Structural Characterisation and Enzymatic Degradation of Exopolysaccharides involved in Paper Mill Slime Deposition

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Abstract

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The chemical fine structures of the EPS produced by 2 bacterial strains isolated from slime deposits on a Finish paper machine were elucidated. The EPS produced by *Brevundimonas vesicularis* consists of the following repeating unit: →4)-α-L-Glc*p*A-(1→4)-α-D-Gal*p*A-(1→4)-β-L-Rha*p*-(1→4)-β-D-Glc*p*(1→. Structural elucidation of the EPS produced by *Methylobacterium* sp. resulted in the recognition of a 3)-[4,6-*O*-(1-carboxyethylidene)]-α-D-Gal*p*-(1→3)[4,6-*O*-(1-carboxyethylidene)]-α-D-Gal*p*-(1→3)-α-D-Gal*p*-(1→ repeating unit. This EPS could be degraded by a cell free culture containing an endo acting enzyme.

The use of multivariate analysis of the sugar and substituent compositions and FT-IR spectra of a large number of EPSs produced by bacterial strains isolated from different Finish, French and Spanish papermills resulted in the recognition of 4 important groups of slime producing species: a group of *Bacillus* and related genera, showing high mannose and/or glucose levels and a group of *Klebsiella* EPSs that showed galactose with rhamnose as major characteristic sugar moieties that could only be clustered on the basis of similarities in their sugar compositions. Furthermore, a group of EPSs produced by *Enterobacter* and related genera similar to the regularly reported colanic acid and a group of *Methylobacterium* EPSs having high galactose and pyruvate levels could be clustered on both their sugar compositions and FT-IR spectra.

A novel colanic acid-degrading β(1,4)-fucanosyl hydrolase that liberates single repeating units of colanic acid with varying degrees of acetylation was purified and characterised. Kinetic studies showed that the enzymes catalytic activity and affinity is dependant on the pyruvate and *O*-acetyl substituents present on both the backbone and side chains of colanic acid.

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Contents

Chapter 1

General introduction

Introduction.

In 2002 the European paper industry employed 250.000 people within 900 companies that operate 1.260 paper mills. With a trade balance of 6,8 million tonnes the European paper industry was responsible for 28% of the world production of paper and a turnover of €73 billion in the year 2002 [1].

The general process of papermaking is explained by the example given in Fig. 1.1. After making the stock suspension of fibers and water the slurry is pumped to the paper machine. The fibers that are used can generally be divided into chemical, recycled and mechanical pulp fibers. All paper machines consist of the same basic elements: headbox, wire section, press section, dryer section and reel. The actual design of these elements altogether largely depends of the type of paper being made. When the slurry has reached the paper machine it first enters the headbox were the mixture is evenly spread over the entire wide of the paper machine. After the headbox the fibers are deposited on a constantly revolving wire mesh or sieve (wire section) and water is run through or is sucked off from below the wire. This way the sheet of paper is formed, however at this moment the sheet of paper still contains about 80 % of water. The water that is disclosed from the wet paper sheet in the wire section is usually referred to as wire or white water. This white water contains 3-18 mg/L of total nitrogen, 2-6 mg/L of total phosphorus and approximately 500 mg/L dissolved organic carbon [2, 3]. The white water formed underneath the wire section is directed back to the stock preparation vessel via a closed loop system. After the wire section the wet paper sheet is directed to the press section and drying section to further dry the paper sheet before the finished paper is reeled on a steel shaft. The reel of paper is then further processed in the coating and cutting section. In the coating section the raw paper is coated with a substance consisting of pigments or binders to further improve the surface of the paper to the needs of the end user [4].

Nowadays the paper industry is challenged to move towards more sustainable or environment friendly processes. As a result most modern paper mills are operating a closed loop process water system that operates under neutral or alkaline conditions with an increased consumption of recycled fibres as raw material [5, 6]. In 2003 54% of the produced paper and board in the EU was derived from recycled fibres [7]. These changes have resulted in an increasing number of problems due to slime deposits caused by an increase in microbiological activity [6]. Slime is the generic name for deposits of microbial origin within the paper process [8]. Slime is defined as the accumulation of microbial cells immobilized and embedded within an

organic polymer matrix of exopolysaccharides (EPS), mixed in different proportions with fibres, fines, fillers and other materials present within the paper process.
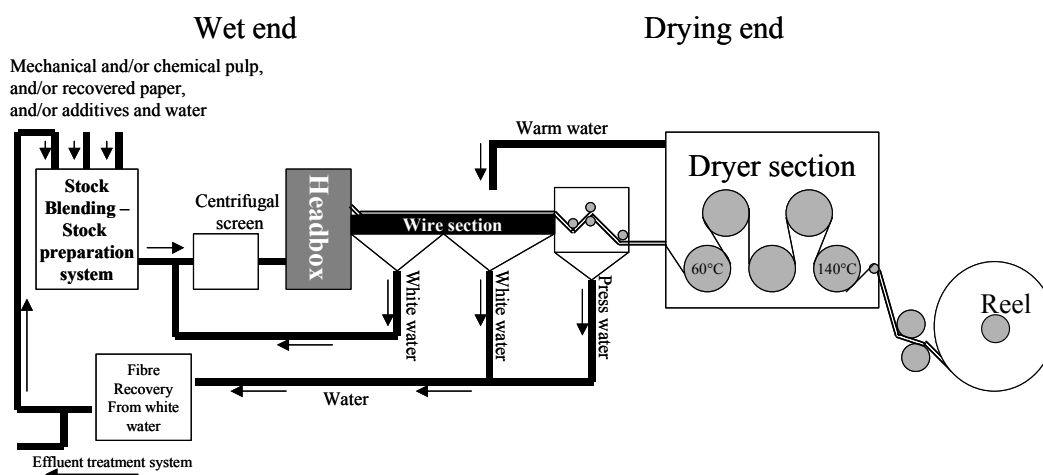
Figure 1.1: Schematic representation of a paper machine (combination of Ref.[3, 4]).

Slime deposition in paper making/manufacturing.

As mentioned above slime is referred to as the generic name for deposits of microbiological origin. The formation of slime is caused by the increased use of closed loop process water systems. Wastewater discharges by paper mills have decreased due to environmental concern resulting in these closed loop process waters. In the 1970s, approximately $100m^3$ water was used per metric ton of paper manufactured, but in 1994 less then $10m^3$ was more usual [2]. This causes the accumulation of nutrients as well as optimal temperatures and pH ranges resulting in serious microbial proliferation, while increased use of recycled fibres causes constant contamination with bacteria. The conditions normally found in paper mills are pH 5-8, temperature ranges between 20-78°C and biodegradable material like celluloses, hemicelluloses, starch and wood extractives [2, 9, 10].

The critical areas of the process are the wet end, coating section and the size emulsion. At the wet end of the machine slimes are usually found underneath the wire frame, on the surface of the foils, the suction boxes, the white water tanks and the clarifiers [11].

Recycling, as part of the paper cycle, plays an important role in the sustainable development of the paper industry. From the approximately 310 million tons of paper and board produced

worldwide in 2003, more than 48% was recovered for recycling. Today about 40% of the raw materials involved in papermaking in the US and Europe is recovered paper, with the rest being mainly wood, virgin fibre, additives (12-15%) and other non-woody fibres (1-2%). It is estimated that, by 2010, about half of the global amount of fibres used in papermaking will be recycled fibres. A direct consequence of moving towards higher recycling rates is the change to more heterogeneous paper sources. This leads to a lower recovered paper quality or, in other words, to more contaminated raw materials, from the point of view of organic, inorganic and microbial content [12]. For example, recycled fibres can contain as many as 1000 times more microorganisms than virgin fibres due to storage and transport conditions of the recycled fibres [13].

Due to both these factors paper machines provide the ideal niche for microorganisms to grow and form microbial biofilms. Biofilms are defined as sessile or attached populations of prokaryotic and eukaryotic microorganisms that exist within a glycocalyx or matrix of extracellular polymeric substances, which they produce themselves [14]. Within paper machines these biofilms could be present mixed in different proportions with fibres, fines, fillers and other materials present within the paper process to form a slime layer.

Slime deposits at critical points within the paper machine cause serious operational problems like clogging of filters, sheet breaks or holes in the paper, foul odours and corrosion [6, 9]. The effects of slime deposits are generally referred to as biofouling.

The use of biocides and extensive cleaning programmes is still commonly used to prevent biofouling resulting in undesired environmental impacts and significant costs due to contamination of raw materials, production downtime and maintenance costs [15].

However due to environmental legislation and the biocidal products directive [16] all biocides should be authorised by providing all relevant data of the biocide to be used.

It has been estimated that the costs for collecting these data are €1-7 million over a time period of 2-5 years. Resulting in a high demand of alternative methods for slime control like the use of for example dispersants, enzymes and mixtures of the latter.

Biofilms

The vast majority of microorganisms live and grow in aggregated forms such as biofilms and flocs. This mode of existence has resulted in the generally accepted expression "biofilm".

Biofilms are accumulations of microorganisms, exopolysaccharides (EPS), multivalent cations, biogenic and inorganic particles as well as colloidal and dissolved compounds [17].

In 1647 Antony van Leeuwenhoek made the first observations of microorganisms living in a sessile or attached state through his microscope by investigating tooth plaque. In 1684 he corresponded the finding of aggregates of 'animalcules' scraped of human teeth to the Royal Society of London [18]. Not until 250 years later, in the first half of the 20th century, the concept of microbes adhering to surfaces was further developed by Claude Zobell. By examining natural marine populations by direct microscopy he concluded that bacteria are attracted to surfaces to which they adhere to form sessile populations [19]. In the late 20th century this led to the general understanding that extrapolations from data obtained on planktonic species to sessile species within a biofilm is not accurate enough to describe bacteria in a natural multi species biofilm [20].

In most natural aquatic environments association with a surface by means of biofilm formation is an efficient tool of the microorganism to linger in a favourable microenvironment rather than being swept away by the current [21].

To form a biofilm bacteria use their flagella or type IV pili to reversibly adhere to the surface. Once settled into a microcolony the production of exopolysaccharides is necessary to ensure the structural integrity of the complex 3D architecture of the biofilm to be formed [21, 22].

Exopolysaccharides

Microbial EPS stands for extracellular polymeric substances, extracellular polysaccharides, exopolysaccharides or exopolymers. Since polysaccharides are assumed to be the most abundant component of EPS it is frequently used as an abbreviation for exopolysaccharides [17]. In this context EPS can be defined as polysaccharides that are either found associated with the microbial cell wall in the form of capsules or completely dissociated from the microbial cell [23]. However, proteins, nucleic acids, as well as amphiphilic compounds including (phospho-) lipids can also be present in EPS [17]. Focussing on polysaccharides, EPS is primarily composed of carbohydrates, but in addition to the various sugars they can be substituted with both organic and inorganic substituents. D-galactose, D-glucose and D-mannose in the pyranose form and both L-fucose and L-rhamnose also in the pyranose form are frequently found within EPS. Furthermore, EPS can be characterised by their often polyanionic nature due to the presence of uronic acids of which D-glucuronic acid is most abundant. Galacturonic and mannuronic acid are found less often [23]. Besides the variety in carbohydrates present in EPS non-carbohydrate ester linked

organic and inorganic substituents can be present of which acetyl, succinyl and phosphate are most commonly encountered. Apart from ester-linked substituents also pyruvate ketals are frequently observed as an organic substituent [23]. EPS are defined as homopolysaccharides if only 1 type of sugar is present and heteropolysaccharides if 2 or more different sugars are present. With respect to their polymeric structures EPS are build of linear or branched repeating units of 2 to 7 different or the same sugars. The majority of microbial polysaccharides are assumed to be heteropolysaccharides [23].

Because different sugars (and substituents) can be present and these sugars can be present in their pyranose and furanose form, in α or β-anomeric configuration and linked to each other with different glycosidic linkages in different sequences there is an enormous variety in possible EPS chemical fine structures.

EPS produced by bacterial species occurring in the paper industry.

With respect to EPS producing species encountered in a paper mill and the structure of the EPS they produce, most research has been directed towards the identification of the species present. Many different genera and species have been detected within paper machine slimes of which the most important are: *Flavobacterium, Enterobacter, Pseudomonas, Bacillus, Klebsiella, Sphaerotilus, Citrobacter* and *Burkholderia Cepacia* [2, 3, 5, 6, 24-30].

With respect to the EPS these bacteria produce, only sugar composition data are available giving no clear indication about the chemical fine structure of these EPSs.

Väisänen et al [2] found a large variety of sugars, other then glucose derived from cellosic fibres and starch used as raw material, by analysing different mixed EPS samples isolated from board machine slimes. In general rhamnose, galactose and mannose were the major neutral sugars that were found within these samples. In addition, the neutral sugars composition of EPS produced by several bacterial isolates from the same board machines grown on defined media was determined. These bacterial isolates included *K. pneumonia*e species that produced an EPS consisting of rhamnose, galactose and mannose, *E. agglomerans* producing an EPS mainly consisting of fucose and galactose, several *Pseudomonas* sp. showing rhamnose, galactose and mannose as the main sugar moieties of the EPS they produced and *Flavobacterium sp., C. michiganese* and *B. licheniformis* were isolated producing EPS composed of mainly mannose and galactose. All of the slime samples and EPS showed the presence of uronic acid, however no distinction between galacturonic, glucuronic and mannuronic acid was made [2]. Similar studies

were preformed on slime samples collected from paper machines by Rättö et al [25]. These studies revealed also rhamnose, galactose and mannose to be the main sugar moieties of the different EPS present. Both *Pseudomonas sp.* and *Citrobacter sp.* were isolated from the slime samples and their EPS was produced under laboratory conditions. The EPS produced by the *Pseudomonas sp.* mainly consisted of glucose and the EPS produced by *Citrobacter sp.* mainly consisted of galactose, glucose and mannose.

The biofilms of different *Burkholderia cepacia* strains were cultivated in paper mill white water-simulating conditions on glass slides or stainless steel coupons by Lindberg et al [31]. Their studies made it evident that glucose, galactose and mannose were the main sugars found in EPS produced by these *Burkholderia cepacia* strains.

Hernandez-Menna et al [32] showed that rhamnose, fucose, galactose, glucose, mannose and glucuronic acid were present in papermill slimes isolated from different paper mills in the US, however no absolute amounts are given making it rather difficult to assign the most important sugar moieties.

The lack of accurate data with respect to the type of uronic acid present and the enormous variation in sequence, linkage type and α or β-anomeric configuration make it impossible to directly translate these sugar compositions in chemical fine structures of EPS.

Furthermore, important substituents like pyruvate ketals, succinyl half esters and *O*-acetyl groups are often not taken into account.

Apart from the importance of uronic acids and non-sugar substituents for the chemical fine structure they also contribute to the physical properties of the EPS. Pyruvate ketals, succinyl half esters and uronic acids contribute to the polyanionic nature of most EPS and in their turn have a large impact on the EPS its physical properties[33, 34] by making interaction possible via divalent cation bridges [17]. On the other hand *O*-acetylation can result in localised hydrophobic regions again important for the physical behaviour of the EPS [33, 34, 17].

As a result of incomplete or inaccurate data on the different EPS building blocks, only a few chemical fine structures of EPS occurring in paper machine slimes are known. An example is levan, which is a fructose containing polysaccharide synthesised from sucrose by several *Bacillus* and *Pseudomonas* species that can grow in paper machine recirculated waters [8]. However, since this EPS can only be produced in the presence of sucrose, which is in short supply in a paper machine environment, it is unlikely that this EPS is important for slime formation in paper machines [28].

The dependency of levan production on carbon source availability is an exception on the general assumption that the EPS produced by a microbial species is independent of the carbon source utilised [23] and therefore it could be possible that EPS structures known from other sources could also be present in a paper mill environment.

Judging from the bacterial species most frequently encountered within paper mill environments some possible EPS could be present.

For example, several *Enterobacteriaceae* species are known to produce an EPS referred to as colanic acid [35] (Fig. 1.2).

Thirteen strains of *Burkholderia cepacia* from various cystic fibrosis clinical isolates, soil and onion samples were shown to produce EPS with the same structural features. It is suggested that the 2 EPSs shown in Fig.1.2. are representative for *B. cepacia* strains [36]. The same sugar moieties were found by Lindberg et al [31] for the EPS produced by different *Burkholderia cepacia* strains cultivated in paper mill white water-simulating conditions.

The genus *Klebsiella* represents a group of slime forming bacteria with fairly uniform and closely related EPS structures. Their sugar composition most commonly comprises up to 3 neutral sugars and an uronic acid, forming repeating units of 3-5 sugars. The differences between the different EPS structures may be as small as the presence or absence of an acetyl group. *O*-acetyl and pyruvate ketals are the most common organic substituents [37] in [34]. As an example the structure of the EPS produced by *Klebsiella* Serotype K70 is given in Fig. 1.2.

With respect to *Pseudomonas sp.* bacterial alginate, composed of mannuronic- and guluronic acid, is commonly encountered. Especially the species *Psuedomonas aeruginosa* is commonly used in laboratory studies on artificial biofilms [33]. *Pseudomonas aeruginosa* is commonly isolated from biofilms in the environment, industrial water systems and infections [38]. Different from the alginates isolated from marine algae bacterial alginate produced by *Psuedomonas aeruginosa* is highly *O*-acetylated and only contains mannuronic acid residues (Fig. 1.2) [23].

EPS degrading enzymes.

Since natural biofilms are composed of mixed species and several different complex EPS structures it is evident that enzymes mixtures able to degrade these complex EPS structures need to have a wide variety of highly specific enzymes and therefore, are not always commercially available. In fact commercially available enzyme preparations have seldom found to be capable of degrading these heteropolysaccharides, however some are active against bacterial alginates

and homopolysaccharides including bacterial cellulose and curdlan [39]. The enzymes are either endoglycanases or polysaccharide lyases and their most common sources are microorganisms or bacteriophages.

Enterobacteriaceae species	→3)-β-L-Fuc*p*-(1→4)-α-L-Fuc*p*-(1→3)-β-D-Glc*p*-(1→ 　　　　4　　　　　　　| 　　　　↑　　　　　　　*O*-Ac 　　　1)β-D-Gal*p*-(3←1)β-D-GlcA*p*-(4←1)-β-D-Gal*p*-4,6pyruvate
Burkholderia cepacia species	→3)-β-D-Glc*p*-(1→3)β-D-Gal*p*(1→ 　　　　　　4　　6 　　　　　pyruvate β-D-Gal*p*-(1→2)-α-D-Rha*p*-(1→4)　　　　　　　(6←1)-β-D-Gal*p* 　　　　　　　　　　|　　　　　　　　　　| 　　　→3)-α-D-GlcA*p*-(1→3)-α-D-Man*p*-(1→3)-β-D-Glc*p*-(1→ 　　　　　　　　　| 　　　　β-D-Gal*p*-(1→2)
Klebsiella Serotype K70	→2)α-L-Rha*p*(1→4)β-D-GlcA*p*(1→4)α-L-Rha*p*(1→2)α-L-Rha*p*(1→ 　　　4　　3　　　　　　　　→2)α-D-Glc*p*(1→3)β-D-Gal*p*-(1→ 　　pyruvate
Psuedomonas aeruginosa	→4)β-D-ManA*p*(1→4)β-D-ManA*p*(1→4)β-D-ManA*p*(1→4)β-D-ManA*p*(1→ 　　　　　　　|　　　　　　　| 　　　　　　*O*Ac　　　　　　*O*Ac

Figure 1.2: Examples of chemical fine structures of the EPS produced by bacterial species occurring in paper mill biofilms: Colanic acid produced by several *Enterobacteriaceae* bacteria [40, 41]; Chemical fine structure of 2 EPS believed to be representative for *Burkholderia cepacia* [36]; Chemical fine structure of the EPS produced by *Klebsiella* Serotype K70 [42]; Bacterial alginate produced by *Psuedomonas aeruginosa* [23].

Due to this, most enzymes acting on EPS have to be isolated from 3 major sources: endogenously from the EPS synthesising microorganism, exogenously from a wide range of other prokaryotic and eukaryotic microorganisms or from bacteriophage particles or phage induced bacterial lysates [39]. Sutherland has given an extended overview of the available EPS degrading enzymes. However, from the enzymes listed it is clear that all of them are highly EPS

specific and the most widespread application of these enzymes was for the study of the EPS structures [39, 43].

With respect to enzymatic removal of slime deposits in paper mill environments several approaches have been suggested resulting in a number of patents and inventions since the 1970's [44-52].

The first approach is based on the prevention of the formation of biofilms by enzymatic interference in the process of adhesion of bacteria to the surface. Therefore slime was attacked at the initial step of slime formation were bacteria use their flagella or type IV pili to adhere to a surface and form a microcolony. All these methods are based on the use of proteases or proteases in combination with endoglucanases that attack the cell wall of bacteria and therefore prevent them from settling and forming a sessile microcolony [46, 47, 50].

Secondly lytic enzymes could be used to cleave the 1,3-glucose linkages in the bacterial cell wall leading to cell lysis that kills the bacteria [44].

A third approach is the use of a single enzyme attacking only one EPS present in the complex slime layer. For example the use of levan hydrolase that degrades a fructose containing homopolysaccharide called levan produced by several *Bacillus* sp. [48, 49].

Furthermore the use of a multi activity enzyme has been claimed to be effective in the removal of slime layers present in industrial processes like paper manufacturing [52]. This enzyme is produced by a newly isolated *Streptomyces* strain capable of degrading colanic acid. Besides its colanic acid degrading activity it is claimed to also have an effect on the biofilms produced by other species like *Klebsiella*, *Pseudomonas* and *Xanthomonas*.

Finally most of the approaches are based upon a mixed enzyme system that combines a whole range of activities. Hernandez-Mena et al [45] reported the use of a combination of galactosidase, galacturonidase, rhamnosidase, fucosidase and α-glucosidase to treat microbial slime within industrial water systems. Furthermore a patent was filed claiming the use of a combination of β-glucanase, α-amylase and protease for removing slime [51]. Also the potential use of the commercial enzyme preparation Pectinex Ultra SP that has a wide range of enzyme activities has been discussed [53]. Apart from the use of commercially available enzyme preparations some studies deal with obtaining enzyme mixtures by culturing bacteria on at least one EPS as primary carbon source [54].

The integrity of complex mixed biofilms is undoubtedly dependant on the presence of different types of macromolecules with polysaccharides and proteins playing a dominant role. Therefore

the use of a complex mixture of enzymes including both proteases and carbohydrases is more likely to be effective then a single enzyme system. However, none of these methods are sufficiently well developed to constitute a full-scale alternative to chemical biocides. Some manufacturers and service companies are of the opinion that the enzymes available are to species-specific and can only be used on a smaller scale or as a supplement to conventional biocidal methods [28]. As a direct consequence knowledge about the different EPSs occurring within a paper mill environment would provide a useful tool for targeting the most important EPS present within this environment. Using this knowledge a treatment method that solely depend on polysaccharases or mixture of proteases and polysaccharases together with the more traditional biocides and bio dispersants could be developed.

Background and aim of the research project.

The work described within this thesis is part of a multidisciplinary EU-project. The objective of the project was to develop novel targeted enzymatic approaches for efficient control of slimes in paper manufacturing. The work has been carried out with financial support from the Commission of the European Communities, specific RTD programme "Competitive and Sustainable Growth", G1RD-CT2000-00387, "Eco-efficient novel enzymatic concepts for slime control in pulp and paper processing (Slimezymes)".

The approach of the project (Fig 1.3) is to isolate slime forming microorganisms from paper machines and produce their EPS on laboratory scale. These EPS are subjected to structural characterization to obtain increased understanding on slime formation in paper machines. This would enable later development of other targeted slime control approaches. The increased understanding mainly refers to the knowledge of paper mill biofilms and important EPS and groups of microorganisms present in paper mills.

To obtain enzymes degrading the EPS produced by the isolated slime producers the same EPS are used as carbon source to grow strains enriched from samples collected from soil, compost and paper mill environments. Since degradation of these polysaccharides is an obligatory prerequisite for the capability to utilize them as carbon source, the organisms capable of growing on these polysaccharides produces the desired EPS degrading enzymes. The enzymes are selected based on their degradation efficiency against slimes isolated from different paper machine environments. In order to obtain sufficient amounts of enzymes and to make their industrial production possible the genes will be cloned into a host suitable for large-scale

production and the enzymes are produced in pilot scale for subsequent purification, characterization and application experiments. The synergism of enzymes and traditional slime control agents will be studied and the optimal combinations of the enzymes and other ingredients identified. The conditions in circulation water systems leading to most efficient slime degradation will be identified.
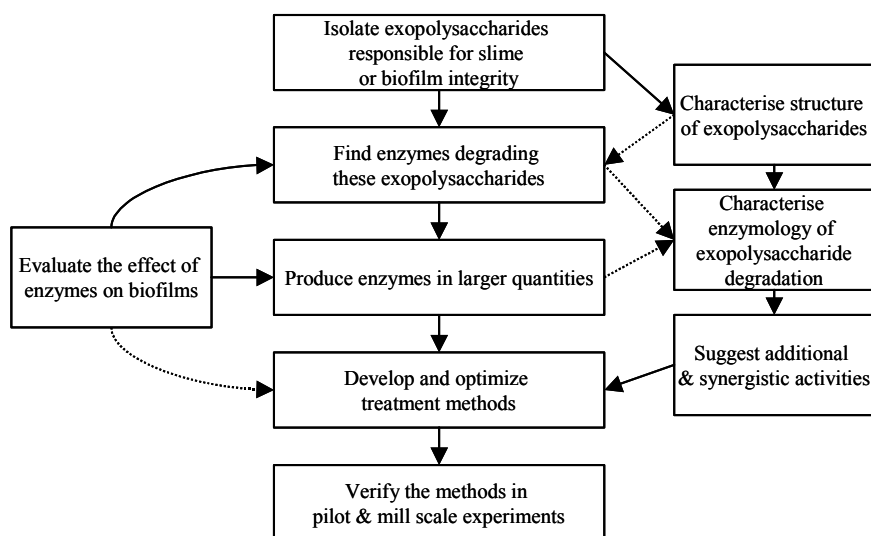

Figure 1.3: Structure of the key tasks and the approach of the multidisciplinary EU-project "Slimezymes".

Aim of this thesis.

The scope of this thesis was to characterise different exopolysaccharides produced by microbial isolates from different paper mills in Finland, Spain and France and to identify the most important exopolysaccharides present. By doing so a targeted approach towards screening for new enzyme activities should be developed. These newly obtained enzymes should be purified and characterised to provide the data for possible cloning into another host.

Chapter 2 and 3 deal with the detailed structural elucidation of the EPS produced by *Brevundimonas vesicularis* and *Methylobacterium* sp both isolated from different Finnish paper mills. In chapter 4 an attempt was made to cluster groups of different bacterial isolates, isolated

from different paper mills in Finland, France and Spain that share chemical characteristics on the basis of the sugar composition and FT-IR spectra of the EPS they produce.

One group, consisting of *Enterobacter* sp., clustered as described in chapter 4 was studied in more detail to obtain unambiguous proof that these bacteria all produce EPS similar to colanic acid (chapter 5).

In chapter 6 the purification and characterisation of a colanic acid degrading enzyme is reported. Finally the results from chapter 2 to 6, combined with additional data on mixed species slime layers isolated from different paper mills, are discussed in chapter 7.

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Chapter 2

Structural elucidation of the EPS of slime producing *Brevundimonas vesicularis sp.* isolated from a paper machine.

Abstract.

The slime forming bacteria *Brevundimonas vesicularis* sp. was isolated from a paper mill and its EPS was produced on laboratory scale. After production, the exopolysaccharide (EPS) was purified and analysed for its purity and homogeneity, HPSEC revealed one distinct population with a molecular mass of more than 2,000 kDa. The protein content was around 9w/w%.

The sample was analysed to determine its chemical structure. The EPS was found to consist of rhamnose, glucose, galacturonic acid and glucuronic acid. Due to the presence of uronic acids the molar ratio between the four sugars found varies from 3:5:2:4 by sugar composition analyses after methanolysis to 1:1:1:1 found by NMR. A repeating unit with a molecular mass of 678Da was confirmed by MALDI-TOF mass spectrometry after mild acid treatment.

^{13}C and ^{1}H hetero- and homonuclear 2D NMR spectroscopy of the native and partial hydrolysed EPS revealed a

→4)-α-L-Glc*p*A-(1→4)-α-D-Gal*p*A-(1→4)-β-L-Rha*p*-(1→4)-β-D-Glc*p*(1→

repeating unit, no non-sugar substituents were present.

Introduction

Slime deposits cause significant operation problems in a paper and board production, such as processing problems and defects in the product quality. Slime is the generic name for deposits of microbial origin in a paper mill [1]. Problematic slimes in the paper and board machines are mixed deposits with thick microbial biofilms as major components. The major structural components of microbial biofilms are polymers excreted by the bacteria present, these polymers largely being heteropolysaccharides. In some cases also proteins and some other substances produced by microbes, e.g. nucleic acids, phospholipids, can be present [2]. The slime deposits may also contain other material derived from the paper manufacture process, such as fibre and organic and inorganic precipitates from process waters [3].

Paper mills, especially those employing increasingly closed loop processes and high use of secondary fibers, have high nutrient levels as well as optimal temperature and pH ranges to support serious microbial proliferation. The conditions normally found in the paper machines are pH 5-8, 20-78ºC and biodegradable materials like cellulose, hemicellulose, starch and wood extractives are widely present [4-7]. Slime formation within the paper industry can cause several problems like off-smells, spots and holes in the end product and may cause severe production delay in the case of web breaks. Nowadays the control of slime problems in paper or board mills is mainly carried out using biocides [4]. Because of environmental and legislative reasons, several alternative slime control methods, such as enzymes, are currently under development. During the last 20 years there have been several reports on the use of enzymes to degrade the slime structure, in order to decrease the use of biocides [8, 9].

Most information about the chemical structure of paper machine biofilm exopolysaccharides has been limited to identifying the different micro-organisms present and to analyses of the sugar residues of which the secreted exopolysaccharides consist. Several articles describe different slime forming micro-organisms commonly found in paper machines. Mattila-Sandholm and Wirtanen [10] report that the most common group of bacteria found in paper machines belongs to the *Enterobactericae, Pseudomonas* sp., *Clavibacter* sp., and *Baccilus* sp.. In addition, Vaïsanen, et al [5] identified the same bacterial species in addition to *Klebseilla* sp. to represent frequently found species in a paper machine. Most of the research has been done only by determining the sugar composition of bacterial slime deposits suggesting that the most common sugars found in paper mill slimes are: glucose, rhamnose, galactose, mannose, fucose and glucuronic acid [3, 5, 11].

Until now hardly any structures for the EPS in paper machine biofilms or from microbes isolated from paper machine biofilms have been published. It is reported that levan is an EPS secreted by several species of *Bacillus* and *Psuedomonas* bacteria in recirculated water of the paper machine [1]. Levan is a specific fructose-containing polysaccharide that is synthesised from sucrose, which is in short supply in paper machine environment. In addition, bacteria producing levan in paper machine conditions are not very common in the process waters [9]. Thus levan is not considered to be a major constituent of paper machine slimes.

Only few attempts have been reported to solve the problem by elucidation of the complete structure of a defined polysaccharide and to apply enzymes on the basis of these data. An example of this is the use of specific levanase against the occurrence of levan described by Chaudhary et al [6]. As indicated above the first step in a logical approach for finding appropriate enzymes to solve slime problems would be by the structural analysis of a number of exopolysaccharides found in bacterial slimes and attacking the structural components of slime deposits. Within this publication we will describe the structural elucidation of an exopolysaccharide produced by *Brevundimonas vesicularis* VTT-E-981024 isolated from a paper mill.

Experimental.

Isolation of bacterial species and extraction and purification of the EPS produced. — The bacterial species *B. vesicularis* VTT-E-981048 was isolated from a slime sample obtained from a paper machine. After isolation and purification of the strain the exopolysaccharide was produced in laboratory scale. The EPS was produced using shale flasks at 30°C in a medium containing glucose (20g/L), yeast extract (0.5g/L), $(NH_4)_2SO_4$ (0.6g/L), KH_2PO_4 (3.18g/L), K_2HPO_4 (5.2g/L), $MgSO_4$ x $7H_2O$ (0.3g/L), $CaCl_2$ (0.05g/L), $ZnSO_4$ x $7H_2O$ (0.2mg/L), $CuSO_4$ x $5H_2O$ (0.2mg/L), $MnSO_4$ x H_2O (0.2mg/L), $FeSO_4$ x $7H_2O$ (0.6 mg/L) and $CoCl_2$ (0.2mg/mL) at pH7.

After cultivation 0.9% NaCl was added to the medium and the medium was slightly homogenised and centrifuged (14687g, 45min). Ethanol (75%(v/v)) was added to the supernatant to precipitate the EPS material. Part of the precipitate was solubilised in water and incubated with protease (Neutrase 0,5L, Novozymes, Denmark) for 1hour at 37 °C, re-precipitated, dialysed (Medicell Visking, MWCO 12,000-14,000 Da) and freeze-dried. The other part was directly dialysed and freeze dried without protease treatment and re-precipitation.

Protein content.— Protein content was measured using the combustion (Dumas) method on a Thermo Quest NA 2100 Nitrogen and Protein Analyzer (Inter Sciences, The Netherlands) according to the instructions of the manufacturer. The sample (5-6mg) was weighed into a sample cup and directly analysed using D-methionine as an external standard. The protein content was calculated using 6.25 as nitrogen to protein conversion factor.

HPSEC of the native EPS.— The EPS (2mg) was dissolved in 1mL of distilled water and analysed by high-performance size exclusion chromatography (HPSEC) using pullulan for calibration. HPSEC was performed on a ThermoQuest HPLC using three Bio-Gel TSK columns in series (60 XL, 40 XL, 30 XL) preceded by an TSK XL guard column (40 x 6mm). Elution took place at 30 °C using 0.8 mL/min 0.2 M $NaNO_3$ as eluent. Detection was performed using a Shodex RI 71 refractive index detector and a Viscotec viscosity and Right Angle Laser Light Scatter (RALLS) detector.

Sugar composition. — The EPS sugar composition was determined using two different methods: Sulphuric acid hydrolysis and methanolysis as described by de Ruiter et al [12].

For methanolysis, the EPS was treated with 2N HCl in dry methanol for 16 hours at 80 °C, followed by 1 h of 2N CF_3CO_2H (TFA) at 121 °C.

For sulphuric acid hydrolysis the EPS was pre-treated with 72% H_2SO_4 for 1 h at 30 °C, followed by hydrolysis with 1 M H_2SO_4 for 3 h at 100 °C.

The released sugars were analysed using high-performance anion exchange chromatography (HPAEC) with pulsed amperometric detection (PAD). HPAEC was performed using a Dionex system containing a ThermoQuest HPLC system, a Dionex EDM Helium degas unit, a Carbopac PA1 column (4 x 250mm) with a Carbopac PA100 guard column and a Dionex ED40 PAD detector. Separation was performed at flow rate 1 mL/min using gradient elution by mixing distilled water, 100 mM NaOH and 1,000 mM NaOAc in 100 mM NaOH. Sample (20 μL) was injected and separated by the following elution program: 26 minutes isocratically with 15 mM NaOH followed by a linear gradient to 100 mM NaOH in 7 minutes. Then a linear gradient was started to 60 mM NaOAc within 12 min followed by a linear gradient to 300 mM NaOAc in another 33 min, keeping the NaOH concentration at 100 mM. The column was washed for 5 min with 1,000 mM NaOAc followed by a 15 min wash step with 100 mM NaOH and equilibrated again for 15 min with 15 mM NaOH.

Absolute configuration.— The absolute configurations of the monosaccharides present in the EPS were determined as described by Gerwig et al. [13]. The GC-FID analyses of the tri-methylsilated (—)-2-butyl glycosides was performed using a Carlo Erba Mega 5160 GC, equipped with a CP-Sil 5 CB column (25 m x 0.32 mm, Chrompack). The temperature programme was: 80 °C → 135 °C at 20 °C/min; 135 °C → 220 °C at 2 °C/min. The injection port and detector temperatures were 200 and 250 °C, respectively. The Helium flow rate was 3 mL/min and the samples (ca. 0.5 μL) were injected directly on the column without a stream splitter.

Sugar linkage analyses.— The EPS sample was methylated according to Hakomori [14] and subsequently dialysed against water and evaporated in a stream of dry air. The methylated samples were hydrolysed using 2 M CF_3CO_2H (1 h, 121 °C). After evaporation in a stream of air (T <20 °C), the partially methylated sample was converted to alditol acetates and analysed by GC-FID. Identification of the compounds was performed using GC-MS as described by van Casteren et al [15].

Partial hydrolyses of the native EPS.— EPS (1 mg) was hydrolysed using 0.5mL 0.05N TFA for 1 h at 100 °C. After cooling, TFA was evaporated in a stream of dry air at 40 °C and the released oligomers were dissolved in 0.5 mL distilled water. HPAEC-PAD was used to analyse the released oligomers using the same Dionex system as described above only with a different gradient. The sample (20 μL) was injected with the following elution program: Separation was started by applying a linear gradient to 500 mM NaOAc in 100 mM NaOH within 30 min, followed by a linear gradient to 1,000 mM NaOAc in 100 mM NaOH within 40 min. Finally the column was equilibrated with 100 mM NaOH for 15 min.

Part of the sample was desalted using Dowex AG 50W-X8 (Biorad) and analysed by matrix-assisted laser desorption /ionisation – time off flight (MALDI-TOF) mass spectrometry. MALDI-TOF MS was performed by mixing 1 μL sample with 1 μL matrix on a plate. The matrix was made by mixing isocarbostyryl (3 mg), 2,5-dihydroxybenzoic acid (9 mg), acetonitril (0.3 mL) and distilled water (0.7 mL). Spectra were recorded using a Voyager-DE RP Biospectrometry Workstation (Applied Biosystems, Framingham, USA) in the positive mode. The laser intensity was set at 2,294, which equals 8.6 μJ per pulse. The pulse delay time was 200 ns, the acceleration voltage was 12,000 V, the grid voltage was 7,200 V and the guide wire

voltage was 9.6 V. The instrument was used in the reflector mode and calibrated with a mixture of maltodextrines.

^{13}C and ^1H NMR. —— Prior to NMR analyses, the samples were exchanged in 99.96% D_2O (Cambridge Isotope Laboratories, USA) and after freeze drying dissolved in 99.996% D_2O (Cambridge Isotope Laboratories, USA). NMR spectra were recorded at a probe temperature of 70 °C on a Bruker AMX-500 spectrometer located at the Wageningen NMR Centre. Chemical shifts were expressed in ppm relative to internal acetone: δ 2.225 ppm for ^1H and δ 31.55 ppm for ^{13}C.

The 1D ^1H proton spectra were recorded at 500.13 MHz using 8-200 scans of 8,192 data points and a sweep width of 3,000 Hz. The 1 D ^{13}C proton decoupled carbon spectra were recorded at 125.77 Hz using 100,000 scans of 32,768 data points and a sweep width of 31,250 Hz.

The 2D COSY spectrum was acquired using the double quantum filtered (DQF) method with a standard pulse sequence delivered by Bruker.

2D TOCSY, 2D NOESY and 2D ROESY spectra were acquired using standard Bruker pulse sequences with 110, 200, 200 ms mixing time, respectively. For all homonuclear 2D spectra 512 experiments of 2,048 data points were recorded using 16-64 scans per increment.

For the 2D HMBC spectrum [16] a standard gradient enhanced 2D- HMQC pulse sequence delivered Bruker was changed into a HMBC sequence by setting the delay between the first proton and carbon pulse to 50 ms. For the HMBC experiment 1,024 experiments of 2,048 data points were performed with 128 scans per increment.

Time domain data were multiplied by phase-shifted (squared-) sine-bell functions.

Results and Discussion

Isolation and purification. —— B. vesicularis VTT-E-981024 was isolated from paper machine slime and grown on laboratory scale to produce extracellular polysaccharide The EPS was precipitated from the culture filtrate by ethanol, treated with protease, re-precipitated, dialysed and freeze-dried. After the extraction and purification it was subjected to further structure elucidation. Part of the sample was not treated with protease in order to detect possible modification of the polymer by side-activities that might be present in the protease preparation.

Prior to the experiments needed for structure elucidation the EPS was analysed for protein content and molecular size distribution. Protein content of the sample without protease and with protease was 9(w/w%) and 8(w/w%), respectively, which indicates that protease treatment did

not have notable effect. On the other hand this result also indicates that the crude EPS seems not to contain major amounts of proteins that would be readily hydrolysable by the protease preparation used, and consequently that the polysaccharide was considerably pure from contaminating proteins.

The molecular size distribution was measured using HPSEC with RI-, RALLS- and viscosity-detection. The EPS was found to consist of only one distinct population with a molecular weight of 2-4 x 10^3 kg/mol and an intrinsic viscosity of around 6.5 dL/g. On the basis of these data it was considered that the polysaccharide was adequately pure for further analysis, and the experiments needed for the elucidation of the EPS structure were carried out without further purification.

Sugar composition and absolute configuration. — The monomer composition of the novel EPS of *B. vesicularis* was determined using HPAEC-PAD (pH > 12) after H_2SO_4 hydrolysis and methanolysis and a specific longer gradient was used to detect oligomeric fragments due to incomplete hydrolyses (Fig. 2.1). Comparing the results found for both hydrolyses methods as shown in Table 2.1 and Fig. 2.1 we could see a significant difference. Hydrolysis with H_2SO_4 yields a considerably lower amount of rhamnose, galacturonic acid and glucuronic acid than found using the methanolyses method (Table 2.1).

Table 2.1

Sugar composition (mol%) of the protease treated and non-protease treated preparations of novel EPS from *B. vesicularis*.

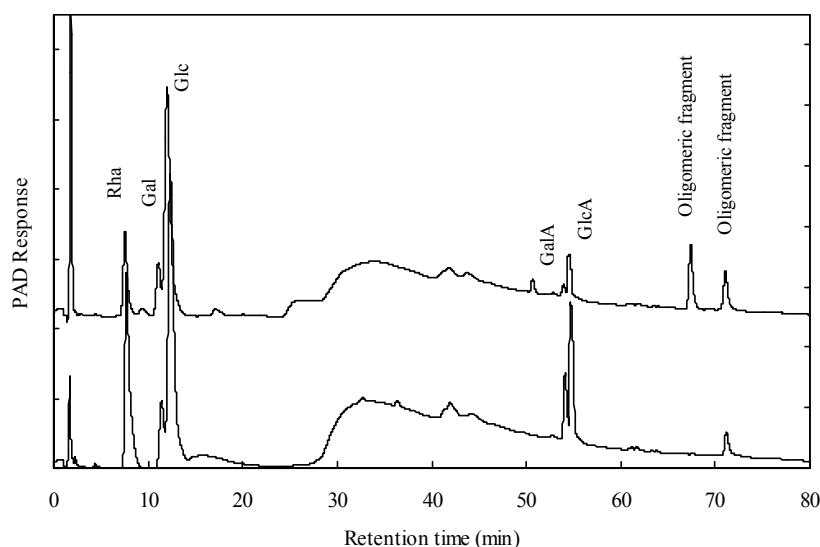| Sugar mol%	EPS		
	Hydrolysis 72% H_2SO_4 + 3h 1M H_2SO_4	Methanolysis 16h 2N HCL in MeOH + 1h 2N TFA	
	Protease treated	Protease treated	Non-protease treated
L-Rhamnose	15.4±2.1	20.4±4.3	30.5±3.7
D-Galactose	11.0±1.0	6.7±1.71	8.2±1.0
D-Glucose	58.1±2.8	32.5±2.9	29.5±3.6
D-Galacturonic acid	4.5±0.8	15.0±1.5	12.6±1.5
L-Glucuronic acid	10.9±1.5	25.4±2.7	19.3±2.3
Total sugar content w/w%	31.7±1.6	79.1±6.8	57.8±5.5

Figure 2.1: HPAEC-PAD elution patterns of the protease treated EPS of *B. vesicularis* after H_2SO_4 hydrolysis and methanolysis followed by TFA hydrolyses, respectively. H_2SO_4 hydrolysis = Upper trace, methanolysis= Lower trac

This is obviously due to incomplete hydrolysis by sulphuric acid resulting in some remaining oligosaccharides (Fig 2.1). Incomplete hydrolyses can be explained by the presence of galacturonic acid and glucuronic acid involved in very stable aldobiuronic acid linkages, which are incompletely hydrolysed by H_2SO_4, a weaker hydrolysing agent than HCl in methanol. It can be concluded that for this kind of EPS containing uronic acids the use of methanolysis would give more reliable results. The higher yield of monosaccharides by methanolysis from the protease treated polysaccharide as compared to the non-treated EPS (Table 2.1) possibly reflects the higher purity of this preparation, due to the two precipitation steps in the isolation procedure of the EPS. Although the sugar compositions of the protease- and non-protease treated EPS are not completely similar, it can be concluded that the former treatment resulted in a rather pure EPS, which was further characterised without further purification.

As shown in Table 2.1 methanolysis revealed the presence of five sugar residues in a molar ratio of Rha:Gal:Glc:GalA:GlcA 3:1:5:2:4. NMR analysis suggests the presence of four hexoses in a molar ratio of GlcA:GalA:Glc:Rha 1:1:1:1. This raises the question why there was a small

amount (6.7%) of galactose and an undisproportional higher amount of glucose. These observations will be explained by the NMR data below. The galacturonic acid to rhamnose ratio by methanolysis was lower then we would expected on the basis of the NMR results this deviation can be explained by the occurrence of an oligomeric fragment even resistant to methanolysis.

After methanolysis the sugar residues released were also converted in trimethylsilylated (—)-2-butyl glycosides and analysed by GLC resulting in recognition of D-glucose, D-galacturonic acid, L-rhamnose L-glucuronic acid and D-galactose.

Partial hydrolysis of the novel EPS of B. vesicularis. — Partial hydrolysis was performed with 0.05 N TFA for 1 hour at 100 °C. This modification of the EPS was performed because rhamnose was present in the repeating unit of the EPS and the first NMR results indicated that rhamnose could be located in the backbone of the polymer. It is assumed that rhamnosyl linkages are weak linkages within the polymeric structure and would make it possible to release oligomeric fractions on the repeating unit level with rhamnosyl residues at the reducing end. Partial hydrolyses was also carried out to decrease the viscosity, which results in the improvement of the resolution in the later shown NMR spectrums. The elution profile of the partially hydrolysed EPS is shown in Fig. 2.2.
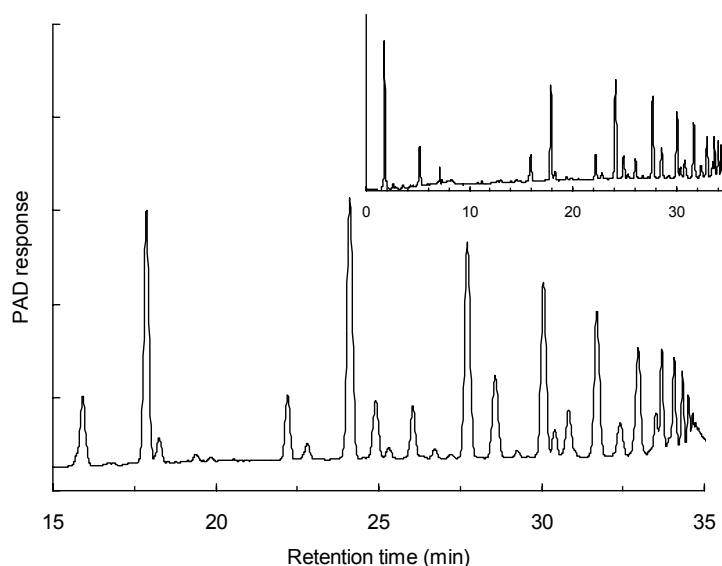

Figure 2.2: HPAEC-PAD elution profile of the mild acid treated (0.05 N TFA) EPS of *B. vesicularis.*

On the basis of the retention time of monogalacturonic acid (12.9 min) in the conditions used it could be concluded that the mild acid treatment of the EPS did not release that many monomeric residues. Taking into account the elution time of monogalacturonic acid it was suggested that the major peaks in the HPAEC elution profile represent a series of oligomeric substances with an increasing number of repeating units.

The same mild acid treated EPS was subjected to MALDI-TOF mass spectrometry, Fig. 2.3 shows the MALDI-TOF mass spectrum also with a series of main peaks. This series would start with the sodium adduct of one tetrameric repeating unit (UA_2, Rha_1, Hex_1) eluting at 17.9 min representing *m/z* 702 in the MALDI-TOF mass spectrum (Figure 2.3), followed by two repeating units (UA_2, Rha_1, Hex_1)$_2$ eluting at 24.1 min representing *m/z* 1,362 and three repeating units eluting at 27.7 min representing *m/z* 2,022 etc. Combining these results with the results found by sugar composition analyses using hydrochloric acid in methanol we could already suggest that the repeating units of the EPS are a tetramer consisting of glucose, rhamnose, galacturonic acid and glucuronic acid.
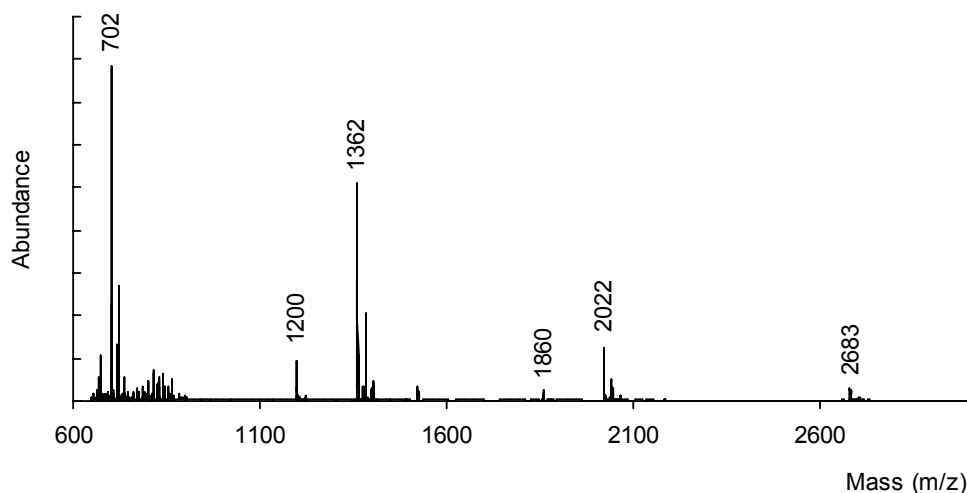

Figure 2.3: MALDI-TOF mass spectrum of the mild acid treated (0.05 N TFA) EPS of
B. vesicularis.

Apart from the oligomeric series of repeating units also another series of minor importance could be found [(UA_2, Rha_1, Hex_1)$_n$+(UA_2, Rha_1)]. This series starts with an heptamer (UA_2,

Rha$_1$, Hex$_1$)+(UA$_2$, Rha$_1$) eluting at 22.2 min representing two repeating units minus one hexose residue *m/z* 1,200 and an undecamer (UA$_2$, Rha$_1$, Hex$_1$)$_2$+(UA$_2$, Rha$_1$) eluting at 26.1 min representing three repeating units minus one hexose *m/z* 1,860 etc.

This indicates that the mild TFA treatment results in partial hydrolysis of the linkage between rhamnose and glucose and that only in a few cases it was also possible to hydrolyse the linkage between glucose and glucuronic acid.

^{1}H- ^{13}C NMR analysis of the native EPS of B. vesicularis. — The signals at δ 5.17, 4.66 and 4.44 ppm of **A** (Table 2.2) belong to the same sugar residue according to the COSY and TOCSY spectra (Fig 2.4 and 2.5). Based on their chemical shifts they can be assigned as the H-1, H-5 and H-4 of α-GalpA, respectively [17-20].

Similarly the signals at δ 5.07 and 4.48 ppm of **B** are assigned as H-1 and H-5 of α-GlcpA, respectively [17, 18].

The signal at δ 1.42 ppm, connected in the TOCSY spectrum with the H-1 at δ 4.48 ppm indicates that residue **C** can be assigned to β-Rha [17, 18, 20].

Finally the chemical shifts found for residue **D** (Table 2.2) are typical for a β-glucose residue.

The total of four anomeric signals confirms the presence of a repeating unit of 4 sugar residues, as suggested by the sugar composition and results from mild hydrolysis experiment.

The ^{13}C- NMR spectrum of the native EPS also showed 4 anomeric signals at δ 107.54, 105.6, 104.74 and 103.96 ppm, respectively. The whole spectrum gave 22 signals where a repeating unit with the four sugar residues mentioned above would normally contain 24 ^{13}C signals. In this case the two expected down field (around 180 ppm) signals for the ^{13}C of the carboxyl- or carboxylate group were not visible; it is known that these signals can have a very low sensitivity [21]. This information also proves the presence of two uronic acid residues.

Table 2.2: 500MHz ^{1}H NMR chemical shifts (δ) in ppm of the residues A, B, C and D or α-GalpA, α-GlcpA, β-Rha and β-Glcp of the native EPS

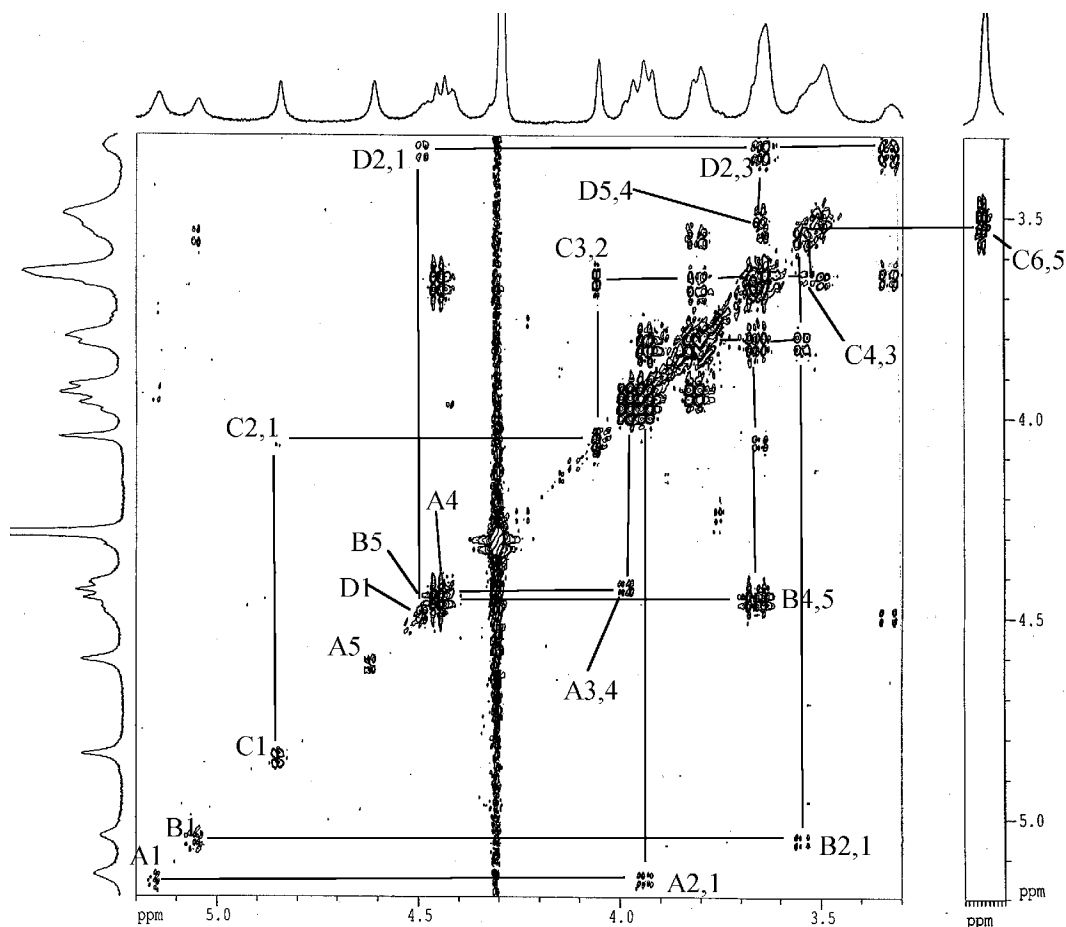| Residue	Proton						
	H-1	H-2	H-3	H-4	H-5	H-6	H-6'
(A) α-D-GalpA	5.17	3.96	4.01	4.44	4.66	-	-
(B) α-L-GlcpA	5.07	3.58	3.83	3.66	4.48	-	-
(C) β-L-Rhap	4.86	4.07	3.65	3.55	3.52	1.42	-
(D) β-D-Glcp	4.5	3.35	3.68	3.65	3.57	3.96	3.88

Figure 2.4: 500-MHz 2D COSY spectrum of the native EPS of *B. vesicularis* recorded in D_2O at 70 ºC. The code A1 stands for the diagonal peak belonging to **A** H-1; A 2,1 indicates the cross peak between **A** H2 and H 1, etc.

^{1}H- ^{13}C NMR analysis of EPS of B. vesicularis after mild acid treatment.— The 0.05 N TFA partially hydrolysed sample could also be used for NMR experiments. The very low amount of monomeric residues present in the hydrolysate might give rise to additional signals in the spectrum due to the rhamnose units at the reducing end and the glucose units at the non-reducing ends of these fragments. However, the partial hydrolysis with 0.05N TFA resulted in only some small fragments and no visible interference with the other residues was observed in the NMR spectra. On the other hand mild acid treatment made it possible to increase the EPS

concentration in the sample improving the resolution in the NMR spectrums. By increasing the EPS concentration in the sample it became possible to record a HMBC 2D NMR spectrum (Fig. 2.6) to assign the glycoside linkage between the different sugar residues and to assign the signals in the ^{13}C –NMR spectrum (Table 2.3).

Using the data obtained by heteronuclear HMBC experiment in Fig. 2.6 it was possible to assign al the signals in the ^{13}C –NMR spectrum. This time it was possible to detect the two down field signals for the carboxylate groups at the GalA and GlcA residues at δ 174.15 and 174.35 ppm respectively. A clear glycosidation effect could be seen by the approximately 10ppm downfield shift of the carbon atoms involved in the glycoside linkages between the different residues (C-4 α-Gal*p*A δ 80.04 ppm, C-4 α-Glc*p*A δ 81.13 ppm, C-4 β-Rha δ 81.86 ppm and C-4 β-Glc*p* δ 78.09 ppm).

Looking at the proton chemical shifts within Table 2.3 and comparing these with the proton chemical shifts found for the native polysaccharide, one could see a clear difference in especially the values found for H-4 and H-5 of the uronic acid residues. It is known that a lower pD results in a downfield shift of the protons of uronic acids [22, 23] and the use of mild acid treatment could have resulted in a lower pD value.

The assignment of the glycosidic linkages between the different residues is based on the HMBC experiment and a homonuclear NOESY experiment (results not shown). Starting with residues **A** and **B** which show a **B** C-1, **A** H-4 and a **B** H-1, **A** C-4 cross-peak supported by a NOESY cross-peak between **B** H-1 and **A** H-4 typical for a 1→4 glycosidic linkage between GlcA and GalA. The inter residual cross-peaks between **A** H-1 and **C** H-5 and **C** H-6 found in the NOESY spectrum and the heteronuclear cross-peak between **A** C-1 and **C** H-4, suggest a GalA-(1→4)-Rha linkage.

The overlap between H-3 and H-4 of the β-Glucose residue (**D**) made it difficult to assign the right glycosidic linkage between rhamnose and glucose. But since it is known that the carbon atoms involved in a glycosidic linkage shift around 10ppm down field there could only be a 1→4 linkage present because **D** C-4 was the only carbon atom that shows a significant down field shift [24]. A Rha-(1→4)-Glc linkage could be proven by the **D** H-4, **C** C-1 and **C** H-1, **D** C-4 cross-peaks shown in the HMBC spectrum. In addition the 2D NOESY spectrum showed a **C** H-1 and **D** H-3, 4 cross peak. The glycosidic linkage between residue **D** and **B** could be revealed by a **D** C-1, **B** H-4 and a **D** H-1, **B** C-4 cross peak in the HMBC spectrum. From the results

found by ^{13}C- and ^{1}H- NMR analysis it could be concluded that the structure of the repeating unit of the novel EPS of *B. vesicularis* is as follows:
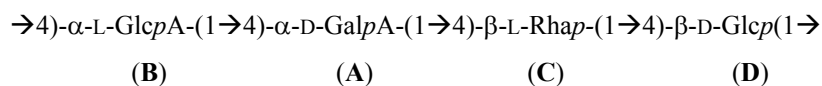
→4)-α-L-Glc*p*A-(1→4)-α-D-Gal*p*A-(1→4)-β-L-Rha*p*-(1→4)-β-D-Glc*p*(1→

      (B)   **(A)**   **(C)**   **(D)**

Table 2.3

^{1}H and ^{13}C chemical shifts (δ) in ppm of the residues A, B, C and D or α-Gal*p*A, α-Glc*p*A, β-Rha*p* and β-Glc*p* of the hydrolysed EPS

Residue	H-1/ (C-1)	H-2/ (C-2)	H-3/ (C-3)	H-4/ (C-4)	H-5/ (C-5)	H-6/ (C-6)	H-6'/ (C-6)
(A)	5.20	3.95	4.02	4.48	4.91	-	-
α-D-Gal*p*A	(100.88)	(69.58)	(69.72)	(80.04)	(71.65)	(174.15)	-
(B)	5.11	3.60	3.86	3.79	4.72	-	-
α-L-Glc*p*A	(100.68)	(72.34)	(72.87)	(81.04)	(71.78)	(174.35)	-
(C)	4.85	4.06	3.67	3.49	3.49	1.40	-
β-L-Rha*p*	(101.81)	(72.65)	(72.65)	(81.86)	(73.10)	(18.29)	-
(D)	4.51	3.33	3.65	3.64	3.54	3.90	3.75
β-D-Glc*p*	(103.51)	(74.53)	(77.01)	(78.09)	(70.98)	(62.17)	(62.17)

Sugar linkage composition.— Sugar linkage analysis combined with mass spectrometry detection confirmed the interpretation of the NMR spectra, but was mainly qualitative. No quantitative assignment could be achieved, because of the presence of unmethylated material and the presence of uronic acids, which were not reduced. Linkage analysis also revealed the presence a 1→4 glucose and a 1→4 linked rhamnose and of minor amounts of terminal galactose, 1,3 linked glucose and 1,3,4 branched glucose. No evidence is present that these residues are connected to the EPS. If this would be the case, this would mean that there is a (3→1)-Glc-(3→1)-Gal side chain present on the glucose residue in one out of five repeating units, but this is not seen by NMR. During the purification steps no residues were lost due to glycosidase activity in the protease used, because no significant differences could be found in the proton NMR spectra of both EPS's. Biochemicaly a suggestion of a side chain on 1 out of 5 repeating units would be surprising, because it is rather rare to find heteropolysaccharides with an irregular composition with respect to the repeating unit backbone and side chain structure [25].
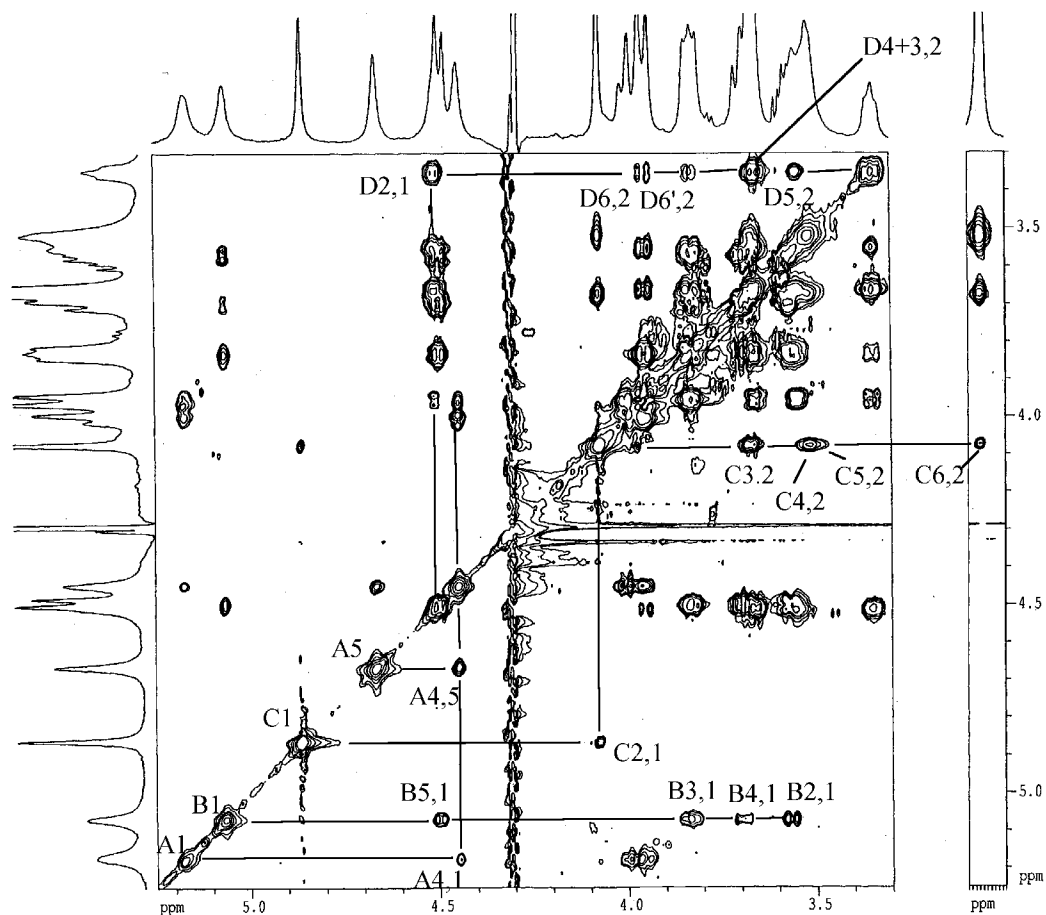
Figure2.5: 500-MHz 2D TOCSY spectrum (110 ms mixing time) of the native EPS of *B. vesicularis* recorded in D_2O at 70 °C. The code A1 stands for the diagonal peak belonging to **A** H-1; A 4,1 indicates the cross peak between **A** H4 and H 1, etc
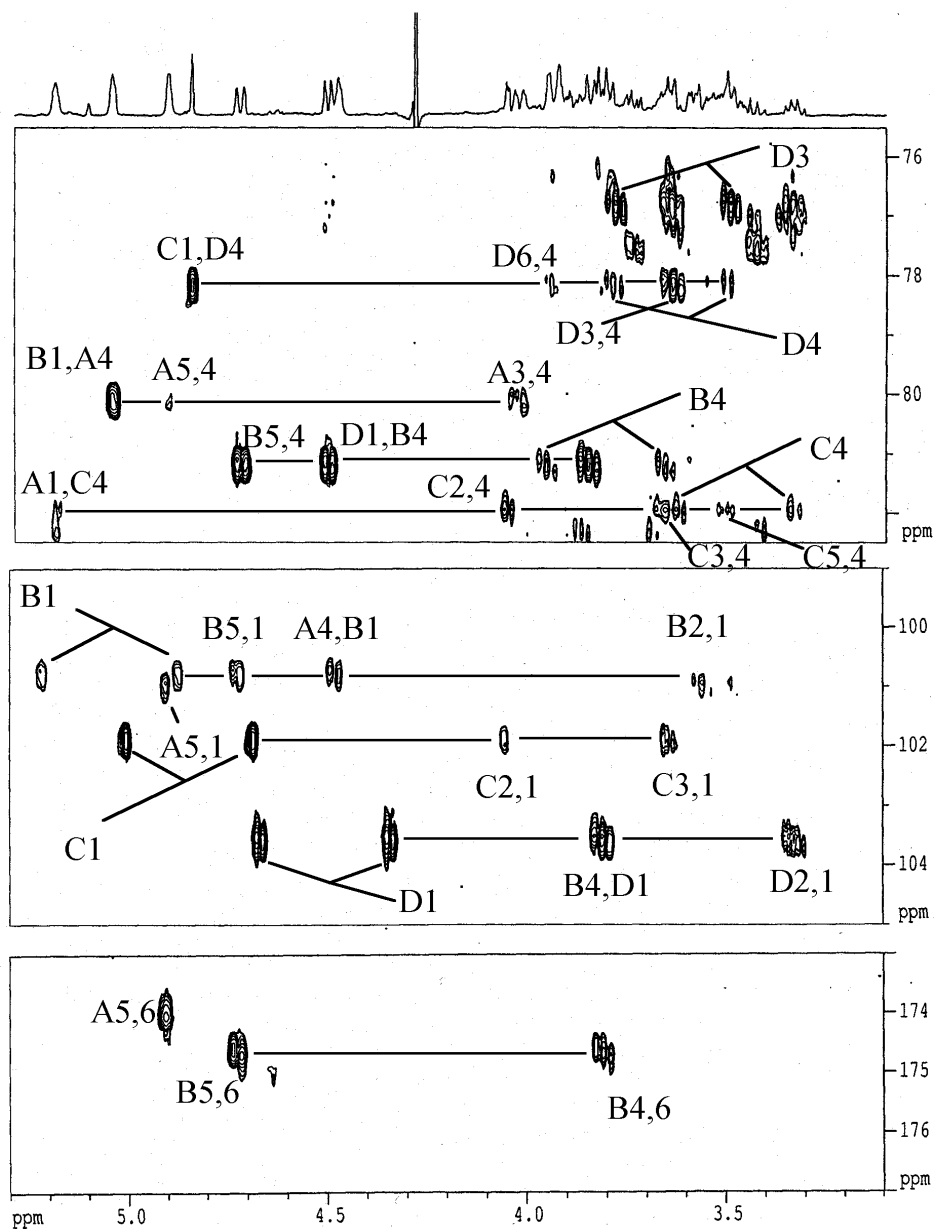
Figure 2.6: 500-MHz 2D ^{1}H ^{13}C undecoupled HMBC spectrum of the partial hydrolysed EPS of *B. vesicularis* recorded in D$_2$O at 70ºC. The code C1 stands for the coupling between **C** H-1 and C-1, C2,1 stands for the coupling between **C** H-2 and C-1 and A4, B1 stands for the long range coupling between **A** H-4 and **B** C1 etcc.

Conclusions.

From the data found by chemical, mass spectrometry and NMR experiments it can be concluded that *B. vesicularis* sp. produces a linear exopolysaccharide without non-sugar substituents containing a tetrasaccharide-repeating unit with the following structure:

→4)-α-L-Glc*p*A-(1→4)-α-D-Gal*p*A-(1→4)-β-L-Rha*p*-(1→4)-β-D-Glc*p*(1→
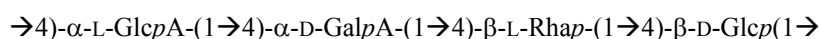
The novel EPS consists of only one distinct homologue population with a molecular weight distributed around 2,000 - 4,000 kDa and an intrinsic viscosity of around 6.5 dL/g.

It also became clear that for our EPS methanolysis with 2 N HCl in dry methanol followed by TFA hydrolysis proved to be more useful than sulphuric acid hydrolysis in determining the sugar composition of the EPS.

The novel EPS found contains a linear backbone consisting of 4 sugar residues. It is obvious that enzymatic degradation may be rather limited due to the variation in sugars, conformation and linkage type present. Further research will be directed towards screening of such enzyme activity in crude enzyme preparations and possibly by screening of cDNA libraries.

Acknowledgement.

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Chapter 3

Methylobacterium sp. isolated from a Finnish paper machine produces highly pyruvated galactan exopolysaccharide.

Abstract.

The slime-forming bacterium *Methylobacterium sp.* was isolated from a Finnish paper machine and its exopolysaccharide (EPS) was produced on laboratory scale. Sugar compositional analysis revealed a 100% galactan (EPS). However, FT-IR showed a very strong peak at 1611 cm^{-1} showing the presence of pyruvate. Analysis of the pyruvate content revealed that, based on the sugar composition, the EPS consists of a trisaccharide repeating unit consisting of D-galactopyranose and [4,6-*O*-(1-carboxyethylidene)]-D-galactopyranose with a molar ratio of 1:2, respectively. Both linkage analysis and 2D homo- and heteronuclear ^{1}H and ^{13}C NMR spectroscopy revealed the following repeating unit: →3)-[4,6-*O*-(1-carboxyethylidene)]-α-D-Gal*p*-(1→3)[4,6-*O*-(1-carboxyethylidene)]-α-D-Gal*p*-(1→3)-α-D-Gal*p*-(1→. By enrichment cultures from various ground and compost heap samples a polysaccharide-degrading culture was obtained that produced an endo acting enzyme able to degrade the EPS described. The enzyme hydrolysed the EPS to a large extent, releasing oligomers that mainly consisted out of two repeating units.

Introduction

Slime deposits in the paper industry are the cause of 70% of all web breaks, blockages and pump failures and therefore are a significant problem within a paper mill [1]. These slime deposits and the problems related to them are generally referred to as biofouling [2]. Paper mills, especially those employing increasingly closed loop processes and high use of secondary fibers, have high nutrient levels as well as optimal temperature and pH ranges to support serious microbial proliferation. The conditions normally found in the paper machines are pH 5-8, 20-78 ºC and biodegradable materials like cellulose, hemicellulose, starch and wood extractives are widely present [2-4]. Under these conditions the bacteria present form microbial biofilms, which are accumulations of microorganisms, exopolysaccharides (EPS), multivalent cations, biogenic and inorganic particles as well as colloidal and dissolved compounds. EPS are the major component of a biofilm and are responsible for the structural and functional integrity of the biofilm [5].

The EPS found in paper mill environments are largely heteropolysaccharides mainly consisting of fucose, rhamnose, glucose, mannose and glucuronic acid [4, 6, 7]. Apart from the biofilm itself slime deposits also contain material derived from the paper manufacture process, such as fibre and organic and inorganic precipitates from the process waters [7].

Prevention of the problems related to slime deposits is still largely performed by the use of more or less toxic biocides. However due to environmental considerations nowadays alternative methods are being investigated for slime control. One of these alternative approaches is the use of enzymes to degrade the EPS responsible for the structural integrity of the slime deposits.

Levanase is an example of an enzyme that degrades the specific EPS called levan [8]. Another example is the use of an enzyme able to degrade colanic acid [9] an EPS that is produced by several bacteria belonging to the *Enterobacteriaceae* family [10]. Furthermore Rättö and co workers screened several bacterial strains that showed enzyme activities towards EPS produced by bacteria isolated from paper machines [11].

For a more targeted search of novel enzymes able to degrade these EPS more knowledge about the chemical fine structure of these EPS is necessary. However only knowing the sugar composition is not sufficient to define a (mixture of) enzymes required to degrade these EPS, since the monosaccharides present can be linked in many different ways.

Furthermore these EPS's can be substituted with several different organic and inorganic substituents like acetyl, pyruvate or phosphate esters [12].

Until now hardly any chemical structures for the EPS's in paper mill biofilms have been published. It has been suggested that a fructose-containing polysaccharide levan is the EPS secreted by several species of *Bacillus* and *Pseudomonas* bacteria in recirculated water of the paper machine [8]. Furthermore, the structure of the EPS produced by *Brevundimonas vesicularis* isolated from a Finnish paper mill has been elucidated. *B. vesicularis* produces a linear polysaccharide containing both glucuronic- and galacturonic acid next to rhamnose and glucose in its repeating unit [13].

Bacteria belonging to the *Methylobacterium* species are known generally to be pink-pigmented bacteria that produce so called pink slime and they were first isolated by Oppong et al [14], but the structure of the EPS produced by *Methylobacterium* strains has not been studied. Therefore this publication deals with the structural elucidation and enzymatic degradation of the EPS produced by a bacterium belonging to the *Methylobacterium* sp. VTT-E-011929 isolated from a Finnish cardboard producing paper mill using chemical pulp as raw material.

Experimental.

Isolation of bacterial species and extraction and purification of the EPS produced. — The bacterial species *Methylobacterium sp.* VTT-E-11929 was isolated from a slime sample obtained from a Finnish cardboard producing paper machine. After isolation and purification of the strain the exopolysaccharide was produced in laboratory scale. The EPS was produced using shale flasks at 30 °C in a medium containing glucose (20 g/L), yeast extract (0.5 g/L), $(NH_4)_2SO_4$ (0.6 g/L), KH_2PO_4 (3.18 g/L), K_2HPO_4 (5.2 g/L), $MgSO_4$ x $7H_2O$ (0.3 g/L), $CaCl_2$ (0.05 g/L), $ZnSO_4$ x $7H_2O$ (0.2 mg/L), $CuSO_4$ x $5H_2O$ (0.2 mg/L), $MnSO_4$ x H_2O (0.2 mg/L), $FeSO_4$ x $7H_2O$ (0.6 mg/L) and $CoCl_2$ (0.2 mg/mL) at pH 7.

After cultivation 0.9 % NaCl was added to the medium and the medium was slightly homogenised and centrifuged (14,687 *g*, 45 min). Ethanol (75% (v/v)) was added to the supernatant to precipitate the EPS material. The precipitate was solubilised in water and incubated with protease (Neutrase 0,5L, Novozymes, Denmark) for 1 hour at 37 °C, re-precipitated, dialysed (Medicell Visking, MWCO 12,000-14,000 Da) and freeze-dried.

Protein content.— Protein content was measured using the combustion (Dumas) method on a Thermo Quest NA 2100 Nitrogen and Protein Analyzer (Inter Sciences, The

Netherlands) according to the instructions of the manufacturer. The sample (5-6 mg) was weighed in to a sample cup and directly analysed using D-methionine as an external standard. The protein content was calculated using 6.25 as nitrogen to protein conversion factor.

HPSEC of the native EPS.— The EPS (2mg) was dissolved in 1 mL of distilled water and analysed by high-performance size exclusion chromatography (HPSEC) using pullulan for calibration. HPSEC was performed on a ThermoQuest HPLC using three TOSOHAAS TSK-Gel columns in series (6000-, 4000-, 3000 PWXL) preceded by an TSK guard column (40x6mm). Enzyme digests were analysed using three TOSOHAAS TSK-Gel columns in series (4000-, 3000-, 2500 PWXL) preceded by an TSK guard column (40 x 6 mm). Elution took place at 30 °C using 0.8 mL/min 0.2 M NaNO$_3$ as eluent. Detection was performed using a Shodex RI 71 refractive index detector.

Sugar composition. —   The EPS sugar composition was determined using methanolysis as described by de Ruiter et al [15]. The EPS was treated with 2 N HCl in dry methanol for 16 hours at 80 °C, followed by 1 h of 2 M CF$_3$CO$_2$H (TFA) at 121 °C. The released sugars were analysed using high-performance anion exchange chromatography (HPAEC) with pulsed amperometric detection (PAD) as described by Verhoef and coworkers.[13]

Absolute configuration.— The absolute configurations of the monosaccharides present in the EPS were determined as described by Gerwig et al. [16]. The GC-FID analyses of the tri-methylsilated (—)-2-butyl glycosides was performed using a Carlo Erba Mega 5160 GC, equipped with a CP-Sil 5 CB column (25 m x 0.32 mm, Chrompack). The temperature programme was: 80 °C→ 135 °C at 20 °C/min; 135 °C→ 220 °C at 2 °C/min. The injection port and detector temperatures were 200°C and 250°C, respectively. The Helium flow rate was 3 mL/min and the samples (ca. 0.5 µL) were injected directly on the column without a stream splitter.

Sugar linkage analyses.— The EPS sample was methylated according to Hakomori [17] and subsequently dialysed against water and evaporated in a stream of dry air. The methylated samples were hydrolysed using 2 M TFA (2 h, 121 °C). After evaporation in a stream of air (T <20°C), the partially methylated sample was converted to alditol acetates and analysed by GC-FID.[18] Identification of the compounds was performed using GC-MS as described by van Casteren et al [19].

Determination of the pyruvate content. — The amount of pyruvate was determined using a method reported by Troyano and coworkers [20]. The EPS (1 mg) were dissolved in 1 mL of 1 M TFA and hydrolysed for 6 h at 120 °C. The hydrolysate was centrifuged and analysed at 40 °C using a Termo Quest HPLC system equipped with both a Shodex RI71 refractive index detector and a Spectra Physics UV2000 UV detector at 220nm. HPLC separation was performed using a Bio-Rad Aminex HPX-87H column using 0.6 ml/min 0,01 N H_2SO_4 as a mobile phase. The amount of pyruvate is calculated using a calibration curve of 0.2 – 2 mM pyruvic acid.

Partial hydrolyses of the native EPS.— Since enzymes are known to facilitate the recognition of repeating units, enzyme-producing bacterial cultures were enriched from compost heap samples by using the EPS produced by VTT-E-011929 as the only carbon source. Culture filtrates from an enrichment culture showing clear reduction of EPS viscosity was used as a crude enzyme preparation to incubate with the EPS in order to obtain oligomers preferably on a repeating unit level.

The EPS (1 mg/mL 50 mM NaOAc buffer) was incubated 50 μL/mL culture filtrate at 30 °C for 15 h in an incubation shaker. The reaction was stopped by keeping the solution at 100 °C for 15min, the sample was centrifuged before analysis.

The released oligomers were analysed using MALDI-TOF ms and HPSEC as described by Verhoef and coworkers. [13]

Auto hydrolyses. — Auto hydrolysis was used to remove pyruvate from the polysaccharide backbone. The native EPS (40 mg) was dissolved in 40 mL water and brought to its H^+ form using Amberlite IR 50 ion exchange resin. A suspension of the polysaccharide solution and Amberlite IR 50 was stirred for 0.5h at 25°C. After filtering of the Amberlite the EPS solution was stirred at 100 °C under reflux for 18 h. The depyruvated EPS was dialysed against water for 24h and freeze-dried.

FT-IR. — The EPS (1 mg/mL) was dried on a crystal and the absorption spectrum between 750 and 4000 cm^{-1} was taken using attenuated total reflectance on a BIO-RAD FTS 6000 FT-IR spectrometer.

^{13}C and ^1H NMR. — Prior to NMR analyses, the samples were exchanged in 99.96% D_2O (Cambridge Isotope Laboratories, USA) and after freeze-drying dissolved in 99.996% D_2O (Cambridge Isotope Laboratories, USA). NMR spectra were recorded at a probe temperature of 70 °C on a Bruker AMX-500 spectrometer located at the Wageningen NMR Centre. Chemical

shifts were expressed in ppm relative to internal acetone: 2.225 ppm for ^{1}H and 31.55 ppm for [13]C.

The 1 D ^{1}H proton spectra were recorded at 500.13 MHz using 64 scans of 8,192 data points and a sweep width of 3,000 Hz. The 2D COSY spectrum was acquired using the double quantum filtered (DQF) method with a standard pulse sequence delivered by Bruker. 2D TOCSY spectra were acquired using standard Bruker pulse sequences with 110ms mixing time. For all homonuclear 2D spectra 512 experiments of 2,048 data points were recorded using 16-64 scans per increment.

For the 2D HMBC spectrum[21] a standard gradient enhanced 2D- HMQC pulse sequence delivered Bruker was changed into a HMBC sequence by setting the delay between the first proton and carbon pulse to 50ms. For the HMBC experiment 1,024 experiments of 2,048 data points were performed with 128 scans per increment.

Results and Discussion

Isolation and purification — Bacterial species *Methylobacterium sp.* was isolated from a Finnish cardboard producing paper mill using chemical pulp as raw material. After isolation the bacterial species was grown on laboratory scale and the EPS produced. Prior to the experiments needed for structure elucidation the isolated and purified EPS was analysed for protein content and molecular size distribution. The protein content was found to be 4% (w/w), while the HPSEC elution profile showed the EPS has a molecular weight of 2,000 kDa. Furthermore the elution profile showed that no other carbohydrate populations were present making it possible to further elucidate the EPS fine structure without further purification.

Sugar composition and absolute configuration — The sugar composition of the EPS was determined by subjecting the EPS to methanolysis followed by TFA hydrolysis. The released sugar residues were analysed by HPAEC and it was found that the EPS only consists of galactose. However the FT-IR spectrum (Fig. 3.1) of the native EPS showed a strong peak at 1,611 cm^{-1} typical for a carboxylate anion indicating the presence of an organic acid substituent. Since pyruvate is very common as an organic acid substituent [12] and is not ester linked to the sugar residues the pyruvate content was measured by HPLC. This revealed that the EPS consisted of both galactose and pyruvate in a molar ratio of 3:2, respectively. The galactose present was found to be in the D absolute configuration. The results above indicate that the

repeating of the EPS would consist of a unit containing 1 D-galactose and 2 pyruvated D-galactoses.
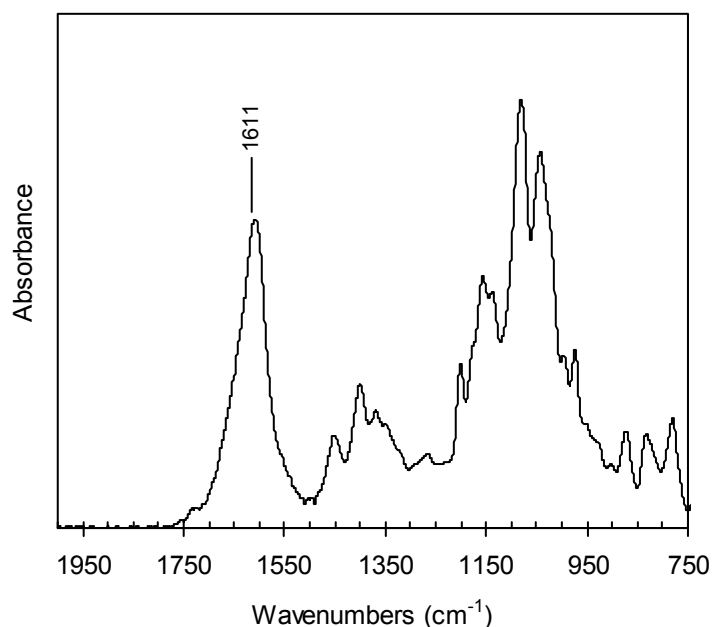

Figure 3.1: FT-IR spectrum of the native EPS produced by *Methylobacterium* sp. after drying 2mg/ml of the sample on an ATR crystal.

Depyruvylation using autohydrolysis. — Pyruvate is a very acid labile group and thus can be removed under very mild acid conditions [22] to obtain a depyruvated sample for both NMR and linkage analysis without degrading the galactan backbone of the EPS. Therefore the EPS was subjected to autohydrolysis, which is known to remove pyruvate ketals [23].
By autohydrolysis the removal of 90% of the pyruvate ketals from the galactan backbone was achieved, resulting in a depyruvated EPS sample necessary for linkage analysis.
The depyruvated sample was analysed by HPSEC revealing a significant decrease in molecular weight, which resulted in a less viscous sample for NMR measurement.

Linkage analysis — Both the native and depyruvated EPS were subjected to permethylation according to Hakamori.[17] Table 3.1 shows the amounts of the different partly methylated alditol acetates released after per methylation followed by hydrolysis of the EPS.
Analysis of the GC-MS spectra of the different partly methylated alditol acetates from the native EPS after hydrolysis resulted in the recognition of 39-mol% of (1→3)-linked galactose and 61-

mol% of 1,3,4,6-linked galactose. This agrees with the conclusions made from the EPS composition that two out of three galactoses found is substituted with a pyruvate ketal. However from the results found for the native EPS it is not possible to distinguish between a 4-6 and a 3-4 bound pyruvate ketal

The GC-MS spectrum of the different partly methylated alditol acetates of the depyruvated EPS revealed the presence of 93-mol% 3-linked-α-galactose and 7-mol% 3,4,6-linked-α-galactose in the EPS. From these results we could conclude that the EPS consists of only 3 linked-α-galactose residues. Furthermore this proved that the repeating unit of the EPS consists of 1 D-galactopyranose and 2 [4,6-*O*-(1-carboxyethylidene)]-D-galactopyranoses, confirmed by the NMR analysis below.

Table 3.1: Glycosidic linkage composition in mol% of the partially methylated alditol acetates of the native and depyruvated EPS of *Methylobacterium* sp.

Glycosyl residue	Yield mol%		Relative retention time[a]
	native EPS	depyruvylated EPS	
3-linked-galactose	39	93	0.643
3-4-6-linked-galactose	61	7	0.889

a) Relative to inositol.

^{1}H NMR analysis of the native EPS.— Fig. 3.2 shows the 1D proton NMR spectrum of both the native and depyruvated EPS. The 1D proton NMR spectrum of the native EPS showed 3 signals (**A, B, C**) in the α anomeric region at 5.36, 5.38 and 5.21 ppm, respectively. Revealing that the 3 D-galactopyranose are in the α anomeric conformation.

Furthermore the 1D proton NMR spectrum showed a signal at 1.48ppm typical for the pyruvate ketals found to be substituted to the galactan backbone.[22, 24]

By recording both a 2D TOCSY (result not shown) and 2D COSY (Fig 3.3) NMR spectrum of the native EPS it became possible to assign the proton chemical shifts of the sugar residues present (Table3.2). The anomeric signal **C** has a chemical shift comparable to the chemical shift found for α-galactopyranoses within literature[25, 26] however the anomeric proton of residue **A** and **B** show a significant downfield shift compared to that residue **C**. This indicates that the neighbouring residue at position 1 of **A** and **B** is substituted with a pyruvate ketal. The chemical shifts of residue **B** indicated that this residue is the unsubstituted α-galactose, because apart from

the downfield shifted anomeric signal the protons show chemical shifts comparable to the chemical shifts reported[25, 26] for 3) -α- galactopyranose. Compared to residue **B**, **A** and **C** show a significant downfield shift with respect to proton 4 and 6 indicating that residue **A** and **C** are both substituted with a pyruvate ketal[25-28].
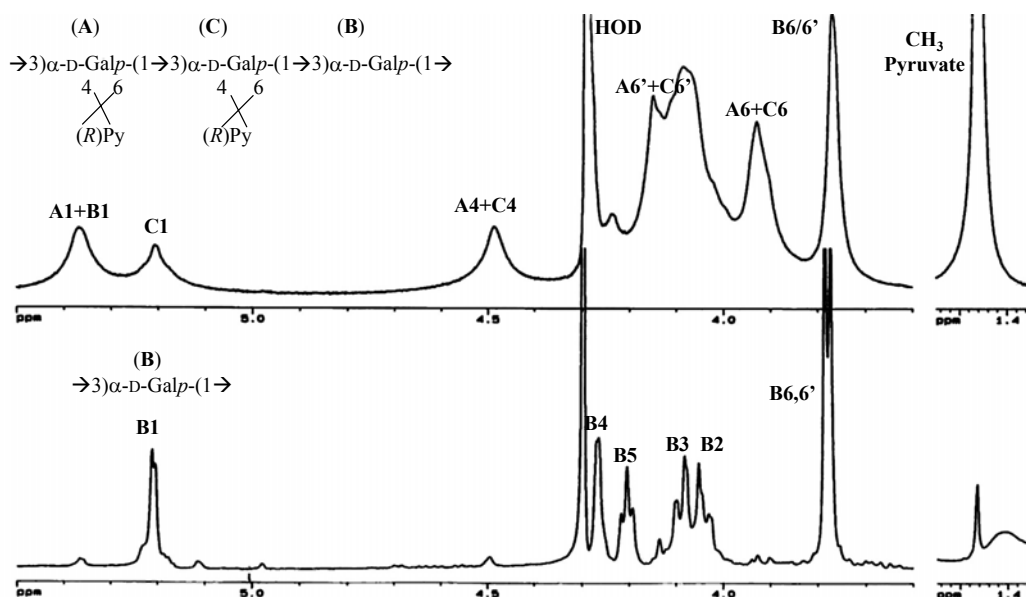

Figure 3.2: 500 MHz ^{1}H NMR spectra of both the native (upper trace) and depyruvated EPS (botom trace) produced by *Methylobacterium* sp.

Fig. 3.2 also shows the proton NMR spectrum of the depyruvated EPS, by recording a 2D COSY spectrum (results not shown) it became possible to assign all the chemical shifts (Table 3.2). Fig. 3.2 shows that after removing the pyruvate ketals only one anomeric signal is left at 5.21 ppm and the disappearance of the pyruvate CH_3 singlet at 1.48ppm.

Furthermore the depyruvated EPS shows similar chemical shifts compared to residue **B** of the native EPS belonging to the non-substituted galactose, apart from the anomeric signal, which is comparable to the one of residue **C** in the native EPS. Residue **C** does not have a substituted galactose at position 1 as neighbouring sugar residue.

Table 3.2: 500MHz proton chemical shift   (ppm) of both the native and depyruvated EPS produced by *Methylobacterium sp.*[*]

Residue	H-1	H-2	H-3	H-4	H-5	H-6	H-6'
Native EPS							
(**A**) →3,4,6)-α-Gal*p*	5.36	4.15	4.15	4.49	nd	3.97	4.08
(**B**) →3)-α-Gal*p*	5.37	4.01	4.11	4.24	4.08	3.76	3.76
(**C**) →3,4,6)-α-Gal*p*	5.21	4.08	4.11	4.47	4.13	3.97	4.08
pyruvate (CH₃)	1.48						
Depyruvated EPS							
→3)-α-Gal*p*	5.21	4.05	4.08	4.27	4.21	3.77	3.77

[*] Chemical shifts relative to internal aceton 2.225ppm
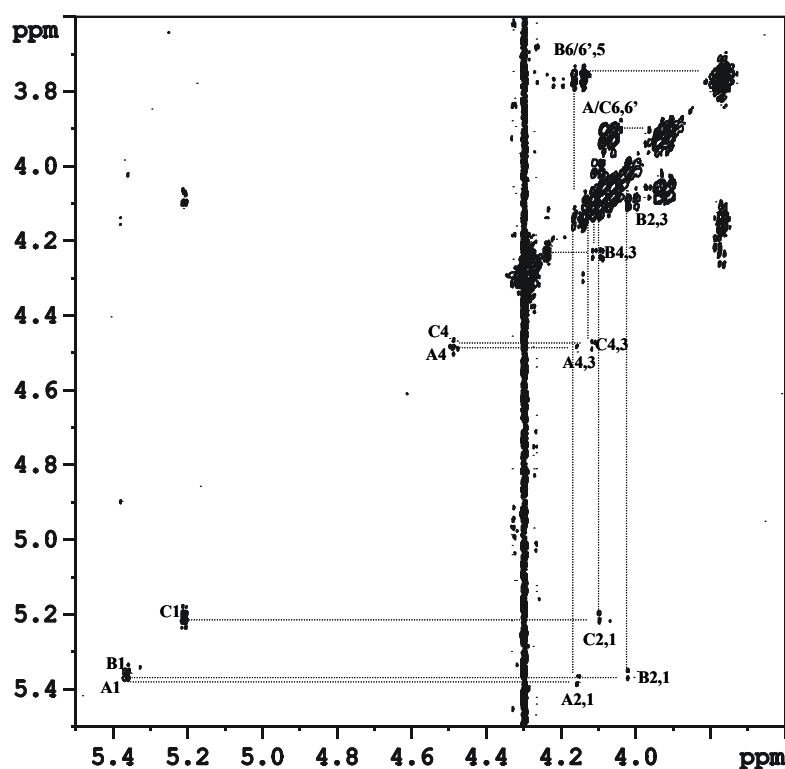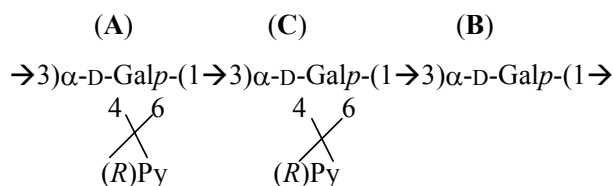
[nd] non determined

Figure 3.3: 500 MHz homonuclear 2D COSY NMR spectrum of the native EPS produced by *Methylobacterium sp.* recorded in D₂O at 70 ºC. The code **A**1 stands for the diagonal peak belonging to **A** H-1; **A** 2,1 indicates the cross peak between **A** H2 and H 1, etc.

A 2D heteronuclear HMBC spectrum was recorded of the oligomers released during enzyme treatment (see below), but it was not possible to solve the spectrum due to low resolution. However, the ^{13}C chemical shifts of the pyruvate ketals could be determined. The carbon of the carboxylic acid group showed a chemical shift of 176.7 ppm and the carbon of the methyl group showed a chemical shift of 26.4 ppm. Together with the proton chemical shift of the methyl group according to Ref. 24 this proves the absolute configuration of the pyruvate ketal is in the *R* form. Furthermore, the heteronuclear crosspeak of the scalar coupling between the quaternary carbon of the pyruvate ketal at δ 102.1 ppm with residue **A** and **C** H-6' at 3.97 ppm could be seen, which is in agreement with the 2 [4,6-O-(1-carboxyethylidene)]-α-D-galactopyranoses found.

Combining the results found by linkage analysis with the results found for NMR analysis it becomes clear that the EPS is a highly pyruvated α-(1-3) galactan with the following repeating unit.

    (A)   **(C)**   **(B)**

→3)α-D-Gal*p*-(1→3)α-D-Gal*p*-(1→3)α-D-Gal*p*-(1→
      4 6   4 6
       ✕   ✕
   (*R*)Py   (*R*)Py

Selective hydrolysis by enzymes from a culture filtrate. — To obtain oligomeric fragments, preferably on a repeating unit level, the native EPS was incubated with a crude enzyme preparation obtained using an enrichment culture isolated from a compost heap. HPSEC (Fig 3.4) was used to determine the decrease in molecular weight after incubating the EPS for 15 h with the culture filtrate. The HPSEC elution profile showed a significant decrease in molecular weight of the enzyme-degraded EPS compared to the native EPS indicating the presence of oligomeric fragments. Fig. 3.5 shows the MALDI-TOF MS spectrum of the enzyme-degraded EPS, one intense peak *m/z* 1294 representing 2 repeating units consisting of 2 D-galactoses (Gal) residues and 4 [4,6-O-(1-carboxyethylidene)]-D-Galactoses (Gal-Py) residues is visible. In addition to the peak at *m/z* 1294 Fig. 3.5 shows minor peaks at *m/z* 830, 1061, 1131 and 1455 due to 4Gal2Gal-Py, 5Gal3Gal-Py, 5Gal4Gal-Py and 7Gal4Py, respectively.

It is evident that the enzyme requires preferably more than 2 repeating units to efficiently split the polymeric backbone. This is supported by the products of enzymatic reaction: The main peak

found representing an hexamer of 2 repeating units accompanied by several minor peaks, which indicates that the enzyme shows a relatively low affinity for this hexamer.

According to these results the culture filtrate contained an endo acting enzyme that degrades the α (1→3) galactan backbone and is tolerant for the two neighbouring [4,6-*O*-(1-carboxyethylidene)]-α-D-galactoses per repeating unit, and mainly releases oligomers consisting out of two repeating units.

Conclusions.

The overall data showed that this *Methylobacterium* sp. strain, representing a species which is known to be responsible for so called pink slime, produces a α-(1→3)- galactan polysaccharide. However, the galactan backbone was found to be highly substituted with pyruvate ketals. This lead to the recognition of a repeating unit consisting of 1 α-D-galactopyranoseand 2 [4,6-*O*-(1-carboxyethylidene)]- α-D-galactopyranoses.

Furthermore an endo acting enzyme that degrades the α (1→3) galactan backbone, obtained by an enrichment culture, was recognised being tolerant to the two neighbouring [4,6-*O*-(1-carboxyethylidene)]-α-D-galactoses per repeating unit, and mainly releasing oligomers consisting of two repeating units.

Further research will be directed towards the purrification an characterisation of the enzyme found within the culture filtrate.
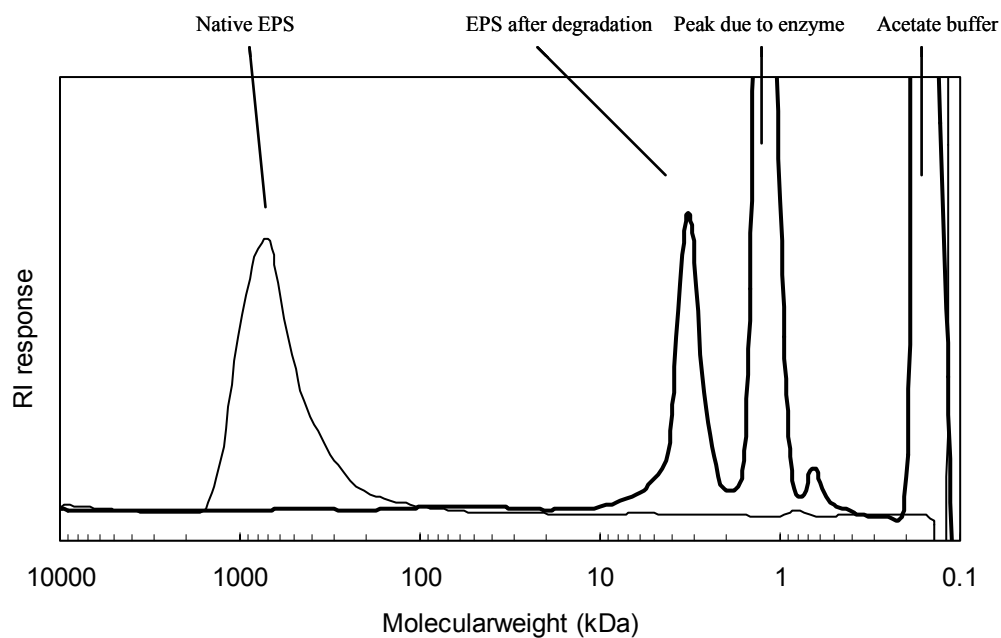
Acknowledgement.

Figure 4: HPSEC elution profile of both the native (—) and enzyme treated EPS (------) produced by *Methylobacterium* sp.
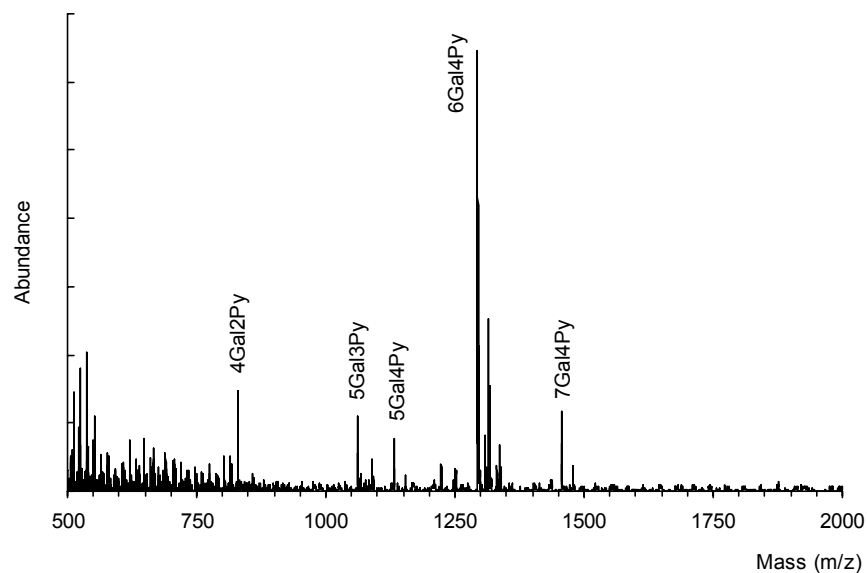

Figure 3.5: MALDI –TOF mass spectrum of the enzyme treated EPS produced by *Methylobacterium* sp. (Py = pyruvate)

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Chapter 4

Sugar composition and FT-IR analysis of exopolysaccharides produced by microbial isolates from paper mill slime deposits.

Summary.

Thirty exopolysaccharides produced by bacteria isolated from biofilms or slimelayers from different paper and board mills in Finland, France and Spain were subjected to size exclusion chromatography and sugar compositional analysis. HPSEC analysis revealed that some samples were composed of several molecular weight populations. These samples were fractionated by size exclusion chromatography and pooled accordingly. Principal Components Analysis of the sugar compositions of the different pools indicated the presence of glucans and mannans. This was caused by insufficient removal of the carbon or nitrogen source (yeast extract) from the bacteria growth medium leading to an overestimation of the glucose and mannose level in the sample, respectively.

From the point of view of slime problems the EPS populations are the most important for multivariate analysis. Four groups of EPSs have been recognised by PCA analysis: a group of EPSs produced by *Enterobacter* and related genera similar to the regularly reported colanic acid; a group of *Methylobacterium* EPSs having high galactose and pyruvate levels and 2 groups that showed less dense clusters produced by *Bacillus* and related genera, showing high mannose and/or glucose levels and *Klebsiella* EPSs that showed galactose with rhamnose as major characteristic sugar moieties.

Fourier Transform Infrared Spectroscopy of the same samples followed by Discriminant Partial Least Squares Regression and Linear Discriminant Analysis showed that, when used with a well-defined training set, FTIR could conveniently be used for clustering instead of time-consuming sugar composition analysis.

The *Enterobacter* and *Methylobacetrium* EPS groups could be recognised clearly. However the fact that this could hardly be done for the other two groups in the data set indicates the importance of a larger and well-defined training or calibration set. The potential to use FTIR, as a tool for pattern recognition and clustering with respect to EPS structures produced by micro organisms isolated from a paper mill environment is discussed.

Introduction.

Nowadays the paper industry is challenged to move towards more sustainable or friendly processes. As a result most modern paper mills are operating a closed loop process water system that operates under neutral or alkaline conditions with an increased consumption of recycled fibres as raw material [1, 2]. In 2003 54% of the produced paper and board in the EU was derived from recycled fibres [3]. These changes have resulted in an increasing number of problems due to deposits caused by an increase in microbiological activity [2].

With respect to microbial deposit problems, paper mills provide an ideal niche for microorganisms to grow and form biofilms. The vast majority of microorganisms live and grow in aggregated forms such as biofilms and flocs. This mode of existence has resulted in the generally accepted expression "biofilm". Biofilms are accumulations of microorganisms, exopolysaccharides (EPS), multivalent cations, biogenic and inorganic particles as well as colloidal and dissolved compounds [4]. In most natural aquatic environments association with a surface by means of biofilm formation is an efficient tool of the microorganism to linger in a favourable microenvironment rather than being swept away by the current [5]. Within a paper mill these biofilms are usually mixed in different proportions with fibres, fines, fillers and other materials that are trapped in this polymer matrix to form a slime deposit. The effects of slime deposits are generally referred to as biofouling. In biofouling, EPSs are responsible for the increase of friction resistance, change of surface properties such as hydrophobicity, roughness, color, etc. [6, 7].

Microorganisms, beside the deposit problems, may also be responsible of spots, bad odours, corrosion, etc. Some microorganisms secrete colour pigments (yellow, pink, orange and red) causing slime formation of different colours e.g. the well known pink-slime. The main characteristic of this specific slime is that it never represents more than 10% of the total slime forming population. However it is very troublesome, because it usually contributes to form a very complex, viscous and rubber slime, that even at low concentrations gives the machine a pink colour in relatively inaccessible places, making it more difficult to detect [8, 9].

Microbial problems have been controlled mainly by the use of biocides [10]. Several surface active agents (surfactants, dispersants, biodispersants, biosurfactants) are also used as a less toxic approach [11, 8]. However due to environmental concerns a new legislation limiting the use of many chemicals and the decrease of their use has been enforced. This has forced the evolution towards more specific control strategies. Enzymes are an interesting alternative to

these traditional chemicals due to their non-toxic character and biological origin. Although the first attempts to enzymatic slime control were taken in the 80s, the use of full-scale enzyme based slime control is limited [8]. This is mainly due to the lack of suitable enzymes that would be active against biofilms and the insufficient understanding of the factors contributing to slime formation in industrial conditions. The identification of problematic EPSs will help with the development of specific enzymatic treatments that directly attack the structure of the biofilm. One of the most important problems concerning enzymes is the great variability in composition of these target polysaccharides in the slimes.

With respect to EPS producing species and the structure of the EPS they produce, most research has been directed towards the identification of the species present. Many different genera and species have been detected within paper machine slimes of which the most important are: *Flavobacterium, Enterobacteriaceae, Pseudomonas, Bacillus, Klebsiella, Sphaerotilus, Citrobacter* and *Burkholderia Cepacia* [1, 2, 8, 12-19].

It is known that EPSs found in paper mill environments are largely heteropolysaccharides mainly consisting of fucose, rhamnose, glucose, mannose and glucuronic acid as reported by Hernandez-Mena and Friend [20], Rättö et al [13], Väisänen et al [14] and Lindberg et al [21]. However, these studies only deal with the sugar compositions of EPS isolates from paper mill environments. The few detailed EPS structures currently known are the ones produced by *Brevundimonas vesicularis* [22], *Raoultella terrigena* [23], *Methylobacterium* sp. [24] and *Sphearothilus natans* [25].

The base for the development of a good strategy to control slime is the knowledge and understanding of the micro flora present at the mill. However this is tedious and time consuming. Therefore new advanced techniques are being used, complementarily to traditional ones, to build up databases allowing, for example, the fast identification of EPS structures and problematic strains in order to define the best anti slime approach. Thus, the aim of this paper is first the characterisation of exopolysaccharides produced by bacteria isolated from different paper and board mills in several countries by the sugar composition of the EPSs they produce; secondly to compare the data with FTIR analysis, a faster technique; and, finally, the clustering of EPS structures on the basis of e.g. different microorganism groups.

Methods.

Isolation of slimy strains from paper machine slime samples— Slime samples were collected from submerged and unsubmerged wet end surfaces of 3 Finnish, 2 Spanish paper mills and 3 French paper mills. Of the slime sample 10 g was homogenized with 90 mL of sterile 0,9% NaCl solution. Spread plate cultures were made of the dilutions 10-2-10-6 on agar plates (Medium I) containing (g/L): glucose (20 g), yeast extract (0.5 g), $(NH_4)_2SO_4$ (0.6 g), KH_2PO_4 (3.18 g), K_2HPO_4 (5.2 g), $MgSO_4$ x $7H_2O$ (0.3 g), $CaCl_2$ (0.05 g), $ZnSO_4$ x $7H_2O$ (0.2 mg), $CuSO_4$ x $5H_2O$ (0.2 mg), $MnSO_4$ x H_2O (0.2 mg), $CoCl_2$ (0.2 mg), $FeSO_4$ x $7H_2O$ (0.6 mg) agar (20 g). The plates were incubated inverted at 30°C and at 50°C. Slimy colonies, appearing as large, shiny and viscous when touched, were picked from the plates and twice purified on same agar medium.

Characterisation and identification of the isolated bacteria— The slimy bacteria isolated were characterised by ribotyping using the automated RiboPrinter® Microbial Characterization System (QualiconTM, Wilmington, DE, USA) following the manufacturer´s instructions with *Eco*RI or PVUII as restriction enzymes. The current commercial Id database of DuPont and the own RiboPrint® -database of VTT Biotechnology were used to identify the genetic fingerprints obtained. The isolates not identified by these databases were subjected to further identification by 16S rDNA sequencing, physiological tests and FAME (fatty acid analysis) [26]. The strains were deposited in culture collection of VTT or CTP.

Production of polysaccharides— The isolates were cultivated in shake flasks in liquid medium with the same composition as the agar medium used for isolation, containing as nitrogen source $(NH_4)_2SO_4$ (0.6 g/L) (Medium I). Some isolates were found to produce more polysaccharide on media with alternative nitrogen sources Bacto tryptone (Difco) (5 g/L) (Medium III) or Bacto tryptone (Difco) (1 g/L) (Medium IV), which were subsequently used. (There are no polysaccharides produced on medium II in table 4.1).

In table 4.1 the medium conditions for each individual microorganism is given. Protein concentrations of the freeze-dried polysaccharides were measured according to [27].

Isolation of polysaccharides— 0.9% NaCl was added to the culture medium, the medium was lightly homogenised for 30-45 seconds and centrifuged chilled at 14700 g for 45 min to separate the cells. 3 volumes of ice-cold ethanol (95 %) were added to the supernatant. If the precipitate did not form at once, the suspension was let to settle at 8°C overnight. The precipitate was either collected with a spoon and a strainer or it was centrifuged chilled at 3900

g for 15 min. The precipitate was solubilised in a small volume of distilled water, dialysed using Medicell Visking (MWCO 12-14000 Da) dialyse tube and freeze dried. In some cases the solubilised polysaccharide after ethanol precipitation was treated with protease (Neutrase 1,5L, Novozymes) (100μL/ 100mL, 37°C, 1h) and re-precipitated with ethanol as described.

Molecular weight distribution and fractionation by HPSEC.— The EPS (2mg) was dissolved in 1mL of distilled water and analysed by High Performance Size Exclusion Chromatography (HPSEC) performed on a ThermoQuest HPLC using three TosoHaas TSK-Gel columns in series (4000-, 3000, 2500,- PWXL) preceded by an TSK guard column (40x6mm). The sample (20μL) was injected and eluted at 30°C using 0.8mL/min 0.2 M NaNO$_3$. Detection was performed using a Shodex RI 71 refractive index detector.

For fractionation 100μL sample of 6mg/mL was injected on the same system and eluted the same way. Fractions of 240μL were collected in 96-well plates using a Gilson FC203B fraction collector. The fractions were pooled according to the carbohydrate populations different in molecular weight and freeze-dried before subjecting them to sugar composition analysis.

Sugar composition. —   The EPS sugar composition was determined using methanolysis as described by de Ruiter et al [28]. Therefore the EPS was treated with 2N HCl in anhydrous methanol for 16 hours at 80°C, followed by 1 h of 2N CF$_3$CO$_2$H (TFA) hydrolysis at 121°C. The released sugars were analysed using High Performance Anion Exchange Chromatography (HPAEC) with Pulsed Amperometric Detection (PAD) using a Dionex system containing a ThermoQuest HPLC system and a Dionex EDM Helium degas unit.   After hydrolysis the samples were injected on a Carbopac PA10 column (4x250mm) with a Carbopac PA10 guard column (4x50mm) preceded with a borate trap (Dionex) and separated at 1mL/min by isocratic elution with water for 40min followed by applying a linear gradient from 0 to 800mM NaOH in 30min. Detection was performed after the post column addition of 0,5mL/min of 1M NaOH using a Dionex ED 40 PAD detector.

Determination of the pyruvate and O-acetyl content. — The amount of pyruvate was determined according to Troyano et al [29]. The EPSs (1mg) were dissolved in 1mL of 1N TFA and hydrolysed for 6 h at 120°C. The hydrolysate was centrifuged and analysed at 40°C using a Termo Quest HPLC system equipped with both a Shodex RI71 refractive index detector and a Thermo Separation Products Spectra System UV2000 UV detector at 220nm.

HPLC separation was performed using a Bio-Rad Aminex HPX-87H column using 0,01N H_2SO_4 (0.6mL/min) as a mobile fase. The amount of pyruvate is calculated using a calibration curve of 0.2-2mM sodium pyruvate.

The acetyl content was determined after saponification with 0.4M NaOH in 50%(v/v) isopropanol/water according to Voragen et al [30].

FT-IR— For FT-IR measurements 100μL of the EPS (1mg/mL) was dried on an ATR crystal and the absorption spectrum between 750 and 4000cm^{-1} was measured by co-adding 100 scans and subtracting both the background and atmospheric water. Spectra were recorded using attenuated total reflectance (ATR) on a Bio-Rad FTS 6000 FT-IR spectrometer.

Multivariate data analysis— All statistical analysis was performed using Unscrambler v.8.0 (CAMO process AS Oslo Norway) and SPSS for Windows v. 10.0.5 (SPSS inc.).

Sugar/substituent compositions in mol% were compared by Principal Components Analysis (PCA) using both cross-validation and calibration /validation data sets without data pre-treatment.

The main goal of PCA analysis is to reduce the dimensionality of a data set in which there are a large number of intercorrelated variables, whilst retaining as much as possible of the information present in the original data. Using PCA the data matrix X (*n* EPSs x *p* sugar moieties) is plotted in *p*-dimensional space and by linear transformation a new set of variables is formed. These variables are called the PC scores and are uncorrelated to each other. Of these newly formed variables the 1st PC describes the direction of the maximum variance present within the X matrix, followed by the 2nd PC and so on [31, 32]. The PC-loadings are factors used to describe the importance or influence of the individual variables or sugar moieties on the newly formed PC-axis and are used to calculate the scores of each individual EPS on the different PC-axis. By plotting for example PC 1 vs. PC 2 samples with similar scores are visualised as a cluster. Using these same PC-loadings the important sugar moieties for each of these clusters can be assigned.

FT-IR spectra were truncated between 2000 and 750cm^{-1} baseline corrected and converted to CSV files using software of BIORAD (Win-IR pro.). After conversion, the spectra were imported into the Unscrambler normalised (maximum normalisation) and the Savitzky-Golay 2nd derivative was taken by averaging over 20 data points for baseline correction and smoothing. After data pre-treatment the spectra were subjected to supervised pattern recognition using Discriminant Partial Least Square (DPLS) regression analysis according to [32]. In contrast to PCA analysis the most commonly used method partial least squares regression (PLS-R) uses both an

X and Y matrix. This way the y-variables in the Y matrix have a direct influence on or guide the decomposition of the X matrix into PCs [31]. The FT-IR spectra obtained within this study were analysed by discriminant (D)PLS-R, this was performed by introducing *dummi variates* indicating group membership as indicated by the clusters formed by PCA analysis of the sugar compositions. These dummi variates were used as y-variables and PLS regression was performed using FT-IR spectra as x-variables (predictors) to facilitate data reduction of the same FT-IR spectra. By using the obtained PC-scores for the different EPS samples the samples could be analysed by linear discriminant analysis (LDA) [32].

The PLS scores (6) were subjected to Linear Discriminant Analysis (LDA) to be able to describe the within and between group variance before and after cross-validation (leave one out). Within and between groups variance was described by the Squared Mahananobis distance from the cluster centre and the number of misclassifications before and after cross-validation. In LDA each sample is assigned to a class (the same as the ones used for DPLS-R) and the class mean scores are calculated from the PC-scores obtained by DPLS-R. The Squared Mahalanobis distance for each individual sample to the class mean score is calculated, and the individual groups are reassigned to the nearest group mean. Using this procedure the number of misclassifications can be determined [32].

Results and discussion.

Isolation of slime producing strains and production of their polysaccharides ▬ The 30 strains used for EPS production were isolated from different paper mills in Finland, Spain and France from different locations at the wet end of the paper machine. Since paper machines are normally cleaned about 1 or 4 times a month it is not sure whether the same species can be isolated from exactly the same spot. However, if the distribution of the species stays the same it can be expected that the same types of slimes will be formed after cleaning the paper machine. Whether the same distribution of species can be found over a long time span remains a question that is rather difficult to address.

Although similar species have been isolated from different paper mills in other studies dealing with paper mill biofilms, indicating that some species seem to be specifically occurring within paper mill slimes. The most commonly detected in paper machine slimes belong *Flavobacterium, Enterobacter, Pseudomonas, Bacillus, Klebsiella, Sphaerotilus, Citrobacter* and *Burkholderia Cepacia* [1, 2, 8, 12-19].

The EPS was produced according to the procedures described in the methods section. To produce polysaccharides in sufficient quantities to be able to screen for enzyme degradability of the same EPSs in future studies a rather rich glucose based medium was used compared to paper machine water typically containing 3-18 mg/L of total nitrogen, 2-6 mg/L of total phosphorus and approximately 500 mg/L dissolved organic carbon [14, 15]. In general the structure of the polysaccharide produced by a microbial species is independent of the carbon substrate used. However, it has been shown that for example nutrient limitation during xanthan production could affect the yield of a specific exopolysaccharide or the uronic acid, acetate or pyruvate content to some extent [33]. Whether exopolysaccharides produced by biofilm species are the same as those produced by planctonic species, remains undetermined [34]. However, Hughes et al [35] has shown that both planktonically prepared and biofilm EPSs could be degraded by the same specific endo-glycanohydrolases releasing the same oligosaccharides. Similarly Costerton et al [36] in [34] used antibodies against planktonically synthesized polysaccharide to demonstrate interaction with material within a biofilm.

Purity and composition of the produced exopolysaccharides — The EPSs produced by 30 different bacteria isolated from different paper mills in Spain, Finland and France, considered as slime producers, were analysed by their sugar composition and substituents present. Before doing so all of the EPSs were subjected to High Performance Size Exclusion Chromatography (HPSEC) analysis to see if the EPS samples consisted of more than one carbohydrate population. Figure 4.1 shows examples of 3 possible situations found and it could be concluded that not all the samples had the same level of purity with respect to the high molecular weight material of interest. Firstly, samples only showing one high molecular weight carbohydrate population. Furthermore samples with a low molecular weight population and samples with both low and intermediate molecular weight populations could be seen. From these results it is obvious that the sugar compositions of some of the produced EPSs could not be used directly, because this would give an overestimate of some of the sugar residues present. Therefore samples with low molecular weight impurities were subjected to extensive dialysis (20kDa cut off) and the samples showing both low and intermediate molecular weight impurities were fractionated on the basis of their molecular weight distribution by HPSEC.
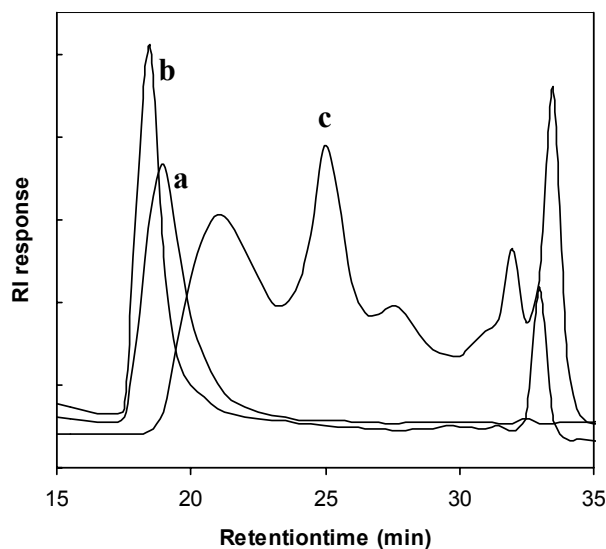
Figure 4.1: HPSEC elution pattern showing examples of 3 possible elution profiles of the EPSs analysed: only 1 High Molecular weight population (a); a HMw and a Low Mw population (b); a HMw, several middle Mw and a LMw population (c).

Sugar and substituent compositions— The sugar- and substituent- compositions of the different EPS samples and their different populations are given in table 4.1. Also the bacterium producing the EPS and the geographical origin of the paper machines are shown. The sugar compositions were subjected to Principal Components Analysis (PCA) to see if the different populations present show common features and can be clustered from each other with respect to their sugar composition.

From the loadings of the different sugar moieties on the first 2 PC's, explaining 80% of the total variance, it could be concluded that a number of the low molecular weight populations show high scores on glucose content. Nearly all of the middle molecular weight populations showed a high score on especially mannose indicating the presence of mannans in several of the EPS samples while also some of the intermediate pools seem to contain glucose. It has been suggested before that these mannans and glucans could be derived from the yeast extract used as nitrogen source [37-39].

To prove this alcohol insoluble solids (AIS) of the yeast extract used were prepared and subjected to HPSEC and sugar composition analysis. Mannose (81 mol%) and glucose (13

mol%) were the major sugar moieties found in the AIS prepared of the yeast extract. Both these sugars accounted for approximately 0,2 w/w% of the yeast extract.

Figure 4.2 shows the HPSEC elution profile of the yeast extract AIS together with an EPS sample containing several molecular weight populations. It is clear that the yeast extract also shows carbohydrate populations in the same molecular weight range as the 2 EPS samples containing several populations. These results show that in the case of a low EPS producer the background of the yeast extract cannot be neglected and one has to be careful when sugar composition of EPSs are directly analysed without first checking whether there is only one or more carbohydrate populations. For further analysis of the sugar and substituent compositions of these EPSs only the HMw pools were used, because the carbohydrates present in the intermediate molecular weight pools are most probably derived from the yeast extract used. But more importantly because EPSs are generally believed to be of high molecular weight and this sample part can be expected to have the highest impact on the slime layers viscosity.

Figure 4.3 shows the appearance of the different sugar and non-sugar moieties over the various EPSs under investigation. It is clear that glucose, galactose and glucuronic acid are present in most of the EPSs. The majority of the EPSs have pyruvate and *O*-acetyl as non-sugar substituents. The fact that 63% of all the EPSs is charged by either uronic acid, mainly glucuronic acid, and/or pyruvate indicates that the polyanionic nature of these EPSs is an important requirement for their appropriate functioning in their environment.

About 50% of all EPSs are substitueted with *O*-Acetyl, which can be seen as a relatively hydrophobic group when compared to the charged groups. Forty percent of the EPSs contains both charged and hydrophobic groups. These characteristics reflect the importance of these groups, capable of ionic interactions and hydrophobic interactions, for EPSs in surface attachment and their important role for the structural integrity of a biofilm [34].
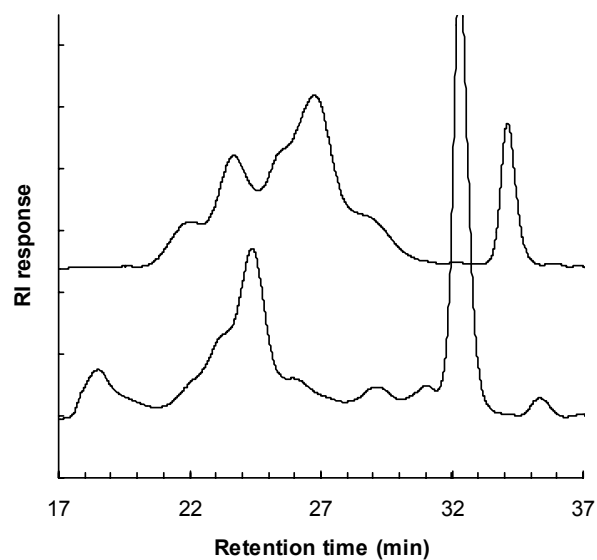
Figure 4.2: HPSEC elution profile of the AIS prepared from yeast extract (upper trace) and example of an EPS sample with more than one carbohydrate populations (lower trace).
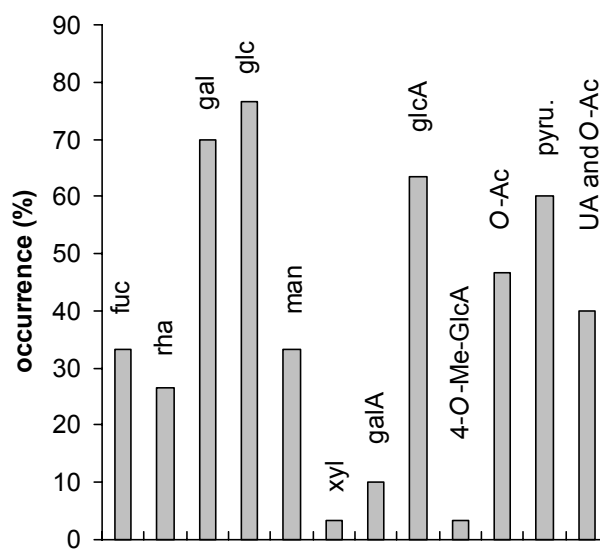

Figure 4.3: Occurrence of the different sugar/substituent moieties as percentage of the total number of EPSs (n=30)

Table 4.1: Geographical origin, bacterial species, different molecular weight populations and sugar/substituent composition of the EPSs produced in mol% [1]

Sample code +Growth medium[2]	Country paper mill1	Bacterial species	Mw Pool	Fucose	Rhamnose	Arabinose	Galactose	Glucose	Mannose	Xylose	GalA	GlcA	4-O-me-GlcA	O-Acetyl	Pyruvate	total sugar
VTT E-011926 (IV)	Finland 3	Enterobacteriaceae (possibly a new genus)	HMw[3]	19	-[4]	-	23	15	16	-	-	13	-	8	5	63
VTT E-011927 (IV)	Finland 3	*Klebsiella pneumoniae*	HMw	-	-	-	13	15	42	-	-	19	-	-	10	80
VTT E-011929 (III)	Finland 1	*Methylobacterium sp.*	HMw	-	-	-	53	-	2	-	-	-	-	-	45	46
VT E-011930 (IV)	Finland 1	*Enterobacter sp.*	HMw	31	-	-	23	15	3	-	1	15	-	8	4	66
VTT E-011935 (IV)	Spain 2	*Enterobacter sp.*	HMw	25	-	-	21	14	2	-	5	12	-	11	9	65
VTT E-011936 (IV)	Spain 1	*Paenibacillus sp.*	HMw	-	-	-	13	17	36	16	3	13	-	3	-	35
VTT E-011937 (IV)	Spain 2	*Rahnella aquatilis*	HMw	-	27	-	41	2	1	-	2	27	-	-	-	71
VTT E-011939 (IV)	Spain 2	*Raoultella terrigena*	HMw	-	41	-	19	17	1	-	-	18	-	1	4	69
VTT E-011940 (IV)	Finland 3	*Enterobacter sp.*	HMw	16	4	-	23	18	9	-	3	11	-	8	6	46
VTT E-011941 (I)	Finland 3	*Enterobacter sp.*	HMw	31	-	-	26	13	2	-	1	16	-	7	5	55
VTT E-011942 (IV)	Finland 3	*Enterobacter sp.*	HMw	20	4	-	22	11	7	-	2	11	-	13	10	64
VTT E-011943 (IV)	Finland 3	*Enterobacter sp.*	HMw	27	1	-	24	15	2	-	2	14	-	10	6	56
VTT E-011944 (IV)	Finland 3	*Enterobacter sp.*	HMw	23	1	-	23	19	4	-	2	14	-	9	5	59
VTT E-981022 (I)	Finland 2	*Paenibacillus sp*	HMw	-	16	-	-	18	49	-	1	16	-	-	-	74
VTT E-981024 (I)	Finland 2	*Brevundimonas vesicularis*	HMw	-	25	-	6	30	-	-	14	23	-	1	-	84
CTP E1112501 (I)	France 3	*Brevibacillus thermoruber*	HMw	-	-	-	-	98	-	-	-	-	-	2	-	10
			ImMw	-	-	-	-	35	48	-	11	6	-	-	-	
			ImMw	-	-	-	-	79	16	-	5	-	-	-	-	
			LMw	-	-	-	2	80	18	-	-	-	-	-	-	

Table 4.4: continued

Sample code +Growth medium	Country paper mill1)	Bacterial species	Mw Pool	Fucose	Rhamnose	Arabinose	Galactose	Glucose	Mannose	Xylose	GalA	GlcA	4-O-me-GlcA	O-Acetyl	Pyruvate	total sugar
CTP E1112506(I)	France 3	*Bacillus smithii*	HMw	-	-	-	6	39	45	-	-	-	-	6	5	13
			ImMw	-	-	-	3	90	8	-	-	-	-	-	-	-
			LMw	-	-	-	-	100	-	-	-	-	-	-	-	-
CTP E11268E1(I)	France 1	*Paenibacillus sp*	HMw	-	-	-	-	8	88	-	-	-	-	4	-	14
			ImMw	-	-	-	2	54	44	-	-	-	-	-	-	-
			LMw	-	-	-	-	100	-	-	-	-	-	-	-	-
CTP E11268E2(I)	France 1	*unidentified*	HMw	-	-	-	52	1	1	-	-	-	-	3	44	28
			ImMw	-	-	-	41	26	33	-	-	-	-	-	-	-
			ImMw	-	-	-	8	72	20	-	-	-	-	-	-	-
			LMw	-	-	-	3	91	7	-	-	-	-	-	-	-
CTPE11268D2(I)	France 1	*Methylobacterium sp.*	HMw	-	-	-	52	1	-	-	6	-	-	3	37	27
			ImMw	-	-	-	40	29	31	-	-	-	-	-	-	-
			ImMw	-	-	-	8	72	20	-	-	-	-	-	-	-
			LMw	-	-	-	2	90	7	-	-	-	-	-	-	-
VTT E-032388(I)	Spain 2	*Klebsiella pneumoniae*	HMw	-	-	-	-	31	4	-	2	15	39	8	-	78
VTT E-032391(I)	Spain 2	*Enterobacter aerogenes*	HMw	-	33	-	11	36	-	-	4	3	-	12	-	13
			ImMw	-	13	-	5	12	70	-	-	-	-	-	-	-
			LMw	-	4	-	37	4	55	-	-	-	-	-	-	-
VTT E-022114 (I)	Spain 2	*Enterobacter sp.*	HMw	26	2	-	21	14	-	-	-	12	-	13	7	25
			ImMw	8	1	-	5	11	74	-	-	-	-	-	-	-
			LMw	-	-	-	-	100	-	-	-	-	-	-	-	-

Table 4.4: continued

Sample code +Growth medium	Country paper mill[1]	Bacterial species	Mw Pool	Fucose	Rhamnose	Arabinose	Galactose	Glucose	Mannose	Xylose	GalA	GlcA	4-O-me-GlcA	O-Acetyl	Pyruvate	total sugar
VTT E-022115 (I)	Spain 2	*Bacillus megaterium*	**HMw**	-	18	-	14	19	30	-	3	15	-	2	-	13
			ImMw	-	19	-	12	7	47	-	14	-	-	-	-	
			ImMw	-	-	-	30	7	64	-	-	-	-	-	-	
			LMw	-	-	-	9	61	30	-	-	-	-	-	-	
VTT E-022116 (I)	Spain 2	*Klebsiella planticola*	**HMw**	-	-	-	18	12	47	-	1	10	-	1	10	61
VTT E-022117 (I)	Spain 2	*Serratia sp.*	**HMw**	-	37	2	-	3	-	-	30	-	-	11	17	38
			ImMw	-	23	-	17	23	14	-	-	23	-	-	-	
			LMw	-	12	-	10	19	52	-	-	8	-	-	-	
VTT E-022118 (I)	Spain 2	*Pantoea sp.*	**HMw**	12	-	-	3	6	67	2	9	-	-	-	-	52
VTT E-022119 (I)	Spain 2	*Pantoea sp.*	**HMw**	-	22	-	13	16	-	-	-	-	-	1	47	22
			ImMw	-	-	-	2	10	88	-	-	-	-	-	-	
			LMw	-	-	-	-	30	70	-	-	-	-	-	-	
VTT E-981023 (I)	Finland 2	*Bacillus sp.*	**HMw**	-	-	-	-	71	6	-	-	-	-	23	-	32
			ImMw	-	5	-	1	60	34	-	-	-	-	-	-	
			LMw	-	-	-	-	100	-	-	-	-	-	-	-	
VTT E-981029 (I)	Finland 2	*Paenibacillus sp.*	**HMw**	-	-	-	15	40	21	-	1	6	-	11	6	72

1) Mol% is defined as [(mol x sugar/ Σ mol all sugars) x 100%)] and therefore is an indicator of the molar distribution of the different sugar moieties found for each sample.
2) Roman numerals indicate the medium used (see experimental section)
3) LMw = Low molecular weight; ImMw= intermediate molecular weight; HMw= High molecular weight
4) Not detected

PCA analysis of the sugar substituent compositions of the high molecular weight pools

All the sugar/substituent compositions of the high molecular weight populations were re-subjected to PCA analysis to be able to form groups of EPSs on the basis of the sugar compositions of the 'pure' EPSs. For these analyses the PCA model developed was validated by sugar compositions found in literature (Table 4.2). Validation was performed by first calculating the PCA model on the basis of the sugar compositions determined for the individual EPSs (calibration set) followed by calculation of the PC scores of the literature EPSs used (validation set) using the developed PCA model. To relate the clusters formed by the use of PCA analysis to the bacterium responsible for the EPS produced the data set was divided in 5 different groups representing different genera of bacteria isolated: 1) *Bacillus* sp., *Paenibacillus* sp. and *Brevibacillus* sp. 2) *Enterobacter* sp. and some other genera of the family *Enterobacteriaceae,* 3)*Methylobacterium* sp., 4) *Klebsiella* sp. and group 5) representing 5 EPSs of various origin. Following this division the symbol indicators in the PCA score plots shown in figure 4.4 could be set according to these groups.

Plotting the 1st versus 2nd PC, explaining 67% of the total variance, two distinctive clusters of EPSs could directly be seen.

The first group, formed by EPSs from mainly *Enterobacter* species (or *Enterobacter* and related genera)*,* shows a dense cluster of EPS's (E-022114, E-011926, E-011930, E-011935, E-011940, E-011941, E-011942, E-011943 and E-011944) with moderate loading on fucose and *O*-acetyl. This group was completely separated from the other EPSs in 3th versus the 4th PC again on the basis of their fucose and *O*-acetyl content. From this plot it was also clear that variation in the composition of these EPSs is mostly accounted for by the difference in *O*-acetyl content. Most of these EPSs show a large similarity in sugar and substituent composition to the well-reported structure of the EPS or M-antigen called colanic acid, known to be produced by members of the *Enterobacteriaceae* family [40-43]. Not all of the EPSs produced by *Enterobacter* species could be assigned to the same dense cluster, but 80% of all the *Enterobacter* EPSs produced share the same sugar/substituent composition (Table 4.1).

The second group could be formed by 3 highly pyruvated galactan EPSs: E-011929, E-112268D2 and E-112268E2. For 2 of these EPS's, E-011929 and E-11268D2, the responsible bacterium has been identified as *Methylobacterium* sp. however for EPS E11268E2 the responsible bacterium still remains unidentified. One could suggest that this last species also belongs to *Methylobacterium* sp. The structure of the EPS produced by this species was

elucidated for sample E-011929 revealing a repeating unit of 3 α(1→3) galactoses with 2 out of the 3 galactoses substituted at position 4 and 6 with a pyruvate ketal [24]. Although the sugar composition of the only other *Methylobacterium* EPS found in literature does not cluster with the 3 EPSs used for calibration it is still characterized by it high galactose loading. More striking was the fact that these EPS were found in both French and Finnish paper mills. Since *Methylobacterium* sp is believed to be one of the species responsible for so called pink slime found in several paper mills[16, 44] it is promising that our approach easily clusters 3 relevant EPSs produced by species isolated from different paper mills in Finland and France.

For the last 2 genera, *Bacillus* and related genera and *Klebsiella*, it was harder to form a dense group or cluster, although more general conclusions could be made. From the first 2 PC's it is obvious that both calibration and validation EPSs produced by *Bacillus* and related genera show high amounts of glucose and/or mannose. However this cluster formed is very broad and shows some overlap with the EPS produced by *Klebsiella* species. On the other hand most of the *Klebsiella* species show loading on galactose but nearly completely overlap the cluster formed for *Enterobacter* sp. EPSs. Looking at PC 3 and 4, part of the EPSs belonging to the *Klebsiella* group could be separated from the other EPSs by the presence of rhamnose. Unfortunately this only accounts for the EPSs produced by the *Klebsiella* species used as validation set. The other EPSs still showed overlap with the *Bacillus* group that now formed a denser group that seems to vary most in their pyruvate content. This overlap is caused by the fact that galactose, glucose and mannose hardly contribute to the variance explained by PC 3 and 4. The results obtained for the EPSs produced by *Bacillus* and *Klebsiella* showed that high glucose and/or mannose are most indicative for EPSs produced by *Bacillus* and related genera. EPSs produced by *Klebsiella* species could be characterized by the presence of high galactose levels and the presence of rhamnose in most of the EPS samples.

Although the results are obtained from a rather limiting data set they show that the determination of the sugar and substituent compositions of the EPSs formed could be used as a first diagnostic tool for detection of troublesome EPSs within slime samples. The composition of the EPSs produced could also assist in assignment of the responsible microorganism. Although an important drawback is the fact that these analyses are time consuming and very dependant on the purity of the sample, because insufficient removal of the carbon/ or nitrogen source could give an overestimate of the glucose and mannose content.
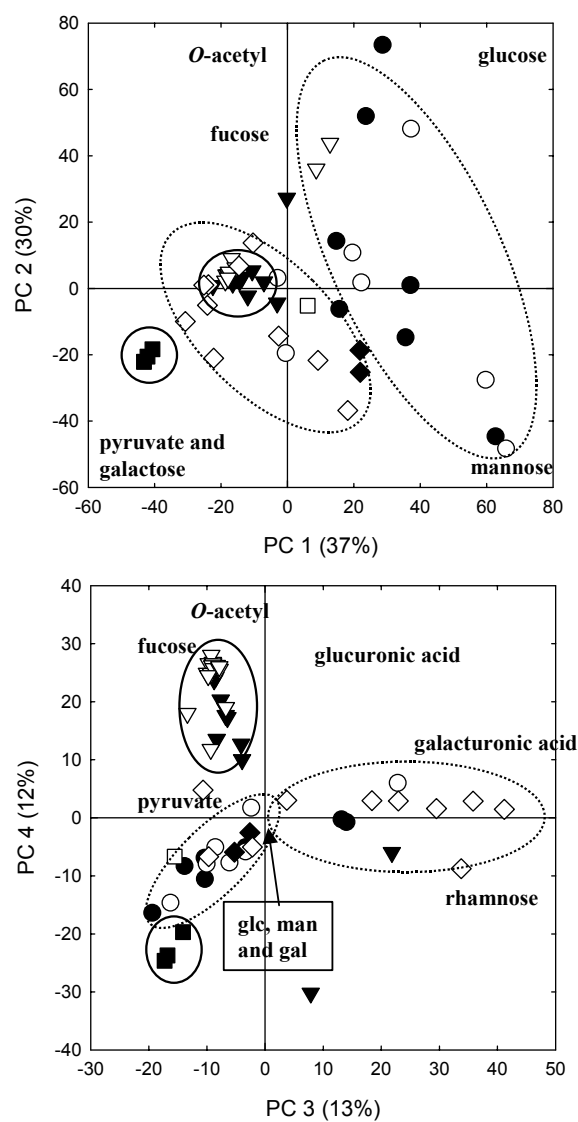
Figure 4.4: PCA score plot of the first 4 PC's after subjecting the EPS sugar substituent compositions to PCA analysis using literature values for validation. The loadings of the mayor sugar moieties on the PCA model are indicated in the score plot.

● - *Bacillus;* ○ - *Bacillus validation;* ▼ - *Enterobacter;* ▽ - *Enterobacter validation;*

■ - *Methylobacterium;* □ - *Metylobacterium validation;* ◆ - *Klebsiella;* ◇ - *Klebsiella validation*

Table 4.2: Sugar compositions (mol%) described in literature of EPSs used for validation of the sugar composition PCA model.

Species	Fuc	Rha	Gal	Glc	Man	GlcA	4-*O*-me-GlcA	Acetyl	Pyruvate
Bacillus polymyxa S-4[45]	-	-	10	30	30	20	10	-	-
Bacillus circulans[46]	-	-	29	29	14	14	-	-	14
Bacillus circulans[47]	-	25	25	-	25	25	-	-	-
Bacillus lichniformis[48]	-	-	-	7	93	-	-	-	-
Bacillus lichniformis[49]	-	-	-	23	77	-	-	-	-
Bacillus Megaterium[50]	-	-	-	80	20	-	-	-	-
Bacillus polymyxa (Strain .NCIB 11429)[51]	-	-	15	41	26	14	-	-	4
Klebsiella strain I-714[52]	-	50	33	-	-	17	-	-	-
Klebsiella serotype 11[53]	-	-	40	20	-	20	-	-	20
Klebsiella serotype K70[23]	-	43	14	14	-	14	-	-	14
Klebsiella type 52[54]	-	33	50	-	-	17	-	-	-
Klebsiella serotype K3[55]	-	-	17	-	50	17	-	-	17
Klebsiella pneumoniae SK1[56]	-	-	56	19	-	19	-	6	-
Klebsiella pneumoniae serotype K40 (3)[57]	-	33	17	-	33	17	-	-	-
Klebsiella pneumoniae serotype K0 (2)[58]	-	40	20	-	20	20	-	-	-
Klebsiella pneumoniae serotype K40 (1)[46]	-	14	57	-	14	14	-	-	-
Klebsiella Type 73[59]	-	25	25	25	-	25	-	-	-
Enterobacter cloacae[60]	25	-	38	25	-	13	-	-	-
Enterobacter (NCIB 11870)[61]	25	-	-	50	-	25	-	-	-
Enterobacter sp.[62]	20	-	-	60	-	20	-	-	-
E.coli S53[42]	30	-	32	14	-	18	-	7	-
E.coli S53C[42]	32	-	29	15	-	17	-	6	-
E.coli S61[42]	29	-	31	15	-	20	-	6	-
E.coli S8[42]	29	-	34	18	-	19	-	-	-
E.coli S22[42]	31	-	32	17	-	19	-	-	-
E.coli S33[42]	30	-	31	17	-	21	-	-	-
E.coli S45[42]	30	-	32	17	-	21	-	-	-
Aerobacter cloacae 5920[42]	28	-	31	17	-	17	-	7	-
Salmonella Bareilly[42]	29	-	32	17	-	21	-	-	-
S. enteriditis[42]	28	-	29	19	-	24	-	-	-
S.paratyphi B[42]	29	-	33	19	-	19	-	-	-
S. typhimurium 1543[42]	28	-	31	15	-	21	-	6	-
Serratia marcescens serotype O14:K12[63]	-	-	17	17	33	17	-	-	17
Rhanella aquatilis[64]	-	2	36	30	22	10	-	-	-
Methylobacterium organophilum[65]	-	-	43	29	29	-	-	-	-

FT-IR Discriminant Partial Least Squares Regression (DPLS) analysis followed by Linear Discriminant analysis LDA analysis—Fourier Transform Infrared Spectroscopy is a fast method that shows the absorption of infra red light by molecular bonds at a given wavelength (λ) usually indicated in wavenumbers (1/λ). This absorption is associated with molecular vibrations; the frequency of the vibration of a specific molecular bond is determined by the masses of the atoms and predominantly the strength of the linkage between them. This way a FT-IR spectrum can be divided into regions that show the IR-bands characteristic for specific functional groups e.g. carboxylic acid. Because FT-IR spectra of polysaccharides show lots of overlap between the different bands and are hard to interpret it is very useful to calculate the 2nd derivative facilitating baseline correction and amplifying maxima within the spectrum, figure 4.5 shows an example of a normal and 2nd derivative FT-IR spectrum. To see if FT-IR has potential to replace sugar/ substituent composition analysis FT-IR spectra of all the EPSs were recorded. This would be favourable because determination of the sugar and substituent compositions of EPSs is time consuming.
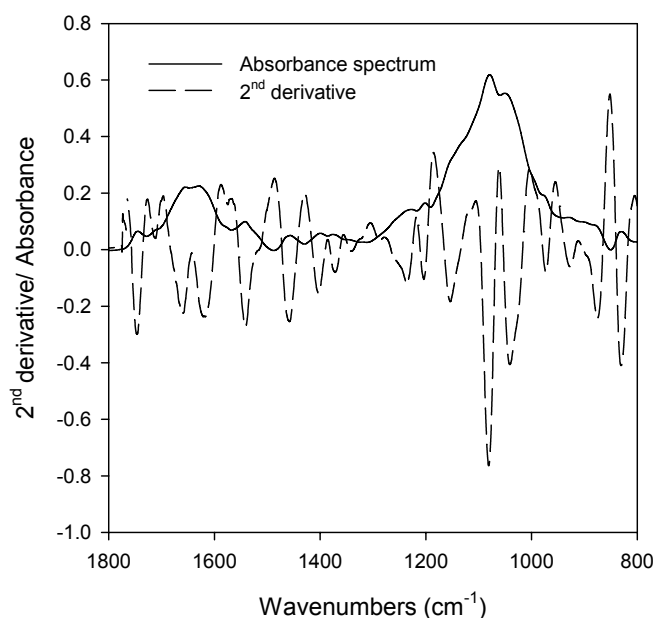

Figure 4.5: Example of a FT-IR absorbance spectrum together with the 2nd derivative of the same spectrum recorded from the EPS produced by *Methylobacterium sp.*

Because the 2nd derivative FT-IR spectra, represented in 1020 dimensions (wavenumbers), were too large (d<n, n=24) to directly apply LDA analysis, first data reduction was performed using DPLS regression.

DPLS regression was performed against a number of dummy variables that indicate group or genera membership by 1 for members and −1 for non-members of a predefined group.

These groups were formed using the results of the PCA analysis applied to the sugar/ substituent compositions as described in the former paragraph: *Bacillus, Enterobacter, Methylobacterium* and *Klebsiella.* Figure 4.6a shows the first 2 PLS scores explaining 43% of the total variance and already from this plot it was possible to recognise the 4 different groups on the basis of their FT-IR spectra. However, the group of EPSs produced by *Bacillus* species is rather broadly distributed and can hardly be considered forming a cluster. A reasonable explanation for this broad distribution is the fact that already upon PCA analysis performed on the sugar compositions of these EPSs only 2 characteristic sugars could be assigned to the cluster of *Bacillus* species. This means that taking into account the other sugars present there can still be a wide variety of possible EPS structures within this cluster of EPS produced by *Bacillus* and related species. Furthermore this could have been caused by the rather low total carbohydrate content observed for most of the EPSs produced by *Bacillus* species. It is reasonable to assume that both these factors result in different FT-IR spectra for all individual EPSs belonging to this group and therefore cannot be clustered. Although detecting several *Bacillus* species is in agreement with the fact that *Bacillus* species are frequently found within paper mill slimes [14, 15, 18, 66], recent studies have shown that *Bacillus* species are incapable of primary colonization but rather depend on coadhesion with other species [67]. These findings might explain the relatively low carbohydrate contents found for the EPS produced by these isolates but also raise the question whether these EPS should be seen as target polysaccharides for enzymatic biofouling control. Figure 4.6b shows the number of PLS scores versus the total explained variance of the first 6 PLS scores explaining 75% of the total variance. The use of more PLS scores would almost certainly result in over fitting in the following LDA analysis. According to Defernez and Kemsley [68] the number of PLS scores used should not exceed *(n-g)/3*, where *g* is the number of predefined groups. In this case n=24 and the number of groups is 4 so a maximum of 6 PLS scores should be used. Figure 4.6 c shows the first 2 canonical discriminant functions of the training set explaining 90% of the total variance. It is obvious that in this case all 4 groups are optimally separated from each other especially because LDA analysis maximises the between

group variances and minimises the within group variance. Therefore to be sure of the suitability or significance of the model it is important to validate the model by cross validation of the model with a leave-one-out method. By doing so the Squared Mahalanobis distance to the cluster centre of each individual sample (figure 4.6d) and the number of misclassifications within each group (table 4.3) could be calculated as an indicator for the suitability of the model for future classification.

From the short distance to the cluster centre of the individual samples belonging to the *Enterobacter* and *Methylobacterium* group compared to the other 2 groups it was clear that these 2 groups of EPSs had the smallest within-group variance. For most of the samples that belong to the *Bacillus* or *Klebsiella* group a larger distance to the cluster centre was observed indicating a large within group variance for these 2 groups. Together with the large within-group variance, 29% of the *Bacillus* EPSs were misclassified after cross validation in comparison to 100% correct classification of the EPSs in the other 3 groups. The results above showed that FT-IR does have potential as a diagnostic tool for EPS identification. It would be preferable to include more cases especially for the *Klebsiella* and *Methylobacterium* group, for this purpose a training set of FT-IR spectra could be developed on the basis of priori knowledge gained from more detailed results derived from other methods e.g. sugar and substituent composition analysis.

With respect to the analysis performed on this data set FT-IR could only be used to cluster the EPSs produced by *Enterobacter* and *Methylobacterium* species although from figure 4.6d it was also clear that especially for the *Enterobacter* group some samples still show a relatively large distance to the cluster centre. To see whether these within group deviations are caused by sample impurities or if some of the samples are really different the next paragraph describes a more detailed analysis of the spectra obtained from the samples within these 2 groups.

> *Detailed within group comparison by FT-IR* ⎯ The FT-IR spectra of the EPSs produced by *Enterobacter* and *Methylobacterium* species were subjected to more detailed analysis to be able to explain that some of the samples show a relatively larger Squared Mahalanobis distance to the cluster centre (Fig 4.6d). To get a clear indication of the differences in these samples the 2nd derivative of their FT-IR spectra are shown in figure 4.7a/b.

The FT-IR spectra in figure 4.7a, of the EPSs produced by *Methylobacterium sp.,* revealed that all theses 3 EPS samples contain the same peaks in their FT-IR spectra and that differences can only be seen in the intensity of specific peaks.

Table 4.3: Classification matrix[1] after applying the first 6 PLS scores generated by DPLS regression of the 2nd derivative FT-IR spectra to LDA analysis with cross-validation.

		Species	Predicted group membership				total
			1	2	3	4	
Original[2]	Count	*Bacillus*	7	0	0	0	7
		Enterobacter	0	11	0	0	11
		Methylobacterium	0	0	3	0	3
		Klebsiella	0	0	0	3	3
	%	*Bacillus*	100	0	0	0	100
		Enterobacter	0	100	0	0	100
		Methylobacterium	0	0	100	0	100
		Klebsiella	0	0	0	100	100
Cross-validated[3]	Count	*Bacillus*	5	2	0	0	7
		Enterobacter	0	11	0	0	11
		Methylobacterium	0	0	3	0	3
		Klebsiella	0	0	0	3	3
	%	*Bacillus*	71	29	0	0	100
		Enterobacter	0	100	0	0	100
		Methylobacterium	0	0	100	0	100
		Klebsiella	0	0	0	100	100

1. A classification matrix shows the number of subjects that is correctly described by pre-defined class using Squared Mahananobis distance to the cluster center to re-assign each subject to the nearest cluster.
2. Assignment based on the original data.
3. Assignment based on cross-validated data set (leave one out)

Samples E-112268D2 and E-112268E2 show a lower intensity than E-011929 for the peaks at 1610 and $1401cm^{-1}$ ($C=O^-$) and 1455 and $1367cm^{-1}$ (CH_3) that belong to the 2 pyruvate moieties present as substituents on 2 out of 3 galactose units per repeating unit [24]. This difference is caused by the presence of protein proven by the fact that these two samples also contain peaks for amide I, II and III stretching vibrations at 1660, 1540 and $1236cm^{-1}$, respectively. By normalising the spectra, the intensity of the bands typical for the carboxyl function within the pyruvate moieties is lowered due to the presence of protein that does not contain these groups, while no large differences could be found in the region typical for the C-C and C-O-C ring vibrations of the different sugar moieties between 1200 and $750cm^{-1}$, because these molecular bonds can be found in both carbohydrates and proteins. These results clearly suggest that the 3 *Methylobacterium* EPSs share the same chemical fine structure. The only differences are accounted for by the sample impurities of samples E-112268D2 and E-112268E2 caused by the presence of protein these findings were confirmed by enzymatic degradation of the EPSs (results not shown).
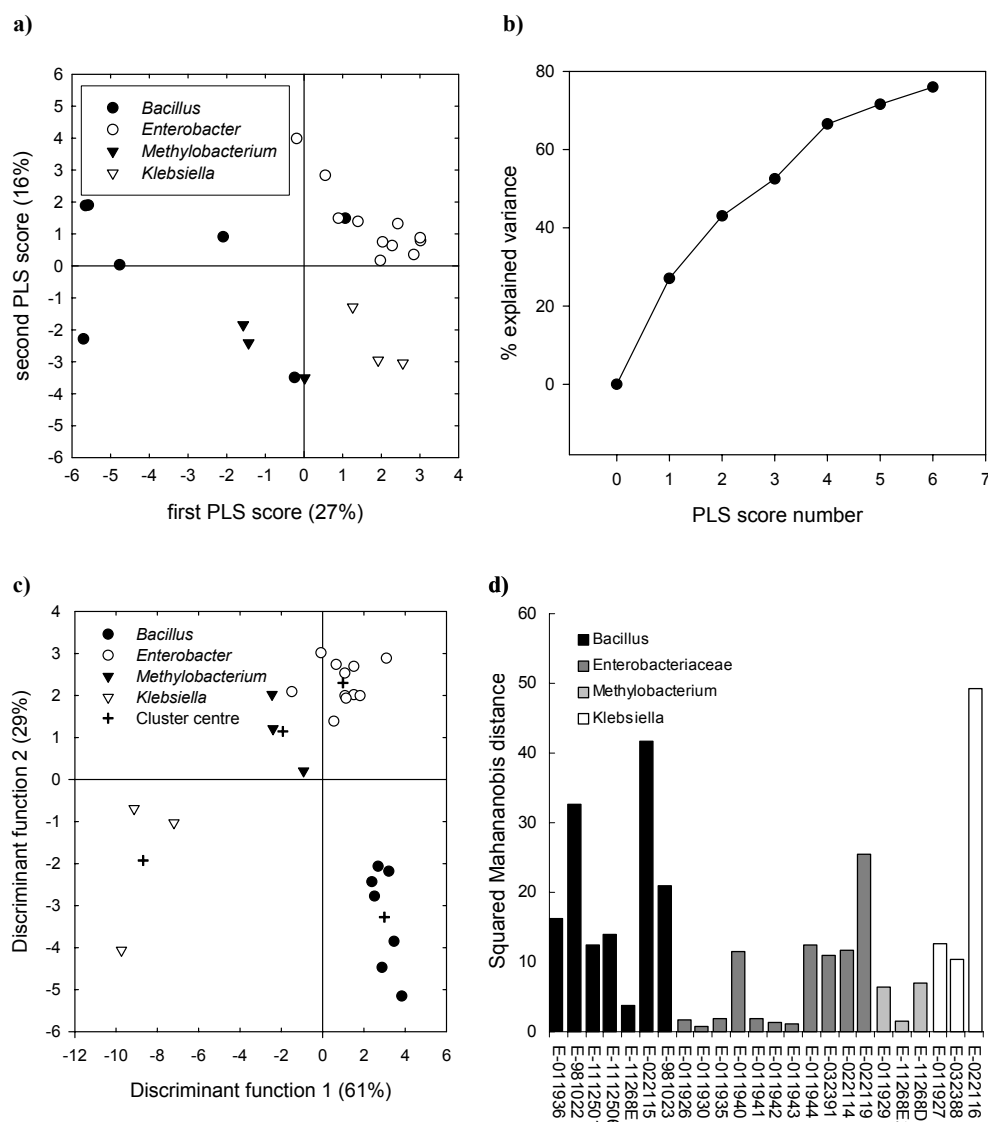
Figure 4.6: a) First vs. second PLS score after applying DPLS regression to the 2nd derivative FT-IR spectra of all EPSs; b) PLS score number vs. the % explained variance after DPLS analysis; c) first vs. second canonical discrimminant function after applying LDA analysis on the first 6 PLS scores (a and b); d) Squared Mahananobis distance to the cluster center of the individual samples belonging to the different pre assigned groups.

Also the FT-IR spectra of the 9 EPS produced by *Enterobacter* and related genera that share the same sugar and substituent moieties as colanic acid were subjected to more detailed analysis. The 2nd derivative FT-IR spectra of the *Enterobacter* EPSs (figure 7b) could be clustered very well. Minor within group deviations were mostly accounted for by the differences in intensity of the different substituents and protein peaks e.g. *O*-acetyl, pyruvate, amide I, II and III. Differences in the C-C and C-O-C ring vibrations between 750 and $1300 cm^{-1}$ are, in this case, only related to differences in intensities of these peaks. This does not account for sample E-011940 showing some very intense additional peaks in its 2nd derivative FT-IR spectrum. Also, from these data, it becomes clear that most of the differences in composition of the *Enterobacter* samples can be accounted for by varying degrees of acetylation, pyruvylation and from differences in protein content.
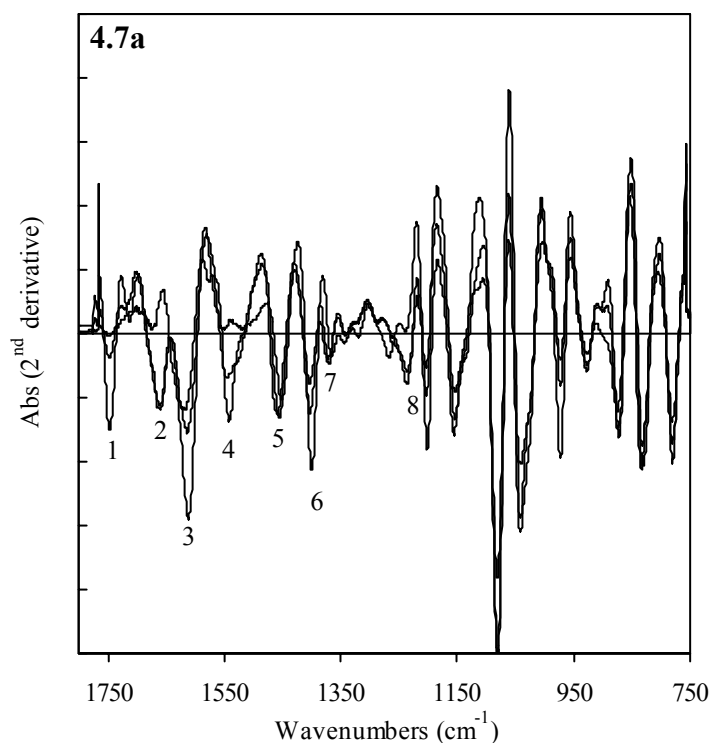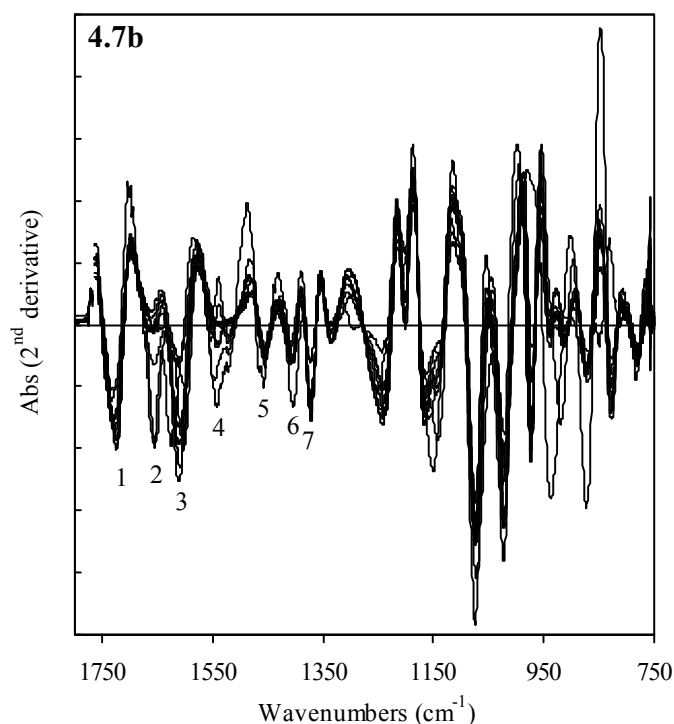
Figure 4.7: 2nd Derivative FT-IR spectra of the EPSs produced by *Methylobacterium* species (a) and *Enterobacter* and related species (b). 1) *O*-acetyl (C=O); 2) amide I ; 3) pyruvate and glcA (C=O); 4) amide II; 5) pyruvate and *O*-acetyl (CH₃); 6) pyruvate and glcA (C-O⁻); 7) pyruvate and *O*-acetyl (CH₃); 8) amide III.

Conclusions.

With respect to the sugar compositions of the EPSs produced by the selected bacteria producing slime from different regions, glucose, galactose and glucuronic acid were concluded to be the most important sugar moieties found. This was in agreement with the studies carried out by Hernandez-Mena and Friend [20], Väisänen et al [14] and Lindberg et al [21].

Apart from these sugar moieties fucose should also be seen as one of the important sugar moieties. Almost all of the EPSs had pyruvate and/or acetyl as non-sugar substituents.

In fact 63% of all the EPSs were charged by uronic acid and/or pyruvate showing a strong polyanionic nature of these EPSs. Together with 50% of all EPSs substitueted with *O*-acetyl,

which can be seen as a relatively hydrophobic group when compared to the charged groups these characteristics could be suggested to be important factors that facilitate the involvement of EPSs in surface attachment and their important role for the structural integrity of a biofilm.

Judging from our studies *Enterobacter* and *Bacillus* sp. could be seen as the major groups of bacteria isolated. These species were reported before but in the earlier studies also *Burkholderia cepacia*, *Klebsiella sp.* and *Pseudomonads* were found to be important[15, 16, 18, 69, 70].

Using PCA analysis of the sugar composition together with a validation data set from literature it became possible to assign fucose to *Enterobacter* sp., glucose and predominantly mannose to *Bacillus* and related genera and galactose together with rhamnose to most *Klebsiella* species. Furthermore, a group of *Methylobacterium* EPSs, consisting only of galactose and pyruvate, was found.

Finally, the presence of impurities caused by low or intermediate molecular weight populations indicated the presence of glucose and mannans, derived most probably from the cultivation medium containing yeast extract. The populations derived from these impurities had to be removed to prevent overestimation of the corresponding sugar moieties.

FT-IR DPLS regression followed by LDA analysis showed that, although it was performed on a rather small data set, there is a potential for FT-IR to replace time-consuming compositional analysis of the EPSs in the future. To achieve this aim a further calibration set of isolated and characterised EPSs is required. By developing this calibration set, FT-IR spectra could be used to define group membership of unknown EPSs using a LDA model.

Overall, our studies revealed that from our limited data set we could already distinguish 2 important exopolysaccharides that can be considered being important for troublesome slime formation within pulp and paper manufacturing. These 2 EPSs were produced by *Enterobacter* and *Methylobacterium* species exhibiting the same structure as colanic acid and a highly pyruvated galactan EPS, respectively. This suggests that detection of sessile or planktonic members of these groups of species or finding their characteristic sugars present in slime could help in determining a targeted anti slime approach.

To prove this future research will be directed towards the detection of these EPSs in samples of biofilms directly taken from a paper machine.

Furthermore, it is important that, independently of the mill type or region, common EPSs have been identified which facilitates common treatment approaches.

Acknowledgement.

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Chapter 5

Colanic acid -producing enterobacteria isolated from paper-machine slimes

Summary

In this study, polysaccharide-producing bacteria were isolated from slimes collected from two Finnish and one Spanish paper mill and the exopolysaccharides (EPSs) produced by 18 isolates were characterised. Most of the of the isolates, selected on the bases of slimy colony morphology, were members of the family *Enterobacteriaceae* most frequently belonging to the genera *Enterobacter* and *Klebsiella* including *Raoultella*. All of the EPSs analysed showed the presence of charged groups in the form of uronic acid or pyruvate revealing the polyanionic nature of these polysaccharides. Further results of the carbohydrate analysis showed that the EPS produced by nine enterobacteria was colanic acid.

Introduction

Slime deposit formation in paper machines can have negative impact on the paper making process and product quality and cause significant economic losses. Slime buid-up is initiated by attachment of micro-organisms on process surfaces and development of surface-attached microbial communities, biofilms, which can further entrap inorganic precipitates and fibres from process waters. Characteristic to microbial biofilms is the formation of an extensive network of highly hydrated exopolysaccharides (EPSs) [1]. In gram-negative bacteria, proteins (flagella, pili) are often involved in the initial attachment of cells to surfaces, in some cases the early attachment is stabilised by EPS. EPS formation is needed in later stages for development of mature three-dimensional biofilm architecture [2].

The bacterial EPSs are heterogeneous, often species- or even strain-specific polymers, the EPSs in mixed-species biofilms can show enormous variability [3]. Bacterial polysaccharides consisting mainly of fucose, rhamnose, galactose, glucose, mannose and glucuronic acid have been detected in paper machine slimes [4-6] but very little data on chemical structures has been published.

A variety of bacteria species, including *Bacillus*, *Citrobacter*, *Deinococcus*, *Enterobacter*, *Flectobacillus*, *Klebsiella*, *Methylobacterium* and *Pseudomonas*, have been isolated from paper machine slimes [7-12]. Some of the isolates have been characterised based on pigment production, filamentous growth characteristics or initial attachment ability but the contribution of different bacteria to the biofilm matrix formation is not known.

This study forms a part of a project aiming at novel enzymatic slime control methods targeted towards matrix polysaccharides in paper machine slimes. Here, isolation of exopolysaccharide-producing bacteria from slimes and characterisation of the polysaccharides produced by the isolates is reported with special attention to colanic acid produced by several enterobacterial isolates from different mills.

Materials and methods

Isolation of polysaccharide-producing bacteria — Slime samples were collected from submerged and unsubmerged wet end surfaces of two Finnish (Fin A, Fin B) and one Spanish (Spa) paper mill. 10 g of the slime sample was homogenised with 90 ml of sterile 0.9% NaCl solution. Spread plate cultures were made of the dilutions 10^{-2}-10^{-6} on agar plates containing

(g/l): glucose (20 g), yeast extract (0.5 g), $(NH_4)_2SO_4$ (0.6 g), KH_2PO_4 (3.18 g), K_2HPO_4 (5.2 g), $MgSO_4$ x $7H_2O$ (0.3 g), $CaCl_2$ (0.05 g), $ZnSO_4$ x $7H_2O$ (0.2 mg), $CuSO_4$ x $5H_2O$ (0.2 mg), $MnSO_4$ x H_2O (0.2 mg), $CoCl_2$ (0.2 mg), $FeSO_4$ x $7H_2O$ (0.6 mg) agar (20 g). The plates were incubated inverted at 30°C and at 50°C. Slimy colonies were picked from the plates and twice purified on same agar medium

Identification of isolates — The isolates were ribotyped using the standard method of the automated RiboPrinter® System (DuPont Qualicon, Wilmington, DE, USA) and *Eco*RI restriction enzyme as described by Bruce [13]. The similarity of the generated patterns were compared with the identification patterns existing in two RiboPrint databases, one of the manufacturer (Release 12.2 (c) 2000 Qualicon, last update in August 2004, includes over 1450 patterns for enterobacteria) and the other created at VTT Biotechnology (e.g. 31, includes over 200 patterns for enterobacteria). In order to form a dendrogram the generated ribogroups were transferred to the BioNumerics programme (Applied Maths, Sint-Martens-Latem, Belgium), where clustering was carried out using Pearson correlation and a Ward dendrogram type. In addition, the patterns of 13 relevant type strains (T) and of *Serratia fonticola* E-991258 (ref) were included from the database of VTT as references. The isolates were also subjected to partial 16S rDNA sequencing (around 450 nucleotides between 1-600 bp of the 16S rRNA gene according to *E. coli* numbering). They were sequenced by the Identification Service of Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) as described by Suihko and Stackebrandt [14]. Finally, the sequences were compared to the GenBank sequences using the BLAST search (1). The physiological properties were performed using API identification strips ID 32 GN and ID 32 E according to the manufacturer´s instructions (bioMérieux SA, Marcy-l´Etoile, France).

Production of polysaccharides — The isolates were cultivated for 5 days at 30ºC in shake flasks in liquid medium with the same composition as the agar medium used for isolation containing $(NH_4)_2SO_4$ (0.6 g/l) (Medium I), Bacto tryptone (Difco) (5 g/l) (Medium III) or Bacto tryptone (Difco) (1 g/l) (Medium IV) as nitrogen source. Culture filtrate viscosities were measured using a Brookfield DV II viscometer at 20 ºC.

Isolation of polysaccharides — 0.9% NaCl was added to the culture medium, the medium was lightly homogenised for 30-45 seconds and centrifuged chilled at 14700 g for 45 min to separate the cells. 3 volumes of ice-cold 100% ethanol was added to the supernatant. If the precipitate did not form at once, the solution was let to precipitate at 8°C overnight. The

precipitate was either collected with a spoon and a strainer or it was centrifuged chilled at 4000 rpm for 15 min. The precipitate was solubilised in a small volume of distilled water, dialysed using Medicell Visking (MWCO 12-14000 dalton) dialyse tube and freeze dried.

Analysis.

Protein concentrations of the freeze-dried polysaccharides were measured with Lowry-method.

Sugar composition — The EPS sugar composition was determined using methanolysis as described by de Ruiter et al [15]. The EPS was treated with 2N HCl in dry methanol for 16 hours at 80°C, followed by 1 h of 2N CF_3CO_2H (TFA) at 121°C. The released sugars were analysed using High Performance Anion Exchange Chromatography (HPAEC) with Pulsed Amperometric Detection (PAD) as described by Verhoef et al [16].

MALDI-TOF MS — For MALDI-TOF MS (Matrix-Assisted Laser Desorption/ Ionisation Time-Of-Flight Mass Spectrometry) a Ultraflex workstation (Bruker Daltronics GmbH, Germany) was used. The mass spectrometer was calibrated with a mixture of malto-dextrins (mass range 365-2309). The samples were mixed with a matrix solution (1 μl of sample in 9 μl of matrix). The matrix solution was prepared by dissolving 9 mg of 2,5-dihydroxybenzoic acid in a 1 ml mixture of acetonitrile:water (300 μl: 700 μl). Of the prepared sample and matrix solutions 2 μl was put on a gold plate and dried with warm air.

Sugar linkage analyses — The EPS sample was methylated according to Hakomori [17] and subsequently dialysed against water and evaporated in a stream of dry air. The methylated samples were hydrolysed using 2M TFA (2h, 121°C). After evaporation in a stream of air (T<20°C), the partially methylated sample was converted to alditol acetates and analysed by GC-FID [18]). Identification of the compound was performed using GC-MS as described by van Casteren et al [19].

Determination of the pyruvate and O-acetyl content — The amount of pyruvate was determined according to Troyano et al [20]. The EPSs (1mg) were dissolved in 1mL of 1N TFA and hydrolysed for 6 h at 120°C. The hydrolysate was centrifuged and analysed at 40°C using a Thermo Quest HPLC system equipped with both a Shodex RI71 refractive index detector and a Thermo Separation Products Spectra System UV2000 UV detector at 220nm. HPLC separation was performed using a Bio-Rad Aminex HPX-87H column using 0.01N H_2SO_4 (0.6mL/min) as a mobile fase. The amount of pyruvate is calculated using a calibration curve of 0.2-2mM sodium

pyruvate. The acetyl content was determined after saponification with 0.4M NaOH in 50%(v/v) isopropanol/water according to Voragen et al [21] using the same chromatographic system.

Partial Hydrolysis ––The EPS (3-4mg) was disolved in 1mL 0.05N TFA and subjected to partial hydrolysis for 1 h at 100°C. After hydrolysis the samples were cooled, dried in a stream of dry air and redisolved in water before MALDI-TOF MS analysis.

Results

Characterization and identification of polysaccharide-producing isolates––A total of 18 slimy isolates were selected for detailed study and they were first characterised by ribotyping. Altogether 16 different ribogroups (ribotypes) were generated, indicating that only three isolates represented the same ribotype (E-011930, E-011940 and E-011943, isolated from two different Finnish paper mills) (Table 5.1). RiboPrint databases could reliably identify to the species level (similarity >0.85) only one isolate, E-011927 as *Klebsiella pneumoniae*. Almost all (16/18, 89%) of the isolates were atypical members of the family *Enterobacteriaceae* belonging to the genera *Citrobacter, Enterobacter, Klebsiella, Rahnella, Raoultella* (formerly *Klebsiella*) or *Serratia* (Table 5.1). One isolate was identified as *Methylobacterium* sp. and the other as *Bacillus simplex*. Many of the isolates are potential members of new species. The clustering (relationship) among the generated RiboPrint patterns and those of relevant type strains is presented in Fig. 5.1.

Production and isolation of EPSs–– EPSs of the 18 isolates were produced for characterization by cultivating the corresponding bacteria in liquid medium. During cultivation, EPS production was estimated by measuring the increase of culture medium viscosity. The viscosities of the culture filtrates of the 18 isolates varied from 1.0 to 44 cP (Table 5.2). The lowest viscosity was detected in *B. simplex* E-022115 culture filtrate and the highest in *Klebsiella/Enterobacter* sp. E-011930 culture filtrate.

Table 5.1. EPS-producing bacteria isolated from paper machine slimes

| VTT code (origin)	Ribogroup, closest similarity to	Partial 16S rDNA sequence, closest similarity (%) to	API results, %id ID 32 GN/ID 32 E	Final identification
E-011926 (Fin B)	1248-S-1 0.67 *Citrobacter freundii*	96.1 *Enterobacter* sp. 95.3 *Salmonella* spp.	low discrimination/ 95.2 *Enterobacter cloacae*	*Enterobacter sp.*
E-011927 (Fin B)	1301-S-6 0.90 *Klebsiella pneumoniae*	99.8 *Klebsiella pneumoniae*[T]	93.8 *Klebsiella pneumoniae*/ 94.7 *Klebsiella pneumoniae*	*Klebsiella pneumoniae*
E-011929 (Fin A)	1303-S-5 0.61 *Methylobacterium sp.*	95.4 *Methylobacterium organophilum*[T] 94.6 *Methylobacterium suomiense*[T]	not done	*Methylobacterium sp.*
E-011930 (Fin A)	502-S-8 0.91 *Enterobacteriaceae*, closest to *Enterobacter*	99.6 (97.3[T]) *Klebsiella pneumoniae* 98.1 *Morganella morganii* 97.5 *Enterobacter cancerogenus*	low discrimination/ 91.9 *Pantoea* spp. (doubtful profile)	*Klebsiella/ Enterobacter sp.*
E-011935 (Spa)	1253-S-2 0.94 *Enterobacter* sp.	99.4 *Enterobacter amnigenus*[T] 99.0 *Buttiauxella izardii*[T]	96.8 *Enterobacter amnigenus*/ 83.1 *Enterobacter amnigenus*	*Enterobacter amnigenus*
E-011937 (Spa)	1253-S-4 0.78 *Serratia marcescens*	99.6 *Rahnella aquatilis*	96.1 *Rahnella aquatilis*/ 94.5 *Rahnella aquatilis*	*Rahnella aquatilis*
E-011939 (Spa)	1253-S-6 0.79 *Raoultella terrigena*	99.2 *Raoultella terrigena*[T]	96.1 *Klebsiella oxytoca*/ 99.9 *Raoultella terrigena*	*Raoultella terrigena*
E-011940 (Fin B)	502-S-8 0.92 *Enterobacteriaceae*, closest to *Enterobacter*	99.6 (97.3[T]) *Klebsiella pneumoniae* 98.1 *Morganella morganii* 97.5 *Enterobacter cancerogenus*	low discrimination/ 88.1 *Pantoea* spp. (doubtful profile)	*Klebsiella/ Enterobacter sp.*
E-011941 (Fin B)	1254-S-2 0.48 *Citrobacter koseri*	99.6 *Citrobacter koseri*[T]	low discrimination/83.5 *Pantoea* spp. (doubtful profile)	*Citrobacter sp.*
E-011942 (Fin B)	1254-S-3 0.78 *Enterobacteriaceae*, closest to *Enterobacter*	99.6 (97.3[T]) *Klebsiella pneumoniae* 98.1 *Morganella morganii* 97.5 *Enterobacter cancerogenus*	low discrimination/ 83.3 *Pantoea* spp. (doubtful profile)	*Klebsiella/ Enterobacter sp.*
E-011943 (Fin B)	502-S-8 0.94 *Enterobacteriaceae*, closest to *Enterobacter*	99.6 (97.3[T]) *Klebsiella pneumoniae* 98.1 *Morganella morganii* 97.5 *Enterobacter cancerogenus*	low discrimination/ 90.6 *Pantoea* spp. (doubtful profile)	*Klebsiella/ Enterobacter sp.*
E-011944 (Fin B)	1254-S-5 0.73 *Enterobacter hormaechei*	99.4 *Citrobacter koseri*	74.5 *Enterobacter cloacae* (not valid)/low discrimination	*Citrobacter sp.*
E-022114 (Spa)	1386-S-3 0.69 *Enterobacter cloacae*	99.2 *Enterobacter cloacae/asburiae*	low discrimination/ 97.4 *Enterobacter cloacae*	*Enterobacter cloacae*
E-022115 (Spa)	1386-S-4 0.84 *Bacillus simplex*	99.8 *Bacillus simplex*	not done	*Bacillus simplex*
E-022116 (Spa)	1386-S-5 0.79 *Raoultella planticola*	100 *Raoultella planticola*[T]/ *ornithinolytica* 100 *Serratia fonticola*	low discrimination/ 99.3 *Raoultella planticola*	*Raoultella planticola*
E-022117 (Spa)	1386-S-6 0.79 *Serratia rubidaea*	98.4 *Yersinia enterocolitica* 98.1 *Serratia entomophila*[T]	99.5 *Serratia rubidaea*/ 99.9 *Serratia rubidaea*	*Serratia sp.*
E-022118 (Spa)	1386-S-7 0.62 *Enterobacter cloacae/ aerogenes*	100 *Enterobacter asburiae*[T]/*cloacae*/ *aerogenes/kobei*	not valid/75.9 *Enterobacter asburiae* (doubtful profile)	*Enterobacter sp.*
E-022119 (Spa)	1386-S-8 0.82 *Enterobacter cloacae*	100 *Enterobacter asburiae*/ *cloacae/kobei*	low discrimination/ 97.4 *Enterobacter cloacae*	*Enterobacter cloacae*

T= Type Strain
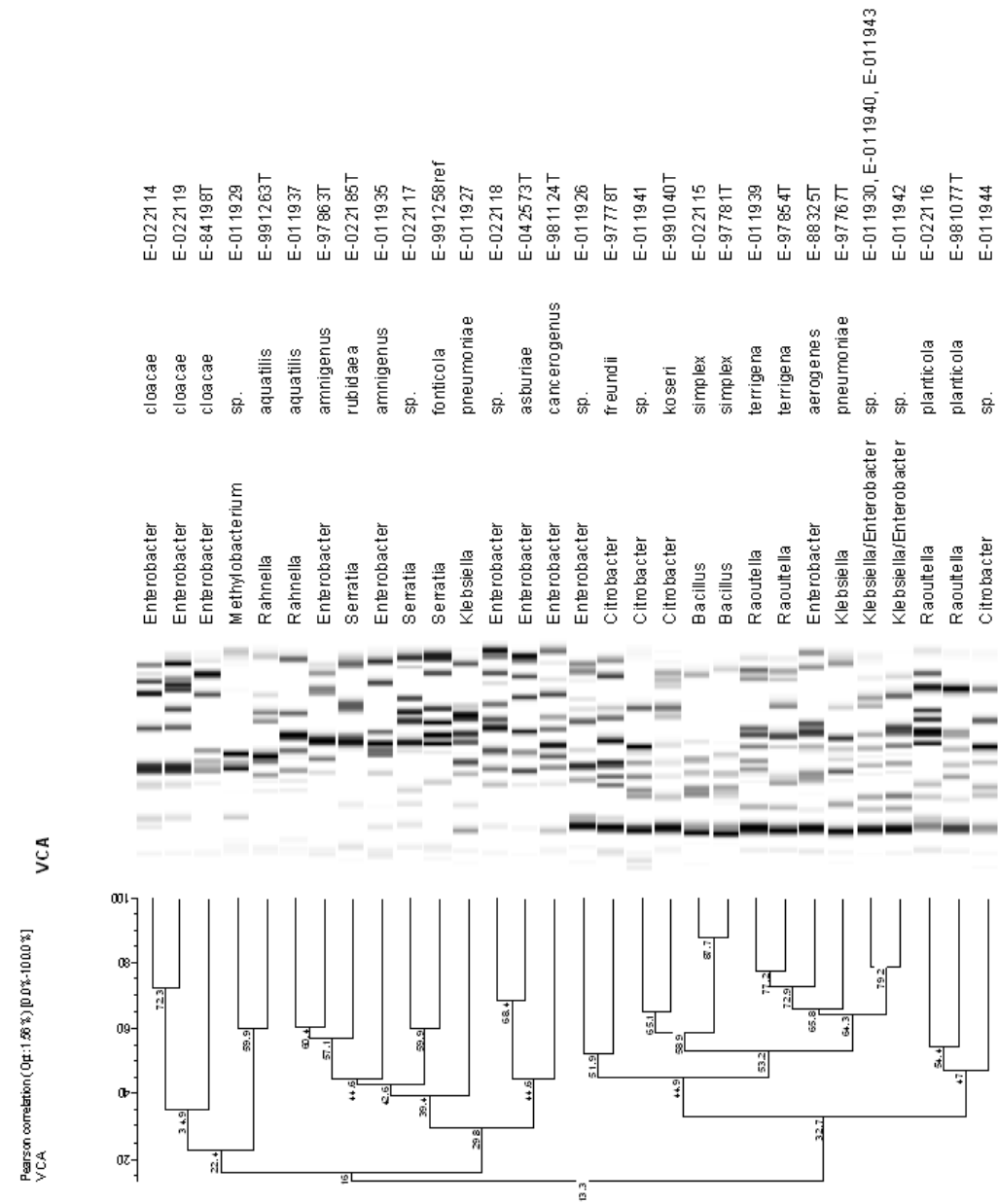
Figure 5.1. Dendrogram of the RiboPrint patterns of isolated strains and 14 reference strains (T and ref)

The amounts of EPSs precipitated from the cultures varied from 0.01 to 2.82 g/l. The highest amount of polysaccharide was produced by *Citrobacter* sp. E-011941 and *Klebsiella/Enterobacter* isolates E-011930 and E-011943 (2.82 g/l, 1.67 g/l, 1.19 g/l, respectively) whereas only 0.12 g/l of polysaccharide was produced by *Klebsiella/Enterobacter* sp. E-011940, belonging to the same ribogroup as E-011930 and E-011943 and isolated from the same mill as E-011943. Due to this difference in the quantity of EPS produced, the isolates E-011940 and E-011943 were both included in further studies together with the third representative of the same ribotype originating from a different mill (E-011930).

Table 5.2: Polysaccharides obtained by ethanol precipitation from liquid cultures of the EPS-producing bacteria isolated from paper machine slimes .

VTT code	Strain	Medium	Culture viscosity (cP)	Yield (total sugars, g/l)	Protein (g/l)
E-011926	*Enterobacter sp.*	IV	2.1	0.13	0.04
E-011927	*Klebsiella pneumoniae*	IV	16.3	0.53	0.02
E-011929	*Methylobacterium sp.*	III	16.1	0.46	0.04
E-011930	*Klebsiella/Enterobacter sp.*	IV	44.0	1.67	0.10
E-011935	*Enterobacter amnigenus*	IV	3.0	0.25	0.02
E-011937	*Rahnella aquatilis*	IV	3.0	0.28	0.02
E-011939	*Raoultella terrigena*	IV	8.0	0.70	0.02
E-011940	*Klebsiella/Enterobacter sp.*	IV	1.3	0.12	0.04
E-011941	*Citrobacter sp.*	I	28.9	2.82	0.43
E-011942	*Klebsiella/Enterobacter sp.*	IV	2.9	0.21	0.03
E-011943	*Klebsiella/Enterobacter sp.*	IV	15.8	1.19	0.03
E-011944	*Citrobacter sp.*	IV	2.4	0.90	0.02
E-022114	*Enterobacter cloacae*	I	1.2	0.03	0.02
E-022115	*Bacillus simplex*	I	1.0	0.02	0.02
E-022116	*Raoultella planticola*	I	2.3	0.36	0.02
E-022117	*Serratia sp.*	I	1.1	0.10	0.03
E-022118	*Enterobacter sp.*	I	1.1	0.10	0.01
E-022119	*Enterobacter cloacae*	I	1.1	0.01	0.00

Characterization of the polysaccharides—The 18 polysaccharides produced were analysed for their sugar and substituent composition as shown in Table 5.3. In general it is interesting to see that all of the EPSs analysed show the presence of charged groups in the form of uronic acid or pyruvate revealing the polyanionic nature of these polysaccharides. Furthermore most of the EPSs analysed are substituted with *O*-acetyl groups. With respect to the sugar compositions half of the isolates produce EPSs with the same sugar composition e.g. EPSs

from isolates E-011926, E-011930, E-011935, E-011940, E-011941, E-011942, E-011943, E-011944 and E-022114 all contain fucose, galactose, glucose, glucuronic acid, pyruvate and *O*-acetyl as their main sugar moieties and substituents in a molar ratio of 2: 2: 1: 1: 1: 1, respectively. Apart from some deviation due to traces of rhamnose, mannose and galacturonic acid all of these EPSs could be suggested to have the same composition. The sugar/substituent compositions of the 9 EPSs described above are the same as the composition found for colanic acid [22, 23], commonly produced by several members of the family *Enterobacteriaceae* [22].

Table 5.3. Sugar and substituent composition (mol%) of the EPSs produced by the isolated strains.

VTT code	Strain	Fucose	Rhamnose	Arabinose	Galactose	Glucose	Mannose	Xylose	GalA	GlcA	O-Acetyl	Pyruvate	total sugar
E-011926	*Enterobacter* sp.	19	-*)	-	23	15	16	-	-	13	8	5	63
E-011927	*Klebsiella pneumoniae*	-	-	-	13	15	42	-	-	19	-	10	80
E-011929	*Methylobacterium* sp.	-	-	-	53	-	2	-	-	-	-	45	46
E-011930	*Klebsiella/Enterobacter* sp.	31	-	-	23	15	3	-	1	15	8	4	66
E-011935	*Enterobacter amnigenus*	25	-	-	21	14	2	-	5	12	11	9	65
E-011937	*Rahnella aquatilis*	-	27	-	41	2	1	-	2	27	-	-	71
E-011939	*Raoultella terrigena*	-	41	-	19	17	1	-	-	18	1	4	69
E-011940	*Klebsiella/Enterobacter* sp.	16	4	-	23	18	9	-	3	11	8	6	46
E-011941	*Citrobacter* sp.	31	-	-	26	13	2	-	1	16	7	5	55
E-011942	*Klebsiella/Enterobacter* sp.	20	4	-	22	11	7	-	2	11	13	10	64
E-011943	*Klebsiella/Enterobacter* sp.	27	1	-	24	15	2	-	2	14	10	6	56
E-011944	*Citrobacter* sp.	23	1	-	23	19	4	-	2	14	9	5	59
E-022114	*Enterobacter cloacae*	26	2	-	21	14	-	-	-	12	13	7	25
E-022115	*Bacillus simplex*	-	18	-	14	19	30	-	3	15	2	-	13
E-022116	*Raoultella planticola*	-	-	-	18	12	47	-	1	10	1	10	61
E-022117	*Serratia* sp.	-	37	2	-	3	-	-	30	-	11	17	38
E-022118	*Enterobacter* sp.	12	-	-	3	6	67	2	9	-	-	-	52
E-022119	*Enterobacter cloacae*	-	22	-	13	16	-	-	-	-	1	47	22

*)   not detected

The EPS produced by *Methylobacterium* sp. E-011929 is a pure galactan with 2 pyruvate ketals substituted at 2 out of 3 galactoses per repeating unit, the chemical fine structure of this EPS was determined by Verhoef et al [24]. Detailed structural analysis of the EPS produced by *Raoultella terrigena* (basonym *Klebsiella terrigena)* E-011939 (unpublished results) showed that this EPS, containing high amounts of rhamnose together with glucose, galactose, glucuronic acid and pyruvate, has the same chemical fine structure as the one reported for *Klebsiella* serotype K70 [25]. *K. pneumoniae* E-011927 and *Raoultella planticola* E-022116 EPSs also have the same sugar and substituent compositions. These latter 2 EPSs show a high mannose level together with glucose, galactose, glucuronic acid and pyruvate similar to the sugars and substituents found typical for *Klebsiella* sp. EPSs [26-28]. No meaningful comparison for the other EPSs could be made on the basis of their sugar and substituent composition.

Based on the results shown above it is clear that colanic acid seems to be one of the major EPSs found to be produced by the slimy bacterial isolates from the paper mills studied. Based on these findings it was decided to look more into detail to the structure of one of the potential colanic acid EPSs (E-011941) and compare the nine potential colanic acid samples with each other using linkage analysis and partial hydrolysis followed by MALDI-TOF MS.

Linkage analysis of colanic acid—The polysaccharide of *Citrobacter* sp. E-011941 was chosen for linkage analysis by per methylation. The results obtained for both the native and carboxyl-reduced sample (Table 5.4) agree with the sugar composition found in Table 3. Linkage analysis of the carboxyl reduced sample revealed the presence of 12 mol% 4-linked fucose, 29 mol% 3,4-linked fucose, 21 mol% 3-linked glucose, 21 mol% 3-linked galactose and 5 mol% 4-linked glucuronic acid. Furthermore 4 mol% terminal and 8 mol% 4,6-linked galactose could be found. It is obvious that these results do not exactly fit the molar proportions of the repeating unit. For the rather high level of 3,4-linked fucose, when compared to the 4-linked fucose, this could be explained by the possible incomplete removal of the *O*-acetyl group, known to be attached to position 2 or 3 of the 4-linked fucose, during methylation leading to an overestimation of 3,4-linked fucose. Furthermore the low amount of 4-linked glucuronic acid found is easily explained by incomplete carboxyl reduction of this moiety to its glucose analogue. Taking into account that bacterial polysaccharides always consist of repeating units it was concluded that this EPS consists of 4-linked fucose, 3,4-linked fucose, 3-linked glucose, 3-linked galactose, 4,6-linked galactose and 4-linked glucuronic acid with a molar ratio of 1:1:1:1:1:1, respectively. The results found agree with previous linkage analysis [10, 29, 30]

performed on colanic acid. The 4,6-linked galactose represents the terminal galactose substituted with a pyruvate ketal at position 4 and 6.

Table 5.4. Glycosidic linkage composition in mol% of the partially methylated alditol acetates of the native and reduced EPS of the isolate E-011941

	native (mol%)	reduced (mol%)	
2,3-di-*O*-Me-Fuc*p*	13	12	→4)Fuc*p*(1→
2-*O*-Me-Fuc*p*	32	29	→3,4)Fuc*p*(1→
2,4,6-tri-*O*-Me-Glc*p*	22	21	→3)Glc*p*(1→
2,3,6-tri-*O*-Me-Glc*p*	-	5	→4)Glc*p*(1→ (GlcA)
2,4,6-tri-*O*-Me-Gal*p*	20	21	→3)Gal*p*(1→
2,3-di-*O*-Me-Gal*p*	9	8	→4,6)Gal*p*(1→
2,3,4,6-tetra-*O*-Me-Gal*p*	3	4	Gal*p*(1→

Partial Hydrolysis of colanic acid—To prove whether the different glycosyl moieties found are linked together in the same sequence as found for colanic acid all the nine potential colanic acid EPSs were subjected to weak acid treatment to be able to release oligomeric fragments. These fragments were analysed by MALDI-TOF MS and the results were compared with the results obtained by the linkage analysis performed and to the known structure of colanic acid. Fig. 5.2 shows an example of the obtained MALDI TOF MS spectra after partial hydrolysis. The proposed structures of the fragments released upon weak acid treatment are summarised in Table 5.5.

The first fragments with *m/z* 495.5 and 537.4 represent a trimer of 2 fucose and 1 hexose with or without *O*-acetyl followed by the fragments at 511.4 and 553.6 representing a trimer of 2 hexose and 1 fucose with or without *O*-acetyl. Judging from acid stability of the different glycosidic linkages present within colanic acid the first fragment most probably is a trimer representing the core of the repeating unit composed of 2 fucoses and 1 glucose with fucose at both the terminal and reducing end of the oligomer. The oligomers at *m/z* 511/553 were more striking to find since its release involves the hydrolysis of the aldobiuronic acid linkage between the glucuronic acid and galactose present in the side chain of the repeating unit. This oligomer represents a trimer with fucose at the reducing end, glucose at the non-reducing end and a terminal galactose attached to the fucose. The fragment found at *m/z* 611.6 was a trimer composed of galactose,

glucuronic acid and pyruvated galactose. This trimer represents the full side chain of colanic acid and has a galactose at the reducing end and a pyruvated galactose at the terminus of the oligomer, no acetylated fragment of this oligomer was found. The hexamers found with mass 1192, 1149, 1107, 1165, 1037 and 995 all represent different forms of the repeating unit (RU) of colanic acid: RU with 3, 2, 1 or no *O*-acetyl group and RU without pyruvate with 1 or 0 *O*-acetyl groups, respectively (Fig. 5.2). Especially with respect to the *O*-acetyl groups the results indicate that colanic acid has more *O*-acetyl substituents then previously assumed [29, 30] and that these *O*-acetyl groups are distributed at given locations over the repeating units forming the polysaccharide.
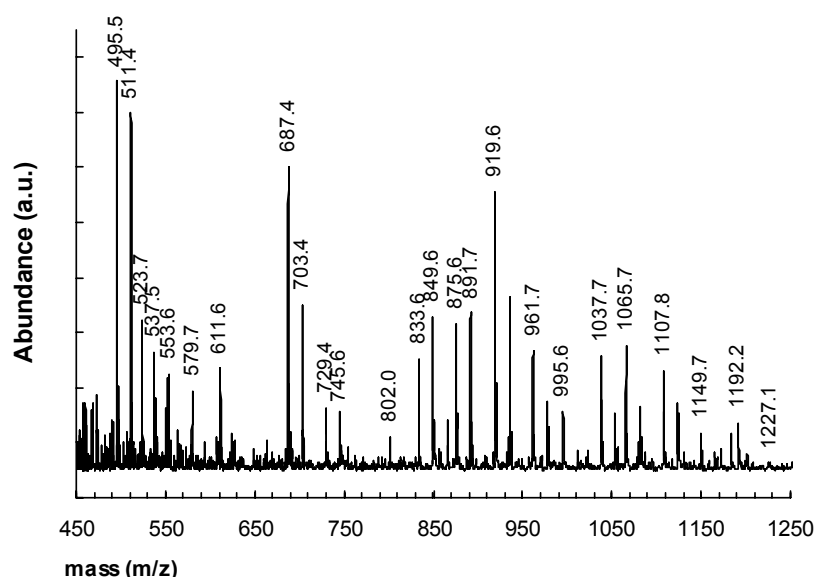
Figure 2. MALDI-TOF ms spectrum of the partially hydrolysed (0.05N TFA) EPS produced by the isolate E-011930.

Since pyruvate is rather acid labile the repeating units without pyruvate could be explained by the removal of this substituent by acid. A mass difference of −146 between the repeating units with 1 or 0 *O*-acetyl groups and without pyruvate showed the release of oligomers representing the latter without fucose at *m/z* 961, 919 and 849, respectively. Furthermore the oligomeric fragment of the repeating unit without the terminal pyruvated galactose (-232) at *m/z* 833 and the oligomeric fragment with *m/z* 875 representing the same oligomer with 1 additional *O*-acetyl

group could be found. Since none of the oligomers found disagrees with the structure known for colanic acid this EPS is considered to be colanic acid. To confirm this for the other 8 EPSs they were treated with the same weak acid conditions to see whether the same fragments were released.

Table 5.5. Fragments released upon mild acid treatment (0,05M TFA 100°C 1h) of the EPS produced by the isolate E-011930 with their corresponding m/z values.

m/z	O- acetyl	pyruvate	oligomer
495,5	-[1]	-	[fuc]-[glc]-[fuc]-OH or [fuc]-[fuc]-OH
537,4	+	-	└[gal]
511,4	-	-	[glc]-[fuc]-OH
553,6	+	-	└[gal]
611,6	-	+	[gal]-[glcA]-[gal]-OH
687,4	-	-	[glc]-[fuc]-OH
729,4	+	-	└[gal]-[glcA]
833,6	-	-	[fuc]-[glc]-[fuc]-OH
875,6	+	-	└[gal]-[glcA]
849,6	-	-	[glc]-[fuc]-OH
919.6	-	+	└[gal]-[glcA]-[gal]
961.7	+	+	
995,6	-	-	
1037,7	+	-	
1065.7	-	+	[fuc]-[glc]-[fuc]-OH
1107.8	+	+	└[gal]-[glcA]-[gal]
1149.7	++	+	
1192.2	+++	+	

1) + = 1 *O*-acetyl or pyruvate moiety present; - = not present
2) OH represents the reducing end based upon preferred hydrolyses according to the most labile glycosidic linkage

Analysis of the fragments released after weak acid treatment of the other 8 EPSs revealed that all of the samples except for the sample of E-022114 contain for 75 % or more of similar fragments as observed in the reference sample. Although the sample of E-022114 only contains 38 % of these fragments, still these fragments do indicate the presence of colanic acid, especially both fragments representing the repeating unit with 1 or 2 *O*-acetyl moieties at *m/z* 1107.8 and 1149.7. Again the between sample variance in fragments released does point to an

inhomogeneous distribution of *O*-acetyl groups over the repeating unit of colanic acid, since 89 % of the samples shows the release of repeating units with 2 *O*-acetyl groups instead of only 1 as always assumed. Future enzymatic studies will be used to determine the positions and distribution of these different *O*-acetyl groups.

Discussion

In this study, polysaccharide-producing bacteria were isolated from slimes collected from two Finnish and one Spanish paper mill based on slimy colony morphology and the EPSs produced by the isolates were characterised. Almost all (16/18) of the slimy isolates were members of the family *Enterobacteriaceae* with *Enterobacter* and *Klebsiella* (*Raoultella*) as the most frequently represented genera. *Enterobacter*, *Klebsiella* and *Citrobacter* species are common in soil, healthy and decaying wood and in plants and also appear to be normal inhabitants in many paper machines [11, 12, 31-33]. According to a Canadian study, these bacteria were continuously growing in almost all pulp and paper mills studied with the most likely growth areas being paper machines, biofilms on machinery and piping and the primary clarifier [34].

The results of the carbohydrate analysis showed that the EPS produced by nine of the enterobacterial isolates is colanic acid consisting of 4-linked fucose, 3,4-linked fucose, 3-linked glucose, 3-linked galactose, 4,6-linked galactose and 4-linked glucuronic acid with a molar ratio of 1:1:1:1:1:1, respectively. The colanic acid –producing isolates belonged to the genera *Enterobacter* or *Citrobacter* or remained unidentified within *Klebsiella/Enterobacter*. Colanic acid is known as the EPS common to many genera in the *Enterobacteriaceae* [22]. Small amounts of colanic acid are normally produced constitutively, production can be increased by osmotic shock [35] β-lactam antibiotics [36] or during growth on solid surfaces [37]. Increase in colanic synthesis has been found to occur in *E. coli* cells growing in biofilms [38]. *E. coli* mutants deficient in colanic acid production are able to attach to abiotic surfaces and form bacterial films of one to two cell layers thick but unable to form the three-dimensional, complex biofilm structures typical of the colanic acid producing parent strain [39, 40].

Colanic acid proved to be the major EPS produced by 9 of the 18 slimy bacteria strains isolated from the paper mills. Future studies will focus on the occurrence of colanic acid in slimes collected from the mills and the potential of enzymatic slime control using colanic acid degrading enzymes.

Acknowledgement

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Chapter 6

Characterisation of a β(1,4)-fucanosyl hydrolase degrading colanic acid

Abstract

A novel colanic acid-degrading enzyme was isolated from a culture filtrate of a mixed population obtained by enrichment culture of a compost sample using colanic acid as carbon source. The enzyme was partially purified resulting in a 17-fold increase in specific activity. Further purification by Native PAGE revealed that the enzyme is part of a high molecular weight multi protein complex of at least 6 individual proteins. The enzyme showed a temperature optimum at 50 °C while after 1 h at 50 °C and pH 7 still 70% of the total activity was left. The pH optimum was found to be pH 7.

Analysis of the degradation products, by PSD/MALDI-TOF MS, showed that the enzyme is a novel $\beta(1,4)$-fucanosyl hydrolase that liberates single repeating units of colanic acid with varying degrees of acetylation.

K_m and V_{max} of the enzyme were determined against the native substrate as well as its de-*O*-acetylated and depyruvated forms. Compared to the native substrate the affinity, measured as $1/K_m$, of the enzyme for the modified substrates was much lower. However for the de-*O*-acetylated sample a dramatic increase in catalytic efficiency (reflected as V_{max}) was observed.

The native form of the substrate showed substrate inhibition at high concentrations, probably due to the formation of non-productive substrate complexes. Removal of the acetyl groups probably prevents this effect resulting in a higher catalytic efficiency.

Introduction.

The formation of slime layers induced by biofilm formation on the surface of paper machines causes significant operational problems within the paper industry. These problems include defects in the final product like spots and holes and process downtime due to web breaks and clogging of the paper machine itself.

Slime is defined as the accumulation of microbial cells immobilized and embedded in an organic polymer matrix of microbial origin, consisting of carbohydrates, proteins, and possibly smaller amounts of nucleic acids (phospho)lipids [1]. The major components of this matrix are usually exopolysaccharides (EPSs), mixed in different proportions with fibres, fines, fillers and other materials that are trapped in this polymer matrix [2].

Previous research by Verhoef et al [3] showed the presence of the EPS colanic acid in several different paper mills within Spain and Finland. Colanic acid is commonly known to be produced by several members of the *Enterobacteriaceae* family [4]. These findings indicate the importance of this EPS with respect to the slime problems as described above.
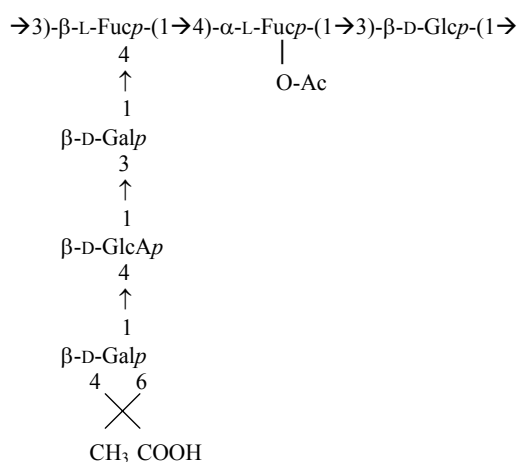
$$\rightarrow3)\text{-}\beta\text{-}\text{L-Fuc}p\text{-}(1\rightarrow4)\text{-}\alpha\text{-}\text{L-Fuc}p\text{-}(1\rightarrow3)\text{-}\beta\text{-}\text{D-Glc}p\text{-}(1\rightarrow$$

```
→3)-β-L-Fucp-(1→4)-α-L-Fucp-(1→3)-β-D-Glcp-(1→
           4                        |
           ↑                       O-Ac
           1
        β-D-Galp
           3
           ↑
           1
        β-D-GlcAp
           4
           ↑
           1
        β-D-Galp
          4   6
           ✕
        CH₃ COOH
```

Figure 6.1: Chemical fine structure of colanic acid[5, 6].

Colanic acid was first discovered by Goebel [7] in 1963 and ever since numerous research has been done towards the chemical fine structure, a better understanding of the formation of this EPS and its role within a developing or already formed biofilm architecture. Only recently it has been discovered that colanic acid is not necessarily a primary biofilm former, but plays an

important role in the formation of the complex three-dimensional structure of the biofilm formed [8]. However apart from previous work done by Verhoef et al [3] and van Speybroeck et al [9] this EPS was never directly associated with slime problems within a paper mill environment.

Colanic acid is composed of fucose, galactose, glucose and glucuronic acid together with acetyl and pyruvate in molar proportions of 2:2:1:1:1:1, respectively [10].

The structure of colanic acid [5, 6] is presented in figure 1 and shows the presence of an *O*-acetyl group at position 2 or 3 of the non-branched fucose and a pyruvate ketal at the terminus of the side chain of the repeating unit. However with respect to the structure it is known that colanic acid produced by different species has the same branched hexasaccharide repeating unit, but per species variation within the substituents may occur. The pyruvate moiety is either linked to position 4 and 6 or 3 and 4 of the galactose terminus of the side chain, furthermore instead of the pyruvate ketal also acetyl forms of formaldehyde or acetaldehyde have been found linked to the same positions [5].

The substituents contribute to the physical properties of the EPS: a negative charge due to the presence of a glucuronic acid moiety and the pyruvate ketal mentioned and slightly hydrophobic properties due to the presence of several *O*-acetyl groups on each repeating unit [11, 12]. These physical properties can be seen as important factors for surface attachment or cell-cell adhesion.

Enzymatic degradation of colanic acid was first achieved by Sutherland et al [13] using a phage induced enzyme hydrolyzing the linkage between the fucose(1→3)glucose linkage. Furthermore, a patent application describing the use of an enzyme, isolated from a novel *Streptomyces* strain, able to degrade colanic acid has been filed by van Speybroeck et al [9]. However, this enzyme does not seem to be very effective since it only shows a molecular weight drop from 2,000 kDa to 400 kDa upon exhaustive degradation.

The present paper describes the characterization and partial purification of a novel β(1,4)-fucanosyl hydrolase that causes a 100% conversion of colanic acid to its corresponding hexasaccharide repeating unit. Furthermore enzymatic research revealed new insights in the *O*-acetylation pattern of colanic acid.

Materials and methods.

Production of enzyme degrading colanic acid — Cultures degrading colanic acid were obtained by enrichment cultivation from a sample of compost, containing paper machine fines, chicken manure and wood bark. The enrichment medium contained 5 g/L colanic acid from a *Citrobacter* sp. (VTT E-011941, produced as described in [14]) and 6.7 g/L Yeast nitrogen base (Difco) in 50 mM potassium phosphate buffer, pH 7. About 1 g of mechanically homogenized compost sample was suspended in 5 mL of physiological NaCl solution, the suspension was mixed and the solids were allowed to settle. 250 µL of suspension was inoculated in 5 mL of enrichment medium. Sterile water was added into reference cultures instead of compost sample. Enrichment cultures were incubated at 50 °C without mixing for 3 days. After correction for the decrease in volume due to evaporation, a 700 µL sample was taken for viscosity measurements and centrifuged to remove the insoluble material. The viscosities of the culture filtrates were measured with Brookfield DV II viscometer (+20 °C, sample size 0.5 mL). Initial viscosity of the enrichment culture was measured and polysaccharide-degrading activity was detected by following the decrease in the viscosity. When the viscosity in the 1st enrichment culture had clearly decreased, 250 µL of culture medium was re-inoculated into 5 mL of fresh enrichment medium. When after 3-day cultivation the viscosity of the second enrichment culture was lowered 500 µLof the culture medium was re-inoculated into 10 mL of fresh medium for the third enrichment culture. After 1-2 day incubation the enrichment cultures were stored in the culture medium at +4 °C for immediate use and recultivated and stored in 5 % glycerol at -80 °C for longer periods.

To produce a batch for enzyme production and purification the enrichment cultures were cultivated for 2 d in 20 mL of medium containing colanic acid from *Citrobacter sp*. (VTT E-011941) as the only carbon source. The cells were separated by centrifugation and the polysaccharide-degrading activity of cell free culture filtrates against colanic acid was measured viscosimetrically. Cell free culture broths were used for further purification of the enzyme degrading colanic acid.

Activity assay — To follow the purification and to measure the kinetic parameters of the enzyme the bicinchoninic acid (BCA) assay according Meeuwsen et al [15] was used.
The substrate solution consisted of 0.5 mg/mL colanic acid in 50 mM phosphate buffer pH 7. This substrate solution was mixed with variable amounts of enzyme until a total volume of 100 µL and incubated at variable temperatures. Enzyme amounts, temperature and time details for

each individual set of incubations will be given in the corresponding section. After incubation 100 μL of the degraded substrate was mixed with 100 μL of BCA reactant and heated at 80 °C for 1 h. Then the sample was cooled on ice and the absorption was measured at 550 nm. Since protein may contribute to this assay the same enzyme solutions without substrate were used as a blank.

The amount of reducing sugars released upon degradation was determined using a fucose calibration curve (5 – 500 μM). Specific activities were expressed as U/mg protein were U stand for μmol reducing sugar released per minute.

The protein content was measured according to Bradford [16] using bovine serum albumin as a standard.

Anion Exchange Chromatography —The crude culture filtrate was ultra filtered (Amicon 10 kDa cut off) and washed with Millipore water (<20 μS/cm^2) followed by 25 mM TRIS/HCl buffer pH=7 and then applied on a Mono Q 5/50 GL (Amersham Biosciences) anion exchange column equilibrated with the same buffer. The protein was eluted from the column by increasing the ionic strength at a flow rate of 1 mL/min from 0 to 1 M NaCl in 20 column volumes. Fractions of 0.5 mL were collected and tested for enzyme activity using the BCA assay.

Gel permeation chromatography — The pool containing enzyme activity after MonoQ separation was applied to a HiLoad 16/60 Superdex 200 Prepgrade (Amersham Biosciences) gel filtration column and eluted at 1mL/min using 100 mM TRIS/HCl buffer pH 7.0. Fractions of 2 mL were collected and activity was measured using the BCA assay.

pH optimum and temperature optimum — The pH optimum was determined in McIlvaine buffers in the pH range from 3 to 8. Colanic acid solution (1 mL; 0.5 mg/mL) was incubated with 20 μL of enzyme solution (pooled after Superdex 200 separation; 23 μg protein/mL) at 30°C for 5h.

The temperature optimum was measured using 0.5 mg/mL colanic acid in 50 mM phosphate buffer pH7 in the range from 20 to 80°C. The colanic acid solutions (1 mL) were incubated with 25 μL of enzyme (pooled after Superdex 200 separation; 23 μg protein/mL) for 5h.

Temperature stability was measured by first heating 20 μl of the same enzyme solution at the same temperature range without substrate for 1h, followed by adding of 1mL 0.5mg/mL colanic acid in 50 mM phosphate buffer pH 7 and 5h incubation at 30°C.

The BCA reducing end essay was used for all activity measurements.

Native and SDS-PAGE — SDS-PAGE was run on a PhastGel[TM] Gradient 4-15% gel (Phastsystem), using low molecular weight protein standards all from Amersham Biosciences. The gel was stained using silver staining.

Native PAGE was run using a BioRad MiniProtean electrophoresis unit on a 5%-15% gradient PAGE gel (ready gel BioRad). Part of the gel was Coomasie stained and 3 unstained lanes were cut into slices of 2 mm. The sections from one lane were incubated with 1 mL 0.5 mg/mL colanic acid (30 °C, 50 mM phosphate buffer pH 7, 18 h) to determine the active protein band. The BCA reducing sugars assay as well as HPSEC were used for analysis of degradation products. The other 2 lanes were used as enzyme and substrate blanks. After incubation of the enzyme blank the enzyme diffused into the buffer. This active enzyme fraction was concentrated and subjected to SDS-PAGE under denaturing conditions with and without prior reduction by β-mercapto-ethanol.

Substrate modifications — Native colanic acid was obtained as described by Verhoef et al [3]. Colanic acid was de-O-acetylated by solving 60 mg of colanic acid in 25 mL 100 mM NaOH and stirring for 18 h at 4 °C. After saponification the de-O-acetylated substrate was dialysed by ultra-filtration using a 10 kDa cut off Amicon filter. The ultra filtrated substrate solution (supernatant) was freeze dried to obtain the de-O-acetylated substrate.

Colanic acid (60 mg) was depyruvated as described by Verhoef et al [17] using autohydrolysis.

MALDI-TOF mass spectrometry — MALDI-TOF MS (Matrix-Assisted Laser Desorption/ Ionisation Time-Of-Flight Mass Spectrometry) and Post Source Decay (PSD)/MALDI-TOF MS was performed on an Ultraflex workstation (Bruker Daltronics GmbH, Germany). MALDI-TOF MS spectra were recorded in the negative mode using a mixture of galacturonic acid oligosaccharides for calibration. PSD/MALDI-TOF MS was recorded in the positive mode using a mixture of malto-dextrins for calibration. The samples were mixed with a matrix solution (1 μL of sample in 9 μL of matrix). The matrix solution was prepared by dissolving 9 mg of 2,5-dihydroxybenzoic acid in a 1 mL mixture of acetonitrile:water (300 μL: 700 μl). Of the prepared sample and matrix solutions 2 μL was put on a gold plate and dried with warm air.

HPSEC of colanic acid. — Colanic acid (2 mg) was dissolved in 1mL of distilled water and analysed by high-performance size exclusion chromatography (HPSEC). HPSEC was performed on a ThermoQuest HPLC using three TOSOHAAS TSK-Gel columns in series (4000-, 3000-, 2500 PWXL) preceded by an TSK guard column (40 x 6mm). Elution took place

at 30°C using 0.8mL/min 0.2 M NaNO$_3$ as eluent. Detection was performed using a Shodex RI 71 refractive index detector.

Results and Discussion.

Production of colanic acid degrading enzyme — A microbial culture producing colanic acid degrading activity was enriched from a sample originating from a compost heap containing paper machine fines, chicken manure and wood bark. The microbes were cultivated in shake flasks and the colanic acid degrading activity was detected viscosimetrically in the cell-free culture filtrate. The enrichment procedure resulted in a mixed culture, and in spite of repeated efforts no single microorganism able to produce activity against colanic acid could be isolated from it. For convenience reasons the colanic acid degrading enzyme is called colanase in the following sections.

Purification of colanase — The crude colanase preparation was obtained from an enrichment culture of composted fines with chicken manure and wood bark on colanic acid as described in the methods section. The purification procedure is summarized in Table 6.1.

Table 6.1: Summary of the colanase purification.

Purification step	V (mL)	Activity ($\mu U^{1)}$/mL)	Protein (mg/mL)	Specific activity (μU/mg)	Activity Yield%	Purification factor
Crude enzyme preparation	15.0	57	0.50	113	100	
MonoQ 5/50 GL	13.5	60	0.17	348	95	3
HiLoad Superdex 200 16/60	6.0	41	0.02	1903	30	17

   1)   1U= 1μmol of reducing sugars released per minute.

The cell free culture was dialyzed by ultra filtration against Millipore water and buffer before applying the crude enzyme preparation to a MonoQ anion exchange column. The specific activity of the crude culture was 113 μU/mg. The active pool after MonoQ anion exchange chromatography showed a 3-fold increase in specific activity (348 μU/mg) retaining 95% of the initial activity.

This purification step was followed by gelfiltration using a prepgrade Superdex 200 column resulting in an active pool with a specific activity of 1903 μU/mg. Upon this purification 30% of the original activity of the crude enzyme preparation was recovered. An apparent molecular weight of 775kDa and 337kDa was found suggesting that the enzyme could be present in 2 different forms. However, upon Native PAGE electrophoresis as shown later it turned out that the enzyme would not migrate into the gel indicating that these molecular weight values are based on non specific interaction of the protein with the column material resulting in a delayed elution of the column.

After gelfiltration further purification of the enzyme was attempted by the use of cation exchange chromatography (MonoS) at pH 4.5 resulting in the irreversible attachment of the enzyme to the column and no activity could be recovered. These results suggest that the isoelectric point of the enzyme should be between pH 7.0 and 4.5. However, chromatofocusing (PBE 94) using a pH gradient between these to values (pH 7.0 to 4.5) did not result in the elution of the enzyme and the enzyme had to be recovered by increasing the pH to 7 followed by a linear gradient to 1 M NaCl to remove the enzyme from the column.

Finally hydrophobic interaction chromatography also resulted in the irreversible attachment of the enzyme to the column.

The fact that native PAGE of the partially purified enzyme mixture still showed 3 major protein bands indicates that it was not possible to fully isolate the enzyme using conventional chromatographic techniques.

Native PAGE — The colanase preparation obtained by conventional chromatographic methods was further purified by means of Native PAGE electrophoresis. According to Native PAGE analysis the enzyme mixture showed 3 major protein bands. In order to determine the position of the protein band belonging to the enzyme the crude enzyme preparation was applied to several lanes on a 5%-15% TRIS/HCL Ready Gel (BioRad) and 3 lanes were cut from the gel before staining. These 3 lanes were divided into slices of 2 mm and lane 1 was incubated with the substrate while lane 2 and 3 was used as a blank. Activity of the proteins present within the individual slices was measured using the BCA reducing sugar assay. For the positive slices degradation of colanic acid was confirmed by HPSEC. The other lanes including the high molecular weight protein standard were stained with Coomassie and compared to the results obtained from the activity tests.

Using this approach it turned out that, during native electrophoresis, colanase did not migrate into the stacking gel, which indicates that the enzyme has a high molecular weight. The extracts from the slices containing the enzyme from the two blank lanes (top slices) were concentrated and analyzed by SDS-PAGE before and after reduction by β-mercapto-ethanol. The SDS-PAGE analysis under non-reducing conditions confirmed that the enzyme could not migrate into the gel due to its molecular weight. However remarkably at least six protein bands were observed upon SDS-PAGE after reduction (Fig. 6.2) indicating that the enzyme is incorporated into a large multi protein complex, in which –S-S- bridges probably play an important role.

Being part of such complex, it was not possible to determine the molecular weight and isoelectric point of the enzyme. The multi protein complex also might explain the difficulties of obtaining a fully purified enzyme preparation. Future work will be directed toward finding out whether 1 of the subunits present in the protein complex is responsible for the colanase activity or whether the full complex is necessary for degradation.
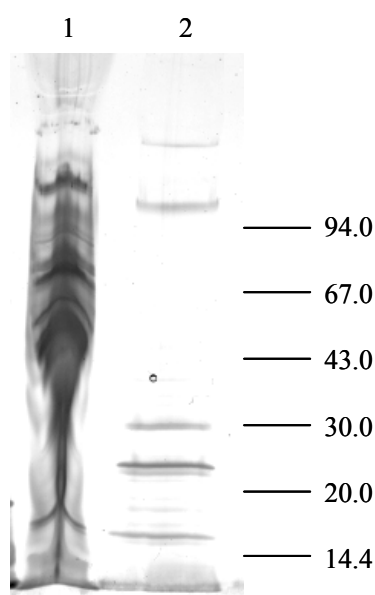
Figure 6.2: SDS-PAGE gel of colanase isolated from a Native PAGE gel: lane1: crude colanase preparation; lane 2: Isolated colanase.

Characterization of colanase — The optimum temperature of the enzyme complex was found at 50 °C in 50 mM phosphate buffer pH 7. Temperature stability tests revealed that the activity of the enzyme starts decreasing at temperatures above 40 °C (Fig. 6.3a). However at 50 °C still 70% of the activity is left. The pH optimum of the enzyme complex was determined using McIlvaine buffers (Fig. 6.3b). The optimum pH of the enzyme complex was found at pH7.
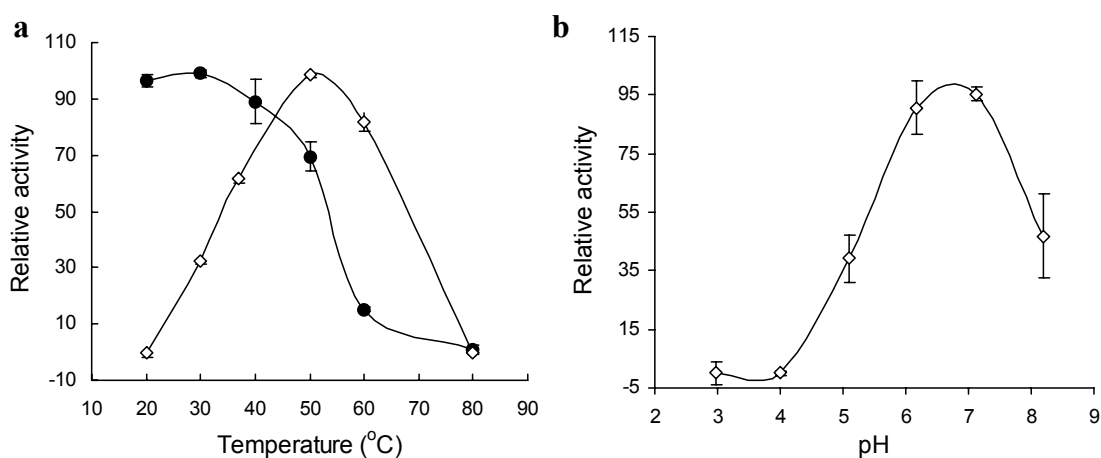

Figure 6.3: Temperature optimum (-◊-) and stability (-•-) (a) as well as pH optimum (b) of partially purified colanase.

Effect of substrate modification — Colanic acid is *O*-acetylated and contains a pyruvate ketal at the terminal galactose of its side chain. Both acetyl and pyruvate could be selectively removed by alkaline or acid treatment, respectively. Hereafter the impact of these side groups on colanase activity could be investigated. The relative activities of the enzyme towards the native, depyruvylated or de-*O*-acetylated were 1.0: 0.3: 3.75, respectively.

Furthermore, the colanase-catalyzed reaction was followed by HPSEC after 18 h and 40 h incubation (Fig. 6.4). From these results it was clear that even after 48 h (outside the linear rate of degradation) the effect of the removal of pyruvate and *O*-acetyl could clearly be observed by the amount of low molecular weight material formed.

The substrate specificity of the enzyme was investigated further by determination of the Michaelis Menten constants K_m and V_{max} of the enzyme for the different modified substrates (Fig. 6.5a/b and Table 6.2).
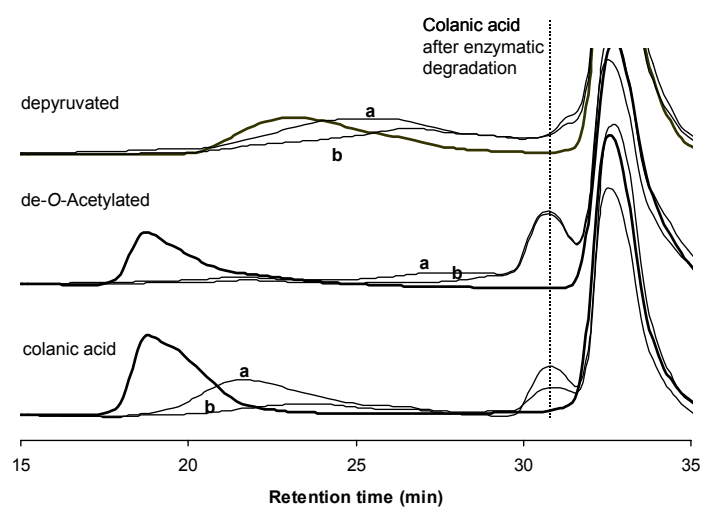
Figure 6.4: **HPSEC elution profile after incubating colanic acid and modified colanic acid (dePyruvated and de-O-Acetylated) with colanase**. The thick trace represents the native substrate **a** and **b** both represent the substrate after degradation for 18 and 48h respectively.
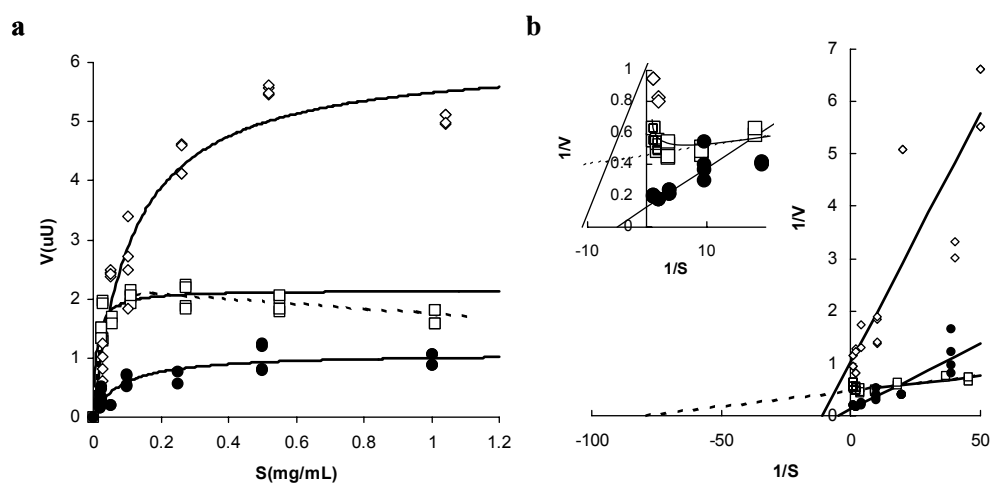

Figure 6.5) a) Reaction rate (V) in μU of colanase against different concentrations of native, de-*O*-acetylated and depyruvated colanic acid (mg/mL) together with the trend lines ($y=V_{max}x/K_m+x$) used for determination of K_m and V_{max}. The dotted line for native colanic acid shows substrate inhibition at higher concentrations; b) Lineweaver and Burk plot of the same experiment (1/V vs. 1/S). -□- native; -◊- de-*O*-acetylated; -•- depyruvated

Table 6.2: $1/K_m$ and V_{max} values for incubation of native, de-*O*-acetylated and depyruvated colanic acid with colanase together with the correlation coefficient for the Michaelis-Menten equation regression fit and the same values determined from the Lineweaver and Burk plot.

Sample	Michaelis-Menten equation			Lineweaver and Burk plot		
	$1/K_m$	V_{max}	R^2	$1/K_m$	V_{max}	R^2
	(mg/mL)	(μU/min)		(mg/mL)	(μU/min)	
Native[1]	100	2.15	0.85	79	2.15	0.79
de-*O*-acetylated	9	6.12	0.94	5	9.87	0.80
depyruvated	12	1.08	0.82	11	0.91	0.80

1. Due to substrate inhibition the
   K_m and V_{max} values for the native substrate should be seen as apparent values.

The removal of the *O*-acetyl moieties by saponification resulted in an increased catalytic activity (V_{max}) of the enzyme with approximately a factor 3 when compared to degradation of the native substrate, while the affinity of the enzyme for the substrate ($1/K_m$) was lowered with a factor ~12.

Removal of the pyruvate moiety lowered both the affinity and the catalytic activity of the enzyme by a factor 8 and 2, respectively.

This indicates that the affinity of the enzyme complex for colanic acid is strongly influenced by the presence of both the pyruvate and *O*-acetyl moieties present on each repeating unit of colanic acid. The decrease in affinity upon removal of the pyruvate ketals obviously shows that the formation of the enzyme substrate complex is driven by electrostatic interaction, which is reflected by the fact that below pH 4 no activity of the enzyme complex could be detected (Fig. 3b). Since pH 4 is below the pK_a of the glucuronic acid and pyruvate moiety these moieties are completely protonated and not available for electrostatic interaction.

Since *O*-acetyl groups frequently introduce steric hindrance and therefore shield the negatively charged groups from interaction with the enzyme complex it seems logical that a 3-fold increase in maximum velocity compared to degradation of the native substrate was observed upon removal of the *O*-acetyl groups. However, in contrast to this assumption, removal of the *O*-acetyl groups also resulted in a decrease in affinity of the enzyme for the substrate and substrate inhibition (dotted line Fig. 6.5a) at higher concentrations of native colanic acid was observed.

Following these observations it was concluded that apart from electrostatic interactions also hydrophobic interaction through the *O*-acetyl groups present plays an important role in the formation of the enzyme substrate complex. Furthermore, both the results for the degradation of the native and de-*O*-acetylated substrate indicate that *O*-acetylation in the native substrate gives rise to substrate inhibition probably by the formation of un-productive substrate binding or by strong (hydrophobic) interactions hindering the release of the formed product and therefore blocking the enzymes availability to form a next enzyme substrate complex.

From these results its obvious that the K_m and V_{max} values for the native substrate should be seen as apparent values determined with the values for which substrate inhibition was neglectable.
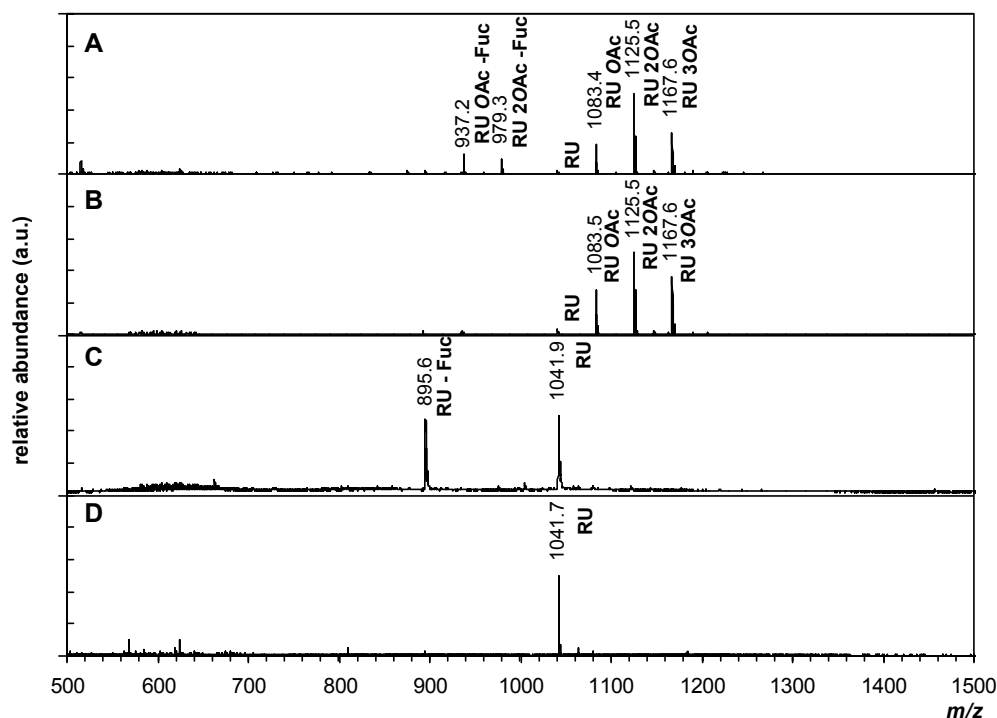

Figure 6.6: MALDI-TOF ms spectra of both native colanic acid and de-*O*-acetylated colanic acid incubated with both crude colanase and purified colanase recorded in the negative mode (M–1): a) Native colanic acid incubated with crude colanase preparation; b) Native colanic acid incubated with purified colanase preparation; c) de-*O*-acetylated colanic acid incubated with crude colanase preparation; d) de-*O*-acetylated colanic acid with purified colanase preparation.

Mode of action — From the HPSEC elution profiles as shown in figure 4 it is clear that the enzyme shows endo-activity towards colanic acid resulting in a significant drop of the molecular weight of the substrate to oligomeric products. The oligomers formed by the cell free culture and the purified enzyme were analyzed by MALDI-TOF MS in the negative mode (Fig. 6.6). This revealed that upon prolonged degradation the enzyme released oligomers representing the repeating unit of colanic acid with varying degrees of *O*-acetylation. Repeating units without and with 1, 2 or 3 *O*-acetyl groups were found at *m/z* 1041, 1083, 1125 and 1167, respectively. The location of these *O*-acetyl groups on the colanic acid repeating units will be discussed later. However by comparing the products from the crude enzyme preparation with the purified preparation it became clear that the crude preparation seemed to contain fucosidase side activity. This can be deduced from the appearance of the same series of oligomers however with the loss of 1 fucose (-146) in the case of the crude enzyme. This side activity was removed during purification proven by the fact that within Fig.6.6b and d, both representing the degradation products after incubation with the purified enzyme, no repeating unit minus fucose could be found. These results prove that only the main de-polymerizing activity was left in the partially purified colanase preparation.

The linkage cleaved by the enzyme on both native and de-*O*-acetylated colanic acid was elucidated using Post Source Decay (PSD)/MALDI-TOF MS analysis in the positive mode. This analysis was preformed in the positive mode to be able to observe neutral fragments released upon PSD analysis that cannot be seen when using the negative mode. The PSD/MALDI-TOF MS spectrum of the de-*O*-acetylated substrate analyzed in the positive mode is shown in Fig. 6.7. Fragmentation shown in Fig 6.7 is annotated according to the systematic nomenclature of Domon and Costello [18].

PSD analysis mainly resulted in the formation of Y_j and Z_j fragments from the terminal end of the side chain. The release of fragment Y_2 and Z_2 at *m/z* 919.5 and 901.5, respectively, was observed. These 2 fragments represent the loss of fucose from the non-reducing end of the core chain of the oligosaccharide indicating that the enzyme splits colanic acid between the two fucoses present in the backbone of colanic acid.

Furthermore all Y and Z fragments expected from the side chain were observed, starting with the loss of the pyruvated galactose at the terminus of the side chain resulting in fragment $Y_{4\alpha}$ and $Z_{4\alpha}$ at *m/z* 833.9 and 815.9. Followed by fragment $Y_{3\beta}$ and $Z_{3\beta}$ (*m/z* 639.3 and 657.3)

representing the release of the glucuronic acid moiety. Finally the loss of galactose from fragment $Y_{2\lambda}$ and $Z_{2\lambda}$ with respectively, *m/z* 477.6 and 495.6 was observed.
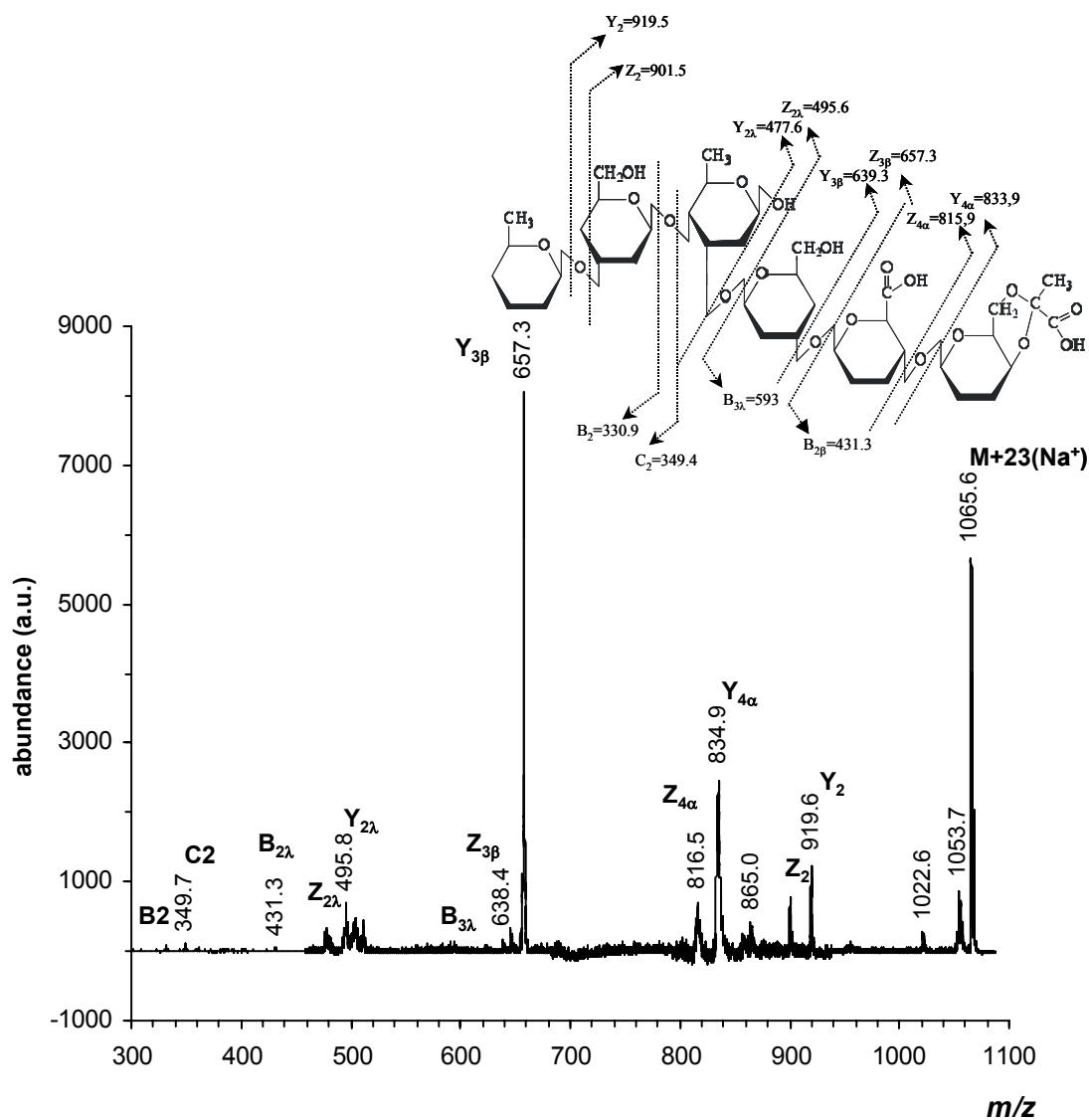

Figure 6.7:PSD/MALDI-TOF ms fragmentation pattern of de-O-Acetylated colanic acid after colanase incubation analysed in the positive mode (M+ 23 (Na$^+$)). Peaks are coded with their mass and corresponding ion. Above the spectrum the structure of the repeating unit of colanic acid is given together with the fragmentation pattern. The alcohol groups and protons in the molecular structure are left out to make the structure less complex.

Due to their low *m/z* value the release of $B_{1 \text{ and } 1\alpha}$ and $C_{1 \text{ and } 1\alpha}$ were not observed, but both B_2 and C_2 showing the release of both fucose and glucose from the non- reducing end of the branched fucose at *m/z* 330.9 and 349.4, respectively, were present in the spectrum. The release of this dimer again proves that the enzyme splits between the two fucoses present in the backbone of colanic acid. A similar fragmentation pattern was found for the native EPS after enzyme treatment and PSD MALDI-TOF MS analysis (results not shown). These findings made it possible to classify the novel enzyme as a β(1,4)-fucanosyl hydrolase.

From the PSD spectra of the native EPS after enzymatic degradation we have been able to confirm the position of 2 of the 3 possible *O*-acetyl groups. Fragment Y_2 was found for the loss of both fucose and fucose substituted with *O*-acetyl at the terminal end of the core oligosaccharide confirming the *O*-acetylation position as found before by Garegg et al. [5, 6]. Besides these fragments we also found the $Y_{2\lambda}$ fragment, in this case representing the loss of the trimeric side chain with an *O*-acetyl group attached to the β-1-3-linked galactose. This result reveals the presence of a second *O*-acetyl group on colanic acid situated on the galactose linked to the branched fucose. The position of the third possible *O*-acetyl group could not be established.

These findings reveal that the non-branched fucose, assumed to be the only acetylation site based upon resistance to β-elimination of this 1,4-linked residue, is not the only O-acetylation site of colanic acid [5, 6].

The variations found in *O*-acetylation patterns on each repeating unit could confirm early observations of Grant et al. [19] that acetylation of colanic acid takes place in a later stage of biosynthesis and that the variations in *O*-acetylation of colanic acid found are derived from incomplete synthesis. It is also possible that *O*-acetylation is part of a random process within the biosynthetic pathway of colanic acid. More than one acetyltranferase could be involved in the attachment of *O*-acetyl groups to an already formed repeating unit linked to a lipid carrier in the same way as found for xanthan biosynthesis [20]. These are also inline with the observation of Stevenson et al [21] who found 2 genes (*wcaB and wcaF*), encoding for different *O*-acetyl transferases, within the *E.coli* K-12 gene cluster responsible for colanic acid biosynthesis.

Combining the results found for the mode of action of the enzyme and the information obtained with modified substrate it is possible to postulate a putative subsite model for the enzyme.

This model is shown in figure 8 and summarizes the characteristics of this enzyme. Although subsite models are normally only used for linear polysaccharide backbones the results obtained

show that in this case the side chain plays an important role in the affinity of the substrate for the enzyme and that a more complex subsite model is necessary. Therefore, unconventionally, the side chain is incorporated within the subsite model showing the terminal pyruvated galactose of the side chain at subsite –4* and the *O*-acetylated fucose in the backbone at subsite +1. No clear evidence for the position of subsite –2, -3, +2 and +3 was obtained leaving the assignment of these subsites on a putative base.
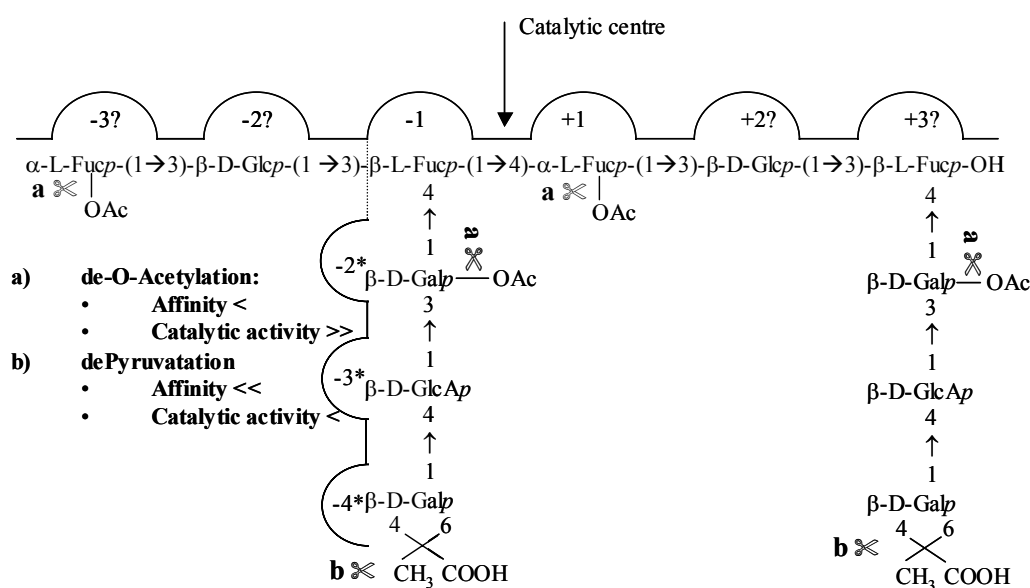

Figure 6.8: Putative representation of the different subsites of colanase and the effects of colanic acid modification upon the enzymes affinity and catalytic activity.

-2*, -3* and –4* are side chain specific subsites

Conclusions.

Purification of colanase obtained from a mixed culture that was enriched from a compost sample using colanic acid as only carbon source resulted in the recognition of a novel β(1,4)-fucanosyl hydrolase. The enzyme could be used in a broad pH and temperature range and therefore could make it applicable in for example paper machines [22-24]. The fucanosyl hydrolase purified was an endo acting, hydrolytic enzyme cleaving colanic acid between the 2 adjacent fucopyranosyl

residues present in the backbone of colanic acid resulting in the formation of single repeating units with varying degrees of *O*-acetylation.

Kinetic studies and substrate modifications revealed that the affinity of the enzyme for colanic acid is based upon electrostatic and hydrophobic interactions and that removal of the *O*-acetyl moieties results in an increase in catalytic activity.

The enzyme found was different from the ones described earlier, because it showed a far more significant drop in molecular weight of the substrate after incubation then the one described by van Speybroeck et al [9], which only showed a molecular weight decrease of approximately 2,000 to 400 kDa instead of 100% release of single hexameric repeating units of about 1 kDa after exhaustive incubation with this enzyme. The enzyme described by Sutherland [13] was phage assisted and different to the enzyme characterized in this work. The latter enzyme was found to be far more effective in the release of repeating units. Furthermore the novel enzyme characterized was found to be a true fucanosyl hydrolase being able to degrade linkages between 2 fucose moieties instead of the linkage between fucose and glucose as in the case of the enzyme described by Sutherland.

The novel β(1,4)-fucanosyl hydrolase obtained has a high molecular weight and seemingly is a part of a multi protein complex consisting of at least 6 different proteins with varying molecular weight.

Future work will be directed towards the determination of partial amino acid sequences of the individual proteins for PCR cloning of the respective genes.

Acknowledgement.

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Chapter 7

General Discussion

Introduction.

The development of enzymatic strategies against biofouling is largely influenced by the structure of the exopolysaccharides secreted by the microorganisms present in paper manufacturing equipment. Because these exopolysaccharides are responsible for the structural integrity of a biofilm and in this way are responsible for the formation of slime layers on paper machines, more detailed knowledge about the EPSs occurring within these biofilms is important.

Based on this knowledge enzymatic strategies can be targeted towards degradation of the most important EPSs present within paper mill slimes.

Therefore, the scope of this thesis was to identify these target EPS structures by detailed structural analysis of the EPS produced by several bacteria isolated from paper mill slime samples. These slime samples were taken from paper mills in Finland, France and Spain using different raw materials.

Firstly the detailed chemical structures of the EPS produced by 4 different bacterial isolates was characterised using different chemical, enzymatic and analytical techniques. Furthermore the EPS produced by 31 different bacterial isolates were grouped into clusters of bacteria that produce the same or similar EPSs. Finally a novel enzyme able to degrade one of the important EPSs, obtained by enrichment of bacteria present in an innoculum from compost heap sample with colanic acid as substrate, was purified and characterized. The results of these studies are successively discussed in this chapter.

EPS produced by bacteria isolated from paper mill environments.

Chemical characterisation of the EPS produced by bacterial isolates from different paper mill environments resulted in the elucidation of 4 EPS structures. Two of these EPSs the EPS produced by *Brevundimonas vesicularis* [1] and *Methylobacterium* [2] (Table 7.1) have not been reported before. The EPS produced by *Enterobacter* and related species [3], colanic acid, has been reported as an EPS commonly produced by several members of the *Enterobacteriaceae* family [4,5] (Table 7.1).

Furthermore a bacterium characterised as *Raoultella terrigena* producing the same EPS as *Klebsiella* Serotype K70 [6] was identified [3] (Table 7.1). *Klebsiella terrigena* has been reclassified as *Raoultella terrigena* [7] and capsule typing of *Klebsiella terrigena* isolates from human faeces revealed serotype K 70 to be present within these isolates [8].

Also, the EPS produced by *Klebsiella pneumoniae* (*Klebsiella* Serotype K3 [9]) [10] and by *Sphearothilus natans* [11], both isolated from paper mill slimes were reported (Table 7.1).

Table 7.1: Chemical fine structures of EPS produced by bacterial isolates from different paper mill slimes.

EPS producing bacterium	Structure of the EPS repeating unit
B.vesicularis [1]	→4)α-L-Glc*p*A(1→4)α-D-Gal*p*A(1→4)β-L-Rha*p*(1→4)-β-D-Glc*p*(1→

Methylobacterium sp. [2]	→3)α-D-Gal*p*(1→3)α-D-Gal*p*(1→3)α-D-Gal*p*(1→
	  4 6 4 6
	  pyruvate pyruvate

Several members belonging to the *Enterobacteriaceae* family: *Enterobacter* sp. and *Citrobacter* sp. [3, 4, 5]	→3)-β-L-Fuc*p*-(1→4)-α-L-Fuc*p*-(1→3)-β-D-Glc*p*-(1→
	     4 |
	     ↑ *O*-Ac
	     1
	β-D-Gal*p*(3←1)β-D-GlcA*p*(4←1)β-D-Gal*p*(4,6)pyruvate

R. terrigena formerly classified as *Klebsiella* Serotype K70 [6, 3]	→2)α-L-Rha*p*(1→4)β-D-GlcA*p*(1→4)α-L-Rha*p*(1→2)α-L-Rha*p*(1→
	    4 3 →2)α-D-Glc*p*(1→3)β-D-Gal*p*-(1→
	    Pyruvate

K. pneumoniae [10] Same structure as *Klebsiella* Serotype K3 [9]	→2)-α-D-Gal*p*A-(1→3)α-D-Man*p*-(1→2)α-D-Man*p*-(1→3)β-D-Gal*p*(1→
	     4
	     ↑
	     1
	α-D-Man*p*(4,6)pyruvate

S. natans [11]	→4)-α-D-Glc*p*-(1→2)-β-D-Glc*p*A-(1→2)-α-L-Rha*p*-(1→3)-β-L-Rha*p*(1→

In general the structures presented in Table 7.1 underline the wide variety of structures that are encountered within biofilms found in the paper industry. In fact half of the bacterial isolates and the EPS they produce have been isolated before from sources outside the paper industry. This indicates that EPS isolated from other sources can also be encountered rather easily in a paper mill environment. These finding justify the assumptions made in chapter 1 about the possible occurrence of the same EPSs in paper mill slime as in biofilms studied from other sources. This especially seems to account for the *Enterobacteriaceae* species isolated from paper mill slime deposits e.g. colanic acid for *Enterobacter* sp. and *Citrobacter* sp. [3] and 2 *Klebsiella* K-type polysaccharides or EPSs *K. pneumoniae* and *R. terrigena* [3, 10].

It is evident that all of the EPSs presented are charged by the presence of either uronic acids or in the form of a pyruvate ketal substituent. Two of the EPSs, colanic acid and *K. pneumoniae*, have a branched repeating unit structure. Furthermore, colanic acid is the only EPS out of the 6 EPSs listed in Table 7.1 that is decorated with *O*-acetyl moieties.

EPS as target substrates for enzymatic biofouling control.

The number of completely characterized EPS structures produced by bacteria is still rather limited and determination of their chemical fine structures is a rather tedious and time consuming task. Therefore the chemical features of a large number EPS were determined and compared with each other using multivariate statistics [12]. This approach made it possible to give a more complete overview of the overall composition of EPSs produced by identified paper mill isolates.

The sugar compositions of the EPSs clearly show that glucose, galactose and glucuronic acid should be considered as important sugar moieties present in EPSs from paper mill environments, which is in agreement with previous studies [13-15]. Apart from these sugar moieties also the non-sugar substituents in the form of acetyl esters and pyruvate ketals were recognized as important components in most of the 30 EPSs analyzed. The recognition of these substituents was in contrast to former studies dealing with paper mill slime, where these substituents were not analyzed.

Figure 7.1 shows a graphical representation of the distribution of the different functional groups and sugar moieties over the EPSs analyzed. It is evident that the negative charge, introduced by mostly glucuronic acid and pyruvate, is an important characteristic of the molecular structure of the EPSs analyzed. In fact 40% of all samples analyzed contained both these moieties. Only 7%

of all the EPSs analyzed did not contain any charged groups. Furthermore 47% of all the samples contained *O*-acetyl esters as non-sugar substituents.

Uronic acids, frequently glucuronic acid, and pyruvate ketals contribute to the polyanionic nature of most EPSs and they have a large impact on the physical properties of the EPSs. On the other hand, *O*-acetylation can result in localised hydrophobic regions also important for the physical behaviour of the EPSs [16, 17]. Within the complex biofilm architecture EPS is responsible for the structural integrity of the biofilm matrix. This is mediated through non-covalent interaction either directly between the polysaccharide chains or indirectly via multivalent cation bridges. Furthermore, lectin like proteins are believed to form indirect cross-links between the EPS present. In addition extra-cellular proteins have also been suggested to play an important role in the formation of indirect cross-links via hydrophobic interactions within the EPS matrix [18].
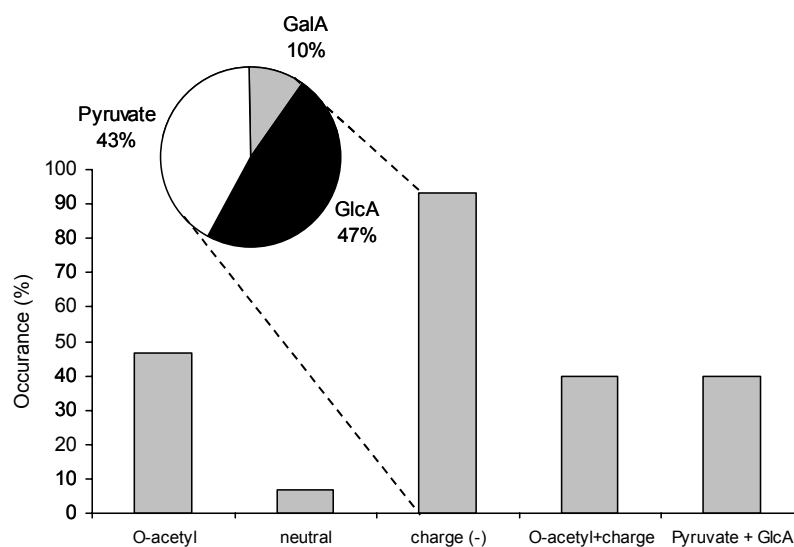

Figure 7.1: Distribution of functional sugar and substituent moieties over all the 30 EPSs analysed on their sugar composition.
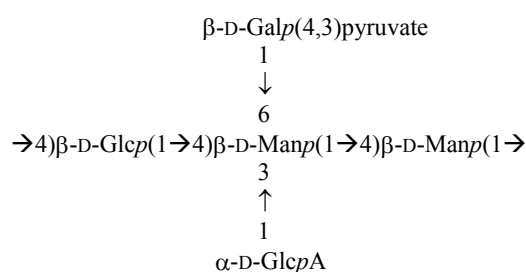
All the sugar and substituent composition data of the EPSs analysed were subjected to Principal Components Analyses (PCA) making it possible to form 4 groups of bacteria that either contain the same or similar EPSs [12].

From the 30 EPSs analysed, 9 EPSs produced by different *Enterobacter* isolates could be clustered into a group of EPS similar to colanic acid (Table 7.1.), an EPS commonly produced by several members of the *Enterobacteriaceae* family.

Secondly, 3 EPSs could be clustered into a group of *Methylobacterium* species all producing the same EPS consisting of a highly pyruvated galactan (Table 7.1.).

Next to 2 groups of clustered EPS with the same structures 2 other EPS clusters could be formed. One contained 7 EPSs of *Bacillus* and related species clustered by their high mannose and/or glucose content. These findings seem to be in agreement with the fact that *Bacillus* species are frequently found within paper mill slimes [15, 19-21]. However, recent studies have shown that *Bacillus* species are incapable of primary colonization but rather depend on coadhesion with other species [22]. These findings might explain the relatively low carbohydrate contents found for the EPSs produced by these isolates (chapter 4, Table 4.1). This makes it questionable whether these EPSs should be seen as target polysaccharides for enzymatic biofouling control.

The second group contained 3 EPSs produced by *Klebsiella* species with high galactose levels in the presence of rhamnose. With respect to the *Klebsiella* species these characteristic sugars are predominantly based upon the EPS used in the validation set (Chapter 4 Table 4.2). The detailed structure of the EPS produced by one of the *K. pneumoniae* (VTT-E-011927) and the *K. planticola* species isolated (chapter 4 Table 4.1) are not known. It would however be interesting to determine their K-serotype, since according to Nimmich [23-25], who determined the sugar composition of the (K) type specific polysaccharides of *Klebsiella* serotype K1 to 80, only *Klebsiella* K10, 26, 30, 31, 33, 35, 39, 46, 59 and 69 contain galactose, glucose and mannose. Comparing the detailed structures reported for these K-types with the sugar composition of the EPSs produced by these 2 *Klebsiella* species only 2 possible structures could apply. The EPS produced by *Klebsiella* K30 or 33 [26], which consists of the following repeating unit:

$$
\begin{array}{c}
\beta\text{-}\text{D-Gal}p(4,3)\text{pyruvate} \\
1 \\
\downarrow \\
6 \\
\rightarrow 4)\beta\text{-}\text{D-Glc}p(1\rightarrow 4)\beta\text{-}\text{D-Man}p(1\rightarrow 4)\beta\text{-}\text{D-Man}p(1\rightarrow \\
3 \\
\uparrow \\
1 \\
\alpha\text{-}\text{D-Glc}p\text{A}
\end{array}
$$

The EPS produced by Klebsiella K35 [27] is rather similar to K30 and 33 apart from the terminal pyruvated galactose, which in this case is incorporated in the backbone of the repeating unit.

$\rightarrow$3)α-D-Gal*p*-(1$\rightarrow$3)α-D-Man*p*-(1$\rightarrow$3)α-D-Man*p*-(1$\rightarrow$3)-β-D-Glc*p*(1$\rightarrow$
　　　4　6　　　　　　　　　　　　　　　　　　　2
　　　pyruvate　　　　　　　　　　　　　　　　 ↑
　　　　　　　　　　　　　　　　　　　　　　　　1
　　　　　　　　　　　　　　　　　　　β-D-GlcA*p*

All the other EPSs were very diverse in structure and their 'producers' could not be grouped on the basis of their EPSs. However, this more diverse group of EPSs did contain the already characterised EPSs produced by *B.vesicularis* and *R. terrigena*. Furthermore this group also contain 2 EPSs produced by *Pantoea* and 1 produced by *Serratia* species.

Together with the *Klebsiella* and *Enterobacter* species present, these 2 species also belong to the *Enterobacteriaceae* family underlining the fact that colanic acid production as mentioned above is not seen for every member of this family of gram-negative bacteria as confirmed by the studies presented in chapter 5 [3].

FT-IR spectra of the same EPS samples followed by DPLS regression and LDA analysis (Chapter 4) could only be used to group the EPS of the first 2 clusters of bacterial isolates, *Enterobacter* and *Methylobacterium* indicating that this method could replace time consuming compositional analysis [12]. However, an important drawback is that a broad database of FT-IR spectra of different EPS samples is a prerequisite for this method to be successful.

The fact that the FT-IR spectra of the EPS samples from the *Methylobacterium* and *Enterobacter* group were the same does prove that these EPS samples are the same. Further prove for this assumption was provided in chapter 5 were the same *Enterobacteriaceae* isolates were subjected to even more detailed structural comparison by ways of partial hydrolysis and linkage analysis. With respect to the *Methylobacterium* EPSs the same enzyme as described in chapter 3 could degrade all 3 of these EPSs.

Enzymes degrading EPS produced by bacterial isolates from paper mill environments.

In this thesis also the purification and characterisation of a β(1,4) fucosyl hydrolase, able to degrade colanic acid produced by a mixed culture isolated from a compost heap sample is

described [28] and since colanic acid appears to be an important target substrate found by the structural elucidation of several exopolysaccharides produced by bacteria isolated from paper mill slime [12, 3] this hydrolase has potential as a anti biofouling enzyme.

The β(1,4)-fucanosyl hydrolase obtained has a high molecular weight and seems to be part of a multi protein complex consisting of at least 6 different proteins with varying molecular weight.

Considering its optimal pH, optimal temperature range and stability the enzyme would have ideal characteristics with regard to a paper mill environment. The optimum pH was found at pH 7, the optimum temperature was found at 50°C, and after 1 h at this temperature still 70% of the activity was retained. Paper machines typically run at neutral to alkaline pH and at temperatures ranging from 30- 55°C [15, 29, 30].

The partially purified fucanosyl hydrolase was an endo-acting hydrolytic enzyme cleaving colanic acid between the 2 adjacent fucopyranosyl residues present in the backbone of colanic acid resulting in the formation of single hexameric repeating units with varying degrees of *O*-acetylation (Chapter 6, Fig.6.6, 6.7 and 6.8).

The affinity of the enzyme complex for colanic acid is strongly influenced by the presence of both the pyruvate and *O*-acetyl moieties present on each repeating unit of colanic acid. In fact removal of the pyruvate ketals resulted in a 10-fold decrease in affinity compared to the native substrate. Obviously this indicates that the formation of the enzyme substrate complex is driven by electrostatic interactions. Since *O*-acetyl groups frequently introduce steric hindrance and therefore shield the negatively charged groups from interaction with the enzyme complex it seems logically that a 3-fold increase in maximum velocity compared to degradation of the native substrate was observed upon removal of the *O*-acetyl groups. However, in contrast to this assumption, removal of the *O*-acetyl groups also resulted in a 10-fold decrease in affinity of the enzyme for the substrate. Also substrate inhibition at higher concentrations of native colanic acid was observed.

Following these observations it was concluded that apart from electrostatic interactions also hydrophobic interaction through the *O*-acetyl groups present plays an important role in the formation of the enzyme substrate complex.

It appears that for this enzyme the non-sugar substituents are an important factor for the ability of the enzyme to degrade colanic acid.

A similar observation was made for the enzyme able to degrade the highly pyruvated galactan EPS produced by *Methylobacterium* sp. [2] firstly considered to be tolerant for both pyruvate

ketals present on the trisaccharide repeating unit. However, recent results (Fig.7.2) showed that the enzyme was not capable of degrading the completely depyruvylated substrate making it evident that in this case the pyruvate ketals are a necessity for degradation.

Another example of a pyruvate dependant enzyme could be xanthan lyase that is exclusively specific for pyruvated mannosyl residues [31].
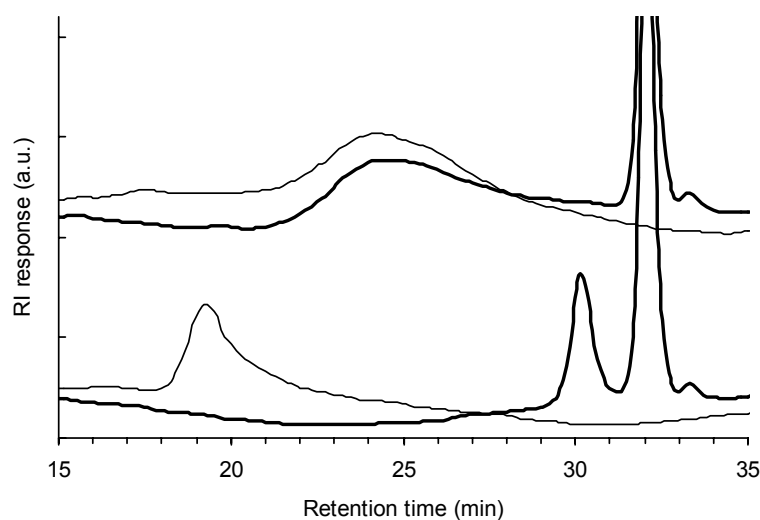

Figure 7.2: HPSEC elution profiles of both the native (bottom traces) and depyruvylated (top traces) EPS of the EPS produced by *Methylobacterium* before (thin line) and after (thick line) enzyme degradation

Due to the high molecular weight protein complex found for the colanic acid-degrading enzyme (>800 kDa) it was not possible to obtain a fully purified enzyme. Other cell free culture solutions able to degrade the EPS produced by *Brevundimonas vesicularis* [1] and *Methylobacterium* sp. [2] were also obtained. Upon purification attempts also these 2 enzymes seemed to be incorporated within a high molecular weight protein making purification of the enzymes rather difficult. The enzyme degrading the EPS produced by *B.vesicularis* eluted in the void of a gel filtration column (>2000 kDa) and the molecular weight of the enzyme degrading the EPS produced by *Methylobacterium* sp. had an estimated molecular weight of approximately 850 kDa (personal communication M. Siika-aho).

Attempts to obtain single species degrading the EPSs were unsuccessful (personal communication M. Rättö). This observation could probably be explained by the fact that in

nature microorganisms coexist in close proximity and are frequently found as consortia capable of degrading complex substrates. These substrates can often not be degraded by the individual species. This explains why only in few cases a pure bacterial culture capable of degrading an exopolysaccharide following normal enrichment procedures could be found [32]. In general the high molecular weigth and unsuccesfull attempts to obtain a single species culture degrading the EPSs have a large impact on the possibility of cloning these enzymes into another host for production on a large scale.

Translation of EPSs produced under laboratory conditions to paper mill slime layers.

For the development of an effective enzyme system preventing biofouling it is important to translate the data obtained from bacterial isolates grown under laboratory conditions back to the situation of the problematic slime layers as they are found within a paper mill. Therefore slime layers from different paper mills in Spain, France and Finland were sampled and pre-treated according to the scheme shown below (Fig. 7.3) with the aim to isolate EPSs from these slime samples. Since slime layers are a mixture of biofilm and fibres, fines, fillers and other materials present within the paper process the slime samples from the different mills were hydrolyzed with cellulose-, hemicellulose- and pectin-degrading enzymes (Econase CE, Ecopulp, Gamanase, Novozyme 188, Pectinex Ultra SPL) for 48 h at 40 °C to remove most of the soluble wood derived fibers. Water-soluble polysaccharides were precipitated before and after the enzyme treatment to produce precipitate **A** and **B** (Fig. 7.3). From the mass balance represented in Fig. 7.4 it is obvious that the water-soluble polysaccharides present in the samples before and after enzyme treatment are only a minor part of the slime sample. In most cases the residue was between 80 and 95 (w/w%) of the sample. It is expected that a large part of the residues consist of lignins and celluloses. The major compounds of the slime samples most probably consist of wood fibre derived polysaccharides like pectins, mannans, lignins and precipitated salts $CaCO_3$.

The different precipitates were analysed for their molecular weight distribution and their sugar composition. From the HPSEC elution profiles (results not shown) it was clear that both precipitate **A** and **B** mainly consisted of high molecular weight material. The high molecular weight material present in these fractions is expected to be EPS material especially in precipitate **B** since most of the wood derived fibres like cellulose, hemicellulose and pectin are completely

hydrolyzed or not soluble at all. The residues left after both precipitation steps were insoluble in water and therefore could not be analysed using HPSEC.
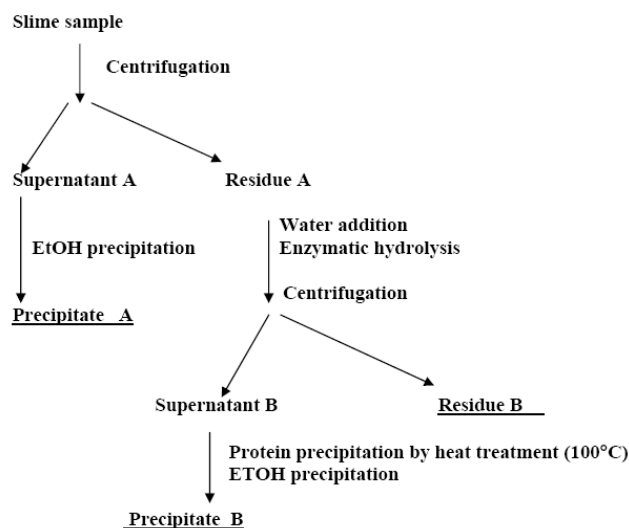

Fig. 7.3 Extraction scheme of the EPS part of different slime samples collected from different paper mills in Spain, Finland and France
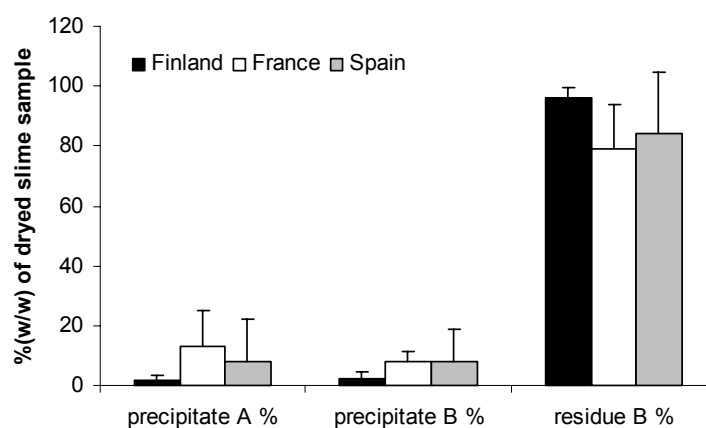

Figure 7.4: Average mass balance after extracting the EPS part of slime samples taken from Finnish, French and Spanish paper mills.

According to the mass balance it is clear that the biofilms (EPSs) formed by the microorganisms present is only a minor part of the slime layers sampled. A large part of the EPSs analyzed could be characterized by their polyanionic nature and several of these EPSs also posses hydrophobic groups in the form of *O*-acetyl esters (chapter 4). We therefore assume that the biofilm components act as a kind of glue that accumulates the raw materials like wood fibers used within the paper mill process through cross linking between different polymers present by divalent cation bridges, H-bridges formed by exposed hydroxyl groups or through localized hydrophobic regions introduced by *O*-acetyl groups or 6-deoxyhexoses [16] to form a slime layer.

To be able to compare the isolated slime samples to the EPSs produced by bacteria isolated from slime layers as analyzed in chapter 2, 3 and 4 both precipitate **A** and **B** were also subjected to sugar composition analysis. The results obtained were rather similar and are summarized in Fig. 7.5 for a few examples.

A wide variety of sugars were found with galactose and glucose as the mayor sugar moieties. This wide variety of sugars found underlines the heterogeneity of the biofilms present and makes it rather difficult to be able to make a clear distinction of the EPS present.
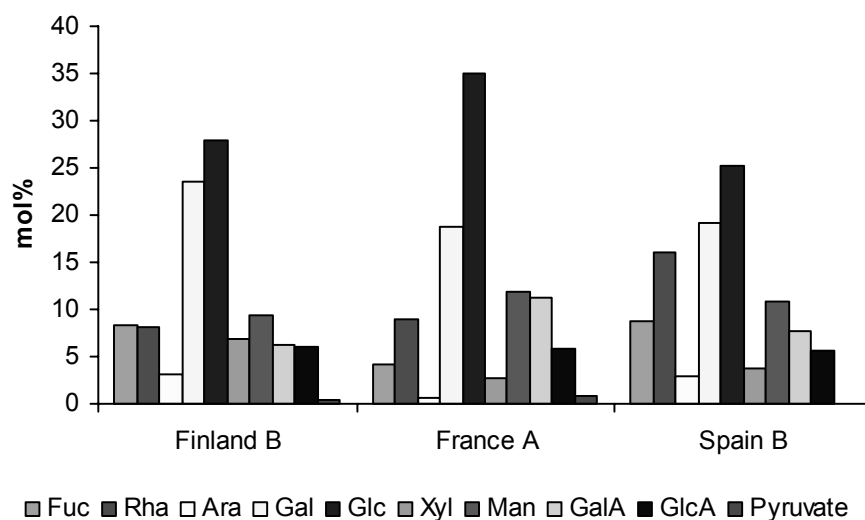

Figure 7.5: Sugar composition of alcohol insoluble, water-soluble polysaccharides isolated from slime layers sampled from different paper mills in Finland, Spain and France. (sequence of the bars is the same as the sequence of the different sugars in the legend)

Because both crude as well as partially purified cell free cultures degrading colanic acid (chapter 6) and the EPS produced by *Methylobacterium* (chapter 3) were available and the sugar moieties and substituents of the isolated slime layers (precipitate **A** and **B**) indicated that these 2 EPSs could be present incubation with these cell free cultures was used to prove the presence of these EPSs. This was done for precipitate **A** and **B** of all the samples by incubation for 48h. After incubation the samples were analyzed by HPSEC, HPAEC and MALDI-TOF MS to see whether degradation took place. However, no visible degradation of the samples was detected by HPSEC or by the both the other more sensitive methods. These findings could mean that, either the enzyme does not work in this system or that the substrate is not available for degradation due to interaction with other polymers present within the samples or that both EPSs are not present. To draw an unambiguous conclusion about the presence of these 2 EPSs they were subjected to partial hydrolysis using 2N TFA at 100ºC for 1h followed by detection of the released oligomers by means of MALDI-TOF MS, because acid hydrolysis is less likely to be influenced by other materials present within the sample matrix. Also these experiments did not provide any clear evidence about the presence of these EPSs. From these results it was concluded that both the EPS produced by *Methylobacterium* sp. and colanic acid were not present in the slime samples subjected to analysis.

The results obtained by analyzing the water soluble polysaccharides extracted from paper machines slime samples on the EPS present indicate that translation of EPS produced by species under laboratory conditions to the EPS present in slimelayers is still a rather complex task. However, Hughes et al [33] have shown that both planktonically prepared and biofilm EPSs could be degraded by the same specific endo-glycanohydrolases releasing the same oligosaccharides. Similarly Costerton et al [34] in [16] used antibodies raised against planktonically synthesized polysaccharides that showed interaction with the same polysaccharides within a biofilm.

With respect to the analysis of heterogeneous glycoconjugates in mixed species biofilms lots of progress has been made by the use of carbohydrate specific lectin binding studies in confocal laser scanning microscopy (CLSM) [35].

By using this non-destructive technique the glycoconjugate distribution within different single and mixed species biofilms could be visualized in fully hydrated living biofilm systems. For example Strathmann et al [36] used fluorescently labeled lectins to visualize the EPS distribution in model *Pseudomonas aeruginosa* biofilms by using concanavalin A (ConA) that binds to alginate. Böckelmann et al [37] reported the use of lectins specifically binding to *N*-acetyl-

D-glucosaminyl, D-galactose, α- D-mannose, α- D-glucose and L-fucose for investigating enzyme-induced detachment of particle associated soil bacteria. A study by Staudt et al [38] shows the use of CSLM in combination with lectin binding for measurements on the development of a fully hydrated multi species biofilm. Using the data obtained, parameters like biofilm thickness, total volume of the bacterial cells and the volume of EPS glycoconjugates could be determined.

Another example was reported by Böckelmann et al [39] by using a combination of fluorescent in situ hybridization (FISH) and lectin binding assays to study the distribution of strain specific EPS in lotic microbial aggregates. Lotic microbial aggregates or "river snow" are mobile biofilms attached to suspended particles in aquatic systems.

Following these examples it would be interesting to combine the studies performed within the scope of this thesis with lectin binding and FISH assays for biofilms grown under paper mill simulating conditions. For example by inoculating a bioreactor with white water from different paper mills followed by growing the biofilms on stainless steel coupons. These biofilms could then be analyzed by determination of the strains present by FISH and the presence of strain specific EPS by the use of lectin binding assays.

Consequences for biofouling control during paper manufacturing.

With respect to the structural features of slime layers on paper mills it is important to realise that the EPS to be targeted with enzymatic biofouling control only form a minor part of an already formed slime layer. This observation indicates that the insoluble fibre precipitates entrapped within the biofilm would almost certainly block the enzyme in effectively degrading the slime layer. This implies that enzymatic biofouling control by targeting the EPS matrix [2, 28, 40-42] would only be effective in an early stage of the formation of a biofilm. But if used in this early stage it could be a valuable contribution to already developed enzymatic control systems that rely on the use of proteases [43, 44], lytic enzymes like lysozyme [45], biodispersants or a combination of the latter [46, 43]. Furthermore, it is important to apply contamination prevention by the use of a strict hygienic regime. All these factors could either prevent bacterial contamination in the first place or severally interfere with the formation of microcolonies or with the first steps of reversible adhesion of the microbes present to the surface of a paper machine. Finally an appropriate multi activity enzyme mixture should be devoid of any cellulase or amylase activety, since both these enzymes are not compatable with the process of paper

manufacturing. This reduces the number of potential enzymes especially since one of the mayor sugar moieties found within the paper mill analysed is glucose.

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Summary

Bacteria that adhere to the surface of a paper machine form a biofilm that entraps the fibres and additives used as raw material to form a slime deposit. The formation of these slime deposits can result in serious problems with respect to the papermaking process itself and the end product. Traditionally these problems are solved by the use of biocides and surfactants, however the use of biocides is not always very effective. Furthermore, the toxicity of most of the biocides used and the impact on the environment and safety concerns with regard to the people that handle them have resulted in more strict legislation towards the use of biocides.

Therefore, there is an increased interest in alternative methods for slime control within paper processing. One of these methods could be the use of enzymes that degrade the exopolysaccharides (EPSs) that form the major structural element of a biofilm. To obtain these enzymes it is important to know which EPSs to target for enzymatic degradation. Therefore the EPSs produced by several species isolated from different paper mills within Spain, Finland or France were subjected to a (detailed) chemical structure elucidation. From these studies several EPSs were selected as target substrates for enzymatic degradation. The enzyme able to degrade one of these substrates was subjected to purification and characterisation studies.
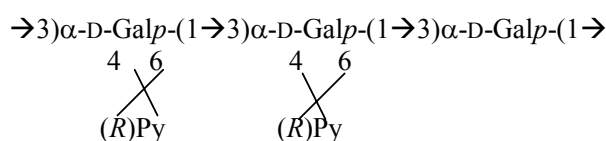
In chapter 1 a general introduction that briefly introduces the important background aspects with respect to the research performed is given. Besides information with respect to paper processing it addresses slime deposit induced problems and the formation of biofilms within a paper machine. Furthermore, structural aspects of EPSs are discussed and the use of enzymes for slime control within a paper process.

Both chapter 2 and 3 deal with the structural elucidation of an EPS produced by bacteria isolated from a slime sample of a Finnish paper mill.

Chapter 2 deals with the detailed structural elucidation of the EPS produced by the slime forming bacterium *Brevundimonas vesicularis* sp. HPSEC analysis revealed one distinct polysaccharide population with a molecular mass of more than 2,000 kDa. The EPS was found to consist of rhamnose, glucose, galacturonic acid and glucuronic acid in a molar ratio of 1:1:1:1, respectively. ^{13}C and ^{1}H hetero- and homonuclear 2D NMR spectroscopy of the native and partial hydrolysed EPS revealed a

→4)-α-L-Glc*p*A-(1→4)-α-D-Gal*p*A-(1→4)-β-L-Rha*p*-(1→4)-β-D-Glc*p*(1→

repeating unit. No non-sugar substituents were present.

In chapter 3 the structure of the EPS produced by the slime-forming bacterium *Methylobacterium* sp is described. Sugar compositional analysis revealed a 100% galactan (EPS). However, FT-IR showed the presence of pyruvate and analysis of the pyruvate content revealed that, based on the sugar composition, the EPS consists of a trisaccharide-repeating unit consisting of D-galactopyranose and pyruvated-D-galactopyranose with a molar ratio of 1:2, respectively. Both linkage analysis and 2D homo- and heteronuclear ^{1}H and ^{13}C NMR spectroscopy revealed the following repeating unit:

→3)α-D-Gal*p*-(1→3)α-D-Gal*p*-(1→3)α-D-Gal*p*-(1→
$\quad\quad$ 4 $\quad$ 6 $\quad\quad\quad\quad$ 4 $\quad$ 6
$\quad\quad\quad$ ✕ $\quad\quad\quad\quad\quad$ ✕
$\quad\quad$ (*R*)Py $\quad\quad\quad\quad$ (*R*)Py

By culturing various soil and compost heap samples on selective media containing this EPS a polysaccharide-degrading culture was obtained that produced an endo acting enzyme able to degrade the EPS described.

Since the procedures for fully elucidating the chemical fine structure of an EPS, exemplified by chapter 2 and 3, can be rather time-consuming chapter 4 deals with the multivariate comparison of sugar compositions and FT-IR spectra of a large number of EPSs to enable clustering of important EPSs on the basis of these features. Therefore, thirty exopolysaccharides produced by bacteria isolated from biofilms or slimelayers from different paper and board mills in Finland, France and Spain were subjected to sugar composition and FT-IR analysis. Based on the sugar compositions four groups of EPSs were recognised by PCA analysis: a group of EPSs produced by *Enterobacter* and related genera similar to the regularly reported colanic acid; a group of *Methylobacterium* EPSs having high galactose and pyruvate levels and 2 groups that showed less dense clusters produced by *Bacillus* and related genera, having high mannose and/or glucose levels and *Klebsiella* EPSs that showed galactose with rhamnose as major characteristic sugar moieties.

FT-IR analysis of the same samples followed by DPLS-R and LDA analysis showed that, if used with a well-defined training set, FTIR could replace clustering based on time-consuming sugar composition analysis. Both the *Enterobacter* and *Methylobacetrium* EPSs could be clustered clearly. However the fact that this could hardly be done for the other two groups in the data set indicates the importance of a larger and well-defined training or calibration set. The potential to

use FTIR, as a tool for pattern recognition and clustering with respect to EPS structures produced by micro organisms isolated from a paper mill environment is discussed.

Of the 30 EPSs discussed within chapter 4 18 EPSs were produced by members of the *Enterobacteriaceae* family. Considering these observations it was decided to study this group of EPS producing bacteria in more detail. Therefore, in chapter 5 the same polysaccharide-producing bacteria were subjected to more detailed species identification and analysis of the EPS they produced. Many of the polysaccharide producing isolates were different *Enterobacter* and *Klebsiella* sp. species. All of the EPSs analysed showed the presence of charged groups in the form of uronic acid or pyruvate revealing the polyanionic nature of these polysaccharides. Further results of the carbohydrate analysis, using linkage analysis and partial acid hydrolysis, proved that the EPS produced by nine of the 18 isolates, all belonging to the family *Enterobacteriaceae*, indeed is colanic acid.

Following the results obtained in chapter 4 and 5, chapter 6 describes a novel colanic acid-degrading enzyme isolated from a culture filtrate of a mixed population obtained by enrichment of compost heap innoculum on a selective medium containing colanic acid as carbon source. The enzyme was partially purified resulting in a 17-fold increase in specific activity. Further purification by Native PAGE revealed that the enzyme is part of a high molecular weight multi protein complex of at least 6 individual proteins. The enzyme showed a temperature optimum at 50 °C while after 1 h at 50 °C and pH 7 still 70% of the total activity was left. The pH optimum was found to be pH 7.

Analysis of the degradation products, by PSD/MALDI-TOF MS, showed that the enzyme is a novel $\beta(1,4)$-fucanosyl hydrolase that liberates single repeating units of colanic acid with varying degrees of acetylation.

K_m and V_{max} of the enzyme were determined against the native substrate as well as its de-*O*-acetylated and depyruvated forms. Compared to the native substrate the affinity, measured as $1/K_m$, of the enzyme for the modified substrates was much lower. However, for the de-*O*-acetylated sample a dramatic increase in catalytic efficiency (reflected as V_{max}) was observed.

The native form of the substrate showed substrate inhibition at high concentrations, probably due to the formation of non-productive substrate complexes. Removal of the acetyl groups probably prevents this effect resulting in a higher catalytic efficiency.

In chapter 7 the results of this thesis are discussed. In combination with unpublished results on the sugar composition and enzymatic degradation of slime samples from different

paper mills the use of EPS degrading enzymes to establish slime control within a paper mills is discussed. Furthermore this chapter addresses the translation of planktonically produced EPSs to their presence within biofilms or slime deposits found in paper mills.

So far results from these additional studies did not provide unambiguous evidence as to whether this translation can be made.

Samenvatting

Het hechten van bacteriën aan het oppervlak van papiermachines door middel van de vorming van een biofilm die papiergrondstoffen als cellulose vezels inkapseld resulteert in de vorming van slijmafzettingen. Deze slijmlagen kunnen ernstige problemen met betrekking tot het papier-productieproces en het eindproduct veroorzaken.

Tegenwoordig tracht men deze problemen op te lossen door het gebruik van biociden, maar deze biociden zijn niet altijd even effectief. Verder is het gebruik van biociden, door de toxiciteit, het effect op het milieu en de veiligheid van het personeel dat ze gebruikt, steeds meer gebonden aan strenge wet- en regelgeving.

Hierdoor is de interesse voor alternatieven voor het gebruik van biociden de laatste jaren sterk toegenomen. Eén van deze alternatieven zou het gebruik van enzymen die het hoofdbestanddeel van een biofilm, exopolysachariden (EPSsen), afbreken kunnen zijn. Voor het formuleren van efficiënte enzym preparaten is het belangrijk te weten welke EPSsen veelvuldig voorkomen.

Daarom zijn verschillende EPSsen geproduceerd door bacteriën geïsoleerd uit slijmmonsters genomen in Spaanse, Franse en Finse papierfabrieken en onderworpen aan een (gedetailleerde) structuuropheldering. Met behulp van deze studies werd het mogelijk om enkele EPSsen aan te wijzen als belangrijke substraten voor enzymatische afbraak. Het enzym dat één van deze substraten kon afbreken werd opgezuiverd en gekarakteriseerd.

Hoofdstuk 1 geeft in het kort de achtergrond van het onderzoek weer. Naast informatie over het maken van papier wordt er ook ingegaan op problemen ten gevolge van slijmdepositie en de vorming van biofilms in papiermachines. Verder wordt de chemische structuur van EPSsen en het gebruik van enzymen ter voorkoming van slijmproblemen besproken.

Zowel hoofdstuk 2 als 3 behandelen de structuuropheldering van een EPS geproduceerd door een bacterie geïsoleerd uit een slijm monster van een Finse papierfabriek.

In hoofdstuk 2 wordt de structuuropheldering van het EPS geproduceerd door *Brevundimonas vesicularis* sp. beschreven. Met behulp van HPSEC analyse werd aangetoond dat het EPS uit een zuivere polysacharidepopulatie met een molecuulgewicht van 2,000 kDa bestaat. Het EPS bestaat uit rhamnose, glucose, galacturonzuur en glucuronzuur in een molaire verhouding van 1: 1: 1: 1. ^{13}C en ^{1}H hetero- and homonuclear 2D NMR spectroscopie van zowel het natieve als een met zwak zuur behandelde EPS resulteerde in de herkenning van de volgende niet-gesubstitueerde repeterende eenheid.

→4)-α-L-Glc*p*A-(1→4)-α-D-Gal*p*A-(1→4)-β-L-Rha*p*-(1→4)-β-D-Glc*p*(1→

In hoofdstuk 3 wordt de chemische structuur van het EPS geproduceerd door *Methylobacterium* sp. beschreven. Aan de hand van de suikersamenstelling werd een 100% galactaan (EPS) verwacht. FT-IR analyse van het EPS toonde echter de aanwezigheid van pyruvaat aan. De pyruvaatbepaling in combinatie met de suikersamenstelling liet zien dat het EPS bestaat uit een trisacharide repeterende eenheid die gevormd wordt door D-galactopyranose en gepyruvateerd-D-galactopyranose in een molaire verhouding van 1: 2. De structuur van de repeterende eenheid werd vastgesteld met behulp van zowel bindingsanalyse als 2D homo- en heteronuclear ^{1}H en ^{13}C NMR spectroscopie:

→3)α-D-Gal*p*-(1→3)α-D-Gal*p*-(1→3)α-D-Gal*p*-(1→
      4 6   4 6
       ✕   ✕
   (*R*)Py   (*R*)Py
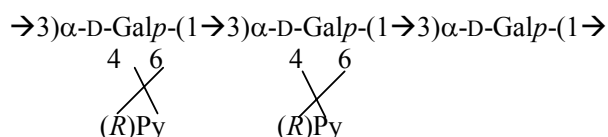
Het kweken van innocula van grond- en compostmonsters in een selectief medium op basis van dit EPS resulteerde in een culture die met behulp van een endo-knippend enzym het EPS geproduceerd door *Methylobacterium* sp. kan degraderen.

Omdat het volledig ophelderen van de chemische fijnstructuur van een EPS erg tijdrovend is, beschrijft hoofdstuk 4 de multivariabele vergelijking van een groot aantal EPSsen op basis van hun suikersamenstelling en FT-IR spectra. Met behulp van deze analyses werd getracht clusters van dezelfde of overeenkomstige EPSsen te vormen. Om dit te bewerkstelligen werden 30 EPSsen uit verschillende papierfabrieken in Finland, Frankrijk en Spanje geanalyseerd op hun suikersamenstelling en FT-IR spectra. Door middel van PCA analyse van de suikersamenstelling werden 4 EPS groepen herkend: een groep met EPSsen geproduceerd door *Enterobacter* en vergelijkbare species vergelijkbaar met het veelvuldig beschreven colanic acid; een groep met EPSsen geproduceerd door *Methylobacterium* met een hoog galactose- en pyruvaatgehalte en 2 groepen met EPSsen die minder compacte clustering vertonen.

Een groep met EPSsen met hoge mannose en/of glucose concentraties geproduceerd door *Bacillus* en daaraan gerelateerde genera en een groep met EPSsen geproduceerd door *Klebsiella* sp. met galactose samen met rhamnose als meest voorkomende suikers.

Analyse van dezelfde EPSsen met behulp van FT-IR gevolgd door DPLS-R en LDA analyse toont aan dat deze methode, mits een goed gedefinieerde kalibratie dataset wordt gebruikt, tijdrovende suikersamenstelling analyses zou kunnen vervangen. Zowel de *Enterobacter* als

Methylobacterium EPSsen konden gemakkelijk worden geclusterd. Dit gold echter niet voor de andere 2 groepen, wat aangeeft hoe belangrijk een grotere en duidelijk gedefinieerde kalibratie dataset is. De potentie van patroonherkenning en -clustering met behulp van FT-IR spectra van EPSsen geproduceerd door micro-organismen geïsoleerd uit papierfabrieken wordt verder besproken.

Van de 30 EPSsen geëvalueerd in hoofdstuk 4 zijn er 18 geproduceerd door bacteriën die behoren tot de *Enterobacteriaceae* familie. Als gevolg van deze resultaten wordt in hoofdstuk 5 in meer detail ingegaan op deze 18 bacteriën. Dezelfde EPSsen werden onderworpen aan meer gedetailleerd onderzoek met betrekking tot identificatie van de micro-organismen op species niveau en het soort EPSsen dat deze micro-organismen produceren. Van deze geïsoleerde bacteriën waren verschillende *Klebsiella* en *Enterobacter* species de meest voorkomende. Zoals op basis van de suikersamenstelling al aangenomen werd in hoofdstuk 4 liet bindingsanalyse en zwak zure behandeling duidelijk zien dat 9 van deze 18 bacteriën het EPS colanic acid produceren.

Uitgaande van de resultaten beschreven in hoofdstuk 4 en 5 wordt in hoofdstuk 6 een colanic acid afbrekend enzym, geïsoleerd uit een cultuurfiltraat, dat werd verkregen door het kweken van een innoculum van een compostmonster in een selectief medium op basis van colanic acid als koolstofbron, beschreven. Gedeeltelijke opzuivering van het enzym resulteerde in een 17-voudige toename in specifieke activiteit. Verdere opzuivering van het enzym met behulp van Native PAGE liet zien dat het enzym deel uitmaakt van een meervoudig eiwitcomplex bestaande uit tenminste 6 individuele eiwitten. De optimale temperatuur voor enzymatische afbraak was 50 ºC terwijl na 1h bij 50 ºC nog 70% van de totale activiteit bewaard bleef. Verder had het enzym een pH-optimum van 7.

Analyse van de degradatieproducten met behulp van PSD/MALDI-TOF MS liet zien dat het enzym een β(1,4)-fucanosyl hydrolase is die repeterende eenheden met variatie in *O*-acetylering vrijmaakt. Voor zowel gede-*O*-acetyleerd en gedepyruvateerd als voor natief colanic acid werden de waardes van K_m en V_{max} bepaald. De affiniteit van het enzym voor de gemodificeerde substraten, gemeten als $1/K_m$, was veel lager dan voor het natieve substraat. Voor het gede-*O*-acetyleerde substraat werd echter een toename in katalytische activiteit gemeten in de vorm van een aanzienlijk hogere V_{max} waarde. Bij hoge concentraties van het natieve substraat werd er substraatremming waargenomen, waarschijnlijk door de vorming van improductieve enzym

substraat complexen. Het verwijderen van de *O*-acetyl groepen voorkomt dit waarschijnlijk waardoor de katalytische activiteit in dit geval toeneemt.

In hoofdstuk 7 worden de resultaten van dit proefschrift bediscussieerd. Gebruik makend van recent verkregen data met betrekking tot de suikersamenstelling en enzymatische afbreekbaarheid van slijmmonsters uit verschillende papierfabrieken wordt het gebruik van EPS afbrekende enzymen ter voorkoming van slijmvorming op papiermachines besproken. Verder wordt in dit hoofdstuk planktonisch geproduceerd EPS en EPS geproduceerd in een biofilm of slijmlaag op papiermachines vergeleken. Tot nu toe hebben deze extra experimenten nog niet aangetoond of het maken van deze vergelijking gerechtvaardigd is.

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Curriculum vitae

René Petrus Verhoef werd geboren op 8 augustus 1977 te Waalwijk. Na het behalen van zijn HAVO diploma aan het Willem van Oranje College te Waalwijk begon hij in 1995 aan de studie Laboratorium Techniek met als specialisatie Levensmiddelenanalyse aan de Internationale Hogere Agrarische School Larenstein te Velp. In het kader van deze studie deed hij een afstudeervak bij het Laboratorium voor Levensmiddelenchemie van de Wageningen Universiteit ('Methode ontwikkeling en validering voor de chromatografische analyse van pectines'). Tevens werd in deze periode ook een onderzoeksstage uitgevoerd bij het voormalige ATO-DLO te Wageningen ('Toepasbaarheid van natuurlijke antioxidanten en gewassen als antioxidant in levensmiddelen'). Na zijn afstuderen in 1999 heeft hij van februari 2000 tot april 2001 als process operator gewerkt bij Givaudan Flavours and Fragrances te Barneveld met als taak de organisch chemische synthese van smaakstoffen.

In april 2001 begon hij aan een promotie-onderzoek onder begeleiding van prof.dr.ir. A.G.J. Voragen en dr. H.A. Schols. Het onderzoek werd uitgevoerd bij het Laboratorium voor Levensmiddelenchemie van de Wageningen Universiteit en de resultaten zijn beschreven in dit proefschrift.

List of Publications

Verhoef, R., P. de Waard, H.A. Schols, M. Rättö, M. Siika-aho, and A.G.J. Voragen, *Structural elucidation of the EPS of slime producing Brevundimonas vesicularis sp. isolated from a paper machine.* Carbohydrate Research, **2002**. 337(20): p. 1821-1831.

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Overview of completed training activities

Discipline specific activities

Courses

VLAG course Advanced Food Analysis, Wageningen (2002).

Summer Course Glycosciences, 7th European Training Course

on Carbohydrates, Wageningen (2002)

2nd Biofilms in Industry, Medicine & Environmental Biotechnology

Euro summer school, Galway, Ireland (2003)

Applied Statistics by Dr. W. Hammers, Wageningen (2002-2003)

Meetings

1st Symposium on Exopolysaccharides from Lactic Acid Bacteria:

from Fundamentals to Applications, Brussels, Belgium, (2001)

11th Gums and Stabilizers for the Food Industry Conference, Wrexham, UK (2003)

Workshop Action Cost E17: Microbiology in Papermaking, Grenoble, France (2004)

X Plant Cell Wall Meeting, Sorrento, Italy (2005)

EU project meetings, 2001-2005

General courses

PhD student week VLAG, 2001,

Food Chemistry PhD trip USA (2002)

Food Chemistry PhD trip Japan (2004)

Career Perspectives Course, Meijer & Meijaard, Wageningen, (2005)

Optionals

Preparation PhD research proposal

Food Chemistry Seminars, 2001-2005, Wageningen University

Food Chemistry Colloquia, 2001- 2005, Wageningen University