Bioaugmentation of Anaerobic Sludge with *Syntrophomonas zehnderi* as a Prospect for Enhanced Methane Production from Oleate

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Abstract
Bioaugmentation of anaerobic sludge with long-chain fatty acids (LCFA)-degrading bacteria can be a feasible strategy to enhance methane production from LCFA. This hypothesis was studied in batch assays with *Syntrophomonas zehnderi* as bioaugmenting strain. This bacterium is able to degrade a wide range of saturated and unsaturated LCFA, and its presence has been reported in several oleate-fed bioreactors. Bioaugmentation assays were performed in the presence and absence of the solid microcarrier sepiolite, and with oleate (unsaturated C18:1 LCFA, 1 mM) as substrate. *S. zehnderi* addition enhanced methane production from oleate, leading to high methane yields in the bioaugmented assays, i.e. 89±5% and 72±1% in the absence and presence of sepiolite, respectively. Comparing bioaugmented assays, a two times faster methane production was observed in the presence of sepiolite, where maximum cumulative methane production was attained after 15 days of incubation. Also, acetate accumulation was prevented by the microcarrier, probably due to a decrease of acetoclastic methanogens inhibition by oleate. Bioaugmentation of anaerobic bioreactors with *S. zehnderi* or other syntrophic LCFA-degraders can be potentially useful for faster reactor start-up or recovery of LCFA-inhibited processes.

Keywords
Bioaugmentation, LCFA, methane, sepiolite, *Syntrophomonas zehnderi*

INTRODUCTION

The introduction of indigenous or allochthonous microorganisms into natural or engineered environments, either singly or in the form of consortia, is known as bioaugmentation. This technique has been frequently applied to fasten the removal of undesired compounds, to improve the performance of on-going biological processes, and to ease the establishment of specific populations in microbial communities (Limbergen et al.,1998; Bouchez et al.,2000; El Fantroussi and Agathos,2005). Successful bioaugmentation of anaerobic waste and wastewater-treating processes with pure or mixed cultures has been reported (Table 1).

Cirne et al. (2006) studied the effects of bioaugmentation as a means of improving the hydrolysis and solubilisation of lipids. Batch assays with a model waste containing 10 % triolein were bioaugmented with *Clostridium lundense*, a lipolytic strain isolated from bovine rumen. The hydrolysis of the lipid fraction was improved, but LCFA degradation appeared to be the limiting step in the complete substrate conversion to methane.

In anaerobic bioreactors, complete LCFA degradation evolves through the coordinated activity of syntrophic bacteria, which convert LCFA to acetate and hydrogen, and methanogenic archaea that utilize these substrates, making the overall conversion energetically possible (Schink,1997).

Table 1. Examples of bioaugmentation studies in anaerobic bioreactors

Bioreactor	Microorganism(s)	Type of waste/wastewater	Ref.
UASBR	*Desulfomonile tiedjei* (as a pure culture and in co-culture)	3-Chlorobenzoate	[1]
UASBR	*Desulfitobacterium hafniense* strain DCB-2	Pentachlorophenol	[2]
UASBR	*Dehalospirillum multivorans*	Tetrachloroethene	[3]
UASBR	Phenol, *o*- and *p*-cresol degrading enriched microbial consortium	Phenolic compounds	[4]
AnSBBR	Enriched culture of sulphate reducing bacteria (immobilized in alginate beds)	Sulphate-rich wastewater	[5]
Batch	*Clostridium lundense* (DSM 17049^T)	Restaurant lipid-rich waste	[6]
Batch	*Azoarcus* sp. strain DN11	Benzene contaminated groundwater	[7]
Batch	*Caldicellulosiruptor lactoaceticus* or *Dictyoglomus* sp.	Cattle manure	[8]
Two stage CSTR	*Caldicellulosiruptor lactoaceticus*	Cattle manure	[8]
Batch	*Ralstonia* sp. HM-1	Cd and Zn contaminated sediment	[9]
UASBR	*Sulfurospirillum barnesii* (immobilized in polyacrylamide gels)	Selenate and nitrate or sulphate	[10]

[1] (Ahring et al.,1992). [2] (Christiansen and Ahring,1996). [3] (Hörber et al.,1998). [4] (Hajji et al.,2000). [5] (Mohan et al.,2005). [6] (Cirne et al.,2006). [7] (Kasai et al.,2007). [8] (Nielsen et al.,2007). [9] (Park et al.,2008). [10] (Lenz et al.,2009). AnSBBR – anaerobic sequencing batch biofilm reactor. UASBR – upflow anaerobic sludge blanket reactor. CSTR – continuous stirred tank reactor.

Presently, there are 7 species of syntrophic bacteria reported as capable of growing on LCFA with more than 12 carbon atoms. Among these bacteria only 4 can utilize unsaturated LCFA, namely *Syntrophomonas sapovorans*, *S. curvata*, *S. zehnderi* and *Thermosyntropha lipolytica* (Sousa et al.,2009). *S. zehnderi* is able to degrade a wide range of saturated and unsaturated fatty acids (with 4 to 18 carbon atoms), which makes it a suitable candidate for using as bioaugmenting strain in LCFA degradation (Sousa et al.,2007c).

The changes in bacterial communities during anaerobic continuous and fed-batch reactors operation with oleate was followed with denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA genes by Sousa (2006). A predominant DGGE-band was identified in all the DGGE profiles and 16S rRNA gene sequences retrieved from the different sludges, and corresponding to this position in the DGGE profiles, where found to be closely related to *Syntrophomonas zehnderi* (99% identity).

Low relative abundance (0.01 to 3%) of syntrophic bacteria has been reported in anaerobic bioreactors (Hansen et al.,1999; Menes and Travers,2006), but extended contact with LCFA appears to stimulate the occurrence of this acetogenic microorganisms (Sousa et al.,2007a).

Thus, the addition of *S. zehnderi* or other LCFA-degrading bacteria to anaerobic sludge can potentially be used to accelerate bioreactors' start-up phase, which is generally time-consuming, or

promote the recovery of disrupted treatment processes when LCFA accumulation/adsorption onto the biomass could not be prevented.

In this work, the potential for improving methane production from LCFA was studied by bioaugmenting anaerobic sludge with *S. zehnderi*. Batch assays were performed with and without the solid microcarrier sepiolite, using 1 mM oleate as sole carbon and energy source. Methane, volatile fatty acids (VFA) and LCFA were monitored throughout the experiment.

METHODS

Source of biomass and mineral media

S. zehnderi DSM 17840T (own culture collection) and *Methanobacterium formicicum* DSM 1535T obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) were used in this study. Non-acclimated granular sludge was collected from a lab-scale reactor treating winery effluent (Santiago de Compostela, Spain). Preparation of the bioaugmenting culture and bioaugmentation assays were performed with a bicarbonate-buffered mineral salt medium made as described by Sousa et al. (2007b).

Preparation of the bioaugmenting culture

S. zehnderi was pre-grown in co-culture with *M. formicicum* in the presence of sodium oleate (≥ 99 %, Fluka). Incubation was performed with 3x 0.5 mM oleate (successive substrate additions) at 37 °C, statically and in the dark. Oleate was added to the medium from a sterile stock solution. Inoculation of *S. zehnderi* and *M. formicicum* active cultures was performed aseptically using sterile syringes and needles. The bioaugmenting culture was centrifuged (1600 *g*, 10 min, 4 ºC) and washed twice with anaerobic medium prior the bioaugmentation assays. Co-culture was distributed in two bottles under N_2 atmosphere and one of this bottles was heat treated (121 ºC, 40 min, 2x) in order to inactivate the culture.

Microcarrier characterization

Sepiolite was the solid microcarrier selected for this work based on the reports of Alves et al. (1999) and Sanchez et al. (1994). Chemically sepiolite is a hydrated magnesium silicate with general formula $Si_{12}Mg_8O_{30}(OH_2)_4(OH)_4 \cdot 8H_2O$. A wet grain density of 1.64±0.02 g cm^{-3} was determined by water picnometry for the calcinated sepiolite. Average values of equivalent diameter (0.64 mm), area (0.35 mm^2) and perimeter (2.34 mm) were determined by image analysis, following the procedure described by Abreu et al. (2007). Before addition to the bottles, sepiolite was submitted to calcination at 550 ºC for 2 hours, washed with distilled water (3x) and sterilized in autoclave (121 ºC, 20 min).

Bioaugmentation assays

Bioaugmentation of anaerobic sludge with the pre-grown *S. zehnderi* co-culture was done in bottles with and without sepiolite. Medium was amended with sludge and *S. zehnderi* co-culture to final concentrations of 13 % and 0.3 % (w/v), respectively. Inactivated *S. zehnderi* co-culture was used in non-bioaugmented controls. In assays with microcarrier 5 g (dry weight) of sepiolite were used. Bottles were incubated with 1 mM sodium oleate at 37 ºC, statically and in the dark. Blank assays containing no oleate were also performed. In order to warrant uniform hydrogenotrophic activity in bioaugmented and non-bioaugmented assays, 5 % (v/v) of an active culture of *M. formicicum* was added to all the bottles. Assays were performed in triplicate. Methane concentration in the headspace of the bottles and VFA in the liquid medium were monitored during the experiment. LCFA were quantified at the end of the assays. Methane yield was calculated as the ratio between

methane concentration measured in bottles' headspace and the theoretical stoichiometric value for complete conversion of 1 mM oleate (i.e. 12.75 mM CH_4).

Effect of oleate on acetoclastic methanogenesis in bioaugmented assays

Bottles with anaerobic sludge and *S. zehnderi* were supplemented with 16 mM acetate (sodium salt ≥ 99 %, Sigma) and 16 mM acetate plus 1 mM oleate. Experimental procedure and monitoring was similar to the described in the previous section. Assays were performed in the absence and presence of sepiolite.

Analytical methods

Methane was measured using a Pye Unicam GC-TCD gas chromatograph and VFA were analyzed by HPLC (Jasco, Japan), as described by Cavaleiro et al. (2009). Mixed liquid and solid phases (cells and microcarrier) withdrawn from the bottles at the end of the experiment were analyzed for total LCFA. Bottles with microcarrier were sonicated for 30 minutes prior sampling. Saturated and unsaturated LCFA were extracted and quantified as previously described by Neves et al. (2009). Esterification of free fatty acids was performed with propanol, in acid medium (3.5 hours at 100 ºC). Propyl esters were further extracted with dichloromethane and analyzed in a gas chromatograph (Varian 3800) equipped with a flame ionization detector and a eq.CP-Sil 52 CB 30 m x 0.32 mm x 0.25 μm capillary column (Teknokroma, TR-WAX).

RESULTS AND DISCUSSION

In anaerobic environments, lipids are easily hydrolyzed to glycerol and LCFA (Hanaki et al.,1981; Pavlostathis and Giraldo-Gomez,1991), but further conversion of these substrates is necessary to maximize methane production. However, LCFA accumulation during the degradation of lipid-rich wastes/wastewaters is well documented (Broughton et al.,1998; Pereira et al.,2002; Cavaleiro et al.,2009). LCFA accumulation has been ascribed to low syntrophic activity in mixed culture systems (Cirne et al.,2006). In this work, we show that bioaugmentation of anaerobic sludge with syntrophic microorganisms can be potentially used to circumvent intermediates accumulation during lipids/LCFA conversion and enhance methane production from these substrates.

Methane production from oleate by bioaugmented and non-bioaugmented sludges, in the absence and presence of sepiolite, is shown in Figure 1. Methane yields are presented in Table 2. Bioaugmentation with *S. zehnderi* enhanced methane production from oleate, leading to high methane yields, i.e. 89±5 % and 72±1 % in the absence and presence of sepiolite, respectively. Moreover, *S. zehnderi* addition to granular sludge resulted in a faster oleate conversion to methane preventing the lag phase observed in non-biaugmented assays (Figure 1).

Comparing bioaugmented assays, a two times faster methane production was observed in the presence of sepiolite, where maximum cumulative methane production was attained after 15 days of incubation (Figure 1; Table 2). Acetate accumulated in the medium only in the absence of sepiolite, reaching a maximum concentration of 5 mM (data not shown). Differences in acetoclastic activity could be the reason for faster oleate to methane conversion in the bioaugmented assay with sepiolite.

Methane production from oleate is dependent on syntrophic relations between acetogenic bacteria and methanogenic archaea (Schink,1997). Successful bioaugmentation is intrinsically dependent on hydrogenotrophic and acetoclastic activities. From these two archaeal groups, acetoclastic methanogens are described as particularly sensitive to LCFA inhibition (Hanaki et al.,1981; Hwu and Lettinga,1997; Shin et al.,2003) and, therefore, the effect of oleate on acetoclastic

methanogenesis was studied using bioaugmented sludge.

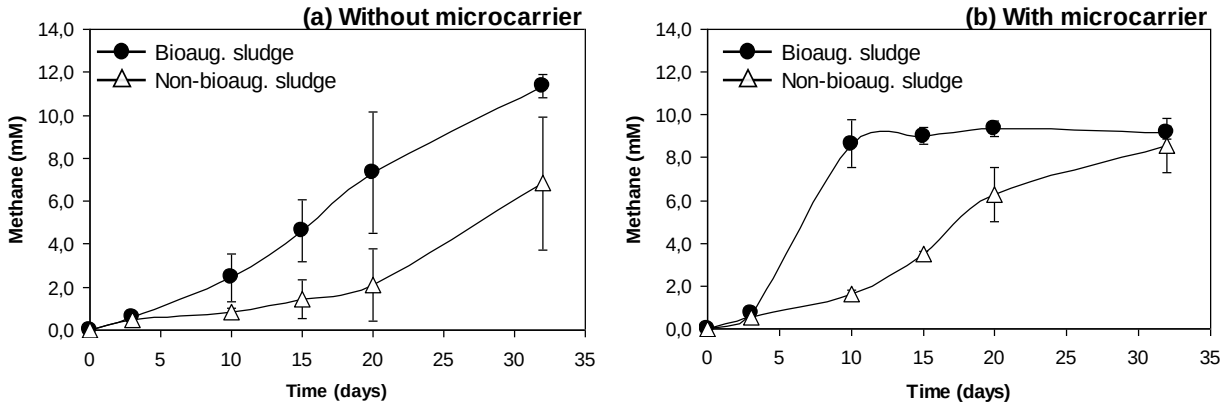

Figure 1. Methane production in the assays (a) without microcarrier and (b) with microcarrier. (●) Bioaugmented sludge; (△) non-bioaugmented sludge.

Table 2: Methane yields (%) in bioaugmented and non-bioaugmented assays

	Without sepiolite		**With sepiolite**	
	t = 15 days	**t = 32 days**	**t = 15 days**	**t = 32 days**
Bioaugmented sludge	36 ± 12	89 ± 5	72 ± 1	72 ± 1
Non-bioaugmented sludge	11 ± 7	54 ± 24	27 ± 1	67 ± 10

A positive effect of sepiolite in methane production from acetate has been previously reported by Sanchez et al. (1994). These authors observed an increase in acetoclastic methanogens numbers in anaerobic sludge incubated with acetate in the presence of sepiolite. In the present study, acetoclastic activity of bioaugmented sludges in the absence and presence of sepiolite, measured through incubation with 16 mM acetate (no oleate added), did not differ significantly. Methane production rates obtained in assays in the absence and presence of sepiolite were similar (i.e. 2.33±0.33 and 2.05±0.24 mM CH_4 day^{-1}, respectively; Table 3). However, when 1 mM oleate was added together with 16 mM acetate, a positive effect of sepiolite in the overall methane production rate was evident. Oleate strongly affected acetoclastic activity in assays without sepiolite lowering methane production rates to 0.69±0.08 mM CH_4 day^{-1} (which corresponds to only 26 % of the methane production rate measured in the presence of sepiolite). The presence of sepiolite seems to prevent acetoclastic activity inhibition as methane production rate was high, i.e. 2.65±0.21 mM CH_4 day^{-1} (Table 3).

Table 3: Effect of oleate addition on methanogenic acetoclastic activity (mM CH_4 day^{-1}) in bioaugmented assays

Oleate concentration (mM)	**Without sepiolite**	**With sepiolite**
0	2.33 ± 0.33	2.05 ± 0.24
1	0.69 ± 0.08	2.65 ± 0.21

This effect might be due to a release of Mg^{2+} ions from the sepiolite to the liquid medium causing the precipitation of oleate in the form of magnesium dioleate. Precipitation of LCFA with divalent ions, such as Ca^{2+} and Mg^{2+}, has been previously used in anaerobic bioreactors as a means of reducing its toxicity (Hanaki et al.,1981; Roy et al.,1985). Adsorption of LCFA on sepiolite surface is also plausible, as this carrier is a good adsorbent for polar molecules (Özdemir et al.,2007).

LCFA bioavailability in the medium can be reduced by this physical mechanism which potentially decreases toxicity effects.

Cell proximity has been referred as a key factor in syntrophic methanogenesis (Stams and Plugge,2009). The use of microcarriers might facilitate interspecies metabolite exchange, enhancing the cooperation between the acetogenic bacteria and the methanogenic archaea. Bioaugmentation assays with sepiolite might also have beneficiated of this effect.

The potential of *S. zehnderi* as bioaugmenting strain during LCFA degradations in the presence of other microcarriers, namely a zeolite, is already being tested in the research group. Also, continuous and sequencing batch experiments are on-going.

CONCLUSIONS

The potential for improving methane production from oleate by bioaugmenting anaerobic sludge with *S. zehnderi* was demonstrated. Higher methane yields were attained in the bioaugmented assays, and a faster methane production was recorded in the presence of sepiolite. The positive effect of sepiolite in oleate to methane conversion might be related with a decrease in LCFA toxicity or/and a potential improvement in metabolites transfer between acetogens and methanogens. Bioaugmentation of anaerobic bioreactors with *S. zehnderi* or other syntrophic LCFA-degraders can be potentially useful for faster reactor start-up or recovery of an LCFA-inhibited bioreactor

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