Appl Microbiol Biotechnol (1992) 37:408–410

Applied and Microbiology Biotechnology

Short contribution

Effect of an anaerobic bacterial consortium isolated from termites on the degradation of olive-mill waste-water

M. Hamdi*, A. Brauman, and J. L. Garcia

Laboratoire de Microbiologie Orstom, Université de Provence, 3 Place Victor-Hugo, F-13331 Marseille cédex 3, France

Received 24 January 1992/Accepted 11 February 1992

Summary. A microbial consortium obtained by enrichment culture on syringate of termite gut material was used to improve the anaerobic degradation of olive-mill waste-water (OMW). Addition of the consortium (1/4 v/v) to the control inoculum originating from waste-water sludge, increased methane production by 50% over the control during anaerobic digestion of OMW prefermented by *Aspergillus niger*. This increase was related to enhanced acetate production in the presence of the consortium. When OMW was not prefermented by *A. niger*, no improvement in methane production was observed, indicating that the aerobic degradation of inhibitory substances is needed for the consortium to express its potential.

Introduction

Olive black water is a waste-water from the manufacture of olive oil. The polluting organic material can lead to 45–55 kg biological oxygen demand per ton of olives, whatever the process used (Balice et al. 1982). The organic fraction includes sugars, tannins, polyphenols, polyalcohols, pectins, and lipids (Fiestas Ros de Ursinos 1981). The anaerobic digestion of this waste is severely inhibited by high concentrations of simple aromatic compounds (Andreoni et al. 1986). The removal of phenolic compounds has already been achieved aerobically using an *Aspergillus niger* strain (Hamdi et al. 1991).

Termites are the only animals in which significant lignin degradation has been shown (Butler and Bucherfield 1979). An anaerobic bacterial enrichment culture on syringic acid was performed from ground hind-guts of soil-feeding termites (Brauman 1989). Since syringate is a predominant aromatic compound in olive waste-water (Balice and Cera 1984), we have tested the effect of the inoculation of such an enrichment culture on the methanogenic fermentation of this waste.

Materials and methods

Source of syringate-degrading consortium. Nests of *Cubitermes* sp., a soil-feeding termite, were collected from Mayombe tropical rain forest of the Congo. Termites were removed less than 2 h after sampling using sterile forceps. Their hind-guts were removed, transferred and kept at 5° C in Hungate tubes (Bellco Glass, Vineland, N. J., USA) containing 5 ml medium of Widdel (1980) and gased with N_2/CO_2 (80/20).

Medium and culture conditions for syringate-degrading consortium. The Hungate tubes were transferred into an anaerobic glove box (La Calhène, Bezons, France). The gut walls were disrupted in a 5-ml sterilized tissue homogenizer for 5 min, then transferred into a 60-ml serum bottle containing 15 ml of Widdel culture medium with the following composition in g/l: KH_2PO_4, 0.2; NH_4Cl, 0.3; KCl, 0.5; NaCl, 1; $CaCl_2 \cdot 2H_2O$, 0.15; $MgCl_2 \cdot 6H_2O$, 0.4; 1 ml/l of trace element solution (Imhoff-Stuckle and Pfennig 1983) and 1 ml/l of resazurin (0.1%, w/v) were added. The medium was adjusted to pH 7.0 with KOH and boiled under O_2-free N_2. After cooling to room temperature, 20 ml medium was transferred into 60-ml serum bottles inside the glove box. The bottles were stoppered with black butyl rubber closures (Bellco) and outgassed with N_2/CO_2. After sterilization (110° C, 35 min), the following sterile solutions were added to each bottle using one-way syringes: 0.25 ml $NaHCO_3$ (10%, w/v); 0.2 ml $Na_2S \cdot 9H_2O$ (4%, w/v); 0.2 ml vitamin solution (10%, w/v) (Pfennig 1978) and 50 µl Na_2SeO_3 (0.3%, w/v). Half of the medium was replaced with 10 mM sterilized and neutralized anoxic syringate solution.

Successive enrichment cultures were obtained by transferring 10–20% of agitated cultures into fresh medium. For kinetic measurements, 1 ml of culture was removed every day in 1.5-ml Eppendorf tubes, centrifuged and frozen before analysis. For experiments on olive-mill waste-water (OMW), the last enrichment culture was transferred into appropriate flasks. All operations were carried out using the technique of Hungate (1960) as modified for the use of syringes (Macy et al. 1972).

To test the physiological properties of the consortium, cells (10%, v/v inoculum) were grown in anaerobic Hungate tubes at 35° C in Widdel medium supplemented with 10 mM substrate. Growth was measured by the optical density at 580 nm. The presence of methanogenic bacteria was detected by optical microscopic observations using the epifluorescence technique at 420 nm (Doddema and Vogels 1978).

* *Present address:* Centre de Biotechnologie, BP W, 3038 Sfax, Tunisia

Correspondence to: J. L. Garcia

Medium and culture conditions for OMW degradation. The medium consisted of filtrate of crude OMW or OMW prefermented by *A. niger* strain A10 (Hamdi and Garcia 1991) during 72 h at 35° C in erlenmeyer flasks. Filtrates of crude OMW are known to contain mainly monomeric phenolic compounds (Balice and Cera 1984); filtrates of prefermented OMW contain metabolic products of the growth of *A. niger* such as carboxylic acids and polyols (Hamdi 1991). The medium was adjusted to pH 7.5 with $Ca(OH)_2$ and used in experiments for methane production in 60 ml serum bottles containing 10 ml medium and 10 ml inoculum with N_2/CO_2 as the gas phase. The inoculum was obtained from a 3-l laboratory anaerobic filter digestor continuously fed with prefermented OMW and inoculated with waste-water sludge (Hamdi et al. 1991).

Analytical methods. Volatile fatty acids and methane were analysed by gas chromatography as described elsewhere (Hamdi 1991; Hamdi and Garcia 1991). Syringate was analysed by HPLC (Brauman 1989). Chemical oxygen demand (COD) was measured by the method of Knechtel (1978).

All chemicals used were of reagent quality.

Results

Physiological properties of syringate-degrading consortium

Within 7 days 5 mM of syringic acid were degraded, mainly to acetate and butyrate, at a rate of 0.8 mM/day. No methane was detected in the enrichment cultures after five transfers. This was confirmed by the lack of fluorescent bacteria in microscopic observations under epifluorescence at 420 nm.

Some aromatic compounds and other characteristic substrates from prefermented OMW (Hamdi et al. 1991) were tested for fermentation by the syringate-degrading consortium. Gallate, syringate, caffeate, vanillate, glycerol, fumarate, malate, pyruvate, glucose and fructose served as substrates. Table 1 compares the methane evolution from nine aromatic substrates after inoculation with (1) sludge from a laboratory digestor and (2) the same sludge enriched with the syringate-degrading consortium (v/v). In all cases, methane production increased in the presence of the syringate-degrading consortium.

Table 1. Comparative methane evolutions from aromatic substrates after inoculation with sludge from a laboratory digestor (S) and the same sludge enriched with the syringate-degrading consortium (SDC) (v/v)[a]

Substrates (1 g/l)	Methane production in 28 days (μM)	
	S	S+SDC
Caffeic acid	81.6	118.9
Gallic acid	176.6	211.3
p-Hydroxyphenyl acetate	8.5	28.5
Protocatechuic acid	30.1	45.2
Pyrogallol	184.7	215.4
Syringic acid	224.1	276.2
Tannic acid	111.3	211.3
Vanillic acid	98.7	145.4
Veratric acid	190.3	224.3

[a] Experiments were performed in 60-ml serum bottles containing 10 ml Widdel medium with an aromatic substrate and 10 ml inoculum. The gas phase was N_2/CO_2

Effect of addition of the syringate-degrading consortium on methane production from OMW

Experiments with a range of consortium/inoculum ratios in serum bottles with prefermented OMW showed that the optimum ratio was between 2 and 6 ml of the consortium for 10 ml total inoculum. For the following experiments, 2 ml of consortium culture was used.

Microscopic observations of the inoculum under epifluorescence at 420 nm showed the presence of methanogenic species similar to the genera *Methanobacterium*, *Methanogenium*, *Methanospirillum* and *Methanosarcina*. The consortium/inoculum mixture was more efficient at decreasing the black colour of prefermented OMW than the inoculum alone.

The effects of the addition of the syringate-degrading consortium on methane production from crude OMW and OMW prefermented by *A. niger*, at two COD concentrations, are reported in Fig. 1. The addition of the consortium only had a positive effect with prefermented OMW. Increase of methane production by 50% was ob-

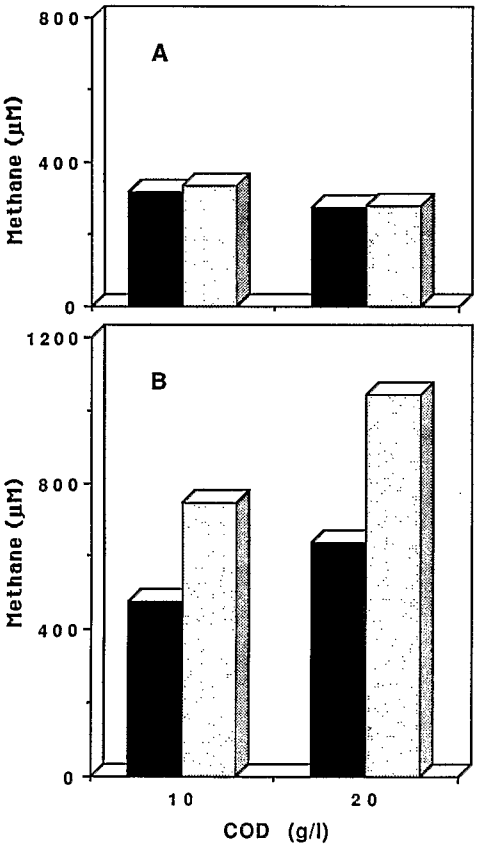

Fig. 1. Methane evolution from crude (**A**) or *Aspergillus niger* prefermented (**B**) olive-mill waste-water, at two chemical oxygen demand (COD) concentrations, in the absence (■) or presence (□) of the syringate-degrading consortium

tained with the consortium on prefermented OMW as compared with this substrate without the consortium.

A strictly anaerobic bacterium using syringate in pure culture has been isolated and is being studied in our laboratory.

Discussion

Syringic acid was the major aromatic compound found in OMW (Balice and Cera 1984); it was degraded by the consortium into acetate, the main precursor of methane (Zehnder et al. 1982), as well as the other aromatics of OMW. A consortium concentration of one fifth of the inoculum was sufficient to obtain the highest methane production on prefermented OMW, less toxic and more biodegradable than crude OMW, as shown previously (Hamdi 1991). So the efficacy of the consortium in degrading aromatic compounds was expressed only when the inhibitory compounds were removed from the culture medium by aerobic growth of the fungus. Indeed, anaerobic digestion of OMW can be carried out only on diluted and/or pretreated substrate because aromatic compounds and lipids are more toxic than is raw waste (Hamdi 1991). The decrease in the black colour of prefermented OMW by the consortium/inoculum mixture may be due to the degradation of darkly coloured polymers such as humic acids by the bacteria of the consortium. The degradation of several other aromatic compounds of prefermented OMW by the consortium, after growth of *A. niger*, accelerated the process of methanization. Acetate produced during the prefermentation of OMW contributed to the additional production of methane.

Our results show that improvement of methanogenesis during the anaerobic degradation of a polluting waste-water is possible using a specific inoculum oxidizing recalcitrant compounds present in the waste.

Acknowledgements. We thank B. Ollivier for helpful discussions, and P. Roger for his comments on the manuscript.

References

Andreoni V, Ferrari A, Ranali G, Sorlini C (1986) The influence of some phenolic acids present in oil mill waters on microbial groups for the methanogenesis. In: Proceedings of International Symposium on olive Byproducts Valorization, FAO, 5–7 March, Spain

Balice V, Cera O (1984) Acidic phenolic fraction of the juice of olives determined by gas chromatographic method. Grasas Aceites 25:178–180

Balice V, Boari G, Cera O, Abbaticchio P (1982) Indagine analitica sulle acque di vegetazione. Nota 1. Inguinamento 7–8:49–53

Brauman A (1989) Etude du métabolisme bactérien de termites supérieurs à régimes alimentaires différenciés. Doctoral Thesis, University of Aix-Marseille II, France, p 168

Butler JHA, Bucherfield JM (1979) Digestion of lignin by termites. Soil Biol Biochem 11:507–513

Doddema HJ, Vogels GD (1978) Improved identification of methanogenic bacteria by fluorescence microscopy. Appl Environ Microbiol 36:752–754

Fiestas Ros de Ursinos JA (1981) Différentes utilisations des margines. In: FAO (ed) Proceedings of Séminaire international sur la valorisation des sous-produits de l'olivier, Monastir, Tunisie, pp 93–110

Hamdi M (1991) Effects of agitation and pretreatment on the batch anaerobic digestion of olive mill wastewater. Biores Technol 36:173–178

Hamdi M, Garcia JL (1991) Comparison between anaerobic filter and anaerobic contact process for fermented olive mill wastewaters. Biores Technol 38:23–29

Hamdi M, Khadir A, Garcia JL (1991) The use of *Aspergillus niger* for the bioconversion of olive mill wastewaters. Appl Microbiol Biotechnol 34:828–831

Hungate RE (1960) Microbial ecology of rumen. Bacteriol Rev 24:353–364

Imhoff-Stuckle D, Pfennig N (1983) Isolation and characterization of a nicotinic acid-degrading sulfate-reducing bacterium, *Desulfococcus niacini* sp. nov. Arch Microbiol 136:194–198

Knechtel RJ (1978) A more economical method for the determination of chemical oxygen demand. Water Pollut Control:25–29

Macy JM, Snellen JE, Hungate RE (1972) Use of syringe methods for anaerobiosis. Am J Clin Nutr 25:1318–1323

Pfennig N (1978) *Rhodocyclus purpureus* gen. nov. and sp. nov., a ring-shaped, vitamin B_{12}-requiring member of the family *Rhodospirillaceae*. Int J Syst Bacteriol 28:283–288

Widdel F (1980) Anaerober Abbau von Fettsäuren und Benzoesäure durch neu isolierte Arten Sulfat-reduzierender Bakterien. Doctoral thesis, University of Göttingen

Zehnder AJB, Ingvorsen K, Marti T (1982) Microbiology of methane bacteria. In: Hughes DE, Stafford DA, Weathley BI, Baader W, Lettinga G, Nyns EJ, Verstraete W, Wentworth RL (eds) Anaerobic digestion 1982, Elsevier Biomedical Press, Amsterdam–New York–Oxford, pp 45–68