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ORIGINAL ARTICLE

Effects of acclimated sludge used as seeding material in the start-up of anaerobic digestion of glycerol

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Abstract Methods for improving the anaerobic digestion of glycerol (propane-1,2,3-triol) were investigated, particularly the effects of using acclimated sludge as seeding material during start-up. Glycerol was supplied to the anaerobic digester at an organic loading rate of 2.5 g-COD L^{-1} day^{-1}. Four experimental runs were carried out with varying mixing ratios of acclimated sludge to unacclimated sludge (0, 10, 20, and 33%). Calculations were performed by employing a numerical model, whose parameters were determined by experimental measurements. Methane production rate (MPR) for all runs attained similar stable values around 21.4 mmol L^{-1} day^{-1}, though more time was required for attaining stable state of methane production with lower mixing ratios of acclimated sludge. The initial MPR calculated was proportional to the mixing ratio of acclimated sludge. Furthermore, molecular biological methods showed that the types of microorganisms observed in all runs were similar. These results indicate that the seeding with different mixing ratios of acclimated sludge did not affect the microbial consortia in the anaerobic digestion approaching stable state, but did affect the cell density of the useful microorganisms at the start of methane fermentation. Consequently, it was confirmed that at a higher mixing ratio of acclimated sludge, the start of methane production became more vigorous.

Keywords Anaerobic digestion · Methane fermentation · Propane-1,2,3-triol · Acclimation · Numerical model

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Introduction

The growing concerns about the environmental impact and the imminent depletion of fossil fuels have led to a fast development of alternative energy, such as biodiesel [1, 2]. The production of biodiesel generates large amounts of glycerol (propane-1,2,3-triol) which is the main by-product of this process [3, 4]. This glycerol must be treated properly before disposal, and it cannot be used in other industries due to the high concentration of impurities [5]. Recently, the anaerobic digestion process has been of interest as an inexpensive and easy to implement method for the treatment of waste glycerol [6–9].

For successful methane production in anaerobic digestion, several factors, such as pH, oxidation reduction potential (ORP), temperature, organic loading rate (OLR), and hydraulic retention time (HRT), must be considered, and their impacts on the process have widely been investigated [10, 11]. In addition to these factors, the seeding material used for the process is considered to be important for successful anaerobic digestion [12–14]. Before achieving stable methane production using a specific substrate, a long lag phase is observed. In the start-up period, the microbial consortia present in the anaerobic sludge changes to adapt to the new source of carbon. This adaptation process is referred to as acclimation.

Using acclimated sludge for the start-up of anaerobic digestion processes can be advantageous in reducing the long lag time observed at the beginning of the process, as the microbial community is already adapted to the substrate. In fact, some studies have focused on the differences of using acclimated and unacclimated sludge as seeding materials. Janeczko and Oleszkiewicz studied the removal of 2-nitrophenol (ONP), which is contained in the effluents of various manufacturing processes, such as

pharmaceuticals, explosives, synthetic rubber, and leather, using both acclimated and unacclimated activated sludge [15]. In their study, they showed that the use of acclimated sludge made it possible to avoid the long lag phase that was observed in the degradation of ONP when unacclimated sludge was used. More recently, Nuchdang et al. investigated the methane yield of Para-grass using two different types of inocula. Unacclimated sludge, or original sludge (OS), was obtained from a domestic wastewater treatment plant. Acclimated sludge, or acclimated microbial consortium (AMC), consisted of sludge previously acclimated to fibrous substrates in palm oil mill effluents [16]. The authors found that the anaerobic digestion of Para-grass using AMC sludge proceeded faster and produced higher yields. This was due to higher acidogenic, acetogenic, and methanogenic activities of the AMC compared with that of the OS.

As mentioned above, the seeding of acclimated sludge was effective in reducing the lag time for the start-up of anaerobic digestion, though the mechanisms for this reduction have not been fully clarified yet. Moreover, there seems to be no previous study comparing the use of acclimated and unacclimated sludge for the degradation of glycerol. In addition, the effect of the amount of seeding material on the performance of methane production is still not clear. If the growth rate of the microorganism is sufficiently high, inoculation size does not affect the lag time required to reach a maximum stable growth level. In fact, microorganisms can increase by a factor of 8 within 1 h using a doubling time of 20 min (similar to that of *E. coli* under favorable conditions). The doubling time of microorganisms, which play an important role in anaerobic digestion, is known to be around 2 days [17]. Therefore, the amount of acclimated sludge seeded in the anaerobic digester may affect the lag time needed to reach vigorous methane production. However, to the best of our knowledge, there is no study dealing with the effects of varying the ratios of seeding with varying ratios of acclimated sludge on the start-up of the anaerobic digestion of glycerol. In this study, sludge acclimated to glycerol was mixed at different ratios with unacclimated sludge obtained from a brewery wastewater treatment plant, and the performance of the anaerobic digestion of glycerol using these mixtures as seeding material was compared.

Materials and methods

Raw sludge and preparation of acclimated sludge to glycerol

Raw granular sludge was obtained from a full-scale upflow anaerobic sludge blanket (UASB) reactor at a brewery wastewater treatment plant, located in Shizuoka prefecture, Japan. The sludge treated effluent consisting mainly of sugars, organic acids, and ethanol at concentrations of approximately 1.5 g L^{-1} COD. Granular sludge was sampled with a 500 mm diameter pipe from the UASB reactor. The granular sludge was stored at 4 °C until use in the fermentation experiments.

The acclimation process was carried out in an anaerobic sequencing batch reactor (ASBR). Raw sludge (300 cm^3) was used as the seeding material and the reactor was filled with distilled water to a volume of 3 L. The contents of the reactor were stirred at 1.7 Hz, and the temperature was maintained at 39 °C throughout the fermentation by keeping the reactor in a water bath. A new batch was started daily by first allowing the sludge to settle during 2 h, then withdrawing 750 cm^3 of liquid medium from the reactor and introducing the same volume of fresh medium. Fresh medium consisted of 4.5 g L^{-1} of $NaHCO_3$, 1.5 g L^{-1} of NH_4Cl, 0.2 g L^{-1} of $(NH_4)_2SO_4$, 0.25 g L^{-1} of K_2HPO_4, 0.3 g L^{-1} of KH_2PO_4, 0.2 g L^{-1} of $CaCl_2{\cdot}2H_2O$, 0.25 g L^{-1} of $MgSO_4{\cdot}7H_2O$, and 6.55 g L^{-1} of glycerol, the sole source of carbon. Every day, the pH of the liquid medium in the reactor was adjusted to 7.6 using 1 M sodium hydroxide. The hydraulic retention time (HRT) of the reactor was set at 4 days, and the organic loading rate (OLR) was adjusted to 2.5 g-COD L^{-1} day^{-1}.

Although not detailed here, we would like to mention that the acclimation, stable state was achieved after 76 days of the experiment, as evidenced by the absence of accumulation of VFAs, the microbial community showed no significant change, and the methane production rate reached a stable average value of 21 mmol L^{-1} day^{-1}. The reason that the relatively short time was enough to obtain a stable state in this experiment has not yet been clarified; however, it might be because the amount of sludge withdrawn daily from the reactor with the liquid sample was quite small compared with the amount of sludge remaining in the reactor (less than 0.3%). The quantity of microorganisms in the reactor sludge, therefore, was kept at a high level in spite of daily liquid sampling. The experiment was run for 97 days, to assure acclimation. This acclimation experiment was denoted as Run A. Additional details can be found in the previous paper [9].

Anaerobic digestion of glycerol using acclimated sludge

Raw granular sludge from the brewery wastewater treatment plant was mixed with acclimated sludge obtained in Run A and used as seeding material for the start-up of new experiments of glycerol anaerobic digestion. Ratios of acclimated to unacclimated sludge in the mixture of the seeding material were prepared at 10% (Run B), 20% (Run

C), and 33% (Run D). In each experiment, 300 cm^3 of seeding material was first put into an ASBR reactor that was then filled with distilled water to a volume of 3 L. The operating conditions and medium composition were the same as those detailed for the acclimation experiment in Sect. 2.1. The experiments were run for about 30 days, to evaluate the effects of seeding on the start-up of the methane production.

Numerical model to estimate the initial methane production rates

To evaluate the effects of seeding on the start-up of glycerol anaerobic digestion, the initial methane production rate (MPR_0) of the experiments was compared. Due to the daily fluctuation of MPR that is inherent to the anaerobic digestion processes, it is difficult to estimate the exact value of MPR_0 experimentally. To obtain a good estimation of the MPR_0, the logistic equation was derived by assuming that the cell density of microorganisms in the reactor increases logistically, and that the MPR is directly proportional to the cell density of the microorganisms. The MPR can thus be expressed by

$$\mathrm{MPR}\ (t) = \frac{K}{1 + \left(\frac{K}{\mathrm{MPRo}} - 1\right) \exp^{-rt}},$$

where K, r, and MPR_0 are parameters defined as the MPR attained at the stable state, the degree of MPR increment, and the initial methane production rate, respectively. To determine MPR_0 more accurately, the integral was taken, thus avoiding errors from MPR fluctuations. Setting the calculated cumulative methane production (CMP) equal to the integral allowed for estimation of the three parameters:

$$\begin{aligned}\mathrm{CMP}\ (t) &= \int_0^t \mathrm{MPR}\ (t)\ \mathrm{d}t \\ &= \int_0^t \frac{K}{1 + \left(\frac{K}{\mathrm{MPRo}} - 1\right) \exp^{-rt}} \mathrm{d}t.\end{aligned}$$

Assuming that K and r were the same for all runs led to six unknowns in total, including MPR_0 for each run. The values were determined using MATLAB to minimize the mean squared error (MSE) between the measured and calculated CMP.

Analytical methods

Samples of the liquid medium in the reactor were withdrawn daily. The pH and oxidation reduction potential (ORP) values were determined with a pH electrode (pH-3P, Mettler Toledo, Greifensee, Switzerland) and an ORP meter (FPH92, Tokyo Garasu Kikai Co., Ltd., Tokyo, Japan), respectively. Gas samples stored in Tedlar bags (Tedlar bag, Omi Odoair Service Co., Ltd., Tokyo, Japan) were analyzed by chromatography-mass spectrometry (GC–MS). The GC–MS used was a Shimadzu QP-5050A equipped with a CP7348 CP-PoraBOND Q column maintained at 40 °C with flow of 1 $cm^3\ min^{-1}$. The volume of produced gas was measured using a dry test gas meter (DC-1, Shinagawa Corporation, Tokyo, Japan). Glycerol and volatile fatty acids (VFA) concentrations were measured by a high-pressure liquid chromatography (HPLC) system equipped with an L-3300 RI monitor (HITACHI, Tokyo, Japan) and a SUGAR SH 1011 column (Shodex, Tokyo, Japan) maintained at 40 °C, with 5 mM H_2SO_4 as the mobile phase and a flow rate of 0.9 $cm^3\ min^{-1}$.

The microbial consortium in the experiments was analyzed by the PCR-DGGE method. Samples of granular sludge were taken directly from the reactor. DNA was extracted from 0.2 g (wet weight) of granular sludge using an ISOIL for Beads Beating kit (Nippon Gene Co. Ltd., Toyama, Japan). PCR was performed using a TaKaRa ex Taqkit and an automated thermal cycler (PCR thermal cycler dice system, TaKaRa, Shiga, Japan). Primers that amplify the 16S rRNA gene of bacteria and archaea consortia were reported in a previous paper [18]. Denaturing gradient gel electrophoresis (DGGE) was carried out using D-code DGGE Complete System (BioRad Laboratories, CA, USA). The PCR product was mixed with an equal volume of 2X gel loading dye (10 mM Tris–HCl at pH 8.0, 20 mM EDTA at pH 8.0, 0.05% [w/v] bromophenol blue, and 70% glycerol) and loaded onto a 10% (w/v) polyacrylamide gel in a 1X TAE buffer (40 mM Tris–acetate at pH 7.4; 20 mM acetate, and 1 mM Na_2EDTA) with a denaturing gradient ranging from 30 to 60%.

Results and discussion

Methane production and volatile fatty acid concentration

Figure 1 shows the courses of CMP for all runs; A through D. Anaerobic digestion was continued for 97 days in Run A; however, the results of only the first 30 days are shown in this figure for comparison with other experimental runs. CMP was strongly dependent on the mixing ratio of the acclimated sludge, and the methane production was accelerated by the addition of acclimated sludge. In Run D, the curve for CMP became almost linear after $\sim$10 days of anaerobic digestion, indicating that the MPR became stable at a value of $\sim$21 mmol $L^{-1}\ day^{-1}$. By contrast, Run A showed a downward convex curve for the CMP. This seems to suggest that the MPR was still increasing at

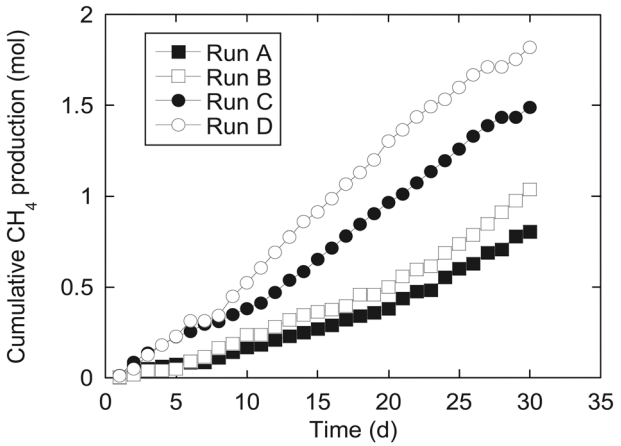

Fig. 1 Time series of cumulative methane production during the experiments. *Filled square* Run A, *unfilled square* Run B, *filled circle* Run C, *unfilled circle* Run D

day 30 of Run A. Overall, the higher the mixing ratio, the larger the CMP became, thus increasing the methane production rate.

Figure 2 compares the courses of concentration of each VFA constituent and the total VFA for all runs. In Run D, the accumulation of VFA was low, with a maximum of 0.3 g L^{-1} total VFA. Methane production was also vigorous from the early stages of Run D (cf. Figure 1). In Run C, higher concentrations of VFA accumulated than those in Run D, reaching a maximum total concentration of 0.5 g L^{-1}. Among all VFA constituents, acetic acid tended to be at the highest concentration during Run C. In Run B, the accumulation of VFA in the early stage of the experiment was even higher than those in Run C, with a maximum total concentration of almost 1 g L^{-1}. In Run A, the concentration of total VFA reached a maximum value of 1.8 g L^{-1} in the early stages of the experiment, with propionic acid dominating throughout the run. No significant accumulation of VFA, however, was observed after day 25 in any of the experiments. In Runs A and B, clear improvement of the methane production was observed after the VFA concentration decreased (cf. Figure 1). These results confirmed that the experiments with larger methane production accumulated lower concentrations of VFA during the first days of the experiment.

In anaerobic digestion, there are two main steps: a given substrate is first transformed to VFA by bacteria, and then the VFA is successively transformed by bacteria and

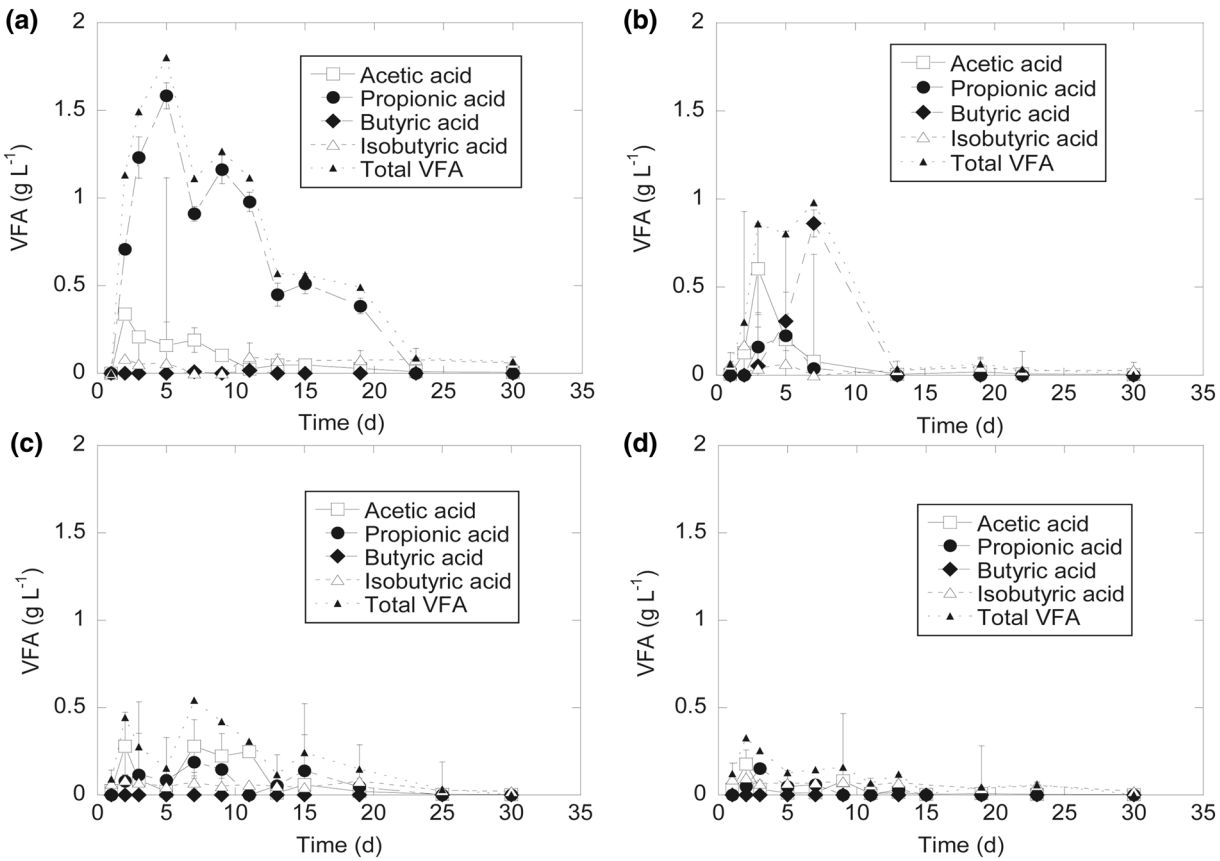

Fig. 2 Time series of VFA concentrations in the liquid medium during the experiments. The *error bar* in each symbol indicates the 95% confidence interval ($n = 3$). **a** Run A. **b** Run B. **c** Run C. **d** Run D. *Unfilled square* acetic acid, *filled circle* propionic acid, *filled diamond* butyric acid, *unfilled triangle* isobutyric acid, *filled triangle* total VFA

archaea to give CH_4 and CO_2 as final products [19]. The accumulation of VFA in the early stages of the experiments indicates an overload of glycerol to the microorganisms that are not yet adapted. In such a situation, the production rate of the VFA was faster than their consumption rate. The results observed in Fig. 2 show that a higher ratio of acclimated sludge in the seeding material leads to a lower accumulation of VFA in the early stage of the experiment. These results suggest that a higher ratio of acclimated sludge more quickly adapts to the overload of the glycerol, and has a better balance between the production and consumption rates of VFA.

Relation between the initial MPR and the mixing ratio of acclimated sludge

Figure 3 compares the calculated and measured CMP values throughout all runs of the experiment. The comparison was made for 97 days in Run A, and for 30 days in Runs B, C, and D. The six parameters (K, r, and MPR_0 for each run) determined by curve fitting using MATLAB are shown in Table 1. The calculated results coincided well with the measurements throughout the experiment of all runs. The K value corresponding to the MPR attained at the stable state was 21.4 mmol L^{-1} day^{-1}, which was similar to that obtained from the slope of the CMP curve in the latest stage of Run D. The r value, which corresponds to the degree of MPR increment, was 0.072 day^{-1}. If this value is large, the MPR becomes stable rapidly, indicating that the r value reflects the growth of microorganisms contributing to the methane production. MPR_0 was 3.9, 4.9, 12.4, and 19.9 mmol L^{-1} day^{-1} for Run A, B, C, and D,

Table 1 Parameters of the logistic equation used for the estimation of the initial MPR in Runs A through D

Run	Parameters of logistic equation		
	r (day^{-1})	MPRo (mmol L^{-1} day^{-1})	K (mmol L^{-1} day^{-1})
A	0.072	3.9	21.4
B	0.072	4.9	21.4
C	0.072	12.4	21.4
D	0.072	19.9	21.4

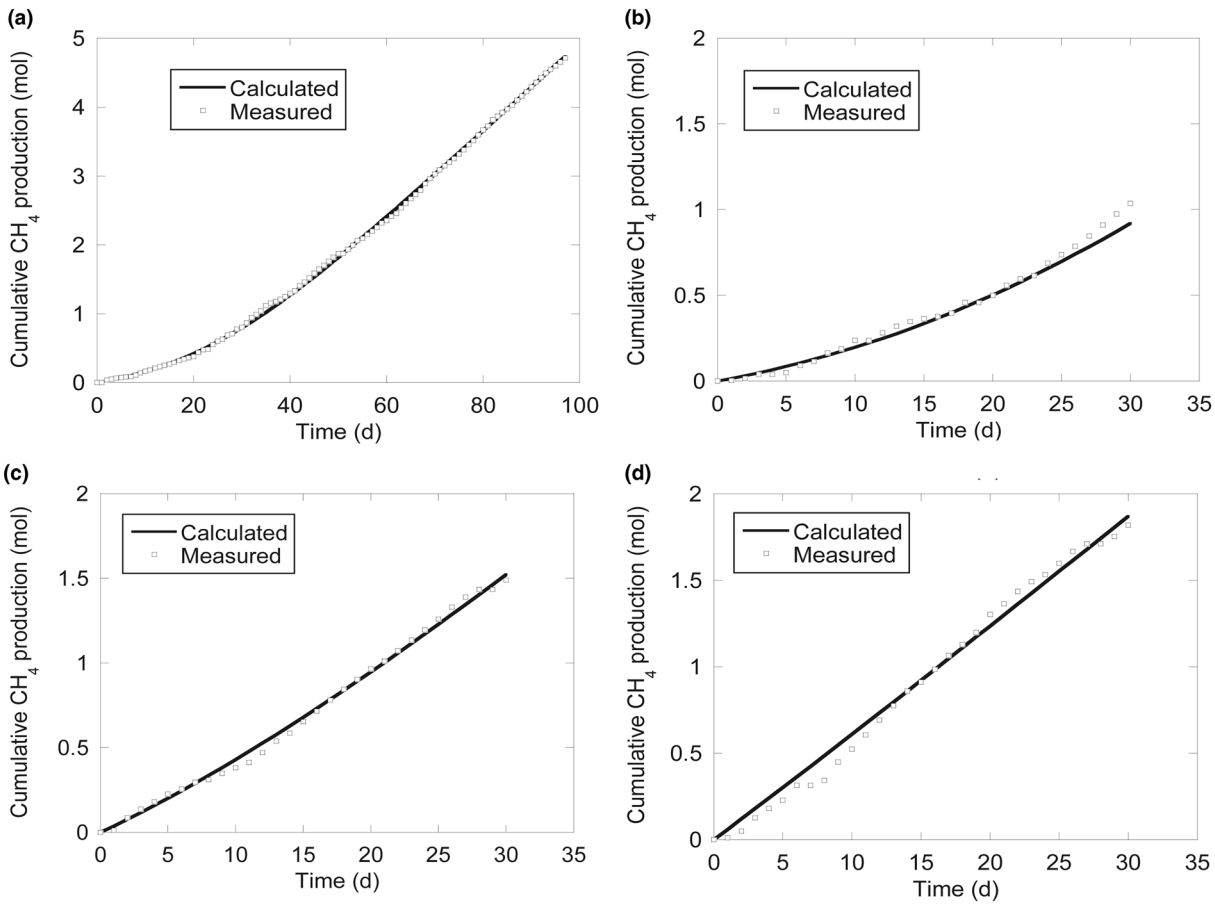

Fig. 3 Time series comparison of calculated and experimentally measured values of the cumulative methane production (CMP) during the experiments. **a** Run A. **b** Run B. **c** Run C. **d** Run D. *Unfilled square* measured, *solid line* Calculated

respectively. The larger the mixing ratio of the acclimated sludge, the higher the MPR_0 value became.

Figure 4 indicates the relationship between the mixing ratio of acclimated sludge and the MPR_0 obtained from curve fitting. A strong correlation was observed between the MPR_0 and the mixing ratio of acclimated sludge. This further supports the hypothesis that the anaerobic digestion process is highly dependent on the amount of the seeding material. Moreover, the proportionality shown in this relationship suggests that the increase of the mixing ratio of acclimated sludge will increase the density of microorganisms that have high activity and are useful for the anaerobic digestion of glycerol.

As mentioned above, final value of MPR was estimated to be the same for all runs, at 21.4 mmol L^{-1} day^{-1}. To compare the lag time to attain MPR, greater than 21.0 mmol L^{-1} day^{-1} (a rather arbitrary choice) for each run was calculated. The calculated time was day 76, 72, 51, and 20, for Run A, B, C, and D, respectively, indicating that the larger the amount of acclimated sludge used as a seeding material, the shorter the lag time for attaining the same vigorous methane production.

Clear evidence for the positive effects of seeding on glycerol digestion was observed. It is known that seeding is not generally effective in accelerating aerobic digestion, and thus the positive effects of seeding are a characteristic feature of anaerobic digestion systems. In many cases of composting, inoculation with external microorganisms was not effective in accelerating the composting [20]. This may be attributed to the microorganisms that play an important role in making the composting process self-sufficient; the microorganisms can grow very fast and attain high cell density only after inhabiting the raw material, even at low cell density (though there are some exceptions). Inoculation with specific microorganisms was effective, however, when they were introduced into the raw composting material of specific organic wastes, such as organic acid [21], furans [22], and biodegradable plastic [23]. In these cases, the microorganisms typically present in the raw material do not possess the ability to degrade and utilize these substrates.

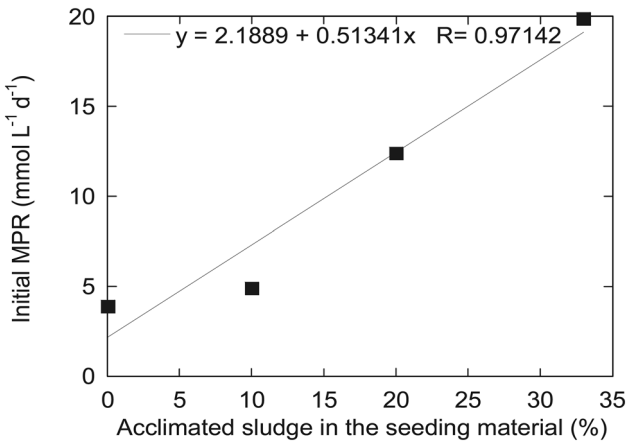

Fig. 4 Relation between the initial methane production rate (MPR_0) and the mixing ratio of acclimated sludge. *Filled square* MPR_0 for the experiments, *solid line* trend line

Microbial consortia

Figure 5 shows the PCR-DGGE fingerprints for bacteria and archaea taken at the end of Runs A, B, C, and D. The microorganisms that were dominant in the microbial consortium, ensuring anaerobic digestion of glycerol in Run A, were identified previously [9]. The dominant bacteria in the acclimated microbial consortium were closely related to an uncultured bacterium clone PEU-90, *Alkalibacter saccharofermentas* strain Z79820, *Garciela nitratireducens* strain Met 79, and *Mesotoga infera* strain VNs100 that were identified as microorganisms B1, B9, B10, and B11, respectively. The dominant archaea in the microbial consortium were mainly related to *Methanobacterium kanagiense, Methanosaeta harundinacea* 6Ac, and *Methanobacterium spp.* These archaea were identified as the microorganisms corresponding to bands A4, A5, and A6, respectively.

As shown in Fig. 5, the microbial consortium at the end of the experimental runs was very similar, though small differences in the intensity of some bands were observed. Different microorganisms could feasibly become dominant after acclimation, if seeding material from different sources was used. Indeed, several studies have shown that using different seeding material can lead to different performances of the anaerobic digestion process [10–12], and that using seeding material from different sources can lead to the development of very different microbial consortia at the end of the processes [14].

In this study, there was a possibility that different microorganisms became dominant because of the varying ratios of acclimated sludge, evidenced by the composition and concentration of the intermediate VFA. Since the VFA accumulation was affected by the mixing ratio of acclimated sludge, the composition of VFA subsequently affected the growth of microorganisms. It is still unclear why the same microorganisms were dominant by the end of each run, irrespective of the different ratio of acclimated sludge seeding. One possibility is that the operational conditions of this study enhanced the growth of microorganisms that were already dominant in the acclimated sludge from Run A. The inoculation of acclimated sludge as a seeding would then accelerate the growth of the same microorganisms. Even so, the fact that all the experiments attained similar microbial consortia and were able to produce methane vigorously from glycerol confirms that the

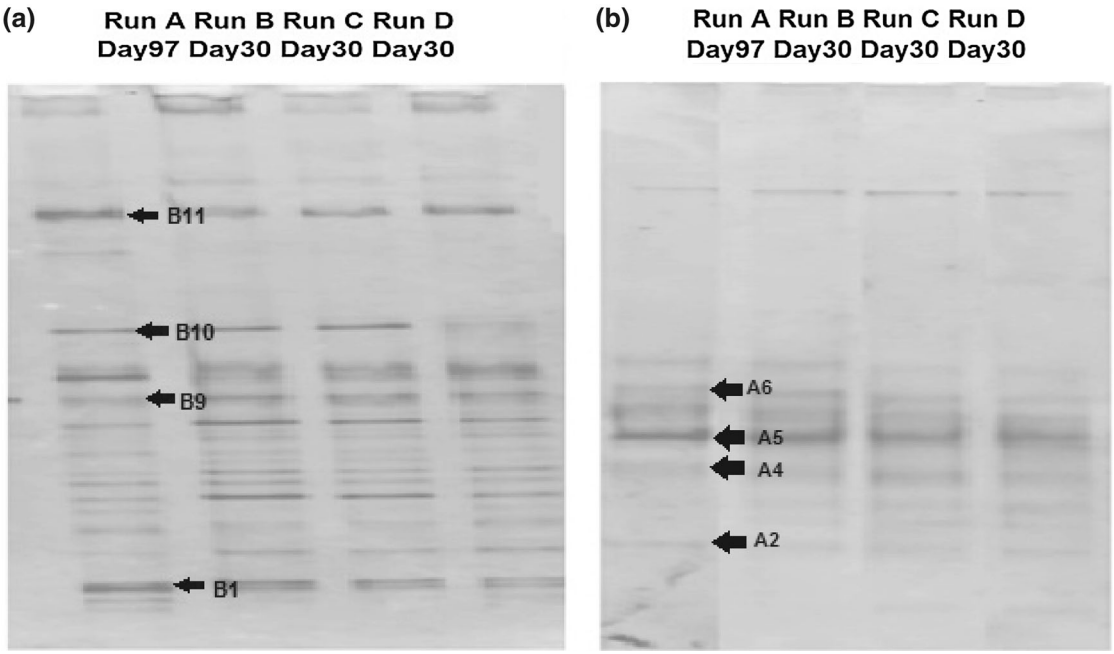

Fig. 5 PCR-DGGE fingerprints at the end of the experiments. **a** Bacteria. **b** Archaea

microorganisms of such consortia were important for the anaerobic digestion of glycerol.

Conclusions

The anaerobic digestion of glycerol was successfully accelerated using acclimated sludge in the seeding material. The experimental results were explained satisfactorily by the numerical model based on a logistic equation, which was then used for the estimation of the MPR_0 and lag time needed before attaining vigorous methane production. The MPR_0 obtained for the different experiments was proportional to the mixing ratio of acclimated sludge. A higher mixing ratio of acclimated sludge in the seeding material allows for a faster improvement in the methane production and also prevents the accumulation of VFA in the medium. The larger the amount of acclimated sludge in the seeding material, the shorter the lag time became.

The most important aspect of the present research was the quantification of the effects of using various mixing ratios of acclimated sludge for seeding material on the performance of anaerobic digestion. Specifically, the effects on lag time, and initial and stable methane production rates, were shown quantitatively. Furthermore, the microbial consortia developed in all runs were similar. These results indicate that the seeding using different mixing ratios of acclimated sludge did not affect the microbial consortium in the anaerobic digestion, once near a stable state. The cell density was affected, however, with the acclimated sludge providing useful microorganisms at the start of anaerobic digestion. These results will help the operators of anaerobic digesters, and the start of a new anaerobic digestion process can be accelerated by introducing acclimated sludge from another source, such as another wastewater treatment plant treating a similar organic matter or a facility that provides acclimated sludge. In these cases, methane production can be started immediately after addition of the acclimated sludge by eliminating the long lag time usually required for sludge acclimation.

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