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The use of agricultural substrates to improve methane yield in anaerobic co-digestion with pig slurry: Effect of substrate type and inclusion level

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ABSTRACT

Anaerobic co-digestion of pig slurry with four agricultural substrates (tomato, pepper, persimmon and peach) was investigated. Each agricultural substrate was tested in co-digestion with pig slurry at four inclusion levels: 0%, 15%, 30% and 50%. Inclusion levels consisted in the replacement of the volatile solids (VS) from the pig slurry with the VS from the agricultural substrate. The effect of substrate type and inclusion level on the biochemical methane potential (BMP) was evaluated in a batch assay performed at 35 °C for 100 days. Agricultural substrate's chemical composition was also analyzed and related with BMP. Additionally, *Bacteria* and *Archaea* domains together with the four main methanogenic archaeal orders were quantified using quantitative real-time TaqMan polymerase chain reaction (qPCR) at the end of the experiment to determine the influence of agricultural substrate on sludge's microbial composition. Results showed that vegetable substrates (pepper and tomato) had higher lipid and protein content and lower carbohydrates than fruit substrates (persimmon and peach). Among substrates, vegetable substrates showed higher BMP than fruit substrates. Higher BMP values were obtained with increasing addition of agricultural substrate. The replacement of 50% of VS from pig slurry by tomato and pepper increased BMP in 41% and 44%, respectively compared with pig slurry only. Lower increments in BMP were achieved with lower inclusion levels. Results from qPCR showed that total bacteria and total archaea gene concentrations were similar in all combinations tested. *Methanomicrobiales* gene concentrations dominated over the rest of individual archaeal orders.

1. Introduction

Anaerobic digestion is an environmentally friendly and could be cost-effective pig slurry treatment process since, in turn of degrading organic matter, it produces energy in the form of methane (CH_4) (González-Fernández and García-Encina, 2009). However anaerobic digestion of pig slurry typically have low solids content (<10% total solids (TS)), and thus, leads to low CH_4 yields in volume basis compared with other organic substrates such as energy crops. In order to overcome these limitations and enhance CH_4 production from slurry, anaerobic co-digestion of pig slurry with different substrates is being widely used (Ward et al., 2008).

Agricultural substrates such as fruits and vegetables with easily biodegradable organic matter content and low nitrogen concentration, are appropriate for co-digestion and can enhance CH_4 production with animal organic wastes (Bouallagui et al., 2009; Dinuccio et al., 2010; González-González et al., 2013). Moreover, the use of agricultural substrates in co-digestion is a viable solution for its disposal in areas of high agricultural production. Therefore, the combination of pig slurry and carbon-rich substrates can result in better digestion performance compared with digestion of pig slurry-only. In addition, co-digestion can reduce the concentration of certain compounds found in the slurry which can be toxic to the anaerobic digestion process such as ammonia (Murto et al., 2004) and volatile fatty acids (VFA).

Agricultural substrates might have adverse effects when added to a stable digester or used in conjunction with other types of residues (Fountoulakis et al., 2008). Gelegenis et al. (2007) stated that olive mill wastewater may inhibit certain microbial groups in anaerobic reactors in co-digestion with diluted poultry manure. These authors reported that the presence of aromatic oils or polyphenols in agricultural substrates could inhibit the development of archaea populations within the methanogenic consortia. Furthermore, methanogen populations and their domains are poorly understood when adding a substrate for co-digestion with pig slurry (Traversi et al., 2011; Yue et al., 2013; Zhang et al., 2011). In pig slurry anaerobic digesters, hydrogenotrophic methanogenesis is the main metabolic pathway for organic matter conversion to CH_4, due to unfavorable environmental factors for acetoclastic

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methanogenes (Song et al., 2010). However, acetogenic methanogenesis is recommended to enhance CH_4 production due to its higher CH_4 yield (Garcia et al., 2000). The addition of a co-substrate could promote acetogenic methanogenesis. Nevertheless, there are few studies that explore the changes in methanogenic population structure of an agricultural substrate to pig slurry in co-digestion. Consequently, it is key to determine the adequate amount of each substrate in pig slurry and agricultural substrate's mixtures to maximize CH_4 production and minimize adverse antimicrobial effects.

Specialist knowledge on not only the type of agricultural substrates which can enhance CH_4 production with pig slurry, but also on their amount in the substrate's mixture could improve pig slurry management in high livestock-density areas; promoting the energetic value of the slurry and the agricultural by-products derived from the fruit and vegetable industry. This information could help farmers to select the best combinations using agricultural substrates and pig slurry in anaerobic co-digestion in these areas and to generate an additional revenue and diversity of the agricultural activity.

The objective of this study was to evaluate the effect of four agricultural substrates (tomato, pepper, peach and persimmon) on the biochemical methane potential (BMP) in anaerobic co-digestion with pig slurry, focusing on the type of substrate and its amount on the final substrate's mixture (inclusion level). The influence of agricultural substrate on the sludge's microbial composition was also studied. This study aims to finally identify the combination that optimizes CH_4 production and can serve as useful input to develop integrated agricultural by-products and pig slurry management systems in high livestock-density areas.

2. Material and methods

2.1. Substrates and inoculum

Pig slurry was created from fresh faeces and urine to avoid the effect of external factors (management, storage time, feeding system or amount of added water) on pig slurry composition (Canh et al., 1998). Pig urine and faeces were collected directly after excretion from six dry sows housed individually. Sows were fed 2.5 kg day^{-1} of a conventional diet for pregnant sows containing, on average, 2.8 Mcal net energy kg^{-1}, 14% crude protein, 2.5% crude fat and 3.5% crude fibre. After collection, the faeces and urine were mixed with distilled water following the procedure described in Møller et al. (2004a), to obtain a fresh slurry with a TS content of 40 g L^{-1}.

The four agricultural substrates assayed were tomato, pepper, peach and persimmon, selected due to their availability and rich content in easily biodegradable carbohydrates. They were obtained from a local market and stored at room temperature for 3 weeks in order to simulate non-marketable products. Before putting into the vials were grounded to homogenize them.

As inoculum, anaerobic sludge was used from an anaerobic digester that treats domestic and industrial wastewater from the wastewater treatment plant in Sagunto, Spain. The inoculum was incubated during 15 days at 35 °C in order to deplete the residual biodegradable organic material (degasification).

2.2. Experimental design and biochemical methane potential determination

The biochemical methane potential (BMP) of each combination of agricultural substrate and pig slurry was determined in a batch assay according to the protocol defined by Angelidaki et al. (2009). The experiments were performed in 120-mL glass bottles, incubated at mesophilic range (35 ± 1 °C) for 100 days.

Bottles were prepared to achieve homogeneity in total volatile solids (VS) (1.3 g VS from manure + agricultural substrates + inoculum), as well as the same amount of inoculum (33.6 mL per bottle). The ratio of inoculum to substrate (pig slurry + agricultural substrates) in all combination was 0.7 on a VS basis (Møller et al., 2004b). Therefore, variations in VS composition were solely attributable to the agricultural substrate.

Each agricultural substrate was tested at four inclusion levels in combination with pig slurry. The inclusion levels consisted of the replacement of the VS from the pig slurry with the VS from the agricultural substrate, expressed as a percentage of the VS coming from the agricultural substrate from the total VS of the mixture (pig slurry + agricultural substrate). The agricultural substrate's inclusion levels tested were: no agricultural substrate addition (inclusion level 0), 15% (inclusion level 1), 30% (inclusion level 2) and 50% (inclusion level 3). Each tested combination was carried out in triplicate. Additionally, three blank bottles containing anaerobic sludge-only were also used in order to determine the anaerobic sludge endogenous CH_4 production which was subtracted from the CH_4 produced by the tested combination at each biogas sampling.

After filling the bottles, they were flushed with nitrogen (99.9%) for one minute to prevent oxygen inhibition, closed with butyl rubber stoppers and then sealed with aluminum crimps. The biogas volume in each bottle was monitored weekly by measuring pressure in the headspace using a manometer (Delta Ohm, HD 9220, Italy). Additionally, a representative sample from the headspace gas volume of each bottle was taken weekly to measure CH_4 content in the biogas. After sampling, the remaining overpressure of the bottle was removed to restore atmospheric pressure.

2.3. Chemical analyses

A representative sample of each separate substrate (agricultural substrate, pig slurry, inoculum) and an initial (day 0) and final (day 100) sample from each tested combination (pig slurry + agricultural substrate + inoculum) was analyzed to determine TS, VS, pH and total ammonium (TAN) (4500 NH_3-B and 4500 NH_3-C procedures) (APHA, 2005). Volatile fatty acids were determined by gas chromatography following the method described by Jouany (1985) with the addition of an internal standard (4-metil valeric). Additionally from agricultural substrates and pig slurry, neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) and total kjeldahl nitrogen (TKN) were determined according to the Van Soest procedure (Van Soest et al., 1991) and APHA (2005) respectively. The lipid content was also analyzed from agricultural substrates and pig slurry (AOAC, 2000).

Methane concentration in the biogas from the batch assay was further analyzed using a Focus Gas Chromatograph (Thermo, Milan, Italy) equipped with a split/splitless injector and a flame ionization detector. The separation was performed in a capillary column GS-Q (J&W Scientific, USA) (30 m × 0.32 mm internal diameter). The carrier gas was helium at a constant flow of 1 mL min^{-1}. Samples were injected with a split ratio of 1:100. The initial oven temperature was set at 50 °C held for 1 min. The temperature was then increased at a rate of 50 °C min^{-1} until 150 °C, which was finally maintained for 2 min. Both detector and injector temperatures were set at 200 °C. An external standard (41.2% carbon dioxide, CO_2 and 58.8% CH_4) was employed for the quantification of CH_4 content in samples.

2.4. Extraction of genomic DNA

On day 100 of the experiment, one sample of each combination under agitated conditions was taken and placed into 20 mL sterile