THE EFFECT OF SUBSTRATE AVAILABILITY ON CELLULOLYTIC ENZYME PRODUCTION BY SELECTED WOOD-ROTTING MICROORGANISMS

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Abstract

Extracellular cellulases have been produced in shake cultures of *Polystictus versicolor*, *Fomes annosus*, *Lenzites trabea*, *Poria monticola*, and *Bacillus polymyxa*, using substrates of cellulose powder and river birch woodmeal. The comparative effect on enzyme production of wet and dry ball milling of the substrates has been examined. Studies were also made to establish the relative amounts of enzyme diffused, bound, or adsorbed during production and the influence of pH on cellulase activity. It was found that wet ball milling reduces the particle size of both materials more rapidly than dry ball milling. There was an overall increase in enzyme released as the particle size of the substrate decreased, but no correlation was established between this phenomenon and growth of the microorganisms. Dry milled substrates of comparable particle size produced higher yields of cellulases than the wet milled substrates, *Lenzites trabea* being more active in enzyme production than any of the other organisms. Diffusion of cellulases occurred more readily in the brown rot fungi than in the two white rot microorganisms and a higher percentage of enzyme remained bound with the latter group.

I. INTRODUCTION

It is generally understood that cellulose can be more readily degraded by microorganisms after it has been modified in some way, either chemically or physically. In many cases organisms which are unable to attack cellulose in its naturally occurring state, such as in the wood cell wall, are found to have cellulolytic abilities when grown on substituted or modified cellulose, in relatively accessible conditions.

The accessibility of cellulose in the case of wood is made complex by its association with lignin and other cell wall constituents. It occurs within the wall as either crystalline or amorphous arrangements (Frey-Wyssling 1937, 1959; Preston, Hermans, and Weidinger 1950).

Where the amorphous regions occur the microfibril is more accessible to the diffusing cellulolytic enzymes of microorganisms. The crystalline regions, by virtue

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of the closer packing and bonding of the cellulose molecules, are relatively resistant to enzymic attack (Norkrans 1950; Walseth 1952). By decreasing the crystallinity, and hence increasing the numbers of amorphous regions, the degree of accessibility and susceptibility to enzymic attack of the cellulose is thereby increased.

There are various ways in which this can be achieved. The method employed in these studies was purely mechanical, utilizing a rotary ball mill. This technique has been used extensively as a method of increasing susceptibility. As far as is known, however, few critical examinations of the duration of ball milling and its effect on cellulolytic enzyme production have been made. The purpose of this investigation was to help meet the need for such a study and at the same time to determine and compare the production of cellulolytic enzymes by five wood-destroying organisms.

II. Materials and Methods

Whatman's standard grade cellulose powder for chromatography purposes (a cotton derivative) and river birch woodmeal (*Betula nigra*) were selected for the cellulosic substrates.

The organisms selected were two brown rot fungi, *Lenzites trabea* (Pers.) Fr. and *Poria monticola* Murr., and a wood-attacking bacterium *Bacillus polymyxa* (Prazmowski) Migula. For the comparative work on the effect of dry and wet ball milling on growth and enzyme production, and the binding and adsorption studies, two white rot fungi, *Polystictus versicolor* (Linn.) Fr. and *Fomes annosus* Fr., were included.

(*a*) *Ball Milling Procedure*

The rotary ball mill consisted of two porcelain grinding jars arranged one above the other on the mill. Each jar contained *c.* 8 lb of flint pebbles (average diameter 0·75 in.) and was rotated at a constant 60 r.p.m. during the experiment.

Approximately 40 g of cellulose powder was introduced into the upper jar and approximately 40 g of 40-mesh birch woodmeal into the lower jar. (The woodmeal was obtained using a Wiley mill and small chips of the seasoned wood.) The cellulose sources were milled either air dry in the jars or as a 3% suspension in distilled water.

The quantity of cellulose source was outlined by Brownell (1965) who based his 40-g aliquots on the degree of purity to be desired for "milled wood lignin" as produced by Bjorkman in 1954. A period of milling of 3 weeks is necessary with such 40-g charges, after which time a high percentage of the "protective" lignin–cellulose bonds (Varossieau 1953; Cowling 1958) will have been disrupted, thus producing a much more susceptible cellulose from the wood. The Whatman's cellulose powder was milled for the same periods as the woodmeal, simply for comparative reasons and uniformity of experiment.

At intervals of 1, 2, 3, 7, 14, and 21 days, 0·5-g samples of the milled cellulose powder and the woodmeal were removed. In the case of the wet-milled material, a homogeneous suspension was first removed, centrifuged, oven-dried, and then weighed at room temperature and humidity. It was calculated from this how many millilitres of suspension would contain 0·5 g air dry cellulose powder or woodmeal. The 0·5-g aliquots were then added to the media in the culture flasks and sterilized by autoclaving at 15 lb/sq. in. for 20 min.

The average particle size of the ball-milled material was determined microscopically at each sampling period.

(*b*) *Cultural Procedures*

250-ml conical flasks containing 100 ml of the sterile liquid media were prepared for the organisms. The fungal flasks were inoculated with 4 by 5 mm plugs of 7-day-old mycelium

growing on 5% malt extract agar; the bacterium with 1 ml suspensions of a 3-day-old nutrient broth culture. The liquid culture media used were as follows:

(1) For *Bacillus polymyxa*:

Phosphate buffer solution (K_2HPO_4 117·7 g/l+KH_2PO_4 44·1 g/l)	10 ml
Ammonium sulphate solution [$(NH_4)_2SO_4$ 132·0 g/l]	10 ml
Magnesium sulphate solution ($MgSO_4.4H_2O$ 40·0 g/l)	5 ml
Zinc sulphate solution ($ZnSO_4.7H_2O$ 1·64 g/l)	1 ml
Ferric citrate solution ($FeC_6H_5O_7$ 2·994 g/l+citric acid 1·916 g/l)	1 ml
Manganese sulphate solution ($MnSO_4.4H_2O$ 1·54 g/l)	1 ml
Sucrose	5 g
Difco yeast extract	1 g
Casamino acids	1 g
Distilled water	1 litre

Calcium carbonate was autoclaved separately and added at the rate of 1 g/100 ml of medium, just prior to inoculation.

(2) For the fungi:

Potassium dihydrogen phosphate	0·6 g
Dipotassium hydrogen phosphate	0·4 g
Ammonium phosphate	2·0 g
Magnesium sulphate	0·89 g
Calcium chloride	55·5 mg
Ferric citrate solution (composition as above)	1 ml
Cobalt chloride solution ($CoCl_2$ 4·0 g/l)	1 ml
Manganese sulphate solution ($MnSO_4.4H_2O$ 3·62 g/l)	1 ml
Zinc sulphate solution ($ZnSO_4.7H_2O$ 4·4 g/l)	1 ml
Thiamine hydrochloride solution (0·1 g/l)	1 ml
Difco yeast extract	0·5 g
Distilled water	1 litre

The resultant pH of medium (1) with dry- or wet-milled cellulose powder was 7·0; with dry- or wet-milled woodmeal was 6·8. The pH of medium (2) was 5·5 and 5·0 respectively. The pH of the wet-milled substrates alone varied slightly with the duration of ball milling; during the early stages cellulose powder was measured at pH 7·0, while woodmeal was pH 6·5. The 14- and 21-day wet-milled cellulose and woodmeal measurements were pH 5·2 and 5·0 respectively.

Preliminary experiments indicated that stationary or fast rotary shaken cultures produced less cellulolytically active filtrates. The culture flasks were therefore slowly shaken on a reciprocal action shaker. All were held at room temperature (24°C). The bacterial flasks were harvested at 5 days' growth and the fungi after 12 days. In the case of the 21-day dry-milled cellulosic samples the brown rot fungi were also harvested after 5 and 19 days' growth and assayed for cellulase activity.

(*c*) *Assay Procedures*

Replicated culture flasks were harvested after the given incubation time. Homogeneous suspensions of cells and substrate within the cultures were obtained by use of a Waring blender followed by a Branson ultrasonic disintegrator. The suspensions were divided into two fractions; one for growth studies, and the other for cellulase assays. A separate set of flasks containing 21-day dry-milled woodmeal as the substrate was used in ascertaining relative amounts of bound and adsorbed cellulase. Throughout these procedures, where "filtrates" were used, the cultures were centrifuged at 15,000 r.p.m. for 30 min to remove cells, substrate, and other insoluble constituents. All stored material was held at 5°C after the addition of 0·01% merthiolate.

(i) *Growth Studies*

Growth was measured in terms of total carbohydrate consumption, using the anthrone procedure of Umbreit, Burris, and Stauffer (1964). The total carbohydrate present before the onset of growth was determined and after the given incubation time the cultures were homogrnized and the total carbohydrate again assayed. The difference in the two values represented a measure of growth.

(ii) *Binding and Adsorption of Cellulases*

Initial cell-free culture supernatants were obtained and stored for assaying as already outlined. Procedures prior to assay were as follows:

(1) The centrifuged cells and woodmeal were washed with sterile distilled water (achieved by gentle agitation) and again centrifuged. The supernatant was stored as outlined.

(2) The cells and woodmeal were then resuspended in sterile distilled water, squashed against the side of the flask with a spatula, and stirred for 3 min at the "½" setting on a Magnestir, using a 1½-in. flea. Centrifuging produced a cell-free and woodmeal-free supernatant.

(3) Stage (2) was repeated. It was felt that this procedure, which was rigidly adhered to, would release more of the loosely bound or adsorbed enzyme than simply steeping in water (or buffer) for a set time.

(4) At this point, attempts were made to release the firmly bound and adsorbed enzyme by the use of ultrasonic vibrations. The centrifuged material from (3) was resuspended, as already described, and the whole subjected to high-frequency vibrations from a Branson portable ultrasonic disintegrator for two separate bursts of 60 sec each (to avoid possible overheating of a single 2-min burst). After standing for 30 min the suspensions were then centrifuged and the supernatant stored in the usual way.

(5) The disintegrated material remaining was finally resuspended in sterile distilled water, merthiolate added, and stored together with the other samples.

All the samples were assayed for cellulase activity as outlined in the appropriate section below.

(iii) *Cellulase Assay Procedures*

The influence of pH on activity of the filtrates was determined for each microorganism; the two brown rot fungi and the bacterium over the pH range 0·6–9·6, and the two white rot fungi at pH 3·0, 4·6, and 7·6. McIlvaine's (1921) buffer was used. The assay systems employed were as follows:

(1) *Loss of Viscosity of Carboxymethyl Cellulose (CMC) Solution.*—The CMC solution was prepared by adding 6 g of air dry CMC (7MP) to 500 ml of 0·05M sodium citrate buffer at the pH's suggested as optimum for each organism. A Waring blender was used to dissolve the CMC and 5 ml of 1% merthiolate was added to the resultant solution as a preservative.

The viscosity of the CMC solution was determined by taking 20 ml of the solution, adding 5 ml of distilled water, and placing in a Brookfield Synchro-Lectric viscometer held at a constant 30°C. From this measurement the value representing 50% loss in viscosity was calulated. The filtrates were assayed by taking 5 ml, mixing with 20-ml aliquots of CMC solution, and determining the time in minutes to reach the reading of 50% loss in viscosity (t_{50}). Cellulolytic activity (CMCase) was expressed in terms of viscosity reduction units per culture, calculated by the formula $(1/t_{50}) \times 100$.

(2) *Increase in Reducing Sugar Value.*—The same solution of CMC was used as in the viscometric determinations. 0·5 ml of filtrate was added to 4·5 ml CMC solution and then incubated at 50°C for 1 hr. Controls were set up using 4·5 ml CMC + 0·5 ml water, and 4·5 citrate buffer + 0·5 filtrate, the latter to determine the amount of reducing substances already present in the filtrate and the former to serve as a blank for setting the colorimeter. After the incubation

period the reactions were stopped by immersing the mixture tubes in iced water. The amount of reducing sugar present in 1 ml of the mixture was determined using the standard procedure for the reduction of 3,5-dinitrosalicylic acid (Sumner and Somers 1944), and a Bausch & Lomb 340 spectrophotometer. Cellulolytic activity (C_x) was expressed in milligrams of glucose equivalents per culture, using a glucose standard curve.

III. Results and Discussion

Figure 1 shows a comparison between the effect of ball milling the substrates in the air-dry state and ball milling them as 3% suspensions in distilled water. In the case of the wet-milled cellulose it was found that two distinct fractions were observed under the microscope, particulate and fibrous. The two appeared to be

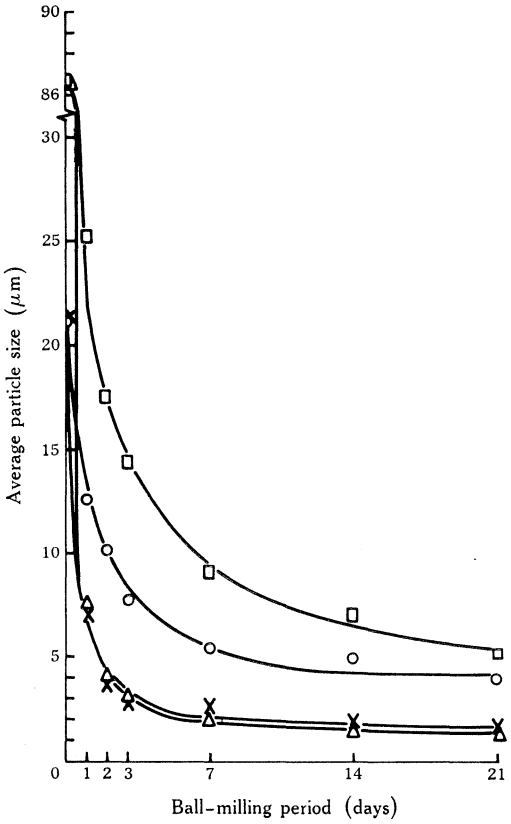

Fig. 1.—Effect of duration of wet (×, △) and dry (○, □) ball milling on average particle size. ○, × Cellulose. □, △ Woodmeal.

distributed in equal proportions and the particles tended to aggregate into small clumps. Howsmon and Marchessault (1959) observed the same occurrence for ball-milled cellulose. It can be seen from Figure 1 that wet milling is a far more effective method of reducing particle size than dry milling, and wet-milled woodmeal is more readily degraded than wet-milled cellulose powder. Leopold and Fujii (1965) report similar findings.

Figure 2 shows the results of different harvesting times with shake cultures of *Lenzites trabea* and *Poria monticola*, using the 21-day dry ball-milled samples of cellulose powder and woodmeal.

With regards CMCase production, it can be seen from Figure 2 that optimum yields for the two fungi were obtained after 12 days' incubation. However, C_x type enzymes were secreted in greater quantities during earlier stages of culturing. This is probably the effect of reducing sugar production, since it is known that as these substances accumulate, inhibition of cellulase occurs. An accumulation of reducing sugar could be accounted for if the fungi reached a maximum rate of cell development in the early stages of incubation and were less active in growth during further

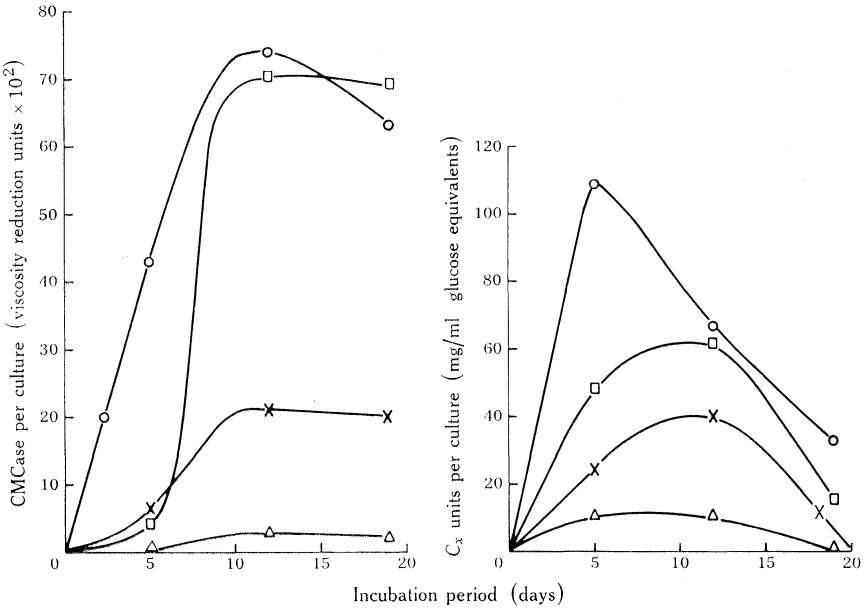

Fig. 2.—Effect of incubation period on cellulolytic enzyme production by *L. trabea* (○,□) and *P. monticola* (×,△). ○,× Cellulose. □,△ Woodmeal.

incubation. Subsequently they would use less of the reducing sugars produced by their metabolism. Alternatively, the fungi were simply unable to deal with all the sugars produced so that an excess developed, giving rise to a feedback inhibition of cellulases.

The apparent effect of substrate particle size on amount of enzyme produced by the cultures can be seen in the composite Figures 3(*a*) and 3(*b*). Both dry- and wet-milled substrates are compared.

It can be seen from these figures that, in general, there is an overall increase in enzyme released as the particle size of the substrate decreases. This was anticipated from the findings of King (1966) and Rautela (1967); the latter found that solubilization (by enzymatic means) of cellulosic substrates containing large particles is slower than corresponding substrates containing smaller particles, while King states that the rate of enzymatic solubilization is directly proportional to the substrate surface area.

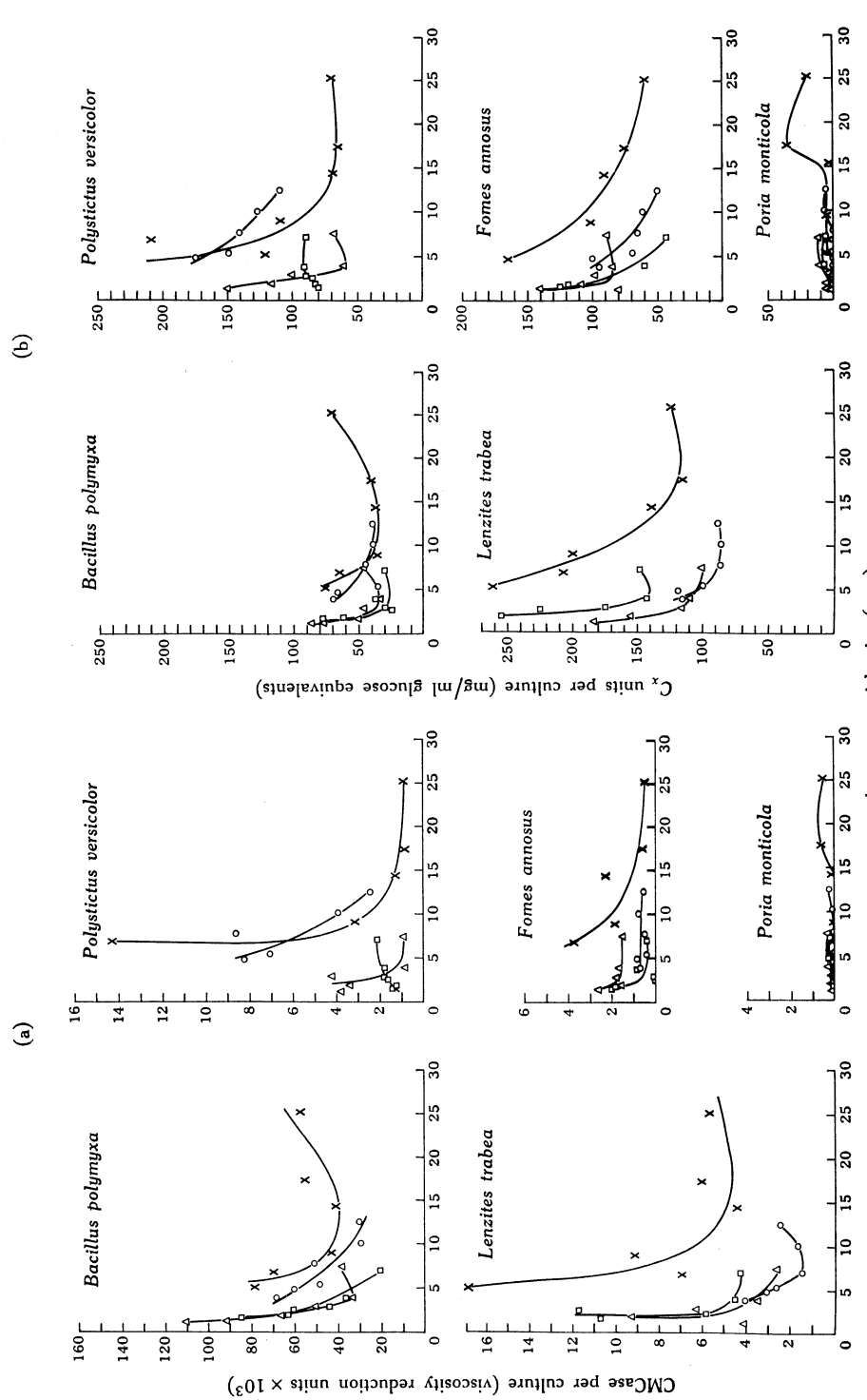

Fig. 3.—Influence of particle size on CMCase production (*a*) and C_x type enzyme production (*b*). ○ Dry-milled cellulose. × Dry-milled woodmeal. □ Wet-milled cellulose. △ Wet-milled woodmeal.

Generally, the dry-milled substrates produced the higher yields of fungal cellulase for an individual culture—a fact not apparently related to the amount of growth within that culture (see later discussion). In this respect, birch woodmeal was more effective than the cellulose powder. However, unlike the fungi, *Bacillus polymyxa* was found to produce better cellulase yields when wet-milled substrates were used. This microorganism was also shown in earlier experiments to produce diffusible cellulases in shake culture in the absence of a cellulosic substrate, although significantly higher yields were obtained when cellulose was added to the medium. It is possible that a different induction or stimulation system is involved with the bacterium than with the fungi, the necessity of which is probably enhanced by the former's thick polysaccharide capsule.

Comparisons of the influence of particle size on growth of the microorganisms are represented in Figure 4.

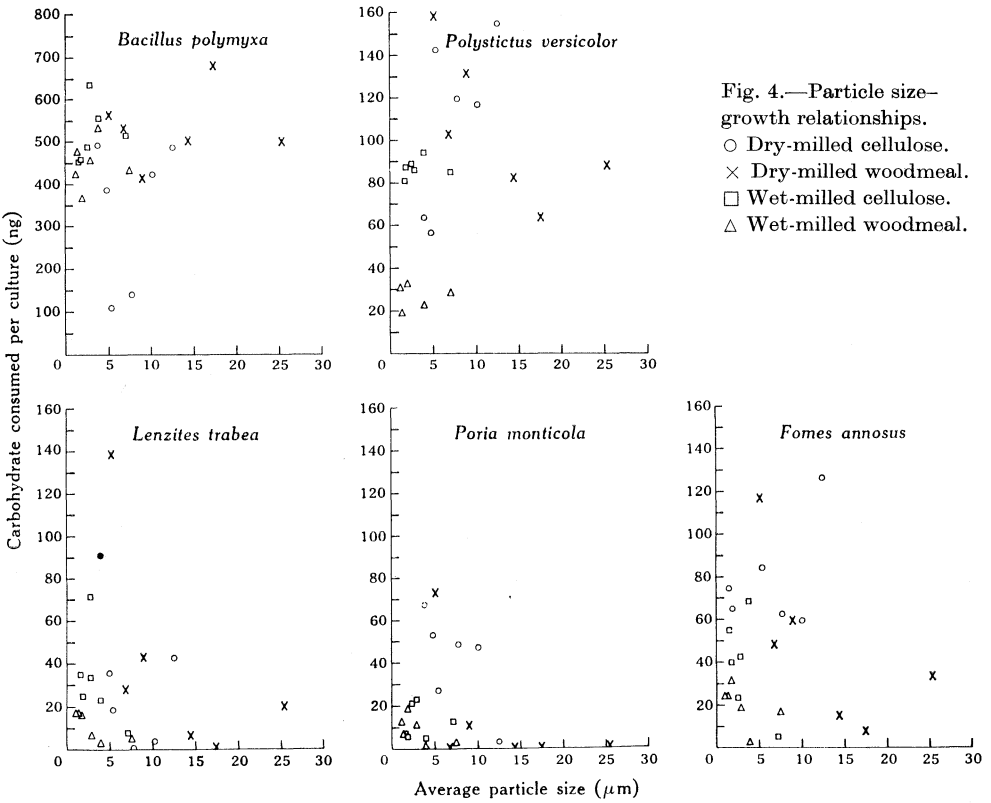

Fig. 4.—Particle size–growth relationships.
○ Dry-milled cellulose.
× Dry-milled woodmeal.
□ Wet-milled cellulose.
△ Wet-milled woodmeal.

As already mentioned the production of higher yields of extracellular cellulases with smaller sizes of substrate particles per culture does not appear to be a direct result of increases in the amount of growth of the culture when measured in terms of total carbohydrate. Rautela (1967) was also unable to establish correlations between growth-supporting and inducing capabilities of his cellulosic substrates.

Dry ball-milled woodmeal supported growth far better than wet-milled woodmeal. Since the basic media and the inocula were the same, this is evidence for a difference in crystal lattice or other structural effects or both produced by the two milling systems. It was expected that trends in growth patterns would not be the

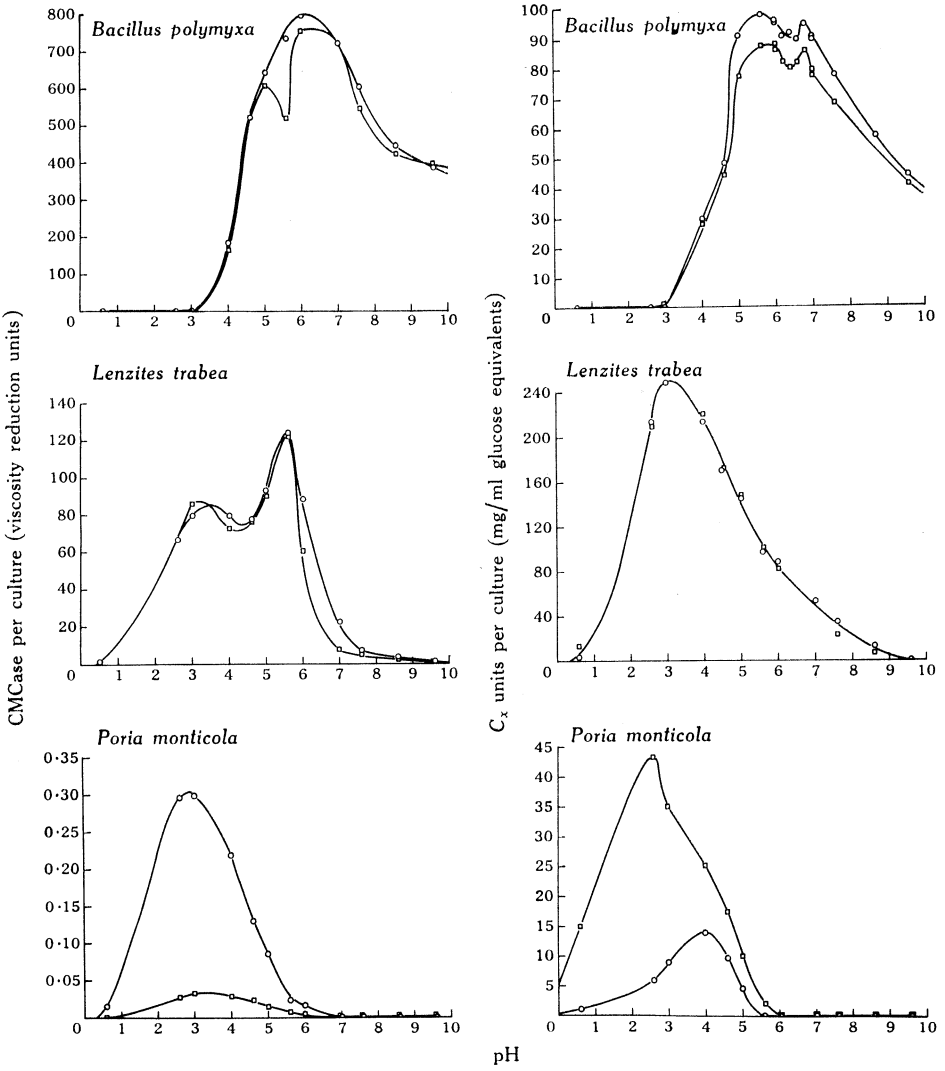

Fig. 5.—Influence of pH on cellulase activity of *B. polymyxa*, *L. trabea*, and *P. monticola*. ○ Cellulose. □ Woodmeal.

same for the two cellulose sources, woodmeal being by far the most complex substrate, even after ball milling. However, the apparent unrelated growth–enzyme production response was more noticeable for the less complex of the cellulosic sources, i.e. cellulose powder.

Lenzites trabea was the most adaptable of the microorganisms in terms of cellulase production; this fungus was able to secrete large quantities of cellulase regardless of the cellulosic substrate or the method of ball milling. The other brown rot fungus, *Poria monticola*, grew very poorly during the entire study, despite earlier observations that this particular fungus was relatively active in extracellular cellulase secretion. The white rot fungi grew well on the test media secreting cellulases on both substrates. However, they were less adaptable than *L. trabea*. It was expected that the white rot fungi would produce the best growth on woodmeal but not necessarily give the highest yields of cellulase: ball milling would remove much of the cellulose protective action of lignin, (which might otherwise have favoured white rot attack) hence providing a more favourable substrate for the brown rot fungi or the bacterium.

The results of the pH–enzyme activity studies are summarized in Figure 5, while Table 1 compares the ratios of the enzymes measured by the two systems (CMC viscosity reduction and dinitrosalicylic acid reducing sugar values) at the different pH's.

TABLE 1

COMPARISON OF RATIOS OF THE ACTIVITY* OF CMCASE TO C_x TYPE ENZYMES AT DIFFERENT pH VALUES FOR EACH ORGANISM

Organism	Substrate	CMCase : C_x Ratio at pH					
		9·6	8·6	7·6	4·6	3·0	0·6
Polystictus	Cellulose	—	—	—	—	—	—
versicolor	Woodmeal	—	—	94 : 1	37 : 1	193 : 1	—
Fomes	Cellulose	—	—	—	—	—	—
annosus	Woodmeal	—	—	28 : 1	18 : 1	59 : 1	—
Poria	Cellulose	—	—	—	25 : 1	41 : 1	0 : 2
monticola	Woodmeal	0	0	0	76 : 1	85 : 1	11 : 1
Lenzites	Cellulose	0	30 : 1	19 : 1	46 : 1	37 : 1	67 : 1
trabea	Woodmeal	0	5 : 1	21 : 1	45 : 1	33 : 1	15 : 1
Bacillus	Cellulose	9 : 1	8 : 1	8 : 1	11 : 1	—	—
polymyxa	Woodmeal	10 : 1	7 : 1	8 : 1	12 : 1	0 : 1	—

* Filtrate only.

It would appear that *B. polymyxa* produced more than one enzyme of both the CMCase type and C_x type when grown on woodmeal. Upon examination of the ratios of CMCase to C_x, when measured over a range of pH values, an almost constant relationship was found. This tends to suggest that either only one enzyme of each type is secreted into the medium or an equal number of each type is produced. The fungal cellulases on the other hand did not exhibit a constant ratio tendency, and it is thought that a more complex system of cellulases is produced by these micro-organisms. It was interesting that the two groups of fungi differed in the relationship of their ratios: the white rot fungi seemed to secrete a more complex cellulase system of the CMCase type, while the brown rots produced a more complex system of the C_x type.

Both groups of fungi possess cellulases which have acidic pH optima. Cellulases from the brown rot fungi have lower pH optima than enzymes from the white rot fungi: *P. versicolor* CMCase 3·0, C_x 4·6; *F. annosus* CMCase 4·6, C_x 4·6; *L. trabea* CMCase 3·0 and 5·6, C_x 3·0; *P. monticola* CMCase 3·0, C_x 2·6. The bacterium as expected produced cellulases with higher optima: CMCase 6·5, C_x 6·8. Furthermore, the enzymes of *B. polymyxa* were still relatively active at pH 9·6, although their range on the acidic side was limited at pH 2·6. Storage of the bacterial filtrates had a pronounced effect on the activity of CMCase. A similar effect has been previously noted by Gascoigne and Gascoigne (1960) and appears to be a function of pH of the stored filtrate.

The distribution within cultures of cellulolytic activity, i.e. the relative amounts of enzymes freely secreted, bound or adsorbed or both can be found in Table 2.

TABLE 2

DISTRIBUTION OF CELLULOLYTIC ACTIVITY IN CULTURES GROWN ON BIRCH WOODMEAL AFTER DRY BALL MILLING FOR 21 DAYS

Cultures subjected to treatments as outlined in Section II(*c*)—treatment A, filtration; treatment B, washing; treatment C, ultrasonic disintegration

Organism	CMCase Activity per Culture (%) for Treatments			Total Activity*	C_x Activity per Culture (%) for Treatments			Total Activity†
	A	B	C		A	B	C	
Polystictus versicolor	59	38	1	12820	48	43	9	420
Fomes annosus	58	38	4	4500	53	38	9	220
Poria monticola	54	46	0	260	98	0	2	20
Lenzites trabea	86	12	2	17300	62	32	6	400
Bacillus polymyxa	67	20	13	2100	69	29	2	130

* As calculated from formula $(1/t_{50}) \times 100$—see Section II(*c*)(iii).

† Expressed as milligrams of glucose equivalents per millilitre culture.

The microscopical observations showed that the ultrasonic treatment was adequate to cause cell wall disruption, and possibly therefore to release bound and adsorbed enzyme.

The most marked difference between the two groups of fungi was that diffusion of cellulases into the filtrate occurred far more readily in the brown rot than in the white rot fungi. This applied to both CMCase and C_x type enzymes. The bacterial enzymes were also more freely secreted into the medium than the white rot enzymes.

If it were correct to assume that substrate-adsorbed enzyme can be released by washing and gentle agitation (and there is dispute about this point) while mycelial-bound enzyme requires more forceful removal, then it could be suggested from the results that most of the percentage cellulase bound or adsorbed is made up of adsorbed enzyme rather than bound. In this respect, more of the C_x component is again bound by the white rot fungi than by either brown rot or bacterium. With regards CMCase, however, slightly more remains attached to the mycelium in *L. trabea* than in *P. versicolor*, although *F. annosus* behaves as for the C_x component.

IV. Acknowledgment

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