Study of methods for the cultivation of anaerobic cellulose-degrading bacteria

K Rampersad, LA Goldstone and GN Tivchev*

Centre for Water and Wastewater Research, Department of Biotechnology, Technikon Natal, PO Box 953, Durban 4000, South Africa

Abstract

A preliminary investigation of the use of different media for isolation and cultivation of anaerobic, cellulolytic bacteria was undertaken. Plant material, paper and leachate samples from a landfill site as well as a soil sample from a compost heap were processed and various media inoculated using anaerobic techniques. Cellulose degradation was obtained using anaerobic cellulolytic media for mesophiles at 10^{-1} and 10^{-2} dilutions at a temperature of 37°C and a pH ranging from 7 to 7.2. Results were obtained after 7 to 14 d.

Introduction

Landfills have become highly engineered facilities designed to contain and separate refuse from the environment, capture leachate, and control gas migration. It is a habitat where microflora are located in a fixed position and subject to the flow of solutions (Barlaz, 1997). Optimising waste degradation hastens stabilisation and reduces long-term liabilities with regard to management responsibilities long after the site has been closed (Cummings and Stewart, 1994).

Municipal refuse contains 40 to 50% cellulose in the form of plant and paper material (Barlaz et al., 1989). Cellulose therefore forms an important part of the decomposition process. The potential for converting cellulosic waste into industrial substrate has stimulated current interest in cellulose fermentation (Sleat et al., 1984). Despite the importance of cellulose hydrolysis for energy generation and possible pollution from landfill leachate and gas production, very little is known about cellulolytic bacteria in landfills (Westlake et al., 1995).

During disposal of refuse, oxygen is rapidly depleted and an anaerobic ecosystem is established (Barlaz et al., 1994). The ecology of cellulose degradation in anaerobic environments is very complex (Leschine, 1995) since anaerobic bacteria are often specialised in terms of the substrates they utilise (Cummings and Stewart, 1994). Cellulose is difficult to mineralise in the natural environment. It consists of repeating glucose units coupled by glycosidic linkages to form a crystalline molecule. Most work on its biodegradation has been conducted with a filamentous fungus *Trichoderma reesei* (Broda, 1992). Decomposition of cellulose with subsequent methane production requires the co-ordinated activity of several trophic groups of bacteria (Barlaz, 1997). Three types of enzymes control the degradative process viz., endoglucanases, cellobiohydrolases and the ß-glucosidases respectively. The first step is hydrolytic cleavage of the cellulose molecule exposing the long chain-reducing ends. The cellobiose is further degraded to glucose monomers (Wood and McRae, 1977). In this ecosystem tolerance to a variety of toxins, efficient substrate transfer utilisation and a range of other factors will select for particular species.

*To whom all correspondence should be addressed.
☎ (031) 204-2299; fax (031) 204-2714; e-mail: tivchev@mariner.ntech.ac.za
Received 9 February 1998; accepted in revised form 5 June 1998

The present study was a preliminary investigation into the isolation and cultivation of cellulolytic bacteria and is intended as a basis for future, in-depth studies performed on anaerobic cellulose-degrading bacteria from landfill sites.

Materials and methods

Sample collection and processing

Collecting of samples

A one-year-old refuse (±30 kg) and leachate (±20 ℓ) sample were obtained from Springfield Park Landfill Site, from a depth of ± 3 m. This site has a natural clay layer, preventing the seepage of leachate to the groundwater and a moisture content within the refuse body of 40%. The refuse sample was collected in a 60 ℓ bucket which was filled, to ensure a minimum air space and subsequently to maintain low oxygen levels. Leachate was filled to the brim of an aspirator. The bulk refuse and leachate sample was stored at 2°C. A 50 g soil sample was obtained from deep layers of a compost heap. All samples were collected with the use of excavation equipment.

Processing of samples for microbiological analysis

A ±20 g plant and paper sample obtained from the one-year-old refuse was weighed and homogenised for 60 s in a 1 ℓ blender previously flushed with oxygen-free nitrogen. It was transferred to an anaerobic stomacher bag containing anaerobic phosphate buffer (23.7 mM, pH 7.2) (Barlaz, 1997). The phosphate buffer was flushed with oxygen-free nitrogen but a reducing agent was omitted because some oxidation occurs during the inoculum formation procedure and the oxidised form of cysteine hydrochloride exerts toxicity (Holdemann et al., 1977). To ensure that the buffer remained anaerobic, it was prepared in a glove-box (10.90% CO_2, 10.30% H_2, 78.08% N_2), flushed with oxygen-free nitrogen, indicator added and stored in the glove-box for 24h. before use. Refuse was homogenised for 60 s and the liquid extract obtained after filtration through cheesecloth was used as an inoculum. It was serially diluted to 10^{-3}. A volume of 9 mℓ of distilled water was added to 1 g of a compost sample, composed mainly of decaying plant material. It was shaken for 5 min and serially diluted to 10^{-3}.

TABLE 1
DEGRADATION OF FILTER PAPER IN ENRICHMENT MEDIUM

Sample	10^{-1}	10^{-2}	10^{-3}
Paper	Degradation and gas production	Degradation, yellow pigmentation and gas production	Degradation, yellow pigmentation and gas production
Plant	Degradation and gas production	Degradation and gas production	Gas production
Leachate	Degradation and gas production	Degradation and gas production	Degradation and gas production
Compost	Slight degradation	Gas production	Gas production

TABLE 2
CELLULOLYTIC ACTIVITY IN PREREDUCED MEDIA

Media	Growth	Temperature (°C)	Cellulolytic activity
Cellulolytic medium for thermophiles	+	65	-
Anaerobic cellulolytic medium	+	37	+
P-A medium	+	37	-
Cellulose Congo-red agar	+	37	-
MS medium	+	33 and 65	-

- no cellulolytic activity
+ sparse growth/cellulolytic activity

TABLE 3
EFFECTS OF DEGRADATION IN ANAEROBIC MEDIUM, pH 7.06, TWO WEEKS AFTER SUB-CULTURING FROM ENRICHMENT MEDIUM

Sample	10^{-1}	10^{-2}	10^{-3}
Paper	Complete degradation	Partial degradation	Partial degradation
Plant	Complete degradation	Partial degradation	Partial degradation
Leachate	Complete degradation	Partial degradation	Partial degradation
Compost	Partial degradation	Partial degradation	No degradation

Media preparation

Media were prepared in a modified Schott bottle implemented by Rogers (1990). The redox indicator, resazurin, 0.1% (w/v) and cellulose powder, Whatman CF 11, were included in the cellulolytic medium. Liquid medium contained Whatman No. 1 filter paper strips. After autoclaving the medium, 1 mℓ 0.05% (w/v) cysteine hydrochloride used as a reducing agent was added through an injection port on the Schott bottle. Media were flushed for 10 min before and after autoclaving with laser CO_2. A change in media colour from blue, when resazurin is added and is in the oxidised state, to colourless denoted reduction of the medium. This should occur after autoclaving and addition of cysteine hydrochloride while gassing.

Cultivation of anaerobic cellulolytic bacteria

Six different media were used for the cultivation process i.e. MS medium (Boone et al., 1989), enrichment medium (Shapton and Board, 1971), cellulolytic medium for thermophiles, anaerobic cellulolytic medium (Atlas and Parks, 1993), cellulose Congo-red agar (Hendricks et al., 1995) and P-A medium (*Standard*

Methods, 1989).

Filter paper strips were added to 20 mℓ of broth in MacCartney bottles. Samples were incubated for 14 d at mesophilic temperatures (30-37°C) in a glove-box and thermophilic temperatures (60 to 65°C) in anaerobic bags, which contained oxygen-free nitrogen. Cellulose degradation was determined by visible observation i.e. the production of a yellow pigment and "fluffing" of the filter papaer. Once cellulolytic activity was evident in the broth medium, 1 mℓ of this inoculum was transferred onto cellulolytic agar plates. Cellulolytic activity was characterised by zones of clearing around colonies on agar plates.

Results and discussion

When enrichment medium was inoculated with paper samples at different dilutions the results showed a yellow pigmentation and fluffing of the filter paper with discrete gas production (Table 1). Similar results were obtained by Weimer and Zeikus (1977), who stated that the above characteristics were initial evidence of cellulose fermentation by *Clostridium thermocellum*. Although this organism is a thermophile the above characteristics were achieved at mesophilic temperatures ranging from 30 to 37°C. The long incubation time (30 d) required to obtain similar results as those obtained at thermophilic temperatures could be attributed to the mesophilic temperatures used.

The results presented in Table 2 demonstrate that of the four media utilised, anaerobic cellulolytic medium for mesophiles was most effective in producing cellulose degradation in broths. When inoculum and control, *Clostridium cellulovorans* (ATCC strain no. 35296), were transferred onto anaerobic cellulolytic medium, no zones of clearing were evident. This may be attributed to factors such as insufficiently reduced media or particle size of the cellulose.

Sodium sulphide added to the medium caused blackening and a sudden increase in pH from 7.06 to 12.00. Several attempts to determine the cause of blackening were undertaken. It was found that sodium sulphide did not react with the cellulose powder but possibly reacted with salt present in the medium. Effects of blackening on cellulolytic growth were not determined as the medium was not initially inoculated. Consequently, addition of the reducing agent was avoided. Successful prereduced media were obtained using the method outlined in **Materials and Methods.**

The cellulose Congo-red agar produced by Hendricks et al. (1995) for the easy visibility of zones of clearing around colonies on agar plates was not previously used under anaerobic conditions. The present study shows that this medium did not perform well in an anaerobic environment as the growth obtained did not show expected morphological characteristics which were red colonies with zones of clearing around them.

According to Sleat et al. (1984), growth may have been more rapid at elevated temperatures but the experiments carried out contradicted these findings as cellulolytic growth was more prevalent at mesophilic temperatures. Further experiments for the optimisation of the solid media are in progress.

Table 3 shows that neutral pH levels are suitable for cellulose degraders. The pH tolerance was as expected for bacteria isolated from a methane-producing landfill as methane production is strongly dependent on pH. It is important that the media are sufficiently buffered as acidic conditions inhibit the growth of the majority of cellulose degrading bacteria (Shapton and Board, 1971).

Ball-milling cellulose powder may increase the extent of degradation (Rosenberg, 1993). This should be investigated in future work. Since cellulolytic activity cannot be determined on solid media, the testing for the presence of cellulase in broths can also be researched, using the cellulose-azure method implemented by Palmisano et al. (1993).

Conclusion

The investigation indicates that anaerobic cellulolytic medium for mesophiles prepared in Rogers modified Schott bottles is suitable for the isolation and cultivation of anaerobic, cellulolytic bacteria. The use of prereduced media facilitated the growth of these bacteria.

Acknowledgements

The authors would like to thank the Water Research Commission for funding the study and to the personnel at the Springfield Park Landfill Site for assistance. Gratitude is also expressed towards Mr V Singh at the University of Durban Westville and Mr G Govender at Tongaat Huletts Sugar for their invaluable assistance.

References

ATLAS RM and PARKS LC (1993) *Handbook of Microbiological Media*. CRC Press, USA. 71-247.

BARLAZ MA (1997) *Microbial Studies of Landfills and Anaerobic Refuse Decomposition*. ASM Press, Washington. 67-69.

BARLAZ MA, SCHAEFER DM and HAM RK (1989) Bacterial population development and chemical characteristics of refuse decomposition in a simulated sanitary landfill. *Appl. Environ. Microbiol.* **55** 55-65.

BARLAZ MA, WANG YS and CAROLINE SB (1994) Anaerobic biodegradation of cellulose and hemicellulose in excavated refuse samples using a biochemical methane potential assay. *J. Ind. Microbiol.* **13** 147-153.

BOONE DR, JOHNSON RL and LIU Y (1989) Diffusion of the interspecies electron carriers H_2 and formate in methanogenic ecosystems and its implications in the measurement of K_m for H_2 or formate uptake. *Appl. Environ. Microbiol.* **55** (7) 1735-1741.

BRODA P (1992) *Biotechnology in the Degradation and Utilization of Lignocellulose.* Kluwer Academic Publisher, Netherlands. 95-114.

CUMMINGS SP and STEWART CS (1994) *A Study of Cellulose Degradation in Landfills.* Rowert Research Institute, Aberdeen. 1-37.

HENDRICKS CW, DOYLE JD and HUGLEY B (1995) A new solid media for enumerating cellulose-utilizing bacteria in soil. *Appl. Environ. Microbiol.* **61** (5) 2016-2019.

HOLDEMANN LV, CATO EP and MOORE WEC (1977) *Anaerobic Laboratory Manual* (4th edn.). Blacksburg, USA. 7.

LESCHINE SB (1995) *Cellulose Degradation in Anaerobic Environments*. Annual Review Inc, Massachusetts. 400 pp.

PALMISANO AC, MARUSCIK DA and SCHWAB BS (1993) Enumeration of fermentative and hydrolytic microorganisms from three sanitary landfills. *J .Gen. Microbiol.* **139** 387-391.

ROGERS GM (1990) Imunolocalisation of Xylanases Produced by *Clostridium xylanolyticum* during Anaerobic Degradation of *Pinus patula*. Ph.D. Thesis, University of Witwatersrand, Johannesburg.

ROSENBERG E (1993) *Microorganisms to Combat Pollution.* Kluwer Academic Publishers, USA. 176 pp.

SHAPTON DA and BOARD RG (1971) *Isolation of Anaerobes*. Academic Press, New York. 67 pp.

SLEAT R, MAH RA and ROBINSON R (1984) Isolation and characterisation of an anaerobic, cellulolytic bacterium, *Clostridium cellulovorans. Appl. Environ. Microbiol.* **48** (1) 88-93.

STANDARD METHODS (1989) *Standard Methods for the Examination of Water and Waste Water* (17th edn.). American Public Health Association. Port City Press, New York. p 75.

WEIMER PJ and ZEIKUS JG (1977) Fermentation of cellulose and cellobiose by *Clostridium thermocellum* in the absence and presence of *Methanobacterium thermoautotrophicum*. *Appl. Environ. Microbiol.* **33**(2) 289-297.

WESTLAKE K, ARCHER DB and BOONE DR (1995) Diversity of cellulolytic bacteria in landfill. *J. Appl. Bact.* **79** 73-78.

WOOD TM and McRAE S (1977) The mechanism of cellulase action. *Ind. Inst. Technol.* **1** 111-141.