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Cellulase production by the Thermophilic Fungus, *Thermoascus aurantiacus*

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Key words: **Cellulase; *Thermascus aurantiacus;* Thermophilic fungus; Isolation; Properties.**

RINGKASAN

Thermoascus aurantiacus *merupakan pengeluar selulase yang paling aktif daripada beberapa kulat termofil yang telah diuji. Suhu optima pertumbuhan untuk T. aurantiacus di dalam medium cecair ialah 45°C. Suhu optima untuk aktiviti β-glukosidase dan karbosil-metilselulase didapati pada 70°C sementara suhu optima untuk aktiviti enzim penghancuran kertas turasan berlaku pada 65°C. Aktiviti maksima didapati pada pH 5.0 untuk enzim penghancuran kertas turasan dan β-glukosidase dan pH 4.3 untuk aktiviti karbosilmetilselulase.*

SUMMARY

Thermoascus aurantiacus *was the most active cellulase producer of several thermphilic fungi tested. The optimum growth temperature for* T. aurantiacus *in liquid medium was 45° C and maximum cellulase production from filter paper occurred at 40° C. The optimum temperatue for β-glucosidase and carboxymethylcellulase activity was 70° C; for filter paper degrading activity it was 65° C. Maximum activity was found at pH 5.0 for the filter paper degrading enzyme and β-glucosidase and pH 4.3 for carboxymethylcellulase activity.*

INTRODUCTION

The crystalline cellulose that occurs in nature is highly resistant to both chemical and microbial degradation. In view of the projected energy shortage there is considerable interest in organisms capable of degrading cellulose. Thermophilic (Ballamy, 1974; Hajny *et al.* 1951; Stutzenberger, 1972; Su and Paulavicius, 1976) bacteria and (Waksman *et al.* 1939) fungi (Barnes *et al.* 1972; Coutts and Smith, 1976; Eriksen and Goksöyr, 1976; Rosenberg, 1978) offer considerable advantages as potential cellulose degraders since the elevated temperature of growth (and lower pH with thermophilic fungi) reduces contamination problems and permits a higher rate of celiulose degradation.

The role of *Thermoascus aurantiacus* as a cellulose degrader has been confused by the claim of Fergus (1969) and Rosenberg (1978) that there was no cellulase produced by this organism. This is in disagreement with the work of Tansey (1971) who demonstrated that *T. aurantiacus* was an active cellulase producer. There is, however, a paucity of information on the hydrolytic breakdown of cellulose or its derivatives by this organism. The purification of the cellulases from *T. aurantiacus* was reported earlier (Tong *et al.* 1980) followed by another paper on the substrate specificity and mode of action of the cellulases (Shepherd *et al.* 1980). This report describes the growth conditions for optimal cellulase production and the general properties of the enzymes from culture filtrates of *T. aurantiacus.*

MATERIALS AND METHODS

Chemical and substrates

Whatman No. 1 filter paper and Whatman cellulose powder (CF 11) were obtained from Whatman Ltd., Maidstone, Kent, U.K. CM-

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cellulose type 7HF was purchased from Hercules, Wilmington, Delaware, U.S.A. Difco powdered yeast extract and Difco Bacto peptone were products of Difco Laboratories, Detroit, Michigan, U.S.A. Davis Bacteriological agar was purchased from Davis Gelatine, Ltd., Christchurch, New Zealand. All other chemicals were of analytical reagent grade.

Isolation of thermophilic fungi

Thermophilic fungi were isolated from samples of sand and decomposed woody materials collected on coastal beaches and compost heaps in Christchurch, New Zealand.

Two isolation techniques were used: (a) direct hyphal isolation after soil or composting materials were incubated at elevated temperatures to promote the growth of thermophiles; and (b) the direct inoculation method (Waksman *et al.* 1931; Waksman *et al.* 1939) in which samples were sprinkled lightly upon the surface of yeast glucose agar (YGA) media prior to incubation.

The hyphal isolates were sub-cultured onto YGA and yeast starch (YpSs) agar (Cooney *et al.* 1964) until pure cultures were obtained. The addition of 30 units of streptomycin per millitre of medium reduced bacterial contamination.

A thermophilic fungus, as defined by Cooney and Emerson (1964), is one that has a maximum temperature for growth at or above 50° C and a inimum temperature for growth at or above

Choice of Thermoascus aurantiacus

The isolated thermophilic fungi were identified as *Chaetomium thermophile* var. *coprophile*, *Humicola lanuginosa*, *Myceliophthora thermophila*, *Penicillium thermophile* and *Thermoascus aurantiacus*.

The cellulolytic ability of these fungi was tested as described below and culture filtrates obtained from *T. aurantiacus* were most active in breaking down filter paper and carboxymethylcellulose (CM-cellulose) (Table 1).

TABLE 1

Degradation of filter paper by culture filtrates of thermophilic fungi

Organism	reducing equivalents (mg/h/ml enzyme)
T. aurantiacus (strain I)	0.009
T. aurantiacus (strain II)	0.004
C. thermophile var. *coprophile*	0.002
M. thermophila	0.003
P. thermophile	0
H. lanuginosa	0

The reaction mixture and the method for estimating reducing sugars are as described in Materials and Methods. Filtrates are from 21 day old cultures grown on Fergus' medium with chopped filter paper as the carbon source.

Inoculum

The inoculum for each growth experiment was taken from cultures grown on YGA at 50° C for 48 h. 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 9 cm diam. petri dish containing agar medium. In liquid culture experiments, one agar-mycelium disc (0.8 cm in diam.) was transferred to a medical flat (Wheaton C-16.500 ml) containing 50 ml of yeast-glucose 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 medium (1969).

Maintenance of stock culture

T. aurantiacus was routinely cultured on YGA slopes which were incubated at 50° C for 48 h and stored at 27° C for no more than four weeks prior to further culturing. For long-term storage, freeze-drying was found to be effective.

Determination of Cellulolytic Ability of Fungi

The isolated thermophilic fungi were initially grown in Reese-Mandels medium (1963) with CM-cellulose as carbon source. Each medical flat containing 60 ml of medium was inoculated with three agar-mycelium discs. After six days incubation at 50° C, the medium was suction filtered through glass fibre paper (Whatman GF/C) and the filtrate centrifuged at 10,000 g for 30 min. The clear supernatant was assayed for enzyme activity against CM-cellulose by the viscometric technique.

The fungi were also grown in Fergus' medium with chopped filter paper as carbon source. After incubation at 50° C for 21 days, the filtrate was collected as described above and assayed for activity against filter paper and CM-cellulose. Cellulolytic activity is expressed in terms of total reducing sugar (as glucose) produced, and is expressed as mg reducing sugar/h/ml reaction mixture.

Determination of Optimum Temperature for Growth

The optimum growth temperature was ascertained by measuring the diameter of colonies on

YGA medium in 9 cm diam. petri dishes containing 30 ml of agar medium. Each agar plate was inoculated with one agar-mycelium disc as described earlier. Quadruplicate colonies were measured after incubation at the temperatures shown in *Fig. 2*, for 24 h and the average diam. of the largest and smallest colony was recorded. A beaker of distilled water was placed in each incubator to reduce dessication of the agar medium. Cultures incubated at 55°C and 60°C were placed in polystyrene bags to further prevent dessication.

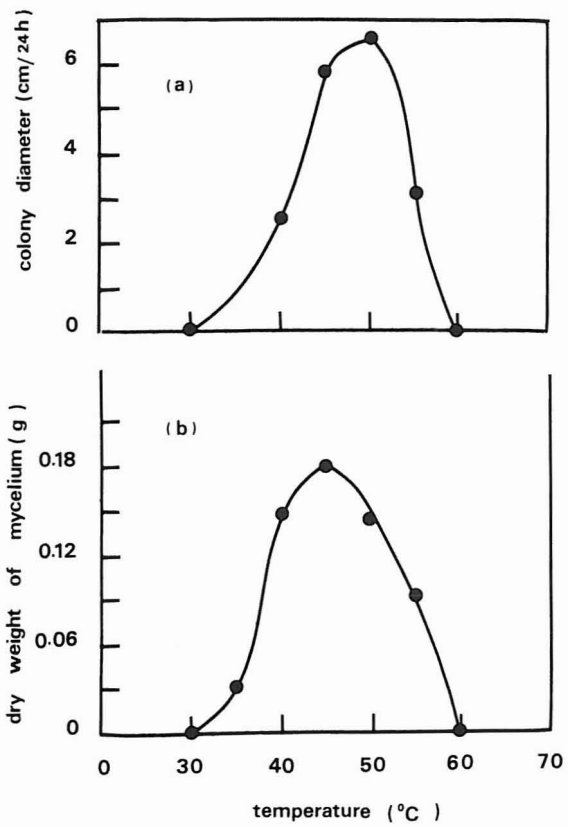

Fig. 2. Growth of T. aurantiacus *on solid (a) and liquid (b) culture medium. Each point represents the average of four replicates.*

Mycelial dry weight from liquid cultures was also determined. Four medical flats each containing 60 ml of yeast-glucose medium were inoculated with one agar-mycelium disc and incubated at the temperatures shown in *Fig. 2* for 24 h. Mycelial mats were harvested by filtration through Whatman No. 1 filter paper, washed three times with distilled water and dried to a constant weight at 60 °C.

Effect of Temperature on Cellulase Production

Medical flats containing 60 ml of Fergus' medium were inoculated and incubated at temperatures shown in *Fig. 3.* Two flats were removed from each temperature after the first, third and fifth day of incubation and then at regular intervals of five days up to 30 days. The contents of

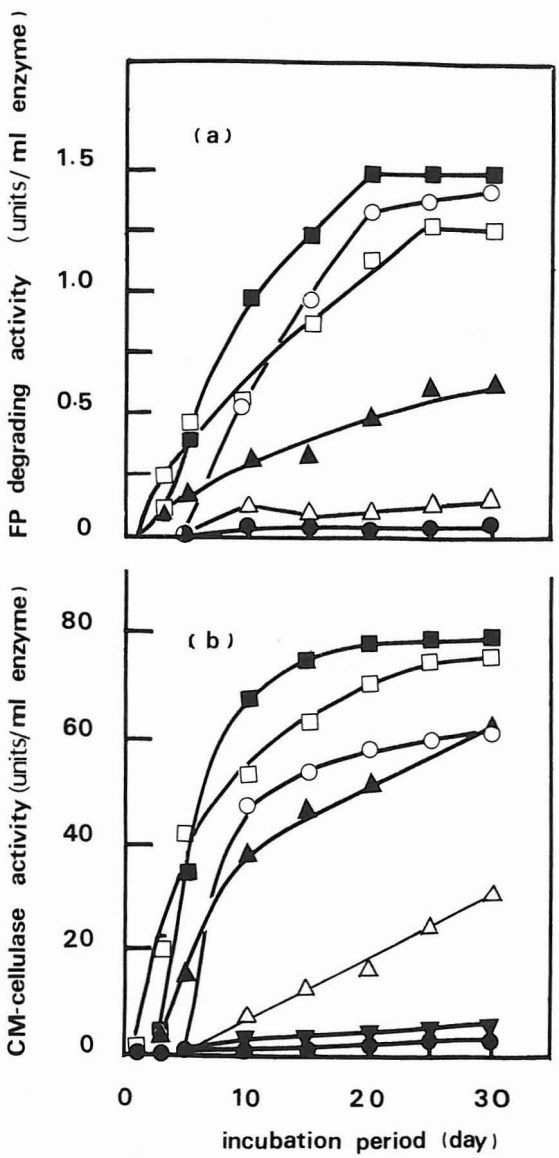

Fig. 3. Influence of Temperature on Cellulase Production. The culture filtrates were tested for activity on (a) filter paper and (b) CM-cellulose as described in Materials and Methods. (●), 30°C; (○), 35°C; (■), 40°C; (□), 45° C; (▲), 50°C; (△), 55°C; (▼), 60° C.

the flats were filtered and cellulase activities assayed on filter paper and CM-cellulose by reducing sugar liberation. The pH of each of the culture filtrates was also measured.

Enzyme Activity Measurements

Cellulase activity was measured by the appearance of reducing end groups liberated from filter paper or CM-cellulose. The number of reducing sugar groups created by hydrolysis of the cellulosic substrates was measured spectrophotometrically using the Nelson-Somogyi procedure (Nelson, 1964, Somogyi, 1952).

An absolute definition of a unit of cellulase activity is difficult. This is because in substrates such as CM-cellulose, the glucose molecules are substituted with carboxymethyl groups, and the products of the enzyme reaction on filter paper and CM-cellulose are heterogeneous polymers; the effect of this on the absorption coefficient of reducing end groups is not known and therefore using a glucose standard to determine the number of μmol of reducing end groups may not represent an accurate estimate.

An indication of total cellulolytic activity was obtained by determining filter paper (FP) degrading activity. Standard reaction mixtures containing 20 mg of filter paper (Whatman No. 1), 0.9 ml citrate-phosphate buffer (0.1 M citric acid, 0.2 M di-basic sodium phosphate) pH 5.0, 0.1 ml enzyme solution and one drop (10 μl) of toluene were incubated at 50° C for 24 h. Mixtures were then analysed for the production of reducing sugar. The toluene added to prevent bacterial growth was found to have no effect on enzyme activity. Reaction mixtures were checked for contamination by withdrawing samples and streaking onto nutrient agar plates and incubated at 37° C and 50° C. A unit of activity is expressed as mg reducing sugar/h/ml enzyme.

In preliminary experiments designed to study the production of cellulolytic enzymes by the isolated thermophilic fungi, carboxymethylcellulase (CM-cellulase) activity was assayed by a modification of the viscometric technique of Horton and Keen (1966). CM-cellulose (0.75% (w/v)) was dissolved in citrate phosphate buffer (0.1 M citric acid; 0.2 M di-basic sodium phosphate), pH 5.0 in a Waring blendor for 5 min. and at 4° C for three days, the viscous but clear supernatant phase was removed and the fibrillar matter at the bottom discarded. Five millilitres of the CM-cellulose solution were pipetted into a Cannon-Fenske viscometer (Type BS/IP/CF size 300) and equilibrated in a water bath at 50° C for 5 min. One ml of enzyme preparation was then added to the viscometer and mixed immediately. The viscosity of the reaction mixture was then determined at regular intervals.

CM-cellulase activity was also measured by reducing group estimation in reaction mixtures containing 0.9 ml of 0.75% (w/v) CM-cellulose in citrate-phosphate buffer, pH 4.5 and 0.1 ml of enzyme solution. Reaction mixtures were incubated for 30 min. at 70° C and the rate of production of reducing sugar determined. A unit of activity is expressed as mg reducing sugar/h/ml enzyme.

β-Glucosidase activity was assayed by a modification of the method of Umezurike (1969). Enzyme solution (0.1 ml) and 0.4 ml 0.001 M ρ-nitrophenyl-glucoside in citrate phosphate buffer (0.1 M citric acid; 0.2 M di-basic sodium phosphate), pH 5.0, was as incubated for 30 min. at 70° C. After incubation, 1.0 ml 1 M sodium carbonate solution was added to 0.5 ml of the reaction mixture, diluted with 10 ml of distilled water and the nitrophenol released estimated from the absorbance at 420 nm. One unit β-glucosidase activity is defined as that amount of enzyme needed to liberate 1 μmol of ρ-nitro phenol per min. under the conditions of the assay.

RESULTS

Comparison of Cellulolytic Ability of Isolated Thermophilic Fungi

Initial experiments were designed to discover which of the culture filtrates obtained from the isolated thermophilic fungi, namely *C. thermophile* var. *coprophile, H. lanuginosa, P. thermophile, M. thermophile* and *T. aurantiacus* (strain I and II) were most active in degrading soluble CM-cellulose, as well as insoluble forms of cellulose, such as filter paper. When grown in Reese-Mandels medium containing CM-cellulose as the main C source, all the isolates except *H. lanuginosa* and *P. thermophile* grew well and the culture filtrates caused a dramatic decrease in the viscosity of a CM-cellulose solution *(Fig. 1). Thermoascus aurantiacus* (strain I) was found to be the most active cellulase producer.

When these organisms were grown in Fergus' medium with filter paper as a carbon source, a similar pattern was observed. *H. lanuginosa* and *P. thermophile* failed to degrade filter paper while *C. thermophile* var. *coprophile, M. thermophila* and *T. aurantiacus* all grew well. The culture filtrates when tested on filter paper showed *T. aurantiacus* to be the most active. Strain I showed almost twice the activity of strain II (Table 1).

Culture fultrates of *C. thermophile* var. *coprophile* and *M. thermophile* also degraded filter paper to a lesser degree. No activity from culture filtrates of *H. lanuginosa* and *P. thermophile* could be detected.

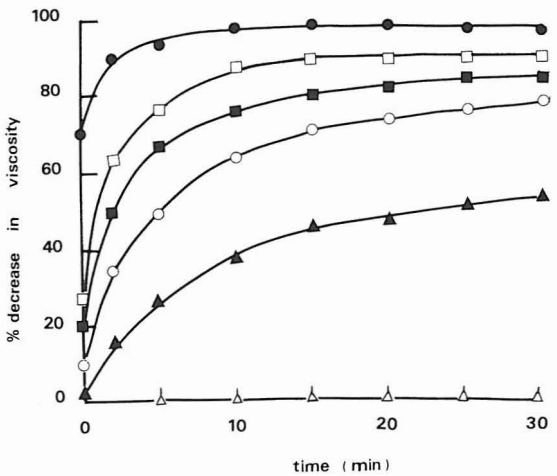

Fig. 1. Hydrolysis of CM-cellulose by Culture Filtrates of Thermophilic fungi. 5.0 ml of 0.75% CM-cellulose (7.5 mg/ml, in 0.1M citric acid; 0.2M di-basic sodium phosphate buffer, pH 5.0) and 1.0 ml of culture filtrate were mixed and the efflux time of the mixture determined with a viscometer at 50° C at the times indicated. The percentage reduction in the viscosity of the CM-cellulose solution after each incubation period was calculated as described by Horton and Keen (1966). The thermophilic fungi tested were: T. aurantiacus *(strain I)* (●); T. aurantiacus *(strain II)* (□); C. thermophile *var.* coprophile (■); *M. thermophila* (○); P. thermophile (▲); *H. lanuginosa* (△).

Growing Thermoascus aurantianus

Optimum temperature for growth on solid medium was 50° C (Fig. 2a); in liquid medium the optimum was lower, at 45° C *(Fig. 2b)*. No growth occurred at 30° C on either media. At 55° C, growth was retarded and at 60° C it was ceased. The fungus produced only 0.1 – 0.2 cm of hyphal extension after one week at 30° C, but there was no growth of the fungus at 60° C even after a prolonged incubation period.

Effect of temperature on cellulase synthesis

The effect of varying growth temperature on cellulase production was measured by determining the ability of culture filtrates to degrade both filter paper and CM-cellulose *(Fig. 3)*.

The general pattern of enzymatic activity on both substrates was similar at the temperatures tested. Optimum temperature for the degradation of both CM-cellulose and filter paper was 40° C and maximum enzyme activity was obtained after 20 days growth. Increasing the incubation temperature from 40 to 55° C resulted in a 90% decrease in the filter paper degrading activity and 60% of the CM-cellulase activity. After 20 days, the integrity of the filter paper in the medium was lost completely and a thin slurry was formed.

The pH of the culture medium changed during growth and these changes were similar for growth between 35 – 55° C. There was a drop within the first day from the initial pH of 6.5 to pH 5.5, followed by a gradual rise to a final pH of between 6.3 and 7.0. Main Cellulase production occurred when the pH was 6.3 – 6.8. The pH of the medium was 6.6 after growth at 40° C for 20 days.

Temperature optima for β-glucosidase and cellulase enzymes

A series of enzyme assays were carried out at pH 5.0 over the temperature range 30 – 80° C *(Fig. 4)*.

Both β-glucosidase and CM-cellulase showed a temperature optimum at 70° C. At higher temperatures, the activities decreased sharply with only 30% of the maximum activity remaining at 80° C. The optimum temperature for FP degrading activity was lower; after a 2 h reaction time, as opposed to the standard reaction time of 24 h, activity was greatest at 65° C compared with the optimum temperature of 60° C for 24 h reaction time. When the incubation temperature was lowered to 30° C, there was a 70 – 90% decrease of the maximum activity for all three enzymes.

Effect of pH on β-glucosidase and cellulase activities

Using a temperature of 70° C for β-glucosidase and CM-cellulase and 60° C for FP degrading enzyme, the pH optimum of the enzymes was determined *(Fig. 5)*. An optimum pH of 5.0 was found for β-glucosidase and FP degrading enzyme and 4.3 for CM-cellulase. If the reaction time of the FP degrading activity was increased from 2 to 24 h, there was a shift in the pH optimum from 4.3 to 5.0.

Effect of pH changes on the stability of β-glucosidase and cellulase activities

Figure 6 illustrates the effect of pH on the stability of β-glucosidase and cellulases. The pH-stability curve for β-glucosidase resembles its pH-activity curve *(Fig. 5)*. The enzyme was stable over a pH range of 4.0 – 6.0. Cellulases exhibited a wider pH range of stability. Thirty percent of the pH 5.0 activity of FP degrading activity and 37%

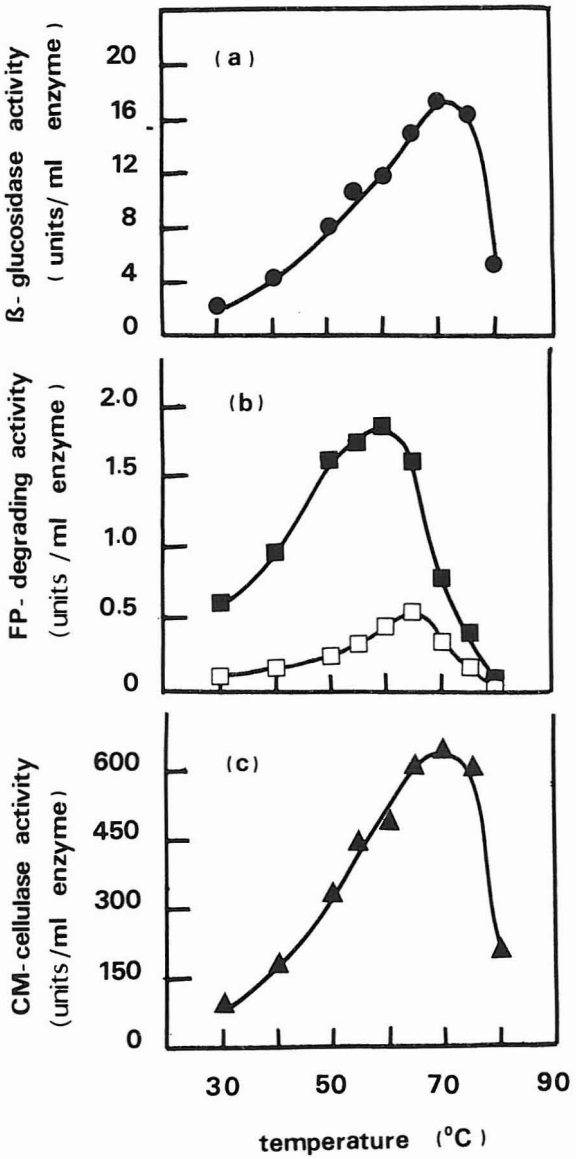

Fig. 4. Temperature Optimum for β-glucosidase and Cellulase Enzymes. Enzyme activities were assayed in the standard systems at pH 5.0 except that the temperatures were varied as shown. The FP degrading enzyme was determined using two different reaction times; (■), 24 h and (□), 2h. Points shown are the average of duplicate determinations.

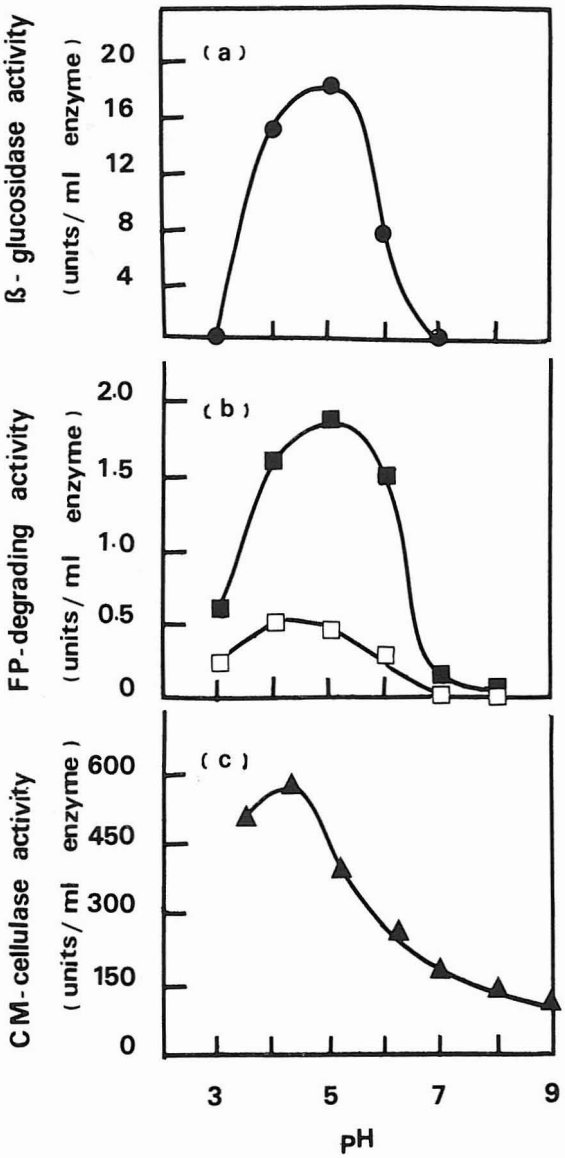

Fig. 5. Effect of pH on β-glucosidase and Cellulase Activities. Enzyme activities were measured in the standard assay systems except that the buffer and the pH were varied. Buffers used were citrate-phosphate (0.1M citric acid; 0.2M di-basic sodium phosphate) buffer (pH 3.0 – 7.0) and 0.2M Tris-HCl (pH 8.0, 9.0). The FP degrading enzyme was determined using two different reaction time; (■), 24 h and (□), 2 h. Points show the average of the determination.

of the maximum CM-cellulase activity still remained at pH 9.0. Both CM-cellulase and β-glucosidase are not stable at pH 7.0 or higher.

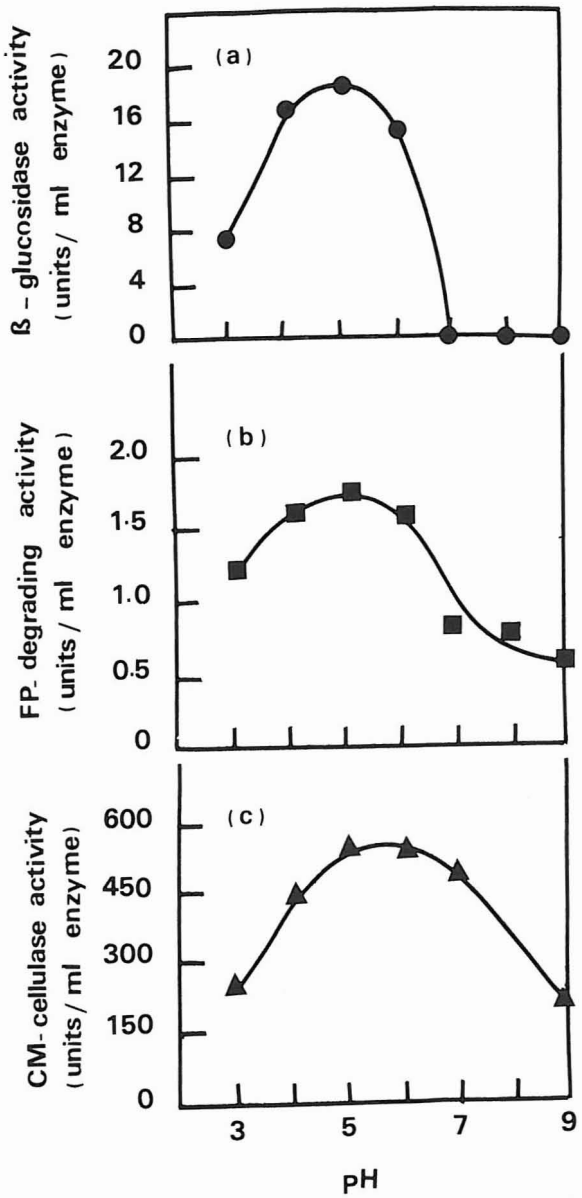

Fig. 6. Effect of pH changes on the Stability of β-glucosidase and cellulase activities. Culture filtrates (0.2 ml) were mixed with the appropriate buffer (1.8 ml) and the resultant pH measured before incubation at 30°C for 3 h. The buffers used were as described for Fig. 4. After 3 h incubation 0.5 ml was withdrawn and assayed for enzyme activity. β-glucosidase and CM-cellulase was assayed at 70°C in citrate-phosphate (0.2M citric acid; 0.4M di-basic sodium phosphate) buffer, pH 5.0 and 4.3, respectively. The FP degrading activity was assayed at 60°C in the same buffer at pH 5.0. The pH of each assay mixture was checked after mixing the component solutions. Points show the average of two determinations.

DISCUSSION

Reports on the ability of *T. aurantiacus* to degrade cellulose have been contradictory. This report confirms the cellulolytic nature of *T. aurantiacus* and culture filtrates of this fungus are found to be the most effective of the themophilic fungi isolated in degrading both filter paper (Table 1) and CM-cellulose *(Fig. 1)*.

The assay procedure using filter paper as a cellulose source has proved to be the most satisfactory for routine use in the estimation of hydrolysis of native cellulose (Folan and Coughlan, 1978; Griffin, 1973; Mandels and Weber, 1969). Filter paper, though partly degraded and more susceptible to hydrolysis by cellulase than cotton, is considered as highly crystalline and difficult to hydrolyse.

Although there are many reports describing fungal extracts capable of degrading soluble cellulosic derivatives only a small number of these appear capable of extensive degradation of highly ordered (crystalline, insoluble) celluloses to soluble sugars (Rosenberg, 1978). Culture filtrates of *T. aurantiacus* exhibited β-glucosidase activity as well as activity on both soluble and insoluble forms of cellulose.

The optimum growth temperature of *T. aurantiacus* on solid media occurred at 46 – 51°C which is in agreement with that reported by Romanelli and co-workers (1975). However, these results differ somewhat from those given by Cooney and Emerson (1964) where an optimum of 40 – 45°C is reported. The difference observed may be attributed to strain variation and the different pH of the media used. The optimum temperature for cellulase production is lower than that for growth, a phenomenon that is commonly observed with thermophilic microorganisms. This may be due to thermal instability of the enzymes at elevated temperatures when long incubation periods are used. This is supported by the observation that the temperature optimum for the FP degrading enzyme decreased from 65°C to 60°C when the incubation period was increased from 2 h to 24 h.

The optimum pH for activity was pH 5.0 for β-glucosidase and FP degrading enzyme and pH 4.3 for CM-cellulase activity. This compares with the high pH optimum of 5.5 for cellulase from *Pyricularia oryzae* (Hirayama *et al.* 1978) and a low pH optimum at 2.5 for *Aspergillus niger* cellulase (Ikeda *et al.* 1973). At neutral pH virtually no activity was detected. The optimum pH for the activity of the FP degrading enzyme could be lower than pH 5.0. When the incubation time of the assay was decreased from 24 h to 2 h *(Fig. 5)* the pH activity optimum moved to pH 4.3. This shift is probably related to the stability of the enzyme.

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