarly high levels of xylan as the chemical methods investigated, with 26 g xylan being extracted per 100 g of *Palmaria palmata*, equivalent to approximately 50% of the original xylan content. This is higher than that reported by other authors when using various chemical methods, wherein xylan yields of 10-35% have been reported [26, 44, 45]. Protein concentrations in the soluble extract (Fig. 2B) were also determined, as a measure of contamination of this extract. It can be seen that sample 1 did result in higher levels of protein contamination than samples 2, 3 and 4,

but, interestingly, aqueous extraction enabled a much reduced protein contamination (10-40-fold lower contamination) as compared to the chemical extraction methods investigated. The high yields of xylan in the soluble fractions and protein in the insoluble fractions following aqueous extraction indicate that the pretreatment conditions used for sample 1 led to structural modifications in the macroalgae, perhaps structural breakdown, which facilitated xylan solubilisation but also induced protein precipitation.

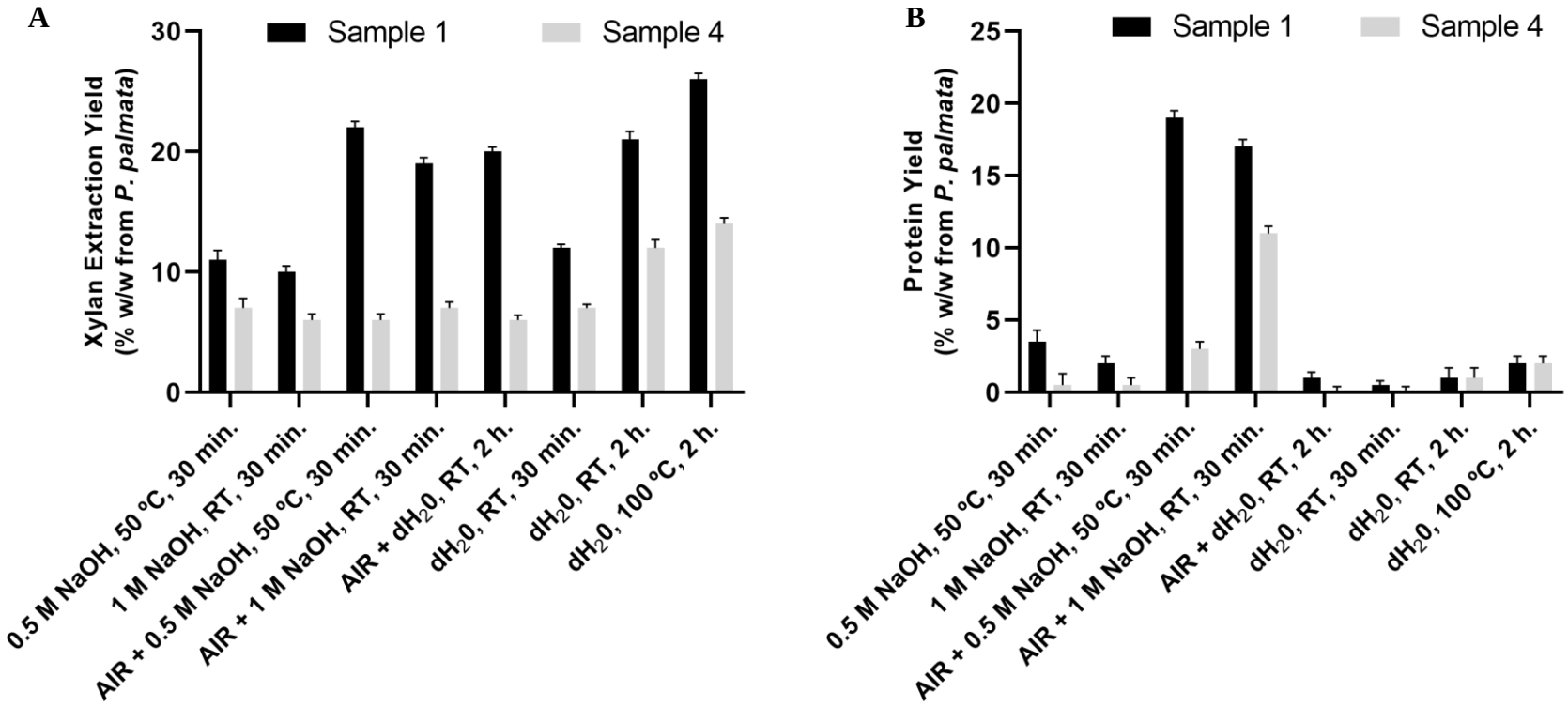


Fig. 2. Bar charts showing the xylan extraction yields (A) and protein contamination (B) in the soluble fractions of samples 1 and 4 prepared using various extraction procedures. Samples 2 and 3 gave similar results to sample 4 (data not shown). RT: room temperature. AIR: alcohol insoluble residue.

3.3. Xylan Aqueous Extraction: Process Optimisation

The high xylan extraction yields and low protein contamination observed for aqueous extraction of macroalgae sample 1 point to the potential of aqueous extraction as a simple, environmentally friendly method for xylan preparation with reduced chemicals usage and reduced costs. The initial results indicated that incubation time and temperature influenced the xylan and protein yields (Fig. 2). Hence, a comprehensive process optimisation aiming at optimisation of all process variables was undertaken. Response surface methodology (RSM) with a central composite design (CCD) was used to maximise xylan extraction and purity. This multivariate statistical technique facilitates rational experimental design and statistical evaluation of results and is commonly used in process optimisation and for a better understanding of process parameters and their effects, especially when multiple parameters are involved and when identification of interactions among process parameters is desired. In the experimental setup, five independent process variables were investigated (incubation temperature, incubation time, salt concentration, initial macroalgae concentration, and mixing speed) at five levels ($-\alpha$, -1, 0, $+1$ and $+\alpha$), with 5 repeats of the central points (0), for a total of 47 runs. Evaluation of the designed experiment indicated an acceptable degree of freedom for lack of fit (22) and pure error (4). The process variables and their units as well as the different level values investigated for each are shown in Table 1 below:

Table 1. Central composite design matrix. Variables investigated, units and values of - α, -1, 0, +1 and $+\alpha$ used to design the RSM-CCD experiment.

	Variable	**Units**	**-α**	**-1 level**	**0**	**+1 level**	**+α**
A	Incubation Temperature	°C	20	43	59	75	98
B	Incubation Time	h	0.5	3	4	6	8
C	Salt Concentration	g/L	0	14	25	36	50
D	Algae Concentration	g/L	10	36	55	74	100
E	Mixing Speed	rpm	0	49	85	121	170

Three different responses were investigated: total xylan extracted (measured as total xylose present in the soluble fraction produced under the experimental conditions used) (g); total protein present in the soluble fraction produced under the experimental conditions used (g);

and total ash present in the soluble fraction produced under the experimental conditions used (g). As the principal *Palmaria palmata* constituents, these three responses will give a better understanding of the process extraction capability as well as the purity of the soluble fractions obtained.

3.3.1. Model Fitting

For all 3 responses investigated, the quadratic polynomial model was found to be the most appropriate for fitting to the experimental data for each response. Indeed, in all cases, this model was characterised by a non-significant lack of fit, low standard deviation, high adjusted R-squared value and a model *p*-value of less than 0.0001 (Table 2) as compared to the other models investigated (i.e., linear, 2 factor interaction, and cubic models). An analysis of variance (ANOVA) was performed for determination of the significance and magnitude of the effects of the variables and all their potential linear and quadratic interactions on the responses.

Table 2. Model summary statistics for each of the three responses investigated. Standard deviation, adjusted R^2, lack of fit values and model *p*-values are listed for the quadratic model.

Response	Std. Dev.	Adjusted R^2	Lack of fit (*p*-value)	Model *p*-value
Total Xylan (g)	0.015	0.95	0.09	<0.0001
Total Protein (g)	0.001	0.97	0.06	<0.0001
Total Ash (g)	0.094	0.84	0.14	<0.0001

3.3.2. Effect of Process Variables on Total Xylan Content of the Soluble Fraction

The quadratic equation for the effects of the process variables on total xylan extraction is shown in Eq. 4 below:

$$Y = 0.22 + 0.017A + 0.006B - 0.002C + 0.064D - 0.009E + 0.001AB + 0.001AC + 0.004AD + 0.012AE - 0.003BC + 0.003BD + 0.002BE - 0.001CD - 0.002CE - 0.003DE + 0.004A^2 + 6\mathrm{E}^{-4}B^2 + 0.002C^2 - 5\mathrm{E}^{-4}D^2 + 0.005E^2 \quad \textbf{(4)}$$

Eq. 4 reveals how the individual variables, double and quadratic interactions affect the amount of xylan extracted using the aqueous extraction process. The negative coefficient

values indicate that individual factors or double interactions negatively affect xylan extraction, whereas positive coefficient values mean that factors increase xylan extraction in the tested range.

ANOVA analysis (Table 3) of the quadratic model for total xylan extracted indicates that temperature, time, macroalgae concentration and mixing speed have significant effects on the total xylan extracted. Macroalgae concentration has the strongest effect, with, as might be expected, higher concentrations of macroalgae enabling higher concentrations of xylan to be extracted. Nevertheless, xylan extraction does not increase proportionally with macroalgae concentration and thereby results in a decrease in xylan yield (i.e., g xylan extracted per g of macroalgae) with increasing macroalgae concentration. Such an observation may have implications in the practical application of this extraction technique as, in the case where xylan yield is deemed more important than total xylan concentration, high volumes of water may be required. Nevertheless, in the case of ocean based aquaculture farms, and in view of the absence of a significant effect of salts (i.e. marine water) on xylan yields, high water utilisation, with possibilities for reutilisation, may not be overly problematic.

Table 3. ANOVA analyses of the significance of each variable on the three different responses studied (a confidence level of 95% was applied). *p*-values below 0.05 are considered significant.

Variable		*p*-value		
		Total Xylan (g)	**Total Protein (g)**	**Total Ash (g)**
A	Temperature (°C)	<0.0001	<0.0001	0.1152
B	Time (h)	0.0144	0.7034	0.4089
C	Salt (g/L)	0.3562	<0.0001	<0.0001
D	Algae (g/L)	<0.0001	<0.0001	0.5704
E	Mixing (rpm)	0.0004	0.9607	0.1683

Temperature was also found to significantly affect the total xylan extracted, and, in fact, it had a significant effect on the total protein extracted as well. High temperatures could be expected to break down the non-covalent interactions of the macromolecular assemblies in the *Palmaria palmata* matrix, leading to component liberation and increased cell wall permeability, while potentially also increasing component solubility, and resulting in the observed increased extraction of components. In the case of xylan extraction, mixing was found to shift from a minor negative effect to a high positive effect on xylan extraction at high

temperatures, showing a significant *p*-value for the interaction of both variables (Fig. 3). The augmentation due to mixing was highest at high temperatures and indeed no increase was observed at lower tempratures. Mixing introduces a physical effect which enhances solution homogeneity and could possibly increase macroalgae permeability and xylan solubility, especially at high temperatures. An increased incubation time could also be seen as increasing xylan extraction while the absence of an effect of salt would suggest that, as previously noted by other authors, electrostatic interactions do not play a strong role in xylan maintenance in the macroalgae matrix [26].

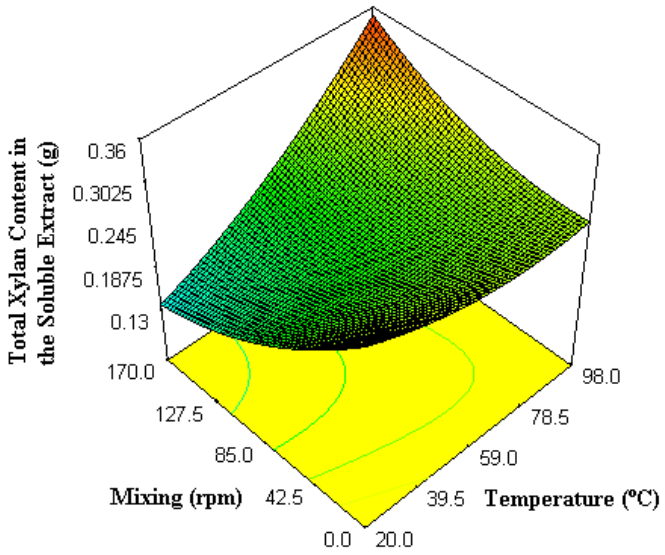


Fig. 3. 3D illustration of the interaction of temperature and mixing and their influence on total xylan extraction. For this surface plot, algae and salt concentration and time were set to 55 g/L, 25 g/L and 4.25 hours, respectively.

3.3.3. Effect of Process Variables on Total Protein Content of the Soluble Fraction

Protein contamination commonly occurs during xylan extraction procedures and requires additional steps and costs for removal, often involving expensive purification protocols based on chemicals use and/or chromatography and that can result in xylan losses. We analysed the effects of process variables on the total protein extracted in the aqueous extract in an attempt to identify the conditions which minimise this problem. The quadratic equation describing the effects of the process variables on the total protein in the soluble fraction is given in Equation 5:

$$Y = 0.012 + 0.004A + 1\mathrm{E}^{-4}B - 0.001C + 0.002D - 1\mathrm{E}^{-5}E + 1\mathrm{E}^{-4}AB - 4\mathrm{E}^{-4}AC - 3\mathrm{E}^{-5}AD + 2\mathrm{E}^{-4}AE - 2\mathrm{E}^{-4}BC - 1\mathrm{E}^{-4}BD - 1\mathrm{E}^{-4}BE + 3\mathrm{E}^{-5}CD - 2\mathrm{E}^{-5}CE - 2\mathrm{E}^{-5}DE + 0.002A^2 + 1\mathrm{E}^{-4}B^2 + 2\mathrm{E}^{-4}C^2 - 1\mathrm{E}^{-4}D^2 - 2\mathrm{E}^{-4}E^2 \quad \textbf{(5)}$$

As mentioned previously, similar to total xylan extracted, temperature has a significant positive effect on protein extractability (Table 3). Nevertheless, in the case of proteins, additional factors must be taken into account. In this case, increased protein release from the cell wall structure would be counterbalanced by reversible/irreversible protein unfolding and precipitation at high temperatures, and hence results in a reduced augmentation with temperature. In fact, the maximum protein solubilisation observed, at 98 °C, was less than 8% of the total protein content of the macroalgae. In addition, temperature was found to interact with salt concentration wherein high salt concentrations reduced the positive effects of temperatures (Fig. 4), possibly due to protein precipitation at high salt concentrations. Also, as for total xylan extracted, total protein extraction also increased significantly with increased macroalgae concentration, but, in contrast, incubation time and mixing had no effect.

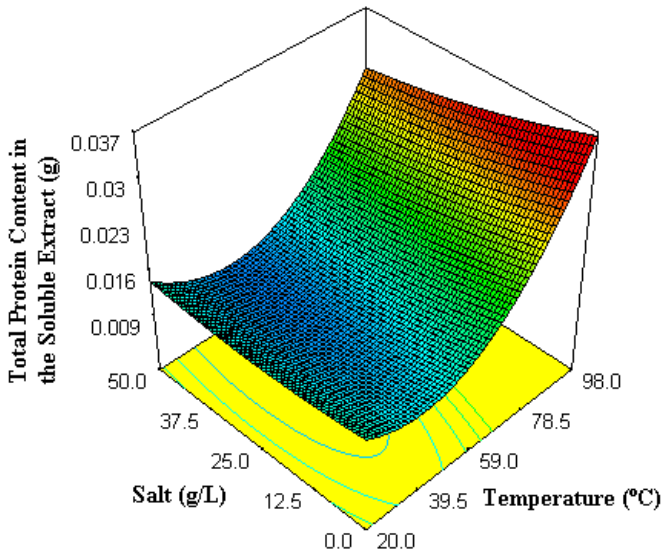


Fig. 4. 3D illustration of the interaction of temperature and salt and their influence on total protein extraction. For this surface plot, algae concentration, mixing and time were set to 55 g/L, 85 rpm and 4.25 hours, respectively.

3.3.4. Effect of Process Variables on Total Ash Content of the Soluble Fraction

The final response analysed was ash content and the quadratic equation for this is shown in Equation 6. This, together with the protein analyses, was analysed as a means for optimising purity of the aqueous soluble xylan extract.

$$Y = 0.45 + 0.018A - 0.009B + 0.18C - 0.006D + 0.015E - 0.022AB - 0.008AC - 0.031\mathrm{E}^{-5}AD + 0.004AE + 0.002BC + 0.014BD - 0.009BE - 0.018CD + 0.005CE - 0.006DE + 0.035A^2 + 0.004B^2 + 0.003C^2 + 0.014D^2 + 0.032E^2 \quad \textbf{(6)}$$

Palmaria palmata already has a high ash content, ≈19% (w/w) in sample 1 of the present study, and this ash is mainly constituted by soluble salts and minerals. Expectedly, addition of soluble NaCl salts significantly increased the soluble ash content. Interestingly, while algae concentration and temperature did not have a significant effect on the total ash extracted in the aqueous extract by themselves, their interaction was revealed to be highly significant, wherein higher algae concentrations reduced the positive effects of temperature (Fig. 5). While this is somewhat difficult to explain, it may be related to the equilibration of salts between the macroalgae matrix and the aqueous solution.

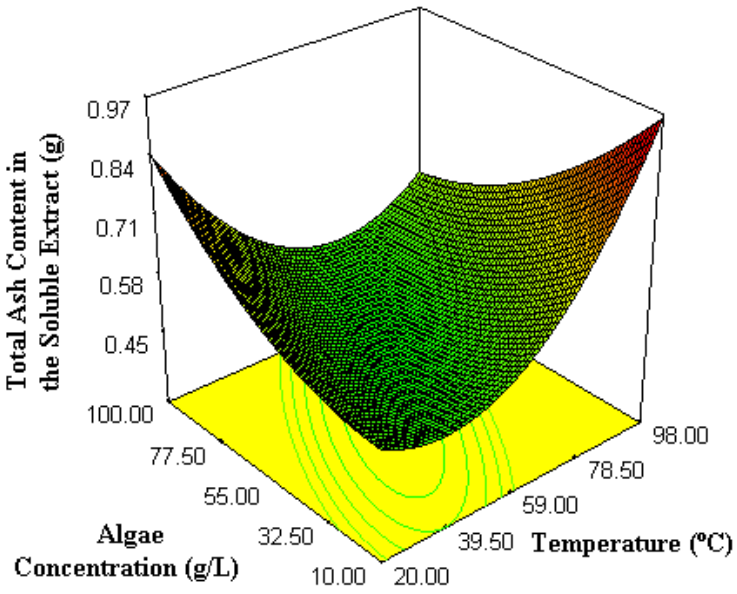


Fig. 5. 3D illustration of the interaction of temperature and algae concentration and their influence on total ash extraction. For this surface plot, salt concentration, mixing and time were set to 25 g/L, 85 rpm and 4.25 hours, respectively.

3.3.5. Optimal Process Conditions

One of the main objectives of using RSM is for determination of the optimum settings of the control variables that result in a maximum (or minimum) of a desired response within a certain region of interest. In our case, we explored the quadratic models developed to optimise the aqueous extraction process with focus on three distinct goals:

A- high xylan extraction, via maximisation of total xylan extracted in the aqueous extract and maintenance of all other responses and process variables within the model range.

B- high xylan extraction and high energy efficiency, via maximisation of total xylan extracted in the aqueous extract, minimisation of energy consuming process variables (temperature, salt, time and mixing) and maintenance of all remaining responses and process variables within the model range.

C- high xylan extraction and high purity, via maximisation of total xylan extracted in the aqueous extract, minimisation of protein and ash content and maintenance of all remaining responses and process variables within the model range.

Table 4 presents the conditions recommended for achievement of the three goals and the predicted responses. Triplicate repeat experiments with the suggested process variables indicated no significant differences between the experimental and predicted response values, thereby confirming the validity of the model. As expected, highest total xylan extracted was observed with process A wherein 0.51 g of xylan was extracted, equivalent to yields of 35 g per 100 g macroalgae (w/w, xylan/macroalgae), and 70 g xylan per 100 g of the total xylan in the macroalgae (w/w, xylan/total xylan in macroalgae). These yields already represent a 35% increase on the values originally observed with our initial xylan extraction studies (see section 3.2) and show the advantage of the optimisation process used in enhancing extraction. Extraction processes B and C do lead to significantly lower yields than process A; yielding 29% (w/w, xylan/macroalgae) and 58% (w/w, xylan/total xylan in macroalgae) for B, and, 30% (w/w, xylan/macroalgae) and 60% (w/w, xylan/ total xylan in macroalgae) for C. Nevertheless, these values are higher than for typical xylan extraction processes from various biomass sources where values between 5-25% (w/w, xylan/macroalgae) and 10-45% (w/w, xylan/ total xylan in macroalgae) have been reported [26, 45].

Table 4. RSM-CCD suggested process variables and predicted values of the three responses for the three different goals indicated, as well as, the desirability values for each goal. Desirability values refer to the proximity of the predicted responses to the inserted desired conditions (scale 0-1). Predicted values are presented as the total predicted g of each component extracted under the experimental conditions used.

Goal	Suggested Process Variables					Predicted Responses (total amount of component extracted)			Desirability
	Temperature (°C)	Incubation Time (h)	Salt Conc. (g/L)	Algae Conc. (g/L)	Mixing speed (rpm)	Total Xylan (g)	Total Protein (g)	Total Ash (g)	
A: High xylan extraction	98	4	0	97	120	0.51	0.04	0.29	1
B: High xylan extraction and energy efficiency	20	6	0	100	0	0.46	0.02	0.61	0.97
C: High xylan extraction and purity	78	8	0	85	100	0.41	0.02	0.1	0.67

In addition, many of the reported processes make use of multi-step, aggressive, chemical and/or physical extraction methods and often result in products of reduced purity. Indeed, a very recently described complex multi-step process making use of hazardous chemicals and high temperatures and pressures to extract xylan from a related Japanese *Palmaria* sp. enabled a xylan yield of only ≈34% (w/w, xylan/total xylan in macroalgae) and a final xylan-rich product of only ≈52% purity [45]. With the simplified aqueous extraction process developed in our study, a xylan purity of up to 73% of the dry weight was obtained for the extract obtained with the 'high xylan extraction' conditions (goal A). Compositional analysis (Table 5), indicates the remaining components as being protein (5 % of dry weight), ash (21% of dry weight) and phenolics (0.4% of dry weight). No lipids were detected in the aqueous extract under the experimental conditions used. Ash content is relatively high, but, depending on the desired application, the presence of ash contaminants may not be a problem and, nonetheless, this can be easily removed by use of an additional ultrafiltration step. Compositions of the extracts prepared with goals B and C conditions were found to be similar to those prepared with goal A conditions.

Table 5. Composition of the soluble and insoluble fractions following aqueous extraction with the 'A: high xylan extraction' conditions (% of the dry weight).

Goal	Fraction	Xylan (%)	Protein (%)	Ash (%)	Lipids (%)	Phenolics (%)
A	**Soluble**	73	5	21	0	0.4
	Insoluble	13	52	27	8	0.1

3.4. Insoluble Extract Composition

The aqueous extraction processes developed here produce two fractions: the soluble xylan-rich fraction and an insoluble fraction and compositional analysis of the latter shows this to be protein-rich (Table 5). It is composed of 52% insoluble protein, 27% ash, 13% xylan, 8% lipids and 0.14% phenolic compounds. This could be used as a protein source for the food and feed industries and/or could be solubilised with solubilising agents or hydrolysed by chemical, physical or enzymatic methods (protease treatment) to produce soluble protein and/or peptides for the food, feed, and healthcare (bioactive peptides) industries. Such a valorisation would considerably enhance the value of the fractionation process developed and would enhance the economic value of *Palmaria palmata* while reducing wastes and contributing to the circular economy concept via utilisation of all major algal components.

3.5. Aqueous Extract: HPLC Analysis

HPLC analysis was used to better characterise the xylan rich fraction produced in this study. SEC-HPLC analyses (Fig. 6A and 6B) indicated the xylan to have an estimated average degree of polymerisation (DP) of 25 xylose units (≈3000 Da) and a dispersion range of between 15 and 45 DP (≈2000-5000 Da). Interestingly, the xylan extracted with the 'goal B: high xylan extraction and energy efficiency' conditions was found to be different to the others (i.e. goals A and C), with a higher molecular weight and avDP of ≈100. Such observations suggest that the more aggressive conditions employed for goals A and C, specifically higher temperatures and mixing speeds and longer incubation times, result in enhanced xylan extraction but also xylan degradation and increased contaminant extraction.

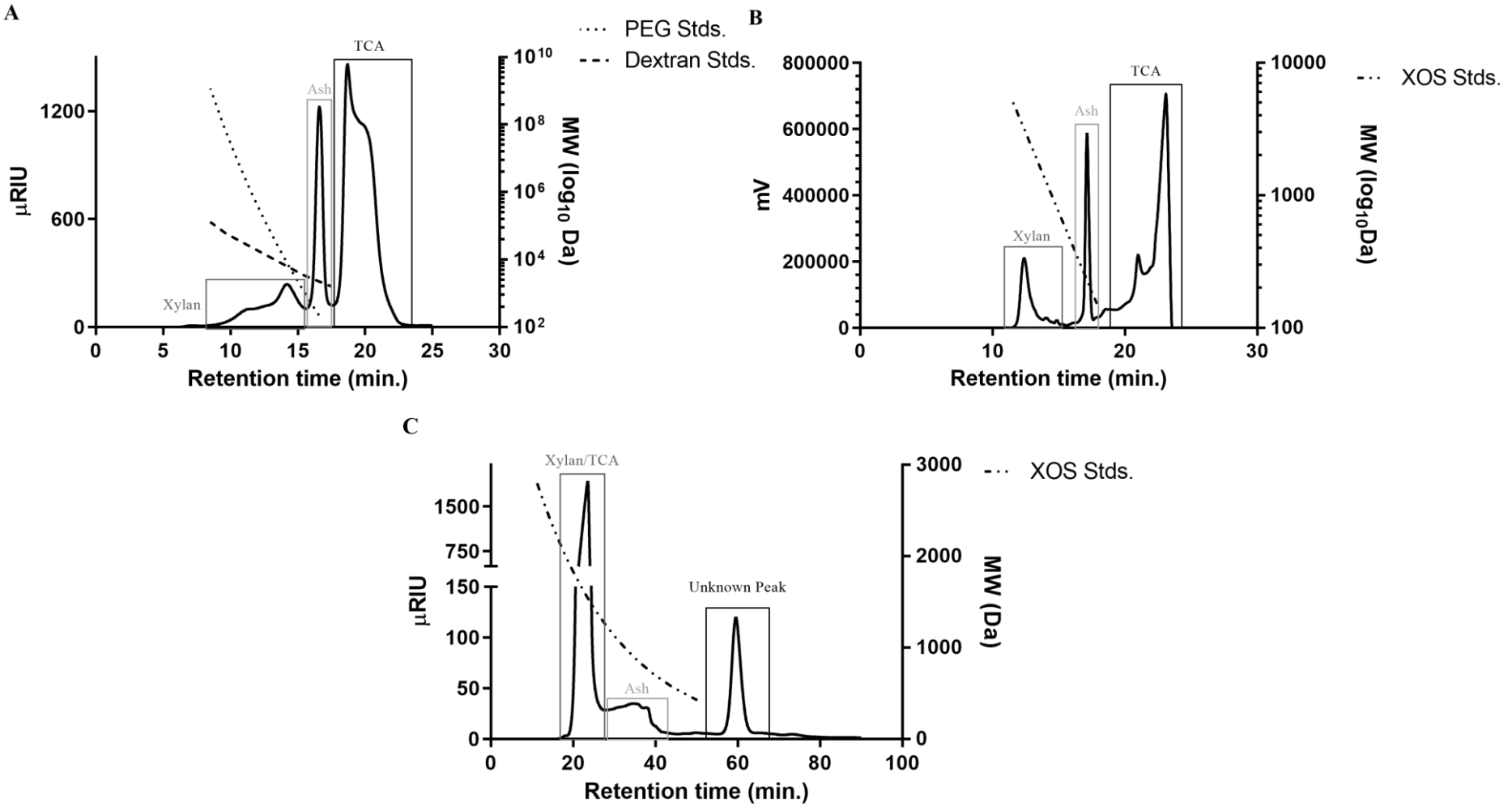


Fig. 6. HPLC analysis of the xylan-rich soluble extract obtained with the 'goal A: high xylan extraction' conditions. Chromatograms of HPLC analysis with a PolySep™-SEC GFC-P linear column (A), a BioBasic™ SEC 60 column (B) and a Rezex™ RSO-Oligosaccharide Ag+ (4)% column (C). Standards curves prepared using polyethylene glycol (dotted line), dextran (long dashed line) and xylo-oligosaccharides-XOS (dash-dotted line) are shown. Note that only xylo-oligosaccharides with degrees of polymerisation from 1 to 6 were analysed and the standard curve obtained was extrapolated to allow estimation of the average degrees of polymerisation of the higher molecular weight xylan.

For determination of the extracts DP by SEC-HPLC xylo-oligosaccharide standards were used. Xylo-oligosaccharide standards with degrees of polymerisation above six are not available and therefore the polymerisation degree was determined by extrapolation of the standard curve, hence the values determined represent estimations. Nevertheless, these values are similar to those observed by reducing end/concentration analysis as described in 2.12 above wherein an average DP of 30 was calculated. Standard curves of dextran and PEG were also examined but these indicated widely different molecular weights for the xylan, 1 kDa to 1.2 MDa with PEG and 4 kDa to 150 kDa with dextran. These compounds are sometimes used in molecular weight determinations of various carbohydrates but their structures are very different to that of xylan and thus not suited for analysis of this. Indeed while xylan is characterised by a random coil configuration of β-1,3/1,4 linkages between d-xylose residues, PEG displays a linear repetition of the monomer H–(O–CH_2–CH_2)n–OH and dextran a "stairs-like" structure of α-D-1,6-glucose-linked glucans. Nevertheless, our analysis indicates the xylan extracted to be of a high molecular weight with no low molecular weight xylo-oligosaccharides, below DP 15, or xylose. This was confirmed by the absence of peaks for such compounds in Rezex™ RSO-Oligosaccharide analysis wherein the xylan eluted as a single peak in the void fraction (Fig. 6C). Additional peaks were also identified in the HPLC analyses and further studies indicated these to be due to the ash present in the sample and an unknown compound. The protein in the extract is believed to have been removed by acid precipitation during the sample preparation steps and total sugar, protein, and reducing sugar analyses, as well as absorption (200-700 nm) studies did not reveal the identity of the unknown peak. Further studies are required to identify this peak, in particular separation with a preparative HPLC column would be beneficial for obtention of greater quantities of material for analyses.

3.6. Soluble Xylan-rich Fraction as an R&D Substrate

The xylan rich fraction prepared in this study has a number of potential applications, either with direct application (e.g. materials, food and feed, cosmetics, R&D) or following hydrolysis to lower molecular weight xylo-oligosaccharides or xylose. In an initial attempt to demonstrate the potential of this xylan-rich fraction, we investigated its use in R&D for the detection and measurement of xylanase activity. The high xylan content, absence of low

molecular weight compounds, high solubility and purity of the soluble xylan-rich fraction indicates its potential in such an application. Indeed, as compared with the commonly used commercial xylan substrate from beechwood xylan, the xylan-rich fraction had similar xylan content. Our analysis indicates the commercial soluble beechwood xylan studied to be composed of 71% xylan (% dw), 5% ash, 4.3% glucose, 2.6% glucuronic acid, 0.3% protein and 0.1% phenolics. Birchwood xylan showed similar component content levels, while oat spelt xylan has lower amounts of xylan, 67% of the dry weight, with 15% glucose, 8% of ash and 10% arabinose. As discussed earlier, the xylan prepared in the present study has a higher ash content but removal of this by ultrafiltration can lead to a final higher purity product composed of ≈90% xylan. Nevertheless, the ash was not found to pose a problem in the use of the xylan as an assay substrate. The additional monosaccharides observed in beechwood xylan are the side-chain substitutions characteristic of this wood sourced xylan. As mentioned earlier and as observed in our studies, *Palmaria palmata* xylan is non-substituted and composed solely of xylose. Furthermore, it is highly soluble, with a maximum solubility of 150 g/L whereas all other currently used commercial xylan sources, i.e. from beechwood, birchwood and oat spelt, are insoluble. Water solubilisation and centrifugation can be used to prepare a soluble fraction, with a maximum solubility of 100 g/L, from commercial beechwood xylan but this requires extra processing steps and results in a loss of 55-60% of the original substrate material. Differences are also observed in the length of the xylan chains, the *Palmaria palmata* xylan has an average DP of 25-45 or 90-110, whereas soluble beechwood xylan has an average DP of 100-120. This implies a higher number of reducing ends in the lower molecular weight xylan prepared in the present study with goal A conditions and this is reflected by a saturation of the DNS and NS assays at lower substrate concentrations. Maximum concentrations of *Palmaria palmata* xylan of 15 g/L are recommended for these assays whereas up to 30 g/L soluble beechwood xylan can be used before assay saturation. Such differences may be important in assays of enzymes with high Michaelis constants (K_M) but solution dilution before the DNS addition and boiling step of the reaction, can be used to overcome this while potentially sacrificing precision.

Comparison of the activities of the glycoside hydrolase family 8 β-1,4-xylanase pXyl on the various xylan substrates at various concentrations (Fig. 7) showed the *Palmaria palmata* xylan to enable a much higher assay precision (4% average coefficient of variation) and

accuracy for measurements as compared to all other substrates tested. Probably resultant of their poor solubility, oat spelt, birchwood and beechwood xylan activity assays are characterised by a poor precision, which negatively affects test accuracy. Average coefficients of variation of 21%, 21% and 15% were observed for oat spelt, birchwood and beechwood xylans, respectively, and this increased significantly at substrate concentrations above ≈30 mg/mL where increased viscosity and coefficients of variation as high as 36%, 54% and 27% were seen, respectively. The better precision seen with the *Palmaria palmata* xylan compared to the other xylan substrates indicates its suitability for use as an assay substrate. Furthermore, the enzyme was shown to successfully enable measurement of activities of endoxylanases belonging to glycoside hydrolase families 8, 10 and 11, as well as β-1,3 xylanases of family 26, thereby demonstrating its widespread applicability for assay of various types of xylanases.

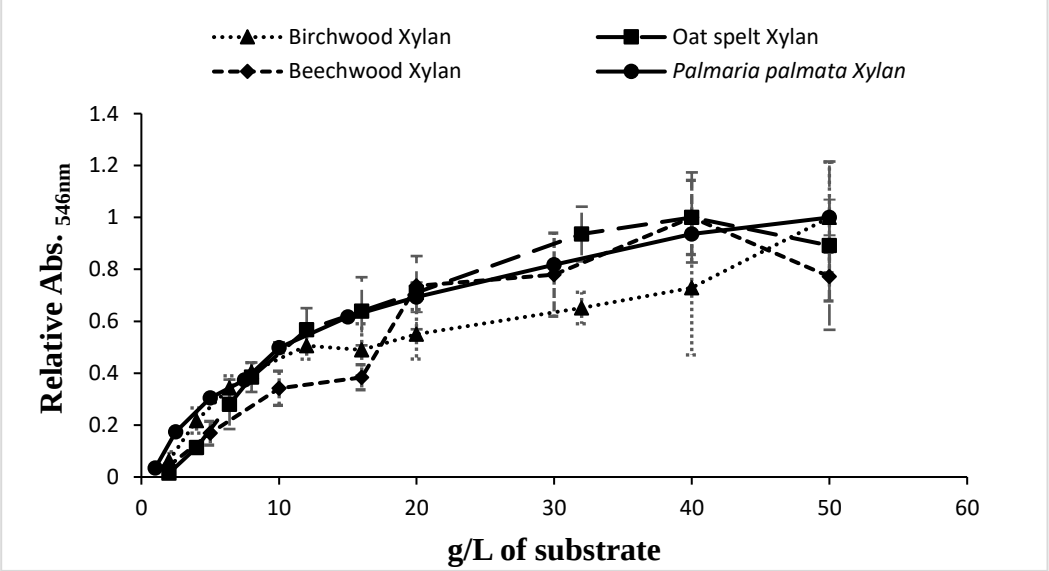


Fig. 7. Comparison of *Palmaria palmata* xylan-rich fraction, oat spelt xylan, birchwood xylan and beechwood xylan as substrates for the DNS reducing sugar assay of the glycoside hydrolase family 8 endoxylanase, pXyl, activity. Values of relative absorption readings at 546 nm for 3 independent test with three replicates each at various substrate concentrations are shown.

The soluble nature of the *Palmaria palmata* xylan inhibits its direct use in the plate screening of xylanase activity and therefore its chemical coupling with the anthraquinone dye remazol brilliant blue (RBB) and incorporation into a solid medium was investigated. Following coupling, product analysis indicated an average of one RBB molecule bound to every 29 molecules of xylose, which is lower than the 1 in 20 reported with RBB-xylan made from

beechwood [35] and it is suggested that in the latter, the methylglucuronic acid side chains could enhance RBB binding with substrate. The *Palmaria palmata* xylan-Remazol Brillant Blue prepared in this study was investigated for use in screening for xylanase activity. As can be seen in Fig. 8, a clear halo is observed around endoxylanase producing colonies and around wells containing endoxylanase solutions whereas no halos are observed in the absence of xylanase activity. Such results clearly point to the potential of this substrate for use in detecting and screening for xylanase activity.

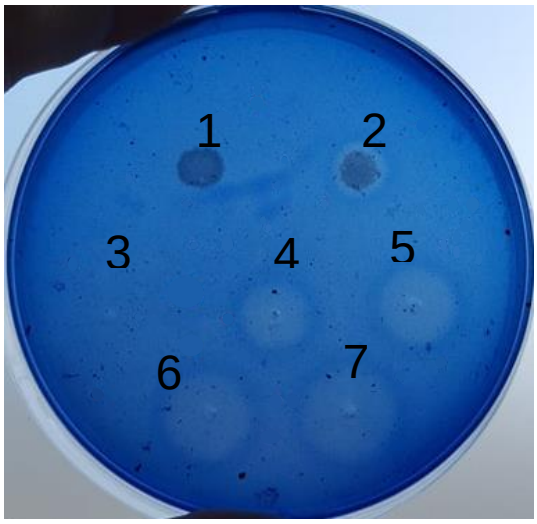


Fig. 8. Use of *Palmaria palmata* xylan-Remazol Brillant Blue as a chromogenic substrate for xylanase activity screening. 1: negative control (*E. coli* BL21(DE3) cells); 2: *E. coli* BL21(DE3) cells expressing the glycoside hydrolase family 8 endoxylanase pXyl; 3: Blank (enzyme buffer: 20 mM MOPS + 100 mM NaCl); 4: 0.01 ug of xylanase pXyl; 5: 0.02 ug of xylanase pXyl; 6: 0.04 ug of xylanases pXyl; 7: 0.06 ug of xylanase pXyl.

4. Conclusion

We have successfully developed a simple, environmentally friendly process for fractionation of the macroalgae *Palmaria palmata* into a xylan rich fraction and a protein-rich fraction. This process involves three principal steps, a proprietary pre-treatment step by the macroalgae supplier involving commonly used macroalgae preservation techniques, an aqueous extraction step, and a final centrifugation or ultrafiltration step to separate the soluble from the insoluble fraction. In the study it was unexpectedly found that aqueous extraction enabled similar to or greater xylan extraction and reduced protein contamination than chemical methods, with the extraction efficiency being dependent on the pre-treatment process used. A multivariate statistical technique with experimental design (RSM-CCD) was

used for characterisation and optimisation of the aqueous extraction step and enabled identification of the principal process factors influencing the process and their interactions. Macroalgae concentration, incubation temperature, time and mixing speed, were found to strongly influence xylan yield whereas salt concentration had no effect. In contrast, salt concentration influenced final xylan product purity, in particular the ash content, while algae concentration and incubation temperature influenced protein contamination.

The optimised, high yield process developed enabled extraction of up to 70% of the total xylan present in the *P. palmata* to give a soluble fraction composed of 73% xylan. These values are higher than those reported for xylan extraction from land plants with chemical and/or physical methods wherein yields of 10-45% (w/w, xylan/total xylan in plant) are common. In addition the process is simpler and more environmentally friendlier that currently used extraction processes. The soluble xylan rich fraction produced in our study contains only minor concentrations of contaminants (proteins, lipids and phenolics), with the exception of ash (21%) which can nevertheless be easily removed by incorporation of an additional ultrafiltration or dialysis step. The optimised process also enables for the preparation of an insoluble fraction, which was found to be mainly composed of protein (52%), and which can have various potential industrial applications and thereby further valorises this red macroalgae and our extraction process while also reducing process wastes. Further characterisation of the xylan produced showed this to have an average degree of polymerisation (avDP) of 25 to 100, depending on the extraction conditions used, and without any low molecular weight xylo-oligosaccharides or xylose being present. The soluble xylan fraction produced herein was shown to be apt for direct use as an assay substrate in xylanase activity measurements and, following chemical coupling with RBB dye, to function as a chromogenic substrate for plate based screening of xylanase activity. Future studies should focus on investigating other applications for the xylan produced herein but also on optimising the pre-treatment step and identifying the unidentified product observed in the HPLC studies.

In this study we clearly demonstrated an environmentally friendly, sustainable, and more efficient alternative for xylan production compared with currently used chemical and/or physical-based processes. The sustainable process developed herein should enable advancements in developing xylan as a low cost commodity product and enhance its commercialisation and use. Indeed, the multi-valorisation of the macroalgae as both a xylan

and protein source will obviously enhance its economic value while also contributing to the circular economy concept by valorising all major algal components and reducing wastes. It will promote a bio-based-economy and leverage the algaculture bioeconomy, greatly enhancing growth in this sector and, through IMTA aquaculture, enhancing the sustainable exploration of endogenous marine resources.

5. References

[1] Y.M. Bar-On, R. Phillips, R. Milo, The biomass distribution on Earth, Proc Natl Acad Sci U S A 115(25) (2018) 6506-6511.
[2] T. Collins, C. Gerday, G. Feller, Xylanases, xylanase families and extremophilic xylanases, FEMS Microbiology Reviews 29(1) (2005) 3-23.
[3] D.S. Naidu, S.P. Hlangothi, M.J. John, Bio-based products from xylan: a review, Carbohydr Polym 179 (2018) 28-41.
[4] K. Petzold-Welcke, K. Schwikal, S. Daus, T. Heinze, Xylan derivatives and their application potential - mini-review of own results, Carbohydr Polym 100 (2014) 80-8.
[5] E.A. Rennie, H.V. Scheller, Xylan biosynthesis, Current Opinion in Biotechnology 26 (2014) 100-107.
[6] B.S. Kayserilioğlu, U. Bakir, L. Yilmaz, N. Akkaş, Use of xylan, an agricultural by-product, in wheat gluten based biodegradable films: mechanical, solubility and water vapor transfer rate properties, Bioresour Technol 87(3) (2003) 239-46.
[7] H. Chakdar, M. Kumar, K. Pandiyan, A. Singh, K. Nanjappan, P.L. Kashyap, A.K. Srivastava, Bacterial xylanases: biology to biotechnology, 3 Biotech 6(2) (2016) 150.
[8] A. Ebringerová, T. Heinze, Xylan and xylan derivatives – biopolymers with valuable properties, 1. Naturally occurring xylans structures, isolation procedures and properties, Macromolecular Rapid Communications 21(9) (2000) 542-556.
[9] C. Dal Farra, N. Domloge, D. Peyronel, Use of purified, water-soluble xylans extracted from *Palmaria,* Patent number: WO2003002085, (2003).
[10] T. Collins, M.A. Meuwis, I. Stals, M. Claeyssens, G. Feller, C. Gerday, A novel family 8 xylanase, functional and physicochemical characterization, J Biol Chem 277(38) (2002) 35133-9.
[11] B.V. McCleary, P. McGeough, A comparison of polysaccharide substrates and reducing sugar methods for the measurement of endo-1,4-β-xylanase, Applied Biochemistry and Biotechnology 177(5) (2015) 1152-1163.
[12] F. Meddeb-Mouelhi, J.K. Moisan, M. Beauregard, A comparison of plate assay methods for detecting extracellular cellulase and xylanase activity, Enzyme and Microbial Technology 66 (2014) 16-19.
[13] C.M. Courtin, K. Swennen, P. Verjans, J.A. Delcour, Heat and pH stability of prebiotic arabinoxylooligosaccharides, xylooligosaccharides and fructooligosaccharides, Food Chemistry 112(4) (2009) 831-837.
[14] C. Amorim, S.C. Silvério, B.B. Cardoso, J.I. Alves, M.A. Pereira, L.R. Rodrigues, In vitro assessment of prebiotic properties of xylooligosaccharides produced by *Bacillus subtilis* 3610, Carbohydrate Polymers 229 (2020) 115460.
[15] R. Maeda, T. Ida, H. Ihara, T. Sakamoto, Induction of apoptosis in MCF-7 cells by β-1,3-xylooligosaccharides prepared from *Caulerpa lentillifera*, Biosci Biotechnol Biochem 76(5) (2012) 1032-4.
[16] A.A. Aachary, S.G. Prapulla, Xylooligosaccharides (XOS) as an emerging prebiotic: microbial synthesis, utilization, structural characterization, bioactive properties, and applications, Comprehensive Reviews in Food Science and Food Safety 10(1) (2011) 2-16.
[17] C. Amorim, S.C. Silvério, K.L.J. Prather, L.R. Rodrigues, From lignocellulosic residues to market: production and commercial potential of xylooligosaccharides, Biotechnology Advances 37(7) (2019) 107397.
[18] M. Perttula, M. Linko, J.-F. Selin, Production of N-butanol and isopropanol from xylose and birch hemicellulose hydrolysate by *Clostridium beijerinckii*, in: O.M. Neijssel, R.R. van der Meer, K.C.A.M. Luyben (Eds.) 4th European Congress on Biotechnology 1987, Elsevier, 1987, p. 285.

[19] L. Canilha, A. Kumar Chandel, T.S. dos Santos Milessi, F.A. Fernandes Antunes, W.L. da Costa Freitas, M. das Gracas Almeida Felipe, S.S. da Silva, Bioconversion of sugarcane biomass into ethanol: an overview about composition, pretreatment methods, detoxification of hydrolysates, enzymatic saccharification, and ethanol fermentation, J Biomed Biotechnol 2012 (2012) 989572.
[20] F.C. de Figueiredo, A.F.A. Carvalho, M. Brienzo, T.S. Campioni, P. de Oliva-Neto, Chemical input reduction in the arabinoxylan and lignocellulose alkaline extraction and xylooligosaccharides production, Bioresource Technology 228 (2017) 164-170.
[21] G. Machado, S. Leon, F. Santos, R. Lourega, J. Dullius, M.E. Mollmann, P. Eichler, Literature review on furfural production from lignocellulosic biomass, Natural Resources 07(03) (2016) 115-129.
[22] P. Sannigrahi, A.J. Ragauskas, G.A. Tuskan, Poplar as a feedstock for biofuels: a review of compositional characteristics, Biofuels, Bioproducts and Biorefining 4(2) (2010) 209-226.
[23] M. Suutari, E. Leskinen, K. Fagerstedt, J. Kuparinen, P. Kuuppo, J. Blomster, Macroalgae in biofuel production, Phycological Research 63(1) (2015) 1-18.
[24] C.M. Keith, L.C.W. Jeffrey, F.J. Simpson, Review of chemical constituents of the red alga *Palmaria palmata* (dulse), Economic Botany 34(1) (1980) 27-50.
[25] S.H.R. Ruth, M.V. Kjell, A.L. Bjørn, M.M. Sverre, Seasonal and geographical variation in the chemical composition of the red alga *Palmaria palmata* (L.) Kuntze, Botanica Marina 47(2) (2004) 125-133.
[26] E. Deniaud, J. Fleurence, M. Lahaye, Interactions of the mix-linked β-(1,3)/β-(1,4)-D-xylans in the cell walls of *Palmaria palmata* (Rhodophyta), Journal of Phycology 39(1) (2003) 74-82.
[27] J.H. Waterborg, H.R. Matthews, The lowry method for protein quantitation, Methods Mol Biol 1 (1984) 1-3.
[28] I. Biancarosa, M. Espe, C.G. Bruckner, S. Heesch, N. Liland, R. Waagbø, B. Torstensen, E.J. Lock, Amino acid composition, protein content, and nitrogen-to-protein conversion factors of 21 seaweed species from Norwegian waters, Journal of Applied Phycology 29(2) (2017) 1001-1009.
[29] E.G. Bligh, W.J. Dyer, A rapid method of total lipid extraction and purification, Canadian Journal of Biochemistry and Physiology 37(8) (1959) 911-917.
[30] L.M. Laurens, T.A. Dempster, H.D. Jones, E.J. Wolfrum, S. Van Wychen, J.S. McAllister, M. Rencenberger, K.J. Parchert, L.M. Gloe, Algal biomass constituent analysis: method uncertainties and investigation of the underlying measuring chemistries, Anal Chem 84(4) (2012) 1879-87.
[31] J.Y. Ahn, D.Y. Kil, C. Kong, B.G. Kim, Comparison of oven-drying methods for determination of moisture content in feed ingredients, Asian-Australas J Anim Sci 27(11) (2014) 1615-1622.
[32] L. Machu, L. Misurcova, J.V. Ambrozova, J. Orsavova, J. Mlcek, J. Sochor, T. Jurikova, Phenolic content and antioxidant capacity in algal food products, Molecules 20(1) (2015) 1118-33.
[33] M. Barroca, G. Santos, B. Johansson, F. Gillotin, G. Feller, T. Collins, Deciphering the factors defining the pH-dependence of a commercial glycoside hydrolase family 8 enzyme, Enzyme and Microbial Technology 96 (2017) 163-169.
[34] Z.-W. Cai, H.-H. Ge, Z.-W. Yi, R.-Y. Zeng, G.-Y. Zhang, Characterization of a novel psychrophilic and halophilic β-1, 3-xylanase from deep-sea bacterium, *Flammeovirga pacifica* strain WPAGA1, International Journal of Biological Macromolecules 118 (2018) 2176-2184.
[35] P. Biely, D. Mislovicová, R. Toman, Soluble chromogenic substrates for the assay of endo-1,4-beta-xylanases and endo-1,4-beta-glucanases, Anal Biochem 144(1) (1985) 142-6.

[36] M. Bjarnadóttir, B.V. Aðalbjörnsson, A. Nilsson, R. Slizyte, M.Y. Roleda, G.Ó. Hreggviðsson, Ó.H. Friðjónsson, R. Jónsdóttir, *Palmaria palmata* as an alternative protein source: enzymatic protein extraction, amino acid composition, and nitrogen-to-protein conversion factor, Journal of Applied Phycology 30(3) (2018) 2061-2070.
[37] A. Naseri, G.S. Marinho, S.L. Holdt, J.M. Bartela, C. Jacobsen, Enzyme-assisted extraction and characterization of protein from red seaweed *Palmaria palmata*, Algal Research 47 (2020) 101849.
[38] H.K. Mæhre, I.-J. Jensen, K.-E. Eilertsen, Enzymatic pre-treatment increases the protein bioaccessibility and extractability in dulse (*Palmaria palmata*), Marine drugs 14(11) (2016) 196.
[39] J. O' Connor, S. Meaney, G.A. Williams, M. Hayes, Extraction of protein from four different seaweeds using three different physical pre-treatment strategies, Molecules 25(8) (2020) 2005.
[40] S.L. Holdt, S. Kraan, Bioactive compounds in seaweed: functional food applications and legislation, Journal of Applied Phycology 23(3) (2011) 543-597.
[41] H.K. Mæhre, M.K. Malde, K.-E. Eilertsen, E.O. Elvevoll, Characterization of protein, lipid and mineral contents in common Norwegian seaweeds and evaluation of their potential as food and feed, Journal of the Science of Food and Agriculture 94(15) (2014) 3281-3290.
[42] D. Lopes, T. Melo, J. Meneses, M.H. Abreu, R. Pereira, P. Domingues, A.I. Lillebø, R. Calado, M.R. Domingues, A new look for the red macroalga *Palmaria palmata*: a seafood with polar lipids rich in EPA and with antioxidant properties, Marine drugs 17(9) (2019) 533.
[43] B. Charrier, M.H. Abreu, R. Araujo, A. Bruhn, J.C. Coates, O. De Clerck, C. Katsaros, R.R. Robaina, T. Wichard, Furthering knowledge of seaweed growth and development to facilitate sustainable aquaculture, New Phytologist 216(4) (2017) 967-975.
[44] A.G. Viana, M.D. Noseda, A.G. Gonçalves, M.E. Duarte, N. Yokoya, M.C. Matulewicz, A.S. Cerezo, β-D-(1→4), β-D-(1→3) 'mixed linkage' xylans from red seaweeds of the order Nemaliales and Palmariales, Carbohydr Res 346(8) (2011) 1023-8.
[45] Y. Yamamoto, H. Kishimura, Y. Kinoshita, W. Saburi, Y. Kumagai, H. Yasui, T. Ojima, Enzymatic production of xylooligosaccharides from red alga dulse (*Palmaria* sp.) wasted in Japan, Process Biochemistry 82 (2019) 117-122.

Chapter II.II

Xylanases for Xylo-oligosaccharides from Macroalgae

Xylan: Process Development and Optimisation

Abstract

Xylo-oligosaccharides are low molecular oligosaccharides composed of D-xylopyranosyl units. They have already found application in health care, pharmaceutics, and the food and feed industries and recent societal trends towards preventative health and wellbeing and an increased focus on functional foods and anti-obesity diets is expected to drive strong future market growth. Currently, the majority of xylo-oligosaccharides studied are β-1,4-linked, with or without side-chain substitutions, and are mainly produced from lignocellulosic xylan by hydrolysis, with a growing preference for enzyme hydrolysis. Presently, endo-β-1,4-xylanases belonging to glycoside hydrolase families 10 and 11 are the most commonly employed, yet xylanases are also found in other glycoside hydrolase families with potential for improved performance.

Xylo-oligosaccharide properties and functionality, and hence application, are related to structure, thus the isolation of novel xylo-oligosaccharides with novel structures could lead to novel and/or improved applications. The red macroalgae *Palmaria palmata* is constituted by a non-substituted mixed linkage β-1,3/β-1,4 xylan, and thus offers a source of novel mixed linkage xylo-oligosaccharides. In the present study, we investigated, compared and optimised the utilisation of enzyme technology, specifically glycoside hydrolase family 8, 10 and 11 xylanases, for the eco-friendly production of xylo-oligosaccharides from *Palmaria palmata* xylan.

A cold-adapted family 8 xylanase was shown to be the most active of the enzymes studied over a large temperature range and enabled for a high extent of substrate hydrolysis. Hydrolysis involved production of mixed-linkage high molecular weight xylo-oligosaccharides intermediates and subsequent hydrolysis to final lower molecular weight products. This enzyme gave rise to final mixed-linkage products of higher molecular weight than family 11 enzymes which also produced high levels of a β-1,4-linked xylotriose, but no xylose. Use of response surface methodology enabled identification of an ambient temperature process with up to 90% product yield and enabling direct utilisation of a soluble xylan-rich fraction produced by aqueous extraction of pre-treated macroalgae.

The process developed herein enables highly efficient, eco-friendly, ambient temperature production of novel non-substituted, mixed linkage β-1,4/β-1-3 xylo-oligosaccharides from *Palmaria palmata*. The process abrogates heating requirements and thereby potentially reduces the process environmental and economic impact. It valorises the red macroalgae studied and further demonstrates the industrial aptitude of the cold-adapted glycoside hydrolase family 8 xylanase.

1. Introduction

Xylo-oligosaccharides are short xylose-backbone constituted oligomers commonly defined as containing between 2 and 10 D-xylopyranosyl units, although average degrees of polymerisation of up to 50 have also been described [1-3]. Most commonly investigated xylo-oligosaccharides have β-1,4-linked backbones but β-1,3 linkages and indeed also mixed β-1,4/β-1,3 linkages have also been reported [4-7]. Furthermore, depending on their source and production method, the backbone chain can be branched to varying degrees with e.g., glucuronyl, methyl-D-glucuronyl, α-L-arabinosyl, galacturonyl, xylosyl, rhamnosyl, galactosyl, glucosyl, acetyl, feruloyl dehydrodiferuloyl, sinapyl and/or p-coumaroyl side-chain groups.

While application of β-1,4-linked xylo-oligosaccharides is still in its infancy, they have already found use in a variety of industries, including health care, pharmaceutics, and the food and feed industries, with a market size of 79 million Euro having been reported for 2017 by Amorim et al. 2019 [8-10]. They have been shown to be effective, safe and stable prebiotics, yet are still classified as emerging prebiotics due to a paucity of information, namely in relation to human experimental studies. Additionally in health care, they have demonstrated various other noteworthy biological activities, including anti-cancer, antibacterial, anti-viral, anti-coagulant, and antidiabetic activities, as well as anti-inflammatory properties and antioxidant potential [11-15]. They have also been shown to be non-cariogenic and characterised by a low calorie value and sweet taste, and thus have potential for incorporation in various foods, feeds and beverages to enhance the organoleptic properties and health aspects of these [16, 17]. In cosmetics and personal care they have been proposed for use in skin and hair regeneration and as moisturisers and anti-oxidants. Finally, their use in the production of platform chemical, biofuels and materials is also recognised wherein their xylose monomers can be used as feedstock [18].

It has been found that the xylo-oligosaccharide structure and composition (i.e., the degree of polymerisation, presence/absence of branching, type(s) of branching and type(s) of linkages) directly affect the properties and functionality of the xylo-oligosaccharides, with, for example, variations in the prebiotic effect, taste, sweetness and/or calorific value being reported. Indeed, as compared to shorter chain xylo-oligosaccharides, higher degrees of polymerisation are associated with decreased sweetness, decreased calorific value, and prebiotic action in the distal part of the colon, as compared to prebiotic action in the more proximal parts for short xylo-oligosaccharides.

Hence, identification of novel xylo-oligosaccharides with novel structures opens the potential for novel or altered properties and functionalities [19-21].

While small amounts of xylo-oligosaccharides are found in some foods, they are usually produced by hydrolysis of xylan, a complex heteropolysaccharide found in plants and constituting the major component of hemicellulose in plant biomass. Currently they are mainly produced from xylan from lignocellulosic land plants, with a recent trend towards industry and agriculture wastes from these so as to reduce process costs and the environmental impact. Due to their plant source, almost all xylo-oligosaccharides studied to date are β-1,4-linked and carry various different side-chain decorations. Inconveniently, most current xylan and xylo-oligosaccharide production processes are characterised by poor yields and low economic and environmental viability [8, 9, 18]. Xylan extraction from lignocellulosic materials usually involves harsh, hazardous and costly multi-step chemical and/or physical treatments with or without enzyme treatment and the isolated xylan is then hydrolysed to xylo-oligosaccharides by high temperature, acid, alkali and/or enzyme treatment. Of these latter hydrolysis processes, hydrolysis with enzymes, and specifically with endo-β-1,4-xylanases (EC 3.2.1.8), has recently gained much interest due to their specificity of action, absence of xylose and other byproduct production, reduced pollutant and waste generation, and reduced environmental impact [8, 22, 23]. Most commonly, glycoside hydrolase family 10 and 11 xylanases are employed as these have historically been the most extensively studied [24]. Importantly, enzymes with xylanolytic activity have also been identified in glycoside hydrolase families 5, 7, 8, 12, 26, 30, 43, 51, 98 and 141. These display various physicochemical and kinetic properties, substrate specificities and product profiles with potential for application in xylo-oligosaccharide production, yet appear not to have been investigated for this.

Recently, we have shown that the red macroalga *Palmaria palmata* is suited as a sustainable xylan source (Chapter II.I). It is a xylan rich, fast growing, carbon neutral, marine resource which does not compete with land plants for arable land or freshwater and can be sustainably produced by aquaculture under the Integrated Multi-Trophic Aquaculture (IMTA) concept. We reported a simple process for a high xylan extraction yield involving use of a combination of alga pre-treatment and water extraction. The isolated xylan rich fraction, composed of ≈75% of a highly soluble xylan, was found to have low levels of contaminants, mainly ash, which facilitates its usage in a variety of applications. In the present study we developed enzyme technology for the eco-friendly production of novel non-substituted, mixed linkage β-1,4/β-1-3 xylo-oligosaccharides from the *Palmaria palmata* xylan-rich fraction. Endoxylanases from glycoside hydrolase families 8, 10, and 11 were

investigated and compared and the xylo-oligosaccharide production process optimised and characterised.

2. Materials and Methods

2.1.Xylan Substrate and Enzymes

Palmaria palmata xylan was prepared as reported in Chapter II.I. 100g of dried pre-treated *P.palmata* was incubated with 1L deionised water at 98 °C for 4 hours at 120 rpm. After incubation the soluble xylan rich fraction was separated from the insoluble fraction by centrifugation at room temperature, 9000 x g for 15 min. The supernatant was then filtered through a grade 1 paper filter (Whatman) and directly used or freeze dried for storage at room temperature.

Xylanases representing three different glycoside hydrolase families from the Carbohydrate-Active enZYmes database (CAZy) were investigated: two glycoside hydrolase family 8 endoxylanases: pXyl and Nzy8A (Genebank accession number: CAD20872.1; code: CpXyn8A, respectively); two glycoside hydrolase family 11 endoxylanases: Mega11A and Nzy11A (Genebank accession number: ABW04217.1 and CAA84537.1, respectively); and a glycoside hydrolase family 10 endoxylanase: Mega10A (Genebank accession number: ACE84499.1). Assays were carried out at the optimal pH for activity in the appropriate buffer recommended for each enzyme:

- pXyl: Mops/NaCl (20 mM MOPS, 100 mM NaCl), pH 7.5
- Nzy8A (NZytech): sodium phosphate (50 mM), pH 6.0
- Mega11A (Megazyme): sodium phosphate buffer (100 mM), pH 6.0
- Nzy11A (NZytech): sodium phosphate (50 mM), pH 6.5
- Mega10A (Megazyme): sodium acetate buffer (100 mM), pH 5.0

pXyl was produced and purified as described in Barroca et al. 2017 [25]. All other enzymes were used as supplied by the suppliers.

2.2. Carbohydrate Quantification

Carbohydrate content was determined by HPLC analysis of samples following hydrolysis to monomers by 4% H_2SO_4 treatment at 121 °C, 1 bar pressure for 20 minutes. Hydrolysed samples were centrifuged at 9000 rpm for 30 minutes, filtered through a 0.22 µm polyethersulfone (PES) filter (Merck) and separated and quantified by HPLC on a ROA-organic acid H^+(8%) column (Phenomenex) at 60 °C. An Elite LaChrom (VWR Hitachi) chromatography system with an Elite LaChrom L-2490 RI detector (VWR Hitachi) at 40 °C was used for all experiments. 2.5 mM H_2SO_4 was used as mobile phase at a flow rate of 0.7 mL/min for the first 7 minutes followed by 0.1 mL/min for a total period of 30 minutes. The EZChrom Elite 3.3.2 SP2 software was employed for data collection and analysis. Sugar monomer concentrations were calculated from respective standard curves of each monomer standard investigated (xylose, glucuronic acid, glucose and arabinose).

2.3. Protein Concentration

Soluble protein content was determined using the Lowry method as described by Waterborg et al. 1984 [26]. 100 µL of 2 M NaOH was added to 100 µL samples before addition of 1 mL of freshly mixed complex-forming reagent (100:1:1 by volume of 2% (w/v) Na_2CO_3: 1% (w/v) $CuSO_4 \cdot 5H_2O$: 2% (w/v) sodium potassium tartrate, respectively) and incubation at room temperature for 10 minutes. 100 µL of 1 N Folin reagent (Themo Fisher Scientific) was then added and vigorous mixed and incubated for a further 30 minutes before absorbance measurement at 750 nm and protein concentration determination from a BSA standard curve.

2.4. Xylo-oligosaccharides: HPLC Analyses

The molecular weight range of the xylo-oligosaccharides produced was analysed with use of two SEC-HPLC columns; a PolySep-SEC GFC-P Linear (Phenomenex) HPLC column and a BioBasic™ SEC 60 (Thermo Scientific) HPLC column with fractionation ranges (pullulans) of, respectively, 1 KDa to 10 MDa and 300 Da to 6 KDa. In both cases, isocratic elution with water as the mobile phase was used with a constant flow rate of 0.5 mL/minute (BioBasic™ SEC 60 column) or 0.6 mL/minute (PolySep-SEC column) for a total period of 30 minutes. Standard curves of β-1,4-linked xylo-oligosaccharides were prepared with 1 g/L xylose, xylobiose, xylotriose, xylotetraose,

xylopentaose and xylohexaose standards (Megazyme). HPLC equipment and software employed were the same as reported for carbohydrate analysis in section 2.2. Prior to HPLC analysis all samples were treated with trichloroacetic acid (TCA) at a final concentration of 10% for at least 2 hours at room termperature, centrifuged at 14000 rpm for 15 minutes and filtered through a 0.22 µm PES filter (Merk) for sample deproteinisation.

A Rezex RSO-Oligosaccharide Ag^{+} (4)% (Phenomenex) HPLC column, which is asserted to enable identification of xylose oligosaccharides with degrees of polymerisation up to 14, was used in identifying the degree of polymerisation of the xylo-oligosaccharides produced. Isocratic elution was used with water as mobile phase at a constant flow rate of 0.15 mL/min for a total period of 90 min. 1 g/L xylose, xylobiose, xylotriose, xylotetraose, xylopentaose and xylohexaose β-1,4 standards were also analysed. HPLC equipment and software employed were the same as reported for carbohydrate analysis (section 2.2). Sample preparation was the same as described above for the SEC-HPLC analysis (section 2.10).

2.5.Activity Assays

The DiNitroSalicylic acid (DNS) and Nelson-Somogyi (NS) assays were utilised in calculating the carbohydrate reducing end content of samples for determination of the average degree of polymerisation of the xylo-oligosaccharide products as well as for determination and comparison of the xylanase activities of the 5 endoxylanase enzymes studied. The DNS assay was performed as previously reported by [27, 28] and the NS assay was performed as described by McCleary et al, 2015 [28].

For the temperature profiles, 30 g/L of *P. palmata* xylan was incubated with 100 µg/L of enzyme at 20 °C and 120 rpm. 50 µL samples were collected at 5 minutes of incubation and directly used for DNS analyses. The same procedure was used for the molecular activity analysis.

For the extent of hydrolyses analysis, 30 g/L of *P. palmata* xylan was incubated with 100 µg/L of enzyme at 20 °C and 120 rpm. 50 µL samples were collected at 2, 6 and 24 hours of incubation and directly used for DNS analyses.

2.6. Average Degree of Polymerisation (avDP)

For calculation of the average degree of polymerisation (avDP) of carbohydrate samples, the DNS and NS assays were performed as described for measurement of xylanase activity [27, 28]. To calculate the initial avDP (0 hours of incubation), the samples were directly used without the addition of enzyme. The avDP of the sample was calculated as follow:

$$avDP = \frac{Total\ \mu M\ of\ xylose\ in\ sample}{Total\ \mu M\ xylose\ equivalents\ measured\ by\ DNS \vee NS} \quad \textbf{(1)}$$

Total μM of xylose was calculated from the measured substrate weight and a D-xylopyranosyl molecular weight of 150 g/L.

2.7. Process Optimisation RSM-CCD

In this study, response surface methodology was employed to optimise the production of xylo-oligosaccharides, to characterise the role of various process variables and identify interactions among variables and their effects. The software program Design-Expert version 7.0.0 (Stat-Ease Inc) was employed for the central composite design (CCD) experimental design, model fitting, diagnosis and interpretation as well as for data plotting and navigation, and identification of optimal conditions. The parameter description, ranges used and the model utilised are described in the results section of this study.

2.7.1. Model Fitting and Validation

The Analyses of variance (ANOVA) was used for the determination of the statistical significance of the model used. Here, the Fisher's statistical test (F-test) was performed to determine the effect of each variable on each response in the study. The significance and the magnitude of the effects estimates of each variable and all their possible linear and quadratic interactions on the responses were determined. A confidence level of 95% significance that is, a *p*-value higher than 0.05, was implemented.

The selection of the model takes into account the significance of the model, evaluated by considering either the F-values or the *p*-values of the model and of the lack of fit. The lack-of-fit test compares the residual error of the selected model with the pure error of the data, calculated though

the analyses of the variance between the 5 central points performed. If residual error significantly exceeds pure error, the model will show significant lack of fit, and another model may be more appropriate. In addition the adjusted R^2, which analyses the distance of the predicted value of the model from the actual data values, was also used for model selection. An adjusted R^2 higher than 85% was considered optimal for this study.

For the RSM-CCD analyses, a final total volume of 5 mL was used for each experimental run and the following response was measured: total xylo-oligosaccharides area. This gives a measure of the quantity of xylo-oligosaccharides produced and was determined by integration of the chromatographic peak areas for all xylo-oligosaccharides identified by Rezex RSO-Oligosaccharide Ag^+ (4)% (Phenomenex) HPLC analysis.

3. Results and Discussion

3.1. Xylanases for Xylo-oligosaccharide Production

As stated above, recently enzyme treatment for production of xylo-oligosaccharides has gained much interest due to its high specificity, mild reaction conditions, safety, and reduced environmental impact. We compared a highly active glycoside hydrolase family 8 psychrophilic xylanase, pXyl, previously isolated and studied by Collins et al. [27, 29, 30], with some of the most active commercially available mesophilic enzymes from glycoside hydrolase families 10 (Mega10A), 11 (Mega11A and Nzy11A), and 8 (Nzy8A). We compared the hydrolytic efficiency as a function of temperature and the extent of hydrolyses of the different enzymes on the *Palmaria palmata* xylan-rich fraction. As this substrate has a high xylan content, ≈73% of the dry weight, and reduced amounts of other components (Table 1), it was used directly in the activity assays without any additional treatment, thereby reducing process steps and costs.

Table 1. Composition (% of the dry weight) and pH of the *P. palmata* xylan substrate used in this study.

pH	Xylan (%)	Protein (%)	Ash (%)	Lipids (%)	Phenolics (%)
5-5.5	73	5	21	0	0.4

3.1.1. Xylanase Temperature Profiles

Comparison of the enzymes molecular activity as a function of temperature (Fig. 1, Table 2), showed that the glycoside hydrolase family 8 xylanase, pXyl, exhibited significantly higher activity at low to moderate temperatures than all other enzymes studied, and maintained ≥90% of its maximum activity between 20 and 40 °C. In fact, at ambient temperature (20 °C), pXyl displays 8- to 82-fold higher molecular activity than the other enzymes studied. This was to be expected as pXyl is a cold-adapted enzyme reported to have an apparent optimal temperature for activity on solubilised birchwood xylan of ≈35 °C [27], whereas all the other xylanases studied are mesophilic enzymes adapted to higher temperatures with apparent maximums for activity at 50-60 °C (Fig. 1, Table 2).

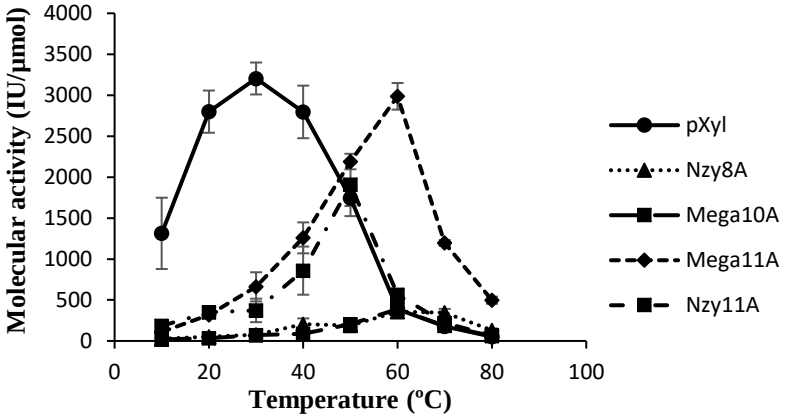


Fig 1. Molecular activity of the various xylanases studied on *P. palmata* xylan as a function of temperature. Activity is expressed as the molecular efficiency (µmol D-xylose reducing ends produced per minute per µmol of enzyme) determined with the DNS assay with a 5 minutes incubation period.

Importantly, the mesophilic family 11 enzyme Mega11A, isolated from *Neocallimastix patriciarum*, has previously been shown to be a highly active enzyme and was in fact designated as the most active xylanase studied [31]. In agreement with this, our study showed Mega11A to display much higher activity than the other mesophilic xylanases studied over the temperature range examined. However, in contrast, under the conditions used, the cold-adapted enzyme pXyl was found to have higher activity than Mega11A at low to moderate temperatures (10-50 °C) and indeed also at their respective maximum temperatures for activity (i.e., 30 °C and 60 °C, respectively) (Fig. 1, Table 2). This high activity observed for the cold-adapted glycoside hydrolase family 8 xylanase on the *Palmaria palmata* xylan at low to moderate temperatures suggests a high potential for its use in the eco-friendly production of xylo-oligosaccharides from this substrate. Indeed, in contrast to use of the mesophilic enzymes where heating is required, use of this enzyme should enable for efficient

ambient temperature processes, avoiding heating requirements and thereby potentially reducing the costs and environmental impact of the macroalgae hydrolysis process.

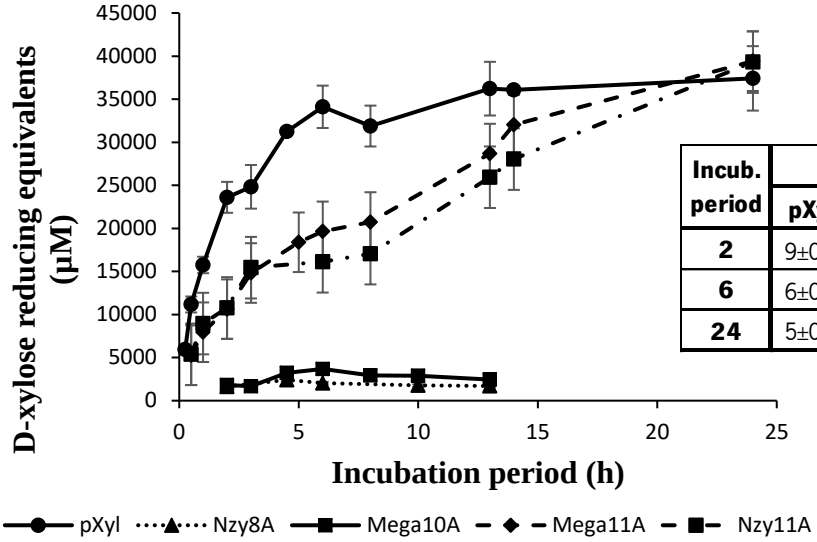
Table 2. Molecular activity at 20 °C (ambient temperature) and at the optimum temperature for activity for all enzymes studied. Activity is expressed as the molecular efficiency (µmol D-xylose reducing ends produced per minute per µmol of enzyme) determined with the DNS assay with a 5 minutes incubation period.

Enzyme	Molecular Activity (IU/µmol) at 20 °C	Molecular Activity (IU/µmol) at Optimum Temperature
pXyl	2799	3204 (30 °C)
Nzy8A	54	356 (60 °C)
Mega10A	34	388 (60 °C)
Mega11A	318	2986 (60 °C)
Nzy11A	349	1906 (50 °C)

3.1.2. Extent of Hydrolyses

In addition to the hydrolytic rate, the extent of substrate hydrolysis is also an important parameter for processes focused on substrate breakdown to high value products such as xylo-oligosaccharides. Analysis of this parameter is critical for this application as a high molecular activity, as determined in 3.1.1 above, does not necessarily equate to a more complete hydrolysis, this latter being determined by the substrate specificity and effects of specific structural features of the substrate on enzyme function, including side chain number and type and/or, as in the present case, the type(s) of backbone linkages. We compared the hydrolysis of the *Palmaria palmata* xylan-rich substrate over time for the five different xylanases of this study. From Fig. 2, it can be seen that pXyl and both family 11 enzymes studied enabled similarly high release of reducing sugar ends, but pXyl achieved maximum hydrolysis more rapidly. Substrate hydrolysis reached maximum within the first 6-8 hours for pXyl, but both Mega11A and Nzy11A required almost 24 hours for this under the conditions used. No significant increases in reducing equivalents was observed for any sample after 24 hours. Conversion of reducing end quantities to average degrees of polymerisation (avDP) indicates hydrolysis to xylo-oligosaccharides of avDP of 9 already at 1.5-2 hours incubation with pXyl, whereas 6-8 hours were required to achieve similar values with the family 11 enzymes (Fig. 2, inset). Further incubation until maximum hydrolysis led to all enzymes yielding final low molecular weight products with apparently similar avDP, estimated as ≈5. The other two enzyme studied, a mesophilic glycoside hydrolase family 8 and family 10 xylanase, displayed low substrate hydrolysis even after extended incubation, possibly indicating the

activity of these enzymes to be severely impeded by the β-1,3-linkages in the substrate backbone or/and the reaction conditions used, possibly the low temperatures and/or high ash content.



Incub. period	Substrate avDP		
	pXyl	Mega11A	Nzy11A
2	9±0.6	19±2.3	19±2.9
6	6±0.4	10±0.4	12±0.7
24	5±0.5	5±0.4	5±0.4

Fig. 2. Extent of hydrolyses of the *P. palmata* xylan by the various xylanases studied at 20 °C. 100 µg/L of each enzyme was used, samples were removed at various time points and the reducing equivalents quantified with the DNS assay. Inset table shows the comparison of the average degree of polymerisation (avDP) of the xylo-oligosaccharide mixes produced by the three most active enzymes of the study following various incubation times.

3.2. Xylo-oligosaccharide Characterisation

HPLC was employed to characterise the xylo-oligosaccharides produced by the different enzymes. Initially size exclusion chromatography with a PolySep™-SEC GFC-P linear column with a fractionation range of 1kDa to 10 MDa was utilised to examine the molecular weight distributions of the xylo-oligosaccharide products. Fig. 3 shows the results for the analysis of hydrolysis over time by the cold-adapted enzyme pXyl. Xylan hydrolysis to lower molecular weight products is already visible following 2 hours incubation with a significant decrease in the xylan peak area and a peak shift to longer retention times (at 14.7 minutes). Following prolonged incubation to 6 hours, this lower molecular weight peak is itself hydrolysed and a peak shift to even lower molecular weights is observed. In fact, two lower molecular weight peaks are clearly formed indicative of two different populations of xylo-oligosaccharide products.

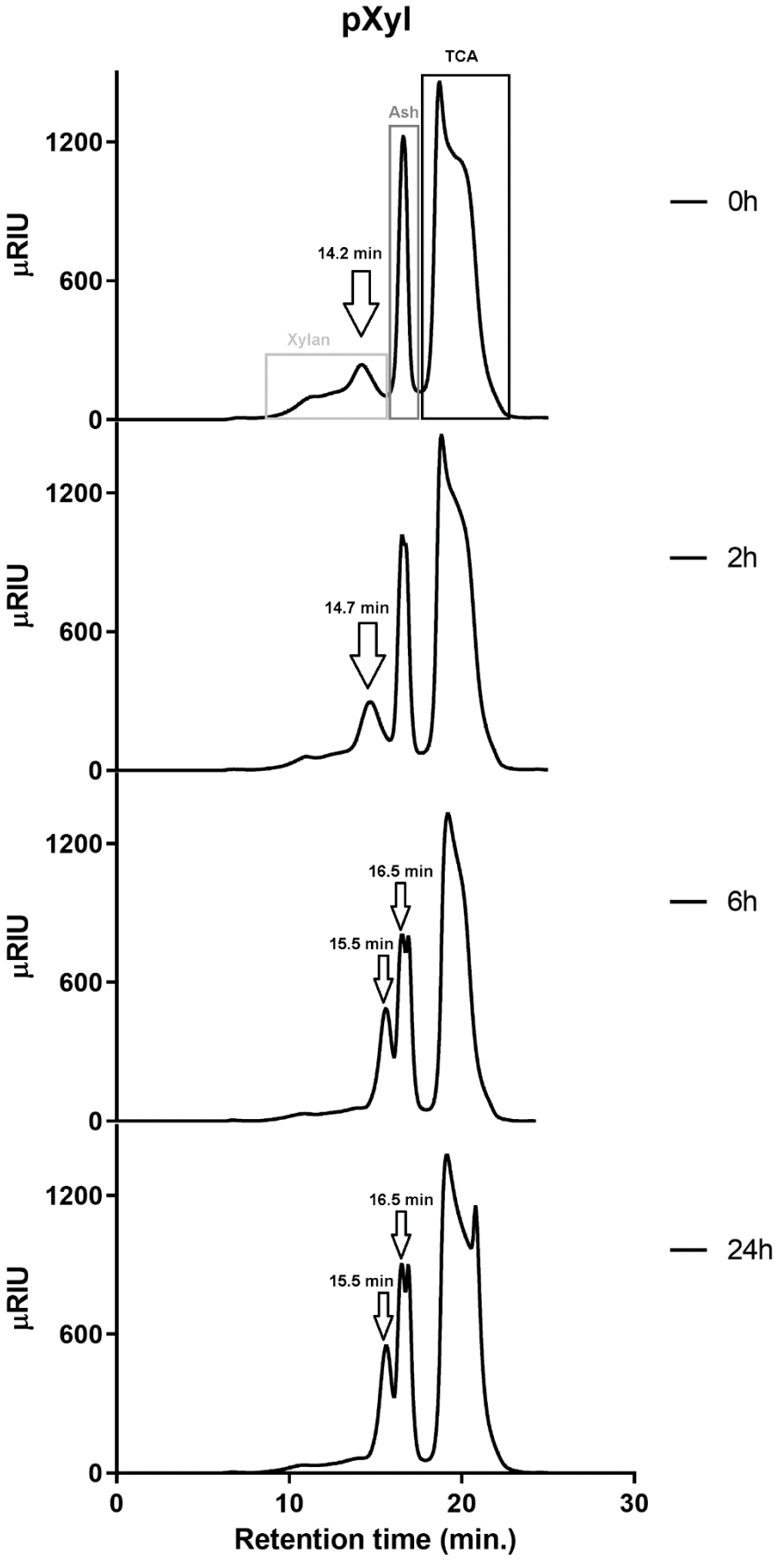


Fig. 3. Chromatograms for the PolySep™-SEC GFC-P linear HPLC analyses of the hydrolysis of the *Palmaria palmata* xylan by the cold-adpated glycoside hydrolase family 8 xylanase pXyl at various incubation times.

It can be suggested that the higher molecular weight peak at 15.5 minutes is constituted by mixed linkage β-1,4/β-1,3 xylo-oligosaccharides which could not be further hydrolysed by pXyl due to hinderance of activity by the presence of β-1,3-linkages. The lower molecular weight peak could be β-1,4-linked or mixed linkage β-1,4/1,3 xylo-oligosaccharides with a different proportion and/or distribution of β-1,3-linkages which permits hydrolysis to a lower degree of polymerisation (DP). 24 hour samples were also analysed, but no further changes were observed with this approach compared to 6 hours incubation, in agreement with Fig. 2 above, which showed maximum hydrolysis at this incubation time under the conditions used. Furthermore, only residual peak area is visible at the retention time of the xylan substrate, indicative of a high degree of hydrolysis.

Fig. 4 shows the chromatograms for the PolySep™-SEC GFC-P HPLC analysis of the 24 hour incubation products for the family 8 enzyme pXyl and the two mesophilic family 11 enzymes Mega11A and Nzy11A. Due to the wide fractionation range of the SEC column used, the xylo-oligosaccharide standards could not be resolved and hence a standard curve for estimation of the hydrolysis products could not be completed. From the chromatograms, it can be seen that highly similar products with two principal populations are observed for all three enzymes. Indeed, the only difference observed is a slight shift in the higher molecular weight peak (15.5 minutes) to lower molecular weights for both mesophilic family 11 enzymes as compared to the family 8 enzyme. This is indicative of a greater degree of hydrolysis and is in agreement with literature reports indicating glycoside hydrolase family 11 enzymes as being more active on shorter chain substrates and releasing smaller DP products than family 8 enzymes [32, 33].

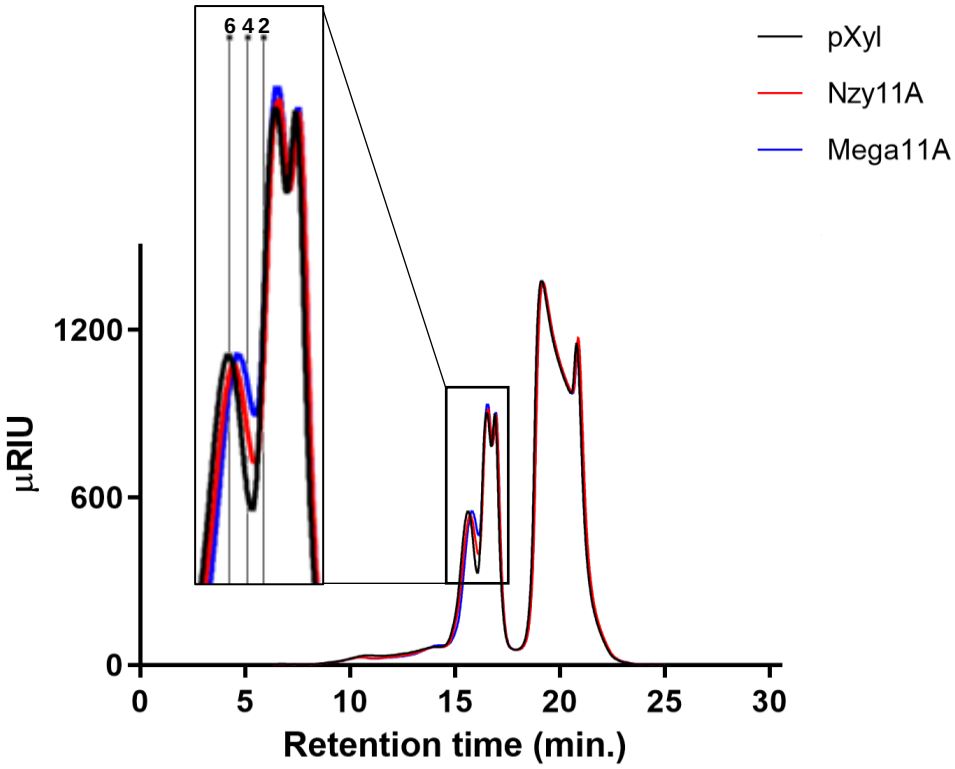


Fig 4. Comparison of the PolySep™-SEC GFC-P linear HPLC chromatograms for the 24 hour hydrolysis of the *Palmaria palmata* xylan by the family 8 enzyme pXyl and the two mesophilic family 11 enzymes Mega11A and Nzy11A. The solid vertical lines labelled 2, 4 and 6 refer to the retention times of xylobiose, xylotetrose and xylohexose standards, respectively.

For a more precise characterisation of the hydrolysis products, both a BioBasic™ SEC 60 HPLC column and a Rezex™ RSO-Oligosaccharide Ag^{+} (4)% HPLC column were investigated for use. The former, like the PolySep™-SEC GFC-P linear HPLC column used above, is also based on size exclusion chromatography separation but here a much narrower fractionation range (300 Da to 6 kDa) should enable for better oligosaccharide separation. The latter column, RSO-Oligosaccharide, is a specific oligosaccharide separation column based on ion-exclusion chromatography which is claimed to separate oligomers of DP 1 to 14. Fig. 5 shows the comparison of the results obtained with these chromatographic columns for analysis of the products of the 24 hour *Palmaria palmata* hydrolysis by pXyl and Mega11A.

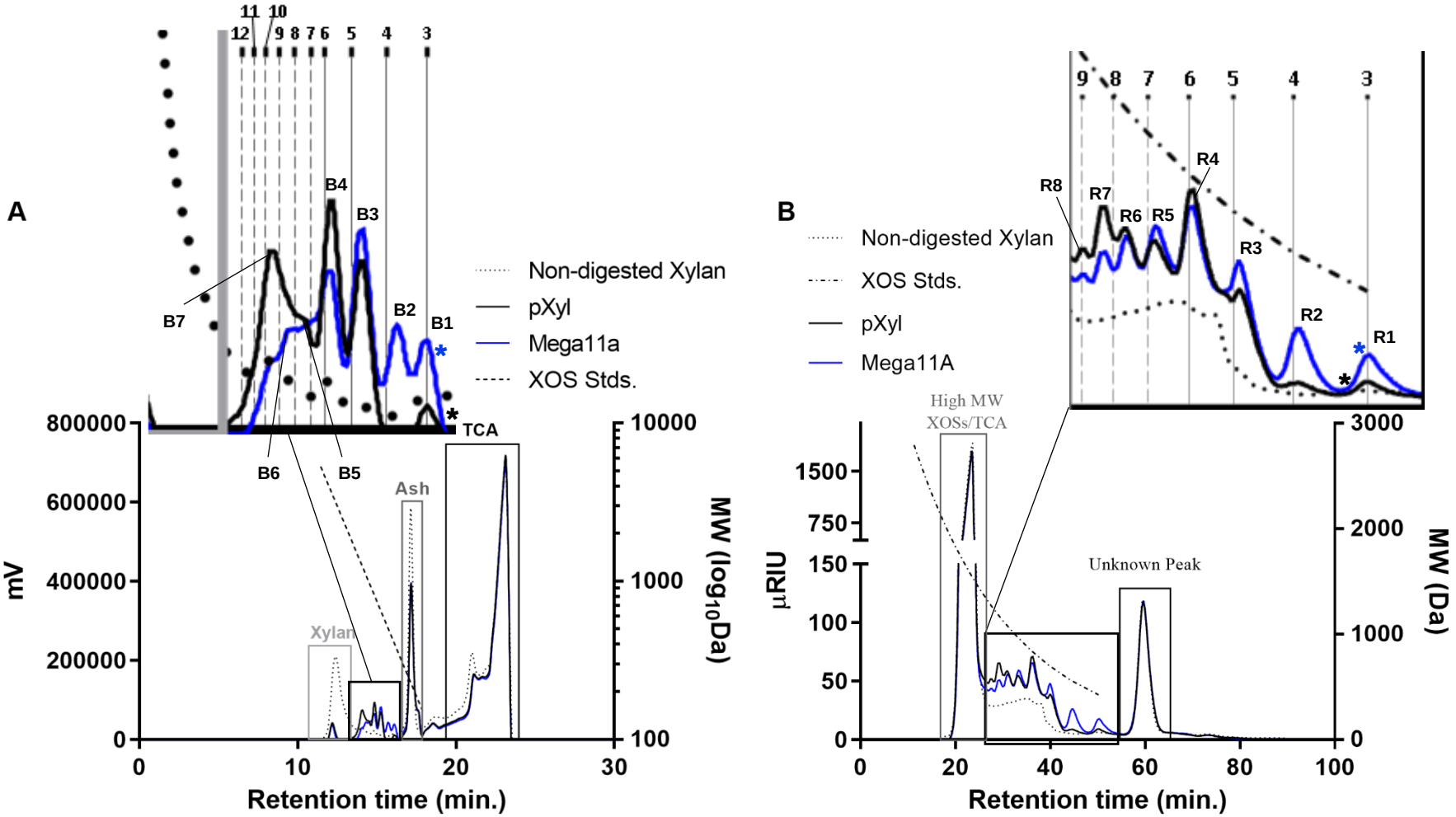


Fig. 5. Comparison of the chromatogram profiles for HPLC analysis with the BioBasic™ SEC 60 column (A) and the Rezex™ RSO-Oligosaccharide Ag+ (4)% column (B) of *Palmaria palmata* xylan 24 hour hydrolysis by the glycoside hydrolase family 8 xylanase pXyl (black lines) and the glycoside hydrolase family 11 xylanase Mega11A (blue lines). Vertical grey lines and numbers 3 to 12 indicate the retention times for linear β-1,4-linked xylo-oligosaccharides. Note that only xylo-oligosaccharide standards with degrees of polymerisation from 1 to 6 were analysed (solid vertical lines), all other retention times (dashed vertical lines) were estimated from an extrapolation of the standard curve and thus are potentially subject to error. *(star) indicates peaks with no-significant difference in retention time with the nearest marked β-1,4-linked xylo-oligosaccharides standard as determined by ANOVA analysis ($p \leq 0.05$).

A total of seven peaks for the SEC column (B1-B7) and eight peaks for the RSO-Oligosaccharide column (R1-R8) are clearly visible, but in addition, slight variations in retention times are seen for specific peaks produced by the different enzymes (i.e., B7, R5, R6), suggestive of potentially additional peaks in these cases. Furthermore, differences in peak resolution are visible between the two chromatographic systems used, with many peaks being residual, peak 'shoulders', or poorly separated peaks, and reflect the different separation basis of the HPLC columns used. Improved peak resolution is in fact evident for the RSO-Oligosaccharide HPLC analysis as compared to the SEC column, suggesting that a smaller molecular weight fractionation range should be employed for future SEC analysis.

Notwithstanding the differences and limitations observed for the two HPLC systems used, they do enable a clear distinction of the final product profiles produced for each enzyme. In the case of pXyl, the major products observed are the relatively higher molecular weight compounds of peaks B/R3, B/R4 and B/R7. In contrast, for Mega11A, a shift in the product profile towards lower DP products is visible wherein peaks B/R1, B/R2, B/R3 and B/R4 are the major end-point products observed. While accepting the limitations associated with extrapolating a standard curve for determination of the retention time of xylo-oligosaccharides with DP>6, it can be seen from Fig. 5 that only peak B1/R1 was found to correlate with the β-1,4-linked xylo-oligosaccharide standards, specifically xylotriose (X3). Peaks B3, B4 and B7, as major pXyl products, are estimated to have a DP equivalent (i.e., equivalent to β-1,4-linked xylo-oligosaccharides) of ≈4-5, ≈5-6 and ≈9-10, respectively. On the other hand, the major Mega11A products are estimated to have DP equivalents of 3, ≈3-4, ≈4-5, and ≈5-6, respectively. Due to the non-decorated mixed β-1,3/1,4-linked nature of the *Palmaria palmata* xylan substrate [6, 34], these peaks could be expected to be non-decorated mixed-linkage β-1,3/1,4 xylo-oligosaccharides of variable DP and/or with variable numbers and/or positioning of β-1,3-linkages which alters chromatographic performance as compared to β-1,4-linked standards. Future studies should aim to fully identify these compounds. The results shown here are in agreement with previous studies [27, 35, 36] where glycoside hydrolase family 8 enzymes are shown to be most active on high molecular weight, low substituted substrates, being strongly impeded by side-chain substitutions and β-1,3-linkages and typically releasing products with DP higher than xylopentose. On the other hand, glycoside hydrolase family 11 enzymes are generally characterised by the release of smaller products, most typically xylobiose and xylotriose even though they are reported to be also impeded by side-chain substitutions and β-1,3-linkages[32].

It must also be noted here that an additional unidentified peak was observed with Rezex™ RSO-Oligosaccharide Ag^{+} (4)% HPLC analysis. This unidentified compound was already observed in the xylan substrate and thus it not a hydrolysis product and previous studies showed it not to be xylose or xylobiose. In addition, total sugar, protein, and reducing sugar analyses, as well as absorption (200-700 nm) studies did not reveal the identity of the unknown peak. Further studies are required to identify this peak, in particular separation with a preparative HPLC column would be beneficial for obtention of greater quantities of material.

As a final step in the investigation of the hydrolysis of the *Palmaria palmata* xylan we used BioBasic™ SEC 60 HPLC analysis to monitor the changes in xylan concentration and xylo-oligosaccharides production with incubation time. From Fig. 6 it can be seen that, in agreement with the other studies discussed above, xylan is initially rapidly (first 2-6 hours) and then more slowly hydrolysed to leave a final residual non-digested xylan concentration of ≈7% of the original substrate quantity. This hydrolyses is accompanied by the initial rapid accumulation of intermediate DP products, specifically a high molecular weight xylo-oligosaccharide(s) (with a retention time close to but not corresponding to that of β-1,4-linked xylo-oligosaccharide standards of DP18-19) and B7 (with a retention time close to but not corresponding to that of β-1,4-linked xylo-oligosaccharide standards of DP9-10). These intermediate DP products are themselves then hydrolysed to give the accumulated lower molecular weight products, with the high MW xylo-oligosaccharide intermediate being completely hydrolysed and B7 being only slowly and incompletely hydrolysed.

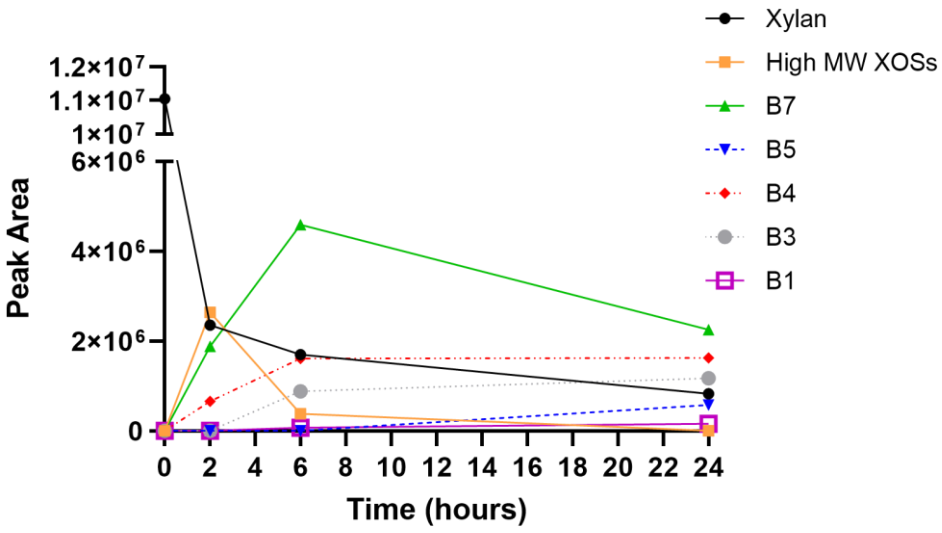


Fig. 6. Changes in xylan and xylo-oligosaccharides concentrations, as measured by peak area, over time for hydrolysis of *Palmaria palmata* xylan by the cold-adapted glycoside hydrolase family 8 xylanase pXyl. Peaks were identified as in Fig. 5A. The high MW XOSs corresponds to a peak observed at a retention time of 13 minutes with BioBasic™ SEC 60 HPLC analysis.

3.3.Xylo-oligosaccharides Production: Process Optimisation CCD

From the results thus far discussed, it can be seen that pXyl has an enormous potential in the efficient, ambient temperature, and thus more economically and environmentally friendly, production of xylo-oligosaccharides from *Palmaria palmata*. An important step in the development of any process for use is process optimisation, and identification of the effects of the different process variables on the product and its productivity. We used response surface methodology with a central composite design (RSM-CCD) to maximise xylo-oligosaccharide production from *Palmaria palmata* xylan. This multivariate statistical technique facilitates rational experimental design and statistical evaluation of results and is commonly used in process optimisation and for a better understanding of process parameters and their effects, especially when multiple variables are involved and when identification of interactions among process parameters is desired. In the experimental setup, six independent process variables were investigated (incubation temperature, incubation time, salt concentration, xylan and enzyme concentration and mixing speed) at five levels ($-\alpha$, -1, 0, $+1$ and $+\alpha$), with 5 repeats of the central points (0), for a total of 79 runs. Evaluation of the designed experiment indicated an appropriate degree of freedom for lack of fit (49) and pure error (4). The process variables and their units as well as the different level values investigated for each are shown in Table 3 below:

Table 3. Central composite design matrix. Variables investigated, units and values of - α, -1, 0, +1 and α used to design the RSM-CCD experiment.

Variable		Units	-α	-1 level	0	+1 level	+α
A	Enzyme concentration	µg/L	2	60	100	140	200
B	Incubation Time	h	0.5	3	5.25	7	10
C	Salt Concentration	g/L	0	14	25	36	50
D	Xylan Concentration	g/L	3	9	13	20	23
E	Mixing Speed	rpm	0	50	85	120	170
F	Incubation Temperature	°C	4	15	22	30	40

The output response measured was xylo-oligosaccharides production, which was calculated and compared for the various experimental runs by integration of the peak areas of all 8 peaks (R1-R8) identified by Rezex™ RSO-Oligosaccharide Ag^{+} (4)% HPLC analysis of products.

3.3.1. Model fitting

The 2 factor interaction model was found to be the most appropriate to describe the effects of the variables on each response. With a value of 0.70, the adjusted R^2 was not optimal as values of or above 0.75 are most typically desired [37]. Nevertheless, the model was characterised by a non-significant lack of fit, a low standard deviation and a model *p*-value of less than 0.0001 (Table 4) as compared to the other models investigated (i.e., linear, quadratic, cubic) and was thus considered adequate for the present study.

Table 4. Model summary statistics for each of the three responses investigated. Standard deviation, adjusted R^2, lack of fit values and model *p*-values are listed for the quadratic model.

Response	Std. Dev.	Adjusted R^2	Lack of fit (*p*-value)	Model *p*-value
XOSs peaks area	$2.3x10^6$	0.70	0.3	<0.0001

3.3.2. Effect of Process Variables on Total Xylo-oligosaccharides Production

The influence of all variables and their interactions on the production of xylo-oligosaccharides as described by the 2 factor interaction model is shown in Equation 2.

$$Y = 1.2\text{E}^7 + 2.9\text{E}^5A + 1.5\text{E}^6B + 4.7\text{E}^4C + 1.7\text{E}^6D + 2.3\text{E}^6E + 1.3\text{E}^6F - 8.6\text{E}^4AB - 3.4\text{E}^5AC - 1.8\text{E}^5AD - 5.4\text{E}^4AE - 8.1\text{E}^4AF - 2.6\text{E}^5BC + 2.0\text{E}^5BD - 6.7\text{E}^5BE - 1.8\text{E}^5BF + 1.0\text{E}^5CD + 2.5\text{E}^5CE + 4.0\text{E}^4CF + 6.2\text{E}^5DE + 4.7\text{E}^5DF - 9.2\text{E}^5EF \quad \textbf{(2)}$$

This equation reveals how the individual variables or double interactions affected the amount of xylo-oligosaccharides produced with the process. Negative coefficient values indicate that individual factors or double interactions negatively affect xylo-oligosaccharides production, whereas positive coefficient values mean that factors increase xylo-oligosaccharides production in the tested range. In the present study, it can be seen that all process variable, i.e., enzyme concentration (A), incubation time (B), salt concentration (C), xylan concentration (D), mixing speed (E) and incubation temperature (F), have a positive correlation with the production of xylo-oligosaccharides. Furthermore, ANOVA analysis (Table. 5) showed that all variables except enzyme concentration and salt concentration had a significant effect on xylo-oligosaccharides production.

Table 5. ANOVA analyses of the significance of each variable on the different responses studied (a confidence level of 95% was applied). p-values below 0.05 are considered significant.

Variable		*p*-value
		XOSs peaks area
A	Enzyme (µg/L)	0.2694
B	Time (h)	<0.0001
C	Salt (g/L)	0.8574
D	Xylan (g/L)	<0.0001
E	Mixing (rpm)	<0.0001
F	Temperature (°C)	<0.0001

The significantly positive effects observed within the ranges investigated in this study for the different variables were to be expected. Longer incubation times and higher xylan substrate concentrations would indeed be expected to lead to greater hydrolysis and higher product formation. Similarly, temperature would be expected to have positive effects on enzyme rate over the temperature range investigated (i.e., 4 to 40 °C) as pXyl has an apparent optimum temperature for activity at ≈30-35 °C [27]. Likewise, an increase in enzymatic rate would be expected with increased mixing rates as this enhances solution homogeneity and enzyme-substrate interactions. The absence of a significant effect of salt concentration within the range examined (i.e., 0-50 g/L) might also be expected as the host organism for this enzyme, *Pseudoalteromonas haloplanktis*, was isolated from a marine environment and has already been shown to be adapted to high salt concentrations [38]. While not having been investigated, it is plausible that pXyl is also adapted to its hosts environment and high salt concentrations. In contrast to the above, the lack of a significant effect for enzyme concentration was less expected and indicates that under the experimental conditions used, increases in enzyme concentrations over the range examined (2 - 200 µg/L) do not lead to a significant increases in product formation. It is tempting to suggest that, under the conditions used, enzyme concentration was saturating, that is to say that substrate concentration, either xylan or/and the xylo-oligosaccharide intermediates produced, was limiting over the range of enzyme concentrations examined. Therefore, lower concentrations of enzyme should be investigated to better characterise its effect on the process. Indeed, lower concentrations of enzyme and higher concentrations of salts as well as larger ranges for the other variables could potentially enable application of a more informative higher order model, e.g., a quadratic model, and enable improved process characterisation.

Three interactions were found to be significant and are shown in Fig. 7. All interactions displayed a positive correlation between variables with mixing being shown to strongly positively interact with incubation time and temperature at low values and with xylan concentrations over the range of values investigated. Mixing would enable a more rapid temperature equilibration, reduce the negative effects of low temperatures such as an increased solution viscosity, increase enzyme-substrate (xylan and xylo-oligosaccharide intermediates substrates) interactions, and enhance substrate dispersion and thereby lead to the observed interactions.

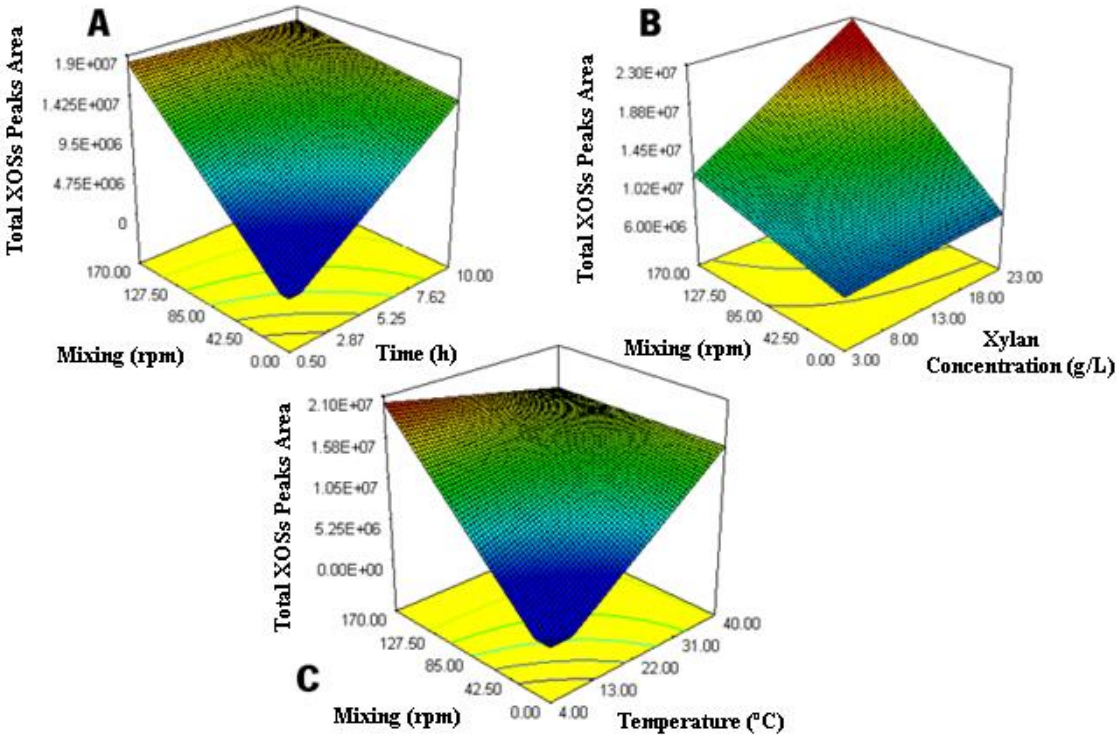


Fig. 7. 3D illustration of the significant interactions of mixing and incubation time (A), mixing and xylan concentration (B) and mixing and incubation temperature and their influence on xylo-oligosaccharides (XOSs) production. For all surface plots, salt and enzyme concentration were set to 25 g/L and 100 µg/L, respectively. For plot A, temperature and xylan concentration were set to 22 °C and 13 g/L, respectively. For plot B, temperature and time were set to 22 °C and 5.25 hours, respectively. For plot C, time and xylan concentration were set to 5.25 hours and 13 g/L, respectively.

3.3.3. Optimal Process Conditions

One of the principal objectives when using RSM is the determination of the optimum settings of the control variables that enable a maximum (or minimum) of a desired response within a certain region of interest. In our case, we explored the 2 factor interaction model developed, while acknowledging its limitations as discussed above, to optimise the xylo-oligosaccharides (XOSs) production process with focus on two distinct goals:

A- high XOSs production, via maximisation of total XOSs area and maintenance of all process variables within the model range.

B- high XOSs production at ambient temperature using the xylan-rich fraction directly as prepared by *Palmaria palmata* aqueous extraction, via maximisation of total XOSs area, targeting temperature to 20 °C and xylan concentration to 30 g/L, minimising enzyme concentration and maintenance of all remaining process variables within the model range

Table 6 shows the process conditions recommended for achievement of the two desired goals. Experimental repetition of the suggested processes indicated higher productivity with process A (27 g/L) than process B (23 g/L). On the other hand, process A has potentially higher environmental and economic costs related to a higher incubation temperature, which is nevertheless counterbalanced in process B by a longer incubation time. Both processes could be applied directly with the xylan-rich fraction prepared by aqueous extraction of the red macroalgae *Palmaria palmata* and avoid the need for additional processing steps for feedstock preparation. Importantly also, the process temperatures employed, i.e., 20 and 38 °C, are significantly lower than those required when using mesophilic enzyme based processes wherein temperatures of 50-60 °C are most typically applied.

Table 6. CCD suggested process variables and predicted values of the XOSs production for the different goals indicated by letters A and B, as well as, the desirability values of each goal. Desirability values refer to the proximity of the predicted response to the inserted desired conditions (scale 0-1). Predicted values are presented as the total predicted area of XOSs produced under the experimental conditions used. Temp.: temperature

Goal	Suggested Process Variables						Predicted Response	Desirability
	Temp. (ºC)	Incubation Time (h)	Salt Conc. (g/L)	Xylan Conc. (g/L)	Mixing speed (rpm)	Enzyme (µg/L)	Total Area of XOSs	
A: High XOSs production	38	8	50	30	170	2	$3.3x10^7$	0.94
B: High XOSs production using the xylan substrate	20	12	10	30	170	2	$2.8x10^7$	0.90

4. Conclusions

Almost all xylo-oligosaccharides studied to date have a β-1,4-linked backbone with/without side-chain substitutions and little is known of the properties, bioactivities or commercial potential of xylo-oligosaccharides with backbones constituted by linkages other than β-1,4-linkages. The red macroalgae *Palmaria palmata* is constituted by a non-substituted mixed linkage β-1,3/β-1,4 xylan [6, 34], the hydrolysis of which would give rise to novel mixed linkage β-1,3/β-1,4 xylo-oligosaccharides with potential for novel and/or improved properties and applications. In this study, we investigated, compared and optimised the utilisation of enzyme technology, specifically xylanases, for the eco-friendly hydrolysis of *Palmaria palmata* xylan to xylo-oligosaccharides.

Both cold-adapted (psychrophilic) and mesophilic enzymes from glycoside hydrolase families 8, 10 and 11 were examined for direct hydrolysis of an aqueous extracted *Palmaria palmata* xylan-rich fraction composed of ≈75% soluble xylan, ≈20% ash and low amount of other components. The cold-adapted family 8 enzyme pXyl and the mesophilic family 11 enzymes Mega11A and Nzy11A were found to display both a high molecular activity and high extent of hydrolysis on this substrate. Importantly, pXyl was found to display the highest activity of all the enzymes studied over a large temperature range, from 4 to ≈45 °C, as well as at the respective optimum temperatures for activity of the enzymes. Mega11A was previously claimed as being one of the most active xylanases known [31] but the higher activity shown by pXyl under the conditions used in this study highlights this enzyme as being among the most active of xylanases currently studied.

HPLC analyses of the xylo-oligosaccharide products from pXyl and Mega11A hydrolysis of *Palmaria palmata* xylan showed a clear difference in the product profiles of each. The family 11 enzyme Mega11A produced xylo-oligosaccharides with lower degrees of polymerisation (DP) than pXyl. Mega11A produced possibly β-1,4-linked xylotriose as the lowest molecular weight product, in addition to a number of other intermediate DP products, possibly mixed linkage β-1,3/β-1,4 xylo-oligosaccharides with estimated DP equivalents of approximately 3 to 6. pXyl produced minute quantities of β-1,4-linked xylotriose, but the major products observed were probably mixed linkage β-1,3/β-1,4 xylo-oligosaccharides with a DP equivalent ranging from 4 to 10. Such observations are in line with the substrate – product profiles generally reported for family 8 and 11 enzymes [27, 32, 33]. Future studies should aim to fully identify the various xylo-oligosaccharides produced with use of technologies such as gas chromatography-mass spectrometry (GC-MS), HPLC-mass

spectrometry (HPLC-MS), nuclear magnetic resonance (NMR) as well as xylo-oligosaccharide hydrolysis studies with specific β-1,3 and/or β-1,4 xylanases.

Due to the favourable characteristics observed for pXyl in the production of xylo-oligosaccharides from *Palmaria palmata* xylan, it was selected for further studies focused on characterisation and optimisation of the xylo-oligosaccharides production process with use of multivariate statistical techniques with experimental design (RSM-CCD). The process variables: incubation temperature, incubation time, xylan concentration and mixing speed were all shown to positively influence xylo-oligosaccharide productivity within the ranges examined, as might be expected. In contrast salt and enzyme concentration were not found to have significant effects over the concentration range examined. It is suggested that the former is related to the putative adaptation to high salt concentrations of the extracellularly produced pXyl isolated from a marine bacterium [38]. The lack of a significant effect of enzyme concentration on product formation is tentatively suggested as being related to substrate limitation under the conditions used, possibly limitation of the intermediate products observed with DP equivalents of ≈18-19 and 9-10.

Optimised processes enabling direct utilisation of the xylan-rich fraction produced by aqueous extraction of pre-treated macroalgae, in combination with reduced, ambient temperature xylanase hydrolysis to xylo-oligosaccharides were identified with up to ≈90% productivity. Such productivities are higher than those reported by previous groups, ranging from 10-80% yield with use in most cases of higher temperatures (≈40 to 70 °C) and/or chemical use [39-43] and hence with a larger environmental and possibly also economic impact. In addition many current process are characterised by xylose contamination which was not produced by the family 8 enzyme pXyl or family 11 enzyme Mega11A of the present study.

Due to its high activity at low to moderate temperatures, enabling for more economically and environmentally acceptable ambient temperature processes, its high extent of hydrolysis of *Palmaria palmata* xylan and its specific product profile for this substrate, pXyl is believed to have a high potential for the production of novel mixed linkage β-1,3/β-1,4 xylo-oligosaccharides. Furthermore, this enzyme is already produced and marketed at an economically viable industrial scale for use in a relatively low-value food industry related application, specifically, in baking. Such characteristics should enable for a more rapid acceptance for use in food and feed industry products and advocates its potential for an economically viable application in the production of novel xylo-oligosaccharides. Such novel products have potential for novel or improved properties

and applications and future studies should focus on investigating the potential of these in such applications as prebiotics, anti-cancer agents, anti-oxidants and anti-microbials.

5. References

[1] P. Moniz, A.L. Ho, L.C. Duarte, S. Kolida, R.A. Rastall, H. Pereira, F. Carvalheiro, Assessment of the bifidogenic effect of substituted xylo-oligosaccharides obtained from corn straw, Carbohydr Polym 136 (2016) 466-73.
[2] D.O. Otieno, B.K. Ahring, A thermochemical pretreatment process to produce xylooligosaccharides (XOS), arabinooligosaccharides (AOS) and mannooligosaccharides (MOS) from lignocellulosic biomasses, Bioresource Technology 112 (2012) 285-292.
[3] J. Delcour, C. Courtin, W. Broekaert, K. Swennen, K. Verbeke, P. Ruteers, Prebiotic preparation, WO 2006/002495 A1, 2006.
[4] Z.-W. Cai, H.-H. Ge, Z.-W. Yi, R.-Y. Zeng, G.-Y. Zhang, Characterization of a novel psychrophilic and halophilic β-1, 3-xylanase from deep-sea bacterium, *Flammeovirga pacifica* strain WPAGA1, International Journal of Biological Macromolecules 118 (2018) 2176-2184.
[5] L. Chen, F. Kong, An efficient and practical synthesis of β-(1→3)-linked xylooligosaccharides, Carbohydrate Research 337(21) (2002) 2335-2341.
[6] E. Deniaud, B. Quemener, J. Fleurence, M. Lahaye, Structural studies of the mix-linked β-(1→3)/β-(1→4)-d-xylans from the cell wall of *Palmaria palmata* (Rhodophyta), International Journal of Biological Macromolecules 33(1) (2003) 9-18.
[7] M. Kiyohara, Y. Hama, K. Yamaguchi, M. Ito, Structure of β-1,3-Xylooligosaccharides Generated from *Caulerpa racemosa* var. laete-virens β-1,3-Xylan by the Action of β-1,3-Xylanase, The Journal of Biochemistry 140(3) (2006) 369-373.
[8] C. Amorim, S.C. Silvério, K.L.J. Prather, L.R. Rodrigues, From lignocellulosic residues to market: Production and commercial potential of xylooligosaccharides, Biotechnology Advances 37(7) (2019) 107397.
[9] A.F.A. Carvalho, P.d.O. Neto, D.F. da Silva, G.M. Pastore, Xylo-oligosaccharides from lignocellulosic materials: Chemical structure, health benefits and production by chemical and enzymatic hydrolysis, Food Research International 51(1) (2013) 75-85.
[10] T. Collins, C. Gerday, G. Feller, Xylanases, xylanase families and extremophilic xylanases, FEMS Microbiology Reviews 29(1) (2005) 3-23.
[11] J. Bian, F. Peng, X.-P. Peng, P. Peng, F. Xu, R.-C. Sun, Structural features and antioxidant activity of xylooligosaccharides enzymatically produced from sugarcane bagasse, Bioresource Technology 127 (2013) 236-241.
[12] P. Christakopoulos, P. Katapodis, E. Kalogeris, D. Kekos, B.J. Macris, H. Stamatis, H. Skaltsa, Antimicrobial activity of acidic xylo-oligosaccharides produced by family 10 and 11 endoxylanases, Int J Biol Macromol 31(4-5) (2003) 171-5.
[13] B. Le, A.P.T. Ngoc, S.H. Yang, Synbiotic fermented soymilk with *Weissella cibaria* FB069 and xylooligosaccharides prevents proliferation in human colon cancer cells, J Appl Microbiol 128(5) (2020) 1486-1496.
[14] J. Zhang, Y.H. Wang, Q.Y. Wei, X.J. Du, Y.S. Qu, Investigating desorption during ethanol elution to improve the quality and antioxidant activity of xylo-oligosaccharides from corn stalk, Bioresource Technology 249 (2018) 342-347.
[15] D. Zhu, Q. Yan, J. Liu, X. Wu, Z. Jiang, Can functional oligosaccharides reduce the risk of diabetes mellitus?, Faseb j 33(11) (2019) 11655-11667.
[16] W.F. Broekaert, C.M. Courtin, K. Verbeke, T. Van de Wiele, W. Verstraete, J.A. Delcour, Prebiotic and other health-related effects of cereal-derived arabinoxylans, arabinoxylan-oligosaccharides, and xylooligosaccharides, Crit Rev Food Sci Nutr 51(2) (2011) 178-94.

[17] C.M. Courtin, K. Swennen, P. Verjans, J.A. Delcour, Heat and pH stability of prebiotic arabinoxylooligosaccharides, xylooligosaccharides and fructooligosaccharides, Food Chemistry 112(4) (2009) 831-837.
[18] D.S. Naidu, S.P. Hlangothi, M.J. John, Bio-based products from xylan: A review, Carbohydr Polym 179 (2018) 28-41.
[19] I. Jain, V. Kumar, T. Satyanarayana, Xylooligosaccharides: an economical prebiotic from agroresidues and their health benefits, Indian journal of experimental biology 53(3) (2015) 131-142.
[20] R.D. Singh, S. Talekar, J. Muir, A. Arora, Low degree of polymerization xylooligosaccharides production from almond shell using immobilized nano-biocatalyst, Enzyme and Microbial Technology 130 (2019) 109368.
[21] M.J. Vázquez, J.L. Alonso, H. Domínguez, J.C. Parajó, Xylooligosaccharides: manufacture and applications, Trends in Food Science & Technology 11(11) (2000) 387-393.
[22] B.Ş. Kayserilioğlu, U. Bakir, L. Yilmaz, N. Akkaş, Use of xylan, an agricultural by-product, in wheat gluten based biodegradable films: mechanical, solubility and water vapor transfer rate properties, Bioresource Technology 87(3) (2003) 239-246.
[23] M. Suutari, E. Leskinen, K. Fagerstedt, J. Kuparinen, P. Kuuppo, J. Blomster, Macroalgae in biofuel production, Phycological Research 63(1) (2015) 1-18.
[24] G. Paës, J.-G. Berrin, J. Beaugrand, GH11 xylanases: Structure/function/properties relationships and applications, Biotechnology Advances 30(3) (2012) 564-592.
[25] M. Barroca, G. Santos, B. Johansson, F. Gillotin, G. Feller, T. Collins, Deciphering the factors defining the pH-dependence of a commercial glycoside hydrolase family 8 enzyme, Enzyme and Microbial Technology 96 (2017) 163-169.
[26] J.H. Waterborg, H.R. Matthews, The lowry method for protein quantitation, Methods Mol Biol 1 (1984) 1-3.
[27] T. Collins, M.A. Meuwis, I. Stals, M. Claeyssens, G. Feller, C. Gerday, A novel family 8 xylanase, functional and physicochemical characterization, J Biol Chem 277(38) (2002) 35133-9.
[28] B.V. McCleary, P. McGeough, A Comparison of Polysaccharide Substrates and Reducing Sugar Methods for the Measurement of endo-1,4-β-Xylanase, Applied Biochemistry and Biotechnology 177(5) (2015) 1152-1163.
[29] F. Van Petegem, T. Collins, M.-A. Meuwis, C. Gerday, G. Feller, J. Beeumen, The structure of a cold-adapted family 8 xylanase at 1.3 A resolution. Structural adaptations to cold and investgation of the active site, The Journal of biological chemistry 278 (2003) 7531-9.
[30] T. Collins, A. Hoyoux, A. Dutron, J. Georis, B. Genot, T. Dauvrin, F. Arnaut, C. Gerday, G. Feller, Use of glycoside hydrolase family 8 xylanases in baking, Journal of Cereal Science 43(1) (2006) 79-84.
[31] M. Vardakou, C. Dumon, J.W. Murray, P. Christakopoulos, D.P. Weiner, N. Juge, R.J. Lewis, H.J. Gilbert, J.E. Flint, Understanding the structural basis for substrate and inhibitor recognition in eukaryotic GH11 xylanases, Journal of Molecular Biology 375(5) (2008) 1293-305.
[32] W. Qu, W. Shao, Cloning, expression and characterization of glycoside hydrolase family 11 endoxylanase from *Bacillus pumilus* ARA, Biotechnology Letters 33(7) (2011) 1407-1416.
[33] S. Nakamura, K. Wakabayashi, R. Nakai, R. Aono, K. Horikoshi, Purification and some properties of an alkaline xylanase from alkaliphilic *Bacillus* sp. strain 41M-1, Applied and environmental microbiology 59(7) (1993) 2311-2316.
[34] E. Deniaud, J. Fleurence, M. Lahaye, Interactions of the mix-linked β-(1,3)/β-(1,4)-D-xylans in the cell walls of *Palmaria palmata* (Rhodophyta), Journal of Phycology 39(1) (2003) 74-82.
[35] A. Pollet, J.A. Delcour, C.M. Courtin, Structural determinants of the substrate specificities of xylanases from different glycoside hydrolase families, Crit Rev Biotechnol 30(3) (2010) 176-91.

[36] S.H. Brawley, N.A. Blouin, E. Ficko-Blean, G.L. Wheeler, M. Lohr, H.V. Goodson, J.W. Jenkins, C.E. Blaby-Haas, K.E. Helliwell, C.X. Chan, T.N. Marriage, D. Bhattacharya, A.S. Klein, Y. Badis, J. Brodie, Y. Cao, J. Collén, S.M. Dittami, C.M.M. Gachon, B.R. Green, S.J. Karpowicz, J.W. Kim, U.J. Kudahl, S. Lin, G. Michel, M. Mittag, B.J.S.C. Olson, J.L. Pangilinan, Y. Peng, H. Qiu, S. Shu, J.T. Singer, A.G. Smith, B.N. Sprecher, V. Wagner, W. Wang, Z.-Y. Wang, J. Yan, C. Yarish, S. Zäuner-Riek, Y. Zhuang, Y. Zou, E.A. Lindquist, J. Grimwood, K.W. Barry, D.S. Rokhsar, J. Schmutz, J.W. Stiller, A.R. Grossman, S.E. Prochnik, Insights into the red algae and eukaryotic evolution from the genome of *Porphyra umbilicalis* (Bangiophyceae, Rhodophyta), Proceedings of the National Academy of Sciences 114(31) (2017) E6361-E6370.
[37] R.U. Owolabi, M.A. Usman, A.J. Kehinde, Modelling and optimization of process variables for the solution polymerization of styrene using response surface methodology, Journal of King Saud University - Engineering Sciences 30(1) (2018) 22-30.
[38] C. Médigue, E. Krin, G. Pascal, V. Barbe, A. Bernsel, P.N. Bertin, F. Cheung, S. Cruveiller, S. D'Amico, A. Duilio, G. Fang, G. Feller, C. Ho, S. Mangenot, G. Marino, J. Nilsson, E. Parrilli, E.P.C. Rocha, Z. Rouy, A. Sekowska, M.L. Tutino, D. Vallenet, G. von Heijne, A. Danchin, Coping with cold: the genome of the versatile marine Antarctica bacterium *Pseudoalteromonas haloplanktis* TAC125, Genome Research 15(10) (2005) 1325-1335.
[39] O. Akpinar, O. Ak, A. Kavas, U. Bakir, L. Yilmaz, Enzymatic production of xylooligosaccharides from cotton stalks, J Agric Food Chem 55(14) (2007) 5544-51.
[40] C. Amorim, S.C. Silvério, R.F.S. Gonçalves, A.C. Pinheiro, S. Silva, E. Coelho, M.A. Coimbra, K.L.J. Prather, L.R. Rodrigues, Downscale fermentation for xylooligosaccharides production by recombinant *Bacillus subtilis* 3610, Carbohydr Polym 205 (2019) 176-183.
[41] A. Purohit, S.K. Rai, M. Chownk, R.S. Sangwan, S.K. Yadav, Xylanase from *Acinetobacter pittii* MASK 25 and developed magnetic cross-linked xylanase aggregate produce predominantly xylopentose and xylohexose from agro biomass, Bioresource Technology 244 (2017) 793-799.
[42] X. Liu, Y. Liu, Z. Jiang, H. Liu, S. Yang, Q. Yan, Biochemical characterization of a novel xylanase from *Paenibacillus barengoltzii* and its application in xylooligosaccharides production from corncobs, Food Chemistry 264 (2018) 310-318.
[43] M.-z. Sun, H.-c. Zheng, L.-c. Meng, J.-s. Sun, H. Song, Y.-j. Bao, H.-s. Pei, Z. Yan, X.-q. Zhang, J.-s. Zhang, Y.-h. Liu, F.-p. Lu, Direct cloning, expression of a thermostable xylanase gene from the metagenomic DNA of cow dung compost and enzymatic production of xylooligosaccharides from corncob, Biotechnology Letters 37(9) (2015) 1877-1886.

Chapter II.III

Deciphering the factors defining the pH-dependence of a commercial glycoside hydrolase family 8 enzyme

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Deciphering the factors defining the pH-dependence of a commercial glycoside hydrolase family 8 enzyme

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ABSTRACT

A prerequisite to the use of any enzyme in any industrial process is an understanding of its activity and stability under process conditions. Glycoside hydrolase family 8 enzymes include many important biotechnological biocatalysts yet little is known of the performance of these with respect to pH. A better understanding of this parameter and its relationship to structure and function in these enzymes will allow for an improved use of these in industry as well as an enhanced ability in their engineering and optimisation for a particular application. An in-depth analysis of the pH induced changes in activity, irreversible inactivation, conformation, stability and solubility of a commercial glycoside hydrolase family 8 xylanase was carried out with the aim of identifying the factors determining the pH dependence of this enzyme. Our study showed that different phenomena play different roles at the various pHs examined. Both reversible and irreversible processes are involved at acidic pHs, with the irreversible processes dominating and being due to protein aggregation and precipitation. At basic pHs, loss of activity is principally due to reversible processes, possibly deprotonation of an essential catalytic residue, but at higher pHs, near the pI of the protein, precipitation again dominates while structure unfolding was discerned at the higher pHs investigated. Such insights demonstrate the complexity of factors involved in the pH dependence of proteins and advances our knowledge on design principles and concepts for engineering proteins. Our results highlight the major role of protein precipitation in activity and stability losses at both low and high pHs but it is proposed that different strategies be used in tailoring the enzyme to overcome this in each case. Indeed the detailed understanding obtained here will allow for a more focused, informed and hence successful tailoring of glycoside hydrolase family 8 proteins for a specific pH and process application.

1. Introduction

One of the principal challenges to the successful integration of an industrial biocatalyst into a commercial process is related to its fitness for the specific application. The enzyme needs to have the desired substrate specificity and selectivity and be highly active and stable under the process conditions. Frequently however, many naturally occurring enzymes do not fulfil all of these requirements, in particular as many industrial processes are carried out under harsh conditions such as extremes of temperature, pH, pressure, salinity and/or in the presence of organic solvents, detergents etc. [1,2]. Xylanases (*endo*-1,4-β-D-xylanase, E.C. 3.2.1.8) are important industrial biocatalysts regularly used in industrial processes in which extreme conditions prevail. They have found application in the technical, food and feed sectors of the enzymes market and are employed under such diverse extreme conditions as high temperatures (pulp and paper, bioconversion), low temperatures (food and beverages), acidic pHs (bioconversion, sulphite pulping, beverages, animal feed), alkaline pHs (bioconversion, kraft pulping, detergents) and/or even in the presence of detergents [3–9]. These enzymes randomly cleave the internal β-1,4-D-xylosidic linkages of the complex plant heteropolysaccharide xylan and are classified into glycoside hydrolase (GH) families 5, 7, 8, 10, 11 and 43 based on

Abbreviations: GH8, glycoside hydrolase family 8; pXyl, psychrophilic glycoside hydrolase family 8 xylanase (UniProtKB entry Q8RJN8); MOPS, 3-(*N*-morpholino)propanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; TAPS, *N*-[Tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid; CHES, *N*-cyclohexyl-2-aminoethanesulfonic acid; CAPS, *N*-cyclohexyl-3-aminopropanesulfonic acid.

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amino acid sequence similarity. Interestingly, while family 10 and 11 members have been extensively studied, much less is known about the other families' members [5,9,10].

We have previously isolated a glycoside hydrolase family 8 (GH8) xylanase, designated pXyl, from the Antarctic bacterium *Pseudoalteromonas haloplanktis* TAH3a [11] and extensively studied this at the physicochemical, functional and structural levels [12–15]. This was shown to be characterised by a specificity for xylan, being most active on long chain xylo-oligomers [11,16], and an insensitivity to xylanase inhibitors [17], as well as typical cold-adapted characteristics of a high activity at low to moderate temperatures and a reduced stability [13,18]. Importantly also, this enzyme has been successfully developed for use as a technological aid in baking where it allows for improved bread quality [19–21] and is currently being commercialised worldwide for use in this application. Indeed, the unique properties of this enzyme point to its suitability for exploitation in various other application areas, in particular in the beverages industry and in animal feeds but also, potentially, in biofuel production. In many of these applications extremes of pH are encountered yet, interestingly, while much is already known of this important industrial enzyme, little is known of its relationship to pH and of the pH dependence of its activity and stability. In fact, GH8 also contains many other industrially important enzymes such as cellulases, licheninases and chitosanases yet it appears that, in contrast to family 10 and 11 enzymes where a large number of studies have been carried out [5,9,10,22–26], pH adaptation in GH8 enzymes has not been studied. Furthermore, in contrast to adaptation of enzymes to temperature, and in particular high temperatures, much less is currently known of adaptation to pH, with variable and sometimes conflicting observations on adaptation strategies being reported [5,9,10,22–27].

Here we addressed these limitations by employing a variety of biochemical and biophysical techniques for an in-depth investigation of the pH dependence of the cold adapted GH8 xylanase pXyl. The detailed knowledge available for this enzyme; of its structure, function, activity and mechanism of action, as well as its commercial value, make it an attractive model for the study. The work described here enables a better understanding of adaptation to pH in this enzyme and in glycoside hydrolase family 8 enzymes in general, but also advances our knowledge on the factors defining the pH-dependent characteristics of enzymes, with important implications for protein science and protein engineering [28].

2. Material and methods

2.1. Xylanase production and purification

The pET22b(+)-*E. coli* BL21(DE3) expression system (Novagen) was used for overexpression of pXyl by batch production at 18 °C using previously described conditions [11] but with optimisation of the induction time and induction period. For optimisation, xylanase production levels following induction with 1 mM IPTG at various growth stages (mid-log, late-log and early-stationary phases) and various induction periods (0–24 h) were compared. Intracellular xylanase production levels were measured by activity measurements with a modified Bernfeld reducing sugar assay [29] with 3% soluble birchwood xylan and 0.1 M McIlvaine's buffer pH6.5, as described previously [11].

The xylanase was purified by a combination of anion exchange, cation exchange and gel filtration chromatographies (GE Healthcare Life Sciences) as previously described [11]. Purified enzyme was dialysed in storage buffer (pH 7.5, 20 mM MOPS + 100 mM NaCl) and stored at 4 °C until use. SDS-PAGE was employed for confirmation of protein purity.

2.2. Buffer mix

Throughout the study the following buffer mix covering the pH range from 3 to 12 was used: 100 mM Citrate (Merck), 20 mM MES (Sigma), 20 mM MOPS (Sigma), 20 mM TAPS (Sigma), 20 mM CHES (Sigma) and 20 mM CAPS (Sigma). The pH was adjusted with HCl or NaOH and the final pH of all solutions was measured after enzyme addition. Unless otherwise stated, all experiments were performed in triplicate.

2.3. pH dependence of activity

Activity of the purified protein as a function of pH was determined with the buffer mix described in 2.2 using a modified Bernfeld reducing sugar assay [29] with 3% soluble birchwood xylan as described previously [11] and assay times of 5, 30 and 60 min.

2.4. Irreversible inactivation

400 μg/mL purified xylanase was diluted 8-fold in the buffer mix at pHs from 3 to 12 and incubated at 25 °C. Samples were taken at 5 min and 24 h, diluted 8-fold in storage buffer (pH 7.5, 20 mM MOPS + 100 mM NaCl) and incubated for one hour at room temperature to allow for reversible refolding/reactivation. Residual activity was measured with a 5 min assay under optimal conditions (pH 6.5 in 0.1 M McIlvaine's buffer) with the Bernfeld reducing sugar assay as described in 2.3 [11,29].

2.5. Tertiary structure analysis: fluorescence spectroscopy

400 μg/mL purified xylanase was diluted 8-fold in the buffer mix at pHs from 3 to 12 and incubated at 25 °C. Samples were taken at 5 min and 24 h and fluorescence emission spectra recorded from 300–400 nm following excitation at 295 nm on a PerkinElmer LS50 fluorescence spectrometer with a scan rate of 100 nm/min and data interval of 0.5 nm. As controls, the fluorescence emission spectra of the xylanase following high temperature and guanidine hydrochloride treatment at pH 7.5 were also measured. The former was prepared by incubating the enzyme diluted in pH 7.5 buffer mix at 80 °C for 20 min before fluorescence spectroscopy analysis as described above. For the latter 6 M guanidine hydrochloride was included in the pH 7.5 buffer mix used for dilution.

2.6. Structural stability: thermal denaturation

400 μg/mL purified xylanase was diluted 8-fold in the buffer mix at pHs from 3 to 12 and incubated at 25 °C for 24 h. The fluorescence intensity at 343 nm with excitation at 280 nm was then measured during a temperature ramp from 25 °C to 70 °C at 1.5 °C/min. A PerkinElmer LS50 Fluorescence Spectrometer connected to a LKB BROMMA 2219 MultiTemp II thermostatic circulator was used. The temperature of the enzyme solution was measured throughout the fluorescence study and data were treated for determination of the denaturation temperature as described by Pace [30].

2.7. Protein solubility

200 μg/mL purified xylanase was incubated in the buffer mix at pHs from 3 to 12 for 24 h at 25 °C, centrifuged at 25000 × *g* for 30 min at 4 °C and supernatants analysed for soluble protein content by absorbance measurements at 280 nm on a UV–vis 1700 spectrophotometer (Shimadzu). To measure the effect of protein concentration on solubility, various concentrations of purified xylanase, from 0.07 to 3.6 mg/mL, in 20 mM MOPs buffer with 100 mM NaCl, pH 7.5 were prepared with an Amicon Ultra-15 Centrifugal Filter Unit, 10 KDa cut-off (Merck Millipore) and incubated

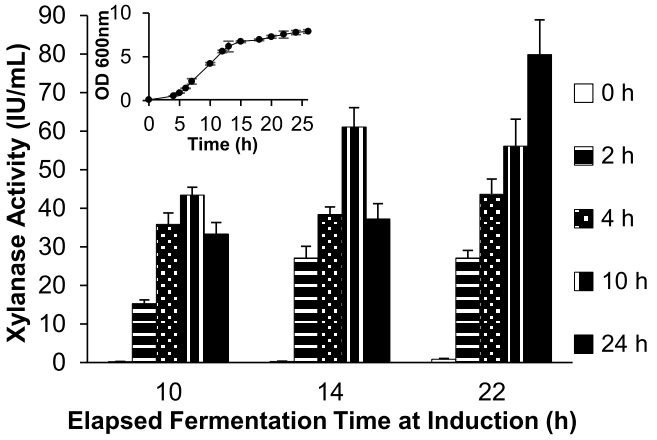

Fig. 1. Effect of elapsed fermentation time at induction and post-induction period on xylanase production. Comparison of xylanase production levels, expressed as international units (IU) of xylanase activity per mL of cell culture, as a function of the elapsed fermentation time at induction (10, 14 and 22 h incubation) and post-induction period (0–24 h). The inset graph shows the growth curve of uninduced *E.coli* BL21(DE3) for assessment of the elapsed fermentation time and stage of growth.

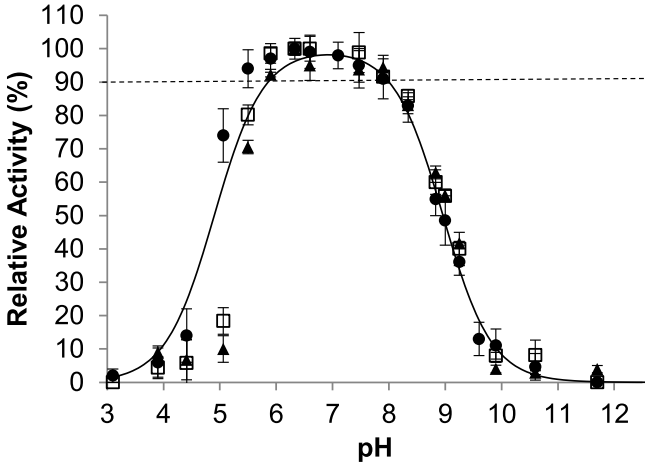

Fig. 2. pH activity profile for the glycoside hydrolase family 8 xylanase. Relative activities for 5 (filled circles), 30 (empty squares) and 60 (filled triangles) minute activity assays are shown. The dashed line indicates the 90% relative activity level. The solid line represents the fitting of the Henderson-Hasselbalch equation to the 5 min data.

at 25 °C. Samples were taken at 0 h and following 24, 72 and 168 h incubation and analysed for soluble protein content as described above.

3. Results and discussion

3.1. Xylanase production

The protein was produced and purified essentially as described previously [11] but with optimisation of the induction time and period. From Fig. 1 it can be seen that, under the conditions used, optimum pXyl production was obtained by induction with IPTG during the early stationary phase (following approximately 22 h incubation at 18 °C) for 24 h. This is in agreement with a recent comprehensive study of batch production approaches with the *E.coli*-pET expression system where it was shown that, in contrast to what is generally believed, optimal recombinant protein production is obtained by induction during the stationary phase [31]. Indeed, protein production is most commonly induced during the exponential growth phase when cells are most actively dividing [32] but our previous studies showed that induction during the stationary phase, where a higher cell density and possibly also a reduced stress on the cells is observed, enables for increased recombinant protein production [31,33]. In accordance with this, and further illustrating the advantages of the approach, in the present study we observed an almost doubling in xylanase production for induction during the stationary phase as compared to induction during the exponential phase. Finally, due to the cold-adapted characteristics and heat instability of the enzyme, an incubation temperature of 18 °C was used, with a resultant reduced growth rate for the *E. coli* host and hence, consequently, an extended induction period of approximately 24 h being required.

3.2. pH dependence of activity

The activity profile for the purified enzyme as a function of pH with 5, 30 and 60 min assays is shown in Fig. 2. Here, activity values greater than or equal to 90% are taken as reflecting a fully active state and it can be seen that under the conditions used optimum activity for pXyl is observed between pHs 5.5 and 8 for the 5 min assay and pHs 6 and 8.0 for the 60 min assay. Outside of this range a rapid loss of activity occurs.

GH8 enzymes catalyse hydrolysis with inversion of the anomeric configuration of the substrate with two catalytic residues, namely the proton donor and proton acceptor, playing essential roles. These two residues have been identified in pXyl as glutamic acid 78 (E78) and aspartic acid 281 (D281), respectively [12]. If one assumes that the pH-dependent activity of the enzyme is defined by the protonation/deprotonation of these two or indeed any two essential residues then the Henderson-Hasselbalch equation can be rearranged to describe this as follows:

$$Relative\ Activity\,(\%) = \left(\frac{1}{1 + 10^{(pKa1 - pH)} + 10^{(pH - pKa2)}}\right) * 100 \qquad (1)$$

Fitting this equation to the 5 min data (filled circles) in Fig. 2 indicates good agreement of the bell shaped curve (black line) with the measured data at neutral and basic pHs while discrepancies are seen for the acidic limb. Such discrepancies indicate that the loss of activity observed at acidic pHs is due to something other than, or in addition to, protonation of an essential residue. Furthermore, it can be seen that use of longer assay periods (30–60 min) resulted in even steeper losses at acidic pHs, at pH 5.5 and below, while the relative activity for the basic limb was little changed. Indeed the pK_a of the residue involved in activity loss at basic pHs can be calculated from Eq. (1) but the poor agreement of data at the acidic pHs invalidates use of the acid pH data. A glutamic acid (E78), as the proton donor, is suggested as being the critical residue governing the basic pH limb in pXyl yet a pK_a of 9 was calculated from equation 1 even though the side chain carboxylic group of glutamic acid in solution has a reported pK_a of ~4.3. Nevertheless pK_a values can vary considerably depending on the environment [25] and in fact high pK_a values for acidic side chains are frequently reported, in particular for the catalytic residues of enzymes e.g. the acid/base residue in Xln A from *Streptomyces lividans* has a pK_a of 9.4 [34] while that of a glycoside hydrolase family 1 β-glucosidase has a pK_a of 8.1 [35]. Structural analysis of pXyl (pdb: 1H13, 1H14) indicates that E78 is located in the active site pocket near numerous hydrophobic (W82, Y380, Y381, W124, Y203) and acidic (D144, D281, D200) residues which could potentially stabilise the protonated form of the side chain and thereby increase the pK_a and allow for the high value observed. Thus taken together, these observations indicate that the loss of activity observed at high pHs may indeed be related to the reversible deprotonation of an essential residue, possibly the proton donor (E78). On the other hand, more complex and time

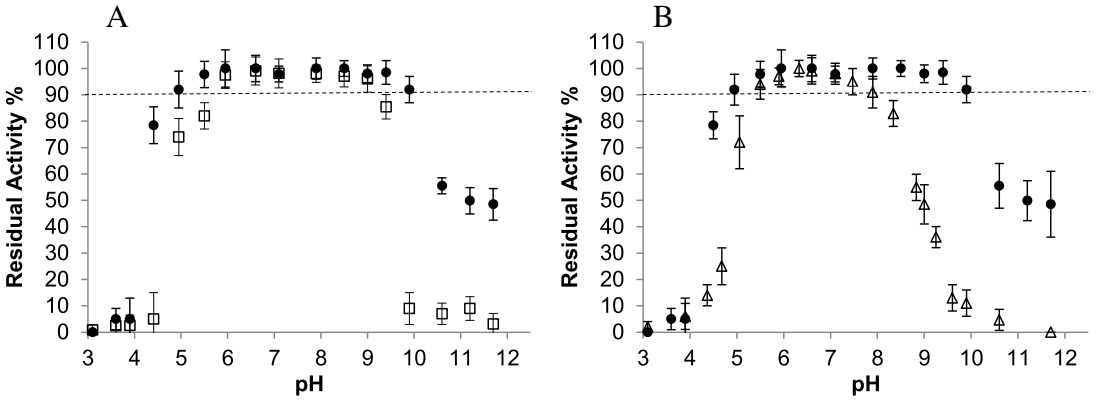

Fig. 3. Irreversible inactivation of the xylanase as a function of pH (A) and comparison of irreversible inactivation with activity (B). Residual activities following 5 min (filled circles) and 24 h (empty squares) incubation at various pHs are shown in (A). The five minute data for irreversible inactivation (filled circles) and activity (empty triangles) as a function of pH are compared in (B). The dashed line indicates the 90% level.

dependent factors, other than simply protonation of an essential residue, play a role in the loss of activity at acidic pHs.

3.3. Irreversible inactivation

Investigation of irreversible inactivation via measurement at optimum pH of the activity remaining after incubation at various pHs gives a measure of the stability of the activity of the enzyme. In this study two incubation periods were used; 5 min, for comparison with the 5 min activity assay, and 24 h, for determination of longer term effects on the enzyme. From Fig. 3A it can be seen that maximum (≥90 %) retention of activity occurs between pHs 5 and 10 following 5 min incubation and that this range is reduced to pHs 6–9 following 24 h. Thus at pHs below 6 and above 9 time dependent irreversible processes which negatively affect enzyme activity occur. Indeed these irreversible processes become dominant at the extremes of pH; at the low pH extremes complete irreversible inactivation already occurs after only 5 min incubation while at pH 10 and above irreversible inactivation dominates for the longer incubation period examined (24 h).

From Fig. 3B, where the results of the 5 min activity and inactivation studies are compared, it can be seen that the activity losses observed between pHs 5 and 10 are solely due to reversible processes as no significant irreversible inactivation was detected. Thus, as previously discussed, the activity loss observed for the basic pH limb, between pHs 8 and 10 with the 5 min assay, are solely due to reversible processes. On the other hand, above pH 10 and for the acidic pH limb at ~pH 4.5, it can be seen that the activity loss measured is greater than that due to irreversible inactivation alone and hence indicates that the observed loss of activity at these pHs is due to both reversible and irreversible processes.

As discussed earlier, the reversible processes at basic pHs likely involve deprotonation of the proton donor (E78) whereas those observed here at acidic pHs could involve protonation of the proton acceptor (D281) or, as previously described for a homologous family 8 chitosanase (ChoK), an irreversible repositioning of the proton donor at an inactive position at acidic pHs [36]. Interestingly, a similar position for the proton donor, near the nucleophilic water molecule, was reported for the wild-type pXyl crystal structure at pH 5 [15]. An induced fit mechanism wherein this proton donor is repositioned on substrate binding was suggested [14] but we propose that the E78 position observed was resultant of the low pH used and hence a crystal structure of wild type pXyl at neutral pHs (~pH 7) is called for. In contrast to the reversible processes, the factors involved in the irreversible processes are as yet unclear and hence, in an attempt to identify these, our studies were focused on characterising the effects of pH on the xylanase structure, stability and solubility.

3.4. Tertiary structure analysis: fluorescence spectroscopy

Fluorescence spectroscopy was used here to probe perturbations induced by pH in the folded state of the protein. The cold adapted xylanase contains 10 tryptophans, 30 tyrosines and 23

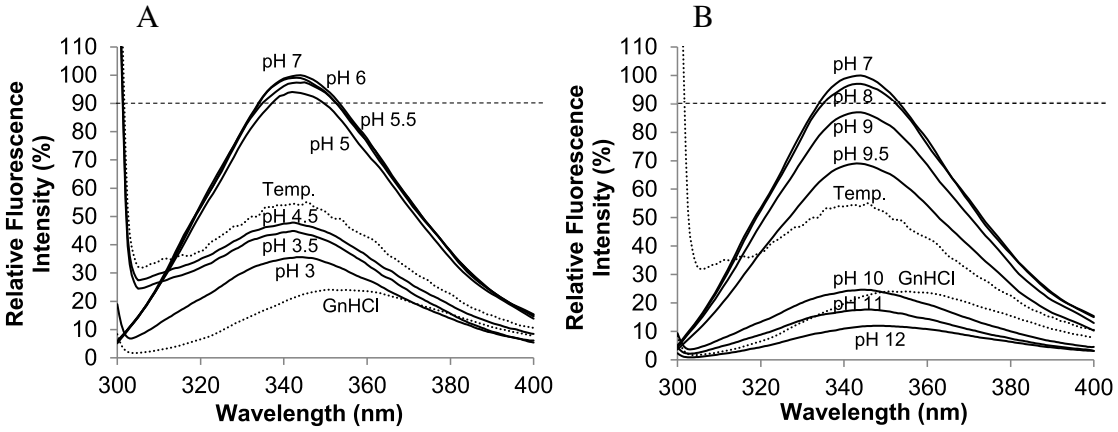

Fig. 4. Fluorescence emission spectra for the xylanase after 24 h incubation at various pHs. The results for incubation at low pHs (A) and high pHs (B) are shown (solid lines) as well as emission spectra for guanidine hydrochloride (GnHCl, dotted line) and temperature (Temp., dotted line) denatured protein. The horizontal dashed line indicates the 90% level.

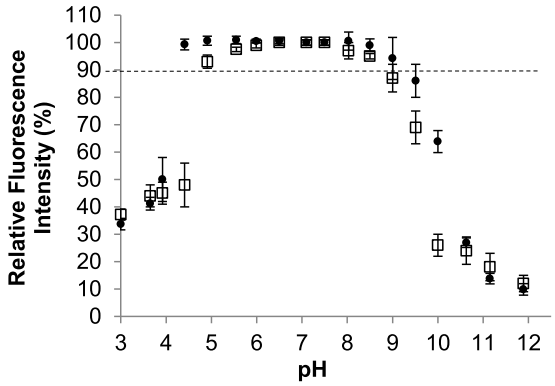

Fig. 5. Relative fluorescence intensity of the xylanase at 343 nm following 5 min (filled circle) and 24 h (empty square) incubation at various pHs. The horizontal dashed line indicates the 90% level.

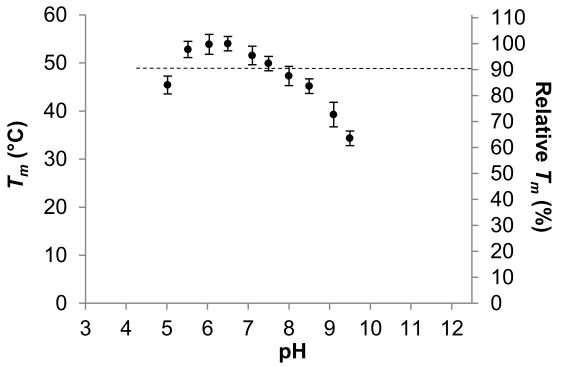

Fig. 6. Apparent denaturation temperature of the xylanase following 24 h incubation at various pHs. The measured apparent melting temperatures (T_m, left axis) and the values expressed as a percentage of the maximum at pH 6.5 (relative T_m, right axis) are shown. No transitions were observed following incubation at pHs ≤pH 4.5 or ≥pH 10. The horizontal dashed line indicates the 90% level.

phenylalanines but as tyrosine fluorescence can be affected by pH [37] and that of phenylalanine is relatively weak, focus was directed to the tryptophans via excitation at 295 nm. Structural analysis indicates that the 10 tryptophans of pXyl are distributed throughout the structure and analysis via the GETAREA server [38] indicates that 2 tryptophans (residues W124 and W249) are strongly exposed (>30% surface exposition), 5 are weakly exposed (5–30% exposition) and the remaining 3 are buried (<5% exposition).

From Figs. 4 and 5 it can be seen that no significant structural modifications were detected with this technique between pHs 4.5 and 9.5 after 5 min and pHs 5 and 9 after 24 h incubation. At pHs 4 (5 min incubation) and 4.5 (24 h incubation) an abrupt decrease in the fluorescence intensity to an acid denatured state with a residual relative fluorescence intensity of ∼50% occurs. In contrast, at the basic pH limb, a more gradual decrease in fluorescence intensity is initially observed at pHs 9 and 9.5 before a strong decrease at pH 10.5 (5 min incubation) or pH 10 (24 h incubation) and then again a more gradual reduction in fluorescence intensity to a denatured state at the higher pHs. The high pH (pH 11–12) denatured state is characterised by a drastically reduced fluorescence intensity and a slight shift in its emission maximum to higher wavelengths (348 nm, as compared to 344 nm at neutral pHs), somewhat similar to the guanidine hydrochloride unfolded protein and characteristic of a denatured state with little residual structure (Fig. 4). On the other hand, the high residual fluorescence for the acid denatured state at pHs 3.5–4.5 and minor shift to lower wavelengths is similar to the high temperature denatured sample (Fig. 4) and indicates an incompletely unfolded state in which the tryptophan residues are at least partially buried, suggestive of an aggregated or molten globule state.

3.5. Stability: thermal denaturation

The effect of pH on the stability of the protein was investigated by measuring the residual thermal stability following 24 h incubation at various pHs. Here thermal denaturation was found to be irreversible and led to protein precipitation. Hence the thermodynamic parameters of stability could not be calculated and only the apparent T_m, as a measure of the kinetics of the irreversible process under the conditions used, could be determined. From Fig. 6 it can be seen that, with an average apparent T_m of 53.9 °C, maximum stability was at pH 6 to 6.5 under the conditions used and that high relative protein stability (≥90% of maximum) was observed between pHs 5.5 and 7.5. Nevertheless, it is important to note that at this 90% level a more than 5 °C reduction in the apparent T_m is already observed, suggestive of a significantly increased rate for the irreversible process even at this level. Furthermore, reductions in the apparent T_m at acidic pHs down to pH 5 and more gradual reductions at the basic pH limb are followed by the absence of transitions for the samples at pH 4.5 and below and at pH 10 and above indicating that here the transition had already occurred during incubation at these pHs.

3.6. Protein solubility

The results of the protein solubility study are given in Fig. 7 where it can be seen that strong precipitation occurs at pHs ∼3.5–4.5 and ∼9.5–10.5. Such results show that the irreversible processes observed earlier at these pHs are dominated by protein precipitation but the increased solubility seen at more extreme pHs suggests that here protein unfolding becomes dominant. Thus the accentuated loss of activity, high irreversible inactivation, abrupt decrease in fluorescence intensity to a denatured state with a high residual relative fluorescence intensity as well as the lack of a thermal transition at acidic pHs (∼<pH 4.5) are all resultant of precipitation of the protein at these pHs. Furthermore, at the basic pHs, the irreversible inactivation observed above pH 9, the relatively high residual fluorescence intensity at ∼pH 10 and the absence of a thermal denaturation transition at this same pH are also due to protein precipitation. Interestingly ∼pH 10 is close to the isoelectric point of pXyl (pI = 9.5–10) where it possesses a net zero charge and where, as seen here, proteins in general tend to have a reduced solubility [39].

Precipitation has been previously observed for pXyl at high concentrations [40] and following heat denaturation [41] and here we investigated this further. Precipitation was found to be dependent

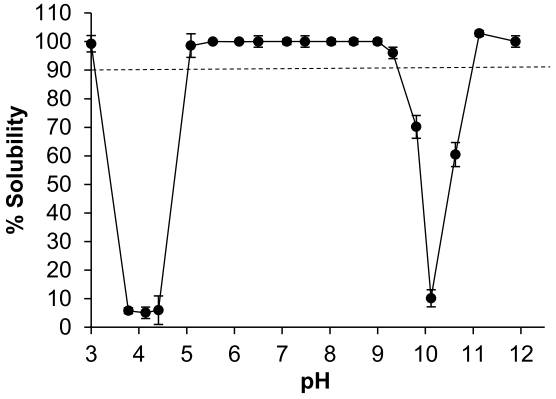

Fig. 7. Xylanase solubility as a function of pH. The concentration of protein remaining in solution was measured following 24 h incubation at various pHs. The horizontal dashed line indicates the 90% level.

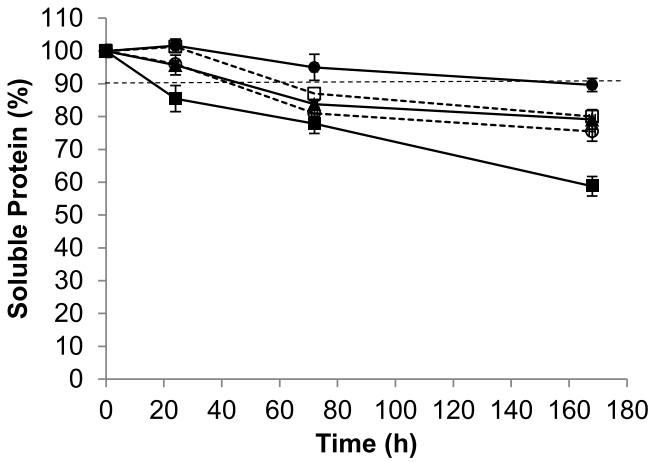

Fig. 8. Solubility of xylanase as a function of initial concentration and incubation time. Initial xylanase concentrations of 0.07 mg/mL (filled circles, solid line), 0.5 mg/mL (empty squares, dotted line), 1.4 mg/mL (filled triangles, solid line), 2 mg/mL (empty circles, dotted line) and 3.6 mg/mL (filled squares, solid line) were examined. The horizontal dashed line indicates the 90% level.

on the protein concentration (Fig. 8) and indeed turbid solutions were clearly visible at concentrations of 2 mg/mL and higher under the conditions used. Thus pXyl has a high propensity for precipitation with this being governed by both initial protein concentration and pH. Hydrophobic effects are believed to play a crucial role in promoting protein aggregation [42] and pXyl has already been reported as being characterised by a relatively high exposure of hydrophobic residues (16.7% of accessible surface area) [15]. This high surface hydrophobicity has been suggested as being related to its adaptation to low temperatures [15] but may also play a role in the high tendency of this enzyme to precipitate as seen here.

4. Conclusions

In this study we produced and purified a GH8 xylanase and carried out an in-depth analysis of the pH induced changes in activity, irreversible inactivation, conformation, stability and solubility so as to identify the factors determining the pH dependence of this industrial enzyme. Different optimal pH ranges were observed with each of the techniques used (see the recapitulative Fig. 9) which reflects both the diverse parameters measured and the distinct sensitivities of the techniques used and highlights the necessity of using a variety of different techniques when characterising proteins.

Highest activity and stability was seen at near neutral pHs and decreased with shifts towards both the acidic and basic pHs. At acidic pHs, the initial loss of activity is due to reversible processes, possibly a repositioning of the proton donor [36] or/and protonation of the proton acceptor, which have little effect on stability or are poorly detected by the techniques used. However, this is rapidly overcome by strong irreversible precipitation which develops over time, with a maximum at pH 4.5 leading to large alterations in all parameters measured. Analysis of the results for the basic pHs indicates that here a different phenomena occurs to that observed at acidic pHs. Initially, conformational alterations accompanied by a reversible loss of activity are observed and allows one to suggest that this may include a reversible deprotonation of the proton donor E78. Subsequently, at pH 10, close to the pI, protein precipitation leads to large modifications being detected in the structure and activity and is followed by protein unfolding at higher pHs leading to a denatured state with little residual structure.

The structure, function, solubility and stability of proteins at a given pH depend on their net charge and the charge state of the individual ionizable residues. Hence, most commonly, approaches for engineering proteins for increased activity and/or stability at acidic or basic pHs involve amino acid changes that alter the pK_a of specific residues, modify the surface charge and/or sometimes also introduce stabilising interactions. From this study it can be appreciated that such approaches would probably have little effect with this GH8 enzyme. At acidic pHs protein precipitation dominates and would mask any positive effects resultant of an engineering strategy focused on activity or stability. Here focus should be placed on reducing precipitation, namely by reducing the surface hydrophobicity via mutation of surface hydrophobic residues to polar residues [43]. At basic pHs, mutations aiming at altering the environment of the proton donor E78 to increase its p*Ka* would probably be effective but at pHs close to 10 precipitation again becomes dominant. This is probably resultant of the net zero charge on the protein near its pI and hence approaches for alleviating precipitation here should be focused on increasing the net charge on the protein at this pH [39]. Finally, at even higher pHs, protein unfolding dominates and hence introduction of stabilising interactions may be relevant.

The insights obtained in this study have implications in protein science and protein engineering and demonstrate the complexity and various factors associated with the pH dependence of proteins and the engineering of these. The identification and detailed description of the various factors determining the pH dependence of pXyl allows for a better understanding of this enzyme and of GH8 enzymes in general and will enable for a more successful tailoring and optimisation of these to a specific process condition.

Conflict of interest

The authors declare that they have no conflict of interest.

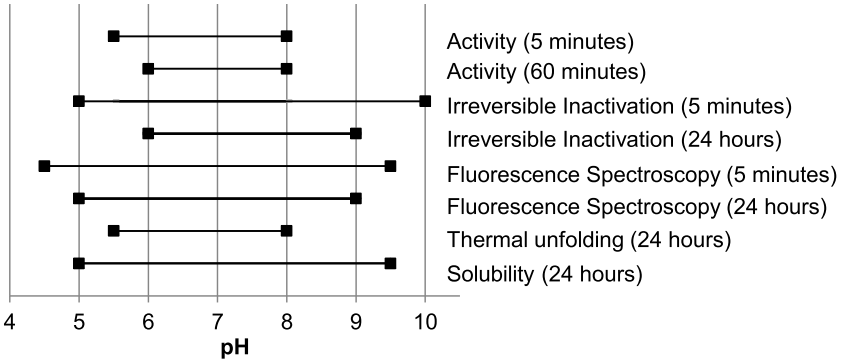

Fig. 9. Summary of the results obtained for pXyl. The optimum pH range (≥90% of the maximum) determined for the activity, irreversible inactivation, stability (fluorescence spectroscopy, thermal unfolding) and solubility are shown.

Author contributions

MB and TC conceived and designed the study and participated in its implementation. MB, GS, BJ, FG, GF and TC carried out the experimental studies. All authors have seen the manuscript and approved its submission.

Acknowledgements

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References

[1] A.S. Bommarius, M.F. Paye, Stabilizing biocatalysts, Chem. Soc. Rev. 42 (2013) 6534–6565.
[2] M.J. Liszka, M.E. Clark, E. Schneider, D.S. Clark, Nature versus nurture: developing enzymes that function under extreme conditions, Annu. Rev. Chem. Biomol. Eng. 3 (2012) 77–102.
[3] O. Akpinar, K. Erdogan, S. Bostanci, Enzymatic production of xylooligosaccharide from selected agricultural wastes, Food Bioprod. Process 87 (2009) 145–151.
[4] F. Beltramino, C. Valls, T. Vidal, M.B. Roncero, Exploring the effects of treatments with carbohydrases to obtain a high-cellulose content pulp from a non-wood alkaline pulp, Carbohydr. Polym. 133 (2015) 302–312.
[5] V. Juturu, J.C. Wu, Microbial xylanases: engineering, production and industrial applications, Biotechnol. Adv. 30 (2012) 1219–1227.
[6] V. Kumar, J. Marín-Navarro, P. Shukla, Thermostable microbial xylanases for pulp and paper industries: trends, applications and further perspectives, World J. Microbiol. Biotechnol. 32 (2016) 1–10.
[7] H. Li, X. Gao, J.D. Demartini, R. Kumar, C.E. Wyman, Application of high throughput pretreatment and co-hydrolysis system to thermochemical pretreatment. Part 2: dilute alkali, Biotechnol. Bioeng. 110 (2013) 2894–2901.
[8] K.K. Wong, L.A. Martin, F.M. Gama, J.N. Saddler, E. de Jong, Bleach boosting and direct brightening by multiple xylanase treatments during peroxide bleaching of kraft pulps, Biotechnol. Bioeng. 54 (1997) 312–318.
[9] T. Collins, C. Gerday, G. Feller, Xylanases, xylanase families and extremophilic xylanases, FEMS Microbiol. Rev. 29 (2005) 3–23.
[10] W. Bai, C. Zhou, Y. Zhao, Q. Wang, Y. Ma, Structural insight into and mutational analysis of family 11 xylanases: implications for mechanisms of higher pH catalytic adaptation, PLoS One 10 (2015) e0132834.
[11] T. Collins, M.A. Meuwis, I. Stals, M. Claeyssens, G. Feller, C. Gerday, A novel family 8 xylanase, functional and physicochemical characterization, J. Biol. Chem. 277 (2002) 35133–35139.
[12] T. Collins, D. De Vos, A. Hoyoux, S.N. Savvides, C. Gerday, J. Van Beeumen, G. Feller, Study of the active site residues of a glycoside hydrolase family 8 xylanase, J. Mol. Biol. 354 (2005) 425–435.
[13] T. Collins, M.A. Meuwis, C. Gerday, G. Feller, Activity, stability and flexibility in glycosidases adapted to extreme thermal environments, J. Mol. Biol. 328 (2003) 419–428.
[14] D. De Vos, T. Collins, W. Nerinckx, S.N. Savvides, M. Claeyssens, C. Gerday, G. Feller, J. Van Beeumen, Oligosaccharide binding in family 8 glycosidases: crystal structures of active-site mutants of the beta-1,4-xylanase pXyl from *Pseudoaltermonas haloplanktis* TAH3a in complex with substrate and product, Biochemistry 45 (2006) 4797–4807.
[15] F. Van Petegem, T. Collins, M.A. Meuwis, C. Gerday, G. Feller, J. Van Beeumen, The structure of a cold-adapted family 8 xylanase at 1.3 A resolution. Structural adaptations to cold and investgation of the active site, J. Biol. Chem. 278 (2003) 7531–7539.
[16] A. Pollet, J. Schoepe, E. Dornez, S.V. Strelkov, J.A. Delcour, C.M. Courtin, Functional analysis of glycoside hydrolase family 8 xylanases shows narrow but distinct substrate specificities and biotechnological potential, Appl. Microbiol. Biotechnol. 87 (2010) 2125–2135.
[17] E. Dornez, P. Verjans, F. Arnaut, J.A. Delcour, C.M. Courtin, Use of psychrophilic xylanases provides insight into the xylanase functionality in bread making, J. Agric. Food Chem. 59 (2011) 9553–9562.
[18] T. Collins, S. D'Amico, J. Marx, G. Feller, C. Gerday, Cold-adapted enzymes, in: C. Gerday, N. Glansdorff (Eds.), Physiology and Biochemistry of Extremophiles, ASM Press, Washington, D.C, 2007, pp. 165–179.
[19] T. Collins, G. Feller, C. Gerday, M.A. Meuwis, Family 8 enzymes with xylanolytic activity, in USPTO Patent (Granted): US8309336 B2 (2012).
[20] T. Collins, A. Hoyoux, A. Dutron, J. Georis, B. Genot, T. Dauvrin, F. Arnaut, C. Gerday, G. Feller, Use of glycoside hydrolase family 8 xylanases in baking, JCS 43 (2006) 79–84.
[21] A. Dutron, J. Georis, B. Genot, T. Dauvrin, T. Collins, A. Hoyoux, G. Feller, Use of family 8 enzymes with xylanolytic activity in baking, in Patents (Granted): US8192772 (2012), EP1549147B1 (2011), CN1681392B (2010), DE60336153 D1 (2011).
[22] B. Al Balaa, K. Brijs, K. Gebruers, J. Vandenhaute, J. Wouters, I. Housen, Xylanase XYL1p from *Scytalidium acidophilum*: site-directed mutagenesis and acidophilic adaptation, Bioresour. Technol. 100 (2009) 6465–6471.
[23] Y.C. Chen, Y.C. Chiang, F.Y. Hsu, L.C. Tsai, H.L. Cheng, Structural modeling and further improvement in pH stability and activity of a highly-active xylanase from an uncultured rumen fungus, Bioresour. Technol. 123 (2012) 125–134.
[24] S. Pokhrel, J.C. Joo, Y.H. Kim, Y.J. Yoo, Rational design of a *Bacillus circulans* xylanase by introducing charged residue to shift the pH optimum, Process Biochem. 47 (2012) 2487–2493.
[25] M.D. Joshi, G. Sidhu, J.E. Nielsen, G.D. Brayer, S.G. Withers, L.P. McIntosh, Dissecting the electrostatic interactions and pH-dependent activity of a family 11 glycosidase, Biochemistry 40 (2001) 10115–10139.
[26] G. Mamo, M. Thunnissen, R. Hatti-Kaul, B. Mattiasson, An alkaline active xylanase: insights into mechanisms of high pH catalytic adaptation, Biochimie 91 (2009) 1187–1196.
[27] T. Beliën, I.J. Joye, J.A. Delcour, C.M. Courtin, Computational design-based molecular engineering of the glycosyl hydrolase family 11 B. subtilis XynA endoxylanase improves its acid stability, Protein Eng. Des. Sel. 22 (2009) 587–596.
[28] Y. Li, P.C. Cirino, Recent advances in engineering proteins for biocatalysis, Biotechnol. Bioeng. 111 (2014) 1273–1287.
[29] P. Bernfeld, Amylases, α and β, Methods Enzymol. 1 (17) (1955) 149–158.
[30] C.N. Pace, Measuring and increasing protein stability, Trends Biotechnol. 8 (1990) 93–98.
[31] T. Collins, J. Azevedo-Silva, A. da Costa, F. Branca, R. Machado, M. Casal, Batch production of a silk-elastin-like protein in *E. coli* BL21(DE3): key parameters for optimisation, Microb. Cell Fact. 12 (2013) 21.
[32] J. Sambrook, D.W. Russell, Molecular Cloning A Laboratory Manual, 3rd ed., Cold Spring Harbor Laboratory Press, New York, 2001.
[33] T. Collins, M. Barroca, F. Branca, J. Padrao, R. Machado, M. Casal, High level biosynthesis of a silk-elastin-like protein in *E. coli*, Biomacromolecules 15 (2014) 2701–2708.
[34] F. De Lemos Esteves, T. Gouders, J. Lamotte-Brasseur, S. Rigali, J.M. Frere, Improving the alkalophilic performances of the Xyl1 xylanase from *Streptomyces* sp. S38: structural comparison and mutational analysis, Protein Sci. 14 (2005) 292–302.
[35] J.E. Nielsen, T.V. Borchert, G. Vriend, The determinants of alpha-amylase pH-activity profiles, Protein Eng. 14 (2001) 505–512.
[36] W. Adachi, Y. Sakihama, S. Shimizu, T. Sunami, T. Fukazawa, M. Suzuki, R. Yatsunami, S. Nakamura, A. Takenaka, Crystal structure of family GH-8 chitosanase with subclass II specificity from *Bacillus* sp. K17, J. Mol. Biol. 343 (2004) 785–795.
[37] J.R. Lakowicz, Protein fluorescence, in: J.R. Lakowicz (Ed.), Principles of Fluorescence Spectroscopy, Springer, Boston, MA, US, 2006, pp. 529–575.
[38] R. Fraczkiewicz, W. Braun, Exact and efficient analytical calculation of the accessible surface areas and their gradients for macromolecules, J. Comput. Chem. 19 (1998) 319–333.
[39] K.L. Shaw, G.R. Grimsley, G.I. Yakovlev, A.A. Makarov, C.N. Pace, The effect of net charge on the solubility, activity, and stability of ribonuclease Sa, Protein Sci. 10 (2001) 1206–1215.
[40] F. Van Petegem, T. Collins, M.A. Meuwis, C. Gerday, G. Feller, J. Van Beeumen, Crystallization and preliminary X-ray analysis of a xylanase from the psychrophile *Pseudoalteromonas haloplanktis*, Acta Crystallogr. D: Biol. Crystallogr. 58 (2002) 1494–1496.
[41] T. Collins, S. D'Amico, D. Georlette, J.C. Marx, A.L. Huston, G. Feller, A nondetergent sulfobetaine prevents protein aggregation in microcalorimetric studies, Anal. Biochem. 352 (2006) 299–301.
[42] J. Gsponer, M. Vendruscolo, Theoretical approaches to protein aggregation, Protein Pept. Lett. 13 (2006) 287–293.
[43] S.R. Trevino, J.M. Scholtz, C.N. Pace, Amino acid contribution to protein solubility: Asp, Glu, and Ser contribute more favorably than the other hydrophilic amino acids in RNase Sa, J. Mol. Biol. 366 (2007) 449–460.

Chapter II.IV

Inverse PCR for Point Mutation Introduction

Inverse PCR for Point Mutation Introduction

Diogo Silva, Gustavo Santos, Mário Barroca, and Tony Collins

Abstract

Inverse PCR is a powerful tool for the rapid introduction of desired mutations at desired positions in a circular double-stranded DNA sequence. Here, custom-designed mutant primers oriented in the inverse direction are used to amplify the entire circular template with incorporation of the required mutation(s). By careful primer design it can be used to perform such diverse modifications as the introduction of point mutations and multiple mutations, the insertion of new sequences, and even sequence deletions. Three primer formats are commonly used; nonoverlapping, partially overlapping and fully overlapping primers, and here we describe the use of nonoverlapping primers for introduction of a point mutation. Use of such a primer setup in the PCR reaction, with one of the primers containing the desired mismatch mutation, results in the amplification of a linear, double-stranded, mutated product. Methylated template DNA is removed from the nonmethylated PCR product by *Dpn*I digestion and the PCR product is then phosphorylated by polynucleotide kinase treatment before being recircularized by ligation, and transformed to *E. coli*. This relatively simple site-directed mutagenesis procedure is of major importance in biology and biotechnology today where it is commonly employed for the study and engineering of DNA, RNA, and proteins.

Key words Site-directed mutagenesis, Inverse PCR, Nonoverlapping primers, Protein engineering

1 Introduction

Site-directed mutagenesis (SDM) is a powerful method for making targeted, predetermined changes in a DNA sequence. It is invaluable in molecular biology and protein engineering for investigating the role of specific nucleotides and amino acids and for engineering desired properties into protein, DNA, and RNA molecules [1–6]. The original, relatively inefficient, SDM methods based on primer extension with single stranded DNA templates [7–9], have evolved over the years, and have been supplanted by the plethora of versatile, highly efficient SDM methods available today. Indeed, currently, a large variety of specific, high-throughput, in vitro [10, 11] and in vivo [12, 13] techniques and manufactured kits with

All authors contributed equally to this work.

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efficiencies up to almost 100% for the site-specific mutation of almost any sequence are available.

The most commonly used in vitro SDM methods employ PCR and are based on either overlap extension PCR [11, 14] or inverse PCR (iPCR) [15, 16] as well as modifications and combinations of these. Overlap extension PCR is more appropriate for linear sequences and requires multiple rounds of PCR, whereas iPCR is designed for circular templates such as vector insert sequences and uses a simplified protocol necessitating only one PCR reaction. Inverse PCR was first reported in 1988 for the identification of flanking regions of a known DNA sequence [15, 16]. Its designation, inverse, comes from the fact that the primers are oriented in the reverse direction, facing "outwards," away from each other, in contrast to regular PCR where "in-facing" flanking primers are employed.

Nonoverlapping-, partially overlapping, or fully overlapping primers can be used for SDM by iPCR. Nonoverlapping, "back-to-back" primers (Fig. 1a) produce a linear mutated sequence which must then be recircularized before transformation to *E. coli* [17]. Partially overlapping primers (Fig. 1b) yield a product with short homologous ends which can be directly transformed for in vivo recombination in *E. coli* [18–20]. Completely overlapping, complementary, inverse primers (Fig. 1c) form part of the widely used QuikChange SDM kit (Strategene), but currently the exact mechanism of action of this is under discussion. It had previously been proposed to progress by linear amplification of template to give a circular product, and not by exponential amplification as for a true PCR chain-reaction [20]. However, recent evidence [21] suggests exponential amplification of a linear product with short homologous ends for recombination, as with partially overlapping primers. Use of partially or fully overlapping primers allows for a more simplified SDM procedure than with nonoverlapping primers, but

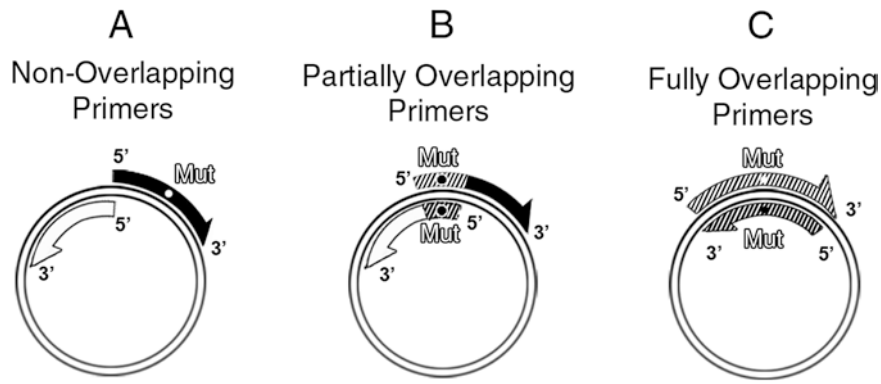

Fig. 1 Illustration of primer design formats for inverse PCR with nonoverlapping (**a**), partially overlapping (**b**) and fully overlapping (**c**) primers. The hatched sections show the overlapping regions of the primers

frequently necessitate longer more complex primers and are sometimes characterized by poor or no amplification of PCR product, formation of primer dimers, and a reduced transformation efficiency [19–21].

In this chapter, we focuse on the iPCR method with nonoverlapping primers for introduction of a point mutation into a DNA sequence. This is composed of three principal steps (Fig. 2): (1) iPCR Mutant Amplification (including primer design, PCR and agarose gel confirmation), (2) Template Removal and Product Recircularization (including template digestion, mutant phosphorylation, and ligation), and (3) Transformation and Mutant Confirmation (transformation, plasmid construct isolation and sequencing). iPCR is relatively easy and rapid to employ and by simple modification of primer design, not only single base changes (point mutations), but also multiple base changes, deletions, and insertion can be carried out (*see* Fig. 2). Currently, a variety of optimized kits based on iPCR with nonoverlapping primers are available, e.g., the Phusion (Thermo Scientific), Q5 (New England Biolabs), and KOD-Plus (Toyobo) site-directed mutagenesis kits.

2 Materials

2.1 iPCR Mutant Amplification

1. PCR Thermal Cycler.
2. Thin-walled PCR tubes.
3. Circular, double-stranded, template DNA. Approximately 1 ng/μL stock, in autoclaved, ultrapure water is recommended, but may vary from 0.1 to 10 ng/μL depending on plasmid size, sequence, and quality (*see* **Note 1**).
4. PCR primers (*see* **Note 2**). For highest SDM efficiency, HPLC or PAGE purified primers are recommended. Resuspend lyophilized primers in 1.2 g/L (10 mM) Tris–HCl (tris(hydroxymethyl)aminomethane, adjust pH with HCl, pH 7) to a concentration of 100 μM and prepare 20 μM working stock solutions by dilution of aliquots in 1.2 g/L (10 mM) Tris–HCl, pH 7. All primer solutions should be stored at −20 °C and repeated freezing and thawing should be avoided.
5. High-fidelity DNA polymerase with proofreading activity (*see* **Note 3**), as supplied, e.g., Phusion High Fidelity DNA polymerase (2 U/μL). Store at −20 °C.
6. DNA Polymerase buffer (as supplied with the polymerase used), e.g., 5× concentrated Phusion HF buffer (*see* **Note 4**). Store at −20 °C.
7. Deoxyribonucleoside triphosphate (dNTP) mix, a stock solution of 10 mM is recommended. Store at −20 °C.
8. Autoclaved, ultrapure water (*see* **Note 5**).

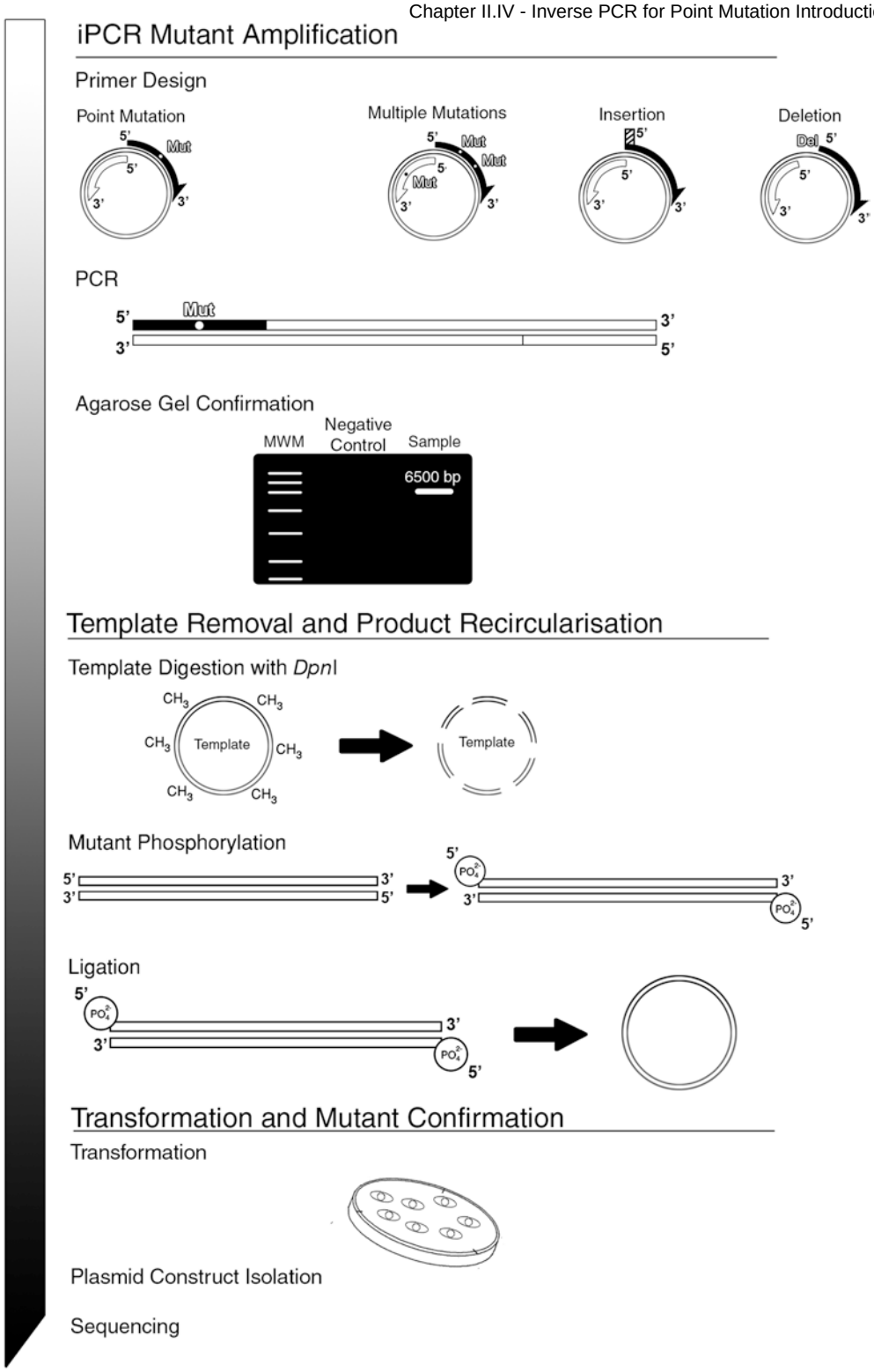

Fig. 2 Flowchart of the protocol for site-directed mutagenesis by inverse PCR with nonoverlapping primers. The primer design formats for introduction of a point mutation, multiple mutations, insertions, and deletions into a double-stranded circular DNA template are shown. Following primer design, the protocol employed for each type of mutation is identical. CH_3: methyl group of methylated DNA; PO_4^{2-}: phosphate group of 5′ phosphorylated DNA

2.2 Agarose Gel Electrophoresis

1. Agarose, molecular biology grade.
2. 50× (50 times concentrated) TAE solution (*see* **Note 6**): 242 g/L (2 M) Tris base, 60 g/L (1 M) glacial acetic acid, 14.6 g/L (50 mM) EDTA (Ethylenediaminetetraacetic acid). Store at room temperature. Dilute in deionized water to 1× (i.e., 50-fold dilution) prior to use.
3. 6× Loading buffer: 500 g/L glycerol, 58 g/L (0.2 M) EDTA, 0.5 g/L bromophenol blue, pH 8.3. Store at room temperature. Prepare 50 mL and store at room temperature for a maximum of 6 months (*see* **Note 7**).
4. Nucleic acid staining solution, e.g., Midori Green Advance (20,000×): 5 µL in 100 mL 1× TAE solution. While being significantly less mutagenic than the traditionally used ethidium bromide stain, appropriate care should be taken to avoid direct contact with midori green or similar nucleic acid stains. Store at −20 °C.
5. Molecular weight marker, as supplied. Store at −20 °C (*see* **Note 8**).
6. Gel casting trays and sample combs.
7. Electrophoresis chamber and power supply.
8. Transilluminator. Always use appropriate safety procedures and wear protective eyewear when using a transilluminator to prevent UV light damage.

2.3 Template Removal and Product Recircularization

1. Restriction enzyme *Dpn*I (as supplied, typically 10 U/µL). Store at −20 °C (*see* **Note 9**).
2. T4 DNA Ligase buffer (as supplied with T4 DNA Ligase, typically 10×, ensure this contains 5–10 mM ATP and 50–100 mM DTT) (*see* **Note 10**). Store at −20 °C.
3. T4 Polynucleotide Kinase (as supplied, typically 10 U/µL). Store at −20 °C (*see* **Note 11**).
4. T4 DNA Ligase (as supplied, typically 5 U/µL). Store at −20 °C (*see* **Note 12**).
5. Incubators at 37 and 25 °C (or room temperature).

2.4 Preparation of Chemically Competent E. coli XL1-Blue

1. *E. coli* XL1-Blue cells (*see* **Note 13**).
2. Transformation Buffer: 3 g/L (10 mM) PIPES, 1.7 g/L (15 mM) $CaCl_2$, 18.6 g/L (250 mM) KCl, 10.9 g/L (55 mM) $MnCl_2{\cdot}4H_2O$. Mix all components except $MnCl_2$ and adjust pH to 6.7 with 112 g/L (2 M) KOH. Add $MnCl_2$ and mix, sterilize solution through a 0.4 µm membrane.
3. 100% dimethyl sulfoxide (DMSO).
4. SOB (Super Optimal Broth): 20 g/L bacto tryptone, 5 g/L yeast extract, 0.6 g/L (10 mM) NaCl, 0.75 g/L (2.5 mM) KCl, 0.95 g/L (10 mM) $MgCl_2$, 1.2 g/L (10 mM) $MgSO_4$.

2.5 Transformation

1. 200 µL aliquots of chemically competent *E. coli* XL1 Blue cells (*see* **Note 13**). Store at −70 °C.
2. pUC18 control plasmid (1 pg/µL). Store at −20 °C.
3. Luria Bertani Broth (LB): 10 g/L bacto tryptone, 5 g/L yeast extract, 10 g/L NaCl. Adjust pH to 7 with a 200 g/L (5M) NaOH solution. Autoclave to sterilize.
4. Ampicillin (*see* **Note 14**): 100 mg/mL stock solution in water. Filter-sterilize through a 0.4 µm membrane and store at 4 °C for no more than 1 month.
5. LB agar plates containing antibiotic (100 µg/mL ampicillin). Prepare LB as described above with addition of 18 g/L agar. Autoclave to sterilize, cool to 50–55 °C, add 1 mL/L of 100 mg/mL ampicillin stock, mix and aseptically pour to petri dishes.

2.6 Mutant Confirmation

1. LB (prepared as described above) + antibiotic (100 µg/mL ampicillin) (*see* **Note 14**).
2. 50 mL polypropylene falcon tubes.
3. Plasmid DNA purification kit, as supplied by manufacturer.

3 Methods

The protocol given is for the introduction of a single point mutation with Phusion high fidelity DNA polymerase in a 1007 bp sequence in the expression vector pET22b(+). The total construct size is 6500 bp and the selection marker is ampicillin resistance. Nevertheless, any templates up to ~10 kb in size and any rapid, high fidelity polymerase can be used with appropriate protocol modification according to manufacturer's recommendations (namely, modifications in the buffer and polymerase concentrations, the PCR cycle and/or antibiotic used) (*see* **Note 3**). PCR process optimization may be required in some cases.

3.1 iPCR Mutant Amplification

1. Primer Design. Inversed primers should anneal to opposite strands of the plasmid, be nonoverlapping and aligned back-to-back with apposing 5′ ends. Ideally, the targeted mismatch mutation should be located in the middle of the primer with 10–15 perfectly matched nucleotides on either side. Mutations can be incorporated closer to the 5′ end but at least ten complementary nucleotides are required at the 3′ end (*see* **Note 15**). Normal considerations for PCR primer design should be adhered to (*see* **Note 16**). Phosphorylation of primers is not required (*see* **Note 2**). For best results, at least HPLC grade purification of primers is required, for primers greater

than 40 nucleotides in length, PAGE purification is recommended (*see* **Note 2**).

2. PCR Reaction Setup (*see* **Note 17**). Add the following components in the order given to a thin walled PCR tube on ice: 13.4 µL autoclaved ultrapure water (for a total final reaction volume of 20 µL), 4 µL of 5× concentrated Phusion HF buffer (1× buffer) (*see* **Note 4**), 0.4 µL of 10 mM stock dNTP mix (200 µM of each dNTP), 0.5 µL of each 20 µM primer stock (0.5 µM of each primer) (*see* **Note 2**), 1 µL of 1 ng/µL plasmid template stock (1 ng) (*see* **Note 1**), and 0.2 µL of 2 U/µL Phusion DNA polymerase (0.4 U) (*see* **Note 3**). Gently mix, briefly centrifuge and immediately place in the thermal cycler. A negative control reaction with all components except the primers, which are substituted with an equal volume of water, should also be set up.
3. PCR Cycle (*see* **Note 18**): 1 cycle at 98 °C for 2 min, 25 cycles of denaturation, annealing and extension at, respectively, 98 °C for 20 s, the calculated primer annealing temperature for 20 s, and 72 °C for 2 min (~20 s/kb of template). A final extension is then carried out at 72 °C for 10 min before cooling to 4–10 °C. The same conditions are used for the sample and negative control.
4. Agarose Gel Confirmation. The results of the PCR are verified by visualizing 5 µL of the sample and negative controls on a 1% agarose gel using the following protocol. To 1 g of agarose add 1× TAE buffer to 100 mL and boil until the agarose is completely dissolved. When cooled to ~50–60 °C, pour into the casting tray, insert comb and leave until completely polymerized. Remove comb and place gel in the electrophoresis chamber, add 1× TAE buffer until gel is covered with solution. To 5 µL of sample and negative control, add 1 µL of 6× loading buffer, mix, and carefully pipette into agarose gel wells. Load molecular weight marker (*see* **Note 8**) into an adjacent well. Run the gel at 7–8 V/cm for 45–60 min, carefully remove and place in nucleic acid staining solution for 30 min before visualizing under a UV transilluminator. A strong band should be visible at 6500 bp for the sample and no band should be observed for the negative control (*see* **Note 19**).

3.2 Template Removal and Product Recircularization

1. Template Digestion. Add 1 µL of *Dpn*I directly to the PCR reaction (15 µL reaction volume remaining), mix by gently pipetting up and down, centrifuge briefly, and incubate for 30 min at 37 °C (*see* **Note 9**).
2. Phospho-ligation. To a new Eppendorf tube add 12 µL of autoclaved, ultrapure water, 2 µL of 10× T4 DNA ligase buffer

(*see* **Note 10**), 4 µL of *Dpn*I treated PCR product (*see* **Note 20**), 1 µL of PNK (10 U/µL) (*see* **Note 11**), and 1 µL of T4 DNA ligase (5 U/µL) (*see* **Note 12**). Mix by gently pipetting up and down, centrifuge briefly to spin down, and incubate for 90 min at 25 °C. Store on ice until transformation or store at −20 °C.

3.3 Transformation and Mutant Confirmation

1. Preparation of chemically competent *E. coli* XL1 Blue for transformation (*see* **Note 13**). Inoculate a single colony of *E. coli* XL1 Blue in 250 mL sterile SOB medium in a 2 L shake flask and incubate at 18 °C, 150 rpm until $OD_{600\ nm}$ = 0.6. Place on ice for 10 min, centrifuge at 4 °C for 10 min at 2500 × *g* and decant the supernatant. Gently resuspend the pellet in 80 mL of ice-cold Transformation Buffer (care should be taken as cells are susceptible to mechanical disruption) and place on ice for 10 min. Centrifuge cells at 4 °C for 10 min at 2500 × *g*. Decant the supernatant and gently resuspend the pellet in 20 mL of ice-cold Transformation Buffer. Add DMSO to a final concentration of 7%, swirl gently and place on ice for 10 min. Dispense 200 µL aliquots in ice cold 1.5 mL Eppendorf tubes and immediately freeze in liquid nitrogen. Store at −80 °C for up to 40 days.
2. Transformation (*see* **Note 13**). Defrost the competent cells on ice (10–20 min) and add 5 µL (*see* **Note 21**) of the phospholigation reaction mix. Swirl the tubes gently and incubate on ice for 30 min. Swirl the tubes gently and heat-shock cells in a water bath at 42 °C for 45 s and immediately transfer to ice for 10–15 min. Add 800 µL of fresh LB medium and incubate for 1 h at 37 °C, 200 rpm. Centrifuge for 3 min at 3000 × *g*, room temperature, remove 850 µL of the supernatant and gently resuspend the pellet in the remaining solution. Spread-plate the remaining ~150 µL solution on LB + ampicillin agar plates and incubate overnight at 37 °C. A positive transformation control with 1 µL of 1 pg/µL pUC18 plasmid and a negative process control with 1 µL of the PCR negative control should also be carried out.
3. Select three transformant colonies and inoculate into 5 mL LB + ampicillin medium (*see* **Note 14**) in a 15 mL Falcon tube. Incubate at 37 °C, 200 rpm overnight. No colonies should be visible for the negative process control. The LB + ampicillin plate for the positive transformation control should have approximately 50 colonies.
4. Isolate plasmid from cultures with a commercial plasmid purification kit and forward for sequencing of the insert in both directions. Greater than 80% of the sequences should contain the desired mutation and no other undesired mutation (*see* **Note 22**).

4 Notes

1. It is essential that the template used for iPCR is purified, circular, double-stranded DNA isolated from a *dam*$^+$*E. coli* strain. The majority of commonly used *E. coli* strains are *dam*$^+$, including *E. coli* XL1-Blue, DH5α and JM109. *E. coli* JM110 and SCS110 are examples of dam$^-$ strains and should not be used. *dam*$^+$*E. coli* strains contain the enzyme Dam methylase which methylates adenine residues in the sequence GATC. This methylated sequence is the target for digestion by *Dpn*I and allows for later removal of template DNA from the nonmethylated in vitro produced iPCR product.

   While best results are achieved with smaller templates, iPCR of templates up to 10 kb is commonplace, with some reports of successes with even larger plasmid constructs.

2. We have successfully used desalted primers for SDM but did encounter an increased number of incomplete product sequences with missing nucleotides at the ligation site. To enhance the yield of full length sequences, HPLC or PAGE purified sequences are recommended. The former augments the content of full length primers (≥85% are full length) while the latter, PAGE, is more apt for ensuring the full length (≥90% are full length) of longer primers (>40 bp).

   Primers are frequently resuspended in water or Tris buffer supplemented with EDTA. Nonsupplemented Tris buffer is preferred as the pH of water is often slightly acidic and can lead to depurination while EDTA can interfere with downstream processes by sequestering essential cations. Do not store oligonucleotides in water at 4 °C. Prepare aliquots of 20 μM working stock, store at –20 °C and avoid repeated freezing and thawing.

   Phosphorylation of the iPCR product with polynucleotide kinase (*see* Subheading 3.2, **step 2**, phospho-ligation) eliminates the need for phosphorylated primers. Nevertheless, if preferred, these may be utilized and the polynucleotide kinase treatment omitted by substitution of this enzyme with 1 μL water during the phospho-ligation step.

3. While the polymerase used here is Phusion High Fidelity DNA Polymerase, any high-fidelity DNA polymerase with a high extension rate and proofreading activity (3′ →5′ exonuclease activity) may be used, e.g., Q5-High Fidelity DNA polymerase, *Pfu* Turbo DNA polymerase, and KOD DNA polymerase. Nevertheless, one should be aware of the particular template size limitations of the polymerase chosen, e.g., KOD DNA polymerase is recommended for templates ≤6 kbp; *Pfu* Turbo and Phusion DNA polymerases are reported to be able to amplify plasmids up to 15 kb. For best PCR results, use the

hot start variants of these polymerases where incorporation of automatic hot start technology permits polymerase activity at high temperatures only. This minimizes nonspecific amplification and primer dimer formation at low temperatures during reaction setup and during the initial PCR cycle, and allows for room temperature reaction setup. Currently commercialized examples include Platinum SuperFi DNA Polymerase, Phusion Hot Start High-Fidelity DNA Polymerase, Q5 Hot Start High-Fidelity DNA Polymerase. In all cases, modify the protocol given in this chapter according to the manufacturers' recommendations for the particular polymerase used.

4. Two buffers are provided with Phusion Polymerase, a HF and a GC Buffer. The former is used as the default buffer for high-fidelity amplification as the error rate with this is lower than with the latter. However, GC Buffer can improve the performance with certain difficult or long templates, such as GC-rich templates or templates with complex secondary structures. For amplification of GC rich templates, use of 3% DMSO with HF buffer should be initially investigated. The GC buffer should only be used when HF buffer gives unsatisfactory results.
5. Autoclave ultrapure water to ensure sterility and inactivate residual nucleases (DNase).
6. The most popular buffers for DNA electrophoresis are TAE and TBE (1 M Tris base, 1 M boric acid, and 0.02 M EDTA). Either may be used here and should give similar results.
7. Bromophenol blue is used as a tracking dye and has an approximate position on a 1% agarose gel equivalent to a 370 bp (TAE buffer) or 220 bp (TBE buffer) fragment. Xylene cyanol FF (0.03% in 6× loading buffer stock) may also be used and has an approximate position on a 1% agarose gel equivalent to a 4160 bp (TAE buffer) or 3030 bp (TBE buffer) fragment.
8. Use a molecular weight marker with component DNA of sizes similar to the expected iPCR product size. We commonly use the GeneRuler 1 kb DNA Ladder.
9. The restriction enzyme *Dpn*I digests template DNA (from *dam*$^{+}$ strains) at the methylated sequence G^{m6}ATC, thereby "enriching for" the nonmethylated in vitro amplified iPCR product. *Dpn*I is highly active in the majority of commonly used polymerase reaction buffers (Phusion, Q5, etc.) and therefore the digestion can be performed directly in the PCR mix without any purification of the DNA. Recently, an optimized, three enzyme mix (*Dpn*I, polynucleotide kinase and ligase) has been reported for a more rapid (5 min) enrichment and phospho-ligation of iPCR products in one step (New England Biolabs).
10. ATP, DTT, and Mg^{2+} are essential buffer components for phospho-ligation. We commonly use T4 DNA ligase buffer

for the double T4 polynucleotide kinase-T4 DNA ligase reaction. Other buffers, such as the T4 polynucleotide kinase buffer, or FastDigest Buffer, or even many of the standard low salt restriction enzyme buffers supplemented with 1 mM riboATP may also be used. Oxidized DTT leads to reduced enzyme activity, avoid repeated freezing and thawing and avoid using solutions more than 1 year old. Addition of 5% polyethylene glycol (PEG) may enhance phospho-ligation but in this case extended ligation should be avoided.

11. T4 polynucleotide kinase phosphorylates the 5′-hydroxyl terminus of double and single stranded DNA and RNA. It is inhibited by ammonium ions and by high salt and high phosphate concentrations, do not use DNA precipitated with ammonium ions.
12. T4 DNA ligases join the 5′ phosphorylated and 3′ hydroxyl ends of the linear product to give a recircularized iPCR product. It is sensitive to high salt and high EDTA concentrations. Rapid ligases (Quick Ligase) which are reported to enable reaction completion in as little as 5 min have recently been marketed.
13. We commonly use in-house prepared chemically competent *E. coli* XL1-Blue as the cloning host. The preparation procedure described here allows for transformation efficiencies of 10^7–10^8 cfu/μL which is normally sufficient for our SDM protocol. *E. coli* XL1-Blue is resistant to tetracycline and hence is not suited for plasmids with tetracycline resistance markers. Other, commercial, higher-efficiency cloning hosts and supercompetent cells for a higher number of transformants may also be used. In addition, use of electrocompetent hosts for transformation by electroporation allows for higher transformation efficiencies and is especially suited for large plasmids (~10 kbp and higher). In this latter case it is essential that the phospho-ligated circular DNA sample is purified (e.g., with a commercial DNA purification kit) to remove salts etc. prior to electroporation.
14. Ensure that the antibiotic/selection agent used is appropriate for the selective marker of the plasmid.
15. The description given is for a point mutation, but a similar primer design strategy may be used for short (1–3 bp) multiple base pair mutations or insertions, which may be included on one or both primers (*see* Fig. 2). Large insertions may be made by adding the nucleotides to be inserted on the 5′ ends of one, or both, of the inverse primers (*see* Fig. 2). Here, the perfectly matched portion of the primers should be 24–30 bp in length and should be used for calculation of the primer melting temperature. For deletions, the inverse primers should be designed to be perfectly matched to the sequences flanking

the fragment to be deleted (*see* Fig. 2). All remaining steps of the iPCR SDM procedure for these different types of mutations are similar to that described.

16. Normal considerations for PCR primer design should be adhered to, i.e., forward and reverse primers should have similar (<5 °C difference) melting temperatures (*see* **Note 11**), a GC content of 40–60%, 1 or 2 Gs or Cs at the 3′ end, and direct repeats, secondary structures, primer dimers and mispriming should be avoided. When designing mutations for introduction of an amino-acid change, the codon usage of the expression host should be taken into consideration for selection of the codon most favoured by the host or/and which requires the least number of base changes. Primer design programs are recommended, e.g., Primer3Plus, OligoCalc, SnapGene, NEBaseChanger, and OligoPerfect.
17. The optimum reaction conditions vary considerably with the polymerase and buffer system used, therefore the reaction conditions recommended by the supplier of the chosen polymerase should always be employed. Mainly, this involves alterations in the amount of polymerase and buffer used.
18. The optimum PCR cycle conditions vary with the polymerase and buffer system used, e.g., the phusion DNA polymerase system is characterized by elevated denaturation and annealing temperatures and high extension rate as compared to the majority of other polymerases. Therefore, the reaction temperatures and times recommended by the manufacturers of the chosen polymerase should always be employed.

    Usually, high-fidelity polymerases are thermostable at temperatures higher than 98 °C. Therefore, denaturation temperatures from 95 to 98 °C can be used. The shortest denaturation time should be used so as to avoid template damage. For most templates a 30-s initial denaturation from 95 to 98 °C is enough. Some templates, due to higher complexity, may require up to 3 min, or up to 5 min for GC-rich templates (>70% GC content).

    The most appropriate annealing temperature varies widely with the polymerase system employed and should be calculated as recommended by the supplier. Free online calculators for determination of the annealing temperatures are provided for the various DNA polymerase systems being currently commercialized, e.g., the Phusion Tm Calculator at ThermoFisher Scientific. For primers with calculated annealing temperatures ≥72 °C with Phusion, a two-step thermocycling protocol is recommended in which the annealing step is eliminated.

    The extension time and temperature depends on the extension rate and optimal temperature of the polymerase utilized, as well as the amplicon length and complexity. Most com-

monly, 72 °C is used. The extension time employed should ensure adequate full-length product synthesis and 15–60 s/kb is usually sufficient.

We recommend using 25 cycles. A higher number of cycles (up to 35) may increase product yield but also increases the probability of secondary, unwanted mutations.

19. If, in addition to a DNA band of the desired size, other nonspecific DNA bands are visible for the sample, then all the remaining 15 μL of the reaction should be run on a 1% agarose gel and the desired DNA band size excised and purified with a commercial gel extraction DNA purification kit. The purified DNA fragment can then be used directly in the step "Template Removal and Product Recircularization" (Subheading 3.2).

    The absence of any visible bands indicates PCR failure and hence typical PCR troubleshooting procedures should be followed, e.g., check primers design, reduce the annealing temperature by 3–5 °C increments, optimize Mg^{2+} concentration in 0.5 mM increments, increase denaturation and extension times, repeat experiment with various concentrations of template. In the case of phusion polymerase, use of both HF and GC buffers as well as addition of 3% DMSO should first be investigated (*see* **Note 4**).

    A weak band may be visible for the negative control if higher template concentrations were used (≥10 ng) but this should be many fold weaker than the sample band.

20. Avoid using large volumes of PCR product as this may interfere with the subsequent phospho-ligation and transformation. If a poor PCR yield leads to the need for larger volumes of PCR product, this should first be purified using a commercially available DNA purification kit.

    Improved phospho-ligation may be attained by use of 5% PEG. Also, following polynucleotide kinase addition, the sample may be incubated at 37 °C for 30 min before cooling to room temperature, adding the T4 DNA ligase, and further incubating at room temperature for 90 min.

21. Avoid using phospho-ligation mix volumes that are more than 10% of the competent cell volume as this leads to a reduced transformation efficiency. Purify phospho-ligation mix by use of a commercial DNA purification kit if transforming by electroporation.

22. The insert sequence should contain the desired mutation only. While it is not feasible to sequence the entire plasmid construct, the use of a high fidelity polymerase reduces the risk of secondary mutations in the vector sequence. To ensure the absence of such mutations, the insert sequence may be recloned into the original non-PCR-amplified vector.

References

1. Bommarius AS, Paye MF (2013) Stabilizing biocatalysts. Chem Soc Rev 42:6534–6565. doi:10.1039/c3cs60137d
2. Collins T, De Vos D, Hoyoux A, Savvides SN, Gerday C, Van Beeumen J, Feller G (2005) Study of the active site residues of a glycoside hydrolase family 8 xylanase. J Mol Biol 354(2):425–435
3. Davids T, Schmidt M, Bottcher D, Bornscheuer UT (2013) Strategies for the discovery and engineering of enzymes for biocatalysis. Curr Opin Chem Biol 17(2):215–220. doi:10.1016/j.cbpa.2013.02.022
4. Sanjuan R (2010) Mutational fitness effects in RNA and single-stranded DNA viruses: common patterns revealed by site-directed mutagenesis studies. Philos Trans R Soc Lond B Biol Sci 365(1548):1975–1982. doi:10.1098/rstb.2010.0063
5. Steiner K, Malke H (1997) Primary structure requirements for in vivo activity and bidirectional function of the transcription terminator shared by the oppositely oriented skc/rel-orf1 genes of *Streptococcus equisimilis* H46A. Mol Gen Genet 255(6):611–618
6. Traboni C, Ciliberto G, Cortese R (1982) A novel method for site-directed mutagenesis: its application to an eukaryotic tRNAPro gene promoter. EMBO J 1(4):415–420
7. Hutchison CA, Phillips S, Edgell MH, Gillam S, Jahnke P, Smith M (1978) Mutagenesis at a specific position in a DNA sequence. J Biol Chem 253(18):6551–6560
8. Hutchison CA 3rd, Edgell MH (1971) Genetic assay for small fragments of bacteriophage phi X174 deoxyribonucleic acid. J Virol 8(2):181–189
9. Edgell MH, Hutchison CA 3rd, Sclair M (1972) Specific endonuclease R fragments of bacteriophage phiX174 deoxyribonucleic acid. J Virol 9(4):574–582
10. Reeves AR (ed) (2016) In vitro mutagenesis. Methods and protocols, series vol 1498, 1 edn. Humana, New York, NY. doi:10.1007/978-1-4939-6472-7
11. Ho SN, Hunt HD, Horton RM, Pullen JK, Pease LR (1989) Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene 77(1):51–59
12. Stuckey S, Storici F (2013) Gene knockouts, in vivo site-directed mutagenesis and other modifications using the delitto perfetto system in *Saccharomyces cerevisiae*. Methods Enzymol 533:103–131. doi:10.1016/B978-0-12-420067-8.00008-8
13. Kren BT, Bandyopadhyay P, Steer CJ (1998) In vivo site-directed mutagenesis of the factor IX gene by chimeric RNA/DNA oligonucleotides. Nat Med 4(3):285–290
14. Higuchi R, Krummel B, Saiki RK (1988) A general method of in vitro preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. Nucleic Acids Res 16(15):7351–7367
15. Triglia T, Peterson MG, Kemp DJ (1988) A procedure for in vitro amplification of DNA segments that lie outside the boundaries of known sequences. Nucleic Acids Res 16(16):8186
16. Ochman H, Gerber AS, Hartl DL (1988) Genetic applications of an inverse polymerase chain reaction. Genetics 120(3):621–623
17. Hemsley A, Arnheim N, Toney MD, Cortopassi G, Galas DJ (1989) A simple method for site-directed mutagenesis using the polymerase chain reaction. Nucleic Acids Res 17(16):6545–6551
18. Qi D, Scholthof KB (2008) A one-step PCR-based method for rapid and efficient site-directed fragment deletion, insertion, and substitution mutagenesis. J Virol Methods 149(1):85–90. doi:10.1016/j.jviromet.2008.01.002
19. Zheng L, Baumann U, Reymond JL (2004) An efficient one-step site-directed and site-saturation mutagenesis protocol. Nucleic Acids Res 32(14):e115. doi:10.1093/nar/gnh110
20. Liu H, Naismith JH (2008) An efficient one-step site-directed deletion, insertion, single and multiple-site plasmid mutagenesis protocol. BMC Biotechnol 8:91. doi:10.1186/1472-6750-8-91
21. Xia Y, Chu W, Qi Q, Xun L (2015) New insights into the QuikChange process guide the use of Phusion DNA polymerase for site-directed mutagenesis. Nucleic Acids Res 43(2):e12. doi:10.1093/nar/gku1189

Chapter III

III. General Conclusions and Future Perspectives

This PhD thesis was centred around a cold-adapted glycoside hydrolase family 8 xylanase that had been previously extensively studied by the group where the present thesis was completed [1-4]. The PhD was divided into two main lines of study: one with a more applied focus (Chapters II.I and II.II), where we developed the use of this enzyme in the production of value added products from a marine biomass source; and another with a more fundamental focus (Chapter II.III), where we characterised the pH-dependence of performance of this xylanase.

For the applied line of the study, the objective was to develop and optimise a green chemistry based process for the production of xylan and xylo-oligosaccharides from the red macroalgae *Palmaria palmata*. Xylan and xylo-oligosaccharides have already been demonstrated to have a vast and growing range of applications with a demonstrated use in the food and feed industries, health care, pharmaceutics and cosmetics, and a rapidly growing use in various materials and as feedstocks for breakdown to monosaccharides and conversion to numerous high-value products.

Currently, xylo-oligosaccharides are mainly produced from xylan originating from land based plants, mostly from lignocellulosic materials, via chemical and physical methods with use of hazardous, corrosive chemicals and processes. In fact, numerous feedstocks are reported for xylan and xylo-oligosaccharide production, including: industry and agriculture wastes, hardwoods, softwoods, cereals, corncob, barley hulls and barley spent grains, brewers' spent grain, almond shells, kenaf stem, corn fiber, rice hulls, sugarcane bagasse, straw, etc. [5-10]. Recently, novel technologies including microwave oven treatment have also been investigated, with varying results [11-13].

Various different multi-step procedures for xylan and/or xylo-oligosaccharide production have been reported and some examples to demonstrate the complexity and limitations of these will now be presented. A method for production from lignocellulosic materials, (sugarcane bagasse, poplar, and eucalyptus) was described involving material crushing and drying, alkali-ethanol treatment at 100-120 °C, filtration, concentration, alcohol precipitation, subcritical water extraction at 120-150 °C, pH adjustment, and enzyme treatment at 45-50 °C, pH 5.4, for 45 to 50 hours [14]. Another example, for production from corncob, cottonseed hull and malt cake feedstocks involved NaClO treatment for 15 hours at room temperature, washing with water and drying, 24% KOH soaking for 17 hours at 37 °C, suction filtering, washing with water, dialysis, acetic acid neutralisation and ethanol drying, followed by endoxylanase treatment for 4 hours at 65-70 °C and pH 5.3 [15]. Moreover, another interesting method for the same feedstocks involved use of high

temperature steam blasting (180 °C at 10 kg/cm^{2}G pressure and blowing to atmospheric pressure) or high temperature incubation (180 °C for 20 minutes), followed by centrifugation and endoxylanase treatment at 55 °C [16]. All these processes above are characterised by multiple steps in addition to the pretreatment processes required for preparation of the biomass feedstocks. They involve use of harsh chemical and/or physical processes and produce hazardous wastes, necessitate specialised equipment and expertise and high energy input, as well as heating during enzyme hydrolysis, and give rise to often impure products with frequent xylose contamination and side product and/or pollutant production.

In all the above processes, a lignocellulosic xylan scource was utilised, which results in the generation of branched or non-branched homolinked β-1,4 xylan and xylo-oligosaccharides. Nevertheless, the isolation of β-1,3 linked xylan and xylo-oligosaccharides have also been described for green algae. Again here a multi-step chemical and physical process was employed, this involved algae drying, grinding, high temperature alkaline treatment, acid treatment, $NaClO_4$ bleaching, alkaline extraction, ethanol precipitation and acetic acid washing, followed by xylo-oligosaccharides preparation by weak acid treatment at high temperature and pressure or by enzyme treatment with a β-1,3-xylanase at pH 6 and 37 °C [17].

In addition to homolinked β-1,3 and homolinked β-1,4 xylans, mixed linkage β-1,3/β-1,4 xylan has also been described, namely in the red macroalgae *Palmaria palmata*. Interestingly, notwithstanding a high xylan content (20-60%), an absence of lignin and a weakly linked cell wall matrix, it has apparently been overlooked as a feedstock for xylan and xylo-oligosaccharide production. In fact, most studies on *Palmaria palmata* xylan have focused on its structural makeup, rather than its use as a source of novel xylo-oligosaccharides. Nevertheless, for the structural analyses, a xylan extraction method was described which involved initial preparation of an alcohol insoluble fraction (prepared by ethanol boiling, filtration, repeated ethanol, acetone, and chloroform:methanol treatments and drying), followed by different sequential extractions with saline, alkaline, and/or chaotropic agents (urea, guanidium thiocyanate) treatment [18]. Indeed, a similar method to this was tested during this PhD study as described in Chapter II.I.

Likewise, more recently, Yamamoto et al, 2019 also used a complex, multi-step process for production of xylan, but also xylo-oligosaccharides, from a related Japanese *Palmaria* sp. Here, the macroalgae was lyophilised and ground to a powder, suspended in chloroform-methanol with stirring for 30 minutes and filtered prior to being treated with 20-volumes of acetone and dried.

The dried powder was then resuspended in 40-volumes of water and autoclaved at 121 °C for 20 minutes. Finally, the xylan was then extracted by 8M urea treatment for 24 hours at room temperature, filtrated and dialysis against water before centrifugation before lyophilisation. This xylan rich fraction was then hydrolysed to various xylo-oligosaccharides and xylose by xylanase treatment for 24 hours at 50 °C, pH 4.5, followed by heating to 100 °C for 5 minutes to stop the reaction. Such a complex, multi-step process making use of hazardous chemicals and high temperatures and pressures does not lend itself to safe and economically and environmentally viable xylan and xylo-oligosaccharides production. In addition, only 33.8% of the original xylan in the macroalgae was extracted to give a xylan-rich fraction which was only 52.2% pure, compared with 70% and 73%, respectively, for the process developed in our study (Chapter II.I). Additionally, only 66.6% of the xylan in the xylan rich fraction was converted to xylo-oligosaccharides which also contained undesired side products such as xylose and glucose [19] whereas our study permitted yields up to 90% with complete absence of xylose (Chapter II.II).

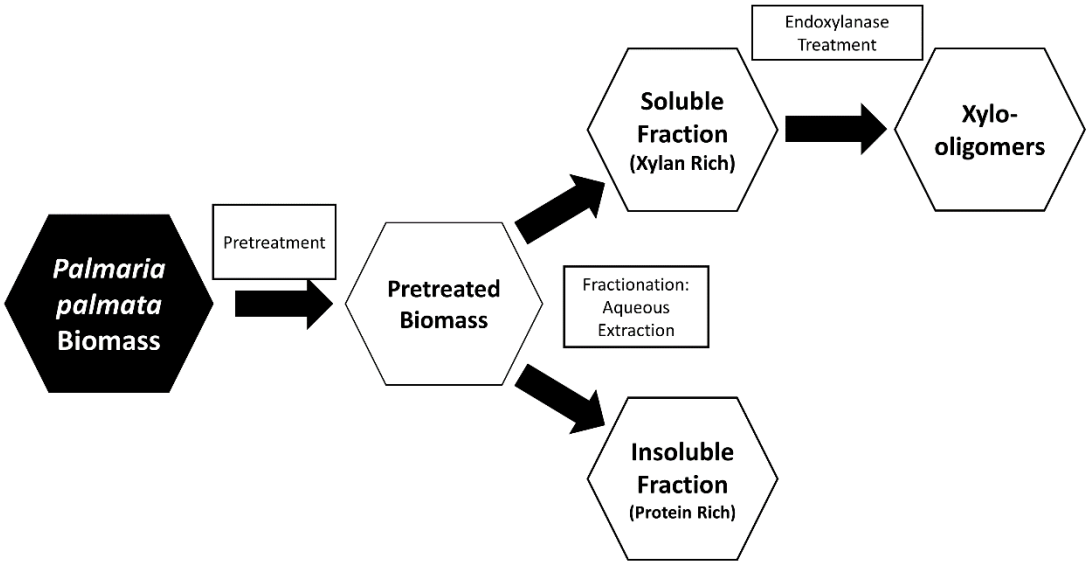

Fig. 1. Schematic representation of optimised process for xylo-oligosaccharides production from the red macroalgae *Palmaria palmata* as developed during this PhD thesis.

The process developed in our study is shown in Fig. 1. It is high yielding, simple, non-hazardous, more eco-friendly and potentially also of lower cost than currently used processes, as described above, while also enabling for production of novel mixed linkage β-1,3/ β-1,4 xylo-oligosaccharides with potentially novel properties and functionalities. In general terms, it can be seen as a 3-step green chemistry based cascading biorefinery system with the first two steps involving *Palmaria palmata* pretreatment and aqueous extraction into a xylan rich fraction and an insoluble, protein

enriched fraction (Chapter II.I). This aqueous-based xylan extraction process was optimised for the different parameters investigated using a statistical multivariate design methodology, resulting in a high yielding soluble xylan extraction of up to ≈70% ± 2.4 of the total original xylan present in *Palmaria palmata* and giving a xylan-rich product composed of 73% ± 3.3 of a mixed-linkage β-1,3/β-1,4 xylan. In contrast to currently available xylan products which are generally poorly soluble, the xylan of the present study is soluble and absent of undesired breakdown (i.e., xylose) and side products and thereby holds the potential for simplifying and enhancing xylan application and opening up new valorisation routes. The xylan product generated was demonstrated to be effective as a soluble substrate in xylanase activity measurements and, following chemical coupling with a suitable dye, to function as a chromogenic substrate for plate based screening of xylanase activity. Future studies should focus on characterising and optimising the macroalgae cultivation and pretreatment processes using procedures similar to those used here for optimisation of the aqueous extraction process. Following optimisation, the whole process, from macroalgae cultivation to xylan and xylo-oligosaccharide production should be submitted to an environmental and economic impact assessment and compared to current production technologies so as to better evaluate the commercial potential. Additionally, studies investigating the utilisation of the xylan produced in further applications such as in materials and high value products preparation should be undertaken.

The final general step of the developed process (Chapter II.II) makes use of highly efficient enzymes, specifically the cold-adapted family 8 xylanase, for the high yielding, highly efficient, ambient temperature conversion of the isolated macroalgae xylan to novel mixed linkage xylo-oligosaccharides. Indeed, comparative studies indicated this enzyme to be the most active of the glycoside hydrolase family 8, 10 and 11 enzymes studied at low to moderate temperatures, while also enabling extensive substrate hydrolysis. Xylo-oligosaccharide production with this enzyme involved initial xylan hydrolyses to an intermediate product before final hydrolysis to the lower molecular weight products. Analyses indicate the products to be principally populated by putative mixed-linkages xylo-oligosaccharides with degrees of polymerisation higher than those observed with the family 11 enzymes studied which also produced high levels of a β-1,4-linked xylotriose. No xylose was produced by the cold-adapted enzyme or the family 11 enzymes studied.

As for the aqueous extraction step, response surface methodology (RSM-CCD) was also employed here to characterise and optimise the xylanase hydrolysis process. This enabled identification of

an ambient temperature process with up to 90% xylo-oligosaccharide product yield directly from the soluble xylan-rich aqueous extracted fraction. The mixed-linkage xylo-oligosaccharides produced have potential as novel structures for improved or even new applications and future studies should focus on better identifying and developing the application of these products. For structure determination, mass spectrometry technologies (GC-MS and HPLC-MS) for the determination of the degree of polymerisation and nuclear magnetic resonance spectrometry and/or hydrolysis studies with β-1,3 and β-1,4 xylanases and/or xylosidases active on short chain substrates, for the analyses of the backbone linkages, should be performed. In relation to application studies, initially the prebiotic potential should be investigated by, for example, examining the effects of these oligosaccharides on human faecal flora and fatty acids production as described previously [20]. The anti-cancer properties of these XOSs should also be investigated, possibly by *in vitro* studies with breast cancer and/or colon cancer cell lines as described by Maeda et al. 2012 [17]. In addition, anti-oxidant and anti-microbial activities could be investigated.

For the fundamental line of research of this thesis, we focused on gaining a better understanding of the physicochemical properties of the cold-adapted family 8 xylanase, and namely the pH dependence of its performance. The most important prerequisite for the application of an enzyme in an industrial application is its stability and activity under process conditions, such as temperature, pH, pressure, salt concentration, composition etc [6, 10, 13]. Interestingly, while much is known about this enzyme, in particular its temperature related characteristics, the effects of pH, another important process parameter, has been little investigated. Importantly also, no studies have as yet investigated the pH dependence of any other glycoside hydrolase family 8 enzyme and, in fact, adaptation of proteins in general to extremes of pH is still poorly studied, with variable and often conflicting observations on adaptation [1, 21].

We investigated the effects of pH on the activity, stability and solubility of the cold-adapted enzyme, with the aim of identifying the factors and their limits defining the pH-dependence of this xylanase and thereby better understanding its suitability for application (Chapter II.III). An in-depth analysis of the pH induced changes in activity, irreversible inactivation, conformation, stability and solubility showed that different phenomena, both reversible and irreversible, play different roles at the various pHs examined. Both reversible and irreversible processes are involved at acidic pHs, with the irreversible processes dominating and being due to protein aggregation and precipitation (Fig. 2). In contrast, at basic pHs, reversible processes are initially involved in the loss of activity, but at

higher pHs, near the pI of the protein, precipitation again dominates whereas structural unfolding is observed at the extreme high pHs investigated.

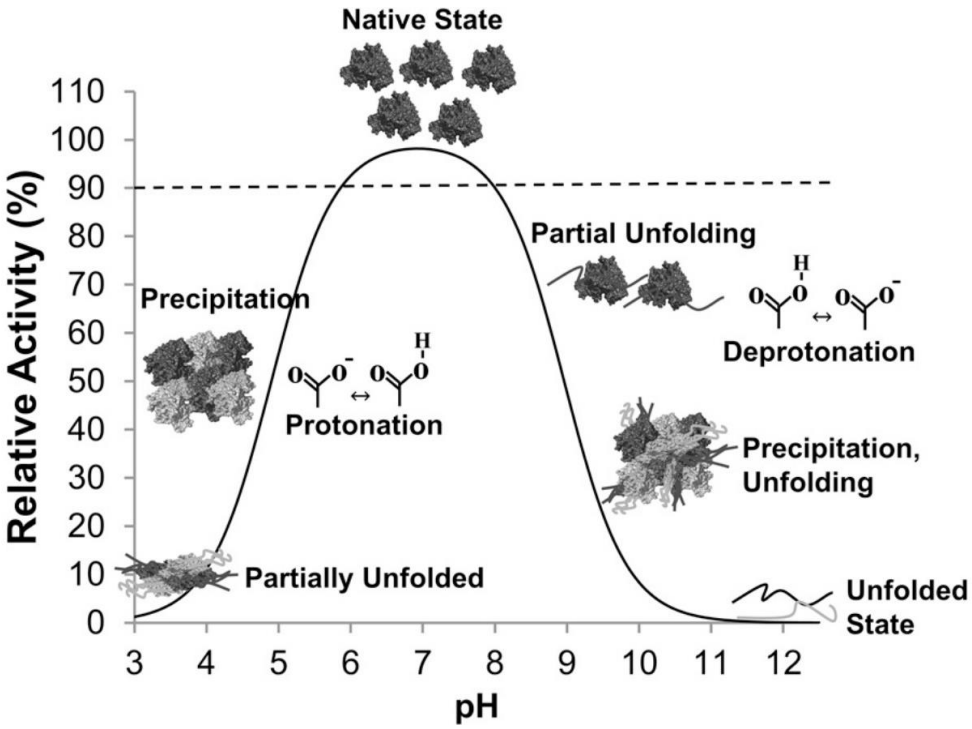

Fig. 2. Representation of the behaviour of pXyl in function of pH and suggested structural modifications. The dashed line represent the range of pHs where pXyl maintain 90% of its activity.

Such insights demonstrate the complexity of factors involved in the pH dependence of proteins and advances our knowledge on design principles and concepts for engineering proteins. While the cold-adapted enzymes has already found successful application in the baking industry and this thesis demonstrates its potential for novel xylo-oligosaccharides production, it is believed to also have potential in further application areas. Such applications include beverages manufacture (≈pH 3.5-5), in animal feeds (≈pH 3-6) and in further biomass valorisation (e.g. fruit and vegetable wastes, with pHs down to pH 2). The results of Chapter II.III clearly show that the pH characteristic of these applications would severely impede the performance of this enzyme. Engineering of the enzyme for enhanced performance under these conditions is thus required and should focus on overcoming the precipitation inherent to acidic pHs for this enzyme. Indeed studies have already been initiated based on reducing the enzymes surface hydrophobicity by mutation of exposed surface hydrophobic residues to the highly polar amino acid serine, and making use of the site-directed mutagenesis approach described in Chapter II.IV. Ongoing studies are focused on production and characterisation of these mutants which is hoped to enable a better understanding of the molecular determinants of acidic pH precipitation in this protein and thereby also advance

our fundamental knowledge of protein structure-function-stability-solubility relationships of proteins in general.

It can be clearly seen that the present thesis enhances the biotechnological potential of the cold-adapted xylanase, further demonstrating the industrial aptitude of this enzyme and opening up new valorisation routes, while also contributing to the multi-valorisation of the macro algae *Palmaria palmata* and promoting growth in blue biotechnology, aquaculture and the seaweed industry.

References

[1] T. Collins, M.-A. Meuwis, I. Stals, M. Claeyssens, G. Feller, C. Gerday, A Novel Family 8 Xylanase, Functional and Physicochemical Characterization, The Journal of biological chemistry 277 (2002) 35133-9.
[2] T. Collins, D. De Vos, A. Hoyoux, S.N. Savvides, C. Gerday, J. Van Beeumen, G. Feller, Study of the active site residues of a glycoside hydrolase family 8 xylanase, Journal of molecular biology 354(2) (2005) 425-35.
[3] T. Collins, A. Hoyoux, A. Dutron, J. Georis, B. Genot, T. Dauvrin, F. Arnaut, C. Gerday, G. Feller, Use of glycoside hydrolase family 8 xylanases in baking, Journal of Cereal Science 43 (2006) 79-84.
[4] A. Dutron, J. Georis, B. Genot, T. Dauvrin, T. Collins, A. Hoyoux, G. Feller, Use of family 8 enzymes with xylanolytic activity in baking, Google Patents, 2012.
[5] A.A. Aachary, S.G. Prapulla, Xylooligosaccharides (XOS) as an Emerging Prebiotic: Microbial Synthesis, Utilization, Structural Characterization, Bioactive Properties, and Applications, Comprehensive Reviews in Food Science and Food Safety 10(1) (2011) 2-16.
[6] C. Amorim, S.C. Silvério, K.L.J. Prather, L.R. Rodrigues, From lignocellulosic residues to market: Production and commercial potential of xylooligosaccharides, Biotechnology Advances 37(7) (2019) 107397.
[7] A. Moure, P. Gullón, H. Domínguez, J.C. Parajó, Advances in the manufacture, purification and applications of xylo-oligosaccharides as food additives and nutraceuticals, Process Biochemistry 41(9) (2006) 1913-1923.
[8] A.K. Samanta, N. Jayapal, C. Jayaram, S. Roy, A.P. Kolte, S. Senani, M. Sridhar, Xylooligosaccharides as prebiotics from agricultural by-products: Production and applications, Bioactive Carbohydrates and Dietary Fibre 5(1) (2015) 62-71.
[9] A.A. Aachary, D. Gobinath, K. Srinivasan, S.G. Prapulla, Protective effect of xylooligosaccharides from corncob on 1,2-dimethylhydrazine induced colon cancer in rats, Bioactive Carbohydrates and Dietary Fibre 5(2) (2015) 146-152.
[10] D.S. Naidu, S.P. Hlangothi, M.J. John, Bio-based products from xylan: a review, Carbohydrate Polymers 179 (2018) 28-41.
[11] A. Teleman, J. Lundqvist, F. Tjerneld, H. Stålbrand, O. Dahlman, Characterization of acetylated 4-O-methylglucuronoxylan isolated from aspen employing 1H and 13C NMR spectroscopy, Carbohydr Res 329(4) (2000) 807-15.
[12] Y. Wang, J. Zhang, A novel hybrid process, enhanced by ultrasonication, for xylan extraction from corncobs and hydrolysis of xylan to xylose by xylanase, Journal of Food Engineering 77 (2006) 140-145.
[13] P.F. Ávila, J.P.L. Franco Cairo, A. Damasio, M.B.S. Forte, R. Goldbeck, Xylooligosaccharides production from a sugarcane biomass mixture: Effects of commercial enzyme combinations on bagasse/straw hydrolysis pretreated using different strategies, Food research international (Ottawa, Ont.) 128 (2020) 108702.
[14] J. Jiang, Y. You, W. Zhang, S. Yang, L. Zhu, A kind of preparation method of xylo-oligosaccharide, CN108251472A, 2018.
[15] Y. Suwa, K. Koga, S. Fujikawa, M. Okazaki, T. Irie, T. Nakada, *Lactobacillus bifidus* proliferation promoting composition, EP 0265970 B1, 1992.
[16] W. Broekaert, C. Courtin, J. Delcour, (Arabino)xylan oligosaccharide preparation, WO/2009/117790, 2009.
[17] R. Maeda, T. Ida, H. Ihara, T. Sakamoto, Induction of apoptosis in MCF-7 cells by β-1,3-xylooligosaccharides prepared from *Caulerpa lentillifera*, Biosci Biotechnol Biochem 76(5) (2012) 1032-4.

[18] E. Deniaud Bouet, J. Fleurence, M. Lahaye, Interactions of the mix-linked β-(1,3)/β-(1,4)-D-xylans in the cell walls of *Palmaria palmata* (Rhodophyta), Journal of Phycology - J PHYCOL 39 (2003) 74-82.
[19] Y. Yamamoto, H. Kishimura, Y. Kinoshita, W. Saburi, Y. Kumagai, H. Yasui, T. Ojima, Enzymatic production of xylooligosaccharides from red alga dulse (Palmaria sp.) wasted in Japan, Process Biochemistry 82 (2019) 117-122.
[20] C. Amorim, S.C. Silvério, B.B. Cardoso, J.I. Alves, M.A. Pereira, L.R. Rodrigues, In vitro assessment of prebiotic properties of xylooligosaccharides produced by Bacillus subtilis 3610, Carbohydrate Polymers 229 (2020) 115460.
[21] M. Barroca, G. Santos, B. Johansson, F. Gillotin, G. Feller, T. Collins, Deciphering the factors defining the pH-dependence of a commercial glycoside hydrolase family 8 enzyme, Enzyme and Microbial Technology 96 (2017) 163-169.