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ORIGINAL PAPER

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Polysaccharide-producing bacteria isolated from paper machine slime deposits

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Abstract Development of novel enzymatic methods for slime deposit control in paper mills requires knowledge of polysaccharide-producing organisms and the polysaccharide structures present in deposits. In this work, 27 polysaccharide-producing bacteria were isolated from slime samples collected from different parts of a paper machine. Most of the isolates produced polysaccharides in liquid culture and nine of them were selected for production of polysaccharides for characterisation. The selected isolates belonged to seven different genera: *Bacillus*, *Brevundimonas*, *Cytophaga*, *Enterobacter*, *Klebsiella*, *Paenibacillus* and *Starkeya*. Using ribotyping, partial 16S rDNA sequencing, physiological tests and fatty acid analysis, four of the nine isolates: *Bacillus cereus*, *Brevundimonas vesicularis*, *K. pneumoniae* and *P. stellifer* were identified to the species level. Production of polysaccharides by the selected isolates varied between 0.07 and 1.20 g L^{-1}, the highest amount being produced by *B. vesicularis*. The polysaccharides were heteropolysaccharides with varying proportions of galactose, glucose mannose, rhamnose fucose and uronic acids.

Keywords Paper machine · Slime · Bacterial polysaccharide · Exopolysaccharide · Biofilm

Introduction

The paper manufacturing process is an open system, which provides good conditions (nutrients, suitable pH and temperature) for microbial growth. Population densities in the process waters vary greatly, the normal range being 10^4–10^8 cfu mL^{-1}[1]. The harmful effects of microbes on machine operation are due mainly to their tendency to grow as biofilms attached to surfaces, which leads to slime deposit formation. Slime formation affects machine runnability by causing plugging and fouling of the machinery. Biofilm bacteria are more resistant to antimicrobial agents than free living cells [3], and thus slime deposits act as constant source of contamination, seeding contaminating organisms into circulating waters and even to the final product. A wide variety of microbial species have been isolated from paper machine slimes [5, 11, 13–15,24, 25], with *Bacillus* spp., *Pseudomonas* spp. and enterobacteria among the most commonly detected bacteria.

Microbial polysaccharides are integral components of biofilms. The biofilm matrix structure varies greatly depending on the microbial cells present and the prevailing conditions [21]. The monosaccharides detected in paper machine slimes include galactose, glucose mannose, rhamnose and uronic acids [12]; in some cases fucose has also been reported [9, 24]. Paper machine slime deposits are normally mixed microbial/chemical deposits containing wood fibres and other process water components in addition to biofilm material [7, 16]. The chemical structures of bacterial polysaccharides in paper machine deposits are poorly characterised. According to current opinion, the bacterial species present in biofilms produce exopolysaccharides (EPS) of the same composition as those formed by the same species living in the planktonic state [20, 21].

Due to growing restrictions on the use of biocides, interest in alternative slime control methods has recently increased. Enzymatic methods based on proteinases are already in use [23]. Protein structures are involved especially in the initial stages of biofilm development, the initial attachment often being stabilised by polysaccharides in more mature biofilms [4]. Further development of methods to break down the deposit structures will require better knowledge of polysaccharide structures. In this study, bacteria producing polysaccharides were isolated from paper machine deposits and the polysaccharides were produced in the laboratory for characterisation.

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Materials and methods

Samples

Slime samples were collected from a paper machine producing printing paper at neutral pH and at 40°C by aseptically removing deposits formed on the wet-end surfaces into sterile flasks. Sampling sites were (1) suction roll, (2) spray water tank screens, (3) disc filter, (4), (5), (6) suction boxes, (7) wire frames. A pulp sample was taken from the machine chest.

Isolation of polysaccharide-producing bacteria

The deposits (10 g) were mixed with 90 ml sterile saline and homogenised (Stomacher 400, Seward Medical, London, UK) for 1 min. Dilutions of the homogenised samples were plated on glucose-ammonium agar plates containing (per litre): 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), $MgSO_4 \cdot 7H_2O$ (0.3 g), $CaCl_2$ (0.05 g), $ZnSO_4 \cdot 7H_2O$ (0.2 mg), $CuSO_4 \cdot 5H_2O$ (0.2 mg), $MnSO_4 \cdot H_2O$ (0.2 mg), $CoCl_2$ (0.2 mg), $FeSO_4 \cdot 7H_2O$ (0.6 mg), agar (20 g). The plates were incubated at 30°C and the total bacteria and the amount of bacteria forming slimy colonies were calculated after 7 days. Slimy bacteria were picked from the plates and purified by serial streaking.

Characterisation and identification of the isolates

The purified isolates were characterised by ribotyping using the automated RiboPrinter System (DuPont Qualicon, Wilmington, Del.) following the manufacturer's instructions [2]. The first choice for restriction enzyme was *Eco*RI (Qualicon), because the identification database (DUP) provided by the manufacturer was based on patterns generated by this enzyme. If the DNA was not digested by this enzyme, the second choice was *Pvu*II (Qualicon) and the third *Pst*I (Qualicon). One isolate from each ribogroup was deposited in the VTT Culture Collection and the isolates not identified by DUP [Version 12.2.(c)2000 Qualicon, last update in June 2002] or VTT RiboPrint databases were subjected to further identification at the identification service of DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany), where partial 16S rDNA sequencing, physiological tests and FAME (fatty acid analysis) were performed [18].

Production of polysaccharides

The isolates were cultivated for 5 days at 30°C in shake flasks. The liquid medium had the same composition as the glucose ammonium agar. Culture medium viscosities were measured at 20°C using a Brookfield DV-II viscometer. NaCl (0.9%) was added to the culture medium, which was lightly homogenised for 30–45 s and centrifuged chilled at 14,700 *g* for 45 min to separate the cells. The polysaccharides were precipitated by adding three volumes of ice-cold ethanol (95%) to the culture filtrates, solubilised in water, treated with proteinase (Neutrase, Novo Nordisk, Espoo, Finland; 100 μL mL^{-1}, 1 h at 37°C), reprecipitated with ethanol, solubilised in water, dialysed and freeze-dried.

Chemical analysis

The total carbohydrate content of the precipitated polysaccharides was determined by the phenol-sulphuric acid method [6], using glucose as standard. The monosaccharide composition was determined by HPLC [8] after hydrolysis with 2 *N* H_2SO_4 at 100°C for 3 h.

Results

Isolation of slimy bacteria and production of polysaccharides

Total amounts of bacteria liberated from slime deposits collected from wet-end surfaces were in the range of 10^6–10^8 cfu g^{-1}, and the counts of slimy bacteria varied from 2×10^2 to $>10^7$ cfu g^{-1} (Fig. 1). In most samples, the count of slimy bacteria was from one to two orders of magnitude lower than the total count. The sliminess was often lost during serial streaking of the isolates on isolation medium: only ca. 30% of slimy colonies picked from the original plates retained their slimy colony morphology when grown as pure cultures.

Slimy bacteria were isolated from all the sampled parts of the paper machine. A total of 27 slimy isolates were obtained. In liquid cultures the increase in culture medium viscosity caused by the production of EPS by

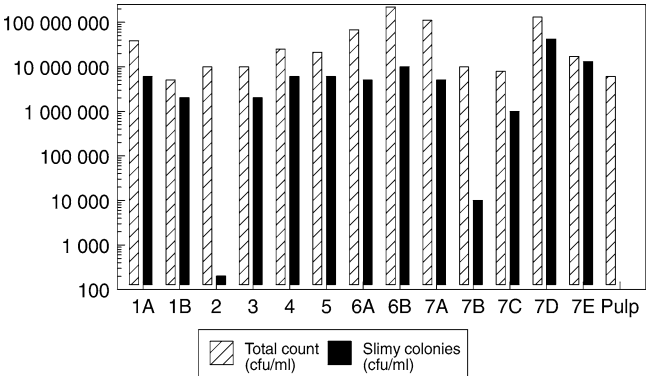

Fig. 1 Counts of total viable aerobic bacteria and slimy aerobic bacteria in the slime deposits

Table 1 Production of polysaccharides by the slimy isolates in liquid cultures: increase in culture medium viscosity (initial viscosity of the culture medium was 1.1 cP), and polysaccharide yield after ethanol precipitation

Isolate	Origin	Sliminess on plates[a]	Viscosity increase (cP)	Polysaccharide yield (total carbohydrates g L^{-1})
1 I	Suction roll	+ +	2.5	0.88
1 II		+ + +	0.3	nd[b]
1 III		+ + +	0.6	nd
1 IV		+	0.1	nd
1 V		+ + + +	1.9	0.31
2 I	Spray water tank screens	+ + +	0.9	nd
3 I	Disk filter	+	0.1	0.43
3 II		+ + +	35.4	1.20
4 I	Suction box	+ + + +	1.5	0.08
4 III		+ +	2.2	0.14
4 IV		+ +	0.1	nd
5 I		+	0.2	nd
6 A I		+	0.3	nd
6 A II		+ + +	0.2	nd
6 A III		+ + +	7.2	0.88
6 A IV		+ +	0.6	nd
6B I		+ +	0.7	nd
6B II		+ + +	0.5	nd
6B III		+ +	0.6	nd
7A I	Wire frames	+ +	0.6	nd
7A II		+ + +	5.8	1.05
7D I		+ + + +	6.8	0.07
7D II		+ + + +	12.6	0.09
7D III		+	0.2	nd
7D IV		+ +	0.3	nd
7E I		+ + +	2.0	0.58
7E II		+	1.0	nd

[a] + Shiny appearance, + + somewhat slimy, + + + clearly slimy, + + + + very slimy
[b] Not determined

the slimy isolates varied from 0.1 to 35.4 cP (Table 1). Slimy colony morphology on plates and viscosity increase in liquid medium did not always exhibit good correlation. However, high (> 5 cP) viscosities were produced only by strains rated clearly slimy or very slimy on plates. Ten isolates causing a viscosity increase of more than 1 cP were selected for further study. In addition, isolate 3 I, originally isolated together with 3 II as a very slimy colony, was included. From 0.07 to 1.20 g L^{-1} polysaccharides were obtained in ethanol precipitation from the culture filtrates of the selected isolates. The highest amount of polysaccharide was produced by isolate 3 II.

Characterisation and identification of the polysaccharide-producing isolates

The selected isolates were further characterised by ribotyping (Fig. 2). Isolates 3 II and 6A III were not digested with *Eco*RI; *Pvu*II was suitable for 6A III and the best digestion of 3 II was obtained with *Pst*I. The isolates 7D I and 7D II, as well as 7A II and 7E I matched to the same ribogroups, respectively, indicating identical isolates. The current available RiboPrint-databases (DUP and VTT) reliably identified isolate 1 I as *Klebsiella pneumonie* (similarity 0.98) and isolate 3 I as *Bacillus cereus* (similarity 0.92). Based on partial 16S

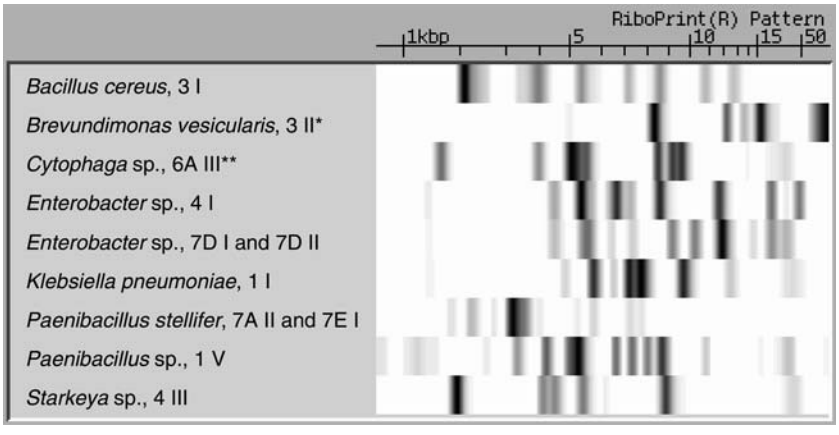

Fig. 2 Characterisation of the polysaccharide-producing isolates by ribotyping using the restriction enzymes *Eco*RI, *Pst*I (*) or *Pvu*II (**)

Table 2 Identification of the polysaccharide-producing isolates

Isolate	VTT-code	Identification			Final result
		Ribotyping highest similarity	Partial 16S rDNA sequence highest similarity	Physiological tests	
3 I	E-981023	0.92% *Bacillus cereus*	100% *B. cereus/anthracis*	*B. cereus*	*Bacillus cereus*
3 II	E-981024	No ID	99.7% *Brevundimonas vesicularis*	*B. vesicularis*	*Brevundimonas vesicularis*
6A III	E-981028	No ID	94.5% *Cytophaga columnaris* = *Flavobacterium columnare*	Point to *Flavobacterium*	*Cytophaga* sp., a new species
4 I	E-981026	0.66% *Enterobacter cloacae*	97.4% *Enterobacter hormaechei*/ 97.1% *E. cloacae*	*E. asburiae/hormachei*	*Enterobacter* sp., a new species
7D II	E-981031	0.89% *Enterobacter cloacae*	97.6% *E. hormaechei*/96.9% *E. cloacae*	*E. cloacae*	*Enterobacter* sp., a new species
1 I	E-981021	0.98% Klebsiella pneumoniae	Not done	Not done	*Klebsiella pneumoniae*
1 V	E-981022	No ID	94.5% *Paenibacillus kobensis/granivorans*	Point to *Paenibacillus*	*Paenibacillus* sp., a new species
7A II	E-981029	0.73% *Paenibacillus stellifer*	99.0% *P. stellifer*	*P. stellifer*	*Paenibacillus stellifer*
4 III	E-981025	No ID	96.9% *Starkeya novella*	Point to *Starkeya*	*Starkeya* sp., a new species

Table 3 Monosaccharide composition (% of identified) of the extracellular polysaccharides (EPS) produced by bacteria isolated from slime samples. *Fuc* Fucose, *Rha* rhamnose, *Ara* arabinose, *Gal* galactose, *Glc* glucose, *Man* mannose, *GlcA* glucuronic acid, *GalA* galacturonic acid

Bacterium	VTT code	Fuc	Rha	Ara	Gal	Glc	Man	GlcA	GalA	$\sum$
Bacillus cereus	E-981023	< 0.1	0.2	5.9	13.5	64.6	15.8	< 0.1	< 0.1	100
Brevundimonas vesicularis	E-981024	< 0.1	27.8	< 0.1	7.2	37.1	1.6	15.9	10.4	100
Cytophaga sp.	E-981028	6.2	< 0.1	< 0.1	20.6	70.6	2.0	0.3	0.3	100
Enterobacter sp.	E-981026	18.6	3.9	< 0.1	24.6	25.5	16.7	2.9	7.8	100
Enterobacter sp.	E-981031	10.4	1.9	< 0.1	39.9	27.8	7.0	12.4	0.6	100
K. pneumoniae	E-981021	< 0.1	< 0.1	< 0.1	32	48.0	7.2	10.9	1.9	100
Paenibacillus sp.	E-981022	< 0.1	24.4	< 0.1	2.9	29.5	38.1	5.1	< 0.1	100
Paenibacillus sp.	E-981029	< 0.1	0.2	< 0.1	22.5	59.7	15.6	2.0	< 0.1	100
Starkeya sp.	E-981025	2.4	2.1	< 0.1	2.4	77.2	14.1	0.9	0.9	100

rDNA sequence and other properties, isolate 3 II was identified as *Brevundomonas vesicularis* and isolates 7A II and 7E I as the recently described *Paenibacillus stellifer* (Table 2). The phylogenetic relationships of the other isolates were so distant from the closest known species that it was impossible to identify them to the species level. One of these belonged to the genus *Paenibacillus*, two to the genus *Enterobacter*, one to *Stackeya* and one to *Cytophaga*.

Polysaccharide composition

The polysaccharides isolated from the culture filtrates of the selected isolates were all heteropolysaccharides with varying proportions of neutral sugars and uronic acids (Table 3). Glucose, galactose and mannose were detected in all samples. The highest uronic acid content was in the *Brevundimonas vesicularis* polysaccharide. The polysaccharides of the two *Enterobacter* spp. were characterised by the presence of fucose, glucose, galactose, mannose and uronic acids. Fucose was also detected in the polysaccharides of *Cytophaga* sp. and *Starkeya* sp. Galactose, glucose and mannose were the main components of polysaccharides of *Bacillus cereus* and *P. stellifer*, and the polysaccharide of *Paenibacillus* sp. contained rhamnose, glucose and mannose.

Discussion

Enzymes that degrade bacterial polysaccharides are potential tools in slime deposit control in paper mills. This study was aimed at increasing knowledge of polysaccharide-producing organisms and on the polysaccharide structures present in deposits to exploit in the development of novel enzymatic methods.

Slime-forming bacteria were frequently obtained from paper machine deposits, representing approximately 1–10% of the total counts. The counts probably underestimate the numbers of bacteria present in slime deposits due to the inadequacy of the homogenisation method used to totally break up the biofilm structures in deposits as well as the selection effect of the laboratory

media and cultivation conditions used. On the basis of ATP measurements and electron microscopy, total bacterial numbers in paper machine slimes have been estimated to be in the range of 10^{10} –10^{12} g^{-1} [24]. Signature lipid biomarker analysis also revealed population numbers around 10^{12} g^{-1} in slimes, 1,000 times higher than the counts obtained with conventional counting methods [22].

The EPS produced by the slimy isolates were heteropolysaccharides composed of varying amounts of the monosaccharides fucose, rhamnose, galactose, glucose, mannose and glucuronic acid. These sugars have been reported as the main constituents of bacterial polysaccharides in paper machine slimes [9, 24]. In addition to the major monosaccharide components, minor amounts of other sugars were detected in almost all polysaccharide samples. These may be impurities liberated from the cells or be derived from the small amount of yeast extract in the medium, or may indicate production of more than one polysaccharide by the isolates.

The polysaccharide-producing isolates identified belonged to seven different genera: *Bacillus*, *Brevundimonas*, *Cytophaga*, *Enterobacter*, *Klebsiella*, *Paenibacillus* and *Starkeya*. Of these, *Bacillus*, *Cytophaga*, *Enterobacter* and *Klebsiella* have also been identified as components of slime flora in earlier studies [5, 14, 17, 24]. *P. stellifer* has been described as a dominant contaminant in food packaging paperboards [19]. On the basis of the analysed monosaccharide compositions, different polysaccharides were produced by the different isolates. Fucose was present in the polysaccharides of *Enterobacter* isolates; one earlier study on slimes from Finnish paper machines also reported fucose in the polysaccharides of enterobacteria [24]. *Brevundimonas vesicularis*, the most efficient polysaccharide producer of the slimy isolates, originated from a deposit collected from the disk filter. Structural elucidation by mass spectrometry and NMR has revealed that the polysaccharide of this *Brevundimonas vesicularis* strain is a linear polymer with a repeating unit containing rhamnose, glucose, glucuronic acid and galacturonic acid [26]. *Bacillus cereus*, originally isolated from the disk filter slime as a co-culture with *Brevundimonas vesicularis*, did not appear slimy on plates or viscous in liquid culture, although some EPS was isolated from the culture broth. It has been reported that *Bacillus* species are not primary biofilm formers in paper machines but that their presence in slimes relates to their ability to colonise existing biofilms [10].

The characterisation of the slimy isolates demonstrated the fact that microbes adapted to the special conditions of paper machines often do not sufficiently resemble earlier described species to permit their identification. Thus, identification is difficult and time consuming work, demanding several methods. Automated ribotyping with the RiboPrinter System is a reliable and rapid method for producing genetic fingerprints of different bacterial isolates. However, identification of the fingerprint patterns requires a large database. A RiboPrint database for bacteria isolated from the pulp and paper industry is being constructed at VTT.

The polysaccharides produced by the isolates will be used for induction of specific polysaccharase enzymes. The effects of these enzymes on biofilms and on slimes in actual process conditions will help to clarify the role of bacterial polysaccharides in biofilms and increase understanding of biofilm build-up in paper machine environments. Detailed characterisation of the polysaccharides is also in progress.

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