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1. EXECUTIVE SUMMARY

Peak oil and peak soil, peak water and peak grain – the beginning of the 21st century marks a crucial transition, from an oil-based economy to a bio-based economy, from exploiting fossil resources to using renewable resources. In many areas such as material sciences or energy, this transition to a knowledge-based bio-economy will heavily rely on the large and diverse group of biopolymers. Bio-based renewable energies mostly rely on the degradation of biopolymers, essentially starch and lignocelluloses, and the consumption of their monomeric building blocks to release the energy stored in them during their photosynthetic biosynthesis. Renewable biomaterials, in contrast, typically rely on the production of biopolymers, either by extraction from biological sources or by biotechnological production means, sometimes combining both. Most biopolymer-based biomaterials such as many polysaccharides, lignin, or rubber are most prominently used for their superior structural properties only. Some biopolymers, however, and in particular polysaccharides, also possess functional properties, and these can be at the basis of a broad spectrum of applications, ranging from food sciences and agriculture over cosmetics and pharmaceutics to biomedical sciences. The potential of such functional biopolymers is evident, as they combine superior material properties with excellent biocompatibility and often highly versatile biological activities, promising advanced applications in many life-science related market fields. However, the potential of polysaccharides is still largely unfulfilled today, mostly as polysaccharides are very demanding both in terms of their chemistry and in terms of their biology. In particular, the typical microheterogeneity, which is a hallmark of natural polysaccharides, is a severe hurdle in establishing reliable structure/function relationships and, eventually, in developing successful applications based on them.

Natural polysaccharides have evolved to fulfill a plethora of functions in their natural biological context, and as each individual polysaccharide typically fulfills multiple roles, their structures represent a balanced compromise to achieve the best overall performance. This, however, is different in biotechnological applications where the polysaccharide used typically has to fulfill a special singe purpose, so that there is room for improvement over the natural compound. Also, the roles attributed to polysaccharides in biotechnological applications may differ from their natural roles, so that modifications may be required to change their structural and functional properties. Today, these modifications aiming at improving the performance of a polysaccharide are typically attempted using chemical means, such as acid or alkaline treatment, or the introduction of additional substituents. These chemical methods, while typically well established and easily upscaled to industrial dimensions, often have severe limitations, in particular regarding their specificity. Also, their environmental burden in terms of energy or water consumption, or in terms of toxic waste production, is sometimes high. The PolyModE project, therefore, aimed at developing enzymatic tools to perform such modifications specifically and in an environmentally benign form.

The central assumption of the PolyModE project was that it is the pattern of substitution of complex functional polysaccharides that fine-tune their physico-chemical properties and/or their biological activities. These could be patterns of e.g. acetylation, sulfation, or methyl-esterification, but they could also be patterns in the sequence of monosaccharide building blocks or their glycosidic linkage type, or even patterns in the distribution of different side chains. We predicted that nature uses enzymes to ‘ write’ these patterns, but also to ‘ read’ them, i.e. to specifically and partially degrade the complex polysaccharides to generate specific oligosaccharides as the individual ‘ words’ of the language of sugars. The PolyModE project targeted writing and reading enzymes for the guluronic acid distribution in alginates from red algae (C5-epimerases, lyases), for the pattern of sulfation in carrageenans from brown algae (sulfatases and sulfurylases), for the pattern of acetylation in chitosans from shrimp and fungi (deacetylases and hydrolases) as well as in the pattern of sulfation in human glycosaminoglycans (sulfotransferases and sulfatases), in the patterns of methyl- and acetyl-esterification in pectins from higher plants (acetyl- and methyl-esterases) and in the distribution patterns of acetylated and/or pyruvylated side chains in bacterial xanthan gums (acetyl-esterases, lyases, and hydrolases). These six polysaccharides represent the most important or most promising functional polysaccharides today, with diverse applications of alginates, carrageenans, pectins, and xanthans as functional food ingredients due to their superior material properties, in particular gelling abilities, and with highly promising applications of glycosaminoglycans and chitosans in biomedical fields due to their versatile and highly specific biological activities.

Six work packages were devoted to these polysaccharides and connected through a central work package focusing on the development of generic techniques in bioinformatics and molecular genetics, heterologous expression and fermentation, enzyme characterization and optimization, as well as structural and functional characterization of enzymatically modified polysaccharides. All seven work packages were highly successful and reached at least one of the two major goals so that in all cases, significant progress beyond the state of the art was achieved. Unfortunately, we were eventually denied a cost-neutral six-month extension of the project which would have allowed us, at no extra cost, to advance these results to the point where they would have been ready to be taken up by industry for further development and integration into their large scale production processes. We now aim at securing alternative funding so that these results will not be lost to European industry and society.

2. SUMMARY DESCRIPTION OF THE PROJECT CONTEXT AND OBJECTIVES

Complex sugars are the most abundant biomolecules, occurring in countless variants. As renewable resources, they are of high priority to man in many areas, from food sciences to medical applications. They can build an enormous diversity of different structures, and information is contained in these structures. Our immune system, for example, often recognizes pathogens by typical sugar structures on their surface. Another example are the gelling properties of pectins which are determined largely by the three dimensional structures of these complex sugars. However, the ' language of sugar' is far more complex than that of genes and proteins; and even today, researchers can hardly read it, much less understand it. So far, we only know a few ' words' of the language of sugars. But if we want to use this ' language' e.g. for medical purposes, we will not only have to be able to read and understand it, we will also need to learn writing it.

The **goal of the PolyModE project** was to develop ' reading and writing tools' for the language of sugars. For this purpose, the PolyModE researchers investigated and used the cells' own machinery. In cells, both processes are performed by enzymes, and these **polysaccharide modifying enzymes** are in the focus of the PolyModE project. We needed to **identify** them first, then to **produce** them in large quantities and in high purity using molecular genetic methods. We have studied their properties, and optimized them for use in the synthesis, modification, and analysis of complex sugar structures in cell free systems. Thus, the research of the PolyModeE project opened up new and hitherto **untapped sources for known hydrocolloids** to be used as functional food additives, but it also aimed at generating **novel complex sugars** with further improved properties. Such specifically acting, complex sugar polymers which are produced in a **completely biological process** will have an **enormous potential** in many fields. As they are well compatible with the human body and as they are easily degraded in the environment, they can play important roles in medical, food, and technical applications. Biologists and chemists, microbiologists and biochemists, molecular geneticists and biotechnologists from three European Universities teamed up with two multinationals as well as half a dozen biotech companies in the PolyModE project. Together, these partners possessed a very **broad spectrum of expertise and experience** in the development and use of state-of-the-art methodology in sugar biology and chemistry, perfectly complementing each other and allowing the direct transfer of results from lab to industry scale. Our consortium consisted of the **whole product chain** from the producers of the **raw materials** to the developers, producers, and users of the **recombinant enzymes**, and further to the analysis and evaluation of the **modified polymers**.

For the PolyModE project, we selected **six polysaccharide classes** with great **economic potential**, namely alginates, carrageenans and agars, chitosans, glycosaminoglycans, pectins, and xanthan gums. Apart from the **fibre and gel forming** polysaccharides such as starch, pectins, alginates, carrageenans, agars, or guar and xanthan gums which are used widely in the food and beverages industry as thickening, gelling, and stabilizing agents, polysaccharide with **biological activities**, such as chitosan, heparin, hyaluronic acids, and other glycosaminoglycans are those with a promising economic potential. The biological activities are highly interesting targets for the **pharmaceutical industry.**
Today, **chemicals** are mainly used to convert these biopolymers. However alternative and/or complementing **enzymatic modifications** will **reduce energy input and environmental impact** while at the same time offering the advantage of **higher specificity** and, thus, potentially yielding **novel polymers** and/or oligomers with **advanced physico-chemical properties and biological functionalities**. Using enzymes involved in the biosynthesis or biodegradation of polysaccharides (i.e. epimerases, deacetylases, sulfurylases) will e.g. allow us to mimic the formation of hydrogels as they occur *in vivo*. We can then expect to discover **biomimetic** and to develop **bioinspired** textures with novel functionalities
The **bottleneck** in enzymatic modifications of polysaccharides today is the **availability of appropriate enzymes** with known **enzymatic properties**, such as e.g. substrate specificities, reaction mechanisms, and product patterns, as well as **suitability** for large scale biotechnological processes, such as e.g. temperature stability, co-factor requirement, product inhibition. To ensure low costs and high reproducibility, **purified recombinant enzymes** are required. These have the added advantage that they can be optimised using state of the art technologies of protein engineering through directed evolution. This process of enzyme optimization relies on a strong basis of appropriate genes coding for a broad diversity of polysaccharide modifying enzymes. Given the estimate that close to 99 % of micro-organism are non-culturable and, therefore, mostly unknown to science today, metagenomic approaches are best suited to try and harness the potential nature has to offer. The success of such an approach largely depends on the educated choice of environmental sample used for the establishment of the metagenomic libraries. An **alternative strategy** was to use transcriptomic approaches to identify transcripts in suitable microorganisms under growth conditions that induce the expression of polysaccharide modifying enzymes (e.g. growth of a marine bacterium in the presence of specific algal polysaccharide as a sole carbon source). Coupled with the genomic data of the same bacterium, this method gives easy access to enzymes that at least metabolise and possibly degrade or modify the polysaccharide of interest. These unbiased **metagenomic and transcriptomic approaches** were complemented by **knowledge-based approaches** in which we mined the genomes and proteomes of organisms known to either produce or degrade the targeted polysaccharide.

The **hydrocolloids associated with polysaccharides** originate from plant (e.g. plant cell walls, tree exudates, seeds, tuber/roots, seaweeds) or animal sources (hyaluronan, chitin, chondroitin sulphate). Additionally, certain types of bacteria and fungi can also produce hydrocolloids (xanthan, gellan, wellan). In general, hydrocolloids ' work with water' ; they thicken, suspend, gel or stabilize a solution. **Functionality** in a given commercial application is controlled by a number of factors, the most important of which are the structure and conformation in aqueous solutions and the way these are influenced by specific or non-specific interactions with other ingredients. The degree of polymerisation and size (molecular weight and dimensions) are other factors that determine their **physical properties**; another crucial factor, and **the one in the focus of the PolyModE project**, are patterns in the fine structure of the hydrocolloids. Hence, considerable efforts to achieve accurate **characterisation** is one important factor to determine the structure-function relationships. In the food industry, their ability to emulsify oil droplets, modify flow properties, gelling ability, stabilisation, prevent sugar crystallisation, impart texture and mouth feel of aqueous foodstuff are often utilised. A range of novel, **nutritional and nutraceutical properties** are being discovered and established for hydrocolloids.

Hydrocolloids are used in **three key segments**: industrial, food and oilfield applications. Overall, food is the dominant application for hydrocolloids, followed by oil field and pharmaceutical applications. The individual hydrocolloids in food can be divided into three major segments, based on overall value. Starches and gelatine are the giant category with over $ 1 billion in sales each. The second group includes five hydrocolloids (pectin, carrageenan, xanthan, alginates, agar) with sales between $ 200-700 million and the remainder are those (CMC, Arabic, Guar, MCC, LBG, MC / HPMC) with sales less than $ 200 million sales each. IMR International reported over 20% value growth in the **total food hydrocolloid market (US$6.1 billion)** in 2011; volume growth remained stable at 3-4%.[1] The value of the guar sector, which rose from $100 million in 2010 to $507 million in 2011, was the central component of this growth. Conventionally, guar was used largely in food, textile, pharmaceuticals, paper, mining and also in oil well drilling. However, over the past few years activity in hydraulic fracturing has increased tremendously and the demand from companies servicing oil- and gas-producing fields has shot up. Guar is a key ingredient in the fluids used to crack open, or hydraulically fracture, shales. Starches and gelatine, which combine to make up over 75% of the volume of the hydrocolloid sector and 40% of the value, were stable. Prices in real terms had been stable or declining for the last two decades. **Pectin, xanthan** and MC / HPMC are expected to be the most rapidly growing hydrocolloids each for their specific reasons in the food hydrocolloid market. **Pectin** is the most **label and consumer friendly** of all hydrocolloids. Consumer concerns and label preferences are paramount in hydrocolloid selection and pectin is a ' no problem' hydrocolloid on a food label. **Xanthan gum** has become the ' **ubiquitous**' hydrocolloid which is appearing on an increasing number of labels worldwide. But despite the specialty functions that they exhibit, hydrocolloids became treated as a commodity. Usage levels for hydrocolloids only form up a fraction of final formulations, yet the stabilizing and texturizing properties that the ingredients exhibit make them an essential component of processed foods as diverse as sauces, yogurts and beverages i.e. hardly a commodity. Opportunities should therefore be exploited to **overcome the commodity image**.[2]

By broadly addressing concrete, **industry defined needs** in the preparation of **novel hydrocolloids** for food and medical applications, the PolyModE project did exactly deliver this. By identifying, characterising, and optimizing polysaccharide modifying enzymes with an emphasis on modification and analysis of patterns of substitution, we even aimed at **opening up a new field for knowledge-based applications in the glycosciences and -technologies.** Well characterised products of enzyme-catalysed bio-transformations will represent novel types of hydrocolloids with unprecedented purity and, thus, known and reliable physico-chemical properties and biological functionalities, promising the development of reliable and innovative applications in the food and medical sciences. KBBE-2007-3-2-07 ' Novel Enzymes' explicitly called for *the search for novel enzymes and micro-organisms from specific or extreme environments, whether by direct isolation or by metagenomics, to create an expanding range of biological catalysts for industrial use.*

Many worldwide corporations have recognized the bio-based technologies as one of the key drivers of sustainable growth. However, the biological process is often considered only when the chemical arsenal has failed to achieve synthesis of the target molecule. This is primarily because of the **unavailability of the desired enzyme** to catalyze the reaction in an efficient manner. The exploitation of new types of enzymes, improvements of enzyme properties and of the production process are overall goals of innovation in the enzyme manufacturing industry. With regard to the PolyModE project we carefully established the most pressing or promising needs in terms of enzymatic modification to (i) reduce the **cost of production**, (ii) reduce the **energy input** required, (iii) reduce the output of potentially **harmful wastes**, (iv) increase the **range of products** available, (v) **add value** to the products, (vi) broaden the **market sector**, and/or (vii) allow analysis of **substitution patterns**.

[1] IMR International, (2012), on http://pubs.rsc.org | doi:10.1039/9781849734554-00001

[2] Weyers, R, (2012), ' Time for a Hydrocolloid Rethink' , The World of Food Ingredients, June 2012: 15-19

Enzymes are applied in various fields, including technical use, food manufacturing, animal nutrition, cosmetics, medication, and as tools for research and development. At present, almost 4000 enzymes are known, and of these, approximately 200 microbial original types are used commercially. However, only about 20 enzymes are produced on truly industrial scale. With the improved understanding of enzyme biochemistry, fermentation processes, and recovery methods, an increasing number of industrial enzymes can be foreseen.

The global enzyme market is estimated to rise 7 percent **to $8.0 billion in 2015** and is **dominated by the food and beverage industry**, which benefits from the expansion of the middle class in rapidly developing economies. Growth came mostly from baking enzymes and other smaller applications such as fat and oil processing. From a regional perspective**, North America** was, by far, the largest consumer of enzyme products followed by the second largest regional market of Western Europe. The world enzyme demand is satisfied by about **12 major producers** and 400 minor suppliers. Nearly 75% of the total enzymes are produced by **three top enzyme companies**, i.e. Denmark-based Novozymes, US-based DuPont (through the May 2011 acquisition of Denmark-based Danisco) and Switzerland-based Roche. The market is highly competitive, has small profit margins and is technology intensive.[3]

By including **world market leaders** as well as **small start-up** and well established **medium-sized SME**, the PolyModE consortium could on the one hand rely on the well-established research base of European companies producing a lion' s share of the world' s industrial enzymes to **quickly reach and penetrate the market**, but it could on the other hand also draw on the **flexibility** and **creativity** of a number of highly successful biotechnology SME involved in discovery and development of micro-organisms and enzymes for specific applications so that we were able to quickly react on problems and opportunities arising in the course of our project, going beyond the state-of-the-art.

3. DESCRIPTION OF THE MAIN S&T RESULTS/FOREGROUNDS

3.1. Work package 1 - Alginate modifying enzymes; WP leader: Mirjam Czjzek, Station Biologique de Roscoff (CNRS)

Context of the project:

Alginate is a linear polysaccharide comprised of 1– 4-linked · -D-mannuronic acid (M or ManA) and its C-5 epimer · -L-guluronic acid (G or GulA).Commercially alginate is harvested from seaweed and has been utilized extensively for a variety of industrial and biotechnological purposes. The annual world-wide alginate production is around **45,000 tons**, which is produced from various types of brown seaweed (*Laminaria, Lessonia, Ascophyllum, Ecklonia* and *Durvillea* species). Alginate with low ManA/GulA ratio has **higher calcium reactivity** and stronger gels can be made from this type. In many different applications, alginates act as **stabilising, thickening, and/or gelling agents**. The main uses are **food additives** (**E400, E401, E402, E403, and E404**), e.g. in baked goods, low-fat spreads, restructured fruit and vegetables, restructured meat and fish, fruit preparations, cream fillings, and milk-based products. In recent years, much attention has been drawn to new and promising applications of alginates in pharmacy and medicine, *e.g.* in drug or protein delivery, cell encapsulation, tissue regeneration, surgery, and wound management. With the entry of alginate-based biomaterials into the field of human medicine, the term ' tailor-made alginate" has been introduced. This means that one ideally wants to produce and use alginate molecules with defined properties optimally suited for a given application. In biological organisms, this is performed by specific enzymes, the mannuronan-C5-epimerases, which convert the synthesized mannuronic acid into its C5-epimer, a reaction that occurs *in planta* after polymerisation only. In principal, the epimerisation can be performed using either the better known **bacterial alginate epimerases** or the less studied **algal enzymes**.

In addition, and due to these novel applications, there is an emerging need for more **detailed information concerning the alginate fine structure.** Although the total monomer composition and the dyad and triad frequencies can be elucidated by, for example, high resolution NMR spectroscopy, there is **limited knowl**edge on the **distribution and the absolute length of the various block types**. A promising approach to the challenge of alginate sequence elucidation is the use of **lyases** to degrade complex alginate molecules followed by **analyses** on the resulting population of **alginate oligomers**. Consequently, the aim of Workpackage 1 consisted in the detailed study of these enzymes, **mannuronan-C5-epimerases** and **alginate lyases**.

[3] Shuang Li , et al (2012), ' Technology Prospecting on Enzymes: Application, Marketing and Engineering, Computational and Structural Biotechnology Journal, 3: 1-11, http://dx.doi.org/10.5936/csbj.201209017

Objectives of the project:

The major objective of the project was to identify, produce, characterize and optimize the use of **algal mannuronan-C5-epimerases** for the production of alginates with **controlled M/G content** (defined as primary targets). The main strategy applied by the beneficiary was to explore and **exploit the natural richness** and diversity of **algal mannuronan-C5-epimerases** that have been identified in the recently published **genomic data** of the **brown algaea** ***Ectocarpus siliculosus*** (around 32 isoforms). After having identified and bioinformatically selected the potential targets, the subsequent tasks consisted in i) understanding and applying a gene model to get access to the **genomic nucleotide sequences** of the targets; ii) experimentally **extract the cDNA** of as many targets as possible iii) finding a **suitable expression system** iv) **purifying and characterizing the enzymes**. All these enzymes were selected as primary targets in order to have access to various isoforms of the major alginate-modifying. Indeed, the presence of the large number of isoforms in brown algal sources suggests that the different isoforms of algal mannuronan-C5 epimerases might yield polymers with different patterns of GulA and ManA distribution. The **recombinant proteins** are then to be used to **modify alginate polysaccharides** in a **controlled**, tailor-made manner.

Specific tasks that were defined on **primary targets**:
1. experimentally extract the cDNA of as many targets as possible
2. finding a suitable expression system
3. purifying and characterizing the enzymes.

The second major objective consisted in the identification of **alginate lyases with new specificities**, and subsequent purification and characterization of various alginate lyases that can be used to **analyze and determine the precise structures of the produced and modified alginate polysaccharides** (defined as secondary targets). The chosen source for exploring enzymes performing alginate degradation was the recently sequenced genome of a major polysaccharide degrading marine Bacteroidetes, *Zobellia galactanivorans*. Several genes, most probably coding for alginate lyases, are organized in potentially co-regulated operons within the genome of this marine bacteria. In order to identify enzymes with differential activity, we have undertaken the experiments necessary to understand the **interplay and expression pattern** of these enzymes in the **context of alginate degradation** by the flavobacteria.

Specific tasks that were defined on **secondary targets**:
4. identification of alginate lyases with new specificities
5. purification and characterization of various alginate lyases
6. use of enzymes to analyse and determine the precise structures of the produced alginate polysaccharides.

Scientific and technical outcome of our research:

The detailed bioinformatics and phylogenetic analyses of the 32 sequences potentially coding for mannuronan-C5-epimerases in *E. siliculosus*, allowed **defining six sub-groups of epimerases**. Our hypothesis is that these sub-groups coincide with fine tuned differences in substrate specificity and/or mode of action. We therefore concentrated on isolating cDNA for at least one epimerase of each sub-group. cDNA of six manuronan-C5-epimerases from *Ectocarpus siliculosus* have been successfully cloned into expression plasmids during the first 2 years (D1.1). However, the heterologous expression revealed to be very difficult and we alternately **switched to the synthesis of the genes identified in the genome data of *E. siliculosus***, with the help of Genart, partner 10. Twenty-two constructions, (i.e. full length and catalytic domain only) covering eighteen individual manuronan-C5-epimerase genes, were synthesized and optimized for various heterologous expression systems. All forces, present in the consortium of PolyModE, with experience in different expression systems contributed to the trials on heterologous expression of the targeted enzymes.

- E. Devich and N. Tremillon, GTPtech, partner 13; expression in *L. lactis*, *P. pastoris* and CHO cells
- M. Krog Larsen, Dupont/Danisco, partner 7; expression in *Trichoderma reesei* strains
- P. Petrova and D. Liutskanova, Bulg.Acad.Sci (BAS), partner 6; expression in *P. Pastoris*
- C. Hervé and R. Fischl, St. Biol. Roscoff CNRS, partner 3; expression in *E. coli*, *P. pastoris* and cell free medium

The various expression vectors were successfully cloned into the respective expression systems by each partner and soluble protein expression was monitored to identify the optimal expression systems. As a result of these efforts, **four different manuronan-C5-epimerase targets** have been expressed at least once in soluble form, although not all with the yield necessary to undertake the biochemical characterization. In two cases, the purified enzymes were successfully submitted to activity tests monitoring the variations of M/G ratio by NMR of various industrial alginates (D1.4). Overall, the 4 years of experience gathered on heterologous expression of members of this enzyme family allowed us to figure out that

i) **expression in *E. coli*** was **possible** but needed to be **followed by a refolding strategy** to obtain **active** enzyme. The advantage of this strategy is that rather **large yields** of pure enzyme can be obtained (20 to 40 mg/L of cell culture) (D1.3)

ii) **soluble expression of active manuronan-C5-epimerase** is obtainable in ***L. lactis***. The advantage of this strategy is that the expression system is directly **compatible for many industrial applications**. (D1.3). The publication of these results in form of scientific articles in peer-reveiwed journals is in preparation.

In conclusion, the PolyModE project allowed figuring out successful **strategies to heterologously produce algal manuronan-C5-epimerase.** The results also show that albeit substantial technological advancements have been made, the heterologous **expression of eukaryotic enzymes still remains a bottleneck** in the strategy of enzymatic biotechnology. Due to this, we were not able to fulfil the announced up-scale of enzyme production and use for industrial processes. At short-term, enriched by our experience obtained within the PolyModE project, further attempts to produce more purified and active enzyme samples are currently ongoing. At mid- and long-term the production of this type of enzymes for **industrial** applications **will need further development of heterolgous expression systems, adaptation of existing technology** and to perform the **up-scale** to test the feasibility at larger scale.

In parallel to the identification and production of **manuronan-C5-epimerase**, we focused on the identification of new alginate lyases with **novel substrate specificities**. This work has successfully been concluded by a transcriptomic approach of the marine bacterium *Z. galactanivorans* through the experimental identification of operons containing **seven alginate lyases** [1].

Subsequently, we have performed the purification and characterization of **three alginate lyases**, which display **different substrate specificities.** While the alginate lyase (belonging to a new family) from *P. alginovora* is highly specific for M-blocks [2], the PI7 enzyme from *Z. galactanivorans* named AlyA1 only cleaves between two G-units. The third enzyme, AlyA5, is an exo-alginate lyase and is able to cleave one by one, any unit starting from the non-reducing end [3]. The enzymes are produced and purified routinely from an *E. coli* heterologous expression and represent a good base to perform **alginate fingerprinting experiments** to detect M/G ratio variations (D1.4). The combined use of **NMR and mass-spectrometry** of the degradation products of various alginate samples, after digestion with the here reported alginate lyases, is actually being established and a ' finger printing' analysis protocol is under preparation. The **alginate ' finger printing' method** will be prepared for publication.

3.2. Work package 2 - Carrageenan modifying enzymes, WP leader: William Helbert (CNRS)

Context of the project:

Carrageenans represent a class of sulphated galactans exhibiting a **wide spectrum of chemical structures**. This class of macromolecules, occurring exclusively in cell wall of numerous **red algal**, shares a common **galactan backbone**, the galactoses residues being **linked alternatively by α (1,3) and β(1,4) glycosidic linkages**. The disaccharide repetition moieties or ' **carrabiose**' units are classified according to the number and position of sulfate ester groups, as well as the occurrence of 3,6-anhydro-ring on the 1,4-linked residue found in gelling carrageenans. For example, the three most industrially exploited carrageenans, namely **kappa-** (κ-), **iota-** (ι-) and **lambda-** (λ-) carrageenans, are distinguished by the presence of one, two and three ester sulfate groups per repeating disaccharide unit, respectively.

The total raw material consumption for carrageenan production is estimated to be about 250,000 tons dry weight of seaweed, yielding close to **50,000 tons of carrageenans with a value of ca. 450 million Euro**. Due to its ability to **interact** with **milk proteins**, carrageenans are widely used in milk-based applications such as in ice cream to prevent whey separation, in milk gels to obtain the desired consistency, in chocolate milk to avoid the precipitation of cocoa particles, and in low-sugar jams and jellies for gelling purposes. In **meat products**, carrageenan' s **gelling properties** bind moisture, reducing cooking loss and improving slicing properties, while pâtés, sausages and similar emulsified products benefit from improved firmness and texture. Applications of carrageenan depends strongly on their **algal origin and extraction procedures**. Up to now, the control of the functional properties of carrageenan is based on the **selection of red algae**, the **formulation** of carrageenan **with other macromolecules** (proteins, polysaccharides) and **method of extraction**. Therefore there is strong need of new process allowing **fine control** of the carrageenan properties and the development of **biotechnological approaches**. Two main classes of enzymes offers interesting perspectives: the **galactose-6-sulfurylases** and the **carrageenan-sulfatases**.

The **conversion** of μ-(mu-) carrageenan into κ–carrageenan, and ν–(nu-) into ι-carrageenan greatly **enhances gelling properties**. This reaction which induces the formation of anhydro-galactose by the removal of the 6-sulfate group is industrially done through the use of **hot alkaline treatment**, but **seaweeds use galactose-6-sulfurylases** for this conversion. Replacing hot alkaline treatment with enzymes has a number of environmental and technical **advantages.** On the one hand, the enzymatic process would **reduce**

the use of **chemicals and energy**. On the other hand, **less molecular degradation** would occur during conversion, yielding higher molecular weights and, thus**, improving functional properties**.

Another enzymatic process which consist to convert **ι– and ι/κ–hybrid carrageenans into pure κ-structures** present also very strong interest. **κ-Carrageenan** is the **main industrial carrageenan** because of its high gel strength. This second type of conversion requires removal of the 2-sulfate-group, however, the sulfate ester linkages are chemically very stable, and there are **no** apparent or practical **chemical methods** to carry out this desulfatation **without also lowering the molecular weight** of the polymer. An enzymatic process would, therefore, be needed, but it is currently not known whether enzymes exist that actually can do this. The main advantage of these conversions would be that they would allow for the use of **many red seaweed species** to produce κ– and ι-carrageenan. In addition, hybrid-carrageenans have composition and distribution of κ- and ι-structures as a function of algal sources, but the use of **sulfatase could result in control distributions** with, therefore, control properties.

What were the objectives when we started the project ?

Because carrageenans are the products of a complex biosynthetic pathway, they are not homopolymers but heteropolymers or hybrid structures composed of sequence of several moieties. This means, for example, that the so-called **κ-carrageenan** defines a family of polysaccharides composed **mainly**, but not only, of **κ-carrabiose units**. The most classical **hybrid structures** of carrageenan are those found in **native or unprocessed κ- and ι-carrageenan chains**. They usually contain fractions of their biosynthetic precursors named μ- and ν-carrageenans, respectively. Other carrabiose combinations have also been demonstrated such as the hybrids κ–/ι–carrageenans in several species of Gigartinales and κ–/β–carrageenans in *Furcellaria lubricallis*.

The **physico-chemical properties** of carrageenans are **directly connected** to their **chemical structures**. It is well-known that the amount of the biosynthetic precursors, μ- and ν-carrabiose units occurring in κ- and ι-carrageenan chains respectively, affects strongly the capability to these macromolecules to make gels. The cyclisation of the μ- and ν-carrabiose units leading to κ- and ι-carrabiose units obtained by hot alkaline treatments strongly enhance the gel strength of the carrageenans. Other hybrid structures of economic interest are the **κ-/ι-carrageenans**, which composition include a wide range of κ-/ι-units ratio. Hybrid κ/ι-carrageenans containing **about 20 to 45% ι-carrabiose units** are also called **κ-2-carrageenans**, because they are κ-carrabiose rich carrageenans but having functional properties that differ strongly to that of κ-carrageenans. Evidences for the hybrid nature of κ-/ι-carrageenans were provided, at first, by comparing dairy and physical performances of κ-/ι-carrageenan extracts with mixtures of standard κ- and ι-carrageenans. More recently, a second line of evidence which support the concept of hybridity comes from **analyses of complex carrageenan** by **enzymatic degradation**. Indeed, the production of hybrid fragments obtained after enzymatic incubation, are likely arising from the **cleavage of hybrid structures**. These observations had allowed addressing not only the **composition** but also the **distribution of the carrabiose units** in hybrid structures.

The **molecular mass**, the **occurrence of anhydrogalactose**, and **sulfate decoration of the galactan chain** are the **main structural parameters** affecting the **physico-chemical properties** of carrageenans. **Chemical** approaches have been described to remove or add sulfate groups on carrageenan. However, these methods lead to the **depolymerization of carrageenan** and are not applicable at industrial scale. Another reaction, the formation of **anhydro-ring** which enhances the gelling ability of carrageenan, is catalyzed by hot alkaline treatment and is applied industrially during carrageenan extraction. This reaction mimics an enzymatic reaction that occurs during the biosynthesis of carrageenan but could be likely **replaced by a more sustainable process**. Therefore, the main objective was to introduce **biotechnological processes** in the field of carrageenan aiming at **improving gelling ability and controlling sulfate decoration of carrageenan using enzymes.** Our objectives were to **identify**, **characterize**, **optimize**, and **express recombinant enzymes**, namely **carrageenan sulfurylases** involved in carrageenan biosynthesis and **carrageenan-sulfatases** involved in their degradation.

What were our research answers ?

The biosynthetic pathways of carrageenan is currently only hypothetic and involve at least three groups of enzymes: the **galactosyl transferases, the sulfotransferases, and the galactose-6-sulfurylases**. The galactosyl transferases catalyse the polymerisation of galactose chains on which the sulfotransferases add sulpfate side-groups. Only the final step of the biosynthesis has been unambiguously demonstrated by the characterisation of the enzymatic activity of the galactose-6-sulfurylases which catalyse the formation of the 3,6-anhydro-bridge. Remarkably, this reaction is **unique in the living world** since it has been described only in the red seaweed. The reactivity of the galactose-6-sulfurylases on the polymeric biosynthetic precursors of carrageenan (i.e μ- and ν-carrageenans) confirms that these enzymes act at the final step of the biosynthesis. Partner 3 had already identified two sulfurylases from the carrageenophyte red seaweed *Chondrus crispus.* The sequencing of the 120 Mbp genome this seaweed was planned in 2008 and will likely provide additional target genes. As sequence information on this class of enzymes was sparse, we proposed to screen for sulfurylase activity in extracts from selected red seaweeds (*Kappaphycus alvarezii*, *Eucheuma*

denticulatum, *Gigartina skottsbergii*), purify enzymes and sequence peptides in order to identify the corresponding genes from the genomes of these algae.

The **biodegradation** of carrageenan **involves carrageenases** which cleave the glycosidic bond and sulfatases which catalyze the removal of sulfate. The evidence of the carrageenan-sulfatases were provided by the discovery of sulfatase acting on the non-reducing end of the oligo-carrageenans: the end products of carrageenases. From a biotechnological point of view, the desulfation of oligo-carrageenans is of little interest because carrageenan loses its gelling properties with the decrease of molecular weight. Therefore, investigation of **de-sulfatation of carrageenan** under its polymeric form and evidencing **endo-acting carrageenan sulfatases** could offer **new perspectives** in the control of carrageenan structure. One of the main sources for the identification of carrageenan-sulfatases and other enzymes involved in carrageenan degradation was the **genomic and transcriptomic data** of *Zobellia galactanivorans;* a bacterium model for marine polysaccharides degradation. The selected gene targets, whether obtained by bacterial genome mining, transcriptomics, or metagenomics, will then be expressed by heterologous expression. The chosen strategies will eventually also lead to the discovery of novel modifying enzymes. Modality of desulfatation (i.e. random, processive) will be investigated by using carrageenan-hydrolysing enzyme. They will be used in the production of specific oligomers and in the fingerprinting analysis of enzymatically modified polymers.

Where are we now at the end of the project ?

We have implemented an **extraction protocol of galactose-6-sulfurylases** from fresh algae and demonstrated a new galactose-6-sulfurylase from *Kappaphycus alvarezii* able to **convert μ- to κ -carrageenan**. The enzyme was biochemically characterized and peptides were sequenced by mass spectrometry. The corresponding hypothetical gene sequence as well as other sequences deduced from previous investigations (4 proteins in total) were cloned and recombinant expression in *E. coli* were attempted. Unfortunately, the targeted proteins were produced **insoluble** and no galactose-6-sulfurylases were measured in the soluble extracts. The targeted genes could not be directly correlated to galactose-6-sulfurylases because they were not the good targets, the activity was not observed because of the insolubility of the protein or also post-translational modification of the enzymes are potentially required as in the case of sulfatase. Altogether, the knowledge-based approaches defined initially **did not allow the confirmation** of the protein sequence for galactose-6-sulfurylase activities.

Galactose-6-sulfurylases activity is measured by chromatographic detection of sulfate ester group released during the enzymatic reaction and by ^{1}H NMR. In both case, the methods are long and fastidious. During the project, we **developed a method of detection of the galactose-6-sulfurylases** at micro-plate scale which was found **very sensitive** and that could be useful for the screening of complex protein extract (from algae and bacteria extracts). We decided to implement this method for the screening cDNA library of growing red algae which likely biosynthesize carrageenan. We selected the algae *Chondrus cripus* which genome has been sequenced and prepared a cDNA library. The library was analyzed by sequencing 200 clones and revealed that 41 % of the clones corresponded to full gene of the algae. The screening of the library was undertaken using the colorimetric method implemented but no sulfurylase activity was detected yet. From these experiments, it seems that the expression of the gene has to be evaluated and better controlled. Also, as for the expression of targeted genes of in *E. coli*, problems of **insolubility and potential post-translational modifications** of the active site may also explained the results.

The second class of enzymes that were investigated were **carrageenan-sulfatases**. Aiming at targeting gene sequences of bacterial sulfatases from **bioinformatic and transcriptomic assays**. The strategy was to achieve an **extensive phylogenetic analysis** of the formyl-glycine dependent sulfatases belonging to bacteria recognized as degraders of sulfated polysaccharides. The first classification was provided using *Rhodopirellula baltica* (104 sequences) and *Zobellia galactanivorans* (71 sequences) together with all characterized fg-sulfatases (fifteen proteins) which revealed that the sulfatases considered diverged into 31 clades coinciding likely with their substrate selectivity. Importantly, eighteen clades do not comprise characterized fg-sulfatases, supporting the existence of subfamilies of fg-sulfatases with novel, unidentified substrate specificities. We have set up and optimized a non-commercial RNA extraction protocol to obtain good quality RNA from *Zobellia galactanivorans* cultivated in minimum media complemented by kappa-, iota- or lambda-carrageenan as sole carbon source. The preliminary analyses have identified **11 sulfatase genes** significantly induced in the presence of the sulfated galactans (porphyrans, kappa- and iota-carrageenans). These sulfatases belong to different subfamilies defined according to our bioinformatic classification (subfamilies S1-7, -11, -15, -16, -19, -20 and -23). Most of these genes are localized in gene clusters which also include glycoside hydrolases and other carbohydrate-related proteins, suggesting that these sulfatases are indeed carbohydrate sulfatases. In parallel, we have used our medium throughput expression strategy in *E. coli* to clone and heterologously express all 72 sulfatases, present in *Z. galactanivorans*. As a result, **52 sulfatases are produced and over-expressed in soluble form**. The activity remains to be determined for all of these enzymes.

In parrallel to bioinformatic and transcriptomic approaches, we have screened **carrageenoloytic bacteria** in order to find strains producing carrageenan-sulfatases. We found that *Pseudoalteromonas atlantica* secreted several carrageenan-sulfatase and three of them were purified. These proteins were sequenced and the associated carrageenan-sulfatase activities were validated heterologous expression in *E. coli*. Interestingly, all these sulfatase were found in gene cluster containing other sulfatases gene which were also tested against carrageenan. Finally, we demonstrated that 6 fg-sulfatase carried carrageenan sulfatase. Four were endo-sulfatase acting on ι-, κ- and α-carrageenan and the three other were exo-sulfases active on oligo-κ- or ι-carrageenan. Combination of the results obtained from bioinformatics, transcriptomics and protein purification, one can predict other genes for carrageenan-sulfatase in other bacterial strains (i.e. *Cellulophaga* sp., *Flavobacterium* sp.).

Enzymology and **mode of action** of **two endo-sulfatases** were investigated in details. The first enzyme can completely converts ι-carrageenanin in α-carrageenan. Applying this enzyme to hybrid ι-/κ–carrageenan gave hybrid α-/ι-/κ-carrageenan and after prolonged incubation α-/κ-carrageenan. Interestingly, pure α-carrageenan, α-/ι-/κ-carrageenan and α-/κ-carrageenan have **never been described in nature**. The second enzymes removes sulfate from κ-carargeenan leading to the neutral β-carrageenan. This enzyme allows preparing **κ-/β-carrageenan** which composition **mimics that of furcellaran, a commercial carrageenan type**. Altogether, enzymology investigations demonstrate that it is possible to **modify** carrageenan under its polymeric form and to **control its structure by adjusting enzyme protocols**. More unexpectedly, we have prepared series of carrageenan that have no equivalent in nature and which could be considered as **' novel' carrageenans**.

Where do we want to be in future and what are the remaining hurdles ?

Classification of sulfatases: The phylogenetic and experimental results drawn from the first marine sulfatase-classification revealed that distinct phylogenetic clades coincide with different substrate specificities. Consequently, it appeared interesting to extend the classification to all fg-dependant sulfatases, reported in genomic databases. The information obtained through such a global classification would also benefit for targeting novel heparan-sulfatases (link with WP4) and even beyond, in analogy to the CAZy database, be of interest to the general scientific community. Our future projects therefore are, at **short-term** and with the help of our bioinformatic platform at SBR, to set up a **global phylogenetic classification of fg-sulfatases**. The bioinformatic analyses of more than 4000 fg-sulfatase genes have actually been achieved, and we are currently working on the establishment of a web interface. At the short-term this database will be accessible to PolyModE partners and at **mid-term this database is intended to be publicly released**. These results will be presented at the Wrexham Gums and Stabilizers conference in Wrexham, june 2013.

Genes for galactose-6-sulfurylases: **Data** on carrageenan **biosynthesis are sparse** and need to be examined in detail to highlight new routes for carrageenan modification using enzymes. Despite, we have definitely **progressed on the enzymology** of these enzymes, the targeted genes for sulfurylases investigated during the project did not lead to unambiguous conclusions. However, we have developed a **new screening method** for these class of enzymes that allowed us envisioning screening a cDNA library also set during the project. **Galactose-6-sulfurylases** are a class of enzymes with **unknown sequence** which makes them a very interesting subject from an academic point of view. For **industry,** these enzymes could replace the chemical modification of carrageenan towards a **more sustainable process**. Therefore, **screening cDNA library** of carrageenophyte algae has to be improved as well as new **technology for the purification** of enzymes have to be developed.

New carrageenan-sulfatases and upscaling production of new carrageenans: We have already evidenced carrageenan-sulfatase with potential in the field of known applications of carrageenan. For example, κ-/β-carrageenan are already used in the industry. Using enzymes that allow **controlling structure and functionality** of this carrageenan has **great interest**. Besides, we have shown we can produce **novel carrageenan** which widens the potential use of carrageenan-sulfatase. Therefore, we scheduled the production of various new carrageenans **at gram scale** in order to **evaluate their potential** in food industry.

Preparation of highly active recombinant sulfatases is also a second line of investigations. Carrageenan-sulfatase required a post-translationnal modification of catalytic amino acid. Controlling this reaction in the expression system and designing efficient **enzymes** for industrial proposes stays a **key challenge** for future exploration of these enzymes. Already, we are evaluating various expression systems and selected series of sulfatases genes to be tested.

3.3. Work package 3 - Chitosan modifying enzymes; WP leader: Bruno Moerschbacher (WWU)

Context of the project:

Chitin isolated from shrimp or crab shell wastes is one of the **most abundant renewable resources** worldwide, and its derivative **chitosan** is among the most interesting and most promising bio-active biopolymers. However, the term chitosan really refers to a family of oligo- and polymers differing in their **degree of polymerisation** (DP), their **degree of acetylation** (DA) and possibly also in

their **pattern of acetylation** (PA). This structural diversity has been implicated in the functional versatility of chitosans, but a detailed knowledge of **structure/function relationships** and, thus, a detailed understanding of the molecular basis of chitosan' s bio-activities are still lacking. This lack of information so far **hindered** the development of reliable **life science applications** of chitosans, such as in plant disease or in scar-free wound healing where promising results have been reported but with rather **poor reproducibilities**. This problem persists partly because reliable ways of production, modification, and analysis for partially acetylated chitosans are missing. A telltale example is the PA: today' s chitosans are produced by partial chemical de-N-acetylation of chitin or by partial re-N-acetylation of fully deacetylated polyglucosamine, invariable leading to chitosans with **random PA;** and the only analytical tool available for PA characterisation is diad analysis using NMR which requires expensive infrastructure and rather large amounts of material, and which yields very limited information on the PA only. This project aimed at the identification of novel chitin and chitosan modifying enzymes (CCME) to provide **alternative or complementary tools** for the **generation and characterisation** of chitosans, including novel chitosans with non-random PA and, thus, potentially more reproducible or even **novel biological activities**.

Objectives of the project:

CCME include enzymes such as endo- or exo-acting chitinases and chitosanases, glucosaminidases and N-acetyl-glucosaminidases, chitobiases and chitin de-N-acetylases. Of particular interest for the generation of chitosan polymers with non-random PA are **processive chitin deacetylases** which would yield chitosans with **block-PA**, while sequence specific chitosan hydrolases might be used to produce chitosan oligomers with non-random PA. In addition, the latter enzymes are also powerful tools for the establishment of **enzymatic / mass spectrometric fingerprinting methods** for the analysis of PA far exceeding the currently available NMR techniques in terms of sensitivity and analytical depth. The major objective of the project was to **identify, produce, characterize and optimize CCMEs to produce and analyse chitosans exhibiting non-random patterns of acetylation.**

Two mutually complementing approaches were used for the identification of potentially novel CCME. The **knowledge based approach** targets organisms which can be expected to harbour CCMEs, such as fungi highly tolerant to antimicrobial chitosans or able to grow on chitosan as a sole carbon source, or pathogenic fungi known to convert the chitin exposed on the surface of their cell walls into chitosan upon penetration into host tissues. If the genome of such organisms is known and available, bioinformatic analyses may identify genes coding for CCME, with the potential of finding novel genes and enzymes. To analyse the novelty, the genes need to be expressed heterologously in a suitable expression host, and the recombinant proteins need to be purified and characterised for their chitin and chitosan modifying abilities.

An **alternative approach** focuses on soils with a long history of exposure to chitin and chitosan which can be expected to be a rich source of genetic diversity of micro-organisms producing CCME. Here, we can either try and isolate chitinolytic/chitosanolytic organisms and proceed as above, or we can use **metagenomic approaches** to also harvest the diversity of the vast majority of non-culturable microorganisms. Typically, this approach starts with the generation of a metagenomic DNA library, either in the form of plasmids carrying small inserts or in the form of fosmids carrying large insert, and its screening using either sequence-based or functional assays. While the sequence based approach relies on prior information on gene sequences for the enzymes targeted, thus limiting its ability to identify truly novel genes and enzymes, the functional approach requires the development of reliable assays based on the different enzymatic activities targeted, which can be very challenging. With the recent developments in next generation sequencing which becomes ever faster and less expensive, **direct sequencing of metagenomic DNA** isolated from an environmental sample becomes feasible. This approach circumvents the cloning step which can be difficult and which can pose regulatory problems, but it is limited to sequence based screening with the inherent limitation of relying on prior sequence knowledge, as discussed above. Also, metagenomic sequencing is prone to a plethora of artefacts and has not yet been used successfully to provide a novel, functional enzyme.

Potentially novel CCME derived from the heterologous expression of the genes so identified need to be characterised to reveal their enzymatic properties such as substrate specificity, mode of action, and **product patterns**. These analyses only can tell whether the enzyme is not only new (i.e. not yet described), but also novel (i.e. different from known enzymes in its properties). The most challenging task of this part of the project is to develop **suitable methods** for the detailed analyses of the products formed. In case of chitosan oligomers, this required the development of **quantitative sequencing techniques** based on mass spectrometry which can give results even when applied to mixtures of oligomers. In the case of chitosan polymers, we needed to develop enzymatic/mass spectrometric fingerprinting techniques to analyse the PA, while methods for the analysis of DP (HP-SEC coupled to RID and MALLS) and DA (NMR) are well established. For this fingerprinting, sequence specific chitosan hydrolases are required which therefore were one target of this work package.

To support the analysis of the enzymatic properties of unknown CCME and their products, we devised a computer programm, **' Chitinator" , a virtual chitosan hydrolase**. This programme mimicks the digestion of a chitosan polymer with a given DP, DA, and PA by an enzyme with a known substrate specificity and mode of action, yielding the oligomers that would be produced. The

programme should be used in two different ways: in the fingerprinting mode, it would compare the oligomers produced when an enzyme with known properties is used on a substrate with unknown properties to the products expected when this enzyme would be incubated with different (virtual) substrates: identifying the substrate that yields products most closely resembling the experimental products reveals the properties of the substrate used. Conversely, in the reverse fingerprinting mode, the programme would compare the oligomers produced when an enzyme with unknown properties is used on a substrate with known properties to the experimental results, thus revealing the properties of the unknown enzyme. Thus, the Chitinator would not only support the enzymatic/mass spectrometric fingerprinting analysis of the PA of chitosan polymers, it would also help in analysing the sequence specificities of the fingerprinting hydrolases required for this analysis. In fact, it might be developed into a tool that could **allow the rapid decision whether a new gene also codes for a novel enzyme**.

Scientific and technical outcome of our research:

Pursuing the knowledge-based approach, we identified **six possibly interesting bacterial chitin deacetylase encoding genes** with potentially novel properties. After heterologous synthesis in *E. coli,* all of the proteins were successfully purified to electrophoretic homogeneity and analysed for their deacetylating activity towards chitin, where two of them ere positive. One of those turned out to be a rather typical chitin deacetylase with activities towards solubilised chitin and chitin oligomers down to the tetramer. The other chitin deacetylase, however, turned out to be very different and appears to be the **first described member of a so far completely unknown family of enzymes**. We are currently performing further experiments on the structural and functional properties of the polymeric and oligomeric products of these two enzymes.

Furthermore, using bioinformatic tools, a promising **sequence of a putative chitin deacetylase (CDA)** was identified in a fungal genome which is similar to known CDA but sufficiently different to promise novel functionalities. A **synthetic gene optimized for the yeast *Hansenula polymorpha*** was successfully expressed and the recombinant protein was purified. The enzyme was active against glycol chitin, chitin oligomers with a degree of polymerization ≥ 2, chitosans, and colloidal chitin. The pH optimum against this latter substrate was 10.5, and the enzyme remained stable within the pH range 5– 8.5. The optimal temperature was 50°C and the enzyme was not inhibited by EDTA or acetate.

To investigate the mode of action of this CDA on chitosans, we applied **enzymatic/mass spectrometric fingerprinting**, using chitosans with the same DP and DA as the CDA products for comparison. Two chemically reacetylated chitosans were determined by ^{1}H-NMR to have DA of 60.8±1.3% and DA 32.4±1.1%, respectively. The chitosan with DA 61% was partially deacetylated using the recombinant CDA to DA ≈ 32%, as determined by quantifying the acetate released, and the product was compared to the chitosan of DA 32% obtained by chemical reacetylation. Fingerprinting was carried out using sequence-specific chitosan degrading enzymes followed by MALDI-TOF-MS analysis. The highly **sequence-specific chitinosanase from *Alternaria alternata*** achieved the most distinct results. The spectra of chemical and enzymatically treated chitosan clearly showed **different sets of peaks**. The novel CDA has, thus, proven to be able to generate chitosans with a non-random pattern of acetylation. Similar experiments were performed with a range of recombinant CDA, and we found distinct patterns in the fingerprinting assays for each one of them.

We consider this to be a **break-through result**: these are the first chitosans ever produced with an other-than-random PA! Clearly, this is the beginning of a **new chapter in chitosan research**, with chitosans of equal DP and DA but differing in PA. We are now in a situation to **analyse** the **biological activities as well as the physico-chemical properties** of these novel chitosans. More blockwise or more even distributions of acetyl groups as compared to the random distributions in conventional chitosans produced using chemical means can be predicted to have a **very significant impact on their bioactivities**. This will be particularly true if a target tissue contains a sequence-specific chitosan hydrolase such as human chitotriosidase which can cleave chitosans only between two adjacent GlcNAc units. Clearly, the degradability and, thus metabolic turnover of the polymer as well as the quantity and quality of oligomeric products formed will greatly depend on the PA of the chitosan polymer applied. Of course, the implications of the availability of chitosans with non-random PA goes **far beyond** their bioactivities. In analogy to polymer chemistry, we can equally expect the material properties of chitosans to be governed to some large extent by their PA. And the entire field of chitosan derivatives will equally profit from such chitosans as the free amino groups made available through the action of CDA are the ideal sites for chemical (or enzymatic) modification. Again, **different patterns of e.g. hydrophobic, cationic or anionic modification will greatly influence the properties of the materials.**

The metagenomic approach focused on **soil samples** with a more than ten-year history of exposure to chitin and chitosans, from the chitin and chitosan producing plant of our partner Gillet Chitosan in Gujarat, North India. To identify the most promising of ten different soil samples collected at different sites of the plant, **chitinolytic and chitosanolytic microorganisms** were isolated from them, and we concomitantly set out to develop a suitable extraction protocol for good yields of high quality environmental DNA from these samples. Two samples turned out to be superior in both respects to the other ones, so that further work concentrated on these. We found that the **chitosanolytic microflora** in these samples was clearly **dominated by fungi** rather than bacteria, and the fungal isolates also possessed the richer biodiversity in terms of CCME compared to the bacteria. A deeper analysis of selected

fungal isolates proved that they also produced a broad diversity of chitosan oligomers when incubated with the same chitosan polymer. Thus, these samples are clearly well suited for the isolation of potentially novel CCME. These results have been submitted for publication.

Two expression libraries were generated from the environmental DNA (eDNA) extracted from a mixture of the two soil samples described above. One was a fosmid library with large inserts of ca. 40 kb, the second one had small inserts (ca. 1 kb) cloned into an expression vector. Concomitantly, we developed **screening protocols** for chitinases, chitosanases, and chitin deacetylases, either based on the release of a fluorescent tag from MUF-substrates, or based on the production of clearing zones around chitinolytic/chitosanolytic colonies. Even though both strategies were established successfully as demonstrated using positive controls into which we had cloned representative target genes, the screenings of both libraries did not yet yield positive clones. Unfortunately, due to changes in the German law on handling GMOs, the screening had then to be discontinued.

The metagenomic approach was then continued using direct sequencing of eDNA from the same soil samples. The eDNA was sequenced using the Roche Genome Sequencer FLX Titanium platform. Several CCMEs were identified by homology to GenBank sequences including a full-length chitinase, a full-length chitosanase, and eight full-length chitin deacetylases, with most of them showing less than 50% identity to already known sequences. As **proof-of-principle**, the chitinase gene obtained from the metagenomic library was codon optimized for *E. coli*, the corresponding sequence synthesized and subsequently transformed into Rosetta 2 (DE3)pLysSRARE using the pET vector system. Purification of the recombinant protein was performed via Strep-tag and verified by SDS page and Western blot experiments. We purified 5 mg of active chitinase as demonstrated by dot blot analysis using glycol chitin at pH 8, proving that sequences obtained from metagenomic soil samples can indeed be used as source for the identification of novel CCCMEs. The further characterisation of the chitinase is ongoing. In addition, the sequences for all eight potential chitin deacetylases and the sequence homolog to chitosanase from the soil metagenome have been codon-optimized for *E. coli* and synthesized. Expression of these genes and production of recombinant enzymes are ongoing.

On the basis of an inactivated chitosanase, a **chitosan-affinity protein (CAP) was developed**. This protein possesses a hexahistidine, a StrepII tag and also a GFP tag, which allow the detection in multiple ways. *In vitro,* this newly developed affinity protein showed high affinity to low DA chitosan, a weaker affinity to high DA chitosan, and no affinity to chitin. We were also able to specifically stain the chitosan bearing parts of infection structures of the wheat stem rust fungus *Puccinia graminis* f. sp. *tritici in vivo*. This work was published (Nampally et al., 2012) and since then, we have had multiple demands for CAP from colleagues aiming to detect chitosan in various systems.

Apart from the chitosanase on which the above described CAP was based, and a number of bacterial chitinases, all of which we have characterized for their cleavage specificities to then use them for the fingerprinting approach, we have also continued working with our new chitosan hydrolase which we have termed chitinosanase, due to its unique cleavage specificity. While our quest for the chitinosanase gene continues to be unsuccessful, we found that this enzyme is best suited to give significant results in the enzymatic/mass spectrometric fingerprinting. In the course of this project, we found that we had to correct the initially assumed cleavage specificity of this enzyme. We now know that it specifically cleaves behind a GlcN followed by a GlcNAc unit. This explains why the enzyme does neither hydrolyse fully deacetylated GlcN oligomers (like any chitosanase would do) nor fully acetylated GlcNAc oligomers (like any chitinase would do). A manuscript describing this enzyme is in preparation.

Over the course of this project, we have described these results to the scientific community on different occasions, most importantly during the Meetings of the European Chitin Society (10th and 11th International Euchis conferences held in in St. Petersburg, Russia, in 2011 and in Porto, Portugal, in 2013, respectively) and during the Meeting of the Ibero-American Chitin and Chitosan Society held concomitantly with the larger International Chitin and Chitosan Conference (6th SIAQ and 12th ICCC held in Fortaleza, Brazil, in 2012). At all occasions, our findings met with **overwhelming interest**, and this was again true during the final dissemination meeting on the occasion of the Food Hydrocolloids conference (17th Gums & Stabilisers for the Food Industry Conference held in Wrexham, U.K., in 2013) where we were able to present our results in the context of all PolyModE results to the wider polysaccharide community..[4]

[4] Nampally, M., Moerschbacher, B.M. and Kolkenbrock, S. (2012) Fusion of a Novel Genetically Engineered Chitosan Affinity Protein and Green Fluorescent Protein for Specific Detection of Chitosan *In Vitro* and *In Situ*

Appl. Environ. Microbiol. 78:3114-3119

3.4. Work package 4 - GAG modifying enzymes; WP leader: Olivier Berteau (INRA)

Context of the project:

Glycosaminoglycans (GAGs) are a family of **heteropolysaccharides** divided into **five major sub-families**, namely chondroitin, dermatan, hyaluronic acid, keratan, and heparan sulfates/heparin. These polysaccharides have major functions into the human body and are involved in many **essential biological processes** from blood coagulation to cell-cell communication, growth, and differentiation. These polysaccharides are also central players in **several pathological conditions** such as cancer, angiogenesis, neurodegenerative diseases such as Alzheimer' s, athero-sclerosis, and microbial infectivity. More than 100 proteins have been identified as interacting with GAGs. These polysaccharides are thus of **major interest for the pharmaceutical industry**. Among them, **heparin** is the one with the most developed and advanced medical applications including the **treatment of thrombosis** and some applications in **cancer therapy**.

The **biological properties** of heparin are intimately connected with its **structur**e which is basically constituted of long unbranched chains build up on a repeating disaccharide unit consisting of an N-acetyl-hexosamine (HexNAc) and an hexose or an hexuronic acid linked by 1,4-linkages. This backbone is further substituted by sulfate groups which introduce further variability and complexity into heparin. This **sulfation pattern** has been shown to be **critical for the interaction of heparin** with many proteins and thus its biological properties. Indeed, the **sulfate groups** not only give a **high negative charge** to the polymer but also greatly **impact heparin conformation** and ability to **interact biological factors** (enzymes, growth factors, etc.).

Objectives of the project:

In the frame of the PolyModE project our main objective was to **identify new enzymes** able to **selectively remove sulfate groups** in order to precisely **control the sulfation pattern of synthetic GAGs**. These enzymes are called **sulfatases** and are commonly found in **Bacteria or higher organisms**, nevertheless very few enzymes able to remove sulfate groups on GAGs were known when we initiated the project. Furthermore, the objective was to identify sulfatase specific for positions on the GAG backbone notably 2-O- and 6-O- sulfatases which are the main sulfated positions on the heparin backbone. We have recently shown that **bacterial sulfatase diversity is broader than hypothesized** and that some **human commensal bacteria** harbour a large number of potential GAG sulfatase. These bacteria were thus an attractive target for the search of specific GAG sulfatases.

Specific tasks that were defined on primary targets:

- **Identify** novel bacterial sulfatase
- Enzyme **expression** and **characterization**
- Determining the **specificity** of these enzymes

The second approach we developed was combining **metagenomics with specific screening strategies** in order to identify novel catalyst able to modify GAGs. After identification of positive clones the main goal was to precisely **identify the gene(s)** involved in this potential novel activity and to **characterize the enzyme(s) responsible**(s). At last, the sulfatases having the appropriate activity and selectivity would be **engineered** in order to improve their catalytic properties and eventually, to change their specificity or mode of action in order to generate novel sulfation patterns thus increasing the diversity of GAG fragments.

Our research answers:

To identify interesting enzymes, we selected several genes candidates based on their sequence and on transcriptomic studies showing expression increase when bacteria were grown with GAGs as sole carbon source. These genes were cloned and further expressed in E. coli. To be active, sulfatases need to undergo an essential post-translational modification which consists of the oxidation of a critical cysteine or serine residue inside the enzyme active site. In E. coli it has been shown that recombinant expressed sulfatases can be active if the critical residue is a cysteine while the activation of sulfatase having a critical serine residue requires the co-expression of a dedicated maturating enzyme. We have thus developed suitable systems allowing the expression and efficient maturation of sulfatase during their expression in E. coli.

Scientific and technical outcome of our research:

We successfully expressed **15 sulfatases** which proved to be active on simple chromogenic substrates. In order to determine if they were good catalyst for GAGs modification we synthesized a broad array of GAG fragments ranging from disaccharide to octasaccharide with a regular sulfation pattern on the position 2-O- and 6-O- of the carbohydrate backbone. After incubation of the enzymes with these synthetic oligosaccharides, the structure modification was analyzed using physico-chemical techniques including HPLC and NMR spectroscopy. Because these analyses required large amounts of product and were time consuming we

also synthesized novel chromogenic substrates that could be analyzed by simple spectrophotometric techniques. This allowed us to screen sulfatases under various conditions using low amounts of enzyme and substrate.

With this strategy we identify several specific GAG sulfatases which proved to have a very strict specificity not only for the nature of the carbohydrate unit but also for the position of the sulfate group. Nevertheless, most of these enzymes were **' exo' -sulfatases** which means they were able only to remove groups on the non-reducing end of the oligo- or polysaccharides assayed. We thus try to express a recently identify mammalian endo-sulfatase to obtain a novel tool for GAGs desulfation. This **endo-sulfatase** was cloned in prokaryotic and eukaryotic expression hosts but proved to be either not soluble or not active whatever was its expression form as a cytoplasmic or an excreted enzyme. In order to circumvent these difficulties, we generated a **truncation library** containing several hundreds of mutants. Unfortunately these constructs proved **to be toxic** when expressed in E. coli and no soluble protein could thus be obtained.

To improve the diversity of available sulfatases, we screened metagenomic libraries from various origins. These metagenomic libraries consisted of E. coli clones containing large **environmental DNA fragments**. Since no methodologies were reported to identify sulfatases in metagenomic libraries, we developed an **efficient screening strategy** allowing to search among ten thousands of clones. We found that the growing conditions were critical to monitor sulfatase activity inside metagenomic libraries. After screening optimization we **successfully identify the first metagenomic sulfatases**.

Where do we want to be in future, what are the remaining hurdles and how to overcome them?

During the PolyModE project we successfully identify **novel sulfatases** specifically active on GAGs from oligo- to polysaccharide. The expression of **active sulfatase** nevertheless **remained challenging** in particular because of the critical post-translational modification which has to be precisely controlled during protein expression. Albeit substantial technological advancements have been made during the project, the **heterologous expression of active sulfatase remains a bottleneck**. We performed some enzyme engineering notably in modifying the critical residue. This strategy proved to be efficient in some cases allowing a 25-times enhancement of the enzyme specific activity. At short-term, further attempts to produce more purified and active enzyme will be performed. At mid- and long-term, we need to **solve the structure of the specific GAG sulfatases** we have identified in order to understand the molecular basis of their specificity and eventually to modify their selectivity and mode of action (i.e. endo vs exo-sulfatase). Furthermore, we will have to **characterize in details the metagenomic sulfatases** identified in order to determine if they can be superior catalysts for industrial application in terms of activity and specificity.

3.5. Work package 5 - Pectin modifying enzymes; WP leader: Henk Schols (WU)

Objectives

The objectives of WP5 pectin modifying enzymes were to provide different and well characterized **pectin acetyl esterases** (PAE) that can **specifically modify sugar beet and other acetyl-rich pectins** having activity with both high and low levels of methylesters. It was intended to compare different and well characterized pectin methylesterases (PME) from various fungi, plants and *Bacillus* species that can modify sugar beet pectins and to **characterize** the enzymatically modified pectin in terms of **distribution of the acetyl groups and methylesters** for specific applications.

Summary

Metagenomic approach versus knowledge-based approach
The identification/selection and production of novel (putative) PAEs and PMEs was done at Münster University (WWU). Identification of novel PAEs and PMEs using a **metagenomic approach** was done by isolating microorganisms from **soil, beet pulp, and rotten sugar beet samples** by growing them in minimal medium with SBP as the sole carbon and energy source. The fermentation liquids were analyzed for PAE and PME activity. Only **minor activity** towards SBP was detected.
The **knowledge-based approach** involved database and literature screening as well as bioinformatic analyses. Several (putative) bacterial PMEs and PAEs were selected for a detailed characterization. The coding sequences of such proteins were cloned, and, if required, codon-optimized synthetic genes were used. Gene expression was done in appropriate inducible *Escherichia coli* strains. The enzymes were produced in fusion with an affinity tag and purified by fast protein liquid chromatography (FPLC). Prior to the enzyme characterization the protein concentration was measured and the enzymes' integrity and purity were verified by SDS-Page and Western blot analysis.

Characterization of selected (putative) PAEs and PMEs: Characterization (pH and temp optimum, activity, substrate specificity and mode of action) of the available PAEs and a PME was done at the Laboratory of Food Chemistry (WU). The five putative pectin

acetylesterases (PAE2-6) exhibited almost the same pH (5-8) and temperature (40-55°C) optimum and showed a significant level of activity towards SBP. PAE4 and PAE6 released acetic acid of 13-58% of the maximum acetic acid from sugar beet pectin with a degree of methylation (DM) ranging from 9-62% and a degree of acetylation (DA) of 15-30%. Moreover, PAE4 and PAE6 were significantly hindered by the amount of methylesters present in the homogalacturonan of SBP. PAE5 was observed to be active towards acetylation within the rhamnogalacturonan region of SBP, while PAE2 was found to **be active mainly towards acetylated SBP galacturonic acid oligomers.**
Since the production of novel PMEs was the secondary target of the PolyModE consortium, a well characterized *A. niger* pectin methyl esterase (fPME) (Van Alebeek, Van Scherpenzeel et al. 2003) and a plant PME (pPME) from orange (Christensen, Nielsen et al. 1998) were also included in the experiments to compare their activity with a novel bacterial PME (PME3). The various PMEs from **different origins were found to be active towards SBPs and lemon pectins** and released 18-68% (PME3), 56-96% (fPME) and 4-72% (pPME) of all methyl esters present, depending on the type of SBP used.

Characterization of unmodified and PAE- or PME-modified SBPs by enzymatic fingerprinting: **Enzymatic fingerprinting** was applied to sugar beet pectins (SBP) produced by either plant (p-PME) or fungal pectin methyl esterases (f-PME) and alkali catalysed de-esterification to reveal the ester distributions over the pectin backbone. So far, the separation, characterization and quantification of all complex oligosaccharides released after the combined endo-polygalacturonase (endo-PGII) and pectin lyase (PL) digestion of SBP has been limited. Using LC-HILIC-MS/ELSD, we now studied in detail all the diagnostic oligomers present, which enabled us to discriminate differently prepared sugar beet pectins having **various levels of methylesterification and acetylation**. Furthermore, distinction of commercially extracted and de-esterified sugar beet pectin having different patterns of substitution was established by using descriptive pectin parameters. The **' degree of hydrolysis"** (DH_{absPG}) of total non-, saturated methylesterified and/or acetylated moieties was introduced as a **new parameter** in addition to DB_{abs} approach for nonmethylesterified and methylesterified sequences degradable by endo-PG and PL. These parameters clearly distinguish the differences of the de-esterification mechanisms performed on SBP (Remoroza, Schols et al. 2013). The study of the **substitution pattern** within the homogalacturonans of SBP is a very **important addition** towards understanding pectin' s structure in relation to pectin esterase modifications.

Mode of action of PAE4: SBP (DM 9%, DA 15%) was digested by a **novel pectin acetyl esterase** PAE4 and the modified pectins were digested with pectolytic enzymes. The generated oligomers were analysed by HPSEC, HPAEC and UHPLC-HILIC-MS according to the method of Remoroza *et al.* (2012; 2013). The detailed analysis of the oligomers generated after endo-PG and PL digestion was performed by MS^n analysis, which provides information of the PAE4 mode of action. The acetylation pattern in unmodified SBP (DM 9%, DA 15%) shows a mixture of O-2/O-3 acetylated galacturonides. PAE4 was **able to remove 40% of all acetyl groups present** and showed to have a preference to remove O-3 substituted acetyl groups.

Mode of action of PME3: Sugar beet pectin SBP (DM 53%, DA 26%) was digested by **PME3** and **released 40% of the maximum methanol in SBP**. After modification of SBP by PME3, the level of nonesterified GalA moieties released after PG/PL incubation increased showing that PME3 remove methylesters in a blockwise fashion. However, the type and amount of oligosaccharides released suggest that the mode of action of PME3 may differ from plant PME which also de-esterify in a blockwise manner.

Conclusions

Novel pectin esterases have been extensively characterized and are able to **modify SBP** having different patterns of substitution. The **PAEs prefer to remove acetyl groups** on the specific position within acetylated oligogalacturonides while **PME is able to remove methylesters** within SBP in a very specific way. The LC-HILIC-MS/ELSD method for separation and characterization of different complex mixtures of diagnostic oligomers before and after modification was proven to be useful. This provides sufficient information to understand the different substitution patterns in SBP either unmodified or PAE/PME-modified. [5]

3.6. Work package 6 - Xanthan gum modifying enzymes; WP leader: Henk Schols (WU)

Objectives

The aim in WP 6 was to find **novel xanthanases**. The primary target was to identify **side chain modifying enzymes** in order to alter the rheology properties of a xanthan solution and thereby **improve xanthan utilization in industry**. Furthermore we searched for backbone degrading enzymes in order to elucidate the xanthan structure of native and enzyme-modified xanthans, especially considering the distribution of the acetyl and pyruvate groups along the side chains.

Summary of the progress

Elucidation of the xanthan structure using backbone degrading enzyme: From literature it is known that xanthan can be present in **different 3-dimensional conformations**: its native ordered helical conformation and its disordered open conformation. The conformation of xanthan in solution is primarily related to the **temperature**, **salt concentration** and the **primary structure** of xanthan. Because these factors also influence **enzyme activity** and since the xanthan conformation itself might affect the enzyme activity as well, a **better understanding** of the correlation between the **xanthan conformation**, the **primary xanthan structure** and the **solution conditions** is necessary before the enzyme activity of xanthan modifying and/or degrading enzymes can be studied.

The conformation of several xanthans differing in molecular composition was investigated using circular dichroism. The conformational change was monitored in solutions with varying ionic strength over a temperature range from 10– 85°C. Based on the obtained results, the ionic strength and/or temperature of the xanthan solutions were varied, to obtain different xanthan conformations. Xanthan solutions at all conditions chosen, were incubated with cellulases from *Myceliophthora thermophila C1* for 48 h. Thereby the influence of the xanthan conformation and the primary xanthan structure on the enzymatic degradation of xanthan by cellulases was studied. The xanthan degradation was analysed by size exclusion chromatography. It was shown that at a fixed xanthan conformation, the backbone degradation by cellulases is equal for each type of xanthan. Complete backbone degradation is only obtained at a fully disordered conformation, indicating that only the secondary xanthan structure influences the final degree of hydrolysis by cellulases. It is thereby shown that, independently on the degree of substitution, xanthan can be **completely degraded to oligosaccharides**. In order to investigate the xanthan structure, the obtained oligosaccharides were further analysed using hydrophilic interaction chromatography (HILIC) with bridge-ethylene hybrid (BEH) amide column coupled to

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[5] **Literature**

Christensen, T. M. I. E., J. E. Nielsen, et al. (1998). ' Pectin methyl esterase from orange fruit: characterization and localization by in-situ hybridization and immunohistochemistry.' Planta **206**(4): 493-503.

Remoroza, C., S. Cord-Landwehr, et al. (2012). ' Combined HILIC-ELSD/ESI-MSn enables the separation, identification and quantification of sugar beet pectin derived oligomers.' Carbohydr. Polym. **90**(1): 41-48.

Remoroza, C., H. A. Schols, et al. (2013). ' Revealing substitution patterns in differently prepared sugar beet pectins by enzymatic fingerprinting.' Biomacromolecules.

Van Alebeek, G. J. W. M., K. Van Scherpenzeel, et al. (2003). ' Partially esterified oligogalacturonides are the preferred substrates for pectin methylesterase of *Aspergillus niger*.' Biochemical Journal **372**(1): 211-218.

electron-spray ionization mass spectrometry (ESI-MS^n) and evaporative light scattering (ELS) detection. The different oligosaccharides could be identified using ESI-MS^n. The results showed the presence of **six different repeating units**. In literature it is generally assumed that xanthan is substituted with an acetyl group on the inner mannose and with a pyruvate group on the outer mannose. We now also observe the presence of an **acetyl group on the outer mannose**. It is therefore concluded that more variations exist in the xanthan repeating structure than generally assumed. The variations existing in the xanthan ' repeating' structure, might explain differences in xanthan functionality. Different xanthans, with similar molecular compositions, were therefore enzymatically degraded and analysed on the repeating units present. The ratio in which the different repeating units coexist in the different xanthan samples was determined based on the ELSD response. The results showed that although the molecular composition of xanthan is similar, the ratio in which the different repeating units coexist within the xanthans is different. Thereby the distribution and position of the acetyl groups within the xanthan samples showed to be different. Because acetyl groups have an important impact on xanthans functionality, differences in functionality of xanthans with similar molecular composition could be explained by **variations in the type of repeating units** present.

Xanthan modifying enzymes: Xanthan acetyl esterases - The **acetyl groups** in the xanthan side chains are known to have a **major impact on xanthans functionality**. Removal of the acetyl groups improves the functionality properties of a xanthan solution. **Xanthan acetyl esterases** would therefore be very **useful** for xanthan modification. Because no xanthan acetyl esterases are described in literature, a targeted database mining for xanthan acetyl esterases is not possible. Therefore several acetyl esterases, known to be active towards various carbohydrates were screened for their activity towards xanthan. A preceding step for this was the cloning of the corresponding genes, the heterologous synthesis of the enzymes in an appropriate *E. coli* expression system, and the protein purification via a StrepII-tag using fast protein liquid chromatography (FPLC). The impact of the xanthan conformation on the enzyme activity was tested by incubating xanthan solution with varying salt concentrations with the different acetyl esterases (AE). The results indicate that of all AEs tested, only two are active towards xanthan. AE-II shows the highest activity in the absence of salts, indicating that the enzyme is only active towards the disordered xanthan conformation. AE-VI is most active in the presence of salts, indicating that this enzyme is not influenced by the xanthan conformation. The two AEs therefore act differently from each other in removing acetyl groups from xanthan**.** The temperature optimum of AE-II and AE-VI showed to be 55°C and 60°C respectively. Because AE-II is not active towards xanthan in the presence of salt, the pH optimum could not be determined. The pH optimum of AE-VI is pH 7.0.*Xanthan lyases* - From literature **several lyases are known that are capable of removing the pyruvylated mannose** from the xanthan side chain through β -elimination. Although the **functionality** of xanthan **will not improve** by such modification, analysis of xanthan before and after modification with lyases might help to **elucidate the distribution pattern of the pyruvate groups** over the xanthan side chains. Therefore effort was directed in the production of pyruvate-specific xanthan lyases. The genes of the two known pyruvate-specific xanthan lyases from *Bacillus* sp. strain GL1 (BAB21059.1) and *Paenibacillus alginolyticus* XL-1 (AAG24953.1) were codon-optimized and synthesized, cloned, and heterologously expressed in an appropriate *E. coli* host. The produced StrepII-tagged enzymes were FPLC-purified. Normal xanthan, pyruvate-free xanthan and highly pyruvylated xanthan were incubated with the produced lyases to check the specificity towards pyruvate groups. The activity was analysed by HPAEC (Fig 4). The results showed that both enzymes were active towards xanthan independent on the degree of pyruvate and both enzymes released mannose and pyruvylated mannose. The produced lyases are thereby different then described in literature. Incubation at different time intervals showed that the release of pyruvylated mannose units are indeed preferred over unsubstituted or acetylated mannose units. It was determined if the differences in activity of the two lyases compared to literature could be explained by the xanthan conformation. However the results showed similar activities of the lyases when xanthan was in a fully ordered or disordered conformation. It was hypothesized that the lyases are active towards all terminal mannose units which are substituted, meaning pyruvylated and acetylated terminal mannose units. To check this hypothesis, the modified xanthan was analysed by HILIC-ELSD-ESI-MS after degradation of the backbone by cellulases and compared to unmodified xanthan. It was shown that all different xanthan side chains are modified by the lyases. The lyases are, therefore, not specific for any type of substitution. The pH and temperature optimum of both enzymes were determined and showed to be pH 5.5/45°C and pH 6.0/40°C respectively for lyase XalA-XL1 and lyase XalA-GL1.

Summary of the progress

A combination of **analytical techniques** have been developed enabling the **elucidation** of the structure of unmodified and enzymatically modified xanthan. The analysis and comparison of different unmodified xanthans showed that six different xanthan side chains exist. The relative abundance of these side chains showed to vary between xanthan samples, even when the molecular composition of the xanthan samples is similar. The xanthan **structure** is thereby **more complex** than generally assumed when studying the structure-function relationship of xanthan. Several xanthan modifying enzymes were found and characterised. Two **novel a-specific xanthan lyases** were produced. Furthermore **two xanthan acetyl esterases** were found and characterised. The enzymes have a different activity towards xanthan.

3.7. Work package 7 - Generic techniques; WP-leader: Corine Sandström (SU)

Context of the project:

WP7, constituted by academic participants and SMEs represented the technological core of the PolyModE project with the aim to support the development of WPs 1 to 6. As such WP7 provided the necessary tools and technologies for the research performed in the other WP.

What were the research questions when we started the project?

The objectives of WP7 were to give support to the other WPs, to develop generic tools and technologies for expression of genes, production of recombinant proteins, establishment and screening of gene libraries and analysis of structures and properties of target proteins and polysaccharides. WP7 was divided into three groups working on analytical tools, gene identification and heterologous expression and fermentation.

What were our research answers?

Geneart supported the academic partners with its expertise in optimizing and synthesizing genes and provided 90 genes (130 kb) optimized for protein production in heterologous expression systems. Furthermore the GeneArt® Truncation technology was developed for producing reading-frame-correct fragment libraries (patent WO2012084923). This technology incrementally trims genes around a defined core region, preserving the open reading frame and avoiding out-of- frame mutations. Since a major challenge of recombinant protein expression in *E. coli* remains poor solubility of numerous proteins of interest, we have expanded the Truncation technology to identify a minimal protein motif to increase the solubility of proteins, which were hitherto not soluble when over-expressed in *E. coli*. In the solubility screen the library variants are expressed in fusion with a reporter protein that mediates antibiotic resistance. Thus only bacteria expressing soluble variants of the library are able to grow on selective media. In the second phase, protein solubility of selected variants without the reporter fusion is verified and the most soluble variant from the library can be used for further applications. Using the combination of GeneArt® Truncation technology and solubility screen we identified variants of lambda-carrageenase from *Pseudoalteromonas carrageenovora* with 59 % increased solubility. The screening service is already available for customers on request.

Artes supported the project through the production of polysaccharide modifying enzymes in cooperation with other project partners and had also the aim to optimize their technology platform, e.g. by developing innovative host strains with superior secretion abilities. The yeast *Hansenula polymorpha* was used as an expression host for expression of several polysaccharide modifying enzymes. Artes investigated whether the chitinase ChiF which performs random hydrolysis of N-acetyl-beta-D-glucosaminide (1-4)-beta-linkages in chitin and chitodextrins could be expressed in *H. polymorpha* as secretory protein. For this, an expression plasmid coding for the structural ChiF-gene was constructed, fused to the commonly used yeast ER signal peptide MFα 1. A C-terminal His epitope tag for detection and purification (‘ MF‧-ChiF-H6”) was introduced. It was shown that ChiF is indeed well expressed in *H. polymorpha*, however, it seems to remain intracellular.In cooperation with INRA, the development of recombinant *Hansenula polymorpha* strains expressing a human sulfatase encoded by the gene ‘ Sulf1” was attempted. In humans, this protein changes binding sites for signalling molecules by removing sulfates from Heparan sulfate proteoglycans (HSPGs). HSPGs are involved in many biological processes, e.g. in transformation of normal to tumour cells. The protein was expressed but was not functional. Therefore, a Sulf1 variant for intracellular expression without signal peptide was produced and positive cells were provided to INRA for protein purification and/or activity testing. In collaboration with WWU, several chitin deacetylases and variants thereof were expressed in *H. polymorpha*: A *Botrytis cinerea* enzyme (‘ BC95”), a glycoprotein of about 70 kDa, and a *Podospora anserina* chitin deacetylase (‘ PaCDA”), an unglycosylated protein of about 42 kDa. Both enzymes were expressed successfully and positive cells were provided to WWU for further testing.

Artes also had the aim to isolate and characterize *H. polymorpha* mutant strains with increased secretion abilities, suitable for high-level production of heterologous proteins. A reporter system to identify strains with increased protein secretion level whose activity can be easily measured was established. A suitable reporter strain was then forwarded to BAS where it was subjected to UV mutagenesis. Mutant strains showing either increased productivity of the reporter enzyme or reduced protease activity were then provided to ARTES for further characterization. These experiments included Western blotting, quantitative amylase tests and copy number determination of the reporter cassette. For an industrial application of the mutant strains, it is necessary to remove the amylase reporter cassette. For that purpose, the reporter protein expression cassette was designed to allow genomic excision by site-specific recombination. Based on a preliminary phenotypic analysis, the cassette removal was successful in case of one strain only. This may be linked to the observation that the majority of the mutant strains display a (partly dramatically) reduced growth. Since good growth is another basic prerequisite for industrial use, it is still an open question whether these strains can be utilized. Further investigation will be needed to clarify this point.

The research of **BAS,** expert on yeast genetics, was concentrated on the development of new host-vector transformation systems and expression of epimerase – encoding genes in methylotrophic yeasts. Two novel *Hansenula polymorpha* (Hp) genes, designated as *HpPUR2,5* and *HpARG4* have been cloned and used for development of such transformation systems. The gene *HpPUR2,5* encodes for bifunctional protein catalyzing steps number two and five of *de novo* purine biosynthetic pathway. Structural analysis revealed several characteristic similarities with other yeasts PUR2,5 genes. Functional tests suggested for crucial role of HpPUR2,5p in *de novo* purine pathway as well as participation in sexual differentiation processes. *HpPUR2,5* and *HpARG4* genes have been used for construction of new sets of vector plasmids carrying these genes as selectable markers. Yeast host strains have been improved by construction of new strains with suitable markers and (in collaboration with ARTES) by isolation of mutants with increased ability for secretion of heterologous proteins and lowered activities of intracellular proteinases. In collaboration with the CNRS three genes encoding for enzymes with epimerase activity were cloned and expressed in methylotrophic yeasts. Cultivation experiments revealed that during incubations on methanol induction medium at least one of these proteins was observed in the culture medium indicating for proper expression and secretion.

SLU supported the project through the development of analytical tools to study the mode of action of the new enzymes discovered by the other WPs as well as the structure and the properties of the oligo- and polysaccharides produced by these enzymes. In collaboration with WP1, CNRS, the complete biochemical and structural characterization of two new alginate lyases from the marine bacterium *Zobellia galactanivorans* were achieved. These two enzymes revealed to have very complementary activities. Thus, together and *in vitro*, the *endo* AlyA7 and *exo* AlyA5 lyases can completely transform natural occurring algal alginate into disaccharide units (· G or · M) and the unsaturated monosaccharide that spontaneously converts into 4-deoxy-L-erythro- 5-hexoseulose uronic acid (· EH). The substrate specificity of the recombinant alginate lyase from the marine bacteria *Pseudomonas alginovora* was also elucidated and these three enzymes will be used to develop a fingerprinting for sequencing of alginates. The use of NMR of hydroxy protons to study the hydrogen bonding and hydration of carrageenans were optimized in an attempt to correlate the structure of the polymers with their gelling properties. NMR methods based on the differences in self-diffusion between water and alginates were also developed allowing obtaining information on the structure of these large polymers without need for preliminary treatment. In collaboration with INRA and Sanofi-Aventis, the specificities of three new sulfatases towards GAGs oligosaccharides and polysaccharides were determined. In collaboration with WWU, the interaction between · -chitin and two purified recombinant non-catalytic chitin-binding proteins (CBP) from two different Bacillus species, Bt-CBP from Bacillus thuringiensis and Bli-CBP from Bacillus licheniformis were investigated using IR, solid-state NMR and scanning electron microscopy. Non-catalytic chitin binding proteins are essential for chitin degradation and one hypothesis is that these proteins disturb the crystallinity of the polysaccharide. Thus the goal was to determine if and how the interaction between the protein and chitin induce conformational changes in chitin. No direct evidence of interaction at the molecular level has been obtained yet but investigation will be continued.

The role of **GTP** was to support the project through the production of polysaccharide modifying enzymes in cooperation with other project partners as well as optimize its alternative heterologous protein expression system based on the bacterial strain *L. lactis*. The Gram+ bacteria *Lactococcus lactis* was used as an expression host to express several polysaccharide modifying enzymes. During the first 18 months of the project we have mainly focused our effort on optimizing our expression system: i/ by completing the characterization and optimizing the expression conditions of our inducible (P_{Zn} *zitR* promoter) and engineered constitutive promoter (PL3 promoter); ii/ by trying, without success, to develop an efficient *E. coli* shuttle vector; iii/ by evaluating the REED technology (Reverse Electro Enhanced Dialysis) in order to increase *L. lactis* biomass production. After this first period we concentrate our effort on expressing polysaccharide modifying enzymes for our project partners. We first investigated whether a list of five C5-epimerases from *E. Silicolusus* (sequences selected by M. Czjzek - WP1) could be expressed in *L. lactis* as secreted proteins. It appeared that one was not secreted, two were produced but with a low secretion yield (< 50 µg/L), the fourth was quite efficiently secreted but difficult to purify, and the fifth (ESI0326) was expressed and purified with a final yield of about 5 mg/L. A second series of five other epimerase sequence candidates were evaluated for expression in L. lactis, but none of them were expressed. We also investigated the expression of the ESI0326 enzyme in the yeast *P. pastoris* as well as in a Mammalian expression system without real success. Finally a *L. lactis* fermentation and purification protocol has been developed in order to provide our Partner with ESI0326 purified enzyme batches with which specific epimerase activity have been measured. The characterisation of this enzyme is still in progress.

In the PolyModE program, the main **LibraGen** achievements are 1. the development of a high throughput functional screen aimed at the detection of a sulfatase activity, 2. a metagenomic library using a selected environmental sample was constructed and 3. already available metagenomic libraries were screened for a sulfatase activity, with, as main result, the identification of 10 different producers from more than 20000 screened metagenomic clones. The strategy followed for the HT sulfatase functional screen started from the demonstration of the capability to detect a sulfatase activity using a control enzyme (for this, the choice of methylumbelliferyl-sulfate as substrate was highly beneficial); it was then demonstrated that a recombinant sulfatase could be produced and detected when a model sulfatase is expressed in the host used for the construction of metagenomic libraries. Indeed,

the particularity of sulfatases is that they require a post-translational modification to be active and the corresponding activating system must be present in the bacterial host. The first chosen model, the *Clostridium perfringens* sulfatase, was proven to be unadapted due to a particular and rather original amino acid sequence. When the *Pseudomonas aeruginosa* sulfatase gene was considered, the sulfatase activity was efficiently detected in a *P. aeruginosa* genomic library and after optimization, the method was applied to the HT sulfatase screening from metagenomic libraries. The work that followed the primary identification of positive clones was first the validation in microplates as well as in flasks of the putative positive clones; then, a sub-cloning was carried out and the sulfatases obtained from isolated sub-clones were characterized by their sequence. In parallel of the functional screening approach, a molecular detection mode was also attempted: for this, we experimented a technical procedure including the immobilization of the target gene onto a membrane in one hand, and the preparation of a labelled DNA probe in the other hand. A control sulfatase gene could be detected by the method but complementary works are required in particular regarding the choice of the DNA probes. In order to discover new chitin/chitosan modifying enzymes, a metagenomic library was constructed from a soil sample from an Indian chitin manufacturing plant. More than 42000 clones (inserts average size: 40 kbp) were obtained from 127 ng DNA. Functional screening for chitinase, chitosanase and chitin deacetylase was attempted but the procedure could not be correctly set up and exploited. Collaboration is envisaged with other partners in order to finalize the screening method of chitin/chitosan modifying enzymes.

To support the development of a NMR methods based on the differences in self-diffusion between water and alginates, developed by SLU, **DuPont** supplied twenty alginate samples with varying content of mannuronic and guluronic acid and measured their respective concentration with FTIR spectroscopy to complement the NMR experiments. A gelling test in 3 ml scale was successfully used to demonstrate increase of viscosity after enzymatic modification of sugar beet pectin and sunflower pectin. A development of a micro plate assay for a gelling test could not be justified by a small number of new enzymes.

4. POTENTIAL IMPACT AND THE MAIN DISSEMINATION ACTIVITIES AND EXPLOITATION OF THE RESULTS

4.1. Impact

Research can be applied, practical and basic at the same time. That was the basic idea behind our PolyModE project. On the one hand our research had a potential **real-world utility**, but on the other hand the PolyModE investigators never lost sight of the desire to **advance scientific understanding**. It was important to promote an effective dialogue between scientists and users. In this way scientists were encouraged to think about how people will **use** their science, right from the outset, before we started the project. Efficient cooperation between partners from science and industry that is between researchers, developers and manufacturers was the **key factor** for our successful project work. Fostering an ongoing knowledge exchange dialogue between knowledge producers and users was a way to produce an atmosphere in which both groups better understood each other' s perspectives, experiences, and **needs** and thus helping the research institutions to **focus their research** on the wider needs of the society and industry. We ensured in our PolyModE project that the **dialogue with stakeholders** was integrated into the scientific research in a seamless way, from conception to completion. The progress, both in fundamental science and in the development of applications, has only been achieved through **international, interdisciplinary, and intersectorial** research. Europe has the **key players**, both in academia and in industry, to further strengthen the already strong background in the fields of polysaccharides and the industrial use of recombinant enzymes. The PolyModE project - by targeting a broad range of economically important polysaccharides from bacterial, fungal, algal, plant, animal, and human origin used in different fields of application - has the potential to support Europe in achieving and maintaining the pole position in the development of a KBBE.

In the frame of the PolyModE project state-of-the-art metagenomic and protein engineering approaches were developed and used in order to identify novel polysaccharide modifying enzymes which allow the **knowledge-based exploitation** of the vast bio-diversity of specific environments to generate novel products from renewable biological resources. This enables the reduction of **energy input and the ecological impact** of traditional chemical production processes, concomitantly creating new opportunities for high tech SMEs as technology providers in industrial biotechnology, food and medical technologies and, thus, **new job** offers for highly qualified young talents in natural sciences and engineering. The PolyModE project aimed at providing an important cornerstone in the building of a Knowledge-Based Bio-Economy. The KBBE strategy is mainly based on the enormous progress made in the biosciences over the past decades, most notable in molecular genetics, genomics, proteomics and related fields. While basic knowledge is rather far advanced in the areas of DNA and protein science and technology, **carbohydrates are far less well studied and understood today.** Therefore the PolyModE project had two parallel ambitions, namely (i) to **increase basic knowledge** and (ii) to **use the new knowledge** for technology transfer and development. We opted with care for polysaccharide modifying enzymes. One reason for this choice was the apparent **lack of basic knowledge** in this field, as exemplified in our lack of understanding the roles of patterns of substitutions in polysaccharides that may lead to the formation of functional oligosaccharide domains within the polymers. Thus, by analysing biosynthesis, modification, and degradation of polysaccharides, and by developing fingerprinting strategies for the analysis of such patterns, we were able to establish detailed **structure-functionality relationships** for the polysaccharides investigated. The second reason for our choice of polysaccharides is their enormous **abundance and diversity**, making them ideal candidates for renewable resources and, thus, for the sustainable development of commercial products. Polysaccharides are used at a large scale in many applications, e.g. as hydrocolloids in the food industry. For these applications, the physico-chemical properties of the polysaccharides, their behaviour in aqueous solutions and emulsions, is of paramount importance. These established applications made it possible for the industrial participants of the PolyModE consortium to exactly pinpoint those modifications which are (i) **economically important** and for which for there is (ii) **no chemical solution**. On the other hand, polysaccharides also lately emerge as the third class of information bearing biomacromolecules, with many physiological roles beyond their well established structural roles, making their **biological activities** highly interesting targets for the pharmaceutical industry. This is a rather novel development, but one with an enormous potential. The PolyModE project was at the forefront of probing and developing the opportunities and challenges in this field. It was an **ambitious task** to deal with polysaccharides which are produced by proteins acting as enzymes, due to the complexity of polysacchrides which, unlike DNA and proteins, are not always linear polymers but can be highly branched and multiply substituted by non-carbohydrate groups. Today, DNA and protein technology are well advanced, and they offered the tools required to successfully match this challenge, as well as the abundance of polysaccharide modifying enzymes offered by nature itself. We used state-of-the-art DNA and protein technology - such as genomics and metagenomics, gene identification and optimisation, heterologous expression and fermentation, protein engineering and directed evolution - to identify, optimise, and use polysaccharide modifying enzymes. We have with care opted for polysaccharide modifying enzymes. One reason for this choice is the apparent lack of basic knowledge in this field.
The second reason for our choice of polysaccharides is their enormous abundancy and diversity, making them ideal candidates for renewable resources and, thus, for the sustainable development of a **commercial products**. Therefore, and through the careful selection of first class academic and industrial participants, allowing **rapid technology transfer** from the academic field into the economy, the PolyModE project was able to contribute significantly for the **transition towards a KBBE**.

The PolyModE project asked relevant questions, brought together key players, created new and significant knowledge, and provided novel and economically viable solutions.
The progress achieved through the PolyModE project both in fundamental science and in the development of applications will support Europe in the development of a KBBE.

4.2. Main dissemination activities

Introduction
Dissemination takes many forms depending on the foreground itself and the different target audiences. Classical activities such as publications in scientific journals and at conferences were maintained in the PolyModE project. But the consortium wanted to take a broader proactive approach. The PolyModE website served as a tool for communication and dissemination not only within the project consortium but also to the scientific and industrial stakeholders and to the general public as well. The consortium had a strong commitment to engage with actors beyond the science communities, to increase interest in science and technology, and to overcome public hesitation to embrace new developments in science and technology on the one hand and on the other hand to raise awareness of the industry communities in order to promote the projects outputs and results which will not be directly used by the commercial project partners. This is an overview of the dissemination strategy, main activities and materials carried out by the consortium of the PolyModE project.

Dissemination strategy
Overall, dissemination activities within the PolyModE project had the objective to deliver relevant project results to **key target groups** in order to promote the exploitation of the project' s results and to ensure the widest dissemination of knowledge arising from the project as well. **Communication is one of the key elements** for a successful strategy to transfer knowledge to the socio economic world. A continuous dialog with stakeholders improved the relevance of the results and enhanced visibility and awareness of the project. Thus dissemination was perceived not only as an ' add on" to the research and development work within the PolyModE project, but as an **integral part of all activities**. The PolyModE consortium pursued a policy of disseminating the results and progress of the project which was consistent but flexible as well.

Target audiences
Effective dissemination actions depend on the clear definition of their target audiences. In order to provide a clear dissemination strategy and enable each partner to work towards promoting the project in the most effective and efficient manner, the consortium defined three key audiences and ranked them according to the importance and influence with respect to the communications objectives, namely the **scientific community**, **potential commercial users** and the **general public**. The main target audiences of the scientific communities were universities and research institutes in order to bring the research results of the project to the relevant scientific stakeholders and resulting from that allowing an **interaction**. On the other hand we wanted to raise public awareness and understanding for science and technology by informing the general public about our project, its background and its progress; raising awareness for the need to research the potential of renewable resources and increasing public understanding of biotechnological processes, including the use of genetically optimised biocatalysts. The general public is in itself a quite heterogeneous target group. For the PolyModE project it was particularly important to **inspire young people**, including **school children and students**, to pursue a career in science. The third important target audience were **commercial actors** in corresponding market segments in order to promote those projects outputs which will not be directly used by the industrial project partners.

Messages
Communication objectives and activities differed in their requirements over the runtime of the project and there were various approaches to address the specific target groups. Therefore the dissemination strategy of the PolyModE project included three consecutive phases, which were: **The awareness-oriented phase** - in the early stage of the project the priority was to set on presenting the project in order to build awareness. The key messages in this phase were: what the project is about, the project' s aims, who are involved in the project and the project activities as well. At a later stage of the project, when results were available, the promotion of the project results to relevant stakeholders were in the focus of the dissemination activities in order to allow potential interested parties to get to know the achievements and the related benefits of the PolyModE project. The key messages in this **result-oriented phase** were more specific; for example: major developments, key milestones in the project, new user communities and applications, etc. In the final phase of the project specific activities were undertaken to start exploitation measures towards potential clients in order to allow project results to be implemented in real products and targeted solutions. Stakeholders should be made interested in the project and its findings and be stimulated to get actively involved. This was the **exploitation-oriented phase**.

Activities

With respect to the dissemination activities, the project pursued a **dual strategy**, taking both an individual and a general focus. All partners involved in the project were responsible to promote active dissemination locally and / or to any other community interested in the developments and results of the PolyModE project. The partners provided contact persons who interfaced with the management team in terms of all dissemination activities. The main challenge was to distribute information and knowledge through a variety of ways, with different purposes to the various target groups. Therefore a **dissemination matrix** was developed to reflect the needs of the different audiences in terms of the amount and types of content that would be of relevance to each target group. The PolyModE management team was responsible to maintain and operate the **infrastructure for building up a dissemination base**, as well as to provide advertising material highlighting the general issues. In this regard different **dissemination tools** were developed. In order to be easily recognisable it was initially imperative for the PolyModE project to build up a strong **corporate image, brand and style.** The style and branding of the project was built around the **PolyModE logo**.

Figure 1: PolyModE logo

The PolyModE communication strategy was not exclusively designed for the scientific world. The consortium tried to communicate the scientific work of the project to the public as well in order to foster the public understanding of science. For this reason it was in a first step essential to set up a **science story** in order to stir up a public interest. The story simplified the complexity of the topic to a legitimate level, but without sacrificing the scientific quality of the research project. **Deciphering the language of complex sugars** acted as a general allegory for the targets of this- from the scientific point of view- most highly ambitious project: So far complex Polysaccharides are only sparely explored information bearing molecules, because they are a lot more complex than genes or proteins for example. Today we only new a few ‘ words’ of the language of sugars. But the premise for any form of recognition is the decoding of information; that means the reading. The aim of the project was to develop new types of specific polysaccharide modifying enzymes. With the help of these reading and writing tools we will be able to decipher and understand the language of complex sugars. And if we want to make use of this language, we will have to learn to write it, too. Furthermore a **corporate design box** was developed. The following collection of tools assisted the PolyModE beneficiaries in adapting the corporate design for their own dissemination activities related to the PolyModE project in order to build up a brand. It included a word document template (to be used for news releases, information sheets and so on), a generic poster template and slide templates. All beneficiaries adopted the corporate design recommendations in a highly satisfactory way and thus the brand became recognisable to a wide range of audiences. The official language of the PolyModE project was English, but in order to ensure that the content of the dissemination materials is widely distributed, it was recommended to translate it into local languages, too. The **PolyModE website** was the primary dissemination route through which the project was presented and it aimed to meet the communication needs of a wide range of users. Other dissemination elements (like leaflets, press releases, scientific publications, news, etc.) were complementary to the website and available for downloading. On the PolyModE website comprehensive information about the project and related information can be found. Apart from advertising our research and achievements, the website was used to promote events and publications, but it was also a platform on which **dialogue and interaction** could take place. This applied equally to internal and external communication. The PolyModE website has two distinct areas. It has a **public part and a restricted part**, accessible in a protected mode for the participants only, guaranteeing complete confidentiality in order to protect intellectual property rights. The restricted area of the site allowed regular and smooth communication between the partners as well as constituting an efficient working tool. The public part of the PolyModE website (http://polymode.eu) contains information such as the general description of the PolyModeE project and of the individual work packages; regularly updated information on the progress of the project and the work packages. Furthermore **background information** on **hydrocolloid polysaccharides**, their current uses and their modifying enzymes are available as well as background information on the **academic and industrial participants** and their contact information. **Abstracts** for presentations at conferences, links to papers and patents published, links to corresponding EC websites and to related projects and websites as well as other news on corresponding subjects are available on the public part of the PolyModE website.

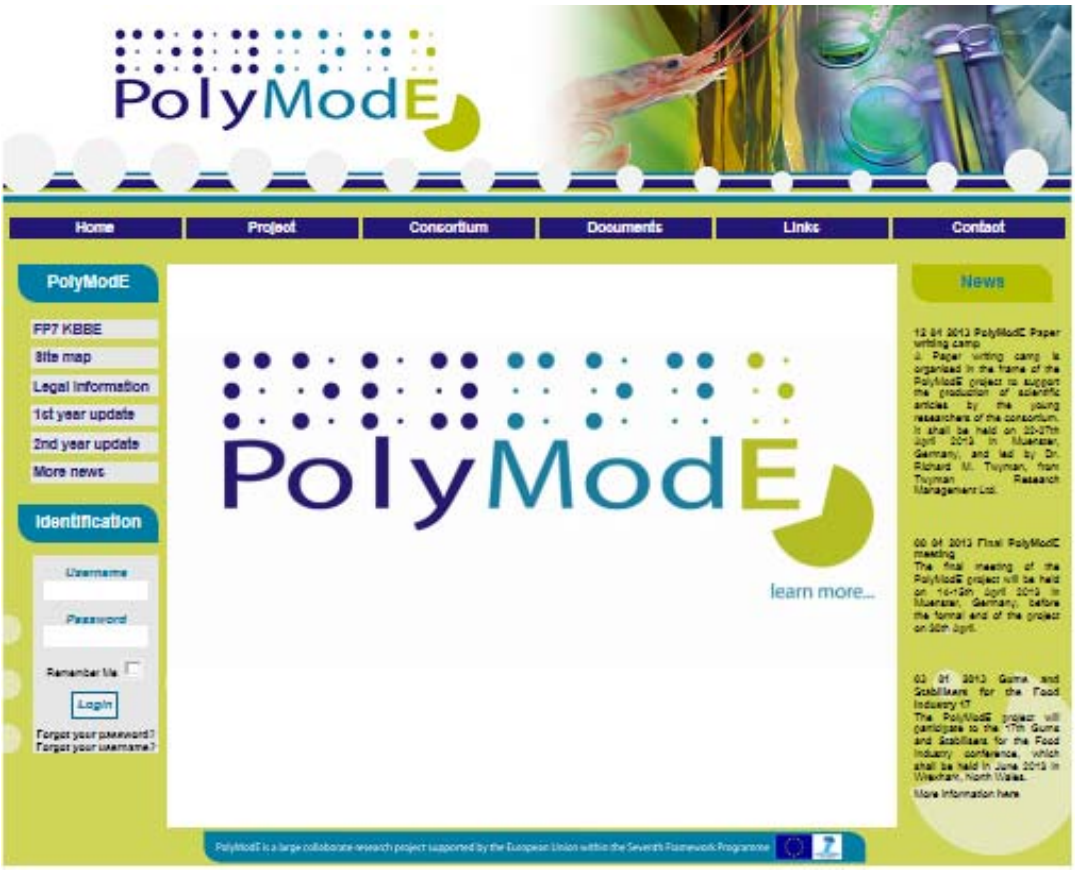

Figure 2: PolyModE website

As a supplementary approach, the original plan was, to offer a PolyModE Blog. But the consortium decided that a blog would not be the right dissemination channel for our project, basically to ensure the confidentiality of sensitive foreground data and even more after we saw that our internal discussion forum was not used and accepted by the partners. By experience the consortium knew that **direct contacts** with the targeted audiences are one of the **most effective dissemination activities**, especially towards the **scientific community and potential industrial users.** Therefore active participation in conferences, workshops and trainings were important dissemination activities of all partners as well as visits with individual presentations, involving external stakeholders, e.g. presentation about our project work at national and industrial fairs like the Gen2bio ((2010, St. Malo) or the Biotechnica (2011, Hannover). In this regard it should also be emphasized in particular that the PolyModE project was presented to prominent companies of the food industry within the scope of two excursions of the Food Chemistry PhD students from WU to Switzerland and Italy in October 2010 and to Malaysia and Singapore, in November 2012. Another dissemination activity, where we had the chance to introduce our project, was for example the ' Open doors of science' initiative at SBR with workshops and presentations about the exploitation potential of macro-algae. The presentation of results were carried through continuously by means of oral and poster communications at major international conferences e.g. ICCC in Taipeh (2009), CBM9 in Lisbon (2011), EUCHIS conference in St. Petersburg (2011), Plant and Seaweed Polysaccharide Workshop in Nantes (2012), CBM10 in Prague (2013), Gums & Stabilizers conference in Wrexham (2013) and Eurocarb in Israel (2013). **The PolyModE international dissemination conference** took place in combination and on the occasion of the **17th Gums and Stabilisers Conference** at Glyndŵ r University, Wrexham, UK on 25th - 28th of June 2013, under the headline**: ' The changing face of food manufacture: the role of hydrocolloids'** This conference is a premier forum for providing closer interaction between academic and industrial scientists in the field of Food Hydrocolloids. Thus it guarantied not only a **highly scientific approach** but also **huge interest of industry**. This event offered a great opportunity to present the non-confidential PolyModE results to a broad industrial audience and an involvement of potential users and stakeholders from outside the consortium. The conference proceedings will be published by the Royal Society of Chemistry. The book will be available electronically on the RSC website as well as in hard copy. This book will also capture the research findings of the PolyModE project. It will be a useful reference for researchers and other professionals in industry and academia, particularly those involved directly in food science. An initial **project leaflet** was produced to describe the project, its objectives, the participating organizations, the expected results and the activities planned. The leaflet was used for raising

awareness and it was intended to support presentations at events as well as individual meetings carried out. In addition a **final general project leaflet** was developed, describing the final results of the project, which served amongst others as a communication tool for the PolyModE dissemination conference in Wraxham.

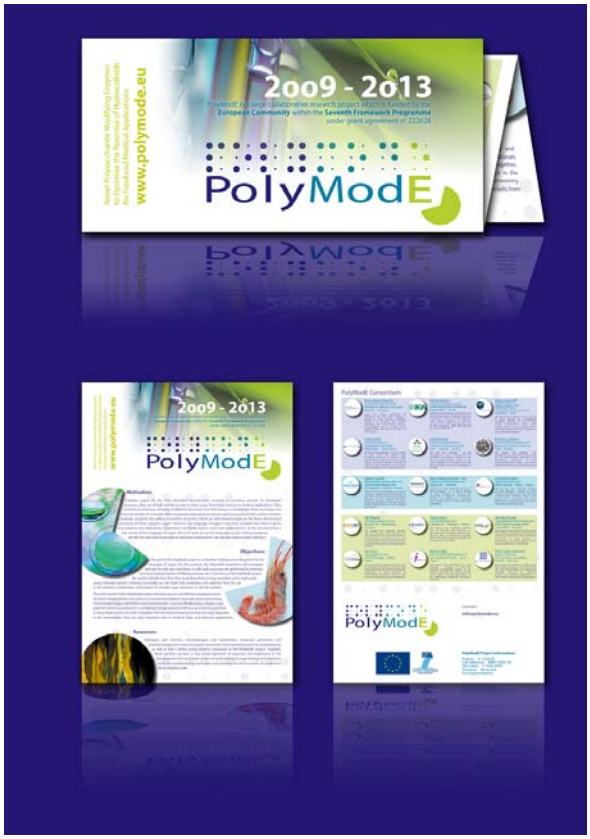

Figure 3: PolyModE project leaflet (2009)

Figure 4: PolyModE project leaflet (2013)

Peer-reviewed scientific articles constitute an excellent dissemination activity to reach a wider scientific audience and make them aware of the projects objectives, activities and results in a detailed manner. The PolyModE project partners made a real effort to disseminate the main findings by means of peer-reviewed articles. These publications involved external review of findings, which is crucial for safeguarding the overall scientific quality of the project. Moreover project findings were also published in **professional and technical journals**, which are an important source of information for other professionals, e.g. working in industry. In this context it should be mentioned that we promoted our project not only to our own core scientific community but also to a wider range of potential stakeholders with the dual aims of potential commercialization /exploitation and future research collaborations. Therefore we cooperated with Research Media who are the publishers of the **International Innovation Journal**. International Innovation is a research journal covering the most important issues in science and research currently. The journal was developed to bridge the gap between science, policy, research, government and the private sector. It is a hard copy journal and also hosted online. International Innovation reaches a dedicated global audience of **39'000** key researchers, policy makers, government and decision makers across both the private & public sectors. The report was also made available online on a dedicated web page for a period of twelve months. The hard copy version of the report was distributed within the EU. The PolyModE project participated in the annual International Innovation publication (2010) which had a focus on food production and quality with a 3 page article.

The Future of complex sugars

Professor Bruno Moerschbacher, explores the possibilities of using polysaccharide modifying enzymes to produce novel functions and new complex sugars for industry and beyond

Can you outline some functions of complex polysaccharides, and explain why they are so important?

How do you hope to optimise enzymes outside of living cells?

Why do PolyModE researchers see a need to isolate the enzymes used by nature, and by what means will you adapt them to environments outside of living cells?

How will PolyModE improve the production of known complex sugars, and will the project generate novel complex sugars with further improved properties?

Could this research lead to the engineering of designer polysaccharides with desired properties?

With reference to biological compatibility, what could this mean for both the human body and the environment?

Learning the Language of Polysaccharides

PolyModE is an exciting new EC funded project investigating the molecular language of complex sugars. Researchers hope to use the enzymes that produce sugars to synthesise and modify them for a myriad of uses

Figure 5: Publication International Innovation, 2010

The **regional and local media** (newspapers, magazines, radio) were also important for reaching the general public, as well as for option formers. By means of press releases, conferences, interviews or other events the PolyModE partners informed about the project' s work and findings. As a main principle, internal general assembly meetings took place every six month. Other internal meetings that involve only a few partners supplemented according to need. Further internal dissemination measures have been completed by means of four different **technical workshops** combined with a high level of attention to participating students.

Special care was taken to transfer the PolyModE messages into general public vocabulary in order to produce a clear idea of the project. In this regard the project tried to be different and to use innovative ways to approach the targeted audience. Young researchers of the **WWU** realised their thoughts to disseminate the PolyModE foreground to the general public, especially in order to **inspire young people**, including **school children and students .** The group developed a computer game, reflecting the aims of the project, which was distributed as a smart phone app in cooperation with the University of Applied Science in Münster, responsible for the technical implementation. This was a great idea, effective and efficient at the same time, to explain the PolyModE story to the general public and to lead especially kids and youngsters to science by learning playfully. The game is also useful to serve teachers as a strong incentive for their lessons. The so-called **PolyModEfyer** is not only a game, **it is science**. The game is to introduce youngsters but also all other interested parties into the **fascinating world of sugars** – and if they enjoy it, they might also want to learn more on www.polymode.eu. The general public was informed by a wide range of different press releases that the PolyModEfyer is available under https://play.google.com/store/apps/details?id=de.se.group4.polymode.

Figure 6: QR Code Contact PolyModE

Figure 7: QR Code PolyModEfyer

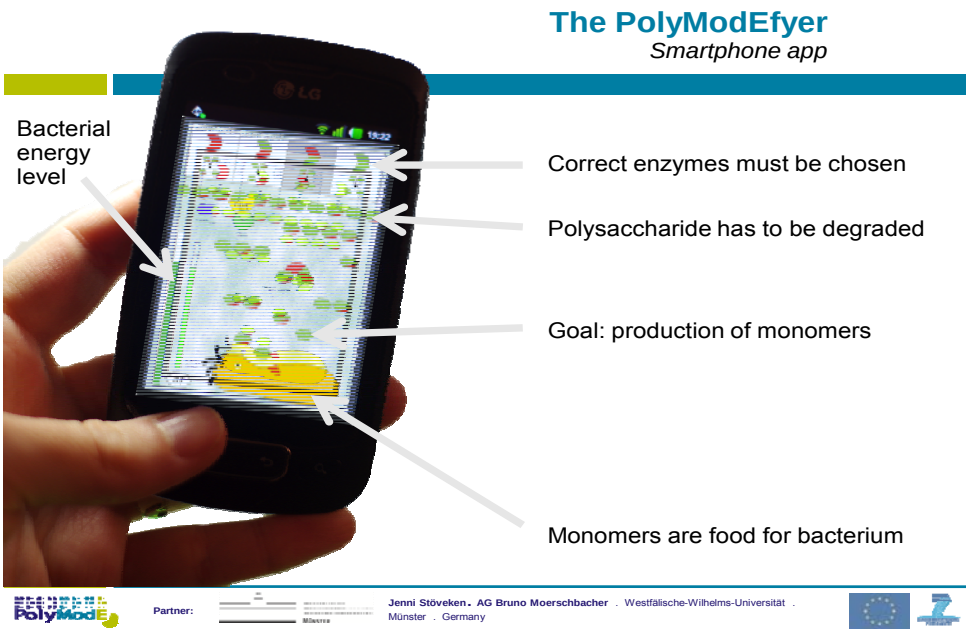

Figure 8: The PolyModEfyer Smartphone app

Furthermore the project was invited to participate in an accompanying exhibition on the occasion of the **opening session** of the new European research framework program ' **Horizon 2020" in North Rhine-Westphalia** on the 30th of September 2013 in Düsseldorf to present its achievements. This is an event for and on behalf of the Ministry of Innovation, Research and Technology as well as the Ministry of Trade and Industry of North Rhine-Westphalia. This event will be a **premium forum** with up to 500 participants from science, industry and politics.

It should be mentioned, that especially at the end or after the completion of research projects a large number of papers are submitted to peer reviewed journals.
Some important papers, generated within the course of the PolyModE project, which have been submitted, but have not been reviewed and accepted for publication yet:

[1] Nampally, M., Rajulu, G., Gillet, D., Suryanarayanan, T.S., and Moerschbacher, B.M. (2013) More fungal than bacterial diversity of chitinolytic and chitosanolytic species and enzymes exist in soil samples with a history of chitin and chitosan exposure. ***World J. Microbiol. Biotechnol.,*** **submitted**
[2] R. Fischl (2013) Bioinformatic and biochemical analyses of the enzymes responsible for the final alginate synthesis in brown algae: the C5-epimerases from E. Siliculosus. ***New Phytologist*****, submitted**
[3] R. Fischl (2013) Heterologous expression of brown algal C5-epimerase. ***Microbial Cell Factories BioMed Central,*** **submitted**
[4] F. Thomas (2012) The mode of substrate recognition by alginate lyases from marine origin differs from those isolated f. rom soil bacteria. ***Journal of Biological Chemistry*****, submitted**
[5] Anton Stoyanov, Penka Petrova, Dimitrinka Lyutskanova, Kantcho Lahtchev (2013) Cloning and characterization of PUR2,5 gene encoding bifunctional enzyme of purine biosynthesis in the yeast Hansenula (Ogataea) polymorpha. ***Microbiological Research,*** **submitted**
[6] C. Remoroza (2013) Innovative approach of characterizing highly substituted sugar beet pectin by enzymatic fingerprinting. ***Biomacromolecules,*** **submitted**
[7] Kool, M.M., Gruppen, H., Sworn G., Schols, H.A.(2013) Comparison of xanthans by the relative abundance of its six constituent repeating units. ***Carbohydrate Polymers,*** **accepted for publication**
[8] Kool, M.M., Schols, H.A., Delahaije, R.J.B.M., Sworn, G., Wierenga, P.A. Gruppen, H. (2013) The influence of the primary and secondary xanthan structure on the enzymatic hydrolysis of the xanthan backbone. ***Carbohydrate Polymers, in press***

[9] F. Thomas (2013) The paralogous PL7 alginate lyases AlyA1 and AlyA5 from Zobellia galactanivorans evolved different active site topologies for complementary modes of action. *Journal of Biological Chemistry,* **accepted**
[10] Remoroza, C., H. A. Schols, Buchholt H.C. and Gruppen H. (2013) Revealing substitution patterns of differently prepared sugar beet pectins by enzymatic fingerprinting**.** *Biomacromolecules,* **submitted**

4.3. Exploitation of the results

4.3.1. Work package 1 - Alginate modifying enzymes; WP leader: Mirjam Czjzek (CNRS)

In the brown algal cell wall alginate is tailored by **specific enzymes** to fulfil **different functional roles** such as stability, resistance and flexibility. These characteristics are tuned by variation of the M/G content and distribution along the polysaccharide chain. By taking inspiration from these biological processes, the foreground generated in WP1 is of two natures: the use of enzymes of algal origin for tailoring the alginate polysaccharides opens a larger diversity of modifications that can be attained, and the **use of marine bacterial enzymes** are particularly well adapted to develop **specific fingerprinting analysis** methods of modified alginate. In addition, enzymatic modifications and degradations of polysaccharides will help develop **white biotechnological processes**, at several stages along the polysaccharide production chain. Enzymes could be integrated into polysaccharide **extraction methods**, thus replacing alkali-treatments, as well as at later stages for the conditioning of the polysaccharide into particular forms for **specific applications**. These processes could also have the advantage to have a more **reproducible and controlled** outcome.

The possibility to control more precisely the **M/G ratio and distribution** allows **fine-tuning of physicochemical properties** of the alginate polysaccharide and therefore opens a wider field of applications, especially in the field of health and cosmetics. Combined use of enzymes modifying and degrading the alginate from various origins could therefore lead to the development of new biotechnologies and the use of alginate with unique patterns in new and promising applications in pharmacy and medicine, *e.g.* in drug or protein delivery, cell encapsulation, tissue regeneration, surgery, and wound management. The access to complementary enzymes of alginate degradation with precisely known **substrate specificities** also gives rise to the application of **enzyme assisted analytical methods**, allowing the setup of quality control procedures for fundamental research purposes, but also for applications in pharmacy and medicine.

An emerging field, to which our results could potentially be of important contribution, is the use of algae as **renewable biomass** in developments of **alternative energy resources**. Indeed, the potential use of brown algal biomass for biofuel or as source for other value added basic molecules (sulfated detergents, lipids, sulphated oligosaccharides, etc) passes by the use of catabolic enzymes, like alginate lyases, but also all other enzymes that have been identified and partially characterized in our research about the marine bacterial alginolytic operons.

Main dissemination activities:

A- Publication of results in peer-reviewed scientific journals:
[1] Thomas F, Barbeyron T, Tonon T, Génicot S, Czjzek M, Michel G. (2012) Characterization of the first alginolytic operons in a marine bacterium: from their emergence in marine Flavobacteriia to their independent transfers to marine Proteobacteria and human gut Bacteroides. *Environ Microbiol*. **14**:2379-2394.
[2] Lundqvist LC, Jam M, Barbeyron T, Czjzek M, Sandström C (2012) Substrate specificity of the recombinant alginate lyase from the marine bacteria Pseudomonas alginovora. *Carbohydr Res.* **352**:44-50.
[3] Thomas F, Lundqvist LC, Jam M, Jeudy A, Barbeyron T, Sandström C, Michel G, Czjzek M (2013) Comparative characterization of two marine alginate lyases from *Zobellia galactanivorans* reveals distinct modes of action and exquisite adaptation to their natural substrate. Accepted in JBC
B- Presentation of results through oral and poster communications at major international conferences:
2011 CBM9 in Lisbon, **2012** Plant and Seaweed Polysaccharide Workshop in Nantes, **2013** CBM10 in Prague, **2013** Gums & Stabilizers conference in Wrexham, **2013** Eurocarb in Israel
C- Presentation about marine biotechnology at national and international industrial fairs:
2010 Gen2bio St Malo - Discovery of novel enzymes from marine bacteria ;
2011 Biotechnica Hannover - Marine genomics as a basis for blue biotechnology
D- Open doors of science' initiative at SBR - workshops and presentations about exploitation potential of macro-algaea

4.3.2. Work package 2 - Carrageenan modifying enzymes, WP leader: William Helbert (CNRS)

What is the unique value proposition of the foreground generated in WP2?

Carrageenans are complex macromolecules which chemical structures modulates their functional properties (gelling, thickening) appreciated in numerous food and cosmetics applications. We have isolated and produced by biotechnology processes **carrageenan modifying enzymes** that allow **controlling the structure** and, therefore, the **functional properties** of carrageenan. For the first time, we have identified, characterized and produced **formyl-glycine** dependant **carrageenan-sulfatase** active on carrageenan under its polymeric form. In addition to the expected control of known carrageenan structures (i.e. kappa/beta-carrageenan), we were able to **synthesize new type of carrageenan** (i.e. alpha-carrageenan) opening **new perspectives** in the **industrial** exploitation of the carrageenans.

What are we going to achieve with the foreground?

Carrageenans are made of various repetition moieties - called **carrabiose units** - classified as a function of the number and the position of sulfate ester groups. The **composition and distribution** of carrabiose units are two **key parameters** that determine the exact **physical properties** of a particular carrageenan. With carrageenan-modifying enzymes, we will prepare new series of carrageenan with **controlled composition** and distribution carrabiose unit. We expect **new properties** of these carrageenans that will find some application in food industry. The use of sulfurylases can improve the sustainability of the carragneenan production process in three ways. One, **reduce** the need for **caustic chemicals**. Two, **reduce** the **energy consumption** of the process, since sulfurylases have shown to be active at low temperature. Three, **increase yield and/or functionality** because of less carrageenan degradation side reactions, leading to a better utilization of the raw materials. Having a range of sulfatase enzymes increases the flexibility of raw materials that can be used. This in turn can lead to better exploiting of algal resources especially of farmed seaweeds (Kappaphycus sp, Eucheuma sp.). In combination, the family of enzymes allows the production of **more sustainable more efficient stabilizers** for the food, personal care and pharmaceutical industries.

Which applications could emerge from the foreground?

One of the unique properties of carrageenans is their ability to **interact with proteins**. As such most carrageenan applications in the food industry are protein containing foods, i.e. dairy and meat. It has been demonstrated that rather small sequences of carrageenan repeat units make up domains that interact with peptide sequences on proteins. As such sulfurylases and sulfatases can be used to tailor specific carrageenans with stronger and/or new protein interactions for namely **dairy and meat applications**. Users can use the enzymes to achieve a more sustainable and potentially more **cost effective production process** and higher **flexibility on raw materials**. Moreover, new functionalities can allow for the development of more efficient products or products with unprecedented **functionalities**.

4.3.3. Work package 3 - Chitosan modifying enzymes, WP leader: Bruno Moerschbacher (WWU)

What is the unique value proposition of the foreground generated in WP3?

In the course of the PolyModE project, we have reached both of our goals, namely the identification of novel chitin and chitosan modifying enzymes which can be used to i) generate and ii) analyse novel chitosans with non-random patterns of acetylation. Such chitosans were not previously available, as today' s chitosans are invariably generated from chitin using chemical ways, either by partial deacetylation or by full deacetylation followed by partial reacetylation. Both procedures generate chitosans with random PA. Our novel chitosans generated by partial enzymatic deacetylation of high-DA chitosans yielding low-DA chitosan are the first ones ever with non-random PA, and we have even found that different chitin deacetylases produce chitosans with different, non-random PAs. This latter observation was made possible by the development of enzymatic/mass spectrometric fingerprinting tools making use of novel sequence-specific chitosan hydrolases characterised during the PolyModE project. Based on theoretical considerations, we predict that both the physico-chemical properties and the biological acitivities of these novel chitosans will differ from those of the conventional chitosans.

What are we going to achieve with the foreground?

Our novel chitosans will open up a completely new phase in chitosan research and, predictably, in chitosan applications. This is true for applications making use of the superior material properties of chitosans as well as for applications based on their versatile biological activities. As an example, the known pattern specificity of e.g. human chitotriosidase, will now allow us to develop designer chitosans with predictable digestability in the target tissue, giving rise to predictable oligomeric degradation products with

predictable biological, e.g. wound healing activities. Similarly, we foresee further improvements of the plant strengthening activities of chitosans on the same grounds. But we also see new possibilities arising for applications of chitosans in material sciences. It is well known from polymer chemistry that the blockiness of co-polymers strongly influences their material, e.g. rheological properties. Consequently, the PA of chitosans which can be considered as co-polymers of GlcN and GlcNAc can be expected to have an equally deep impact on e.g. the gel and film forming properties of chitosans. It is important to realise that chemical modifications of chitosans which are often used to improve the material properties of chitosans typically rely on the free amino groups as anchor points. Thus, PA will also significantly influence the performance of the many chitosan derivatives described in the literature.

Which applications could emerge from the foreground?

As discussed above, we are convinced that our novel chitosans with different, non-random PA will open up new possibilities for chitosan-based applications. We can predict that this will be true for almost all type of applications, whether in the form of a hydrogel or film e.g. in food packaging, as a seed dressing or leaf spray for plant growth promotion and plant disease protection, as a nanoparticulate drug delivery system, or as a wound dressing to promote scar-free healing.

A: Publication of results in peer-reviewed scientific journals:
[1] Nampally, M., Rajulu, G., Gillet, D., Suryanarayanan, T.S., and Moerschbacher, B.M. (2013) More fungal than bacterial diversity of chitinolytic and chitosanolytic species and enzymes exist in soil samples with a history of chitin and chitosan exposure. ***World J. Microbiol. Biotechnol.*** **Submitted**
[2] Kolkenbrock, S. and Moerschbacher, B.M. (2013) Generation and evaluation of pPolyREPII, a synthetic, modular, broad host range expression vector for diverse Gram(+) and Gram(-) prokaryotes. ***PloS one*** **submitted**
[3] Stöveken, J., Kolkenbrock, S., Zakrzewski, M., Pühler, A., Schlüter, A. and Moerschbacher, B.M. Gene mining for chitin and chitosan modifying enzymes in a taxonomically-diverse metagenomic soil library yields a novel active chitinase (2013) ***PloS one*** **to be submitted**
[4] Hoßbach, J., Kranz, A. and Moerschbacher, B.M. Novel chitin deacetylase from *Podospora anserina* with two carbohydrate binding domains produces chitosans with non-random patterns of acetylation. ***Carbohydr. Polym.*** **to be submitted**
B: Non-peer reviewed (proceedings of conferences)
[5] Honorato, T.L., Rodrigues, S., Moerschbacher, B.M., and Franco T.T. (2011) Chito-oligosaccharides produced by crude enzyme extract of *Trichoderma polysporum*. ***Adv. Chi. Sci.*** **13:114-120**
[6] Moerschbacher, B.M., Bernard, F. , and El Gueddari, N.E. (2011) Enzymatic / mass spectrometric fingerprinting of partially acetylated chitosans. ***Adv. Chi. Sci.*** **13:185-191**
[7] Kolkenbrock, S., El Gueddari N.E. and Moerschbacher, B.M. (2013) Towards biotechnological production of chitosans. ***Adv. Chi. Sci.*** **submitted**
[8] Wagenknecht, M., Kolkenbrock, S. and Moerschbacher, B.M. (2013) From gene to protein – Gene selection, cloning, and appropriate strategies for heterologous synthesis of polysaccharide-modifying enzymes. Royal Society of Chemistry **submitted**

4.3.4. Work package 4 - GAG modifying enzymes, WP leader: Olivier BERTEAU (INRA)

What is the unique value proposition of the foreground generated in WP4?

Glycosaminoglycans are **highly complex** polysaccharides which **pharmaceutical applications** rely on a precise **control of their structure and composition.** We have identified and characterized **novel sulfatases able to selectively modify** a wide range of GAGs from oligo- to polysaccharides. These enzymes should allow producing **novel GAGs derivatives** and thus increasing the repertoire of available compounds with promising application from health to cosmetic. In addition to selectively control and modify the sulfation pattern of GAGs, these enzymes should be **valuable tools to sequence** complex GAGs.

What are we going to achieve with the foreground?

Naturally produced GAGs are made of various repetition motifs with intrinsic sulfation heterogeneity. This **sulfation pattern** is known to be a **critical parameter** for the **biological properties** of GAGs. Indeed, incorrect sulfation pattern not only can lead to inactive compounds but can have also result in compounds with detrimental properties. With these **novel sulfatases**, we will be able to prepare **novel libraries** of GAGs compounds for either **the synthesis** of novel bioactive products or to **develop** novel **research tools** including novel carbohydrate-based chips. We expect that these novel GAGs compounds will interact with interesting pharmaceutical targets and thus increase the number of application of GAGs in therapeutic.

Which applications could emerge from the foreground?

These novel enzymes, with characterized specificity, are also a unique opportunity to **rationalize** the **molecular determinants** that govern their specificity and selectivity. From this detailed understanding, **novel opportunities** for enzyme design and engineering should emerge to develop **environmentally friendly catalysts** for a wide variety of application. Indeed, we should be able to re-engineer these enzymes active sites to modify their selectivity and thus obtain compounds with novel structures and properties. A more ambitious goal would be to modify the type of polymers they recognized this will dramatically **broaden their applications** and will open **new possibilities in the biotechnology** of polymers which currently lack such type of catalysts for the engineering of sulfated polymers.

4.3.5. Work package 5 - Pectin modifying enzymes, WP leader: Henk Schols (WU)

Pectin is a polysaccharide frequently occurring in nature, as it is a constituent of the **cell wall of all terrestrial plants**. In the food industry, pectin plays an important role due to its use as **gelling, stabilizing, and thickening agent**. It is extracted from **lemon, apple**, and **sugar beet**, the latter source gaining increasing interest because of the significant amount of sugar beet produced worldwide. Other than, e.g., pectin originating from lemon, **sugar beet pectin** (SBP) contains **acetyl groups** (degree of acetylation (DA) 30%) predominantly located at the homogalacturonan (HG) regions. Earlier it was shown that **deacetylation enhances** the **gelling ability** of SBP. Such modification may be achieved by **saponification** using **chemicals**. However, chemical deacetylation comes along with the **hydrolysis of methyl esters** which are likewise present in pectin and positively influence pectin' s **rheological properties**. Hence, the necessity of an **alternative approach**, selectively removing only acetyl groups from the pectin backbone, was obvious. **Pectin acetylesterases** (PAEs) represent a group of enzymes providing such possibility. One major focus of WP 5 was the **identification and characterization** of PAEs capable to **specifically modify SBP** and other acetyl-rich pectins showing activity in the presence of both high and low levels of methylesters. A subordinated focus was put on **pectin methylesterases** (PMEs) able to **convert SBP into low-methyl pectin with a high DA**, the functionality of which is completely **unknown** at present.
During the project several PAEs have been produced and characterized. Two of them showed a significant release of acetyl groups (20-40%) using SBP as the substrate. Both enzymes were active towards the HG regions only and preferred to deacetylate the galacturonic acid (GalA) residues at the *O*-3 position. It became also evident that the activity of both PAEs was hindered by the presence of methyl groups located at the same or adjacent GalA residues. A PAE specifically deacetylating the rhamnogalacturonan (RG) regions was likewise found. A **novel bacterial PME** was identified that demethylated SBP (60-85%), interestingly in a **blockwise** manner. As hindered by the presence of acetyl groups, such enzyme almost completely demethylated the non-acetylated lemon pectin. Moreover, we established a **new fingerprinting method** for the detailed structural analysis of SBP-derived oligomers generated after the treatment of the polymer with backbone-degrading enzymes.

Our findings have the potential to enzymatically turn SBP into low-acetyl but still methylesterified pectin being structurally similar and, therefore, an **alternative to lemon pectin**. Along with that, it would allow the **reasonable use of an agro industrial by-product**, originating from the beet sugar production, thus, preserving resources. The **new enzymatic pectin-fingerprinting** HILIC-ELSD MS technique allows the analysis of the **chemical fine structure** of SBP such as the substitution patterns of methyl and/or acetyl groups and, based on this, the **investigation of the mode of action** of PAEs and PMEs. The method has the potential to be used by pectin producers to **investigate (new) raw materials** for pectin production as well to **monitor the isolation process and down-stream processing**. Linking the new findings allow **detailed structure-function relationships** leading to a better understanding of the **rheological properties** of enzymatically modified sugar beet pectin.

Main dissemination activities:
A- Publication of results in peer-reviewed scientific journals:
[1] Remoroza, C., S. Cord-Landwehr, et al. (2012). Combined HILIC-ELSD/ESI-MSn enables the separation, identification and quantification of sugar beet pectin derived oligomers. Carbohydr. Polym. 90(1): 41-48.
[2] Remoroza, C., H. A. Schols, et al. (2013). Revealing substitution patterns in differently prepared sugar beet pectins by enzymatic fingerprinting. Submitted for publication.
B- Presentation of results through oral and poster communications at major international conferences:
2013 Gums & Stabilizers for the Food Industry, symposium, Wrexham, UK, June 2013
2012 11th International hydrocolloids conference, Purdue University, Indiana, USA, May 2012
2011 Gums and Stabilizers for the Food Industry, symposium, Wageningen, NL June 2011
2011 2nd European Polysaccharide Network of Excellence International Conference, August 2011, Wageningen, the Netherlands

4.3.6. Work package 6 - Xanthan gum modifying enzymes, WP leader: Henk Schols (WU)

Xanthan is a polysaccharides produced by *Xanthomonas campestris.* The xanthan structure is rather repetitive, however the use of different production strains or fermentation conditions give rise to xanthans with **different degree of acetylation and pyruvylation**. These substituents have a **major impact on the functional properties** of xanthan. The exact structure-functional relationship, however, is **poorly understood**, especially since no suitable methods are available for the analysis and comparison of the structure of various xanthans. Until the start of the PolyModE project, the functionality of xanthan was mainly explained from a more **physical point of view**, assuming the structure of xanthan as regular with only one major repeating unit as established in the nineteen seventies. In WP6 we developed a method to **analyse the xanthan structure in more detail** using cellulases to degrade the xanthan backbone, which allowed the analysis of individual xanthan repeating units. We have identified six different xanthan side chains and indicated that the position of the acetyl group can vary from the position in the generally excepted xanthan structure. Comparison of various xanthans showed that the ratio in which the six different side chains are present in xanthan differs between xanthan samples, even when the molecular composition is the same. Furthermore we correlated the **transitional behaviour** of xanthan to the different side chains and we showed that especially the substitution pattern of the outer mannose unit mainly influences the xanthan conformation and thereby the xanthan functionality. In addition to the development of a **xanthan fingerprinting method**, two **acetyl esterases** were characterised for their activity towards xanthan. One acetyl esterase specifically removes the acetyl groups on the outer mannose and the other specifically removes the acetyl group on the inner mannose.
Linking these new findings in xanthan structure and in the variability of xanthan' s structure will allow a **better understanding and control of the production of xanthan and it' s downstream processing**. Revealing the differences in chemical fine structures between different batches of xanthan having different functionality will lead to a better understanding of the **rheological properties** of xanthan and consequently will lead to **improved** or even **novel applications** of xanthan. The availability of novel enzymes able to specifically remove (part of) the acetyl substitution of xanthan may open a new field of functionality and applications. The use of enzymes to modify the xanthan structure will enable the xanthan producers to **omit the use of chemical treatment** of xanthan and genetically modified xanthan producing organisms which is in line with modern trends in society (green label, non-GMO, increased sustainability, ecological-, carbon- and water footprint, etc).

Main dissemination activities:
A- Publication of results in peer-reviewed scientific journals:
[1] Kool, M.M., Schols, H.A., Delahaije, R.J.B.M., Sworn, G., Wierenga, P.A. Gruppen, H. (2013) The influence of the primary and secondary xanthan structure on the enzymatic hydrolysis of the xanthan backbone. *Carbohydrate Polymers*, in press, June 2013
[2] Kool, M.M., Gruppen, H., Sworn, G., Schols, H.A. (2013) Comparison of xanthans by the relative abundance of its six constituent repeating units. *Accepted for publication in Carbohydrate Polymers, June 2013*
B- Presentation of results through oral and poster communications at major international conferences:
2011 2nd European Polysaccharide Network of Excellence International Conference, August 2011, Wageningen, the Netherlands
2012 11th International hydrocolloids conference, Purdue University, Indiana, USA, 2012
2013 Gums & Stabilizers for the Food Industry, symposium, Wrexham, UK, 2013

4.3.7. Work package 7 - Generic techniques, WP leader: Corine Sandström (SLU)

All partners of **WP7** were fully integrated into the other six WPs and have successfully contributed to their progress. For example, **Geneart** has developed the GeneArt® Truncation technology for producing reading-frame-correct fragment libraries (patent WO2012084923) and a screening service is now available for customers**. Artes and BAS** have optimized expression and fermentation systems and successfully express several enzymes such as sulfatases and chitin-deacetylaes in *Hansenula polymorph.* **SLU** in collaboration with CNRS has identified and characterized the mode of action and substrate specificity of a set of three new and complementary lyases that will be used for fingerprinting of alginates. **GTP** has mainly worked on expressing several epimerase enzymes and successfully expressed three of them in *L. Lactis.*

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