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Colonization of Supports by Methanogenic and Sulfate Reducing Bacteria in Anaerobic Filter-Type Reactors

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ABSTRACT. Competition between methanogenic bacteria (BPM) and sulfate reducing bacteria (BRS) for acetate and hydrogen has been a limiting factor in the process of anaerobic digestion. Since has been shown that BPM have a comparatively greater capacity for adhering to surfaces, immobilization of these bacteria on support media may be a method for favoring their presence. This work has studied the formation of biofilms by BPM and BRS trophic groups on ceramic and polyethylene support materials. The results suggested that the hydrogenotrophic BPM attained growth rates on ceramic of 0.061 h^{-1} and on polyethylene of 0.030 h^{-1}, with counts of 5.5 x 10^7 Most Probable Number (MPN) ml^{-1} on ceramic and 1.1 x 10^8 MPN ml^{-1} on polyethylene. These values were significantly higher than those of other trophic groups among the methanogens which were studied (acetotrophs, formatotrophs, and methylaminotrophs). In all cases the BRS attained lower values of maximum growth rate than the BPM. Rates for acetotrophic BRS were 0.009 h^{-1} on ceramic and 0.008 h^{-1} on polyethylene, with counts of 6.7 x 10^6 MPN ml^{-1} on ceramic and 4.2 x 10^6 MPN ml^{-1} on polyethylene. Statistical analyses showed these values to be significantly higher ($p \leq 0.05$) than values for hydrogenotrophic BRS. In both BPM and BRS communities, the proportions of their respective trophic groups in suspension (no supports) were different from those observed in systems containing supports, on which they formed biofilms. It was observed that hydrogenotrophic and methylaminotrophic BPM were those best retained on ceramic and polyethylene.
Keywords: methanogenesis, sulfate reduction, biofilms, anaerobic filter

ABSTRACT. La competencia entre bacterias metanógenas (BPM) y bacterias sulfato reductoras (BRS) por el acetato e hidrógeno ha sido un factor limitante en el proceso de la digestión anaeróbica. Desde que se demostró que las BPM tienen una mayor capacidad para adherirse a superficies, la inmovilización de esas bacterias sobre soportes puede ser un método para favorecer su presencia. Este trabajo estudió la formación de biofilms por grupos tróficos de BPM y BRS sobre soportes de material cerámico y polietileno. Los resultados sugieren que las BPM hidrogenotróficas alcanzó velocidades de crecimiento sobre cerámica de 0.061 h^{-1} y sobre polietileno de 0.030 h^{-1}, con cuentas de 5.5 x 10^7 Número Más Probable (MPN) ml^{-1} sobre cerámica y 1.1 x 10^8 MPN ml^{-1} sobre polietileno. Esos valores fueron significativamente más altos que los de otros grupos tróficos metanógenos que fueron estudiados (acetótrofos, formatótrofos y metilaminótrofos). En todos los casos los BRS alcanzaron valores más bajos de la velocidad de crecimiento máxima que los BPM. Las velocidades para acetotróficos BRS fueron 0.009 h^{-1} sobre cerámica y 0.008 h^{-1} sobre polietileno, con cuentas de 6.7 x 10^6 MPN ml^{-1} sobre cerámica y 4.2 x 10^6 MPN ml^{-1} sobre polietileno. Análisis estadístico demostró a esos valores ser significativamente más altos ($p \leq 0.05$) que los valores para BRS hidrogenotróficos. En comunidades de BPM y BRS, las proporciones de sus respectivos niveles tróficos en suspensión (sin soporte) fueron diferentes de los observados en sistemas que contienen soportes, sobre los cuales formaron biofilms. Se observó que BMP hidrogenotróficas y metilotróficas fueron las que se retuvieron mejor sobre cerámica y polietileno.
Palabras clave: metanogénesis, reducción de sulfato, biofilms, filtro anaeróbico.

INTRODUCTION

The treatment of anaerobic sulfate-containing effluents may be accomplished by one of, or a combination of the two following bacteria-based methods: a) sulfate reduction, and b) methanogenesis.[10] The result of competition between sulfate reducing (BRS) and methanogenic (BPM) trophic groups may on one hand define the biogas produced, and on the other, determine the feasibility of methanogenic treatment of a given wastewater.[18] The concentration of sulfate in fisheries wastewaters (>1.2 gl^{-1}) favors sulfate reduction and may cause problems of corrosion given the presence of H_2S during application of the anaerobic process most suited to the treatment of these ef-

fluents.[2,4] The permanence and activities of the BRS and BPM groups in biomass batch reactors, among other things, depends on the characteristics of the support component (nature, hydrophobicity, ability to add micronutrients) as well as on the adherence characteristics of the bacteria which form these communities (capacity for formation of exopolysaccharides and membrane proteins).[15]

Studies in the literature have demonstrated that the reduction in Chemical Oxigen Demand (COD) and methanogenic activity during anaerobic treatment of fisheries effluents are feasible[2,18] and have established the presence of BPM in systems with biofilms in sulfide-generating environments.[6,7] The preceding implies that BPM may subsist and compete with BRS in the presence of sulfates. It has also been reported that the methylamine consuming BPM may survive and compete, given that the BRS do not consume methylamine.[6,9] The proportion of each group (BRS, BPM) within support-immobilized or planktonic populations in the presence of sulfate is unknown.

The objective of the present study was to determine the effect of the type of support on the adhesion of the BPM and BRS trophic groups. To this end, two support materials were tested including, a) a ceramic, representing a material of natural origin which was hydrophobic and capable of releasing micronutrients, and, b) polyethylene, an artificial, hydrophobic material.

MATERIALS AND METHODS

Inoculum. The anaerobic inoculum was obtained from a reactor which had been operative for 24 months in the Department of Microbiology (University of Concepción, Chile). This anaerobic suspended biomass reactor is maintained at 37°C and functions continuously, with agitation at 120 rpm. System feed includes fisheries waste products and presently functions with a residence time of 10 days, with 90% removal of DQO. It produces a biogas with methane fractions above 0.8 .

Assay systems and model culture media. Amber-colored 50 ml vials were employed, containing 25 ml each of model effluent and closed with butyryl caps and aluminum seals. Support material tested included ceramic spheres of 0.5 cm diameter and pieces of polyethylene 0.5 cm in diameter and 1 cm in length (Bioblock, Denmark), at a displacement of 1 ml per culture vial. The model effluent was prepared on the basis of Balch-3 medium[4] and on the fisheries effluent characteristics of Aspé *et al*[2] to give the following composition: 7.0 g COD l^{-1}; 2 g SO_4^{-2} l^{-1}; 9 g Na^+ l^{-1}; 18 g Cl^- l^{-1}; 250 g NH_4^+ l^{-1}; 900 g of total N l^{-1}; and 66.2 g PO_4^{-3} l^{-1}. Preparation of the material was carried out in an anaerobic environment, continuously gassed with a mixture of 80% N_2 and 20% CO_2. Redox potential below -300 mV was obtained by the addition of Na_2S x 9 H_20 (final concentration 0.025%). Control systems were run not containing support materials. Systems were inoculated for 10 days at 37°C with constant agitation at 120 rpm, in an atmosphere of 80% CO_2:20% H_2 .

Counts of planktonic bacteria and sessile bacteria on support materials. Planktonic bacteria not associated with support materials were counted by obtaining serial dilutions from the liquid phase in the test vials. For analysis of sessile bacteria, each support material was washed with the anaerobic solution described by Kataoka *et al*[8] to eliminate non-adherent bacteria. Adhered biomass was resuspended into 10 ml of the same solution by means of ultra-sonication (Ultrasonic Cleaner 10-W) for 30 s.[21] Serial dilutions were made from this suspension, employing the Most Probable Number (MPN) technique.[1] Balch-3 media was used for counts of bacteria of the BPM trophic group, omitting tripticase and adding acetate, formate, trimethylamine, and hydrogen as sole electron donors for determination of acetotrophic, methylaminotrophic, formatotrophic and hydrogenotrophic BPMs.[14] Total counts of BRS were obtained using the selective, differential medium described by Sharma and Hobson[14] plus acetate to determine acetotrophs, and 80% H_2:20% CO_2 to determine hydrogenotrophs.

Measurement of methane. Detection and quantification of methane was carried out by gas chromatography using a HACH-Carle ® Series 100 AGC instrument (HACH Co. , Loveland, Co., USA).

Data analyses. Duration of the lag phase (λ) , growth rate (μ) and maximum count in the stationary phase (A) was carried out on counts over time of planktonic cells and cells associated with supports of each trophic group using the Gompertz model.[12] The treatment effect was studied using growth rate of the biofilm by means of analysis of variance. Adjustment of the growth model and analysis of variance were computed using a Systat version 5.0 program.[20]

RESULTS

Results on growth rates (μ) and maximum counts (A) obtained for the BPM group are given in Table 1 and for the BRS group in Table 2. The results showed there were no significant differences among the growth rates of methanogenic groups without supports (suspended cells).

In contrast, there were differences in counts at the beginning of the stationary phase (A) where the methylaminotrophic and hydrogenotrophic BPM reached values in excess of 10^8 MPN l^{-1} (Table 1). In systems where bacteria were adhered to ceramic and polyethylene supports, the growth rates and total counts were greater for the hydrogenotrophic BPM, followed by the acetotrophic BPM (Table 1). Methanogenic formatotrophs and acetotrophs did not constitute more than 10% of the total methanogenic population, estimated as a simple sum of the counts of all the trophic groups at the beginning of the stationary phase (Table 1).

Within the BRS group, the acetotrophs were dominant in suspended biomass systems (2.1 X 10^7 MPN ml^{-1}); a

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Table 1. Growth rates (m± 95% confidence interval) and maximum stationary-phase counts (A ± 95% confidence interval) of trophic groups of suspended and support-attached methanogenic bacteria (BPM) obtained by fit to the Gompertz model.[12]

	Treatement					
	No Support		Ceramic		Poliethylene	
Trophic Group	μ (h^{-1})	A (NMP) Resuspended Cells ml^{-1}	μ (h^{-1})	A (NMP) Resuspended Cells ml^{-1}	μ (h^{-1})	A (NMP) Resuspended Cells ml^{-1}
BPM-Ac	0.028 ± 0.006	$5.1 \times 10^7 \pm 1.8 \times 10^7$	0.014 ± 0.004	$2.9 \times 10^6 \pm 1.0 \times 10^6$	0.020 ± 0.005	$9.7 \times 10^6 \pm 3.7 \times 10^6$
BPM-F	0.025 ± 0.012	$1.9 \times 10^6 \pm 6.7 \times 10^5$	0.012 ± 0.005	$3.6 \times 10^6 \pm 7.9 \times 10^5$	0.020 ± 0.005	$1.3 \times 10^7 \pm 5.5 \times 10^6$
BPM-H	0.028 ± 0.009	$4.4 \times 10^8 * \pm 1.5 \times 10^8$	0.061*± 0.022	$5.5 \times 10^7 * \pm 1.4 \times 10^7$	0.030 ± 0.004	$1.1 \times 10^8 * \pm 3.0 \times 10^7$
BPM-M	0.029 ± 0.001	$5.6 \times 10^8 * \pm 2.9 \times 10^8$	0.033 ± 0.006	$1.8 \times 10^7 \pm 5.8 \times 10^6$	0.029 ± 0.007	$1.9 \times 10^7 \pm 1.2 \times 10^7$

Ac, Acetotrophic; F, Formotrophic; H, Hydrogenotrophic; M, Methylaminotrophic; (*), significant difference ($p \leq 0.05$)

Table 2. Growth rates (μ ± 95% confidence interval) and maximum stationary-phase counts (A ± 95% confidence interval) of trophic groups of suspended and support-attached sulfate reducing bacteria (BRS) obtained by fit to the Gompertz model.[12]

	Treatement					
	No support		Ceramic		Poliethylene	
Trophic Group	μ (h^{-1})	A (NMP) Resuspended Cells ml^{-1}	μ (h^{-1})	A (NMP) Resuspended Cells ml^{-1}	μ (h^{-1})	A (NMP) Resuspended Cells ml^{-1}
BRS-Ac	0.004*± 0.0008	$2.11^7 \pm 1.8 \times 10^7$	0.009 ± 0.001	$6.7 \times 10^6 \pm 1.0 \times 10^6$	0.008 ± 0.002	$4.2 \times 10^6 \pm 3.7 \times 10^6$
BRS-H	0.002 ± 0.0007	$3.4 \times 10^5 \pm 6.7 \times 10^5$	0.004 ± 0.002	$1.3 \times 10^6 \pm 7.9 \times 10^5$	0.004 ± 0.0002	$9.4 \times 10^6 \pm 5.5 \times 10^6$

Ac, Acetotrophic; F, Formotrophic; H, Hydrogenotrophic; M, Methylaminotrophic; (*), significant difference ($p \leq 0.05$)

similar trend was observed in systems with ceramic and polyethylene supports, with the acetotrophic group 95% higher than recorded for the hydrogenotrophs (Table 2).

No significant differences were observed between growth rates of acetotrophic BRS attached to ceramic and polyethylene supports, where they showed magnitudes of 0.008 to 0.009 h^{-1} . Similarly, the values of (m) for the hydrogenotrophic BRS (0.004 h^{-1}, Table 2) were not different between biofilms on ceramic and polyethylene substrates, although they were significantly less than values for acetotrophic BRS (Table 2).

DISCUSSION

The analyses of kinetic parameters and growth of the BPM and BRS trophic groups showed that the methylaminotrophic and hydrogenotrophic populations were those which more readily colonized the ceramic and polyethylene surfaces (Tables 1, 2). Efficacy of the supports for retention of biofilms may be due to the high specific surface and hydrophobicity of the polyethylene, and the capacity for liberation of micronutrients by the ceramic substrate[11,16]

These results suggested that in spite of the high sulfate concentration described for fisheries effluents[2,3] the activity of the methanogenic bacteria in these sulfhydryl-generating environments may be sustained by the presence of non-competitive substrates such as the methylated amines.[9] This is in agreement with observations made on marine sediments by Ollivier *et al.*,[12] where microorganisms isolated were mostly methylaminotrophic BPM species. The preceding suggests that although the methanogenesis may not be sustained exclusively by methylaminotrophic bacteria, these may indeed form an important part of the methanogenic community in sulfide-generating environments, particularly in those where sulfate-rich resi-

dues are treated anaerobically.[16,18]

The principal precursor source of methylated amines in marine ecosystems is that of the degradation of glycine-betaine and trimethylamine oxide , which are both typically found in the tissues of marine organisms, including fishes.[9,16]

In conclusion, and in accordance with present results, use of ceramic material as inert material in fixed-bed reactors may permit the formation of biofilms enriched with methylaminotrophic BPM, and may be a good alternative for improving methanization and diminishing the activity of BRS.

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REFERENCES

1. APHA. 1985. Standard methods for the examination of water and wastewater. 16th edition, American Public Health Assoc. Washington D.C.
2. Aspé, E., M. C. Martí and M. Roeckel. 1997. Anaerobic treatment of fishery wastewater using a marine sediment inoculum. Water Res. 31:2147-2160.
3. Aspé, E., M. Roeckel, M. Martí, M. Durán, and S. Fuentes. 1994. Actas del XI Congreso de Chileno de Ingeniería Química. 18-11 de Noviembre 1994. Universidad de Concepción. Chile
4. Balch, W. E., G. E. Fox, L. J. Magrum. C. R. Woese, and R. S. Wolfe. 1979. Methanogens: reevaluation of a unique biological group. Microbiol. Rev. 43:260-296.
5. Colleran, E., S. Finnegan, and P. Lens. 1995. Anaerobic treatment of sulfate containing waste streams Antonie van Leeuwenhoek 67:29-46.
6. Gibson, G. R. 1990. Physiology and ecology of the sulphate-reducing bacteria. J. Appl. Bacteriol. 69:769-797.
7. Isa, Z., S. Grusenmeyer, and W. Verstraete. 1986. Sulfate reduction relative to methane production in high-rate anaerobic digestion: microbiological aspects. Appl. Environ. Microbiol. 51:580-587.
8. Kataoka, N., Y. Tokiwa and K. Takeda 1991. Improved technique for identification and enumeration of methanogenic bacteria colonies on roll tube by epifluorescence microscopy. Appl. Environ. Microbiol. 57:3671-3673.
9. King, G. M. 1988. Methanogenesis from methylated amines in a hypersaline algal mat. Appl. Environ. Microbiol. 54:130-136.
10. Lettinga, G. 1995. Anaerobic digestion and wastewater treatment systems. Antonie Van Leeuwenhoek 67:3-28.
11. Muñoz, M. A., J. M. Sanchez, J. M. Rodriguez-Maroto, M. A. Moriñigo and J. Borrego. 1994. Evaluation of the use of sepiolite to optimize the methanogenesis from anaerobic domestic sludges in laboratory conditions. Water Res. 28:195-200.
12. Ollivier, B., P. Caumette, J. E. García, and R. A. Mah. 1994. Anaerobic bacteria from hypersaline environments. Microbiol. Rev. 58:27-38.
13. Oren, A. 1990 Formation and breakdown of glycine betaine and trimethylamine in hypersaline environments. Antonie Van Leeuwenhoek 58:291-8.
14. Sharma, V. K., and P. N. Hobson. 1987. A convenient method for detecting sulfate-reducing bacteria. Lett. Appl. Microbiol. 5:9-10.
15. Sticker D. 1999. Biofilms. Curr. Opin. Microbiol. 2:270-275.
16. Urrutia H., R. Vidal, M. Baeza, J. Reyes, and E. Aspe. 1997. Growth of methylaminotrophic, acetotrophic and hydrogenotrophic bacteria isolated from anaerobic fermentors using artificial supports. Microbiología SEM. 13:209-240.
17. Veiga, M. C., R. Méndez, and J. M. Lema. 1992. In Melo, L.F., Bott, T.R., Fletcher, M. and Capdeville, B. (eds) Biofilms Science and Technology. Kluwer Academic Publishers (Netherlands), pp 421-434.
18. Vidal, G., E. Aspé, M. C. Martí, and M. Roeckel. 1997. Treatment of recycled wastewaters from fish meal factory by anaerobic filter. Biotechnol. Lett. 19:117.
19. Visser, A., I. Beeksma, F. Van Der Zee, A. J. Stams, and G. Lettinga. 1993. Anaerobic degradation of volatile fatty acids at different sulphate concentrations. Appl. Environ. Biotech. 40: 549-556.
20. Wilkinson, L. 1990. Systat. The system for statistics. Evanston II: Systat, Inc.
21. Yoon, W. B. and R. A. Rosson. 1990. Improved method of enumeration of attached and free-living bacteria in response to a diel variations in seawater turbidity Appl. Environ. Microbiol. 56:595-600.
22. Zwietering, M. H., F. M. Rombouts and K. van't Riet 1992. Comparison of definitions of the lag phase and the exponential phase in bacterial growth. J. Appl. Bact. 72:139-145.