Metabolism of Long-Chain Fatty Acids (LCFAs) in Methanogenesis

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Abstract

Anaerobic digestion (AD) is an important process for generating third-generation fuel in the form of methane from a variety of organic wastes. Efficiency of AD is dependent on inhibitory effect exerted by the substrate on methanogenic pathway. Here we assess the potential of utilizing lipid-rich waste as a suitable substrate for methane production. Anaerobic digestion of lipids leads to production of long-chain fatty acids (LCFAs) which are known to inhibit acetoclastic methanogens. The problems faced during AD of lipid-rich waste, strategies for overcoming the problems, and application of genomic tools for characterization of microbial community involved in biomethanation of this substrate are also discussed.

16.1 Introduction

Anaerobic digestion (AD) is a process of decomposition of organic matter by mixed microbial inoculum in the absence of oxygen in four steps: hydrolysis, acidogenesis, acetogenesis, and methanogenesis. Among various substrates, significant amount of lipid-rich waste produced each year from different industries such as food processing, dairy, olive oil mills, slaughterhouses, and edible oil processing can serve as alternative source for anaerobic conversion to methane. The utilization of lipid-rich wastes as suitable substrates for biomethane production, the associated problems which negatively affect the AD process, and the methods for overcoming these problems have been reported by many researchers (Table 16.1). Wastes from these industries contain fats, oil, and grease (FOG) which together represent the lipid-rich layer. The tendency of FOG layer to float on the surface of water leads to clogging within the discharge pipes due to its accumulation. In anaerobic treatment systems, hydrolysis of such lipids by extracellular lipases leads to formation of glycerol along with long-chain fatty acids (LCFAs), followed by β-oxidation resulting in formation of acetate and hydrogen. LCFAs in FOG are mainly of saturated (37–48 %) and unsaturated (51–58 %) nature whose reduced state makes FOG an ideal

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Table 16.1 Methane production from various sources of long chain fatty acids (LCFAs)

Lipid-rich waste	Lipid/LCFA composition/conc	HRT/strategy of operation	Time	Lag phase	Methane/biogas potential	Methane/biogas yield	Inhibitory conc.	Reference
Simulated lipid waste	Triolein: 5–47 % (COD basis) Abundant palmitate followed by oleate, myristate, and stearate	Batch	120 days	5–40 % = 10 days; 47 % = 60 days	93–100 % of theoretical yield	800–900 ml/g of VS added	31 %	Cirne et al. (2007)
(i) Landfill leachates	19 % (VS basis)	Semicontinuous pilot plant	47 days	–	23.5 % increase over period without co-digestion	12.4 Nl/kg	–	Pastor et al. (2013)
(ii) Used oil						970.6 Nl/kg		
(i) Cattle manure	–	Batch BMP	17 days	–	78 % of theoretical yield	509 mL CH_4/g VS added	–	Kougias et al. (2014)
(ii) Antifoam agents (oils)	0.05–0.5 % (v/v_{feed})	Batch BMP	25 days	–	70–83 %	623–837 mL CH4/g VS added	Complete inhibition for tributyl phosphate	
	Rapeseed oil	CSTR	110 days	–	–	284–377 mL CH4/g VS added		
	Oleic acid							
	Octanoic acid							
	Tributylphosphate							
(iii) Co-digestion of antifoam with cattle manure	0.05–0.5 % (v/v_{feed})	Batch BMP	25 days		89–91 %	498–655 mL CH4/g VS added	–	
	Rapeseed oil				82–94 %	528–600 mL CH4/g VS added	0.5 %	
	Oleic acid				14–92 %	92–192 CH4/g VS added	0.5 %	
	Octanoic acid				0	0	0.05 %	
	Tributyl phosphate							
	Individually with cattle manure							
Co-digestion of sewage sludge with trapped grease waste	12–37 %; OLR: 2.2–2.8 kg COD/ m^3.d (COD basis)	Bench-scale CSTR	550 days	–	1.2 and 2.2 times increase over sewage sludge alone	–	37 %	Silvestre et al. (2014)

(i) Simulated OFMSW	Diluted pet food; OLR: 0.55–2.57 kg TVS/m^3.d (VS basis)	17 days; Pilot scale semicontinuous, semicontinuous, completely mixed liquid reactor	~275 days	10 days	–	0.8 m^3 biogas or 0.5 m^3 CH_4/kg TVS removed	—	Fernández et al. (2005)
(ii) Co-digestion of OFMSW with	0.97 kg TVS/m^3.d						–	
(a) Animal fats	4–28 % of OLR (predominant palmitic, stearic, oleic, and linoleic acids)					0.55–0.75 m^3 biogas or 0.35–0.5 m^3 CH_4		
(b) Vegetable fats (coconut oil)	28 % of OLR (predominant lauric, myristic, palmitic acids)					0.7 m^3 biogas or 0.45 m^3 CH_4	–	
(i) Cow manure and food waste	4.6 g COD/ $L_{reactor}$·d	15 days; CSTR	148 days	–	–	–		Neves et al. (2009)
(ii) Co-digestion of cow manure and food waste with increasing pulses of canned fish industry oily waste	4.6 g COD/ $L_{reactor}$·d+9–18 g COD/ L.d		203 days	–	70 % increase over cow manure and food waste alone	Offline BMP 0.4–0.9 g COD-CH_4/g VS	18 g COD/ $L_{reactor}$·d	
			224 days	–		0.6-0.9 gCOD-CH4/g VS		
Oleate acclimatized sludge with LCFAs	OLR: 6 kg COD/m^3/ day	1.18–2.7 days; EGSB	~800 h	500 h	–	5200 mg COD–CH_4 g VSS measured at end of assay	–	Pereira et al. (2005b)
(i) Oleic acid								
(ii) Palmitic acid		1.18–2.7 days; EGSB	~800 h	50–60 h		4570 mg COD–CH4 g VSS measured at end of assay	–	
(i) Waste- activated sludge (WAS)	Substrate/inoculum (S/I) = 0.08	BMP	750 h	81.8 h	–	117 mL CH_4/g TVS	–	Liet al. (2011)
(ii) Co-digestion of WAS with	3–12 g (S/I = 0.46–1.59)			3.9 h		124–134 mL CH4/g TVS	12 g (S/I = 1.59)	
(a) Synthetic kitchen waste (KW)								
(b) Fat, oil, and grease (FOG)	0.2–1.4 g (S/I = 0.3–1.61)			28.3 h		31.9–418 mL CH4/g TVS	0.7–1.4 g (S/I = 0.85–1.61)	

substrate for anaerobic digestion with a potential for increased biogas production owing to the abundant carbon content (Suto et al. 2006; Canakci 2007). The LCFAs so formed have also been suggested to inhibit the anaerobic digestion system (Pereira et al. 2005a, b).

Though the basic degradation pathway of saturated LCFAs involving β-oxidation has been known for a long time, various studies report different pathways for degradation of unsaturated LCFAs. Some studies have suggested saturation of the unsaturated LCFAs prior to β-oxidation, while other studies proposed β-oxidation of unsaturated LCFAs before they were completely saturated (Lalman and Bagley 2000, 2001). Irrespective of the type of LCFAs (saturated or unsaturated), the primary route followed for microbial degradation of LCFAs consisted of the following steps: (1) adsorption of LCFA onto the cell surface, (2) uptake of LCFA, and (3) β-oxidation and formation of lower molecular weight products (Sousa et al. 2010). One unique feature of LCFA decomposition was demonstrated by Sousa et al. (2010), which suggested that bacteria with capacity for degrading unsaturated fatty acid also possessed the ability to degrade saturated fatty acids, but not vice versa.

16.2 LCFA Detection Methods

One of the problems encountered during biomethanation studies with LCFAs is the lack of appropriate methods for detection of the LCFAs which is attributed to the low solubility of LCFAs, especially the saturated ones. Most of the methods for quantification of LCFAs involve extraction in suitable solvents, followed by sample preparation by derivatization. The processed samples could then be subjected to LCFA quantification by various methods, majority of which are based on chromatography.

Tarola et al. (2012) quantitatively determined the composition of fatty acids produced by the action of lipase on drying oils. The procedure involved extraction of fatty acids with n-heptane and derivatization with ɠ-bromoacetophenone followed by quantification by HPLC. The analytical enzymatic procedure provided advantage of separation of analytes with greater efficiency and sensitivity for <0.5 μg/mL of free fatty acids (FFAs).

Free fatty acids (FFAs, C_4–$C_{18:3}$) were quantified from bovine milk by Amer et al. (2013). A novel GC–MS (gas chromatography–mass spectrometry)-based method was developed, which consisted of derivatization of FFAs with ethyl chloroformate and validation by using appropriate standards. The major advantage of this method was the absence of solvent extraction and evaporation steps which reduced the loss of short-chain free fatty acids (SCFFAs) during sample preparation.

Salimon et al. (2014) compared two methods for analysis of FAs and *trans* fatty acids in bakery products with the help of gas chromatography. Methods were based on the use of KOCH3/HCl and trimethylsilyl diazomethane (TMS-DM) as reagents for derivatization which showed that KOCH3/HCl method required shorter time and was more convenient than TMS-DM method for analysis of *cis/trans* fatty acid samples. However, the use of highly polar capillary column and flame ionization detector can provide the benefit of analyzing the LCFAs without the need for derivatization step as was demonstrated by Jiang et al. (2012) in the case of palmitic, stearic, and oleic acids.

Capillary gas chromatography method was developed by Neves et al. (2007) in which linear calibration curves for C12–C18 LCFAs were constructed in the range 25–1270 mg/L. Pentadecanoic acid (C15:0) was used as internal standard for quantifying all the acids with response factors ranging from 0.79 to 1.09. Owing to the time-consuming and tedious nature of conventional methods of sample processing, Kang and Wang (2005) devised a rapid and simple method for the analysis of long-chain polyunsaturated fatty acid contents by combining the two steps of conventional analysis, i.e., extraction and methylation, into a single step.

16.3 Mathematical Modeling and Kinetics of LCFA Degradation

A number of studies have evaluated the improved methane production observed under varying operational conditions with application of linear regression models for indicating the first-order production rates (Hansen et al. 2004; Heo et al. 2004; Carucci et al. 2005). Angelidaki et al. (1999) developed a dynamic model for prediction of process performance of AD of complex material and co-digestion of different wastes. Detailed description of physiological conditions was incorporated in the model along with other primary modulating factors such as free ammonia, acetate, volatile fatty acids (VFAs), and LCFAs to describe the effect of co-digestion on methane production.

Various configurations of reactors could also be used to study the mathematical model of LCFA degradation. One such model was developed by Knobel and Lewis (2002), wherein mathematical modeling was used for discussing all reactions occurring before sulfate including hydrolysis of solid substrates, β-oxidation of long-chain fatty acids, acidogenesis, and acetogenesis. Using this model, prediction of the dynamic and steady state behavior of different types of reactors was arrived at for simulation using both simple and complex carbon sources.

In addition to linear models, nonlinear regression models were applied for describing the co-digestions of FOG and kitchen waste (KW) for demonstrating amelioration of the methane production along with shortening of lag phases of biodegradation (Li et al. 2011). However, accumulation of LCFAs on the sludge during digestion of FOG induced a delay in initial methane production by creating a physical barrier which hindered the transfer of substrates and products. This phenomenon was shown to be reversible which could be eliminated after biomass-associated LCFA was completely mineralized (Pereira et al. 2005b). The decomposition of LCFAs is dependent on the enthalpy-driven energy released from methanogenesis by release of protons and electrons which are used during spontaneous methanogenesis for methane production. Thus, LCFAs could be efficiently decomposed into methane, carbon dioxide, and SCFAs when sufficient thermal energy was supplied (Oh and Martin 2010).

Zonta et al. (2013) applied mathematical modeling for studying the dynamics of LCFA inhibition on AD in the presence of synthetic adsorbent (bentonite) and synthetic substrate (sodium oleate). Validation of the model provided an insight into the biophysics of the inhibitory process. The model also confirmed the higher sensitivity of the acetoclastic population than the acidogenic population to the LCFA inhibition and highlighted the significance of distribution of saturated/unsaturated LCFAs degraders in the evolution of the system.

16.4 Inhibition of Methanogenesis by LCFAs

Detailed studies on the pathways involved in inhibition of AD process by LCFAs can provide deeper insight into the mechanism of inhibition. β-oxidation, the rate-limiting step in LCFA degradation, initially reaches a maximum with increase in LCFA concentration, followed by a decrease thereafter, indicating that LCFA acts as a substrate inhibitor for β-oxidation. Shin et al. (2003) compared the inhibitory effects of LCFAs (16–18 carbons) on β-oxidation and VFA degradation using acclimated granular sludge. Assessment of VFA degradation rates and β-oxidation by applying Gompertz equation indicated maximum methane production of 86–90 % of the theoretical values for VFA and 60–70 % for LCFAs. Acetate degradation was affected to a greater extent in the presence of unsaturated LCFAs (oleate, linoleate) than saturated (stearate, palmitate) ones, while the effect of LCFA inhibition on propionate degradation was less pronounced than on acetate degradation. Degree of saturation of LCFAs was shown to effect the AD process, and the observation that unsaturated LCFAs contributed to additional solids removal in comparison to saturated LCFAs was attributed to higher solubility of unsaturated LCFAs than the saturated LCFAs (Shin et al. 2003).

There are different opinions on the rate-limiting step in formation of methane from LCFAs. Cavaleiro et al. (2013) suggested β-oxidation to be the limiting step in the above pathway by demonstrating that the first steps of unsaturated LCFA degradation were not obligatory and syntrophic. Different reactors were fed with saturated and unsaturated C16 and C18 LCFAs in the presence or absence of selective inhibitor of methanogens, bromoethanesulfonate, followed by analysis of bacterial community composition by denaturing gradient gel electrophoresis (DGGE). Degradation of oleate (C18:1) resulted in the higher accumulation of myristate (C14:0) and palmitate (C16:0) in the bioreactors in which methanogenesis was inhibited than compared to non-inhibited methanogenic bioreactors.

The inhibitory effect of LCFAs on AD process was reportedly caused by limitations for transport of LCFA accumulated onto anaerobic sludge (Pereira et al. 2005b). Comparison of specific methanogenic activity of two sludges before and after mineralization of LCFAs showed accumulation of palmitic acid. White spots of precipitated palmitic acid were observed between the nonencapsulated sludge, and very high initial methanogenic activity was observed in the case of all tested substrates except butyrate. In the encapsulated sludge, it was adsorbed into the surrounding, and methane production was reported only from ethanol and H_2/CO_2 after a lag phase of 50 h. Thus, it was concluded that LCFA caused hindrance in the transfer of substrates by creating physical barrier, thereby leading to a delay in initial methane production.

16.5 Effects of LCFAs on Anaerobic Digestion Process

Lipid-rich wastes are generated from different sources including slaughterhouse, meat-processing plants, dairy, edible oil, grease trap, and food wastes from restaurants. Inhibition of anaerobic process by lipids is known to be caused due to sludge flotation and washout in the presence of LCFAs which are hydrolyzed products of lipids. FOG is a major component of lipid-rich surface layer of wastewater generated by food processing and cooking, which is primarily composed of LCFAs (palmitic, stearic, oleic, and linoleic acids) (Suto et al. 2006; Canakci 2007). Problems associated with AD of excessive FOG include inhibitory effects such as lag phase for decomposition, cessation of biogas production, and digester washout.

Though excessive addition of LCFAs causes inhibition in the AD process, Zhu (2013) observed an increase in the production of biogas in the reactors fed with high concentration of LCFAs. Among all the LCFAs added in same dosage, highest increase in digester performance was achieved with linoleic acid, while a mixture of oleic acid and stearic acid was reported to be least effective. From the study, the authors suggested that reactor stability could be improved and LCFA accumulation could be avoided by keeping the dosage of oleic acid below 30 %. The finding that although methane production was not completely inhibited by either of the LCFAs, oleate was more inhibitory to methanogens than palmitate was confirmed by Sousa et al. (2013). The authors studied the effects of saturated (palmitate [16:0]) and unsaturated (oleate [18:1]) LCFAs on hydrogenotrophic methanogens by adding *Methanospirillum hungatei* and *Methanobacterium formicicum* to oleate- and palmitate-degrading enrichments. The survival of the two cultures in enrichments was monitored by DGGE analysis which showed higher resistance and presence of *M. formicicum* in both oleate and palmitate enrichments. On the other hand, *M. hungatei* was detected only in palmitate enrichment and viability tests, confirmed the presence of higher percentage of damaged cells of *M. hungatei* indicating higher sensitivity of this culture to oleate than *M. formicicum*.

The inhibitory effect exerted by LCFAs is reported to be stronger for gram-positive organisms even at low concentrations than gram-negative organisms. The inhibition was linked to the adsorption of the LCFAs on the cell wall of anaerobic consortium, thus causing interference in the transport of substrate and products. Cell

wall composition of methanogens played a role in sensitivity of the methanogens to LCFAs. Thermophiles were more sensitive to LCFAs than mesophiles owing to their variable cell wall composition. Zeitz et al. (2013) studied the inhibitory effects of saturated fatty acids on methanogenesis by *Methanosarcina barkeri, M. mazei, Methanococcus voltae* (at 37 °C), and *Methanothermobacter thermoautotrophicus* (at 65 °C). The effect of the methanogenic coenzyme M (HS-CoM) on inhibition by LCFAs showed that methanogens were susceptible to C10 and C11 and less to C14 LCFAs. C18 LCFA affected *M. thermoautotrophicus*, while in *M. mazei* cultures, the action of C14 was not prevented by HS-CoM, thereby suggesting that the SFA-induced effect on methanogenesis was not due to the inhibition of HS-CoM.

16.6 Strategies for Overcoming Inhibitory Effect of LCFAs

In an anaerobic environment, extracellular lipases produced by acidogenic bacteria hydrolyze lipids to generate glycerol and free LCFAs of which glycerol is further converted in the bacterial cell to acetate by acetogenesis and acetate and hydrogen were produced from LCFAs through β-oxidation pathway (syntrophic acetogenesis). The process efficiency depends on the utilization of generated hydrogen by hydrogenotrophic methanogens. Along with hydrogen, low solubility of saturated LCFAs creates limitation in the degradation of 16 and 18 carbon-saturated acids, while five times higher degradation has been observed in case of unsaturated fatty acids. These process limitations have been shown to be overcome by application of co-digestion strategy which is considered to be an effective, cheap, and suitable method for reducing the process limitations and improving methane yields (Alatriste-Mondragón et al. 2006).

Fernández et al. (2005) demonstrated the advantage of co-digestion strategy during AD of fats of different origins with organic fraction of municipal solid waste (OFMSW) in a semicontinuous reactor. Under mesophilic conditions, 88 % total fat removal occurred at corresponding biogas yield of 0.8 m^3/kg total volatile solids (TVS) removed and 60 % as methane, with neither LCFAs nor VFAs being detected irrespective of the source of the fat (animal or plant origin).

Improved process efficiencies have also been shown to be achieved by co-digestion of FOG with 25 % reduction in biosolids being reported in addition to 60–70 % biogas production (Bailey 2007; York et al. 2008). In addition to co-digestion, effect of co-substrates was also investigated on the degradation of LCFAs in single- and two-stage upflow anaerobic sludge blanket (UASB) digesters (Kuang 2002). Glucose was found to be better co-substrate than cysteine for promoting granule formation in a single-stage UASB resulting in decreased toxicity of sodium oleate. On the other hand, granule formation was severely affected on addition of cysteine and sodium oleate in combination, while different microbial communities were recovered from LCFA-inhibited digester on addition of combination of glucose and cysteine. However, similar strategy of using co-substrates consisting of two LCFAs led to conflicting results as was demonstrated by Cavaleiro et al. (2008) who incubated two anaerobic sludges with oleate and palmitate in batch mode. An initial decrease in methane production was noted due to the presence of biomass-associated LCFAs, which improved significantly when sufficient time was given for the degradation of this LCFA. This created a lag phase for methane production and simultaneously increased the tolerance of the acetotrophic methanogens to LCFAs. Alternatively, the authors suggested that pulsed addition of lipid-rich waste (dairy wastewater) containing 53 % fat could improve the efficiency of cumulative methane production and COD removal in each new pulse, along with significant decrease in VFA levels.

The lipid concentration in lipid-rich waste was shown to have a strong correlation with efficiency of methane production. An initial lag phase of 6–10 days is detected before degradation of lipids starts. Enzymatic hydrolysis of lipids could reduce the lag phase and enhance the

hydrolysis, but the intermediates produced during the hydrolysis have been shown to be inhibitory to latter steps of the degradation process (Cirne et al. 2007). This drawback could be overcome and methane production could be enhanced by controlled, intermittent input of an increasing concentration of fat as was demonstrated in the co-digestion of manure and food waste by controlled, intermittent inputs of oil (Neves et al. 2009).

In another study of co-digestion, Pastor et al. (2013) did a comparative evaluation of biogas production from sludge generated in wastewater treatment plant (WWTP), landfill leachates, and used oil. The biogas production was highest (970.6Nl/kg) in the case of used oil during co-digestion with sludge in a pilot plant operated under semicontinuous conditions. Silvestre et al. (2014) co-digested sewage sludge with different doses of grease waste in the bench-scale CSTR under thermophilic conditions. Addition of grease waste up to 27 % resulted in increased methane yield, but higher doses of grease waste led to instability and LCFA accumulation. Thus, though, the addition of grease waste promoted acetoclastic activity by inhibiting the hydrogenotrophic activity, it suggested that tolerance to LCFAs could be enhanced by slow addition of lipid-rich waste.

Inhibition of biogas production has been observed in the presence of LCFAs even under varying physiological conditions such as digestion in batch and semicontinuous experiments under thermophilic conditions at LCFA concentration exceeded 1.0 g/L. However, recovering strategies such as adsorbing the LCFAs, increasing the biomass/LCFA ratio by diluting with active inoculum, and reducing the bioavailable LCFA concentration could be used for overcoming the inhibition (Palatsi et al. 2009). Alternatively, anaerobic biomass could be acclimatized to an inhibitory concentration of LCFA by repeated exposure leading to enhancement in degradation rates.

In spite of various reports on the sensitivity of methanogens to LCFAs, acclimation strategy could result in increased resistance as observed in case biofilm formed in the presence of oleic acid in comparison to control biofilm produced in the absence of lipids (Alves et al. 2001).

Qian (2013) studied the effect of four different LCFAs (stearic, oleic, linoleic, and linolenic acids) and showed improved degradation efficiency when a mixture of stearic and oleic acid (1:2 by mass ratio) was fed in the digester compared to stearic acid alone due to lower metabolic capacity of bacteria for saturated LCFAs on account of their lower solubility. Increase in solubility of stearic acid by dissolving it in oleic acid could result in enhanced degradation efficiency of stearic acid.

Foam formation is an important factor in the degradation of lipid-rich waste. Lienen et al. (2013) investigated the microbial community in a full-scale biogas plant fed with sewage sludge and FOG as substrate together with *Microthrix parvicella* (which promoted the formation of foam) over a 15-month period. QPCR indicated the presence of higher number of *M. parvicella* following an excessive foaming in comparison to reference digesters. Shift in the number of *M. parvicella* was observed corresponding to its seasonal abundance in the sludge of the WWTP.

In addition to foaming promotion by lipid-rich waste, different oils have also been known to serve as agents for foam control/reduction as demonstrated by Kougias et al. (2014). The study compared the role of oils on foam reduction in manure-based biogas reactors which indicated that though all the oils studied (octanoic acid, oleic acid, rapeseed oil, and tributyl phosphate) efficiently suppressed foaming in reactors, only rapeseed oil had a synergistic effect on methane yield, while tributyl phosphate was inhibitory to the biogas process.

16.7 Characterization of LCFA Degrading Methanogenic Community by Genomic Tools

Combination of enrichment studies with molecular approaches has revealed the classification of LCFA-degrading microbes in phylogenetically diverse bacterial groups (Hatamoto et al. 2007a;

Sousa et al. 2007a, b). Enrichment, isolation, and SIP [RNA-based stable isotope probing] have enabled characterization of bacterial community involved in AD of diverse LCFAs as sole energy source (Hatamoto et al. 2007a). Bacteria associated with the family *Syntrophomonadaceae* were found to be dominant along with evidence for existence of the bacteria belonging to the phylum *Firmicutes* in the enrichment cultures (Hatamoto et al. 2007a). SIP has also been employed for determining the microbial diversity in methanogenic sludges involved in LCFA degradation using ^{13}C-labeled palmitate (Hatamoto et al. 2007b). The study revealed the bacterial diversity actively involved in the degradation of LCFA including members from families *Syntrophaceae* and *Syntrophomonadaceae*, phyla *Bacteroidetes* and *Spirochaetes*, and clone cluster from class *Deltaproteobacteria*.

Dominance of uncultured bacteria belonging to the family *Firmicutes* and *Proteobacteria* phyla was reported from stable oleate and palmitate enrichment cultures by DGGE (Sousa et al. 2007a, b). Syntrophic fatty acid-oxidizing bacteria belonging to *Syntrophomonas* were found to be prevalent in the identified, predominant DGGE bands which also showed the ability of oleate enrichment culture to utilize palmitate without any change in DGGE profile. However, reverse study with palmitate-specialized culture demonstrated a lag phase of 3 months for degradation of oleate after which a change in DGGE profile was observed.

Immunological probes have been used for targeting saturated fatty acid-β-oxidizing syntrophic bacteria which included three mesophilic *Syntrophomonadaceae* species. The methanogenic rRNA was found to be comprised of *Methanomicrobiales* which were found to be the main hydrogen-utilizing microorganisms. 0.2–1 % rRNA belonged to family *Syntrophomonadaceae*, but majority belonged to the genus *Syntrophomonas* (Hansen et al. 1999).

Microbial diversity of methanogenic sludges degrading LCFAs has been characterized by SIP (Hatamoto et al. 2007b). Sludge was incubated with ^{13}C-labeled palmitate (1 mM), and ^{13}C-labeled bacterial rRNA was detected after 8–19 days of incubation. Sequencing of clones carrying reverse transcribed 16S rRNA suggested the occurrence members of *Syntrophaceae*, *Deltaproteobacteria, Clostridium*, *Bacteroidetes*, *Spirochaetes*, and *Syntrophomonadaceae* showing the involvement of varied bacterial groups in the anaerobic degradation of LCFAs. Other molecular tools such as cloning of 16S rRNA gene and in situ hybridization were also used by the authors for analyzing LCFA-degrading microbes (Hatamoto et al. 2007a). Palmitate, stearate, oleate, and linoleate were used as substrates, and predominant bacteria belonging to *Syntrophomonadaceae* were detected in addition to members belonging to phylum *Firmicutes* and class *Deltaproteobacteria*. The authors were also able to detect and characterize a strain from family *Syntrophomonadaceae*, which possessed the capacity of generating acetate and methane from palmitate in syntrophic association with *Methanospirillum hungatei* along with a strain from the phylum *Firmicutes*.

Strain OL-4^{T} was isolated from anaerobic expanded granular sludge bed reactor treating an oleate-based effluent, which was found to degrade oleate in co-culture with *Methanobacterium formicicum* Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM) 1535^{NT} (Sousa et al. 2007a, b). Phylogenetic analysis of 16S rRNA revealed the identity of this isolate which was found to be closely related to *Syntrophomonas erecta* DSM 16215 T, *Syntrophomonas wolfei* subsp. *wolfei* DSM 2245 T, and *Syntrophomonas sp.* TB-6; hence, it was named *Syntrophomonas zehnderi* sp. nov.

Kazakov et al. (2009) explored the ability of bacteria for utilizing branched-chain amino acids and fatty acids as the sole carbon sources and converting them into acetyl-coenzyme A, propanoyl-CoA, and propionyl-CoA by comparative genomics approach. DNA motifs and transcriptional factors that controlled the FA and ILV utilization pathways in *Proteobacteria* were identified by this approach, which revealed existence of remarkable variability in the regulatory systems controlling genes associated with the fatty acid degradation pathway.

Though numerous molecular tools have enabled characterization of LCFA-degrading community, the rarity of complete genome sequences available in database has hindered protein identification due to which studies on metaproteomes of complex microbial communities have remained a big challenge (Sousa et al. 2013). Still, proteomic analysis can play a key role in understanding the cellular responses to stimuli during degradation applications. Han et al. (2008) studied gene expression changes in *Escherichia coli* in response to LCFA (oleic acid) which revealed an altered expression level of proteins and synthesis of nine new proteins (AldA, Cdd, FadA, FadB, FadL, MalE, RbsB, Udp, YccU) compared to glucose.

The significance of fadBA5 operon of *Pseudomonas aeruginosa* in utilization of LCFAs as sole carbon source was demonstrated by Kang et al. (2008). The regulation of fadBA5 by LCFAs was validated by constructing a P_{fadBA5}–lacZ fusion using the promoter region of fadBA5, which led to the identification of *PsrA* as regulator for derepressing fadBA5 operon during binding of LCFA.

The presence of *Desulfovibrionales* and *Syntrophobacteraceae* groups in sulfate-reducing enrichment culture was identified by Sousa et al. (2009). Authors studied the diversity of the palmitate- and oleate-degrading anaerobic bacteria in the presence of sulfate as electron acceptor, by DGGE. Inhibition of methanogenic and acetogenic syntrophic bacteria was observed when the LCFA-degrading methanogenic communities were subcultured in the absence of sulfate. Since archaea were not detected by real-time PCR, it was concluded that the bacterial degradation of LCFAs was also influenced by the substrate.

The significance of understanding the microbial interactions essential for optimizing methane formation from LCFA-containing waste streams in bioreactors was highlighted by Sousa et al. (2010). It was proposed that interspecies hydrogen transfer played a key role in the LCFA conversion to methane for which the syntrophic cooperation of acetogenic bacteria and methanogenic archaea was required.

Salvador et al. (2013) used a combination of metaproteomics approach with 16S rRNA gene pyrosequencing for revealing the microbial composition of the sludge incubated with stearate and oleate. Analysis of proteins by LC-MS/MS showed that archaeal proteomes were better identified than bacterial ones. Organisms from the class *Deltaproteobacteria* were reported to be abundant and dominant being the *Syntrophobacter fumaroxidans*, while the methanogenic population was dominated by *Methanosaeta concilii*.

16S rDNA DGGE profiling has also been used for characterizing the eubacterial and archeal community structure at the start and at the end of operation of thermophilic anaerobic digesters fed with manure (Palatsi et al. 2010). Exposure of the reactor biomass to inhibitory pulses of LCFA resulted in improvement in hydrogenotrophic and acidogenic activity though no change in the microbial community upon exposure to LCFA was detected. DGGE profiles revealed several uncultured ribotypes from β-oxidation bacterial genera (*Clostridium* and *Syntrophomonas*) and syntrophic archaeae related to genus *Methanosarcina*. Physiological nature of biomass adaptation was tested by mathematical model IWA IDM1 which the explained mechanism of the LCFA inhibition by considering inhibitory concentration of substrate and specific biomass content.

16.8 Perspectives

Lipid-rich waste from different industries provides an alternative feed source with potential in biomethanation. However, the inhibitory effect of LCFAs generated during lipid hydrolysis limits the wide-scale use of this feed source for methane production. Biotechnological approach can aid in circumventing the problems associated with anaerobic digestion of LCFAs, and enhancement in efficiency of AD process can be achieved by application of genomic tools.

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