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Cellulose degradation by a mixed bacterial culture

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SUMMARY

An integrated mixed bacterial culture consisting of four strains has been isolated by a batch enrichment technique. The cellulolytic member (strain D) is a *Cellulomonas* sp. and the others are non-cellulolytic. The interaction between strains D and C is pronounced and appears to involve an exchange of reducing sugars and growth factors. The symbiotic relationship of this naturally occurring mixed culture is therefore one of mutualism. The filter paper cellulase and carboxymethyl cellulase activities in extracellular fluid are high, while β-glucosidase activity is low. The mixed culture digests a variety of lignocellulosics efficiently and is of fundamental interest in the study of microbial interrelationships.

INTRODUCTION

In the last decade, intensive research has been focussed on the utilization of cellulosic wastes as raw material for the production of single cell protein (SCP), ethanol, sugars, biogas and liquid fuels by employing bacterial fermentation processes [8,21,22]. Development of mixed cultures has been attempted to increase the production of biomass, fuels and enzymes and the digestion of cellulose [11,15,16,23]. Mixed cellulolytic cultures isolated by enrichment techniques were shown to be more advantageous than those produced by mixing pure strains in the laboratory. The naturally selected mixed cultures were more stable owing to their synergistic metabolic interactions [6,14]. These metabolically coupled systems overcome feedback regulation and metabolite repression as one organism consumes the product produced by the other. We report here the isolation and characterization of a new cellulolytic bacterial mixed culture, the nature and mechanism of interaction, and the scope for utilizing this new culture for the production of SCP from natural lignocellulosic substrates.

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MATERIALS AND METHODS

Chemicals

Chemicals used and their sources include: filter paper (Whatman No. 1); yeast extract (Oxoid Ltd., U.K.); tryptone bacteriological (Loba-Chemie, India); carboxymethyl cellulose (high viscosity, BDH Chemicals Ltd., U.K.); cellulose pulver for

chromatography (Schleicher & Schull, F.R.G.); 3,5-dinitrosalicylic acid (Loba-Chemie, India); anthrone (E. Merck, India); p-nitrophenyl β-D-glucoside (Bioorganics, India).

Enrichment and isolation of cellulolytic organisms

For isolation, screening and other experiments the mineral medium described by Chandra and Shethna [5] was used. One percent (w/v) filter paper (Whatman No. 1) cut into 1 cm^2 pieces was used as sole carbon source. Wet soil samples collected from Ooty, Tamil Nadu, India from an area exposed to decayed leaves and wood in the forest, were used as source of inoculum (about 1 g soil) for 100–200 ml medium in 500-ml Erlenmeyer flasks which were incubated on a rotary shaker at 200 rpm at room temperature (33–35°C). Six to eight serial transfers were made at 10-day intervals on filter paper (FP) cellulose agar plates containing 4–5 cm diameter FP discs. An isolated colony on FP cellulose agar was picked. On plating this single colony on nutrient agar, four types of distinct colonies were observed and these were separated based upon differences in colony morphology. Purity of the strains was confirmed by microscopic examination. A cellulolytic strain grown for a week on FP cellulose agar and non-cellulolytic strains grown for 48 h on nutrient agar slants were stored at 10°C and subcultured monthly.

Identification and characterization

Morphological features were tested as described by Doetsch [7]. The procedures of Smibert and Krieg [24] were used for biochemical characterization. Ten milliliters of mineral medium without yeast extract (YE) were used to test for the utilization of different carbohydrates, organic acids and alcohols (0.5%, w/v) as sole carbon sources by strain C; these cultures were incubated at room temperature without agitation. For strain D, 0.05% (w/v) YE was added to mineral medium. The substrates were autoclaved at 120°C for 10 min or filter-sterilized and added separately. Inoculum was cells of strains A, B or C grown on nutrient broth for 24 h, then washed twice with mineral medium. For strain D, cells were grown on 0.5% (w/v) cellobiose with YE (0.05%, w/v) for 48 h and then washed twice. Bergey's Manual [2] was used for the identification of the strains.

Growth measurement

Growth was followed by measuring optical density (OD) at 560 nm. For insoluble cellulosic substrates, samples were filtered through Whatman No. 1 filter paper to remove residual cellulose and the OD of the filtrate containing cells was measured. OD was related to viable count by plate counts on nutrient agar.

Effect of temperature and pH on growth

Cultures in YE (0.05%, w/v)-supplemented FP cellulose (0.5%, w/v) medium (200 ml) were incubated at selected temperatures without agitation. To determine the effect of pH, the initial pH of the medium was adjusted aseptically with 1 N NaOH. Cultures were incubated at room temperature with agitation. pH was not corrected during growth. Growth was estimated by measuring OD.

Effect of growth factors on cellulose and cellobiose utilization

YE (0.05%, w/v), peptone (0.1%, w/v), tryptone (0.1%, w/v) and 250 μg each of vitamins (vitamin B_{12}, calcium pantothenate, thiamine-HCl, nicotinic acid, vitamin B_6, *p*-aminobenzoic acid, folic acid, biotin and riboflavin) were added to 200 ml of 0.5% (w/v) FP cellulose medium or 0.5% (w/v) cellobiose medium. Cultures were incubated at room temperature (33–35°C) with agitation. Inocula of strain D and strain C were cultured for 2 days on cellobiose – YE medium, and cells were washed twice in mineral medium before using them as inoculum.

Digestion of cellulosic substrates

Alkaline-pretreated lignocellulosic substrates (0.5%, w/v) (Table 3) or 1% (w/v) processed cellulosic substrates with YE (0.05%, w/v) were added to the mineral medium used for isolation. The cultures (100 ml in 500-ml Erlenmeyer flasks) were incubated with agitation at room temperature. Pretreatment was carried out by the addition of 40 ml of 1% (w/v) NaOH to 1.0 g of lignocellulosic sub-

strate for 1 h at 120°C; the suspension was washed to neutrality and dried at 95°C to constant weight. To estimate the quantity of lignocellulosic substrate digested, the entire contents of the flask were filtered through preweighed Whatman No. 1 filter paper and the residue was washed twice with distilled water to remove cells. The filter paper with contents was dried at 95°C for 48 h. The percentage of substrate digestion was calculated from the difference in weights of substrate before and after growth. For processed cellulosic substrates the cellulose content was assayed by the anthrone-H_2SO_4 method [25] after filtering and collecting residue. Since carboxymethyl cellulose (CMC) is a soluble substrate, it was assayed without filtration by the same method [25].

Estimation of biomass

The entire contents of cultures (100 ml) grown on lignocellulose were filtered to remove undigested insoluble substrates. Cells were harvested by centrifugation at 12 000 × *g* for 20 min at 4°C. The cell pellet was digested with 1 N NaOH at 90°C for 20 min and protein was assayed by the method of Lowry et al. [17].

Measurement of enzyme activity

Cultures were grown in 100 ml of 1% (w/v) FP cellulose medium on a rotary shaker at room temperature. Cultures were centrifuged at 12 000 × *g* for 20 min at 4°C. The supernatant was used for the assay of cellulase enzymes. Reducing sugar was determined by the modified method of Miller [19] using 3,5-dinitrosalicylic acid. Extracellular protein was assayed by the method of Lowry et al. [17]. Carboxymethyl cellulase (CMCase) as endo-1,4-β-D-glucanase (1,4-(1,3;1,4)-β-D-glucan 4-glucanohydrolase, EC 3.2.1.4) and filter paper cellulase (FPase) were determined by the method of Andreotti [1] and their activities were expressed as mg reducing sugar released per ml per h. β-Glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) was assayed by the method of Wood [26] and its activity was expressed as mg *p*-nitrophenol (PNP) released per ml per h.

RESULTS

Screening of cellulolytic mixed culture

On initial screening for cellulolytic colonies on FP cellulose agar, a single colony was isolated. When cells from this colony were diluted and streaked on nutrient agar, four types of distinct colonies (A, B, C and D) were observed. These colonies were picked and purified separately. All these were tested for their cellulolytic ability. Strains A, B and C in pure culture did not grow on FP cellulose even in the presence of YE, while strain D could grow with YE (0.05%, w/v) to 2.99 OD (3.4×10^{10} cells/ml). Good growth (2.71 OD) was also achieved by growing a mixture of strains A, B, C and D on FP cellulose without YE.

Characteristics of the organisms in the cellulolytic mixed culture

Some morphological and biochemical characteristics of isolates D and C are shown in Table 1. Strain D utilized the sugars glucose, fructose, sucrose, xylose, cellobiose, starch, xylan, galactose, mannose, trehalose, inulin, salicilin, glycogen, maltose and cellulose but did not utilize arabinose, lactose, ribose, rhamnose, erythritol, mannitol, melezitose, raffinose, sorbitol, inositol, esculin and amygdalin. Strain D did not use D-ribose, 3,4-dihydroxybenzoate, sodium succinate, *n*-butanol, ethanol, sodium butyrate and glycerol as carbon sources, but used sodium acetate and sodium lactate. The optimum temperature for growth was 30°C and the temperature range was 30–37°C. Growth occurred over the pH range 6.5–7.2 with the optimum at 7.0.

Utilization of sugars by strain C was also tested and it was found to utilize glucose, fructose, arabinose, xylose, cellobiose, ribose, galactose, mannose, rhamnose, trehalose, mannitol, sorbitol, inulin, salicilin, inositol and maltose. It did not utilize lactose, sucrose, starch, erythritol, melezitose, raffinose, esculin, cellulose and amygdalin, and weakly utilized xylan and glycogen.

Table 1

Morphological and biochemical characteristics of strains C and D

Characteristic	Strain	
	C	D
Cells	Short, straight rods (1.65 × 5.4 μm) in young cultures and coccoid cells (0.5–0.8 μm) in old, weakly Gram-positive, non-motile and non-sporing	Short, straight and curved rods (1.65 × 2.5–4.5 μm) Gram-positive, non-motile and non-sporing
Nutrient broth	Moderate turbid and odorless	Poor growth and odorless
Colonies on		
(a) nutrient agar (3 days)	4–5 mm diameter, irregular, entire, opaque, convex and mucoid	1 mm diameter, circular, convex, smooth, opaque and whitish-yellow pigmented
(b) starch agar (4 days)	No growth	4–5 mm diameter and yellow pigmented
Filter paper in peptone broth	–	+
Voges-Proskauer test	–	–
Methyl red test	–	+
Gelatin hydrolysis	–	Slow liquefaction
Nitrate reduction	+	+
Nitrite reduction	+	–
Indole production	+	–
H_2S production	+	–
Urea hydrolysis	–	+
Citrate utilization	+	+
Catalase	+	+
Hugh and Leifson's test		
(a) aerobic growth		
glucose	–	Acid
sucrose	–	Acid
lactose	–	Weakly acidic
maltose	–	Acid
(b) anaerobic growth		
glucose	–	Acid
sucrose	–	Acid
lactose	–	Weakly acidic
maltose	–	Acid

Nature of interactions between strains in the mixed culture

To determine the kind of interactions among the strains, different combinations of strains A, B, C and D were tested for growth on FP cellulose (1%, w/v). Strain D interacted only with strain C (Fig. 1A). The total growth reached by the mixed culture (strain D + C) was several times higher than that of pure cultures of strains C or D (Fig. 1A). With strain A and B only negligible growth resulted.

To explore further whether this interaction is confined to only cellulose, growth on another substrate, cellobiose (0.5%, w/v), was also tested (Fig. 1B). Again, strain D grew only with YE or with strain C. Strain C, although non-cellulolytic, could grow on cellobiose without YE (Fig. 1B).

Effect of growth factors

Having estabished that YE could take over the role of strain C in mixed culture, further experiments were carried out to find out the specific growth factors required by strain D. The growth of strain D with YE in four days yielded 2.6 × 10^{10} cells/ml, while with vitamin mixture, peptone and tryptone growth resulted in 2.2 × 10^{10}, 9.0 × 10^{9} and 4.6 × 10^{9} cells/ml, respectively. In another experiment, the effect of B-complex vitamins on growth was tested. Deficiency of the vitamins thiamine and biotin caused a ten-fold decrease in growth (2.1 × 10^{9} cells/ml) indicating that these vitamins stimulated cellulolysis and growth by strain D.

Enzyme activities of strain D in pure and mixed culture

Activities of FPase and CMCase of strain D are shown in Fig. 2. These enzymes are extracellular and are released into the medium during the growth of strain D. Accumulation of reducing sugars was detected in cultures of strain D with YE and in mixed culture of strain C with strain D (Table 2).

Digestion of cellulosic residues and production of biomass

The *Cellulomonas* isolate (strain D) was able to digest many processed cellulosic substrates as sole carbon source. In 4 days' growth, the degree of digestion of crystalline cellulose (TLC grade) was 32.54%, tissue paper 42.4%, filter paper (Whatman No. 1) 49.2%, news print 41.00%, packing material

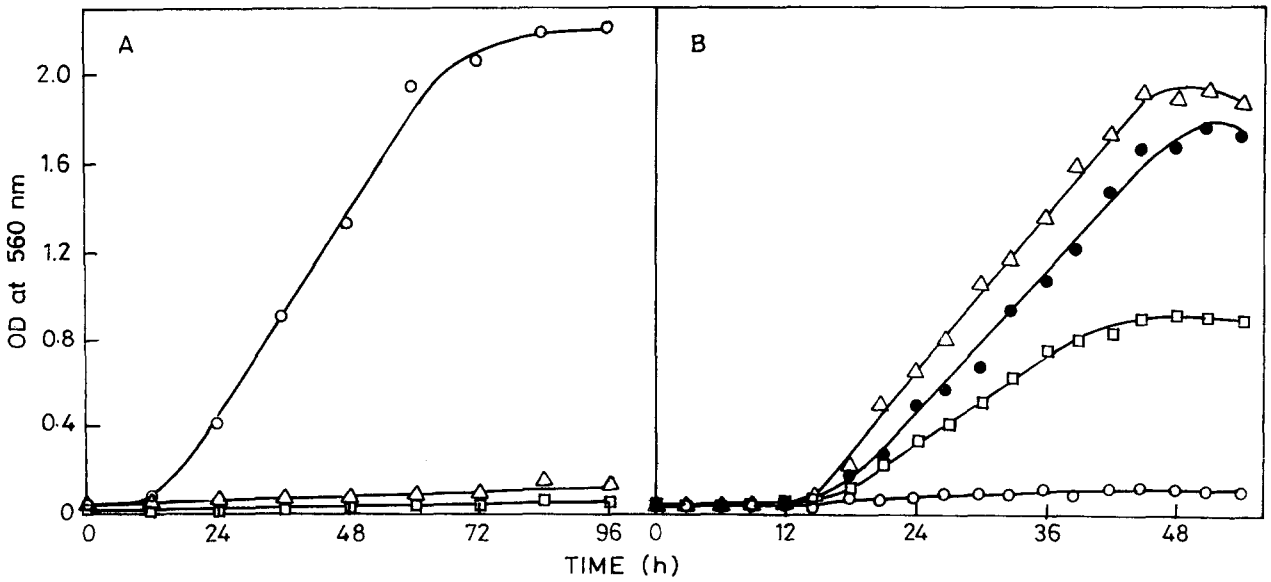

Fig. 1. (A) Growth of strain D in pure culture and in mixed culture with strain C on FP cellulose without YE: O, strains D+C; △, strain D; □, strain C. (B) Growth of strain D in pure and in mixed culture with strain C on cellobiose: △, strains D+C without YE; ●, strain D with YE; □, strain C without YE; O, strain D without YE.

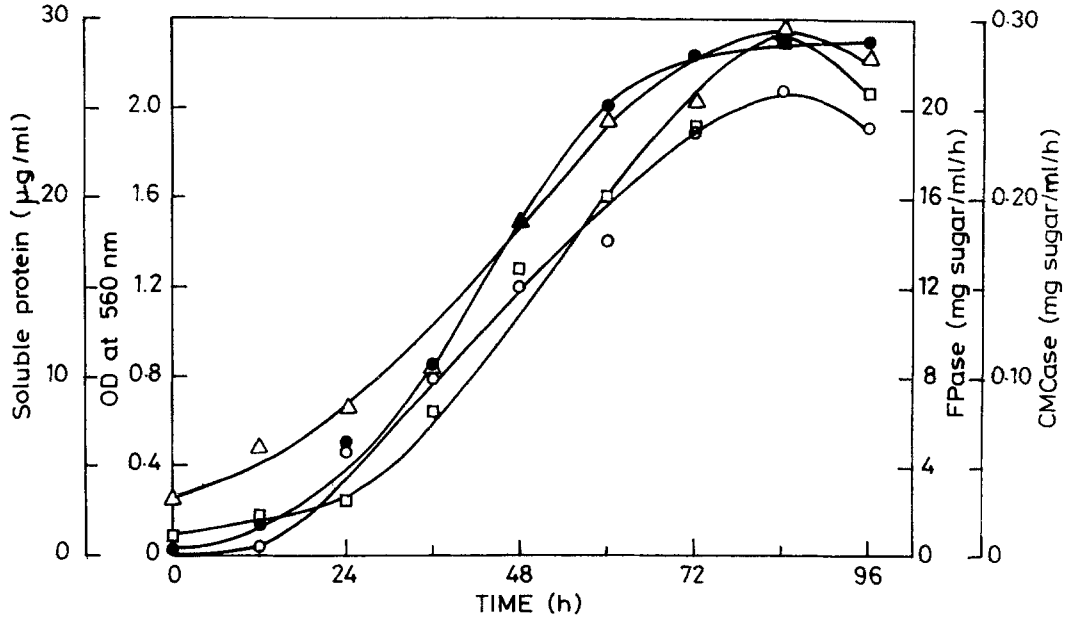

Fig. 2. Growth and production of FPase and CMCase by strain D grown on FP cellulose: ●, growth; □, soluble extracellular protein; △, FPase; ○, CMCase.

Table 2

Enzyme production by strain D in pure and mixed culture

Culture medium and inoculum	Percent cellulose utilization	Reducing sugar in culture filtrate (mg/ml)	Extracellular protein (mg/ml)	FPase (mg sugar/ml/h)	CMCase (mg sugar/ml/h)	β-Glucosidase (mg PNP/ml/h)
1% w/v FP cellulose + YE (0.05%, w/v), steriie	NT	0	0.001	NT	NT	NT
1% w/v FP cellulose + strain D	0.05	0.002	0.001	ND	ND	ND
1% w/v FP cellulose + YE (0.05%, w/v) + strain D	49.00	0.048	0.029	27.10	0.19	0.003
1% w/v FP cellulose + strain D + strain C	62.02	0.031	0.044	32.42	0.23	0.005

NT, not tested; ND, not detectable.

Table 3

Biomass production and digestion of lignocellulosic substrates by mixed culture

The inoculum consisted of 2 ml each of cells of strain A, B, C and D grown as given in Methods. Digestion and biomass were determined after 7 days growth.

Substrate (0.5%, w/v)	Degree of digestion (%)		Biomass (g/l)	
	untreated	alkali-treated	untreated	alkali-treated
Sugarcane bagasse[a]	46.18	51.97	1.09	1.37
Cotton linters	26.57	39.40	0.78	0.84
Banana stalk[a]	31.18	40.00	0.82	0.92
Coconut fibers[a]	25.25	28.11	0.42	0.50
Rice husk[b]	33.80	48.46	0.67	0.75

[a] Wet materials were cut into 1-inch pieces, dried at 100°C for 48–72 h, ground and sieved to 500–200 μ diameter.
[b] Substrate was a mixture of husk with bran.

(cardboard) 39.71%, and CMC 31.72%. In addition, the mixed culture had good efficiency in digesting lignocellulosic residues (Table 3). Alkali-pretreated substrates were well utilized by the mixed culture. Production of biomass was higher from alkali-treated substrates than from untreated substrates.

DISCUSSION

Of the four strains isolated, strain D alone was found to be cellulolytic in the presence of either YE or strain C. Others were non-cellulolytic even in the presence of YE.

Strain D resembles the genus *Cellulomonas* as described in Bergey's Manual [2]. The non-cellulolytic strain C was identified as an *Arthrobacter* sp. The observation that strain C does not require growth factors to grow on cellobiose as a pure culture is in contrast to the report of De la Torre and Campillo [6] in which an interacting *Xanthomonas* sp. found in a mixed cellulolytic culture required growth factors on cellobiose. Further, they did not show the extent of growth that could be reached by *Cellulomonas flavigena* in pure culture in the presence of growth factors. Han [11] also investigated the interactions of different cellulolytic mixed systems of *Cellulomonas* sp. with *Alkaligenes faecalis* and with cellobiose-utilizing yeasts. He showed that the effect of interaction was more pronounced in the growth of mixed culture containing *A. faecalis* than with yeasts as interacting species. The nature of the interaction has not been fully established. Other mixed cultures of *Cellulomonas* sp. and *Bacillus subtilis* [20], and *Cellulomonas* sp. and *A. faecalis* [4] have been reported but the mechanisms of interaction were not studied. Nitrogen fixation associated with cellulose breakdown has been demonstrated by mixing cultures of *Azospirillum brasilense, Azo. lipoferum, Bacillus macerans* and *Cellulomonas gelida* [10]. We have reported here a new natural system of mixed culture of *Cellulomonas* sp. and *Arthrobacter* sp. and the nature of the interaction mechanism.

Activities of FPase and CMCase increased with growth of strain D, and hence these are extracellular and released into the medium. Sugar accumulated in the cellulose medium. The sugar(s) has not yet been identified, but is likely that this is cellobiose, since the level of extracellular β-glucosidase in this system is low. It is known that cellobiose can be transported across the cell membrane [8,12].

The stimulating effects of the vitamins thiamine and biotin on cellulolysis has also been reported by others [6]. Thus, a complementary exchange of nutrients, such as sugars and some growth factors, may take place between cells of strains C and D.

This interaction appears to be obligatory on cellulose and is similar to the interactions of the mixed system reported by De la Torre and Campillo [6]. According to the definitions of Fredrickson [9], Harrison [13] and Bungay and Bungay [3], this mechanism of interaction on cellulose can be described as mutualism. The fact that on cellobiose one of the partners (strain C) could grow, while strain D could not, indicated that this interaction can be defined as protocooperation according to Fredrickson [9] where mutualism is not obligatory, or as commensalism as defined by Harrison [13].

The cellulolytic strain D utilized a variety of processed cellulosic materials. The extent of digestion of filter paper, cellulose powder and CMC is comparable to that obtained by Han and Srinivasan [12]. Various lignocellulosic substrates were digested well by this new mixed culture. The extent of digestion and efficiency of biomass production by our mixed culture is comparable with that reported by De la Torre and Campillo [6], who reported that a mixed culture degraded 48.7% of alkali-treated sugarcane bagasse and yielded a biomass of 1.3 g/l. This new mixed culture used 52% of the same substrate and produced a biomass of 1.37 g/l . Evaluation of the nutritional and toxicological aspects of this cell protein is to be assessed according to Mateles [18].

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