SHORT COMMUNICATIONS

Identification and Characterization of a Cellulolytic Isolate

By A. KAUFMANN, J. FEGAN, P. DOLEAC, C. GAINER,
D. WITTICH AND A. GLANN

Department of Life Sciences, St Mary's University, San Antonio, Texas 78284, U.S.A.

(Received 14 July 1975; revised 21 November 1975)

INTRODUCTION

One method proposed for the disposal of solid waste is composting, which involves grinding the material, mixing it with sewage sludge and allowing it to undergo microbial decomposition. This method permits disposal without additional danger to our environment. Since refuse contains as much as 50 % paper (*Municipal Refuse Disposal*, 1961), cellulose degradation is an important part of the decomposition process. This paper describes the identification and characterization of a cellulolytic bacterium isolated from solid waste. The conditions necessary for the maximum production of the cellulolytic enzyme, susceptibility of various substrates to the enzyme action, and the end products of cellulose hydrolysis are also reported.

METHODS

Isolation and maintenance media. For preliminary screening of refuse material for cellulolytic bacteria, the mineral salts medium described by Han & Srinivasan (1968) was used. Platings were done on the cellulose mineral salts agar medium described by Skinner (1960), modified by substituting microcrystalline cellulose (Avicel, FMC Corporation, Newark, Delaware, U.S.A.) for filter-paper powder and by adding 0.1 % (w/v) yeast extract. The cellulolytic isolate was maintained at 4 °C on cellulose agar.

Isolation procedure. Samples of solid waste material (5.0 g) obtained from a composting plant (Johnson City, Tennessee, U.S.A.) were macerated and dispensed into flasks containing 95 ml of sterile saline. After overnight incubation at 30 °C 10 ml portions of supernatant fluid were inoculated into mineral salts medium (100 ml) with filter-paper strips (100 mg) as carbon source. Samples were shaken on a reciprocal shaker for 3 to 10 days or until visible breakdown of the strip was observed. Dilutions of the supernatant were plated on cellulose agar, and plates were incubated at 31 °C and examined periodically for colonies which showed areas of clearing. Representative colonies were transferred to nutrient broth, allowed to grow for 24 h, diluted and plated again on cellulose agar. This procedure was repeated until a pure culture of the cellulolytic bacterium was obtained.

Cellulose substrates. The following substrates were used: carboxymethylcellulose (CM-cellulose: type 7HF, Hercules Inc., Wilmington, Delaware, U.S.A.), cellulose powder (Whatman chromedia CF11), cellobiose (Difco), filter paper (Whatman no. 1), paper bags, newspaper and paper towel.

Characterization and identification of the organism. The isolate was characterized by standard biochemical and morphological techniques. The procedure of Keddie, Leask & Grainger (1966) was used to test for vitamin requirements, and carbon substrate utilization

Table 1. *Morphological and biochemical properties of C. flavigena and the cellulolytic bacterium*

Property	<i>C. flavigena</i>	Cellulolytic isolate
Morphological characteristics:		
Shape	Irregular rod	Irregular rod
Size (μm)	$0.5 \times 0.7-2.0$	$0.4 \times 2-2.5$
Growth in broth	Moderate growth	Moderate growth
Characteristic growth on agar slant	Yellow pigment	Scant grey
Motility	Positive	Positive
Optimal growth temperature	30°C	$28-33^\circ\text{C}$
Gram stain	Variable	Variable
Filter-paper degradation	Fibres separate on agitation	Fibres separate on agitation
Gelatin stab	Slowly hydrolysed	Slowly hydrolysed
Biochemical characteristics:		
Glucose	Acid	Acid
Lactose	—	Acid
Maltose	—	Acid
Sucrose	—	Acid
Arabinose	—	Acid
Xylose	—	Acid
Dulcitol	—	Negative
Glycerol	—	Negative
Mannitol	—	Negative
Acetyl methyl carbinol	Negative	Negative
Starch hydrolysis	Positive	Positive
Nitrate reduction	Positive	Positive
O_2 requirement	Facultative	Facultative
Catalase	Positive	Positive
Ammonia production	—	Negative
Biotin and thiamine required	Positive	Positive
D-Ribose	Negative	Negative
Acetate	Positive	Negative
Succinate	Negative	Negative
Ethanol	Negative	Negative
Propionate	Negative	Negative

was determined by the method of Stainer, Palleroni & Doudoroff (1966). The organism was identified using *Bergey's Manual of Determinative Bacteriology* (1974).

Crude enzyme preparation. Cell-free filtrates containing the enzyme were prepared by the procedure of Han & Srinivasan (1968) and stored at 4°C until used.

Gravimetric assay of enzyme activity. A culture of the organism was added to flasks containing the basal salts medium and 100 to 300 mg of various cellulosic materials. Flasks were incubated for 10 to 14 days with shaking at 31°C , and then processed using the procedure of Lembeck & Colmer (1967).

Colorimetric assay of enzyme activity. Cellulase activity in cell-free filtrates were determined by the procedure of Nelson (1944). Reducing sugars were measured as glucose and all reaction mixtures consisted of 9.8 ml of 1.0% (w/v) CM-cellulose and 0.2 ml of crude enzyme solution. A unit of enzyme activity is defined as the amount of enzyme that releases 1.0 μequiv of hydrolysed glycosidic bonds/min per ml of filtrate. Extinction measurements were made with a Spectronic 20 colorimeter (Bausch & Lomb) at 550 nm.

Determination of the end products of cellulose utilization. Reaction mixtures consisting of a cellulosic substrate and crude enzyme were analysed for hydrolytic products using paper chromatography. Before spotting, hydrolysates were passed through two ion-exchange resin columns (Dowex 20W-X8, Dowex 1-8) to minimize streaking. Samples from the

Table 2. *Degradation of various cellulosic substrates by the soil isolate*

The organism was inoculated into 100 ml of media containing various substrates (100 to 300 mg) as the sole carbon source, and incubated at 31 °C for 10 to 14 days on a reciprocal shaker. The degree of substrate solubilization was measured gravimetrically.

Substrate	Incubation time (days)	Initial wt (mg)	Final wt (mg)	Solubilization of cellulose (%)
Avicel (microcrystalline cellulose)	12	103.8	55.0	47.0
Cellulose powder	10	116.4	110.4	5.2
Filter-paper strips	10	114.0	88.3	22.5
Newspaper	12	283.5	187.8	33.7
Biodegradable bag	14	121.5	100.3	17.5
Paper towel	12	103.3	62.0	39.8

Fig. 1. The effect of temperature (●) and pH (○) on the cellulase activity of the cellulolytic isolate.

column were evaporated to dryness and resuspended in 1.0 ml water. Approximately 15 to 30 μ l of material was spotted on the filter paper (Whatman no. 1), dried and developed ascendingly for 12 to 16 h with tertiary butanol–glacial acetic acid–water (3:1:1, by vol.). The chromatogram was dried at room temperature, sprayed with an aniline–oxalate mixture (0.9 g oxalic acid in 200 ml water and 1.8 ml alinine), and heated to 80 °C for 3 min. Reducing sugars were detected as brown spots.

RESULTS AND DISCUSSION

Until recently, 10 species of *Cellulomonas* were recognized (*Bergey's Manual of Determinative Bacteriology*, 1957), but at present, *Cellulomonas flavigena* is the only accepted species (*Bergey's Manual of Determinative Bacteriology*, 1974). Table 1 shows a limited comparison of the morphological and biochemical properties of the isolate and *C. flavigena*. There are some differences between the two organisms, but agreement is generally good. The susceptibility of the various cellulosic substrates to the action of the cellulolytic strain is shown in Table 2: Avicel showed the greatest degree of solubilization (47 %) and cellulose powder the least (5.2 %).

Figure 1 shows the effect of pH and temperature on cellulase activity; pH and temperature ranges of 6.5 to 7.5 and 45 to 50 °C respectively were necessary for maximum enzyme activity. Stutzenberger, Kaufmann & Lossen (1970) have shown that the temperature and pH optima of compost extracts are 65 °C and 6.0. It seems likely that only organisms possessing enzymes active at these values would make a significant contribution to the breakdown of cellulosic materials, so our cellulolytic isolate probably plays only a minor role in cellulose degradation during composting.

The effect of substrate concentration was determined by incubating, at 50 °C, 0.2 ml of crude enzyme (0.25 mg protein/ml) with 9.8 ml of CM-cellulose at concentrations up to 14.7 mg/ml. After 60 min, 1.0 ml samples were removed and the reducing sugar measured as glucose. Substrate concentrations in excess of 4.9 mg/ml (0.5 %, w/v) saturated the enzyme and gave a maximum rate of 38 µg glucose/h per 0.05 mg enzyme protein.

End products of cellulose hydrolysis were identified by ascending paper chromatography. In hydrolysates of filter paper and cellulose powder, both glucose and cellobiose were detected, suggesting that the initial hydrolytic attack results in the production of cellobiose, and this is further degraded to glucose and transported and metabolized by the cell.

This investigation was supported by a grant from the Texas Natural Resources Foundation. The authors wish to thank Dr O. W. Van Auken for technical assistance and Dr B. Machia for his photographic assistance.

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