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Anaerobic digestion of cellulose by pure and mixed bacterial cultures

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SUMMARY

By enrichment technique, nine anaerobic mixed bacterial cultures were isolated, five of which showed stable cellulolysis. All cultures fermented cellulose and produced different fermentative products. Mixed culture BOC 25 yielded major levels of acetate and ethanol (39.6 and 12.0 mmol/l, respectively) and minor levels of propionate (2.5 mmol/l) and digested filter paper cellulose to the extent of 32.5% w/v. BOC 25 digested cellulosic and lignocellulosic substrates and produced filter paper cellulase, carboxymethyl cellulase, Avicelase and β-glucosidase. Strain DC 25, a cellulolytic *Clostridium* was purified from one of the mixed cultures. The fermentation products of DC 25 from cellulose, cellobiose or glucose were ethanol, acetate, formate, H_2 and CO_2.

INTRODUCTION

Anaerobic digestion is involved in sewage and industrial waste treatment and is proved to be an efficient way of converting organic matter to methane and carbon dioxide. Development of anaerobic mixed cultures was attempted for the production of commercially important acids, alcohols, enzymes, etc. from cellulosic wastes [7,13,20]. However, there have been few detailed studies to date on the isolation and characterization of mixed anaerobic cellulolytic culture systems. Among the pure cellulolytic organisms, the fungus *Trichoderma reesei* [24], the thermophilic anaerobic bacterium *Clostridium thermocellum* [23,24] and a few rumen cellulolytic organisms have been extensively studied. A few pure mesophilic cellulolytic anaerobes are important in industrial applications involving coupled fermenta-

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tions with non-cellulolytic *C. acetobutylicum* [30] and *Zymomonas mobilis* [14] to produce solvents.

In this paper, we describe a variety of cellulose enrichments, pattern of production of different cellulose digesting enzymes on various cellulosic and lignocellulosic substrates and isolating/preliminary characterization of a cellulolytic *Clostridium* from one of the enrichments designated BOC 25.

MATERIALS AND METHODS

Chemicals

Filter paper (Whatman No. 1); yeast extract (Oxoid Ltd., U.K.); carboxymethyl cellulose (high viscosity, BDH Chemicals Ltd., U.K.); cellulose pulver for chromatography (Schleicher and Schull, F.R.G.); Avicel (PH 105, Serva, F.R.G.); anthrone (E. Merck, India); *p*-nitrophenyl β-D-glucoside (Bioorganics, India).

Enrichment and isolation of cellulolytic organisms

For most experiments mineral medium supplemented with yeast extract (YE) (0.1% w/v) as described by Madden [16] was used. Liquid and solid media were anaerobically prepared in serum vials as described by Miller and Wolin [19]. Deoxygenated nitrogen gas was used for flushing the vials and filter sterilized solutions of cysteine hydrochloride and sodium sulphide each at 0.025% w/v were used for reducing the medium. 1% w/v filter paper (Whatman No. 1) cut into strips of 4.5 cm by 1.0 cm was used as sole carbon source. Pre-reduced and autoclaved media were adjusted to pH 7.0 with 1 N NaOH.

Samples were collected from the following sources: Dungs of herbivorous animals (buffalo, elephant, horse); decayed grass, leaf, dung, saw dust and scrap, municipal garbage composts which were subject to anaerobic decomposition; decayed garbage mixed with sewage water, etc. About 500 mg of samples were added as inoculum to 50 ml medium in 100 ml serum vials and incubated at 37°C without agitation for 13–20 days or until visible gas formation, appearance of turbidity and acidity in the culture and a visible decrease in the amount of cellulose occurred. Inoculum (1–2 ml) was transferred into 50 ml fresh liquid cellulose medium in 100 ml serum vials once in two weeks and were subcultured about 15–21 times. Cultures showing stable cellulolysis after several subcultures in broth were spread on cellulose and cellobiose agar slants in serum vials. Single colonies were picked and tested for utilization of cellulose and cellobiose. Cellulolytic pure and stable mixed cultures grown for 15 days in FP cellulose broth were stored at 10°C and subcultured monthly.

Identification and characterization of cellulolytic pure culture

Morphological features were tested as described by Doetsh [4]. The procedures of Holdeman, et al. [9] were used for biochemical characterization. Utilization of different carbohydrates by the pure cellulolytic strain was tested in 10 ml mineral medium [16] with YE (0.1% w/v) and 0.5% w/v sugar incubated at 37°C without agitation. The carbohydrates were autoclaved at 120°C for 10 min. or filter sterilized and added to the medium separately. Cells grown on cellobiose (0.5% w/v) broth for 30 h were used as inoculum. Bergey's Manual [3] and Anaerobe Laboratory Manual [9] were used for the identification of the strain.

Growth measurement

Growth was followed by measuring optical density (OD) at 560 nm in a colorimeter (Baush and Lomb Spectronic 20 with 1 cm light path). Cultures grown on insoluble cellulose were filtered through Whatman No. 1 filter paper to remove undigested substrate and cells were harvested by centrifugation at 12 000 × *g* for 20 min at 4°C. The cell pellet was dried at 90°C for 48 h and dry cell weight (g/l) was determined. From control experiments it was observed that there was no significant adherence of cells to substrates.

Cellulose utilization

1% w/v processed cellulosic substrates and lignocellulosic substrates with YE (0.1% w/v) were added to mineral medium. The cultures (30 ml in 100 ml serum vials) were incubated at 37°C with agitation.

To estimate the quantity of processed cellulosic substrate digested, the cellulose content was assayed by the Anthrone-H_2SO_4 method [32] after filtering and collecting the residue. Carboxymethyl cellulose (CMC) being soluble substrate was assayed without filtering by the same method [32].

Analysis of fermentation products

Hydrogen gas in the head space of culture vials was analyzed using Gas chromatograph (Aimil-Nucon Series, India) fitted with a Molecular Sieve 13X (60/80 mesh) column, 1.8 m by 2 mm (ID) and thermal conductivity detector. The column temperature was 80°C and the carrier gas was nitrogen at a flow rate of 35 ml/min. Injector and detector temperatures were 150°C. CO_2 gas was determined by trapping it as Na_2CO_3 in 0.1 N NaOH and titrated against standard acid [2].

To determine volatile acids and alcohols, acidified cell free broth was analysed using Chromosorb 101 (60/80 mesh) column, 1.8 m by 2 mm (ID) and flame ionization detector. The injector, column and detector temperatures were 180°C, 160°C and 180°C respectively. The carrier and fuel gases were nitrogen and hydrogen with flow rates of 30 and 35 ml/min, respectively. Lactate was determined colorimetrically as described by Muller [21]. Formate was assayed by the method of Sleat and Mah [25].

Enzyme assays

Cultures were centrifuged at $15\,000 \times g$ for 20 min at 4°C. The supernatant was used for the assay of cellulase enzymes. Carboxymethyl cellulase (CMCase) as endo-1,4-β-D-glucanase (1,4-(1,3;1,4)-β-D-glucan 4-glucano hydrolase, EC 3.2.1.4) and filter paper cellulase (FPase) were determined by the method of Andreotti [1]. β-glucosidase (β-D-glucoside gluco hydrolase, EC 3.2.1.21) was assayed by the method of Wood [33] using *P*-nitrophenyl β-D-glucoside as substrate. Avicelase was determined as described by Ng et al. [23] using 0.5 ml of 0.05 M ammonium acetate buffer (pH 5.0), 0.5 ml culture filtrate, 2 mg Avicel and incubated at 50°C for 2 h. The sugar released was assayed by DNS method. The units of CMCase and β-glucosidase activities are expressed as 1 μg glucose or *p*-nitrophenol equivalents released per min. and FPase and Avicelase activities as 1 μg glucose equivalent released per hour. Reducing sugar was determined using 1 ml sample; 1 ml of DNS reagent and 10 ml distilled water by the method of Miller [18]. Soluble protein was assayed by the method of Lowry, et al. [15].

Effect of pH and temperature on growth

Cultures on cellobiose medium (0.5% w/v) supplemented with YE (0.1% w/v) were incubated at different temperatures. To determine the effect of pH, the initial pH of the medium was adjusted aseptically with 1 N NaOH. Cultures were incubated at 37°C without agitation. pH was not corrected during growth. Growth was estimated by measuring OD.

RESULTS

Isolation of cellulolytic mixed cultures

By enrichment technique, nine cellulolytic mixed cultures were established initially, of which five cultures (HDE 33, BOC 25, BFH 16, LCE 18 and DGE 4) utilized cellulose consistently even after three years of maintenance. These cultures were examined microscopically and were found to contain several morphotypes. These organisms when seperated from the mixed cultures could not grow on cellulose as the sole energy and carbon source. A few organisms could grow on cellobiose but not on cellulose in pure culture.

Cellulose fermentation by mixed cultures

BOC 25, one of the mixed cultures was chosen for further studies as it utilized more FP cellulose and produced high levels of fermentation products, viz., acetate, (39.6 mmol/l) and ethanol (12.0 mmol/l) compared to other cultures (Table 1). BOC 25 contained a short, straight, Gram negative, sporulating, motile rod and two other cell types that were short, Gram positive, non-motile and non-sporulating curved rods. The pH of the mixed culture decreased to 5.5 from an initial pH of 7.0.

BOC 25 also utilized newsprint, Avicel, cellulose powder (TLC grade) packing material, tissue paper

Table 1

Fermentation of cellulose by mixed cultures

Mixed culture	Origin	% FP cellulose utilization	Dry cell weight g/l	Fermentative end products (mmol/l)							
				Ethanol	Acetate	Propionate	Formate	Lactate	Butyrate	CO_2	H_2
HDE 33	Horse dung	31.8	0.29	ND	24.8	14.2	1.9	Traces	nil	15.6	5.0
BOC 25	Buffalo dung compost	32.5	0.38	12.0	39.6	2.5	Traces	ND	nil	13.5	16.0
BFH 16	Buffalo dung	31.0	0.20	ND	13.8	9.3	0.8	0.3	nil	14.8	8.0
LCE 18	Leaf compost	25.0	0.20	ND	22.8	12.7	0.2	1.2	Traces	17.1	13.4
DGE 4	Decayed grass compost	27.3	0.19	9.4	5.3	ND	Traces	2.2	nil	13.0	14.9

ND, not detectable.

and CM cellulose to the extent of 20–46% with production of ethanol (9.0–11.0 mmol/l) acetate (7.0–29.0 mmol/l) and propionate (0.6–1.8 mmol/l) respectively (data not shown).

Production of cellulase by mixed culture BOC 25

On insoluble crystalline cellulosics, viz., FP cellulose, cellulose powder and Avicel, higher levels of FPase, CMCase and β-glucosidase were produced

Table 2

Production of enzymes by mixed culture BOC 25 on different growth substrates

Substrate (1% w/v)	Culture filtrate					
	Soluble protein (μg/ml)	Reducing sugar (μg/ml)	CMCase (U/ml)	FPase (U/ml)	Avicelase (U/ml)	β-glucosidase (U/ml)
Filter paper (Whatman No. 1)	360	48	9.7	15.4	19	0.81
Cellulose Powder	432	56	8.1	13.4	15	0.76
Avicel	416	56	8.2	11.4	16	0.68
Packing material	288	28	5.7	12.9	14	0.62
News print	328	36	7.7	12.2	12	0.64
Tissue paper	432	60	6.6	10.4	11	0.64
CMCellulose	240	36	2.1	8.4	ND	0.36
Banana stalk	245	60	6.3	9.9	14	0.72
Cotton linter	240	28	3.4	7.0	8	0.47
Sugarcane bagasse	300	48	6.7	9.1	12	0.69
Holo cellulose	360	60	7.7	11.7	13	0.67
Rice husk	250	36	3.6	7.4	9	0.49

ND, not detectable.

than on soluble substrate such as CM cellulose, impure substrates such as newsprint, packing material and lignocellulosics (Table 2).

Fig. 1 shows the time course of production of enzymes and utilization of tissue paper cellulose (1% w/v) by BOC 25. Production of all enzymes increased progressively till 12–14 days of growth and declined thereafter. During this period there was correlation between the rate of cellulose utilization and the rate of increase in cellulolytic activities.

Morphological and biochemical characteristics of cellulolytic pure anaerobic bacterium DC 25

BOC 25 was plated on cellobiose-agar. Three different single colonies were picked and purified on cellobiose. Only one of these three pure cultures grew on cellulose and was designated as strain DC 25. The purity of this culture was also assessed microscopically. DC 25 was obligately anaerobic. Cells were short, straight rods and 1.5–3.7 by 0.4–0.7 μM in size when grown on cellobiose medium. The strain formed central, spherical to oval endospores. Spores were observed rarely and only in very old cultures grown in soluble sugars. Cells were motile and stained Gram negative at different stages of growth. Colonies on cellobiose medium were circular, opaque, creamy, convex with entire margin and measured 1.8 mm in diameter.

Good growth occurred in nutrient broth only when supplemented with fermentable sugar. Acetyl methyl carbinol, indole and H_2S gas were not produced. Nitrate and nitrite were not reduced and gelatin was not hydrolyzed. Milk and meat were not fermented. Gas production and sediment were observed in the broth. Catalase was negative. Glucose, fructose, arabinose, lactose, xylose, cellobiose, cellulose, ribose, galactose, mannose, mannitol, maltose, raffinose and sorbitol were fermented. Weak growth was observed on sucrose, starch, rhamnose, trehalose, melizitose and inulin. There was no growth on xylan, erythritol, amygdalin and inositol.

Rates of growth

Strain DC 25 grew on glucose and cellobiose with the doubling times of 14 h and 12 h respectively at 37°C. Yield of dry cell weights was similar on both sugars. The doubling time of DC 25 on FP cellulose was 56 h. The optimal temperature and pH for growth on cellobiose was 37°C and 7.0 respectively.

Fermentation products and cellulase enzymes of DC 25

Strain DC 25 fermented cellulose, cellobiose, or

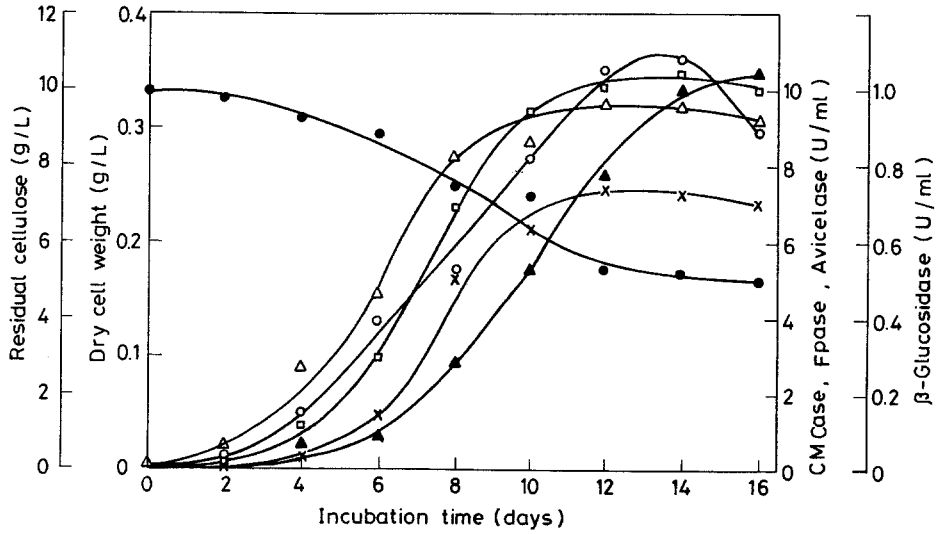

Fig. 1. The time course of cellulase enzyme production during growth of mixed culture BOC 25 on tissue paper cellulose. (△, CMCase; □, FPase; ▲, Avicelase; X, β-glucosidase; ●, residual cellulose, ○, dry cell weight.)

glucose and produced ethanol, acetate, CO_2 and H_2 as the major products of fermentation; small amounts of formate were also consistently produced. The time course of acetate and ethanol production by DC 25 was followed on FP cellulose. The highest amounts of ethanol were produced after 10 days of growth with utilization of cellulose to 29% (w/v). pH of the broth decreased from 7.0 to 5.3.

Different cellulosic growth substrates such as MN 300 cellulose, Avicel, newsprint, packing material, tissue paper, sugarcane bagasse, holo cellulose (wheat straw), and CM cellulose induced CMCase, FPase, Avicelase and β-glucosidase in DC 25. The highest levels of CMCase (7.2 U/ml); FPase (13.1 U/ml); Avicelase (16.8 U/ml) and β-glucosidase (0.62 U/ml) were induced on FP cellulose. Cellobiose and glucose induced these enzymes poorly.

DISCUSSION

Considering the fact that there is relatively little information on pure and mixed cultures of cellulolytic anaerobes from habitats other than rumen and gastro-intestinal tract of non-ruminants and sewage/anaerobic digestor, the work reported here was undertaken to isolate and characterize anaerobic consortia from different environmental sources. Mixed cultures were attempted to produce fuels and enzymes from cellulose [7,8,13,20]. There are only few reports on anaerobic natural mixed cellulolytic cultures in literature [11,13,31]. The naturally selected cellulolytic mixed cultures are advantageous and more stable than artificially constituted co- and mixed cultures, owing to their inherent synergistic metabolic interactions [5,8,13,28].

Five stable anaerobic consortia which could ferment cellulosics for over 3 years were isolated. They appear to be obligate and their different patterns of production of enzymes and fermentation products are reported. These mixed cultures were used directly as inoculum without any further processing in all our studies. This new method of using stable mixed culture as inoculum has advantages such as easy handling; less expensive maintenance of consortia over use of chemostat devices or fermentor; less chance of contamination and spurious results unlike possibility in chemostat devices; and avoidance of encountering a different environment by processing of chemostat grown mixed culture inoculum and consequent variation in cellulose utilization and product formation [5,11,12,13].

The yields of acetate, propionate and gases by mixed culture BOC 25 on cellulose is comparable to the product levels of the heat treated natural mixed culture reported by Khan, et al. [13], while that culture [13] produces only traces of ethanol, BOC 25 produces12 mmol/l of ethanol which is advantageous for further industrial application. The cellulase enzymes produced by BOC 25 are extra cellular and are comprised of endo- and exoglucanase and β-glucosidase activities whose levels appear to be regulated by the nature of growth substrates as also observed in other systems [29]. These enzymes act synergistically to hydrolyze native cellulose [33].

In order to understand how this consortium BOC 25 works, we undertook separation of the individual organisms. Of the three different morpho types constituting BOC 25, only DC 25 a pure culture, was cellulolytic. This strain DC 25 appears to be a *Clostridium* species. Characteristic properties of DC 25 was compared with the literature data available on the pure mesophilic cellulolytic clostridia, viz., *C. lochheadii, C. longisporum, C. polysaccharolyticum* [10] *C. cellulovorans,* [27], *C. populeti* [26], *C. papyrosolvens* [17] *C. cellulolyticum* [6], *C. lentocellum* [22], *C. chartatabidum* [10] and thermophilic bacteria such as *C. thermocellum* [23] and *C. stercorarium* [16]. However, DC 25 differs from all these in morphological, cultural, cellulolytic properties, utilization of sugars and in fermentative end products. Properties of DC 25 is closest to that of *C. cellobioparum* [3,9], the original type strain of which appears extinct. Fermentation by DC 25 differs from the parent mixed consortia BOC 25 in that propionate is not detectable in pure culture and levels of enzymes are slightly different.

Recent studies on production of ethanol from cellulose by micro-organisms have dealt with thermophilic bacteria [6,23,24]. There is relatively little information for comparing the mesophilic with

thermophilic fermentation systems. Furthermore, in nature, the occurrence and contribution of mesophilic bacteria to the anaerobic degradation of cellulosic wastes is more than that of thermophiles. Therefore, the pure and mixed mesophilic cultures isolated and described here are of interest for a better understanding of anaerobic cellulolysis in nature and for their potential utilization for bioconversion of cellulosic wastes to useful products.

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