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EXECUTIVE SUMMARY

Nano3Bio has been a huge challenge, exciting research, and a rewarding experience! We had a large international and interdisciplinary consortium of academic and industrial partners to cover all relevant aspects of the subject, to allow us to successfully and efficiently address the different aspects associated with it. We aimed to develop biotechnological production techniques for chitosan polymers and oligomers which are better defined and more closely resembling natural chitosans than today's conventional chitosans produced from natural chitin using chemical means.
Chitosans are one of the most promising classes of functional biopolymers, with many different possible applications. Some chitosans can protect plants from disease and some chitosans can promote growth and fruit development in plants; some chitosans can support wounds to heal without scar formation and some chitosans can be used to deliver drugs to their target tissues; some chitosans can be used for waste water purification or drinking water clarification, and some chitosans can be used as food conservatives or stabilisers in cosmetics. Today, chitosans are produced from the enormously widespread biopolymer chitin which is mainly sourced from shrimp and crab wastes of the fishery industries. However, the chemical conversion of chitin to chitosan leads to random mixtures of chitosans whose properties and functionalities are difficult to predict and even more difficult to reproduce. This has long hindered the development of chitosan-based products and applications. Research of the past twenty years, in which some of the Nano3Bio partners were centrally involved, has shed light on the structure-function relationships of chitosans so that today, the chemical conversion process can be steered in a reproducible way to yield rather well-defined "second generation" chitosans with known functionalities. These are currently becoming available on the markets at industrially relevant volumes, and products based on them are increasingly appearing in different end-markets, such as in agriculture. These products are fully biocompatible and biodegradable, they are non-toxic, non-allergenic, environment-safe and consumer-friendly. They are, thus, ideally suited to meet the needs of the ongoing conversion from a petrol-based to a bio-based industry in an interest of sustaining the basis of life on Earth, and to satisfy the demands of increasingly critical, health-oriented customers.

However, even these second generation chitosans are probably not truly representative of natural chitosans which are produced by some fungi from the chitin in their cell walls using enzymes called chitin deacetylases. In contrast to chemical methods, enzymatic tools can be expected to yield much more closely defined chitosans, and we had predicted based on preliminary evidence that these natural or biotechnologically produced chitosans would differ from their chemically produced counterparts. In a small previous project, the core Nano3Bio partners had already provided proof-of-principle that a biotechnological production of different, fully defined chitosan oligomers is possible. To this end, bacterial chitin synthase encoding genes were transferred to the bacterium E. coli, the standard working horse for biotechnology and genetic engineering. This allowed E. coli to produce chitin oligomers, and adding genes for different chitin deacetylases converted these into different chitosan oligomers. The Nano3Bio project now convened a consortium of experts from Academia and Industry to try and extend these results with the goal to

- identify and characterise chitin synthase and chitin deacetylase genes and enzymes from different organisms;
- biotechnologically produce a broader diversity of fully defined chitosan oligomers;
- improve the yield and purity of the biotechnologically produced chitosan oligomers;
- biotechnologically produce novel chitosan polymers differing from conventional, chemically produced chitosans;
- improve analytical techniques to investigate the structures of the novel chitosan oligomers and polymers;
- determine biological activities of the novel chitosans in comparison with conventional chitosans;
- develop nano-formulations of the novel chitosans in comparison with conventional nano-chitosans.

All of these goals were achieved:

- We produced the first chitin polymers in vitro using a recombinant chitin synthase.
- We produced all fourteen possible chitosan tetramers using a range of recombinant chitin deacetylases.
- We improved the efficiency of biotechnological production of chitosan oligomers by a factor of 20.
- We engineered transglycosylating chitinases and converted them into chito-synthases, yielding chitin polymers.
- We provided the first experimental evidence that different mono-acetylated chitosan tetramers have different bioactivities.
- We identified the first non-fungal natural chitosan in green algae, offering a source for the first plant chitosan.
- We developed enzymatic/mass spectrometric fingerprinting for chitosan analysis in unprecedented molecular detail.
- We found that green-algal chitosan differs from all known conventional chitosans in its fine-structure.
- We showed that green-algal chitosans are at least as good as conventional chitosans in different bioactivities.
- We developed an enzymatic process to produce chitosan polymers which resemble natural chitosans more closely than conventional chitosans.
- We developed chitosan-based nano-formulations for improved drug delivery and bio-mineralised bone implants.

Moreover, we provided

- the first comprehensive life cycle assessments for chitosans, also showing that the biotechnological production can already compete with and in some aspects beat the conventional production in terms of sustainability;

- an exhaustive report on the legal aspects of production and registration of products based on biotechnologically produced chitosans - as a case study for other functional biopolymers that will be essential players in the conversion to a sustainable, circular bio-economy.

We are convinced that the Nano3Bio project, thus, has made more than just the first steps towards the development of "third generation" chitosans. These will be chitosans that are even better defined than the current second generation of chitosans, they will be narrower in their size distribution and they will have different, well-defined sequences of acetylated and non-acetylated monomeric units - unlike the current conventional chitosans in which due to their chemical means of production, the acetylated and non-acetylated monomers are randomly distributed. They will be of non-animal origin and, thus, will be suited for sensitive markets such as cosmetics and biomedicine. Some of the novel biotech chitosans can already be produced at pilot scale and, hence, are available for benchmarking and integration into existing processes and products. All-in-all, we can certainly state with pride and without exaggeration that the Nano3Bio project has been successful even beyond high initial expectations - both on the scientific and on the economic level.

1. SUMMARY DESCRIPTION OF THE PROJECT CONTEXT AND OBJECTIVES

The Challenge:

Chitosans are an amazing family of highly promising functional biopolymers with superb physico-chemical properties and a host of interesting biological activities. Chitosans are functional biopolymers combining superior material properties with excellent biocompatibility and highly versatile biological activities with promising advanced applications e.g. in biomedicine and agriculture, but also in cosmetics, food sciences and many other life-science related market fields. So-called **first generation chitosans** can be produced by a chemical process from natural chitin, one of the most abundant renewable resources on earth, derived from the exoskeletons of shrimp, crab, crawfish and other crustaceans' wastes by partial de-N-acetylation in a relatively simple way. These rather harsh chemical procedures require high energy input and employ e.g. concentrated acid and alkali solutions, leading to large volumes of salt effluents which in turn require sophisticated and expensive waste-water treatment, and they yield mixtures of chitosans which differ in composition from batch to batch unless strict measures of quality control are employed. These industrial grade products are sufficient for many applications where the material properties of chitosans are relevant rather than their biological activities (e.g. wastewater treatment, textile finishes). However, they are ill-suited for more demanding applications which are based on the biological functionalities of chitosans, such as in agriculture or medicine.

Currently, chitosans are **applied in various fields**. The global chitosan market is classified according to applications such as water treatment, biomedicine & pharmaceutical, industrial, food & beverages, cosmetics, agrochemical, and others (including fuel cells, photographic products, etc.). Chitosans are marketed under **various product grades** such as industrial, pharmaceutical, and food & beverage depending upon product purity and according to **different end-user demands**. **Shrimp** is globally used as the **primary source** for chitosan production (80% of the total chitosan market volume in 2015), followed by crab. Apart from crustacean shell waste, chitosan can also be produced from **certain fungi**. However, low amount of chitin in the cell walls of fungi and the absence of an optimal procedure for extraction of chitin on a commercial scale make it a less used source for obtaining chitosan. According to the Zion Market Research report "Chitosan Market for Water Treatment, Biomedicine & Pharmaceutics, Industrial, Food & Beverages, Cosmetics, Agrochemical and Other Applications - Global Industry Perspective, Comprehensive Analysis, and Forecast, 2015 – 2021", the global chitosan market was valued at over USD 1.84 billion in 2015 and is **expected to reach USD 4.74 billion in 2021, growing at a CAGR of slightly above 17.1% between 2016 and 2021**. Apart from conversion to glucosamine by acid hydrolysis, the use as a **flocculating agent** to treat wastewater is the largest application of chitosan covering one-third of the global market. The major reason for the growth in the global chitosan market is the rise in demand for products used for the treatment of contaminated water. Rapid industrialization in the BRIC nations accompanied with a growing urban population has propelled the clean water demand across the globe and will further complement the chitosan industry growth. Chitosan is a coagulant for organic and inorganic compounds present in water and is also used as chelating agent to bind toxic heavy metals that are present in industrial wastewater. Chitosan as a renewable resource is more effective and costs less than conventional flocculants in water treatment and will therefore lead the chitosan market in future as well, at least in terms of mass volume. In terms of mass, the **biomedical and pharmaceutical market** segments are much smaller. However, as mentioned above, very different qualities of chitosans are required for these different market sectors, with very different price tags also. Thus, in spite of low volumes, these market sectors are highly interesting. The demand for chitosans in this biomedicine and pharmaceutical sector is also expected to increase. The chitosan market for biomedicine and pharmaceutical applications is projected to grow at an annual rate of more than 17% over 2016-2024. Chitosans are used in manufacturing pharmaceuticals, as filler and carrier for controlled release drugs and as coating on pharmaceutical products (https://www.gminsights.com/industry-analysis/chitosan-market, 2017). Chitosan based hydrogels and **wound healing bandages** have found a market in the field of medicine in the form of **non-wovens, nanofibers, composites, films, and sponges**, **hemostatic** bandages in particular are widespread. Similar products are temporary surgical dressings, stuffable dressings and gauze dressings. Further, **wound dressings** are used for protective treatment of partial and full thickness dermal ulcers, leg ulcers, superficial wounds, abrasions, burns, as well as donor sites. Today, these applications focus in particular on the **physical properties** of chitosans. The **ongoing R&D activities** are directed towards the various **biological activities and novel applications** of chitosans by developing new, safer, more environment-friendly production technologies. This will lead to a considerable, positive influence on the market growth in this field. The results of the Nano3Bio project will contribute to this positive development.

Increasing applications of chitosans as **agricultural biologics**, by helping beneficial microorganisms in soil to thrive and inhibiting the growth of harmful microorganisms, as environmental-friendly **bio-fungicides** and bio-pesticides, and as **preservative coatings** are expected to have a positive impact on the market growth as well. The agrochemical segment is likely to report the **fastest growth** among all the applicationsegment. At present, it holds around 5% of the demand generated for chitosan globally (https://www.transparencymarketresearch.com/pressrelease/chitosan-market.htm, 2015). The results of the Nano3Bio project will have a positive impact on the future development of this market segment, too.

The demand for chitosans is also considerable in the **food and beverage industry** as it is used as a food stabilizer, preservative, and a crucial coagulation agent. In addition to this, chitosans are also used as a dietary supplement due to the alleged ability to reduce body weight through the fat binding mechanism. Moreover, chitosan has been approved for its application as a dietary supplement in several countries (https://www.gminsights.com/industry-analysis/chitosan-market, 2016.) Chitosans are also commercially used in the production of **cosmetics**,

e.g. as a substitute for hyaluronic acid in anti-aging cosmetics. Furthermore, apart from skin care products, they are also used to manufacture hair care products. They possess the ability to form an elastic film on hair, thereby imparting softness and physical strength to hair fiber. In addition, chitosans are used in **dental care** products such as toothpaste and mouthwashes. The increased usage of chitosans in the manufacturing of cosmetics and rising demands of different cosmetic products in developed and developing nations are major driving factors for the global chitosan market. **However,** there is also a sizeable rise in the number of consumers opting for **non-animal based, bio-derived** products. This will have a negative impact on the market growth, because the primary source for today's chitosan production is crustaceans shell wastes. This affects not only applications in the cosmetics industry, but also in the biomedical, pharmaceutical and in the food and beverage industry. Nano3Bio will contribute to the solution of this problem.

Geographically, the global chitosan market was dominated by the Asia Pacific region which accounted for over 55% shares of the global market in 2015. Asia Pacific is followed by North America and Europe. Japan and China are the major producers of chitosans in the Asia Pacific region. However, the **demand for chitosan** is the **highest in North America and developed economies of Europe and Japan** due **to large demands for high-quality grades of chitosan for cosmetics, biomedical and pharmaceutical applications**. However, environmental concerns associated with the commercial manufacturing of chitosan and high production costs are expected to curtail the growth of this industry. The chitosan market price trend, due to high cost of manufacturing from raw materials, is a key restraint. The chemical manufacturing technique has been continuously improved, but remains conceptionally unchanged from the time it was first introduced. Moreover, the cost of treating environmentally-hazardous waste produced during the manufacturing process is substantial. In addition, the industry faces **competition from animal feed manufacturers** who source the same raw materials (http://www.reportsnreports.com/reports/404544-global-chitosan-market-2015-2019.html, 2015).

The **market** for chitosan is very **competitive** due to the presence of several small and large scale players in the industry. The industry is highly integrated and most of the manufacturers produce chitosans as well as the end products. However, certain food supplement makers, cosmetics and biomedical product makers, pharmaceutical companies, cosmetic manufacturing companies, and natural diet supplement manufacturing companies procure chitosans from manufacturers and use them as an ingredient to manufacture the end products.

The chitosan markets have been impacted in recent years by a lack of quality, purity, reproduceability, traceability - which is essential primarily in the pharmaceutical and medical industry - and by high production costs, production shortages as well as heavy pollution during the production process. All of these issues can be addressed - are are being addressed successfully by a few responsible large and small scale chitosan producers who use highly sophisticated techniques optimised in terms of product quality and environmental impact and who produce under high quality management standards, but most production processes globally are still rather crude and often massively polluting, thus tarnishing the reputation of the whole industry.

Moreover, initial over-optimistic promises reported in the scientific literature could not be kept; especially with regard to results on bioactivities. They did not lead to the promised development of highly sophisticated applications because the results were not reliably reproducible. Chitosans differ in their structures and functions. Some special chitosans can protect plants from specific diseases, some can be used for targeted drug delivery, some can protect food from spoilage, and some can support wound healing. Three main factors, the degree of polymerisation (DP), the degree of acetylation (DA), and the pattern of acetylation (PA) of chitosan oligomers and polymers have been shown (DP, DA) or suggested (PA) to determine their physico-chemical properties as well as their biological activities. Two decades of fundamental research on structure-function relationships have led to the development of a limited collection of high quality chitosans which are well-defined in terms of DP and DA and which have reliably random PA, with known physico-chemical properties and reproducible biological functionalities. Today, these **second-generation chitosans** are ready for the markets, becoming available in sufficient quantities for the development of successful products which begin to appear on the markets. Just recently, the European Commission registered chitosan hydrochloride as a 'basic substance' which can now be used e.g. in agricultural products without the need for lengthy and costly toxicity studies and registration processes. However, the potential for life science applications of chitosans is **far from being fully exploited**. Even the highest quality, best-characterised chitosans – whether generated chemically or chemo-enzymatically – are mixtures of polymers differing greatly in their DP and DA, and in the case of enzymatic deacetylation or depolymerisation for the production of chitosan oligomers, also PA. This inherent heterogeneity, which is a hallmark of biopolymers, is a very significant hurdle to the development of marketable chitosan-based products for highly sophisticated applications, not least due to regulatory rules which are defined based on the properties of synthetic chemicals. The lack of reliability and reproducibility of chitosans is due to a **fundamental lack in understanding molecular structure/function relationships and cellular modes of actions** of partially acetylated chitosans. Only if it were known which chitosan exactly is exhibiting which specific biological activity, we would be able to successfully exploit the full, fascinating potential of this family of biopolymers. Progress towards such a detailed understanding is currently ongoing, and the Nano3Bio project has again significantly contributed to it. Another problem encountered with some life science applications of chitosans is the **animal origin** of almost all commercially available chitosans today, as in the eyes of some customers of e.g. bio-cosmetics, this animal origin appears to be associated with assumed problems of allergenic or viral contaminations. This is an issue that is very difficult to address scientifically as it based on false assumptions - the rigorous chemical processes involved in chitin extraction and conversion to chitosan safely remove all possible contaminations of allergenic proteins or viruses, as has been experimentally proven many times. Still, the fear is there, both with customers and with parts of industry also, in the latter case sometimes based on poor experiences made in the past with ill-defined first generation chitosans or chitosans procured even today from producers lacking rigorous quality control measures in their production processes. For sensitive markets such as bio-cosmetics which are governed by emotions rather than by scientific evidence, biotechnologically produced Nano3Bio chitosans may also offer a solution.

However, Nano3Bio goes beyond emotions: biotechnologically produced chitosans which are more closely resembling natural chitosans and which differ significantly from conventional, chemically produced chitosans, with their novel structural properties and biological functionalities, will open up new markets for chitosans, complementing and not competing with high quality conventional chitosans.

Addressing the challenge

The EU funded Nano3Bio project, therefore, aimed to design **biotechnological ways** by using nature's tools to produce **well-defined natural third generation chitosans of animal and non-animal origin,** which differ from their conventional, chemically produced counterparts by having **better defined and different structures**, and **more reliable functions**. They promise to allow the development of novel, sustainable, environment-friendly and customer-safe solutions e.g. in plant disease protection, food preservation, targeted drug delivery for instance for tumor therapy, and improved wound healing even of large, third degree burns and chronic wounds.
A strong consortium of 22 appropriately experienced and highly motivated partners from academia and industry teamed up for the Nano3Bio 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 glyco-biology and chemistry, perfectly complementing each other and allowing the direct transfer of results from lab to pilot plant scale. Our consortium consisted of the **whole product chain** from the **raw materials producers** to the developers, suppliers, and users of the **chitosans**, and further to the analysis and evaluation of the **developed products**. Furthermore, the project included a thorough investigation of the legal requirements for their registration, in order to identify constraints and bottlenecks impeding market entry of these biopolymer-based biomaterials and bio-actives. In parallel, comprehensive life cycle assessments of the novel biotechnologically produced chitosans were performed in comparison to conventional chitosans available on the market today, to ensure compliance with the overall objectives of the transition to a bio-economy, namely reduced energy input, reduced environmental burden, and concomitantly improved product quality and increased consumer safety.
The Nano3Bio project pursued **two different, parallel approaches towards the biotechnological production of novel, natural, custom-made chitosans**. Both approaches made use of nature's own tools for the biosynthesis of chitosans. The first approach targeted an **enzymatic conversion of chitin into chitosans**, using chitin deacetylases. The raw material used in this process was **natural chitin** isolated from shrimp and crab shell wastes of the fishery industries - the same that is used today for the chemical conversion into chitosan. Chitin deacetylases can be found in a range of fungi, which are rare natural producers of chitosan, as well as in some bacteria - but we now also discovered them in some viruses and microalgae, which we found to also be natural producers of chitosans. Also, we used chitinases and chitosanases to convert chitosan polymers into chitosan oligomers, adding structural and functional specificity in the process. Interestingly, we found that some of these enzymes can also be used in reverse when applying appropriate conditions in vitro, allowing synthesis rather than degradation. However, we had to realise that these enzymes often do not perform well in biotechnological processes so that we had to **optimise them through bioinformatics-based protein engineering**. Genes for chitin synthases, chitin deacetylases, and transglycosylating chitinases were mined from different sources and heterologously expressed, and the recombinant enzymes were characterized and optimized by protein engineering through rational design and molecular evolution, e.g. targeting engineered glycosynthases. At the end of the Nano3Bio project, we have achieved the first production of biotechnologically produced, natural chitosans the **structure of which differs significantly from all known conventional chitosans**, and the biological functionalities of which are currently investigated.
The **second approach** for the biotechnological production of chitosans went one step further by even **producing the chitin in an enzymatic process**. Nature's enzymes for this step - chitin synthases - are widespread e.g. in fungi and insects, but we again also found them in some viruses, bacteria, and microalgae. These are mostly large, complex enzymes naturally embedded in cell membranes and once isolated, they exhibit extremely poor performance. Therefore, we used these enzymes in their natural environment, i.e. in living cells. Combining different, natural or optimised chitin synthases and chitin deacetylases in bacteria, we **achieved the production of a range of fully defined, small chitosan oligomers**. Sophisticated metabolic engineering of the production strains led to high purities and yields of the target chitosans. Upscaling of fermentation and down-stream processing led to the production of **sufficient amounts** of these **novel chitosans**, which are now being studied for their biological activities.
The bioinspired chitosans obtained from both of these approaches were analysed and formulated into bio-mineralised hydrogels, nanoparticles, nanoscaffolds, etc., to impart novel properties, including by surface nano-imprinting, electro-spinning and -spraying as well as 3D-plotting, and were bench-marked against their conventional counterparts in a variety of cell based assays and routine industrial tests for e.g. cosmetics and pharma markets.

The eight key objectives

The Nano3Bio project was subdivided into eight closely cooperating work packages, which are reflecting its key objectives**:**

Work package 1 was devoted to identifying and characterizing suitable genes and enzymes for the biotechnological synthesis and modification of chitin and chitosan oligo- and polymers from a broad range of sources to harvest the potential of natural biodiversity.

Work package 2 was devoted to using the enzymes identified and characterized in WP1 for the in vitro production of defined chitosan oligomers and polymers in a biorefinery approach.

Work package 3 was devoted to using the genes identified and characterized in WP1 for the in vivo production of defined chitosan oligomers and polymers in a cell factory approach.

Work package 4 was devoted to the structural and biological analysis of biotechnologically produced chitosans delivered by WP2 and WP3, in comparison to the best conventional chitosans available on the markets today, and to a wide range of speciality chitosans produced at lab scale.

Work package 5 was devoted to the evaluation of the application potential of the novel, biotechnologically produced chitosans delivered by WP2 and WP3, as compared to that of the best conventional chitosans available on the markets today, and to a wide range of speciality chitosans produced at lab scale in WP4. As the biotech chitosans were not yet available during the first half of the project, we initially developed new nanoformulations of well-defined conventional chitosans, to set the benchmarks for the biotech chitosans.

Work package 6 was devoted firstly to the first ever comprehensive Life Cycle Assessment of chitosan production,including all pertinent data for different conventional and biotech chitin and chitosan polymer and oligomer production pathways. Secondly, this WP provided a comprehensive overview of legal demands and constraints for the biotechnological production and use of functional biopolymers such as chitosans in a range of pertinent markets.

Work package 7 was devoted to the administrative, legal, and financial management of the large project, which comprised 24 partners from 21 European and 1 Indian institutions.

Work package 8 was devoted to dissemination and exploitation of the goals, strategies, and results of the Nano3Bio project.

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

2.1 WORK PACKAGE 1 - GENES AND ENZYMES

Objectives

The main objective of WP1 was to identify, clone, heterologously express, characterise, and optimise genes and enzymes involved in the biosynthesis and modification of chitin and chitosan, to be exploited in WP2 and 3 for the production of chitin/chitosan molecules with finely tuned structural properties. The target genes were (1) novel soluble chitin synthases (CS) yielding chitin oligomers of different, defined degrees of polymerization (DP); (2) novel transmembrane CS with different rates of processivity yielding chitin polymers of different DP; (3) novel chitin deacetylases (CDA) with unique deacetylation specificity yielding chitosan oligomers with different, defined degrees of acetylation (DA) and patterns of acetylation (PA); (4) novel CDA with different rates of processivity yielding chitosan polymers with different blockiness; (5) novel chitosan hydrolases cleaving chitosans at narrowly defined, specific sequences; and (6) novel chitosan hydrolases with strong transglycosylating activities. WP1 relied on a multidisciplinary approach in which fundamental research was combined with the development of new technologies to serve the translational activities of the project and the development of concrete chitin/chitosan-based products with potential and, where possible, short-term commercial applications in a whole range of sectors.

Outcomes

This WP successfully delivered its planned outcomes with, in the first instance, the discovery of new chitin/chitosan active enzymes through the mining of public and in-house gene databases at an unprecedented scale. The work included the use of state-of-the-art gene mining approaches to screen fungal, oomycete, diatom, microalgal, viral and bacterial genomes. Following the implementation of these activities during the first year, multiple newly uncovered enzymes were expressed in heterologous systems to demonstrate their biochemical activity, and some were engineered to improve their potential use for the production of chitin/chitosan/chito-oligosaccharides with well-defined structural and biological properties. This part of the work required the tuning of existing expression systems to facilitate and enhance the expression of challenging proteins such as the membrane-bound CS and other challenging proteins that do not express easily in traditional prokaryotic systems. In parallel to the characterization and engineering of new enzymes, WP1 involved the further optimization of multiple enzymes that were available at the beginning of the project from previous research led by the different partners.

An important task in this WP has been the identification of suitable genes that encode CS and CDA with an emphasis on genes that are likely to be involved in chitosan biosynthesis and modification (Task 1a). Two main approaches were successfully used for gene identification: i) analysis of the expression profiles of CS and CDA genes in bacteria, fungi, oomycetes, diatoms and microalgae that were expected to produce and/or modify chitin and/or chitosan and identification of co-expressed genes; ii) bioinformatics analysis of CS and CDA genes from the abovementioned organisms. Complementary analytical work has also been performed in WP1, in collaboration with WP2 and WP4, to confirm the occurrence of chitin/chitosan in selected diatoms and algae. This involved the use of a combination of analytical approaches available across the partnership, including, but not limited to: monosaccharide and linkage analysis by GC/MS; determination of molecular weight and degree and patterns of acetylation using a combination of techniques such as viscometry, light scattering and enzymatic fingerprinting including LC-MS/MS approaches; NMR spectroscopy; cell biology and optical microscopy underpinned by the availability of specific fluorescent probes such as chitin or chitosan binding proteins. A high-throughput screening technology was also developed to detect chitin/chitosan in microalgae from the proprietary collection of one of the industry partners involved. Methods adapted to the extraction of these polymers and chitin/chitosan oligosaccharides were also developed. This was critical for the characterization of so far poorly characterized organisms such as the diatoms and microalgae.

From the gene mining approach, suitable CS and CDA genes were cloned in different expression systems, in particular *Escherichia coli*, *Saccharomyces cerevisiae*, *Hansenula polymorpha,* and diatoms (Tasks 1b and 1c). In several instances, it was necessary to generate new expression systems to facilitate screening of biochemical activities and enzyme production prior to characterization. For example, *S. cerevisiae* mutant strains that show virtually no CS activity in vitro were engineered to decrease background activity and facilitate the expression of new heterologous CS. The strain SK53 was mutated in two of its CS genes (chs1Δ::KanMX4 and chs3Δ::HIS3) and in a protease (pep4Δ::LYS2) to limit protein degradation during heterologous expression. Several CS from different organisms, e.g., oomycetes, the Zygomycete fungus *Mucor*, the *Chlorella* virus, bacteria, and diatoms were successfully expressed in this strain and biochemically characterized. This tool was also crucial to engineer CS activities for the production of compounds with specific properties. A whole range of CS mutants were successfully produced in an active recombinant form that produced chito-oligosaccharides of different molecular masses as well as insoluble chitin crystallites with crystallinity indices as high as 75%. Completely new systems were also devised for the expression of CS and CDA with the objective of converting these organisms into engineered systems for the large-scale production of chitin/chitosans *in vivo* (WP3). Some diatoms were successfully shown to be natural producers of chitin and potential factories for the production of chitin/chitosan products *in vivo*. Multiple microalgal species were also shown to be natural producers of chitin/chitosan polymers and oligomers. Screening of tens of species from an in-house library from one industry partner, combined with the characterization of the products formed naturally by some of these species, revealed the potential to produce specific compounds in

quantitative amounts by scaling up cultures of microalgae.

Available and new recombinant CDAs were analyzed for their enzymatic properties (Task 1d). A CDA from *Cryptococcus neoformans* is one of several workhorses that arose from the project. It was successfully expressed in optimised *Hansenula* strains where it was also co-expressed with a CS protein from *C. neoformans* to produce deacetylated forms of chitin. To identify non-homologous CDA genes in bacteria, Zygomycetes, Basidomycetes, viruses, and algae, a detailed bioinformatic analysis was carried out. As a result, based on the similarities and differences among the CDA, phylogenetic relationships were established and six classes in two divisions were defined at the taxonomic level. Further, in each class, the presence and organization of carbohydrate-binding domains as well as the presence of a GPI anchor suggesting membrane association were assessed. Several crystals were obtained from the truncated form of a bacterial CDA and the first 3D structure of the enzyme was solved and shown to form a dimer. Analysis of the loops structure demonstrated that the protein contains short loops providing an open binding cleft where the substrate may bind in different modes. This is only one of several examples where mechanistic details were obtained from recombinant and/or engineered enzymes. Deacetylase activity of expressed CDA proteins on chitooligosaccharides was monitored by HPLC-MS. For example, one of the characterized enzymes had no activity towards DP2 but it was active on DP3, DP4 and DP5, the activity increasing with the DP of the substrate. This enzyme is characterized by a processive behaviour or multiple chain attack mechanism. Enzyme specificity was analyzed by a new labelling technique and MS-analytic methodologies developed within the project.

Another aim that was successfully addressed was the identification and engineering of transglycosylating chitinases (Task 1e). Genome searches of multiple bacterial species, e.g., *Flavobacterium johnsoniae*, *Paenibacillus elgii* SMA-1-SDCH02, *Stenotrophomonas maltophilia*, *Bacillus amylo-liquefaciens* and *Nocardia dassonvillei* revealed the presence of genes for chitin and chitosan degrading/modifying enzymes. The characterization of chitinolytic enzymes from these bacteria revealed the presence of chitinases (*Fj*ChiC, *Pe*Chi1 and *Stm*ChiA) with very low or no transglycosylation (TG) activity. A family 46 glycoside hydrolase (GH46) enzyme showed activity on both chitin and chitosan oligomers, and also showed TG activity on chitin tetramers. The characterization of chitinases mainly included determination of optimum conditions for activity with polymeric and oligomeric substrates. Template based homology modeling was performed for several chitosanases using the crystal structure of *Streptomyces* N174 chitosanase. The residues essential for catalytic activity were identified after developing the model and protein engineering approaches were implemented.

Based on the data obtained in this WP, the Partners involved in WP1 jointly selected through a continuous process the most relevant gene candidates to be further assessed and evaluated for *in vitro* chitosan production and/or modification in WP2 and for *in vivo* production in WP3.

In addition to gene discovery and enzyme expression and engineering, WP1 has included the development of novel techniques for the generation and characterization of combinatorial libraries for directed evolution experiments. Specifically, a new type of combinatorial library based on Gene-Art™ Strings™ DNA Fragments was developed and commercialized by one of our industry partners, Thermo Fisher Scientific GENEART GmbH. This development enables to deliver libraries much faster and at significantly reduced cost. In addition, a next generation sequencing technology was developed. It allows the characterization of tens to hundreds of thousands of specimens from a given library as opposed to a peer group of dozens to hundreds, which has been the latest standard until this new development.

Outlook

WP1 has generated a wealth of fundamental information that has been exploited in WP2 and WP3 for the production of chitin and chitosan products *in vitro* and *in vivo*, respectively. Several organisms that were not expected to be producers of chitin and/or chitosan polymers and oligomers have been identified and their potential for exploitation has been evaluated. Some of the newly identified producers have even been used as expression systems for enzyme characterization. These include some diatom species. It remains to be determined whether these organisms can be commercially exploited as such and/or what approaches could be implemented to increase their product yield. Very promising classes of organisms for this purpose are several microalgal species. In this case, transformation approaches for genetic engineering have yet to be tested, but the project has already demonstrated that non-modified species are excellent candidates for commercial production of chitin/chitosan molecules. The gene mining approaches have also revealed new chitin/chitosan active enzymes, many of which were successfully characterized and even engineered to the extent where specific products with defined structural properties have been obtained. However, the enzyme discovery activities were part of a continuous approach during several years and, to ensure delivery of the expected and planned outcomes, focus was given in the last part of the project to the full characterization and engineering of the most promising enzyme targets. This means that there is still a wealth of proteins that have been identified and preliminarily characterized in the project that would deserve to be further studied and evaluated for the production and engineering of tailored chitin/chitosan products.

2.2 WORK PACKAGE 2 - *IN VITRO* BIOSYNTHESIS

Objectives of the work package

This WP aimed at developing biocatalytical approaches for the *in vitro* biosynthesis of chitosans with a range of defined degrees of polymerization (DP), degrees of acetylation (DA), and patterns of acetylation (PA). This was to be achieved by using the enzymes delivered by WP1 (some already available at the beginning and others obtained during the course of the project) and engineered variants suitably adapted to biocatalysis in

this WP. Suitable chitosan oligomers and derivatives needed to be prepared for enzymatic polymerization reactions, and the reaction conditions for preparative scale production of the target products were to be optimised. Different complementary strategies had to be developed to access a range of defined chitosans by using:

a) specific wild-type chitin deacetylases (CDA) acting on chitin/chitosan oligo/polymers alone or in combination;
b) engineered chitin deacetylases for improved efficiency and novel deacetylation patterns;
c) transglycosylating (TG) chitinases engineered to improve transglycosylation over hydrolytic activity and using defined chitosan oligomers as donor substrates; d) specific chitinases engineered as glycosynthases (GS) in combination with chemically activated donor substrates derived from defined chitosan oligomers.

The work package in the context of the project

WP2 was initially focused on CDA acting on chitosan oligomers, with IQS (P3) studying chimeric CDA to understand subsite specificities, and WWU-1 (P1) using combinations of CDA to produce as many chito-tetraoses as possible for bioactivity studies in WP4. These approaches relied on the supply of rather large amounts of fully acetylated chitin oligomers produced *in vivo* by BBEPP (P12) using *E. coli* clones supplied by WWU-1 (P1) and UGent-1 (P2). Protein engineering of CDAs (and related carbohydrate esterases of the same family active on chitooligosaccharides) were conducted by IQS (P3) both to understand the structure-function relationships defining the different deacetylation patterns exhibited by this family of enzymes and to engineer specificity for efficient preparation of chitosan oligosaccharides. Then, two strategies were developed to polymerize the chitin and chitosan oligosaccharides to access oligomeric/polymeric products: transglycosylating chitinases with unmodified oligomers as donor substrates by HYDER (P9), and glycosynthases derived from chitinases by IQS (P3), the latter using chemically activated oxazoline oligomers as donor substrates, which were prepared by ENANTIA (P21). Synthetic genes and gene libraries were synthesized and provided by GA/LT (P9), some using their newly developed “DNA Strings” technology.

Results achieved during the project

Task 2a: Chitosan oligosaccharides by enzymatic deacetylation.

A) Specific wild-type chitin deacetylases (CDA) acting on chitin/chitosan oligo/polymers alone or in combination. Some CDAs have the salient ability to deacetylate chitin oligomers at specific positions, thereby generating chitosan oligomers with a defined pattern of acetylation (PA). Bacterial chitin deacetylases such as NodB or *Vc*CDA are specific for deacetylating a single residue within a chitin oligomer, thus producing mono-deacetylated chitosan oligomers. NodB deacetylates exclusively the GlcNAc unit at the non-reducing end (yielding DA[A]$_{0-4}$), whereas *Vc*CDA deacetylates the second unit from the non-reducing end (yielding AD[A]$_{0-4}$). WWU-1 (P1) identified and characterized several fungal CDAs yielding paCOS (**p**artially **a**cetylated **C**hitosan **O**ligo**S**accharides) with different patterns of acetylation in WP1. Both bacterial and fungal CDAs have been here exploited by WWU-1 (P1) to successfully produce a library of chitosan tetramers in mg scale. Unfortunately, chitosan oligomers having only the reducing end deacetylated cannot be produced by any of these enzymes. Enzymatic *N*-acetylation of chitosan oligomers was therefore developed since CDAs are able to catalyze the reverse reaction in the presence of excess acetate. In this way, WWU-1 (P1) was able to produce a broad range of chitosan oligomers with specific PA by enzymatic deacetylation of chitin oligomers or enzymatic N-acetylation of chitosan oligomers using different combinations of enzymes. In fact, using these approaches, we can now produce e.g. all fourteen possible partially acetylated chitosan tetramers in a pure form regarding their DP, DA, and PA. This enables for the first time to investigate the influence of the PA on the bioactivity of the paCOS without the problem of contamination with other paCOS, as detailed in WP4.

Besides chitin deacetylases, some peptidoglycan deacetylases (PG) are also active on COS. IQS (P3) showed that a *Bacillus* PG is specific for deacetylating chitin tetramer and pentamer in all positions except the reducing end. When catalyzing the reverse reaction, *N*-acetylation of fully deacetylated oligomers, a chitosan oligomer with only the reducing end left deacetylated was produced, thus having a new enzyme able to generate those difficult-to-produce mono-deacetylated paCOS at the reducing end with a single enzyme.

The strategy of enzymatic deacetylation has also been applied to polymers to generate chitosan polymers with a non-random PA. In this way, WWU-1 (P1) was able to produce different chitosan polymers with DA 30% using different recombinant chitin deacetylases. Structural analysis (^{1}H-NMR and the enzymatic / mass spectrometric fingerprinting method described in WP4) revealed a more block-wise or a more regular pattern of acetylation in these polymers, depending on the chitin deacetylase used, unlike the random pattern observed in all conventional, chemically produced chitosans.

B) Engineering specificity of chitin deacetylases

Based on the crystal structure of *Vc*CDA solved by P3 and the proposed “subsite capping model” (see WP1), protein engineering experiments have been designed to evaluate structure/function relationships and introduce novel specificities aimed at the production of chitosan oligosaccharides with defined patterns of acetylation.

B.1.-Chimeric CDAs.-The initial strategy designed by IQS (P3) to engineer CDA enzymes was the construction of chimeric proteins. Shuffling the N-t and C-t moieties of four selected CDAs with different specificity gave twelve chimeric constructs that were recombinantly expressed. The

most soluble and active proteins were purified and characterized. Remarkably, one chimera based on the scaffold of a fungal processive CDA and the N-t part from a bacterial single-site specific CDA acquired the bacterial specificity for single site deacetylation. These results are in agreement with the "subsite capping model" which proposes that the deacetylation pattern exhibited by different CDAs is governed by critical loops that shape and differentially block accessible subsites in the binding cleft of CE4 enzymes.

B.2.- VcCDA.- Using the *V. cholerae* CDA as a model, P3 designed experiments to analyse and engineer separately the "reducing end" (or positive) and the "non-reducing end" (or negative) subsites of the enzyme's binding cleft.

*"Reducing end subsites".- Vc*CDA prefers N.N'-diacetyl chitobiose (DP2) as substrate and has a strongly reduced activity on longer substrates. Modelling studies based on the "subsite capping model" suggested that Loop 5 has a major contribution to prevent binding of substrates longer than DP3 by partially blocking the positive subsites of the binding cleft. P3 conducted extensive mutagenesis work to prove the subsite capping model and to engineer substrate specificity. First generation mutants included the engineering of a disulfide bond to block Loop 5 dynamics, and reduction of the loop size by replacing Loop 5 by a shorter loop to expose new positive subsites. The "disulfide" mutant retained the same wt activity on DP2 substrate, but remarkably had strongly reduced activity with DP4 and DP5, concluding that Loop 5 has been locked in a close conformation that prevents binding of long substrates as predicted by the proposed subsite capping model. The "shorter loop" mutant resulted in a rather unstable protein prone to precipitation and with low deacetylase activity on any substrate. However, the activity ratio on DP4 *vs.* DP2 substrates has been reverted relative to the wt enzyme, again in accordance to the model. Second generation mutants involved the fine tuning of Loop 5 to obtain new variants with high activity on long substrates: increase Loop 5 flexibility by mutation of prolines to alanines, reduce the loop size up to the native S-S bridge at the beginning of the loop, remove either or the two α-helices in Loop 5. All constructs were highly active and showed a remarkably increased activity with long substrates while having reduced activity with DP2. Activity with DP5 (in terms of k_{cat}) was about 7 to 10 fold higher than the native wt activity with its preferred substrate DP2. The PG mutant (proline residues replaced by glycine) is the most relevant for applications due to its high expression yield, catalytic properties, and stability, and it retains the deacetylation pattern [ADA_{n-2}] with long substrates.

"Non-reducing end subsites".- The negative subsites of CDAs are shaped by Loops 1, 6, and 2. In *Vc*CDA, Loop 6 and Loop 1 establish strong interactions which maintain the loops in a position that caps the non-reducing end of the substrate binding cavity and define the specific deacetylation pattern for the penultimate GlcNAc residue from the non-reducing end of the substrate. P3 showed that simple approaches to delete these interactions were highly detrimental, such as partial deletion of Loop 1, point mutations to disrupt H-bond interactions or a double mutant to eliminate key interactions between Loop 6 and Loop 1. Therefore, a different strategy was considered: directed evolution by an Iterative Saturation Mutagenesis (ISM) method carefully selecting four sites for the combinatorial approach.

To screen large mutant libraries, a suitable high throughput screening (HTS) method was first required. To this end, IQS (P3) developed and validated an HTS assay based on capture of the mutant proteins from an expression library (random or targeted directed evolution libraries) with functionalized magnetic beads and detection of product formation from COS substrate. Validation using wt as positive and an inactive mutant as negative controls gave good sensitivity (Z-factor as quality parameter) to discriminate active and inactive mutants. The HTS method was applied to the screening of a random library of the *Vc*CDA catalytic domain looking for active mutants at 50ºC, finding one hit that came to be a new variant with 10-fold higher activity than the wt enzyme with long oligomers. Next, synthetic combinatorial libraries on the non-reducing end subsites were prepared using the "DNA String" technology by GA/LT (P9) and are currently screened for novel and improved deacetylase mutants.

B.4.- Fungal CDAs.- Fungal CDAs that show a multiple chain mechanism deacetylate more than one position, yielding paCOS with defined patterns. WWU-1 (P1) evaluated the preference for acetylated or deacetylated units at defined subsites in the active site. Structural analysis on the modelled structures of three selected fungal CDAs revealed that they differ in the amino acids which interact with the unit bound at subsite -1 in the enzymes' active site. Exchange of these different residues between enzymes resulted in different behaviors, with partial reversal of specificities, which will be further studied. WWU-1 also used three different recombinant fungal CDAs to enzymatically convert a conventional high DA chitosan (DA 60%) into three low DA chitosans (DA 30%). ^{13}C-NMR diad analyses as well as the newly developed enzymatic / mass spectrometric fingerprinting analysis in WP4 revealed that one of these low DA chitosans resembled a conventional chitosan of DA 30% with random PA, while another one had a more block-wise PA and the third one a more regular PA. These are, thus, the first chitosan polymers with proven non-random PA which will allow to investigate the influence of PA on biological activities (see WP4).

B.5.- PG deacetylase.- A bacterial peptidoglycan deacetylase showed a multiple chain mechanism deacetylating all positions except the reducing end of COS substrates (above). IQS (P3) solved the 3D structure of the catalytic domain, and docking studies provided further insights into the enzyme's specificity. Initial deacetylation occurs at any of the central residues of the COS substrate. A key arginine residue was proposed to stabilize one of the binding modes relative to others for initial binding leading to the first deacetylation event. Mutational studies conducted by P3 demonstrated that replacement of that Arg by smaller and more hydrophobic residues shifts the binding preference to one preferred binding mode. This strategy will by further studied to engineer specificity.

Task 2b: Polymerization by transglycosylating chitinases

HYDER (P8) has characterized the chitinases *Sp*ChiD, *Stm*ChiA, *Fj*ChiC, and *Pe*Chi1 which revealed to have transglycosylation (TG) ability with chitin oligosaccharides (COS) as substrates. Hence, attempts were made to improve TG activity of these enzymes through a 3D model-guided site-directed mutagenesis with the aim of identifying "hyper-transglycosylating" mutants for synthesis of longer chain COS. Single mutants of the

selected chitinases showed improved transglycosylation/hydrolysis ratios relative to the parental wt enzymes. The analysis was extended to a number of double mutants on the best performing single mutants of *Sp*ChiD and *Pe*Chi1 enzymes. All of them were characterized for residual hydrolase activity and for transglycosylation activity using acetylated chitopentaose (DP5, A5) as substrate. Main transglycosylation products detected by HPLC were DP6 and DP7, with variable product yields and stabilities over time due to further hydrolysis by the still high hydrolase activity of the mutants. Out of them, a point tyrosine mutant of the *Serratia proteamaculans* ChiD chitinase gave best results producing COS with the maximum detectable chain length of DP10, and major TG products being DP6 and DP7. Different reaction conditions were investigated: effect of substrate concentration, pH and reaction buffer, effect of osmolytes, ionic liquids, and other additives. Addition of ionic liquids up to 15% in the aqueous buffer reduced the hydrolase activity but also had a significant effect reducing TG activity. Likewise, osmolytes and humectants did not improved TG yields. In a large scale reaction using the tyrosine mutant of SpChiD enzyme, P8 reported the production of an oligomeric mixture mainly containing DP6 and DP7 in 11% and 6%, respectively, with a total transglycosylation products concentration of ~17%.

P8 also evaluated the transglycosylation ability of *Sp*ChiD, wt and tyrosine mutant, with chitosan oligomers with different patterns of acetylation as substrates, namely tetramers produced by P1 (WWU), and a pentamer supplied by P12 (BBEPP). The enzymes only exhibited TG with 'DADA' chitosan tetramer, probably reflecting the specificity of this chitinase for acetylated and deacetylated residues in the different subsites of the binding site cleft. The results envision the potential of *Sp*ChiD to produce longer chain COS from chitosan oligosaccharides of different patterns.

Task 2c: Chemical activation of chitosan oligomers

For the glycosynthase-catalyzed polymerization strategy (below, task 2d), the chitin and chitosan oligomers have to be activated. ENANTIA (P21) was engaged in the preparation of chitooligosaccharides having an oxazoline ring at the reducing end to be used as glycosyl donors by P3 in Task 2d. P21 developed analytical HPLC/MS methods using a PGC (porous graphitic carbon) column instead of the usual BEH Amide column for analysis of both *O*-acetylated and *O*-deacetylated compounds. Different purification protocols of the DP5/DP4 mixtures supplied by P12 (BBEPP) to obtain pure DP5 were studied. The initial procedure based on peracetylation/purification/de-O-acetylation was discontinued. Alternatively, repeated dispersion/crystallization of the DP5/DP4 materials from organic solvent (acetone, IPA):H2O mixtures in the presence of tributylamine were also found to partially remove DP4. Purification at mid-scale (hundreds of mg) affording pure DP5 was developed by semipreparative HPLC using also a PGC, Hypercarb™ column (>99% HPLC/MS) in a ca. 60% yield. Oxazoline formation from O-unprotected hemiacetalic compounds (either fully or partially N-acetylated) using 2-chloro-1,3-dimethyl-1H-benzimidazol-3-ium chloride (CDMBI) was investigated and optimized. Reactions proceeded with good yields, but the difficulty was the final purification step to remove salts. Other conditions for oxazoline formation allowing the coupling with the next enzymatic polymerization reaction were also investigated. Replacement of the base usually employed (trimethylamine) by a combination of an inorganic soluble base (Na_3PO_4, the same base to be used for buffering the enzymatic reactions) and a polymer-supported base, which can be removed from the reaction medium by filtration, gave good results. The final oxazoline products were transferred to IQS (P3) for task 2d.

Task 2d: Polymerization by engineered glycosynthases

The second proposed strategy for in vitro biosynthesis of chitosan oligomers/polymers with defined acetylation pattern was to engineer GH18 chitinases into glycosynthases for the controlled polymerization of activated chitosan oligosaccharides. To this end, IQS (P3) selected nine GH18 chitinases, from which six could be expressed as soluble and active proteins. Since GH18 chitinases act by a substrate assisted catalytic mechanism, their engineering into glycosynthases was initially based on removal of the assisting residue involved in the first step of the hydrolase mechanism with the aim of abolishing or reducing hydrolytic activity and catalyzing the controlled polymerization of activated chitosan oligosaccharides. Alanine mutants at the assisting residues were prepared for the six selected chitinases, and initial work was performed on the *Serratia proteamaculans ChiD*, The mutant showed glycosynthase activity by polymerization of acetylated chitopentaose oxazoline (DP5-Ox). A precipitated polymer was obtained in low yield, which contained mainly DP10 oligomer. Although reaction conditions were improved, the polymer was partially re-dissolved due to the still high residual hydrolase activity of the mutant (about 1% of the wt enzyme). A systematic analysis of first generation (single point mutants at the assisting residue) from the other selected chitinases did not give better results, as all retained significant hydrolase activity. Then, further engineering was conducted on the *Sp*ChiD enzyme. A second generation of mutants was designed by adding a second mutation at residues in the neighborhood of the assisting catalytic residue. Hydrolase activity was further decreased, and the mutants showed enhanced glycosynthase activity. In particular, a double alanine mutant showed the best performance yielding polymers (mainly DP10) which increased in time and which were not hydrolyzed. However, the yield in polymer was low (30%). A third generation for the SpChiD chitinase was designed, in which a third residue was mutated on the best double mutant. From a small library of triple mutants, one performed the best as glycosynthase, yielding an insoluble polymer (mainly composed of DP10) in up to 60% yield, and retaining very low hydrolase activity (5 orders of magnitude lower than the wild type enzyme).

Task 2e: Analysis and optimization of polymerization reaction conditions

The proof-of-concept to produce chitin oligomers/polymers by the two strategies, transglycosylating chitinases (TG) with unmodified COS donors, and glycosynthases derived from chitinases (GS) with activated oxazoline COS, has been established.

a) The TG strategy renders mainly oligomers of low degree of polymerization and low product yields for preparative production. Work is in progress to engineer new enzyme variants to improve efficiency. (P8, HYDER)

b) The GS strategy renders longer oligomers, DP10 being the major compound. In triple mutants with glycosynthase activity, the residual hydrolase activity has been reduced 5 orders of magnitude, and long oligomers were obtained in 60% yield (analytical scale). Work is in progress to increase the glycosynthase activity and upscale the reactions. (P3, IQS)

In both strategies, the use of chitosan oligosaccharides (DADA for TG, ADA-Ox and ADAAA-Ox for GS) also showed oligomer/polymer formation, but so far with low yields only. Improved production of chitosan oligomers and polymers is under investigation with a new generation of TS and GS mutants.

Outlook

In this WP, the in vitro bio-refinery approach towards the production of novel chitosan oligomers and polymers was successfully pursued, using the enzymes provided by WP1. A range of recombinant and engineered chitin deacetylases were used to produce a large range of fully defined chitosan oligomers, i.a. a ful set of the fourteen possible partially acetylated tetramers. Some of these chitin deacetylases were also used to convert high DA conventional chitosans with random patterns of acetylation into biotechnologically modified low DA chitosans with non-random patterns of acetylation. Interestingly and unexpectedly, this yielded polymers with both more block-wise and more regular distribution of the GlcN and GlcNAc monomeric units than their random distribution in all chitosan polymers known today. These novel chitosans have significantly different solution properties and biological functionalities, e.g. in terms of digestability by human chitinases, as shown in WP4 and WP5, making them interesting candidates for biomedical applications. The use of optimized transglycosylsating chitinases gave access to longer chain chitin oligomers produced from short chain chitin oligomers obtained from the cell factory approach of WP3. These higher DP chitin oligomers are particularly interesting for research into plant strengthening activities of chitosans and they are, thus, potentially interesting for agricultural applications, as shown in WP4. Glyco-synthases engineered based on these hyper-transglycosylating chitinases gave access to even longer chitin oligomers when acting on chemically activated chitin oligomers obtained in good yields from the natural oligomers provided by the cell factories of WP3. For the time being, the amounts of large oligomers and small polymers available through glyco-synthases are too low to be tested for bioactivities, but first results already indicate that the approach can be used on chemically activated chitosan oligomers, too, which can be expected to yield chitosan polymers with fully defined structure, an exciting project for the future. Finally, one unexpected result which was not foreseen in the project proposal was the in vitro use of recombinant chitin synthases to yield chitin polymers. We did not dare to hope that these transmembrane enzymes would remain their activity and sufficient processivity to yield polymers, but this was indeed the case. The amounts were minute, but with a value of 150, the degree of polymerization reached was amazing. Whether or not this will ever be useful for the production of sufficient amounts of biotechnologically produced chitin polymer to be used in any bioassay may remain to be seen, but the proof-of-principle was achieved beyond the initial expectation.

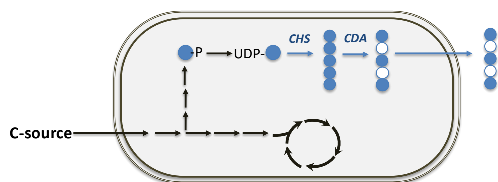
2.3 WORK PACKAGE 3 - *IN VIVO* BIOSYNTHESIS

The overall objective of Work Package 3 (WP3) “In Vivo Biosynthesis” was to produce a wide range of chitosans with specific degree of polymerization (DP), degree of acetylation (DA) and pattern of acetylation (PA). To this end, two approaches were followed:

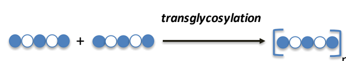
- **Approach 1:** Consisted in the in vivo production of chitin/chitosan oligomers by bacterial chitin synthases (CHS) and chitin deacetylases (CDA) in microbial cell factories. Then, by the use of transglycosylating chitinases or engineered chito-synthases, these oligomers were to be linked together to produce specific polymers in vitro in the framework of WP2 (Figure 3.3.1, left).
- **Approach 2:** Consisted in the production of specific polymers directly by transmembrane chitin synthases combined with extracellular chitin deacetylases (Figure 3.3.1, right).

Approach 1: *In vivo* production of chitosan oligomers which are subsequently converted to specific polymers by action of transglycosylating enzymes (WP2)

In vivo

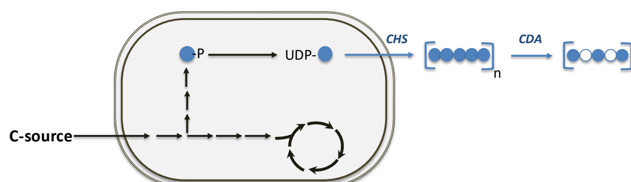


In vitro (WP2)



Approach 2: *In vivo* production of chitosan polymers by transmembrane CHS combined with extracellular CDA

In vivo



- **Screening** of natural chitin/chitosan production strains
- Development of **metabolic engineering tools** for selected strains
- **Metabolic engineering** of natural and non-natural chitin/chitosan production strains

Figure 3.3.1. Approaches followed in Work Package 3 (WP3) to produce a wide range of chitosans. Approach 1: In vivo production of chitosan oligomers which are subsequently converted to specific polymers by action of transglycosylating enzymes in vitro in WP2 (left). Approach 2: In vivo production of chitosan polymers by transmembrane chitin synthase (CHS) combined with extracellular chitin deacetylase (CDA) (right). *N*-acetyl glucosamine is represented by a filled blue circle, *N*-glucosamine is represented by an open circle.

To this end, different hosts, such as bacteria, fungi/yeast, and algae were to be used. Fermentations were to be performed both at lab-scale and pilot-scale to produce sufficient quantities of these specific chitosans.

Image 1: Pilot plant BBEPP

To obtain this objective, WP3 closely interacted with other WPs in the Nano3Bio project. WP3 depended on the delivery of novel genes (CHSs and CDAs) by WP1. The chitins and chitosans produced in this WP were analysed in WP4, used in WP2 as well-defined starting material (chitosan oligomers), and used to identify suitable (nano)biotechnological applications in WP5. Furthermore, the developed production processes were evaluated in WP6 by using life cycle assessment (LCA) methodology.

Approach 1: *In vivo* production of chitosan oligomers which are subsequently converted to specific polymers by action of transglycosylating chitinases or engineered chito-synthases in vitro in WP2

At the start of Nano3Bio, WWU-1 (P1) and UGent-1 (P2) had an *Escherichia coli* platform at their disposal to produce chitosan oligomers. During the Nano3Bio project, this platform was improved by:

- increasing product yield and productivity,
- improving product purity, and
- expanding the library of well-defined chitooligosaccharides produced in vivo.

More specifically, this available *E. coli* platform was extended with other CHSs and CDAs (selected from WP1) to increase our spectra of highly specific chitosan oligomers in terms of DP, DA, and PA. Further optimization was focused on increasing product titres, narrowing product specificities, and facilitating product purification.

These improvements were realized by applying newly developed metabolic engineering tools. UGent-1 (P2) has developed a method called SSA to easily and reliably fine tune gene expression. This method was further extended with the Golden Gate assembly method for directed combinatorial engineering of pathways, called SSA^P, to easily and reliably construct and fine tune pathways. Furthermore, genetic circuits were built to control and monitor pathways.

In addition to *E. coli,* alternative microorganisms were investigated for their applicability as chitosan oligomer production host. The microorganisms *Corynebacterium glutamicum* and *Bacillus* sp. seemed to be attractive candidates. Also for these hosts, the metabolic engineering and synthetic biology toolbox was extended. To this end, a versatile genome editing tool was developed based on the CRISPR/Cas9 system. The developed system allows scarless genomic editing (knock-outs, knock-ins, and point mutations) in an iterative or combined fashion. In addition, in order to vary gene expression, promoter libraries were evaluated for these alternative hosts.

Using these novel tailor-made microbial cell factories for the production of well-defined and pure chitosan oligomers, fermentations were performed and optimized both at lab-scale and pilot-scale. In addition, adequate methods for product recovery were developed and optimized. As such, sufficient quantities of these specific chitosan oligomers were produced for WP2 (deliverable D3.1), WP4 for analysis, and WP5 for (nano)biotechnological applications.

Approach 2: In vivo production of chitosan polymers by transmembrane chitin synthase (CHS) combined with extracellular chitin deacetylase (CDA)

In this approach, the in vivo production of chitosan polymers was assessed by:

- evaluation of chitin and chitosan polymer production hosts,
- development of metabolic engineering tools for selected hosts,
- metabolic engineering and screening of selected chitin and chitosan producers, and
- upscaling and downstream processing.

From a literature research and preliminary data, the following hosts were selected to evaluate for chitin and chitosan polymer production:

- Bacteria: *E. coli*, *C. glutami*cum and *Bacillus* sp.
- Yeast: *Hansenula polymorpha* and *Saccharomyces cerevisia*e
- Diatoms: *Thalassiosira pseudonana* and *Phaeodactylum tricornutum*
- Microalgae: *Chlamydomonas reinhardtii*, *Chlorella variabilis*

For all these hosts, novel metabolic engineering tools were designed and optimized:

- Bacteria: see under approach 1
- *H. polymorpha*: Artes (P16) evaluated new selection markers in order to avoid resistance genes. Although the *Saccharomyces CUP1* gene has been used before, e.g. for genetic manipulation of brewer's yeast, it turned out that selection in *Hansenula* did not work, among other reasons due to a high and varying basic copper resistance of the host strains. However, the *Saccharomyces cerevisiae SUC2* gene seems promising as selection marker in *Hansenula*. The selection is based upon the fact that expression of *ScSUC2* in *Hansenula* renders *Hansenula* raffinose$^+$. Concluding experiments are carried out to test whether multi-copy integrants lead to a stable raffinose$^+$ phenotype.
- Microalgae: Greenaltech (P13) initially continued to focus on developing metabolic engineering tools for C*. reinhardtii*, the model microalga in terms of molecular biology, resulting in a good set of molecular biology tools for *C. reinhardtii*. In addition, Greenaltech has started to develop the metabolic engineering toolbox for *Chlorella* strains. They developed a transformation method based on the preparation of protoplasts, they evaluated several antibiotics which can work as a selection agent, they have also adapted a protocol to extract RNA from the extremely difficult to break *Chlorella* cells, they have designed an infection protocol and produced enough viral particles of CvsA1 and MN0810.1 for infections at scales in which the production of chitin or chitosan can be quantified.

These metabolic engineering tools were successfully applied to evaluate the selected natural and non-natural producers (deliverable 3.2 and 3.3).

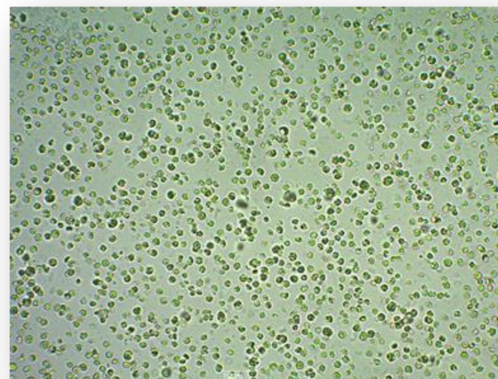


Image 2: *Chlorella* sp. cells

Based on the experience from the former project PolyModE, Artes (P16) in collaboration with WWU-1 started to use natural producer *H. polymorpha* as expression organism for new, previously uncharacterized CDAs (from WP1) to produce chitosan polymers. In addition, to increase the chitosan polymer production, CHSs (from WP1) were co-expressed in CDA expression strains. Unfortunately, cell wall analysis revealed no hints for specific changes in cell composition. Alternatively, gene knock-outs can be on option to increase the chitin level in yeast. To test this, several deletion strains covering e.g. protein processing available at Artes were provided to WWU-1 for analysis of the cell wall composition. Unfortunately, the results of the cell wall analysis were not conclusive, likewise due to experimental uncertainties. Furthermore, some attempts were made to create these deletions in the CHS/CDA expressing *Hansenula* strains which were not successful; likewise, the combination is harmful to the cell.

CNRS (P5) assessed the possibility of diatoms as potential natural chitosan polymer production host in collaboration with WWU-1. According to an optimized chitin localization assay using confocal microscopy and flow cytometry, *T. weissflogii*, *T. pseudonana* and *T. rotula* appeared to be good candidates for chitin nanofiber production. More specific, results indicate that chitinous fibres in these species are extracellular. Furthermore, chitin synthases, chitin deacetylases and chitinases, found in diatoms, as well as a range of genes from other organisms including bacteria and fungi (selected in WP1), were manipulated in diatoms, both *T. pseudonana* and *P. tricornutum*, using available technologies for gene overexpression and for gene silencing. Expression of CHS genes in *P. tricornutum* resulted in higher synthesis of chitosans. Interestingly, localization of the protein was observed to change during cell division.

Of the non-natural producers, bacteria were assumed to be the most challenging. Remarkably, the pellet of *E. coli* expressing a viral CHS from CVK displayed an increased GlcNAc content which suggests a functional expression of CHS for chitin polymer production. However, follow up research is required to confirm the product and optimize the yield.

At the start of Nano3Bio, it was thought that the expression of enzymes from other chitosan producing species would be the best strategy to produce chitosan oligomers in microalgae, as there was no evidence of the presence of chitosan or even chitin in microalgae apart from diatoms and *Phaeocystis*. Therefore, *C. reinhardtii* was transformed with CHSs (from WP1) using the developed and optimized tools. Unfortunately, the formation of chitosan oligomers or polymers was not detected in any of the *C. reinhardtii* extracts, meaning that it had no chitin synthase activity. Interestingly, using a newly developed and optimized technique that allows to easily and quickly detect chitin/chitosan in the exterior and interior of microalgal cell walls indicated that some of the microalgal species tested were capable of producing chitosan polymers naturally. These strains were further characterized in depth. In addition, optimizing the synthesis of chitin and chitosan polymers in these natural production hosts was started using the developed metabolic engineering tools.

In order to obtain enough quantities of microalgal chitosan polymers for its characterization and the determination of its bioactivities, the production process was optimized, scaled-up and an optimized recovery process was developed (deliverable 3.4). At the end, Greenaltech has been able to develop a protocol with which batches of multi-gram of natural microalgal chitosan polymers are obtained. These microalgal chitosan polymers were provided to WP4 for complete analytical and physical characterization (DP, DA, PA, purity, etc.) and the study of its bioactivities in WP4 and WP5.

Using the developed technology and know-how during the Nano3Bio project, we want to further explore the various applications of chitosan oligomers and polymers in different fields such as human, animal and plant health. Still, more understanding of specificity of bioactivities of chitosan oligomers and polymers will be required to develop applications.

Applying the developed environmental friendly, fully controllable, robust and reproducible methods for the production of well-defined and pure chitosan oligomers and polymers in terms of DP, DA and PA, we can provide the required amounts of products for application development. However, to fully exploit the potential applications of chitosan oligomers and polymers, further research and development should focus on:

- expanding the chitosan oligomer and polymer portfolio in view of understanding the structure/bioactivity relation of chitosan oligomers and polymers, and
- optimizing process parameters in view of reducing the production cost.

Outlook

In this WP, the in vivo cell factory approach towards the production of novel chitosan oligomers and polymers was successfully pursued, using the enzymes provided by WP1. A range of wildtype and engineered chitin synthase genes were expressed in *E. coli* and *C. glutamicum*, yielding cell factories for the production of fully acetylated chitin oligomers of different degrees of polymerization. These were tested for their biological activities in WP4, and provided to WP2 for their in vitro conversion into fully defined chitosan oligomers and longer chain chitin oligomers, and for their chemical activation to be converted into very large chitin oligomers and even small chitin polymers using engineered chito-synthases.

Adding different wild type and engineered chitin deacetylase genes to these chitin oligomer producing bacteria yielded cell factories for the production of fully defined, partially acetylated chitosan oligomers. Again, these were tested for their bioactivities in WP4 and made available to WP2 where first evidence for their in vitro chain elongation and polymerization were already obtained. Comprehensive metabolic engineering allowed to strongly increase the yield and purity of the oligomeric products, and optimized down-stream processing allowed their pilot scale production in qualities already sufficient to be tested in drop-in experiments of industrial processes.

In spite of numerous attempts using a range of microbial and microalgal systems, the production of chitin or chitosan polymers in cell factories was not yet successful. This was clearly the most ambitious part of our project, and the progress we made allows to hope that it will be achievable in future. Both in yeasts and in diatom algae, we were successful in heterologously over-expressing chitin synthase and chitin deacetylase genes, and the resulting recombinant enzymes were shown to be catalytically active in vitro and in vivo. An exciting results that we had not expected, at least not with such an amazing impact - namely the discovery of green algae as the first natural non-fungal chitosan producers put an end to our attempts of engineering such a chitosan polymer producing cell factory. Instead, we focused on this natural cell factory and optimized the extraction and purification processes required for the isolation of this highly precious biopolymer. This novel and natural, non-animal and non-GMO chitosan was provided in sufficient quantities to WP4 for structural and biological characterization, and to WP5 for nano-formulation and benchmarking against structurally similar, conventional chitosans, including drop-in experiments for use as a dual action gelling and preservation agent in hand cream formulations.

2.4 WORK PACKAGE 4 - PRODUCT ANALYSIS

The WP in the context of the project

This work package served to analyse the potentially novel chitosan oligomers and polymers - biotechnologically produced in WP 2 and WP 3 using enzymes provided by WP 1 - regarding their chemical structures and their biological activities, to guide the development of nano-formulations and the identification of promising applications in WP 5. To this end, techniques for the analysis of the structural properties and the biological functionalities of the chitosans needed to be developed or optimized, to make them suited for the biotech chitosans. Properties and functionalities of the biotech chitosans needed to be benchmarked against their conventional, chemically produced counterparts. Therefore, an additional important task of the WP was to produce and characterise a large set of conventional chitosans differing widely in their structures, namely in terms of their degree of polymerization (DP) and their degree of acetylation (DA). While these conventional chitosans are known to have random patterns of acetylation (PA), the prediction was that the biotechnologically produced chitosans might have other, non-random PAs.

The objectives of the WP when we started the project

This WP, thus, aimed to

- produce and characterize a large set of conventional chitosans using established chemical technologies;
- develop or optimize techniques for the analysis of the structural properties of chitosan polymers and oligomers;
- compare the structural properties of chemically and biotechnologically produced chitosan polymers;
- compare the structural properties of chemically and biotechnologically produced chitosan oligomers;
- develop techniques for the analysis of the biological functionalities of chitosan oligomers and polymers;
- determine the antimicrobial activities of chitosan oligomers and polymers against bacteria and fungi;
- determine the plant growth promoting and plant disease resistance inducing activities of chitosan oligomers and polymers;
- determine the cytocompatibility and bioactivities of chitosan oligomers towards human cells.

Where are we now at the end of the project; what are the expected and unexpected results of the WP?

All of these objectives were achieved, as detailed below.

Production and characterization of a large set of conventional chitosans using established chemical technologies

The SME partners GILLET (P10) and HMC (P20) produced a large set of chitosans with different DP and different DA, by chemical de-N-acetylation of α-chitin extracted from shells of shrimp (P10) or white snow crabs (P20) or β-chitin from squid endoskletons, and/or chemical depolymerisation of the high MW chitosans thus obtained. In addition, WWU-1 (P1) and HMC (P20) produced series of chitosans with different DA by chemical re-N-acetylation of a high MW polyglucosamine obtained from the above chitins by repetitive, complete alkaline de-N-acetylation,

using acetic anhydride. Together, these chitosans were aimed to span a DP range from ca. 100 to 3500, and a DA range from 0 to 70%. As chemical methods were used, the PA of all of these conventional chitosans was expected to be random. In addition to these chitosan polymers, HMC (P20) also supplied one mixture of chitosan oligomers from their range of commercial products that was described as containing water soluble oligomers of a MW < 5 kDa, i.e. a DP < 25.

Development or optimization of techniques for the analysis of the structural properties of chitosan polymers and oligomers

The DP of chitosan polymers can be analysed using a number of established techniques, the current gold standard being HPSEC-RI-MALLS. This method separates the polymers according to their size and allows absolute quantification of the molecular weights based on refractive index and light scattering determinations. Thus, the technique gives not only average values but also MW distributions, indicated as MW dispersity Đ (formerly polydispersity index I_P) - unlike e.g. viscometry, a widely adopted method because it does not require expensive infrastructure, which gives average values only. We analysed all chitosans using both techniques and initially found discrepancies in the results obtained for some chitosans. These allowed us to refine the analysis of the MALLS data, eventually giving good agreement between both techniques.

Equally, the DA of chitosan polymers can be analysed using a wide variety of techniques, the current gold standard being ^{1}H-NMR which we also used to characterise our chitosans. Again, as this method requires sophisticated infrastructure, different titration techniques are more widely used, particularly by chitosan producing companies with no access to NMR. Like viscometry, titration-based techniques are prone to yield unreliable results unless performed by skilled personnel. WWU-2 (P1) developed a new variant of a titration-based assay which is fast and reliable and which can be used to follow e.g. the progress of enzymatic de-N-acetylation of a chitosan sample. However, all of the available techniques for DA determination require rather large amounts of sample (in the mg range, i.e. large when dealing with novel chitosans produced on a lab scale, but small when thinking of commercial chitosan production). Also, all methods yield average values for DA even though chitosan samples are always mixtures of polymers differing in DP and DA. This is particularly important as (i) the polydispersity in DA (for which there is not only no way of measuring it, but for which not even a symbol has been defined so far) is expected to differ greatly between samples produced using de-N-acetylation versus re-N-acetylation, and (ii) the DA has been shown to greatly influence the biological activity of chitosan polymers, apparently more so than the DP. We have developed two new methods for enzymatic / mass spectrometric fingerprinting analysis of the DA of chitosan polymers. The first one involves the complete enzymatic degradation of the polymer sample using a mixture of recombinant chitinases and chitosanases deliverered by WP 1, and mass spectrometric quantification of the monomeric and dimeric products obtained. This allows the calculation of GlcN and GlcNAc units present and, thus, of the average DA of the sample. In addition, the method allows to quantify the amount of chitosan in a sample and, thus, its purity. Surprisingly, there is no accepted standard method for this important analysis and in fact, no reliable method seems to exist so far. The best method currently available is elemental analysis, but this is error-prone in the presence of organic contaminants such as proteins. The second fingerprinting method we developed uses a novel chitosan hydrolase named chitinosanase recently discovered and characterized by WWU-1 (P1) which cleaves chitosan polymers into products each containing one block of consecutive GlcN units along with the neighboring block of consecutive GlcNAc units. Again, these oligomers are quantified using mass spectrometry, and the results are analysed using sophisticated multiparametric statistics. Both methods can be used on very small chitosan samples (in the µg range) and the DA values obtained are as accurate as those determined using ^{1}H-NMR. In theory, the second technique even has the potential to inform about the DA polydispersity - for which no method currently exists, as discussed above - but this is not yet an established, reliable technique.

The least studied structural aspect of chitosan polymers is their PA. The only method currently available to analyse PA is diad analysis using ^{13}C-NMR, a method requiring both sophisticated, expensive infrastructure and rather large amounts of sample (10-100 mg). This method has been used to establish that all chitosans produced using chemical means, be it heterogeneous or homogeneous de-N-acetylation of chitin or homogeneous re-N-acetylation of polyglucosamine, yield chitosan polymers with random PA. The P_A value of these chitosans typically ranges from ca. 0.8 to 1.1 (surprisingly, unexpectedly, and not yet understood: increasing with increasing DA, starting at values as low as ca. 0.5 for chitosans with a DA close to 0%, reaching the theoretically expected value of 1 at around DA 20%, and increasing to about 1.1 for higher DAs), where a value of 1 would indicate perfect random PA, a value of 0 perfect blockwise PA, and a value approaching 2 perfect regular PA (unlike suggested in literature, this value of 2 can only be reached when the chitosan has a DA of 50%, while the maximum possible value decreases for lower and higher PAs). The second enzymatic / mass spectrometric fingerprinting analysis described above also has the potential to inform about the PA of a chitosan sample and it could provide even more information on the sizes of GlcNAc and GlcN blocks within the chitosan polymers than diad analysis. However, both methods can only safely establish whether the PA of a chitosan can be or cannot be random. Thus, if a chitosan has a non-random PA, this can be determined with certainty by exclusion of the possibility of randomness, while a random PA can never be determined with certainty.

Analysing DP, DA, and PA of chitosan oligomers is much more straightforward since the development of mass spectrometric methods. The MW of a chitosan oligomer gives accurate information on both DP and DA, and as the analytes are separated according to their MW, the methods give not only average values but also inform about the distribution of both DP and DA in the sample. However, isomeric paCOS of identical DP and DA but different PA cannot be distinguished using simple one-dimensional (MS^1) techniques. However, two-dimensional (MS^2) techniques can allow sequencing of paCOS by fragmentation, and WWU-1 (P1) has developed a quantitative, combined MS^1/MS^2 technique which allows to sequence paCOS even in mixtures, as long as the mixtures are not too complex and the oligomers are small enough, i.e. with a DP ≤ 6, and to quantify the amounts of the different paCOS in the mixture.

Comparison of the structural properties of chemically and biotechnologically produced chitosan oligomers

Surprisingly, mass spectrometric analysis performed by WWU-1 (P1) on the chemically produced chitosan mixture provided by HMC (P20) showed the presence of only minute amounts of mostly fully deacetylated GlcN oligomers in the sample. The result was corroborated by NMR analysis which found no evidence for chitosans in the sample. These results are consistent with previous analyses performed by others (including our Scientific Advisory Panel member Prof. Dr. Martin Peter) for the company who also had been unable to detect chitosan in the sample. The reason for this unexpected result remains elusive. WWU-1 (P1) produced several samples of chitosan oligomers by acid hydrolysis of chitosan polymers on lab scale, and mass spectrometric analyses of these samples allowed their full characterisation.

Mass spectrometry was also used by WWU-1 (P1) to analyse the chitosan oligomers produced in WP2 using the bio-refinery approach, and in WP3 using the cell factory approach. The bio-refinery approach of WP2 yielded a large variety of fully defined paCOS, in particularly by partial enzymatic de-N-acetylation of fully acetylated GlcNAc oligomers and partial re-N-acetylation of fully deacetylated GlcN oligomers, or a combination of both, performed by WWU-1 (P1). As an example, this approach allowed to prepare all 14 possible different tetrameric paCOS. Unusually large chitin oligomers, namely up to DP 8, were obtained by HYDER (P8) using optimized transglycosylating chitinases, and particularly by IQS (P3) using engineered chito-synthases, reaching up to DP 15 (which according to some definitions might be considered small polymers already). The portfolio of different paCOS available through the cell factory approach was broadened somewhat, but the main focus of WP3 was on metabolic engineering to improve titers and yields by UGHENT (P2) and on upscaling fermentation and down-stream processing by BBEPP (P12). Here, the quantitative sequencing method allowed to analyse in detail the purity of the samples, thus steering the optimization efforts of WWU-1 (P1) in purifying defined paCOS produced using recombinant chitin deacetylases through HILIC, of IQS (P3) in engineering chitin deacetylases for changed substrate and product profiles, of UGHENT (P2) in improving yield and purity of paCOS produced in vivo, and of BBEPP (P12) in enhancing yield and purity of paCOS during down-stream processing after fermentation of the cell factories.

Comparison of the structural properties of chemically and biotechnologically produced chitosan polymers

The DP analyses performed by WWU-1 (P1) showed that the conventional chitosan polymers produced ranged in DP from ca. 100 to ca. 3200, i.e. in the target range. Also, in most cases, the results confirmed the expectations or measurements of the SME partners which had produced and provided them. The Đ values ranged from 1.1 to 2.2, confirming that the chitosans had low to very low dispersities and, thus, were of high to very high quality. Similarly, the DA analyses revealed values in the range of 0.2 to 28% for the chitosans produced by de-N-acetylation of chitin, and of 0.2 to 65% for the chitosans produced by re-N-acetylation of polyglucosamine. Again, the values determined were in good agreement with the values reported by the producing SME partners GILLET (P10) and HMC (P29), providing evidence of the high quality of the samples. In all cases measured, the PA of these chitosans was consistent with the assumption of randomness.

Both ^{13}C-NMR diad analysis and enzymatic / mass spectrometric fingerprinting analyses, both performed by WWU-1 (P1), established beyond doubt that the chitosan polymers produced using chitin deacetylases in bio-refinery in vitro approaches had non-random PA. Interestingly, when the enzymes were used to partially de-N-acetylate chitosans with high DA (e.g. DA 60%) to yield chitosans with low DA (e.g. DA 30%), the products obtained differed in PA depending on the chitin deacetylase used. As hoped, one CDA yielded a chitosan with a more block-wise PA (P_A 0.85) while another one yielded a chitosan with a more regular PA (P_A 1.33) than the random PA seen with chemically produced chitosans (P_A 1.11). Interestingly, a third CDA yielded a chitosan which according to ^{13}C-NMR analysis appeared to have a random PA (P_A 1.19) but which was clearly distinguished from the chemically produced conventional chitosans as well as from the two other biotechnologically produced chitosans of the same DA as revealed by enzymatic / mass spectrometric fingerprinting analysis followed by multivariate statistics. This unexpected result shows on the one hand the superior power of our new analytical technique, but on the other hand that there is still a need for deeper analyses and for the development of even more powerful techniques.

Perhaps the most remarkable result in terms of structural comparison of novel versus conventional chitosan polymers is the observation of a non-random PA of the newly discovered natural chitosan in green algae. When WWU-1 (P1) performed enzymatic / mass-spectrometric fingerprinting analyses on the green algal chitosan supplied by GREENALTECH (P13) using chitinases and chitosanases followed by quantitative sequencing of the paCOS produced, it became apparent that diads of adjacent GlcNAc residues were strongly over-represented compared to expectation based on a random distribution. This is the first and so far only natural chitosan that has been analysed in such detail, in fact the first one ever to be analysed for its PA. For the time being, thus, it must be concluded that all conventional chitosans, whether produced from chitin by alkaline deacetylation of from polyglucosamine by chemical reacetylation, are pseudo-natural biopolymers, not natural ones.

Development of techniques for the analysis of the biological functionalities of chitosan oligomers and polymers

WWU-1 and WWU-2 (P1) had already developed quantitative, microtiter-plate based bioassays to measure the antimicrobial activities of chitosans against bacteria and fungi, and these were used as a basis by GREENALTECH (P13) to develop an in-house assay for the antimicrobial activities of green algal chitosan. These lab-scale assays were complemented by industry-standard challenge assays performed by COSPHATEC (P11).

WWU-1 (P1) and HYDER (P8) also had already established a number of bioassays to test the bioactivities of chitosans towards plant cells and intact plants. HYDER expanded this portfolio by a number of assays using intact plants, regarding both plant growth and plant disease resistance. One of the most widely used cell-based assays is an elicitor assay quantifying the rapid and transient production of hydrogen peroxide in suspension-cultured plant cells. Such cell lines are available for a range of plant species, including important crop plants such as rice. These assays are fast and convenient, but they are often criticized because they are using rather rapidly dividing, undifferentiated cells which have often been cultured in suspension for several decades during which time any number of mutations and chromosomal rearrangements will have occurred.

WWU-1 therefore developed and standardized new assays based on Arabidopsis seedlings or plant leaf discs that can also be performed in microtiter plates. These assays allow to estimate the potential of a chitosan - and possibly other compounds - to be used as a plant biostimulant - i.e. a compound stimulating growth and development, inducing tolerance towards abiotic stress, and/or induce a state of systemic resistance to pathogens. Based on these assays, Dr. Rebecca Melcher from WWU-1 (P1) is currently about to set-up a start-up biotech company for which seed financing has already been obtained from public sources.

Finally, UHEI (P7) and WWU-2 (P1) had already established a few cell-based bioassays to determine cytocompatibility and bioactivities of chitosans against a range of human cells. During the Nano3Bio project, both partners have expanded their portfolio of bioassays. To measure chitosan-induced changes in tight junction integrity of mammalian cells, WWU-2 has developed an assay based on automated long-term transepithelial impedance measurements as an improvement over previously performed low-throughput assays that had to be performed manually using hand-held electrodes and often damaged cell layers. In cooperation, UHEI and WWU-2 developed an assay to detect the formation of a protein corona around chitosan coated nanocapsules and its influence on their *in vitro* cellular uptake.

Determination of the antimicrobial activities of chitosan oligomers and polymers against bacteria and fungi

WWU-1 (P1) had already performed a comprehensive and systematic investigation of the influence of DP and DA on the anti-bacterial and anti-fungal activities of conventional chitosan polymers. The results showed that the highest bacteriostatic and fungistatic activities were associated with small, highly deacetylated chitosan polymers. Best activities were seen for chitosans of DP 20-50 and DA 0-25%. In the past, the elucidation of structure-function relationships concerning the antimicrobial activities of chitosans had been performed using well-defined chitosans produced by re-N-acetylating polyglucosamine under laboratory conditions. We now repeated the experiments with conventional chitosans obtained either from α-chitin (provided by HMC, P20) or from β-chitin (provided by GILLET, P10), both produced by heterogeneous deacetylation. Due to the production process, these commercial chitosans are expected to have much broader DA polydispersities than the earlier used lab-chitosans. However, we found that *cum grano salis,* the same results were obtained as described before, evidencing that the results obtained in the lab are relevant for commercial chitosans produced on industrial scale, too.

There is a plethora of literature on the antimicrobial activities of chitosan oligomers, but the conclusions range from “completely inactive” to “more active than the polymers”. This is why we complemented our earlier studies on the bioactivities of chitosan polymers with an equally systematic investigation into the antimicrobial activities of chitosan oligomers, under identical conditions. We found that chitosan oligomers clearly also possess antimicrobial activities, but these were much lower than those of chitosan polymers. Antimicrobial activities decreased with decreasing DP and increased with decreasing DA, i.e. large, fully or very highly de-N-acetylated chitosan oligomers were the most active ones. Viewed together, the best antimicrobial activities are still shown by small, highly deacetylated chitosan polymers. Interestingly, the novel natural chitosan sourced from green algae possess exactly these properties, and its antimicrobial activity also is at par with that of conventional chitosans of similar structure in terms of DP and DA. The challenge tests performed by COSPHATEC (P11) are reported in WP5 below.

Determination of the plant growth promoting and plant disease resistance inducing activities of chitosan oligomers and polymers

WWU-1 (P1) and HYDER (P8) have ample experience in determining the resistance inducing activities of chitosans in plants, and both partners have recently also started to analyse the growth promoting activities of chitosans. In a nutshell, these trials performed over the past two decades had shown that the best chitosan to induce disease resistance reactions in plants differ from species to species and from disease to disease, making it difficult to draw general conclusions. However, as a general trend, it may be concluded that large chitosan polymers with intermediate DA seem to be best suited to induce plant disease resistance. Again, as described above for the antimicrobial activities, this conclusion was drawn from well-defined lab-chitosans, and we now repeated the experiments using the commercial chitosans of GILLET (P10) and HMC (P20), obtaining similar results. Of course, these experiments were performed with a limited set of chitosans only and using the leaf disk assay on potato leaves only, but overall, we feel confident to conclude that the results obtained using lab-chitosans can, *cum grano salis,* again be transferred to commercial chitosans.

Recent evidence tends to suggest that chitosan polymers applied to plants are partially degraded to chitosan oligomers by the chitosanolytic enzymes present in the plants, namely chitinases secreted by the plant cells into the apoplast and, possibly, chitosanases provided by endophytic fungi living in the intercellular spaces of the host plant. Therefore, in the Nano3Bio project, we focused on the bioactivities of chitin and chitosan oligomers towards plant cells and intact plants. A first set of experiments was performed by HYDER (P8) using chitin hexamer and heptamer produced in WP2 from chitin tetramer, itself from the cell factory approach of WP3 provided by BBEPP (P12), using a hyper-transglycosylating chitinase engineered by HYDER (P8) in WP1. In addition, the mono-deacetylated chitosan hexamer with a GlcN residue at its non-reducing end, also from the cell factory approach and provided by BBEPP, was included in the comparison. In all tests performed on rice plants, the chitin heptamer had the strongest effect. Gene induction studies indicated that this elicitor may induce both the salicylic acid-dependent pathway of Systemic Acquired Resistance (SAR) and the jasmonic acid-dependent pathway of Induced Systemic Resistance (ISR).

A second set of experiments was performed by WWU-1 (P1) using paCOS produced in WP2 by partial enzymatic digestion of conventional chitosans of different DA, using different recombinant chitinases and chitosanases characterized in detail in WP1. When these paCOS were tested for their elicitor activities in rice cells, only those with higher DA proved active. When separated according to their DP, the bioactivity decreased with decreasing DP, as expected. In a second series of bioassays, the paCOS were tested for priming activity in the same cells, i.e. for the induction of a state of alert allowing the cells to react more efficiently to a second eliciting trigger given at sub-optimal concentration. Also in these experiments, paCOS of higher DA and higher DP were more active but surprisingly, the tetrameric products of chitosanase digestion proved priming-active while the tetrameric chitinase products did not. Detailed studies finally revealed that the mono-acetylated tetramer with the GlcNAc

residue at its non-reducing end was the most active one. This is the first evidence ever that the PA of chitosan is crucial for its bioactivity, clearly proving that this difficult to control and difficult to analyse factor needs to be taken into account when optimizing bioactivities.

Finally, WWU-1 (P1) also analysed in detail the composition of a chitosan sample which GILLET (P10) had developed based on the results of previous joint research projects and which has proven superior performance as a plant biostimulator. It turned out that this sample was a mixture of small chitosan polymers and large chitosan oligomers, both of which possess antimicrobial and plant strengthening activities. Interestingly, we found that these two parts of the original mixture exhibit synergistic activities, both towards microbes and towards plants, possibly explaining its superior performance.

Determination of cytocompatibility and bioactivities of chitosan oligomers towards human cells

Most of the experiments related to bioactivities of chitosans towards human cells were performed using nano-formulated chitosans to overcome the problems of solubility at physiological, near-neutral pH values, and are therefore reported in WP5. However, some experiments were performed using defined chitosan oligomers provided by WP2 and 3. UHEI (P7) used a series of fully defined paCOS prepared by WWU-1 (P1) in a tryptophan fluorescence assay and quantified their binding affinity to YKL-40, a human chitin binding protein possibly involved in tumour progression. Binding affinities decreased with decreasing DA, but were influenced by the PA of the paCOS, in particular for the double-deacetylated ones, again corroborating the importance of PA for bioactivities.

UHEI (P7) then performed a functional assay using recombinant YKL-40 producing cells mimicking neutrophils to attract melanoma cells in a migration assay. In this assay, the migration of melanoma cells towards the YKL-40 producing cells was inhibited by the presence of chitin and, less strongly, chitosan oligomers in the medium. At the rather high concentration used, no PA-dependence of this inhibitory effect was seen. While chitin oligomers had a stronger anti-migratory effect in this cellular assay, these oligomers are expected to have a rather short half-life in the blood or other human tissues due to the presence of chitinolytic enzymes such as human chitotriosidase. In contrast, partially acetylated chitosan oligomers are not degraded by these enzymes and might, thus, persist longer in a patient.

Outlook

In spite of the breakthrough results obtained regarding the analysis of structure and bioactivities of third generation chitosan oligomers and polymers with non-random PA, a number of important analytical questions remain. The most important ones are thought to be:

- developing a method to analyse the dispersity in DA of chitosan polymer samples;
- improve the mass spectrometric sequencing analyses of chitosan oligomers to extend beyond hexamers;
- improve the enzymatic / mass spectrometric fingerprinting analyses of chitosan polymers with non-random PA.

Beyond these tools that are required to investigate molecular structure-function relationships in even deeper detail, future work will no doubt focus on elucidating cellular modes of action of chitosans, to understand how chitosans inhibit bacterial and fungal growth, how they promote plant growth and development, how they induce stress tolerance and disease resistance in plants, how they are taken up and how they are digested by human cells, how they support scar-free wound healing, if and how they exhibit anti-tumour and the many other biomedical activities that have been reported anecdotally in literature, but that are discussed controversially and that have not been studied in a systematic and comprehensive way. A prerequisite for performing such analyses is the availability of fully characterized chitosan oligomers and well-characterised chitosan polymers with not only different DP and DA, but also different PA. The Nano3Bio project has finally delivered such third generation chitosans through its WP2 and 3, using enzymes provided by WP1, as evidenced by the analyses performed in this WP4. WP4 has also already provided proof-of-concept that the PA of chitosan oligomers crucially determines their biological activities, both towards plant and human cells. We are expecting similar results for the influence of PA of chitosan polymers on bioactivities, and we are confident that these are forthcoming. However, the biggest hurdle here is the still extremely limited amount of such chitosans that can currently be provided. Clearly, up-scaling their production is a non-trivial task that the partners will continue to address, in continuation of WP2 and 3.

2.5 WORK PACKAGE 5 - FORMULATION AND APPLICATIONS

The WP in the context of the project

WP5 was devoted to the development of chitosan-based biomaterials, biopharmaceutical formulations and applications in life sciences and biotechnology. This involved evaluating the potential of the novel, biotechnologically produced chitosans delivered by WP2 and WP3, as compared to that of the best conventional chitosans available on the markets today, and to a wide range of specialty chitosan produced at lab scale in WP4. As the biotech chitosans were not available until the last year of the project, we developed new nanoformulations using well-defined conventional chitosans, to set the benchmarks for the biotech chitosans. The formulations and potential applications of the chitosans relied on the use of the polymers in aqueous solution, enzymatically mineralized physical hydrogels, or in the form of nanostructures such as nanoparticles, nanocapsules or nanofibers. We have developed enzymatic methods for the biomineralization of chitosan hydrogels potentially useful for bone repair. We

have found a way to stabilize chitosan nanofibers produced by electrospinning in aqueous solvents, so that they can be used as drug delivery agents in biomedical systems. We prepared nanocapsules to interfere with bacterial quorum sensing as a future potential alternative to suppress the virulence of pathogenic bacteria and ameliorate antibacterial resistance to antibiotics. We have developed chitosan nanoparticles for the efficient delivery of genes to mammalian cells. We have, for the first time, followed the uptake and intracellular transport of chitosan nanoparticles into and in mammalian cells. In the long run, perhaps the most influential discovery we made relates to a method to use chitosan particles for the diagnosis and possibly even treatment of cancer pre-metastases. A more immediate potential of chitosans we are developing is their dual use as thickener and preservative in hand cream and body wash formulations.

Where are we now at the end of the project; what are the expected and unexpected results of the WP?

At the outset of the project, we aimed at comparing biotechnologically-produced bioinspired chitosans with fully characterized standard chitosans obtained by chemical methods. We expected that the newly available “third generation” chitosans and oligomers obtained by sustainable biotechnological approaches (bio-refinery and cell factories) would perform at least equally well as the standard counterparts, and perhaps in some instances outperform them or lead to completely unexpected functionalities (e.g. in biological activities or material properties). For the development of cosmetic and biomedical formulations, we required polymers, rather than oligomers, as the material properties, namely thickening of solutions, hydrogels, nanofibers, nanoparticles, and films, all rely on the polymeric characteristics of chitosan. We succeeded in this aim, thanks to the advent of chitosan polymers from green microalgae and enzymatically de-acetylated ones that became available in sufficient amounts towards the end of the project. This enabled us to establish the necessary comparisons with the standard chitosans in the different systems. In all the tested systems so far, the biotechnological chitosans have performed up to the same standard as their conventional counterparts of similar characteristics (molecular mass and degree of acetylation) per the material properties (e.g, particle size and zeta potential of nanocapsules, mechanical properties of biomineralized hydrogels, characteristics of electrospun nanofibers). Unexpectedly, we also discovered that chitosan oligomers can be used to generate nanofiber scaffolds in aqueous conditions by harnessing the electrostatic self-assembly capacity upon interacting with xanthan gum. In terms of antimicrobial properties, the microalgal chitosan has shown to perform equally effective as the standard counterpart. This is a great achievement of the project, as it paves the way towards the future availability of non-animal and non-GMO chitosans for the high-price sector of bio-cosmetics and medical devices, and probably food too. Yet to be fully established are the differences in biological properties that the new generation of chitosans generated in the project entails. The activities of the WP have enabled us to conclude that the biotech chitosans performed in fully comparable manner with their conventional counterparts of similar structural characteristics. This has been undoubtedly one of the major achievements of the project. With a perspective to future developments, we can safely predict that these novel chitosans will open up a completely new chapter of chitosan research, significantly boosting the claim of chitosans being the most promising and most versatile functional biopolymers for a circular bioeconomy.

The main formulations and applications developed throughout the project can be summarized as follows

1. Thickening and antimicrobial chitosan cosmetic formulations.
2. Hydrogels and mineralized hydrogels as biomaterials and drug delivery.
3. Surface functionalized poly-ε-caprolactone electrospun fibers and 3D printed structures for tissue engineering.
4. Chitosan-based nanofibres for drug delivery.
5. Chitosan-based nanoparticles for vaccine delivery.
6. Chitosan-based nanocapsules to target bacterial quorum sensing signalling in gram negative bacteria.
7. Chitosan-based nanocapsules to target cancer tumor cells.
8. Chitosan-based nanoparticles produced by electrospraying for vaccine delivery;
9. Non-viral particles for delivery of miRNA intracellularly to breast cancer cells comprised by specific chitosans.

Each of these items is explained in further detail:

1. *Conventional and biotech chitosan solutions for use in the cosmetic sector.* Throughout the project, Cosphatec (P11) has overseen the application of the chitosans generated by the consortium about their rheological behaviour and antimicrobial activities in two cosmetic formulations, namely emulsified hand creams and liquid shower gels (Cosphaderm® Liquid Soap). The viscosity of both formulations was optimized using conventional and microalgal chitosans to the level that full replacement of other thickening agents (e.g. xanthan or konjac gums) can be realized. Antimicrobial challenge tests of the various formulations also enabled to identify chitosan combinations that can suppress the growth rate of gram positive (*S. aureus*) and gram negative (*E. coli, P. aeruginosa*) bacteria, yeast (C. *albicans*), and to a certain extent, also the fungus *A. brasiliensis*. Demonstration of the feasibility of replacement of the thickener and preservative systems by a biotechnologically produced chitosan constitutes one of the major achievements of the project. This was a basic prerequisite necessary to transfer previously acquired knowledge of conventional chitosan application to the future use of a novel biotechnologically-produced chitosan in cosmetic formulations, as it was shown for hand creams and liquid shower gels. Here, the non-animal and non-GMO origin of the algal chitosan is a crucial asset, particularly for the high-price sector of bio-cosmetics.
2. *Biomineralized thermosensitive chitosan hydrogels for bone regeneration.* UGHENT (P2) developed chitosan temperature sensitive hydrogels for use as biomaterials for bone regeneration. The strategy was based on using β-sodium glycerol phosphate (NaGP)

along with ALP (alkaline phosphatase) to induce enzyme-mediated formation of apatite minerals (CaP) when incubated in calcium glycerol phosphate (Ca-GP) in the absence of osteogenic growth factors. Several formulations suitable to produce chitosan hydrogels were identified. Osteoblast cells encapsulated within the hydrogels were found to be viable at the different time points investigated. These results suggest that the developed hydrogels are suitable for cell encapsulation. Also, conventional chitosans were compared in their gelation ability with microalgal chitosan. The results showed that thermosensitive hydrogels using chitosan sourced from microalgae leads to scaffolds that exhibit almost identical properties in viscosity, gelation time, mechanical properties and biomineralization ability when compared to those furnished from conventional chitosans.

3. *Chitosan surface-functionalised poly-ε-caprolactone electrospun fibers and 3D printed structures.* Electrospun fibers and 3D printed polycaprolactone (PCL) scaffolds were also developed by UGHENT (P2). These fibres were coated with two different chitosans of varying molar mass. The antimicrobial activity of the materials against *Staphylococcus aureus* ATCC 6538 and *Staphylococcus epidermidis* ET 13 was investigated. The fabricated scaffolds were evaluated *in vitro* to study their cytotoxicity and cell adhesion ability by UHEI (P7). PCL surface-modified scaffolds showed no cytotoxicity. PCL surface-modified fibres using biotech chitosan led to fibers that exhibited similar results in coverage of the surface as probed by FTIR and XPS, when compared to those modified with conventional chitosans, thus offering the proof-of-concept of the utilization of the novel chitosans in biomaterials functionalization.
4. *Nanofibers by electrospinning and by electrostatic self-assembly*. The feasibility of fabricating chitosan-based nanofibers by electrospinning was thoroughly investigated throughout the project by DTU (P6). To this end, the series of conventional fully characterised chitosans with different Mw and DP provided by GILLET (P10) and HMC (P20) were evaluated.

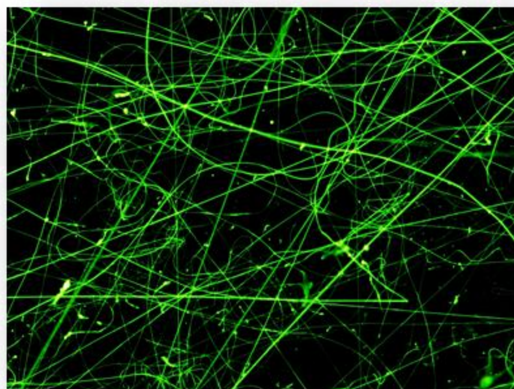


Image 3: Fluorescent Chitosan electrospun fibers

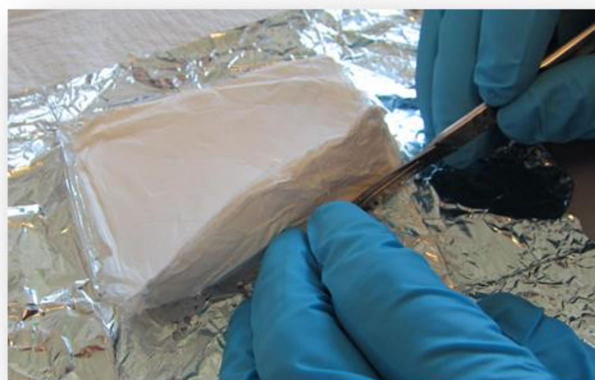


Image 4: Electrospun chitosan fiber mat

This entailed to determine the optimal solvents prior to the electrospinning process. To increase stability of the electrospun chitosan fibers, several strategies were explored, including chemical crosslinking with genipin, as well as neutralization using NaOH, Na_2CO_3 and NH_3. Further, the inclusion/blending ofphospholipids solutions with chitosan was assessed as a novel and alternative stabilization method. Among all of the stabilization methods tested, the inclusion of phospholipids was shown to be the most promising protocol to fabricate electropsun chitosan fibers. Homogeneous and uniform nanofibers, made with GILLLET (P10) Chitosan112, were obtained and the diameters tended to increase with the increase of phospholipid content. FTIR spectroscopy offered finger prints of the nanofibers and showed bands centred at 2935 and 2856 cm^{-1}, indicative of the presence of aliphatic methylene groups from phospholipids ($-CH_3$ and $-CH_2$, respectively) and an attenuated band at 1537 cm^{-1} (corresponding to NH3+), the result of the interaction with chitosan. DLS measurements showed that the zeta potential from chitosan solution becomes less positive with the increase of phospholipid content also as result of the interaction of positively charged amine groups of chitosan with phospholipids. The size analysis of data from DLS showed that the average size increased drastically (exceeding the limits of detection of the equipment). By optical microscopy it was observed that these fibers remained viable in PBS at least for a period of seven days with reduced water uptake and weight loss. The potential of the chitosan/phospholipid fiber matrix to be used as delivery carrier for lipophilic drugs (e.g. curcumin and diclofenac) and vitamins (e.g. vitamin B12) was investigated. The encapsulation of vitamin B12 was successfully accomplished without changing significantly fiber properties. Both FTIR spectra display the same signature with and without encapsulated drug. Sustained release of the vitamin B12 from nanofibers was registered over a period of seven days. The same fibers were shown to be non cytotoxic when applied to fibroblast cells (in collaboration with UHEI, P7). Additionally, the fibroblast seeded on top of the fibers were shown to adhere and grow at the top of this matrix. The potential of nanofibers to be used in bone tissue engineering was also investigated in collaboration with UGHENT (P2). Miner-

alization of the fibers was achieved by soaking in a solution of the enzyme urease, followed by the incubation in a solution containing enzyme substrate (urea) and Ca^{2+} ions, demonstrating the potential of this matrix to be used in bone regeneration therapies. Electrospun nanofibers produced with HMC 90/2500 chitosan (P20) were tested with MDCK cells in terms of cytotoxicity (collaboration WWU-2, P1). Herein it was concluded that cytotoxicity is mediated by the density of fibers used in cell culture media. With regard to the influence of chitosan structure on the mechanical strength of the nanofibers, it was possible to establish that chitosans with higher Mw and lower DA increased the modulus of elasticity of Ch/P systems. The results on chitosan-based electrospun fibres achieved throughout the project have provided systematic structure-function relationships between different chitosans of varying Mw and DA, and the physicochemical properties of the nanofibers. These results are of enormous relevance for the subsequent development of biomaterials and medical devices to be realized in wound healing, tissue engineering and other applications. On a different front, in collaboration with WWU-2 (P1), DTU (P6) discovered a protocol whereby mixing chitosan oligomers (from HMC, P20) with xanthan gum (from COSPHATEC, P11) yielded self-assembled nanofibers in aqueous solution. The morphology of these nanofibers was observed to be controlled by the ionic strength, concentration and position of both components and by the time of incubation. This was a result that offered a simple new route to obtain biopolymer-based nanostructured materials. It also provided the proof-of-concept that it is possible to use not only long polymeric chitosans, but also its oligomers, to trigger the self-assembly of xanthan gum and to generate a novel 3D nanostructured scaffolds. The realization of this process to generate directly hydrated nanofiber scaffolds for the fabrication of novel biomaterials is yet to be established in future studies.

5. *Chitosan-based nanoparticles by electrospraying.* Chitosan particles for oral vaccine delivery have been generated using electrospraying by DTU (P6). Mixtures of acetic acid and ethanol were used as solvent and different concentrations and chitosans with different DA and DP obtained from HMC (P20) were tested. Low DP chitosans were found to facilitate particle formation. An adjuvant, Quil-A, was added and the release of ovalbumin, used as a surrogate antigen, from chitosan microparticles was tested for the different series of chitosans. These particles displayed mucoadhesive properties and the production of biomarker IL-6 from dendritic cells was also investigated to assess immune responses triggered by this system.
6. *Chitosan-genipin mesoscopic gels.* At WWU-2 (P1), the feasibility to prepare gel particles with chitosans provided by GILLET (P10) and HMC (P20) of varying Mw and DA (ranging from 34 to 213 kg/mol and from 9 to 20%, respectively), was established. For the first time, irregular gelation kinetics at below critical chitosan concentrations and for chitosans with low molar mass were documented. Under these conditions, critical crosslinking times can be measured by dynamic light scattering (DLS), thus showing they occur earlier than the onset of macroscopic gelation measured with other rheological techniques. We hypothesized that so-called mesoscopic gels formed by the sequential connection of individual polymer chains that connect at their outer contact points. Eventually, the growing polymer network resembles the appearance of gel-like structures. This work offered a novel route to generate chitosan particles of varying size and morphology.
7. *Chitosan-based nanocapsules for bacterial anti-quorum sensing.* At WWU-2 (P1), chitosan-coated oil-core nanocapsules were investigated in terms of their interaction with an *E. coli* Top 10 biosensor, and their influence in bacterial quorum sensing activity. It has been possible to quantify experimentally and in silico in a very precise manner the ratio of nanocapsules to bacteria that achieve a maximum degree of adsorption of 145 nanocapsules/bacterium. At this maximum ratio, the bacteria aggregate, whereas at lower or higher ratios, bacteria remain unaggregated. The influence of aggregation on the quorum sensing response was evaluated and we found that at the greatest aggregation point, the quorum sensing signal is also minimal.
8. *Chitosan nanocapsules in interaction with mammalian cells.* A full analysis of the interaction of chitosan nanocapsules with mammalian cells was performed between UHEI (P7) and WWU-2 (P1). It was found that the chitosan's DA influences the interaction with lipids and cell membranes in serum-free conditions. In the presence of serum proteins, we found a strong quenching of the particle uptake suggesting the formation of a protein corona at the particle surface. As model system, we used artificial lipid vesicles (DPPC-vesicels), HEK293 cells, and human umbilical vein endothelial cells (HUVECs). Experiments were performed under static cell culture conditions and under physiological blood flow conditions. Accordingly, we confirmed that chitosan nanocapsules formulated with a chitosan of DA 34% are most hemocompatible, suggesting a prolonged circulation in blood.
9. *Non-viral nanoparticles for gene delivery to mammalian cells.* At WWU-2 (P1), non-viral strategies for microRNA delivery to breast cancer and pEGFP–siRNA co-delivery to cystic fibrosis cells systems were addressed. Chitosan samples of biomedical grade purity were obtained from GILLET (P10), and fully characterized in their Mw and DA features. These chitosans were used to prepare either nanocomplexes (NCXs) or nanocapsules (NCs) microRNA-hsa-145, known to be downregulated in MCF 7- breast cancer tumor cells. Self-assembled chitosan–miRNA nanocomplexes (NCXs) and chitosan-based nanocapsules were furnished at varying molar charge equivalent (amino/phosphate, [n+]/[n-]) ratios (from 0.6 to 8). As a general trend, the Z-average particle diameter of the NCXs was 80–190 nm. The binding affinity was studied using Surface Plasmon Resonance (SPR) and fluorescence spectroscopy. Transfection was carried out, targeting Junction Adhesion Molecule A (JAM-A), which was successfully down-regulated in presence of chitosan with high-DP and intermediate DA of 30%, indicating reduction in breast cancer cell motility and invasiveness. In parallel, we evaluated chitosan–pEGFP and chitosan–pEGFP–siRNA complexes using conventional chitosan from HMC (HMC Sample 70/5). Cell viability studies did not show any significant cytotoxic effect of the chitosan-based complexes in a human bronchial cystic fibrosis cell line (CFBE41o-). We evaluated the transfection efficiency of this cell line with both chitosan-pEGFP and co-transfected with chitosan-pEGFP–siRNA complexes at (amine/phosphate, N/P) charge ratio of 12. We reported an increase in the fluorescence intensity of chitosan-pEGFP and a reduction in the cells co-transfected with chitosan-pEGFP–siRNA. These studies provided proof-of-principle that co-transfection with chitosan might be an effective delivery system in a human cystic fibrosis cell line.

Outlook

The Nano3Bio project has been a unique and instrumental platform that has enabled to address the influence of chitosan structural features of a series of second generation chitosans on the properties of every category of the major nanostructured materials for healthcare and biomedical application. These materials span polymeric solutions, nanoparticles (matrix and oil core nanocapsules), nanofibers, fibres, biomineralized hydrogels, and non-viral gene delivery particles. The achieved results have enabled in most cases to help answering the question **"which chitosan is best suited for a given application"?** When the biotech chitosan became available during the second half of the project, with a limited set of characteristics (Mw and DA), and in small amounts, initial benchmark comparisons were possible between this chitosan and the conventional ones. As expected, the best-suited chitosan for a given application did not always match those of the first biotech chitosan polymers available. Hence, future studies will be necessary to continue the initiated benchmarking between conventional and biotech chitosans produced at large scale, and possibly with additional, different structural characteristics.

For most of the different formulations and applications addressed, we have achieved in vitro proof-of-concept of the biological performance of the different systems. Future studies will be needed to address the in vivo proof-of-principle of pre-clinical value particularly for the biomaterials for bone regeneration and for gene delivery.

2.6 WORK PACKAGE 6 - LIFE CYCLE ASSESSMENT

The objective of WP6 was to provide a detailed environmental assessment of biotechnologically and conventionally produced chitosans by using **life cycle assessment (LCA)**. Another major objective of this WP was to clarify the legal constraints for market entry of biotechnologically produced biosimilars using the novel chitosans as a case study, to identify bottlenecks in registration procedures, and to draft possible scenarios to overcome such legal hurdles. Below, we provide the main findings of the two assessments.

LCA was applied to the production of chitosans obtained from chitin from crab shells, chitosans obtained from chitin from shrimp shells and chitosans obtained from chitin from squid pens. We also assessed production of chito-oligosaccharides (COS), the 'building blocks' of chitin. In particular, we assessed production of COS obtained through biotechnology as well as from conventional sources, namely from chitin from crab shells, shrimp shells and squid pen.

Production of chitin and chitosans was assessed based on primary data at industrial scale. For chitin and chitosan from shrimp shells and squid pen, data were provided by Gillet Chitosan, a commercial producer of these materials in India. Both chitin and chitosan are produced in the state of Gujarat, using by-products from a nearby fish processing factory. For chitin and chitosan from crab shells, the supply chain is more complex. Shells are obtained from Canada's Atlantic coast fisheries. Dry shells are shipped to China, where chitin is produced. Subsequently, chitin is shipped to Europe, where the chitosan producer is located. Data for chitin and chitosan productions were obtained from the actual producers, whereas crab shell waste processing data were obtained from literature.

Biotechnological production of COS was assessed based on pilot scale production by the BioBase Europe Pilot Plant (BBEPP), considering scaling up of the process to a large fermentation tank of 4,500 L. Finally, production of COS from chitin was assessed based on estimated data by one of the commercial chitosan producers in the Nano3Bio consortium, using as main data source a patent on the subject.

The system under study is a 'cradle to gate' one, whereby the supply chain of the different products was followed from the extraction of natural resources and raw materials up to product's manufacture. Distribution, use, and disposal of the product itself is excluded, however management of waste and by-products generated in the supply chain, including manufacturing, is included. We also included an estimate of the environmental impact associated to indirect land use changes (iLUC), that is, an estimate of how the demand for bio-based products like chitosans and COS induce deforestation and intensification of agricultural land currently in use.

For chitosans from crab shells, one of the identified key drivers was energy use: the use of coal as fuel during chitin production in China as well as the overall electricity use dominate several environmental impacts, such as greenhouse gas (GHG) emissions. A beneficial effect to the environment was identified, related to the supply of crab shells for chitosan production. These shells are diverted from a composting process, thereby avoiding the ammonia emissions associated to composting. As for transports, their influence is not a key driver, even though crab shells and chitin are transported over long distances.

For chitosans from shrimp shells, one of the key contributions to the impact of its supply chain in India is the production of the hydrochloric acid consumed during chitin production. Overall, the contribution of energy use (heat and electricity) for this chitosan supply chain is relatively low. This is to a large extent due to the fact that both chitin and chitosan are dried using solar energy. Overall, we find that the shrimp-shell chitosan supply chain is a very efficient one, as it is local, recycles internally many of its by-products, such as caustic soda and water, uses solar energy for product drying, and has substantial economies of scale due to its relatively high production capacity. On the other hand, in this supply chain, shrimps are diverted from animal feed production. This diversion creates an additional demand for animal feed crops such as soy, thus creating pressure to either put new land into cultivation, via deforestation, or by increasing yields in currently cultivated land. This has a substantial effect

on the results.

Production of chitosans from squid pen has as key environmental impact contribution the production of electricity, mostly used to dry the final product. In terms of GHG emissions, electricity production accounts for more than 90% of the total emissions attributable to this production route. Production of chemicals, most notably caustic soda and sodium chloride, have a substantial contribution in several impact categories. It must be highlighted that production of chitosans from squid pen does not require hydrochloric acid, as opposed to production from crustacean shells.

Biotechnological production of COS appears to have two key contributions to most environmental impacts included in the LCA, namely production of electricity and production of glucose. Both products are used in the fermentation process, which is the step in the process chain which shows the biggest impact. Electricity is also used in all the other processes taking place before and after fermentation. However, taking all of those processes together (excluding fermentation), their contribution is relatively low. Regarding the production of chemicals, caustic soda and ammonium chloride are the most important ones in the results. While the first substance is used mainly for cleaning the equipment, the latter is needed as a nutrient during inoculum development and the following fermentation process.

Besides identifying the key environmental aspects of the different chitosan and COS production routes, it was also a goal of the project to compare COS production following the Nano3Bio approach (fermentation) with the conventional approach (chitin hydrolysis: breaking down chitin into COS). This comparison was performed only with a preliminary character. This is because 1) production of COS by fermentation has been modelled based on data at pilot scale, even though an effort has been made to scale up the data for a higher production capacity; 2) production of COS from the hydrolysis of chitin is not based on real production but on a patent description and expert knowledge by the Nano3Bio consortium; 3) the COS obtained by the two routes are different in composition, therefore they might not be functionally equivalent. In spite of these limitations, it was decided to perform this comparative analysis in order to have a first look at the relative sustainability of the new biotechnological process. The results showed that COS produced by fermentation have lower impact scores than any of the chitin alternatives in nine out of 16 impact categories. As a summary, we can say that as a general trend, in many impact indicators, COS produced by fermentation perform better than COS production from chitin, except when the chitin source is shrimp by the Indian producer. These results are encouraging for the technology developed by the Nano3Bio project, since at an early stage of process development we can see that in many impact indicators, its performance is comparable to the current technology.

Future research on the life cycle impacts of chitosans should focus on the following areas, which we could not reach in Nano3Bio: 1) to assess COS/chitosan production using data from full industrial scale, in order to reduce uncertainties induced by the use of pilot scale or literature data; 2) to expand the scope of the study to account for the remaining phases of the life cycle, namely the use phase and end of life; 3) to compare, on a full life-cycle basis, chitosans/COS with alternative products fulfilling the same functions, in order to determine potential environmental advantages or drawbacks.

The **regulatory analysis** carried out in this WP addressed three fundamental areas: operations, products, and a gap/bottleneck analysis. The regulatory analysis started with a process mapping of chitosan sources leading to the various products. The traditional production of chitosans was examined versus biotechnological production methods of defined COS and polymers. Different cases were identified that allowed to investigate legal requirements and possible differences between conventionally produced and biotechnology based chitosans. Where needed, both national and EU legislation was included in the analysis. Finally, also some non-legal requirements were reviewed in order to picture the potential impact.

On the operational aspects, there are general requirements such as those related to environmental protection and occupational health that are applicable for all cases. They are very general in nature, yet nanomaterials may require specific attention. The next determining factor for operational aspects is the use of genetically modified organisms (GMO), which imposes specific measures. Finally, specific testing on animals and/or humans may be required depending on the development objective. Such testing is subject to legal obligations, which may differ when dealing with nanomaterials. Most of these legislations are based on implementations of EU Directives.

In addition, one of the goals of the project was to identify if differences in implementation between member states lead to competitive (dis-) advantages. Project partners did not identify any specific bottleneck. In consequence, the national implementations of Directives covering operational aspects did not reveal to hinder the implementation of a typical cross-national consortium.

When considering legal requirements related to product types, legislation on chemical compounds, in particular REACH, is highly relevant as it addresses nanomaterials and provides guidance for assessment. It would be applicable irrespective of the production method. No new EU legislation for nanomaterials has been introduced. Either provisions are included in other legal instruments (e.g. product specific legislation) or introduced via national legislation. The main issue raised in this context is the difficulty of applying the nanomaterial definition to complex and diverse products such as chitosan.

Concerning the use of GMO, given that biotechnological production of COS involves extracting the product from the fermentation volume, no GMO will be present. As such, it would not be considered a GM product, but rather a product developed with a GM processing aid. While downstream non-food/non-feed uses are in any case not covered by legislation, the absence of the GM material in COS/chitosan would also place it out of scope of the GM legislation.

In addition, legislation defining organic production was reviewed. The legislation covers specifically agricultural production and then excludes any involvement of GMO. This would also include chitosan produced with GM microorganisms. Some organic certification schemes have expanded the concept to cover other sectors such as cosmetics. In that case, both GMO as well as nanomaterials are potentially excluded from the certification scheme. Although such schemes are voluntary, they are usually requested by a specific client or market.

With very diverse cases for medical applications, regulatory requirements relating to medicinal products for human use, medical devices, combined advanced therapy medicinal product and nanotechnology-based medicinal products/medical devices were reviewed. One of the challenges remains the determination of the category in which a product will fall, in particular for products that represent a combination of functionalities. Furthermore, the evaluation stressed the need to have similar approaches for similar products: the information required for a conventional chitosan-based wound healing application should not differ from those required for a biotechnology based or from one-termed nanomaterials.
Placing on the market of other products (cosmetics, functional feed ingredients, feed additives and crop protection) follows the sector-specific legislation. In some cases, nanomaterials are clearly covered and specific requirements are indicated.
Although we lack actual nanomaterial products to compare regulatory data requirements, it can be concluded that 1) many of the product legislations incorporate provisions for addressing concerns related to nanomaterials; 2) whenever nanomaterials are covered, this usually results in additional data requirements and/or a default higher risk classification (see e.g. medical devices); 3) the indication 'nano' on labels is already required in the legislation on food, cosmetics and biocides.
To conclude, Europe's Bio-economy Strategy addresses the production of renewable biological resources and their conversion into vital products and bio-energy. The Nano3Bio project analysed the framework in which example cases of such applications should strive. Combining the information with the experience of the project partners, the following recommendations were formulated: 1) creating fast-track and reduced data requirements for biotechnology products replacing conventional products and harnessing the full potential of designed products; 2) applying the nanomaterial definition in a pragmatic way, ensuring that products are not stigmatised and subject to more requirements only because of a matter of definition; 3) further harmonisation of legislation and its implementation, in particular for aspects related to market introduction; 4) bridging the knowledge gap of hazards related to nanomaterials and establishing realistic regulatory study designs that deliver information adapted to the competent authorities' needs.

Outlook

This WP has provided a wealth of precious data required to calculate environmental impacts of industrial chitosan production, and it has produced the first set of comprehensice Life Cycle Assessments for different conventional production processes yielding chitin and chitosan polymers and oligomers. We see that our first publication on the topic has been very well received and we are confident that it will have a large impact on making chitosan production more environmentally friendly, hopefully far beyond the two commercial chitosan producers in the Nano3Bio consortium who already operate under strictly quality controlled, largely optimised conditions. The first Life Cycle Assessment for the biotechnological production of chitin oligomers, and its comparison to a conventional process yielding a similar product - though not yet fully reliable as both were based on pilot scale experiments, written descriptions and educated assumptions - already suggtest that the biotechnological processes can be on par with the conventional chemical processes. It will be important to accompany the further development of the biotechnological processes with parallel LCA investigations to steer the process in the best possible direction.
Similarly, the comprehensive regulatory analysis will be influential in steering future developments towards the biotechnological production and use of functional biopolymers such as chitosan which in this context served as an example only. Not unexpectedly, it became clear that current legislation which was developed in the past to deal with the production and use of synthetic chemicals, including divers polymers, sometimes clashes with the characteristics of biopolymers. The analysis, thus, serves on the one hand to select possible application fields for biotechnologically produced chitosans that are less prone to create problems during manufacturing and registration. On the other hand, it may also serve as a guide to policy makers identifying gaps and needs for modification of current legislation to adapt the rules to empower biomaterials and bioactives in the interest of a smooth transition to a knowledge-based, circular bioeconomy. One emerging field of particular interest and of clear need of action in this respect is a pragmatic definition of nanomaterials.

3 POTENTIAL IMPACT (INCLUDING THE SOCIO-ECONOMIC IMPACT AND THE WIDER SOCIAL IMPLICATIONS OF THE PROJECT SO FAR) AND THE MAIN DISSEMINATION ACTIVITIES AND EXPLOITATION OF THE RESULTS

3.1 IMPACT

The EU funded Nano3Bio project aimed to develop biotechnological ways of producing well defined polysaccharides and nanotechnological tools for their formulation and functionalization, yielding products with known physico-chemical properties and reliable biological activities. The Nano3Bio project offered a fresh approach towards realising the ecological and economical potential of these functional biopolymers, opening new opportunities for the knowledge-based development of high added-value products utilisable e.g. in pharmaceutics, medicine, or cosmetics.

We used chitosans, possibly the most versatile and promising polysaccharides available, for the implementation of the objectives listed in the corresponding work programme by broadly addressing concrete, industry defined needs. The outcomes of Nano3Bio are addressing certain **societal challenges** like health, demographic change and wellbeing; food security, sustainable agriculture and forestry, marine and maritime and inland water research, and the Bioeconomy; climate action, environment, resource efficiency and raw materials.

The Nano3Bio project aimed at providing an important cornerstone in the building of a **Knowledge-Based Bio-Economy**. Therefore, the Nano3Bio project had two parallel ambitions, namely (i) to **increase basic knowledge** and (ii) to **use the new knowledge** for technology transfer and development. The progress, both in fundamental science and in the development of applications, has only been achieved through **international, interdisciplinary, and intersectorial** research. This is demonstrated e.g. by a number of scientific publications produced by the project and invited presentations. The efficient cooperation between partners from science and industry that is between researchers, developers, manufacturers and project management services was the **key factor** for our successful project work and will allow **rapid technology transfer** from the academic field into economy. The great **economic potential** of the generated project foreground is demonstrated by the relatively **high number of patent applications** resulting from the project. One patent application has already been made. Four further patent applications have been drafted and are currently in the approval phase carried out by the project partners.

Nano3Bio **engaged industry and SMEs** strategically. Besides the highly interdisciplinary academic team, our consortium consisted of the whole product chain from the raw material producers to the developers, suppliers, and users of chitosans. Furthermore, an experienced team carried out **a regulatory analysis** for the novel bio-engineered, smart chitosans in order to investigate the legal requirements for their registration, to identify constraints and bottlenecks impeding market entry of biopolymer based biomaterials and, thus, the rational advancement of the KBBE. In parallel, comprehensive **life cycle assessments** of the novel biotechnologically produced chitosans were performed in comparison to conventional chitosans available on the market today, to ensure compliance with the overall objectives of the **transition to a bio-economy**, namely reduced energy input, reduced environmental burden, and concomitantly improved product quality and increased consumer safety. These and all other non-confidential project outcomes will be offered as **open access** publications for a wide public use and will be extremely helpful for informing especially SMEs, allowing them to anticipate regulatory requirements as well as on the environmental impacts during the life cycle of their products. The **identified policy gaps** can as well support decision-makers in **improving legislation** to facilitate the valorisation of products produced with biotechnology.

In line with the topics of the call, Nano3Bio converged biotechnological production and nanotechnological formulation of innovative, bio-active, bio-mimetic, bio-inspired, and bio-engineered chitosans as high added value materials for a range of applications in the high price market sectors of the life sciences. The **potentials of molecular engineering, bioinformatics, systems biology, and nanobiotechnology** were used to design and engineer improved biocatalysts and bio-catalytic processes, improving their performance in the production of high value primary materials, deploying bio-processes with increased yield, quality, and purity of the conversion products, i.e. narrowly or even fully defined chitosan polymers with known and tested biological functionalities. The Nano3Bio approach entailed both, the **in vivo production** of bio-mimetic materials as well as their **in vitro production based on innovative biotechnology** processing using chemo-enzymatic technologies. Nano3Bio included under the same umbrella different approaches to **exploit current progress in nanobiotechnology**, i.a. biologically produced bioinspired and nanostructured polymers with novel and **improved bio-functionalities, biomineralization of physical chitosan hydrogels** for bone repair, and the development of chitosan **nanoparticles, nanocapsules, and nanofibres** to overcome solubility limitations of chitosans and to improve their bio-accessibility and bio-compatibility.

Furthermore, the project **stimulated the mobility** of researchers not only within the European Union but also beyond its borders, because one Indian institution participated in the project, too. The KBBE will not only play an important role because it is an extremely valuable economic resource, but will also help to find **solutions for the most pressing challenges facing our and the future generations**. Therefore, a **global working environment** is a fundamental requirement, especially for **early stage researchers and doctoral students**. Nano3Bio contributed also particularly to broaden their **skills** and know how. Thus **Technical Training Workshops**, focusing on different aspects of the project, like e.g. “Metabolic engineering”, “IPR Management” or “Scientific paper writing” were organized in conjunction with consortium meetings. Furthermore, especially young scientists were encouraged to visit the labs of other partners to make use of their expertise and infrastructure in order to establish lasting

and trusted links between the partners of the consortium - and they made ample use of this opportunity. The crucial role of **personnel exchange** will have a beneficial impact on their future careers.

The Nano3Bio project aimed at developing biotechnological biosynthesis strategies for partially acetylated chitosan oligomers and polymers with narrowly defined quality. **Two different approaches** were adopted in parallel: an **in vitro biorefinery approach** and an **in vivo cell factory approach**. In the biorefinery approach, we used bioengineered and optimized chitin synthesizing, modifying, and degrading enzymes on conventionally produced chitin and chitosans or their chemically activated building blocks, to yield novel chitosans with more defined, non-random patterns of acetylation not achievable using chemical protocols. In the cell factory approach, we used genes coding for chitin synthases and chitin deacetylases towards the production of monoclonal oligo-chitosans. **Both approaches were successful, partly beyond expectation.** As an example, we have identified several natural chitosan-producing organisms outside the kingdom of fungi to which natural chitosan production was thought to be limited. This **offers unprecedented opportunities for the biotechnological production of pure chitosans in their native forms** for which we already have strong reasons to believe that they differ from today's conventional chemically produced chitosans. Moreover, the non-animal origin of these novel chitosans will allow rather **swift entry** into sensitive, **high-price markets** such as bio-cosmetics. The novel biotech-chitosans are not only **different from conventional chitosans** produced chemically, e.g. in having different, non-random patterns of acetylation, they are also more defined in terms of their chemical properties and - most likely, as this remains to be proven - **biological functionalities**, and the ones produced in cell factories will in addition equally avoid the problems of animal origin. We started to characterize these novel chitosans and their nano-formulations more thoroughly, and assessed their performance in a range of benchmarking application tests, in particular for cosmetic products, but also for other markets.

In the past, our series of highly successful European research projects **CARAPAX, NanoBioSaccharides**, and **PolyModE** has paved the way from yesterday's ill-defined and poorly reproducible "first generation chitosan" to today's well-defined "second generation chitosans" which begin to appear on the markets in sufficient quantities for the development of reliable chitosan-based products, firstly in the agricultural market sector and increasingly also in the biomedical sector. We are confident that **the Nano3Bio project will guide the way towards tomorrow's "third generation chitosans"** which due to their biotechnological production processes will be different and more natural than the conventional chitosans derived by chemical processes from shrimp and crab shell wastes. The comprehensive Life Cycle Assessment, which we performed in the Nano3Bio project, will allow to **further optimizing the conventional production processes**, lowering their environmental impact, and will guide the way to the development of sustainable biotechnological production processes. In parallel, the thorough analysis of the **legal constraints** hindering the commercial success of bio-based products, in particular products based on functional biopolymers such as chitosans, will point towards hot spots of required future legal activities to foster the transition to a sustainable, circular bio-economy.

3.2 MAIN DISSEMINATION ACTIVITIES

Efficient dissemination and use of the results generated by Nano3Bio were among the fundamental requirements for the participants of this project since the success of dissemination activities decisively contributes to the **overall success of a research project** as measured by usage of its outcomes through external entities from science, industry, education and the public health sector. Dissemination takes many forms depending on the foreground to be communicated and on the different target groups. On the one hand, classical activities such as publications in scientific journals and at conferences were performed. On the other hand, the consortium pursued a **broader approach** incorporating relatively new media like smartphone apps and Social Media. The Nano3Bio consortium had a commitment to engage target groups beyond science and industry communities to increase a broader interest in bio-sciences and bio-technology and to get into a dialogue as to public hesitation concerning new developments in these fields. This summary provides an overview of the Nano3Bio dissemination strategy and selected activities performed by the consortium.

Strategy

Dissemination activities within the Nano3Bio project were designed to communicate relevant project results to **key target groups** in order to exploit the project's results and to ensure the widest dispersion of advanced knowledge arising from the project. Nano3Bio's motivation for joint action in this field were profiling the consortium members and the project itself, generating contacts to relevant scientific and non-scientific players and increasing opportunities for future activities based on Nano3Bio project results. Not least, Nano3Bio aimed to explain the project approach and its results to the interested public in order to explain the included perspectives for society, the environment and industry and in order to underline that the EU's investment in this project brought out significant benefits for these fields.
Overall, the consortium pursued a **target-oriented policy** of disseminating the progress of the project. It incorporated the following goals: to disseminate new scientific knowledge, to use the new knowledge for developing novel technologies as well as to raise public awareness and understanding for science and technology.

Target audiences

During the project, the Nano3Bio consortium had the following target audiences for the fields of dissemination and communication in focus: potential industrial partners respectively clients, policy makers, scientific networks and communities, young academics, the interested general public and the internal staff of participating organisations. Accordingly, the Nano3Bio 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 a **broad understanding of related scientific matters** and Nano3Bio's achievements.

Messages

To focus target group oriented communication, the following strategic key benefits were defined for different target group related key messages: a) Benefits for science. Nano3Bio stands for highly forward-looking innovation in the field of bio-engineering related to chitin and chitosans. b) Benefits for young academics: Nano3Bio offers a lot to discover particularly towards students and young scientists. c) Benefits for industry: Economic use cases are part of the Nano3Bio approach. Smart chitosans are expected to open up new market sectors. d) Benefits for society: Nano3Bio aims to create beneficial impact for society as a whole. e) Benefits for environmentally interested target groups: Nano3Bio aims at producing chitosans more efficiently. The accordant benefits were specified in detail in the 'Benefits' page of the Nano3Bio website and its target group related subpages.
Furthermore, within its communications, the consortium made use of the following main and unique characteristics of Nano3Bio, which were implemented and repeated within the project's dissemination activities in a deliberately simplified manner: a) Nano3Bio underlines that chitosans are rightfully regarded as the most versatile and promising class of functional biopolymers. b) Nano3Bio delivers third generation chitosans with known and defined patterns of acetylation. These will come up with clear properties and functionalities and will be highly interesting for various applications. c) Nano3Bio establishes a detailed life cycle assessment of chitosan production processes, allowing comparison of different production approaches as to environmental effects. d) Nano3Bio has reached a number of impressive achievements (see below).
Not least, the dissemination strategy of Nano3Bio included three consecutive phases.
The first phase was **awareness-oriented**. In the early stage of the project, the priority was to present the project in order to build awareness for its existence and its general approach. Key content during this phase was: What the project is about, what it aims at, who is involved, and what are its potentials. The most important key messages for this phase were: chitosans are an amazing class of functional biopolymers, perhaps the most versatile and most promising one, chitosans can be used in medicine, agriculture, food industry, cosmetics, and many other application fields, there are significant differences between first, second and third generation chitosans, Nano3Bio will meet new market requirements, which cannot be complied with traditional chitosans, examples for general benefits that can be realised by implementing biotechnologically generated chitosans, a description of the capacity aggregated within the Nano3Bio consortium and its European funding.
The second phase was rather **result-oriented**. At a later stage, when results were available, the communication of these achievements was in focus in order to involve interested parties. The key messages in this phase were more specific and particularly related to the project's achievements and their possible applications. Accordingly, today e.g. an impressing list of Nano3Bio major achievements including related press releases can be found in the press section of the Nano3Bio project website.

The third phase was mainly **exploitation-oriented**: in this final phase, specific activities were undertaken to start the actual exploitation targeted to potential clients in order to allow project results to be implemented in specific products and subsequent research activities. Accordingly, the related messages were very specific and application or research field oriented. Furthermore, if applicable, potential future partners, showing interest in Nano3Bio, were contacted and provided with information about the project's outcomes and its novel opportunities.

Activities

All consortium partners were involved in Nano3Bio's dissemination and communication activities. The main challenge was to distribute information and knowledge through a variety of ways with different purposes to the various target groups. In this process, the project's messages mentioned above were implemented in order to reach the different target groups with an appropriate level of information as to technical qualification and depth of details. The different activities are specified below:

- Website

The Nano3Bio website www.nano3bio.eu is the primary dissemination tool presenting the project. Comprehensive information about the project and related information can be found here. The website aims to meet the communication needs of a wide range of users. The website serves for communication and dissemination to all target groups mentioned above. Other dissemination elements (like the factsheet, press releases, video clips etc.) are available for download.
Apart from communicating the general project approach, its targets and expected benefits, research activities and achievements, the website is used to promote events and publications. When creating the website, it was one of the main goals to direct the content to the target groups of the project. For example, the website contains explicit subpages clearly directed to those target groups (potential partners from industry, young academics, press and media, the general public etc.) and showing their potential benefits through Nano3Bio.
The website provides easily accessible information about Nano3Bio basics and invites users to get into continuing issues intuitionally. It incorporates the basic rules of contemporary web usability as well as search engine optimisation and a responsive design allowing easy and comfortable access via desktop computers, tablet computers and smartphones. Not least, the website is the background information channel for all activities realised on the project's social media channels (see below). Furthermore, the website is implemented as a platform to provide the video clips produced by the project.

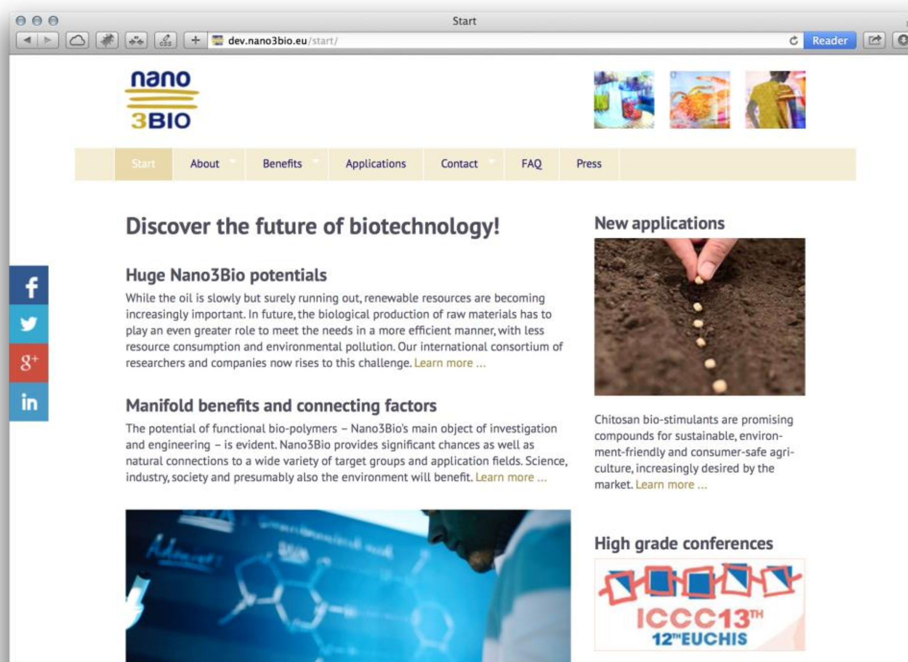


Image 3: Nano3Bio website

- Social Media

Nano3Bio is represented with a project specific profile on the popular Social Media platforms LinkedIn, Google+, Facebook, Twitter and YouTube as well as on the topic-oriented platform Nanobay. A Nano3Bio account on these platforms was created and provided with the project logo and other specific design elements and descriptions. These channels were used to deliver all kinds of updates about the project.

Additionally, due to the relevance of Wikipedia on the internet in the information search process nowadays, the project created a Wikipedia entry about itself.

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Additionally, due to the relevance of Wikipedia on the internet in the information search process nowadays, the project created a Wikipedia entry about itself.

Image 6: Selected Social Media platforms incorporated by Nano3Bio

-Smartphone Apps

Three **project specific smartphone apps** were developed and distributed by the project. Ideas and requirements regarding these apps were collected during project meetings.
The first app, the **conference/meeting app** is a comprehensive tool for managing and accompanying Nano3Bio related events. The app is directed to project partners and external visitors of Nano3Bio meetings or conferences and it is a beneficial tool for realising and accompanying the project's dissemination meetings. For that purpose, it provides all relevant information for participants of such events including up-to-date information about aspects like a complete agenda including scientific agenda points, meals, breaks, social events and background information. Furthermore, it contains a tool for organising one-to-one appointments of participants (match-making tool).

Image 7: Selected smartphone app views of the Nano3Bio conference app

The second app provides the functionalities and content for a **comprehensive information base on chitin and chitosans** including their contemporary production methods. The app allows reading and editing a specific wiki, '**The Chitosan Wiki App'**, powered by Nano3Bio. In general a wiki is a media type that provides collaborative modification of its content by a defined or open community by the aid of digital platforms. Any person or defined members can contribute new content or enter existing content any time. 'Wikipedia' probably is the most popular example for a successful public Wiki. Initially, the content of 'The Chitosan Wiki App' is managed, maintained and equipped by the Nano3Bio consortium. Hence, it is guaranteed that the Nano3Bio project contribution is visible to related target groups very well. The future opportunity for involving a broader community to edit the wiki, driven by the so-called 'collective intelligence', provides a great opportunity to **keep up the project's visibility even beyond the project duration**. Furthermore, active usage by potential follow-up projects is possible and intended, so that the wiki app can be understood and further upgraded towards a comprehensive European knowledge base related to chitin and chitosan.
The third smartphone app is the **Chitosan Tutorial 'ChitoTutorial'**. Its target group mainly consists of **young academics and particularly students** on an upper secondary school intensive course level. The Chitosan Tutorial App offers a visually appealing interface containing a manageable amount of text o provide an easily understandable introduction into scientific work as well as application areas of chitin and chitosans. Its handling is possible intuitively and playfully without any basic knowledge by an interactive selection of topics and levels. The architecture of information allows target group and situation related 'information lanes': a 'fast lane' for a very brief introduction and a 'detailed lane' providing significantly more detailed information displayed in additional steps (available by tapping on 'more details' in the footer line of numerous app views). In this manner the app explains chitosan science and applications step by step at which it provides the opportunity to choose between two degrees of difficulty. The three smartphone apps are easily accessible via the Nano3Bio project website.

nano 3BIO
Factsheet
Nano3Bio: Tapping the potential of chitosans
Traditional chitosan and new market requirements
What chitosans can do for you
New approach, advanced opportunities
International capacity aggregated, breakthrough aimed
www.nano3bio.eu

- Factsheet
The Nano3Bio factsheet provides a brief summary describing the project, its objectives, participating organizations, expected results and planned activities. It was made available for download and as a printed version as a supporting tool for presentations, at events and for individual meetings. In addition, it served as a communication tool providing **essential background information** within mailings to target groups.
The Nano3Bio factsheet was designed as a flexible internet-based and printable medium that could be adapted for many occasions and updated due to new project outputs. Its several updates contain a brief description of the Nano3Bio overall approach, new major achievements and specific aspects related to the respective occasion of usage. Not least the factsheet gives impressions as to the multiple application areas of chitosans; it underlines the generated benefits in line with the communication strategy (see above) as well as the European funding of the project.

- Scientific publications
Peer-reviewed scientific articles constitute an excellent dissemination activity to reach a **wider scientific audience** and make it aware of the project's activities and results. During the duration of the Nano3Bio project, its partners disseminated many findings by means of peer-reviewed articles. Furthermore, numerous scientific posters and lectures were presented at conferences and other scientific events. These publications involve external review of findings, which is crucial for safeguarding the overall scientific quality of the project. Moreover, project findings were published in professional and technical journals, which are an important source of information for stakeholders from science and industry.

- Classical press and media work
The **regional and local media are important for reaching the general public**. By means of press releases, interviews and events, the Nano3Bio partners informed these media about the project's work and findings. For this purpose, scientific news and Nano3Bio's key messages were transferred into a rather **easy and general vocabulary** in order to produce a clear idea of the project for a broad public. Whenever appropriate due to project results or public activities, classical press and media work was carried out in cooperation with the consortium and the leading partner in charge of the respective news or action. New and relevant Nano3Bio issues were regularly processed as classical press releases and published accordingly. All media releases are available on the respective 'Press' subpage of the project website.

- Networking with related EU projects and conferences
Nano3Bio used several occasions to create and to practise **networking with external projects, conferences and events**. This cooperation included communicating each other's internet and social media channels, participation in events and conferences, dissemination meetings, appearance on a trade fair as well as support in the field of press and media communication. Amongst others, the following organisations, projects and conferences were involved in these networking activities: CommNet, CommBeBiz, ProBio, VDI and Nanotech 2015, European Symposium on Biopolymers, ICCC and EUCHIS, NRW Nano Conference, EUROCARB Conference, Indian Chitin and Chitosan Society. Moreover, many scientific presentations of project partners on further conferences and meetings spread the word about Nano3Bio. Not least, the involvement of the project's Advisory Panel Members was in line with the networking approach focussing on science and potential application partners.

Image 9: NRW Nano Conference, 2016

Image 10: Exhibition, ICCC13th and 12th EUCHIS Conferences 2015

- Conferences and other events
Active participation in **conferences and workshops** is an important dissemination activity, offering excellent opportunities to present the project to different target groups and to get in touch with other players particularly from the fields of research, education as well as industry. Hence, the consortium made use of opportunities to participate in conferences in the role of a speaker, (poster) presenter, moderator or exhibitor. Not least, Nano3Bio held several international scientific dissemination meetings (see below). As a specific tool for creating awareness for Nano3Bio on conferences, the Nano3Bio meeting/conference app (see below) was created.

Image 11: 1st Nano3Bio dissemination conference, 2015

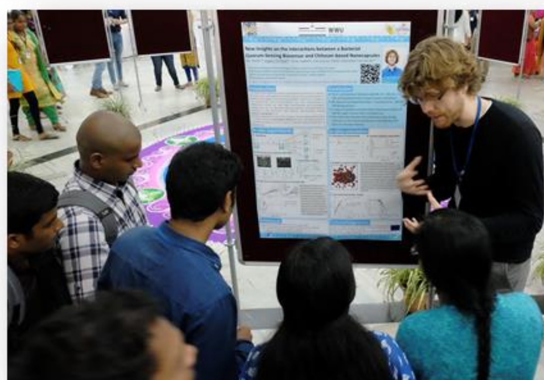
Image 12: 2nd Nano3Bio dissemination conference, 2017

-Short video clips

Video clips generally are an appropriate and promising communication instrument, which can be applied on multiple channels via the internet as well as within presentations and trade fairs. Hence, numerous **short video clips were produced for Nano3Bio**. These are particularly important for the project's web based activities, since they provide a brief introduction into Nano3Bio's approach and they briefly explain the project's achievements. The first video is a deliberately short trailer introducing Nano3Bio within 1:38 minutes only, referring to the two following and more detailed clips. The second and the third video clip are longer and concerted videos (7:16 and 5:50 min, respectively). They provide more detailed explanations including **interviews with project partners** and background information about the projects potentials, objectives and activities. Subsequently, in the second half of the project, several video clips about Nano3Bio's major achievements were created. Each of these clips contains a short interview based message describing a **specific project achievement**. The person explaining this achievement in each case represented the project partner in charge for this respective outcome. All video clips are available on the project website and they were published on Nano3Bio's Social Media platforms. The longer clips (second and third video, see above) were also implemented in a presentation loop used at Nano3Bio stands at conferences and other public events.

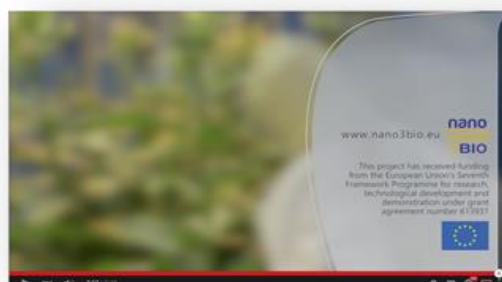

Image 13: Previews of selected Nano3Bio video clips

- Dissemination meeting and dissemination conference.

For a strongly science-related project like Nano3Bio, **scientific meetings and conferences** were an appropriate and highly relevant platform to spread the word about project activities, outcomes and economic potentials, allowing to reach a broad target community and to get in touch with relevant stakeholders. Hence, the first **Nano3Bio dissemination session** was held in the framework of the **12th International Conference of the European Chitin Society and 13th International Conference on Chitin and Chitosan (ICCC/EUCHIS 2015)** in Germany. This was an ideal platform for meeting and interacting with academic and industrial scientists as well as with producers and users of chitosans. In this respect, the conferences were an excellent networking occasion for bridging the gap between academic research and industrial product or process development. During the conferences, activities and background information from Nano3Bio and selected project achievements were presented. Furthermore, the project was advertised during an implemented young researcher symposium, in associated technical workshops, and at the corresponding exhibition. A Nano3Bio stand presented the project, its partners and activities to scientists from academia and industry and towards press and media. Furthermore, at the conference, the consortium performed the following activities: permanently showing an infinite loop presentation (sound provided via headphones) at the Nano3Bio stand in the exhibition area, providing the Nano3Bio meeting/conference app (see above) including immediate access to comprehensive information about Nano3Bio for all participants, being acknowledged as an official sponsor of the conferences including multiple appearance of the project logo on conference media, participating in the poster exhibition with scientific posters from several project partners, providing the Nano3Bio factsheet (see above) to all conference participants via the official conference bag, using the Nano3Bio appearance for social media postings, applying two Nano3Bio roll-up posters at the Nano3Bio stand and not least, presence of many Nano3Bio partners, allowing broad networking activities (specifically by dint of the match-making tool provided in the meeting/conference app).

A dedicated session entitled "NanoBioEngineering of Bioinspired Chitosans and Chitooligomers" (EU 7th EUFP Project Nr. 613931)", was entirely focussed on the results of Nano3Bio during the **European Chitin Society (EUCHIS) Conference and Iberoamerican Chitin Society (SIAQ) Symposium**, the globally most significant conferences in the field of chitin and chitosan, held in Sevilla, Spain on 31st May to 3rd June 2017. A Young Researchers Symposium was held in conjunction with the conference to foster the involvement of young scientists in the communication of the Nano3Bio project outcomes.

Another successful Nano3Bio dissemination session attended by participants from all over the world was organised at the 19th Carbohydrate Symposium (EUROCARB) held on July 2nd – 6th 2017 (**EUROCARB 2017)** in Barcelona. It included lectures by Nano3Bio project partners, e.g. explaining why Nano3Bio aims to develop biotechnological ways to produce well-defined chitosans as well as accordant project achievements and promising application potentials. The Nano3Bio session was well-visited and included lively discussions amongst scientists from around the world who were impressed by the project results and interested in future collaborations – an excellent result of this Nano3Bio dissemination meeting.

Not least, at the very end of the project, **the final dissemination meeting** took place in Hyderabad, India. The consortium held this conference under the title **'The Future of Chitosans'** and used it to present Nano3Bio's outcomes to a broad public, mainly focused on **young researchers**. Since in important fields, the consortium achieved or prepared a breakthrough from basic research to biotechnological applications, the conference generated high interest; more than 500 participants visited the event.

Image 14: Nano3Bio final dissemination conference, Hyderabad, India,

The lectures by the Nano3Bio project partners were designed in rather easy language in order to particularly involve young and future researchers, explaining the novel and complex project activities and outcomes in a catchy manner in order to generate maximum interest for this important and promising field of biotechnology. For example, the discovery of a protocol for production of chitosans with defined structures, the development of a low-cost protein engineering technology as well as the isolation and identification of the first chitosans generated by microalgae were introduced. Moreover, Nano3Bio speakers explained significant research results on the internalisation of chitosan nanocapsules into human cells, which is especially relevant for therapies of cancer with chemotherapeutics and could lead to more effective therapies with reduced adverse effects and better life quality for patients. Also, the identification of genes from bacteria, fungi and algae that can be used to drive the biotechnological production of encoded enzymes and the so-called electrospun chitosan nanofibers and electrosprayed chitosan nanoparticles as platforms for the encapsulation and efficient release of vaccines and drugs in human medicine as well as thermo-sensitive chitosan hydrogels as promising materials for regenerating damaged tissues were introduced. Not least, the Nano3Bio speakers underlined the emphasis on sustainability by presenting the first detailed life cycle assessment of chitosan production in order to evaluate and to compare newly invented approaches to traditional ones in terms of their environmental impact, concerning topics such as greenhouse-gas emissions, water use or land use. In order to achieve a maximum of visibility, the consortium flanked its final dissemination event by an accordant press and media release, involving the press offices of several project partners. Banners in the hall of the meeting venue and on stage underlined the clear visibility of Nano3Bio and its EU funding. The most important conference related media were provided on the project website during the event itself: the event booklet containing background information and abstracts was and still is available for download and many pictures and short news were provided in real-time during the conference. Not least, social media postings flanked the public relation activities before and during the event.

Finally, the consortium presented numerous scientific communications on Nano3Bio outcomes in the frame of the **6th Indian Chitin and Chitosan Society Symposium** organised in conjunction with the Nano3Bio dissemination day, on 21st – 22nd September 2017 in Hyderabad, India.
Overall the dissemination meetings were excellent occasions to stimulate discussions between scientists from academia and industry, especially in terms of possible applications as well as new and upcoming chitosan-based products and markets. Furthermore, all dissemination meetings significantly spread information about the Nano3Bio project amongst leading international experts from the fields of chitin and chitosans.

- Give-away articles
For all kinds of events and face-to-face meetings a Nano3Bio ball pen was created and printed with the project logo and its internet address as a nice giveaway helping to keep Nano3Bio in mind. Furthermore, for the final dissemination meeting (see above), notepads and conference bags were printed with the Nano3Bio corporate design and distributed to all participants.

By providing appropriate information for external target groups WP8 contributed to the practical application of the project achievements.

3.3. EXPLOITATION OF THE RESULTS

3.3.1 Work package 1 - Genes and Enzymes

Chitin and chitosan represent altogether some of the most abundant biopolymers in nature. The mechanisms underlying their formation and structural modifications are, however, not well understood. Gaining insight into these fundamental processes requires the characterization of the proteins and genes involved. WP1 has successfully addressed these fundamental scientific questions by characterizing available and newly discovered enzymes with different types of catalytic activities. With this knowledge, it has been possible in WP1 to develop several engineered enzymes that can be used for the production of chitin and chitosan molecules with defined structural and functional (including bioactivity) properties for the further design of tailored chitin-based commercial products. Examples of the types of products that can be derived from the knowledge gained in WP1 through *in vitro* (WP2) and *in vivo* (WP3) approaches are illustrated in section 4.2.5. 'Formulation and Applications'. The results of WP1 are expected to lead to additional longer term applications through the further evaluation of the natural producers of chitin and chitosan identified in WP1, such as some of the microalgal and diatom species, and their engineering through genetic approaches. Beneficiaries will be biotech companies and end-users from the biopharmaceutical, agricultural, materials and biomaterial composites. In addition, the library of enzymes that was generated has the potential to lead to a larger number of new commercial products in the mid and longer term.

A new type of combinatorial library based on GeneArt™ Strings™ DNA Fragments was developed and is already commercialized by Thermo Fisher Scientific GENEART GmbH. This development enables to deliver libraries much faster and at significantly reduced cost. A next generation sequencing technology that allows the characterization of tens to hundreds of thousands of specimens from a given library has also been developed and is being commercialized. These technologies have immediate beneficial impacts to academic and industry R&D sectors concerned by the engineering of new biocatalysts and their application to bioproduct development ('Industrial Biotechnology').

3.3.2. Work package 2 - *in vitro* Biosynthesis

Proof-of-concept with regard to the main objectives of the work package has been achieved.
Expected exploitation of the main results achieved in this work package includes:

a) Use of wild-type chitin deacetylases (alone or in combination) acting on chitin oligomers (for specific de-*N*-acetylation) or on chitosan oligomers (for *N*-acetylation) to produce libraries of pure chitosan oligomers with a defined pattern of acetylation. The methods and purification protocols developed at laboratory scale by WWU-1 (P1) are amenable to upscaling. The same enzymes can also be used for de-*N*-acetylation of chitosan polymers of high DA to produce chitosan polymers with low DA and non-random patterns of acetylation.
Exploitation: The foreground will be used in further research activities. Currently, the fully defined oligomers produced on small scale are being implemented in a number of bioassays in collaboration with partners, in order to identify the most promising exploitation strategy. This must not necessarily be the use of the fully defined, fully purified oligomers but might also be the use of the knowledge on the most active oligomer to tailor the production process of an oligomer mixture reliably containing a known amount of it and, thus, reliably delivering a desired activity.

b) Use of engineered chitin deacetylases and peptidoglycan deacetylases for efficient preparation of chitosan oligosaccharides with defined deacetylation patterns. Several mutant enzymes have shown good performance in terms of activity, specificity, and stability to be claimed as novel biocatalyst for preparation of pure and structurally defined chitosan oligosaccharides.
The technology has been developed at laboratory scale by IQS (P3). The target chitosan oligosaccharides were obtained at the mg scale. Upscaling and validation will be required for eventual commercialization of the enzymes and/or the products.
Exploitation: The foreground will be used in further research activities. The know-how will be used as a service to develop engineered enzymes for specific product requests. Commercialization of the service to be done through the Technology transfer office of the University. Eventually, IPR measures to transfer the technology to industry might be considered. At present, no specific actions to seek industrial partners for development or commercialization have been undertaken.

c) Use of transglycosylating chitinases for the preparation of large chitin oligomers and chitosan polymers. Detailed scientific knowledge has been gained in the engineering of this family of enzymes to modulate transglycosylation and hydrolysis activities. Product yields are still low for attempting upscaling of the processes, and the strategy requires further development.
Exploitation: The foreground will be used in further research activities. Small quantities of oligomers generated by transglycosylation of chitinases at lab scale were tested for plant strengthening activities. The knowledge generated can be used further, for better yields of the long chain chitooligosaccharides, using mutants that show improved transglycosylation activity.

d) A procedure to synthesize activated oxazoline compounds from chitin and chitosan oligosaccharides. The reaction conditions and purification protocols have been developed at laboratory scale by ENANTIA (P21) and are amenable to scale up.

Exploitation: The foreground has been transferred to IQS (P3) for the preparation of substrates for glycosynthases.

e) Use of glycosynthase mutants derived from chitinases in combination with activated chitin/chitosan oligomers for the preparation of large chitin oligomers and chitosan polymers. The proof-of-concept to produce low molecular weight chitin/chitosan polymers has been established by IQS (P3).
Exploitation: The foreground will be used in further research activities. The know-how will be used as a service to develop engineered enzymes for specific product requests. Commercialization of the service to be done through the Technology transfer office of the University. Eventually, IPR measures to transfer the technology to industry might be considered. At present, no specific actions to seek industrial partners for development or commercialization have been undertaken.

3.3.3. Work package 3 - *in vivo* Biosynthesis

The main results of WP3 are:

- the newly developed **metabolic engineering tools**
- **strains** optimized for chitosan oligomers and polymers production
- **methods** to produce well-defined and pure chitosan oligomers and polymers, and
- a **product portfolio** of well-defined and pure chitosan oligomers and polymers in terms of degree of polymerisation (DP), degree of acetylation (DA) and pattern of acetylation (PA).

Generic metabolic engineering tools

In our view, these novel generic metabolic engineering tools and technologies for the different assessed production hosts will be a major contribution with a view to the reliable engineering of biological systems. Hence, this will contribute to boost technological innovations, derisking the development of novel bioprocesses, and support the industrial biotechnology as key-enabling technology for the bio-based economy.

The developed metabolic engineering tools will be predominantly used by the Nano3Bio partners as well by the metabolic engineering field for the tools and technologies which are published.

Optimized strains and methods for chitosan oligomers and polymers production

The developed and optimized strains for chitosan oligomers and polymers production typically show a value multiplayer effect, since these are ideal starting points for the development of product-specific microbial cell factories (MCFs), e.g., by plugging in the desired product pathway. Obviously, more optimisation is required than this, nevertheless, using such chassis strains as starting point for extending the chitosan oligomers and polymers portfolio allows to significantly reduce the development cost and risks, which is of major importance for commercial success.

The microbial strains are a gateway to the biotechnological production of numerous well-defined and pure chitosan oligomers in terms of DP, DA and PA. These strains will be mainly used by WWU-1 (P1) and UGent-1 (P2) to expand the set of product-specific MCFs and improve process parameters. These product-specific MCFs can be used by industrial biotech companies or contract manufacturing organisations (CMOs) to produce the specific well-defined and pure chitosan oligomers. Similarly, the microalgae strains are a gateway to the biotechnological production of numerous well-defined and pure chitosan polymers in terms of DP, DA and PA. These strains will be mainly used by Greenaltech (P13) to further optimize the strain and extend the set of product-specific microalgal production hosts. Next step will be licensing the technology, establishing partnering deals with industrial biotechnology companies or CMOs, etc.

The developed fermentation processes and product recovery methods will be applied to produce sufficient amounts of chitosan oligomers and polymers for application development and as such to fully explore the potential of chitosan oligomers and polymers.

The preferred scenario for valorisation would be licensing the technology, establishing partnering deals with industrial biotechnology companies or CMOs, application partners, etc.

Product portfolio of well-defined and pure chitosan oligomers and polymers in terms of DP, DA and PA

The developed strains and methods will generate a broad portfolio of well-defined and pure chitosan oligomers and polymers in terms of DP, DA and PA to evaluate applications in human, animal and plant health. Furthermore, this broad portfolio will facilitate to unravel the structure/bioactivity relation of chitosan oligomers and polymers which will significantly boost specific application development and subsequently the full market penetration of these molecules.

The current product portfolio will be used by the Nano3Bio partners to further develop applications (WP5). In addition, partnering deals with applications partners in the various application fields such as animal nutrition, cosmetics, etc., are envisaged.

3.3.4. Work package 4 - Product Analysis

The main results of WP4 are on the one hand new analytical techniques for the structural and functional characterisation of chitosan oligomers and polymers, and on the other hand new insights into structure-function relationships of partially acetylated chitosans towards microbial, plant, and human cells. In both areas, the results obtained will be the basis of future research projects, to further improve the techniques and to use the results towards the optimisation of production processes, the implementation of quality control measures, and the development of knowledge-based applications.

Most of the analytical tools were developed by WWU-1 (P1), in particular regarding the structural characterisation of chitosans. This rather unique expertise is currently in growing demand from different academic groups and from different industries. As the expertise is not available with common commercial analytical laboratories - as evidenced by the growing number of enquiries particularly from industry - WWU-1 has recently set up a service unit under the name “ChitoProf - Professional Chitosans for the Life Sciences” which allows to offer these services under market conditions. This service is in growing demand, so that ChitoProf might be further developed into a spin-off biotech start-up. Similarly, the tools for functional analysis of chitosans have been improved by WWU-1 (P1) and HYDER (P8), particularly regarding plant strengthening activities, and by WWU-2 (P1) and UHEI (P7) regarding biomedically relevant bioactivities towards human cells. In case of WWU-1, a spin-off biotech start-up will be founded in early December 2017 as seed financing has already successfully been applied for by a former PhD student of the group. Similarly, for potential biomedical applications, a second spin-off biotech start-up is currently planned to be set up by UHEI (P7), in collaboration with WWU (P1).

The knowledge gained on structure-function relationships of chitosans can best be used on a short term basis for the optimisation of known potential applications of chitosans which so far have failed to achieve market introduction or penetration due to insufficient efficiency or reproducibility of the biological functionalities. The first target could be agricultural applications in which chitosan-based products are currently already winning market shares. The identification by WWU-1 (P1) of synergistic activities between chitosan oligomers and polymers in a chitosan preparation developed by GILLET (P10) based on previous successful joint research projects of the two partners will be further pursued to identify optimal mixing ratios, and these will eventually be used for the further optimisation of the product. Increased performance will support the product’s success on the markets which is already upcoming, particularly in countries such as India or Morocco where production is not at the enormously high, environment-damaging level of Western agriculture and where at the same time, a sufficiently educated customer base can be found who on the one hand understands the need for environment protection and is willing to implement measures to guarantee it, and who on the other hand has the knowledge and the resources to do so.

A second market that might be targeted would be cosmetics. COSPHATEC (P11) as a commercial supplier of cosmetics ingredients is in a perfect position to open up this market for chitosans. COSPHATEC has specifically tested possible drop-in scenarios for the Nano3Bio chitosans, as stabilisers and/or preservatives, as outlined in WP5. Importantly, the newly discovered green algal chitosan described in WP3 is of potential interest particularly for the high-price market sector of organic cosmetics as this is the first and only plant chitosan and as the results of this WP4 have shown that the performance of the natural green algal chitosan is as good as that of a conventional chitosan with similar structure in terms of DP and DA. Most interestingly, the structural parameters of this natural chitosan match those of the most efficiently antimicrobial conventional chitosans, again as evidenced by the results of this WP4.

Another highly interesting market potentially to be opened up for chitosans based on the knowledge i.a. provided by this WP4 on structure-function relationships of chitosans would be the animal feed market. First reports already suggest that chitosan oligosaccharides could have prebiotic activities and might also be used as substitutes for antibiotics still used (illegally) as a growth promotor, even in Europe. In fact, the antibiotics use in European animal husbandry, apparently still often given pre-emptively e.g. during early weaning of piglets to prevent disease caused by their still under-developed immune system, is enormous and most alarming. Functional cellular assays to screen paCOS for their performance as prebiotics still need to be developed, but the analytical tools provided by this WP4 and our experience in developing such assays represent a sound basis for much-needed future research and development in this area.

In the longer run, biomedical applications in e.g. wound healing, drug delivery, tissue engineering, or anti-tumour therapy can be envisaged for the biotechnologically produced chitosans. However, these will require more intensive investigations into the biomedically relevant bioactivities of the chitosans. The methods developed in this WP4 by UHEI (P7) and WWU-2 (P1) to follow cellular uptake of chitosans and their intracellular fate including degradation represent a crucial step towards medical applications of chitosans. So far, it is believed that human cells easily take up chitosans and, particularly, chitosan nano-particles via endocytosis, but that the chitosan-containing endosomes fail to develop into functional phagosomes which would eventually digest the chitosan. We believe that this is most likely due to the inability of human chitotriosidase to efficiently digest conventional chitosans with low DA. Further research will indicate whether any of our novel biotech chitosans can be a solution to this problem. Developing a chitosan that can be degraded by human chitotriosidase may well turn out to be a game changing event in the already decades-long pursuit of e.g. developing chitosan-based sustained release drug carriers.

Finally, as the third generation chitosans developed in the Nano3Bio project are completely novel compounds never identified, analysed, or used before, we should not exclude the possibility that eventually, even completely new applications for these chitosans may be found. Our future projects will have to carefully avoid a bias towards already known functionalities and already proposed applications.

3.3.5. Work package 5 - Formulation and Applications

Throughout the various tasks of WP5, it has been possible to generate novel formulations based on **"second generation"** chitosans, and to identify which chitosans are best suited for a given application. The dissemination of this knowledge will resonate at various levels. On the one hand, it will provide potential users in chitosan markets, such as healthcare (e.g. cosmetics) and biomedical devices, extremely valuable information about the type of chitosan that can suit best specific needs. On the other hand, it will guide the future of new sustainable sources of chitosan to provide specific chitosans for the fastest growing markets.

The major applications that the project has contributed to propel can be summarized in the following:

1. Cosmetic formulations. COSPHATEC (P11) has succeeded to show the feasibility of using biotech chitosan in hand cream and liquid soap gels, as an alternative multifunctional agent, with thickening and antibacterial activity. This will enable to replace triclosan already banned in hand liquid soaps, and to reduce the amount of parabens in these types of products, which have fallen in the perception of consumers seeking for more alternative options, particularly for the niche bio-market.
2. Biomineralized scaffolds for bone regeneration. The results achieved by UGHENT (P2) on chitosan hydrogels enzymatically biomineralized are likely to have great possibilities of further commercialization in the market of dental implants. The material and antimicrobial properties of chitosan in these applications will prove to be unique.
3. Nanofibers for wound dressing and for biomaterials coating. With the advent of the technology of electrospinning, currently scaled up to industrial level, chitosan-phospholipid nanofibers developed by DTU (P6) are bound to find application in the biomedical sector, particularly for wound healing devices, in which the antimicrobial and wound healing properties of chitosan combine to offer a unique biomaterial. Chitosan fibres with antibacterial activity are already available in the textile sector. In the sector of biomaterials, chitosan-phospholipid nanofibers can also find new markets, particularly when a drug needs to be slowly released locally from the surface of the biomaterial.
4. Nanocapsules for vascular tumor accumulation. In the field of therapy and diagnostics for cancer, nanocapsules coated with hemocompatible chitosan accumulate in the lumen of tumor vessels, as the consequence of targeting VWF fibres. This biotargeting capacity outperforms commercial liposome formulations while it also exploits a novel route to potentially target cytostatic drugs to cancer cells. This alternative approach to the enhanced permeability and retention (EPR) claimed to work for smaller nanoparticles, offers to be a promising novel nanomedicine platform. A new project is currently being pursued directed to explore the feasibility of this approach between UHEI and WWU groups. The estimated time to exploit this result is about two years.
5. Nanocapsules and nanoparticles for attenuation of bacterial virulence via quorum sensing inhibition. In the field of antibacterial products for use in biomedical and veterinary sectors, the accelerated emergence of antibiotic resistant pathogenic bacterial strains demands to seek for alternative therapies. Future projects are necessary to explore the potential of chitosan-based particles to exert anti-quorum sensing activity and target bacterial antibiotic strains and thus reduce the MICs of conventional and new generation antibiotics. The potential disruption of bacterial biofilms as a consequence of inhibition of quorum sensing is another key focus. The time horizon to see the commercial application of these results is bound to future projects that address these issues. In vivo validations of the efficacy of these novel approaches are also necessary.
6. Chitosan-based non-viral particles for gene delivery to be used in breast cancer therapy. WWU developed a formulation based on chitosan of specific Mw and DA effective to deliver a therapeutic gene (e.g. microRNA) intracellularly to breast cancer cells, at no expense of the cell viability. To bring these results closer to the bedside, in vivo pre-clinical studies are necessary. The road to the eventual exploitation of this result depends entirely on the future in vivo proof-of-concept and further clinical trials that could follow. These studies would depend on large capital investments and engagement of the pharmaceutical industry.
7. Chitosan-based non-viral particles for gene co-delivery to be used in cystic fibrosis therapy. In the sector of rare diseases, gene therapy is progressing in accelerated steps. However, most of the researched therapies are seeking the use of attenuated adenoviruses. Non-viral vectors that pose no toxic or immunogenic risks are particularly attractive, and chitosan is a unique player in this arena. The sector of rare diseases is among the most promising ones, where gene therapies may see the first applications in the medicine of the future. The advent of gene editing technologies like CRISPR-CAS9, holds great potential in this regards, and future "third generation" chitosans may be key players.

3.3.6. Work package 6 - Life Cycle Assessment

WP6 has achieved a list of results that will be publicly available. The outcome of the life cycle assessment (LCA) work has included the first peer-reviewed publication on the environmental impacts of chitosan production based on crustacean shells (shrimp and crab). Besides this peer-reviewed publication, available in the International Journal of Life Cycle Assessment, we have submitted several data sets on chitosan and COS production to the European Life Cycle Database. These data sets (one on chitosan produced from shrimp shells, one on chitosan produced from crab shells, and one on biotechnological production of COS) will be available to LCA practitioners to use in studies where chitosan or COS are involved. All the underlying life cycle data on the above-mentioned chitosan and COS productions, plus an additional production route based on squid pen, are also publicly available through Deliverable 6.4. All these resources make Nano3Bio the first research project to put detailed chitin, chitosan and COS life cycle data out in the public domain.

WP6 has also produced the first regulatory analysis on the introduction to the market of biotechnologically produced chitosans and COS. The results of this analysis, publicly available through Deliverable 6.3 and currently submitted to the journal New Biotechnology, will inform future product developers, allowing them to anticipate regulatory requirements during the life cycle of the products. The identified policy gaps can as well support decision-makers in improving legislation to facilitate the valorisation of products produced with biotechnology.

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