BIOGAS PRODUCTION BY ENCAPSULATED METHANE-PRODUCING BACTERIA

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Encapsulation of methane-producing bacteria was carried out with the objective of enhancing the rate of biogas production. Encapsulation with a one-step liquid-droplet-forming technique was employed for the natural membrane, resulting in spherical capsules with an average diameter and a membrane thickness of 4.3 and 0.2 mm, respectively. The capsules were made from alginate, using chitosan or Ca^{2+} as counter-ions, together with the addition of carboxymethylcellulose (CMC). A Durapore® membrane (hydrophilic PVDF) with a pore size of 0.1 µm was used for synthetic encapsulating sachets having width and length dimensions 3×3 and 3×6 cm^2 for holding the bacteria. During the digesting process, the dissolved substrates penetrated through the capsule membrane, and biogas inside the capsules was able to escape by diffusion. The results indicate encapsulation to be a promising method of digestion, with a high density of anaerobic bacteria. The method holds considerable potential for further development of membranes and their applications.

Keywords: Encapsulation; Immobilization; Biogas production; Digestion; Methane

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INTRODUCTION

Biogas is a renewable energy source with several applications, e.g. car fuel, heating, cooking, electricity production, etc. Biogas consists mainly of methane and carbon dioxide, but it may also contain minor impurities of other components such as hydrogen sulphide (Deublein and Steinhauser 2008). The anaerobic digestion process and production of methane consists of bacterial hydrolysis, acidogenesis, acetogenesis, and methanogenesis. The doubling time of the hydrolysis and acidogenesis bacteria is about 1.0 to 1.5 days, while acetogens and methanogens need about 1 to 4 and 5 to 15 days, respectively (Gerardi 2003). This means that methane-forming bacteria require a long time inside the digester and may easily be washed out. In addition, the methanogens are very sensitive to the process conditions; their low growth rate results in a relatively long start-up period of up to 3 months to have a stable operation (Deublein and Steinhauser 2008). Consequently, a high dilution rate of the digester or early withdrawal of digester sludge results in a greatly reduced methane-forming bacterial population. Retaining the bacteria inside the digester by encapsulation or immobilization within a membrane may be a solution to these problems. In this article the term "encapsulation" will be used in a broad sense, not restricted to colloidal-sized microcapsules. Thus, it will be shown that a

macro-sized sachet, prepared from permeable membrane, can be used to achieve effects that are analogous to other forms of encapsulation.

The traditional digestion system used for biogas production can be described as a "one-stage" system, in which all biological reactions occur within a single sealed reactor (Griffin et al. 1997). However, serial reactions can be carried out in a "two-stage" digestion system, in which the digestion occurs in serial reactor stages, each of which can be optimized for better control over the different bacterial communities living within the digesters, e.g. the hydrolytic or methanogenic bacteria.

Cell retention by filtration, immobilization, encapsulation, or recycling by centrifugation has been extensively studied in connection with other processes such as ethanol production (Najafpour et al. 2004; Talebnia et al. 2005; Talebnia and Taherzadeh 2006). Among these methods, encapsulation has been widely applied to various bioprocesses such as whole cell enzymes, artificial cells, and biosorbents (Kourkoutas et al. 2004; Park and Chang 2000), but no previous reports on the use of encapsulation technology for anaerobic digestion are available.

Cell immobilization has been shown to be an attractive method, as it helps in maintaining a high cell concentration in the reactor. The cells in this method are retained in a capsule made of a membrane that is permeable to the nutrients and the metabolites (Talebnia et al. 2005), but with no cell leakage, making it possible to obtain higher cell concentrations. The high cell density not only improves the productivity of a bioreactor, but it also provides many other benefits compared to free cells. The microbial cells immobilized in a hydrogel matrix are, for example, protected from harsh environmental conditions such as pH, temperature, organic solvents, and toxic components. Immobilized microbial cells can also be handled more easily and are recovered from the solution without difficulty. Continuous processes can be operated with high cell densities without any loss of microbial cells even at high dilution rates, resulting in a higher bioreactor volumetric productivity.

The aim of the present work was to develop an encapsulation system for bacterial digestion to be used in biogas production. The purpose of the experiments was to investigate the encapsulation process at the second stage of a two-stage digestion system, in which the methanogenesis occurs and encapsulated methane is produced. A synthetic medium including acetate, propionate, butyrate, methanol, and glucose, a typical composition of a bacterial hydrolysis stream, was used as carbon source. The methane production of encapsulated digesting bacteria was examined, using natural as well as synthetic membranes.

MATERIALS AND METHODS

Anaerobic Culture and Medium

Anaerobic culture for the encapsulation process was obtained from a 3000-m^3 municipal solid waste digester, operating under thermophilic (55 ºC) conditions (Borås Energi och Miljö AB, Sweden). The inoculum was maintained in an incubator at 55 ºC for 3 days to keep the methanogenic bacteria active. Then, the culture was mixed and filtered through the screen with pore size of 1 mm. The methane production by the

methanogens was studied, using acetate (300 g/L), propionate (100 g/L), butyrate (100 g/L), methanol (100 g/L), and glucose (100 g/L) as carbon and energy sources, buffered at pH 7.0±0.2 with $NaHCO_3$. The basal medium (BM) in all experiments, used for optimum anaerobic microbial growth including micro- and macro-nutrients, were as follows (in mg/L): NH_4Cl (1200), $MgSO_4 \cdot 7H_2O$ (400), KCl (400), $Na_2S \cdot 9H_2O$ (300), $CaCl_2 \cdot 2H_2O$ (50), $(NH_4)_2HPO_4$ (80), $FeCl_2 \cdot 4H_2O$ (40), $CoCl_2 \cdot 6H_2O$ (10), KI (10), $MnCl_2 \cdot 4H_2O$ (0.5), $CuCl_2 \cdot 2H_2O$ (0.5), $ZnCl_2$ (0.5), $AlCl_3 \cdot 6H_2O$ (0.5), $Na_2MoO_4 \cdot 2H_2O$ (0.5), H_3BO_3 (0.5), $NiCl_2 \cdot 6H_2O$ (0.5), $Na_2WO_4 \cdot 2H_2O$ (0.5), Na_2SeO_3 (0.5), cysteine (10), and $NaHCO_3$ (6000) (Isci and Demirer 2007).

Encapsulation Procedure Using Natural Membrane

Among the various techniques available for cell encapsulation using natural membranes, the liquid-droplet-forming technique (Talebnia 2008) was chosen for this experiment. The digesting sludge, including methane-forming bacteria, was suspended in a 1.3 or 2.6 % (w/v) $CaCl_2$ solution, containing 2.6% carboxymethylcellulose (CMC) (Sigma-Aldrich), with a volume ratio of digesting sludge to $CaCl_2$ and CMC solution of about 1:1. The CMC had 0.7 and 0.9 degree of substitution (DS, testing optimal substitution degree) and was added to increase the viscosity (Talebnia et al. 2005). Tween 20 improves the permeability of the capsule membrane. Thus the initial solution (containing bacteria + CMC + $CaCl_2$) was dropped through an extruder into a sterile 0.6% (w/v) sodium alginate solution (SIGMA-ALDRICH), containing 0.1% (v/v) Tween 20. The resulting solution was stirred at 330 rpm during capsule production. After 10 minutes of gelation, the capsules were washed with sterile water for 10 min, and then allowed to harden in a 1.3% (w/v) $CaCl_2$ solution for 20 min (Fig.1).

The effect of the different $CaCl_2$ concentrations (see above) on the encapsulation process was also investigated. Adding $CaCl_2$ develops wall thickness, pore size, surface charge, and the mechanical strength of the capsules (Park and Chang 2000). Anaerobic digestion in batch reactor was performed for 6 days under thermophilic conditions on encapsulated anaerobic cultures, produced from the different concentrations of $CaCl_2$, and the methane production was measured.

In another set of experiments, chitosan-alginate capsules were prepared, using a method similar to the one used for the production of the alginate beads. The capsules were washed after hardening with the 1.3% $CaCl_2$ solution and then deposited into a low-molecular-weight chitosan solution, including 0.2% chitosan and 0.3 M $CaCl_2$ dissolved in 0.04 M acetate buffer at pH 5. The volume ratio of the capsules to the chitosan solution was 1:5. The chitosan replaced calcium ions in the capsule. The coating process was performed at 30 °C for 24 h in a shaker bath at 130 rpm.

Encapsulation Procedure Using Synthetic Membrane

The digesting sludge obtained from the municipal solid waste digester was first centrifuged for 10 minutes at 14,000×g to separate the solids from the liquid. The purpose was to use only the solids for inoculums, since the solids can be handled easily. Durapore® filter membrane with 0.1 µm pore size and 125 µm thickness (Millipore AB, Sweden) was used for the encapsulation. The membrane consisted of hydrophilic polyvinylidene fluoride (PVDF), which provides high flow rates and throughput, low

extractability, as well as a broad chemical compatibility and a high operating temperature. For the encapsulation, the membrane was first made into sachets of the sizes 3×3 cm^2 and 3×6 cm^2 (Fig. 2). To each sachet, 3 g of inoculum was added, after which the sachet was sealed immediately. The sealing time was 4.5 sec and the cooling time 5.0 sec (ADMEDICA, Germany). Four different sets of treatments were executed, after which the digesting process of the encapsulated bacteria proceeded for 15 days in a batch reactor, designed for biogas production.

Fig. 1. Capsules made of natural membrane, containing digesting bacteria

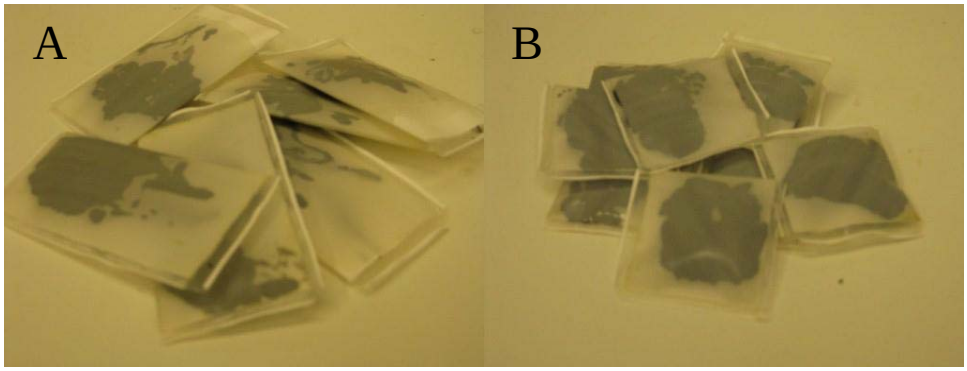

Fig. 2. Sachet capsules made of synthetic membrane, containing digesting bacteria cells: A= Sachets size 3×6 cm^2, B= Sachets size 3×3 cm^2

Biogas Production

The anaerobic digestion experiments were carried out at 55 °C in batch digesters, according to a previously described method (Hansen et al. 2004). For the biogas production using digesting bacteria encapsulated in natural membrane, the digesters used were serum glass bottles with 118 mL working volume, closed with butyl rubber seals and aluminium caps. To each bottle, 15 mL of capsules together with 20 mL of synthetic medium were added. For the experiments using synthetic membranes, serum glass bottles with 250 mL working volume and closed with butyl rubber seals and plastic caps, were used as digesters. For each reactor, 3 capsules along with 100 mL synthetic medium were added. The headspace of each bottle was flushed with 80% nitrogen, 20% carbon dioxide gas mixture, thereby obtaining anaerobic conditions. To determine the methane produc-

tion during the digestion process, gas samples from the headspace of each bottle were regularly withdrawn and analysed by gas chromatography.

Analytical Procedure for the Biogas

The biogas was analysed using a gas chromatograph (Auto system, Perkin-Elmer, USA) equipped with a prepacked column (Perkin-Elmer, 6'x1.8" OD, 80/100 Mesh, USA), a thermal conductivity detector (Perkin-Elmer, USA), and at an injection temperature of 150 °C. The carrier gas was nitrogen at 60 °C, with a flow rate of 20 mL/min. The biogas samples were withdrawn and analysed on a daily basis, using a 0.25 mL pressure-tight gas syringe (VICI, precisions sampling Inc., USA), and the results presented at standard conditions (temperature 273.15°K and 101.325 kPa), including standard deviations of the measurements.

RESULTS AND DISCUSSION

Biogas is probably one of the oldest biological products in the world, and it has a considerable potential to substitute for at least part of the global oil consumption. The current practice of biogas production is to feed the substrates continuously into the digesters at flow rates allowing retention times of about 30 days. The long retention time demands large reactors, a major drawback to industrial development of biogas processes. Hence, efforts to reduce retention time are continuously attempted. In the present study, novel methods of encapsulation were tested to reduce retention time while retaining high cell density in the reactors.

Digestion by Bacteria Encapsulated in Natural Membranes

The capsules formed with the liquid-droplet-forming technique and natural membrane (Talebnia 2008), were spherical with an average diameter of 4.3 mm and a membrane thickness of about 0.2 mm.

Carboxymethyl cellulose (CMC), the added anionic polymer, strengthens the beads formed (Jokinen et al. 2006; Watanabe et al. 2008). CMC is frequently used as a thickener, binder, stabilizer, suspending, and water-retaining agent in many applications (Pilizota *et al.* 1996). CMC and $CaCl_2$ solutions are usually used together with sodium alginate in encapsulation techniques to improve capsule stability and membrane structure (Park and Chang 2000; Talebnia *et al.* 2005). It was expected that CMC with different degrees of substitution of the cellulose structure as well as different concentrations of $CaCl_2$ solution, would exhibit different characteristics. Hence, the digesting bacteria were in this experiment suspended in a $CaCl_2$ solution, containing CMC of DS 0.7 and 0.9 to produce capsules. Anaerobic digestions were then performed, using the bacteria encapsulated in both types of capsules.

Chitosan is a positively charged polymer, and thus it can replace Ca^{2+}, functioning as counter-ions during the formation of the capsules. Chitosan has many applications in the food industry, agriculture, pulp and paper industry, cosmetic, and toiletries, as well as wastewater treatment (Ilium 1998). As a functional material, chitosan offers a unique set of characteristics. These include biocompatibility, biodegradability to harmless products,

nontoxicity, physiological inertness, antibacterial properties, chelation of heavy metal ions, gel forming properties, hydrophilicity, and a remarkable affinity to proteins (Krajewska 2004). In addition, chitosan has been applied for the encapsulation technology in combination or as second layer coating with another polymer in order to improve the stability of capsules (Talebnia et al. 2005; Yoo et al. 1996).

In order to study the effect of chitosan on the digestion process, another setup of the experiment was performed. The capsules were placed in a chitosan solution for 24 h, allowing Ca^{2+} to be replaced by the chitosan, acting as counter ions to the alginate. After this procedure, digestions were carried out by both Ca-alginate and chitosan-alginate encapsulated bacteria.

Methane production by the encapsulated bacteria during 6 days in batch reactors was analysed. A synthetic medium, containing acetate, butyrate, propionate, methanol, and glucose as carbon sources (at pH 7), was used. The results are summarized in Fig. 3.

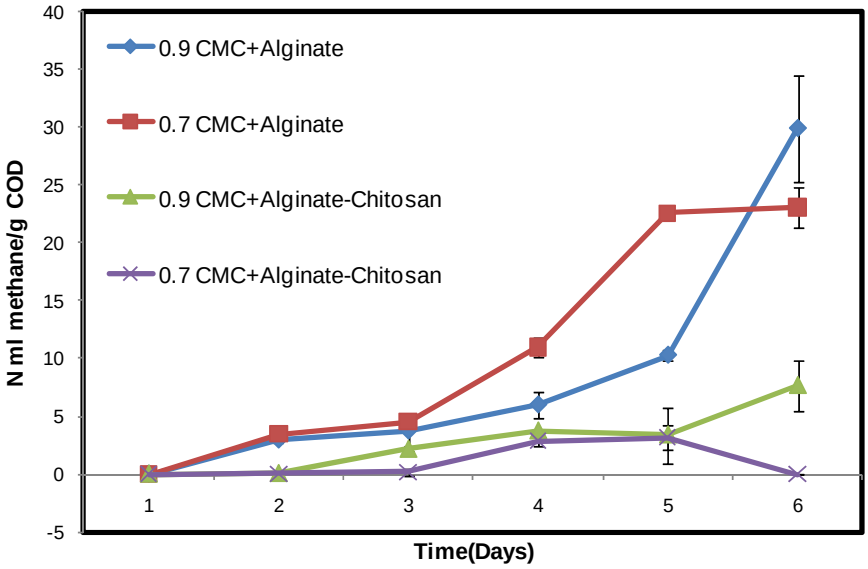

Fig. 3. Accumulated methane production by digesting bacteria, encapsulated in different natural membranes

Among the different treatments of Ca-alginate capsules, the effect of the cellulose substitution degree was of specific interest. Carboxymethyl cellulose (CMC) was added to increase the viscosity of the initial solution so as to facilitate the formation of spherical capsules. The methane production of encapsulated bacteria was affected by the degree of substitution of the cellulose structure. On the 6^{th} day the methane production by encapsulated beads, with CMC of DS 0.7 and 0.9 was 23.06 mL/g COD and 29.92 mL/g COD, respectively. CMC has previously been used for encapsulation as a supporting material (Yoshioka et al. 1990). CMC is a cellulose derivative with carboxymethyl groups (-CH_2-COOH) bound to some of the hydroxyl groups of the glucopyranose monomers. The functional properties of CMC are determined by the DS and the chain length of the cellulose backbone structure as well as by the degree of clustering of the carboxymethyl substituent (FAO/WHO Food standard 2011). The results show that the digesting bacteria

in capsules produced using higher DS have higher production rates. This may be attributed to the higher stability of these capsules. In contrast, the digestion by bacteria in chitosan-alginate capsules was not as successful (Fig. 3). This may be explained by the fact that the chitosan had an inhibitory effect on the bacteria (Kong et al. 2010; Krajewska 2004; Liu et al. 2004; Tikhonov et al. 2006).

The study of the effect of different $CaCl_2$ concentrations on the encapsulation process revealed that the methane production performed by the encapsulated bacteria increased in all experiments during the digestion period (Fig. 4), and the gas bubbles developing during incubation resulted in a sharp increase of methane production.

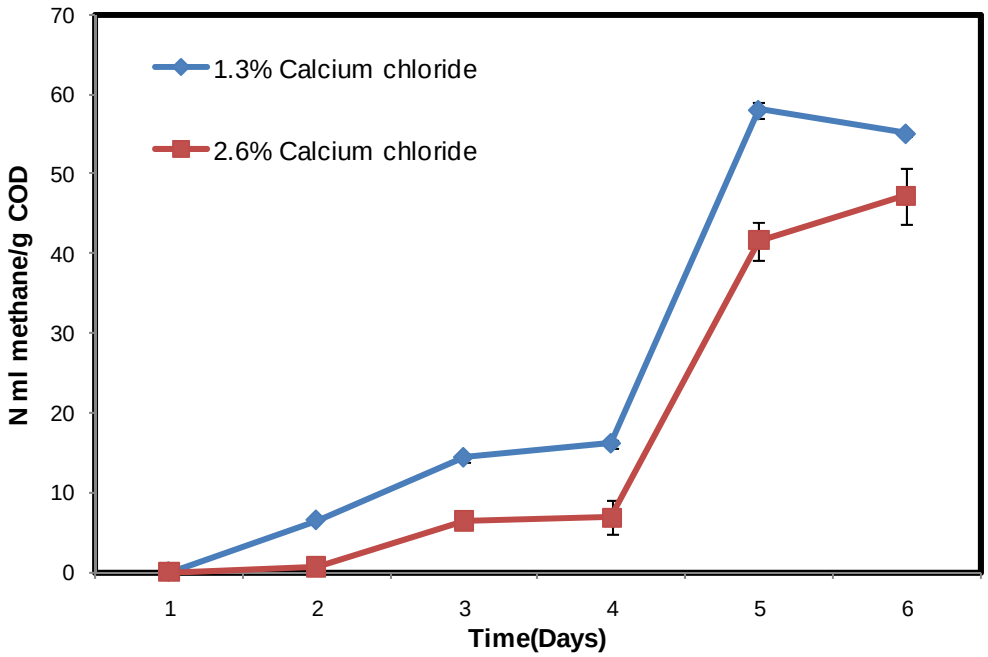

Fig. 4. The effect of different calcium chloride concentrations on the methane production by bacteria in CMC-Alginate capsules under thermophilic conditions

The addition of 1.3% $CaCl_2$ resulted in the largest methane volume of 58.04 mL/g COD on the 5^{th} day of incubation, but the capsules had less stability than the ones in 2.6% $CaCl_2$. After the 5^{th} day, methane started to be produced slowly due to the limitation of substrate added, and their membrane stability decreased. These results show the $CaCl_2$ concentration to have an effect on the membrane structure and consequently on the methane production.

Digestion by Bacteria Encapsulated in Synthetic Membranes

The purpose of using synthetic membrane filters as supporting material for methane production was to increase the methane production and to extend the stability of the produced capsules during digestion. No leakage of inoculum was observed after sealing and during the digestion. The results of this experiment are summarized in Fig 5.

The capsules swell immediately after the first day in the digesters. This means that the biogas was produced inside the capsules and then released out. The results

illustrate that methane was produced continuously from the beginning until the last day of the digestion experiment. The biogas increased continuously up to the 6th day, and then slightly decreased after that until the 15th day of digestion (Fig 5). Depending on the substrate used, 80 to 90 percent of methane potential is typically produced during the first week. However, some organic matter may be slowly degradable (Hansen et al. 2004). The maximum volume of methane was observed on the 6th day of digestion and was produced by encapsulated bacteria with the sachet capsule size of 3×6 cm^2. The maximum volume (173.77 ml/g COD) was larger than what was produced by bacteria in the other capsules that day (133.56, 13.73 and 12.74 ml/g COD for 3x3, 3x3 blank and 3x6 blank, respectively). The results reveal that methane was produced in the capsules of different sizes made of Durapore® membrane filter, and that the capsules remained stable after 15 days of digestion.

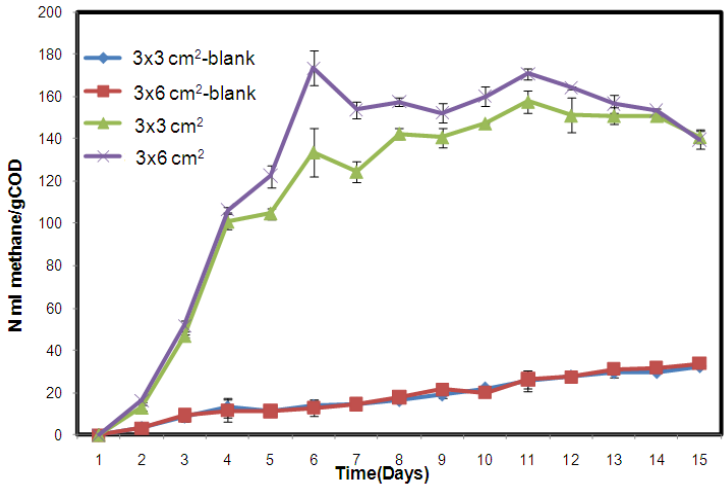

Fig. 5. Accumulated methane production using different sachet sizes of PVDF filter membranes

CONCLUSIONS

1. Digestions using natural and synthetic membranes capsules were both successful. This method can be used to avoid washout of the slow-growing methanogens from the digester during rapid digestions.
2. The largest volume of methane was produced by bacteria encapsulated in alginate-based membrane, as compared to alginate-chitosan-based material in their capsule membrane.
3. Using CMC with a DS of 0.9 in the alginate-based capsules increased the methane volume more than CMC with a DS of 0.7. Treatment with 1.3% $CaCl_2$ as the counter-ion resulted in higher methane volume in comparison to 2.6% $CaCl_2$.
4. The larger methane volume was produced from encapsulated bacteria in capsule size of 3×6 cm^2, as compared to capsule size of 3×3 cm^2.

5. The results show that methane production from encapsulated digesting bacteria using natural and synthetic membranes was successful, but the capsules from Durapore® membrane filter exhibited a higher stability in the digester than the alginate-based capsule, and that it is feasible to develop the encapsulation technology for biogas production. However, further research is required to investigate the potential of different kinds of membranes for cell encapsulation, in order to improve the industrial development of biogas production.

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