The Role of Cellulose-Hydrolyzing Bacteria in the Production of Biogas from Plant Biomass

Vladimir V. Zverlov, Daniela E. Köck, and Wolfgang H. Schwarz

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Abstract Biological decomposition of biomass, i.e., the abundant and renewably produced whole plant biomass, is the basis for the production of bioenergy and platform chemicals in a biorefinery. Biogas formation is presently the most energy-efficient, versatile, and mature technology of producing energy and (potentially) a number of useful by-products. It can use a wide range of dedicated energy crops and by-products from the biorefinery. Biogas is easily stored and distributed by the existing infrastructure and can be used directly by the end consumers. Although biogas fermentation from plant biomass uses mature technology, the efficiency and yield of biogas plants can however still be increased. Little is, for instance, known about the underlying biology, and the biological basis of the process is not completely understood. This review deals with the first step of biogas fermentation, the hydrolysis of the polysaccharides in plant biomass. It is regarded as one of the

V.V. Zverlov • D.E. Köck • W.H. Schwarz (✉)
Department of Microbiology, Technische Universität München, Emil-Ramann-Str. 4, 85350 Freising-Weihenstephan, Germany
e-mail: wschwarz@wzw.tum.de

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rate-limiting steps in the process. It also determines the overall efficiency of the process. Cellulose is recalcitrant to enzymatic hydrolysis and needs special enzyme systems which are produced by a limited number of specialized microorganisms. Various bacterial enzyme systems for cellulose degradation are discussed. The bacteria in biogas fermenters are analyzed, and potential key players for cellulose degradation are pointed out. The principles of their enzyme systems could be used for developing new cellulases for cellulosic biomass as a basic substrate in a future biotechnology.

1 Introduction

Biorefinery intends to make comprehensive use of all material and energy streams derived from biomass "by complex systems based on ecological technology" (Kamm and Kamm 2004). It produces a variety of carbon-based products as well as energy—based on renewable material and thus sustainable. However, whereas industrial biotechnology often depends on clean and well-defined biomass (or purified fractions thereof) as substrate for separation and conversion processes, the production of biogas does not necessarily require to grow dedicated biomass (Fig. 1). Waste material and by-products can be used—they do frequently have no further use or are too expensive to reprocess. This material may be worthless in a chemical sense (useless compounds) or bound in a material complex such as a lignin matrix which is recalcitrant to further separation processes. Such material often occurs in too small amounts to set up a special process, and its further use is therefore not economically feasible. This is also often the case for waste material

Fig. 1 A mixture of grass and maize silage fed into a biogas fermenter

such as biological municipal solid waste, garden waste, road cuttings, and other waste material.

For these and other kinds of heterogeneous material, the biogas process may be installed as a channeling reaction to produce a relatively homogeneous, low-value but energy-rich gas consisting mainly of methane (CH_4) and carbon dioxide (CO_2) (Bochiwal et al. 2010). This gas mixture can be used for chemical synthesis of higher value products—and thus for refeeding into the biorefinery process. After purification it can also be used for energetical purposes (by combustion or fuel cell technology), for instance, as process energy for the biorefinery or filled in cars for transportation. On the other hand, it can be fed into the gas grid for a variety of purposes (Antoni et al. 2007). The gas grid needs purification of the biogas; however, it has the additional value of energy storage and utilization at the point of use. The separation of methane gas from the other constituents of biogas such as H_2S or CO_2 is state of the art and even in large scale economically feasible. Other constituents of biogas such as NH_4 or N_2 are avoidable by adapting the process appropriately or are harmless for the intended use. Biogas has advantages over the production of hydrogen gas from biomass, including the versatility of production, the energy content of the gas, and the storability and transportability for which the infrastructure is already existing (Fehrenbach et al. 2008).

Biogas production from waste material and by-products as well as from dedicated energy plants has also the advantage of producing a high-value organic fertilizer, either as a liquid manure, or dried and pelleted, or otherwise formulated. This fertilizer retains from the feed material most of the nitrogen as natural and biologically active ammonium compounds, all of the macro- and microelements contained in (and necessary for the growth of) plant biomass, and, probably most important, all of the phosphorous in natural and biologically accessible form. Whereas lost ammonium/nitrate can be regained by energy-intensive synthetic processes from aerial nitrogen gas, and all macro- and microelements are abundant in minerals, the agriculture of the near future will depend on recycled phosphorous compounds, free (or almost free) of heavy metal contaminants which are unavoidable when natural mineral deposits for phosphor salts will come to exhaustion in the near future (Lebuf et al. 2012).

It is often discussed that biogas fermentation is in competition with the plant material which has to be left on the fields to improve soil quality or at least to avoid soil depletion of humic substances. However, the carbon compounds used for the production of biogas would be lost quickly anyway due to natural aerobic decay when the plant material was distributed to or worked in the soil of fields. The digestate from biogas plants contains all the lignin and a part of other recalcitrant carbon compounds which cannot be used for biogas formation and thus lead at the end to humification and soil improvement when brought back to the fields. The biogas process is thus part of a complete recycling system in agriculture and forestry. The biogas process can therefore become an important stepstone for a biomass-based society built on sustainable energy and material supply; it is recycling as much of its resources as possible (Weiland 2006).

To install such a promising technology on a much broader basis, the technology—which is already mature and economically feasible in its application—still has to be considerably improved. There is potential for improvement on many stages of the process. Its material (substrate) basis should be broadened and its reliability optimized. This will also save the resources and reduce the energy necessary to produce the biomass fed to the process. To achieve these improvements, it will be essential to switch from an empirical approach of optimizing biogas technology toward a knowledge-based biological engineering, including a thorough understanding of the biological processes underlying the system biology of a biogas production plant. Such knowledge is now accumulating by scientifically raveling up the different aspects of substrate decomposition, metabolism, and energy as well as carbon flow within the reactor.

2 The Various Steps in Biogas Production

Biogas is produced by a natural consortium of interacting bacteria and archaea, possibly also involving anaerobic fungi (Fliegerová et al. 2010; Griffith et al. 2010). In contrast to the rumen microbial consortium, ciliates are obviously absent and the role of anaerobic fungi is so far largely unknown. So it seems to be the bacteria which degrade the constituents of the biomass fed into the fermenter vessel, particularly the polysaccharides. They use them for the buildup of cell biomass and the production of enzymes to hydrolyze the polymers in biomass; they release sugars for other bacteria, use up all residual oxygen to make the microenvironment completely anaerobic, and release fermentation products which are in turn utilized by other bacteria and by archaea for biogas production.

Biogas production is a cooperation of basically three types of bacteria, working in a succession of events, to name the most relevant steps: hydrolytic bacteria degrade the polymers in the biomass and produce—together with the saccharolytic bacteria—organic acids, alcohols, CO_2, and H_2 (the hydrolytic and acidogenic step); these products are converted by syntrophic bacteria, the acetogenic bacteria, to acetate; the acetate and the gases CO_2 and H_2 are converted to methane and CO_2 by the acetotrophic and the hydrogenotrophic methanogens, respectively. Other reactions such as acetate oxidation are also involved (Fig. 2).

The low amount of biological energy produced by these processes in the absence of oxygen (and thus in the absence of respiration) leads to massive turnover of substrate with the production of oxidized (such as CO_2) and reduced compounds (such as H_2 and CH_4) through disproportionation. The first step in the biogas process, the hydrolysis, is regarded as crucial for the efficiency—the more of the substrate is utilized, the greater is the amount of methane produced. Moreover, it is regarded as rate limiting because all downstream processes depend completely on the yield and production rate of the initial hydrolysis. The rate of decomposition during the hydrolysis stage depends greatly on the nature of the substrate. The transformation of cellulose and hemicellulose generally takes place more slowly

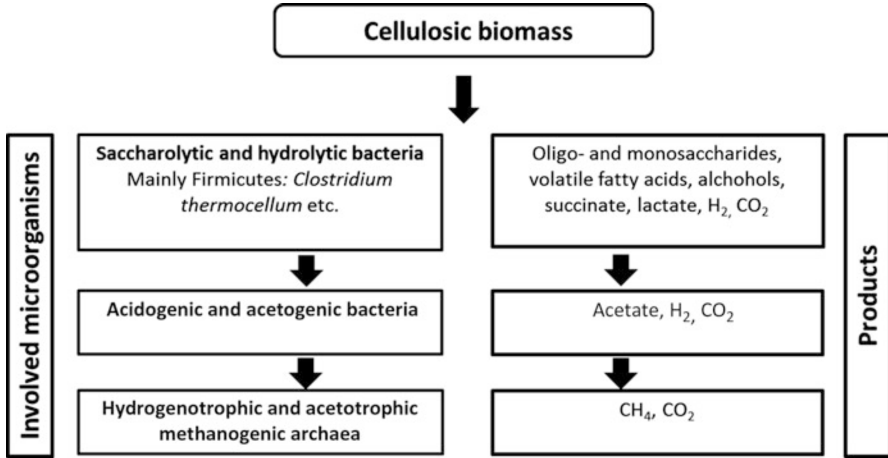

Fig. 2 Biological and metabolic processes during biogas fermentation, based on cellulosic biomass

than the decomposition of proteins (Boone et al. 1993; Lynd et al. 2002; Noike et al. 1985).

The processes downstream from hydrolysis (acidogenesis and acetogenesis) are basically the channeling of all fermentation products into acetate and the gases CO_2 and H_2; these are finally used by the acetotrophic and the hydrogenotrophic archaea which finally produce methane and carbon dioxide, the biogas (Schnürer and Jarvis 2009) (Fig. 2).

The production of biogas from organic materials is widespread in nature and can be found, for instance, in the intestine of plant-feeding animals or insects, in compost formation, in marshes and swamps, or in the debris on the ground of lakes where plant biomass is degraded anaerobically (Görisch and Helm 2006). A number of studies have been carried out on biogas formation, beginning with studies on wastewater and manure, later on plant biomass (Zverlov et al. 2010; Kampmann et al. 2012; Cirne et al. 2007). However, although cellulose is the richest source of organic carbon compounds on earth (Cox et al. 2000), studies on cellulose as substrate and its degradation for biogas production are still insufficient.

Whereas a number of microorganisms are known to be able to degrade natural cellulose, and some bacteria have been studied intensively which degrade cellulose in the rumen of cattle or in the environment, the identity and obvious variety of the cellulose-degrading bacteria in biogas plants is still largely untapped. In general there is a lack of information on the microbiology in various steps of the biogas process, and particularly in the first, the hydrolytic step.

3 The Recalcitrance of Cellulose

The polysaccharides in plant cell walls give the plants structure and sturdiness and strength in stems and stalks. They make up for the greatest part of plant biomass and therefore represent a large potential for biogas production. The intertwined composition of various polymers makes the material resistant to microbiological attack—only a few saprophytic organisms are able to degrade this material relatively efficiently. Some of these microorganisms are degrading it in coexistence or even in symbiosis with plant-feeding animals and insects, such as cattle, camels, roaches, or termites.

There are three major polymeric, interwoven components of biomass: cellulose, hemicellulose/pectin, and lignin. Fundamentally different types of enzyme sets are necessary for their degradation, and no single microorganism is able to degrade all polymers in plant cell walls completely without the help of others.

The slow hydrolysis rate of plant cell wall degradation is mainly related to the presence of lignin, which cannot be substantially degraded under anaerobic conditions. The amount of lignin diminishes degradation rate and degradability, obviously by occluding accession sites for hydrolyzing enzymes (Lynd et al. 2002; Schnürer and Jarvis 2009).

The major cell wall component (in mass) is cellulose. It is an extremely recalcitrant material to degrade and is only degraded slowly. Consistent of long parallel, unbranched, homogeneous chains of β-1,4-linked glucose monomers, cellulose is highly crystalline, interrupted only by short amorphous regions (Fig. 3). Cellulose is thus an extremely tough material for enzymatic degradation, being not hydrated and completely insoluble, with a hydrophobic surface. In addition it is very large compared to the size of an enzyme. To fit into an enzyme substrate pocket for degradation, a cellulose molecule has to be pulled out from the crystalline surface where it is linked with numerous interchain and interlayer hydrogen bonds. Moreover, only a small part of the substrate is "visible" to an enzyme at the surface of the crystal and thus accessible to enzymatic attack; most of the substrate is hidden within the crystal (Schwarz 2004).

These effects make cellulose relatively resistant to degradation; its degradation speed is extremely slow and necessitates the presence of a consortium of different and specialized enzymes which act cooperatively to break up the surface with binding modules (non-catalytic "activity") and a multitude of synergistically acting β-glucanase modules (catalytic activity). These enzymes have different modes of

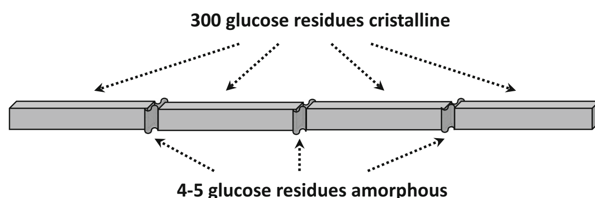

Fig. 3 Approximate structure of a cellulose crystal in ramie wood (according to Nishiyama et al. 2002, 2003a, b)

activity, such as endo-active endoglucanases and processively active exoglucanases (Schwarz et al. 2004). They degrade cellulose to a mixture of cellobiose, cellotetraose, and various other cellodextrins, which are taken up by the cells and further degraded by β-glucosidases/cellobiases and/or cellobiose or cellodextrin phosphorylases.

4 Cellulose-Degrading Bacteria Are Rare

So far only a relatively small number of cellulolytic bacterial species have been isolated and characterized. A list restricted to bacteria which can utilize cellulose in its natural (=crystalline) configuration as sole carbon and energy source is compiled in Table 1. Many more bacteria produce extracellular β-glucanases, usually endoglucanases, which hydrolyze the β-1,4-glucosidic bond only in soluble (mixed linkage) β-glucans or artificial cellulosic compounds such as carboxymethylcellulose (CMC). Although CMC is chemically a cellulose with its typical β-1,4-glucosidic linkages, its degradation is a precondition but not a sufficient indication for degradation of natural (i.e., crystalline) cellulose. The literature was checked carefully for indication of traits such as growth on filter paper or crystalline cellulose powder (such as Avicel) or the like (Fig. 4). Such strains are also called "true cellulolytic" bacteria. Due to their potential importance for biotechnology, the genomes of a number of the species listed in Table 1 are sequenced and deposited in databases.

From the strains listed in Table 1, two general physiological groups of cellulolytic bacteria can be formed: the anaerobic and the aerobic cellulolytic bacteria. The difference is the gain of energy from the sugars obtained from the cellulose. The anaerobic bacteria (e.g., the *Clostridia*) can produce from the same amount of glucose roughly only 1/10 of the Adenosine triphosphate (ATP) by fermentation as aerobic bacteria by respiration. Therefore, the enzymes producing the glucose from cellulose under anaerobic conditions have to be at least ten times as active to compensate for the energy used for their production (Lynd et al. 2002).

It is obvious that the majority of the so far known truly cellulolytic species belong to the bacterial phyla *Firmicutes* and *Actinobacteria*. However, the number of species in an ecosystem does not reflect the prevalence of certain bacteria in a habitat—the number of individual cells of a certain function (such as being truly cellulolytic) in a population is by far more important. This number was estimated for some samples by culture-independent methods such as high-throughput pyrosequencing of 16S rRNA sequences found in a population or by assigning sequence tags to known genes in a database and counting the next known neighbors in a phylogenetic or a similarity tree.

Total DNA or RNA isolated from biogas plant sludge was used for these investigations (Fig. 5). By these methods, *Firmicutes* were found to be the majority of bacteria in saccharolytic bacterial communities such as a biogas fermenter (see

Table 1 List of bacteria reported to grow on crystalline cellulose as carbon and energy source. Utilization of crystalline cellulose t = thermophile (optimum growth above 50 °C), m = mesophile. Material or habitat for isolation and phylogenetic position are indicated

Phylogeny	Species	Temp	Source	Reference
Phylum *Firmicutes*				
Class *Clostridia* Order *Thermoanaero-bacterales* Family *incertae sedis*	*Caldicellulosiruptor bescii*	t		Svetlichnyi et al. (1990)
	Caldicellulosiruptor hydrothermalis	t	Hot spring	Miroshnichenko et al. (2008)
	Caldicellulosiruptor kristjanssonii	t	Hot spring	Bredholt et al. (1999)
	Caldicellulosiruptor kronotskyensis	t	Hot spring	Miroshnichenko et al. (2008)
	Caldicellulosiruptor lactoaceticus	t		Mladenovska et al. (1995)
	Caldicellulosiruptor obsidiansis	t	Hot spring	Hamilton-Brehm et al. (2010)
	Caldicellulosiruptor saccharolyticus	t	Hot spring	Rainey et al. (1994)
	Thermoanaerobacter cellulolyticus	t	Hot spring	Bergquist et al. (1999)
Class *Clostridia* Order *Clostridiales* Family *Lachnospiraceae*	*Butyrivibrio fibrisolvens*	m	Rumen	Berger et al. (1990)
	Cellulosilyticum lentocellum	m	Rumen	Cai and Dong (2010)
	Cellulosilyticum ruminicola	m	Rumen	Cai and Dong (2010)
	Ruminococcus succinogenes	m	Rumen	Fields et al. (2000)
Class *Clostridia* Order *Clostridiales* Family *Eubacteriaceae*	*Eubacterium cellulosolvens*	m	Rumen	Anderson and Blair (1996)
Class *Clostridia* Order *Clostridiales* Family *Clostridiaceae*	*Clostridium aldrichii*	m	Wood fermenter	Yang et al. (1990)
	Clostridium alkalicellulosi	m	Soda lake	Zhilina et al. (2005)
	Clostridium caenicola	t	Sludge	Shiratori et al. (2009)
	Clostridium celerecrescens	m	Manure	Palop et al. (1989)
	Clostridium cellobioparum	m	Rumen	Lamed et al. (1987)
	Clostridium cellulofermentans	m	Manure	Yanling et al. (1991)
	Clostridium cellulolyticum	m	Compost	Pagés et al. (1997), Bélaich et al. 1997
	Clostridium cellulosi	t	Manure	Yanling et al. (1991)

(continued)

Table 1 (continued)

Phylogeny	Species	Temp	Source	Reference
	Clostridium cellulovorans	m	Wood	Shoseyov and Doi (1990), Tamaru et al. (2000)
	Clostridium chartatabidum	m	Rumen	Kelly et al. (1987)
	Clostridium clariflavum	t	Sludge	Shiratori et al. (2009)
	Clostridium herbivorans	m	Pig intestine	Varel et al. (1995)
	Clostridium hungatei	m	Soil	Monserrate et al. (2001)
	Clostridium josui	t	Compost	Kakiuchi et al. (1998)
	Clostridium longisporum	m	Rumen	Leschine (1995)
	Clostridium papyrosolvens	m	Paper mill	Pohlschröder et al. (1995)
	Clostridium phytofermentans	m	Soil	Warnick et al. (2002)
	Clostridium populeti	m	Wood fermenter	Leschine (1995)
	Clostridium sp. *C7*	m	Mud	Cavedon et al. (1990)
	Clostridium stercorarium	t	Compost	Schwarz et al. (1995)
	Clostridium straminisolvens	m	Rice straw	Kato et al. (2004)
	Clostridium sufflavum	m	Cattle waste	Nishiyama et al. (2009)
	Clostridium termitidis		Termite	Hethener et al. (1992)
	Clostridium thermocellum	t	Sewage + soil	Lamed et al. (1991)
	Clostridium thermocopriae	t	Hot spring	Jin and Toda (1989)
	Clostridium thermopapyrolyticum	h	Mud	Méndez et al. (1991)
Class *Clostridia* Order *Clostridiales* Family *Ruminococcaceae*	*Acetivibrio cellulolyticus*	m	Sewage	Ding et al. (1999)
	Acetivibrio cellulosolvens	m	Sewage	Khan et al. (1984)
	Ruminococcus albus	m	Rumen	Ohara et al. (2000)
	Ruminococcus flavefaciens	m	Rumen	Aurilia et al. (2000)
Class *Clostridia* Order *Halanaerobiales* Family *Halanaerobiaceae*	*Halocella cellulosilytica*	m	Saline lake	Simankova et al. (1993)

(continued)

Table 1 (continued)

Phylogeny	Species	Temp	Source	Reference
Class *Bacilli* Order *Bacillales* Family *Thermoactinomycetaceae*	*Thermoactinomyces* sp. *YX*	t		Hägerdahl et al. (1979)
Class *Bacilli* Order *Bacillales* Family *Alicyclobacillaceae*	*Caldibacillus cellulovorans*	t		Sunna et al. (2000)
Class *Bacilli* Order *Bacillales* Family *Bacillaceae*	*Bacillus circulans*	m		Kim (1995)
	Bacillus pumilus	m		Ariffin et al. (2006)
Phylum *Actinobacteria*				
Class *Actinobacteria* Subclass *Actinobacteridae* Order *Actinomycetales* Suborder *Frankineae* Family *Acidothermaceae*	*Acidothermus cellulolyticus*	t	Acidic hot spring	Eppard et al. (1996), Maréchal et al. (2000)
Class *Actinobacteria* Subclass *Actinobacteridae* Order *Actinomycetales* Suborder *Micrococcineae*	*Cellulomonas biazotea*	m		Lednicka et al (2000)
	Cellulomonas cartae	m		Thayer et al. (1984)
	Cellulomonas cellasea	m		Lednicka et al. (2000)
	Cellulomonas cellulans	m	Soil	Lednicka et al. (2000)
	Cellulomonas fimi	m	Soil	Lednicka et al. (2000)
	Cellulomonas flavigena	m	Soil	Lednicka et al. (2000)
	Cellulomonas gelida	m		Thayer et al. (1984)
	Cellulomonas iranensis	m	Forest soil	Elberson et al. (2000)
	Cellulomonas persica	m	Forest soil	Elberson et al. (2000)
	Cellulomonas uda	m	Sewage	Thayer et al. (1984)
Class *Actinobacteria* Subclass *Actinobacteridae* Order *Actinomycetales* Suborder *Micrococcineae* Family *Microbacteriaceae*	*Curtobacterium flaccumfaciens*	m	Soil	Lednicka et al. (2000)
Class *Actinobacteria* Subclass *Actinobacteridae* Order *Actinomycetales* Suborder *Micrococcineae* Family *Promicromonosporaceae*	*Xylanimonas cellulosilytica*	m	Decayed tree	Anderson et al. (2012)

(continued)

Table 1 (continued)

Phylogeny	Species	Temp	Source	Reference
Class *Actinobacteria* Subclass *Actinobacteridae* Order *Actinomycetales* Suborder *Micromonosporineae* Family *Micromonosporaceae*	*Actinoplanes aurantiaca*	m	Soil	Coughlan and Mayer (1992)
	Micromonospora melonosporea	m	Compost	Wilson (1992)
	Micromonospora chalcae	m	Soil	Gallagher et al. (1996)
	Micromonospora propionici	m	Termite	Leschine (1995)
	Micromonospora ruminantium	m	Rumen	Leschine (1995)
Class *Actinobacteria* Subclass *Actinobacteridae* Order *Actinomycetales* Suborder *Pseudonocardineae* Family *Pseudonocardiaceae*	*Actinosynnema mirum*	m	Grass blade	Anderson et al. (2012)
Class *Actinobacteria* Subclass *Actinobacteridae* Order *Actinomycetales* Suborder *Streptomycineae* Family *Streptomycetaceae*	*Streptomyces albogriseolus*	m		Van Zyl (1985)
	Streptomyces aureofaciens	m	Compost	EL-Din et al. (2000)
	Streptomyces cellulolyticus	m		Li and Gao (1997)
	Streptomyces flavogriseus	m	Soil	MacKenzie et al. (1984)
	Streptomyces lividans	m		Kluepfel et al. (1986)
	Streptomyces nitrosporeus	m		Van Zyl (1985)
	Streptomyces olivochromogenes	m		Coughlan and Mayer (1992)
	Streptomyces reticuli	m	Soil	Schrempf and Walter (1995)
	Streptomyces rochei	m	Termite gut	Perito et al. (1994)
	Streptomyces thermovulgaris	m		Coughlan and Mayer (1992)
	Streptomyces viridosporus	m		Coughlan and Mayer (1992)
Class *Actinobacteria* Subclass *Actinobacteridae* Order *Actinomycetales* Suborder *Streptosporangineae* Family *Nocardiopsaceae*	*Thermobifida alba*	m		Kukolya, pers. commun.
	Thermobifida cellulolytica	t	Compost	Kukolya et al. (2002)
	Thermobifida fusca	t	Soil	Wilson (1992), Kukolya, pers. commun. (2003)

(continued)

Table 1 (continued)

Phylogeny	Species	Temp	Source	Reference
Class *Actinobacteria* Subclass *Actinobacteridae* Order *Actinomycetales* Suborder *Streptospor-angineae* Family *Thermomonosporaceae*	*Thermomonospora curvata*	t		Coughlan and Mayer (1992)
Class *Actinobacteria* Subclass *Actinobacteridae* Order *Actinomycetales* Suborder *Streptospor-angineae* Family *Streptosporangiaceae*	*Thermobispora bispora*	t	Soil	Wilson (1992)
	Streptosporangium subroseum	m	Soil	Zhang et al. (2002)
Superphylum *Fibrobacteres/Acidobacteria* group, phylum *Fibrobacteres*				
Class *Fibrobacteria* Order *Fibrobacterales* Family *Fibrobacteraceae*	*Fibrobacter succinogenes*	m	Rumen	Schellhorn and Forsberg (1984)
Superphylum *Bacteroidetes/Chlorobi* group, phylum *Bacteroidetes*				
Class *Cytophagia* Order *Cytophagales* Family *Cytophagaceae*	*Cytophaga aurantiaca*	m	Soil	Li et al. (1997)
	Cytophaga haloflava	m	Soil	Li et al. (1997)
	Cytophaga hutchinsonii	m	Soil	Li et al. (1997)
	Cytophaga krzemieniewskae	m	Soil	Li et al. (1997)
	Cytophaga rosea	m	Soil	Li et al. (1997)
	Sporocytophaga myxococcoides	m	Soil	Coughlan and Mayer (1992)
Class *Flavobacteriia* Order *Flavobacteriales* Family *Flavobacteriaceae*	*Flavobacterium johnsoniae*	m	Soil	Lednicka et al. (2000)
Class *Bacteroidia* Order *Bacteroidales* Family *Bacteroidaceae*	*Bacteroides cellulosilyticus*	m	Human fecal sample	Robert et al. (2007)
	Bacteroides cellulosolvens	m	Sewage	Lamed et al. (1991)
	Bacteroides sp. P-1	t	Rotting biomass	Ponpium et al. (2000)
Class *Bacteroidia* Order *Bacteroidetes*, order II. *incertae sedis* Family *Rhodothermaceae*	*Rhodothermus marinus*	t	Hot spring	Bergquist et al. (1999)

(continued)

Table 1 (continued)

Phylogeny	Species	Temp	Source	Reference
Phylum *Proteobacteria*				
Class *Betaproteobacteria* Order *Burkholderiales* Family *Alcaligenaceae*	*Achromobacter piechaudii*	m	Soil	Lednicka et al. (2000)
Class *Gammaproteo-bacteria* Order *Enterobacteriales* Family *Enterobacteriaceae*	*Dickeya dadantii*	m	Plant biomass	Rabinovich et al. (2002)
Class *Gammaproteo-bacteria* Order *Xanthomonadales* Family *Xanthomonadaceae*	*Xanthomonas* sp.	m	Brack water	Mullings and Parish (1984)
Class *Gammaproteo-bacteria* Order *Pseudomonadales* Family *Pseudomonadaceae*	*Cellvibrio gilvus*	m	Soil	Coughlan and Mayer (1992)
	Cellvibrio mixtus	m	Soil	Lednicka et al. (2000)
	Cellvibrio vulgaris, *C. fulvus*	m	Soil	Blackall et al. (1985)
	Pseudomonas fluorescens (cellulosa)	m	Plant biomass	Dees et al. (1995)
	Pseudomonas mendocina	m	Soil	Lednicka et al. (2000)
Subphylum *delta*/*epsilon subdivisions* Class *Deltaproteo-bacteria* Order *Myxococcales* Family *incertae sedis*	*Myxobacter* sp. AL-1	m	Soil	Avitia et al. (2000), Pedraza-Reyes, pers. commun.
Phylum *Thermotogae*				
Class *Thermotogae* Order *Thermotogales* Family *Thermotogaceae*	*Fervidobacterium islandicum*	t	Hot spring	Huber et al. (1990)
	Thermotoga maritima	t	Hot spring	Bergquist et al. (1999)
	Thermotoga neapolitana	t	Hot spring	Bergquist et al. (1999)

below). It should be kept in mind that the source of the first described isolation is only an indication of the environment in which these bacteria can be found. Most of them have later been isolated from or identified in other environments. For example, *Clostridium thermocellum*, originally isolated from sewage and from soil, has been shown to be a very common environmental bacterium, present in a variety of habitats where plant biomass is degraded, such as cattle manure, garden soil,

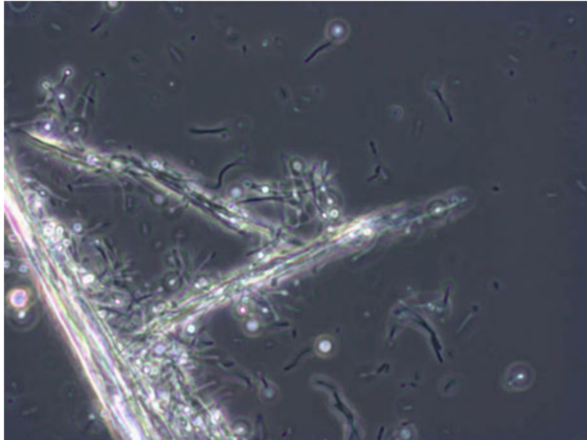

Fig. 4 Cellulose-degrading bacteria on cellulose fibers (phase contrast microscopy, ×1,000 magnification)

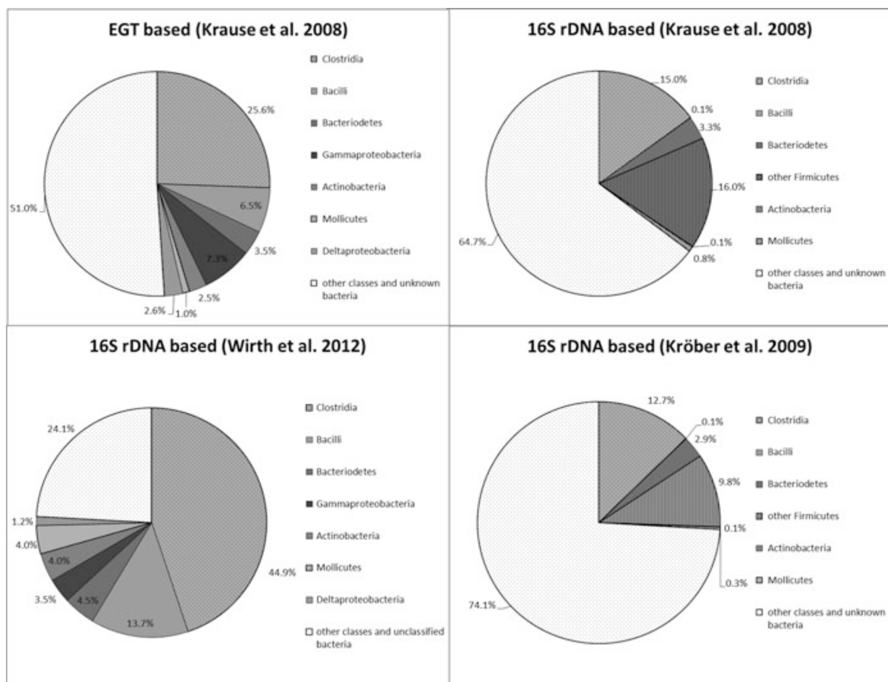

Fig. 5 Taxonomic profiles from the superkingdom bacteria of different biogas microbial communities based on analysis of metagenome sequence reads created by next-generation sequencing techniques. Phylogenetic class is indicated. The authors compared either 16S-rDNA sequences or environmental gene tags (EGTs) with different reference data sets, e.g., Ribosomal Database Project Classifier or ARB Database (modified after Wirth et al. 2012; Kröber et al. 2009; Krause et al. 2008)

bushes, compost, agriculturally used plots, and biogas fermenters run with energy crops (Zverlov et al. 2010; unpublished data).

Most of the cellulolytic species listed in Table 1 are saprophytic, saccharolytic bacteria (especially the thermophilic bacteria); however, some are pathogenic to humans or plants such as *Bacillus circulans* (Leary et al. 1986) or *Pseudomonas mendocina* (Aragone et al. 1992). Whereas cellulolytic activity is apparent for plant pathogenic bacteria, this is not obvious for human pathogens. Most plant pathogens have some cellulolytic activity for loosening the cell wall and getting access to the cell lumen; however, this activity is often not sufficient for providing the sugars from cellulose to support growth. Plant pathogenic bacteria do not seem to play a role in biomass degradation during the biogas process.

5 Taxonomic Composition of Biogas Plants Obtained by Clone Library 16S-rDNA Sequences and Metagenome Sequences Generated by Next-Generation Sequencing

Only a small fraction of the bacterial species present in a biogas fermenter has been isolated in pure culture. Strain purification, however, is a precondition for a thorough characterization of their catabolic and metabolic traits, i.e., the use of substrates and the formation of fermentation products—and hence their role in the biogas process chain in a fermenter. However, culture-independent methods are necessary to analyze the complete composition of the bacterial biogas community. Currently next-generation sequencing methods, like 454 pyrosequencing, are used to analyze bacterial (and archaeal) communities by generation of environmental gene tags (EGTs) and clusters of orthologous groups of proteins (COGs) or 16S-rDNA libraries (Krause et al. 2008; Schlüter et al. 2008; Kröber et al. 2009; Wirth et al. 2012). These methods are however severely hampered by the lack of reference genomes for most of the species involved, often even genera. This is reflected in the high percentage of “unknown bacteria” or “other classes” in Fig. 5.

As shown in Table 1, most of the hitherto known bacteria able to efficiently degrade natural cellulose belong to the phylum *Firmicutes*, particularly to the class *Clostridia*. The overwhelming majority of the identified species in the biogas fermenters were also members of the phylum *Firmicutes*, class *Clostridia* (Fig. 5; Wirth et al. 2012; Krause et al. 2008). Among the EGTs, coding for proteins involved in the hydrolysis of poly- and oligosaccharides, *Firmicutes* are again with over 50 % the dominant phylogenetic group and again mainly represented in the class *Clostridia* (Krause et al. 2008). Consequently, most of the identified species with known cellulolytic members also belong to the class *Clostridia*. Cellulolytic species of the other phyla are only rarely identified.

Although most known cellulolytic bacteria belong to the *Clostridia*, this argument does not work if turned around: most clostridial species do not contain cellulose degraders, and thus the affiliation to the clostridia does not work as an

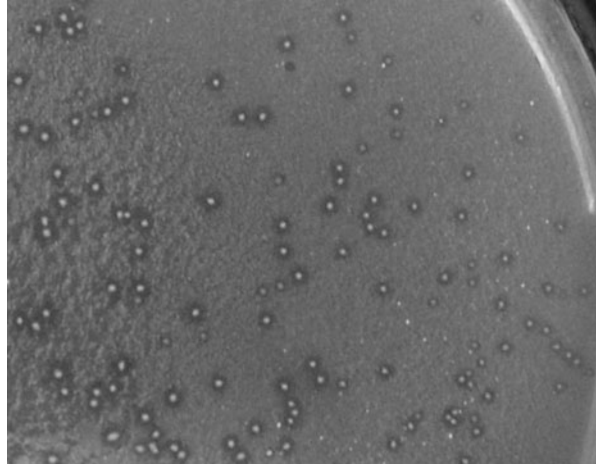

Fig. 6 Colonies of a new isolate of *C. thermocellum* (*white spots*) from biogas plants was plated on an agar plate overlayed with a thin agar layer containing cellulose powder (Avicel). The *hazy background* (from the cellulose fibers) is cleared around the colonies (*darker halo*) which produce cellulases and dissolves the cellulose

argument for a cellulolytic trait—for each single species, it has to be shown that its members are able to utilize crystalline cellulose. Even closely related bacteria (on the 16S-rDNA sequence level) differ in this ability. And the use of genomic sequence data for strain assignment to a functional group can also sometimes be misleading. In the case of *Clostridium acetobutylicum*, all the sequences necessary for expression of cellulosomes (very efficient extracellular cellulase complexes) were present (Sabathé et al. 2002). However, the encoded proteins turned out to be defective in expression and/or activity and did not show sufficient cellulose hydrolysis capability for supporting the bacterium to grow on cellulose. Hence this species is not included in the "cellulolytic" bacterium species listed in Table 1.

Bacteria of the species *Clostridium thermocellum* degrade crystalline cellulose efficiently (Fig. 6). They occur most frequently in metagenome analysis data (Wirth et al. 2012; Krause et al. 2008). Other cellulolytic bacteria with high sequence abundance (within the 40 most frequently found species) were *Clostridium cellulolyticum*, *Ruminococcus albus*, *Clostridium saccharolyticum*, and *Caldicellulosiruptor saccharolyticus* (Wirth et al. 2012). The high abundance of cellulolytic members in the *Clostridia* indicates the important role of these bacteria for degradation of complex substrates in the natural remineralization of biomass.

The metagenomic analysis created a high number of sequences which could not be allocated to any microbial species (Fig. 5: up to 74 % are unclassified bacteria; Kröber et al. 2009). This implies the presence of many still unidentified microorganisms in biogas plants. And it indicates also that probably important bacteria involved in the degradation of cellulose are still unknown and cannot be identified due to the lack of reference sequences. This lack of knowledge can only be overcome by isolating and characterizing new cellulolytic bacteria.

A question still untouched is the cooperation of different bacteria for the degradation of resilient substrates. It was shown that a combination of enzymes of similar but not identical substrate specificity can be more effective in substrate

degradation (Zverlov et al. 2010; see below: cellulosomes). A similar effect could be generated by exoenzymes which work together but are produced by different bacteria (Kato et al. 2005). This is difficult to investigate with the methods at hand and has not been systematically investigated so far. One example is the degradation of cellodextrins to glucose which could be effected by extracellular β-glucosidases. However, most saccharolytic bacteria seem to possess uptake systems for cellodextrins, and these are degraded by intracellular enzymes, either by phosphorolysis leading to glucose-1-phosphate (as was described for *C. thermocellum*) or hydrolytically by β-glucosidases. There is the possibility that free glucose might not be an intermediate in biogas formation from lignocellulosic biomass. Another example is the combination of the cellulose specialist *C. thermocellum* and the hemicellulose specialist *C. stercorarium* which has been shown to degrade maize silage more efficiently than did any of the species alone (Zverlov et al. 2010).

6 The Enzyme Systems Used by Cellulolytic Bacteria in Biogas Plants

The addition of bacteria with superior cellulose-degrading ability could speed up the hydrolytic processes and make biogas production faster and thus more cost-efficient, among others, by utilizing more of the substrate in a shorter time. Addition of external enzyme, majorly consisting of cellulases, has only limited effect and is costly. Added enzymes would only have an effect when they would supplement with types of hydrolytic activity which are not (sufficiently) produced by the bacterial community in the process. The added hydrolytic bacteria would grow on the biomass are adapted to the substrate composition present, and produce sufficient amounts of enzymes of all types to degrade the various polysaccharides in the substrate so that they get at the end (after using up starch, hemicellulose, and pectin) access to the sugars in cellulose. Through the secretion of all necessary enzymes, the sugars released by their activity feed as well the other saccharolytic bacteria which produce the substances needed at the end of the complex biogas process by the archaea for methane production (Fig. 3). The overall methane production has been improved by repeated addition of a selected inoculation culture (Schmack and Reuter 2010). The addition of a selected cellulolytic culture improved the efficiency in a thermophilic technicum scale process (paper in preparation).

There are bacteria with fairly moderate cellulose degradation ability such as *Clostridium stercorarium*, which is more a specialist for the degradation of hemicellulose, but can thrive on filter paper as sole carbon source (Adelsberger et al. 2004). This species produces two cellulolytic enzymes of glycosyl hydrolase family 9 and 48 (GH9 and GH48) (Zverlov and Schwarz 2008). These two separate enzymes, an endoglucanase and a cellobiohydrolase, respectively, act synergistically to degrade the crystalline cellulose, however, slowly and incomplete. It does

this best at moderately thermophilic to thermophilic conditions. This species is found widespread in decaying biomass and has also been identified repeatedly in biogas fermenters (Madden 1983; Schwarz et al. 2004; Zverlov et al. 2010 and unpublished results).

Better cellulose degraders than *C. stercorarium* are thermophilic, anaerobic species within the genus *Caldicellulosiruptor*. The major cellulolytic enzyme of *C. bescii* (former *Anaerocellum thermophilum*) is the extracellular cellulase CelA which consists of a GH9 and a GH48 module combined within one polypeptide (Zverlov et al. 1998). The close neighborhood between the two enzymatic activities seems to enhance the synergistic effect which makes this enzyme system superior to the simple additive mixture of separate activities as is found with *C. stercorarium*.

The best cellulase system so far known is that of *Clostridium thermocellum*. It produces an extracellular, macromolecular complex which is called cellulosome (Schwarz 2001). The cellulosome is also the most elaborate extracellular enzyme system known in bacteria. It was used as a model system for an extracellular bacterial enzyme complex as well as an exemplary cellulase (Arai et al. 2001; Durrant et al. 1991; Kruus et al. 1995; Kurokawa et al. 2002; Zverlov et al. 1998, 2002, 2003, 2005a, b). In this enzyme complex the enzyme components bind via high-affinity and highly specific protein–protein interactions between the dockerins on the enzymatic components which bind to one of eight or nine cohesin modules in a so-called cellulosome-integrating protein, the CipA protein. These complexes stay cell wall bound on the surface of an actively growing bacterial culture (Bayer et al. 1985). The complexes seem to be shed off in later growth stages, where they can be isolated easily for investigation. Besides the cohesin modules, the CipA protein contains a substrate-binding module, a carbohydrate-binding module of type CBM3 which binds the complex (and hence the bacteria) to the surface of crystalline cellulose. This causes on the one hand a high local density of enzymes on the substrate surface (the insoluble cellulose) near the site of binding; this high enzyme density is essential for the high activity on the crystalline substrate. On the other hand, the localization on the cell wall allows direct take up of the degradation products of cellulose, the cellodextrins, by the bacterial cell. Other enzymes in the cellulosome degrade xylan and other hemicellulosic substances, pectin or chitin, and thus make the cellulose accessible for the highly specialized and effective cellulases (Schwarz 2001) (Fig. 7).

Cells of *C. thermocellum* do not use other sugar substrates than cellodextrins—glucose is hardly and other sugars (mono- or oligosaccharides) are not at all metabolized by this bacterium. The degradation products of hemicellulose, cellulose, etc., are left to the other bacteria within the bacterial community. *C. thermocellum* (and probably other similar bacteria) are thus the feeding machines driving the whole biogas process.

Cellulase complexes similar to the cellulosomes of *C. thermocellum* were also identified in other cellulolytic bacteria such as the mesophilic species *C. cellulovorans*, *C. cellulolyticum*, *C. papyrosolvens*, *Bacteroides cellulosolvens*, *Acetivibrio cellulolyticus*, *Ruminococcus albus*, and *Ruminococcus flavefaciens* and the thermophilic species *Clostridium clariflavum* and *Clostridium josui*. They

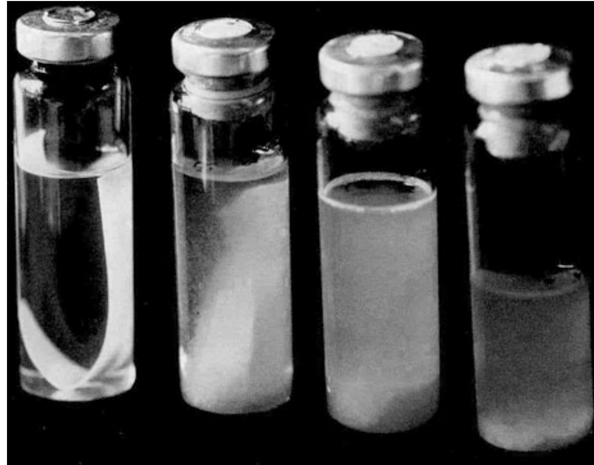

Fig. 7 A culture of *C. thermocellum* is inoculated to an anaerobic flask (rubber stoppered) containing a filter paper strip. The paper is increasingly dissolved after 1, 2, and 3 days (flask 2, 3, and 4 from *left* to *right*). Gas is formed under vigorous growth on day 2 (*bubbles*)

partially have a more or less different cellulosome architecture, and those of, for instance, the ruminococci are even more elaborate than in *C. thermocellum* (Bayer et al. 2013). Of them *C. thermocellum*, *C. cellulolyticum*, and *R. albus* had been detected in biogas plants (Wirth et al. 2012), underscoring their potentially important role for cellulose degradation in the industrial process.

7 The Cellulosome, an Efficient Cellulase Complex

The cellulosomes of *C. thermocellum*, *C. cellulolyticum*, and *C. cellulovorans* have been intensively investigated and still are subject to mechanistic studies including the in vitro reconstitution of complexes (Blouzard et al. 2010; Krauss et al. 2012; Vazana et al. 2012). The potential of complex formation for the degradation of the recalcitrant crystalline cellulose was made obvious by complete abolishment of CipA formation by mutagenesis (Zverlov et al. 2008; Olson et al. 2013). Mutants lacking the non-catalytic CipA protein are defective in cellulose degradation and not able to use filter paper as carbon source for growth anymore, although all the enzyme components of the cellulosome are still produced; i.e., although the cellulases are present in about the same amount, their activity is restricted to the hydrolysis of the soluble glucans CMC and barley β-glucan (Zverlov et al. 2008). Hence, the complex formation is a way of producing a cellulase enzyme system with considerably enhanced activity without having to produce ultra-large proteins as is the case with the enzyme systems of *Caldicellulosiruptor* strains.

The combination of various enzyme types in large protein molecules or protein complexes and with binding modules for the substrate enhances the activity by neighboring effects, a high local concentration of enzymatic activities on the site of binding, and the cooperation between enzymes of different mode of action, such as

endo- and exo-mode. This results in a synergistic effect which has been shown to be as high as a 15-fold activity, when the activity of complexed and comparable noncomplexed system are compared (Zverlov et al. 2008; Krauss et al. 2012).

The production of enzyme systems with enhanced efficiency is a necessity for anaerobic bacteria which can generate only a limited amount of energy in the form of ATP from the glucose produced.[1] This is in line with the general observation that anaerobic organisms use more energy-saving mechanisms than aerobic organisms.

8 The Biogas Process in Biorefinery Context

Although the biogas process, as it is widely established by now, is not a classical part of a biorefinery process chain, it often utilizes by-products or end products which have no further value for other technologies. Biogas formation may produce methane and carbon dioxide from recalcitrant or mixed material too “dirty” (too impure) to be used in the production of clean materials. However, biogas itself can be fed into the production of various chemicals by catalytic technologies using heterogeneous catalysts (Lunsford 2000) and thus making otherwise useless raw materials accessible for biorefinery. On the other hand, the sludge from the biogas process (the digestate) has been extracted for producing considerable amounts of vitamins B2 and B12 (riboflavin and cobalamin) in a complete biorefinery approach of utilizing lignocellulosic agricultural residues via clostridial acetone–butanol fermentation, using the fermentation gas, the biogas sludge for methane and vitamin production, and the biogas digestate as feed for yeast to single-cell protein in husbandry fodder (Zverlov et al. 2006). However, the full chain of biorefinery in this innovative Russian process scheme has not been realized due to economic restrictions and lack of scale.

It can be speculated that advanced membrane technology could separate carbonic acids, higher alcohols, or other intermediate fermentation products from the sludge during biogas fermentation. But none of these processes is so far developed enough to calculate cost-effectiveness, and integration in an economically viable biorefinery process is not foreseeable.

Conclusion

Identification of key players for cellulose degradation in the biogas fermenter is hampered by the limited knowledge on truly cellulolytic bacteria. Some important cellulose-degrading bacteria in nature seem to be still undetected,

(continued)

[1] Only about 1/10 of the amount of ATP can be produced from a glucose molecule by anaerobic metabolism compared to respiration. However, the same amount of energy has to be expended for protein synthesis and secretion.

especially for biogas fermenters. Isolation and thorough characterization of new cellulolytic bacteria from anaerobically decaying plant material will help greatly to develop methods for monitoring the number of cellulolytic bacteria in the fermenters. To know the key players will also help to define the optimal conditions for substrate hydrolysis and to identify the optimal bacteria for inoculating biogas fermenters with the result of an increased space time yield in addition to a better substrate utilization yield.

To identify the mechanisms underlying the extraordinarily effective hydrolysis of recalcitrant substrates such as crystalline cellulose will help to monitor the state of commercial biogas plants and to improve the yield of the process by adjusting to optimized conditions for biomass utilization. Moreover, it will give hints to improve the activity of commercially produced cellulase preparations and thus a crucial leap forward to the biotechnology of the second and third generation which intends to use cellulosic biomass as substrate.

Downstream processes in the biogas formation will have to be improved to take up the increased carbon flow from substrate hydrolysis. This could lead to improved biogas production efficiency and thus a better eco footprint as well as an improved process economy.

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