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Mixed inoculum origin and lignocellulosic substrate type both influence the production of volatile fatty acids during acidogenic fermentation

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ABSTRACT

Volatile fatty acids (VFAs) are increasingly viewed as platform substances for the production of sustainable biofuels and green chemicals. Optimising the acidogenic fermentation requires determining which environmental parameters (temperature, pH, substrate, inoculum, etc) have the largest impact on the metabolite profile. The present study investigates the influence of substrate and mixed inoculum origin. Nine combinations of three complex lignocellulosic substrates and three different mixed inocula were monitored under batch conditions (35 °C; pH 6.5; triplicates, monitoring of pH, soluble COD and VFAs) during 30 days. All nine combinations led to a significantly different fermentation profile, and each combination represented a unique situation. The metabolite profiles were highly reproducible within each substrate–inoculum combination. The substrate COD conversion yield to VFAs was up to 64% (average 31%). VFAs represented most (between 61 and 98%) of the soluble COD. Acetic, propionic and butyric acids were always the major VFAs.

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1. Introduction

Anaerobic digestion (AD), i.e. the conversion of organic matter under anaerobic conditions, can be used to treat most liquid or solid organic wastes and residues from agricultural and agro-industrial origins [1]. The AD process takes place in four phases: hydrolysis, acidogenesis, acetogenesis and methanogenesis. Acidogenesis converts the hydrolysed substrates to short chain, volatile fatty acids (VFAs), that are usually further converted to methane. These VFAs intermediates are seen as potentially interesting platform substances in the biorefining of biowastes and residual biomass (e.g. lignocellulosic agro-industrial residues) to chemical building blocks with industrial relevance for the production of biofuels and green chemicals [2]. The production of VFAs through biomass fermentation is of interest as it provides a more sustainable solution than the use of fast diminishing petrochemicals.

Overall, one can consider that three groups of microorganisms succeed each other in the AD process: acidogenic bacteria, followed by acetogenic bacteria and finally archaeal methanogens [3,4]. The acidogenic bacteria are responsible for the fermentation of hydrolysis end products into VFAs, such as acetate, propionate, butyrate or iso-butyrate. These fermentation products, together with ethanol and lactate, are further oxidised to acetate, formate and CO_2 by the acetogenic bacteria, with concomitant transfer of electrons to produce H_2. These substances are the main substrates for the last phase methanogenesis, where methane is formed [4]. A recent meta-analysis investigating the diversity of the microorganisms that are involved in AD shows the potential role of a large number of bacteria and archaea [5]. In the same study, it is estimated that so far nearly 60% of the bacterial diversity and 90% of the archaeal diversity involved in AD have been identified.

It has been shown that AD, including hydrolysis and acidogenesis, is influenced by many parameters. These parameters are among others temperature [6–8], pH [7,9–12], organic load [13–15], substrate composition [16–20] and inoculum [21–23]. In order to optimise biomass fermentation at the industrial scale, it is necessary to determine which parameters have the largest influence.

Several studies reported the fermentation of simple substrates and with pure microbial strains. However, mixed cultures based

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on natural microbial communities with a high diversity are currently increasingly used as inocula. Such mixed inocula enable the fermentation to proceed under non-sterile conditions without the risk of strain degeneration [18]. Temudo et al. [18] compared the fermentation of several simple substrates by mixed cultures and they obtained the same catabolic products from xylose and glucose fermentation, whereas glycerol gave rise to another pattern of fermentation products. Another study by Akutsu et al. [19] tested eight different mixed inocula, and obtained similar fermentation patterns for a glycerol containing medium with all inocula. However, when they used a starchy medium, the inocula origin had a significant effect on the fermentation pattern. Moreover, another study using complex substrates such as sugar beet silage and grass/clover silage showed similar evolution of substrate hydrolysis when inoculated with one and the same mixed inoculum, whereas their microbial populations developed differently [24]. Their VFA profiles differed as well. This shows that substrate fermentation by mixed inocula is a complex topic.

The present study examined the impact of complex lignocellulosic substrate composition and mixed inoculum origin on the metabolic fermentation profile of the acidogenesis as well as the relative importance of those two parameters. It was hypothesised that if the type of substrate is the most important factor influencing the fermentation, the evolution of the VFAs over time would be reproducible regardless of the origin of the mixed inoculum. On the other hand, if the origin of the mixed inoculum is the most influencing factor, then a change of substrate would not greatly affect the fermentation profile. Special focus was put on hydrolysis efficiency and the conversion yield of the biomass substrate, in particular on the net production and consumption of VFAs. The identification of factors that mostly influence the fermentation of lignocellulosic substrates would greatly enhance the control of future biorefining processes at industrial scale.

2. Materials and methods

2.1. Substrates and inocula

Three complex lignocellulosic substrates with different lignocellulosic fibre content were used: dried sugar beet pulp (DSBP), wheat bran (WB) and distiller's dried grains with solubles (DDGS). DSBP was produced by Raffinerie Tirlemontoise after sugar extraction (Tienen, Belgium); it was mechanically pressed and then air-dried in a rotating drum drier at the sugar factory. WB was produced by Moulins de Statte (Huy, Belgium) and DDGS originated from the ethanolic fermentation of cereals produced by Alco Bio Fuel (Ghent, Belgium). Characterisation of the substrates (Table 1a) shows that they were similar in dry and volatile matter, with total solids (TS) of approximately 0.9 kg kg_{FW}^{-1} and more than 90% of the dry matter consisting of volatile solids (VS). The non soluble dry and volatile matter (TS_{ns} and VS_{ns}, respectively) were higher for DSBP than for DDGS, whereas the total chemical oxygen demand (COD_t) was lower. The soluble COD (COD_s) ranged from 1/6th of the total COD (COD_t) for WB up to 1/4th of the COD_t for DDGS. Lignocellulosic fibres constitute more than 50% of the dry matter of all substrates, with DBSP being richer in cellulose and WB being richer in hemicellulose and lignin. With respect to the most digestible fractions, DDGS is richer in proteins, DBSP in pectins and WB in starch.

Mixed inocula originated from anaerobic sludge (AS), granular sludge (GS) and acidogenic fermentation broth (AFB). The AS inoculum was produced in a semi-continuous laboratory-scale reactor maintained at 35 °C and fed weekly through the addition of concentrated fresh activated sludge at a rate of 10% of the inoculum COD_t. Fresh activated sludge was collected in the recirculation loop, after the secondary clarifier, of a waste water treatment plant (Basse-Wavre, Belgium) and concentrated by decanting for 1 day in the dark at 4 °C. The GS inoculum was obtained from a UASB reactor for the treatment of waste water from the brewery of Carlsberg (Celarevo, Serbia). The AFB inoculum was collected from the acidogenic reactor of the two-stage biomethanation plant of the farm l'Hoste (Basse-Wavre, Belgium) owned by Greenwatt S.A. (Louvain-la-Neuve, Belgium) and using maize silage as substrate. The inocula were stored in the dark at 4 °C until use. Their composition is summarised in Table 1b. The inocula varied largely in TS and VS, which were lowest for AFB and highest for GS. The TS_{ns} and VS_{ns} were larger for AS than for the other two inocula, whereas the COD_t was lower for GS. The COD of the inoculum AFB was the most soluble and that of AS the least soluble.

2.2. Experimental set up and determination of the fermentation profiles

Fermentation was conducted over 30 days for the nine possible combinations of the three complex lignocellulosic substrates and the three mixed inocula. The fermentation was performed in batch bioreactors of 250 mL (Schott Duran GL 45). The bioreactor bottle was closed with a PBT screw-cap, containing a PTFE coated silicone seal and the lateral tube was closed with a conical rubber stopper. Each bioreactor was filled with 0.2 L of a mix consisting of substrate, inoculum and water according to the proportions indicated in Table 2. The total COD in the bioreactor varied between 12.5 and 21.7 g L^{-1}, though the COD ratio of substrate and inoculum was set to approximately 4 for all conditions (Table 2). The pH of this fermentation broth was decreased to 6.5 through the addition of a known volume of 1 M HCl. Before hermetic closure of the bioreactors, the headspace was flushed for two minutes with a constant flow of nitrogen gas in order to ensure the absence of oxygen in the bioreactors. All substrate–inoculum combinations were set up in triplicate.

The bioreactors were transferred to a 35 °C incubation room and placed on a shaker orbiting at 120 rpm. The fermentation was monitored for 30 days and samples and measurements were taken on 14 occasions, i.e. at day 0, 2, 5, 7, 9, 12, 14, 16, 19, 21, 23, 26, 28 and 30, in order to determine the fermentation profile. The analysed parameters were: pH, COD_s, ethanol and volatile fatty acids (VFAs) concentration. At each sampling day, the fermentation broth was mixed with a magnetic bar and 9 mL were sampled from each bioreactor. The bioreactor pH was measured and adjusted to 6.5 with known volumes of 1 M HCl or 1 M NaOH. Sampling time was reduced as much as possible and the bioreactors headspace was flushed for two minutes with nitrogen gas before hermetic closure. The 9 mL samples were centrifuged (Beckmann Coulter TJ-25Centrifuge) at 15317× *g* for 15 min and the supernatant was filtered through 0.3 μm filters (Millipore, cellulose ester membranes) and stored at −20 °C. This filtered supernatant was used to measure the COD_s and metabolites.

2.3. Analytical methods

TS content of the various substrates and inocula was determined by drying representative samples at 105 °C until a stable mass was obtained. VS was obtained from the same samples after burning the dry mass to ashes at 550 °C for 5 h. The non-soluble total and volatile fractions (TS_{ns} and VS_{ns}, respectively) were determined after elimination of the soluble matter from the sample and represent the particle fraction of the substrates and inocula. These non soluble fractions were obtained by suspending a known sample mass in a known volume of demineralised water. The suspension was vortexed for 1 min, centrifuged at 15317× *g* for 15 min and the supernatant was eliminated. The pellet was re-suspended in distilled water and the procedure was repeated three times. The final

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