

Isolation of a Cotton Wool Degrading Strain of *Cellulomonas*: Mutants with Altered Ability to Degrade Cotton Wool

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Abstract

A *Cellulomonas* strain (CS1-1) which could readily degrade cotton wool was isolated from soil. Production of cell-bound and extracellular carboxymethylcellulase (CMCase), β -glucosidase and avicelase during growth on different substrates was determined. Methods for the isolation of mutants were assessed and mutants were isolated which were altered in their ability to degrade cotton wool when compared to CS1-1. Studies on one mutant (CS1-7) which was able to degrade cotton wool more rapidly than the parent revealed large differences in the levels of cell-bound and extracellular CMCase when compared to CS1-1.

Introduction

The best characterized cellulolytic bacteria belong to the following genera or groups: the *Pseudomonas*, which include some of those strains which were previously classified as *Cellvibrio* (Yoshikawa *et al.* 1974; Berg 1975); the *Cellulomonas* (Han and Srinivasan 1968; Stewart and Leatherwood 1976; Béguin and Eisen 1977); the *Sporocytophaga* (Berg *et al.* 1972) and those broadly classified as the anaerobic digesters (Leatherwood 1965; Lee and Blackburn 1975; Weimer and Zeikus 1977). Considerable work has also been reported on a strain called *Cellvibrio gilvus* (Storvick *et al.* 1963; Carpenter and Barnett 1967) but the complete *Cellvibrio* genus is no longer recognized in the eighth edition of 'Bergey's Manual of Determinative Bacteriology'. Several species have been assigned to the genus *Pseudomonas* (Buchanan and Gibbons 1974) but available information on *Cellvibrio gilvus* suggests that it should be classified as a *Cellulomonas* species.

Most cellulolytic bacteria studied seem to cause little or no degradation of either highly crystalline cellulose such as cotton wool or lignocellulosic material which has not been subjected to some pretreatment (Han and Srinivasan 1968; Berg *et al.* 1972). An exception to this seems to be the *Cellvibrio* strains isolated from polluted water and reported by Berg *et al.* (1968). Some of these strains appeared fairly active in degrading cotton fibres but the exact method of testing was not reported and we are not aware of any additional information published on them. Generally, however, bacterial growth and cellulolytic properties are studied using highly processed essentially pure cellulose or filter paper.

The potential of bacterial systems for the degradation of highly crystalline or natural cellulosic material may not have been fully explored. This paper reports on an extensive screening program resulting in the isolation of a *Cellulomonas* strain capable of degrading commercial absorbent cotton wool and on the selection of a range of mutants, of which one is able to degrade cotton wool more efficiently than can the parent strain.

Materials and Methods

Commercial cellulose (avicel SF) was obtained from Ashai Kasai Industrial Co., Japan; sodium carboxymethylcellulose (CMC) with 0.7–0.8 degree of substitution was from B.D.H., United Kingdom; absorbent cotton wool was obtained from Johnson & Johnson Pty Ltd, Australia, and toilet paper was from Gibson Chemicals Ltd, Australia.

The basic salts medium was a Dubos salts medium which consisted of 0.05 g NaNO_3 , 0.1 g K_2HPO_4 , 0.05 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05 g KCl and 0.001 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ per 100 ml solution at pH 7.0–7.3 (Dubos 1928). Yeast extract was always added at a final concentration of 0.02% (w/v) unless otherwise stated. In liquid medium carbon sources were added at the following concentrations: glucose, 10 mM; cellobiose, 5 mM; CMC, 1% (w/v); avicel, 1% (w/v) and cotton wool, 0.5% (w/v). Cotton wool was not cut up or specifically preconditioned, but medium containing cotton wool was sterilized by autoclaving for 20 min at 138 kPa above atmospheric pressure. Soluble carbon sources were used in solid media at the same concentrations as in liquid media whilst insoluble cellulose was utilized by incorporation into a 4-ml overlay as follows: avicel, 1.25%; cotton wool, cut to approximately 1-mm lengths and used at 0.4%; toilet paper, macerated in distilled water using a Kolloid-technik 'Puc-Vikosator' type JV10 mill and used at 1.6%. The solid media were prepared by addition of 1.2% agar.

Preparation of Inoculum for Use in Growth Experiments

A single colony from a Dubos+1% CMC+0.02% yeast extract plate was inoculated into Dubos+5 mM cellobiose+0.02% yeast extract and grown for 18 h at 30°C on a reciprocal shaker. The culture was centrifuged and washed twice in 0.9% saline and then the absorbance was adjusted to 0.6 at 610 nm. A 1.0% inoculum of this culture was used except in cotton wool digestion experiments where a 0.25% inoculum was used.

Growth of Cultures

Cotton wool digestion experiments were carried out in a 1-litre conical flask containing 200 ml of Dubos+0.02% yeast extract liquid medium with 1.0 g of absorbent cotton wool as carbon source. The flasks were incubated stationary at 30°C.

To study enzyme distribution in the parent strain CS1-1 (Fig. 1), growth was carried out in a Quickfit FVIL 1-litre vessel. The initial volume of culture was approximately 820 ml. The air supply system consisted of an adjustable pressure reducing valve, a needle valve for control of air flow, a flow meter and a Whatman Gamma 12 air filter fitted with a 03 grade filter tube. The air fed into the hollow Vibramix shaft and discharged at the base of the stirrer plate. The air escaped from the fermenter through a cooling condenser connected with a Mackley 10L filter to prevent back contamination. Dissolved oxygen tension was measured using a Johnson-type electrode (Johnson *et al.* 1964). In the experiments reported in this paper the air supply was set to give 100% dissolved oxygen for each medium prior to inoculation and then the rate of air supply was not varied throughout the growth experiment. Temperature was controlled by means of the Ether Mini model 19-90/1 using a resistance thermometer type P5 as a sensor and operating a 250-W infrared lamp. pH was continuously monitored using a pH probe connected to a Dynaco 21A pH meter. No pH control was employed. Culture samples were taken through a steam sterilizable connection at the base of the fermentor.

When studying enzyme distribution in CS1-7 compared to CS1-1 (Table 3) cultures were grown in shaker flasks with 160 ml culture in a 1-litre conical flask and these were shaken at 30°C on a reciprocal shaker.

Determination of Residual Cotton Wool

A gravimetric method similar to that described by Fahraeus (1947) was employed to determine the extent of cotton wool digestion. Controls were prepared in which growth medium was inoculated with a heat-killed culture and then incubated and tested with the sample cultures. The weight loss in controls was subtracted from that in sample cultures to determine the amount of cotton wool digestion by the cellulolytic bacteria.

Determination of Enzyme Activity

(i) Buffer used in enzyme studies

The buffer used throughout the enzymic studies was prepared by mixing 0.05 M citric acid and 0.1 M Na_2HPO_4 in appropriate proportions to give buffer solutions ranging from pH 2.2 to pH 8.0. This buffer is referred to as the citrate-phosphate buffer.

(ii) Preparation of enzyme extracts

Culture samples (20–40 ml) were taken at the appropriate time intervals and centrifuged at 10000 *g* for 20 min at 2°C. The supernatant was then used as the extracellular enzyme extract. If the reducing sugar content was significant it was removed by dialysis against a citrate-phosphate buffer at pH 7.0. The cells were then broken by sonication for a total of 4 min using a Bronwill Biosonik IV Sonicator. The extracts were centrifuged at 15 000 *g* for 20 min at 2°C and the supernatant was used as the cell-bound enzyme preparation.

(iii) Estimation of reducing sugars

The reducing sugars accumulating in the medium during cotton wool digestion experiments were determined using a dinitrosalicylic acid reagent (Miller *et al.* 1960) with a glucose standard. In all other estimations of reducing sugar equivalents the method of Somogyi (1952) and the chromogen developed by Nelson (1944) were used, with glucose as a standard.

(iv) Assay of carboxymethylcellulase (CMCase) based on the formation of reducing sugar

The method employed was based on that developed by Reese *et al.* (1950) for measurement of 'C_x' activity. The enzyme preparation (1.0 ml), at an appropriate dilution, was added to 1.5 ml of 1% CMC and incubated at 40°C for 20 min. The reaction was stopped by addition of the reagents for estimation of the reducing sugars. If the protein content was high in the enzyme extracts, deproteinization was achieved by adding 1.0 ml of 5% $\text{ZnSO}_4 \cdot 6\text{H}_2\text{O}$ followed by 1.0 ml of 0.15 M barium hydroxide and then removing the precipitate by centrifugation at 2500 *g* for 10 min.

One unit of enzyme activity is defined as the amount of enzyme that will produce reducing equivalents equal to 1 μmol of glucose per minute under the assay conditions given above. Activity is expressed as units per millilitre of culture medium for both cell-bound and extracellular CMCase. We are specifically calling this enzyme CMCase as we do not know how many enzymes are involved or the substrate specificity of the enzyme(s) active on CMC.

(v) Assay of avicelase activity

Enzyme solution (1 ml) was added to 1.5 ml of a 1% suspension of avicel in citrate-phosphate buffer, pH 7.0, in a 28-ml McCartney bottle and the mixture was incubated on a reciprocal shaker at 30°C for 8 h. The reaction was stopped by addition of the reagents for estimation of reducing sugar. Avicel remaining in suspension was removed by filtration and if necessary protein was removed as described for the CMCase assay. One unit of avicelase activity is defined as that amount of enzyme required to give 1 μmol of reducing sugar per minute.

(vi) Assay of β -glucosidase (β -D-glucoside glucohydrolase, EC 3.2.1.21) activity

β -Glucosidase was estimated by measuring the release of p-nitrophenol (PNP) from p-nitrophenyl- β -D-glucoside (PNPG) (Han and Srinivasan 1969). An appropriately diluted enzyme extract (1 ml) and 0.5 ml of 0.005 M PNPG were added to 1.5 ml of citrate-phosphate buffer at pH 7.0. After incubation for 20 min at 37°C the reaction was stopped by adding 2.0 ml of 1 M sodium carbonate. The absorbance of the yellow solution produced was read at 400 nm and compared with a standard curve constructed using PNP.

One unit of enzyme activity is defined as the amount of enzyme which liberates 1 μmol of PNP per minute. Specific activity is represented as units per milligram protein.

(vii) Protein determination

Protein was estimated using the method reported by Lowry *et al.* (1951).

Mutagenesis

For u.v. mutagenesis bacterial suspensions were grown to late log phase in Dubos+5 mM cellobiose+0.05% yeast extract, centrifuged and resuspended in an equal volume of saline. The suspension (10 ml) was placed in a sterile Petri dish and irradiated sufficiently at 254 nm to give approximately a 3 log kill. The irradiated culture was centrifuged and resuspended in Dubos+5 mM cellobiose+0.05% yeast extract and then incubated for 6 h at 30°C on a reciprocal shaker. This population was then screened for mutants with altered ability to degrade avicel. Screening was

achieved by plating for single colonies on a Dubos+1.25% avicel medium or a Dubos+10 mM glucose+1.25% avicel medium. Mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NG) at 150 µg/ml was carried out by the method described by Fargie and Holloway (1965).

Results

Isolation and Characterization of the Cellulomonas sp. (CS1-1)

(i) Isolation, classification and growth responses of CS1-1

An extensive screening program was undertaken to obtain cellulolytic bacteria capable of degrading cotton wool and which could be used in a mutation study; the samples screened were taken from different soil types, composting garden refuse, rotting logs and leaf humus.

The chosen organism was found to be rod shaped (0.3–0.5 by 0.8–1.2 µm), gram positive, oxidase negative, catalase positive, fermentative, a facultative aerobe, non-motile and to form yellowish colonies on a variety of media. In addition the organism had a yeast extract growth requirement. The classification tests carried out suggest that this organism is a *Cellulomonas* species, and consequently the strain was called CS1-1. Attempts were made to define the growth factor requirement of CS1-1 but no combination of vitamins and/or amino acids was found that could substitute for yeast extract itself. In an earlier study Keddie *et al.* (1966) reported that the *Cellulomonas* strain NCIB8077 had a growth requirement of biotin and thiamine. Biotin and thiamine were therefore tested as growth supplements for NCIB8077 and CS1-1 but only slight growth stimulation was observed for each organism; these specific supplements could not substitute for yeast extract. Since growth responses of CS1-1 were to be determined on a variety of different carbon sources it was necessary to establish the yeast extract concentration which could provide the growth requirements but which would not interfere with the determination of growth responses. The yeast extract concentration effective for this purpose was found to be 0.02%. At this concentration in both solid and liquid medium growth responses could be clearly determined without significant interference from the added yeast extract. Yeast extract at 0.02% was therefore added to all media unless otherwise stated.

Growth responses of CS1-1 were determined on a variety of media. After incubation for 2 days CS1-1 was found to grow well in liquid Dubos medium supplemented with cellobiose, CMC, or in nutrient broth. Under similar conditions slower growth was obtained in Luria broth and in Dubos medium supplemented with glucose and sucrose. On the insoluble cellulosic materials avicel and macerated toilet paper, discrete colonies were observed after incubation for approximately 2 days. Distinct clearing of the avicel and macerated toilet paper layers was observed after incubation for 4 and 10 days respectively. After incubation of CS1-1 on Dubos+cotton wool plates for 8 days, bacteria were seen to be growing along the fibres both on the surface of the plate and down into the medium. From microscopic examination it was apparent that significant biodegradation of this highly crystalline cellulosic material had occurred.

(ii) Effect of growth conditions on production of enzymes associated with the cellulolytic process

The production of enzymes involved in the cellulolytic process was studied under a variety of growth conditions. Assays were carried out with both cell-bound and

extracellular fractions using avicel (for avicelase), CMC (for CMCase), and p-nitro-phenyl- β -D-glucoside (for β -glucosidase) as substrates.

Only very low and probably insignificant levels of avicelase were detected. In addition β -glucosidase was never detected as an extracellular enzyme. Consequently only the cell-bound and extracellular levels of CMCase and the cell-bound levels of β -glucosidase are reported. Fig. 1 presents data on production of these enzymes during growth on cellobiose, CMC and avicel. In addition viable counts, pH and accumulation of reducing sugar were monitored during growth.

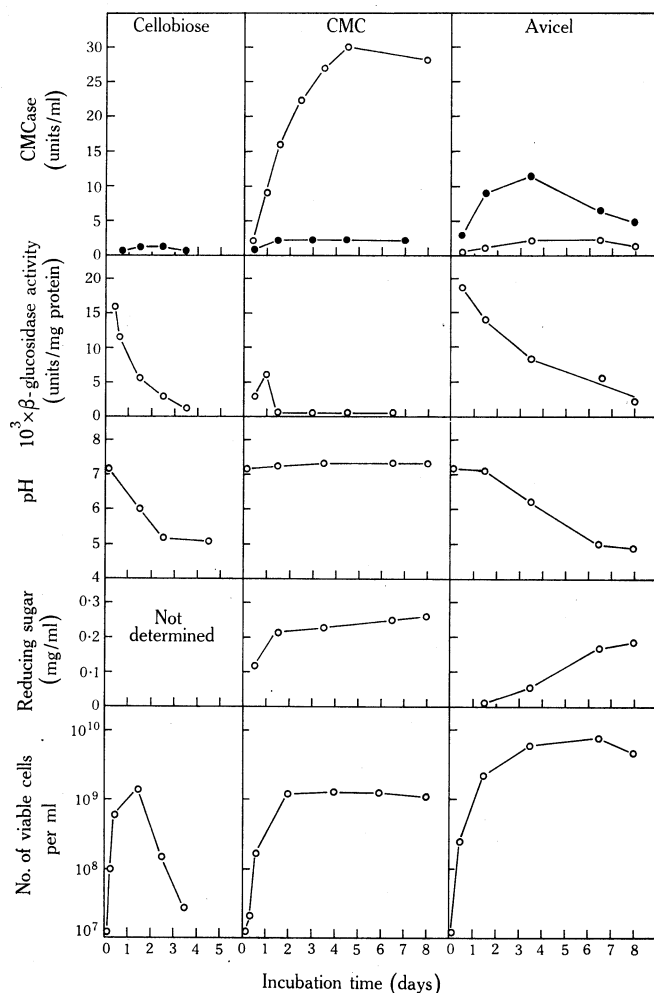


Fig. 1. Growth characteristics and enzyme production using CS1-1 on cellobiose, CMC and avicel during growth in the fermentor. ● Cell-bound CMCase. ○ Extracellular CMCase.

During growth on both avicel and cellobiose the pH of the culture medium decreased from 7.2 to a pH of *c.* 5.0. On cellobiose the decrease was much more rapid. On CMC, however, pH remained high and in fact rose slightly from the starting pH of 7.2. The viable cells in the population on cellobiose appeared to be

dependent on the pH in the culture. If the pH decreased rapidly to less than 6.0 then viability of the culture also decreased quickly. Reducing sugar was found to accumulate from growth on avicel and CMC.

Following growth on the insoluble substrate avicel, it was predominantly the cell-bound CMCase that was detected. The reverse was the case for the soluble substrate CMC—very high levels of extracellular CMCase were detected compared with the cell-bound enzyme levels. On cellobiose no extracellular CMCase was detected but low levels of the cell-bound enzyme were detected. Low levels of β -glucosidase were detected during growth on each of the three substrates.

The level of cell-bound β -glucosidase is shown as the specific activity, so enzyme levels produced during growth on the different substrates can be compared directly. For CMCase the activity is expressed as units per millilitre of culture medium. This provided a reasonable method for comparison of cell-bound and extracellular levels of CMCase on a particular growth substrate. Although levels of cell-bound CMCase could be shown as the specific activity, extracellular CMCase levels could not be presented in this fashion because of the yeast extract additive and the extremely low levels of extracellular protein synthesized. It is therefore difficult to compare CMCase levels produced under different growth conditions. Since the total viable count varies considerably between each culture (8×10^9 /ml on avicel and 2×10^9 /ml on CMC), the number of viable cells contributing to the level of CMCase varies between experiments. It is therefore possible to calculate the enzyme activity for a certain number of viable cells in the culture and to obtain a more accurate comparison between the different growth conditions. During the initial growth period and when cell viability decreases as a result of the decrease in pH the comparative data are misleading. However, between these two regions comparative data can be obtained. From these calculations it is clear that the extracellular CMCase detected during growth on CMC is, per viable cell, approximately 30-fold higher than that detected during growth on avicel.

(iii) *Properties of the extracellular CMCase produced during growth on CMC*

No significant loss in activity of the extracellular CMCase was detected as a result of standing in a citrate-phosphate buffer at 4°C for 48 h at pH 7.0, at 30°C for 24 h at pH 7.0, and at 30°C for 12 h at pH 5.0. Standing for 24 h at pH 5.0 resulted in a 10% loss in activity. The enzyme was stable to freeze-drying and to dialysis against tap water for 48 h at 4°C in Visking cellulose casing. The pH optimum of the enzyme was found to be 6.5–7.0. At pH 4.5, 5.0, 6.0 and 8.0 there was 20, 30, 75 and 65% activity detected relative to the total recorded at the optimum pH.

Loss of CMCase activity was observed in the presence of a 1% suspension of avicel in citrate-phosphate buffer at pH 7.0 and 30°C. 20% of the CMCase activity was lost after incubation for 4 h. Incubation for an additional 8 h did not result in any further loss in activity. Inhibition of the CMCase activity by cellobiose was determined following preincubation at pH 7.0 for 15 min with cellobiose at various concentrations. No inhibition of CMCase was detected up to 10 mM cellobiose but a 30% inhibition was observed at 20 mM and 55% inhibition at 50 mM cellobiose.

Isolation of Mutants with Altered Cellulolytic Properties

CS1-1 underwent mutation with NG at 150 μ g/ml and this resulted in a 1 log kill. Approximately 6000 survivors were plated onto Dubos+1.25% avicel plates. There

appeared to be two classes of mutants—those able to degrade the avicel layer more rapidly than the parent CS1-1, of which mutant CS1-2 was chosen as representative, and those degrading the avicel layer much more slowly than the parent. The colony morphology of CS1-2 was quite different from that of CS1-1. The mutant CS1-2 had watery, glistening colonies which were more translucent than the clearly defined yellowish colonies of CS1-1.

On the basis that CS1-2 appeared to be a more active cellulolytic strain, this culture was further irradiated with u.v. light in an attempt to isolate mutants with further improvements in cellulolytic properties. In this case 10 000 colonies were screened on Dubos+1.25% avicel medium. In total 63 potential mutants were selected that appeared to degrade avicel faster than did CS1-2. These mutants could be placed into two classes based on their distinctive colony morphology. CS1-4 was chosen as being representative of the class which formed very small distinct white colonies. CS1-5 was chosen as being representative of the second class which characteristically formed large watery colonies similar to the parent CS1-2.

Table 1. Clearing of avicel in solid medium with and without added glucose by CS1-1 and the mutant derivatives

Strain No.	Derivation	Time (days) to clear 1.25% avicel	
		No added glucose	With 10 mM glucose
CS1-1	Wild type	3.5	10.0
CS1-2	NG mutagen. of CS1-1	2.5	10.0
CS1-4	u.v. mutagen. of CS1-2	2.5	n.t. ^A
CS1-5	u.v. mutagen. of CS1-2	2.5	n.t.
CS1-7	u.v. mutagen. of CS1-1	2.0	6.0

^A Not tested.

In a third mutation experiment screening was carried out on avicel in the presence of glucose. Both CS1-1 and CS1-2 were irradiated with u.v. light and then approximately 10 000 survivors from each culture were plated for single colonies on Dubos+1.25% avicel+10 mM glucose+0.05% yeast extract. Six mutants of CS1-1 were selected. Of these, three had a colony morphology similar to CS1-1; the other three had a colony morphology similar to CS1-2 although they grew much better than that strain. Those mutants with colony morphology like CS1-2 were distinctly superior to those like CS1-1 both in degrading avicel by itself and in degrading avicel in the presence of 10 mM glucose. One of the more active strains was chosen for further study and was called CS1-7. Mutants of CS1-2 were also selected. These appeared to be marginally superior to the parent in degrading cellulose but they were much slower than the mutants isolated from CS1-1.

For comparative purposes the growth responses of the parent and chosen mutants are given in Table 1. By this test method all the mutants appear significantly superior to the parent in the degradation of an avicel layer in solid medium. The mutant CS1-7 appears to be slightly more active than the other mutants. When 10 mM glucose is incorporated into the test medium there is a marked increase in the time period required for clearing of the avicel layer. The mutant CS1-7 is much less sensitive to this glucose effect than is the parent strain.

Growth Characteristics of CS1-1 and Mutants on Cotton Wool

To obtain a quantitative assessment of the cellulolytic activity of CS1-1 and the mutant derivatives, degradation of cotton wool was studied in liquid culture. The action on cotton wool was assessed by determination of residual cellulose, culture pH and viable counts following stationary incubation for 3 and 5 days. The results of these experiments are given in Table 2. It is clear that CS1-1 is a very active cellulolytic strain, resulting in 9% degradation of cotton wool after incubation for 3 days and

Table 2. Growth of *Cellulomonas* mutants on 0.5% cotton wool in stationary culture

Parameter	Strain	Incubation time (days)		
		0	3	5
Viable count (cells/ml)	CS1-1	8×10^6	1×10^9	6×10^8
	CS1-2	2×10^6	6×10^8	4×10^8
	CS1-4	8×10^6	1×10^9	5×10^8
	CS1-5	6×10^6	5×10^8	7×10^8
	CS1-7	6×10^6	1×10^9	2×10^8
Culture pH	CS1-1	7.3	6.1	5.9
	CS1-2	7.3	6.3	5.6
	CS1-4	7.3	6.5	6.0
	CS1-5	7.3	6.5	5.5
	CS1-7	7.3	5.8	5.4
Cotton wool digestion (%)	CS1-1	0	9	16
	CS1-2	0	6	13
	CS1-4	0	8	16
	CS1-5	0	6	13
	CS1-7	0	13	24
Reducing sugar (mg/ml culture medium)	CS1-1	<0.10 ^A	<0.10	0.16
	CS1-2	<0.10	<0.10	<0.10
	CS1-4	<0.10	<0.10	0.14
	CS1-5	<0.10	<0.10	<0.10
	CS1-7	<0.10	0.22	0.57

^A Reducing sugar (as glucose) present in culture medium at less than 0.10 mg/ml.

16% after incubation for 5 days. As a result of the growth on cotton wool the pH of the medium decreased and, between 3 and 5 days incubation, this resulted in a decrease in cell viability. The cotton wool degradation observed did not represent the limit of breakdown. If the residual cotton wool was extracted after digestion for 5 days and then used as substrate in fresh growth medium, 14% additional digestion was obtained after incubation for a further 5 days.

Surprisingly the only mutant which is clearly superior to the parent CS1-1 in degrading cotton wool is CS1-7 which degraded 24% after incubation for 5 days. It is therefore apparent the plate selection on avicel medium did not necessarily produce mutants which were more active than the parent on cotton wool. For all strains a marked drop in pH was observed in liquid culture, and for both CS1-1 and the more active mutant CS1-7 significant accumulation of reducing sugar equivalents was observed after growth on cotton wool for 3 days.

Enzyme Production by the Mutants Compared to CS1-1 when Grown on Avicel

CS1-7 was clearly more cellulolytic than CS1-1, so experiments were conducted to determine whether there was any significant change in distribution or levels of avicelase, CMCase and β -glucosidase during growth on the insoluble substrate avicel. As with the parent only very low and probably insignificant levels of avicelase were detected and no extracellular β -glucosidase was detected. Levels of cell-bound and extracellular CMCase, cell-bound β -glucosidase, viable counts and pH changes during growth of CS1-1 and CS1-7 on avicel are recorded in Table 3. In contrast to the experiments conducted to characterize CS1-1 (Fig. 1), these experiments were conducted in shaken flasks and slight differences in various parameters are apparent. In each case the viable counts of CS1-1 and CS1-7 and the pH changes resulting from growth of these strains are very similar. These make comparison of enzyme levels relatively straightforward. As a result of mutation there are very obvious differences in the levels of both cell-bound and extracellular CMCase. Increased levels of each enzyme were produced and the ratio was changed such that higher levels of the extracellular enzyme were produced by CS1-7. In addition to changes in CMCase levels and distribution, approximately twice the amount of β -glucosidase was detected in CS1-7 as in CS1-1.

Table 3. Growth characteristics and enzyme production of CS1-7 and CS1-1 grown on avicel in shaken flask cultures

Parameter	Strain	Incubation time (days)		
		0	2	4
Viable count (cells/ml)	CS1-1	2×10^7	6×10^9	7×10^9
	CS1-7	2×10^7	5×10^9	7×10^9
Culture pH	CS1-1	7.2	7.0	6.2
	CS1-7	7.2	7.1	6.1
β -Glucosidase (units/mg protein)	CS1-1	—	0.01	0.01
	CS1-7	—	0.02	0.01
Extracellular CMCase (units/ml culture)	CS1-1	—	3	5
	CS1-7	—	15	40
Cell-bound CMCase (units/ml culture)	CS1-1	—	6	8
	CS1-7	—	15	22

For the three mutants CS1-2, CS1-4 and CS1-5 the maximum viable count observed on avicel medium was 2×10^9 /ml compared to 8×10^9 /ml for CS1-1 grown under the same conditions. The decrease in pH of the culture medium was more rapid for all these mutants, reaching pH 4.5 after incubation for 5 days, compared to pH 5.5 for CS1-1 after the same incubation period. Since the total number of viable cells is much lower for the mutants than for CS1-1, this rapid pH change is more significant. The cell-bound CMCase activity per viable cell for these mutants between days 2 and 5 was always about half that for CS1-1, whereas the extracellular CMCase activity per viable cell was approximately three times higher than for CS1-1.

Discussion

A strain of *Cellulomonas* was isolated from the soil which could degrade commercial absorbent cotton wool. Although this strain is active in the degradation of highly crystalline cellulose in cotton wool, no significant enzyme activity against the

insoluble substrate avicel was detected in the extracellular culture medium or the cell-bound enzyme extract. This characteristic is common amongst the cellulolytic bacteria which have been characterized. It seems that factors other than the *in vitro* enzyme activity of CMCase and β -glucosidase may be involved in permitting effective degradation of crystalline cellulose.

In studying enzyme distribution during growth on various substrates it is apparent that this varies markedly depending on the carbon source. Cellobiose as substrate appears to cause repression of synthesis of CMCase. Growth on the soluble substrate CMC resulted in formation of high levels of extracellular CMCase when compared to cell-bound CMCase. During growth on avicel the reverse situation was observed in that the cell-bound CMCase level was higher than the extracellular level. There may have been some adsorption and/or inactivation of the extracellular CMCase on the avicel and this may have reduced the amount detected during growth. However, this is probably not a major factor since only a 20% loss of extracellular CMCase activity has been observed in the presence of avicel.

The degree of cotton wool digestion and enzyme production during growth on various substrates provides a convenient way to characterize any mutants with alterations in cellulolytic properties. However, a study of enzyme distribution only gives an indication of gross changes. It is not known how many enzymes contribute to the various activities being assayed or whether entirely different enzymes having activity against a particular substrate are produced under different growth conditions. This limitation is evidenced by the complexity of the cellulases produced by *Pseudomonas* (Yoshikawa *et al.* 1974) and by *Cellvibrio gilvus* (Storvick and King 1960).

Two selection methods were used in attempting to isolate mutants which were more efficient than the parent in their ability to degrade cotton wool. Avicel, not cotton wool, was used in the selection medium primarily because it is more convenient. On solid medium with avicel as substrate CS1-2 appeared to be a more active cellulolytic strain than the parent CS1-1. However, the ability of this strain to degrade cotton wool was reduced compared to CS1-1. Therefore it became apparent that CS1-2 was not a good parent strain for the additional mutation experiment which resulted in the isolation of CS1-4 and CS1-5. Although these strains seemed as good or marginally better at degrading cotton wool than CS1-2 when tested in liquid medium, they were no better than CS1-1. It is possible, however, that these mutants are more active than CS1-1 against avicel in liquid medium. We consider it more likely that the apparent avicel degradation on the selection plates was misleading. The mutant colonies were more translucent than CS1-1 and consequently they only appeared to be more highly cellulolytic. In addition, when using avicel medium CS1-1 caused initial clearing after incubation for 3-5 days whilst the mutants showed clearing after 2-5 days. The time differential for clearing is therefore slight. With incorporation of glucose into the medium no lysis of avicel was observed until 10 days for CS1-1 compared with 6 days for CS1-7. The time differential was thus increased so that in isolation of mutants such as CS1-7 a very obvious difference was apparent when compared with the parent strain. For CS1-7 the ability to degrade cotton wool was significantly improved. Marked differences in enzyme levels and distribution were also observed when CS1-7 was grown on avicel and compared with CS1-1 grown on the same substrate.

This work illustrates that mutation can be employed to affect the cellulolytic process in *Cellulomonas* in terms of the enzymes synthesized and the rate of degrad-

ation of cotton wool. To date only limited information is available concerning the effect of mutation on the cellulolytic process in bacteria. The only other data for a strain of *Cellulomonas* is that in which mutants exhibiting derepressed synthesis of extracellular CMCase are reported (Stewart and Leatherwood 1976). Currently we are continuing the mutation program to determine how effective it will be in further increasing rates of cellulose degradation.

Acknowledgments

W. Y. Choi was supported by a Colombo Plan Scholarship and K. D. Haggett by a Commonwealth Post Graduate Award.

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Manuscript received 20 January 1978