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Dynamics of biofilm formation during anaerobic digestion of organic waste

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ABSTRACT

Biofilm-based reactors are effectively used for wastewater treatment but are not common in biogas production. This study investigated biofilm dynamics on biofilm carriers incubated in batch biogas reactors at high and low organic loading rates for sludge from meat industry dissolved air flotation units. Biofilm formation and dynamics were studied using various microscopic techniques. Resulting micrographs were analysed for total cell numbers, thickness of biofilms, biofilm-covered surface area, and the area covered by extracellular polymeric substances (EPS).

Cell numbers within biofilms (10^{11} cells ml^{-1}) were up to one order of magnitude higher compared to the numbers of cells in the fluid reactor content. Further, biofilm formation and structure mainly correlated with the numbers of microorganisms present in the fluid reactor content and the organic loading. At high organic loading (45 kg VS m^{-3}), the thickness of the continuous biofilm layer ranged from 5 to 160 μm with an average of 51 μm and a median of 26 μm. Conversely, at lower organic loading (15 kg VS m^{-3}), only microcolonies were detectable. Those microcolonies increased in their frequency of occurrence during ongoing fermentation. Independently from the organic loading rate, biofilms were embedded completely in EPS within seven days. The maturation and maintenance of biofilms changed during the batch fermentation due to decreasing substrate availability. Concomitant, detachment of microorganisms within biofilms was observed simultaneously with the decrease of biogas formation.

This study demonstrates that biofilms of high cell densities can enhance digestion of organic waste and have positive effects on biogas production.

1. Introduction

The production of biogas provides a versatile carrier of renewable energy, as methane can replace fossil fuels partly in both heat and power generation and as vehicle fuel [1]. Besides technical improvements of biogas plants the efficiency of the biogas process can be further improved by engineering the microbial community.

A possible approach to improve the biogas process is the addition of biofilm carriers (e.g. plant material) to the biogas reactors. Bacteria and archaea involved in the methane production during anaerobic digestion could attach to biofilm carriers and form biofilms. Biofilms are assemblages of microorganisms, attached to a surface and encased in an extracellular polymeric substances (EPS) matrix, that functions as a cooperative consortium [2]. The structure of microbial communities ranges from monolayers of scattered single cells to thick, mucous structures of macroscopic dimensions [3].

The biofilm life cycle can be divided into three stages: the attachment of single cells to a surface, the maturation of the biofilm to complex microcolonies and the cell dispersal of highly motile planktonic cells. The biofilm mode of life is a feature common to most microorganisms in natural habitats [2]. Biofilms are ubiquitous in almost every aqueous interface, such as solid–liquid or air–liquid interfaces [4]. In most instances where biofilms are a nuisance, the term microbial fouling or biofouling is widely used [5]. For example, biofouling can be a problem in the food industry, it contributes to human infections [6] and it can lead to biocorrosion [7]. However, biofilms do not only reveal negative effects. The

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application of biofilms can be found in many anaerobic systems, especially in the disposal of organic material (e.g. in sewage treatment or biogas production). In wastewater treatment, biofilms play an important role as they create the basis of diverse aerobic and anaerobic reactors [8]. Biofilms contribute to a more efficient degradation of organic substrates and to a higher biogas or methane yield. Moreover, biofilm formation can result in a more stable degradation process. There are several explanations for these positive effects of biofilms on anaerobic digestion. Microorganisms attach to surfaces and build up complex aggregates. Thereby, the biomass increases, due to higher cell densities within the biofilms. Thus, more efficient degradation of organic substrates is shown [9,10]. For instance, Zak [9] demonstrated that the addition of a plant-based biofilm carrier improves biogas formation. The specific methane yield and the organic dry matter degradation increased by up to 7% and 10%, respectively, due to the microbial biomass on the biofilm carriers.

The biofilm mode of life offers advantages like syntrophic interactions due to the physical vicinity of microorganisms within biofilms. Syntrophism is a special case of cooperation between two metabolically different types of microorganisms, which depend on each other for degradation of a certain substrate, typically through transferral of one or more metabolic intermediate(s) between the partners [11]. Due to syntrophic interactions, the pool size of the shuttling intermediate can be kept low, resulting in an efficient cooperation [12]. Further, microorganisms attached to a biofilm carrier form an EPS matrix that offers protection. This EPS matrix provides mechanical stability and serves as a diffusion barrier [13]. The matrix entraps extracellular enzymes, and prevents the wash off of these enzymes improving the efficiency of substrate degradation [14]. The diffusion barrier also prevents the entry of harming substances into the biofilm. Thus, cells within biofilms are less strongly affected than suspended cultures from changes in environmental conditions such as temperature, pH, nutrient concentrations, metabolic products and toxic substances [15,16]. Those substances can be introduced by substrate addition or produced during anaerobic digestion [17].

The aim of this study was to investigate the dynamics of biofilm formation in respect to different organic loading rates. Therefore, biogas reactors with low and high organic loading rates were set up. Biofilm carriers were incubated in these biogas reactors and removed after certain periods to investigate the biofilms. Moreover, cell numbers within the formed biofilms and fluid reactor contents of the biogas reactors were quantified. The formation of biofilms is influenced by different factors like genotypic and physico-chemical factors [3]. Consequently, substrate composition greatly influences the biodiversity, physiology and structure of biofilms [18]. Thus, the biofilm structures were also investigated in respect to different organic loading rates. Moreover, biogas production of biogas reactors were measured and compared with biofilm formation and development of cell numbers within biofilms and fluid reactor contents.

2. Materials and methods

2.1. Experimental set up

Two lab-scale biogas reactors with different organic loadings were set up. The lab-scale biogas reactor with a high organic loading (H-OL, 12 L) was set up at Ulm University (Germany) and contained 8 L inoculum from a full-scale biogas reactor supplied with swine manure, food leftovers, stale bread, corn silage and potato peelings [19]. H-OL was fed with 2 L dissolved air flotation (DAF) sludge collected from slaughterhouse wastewater (Ulmer Fleisch GmbH, Ulm, Germany). The organic loading amounted to 45 kg VS m^{-3} (VS, volatile solids). The reactor was incubated in a water bath at 38 °C and mixed every 15 min for 3 min at 60 rpm by an agitator. During fermentation, biogas production and methane formation were measured by a Milligascounter (Dr. Ing. Ritter Apparatebau GmbH & Co. KG, Germany) and a methane sensor (BlueSens gas sensor GmbH, Germany) as described by Schropp [20].

The lab-scale biogas reactor with a low organic loading (L-OL, 0.5 L) was set up at RMIT University (Melbourne, Australia) with 0.28 L anaerobic digested sludge from a municipal wastewater treatment plant (Melbourne, Australia). L-OL was fed with 0.12 L of DAF sludge. The organic loading amounted to 15 kg VS m^{-3}. The reactor was operated at 35 °C and not mixed. Biogas production of reactor L-OL in batch experiments was measured volumetrically with a gas burette as described by Procházka et al. [21].

Special biofilm carriers made from polypropylene (PP) foil were used for microscopical analysis of biofilm characteristics. PP-discs (Ø 9 mm) were punched out of a polypropylene foil (Ø 0.5 mm) and a hole was made in the middle of each PP-disc to slide several PP-discs on a stainless steel wire (Ø 1 mm) with a length of 20 cm. One end of the wire was formed to a loop in order to fix a nylon line to hang the biofilm carrier in the reactor and to enable an easy removal. The PP-discs were rinsed with double distilled water and ethanol 70% to remove particles and were autoclaved for 20 min at 120 °C prior addition to reactors. These biofilm carriers were incubated in the biogas reactors for certain periods (H-OL: 1–7 days; L-OL: 1–28 days).

2.2. Fixation of samples

In order to determine total cell numbers, samples from fluid reactor contents (frc) were fixed according to the protocol of Daims et al. [22]. Therefore, 0.5 ml of frc was mixed with 1.5 ml paraformaldehyde solution (4%) [23]. After 4 h of fixation samples were centrifuged at 5000× *g* for 3 min, the supernatant was removed and the cell pellet washed using 2 ml of phosphate buffered saline (PBS) [23] to remove the toxic paraformaldehyde and substrate residues. This step was repeated three times. Finally, cell pellets were mixed with 0.5 ml PBS solution and 0.5 ml ethanol (100%) and stored at −20 °C.

Biofilms attached to PP-discs were removed from the reactors and fixed in FPA solution (100 ml formalin, 100 ml propionic acid, 1800 ml ethanol (70%)) for one day to ensure stable fixation. Further processings of those samples was dependent on the subsequent microscopic techniques.

2.3. Sample preparation and microscopy

2.3.1. Epifluorescence microscopy

Total cell numbers of microorganisms in frc and in biofilms attached to PP-discs were analysed by epifluorescence microscopy. Therefore, fixed cells were scratched of the PP-discs. Samples were diluted in PBS. Due to the aggregation of microorganisms the samples were homogenised by either using a RiboLyser (Hybaid Ltd., Middlesex, UK) or a grinder and sterile glass beads (Ø 0.1 mm). 20 µl of the homogeneous cell suspension was dropped onto each well of a Teflon-coated slide (8 wells, Ø 6 mm; Menzel GmbH & Co. KG, Germany) and dried for 15 min at 60 °C. In order to fix cells, the slide was pulled through the flame of a Bunsen burner for 1–2 s and further dehydrated in 50, 80 and 100% ethanol for 3 min each time. Cells were stained with 20 µl of 1 × SYBR® Gold Nucleic Acid Gel Stain (Invitrogen GmbH, USA) per well for 10 min in the dark at room temperature, flushed with cold double-distilled H_2O, and immediately dried with compressed air. Before microscopy, two drops of Citifluor™ AF1 (Citifluore Ltd., UK) were applied to the