Solid Media Containing Carboxymethylcellulose to Detect C_x Cellulase Activity of Micro-organisms

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(Received 17 May 1976; revised 15 July 1976)

SUMMARY

Solid media containing carboxymethylcellulose (CMC) were developed to detect C_x cellulase-producing micro-organisms. Hydrolysis of CMC was seen as a clear zone around colonies after flooding plates with 1 % aqueous hexadecyltrimethylammonium bromide. Tests with ten bacterial and four fungal species showed that the degree of substitution (DS) of the CMC affects both growth and enzyme production. Most of the organisms produced more C_x cellulase on CMC with a DS of 0.9, but CMC with a DS of 0.4 was better for one fungus. A qualitative measure of cellulase production may be obtained by calculating the ratio of zone size to colony diameter. Solid media containing CMC provided a more rapid assay of C_x cellulase production than a medium containing native cellulose.

INTRODUCTION

A solid medium providing rapid assays would be useful for the direct enumeration and isolation of cellulase-producing organisms from natural materials, and for the selection of cellulolytic mutants of bacteria and fungi. When native cellulose, in the form of paper or even finely-divided cellulose fibre, is used as the substrate, many weeks may be required to determine whether test cultures produce a cellulase, but using treated celluloses the incubation times required may be much shorter.

Powdered cellulose, treated with phosphoric acid and reprecipitated, has been used for testing the relative cellulolytic ability of fungi (Rautela & Cowling, 1966): results can be obtained in about 7 days. Eggins & Pugh (1962) used cellulose, ball-milled for 72 h, to isolate cellulose-decomposing fungi from soil. The milling increased the susceptibility of the native cellulose to enzyme attack. Using a modification of the Eggins & Pugh (1962) technique, Park (1973) isolated and enumerated cellulose-decomposing fungi in 8 days; sometimes only after 3 to 5 days. The particle size of cellulose can affect cellulase production by micro-organisms (Greaves, 1971). Release of the enzyme increased as the particle size of the substrate decreased but no correlation was established between this phenomenon and growth of the micro-organisms. Bravery (1968) found that asparagine and yeast extract can, under some conditions, inhibit cellulolytic activity of fungi.

Carboxymethylcellulose (CMC), a water-soluble cellulose derivative, is a useful substrate for detection of C_x cellulase production because it is degraded quickly by micro-organisms (Mandels, Andreotti & Roche, 1976). In this paper, we report the development of media with CMC as the cellulose substrate for use with bacteria and fungi. Various parameters relating the degree of substitution to production of C_x cellulase were studied. The rates of hydrolysis of different CMCs by cell-free culture filtrates were also examined.

METHODS

Organisms. *Streptomyces albidoflavus*, QMB1588 and QMB1634, *Streptomyces* sp. QMB1635, *Streptomyces parvus* QMB1632, and *Trichoderma viride* QMB9123 were from U.S. Army Natick Laboratories, Natick, Massachusetts, U.S.A.; *Streptomyces satsumaensis* PRL2256 and *Streptomyces* sp. PRL2527 from Prairie Regional Laboratory, Saskatoon, Saskatchewan, Canada; *Cellulomonas biazotea* and *Cellulomonas flavigena* from Biochemical Corporation of America, Salem, Virginia, U.S.A.; *Streptomyces* sp. ELQ-2 and ELQ-3, *Penicillium* sp. and *Peziza ostracoderma* were isolated by us from soil and the *Fusarium* sp. was isolated from leaf compost.

To inoculate solid media, bacteria (from agar slants) were suspended in distilled water to produce a slight turbidity, and fungi were grown on potato dextrose agar (Difco) and their spores were suspended in water. Drops of each suspension were applied to the surface of solidified test media. Bacteria were incubated for 4 days at 30 °C and fungi for 2 to 4 days at 28 °C unless otherwise stated. For single colonies, fungal spores were counted and diluted so that 0.1 ml spread on the surface of solidified media provided 10 to 100 colonies.

Measurement of growth and C_x cellulase production on solid media. After incubation, the size of the colony was measured and the surface of the medium was flooded with 1% (w/v) aqueous hexadecyltrimethylammonium bromide (Hankin, Zucker & Sands, 1971). This reagent precipitates undegraded CMC (or other long-chain polysaccharides) leaving a clear zone where CMC has been degraded, sometimes with a zone of dense precipitation. The size of the zone was measured and the ratio of zone size to colony diameter was calculated.

Test media. The best medium for showing C_x cellulase production by bacteria contained mineral salts (Zucker & Hankin, 1970), 0.1% (w/v) yeast extract, 0.5% (w/v) CMC-9M8F, and 1.0% (w/v) agar, at pH 7.0. As well as trace elements and phosphate salts, the mineral solution contained ammonium sulphate (1 g l^{-1}) as the nitrogen source. The same medium was used for the fungi, except *T. viride*, for which CMC-4M6F was substituted for CMC-9M8F and the medium was adjusted to pH 5.0. The mineral salts and yeast extract were adjusted to the selected pH and the CMC was added very slowly with continuous mixing. The agar was then added and the mixture was heated to effect solution. Media, sterilized by autoclaving, were cooled to 48 °C before plates were poured. Experiments detailing selection of above media are described in Results. For liquid media, the agar was omitted and cultures were grown with shaking at 30 °C.

Restriction of fungal colonies. An aqueous solution of sodium desoxycholate (40 mg ml⁻¹, sterilized by autoclaving) was added to sterile media to give 200 or 400 µg ml⁻¹. Sorbose was added to the media before autoclaving to give 1% or 3% (w/v).

Carboxymethylcellulose. Carboxymethylcellulose samples were supplied by Hercules, Inc., New York, U.S.A. The types of CMC used, their degrees of substitution, and the viscosities of their solutions are listed in Table 1. Cellulose powder (standard grade, ashless; Whatman) was also used.

Commercial enzymes. The following partially purified enzymes were tested to see if any reaction occurred on solid media with CMC: α -amylase, 50 to 100 units mg⁻¹ (Sigma); β -amylase, crude, approximately 10 units (mg protein)⁻¹ (Sigma); cellulase from *Aspergillus niger*, 0.7 to 1.0 units mg⁻¹ (Sigma); cellulase from *T. viride*, (Onozuka SS, Japan Biochemical Co.); chitinase from *Streptomyces griseus*, 40 units mg⁻¹ (Nutritional Biochemical Co., Cleveland, Ohio, U.S.A.); chitinase, 1 mg yielded approximately 0.4 mg glucose (Calbiochem); collagenase, 125 units mg⁻¹ (Sigma); invertase, K value 1.0 (Nutritional Biochemical Co.); lipase from wheat germ (Nutritional Biochemical Co.); lysozyme from

Table 1. Carboxymethylcellulose samples used*

CMC no.	Degree of substitution†	Specific viscosity range (mPa s)
4M6F	0.4	300–600 (2% soln)
4H1	0.4	400–1000 (1% soln)
7MF	0.7	300–600 (2% soln)
7H	0.7	1500–2500 (1% soln)
7L	0.7	25–50 (2% soln)
7L2	0.7	≤ 18 (2% soln)
9M31	0.9	800–3100 (2% soln)
9M8F	0.9	400–800 (2% soln)
9H4	0.9	2500–4500 (1% soln)
12M8P	1.2	400–800 (2% soln)

* Data from *Chemical and Physical Properties* (1971).

† Degree of substitution indicates the average number of hydroxyl groups (up to a maximum of three) per anhydroglucose unit in cellulose substituted by a carboxymethyl group.

Table 2. Ratios of the size of CMC hydrolysis zones to colony diameters

Organisms were incubated on solid media containing 0.5% w/v CMC at pH 7.0 for 4 days at 30 °C. Standard deviations are shown where three or more tests were done.

Organism	Type of CMC						
	4M6F	7L	7MF	9M31	9M8F	9H4	12M8P
<i>Streptomyces</i> sp. ELQ-3	2.03 ± 0.1	2.51	2.70 ± 0.2	2.69	2.80 ± 0.1	2.84	1.63 ± 0.2
<i>Streptomyces</i> sp. ELQ-2	1.97 ± 0.1	2.43 ± 0.1	2.46 ± 0.1	2.46	2.75 ± 0.2	2.85 ± 0.6	NM
<i>S. satsumaensis</i>	1.98 ± 0.3	2.29 ± 0.2	2.48 ± 0.1	2.48	2.68 ± 0.2	2.57 ± 0.3	1.50 ± 0.3
<i>Streptomyces</i> sp. PRL2527	1.33 ± 0.1	1.40	1.53 ± 0.1	1.37	1.47 ± 0.1	1.39	1.28 ± 0.1
<i>S. parvus</i>	1.97 ± 0.4	2.43	3.04 ± 0.3	2.37	2.87 ± 0.1	2.74	NM
<i>Streptomyces</i> sp. QMB1635	1.53 ± 0.1	NM	1.76 ± 0.1	2.31	1.54 ± 0.1	1.40	NM
<i>Cellulomonas biazotea</i>	2.47 ± 0.1	3.06	2.75 ± 0.3	3.12	2.89 ± 0.4	2.96	2.59 ± 0.5
<i>C. flavigena</i>	3.13 ± 0.3	3.32 ± 0.2	3.57 ± 0.4	3.28	4.12 ± 0.7	3.81	2.70 ± 0.8

NM, Zone very diffuse and not measurable.

Micrococcus lysodeikticus, Grade I, 30000 units mg⁻¹ (Sigma); papain, Type II (Sigma); pronase, B grade, 45000 proteolytic units g⁻¹ (Calbiochem). Individual enzymes were tested by sprinkling them on the surface of solid CMC medium.

Liberation of glucose from CMC. To test for C_x cellulase production by growing cultures, 0.5 ml cell-free culture filtrate (or a suitable dilution) was incubated with 1.5 ml 1% (w/v) CMC-9M8F or CMC-4M6F (as described in text) in citrate buffer (0.05 M), pH 5.8, for 1 or 3 h. Liberation of glucose from the CMC was assayed by the method of Nelson (1944) as modified by Somogyi (1952).

RESULTS AND DISCUSSION

Bacterial studies

Bacteria were tested for their ability to produce C_x cellulase on solid media containing each CMC listed in Table 1 (at 0.5%, w/v) and 1.5% (w/v) agar, and the ratios of the size of the zone where CMC was degraded to colony size were calculated (Table 2). Neither *Streptomyces albidoflavus* QMB1588 nor QMB1634 produced measurable zones of cellulolytic activity on these media. Media at pH 5.0 and 7.0 were tested but only data for pH 7.0 are shown since most bacteria grew very poorly at pH 5.0. The *Streptomyces* species isolated from soil (*Streptomyces* sp. ELQ-2 and ELQ-3) grew well and produced C_x cellulase on media at both pH values.

In general, bacteria growing on CMCs with a degree of substitution of 0.7 or 0.9 gave the largest ratios of zone size to colony diameter. We adopted CMC-9M8F as our standard since it allowed good cellulase production by these bacteria, and the zones of degradation were clearer and easier to measure than with the other CMCs. The effect of different viscosities within a CMC group on cellulase production was negligible.

Media were prepared with cellulose powder instead of CMC, but even after 8 weeks incubation no degradation of it was observed.

Since the combination of 0.5% CMC with 1.5% agar provides a fairly rigid matrix that could slow the diffusion of the enzyme, we tested various concentrations of agar and CMC-9M8F. Larger zone size to colony diameter ratios were obtained with lower concentrations of CMC or agar. However, media with 0.25% CMC provided barely enough precipitate, when the medium was flooded with the precipitating reagent, for the zone of degradation to be visible. Media with less than 0.8% agar were difficult to use when inocula were spread over the surface unless Ionagar (Colab) was used, although such media could be used for pour-plates. The optimum ratio of zone size to colony diameter was provided by 1.0% agar and 0.5% CMC, a combination used in all subsequent experiments.

Since Tween 80 enhances cellulase production by *Trichoderma viride* (Reese & Maguire, 1969), we tested it at 0.1 and 0.3% (w/v) in a medium at pH 7.0 that contained 0.5% CMC-9M8F, 0.1% yeast extract and 1.0% agar. Only *S. albidoflavus* strains QMB1588 and QMB1634 responded to Tween 80, producing a C_x cellulase with both concentrations, though 0.3% Tween 80 gave the better response. Without Tween 80 no C_x cellulase was produced. When 1.0% (w/v) glucose was added to the medium, C_x cellulase production was suppressed in *Streptomyces* spp. QMB1635, ELQ-2, ELQ-3, and PRL2527, and in *S. parvus*.

The optimal incubation time to give a high ratio of zone size to colony diameter was determined by testing four bacterial species on three different CMCs. Two days incubation provided only faintly visible zones and the maximal ratios of zone size to colony diameter occurred between 4 and 9 days. Colony diameters did not increase substantially after 4 days although the ratios increased slightly, probably because of diffusion of the C_x cellulase through the medium. Commercial enzymes were tested for their ability to degrade CMC-9M8F. A crude chitinase preparation gave a positive response but a purified preparation did not. Enzymes giving negative results were pronase, collagenase, papain, α - and β -amylase, lipase, lysozyme and invertase. The action of commercial cellulases is discussed below.

Fungal studies

Four species of fungi were tested on solid media containing the CMCs listed in Table 1, at both pH 5.0 and 7.0. Although it was difficult to see zones of CMC degradation on these plates because of the spreading nature of the fungal colonies, distinct differences in C_x cellulase production were observed. *Peziza*, *Fusarium* and *Penicillium* species grew and produced cellulase at both pH values with all CMCs. *Trichoderma viride* QMB9123 degraded little or none of the CMC at pH 7.0. Commercial cellulases, from *T. viride* and *A. niger*, produced clear zones on all the CMCs at both pH values, indicating that the failure of our *Trichoderma* culture to degrade CMC at pH 7.0 was probably due to lack of production of a C_x cellulase.

The clearest zones (not necessarily the largest) were seen with *Peziza* and CMC-9M8F at pH 5.0, *Fusarium* and CMC-9M8F at pH 7.0, *Penicillium* and CMC-9M8F at both pH 5.0 and 7.0, and *Trichoderma* and CMC-4M6F at pH 5.0. Commercial cellulase from *Trichoderma* and *Aspergillus* produced the clearest zones on CMC-7H and CMC-9M8F

at pH 7.0. With *Trichoderma*, *Peziza* and *Fusarium*, clear areas were seen under the colonies but they did not extend much beyond the edge of the colony. The slower-growing *Penicillium* colonies showed zones of cellulolytic activity beyond the edge of the colony.

Two attempts were made to limit the size of the fungal colonies to allow more time for cellulase diffusion. Sodium desoxycholate was tested at 200 and 400 $\mu\text{g ml}^{-1}$ with CMC-9M8F at pH 7.0 for *Penicillium*, *Fusarium* and *Peziza*, and with CMC-4M6F at pH 5.0 for *Trichoderma*. Some difficulties in using desoxycholate have been discussed previously (Hankin & Anagnostakis, 1975). *Peziza* spores did not germinate on the medium with desoxycholate at 400 $\mu\text{g ml}^{-1}$ but did so with 200 $\mu\text{g ml}^{-1}$. This latter concentration did not restrict the growth of *Penicillium* and *Fusarium* enough to make it useful. *Trichoderma* degraded the desoxycholate.

Sorbose, used by fungal geneticists to restrict the size of colonies (Tatum, Barratt & Cutter, 1949), was also tested. In a medium at pH 5.0 which contained CMC-4M6F, the colony diameters of all fungi except *Peziza* were restricted more by 1% (w/v) sorbose than by 3%. Clear zones of C_x cellulase activity were detected after 7 days around all colonies restricted by 1% sorbose in this medium, except *Fusarium*. In a medium at pH 7.0 with CMC-9M8F and 1% sorbose, cellulase activity was detected after 3 days growth of *Fusarium*, *Peziza* and *Trichoderma*. *Penicillium* grew more slowly and produced colonies with large zones only after 5 days incubation. The 1% sorbose did not prevent germination of *Peziza*, *Fusarium* and *Trichoderma*.

We conclude that CMC-9M8F and CMC-4M6F are satisfactory substrates for detecting C_x cellulase production by most higher fungi, and that the addition of 1% sorbose is useful because it restricts colony diameters.

Effect of degree of substitution of CMC on growth

Although CMC has been used as a substrate for bacteria and fungi for C_x cellulase production, no extensive investigations have been made of which CMC provides the best carbon source for growth. CMCs with a degree of substitution (DS) in the range 0.5 to 1.0 have been used on the assumption that the lower the DS value the easier it would be for the organism to attack the molecule (Reese, Siu & Levinson, 1950; Levinson & Reese, 1950; 1957). This has been based on work with partially purified cellulases, usually derived from fungi. It was found that the DS of cellulose considerably influences the rate of hydrolysis by cellulases; the higher the degree of substitution, the lower the rate of hydrolysis. We also observed that the DS affects utilization of CMC by micro-organisms. However, the CMCs with the lowest DS values (0.4 and 0.7) were not as effective as CMC with a DS of 0.9 for our assay (except for *Trichoderma*). We also found, as did Reese *et al.* (1950), Levinson & Reese (1950) and Reese (1957) with isolated cellulases, that the degree of polymerization did not substantially affect the breakdown of CMC.

Studies with cell-free extracts

Almin & Eriksson (1968) tested 18 different CMCs against four different fungal cellulase preparations. They found, as did Reese *et al.* (1950), Levinson & Reese (1950) and Reese (1957), that increasing the DS increased the resistance of the CMC to enzymic hydrolysis. Both Almin & Eriksson (1968) and Eriksson & Hollmark (1969) assumed that the number of adjacent unsubstituted glycosyl residues was the only factor influencing the rate of hydrolysis of the CMC chain. Thus, hydrolysis occurs rapidly when three adjacent unsubstituted glycosyl residues are present, and slowly when only two are present. Our findings with pure cultures of bacteria grown with CMC as a carbon source suggest that some other mechanism

Table 3. *Liberation of glucose from CMC by cell-free culture filtrates*

Streptomyces sp. ELQ-2 and *Cellulomonas biazotea* were grown on CMC-9M8F, pH 7.0, for 3 days, and *Trichoderma viride* was grown on CMC-4M6F, pH 5.0, for 14 days (see Mandels, Weber & Parizek, 1971). Cell-free culture filtrates were tested for C_x cellulase activity by measuring the release of glucose from 1% (w/v) CMC, pH 5.8, at 40 °C. Results are expressed as μg glucose released $\text{min}^{-1} \text{ml}^{-1}$.

CMC tested*	<i>Streptomyces</i> sp. ELQ-2	<i>C. biazotea</i>	<i>T. viride</i>
4M6F	5.4	4.4	127.8
7MF	5.2	4.1	188.8
9M8F	3.2	2.7	94.4
12M8P	1.5	1.6	50.0

* Tests were conducted for 3 h, during which time the rates were linear.

may regulate the attack on the CMC molecule by the growing organism since larger colonies were obtained as the DS increased up to 0.9.

One possible explanation for this is that the cellulases produced by organisms growing on different CMCs are significantly different from each other. If this were true, a cellulase produced on one CMC might not work well on another. To test this, three micro-organisms were grown in liquid medium on CMCs and the cell-free culture filtrates were tested for ability to hydrolyse glucose from CMCs with different degrees of substitution (Table 3). There was essentially no difference between CMCs of DS 0.4 and 0.7 except with *T. viride* filtrates. Rates were slightly lower for CMCs of DS 0.9 and 1.2. Hence, a cellulase produced on one CMC can hydrolyse others. The bacterial cellulases produced on CMC-9M8F hydrolysed the two CMCs with lower DS (0.4 and 0.7), and the fungal cellulase produced on CMC-4M6F hydrolysed the CMC with a higher DS (0.7). No statistically significant correlation (F test) was found between the ratios of zone size to colony diameter on solid media and the amount of CMC hydrolysed to glucose by cell-free culture filtrates from the bacteria listed in Table 2.

We previously used a solid medium that contained a CMC of DS 1.2 to detect cellulase-producing organisms in soil and sewage (Hankin, Sands & Hill, 1974; Hankin & Sands, 1974). In this study we have found that a CMC of DS 0.9 is a better substrate for a wider range of micro-organisms. Media containing CMC of DS 0.4 will allow detection of cellulase production by some fungi for which CMC of DS 0.9 is not as effective.

Our data show the usefulness of a solid medium containing CMC as substrate to detect C_x cellulase production by micro-organisms. Results are obtained more quickly than when less readily hydrolysable cellulosic substrates such as paper or cotton fibre are used. Even though our solid medium provides only qualitative data, it could be useful for screening mutants with greater cellulolytic activity where large changes in enzyme production are sought.

We thank Margaret Staba for skilful technical help.

REFERENCES

- ALMIN, K. E. & ERIKSSON, K. E. (1968). Influence of carboxymethylcellulose on the determination of cellulase activity in absolute terms. *Archives of Biochemistry and Biophysics* **124**, 129–134.
- BRAVERY, A. F. (1968). Microbiological breakdown of cellulose in the presence of alternative carbon sources. *Journal of the Science of Food and Agriculture* **19**, 132–135.
- CHEMICAL AND PHYSICAL PROPERTIES. (1971). Cellulose gum, sodium carboxymethylcellulose. Wilmington, Delaware, U.S.A.: Hercules Inc.
- EGGINS, H. O. W. & PUGH, G. J. F. (1962). Isolation of cellulose-decomposing fungi from soil. *Nature, London* **193**, 94–95.
- ERIKSSON, K. H. & HOLLMARK, B. H. (1969). Kinetic studies of the action of cellulase upon sodium carboxymethyl cellulose. *Archives of Biochemistry and Biophysics* **133**, 233–237.
- GREAVES, H. (1971). The effect of substrate availability on cellulolytic enzyme production by selected wood-rotting microorganisms. *Australian Journal of Biological Sciences* **24**, 1169–1180.
- HANKIN, L. & ANAGNOSTAKIS, S. L. (1975). The use of solid media for detection of enzyme production by fungi. *Mycologia* **47**, 597–607.
- HANKIN, L. & SANDS, D. C. (1974). Bacterial production of enzymes in activated sludge systems. *Journal of the Water Pollution Control Federation* **46**, 2015–2025.
- HANKIN, L., ZUCKER, M. & SANDS, D. C. (1971). Improved solid medium for the detection and enumeration of pectolytic bacteria. *Applied Microbiology* **22**, 205–209.
- HANKIN, L., SANDS, D. C. & HILL, D. E. (1974). Relation of land use to some degradative enzymatic activities of soil bacteria. *Soil Science* **118**, 38–44.
- LEVINSON, H. S. & REESE, E. T. (1950). Enzymatic hydrolysis of soluble cellulose derivatives as measured by changes in viscosity. *Journal of General Physiology* **33**, 601–628.
- MANDELS, M., WEBER, J. & PARIZEK, R. (1971). Enhanced cellulase production by a mutant of *Trichoderma viride*. *Applied Microbiology* **21**, 152–154.
- MANDELS, M., ANDREOTTI, R. & ROCHE, C. (1976). Measurement of saccharifying cellulase. *Symposium on Enzymatic Conversion of Cellulosic Materials*, Newton, Massachusetts, U.S.A. *Biotechnology and Bio-engineering*, symposium no. 6, (in the Press).
- NELSON, N. (1944). A photometric adaptation of the Somogyi method for the determination of glucose. *Journal of Biological Chemistry* **153**, 375–380.
- PARK, D. (1973). A modified medium for isolation and enumeration of cellulose-decomposing fungi. *Transactions of the British Mycological Society* **60**, 148–151.
- RAUTELA, G. S. & COWLING, E. B. (1966). Simple cultural test for relative cellulolytic activity of fungi. *Applied Microbiology* **14**, 892–898.
- REESE, E. T. (1957). Biological degradation of cellulose derivatives. *Industrial and Engineering Chemistry* **49**, 89–93.
- REESE, E. T. & MAGUIRE, A. (1969). Surfactants as stimulants of enzyme production by microorganisms. *Applied Microbiology* **17**, 242–245.
- REESE, E. T., SIU, R. G. H. & LEVINSON, H. S. (1950). The biological degradation of soluble cellulose derivatives and its relationship to the mechanism of cellulose hydrolysis. *Journal of Bacteriology* **59**, 485–497.
- SOMOGYI, M. (1952). Notes on sugar determination. *Journal of Biological Chemistry* **195**, 19–23.
- TATUM, E. L., BARRATT, R. W. & CUTTER, V. M., JR. (1949). Chemical induction of colonial paramorphs in *Neurospora* and *Syncephalastrum*. *Science* **109**, 509–511.
- ZUCKER, M. & HANKIN, L. (1970). Regulation of pectate lyase synthesis in *Pseudomonas fluorescens*. *Journal of Bacteriology* **104**, 13–18.