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*review*

# Potential of Selected Rumen Bacteria for Cellulose and Hemicellulose Degradation

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**Summary**

Herbivorous animals harbour potent cellulolytic and hemicellulolytic microorganisms that supply the host with nutrients acquired from degradation of ingested plant material. In addition to protozoa and fungi, rumen bacteria contribute a considerable part in the breakdown of recalcitrant (hemi)cellulosic biomass. The present review is focused on the enzymatic systems of three representative fibrolytic rumen bacteria, namely *Ruminococcus flavefaciens*, *Prevotella bryantii* and *Pseudobutyrivibrio xylanivorans*. *R. flavefaciens* is known for one of the most elaborated cellulosome architectures and might represent a promising candidate for the construction of designer cellulosomes. On the other hand, *Prevotella bryantii* and *Pseudobutyrivibrio xylanivorans* produce multiple free, but highly efficient xylanases. In addition, *P. xylanivorans* was also shown to have some probiotic traits, which makes it a promising candidate not only for biogas production, but also as an animal feed supplement. Genomic and proteomic analyses of cellulolytic and hemicellulolytic bacterial species aim to identify novel enzymes, which can then be cloned and expressed in adequate hosts to construct highly active recombinant hydrolytic microorganisms applicable for different biotechnological tasks.

*Key words:* rumen bacteria, *Ruminococcus flavefaciens*, *Prevotella bryantii*, *Pseudobutyrivibrio xylanivorans*, cellulosome, xylanase, probiotics

## Introduction

Rumen represents one of the best studied anaerobic ecosystems and constitutes a basis for the current understanding of microbial ecology in anaerobic digestive systems, including human intestinal tract and anaerobic biogas reactors. Due to the lack of (hemi)cellulolytic enzymes, the digestion of plant biomass in herbivorous animals strongly depends on rumen microbiota. The primary role of microorganisms in this complex food chain is the initiation of a plant biomass breakdown by cellulolytic and hemicellulolytic microorganisms.

### *The structure of plant cell wall*

The major component of plant cell wall is cellulose, which comprises from 20 % (in certain grasses) up to 90 % (in the cotton fibres) of the dry mass of plant tissue. It is a homopolysaccharide consisting of 100 to 20 000 D-glucopyranose residues linked by 1,4-β-glycosidic bonds. Parallel glucan chains are tightly packed to form microfibrils and larger macrofibrils. In most natural substrates cellulose fibrils are dominated by highly ordered crystalline areas, interrupted by less organized amorphous sections. In plant cell walls, cellulose fibrils are embedded in a matrix of other polysaccharides: pectin (as homogalacturonan and rhamnogalacturonan) and hemicellulose (xyloglucans, xylans, mannans and glucomannans, and 1,4-β- and 1,3-β-glucans). Among hemicellulosic substrates, xylan is the most abundant. The xylan structure is more heterogeneous than cellulose and consists of 1,4-linked β-D-xylopyranose backbone that bears many

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different side chains. The backbone is often partially acetylated and the type of substitution depends on botanical origin. Arabinoxylan (AX) is common type of xylan found in grasses and cereals (*e.g.* oat spelt xylan). It carries L-arabinose side-chains that can be esterified by ferulic or coumaric acid. These enable cross-linking of xylan backbone and enhance cell wall recalcitrance against enzymatic attack. Hardwood xylan (*e.g.* birchwood xylan) is glucuronoxylan (GX) with methylated or non-methylated D-glucuronic acid side-chains. Most of the D-xyloses are acetylated. Softwood arabinoglucuronoxylan (AGX) is more heavily substituted by methylated glucuronic acid than GX and additionally carries arabinofuranose branches (*e.g.* beechwood xylan). Usually, AGX chains are shorter than GX (*1*).

### *Enzymes that degrade (hemi)cellulose – glycoside hydrolases*

Enzymes that catalyze the hydrolysis of glycosidic bonds and degrade polysaccharides into smaller structural subunits are known as glycosidases or *O*-glycoside hydrolases (GH). According to their primary structure (amino acid sequence similarity), the GHs are currently classified into 133 families. Some of these families are further grouped in clans according to their 3D structure homologies (*2*). In addition to catalytic modules, many GH also contain one or more carbohydrate-binding modules (CBM). Typically, the CBMs and catalytic modules are connected by linker regions, allowing more flexibility to the catalytic part. The primary function of CBM is selective binding of the substrate and enzyme accumulation on its surface (*3*).

Cellulases are glycoside hydrolases with the primary role of cleaving the cellulose chains into shorter cellodextrines with two to six units. According to their mode of action, they are divided to endoglucanases and exoglucanases (also cellobiohydrolases). Endoglucanases randomly attack the internal glycosidic bonds in the amorphous regions of the cellulose chains and increase the number of loose ends, which represent the substrates for exoglucanases. The latter are generally processive enzymes that bind to the substrate and then progress to the end of the chain by releasing shorter saccharides. On the other hand, majority of the endoglucanases detach from the substrate after each cleavage and then randomly bind to another location (*3*).

Due to xylan's complex structure with diverse nature of side-chains, degradation is also carried out by the synergistic action of many different enzymes. The enzyme system usually consists of endoxylanases and β-xylosidases that cleave the polyxylopyranosyl backbone and many different side-chain-removing enzymes like α-L-arabinofuranosidases, α-glucuronidases, acetylxylan esterases, *p*-coumaric and ferulic acid esterases. Microbes usually possess the whole set of enzymes, often producing multiple forms of certain catalytic property with subtle differences in order to facilitate the solubilization of a variety of xylans (*4,5*).

Synergistic action of different enzymes with specialized roles is needed for efficient degradation of natural cellulosic substrates. Some anaerobes developed more effective cellulolytic strategy by spatially combining enzymes in extracellular multienzyme complexes, termed cellulosomes. These comprise a set of components with structural proteins (scaffoldins) binding different catalytic modules. The main interactions in the complex are the ones between the cohesion modules of the scaffoldins and dockerin module of the enzymes. Cellulosome structure varies among the species in variable number of the scaffoldins and diverse types of cohesin-binding sites that define the nature of the attached enzymes. Cellulosomes have so far mainly been described in some species from the bacterial order *Clostridiales* and in some rumen anaerobic fungi (*6*).

Degradation of a plant cell wall is usually conducted by the coordinated action of diverse members of the microbial community, acting as enzyme producers and/or end-product utilizers. In nature, such communities are found in different environments where intensive plant biomass degradation takes place, rumen being one of the richest ones. Not only cellulolytic and xylanolytic bacteria represent valuable sources of hydrolytic enzymes and other metabolites, but some of them also exhibit promising probiotic traits. The characterization of the enzymes and their catalytic mechanisms, and understanding of the induction triggers promote their biotechnological applications. Additionally, genomic analysis of efficient cellulolytic and hemicellulolytic communities may identify genes coding for novel and/or more efficient enzymes. Their subsequent cloning and expression in adequate hosts may lead to the production of recombinant microorganism with optimized properties for the degradation of recalcitrant plant biomass.

The following article is focused on the cellulolytic and hemicellulolytic machinery of the three prominent bacterial strains from rumen that were comprehensively studied in our research group (Chair of Microbiology and Microbial Biotechnology, Biotechnical Faculty, University of Ljubljana, Slovenia) in collaboration with some foreign institutes (see references): *Ruminococcus flavefaciens*, *Prevotella ruminicola* and *Pseudobutyrivibrio xylanivorans*.

## *Ruminococcus flavefaciens*

### *The first insight in R. flavefaciens cellulosomes (strains FD-1 and 17)*

*Ruminococcus flavefaciens* is a Gram-positive, strictly anaerobic coccus-shaped bacterium, phylogenetically classified in clostridial cluster IV (phylum Firmicutes). It is one of the most abundant cellulolytic bacteria in the gastrointestinal tract of the herbivores and currently the only rumen bacterium in which cellulosomes were experimentally confirmed (*7*). Comparative nucleotide sequence analysis in strains 17 and FD-1 revealed a *sca* gene cluster encoding four scaffoldins, namely ScaA, ScaB, ScaC and ScaE. The arrangement of the cluster and spacer regions were well conserved within the species, whereas structural genes showed a considerable diversity, which may be associated with the ability of individual strains to degrade different types of (hemi)cellulosic substrates (*6,8*). The core structural (enzyme-binding) proteins of *R. flavefaciens* cellulosomes proved to be two glycosylated scaffoldins, ScaA and ScaB. ScaB is the largest of the

scaffoldins and provides a basis for anchoring up to seven ScaA subunits, resulting in a multi-scaffoldin complex. Each ScaA protein binds two (in strain FD-1) or three (in strain 17) enzyme subunits directly or *via* the smallest scaffoldin, ScaC (*9*). Acting as an adapter protein, ScaC contributes to the variability of the cellulosome structure and substrate specificity (*10*). ScaE is an anchoring scaffoldin, covalently bound to the cell wall. It has only one cohesin that binds to ScaB and anchors the whole complex to the cell (*11*).

Interestingly, no CBMs similar to the previously described ones were found in *R. flavefaciens*. Instead, another protein with cellulose-binding capacity, CttA, was suggested to play this role in strain 17. The newly described protein is encoded by gene *cttA*, which is located adjacent to previously described *sca* genes and is considered part of the *sca* cluster. Its role was proposed after two regions of the recombinant protein were found to strongly bind to microcrystalline cellulose. The CttA also bears a ScaB-like dockerin which binds a cohesin of the cell-anchoring scaffoldin ScaE (*12*).

### *Genomic and expression studies of R. flavefaciens 007C and 007S*

Further insight in the mechanisms of cellulose degradation was gained by studying mutants of *R. flavefaciens* 007 (*13*). The first mutant, namely 007S, lost the ability to degrade cotton cellulose after being repeatedly subcultivated in cellobiose medium. After transferring 007S to the medium with cotton as a sole source of energy, the strain regained cotton degradation ability. The second mutant was named 007C (*14*). The mutants do not differ in the degradation of other substrates (cellobiose, xylan, Avicel), and can therefore be used as a model for elucidating a mechanism of cotton cellulose degradation.

Analysis of the 007C draft genome has revealed the same *sca* cluster arrangement as in other strains of *R. flavefaciens*: *scaC-scaA-scaB-cttA-scaE* (*13*). Since the amino acid sequences of the *sca* genes in strains 007C and 007S were nearly identical to scaffoldins of strain 17, the cellulosome structure was predicted to be the same (Fig. 1).

The 007C draft genome analysis also revealed several genes for putative cellulases, namely one for a glycoside hydrolase GH48 and GH44 and a wide range of enzymes from families GH9 and GH5 (*13*). It is possible that multiple putative endoglucanases from families GH5 and GH9 evolved from gene duplication and point mutation accumulation in the two origin genes. Minor differences in specificity and kinetic properties of these enzymes may offer the bacterium a competitive advantage in its natural environment. In most 007C cellulases, dockerin modules were also identified, confirming the enzyme association in the cellulosome. As in strain FD-1, substrate-binding modules were detected exclusively with the catalytic modules of GH9 and were mainly type CBM_3a (binding crystalline cellulose).

Zymograms confirmed the existence of a wide range of different endoglucanases in *R. flavefaciens* 007C and 007S: four were produced constitutively, while at least seven appeared to be induced exclusively when the strains were grown on insoluble cellulose (*15*). This is consistent with previous finding of Berg Miller *et al.* (*16*), who also reported cellulose-associated inducible transcription of only part of the GH consortium, while the rest of the enzymes appeared to be similarly expressed on cellobiose and cellulose. Some recent findings argue that differential expression of cellulolytic enzymes may not be mediated by the substrate directly, but rather *via* transcriptional regulators that respond to differences in growth rate on different carbon sources (*17*).

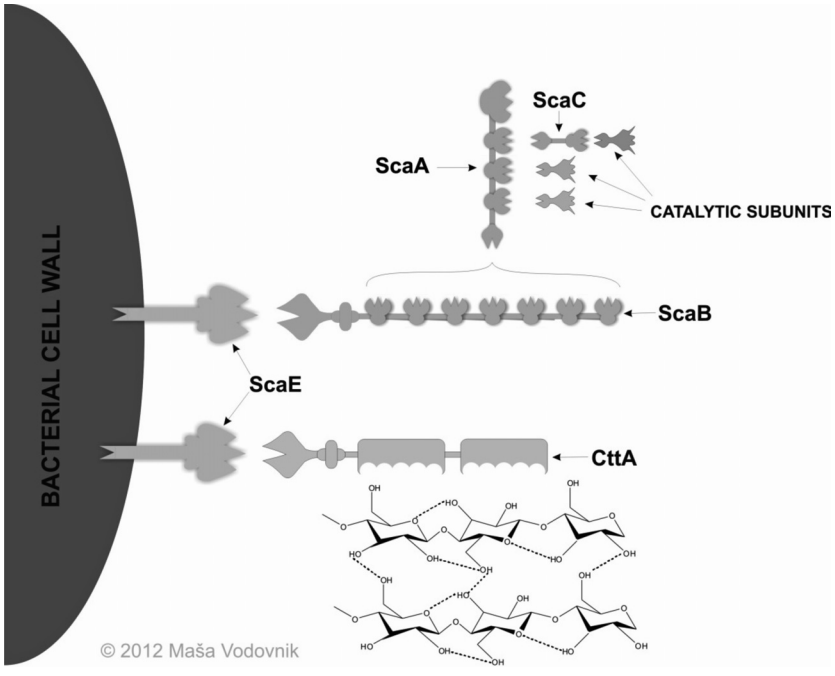


**Fig. 1.** Putative model of cellulosome organization in *Ruminococcus flavefaciens* 007 mutants. ScaB is the largest scaffoldin and carries several ScaA subunits. Different enzymes are attached to ScaA directly or *via* small scaffoldin ScaC. ScaE anchors the whole structure to the cell surface. CttA is cellulose-binding protein linked to the cell *via* ScaE (by M. Vodovnik)

In the initial stage of growth of *R. flavefaciens* 007C and 007S, the majority of the endoglucanases were cell-bound and their molecular mass corresponded mainly to cellulosomal GH9. In the later phases, additional smaller endoglucanases were detected predominantly in the supernatant fraction. Their molecular mass corresponded mainly to the enzymes from family GH5. It was proposed that GH9 carrying their own CBMs are the first to attack intact crystalline cellulose. The released shorter chains were then hydrolyzed by other enzymes among which are smaller, non-cellulosomal GH5 (*13*).

Proteomic analysis of *R. flavefaciens* 007C and 007S extracellular fractions revealed two dominant cellulosomal enzymes – a GH9 (homologue of *R. albus* endoglucanase Cel9B) and a GH48 (homologue of *R. albus* exoglucanase Cel48A) (*18,19*). The complementary action of the enzymes by the following scenario was suggested: after the GH9 endoglucanase cleaves cellulose chains in the amorphous regions, the GH48 exoglucanase binds to newly generated loose ends and travels along the chain, processively cleaving off the saccharides. Among the most abundantly expressed proteins during growth on the cellulose were not only a GH48 exoglucanase and some putative endoglucanases from family GH9, but also three products of *sca* gene cluster (ScaA, ScaB and CttA) and some ABC-transporters (*18*).

In further work, the extracellular protein profiles of *R. flavefaciens* 007C and 007S were compared in order to find protein candidates that may be crucial for the degradation of highly crystalline cellulose such as cotton (*13,18,20*). In contrast to the previous study of Rincón *et al.* (*11*), the analysis revealed no differences in relative amounts of CttA protein (*12*). However, two other proteins were highly upregulated in a cotton-degrading strain 007C. The first of these was a type IV pili-related (T4P) protein, subsequently named Pil3. Its relative volume was more than 19-fold higher during growth on Avicel cellulose than on cellobiose. The analysis of genetic neighbourhood of the gene coding Pil3 revealed the whole cluster of nine genes coding for different components of T4P biogenesis machinery. T4P are well studied in Gram-negative species, where they play diverse roles, especially in cell adherence and motility (twitching motility, for example). On the other hand, not much is known about these structures in Gram-positive bacteria. Only recently, analogues of a Gram-negative type IV pilus gene cluster were found in some Gram-positive strains, particularly in the most ancient bacterial class *Clostridia*, which implies a common evolutionary origin (*21*). Since our study revealed a T4P-related protein to be highly upregulated during growth on cellulose exclusively in cotton-degrading mutant 007C, it is possible that their role in *R. flavefaciens* is associated with the attachment to the highly crystalline regions of cellulose (*13*). It is also possible that pili have a role in processive movement of the cells along the cellulose polymers and/or cellulose amorphogenesis (*13*).

The second protein that was exclusively upregulated in *R. flavefaciens* 007C was a rubrerythrin-like protein (Rub) (*13,18*). This is a small protein (20 kDa) with an N-terminal $Fe^{2+}$ binding module and a C-terminus homologous to rubredoxin. Although it is widely distributed in anaerobic bacteria and archaea, its physiological role has not been identified yet. Interestingly, an open reading frame encoding a putative GH3 was found adjacent to the gene encoding Rub in *R. flavefaciens*, indicating its possible association with cellulolytic system (*13*). A novel mechanism of oxidative cellulose degradation involving copper-dependent polysaccharide monooxygenases was recently described in fungi (*22*). These enzymes were recently reclassified as auxiliary activity family 9 (AA9, former GH61) and are able to cleave strongly packed cellulose microfibrils in the crystalline areas with their strong oxidative action. It can be speculated that rubrerythrin in *R. flavefaciens* may play a role in oxygen detoxification system as found in other anaerobic microorganisms (*23*). However, its involvement in similar oxidative reactions of recalcitrant cellulose breakdown is an interesting area for future studies.

### *Yellow affinity substance – an auxiliary factor involved in cellulose degradation?*

Several strains *of R. flavefaciens* have long been known to produce high amounts of a yellow affinity substance (YAS) that strongly binds the cellulose (*24*). However, its chemical structure and function still remain unknown. In a study of Kopečný and Hodrová (*24*), YAS was also found to enhance the attachment of cellulases to the substrate. In our study, the majority of YAS was not associated directly with catalytic modules, but rather with the three large, heavily glycosylated scaffoldins, namely ScaA, ScaB and CttA (*13*). This suggests its role in the attachment of the complex to the substrate, which was supported by the fact that the addition of YAS to the washed supernatant proteins of *R. flavefaciens* 007C increased the microcrystalline cellulose breakdown.

### *Potential for applications in biotechnology*

The study of *R. flavefaciens* cellulolytic system revealed one of the most complex cellulosomal machineries with multimodular proteins and several putative auxiliary components (type IV pili, YAS, *etc.*) that allow efficient cellulose degradation in anaerobic environments. Newly gained knowledge may be usefully applied in many different fields of biotechnology. Besides traditional applications in paper, food and textile industry, the biotechnological potential of cellulosomes is now becoming increasingly recognized in bioenergy production (mainly biogas and biofuel production from plant biomass) (*3*). Together with novel molecular biology tools, knowledge of efficient cellulolytic systems can enable the construction of artificial cellulosomes with desired properties, such as increased capacity for the degradation of lignocellulose, better stability in process conditions, *etc*. These chimeric complexes are called 'designer cellulosomes' and are eventually meant to be expressed in industrial microorganisms. So far, solventogenic clostridia are the major object for heterologous expression of cellulosomal proteins and functional minicellulosome produced in otherwise non-cellulolytic *Clostridium acetobutylicum* (*25*). Due to phylogenetic relatedness, the knowledge about ruminoccocal cellulolytic machinery may be of great applicative value.

### *Prevotella bryantii*

*Prevotella* spp. are one of the dominant xylanolytic bacterial genera detected in the rumen (*26*), *P. bryantii* being prevalent in liquid fraction of rumen samples (*27*). Rumen isolates were formerly classified in one species, namely *P. ruminicola*. However, interstrain variability in xylanase gene distribution, zymogram patterns (*28*) and 16S rDNA sequences (*29*) initiates full assessment of the strains and eventually four species in the genus *Prevotella* were established (*30*). Rumen *Prevotella* strains are important in protein and peptide metabolism, some possess extracellular DNase activity and, nevertheless, they are able to degrade several polysaccharides commonly present in rumen digesta like xylan, cellulose and starch (*30*). Among xylanolytic strains, redefined *P. ruminicola* and *P. bryantii* with type strain $B_14$ were constituted. Strains of *P. bryantii* are strictly anaerobic Gram-negative bacteria belonging to the large *Bacteroidetes* phylum, and are capable of xylan, soluble cellulose (carboxymethylcellulose, CMC), starch and protein breakdown (*30*).

### *Xylanolytic activity of P. bryantii B14*

As many rumen xylanolytic bacteria, strain $B_14$ possesses multiple xylanase genes and has the highest xylanolytic activity of all *Prevotella* spp. (*31*). The xylanolytic activity was clearly inducible with growth in xylan-containing medium and the activities were 20-times higher compared to the medium containing only glucose (*32,33*). Xylanase production was found to be primarily induced by medium (longer than five xylopyranose units) to large-sized xylooligosaccharides (*34*). It was believed that oligosaccharides are generated by the action of *P. bryantii* extracellular xylanases or in the rumen by hydrolytic activity of other microorganisms. Monosaccharides including glucose, arabinose and xylose repressed xylanase production in ratios as little as 0.1 % (by mass per volume) when associated with xylan (*35*), pointing to catabolite repression of monosaccharides in *P. bryantii* $B_14$.

Xylanolytic activity of the strain $B_14$ was predominantly cell-bound and less than 5 % was detected in the culture supernatant (*36*). The CMCase was previously suspected to be cell-surface bound (*37*), but cell fractionation with osmotic shock released the majority of the CMCase and xylanase activities in the periplasmic fraction, the β-xylosidase and α-L-arabinofuranosidase remaining cell-membrane associated (*38*). The polysaccharolytic enzyme localization in the periplasm, where enzyme-polymer interaction is obstructed by the outer membrane, was surprising at the time. It was assumed that periplasmic position protects the hydrolytic enzymes against environmental detrimental factors and offers the bacterium competitive fitness with segregation of the enzymes and the degradation products from the extracellular matrix (*36*).

Despite the apparent substrate inaccessibility, *P. bryantii* $B_14$ was able to effectively utilize xylans (*36*). The majority of the produced xylooligosaccharides were rapidly utilized and also larger water-soluble xylan fragments were consumed to a high degree (*36*). Degradation of less-accessible water-insoluble part of xylans was the main limitation in utilization of isolated xylans in strain $B_14$. It was suspected that in nature *P. bryantii* xylanolytic system might be more efficient because native form of xylan bears more acetyl side-chains that increase its solubility (*36*). The preference towards less substituted type of GX over AX was also detected, indicating that side-chain decoration might hinder polymer solubilization (*36*). On the other hand, among the pentose sugars derived from xylan, uronic acid was utilized with the greatest efficiency, exceeding xylose and arabinose levels (*36*). The preference of GX type of xylan is probably a consequence of higher uronic acid content and more potent side-chain-cleaving enzyme, α-glucuronidase, recently found to be highly induced in xylan medium (*39*).

### *Identification and characterization of P. bryantii B14 xylanolytic system*

In *P. bryantii* $B_14$ four coding regions for xylanolytic activity were identified and cloned (*40*), and each corresponds to a clearing band detected by zymogram analysis (Fig. 2) (*31*). Briefly, zymograms were developed with denatured proteins on gels with incorporated xylan. The resulting gel with separated proteins was soaked in renaturating solutions overnight and then incubated at 38 °C. The xylanolytic activity of enzymes was detected as clearing areas by Congo red staining (*31*).

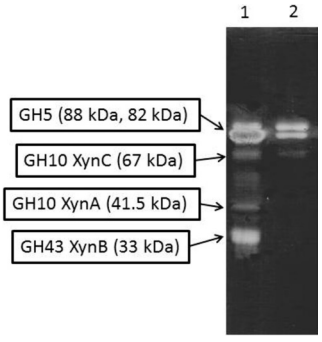


**Fig. 2.** Typical xylanogram of *Prevotella bryantii* $B_14$. GH5 endoglucanases have additional xylanolytic activity, XynA and XynC are GH10 endoxylanases and XynB is GH43 exoxylanase/β-xylosidase. 1 – cell fraction, 2 – periplasmic fraction (by R. Marinšek-Logar)

The first region corresponds to a previously described broad-specificity GH5 endoglucanase (*41*). The zymograms revealed two proteins (of 82 and 88 kDa), being primarily CMCases with weak endoxylanolytic activity (*31,33*). Later, Matsushita *et al.* (*42*) found out that GH5 endoglucanase is encoded in two overlapping reading frames and the obtained double band is a result of reading frame shift.

The other three xylanases lack CMCase activity. The second clone exhibited β-xylosidase and α-L-arabinofuranosidase activity in addition to endoxylanase (*40*). In this region two genes were found to be closely linked – *xynA*, coding for GH10 endoxylanase, and *xynB*, an oxygen sensitive GH43 exoxylanase with associated β-xylosidase and α-L-arabinofuranosidase activity (*40*). The XynB enzyme activities were severely affected by elevated temperature and oxygen presence (*40*). The preference for GX over AX was detected and was most likely due

to better solubility (*40*). The cloned XynB processively releases xylose from non-reducing ends of xylooligosaccharides and xylan, exhibiting exoxylanase action (*40*). It cleaves also xylobiose, but no arabinose was released from native xylans although XynB degrades the artificial substrate *p*-nitrophenylarabinofuranoside (*32,40*). On xylanograms, the 33-kDa clearing band corresponds to *xynB* gene product and its activity was better detectable on zymograms prepared with soluble xylan (less substituted form with lower degree of polymerization) and addition of reducing agent to provide anaerobic conditions, confirming its free-end chain dependence and oxygen sensitivity also in native form (*31*).

Cloned XynA enzyme was also thermolabile, but the activity was not affected by oxygen (*40*), which represents an advantageous trait in biotechnological applications. With XynA enzyme xylan degradation products were mainly xylobiose and xylotriose, proving its endoxylanolytic function (*40*). Again, cloned XynA exhibits higher activity on GX compared to AX as a substrate (*32*). On xylanograms *P. bryantii* $B_1$4 native XynA was presumably detected as a faint band at 41.5 kDa (*31*).

In AX breakdown XynB acts synergistically with XynA endoxylanases and up to 10-fold enhancement of reducing sugar release was determined (*40*). Synergism in xylan degradation is frequently recorded because the efficient action of exoxylanases/β-xylosidases depends on the availability of xylan loose ends generated by endoxylanases.

The clone carrying *xynC* gene was found to code for another GH10 endoxylanase. On AX zymograms the endoxylanase XynC was identified as clearing band at 67 kDa (*31,33*). XynC and the smallest, 33-kDa XynB were detected also in cultures grown in glucose-containing medium and therefore considered to be constitutively produced in low quantities (*31*). In xylan-grown cells the clearing bands were larger and more pronounced, indicating their inducibility (*31*).

The XynC enzyme was partially purified (*31,43*). Several methods were applied in enzyme isolation, most of them resulting in heavily aggregated states of periplasmic extract (*31*). The isolation of native xylanolytic enzymes from rumen bacteria is rarely reported, probably due to their high tendency to form aggregates. Finally, successful HPLC separation of crude periplasmic proteins using DEAE-based compact porous discs (CIM DEAE-8) resulted in four peaks with xylanolytic activity (*31*). The fraction including 67-kDa endoxylanase exhibits the highest hydrolytic activity on soluble AX and showed good temperature stability with optimum at 39 °C and pH=5.5 (*31*). Also with trypsin and pepsin concentrations exceeding the physiological levels, XynC retains its activity (*31*), indicating their possible application as feed additives for monogastric animals (*35*).

A unique insertion of 160 amino acid residues in the catalytic module of XynC was identified and no similar sequence was retrieved in the database at the time (*44*). Due to its position next to the catalytic module, it was suspected to be a novel type of substrate-binding module (*44*). Later it was assigned to CBM family 4.

Recently, XynC was found to be part of xylan utilization system located on the outer membrane (*45*). Transcriptomic analysis in *P. bryantii* $B_1$4 revealed homologues of starch utilization system in *Bacteroides thetaiotamicron* (*46*). In the predicted model the proteins of xylan utilization system (Xus) together with XynC are responsible for xylan binding, cleavage and transport of xylooligosaccharides across the outer membrane into the periplasm (*45*). The presence of an N-terminal signal peptidase II cleavage site in XynC indicates its position on the outer leaflet of outer membrane (*45*) and explains the enzyme low pH and oxygen tolerance in addition to resistance to proteolytic attack (*31*). The electrophoretic analysis of the partially purified 67-kDa enzyme (XynC) displayed other four to five non-xylanolytic proteins (*31*), which is in accordance with the five genes identified in the *xus* cluster (*46*). The predicted xylan-utilization system consists of XusA and XusC spanning the outer membrane most likely involved in oligosaccharide transport, XusB and XusD on the outer leaflet binding the xylan, XynC with the catalytic action and XusE with yet unknown function (*45*). The proteins seem to be strongly inter-linked and identification of high molecular xylanolytic complexes raised a speculation of xylanosome existence (*47*). The previously observed periplasmatic location of XynC was most likely a result of firm association with the outer membrane and consequent release with periplasmic marker. Furthermore, membrane association explains the formation of protein aggregates upon isolation. On membrane disruption, the released protein complexes are apparently bound along the remaining xylan polymer, hence forming an inseparable aggregate. However, the aggregates retain xylanolytic activity (*31*).

The comprehensive transcriptomic study discloses many upregulated genes during growth of the strain $B_1$4 on AX (*46*). Most of them were annotated as coding for proteins related to xylan metabolism and many were designated as hypothetical proteins (*46*). The study revealed the major xylanolytic gene cluster with centrally positioned *xynR* regulator gene, including previously identified *xynC*, *xynB* and *xynA*. Upstream to *xynR*, all of the *xus* genes are located followed by *xynC*. This group contains six of the seven most highly induced genes, indicating their crucial role in xylan metabolism (*46*). Regarding the highly conserved xylan utilization operon across xylanolytic Bacteroidetes (*46*), the hypothetical model for signal transduction analogous to *B. thetaiotamicron* mucin oligosaccharide induction was proposed (*45*). In strain $B_1$4 signal transduction is presumably initiated by xylooligosaccharide transfer by transmembrane protein XusC/D that triggers an unknown signalling cascade leading to activation of XynR and inducing the transcription of xylan-utilization gene (*45*).

The elaborated xylanolytic system of *P. bryantii* $B_1$4 was complemented with a characterization of a novel GH5 endoxylanase (*46*) and three additional GH3 β-xylosidases (*48*). Substrate affinity in multiple GH3 enzymes revealed their unique role in the hydrolysis of xylosidic linkages dependent on the type of substitution (*48*). The side-chain preference was not determined for GH43 due to lack of appropriate substrates (*36*), but the production of several enzymes hydrolyzing the identical chemical bond is substantiated by the heterogeneity of the substrate with side chains hindering the access.

### *Potential biotechnological applications of xylanases*

There are many areas of potential applications of xylanases, especially cellulase-free enzymes, which are of great value as they specifically remove xylan and leave cellulose intact. The enzymatic treatment is often a better alternative to chemical processing in regard to environmental pollution and energy conservation. Xylanases are routinely used in development of environment-friendly technologies in the pulp and paper industry (biobleaching and biopulping) and for processing flour in bakery industry. Xylanases are also efficiently used as feed additives in non-ruminant livestock (poultry, pigs) as they release simple sugars from polymers that otherwise resist degradation by indigenous digestive enzymes. Additionally, xylanases reduce the viscosity of the intestinal content (*e.g.* arabinoxylan of wheat and rye) and by that improve the absorption of digested food, prevent colonization by pathogens and overall benefit the animal (*49*). Moreover, xylanases can be used to produce diverse xylooligosaccharides with prebiotic properties (*50*) and their application in functional foods industry is encouraged. Recently, the most interesting area where xylanases and xylanase-producing microorganisms are employed is the biofuel production, where they are used for the degradation of lignocellulose material (*51,52*).

Currently, mainly fungal xylanases are used in the industrial applications, as fungi are considered more efficient producers than bacteria and archaea. However, occupational exposure to enzymes of fungal origin was shown to trigger diverse allergic reactions (*53,54*). Bacterial sources of potent xylanases are therefore gaining increased interest and rumen microbiota can be efficiently employed in that manner.

## *Pseudobutyrivibrio xylanivorans*

One of the most xylanolytically active rumen bacteria are also the members of the *Pseudobutyrivibrio-Butyrivibrio* group. Historically, all Gram-negative staining strains of rumen origin producing butyrate in glucose medium were classified as *Butyrivibrio fibrisolvens*, later *Butyrivibrio* sp. considering the increasing phenotypic diversity among the isolates (*55–57*). The full revision of *Butyrivibrio*-like bacteria resulted in strain distribution into four different phylogenetically coherent species, one of them newly described *Pseudobutyrivibrio xylanivorans* with type strain Mz5 (*58*), isolated in our lab. The species are classified to Lachnospiraceae family in the Firmicutes phylum and their Gram-negative staining was found to be a result of a very thin peptidoglycan layer (*59*).

### *Identification and characterization of Pseudobutyrivibrio xylanivorans Mz5 xylanolytic system*

On isolation, strainMz5 exhibited the highest xylanolytic activity at least 1.6 times higher than other rumen xylanolytic species, including several *Butyrivibrio*-like strains (*60,61*). Based on the ability of strain Mz5 to grow on xylan as a sole carbohydrate source (*62*), we assumed that a wide range of enzymes capable of efficient xylan degradation must be present.

The active xylanolytic system consisted of at least seven electrophoretically distinct xylanases that were detected on zymograms with AX (Fig. 3) (*61,63*). Multiple clearing bands (up to 14 were detected in certain growth phase) representing xylanolytic activity ranged from 30 to 146 kDa (*61,64*). Multiplicity together with high xylanolytic activity offers the bacterium a dominant position in xylan breakdown process and not surprisingly, the butyrivibrios are among the most frequently isolated bacteria from ruminants fed on forages (*65*). Multiple

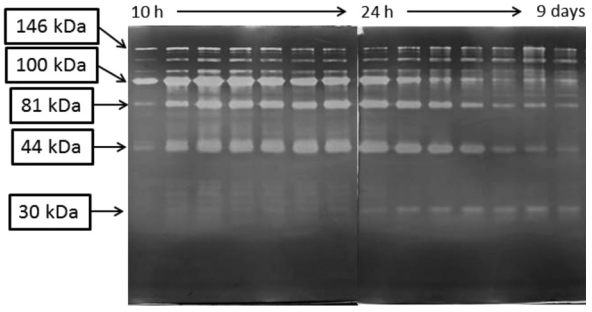


**Fig. 3.** The xylanogram of cell-associated xylanases in *Pseudobutyrivibrio xylanivorans* Mz5. Four major xylanases are indicated (146, 100, 81 and 44 kDa) and the isolated Xyn11A (30 kDa). Lanes represent time-dependent emergence of the enzymes (10 hours to 9 days) (by M. Zorec)

forms of an enzyme could be a result of several distinct genes, or proteins of a single gene subjected to differential post-translational modifications (like glycosylation or proteolysis). In later growth phases, the detected multiplicity can also be a consequence of partial proteolysis or enzyme association with disintegrated extracellular polysaccharide (EPS) layer as reported for proteinases (*66*). The molecular masses of the four major enzymes identified as large clearing bands on xylanograms were 44, 81, 100 and 146 kDa (*67*). The majority of the xylanolytic activity was cell-associated (*61,63*). The production of EPS in butyrivibrios is a common trait and the presence of a thick EPS layer was suspected also for strain Mz5 as it formed mucoid colonies with emanating bubbles (*61,68*). The detected cell-bound activity was therefore most likely a result of extracellular enzymes retained in cell proximity because the glycocalyx prevented their diffusion. In the later growth phases when pH dropped below 5.8, the heteropolysaccharide matrix probably disintegrated and released the xylanolytic enzymes from the cell surface, when a decrease in cell-associated xylanolytic activity with simultaneous increase in the supernatant activity was observed (*61,64*). The electrophoretic equivalence of supernatant and cell-associated xylanases further supports these assumptions.

Strain Mz5 also possesses β-xylosidase and α-L-arabinofuranosidase activity that were mostly cell-bound (*61*). The peak of cell-associated xylanase activity was detected in the mid-exponential phase at point where the 100- and 146-kDa xylanases were the most prominent (*61,63*). After the peak, xylanolytic activity declined rapidly although the clearing bands on the xylanograms intensified in brightness and in number (*61*). This discrepancy is due to method design as the majority of

xylanases are inhibited by low pH that persists for the time of reducing sugar test, while xylanograms are developed in the neutral pH that reactivates the enzymes to some extent.

The production of xylanolytic enzymes in strain Mz5 is highly inducible with AX and specific activity increased as much as 33-fold in comparison with glucose (*60*,*61*). The AGX and GX xylan induced the xylanolytic activity, too, but the levels were approximately fivefold lower (*61*). On xylanograms two faint xylanolytic bands were detected at 44 and 146 kDa in cells grown in glucose--containing medium (*61*,*63*). The major part of xylanolytic activity in glucose medium was detected in the culture supernatant (*61*), which points to the fact that constitutive enzymes are released to the extracellular space and by that expand the area of possible substrate contact. These two xylanases are therefore considered to be constitutively produced with the larger one being more abundant as seen on xylanograms. The degradation products generated by the action of 44- and 146-kDa xylanases can then enter the cell and induce the synthesis of other enzymes.

The partially purified 44-kDa enzyme efficiently degraded AX and xylooligosaccharides to xylobiose and xylose, but was inactive towards xylobiose and was thus considered to be exoxylanase without β-xylosidase activity (*67*,*69*). The molecular mass of the protein indicated homology to same size XynA xylanase found in *Pseudobutyrivibrio* strain 49, which was classified into GH10 family (*70*). The presence of GH10 enzymes was suspected also in Mz5 as they are widely distributed across the *Butyrivibrio-Pseudobutyrivibrio* group (*71*). Additionally, an identical gene sequence of related strain CF3 *xynI2* gene encoding for GH10 xylanase was found (*72*). The exact species designation of strain CF3 is pending, but its close relatedness to the other strain of *Pseudobutyrivibrio xylanivorans*, DSM 10317 (*58*,*73*) relates strain CF3 to Mz5.

Additionally, the smallest enzyme (30 kDa) was successfully purified (*67*,*74*). During growth in batch culture, the enzyme emerged on xylanograms at the beginning of the stationary phase and persisted into the prolonged cultivation (*61*). The 30-kDa xylanase, XynT (later designated as Xyn11A) was classified into GH11 family and it was the first family 11 xylanase found in the *Butyrivibrio--Pseudobutyrivibrio* group (*75*). Since the activity of Xyn11A was at least threefold higher in the soluble fraction of xylan, the absence of the xylan-binding module was suspected. Xyn11A action on AX resulted in xylotetraose or higher xylooligosaccharides placing the enzyme at the beginning of the xylan breakdown process. The cleavage site of Xyn11A was predicted to be near substitutions, because the activity towards less substituted types of xylan was lower (*76*). Similar amino acid sequences to Xyn11A were also found in xylanases from strains of *Clostridium*, *Bacillus* and *Ruminococcus* (*76*).

On xylanograms the concurrent loss of visible bands at 81 and 100 kDa with 30-kDa band emergence (*61*,*63*) indicated that the smallest enzyme could be a proteolytic fragment of the larger xylanases, retaining catalytic function. When the *xyn11A* gene sequence was analyzed, a multimodular structure of the enzyme was revealed (*77*,*78*). The gene consists of previously isolated catalytic module from GH11 family, followed by a binding module (CBM6) and a NodB-like module (*78*). Both non-catalytic modules enhance xylan breakdown in a different manner. Binding subunit facilitates the activity towards insoluble substrate with attaching and concentrating the enzymes in proximity to xylan. Further on, NodB-like subunit removes acetyl side chains and exposes the xylan backbone to xylanase attack. Similar module arrangement was found also in *Clostridium thermocellum*, *Ruminococcus albus* and soil bacterium *Clostridium fimi* (*78*). The conservation level was particularly high in the catalytic part and homologues of *xyn11A* of Mz5 were found also in other *Pseudobutyrivibrio* strains (*75*). High level of similarity can be explained by the existence of common ancestral origin and subsequent horizontal gene transfer, which occurs frequently in the rumen (*79*). Nevertheless, combining the catalytic module with binding and deacetylating subunit offers a bacterium competitive advantage in xylan utilization in diverse environments. In certain species, association of several complementary catalytic modules with binding modules evolved in more elaborated multiprotein complexes such as cellulosomes.

The linkage of the catalytic module to non-catalytic proteins could contribute to several unsuccessful attempts in isolation of other xylanases (*67*,*69*). The presence of CBM as pointed out earlier, additionally promotes native enzyme aggregation by accumulating them on the substrate residues. Additionally, membrane transporter proteins could be associated with enzyme complexes as it was revealed in *Prevotella bryantii* $B_1$4. By recent proteomic analysis, several putative carbohydrate transporters were identified in closely related rumen species, *Butyrivibrio proteoclasticus* (*80*). Most of them were members of ABC transport system, some predicted to be involved in oligosaccharide transport (*80*). Like in *Prevotella bryantii* $B_1$4, upregulation with xylan medium and clustering with other xylan utilization genes was identified (*80*).

Therefore, only partially isolated 81-kDa xylanase was obtained and the fraction exhibited a single clearing band on xylanogram with many other proteins still detected on electrophoretograms (*67*). The 81-kDa enzyme is presumably a complete multimodular complex carrying a 30-kDa xylanase subunit and thus displays almost identical degradation pattern to 30-kDa enzyme. The 81-kDa xylanolytic activity on AX generates different xylooligosaccharides, xylopentaose being the largest (*67*). On the other hand, the 30-kDa enzyme smallest product was tetraoligosaccharide, which might be a result of a minor active site alteration upon proteolytic cleavage.

The putative mechanism for xylan degradation has been proposed (*67*,*72*), starting by the action of constitutively synthesized 145-kDa endoxylanase, which releases smaller fragments of xylan and generates free ends of xylan. The 44-kDa exoxylanase binds to newly formed chain termini and produces wide range of different xylooligosaccharides including xylose. The enzymes are most likely detached from the cell to increase the chances for coming into contact with xylan. Next, the xylooligosaccharides are transported across the membrane by similar membrane transporters as found in *B. proteoclasticus* and induce the synthesis of other xylanolytic enzymes by unknown mechanism. The induced xylanases are transported across the membrane and trapped in the EPS layer that retains the bulk of enzyme activity close to the

cell surface. The attachment of the enzymes to the cell membrane is still unclear, but similar associations to membrane transporters as reported for *B. proteoclasticus* are not excluded. The amount of constitutively synthesized xylanases also increases and joint performance of all enzymes results in xylooligosaccharides of variable lengths, some also substituted, and xylose. The products are then transported into the cell, where further degradation proceeds with the cell-associated β-xylosidase and arabinofuranosidase concluding with a variety of pentoses and hexoses available for cell metabolism.

### *Potential use of Pseudobutyrivibrio xylanivorans Mz5 as a probiotic*

Probiotics are live microorganisms with beneficial effects to the host in many ways: pathogen growth inhibition with production of organic acids and bacteriocins, impeding pathogen adhesion and colonization by competitive exclusion, neutralizing the toxins and boosting up the host immune system (*81*). Besides its use as a source of enzymes, the strain Mz5 could potentially be applied as a probiotic due to several other advantageous traits. First, the strain Mz5 is a potent xylanolytic producer and abundant rumen inhabitant. Therefore, butyrivibrios as rumen indigenous bacteria are promising hosts for recombinant genes that could improve fibre degradation in rumen. Even though genetically modified strategies are heavily opposed by consumers, several solutions exist to minimize the risks and produce the microorganism safe enough to release (*82*). Then, it produces high amounts of butyrate and lactate with concurrent acetate utilization from the medium (*68*,*83*). The butyrate together with lactate induce decline in pH level, hence inhibiting the growth of many pathogens. In monogastric animals, butyrate was proved to have a primary role in metabolism and normal development of colonocytes and in having a detrimental effect on cancer cells (*84*). Mz5 also showed bacteriocin-like inhibition of some rumen bacteria and several strains of *Salmonella enteritidis* and *E. coli* isolated from poultry meat (*85*). The production of inhibitory substances additionally contributes to competitive fitness of the bacterium in gastrointestinal tract of animals. Another trait seems favourable at the time, but is currently disputed about – beneficial effects of the conjugated linoleic acid (CLA) in treating obesity, cancer and preventing cardiovascular diseases in humans (*86*). Still, *Butyrivibrio* species are the principal CLA producers in the rumen from linoleic acid, and Mz5 also produces the CLA-precursor *trans*-vaccenic acid (TVA) (*87*). CLA was detected only as an intermediate and TVA was the final product in biohydrogenation process (*87*). Further on, adhesion test to human cell culture was adapted for anaerobic bacteria (*88*). Cells of Mz5 successfully attach to model Caco-2 cells and this finding additionally supports further *in vivo* studies. Nevertheless, oxygen tolerance was also detected as Mz5 retained viability in partially oxygenated medium (*89*). Retaining the viability upon oxygen exposure facilitates handling of the cultures in different biotechnological applications.

Efficient application of *Butyrivibrio*-like strain as probiotic has been shown in mice. Oral administration of live cultures increased the level of butyrate produced and reduced the extent of preneoplastic lesion in the colon (*90*). Additionally, feeding mice with probiotic strain lessened the colitis symptoms (*91*) and recently, an EPS extract from the same strain has been shown to alleviate the atopic dermatitis lesions (*92*). Taken together, the *Pseudobutyrivibrio xylanivorans* strain Mz5 is a promising probiotic candidate having many beneficial characteristics like butyrate, CLA and EPS production together with oxygen tolerance and an ability to attach to human epithelial colorectal cells.

### *Potential use of rumen bacteria in biogas production*

Recent studies of rumen-derived cellulolytic and xylanolytic bacteria are currently focused towards the biological pretreatment of recalcitrant lignocellulosic substrates in biogas production (*93*). Utilization of low-cost material such as waste plant residues is gaining attention in a sense of consolidated bioprocessing. Other pretreatment methods like physical and chemical demand certain energy input and have high environmental impact. Therefore, the biological pretreatment by appropriate microorganisms is preferred to improve the hydrolysis rate of lignocellulose biomass, which is the rate-limiting step in biogas production (*94*). The promising results by utilization of cellulolytic *R. flavefaciens* 007C and xylanolytic *Pseudobutyrivibrio xylanivorans* Mz5 in substrate pretreatment expand the area of biotechnological application of fibrolytic rumen bacteria (*93*).

## Conclusion

Rumen is a rich source of fibrolytic enzymes and different microorganisms producing them, and thus represents a good source of productive bacteria for different biotechnological purposes. *Ruminococcus flavefaciens* carries one of the most elaborated architectures of the cellulosome and provides vast spectra of possibilities in construction of designer cellulosomes. At the same time, it represents an interesting pool of genes for genetic manipulation of different host bacteria for high production of cellulases and hemicellulases. The xylanolytic *Prevotella* and *Pseudobutyrivibrio* produce multiple forms of highly potent enzymes, but upscaling their isolation might be difficult as they are linked to other non-catalytic accessory proteins enhancing their function *in vivo*. The promising areas of application are as probiotics for monogastric animals (especially pigs and poultry) and utilization in pretreatment bioprocesses for biogas production from lignocelluloses waste substrates originating mostly from agro-food industry. Due to the high applicative potentials of the three selected rumen bacteria, their ecology, physiology and biochemistry were thoroughly studied by our research group and our collaborators.

## References

1. A. Heredia, A. Jiménez, R. Guillén, Composition of plant cell walls, *Z. Lebensm. Unters. Forsch. 200* (1995) 24–31.
2. Carbohydrate Active Enzymes Database, AFMB, Marseille, France (http://www.cazy.org).
3. M. Vodovnik, R. Marinšek-Logar, Cellulosomes – Promising supramolecular machines of anaerobic cellulolytic microorganisms, *Acta Chim. Slov. 57* (2010) 767–774.

4. R. Marinšek-Logar, F.V. Nekrep, Microbial degradation of xylan, *Zb. Biotehn. Fak. Univ. Ljubl., Kmet. Zooteh. 62* (1993) 243–255 (in Slovene).
5. R. Marinšek-Logar, Arabinofuranosidases – The debranching enzymes in xylan degradation, *Zb. Biotehn. Fak. Univ. Ljubl., Kmet. Zooteh. 64* (1994) 235–242 (in Slovene).
6. D.B. Wilson, Microbial diversity of cellulose hydrolysis, *Curr. Opin. Microbiol. 14* (2011) 259–263.
7. J.M. Brulc, C.J. Yeoman, M.K. Wilson, M.E. Berg Miller, P. Jeraldo, S. Jindou *et al.*, Cellulosomics, a gene-centric approach to investigating the intraspecific diversity and adaptation of *Ruminococcus flavefaciens* within the rumen, *PLoS ONE, 6* (2011) e25329.
8. S. Jindou, J.M. Brulc, M. Levy-Assaraf, M.T. Rincon, H.J. Flint, M.E. Berg *et al.*, Cellulosome gene cluster analysis for gauging the diversity of the ruminal cellulolytic bacterium *Ruminococcus flavefaciens*, *FEMS Microbiol. Lett. 285* (2008) 188–194.
9. S.Y. Ding, M.T. Rincon, R. Lamed, J.C. Martin, S.I. McCrae, V. Aurilia *et al.*, Cellulosomal scaffoldin-like proteins from *Ruminococcus flavefaciens*, *J. Bacteriol. 183* (2001) 1945–1953.
10. M.T. Rincón, J.C. Martin, V. Aurilia, S.I. McCrae, G.J. Rucklidge, M.D. Reid *et al.*, ScaC, an adaptor protein carrying a novel cohesin that expands the dockerin-binding repertoire of the *Ruminococcus flavefaciens* 17 cellulosome, *J. Bacteriol. 186* (2004) 2576–2785.
11. M.T. Rincón, T. Čepeljnik, J.C. Martin, R. Lamed, Y. Barak, E.A. Bayer *et al.*, Unconventional mode of attachment of the *Ruminococcus flavefaciens* cellulosome to the cell surface, *J. Bacteriol. 187* (2005) 7569–7578.
12. M.T. Rincón, T. Čepeljnik, J.C. Martin, Y. Barak, R. Lamed, E.A. Bayer *et al.*, A novel cell surface-anchored cellulose--binding protein encoded by the sca gene cluster of *Ruminococcus flavefaciens*, *J. Bacteriol. 189* (2007) 4774–4783.
13. M. Vodovnik, Cellulolytic system of bacterium *Ruminococcus flavefaciens* strains 007C and 007S, *PhD Thesis*, University of Ljubljana, Ljubljana, Slovenia (2012) (in Slovene).
14. C. Stewart, S. Duncan, C.A. McPherson, A. Richardson, H.J. Flint, The implications of the loss and regain of cotton-degrading activity for the degradation of straw by *Ruminococcus flavefaciens* strain 007, *J. Appl. Microbiol. 68* (1990) 349–356.
15. M. Vodovnik, R. Marinšek-Logar, Expression patterns of *Ruminococcus flavefaciens* 007S cellulases as revealed by zymogram approach, *Folia Microbiol. 57* (2012) 367–370.
16. M.E. Berg Miller, D.A. Antonopoulus, M.T. Rincon, M. Band, A. Bari, T. Akraiko *et al.*, Diversity and strain specificity of plant cell wall degrading enzymes revealed by the draft genome of *Ruminococcus flavefaciens* FD-1, *PLoS ONE, 4* (2009) e6650.
17. A. Riederer, T.E. Takasuka, S. Makino, D.M. Stevenson, Y.V. Bukhman, N.L. Elsen, B.G. Fox, Global gene expression patterns in *Clostridium thermocellum* as determined by microarray analysis of chemostat cultures on cellulose or cellobiose, *Appl. Environ. Microbiol. 77* (2011) 1243–1253.
18. M. Vodovnik, S.H. Duncan, M.D. Reid, L. Cantlay, K. Turner, J. Parkhill *et al.*, Expression of cellulosome components and type IV pili within the extracellular proteome of *Ruminococcus flavefaciens* 007, *PLoS ONE, 8* (2013) e65333.
19. M. Vodovnik, S.H. Duncan, G.J. Rucklidge, M.D. Reid, L. Cantlay, R. Marinšek-Logar *et al.*: Expression of the Extracellular Proteome of R*uminococcus flavefaciens* 007. In: *Gut Microbiology: New Insights into Gut Microbial Ecosystems*, *Proceedings of the 7th Joint Symposium Rowett-INRA & University of Aberdeen*, Aberdeen, UK (2010) p. 93.
20. M. Vodovnik, S.H. Duncan, G.J. Rucklidge, M.D. Reid, L. Cantlay, R. Marinšek-Logar *et al.*, *Ruminococcus flavefaciens* 007C and 007S differ in expression of several proteins associated with growth on cellulose, *9th Carbohydrate Bioengineering Meeting*, Lisbon, Portugal (2011) p. 188.
21. S. Imam, Z. Chen, D.S. Ross, M. Pohlschröder, Identification of surprisingly diverse type IV pili across a broad range of Gram-positive bacteria, *PLoS ONE, 6* (2011) e28919.
22. R.J. Quinlan, M.D. Sweeney, L. Lo Leggio, H. Otten, J.C. Poulsen, K.S. Johansen *et al.*, Insights into the oxidative degradation of cellulose by a copper metalloenzyme that exploits biomass components, *Proc. Natl. Acad. Sci. USA, 108* (2011) 15079–150784.
23. O. Riebe, R.J. Fischer, D.A. Wampler, D.M. Kurtz, H. Bahl, Pathway for $H_2O_2$ and $O_2$ detoxification in *Clostridium acetobutylicum*, *Microbiology, 155* (2009) 16–24.
24. J. Kopečný, B. Hodrová, The effect of yellow affinity substance on cellulases of *Ruminococcus flavefaciens*, *Lett. Appl. Microbiol. 25* (1997) 191–196.
25. F. Sabathé, P. Soucaille, Characterization of the CipA scaffolding protein and *in vivo* production of a minicellulosome in *Clostridium acetobutylicum*, *J. Bacteriol. 185* (2003) 1092–1096.
26. D.M. Stevenson, P.J. Weimer, Dominance of *Prevotella* and low abundance of classical ruminal bacterial species in the bovine rumen revealed by relative quantification real-time PCR, *Appl. Microbiol. Biotechnol. 75* (2007) 165–167.
27. N.M. Nathani, A.K. Patel, P.S. Dhamannapatil, R.K. Kothari, K.M. Singh, C.G. Joshi, Comparative evaluation of rumen metagenome community using qPCR and MG-RAST, *AMB Express, 3* (2013) 55.
28. G. Avguštin, H.J. Flint, T.R. Whitehead, Distribution of xylanase genes and enzymes among strains of *Prevotella* (Bacteroides) *ruminicola* from the rumen, *FEMS Microbiol. Lett. 99* (1992) 137–144.
29. G. Avguštin, F. Wright, H.J. Flint, Genetic diversity and phylogenetic relationships among strains of *Prevotella* (Bacteroides) *ruminicola* from the rumen, *Int. J. Syst. Bacteriol. 44* (1994) 246–255.
30. G. Avguštin, J.R. Wallace, H.J. Flint, Phenotypic diversity among ruminal isolates of *Prevotella ruminicola*: Proposal of *Prevotella brevis* sp. nov., *Prevotella bryantii* sp. nov., and *Prevotella albensis* sp. nov. and redefinition of *Prevotella ruminicola*, *Int. J. Syst. Bacteriol. 47* (1997) 284–288.
31. R. Marinšek-Logar, Characterization of the xylanolytic enzyme system of rumen bacterium *Prevotella bryantii* B14, *PhD Thesis*, University of Ljubljana, Domžale, Slovenia (1999) (in Slovene).
32. A. Gasparič, R. Marinšek-Logar, J. Martin, J.R. Wallace, F.V. Nekrep, H.J. Flint, Isolation of genes encoding β-D-xylanase, β-D-xylosidase and α-L-arabinofuranosidase activities from the rumen bacterium *Prevotella ruminicola* $B_1$4, *FEMS Microbiol. Lett. 125* (1995) 135–142.
33. R. Marinšek-Logar, A. Gasparič, F.V. Nekrep, Xylanolytic enzyme system of rumen bacterium *Prevotella bryantii* B14, *Zb. Biotehn. Fak. Univ. Ljubl., Kmet. Zooteh. 72* (1998) 63–67.
34. K. Miyazaki, T. Hirase, Y. Koyima, H.J. Flint, Medium- to large-sized xylo-oligosaccharides are responsible for xylanase induction in *Prevotella bryantii* B14, *Microbiology, 151* (2005) 4121–4125.
35. R. Marinšek-Logar, A. Gasparič, The role of bacterial xylanases in ruminal digestion of plant polysaccharides, *Krmiva, 38* (1996) 73–79.
36. K. Miyazaki, J.C. Martin, R. Marinšek-Logar, H.J. Flint, Degradation and utilization of xylans by the rumen anaerobe *Prevotella bryantii* (formerly *P. ruminicola* subsp. *brevis*) B14, *Anaerobe, 3* (1997) 373–381.
37. R.G. Gardner, J.E. Wells, J.B. Russell, D.B. Wilson, The effect of carbohydrates on the expression of the *Prevotella ruminicola* 1,4-β-D-endoglucanase, *FEMS Microbiol. Lett. 125* (1995) 305–310.

38. R. Marinšek-Logar, F.V. Nekrep, On the localization of xylanolytic enzymes in *Prevotella bryantii* B14, *Zb. Biotehn. Fak. Univ. Ljubl., Kmet. Zooteh. 70* (1997) 31–38.
39. D. Dodd, Y.H. Moon, K. Swaminathan, R.I. Mackie, I.K. Cann, Transcriptomic analyses of xylan degradation by *Prevotella bryantii* and insights into energy acquisition by xylanolytic bacteroidetes, *J. Biol. Chem. 285* (2010) 30261–30273.
40. A. Gasparič, J. Martin, A.S. Daniel, H.J. Flint, A xylan hydrolase gene cluster in *Prevotella ruminicola* B14: Sequence relationships, synergistic interactions, and oxygen sensitivity of a novel enzyme with exoxylanase and β-(1,4)-xylosidase activities, *Appl. Environ. Microbiol. 61* (1995) 2958–2964.
41. O. Matsushita, J.B. Russell, D.B. Wilson, Cloning and sequencing of a *Bacteroides ruminicola* B14 endoglucanase gene, *J. Bacteriol. 172* (1990) 3620–3630.
42. O. Matsushita, J.B. Russell, D.B. Wilson, A *Bacteroides ruminicola* 1,4-β-D-endoglucanase is encoded in two reading frames, *J. Bacteriol. 173* (1991) 6919–6926.
43. R. Marinšek-Logar, F.V. Nekrep, M. Barut, A. Štrancar, Very fast purification of a 66 kDa-endoxylanase and a broad specificity endoglucanase/xylanase from *Prevotella bryantii* B14 by anion-exchange HPLC using CIM (convective interaction media) DEAE-8 tubes, *Book of Abstracts – Biotechnology 2000: The World Congress on Biotechnology, 11th International Biotechnology Symposium and Exhibition*, Berlin, Germany (2000) p. 69.
44. H.J. Flint, T.R. Whitehead, J.C. Martin, A. Gasparic, Interrupted catalytic domain structures in xylanases from two distantly related strains of *Prevotella ruminicola*, *Biochim. Biophys. Acta, 1337* (1997) 161–165.
45. D. Dodd, R.I. Mackie, I.K. Cann, Xylan degradation, a metabolic property shared by rumen and human colonic Bacteroidetes, *Mol. Microbiol. 79* (2011) 292–304.
46. D. Dodd, Y.H. Moon, K. Swaminathan, R.I. Mackie, I.K. Cann, Transcriptomic analyses of xylan degradation by *Prevotella bryantii* and insights into energy acquisition by xylanolytic Bacteroidetes, *J. Biol. Chem. 285* (2010) 30261–30273.
47. R. Marinšek-Logar, F.V. Nekrep, Cellulosomes – or even xylanosomes?, *Zb. Biotehn. Fak. Univ. Ljubl., Kmet. Zooteh. 68* (1996) 19–26.
48. D. Dodd, S. Kiyonari, R.I. Mackie, I.K. Cann, Functional diversity of four glycoside hydrolase family 3 enzymes from the rumen bacterium *Prevotella bryantii* $B_1$4, *J. Bacteriol. 192* (2010) 2335–2345.
49. O. Esmaeilipour, H. Moravej, M. Shivazad, M. Rezaian, S. Aminzadeh, M.M. Van Krimpen, Effects of diet acidification and xylanase supplementation on performance, nutrient digestibility, duodenal histology and gut microflora of broilers fed wheat based diet, *Br. Poultry Sci. 53* (2012) 235–244.
50. P. Falck, S. Precha-Atsawanan, C. Grey, P. Immerzeel, H. Stalbrand, P. Adlercreutz *et al.*, Xylooligosaccharides from hardwood and cereal xylans produced by a thermostable xylanase as carbon sources for *Lactobacillus brevis* and *Bifidobacterium adolescentis*, *J. Agric. Food Chem. 61* (2013) 7333–7340.
51. M. Sharma, A. Kumar, Xylanases: An overview, *Brit. Biotechnol. J. 3* (2013) 1–28.
52. X. Meng, A.J. Ragauskas, Recent advances in understanding the role of cellulose accessibility in enzymatic hydrolysis of lignocellulosic substrates, *Curr. Opin. Biotechnol. 27* (2014) 150–158.
53. B.J. Green, D.H. Beezhold, Industrial fungal enzymes: An occupational allergen perspective, *J. Allergy, 2011* (2011) 1–11.
54. M.L. Caballero, M. Gomez, M. González-Muñoz, L. Reinoso, R. Rodríguez-Pérez, E. Alday *et al.*, Occupational sensitization to fungal enzymes used in animal feed industry, *Int. Arch. Allergy Immunol. 144* (2007) 231–239.
55. R. Marinšek-Logar, J. Kopečný, M. Zorec, Variability of *Butyrivibrio fibrisolvens* estimated by cellular fatty acid profiles, DNA restriction analysis and fermentation patterns, Second Joint INRA-RRI Gastrointestinal Tract Microbiology Symposium: Challenges for Microbial Digestive Ecology at the Beginning of the Third Millennium, *Reprod. Nutr. Dev. 40* (2000) 182–183.
56. J. Kopečný, R. Marinšek-Logar, Y. Kobayashi, Phenotypic and genetic data supporting reclassification of *Butyrivibrio fibrisolvens* isolates, *Folia Microbiol. 46* (2001) 45–48.
57. T. Čepeljnik, R. Marinšek-Logar, *Butyrivibrio* strains-important butyrate producing bacteria from animal gastro-intestinal tract and current taxonomic status of strains in this genus, *Zb. Biotehn. Fak. Univ. Ljubl., Kmet. Zooteh. 80* (2002) 19–28.
58. J. Kopečný, M. Zorec, J. Mrázek, Y. Kobayashi, R. Marinšek-Logar, *Butyrivibrio hungatei* sp. nov. and *Pseudobutyrivibrio xylanivorans* sp. nov., butyrate-producing bacteria from the rumen, *Int. J. Syst. Evol. Microbiol. 53* (2003) 201–209.
59. R.B. Hespell, K. Kato, J.W. Costerton, Characterization of the cell wall of *Butyrivibrio* species, *Can. J. Microbiol. 39* (1993) 912–921.
60. M. Zorec, R. Marinšek-Logar, G. Avguštin, Isolation of butyric acid producing xylanolytic bacteria from the rumen, *Zb. Biotehn. Fak. Univ. Ljubl., Kmet. Zooteh.70* (1997) 21–29.
61. M. Zorec, Isolation of highly xylanolytic rumen bacterium and characterization of its enzymatic system for xylan degradation, *MSc Thesis*, University of Ljubljana, Ljubljana, Slovenia (2002) (in Slovene).
62. R. Marinšek-Logar, M. Zorec, F.V. Nekrep, Isolation and characterization of *Butyrivibrio-like* rumen bacteria possessing high xylanolytic activity, Second Joint INRA-RRI Gastrointestinal Tract Microbiology Symposium: Challenges for Microbial Digestive Ecology at the Beginning of the Third Millennium, *Reprod. Nutr. Dev. 40* (2000) 191–192.
63. M. Zorec, R. Marinšek-Logar, T. Čepeljnik, F.V. Nekrep, The influence of substrate concentration and growth phase on expression of *Butyrivibrio* sp. Mz5 endoxylanases, *Zb. Biotehn. Fak. Univ. Ljubl., Kmet. Zooteh. 76* (2000) 199–206.
64. M. Zorec, T. Čepeljnik, F.V. Nekrep, R. Marinšek-Logar, Multiple endoxylanases of *Butyrivibrio* sp., *Folia Microbiol. 46* (2001) 94–96.
65. D.J. Grilli, M.E. Cerón, S. Paez, V. Egea, L. Schnittger, S. Cravero *et al.*, Isolation of *Pseudobutyrivibrio ruminis* and *Pseudobutyrivibrio xylanivorans* from rumen of Creole goats fed native forage diet, *Folia Microbiol. 58* (2013) 367–373.
66. E. Strydom, R.I. Mackie, D.R. Woods, Detection and characterization of extracellular proteases in *Butyrivibrio fibrisolvens* H17c, *Appl. Microbiol. Biotechnol. 24* (1986) 214–217.
67. T. Čepeljnik, K. Fidler, R. Marinšek-Logar, Xylan degradation by different xylanases of rumen bacterium *Pseudobutyrivibrio xylanivorans* $Mz5^T$, *Acta Agric. Slov. 84* (2004) 141–151 (in Slovene).
68. M. Zorec, J. Kopečný, Y. Kobayashi, F.V. Nekrep, R. Marinšek-Logar, Isolation and characterization of a highly xylanolytic rumen bacterial strain Mz5, type strain of a new species *Pseudobutyrivibrio xylanivorans*, *Book of Abstracts of the 1st FEMS Congress of European Microbiologists*, Ljubljana, Slovenia (2003) p. 239.
69. T. Čepeljnik, M. Zorec, F.V. Nekrep, R. Marinšek-Logar, Isolation of endoxylanases from anaerobic bacterium *Butyrivibrio* sp. strain Mz5 is possible by anion exchange chromatography on CIM DEAE-8 monolithic column, *Acta Chim. Slov. 49* (2002) 401–408.
70. B.M. Mannarelli, S. Evans, D. Lee, Cloning, sequencing, and expression of a xylanase gene from the anaerobic ruminal bacterium *Butyrivibrio* fibrisolvens, *J. Bacteriol. 172* (1990) 4247–4254.

71. B.P. Dalrymple, Y. Swadling, I. Layton, K.S. Gobius, G.P. Xue, Distribution and evolution of the xylanase genes *xynA* and *xynB* and their homologues in strains of *Butyrivibrio fibrisolvens*, *Appl. Environ. Microbiol. 65* (1999) 3660–3667.
72. T. Čepeljnik, Multiple xylanases of rumen bacterium *Pseudobutyrivibrio xylanivorans* Mz5$^T$ and its possible use as a probiotic, *PhD Thesis*, University of Ljubljana, Ljubljana, Slovenia (2005) (in Slovene).
73. C.D. Moon, D.M. Pacheco, W.J. Kelly, S.C. Leahy, D. Li, J. Kopečný, G.T. Attwood, Reclassification of *Clostridium proteoclasticum* as *Butyrivibrio proteoclasticus* comb. nov., a butyrate-producing ruminal bacterium, *Int. J. Syst. Evol. Microbiol. 58* (2008) 2041–2045.
74. T. Čepeljnik, Isolation and characterization of xylanase XynT from rumen bacterium *Pseudobutyrivibrio xylanivorans* Mz5$^T$, *MSc Thesis*, University of Ljubljana, Ljubljana, Slovenia (2003) (in Slovene).
75. T. Čepeljnik, R. Marinšek-Logar, Do rumen butyrivibrio really possess only family 10 xylanases?, Fourth Joint INRA--RRI Symposium Gut Microbiology: Concerns and Responses to Food Safety, Health and Environmental Issues, *Reprod. Nutr. Dev. 44* (2004) p. S57.
76. T. Čepeljnik, I. Križaj, R. Marinšek-Logar, Isolation and characterization of the *Pseudobutyrivibrio xylanivorans* Mz5 xylanase XynT – the first family 11 endoxylanase from rumen *Butyrivibrio-related* bacteria, *Enzyme Microb. Technol. 34* (2004) 219–227.
77. T. Čepeljnik, M. Rincón, H.J. Flint, R. Marinšek-Logar, GH11 catalytic domain is only a part of multidomain enzyme xynT from *Pseudobutyrivibrio xylanivorans* Mz5$^T$, *4th International Symposium of Anaerobic Microbiology – ISAM*, Jablonna, Poland (2005) p. 17.
78. T. Čepeljnik, M. Rincón, H.J. Flint, R. Marinšek-Logar, XYN11A, a multidomain multicatalytic enzyme from *Pseudobutyrivibrio xylanivorans* Mz5T, *Folia Microbiol. 51* (2006) 263–267.
79. M. Peterka, T. Accetto, G. Avguštin, Horizontal gene transfer in bacteria, *Zb. Biotehn. Fak. Univ. Ljubl., Kmet. Zooteh. 76* (2000) 163–173.
80. J.J. Bond, J.C. Dunne, F.Y. Kwan, D. Li, K. Zhang, S.C. Leahy *et al.*, Carbohydrate transporting membrane proteins of the rumen bacterium, *Butyrivibrio proteoclasticus*, *J. Proteomics, 75* (2012) 3138–3144.
81. M. Vodovnik, R. Marinšek-Logar, Probiotics in animal nutrition, *Acta Agric. Slov. 92* (2008) 5–17 (in Slovene).
82. E. Forano, H.J. Flint, Genetically modified organisms: Consequences for ruminal health and nutrition, *Ann. Zootech. 49* (2000) 255–271.
83. M. Zorec, F.V. Nekrep, R. Marinšek-Logar, Butyrate production of anaerobic rumen bacterium *Pseudobutyrivibrio xylanivorans* Mz5$^T$, *Zb. Biotehn. Fak. Univ. Ljubl., Kmet. Zooteh. 82* (2003) 17–24 (in Slovene).
84. G.T. MacFarlane, S. MacFarlane, Bacteria, colonic fermentation, and gastrointestinal health, *J. AOAC Int. 95* (2012) 50–60.
85. T. Čepeljnik, M. Zorec, R. Kostanjšek, F.V. Nekrep, R. Marinšek-Logar, Is *Pseudobutyrivibrio xylanivorans* Mz5T suitable as a probiotic?: An *in vitro* study, *Folia Microbiol. 48* (2003) 339–345.
86. T.A. McCrorie, E.M. Keaveney, J.M. Wallace, N. Binns, M.B. Livingstone, Human health effects of conjugated linoleic acid from milk and supplements, *Nutr. Res. Rev. 24* (2011) 206–227.
87. T. Čepeljnik, E. Devillard, Capability of biohydrogenation of linoleic acid in rumen bacterium *Pseudobutyrivibrio xylanivorans* Mz5T, *Acta Agric. Slov. 88* (2006) 75–81 (in Slovene).
88. T. Čepeljnik, B. Lah, M. Narat, R. Marinšek-Logar, Adaptation of adhesion test using Caco-2 cells for anaerobic bacterium *Pseudobutyrivibrio xylanivorans*, a probiotic candidate, *Folia Microbiol. 52* (2007) 367–373.
89. T. Čepeljnik, M. Zorec, A. Lenassi Zupan, F.V. Nekrep, R. Marinšek-Logar, A new highly xylanolytic butyrate-producing probiotic candidate bacterium, *Book of Abstracts of the 53rd Annual Meeting of the European Association for Animal Production*, Cairo, Egypt (2002) p. 129.
90. S. Ohkawara, H. Furuya, K. Nagashima, N. Asanuma, T. Hino, Oral administration of *Butyrivibrio fibrisolvens*, a butyrate-producing bacterium, decreases the formation of aberrant crypt foci in the colon and rectum of mice, *J. Nutr. 135* (2005) 2878–2883.
91. S. Ohkawara, H. Furuya, K. Nagashima, N. Asanuma, T. Hino, Effect of oral administration of *Butyrivibrio fibrisolvens* MDT-1 on experimental enterocolitis in mice, *Clin. Vaccine Immunol. 13* (2006) 1231–1236.
92. T. Hino, K. Suzuki, S. Ohkawara, T. Miwa, N. Asanuma, Effects of oral administration of *Butyrivibrio fibrisolvens* MDT-1 on the development and healing of atopic dermatitis in NC/Nga mice, *Eur. J. Dermatol. 22* (2012) 211–217.
93. M. Čater, L. Fanedl, M. Zorec, R. Marinšek-Logar, Enhancement of biogas production from brewery spent grain by adding *Ruminococcus flavefaciens* 007C and *Pseudobutyrivibrio xylanivorans* Mz5$^T$, *13th World Congress on Anaerobic Digestion: Recovering (Bio) Resources for the World*, Santiago de Compostela, Spain (2013) iWA–11123.
94. M. Čater, M. Zorec, R. Marinšek Logar, Methods for improving anaerobic lignocellulosic substrates degradation for enhanced biogas production, *Springer Sci. Rev.* (in press).