Xylan degradation by the anaerobic bacterium *Bacteroides xylanolyticus.*

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Xylan degradation by the anaerobic bacterium *Bacteroides xylanolyticus*.

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1. Voor een beter begrip van de regulering van de xylanasevorming is het nodig fysiologische studies te combineren met in vivo genetisch werk.

2. Het is te simplistisch om te beweren dat de hemicellulolytische enzymen van *Fibrobacter succinogenes* constitutief geproduceerd worden.
Malburg et al. 1992 In: Microbial degradation of natural products (Winkelmann ed.) pp127

3. Het taalgebruik van Britse wetenschappers bevordert het begrip van de Engelstalige wetenschappelijke literatuur niet.

4. Het lijkt erop dat de hervormingen die nodig zijn om te voldoen aan de voorwaarden voor de Euro, Europa eerder verdelen dan verenigen.

5. De conclusie dat de cellulose depolymeriserende activiteit van de SFGF-peptide van niet-enzymatische aard is, is voorbarig.
Liu and Gao, 1996, ACS symp. 655:166

6. Literatuurwaarden voor glycanase-activiteiten, bepaald door de vorming van reducerende suikers te meten, zijn niet vergelijkbaar.

7. Het getuigt niet van emancipatie, dat trouwen voor een vrouw in Duitsland al voldoende kan zijn om met Frau Doktor aangesproken te worden.

8. Een kind komt sneller ter wereld dan een proefschrift.

9. Het is voor een Amerikaans bedrijf moeilijk te begrijpen dat de Europese Unie slechts een verzameling van landen is, ieder met afzonderlijke voorschriften en regels.

10. De schaakwedstrijd tussen Deep Blue en Kasparov had beter uitgezonden kunnen worden in het reclameblok.

11. De beslissing om 250.000 gezonde pasgeboren biggen te vernietigen, doet vermoeden dat er in Den Haag te veel rundvlees wordt gegeten.

12. Beter laat dan nooit.

Stellingen behorende bij het proefschrift "Xylan degradation by the anaerobic bacterium *Bacteroides xylanolyticus*".

Philippe Schyns — Wageningen, 9 Juni 1997

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Chapter 1

General Introduction

THE BACTERIAL DEGRADATION OF XYLAN

n interest in transforming vast reserves of renewable plant biomass to fuel and chemical feedstock was generated by the oil crisis of the mid seventies. Lignocellulose represents an important renewable energy source. Forestry, agricultural and food residues represent an immense resource for the production of a variety of feedstock chemicals (85). The conversion of wood, municipal wastes or agricultural residues to fermentable sugars is accomplished by hydrolysing the polysaccharide components in the lignocellulosic fractions. This can be done by either thermochemical or biological means. The soluble sugars can than be fermented to desired end products, like ethanol. Some type of pretreatment is generally required to render the carbohydrate fraction accessible to enzymatic and microbial attack, because such materials are only partially degradable in their native form. The annual production of cellulose, the major component of biomass, is estimated at around 8.5×10^{10} tons (35). After cellulose, hemicellulose is the second most abundant source of renewable organic material available in nature.

An important part of the microbial carbon cycling is the biodegradation of plant polymers. In nature, a large number of organisms cooperate in the efficient recycling of carbon of lignocellulose. Lignocellulose, because of its complex structure, is relatively slowly degraded biologically. Therefore, extensive research activities have focused on the biochemistry and the genetics of enzyme systems which are involved in the degradation of lignin, cellulose and hemicellulose. The combination of approaches based on molecular genetics, biochemistry and structural biology has led to a fast accumulation of data about the structure and function of xylanolytic enzymes in the past 5 years.

Hemicellulose : Composition and structure

Plant cell walls is the major reservoir of fixed carbon in nature. The cell wall of terrestrial plants is a composite material, called lignocellulose, in which cellulose, hemicellulose and lignin are in tight association. The close association of hemicellulose with cellulose and lignins contributes to cell wall rigidity and flexibility. The name hemicellulose was first introduced by Schulze in 1891 for the fractions extracted from plant materials with dilute alkali (116). It is a collective term for a group of polysaccharide found in higher terrestrial plants. Hemicelluloses can generally be defined as non-cellulosic cell wall polysaccharide. Cellulose is a homopolymer of up to 14000 β-1,4-linked glucosyl residues and occurs naturally mainly in crystalline form. Lignin is a complex aromatic polymer consisting of different phenylpropane units connected to each other by both ether and carbon linkages

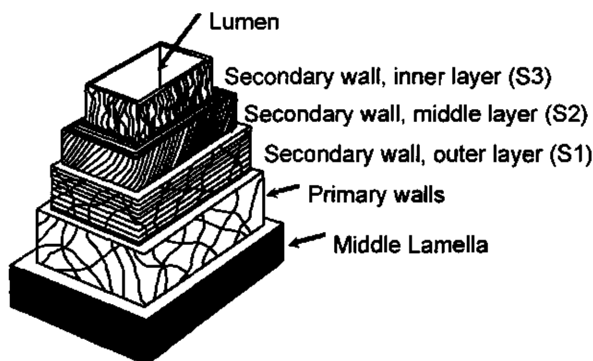

Figure 1. Schematic representation of a plant cell wall (adapted from ref. T3). Each layer has a different fiber orientation

(69). It is now generally accepted that a part of the lignin is chemically linked to the cell wall hemicelluloses (140). It was shown that in softwood acetylglucomannan and β-1,4-galactan were bound to lignin through their primary hydroxyl group, while arabinoglu-curonoxylan was linked through the C-2 and C-3 positions of the xylan main chain. Ferulic

acid residues of lignin were shown to be bound to the *O*-5 position of L-arabinofuranosyl of hemicellulose of grasses (57,73). The interactions between cellulose and hemicellulose in plant cell walls are believed to be of non covalent nature (137). Hemicellulose interacts with cellulose as well as to itself by hydrogen bonds. This is thought to stabilize the cell wall matrix and renders the structure insoluble to water. Hemicelluloses compared with cellulose are low molecular weight polymers (approximately 20 kDa) (2). Hemicellulose can be classified in four major groups: xylans; mannans; arabinogalactans and arabinans (Table 1).

Table 1. Polysaccharides in four different lignocellulosic materials.

	Cottonwood	Pine	Straw	Bagasse
	(Percentage based on dry raw material)			
Glucan	43.4	42.4	31.9	38.8
Xylan	13.0	5.9	19.0	21.4
Mannan	2.0	11.0	0.2	0.2
Arabinan	0.3	1.3	2.1	1.4
Galactan	0.3	2.3	0.6	0.3
Total	58.3	62.9	53.8	62.5

(Data from ref. 108)

Hemicellulose can represent up to 48% of the dry matter of plant cell walls (25). Hardwoods are composed of about 50% cellulose, 23% hemicellulose and 22% lignin. Herbaceous materials and agricultural residues generally contain a higher proportion of hemicellulose (up to 33%). Most hemicelluloses occur as heteropolysaccharides containing different carbohydrate residues (D-xylose, L-arabinose, D-mannose, D-glucose, D-galactose, D-glucuronic acid, D-galacturonic acid) in the backbone chain and/or side chains, thus generating extremely variable polysaccharides (105). The predominantly alkali-soluble polysaccharide of primary (young) monocot cell walls are composed of (4-O-methyl)glucuronoarabinoxylans and to a lesser extent of mixed-linked glucan (144). Xyloglucans form the major hemicellulosic polymer of primary dicot cell wall (22).

Substantial amounts of additional hemicellulose are laid down during secondary thickening of plant cell walls (Fig. 1). In both monocots and dicots these are predominantly glucuronoarabinoxylans. These contain a lower level of substitution with glucuronic acid and arabinose residues compared to the polymer formed by the primary monocot wall (24). Glucuronoarabinoxylans from both primary and secondary walls are substituted with ester-linked non-carbohydrate compounds, notably with acetyl groups and phenolic acid residues.

The chemical structure of xylans.

Xylan, one of the major components of hemicellulose, occurs widely in nature and is probably present in all higher land plants (40). It is a β-1,4-linked polymer of xylose substituted with side chains of other pentoses, hexoses, some short oligosaccharides, uronic acids, cinnamate-based esters, and acetyl esters (Fig. 2 and Table 2) (2,127,128).

Table 2. Composition of xylans isolated from three different plant types.

Polysaccharide	Source	Percentage of dry weight	Composition	Ratio
Arabinoxylan	Grasses	20-40	Xyl:Araf: Glc:fer: pCou:Ac	varying depending on tissue
O-acetyl-4-O-methyl-glucuronoxylan	Hardwoods	10-35	Xyl:mGlc: Ac	10:1:7
Arabino-4-O-methylglucuronoxylan	Softwoods	10-15	Xyl:mGlc: Araf	10:2:1.3

Xyl=xylose, Araf=arabinofuranose, mGlc=4-methyl-glucuronic acid, fer= ferulic acid, pCou=p-coumaric acid, Ac=acetic acid.

(Data from ref. 108)

Xylan structure varies according to the botanical origin. However, the composition can also vary within a single plant, depending on its origin of tissue and cell, with age and state of growth and with the conditions under which the plant grows (2,23,127,128,144). The

glucuronoxylans which are found in hardwoods and graminaceous plants, are composed of β-1,4-linked β-D-xylopyranosyl units forming the backbone of the polymer.

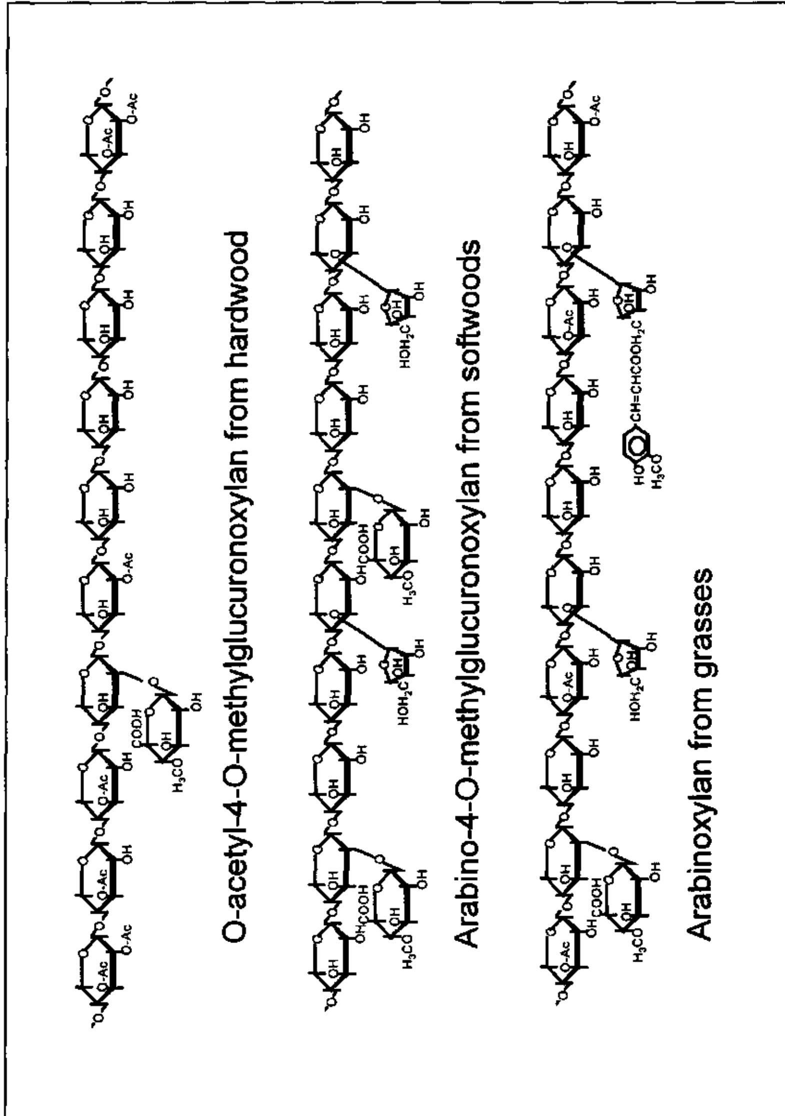

Figure 2: Structure of different types of xylan.

About 10% of the xylosyl residues are substituted by 1,2-linked α-D-glucuronic acid or 4-methyl-α-D-glucuronic acid. About 60% to 70% of the xylose residues in the glucuronoxylans of hardwoods are acetylated. Grass xylans are also acetylated. The *O*-acetylated groups are located at the C-3 or C-2 of the xylopyranosyl residue. Softwood xylans and xylans from most graminaceous plants have single L-arabinofuranosyl units attached through α linkages to some O-3 positions of the main chain (144).

The amounts of feruoyl and p-coumaric acids linked via L-arabinose residues in xylan is relatively small. In barley straw arabinoxylan every 15th arabinose unit is estimated to be esterified with ferulic acid, and every 31st with p-coumaric acid (100). The coumaryl and feruloyl moieties are thought to anchor the xylan chains to the lignin matrix by cross-linking the two polymers (40,100). The degree of substitution of xylose-backbone chains varies greatly and has a major influence on the solubility, functionality and degradability of the xylan. The more substituted xylan cannot form hydrogen bonds with itself and becomes more soluble in water.

Xylanolytic Bacteria

Hemicellulosic compounds are subject to degradation by various fungal and bacterial populations. The presence of microorganisms that are able to degrade xylan, was reported over 100 years ago by Hoppe-Seyler (63), who described a gas production by river mud microbes using wood xylan suspensions. Bacteria play a role in both aerobic and anaerobic degradation of hemicelluloses. Bacteria degrading xylan have been frequently isolated from compost, litter, soils, and sludges, the rumen system and other gastro-intestinal tracts. Some plant pathogenic *Pseudomonas* sp., *Xanthomonas alfalfae*, and *Achromobacter* species have been isolated that also produce hemicellulases as a part of their enzyme system (89,109). A large number of actinomycetes and *Bacillus* species isolated from soils and compost can degrade xylans (95,146). Herbivorous higher animals and insects depend on microbial activity in some part of their gut for the conversion of polysaccharide of plants to substances which the animal can metabolize (62). Hemicellulolytic *Aerobacter,*

Aeromonas, Bacteroides, Butyrivibrio, Ruminococcus and *Clostridium* species have been isolated from human and animal intestine (110,114,155). Anaerobic *Clostridia* species have been isolated from mesophilic and thermophilic methane sludges (126).
Plant cell walls are particular resistant to microbial degradation. Physical access to the polymer can be restricted by the surrounding lignocellulosic components. Most xylanolytic microorganisms grown under natural conditions produce both cellulolytic and xylanolytic enzymes to efficiently degrade plant cell walls. However, some of them, like *Fibrobacter* and *Ruminococcus*, are not able to grow on xylose, the degradation end-product of xylan (31). This means that growth of these organisms on xylan is poor. It has been shown recently that some of these bacteria are able to utilize short xylo-oligomeric substrates (26). Despite the fact that xylan never occurs in nature in the absence of cellulose, there are microorganisms that produce only xylanolytic enzymes. Some of these organisms are endo-(1,4)-β-glucanase positive but they are unable to grow on cellulose or its soluble derivatives. It is also possible that some xylanases show cellulase activity (5,46). In general, non-cellulolytic micro-organisms, possessing some glucanase activity, are unable to hydrolyse crystalline cellusoses.

Anaerobic bacterial hemicellulose degradation.

Anaerobic cellulolytic and hemicellulolytic bacteria are responsible for the initial stage in the overall conversion of lignocellulosic material to methane and carbon dioxide (52). The anaerobic digestion process may be thought in simple terms to be composed of three steps, which include hydrolysis of polymeric substrates to monomers, fermentation of monomers to organic acids, hydrogen and carbon dioxide and conversion of organic acids, hydrogen, and carbon dioxide to methane. For highly polymeric feedstocks the rate limiting step in the overall anaerobic digestion has been identified as the hydrolysis of polymers to soluble monomers (16,101). For a thorough comprehension of anaerobic digestion it is therefore important to obtain a better insight into the microbiology of hydrolytic bacteria. Anaerobic degradation of hemicellulose occurs in a variety of anaerobic biota, such as manure, compost, sludges of waste water treatment plants and marine or freshwater sediments. In

addition, the hydrolysis of xylan by anaerobic microorganisms present in the rumen and gastro-intestinal tract plays an essential role in the nutrition of herbivorous animals. Most of our knowledge with respect to anaerobic digestion of plant material originates from studies of rumen fermentation (90). The rumen flora harbors a variety of anaerobic bacteria utilizing plant cell wall polysaccharide. Their xylanolytic activities play an important role in the overall rate of degradation of plant cell wall material in the rumen system. *Ruminococcus, Butyrivibrio, Bacteroides, Prevotella* and *Fibrobacter* species are rumen bacteria with xylanolytic activities (90). Several xylanolytic enzymes produced by these organisms have been identified and characterized and the genes of some xylanolytic enzymes from *Fibrobacter succinogenes, Prevotella (Bacteroides) ruminicola, Butyrivibrio fibrisolvens, Ruminococcus albus, and Ruminococcus flavefaciens* were recently cloned (38,43,82,83,90,92,93,104, 135,152,153). Xylanolytic enzymes of microorganisms have received much attention over the last ten years mainly due to their potential biotechnological application. The best studied anaerobic xylanolytic bacteria are presented below.

Fibrobacter succinogenes is a predominant rumen cellulolytic, gram-negative, rod-shaped bacterium. It grows optimally between pH 6 and 7 and a temperature between 37°C and 40°C. It can ferment cellulose, cellobiose, starch, glucose, dextrin, pectin, maltose, and trehalose to succinate, acetate formate and CO_2 as the main products. It cannot grow on xylose. Cellulosome type of structures have been detected (4). The organism produces several endoglucanases, cell bound exoglucanase and cellobiase, periplasmic cellodextrinase and at least four xylanases as well as an acetylxylan esterase (90,91). Furthermore glucuronidase-, arabinofuranosidase- and ferulic acid esterase activities were detected (90,96,124).

Ruminococcus albus and *Ruminicoccus flavefaciens* are strictly anaerobic, cellulolytic, gram-positive/variable cocci that are important in the degradation of plant cell wall material in the rumen of cattle and sheep. Most isolated strains ferment cellulose, xylan and pectin and all ferment cellobiose. The main fermentation products are acetate, formate, ethanol (*R. albus*), succinate (*R. flavefaciens*), CO_2 and H_2 (90). *R. flavefaciens* can degrade the highly ordered crystalline cellulosic materials, *R. albus* cannot. *R. flavefaciens*

cells produce both xylanases and cellulases. Both *Ruminococci* produce structures resembling cellulosomes (4).

Butyrivibrio fibrisolvens are strictly anaerobic, curved rod-shaped bacteria. It has a gram-positive cell wall structure, which stains gram-negative, because of its thin cell wall. Most strains ferment xylan, pectin, arabinose, glucose, fructose, and galactose. Some strains also ferment cellulose, cellobiose, starch, maltose and/or xylose. The main fermentation products are acetate, butyrate, formate, lactate, ethanol, CO_2 and H_2 (90). Genes encoding for an endoglucanase, cellodextrinase, ß-glucosidase, two xylanases and a dual ß-xylosidase/α-L-arabinofuranosidase were cloned (83,93,135).

Prevotella (Bacteroides) ruminicola are strictly anaerobic, gram-negative, rod-shaped bacteria found in large amounts in the rumen. Depending on the strain they can ferment starch, xylan, pectin, maltose, cellobiose, xylose and arabinose. All ferment glucose, fructose, galactose and lactose. The main fermentation products are acetate, propionate, succinate and formate (90). Endoglucanase, ß-xylosidase, α-L-arabinofuranosidase and xylanase genes were cloned (43,90).

Thermophilic anaerobic xylan degrading bacteria include:

Clostridium thermocellum Several related clostridial species are thermophilic, cellulolytic bacteria which grow at pH 7 and 60°C. They are able to ferment cellulose, cellobiose and several other monomeric sugars. Although *C. thermocellum* degrades xylan, the resulting xylose and xylobiose are not fermented (143). The organism produces a complex of cellulolytic enzymes organized in so called cellulosomes (4,5). These are cell bound during growth. These complexes also may contain one or several xylanases (4).

Clostridium stercorarium and *Clostridium thermolacticum* are closely related to each other and belong to one cluster of *Clostridia* together with *C. thermocellum* (28). *C. thermolacticum* ferments xylan, starch, cellobiose, glucose and xylose, but not arabinose. Depending on the strain cellulose is fermented as well. One xylanase of this organism is well characterized (29,30). Several xylanolytic enzymes and cellulolytic were isolated from cultures of the cellulolytic thermophilic anaerobe *C. stercorarium*. Three xylanases from

C. stercorarium were purified and characterized (6). Two xylanase, six ß-xylosidase and α-L-arabinofuranosidase genes were sequenced and/or cloned (41,113,117).
Thermoanaerobacterium saccharolyticum is a thermophilic anaerobic bacterium growing in the pH range of 4 to 6.5 and up to temperatures of 66°C. From one *Thermoanaerobacterium* strain a xylanase, two xylosidases, two acetyl xylan esterases and a α-glucuronidase were identified recently (120,121).

Biochemical properties of xylanolytic enzyme systems

Because of the complex nature of the polymer, complete hydrolysis of xylan requires the action of several hydrolytic enzymes. Not only enzymes that attack the internal *β*-1,4-xylosidic bond, called endo-*β*-(1,4)-xylanases (EC 3.2.1.8) are required. Enzymes that hydrolyse the substituents and *β*-xylosidases (EC 3.2.1.37) that convert the small xylooligosaccharides produced by the endo-xylanases into xylose, are equally essential (Fig. 3). Not all xylosidic linkages in the hemicellulose are equally accessible to the endo-xylanases. The substituents on the xylose backbone, can prevent the hydrolysis of particular xylose-linkages. During xylan hydrolysis, the accessibility of some linkages change due to the action of enzymes involved in the removal of the substituents (105). Enzymes involved in the hydrolysis of xylan substituents are acetyl esterases (EC 3.1.1.6), *α*-L-arabinofuranosidases (EC 3.2.1.55) and *α*-glucuronidases (EC 3.2.1). Furthermore, esterases can be involved in hydrolyzing the bond between xylan and lignin, such as feruloyl esterases and coumaryl esterases.
The enzymes are classified according to the substrates they act upon, by the bonds they cleave and their patterns of product formation. The xylanolytic enzymes are mostly characterized by their action on defined substrates. It has to be kept in mind that their natural substrate can differ considerably from the commercially available substrates. For example, xylans are often acetylated or otherwise esterified. The most common method for xylan recovery - solubilization in alkali - readily cleaves ester linkages. Deacetylation makes the xylans much less soluble in water, but generally makes the substrate more susceptible to

enzymatic attack. Because of the heterogeneous properties of the xylans in nature, the different xylanolytic enzymes will exhibit the ability to hydrolyse the different substrates to varying extents. Combined with the difficulties encountered in isolating and characterizing defined homogeneous substrates, the substrate specificities of xylanolytic enzymes are often very difficult to define. Many xylanolytic organisms produce a whole array of xylanolytic enzymes involving several debranching enzymes and multiple xylanases each with their own specificity. The degree of substitution of a particular type of xylan and the nature of the substituents may favor the action of specific hydrolases over others (43,60,61,

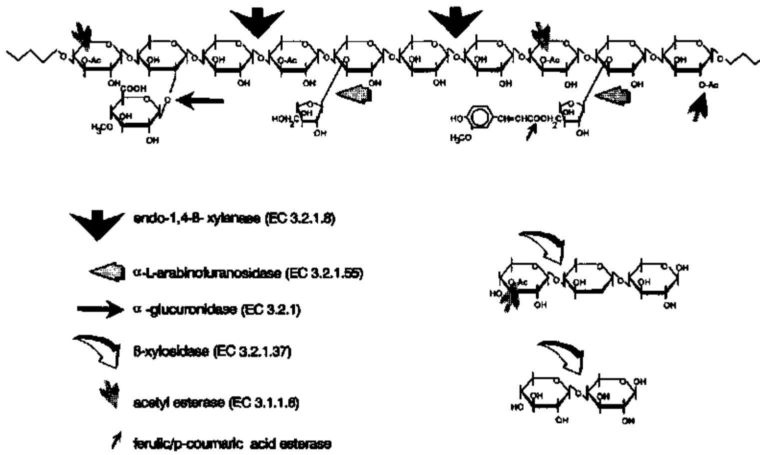

Figure 3. Schematic representation of the sites of action of the enzymes involved in xylan degradation (redrawn from ref. 8).

82,83,84,90,91,93,94,96,117,124, 135,146,154). With a few exceptions xylan- degrading micro-organisms are cellulolytic and often produce complex mixtures of cellulolytic and xylanolytic enzymes.

Xylanases

The most important enzymes in the xylan degradation are the endo-1,4-ß-xylanases (1,4-ß-D-xylan xylanohydrolases, EC 3.2.1.8), which hydrolyse β-1,4-glycosidic linkages between xylopyranose units. From all the xylanolytic enzymes, by far most is known about endo-1,4-ß-xylanases. A very large number of reports on the production, properties and applications of these enzymes has been published in the last 25 years. The characteristics of xylanases from plant, bacterial and fungal sources have been described in detail in several books and review articles (27,33,111,139,146). Xylanases from different microbial origin have been purified and they are almost exclusively single subunit proteins with molecular weight values ranging from 10 to 90 kDa (146). The smallest endoxylanase (5.5-6.0 kDa) was isolated from *Chania* sp. (3). Larger xylanases with molecular weight of 130 to 180 kDa were isolated from thermophilic anaerobic *Thermoanaerobacterium* species (80,120). Wong and coworkers noted that many endoxylanases can be classified according to their pI and molecular weight. A distinction can be made between those with M_r values of less than 30 kDa which are usually basic proteins, while those with M_r values higher than 30 kDa are usually acidic. There are, however, exceptions to this pattern. Xylanolytic microorganisms often produce more than one xylanase (146). In order to attack the different linkages in xylan efficiently, microorganisms have different xylanases with different specificities. However, these different specificities are not easily distinguished at biochemical level. The xylanases are usually the product of multiple genes (table 5 and 6). In some cases different xylanases can be recovered from microbial cultures that are the result of degradation products of a single major xylanase (30,146). The extent of multiplicity of xylanases in microorganisms is obscured by the fact that some xylanases have minor roles and are not produced in quantities large enough to allow purification and characterization. Recently it has been shown that some bacteria possess at least 3 to 5 different genes producing active distinct xylanases (1,37,43,91). Some of these enzymes could only be studied by over-expressing their corresponding genes, in order to obtain them in large enough quantities. An other difficulty in the biochemical analysis of xylanases is the fact that cleavage of β-1,4-glycosidic bonds within the xylan backbone can

also be affected by bifunctional endoglucanases that are able to hydrolyse both the 1,4-linkages in cellulose and xylan. The recent advances in genetical analysis made it possible to classify these enzymes as either xylanases or cellulases. Table 4 lists the xylanases isolated and purified from a number of anaerobic bacteria.

Arabinose-liberating endoxylanases.

In the past 20 years a number of xylanases have been reported not only to cleave the xylose-backbone, but also to liberate the α-arabinose residues on the side chain of arabinoxylan as free arabinose (table 3)(146). Thus, endoxylanases have been classified as debranching or non-debranching (33,111). This activity, although well reported in literature, is still controversial. Some researchers believe that this activity is due to a conta-

Table 3. Characterized debranching xylanases.

Organism	M.W. (kDa)	pI	Ref
Aspergillus niger	33	4.2	146
	31*	n.d.	
	50*	n.d.	
Ceratocytis paradoxa	?	9.2	32
Fibrobacter succinogenes	53.7	8.9	94
Trichoderma reesei QM9414	8.5*	10.3	146
	11.7*	8.6	
	14.4*	6.6	
	40.7*	8.5	
Trichoderma viride	17.8*	9.2	146
	53	5.3	
Trichoderma koningii	29	7.2	146

* molecular weight not estimated by SDS-PAGE, but gel filtration, or not specified method.
n.d. not determined.

mination of the xylanase preparation with α-L-arabinofuranosidase. Whether this dual activity is the result of a broad substrate specificity of one active site or the action of two

Table 4. Properties of some purified endoxylanases from anaerobic bacteria.

Organism	Strain	Xylanase	Corresponding gene cloned	Nr. of identified genes or proteins	M_r (kDa)	T opt. (°C)	pH opt.	Arab. release	X_3 cleaving[c]	Ref.
Fibrobacter succinogenes	S85	1		4 or 5	53.7			yes	?	94
		2	xynB?		66			no	?	92
			xynC		63	50		no	?	154
Butyrivibrio fibrisolvens	49		xynA	2	45[b]		5.4			93
Butyrivibrio fibrisolvens	H17c		xynB		73[b]	55	5.4-6			82
Clostridium acetobutylicum	ATCC 824	A			29	60	5.5-6.0			79
		B			65	50	5.0			
Clostridium stercorarium		A		3	44	75	6.5	no		6
		B			72	75	6.0	no		
		C			62	75	6-7	no		
Clostridium thermolacticum				4	39	80	6-6.5	no	no	29
Thermoanaerobacterium saccharolyticum	B6A-RI		xynA	1	130	70	5.5		yes	80
Thermoanaerobacterium sp.	JW/SL-YS485				234[a]	80	6.2		yes	120
Caldocellum saccharolyticum			xynA		42	70	5.5-6			84

[a]: native PAGE: subunits= 180 and 24 kDa
[b]: the xylanases were cloned, not purified.
[c]: X_3 cleaving: Ability of xylanases to hydrolyse xylotriose

different active sites is not yet known. The xylanase I from *Fibrobacter succinogenes* would suggest the second, because this enzyme releases the arabinose residues directly from the xylan backbone prior to hydrolysis of the xylan backbone to xylooligosaccharides (94). Since the xylanases classified so far on the basis of their primary structure have not been shown to release arabinose residues from xylan these debranching xylanases may belong to a distinct family. They may also be part of one of the existing families and the debranching activity could be a result of a less specific active centre within the enzyme, allowing the release of arabinose from xylooligomers. The ultimate answer whether or not debranching xylanases are the result of one single enzyme or of contamination, will be obtained when cloned xylanases turn out to possess the debranching activity. To my knowledge such research has not yet been reported.

Structural elements in xylanases

Many cellulases and xylanases have been characterized at both the biochemical and molecular level. The amino acid sequences of a great number of ß-1,4-glycanases have been determined (46,58,59). Analysis and comparison of the primary sequences and comparison of putative secondary structure by means of hydrophobic cluster analysis have revealed conserved stretches which are common to both cellulases and xylanases. Many of the putative polypeptides encoded by the genes are comprised of two or more domains, one of which is the catalytic domain. The glycanases can be grouped in families of related catalytic-domain sequences on the basis of amino acid sequence similarities (5,46,58,59). Until now 45 different families of structurally related glycosyl hydrolases were identified (59). Common structural features frequently found in such enzymes can be related to the fact that they all have general catalytic activities as glycosyl hydrolases. The variations may be related to the individual specificities of the enzymes. Almost all known xylanases were classified into two different families denoted G and F by Gilkes and coworkers (46) or 10 and 11 according to Henrissat (58) based on their catalytic domain. The utility of this classification is the ability to predict the general three dimensional structure and active site topology of all family members, if this is known for one or a few representatives.

Although the catalytic domains of xylanases and many cellulases exhibit little homology, suggesting that cellulases and xylanases have evolved from distinct ancestral genes, some families can be grouped into superfamilies with conserved folds and similar cleaving mechanism. It was reported recently that families 1 (ß-glucosidases), 2 (ß-galactosidases), 5 (family A cellulases), 10 (family f xylanases) and 17 (ß-glucanases) form such a superfamily (70). Distant but significant relationship between family G xylanases and family H cellulases was also demonstrated recently by hydrophobic cluster analysis (131). In addition to a catalytic domain, the enzymes may contain a discrete, independently functioning substrate-binding domain, like the cellulose-binding domain separated from the catalytic domain by a short linker region rich in proline and hydroxyaminoacids (5,46). These inter-domain linker regions may be important in rendering the enzyme flexible enough to attack the native insoluble substrate. Although xylanases generally cannot hydrolyse insoluble cellulose, some xylanases have cellulose binding domains, which enables them to specifically adsorb to cellulose (98). It seems that cellulose binding xylanases play a role in the synergistic cooperation of various glycanases necessary for the complete degradation of complex cellulosic materials. Recently, a xylan-specific binding domain was reported (15). Xylanase D of *Cellulomonas fimi* contains a cellulose binding domain and a specific xylan binding domain, with negligible affinity for other polysaccharides. Other conserved domains may be non-catalytic thermostabilizing domains (39,81), and domains comprised of two or three tandem repeats (5,97) which may participate in protein-protein interactions (129). In some bacterial species, notably species from the genus *Clostridium*, the principal parts of the cellulolytic system (containing xylanases) are secreted and organized into a multicomponent high molecular mass multienzyme complex called cellulosome (4,77). *Ruminococcus flavefaciens* FD-1 secretes two multiprotein complexes in which all the endoglucanases have xylanase activity (see also below)(34). The cellulosomes are generally associated with the cell surface. A central polypeptide component is responsible for integrating the catalytic subunits into a cohesive enzyme complex (112). This non-catalytic subunit also contains a cellulose binding domain which mediates the attachment of the cellulosome and bacterial cell to the substrate. This is in

contrast to normal cellulolytic systems where the cellulose binding domain is part of a catalytic subunit (47). *Butyrivibrio fibrisolvens* was reported to produce two multi-enzyme complexes, one having cellulase activity and the other predominantly xylanase activity. This secreted enzyme complex was called a xylanosome, in analogy with the cellulosome (83).

A classification on the structural properties of enzymes gives a good reflection of the relationship and molecular evolution of the enzymes. It is now obvious that xylanases evolved by domain shuffling, with subsequent modification of the domains.

Table 5 and 6 lists the xylanase genes that have been classified so far. Some bifunctional xylanases belong however to families containing mainly cellulases. CelD (44631 Da) from *R. flavefaciens* FD-1 encodes for endoglucanase activity and xylanase activity and belongs to family E cellulases (138). The *Prevotella ruminicola* strain 23 xylanase belongs to family A (142). This family contains mainly cellulases. Wong and coworkers (146) have divided xylanases on other grounds into two categories: the low molecular weight basic xylanases and the high molecular weight acid xylanases. Many organisms produce both alkaline and acid xylanases. The low molecular weight basic xylanases are usually endo-1,4-β-xylanases with specific activity on xylan only, whereas the other group seems to contain endo-xylanases with cellulase activity. These groups roughly correspond to the families G and F, respectively. Family G xylanases are usually very specific towards xylan and do not have any activity on carboxymethyl cellulose. With few exceptions, the family G acid-xylanases are all of fungal origin. Xylanase C of *Fibrobacter succinogenes*, an enzyme with two catalytic domains, showing high homology to each other, belongs to family G and has an isoelectric point of 6.2 (154). The acid xylanase A of thermophilic bacteria *Clostridium stercorarium* and the related *Clostridium thermolacticum* also belong to family G.

The molecular architecture of xylanases varies between microorganisms. Many filamentous fungi and several bacteria synthesize single-domain xylanases (46). In

contrast, the anaerobic fungus *Neocallimastix patriciarum* and the rumen bacteria *Ruminococcus flavefaciens* and *Fibrobacter succinogenes* synthesize xylanases which are comprised of multiple catalytic domains (38,45,104,152).

Table 5. Family F xylanase catalytic domains:

Enzyme	Origin	total Nr of genes	Catalytic domain[a]	No. of amino acids	Reference
XYND	*Bacillus polymyxa*	1			48
XYNA	*Bacillus* sp. C-125			396	55
XYNA	*Butyrivibrio fibrisolvens* 49		N	378	93
XYNB	*Butyrivibrio fibrisolvens* H17c		N	635	82
XYNA[b]	*Ruminococcus flavefaciens*	4	C	925	152
XYNY	*Clostridium thermocellum*	2-3	N	1030	39
XYNZ	*Clostridium thermocellum*	2-3	C	809	50
XYNB	*Clostridium stercorarium*	3		387	41
XYNA	*Thermoanaerobacterium saccharolyticum* B6A-RI			1157	80
XYNA	*Caldocellum saccharolyticum*	1		312	84
XYNB	*Fibrobacter succinogenes*	4-5	N	586	92
XYNA	*Streptomyces lividans*	3		436	122
XYNI	*Streptomyces thermoviolaceus*	2			134
XYNA/B	*Pseudomonas fluorescens* subsp. *cellulosa*	2	C	585 555	74/54
XYN	*Thermoascus aurantiacus*			269	125
XYN	*Cryptococcus albidus*	1		311	19
XYNA	*Aspergillus kawachii*				67
XYN	*Penicillium chrysogenum*[c]				53

[a]: C or N denotes the location of the catalytic domain at the C- or N-terminal of the gene respectively.
[b]: A second catalytic domain belongs to family G.
[c]: Based on experimentally determined partial or full amino acid sequences.

Table 6. Family G xylanase catalytic domains:

Enzyme	Origin	Total Nr. of genes	Catalytic domain[a]	No. of amino acids	Reference
XYNA	*Bacillus circulans*	2		185	150
XYNA	*Bacillus pumilus*	1		201	42
XYNA	*Bacillus subtilis*			182	103
XYNB	*Streptomyces lividans*	3		293	122
XYNC	*Streptomyces lividans*	3		191	122
XYNII	*Streptomyces thermoviolaceus*	2			134
XYNB	*Clostridium acetobutylicum*	2		234	151
XYNA[b]	*Ruminococcus flavefaciens*	4	N	925	152
XYNB	*Ruminococcus flavefaciens*	4	N	781	153
XYND[d]	*Ruminococcus flavefaciens*	4	N	802	38
XYNC	*Fibrobacter succinogenes*	4-5	two	608	104
XYNA	*Clostridium stercorarium*	2	N	511	113
XYNA	*Clostridium thermolacticum*[c]	1	N	340<>570	30
XYND	*Cellulomonas fimi*	4	N	644	98
XYN	*Neocallismastix patriciarum*		N		
XYNA	*Neocallismastix patriciarum*	1	C		45
XYNA	*Thermomonospora fusca*		N	338	66
XYNA	*Schizophyllum commune*[c]				102
XYNA	*Chania* sp.[c]				3
XYN1/2	*Trichoderma reesei*	2			130
XYN	*Trichoderma harzianum*[c]				149
XYN	*Trichoderma viride*[c]				148
XYNA	*Aspergillus niger var awamori*				86
XYNA	*Aspergillus tubigensis*				49
XYNC	*Aspergillus kawachii*				68

a: C or N denotes the location of the catalytic domain at the C- or N-terminal of the gene respectively.
b: A second catalytic domain belongs to family F.
c: Based on experimentally determined partial or full amino acid sequences.
d: The enzyme contains a second catalytic domain at the C-terminal with ß(1,3-1,4)-glucanase activity.

Mode of action of xylanases

In general, endo-1,4-β-xylanases degrade xylans by attacking the internal β-xylosidic linkages of the xylan backbone chain resulting in multiple scission. The sites attacked along the xylan chain and the frequency of bond cleavage is governed by the structure of the heteroxylan, and by the number of subsites in the active site of the enzyme which affects the free energy of binding to the glycosyl residues. The degradation products during early course of hydrolysis are xylooligosaccharides, usually containing arabinose and/or uronic acid and acetic acid substituents. As hydrolysis proceeds these oligosaccharides are usually progressively converted to xylose and xylobiose and/or xylotriose (33,111,146). The ultimate size of the final end-product is determined by the specificity of the enzyme, and this in turn, may be affected by the frequency and spacing of the side-chain substituents on the xylan chain. Family G xylanases, at high substrate concentration, also possess transglycosylation activities which leads to products larger than the starting substrate (7). It was shown for the xylasase from *Cryptococcus albidus* that the active site is comprised of four subsites and that the catalytic site is located in the middle (7). In contrast, the active site of *Pseudomonas fluorescens* includes at least five subsites, and the cleavage occurs between the first and second subsite from the non-reducing end of the substrate (Fig. 4) (56).

All glycosyl hydrolases are thought to catalyze the transfer of a glycosyl group to water by a general acid catalysis mechanism in which two amino acid residues participate in a single-displacement or double-displacement reaction. This enzymatic breakdown of a glycosidic bond is a stereoselective process, in which the conformation of the anomeric centre (C-1 carbon) can either be inverted or retained (123). The hydrolysis reaction catalyzed by family F and G xylanases has recently been shown to proceed with retention of anomeric configuration at the newly formed reducing end (44). The catalytic mechanism proposed resembles that of henn-egg white lysozyme. This acid catalytic mechanism involves most probably two glutamate residues that seem to be 100% conserved in these protein families (56,88,99,132). One glutamate acts as the nucleophile the other as the acid/base catalyst (Fig. 5).

Amino acid sequence analysis suggested that the structure of the two xylanase families are different (46). Recently several three-dimensional structures of bacterial and fungal xylanases, with representatives from both families, have been resolved (56,132). The family G enzymes are low-molecular weight xylanases with highly conserved amino acid sequences, indicating similar structural folds (131). These enzymes are single-domain ß-sheet proteins. The active site is in a cleft at the intersection of two sheets. The two glutamate catalytic residues are located in the middle of the cleft. The family F xylanase catalytic domain has a 8-fold ß/α-barrel architecture (56). The substrate binding cleft is formed by long loops at the carboxy-terminal end of ß-strands 4 and 7 and short loops at the carboxy-terminal of ß-strands 5 and 6. The two catalytic glutamate residues are located at the carboxy-terminal end of ß-strands 4 and 7 and have been identified as the acid/base catalyst and nucleophile, respectively.

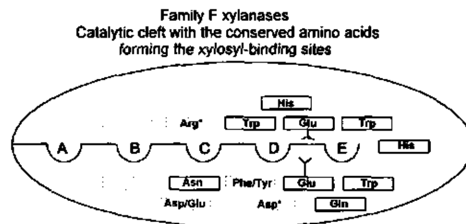

Figure 4. Active site of Pseudomonas fluorescens xylanase A showing the two catalytic glutamate residues and the proposed substrate binding sites for the xylosyl units (based on data from ref. 51).

Because of strong hydrogen-bonding properties of the phenolic hydroxyl of tyrosine (Tyr) and the capacity of tyrosine and tryptophan residues to act as docking residues for the hydrophobic part of a sugar residue, these amino acids are thought to play an important role in carbohydrate binding proteins. By alignment of the sugar residues, these amino acids contribute to selectivity of fit of the substrate to the binding site of the enzyme.

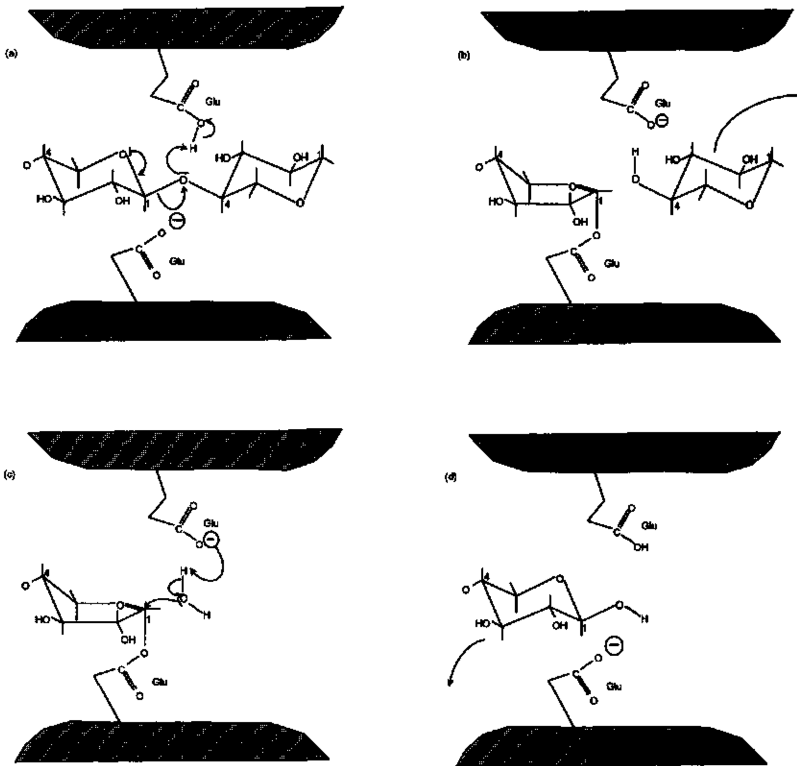

Figure 5. Hydrolysis of xylan by xylanases with retention of the anomeric configuration. The role of the two catalytic glutamate residues (based on data from ref. 56).

Tryptophan and tyrosine have been shown to be essential for substrate binding in most of the glycoside hydrolases (21,56,132). At least 5 binding sites were identified in xylanase A (Fam. F) of *Pseudomonas fluorescens* (56) (Fig. 4). Xylanase II (Fam. G) of *Trichoderma*

reesei has putative binding sites for five xylose residues inside the catalytic cleft, whereas the closely related xylanase I has only three subsites (133). The binding site cleft of xylanase A (Fam. G) from *Schizophyllum commune* spans about seven xylose units and the catalytic site is located asymmetrically within it (20). It seems that this xylanase uses mainly tyrosyl residues as docking site for the xylosyl units. This in contrast with the family F xylanase of *Pseudomonas fluorescens*, which has tryptophan and other amino acids involved in substrate binding (Fig. 4) (21,56). If this difference holds true for other members in these families, this may explain the difference in substrate selectivity between these two families. Family G xylanases normally only hydrolyze xylan, whereas family F xylanases often have some endo-glucanase side activity. The only difference in substrate being the environment around the C-5 atom, one could expect an important role for the docking site amino acids of the enzymes.

β-Xylosidases

β-Xylosidases (1,4-ß-D-xylan xylohydrolases, EC 3.2.1.37) hydrolyse xylooligosaccharides and xylobiose to xylose. They attack short chain xylo-oligosaccharides by liberating xylose from the non-reducing end of the substrates. The activity normally increases with decreasing chain length, and is highest towards xylobiose. *β*-Xylosidases generally exhibit little or no action against polymeric xylans. Most *β*-xylosidases catalyse the cleavage of artificial substrates such as p-nitrophenolxylopyranoside. *β*-Xylosidases are less common than endo-xylanases, because they are only necessary in order to produce xylose. This allows xylanolytic organisms to grow efficiently on xylan. Xylanases on the other hand are also produced by a range of cellulolytic organisms in order to make the cellulose more accessible. These cellulolytic organisms do not grow on xylan and do not produce *β*-xylosidases.

Most *β*-xylosidases are large relatively to the endo-xylanases. These enzymes may be monomeric, dimeric or tetrameric, with Mr values ranging from 26000 to 360000. They are produced by a variety of bacteria and fungi and may be found in the culture fluid, associated with the cell or both (36,146). Some organisms produce bifunctional

β-xylosidases that also possess α-L-arabinofuranosidase activity (135). Many have transferase activity in addition to direct hydrolase action, and they can produce transfer products with higher molecular weight than that of the substrate (65). Some β-xylosidases have also been reported to possess ß-glucosidase activity (136).

Debranching enzymes:

Arabinofuranosidases, glucuronidases and esterases.

One of the problems in the degradation of hemicelluloses is the high degree of substitution found on the hemicellulose backbone. These substituents interfere with the enzymatic degradation of the polymer through steric hindrance. Removal of these side-chain groups generally facilitates the decomposition of hemicellulose (105).

α-L-Arabinofuranosidases

α-L-Arabinofuranosidases (EC 3.2.1.55) hydrolyse terminal non-reducing α-L-arabinofuranoside groups of arabinoxylans or arabinose substituted xylo-oligomers. Some α-L-arabinofuranosidases are only active on short oligomeric substrates, while others have the ability to cleave arabinosyl residues not only from arabinoxylan but also from arabinans and arabinogalactans. The enzyme activity is routinely assayed using the *p*-nitrophenyl- or methylumbelliferyl-α-L-arabinofuranosides as artificial substrates. The enzymes are found both intracellularly and extracellularly, depending on the organism. The α-L-arabinofuranosidases are produced by many anaerobic bacteria (145), and have been purified from *Ruminococcus albus* 8, *Clostridium acetobutylicum* and *Butyrivibrio fibrisolvens* (51,61,78). These enzymes may be monomeric, dimeric, tetrameric or even octameric, with Mr values ranging from 54,000 to 495,000 depending on the microbial source (72,76).

α-Glucuronidases

α-Glucuronidases are required for hydrolysis of the α-1,2-glycosidic linkage between xylose and D-glucuronic acid or its 4-*O*-methyl ether. This enzyme has been purified to homogeneity and characterized only from a few sources (75,107,121). The α-Glucuronidase from *Agraricus bisporus* is a large protein (450 kDa) with an acidic isoelectric point and a pH optimum of about 3.3. The enzyme has no activity towards 4-*O*-methylglucuronic acid-xylan but cleaves 4-*O*-methylglucurono-substituted xylo-oligomers with degree of polymerization (DP) values ranging from 2 to 6, with highest activity towards the dimer (107). The enzyme was inhibited by the presence of acetyl substituents on xylan. It seems that acetylxylan esterase - removing the acetyl groups -, endoxylanase - producing short 4-*O*-methylglucurono-substituted xylo-oligomers -, and α-glucuronidase interact synergistically to effectively hydrolyse the polymeric xylan. In contrast, the α-D-glucuronidase from a thermophilic fungus *Thermoascus aurantiacus* is a single polypeptide chain with a M_r of 118 kDa. It hydrolyzed 4-*O*-methylglucurono-substituted xylo-oligomers with DP 1 to 7 and also had activity towards 4-*O*-methylglucuronic acid-xylan. The α-glucuronidase from *Fibrobacter succinogenes* is inactive towards the polymeric substrate, but also towards the 4-*O*-methylglucurono-substituted xylobiose. However, when xylan was pretreated with xylanase, activity was enhanced, suggesting that substituted xylo-oligomers must be generated with a DP>2 (124). Very recently, the purification of a α-D-glucuronidase from a thermophilic anaerobic *Thermoanaerobacterium* sp. was reported (121). The dimeric enzyme had a M_r of 130 kDa. The enzyme was active on the polymeric xylan and acted synergistically with a ß-xylosidase to hydrolyze 4-*O*-metylglucurono-substituted xylo-oligomers.

Esterases

In addition to enzymes hydrolyzing the glycosidic linkages in xylans, esterases are needed to remove esterified acids from xylan.

Acetyl Esterases

Acetyl esterase is a general term for an enzyme that cleaves an esterified acetic acid residue from a compound. In this case acetyl esterase refers to the enzyme that cleaves acetic acid from a short acetylated xylan oligomer and is inactive against acetylated long-chain xylan polymers. This enzyme acts on the short acetylated end-products of xylan degradation, removing any remaining acetyl groups and allowing access of ß-xylosidase to the xylose oligomer. The enzymes are normally active against small degradation products such as acetylated xylose, xylobiose, and xylotriose. In contrast to acetyl esterases, acetylxylan esterases are active against long-chain intact acetylated xylan

Acetyl xylan esterases

Acetyl xylan esterases remove O-acetyl groups from the C-2 and C-3 positions of xylose residues in both xylan and xylooligomers. The production of acetyl xylan esterases by various fungi was first reported by Biely and co-workers (9). Acetyl xylan esterases have been detected in several bacteria, like *Fibrobacter succinogenes*, *Butyrivibrio fibrsolvens,* and several *Streptomyces* strains (60,71,87,96,106)

Coumaryl and feruloyl Esterases.

Coumaric acid and ferulic acid are cinnamate-based acids that occur in an ester linkage to α-L-arabinofuranose units attached to the xylan backbone (100). They form the cross-links to the lignin matrix, giving structural support to the cell wall and protect the xylan chain from degradation by preventing access of xylanase to the polymer. Coumaryl and feruloyl esterases remove these moieties, opening up the cell wall structure and allowing xylanase easier access to the xylan polymer. Ferulic acid and p-coumaric acid appendices have been found to reduce the digestibility of graminaceous plants, either because of inhibition of the growth of rumen bacteria by the hydroxycinnamic acids (17), or because of limitations in the hydrolysis due to lack of hydroxycinnamic acid-cleaving esterases. An esterase capable of hydrolysing ester-linked ferulic acid from wheat bran hemicelluloses was first detected in culture filtrates of *Streptomyces olivochromogenes* (87). These enzymes were also

shown to be produced by anaerobic fungi (18). In the culture fluid of the anaerobic bacterium *Fibrobacter succinogenes* low activities of both ferulic acid esterase and cinnamic acid esterase were detected (124)

Regulation of xylanolytic enzyme synthesis

Although a considerable amount of work has been done about the molecular structure, function and application of xylanases, comparatively little is known about the regulation of their formation. The study of the regulation of the synthesis of xylanolytic enzymes is particularly difficult to elucidate: First of all, most xylanolytic microorganisms are also cellulolytic. This signifies that these organisms not only produce a whole array of xylanolytic enzymes but also a complex mixture of cellulolytic enzymes. This complicates the study of regulation of xylanases synthesis. Both cellulases and xylanases cleave β-1,4-glycosidic bonds and are closely related at the functional and structural level (46). Some xylanases have multi domain catalytic activities, and some of these enzymes are bifunctional, in that they not only possess cellulase activity but also xylanase activity. Many microorganisms produce as mentioned earlier an array of xylanases with only minor differences in catalytic activity or specificity. Some of these enzymes are produced in amounts too little to allow a good biochemical characterization. In addition, because of the complex structure of the substrate, enzymatic degradation products, which may play a role as inducer, are very difficult to determine. This is even more complicated because some xylanases also possess transglycosylating activities enabling them to form hetero-oligosaccharides which are believed to play a role in induction (64). All these factors together make it almost impossible to determine a general mechanism describing the regulation of xylanolytic enzymes. The recent developments in genetical characterization of the different xylanase genes and the possibility to study the genes in the xylanolytic organism, have given a potent tool to eliminate some of the difficulties mentioned above (1). Expression of the xylanolytic enzymes of several bacteria in *E. coli* has given very little information on the mechanisms of regulation of the individual genes and corresponding enzymes, because

the host promoter was used. Furthermore, the sugar uptake mechanisms of the microorganism may play an important role in delivering the possible inducers. A xylanase gene of *Prevotella ruminicola* was cloned into similar organisms, *Bacteroides fragilis* and *Bacteroides uniformis*, but it seemed that the enzymes were produced constitutively in these organisms (141).

Although there appears to be no single regulatory mechanism in bacteria, in general, the xylanolytic enzyme production is repressed in the presence of easily metabolized carbon sources, like for example the end-products of xylan degradation, xylose and arabinose. In some bacteria, the xylanase synthesis seems to be constitutive, but in many bacteria the xylan-degrading enzymes are inducible. Low activities, resulting from constitutively produced enzymes, are sometimes found. These low activities are believed to be necessary to generate low-molecular-weight inducers from the polymeric xylan. It is commonly assumed that the polymeric substrate, due to its size, itself is not directly involved in the induction mechanism. The low molecular weight inducers can be direct degradation products from the polymeric substrates or products generated through transglycosylation of mono- or oligomeric- saccharides derived from the polymer.

Application of cellulases and hemicellulases

The past 10 years there has been a growing interest in microbial enzyme systems that degrade the major plant hemicellulose, xylan. Such enzymes have great biotechnological potential. Traditionally, the application of xylanases was thought to be in the bioconversion of lignocellulosic materials to produce products of higher value, such as single cell protein, fuel and other chemicals (85). Research in this field was mainly focussed on the use of cellulolytic enzymes in combination with hemicellulases. The economical outlook for such processes is low and the bioconversion of massive accumulating agricultural, forestry and municipal solid waste residues is now viewed mainly from the perspective of waste management.

Hemicelluloses tend to be hydrolysed easily by acid, which makes enzymic degradation a less attractive alternative. However, hemicellulases will probably be more useful when selective removal of hemicellulose is required for the production of certain materials that have high value, or to replace environmentally harmful processes. Such applications may be found in the food, feed, and pulp and paper industries. Cellulase-free xylanases can be applied in pulp and paper industry for bleaching of kraft pulp, essentially removing lignin from pulp. In the following table some applications of xylanolytic enzymes are listed.

Table 7. Applications of xylanolytic enzymes, alone or in combination with cellulolytic and pectinolytic enzymes.

Application
Extraction of fruit juices, flavours, spices, oils and pigments
Clarification of fruit juices, beer and wines
Production of modified xylans as bulking agents for food processing
Conversion of xylans into monomeric products for conversion into sweetener (xylitol) or flavours
Modification of cereal flours so as to enhance volume, textural and staling properties of bread
Improvement of feed digestibility for cattle, pigs and poultry
Retting of flax, hemp, jute, sisal and bast
Refining of dissolving pulps for production of viscose rayon, cellulose esters and cellulose ethers
Saccharification of agricultural and forestry wastes and residues for fermentation to fuels and chemical feedstocks, like aceton, butanol, ethanol etc.
Improvement of fibre quality, and changing of water retention properties of paper pulp.
Prebleaching of pulps for paper manufacture

(for more details see ref. 147)

Bacteroides xylanolyticus X5-1.

The organism used in this study was *Bacteroides xylanolyticus* X5-1 (DSM 3808). As part of a study on the production of biogas from agricultural waste, anaerobic xylanolytic bacteria were isolated from fermenting cattle manure at the Department of Microbiology of the Wageningen Agricultural University (115). *Bacteroides xylanolyticus* is a strictly

anaerobic non-spore forming, Gram-negative rod-shaped bacterium. This organism grows rapidly on xylan and a wide range of soluble sugars like glucose, xylose, arabinose, fructose, cellobiose and mannose. Cellulose, carboxymethyl cellulose and the hemicelluloses, xanthan, laminaran, galactomannan, arabinogalactan and gum arabic are not fermented by this organism. The optimal growth temperature is between 25 and 40°C at pH values between 6.5 and 7.5. The GC content of the DNA was measured 34.8%, but a GC content of 45% has also been reported (119).
The main fermentation products when grown on xylan or xylose are acetate, ethanol, formate. In addition, small amounts of 1,2-propanediol and lactate are produced (10). The biochemistry and physiology of product formation from xylose by *Bacteroides xylanolyticus* was studied in detail by Biesterveld (10). The xylose catabolism pathway, the xylose uptake system, the induction mechanism of key enzymes of the xylose catabolism, the regulation of the xylose metabolism by interspecies electron transfer and the effect of external electron acceptors on the physiology of the xylose metabolism were investigated (10-14).

Scope and outline of this thesis.

Anaerobic bacteria have been shown to produce a range of cell wall-degrading enzymes. The best studied mesophilic anaerobic bacteria have been isolated from the rumen. Because of the complexity of plant cell wall polymers, most of these organism are able to degrade and grow on cellulose. Although during the last five years a great number of studies have been devoted to the subject of xylan degradation, there is still little known about the regulation of the individual enzymes responsible for xylan degradation by anaerobic bacteria. To be able to study the xylanolytic enzymes and the regulation of their synthesis we chose a the mesophilic anaerobic bacterium *Bacteroides xylanolyticus* X5-1, as a model organism. *B. xylanolyticus* is together with *B. ruminicola* an important xylan degrader (119). It grows rapidly on xylan and it is not able to grow on cellulose or other hemicelluloses. This made it possible to study the xylanolytic enzymes and the regulation of their synthesis without interference of cellulolytic enzymes. This bacterium produced a

range of xylanolytic enzymes when grown on xylan, but no activities were found on glucose (118).

The aim of this research was to understand the strategy used by *Bacteroides xylanolyticus* X5-1 to degrade the complex substrate xylan. The most important enzymes involved in the degradation of xylan were identified and by purifying them, their properties and substrate specificities could be resolved. Next, the regulation of the formation of these xylanolytic enzymes was studied. Since many reports are published monthly on the subject of xylan degradation, I attempted in the general introduction to give an overview on this subject. In chapter 2, the purification and regulation of the two most important xylanolytic enzymes of *B. xylanolyticus* X5-1, xylanase I and II is presented. Although the substrate (xylan) for both enzymes is the same, their properties are quite different. This organism uses different mechanisms to regulate the production of these distinct enzymes. Chapter 3 describes the purification, characterisation and regulation of the ß-xylosidase. This enzyme transforms the final products of the two xylanases into xylose, which the bacterium can use as a growth substrate. The purification, properties and regulation of a cell-associated α-L-arabinofuranosidase are presented in chapter 4. Depending on the source from which xylan is isolated, this polysaccharide can contain acetyl side chains. In chapter 5 the purification and characterisation of an acetyl esterase, involved in the removal of acetyl side chains from xylose units is reported. The next two chapters, 6 and 7, contain the summary in English and Dutch with a schematic representation of the xylan degradation and enzyme regulation by *B. xylanolyticus* X5-1.

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Chapter 2

Purification, Mode of Action and Regulation of two Xylanases of *Bacteroides xylanolyticus* X5-1.

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Abstract

Two different xylanases from the anaerobic bacterium *Bacteroides xylanolyticus* X5-1 were purified by column chromatography to apparent homogeneity and partially characterized. The organism had a different regulation for the synthesis of the two xylanases. Xylanase I, a 38 kDa protein, was constitutively synthesized when enough energy was available to the cells and no easily metabolisable sugars were present. In resting cell suspensions, pyruvate induced high levels of this enzyme. Under growing conditions, with pyruvate as sole energy and carbon source, xylanase was not synthesized. Monomeric sugars as well as the non-metabolisable sugar D-arabinose repressed the induction of xylanase I by pyruvate. Short xylooligosaccharides also induced xylanase I synthesis. Xylanase I degraded xylan and xylo-oligomers with a degree of polymerization of 4 and higher. It also had arabinose cleaving ability on smaller oligomers. Xylanase II was a 63 kDa protein that was only produced in larger amounts when the organism was grown on xylan. Xylanase II degraded xylan and xylo-oligomers with a degree of polymerization of 3 and higher. No arabinose was released from oat spelt arabino-xylan or arabinose substituted xylo-oligomers by this enzyme. During growth the two xylanases were cell associated.

Introduction

Xylans are heterogeneous polysaccharides found in plant cell walls as part of the ligno-cellulosic complex. The amount and the composition of xylans depend on the type of plant, plant tissue and age. These polymers consist of ß-1,4-linked D-xylose pyranose chains containing several side chains at the O-2 and O-3 position. The substituents include acetyl, arabinosyl and uronyl groups (2). The principal hemicellulose in forages consumed by ruminants is arabinoxylan (34). Anaerobic mesophilic *Bacteroides, Prevotella, Fibrobacter, Ruminococcus* and *Clostridium* species have been shown to degrade xylan. Many cellulolytic ruminal bacteria can degrade xylan, but few are capable to grow on these substrates (11). For the complete breakdown of xylan several enzymes are required,

which act on the polyxylose chain or cleave of the side chain substituents. Endo-1,4-ß-xylanases are produced by a variety of fungi and bacteria (2, 12, 25). These enzymes hydrolyse glycosidic bonds in xylan. Due to the complex structure of xylan multiple xylanases with different specificities are likely to be required for an efficient degradation. Multiple xylanase genes have been detected in several bacteria like *Ruminococcus flavefaciens, Fibrobacter succinogenes* and *Prevotella ruminicola* (14,16, 21). In anaerobic environments like the rumen, the biosynthesis of polysaccharide degrading enzymes is often regulated. In these highly competitive environments this is essential for survival. In general, xylanase formation seems to be controlled by two basic regulation mechanisms The xylanase synthesis is repressed in the presence of easy metabolizable carbon sources and is induced in the presence of the polymeric substrate or its degradation products. Due to its large molecular size, it is not probable that xylan will act as inducer since it cannot penetrate the microbial cell membrane. Soluble oligosaccharides are therefore thought to play an important role in xylanase induction. In the yeast *Cryptococcus*, which has been well characterized (2), basic levels of a constitutive enzyme give rise to soluble hydrolysis products of xylan. They can enter the cells and function as inducers. As part of a study on the production of biogas from agricultural waste, several xylanolytic bacteria were isolated from fermenting cattle manure from different sources (26). A typical strain, *Bacteroides xylanolyticus* X5-1, was investigated in more detail (4,5,6,7,27,28). This organism, a strictly anaerobic, non-spore forming, motile rod-shaped bacterium, ferments a wide range of soluble sugars and grows on xylan, but it does not grow on cellulose or other hemicelluloses. These properties make it a suitable organism to study xylan degradation, ensuring that hydrolysis of xylan is due to the action of xylanases and is not non-specific activity of cellulases. *B. xylanolyticus* produce xylanase, xylosidase and arabinofuranosidase activities during growth on xylan (27,28). Xylose, the main end product of xylan hydrolysis, is intracellularly converted by the successive action of xylose-isomerase and xylulokinase to xylulose 5-phosphate which is then further metabolised via the pentose phosphate pathway and glycolysis to acetate, ethanol, formate, H_2 and CO_2 as

principal fermentation products (4).The aim of this research was to characterize the properties of the xylanases and to study the regulation of their synthesis.

Materials and methods

Organism and growth conditions. *Bacteroides xylanolyticus* X5-1, was obtained from the German collection of microorganisms (DSM 3808) and cultured as described previously (28). Unless stated otherwise, oat spelt xylan (lot nr. 38F-0722, Sigma, St. Louis, Mo, USA) was used as the substrate. Routinely, 4 g/l was added prior to heat sterilization. Mass-cultivation of *B. xylanolyticus* X5-1 for the purification of xylanase I was performed at 37°C in 10-l bottles, containing 8 l medium. Cells from two 8-l cultures, grown during 18 hours on 0.5 % (w/v) glucose, were harvested anaerobically by continuous centrifugation (Heraeus Sepatech Biofuge 28RS, Osterode, Germany), washed with anaerobic fresh carbon source-free medium and incubated in 1.75 litre medium containing 80 mM pyruvate for 9.5 hours. The cells were harvested by centrifugation 10 min. at 10000 *g*, washed and resuspended in 50 mM Tris/HCl, pH 7.5. To the cell suspension, CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) 0.1% (w/v) was added and incubated for 18 hours at 4°C. This suspension was processed further for enzyme isolation. Mass-cultivation of *B. xylanolyticus* X5-1 for the purification of xylanase II was performed in 3-l bottles. 4.5 l *B. xylanolyticus* X5-1 culture, grown on 4 g/l Birch wood xylan (charge number: 14210983, Roth, Karlsruhe, Germany), was harvested after 24 hours of incubation by centrifugation for 10 min at 10.000 x g, and the cell pellet was resuspended in 50 ml 50 mM Tris/HCl pH 7.5.

Continuous cultivation was performed at 37 °C in 1-l chemostats with a working volume of 500 ml. The same medium as for batch cultivation was used except that 2 g/l of yeast extract was added instead of 0.5 g/l and that tryptone was omitted. The pH was maintained at 7.0 ± 0.1 with 2 N NaOH. A continuous stream of N_2/CO_2 (80%/20%) at a flow rate of 130 ml per hour was led over the cultures. To guarantee steady-state conditions, cells were

analyzed after at least 6 volume changes. Bacterial dry weight was quantified as described by Biesterveld et al. (6).

Enzyme induction experiments. Cells (0.5 - 2 l) were grown on 20 mM glucose, harvested anaerobically in the late exponential phase by centrifugation at 10,000 x *g* for 10 min, washed with anaerobic medium without substrate, and resuspended in a 10 % volume of fresh medium containing the appropriate carbon source. The cell suspensions were incubated at 37°C. At different time intervals 1-ml samples were taken. Cells were centrifuged at 10,000 x *g* for 5 min, washed with 50 mM potassium phosphate buffer (pH 6.5) and resuspended in 500 µl of 50 mM Tris/HCl buffer (pH 7). Thereafter, xylanase activities were determined in crude cell free extracts.

Preparation of cell free extract. Cell suspensions were disrupted by sonification at 0°C (5 times for 20 s at 40 W, using a Branson Sonic Sonifier, Danbury, CT, USA). The broken cells were centrifuged for 20 min at 10000 *g*. The supernatant was designated crude cell-free extract.

Enzyme purification. Unless otherwise stated all procedures were carried out aerobically at room temperature.

Xylanase I: The crude cell free extract was centrifuged at 100,000 x *g* for 1 h at 4°C. The supernatant was applied to a Q-sepharose fast flow column (3.2 by 12 cm) equilibrated with 50 mM Tris/HCl pH 7.5 (buffer A). The column was developed with 225 ml of a linear gradient of 0 to 0.3 M NaCl in buffer A. The active xylanase I eluted in the void volume. The fractions with xylanase activity were loaded onto a column (3.2 by 8 cm) packed with S-sepharose, equilibrated with buffer A at 3 ml/min. The xylanase activity was not retained by the column. Xylanase containing fractions were pooled and applied to a hydroxyapatite column (2.2 by 18 cm) equilibrated with potassium phosphate buffer (10 mM, pH 7). The adsorbed protein was eluted from the column in a 250 ml-linear gradient of 10 mM to 150 mM potassium phosphate buffer pH 7. The xylanase eluted at 90 mM potassium phosphate. Fractions with activity were combined, concentrated by ultrafiltration with an Amicon ultrafiltration cell (Grace, Rotterdam, the Netherlands) equipped with a YM 5 filter (molecular cut off 5000 Da) and applied to a sephadex G-75 gel

filtration column (V_0=34 ml; V_t=90 ml) equilibrated in buffer A plus 140 mM NaCl. The column was developed at a flow rate of 0.2 ml/min. The colourless xylanase eluted as one symmetrical peak at 54.5 ml.

Xylanase II: The crude cell extract was centrifuged at 100,000 x *g* for 1 h at 4°C and the supernatant was applied to a Q-sepharose fast flow column (3.2 by 12 cm) equilibrated with 50 mM Tris/HCl pH 7.5 (buffer A). The column was developed with 400 ml of a linear gradient of 0 to 0.75 M NaCl in buffer A. Two xylanase activity peaks could be detected, one eluted in the void volume and the other eluted at 0.33 M NaCl. The xylanase activity peak, eluting at 0.33 M NaCl, was collected and applied to a hydroxyapatite column (2.2 by 18 cm) equilibrated with potassium phosphate buffer (10 mM, pH 7). The adsorbed protein was eluted from the column in a 200-ml linear gradient of 10 mM to 130 mM potassium phosphate buffer pH 7. The xylanase activity eluted at 105 mM potassium phosphate. Fractions with activity were combined and adjusted to 1.25 M ammonium sulphate by slow addition of granular ammonium sulphate. The enzyme preparation was centrifuged for 20 min at 10000 *g* and loaded onto a phenylsuperose HR5/5 column. The column was equilibrated with 1.25 M ammonium sulphate in buffer A and eluted with a 50 ml linear gradient of 1.25 M to 0 M ammonium sulphate. The enzyme eluted from the column at 0.4 M ammonium sulphate. The xylanase-containing fractions were concentrated 10 times by ultrafiltration with an Filtron microsep microconcentrator (Filtron technology corp., Northborough, MA, USA) equipped with a membrane with a nominal molecular weight cut off of 10 kDa. 250 μl was injected onto a Superose 6 HR 10/30 gel filtration column (V_0=7.9 ml; V_t=23.4 ml) equilibrated in buffer A plus 100 mM NaCl. The column was developed at a flow rate of 0.25 ml/min. The colourless xylanase eluted as one symmetrical peak at 16.1 ml. To determine the native molecular mass of the protein, the purified xylanase II was applied to a sephadex G-75 gel filtration column (V_0=34 ml; V_t=90 ml) equilibrated in buffer A plus 140 mM NaCl. The column was developed at a flow rate of 0.2 ml/min. The colourless xylanase eluted as a symmetrical peak at 47 ml.

The following standards (Pharmacia Fine Chemicals, Uppsala, Sweden) were used: Ribonuclease A 13.7 kDa, chymotrypsinogen A 25 kDa, ovalbumin 43 kDa and bovine serum albumin 67 kDa (Pharmacia Fine Chemicals, Uppsala, Sweden).

Xylanase assays. Xylanase activity was determined by monitoring the release of reducing sugar from oat spelt xylan (lot nr. 12070, Serva, Heidelberg, Germany) by the Nelson-Somogyi method (29), with xylose as the standard. The assay mixture consisted of 1% (wt/vol) xylan supplemented with 50 mM potassium phosphate buffer (pH 6.5) and enzyme to give a final volume of 0.5 ml. The reaction mixture was incubated for 20 min, and stopped by boiling for one min. One unit (U) of xylanase activity was defined as the amount of enzyme that released 1 µmol of reducing sugar (expressed as xylose equivalents) per min. Because of the negative effect of high ammonium sulphate concentrations on the Nelson-Sompogyi method, the xylanase activity of samples containing this salt were determined using Remazol Brilliant Blue R-D-xylan (lot nr. 119FO148, Sigma, St. Louis, MO, USA) as a substrate with the method described by Biely (3). The assay was performed at 37°C in a 50 mM potassium phosphate buffer (pH 6.5). The xylanase activity was determined by photometrically measuring (595 nm) the enzyme-released dyed-xylan fragments soluble in 2 volumes of ethanol.

Xylanase activity was also assayed using xylo-oligosaccharides as substrate. The hydrolysis experiments were performed at 37 °C in 50 mM Potassium phosphate buffer pH 6.5 (xylanase I) or 50 mM Tris/HCl buffer pH 7 (xylanase II). Purified xylanase I (0.05 Units) was incubated during 2 hours with respectively 4.5 mM xylobiose, 2.3 mM xylotriose and 0.4 mM xylotetraose plus 0.6 mM arabinose-substituted xylotetraose. The hydrolysates were analyzed by HPLC equipped with a Biorad Aminex 87P carbohydrate column and RI-detector as described previously (13). Purified xylanase II (0.05 Units according to the reducing sugar assay) was incubated during one hour with 1.5 mM xylo-oligomers ($X:X_2:X_3=1:4:3$). The hydrolysates were analyzed by HPLC equipped with a Carbopac PA1 column, with puls-amperometric detection with a gold electrode (Dionex, Sunnyvale, CA, USA). The column was eluted with a flow rate of 1 ml/min with 100 mM NaOH plus 25 mM sodium acetate.

Protein determination. Protein was determined with Coomassie brilliant blue G250 as described by Bradford (1976). Bovine serum albumin was used as a standard.

Polyacrylamide gel electrophoresis *(PAGE).* SDS PAGE was performed on 12.5% (w/v) gels according to the method of Laemmli (1970). Protein was stained with Coomassie brilliant blue. Molecular weight of the subunits was estimated by comparison to protein standards: trypsin inhibitor (M_r 20100), carbonic anhydrase (M_r 30000), ovalbumin (M_r 45000), bovine serum albumin (M_r 67000) and phosphorylase b (M_r 94000) (Pharmacia Fine Chemicals, Uppsala, Sweden).

Preparation of xylo-oligosaccharides. Xylo-oligosaccharides were prepared as described previously (28). Sugars were analyzed by HPLC equipped with an Aminex HPX-87P column (Biorad) maintained at 80°C. Degassed water was the mobile phase. The degree of polymerization of the oligosaccharides was estimated by determining the amount of reducing sugars before and after chemical hydrolysis by the method described by Lee et al. (20).

Results

Effect of carbon sources on xylanase synthesis in batch.

Bacteroides xylanolyticus was cultivated and transferred at least three times in media containing as substrate either glucose, arabinose, xylose, pyruvate or xylan. Endo-ß-1,4-xylanase of *B. xylanolyticus* appeared to be an inducible extracellular enzyme activity. The highest level of enzyme in the culture occurred with xylan as carbon source (Table 1). Significantly lower xylanase activities were detected in the cultures after growth on glucose. Fastest growth was observed with D-glucose, D-xylose and L-arabinose. The highest cell yield was obtained with glucose as carbon source. When cells were grown on the pentoses D-xylose or L-arabinose, the bacteria established xylanase activities of about 10% of the activity found with xylan-grown cultures. *B. xylanolyticus* showed poor growth with pyruvate as sole carbon and energy source. No significant xylanase activity could be

detected in these pyruvate grown cultures. Maximal biomass formation with xylan was obtained after 24 h, when cultures were inoculated with 1% of an exponentially growing culture. Xylanase activity was extracellular but mainly associated with the cell fraction during exponential growth. After prolonged incubation, xylanase activity appeared in the cell free medium. The xylanase could be partially released from the cells by extensive washing with 20 mM phosphate buffer.

Table 1. Xylanase activities of *Bacteroides xylanolyticus* on different growth substrates after 24 hours incubation.

Growth substrate	xylanase $\mu mol.min^{-1}.mg^{-1}$
D-glucose (20 mM)	0.02±0.02
L-arabinose (20 mM)	0.03±0.02
D-xylose (20 mM)	0.05±0.02
pyruvate (50 mM)	0.005±0.005
oat spelt xylan (0.4%)	0.35±0.1
birch wood xylan (0.4%)	0.25±0.1
larch wood xylan (0.4%)	0.35±0.1

Effect of carbon sources on xylanase activity in glucose pregrown concentrated cell suspensions.

Since only xylan-grown cells showed significant xylanase activities, the effect of possible xylan degradation products, xylo-oligomers and xylose, on the xylanase formation in concentrated cell suspensions was tested. Cells pregrown on glucose were concentrated 10 times and incubated in fresh medium with various carbon sources. Cells grown on glucose always showed low xylanase activity. This activity varied in the different batches between 0.005 and about 0.05 $\mu mol.min^{-1}.mg\ protein^{-1}$. When no carbon source was added to the concentrated cell suspensions, no increase in xylanase activity could be observed (data not shown). Incubation of the concentrated cells with a mixture of short xylooligomers (2.2

mM xylose, 3 mM xylobiose and 0.8 mM xylotriose) resulted in a rapid 10-fold increase in xylanase activity (Figure 1A).

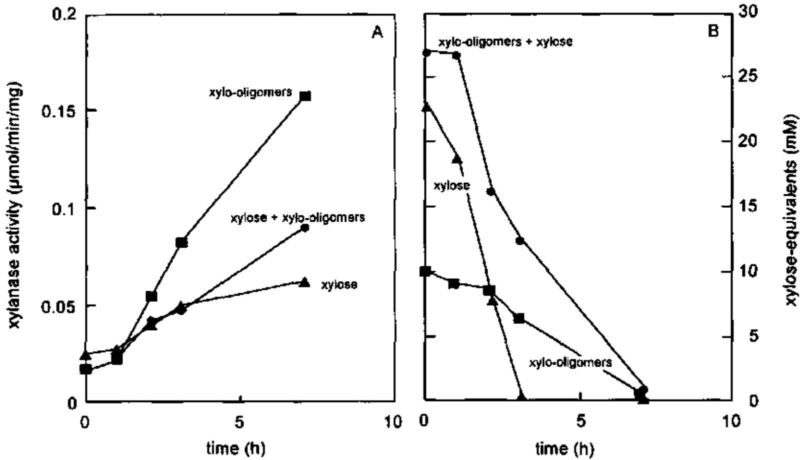

Fig. 1. A: Xylanase induction in concentrated cell suspensions of *B. xylanolyticus* X5-1, pregrown on glucose, in the presence of short xylo-oligomers (■), xylo-oligomers plus 20 mM xylose (●), and 22 mM xylose alone (▲). B: Concentration of the xylo-oligomers (■), xylo-oligomers + xylose (●), and xylose (▲) expressed in total xylose-equivalents.

Addition of xylose (20 mM) together with the xylo-oligomers resulted in a significant lower increase in xylanase activity. In the presence of only xylose (22 mM) the cells produced xylanase at a comparable rate. After about 3.5 hours of incubation, when all the xylose was consumed, xylanase production stopped. Consumption of the xylo-oligomers by the concentrated cell suspensions was slow compared to the consumption of xylose in the absence of oligomeric sugars (Figure 1B). Increasing the initial xylose concentration did not result in a higher production of xylanase. In cell suspensions incubated with 15 mM or 40 mM xylose, the sugar was consumed at about the same rate in both incubations, but no significant xylanase activity increase could be observed in the 40-mM incubation.

Xylose, in this case, apparently repressed the xylanase synthesis. To test if the increase in xylanase activity in the presence of low concentrations of xylose or xylooligomers, was just an effect of energy- or carbon-source in the absence of a catabolite repressor, we incubated the concentrated cell suspensions, pregrown on glucose, with a non-sugar carbon source. When pyruvate was added to glucose pregrown concentrated cell suspensions a considerable increase in xylanase activity could be observed (Figure 2). The increase in xylanase activity was proportional to the decrease of pyruvate and continued until pyruvate was completely consumed. When rifampicin at a concentration of 5 µg/ml was added together with pyruvate, xylanase induction was significantly lower (Figure 2). Pyruvate consumption was not inhibited by this concentration of rifampicin.

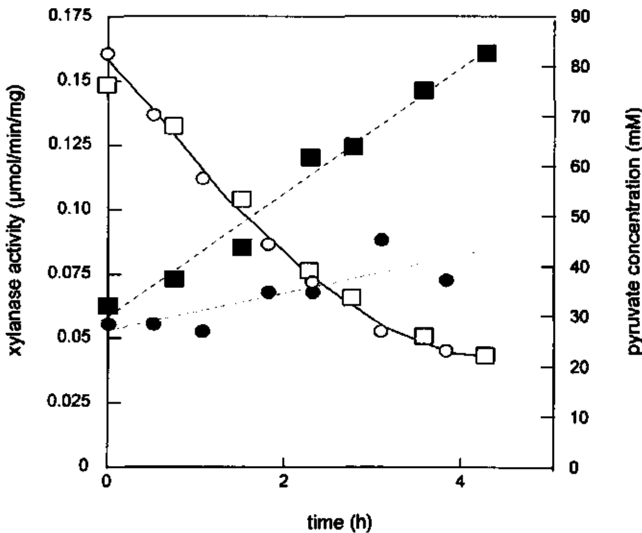

Fig. 2. Xylanase induction in concentrated cell suspensions of *B. xylanolyticus* X5-1 by pyruvate in the presence and absence of 5 µg/ml rifampicin. Cells were pregrown on glucose. ■, xylanase activity induced by pyruvate; ●, xylanase activity induced by pyruvate in the presence of rifampicin; □, pyruvate concentration; ○, pyruvate concentration in the presence of rifampicin.

Addition of 10 mM xylose together with 75 mM pyruvate resulted in a decrease of xylanase production to a certain minimum level (Figure 3), comparable to the xylanase

production level obtained with 17 mM xylose alone (Figure 4). The readily metabolisable sugars like D-glucose, L-arabinose and cellobiose (all 10 mM) also repressed the xylanase synthesis induced by pyruvate.

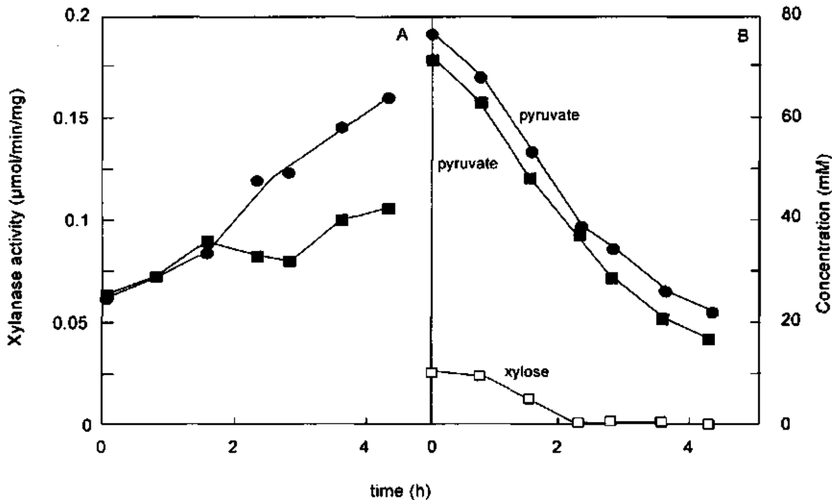

Fig. 3. Xylanase induction in concentrated cell suspensions of *B. xylanolyticus* X5-1, pregrown on glucose, by pyruvate in the presence and absence of 10 mM xylose. A: ●, xylanase activity induced by pyruvate; ■, xylanase activity induced by pyruvate plus xylose. B: pyruvate concentration in the absence of xylose (●), and in the presence of xylose (■); xylose concentration (□).

D-arabinose repressed the xylanase induction by pyruvate in the same way although this sugar is not metabolised by *B. xylanolyticus* X5-1 (Figure 4). The xylanase induction by pyruvate plus D-arabinose was comparable to the induction by xylose alone. In all these cases, the presence of sugars had no effect on the pyruvate consumption. When xylo-oligomers (5 mM) were incubated together with pyruvate, the xylanase induction was not repressed.

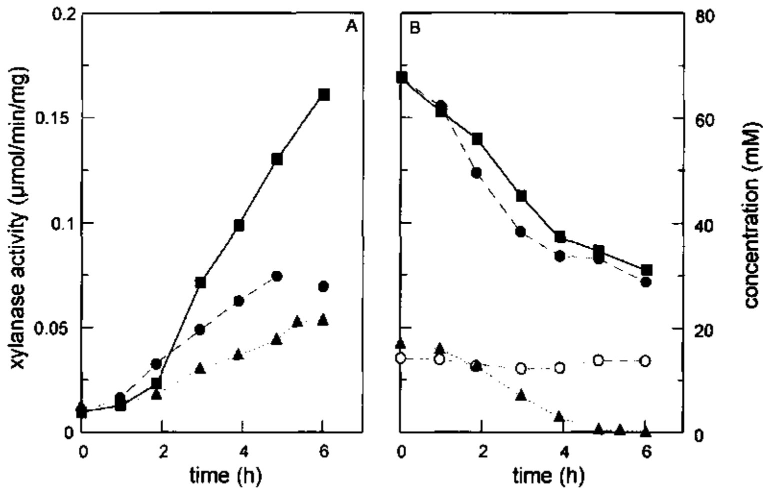

Fig. 4. Xylanase induction in concentrated cell suspensions of *B. xylanolyticus* X5-1, pregrown on glucose, by pyruvate in the presence and absence of 15 mM D-arabinose. A: ■, xylanase activity induced by pyruvate; ●, xylanase activity induced by pyruvate plus D-arabinose; ▲, xylanase activity induced by 17 mM xylose. B: pyruvate concentration in the absence of D-arabinose (■), and in the presence of D-arabinose (●); D-arabinose concentration (O); xylose concentration (▲).

Fumarate is not a growth substrate for *B. xylanolyticus* X5-1. However, concentrated cell suspensions of *B. xylanolyticus* X5-1 can convert fumarate to mainly acetate and ethanol, with malate as an accumulating intermediate (data not shown). Concentrated cell suspensions incubated with 20 mM fumarate showed no significant increase in xylanase activity. In contrast, when both fumarate and acetol were present, a 4-fold increase in xylanase activity could be observed. Acetol, which is converted to propanediol, can act as an electron acceptor (5), regenerating NAD^+ and preventing the accumulation of malate and consequently facilitating the conversion of fumarate to pyruvate. The end product shifts from ethanol to acetate.

Table 2. Xylanase activities of *Bacteroides xylanolyticus* on different growth substrates at steady state conditions in chemostat cultures.

dilution rate (h^{-1})	Carbon source	$conc._{in}$ (mM)	$conc._{out}$ (mM)	dry weight (g/l)	xylanase activity ($\mu mol.min^{-1}.mg^{-1}$)
0.015	pyruvate	25.7	0	0.09	0.002
0.086	pyruvate	43.1	2	0.25	0.019
0.20	pyruvate	43.1	12.1	0.19	0.023
0.084	pyruvate*	52.9	15.2	0.24	0.003
0.24	pyruvate*	52.9	34.9	0.096	<0.001
0.015	xylose	20	0	0.30	0.074
0.05	xylose	20	0	0.39	0.045

*: Nitrogen-limited cultures

Effect of pyruvate on xylanase synthesis in continuous culture.

B. xylanolyticus X5-1 grown on pyruvate in batch culture did not produce xylanase activity. Concentrated cell suspensions produced high amounts of xylanase when incubated with pyruvate. Under these conditions no net growth was observed. To test if the growth rate of the culture is of influence on the production of xylanase, *B. xylanolyticus* X5-1 was cultivated in a chemostat with pyruvate as carbon and energy source. Two limitation were imposed on the culture: carbon/energy limitation and nitrogen-limitation. Further more several growth rates were tested: 0.015 h^{-1}, 0.08 h^{-1} and 0.2 h^{-1}. Xylanase activity in the culture was measured for the five different conditions (Table 2). Under none of these conditions significant amounts of xylanase were produced. The xylanase activities of xylose-limited cultures was also determined. Chemostats were run under the same conditions as the pyruvate-limited cultures, with xylose instead of pyruvate as the limiting carbon and energy source. The dilution rates were 0.015 h^{-1} and 0.05 h^{-1}. The xylanase activities of these cultures were 0.074 and 0.045 $\mu mol.min^{-1}.mg^{-1}$ respectively.

Multiplicity of xylanases from *B. xylanolyticus* X5-1.

Cells grown on birch wood xylan, showed two distinct xylanase activity peaks when cell extracts were applied to an anionic exchange chromatography column (Figure 5). The first activity peak, eluting in the void volume fraction was denoted xylanase I. The second activity peak eluted at 200 mM NaCl and was referred to as xylanase II. The xylan source played an important role in the distribution between xylanase I and II. On oat spelt xylan the distribution between xylanase activity peak I and II was in the range of 70-80% to 30-20% respectively.

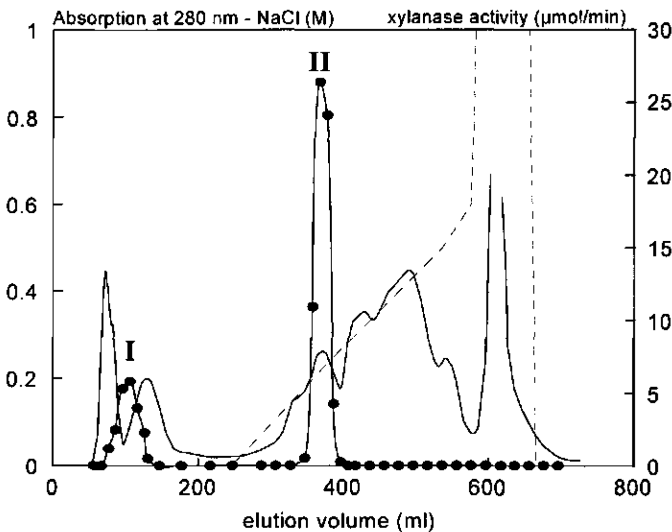

Fig. 5. Typical example of Q-sepharose anion exchange chromatography of cell free extract of *B. xylanolyticus* X5-1, grown on birch wood xylan. Chromatographic conditions are described in Materials and methods. —: Absorption spectrum at 280 nm, - - -: NaCl gradient, —●—: xylanase activity. I: first xylanase activity peak; II, second xylanase activity peak.

However, on birch and larch wood xylan about 60% to 80% of the xylanase activity recovered was xylanase II. Since it was demonstrated that *Bacteroides xylanolyticus* X5-1 produces high levels of xylanase activity when grown on xylan or after incubation of concentrated cell suspensions with pyruvate, it was investigated if there was a difference in the type of xylanase produced under these conditions.

For this purpose the xylanases were purified after growth on birch wood xylan or after induction by pyruvate and xylose, and it was partially purified after induction by short xylo-oligomers, after growth on oat spelt xylan or larch wood xylan. *B. xylanolyticus* X5-1 cells pregrown on glucose, and induced by pyruvate produced high amounts of xylanase I. The purification of xylanase I of pyruvate induced cells is summarized in table 3. The cell free extract of cells induced by pyruvate was fractionated by anionic exchange-, cationic exchange- and hydroxyapatite- column chromatography. The fractions eluting from the hydroxyapatite column, were concentrated by ultrafiltration with a membrane with a molecular weight cut-off of 5000 Da. The enzyme was not retained by a membrane with molecular weight cut-off of 30,000 Da and only in part by a membrane with a cut-off of 10,000 Da. The enzyme fraction eluted as one symmetrical peak from a G-75 gel filtration column.

Table 3. Purification of xylanase I from glucose grown and pyruvate induced cells of *B. xylanolyticus* X5-1.

Purification Step	Total Protein (mg)	Total activity (U)	Specific Activity (U/mg)	Purification (fold)	Recovery (%)
Cell extract	1755	185	0.105	1	100
Q-Sepharose	42	160	3.8	36	86
S-Sepharose	16	140	8.8	84	76
Hydroxyapatite	4.3	72	16.7	159	39
YM5-ultrafiltration	2.3	59	25.4	242	32
Sephadex G75	0.8	27	33.8	322	15

The first xylanase activity peak of the xylan cultures was identical in properties and size to xylanase I, purified after induction by pyruvate. Cells pregrown on glucose and there after induced with 20 mM xylose or short xylooligomers (containing mainly xylose, xylobiose

and xylotriose) (see induction experiments) also predominantly produced xylanase I. No significant amounts of xylanase II could be detected.

The molecular weight of xylanase I was estimated to be 38 kDa in polyacrylamide gel in the presence of sodium dodecyl sulphate (Figure 6) and 29 kDa by gel filtration.

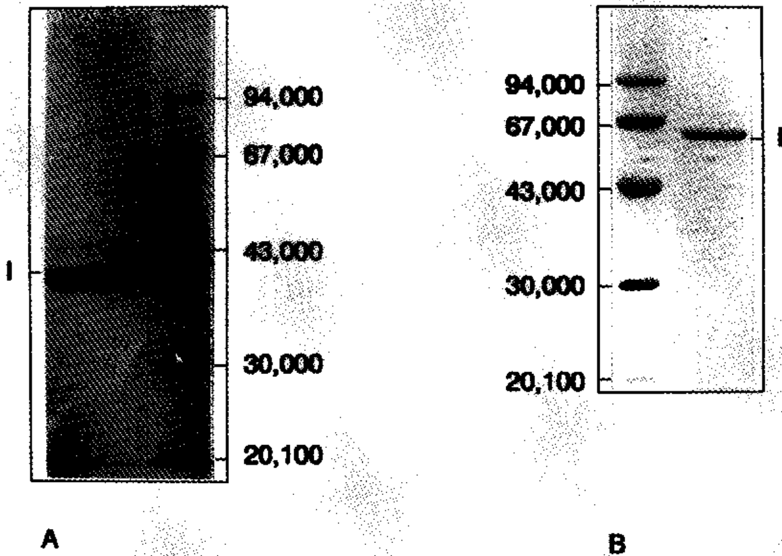

Fig. 6. 12.5% SDS-polyacrylamide gel electrophoresis of purified xylanase I (A) and xylanase II (B). I, 2.3 µg purified xylanase I. II, 1.2 µg purified xylanase II. Molecular size standards 94, 67, 45, 30 and 20.1 kDa respectively.

Xylanase I degraded the polymeric substrate xylan. However, after prolonged incubation of oat spelt xylan with xylanase I, arabinose appeared as hydrolysis product. The release of arabinose by the enzyme coincided with the appearance of xylose. The enzyme hydrolysed a mixture of xylotetraose and arabino-xylotetraose to arabinose, xylose, xylobiose and xylotriose (Figure 7). Xylobiose and xylotriose were not hydrolysed by xylanase I.

The second xylanase was purified from *B. xylanolyticus* cells grown on birch wood xylan. The enzyme purification is summarized in table 4.

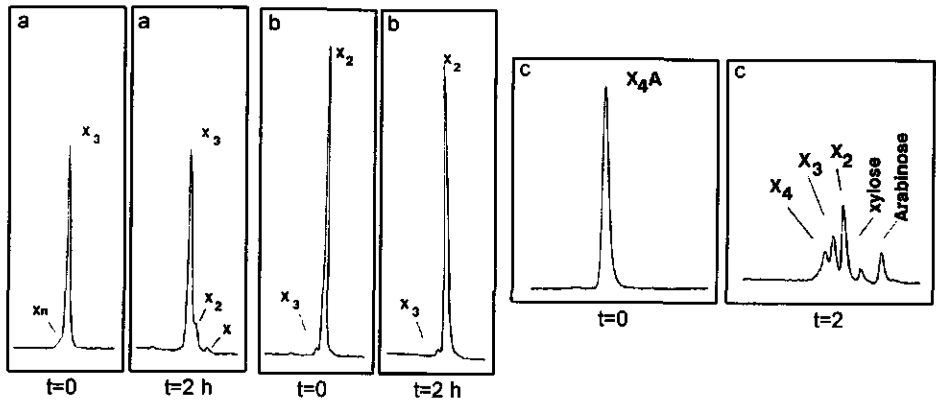

Fig. 7. High-pressure liquid chromatography analysis of the products from hydrolysis of xylo-oligosaccharides by the purified xylanase I of *B. xylanolyticus* X5-1. HPLC analysis was carried out on aliquots of the reaction mixture taken after 0 and 2 hours. a: hydrolysis of xylobiose, b: hydrolysis of xylotriose, c: hydrolysis of a mixture of xylotetraose and arabinose-substituted xylotetraose.

Table 4. Purification of xylanase II from Birch xylan grown cells of *B. xylanolyticus* X5-1.

Purification Step	Total Protein (mg)	Total activity (U)	Specific Activity (U/mg)	Purification (fold)	Recovery (%)
Cell extract	491	148	0.3	1	100
Q-Sepharose	34	111	3.25	10.8	75
Hydroxyapatite	6.5	79	12.1	40.3	53
Phenylsuperose	3.5	68	19.4	64.7	46
Superose 6	1.5	41	27.2	90.7	28

The supernatant of the cell free extract, after centrifugation for 1 h at 100,000 *g* was fractionated by Q-sepharose anionic exchange chromatography. The first of the two xylanase activity peaks (xylanase I) eluted in the void volume and comprised of about 16 % of the xylanase activity (22 Units). The second peak eluted at about 0.3 M of NaCl and accounted for 83% of the recovered xylanase activity. This second peak, denoted xylana-

se II, was used for further purification. The enzyme was purified in three additional steps. The enzyme eluted as one symmetrical peak on chromatography on a suparose-6 gel filtration column. However, the protein eluted outside of the calibration curve. Therefore the molecular weight of the enzyme was estimated by gel filtration on a Sephadex G-75 column.

The molecular weight of xylanase II was estimated to be 63 kDa by SDS-PAGE (Figure 6) and 50 kDa by gel filtration.

Even though the two xylanases showed similar temperature and pH optima (40°C and pH 6.0-6.5 respectively), their final hydrolysis products of oat spelt xylan differed. Xylanase II hydrolysed xylan to small xylooligosaccharides, but was not able to release arabinose from oat spelt xylan or xylo-oligosaccharides. Xylanase II did hydrolyse xylotriose, but not xylobiose (Figure 8). The activities towards these short oligosaccharides was about 5 % of the activity towards the polymeric substrate.

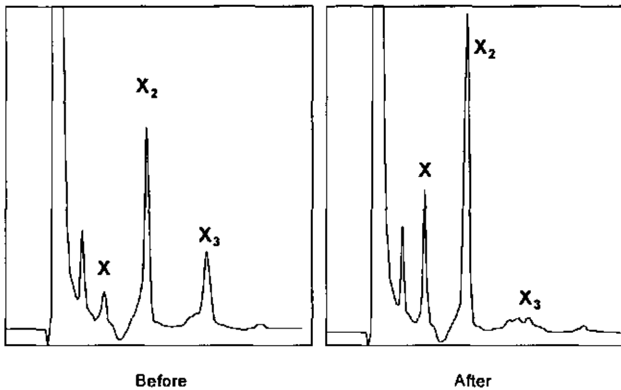

Fig. 8. High-pressure liquid chromatography analysis of the products from hydrolysis of a mixture of xylose (X), xylobiose (X2) and xylotriose (X3) by the purified xylanase II of *B. xylanolyticus* X5-1. HPLC analysis was carried out on aliquots of the reaction mixture taken after 0 and 1 hour.

Discussion

During growth on xylan, *Bacteroides xylanolyticus* X5-1 produces two xylanases. The xylanase activities found with xylan grown cells is comparable to xylanase activities produced by other related *Bacteroides* and *Prevotella* species (15,31,32). The two purified endo-ß-1,4-xylanases from *B. xylanolyticus* are monomeric enzymes with a molecular weight of 38000 (xylanase I) and 63000 Da (xylanase II), respectively. Xylanase I could hydrolyse xylan and xylo-oligomers with a polymerization degree of 4 and higher. Furthermore, this enzyme was able to cleave of arabinose side-chains. The purified enzyme also showed some endo-glucanase activity, although it was not active on crystalline cellulose. Therefore, it is an enzyme with a broad substrate specificity. The final products of the enzymatic degradation of xylan were xylotriose, xylobiose, xylose and arabinose. Xylanase II degraded xylan and xylo-oligomers with a polymerization degree of 3 and higher. Arabinose was not released from oat spelt xylan or substituted oligosaccharides during incubation with xylanase II. During growth the xylanases of *B. xylanolyticus* were cell associated. Polysaccharases of related *Bacteroides* and *Prevotella* strains, and of *Ruminococcus* strains were also found to be cell-associated (15,17,24,33,35).

P. ruminicola, a non-cellulolytic xylan degrading rumen bacterium, has 4 distinct genes coding for xylanase activity. Xylanase A, belonging to family F (18) has a Mw of around 40 kDa and hydrolyses xylan and xylopentaose to xylobiose and xylotriose, similar to xylanase I of *B. xylanolyticus*, but it is not able to release arabinose from oat spelt xylan (17). Most reports on arabinose releasing activity concern xylanases of fungal origin (37). One other mesophilic anaerobic bacterium, *Fibrobacter succinogenes,* was reported to produce a 53.7 kDa xylanase with arabinose releasing activity (22). This enzyme released the arabinose from the polymeric substrate, where xylanase I of *B. xylanolyticus* released arabinose after prolonged incubation with xylan, and after incubation with arabinose containing xylo-oligomers. Since arabinose release coincided with xylose appearance in the assays, this side activity could be the result of a-specific cleavage. On the other hand,

this activity may also be the result of a second catalytic domain with arabinosidase activity. Xylanases with multiple catalytic domains have been described for *Fibrobacter succinogenes* and other anaerobic bacteria (38, 23).

B. xylanolyticus cells grown on glucose produced only negligible levels of xylanase activity. The synthesis of the xylanolytic enzymes was regulated in this organism. *B. xylanolyticus* had a different regulation for the synthesis of the two xylanases. Xylanase I production did not seem to be induced by a direct product of xylan degradation. It could be detected with cells grown on xylan and with resting cells induced by xylo-oligomers and low concentrations of xylose. Xylose had a dual role in that it induced the formation of low levels of xylanase at low concentrations, but at high concentrations (40 mM) it completely repressed the enzyme synthesis. The good induction of xylanase formation in the presence of short xylo-oligosaccharides probably best mimicked the natural situation, although the xylobiose and xylotriose probably are not direct inducers. This particularly because pyruvate was a very good inducer, when added to resting cells. Pyruvate, an intermediate of the sugar metabolism, probably acted as a good inducer because it circumvents the catabolic repression normally occurring when easily metabolisable sugars are available for the cells. The effect of the added antibiotic rifampicin indicated a possible transcriptional control of xylanase I synthesis. Slow growth alone, as observed during growth on pyruvate, was insufficient to promote xylanase formation. Under growing conditions with pyruvate as sole carbon source and in chemostat cultivations, under carbon-source or nitrogen-limitations, *B. xylanolyticus* did not produce significant levels of xylanase activity, probably because of the slow uptake rate and energy yield under these conditions. In several anaerobic bacteria a lower level of xylanase activity is found at higher dilution rates (24,35). A derepression under conditions of substrate limitation, as reported for some other anaerobic bacteria, was not observed in xylose-limited cultures (9, 20). In a *Butyrivibrio fibrisolvens* strain grown in a chemostat, appreciable levels of xylanase were detected only when xylan was used as the substrate (36). *B. xylanolyticus* could not grow on fumarate but resting cell suspensions were able to consume this substrate. Significant levels of xylanase activity were not detected under these conditions, although

fumarate, like pyruvate, is also a non-sugar substrate. The conversion of the accumulating malate and the regeneration of NAD^+ was a limiting factor, which leads to the production of mainly ethanol resulting in a very low net energy yield. Acetol could act as an external electron acceptor, and by conversion to 1,2-propanediol NAD^+ was regenerated (5). In the presence of acetol, fumarate was converted mainly to acetate yielding more ATP and this resulted in a 4-fold increase in xylanase activity. The induction of xylanase I by pyruvate was repressed by readily metabolizable sugars like D-glucose, cellobiose, D-xylose and L-arabinose (all 10 mM). The formation of xylanase was not completely repressed by the addition of these sugars, but was reduced to a level of about 25 % of the xylanase activity obtained in incubations with pyruvate only. *B. xylanolyticus* was able to produce xylanase activity at a low level even in the presence of easily metabolisable pentoses, which are released during the xylan degradation. An interesting feature was the repression of xylanase synthesis, induced by pyruvate, in the presence of the non-metabolisable sugar D-arabinose. The pyruvate consumption was not inhibited, when D-arabinose was present. Assuming that D-arabinose was taken up, and not further metabolised, the repression of the xylanase I formation by sugars must involve the sugar-uptake system. Xylooligosaccharides did not repress the induction of xylanase by pyruvate. Regulation of xylanase I can be interpreted as constitutive synthesis under catabolite control. The availability of enough metabolic energy is essential for xylanase I formation, while sugars present in the medium repress xylanase I formation. The repression likely is at the level of the sugar uptake, but does probably not involve cyclic-AMP and PTS-mediated repression, because *B. xylanolyticus* and related bacteria have a non-PTS active uptake system for pentoses and the levels of cyclic nucleotides in these bacteria are very low (4, 30, 10).

Xylanase II was only produced in significant amounts when the organism was grown on xylan. On monomeric sugars, as well as short xylo-oligomers, no xylanase II was produced. The formation of this enzyme was also catabolite repressed by easy metabolisable substrates. Pyruvate did not induce xylanase II. There was a big difference between the amount of xylanase II produced on the three xylan types tested. Xylanase II was produced in much higher amounts on birch- or larch-wood xylan, compared to oat spelt

xylan. We were not able so far to identify the exact nature of the inducer of this enzyme. In *Streptomyces lividans* one of the xylanases is only produced when a xylo-oligomer with a polymerisation degree of 11 is present, or when one of the other xylanases is active and produces a specific xylan degradation product (1). In the case of *B. xylanolyticus* one could think of a xylooligomer containing specific side chains, found in wood-xylan more than in grass xylan.

Bacteroides xylanolyticus X5-1 is able to fine tune its xylanase synthesis according to demand. It is possible that the difference in substrate specificities of the two purified xylanases reflect their role in xylan degradation. Xylanase I has a broad specificity and is produced constitutively. Xylanase II may be better adapted to attack wood xylan, because it is preferentially produced when grown with these substrates. It is possible that this organism is even able to produce more than two different xylanases. The genetic analysis of related bacteria revealed that genes encoding for several different xylanases may be present in the genome (21, 16).

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Chapter 3

Regulation, Purification and Characterization of the *β*-Xylosidase of *Bacteroides xylanolyticus* X5-1.

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Abstract

The regulation of the formation of a *β*-xylosidase by *Bacteroides xylanolyticus* X5-1 was investigated. The enzyme was produced after growth on xylan or the pentoses D-xylose or L-arabinose. No enzyme was detected after growth on glucose or cellobiose. High concentrations of xylose, other easy metabolisable sugars and pyruvate repressed the enzyme synthesis. The *β*-xylosidase production is induced by xylose or arabinose and repressed by an intermediate of the metabolism of sugars and pyruvate. The *β*-xylosidase induced by xylose was purified by column chromatography. The enzyme had an apparent molecular weight of 165 kDa as estimated by gel filtration. SDS-polyacrylamide gel electrophoresis revealed one single protein band corresponding to a subunit of 85 kDa. The enzyme exhibited optimal activity at pH 6 and 40°C. The isoelectric point was 6.3. It hydrolyzed *p*-nitrophenyl-xyloside with a K_M of 0.125 mM. The activity was strongly inhibited by Hg^{2+} at concentrations as low as 10 μM. The enzyme hydrolyzed short xylo-oligosaccharides into xylose; xylan and other *p*-nitrophenylglycosides were no substrates for the enzyme.

Introduction

Xylan is a widely distributed type of hemicellulose located in the cell walls of plants. In some plants xylan can account for as much as 20 to 30 % of the dry weight. Effective utilization by microbial processes would be of great economical interest. Complete breakdown of branched xylan requires the action of several hydrolytic enzymes (4,29): endo-1,4-*β*-D-xylanases (EC 3.2.1.8), *β*-D-xylosidases (EC 3.2.1.37), and several enzymes involved in the breakdown of substituents attached to the xylan backbone. *β*-Xylosidases cleave of xylose residues from the non-reducing end of short xylooligosaccharides (29). Xylan-degrading enzymes are produced by a wide variety of microorganisms, including aerobic and anaerobic mesophiles and thermophiles (4,29,42). Although many fungal

β-xylosidases have been isolated and characterized (15,20,21,34,36), and several β-xylosidases from thermophilic bacteria have been isolated (1,14,23), relatively little is known about the enzyme of mesophilic anaerobic bacteria (18). The substrate range of the described β-xylosidases varies; some have an additional α-L-arabinofuranosidase or β-glucosidase activity (7,18,28,33,35,37), whereas others are not able to hydrolyse the natural substrate xylobiose (14).

Xylanases and xylosidases are produced in high amounts during growth on xylan and the synthesis of these enzymes is often catabolite repressed by monomeric sugars (4,39,40). High molecular weight xylan can not enter the cells, and consequently can not directly induce the synthesis of xylanolytic enzymes. Low molecular weight sugars are often involved in either the induction and/or the repression of xylan degrading enzymes (3,12,22,39,41,43). The β-xylosidase affects the concentration of its substrates, short xylo-oligomers like xylobiose and xylotriose, as well as its final product xylose. Therefore, the enzyme can play an important role in the regulation of the different components of the xylan degrading complex. The β-xylosidases of some microorganisms also show glycosyl transferase activity (14,29), leading to the formation of specific oligosaccharides. This activity possibly plays an important role in the regulation of xylan degrading enzymes in these organisms. Hrmova et al. (13) showed for example that these enzymes are induced by heterodisaccharides composed of glucose and xylose in *Aspergillus terreus*.

The mechanism of control of xylanolytic enzyme synthesis varies considerably among different microorganisms as is revealed by an analysis of literature data (4,12,17,22,39,40,41,43). Induction, catabolite repression, growth rate and other environmental factors can influence the activity of the xylanolytic enzymes. The present paper deals with the regulation, and the purification and characterization of the β-xylosidase of *Bacteroides xylanolyticus* X5-1. *Bacteroides xylanolyticus* X5-1, an anaerobic bacterium isolated from fermenting cattle manure by Scholten-Koerselman et al. (30), grows efficiently on xylan but not on cellulose. Several xylanolytic enzyme activities have been detected in cultures of *Bacteroides xylanolyticus* X5-1 grown on xylan (31).

Materials and methods

Growth conditions. *Bacteroides xylanolyticus* X5-1 (DSM 3808) was isolated and described by Scholten-Koerselman (30). The organism was cultured at 37°C in a bicarbonate buffered medium as described by Biesterveld et al. (5) supplemented with 0.4 g of tryptone (Oxoid ltd, Basingstoke, England) per liter. When xylan was used as the substrate, it was added to the medium prior to autoclaving the culture-bottles. Other substrates for growth were added separately as filter-sterilized solutions. Growth was monitored by measuring the hydrogen production, or by analyzing the clarified growth medium by HPLC as described before (8). The latter method enabled the simultaneous determination of sugar consumption and the formation of fermentation products ethanol and acetate. In batch the organism was routinely cultured in 120-ml bottles containing 50 ml medium and a gas phase of 180 kPa N_2/CO_2 (80%/20%).

Mass-cultivation was performed at 37 °C in 8-l bottles. Cells were harvested anaerobically with a Heraeus Sepatech Biofuge 28RS continuous-flow centrifuge, yielding 0.5-1.0 g wet cells per liter.

Continuous cultivation was performed at 37 °C in 1-l chemostats with a working volume of 500 ml. The same medium as for batch cultivation was used except that 2 g/l of yeast extract was added instead of 0.5 g/l and that tryptone was omitted. The pH was maintained at 7.0 ± 0.1 with 2 N NaOH. A continuous stream of N_2/CO_2 (80%/20%) at a flow rate of 130 ml per hour was led over the cultures. To guarantee steady-state conditions, cells were analyzed after at least 6 volume changes. Bacterial dry weight was quantified as described by Biesterveld et al. (5).

Preparation of cell-free extract. Cells were suspended in 50 mM Tris-HCl pH 7.5 (0.2 g wet cells/ml buffer) and disrupted by sonification at 0°C (5 times 20 s at 40 W), using a Branson Sonic Sonifier (Danbury, CT, USA). The broken cells were centrifuged for 20 min at 10,000x*g* and the supernatant was centrifuged again at 100,000x*g* for 1 h at 4°C. The supernatant contained about 20 mg protein per ml and was designated as cell free extract.

Enzyme assay. *β*-xylosidase (EC 3.2.1.37) was assayed routinely using *p*-nitrophenyl-*β*-D-xylopyranoside as an artificial substrate. Enzyme activity was determined by measuring the amount of *p*-nitrophenol released from the substrate. The assay mixture (0.4 ml) contained 1 mM substrate in 50 mM potassium phosphate (pH 6.5). The incubation temperature was 40°C unless indicated otherwise. The reaction was terminated by the addition of 0.8 ml of 0.5 M sodium carbonate. The released *p*-nitrophenol was determined spectrofotometrically at 405 nm. One unit of activity was defined as the amount of enzyme releasing 1 µmol of *p*-nitrophenol per min.

β-Xylosidase was also assayed using xylo-oligomers as substrate. The hydrolysis experiments were performed at 37 °C in 1 ml 50 mM potassium phosphate buffer (pH 6.5) supplemented with 1 mM DTT. Purified *β*-xylosidase (0.1 U according to the *p*-nitrophenol assay) was incubated during 2 hours with 0.8 mM xylobiose or xylotriose. 1.75 mM Xylotetraose and bigger oligomers were incubated with 10 times more *β*-xylosidase during 3.5 hours. The reactions were stopped by boiling the reaction mixture for one min. The hydrolysates were analyzed by HPLC equipped with a Biorad Aminex 87P carbohydrate column as described previously (8). One unit of *β*-xylosidase activity is the amount of enzyme releasing 1.0 µmol of xylose per min.

Protein determination. Protein was determined with Coomassie brilliant blue G250 as described by Bradford (6). Bovine serum albumin was used as a standard.

Enzyme induction experiments. Cells (0.5 - 2 l) were grown on 20 mM glucose, harvested anaerobically in the late exponential phase by centrifugation at 10,000 x *g* for 10 min, washed with anaerobic medium without substrate, and resuspended in a 10 % volume of fresh medium containing the appropriate carbon source. The cell suspensions were incubated at 37°C and at different time intervals 1-ml samples were taken. Cells were centrifuged at 10,000 x *g* for 5 min, washed with 50 mM potassium phosphate buffer (pH 6.5) and resuspended in 500 µl of 50 mM Tris/HCl buffer (pH 7). Thereafter, *β*-xylosidase activities were determined in cell free extracts.

Polyacrylamide gel electrophoresis (PAGE). SDS PAGE was performed using 12.5% (w/v) gels according to the method of Laemmli (16). Protein was stained with Coomassie

brilliant blue. Molecular weight of the subunits was estimated by comparison with protein standards; trypsin inhibitor (M_r 20100), carbonic anhydrase (M_r 30000), ovalbumin (M_r 45000), bovine serum albumin (M_r 67000) and phosphorylase b (M_r 94000) (Pharmacia Fine Chemicals, Uppsala, Sweden).

Preparation of xylo-oligosaccharide mixture. Oat spelt xylan (lot no. 38F-0722, Sigma chem. co., St. Louis, MO, USA) was pretreated with amyloglucosidase (from *Rhizopus*, lot no. 48B-2186, Sigma chem. co., St. Louis, MO, USA) to remove contaminating starch. After ethanol precipitation, the xylan was resuspended (50 g/l) in 20 mM acetate buffer (pH 5.0) and partially hydrolysed with a xylanase preparation from *Trichoderma viride* (lot no. 95595, Fluka Biochemika, Buchs, Switzerland) at 37°C. The reaction was stopped by boiling for 5 min. The soluble fraction was concentrated by rotary evaporation. The oligosaccharides were separated by repeated gel filtration on a column (100 x 2.2 cm) of Bio-gel P2 (Bio-Rad, Richmond, CA, USA) (fractionation range 100-1800). Sugars were analyzed by HPLC as described previously (8). The degree of polymerization of the oligosaccharides was estimated as described by Lee et al. (17).

Enzyme purification. All purification steps were performed at room temperature. Cell-free extract was applied to a Q-Sepharose Fast Flow (Pharmacia Fine Chemicals, Uppsala, Sweden) column (3.2x17 cm) equilibrated with 50 mM Tris-HCl (pH 7.8) containing 1 mM DTT (buffer A). The column was developed (2 ml/min) with a 400-ml linear gradient (0-0.7 M NaCl in buffer A). The active β-xylosidase eluted at 0.5 M NaCl. Fractions with activity were combined, concentrated by ultrafiltration on an Amicon PM 30 membrane (Grace, Rotterdam, The Netherlands). Ammonium sulfate was added to the enzyme preparation to a final concentration of 0.5 M. Then, it was applied to a phenyl sepharose column equilibrated with buffer A with 0.5 M ammonium sulfate and eluted with a linear gradient of 0.5 M to 0 M ammonium sulfate in buffer A. The activity peak eluting at 0.25 M ammonium sulfate was pooled, diluted with 4 volumes of water and applied in fractions of 10 ml onto a Mono-Q HR 5/5 column, equilibrated with buffer A (1 ml/min). The enzyme eluted from the column as one concentrated peak at 0.45 M NaCl in a linear gradient of 15 ml (0-0.7 M NaCl in buffer A). Fractions with β-xylosidase activity

were combined concentrated in a PM 30 Centricon unit (Grace, Rotterdam, The Netherlands), and 250 μl was injected onto a Superose 6 HR 10/30 gelfiltration column (V_0=7.64 ml; V_t=23.4 ml) equilibrated in buffer A plus 100 mM NaCl. The column was developed at a flow rate of 0.25 ml/min. The colourless *β*-xylosidase eluted as one symmetrical peak. The same procedure was also used for molecular mass determination of the native enzyme. The following standards (Pharmacia Fine Chemicals, Uppsala, Sweden) were used: thyroglobulin 669 kDa, ferritin 445 kDa, catalase 232 kDa and aldolase 158 kDa.

Determination of pI. Isoelectric focusing was performed at 25 °C from pH 4 to 10 as described by O'Farell (25). The following pI markers were used (Pharmacia Fine Chemicals, Uppsala, Sweden): C-phycocyanin from *A. nidulans* (pI-4.75 and 4.85), azurin from *P. aeruginosa* (pI-5.65), trifluoroacetylated porcine myoglobin met (pI-5.92), porcine myoglobin met (pI-6.45), equine myoglobin met (pI-7.3), sperm whale myoglobin met (pI-8.3), horse heart cytochrome-c (pI-10.6). Proteins were stained with Coomassie Brilliant Blue.

Temperature stability. For determination of the temperature stability the purified enzyme preparation was incubated for 10 min. in 50 mM potassium phosphate buffer (pH 6.5) at different temperatures (20-50°C), and at different time intervals the samples were assayed for residual *β*-xylosidase activity at 40°C.

pH optimum. The dependence of the purified *β*-xylosidase on pH was determined in Tris/maleate, Tris/HCl and sodium acetate (50 mM) buffer between pH 4.3-8.9 at 37°C.

Results

Effect of carbon sources on the *β*-xylosidase production by *Bacteroides xylanolyticus* X5-1 grown in batch culture. *B. xylanolyticus* was grown on various carbon sources. After 24 hours of growth cells were harvested and *β*-xylosidase activity levels in cell extracts were measured (Table 1). When cells were grown on xylan the *β*-xylosidase activity was cell associated. Only a very low activity was found in the cell free medium.

Table 1. ß-Xylosidase activities of *Bacteroides xylanolyticus* X5-1 grown on various carbon sources.

carbon source	concentration	ß-xylosidase activity (μmol min^{-1} mg^{-1})
glucose	20 mM	< 0.01
pyruvate	60 mM	< 0.01
xylan	0.4% (w/v)	0.5 ± 0.2
D-xylose	20 mM	2.5 ± 0.2
D-xylose	30 mM	1.3 ± 0.2
D-xylose	40 mM	0.25 ± 0.2
L-arabinose	20 mM	2.75 ± 0.5

The organism grew efficiently on glucose, but only negligible *β*-xylosidase activity was detected in cell extracts of glucose-grown cells. Growth on pyruvate was slow; in pyruvate-grown cells also no *β*-xylosidase activity was detected. High levels of *β*-xylosidase were produced during growth on 20 mM D-xylose or 20 mM L-arabinose. When higher concentrations (30 mM or 40 mM) of D-xylose were used, xylose was not completely consumed; only about 18 to 20 mM xylose was consumed in 24 hours. Increasing the initial xylose concentration had a dramatic effect on the *β*-xylosidase activity in the cultures. A 10-fold reduction of the enzyme activity was observed when the xylose concentration in the growth medium was increased from 20 to 40 mM.

Effect of the growth rate on the production of *β*-xylosidase in a chemostat. *B. xylanolyticus* was grown in a chemostat with xylose (10 mM) as substrate. At low dilution rates xylose was growth limiting, and no *β*-xylosidase activity could be detected in the culture. An increase in the dilution rate had no effect on *β*-xylosidase production as long as the substrate remained limiting (Fig. 1). At growth rates higher than 0.2 h^{-1}, xylose was not completely consumed. Under these conditions *β*-xylosidase was produced by the organism. When 20 mM glucose was used as carbon source in a similar experiment, *β*-xylosidase activity was never detected in the cultures (data not shown).

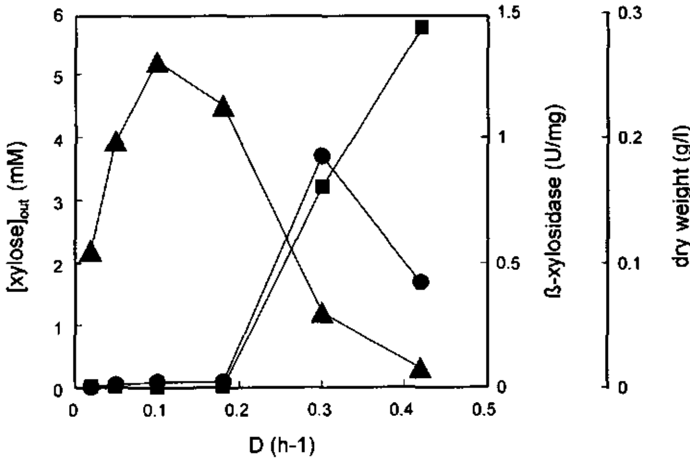

Fig. 1. Effect of the dilution rate (growth rate) on the specific activity of *β*-xylosidase (●), residual xylose concentration (■) and dry weight (▲) in a xylose-limited continuous culture of *Bacteroides xylanolyticus* X5-1.

Enzyme induction in the presence of various glycosides and oligosaccharides. Concentrated cell suspensions of glucose-pregrown cells were incubated in fresh medium containing various carbon sources. After 6 hours cells were harvested and *β*-xylosidase activities were measured in cell extracts (Table 2). Only the pentoses D-xylose and L-arabinose efficiently induced *β*-xylosidase activity. D-ribose was only slowly consumed and did not yield an increase in *β*-xylosidase activity. D-arabinose, which was not consumed, did not lead to *β*-xylosidase production. Mono-, di- or trimers from hexoses added together with xylose almost completely repressed the induction of *β*-xylosidase. Addition of glucose had an immediate repressive effect on the *β*-xylosidase induction by xylose (Fig. 2). In this experiment both sugars were consumed simultaneously.

Table 2. Effect of different sugars on the induction of ß-xylosidase activity in concentrated cell suspensions of glucose pregrown cells of *B. xylanolyticus*.

inducer	percentage ß-xylosidase activity
13 mM xylose	100 %
13 mM L-arabinose	80 - 100
13 mM D-arabinose	< 1
13 mM D-ribose	4
10 mM glucose	1
13 mM xylose +	
+ 12 mM fructose	6
+ 12 mM glucose	3
+ 6 mM trehalose	2
+ 3.4 mM raffinose	9
no addition	< 1

[a] 100% = 0.8 μmol min^{-1} mg^{-1}

Effect of the xylose concentration on the *β*-xylosidase production. Concentrated cell suspensions were incubated with different amounts of xylose. Culture samples were periodically taken, and *β*-xylosidase activity in cell extracts was measured (Fig. 3). The rate of *β*-xylosidase production as well as the ultimate amount of *β*-xylosidase were affected by the xylose concentration in the incubation. The incubations with an initial xylose concentration of 8 mM, showed a fast increase in *β*-xylosidase activity. After about three hours, xylose had been completely consumed and the increase in *β*-xylosidase activity stopped. The highest final level of *β*-xylosidase activity was reached with 18 mM xylose. Incubations with 40 mM xylose showed a markedly lower increase in *β*-xylosidase activity. The induction of *β*-xylosidase apparently was repressed by high concentrations of the inducer. A similar repression of *β*-xylosidase synthesis was found when the cell suspensions were incubated with pyruvate together with 18 mM xylose. The addition of 90 mM of pyruvate almost completely repressed the *β*-xylosidase induction by 18 mM xy-

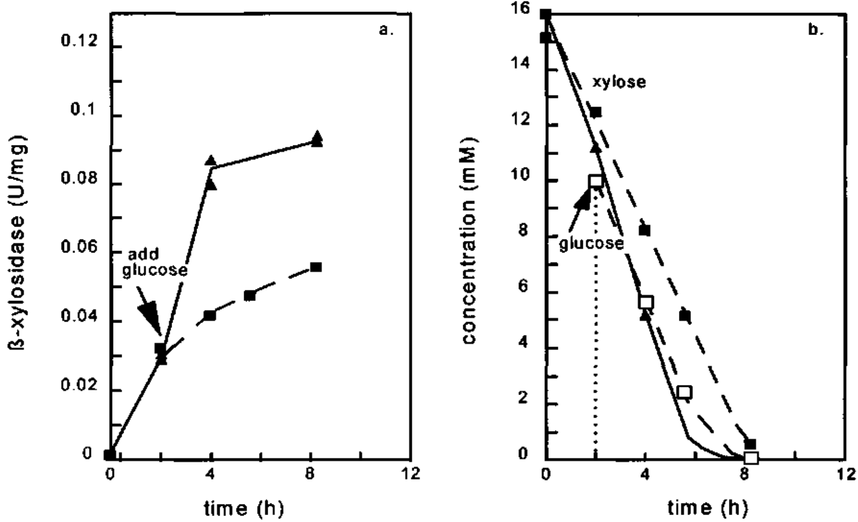

Fig. 2. Effect of glucose addition on the induction of *β*-xylosidase activity in cell suspensions of glucose grown *B. xylanolyticus* cells. At time zero the cells were supplied with 16 mM of xylose. After 2 hours glucose was added. a: *β*-xylosidase activity in absence of glucose (—▲), and after addition of 10 mM glucose (--■). b: Xylose consumption in the presence (--■) and the absence (—▲) of glucose, and (--□) glucose consumption.

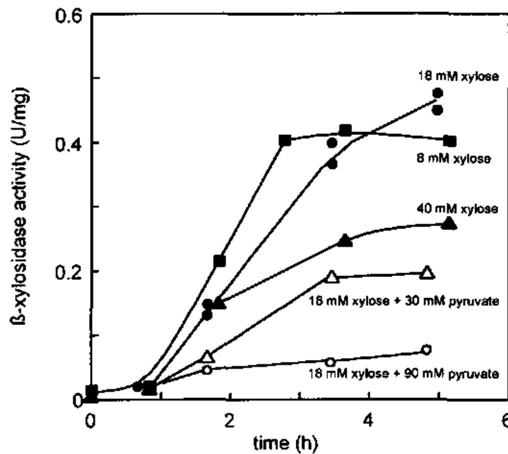

Fig. 3. *β*-Xylosidase induction in concentrated cell suspensions of *B. xylanolyticus* X5-1 by different concentrations of xylose or xylose plus pyruvate. Cells were pregrown on glucose.

lose, whereas 30 mM pyruvate partially repressed the *β*-xylosidase induction. When only pyruvate was present in the incubation, *β*-xylosidase activity was not induced.

Table 3. Purification of ß-xylosidase from a 8-l culture of *Bacteroides xylanolyticus* X5-1, pregrown on glucose, and then induced by xylose.

	Total protein (mg)	Total activity (μmol min^{-1})	Specific activity (μmol min^{-1} mg^{-1})	Recovery (%)	Purification (fold)
Cell extract	1050	213	0.2	100	1
Q-sepharose	45	148	3.3	69	16
Phenyl-sepharose	3.6	25	7	12	35
Mono Q	0.37	10	28	5	140
Superose 6	0.06	2	31	0.9	154

Purification of *β*-xylosidase induced by D-xylose. Results of a typical enzyme purification are summarized in table 3. An 8-l culture of *B. xylanolyticus* grown on glucose was harvested anaerobically and incubated during 6 hours at 37 °C in 800 ml fresh medium containing 20 mM xylose. The cells were harvested, washed and cell extracts were made by sonification. The cell free extract of induced cells was fractionated by Q-sepharose anion exchange column chromatography. The *β*-xylosidase activity eluted from the column as one peak at about 0.5 M NaCl. Although essential for the purification, the use of a hydrophobic interaction column greatly reduced the recovery and the stability of the enzyme. When chromatographed on superose-6, the enzyme eluted as a symmetrical peak. The enzyme was purified about 154 fold with a recovery of only 0.9 %. The low recovery was mainly due to the instability of the enzyme.

Properties of the purified *β*-xylosidase. The *β*-xylosidase eluted from the suparose 6 HR10/30 column at a volume which corresponded to a molecular mass of 165 kDa, as compared to standards of known molecular mass. SDS/polyacrylamide gel electrophoresis of the purified *β*-xylosidase revealed one subunit with molecular mass of 83 kDa, which suggest an α_2 subunit stoichiometry for the native enzyme (Fig. 4).

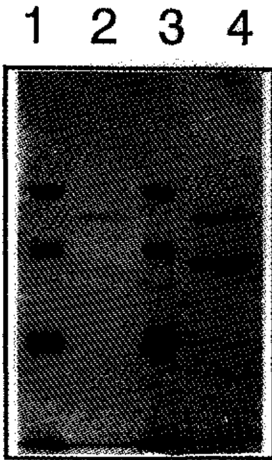

FIG. 4. 12.5 % SDS-PAGE gel of the purified *β*-xylosidase. Lanes 1 and 3, molecular size standards (94000, 67000, 45000 and 30000 respectively); lane 2, 0.5 μg of purified *β*-xylosidase; Lane 4, 1 μg of enzyme preparation prior to final gelfiltration step.

It was not possible to determine the native molecular mass or the isoelectric point using native polyacrylamide gel electrophoresis. The purified *β*-xylosidase easily aggregated causing it to migrate through the gel as a high molecular weight complex (> 800 kDa).

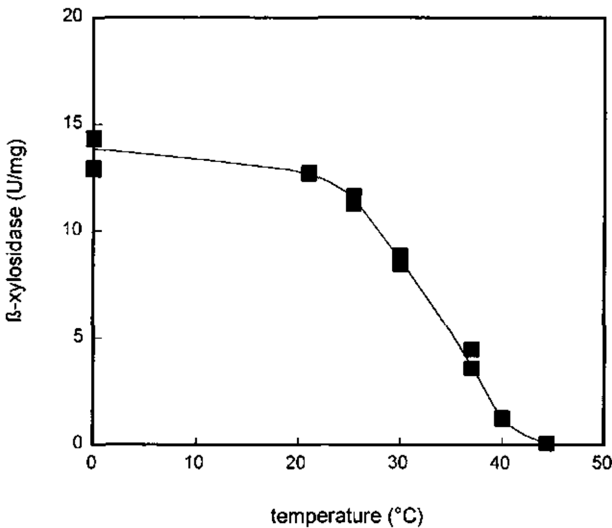

Fig. 5. Heat denaturation of the purified *β*-xylosidase incubated during 10 min at the indicated temperature in potassium phosphate buffer (pH 6.5). The residual activity was measured in a 30-second assay at 40 °C.

The pI of the purified enzyme was 6.3 as determined by denatured two dimensional polyacrylamide gel electrophoresis according to O'Farrell (25). Highest enzyme activity was found between pH 5.8 and 6.2. The enzyme was most active at 40 to 45°C, when measured with a one minute assay. Incubation of the enzyme during 10 minutes at temperatures above 20°C resulted in a decrease of activity (Fig. 5). Heating the enzyme for 10 minutes at 45°C resulted in a complete loss of activity. The half life of the purified enzyme was 9 min at 40°C and 11 min at 37°C. To test the effect of various chemicals on the enzyme activity, the enzyme was purified as described in the materials and methods section with the omission of DTT in the buffers. Addition of 1 mM DTT plus 1 mM $MgCl_2$ increased the half life of the enzyme at 40°C to 30 min. Addition of 5 mM of DTT and 5 mM $MgCl_2$ led to a slightly higher stability, but the initial activity decreased with 60%. Addition of EDTA (1 mM or 5 mM) did not affect the activity, indicating that bivalent cations were not required. Moreover, addition of 1 mM Mg^{2+}, K^{+}, or Ca^{2+} had no effect on *β*-xylosidase activity. However, addition of 5 mM $MgCl_2$ resulted in an activity decrease of 40%. Addition of *N*-ethylmaleimide (5 mM) to the standard reaction mixture did not affect the reaction rate. Complete inhibition of the *β*-xylosidase was achieved by addition of 0.01 mM Hg^{2+}. Normal Michaelis-Menten kinetics was followed by the enzyme. The K_M of the enzyme for the artificial substrate *p*-nitrophenyl-*β*-D-xylopyranoside was 0.125 mM, and the V_{max} was 33 μmol.min^{-1}.mg^{-1}.

Substrate specificity. The purified *β*-xylosidase was tested for its substrate specificity. No hydrolysis was found when the enzyme was assayed with the aryl-glycosides *p*-nitrophenyl-*β*-D-glucopyranoside, *p*-nitrophenyl-*β*-D-galactopyranoside, *p*-nitrophenyl-*β*-D-mannopyranoside or *p*-nitrophenyl-*α*-L-arabinopyranoside. When *p*-nitrophenyl-*α*-L-arabinofuranoside was used as substrate the reaction rate was about 3% of the *β*-xylosidase activity found with *p*-nitrophenyl-*β*-D-xylopyranoside. The activity of the *β*-xylosidase towards its natural substrates was also tested. Xylobiose, xylotriose and xylo-oligomers with polymerization degree of 4, 5 and higher were isolated from hydrolysed xylan by repeated gel permeation chromatography. Activity of the purified *β*-xylosidase towards xylobiose and xylotriose was about the same (Fig. 6 a,b). The oligosaccharides were

completely hydrolysed by the enzyme to yield xylose as final product. Activity for xylo-oligomers with a polymerization degree higher than 3 was much lower. Only minor amounts of xylose were released from these substrates (Fig. 6 c,d). The enzyme was inactive towards oat spelt xylan.

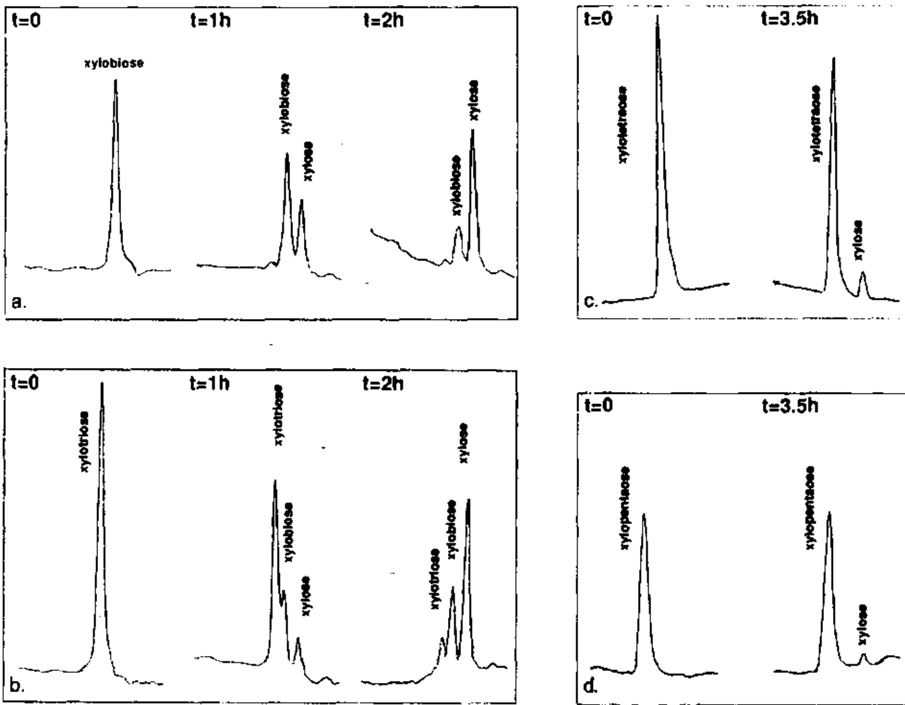

Fig. 6. High-pressure liquid chromatography analysis of the products from hydrolysis of xylo-oligosaccharides by the purified *β*-xylosidase of *B. xylanolyticus* X5-1. HPLC analysis was carried out on aliquots of the reaction mixture taken after 0, 1 and 2 hours (a,b) or 0 and 3.5 hours (c,d). a: hydrolysis of xylobiose, b: hydrolysis of xylotriose, c: hydrolysis of xylotetraose, and d: hydrolysis of xylopentaose.

Discussion

Batch growth of *Bacteroides xylanolyticus* X5-1on xylan, xylose and arabinose yielded *β*-xylosidase activity. No *β*-xylosidase activity was found in the cultures after growth on glucose or pyruvate. In many anaerobic rumen bacterial isolates, such as *Clostridium acetobutylicum*, and some strains of *Butyrivibrio fibrisolvens*, in the thermophile *Thermoanaerobacterium saccharolyticum* B6A, *Bacillus pumilus* and the yeast *Pullularia pullulans* either xylose or pentose-polymers were the most effective substrates for the production of *β*-xylosidase (10,17,19,26,27,40,41). In some of these organisms the pentose L-arabinose could also induce *β*-xylosidase production. In *Bacteroides ovatus, Bacteroides amylogenes, Thermomonospora fusca* and some strains of *Butyrivibrio fibrisolvens* *β*-xylosidase was produced after growth on xylan, but not on xylose or other monomeric sugars (2,11,32,38). The *β*-xylosidase of *Bacteroides amylogenes* was also produced after growth on xylobiose (11). In all these micro-organisms *β*-xylosidase was not produced during growth on glucose. The *β*-xylosidase produced by *B. xylanolyticus* was mainly cell-associated and was only released into the medium in trace amounts. This is also the case for the *β*-xylosidase from many other sources (1,3,9,10,19,32).

The high activities of *β*-xylosidase in *B. xylanolyticus* found after growth on L-arabinose and D-xylose, compared with the activity when grown on xylan, suggest a role of these pentoses in the induction of *β*-xylosidase. Experiments with concentrated cell suspensions showed that only the pentoses D-xylose and L-arabinose were efficient inducers of *β*-xylosidase synthesis. Glucose as well as other mono-, di- and trimers from hexoses severely repressed the enzyme production. The repressive effect of glucose on the *β*-xylosidase induction by xylose was immediate, and it can be interpreted in terms of catabolite repression. A regulation by inducer exclusion, by which glucose prevents the entry of inducers, is not likely since both xylose and glucose were consumed simultaneously by the cell suspensions. Catabolite repression occurred when easy metabolizable substrates like sugars or pyruvate were available to the cells. The inducer xylose also gave rise to catabolite repression. The repression by high concentrations of

xylose on the enzyme induction could be mimicked by incubating the cell suspensions with pyruvate together with xylose. The inducer concentration had an effect on both the induction rate as well as the extent of induction of *β*-xylosidase. At low xylose concentrations the rate of *β*-xylosidase production was highest and stopped as soon as all the inducer was depleted. High concentrations of xylose strongly inhibited the synthesis of *β*-xylosidase in batch cultures as well as in concentrated cell suspensions. A simultaneous induction and repression of the *β*-xylosidase synthesis by xylose was observed, which resulted in an optimal inducer concentration of about 20 mM. Shao and Wiegel (33) also found a dependence of *β*-xylosidase activity in *Thermoanaerobacter ethanolicus* on the concentration of the growth substrate xylose. At a concentration of 27 mM (0.4 %) xylose the *β*-xylosidase activity in *T. ethanolicus* was maximal. In contrast, the yeast *Pullularia pullulans* *β*-xylosidase production was induced by xylose, but no repression could be observed at high concentrations (27). Metabolisable pentoses and hexoses as well as the glycolytic intermediate pyruvate (5) can act as efficient repressors of *β*-xylosidase induction in *B. xylanolyticus.* This suggests that the repression of *β*-xylosidase synthesis occurs through a metabolite generated in a condition of carbon and energy excess.

In chemostat experiments with xylose as limiting substrate the enzyme was only produced by *B. xylanolyticus* at higher dilution rates when the carbon source accumulated in the medium. At low growth rates the culture was energy limited, which might explain the lack of *β*-xylosidase production.

The purified enzyme of *B. xylanolyticus* hydrolysed *p*-nitrophenyl-*β*-D-xyloside and can be denoted as a typical *β*-D-xylosidase in the sense that it could cleave of single xylose units from short xylo-oligosaccharides. The activity for xylobiose and xylotriose was much higher than for the longer xylo-oligomers. This effect of decreasing activity towards xylo-oligosaccharides with increasing chain length was also found for the *β*-xylosidase from other sources (18,20,21,34). The *β*-xylosidase from *B. xylanolyticus* lacks activity towards xylan and most aryl-glycosides except *p*-nitrophenyl-*β*-D-xyloside and showed low activity towards *p*-nitrophenyl-*α*-L-arabinofuranoside. Very low levels of *α*-L-arabinofuranosidase activity were also found in several other bacterial *β*-xylosidase

preparations (1,14,18). *p*-Nitrohenyl-α-L-arabinofuranosidase activity has been reported for a *Trichoderma reesei* and *Butyrivibrio fibrisolvens* β-xylosidase (28,35) and *p*-nitrophenyl-α-L-arabinopyranosidase has been reported for the *Penicillium wortmanni* β-xylosidase (7). The β-xylosidase of *Thermomonospora ethanolicus* showed both α-arabinosidase activities (33). The general properties of the β-xylosidase from *B. xylanolyticus* are comparable to those reported for other bacteria (Table 4).

Table 4. General properties of the purified β-xylosidases from several microorganisms.

Organism	Mol wt (Da)	Mol wt of subunits (Da)	Sp act (µmol. $min^{-1}.mg^{-1}$)	pH opt	T opt (°C)	K_M (mM pNPX)	V_{max} (µmol. $min^{-1}mg^{-1}$)	pI	Ref.
Bacteroides xylanolyticus	165,000	85,000	31	5.8-6.2	40-45	0.125	33	6.3	
Clostridium acetobutilicum	224,000	85,000 63,000	16	6-6.5	45	3.7	19.6	5.85	18
Bacillus pumilus	130,000	70,000	3.4	7		2.4	3.4	4.4	26
Bacillus stearo-thermophilus	150,000	75,000	34.2	6	70	1.2		4.2	23
Thermomono-spora fusca	165,000	56,000	8	5-9	40-60	0.89		4.37	1
Thermoanaeroba cter ethanolicus	165,000	85,000	66	5-5.2 5.8-6	82 93	0.018 0.038	122 183	4.6	33
Caldocellum saccharolyticum		53,000	49.2	5.7	70	10	64	4.3	14
Chaetomium trilaterale	240,000	118,000		4.5		2.8	2.6	4.86	36
Aspergillus niger	78,000		5.2	6.7-7	42				15
Aspergillus niger			4.2	3-4		0.362	30.9	4.3	24,34
Penicillium wortmanni	100,000		11.4	3.3-4		0.12		5	7
Trichoderma viride	101,000		10.8	4.5	55	5.8	-	4.45	20
Trichoderma reesei	100,000		28.2	4	60	0.08		4.7	28
Emericella nidu-lans	240,000	116,000	62.9 **	4.5-5	55	1		3.25	21

The β-xylosidases from *Bacillus pumilus*, *Bacillus stearothermophilus* and *Thermoanaerobacter ethanolicus* are also dimeric enzymes of comparable size. The K_M of 0.125 mM for *p*-nitrophenyl-β-D-xyloside is relatively low. A big difference between the β-xylosidase of *B. xylanolyticus* and the enzymes from different sources is the extreme low

thermostability of the purified protein. In vivo, the enzyme would probably be more stable when located in the cytosol. The pH optimum of *B. xylanolyticus* like that of most bacterial *β*-xylosidases is around 6, whereas the optimal pH of fungal enzymes is usually below 4.5 (Table 4). The pI of the *β*-xylosidase of *B. xylanolyticus* was near pH 6, as was found for the enzyme of *Clostridium acetobutylicum* (18). Most other *β*-xylosidases characterized thus far have a pI close to 4 (Table 4).

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Chapter 4

Production, Purification and characterization of an α-L-arabinofuranosidase from *Bacteroides xylanolyticus* X5-1.

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Abstract

Cell-free extracts of L-arabinose- and D-xylose- grown cells of the mesophylic anaerobic bacterium *Bacteroides xylanolyticus* X5-1 contained high activities (2 U/mg) of an α-L-arabinofuranosidase (EC 3.2.1.55). The enzyme was also produced during growth on xylan, but not during growth on glucose or cellobiose. The enzyme was mainly extracellularly attached to the cell when the organism was grown on xylan and was not released into the medium. The enzyme was purified 41-fold to apparent homogeneity. The native enzyme had an apparent molecular mass of 364 kDa and was composed of six polypeptide subunits of 61 kDa. The enzyme displayed a pH optimum of 5.5 to 6.0, and a pH stability of 5.5 to 9.0. The temperature optimum was 50°C and the enzyme was stable up to 50°C. Thiol groups were essential for activity, but the enzyme activity was not dependent on divalent cations. The K_m and V_{max} for *p*-nitrophenyl-α-L-arabinofuranoside were 0.5 mM and 155 U/mg of protein, respectively. The enzyme was specific for the α-linked arabinoside in the furanoside configuration. The enzyme displayed activity with arabinose-containing xylo-oligosaccharides with a polymerization degree of 2-5, but not with the polymeric substrates oat spelt xylan or arabinogalactan. The enzyme belongs to the *Streptomyces purpurascens*-type of α-L-arabinofuranosidase.

Introduction

Xylan is a widely distributed type of hemicellulose of plant cell walls. This complex polymer consists of a ß-D-1,4-linked xylopyranoside backbone substituted with arabinosyl, acetyl, uronyl and glucosyl side chains. The nature of the substituents is dependent on the source from which the xylan has been isolated. Xylans of softwoods and monocotyls such as grasses and cereals are generally characterized by the presence of L-arabinose residues, linked α-glycosidically to O-3 positions of D-xylose (Timell 1967; Wilkie 1979; Biely 1985). Many cellulolytic rumen bacteria have been shown to degrade xylan, but only a few are able to grow on this substrate (Dehority 1965; 1967; Williams and Withers 1982; Hespell et al.

1987). Anaerobic bacteria able to grow on xylan predominantly belong to the genera of *Bacteroides* (Scholten-Koerselman 1986; Salyers et al. 1982), *Butyrivibrio* (Hespell et al. 1987; Sewell et al. 1988) and *Clostridium* (Madden 1983; Berenger et al. 1985).

The complete microbial degradation of branched xylan involves the action of several hydrolytic enzymes; endo-ß-1,4-xylanases which hydrolyse the internal ß-1,4-xylosic linkages of the xylan backbone, ß-D-xylosidases which release xylose residues from small oligomeric substrates, and several enzymes capable of hydrolysing substituents from the xylan backbone such as arabinofuranosidases, acetyl esterases, uronidases and glucosidases (Biely 1985). α-L-arabinofuranosidases (AF), which remove L-arabinose side chains from polymeric or oligomeric substrates, have been purified from fungi (Kaji et al. 1970) and bacteria like *streptomyces* (Kaji et al. 1981; Komae et al. 1982), *Bacillus subtilis* (Weinstein and Albersheim 1979), *Ruminococcus albus* 8, *Clostridium acetobutylicum* and *Butyrivibrio fibrisolvens* (Greve et al. 1984; Lee et al. 1987; Hespell and O'Brian 1992).

Bacteroides xylanolyticus X5-1, a predominant strain isolated from fermenting cattle manure, grows efficiently on xylan. Unlike many other anaerobic xylanolytic bacteria, it is unable to use cellulose or other hemicelluloses for growth (Scholten-Koerselman et al. 1986). Several xylanolytic enzyme activities could be detected in cultures from *B. xylanolyticus* grown on xylan (Schyns and Stams 1992). These enzyme activities include xylanase, ß-D-xylosidase, acetyl-esterase and α-L-arabinofuranosidase activities. Here we report the purification and characterization of the α-L-arabinofuranosidase from *B. xylanolyticus*, expressed in high amounts during growth on xylose and arabinose.

Materials and methods

Chemicals. Unless stated otherwise, chemicals used were of analytical grade. L-arabinose, D-cellobiose, oat spelt xylan, larch wood arabinogalactan, Tris(hydroxymethyl)aminomethane, all *p*-nitrophenyl-glycosides, except *p*-nitrophenyl-*β*-D-glucopyranoside, and amyloglucosidase from *Rhizopus* (lot no. 48B-2186) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). *p*-nitrophenyl-*β*-D-glucopyranoside was obtained from Boehrin-

ger GmbH (Mannheim, FRG). Xylanase from *Trichoderma viride* (lot no. 95595) was obtained from Fluka Biochemika (Buchs, Switzerland). Sodium dodecylsulfate, acrylamide, hydroxylapatite and Biogel P2 were from Biorad (Veenendaal, the Netherlands). Q-Sepharose Fast Flow, Sepharose CL-6B, Mono-Q HR 5/5, Superose 6 HR 10/30, molecular mass markers for SDS-PAGE and for gel filtration were purchased from Pharmacia LKB Biotechnology (Uppsala, Sweden). All other chemicals were purchased from Merck (Darmstadt, FRG).

Media and cultivation. *Bacteroides xylanolyticus* X5-1 (DSM 3808) was isolated and described by Scholten-Koerselman et al. (1986). The organism was cultured in a bicarbonate buffered medium containing (g/l): $Na_2HPO_4.2H_2O$, 0.60; KH_2PO_4, 0.45; NH_4Cl, 0.3; $CaCl_2.2H_2O$, 0.11; $MgCl_2.6H_2O$, 0.11; NaCl, 0.3; $NaHCO_3$, 4.0; $Na_2S.9H_2O$, 0.24; yeast extract, 0.5; trypton, 0.4; resazurin, 0.0005; 1 ml of a trace element solution according to Wolin et al. (1963) per liter and 1 ml of a vitamin solution according to Zehnder et al. (1980) per liter. The vitamin solution was filter sterilized. Xylan was added to the medium prior to autoclaving. Sugars were added from 2M filter-sterilized stock solutions.

B. xylanolyticus was cultivated routinely at 37°C in the dark in 120-ml serum vials with 50 ml of medium. Vials were sealed with butyl rubber stoppers (Rubber BV, Hilversum, Holland) and aluminium caps. The gas phase in the vials consisted of N_2/CO_2 (80:20 v/v; 170 kPa.). Mass-cultivation was performed in 8-l bottles at 37°C. Cells were harvested aerobically at the late logarithmic phase by continuous centrifugation (Heraeus Sepatech Biofuge 28RS, Osterode, FRG).

Preparation of cell-free extract. Cells were suspended in 50 mM Tris-HCl pH 7.6 (0.2 g wet cells/ml buffer) and disrupted by sonification at 0°C (5 times 20 s, at 40 W, using a Branson Sonic Sonifier, Danbury, CT, USA). The broken cells were centrifuged for 20 min at 10,000 x *g* and the supernatant was centrifuged again at 100,000 x *g* for 1 h at 4°C. The supernatant contained about 20 mg protein per ml and was designated as cell free extract.

Enzyme purification. Unless otherwise stated all procedures were carried out aerobically at room temperature. The crude extract was applied to a Q-sepharose fast flow column (3.2 by 12 cm) equilibrated with 50 mM Tris/HCl pH 7.6 (buffer A). The column was developed

with 350 ml of a linear gradient of 0 to 0.6 M NaCl in buffer A. The active *α*-L-arabinofuranosidase eluted at 0.45 M NaCl. The AF-containing fractions were concentrated by ultrafiltration with an Amicon ultrafiltration cell (Grace, Rotterdam, the Netherlands) equipped with a PM 30 filter. The ultrafiltration retentate was adjusted to 0.25 M $(NH_4)_2$-(SO_4) by slow addition of granular ammonium sulfate and loaded onto a column (2.2 by 10 cm) packed with phenylsepharose. The column was equilibrated with 0.25 M ammonium sulfate in buffer A and eluted with a linear gradient of 0.25 M to 0 M ammonium sulfate at a flow rate of 1 ml/min. AF activity-containing fractions were pooled, diluted with sodium phosphate buffer (10 mM, pH 7), and then concentrated and desalted by repeated dilution and ultrafiltration. This enzyme preparation was applied to a hydroxylapatite column (2.2 by 18 cm) equilibrated with sodium phosphate buffer (10 mM, pH 7). The adsorbed protein was eluted from the column in a 220 ml-linear gradient of 10 mM to 150 mM sodium buffer pH 7. The AF eluted at 100 mM sodium phosphate. Fractions with activity were combined and applied to a Mono-Q HR 5/5 column, equilibrated with 50 mM sodium phosphate buffer (pH 6.5) at a flow rate of 1 ml/min. The enzyme eluted from the column at 0.35 M NaCl in a 20 ml-linear gradient of 0 to 0.5 M NaCl. Fractions with AF activity were combined and 250 µl was injected onto a Superose 6 HR 10/30 gelfiltration column (V_0=7.9 ml; V_t=23.4 ml) equilibrated in buffer A plus 100 mM NaCl. The column was developed at a flow rate of 0.25 ml/min. The colourless AF eluted as one symmetrical peak at 13.8 ml.

α-L-arabinofuranosidase assay. Routinely *α*-L-arabinofuranoside (EC 3.2.1.55) was assayed using *p*-nitrophenyl-*α*-L-arabinofuranoside (Araf*α*Np) as an artificial substrate (aryl-*α*-arabinofuranosidase). Enzyme activity was determined by measuring the amount of p-nitrophenol (*p*NP) released from the substrate. The assay mixture contained 1 mM substrate in 50 mM sodium phosphate (pH 6.5) in a final reaction volume of 0.25 ml. The incubation temperature was 50°C unless indicated otherwise. The reaction was terminated by the addition of 0.5 ml of 0.1 M sodium carbonate. The released *p*NP was determined spectrophotometrically at 405 nm, with *p*NP as a standard. One unit of activity was defined as the amount of enzyme releasing 1 µmol of *p*NP per min. AF was also assayed using xylan or xylan oligomers as substrates. The assay mixture contained 0.5% (w/v) substrate in 50

mM Tris/HCl (pH 7.0) containing 100 mM NaCl in a final volume of 1 ml. The assay mixture was incubated at 37°C during 30 min. Assays containing xylan were incubated for 2 hours with 10 times as much enzyme. The reaction was stopped by boiling during 1 min. The reaction was followed by measuring the release of arabinose by HPLC equipped with a Chrompack organic acid column (30 cm x 6.5 mm). The mobile phase was 0.005 M H_2SO_4 at a flow rate of 0.6 ml/min. The column temperature was 60°C. Samples (20 μl) were injected using a Spectra Physics autosampler (SP 8775). Compounds eluting were quantified by differential refractometry using a LKB 2142 refractometer. One unit of AF activity is the amount of enzyme releasing 1.0 μmol of arabinose per min.

Protein determination. Protein was determined with Coomassie brilliant blue G250 as described by Bradford (1976). Bovine serum albumin was used as a standard.

Polyacrylamide gel electrophoresis (PAGE). SDS PAGE was performed on 12.5% (w/v) gels according to the method of Laemmli (1970). Protein was stained with Coomassie brilliant blue. Molecular weight of the subunits was estimated by comparison to protein standards.

Preparation of xylo-oligosaccharide mixture. Oat spelt xylan was pretreated with amyloglucosidase to remove the contaminating starch. After ethanol precipitation (3 volumes), the xylan was resuspended (50 g/l) in 20 mM acetate buffer pH 5.0 and partially hydrolysed with a xylanase preparation from *Trichoderma viride* at 37°C. The reaction was stopped by boiling for 5 minutes. The mixture was centrifuged at 5000 x *g* for 10 min and the supernatant was concentrated by rotary evaporation. The oligosaccharides were separated by a repeated gel filtration on a column (100 x 2.2 cm) of Bio-gel P2 (fractionation range 100-1800). Sugars were analyzed by HPLC equipped with an Aminex HPX-87P column (Biorad) maintained at 80°C with degassed water as the mobile phase. The degree of polymerization of the oligosaccharides was estimated as described by Lee et al. 1985.

Results

Growth conditions and enzyme activities. When *Bacteroides xylanolyticus* X5-1 was grown on glucose or cellobiose only low AF activities were detected in the cultures (Table 1). Cells grown on L-arabinose or D-xylose displayed AF activities of up to 2.5 U/mg. When oat spelt xylan was used as a substrate the specific AF activities were about 0.35 U/mg.

Table 1. The effect of the growth substrate on the specific activity and the total activity per culture volume of the α-L-arabinofuranosidase from *Bacteroides xylanolyticus* X5-1.

	α-L-arabinofuranosidase	
Carbon source	(U/mg)	(U/ml culture)
Glucose	0.05	0.01
Cellobiose	0.08	0.01
L-Arabinose	2.4	0.3
Xylose	1.9	0.25
Xylan (oat spelt)	0.35	0.05

Data are the mean values of triplicate determinations.

During batch growth on L-arabinose or D-xylose, the enzyme was produced throughout the logarithmic growth phase until all the substrate had been consumed. Only about 5% of the total AF activity was found in the cell free growth medium at the end of growth. Even in the late stationary phase no increase in AF activity in the culture supernatant could be observed. The AF activity could readily be measured with intact whole cells. When grown on L-arabinose the AF activity of whole cells was about 50% of the activity measured in cell extracts, suggesting that 50 % of the enzyme resides intracellularly and the rest is extracellularly bound to the cells. The extracellular AF activity could not be released from the cells by an osmotic shock treatment (Neu and Heppel 1965). After disruption of the cells by sonification the major part of the AF activity was released from the cells and cell debris. The activity did not remain membrane bound as was shown by fractionation of crude

extracts of cells grown on L-arabinose by ultra-centrifugation at 100,000 x *g* (1 h). About 95% of the activity remained in the supernatant. When grown on xylan the whole cells exhibited approximately the same AF activities as cell free extracts. This suggests that the AF activity was mainly located extracellularly and cell bound when the organism was grown on xylan.

Table 2. Purification of α-L-arabinofuranosidase from *Bacteroides xylanolyticus* X5-1.

	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Recovery (%)	Purification (fold)
Cell extract	1045	2758	2.6	100	1
Q-sepharose	137	1290	9.4	46	3.6
Phenyl-sepharose	50	821	16.4	29.8	6.2
Hydroxyapatite	15	414	27.5	15	10.4
Mono Q	1.7	138	78.6	5	29.8
Superose 6	0.9	97	108	3.5	41.5

Purification. The purification of the oxygen stable AF was performed aerobically at room temperature. The enzyme purification is summarized in Table 2. The cell free extract of cells grown on 20 mM L-arabinose was fractionated by Q-sepharose anion exchange column chromatography (Fig. 1). The AF activity eluted in a major peak at 0.45 M NaCl and in a minor peak at 0.5 M NaCl from column. The minor peak coeluted with a peak containing high ß-xylosidase activity. The major peak, which accounted for 80% of the total activity was selected for further purification. The hydroxylapatite column was used to separate the AF from contaminating ß-xylosidase activity. When chromatographed on superose-6, the enzyme eluted as one symmetrical peak. The volume at which the enzyme eluted corresponded to a size of 364000 dalton. The enzyme was purified about 40 fold with a recovery of 3.5 %.

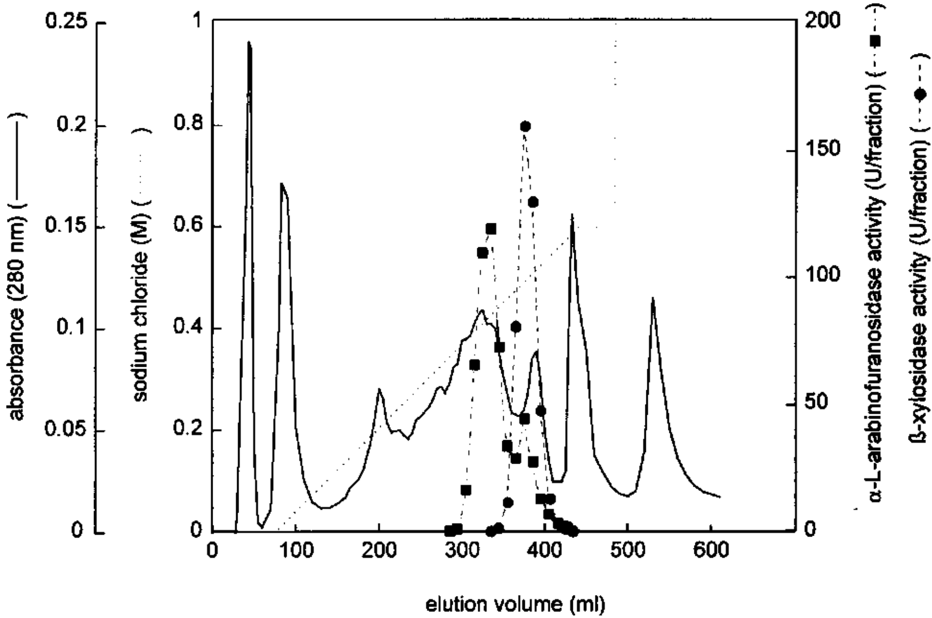

Fig. 1. Q-sepharose anion exchange chromatograph of cell free extract of L-arabinose-grown *Bacteroides xylanolyticus* cells. Chromatographic conditions are described in Materials and Methods.

Properties. The purified enzyme exhibited a single protein band of 61000 Da on SDS-PAGE (Fig. 2). The enzyme showed optimal activity at pH 5.5 to 6.0 and it was stable from pH 5 to 8.9 (Fig. 3). The highest initial AF activities could be measured at temperatures between 45 and 55°C, using a one minute assay. The thermal stability (20 min. incubation at given themperature) of the enzyme was low at temperatures higher than 45°C (Fig. 4.).

The enzyme followed normal Michaelis Menten kinetics, when the artificial substrate ArafαNp was used. The K_m value was 0.5 mM ArafαNp and the V_{max} 155 U/mg.

The effects of various chemicals on the AF activity was determined using the purified enzyme (dissolved in buffer A with 100 mM NaCl). Results are shown in Table 3. Enzyme activity was not stimulated by divalent cations, and EDTA had no inhibitory effect. $HgCl_2$ had a strong inhibitory effect on the enzyme activity. Addition of 1 mM Cu^{2+} resulted in 15

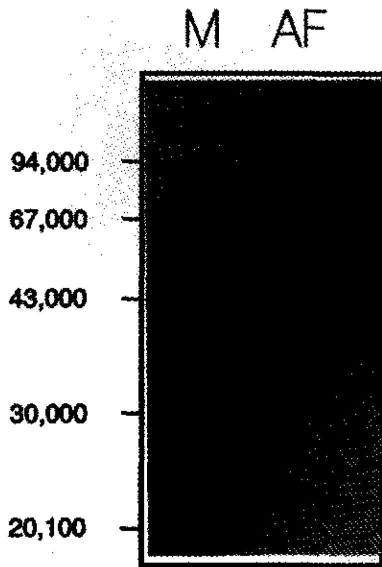

Fig. 2. SDS-polyacrylamide gel electrophoresis of purified α-L-arabinofuranosidase. (M) molecular weight standards. (AF) 1 μg purified enzyme.

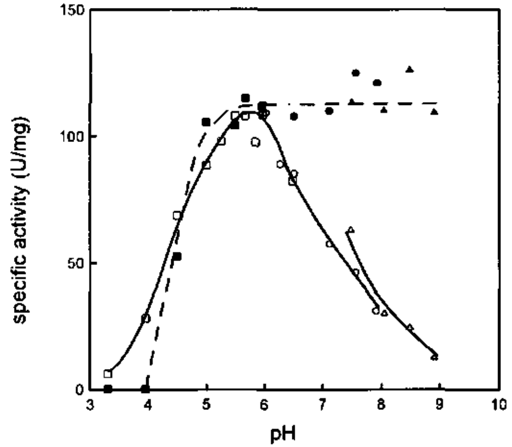

Fig. 3. Effect of pH on activity (solid line) and stability (dotted line) of the purified α-L-arabinofuranosidase. Buffers: citrate-phosphate (□ ,■), sodium-phosphate (O,●) and Tris/HCl (Δ,▲). For activity the enzyme was assayed at 50°C at the indicated pH. For stability the enzyme was exposed to the indicated pH for 20 min at 37°C and the residual activity was measured at pH 5.7 and 50°C.

Table 3. Effect of several chemical compounds on the α-L-arabinofuranosidase activity.

Compound added	Relative activity (%)
None	100
DTT (0.1 mM)	70
ß-mercaptoethanol (1 mM)	98
EDTA (1 mM)	102
$MgCl_2$ (1mM)	98
$CaCl_2$ (1 mM)	106
$CuCl_2$ (1 mM)	85
$HgCl_2$ (0.001 mM)	12
$HgCl_2$ (0.1 mM)	0
DTT (0.1 mM)+ $HgCl_2$ (0.001 mM)[a]	69
DTT (0.1 mM)+ $HgCl_2$ (0.1 mM)[a]	8

[a] the enzyme was preincubated for 5 min with DTT prior to the $HgCl_2$ addition.

% inhibition, and 0.1 mM DTT in 30 % inhibition of the activity. Preincubation of the enzyme with DTT (0.1 mM) prior to the addition of $HgCl_2$ (1 μM) protected the enzyme against inactivation.

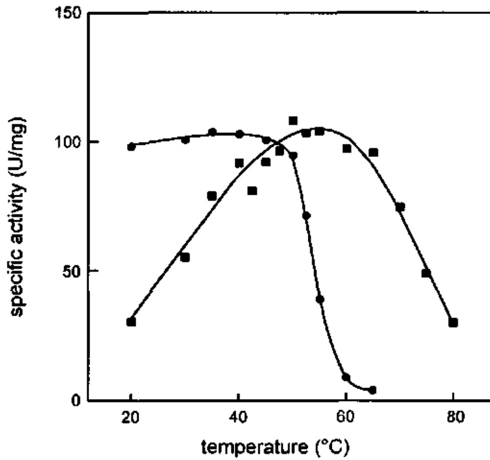

Fig. 4. Effects of temperature on activity (■) and stability (●) of the purified *α*-L-arabinofuranosidase. For activity the enzyme was assayed at the indicated temperature. For stability the enzyme was incubated at the indicated temperature for 20 min at a pH of 7.5 and the residual activity was measured at 50°C and pH 5.7.

Table 4. Activity of *α*-L-arabinofuranosidase with various substrates.

Substrates	Specific activity (U/mg)
p-nitrophenyl-*α*-L-arabinofuranoside	124
p-nitrophenyl-*α*-L-arabinopyranoside	<0.01
p-nitrophenyl-*β*-D-xylopyranoside	0.6
p-nitrophenyl-*β*-D-glucopyranoside	<0.01
p-nitrophenyl-*β*-D-galactopyranoside	<0.01
p-nitrophenyl-*β*-D-mannopyranoside	<0.01
arabinogalactan	<0.01
xylan from oat spelt	<0.01
xylan from birch wood	<0.01
xylobiose, xylotriose	<0.01
xylo-oligomers with a polymerization degree less than 5	14

Substrate specificities. The purified enzyme showed highest activity with *p*-nitrophenyl-*α*-L-arabinofuranoside, but was not able to hydrolyse *p*-nitrophenyl-*α*-L-arabinopyranoside (Table 4). Only 0.5 % of that activity was found with *p*-nitrophenyl-*β*-D-xylopyranoside as substrate. No activity could be detected with the other aryl-glycosides. The enzyme was unable to release arabinose from the polymeric substrates arabinogalactan and xylan. When incubated with xylo-oligosaccharides with a polymerization degree of 2 to 5, the enzyme was able to cleave off the arabinose side chains. Xylobiose and xylotriose were not hydrolysed by the enzyme.

Discussion

The synthesis of AF in *Bacteroides xylanolyticus* X5-1 seems to be regulated. The very high activities found after growth on L-arabinose and D-xylose, compared to xylan, suggest that the synthesis of AF is induced by these pentoses or by metabolites directly derived from these sugars, whereas hexoses like glucose and cellobiose may act as catabolite repressor. Comparable results have been found in some of the rumen bacterial isolates investigated by Williams and Withers (1982). These strains exhibited high AF activities when grown on L-arabinose or arabinose-containing polysaccharides, but low activities when grown on glucose or cellobiose. Only one of these strains, a spore-forming isolate, yielded such high AF activities as found with *B. xylanolyticus* X5-1. The AF synthesis by *Butyrivibrio fibrisolvens* is strain dependent. Williams and Withers (1992) reported high activities of this enzyme when strain NCFB 2249 was grown on xylose or xylose-containing saccharides. In contrast, strain GS 113 contained only low activities after growth on monomeric sugars but high cell bound extracellular AF activities after growth on xylan (Hespell and O'Bryan 1992). *Ruminococcus albus* 8 and *Clostridium acetobutylicum* produced an AF that is released into the medium mainly when the organisms were grown on cellobiose or polymeric saccharides (Greve et al. 1984; Lee et al. 1985). The *α*-L-arabinofuranosidase produced by *B. xylanolyticus* X5-1 is only released into the medium in trace amounts. Most of the enzyme remains extracellularly cell bound when grown on xylan. This suggests that the location of

the AF taking part in the xylan degradation is cell associated and extracellular. In this way the enzyme can cooperate with the xylanases of this organism. However, when the cells are grown on L-arabinose or D-xylose, and thus high amounts of the enzyme are synthesized, about half of the AF activity seems to reside intracellularly. The overproduction of the enzyme leads to only a partial excretion of the activity. The extracellular AF activity was tightly associated to the cells. In *Butyrivibrio fibrisolvens* similar extracellularly cell-bound glycosidases are found (Hespell and O'Bryan 1992). The *β*-xylosidase and the extracellular part of the AF activity of this organism could not be released by treatment with Triton X-100 or other mild detergents. The *β*-glucosidase of *Cellulomonas uda* was found to be cell bound and the major part of the activity could be released by disrupting the cells by sonification (Stoppok et al. 1982).

The amount of AF (0.3 % of the total protein) that is produced when *B. xylanolyticus* X5-1 is grown on xylan is comparable with values found for *Butyrivibrio fibrisolvens* GS113 (Hespell and O'Bryan 1992). The molecular weight of the purified enzyme determined by gel filtration (365000 da) and the single protein band (61000 da) after SDS-PAGE indicate that the AF of *B. xylanolyticus* X5-1 consists of a hexamere. In that respect the enzyme differs from the octameric, tetrameric and monomeric nature of AFs from other microbial sources (Kaji et al. 1970, 1981; Komae et al. 1982; Greve et al. 1984; Lee et al. 1987; Hespell et al. 1992). The enzyme is stable under the conditions found in the extracellular environment. The pH optimum (5.5-6.0) lies in the normal range for the bacterial enzymes and the enzymes from several *Streptomyces* strains (Kaji 1984). The enzyme was not affected by divalent cations. The enzyme was very sensitive to sulfhydryl inhibitors like mercury indicating the presence of essential thiol groups in the enzyme. The anaerobic growth environment of the organism probably ensures that the sulfhydryl groups remain reduced. A similar sensitivity for mercury was reported for the AFs of the rumen bacteria *Butyrivibrio fibrisolvens* GS113 and *Ruminococcus albus* 8 (Hespell et al. 1992; Greve et al. 1984).

The substrate specificity of the purified AF was very narrow. It was only able to release the *α*-linked L-arabinose in the furanose form from the synthetic substrates tested. Compared with the high activities found with the artificial substrate *p*-nitrophenyl-*α*-L-arabino-

furanoside the activities with potential natural substrates were low. The enzyme was unable to release L-arabinose from arabinogalactan or oat spelt xylan, it could however cleave off arabinose residues from arabinose containing xylo-oligosaccharides with a polymerization degree of about 2 to 5. The products that could be identified were arabinose, xylobiose and xylotriose. Kaji (1984) divided the AFs in two groups according to the substrate specificity of the enzyme. An *Aspergillus niger* type of AF and a *Streptomyces purpurascens* type. The first group releases the L-arabinosyl residues not only from polymeric L-arabinan, arabinoxylan and arabinogalactan but also from low molecular weight L-arabino-oligosaccharides and synthetic substrates. The second group only acts on low-molecular-weight L-arabinofuranose containing oligo-saccharides and synthetic substrates. Most of the microbial AFs purified thus far belong to the first group (Greve et al. 1984; Kaji 1984; Lee and Forsberg 1987; Hespell and O'Bryan 1992). According to the substrate specificity the AF from *B. xylanolyticus* X5-1 belongs to the second group.

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Chapter 5

Purification and characterization of a xylose acetyl esterase from *Bacteroides xylanolyticus* X5-1 and its role in xylan degradation.

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Abstract

During growth on xylan *Bacteroides xylanolyticus* X5-1 produced high activities of an acetyl esterase. Very low activities of this enzyme were found after growth on glucose. The acetyl esterase was purified by column chromatography from cell extracts of *Bacteroides xylanolyticus* X5-1 grown on xylan. The enzyme had an apparent molecular mass of 245 kDa. SDS-polyacrylamide gel electrophoresis revealed one single protein band corresponding to a subunit of 62 kDa. The temperature and pH optima were 45°C and 6.3 respectively. The activity dropped dramatically at temperatures above 55°C. The activity was inhibited by Hg^{2+}. The purified enzyme exhibited a K_m of 0.09 mM and a V_{max} of 200 U/mg for the hydrolysis of *p*-nitrophenylacetate. The enzyme showed highest activity for acetylated monosaccharides and acetylated aromates. It was also active with methylacetate, ethylacetate and glyceryl triacetate, but could not liberate acetate from acetylated xylan.

Introduction

Plant cell walls consist for 8 to 38 % of hemicellulose. Hemicelluloses constitute of a group of polysaccharides (excluding pectin) which remain associated with the cellulose after removal of lignin. In hardwood, grasses and cereals the major hemicelluloses are substituted xylans (1,29). Xylans are characterized by a ß-1,4-linked-D-xylopyranoside chain which carries a variable number of substituents consisting of O-acetyl groups, L-arabinofuranosyl groups and uronic acids. In hemicellulose from hardwood and grasses about half of the xylose residues are substituted at the O-2 or O-3 positions, and 50 to 70% of the substituents are acetyl groups (3,9). As a result of the preparation of xylan under alkaline conditions, deacetylated xylans have been used in many enzymological studies. Investigations with ruminants have shown that acetyl groups can greatly restrict the polysaccharide degradation (9,21). Chemical removal of acetyl groups from cell wall fractions of dried grasses resulted in an increased digestibility (21,33).

Biely and coworkers (4,5) first reported the presence of acetylxylan esterases in fungal cellulolytic systems and showed a cooperative action of fungal esterase and xylanase in the

hydrolysis of acetyl xylan. Initial observations of Williams and Withers showed that aryl acetyl esterase activity is also present in several hemicellulolytic anaerobic rumen bacterial isolates, but it is was not investigated if these isolates could deacetylate xylan (30,31). Acetyl esterase and acetylxylan esterase activities were also reported in several *Butyrivibrio fibrisolvens* strains and in *Fibrobacter succinogenes* (11,18). The enzymatic release of acetyl groups, which plays a role in the complete degradation of xylan, can occur in two different ways. In the case an acetylxylan esterase is involved, the enzyme acts on the polymeric substrate and increases the accessibility of the polymer for the xylanase. It is also possible that the acetyl esterase primarily acts on short acetyl substituted xylooligomers, resulting in unsubstituted substrates for the ß-xylosidase. In the latter case the enzyme does not necessarily have to be active on the polymeric acetyl xylan. Only a few microbial acetyl esterases participating in xylan degradation have been purified and characterized. Among these are an acetyl esterase (23) and two acetylxylan esterases (23,28) from *Trichoderma reesei* and acetylxylan esterases from the actinomycete *Thermomonospora fusca* (2) and the anaerobic cellulolytic bacterium *Fibrobacter succinogenes* (19).

In this paper we report the purification and characterization of a xylose acetyl esterase of *Bacteroides xylanolyticus* X5-1. This anaerobic bacterium grows efficiently on xylan and many soluble sugars, but not on cellulose (25). When grown on xylan, the organism produces xylanase, ß-xylosidase and α-arabinofuranosidase activities (26). Although no acetylxylan esterase activity could be detected in these cultures, they produced considerable amounts of a xylose acetyl esterase.

Materials and methods

chemicals. Unless stated otherwise chemicals used were of analytical grade. Birch wood xylan (charge number: 14210983) was obtained from Roth (Karlsruhe, Germany). Oat spelt xylan (lot number: 38F-0722), β-D-xylose tetraacetate, β-D-glucose pentaacetate, α-naphtyl acetate, *p*-nitrophenyl-acetate, Tris(hydroxymethyl)aminomethane were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Larch wood xylan (lot number: 23262) was obtained from ICN Biomedicals (Costa Mesa, CA, USA). Sodiumdodecylsulfate, acryl-

amide, hydroxyapatite and Biogel P2 were from Biorad (Richmond, VA, USA). Q-Sepharose Fast Flow, Sepharose CL-6B, Mono-Q HR 5/5, Superose 6 HR 10/30, molecular mass markers for SDS-PAGE and for gelfiltration were purchased from Pharmacia LKB Biotechnology (Uppsala, Sweden). All other chemicals were from Merck (Darmstadt, Germany).

Media and cultivation. The organism used in this study, *Bacteroides xylanolyticus* X5-1 (DSM 3808), was isolated and described by Scholten-Koerselman et al. (1986). The organism was cultured in a basal bicarbonate buffered medium as previously described by Biesterveld et al. (1994). Additionally, the medium contained 0.4 g trypton per liter. Xylan was added to the medium prior to autoclaving. Sugars were added separately from 2M filter-sterilized stock solutions.

B. xylanolyticus X5-1 was cultivated in 120-ml serum vials (Aluglas Verenigde Bedrijven bv, Amersfoort, the Netherlands) with 50 ml medium or in 3-l serum bottles with 1.5 l of medium. Vials and bottles were sealed with butyl rubber stoppers (Rubber BV, Hilversum, The Netherlands) and aluminum caps. The gas phase in the vials and bottles consisted of N_2/CO_2 (80:20 v/v; 170 KPa.). After inoculation (1%) cultures were incubated at 37°C in the dark. Cells were harvested at the end of the logarithmic growth phase by centrifugation.

Preparation of cell-free extract. Cells were suspended in 50 mM Tris-HCl (pH 7.5) and disrupted by sonification at 0°C (5 times 20 s, at 40 Watt, using a Branson Sonic Sonifier (Danbury, CT, USA). The broken cells were centrifuged for 20 min. at 10,000 x *g* and the supernatant (crude cell extract) was centrifuged again at 100,000 x *g* for 1 h at 4°C. The supernatant contained about 9 mg protein per ml and was designated as cell free extract.

Enzyme purification. Unless otherwise stated all procedures were carried out aerobically at room temperature. All columns were equilibrated with the starting buffers of the gradients, and developed with linear gradients in buffer. The cell extract was applied to a Q-sepharose fast flow column (3.2 by 12 cm) equilibrated with 50 mM Tris/HCl (pH 7.5) (buffer A). A 345-ml gradient from 0 to 0.7 M NaCl in buffer A was applied to the column. Fractions with *p*-nitrophenylacetyl esterase activity were pooled and assayed for the ability to release acetate from *β*-D-xylose tetraacetate. The xylose-acetyl esterase activity-containing fractions were concentrated by ultrafiltration with an Amicon ultrafiltration cell (Grace, Rotterdam, the

Netherlands) equipped with a PM 30 filter. The ultrafiltration retentate was diluted (1:1) with milliQ water (Millipore, MA, USA) and loaded on a hydroxyapatite column (2.2 by 18 cm) equilibrated with 0.01 M potassium phosphate buffer (pH 7). The adsorbed protein was eluted from the column in a 220-ml linear gradient of 10 to 150 mM potassium phosphate buffer (pH 7). The acetyl esterase activity eluted at 85 mM potassium phosphate. Fractions with activity were combined and concentrated by ultrafiltration (Amicon PM-30 membrane). Ammonium sulfate was added to the concentrated enzyme solution to a final concentration of 1 M. The protein solution was then applied to a phenyl-Sepharose CL-4B column (2.2 by 10 cm) equilibrated with 1 M ammonium sulfate in buffer A. A 100 ml linear gradient of 1 to 0 M ammonium sulfate in buffer A was applied at a flow rate of 1 ml/min. The acetyl esterase-containing fractions were pooled and applied to a Mono-Q HR 5/5 column, equilibrated with 50 mM sodium phosphate buffer (pH 6.5) at a flow rate of 1 ml/min. The enzyme eluted from the column at 0.40 M NaCl in a 25-ml linear gradient of 0 to 0.5 M NaCl in sodium phosphate buffer (pH 6.5). Fractions with acetyl esterase activity were combined and injected on to a Superose 6 HR 10/30 gelfiltration column (V_0=7.9 ml; V_t=23.4 ml) equilibrated in buffer A plus 100 mM NaCl. The column was developed at a flow rate of 0.25 ml/min. The colorless acetyl esterase eluted as one symmetrical peak at 15.3 ml.

Acetyl esterase assay. Acetyl esterase (EC 3.1.1.6) was routinely assayed using *p*-nitrophenyl-acetate as an artificial substrate (aryl-acetyl esterase). Enzyme activity was determined by measuring the amount of *p*-nitrophenol released from the substrate. The assay mixture contained 1 mM substrate in 50 mM sodium phosphate (pH 6.5) in a final reaction volume of 1 ml. The incubation temperature was 45°C unless indicated otherwise. The liberation of *p*-nitrophenol was followed spectrophotometrically at 405 nm (ε=5.15 $mM^{-1}.cm^{-1}$) in time. One unit of activity was defined as the amount of enzyme releasing 1 µmol of *p*-nitrophenol per min. The assay conditions were the same when α-naphtyl acetate was used as a substrate. In this case the release of α-naphtol was followed at a wavelength of 323 nm (ε= 2.0 $mM^{-1}.cm^{-1}$). Acetyl esterase was also assayed by measuring the fatty acid release from several substrates (e.g. β-D-xylose tetraacetate, glucose tetraacetate, glyceryl triacetate, glyceryl

tributyrate, methylacetate, ethylacetate, ethylpropionate). The assay contained 2 mM substrate (concentrations of 20 mM were used for glyceryl triacetate, methylacetate, ethylacetate and ethylpropionate) in a total volume of 1 ml of 50 mM potassium phosphate buffer (pH 6.5). The assay mixture was incubated at 37°C during 10 to 30 minutes. The reaction was stopped by boiling for 1 min, and the released acetate (or other fatty acid) was measured by HPLC equipped with a Chrompack organic acid column (30 cm x 6.5 mm) (10). One unit of acetyl esterase activity is the amount of enzyme releasing 1 μmol of acetate per min.

To investigate the effect of activators and inhibitors on the acetyl esterase, the enzyme was preincubated for 30 min at room temperature in 50 mM citrate-phosphate buffer (pH 6.5) to which the test substance was added in concentrations of 1 to 5 mM. The preincubated enzyme was then diluted 8 times in the same buffer and assayed for residual activity at 45 °C.

Acetylxylan esterase assay. Acetylated oat spelt xylan was prepared as described by Johnson et al. (12). The degree of substitution of acetyl xylan was 1.1 as estimated by the method of Mitchell et al. (1990). The assay mixture contained 1% (w/v) xylan or acetyl-xylan in 50 mM Tris/HCl (pH 7.0) in a final volume of 1 ml. The assay mixture was incubated at 37°C for 30 min to 5 hours with an appropriate amount of enzyme solution. The reaction was stopped by boiling during 1 min, and the amount of acetic acid released was measured as described above.

Xylanase assays. The xylanase assays were performed as described previously (25). The release of reducing sugar was determined by the modified method described by Nelson (1944), with xylose as the standard (Somogyi 1952).

Protein determination. Protein was determined with Coomassie brilliant blue G250 as described by Bradford (1976). Bovine serum albumin was used as a standard.

Polyacrylamide gel electrophoresis (PAGE). SDS PAGE was performed on 12.5% (w/v) gels according to the method of Laemmli (1970). Protein was stained with Coomassie brilliant blue. Molecular weight of the subunits was estimated by comparison with protein standards; trypsin inhibitor (M_r 20100), carbonic anhydrase (M_r 30000), ovalbumin (M_r

45000), bovine serum albumin (M_r 67000) and phosphorylase b (M_r 94000) (Pharmacia Fine Chemicals, Uppsala, Sweden).

Results

The effect of growth substrate on the xylose acetyl esterase activity.

The organism was grown on three different types of xylan and on glucose, xylose or pyruvate. The xylanase activities and acetyl esterase activities were measured in the crude cell extract (table 1). To measure the acetyl esterase activity, β-D-xylose tetraacetate was used as substrate. Both the xylanase activity and the xylose acetyl esterase activity are mainly produced when the cells are grown on xylan. On glucose hardly any xylose acetyl esterase activity was found in the cell extracts.

Table 1. The effect of the growth substrate on the specific activity of the xylanase and xylose acetyl esterase of *B. xylanolyticus* X5-1.

growth substrate	xylanase[a] (U/mg)	xylose acetyl esterase[a,b] (U/mg)
glucose (20 mM)	<0.01	< 0.05
xylose (20 mM)	0.02	0.2 ± 0.15
pyruvate (60 mM)	0.01	0.15 ±0.1
Larch xylan (0.4 %)	0.37	2.3 ± 0.3
Birch xylan (0.4 %)	0.30	1.8 ± 0.3
Oat xylan (0.4 %)	0.15	2.1 ± 0.3

[a] activities are average of three cultures.
[b] activity was measured as the release of acetate from xylose tetraacetate after 15 min.

The acetyl esterase activity is predominantly cell associated. Only 5 to 7 % of the acetyl esterase activity could be detected in the cell free culture supernatant at the end of the growth phase of xylan-grown cells. The acetyl esterase could be measured with whole cells as well as with cell extracts. Whole cells yielded about 75% of the activity found in the corresponding cell extract.

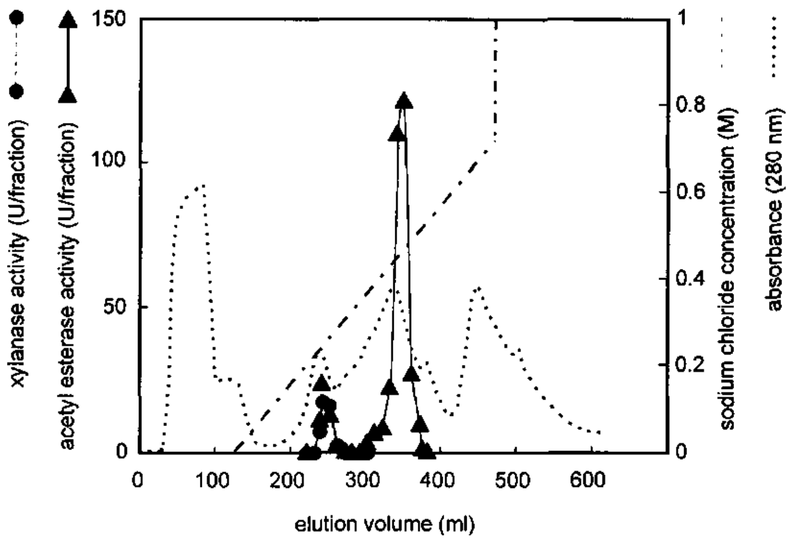

Fig. 1. Q-sepharose anion exchange chromatography of cell free extract of xylan grown *Bacteroides xylanolyticus* cells. Chromatographic conditions are described in Materials and Methods. ▲-▲: aryl-acetylesterase activity.●-●: xylanase activity.

Table 2. Purification of the xylose acetyl esterase from *Bacteroides xylanolyticus* X5-1.

	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Recovery (%)	Purification (fold)
Cell extract	491	400	0.8	100	1
Q-sepharose	71	289	4.1	72	5
Hydroxylapatite	24	257	10.7	64	13
Phenyl-sepharose	3.3	240	72	60	88
Mono Q	1	172	169	43	207
Superose 6	0.34	66	190	16.5	232

Purification

The purification of the acetyl esterase was performed aerobically at room temperature. Cell extract was applied to a Q-sepharose anion exchange column. Three peaks could be detected with activity against *p*-nitrophenylacetate (fig. 1). The first peak coeluted with a peak containing xylanase activity. The second peak eluted as a small shoulder of the third main activity peak. No deacetylation of birch wood xylan or chemically acetylated oat spelt xylan could be achieved with any of the three activity peaks. Only the major acetyl esterase activity peak, eluting at about 0.45 M NaCl could deacetylate ß-D-xylose tetraacetate. This activity peak was used for further purification. The xylose acetyl esterase was purified about 230 times in 5 steps, with a recovery of 17 % (table 2).

Properties of the acetyl esterase.

The molecular mass of the native acetyl esterase was estimated by gelfiltration on superose 6 HR 10/30 and appeared to be 245 ± 10 kDa, when compared to standards of known molecular mass. Sodium dodecyl sulfate gel electrophoresis of the purified enzyme revealed one band with a molecular mass of 62 kDa (Fig. 2).

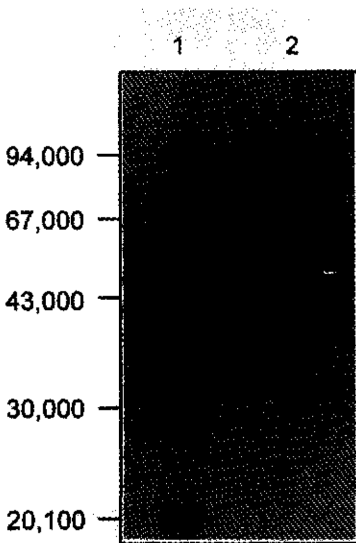

Fig. 2. SDS-polyacrylamide gel electro-phoresis of purified acetyl-esterase; lane 1: molecular weight standards; lane 2: 1 µg purified acetyl esterase after superose 6 gelpermeation.

The pH optimum was determined using *p*-nitrophenylacetate as substrate in the assay performed at 45°C. The extinction coefficient of *p*-nitrophenol was determined at 45°C for each of the buffers used. The acetyl esterase activity was highest at a pH between 5.9 and 6.7 (Fig. 3). The enzyme was stable between pH 6 and 8 when incubated at 37°C for 20 minutes in the appropriate buffer. The residual activity was measured at 45°C in 50 mM potassium phosphate buffer at pH 6.5 (fig 4). The acetyl esterase was most active between 40 and 50°C. At temperatures higher than 55°C the activity decreased rapidly. The enzyme was stable during 30 min in 50 mM Tris/HCl buffer (pH 7.5) at temperatures up to 37°C. Hardly any activity was recovered after 30 min incubation at 50°C (Fig. 4). At 4°C the enzyme remained stable for several months. Addition of 1 mM and 5 mM EDTA, magnesium chloride, copper chloride, calcium chloride, N-ethylmaleimide, DTT, and ß-mercaptoethanol had no effect on the enzyme activity. 1 mM of mercurous chloride caused a loss of acetyl esterase activity of 98 %. The kinetic properties of the enzyme were determined using *p*-nitrophenylacetate as a substrate. The purified enzyme exhibited a K_m of 0.09 mM *p*-nitrophenylacetate and a V_{max} of 200 U/mg when assayed at 45°C and pH 6.5.

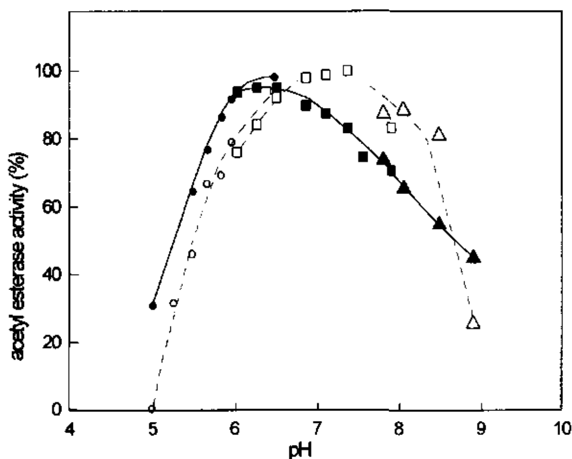

Fig. 3. Effect of pH on activity (solid line) and stability (dotted line) of the purified acetyl-esterase. Buffers: citrate-phosphate (○,●), sodium-phosphate (□,■) and Tris/HCl (△,▲). For activity the enzyme was assayed at 45°C at the indicated pH. For the stability test the enzyme was exposed to the indicated pH for 20 min at 37°C and the residual activity was measured at pH 6.5 and 45°C.

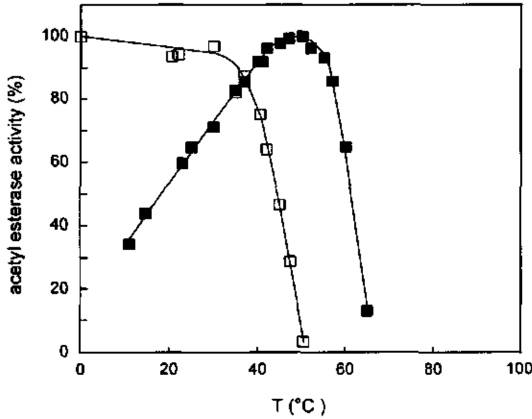

Fig. 4. Effects of temperature on activity (■) and stability (□) of the purified acetyl-esterase. For activity the enzyme was assayed at the indicated temperature. For stability the enzyme was incubated at the indicated temperature for 20 min at a pH of 7.5 and the residual activity was measured at 45°C and pH 6.5.

Table 3. Activity of the purified acetyl esterase with various substrates.

Substrates	Specific activity (U/mg)
p-nitrophenyl acetate	175
α-naphtyl acetate	182
xylan from birchwood	< 0.1
acetylated xylan	< 1
β-D-xylose tetraacetate	174
β-D-glucose pentaacetate	135
methylacetate	13
ethylacetate	24
ethylpropionate	1
glyceryl triacetate	41
glyceryl tributyrate	< 0.1

Substrate specificities.

The purified acetyl esterase from *Bacteroides xylanolyticus* X5-1 cleaved the acetyl-ester bonds from several substrates like α-naphthyl acetate, p-nitrophenyl acetate, methyl acetate, ethyl acetate, glyceryl triacetate (triacetin, β-D-glucose pentaacetate and β-D-xylose tetraacetate (table 3). The enzyme had practically no activity against birch wood xylan, or acetylated xylan. The activity of the enzyme was highest for the aryl-substrates like α-naphthyl acetate, p-nitrophenyl acetate and for β-D-xylose tetraacetate. β-D-xylose tetraacetate could be completely deacetylated after prolonged incubation (fig. 5). Propionate and butyrate were not released from the substrates ethylpropionate and glyceryl tributyrate (tributyrin), respectively. Boiled enzyme exhibited no activity.

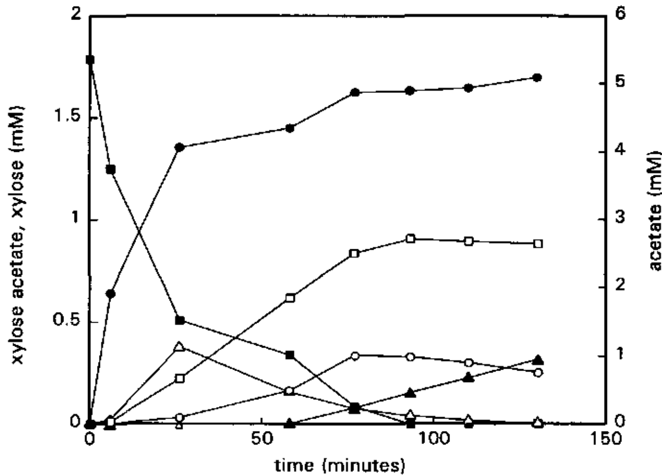

Fig. 5. Formation of acetic acid and xylose from xylose tetraacetate by the purified acetyl-esterase of *B. xylanolyticus* X5-1. Conditions: substrate concentration 1.8 mM, initial pH 6.5, temperature 37°C, 0.2 U acetyl-esterase. ■-■: xylose tetraacetate, ●-●: acetate, ▲-▲: xylose. The intermediates of the reaction: xylose tri-, di-, and mono-acetate were not identified. The concentration of these intermediates were estimated based on the relative peak area (Rf-values) of xylose tetraacetate and xylose. Δ-Δ intermediate 1, □-□ intermediate 2, O-O intermediate 3.

Discussion

The synthesis of the acetyl (xylose) esterase in *Bacteroides xylanolyticus* X5-1 is regulated. The acetyl (xylose) esterase was mainly produced when the organism was grown on xylan, but not when grown on glucose. The production of acetyl esterase on different growth substrates has been investigated for several microorganisms. In several anaerobic bacteria the synthesis of acetyl esterase is constitutive, like in *Thermoanaerobacterium saccharolyticum*, *Fibrobacter succinogenes* and in some *Butyrivibrio fibrisolvens* strains (11,16,18). Although substantial activities could be found during growth on glucose in most *Butyrivibrio fibrisolvens* strains, in some xylanolytic *Butyrivibrio fibrisolvens* strains acetyl esterase levels were highest in cultures grown on xylan (11,32). Low constitutive levels of acetylxylan esterase were found in cultures of several *streptomyces* spp. (13,17). However, these activities were greatly elevated when the organisms were grown on hemicellulose. In *Thermomonospora fusca* the extracellular acetylxylan esterase activity was low and only detected in cultures grown on xylan (2). Several cellulolytic fungi produce acetylxylan esterases (6,14); the presence of xylan or cellulose in the growth medium induced the formation of the acetylxylan esterase.

Table 4. General properties of the purified acetyl esterases from several microorganisms.

Organism	Molecular weight (kDa)	Mol wt of subunits (kDa)	AE[b] Specific activity (μmol. $min^{-1}.mg^{-1}$)	AXE[b] Specific activity (μmol. $min^{-1}.mg^{-1}$)	pH opt	T opt (°C)	K_M (mM aryl acetate)	V_{max} (μmol. $min^{-1}mg^{-1}$)	Ref.
Bacteroides xylanolyticus	245	62	190	ND[a]	5.9-6.7	45	0.09	200	
Fibrobacter succinogenes	55	55	2930	8.6	7.0	47	2.66	9100	19
Thermomonospora fusca	80	40	0.06						2
Trichoderma reesei	34	34	4.8	78	5.0-6.0	60-65			24,28
Trichoderma reesei	45	45	108	ND[a]	5.5	60-65			23

[a]ND= not detected
[b]AE= acetyl esterase , AXE= acetyl xylan esterase

Only a few acetyl esterases have been purified from xylan-degrading microorganisms (Table 4.). *Trichoderma reesei* produces three extracellular acetyl esterases, that play a role in xylan degradation (23,24,28). Two acetyl esterases (34 kDa), isoenzymes with very similar properties, had a preference for high molecular weight substrates. These enzymes deacetylate acetyl xylan and acetylated xylooligomers. The other acetyl esterase (45 kDa) could not deacetylate the polymeric substrate, but was only active on acetylated xylobiose and xylose. The extracellular acetyl esterase of *F. succinogenes* had very high aryl acetyl esterase activity and was capable of hydrolysing the acetyl groups from acetyl xylan (18,19). *T. fusca* produced a dimeric (80 kDa) intracellular acetyl esterase that is excreted as a 40 kDa monomer and is able to deacetylate acetyl xylan (2). The acetyl esterase from *B. xylanolyticus* X5-1 differs markedly from these enzymes. The apparent molecular mass of the enzyme was estimated to be 245 kDa. Electrophoresis on SDS-page indicated that the acetyl esterase from *B. xylanolyticus* X5-1 is composed of 4 identical subunits of 62 kDa. The extracellular acetyl esterases described in the other micro-organisms were all monomeric enzymes with a molecular mass varying from 34 kDa to 55 kDa. The arylacetyl esterase activity of the purified enzyme was comparable to the activity found for the enzyme of *T. reesei* (100 U/mg), but much higher than that of *T. fusca* (0.06 U/mg) and much lower than that of *F. succinogenes* (2930 U/mg). The pH optima of the different purified acetyl esterases were comparable. They also had similar temperature optima of about 45°C, whereas the enzymes rapidly lost activity at temperatures of 55°C and higher. Except for Hg^{2+}, metal cations or chelators did not influence the esterase activity significantly, suggesting that, as for the acetyl esterase of *F. sucinogenes*, no metal ions were required for activity. In contrast, the aryl acetyl esterase activity in cultures of several *B. fibrisolvens* strains was inhibited in the presence of calcium ions and sodium EDTA (11).

The acetyl esterase from *B. xylanolyticus* X5-1 is not active on acetyl xylan and consequently cannot be designated as an acetylxylan esterase. The enzyme hydrolyses several low molecular weight acetyl esters, but not esters of fatty acids with a longer chain length. This enzyme can therefore be classified as an acetyl esterase (E.C. 3.1.1.6.). Compared to other acetyl esterases this enzyme has a high activity on *β*-D-xylose tetraacetate. Furthermore, this

enzyme hydrolyses all four acetyl groups from the *ß*-D-xylose tetraacetate. *T. reesei* also produces an acetyl esterase, active on short acetylated glycosides. However, this latter enzyme showed regioselective cleavage of only the acetyl groups from the C-3 position of the xylopyranose ring (24).

Acetyl esterase activity acting on acetyl xylan could not be detected in cultures of *B. xylanolyticus* X5-1. The fact that the acetyl esterase is mainly synthesized when the organism is grown on xylan, makes it probable that it plays a role in the degradation of xylan. However, the enzyme can not act synergistically with the xylanase thus creating un-substituted xylose residues, promoting the hydrolysis of the interglycosidic linkages by the xylanase as has been reported for *T. reesei, Schizophyllum commune* and the actinomycete *T. fusca* (2,5,24). The cell associated acetyl esterase of *B. xylanolyticus* X5-1 could be involved in delivering unsubstituted xylose and xylobiose to the cell, thus facilitating the uptake of the sugars and subsequent hydrolysis of xylobiose by the ß-xylosidase.

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Chapter 6

Summary and general conclusions

Plant cell walls are the major reservoir of fixed carbon in nature. The mineralization of the fiber material, the so called lignocellulosic complex, proceeds almost exclusively by microbial processes in both aerobic and anaerobic environments. In anaerobic microbial processes the energy of the plant polymers can be conserved in fermentation products. The valorization of agricultural waste plant materials can consist of low and high technological processes. These include the production of biogas, ethanol, solvents and enzymes.

The first step in the anaerobic conversion of plant cell wall material is enzymatic degradation of the polysaccharides to soluble sugars. This is the rate limiting process and it is often incomplete. The hemicellulose xylan is one of the major constituents of plant cell walls. The structure of this polysaccharide is dependent on the source from which it has been isolated. The diversity of xylan is one of the reasons why the hydrolysis of this polysaccharide can be slow or incomplete. For a thorough comprehension of anaerobic digestion it is therefore important to obtain a better insight into the microbiology of xylan-degrading bacteria.

The aim of this thesis was to examine the nature of the enzymes needed for a complete degradation of xylan and to study their regulation. In parallel a research was conducted by Steef Biesterveld to investigate the bacterial fermentation of xylose, the major constituent of xylan. The xylose uptake system as well as the xylose metabolic pathways of the model organism *Bacteroides xylanolyticus* X5-1 were investigated. The main topics of his research are summarized here. Xylose enters the cell through an active uptake system (Fig. 1C and 1D) and is converted via the pentose phosphate pathway followed by the glycolysis to acetate, ethanol, H_2, CO_2 and formate as the main fermentation products. The first two enzymes in the xylose metabolism, xylose-isomerase and xylulose-kinase were inducible, whereas the xylose transport was constitutive. The regulation of product formation and the regulation of the formation of some key enzymes by H_2 were investigated. No diauxic growth, that is no preference for glucose over xylose or arabinose was observed. External electron acceptors could be used to shift the metabolic pathways. It was shown that it is possible to modulate the xylose metabolism by several methods and at different levels.

The complete microbial degradation of branched xylan involves the action of several hydrolytic enzymes: endo-1,4-β-D-xylanases (EC 3.2.1.8) which hydrolyze the internal ß-1,4-xylosic linkages of the xylan backbone, β-D-xylosidases (EC 3.2.1.37) which release xylose residues from small oligomeric substrates, and several enzymes capable of hydrolyzing substituents from the xylan backbone such as arabinofuranosidases and acetyl esterases.

Bacteroides xylanolyticus X5-1, a predominant strain isolated from fermenting cattle manure, grows efficiently on xylan. The organism produces at least five different enzymes to degrade this polymeric substrate. These enzyme activities include xylanase, ß-D-xylosidase, acetyl-esterase and α-L-arabinofuranosidase activities. The enzymes are not secreted into the medium, but stay associated to the cell during exponential growth (Fig. 1A and 1B). This enables this organism to efficiently use the degradation products of the different enzymes in a highly competitive environment. The production of the enzymes is tightly regulated. The mechanism of control of xylanolytic enzyme synthesis varies considerably among different microorganisms as is revealed by an analysis of literature data. Induction, catabolite repression, growth rate and other environmental factors can influence the activity of the xylanolytic enzymes. High molecular weight xylan can not enter the cells, and consequently can not directly induce the synthesis of xylanolytic enzymes. Low molecular weight sugars are often involved in either the induction and/or the repression of xylan degrading enzymes.

In chapter 2 the regulation, purification and some properties of two endo-β-1,4-xylanases were presented. During growth on xylan, *B. xylanolyticus* X5-1 produces two different endo-ß-1,4-xylanases. These enzymes were purified by column chromatography to apparent homogeneity. Both enzymes are monomeric with a molecular weight of 38000 (xylanase I) and 63000 Da (xylanase II), respectively. Xylanase I degraded xylan and xylo-oligomers with a polymerization degree of 4 and higher. Xylanase I of *B. xylanolyticus* released arabinose after prolonged incubation with xylan, and after incubation with arabinose containing xylo-oligomers. Since arabinose release coincided with xylose appearance in the assays, this side activity seems to be the result of unspecific cleavage. The final products of

the enzymatic degradation of arabinoxylan by xylanase I are xylotriose, xylobiose, xylose and arabinose. Xylanase II degraded xylan to xylose and xylobiose. Small xylo-oligomers were degraded much slower than the polymeric substrate. Arabinose was not released from oat spelt arabinoxylan or smaller oligosaccharides by this enzyme.

The regulation of the formation of the two xylanases was investigated. Little attention has so far been paid to the possibility that the formation of individual xylanases might be under different control. *B. xylanolyticus* has a differential regulation for the synthesis of the two xylanases. Xylanase I production did not seem to be induced by a direct product of xylan degradation, but was constitutively synthesized when no easily metabolizable sugars were present, and enough energy was available for the cells (Fig. 1A). The formation of xylanase I was repressed by readily metabolizable sugars as well as by the non-metabolisable sugar D-arabinose. The uptake of D-arabinose and the other sugars must therefore be involved in the repression of xylanase synthesis or the sugars themselves are direct repressors (Fig. 1C). Short xylo-oligosaccharides were good inducers of xylanase I. In resting cell suspensions pyruvate induced high levels of this enzyme. Pyruvate, an intermediate of the sugar degradation, probably acted as a good inducer because it circumvented the catabolic repression normally occurring when easily metabolisable sugars are available to the cells. Under growing conditions with pyruvate as sole carbon source, *B. xylanolyticus* did not produce xylanase activity. *B. xylanolyticus* produced low levels of xylanase activity in the presence of low concentrations of pentoses, which are released during xylan degradation. Regulation of xylanase I can be interpreted as constitutive synthesis under catabolite control. The availability of enough energy was the main factor responsible for xylanase I formation, but sugars present in the medium repressed the xylanase I formation. The uptake of the sugars is likely to be involved in the xylanase repression (Fig. 1C).

Xylanase II was only produced in significant amounts when the organism was grown on xylan. It was produced in higher amounts on birch- or larch-wood xylan, compared to oat spelt xylan. On monomeric sugars, as well as short xylo-oligomers, no xylanase II was produced. The formation of this enzyme was also catabolite repressed by easy metabolisable substrates. The exact nature of the inducer of this enzyme was not identified, but one could

think of a xylooligomer containing specific side chains, found in wood-xylan more than in grass xylan (Fig. 1A). *B. xylanolyticus* X5-1 is able to fine tune its xylanase synthesis according to demand. It is possible that the difference in substrate specificities of the two purified xylanases reflect their role in xylan degradation. Xylanase I has a broad specificity and is produced constitutively. Xylanase II may be better adapted to attack wood xylan, because it is preferentially produced under these conditions. The different regulation mechanism and the substrate specificities of the two xylanases allows a flexible response to changes in nutritional conditions.

In chapter 3 the regulation, purification and properties of a β-xylosidase of *B. xylanolyticus* X5-1 was presented. The formation of β-xylosidase activity is induced by the pentoses D-xylose and L-arabinose and repressed by intermediates of the sugar metabolism and pyruvate (Fig. 1B). A simultaneous induction and repression of the β-xylosidase synthesis by xylose was observed, which resulted in an optimal inducer concentration of about 20 mM. The repressive effect of glucose on the β-xylosidase induction by xylose was immediate and can be interpreted in terms of catabolite repression. A regulation by inducer exclusion, by which glucose prevents the entry of inducers, is not likely since both xylose and glucose were consumed simultaneously by cell suspensions.

The β-xylosidase induced by xylose was purified by column chromatography. The purified enzyme had a very low thermostability. In vivo, the enzyme would probably be more stable when located in the cytosol. It had an apparent molecular weight of 165 kDa and was composed of two subunits of 85 kDa. The enzyme exhibited optimal activity at pH 6 and 40°C. The isoelectric point was 6.3. It hydrolyzed *p*-nitrophenyl-β-D-xyloside with a K_M of 0.125 mM. The activity was strongly inhibited by Hg^{2+}. The β-xylosidase of *B. xylanolyticus* hydrolyzed *p*-nitrophenyl-β-D-xylopyranoside and can be denoted as a typical β-D-xylosidase in the sense that it could cleave of single xylose units from short xylo-oligosaccharides. The activity for xylobiose and xylotriose was much higher than for the longer xylo-oligomers. Xylan and other *p*-nitrophenylglycosides were no substrates for the enzyme.

In chapter 4 the purification of a cell-associated α-L-arabinofuranosidase of *B. xylanolyticus* X5-1 is described. The enzyme was purified 41-fold to apparent homogeneity. The native enzyme had an apparent molecular mass of 364 kDa and was composed of six polypeptide subunits of 61 kDa. The enzyme was stable under the conditions found in the extracellular environment. The enzyme was not affected by divalent cations and was very sensitive to sulfhydryl inhibitors like mercury indicating the presence of essential thiol groups in the enzyme. The anaerobic growth environment of the organism probably ensures that the sulfhydryl groups remain reduced. The substrate specificity of the purified α-L-arabinofuranosidase was very narrow. It was only able to release the α-linked L-arabinose in the furanose form from the synthetic substrates tested. The K_m and V_{max} for *p*-nitrophenyl-α-L-arabinofuranoside were 0.5 mM and 155 U/mg of protein, respectively. Compared with the high activities found with the artificial substrate *p*-nitrophenyl-α-L-arabinofuranoside the activities with potential natural substrates were low. The enzyme was unable to release L-arabinose from arabinogalactan or oat spelt arabinoxylan, it could however cleave of arabinose residues from arabinose containing xylo-oligosaccharides with a polymerization degree of about 2 to 5. The enzyme belongs to the *Streptomyces purpurascens*-type of α-L-arabinofuranosidase.

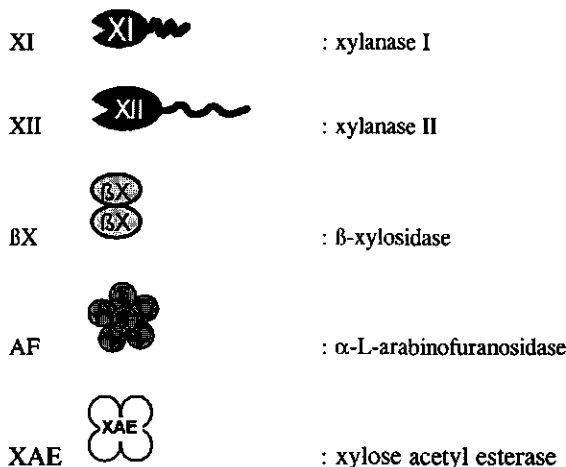
The synthesis of the multimeric α-L-arabinofuranosidase in *B. xylanolyticus* X5-1 was regulated. High activities were found after growth on L-arabinose and D-xylose, compared to growth on xylan (Fig 1B). This suggest that the synthesis of α-L-arabinofuranosidase is induced by these pentoses or by metabolites directly derived from these sugars. The enzyme was not produced when the organism was grown on glucose or cellobiose. These hexoses acted as catabolite repressor. The enzyme was mainly extracellularly attached to the cell when the organism was grown on xylan and was not released into the medium. In this way the enzyme can cooperate with the xylanases of this organism.

Chapter 5 describes the purification and characterization of a xylose acetyl esterase of *B. xylanolyticus* X5-1. No acetyl-xylan esterase activity could be detected in cultures grown on

xylan, but *B. xylanolyticus* X5-1 produced high activities of an acetyl-xylose esterase. The synthesis of the acetyl-xylose esterase in *B. xylanolyticus* X5-1 was regulated. The acetyl xylose esterase was only produced in significant amounts when the organism was grown on xylan (Fig. 1A). The acetyl esterase was purified by column chromatography from cell extracts of *B. xylanolyticus* X5-1 grown on xylan. The enzyme had an apparent molecular mass of 245 kDa and was composed of 4 identical subunits of 62 kDa. No metal ions were required for activity. The enzyme was stable under the conditions found in the extracellular environment. The acetyl esterase from *B. xylanolyticus* X5-1 was not active on acetylated xylan, but hydrolyzed several low molecular weight acetyl esters, but not esters of fatty acids with a longer chain length. This enzyme could therefore be classified as an acetyl esterase (E.C. 3.1.1.6.). Compared to other acetyl esterases this enzyme had a high activity on *β*-D-xylose tetraacetate. Furthermore, this enzyme hydrolyzed all four acetyl groups from the *β*-D-xylose tetraacetate. The fact that the acetyl esterase was mainly synthesized when the organism was grown on xylan, makes it probable that it plays a role in the degradation of xylan. The cell associated acetyl esterase of *B. xylanolyticus* X5-1 could be involved in delivering unsubstituted xylose, xylobiose and xylotriose to the cell, thus facilitating the uptake of these sugars and the subsequent hydrolysis by ß-xylosidase.

B. xylanolyticus X5-1 has a set of enzymes enabling it to efficiently grow on xylan. All the xylanolytic enzymes studied in this research do not seem to be located in one regulon. The *α*-L-arabinofuranosidase and *β*-xylosidase could be located in one operon. By having different induction mechanisms for the synthesis of the xylanolytic enzymes, this organism is able to adapt specifically to the environmental conditions. In figure 1, the xylan degradation and enzyme regulation by *B. xylanolyticus* X5-1 is represented schematically.

Figure 1. Schematic representation of the xylan degradation by the anaerobic bacterium *Bacteroides xylanolyticus* X5-1.



Schemes A to D represent events happening inside and outside of the bacterial cell during xylan degradation, that influence the production of xylanolytic enzymes:

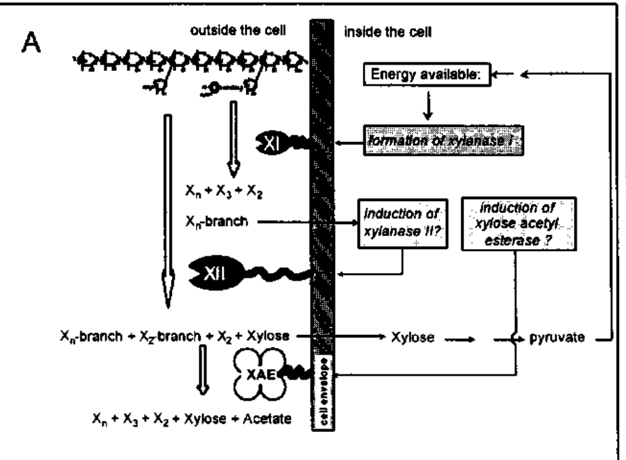
A: Low levels of xylanase I are produced by the organism, when energy is available and no sugars are present in large amounts. This enzyme is exported and xylan is hydrolyzed into branched oligosaccharides and short xylooligomers. When these oligomers enter the cell, they induce the formation of the second xylanase and the xylose acetyl esterase. These enzymes degrade xylan further and larger amounts of xylo-oligosaccharides are produced. The acetyl side chains are removed by the xylose acetyl esterase and xylose and arabinose are metabolized by the cell to yield energy. This in turn induces the formation of the first xylanase, repeating the whole cycle.

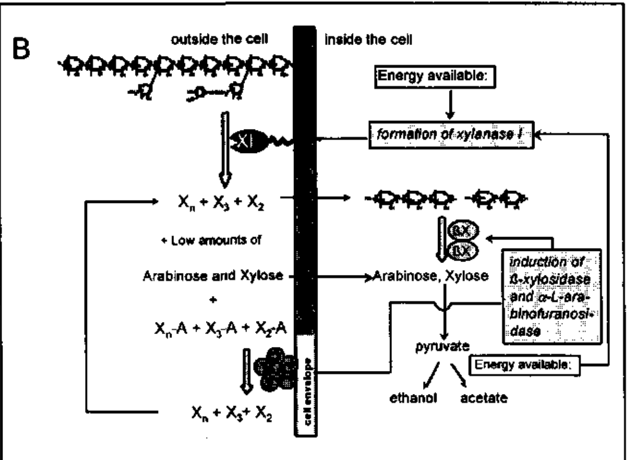
B: By the action of xylanase I, xylanase II and xylose acetyl esterase, arabinose and xylose are formed. These are transported into the cell and induce the formation of ß-xylosidase and α-L-arabinofuranosidase. The α-L-arabinofuranosidase removes the arabinose side chains from branched xylooligosaccharides generated from xylan by the xylanases. The short xylooligosaccharides are then converted to xylose by the ß-xylosidase. Xylose catabolism in turn yields energy and xylanase I is synthesized.

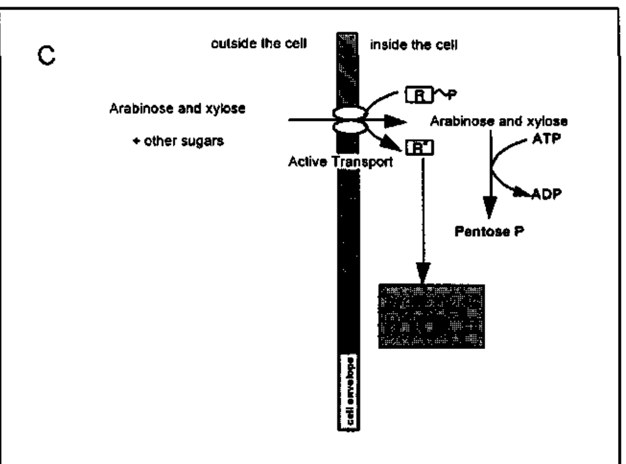
C: The degradation of xylan proceeds as mentioned above and increasing amounts of monosaccharides are produced. These pentoses are taken up by the cells and by catabolite repression the synthesis of the xylanases is repressed.

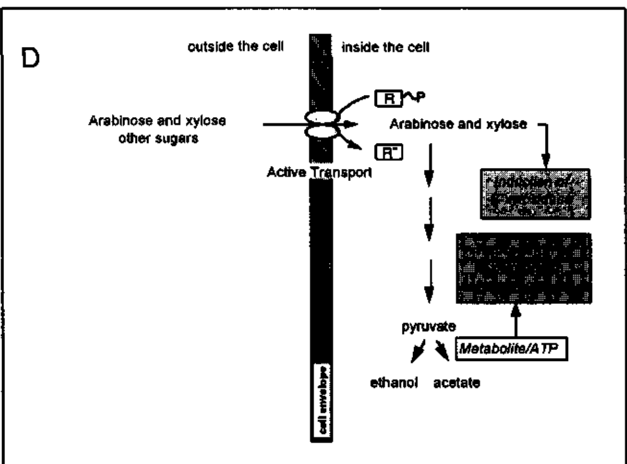
D: Xylose and arabinose, the major end products of xylan hydrolysis, are metabolized and yield mainly ethanol, acetate, CO_2, H_2 and formate. During this process a metabolite is formed which represses the ß-xylosidase synthesis.

In short, the xylanolytic enzymes are produced as long as the concentration of the xylan degradation products, xylose and arabinose, are not to high. At the end of the xylan hydrolysis, high amounts of monomeric sugars directly or indirectly cause a repression of the xylanolytic enzyme formation.


A
outside the cell
inside the cell
Energy available:
formation of xylanase I
XI
$X_n + X_3 + X_2$
X_n-branch
induction of xylanase II?
induction of xylose acetyl esterase ?
XII
X_n-branch + X_2-branch + X_2 + Xylose
Xylose
pyruvate
XAE
$X_n + X_3 + X_2$ + Xylose + Acetate
cell envelope


B
outside the cell
inside the cell
Energy available:
formation of xylanase I
XI
$X_n + X_3 + X_2$
+ Low amounts of
Arabinose and Xylose
+
X_n-A + X_3-A + X_2-A
Arabinose, Xylose
induction of ß-xylosidase and α-L-arabinofuranosidase
pyruvate
Energy available:
ethanol
acetate
$X_n + X_3 + X_2$
cell envelope


C
outside the cell
inside the cell
Arabinose and xylose
+ other sugars
Active Transport
Arabinose and xylose
ATP
ADP
Pentose P
cell envelope


D
outside the cell
inside the cell
Arabinose and xylose
other sugars
Active Transport
Arabinose and xylose
pyruvate
Metabolite/ATP
ethanol
acetate
cell envelope

Chapter 7

Samenvatting

Plantecelwanden zijn de belangrijkste bron van gebonden koolstof in de natuur. De mineralisatie van vezelachtig materiaal uit deze plantecelwanden, het zogenoemde lignocellulose complex, komt vrijwel volledig voor de rekening van microbiële processen, zowel in aërobe als anaërobe milieus. In anaërobe microbiologische processen kan de energie, die opgeslagen ligt in de plantaardige polymeren, geconserveerd worden in fermentatieproducten. De opwaardering van plantaardig landbouwafval kan via laag- en hoogwaardige technologische processen verlopen. Hieronder vallen onder andere de produktie van biogas, ethanol, oplosmiddelen en enzymen.

De eerste stap in de omzetting van plantecelwandbestanddelen is de enzymatische afbraak van de polysacchariden tot oplosbare suikers. Dit proces is de snelheidsbeperkende stap en is vaak onvolledig. Het hemicellulose xylaan is een van de belangrijkste bestanddelen van plantecelwanden. De structuur van deze polysaccharide is afhankelijk van de bron waaruit deze geïsoleerd is. De diversiteit in structuur van xylaan is een van de redenen waarom de hydrolyse van deze polysaccharide langzaam of onvolledig kan zijn. Voor een grondig begrip van de anaërobe afbraak van dergelijke polysacchariden, is het belangrijk een beter inzicht te krijgen in de microbiologie van xylanolytische bacteriën.

Voor de volledige microbiële afbraak van vertakt xylaan zijn verschillende hydrolytische enzymen noodzakelijk: endo-1,4-β-D-xylanasen (EC 3.2.1.8) hydrolyseren de interne ß-1,4-xylosidische bindingen van de xylaan hoofdketen, β-D-xylosidasen (EC 3.2.1.37) splitsen xylose eenheden af van korte oligomere xylose ketens en tenslotte verscheidene enzymen, waaronder arabinofuranosidasen and acetyl esterasen, die betrokken zijn bij de afsplitsing van de verschillende zijketens van xylaan.

De produktie van xylanolytische enzymen door micro-organismen is in het algemeen strak gereguleerd. Welk mechanisme verantwoordelijk is voor deze regulering hangt af van het micro-organisme. Inductie, kataboliet repressie, groeisnelheid en omgevingsfactoren kunnen de activiteit van de xylanolytische enzymen beïnvloeden. Xylaan, een polymeer met een hoog molecuulgewicht, kan de cellen niet binnendringen. Daarom gaat men ervan uit dat het polymere xylaan geen directe rol speelt bij de inductie van de xylanolytische enzymen.

Monomere en oligomere oplosbare suikers zijn echter vaak betrokken bij de inductie en/of de repressie van xylaan-hydrolyserende enzymen.

Bacteroides xylanolyticus X5-1, een stam geïsoleerd uit gistend rundermest, groeit goed op xylaan. Dit organisme produceert ten minste vijf verschillende enzymen die noodzakelijk zijn om dit polymere substraat af te breken. Deze enzymactiviteiten omvatten xylanase, ß-D-xylosidase, acetyl-esterase en α-L-arabinofuranosidase activiteiten. De enzymen worden niet in het kweekmedium uitgescheiden, maar blijven gedurende de groeifase van de bacteriën geassocieerd aan de cel (Hoofdstuk 6, figuur 1A en 1B). Hierdoor is dit organisme, dat moet overleven in een zeer competitief milieu, in staat efficiënt gebruik te maken van de hydrolyseproducten van de verschillende xylanolytische enzymen.

Het doel van dit proefschrift is de aard en regulering van de enzymen, die noodzakelijk zijn voor een volledige afbraak van xylaan, te bestuderen. Een parallel onderzoek werd verricht door Steef Biesterveld naar de bacteriële fermentatie van xylose, de belangrijkste bouwsteen van xylaan. De belangrijkste onderwerpen van dit parallelle onderzoek zijn hier samengevat. Het xylose opname systeem, alsook de fermentatie route van xylose, van het modelorganisme *Bacteroides xylanolyticus* werden onderzocht. Xylose wordt via een actief transportsysteem in de cel opgenomen (Hoofdstuk 6, fig. 1C en 1D) en wordt vervolgens via een combinatie van de zogenaamde pentosefosfaat route en de glycolyse, omgezet in acetaat, ethanol, H_2, CO_2 en formiaat als belangrijkste fermentatieprodukten. De eerste twee enzymen in het xylose metabolisme, xylose-isomerase en xylulose-kinase, zijn induceerbaar, terwijl het xylose transport constitutief is. Diauxie, d.w.z. een voorkeur voor glucose boven xylose of arabinose, kon niet worden waargenomen. Externe electronen acceptoren konden worden gebruikt om bepaalde metabole routes te verschuiven.

In hoofdstuk 2 wordt de regulering, zuivering en een aantal eigenschappen van twee verschillende endo-β-1,4-xylanasen gepresenteerd. Gedurende de groei op xylaan produceerde *B. xylanolyticus* X5-1 twee verschillende endo-β-1,4-xylanasen. Deze enzymen werden gezuiverd met behulp van kolomchromatografische methoden. Beide enzymen zijn monomere eiwitten met een molecuulgewicht van respectievelijk 38000 (xylanase I) en

63000 Da (xylanase II). Xylanase I is in staat xylaan en xylo-oligomeren, met een polymerisatiegraad van 4 of meer, af te breken. Xylanase I splitst, indien het voor langere tijd geïncubeerd wordt met arabinoxylaan of arabinose-bevattende xylo-oligomeren, arabinose af van deze vertakte substraten. Daar de afsplitsing van arabinose samenvalt met de verschijning van xylose in de activiteitstesten, lijkt deze nevenactiviteit van xylanse I te berusten op a-speciefieke hydrolyse. De eindproducten van de enzymatische afbraak van arabinoxylaan door xylanase I zijn xylotriose, xylobiose, xylose en arabinose. Xylanase II hydrolyseert xylaan tot xylobiose en xylose. Korte xylo-oligomeren worden veel langzamer door dit enzym afgebroken dan het polymere substraat. Arabinose wordt niet afgesplitst van arabinoxylaan of kortere arabino-xylo-oligosacchariden door xylanase II.

In *B. xylanolyticus* wordt de synthese van de twee geïsoleerde xylanasen op verschillende wijze gereguleerd. Xylanase I lijkt niet geïnduceerd te worden door een direct product van de xylaan afbraak. Dit enzym werd constitutief geproduceerd in afwezigheid van gemakkelijk metaboliseerbare suikers mits tegelijkertijd voldoende energie beschikbaar was voor de cellen (Hoofdstuk 6, Fig. 1A). De vorming van xylanase I werd onderdrukt door zowel gemakkelijk metaboliseerbare suikers alsook door de niet-metaboliseerbare suiker D-arabinose. Een mogelijke verklaring hiervoor is, dat ofwel de opname van D-arabinose of andere suikers betrokken is bij de repressie van de xylanase synthese of de suikermoleculen zelf fungeren als direkte repressoren (Hoofdstuk 6, Fig. 1C). Korte xylo-oligosacchariden induceerden xylanase I. In rustende celsuspensies induceerde pyruvaat grote hoeveelheden van dit enzym. Pyruvaat, een intermediair van de suikerafbraak, was in staat als inductor te fungeren, omdat dit substraat geen cataboliet repressie veroorzaakt. Cataboliet repressie trad normaal op wanneer gemakkelijk metaboliseerbare suikers voor de cellen beschikbaar zijn. *B. xylanolyticus* produceerde geen xylanase activiteit tijdens groei op pyruvaat als enige koolstof- en energiebron. In aanwezigheid van lage concentraties pentoses, die vrijkomen tijdens de xylaan afbraak, maakte *B. xylanolyticus* lage hoeveelheden xylanase aan.

De regulering van xylanase I kan beschouwd worden als constitutieve synthese, gecontroleerd door cataboliet repressie. De beschikbaarheid van genoeg energie is de voorwaarde voor xylanase I vorming. Suikers aanwezig in het kweekmedium remmen

xylanase I vorming. De opname van deze suikers is naar alle waarschijnlijkheid betrokken bij de repressie van xylanase I (Hoofdstuk 6, Fig. 1C).

B. xylanolyticus X5-1 produceerde xylanase II alleen in significante hoeveelheden tijdens groei op xylaan. Meer xylanase II werd gevormd na groei op berken- en larix-xylaan, dan na groei op haverspelt xylaan. Tijdens groei op monomere suikers of korte xylo-oligomeren werd geen xylanase II gevormd. De synthese van dit enzym was eveneens onder cataboliet controle door gemakkelijk metaboliseerbare suikers. De exacte inductor is niet bekend. Mogelijke kandidaten zijn xylo-oligomeren van bepaalde lengte met specifieke zijketens, die in grotere hoeveelheden voorkomen in xylaan afkomstig uit hout, dan in xylaan uit grassen (Hoofdstuk 6, Fig. 1A). *B. xylanolyticus* X5-1 is in staat de xylanase synthese aan te passen aan de milieu omstandigheden waarin het verkeerd. Het verschil in substraatspecificiteit van de twee gezuiverde xylanasen geeft hun rol aan in de xylanase afbraak. Xylanase I heeft een brede substraatspecificiteit en wordt constitutief geproduceerd. Xylanase II is daarentegen beter geschikt om xylaan afkomstig uit houthoudende gewassen aan te grijpen, daar het voornamelijk tijdens groei op dit substraat gevormd wordt. De verschillende reguleringsmechanismen en substraatspecificiteiten van de twee xylanasen maken een flexibele reactie mogelijk op veranderingen in groeicondities.

In hoofdstuk 3 werden de regulering, zuivering en eigenschappen van een β-xylosidase van *B. xylanolyticus* X5-1 gepresenteerd. De vorming van β-xylosidase werd geïnduceerd door de pentoses D-xylose en L-arabinose en geremd door tussenproducten van het suiker metabolisme en door pyruvaat (Hoofdstuk 6, Fig. 1B). Een gelijktijdige inductie en repressie door xylose kon worden waargenomen, hetgeen resulteerde in een optimale inductor concentratie van ongeveer 20 mM. De remmende werking van glucose op de inductie van β-xylosidase door xylose was onmiddelijk en kan worden uitgelegd als cataboliet repressie. Een reguleringsmechanisme berustend op zogenaamde “inducer exclusion”, waarbij glucose de opname van de inductor verhindert, is niet waarschijnlijk, daar xylose en glucose beiden gelijktijdig door de celsuspensies werden geconsumeerd.

Het β-xylosidase geïnduceerd door xylose werd gezuiverd m.b.v. kolomchromatografie. Het gezuiverde enzym had een zeer lage thermostabiliteit. Het had een schijnbare molecuulmassa

van 165 kDa en bestond uit twee ondereenheden van 85 kDa. Het enzym had de hoogste activiteit bij pH 6 en bij 40°C. Het isoëlectrisch punt was 6.3. De K_M voor *p*-nitrophenyl-*β*-D-xyloside als substraat was 0.125 mM. De activiteit werd sterk geremd door Hg^{2+}, zelfs bij concentraties van 10 μM. Het *β*-xylosidase van *B. xylanolyticus* hydrolyseerde *p*-nitrophenyl-*β*-D-xylopyranoside en valt onder de groep van *β*-D-xylosidasen, omdat het enkelvoudige xylose eenheden afsplitst van korte xylo-oligosacchariden. De activiteit voor de substraten xylobiose en xylotriose was veel hoger dan voor langere xylo-oligomeren. Xylaan en andere *p*-nitrophenylglycosides waren geen substraat voor dit enzym.

In hoofdstuk 4 werd de zuivering bescheven van een celgebonden *α*-L-arabinofuranosidase van *B. xylanolyticus* X5-1. Dit enzym werd 41 maal gezuiverd, resulterend in schijnbare homogeniteit. Het natieve enzym had een schijnbare molecuulmassa van 365 kDa en bestond uit 6 polypeptide ondereenheden van 61 kDa. Het enzym was stabiel onder de omstandigheden, die gevonden worden in de extracellulaire omgeving van de bacterie. Diverse divalente kationen hadden geen effect op de enzymactiviteit. Het enzym was zeer gevoelig voor sulfhydryl remmers, zoals kwik. Dit wijst op de aanwezigheid van essentiële thiolgroepen in het enzym. Het anaërobe milieu van dit organisme zorgt ervoor dat de sulfhydryl groepen gereduceerd blijven. De substraatspecificiteit van het *α*-L-arabinofurano-sidase was zeer smal. Het enzym was alleen in staat *α*-gebonden L-arabinose in de furanose vorm te splitsen van de geteste substraten. De K_m en V_{max} voor *p*-nitrophenyl-*α*-L-arabinofu-ranoside was respectievelijk 0.5 mM en 155 U/mg eiwit. Vergeleken met de hoge activiteiten die gevonden werden voor het kunstmatige *p*-nitrophenyl-*α*-L-arabinofuranoside, waren de activiteiten voor de mogelijke natuurlijke substraten laag te noemen. Het enzym was niet in staat L-arabinose af te splitsen van de polymeren arabinogalactan of haverspelt arabinoxylaan. Het was echter wel in staat arabinose af te splitsen van arabinose-houdende xylo-oligosacchariden met een polymerisatiegraad van ongeveer 2 tot 5. Het enzym behoort tot de groep van *Streptomyces purpurascens*-type *α*-L-arabinofuranosidasen.

De vorming van het multimere *α*-L-arabinofuranosidase door *B. xylanolyticus* X5-1 wordt gereguleerd. Vergeleken met de activiteiten van dit enzym, die gedetecteerd werden na groei

op xylaan, waren de activiteiten na groei op L-arabinose en D-xylose zeer hoog (Hoofdstuk 6, Fig 1B). Dit lijkt erop te wijzen dat deze pentoses, of directe stofwisselingsproducten van deze suikers, verantwoordelijk zijn voor de inductie van α-L-arabinofuranosidase. Het enzym werd niet gevormd wanneer de bacterie gekweekt werd op glucose of cellobiose. Deze suikers waren cataboliet repressors. Het enzym was voornamelijk extracellulair gebonden aan de cellen na groei op xylaan en werd niet losgelaten in het medium. Dit maakt het mogelijk voor het α-L-arabinofuranosidase om samen te werken met de xylanases van dit organisme. Het gevormde arabinose kan ook direct worden opgenomen door de cellen.

In hoofdstuk 5 werd de zuivering en karakterisering van een xylose acetyl esterase van *B. xylanolyticus* X5-1 beschreven. Hoewel geen acetyl-xylan esterase activiteit kon worden gedetecteerd in culturen gekweekt op xylaan, produceerden zij hoge activiteiten van een acetyl-xylose esterase. De synthese van het acetyl-xylose esterase in *B. xylanolyticus* X5-1 was gereguleerd. Dit enzym werd alleen in significante hoeveelheden geproduceerd wanneer het organisme werd gekweekt op xylaan (Hoofdstuk 6, Fig. 1A). Het acetyl esterase werd gezuiverd door middel van kolomchromatographie. Het enzym had een schijnbare molecuulmassa van 245 kDa en bestond uit 4 identieke ondereenheden van 62 kDa. De noodzaak van metaalionen voor de werking van het enzym kon niet worden aangetoond. Het enzym was stabiel onder condities die gevonden worden in het extracellulair milieu van *B. xylanolyticus* X5-1. Het acetyl esterase was niet actief op geacetyleerd xylaan, maar was in staat acetaat af te splitsen van verscheidene acetylesters met een laag molecuulgewicht. Esters van vetzuren met een langere ketenlengte werden niet gehydrolyseerd. Dit enzym kan daarom worden geclassificeerd als een acetyl esterase (E.C. 3.1.1.6.). In vergelijking met andere acetyl esterasen had dit enzym een hoge activiteit voor β-D-xylose tetraacetaat en was in staat alle vier acetylgroepen β-D-xylose tetraacetaat af te splitsen. Daar het enzym voornamelijk werd gevormd wanneer de bacterie werd gekweekt op xylaan, en zeer actief was op acetyl-gesubstitueerde xylose, maken het waarschijnlijk dat dit enzym betrokken is bij de xylaan afbraak. Het celgebonden acetyl esterase van *B. xylanolyticus* X5-1 zou zo xylose, xylobiose en xylotriose aan de bacterie kunnen leveren, welke eventueel door het ß-xylosidase gehydrolyseerd zouden worden tot xylose.

B. xylanolyticus X5-1 heeft een set van enzymen die het in staat stellen om efficiënt te groeien op xylaan. Alle hier beschreven enzymen lijken niet te vallen onder één enkel regulon. Het α-L-arabinofuranosidase en β-xylosidase zouden kunnen liggen op een operon. Met behulp van verschillende reguleringsmechanismen voor de synthese van de xylanolytische enzymen, is *B. xylanolyticus* X5-1 in staat zich aan te passen aan de heersende omstandigheden. Figuur 1 in hoofdstuk 6 geeft de afbraak van xylaan en de enzymregulering door *B. xylanolyticus* X5-1 schematisch weer.

Nawoord

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Curriculum Vitae

Philippe Jean Yves Marie Joseph Schyns werd op 3 december 1962 geboren te Marche-en-Famenne in België. Na het behalen van het VWO diploma aan het gymnasium Rolduc te Kerkrade in 1980, begon hij aan de studie Moleculaire Wetenschappen aan de toenmalige Landbouwhogeschool te Wageningen. De doctoraalstudie omvatte de vakken Microbiologie, Moleculaire Biologie en Proceskunde. Vanaf 1987 was hij 8 maanden verbonden aan het Massachussets Institute of Technology als visiting scientist bij de vakgroep Chemical Engineering van professor C.L. Cooney.

Na het behalen van zijn inginieursdiploma, begon hij eind 1988 als assistent in opleiding aan de vakgroep Microbiologie van de Landbouwuniversiteit te Wageningen. De resultaten van het promotie onderzoek aan de xylaanafbraak door anaërobe bacteriën zijn beschreven in dit proefschrift. Na het beëindigen van zijn werkzaamheden aan de vakgroep Microbiologie, was hij gedurende twee en een half jaar werkzaam bij de 'Zentrale Forschung' in de afdeling Biotechnologie van Bayer AG in Leverkusen, Duitsland. Sinds Januari 1996 is hij in dienst bij Nalco Europe B.V. in de Pulp and Paper Research afdeling.