Biologie

“Metagenomics and bio-engineering of chitin and chitosan modifying enzymes for biotechnological applications”

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Dedicated to my family and best friends

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List of publications

Parts of my thesis have been or will be submitted for publication in scientific journals or as a patent, or have been presented at seminars, courses or meetings, as follows:

1 Manuscripts

1.1 Malathi Nampally, Bruno M. Moerschbacher, Stephan Kolkenbrock. A novel genetically engineered chitosan affinity GFP-fusion protein to specifically detect chitosan *in vitro* and *in situ*. (submitted to "Applied Environmental Microbiology")

1.2 Malathi Nampally, Sven Basa, Bruno M. Moerschbacher. Enzymatically generated, partially acetylated chitosan oligomers differentially acting as priming agents or as elicitors of an oxidative burst in suspension cultured plant cells. (to be submitted after IPR protection)

1.3 Malathi Nampally, Govinda Rajulu, T. S. Suryanarayanan, Dominique Gillet, Bruno M. Moerschbacher. More fungal than bacterial diversity of chitinolytic and chitosanolytic species and enzymes found in soil samples with a history of chitin and chitosan exposure. (submitted to "Fungal Diversity")

1.4 Malathi Nampally, Stephan Kolkenbrock, Daniel Auriol, Bruno M. Moerschbacher. Metagenomics to identify novel chitin and chitosan modifying enzymes from soil samples with a history of chitin and chitosan exposure. (unpublished results)

2 Patent

Malathi Nampally, Stephan Kolkenbrock, Bruno Moerschbacher. Chitosanase variants and methods of using the same (*European patent* No. 11 179 878.1 – 1223; application date September 2, 2011)

3 Oral and poster presentations

3.1 Institut für Biologie und Biotechnologie der Pflanzen (04.2008)

3.2 Institut für Biologie und Biotechnologie der Pflanzen (07.2009)

3.3 Malathi Nampally, Neeraja Chilukoti, Bruno M. Moerschbacher. Genetic engineering of chitosan hydrolysing enzymes. PolyModE meeting September 2009, Toulouse, France.

4 Paper originating from work done in preparation for my PhD project in the group of Prof. Dr. Appa Rao Podile, University of Hyderabad, India

Podile, A. R., Das, S. N., Sarma, P. V. S. R. N., Neeraja, C., & Malati, N. (2010). Members of Gammaproteobacteria and Bacilli represent the culturable diversity of chitinolytic bacteria in chitin-enriched soils. World Journal of Microbiology & Biotechnology, 26(10), 1875-1881

Summary

Chitin isolated from shrimp waste is one of the most abundant renewable resources worldwide, and its derivative chitosan is among the most interesting bio-active biopolymers. Shrimp waste is generated in tremendous amounts in industries where shrimp is processed, only the meat is taken out and all the rest is considered waste. As the shrimp waste is a rich source of chitin, some of it is used commercially for the chemical generation of chitin and chitosan. However, a detailed understanding of the molecular basis of chitosan's bio-activities is still lacking, and reliable ways of production and modification are missing. These problems have so far prevented the successful development of chitosan based applications that have commercial importance, especially in fields of the life sciences such as agriculture or bio-medicine. Chitin and chitosan modifying enzymes (CCME) such as chitinase, chitin de-acetylase, and chitosanase could be used as powerful tools for biotechnologically addressing these challenges The current work describes some biotechnological approaches to use such enzymes for the in situ analysis of chitin and chitosan in biological samples, for the in vitro generation of biologically active chitosan oligomers, and for the identification of novel CCME using a metagenomic approach.

Some pathogenic fungi are known to produce chitin-deacetylase when invading their host tissue to convert the chitin into chitosan to prevent cell wall degradation by host chitinases. Also, the non-self surveillance machinery of plants is geared towards recognising chitin, hence biotrophic pathogens may disguise by deacetylating the surface exposed chitin into chitosan. Chitin and chitosan specific histochemical stains are required to investigate whether this chitin deacetylation is a pathogenicity strategy of any given pathogen. However, no antisera and no lectins are available commercially to detect the chitosan produced in fungal cell walls during infection of host tissues Chitinases and chitin binding domains have been successfully used for histochemical staining of chitin in fungal cell walls, and we have now developed a chitosan affinity protein (CAP) engineered from a mutated chitosanase fused to green fluorescent protein (GFP) and two affinity tags for purification and detection. CAP was successfully used for the histochemical detection of chitosan in the cell walls of the wheat pathogenic fungus *Puccinia graminis tritici* and is now being tested towards human pathogenic fungi. CAP is currently being further developed into a tool for estimatig the degree of acetylation (DA) of different chitosans.

Chitosan is know to be able to induce disease resistance in plants, but a detailed understanding of the mechanism of action of chitosan recognised by the plant cells at the molecular level still needs extensive research. The degree of polymerisation (DP), the degree of acetylation (DA) and maybe the pattern of acetylation (PA) play important roles in biological activities. Hydrolysis of chitin and chitosan with CCME may produce chitin and chitosan oligomers with specific DP and DA, and non-random PA. Chitosans with different DA were hydrolysed with purified recombinant *Bacillus* chitinases and chitosanase to obtain broad mixtures of chito-oligomers (COS). COS were analysed using HPLC, HP-TLC and MALDI-TOF-MS. COS produced by chitosanase and chitinase hydrolysis turned out to be elicitors and priming agents, respectively, inducing defense responses in suspension cultured cells of *Medicago truncatula* and *Oryza sativa*. This result not only implies that the resistance-inducing activities of chitosans towards plants are mediated by oligomers rather than polymers, but it also reveals that enzymes can be used to specifically generate COS with distinct bio-activities.

Therefore, identification of novel enzymes is a promising way to produce defined oligomers which may have interesting applications. A metagenomics approach would be an interesting tool to identify novel CCME which have unusual functions and which may produce products which have interesting biological activities.Soil samples obtained from a chitosan producer in Gujarat with a long history of exposure to chitin and chitosan showed to be rich in genetic diversity of chitin and chitosan degrading and modifying bacteria and fungi when screened for CCME. Oligomers produced by chitinolytic and chitosanolytic fungi showed broad diversity, which may have potential biological activities. As much of the microbial diversity cannot be cultured, metagenomic DNA was directly isolated from soil and also from cultivable and non cultivable bacteria and used to generate a small insert and a large insert metagenomic DNA library in *E. coli* host. Two types of analysis can be used to obtain information from the metagenomic library, function driven approaches and sequence driven approaches. Strategies for screening CCME using function driven approaches were established successfully using known CCME from *Bacillus* as positive controls. Both large and small insert libraries were screened for chitinases using fluorogenic and colloidal chitin substrates; however, no positive clones were obtained yet. The screening will need to be continued for chitosanases and chitin deacetylases using the assays I have established. A sequence driven approach has now also been planned and would be the next step to try and identify novel CCME.

Introduction

Structure, Sources and Production of Chitin and Chitosan

Chitin, a β-(1→4)-linked homopolymer of N-acetyl-D-glucosamine, is an abundant structural polysaccharide. It is a constituent of the exoskeletons of zooplankton and invertebrate larvae, insects and crustaceans, it is present in fungal cell walls and in the extracellular material of some diatoms as well as in the endoskeleton of squid. Chitin can be partially or fully de-N-acetylated to yield chitosans. Chitosan is the only known natural polycationic polymer and has numerous unique and biotechnologically interesting properties. The term chitosan really refers to a family of oligo- and polymers differing in their degree of polymerisation (DP), their degree of acetylation (DA) and possibly also in their pattern of acetylation (PA).

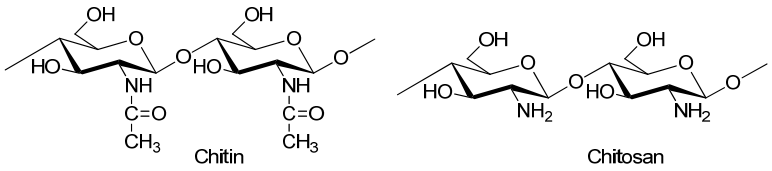

Fig.1: chemical structure of chitin and chitosan

Chitosans are highly basic polysaccharides whereas most other naturally occurring polysaccharides like chitin, cellulose, dextran, pectin, alginate, agar, agarose and carrageenans are neutral or acidic in nature (Pradip Kumar Dutta, 2004). Chitin is produced commercially mainly from shrimp shell wastes (Percot et al., 2003). Shrimp waste is generated in tremendous amounts in industries where shrimp is processed, only the meat is taken out and all the rest is considered waste and hence is dumped. This creates a lot of environmental problems that in more than one way affect the social well-being (Nicol, 1991). As the shrimp waste is a rich source of chitin, industries have been created to isolate this precious raw material from the shells. However, demineralisation and deproteination require large amounts of acid and soda and, thus, a lot of fresh water. The de-acetylation of chitin to generate chitosan requires even larger amounts of soda and, in addition, a lot of energy to heat the process. Alternative ways of chitin isolation and chitosan generation are thus required to help realise the potential of these biopolymers without undue environmental stress. Enzymes might offer opportunities for a more environmentally benign approach to chitin and chitosan production. Moreover, enzymatic methods might yield chitosans with different physico-chemical properties, such as blockwise instead of random PA, and thus, potentially other biological activities.

Bio-Activities and Life Science Applications of Chitosans

Chitosan has been used to protect crop plants from disease, chitosan has antimicrobial properties which can be used in tissue and paper coating, chitosan can bind heavy metals and proteins in drinking water purification and in waste water treatment, and chitosan can be used to produce wound dressings to promote scar-free wound healing or as a carrier for genes, drugs, and vaccines. Chitosans differ in their DA, DP and PA, this structural diversity has been implicated in the functional versatility of chitosans, but a detailed knowledge of structure/function relationships and, thus, a detailed understanding of the molecular basis of chitosan's bio-activities are still lacking (Kim and Rajapakse, 2005; EI Gueddari et al., 2007; Trombotto et al., 2008; Moerschbacher et al., 2011). This lack of information so far hindered the development of reliable life science applications of chitosans, such as in plant disease protection (Ortmann et al., 2004; Aziz et al., 2006; Dos Santos et al., 2008), or in scar-free wound healing (Rinaudo, 2006; Minagawa et al., 2007; Zhang et al., 2010) where promising results have been reported but typically with rather poor reproducibilities. This problem persists partly because reliable ways of production, modification, and analysis for partially acetylated chitosans are missing. A telltale example is the PA: today's chitosans are produced by partial chemical de-N-acetylation of chitin or by partial re-N-acetylation of fully deacetylated polyglucosamine, invariably leading to chitosans with random PA (Moerschbacher et al., 2011); and the only analytical tool available for PA characterisation is diad analysis using NMR which requires expensive infrastructure and rather large amounts of material, and which yields very limited information on the PA only (Chen et al., 2010).

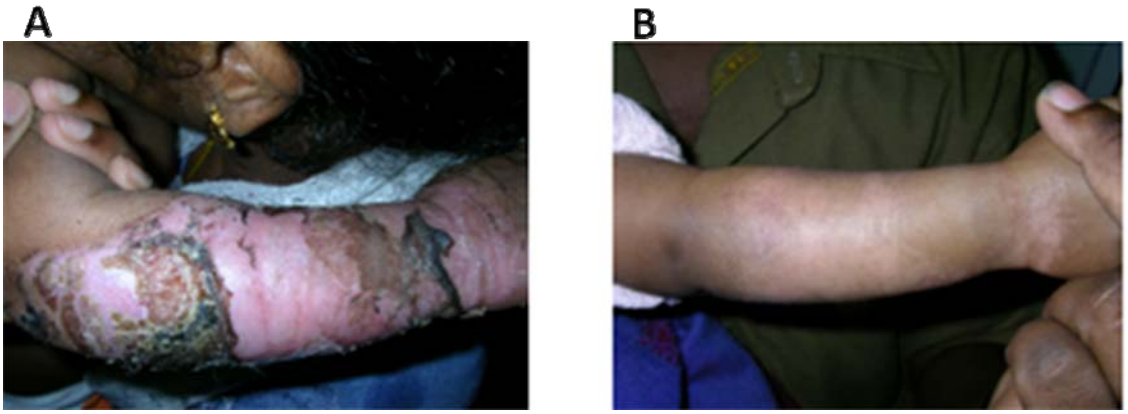

Fig.3: Chitosan as a wound dressing for scar-free wound healing. Burnt skin was applied with a chitosan film (A), wound healing after one month (B) (Courtesy of Dr. Gillet).

However, the biological activities of chitosan polymers and oligomers may depend on specific patterns of acetylation, e.g. if the chitosan polymer needs to be degraded into biologically active oligomers by chitinolytic enzymes present in the target tissue. Such enzymes are known to have more or less strict substrate specificities, e.g. requiring a number of consecutive, non-acetylated residues to bind and cleave (Moerschbacher et al, 2007). Also, biologically active chitosan oligomers might act by interacting with receptors e.g. on human endothelial cells, and these receptors also might have narrow ligand binding specificities. Enzymes may allow us to design more specific ways of generating chitosan polymers and oligomers, with defined physico-chemical properties and known biological activities. The work described here aimed at the identification of novel CCME to provide alternative or complementary tools for the generation and characterisation of chitosans, including novel chitosans with non-random PA and, thus, potentially more reproducible or even novel biological activities. Chitin and chitosans can also be partially or fully depolymerised to yield N-acetylglucosamine and glucosamine oligomers or monomers, respectively. Monomers are used successfully to treat arthritis (Tamai et al., 2002); the medical potential of the oligomers is highly promising but still requires extensive research. Again depolymerisation is done chemically in today's commercial processes, often yielding chitosans with large polydispersity index, but enzymes may be used to generate more defined mixtures of specific chitosan oligomers.

Chitin & Chitosan Modifying Enzymes and their Applications

A number of CCME have been described. These include hydrolytic enzymes such as chitinases and chitosanases which can degrade chitin and chitosan polymers into oligomers (Kim and Rajapakse, 2005; Dahiya et al., 2006). A different class of chitin & chitosan modifying enzymes are chitin de-N-acetylases which may convert chitin polymers and oligomers into chitosans (Zhao et al., 2010). By far the best studied CCME today are bacterial endo-acting chitinases (Bhattacharya et al., 2007). However, novel CCME are necessary for chitosan bio-engineering. A great deal of interest has been generated on chitinases because of their applications in the biocontrol of plant pathogenic fungi, mosquito control, production of chito-oligosaccharides, and mycolytic enzyme preparations (Dahiya et al., 2006). Many researchers have concentrated their attention on the utility of chitinase in biological control but a limited number of reports indicate that much more extensive research is needed to investigate the importance of chitinase in environmental and biomedical biotechnology (Felse and Panda, 2000).

Role of CCME in histochemical staining of pathogenic fungi

Another biotechnological use of CCME is their use as histochemical tools. Biotrophic pathogens need to prevent triggering of active defense reactions of their host tissues as they depend on living host cells for growth and development. As the non-self surveillance machinery of plants and animals including humans is geared towards recognising chitin as a principal component of fungal cell walls, some biotrophic fungal pathogens disguise by deacetylating the surface exposed chitin into chitosan (El Gueddari et al., 2002). This strategy helps them to escape firstly the attack by host chitinases, and secondly the host recognition mechanisms. It is, therefore, highly interesting and promising in terms of disease prevention to understand the host pathogen interactions.

Lectins may be used to detect the presence of chitin or chitosan on the hyphal wall, allowing a better understanding of the molecular mechanism of biotrophic fungal infection of plants, animals, and humans. Since lectins with specificity to chitosan are not commercially available (Lienart et al., 1991), alternative chitosan-specific probes are needed to test for chitosan on the surfaces of hyphae (Hadwiger and Line, 1981; Grenier et al., 1991). However, chitosan also is a notoriously poor antigen. To detect the chitosan produced in a fungal cell wall during infection of the host tissue, thus, no commercial antisera is available. Clearly, production of antibodies against chitosan is of great importance and has good commercial value. Many attempts of generating anti-chitosan antisera were unsuccessful or yielded rather poor antibodies with low affinity (Sorlier et al., 2003; Baker et al., 2007). Chitinase, chitosanase and chitin binding domains were used for histochemical staining of chitin and chitosan in plant and human pathogenic fungi (Benhamou and Asselin, 1989; Grenier et al., 1991; Manocha and Zhonghua, 1997; Hardt and Laine, 2004). Staining of chitosan using chitosan antisera was done in plant pathogenic fungi (El Gueddari et al., 2002; Tucker et al., 2010). The anionic dye eosin Y was used to stain the chitosan produced in the human pathogenic fungus *Cryptococcus neoformans* (Baker et al., 2007); however, this stain is rather non-specific, relying on electrostatic interactions only. We have now improved the enzyme-based approach for the histochemical detection of chitosan using a chitosan affinity protein (CAP) engineered from a mutated chitosanase fused to GFP, and with two affinity tags for purification and detection. *Bacillus spec* chitosanase belonging to the glycosyl hydrolase family 8 was used for the construction of CAP. Site directed mutagenesis (SDM) was done for the chitosanase to mutate a glutamic acid residue which is important for catalysis to glutamine(Ozaki et al., 1994; Alzari et al., 1996; Adachi et al., 2004). Mutated

chitosanase was fused to GFP and two affinity tags StrepII and HIS_6 which allowed the use of several detection methods. CAP was successfully used to stain the chitosan in substomatal vesicles and infection hyphae in the plant pathogenic fungus *Puccinia graminis tritici*. Hence, CAP can be used for histochemical staining of chitosan in pathogenic fungi. (refer chapter 1)

Chitosan and chitosan oligomers and their role in the defense response of plants

As mentioned above, CCME, may also be used to generate specific mixtures of chitosan oligomers, with the aim to obtain reliable biological activities. COS were produced chemically in the past, but enzymatic preparation methods captured a great interest due to concerns on safety and toxicity (Kim and Rajapakse, 2005). Hydrolysis of chitin by chitinase is the key step in the solubilization and mineralization (Cottrell et al., 1999) though bacteria employ several proteins, including chitin-binding proteins to degrade chitin (Montgomery and Kirchman, 1994; Suzuki et al., 1998). The first step in chitin degradation, which is primarily done by microbes, is the hydrolysis of the glycosidic bonds between N-acetyl-D-glucosamine residues by chitinases. This can be achieved in different modes of action, namely by endo- or exo-acting enzymes. While endo-enzymes cleave glycosidic linkages in the middle of polymer chains thus leading to successively smaller polymers and, eventually, oligomers, exo-acting enzymes degrade polymers from their reducing or non-reducing ends, generating dimers in the process. Chitinases and chitosanases were successfully used for enzymatic hydrolysis of partially acetylated chitosans to obtain chitosan oligomers (Bahrke et al., 2002; Lee et al., 2002). In the present study, chitinases from *Bacillus licheniformis*, and *Bacillus thuringiensis* and a chitosansase from *Bacillus spec* were used to hydrolyse chitosans with different degrees of acetylation (DA 10%, DA 35% and DA 56%) to obtain mixtures of chitosan oligomers (COS) which may have potential biological activities. Oligomers obtained by enzymatic hydrolysis were analysed by HP-SEC, HP-TLC and MALDI-TOF MS to check for the DP and DA. HP-SEC and HP-TLC analyses showed that the different chitosans were completely hydrolysed by *Bacillus* chitinases and chitosanase. These results also showed that the obtained COS differs in their DP and DA. All COS obtained had DP ranging from 2-20. When chitosans of DA 35% and DA 50% were hydrolysed by chitinases, MALDI-TOF MS analysis showed that COS obtained having DP ranging from 5-8 were major products. When chitosans of DA 10% and DA 35% were hydrolysed by chitosanase, smaller oligomers were obtained with chitosan DA 10% as chitosans with low DA are best degraded by chitosanases, and COS having DP ranging from 2-15 were major products (refer chapter 2).

COS obtained by enzymatic hydrolysis were used to test for biological activities in suspension cultured *Medicago truncatula* and *Oryza sativa* cells. Plants have efficient defense machinery against potentially pathogenic microorganisms which attack them constantly. They have developed different defense responses including production of reactive oxygen species termed as oxidative burst (Ree et al., 1994; Wojtaszek, 1997). The oxidative burst is one of the earliest and possibly one of the most crucial reactions possessed by plants to fight against biotic and abiotic stresses (Wojtaszek, 1997). Other defense mechanisms include cell wall reinforcement, phytoalexin production, and deployment of antimicrobial proteins (Kombrink et al., 1995; Mahalingam and Fedoroff, 2003). Resistance is also often associated with programmed cell death of cells at the site of infection, known as hypersensitive response (Jones and Dangl, 1996). Often, the resistance is triggered by signal molecules which are either produced by the host or by the pathogen when these invade the plant tissue, and such signal molecules are termed as elicitors. Besides being genetically programmed, resistance can also be induced which gives protection to the plant in future against pathogens. The process of induced resistance can be classified as systemic acquired resistance (SAR) or induced systemic resistance (ISR) based on their signalling pathways and their effectiveness. One mechanism of both processes appears to be sensitisation of the defense response which occurs not only in plants but also in animals, and which is known as priming. Priming agents are signal molecules which act as kind of alarms to prepare and protect host cells from pathogens (Jung et al., 2009; Tsai et al., 2011).

Several reports have shown that chitosan elicits a variety of defense reactions in higher plants; the biological activity of chitosan polymers and oligomers depends on their molecular weight and degree of acetylation (Gueddari and Moerschbacher, 2004). It has been shown that chitosan oligomers with high molecular weight were more active than those of lower molecular weight in inducing defense responses (Vander et al., 1998; Aziz et al., 2006). The interaction of chitosans with membrane proteins at the molecular level is still not so clear, but early responses of the Mitogen-Activated Protein kinase pathway suggest this to be the signalling pathway for both chitin (Wan et al., 2004) and chitosan (Vasconsuelo et al., 2003) elicitors. A receptor for chitin oligomers has been identified (Okada et al., 2002; Petutschnig et al., 2010), whereas the mechanism of chitosan oligomers recognition by plant cells is still unknown. It has been suggested that chitosan which is a polycationic polymer interacts with the negatively charged plasma membrane phospholipids, thus activating a common MAP kinase-dependent defense response (Kauss et al., 1989; Shibuya and Minami, 2001). Chitosan

oligmers which were obtained by enzymatic hydrolysis were checked for elicitor and priming activities in suspension cultured *Medicago truncatula* and *Oryza sativa* cells in the present study. The induction of an oxidative burst, which is a measure of elicitation of defense response, was assessed by estimating the amount of H_2O_2 released by luminol-dependent chemiluminescence method. Interestingly it was observed that the COS produced by chitosanase induced a faster oxidative burst in suspension-cultured *Medicago* cells compared to rice cells. COS from chitosans (DA 35% and DA 10%) generated by chitosanase turned out to be elicitors and showed differences in their elicitor activity in Medicago and rice cells, whereas COS produced by chitinases from chitosans (DA 50% and DA 35%) showed priming activity in suspension-cultured Medicago cells (refer chapter 2).

It was proposed that differences in the activity of elicitors between cell cultures from different plant species and between cells grown in culture versus cells in plant tissues in general may be due to the fact that the extracellular matrix of cells grown in culture medium may be different from that found in tissue of whole plants in terms of their porosity, polysaccharide composition, and enzymatic activities (Cabrera et al., 2006). The recognition of polycationic chitosan and chitin oligomers by the receptors in plant cells may, thus, vary and also depend on the availability of host hydrolytic enzymes (Cabrera et al., 2006). It has been shown that the carrot, wheat, barley, rice and tobacco BY-2N cells responded differently to chitin oligomers, although their plasma membranes all contained a high-affinity binding site for N-acetyl-chitooligosaccharides (Okada et al., 2002). This might be one reason to explain the dissimilar activities of mixture of COS towards the Medicago and rice cells. Specific chitin and chitosanolytic enzymes would be necessary to obtain the desired oligomers. On the other hand, recent findings appear to suggest that the signal molecules perceived and the signal pathways triggered in monocot and dicot cells may differ substantially (Ortmann et al., 2006; Paulert et al., 2010), and this may be another reason for the differences observed. To answer this question, more detailed investigations of enzymatically generated, well characterised chitosan oligomers will be required. To identify new or novel enzymes required for this, it is necessary to explore the biodiversity of chitino- and chitosanolytic microorganisms.

Biodiversity of chitinolytic and/or chitosanolytic microorganisms in soil

To investigate the diversity of CCME, it becomes essential to trace out the biodiversity of the tremendous population of chitin and chitosan modifying microorganisms. Chitinases could be used as a powerful tool to utilize the shrimp shell waste in order to generate products that have commercial importance. Little, however, is known about the chitinolytic bacteria and fungi that can degrade the crystalline chitin present in native shrimp shells (Manucharova et al., 2008). One promising source for such chitinolytic bacteria and fungi might be soil (Brzezinska et al., 2010; Das et al., 2010), as the chitin present in insect cuticles is thought to be degraded by soil dwelling bacteria and fungi. However, it has been estimated that only a very small fraction of microorganisms present in any environmental sample can be analysed using culture-based methods.It has been estimated that a single gram of soil contain several thousands of bacteria (Torsvik et al., 1994) and 1.5 million of fungal species exist worldwide (Hawksworth, 1991), but the vast majority of both are unknown to science. It has been estimated that 99% of bacteria present in soil are not culturable (Amann et al., 1995; Hugenholtz and Pace, 1996), and that only 5% of the fungal species have been identified so far (Naveen kumar et al., 2011). Novel detection methods not depending on culturing the bacteria and fungi have identified microbes differing from any known bacteria and fungi so that they may represent a whole new classification. Therefore, a screening of the bacterial and fungal population from soil could be promising, using shrimp waste as the sole carbon source. The identification of such able bacteria and fungi may give us some new and effective strains that produce new or novel CCME which can be used in chitin and chitosan biotechnology.

Different types of soil have been investigated in the past for their chitinolytic potential (De Boer et al., 1999; Manucharova et al., 2011). We used unique soil samples in this study which were derived from a chitin and chitosan producing company in Northern India. Mahtani Chitosan has been operating close to Veraval in Gujarat for ten years, and it is today one of the most important chitin and chitosan producers of India. The soil at different places in this company has been in contact with fresh and dry shrimp shells as well as with chitin and chitosan in different stages of processing. In an exploratory study, the University of Hyderabad, the chitosan producing company from Gujarat and the University of Münster have collaborated to obtain a first idea on the diversity of chitin and chitosan modifying bacteria in some soil samples from the company. For isolation of chitinolytic bacteria, we initially tried culture-based screenings as e.g. described by (Maltseva and Oriel, 1997).

Chitinolytic bacteria are typically detected by either the production of clearing zones on agar containing chitin or by hydrolysis of glycol chitin and different chitosans. Using such methods, we demonstrated that the prevalence and diversity of chitinolytic bacteria are much larger in soil samples with a history of chitin or chitosan exposure compared to control soil samples (Das et al., 2010).

In search of novel CCME, we have now isolated chitinolytic and chitosanolytic bacteria and fungi from these soil samples. Fungal diversity clearly exceeded bacterial diversity both in terms of species and in terms of chitinolytic and chitosanolytic enzymes making these fungi a promising source of novel CCME for chitosan bio-engineering. Fungi are reported to contribute more than bacteria to environmental degradation of chitin (Brzezinska, 2007). When the spent culture media of selected fungal species were used as crude enzyme extracts for incubation with different chitosans as substrates, broadly diverse mixtures of oligosaccharides were obtained (refer chapter 3). These can now be analysed for their biological activities, e.g. concerning eliciting or priming activities in plants.

The oligomers identified in these experiments will be the products of the joint activities of different CCME present in the spent culture media, including various types of chitinases, chitosanases, and chitin de-N-acetylases. However, culture based techniques cannot tap the genes from non-culturable microbes and therefore a new methodology termed metagenomics has been developed to isolate genes encoding various enzymes directly from soil samples, bypassing isolation of soil microbes into pure culture. For this reason, we opted for a metagenomic approach. Eventually, this metagenomic approach will hopefully allow identification of novel enzymes which have unusual functions and which may produce products with novel and interesting biological activities.

Metagenomics

The term metagenomics was introduced by Jo Handelsman, Jon Clardy, and Robert M. Goodman (Handelsman et al., 1998). Metagenomics is the study of the sum of all genomes isolated from any environmental samples independent of culturing the microorganisms which may lead to identify new or novel enzymes. One such promising environmental sample to identify novel enzymes is soil (Cowan et al., 2005; Daniel, 2005; Schmeisser et al., 2007) Soil microbes act as scavengers by participating in mineralization of organic matter, thereby maintain the ecological balance of soil. Hence, soil microbes must be producing a broad variety of enzymes used for degrading polysaccharides, proteins and lipids. Chitin and

chitosan modifying enzymes are one of the important classes of enzymes which can be isolated from soil microbes. The genetic diversity is assessed by isolation of DNA followed by direct cloning of functional genes from the environmental samples. The extracted metagenomic DNA has to be modified and inserted into a model organism. The model organism then expresses this DNA where it can be studied using standard laboratory techniques (Henne et al., 1999). Metagenomics, thus, is a multistep process that relies on the efficiency of four main steps.

1. Isolation of the genetic material
2. Manipulation of the genetic material
3. Library construction
4. Analysis of the genetic material in the metagenomic library

The importance of metagenomics is to effectively characterize the genetic diversity present in samples under laboratory culturing techniques. Metagenomics enrich the knowledge and applications of many aspects of industry, therapeutics, and environmental sustainability. Metagenomics is a new and exciting field of molecular biology that is likely to grow into a standard technique for understanding biological diversity.

Both sequence and function dependent methods can be used for screening the chitin and chitosan modifying enzymes existing in the metagenomic DNA library (Daniel, 2004; Voget et al., 2005). A number of soil samples were screened and two samples best suited to yielding a high biodiversity of chitin / chitosan degrading micro-organisms were selected. Protocols for the isolation of high quality metagenomic DNA were then optimised in collaboration with an industrial partner, LibraGen from Toulouse, France. Two types of libraries have been constructed; a large insert (ca 42 kbp) fosmid library which was generated by LibraGen, and a small insert (ca 2.5 kbp) plasmid library by us. Both libraries were screened using functional screening assays established and optimised by us. The large insert library was constructed only by using the DNA isolated from cultivable and uncultivable bacterial DNA isolated from soil. On the other hand, the small insert library was constructed by using total metagenomic DNA isolated from soil to exploit also the genetic diversity of fungal CCME.

In a metagenomic screening process, the target genes typically represent only a small portion of the total nucleic acid fraction. Gene mining in complex metagenomic libraries, thus, represents the most crucial step of the whole project. Two types of analysis can be used to

obtain information from metagenomic libraries, namely a sequence driven approach and a function driven approach. In sequence driven approaches, the targeting of a specific gene can be achieved by PCR using primers derived from known genes for chitin and chitosan modifying enzymes. Amplicons can then be labeled as probes to identify the putative full-length gene(s) in the metagenomic library. In principle, sequence driven methods can be used to complement the below described function driven approach. Large scale sequencing of a metagenomic library poses serious financial constraints and therefore was not undertaken for the time being but with the advent of novel sequencing technologies, they are now planned for the future. The ability to clone large fragments of metagenomic DNA allows entire functional operons to be targeted with the possibility of recovering entire metabolic pathways. Consequently, it will be interesting to completely sequence the inserts of clones expressing chitin or chitosan modifying enzymes. Additional, yet unknown genes related to chitin and chitosan modification may be identified in this way.

Functional approaches have the advantage of being less costly and less dependent on prior knowledge of sequence information, so that chances of identifying truly novel genes are much higher (Schloss and Handelsman, 2003). On the other hand, the functional approach requires the availability of enzyme activity assays that are suitable for large scale screening. Therefore, in this project, screening methods for different chitin and chitosan modifying enzymes were developed and the libraries were screened for expression traits using function driven approach. Such an approach has previously been described to identify novel genes and antibiotics (Handelsman et al., 2002; Banik and Brady, 2010), lipases (Henne et al., 2000), and the ecology of chitin degrading bacteria and chitin degradation in aquatic systems (Cottrell et al., 1999).

Clones expressing chitin or chitosan modifying enzymes can be identified using simple stratagies that were established and that can be run in large quantities. Chitinase producing clones can easily be detected by the generation of clearing zones in chitin containing agars. The same principle can be used to detect chitosanase containing clones. Both metagenomic libraries constructed were screened for chitinases using colloidal chitin as a substrate and also using fluorogenic substrate analogs of chitin [4-methylumbelliferyl-D-*N*,*N*-diacetylchitobioside (MUFdiNAG) and 4-methylumbelliferyl-D-*N*,*N*,*N*-triacetylchitotrioside (MUF-triNAG)]. In addition, the plasmid library clones were also screened using a KongoRed-based assay using colloidal chitin as a substrate. So far, no positive clones were obtained from either of the libraries. Dot blot assays were established to screen the libraries

for clones containing chitinase, chitosanase and chitin-deacetylase, using clones expressing known *Bacillus* genes as positive controls. However, it is unclear at present whether these screening strategies will be sensitive enough when libraries are screened using function driven approach. The identification of clones expressing chitin de-acetylases most likely will require development of a new strategy that makes use of the Chitosan Affinity Protein (CAP) described above (refer chapter 4).

Outlook

Biodiversity of chitinolytic and/or chitosanolytic bacteria and fungi was high in soil samples from Mahtani Chitosan Company. Spent culture media of chitinolytic and/or chitosanolytic bacterial and fungal species can be used as crude enzyme extracts for incubation with different chitosans as substrates, and this gave broadly diverse mixtures of oligosaccharides. These can be analysed for their biological activities like antimicrobial activities and eliciting or priming activities in plants. To exploit the genetic diversity of CCME, both small insert and large insert metagenomic libraries were successfully constructed using metagenomic DNA isolated from soil samples. Both large insert and small insert libraries were screened only for chitinases. However, no positive clones were obtained so far. Due to the time limit different screening strategies which were established could not be performed to identify CCME's from metagenomic libraries. Due to financial constraints sequence driven approach was not tried in the present study. However, several methods which were discussed in (refer chapter 4) could be tried to identify new or novel CCME from the metagenomic libraries.

Finally, the recombinant CCME needs to be characterised e.g. in terms of their substrate specificities, their cleavage mechanism, their processivity, or their product patterns, in order to identify novel enzymes. We are currently developing a versatile and powerful computer program which we call "chitinator", mimicking the enzymatic degradation of partially acetylated chitosan polymers by sequence specific chitosan hydrolases. This program was initially designed for the fingerprinting analysis of PA: if a chitosan with known DP and DA but unknown PA is hydrolysed using a chitosan hydrolase with a known substrate and cleavage specificity, and the products generated are quantitatively analysed for their DP and DA, the chitinator can easily determine whether the chitosan used as a substrate had a random or a non-random PA. And with the availability of more well characterised, sequence specific chitosan hydrolases, the chitinator will in future also be able to suggest possible distributions of the acetyl groups in a chitosan with non-random PA. Recently, the chitinator has been

expanded to include a reverse fingerprinting analysis: if a chitosan of known and defined DP, DA, and random PA is degraded by a novel chitosan hydrolase and the products generated are analysed by mass spectrometry, the chitinator can employ evolutionary algorithms to generate a virtual chitosan hydrolase with substrate and cleavage specificities yielding just these products from this substrate. In future, a deacetylase module is to be added to the program which will also allow the characterisation of possible processivity in an unknown chitin deacetylase. In this reverse fingerprinting mode, the chitinator can thus be used to analyse new and potentially novel CCME, e.g. as identified in the metagenomic sequencing approach described.

The final step will be the characterisation of the products of enzymatic conversion, both in terms of their physico-chemical properties and of their biological activities. The products will e.g. be tested for their antimicrobial activities and for stimulatory or inhibitory activities towards plant and human cells.

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CHAPTER 1

"This chapter has been submitted for publication with the following co-authors: Stephan Kolkenbrock (who was involved in designing the strategy of the project and in discussing the results), Bruno M. Moerschbacher (who supervised my work and who was involved in designing the strategy of the project and in discussing the results)."

A novel genetically engineered chitosan affinity GFP-fusion protein to specifically detect chitosan *in vitro* and *in situ*

Abstract

Chitin is the second most abundant organic molecule, persent e.g. in insect and arthropod exoskeletons and fungal cell walls. In some species or under specific conditions, chitin appears to be enzymatically de-N-acetylated to chitosan, e.g. when pathogenic fungi invade their host tissues. Here, the deacetylation of chitin is assumed to represent a pathogenicity mechanism protecting the fungus from the host's chitin-driven immune response. While chitin specific lectins are well known, no comparable tools are available to histologically detect the presence of chitosan. Also, chitosan is poorly antigenic so that attempts at generating high affinity antibodies directed against chitosan have failed repeatedly. We have, therefore, generated a fusion protein between a chitosanase inactivated by site directed mutagenesis, the green fluorescent protein, and strepII as well as his_6 tags for purification and detection. The recombinant chitosan affinity protein (CAP) expressed in *E. coli* was shown to specifically bind to chitosan, but not to chitin, and the affinity increased with decreasing degree of acetylation. In vitro, CAP detection was possible either based on GFP fluorescence or using streptavidin or anti-his_5 antibodies. CAP fluorescence microscopy revealed binding to the chitosan exposing endophytic, but not the chitin exposing ectophytic infection structures of the wheat stem rust fungus, verifying its suitability for *in situ* chitosan staining.

Introduction

Biotrophic pathogens need to prevent triggering of active defense reactions of their host tissues as they depend on living host cells for growth and development. Typically, fungal cell walls contain chitin as a fibrillar element, but many fungi are known to produce chitin deacetylases during specific developmental stages (15). Some plant pathogenic fungi are

known to produce chitin deacetylase to convert the chitin on the surface of their infection structures into chitosan to evade the chitin-driven immune response of their hosts (12). On the one hand, chitinases and β-1,3-glucanases act as antifungal enzymes in most plants (14). By converting chitin into chitosan when invading the plant tissue, the fungus avoids the degradation of its cell walls by host enzymes. On the other hand, the non-self surveillance machinery of plants is geared towards recognising chitin (40). Conversion of chitin into chitosan prevents the generation of elicitor active chitin oligomers which would reveal the presence of the pathogen to the plant, triggering active defence responses. Hence, the deacetylating of surface-exposed chitin into chitosan acts as a molecular disguise strategy. and, consequently, chitin deacetylases may constitute crucial pathogenicity factors. Pathogenicity factors are perfect targets for broad-spectrum antifungal agents as they can be expected to be important for different types of pathogens, but may not be present in non-pathogenic endophytic or mutualistic fungi.

A similar disguise strategy likely acts as a pathogenicity mechanism in fungal pathogens of human tissues, too. Chitin deacetylase was shown to be present at the plasmamembrane of the early stages of the life cycle in the human pathogenic fungus *Encephalitozoon cuniculi* (9). *Cryptococcus neoformans*, the causal agent of cryptococcosis in humans, was shown to possesses four putative chitin deacetylase genes, and two of its chitin deacetylases have been reported to induce protective immune responses in the host, indicating that they are expressed during host tissue colonisation (3, 7, 24). Deletion mutants of the chitin deacetylases in this opportunistic fungal pathogen showed that chitosan is important during vegetative growth, it appears to help in maintaining cell integrity, and to aid in bud separation (3). Paracoccidioidomycoses, a disease affecting ten million people in Latin America, is caused by the fungus *Paracoccidioides brasiliensis,* and transcriptional analysis showed that a chitin deacetylase gene is upregulated in the pathogenic yeast form of the fungus (13). Similarly, the cyst wall of *Entamoeba invadens,* a reptile-pathogenic relative of the human pathogen *Entamoeba histolytica,* was shown to contain chitosan, consistent with the presence of a functional chitin deacetylase (11).

Thus, conversion of the more typical structural polysaccharide chitin present in the endo- or exoskeletons and in the cell walls or extracellular matrices of so many organisms, into the structurally less resilient but also less telltaling chitosan emerges as a widespread pathogenicity strategy not only of fungal pathogens. However, the evidence so far relies mostly on molecular genetic detection of the expression of a chitin deacetylase gene, while

the presence of the product of the enzyme chitin deacetylase, namely chitosan, has only been achieved in very few instances (8, 12, 22). This is due to the fact that neither lectins (10, 25) nor antibodies with specificity to chitosan (3, 20) are commercially available. In fact, chitosan is a rather poor antigen, and attempts to generate antisera with reasonable specificity and affinity have failed more often than not. Hence, alternative chitosan-specific probes are needed to test for chitosan on the surfaces of e.g. pathogenic fungal hyphae (18). One possibility for the specific detection of polysaccharides are polysaccharide hydrolases or other polysaccharide modifying enzymes with an affinity to the targeted molecule, or specific polysaccharide binding proteins or modules (16, 19, 28, 32, 34), an approach already successfully employed for the detection of chitosan (17). Such proteins can either be detected using specific antibodies directed against them (12, 37), or by tagging the proteins chemically, e.g. using fluorescence tags such as FITC or using gold particles (4-6, 17, 26), or genetically, by generating fusion proteins, e.g. with the fluorescent protein GFP (19) or with a peptide tag against which commercial antibodies are available. We here describe an extension of this latter method, generating by genetic engineering a chitosan affinity protein (CAP) based on a bacterial chitosanase which we inactivated using site directed mutagenesis, and to which we fused three different tags, namely GFP and the affinity tags strepII and his_6, for purification and detection. This is a versatile, generic technique that can easily be transfered to other substrates, and which allows multiple staining of different substrates if GFP variants are used which absorb and emit light at different wavelengths.

Materials and Methods

Chemicals

Wheat Germ Agglutinin lectin coupled to Texas Red was purchased from Molecular Probes (Eugene, Oregon, USA). Restriction enzymes, Fast rapid T4 DNA ligase, and Phusion DNA polymerase were purchased from Fermentas (St. Leon-Rot, Germany).

Bacterial strains, plasmid and culture conditions

Escherichia coli DH5α was used as storage host for recombinant plasmids. *E. coli* Rosetta 2 (DE3) [pLysSRARE2] was used for recombinant protein expression (purchased from Merck, Darmstadt, Germany). The pET-22b(+) vector was purchased from Merck, Germany. Plasmids were prepared for sequencing and sequenced at MWG eurofins (Ebersberg, Germany). Sequences were analysed by clone manager. *E. coli* DH5α and *E. coli* Rosetta 2

(DE3) [pLysSRARE2] with pET-22b(+) constructs were grown in LB at 37^{o}C with 100 µg/ml ampicillin and 100 µg/ml ampicillin plus 34 µg/ml chloramphenicol, respectively, for the selection of transformants. Auto induction medium (AIM) (33) was added to induce the cells for expression of the target protein, cultures were grown at 28^{o}C. For long term storage, liquid cultures were supplemented with 30% (v/v) glycerol and stored at -70 ^{o}C.

Cloning of the Bacillus chitosanase (CSN)

Details of primers used in the present study are given in supplementary data. From soil, a *Bacillus* sp. strain was isolated which served as template for CSN gene amplification using CSN_pET_for and CSN_pET_rev. The CSN gene was cloned into pET-22b(+) via *Eco*RI and *Hind*III without the native signal peptide encoding sequence generating pET-22b-CSN. Using this plasmid as a template, the CSN gene was amplified using primer pairs CSN_StrepII_for and CSN_StrepII_rev. In doing so, an amplificate containing an upstream located StrepII coding sequence was generated and subsequently cloned in pET-22b via *Nde*I and *Hind*III sites, resulting in pET-22b-StrepII-CSN.

In the following step, the gene encoding eGFP was amplified from pEGFP vector (Clontech, Saint-Germain-en-Laye, France, Catalog #6077-1) using the eGFP_for and eGFP_rev primers. The amplified eGFP gene was cloned in pET-22b(+)-StrepII-CSN via *Hind*III and *Xho*I restriction sites, generating the expression plasmid pET-22b-StrepII-CSN-eGFP-His_6 (Fig. 1).

***Site-directed mutagenesis of* CSN**

In order to obtain a mutein of CSN which binds to chitosan with high affinity and specificity but is devoid of hydrolytic activity, a bioinformatical approach was chosen to identify an amino acid which is essential for CSN catalytic activity. At position 122 of the polypeptide chain, an amino acid was identified which represents a catalytically active glutamate (E) of the CSN that was then replaced by the structurally related but chemically distinct glutamine (Q) (1). Therefore, the vector pET-22b-StrepII-CSN-eGFP-His_6 was subjected to site directed mutagenesis using the 5'-phosphorylated primers CSN_E122Q_for and CSN_E122Q_rev to obtain pET-22b-StrepII-CSN-eGFP-His_6 E122Q (termed as CAP).

Expression and purification of CAP

Both CAP and its active form were synthesized in *E. coli* Rosetta 2 (DE3) [pLysSRARE2]. Cell suspensions in 20 mM triethanolamine (pH 8) containing 400 mM NaCl, 1 mM phenylmethylsulfonylfluoride, 1 mM $MgCl_2$ and 10 U/ml benzonase were incubated for 30 min at room temperature, then sonicated briefly. Crude extracts (50 ml from 15 g net biomass) were obtained by centrifugation and applied to a streptactin column (Strep-Tactin superflow Plus, 1 ml bed volume, Qiagen, Hilden, Germany), which was then washed with 20 mM triethanolamine (pH 8) containing 400 mM NaCl, and finally eluted in the same buffer additionally containing 2.5 mM D-desthiobiotin. The eluted fractions were concentrated in ultrafiltration devices (Sartorius stedim biotech, Göttingen, Germany) with a molecular weight cut off at 10 kDa, supplemented with 10% (v/v) glycerol, and stored at 4 ^{o}C.

Preparation of chitosans

Chitosan (average degree of acetylation (DA) 3%, average degree of polymerization (DP) 2,124) was generously provided by Dr. Gillet, Mahtani Chitosan, Veraval, India. This raw chitosan was dissolved in an aqueous acetic acid solution and purified by successive filtration and extensive washing steps involving repeated precipitation and centrifugation, before chitosans with different DA were prepared by partial re-N-acetylation using acetic anhydride in 1,2-propanediol, as described previously (38). The DA of the resulting chitosans was determined using ^{1}H NMR spectroscopy (21).

Analysing CAP enzyme activity

In a dot activity assay, the hydrolytic activity of CAP towards chitosan was assessed. The crude extracts of *E. coli* Rosetta 2 (DE3) [pLysSRARE2] bearing pET-22b(+), pET-22b-StrepII-CSN-eGFP-HIS_6 or pET-22b-StrepII-CSN-eGFP-HIS_6 E122Q were applied on a polyacrylamide gel containing chitosan DA 35% (0.1 mg/ml) as a substrate. The gel was incubated at 37°C overnight. Later, the gel was stained with 0.01% (w/v) calcoflour white (Sigma, Steinheim, Germany) in 0.5 M Tris (pH 8.9) for 5 min; the gel was washed in deionized water for 1 h and dark spots indicating enzyme activity were visualized on a UV transilluminator (29).

Binding specificity of CAP

Fusion to GFP and two affinity tags allow the usage for several detection strategies, namely by chemiluminescence, under UV light, by transluminator (also known as Dark reader), and fluorescence microscopy. Excitation wavelength for GFP is 488 nm and emission wavelength is 510 nm. In order to analyze the affinity of CAP, different chitosans with DA ranging from 10% to 56% as well as glycol-chitin were spotted in dilution series (1000 ng to 2 ng) onto a nitrocellulose membrane (GE Healthcare, Munich, Germany). Membranes were then incubated at 70^{o}C to allow the different chitosans and glycol-chitin to stick firmly to the membrane. The membrane was blocked using 5% (w/v) BSA in 1X TBS buffer for 1 h at room temperature; later, the membrane was washed with 1X TBS buffer for 15 min. After washing, the membrane was incubated with CAP (0.1 mg/ml in TBS containing 5% (w/v) BSA) for 1 h at room temperature. Washing steps were continued with 1X TBS-TT buffer twice for 15 min each, then the membrane was incubated with streptactin-HRP (IBA, Göttingen, Germany). In an independent experiment, the same procedure was followed as explained above, but anti-His_5 antibody (Qiagen, Hilden, Germany) was used for detection. In both cases, the signal was detected by chemiluminescence. Later, membranes were used to observe fluorescence on a transiluminator (Dark Reader, MoBio, Goettingen, Germany) or under a UV lamp to check for fluorescence. Although the excitation wavelength of a UV transiluminator is not optimal to excite eGFP, the fluorescence was also observed when using such a device instead of the Dark Reader.

In situ *staining of chitosan using CAP*

In a further experiment, CAP was employed for specific staining of chitosan in the cell walls of a plant pathogenic fungus, in analogy to the chitosan specific antibody used in El Gueddari et al. (2002). Urediniospores of the wheat stem rust fungus *Puccinia graminis* (Pers.) f.sp. *tritici* (Eriks. & E. Henn) (race 32) were used for the experiment. Differentiation of infection structures was induced by a mild shock (2 h, 30°C) starting 2 h after sowing urediniospores into polystyrene Petri dishes (Ø 5 cm) and adding distilled water (5 ml), as previously described (12). Then, incubation was continued at 23°C over night for the development of infection structures.

After allowing the spores to produce different infection structures, fungal germlings were incubated with 2% (w/v) BSA in 1X PBS buffer for 2 h at room temperature. Incubation was followed by three washing steps with 1X PBS/Tween 20 of 15 min each. Spores were further

incubated with the chitin specific lectin Wheat Germ Agglutinin (WGA) coupled to Texas Red (Life Technologies GmbH, Darmstadt, Germany) and CAP (both at 0.1 mg/ml in TBS containing 5% (w/v) BSA) for 1 h at room temperature. After incubation, washing was done with 1X PBS/Tween 20 three times for 15 min each. GFP and Texas Red was monitored with a confocal laser scanning microscope (Leica TCS SP5 X, Wetzlar, Germany) with excitation / emission wavelengths of 488 / 595 nm and 500-545 / 608-700 nm, respectively.

Results

Cloning, site-directed mutagenesis, heterologous expression, and purification of CAP

An enzymatically inactive construct of a bacterial chitosanase was engineered (Fig. 1) using site directed mutagenesis as described above. The wild type chitosanase gene was amplified from genomic DNA of a *Bacillus* sp. strain isolated from soil, using primers derived from conserved regions of *Bacillus* chitosanases. The chitosanase gene was amplified with an upstream StrepII coding sequence. An eGFP encoding sequence was cloned downstream from the chitosanase gene before the His_6 encoding sequence in the expression vector. This construct was termed wildtype chitosanase fusion protein. After achieving the whole construct, site directed mutagenesis was done by exchanging the catalytically active glutamic acid residue into glutamine at position 122 (1). This second construct was termed chitosan affinity protein (CAP).

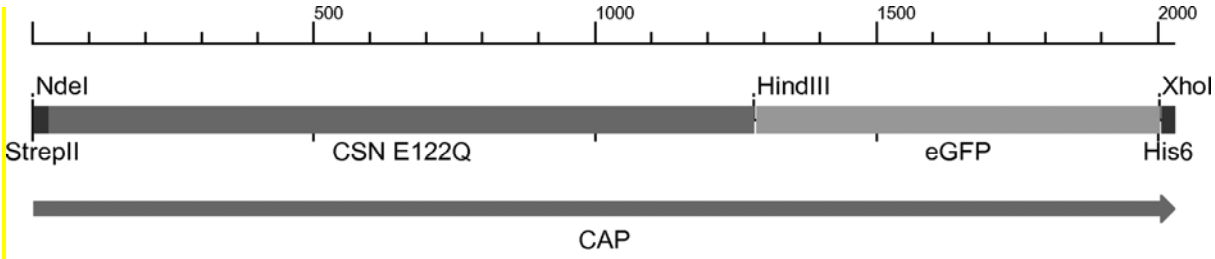

Fig.1 Schematic represantation of CAP gene. The scale above indicates the nucleotide position.

Both the wild type chitosanase fusion protein and CAP were synthesized in *E. coli* Rosetta2 [pLysSRARE2]. Cultures were incubated at 37°C for 48 hours. Recombinant proteins were purified by affinity chromatography. SDS-PAGE analysis (Fig. 2A) showed both proteins at the expected size of 75 kDa. This was confirmed by Western blot analysis (Fig. 2B). A minor band was observed at lower size in both SDS-PAGE and Western blot potentially indicating partial degradation of the fusion protein.

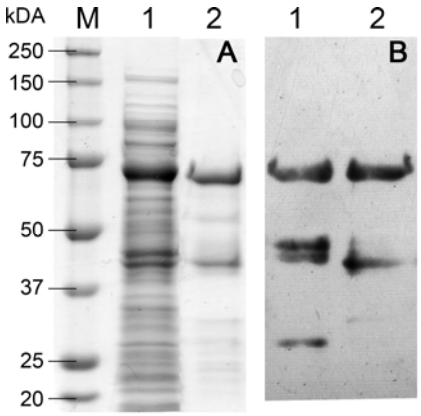

Fig. 2: SDS-PAGE of the crude extract from *E. coli* Rosetta2 [pLysSRARE2, pET-22b-StrepII-CSN-eGFP-HIS_6 E122Q] (60 µg, lane 1) and of CAP purified using affinity chromatography (6 µg, lane 2), either stained using Coomassie Brilliant Blue G-250 (A) or Western blotted and detected by chemiluminescence using StrepII affinity protein (B). The band at ca. 75 kDa represents CAP.

Both the wildtype chitosanase fusion protein and CAP were checked for enzymatic activity in a dot activity assay using a polyacrylamide gel containing chitosan with a DA of 35% as a substrate. Activity, revealed as a dark spot in the Calcofluor White stained gel due to the degradation of the chitosan embedded in the gel, was seen with the wild type chitosanase fusion protein, but not with the mutated chitosanase fusion protein, CAP (Fig. 3).

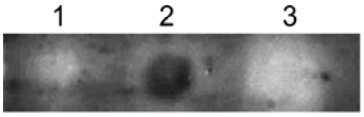

Fig. 3: Dot activity assay to check the enzymatic activity of wildtype chitosanase and CAP.
1. Crude extract from *E. coli* Rosetta 2 [pLysSRARE2, pET-22b(+)],
2. Purified wildtype chitosanase,
3. Purified CAP. Chitosanase activity is revealed as a dark spot in the Calcofluor White stained gel.

Binding specificity of CAP

To assess the binding specificity of CAP, different chitosans with a DA ranging from 10% to 56% as well as glycol-chitin (DA 100%) were spotted in dilution series (1000 ng to 2 ng) onto a nitrocellulose membrane. CAP binding to the chitosans can be detected using GFP fluorescence or by any of the tags attached to it (Fig. 4). GFP fluorescence was visualised using a transiluminator or under a UV lamp, StrepII and His_6 tags e.g. using appropriate antibodies coupled to horseradish peroxidase and a chemiluminescence assay. All three detection methods clearly showed that the affinity of CAP decreases with increasing DA, and that CAP has no affinity to fully acetylated glycol-chitin.

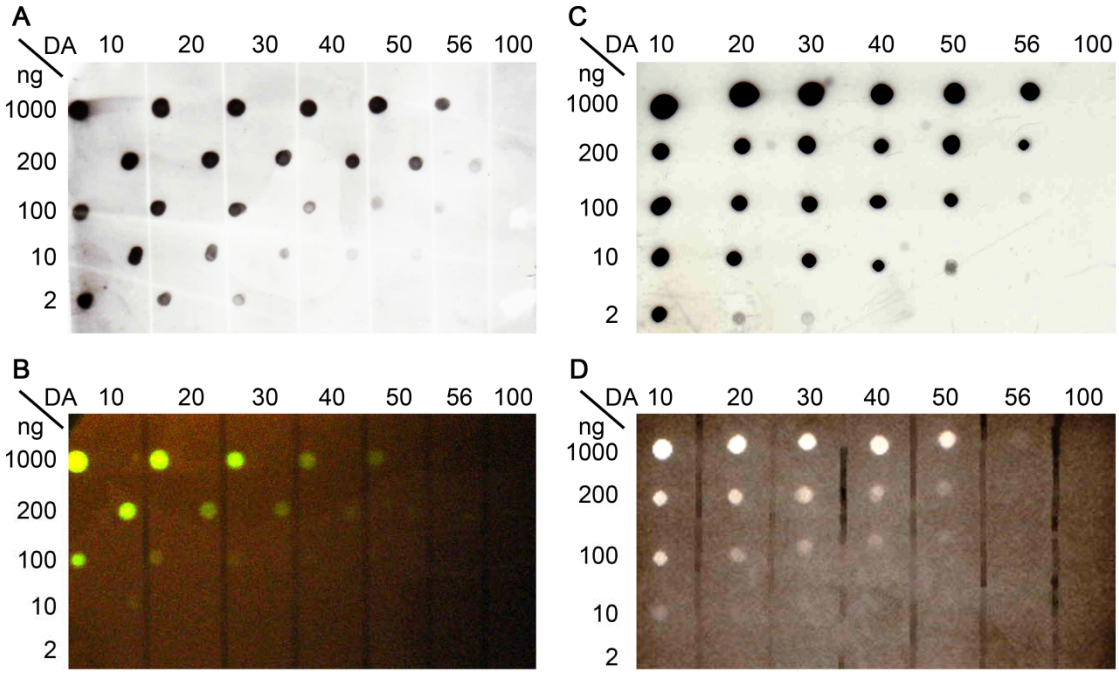

Fig. 4: Binding specificity of CAP. Different chitosans (DA 10% - DA 56%) and glycol-chitin (DA 100%) were spotted in dilution series (starting with 1000 ng) onto nitrocellulose membrane, the membrane was blocked with BSA, washed, and incubated with CAP (0.1 mg ml^{-1} in TBS containing 5% (w/v) BSA) for 1 h at room temperature before excess CAP was washed off.
Bound CAP was detected using HRP-coupled StrepII affinity protein (A) or anti-His_5 antibody (C) or by the fluorescence of GFP on a transiluminator (Dark Reader, MoBitech, Germany; excitation wavelength of 420-500 nm) (B) or under a UV lamp (AlphaImager, Alpha Innotech Corp., USA set to 365nm) (D).

In situ *staining of chitosan using CAP*

Uredospores of the wheat stem rust fungus were germinated in a petri dish, and differentiation of infection structures was induced using a mild heat shock. It has been shown previously that the ectophytic infection structures, namely germ tube and appressorium, expose mainly chitin, while the endophytic infection structures, namely substomatal vesicle and infection hyphae, expose mainly chitosan on the surface of their cell walls; growing tips of the infection hyphae are known to expose chitin rather than chitosan (12). Germlings were double-stained using the chitin-specific lectin WGA coupled to Texas Red (4, 35) and the chitosan-specific CAP, and fluorescence was observed by confocal laser scanning fluorescence microscopy (Fig. 5). eGFP and Texas Red fluorescence was monitored using excitation / emission wavelengths of 488 / 595 nm and 500-545 / 608-700 nm, respectively. WGA bound to the germ tubes and appressoria as well as to the tip of the infection hyphae (red fluorescence), while CAP strongly stained the substomatal vesicle and weakly the infection hyphae (green fluorescence).

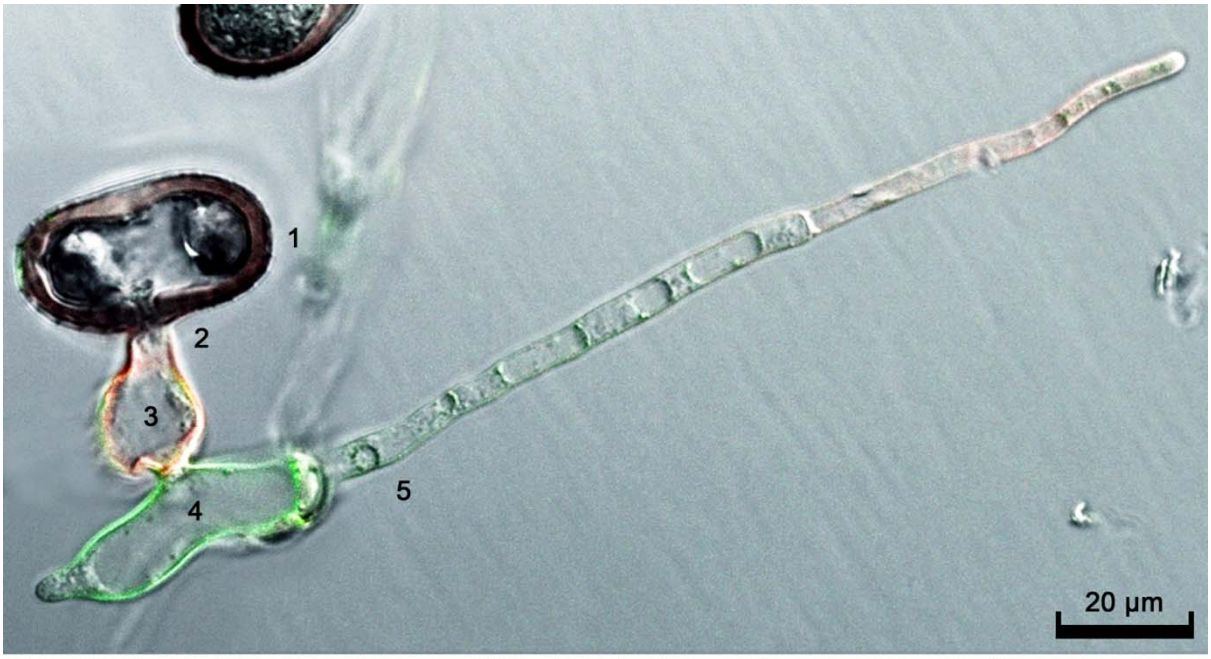

Fig. 5: Double staining of *in vitro* induced infection structures of the wheat stem rust fungus, *Puccinia graminis* f. sp. *tritici*. 1, spore; 2, germ tube; 3, appressorium; 4, substomatal vesicle; 5, infection hypha. Germ tube, appressorium, and the tip of the infection hypha were labelled by WGA conjugated to Texas Red (red fluorescence) indicating the presence of chitin, while CAP staining revealed the presence of chitosan in substomatal vesicle and, less marked, infection hypha (green fluorescence).

Discussion

Conversion of surface-exposed cell wall chitin into chitosan during penetration and colonisation of the host tissue appears to be a wide-spread pathogenicity mechanism in biotrophic fungi, both for plant and human pathogens (3, 12). This conversion was often deduced from a concomitant induction of a chitin deacetylase rather than by direct histochemical proof of the presence of chitosan due to lack of a commercially available specific stain for chitosan (15). Polyclonal chitosan-specific antisera have been produced repeatedly and recently, the generation of a monoclonal antibody specifically directed against chitosan has been described (12, 18, 20, 23, 30, 31, 39), but they have rarely been used in histochemical staining due to their typically low affinity towards the antigen (12, 37). Lectins with specificity to chitosan have been described (10, 25) but are not commercially available and our attempts at reproducing the findings were not successful. The anionic dye eosin Y was used to stain chitosan in the human pathogen *Cryptococcus neoformans* (3) and in the maize pathogen *Ustilago maydis* (36). However, this anionic dye, which is typically used as a counterstain to hematoxylin, binds to cationic chitosan presumably through electrostatic

interactions only, limiting its specificity. Clearly, alternative chitosan-specific probes are needed to test for chitosan on the surfaces of pathogenic hyphae.

Even though lectins with high affinity and good specificity for chitin such as WGA are easily available, a number of alternative chitin-specific probes have been described. A wild-type chitinase was successfully used to stain chitin in pathogenic fungi and fungal infected body fluids (6). A chitin binding domain fused to GFP has been used for staining chitin in *Saccharomyces cerevisiae* and in human tissue with various fungal infections (19). In spite of one report of using a gold-complexed chitosanase for the cytochemical detection of chitosan (17), this approach does not seem to have been followed up. In the present work, we have extended this enzyme-based strategy by engineering a chitosan-specific probe from a SDM-inactivated chitosanase gene fused to GFP and two affinity tags for purification and detection. The specificity of the chitosanase chosen for the degradation of highly deacetylated chitosans (unpublished) confers the same binding specificity to the mutein, and exchanging a catalytically active residue prevents hydrolysis of the bound substrate. Active side residues have been investigated in detail in three bacterial hydrolases belonging to CAZY glycoside hydrolase family 8, namely in a chitosanase from *Bacillus sp.17*, in endoglucanase CelA from *Clostridium thermocellum,* and in endoglucanase K from *Bacillus sp. KSM-330* (1, 2, 27). In all three einzymes, a glutamic acid residue acts as a proton donor and an aspartic acid residue acts as a nucleophile in catalysis. These amino acids are conserved in all GH-8 family members. Previously, both the glutamic acid and the aspartic acid residues in the *Bacillus sp.17* chitosanase have been exchanged to glutamine and asparagine, respectively, to show that both are important in catalysis, and that the aspartic acid residue is involved in substrate binding, too (1). In the present work, we therefore exchanged only the glutamic acid residue to glutamine, leaving the aspartic acid residue unchanged. This abolished the hydrolytic activity completely but retained the substrate affinity.

The SDM-inactivated chitosanase was fused to GFP as well as to two affinity tags, allowing easy one-step purification via affinity chromatography, and providing alternative ways of detection. GFP can be visualised directly due to its fluorescence, and all three tags can be detected using commercially available antibodies coupled to a large variety of markers. As an example, we have used the very sensitive detection method based on horse radish peroxidase-coupled antibodies and chemoluminescence of its enzymatic product. This method is clearly more sensitive than fluorescence detection of GFP, but the latter is the easiest way of *in situ* detection of chitosan, e.g. in the cell walls of pathogenic fungi, using fluorescence microscopy.

It is interesting to note that CAP gave a binding pattern which slightly differed from the one seen previously using a polyclonal anti-chitosan antiserum (12) (Gueddari et al., 2002). In both cases, chitin was seen to be exposed in the epiphytic infection structures of the wheat stem rust fungus, namely germ tubes and appressoria, and chitosan was detected on the surface of endophytic infection structures, namely substomatal vesicles and infection hyphae. However, while both chitosan-specific probes stained the substomatal vesicle strongly, the antibody bound more strongly to the infection hyphae than the antiserum. We assume that this differential staining pattern is due to subtle differences in the specificities of the two probes. While we have shown in this work that the affinity of the chitosanase based CAP is highest for chitosans with very low degrees of acetylation, the antiserum appears to bind best to chitosans with intermediate degrees of acetylation (unpublished). We may, thus, conclude that the chitosan in the substomatal vesicle is more strongly deacetylated than the one exposed on the surface of the infection hyphae. To date, no methods exist for the *in situ* analysis of the degree of acetylation of chitosans in a cell wall, and the degree of acetylation of chitosans present in fungal cell walls has not been determined accurately. The differential binding of the two chitosan-specific probes may guide the way to the development of a histochemical assay for the determination of DA.

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40. **Wan, J., X. C. Zhang, and G. Stacey.** 2008. Chitin signaling and plant disease resistance. Plant Signal Behav. **3:**831-833.

Supplementary data

List of primers used for cloning

CSN_pET_for: 5'TCAGAATTCGGCTGCAAAGGAAATGAAACCATTTC3'
CSN_pET_rev: 5'GTCAAGCTTATTATCGTATCCTTCATAGATTG3'

CSN_StrepII_for:
5'CCCCATATGTGGTCACATCCTCAATTTGAAAAAGATATCGGAATTAA
TTCGGATCC3'
CSN_ StrepII_rev: 5'CCCAAGCTTTTAATTATCGTATCCTTCATAGATTGC3'

eGFP_for: 5'CCCAAGCTTGTGAGCAAGGGCGAGGAG3'
eGFP_rev: 5'CCGCTCGAGCTTGTACAGCTCGTCCATGC 3'

CSN_E122Q_for: 5'TGAAGTTCCAAGTGGCTTAAACCC3'
CSN_E122Q_rev: 5'CAAGGTCAAGGGTATGGGATGATAATTACAG3'
wherein both the primers were phosphorylated at the 5'-end

CHAPTER 2

"This chapter will be submitted for publication with the following co-authors: Sven Basa (who was involved as a MSc student in performing the experiments under my direct guidance), Bruno M. Moerschbacher (who supervised my work and who was involved in designing the strategy of the project and in discussing the results)."

Enzymatically generated, partially acetylated chitosan oligomers differentially acting as priming agents or as elicitors of an oxidative burst in suspension cultured plant cells

Abstract

Chitin is a polysaccharide of *β*-1-4-linked *N*-acetyl-D-glucosamine (GlcNAc) units which is highly abundant in nature. As a sustainable resource, it mainly occurs in fungal cell walls and exoskeletons of insects and crustaceans. Chitosan, the fully or partially deacetylated counterpart of chitin, possesses remarkable biological properties such as anti-microbial and plant disease resistance inducing activity. Thus, chitosan can be suitable for different applications in agriculture, cosmetics, water treatment, and medicine, but its mode of action is not known. Thus, it is unclear at present to which extent e.g. the plant protective bio-activities are carried by chitosan polymers or oligomers produced in the target tissue by chitosanolytic enzymes present. Therefore, chitosan oligomers were generated by enzymatic treatment of chitosan polymers having different degrees of acetylation (DA 50%, 35%, 10%) using recombinant, purified chitosanase or chitinases from *Bacillus* expressed in *E. coli.* The oligomer mixtures produced were analysed using mass spectrometry, and were then used for treating suspension cultured rice and *Medicago* cells. As expected, MALDI-TOF mass spectrometry revealed differences in DP and DA of chitosanase and chitinase products. In particular, the potentially biologically active oligomers in the DP range 5-10 were characterised by higher DAs in the chitosanase products, and by lower DAs in the chitinase products. Elicitor activity was quantified by measuring the induction of a chitosan oligomer triggered oxidative burst, assessed by estimating the amount of H_2O_2 released by luminol-dependent chemiluminescence. Priming activity was assessed by pre-treating cells with putative priming active chitosan oligomers prior to stimulating the cells with a low dose of a known elicitor and quantifying the induced oxidative burst. Interestingly, the products of

chitinase and chitosanase digestion exhibited differential biological activities: while chitosanase products were elicitor active, chitinase products showed priming activity. The results suggest that chitosan oligomers with higher DA possess eliciting activity, probably because they resemble known elicitor active chitin oligomers, while chitosan oligomers with lower DA have priming activity. The potential significance of these results for the development of reliable chitosan based plant protectants is discussed.

Introduction

Plants have evolved effective defense mechanisms against potentially pathogenic microorganisms which attack them constantly. These include different defense responses such as the rapid and transient production of reactive oxygen species (ROS) in what is called an oxidative burst (Wojtaszek, 1997). The oxidative burst is one of the earliest reactions exhibited by plant cells and tissues fighting against biotic and abiotic stress (Wojtaszek, 1997), and it appears to be centrally involved in programmed cell death at the site of infection known as hypersensitive response (Jones and Dangl, 1996). Other defense mechanisms typically integrated with the hypersensitive response include cell wall reinforcement, phytoalexin biosynthesis, and the production of antimicrobial proteins (Kombrink and Somssich, 1995; Mahalingam and Fedoroff, 2003). These resistance responses are triggered by the recognition of signal molecules called elicitors which are either derived from the pathogen or from the host itself (Kombrink and Somssich, 1995). In recent years, it has become apparent that resistance responses such as the hypersensitive reaction are typically triggered by the concomitant or sequential recognition of several different elicitors (Moerschbacher and Reisener, 1997) together forming what is now called a pathogen associated molecular pattern (PAMP) (Nurnberger and Brunner, 2002; Nurnberger and Kemmerling, 2009).

In addition to acute resistance responses directed against an attacking pathogen, induced resistance can also be triggered giving protection to the plant in future against pathogens. At least two different types of induced resistance can be distinguished based on differences in their signalling pathways and their spectra of effectiveness, namely systemic acquired resistance (SAR) and induced systemic resistance (ISR) (Durrant and Dong, 2004; Van Wees et al., 2008; Jung et al., 2009). On the cellular level, induced resistance appears to involve priming, i.e. a sensitisation of host cells allowing them to react more quickly and/or more strongly to the recognition of an elicitor active signal molecule compared to naive, non-

primed cells (Conrath et al., 2002; Ortmann et al., 2004). Such a sensitisation of stress responsiveness during induced resistance occurs not only in plants but also in animals, indicating that this may be an evolutionary old strategy to fight against pathogens (Tsai et al., 2011). Priming-active and elicitor-active signal molecules may be considered as synergistically acting elements of a PAMP.

Priming-active compounds such as salicylic acid can be used as lead structures for the development of plant protectants, with the advantage of not inducing a full fledged, energy consuming and potentially destructive resistance response such as the hypersensitive response, but rather prepare the plant for improved defense should the need arise (Goellner and Conrath, 2008; Jung et al., 2009; Paulert et al., 2010). Conversely, it might be argued that the mode of action of compounds known to induce disease resistance in plants might have priming activity on the cellular level. This assumption was verified in the case of ulvan, a complex polysaccharide produced by green algae (Lahaye and Robic, 2007) which is known to induce disease resistance in plants (Paulert et al., 2009; Jaulneau et al., 2010) and which we recently showed to have priming activity in monocot cells (Paulert et al., 2010). Another compound which has been used for a long time as a plant protectant is chitosan produced by partial deacetylation of chitin (Terry & Joyce, 2004; El Hadrami et al., 2010), but whose mode of action is not well understood (Moerschbacher, 2005). It might be speculated that chitosan also has priming activity, but only elicitor activity has been reported so far.

Interestingly, chitosan is a natural component of the cell walls of some pathogenic fungi where chitin is deacetylated to chitosan during penetration and colonisation of the host tissue to protect it from the host chitinases and other hydrolytic enzymes (El Gueddari et al., 2002; Baker et al., 2007; Treitschke et al., 2010; Tucker et al., 2010). Although the cell walls of phytopathogenic fungi, thus, are rich in chitin and chitosan, and both have been implicated as members of PAMPs (Hadwiger and Beckman, 1980; Kohle et al., 1984; Kurosaki et al., 1986; Notsu et al., 1994; Vander et al., 1998; Lee et al., 1999; Ortmann et al., 2004; Cabrera et al., 2006; Dos Santos et al., 2008; Oliveira et al., 2008) their detailed role in the triggering of plant disease resistance remains to be understood. Chitin, as an insoluble, crystalline polymer, most likely needs to be enzymatically degraded by plant apoplastic chitinases yielding water soluble chitin oligomers to exhibit elicitor activity (Ride and Barber, 1990; Vander et al., 1998). Indeed, membrane bound receptor proteins specifically binding chitin oligomers have been identified in a number of plants (Okada et al., 2002; Kaku et al., 2006; Petutschnik et al., 2010;). The interaction of chitosan oligomers with membrane proteins at

the molecular level is still not clear, but early activation of a MAP kinase pathway seems to be triggered by both chitin and chitosan elicitors (Shibuya and Minami, 2001; Vasconsuelo et al., 2003; Wan et al., 2004). However, it has also been speculated that chitosan, due to the positive charges conveyed by the free amino groups at the slightly acidid pH of the plant cell wall, might interact directly with the negatively charged phospholipids of the plasmamembrane rather than with a proteinaceous receptor (Kauss et al., 1989). Even though plants appear to lack chitosanase enzymes, partially acetylated chitosans can also be degraded by chitinases, generating partially acetylated chitosan oligomers. While the influence of the degree of polymerisation (DP) on the elicitor activity has been studied extensively for chitin oligomers (Barber et al., 1989; Yamada et al., 1993; Vander et al., 1998) and, rarely, also for chitosan oligomers (Aziz et al., 2006), few studies have investigated the influence of the DA on the elicitor activity of chitosan oligomers (Cabrera et al., 2006; Dos Santos et al., 2008).

We here report on the generation of chitosan oligomers from partially acetylated chitosan polymers using recombinant enzymes, the mass spectrometric characterisation of the oligomers produced, and the evaluation of their biological activities towards suspension cultured plant cells. We found that the products of chitosanase and chitinase catalysed depolymerisation differed in their biological activities, the former eliciting an oxidative burst and the latter acting as a priming agent.

Materials and Methods

Chemicals

Luminol was purchased from Sigma, potassium hexacyanoferrate(III) from Fluka (Buchs, Germany). All the chemicals used for preparation of buffers were purchased from Roth.

Preparation of chitosans

Purification of the initial chitosan

Initial chitosan (batch 113) with DA's of 3,6% and DP_w 2124 was purchased from Mahtani Chitosan Pvt. Ltd. (India). The chitosan powder was dissolved (5 g/L (w/v)) in an aqueous acetic acid solution, filtered successively through 3, 0.8, and 0.45 µm pore size membranes (Millipore), and precipitated by means of a dilute aqueous solution of ammonia. After repeating washings with deionized water and centrifugations cycles (until the conductivity of the supernatant reached that of water), purified chitosan was then lyophilized.

Acetylation of chitosan

Acetylation was performed according to a modified protocol from (Vachoud et al., 1997). Purified chitosans were dissolved to 1g/L in a 0.1% acetic acid/1,2-propanediol mixture. Different amounts of a solution of pure and fresh acetic anhydride in 1,2-propanediol were added during stirring thoroughly and gently, in stoechiometric conditions, to reach the desired DA. At the end of the reaction, reacetylated chitosans were fully precipitated by addition of dilute aqueous ammonia until pH of around 11 was reached and then washed several times with deionized water until pH 7.5 was reached in order to preserve the $-NH_2$ form.

Characterization of chitosans

Degree of acetylation, DA%

Reacetylated chitosans were dissolved in dilute acidic D2O (at pD 3-4), and their DAs were analyzed by ^1H NMR spectroscopy. Spectra were recorded on a Bruker UltraShield^{TM} Spectrometer (300 MHz, 128 numbers of scans).

Number-average degree of polymerization, DP_w

The DP_w was determined using the HP-SEC (High-Performance Size Exclusion Chromatography). The Separation was performed on a system coupling on line: two serially connected columns (TSK G3000-PW and TSK G6000-PW, i.d. = 7.8 mm, l = 300 mm) with a differential refractometer (Waters R 410, from Waters-Milipore) and a multiangle laser-light scattering detector operating at 632.8 nm (Wyatt Dawn DSP). A 0.15 M ammonium acetate/0.2 M acetic acid buffer (pH = 4.5) was used as eluent at a flow rate of 0.5 mL/min. This online setup allowed the simultaneous determination of the concentration and molecular weight on each eluting time.

Preparation of enzymes

Recombinant chitinases from *Bacillus licheniformis*, *Bacillus thuringiensis* and chitosanase from *Bacillus sp* were expressed heterologously in *Eschericia coli* and purified by affinity chromatography using C-terminal StrepII-tag and anionic exchange chromatography (data not shown). Pure enzymes were rebuffered in ammonium acetate buffer (50 mM, pH 5.2) according to enzymatic characterization (Neeraja et al., 2010).

Enzymatic hydrolysis of chitosans / Preparation of elicitors and priming agents

Hydrolysis of chitosans with chitinases and chitosanase were done in ammonium acetate buffer (50 mM, pH 5.2) at 37˚C for 48 h. To obtain COS, 1 mg of chitosans (DA10/35/50%) were hydrolyzed by 5 µg of enzymes. Samples were lyophilized overnight which were then dissolved in sterile MilliQ water to a concentration of 1 mg ml^{-1} to characterize them. Abbreviations for COS produced by *Bacillus* chitinases and chitosanase used in the present study were given below (Table 1).

Table.1 Enzymes and substrates used for generation of COS

S. No	Enzyme	Substrate	Name of the COS
1	*B. licheniformis* chitinase	Chitosan DA 50%	BliCHI50
2	*B. licheniformis* chitinase	Chitosan DA 35%	BliCHI35
3	*B. thuringiensis* chitinase	Chitosan DA 50%	BthCHI50
4	*B. thuringiensis* chitinase	Chitosan DA 35%	BthCHI35
5	*B. sp* chitosanase	Chitosan DA 35%	BspCHO35
6	*B. sp* chitosanase	Chitosan DA 10%	BspCHO10

Culture and maintenance of Medicago cell suspension culture

Medicago truncatula jemalong (dicot) cell suspension cultures were obtained as described by Baier et al. (1999) and Scheidle et al. (2005) and cordially supplied by Prof. Dr. Karsten Niehaus (University of Bielefeld, Germany). The cells were maintained in MS medium (Murashige and Skoog, 1962) supplemented with sucrose (30 g l^{-1}) and with phytohormones 2,4-dichlorophenoxyacetic acid (1 mg l^{-1}) and kinetin (0.1 mg l^{-1}), and subcultured every 7 d in 15 ml medium. The cells cultures were kept at 26°C in the dark under agitation.

Culture and maintenance of Rice cell suspension culture

Suspension cultured rice cells (*Oryza sativa* L.) were kindly provided by Dr. Burkhard Schmidt (University of Aachen, Germany). The cells suspension cultures were grown in MS medium (Murashige and Skoog, 1962) supplemented with sucrose (30 g l^{-1}) and 2,4-dichlorophenoxyacetic acid (1 mg l^{-1}) and subcultured every 7 d in 15 ml medium. The cells cultures were kept at 26°C in the dark under agitation.

Measurement of H_2O_2 by oxidative burst reaction in plant cell suspension cultures

COS produced by chitinases and chitosanase were tested in both medicago and rice cells. The cells were used 3-4 days after subcultivation into fresh medium, gently separated from the medium through a sintered glass filter. Aliquots of 300mg of cells were suspended in 5ml of pre-incubation medium (3% sucrose [w/v] and 10mM MES in 5% [v/v] culture medium, pH 5.8) in a six-well-plate and incubated under culture conditions for 5 h as described by Ortmann and Moerschbacher (2006). Determination of oxidative burst is based on the method of Warm and Laties (1982) using a luminometer (Lumat LB 9501/16 Berthold, Wildbad, Germany). The chemiluminescent assay is based on peroxidation of luminol to an aminophthalate dianion by potassium hexacyanoferrate(III) and hydrogen peroxide released from cells after stimulation. The chemiluminescence can be measured as relative light units (RLU) which is proportional to the amount of H_2O_2 released. Cells were treated with COS (10 µg ml^{-1}) hydrolysate. Then 200 µl of cell culture were mixed with 700 µl of potassium phosphate buffer (50 mM, pH 7.9). The reaction was activated by addition of 100 µl of 1.21 mM Luminol and 100 µl of 14 mM potassium hexacyanoferrate(III) and measurement of H_2O_2 was done by light detection (10 s integration time, 430 nm). The micromolar (µM) H_2O_2 concentration was determined using a standard calibration curve.

Elicitation of cells with COS

For testing elicitor activity COS were added right before measurement to 5 h pre-incubated suspension-cultured cells at 26°C to get a final concentration of 10 µg ml^{-1} and the cells were incubated with moderate shaking in dark.

Priming of cells with COS

When COS were tested for priming activity, Ulvan (10 µg ml^{-1}) was used as elicitor, Salycilic acid (SA) as appositive control which is a known inducer in plants. Distilled water was used as negative control. To investigate the priming activity of COS, cells were treated with COS at a final concentration of 10 µg ml^{-1} 2 h after incubation at 26°C with moderate shaking in dark. SA with a final concentration 5 µg ml^{-1} was added to cells in one well. Incubation was extended for another 3 h then the cells were elicited with Ulvan to measure the H_2O_2 released. After every four minutes 200 µl of cell suspension were taken to measure released H_2O_2.

Characterization of chitosan oligomers

High performance-thin layer chromatography (HP-TLC)

Aliquots (20 µL) from each sample (1 mg/ml CSN35, CSN10, B.li50, B.li35, B.t50, B.t35) were concentrated under reduced pressure to scale down the volume to 10 µL. Aliquots (10 µL) were applied on TLC plates (Merck, Berlin, Germany), then run in butanol:methanol:ammonia:water (5:4:2:1, v/v/v/v) and stained using aniline-diphenylamine reagent (4 mL of aniline, 4 g of diphenylamine, 200 mL of acetone, and 30 mL of 85% phosphoric acid). Oligomers were visualised by heating the plate at 180˚C for 3-5 min. Oligomers were compared with authentic N-acetylglucosamine (DP 1, 2, 3, 5, 6) and D-glucosamine (DP 1, 3, 4) standards (Seikagaku, Tokyo, Japan).

High performance-Size exclusion chromatography (HP-SEC)

To analyze whether the hydrolysis was complete, HP-SEC was performed using a PSS SECcurity GPC System. Hydrolyzates were sterile filtered using 0.22 µm filters. A Volume of 100 µl from each sample (1 mg/ml) and chitosan polymer as controls was loaded on a set of Novema columns (1 x 30 A, 2 x 3000 A) after equilibrating with elution buffer (150 mM ammonium acetate, pH 4.5 filtered and degassed). Elution flow rate was set to 0.5 ml min^{-1} for 85 min per sample.

MALDI-TOF Mass spectrometry

Mass spectra were obtained for all six different samples using UV laser emitting at 337 nm with a pulse rate of 30 Hz over 60 s and 2,5-dihydroxybenzoic acid (DHB) was used as matrix (Loboda et al. 2003). A sample volume of 1 µl (1mg ml^{-1}) was mixed with 1 µl DHB spotted onto the MALDI sample plate and gently dried with a hot air gun. Mass spectra of positively charged ion adducts were analyzed with MoverZ.

Results

Enzymatic production and chemical analysis of partially acetylated chitosan oligomers

Three different recombinant chitosanolytic enzymes were produced in *E. coli* by the heterologous expression of the corresponding genes isolated from three different *Bacillus* species, namely a chitinase from *B. licheniformis* (BliCHI), a chitinase from *B. thuringiensis* (BthCHI), and a chitosanase from *B. spec.* (BspCHO). Cloning and characterisation of the enzymes have been described previously (Neeraja et al., 2010; Chapter1). Three different

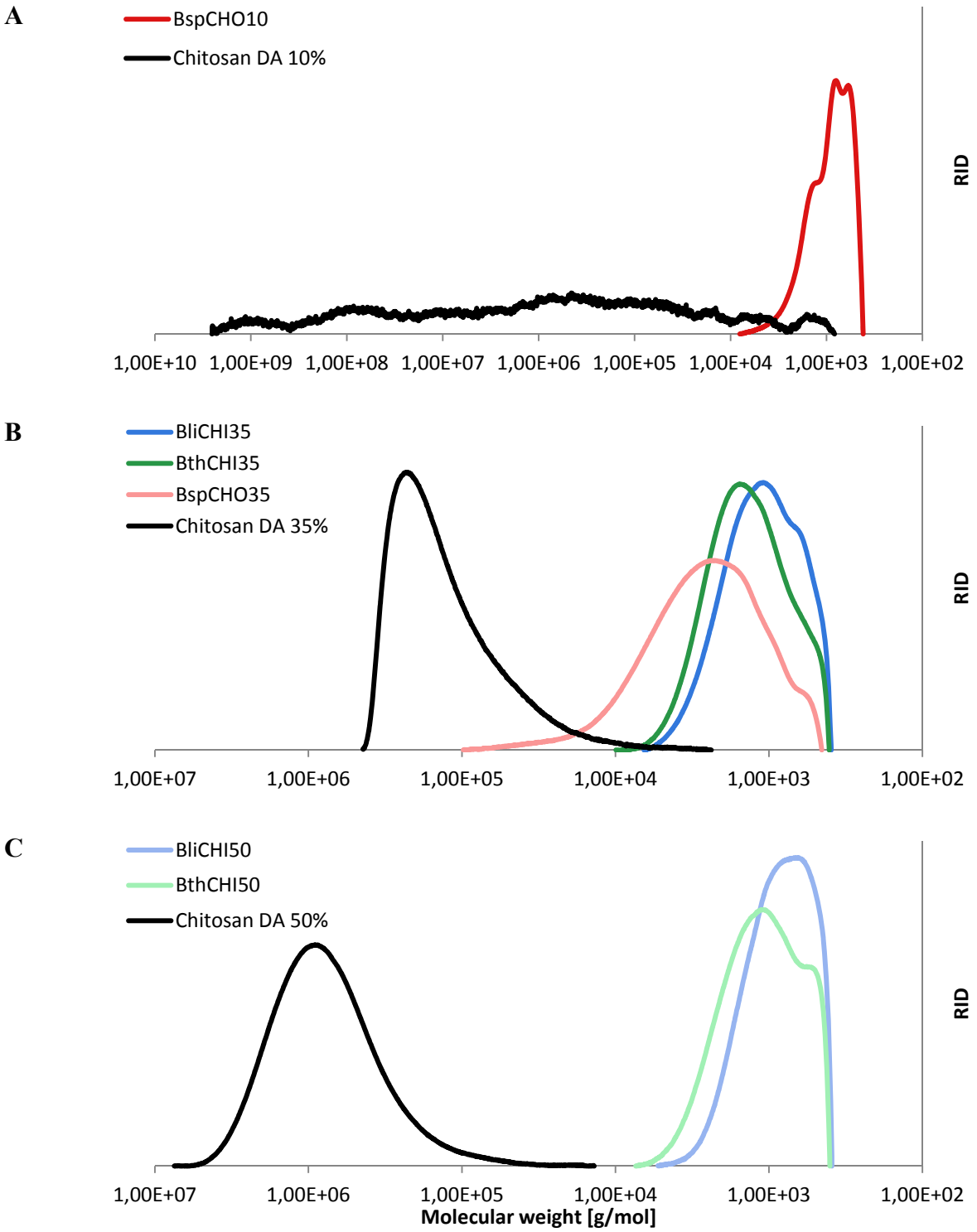


Fig. 1 Mass overlay after HP-SEC of hydrolysates shows complete hydrolysis of chitosan DA 10%, DA 35%, DA 50% into smaller oligomers after 48 h of incubation with chitosanolytic enzymes, as indicated. BliCHI, B. licheniformis chitinase; BthCHI, B. thuringiensis chitinase; BspCHO, B. spec. chitosanase.

chitosans with DA 10%, DA 35%, and DA 50% were used as substrates, in the following combinations: DA 10% / BliCHI, DA 10% / BthCHI, DA 35% / BliCHI, DA 35% / BthCHI, DA 35% / BspCHO, DA 50% / BspCHO; the other combinations did not yield oligomers in

the biologically active range of DP 5 to 10. End point hydrolyses were carried out by incubation over night, to obtain reproducible mixtures of partially acetylated chitosan oligomers. HP-SEC analysis revealed that the polymers were degraded completely.

HP-TLC analysis showed that mixtures of chitosan oligomers were produced in the biologically active range of DP > 4 in all enzyme/substrate combinations (Fig. 1). Mass spectrometric analyses using MALDI-TOF-MS allowed us to identify the DP and DA of the enzymatically produced oligomers (Table 1 and suppl. Fig. 2). As expected, chitinases produced oligomers with lower DP from chitosans with higher DA, while chitosanase produced oligomers with lower DP from chitosans with lower DA. Also, the larger chitinase products were characterised by lower DA, containing never more than three acetylated residues, while the larger chitosanase products had higher DAs, containing up to eight GlcNAc residues.

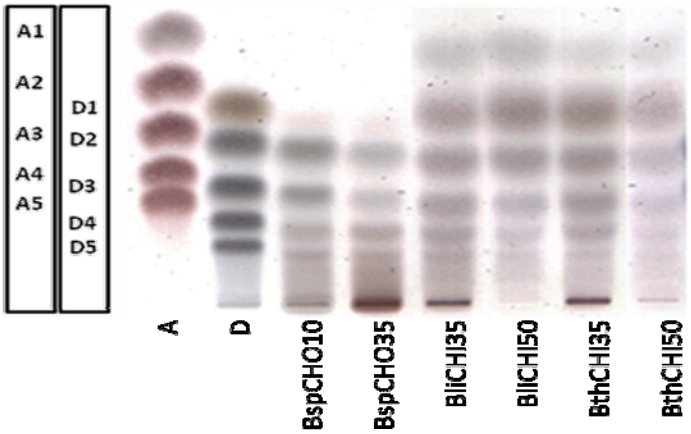


Fig. 2: HP-TLC analysis of the products of chitosans (DA 10%, DA 35%) incubated with chitosanase (BspCHO10, BspCHO35) and chitosans (DA 35% and DA 50%) incubation with chitinases (BliCHI35, BliCHI50, BthCHI35 and BthCHI50). Oligomers of GlcNAc (A) and GlcN (D) were used as standards. The DP of the standards is given on the left sides of the plates.

Table: 1 MALDI-TOF-MS analysis of oligomers after hydrolysis of chitosans (DA 10%, DA 35% and DA 50%) with *Bacillus* chitosanase (BspCHO10 and BspCHO35) or *Bacillus* chitinases (BliCHI35, BliCHI50, BtCHI35, BtCHI50). Products are arranged in a decreasing order according to their total intensity (H^+- and Na^+-adducts of individual oligomers were summed up) (actual spectra are found in the supplementary data).

BliCHI35	BliCHI50	BthCHI35	BthCHI50	BspCHO10	BspCHO35
D6A1 D1A2	D3A2 D3A3	D6A2 D11A2	D4A2 D5A4	D2	D6A3 D5A1
D7A1 D11A1	D5A2 D9A1	D5A2 D3A1	D3A2 A2	D5A1	D4A2 D6A6
D5A1 D12A1	D4A2 D1	D7A2 D9A1	D5A2 D6A4	D4A1	D5A3 D3A2
D4A1 D9A2	D2A2	D8A2 D4A3	D2A2 D5A1	D3	D5A2 D9A6
D2A1 D13A1	D2A1	D4A2 D7A3	D5A3 D9A2	D6A2	D4A1 D4A4
D5A2 D10A2	D1A1	D9A2 D5A3	D4A3 D7A4	D5A2	D6A4 D1
D8A1 D2	D6A2	D3A2 A1	D6A3 D6A1	D3A1	D3A1 D3A3
D3A1 D1	A1	A2 D9A3	D6A2 A1	D4A2	D7A4 D9A5
D4A2	D1A2	D2A1 D8A3	D7A3 D2	D7A2	D5A4 D8A3
D3A2	D3A1	D10A2 D12A2	D3A3	D6A3	D4A3 D4A5
D1A1	D7A2	D4A1 D10A1	D1A2	D7A3	D7A5 D9A8
D9A1	D5A1	D5A1 D2A3	D7A2	D5A3	D6A5
D6A2	D4A1	D6A1 D3A3	D8A3	D8A3	D6A2
A1	D6A1	D1A1 D10A3	D2A1	D7A4	D8A5
A2	D7A1	D7A1 D11A1	D4A1	D4A3	D7A3
D10A1	A2	D8A1 D11A3	D2A3	D6A4	D8A6
D7A2	D8A2	D2A2 D12A1	D1A1	D8A4	D7A6
D2A2	D8A1	D1A2 D1	D8A2		D8A4
D8A2	D9A2	D6A3	D3A1		D5A5

Elicitor activities in suspension cultured cells of Medicago truncatula

Elicitor activity of the enzymatically produced mixtures of partially acetylated chitosan oligomers were tested on suspension cultured cells of *Medicago truncatula* by measuring ROS production in an oxidative burst. When tested at a concentration of 50 µg ml^{-1}, only the chitosanase products induced an oxidative burst while all chitinase products were inactive (Fig. 3). The oxidative burst induced was an unusually rapid one, reaching its peak 4-8 min after elicitation, then starting to decrease immediately and returning to its initial level after

about 30 min. The products obtained by chitosanase digestion were also elicitor active at a concentration of 10 µg ml^{-1}, the products of the DA 35% chitosan were the most active elicitors.

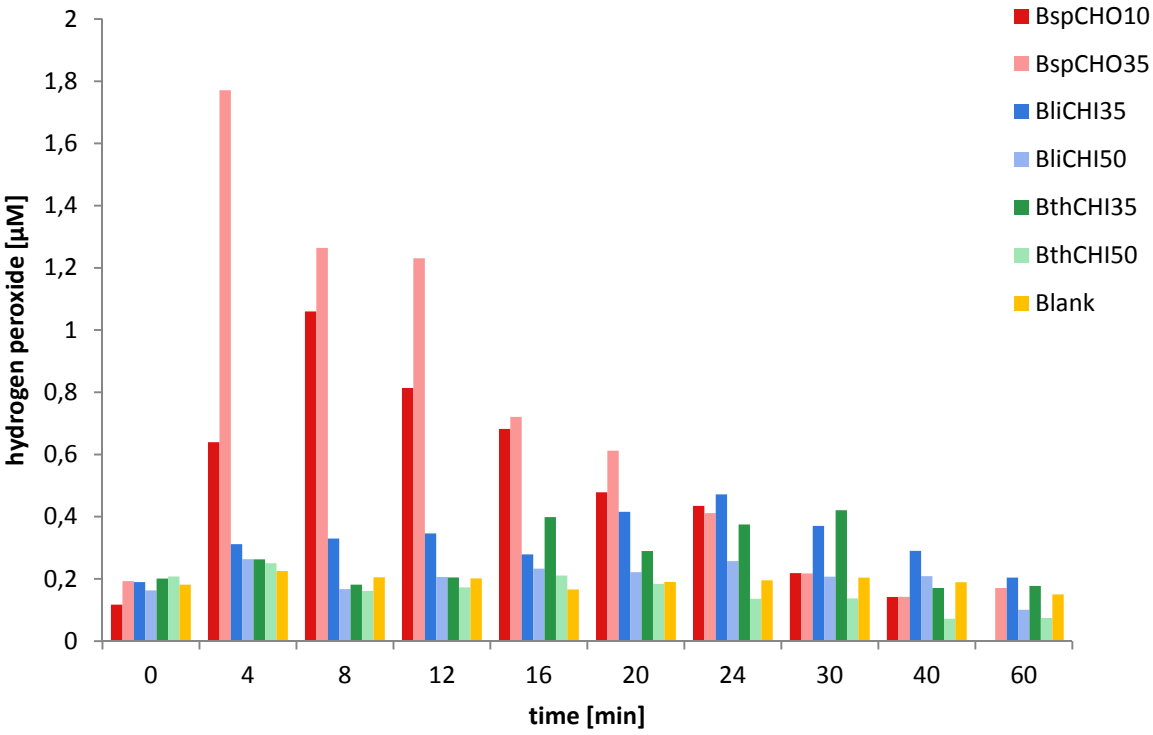


Fig. 3: Elicitation of an oxidative burst in suspension cultured *M. truncatula* cells induced by enzymatically generated, partially acetylated chitosan oligomers (50 µg ml^{-}) obtained by incubating partially acetylated chitosan polymers (DA 10, 35, 50%) with recombinant *Bacillus* chitosanase (BspCHO) or chitinase (BliCHI, BthCHI). The concentration of extracellular H_2O_2 was measured by luminol-dependent chemiluminescence. Data shown are from one representative of three independent experiments with similar results. Sodium acetate buffer was used as a negative control.

Priming activities in suspension cultured cells of Medicago truncatula

To test for priming activity, suspension cultured *Medicago* cells were pre-treated with the elicitor-inactive chitosan oligomer mixtures obtained after digestion with chitinases at a concentration of 10 µg ml^{-1} two or three hours prior to elicitation using the known elicitor ulvan (10 µg ml^{-1}). Pre-treatment with the known priming agent salicylic acid (5 µg ml^{-1}) was used as a positive control, pre-treatment with distilled water served as a negative control. Chitosanase products were not tested as their potential priming activity would be masked by their elicitor activity.

Naive cells pre-treated with water only exhibited a rather slow ulvan-induced oxidative burst, with an onset around 20 min, a peak around 60 min, and return to basal levels around 90 min after elicitation (Fig. 4). Primed cells pre-treated with salicylic acid started producing H_2O_2 already around 10 min and reached a peak at around 30 min after elicitation, and the maximum concentration of H_2O_2 reached was higher than in the naive cells. Similarly, cells pre-treated with any of the chitinase products were clearly primed, showing a rapid onset of the oxidative burst and a slightly longer lasting peak of high H_2O_2 production. The products obtained by chitinase digestion of the DA 35% chitosan were the most active priming agents, being at least equally active as salicylic acid.

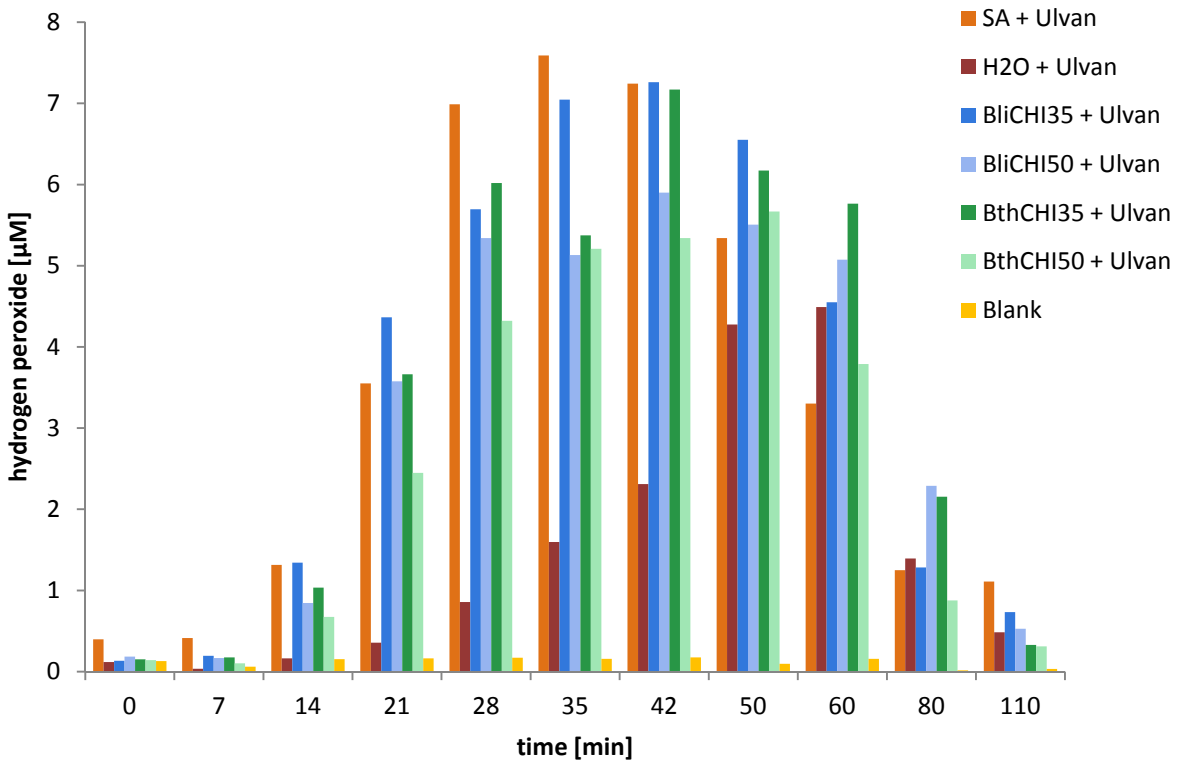
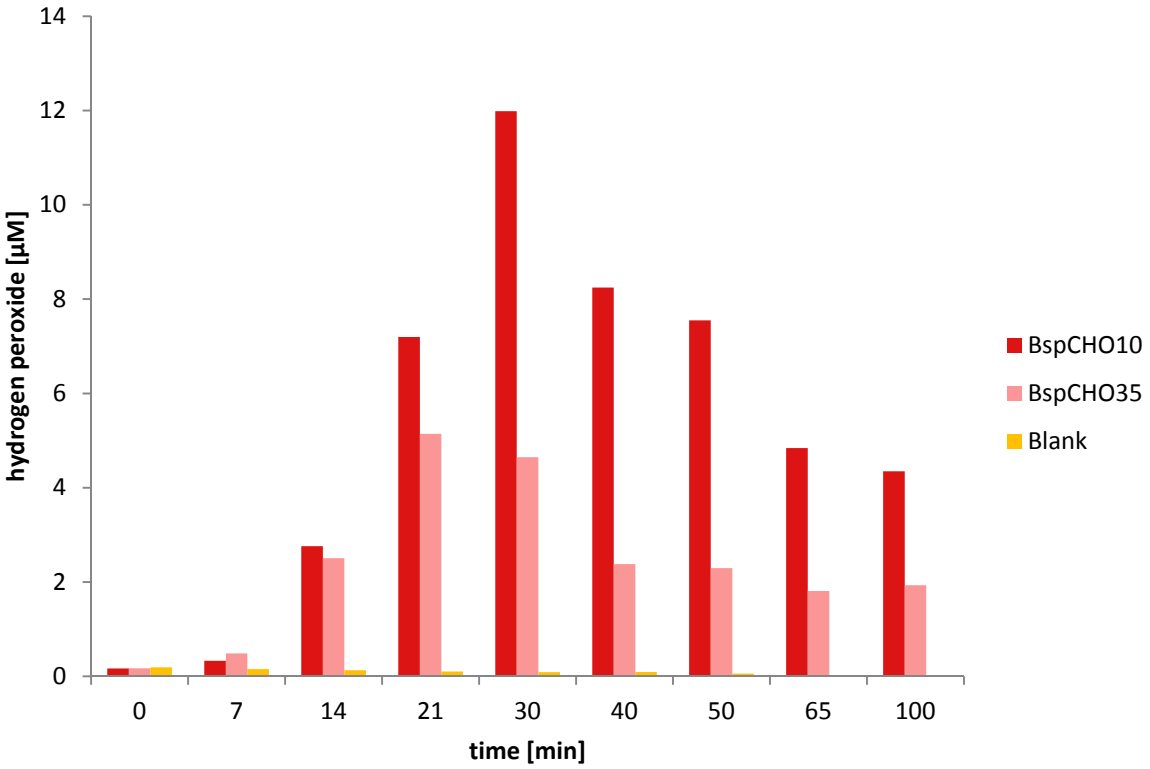


Fig. 4: Priming of an ulvan-induced oxidative burst in suspension cultured *M. truncatula* cells by enzymatically generated, partially acetylated chitosan oligomers (50 µg ml^{-}) obtained by incubating partially acetylated chitosan polymers (DA 35, 50%) with recombinant *Bacillus* chitinase (BliCHI, BthCHI). Cells were pre-treated for 3 h with the chitosan oligomers (10 µg ml^{-}), then elicited by the addition of ulvan (10 µg ml^{-}). The concentration of extracellular H_2O_2 was measured by luminol-dependent chemiluminescence. Data shown are from one representative of three independent experiments with similar results. Salicylic acid (5 µg ml^{-}) was used as a positive control.

Elicitor activities in suspension cultured cells of Oryza sativa

When the enzymatically produced mixtures of partially acetylated chitosan oligomers (10 µg ml^{-1}) were tested for elicitor activity on suspension cultured cells of rice, only the

chitosanase products were active while products of all chitinase digestions were inactive (data not shown). The products obtained by chitosanase digestion of the DA 10% chitosan were the most active elicitors, inducing the production of H_2O_2 around 10 min and reaching a very high peak level around 30 min after elicitation (Fig. 5). Levels of H_2O_2 then started dropping



immediately but did not reach basal levels during the 100 min of the experiment.

Fig. 5: Elicitation of the oxidative burst of *Oryza sativa* cells by enzymatically generated, partially acetylated chitosan oligomers (50 µg ml^{-}) obtained by incubating partially acetylated chitosan polymers (DA 10, 35%) with recombinant *Bacillus* chitosanase. Cells were elicited by ulvan (10 µg ml^{-}) culture induced with COS (10 µg ml^{-1}) obtained by chitosanase. The concentration of extracellular H_2O_2 was measured by luminol-dependent chemiluminescence. Data shown are from one representative of three independent experiments with similar results.

Discussion

The mixtures of partially acetylated chitosan oligomers used in this study were produced enzymatically through the digestion of chitosan polymers by chitinases and chitosanases obtained from *Bacillus* species. *Bacilli* are well known chitinolytic microorganism and they produce different chitinases and chitosanases which have been used for the generation of

chitosan oligomers (Somashekar & Joseph 1996; Yeon Jin Choi et al., 2004; Dahiya et al., 2006; Neeraja et al., 2010). Oligomers produced from a non-defined chitosan using a crude enzyme extract from *Bacillus amyloliquefaciens* have been reported to have biological activity, inducing apoptosis in mouse colon carcinoma cells and possessing antitumor activity (Liang et al., 2007). To our knowledge, our work is the first report of the biological activities of well characterised chitosan oligomers produced from defined chitosan polymers using pure chitosanolytic enzymes. As a consequence of the different DAs of the substrates and of the different cleavage specificities of the enzymes used, the resulting product mixtures differed in their composition, both concerning their DP and their DA. As expected, the chitinase products were dominated by very small highly acetylated oligomers and larger oligomers with lower average DAs. In contrast, the chitosanase products contained many very small oligomers with a low DA and larger oligomers with higher average DA.

We have then shown that these products also strongly differ in their biological activities: chitinase products were elicitor active, while chitosanase products had priming activity. It can be assumed that these differences in biological activities of the partially acetylated chitosan oligomers reflect the differences in both DP and DA of the chitosan oligmers produced by the two enzymes. Typically, oligosaccharides need a minimum DP of ca. 5 to be biologically active, and this has also been shown previously for fully acetylated chitin oligomers (Vander et al., 1998). On the other hand, the influence of DA on biological activities of chitosan oligomers is far less well studied. It would appear from our results that chitosan oligomers with higher DA tend to be elicitor active, as also shown recently in *Araucaria* cells (Dos Santos et al., 2008), while those with lower DA tend to have priming activity.

We assume that the differential activities of chitinase versus chitosanase released chitosan olgomers are related to differential binding of partially acetylated chitosan oligomers to receptors in the plant plasma membranes (Moerschbacher & Gueddari 2004; Aziz et al., 2006). While potential receptors for chitin oligomers have been described in a number of species, including monocot and dicot plants (Okada et al., 2002; Petutschnik et al., 2010), the mode of perception of chitosan is less clear. While a rather non-specific, disruptive effect of polycationic chitosan polymers towards the polyanionic plant plasmamembrane has been deduced from the observation that the elicitor activity of chitosan polymers increased with decreasing DA and, hence, increasing positive charge density (Kauss et al., 1989), our results clearly suggest an additional more specific, presumably receptor-based perception of chitosan oligomers. While chitosan-specific lectins have been described occasionally (Lienart et al.,

1991; El Gueddari et al., 2002; Chen and Xu, 2005), their role in chitosan perception is unclear. It might be assumed that the chitosanase products with their higher DA elicit the oxidative burst by binding to the chitin receptor, as binding of chitin oligomers to this receptor also typically elicits an oxidative burst. This is also in agreement with the observation that the elicitor activity of partially acetylated chitosan oligomers increased with increasing DA (Dos Santos et al., 2008). However, the perception of the priming active chitinase products with their low DA is less obvious. They might induce a different reaction upon binding to the same receptor, or they might be recognised by a different receptor.

Comparing the elicitor activities of the chitosanase products in *Medicago* and rice, it also becomes apparent that the two plant species differ in their responses: while in *Medicago,* the oligomers derived from chitosan with a DA of 35% were more active than those derived from the DA 10% chitosan, the reverse was true in rice. Fundamental differences in the resistance related signal perception between monocot and dicot plants have been reported repeatedly in the past. In dicot, but not in monocot plants, bacterial flagellin (Felix et al., 1999) and plant cell wall derived oligogalacturonans (Baier et al., 1999; Aziz et al., 2007) act as exogenous and endogenous elicitors, respectively. In contrast, a small oligogalacturonan oligomer possessed suppressor activity in monocot, but not in dicot plants (Moerschbacher et al., 1999). A bacterial exopolysaccharide (Ortmann et al., 2006) and ulvan from green algae (Paulert et al., 2010) were shown to act as elicitors of an oxidative burst in dicot plant cells, but as priming agents in monocot cells, and different signal transduction pathways appear to be involved in induced resistance (Kogel and Langen, 2005). Also, the elicitor-active fragment of fungal ß-(1,3)-ß-(1,6)-glucan differs between dicot and monocot plants (Yamaguchi et al., 2000). The molecular basis for these differences is unclear at present, but it is tempting to speculate that it might be related to the fundamental differences in plant cell wall composition and architecture between dicot and commelinoid monocot plants (Carpita and McCann, 2002). To our knowledge, our observation of priming active chitosan oligomers is the first report of a carbohydrate-based priming agent active in dicot plants.

The rapid and transient release of H_2O_2 in an oxidative burst is generally used as a sensitive indicator of the induction of disease defense responses and common stress responses in plant cells as it has e.g. been assumed to ‘orchestrate’ the hypersentitive reaction (Levine et al., 1994). Also, the ability of a signal compound to prime suspension cultured plant cells for an elicitor-triggered oxidative burst appears to be correlated with its ability to induce disease resistance in intact plants (Klarzynski et al., 2000; Paulert et al., 2010). In the light of this,

our finding of priming active chitosan oligomers can be of fundamental importance in leading a way into reliable, chitosan-based plant disease protecting agents. Moreover, the present study serves as proof of principle that the biological activity of chitosan polymers will depend strongly on the chitosanolytic enzymes present in a target tissue, as predicted earlier (Cabrera et al., 2006; EI Gueddari et al., 2007). It is interesting to note that plants appear to have evolved a multitude of chitinases (Kasprzewska, 2003) but probably no chitosanases. Some plant pathogenic fungi appear to have evolved an avoidance and disguise strategy to escape the chitin-geared plant immune system by deacetylating the chitin present in their cell walls to form chitosan when penetrating and colonising their host tissues (OConnell et al., 1996; El Gueddari et al., 2002). It would now appear that plants have counteracted this strategy by developing a recognition machinery for the oligomers produced by chitinase digestion of the fungal chitosan, priming them for a rapid and efficient resistance reaction. We have recently reported on the surprising finding of a multitude of chitosanases produced by fungi living endophytically in the leaves of some tropical plant species (Rajulu et al., 2011), apparently without doing harm to the plants, but also without apparent profit for the plant. Perhaps, the chitosanases secreted by these fungi complement the plant's own defensive enzyme machinery, weakening chitosan-containing plant pathogens and concomitantly strengthening the plant by producing elicitor active chitosan oligomers.

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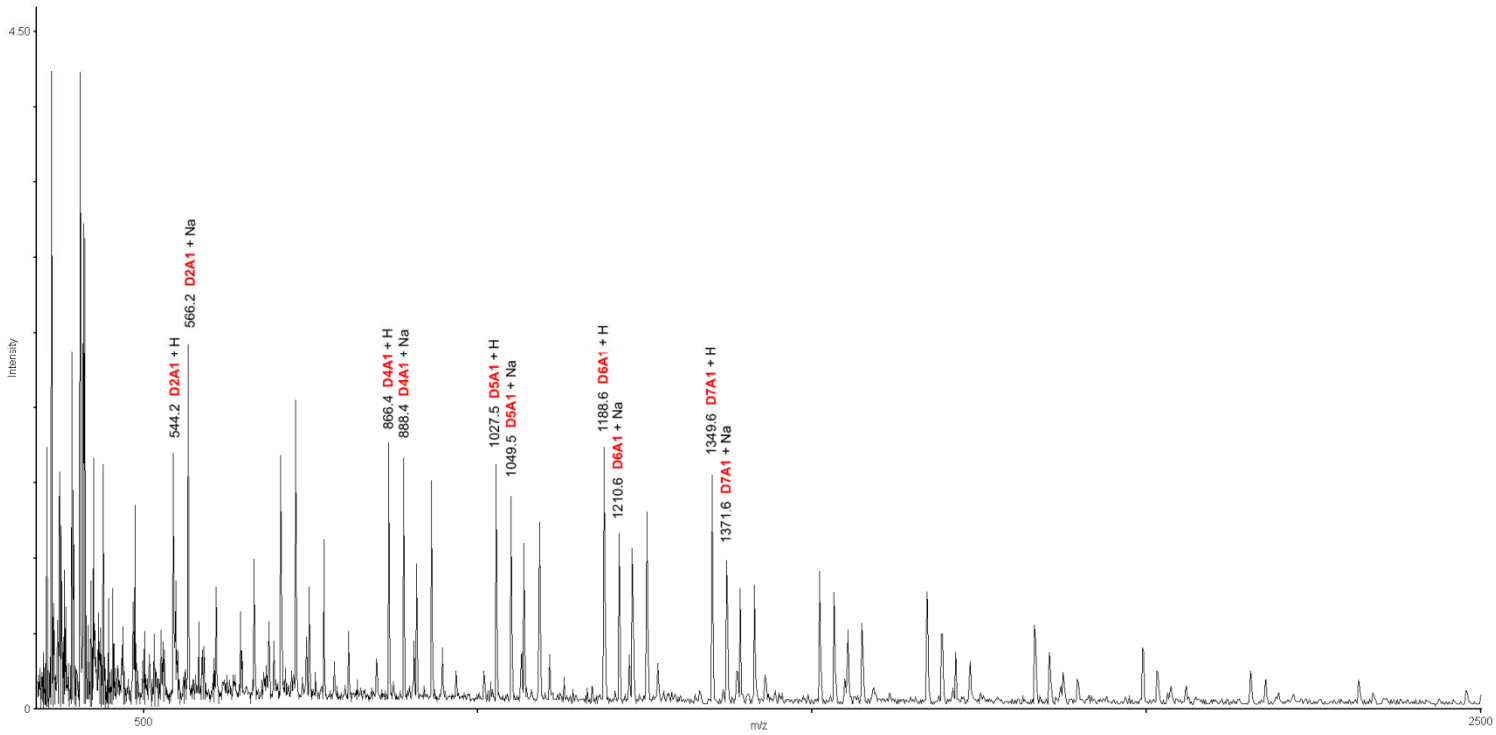
Supplementary data



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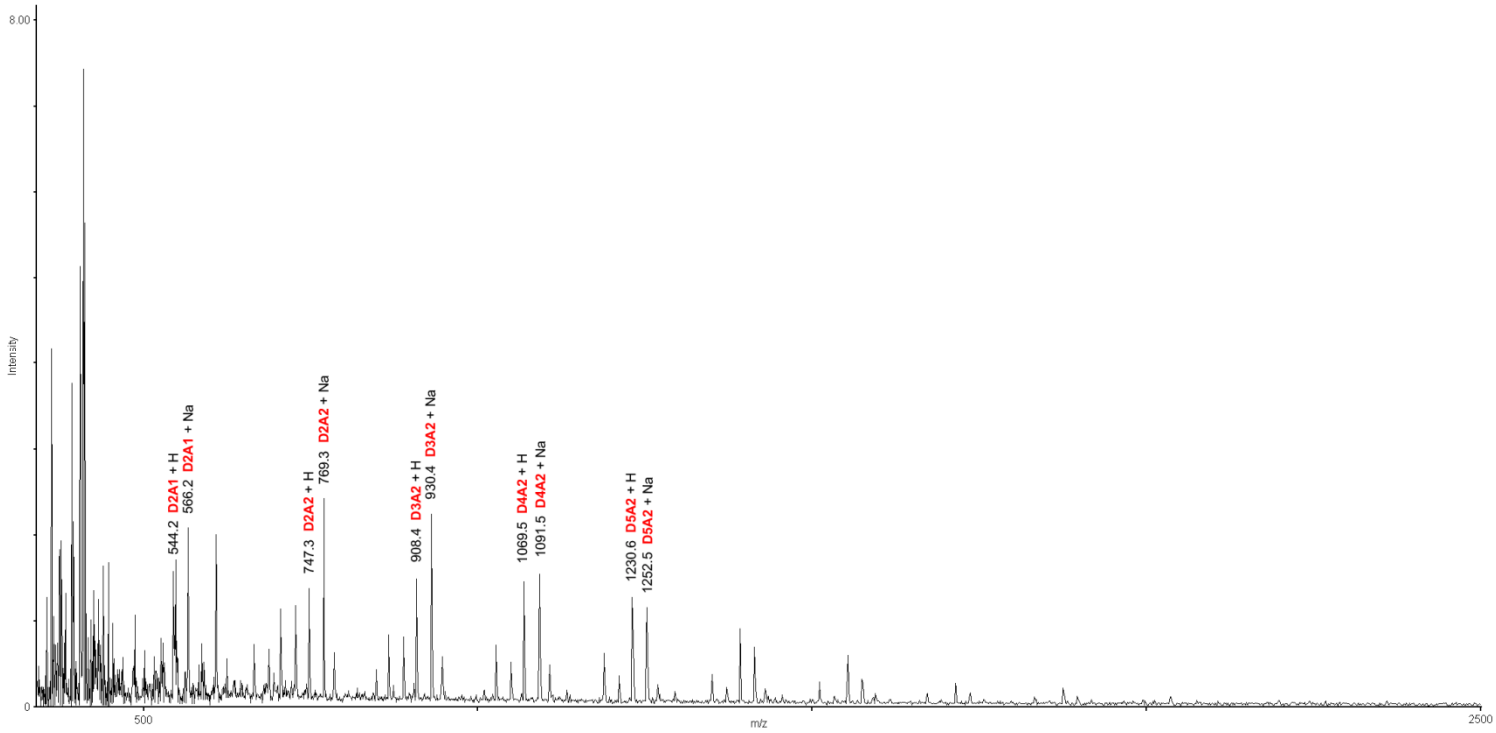

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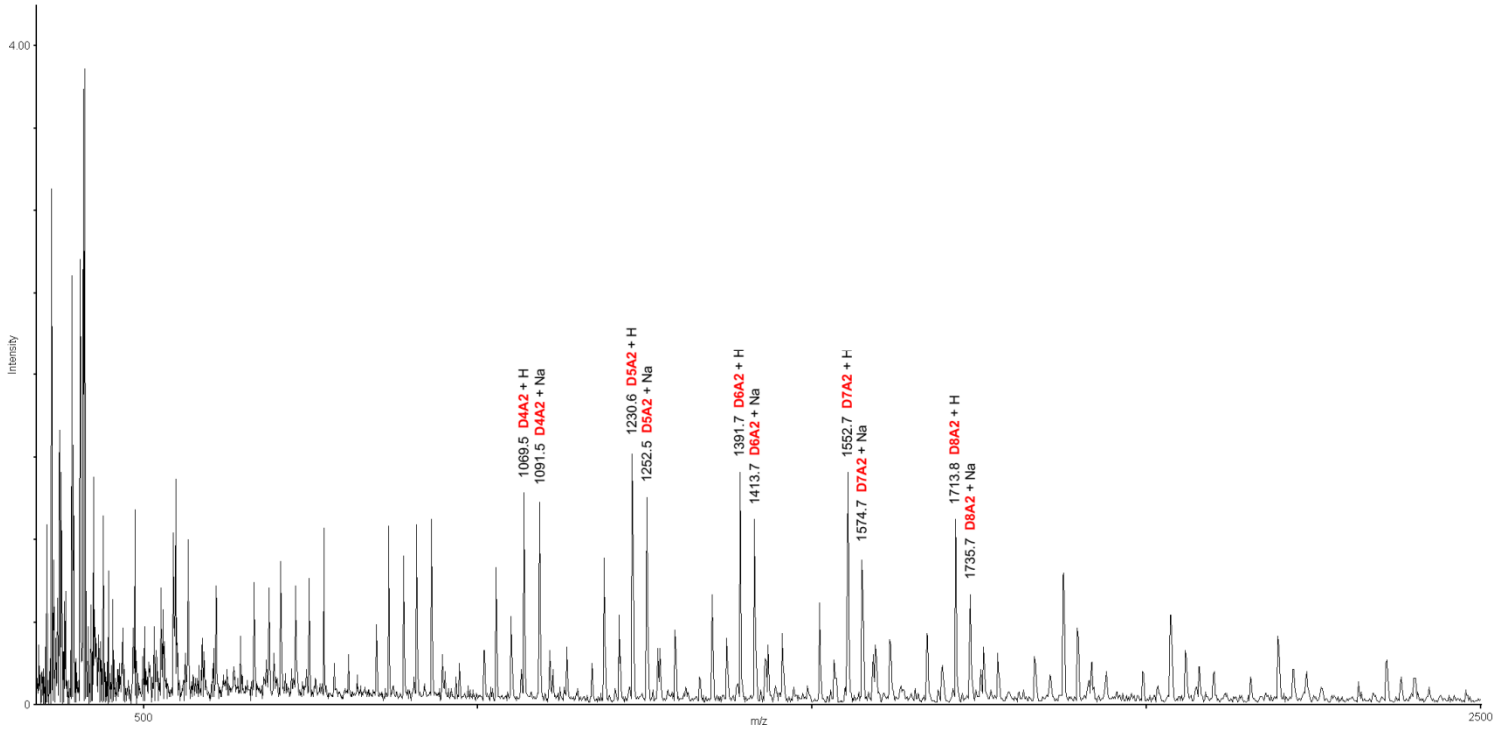

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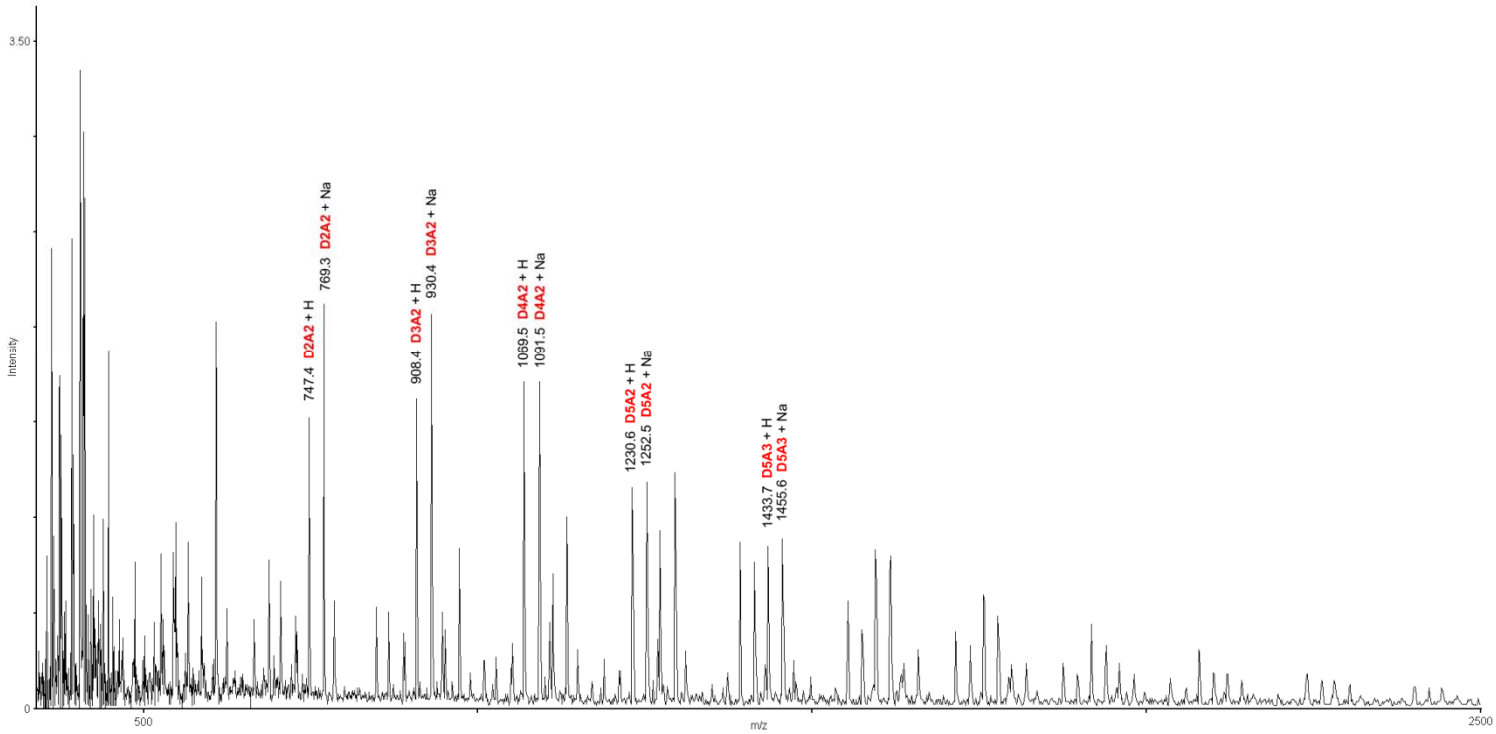

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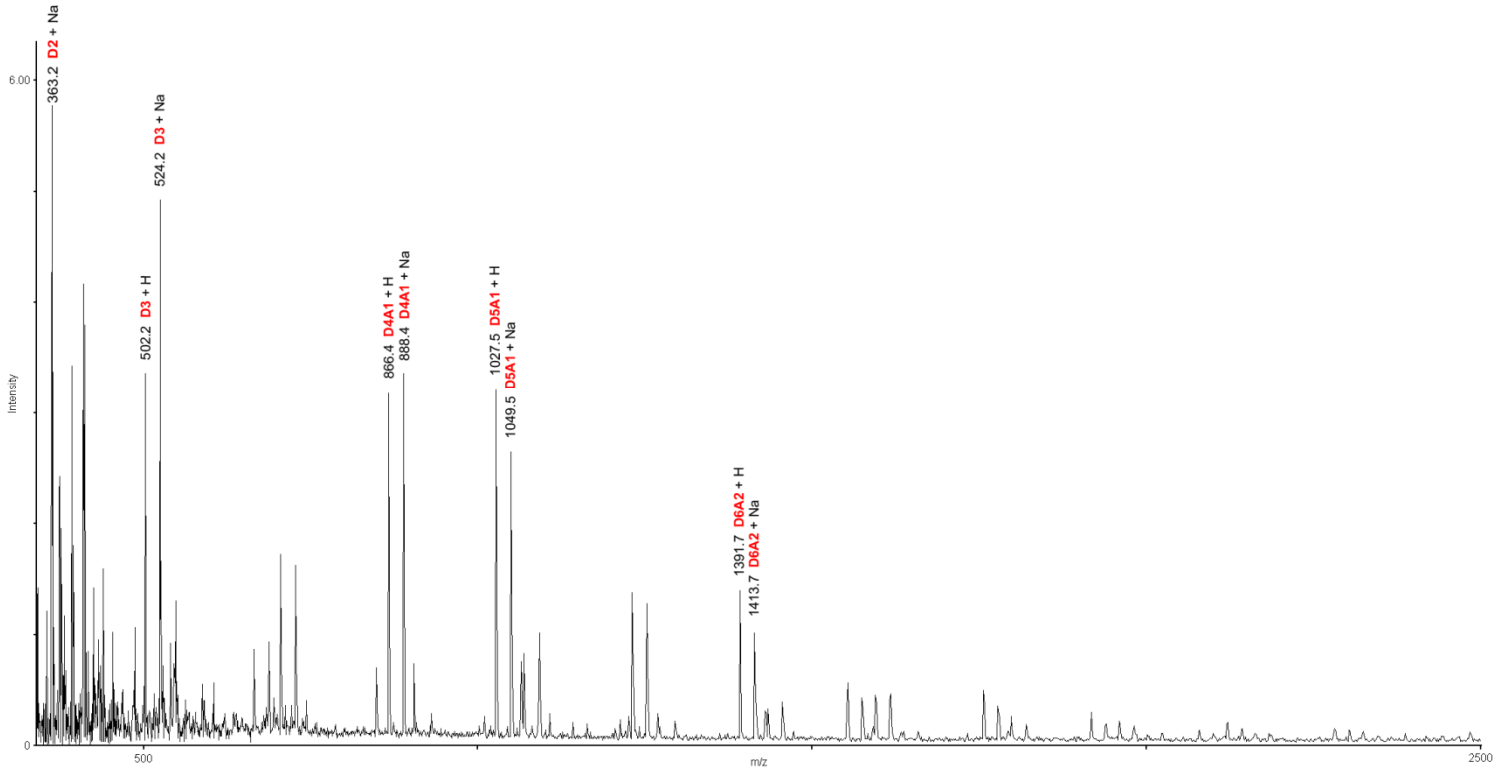

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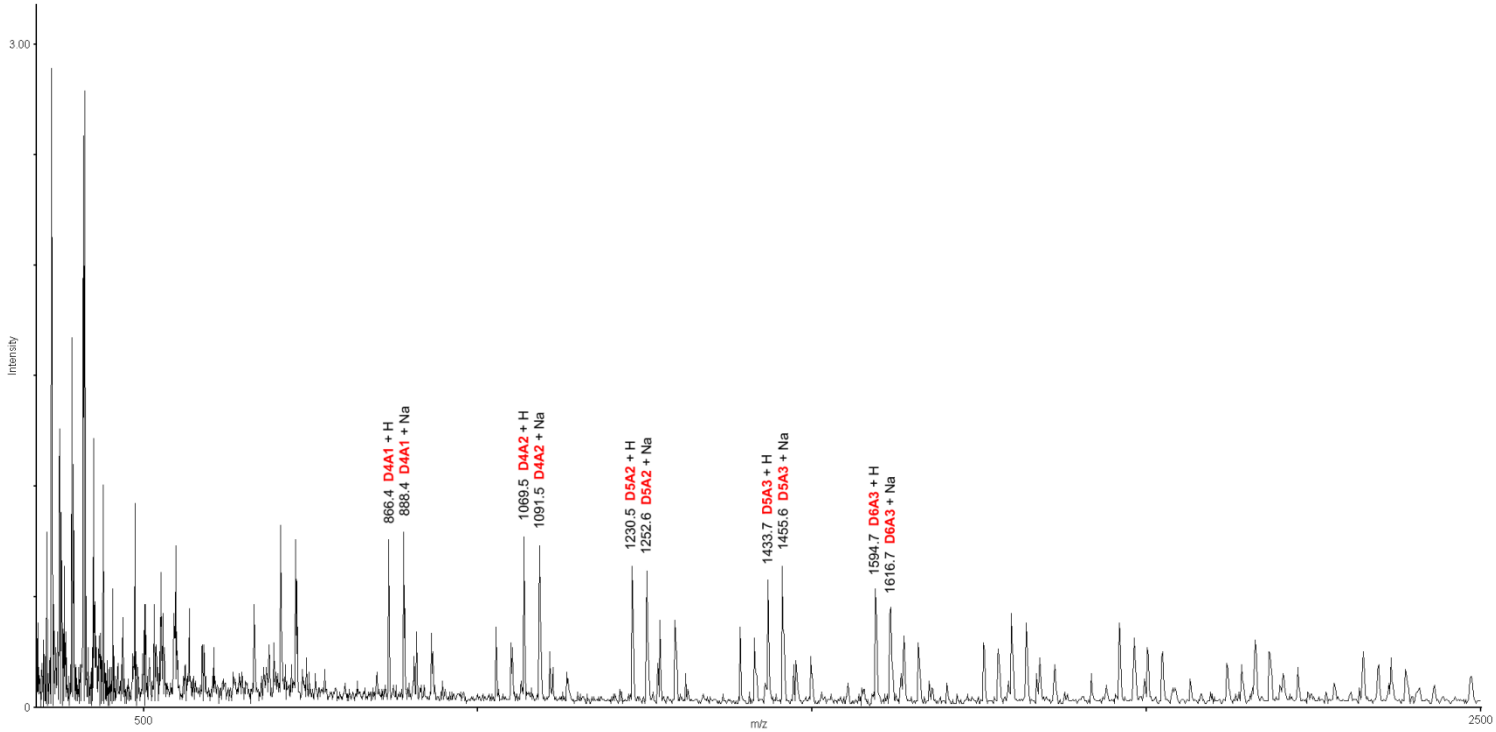

MALDI-TOF-MS analysis of oligomers after hydrolysis of chitosan DA 35% with *Bacillus spec.* chitosanase (BspCHO35).

CHAPTER 3

"This chapter has been submitted for publication with the following co-authors: Govinda Rajulu (who performed the taxonomic identification of fungal isolates; this part of the work is indicated by italics in the text below), D. Gillet (who was involved in discussing the strategy of the project and who supplied soil samples, chitin and chitosan), T. S. Suryanarayanan (who supervised Govinda Rajulu's work and who was involved in discussing the results), Bruno M. Moerschbacher (who supervised my work and who was involved in designing the strategy of the project and in discussing the results)."

More fungal than bacterial diversity of chitinolytic and chitosanolytic species and enzymes found in soil samples with a history of chitin and chitosan exposure

Abstract

Chitin is one of the most abundant biomolecules on earth, and its partially de-N-acetylated counterpart, chitosan, is one of the most promising biotechnological resources due to its diversity in structure and function. Recently, chitin and chitosan modifying enzymes (CCME) have gained increasing interest as tools to engineer chitosans with specific functions and reliable performance in biotechnological and biomedical applications. In search of novel CCME, we isolated chitinolytic and chitosanolytic microorganisms from soils with a more than ten years history of chitin and chitosan exposure, and screened them for chitinase and chitosanase isoenzymes and for the oligomeric product patterns by incubating their spent culture media with chitosan polymers. Of 60 bacterial strains isolated, only eight were chitinolytic and/or chitosanolytic; 20 out of 25 fungal isolates proved to be chitinolytic and/or chitosanolytic. The bacterial isolates produced rather similar patterns of chitinolytic and chitosanolytic enzymes, while the fungal isolates produced a much broader range of different isoenzymes. Diverse mixtures of oligosaccharides were formed when chitosan polymers were incubated with the spent culture media of select fungal species. In these soil samples, thus, fungal diversity clearly exceeded bacterial diversity both in terms of species and enzymes and their products. It appears that soils with prolonged chitin and chitosan exposures are a good source of fungi with novel CCME for chitosan bio-engineering.

Introduction

Chitin isolated from shrimp waste is one of the most abundant renewable resources worldwide, and its derivative chitosan is among the most interesting bio-active biopolymers. As shrimp and crab shell wastes are rich sources of chitin, some of it is used commercially for the extraction of chitin and for its partial chemical de-N-acetylation to yield chitosans. The term chitosan in fact describes a family of molecules differing in respect to their degree of polymerisation (DP), their degree of acetylation (DA), and potentially also their pattern of acetylation (PA). These factors appear to influence both the physico-chemical solution properties of the chitosans as well as their biological functionalities (El Gueddari et al. 2002; EI Gueddari & Moerschbacher, 2004; Lamarque et al. 2005; Moerschbacher 2005; Domard 2011). Detailed structure-function analyses, therefore, crucially depend on the availability of well characterised chitosans with broad ranges of known DPs, DAs, and PAs. Chitin and chitosan modifying enzymes (CCME) such as chitinase, chitin de-acetylase, and chitosanase could be used as powerful tools complementing the chemical methods currently used for this purpose (Kim and Rajapakse 2005; EI Gueddari et al. 2007; Kohlhoff et al. 2009). Similarly, reliable analytical methods are needed to characterise the chitosans in detail and again, enzymatic fingerprinting methods promise to become a powerful tool to complement the existing chemical methods of analysis (Chen et al. 2010; Moerschbacher et al. 2011).Thus, CCME can help to better utilize the chitin containing waste materials in order to generate products that have commercial importance. Soils with a long history of exposure to chitin and chitosan should be a rich source of genetic diversity of chitin and chitosan degrading and modifying micro-organisms (Manucharova et al. 2008; Das et al. 2010; Brzezinska et al. 2010).

Chitin has been estimated to be the second most abundant organic molecule on earth and consequently, chitin degradation is of vital importance to all ecosystems. Several reports showed that terrestrial soil and marine sludges are major sources of chitinolytic micro-organisms, including fungi, bacteria, and actinomycetes. Chitin degradation appears to involve endo- and exo-acting chitinases and chitobiases (Martinez. 2001); whether chitin deacetylases and chitosanases are also involved in the environmental degradation of chitin is a matter of debate (Li et al. 2007). In addition to being involved in chitin decomposition, CCME are also involved in other processes. Chitinases belong to the evolutionarily old repertoire of disease resistance mechanisms in plants and animals, including humans

(Gorzelanny et al. 2010; Taira et al. 2002). Chitinases as well as chitin deacetylases and, possibly, chitosanases are also involved in morphogenesis in fungi, protozoa, and invertebrates (Gooday et al. 1995; El Gueddari et al. 2002; Patil et al. 2000).

While fungi are reported to contribute more than bacteria to environmental degradation of chitin (Swiontek-Brzezinska. 2007), a lot less is known about the fungi involved compared to chitinolytic bacteria, and bacterial CCME have been studied in much more detail than fungal enzymes. In terrestrial soils, the most prevalent chitin degrading bacteria belong to the genera *Bacillus, Stenotrophomonas, Gammaproteobacteria,* and *Arthobacter* (Manucharova et al. 2008; Das et al. 2010), while *Actinobacterium, Pantoea,* and *Pseudomonas* were major chitinolytic bacterial genera found in marine sludges (Metcalfe et al. 2002; Gohel et al. 2004). The most abundant among chitinolytic bacteria in water and bottom sediments were *Achromobacter, Bacillus,* and *Enterobacteriaceae* (Donderski and Brzezinska 2001). Brzezinska (2010) showed that fungi isolated from water, soils, and sludges were chitinolytic. *Trichoderma viridae* isolated from soil exhibited the highest chitinolytic activities when chitin was included in the culture medium to induce chitinases (Manucharova et al. 2008). *Mortierella* and *Fusarium* are involved in chitin decomposition in chitin amendments of dune soils (De Boer et al. 1999).

The present work looks at the diversity of chitinolytic and chitosanolytic fungi and bacteria in soils of a chitin and chitosan producing company in Gujarat, India. These soils had been exposed to dry or fresh shrimp shells or to chitin or different types of chitosan for more than ten years. In addition to species diversity, we also analysed the diversity of CCME present in these organisms, as well as the diversity of products produced by these CCMEs.

Materials and Methods

Selection of soil samples

Seven soil samples were collected from different sites of a chitin/chitosan producing company, Mahtani Chitosan Pvt. Ltd., Veraval, Gujarat, India. Soil samples were collected from a depth of 5 to 10 cm. They differed in their texture ranging from sandy to clay. Samples were sent to Germany via airmail, checked for consistency, and stored at 4°C for a maximum of two months until further processing.

Preparation of colloidal chitin and chitosans

Colloidal chitin was prepared according to the method described by Berger and Reynolds (1988) with slight modifications. To 10 g of β-chitin isolated, 500 mL of conc. HCl was added and mixed under stirring until the mixture was homogeneous and incubated at 4°C over night. Two litres of double distilled water was then added and stirred for 48 hours at 4°C. The colloidal chitin thus prepared was washed with double distilled water until the pH was neutral.

Chitosan (average DA 3%, average DP 2,124) was dissolved in an aqueous acetic acid solution and purified by successive filtration and extensive washing steps involving repeated precipitation and centrifugation; following this, chitosans with DA 35%, DP 900 and DA 50%, DP 820 were prepared by partial re-N-acetylation using acetic anhydride in 1,2-propanediol, as described previously (Vachoud et al. 1997). The DA of the resulting chitosans was determined using ^{1}H NMR spectroscopy (Hirai et al. 1991), and the DP using HP-SEC coupled to RI and MALLS detectors (Lamarque et al. 2005).

Preparation of agar plates containing colloidal chitin or chitosan

Luria–Bertani and M9 minimal media were prepared and used as described (Sambrook and Russell, 2001). To M9 minimal medium, 0.5% of colloidal chitin was added as a sole carbon source to prepare agar plates to screen for chitinolytic activity. LB medium agar plates with 0.9% chitosan DA 3% were prepared to identify chitosanolytic fungi.

Isolation of chitinolytic* or *chitosanolytic fungi and bacteria from soil samples

*Fungi were isolated by dilution plating method (*Waksman*,* 1927*) and Warcup soil-plate method (Warcup 1950). For dilution plating, 2 g of soil was suspended in 1 mL of sterile distilled water and ten-fold dilutions of this were spread on PDA (Difco Potato Dextrose Agar medium, Becton & Dickinson, Sparks, USA) agar plates containing chloramphenicol (0.15 mg/mL) to obtain individual fungal colonies. For soil plates, 2 g of soil was placed in each sterile Petri dish, cooled PDA medium (10-12 mL) was added and soil particles were spread in the medium. All plates were prepared in replicates. One set was incubated at room temperature (20-22°C) and another set was incubated at 28°C for 15-20 days in the dark to obtain fungal colonies.*

Fungi that appeared in serial dilution and soil-plate methods were isolated, purified and identified using standard taxonomic keys before being screened for chitinolytic and chitosanolytic activities.

Bacteria were isolated using a modified serial dilution method of Maltseva et al. (1997). Initially, 10 g of soil was inoculated in LB (Luria-Bertani) medium and another 10 g of soil was inoculated in M9 minimal medium with colloidal chitin (0.5%) to enrich chitinolytic microorganisms. Later, a few microlitres of the enriched cultures were spread on LB agar plates to obtain isolated colonies. Concomitantly, 1 g of soil was suspended in 1 mL of sterile distilled water and ten-fold dilutions of this were spread on LB agar plates and incubated at 37°C for isolating bacteria. Pure cultures were obtained by re-streaking the colonies several times until single colonies were obtained. Pure bacterial isolates were then screened for chitinolytic activity on colloidal chitin (0.5%) in minimal medium agar plates.

Preparation of samples for zymography and Thin Layer Chromatography

Each fungal isolate was grown in potato dextrose medium for 7 days as static culture at 28°C in the dark, and the mycelium was removed by filtration. One hundred ml of the culture filtrate was dialyzed (MWCO 1,000 Da) for 24 h against distilled water at 4°C. The dialyzed culture filtrate was lyophilized and used as a source of enzyme; 10 mg of the lyophilized culture filtrate was mixed in 1 mL of 50 mM sodium acetate buffer (pH 5.2) and centrifuged at 16,000 g for 5 min (20˚C). From this preparation, 5-10 µL of sample was used for dot activity assay or zymography.

Bacterial isolates were grown in 10 mL of LB medium for 48 h at 37°C. Then, cell pellets were spun down and the culture supernatants were lyophilised. Lyophilised samples were dissolved in 1 mL of 5 mM sodium acetate buffer (pH 5.0) and used for assessing enzyme activities.

Detection of chitinase and chitosanase enzyme activity dot assay or zymography

Chitinolytic and chitosanolytic enzyme activities were detected using a dot activity assay as described previously (Rajulu et al. 2011). Briefly, 5 µL from each fungal culture filtrate was applied on the gels prepared with glycol chitin (0.3 mg/mL) and chitosan DA 35% (0.1 mg/mL). Gels were incubated at 37˚C overnight, activity was detected by staining the gel with calcofluor and enzyme activity was visible as dark spot under UV transilluminator.

For detecting the activity of chitinase and chitosanase isoenzymes, SDS-PAGE (12%) was performed in gels containing 0.3 mg/mL of glycol chitin for chitinase or 0.1 mg/mL of one of two chitosans (DA 50 % or DA 35%) (Trudel and Asselin 1989). After electrophoresis (50 mA for 4 h), the gel was washed twice for 20 min each in 50 mM sodium acetate buffer (pH 5.2, with 1% Triton X-100). It was then washed twice in the same buffer for 20 min each (without 1% Triton X-100). The gel was incubated at 37˚C for 12 h under shaking in 50 mM sodium acetate buffer (pH 5.2) and stained with 0.01% Calcofluor White (Sigma, Steinheim, Germany) in 0.5 M Tris-buffer (pH 8.9) for 5 min; finally, the gel was washed in deionized water for 1 h and the enzyme activity was visualized on a UV transilluminator.

Detection of chitosan oligomers by thin layer chromatography

Aliquots (20 µL) of spent culture media of selected fungal isolates were mixed with 20 µL of chitosan DA 35% solution (1 mg/mL) and incubated overnight at 37˚C in 50 mM sodium acetate buffer (pH 5.5). Samples were concentrated under reduced pressure to scale down the volume to 10 µL. Aliquots (10 µL) were applied on TLC plates (Merck, Berlin, Germany), then run in butanol:methanol:ammonia:water (5:4:2:1, v/v/v/v) and stained using aniline-diphenylamine reagent (4 mL of aniline, 4 g of diphenylamine, 200 mL of acetone, and 30 mL of 85% phosphoric acid). Oligomers were visualised by heating the plate at 180˚C for 3-5 min. Oligomers were compared with authentic N-acetylglucosamine (DP 1, 2, 3, 5, 6) and D-glucosamine (DP 1, 3, 4) standards (Seikagaku, Tokyo, Japan).

16S-rDNA analysis of bacterial isolates

Colony PCR was performed on the bacterial soil isolates using bacterial universal primers (Hogg and Lehane 1999): forward primer (5´AGAGTTTGATC(AC)TGGCTCAG3´); reverse primer (5´AAGGAGGTGATCCA(AGCT)CC(AG)CA3´). Amplicons were cloned into PCRII-TOPO vector and sent for sequencing (MWG, Ebersberg, Germany). Blast analyses were performed with the sequences obtained in the NCBI database. Sequences obtained were deposited in NCBI under Gene Bank with sequence id's JN593073-JN593080.

Results

Isolation and screening for chitinolytic or chitosanolytic bacteria and fungi

A total of sixty bacterial strains were isolated from eight soil samples collected from different sites of the chitin/chitosan producing company. When grown on minimal medium agar plate containing 0.5% colloidal chitin as a sole carbon source, eight strains produced clear zones consistently around their colonies indicating chitinolytic activity; one strain showing weak activity failed to show repeated activity and was excluded from further study (Fig. 1A).

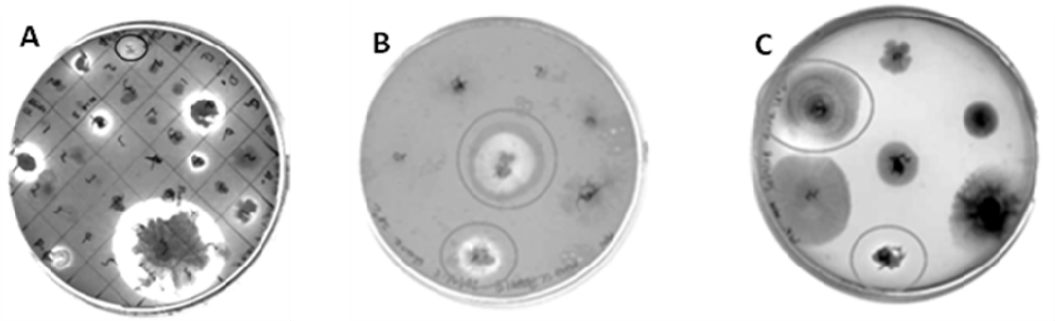

Fig. 1: Screening for chitinolytic and chitosanolytic activities in bacterial and fungal isolates from soil samples. A, bacterial strains showing clearing zones on minimal medium agar plates containing colloidal chitin; one strain showing weak chitinolytic activity (top, marked with circle) was excluded from further studies as it did not show the activity consistently. B and C, examples of fungal strains showing clearing zones on agar plates containing colloidal chitin in minimal medium (B) or chitosan DA 3% in LB medium (C).

The eight strains were grown in liquid LB medium and observed under the microscope to check for morphological characters. Microscopic observations initially suggested that all of them were *Bacillus* species, differing in their motility, sporulation and arrangement of spores. 16S-rDNA analyses corroborated this identification for seven of these strains which all belonged to the *cereus/anthracis/thuringiensis* group of *Bacillus*. The eighth strain was identified as *Gammaproteobacteria,* having high similarity with *Enterobacter cloacae* (99%), *Klebsiella pneumoniae* (97%), *K. variicola* (98%), and *Salmonella enterica* (98%).

A total of 25 different fungal strains were isolated from the two different soil samples by dilution and soil-plate method. These strains were tentatively classified based on morphology. The fungal isolates were dominated by Aspergillus species; other genera included Acremonium, Aureobasidium, Cladosporium, Curvularia, Drechslera, Fusarium, Penicillium, and Sporormiella (Table 1). Of seven randomly chosen fungal isolates, two showed clearing zones on chitin (Fig. 1B), and two others on chitosan (Fig. 1C), suggesting an abundance of chitinolytic and chitosanolytic fungi in these soil samples. When crude

enzyme extracts of all 25 isolates were screened for chitinolytic and chitosanolytic activities in a dot assay, most of the isolates showed activity on one or both of the substrates tested, namely glycol-chitin and chitosan DA 35 % (Table 1).

Table 1: Screening of crude extracts of 25 different fungal soil isolates using dot activity assays on glycol chitin or chitosan DA 35%. + = positive; - = negative

isolate	name of the fungus	activity in dot assays	
		glycol chitin	chitosan DA 36%
1	*Fusarium sp*	+	-
2	not identified	-	+
3	*Penicillium sp*	+	+
4	*Aspergillus sp*	+	-
5	*Acremonium sp*	-	-
6	*Cladosporium sp*	+	+
7	*Cladosporium sp*	-	+
8	*Aureobasidium pullulans*	+	-
9	*Aureobasidium pullulans*	+	-
10	not identified	+	+
11	not identified	-	+
12	not identified	-	+
13	not identified	+	-
14	*Aspergillus sp*	-	-
15	*Curvularia sp*	-	-
16	not identified	-	-
17	*Aspergillus sp.*	+	+
18	*Aspergillus niger*	+	+
19	*Sporormiella intermedia*	-	-
20	*Cladosporium cladosporioides*	-	+
21	*Drechslera sp*	+	+
22	*Drechslera sp*	+	-
23	*Aspergillus sp*	-	+
24	*Acremonium sp*	+	+
25	not identified	-	+

Chitinolytic and chitosanolytic enzymes from bacterial and fungal soil isolates

Crude extracts of the eight chitinolytic bacterial strains isolated were separated on semi-native–SDS-PAGE gels to analyse chitinolytic and chitosanolytic isoenzymes. Different substrates were co-polymerised into the zymography gels, namely glycol chitin (Fig. 2A), chitosan DA 56% (Fig. 2B), or chitosan DA 35% (Fig. 2C). A crude extract of a known chitinolytic strain of *Bacillus licheniformis* (Songsiriritthigul et al. 2010) was loaded as a positive control. As expected, the crude extract of *B. licheniformis* showed activity on all three substrates whereas the crude extracts from the different soil bacterial strains showed differences in their activities. Isolates 2, 3, 5, and 7 showed the same two high-molecular weight chitinases as *B. licheniformis,* while the extracts from isolates 1, 4, 6, and 8 were not active towards glycol chitin (Fig. 2A). All isolates including *B. licheniformis* produced one high MW isoenzyme degrading chitosan DA 56%, and isolates 2, 4, and possibly 7 possessed an additional isoenzyme with a MW between 50 and 75 kDa capable of degrading this chitosan (Fig. 2B). Also, all of the strains including *B. licheniformis* possessed isoenzymes degrading the chitosan DA 35% (Fig. 2C), where isolates 1 and 5 produced the fewest and most weakly active isoforms and isolates 4 and 7 produced the highest chitosanase activities. Considering activities on all three substrates, it becomes clear that all eight isolates differ between each other and from *B. licheniformis* in their chitinolytic and chitosanolytic isoenzymes, but the diversity is limited.

PCR was performed on genomic DNA of the eight bacterial soil isolates using primers designed from conserved regions of known *Bacillus* chitosanases. Amplicons were observed at 1.3 Kb only in strains 1, 3, 6, and 7 (data not shown), the other strains did not show any amplification. Amplicons were sequenced, and Blast results showed that the sequences were identical to the known chitosanase sequence of *Bacillus sp. strain KCTC 0377BP (*Choi et al. 2004).

To analyse chitosanolytic isoenzymes of fungi, crude extracts of fungal isolates which were positive in dot assay with chitosan DA 35% as a substrate, were subjected to semi-native SDS-PAGE in a gel containing chitosan DA 35% (Fig. 3). Isoenzyme activity was seen in all isolates, but the isolates differed in the number of isoforms present and in the overall activity. The number of isoenzymes found ranged from one to three, and their MW ranged from very low to very high. Isolates 3, 10, 17, 18, and 23 (*Penicillium* and all three chitosanolytic *Aspergillus* isolates) had one very strong activity at MW of ca. 250 kDa; isolates 7 and 20

(two of the three *Cladosporium* isolates) showed one sharp band around 50 kDa, and isolates 11, 12, and 18 were characterised by one or two low MW isoforms between 10 and 20 kDa.

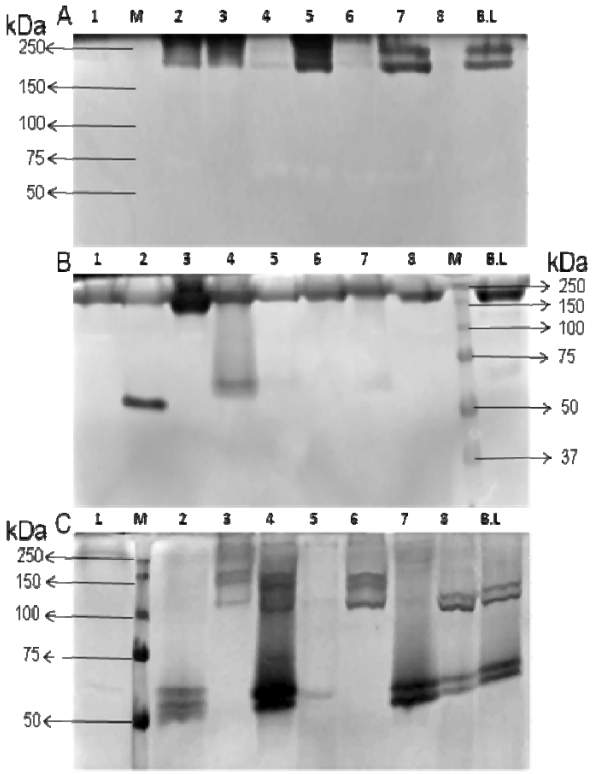

Fig. 2: Semi-native SDS-PAGE of crude extracts of the bacterial soil isolates (1-8), followed by zymography using glycol chitin (A), chitosan DA 56% (B), or chitosan DA35% (C) as a substrate. A known chitinolytic strain of *Bacillus licheniformis* (B.l.) was used as a positive control. The positions of marker proteins (M) are given on the sides of the gels.

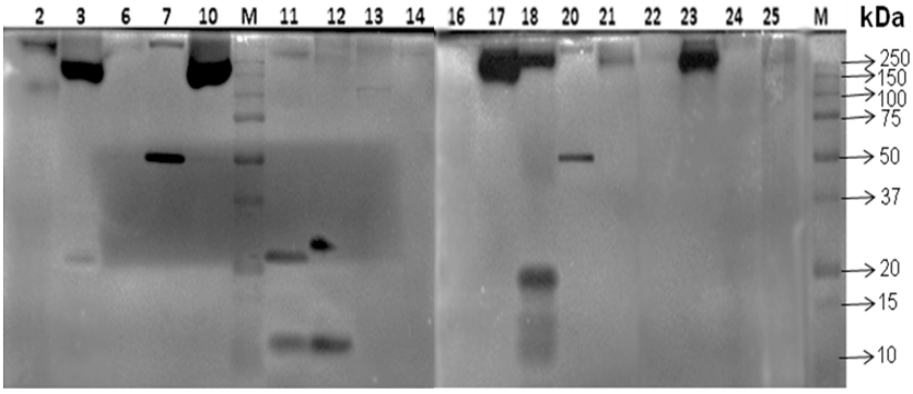

Fig. 3: Semi-native SDS-PAGE of crude extracts of selected fungal soil isolates (numbers correspond to Table 1), followed by zymography using chitosan DA 35% as a substrate. The positions of marker proteins (M) are given on the right side.

Samples which differed clearly in their isoenzyme spectrum were selected and separated using isoelectric focusing (IEF). For zymography, polyacrylamide gels containing different

chitosans with DA 35% and DA 56% were overlaid on the IEF gel after the run (Fig. 4). Gels were incubated at 37°C overnight, stained using Calcofluor White to detect chitosanolytic activity. All fungal isolates tested possessed one to four chitosanolytic isoenzymes with isoelectric points in the range of pH 4 to 8. While few differences were seen between the two substrates, clear differences were obvious between the different isolates.

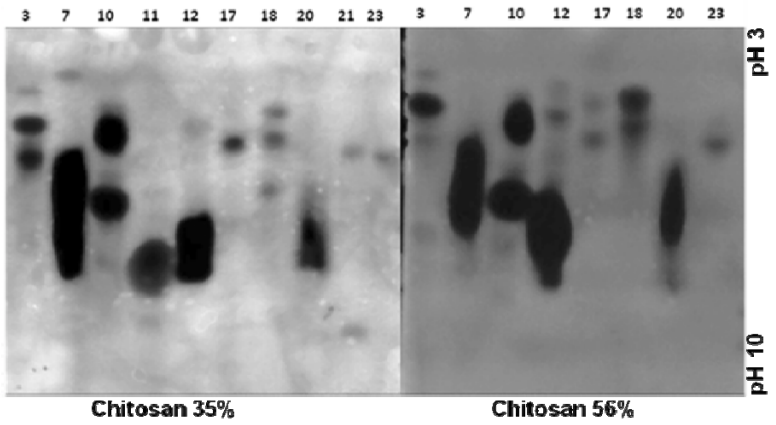

Fig. 4: Isoelectric focussing of crude extracts of selected fungal soil isolates (numbers correspond to Table 1), followed by zymography using overlay gels containing chitosan DA 35% (A) or chitosan DA 56% (B) as a substrate. The pH range of the gels is indicated at the right side.

Chitosan oligomers produced by chitosanolytic enzymes of selected fungal soil isolates

Spent culture filtrates from fungal isolates which showed a single dominant isoenzyme in zymography (isolates 3, 7, 10, 12, 17, 20, 23) were incubated with chitosan DA 35% at 37°C, and the chitosan oligomers produced were analysed using TLC (Fig. 5). This preliminary analysis showed that different oligomer mixtures were produced by each fungal isolate, ranging from only the monomers GlcN and GlcNAc (isolates 3, 23) to a mixture of small oligomers ranging in degree of polymerization from 2 to 6 (isolates 7, 10, 12, 20). Isolate 17 produced only larger oligomers.

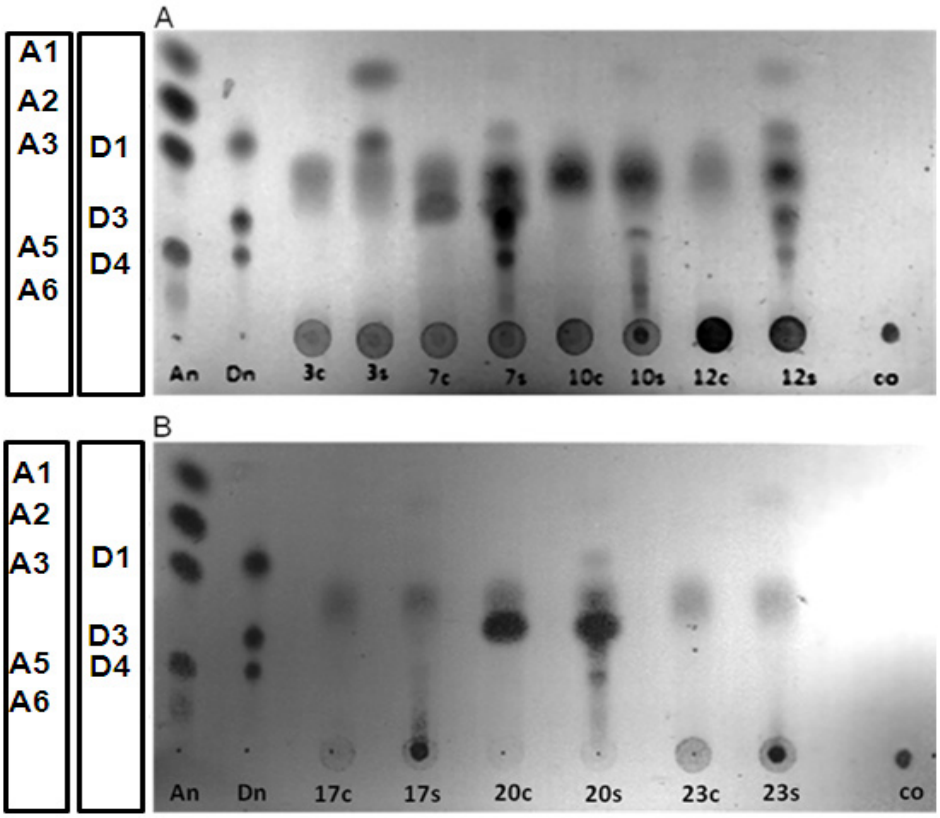

Fig. 5: TLC analysis of the products of chitosan (DA 35%) incubation with crude extracts of selected fungal soil isolates (numbers correspond to Table 1). Crude extracts were incubated with (samples labeled 3s, 7s, 10s, 12s, 17s, 20s, 23s) or without chitosan (samples labeled 3c, 7c, 10c, 12c, 17c, 20c, 23c) as a substrate. Chitosan incubated without any crude extract (co) was used as a control, and oligomers of GlcNAc (An) and GlcN (Dn) were used as standards. The DP of the standards is given on the left sides of the plates.

Discussion

This study is part of a larger project aiming at detecting novel chitin and chitosan degrading and modifying enzymes. To this end, we first identified soil samples with a long history of exposure to chitin in the form of shrimp shells, chitin, and chitosan, which we found on the premises of the chitin/chitosan producing company Mahtani Chitosan in Veraval, Gujarat, India. Here, ca. 5,000 tons of fresh and dried shrimp shells are being processed annually since 1995, to produce ca. 150 tons of chitin, of which ca. 36 tons are partially deacetylated to yield ca. 25 tons of chitosan, per year. From such soils, we isolated microorganisms to screen them for chitin and chitosan degrading enzymes.

In a previous study, our collaborators from India have reported on the bacterial biodiversity and its chitinolytic potential in these soil samples (Das et al. 2010). While in the former study, the bacterial soil isolates were dominated by *Gammaproteobacteria,* we found mainly

Bacillus species in our study, possibly becausen the latter are more stable in the soil and could have survived during storage and transport of soils from India. This was not unexpected as *Bacillus* species are well known chitin degraders in soils (Cody 1989; Nishijima et al., 2005). Interestingly, we found seven different species of *Bacillus* which differed in their chitinolytic and chitosanolytic enzymes. All the seven belonged to the *cereus/anthracis/thuringiensis* group of *Bacillus* (Alcaraz et al. 2010) which is well known for its potential to degrade chitin and chitosan (Ivanova et al. 2003). While the only chitinase isoenzymes seen were two high MW bands, also seen in a refernce bacterial strain *B. licheniformis* strain with known chitinolytic potential (Tantimavanich et al. 1998; Chuang et al. 2008; Neeraja et al. 2010b; Neeraja et al. 2010c), the diversity of chitosanases was more pronounced. When using degenerate primers based on known *Bacillus* chitosanases (Su et al. 2006), we were able to amplify a chitosanase gene from four of the seven strains, but it turned out to be identical to a known chitosanase gene from *Bacillus sp. strain KCTC 0377BP* (Choi et al. 2004). We have now set up a pooled genomic DNA library of these strains which is currently being screened for chitinase and chitosanase genes.

While chitinolytic and chitosanolytic fungal species isolated from soil have been reported often (Sherief et al. 1991; Sharaf 2005; Shindia, 2007), this to our knowledge is the first report on fungal diversity in soils with a history of chitin/chitosan exposure. Chitinolytic and chitosanolytic enzymes can be expected to be found in fungi firstly for chitin and chitosan degradation and, thus, to use the polysaccharides as a carbon and nitrogen source (Battaglia et al. 2011). Secondly, fungi are known to produce chitinolytic enzymes for cell wall remodeling during developmental processes (El Gueddari et al. 2002; Lindahl and Finlay 2006). This may be the reason why almost all of the fungal isolates screened were positive for chitin and/or chitosan degrading enzymes: 13 were chitinolytic, 14 were chitosanolytic, and seven isolates were both chitinolytic and chitosanolytic.

Out of 20 fungal isolates which were chitinolytic and/or chitosanolytic, 14 belong to seven different genera, namely *Acremonium, Aspergillus*, *Aureobasidium*, *Cladosporium*, *Drechslera, Fusarium*, and *Penicillium*, the remaining six were not identified. All of these genera are known saprotrophic often found in soils (Satish, 2007). *Aspergillus* and *Fusarium* are known to produce chitinases and chitosanases and these enzymes have been characterised extensively (Mathivanan et al. 1998; Jaques et al. 2003; Shindia, 2007). *Penicillum* is also known to produce both chitinase and chitosanase (Fenton and Eveleigh 1981; Binod et al.

2005). The production of different chitinases and/or chitosanases has also been reported for plant endophytic fungal isolates belonging to the genera *Aureobasidium, Cladosporium*, *Drechslera*, and *Fusarium* (Rajulu et al. 2011). *Aureobasidium pullulans* produces exo- and endo-type chitinases and exhibits antagonistic properties towards post-harvest pathogens (Zhang et al. 2010). Anamorphic fungi were more frequently isolated (Rajulu et al. 2011).

Interestingly, even isolates from the same genus differed significantly in their chitinolytic and chitosanolytic potential. A typical case is the genus *Aspergillus* which dominated the fungal soil isolates. Of the five *Aspergillus* isolates, one was chitinolytic but not chitosanolytic, one was chitosanolytic but not chitinolytic, two were both chitinolytic and chitosanolytic, and one was neiter chitinolytic nor chitosanolytic. Similarly, of two *Acremonium* isolates, one had neither, one had both activities, all three *Cladosporium* isolates were chitosanolytic but only one was also chitinolytic, and both *Drechslera* isolates were chitinolytic but only one was also chitosanolytic. Clearly, these soils with their rich fungal biodiversity are a promising source for potentially novel chitinolytic and chitosanolytic enzymes.

This assumption was corrobrated by the analysis of isoenzyme patterns using zymography, both using semi-native SDS-PAGE separating isoenzymes according to size and using IEF separating them according to their isoelectric point. As we were more interested in chitosanases, semi-native SDS-PAGE and IEF zymographies were perfomed using chitosans as substrates. Some fungal isolates that had tested positively in the dot activity assay using chitosan DA 35% as a substrate showed only weak activities in the zymography with a similar substrate, possibly because some enzymes failed to renature properly when the SDS was washed out. In terms of isoenzymes detected, the resolving power of IEF was superior to that of native PAGE. For example, sample number 12 showed one isoform on SDS-PAGE, but four isoforms were resolved in IEF, and similar results were seen for samples 3, 10, 17, and 20. Most fungal isolates produced more than one chitosanolytic isoenzyme, adding to the diversity. Inspite of this multiplicity of enzymes, chitosan DA 35% was not fully degraded to monomers or very small oligomers by all crude spent fungal culture media. Instead, oligomers of the biologically interesting size of pentamer and larger were produced in some cases, e.g. by isolates 7, 10, 12, 17, and 20 - i.e. including one *Aspergillus* and two *Cladosporium* isolates.

In conclusion, the present study revealed strong and highly diverse chitinolytic and chitosanolytic activities in the microorganisms isolated from soil samples with a history of

chitin/chitosan exposure, and the fungal diversity by far exceeded the bacterial diversity. To our knowledge, analysing the biodiversity of microorganisms in an enivronmental sample by screening for the diversity of isoenzymes and for the oligomers produced by these enzymes is a novel but promising approach. The high diversity found is of biotechnological relevance as isolated bacterial and fungal chitinases and chitosanases (Dahiya et al. 2006; Neeraja et al. 2010a) as well as the oligomers produced by purified or crude chitinolytic and chitosanolytic enzymes (Cheng and Li 2000; Choi et al. 2004; Liang et al. 2007) may have interesting and diverse biological activities and may, thus, be useful in a wide range of applications. We believe that our ongoing screening of metagenomic libraries established from these soil samples will yield even more and even more diverse chitin and chitosan modifying enzymes.

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CHAPTER 4

"This chapter will be submitted for publication with the following co-authors: Stephan Kolkenbrock (who was involved in designing the strategy and in discussing the results), Daniel Auriol (who constructed the large insert fosmid library; this part of the work is indicated by italics in the text below), Bruno M. Moerschbacher (who supervised my work and who was involved in designing the strategy of the project and in discussing the results)."

Metagenomics to identify novel chitin and chitosan modifying enzymes from soil samples with a history of chitin and chitosan exposure

Abstract

Novel genes can be identified by the construction and screening of metagenomic libraries. In the present study we describe the construction of *large insert (using fosmid)* and small insert (using plasmid) metagenomic libraries in *Escherichia coli* using metagenomic DNA isolated from soil that were screened for chitin and chitosan modifying enzymes. The *large insert library consists of 42,000 clones with an insert size of 40 kb* and the small insert library consists of 14,200 clones with an average DNA insert size of 1-5 kb. Function driven screening strategies were established to screen the libraries for chitin and chitosan modifying enzymes, using clones containing a chitinase, chitosanase or chitin-deacetylase gene from *Bacillus* as positive controls. Both metagenomic libraries were screened for chitinases using clearing zone assays on colloidal chitin as a substrate and also using fluorogenic substrate analogs of chitin [4-methylumbelliferyl-D-*N*,*N*-diacetylchitobioside (MUFdiNAG) and 4-methylumbelliferyl-D-*N*,*N*,*N*-triacetylchitotrioside (MUF-triNAG)]. In addition, a congo red assay was performed for the plasmid library clones using colloidal chitin as a substrate. Screening is ongoing but so far, have not yet yielded positive clones.

Introduction

Chitin is considered to be the second most abundant polymer existing on earth. It is estimated that worldwide annual production of chitin is around 100 billion tons (Tharanathan and Kittur, 2003). Chitin is produced commercially mainly from shrimp and crab shell wastes (Percot et al., 2003). This waste fraction is generated in tremendous amounts in industries where shrimp is processed; the meat is used for food purposes, but the rest is too often

considered waste, and hence dumped, creating environmental problems (Nicol, 1991). Commercially, a small portion of the chitin is converted into chitosan, the only naturally occuring polycationic polysaccharide with many potential applications e.g. in agriculture, pharmaceutics, food sciences, pharmacology, and bio-medicine (Lamarque et al., 2005; Zhang et al., 2010). However, the term chitosan really refers to a family of oligo- and polymers differing in their degree of polymerisation (DP), their degree of acetylation (DA), and possibly also in their pattern of acetylation (PA). This structural diversity has been implicated in the functional versatility of chitosans, but a detailed knowledge of structure/function relationships and, thus, a detailed understanding of the molecular basis of chitosan's bio-activities are still lacking (Schatz et al., 2003; EI Gueddari et al., 2007; Trombotto et al., 2008; Gorzelanny et al., 2010; Moerschbacher et al., 2011). This lack of information has been a major hindrance in the development of reliable life science applications of chitosans.

Increasingly, oligomers have become the focus of research on the biological activities of chitosans in recent years (Kim and Rajapakse, 2005; Yin et al., 2010) not least because structure/function-analyses are more feasible with oligomers than with polymers. Also increasingly, enzymatic production methods for chitosan polymers and, especially, oligomers are being explored as alternatives or, more reasonably, to complement and extend the currently prevailing chemical methods (El Gueddari et al., 2007). Chemical methods of deacetylation and depolymerisation have the drawback of risking environmental pollution even though this can be kept to a minimum with appropriate technologies (Kim and Rajapakse, 2005). But chemical methods, if carried out carefully, invariably and reproducibly lead to random distributions of DP and DA, and, potentially most importantly, to random PAs. While a reliably random PA is considered a quality criterion for today's commercial chitosans, we have argued that the PA of a chitosan will greatly influence its biological activities; in particular if a sequence specific chitosan hydrolase is present in the target tissue (Gorzelanny et al., 2010). In that case, both the half-life of the polymer and the quantity and quality of the oligomers produced as well as their rate of production and further degradation will depend on the PA of the chitosan applied.

We have argued that chitin and chitosan modifying enzymes (CCME) may offer powerful tools for the generation of novel chitosans with other-than-random PA (El Gueddari et al., 2007; Kohlhoff et al., 2009). For instance, processively acting chitin deacetylases may generate chitosans with blockwise PA, in analogy to processive pectin methyl-esterases

generating pectins with blockwise patterns of methyl-esterification (Daas et al., 1999). On the other hand, sequence-specific chitosan hydrolases will produce oligomers with defined residues at their reducing and non-reducing ends. As an example, the novel chitinosanase enzyme we have recently described, which specifically cleaves the glycosidic linkage between an acetylated and a non-acetylated unit within a chitosan chain, will yield oligomers where all the acetylated residues will form a block at the reducing end of the oligomer, whereas all the non-acetylated residues will be blocked at its non-reducing end (Kohlhoff et al., 2009).

But CCME may offer even further opportunities beyond a more environmentally benign approach to chitin and chitosan production and the prospect of allowing the preparation of novel chitosans with non-random PA. Enzymes such as chitinases and chitosanases themselves may have a direct potential for applications because of their bio-activities, such as antimicrobial properties (Grenier and Asselin, 1990; Patil et al., 2000; Ghormade et al., 2010). Lastly, again in analogy to what has been developed in pectin technology (Voragen et al., 2009; Westphal et al., 2010), CCME may be used to develop enzymatic/mass spectrometric fingerprinting analytical methods, e.g. for the analysis of the PA of chitosans (Moerschbacher et al., 2011).

In spite of substantial work on CCME to date, it can be expected that a large diversity of novel, potentially biotechnologically precious CCME remain to be discovered. To tap into this presumed diversity, it is advisable to try and harvest the potential of the vast majority of non-culturable micro-organisms which have so far mostly escaped the attention of researchers. One promising source for such chitinolytic and chitosanolytic micro-organisms might be soil, and in particular soil with a history of exposure to chitin and chitosan containing materials. We have shown that soil samples collected from the premises of a chitin and chitosan producing company (Mahtani Chitosan Pvt. Ltd. India) are rich in chitinolytic bacteria and fungi (Das et al., 2010; Chapter 3). We have now used these same soil samples for the extraction of DNA and the construction of metagenomic DNA libraries.

High quality soil DNA free of contaminants such as humic substances and phenolics (Yeates et al., 1997; Harry et al., 1999; Kauffmann et al., 2004) is necessary for the construction of promising metagenomic libraries. Numerous manual DNA extraction methods have been developed for soil samples and alternatively, several commercial kits optimised for soil DNA extraction are available (Zhou et al., 1996; Ikeda et al., 2004; Mahmoudi et al., 2011), but a

suitable method needs to be established for each individual case. Once pure DNA is available, metagenomic libraries can be constructed using plasmids or fosmids (Schmeisser et al., 2007). The classical approach, i.e. the construction of small insert libraries with an insert size of 1-10 kb using plasmids, does not allow the detection of large gene clusters which, however, can be obtained when a fosmid library is constructed cloning large fragments of DNA with an insert size of ca. 40 kb. Both approaches have been used successfully to identify novel enzymes from environmental samples (Cottrell et al., 1999; Henne et al., 1999; Rondon et al., 2000; Gillespie et al., 2002; Sharma et al., 2010).

Metagenomic libraries can be screened for the genes of interest using two strategies, namely function-driven approaches and sequence-driven approaches (Schloss and Handelsman, 2003). Both stratagies have their advantages and limitations. The limitation of all function-driven approaches is that complete genes must be cloned in the correct direction and in the correct reading frame, and then must be transcribed and translated successfully into functional proteins in a heterologous host, typically *E. coli.* Sequence driven approaches are limited in so far as the probes or the PCR primers used for screening are dependent on the existing sequence data of known genes, so that chances of finding truly novel genes are lower than with functional approaches(Cottrell et al., 1999; Schloss and Handelsman, 2003). Metagenomic approaches have been successfully employed in identifying different polysaccharide modifying or degrading enzymes, e.g. amylases, cellulases, xylanases and xylosidases, agarases, and chitinases (Schmeisser et al., 2007).

In this study, we describe the development of DNA extraction protocols optimised for the soil samples of the chitin and chitosan manufacturing company Mahtani Chitosan in Veraval, Gujarat, India, which we have shown to possess a rich diversity of chitin and chitosan modifying bacteria and fungi (Das et al., 2010; Chapter 3). We then describe the construction of a large insert fosmid libray with an average DNA insert size of 40 kb, and of a small insert plasmid library with an average DNA insert size of 1-5 kb as well as the development of functional screening protocols for chitinases, chitosanases, and chitin deacetylases, including the construction of positive controls for these enzymes using known genes in appropriate vectors. The functional screening of both libraries is ongoing but has not yet yielded positive clones, and a sequence-driven screening is planned.

Materials and Methods

Chemicals

Restriction enzymes, T4 DNA ligase and pUC19 plasmid were purchased from Thermo Scientific, Germany. Robot Qpix2^{XT} (Genetix, Hampshire, UK) was used for colony picking. Nunc Petriplates (Catalog no 240835) were purchased from Thermo Fisher Scientific, Roskilde, Denmark. Microtiter plates with 384 wells were purchased from Greiner (Germany) for growing clones and storage of library. All the chemicals used were molecular biology grade purchased from Roth or Sigma Aldrich (Germany). Chitin analogs 4-methylumbelliferyl-D-*N*,*N*-diacetylchitobioside (A2-MUF) and 4-methylumbelliferyl-D-*N*,*N*,*N*-triacetylchitotrioside (A3-MUF) were purchased from Sigma Aldrich. Nitrocellulose membrane was purchased from GE Healthcare, Germany.

Bacterial strains, plasmids and culture conditions

Escherichia coli DH5α was used as a storage host for recombinant plasmids. *E. coli* BL21 (DE3) [pRARE2] in combination with the expression vector pET-22b(+) (Novagen, Germany) was used as the host for over expression. Plasmids with inserts were sequenced at MWG eurofins. *E. coli* BL21(DE3) [pRARE2] as host and pET-22b(+) expression vector (Novagen) were used for construction of small insert library and *E. coli* EPI100 was used as host for large insert fosmid library. *E. coli* DH5α and *E. coli* BL21(DE3) [pRARE2] with pET-22b(+) constructs were grown in lysogeny broth (LB) at 37^{o}C with 100 µg/ml ampicillin and 50 µg/ml carbenicillin plus 34 µg/ml chloramphenicol, respectively for the selection of the transformants. For expression, cultures were grown in LB at 37^{o}C with auto induction medium "M" and "5052" (AIM) (Studier, 2005). For long term storage liquid cultures were supplemented with 30% (v/v) glycerol and stored at -70^{o}C. Libraries were stored in microtiter plates with 384 wells containing LB plus 10% (v/v) glycerol in the culture medium. The constructed plasmids (Table 1), primers (Table 2) and their details used in the present study are given below.

Table 1: List of plasmids used for establishing the positive controls

Plasmid	**Purpose**	**Description**	**Gene bank accession number**	**Source**
pUC19	Cloning&expression	Commercially available	L09137	Thermo Scientific
pET22b(+)	Cloning&expression	Commercially available		Novagen
pUC19+chi	Positive control for screening strategies	*Bacillus licheniformis* DSM13 chitinase in pUC19	GQ899144	Present study
pUC19+csn	,,	*Bacillus sp* chitosanase in pUC19	AF334682	,,
pUC19+ CDA5	,,	*Bacillus licheniformis* DSM13 chitin deacetylase5 in pUC19	AAU41328	,,
pET-22b+*B.lichi*	,,	*Bacillus licheniformis* DSM13 chitinase in pET-22b(+)	GQ899144	,,
pET-22b+*B.tchi*	,,	*Bacillus thuringiensis* chitinase in pET-22b(+)	GQ899142	,,
pET-22b+*B.sp* csn	,,	*Bacillus sp* chitosanase in pET-22b(+)	AF334682	,,
pET-22b+ CDA5	,,	*Bacillus licheniformis* DSM13 chitindeacetylase5 in pET-22b(+)	AAU41328	,,
pET-22b+ CDA1	,,	*Bacillus icheniformis* DSM13 chitindeacetylase1 in pET-22b(+)	AAU39593	,,

Table 2: List of the primers used to construct positive controls. *E. coli* DH5α was used for plasmid storage while *E. coli* BL21(DE3) [pRARE2] was used as positive control, since they are capable to express the cloned genes.

S. No	Name of the primer	Sequence 5'-3'	Restriction sites	Name of the gene	Name of the clone	Size of amplicon (Kb)
1	B.li for	gataagcttggattccggaaaaaactataaaatc	*Hind*III	*Chi* from B.li GQ899144	Puc19D chi	1.7
2	B.li rev	acatctagactatccatttgactttctgttattc	*Xba*I			
3	csn for	agcaagcttgatgaatggaaaaagaaatatttttac	*Hind*III	*Csn* from B.sp AF334682	Puc19 csn	1.3
4	csn rev	actgaattcttaattatcgtatccttcatag	*Eco*RI			
5	CDA5 for	aactgcaggggcccaaagcaggaacc	*Pst*I	*Cda5* from B.li AAU41328	Puc19 CDA5	0.7
6	CDA5 for	cccgagctcctatttttcaaattgaggatgtgacc	*Sac*I			
7	B.li for	cgcggatccggattccggaaaaaactataaaatcatc	*Bam*HI	*Chi* from B.li GQ899144	R2Blichi	1.7
8	B.li rev	cgctcgagttcgcagcctccgatcagccg	*Xho*I			
9	B.t for	cggaattcggattcaccaaagcaaagtcaaaaaattg	*Eco*RI	*Chi* from B.t GQ899142	R2Btchi	1.9
10	B.t rev	ccaagcttgttttcgctaatgacggcatttaaaag	*Hind*III			
11	csn for	tcagaattcggctgcaaaggaaatgaaaccatttc	*Eco*RI	*Csn* from B.sp AF334682	R2csn	1.3
12	csn rev	gtcaagcttattatcgtatccttcatagattg	*Hind*III			
13	CDA5 for	ccatggaattccatatgggcccaaagcaggaac	*Nde*I	*Cda5* from B.li AAU41328	R2CDA 5	0.7
14	CDA5 for	cgagctcgaaattccatctgaaatgaaccg	*Sac*I			
15	CDA1 for	ccatggaattccatatgttgatttttttaacgatattaataggtttgg	*Nde*I	*Cda1* from B.li AAU39593	R2CDA 1	1.3
16	CDA1 for	cgagctcgaaatagcctctctgcttcttcac	*Sac*I			

Preparation of colloidal chitin and chitosans

Colloidal chitin was prepared according to the method described by Berger and Reynolds, (1988) with the following modifications. To 10 g of β-chitin (Mahtani Chitosan Pvt Ltd, India) 500 ml of conc. HCl was added and mixed under stirring until the mixture was homogeneous, the sample was then incubated at 4°C over night. Two Liters of double distilled water was then added and kept for stirring at 4°C for another 48 hours.

Subsequently, colloidal chitin was washed with ddH_2O until the pH became neutral. Chitosan (average DA 3%, average DP 2,124) was generously provided by Dr. Gillet, Mahtani Chitosan, Veraval, India. This raw chitosan was dissolved in an aqueous acetic acid solution and purified by successive filtration and extensive washing steps involving repeated precipitation and centrifugation, before chitosans with DA 35%, DP 900 and DA 50%, DP 8200 were prepared by partial re-N-acetylation using acetic anhydride in 1,2-propanediol, as described previously (Vachoud et al., 1997). The DA of the resulting chitosans was determined using ^{1}H NMR spectroscopy (Hirai et al., 1991), and the DP using HP-SEC coupled to RI and MALLS detectors (Lamarque et al., 2005).

Preparation of colloidal chitin agar plates

LB and M9 minimal media were prepared as described in Sambrook and Russell (2001). M9 minimal medium was supplemented with 0.5% (w/v) of colloidal chitin was used to prepare agar plates to screen for chitinolytic activity. AIM "5052" and "M" (Studer, 2005) were prepared and included in the minimal medium and LB medium agar plates.

Sample collection

Seven soil samples were collected from different sites of a chitin/chitosan producing company (Mahtani Chitosan (MC) Pvt. Ltd., Gujarat, India). Soil samples were collected from depths of 5-10 cm. Samples were sent to Germany via airmail (export permit of the National Biodiversity Authority of India), and stored at 4°C for a maximum of two months until they were processed. Two of these soil samples were collected close to the chitin drying platform (labelled as C), and one close to the dry shells drying area (labelled as E). Rest of the soil samples were not considered for the library construction as the yield of isolated DNA was very low in preliminary experiments (data not shown).

Cloning strategy for construction of small insert metagenomic DNA library

Metagenomic DNA extraction from soil samples

Two methods were chosen to isolate metagenomic DNA. Initially, DNA isolation was performed according to the method described by Zhou *et al.* (1996). Seven different soil samples were considered for metagenomic DNA isolation; two samples (C and E) gave good yield of DNA in comparison to other soil samples. Several purification methods were tried to purify the obtained metagenomic DNA (Zhou et al., 1996; Ikeda et al., 2004) Ultra clean soil

DNA kit and Power soil DNA kit, Mobio, Hamburg, Germany), however, DNA quality was not sufficient for performing restriction digestion for cloning to construct the metagenomic library (data not shown). Good concentration of metagenomic DNA was obtained by using ultraclean soil DNA isolation kit (Mobio, Hamburg, Germany). Obtained metagenomic DNA was of good quality to perform restriction digestion and cloning to construct the library.

Amplification of pET-22b(+) vector

To avoid relegation of blunt ended pET-22b(+) expression vector, it was amplified with different sets of primers (Table 3) without multiple cloning site. But the start codon was included as the whole library is constructed under inducible T7 promoter to get high expression of cloned genes. Extra bases were added at the 5' end of the primer for pET22-for2 and pET22-for3 to get different open reading frames to clone the inserts. But the reverse primer used was the same for all the three PCR reactions. Circular vector DNA (pET-22b(+)) was used as template. After amplification, the PCR products were treated with restriction endonuclease *Dpn*I to remove the methylated template DNA. The amplified, unmethylated DNA is not a substrate for *Dpn*I. Product DNA was ethanol precipitated, resuspended in 10 mM TAE (pH 8.0), and subsequently used for construction of library.

Table 3: Details of the primers used for PCR to obtain linear pET-22b(+) vector in all three reading frames.

S. No	Name of the primer	Sequence 5'-3'
1	pET22-for1	catatgtatatctccttcttaaagttaaac
2	pET22-for2	ccatatgtatatctccttcttaaagttaaac
3	pET22-for3	cccatatgtatatctccttcttaaagttaaac
4	pET22-rev	ctagcataaccccttggg

Preparation of inserts for plasmid library

Metagenomic DNA was subjected to restriction digestion by different restriction endonucleases which can generate blunt ends were tested to get the partially digested metagenomic DNA; *Msc*I and *Sma*I were found to be more effective to achieve the desired insert sizes. Metagenomic DNA was digested with fast digest *Msc*I and *Sma*I restriction enzymes individually at 37°C for 30 min. Digested metagenomic DNA fragments were pooled and run in 1% agarose gel for 1 hour at 100 volts. DNA fragments ranging from 1-5 kb were cut out and collected by electroelution. Subsequently the DNA fragments were precipitated with isopropanol and washed with 70% ethanol. Later the pellet was air-dried and dissolved in milliQ water.

Preparation and tRNA precipitation of ligation mixture

Electrocompetent cells of *E. coli* BL 21(DE3) [pRARE2] were prepared as described by Hanahan (1999). For Plasmid DNA ligations, 300-400ng of size-selected DNA (1-5kb) electroeluted from TAE agarose gel matrix (5-10 µl) and 100 ng of linearised pET-22b(+) vector DNA were mixed (pET-22b(+) linearised plasmid amplified by three set of primers was mixed in equal ratio to obtain 100 ng). Two microliters of ligation buffer (10X) and 12 U of T4 ligase were added to a final volume of 20 µl. Incubation of ligation mixture and tRNA precipitation of ligation mixture to improve the transformation efficiency was performed according to the method described by Zhu and Dean (1999) with slight modifications as follows. After ligation, 20 µl of ligated product was mixed with 5µl of 1µg/µl yeast RNA (dissolved in ultrapure water, Roche Diagnostics, Mannheim, Germany) and 250 µl of cold ethanol were added to precipitate the DNA. After 15 min incubation at -20°C, the sample was centrifuged at 14000 (× *g*) for 10 min at 4°C. The pellet was washed once with 100 µl of 70% (v/v) ethanol, air-dried, and suspended in 20 µl of ultrapure water. Fifty microliters of competent cells were mixed with suspended DNA and transformed using an Electroporator (Biorad, Germany) with the following conditions: 10 kv/cm for field strength and 2.4 ms for time constant. Transformed cells were regenerated in 0.5 ml of SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl2, 10 mM $MgSO_4$, 20 mM glucose pH 7.0) and grown at 37°C for 1 h without shaking. Cells were then plated on LB agar plates containing 34 µg/µl chloramphenicol, 50 µg/µl carbenicilin and incubated at 37°C overnight for the selection of transformants.

DNA extraction from cultivable and noncultivable bacteria for construction of large insert library

DNA was isolated from cultivable and non cultivable bacteria enriched from the soil samples. Isolated DNA was used for the construction of large insert fosmid library. E. coli EPI100 was used as a host. This work was performed by Dr. D. Auriol, Libragen, Toulouse.

Screening strategies for chitin and chitosan modifying enzymes

Chitinase assay with chitin analogs

Oligomers in which MUF was conjugated to the reducing end of the chitin dimer, or trimer were used to identify chitinases (Howard et al., 2003b). The assay has been established in microtiter plates with clones expressing chitinase from *B. licheniformis* DSM13 using as positive control, as the whole library was in micro titer plate format. The MUF-assay has been established with A2-MUF and A3-MUF by using the positive controls (Puc19Dchi, Puc19Echi, R2Blichi). The MUF-assay was performed by growing clones expressing chitinase in minimal medium (pH 7.0) containing ampicillin 100 µg/ml. *E. coli* DH5α, *E. coli* BL21 (DE3) [pRARE2] cells harbouring pUC19 and pET-22b(+)respectively were used as negative control. AIM was supplied as carbon source and also to induce the cells for expression. Initially cells were incubated for 24 hours at 37°C, and then A2-MUF and A3-MUF were added to a final concentration of 1.5 µM in 50µl medium. After adding MUF-substrates, incubation was extended for another 24 hours and release of MUF was observed by checking for fluorescence under UV light.

Assay of chitinase activities using colloidal chitin plates and congored staining

Before constructing the small insert metagenomic DNA library in pET-22b(+) system using *E. coli* BL21(DE3) [pRARE2] as a host, positive controls were constructed in pET-22b(+) systems to use them for establishing the activity-based screening assay. Chitinase from *B. licheniformis* DSM13 was cloned into pUC19 vector and transformed into *E. coli* DH5α and *E. coli* EPI100 hosts and grown on LB agar plates at 37˚C containing 50µg/ml carbenicillin. Clones were named as Puc19Dchi and Puc19Echi (Table 2). Chitinases from *B. licheniformis* and *B. thuriengensis* were cloned in pET-22b(+) vector and transformed into *E. coli* BL21 (DE3) [pRARE2] host and grown on LB agar plates containing 50 µg/ml carbenicillin and 34 µg/ml chloramphemicol, clones were named as R2Blichi and R2Btchi (Table 2). Clones expressing chitinase in different *E. coli* hosts (explained above) were checked for their activity using agar plates containing minimal medium supplemented with 0.5% colloidal chitin as a major carbon source. The plate was also spotted with *E. coli* hosts (explained above) harbouring pUC19 and pET-22b(+) plasmids as negative controls. Plate containing clones was incubated at 37˚C for 48 hours. After checking for clearing zones plates were washed with water extensively and stained with congo red staining solution (2mg/ml) for 20 minutes. Later plates were washed with 1 M NaCl three times each for 20 minutes.

Screening strategy for chitindeacetylases using chitosan affinity protein (CAP)

Clones expressing different chitin deacetylases (CDA) (R2CDA5 & R2CDA1) from *Bacillus* were grown on LB agar plates containing 50 µg/ml carbenicillin and 34 µg/ml chloramphenicol including AIM for the expression of protein. *E. coli* BL21(DE3) [pRARE2] harbouring the empty vector was used as negative control (R2 0). After growing the clones overnight at 37°C, nitrocellulose membrane coated with 0.1% (v/v) glycol chitin was overlaid on the plate containing CDA clones. Then the membrane was removed and incubated at 37°C overnight by placing the blotting papers buffered with 20 mM TEA buffer (pH 8.0) underneath. During this time the produced CDA degrade the glycol chitin available on the membrane and converts it in to chitosan. Later the membrane was washed with ddH_2O several times to make sure the colonies sticks to the membrane were washed properly. Membrane was then blocked with 5% BSA (w/v) in 1X TBS buffer for 1 hr at room temperature, then the membrane was washed with 1X TBS buffer for 15 minutes. After washing, the membrane was incubated with Chitosan Affinity Protein (CAP, which specifically binds to chitosan (Chapter 1) (0.1 mg/ml in TBS, 5% (w/v) BSA) for 1 hr at room temperature. Washing steps were proceeded with 1X TBS-TT buffer two times each 15 minutes then the membrane was incubated with streptactin HRP conjugate to detect the signal for CAP using chemiluminiscence method as the StrepII tag was fused at the N-terminus of the CAP.

Dotblot assay for screening chitin/chitosan modifying enzymes

Positive controls containing chitinase, chitosanase and chitindeacetylase (R2Blichi, R2csn, R2CDA5, Table 2) were grown in LB broth containing containing 50 µg/ml carbenicillin and 34 µg/ml chloramphemicol. AIM was supplemented in the medium for expression of proteins. Cultures were incubated at 37°C for 48 hours. Culture containing only vector (R2 0) and pure enzymes were used as negative and positive controls respectively. After incubating for 48 hours 5 µL-drops of the spent media were then spotted onto a polyacrylamid gel containing glycol-chitin and two chitosans with DAs of 10% and 35% (composition of the gel was given below). The gel was incubated at 37˚C overnight and stained with 0.01 % Calcofluor in 0.5 M Tris-buffer (pH 8.9) solution for 5 min; finally, the gel was washed in deionized water for 1 h and the enzyme activity was visualized on a UV transilluminator. To detect CDA activity gel was depolymerised with HNO_3 treatment (7.5 g of Sodium Nitrite (HNO_2), 20 ml of water and 550 µl of Conc. Sulfuric Acid (H_2So_4)) 15-20 minutes. After

depolymerisation gel was washed with water several times, activity of CDA was seen as dark spots.

Compositon to prepare a polyacrylamide gel for dotblot assay (6 cm x 8.5 cm gel)

Acrylamide (30%)	2 ml
Chitosan DA10% (1 mg/ml)	300 µl
Chitosan DA50% (1 mg/ml)	300 µl
Glycol Chitin (1 mg/ml)	200 µl
Buffer (50 mM sod.phosphate pH 6.2)	3.2 ml
40% (w/v) APS	3 µl
TEMED	3 µl

Results

Construction of small insert metagenomic DNA library

For the isolation of metagenomic DNA, several manual protocols and kits were compared, and one kit was eventually chosen which yielded the best DNA based on electrophoretic mobility and purity, and on digestibility with restriction enzymes as quality criteria. The metagenomic DNA was digested with *Msc*I and *Sma*I, individually and in combination. After restriction digestion, the three samples were pooled and separated using gel electrophoresis (Fig. 1). Inserts ranging from 1-5 kb in size were selected for electro-elution, then cloned in pET-22b (+) vector to construct the library using electro-transformation of *E. coli* BL21 (DE3) [pRARE2] cells. After regeneration, cells were plated on LB agar plates containing 50 µg/ml carbenicillin and 34 µg/ml chloramphenicol for selection of transformants. Clones were picked by a robot and inoculated into 384 microtiter well plates containing LB medium containing the same antibiotics. Inoculated microtiter plates were incubated at 37°C overnight. A total of 14,000 clones were obtained for the library. The whole library was stored in the LB culture medium supplemented with 10% glycerol at -80^{o}C in 384 microtiter well plates.

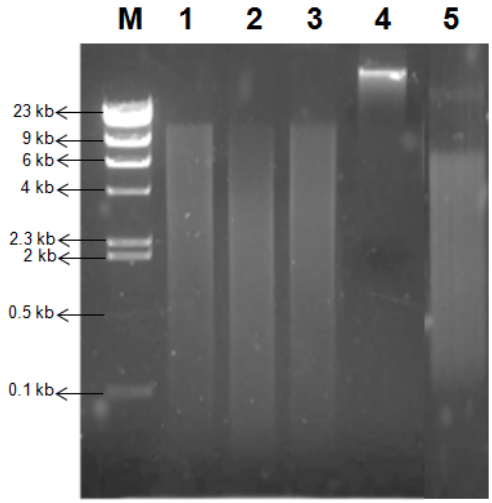

Fig. 1: Metagenomic DNA isolated from soil samples. M: HindIII-digested ʎ 23Kb marker; 1: metagenomic DNA digested with *sma*I; 2: metagenomic DNA digested with *Msc*I; 3: metagenomic DNA digested with *Msc*I and *Sma*I; 4: undigested metagenomic DNA; 5: pooled digested metagenomic DNA after electro-elution.

Construction of large insert metagenomic DNA library

Both cultivable and non-cultivable bacteria were enriched from the selected soil samples according to established protocols. Metagenomic DNA was isolated from the enriched bacteria and then used for the construction of a large insert fosmid library with an average DNA insert size of 40 kb in E. coli strain EPI100. Chloromphenicol (12 µg/ml) was used for the selection of transformants, and a total of 42,000 clones were obtained. The quality of the library was assessed by performing restriction digestion for ten randomly picked clones which revealed ten different restriction patterns (Fig. 2). The whole library was stored in LB culture medium supplemented with 10% glycerol at -80^{o}C in 384 microtiter well plates.

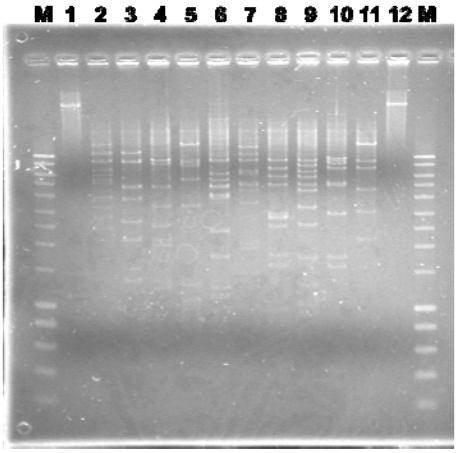

Fig. 2: Restriction patterns of fosmids isolated from ten randomly picked clones of the large insert fosmid libray. M: DNA marker; lanes 1 and 12: undigested metagenomic DNA; lanes 2 to 11: restriction-digested fosmid DNA from ten different clones.

Construction of positive controls

In order to establish function-based screening strategies, positive controls were constructed using appropriate *Bacillus* genes (Table 1 and 2) in pET-22b(+) and pUC19 vectors transformed into *E. coli* BL21(DE3) [pRARE2], *E. coli* EPI100, and *E. coli* DH5α. In all combinations, clones producing active recombinant enzyme were obtained (data not shown).

Chitinase assay using fluorogenic chitin analogs

Screening for chitinases can be performed in microtiter plates using fluorogenic, MUF-coupled chitin oligomers A2-MUF and A3-MUF; the former is sometimes indicated to be specific for exo-acting and the latter for endo-acting chitinases. Conditions were optimised so that the chitinase containing positive control clones Puc19Dchi, Puc19Echi, and R2Blichi released fluorescent MUF from both substrates, while empty vector controls were negative (Fig. 3). Using this assay, both the small insert plasmid library and the large insert fosmid library were screened for chitinases.

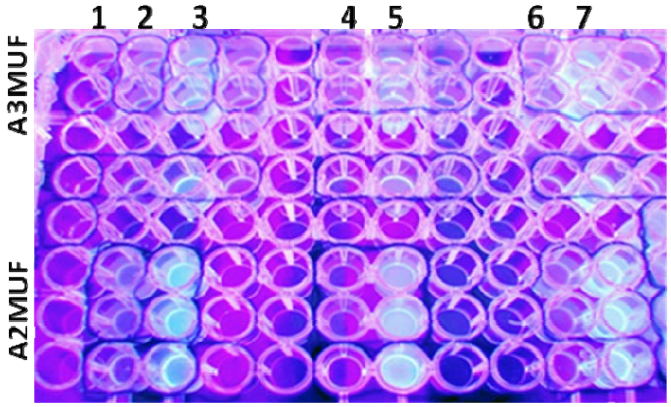

Fig. 3: *Bacillus licheniformis* DSM13 chitinase constructs in different hosts showing fluorescence on incubation with chitin dimer and chitin trimer conjugated to MUF. 1: minimal medium with substrate alone; 2: Puc19D (vector control); 3: Puc19Dchi; 4: Puc19E (vector control); 5: Puc19Echi; 6: R2 0 (vector control); 7: R2Blichi.

Chitinase assay using colloidal chitin plates and congo red staining

An alternative screening method for chitinases employed the creation of clearing zones in agar plates containing colloidal chitin around clones producing an active chitinase. Again, conditions were optimised so that the chitinase containing positive control clones Puc19Dchi, Puc19Echi, R2Blichi, and R2Btchi showed clearing zones but an empty vector control did not (Fig. 4A). Using these assays, both the small insert plasmid library and the large insert fosmid library were screened for chitinases.

In a variant of this assay, the contrast of the clearing zones was increased by congo red staining of the surrounding chitin (Teather and Wood, 1982). Even under optimised conditions, this assay appeared to be slightly less sensitive than the simple clearing zone assay described above, as the positive control clone Puc19Echi which showed weak activity in the former assay did not appear active in the latter assay (Fig. 4B). The small insert plasmid library was also screened using this method.

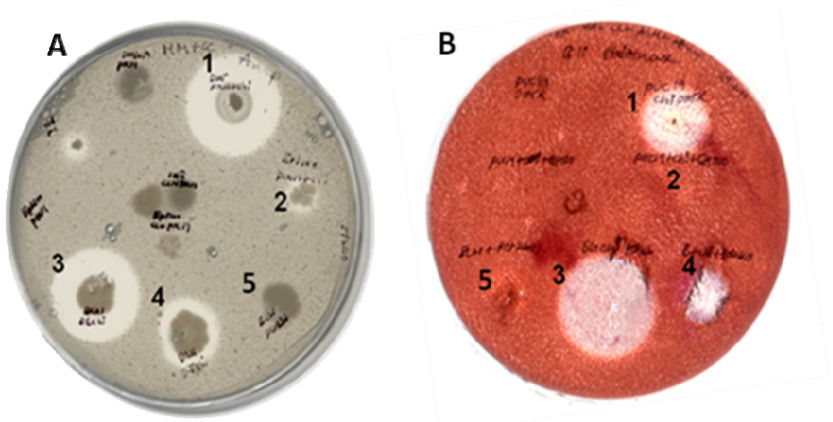

Fig. 4: Screening for chitinase activity on agar plates containing colloidal chitin, without (A) and with (B) congo red staining of chitin. 1: *E.coli* clone expressing chitinase Puc19Dchi; 2: Puc19Echi; 3: R2Blichi; 4: R2Btchi; 5: R2 0 (vector control).

Chitin deacetylase assay using chitosan affinity protein (CAP)

A new screening strategy for detecting chitin deacetylases was established using positive control clones expressing CDA genes as positive controls (Table 1 and 2). Clones were grown on LB agar plates over night, then overlaid with a membrane containing the water soluble chitin derivative glycol-chitin, as CDA typically exhibit very low activities towards crystalline or colloidal chitin. After incubation, the membrane was stained using CAP, a chitosan-specific binding protein engineered from a SCM-inactivated bacterial chitosanase fused to different tags for detection. CAP was visualised immunologically using chemiluminescence based on the N-terminal StrepII tag of CAP. A strong signal was detected for the positive control clone expressing R2CDA5 and a slightly weeker signal for R2CDA1, while the empty vector control was negative (Fig. 5). The small insert plasmid library was also screened using this method.

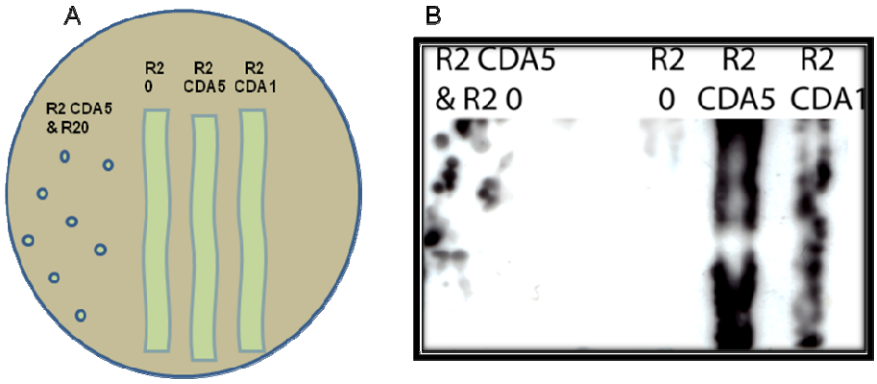

Fig. 5: Screening for chitin deacetylases using the chitosan affinity protein CAP. R2CDA5 & R2 0: mixture of clones with and without CDA5; R2 0: *E. coli* BL21 (DE3) [pRARE2] with pET-22b(+); R2CDA5: *E. coli* BL21 (DE3) [pRARE2] clone expressing CDA5; R2CDA1: *E. coli* BL21 (DE3) [pRARE2] clone expressing CDA1.

Dot activity assay to screen for chitin and chitosan modifying enzymes

Finally, a combined activity assay was developed for the detection of clones expressing chitinase, chitosanase, or chitin deacetylase, where the clones were grown in microtitre plates and 5 µL-drops of the spent media were then spotted onto a polyacrylamid gel containing glycol-chitin and two chitosans with DAs of 10% and 35%. The gel was incubated overnight followed by Calcofluor staining of chitin and chitosan, revealing dark spots for chitinase and chitosanase activities. In a subsequent step, chitosans were depolymerised chemically using nitrous acid, revealing chitin deacetylase activity through the appearance of additional dark spots where glycol-chitin had been deacetylated into chitosan. This combined activity assay was developed using chitinase, chitosanase, and chitin deacetylase expressing positive control clones, namely R2Blichi, R2csn, and R2CDA5, respectively. Purified chitinase, chitosanase and chitin deacetylases were used as additional positive controls (Fig. 6). Chitinase and chitosanase gave dark spots before nitrous acid treatment, whereas chitin deacetylase gave dark spots only after the chitosan depolymerising treatment. Dark spots caused by chitosanase disappeared after the treatment while spots caused by chitinase remained visible. CDA1 is a novel dual-activity enzyme having both chitin deacetylase and chitosanase activity (unpublished), hence its activity was seen before and after depolymerisation. The large insert fosmid library was screened using this method.

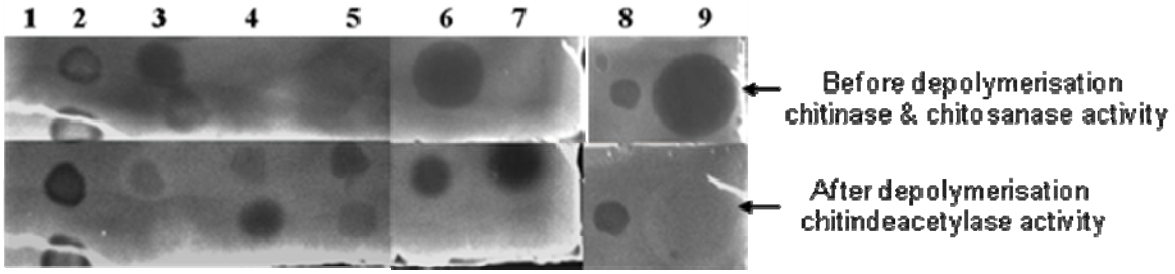

Fig. 6: Combined dot activity assay to screen for chitin and chitosan modifying enzymes. Spent media of *E. coli* clones expressing chitinase, chitosanase, or chitin deacetylase as well as the purified enzymes were spotted onto a polyacrylamide gel containing glycol-chitin and two chitosans with DA of 10% and 35%, and substrates remaining after incubation were stained using Calcofluor white either directly (upper gel) or after chitosan depolymerisation using nitrous acid. 1: R2 0 (empty vector); 2: R2 Blichi; 3: R2 csn; 4: R2CDA5; 5: R2CDA5 (CDA5 was applied twice); 6: purified CDA1; 7: purified CDA5; 8: purified chitinase; 9: purified chitosanase.

Screening the small insert plasmid library for chitinases and chitin deacetylases

The small insert plasmid library was initially screened for chitinases on minimal medium agar plates supplemented with 0.5% colloidal chitin, and using the fluorogenic MUF-substrates as detailed above. The whole library was stamped on colloidal chitin substrate to check for clearing zones, and also into sterile 384-well microtiter plates containing minimal medium supplemented with carbenicillin and chloromphenicol. Auto-induction medium (AIM) was added as carbon source and also to induce the cells for expression of insert. Plates were then incubated at 37°C for several days and checked for clearing zones daily. Microtiter plates were also incubated at 37°C, and checked for cleavage of the MUF-substrates under UV light. Alternatively, after incubating the library on colloidal chitin substrate plates for several days, plates were then stained with congo red solution to enhance the contrast for chitinases. However, no positive clones were obtained from the small insert library when screened by these three different methods. The screening strategy which was established for CDA using CAP was also tried; however, no positive clones were obtained so far after screening about 5,760 clones from the library. This strategy still has to be optimised to upscale the method for screening the whole library.

Screening the large insert fosmid library for chitinases

For the fosmid library, clones were initially pooled into seven sub-libraries each corresponding to about 16 microtiter plates, together representing the whole library. The seven pools were grown and transfered to one large agar plate each for growing. When growth was completed, cells were collected, and the seven different pools were then grown in minimal medium containing MUF-substrates to screen for chitinases. The same pools were

also streaked on agar plates containing minimal medium supplemented with 0.5% colloidal chitin to check for clearing zones. Also, the dot activity assay was performed with the pools to screen for chitin and chitosan modifying enzymes on gels containing glycol-chitin and two chitosans. However, no positive clones were obtained with any of these screening methods.

Discussion

Soil is generally a rich source for micro-organisms and for hydrolytic enzymes and, thus, the selected soil samples from Mahtani Chitosan Company with a long history of chitin and chitosan exposure are promising sources of novel CCME. In fact, we have shown that these soils possess a rich biodiversity in chitin and chitosan degrading bacteria and fungi ; Chapter 3). Construction of small insert DNA libraries and recovery of novel genes have been reported for different soil samples (Cottrell et al., 1999; Henne et al., 1999; Henne et al., 2000; Sharma et al., 2010). To overcome the limitations of small insert libraries, construction of large insert DNA libraries using fosmids or cosmids with an insert size of approximately 40 kb has been employed to develop a metagenomic library (Kimura, 2006). BAC vectors were used to construct very large insert (>200 kb) metagenomic libraries using DNA extracted from environmental samples (Rondon et al., 1999). In the present study, *E. coli* was used as a host to construct both the small insert and the large insert metagenomic DNA library, using plasmids and fosmids, respectively. While the large insert fosmid library has the advantage of potentially yielding whole chitin or chitosan degrading gene clusters, it has the drawback of being constructed using only bacterial DNA. As we had shown that the chitinolytic and chitosanolytic fungal diversity in these soil samples is richer than the bacterial one, we decided to also construct the small insert plasmid library using metagenomic DNA isolated directly from the soil samples.

Both the small insert library of 14,200 clones and the large insert library of 42,000 clones were screened using colloidal chitin as a substrate and fluorogenic substrate analogs. However, no positive clones have yet been identified based on these function driven approaches. It has been estimated that more than 10^7 clones carrying inserts of 5 kb each would be required to represent the whole metagenome of a given soil sample (Handelsman et al., 1998). Clearly, thus, the libraries do not represent the full metagenome of the soils investigated. More clones for the plasmid library can easily be obtained to continue the screening, and the fosmid library is currently being screened using individual clones.

Screening of uncultured marine microorganisms for chitinases using fluorogenic substrates has been successful(Cottrell et al., 1999), and this was the reason why we opted for this screening method first. Using the MUF-substrates may favour the identification of chito-oligosacharide modifying enzymes. In order to also screen for polysaccharide modifying enzymes, we therefore used polymer-based screenings looking for the generation of clearing zones around positive colonies or their spent media. If more extended screenings of the libraries using the established protocols will not yield novel CCME genes, several other methods can be tried, e.g. using reducing end assays, chitin-azure, chitin analogs (Howard et al., 2003a), or RBB-chitin (Ramirez et al., 2004) to identify chitinases.

Using function driven approaches, finding positive clones is challenging as the insert which got cloned needs to be expressed into functional protein in the particular host species chosen. For this reason, the pET-22b(+) plasmid for the small insert library was amplified with primers including extra bases to increase the chance of cloning the insert into the correct reading frame. Generating pET-22b(+) vector by PCR also helped in avoiding false positive transformants as the PCR-generated vector cannot be re-ligated due to the un-phosphorylated oligos. An additional problem which is inherent to both plasmid and fosmid libraries is the specificity of type II secretion systems making it unlikely that the *E.coli* strain used as a host is secreting the expressed proteins (Pugsley, 1993). Hence, longer incubations are needed for host cell lysis to occur, to allow the enzyme to react with the extracellular substrate to identify positive clones from genomic libraries.

A particular problem is the screening for chitosanases, firstly because fluorogenic substrates for these enzymes are not available and attempts at converting the MUF-coupled chitin oligomers into MUF-coupled chitosan oligomers using CDA (Howard et al., 2003a) failed when we tried several recombinant CDAs (unpublished), and secondly because chitosan has antimicrobial properties (Kong et al., 2010) so that growth of the *E. coli* clones is hindered. It may be assumed that chitosanase expressing clones become resistant to the antibacterial activity of chitosan (Zivanovic et al., 2007) and in that case, selective growth based screenings on chitosan containing media could be performed. While this was indeed the case for the chitosan producing positive control clones, the growth of these clones was too poor to promise successful screening of a library (unpublished). A solution for this problem could be the use of culture supernatants of the library clones in a zymography based screening where the samples are applied to a substrate containing gel to generate clearing zones. This approach allows to screen for chitin deacetylases also, and we have begun to develop a

method which would allow for the simultaneous screening of chitinases, chitosanases, and chitin deacetylases. The assay works well with the positive controls but cannot yet be applied to the screening of individual clones of a whole library as the gels dry up too quickly while applying the samples onto the gel. Similarly, the newly developed screening methods for chitin deacetylases based on the chitosan affinity protein CAP (refer chapter 1) was successfully tested on the positive controls, but still requires optimisation before it can be used for screening libraries on a large scale.

An alternative approach for screening the metagenomic libraries would be a sequence driven approach in which known sequence information is used to design primers or probes to identify novel genes. Significant discoveries have been reported based on sequence driven approaches but the method is more expensive compared to the functional based screening. What is more important, chances of finding novel genes are low as known sequence information is used for designing the primers or probes (Schloss and Handelsman, 2003). However, the newly emerging techniques of high throughput sequencing now allow deep metagenomic sequencing, offering new possibilities (Petrosino et al., 2009). On the one hand, such large scale metagenomic sequence data may be searched making use of Hidden Markov-Model algorithms allowing identification of functional domains, such as CAZY family GH18 or GH19 chitinase domains, polysaccharide deacetylase domains, polysaccharide binding domains, etc. On the other hand, such sequencing might allow the identification of genes clusters if applied to high molecular weight metagenomic DNA such as used for the construction of the fosmid library, and this might lead to the identification of unknown genes in a chitin or chitosan degrading gene cluster. Such an approach of metagenomic sequencing is currently under consideration and will be performed once financial support can be secured.

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