Hydrolysis of Fibrous Cotton and Reprecipitated Cellulose by Cellulolytic Enzymes from Soil Micro-organisms

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1. The action of cell-free filtrates from *Trichoderma koningii* was examined on undegraded cellulose in the form of cotton fibres, on degraded cellulose in the form of cellulose powder reprecipitated from phosphoric acid and on the soluble cellulose derivative CM-cellulose. 2. The cell-free filtrates compare favourably with intact cellulolytic micro-organisms in producing complete solubilization of undegraded as well as of degraded types of cellulose. Enzymic solubilization of cotton fibres gives quantitative conversion into glucose. Cellobiase is present. 3. The early enzymic breakdown of cotton fibres is characterized by the formation of very short fibres that increase to a maximum and disappear gradually by conversion into glucose. Disintegration of cotton fibres to short fibres is assisted by shaking, and within 20 hr. the enzyme converts a minor fraction (up to 16%) of substrate into soluble products and a major portion (80%) into insoluble short fibres. 4. Maximum enzymic activity on cotton fibres occurs at about pH 5.0, measured by the formation of short fibres, and at about pH 3.8 on reprecipitated cellulose, measured by solubilization of the substrate. 5. Gluconolactone, glucose and cellobiose fail to produce marked inhibition of the enzymic hydrolysis of cotton fibres to short fibres or of the solubilization of reprecipitated cellulose unless present in amounts comparable with or greater than the initial weight of these two forms of cellulose. 6. The heavy-metal ions Cu^{2+} , Hg^{2+} and Fe^{3+} at 2 mM concentration give 80–100% decrease in the enzymic breakdown of cotton fibres, measured by the formation of short fibres. At the same concentration Hg^{2+} and Fe^{3+} but not Cu^{2+} also produce 70–100% inhibition of the solubilization of reprecipitated cellulose. 7. The ability to hydrolyse cotton fibres to short fibres and CM-cellulose to sugars is completely lost after heating enzyme preparations for 10 min. at 71°.

Many micro-organisms catabolize cellulose in the immature or naturally swollen state as found in the unopened cotton-boll, or after maturity, only when the cellulose has suffered prior degradation. However, a few fungi and bacteria not only metabolize the less resistant forms of cellulose described above, but also the undegraded forms found in mature dried cotton fibres. *Myrothecium verrucaria*, for example, dissolves 80% or more of de-waxed cotton fibres in 14 days (Fig. 1 in Halliwell, 1961); likewise, mixed rumen bacteria and a few isolated pure strains utilize up to 97% of fibrous cotton in 3–7 days (Halliwell, 1957b; Halliwell & Bryant, 1963). Numerous unsuccessful attempts have been made to isolate cell-free enzymic preparations that could emulate the very extensive solubilization of undegraded cellulose characteristic of these truly cellulolytic micro-organisms.

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Our attention has been extended to other micro-organisms than *M. verrucaria* and rumen bacteria. Under optimum growth conditions it was possible to obtain from *Cellvibrio vulgaris* filtrates capable of severing the long fibres of mature fibrous cotton to give numerous short fibres, with only small production of soluble material. Search for filtrates with yet greater solubilizing power included *Aspergillus niger*, *Aspergillus terreus*, *Cellvibrio vulgaris* (two strains), *Chaetomium globosum*, *Coniophora cerebella*, *Polystictus versicolor*, *Sporocytophaga myxococcoides* (two strains), *Trichoderma koningii* and *Trichoderma viride* (two strains). The most promising of these was *T. koningii*, from which cell-free filtrates were prepared capable of completely dissolving mature fibrous cotton. The present paper describes the total enzymic conversion of cotton fibres either into short fibres and a little glucose or completely into glucose by cell-free culture filtrates from *T. koningii*, according to the conditions employed. The first process has been

found to proceed at least partially with filtrates from *M. verrucaria* as well as *C. vulgaris*. (cf. Halliwell, 1965).

MATERIALS AND METHODS

Source of enzymes. Cultures of *M. verrucaria* (Halliwell, 1961) and *T. koningii* (Commonwealth Mycological Institute) were grown on non-de-waxed Texas cotton fibres in shaken or stationary flasks as described in Fig. 1 in Halliwell (1961) on the same or a larger scale. Cell-free filtrates were prepared by filtering the culture medium through sintered glass (porosity 3), and centrifuging at 66000g for 20 min. at 3°. The supernatant phase was passed through a sterile sintered-glass bacteriological filter (porosity 5/3) when required for experiments involving prolonged incubation at 37°.

Substrates. Native cotton fibres in the form of non-de-waxed and de-waxed Texas cotton fibres, cellulose powder (Whatman) and sodium CM-cellulose were as described by Halliwell (1961, 1962).

Reprecipitated cellulose (cellulose reprecipitated from H_3PO_4), in contrast with undegraded native cotton fibres, is a highly degraded cellulose, prepared by dissolving cellulose in hot H_3PO_4 followed by reprecipitation in ice-water to arrest further degradation. Whatman cellulose powder (5g.) was added with stirring over a 7 min. period to 100 ml. of conc. H_3PO_4 (A.R. 90%; Hopkin and Williams Ltd., Chadwell Heath, Essex.) in a beaker at about 20°. The beaker was then transferred to a vigorously-boiling-water bath for exactly 4 min., the minimum time required for the liquid to clarify and for most of the particulate matter to dissolve. The solution was immediately decanted into 2 l. of ice-water. Precipitation occurred slowly, becoming obvious after about 3 min., and was allowed to continue in the cold for some hours to give a clear supernatant, which was decanted. The residual suspension was centrifuged at 1800g for 20 min. to give a loosely packed sediment, and this was washed repeatedly with water until neutral by centrifuging at 15000g. The powder was resuspended in water. Dry weight of the reprecipitated cellulose was determined on a sample after heating at 105° for 18 hr. This material should be distinguished from cellulose merely 'swollen' by contact with cold conc. H_3PO_4 (Halliwell, 1961).

Other materials were cellobiose (L. Light and Co. Ltd., Colnbrook, Bucks.), glucono-(1 → 5)-lactone [gluconic acid (lactone), Laboratory Reagent; British Drug Houses Ltd., Poole, Dorset], glucose (A.R.; Hopkin and Williams Ltd.).

Determination of reducing sugars. This was carried out colorimetrically with $K_3Fe(CN)_6$ (Halliwell, 1961).

Method of enzyme assay (a) with the soluble substrate, CM-cellulose. The estimation of reducing sugars (see above) formed from CM-cellulose was done in conjunction with determinations based on the glucose oxidase-peroxidase reagents normally used for glucose estimation (cf. Huggett & Nixon, 1957a,b; Bergmeyer & Bernt, 1963). CM-cellulase, present in cell-free filtrates from *T. koningii*, hydrolyses its soluble substrate to cellobiose and glucose (detected chromatographically). Glucose in the presence of glucose oxidase is oxidized at once to gluconic acid and H_2O_2 . This in turn reacts with *o*-dianisidine in the presence of peroxidase, yielding a pink solution suitable for colorimetric determinations. All the reagents are present in the incubation with the cellulolytic enzyme, as described below.

Colorimetric estimation of glucose and cellobiose with glucose oxidase and peroxidase. Glucose oxidase solution was the supernatant phase obtained from a suspension of glucose oxidase (crude powder; Sigma Chemical Co. Ltd., St Louis, Mo., U.S.A.) prepared at 6 mg./ml. in water and centrifuged at 1800g for 5 min. The peroxidase-anisidine reagent consisted of 0.5 mg. of peroxidase (C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany) plus 99 ml. of water plus 1 ml. of 0.5% *o*-dianisidine (Laboratory Reagent; British Drug Houses Ltd.). The anisidine solution was prepared by dissolving the solid in 95% (v/v) ethanol at 37°.

Glucose (0.0–6 ml. of 0.025% solution), or cellobiose containing 0–150 μ g. of sugar, plus 0.9 ml. of 0.2M-sodium acetate–0.2N-acetic acid buffer, pH 5.4, was made up to 3.6 ml. with water. Glucose oxidase solution (0.5 ml.) was added, followed immediately by 2.5 ml. of peroxidase-anisidine reagent, giving a final volume of 6.6 ml. (pH 5.5). All tubes were incubated for exactly 1 hr. at 37°, removed, mixed and read immediately against water in 8 ml. cuvettes with a 1 cm. light-path in a Spekker absorptiometer with Ilford blue filters (no. 602; peak transmission 470 m μ).

Under these conditions cellobiose, as well as glucose, in amounts up to 150 μ g. gave a linear calibration curve that intersected the axis at about 6 μ g. of the sugar. In estimations by the above method at pH 5.5 and 7.2 cellobiose gave 23 and 15% respectively of the colour given by the same weight of glucose. Readings obtained with glucose after 1 hr. at pH 5.5, corrected as usual for the 'reagent blank', increased by less than 10% after a further hour at 37°. Readings obtained with cellobiose after 18 hr. at 37° and pH 5.5 were almost identical with those given by glucose in 2 hr.

Estimations were also done with the glucose oxidase-peroxidase-anisidine reagents under similar conditions at pH 7 in tris buffer (Dahlqvist, 1961). This inhibited the cellobiose activity of the reagents.

Enzymic assay with CM-cellulose by using glucose oxidase and peroxidase. CM-cellulose (1.8 ml. of 1% solution), 0.9 ml. of acetate buffer, pH 5.4, and 0.8 ml. of water were mixed. Then 0.5 ml. of glucose oxidase solution and 2.5 ml. of peroxidase-anisidine reagent (see above) were added and mixed. The reaction was started by pipetting in 0.1 ml. of cell-free filtrate, diluted beforehand if necessary. Appropriate substrate and enzyme controls were included. After exactly 1 hr. at 37° all tubes were removed and read immediately, without inactivation, in 8 ml. cuvettes, as described above, commencing with the tubes containing both enzyme and substrate.

Method of enzyme assay (b) with the degraded insoluble substrate, cellulose reprecipitated from phosphoric acid. In dilute suspension this cellulose formed a stable medium whose extinction was proportional to its concentration. The decrease in extinction due to solubilization by cellulolytic preparations was used to measure enzymic activity.

Turbidimetric estimation of cellulose reprecipitated from phosphoric acid. Cellulose suspensions containing 0–36 mg. dry wt. of reprecipitated cellulose were made up to 80 ml. in a measuring cylinder and mixed by inversion, unnecessary aeration of the suspension being avoided. Samples were decanted into an 8 ml. cuvette with a 1 cm. light-path and read against water with Ilford orange filters (no. 607; peak transmission 600 m μ). The orange filters gave lower blank values than did neutral filters with equally good transmission.

Standard assay on degraded insoluble substrate, cellulose reprecipitated from phosphoric acid (reprecipitated cellulose). Approx. 31 mg. of reprecipitated cellulose in suspension (1.0 ml.) was added to 1 ml. of 0.2 M-sodium acetate-0.2 N-acetic acid buffer, pH 4.0, and water in a total volume of 3 ml. Then 1 ml. of cell-free enzyme solution was added with mixing and the reaction was allowed to proceed at 37° for 1 hr. Each tube was then washed out rapidly and thoroughly with up to 70 ml. of water and the total volume was made up to 80 ml. in a cylinder. After mixing a portion was read as above against water on a Spekker absorptiometer. Tubes containing both enzyme and substrate were removed from the water bath, diluted and read before tubes containing only one of these reagents. The whole procedure from removal after incubation to dilution, mixing and reading occupied less than 3 min. for any tube. For a series of tubes it was convenient to add the enzyme at suitably spaced intervals.

Method of enzyme assay (c) with the undegraded insoluble substrate, fibrous cotton. The new enzyme preparations from *T. koningii* described below hydrolysed cotton fibres completely to soluble reducing sugars or to very short insoluble fibres. Enzymic action was followed by: (1) the loss in weight of cotton fibres determined gravimetrically (Halliwell, 1957a), or colorimetrically with dichromate on filter-sticks (Halliwell, 1958), from measurements on the residual fibres after incubating in the presence and absence of the enzyme; (2) the formation of reducing sugars, by the colorimetric ferricyanide procedure (Halliwell, 1961) and by the glucose oxidase-peroxidase method described above.

The production of insoluble short fibres was followed in the 'extract of short fibres' (see below) separated from the enzymic reaction mixture. The amount of short fibres in suspension was determined turbidimetrically by measuring extinctions in the absorptiometer or colorimetrically. Turbidimetric measurements were done either on whole suspensions of the 'extract of short fibres' or, in certain cases (e.g. Fig. 3), on the washed material that was sedimented on the centrifuge. The colorimetric estimations were made on the 'extract of short fibres' as follows. The fibres were washed with water (to remove soluble material) either (a) once on the centrifuge or (b) washed well on a sintered-glass filter-stick, before oxidation with the dichromate reagent. They were then suspended in a suitable volume of water and dichromate reagent. The volumes of the latter two liquids, originally described for about 1.4 mg. of cellulose, were increased up to 4.5-fold and the heating time was extended to 1.75 hr. to compensate for the larger weight of fibres involved, up to 5 mg. (Halliwell, 1962).

Thus with short-fibre production the 'extract of short fibres' is used to measure: (1) all the material in suspension turbidimetrically; (2) centrifuged material therefrom either colorimetrically with dichromate or turbidimetrically; (3) short fibres, freed from filter-passing non-fibrous cellulosic material of milky appearance (see below), by the dichromate method.

Standard cellulase assay on cotton fibres as measured by the formation of short fibres. Cell-free filtrates from *T. koningii* acting on cotton fibres produce numerous very short fibres in 1-2 days. Shaking achieves the same effect in a few hours. In the standard cellulase assay for the production of short fibres screw-capped bottles were used with 25 mg. of non-de-waxed or de-waxed cotton fibres, 3-5 ml. of 0.2 M-sodium acetate-0.2 N-acetic acid buffer, pH 4.85,

about 3 ml. of enzyme and water to give a final volume of 9 ml. These were shaken vigorously, by hand a few times to wet the fibres, and then mechanically for 2 hr. at 37°. The bottles were then removed for extraction of the short fibres formed as follows. The residual mass of apparently un-attacked fibres was carefully transferred with fine forceps from the enzymic digest in the bottle to the inner surface of the cap of the bottle on the bench. The liquid medium with its content of short fibres was decanted through a small funnel with a short stem (internal diam. 2.5 mm.) sitting in a 25 ml. measuring cylinder. The cap and its fibrous mass were returned to the bottle along with 5 ml. of water; further amounts of short fibres were extracted from the residual cotton fibres by shaking vigorously 30 times by hand. This first 'aqueous extract' was transferred to the cylinder as described above, and the extraction repeated with another 5 ml. of water. After adding the second 'aqueous extract' to the cylinder the volume was made up with water to 20 ml. The combined 'extract of short fibres' consisted of short fibres and also, in some cases, of material of smaller size, called non-fibrous cellulose. The 'extract of short fibres' was sufficiently stable to permit extinction measurements to be made against water on a Spekker absorptiometer in 33 ml. cuvettes with a 4 cm. light-path and Ilford orange filters (no. 607; peak transmission 600 mμ).

Enzymic assays were initiated at suitably spaced intervals and removed after 2 hr. incubation for immediate separation of the short fibres. Extinction measurements on the 'extract of short fibres' were made immediately after preparation, or alternatively all extracts were put aside and read when the experiment had been completed. The 'extracts of short fibres' were stable enough for assay in this manner. Although the short fibres sedimented on standing they were readily resuspended by inverting the suspension and gave the same reading, even after 24 hr. at 3°.

RESULTS

CM-cellulase. The glucose oxidase-peroxidase procedure for estimating hydrolysis of soluble substrates, such as CM-cellulose, has the advantage that once the cellulolytic enzyme and two reagents are added no further treatment is necessary until the colorimetric readings are made. Colour develops gradually after addition of the CM-cellulase and may be read in less than the standard 1 hr. incubation period if desired. It could be made a continuous-recording method. Preliminary experiments showed maximum activity with 1.8 ml. of substrate.

As shown above, the glucose oxidase-peroxidase reagent was unspecific. Cellobiose also reacts under these conditions, the effect being 50% greater at pH 5.5 than at pH 7.2, suggesting the participation of cellobiase as an impurity in the glucose oxidase preparation. When the glucose oxidase-peroxidase-anisidine method was used to estimate the glucose content of enzymic hydrolysates of cotton fibres the same value was obtained in acetate buffer, pH 5.4, as in tris buffer, pH 7.0, at all stages up to complete hydrolysis. As shown below, *T.*

Fig. 1. Calibration curve of reprecipitated cellulose (cellulose reprecipitated from H_3PO_4) and the effect of substrate concentration on enzymic activity. Δ , Calibration curve relating extinction to dry weight of reprecipitated cellulose. $\circ$, Standard assay procedure at pH 4 on reprecipitated cellulose with cell-free filtrates from *T. koningii*, showing the effect of reprecipitated cellulose concentration on enzymic activity, measured by the decrease in extinction.

Fig. 2. Enzymic hydrolysis of cotton fibres and cellobiose. $\circ$, De-waxed cotton fibres (2mg.) were incubated in the standard cellulase assay system but without agitation at pH 4.9 with 5 ml. of cell-free culture filtrate from *T. koningii* for 17 days. Residual cotton fibres were estimated by oxidation with dichromate on filter-sticks. $\bullet$, Cellobiose (2mg.) incubated under the same conditions for 18 hr. Glucose formation was measured by the glucose oxidase-peroxidase reaction in tris buffer, pH 7.0.

koningii filtrates contain cellobiase and the end product is in fact glucose.

Enzymic hydrolysis of reprecipitated cellulose. Provided that reprecipitated cellulose is washed thoroughly, as described, to remove irregular-sized particles, the material readily forms an aqueous suspension stable enough for turbidimetric estimations. The calibration curve relating cellulose concentration to extinction departs slightly from linearity (Fig. 1). Enzymic solubilization of reprecipitated cellulose at pH 4 as revealed by partial clarification of the suspension is also illustrated in Fig. 1 and saturation of the enzyme is approached asymptotically.

In the standard assay at pH 4 with 31mg. of cellulose, cell-free filtrates from *M. verrucaria* as well as from *T. koningii* readily achieved complete dissolution of the substrate; for example, filtrates from the latter organism solubilized 15 and 100% of the cellulose in 1 hr. and in 18–24 hr. respectively.

Cellulase. The most noteworthy difference between previous cellulolytic enzyme preparations and the present cell-free cellulase is the ability of the latter to break down undegraded cellulose, such as that found in cotton fibres, as completely as do intact cellulolytic micro-organisms. Fig. 2 illustrates the total solubilization of cotton in 17 days by a single application of enzyme and the complete

hydrolysis of cellobiose. In another experiment, when 98% of the original fibrous cotton had been solubilized, measured by loss of weight on filter-sticks with the dichromate reagent, the reducing sugar (estimated as glucose) in the filtrate corresponded to 95% hydrolysis by both the ferricyanide and the glucose oxidase-peroxidase methods. Similar results have been achieved with non-de-waxed cotton: 3–4 weeks were required for virtually complete conversion into glucose.

Sterility tests were done after all prolonged incubations. The accumulation of reducing sugar and the unaltered acid pH confirmed the absence of viable cellulolytic organisms.

The most obvious feature of the solubilization of fibres shown in Fig. 2 was the appearance in 1–2 days of very short fibres up to about 3mm. in length. Their production was used to follow this primary stage in the enzymic degradation of cellulose. The very short fibres were estimated directly from the extinction of their suspension and subsequently from their cellulose content determined by oxidation with dichromate. The proportionality between extinction and the weight of cellulose is shown in Fig. 3.

Fig. 3. Relationship between extinction of the short fibres and their cellulose content. Non-de-waxed cotton fibres were hydrolysed enzymically by cell-free filtrates from *T. koningii* in the standard cellulase assay at pH 4.9. The short fibres were extracted in the usual manner and washed well with water by centrifuging. Samples were diluted with water to the standard volume for measuring extinctions, after which the cellulose content of the same samples was estimated by oxidation with dichromate of filter-sticks.

Effect of substrate concentration on the enzymic formation of short fibres from cotton fibres. Filtrates from *M. verrucaria* and from *T. koningii* gave similar curves, attaining maximum activity in the presence of 25–30 mg. of fibres, depending on the nature of the initial cotton (Fig. 4). With these amounts of fibres the waxy layer in non-de-waxed cotton discouraged enzymic formation of short fibres at a lower concentration than it did with de-waxed fibres. This effect appeared to be associated with the difficulty of wetting larger amounts of non-de-waxed fibres. Quantities above 30 mg. of either substrate, however, produced increasing inhibition of enzymic activity.

In Fig. 4, the short fibres were separated from soluble carbohydrates in the aqueous extract by centrifuging before oxidation with dichromate. This technique was also used in the experiment (○ in Fig. 5); the formation of short fibres from 25 mg. of non-de-waxed cotton fibres in periods up to 4 hr. was followed. The extinction of the whole extract of short fibres was measured, after which soluble products were removed by centrifuging and the sedimented short fibres were oxidized with dichromate. In 2 and 4 hr. the amount of short fibres produced in the extract corresponded to 9 and 26% breakdown respectively of the weight of cotton fibres taken.

Fig. 4. Effect of weight of cotton fibres on the enzymic formation of short fibres. Standard cellulase assay. De-waxed and non-de-waxed cotton fibres were treated with cell-free filtrates from *M. verrucaria*, after which the formed short fibres were separated from the extract by centrifuging and estimated by oxidation with dichromate. ●, Short fibres formed from de-waxed cotton; ○, with a different enzyme filtrate, short fibres formed from non-de-waxed cotton. Non-de-waxed cotton fibres were treated with cell-free filtrates from *T. koningii*, after which (a) the extinction of the whole extract of isolated short fibres was measured (▲), (b) the short fibres were then separated from the extract by centrifuging and estimated by oxidation with dichromate (■), and (c) the total loss of weight of the cotton fibres was determined gravimetrically on the residual cotton fibres after removing the extract of short fibres (△).

Total enzymic hydrolysis of cotton fibres to short fibres and soluble products. The enzymic formation of short fibres from the long cotton fibres was followed after first removing the small fraction of non-fibrous cellulose (see below) as well as soluble carbohydrates. This separation was accomplished by extensive washing on the centrifuge (as in Fig. 3) or more conveniently by washing the extract of short fibres on a filter-stick. All material not retained on the filter-sticks is therefore included in the fraction described as soluble.

Filter-sticks were used to measure the formation of short fibres from 4.5 mg. of non-de-waxed and de-waxed cotton fibres incubated with cell-free filtrates from *T. koningii* for up to 12 hr. (Fig. 6). At the end of this period, the transformation of cotton fibres into short fibres and soluble products appeared to be visibly complete. After removal of soluble products and the extract of short fibres by the standard procedure, estimation of the residual non-de-waxed and de-waxed cotton indicated 98 and 96% breakdown respectively. Of the 96%

Fig. 5. Effect of duration of incubation on the enzymic breakdown of cotton fibres to soluble products and to insoluble short fibres. The standard cellulase assay with cell-free filtrates from *T. koningii*. Non-de-waxed cotton fibres (25mg.) were incubated with enzyme for 1-4hr., after which (a) the extinction of the whole extract of short fibres was read (●), and (b) mg. of cellulose of the short fibres was measured by oxidation with dichromate after removing the aqueous phase by centrifuging (○).

Fig. 6. Total enzymic hydrolysis of cotton fibres to short fibres and soluble products in the standard cellulase assay with cell-free filtrates from *T. koningii*. Non-de-waxed and de-waxed cotton fibres (4.5mg.) were incubated with enzyme for 2-12hr. until conversion into short fibres appeared to be complete, after which (a) the extinction of the whole extract of short fibres, from de-waxed cotton fibres, was read (●), (b) cellulose of the short fibres (□, formed from non-de-waxed cotton; Δ, formed from de-waxed cotton) was determined (as a percentage of the weight of cotton fibres taken) by oxidation with dichromate on filter-sticks, and (c) the total solubilization (all filter-passing products) of the original cotton (○, non-de-waxed cotton fibres; ▲, de-waxed cotton fibres) was estimated from the formula $100\% - (\text{short fibres} + \text{residual cotton fibres})$.

total breakdown of de-waxed fibres achieved in 12hr., 80% was converted into short fibres and 16% (by difference) into soluble carbohydrates (including filter-passing non-fibrous material). In 7hr., 71% of the cotton was converted into short fibres and only 10% solubilized. Similar values were obtained with 4.5mg. of non-de-waxed cotton fibres, which were transformed into 73% of short fibres and only 25% (by difference) of soluble products in 12hr. Thus the amount of insoluble cellulose produced as short fibres from either de-waxed or non-de-waxed cotton fibres greatly exceeded the fraction solubilized (cf. Halliwell, 1962).

Variation in size of the cellulose particles apparently occurs with 4.5mg. of cotton in the experiment in Fig. 6 between 2.3 and 7hr., in which interval the extinction of the relatively clear extracts of short fibres from non-de-waxed (not illustrated) and de-waxed fibres (●) increased two- and three-fold respectively, whereas the cellulose content of the short fibres (□ and Δ) increased six- and ten-fold respectively in the same period despite the probable loss of filter-passing material. This evidence and the formation of mainly non-fibrous insoluble cellulosic products in the first hour of incubation suggest that the primary enzymic attack was on the smaller, more accessible,

molecular chains in cellulose. These were subsequently truly solubilized more rapidly than the longer chains that were liberated later as distinct short fibres. This conforms with the extensive fragmentation and small degree of solubilization that occurred in the later periods of incubation, as described above.

Effect of enzyme concentration on the formation of short fibres. Over the range of concentration of cell-free filtrates used in the standard assay system enzymic activity was linearly related to the extinction of the extract of short fibres and also to the cellulose content of these fibres determined colorimetrically on filter-sticks (Fig. 7). The latter measurements in this experiment indicate a 16% conversion of fibrous cotton into short fibres in 2hr. with the largest quantity of enzyme. Cell-free filtrates from *M. verrucaria* gave similar results under the same assay conditions. Such curves contrast with those obtained with similar enzyme preparations from *M. verrucaria* acting on swollen

Fig. 7. Effect of enzyme concentration on the formation of short fibres from non-de-waxed cotton fibres by cell-free filtrates from *T. koningii*. The standard cellulase assay was used. ○, Extinction of the whole extract of short fibres; Δ, mg. of cellulose of the short fibres determined by oxidation with dichromate on filter-sticks.

Fig. 8. Effect of pH on the hydrolysis of reprecipitated cellulose by cell-free filtrates from *T. koningii*. Enzymic solubilization of cellulose was measured by the decrease in extinction of the suspension in citric acid-phosphate buffer and below pH 2.5 in (a) citric acid-phosphate buffer adjusted with H_2SO_4 or in (b) acetate-HCl buffer. The results below pH 2.5 were indistinguishable with the different buffers.

cellulose powder, where loss of weight of the substrate was proportionate only at smaller concentrations of enzyme (Halliwell, 1961).

Activity of cell-free filtrates at different pH values on reprecipitated cellulose. Citric acid-phosphate buffer was used to cover the range pH 2-8 in mixtures containing cell-free culture filtrates from *T. koningii* and reprecipitated cellulose. At pH values below 2.5, citric acid with and without phosphate was adjusted to the required pH with dilute sulphuric acid to retain both of the original ions. Fig. 8 indicates little activity above neutrality, whereas some solubilization took place at a pH as low as 2. At higher pH values enzymic activity increased rapidly to a maximum about pH 3.5-4.0.

Activity of cell-free filtrates at different pH values on non-de-waxed cotton fibres. Under standard assay conditions breakdown of cotton fibres by cell-free filtrates from *T. koningii* showed two reproducible optima at pH 3.6 and 5.0 in acetate buffer when extinction measurements were made on the whole extract of short fibres (○ in Fig. 9). In citrate buffer the optima were at pH 3.1 and 4.8. When, however, the short fibres in the same whole extracts were measured colorimetrically after washing and oxidation with dichromate on filter-sticks, the lower pH optima was absent and left only a single peak at pH 5.0 in acetate buffer (in Fig. 9) or at pH 4.8 in citric acid-phosphate buffer.

Fig. 9. Effect of pH on the formation of insoluble non-fibrous cellulose and insoluble short fibres from non-de-waxed cotton fibres by cell-free filtrates from *T. koningii*. The standard cellulase assay was used. Enzymic activity was measured in acetate-acetic acid buffer or in acetate-HCl buffer for the lower pH values. Each whole extract of short fibres was examined for (a) extinction (○), and then (b) for cellulose content by oxidation with dichromate on filter-sticks (□).

In the latter buffer, enzymic formation of short fibres, measured colorimetrically on filter-sticks, was limited to pH 2.3–7.3 (cf. acetate; □ in Fig. 9).

Enzymic activity at about pH 3.1–3.6 favoured the formation of non-fibrous cellulosic particles that were responsible for the milky appearance of the extracts and the high turbidity. Such particles, however, were small enough to permeate the filter-stick. Similar results were obtained with de-waxed cotton fibres tested enzymically at pH 3.2 and 4.8 when the whole extract of short fibres produced at each pH value was examined by extinction measurements and subsequently by oxidation with dichromate on filter-sticks.

Enzymic degradation of cotton fibres to short fibres and soluble products at pH 3 compared with that at pH 5. In Fig. 9 it was possible that the amount of cotton fibres degraded enzymically at pH 3.6 to soluble and insoluble products, and found

in the extract of short fibres, was less than that degraded enzymically at pH 5.0. It may, however, have been more dispersed, resulting in loss through the filter-stick of the smaller cellulosic particles that contributed highly to the extinction measurements.

This possibility was tested in the standard assay procedure at pH 4.8 to encourage enzymic formation of discrete short fibres and at pH 3.1 to encourage production of filter-passing non-fibrous degradation products. The standard weight, 25 mg. of non-de-waxed cotton fibres, as well as 5 mg., was shaken for the standard period of 2 hr. or for 20 hr. At the latter period the breakdown of fibres at pH 4.8 to short fibres and soluble products appeared complete (Table 1). The extracts of short fibres contained cellulosic degradation products of three types: (1) truly soluble; (2) insoluble filter-passing non-fibrous cellulose particles; (3) insoluble non-filter-

Table 1. *Effect of pH on the formation of soluble and insoluble cellulosic breakdown products from cotton fibres by cell-free filtrates from T. koningii*

The standard cellulase assay for 2 or 20 hr. was used. Non-de-waxed cotton fibres were shaken with enzyme for the periods shown at pH 3.1 or 4.8. The whole aqueous extract of short fibres was prepared as normally to measure its extinction; the cellulose content of the extract was determined (1) on the sedimented cellulose after centrifuging off soluble carbohydrates and (2) on filter-sticks after washing. The loss in weight of the original cotton was determined before and after extracting the short fibres.

Expt.	Experimental conditions	Extinction of extract of short fibres	Short fibres in extract [% of initial weight of cotton fibres (after incubation), determined by oxidation with dichromate]		Total loss of weight of original cotton fibres [% of initial weight (after incubation) determined gravimetrically on the residual cellulose after removing extract of short fibres]	
			After centrifuging	After washing on a filter-stick		
Expt. 1	25 mg. of cotton fibres; 2 hr.; pH 3.1	0.43	4.4	0	5.6	
	25 mg. of cotton fibres; 2 hr.; pH 4.8	0.10	4.1	3.0	10.8	
Total loss of weight of original cotton fibres [% of initial weight (after incubation), determined by dichromate oxidation of residual cellulose on filter-sticks]						
Expt.					Before removing extract of short fibres	After removing extract of short fibres
Expt. 2	5 mg. of cotton fibres; 2 hr.; pH 3.1	0.14	8	—	9	9
	5 mg. of cotton fibres; 2 hr.; pH 4.8	0.06	15	—	9	23
Expt. 3	5 mg. of cotton fibres; 20 hr.; pH 3.1	0.26	76	59	—	81
	5 mg. of cotton fibres; 20 hr.; pH 4.8	0.20	79	74	—	94

passing short fibres. Truly soluble material in the extracts was removed by centrifuging or by filtering. Insoluble cellulosic degradation products of types (2) and (3) were removed together by centrifuging. Insoluble non-filter-passing short fibres were isolated from the other two types of cellulosic degradation products by filtering the extracts of short fibres.

In Expt. 1 in Table 1 total enzymic cellulolysis was measured gravimetrically from the loss of weight of the cotton fibres that followed removal of the extract of short fibres and subsequent washing of the residual cotton fibres. Total cellulolysis at pH 4.8 was almost twice as great as that at pH 3.1, where about 6% total breakdown of substrate took place, of which 80% was found as insoluble sedimentable cellulose (measured colorimetrically) in the extract of short fibres and 20% (by difference) was truly solubilized. At pH 4.8, 11% total cellulolysis occurred, of which 40% was found in the insoluble sedimentable fraction of short fibres and the rest was solubilized. Extinction measurements of whole extracts of short fibres produced enzymically at pH 3.1 showed a four-fold greater activity than those produced at pH 4.8, although the cellulose of the sedimented short fibres was about the same under both conditions (Expt. 1 in Table 1). This apparent anomaly was due to the presence, in extracts of short fibres produced enzymically at pH 3.1, of much non-fibrous sedimentable cellulose, in contrast with the discrete short fibres formed at about pH 4.8. The amount of this non-fibrous small particulate material present was shown by difference after filtering off the extracts of short fibres before the colorimetric estimation of the discrete short fibres retained on the filter. When this was done 25 and 100% respectively of the cellulose in the extracts produced enzymically at pH 4.8 and 3.1 escaped through the filter (Expt. 1 in Table 1).

Smaller amounts (5mg.) of cotton fibres were examined at the two pH values in the same incubation periods in experiments designed to permit measurements on the residual cotton fibres before and after removing the extract of short fibres (Expt. 2 in Table 1). The total loss of weight of the enzymically treated cotton fibres before removal of the extract of short fibres consists of truly soluble products plus filter-passing non-fibrous products; the total loss of weight after removing the extract of short fibres consists of the truly soluble and non-fibrous fractions just mentioned plus non-filter-passing short fibres.

Total cellulolysis was again greater at pH 4.8 than at pH 3.1, where almost all hydrolytic products were present in suspension in the extract of short fibres as smaller sedimentable particles. This non-fibrous small-particulate matter was readily washed through the filter, so that the weight of the

residual cotton was the same whether or not their extract of short fibres had first been removed. In 2hr. at pH 4.8, 9% of the cotton fibres were converted into filter-passing products, and after the short fibres had been extracted from the residual cotton a further 14% loss of weight occurred. This loss corresponded almost to that amount of cellulose (15%) found directly by estimating the sedimentable short fibres, indicating that essentially all the cellulose particles in the extract of short fibres were large enough to be retained on the filter-stick.

In 20hr. at pH 4.8 enzymic breakdown of 5mg. of cotton fibres was judged to be complete by the absence of long fibres characteristic of the initial substrate. Analysis showed 94% conversion into soluble and insoluble products, with 74% of the substrate being recovered in the insoluble non-filter-passing short-fibre fraction (Expt. 3 in Table 1). No more than 15% true solubilization accompanied the almost-complete breakdown of the substrate and only 5% of the original cotton fibres was converted into the filter-passing non-fibrous type of cellulose. At pH 3.1 the extent of cellulolysis (81%) was again less than at pH 4.8, and only 5% of the cotton fibres was truly soluble. The formation of insoluble cellulosic degradation products (76 and 79%), however, was almost identical at both pH values, but at pH 3.1 much more of this fraction (17% of the initial substrate) consisted of filter-passing non-fibrous cellulose.

Nature of the insoluble filter-passing non-fibrous cellulosic degradation products. This non-fibrous material produced at the lower pH values was sedimented by centrifuging and lost by filtering. Attempts were made to establish its nature by incubating non-de-waxed cotton fibres in the standard cellulase assay but with citric acid-phosphate buffer, pH 2-7. Enzymic activity was obtained from extinction measurements of the whole extract of short fibres prepared in the usual manner in water. Under those conditions activity extended over the range pH 2.3-7.2 and, as reported above, two optima were present, at pH 3.1 and 4.8. When the experiment was repeated and the reaction terminated by adding N-sodium hydroxide to give pH 11, before preparing the extracts of short fibres (final pH 10.8) for turbidimetric readings, the optimum at pH 3.1 was absent. Activity fell gradually from the remaining peak at pH 4.8 to zero at pH 2, suggesting that filter-passing particles were alkali-soluble (cf. β -cellulose).

Effect of carbohydrates and metals on the enzymic degradation of non-de-waxed cotton fibres to short fibres and on the solubilization of reprecipitated cellulose. Whereas glucose had no marked inhibitory action on the enzymic solubilization of reprecipitated cellulose or the formation of short fibres from cotton fibres, unless present in amounts at least

Table 2. *Action of various reagents on the enzymic breakdown of cotton fibres to short fibres, and the solubilization of reprecipitated cellulose, by cell-free filtrates from T. koningii*

Standard cellulase assay in acetate buffer, pH 4.8, on non-de-waxed cotton fibres was used. Whole aqueous extracts of short fibres were prepared in the usual manner and examined for extinction and subsequently for cellulose content by colorimetric estimation with dichromate on filter-sticks. Standard assay for reprecipitated cellulose was in acetate buffer, pH 4.0. Results are given as percentage inhibitions or activations relative to the control assay incubated without the reagent. All reagents in the Table were added immediately before the enzyme.

Reagent (final concn.)	Substrate.....	Percentage inhibition (–) or activation (+)		
		Reprecipitated cellulose	Cotton fibres	
			Activity measured by extinction	Activity measured by dichromate on filter-sticks
Carbohydrates				
Glucose (100 mm, 10 mm)		–46, 0	–60, 0	–50, 0
Cellobiose (10 mm, 1 mm, 0.2 mm)		–30, 0, 0	–50, 0, 0	–83, +11, 0
CM-cellulose (0.33%, 0.07%)		–86, –23	–31, 0	—
Gluconic acid (lactone) (25 mm, 2.5 mm)		–24, –16	–32, 0	–31, 0
Metal salts				
CuSO ₄ (20 mm, 2 mm, 0.2 mm, 0.02 mm)		–28, –13, 0, 0		–100, –80, –30, 0
HgCl ₂ (2 mm, 0.2 mm)		–70, –41		–100, –40
FeCl ₃ , (2 mm, 0.2 mm, 0.02 mm)		–100, –54, –30		–100, –15, 0
MnSO ₄ (20 mm, 2 mm)		0, 0		–35, –11
Zinc acetate (20 mm, 2 mm)		0, 0		–55, 0

two and six times as great respectively as the initial amount of each substrate, cellobiose at 0.01 M was slightly more effective, giving 30 and 50–83% inhibition respectively when present in amounts approximately equivalent to the initial weight of the two forms of cellulose (Table 2). CM-cellulose, a substrate sometimes used for cellulolytic enzymes, produced less inhibition of the enzymic hydrolysis of cotton fibres to short fibres than it did of the solubilization of reprecipitated cellulose. Gluconolactone also was not very effective in preventing the breakdown of either substrate.

The heavy-metal ions Hg²⁺, Cu²⁺ and Fe³⁺ were significantly inhibitory in that order at 0.2 mM final concentration, whereas Mn²⁺ and Zn²⁺ required approximately 100-fold this concentration to effect a comparable inhibition of the breakdown of cotton fibres to short fibres when measured colorimetrically on filter-sticks. In general the action of metal ions on the enzymic solubilization of reprecipitated cellulose was similar to that on cotton fibres, except with Mn²⁺ and Zn²⁺. These failed to produce any inhibition of the enzymic solubilization of reprecipitated cellulose at concentrations that gave 35–55% decrease in the hydrolysis of cotton fibres to short fibres.

Effect of heating and shaking on the enzymic degradation of non-de-waxed cotton fibres to short fibres and on the enzymic hydrolysis of CM-cellulose. After heating cell-free filtrates from *T. koningii* in the absence of substrate at pH 6.4 for 10 min. at

71° no residual activity was present when tested on CM-cellulose as measured by the glucose oxidase-peroxidase method, or on cotton fibres when the extract of short fibres was measured colorimetrically on filter-sticks. Of the original enzymic activity 100 and 94% respectively were retained against the same substrates after heating for 10 min. at 51°. No loss of activity occurred after cell-free culture filtrates at pH 6.4 were shaken (as in the standard cellulase assay) for 2 hr. at 37° and then assayed on cotton for the production of short fibres or on CM-cellulose by the method just described.

DISCUSSION

The most suitable substrate for stringent enzymic work on cellulase is native fibrous cotton purified by minimal treatment to remove non-cellulosic impurities. Organic solvents are used to remove wax, and hot alkali under nitrogen to separate pectins (Corbett, 1963; Halliwell, 1963). If the purification is not rigorously controlled, considerable degradation of cellulose may occur by dilute alkali without participation of oxygen (Corbett, 1959; Richards, 1963) and particularly by alkali in the presence of oxygen (Nevell, 1963).

Unlike the parent organism, cell-free cellulolytic filtrates in the past failed to produce more than minor breakdown of undegraded cellulose such as that found in carefully purified cotton fibres. As a result some workers have used alternative forms of

cellulose as enzymic substrates, including cellulose powders or cotton fibres subjected to grinding or chemical treatments or both. These procedures promote degradation of cellulose to a minor or major extent, forming molecular regions more susceptible to attack by enzymic preparations. Unless an enzyme preparation has previously been shown to break down effectively any residual undegraded areas, as well as the more susceptible degraded portions of cellulosic substrates, it is difficult to assess the significance of the results. An indication of the extent of degradation of a cellulose is shown, for example, by the β - plus γ -cellulose content [the fraction of cellulose soluble in 17.5% (w/w) sodium hydroxide at 20°], which with cellulose powder (Whatman) amounted to 30%, in contrast with 2% in de-waxed cotton fibres (Halliwell & Bryant, 1963).

Cellulose powder (Whatman) and acid-hydrolysed ball-milled cotton wool are degraded types of cellulose used by Abou Akkada, Eadie & Howard (1963). Extracts from rumen protozoa solubilized 25–30% of these degraded cellulosic substrates, an amount corresponding to the degraded fraction present as β - plus γ -cellulose in this type of cellulose. Undegraded cellulose in the form of de-waxed Texas cotton fibres was not attacked by the same protozoal extracts (B. H. Howard, personal communication).

Selby, Maitland & Thompson (1963) preferred to use processed cotton fibres in the form of yarn rather than untreated cotton fibres. Yarns were autoclaved at 140° in 1% sodium hydroxide for 6 hr., to remove impurities, before being used as a substrate for filtrates from *M. verrucaria*. Complete loss of yarn strength was achieved but less than 30% loss of weight in 16–35 days, although the yarn was treated daily with fresh replacements of culture filtrate: this restricted solubilization was attributed to localized attack on cellulose by the enzyme and its immobility in the crystalline regions of cellulose (Selby *et al.* 1963; Selby, 1961). Such problems, however, present no difficulty to the intact cellulolytic organism.

A form of cellulose that is readily hydrolysed by culture filtrates is the undried cotton fibre in the naturally swollen state in unopened cotton bolls. When removed from early mature bolls and kept constantly in water, such cotton fibres readily disintegrate and lose up to 52% of their weight when shaken with culture filtrates of various fungi (Marsh & Reese, 1963). When allowed to dry these same fibres become resistant to the culture filtrates. *Trichoderma* species have also been used with other cellulosic substrates (for reviews see Reese, 1959, 1963; Toyama, 1963). Using *T. koningii* filtrates, Toyama (1953) found that regenerated celluloses were more readily hydrolysed than was cotton.

Whereas activity on ground cotton ceased after 3 days culminating in 12% solubilization as reducing sugar (Toyama, 1960), that on filter papers caused complete disintegration to fine fibres with 7% saccharification (Toyama, 1963).

In the present work non-de-waxed cotton fibres were used directly, or were de-waxed by the purification procedures recommended above, by employing organic solvents and alkali under nitrogen to minimize degradation. Though previous culture filtrates and purified 'cellulase' preparations attacked only degraded celluloses or minor fractions of undegraded cellulose, the new cell-free enzyme preparations readily hydrolysed degraded (reprecipitated) cellulose and also produced complete solubilization of undegraded cellulose of cotton fibres, in a manner similar to that achieved by intact micro-organisms. The loss of weight of cotton fibres in the presence of cell-free filtrates was recoverable as glucose. In the early stages of breakdown with the conditions shown in Table 1 and Fig. 6 extensive transformation of de-waxed and non-de-waxed cotton to short fibres was accompanied by relatively little solubilization.

The cell-free culture filtrates from *T. koningii* described act on all types of cellulose from undegraded cotton fibres through degraded insoluble cellulose in the form of reprecipitated cellulose to the soluble cellulose derivative, CM-cellulose. So far, inactivation studies have not separated these enzymic activities. The pattern emerging from the present work on the cellulolysis of undegraded cellulose in the form of carefully purified cotton fibres suggests a random breakdown of the substrate releasing: (1) insoluble non-filter-passing very short fibres; (2) insoluble filter-passing cellulosic products; (3) truly soluble products, mainly glucose. Formation of glucose and of the short fibres proceeds simultaneously. Cellobiose is rapidly hydrolysed to glucose.

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Note added in proof. Since this paper was submitted for publication, Mandels & Reese (1964) have described enzyme preparations from *Trichoderma viride* that produce 70–80% hydrolysis of cotton but do not apparently liberate the short fibres described in the present paper.

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