

Degradation of cellulose by *Aspergillus* sp *Trichoderma koningii*, and *Myriococcum* sp.

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RINGKASAN

Turasan kultur Aspergillus sp, Trichoderma koningii dan Myriococcum sp menunjukkan bahawa organisma-organisma tersebut adalah aktif dalam pemecahan kapas dan kertas turas. Suhu optima bagi tumbuhan Aspergillus sp dan T. koningii adalah pada 37° C sementara bagi Myriococcum pada 45° C-50° C. Suhu optima bagi aktiviti T. koningii didapati pada 45° C, dan 40° C bagi Aspergillus sp dan Myriococcum; pH optima bagi aktiviti berlaku pada pH 5.0, 4.0 dan 7.0 masing-masing. Kajian ke atas sisa selulosa menunjukkan bahawa sisa nenas adalah yang paling mudah dipecahkan. Dengan menggunakan ekstrak enzyme mentah untuk kajian sinergistik didapati hanya sedikit penambahan dalam aktiviti enzim (<40%).

SUMMARY

Culture filtrates obtained from Aspergillus sp, Trichoderma koningii and Myriococcum sp were shown to be active in degrading cotton wool and filter paper. The optimum temperature for growth for Aspergillus sp and T. Koningii occurred at 37° while that of Myriococcum at 45°-50° C. Optimum temperature for activity was recorded as 45° C for T. koningii and 40° C for Aspergillus sp and Myriococcum sp; the pH optima occurred at pH 5.0, 4.0 and 7.0 respectively. The action of these organisms on various cellulosic wastes was tested and pineapple waste was the most amenable to degradation. Synergistical studies using crude enzyme extracts indicated only a small increase in enzyme activity (40%).

INTRODUCTION

Owing to the depleting reserves of fossil fuel and shortage of oil, much interest has been prompted in the use of cellulosic materials as alternative sources of energy and food. Since the major part of photosynthetic activity ends up as cellulosic materials, they represent the largest renewable resource available for utilization by cellulolytic microorganisms. Hydrolysis can be effected either by enzymic or acid hydrolysis; acid hydrolysis suffers from the problems of formation of decomposition products and corrosion of process equipment whereas the enzymic route offers sugar monomers under mild processing conditions as the endpoint. The main cost factor in enzymic hydrolysis is the high cost of the cellulase enzymes; reduction of costs will involve the proper selection of strains yielding high levels of the enzymes and a thorough knowledge

of their properties. The main aims of this work include the isolation and identification of cellulolytic organisms, determination of their optimum conditions in cellulose breakdown and also a study of their activities on different organic wastes as substrates.

MATERIALS AND METHODS

Materials

Various isolates of fungi were made from compost heaps in Sungei Besi, Kuala Lumpur, and a wood factory in Jalan Klang Lama, Kuala Lumpur using the methods described below. The most active cellulolytic organism was an *Aspergillus* sp from a compost heap. This was selected for further study and comparison with other known cellulolytic fungi. The latter were obtained from a culture collection in the Department of Biochemistry and Microbiology, Universiti

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Pertanian Malaysia, Serdang, Selangor. Whatman no. 1 filter paper was obtained from Whatman, Maidstone, Kent ME14 2LE, U.K. Cotton wool was purchased locally. Palm oil fibres were kindly supplied by Jabilee Estate, Malacca. Pineapple wastes were from Pineapple Cannery, Malaysia Sdn. Bhd., Johore and cocoa pods were from Devon Estate, Sime Darby, Malacca.

Difco powdered yeast extract was a product of Oxoid Limited, England. Agar was obtained from Sigma Chemical Company, Missouri, U.S.A. Difco Bacto peptone was purchased from Difco Laboratories, Detroit, Michigan, U.S.A.

Buffers

The citrate/phosphate buffers from pH 3.0 to 8.0 were all prepared from 0.1M-citric acid and 0.2M-dibasic sodium phosphate. 0.2M-Tris/HCl buffer was used for pH 8.0 and 9.0.

Methods

Isolation of fungi. The isolation techniques used were the direct method of Waksman *et al.* (1939), in which samples were sprinkled upon the surface of the media, and the dilution method. Hyphal isolates were subcultured onto fresh yeast-glucose agar (YGA) until pure cultures were obtained.

Preparation of culture filtrate. Fungi were subcultured on YGA and grown for three days at 44°C for *Aspergillus* and *Myriococcum* sp and 25°C for *T. koningii*. Three agar-mycelium discs (0.8 cm diam.) were transferred to a Wheaton medical flat (C-16, 500 ml) containing 60 ml of Fergus (1969) medium with 2.0 g filter paper as carbon source. After 16 days incubation at the above mentioned temperature for each organism the culture was filtered through glass-fibre paper (Whatman GF/C) to remove hyphal fragments and residual insoluble cellulose. The clear culture filtrate obtained was stored in the deep freeze for later use.

Choice of Organisms for Further Studies

The actively cellulolytic fungus from the initial survey was identified as an *Aspergillus* sp. The cellulolytic ability of this fungus together with those obtained from the departmental collection was tested in the manner as described below. Culture filtrates from *Aspergillus* sp., *Myriococcum* sp. and *T. koningii* were most active in degrading cotton wool (Table 1) and thus they were chosen for further studies.

Table 1
Degradation of cotton wool by fungi.¹

Organism	Specific activity (unit/mg) × 10 ⁻²	
	25°C	44°C
<i>Aspergillus</i> sp.	5.06	5.95
<i>Chaetomium</i> sp.	0.89	1.30
<i>Humicola insolens</i>	0.55	0.90
<i>Myriococcum</i> sp.	3.74	4.95
<i>Myrothecium verrucaria</i>	1.36	1.87
<i>Sporotrichum pulverulentum</i>	0.30	1.29
<i>Thermoascus aurantiacus</i>	3.16	3.32
<i>Trichoderma koningii</i>	2.26	5.08

¹ Standard assay system was used except that the temperatures of incubation were 25 and 44°C. Reducing sugars formed were determined as described in Materials and Methods.

Inoculum. The inoculum for each growth experiment was taken from cultures grown on YGA at 25°C or 44°C for three days. An agar-mycelium disc (0.8 cm in diam) was cut from the perimeter of the colony with a sterile cork borer, inverted and placed at the centre of a 9cm diam. petri dish containing agar medium.

For seeding liquid media, three agar-mycelium discs (0.8 cm in diam) were placed into each medical flat containing 60 ml of Fergus (1969) medium.

Maintenance of stock cultures. *Aspergillus* sp., *Myriococcum* sp. and *T. koningii* were cultured onto YGA slopes and incubated for three days at their optimum temperature for growth (Fig. 1) and stored at 27°C for no more than four weeks prior to further culturing.

Determination of cellulolytic ability of fungi. The organisms were grown in Fergus (1969) medium with chopped filter paper as carbon source. After incubation for 16 days at the temperature indicated above for each organism, the filtrate was collected in the manner as described above and assayed for activity against filter paper. Cellulolytic activity is as defined below.

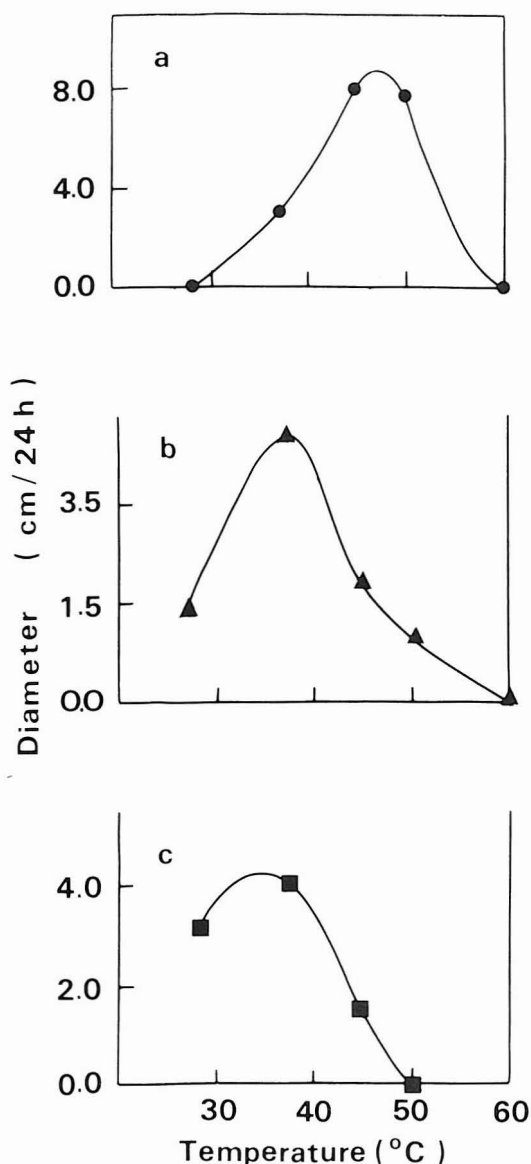


Fig. 1. Optimum temperature for growth on YGA. Each point represents the average colony diameters in triplicates.
 (a) *Myriococcum*
 (b) *Aspergillus*
 (c) *Trichoderma koningii*

Determination of temperature optimum for growth.

The optimum growth temperature was ascertained by measuring the diameters of colonies on YGA medium in 9 cm diam. petri dishes containing approximately 30 ml of agar medium.

Each agar plate was inoculated with one agar-mycelium disc as described earlier. Triplicate colonies were measured after incubation at the temperatures shown in Fig. 1 for 48 h and the average of the longest and shortest diameter of each colony was recorded. To reduce desiccation of the agar medium, a beaker of distilled water was placed in each incubator.

Effect of incubation period on the production of enzyme. Medical flats containing 60 ml of Fergus medium were inoculated and incubated at temperatures mentioned above for different times. The contents of two flats from each culture were harvested at interval of two to three days up to 16 days by suction through a Whatman GF/C filter. The culture filtrates were then assayed for cellulase activity on cotton wool determined by estimating the reducing sugars formed using the Nelson-Somogyi method (Nelson, 1944; Somogyi, 1952) as described below.

Determination of protein. Protein was determined by the Method of Lowry *et al.* (1951). Crystalline bovine serum albumin was used as a standard.

Determination of reducing sugars. The number of reducing sugar groups created by hydrolysis of the cellulosic substrates was measured spectrophotometrically by using the Nelson-Somogyi procedure (Nelson, 1944; Somogyi, 1952).

Assay of cellulolytic activity. An indication of total cellulolytic activity was obtained by the determination of filter paper degrading activity or cotton wool degrading activity. The standard reaction mixture contains 20 mg of filter paper (Whatman no. 1) or 3.0 mg of cotton wool, 0.5 ml of citrate-phosphate buffer, pH 5.0, 0.5 ml of enzyme solution (culture filtrate) of appropriate dilution (if necessary) and one drop (10 μ l) of toluene. After incubation at 44°C for three days, 0.5 ml of the reaction mixture was withdrawn and assayed for reducing sugars using the method mentioned above.

An absolute definition of a unit of cellulase activity is difficult. There is little to be gained by expressing the activity in terms of glucose equivalents, since glucose is not the only product of the enzyme reaction (Tong *et al.* 1980). A unit of cellulase activity is defined as that amount of enzyme that produces an increase in absorbance of 0.10 at 560nm under the conditions defined. A change in absorbance of 0.10 is equivalent to 30 μ g of glucose under the conditions given, and

thus cellulase preparations with units quoted in glucose equivalents can be compared.

Preparation of treated natural cellulosic substrate for hydrolysis.

Physical pretreatment. The cocoa pod and bagasse were cut and ground using a mortar and pestle. The solid pineapple waste was blended for one minute. The treated substrate was suspended in distilled water (500 ml) and suction filtered through two layers of muslin cloth with several changes of distilled water. The substrate was then resuspended in 250 ml of distilled water and shaken for two hours. Aliquots of the suspension were withdrawn and analysed for the presence of reducing sugars. Where necessary, the process of washing was repeated until no more reducing sugars were detected in the suspension. The following treatments were carried out after the physical pretreatment.

Alkaline pretreatment. Ten grams of cellulosic substrate were dispersed in 100 ml of 2M NaOH and left under vacuum for three hours. After this, the treated substrate was washed thoroughly in distilled water and suction filtered until the filtrate gave the pH of distilled water (pH 5.9).

Acid pretreatment. Fifteen grams of cellulosic substrate were suspended in 200 ml of 15.2M (85% v/v) ortho-phosphoric acid with occasionally stirring. After three hours, one litre of distilled water was added and the mixture was stirred and suction filtered through two layers of Whatman no. 1 filter paper. 500 ml 2% (w/v) sodium carbonate was then added and the mixture homogenised for 5 min in a waring blender. It was suction filtered and washed with 2 litres of distilled water until the filtrate gives the pH of distilled water (pH 5.9).

Autoclaving pretreatment. Ten grams of the substrate was autoclaved for 60 min at 15 psi.

RESULTS

Comparison of cellulolytic ability of fungi

Initial experiments were designed to find out which of the culture filtrates obtained from the organisms (Table 1) tested were active in degrading cotton wool. When grown in Fergus (1969) medium, *Aspergillus sp.*, *T. koningii* and *Myriococcum sp* grew well on filter paper as a main carbon source. Visible breakdown of the filter paper by these organisms occurred after four days of incubation and by the eleventh day, the integrity of the filter paper was almost completely lost. The culture filtrates obtained from the various

organisms were tested on cotton wool at 25°C and 44°C. Results (Table 1) showed that all activities were higher at 44°C than 25°C. Also, the culture filtrates from the three organisms mentioned above were most active in degrading cotton wool. They were thus chosen for further studies.

Temperature optimum for growth

The extent of growth of *Aspergillus sp.*, *T. koningii* and *Myriococcum sp.* on YGA was determined at the temperatures indicated in Fig. 1. The optimum temperature for *T. koningii* occurred at 37°C and no growth took place at 50°C. *Aspergillus sp.* has an optimum temperature for growth at 37°C but growth did not cease at 50°C. According to Cooney and Emerson (1964), a thermophilic fungus is defined as one that has a maximum temperature for growth at or above 50°C and a minimum temperature for growth at or above 20°C. *Myriococcum sp.* is a known thermophile and from this study, the optimum temperature for growth occurred at 45–50°C.

Effect of incubation period on the production of enzymes

Fig. 2 demonstrates the production of cellulase enzymes over a period of 16 days as measured by the ability of the culture filtrate to degrade cotton wool. Initially, the activity was low in each case and fluctuated until the eleventh day, after which, the activity produced by *Aspergillus sp.* and *T. koningii* increased sharply and by this time the integrity of the filter paper in the medium was almost lost completely and a thin slurry was formed. However, the activity produced by

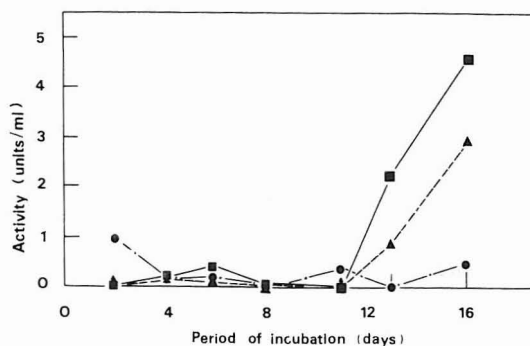


Fig. 2. Effect of incubation period on the production of enzymes.

Culture filtrates were harvested and assayed for activity towards cotton wool at the intervals shown.

Myriococcum sp.
Aspergillus sp.
T. koningii

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Myciococcus sp. in the culture filtrate was comparatively lower.

pH optimum for cellulase enzymes

A series of enzyme assays was carried out at 44°C over the pH range 2.0–9.0 using citrate-phosphate buffer (pH 2.6–2.0) and Tris-HCl buffer (pH 8.0 and 9.0). The pH of the mixture was measured before and after addition of culture filtrate.

Results (Fig. 3) obtained indicated that a sharp optimum pH of 5.0 was found for *T. koningii*. At pH lower than 2.6 and higher than 8.0, the activity was lost completely. Cellulase enzymes produced by *Myriococcus* showed a pH optimum of 7.0 and still retained 50% of the activity at pH 8.0. In contrast, cellulases from *Aspergillus sp.* showed a double pH optimum at pH 4.0 and 8.0. The activity declined sharply at pH below 4.0 and above 8.0.

Temperature optimum for cellulase enzymes

Enzyme activity was tested at the optimum pH for each organism at temperatures of 30, 37, 45, 55, 65, 70 and 80°C (Fig. 4).

For *T. koningii*, the optimum temperature for cellulase activity was recorded as 45°C. At 30°C, there was a 60° decrease of activity and at 65°C, the activity was almost nil. Both *Aspergillus sp.* and *Myriococcus sp.* had an optimum temperature of activity towards filter paper at 40°C and activity was less stable at higher temperature compared to that of *T. koningii*.

Enzyme activity on different cellulosic wastes as substrate

The results in Table 2 indicate that high enzyme activities were obtained using pineapple waste as substrate. There is possibly a lower percentage of lignin and hemicellulose in the pineapple waste as compared to those in the sawdust, cocoa pod, bagasse and palm oil waste. Pretreatment by acid or alkaline appeared to be more effective in loosening the highly crystalline structure of the cellulose in pineapple waste, bagasse and palm oil fibres than the other substrates tested. From the results obtained the culture filtrate from *T. koningii* exhibited the highest hydrolytic activity on these cellulosic

Table 2
Effect of pretreatment on enzymatic hydrolysis of cellulosic waste materials.¹

Treatment		Activity (unit/ml)					
		Palm oil fibre	Rice husk	Pineapple waste	Cocoa pod	Sawdust	Bagasse
Acid	<i>T.k.</i>	190	36	423	22	16	211
	<i>Asp.</i>	26	20	77	14	9	35
	<i>Myrio.</i>	12	16	64	9	4	18
Alkaline	<i>T.k.</i>	139	79	242	36	31	287
	<i>Asp.</i>	19	21	57	5	8	35
	<i>Myrio.</i>	13	10	48	6	5	35
Autoclave	<i>T.k.</i>	97	30	246	18	4	128
	<i>Asp.</i>	11	18	38	4	15	17
	<i>Myrio.</i>	6	3	26	2	1	8
Untreated	<i>T.k.</i>	23	27	231	14	2	96
	<i>Asp.</i>	4	17	17	2	5	17
	<i>Myrio.</i>	2	0	20	1	0	5

¹Enzyme activities were assayed in the standard assay system at the optimum temperature and pH for each organism except that the substrate is different. *T.K.* = *Trichoderma koningii* ; *Asp.* = *Aspergillus sp.* ; *Myrio.* = *Myriococcus sp.* ;

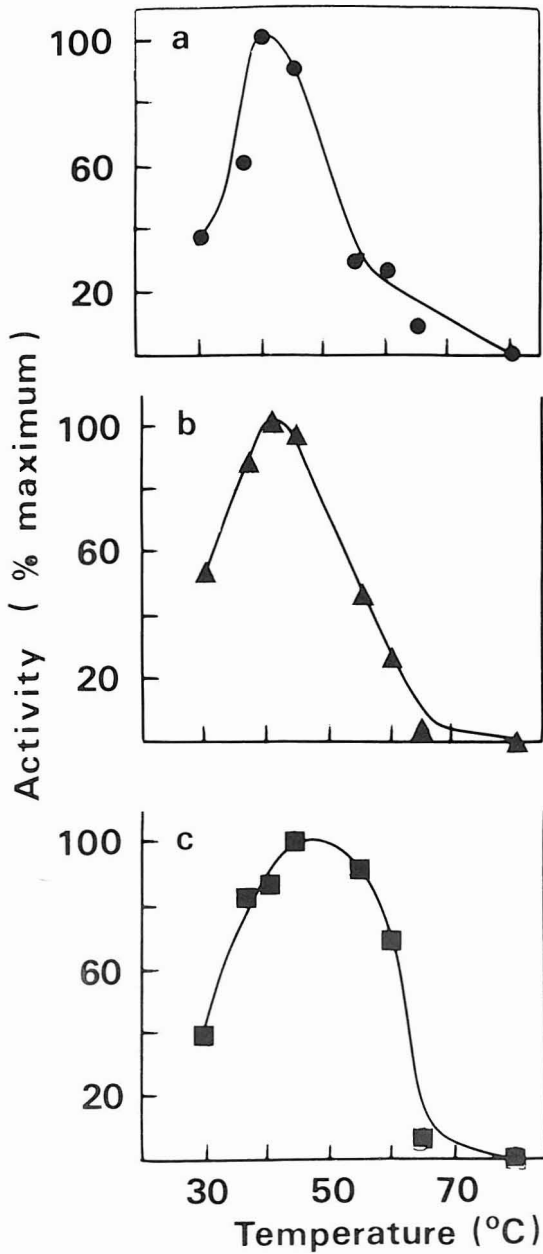


Fig. 3. Effect of pH on enzymatic activity towards cotton wool.

Assays were carried out using standard assay system in citrate-phosphate buffer (0.2 M citric acid; 0.2 M dibasic sodium phosphate) for pH 2.6–7.0 and 0.2 M Tris-HCl for pH 8.0–9.0. Points represent the average of duplicates.

- (a) *Myriococcum* sp.
- (b) *Aspergillus* sp.
- (c) *T. koningii*

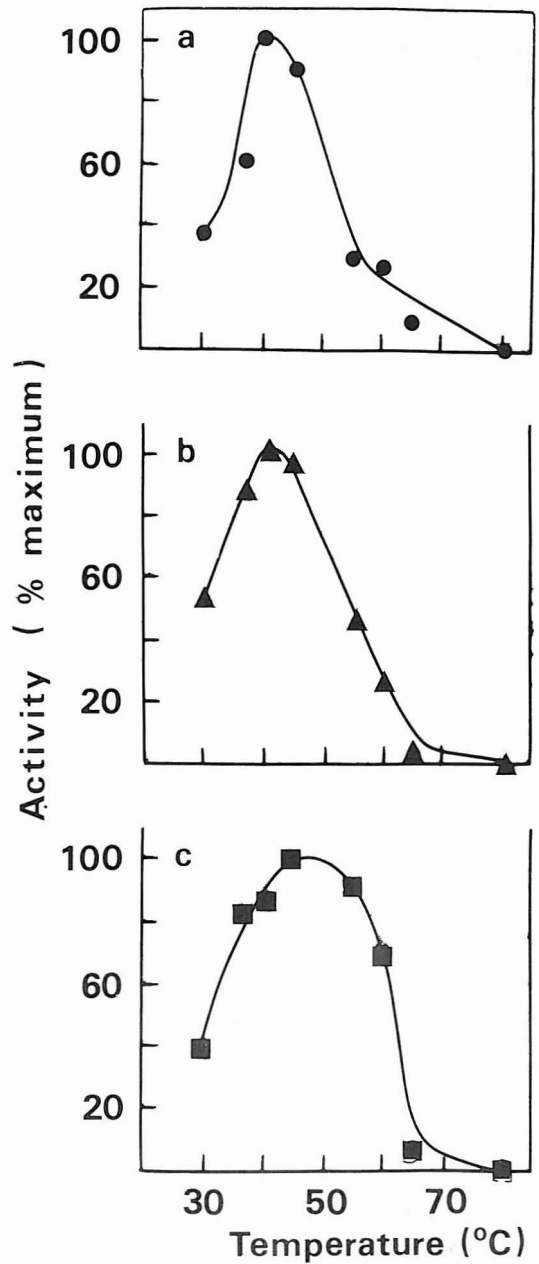


Fig. 4. Effect of temperature on enzymatic activity towards filter paper.

Standard assay system was used at the optimum pH for each organism, except that the incubation temperatures were varied. Points represent the average of duplicates.

- (a) *Myriococcum* sp.
- (b) *Aspergillus* sp.
- (c) *T. koningii*

wastes whereas culture filtrate from *Myriococcum sp.* was the least active.

Enzyme synergism

Synergism between different components of the cellulases of a number of fungi has been reported by a number of Workers (Olutiola and Ayers, 1973; Wood, 1968; Selby and Maitland, 1967).

Preliminary studies were carried out to determine whether synergistical effect exists between enzymes from these organisms. Release of reducing sugars from the pineapple waste was used as an indicator of enzymic activity. Results (Table 3) showed that by combining culture filtrates from *Aspergillus sp.* and *T. koningii* an increase of 37.5% in reducing sugar formation was obtained. Similarly, an increase of 32.6% was recorded by pooling culture filtrates from *T. koningii* and *Myriococcum sp.* whereas a 10% increase was noted when enzymes from *Aspergillus sp.* and *Myriococcum sp.* were pooled.

DISCUSSION

The assay procedure using filter paper as a cellulose substrate has proved to be the most satisfactory for routine use in the estimation of hydrolysis of cellulose (Mandels and Weber, 1969; Griffin, 1973; Folan and Coughlan, 1978).

Filter paper, though partly degraded and more susceptible to hydrolysis by cellulase than cotton, is considered as highly crystalline and difficult to hydrolyse. Its simplicity and reproducibility have established the filter paper assay in many laboratory for characterising cellulase produced by microorganisms. This method, with minor differences, is also in use in those laboratories most active in investigating cellulose to glucose conversion, e.g., U.S. Army Natick Laboratories, Natick, Northern Regional Research Laboratory, Peoria and Lawrence Berkeley Laboratory, Berkeley. In this work, the filter paper assay measures the amount of reducing sugar expressed as glucose produced from 20 mg Whatman No. 1 filter paper with a reaction time of three days. Where necessary, dilution of the enzyme used in each assay was made so that the measurement taken was within the linear portion of the standard curve. Direct comparison of the results of this study with those from other workers in difficult, because there are often differences in the choice of cellulose substrate or the experimental conditions used.

Many fungi synthesise enzymes that can degrade soluble cellulosic derivatives. However, the number of fungal species that produce enzymes capable of the extensive degradation of highly ordered (crystalline, insoluble) celluloses to insoluble sugars is much less. Preliminary investi-

Table 3
Cellulolysis of pineapple waste by culture filtrate from each organism and in combination.

Culture filtrate	Activity (unit/ml)	Expected value if no synergism (× 2)	Increase in activity (%)
<i>T. koningii</i>	291.2		
<i>Aspergillus sp.</i>	51.5		
<i>Myriococcum sp.</i>	26.5		
<i>T. koningii</i> + <i>Aspergillus sp.</i>	465.2	342.7	35.7
<i>T. koningii</i> + <i>Myriococcum sp.</i>	421.2	317.7	32.5
<i>Aspergillus sp.</i> + <i>Myriococcum sp.</i>	85.8	78.0	10.0

0.15 ml of culture filtrate of 0.075 ml from each of the two culture filtrates was added to 20 mg of substrate in 0.85 ml citrate phosphate buffer pH 5.0 and incubated at 45°C for 72 hours. The standard assay was used as described in Materials and Methods.

gation of a number of cellulolytic organisms tested had shown that culture filtrates obtained from *Aspergillus sp* *Trichoderma viride* and *Myriococcum sp.* were most active in degrading cotton wool and filter paper.

Growth on solid medium was determined by measuring the diameters of the colonies because it has been shown to be a reliable method to determine the rate of growth of fungi (Trinci, 1971; Evans, 1971; Tansey, 1972; Prodromon and Chapman 1974). The optimum growth temperature on solid medium for *Aspergillus sp.* and *T. koningii* occurred at 37°C while that of *Myriococcum sp.* was found to be 45–50°C.

Fungal cellulases, in general, are stable from pH 3 to 8 at 30°C, active from pH 3.5 to 7.0, and usually show optimal activity at pH 4.0 to 6.0. The optimal pH of 5.0 observed for cellulases produced by *T. koningii* seems consistent with the data reported by Iwasaki and co-workers (1965). Wood (1968) and Halliwell (1965) reported two pH optima for cellulases from *T. koningii*. Two pH optima were found in cellulases of *Aspergillus sp.* at pH 4.0 and 8.0. Double pH optima have also been reported for the cellulase enzymes of *Aspergillus havus* (Olutiola, 1977), and *Verticillium albo-atrum* Whitney *et al.*, (1969). In the case of *Myriococcum sp.*, a higher pH optimum of 7.0 was found in the present work.

Studies on the production of cellulase enzymes over a period of 16 days indicated that initially the activity was low and started to increase after 11 days of incubation. Wood (1968) working on *T. koningii* reported that the activity reached a plateau after 20 days of incubation. In this study the optimum period for cellulase production was not ascertained because the period of incubation was carried on only for 16 days.

Of the cellulosic wastes studied pineapple wastes was found to be more amenable to attack by cellulolytic enzymes and thus would be the appropriate substrate for further studies to examine its potential. Physical and chemical pretreatment were done to ensure that each substrate would be more accessible to enzymatic action since lignin and hemicellulose were present. Chemical pretreatment by alkali or acid was found to be more effective compared to autoclaving which requires an external energy source and is thus prohibitively expensive to operate on a large scale. Economically, sodium hydroxide would be cheaper than orthophosphoric acid.

Synergistic studies using crude enzyme extracts indicated only a small increase in activity

(<40%) using pineapple waste as substrate. It would certainly be an advantage if one can find a good combination of enzymes and in this area there is a great potential for genetic manipulation to increase enzyme activity.

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