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EFFECTS OF TEMPERATURE ON AGEING CULTURES OF A CYANIDE-PRODUCING LOW-TEMPERATURE BASIDIOMYCETE, ISOLATE W_2

by

© AWATAR SINGH SEKHON

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

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FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "EFFECTS OF TEMPERATURE ON AGEING CULTURES OF A CYANIDE-PRODUCING LOW-TEMPERATURE BASIDIOMYCETE, ISOLATE W_2" submitted by Awatar Singh Sekhon in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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Date June, 1969

ABSTRACT

Physiological and biochemical studies on the effects of temperature of a cyanide-producing low-temperature basidiomycete, isolate W_2 were undertaken to obtain a better understanding of the metabolic processes during growth and autolysis.

For determining growth, different culturing techniques using a defined medium were employed. In these studies a greater yield of mycelium was obtained for cultures incubated at 1°C than at other temperatures in liquid shake cultures. Diameters of colonies in petri plates and yields of mycelium in liquid static cultures were greater for cultures incubated at 15°C. Cultures incubated at 15°C and transferred to lower temperatures resulted in yields of mycelium which were intermediate to those of cultures incubated at 15 and 1°C respectively. Autolysis of mycelium grown at 1°C was much slower than for cultures incubated at higher temperatures.

Total protein values were higher for the mycelium grown at 1°C than at 15°C. Maximum numbers of protein bands, as determined by disc gel electrophoresis, were observed for cultures incubated at 1°C. With changes in incubation temperatures, not only protein patterns changed, but intensities of stain uptake by protein bands varied considerably. Similar changes were also found to occur in peroxidase isozyme patterns.

Protease activity of cell-free mycelial extract was higher for cultures incubated at 1°C than at 15°C.

Maximum levels of ribonucleic acid of cell-free mycelial

extracts occurred in very young cultures incubated at 15°C, whereas, for the cultures incubated at 1°C, maximum amounts were recorded in autolysing cultures. There was a correlation between the ribonuclease activity and ribonucleic acid contents for cultures incubated at 15°C, but this relationship did not occur at 1°C, even though the activity of ribonuclease was higher.

The first and maximum release of hydrogen cyanide occurred during autolysis in cultures incubated at 15°C, and also for cultures incubated at 15° and then transferred to 10° and 1°C respectively three days prior to harvesting. At 1°C, the maximum value of hydrogen cyanide was approximately one-half that obtained for cultures incubated at 15°C. Near maximum levels were maintained for a longer period for cultures incubated at 1°C than at higher temperatures.

β-glucosidase activity of culture medium and cell-free mycelial extracts for ageing cultures incubated at 15°C followed the same trend as noticed for hydrogen cyanide release in cultures incubated at 15°C. The maximum activity of β-glucosidase of cell-free mycelial extracts of mycelium grown at 1°C was higher than the maximum value obtained for cultures incubated at 15°C. The rates of increase and decrease in activity for cultures incubated at 1°C were slower than those observed for cultures incubated at 15°C.

The presence of 'free' hydrogen cyanide in culture medium and cell-free mycelial extracts for cultures incubated at 15° and 1°C was noticed only after a decrease in dry weight of mycelium occurred. Somewhat similar amounts of 'free' hydrogen cyanide, collected from non-autoclaved and autoclaved fractions of culture medium and cell-free mycelial extracts, indicated that the release of hydrogen cyanide is not an enzymatic process.

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INTRODUCTION

Physiological processes of microorganisms, such as growth and development, are intricately related to the environment. In order that these processes proceed at their optimal rate, energy is required, and this energy is derived from the oxidation of substrates on which microorganisms grow. Oxidative processes proceeding through a chain of reactions are affected by temperature, which is one of the most important of environmental factors.

In relation to temperature, microorganisms have been classified into three main categories: (a) thermophiles, (b) mesophiles, and (c) psychrophiles, depending on whether they grow at high, moderate, or low temperatures respectively. Precise limits of the temperature range for each group appear to be arbitrary. The first two groups can be easily distinguished by their growth-temperature optima (Ingraham and Stokes 1959).

The term 'psychrophile' was first introduced by Schmidt-Neilsen (1902) for bacteria which grow between 5°and 20°C. Ingraham and Stokes (1959) suggested that psychrophiles should be distinguished from mesophiles not on the basis of their optimum growth temperature, but their ability to grow at 0°C within a two-week period. This definition has been accepted very widely in the field of bacteriology (Farrell and Rose 1965, 1967). Psychrophilic fungi are those which are considered to have an optimum temperature for growth near 10°C or below (Deverall 1968).

A number of fungi have been reported to cause spoilage of food products stored at low temperatures. Brooks and Hansford (1923) found

that the fungi namely Cladosporium herbarum, Mucor racemosus, M. mucedo, M. lucitanicus, Penecillium expansum, P. anomalum, Sacchromyces spp., Sporotrichum carnis, Torula botryoides, Wardomyces anomala, Hormodendron cladosporioides, and Thamnidium spp., occurred on meat stored at zero or subzero temperatures. Of these Cladosporium herbarum caused 'Black spot' on cold stored meat. Other fungi which have been isolated by Gunderson (1961) from frozen meat products were Aureobasidium (=Pullularia) pullulans, Botrytis cinerea, Phoma spp., Geotrichum candidum, and Chrysosporium pannorum. All of these fungi were able to grow at 0°C.

Plant pathogenic fungi are known to incite disease at low temperatures. One of the 'snow mold' pathogens of turf grasses and winter cereals, Fusarium nivale, was studied by Dahl (1934). The disease development was rapid between 0°C and 5°C, and slow between 15°C and 20°C. He found that in culture, the fungus grew between 2°C and 32°C with an optimum of 20°C. Remsberg (1940) reported that Typhula idahoensis, which caused 'snow mold' of grasses and winter cereals, grew in cultures between 0°C and 18°C with an optimum between 9°C and 12°C. Also T. variabilis was found to be responsible for the destruction of sugar beets, potatoes, asparagus rhizomes, and celery in storage. A fungus which causes serious damage to winter cereals and grasses in Northern British Columbia, Alberta, Yukon, and Alaska is Sclerotinia borealis (Cormack 1951, and Lebeau and Logdson 1958). The fungus has also been reported to cause severe damage to winter cereals in Finland (Jamalainen 1949), Norway (Roed 1956), and State of Washington (Sprague et al. 1961). Ward (1966) found that the optimum and maximum temperatures for growth of S. borealis was 0°C and 15°C respectively. The fungus Phacidium infestans which causes a blight of pines in Northern Sweden, grew between 0°C and 3°C in cultures, although

its optimum growth temperature was 15°C (Pehrson 1948).

An unidentified low-temperature basidiomycete, later shown to produce hydrogen cyanide in cultures (Lebeau and Dickson 1953, 1955, Ward et al. 1961, and Ward and Lebeau 1962), considered to be responsible for spring killing of grasses and legumes in Alberta was characterized by Broadfoot and Cormack (1941) as a 'snow mold' pathogen. They found that the fungus grew readily in culture between 0°C to 18°C.

This low-temperature basidiomycete occurred on grasses in Alaska. The isolates from Alaska had a similar cultural appearance as those obtained in Canada (Lebeau and Logdson 1958).

In 1953 Lebeau and Dickson considered that the unidentified low-temperature basidiomycete produced sufficient amounts of hydrogen cyanide to kill buds and crown tissues of alfalfa plants. Subsequently, in 1955 they associated the development of winter crown rot of alfalfa with lethal amounts of hydrogen cyanide present in crown tissues. In addition, Lebeau et al. (1959) demonstrated that diseased alfalfa plants contained hydrogen cyanide, in concentrations proportional to the damage to the host, and that there was a relationship between the amount of hydrogen cyanide produced and the severity of disease.

Isolates of low-temperature basidiomycetes, were grouped into A, B, and C categories, on the basis of their cultural behaviour on common laboratory medium. The type A isolates (W_1 and W_{10}) were characterized by having very slow-growing compact colonies, with mycelium appressed to the substratum. Type A isolates produced hydrogen cyanide in large amounts in host plants and none in culture, and are highly pathogenic on alfalfa plants. Type B isolates (W_2 and W_5) grew rapidly in cultures,

produced smaller quantities of hydrogen cyanide in alfalfa plants, but released large amounts in cultures. Type C isolates (W_{14} and W_{17}) grew faster than the other two types and did not produce hydrogen cyanide under any conditions, and were not pathogenic (Ward et al. 1961).

In relation to cyanogenesis, Ward and Lebeau (1962) reported that isolate W_1 did not produce hydrogen cyanide in shake cultures, but isolate W_2 released hydrogen cyanide, only when there was a decline in the yield of the mycelium. These workers concluded that hydrogen cyanide was the product of autolysis.

Working on the aspect of cyanogenesis, Ward (1964b) demonstrated the presence of a cyanogenic compound from the mycelium of isolate W_2. He observed that hydrogen cyanide occurred even before autolysis began, but this hydrogen cyanide was in a bound form. The bound hydrogen cyanide increased rapidly with the age of culture and reached a maximum shortly after the maximum yield of mycelium was obtained in 9-day old cultures, after which autolysis began and hydrogen cyanide was released in a free form.

Hydrogen cyanide, which is normally released during the onset of autolysis in shake cultures, was also released prior to autolysis when l-glycine and l-serine were used as nitrogen sources in media (Ward and Thorn 1966). However, Stevens and Strobel (1968) concluded that valine and isoleucine were precursors of the cyanogenic glucosides, linamarin and lotaustralin respectively, and they found these glucosides to be present in fungus mycelium.

A scheme for hydrogen cyanide fixation in isolate W_2 was proposed by Strobel (1966). In this study the cultures fed with $K^{14}C^{15}N$ showed an increase of ^{14}C labeling in α-aminopropionitrile. He demonstrated

the presence of a nitrile synthetase in cell-free extracts and concluded that the enzyme was responsible for the condensation reaction of acetaldehyde, ammonia, and hydrogen cyanide to form α-aminopropionitrile. A year later (1967), he regarded that the compound 4-amino-4-cyanobutyric acid was a precursor in glutamate biosynthesis.

On the mechanism of hydrogen cyanide formation in the host, Colotelo and Ward (1961) reported that a crude preparation of B-glucosidase from isolate W_1 released hydrogen cyanide when reacted with aqueous solution of a cyanogenic glucoside amygdalin. Also, when these workers reacted crude enzyme preparation with Medicago sativa homogenates, hydrogen cyanide was observed. These findings led to the conclusion that the host plant supplies the necessary substrate for cyanogenesis. Another interesting aspect of cyanogenesis was found by Lebeau and Atkinson (1967). In these studies, hydrogen cyanide release was inhibited when borax (as Sodium tetra borate) was incorporated into medium. However, the growth of the fungus was only slightly retarded. These workers postulated that borax inhibited the synthesis of a cyanogenic compound in cultures, because it did not inhibit the activity of B-glucosidase in in vitro.

Studies on the physiology and biochemistry of low-temperature bacteria have received much attention (Mossel and Ingraham 1955, Ingraham and Bailey 1959, Ingraham and Stokes 1959, Witter 1961, Rose 1962, Stokes 1962, and Farrell and Rose 1965, 1967). However, studies with low-temperature fungi have been almost neglected.

The fungus used for the present physiological and biochemical investigations was the cyanide-producing basidiomycete which is able to

grow and cause disease in turf and legume plants at low-temperatures. These studies were undertaken to obtain a better understanding of the metabolic processes at low temperatures since there is no information regarding metabolic activities of fungi grow at low temperatures. In these studies the following were investigated: (a) growth, (b) quantitative and qualitative studies on protein metabolism, (c) protease activity, (d) total ribonucleic acid, (e) ribonuclease activity, (f) hydrogen cyanide production in cultures, (g) B-glucosidase activity, and (h) evolution of 'free' hydrogen cyanide from culture medium and cell-free mycelial extracts.

MATERIALS AND METHODS

Organism and Growth Medium

The fungus used for these studies was a cyanide-producing low-temperature basidiomycete, isolate W_2. This isolate has been studied extensively for cyanogenesis (Ward and Lebeau 1962, Ward 1964b, Strobel 1966, 1967, and Stevens and Strobel 1968), and limited growth studies and pathogenesis have also been investigated (Lebeau and Dickson 1955, and Ward 1964a).

Stock cultures of this fungus were maintained in test tubes on agar slants using synthetic medium, described below, at 3-5°C.

The synthetic medium was described by Ward and Colotelo (1960). The composition of this medium included the following ingredients:

Major nutrients:

D-glucose	15.00 g
l-asparagine	2.36 g
KH_2PO_4	1.00 g
$MgSO_4.7H_2O$	0.50 g

Minor nutrients:

$Fe_2(SO_4)_3$	0.715 mg
$ZnSO_4.H_2O$	0.540 mg
$Co(NO_3)_2.6H_2O$	0.245 mg
$MnCl_2.4H_2O$	0.135 mg
$H_2MoO_4.H_2O$	0.085 mg
$CuSO_4.5H_2O$	0.195 mg
$CaCl_2$	4.000 mg

Distilled water to make 1 liter

Vitamins:	Thiamine. HCl	100 ug
	Ca-pantothenate	100 ug
	Pyridoxine. HCl	100 ug
	d-Biotin	10 ug
	i-Inositol	5 mg

The minor nutrients and vitamins were made in a stock and the inclusion of 1 ml of each in 1 liter gave the required concentrations.

N KOH to adjust pH to 6.0.

A. Growth Studies

Growth studies were carried out using three different techniques (a) petri dish culture, (b) liquid static culture, and (c) liquid shake culture. The procedures used here for each technique are described as follows:

1. Petri dish culture

In the centre of petri dishes (13 cm internal diam.), containing 50 ml of the above synthetic medium solidified with 1% agar (Difco-Bacto), was placed a disc (5 mm diam.) of inoculum taken from the periphery of 10-day old culture incubated at 15°C. These petri dishes were incubated at 15°C and 1°C. The diameters of colonies (mm) were measured every two days over a 24-day period. The experiment was repeated once. Each point in Fig. 1. is the average value of six replicates.

2. Liquid static culture

250 ml Erlenmeyer flasks containing 50 ml of synthetic medium were inoculated with 5 mm discs from 10-day old (see above) cultures. These flasks were incubated at 15°C and 1°C. Cultures were harvested at 8,12,16,24, and 32 days after inoculation. For the cultures incubated

at 1°C, it was necessary to harvest at 48 and 56 days after inoculation due to the slow growth of the fungus. Fungus mycelium was separated from the culture medium by filtration using No. 615 Eaton-Dickman tared filter paper. These mycelial mats, while still on the filter paper, were washed thoroughly with distilled water to remove residual medium. The mats were dried at 95°C for 18-24 hours, and then cooled in a desiccator. Results are expressed as mg dry weight of mycelium/flask. Results for each harvest are the average value of six replicate flasks, and the experiment was repeated once.

3. Liquid shake culture

The amount of mycelium produced in shake culture was determined using 250 ml wide-mouth Erlyenmeyer flasks with centre well each containing 50 ml of synthetic medium. These flasks were inoculated with one ml aliquot of homogenized mycelium per flask. This one ml aliquot contained approximately 2.0 mg (2.00 - 2.25 mg) dry weight of homogenized fungus mycelium. Inoculum preparation was carried out as described by Ward and Colotelo (1960). Cultures were incubated at 15° and 1°C on a gyrotary shaker having 270 oscillating motions/min. Cultures were harvested at 8, 12, 16, 24, and 32 days after inoculation. In addition, yields of mycelium for cultures harvested 48 and 56 days after inoculation were determined for cultures incubated at 1°C. Mycelium was separated from the culture medium by means of centrifugation at 32,000 x g for 4 min. The mycelial pellet was washed three times by suspending the pellet in cold deionized water. In each cycle, water was removed after centrifugation. The mycelial pellet was placed on a previously dried and weighed filter paper, and dried at 95°C for 18-24 hours. The results for yield of mycelium (mg dry weight/flask) represent the average value for 9 culture flasks. In each experiment 3 replicate flasks were used and the experiment

was repeated twice.

The measurements for pH of culture medium and non-buffered cell-free mycelial extracts for cultures incubated at 15°C and 1°C, and harvested at their respective ages were made.

To note the effect of change of temperature on yield of mycelium, liquid shake cultures incubated at 15°C were transferred and incubated at 10°C(15---10°C) and 1°C(15---1°C) respectively, three days prior to harvesting at the above mentioned ages. At these incubation temperatures experiments were repeated three times and once respectively. The dry weights of mycelium were determined from 13 to 17 days after inoculation, in order to note the first drop in yield of mycelium grown at 15°C, are presented in Table 1. Each figure in the table is the average value of 9 replicate flasks, and this experiment was repeated twice.

Note: In this thesis the abbreviations 15---10°C and 15---1°C will be used to indicate a transfer of cultures for 15°C to 10°C and to 1°C respectively.

B. Protein Analyses

1. Extraction

Approximately 5 g fresh weight of washed mycelium harvested (see above for harvesting procedure) 6, 8, 12, 16, 24, and 32 days after inoculation for the cultures incubated at 15°C, 1°C, 15---10°C, and 15---1°C, were frozen at -23°C for 15-18 hours. Additional harvestings were carried out at 48 and 56 days after inoculation for the cultures incubated at 1°C. The frozen mycelium was put through a chilled Hughes press (-23°C) to disrupt the mycelium. To the disrupted mycelium was added 0.1 M Tris-HCl buffer, pH 7.5(1:4 w/v). The buffered slurry was centrifuged at 32,000 x g for 30 min. After centrifugation the supernatant

was carefully removed using a pipette fitted with a pro-pipette and the precipitate discarded. Three separate 0.1 ml aliquots of the supernatant were used for protein determinations. The soluble proteins in the remaining supernatant were precipitated by adding $(NH_4)_2SO_4$ (662 mg/ml), and the sample was agitated for 3 hours by means of a magnetic stirrer at 3-5°C. This mixture was centrifuged at 32,000 x g for 30 min and the supernatant discarded. The pellet was dissolved in 3-5 ml of 0.1 M Tris-HCl buffer, pH 7.5. The $(NH_4)_2SO_4$ was removed by dialyzing the sample against the above buffer for four hours. To hasten dialysis, the buffer was replaced every hour, and the dialysis sac was agitated with a magnetic stirrer. The extracted protein was analyzed when the dialyzing buffer gave a negative test for SO_4 when $BaCl_2$ was used as an indicator. All extraction procedures were carried out between 3-5°C.

2. Total protein determinations

Total protein determinations of the cell-free mycelial extract fractions were carried out using the method of Lowry et al. (1951). In this procedure quantitative results were obtained from a standard curve (see below for standard curve) for which Bovine Serum Albumin, Nutritional Biochemical Corporation (N.B.C.), was used as the protein source. Results are expressed as mg of total protein/g dry weight of mycelium.

3. Standard curve

To test tubes each containing 1.0 ml aliquot of different dilutions of Bovine Serum Albumin (N.B.C.) dissolved in 0.1 M Tris-HCl buffer, pH 7.5, was added 5 ml of a mixture of 1.0 ml of 0.5% $CuSO_4.5H_2O$ in 1% potassium tartrate, to 50 ml of 2% Na_2CO_3 in 0.1 N NaOH, and after 10 min, 0.5 ml of Folin-phenol reagent (1:1 v/v) aqueous was added. The intensity of the blue colour, developed after an incubation of 30 min

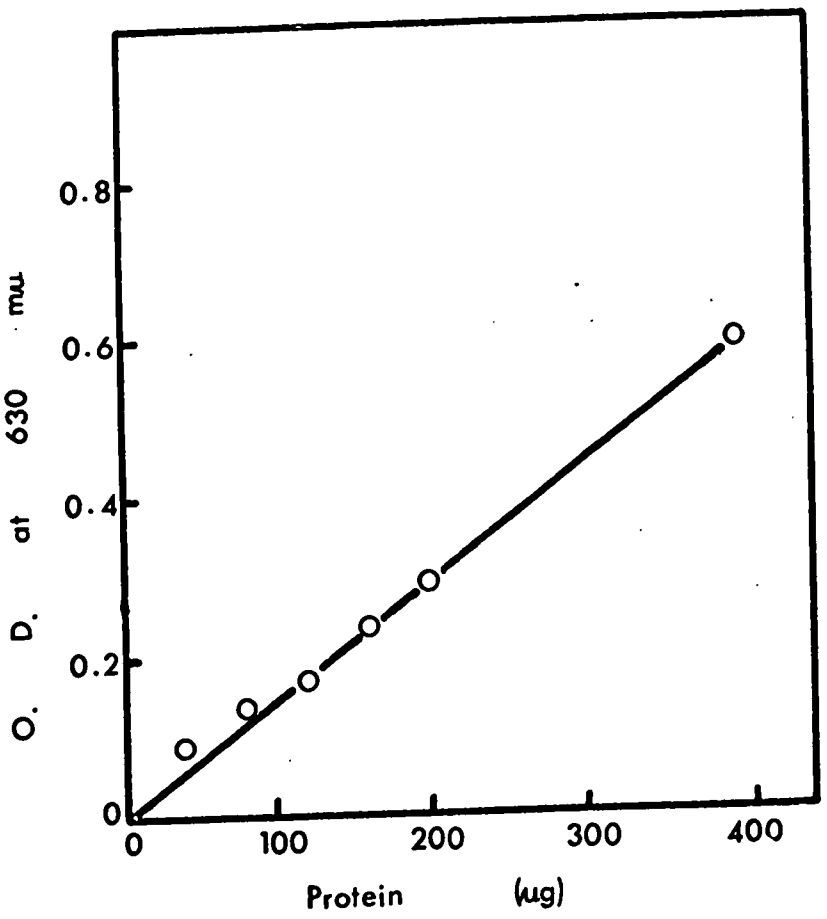

Figure 1. Standard curve for total protein concentration.

at room temperature, was read at 630 mu using a Hitachi Perkin-Elmer (HPE) spectrophotometer, Model 139. Standard curve results are shown in Fig. 1. Each point on the curve is the mean value of nine readings. In this procedure, for the preparation of standard curve, one determination represents three readings and the procedure was repeated twice.

4. Disc gel electrophoresis of proteins and peroxidase isozymes

The procedure for disc gel electrophoresis using standard 7.5% polyacrylamide gels, described by Davis (1964), for the separation of total proteins and peroxidase isozymes, was followed. Duplicate and/or triplicate determinations were made on aliquots of $(NH_4)_2SO_4$ precipitated proteins. Each aliquot contained 300 ug protein (as determined by Lowry et al. 1951) using a current of 3 mAmp/gel applied for 60 min.

Total proteins on the gels were detected with one percent Amido black 10 B, in 7% acetic acid. Peroxidase isozymes on the gels, run at the same time as those for proteins, were stained with Benzidine.2HCl reagent (Bulletin-Special subject, Enzyme analysis, Canal Industrial Corporation, Rockville, Maryland). The electrophoretic experiments for total proteins and peroxidase isozymes for cultures incubated at 15°C, 15---10°C, 15---1°C, and 1°C were repeated twice, three times, once, and twice respectively.

C. Protease Activity

Protease activity was determined for mycelium of cultures grown at 15°C and 1°C, using the procedure described by Keen et al. (1967). Harvestings were carried out a 8, 12, 16, 24, and 32 days after inoculation, and it was found necessary to make two additional harvestings at 48 and 56 days after inoculation due to the slow growth of the fungus at 1°C. The extraction procedure used was as follows:

Mycelium was frozen at -45°C for 15-18 hours, expressed through the chilled Hughes press (-45°C), and to the disrupted mycelium was added 0.1 M Tris-HCl buffer, pH 7.5(1:4, w/v). The slurry was centrifuged at 32,000 x g for 30 min, and the cell-free mycelial extract was used for protease determinations.

The substrate protein used in these determinations was 1% casein (Hammarsten, N.B.C.) dissolved in 0.1 M Tris-HCl buffer, pH 8.5, containing 0.0025% Thimerosal (Sigma Chemical Co.), and then denaturated by placing the solution in a boiling water bath for 15 min. After cooling, the solution was adjusted to a pH of 7.5 with a few drops of 1N HCl. One ml aliquots of this solution were transferred to test tubes and then placed in a water bath at 35°C for 5 min. To this solution of casein was added 1.0 ml of the above cell-free mycelial extract, and the mixture was incubated for 20 min at 35°C. The reaction was terminated with the addition of 3.0 ml of 10% trichloroacetic acid to each test tube. After incubating at room temperature for 60 min, the precipitates formed were removed by filtering through Whatman No. 2 filter paper. The optical densities of the clear filtrates were measured at 280 mu using a HPE spectrophotometer, and the changes in optical density were related to protease activity. The final readings for protease activity were obtained after subtracting the O.D. of blanks i.e. those solutions containing inactivated protease, from those obtained using the active enzyme preparations. The blanks were prepared by adding trichloroacetic acid to the casein solution prior to the addition of cell-free mycelial extracts.

For the quantitative estimation of protease activity, two standard curves were necessary. The first curve represents the optical density values obtained after reacting known amounts of crude fungal protease

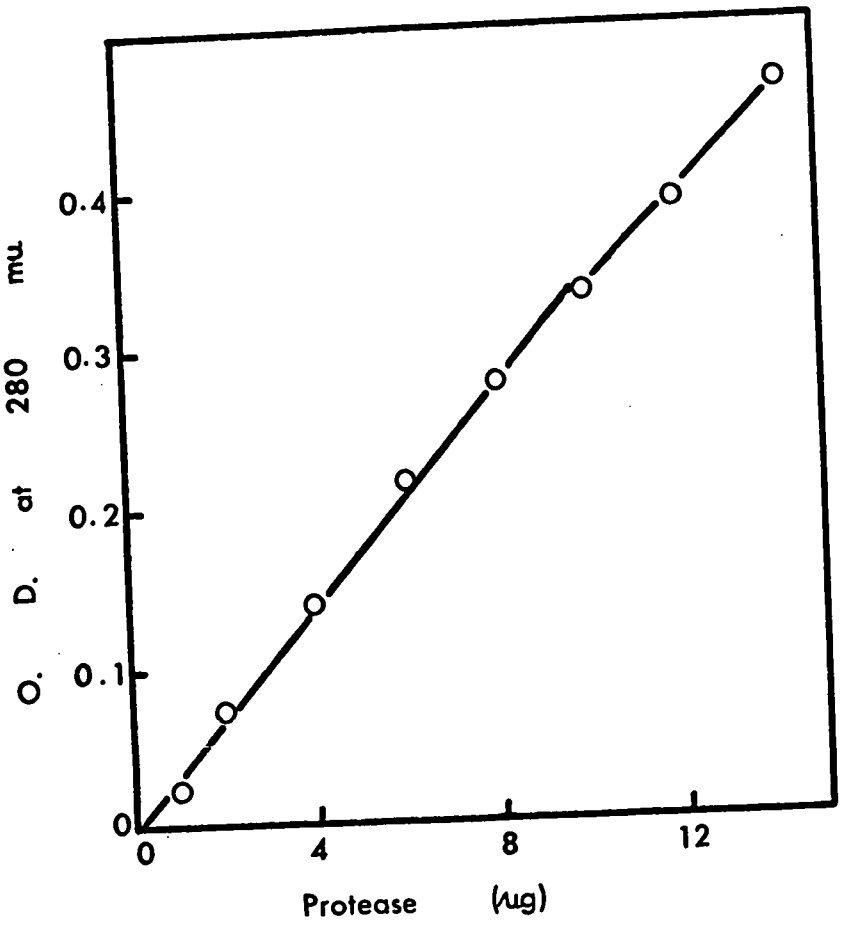

Figure 2 (a). Standard curve used for protease activity.

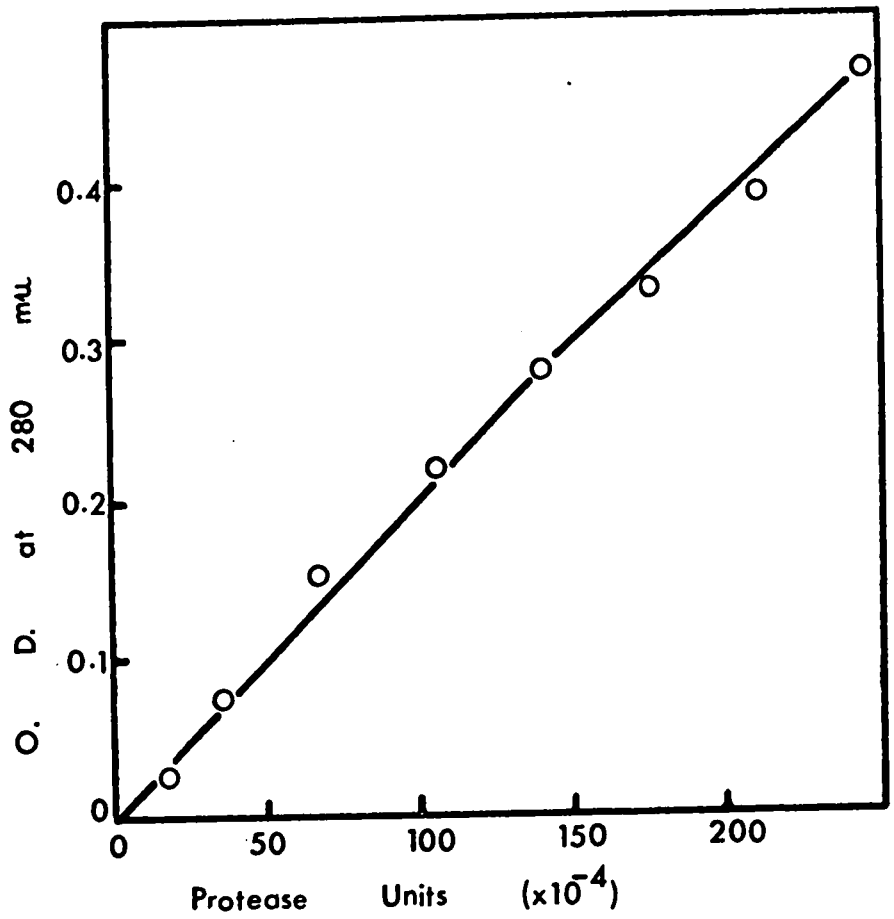

Figure 2 (b). Standard curve for crude fungal protease activity.

(N.B.C.) with the above casein solution. The second curve was necessary to convert the O.D. values to enzyme units for protease activity which was carried out following the procedure of Kunitz (1947). Kunitz defined a protease unit as the activity which gives rise to an increase of one unit of O.D. at 280 mu per minute digestion. Each point on the standard curves (Figs.2a,b)is the average of six readings resulting from two separate determinations. Each determination was comprised of three replicates.

The experiments for protease activity of cell-free mycelial extracts of the cultures incubated at 15°C and 1°C were repeated twice and once respectively. Each experiment consisted of three replications.

D. Total Ribose Nucleic Acid (RNA)-Ribonuclease (RNase) Determinations

1. Total RNA

RNA is well known to be directly involved with protein synthesis, therefore, the determinations of RNA were necessary for these studies.

(a) Extraction

RNA was extracted using the procedure of Bolton (1966), with slight modifications. For these studies one part of the cell-free mycelial extracts (see protease extraction procedure) was mixed with one part of preservative free 85% phenol equalibrated with 0.02 M Na-phosphate buffer, pH 7.0, containing 0.5% Sodium lauryl Sulphate (SDS).

After shaking the cell-free mycelial extract-phenol mixture vigorously for 15 min, the resulting emulsicn separated into three phases (upper, middle, and lower) during centrifugation at 13,000 x g for 15 min. The upper (aqueous) phase was removed very carefully with a syringe. The remaining fractions were discarded. The aqueous portion was again mixed with the above phenol solution, shaken again for 10 min,

and centrifuged for 15 min. The aqueous phase was again removed and extracted once more with the phenol reagent. To this aqueous phase was added an equal portion of ethyl ether to remove traces of phenol. The procedure for phenol removal was repeated once. In each case the ether layer was discarded after centrifugation. To the aqueous phase was added sufficient NaCl to make the solution 0.1 M. Two volumes of chilled 95% ethanol were added. This solution was let stand at 3°C for 18 hours. RNA precipitated and was pelleted by centrifugation at 32,000 x g for 15 min. The supernatant was discarded and the pellet dissolved in 25 ml of M Tris-HCl buffer, pH 7.5.

(b) RNA determinations

Amounts of the above extracted RNA were determined by the colorimetric procedure of Dische (1955). To 1.5 ml of RNA was added 3.0 ml of Orcinol reagent. The reagent was prepared by dissolving 100 mg of $FeCl_3.6H_2O$ in 100 ml of HCl sp. gr. 1.19, and adding 3.5 ml of 6% solution of Orcinol (Reagent grade, Fisher Scientific Co.) in ethanol. The reaction mixture, in test tubes, was heated in a boiling water bath for 3 min, and then cooled under tap water. The optical densities of the solutions were read at 665 mu against the blank which was prepared with 0.1 M Tris-HCl buffer, pH 7.5.

(c) Standard curve

A standard curve was prepared after reacting various concentrations of RNA (Reagent grade, N.B.C.) with Orcinol reagent using the above procedure. Each point on the standard curve (Fig. 3) is an average of nine readings, in which three determinations were made. Each determination consisted of three readings. Results for RNA determinations of cell-free mycelial extracts for cultures incubated at 15°C and 1°C are

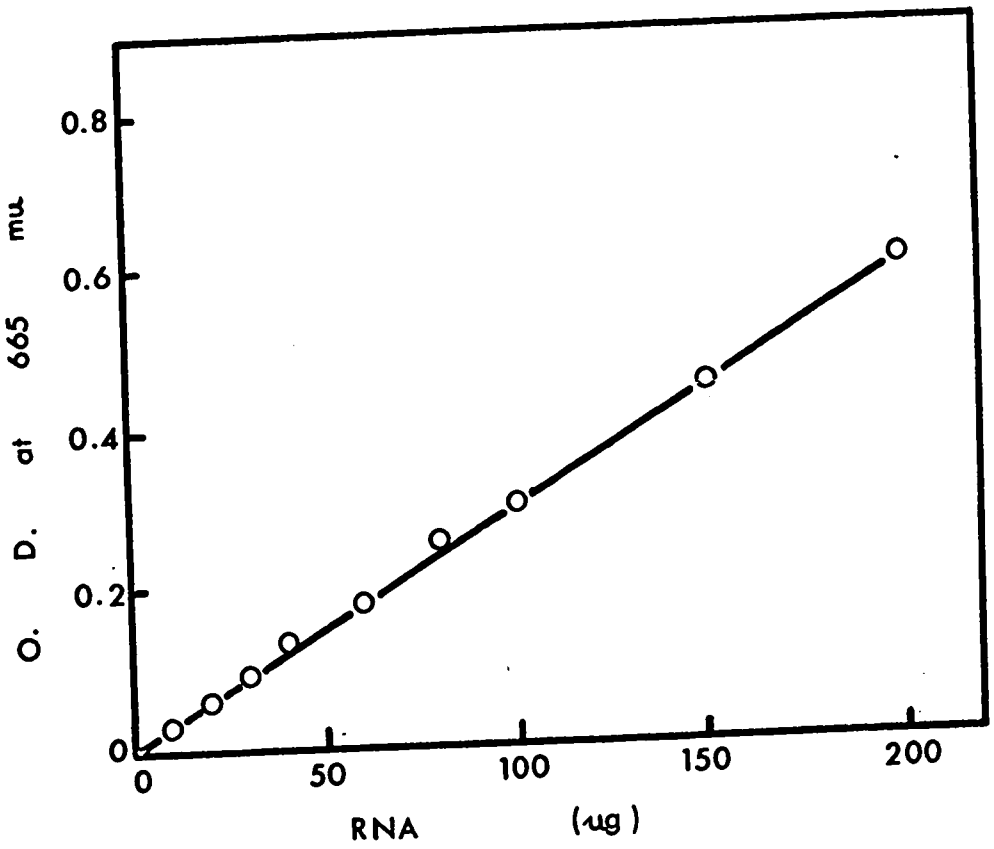

Figure 3. Standard curve for total RNA.

shown in Table 8, and the experiments were repeated twice and once respectively. In each experiment three replicate readings were obtained.

(d) Deoxyribonucleic acid (DNA)

To determine the extent of DNA which may have been present in the above RNA preparations, the colorimetric method for DNA estimation by Burton (1955) was employed. In this procedure 1.0 ml of the above RNA sample was added to an equal volume of 1 N $HClO_4$, after which 4.0 ml of diphenylamine reagent was added. The diphenylamine reagent was prepared by dissolving 1.5 g of diphenylamine (Fisher Scientific Co.) in 100 ml of glacial acetic acid and to this solution was mixed 1.5 ml of H_2SO_4. Before use, 0.1 ml of aqueous acetaldehyde (1.6 mg) was mixed in each 20 ml of reagent. After incubating the reaction mixture for 16-20 hours at 30°C, the optical densities of the blue colour were recorded at 600 mu using the HPE spectrophotometer.

A standard curve for DNA (highly polymerized, N.B.C.) is shown in Fig. 4. Each point on the standard curve is the mean value of nine readings. This experiment, consisting of three replications, was carried out three times.

2. RNase determinations

To find out the extent of RNase activity in the cell-free mycelial extracts, which were also employed for RNA extractions, the following determinations were carried out:

The cell-free mycelial extracts of mycelium from cultures incubated at 15°C and 1°C were assayed for RNase activity. The procedure of Wilson (1963) was followed for these determinations. In this procedure 0.1 ml of cell-free mycelial extracts were reacted with 1.0 ml of substrate-buffer solution (0.4% RNA (N.B.C.) - 0.125 M cacodylic acid,

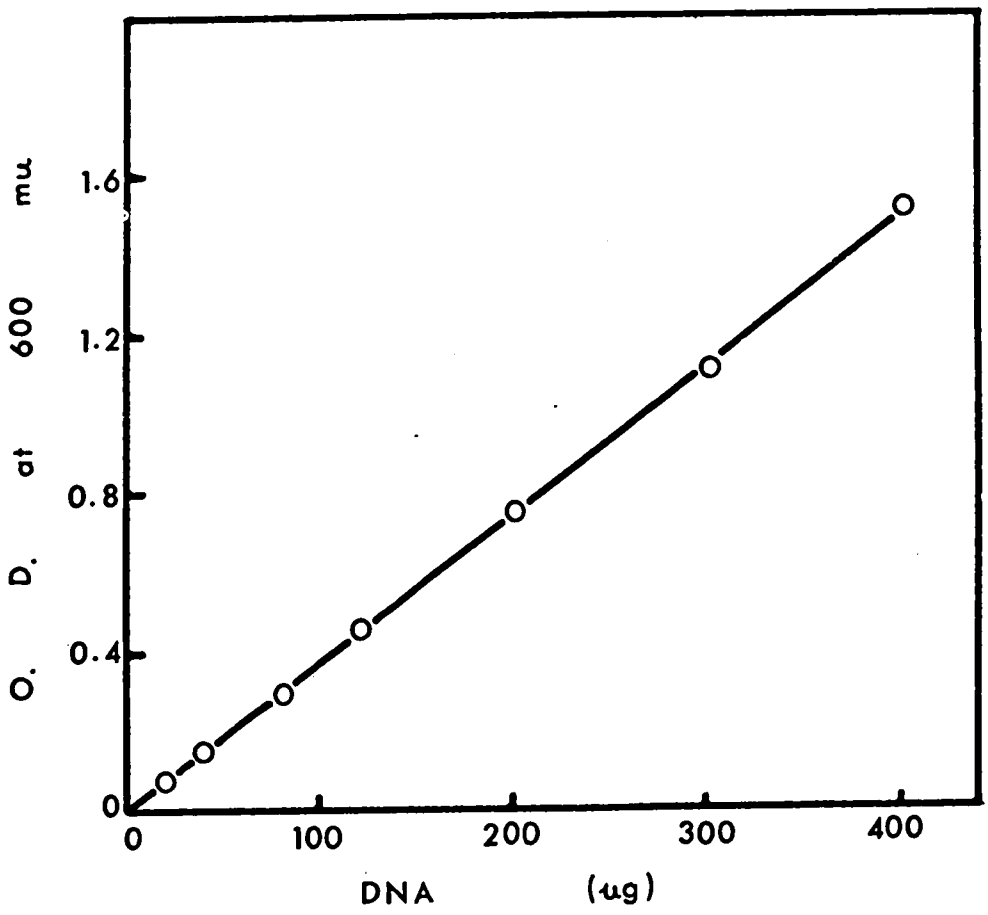

Figure 4. Standard curve for DNA.

the pH of this solution was raised to 5.8 by adding small amounts of imidazole), 0.4 ml of 1 M KCl, and 1.0 ml of distilled deionized water. This reaction mixture was incubated in a 12 ml centrifuge tubes for 30 min at 37°C. The reaction was stopped by adding 0.5 ml of 0.75% uranyl acetate in 25% $HClO_4$. The reaction mixture was chilled by placing the centrifuge tubes in an ice bath and then centrifuged at 10,000 x g for 15 min. An aliquot of 0.1 ml of the supernatant was removed and diluted 30 times with deionized distilled water and the optical density measured at 260 mu with a HPE spectrophotometer. These readings were corrected for the blank samples which were prepared using the above procedure except that the uranyl acetate in $HClO_4$ was added at zero time.

The results for RNase activity in terms of units/g dry weight of mycelium are presented in Table 8 . Each value in the table is the average of nine and six replicates for mycelium grown at 15°C and 1°C respectively. The experiment for RNase activity was repeated twice and once for the cultures incubated at 15°C and 1°C respectively. Each experiment was comprised of three replicates.

E. Hydrogen Cyanide (HCN) Determinations

1. HCN release from cultures

Hydrogen cyanide (HCN) was collected in the centre well of each wide-mouth flask in a 24-hour period before harvesting of mycelium in liquid shake culture using the procedure described by Ward and Lebeau (1962). In this procedure, the method of HCN determination is somewhat similar to the Conway microdiffusion method (1962). A 5.0 ml aliquot of alkaline sodium picrate reagent (5 g picric acid, 25 g Na_2CO_3 and deionized distilled water to make up to one liter) in the centre well of a wide-mouth flask was used to absorb the HCN released by the culture.

For HCN determinations, the fungus was cultured at 15°C and 1°C. Also to note the effect of temperature change on HCN release, cultures were incubated 15---10°C and 15---1°C. After the absorption of HCN for a 24-hour period, one ml of distilled water was added to the centre well containing sodium picramate. The solutions were thoroughly mixed, and brought up to a total volume of 6.0 ml with deionized distilled water. An aliquot of 1.0 ml was withdrawn with a pipette and this sample was diluted with H_2O to a final volume of 10 ml in a test tube. Test tubes were heated for 5 min in a boiling water bath. After cooling, the optical densities of sodium picramate solutions were determined at 490 mu using a HPE spectrophotometer, Model 139. Quantitative determinations for the amount of HCN collected were obtained from a standard curve prepared after reacting known quantities of potassium cyanide (KCN) with sodium picrate.

Results from the HCN determinations (p.p.m/flask) for the cultures incubated at the temperatures mentioned above are shown in Fig. 21. Each point on the graph is the average value of nine, twelve, six, and nine replicate flasks. For the cultures incubated at 15°C, 1°C, 15---10°C, and 15---1°C respectively, the experiments were repeated twice, three times, once, and twice respectively.

The determinations for HCN collection on daily basis from 13 to 17 days, to ascertain the first release of HCN in cultures incubated at 15°C, are shown in Table 9. This experiment was repeated once and each figure in the table is the average value of six replicates.

2. Standard curve

The procedure for obtaining the standard curve for HCN determinations was essentially the method described by Ward and Lebeau (1962).

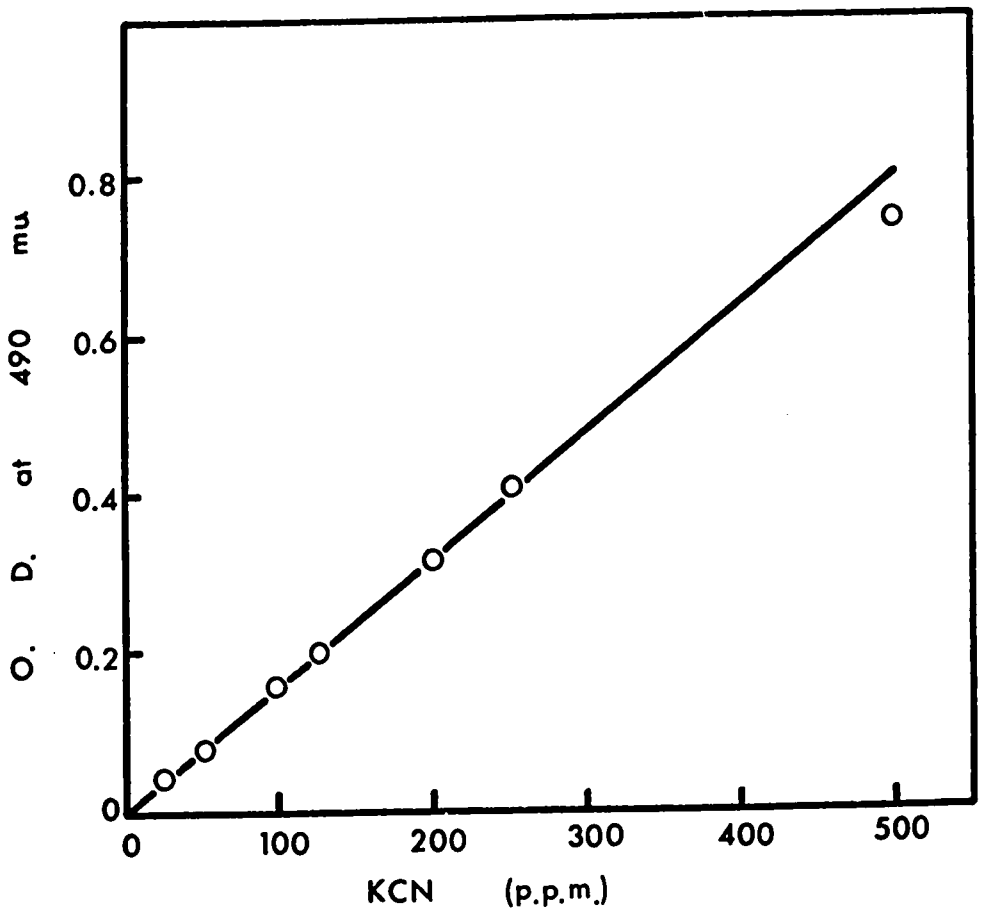

Figure 5. Standard curve for hydrogen cyanide used for hydrogen cyanide release in ageing cultures.

In this method, 1.0 ml aliquots of KCN each containing 25, 50, 100, 125, 250, and 500 p.p.m. were reacted with 5.0 ml of alkaline sodium picrate reagent. The blanks were prepared by substituting 1.0 ml of water for the KCN solutions. The reaction mixture was heated 5 min in a boiling water bath and then cooled in running tap water. One ml aliquot of the reaction mixture was diluted ten times with deionized distilled water and the solution thoroughly mixed. The optical densities of these solutions were measured at 490 mu with HPE spectrophotometer, Model 139. Each point on the curve (Fig. 5) is the average value of nine replicates. Determinations for standard curve were reproduced twice, and each determination consisted of three replicates.

3. β-glucosidase activity and 'free' cyanide (HCN) determinations

(a) β-glucosidase activity and 'free' HCN

Associated with HCN release from cultures (Fig. 21), the release of 'free' HCN from culture medium and cell-free mycelial extracts was investigated. The enzyme β-glucosidase has been shown to be involved with cyanogenesis (Colotelo and Ward 1961). However, the activity of β-glucosidase as related to age of culture and incubation temperatures has not been presented. In these studies, β-glucosidase activity and 'free' HCN of culture medium and cell-free mycelial extracts were determined using a modified version of the Conway microdiffusion technique (1962). In this modified procedure, somewhat similar to that used by Colotelo and Ward (1961), β-glucosidase was reacted with a cyanogenic glucoside, and the HCN released was collected by an absorbent, alkaline sodium picrate, in the centre well. β-glucosidase activity of culture medium and cell-free mycelial extracts of cultures incubated at 15°C and 1°C, harvested 8, 12, 16, 24, and 32 days after inoculation was determined.

For cultures incubated at 1°C the first determination was carried out at 12 days after inoculation due to the slow growth of the fungus. Also, for cultures incubated at 1°C, it was found necessary to carry out further determinations at 48, 56, and 72 days after inoculation due to a continual increase in B-glucosidase activity in cell-free mycelial extracts. The cell-free mycelial extracts were similar to those employed for protease, RNA, and RNase determinations. The culture medium was that portion of the cultures separated from the mycelium after centrifugation at 32,000 x g for 4 min.

The cyanogenic glucoside used for determining β-glucosidase activity was 0.02 M amygdalin (Calbiochem.). Note: the amygdalin preparation from N.B.C. released 'free' HCN without the presence of the enzyme.

In the experiments using the modified Conway units (Obrink 1955) for collecting HCN, deionized water or 0.1 M Tris-HCl buffer, pH 7.5, were substituted for the culture medium, cell-free mycelial extracts and/or amygdalin wherever the appropriate control was required. It was necessary to subtract the values for 'free' HCN from those for total HCN to obtain the amounts of HCN released as a result of β-glucosidase activity. 'Free' HCN was considered to be the HCN collected in the centre well of Conway units when the reaction chamber contained either reaction mixture minus amygdalin or aliquots of either culture medium or cell-free mycelial extracts which had previously been autoclaved at 121°C for 30 min.

The following procedure was used for determining the total amount of HCN as related to β-glucosidase activity and the release of 'free' HCN:

(i) 3.0 ml of alkaline sodium picrate placed in the centre well of the modified Conway units.

(ii) 2.0 ml of 0.02 M amygdalin (substrate/or H_2O) added to the outer chamber.

(iii) 4.0 ml of culture medium/or H_2O, or cell-free mycelial extracts/ or buffer added to the outer diffusion chamber containing the substrate/or H_2O.

(iv) 3.0 ml of substrate added to the outer closing chamber (this chamber acts as a seal).

(v) The plastic top of the Conway unit was placed so that the edges were immersed in the substrate of the outer closing chamber.

(vi) Microdiffusion units were incubated for 22 hours at room temperature ($24^{o}C$) after being placed in a plastic bag containing wet cotton swabs.

(vii) To the centre well, 1.0 ml of deionized water was added and mixed thoroughly.

(viii) 2.0 ml aliquot from the centre well was removed, and the volume made up to 10 ml with deionized distilled water in a test tube.

(ix) The solution was heated for 5 min in a boiling water bath followed by cooling under running tap water.

(x) The O.D. of the sodium picramate was recorded with HPE spectrophotometer, Model 139, at 490 mu.

The amount of HCN collected in the centre well was related to β-glucosidase activity and/or 'free' HCN. Quantitative estimations for HCN collected were obtained by relating the optical densities of sodium picramate with those of standard curve prepared after reacting known quantities of KCN with sodium picrate and read at 490 mu.

Results for β-glucosidase activity are expressed as the amount of HCN (p.p.m.) released by per ml of culture medium or cell-free mycelial extract equivalent to 10 mg dry weight of mycelium. The presence of 'free' HCN is also expressed in a similar manner.

Each point in the Fig. 22 and Table 11 is the average value of nine and six replicates for β-glucosidase and 'free' HCN determinations for the cultures incubated at 15°C and 1°C respectively. At these incubation temperatures the experiments were repeated twice and once respectively. However, the determinations for B-glucosidase and 'free' HCN on a daily basis, from 13 to 17 days after inoculation, were carried out twice only for the cultures incubated at 15°C. Results for these determinations are expressed on the same basis as mentioned above and are presented in Tables 10,12.

(b) Standard curve for HCN determination

The standard curve (Fig. 6) for determining β-glucosidase activity against amygdalin and 'free' HCN was prepared by reacting various amounts of KCN in 1.0 ml of deionized distilled water with 3.0 ml of alkaline sodium picrate reagent. A 2.0 ml aliquot of the above was removed, diluted to 10 ml with deionized distilled water, heated in a boiling water bath for 5 min, and then cooled under tap water. The optical densities of the resulting sodium picramate solutions were read against a blank at 490 mu. The blanks were prepared from the sodium picrate withdrawn from the outer diffusion chamber of Conway units containing amygdalin + H_2O. Results for the standard curve were reproduced twice and each determination consisted of three replicates.

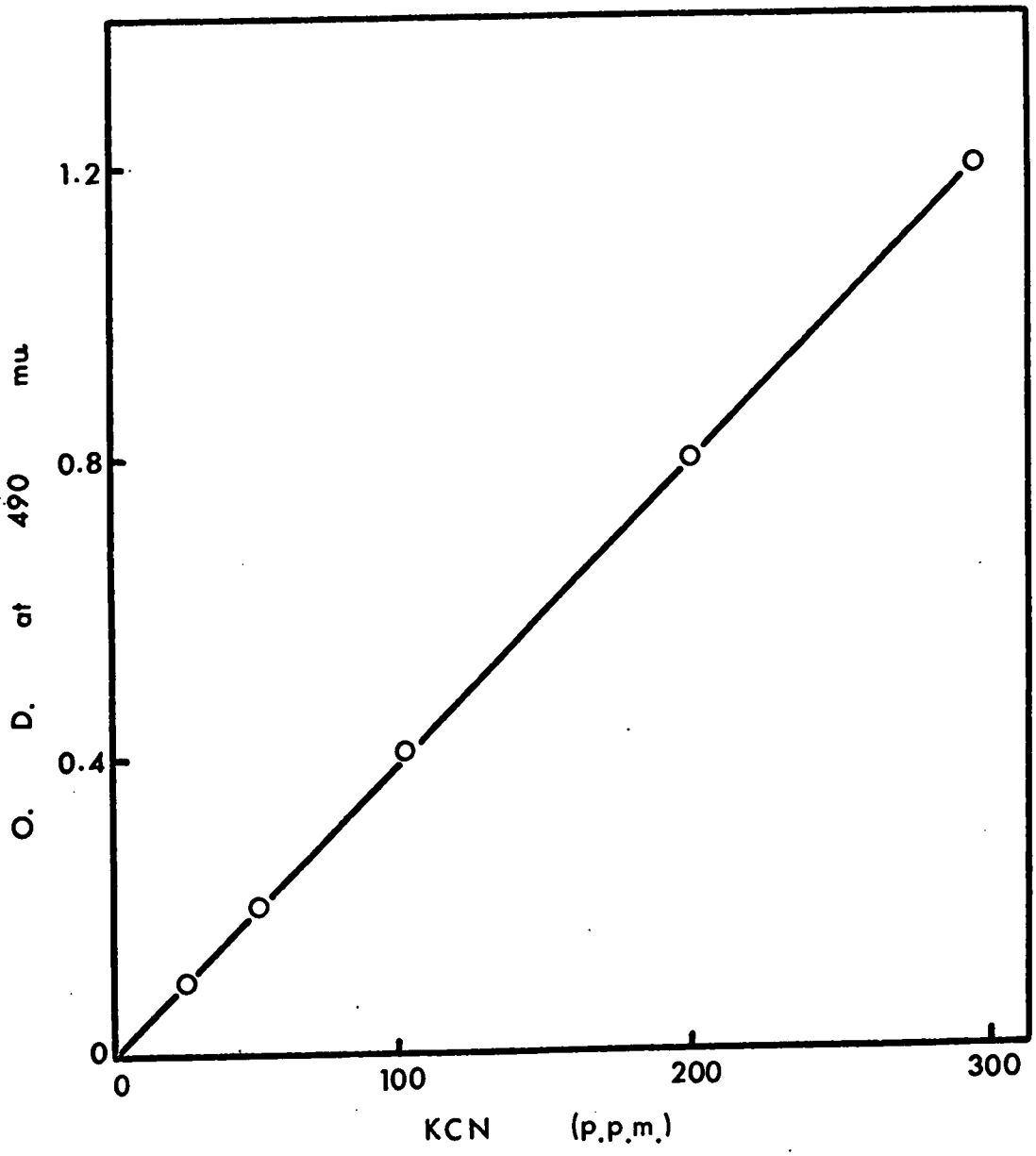

Figure 6. Standard curve for hydrogen cyanide used for determining the release of hydrogen cyanide from amygdalin due to β-glucosidase activity and 'free' hydrogen cyanide.

EXPERIMENTAL RESULTS

A. Growth of W_2 in Petri Dish, Liquid Static, and Liquid Shake Cultures at Various Temperatures

The results for growth of W_2 on a synthetic medium in petri dish, liquid static, and liquid shake cultures are presented.

1. Petri dish cultures

The growth response (diam. of colony) of isolate W_2, on synthetic medium plus agar at 15°C and 1°C is shown in Fig. 7. The diameter of colonies (mm) of cultures incubated at 15°C were much greater than those incubated at 1°C. Colonies grown at 15°C were fluffy white or cottony and appressed to the agar medium. At 24 days, at which time the experiment was terminated, the diameters of colonies at 1°C were less than one-half those grown at 15°C.

2. Liquid shake cultures

The results for growth using liquid static cultures are presented in Fig. 8. In these studies maximum yield (dry weight, mg /flask), for mycelium grown at 15°C, was obtained 20 days after inoculation. However, at 1°C, the yield of mycelium increased up to 56 days after inoculation, at which time the experiment was terminated. It should be noted that, for cultures incubated at 1°C, there was a lengthy lag phase before rapid growth occurred.

3. Liquid shake cultures

The amount of mycelium (dry weight, mg /flask) obtained from cultures grown at 15°C, 1°C, 15---10°C and 15---1°C (grown at 15°C and transferred to 10°C and 1°C respectively three days prior to harvest) are shown in Fig. 9. The maximum dry weights from cultures incubated at 15°C, 15---10°C, and 15---1°C were obtained 12 days after inoculation and

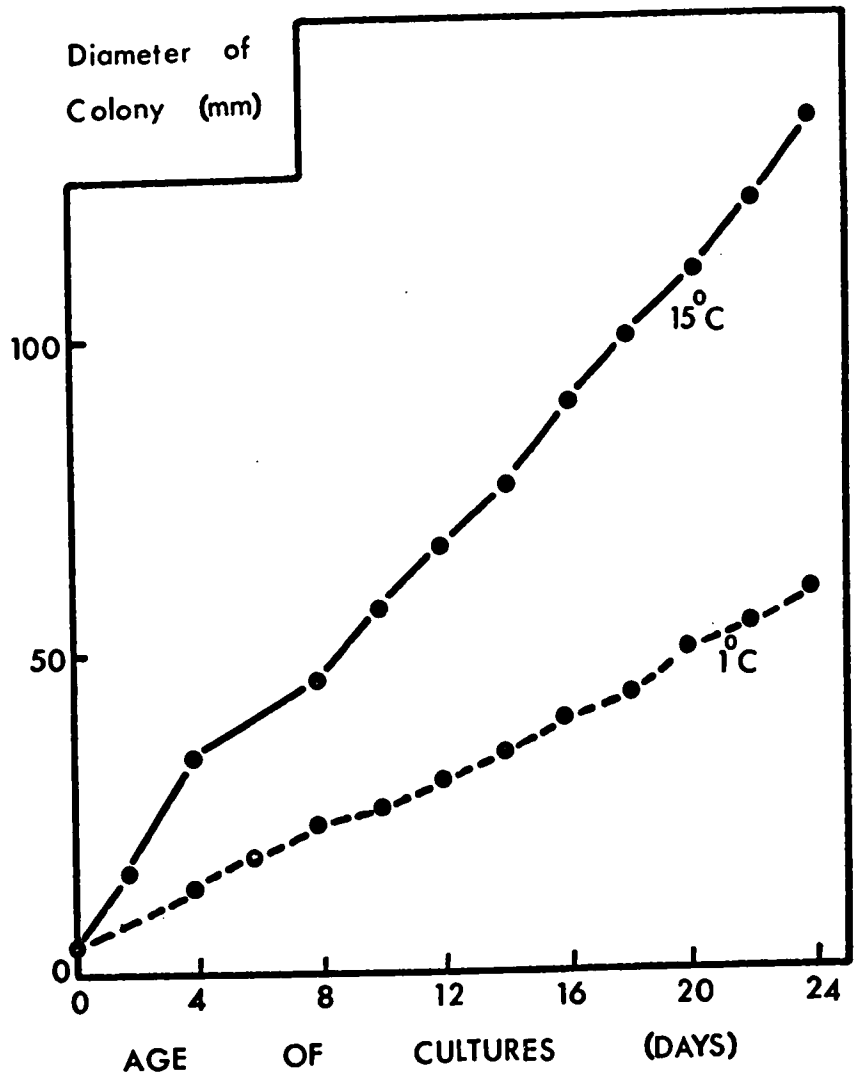

Figure 7. Growth of the low-temperature basidiomycete, isolate W_2, incubated at 15°C and 1°C on a synthetic medium in petri dishes.

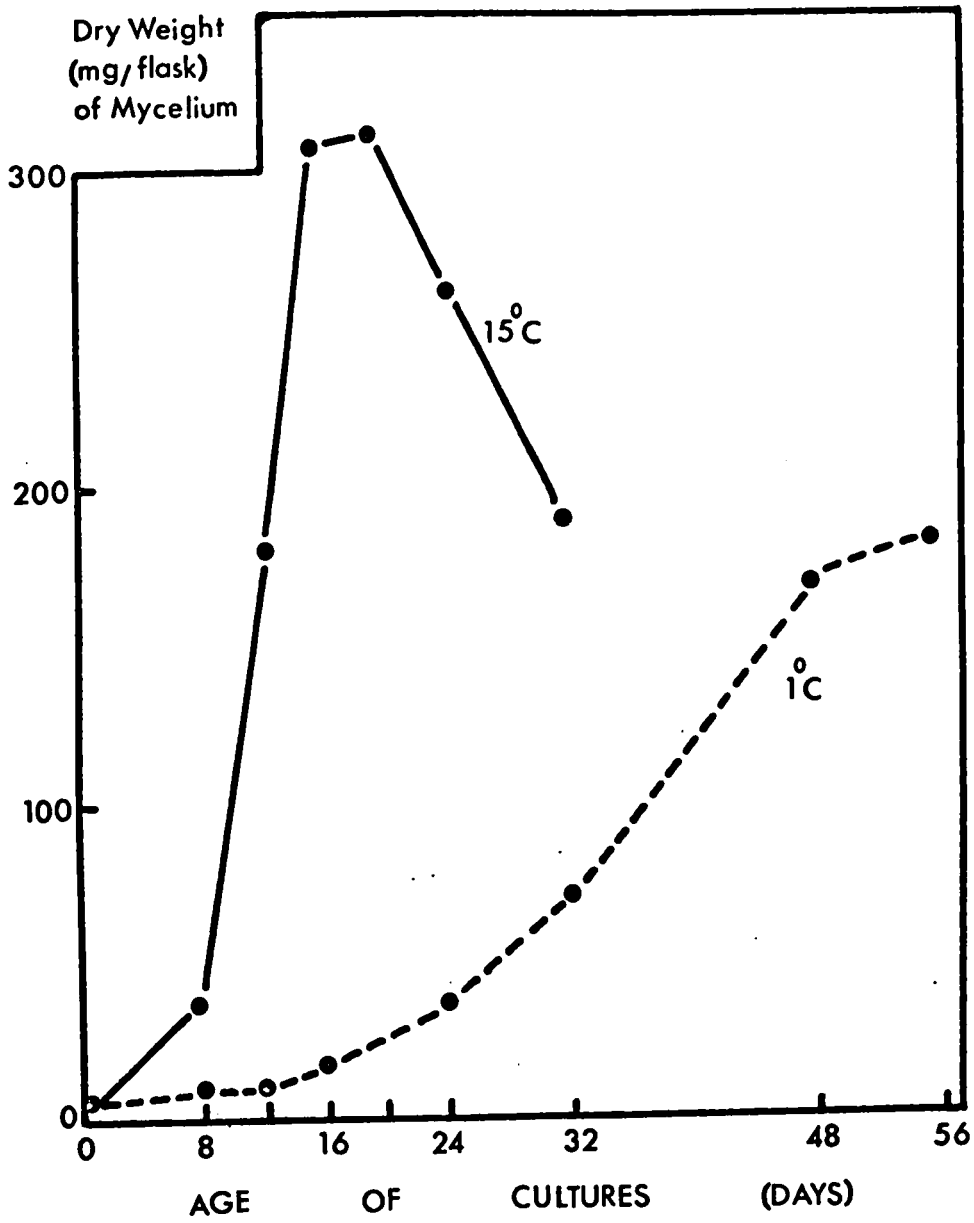

Figure 8. Yield of mycelium of the low-temperature basidiomycete, isolate W_2, on a synthetic medium in liquid static cultures incubated at 15°C and 1°C.

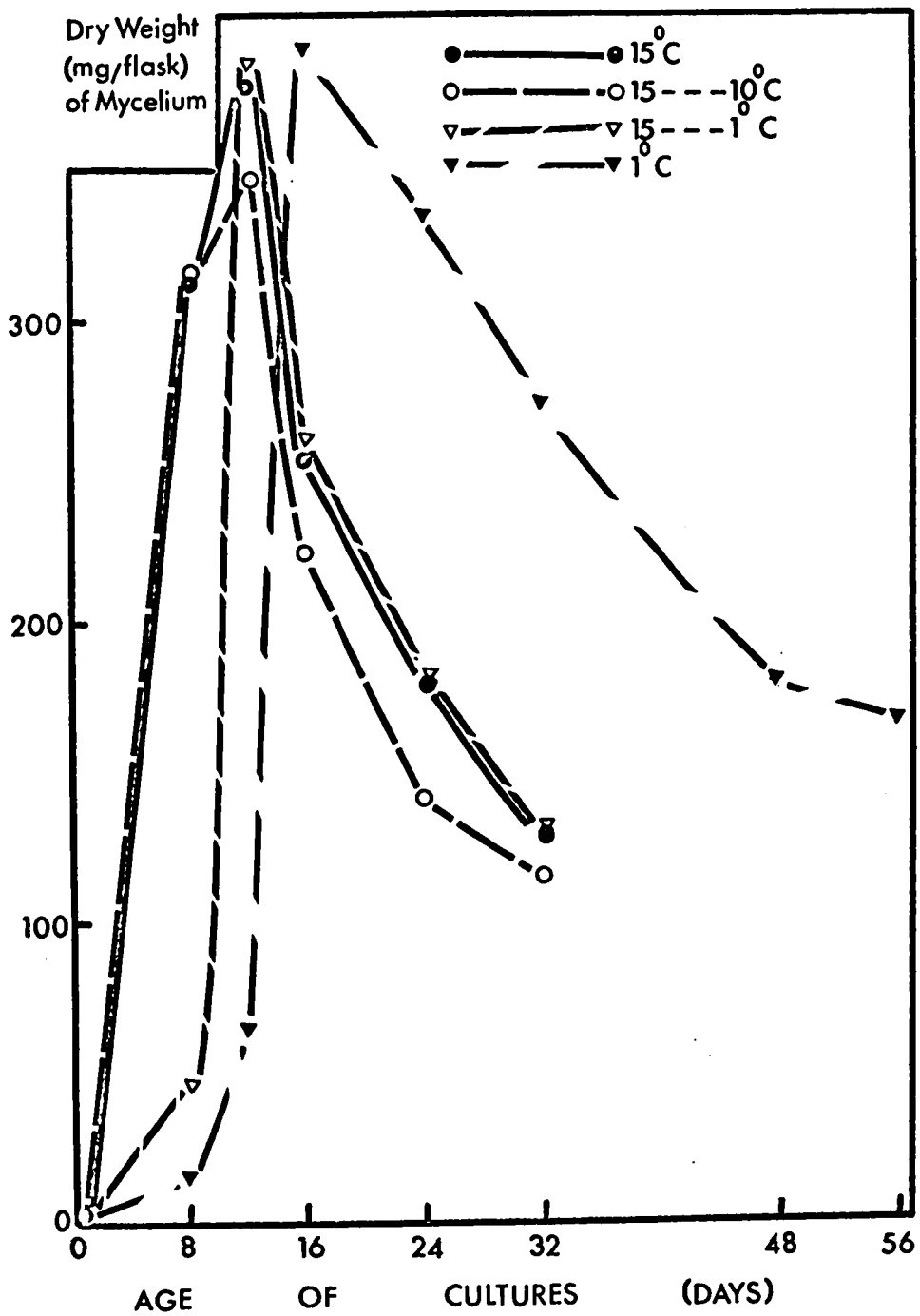

Figure 9. Yield of mycelium of a low-temperature basidiomycete, isolate W_2, using a synthetic medium in liquid shake cultures incubated at 15°C, 1°C, and at 15°C and then transferred to 10°C and 1°C respectively three days prior to harvest (15---10°C, and 15---1°C).

at 16 days for cultures incubated at 1°C. The dry weight of mycelium was greater in those cultures incubated at 1°C than at the other temperatures. Prior to obtaining maximum amount of mycelium, the rates of growth for cultures incubated at 15°C, 15---10°C, and 15---1°C were quite rapid; however, those cultures incubated at 1°C showed a slight lag phase from zero time to 8 days after inoculation.

A rapid decline in dry weight was obtained for all cultures after maximum growth was reached. However, the decrease in dry weight was less for cultures grown at 1°C than those incubated at the other temperatures, even when the harvestings were extended to 56 days after inoculation.

Since there was such a great difference in values for dry weights of mycelium obtained at 12 and 16 days after inoculation at 15°C, it was necessary to harvest cultures at 13, 14, 15, 16, and 17 days after inoculation. The results for these studies are shown in Table. 1.

Table1 : Yield of mycelium of a low-temperature basidiomycete, isolate W_2 grown on synthetic medium in liquid shake cultures incubated at 15°C. Cultures were harvested from 13 to 17 days after inoculation.

Age of Cultures (days)	Yield of mycelium (dry weight, mg /flask)
13	303.50
14	296.12
15	272.64
16	255.32
17	225.15

4. pH changes (culture medium and non-buffered cell-free mycelial extracts)

Values for pH of culture medium and non-buffered cell-free mycelial extracts for cultures of various ages incubated at 15°C and 1°C respectively, are shown in Table 2. The lowest pH values for culture

medium and cell-free mycelial extracts corresponded with maximum growth i.e. dry weight of mycelium which occurred at 12 and 16 days after inoculation for cultures incubated at 15°C and 1°C respectively. The greatest drop in pH occurred in the culture medium on 12 and 16 days after inoculation at 15°C and 1°C respectively.

The final pH of the medium for cultures incubated at 1°C was about one pH unit higher than those incubated at 15°C; however, cultures incubated at 1°C were harvested for a longer period of time than those incubated at 15°C.

In relation to temperature, the final pH values of non-buffered cell-free mycelial extracts for cultures incubated at 15°C and 1°C were very similar, and the variation was not as great as for culture medium.

Table 2 : Changes in pH of culture medium and non-buffered cell-free mycelial extracts of ageing cultures* of a low-temperature basidiomycete, isolate W_2. Cultures were grown on a synthetic medium and incubated at 15°C and 1°C.

	Incubation Temperature			
	15°C		1°C	
	pH			
Age of Cultures (days)	Culture medium	Cell-free mycelial extracts	Culture medium	Cell-free mycelial extracts
0	6.20	--	6.20	--
8	5.47	6.38	6.15	--
12	4.56	6.31	5.70	6.52
16	6.29	6.43	4.85	6.27
24	7.38	6.92	6.15	6.40
32	7.44	6.86	6.48	6.43
48			7.46	6.96
56			7.61	6.98
72			7.78	6.97

* pH of sterile culture medium + inoculum, at zerotime 6.20.

B. Protein Analyses

1. Total proteins

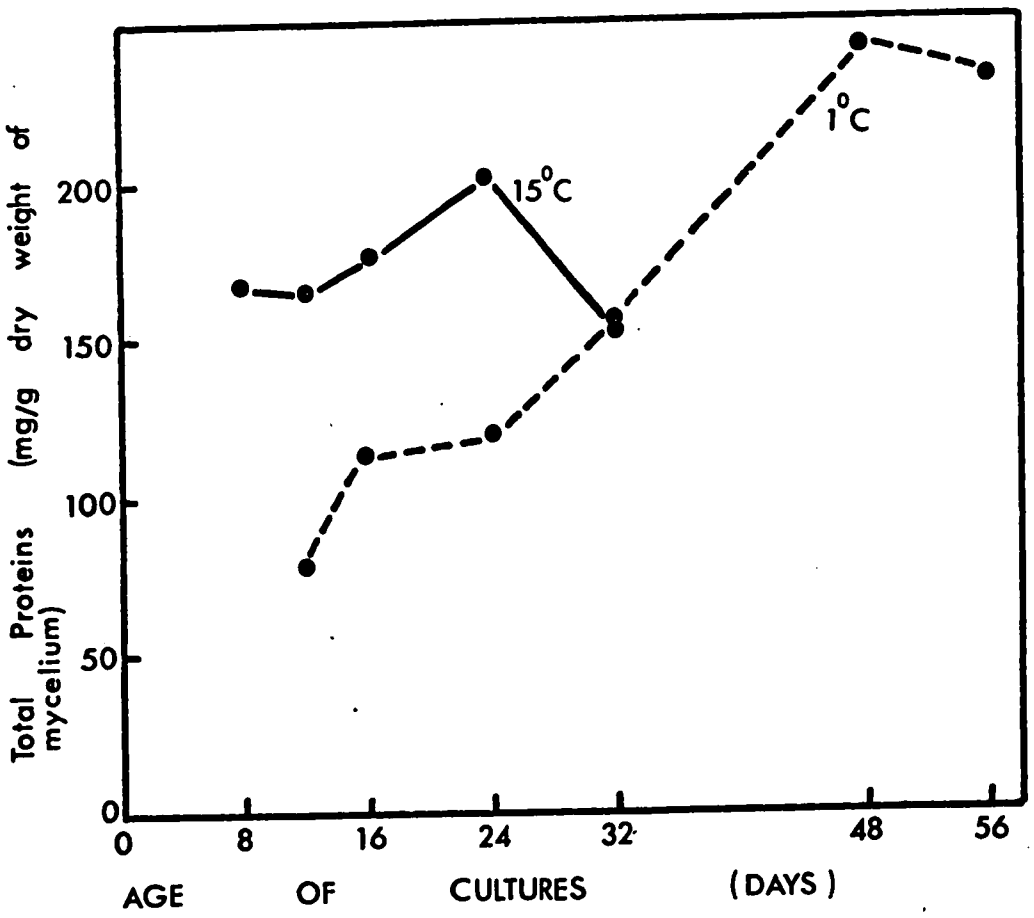

Figure 10. Total proteins of cell-free mycelial extracts from mycelium of ageing cultures of a low-temperature basidiomycete, isolate W_2, incubated at 15°C and 1°C.

The results for total protein determinations for cell-free mycelial extracts, of mycelium grown at 15°C and 1°C and harvested 8, 12, 16, 24, and 32 days after inoculation are presented in Fig. 10. As in other phases of these studies for cultures incubated at 1°C, it was found necessary to do two additional harvests i.e. 48 and 56 days after inoculation due to the slow growth of the fungus.

The maximum values for total proteins for cultures grown at 15°C and 1°C were obtained 24 and 48 days after inoculation respectively.

In general there was a rapid increase in protein, although somewhat slower for cultures incubated at 1°C as compared to cultures incubated at 15°C, before the maximum value was reached.

There was a significant reduction in total protein values for mycelium harvested 32 and 56 days after inoculation for cultures grown at 15°C and 1°C respectively.

2. Gel electrophoretic patterns of proteins and peroxidase isozymes

The protein and peroxidase patterns of cell-free mycelial extracts obtained on polyacrylamide gels are shown in Figs. 12-19. Protein bands varied in their staining properties, therefore an arbitrary scheme was used to designate the relative intensities of stain uptake. Patterns on the gels are shown as in the above Figs.12-15 and not as photographs because good photographic definition of the lightly stained protein bands in the photographic prints was not possible. Prominent bands are shown as solid lines, faint bands as broken lines, and diffuse zones as diagonal lines.

To help interpret the protein patterns on the gels the gels were divided into four quarters (Figs. 12-15), and the numbers of bands in each quarter noted. Also, the numbers of bands in each quarter of the

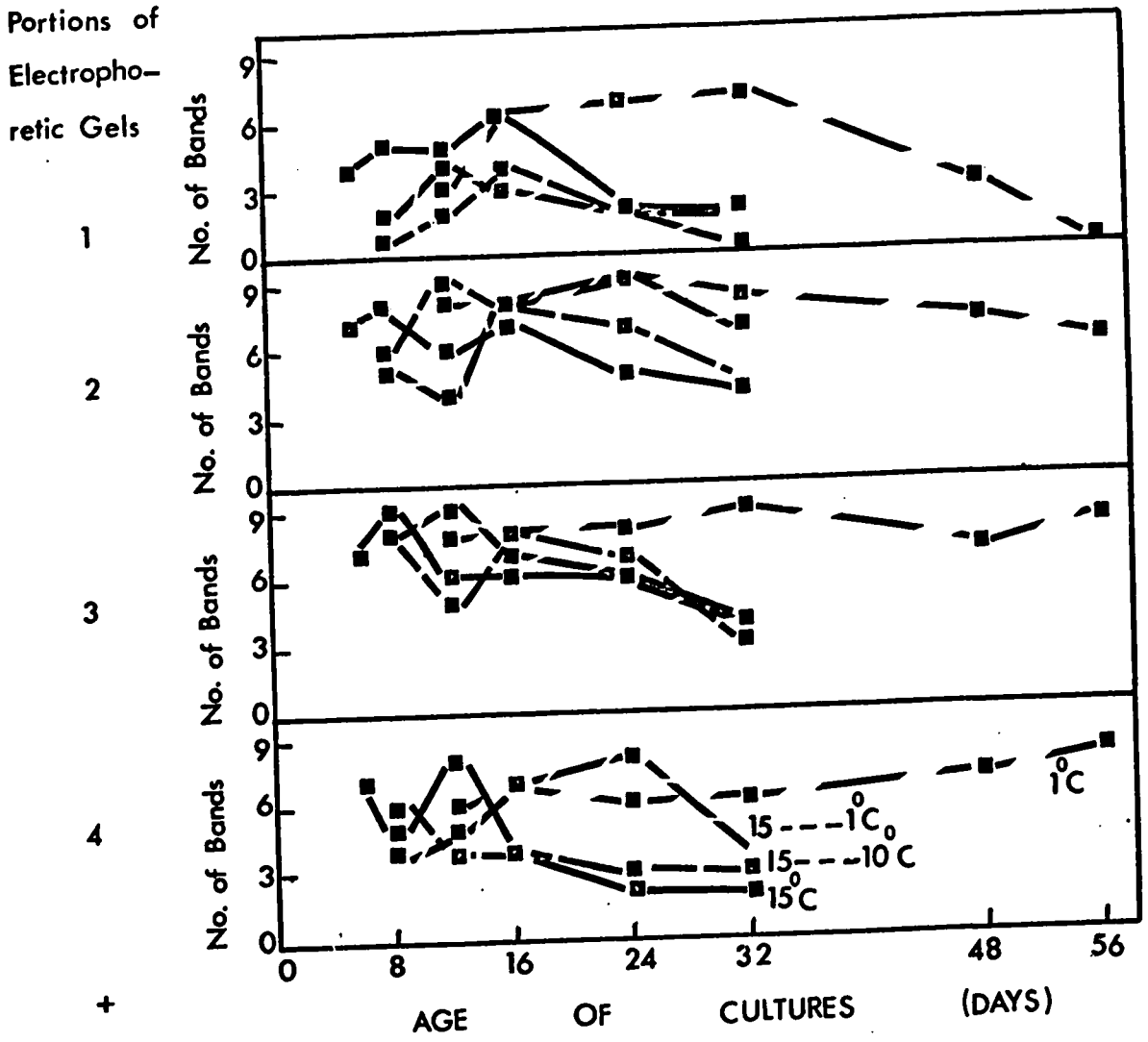

Figure 11. Distribution of protein bands in each quarter of electrophoretic gels as related to the age of cultures and various incubation temperatures.

gels were plotted against the age of cultures, grown at various temperatures (Fig. 11). In several studies (Clements 1965, Gottlieb and Hepden 1966, Hart and Bhatia 1967, Smith and Powell 1968, and Whitney et al. 1968), Ef values were used as criteria for identifying individual electrophoretic bands. In these studies, Ef values for individual protein, were also calculated and are presented in Tables 3-6.

3. Effects of Temperature on Proteins Extracted from Mycelium and Separated by Disc gel Electrophoresis

(a) Protein patterns at 15°C

The numbers of protein bands from mycelium extracts of cultures grown at 15°C and harvested 6, 8, 12, 16, 24, and 32 days after inoculation were 25, 27, 25, 23, 15, and 12 respectively (Fig. 12). There was not only a change in the protein patterns of cultures varying in age, but there was a wide variation in protein stain uptake by individual protein bands.

The numbers of protein bands were greater in cell-free mycelial extracts of 6-, 8-, and 12-day old cultures than for those harvested 16, 24, and 32 days after inoculation. In 24-, and 32-day old cultures there were approximately one-half the number of bands visible on the gels as compared to cultures harvested previously. Of these, only three bands in 24-day old and one in 32-day old cultures were sharply defined.

When the gels were divided into four quarters, several significant features were noted. The number of slower moving bands (slower due to their molecular weight, size, and/or charge) of 24 and 32 days old cultures in the top quarter decreased from one-half to one-third that observed for cultures harvested earlier. The faster moving protein bands located in the lower quarter of the gels were much fewer in numbers one-half to one-fourth, for cultures harvested 24 and 32 days after inoculation

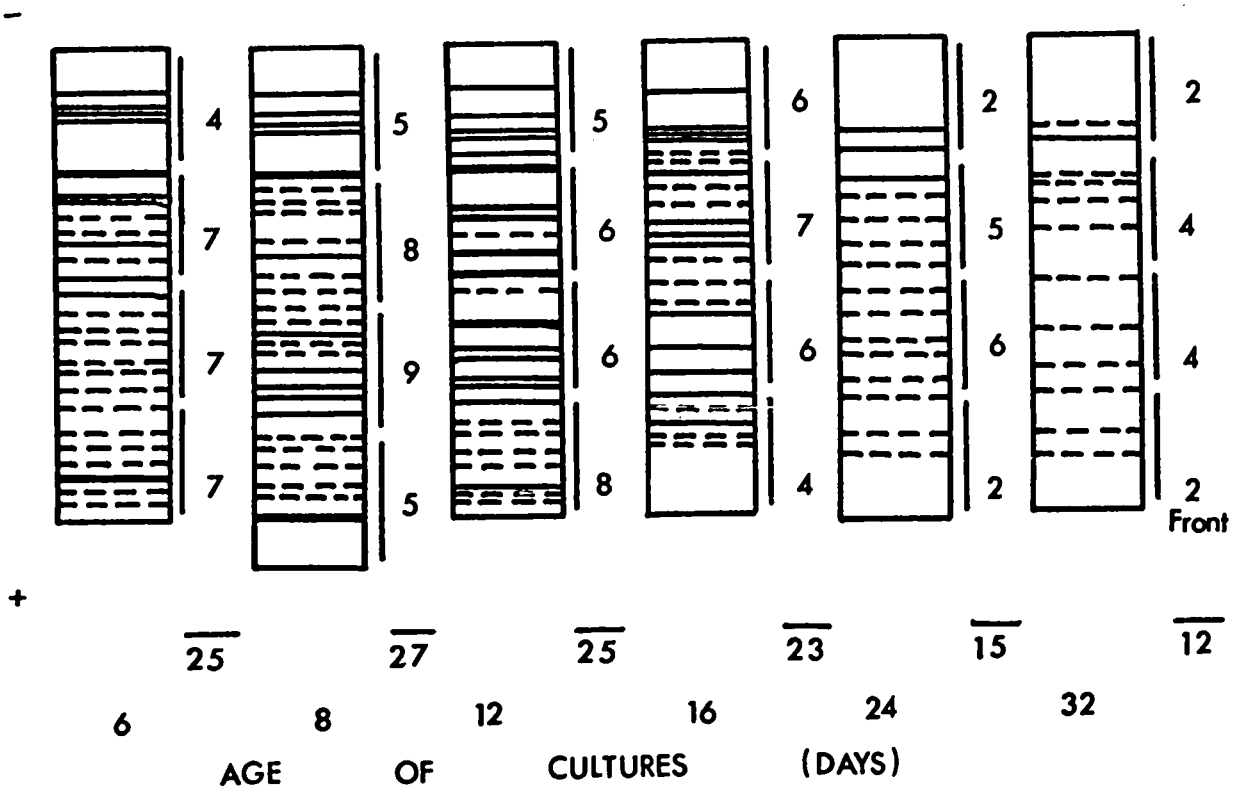

Figure 12. Gel electrophoretic patterns of soluble proteins from mycelium of ageing cultures of a low-temperature basidiomycete, isolate W_2, grown on synthetic medium in liquid shake cultures incubated at 15°C.

as compared to those harvested earlier.

Protein bands with intermediate migration rates, i.e. in the central quarters of the gels, also decreased in numbers but the decrease was not as great as in the first and fourth quarters.

(b) Protein patterns at 1°C

The numbers of protein bands obtained for cultures incubated at 1°C were 25, 29, 30, 30, 24, and 22 for 12-, 16-, 24-, 32-, 48-, and 56-day old cultures respectively.

The total numbers of bands in cell-free mycelial extracts were more often greater and sometimes the same as compared to cultures incubated at 15°C, and harvested at similar times after inoculation. For cultures incubated at 1°C there was only a slight decrease in the total numbers of protein bands for mycelium harvested at 48 and 56 days; whereas, the decrease was very sharp for 24- and 32-day old cultures incubated at 15°C. However, as at 15°C, the numbers of protein bands in each quarter on the gel varied. In the first quarter particularly, the numbers of protein bands of cell-free mycelial extracts for cultures harvested 48 and 56 days after inoculation decreased markedly (Fig. 13). However, when the fourth quarter of the gels are compared, there was no drastic change in numbers of bands of cell-free mycelial extracts from mycelium incubated at 1°C as compared to those incubated at 15°C, even though the harvestings of cultures were extended to 56 days after inoculation for cultures incubated at 1°C.

With regard to intensity of stain uptake, there were more sharply defined bands observed in the cell-free mycelial extracts from 48- and 56-day old cultures than observed for mycelium grown at 15°C and harvested at 24 and 32 days after inoculation.

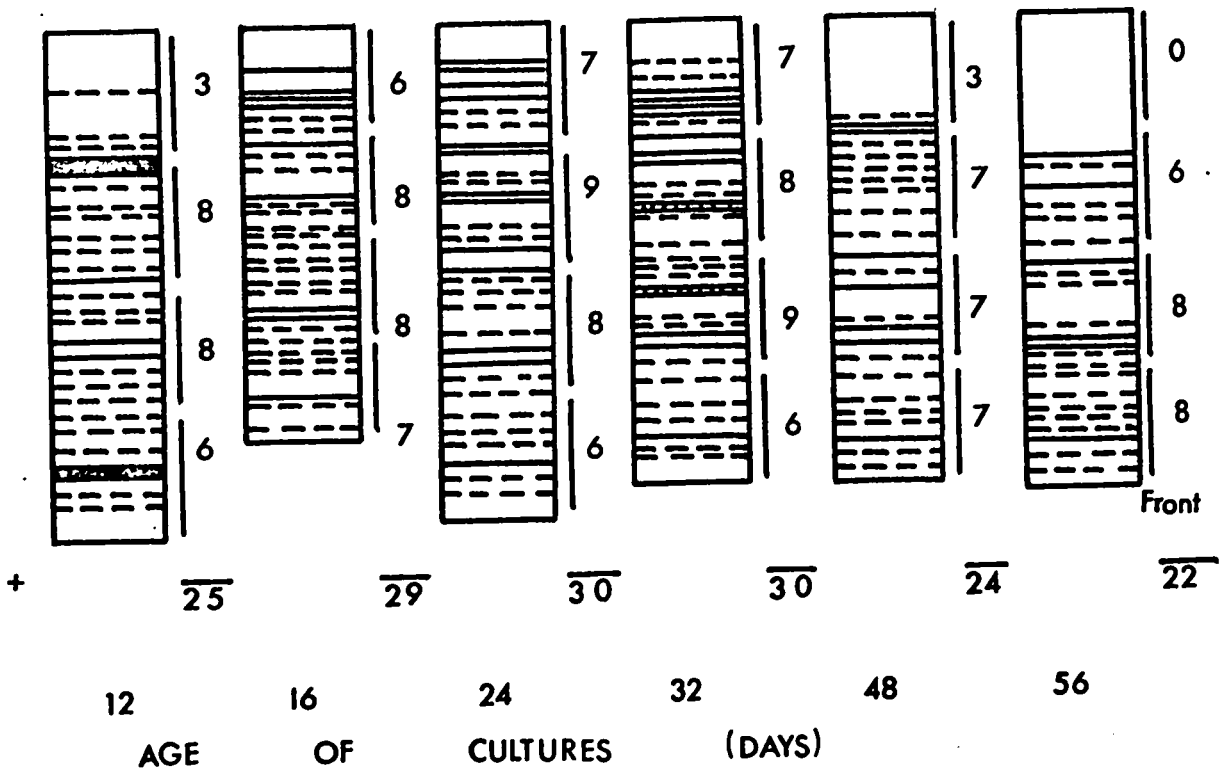

Figure 13. Gel electrophoretic patterns of soluble proteins from mycelium of ageing cultures of a low-temperature basidiomycete, isolate W_2, grown on synthetic medium in liquid shake cultures incubated at $1^{o}C$.

In the central quarters of the gels the most noticeable difference between cell-free extracts of mycelium grown at 15°C and 1°C is that, there were fewer heavily stained bands from mycelium grown at 1°C as compared to mycelium grown at 15°C for cultures harvested at 12 and 16 days after inoculation.

(c) Protein patterns at 15---10°C

The total numbers and patterns of protein bands in cell-free mycelial extracts of mycelium grown at 15---10°C are shown in Fig. 14. The numbers of bands observed were 21, 20, 24, 19, and 12 for cultures harvested 8, 12, 16, 24, and 32 days after inoculation. The numbers of protein bands decreased rapidly as found in 24- and 32-day old cultures. A similar decrease in band number was also found for 24- and 32-day old cultures incubated at 15°C. For 8- and 12-day old cultures the numbers of protein bands were lower than for corresponding cultures incubated at 15°C, but 16-day old cultures showed one more band than for cultures incubated at 15°C. The total number of protein bands in gels representing each harvest was generally the same or less than observed for cultures grown at 15°C with an exception to the cultures harvested 24 days after inoculation at 15---10°C, when more protein bands were noticed as compared to those cultures incubated at 15°C.

The distribution of protein bands on the gels differed from those of cultures incubated at 15°C. In the first quarter there were fewer bands than observed for cultures incubated at 15°C. Also, in the first quarter of the gels, there were more bands observed for cultures incubated at 15---10°C except on the twelfth day, at the time one-half the number of bands was observed as compared to those incubated at 15°C.

It should be noted that the pattern, in terms of distribution

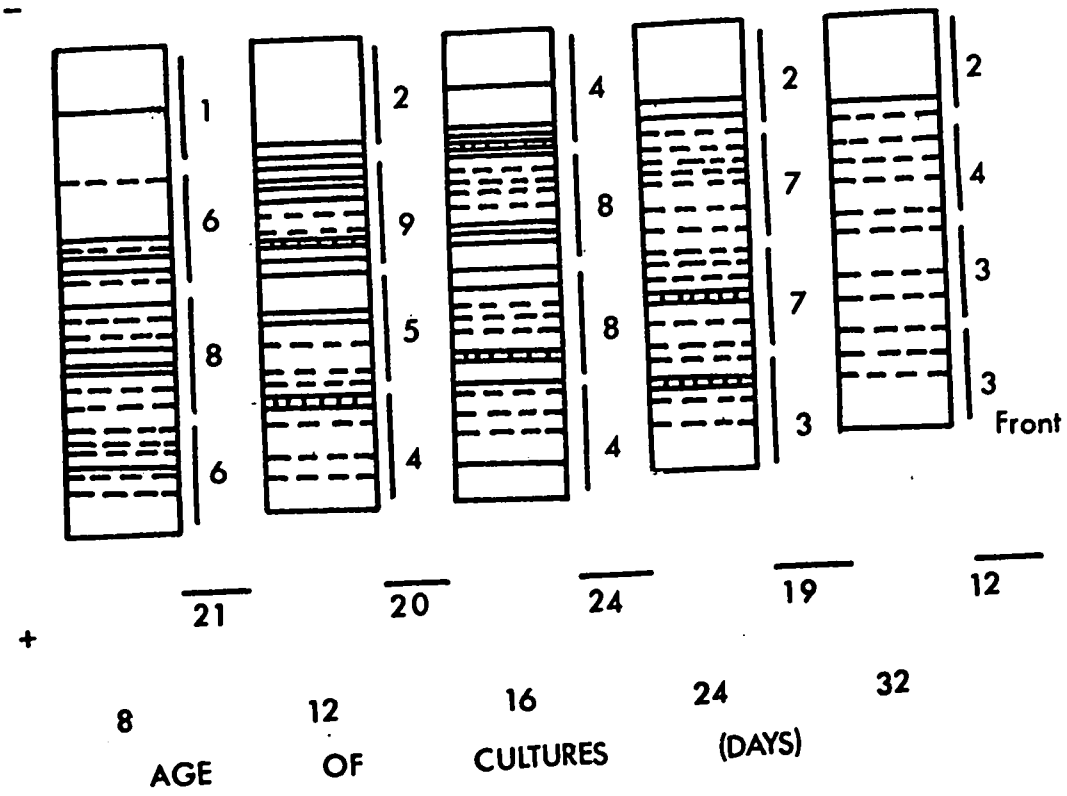

Figure 14. Gel electrophoretic patterns of soluble proteins from mycelium of a low-temperature basidiomycete, isolate W_2, grown on synthetic medium in liquid shake cultures incubated at 15---10°C.

of bands and stain uptake in each quarter, differed considerably from those observed for cultures grown at 15°C except for 32-day old cultures in which the distribution was very similar.

(d) Protein patterns at 15---1°C

The total numbers of protein bands observed for cultures incubated at 15---1°C and harvested 8, 12, 16, 24, and 32 days after inoculation were 19, 22, 25, and 14 respectively (Fig. 15). As already noted above, for the other cultures incubated at various temperatures, the pattern, i.e. numbers and distribution of bands and stain uptake in each quarter of the gel varied with age of culture. It should be noted that for the cultures incubated at 15---1°C, there were more protein bands for 16-, 24-, and 32-day old cultures as compared to those incubated at 15°C; however, there were fewer bands observed than for the corresponding cultures incubated at 1°C. In the younger cultures, i.e. 8 and 12 days, there were fewer bands observed than noted for mycelium grown at 15°C.

4. Effects of Temperature on Peroxidase Isozymes Extracted from Mycelium and Separated by Disc Gel Electrophoresis

Disc gel electrophoretic patterns for peroxidase isozymes, as indicated by staining with Benzidine. 2 HCl reagent, for cultures incubated at various temperatures are shown in Figs.16-19. The patterns changed with age of culture as well as with incubation temperatures. Peroxidase isozyme bands also showed variation in stain uptake. On the basis of stain uptake, bands were classified into three categories: sharp i.e. well-defined bands are represented by dark lines, faint bands by broken lines, and diffuse bands by diagonal lines. Isozymes generally appeared in the top and/or lower portions of gels, and their presence in central portions was rare.

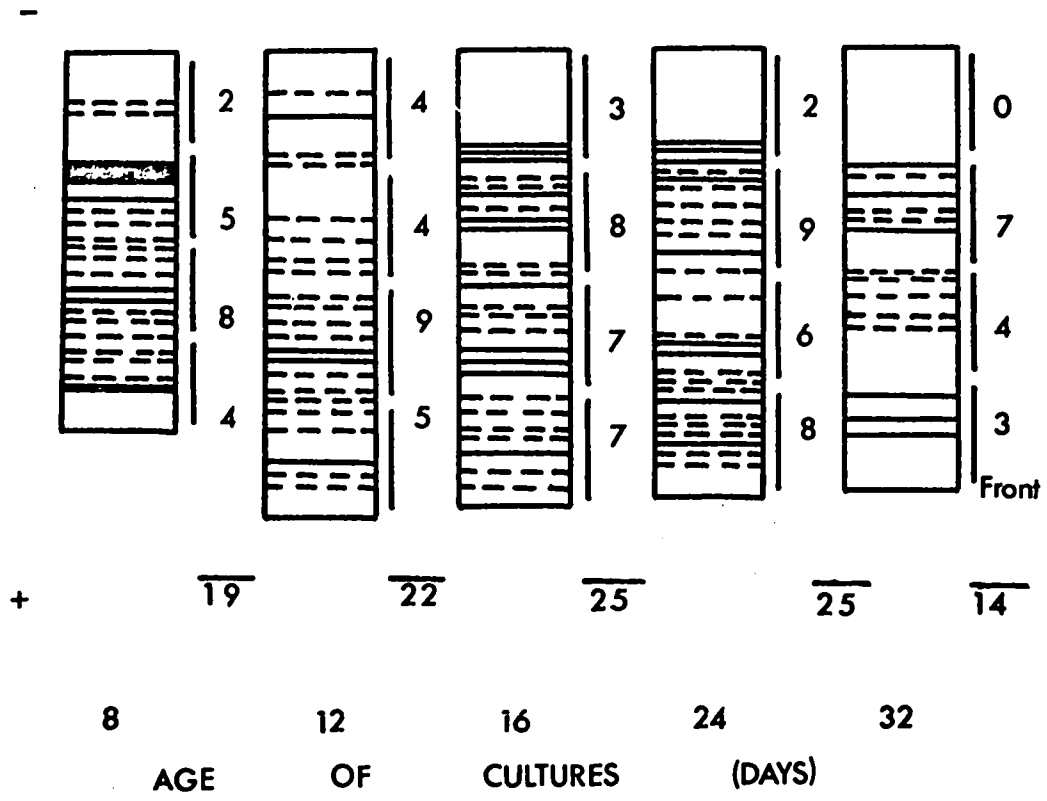

Figure 15. Gel electrophoretic patterns of soluble proteins from mycelium of a low-temperature basidiomycete, isolate W_2, grown on synthetic medium in liquid shake cultures incubated at 15---1°C.

Table 3 : Ef values of protein bands of various ages of cultures incubated at 15°C.

Quarters of Gel	Age of Cultures (days)					
	6	8	12	16	24	32
1	.09 .12 .13 .15	.09 .12 .14 .15 .24	.09 .15 .18 .2 .23	.09 .17 .18 .19 .22 .24	.18 .22	.18 .22
2	.26 .33 .35 .38 .41 .44 .48	.26 .29 .31 .36 .39 .43 .46 .49	.26 .34 .36 .40 .43 .48	.26 .29 .33 .36 .39 .40 .44 .49	.28 .32 .37 .41 .46	.29 .31 .35 .41
3	.52 .56 .59 .62 .66 .68 .72	.52 .55 .56 .57 .61 .64 .66 .69 .73	.51 .68 .64 .66 .70 .72 .75	.53 .56 .62 .68 .72 .75	.51 .56 .61 .65 .69 .73	.51 .62 .69 .75
4	.76 .81 .84 .87 .90 .93 .96	.76 .79 .82 .84 .88	.79 .83 .86 .89 .92 .94 .96	.78 .81 .82	.80 .84	.83 .88

Table 4 : Ef values of protein bands of various ages of cultures incubated at 1°C.

Quarters of Gel	Age of Cultures (days)					
	12	16	24	32	48	56
1	.12	.10	.07	.09	.21	
	.2	.16	.09	.12	.22	
	.23	.17	.12	.16	.24	
		.19	.15	.17		
		.22	.17	.18		
		.25	.21	.20		
			.25	.22		
2	.26	.28	.26	.26	.27	.30
	.30	.31	.30	.28	.30	.32
	.34	.35	.31	.31	.33	.36
	.36	.40	.34	.35	.34	.40
	.40	.42	.35	.38	.37	.43
	.43	.44	.41	.40	.41	.48
	.46	.48	.43	.42	.46	
	.49	.50	.46	.48		
			.50			
3	.53	.53	.51	.51	.51	.52
	.55	.56	.54	.53	.54	.55
	.56	.58	.57	.55	.57	.57
	.60	.62	.62	.58	.64	.65
	.63	.64	.66	.64	.66	.68
	.66	.68	.69	.66	.69	.70
	.69	.70	.71	.67	.72	.72
	.72	.73	.74	.71		.75
				.74		
4	.76	.76	.79	.77	.76	.80
	.78	.77	.82	.83	.81	.83
	.81	.80	.84	.86	.84	.85
	.86	.83	.88	.89	.86	.87
	.90	.89	.92	.93	.89	.90
	.93	.91	.95	.94	.93	.93
		.96			.95	.96

Table 5 : Ef values of protein bands of various ages of cultures incubated at 15---10°C.

Quarters of Gel	Age of Cultures (days) 8	12	16	24	32
1	.13	.23 .25	.12 .2 .22 .24	.18 .21	.21 .25
2	.27 .39 .41 .43 .46 .48	.27 .30 .31 .34 .37 .41 .43 .47 .50	.26 .28 .32 .34 .37 .41 .43 .45	.26 .28 .31 .32 .35 .41 .46	.3 .35 .40 .48
3	.53 .56 .59 .62 .65 .67 .70 .74	.58 .60 .65 .71 .72	.51 .54 .58 .61 .63 .67 .68 .74	.51 .53 .57 .61 .66 .71 .75	.52 .63 .68
4	.78 .81 .83 .86 .88 .91	.77 .82 .89 .94	.76 .81 .85 .91	.80 .84 .89	.76 .81 .90

Table 6 : Ef values of protein bands of various ages of cultures incubated at 15---1°C.

Quarters of Gel	Age of Cultures (days)				
	8	12	16	24	32
1	.12 .15	.08 .13 .22 .23	.20 .22 .23	.21 .22	
2	.31 .38 .41 .44 .48	.35 .40 .44 .47	.27 .29 .31 .34 .37 .38 .46 .48	.26 .27 .28 .31 .35 .38 .41 .45 .49	.26 .28 .33 .36 .38 .41 .50
3	.51 .54 .58 .62 .65 .67 .70 .75	.52 .55 .58 .61 .64 .66 .69 .72 .74	.51 .56 .57 .61 .65 .68 .70	.55 .64 .65 .68 .71 .73	.51 .55 .59 .63
4	.79 .81 .85 .88	.77 .81 .88 .90 .93	.76 .79 .82 .84 .87 .91 .94	.76 .78 .82 .83 .85 .87 .90 .92	.78 .83 .87

(a) Peroxidase isozyme patterns at 15°C

The numbers of peroxidase isozyme bands from cell-free mycelial extracts of mycelium at 15°C and harvested 6,8,12,16,24, and 32, days after inoculation were 11, 7, 4, 2, 4, and 3 respectively (Fig. 16). The maximum number of peroxidase isozyme bands was detected in 6-day old cultures. Only one isozyme band, represented by diagonal lines, was present in all gels except that which represents the 6-day old culture. In general, the number of isozymes in the lower portion of the gels disappeared as cultures aged. Well-defined sharp, diffuse zones, and/or faint bands were observed in extracts for mycelium harvested at later stages.

(b) Peroxidase isozyme patterns at 1°C

The isozyme patterns for cultures incubated at 1°C are presented in Fig. 17. The number of bands for cell-free mycelial extracts were 4, 8, 3, 4, 1, and 2 for the mycelium harvested 12, 16, 24, 32, 48, and 56 days respectively after inoculation. Two significant features noted for these gels were: (a) diffuse zones were present in extracts from mycelium harvested from 12 to 32 days after inoculation, and (b) almost complete absence of isozyme bands in extracts from mycelium harvested 48 and 56 days after inoculation.

(c) Peroxidase isozyme patterns at 15---10°C

The peroxidase isozyme patterns of mycelium grown at 15---10°C are shown in Fig. 18. The numbers of bands observed were 11, 11, 4, 6, and 2 for cultures harvested 8, 12, 16, 24, and 32 days respectively after inoculation. Peroxidase isozyme bands showed a variation in their stain uptake property. Several important features of these gels should be noted. In addition to there being more bands in the 15---10°C cultures than those incubated at 15°C, the distribution of bands is quite different

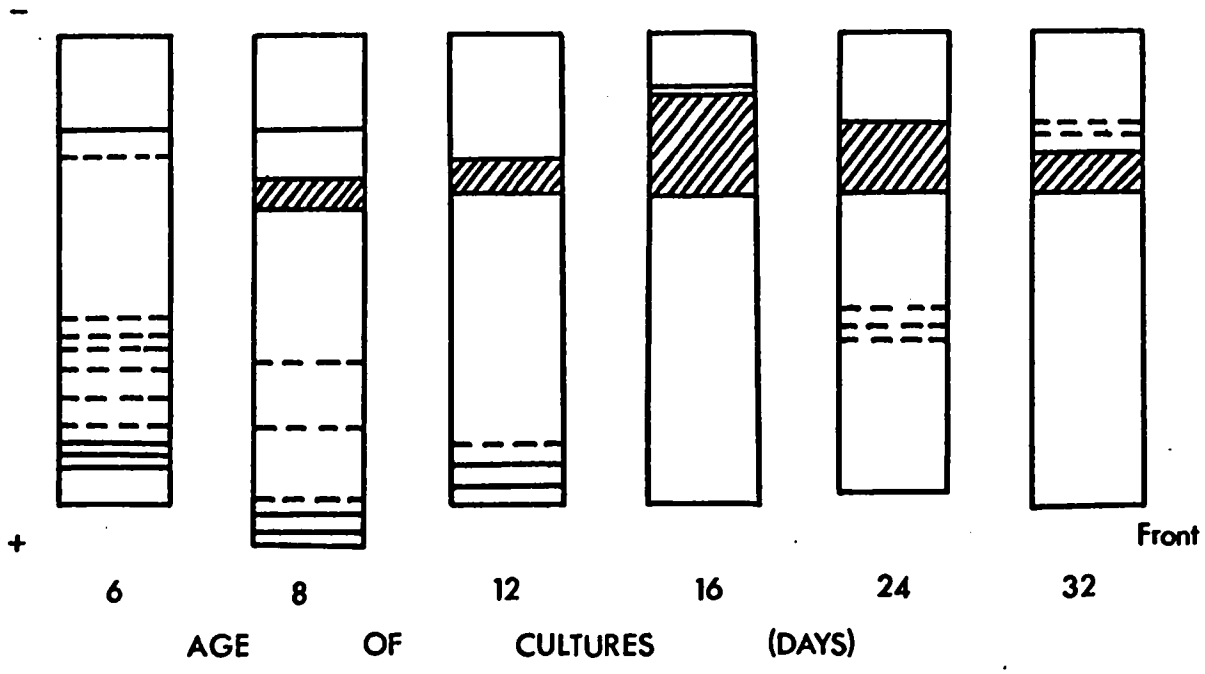

Figure 16. Gel electrophoretic patterns of peroxidase isozymes from mycelium of a low-temperature basidiomycete, isolate W_2, grown on synthetic medium in liquid shake cultures incubated at 15°C.

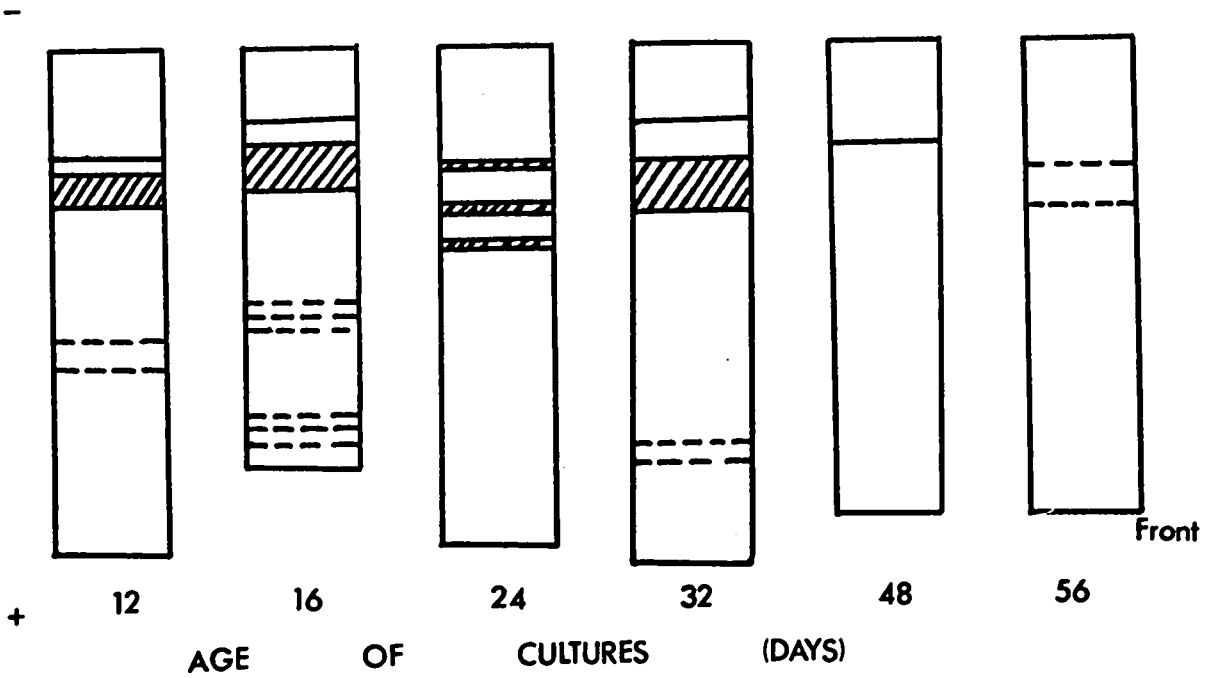

Figure 17. Gel electrophoretic patterns of peroxidase isozymes from mycelium of a low-temperature basidiomycete, isolate W_2, grown on synthetic medium in liquid shake cultures incubated at $1^{o}C$.

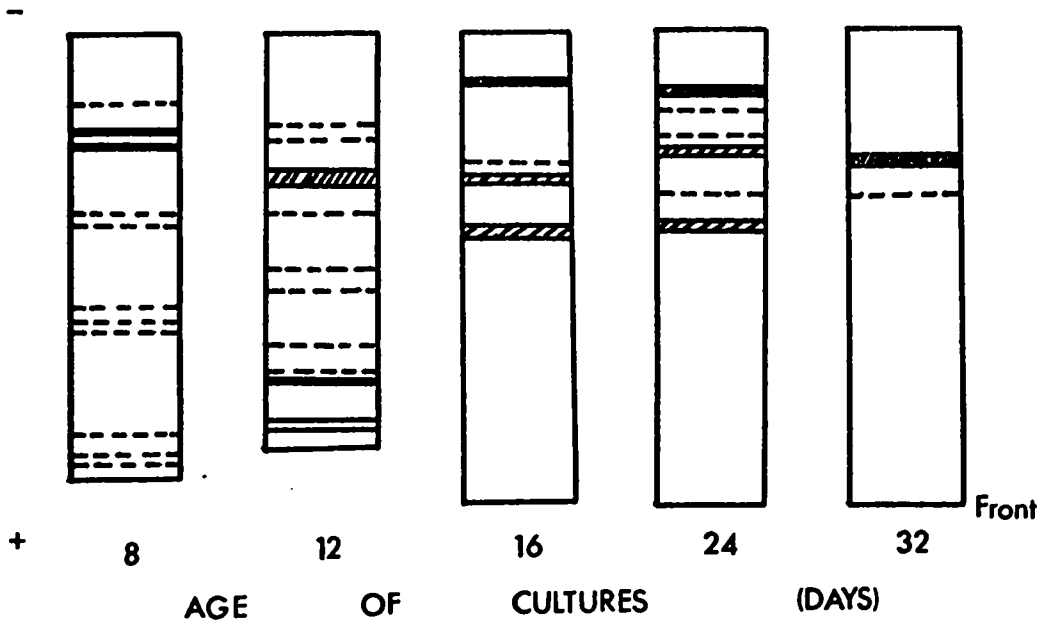

Figure 18. Gel electrophoretic patterns of peroxidase isozymes from mycelium of a low-temperature basidiomycete, isolate W_2, grown on synthetic medium in liquid shake cultures incubated at 15---10°C.

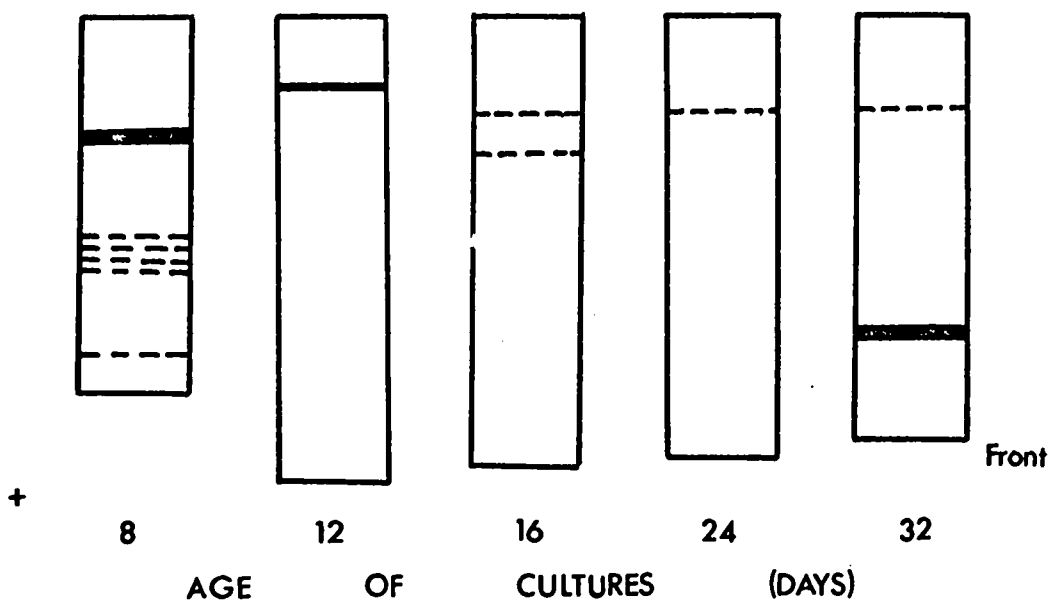

Figure 19. Gel electrophoretic patterns of peroxidase isozymes from mycelium of a low-temperature basidiomycete, isolate W_2, grown on synthetic medium in liquid shake cultures incubated at 15---1°C.

in the 8- and 12-day old cultures. As noted for 16-, 24-, and 32-day old cultures incubated at 15---10°C, there were no bands present in the lower portion of the gels. The wide diffuse zones observed on gels for cultures incubated at 15°C and harvested from 8 to 32 days after inoculation were very narrow for cultures incubated at 15---10°C.

(d) Peroxidase isozyme patterns at 15---1°C

The peroxidase isozyme patterns for the cultures incubated at 15---1°C are shown in Fig.19. The number of isozyme bands detected were 6, 1, 2, 1, and 2 from the cell-free mycelial extracts of the mycelium grown at 15---1°C and harvested 8, 12, 16, 24, and 32 days respectively after inoculation. The maximum number of bands were observed for mycelium harvested from 8-day old cultures, and for subsequent harvestings there was either one or two bands present. The total numbers of bands were less than for cultures incubated at 15°C, 15---10°C, and 1°C. The diffuse zone was not evident in cultures incubated at 15---1°C as noted for cultures incubated at the other temperatures. In 32-day old cultures there was a wide well-defined band in the lower portion of the gel which was not evident in any of the other gels regardless of age of cultures or incubation temperatures.

C. Protease Activity

The above results for protein patterns, using disc gel electrophoresis, indicated fewer protein bands in the mycelial extracts from older cultures as compared to younger cultures. This decrease in number of bands was thought to be due to protein breakdown. In order to determine a cause for proteolysis, protease studies were carried out.

Protease activities of cell-free mycelial extracts of mycelium grown at 15°C and 1°C, and harvested 8, 12, 16, 24, and 32 days after

inoculation respectively are shown in Fig 20. Two additional harvests were made at 48 and 56 days after inoculation for cultures incubated at 1°C. The activity for protease at 8 days after inoculation was not determined at 1°C, due to the slow growth of fungus. For the cultures incubated at 15°C, maximum protease activity was reached 12 days after inoculation. Up to this stage i.e. 8 days, there was considerable protease activity and after 12 days a rapid decline occurred.

Protease activity was approximately 25 percent higher in cultures incubated at 1°C as compared to those incubated at 15°C, when maximum activities are compared. For cultures incubated at 1°C, maximum protease activity was obtained between 24 and 32 days after inoculation, whereas, it occurred at 12 days for mycelium grown at 15°C. Protease activity for 12-day old cultures incubated at 1°C was approximately one-half that for cultures incubated at 15°C. In the 32-day old cultures, incubated at 1°C, protease activity was approximately three times as great as that for culture incubated at 15°C. Even for 48- and 56-day old cultures incubated at 1°C, protease activity, was higher than that for 32-day old cultures incubated at 15°C.

D. Total RNA - RNase Activity

The results for total RNA and RNase determinations of cell-free mycelial extracts for cultures incubated at 15°C and 1°C, and harvested 8, 12, 16, 24, and 32 days after inoculation respectively are shown in Table 8. As in other phases of these studies, determinations were done on mycelium harvested 48 and 56 days after inoculation due to the slow growth of the fungus at 1°C.

1. Total RNA

The maximum value for total RNA was obtained in 8-day old cultures for mycelium grown at 15°C; whereas, for cultures incubated at 1°C,

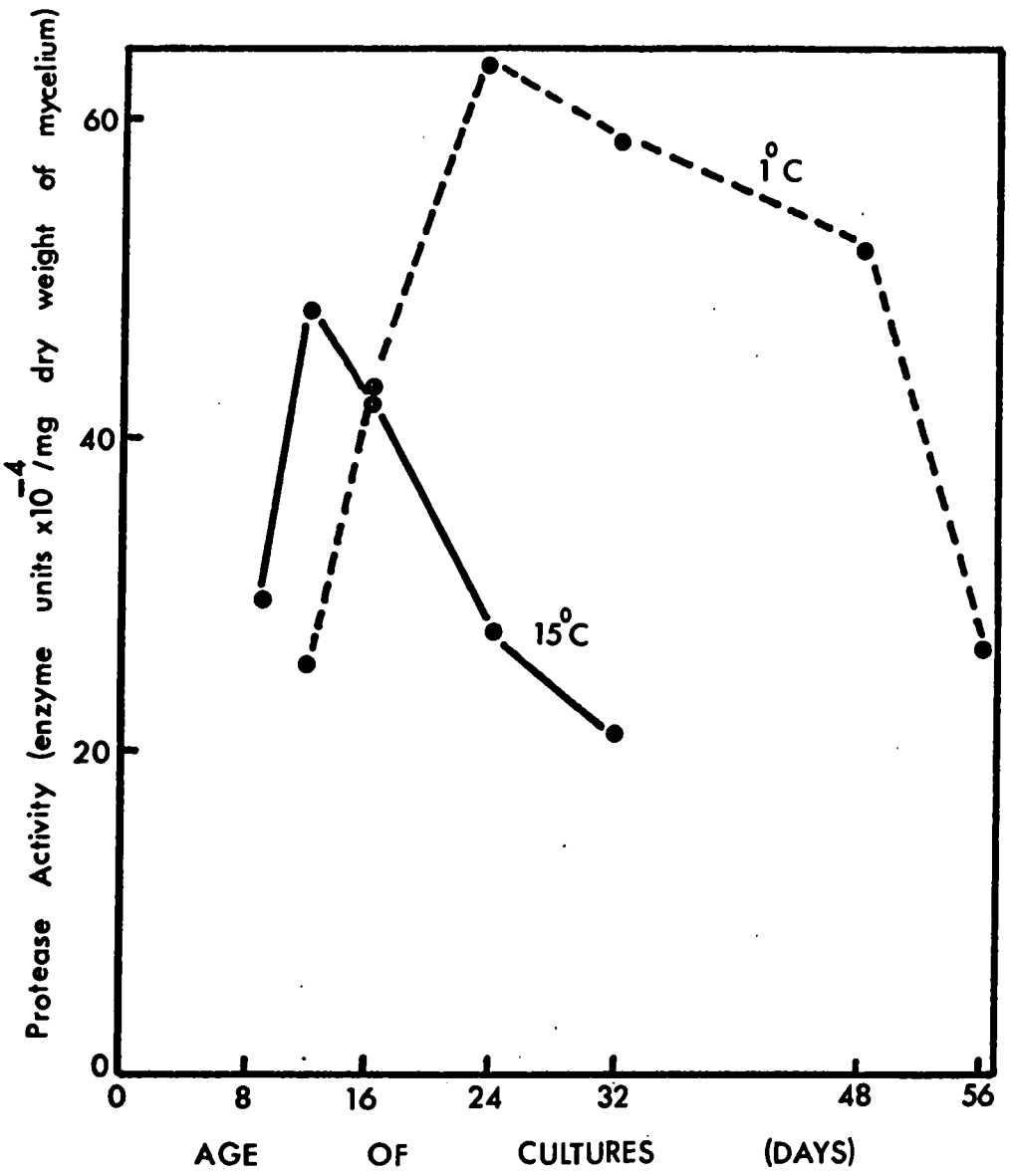

Figure 20. Protease activity of cell-free mycelial extracts from mycelium of ageing cultures of a low-temperature basidiomycete, isolate W_2. Cultures were grown on synthetic medium in liquid shake flasks incubated at 15°C and 1°C.

the maximum value was obtained 32 days after inoculation. It should be noted that the 8-day old cultures, incubated at 15°C were the first to be harvested. There was a small decrease in the total RNA value for the 12-day old cultures, followed by a rapid decline, and then levelling off or small drop in the older cultures. In the younger cultures incubated at 1°C, there were low levels of total RNA followed by a sharp increase for 32-day old cultures. There was a gradual decline in RNA levels for cultures harvested at 48 and 56 days after inoculation.

Table 7 : Amounts of DNA in the preparations used for RNA determinations for ageing cultures of a low-temperature basidiomycete, isolate W_2 incubated at 15°C and 1°C.

Age of Cultures (days)	DNA (mg /g dry weight of mycelium) 15°C	1°C
8	0.29	--
12	0.30	0.25
16	0.15	0.33
24	0.17	0.25
32		0.26
48		0.96
56		0.50

Table 8 : Total RNA - RNase activity of cell-free mycelial extracts from mycelium of ageing cultures of a low-temperature basidiomycete, isolate W_2. Cultures were grown using a synthetic medium in liquid shake culture at 15°C and 1°C.

	Incubation Temperature 15°C		1°C	
Age of Cultures (days)	RNA (mg/g dry weight of mycelium)	RNase activity (units/g dry weight of mycelium)	RNA (mg/g dry weight of mycelium)	RNase activity (units/g dry weight of mycelium)
8	9.83	11066		
12	8.58	13230	4.05	1146
16	5.75	16405	5.85	2421
24	6.06	12636	5.73	11461
32	4.07	5799	14.07	21308
48			9.32	13107
56			8.55	17177

(a) Note: The amounts of DNA present in RNA preparations from cultures incubated at 15°C and 1°C are shown in Table 7. These results indicate that the total RNA preparations were almost free of DNA.

2. RNase activity

The maximum activities for RNase were obtained for 16- and 32-day old cultures incubated at 15°C and 1°C respectively. The value for 32-day old cultures, incubated at 1°C, was considerably higher (approximately 25 percent) than the maximum obtained for the 16-day old cultures incubated at 15°C. It should be noted that RNase activity of 16-day old cultures incubated at 1°C was approximately twice that of 12-day old cultures, but the activity for 24-day old cultures was approximately five times that for the 16-day old cultures. RNase values for 48- and 56-day old cultures were approximately two-thirds that of the 32-day old cultures.

For cultures incubated at 15°C, the RNase activity of the 32-day old cultures was approximately one-third that of the maximum which was obtained for the 16-day old cultures. RNase activities for the other harvests, i.e. at 8, 12, and 24 days, were approximately three quarters that of the 16-day old cultures.

E. HCN Studies

The results for HCN released from cultures, β-glucosidase activity of culture medium and cell-free mycelial extracts, and 'free' HCN from culture medium and cell-free mycelial extracts, are presented.

1. HCN release from cultures

The amounts of HCN (p.p.m./flask) released by the cultures incubated at 15°C, 1°C, 15---10°C, and 15---1°C, and harvested at 8, 12, 16, 24, and 32 days respectively after inoculation are presented in Fig. 21.

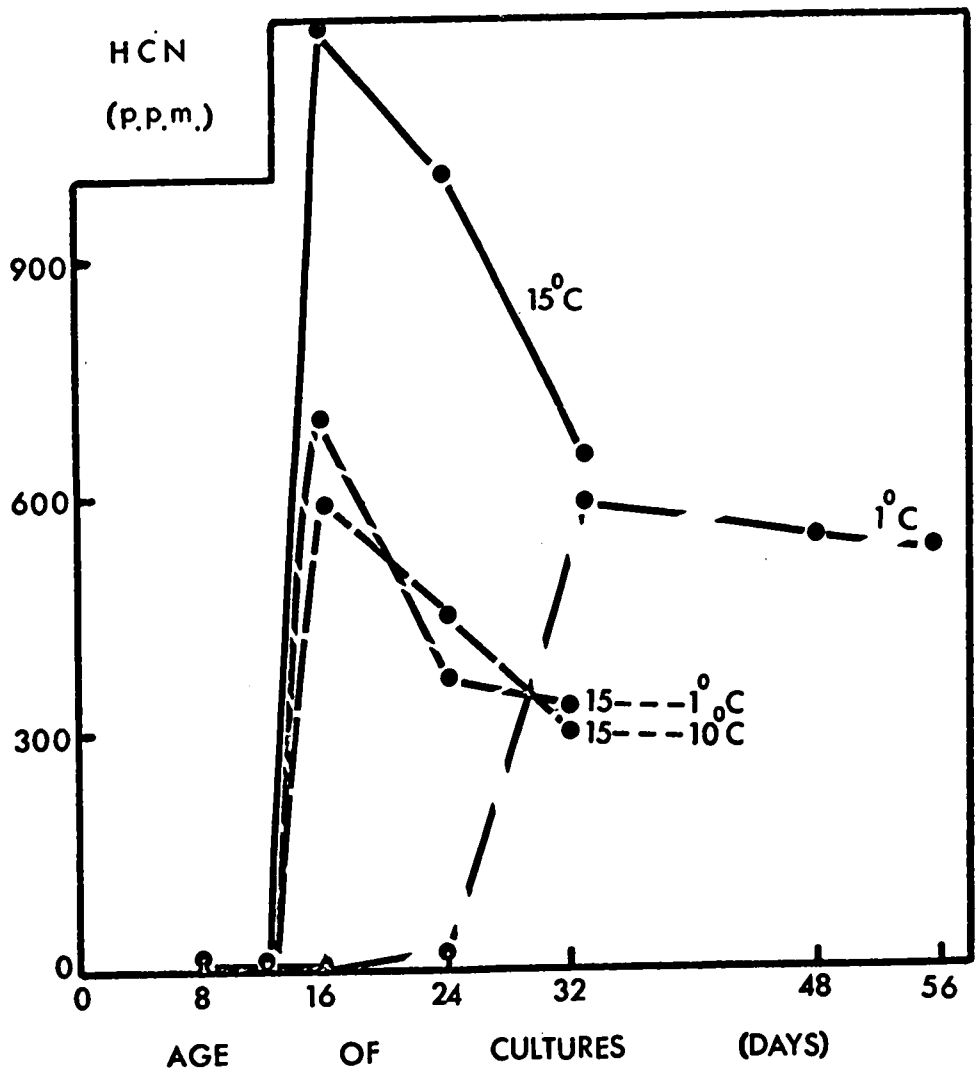

Figure 21. Hydrogen cyanide release by ageing cultures of a low-temperature basidiomycete, isolate W_2, grown on a synthetic medium in liquid shake culture in a 24-hour period prior to harvest at various incubation temperatures (Cultures incubated at 15°C and transferred to 10°C (15---10°C) and 1°C (15---1°C) respectively three days prior to harvest).

For cultures incubated at 1°C, two additional harvests at 48 and 56 days after inoculation were made due to the slow growth of the fungus.

At 15°C, 15---10°C, and 15---1°C the maximum amounts of HCN collected over a 24-hour period occurred in 16-day old cultures. For cultures incubated at 1°C the peak was reached 32 days after inoculation. HCN was not detected for cultures incubated at 15°C, 15---10°C, and 15---1°C, and harvested at 8 and 12 days. For cultures incubated at 1°C, HCN was first detected in 24-day old cultures. It should be noted that the maximum amounts of HCN collected for 16-day old cultures incubated at 15---1°C, and 32-day old cultures at 1°C, were approximately the same. Accompanying a decrease in incubation temperature there was a corresponding decrease in HCN (at 15°C, 15---10°C, and 15---1°C), and once started, this decline was very rapid. After the maximum levels had been reached, the amounts of HCN collected were much lower than the peak values except for cultures incubated at 1°C. At 1°C, the decrease in HCN, after the maximum was reached at 32 days after inoculation, was very slight for cultures harvested 48 and 56 days after inoculation.

Table 9 : Release of HCN from cultures of a low-temperature basidiomycete, isolate W_2 incubated at 15°C, and harvested from 13 to 17 days after inoculation.

Age of Cultures (days)	HCN (p.p.m./flask)
13	9.16
14	30.75
15	492.71
16	1138.70
17	969.75

When the measurements for HCN for cultures incubated at 15°C were carried out on a daily basis from 13-17 days, to elaborate on that interval

at which HCN is first released, it was found that HCN was first released 13 days after inoculation (Table 9). After 14 days, the rate of HCN evolved was very rapid, reaching a peak value at 16 days, following which, there was a sharp decline.

2. β-glucosidase and 'free' HCN studies

(a) β-glucosidase

The results for β-glucosidase activity in culture medium and cell-free mycelial extracts of mycelium grown at 15°C and 1°C respectively, and harvested 8, 12, 16, 24, and 32 days after inoculation are shown in Fig. 22. Due to a continual increase in β-glucosidase activity in mycelial extracts of mycelium harvested up to 32 days after inoculation at 1°C, and also due to the slow growth of the fungus at this temperature, it was found necessary to carry out additional harvestings at 48, 56, and 72 days after inoculation.

At 15°C, β-glucosidase activities were highest in culture medium and cell-free mycelial extracts of cultures harvested 16 days after inoculation. However, for cultures incubated at 1°C, the maximum β-glucosidase activities for culture medium and cell-free mycelial extracts were found in 24- and 56-day old cultures respectively.

In the studies carried out at daily intervals from 13-17 days after inoculation, for the cultures incubated at 15°C, β-glucosidase activity reached a maximum in the culture medium and cell-free mycelial extracts of 16-day old cultures (Table 10). These studies agree with the results reported above for those determination which were carried out for longer intervals. Cultures harvested 17 days after inoculation showed a drop in β-glucosidase activity both for culture medium and cell-free mycelial extracts.

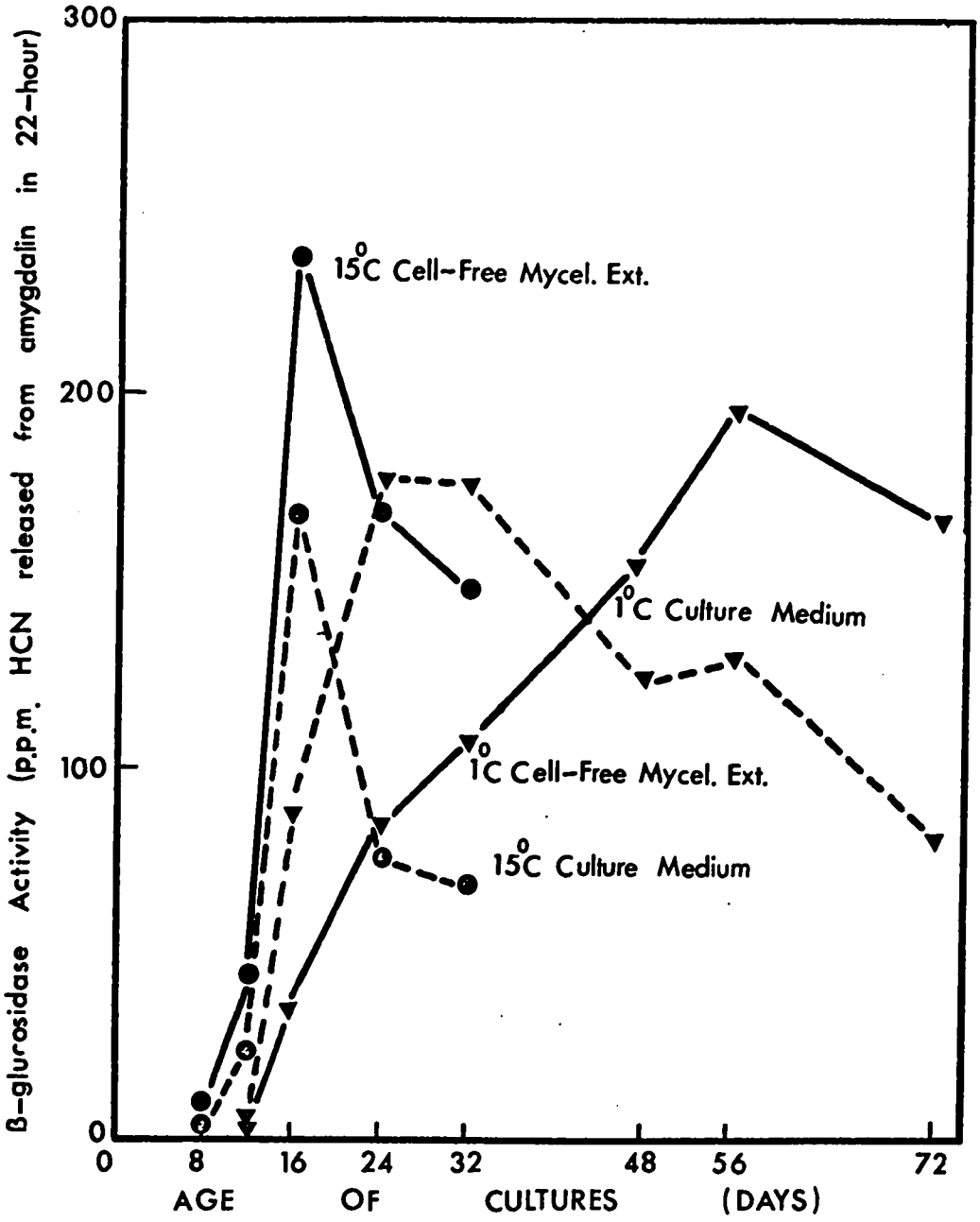

Figure 22. β-glucosidase activity in terms of HCN released from amygdalin in a 22-hour period, of culture medium (1.0 ml) and cell-free mycelial extracts (10 mg equivalent dry wt. of mycelium) of a low-temperature basidiomycete, isolate W_2, in ageing cultures incubated at 15°C and 1°C.

Table 10: β-glucosidase activity, in terms of HCN released from amygdalin in a 22-hour period, of culture medium and cell-free mycelial extracts of a low-temperature basidiomycete, isolate W_2. Cultures grown on a synthetic medium in liquid shake flasks at 15°C were harvested from 13 to 17 days after inoculation.

	β-glucosidase activity	(release of HCN in p.p.m.)
Age of cultures (days)	Culture medium (1.0 ml)	Cell-free mycelial extracts (10 mg equivalent dry weight of mycelium)
13	23.98	49.60
14	126.21	200.90
15	158.12	188.90
16	162.54	235.20
17	114.16	202.10

(b) 'free' HCN

The values for 'free' HCN from autoclaved and non-autoclaved fractions of culture medium and cell-free mycelial extracts for cultures incubated at 15°C and 1°C are presented in Table 11.

Results for 'free' HCN for non-autoclaved culture medium of cultures grown at 15°C indicate that HCN was first detected for 16-day old cultures and reached a maximum level 24 days after inoculation. For cultures incubated at 1°C, 'free' HCN in the culture medium, was not detected until after 16 days at which time 2 p.p.m. were noted for the 24-day old cultures.

At 32 days, the value for 'free' HCN was seven times greater than that obtained at 24 days. For 48-, 56-, and 72-day old cultures the values for HCN were approximately one-half that obtained for cultures incubated at 15°C and harvested 24 and 32 days after inoculation.

For the non-autoclaved cell-free mycelial extracts, for cultures incubated at 15°C, 'free' HCN was first detected 16 days after inoculation (Table 11), and the maximum value obtained for 24-day old cultures. The

'free' HCN value, for cultures harvested at 32 days were approximately one-third less than that obtained at 24 days. A 1°C, 'free' HCN was first detected 32 days after inoculation and the values obtained were very low as compared to those obtained for 24-, and 32-day old cultures incubated at 15°C.

The trend of 'free' HCN from autoclaved fractions of culture medium and cell-free mycelial extracts was similar to those for non-autoclaved aliquots. However, the variations which correspond to lower values for autoclaved fractions may be due to a loss of the volatile HCN during autoclaving, but in some of the determinations the difference in values between autoclaved and non-autoclaved fraction was very slight.

In the studies concerning 'free' HCN released from non-autoclaved and autoclaved fractions of culture medium and cell-free mycelial extracts which made on a basis from 13 to 17 days after inoculation for the cultures incubated at 15°C (Table 12), the first detection of 'free' HCN in the non-autoclaved culture medium was made 14 days after inoculation. In the non-autoclaved cell-free mycelial extracts 'free' HCN was noticed 15 days after inoculation. It should be noted that the average value for 'free' HCN released on a daily basis from cell-free mycelial extract was approximately three times higher than that obtained when cultures were harvested at 4-, and 8-day intervals (Table 11). These results were consistent for repeated experiments. In the autoclaved fractions 'free' HCN was released and the amounts collected were complimentary to those for the representing non-autoclaved fractions. In these experiments the values were slightly lower than for the non-autoclaved fractions and this may have been due to a loss of HCN during autoclaving.

Table 11: 'Free' HCN released from non-autoclaved and autoclaved fractions of culture medium and cell-free mycelial extracts of mycelium from ageing cultures. of a low-temperature basidiomycete, isolate W_2. Cultures were grown on a synthetic medium in liquid shake flasks at 15°C and 1°C.

Age of Culture (days)	'Free' HCN (p.p.m.) 15°C				1°C			
	Culture medium (1.0 ml)		Cell-free mycelial extracts (10 mg equivalent dry weight of mycelium)		Culture medium (1.0 ml)		Cell-free mycelial extracts (10 mg equivalent dry weight of mycelium)	
	non-autoclaved	autoclaved	non-autoclaved	autoclaved	non-autoclaved	autoclaved	non-autoclaved	autoclaved
8	--		--	--				
12	--		--	--	--	--	--	--
16	24.80	23.85	1.70	0.95	--	--	--	--
24	61.80	63.50	13.20	7.60	2.10	1.25	--	--
32	60.90	65.07	8.90	2.94	15.70	3.75	2.60	--
48					28.60	20.50	2.40	0.88
56					28.10	15.50	1.70	--
72					26.50	19.06	2.20	0.87

Table 12: 'Free' HCN from non-autoclaved and autoclaved fractions of culture medium and cell-free mycelial extracts of mycelium in liquid shake cultures of a low-temperature basidiomycete, isolate W_2. Cultures grown on a synthetic medium at 15°C were harvested from 13 to 17 days after inoculation.

Age of Culture (days)	'Free' HCN (p.p.m.)			
	Culture medium (1.0 ml)		Cell-free mycelial extracts (10 mg equivalent dry weight of mycelium)	
	non-autoclaved	autoclaved	non-autoclaved	autoclaved
13	--	--	--	--
14	2.50	2.18	--	--
15	3.90	3.12	2.30	--
16	24.50	20.00	5.00	2.66
17	37.50	24.06	5.70	2.78

DISCUSSION

The cultural characteristics of a number of fungi, including some plant pathogens, which grow at low-temperatures, have been reviewed by Deverall (1968); however, studies on the physiology and biochemistry of low-temperature plant pathogens have been relatively neglected (Farrell and Rose 1967). Deverall (1968) considered that psychrophilic fungi are those which have an optimum temperatures for growth near 10°C or below. Broadfoot and Cormack (1941) reported the cardinal temperatures for a low-temperature basidiomycete, which was later shown to produce HCN in cultures and diseased plants (Lebeau and Dickson 1953, 1955, Ward et al.1961, and Ward and Lebeau 1962), to be -4, 15, and 26°C (minimum, optimum, and maximum) on a nutrient medium; however, the cultural technique was not given. In the liquid static cultures, using complex and synthetic media, greater amounts of mycelium were produced at 12°C (Lebeau and Dickson 1955). Other workers (Ward et al.1961) did not find a significant difference in growth of isolate W_2 in test tubes incubated between 12.5 to 17°C. Also, in the same studies, these workers observed very little growth at 0°C over a 27-day incubation period. Most of the other reports for this fungus have been concerned with cyanide studies (Lebeau and Dickson 1953, 1955, 1959; Ward and Lebeau 1962, Ward 1964b, Strobel 1964, Ward and Thorn 1967, Strobel 1967, and Stevens and Strobel 1968).

The above workers considered the optimum temperature for growth to be near 15°C using various media and different cultural techniques. Most of the growth (Ward and Colotelo 1960, and Ward and Lebeau 1962) and cyanide studies (Ward and Lebeau 1962, and Ward and Thorn 1966) were carried out with liquid shake cultures using a synthetic medium. However, in their cyanogenesis studies, Ward (1964b) used malt yeast extract

in liquid shake cultures, and Stevens and Strobel (1968) employed the synthetic medium in liquid static cultures, at 15°C and 13°C respectively. In this thesis, the yields of mycelium for cultures incubated at 1°C were found to be as much, and slightly higher, as those for cultures incubated at 15°C using the synthetic medium which was also used by Ward and Colotelo (1960), Ward and Lebeau (1962), Ward and Thorn (1967), Strobel (1967), and Stevens and Strobel (1968).

It should be noted that at 1°C, there is a considerable lag period prior to rapid growth regardless of the cultural technique used. The growth in petri dish and liquid static cultures was comparatively slower at 1°C to that of cultures incubated at 15°C. Therefore, the response of the fungus varied with the incubation temperature and culturing technique, even when one defined medium was employed. The previous workers overlooked the criterion of using one medium for their growth experiments employing various culture techniques to define optimum temperature for growth. Various culture techniques, different media, and incubation temperatures would be required to determine the optimum growth temperature. Previous findings, and present studies as well did not meet above requirements fully.

On the underside of mycelial mats of liquid static cultures can be seen a gelatinous substratum which could not be removed by washing with water or other means i.e. scraping, without removing some of the mycelial hyphae embedded in it. In determining the amounts of mycelium produced, as fungal mats in liquid static cultures, previous workers (Lebeau and Dickson 1955, Ward and Colotelo 1960, Strobel 1964, and Stevens and Strobel 1968) had not mentioned the association of mycelium and gelatinous substratum; therefore, their values and those presented in

Fig. 8 for dry weights of mycelium would be an excess by the amount(s) of gelatinous material present. It was also impossible to determine whether the gelatinous material is produced in petri dish cultures. The results for dry weight of mycelium in liquid shake culture (Fig. 9 and Table 1) represent more accurately the yield of mycelium, because, the gelatinous material coating on the mycelium can be removed, to a large degree, by washing with water. Colotelo (unpublished data) determined the amounts of the gelatinous material of culture medium for cultures of various age, but the nature of this material was not determined. Martin and Adams (1956) carried out a survey of fungal polysaccharides found in culture medium.

The present studies indicated that the loss in dry weight or autolysis began soon after the maximum growth occurred for the cultures incubated at 15°C. The loss in dry weight was comparatively slower for the mycelium grown in liquid shake cultures at 1°C. Farrell and Rose (1965) mentioned that at temperatures below the optimum for growth, for some low-temperature bacteria, nutrients, particularly sugars, are chanelled less into catabolic pathways, and to a greater extent into pathways that lead to the synthesis of storage products. The growth curves as related to the change in temperature followed the same trend as noted for cultures incubated at 15°C. These experiments showed that the change of temperature did not affect the dry weights of the mycelium appreciably, especially during the autolytic phase when cultures were transferred from 15°C to lower temperatures three days prior to harvesting.

Studies on the effects of changes in incubation temperature of a low-temperature yeast have received considerable attention (Hagen and Rose 1962). However, this approach in the study of growth of low-temperature fungi has generally not been used.

Autolysis of the mycelium was associated with an increase in pH values both in culture medium and mycelium. There was a decrease in pH of the culture medium during the rapid growth phase but an increase in pH of non-buffered mycelial sap and culture medium during the autolytic phase. The decrease in dry weight or autolysis may be considered due to the lack of amino nitrogen in the medium. Leal et al. (1967) demonstrated that asparagine at the rate of 2 g/l was completely deamidated and deaminated by Phythophthora hevae in culture medium 11 days after inoculation, and also, the autolysis of mycelium was more rapid at the above concentration. However, no studies on the amounts or nature of nutrients in the culture medium were carried out. Leal et al. (1967) during their course of investigations found a similar trend in pH values as noted for these studies.

For cultures incubated at 15°C and 1°C, there was an increase in total proteins (of cell-free extracts not precipitated with ammonium sulphate) as measured by the method of Lowry et al. (1951), even after a decrease in dry weight of mycelium was noticed. This may be due to first, some specific proteins or specific non-protein compounds which are responsible for higher colour development when reacted with Folin reagent. Lowry et al. (1951) stated that tyrosine, tryptophane, and some non-protein compounds i.e. uric acid, xanthine, and guanine, when reacted with Folin phenol reagent, resulted in higher colour development. The presence of these compounds which gives higher colour development may

account for the increase in amounts of mycelium from 5 to 8 g fresh weight, required to obtain discernible protein bands on the electrophoretic gels for extracts from mycelium harvested 32 and 56 days. Hall (1967) also observed that the protein patterns of the gels containing the extracts from his oldest cultures were weakly stained and poorly resolved as compared to younger cultures. Secondly, the total protein content increased in ageing cultures even though there was a decrease in dry weight of mycelium. However, the electrophoretic studies clearly showed that in the autolysing cultures, there was a decrease in numbers of protein bands.

Higher total protein values were obtained from the cultures incubated at 1°C, as compared to those incubated at 15°C (Fig.10), and this suggests that more proteins were synthesized at a lower temperature. Gerloff et al. (1967) observed that, on a dry weight basis, the soluble protein content of alfalfa roots increased during hardening. Electrophoretic studies indicated that during the early phase of growth, cultures incubated at 15°C and transferred to lower temperatures, protein synthesis was not as rapid as noted for the cultures incubated only at 15°C. Therefore, it would appear that the lower temperatures were responsible for maintaining and/or increasing protein levels or that the rate of protein breakdown was slower.

On the basis of age for cultures incubated at any one temperature, direct correlation can not be made between total protein values (as obtained by using the method of Lowry et al. 1951) and those for protein extracts which were used for the electrophoretic studies. Data presented by Gottlieb and van Etten (1966) indicated a decrease in protein content and an increase in carbohydrates on per unit dry weight basis with an increase in age of cultures of Rhizoctonia solani and Sclerotium

bataticola grown at 26°C.

In the cultures incubated at 15°C, the disc electrophoresis studies indicated that the maximum numbers of protein bands were present in growing cultures. At this temperature there were fewer protein bands present and this coincided with a decrease in the dry weight of mycelium. This decrease in numbers of bands was quite apparent in older cultures i.e. 24 and 32 days after inoculation. For those cultures incubated at 1°C, the maximum numbers of protein bands were detected only after the maximum yield of mycelium was obtained. Also, in older cultures incubated at 1°C, there was a slower breakdown and/or disappearance of protein bands as compared the older cultures incubated at 15°C. It may be considered that the first decrease in dry weight, after the maximum was reached, was not due to a decrease in protein content, but may be due to the disappearance of other compounds. As regards to temperature effects, it is clear that the breakdown of proteins, as evident from the electrophoretic studies, was much slower for cultures incubated at 1°C, as compared to those for 15°C. These changes for proteins followed somewhat the same pattern as noted for changes in dry weights of ageing cultures. Moreover, there were the same or more protein bands noted for those cultures which had been grown at 15°C and then transferred to lower incubation temperatures. These findings supported the data noted for cultures incubated at 1°C.

In their autolytic studies of Colletotrichum gloeosporioides, Tandon and Chandra (1962) noted that there was a decrease in bound amino acids in acid hydrolysates of very old cultures. Bent (1967) reported that the composition of the soluble proteins changed, and protein content decreased considerably during incubation for three species of Penicillium.

Whitney et al. (1968) found that the protein patterns of Verticillium albo-atrum, V. dahliae, and Fusarium oxysporum varied considerably in their composition with regard to the age of cultures.

When electrophoretic gels were divided into four quarters (Fig.12-15), and the numbers of protein bands in each quarter were plotted against the age of the culture (Fig. 11), it was found that the distribution of protein bands for cultures incubated at 15°C and 15---10°C, in the first quarter of the gels was quite similar. This distribution of bands in this first quarter for cultures incubated at 15---1°C followed the same trend as noted for cultures incubated at 1°C. The distribution of protein bands in the second quarter of the gels, for cultures incubated at 15---10°C and 15---1°C, followed the trend as noted for cultures incubated at 15°C and 1°C respectively. It should be noted that, with an increase in age of cultures, there was a decrease in numbers of protein bands in the first two quarters of the gels, but the decrease was very rapid for those cultures incubated at 1°C and 15---1°C harvested 56 and 32 days respectively, as compared to those cultures incubated at 15°C and 15---10°C. The decrease in the numbers of protein bands in the first quarter for the cultures incubated at 1°C and 15---1°C may be considered due to a change and/or breakdown of high molecular weight proteins. This breakdown of high molecular weight proteins may result in smaller protein units. The results in Fig.11 indicate that for cultures incubated at 1°C and harvested at 48 and 56 days, the numbers of protein bands in the first and second quarters of the gels decreased. Associated with the decrease of protein bands (the top halves of the gels), ther was an increase in numbers of protein bands in the lower halves. This increase may have resulted due to a breakdown of proteins observed in the upper halves.

The protein patterns for mycelium grown at 15°C, 15---10°C, and 15---1°C and harvested after 12 days i.e. 16 to 32 days, showed similar trends. An exception occurred for the 24-day old cultures incubated at 15---1°C, when the numbers of protein bands increased. This increase may be considered due to a slower breakdown of proteins at the lower temperature as compared to those cultures incubated at 15°C and 15---10°C.

The protein changes found in these studies not only indicated an increase or decrease in numbers of bands as the cultures aged. Significantly, there were variations in the stain uptake by the proteins. The varying intensities of stain uptake by individual bands may be related to the amount of proteins present. In the present studies, Figs. 12-15 show that there were more heavily stained protein bands in younger cultures then older cultures. In older cultures very few heavily stained bands were observed.

The quantitative total protein determination using the method of Lowry et al. (1951) are reflected in the electrophoretic studies in the latter part of autolytic phase. Bent (1967) considered that some of proteins may (a) aggregate, (b) become bound into structures which are too large or carry insufficient charge to migrate into gels, and (c) are not extractable from mycelium. The changes noticed in the gels from younger to older preparations of cell-free mycelial extracts, may also correspond due to a change in various enzyme levels. Some of the protein bands on the gels may be attributed to enzymes.

In these studies peroxidase isozyme patterns changed with the age of cultures and incubation temperatures. There was not only a change in patterns but also stain uptake (Benzidine. 2 HCl). The numbers of peroxidase isozymes were always higher in growing cultures at all the

incubation temperatures, and this number decreased as the amount of mycelium decreased. The variation in peroxidase bands may be due to the age of tissue or associates with other metabolic activities. It has been shown that there were seasonal fluctuations in horseradish peroxidase activity (Jermyn and Thomas 1954). Roberts (1969) reported that there was a higher peroxidase isozymic complement in wheat leaves grown at 6°C than those grown at 20°C.

At 15°C, protease, the enzyme which splits protein into peptides (Whitaker 1961), of cell-free mycelial extracts was found to have maximum activity at the time maximum yields of mycelium were obtained. This activity decreased rapidly during the autolytic phase. However, protease activity, for cultures incubated at 1°C, did not follow the curve, shown in Fig. 9 for yield of mycelium. Rather, it followed the curve for B-glucosidase of cultures incubated at 1°C. Also, protease activity for cultures incubated at 1°C was higher than those incubated at 15°C. These results for protease activity followed the same trend as that found by Peterson and Gunderson (1960) for Pseudomonas fluorescence. These workers demonstrated higher endo- and extracellular proteolytic activity when the cultures were incubated at 10°C as compared to 20°C.

In relating protease activities to total protein values, maximum protease activities were reached before maximum protein values occurred, see Figs. 10,20 for cultures incubated at 15°C and 1°C.

These in vitro studies may not reflect accurately what is happening in the in vivo systems since the cultures were incubated at 15°C and 1°C respectively but protease activity was measured at 35°C. It would be of considerable interest, therefore, to measure the protease activity at the temperature at which the fungus was cultured. At the lower temperature, the enzyme may be present, but not as active as at 35°C. Of course,

other proteolytic enzymes such as pronase, proteinases may also be involved in protein breakdown. As well, enzymes which breakdown peptides may release some compounds i.e. tyrosine, tryptophane, etc. which react with Folin phenol reagent resulting in high colour development, and this could account in part, for the high total protein values obtained in very old cultures.

The maximum RNA content in the cell-free mycelial extracts of the mycelium grown at 15°C occurred before the maximum yield of mycelium was obtained. However, at 1°C, the maximum RNA values occurred well after maximum yields of mycelium were recorded. For cultures incubated at 15°C, there appears to ba a direct RNA-RNase correlation which was not found for cultures incubated at 1°C. Gottlieb and van Etten (1966) found that the percentage of RNA per unit of dry weight of mycelium decreased from young to older portions from fungal mats of liquid static cultures of Rhizoctonia solani and Sclerotium bataticola; however, these workers did not carry out complementary RNase studies.

It is puzzling that maximum amounts of RNA and RNase activity occurred at the same time. At 1°C, it seems that RNA is not enzymatically broken down as noted for cultures incubated at 15°C. RNase may be present in large amounts but inactive at the lower temperature. Also, it is possible that at 1°C, RNase in an in vivo system is inactive, but when measured at 37°C for the in vitro studies, RNase in some way, is activated. As for protease activity, it would be of considerable interest to assay for RNase activity at the temperature at which fungus was grown.

Tempest and Hunter (1965) considered that when cultures of Aerobacter aerogenes were transferred from 40°C to 25°C, the rate of

protein synthesis per unit of ribosome, was constant at a particular temperature, but due to lowering the temperature, ribosomal activity was decreased, so that, in order to maintain a constant growth rate, the bacteria compensated by synthesizing additional ribosomal RNA.

Since the RNA represents ribosomal, soluble, and messenger RNA, it is possible that variation in one of these RNA components could alter considerably RNA-RNase relationship(s).

The results for HCN production by cultures incubated at various temperatures indicated that HCN was not released by young cultures. The release of HCN in cultures was noticed only after there was a decrease in mycelial dry weights. These results support the data of Ward and Lebeau (1962) who concluded that HCN was released during the autolytic phase. Furthermore, the data for experiments in which HCN was collected on a daily basis confirmed that HCN was released only after a decrease in dry weight of mycelium started. In these studies, the release of HCN for the cultures incubated at 15°C was greater than reported by Ward and Lebeau (1962); but, the trend for HCN release was the same. The higher amounts of HCN noted, for these studies, may have been due to slight cultural conditions. Except for Lebeau and Dickson (1955), who determined the amounts of HCN in 30-day old mycelial mats grown on an alfalfa-crown meal medium at 4°C, 8°C, 12°C, and 20°C; no studies have been carried out to determine the effects of various incubation temperatures by ageing cultures on HCN production. For this thesis, using the liquid shake culture technique and one defined medium, the maximum amounts of HCN were liberated at 15°C. At the other incubation temperatures, the amounts were less than observed at 15°C. For cultures incubated at 1°C, the maximum amount of HCN released was much

less than that at 15°C, but under natural conditions the fungus has not been observed to be active at 15°C. Therefore, culturing the fungus at 1°C instead of 15°C is closer to natural low-temperature conditions since the fungus is pathogenic on alfalfa and forage plants during the winter months. It has been considered that a definite period of association between host and fungus is required in the fall and early winter before the host becomes susceptible to the disease, and dormant plants appear to become progressively more susceptible (Cormack 1942, and Lebeau and Dickson 1955). The possible explanation for requiring a period of association is that at subzero temperatures, fungus growth is slow. Lebeau and Dickson (1955) considered that for the expression of disease symptoms lower temperature (2-4°C) was essential. The relation to lower temperature to disease development is that under snow covers (2-4°C) HCN released by the fungus becomes in contact with the host tissues, and the HCN makes the tissues susceptible for disease injury. At higher temperature, HCN released by the fungus, is not absorbed by the host tissues, and therefore, are not damaged. In these studies, at 1°C, the amounts of HCN released were less than at 15°C, but near maximum amounts of HCN were maintained for a longer period than for cultures incubated at higher temperatures. Also, there was less decrease in dry weight of mycelium grown at 1°C as compared to cultures incubated at 15°C, indicating that autolysis was much slower.

The nature of the substrate in living dormant host tissue may play a large role in cyanogenesis. The results of Ward and Thorn (1964) and Stevens and Strobel (1968) should not be overlooked. Ward and Thorn (1966) were able to detect HCN when they substituted glycine for asparagine in liquid medium, even before the autolysis occurred. Stevens and Strobel (1968) found that amino acids namely glycine, alanine, asparagine,

valine, isoleucine, phenylalanine, serine, and tyrosine were directly involved in the formation of HCN. They also proposed a scheme for cyanogenesis and concluded that isoleucine and valine were precursors for the cyanogenic glucosides i.e. lotaustralin and linamarin respectevely. These glucosides are generally considered to release HCN as a result of enzymatic activity.

In these studies there was no direct relationship between HCN release and proteolysis. Protease activity reached a maximum before the HCN release occurred, and there was even considerable protease activity in 8-day old cultures incubated at 15°C. Indirectly, protease and possibly other proteolytic enzymes may play a role in cyanogenesis.

Preliminary experiments (Colotelo, Unpublished data) showed that HCN was released when cell-free culture medium was reacted with the cyanogenic glucoside amygdalin. In their studies, Colotelo and Ward (1961) reacted cell-free culture medium with alfalfa root homogenates and found that HCN was released. This provided positive evidence that (a) the culture medium had β-glucosidase activity and (b) the host provided a cyanogenic glucoside.

Ward (1964b) demonstrated β-glucosidase activity in homogenates of W_2 mycelium. Very recently Stevens and Strobel (1968) separated two β-glucosidases from 3-week old W_2 mycelial mats on a DEAE cellulose column and determined their properties i.e. optimum pH, substrate concentrations, substrate specificity, and energy of activation. Lebeau (1966) proposed a hypothetical scheme for cyanogenesis involving β-glucosidase in HCN production. However, these workers did not determine the activity of β-glucosidase in relation to the age of cultures and incubation temperatures. In these investigations, β-glucosidase

levels in culture medium and cell-free mycelial extracts were quite low and reached a maximum only after autolysis began. The β-glucosidase activity for ageing cultures incubated at 15°C respectively followed the same trend as HCN production. At the higher temperature there was a rapid increase in activity to a maximum level, after which there was a sharp decline. At 1°C, there was a slow increase in activity, and after maximum was reached, well after the time that the dry weight of mycelium had started to decrease, there was a slight decline. This phenomenon raises the question whether at low temperature, HCN release resulting from β-glucosidase activity, can be correlated with a decrease in dry weight of mycelium.

In considering the release of HCN, β-glucosidase activity and pathogenecity, other workers considered that the HCN released by the fungus affected host tissues making it more susceptible (Lebeau and Dickson 1955). It has been shown that the fungus synthesizes cyanogenic glucosides and their hydrolysing enzymes (Stevens and Strobel 1968). It has also been shown that a low temperature basidiomycete, isolate W_1, which does not produce HCN in culture but in association with host tissues HCN is released (Ward et al. 1961); therefore, the host can provide substrate for β-glucosidase activity to release HCN.

On the basis of the evidence provided in this thesis, pathogenecity at low temperatures involving HCN may be associated with the age of mycelium. In the preautolytic phase, intra- and exocellular β-glucosidase activity is very low and possibly insufficient to hydrolyse cyanogenic glucoside of the fungus and/or host. In the autolytic phase β-glucosidase activity is very high, maintained for a longer period of time, and could

be sufficient to cause the release of HCN in such amounts as to injure host tissues.

In these studies, 'free' HCN was released from non-autoclaved and autoclaved culture medium and cell-free mycelial extracts from cultures incubated at 15°C and 1°C. The studies for 'free' HCN measured on a daily basis for the cultures incubated at 15°C for culture medium and cell-free mycelial extracts, confirmed that 'free' HCN was released only after autolysis began. These results differ somewhat from those presented by Ward (1964b) who found that boiled extracts of an acetone powder preparation, from W_2 mycelium, did not release 'free' HCN. He used 17-day old mycelial mats grown on malt yeast-dextrose medium as static cultures. Notwithstanding medium and cultural technique, it would appear that Ward's cultures were not sufficiently aged. The studies in this thesis for 'free' HCN released from non-autoclaved and autoclaved culture medium and cell-free extracts indicated that this is not necessarily an enzymatically controlled process. These investigations, to some extent, support Ward's (1964b) findings who postulated that the release of HCN is not an enzymatic phenomenon.

SUMMARY

In summary, the studies for this thesis have resulted in the following new information on the effects of temperature on various aspects in the physiology and biochemistry of the cyanide-producing low-temperature basidiomycete, isolate W_2:

1. It was found necessary to use one medium using various cultural techniques for growth studies.
2. W_2 grew better at 1°C than at higher temperatures.
3. Changes in temperature during incubation altered yield of mycelium and other physiological and biochemical processes.
4. Total protein content of mycelium was higher at lower temperature.
5. The numbers of protein bands as measured by disc gel electrophoresis were higher at the lower temperature and the protein patterns varied with incubation temperature and age of cultures.
6. Changes in incubation temperature also affected numbers and patterns of peroxidase isozymes.
7. Protease activity was highest at the lower temperature and did not complement total protein determinations.
8. RNA - RNase were higher at the lower incubation temperature, but maximum values occurred later at the lower temperature as compared to cultures incubated at the higher temperature.
9. Incubation temperature affected HCN release. Less HCN produced in autolysing cultures at low temperature as compared to that released at the higher temperature, but HCN production maintained for a longer period of time at the lower temperature.

10. Low incubation temperature increased β-glucosidase activity in cell-free mycelial extracts.

11. 'Free' HCN release determined from non-autoclaved and autoclaved culture medium.

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APPENDIX

Table A-1: Diameter of W_2 colonies grown at 15°C and 1°C in petri dish culture on an agar-synthetic medium.

Age of Cultures (days)	Diam. of colonies (mm)					
	15°C			1°C		
	Experiments		Average	Experiments		Average
	I	II		I	II	
2	16	16	16			
4	34.33	34.12	34.22	12.50	12.50	12.50
6				18.00	18.08	18.04
8	47.00	45.75	46.37	23.16	22.91	23.03
10	57.33	58.25	57.76	26.33	25.07	25.70
12	68.00	67.5	67.75	30.16	29.75	29.90
14	78.83	76.5	77.66	35.33	34.25	34.79
16	91.16	90.62	90.89	41.00	40.50	40.75
18	102.00	100.37	101.18	45.25	44.50	44.87
20	112.91	109.62	111.26	48.66	49.83	49.24
22	123.83	123.00	123.41	55.08	55.33	55.20
24	134.5	136.87	135.68	61.08	61.41	61.24

Note: Each value in experiments I and II is the average of three determinations.

Table A-2: Yield of mycelium of the low-temperature basidiomycete, isolate W_2, grown on a synthetic medium in liquid static cultures incubated at 15°C and 1°C.

Age of Cultures (days)	Yield of mycelium (dry weight mg/flask)					
	15°C			1°C		
	Experiments		Average	Experiments		Average
	I	II		I	II	
8	34.78	39.10	36.93	10.66	12.00	11.03
12	175.66	185.90	180.78	10.93	11.70	11.31
16	299.70	309.40	305.55	15.70	16.20	15.95
20	310.36	311.76	311.06			
24	265.96	266.86	266.41	32.53	36.66	34.59
32	182.73	190.50	186.61	66.50	69.10	67.80
48				161.80	166.26	164.03
56				174.40	178.16	176.28

Note: Each value in experiments I and II is the average of three determinations.

Table A-3: Yield of mycelium of a low-temperature basidiomycete, isolate W_2, using a synthetic medium in liquid shake cultures incubated at various temperatures.

Incubation temperature (C)	Age of Culture (days)	Yield of mycelium (dry weight, mg/flask) Experiments I	II	III	IV	Average
15°	8	316.60	316.00	314.06		315.55
	12	377.50	374.80	376.20		376.16
	16	253.80	254.20	252.20		253.40
	24	176.50	178.90	180.90		178.70
	32	125.50	128.90	129.05		127.81
1°	8	--	13.30	12.70		13.00
	12	--	64.90	62.90		63.90
	16	389.30	386.30	396.70		390.70
	24	332.10	337.50	334.30		334.60
	32	--	276.10	271.40		273.70
	48	174.60	182.40	180.40		179.10
	56	163.30	165.50	168.70		165.60
15---10°	8	315.20	314.30	316.35	318.25	316.02
	12	256.60	253.80	252.70	253.80	254.20
	16	220.80	223.80	221.20	220.10	221.20
	24	140.70	141.70	142.40	139.90	141.10
	32	114.70	116.10	113.80	115.50	115.00
15---1°	8	46.30	46.80			46.50
	12	383.70	388.70			386.20
	16	259.70	260.80			260.20
	24	178.60	179.00			178.80
	32	129.40	127.10			128.20

Note: Each value in experiments I, II, III, and IV is the average of three determinations.

Table A-4: Hydrogen cyanide release by cultures of a low-temperature basidiomycete, isolate W_2, grown on a synthetic medium in liquid shake culture in a 24-hour period prior to harvest. Cultures incubated at 15°C were harvested from 13 to 17 days after inoculation.

Age of Cultures (days)	HCN (p.p.m./flask) Experiments I	II	Average
13	9.58	8.75	9.16
14	31.30	30.20	30.75
15	493.75	491.68	492.71
16	1137.50	1139.91	1138.70
17	966.60	972.91	969.75

Note: Each value in experiments I and II is the average of three determinations.

Table A-5: Yield of mycelium of a low-temperature basidiomycete, isolate W_2, grown on a synthetic medium in liquid shake cultures incubated at 15°C. Cultures were harvested from 13 to 17 days after inoculation

Age of Culture (days)	Yield of mycelium (dry weight, mg/flask) Experiments I	II	III	Average
13	310.50	300.06	299.40	303.32
14	298.00	295.10	295.26	296.12
15	271.20	273.06	273.66	272.64
16	255.00	256.03	254.93	255.32
17	226.16	224.10	225.20	225.15

Note: Each value in experiments I, II, and III is the average of three determinations.

Table A-6: Changes in pH of culture medium and non-buffered cell-free mycelial extracts of ageing cultures of a low-temperature basidiomycete, isolate W_2. Cultures were grown on a synthetic medium and incubated at 15°C and 1°C.

Age of Cultures (days)	pH 15°C Culture medium Experiments* I	II	III	Average	Non-buffered mycelial extracts Experiments I	II	III	Average	1°C Culture medium Experiments* I	II	Average	Non-buffered mycelial extracts Experiments I	II	Average
8	5.45	5.43	5.53	5.47	6.40	6.35	6.40	6.38	6.15	6.15	6.15	-	-	-
12	4.55	4.55	4.59	4.56	6.30	6.35	6.35	6.31	5.73	5.68	5.70	6.50	6.55	6.52
16	6.30	6.23	6.36	6.29	6.40	6.45	6.45	6.43	4.91	4.79	4.85	6.30	6.25	6.27
24	7.40	7.33	7.43	7.38	6.82	6.95	7.00	6.92	6.17	6.13	6.15	6.40	6.40	6.40
32	7.40	7.46	7.48	7.44	6.85	6.82	6.92	6.86	6.46	6.50	6.48	6.42	6.45	6.43
48									7.43	7.50	7.46	6.95	6.97	6.96
56									7.65	7.58	7.61	6.97	7.00	6.98
72									7.83	7.73	7.78	6.97	6.97	6.97

* Each value is the average of three determinations.

Table A-7: Total proteins of cell-free mycelial extracts from mycelium of ageing cultures of a low-temperature basidiomycete, isolate W_2, incubated at 15°C and 1°C.

Age of Cultures (days)	Total proteins (mg/g dry wt of mycelium) 15°C Experiments* I	II	III	Average	1°C Experiments* I	II	Average
8	175.92	167.02	160.10	167.68			
12	168.44	166.24	157.44	164.04	76.56	83.82	80.19
16	165.94	170.57	173.73	176.41	119.16	107.92	113.54
24	202.86	197.94	205.03	201.27	116.82	121.80	119.31
32	148.00	152.75	155.82	152.19	155.35	156.98	156.16
48					253.35	249.16	251.25
56					231.66	233.84	232.75

* Each value in experiments is the average of three determinations.

Table A-8: Protease activity of cell-free mycelial extracts from mycelium of ageing cultures of a low-temperature basidiomycete, isolate W_2. Cultures were incubated at 15°C and 1°C.

Age of Cultures (days)	Protease activity (Units [a] /mg dry weight of mycelium) 15°C Experiments* I	II	III	Average	1°C Experiments* I	II	Average
8	30.62	31.31	28.55	30.16			
12	50.89	48.02	46.11	48.34	26.78	26.00	26.39
16	45.18	41.13	41.80	42.7	44.31	43.17	43.74
24	29.18	25.61	29.46	28.08	64.89	62.62	63.75
32	23.18	19.55	21.30	21.49	56.93	61.43	59.18
48					53.62	51.49	52.55
56					28.23	26.78	

a $\times 10^{-4}$

* Each value in experiments is the average of three determinations.

Table A-9: Total RNA - RNase activity of cell-free mycelial extracts from mycelium of ageing cultures of a low-temperature basidiomycete, isolate W_2. Cultures were grown using a synthetic medium in liquid shake culture at 15°C and 1°C.

Age of Culture (days)	RNA (mg/g dry weight of mycelium)							RNase activity (units/g dry weight of mycelium)						
	15°C				1°C			15°C				1°C		
	Experiments*			Average	Experiments*		Average	Experiments*			Average	Experiments*		Average
	I	II	III		I	II		I	II	III		I	II	
8	9.80	9.88	9.81	9.83				12170	11210	9820	11066			
12	9.02	8.65	8.09	8.58	4.06	4.04	4.05	14280	12540	12870	12705	1107	1185	1146
16	5.47	5.68	6.10	5.75	5.56	6.13	5.84	16690	16320	16200	16405	2395	2448	2421
24	5.87		6.25	6.06	5.27	6.18	5.72	12430	13180	12270	12636	11338	11585	11461
32	4.06	4.11	4.04	4.07	14.22	13.93	14.07	6130	5416	5852	5799	21484	21132	21308
48					9.27	9.38	9.32					13206	13008	13107
56					8.68	8.83	8.75					16701	17654	17177

* Each value in experiments is the average of three determinations.

Table A-10: Hydrogen cyanide release by ageing cultures of a low-temperature basidiomycete, isolate W_2, grown on a synthetic medium in liquid shake culture in a 24-hour period prior to harvest at various temperatures (Cultures incubated at 15°C and transferred to 10°C (15---10°C) and 1°C (15---1°C) respectively three days prior to harvest)

Incubation temperature (C)	Age of Cultures (days)	HCN (p.p.m./flask) Experiments I	II	III	IV	Average
15°	8	--	--	--		--
	12	--	--	--		--
	16	1043.50	1103.00	1130.00		1092.16
	24	979.25	1001.25	1029.25		1003.25
	32	631.75	629.75	698.50		653.33
1°	8	--	--	--		
	12	--	--	--		
	16	--	--	--		--
	24	14.00	19.00	14.10		15.70
	32		591.00	583.8		587.40
	48	557.30	531.00	539.80		542.70
	56	526.00	530.00	522.50		526.10
15---10°	8	--	--	--	--	--
	12	--	--	--	--	--
	16	732.5	687.5	674.00	699.75	699.18
	24	368.25	367.00	365.25	363.60	366.00
	32	330.00	324.40	345.00	324.10	330.75
15---1°	8	--	--			
	12	--	--			
	16	601.50	584.00			592.70
	24	436.00	450.30			443.10
	32	300.00	295.30			297.60

Note: Each value in experiments I, II, III, and IV is the average of three determinations.

Table A-11: β-glucosidase activity of culture medium of ageing culures of a low-temperature basidiomycete, isolate W_2, grown on a synthetic medium. Cultures were incubated at 15°C.

Age of Cultures (days)	Release of HCN from amygdalin in p.p.m./ml culture medium. Experiments			Average
	I	II	III	
8	2.91	3.25	4	3.38
12	24.37	24.16	23.23	23.92
16	164.58	172.50	165.41	167.49
24	74.56	77.91	77.00	76.49
32	67.08	69.58	72.50	69.72

Note: Each value in experiments I, II, and III is the average of three determinations.

Table A-12: β-glucosidase activity of cell-free mycelial extracts of mycelium of ageing cultures of a low-temperature basidiomycete, isolate W_2, grown on a synthetic medium. Cultures were incubated at 15°C.

Age of Cultures (days)	Release of HCN from amygdalin in p.p.m./10 mg equivalent dry weight of mycelium Experiments			Average
	I	II	III	
8	10.30	10.40	9.00	9.90
12	49.10	43.10	42.50	44.90
16	248.23	230.50	230.10	236.27
24	164.10	157.84	182.60	168.18
32	147.10	147.60	151.40	148.70

Note: Each value in experiments I, II, and III is the average of three determinations.

Table A-13: β-glucosidase activity of culture medium of ageing cultures of a low-temperature basidiomycete, isolate W_2, grown on a synthetic medium. Cultures were incubated at $1^{o}C$

Age of Culture (days)	Release of HCN from amygdalin in p.p.m./ml culture medium		
	Experiments		Average
	I	II	
12	1.5	1.62	1.56
16	88.7	87.07	87.38
24	180.25	175.07	177.66
32	178.00	172.50	176.25
48	126.00	123.40	124.7C
56	135.75	125.82	130.78
72	82.95	83.14	83.14

Note: Each value in experiments I and II is the average of three determinations.

Table A-14: β-glucosidase activity of cell-free mycelial extracts from mycelium of ageing cultures of a low-temperature basidiomycete, isolate W_2, grown on a synthetic medium. Cultures were incubated at $1^{o}C$.

Age of Culture (days)	Release of HCN from amygdalin in p.p.m./10 mg equivalent dry weight of mycelium.		
	Experiments		Average
	I	II	
12	4.61	5.23	4.92
16	33.94	35.87	34.90
24	84.51	83.99	84.25
32	103.57	112.38	107.97
48	151.82	156.32	154.07
56	201.61	189.17	195.39
72	168.31	167.46	167.88

Note: Each value in experiments I and II is the average of three determinations.

Table A-15: 'Free' HCN released from non-autoclaved and autoclaved fractions of culture medium of ageing cultures of a low-temperature basidiomycete, isolate W_2. Cultures were grown on a synthetic medium in liquid shake flasks at 15°C.

Age of Cultures (days)	'Free' HCN (p.p.m.) Culture medium (1.0 ml)						
	non-autoclaved Experiments*			Average	autoclaved Experiments*		Average
	I	II	III		I	II	
8	--	--	--	--	--	--	--
12	--	--	--	--	--	--	--
16	26.50	25.00	22.91	24.80	25.20	22.50	23.80
24	60.00	62.08	63.32	61.80	63.25	63.75	63.50
32	62.91	60.41	59.57	60.96	72.25	57.90	65.07

* Each value in experiments I, II, and III is the average of three determinations.

Table A-16: 'Free' HCN released from non-autoclaved and autoclaved fractions of cell-free mycelial extracts of mycelium from ageing cultures of a low-temperature basidiomycete, isolate W_2. Cultures were grown on a synthetic medium in liquid shake flasks at 15°C.

Age of Cultures (days)	'Free' HCN (p.p.m.) Cell-free mycelial extracts (10 mg equivalent dry weight of mycelium)						
	non-autoclaved Experiments*			Average	autoclaved Experiments*		Average
	I	II	III		I	II	
8	--	--	--	--	--	--	--
12	--	--	--	--	--	--	--
16	1.47	2.50	1.30	1.75	1.28	0.63	0.95
24	11.10	11.60	17.10	13.26	0.93	14.28	7.60
32	10.30	7.80	8.70	8.90	1.28	4.60	2.94

* Each value in experiments I, II, and III is the average of three determinations.

Table A-17: 'Free' HCN released from non-autoclaved and autoclaved fractions of culture medium of ageing cultures of a low-temperature basidiomycete, isolate W_2. Cultures were grown on a synthetic medium in liquid shake flasks at 1°C.

Age of Cultures (days)	'Free' HCN (p.p.m.) Culture medium (1.0 ml)					
	non-autoclaved			autoclaved		
	Experiments*		Average	Experiments*		Average
	I	II		I	II	
8	--	--	--	--	--	--
12	--	--	--	--	--	--
16	--	--	--	--	--	--
24	2.25	2.0	2.12	1.25	1.25	1.25
32	14.50	17.07	15.78	2.50	5.00	3.75
48	27.75	29.50	28.62	20.00	21.00	20.50
56	27.50	28.75	28.12	11.00	20.00	15.50
72	26.05	27.07	26.56	18.12	20.00	19.06

* Each value in experiments I and II is the average of three determinations.

Table A-18: 'Free' HCN released from non-autoclaved and autoclaved fractions of cell-free mycelial extracts of mycelium of ageing cultures of a low-temperature basidiomycete, isolate W_2. Cultures were grown on a synthetic medium in liquid shake flasks at 1°C.

Age of Cultures (days)	'Free' HCN (p.p.m.) Cell-free mycelial extracts (10 mg equivalent dry weight of mycelium)					
	non-autoclaved			autoclaved		
	Experiments*		Average	Experiments*		Average
	I	II		I	II	
8						
12	--	--	--	--	--	--
16	--	--	--	--	--	--
24	--	--	--	--	--	--
32	2.65	2.59	2.62	--	--	--
48	2.50	2.33	2.41	0.89	0.87	0.88
56	1.63	1.81	1.72	--	--	--
72	2.36	2.07	2.21	0.89	0.85	0.87

* Each value in experiments I and II is the average of three determinations.

Table A-19: 'Free' HCN released from non-autoclaved and autoclaved fractions of culture medium and cell-free mycelial extracts of mycelium of cultures of a low-temperature basidiomycete, isolate W_2. Cultures grown on a synthetic medium at 15°C were harvested from 13 to 17 days after inoculation.

Age of Cultures (days)	'Free' HCN (p.p.m.) Culture medium (1.0 ml)						Cell-free mycelial extracts (10 mg equivalent dry weight of mycelium)					
	non-autoclaved			autoclaved			non-autoclaved			autoclaved		
	Experiments*		Average	Experiments*		Average	Experiments*		Average	Experiments*		Average
	I	II		I	II		I	II		I	II	
13	--	--	--				--	--	--	--	--	--
14	2.60	2.50	2.55	2.50	1.87	2.18	--	--	--	--	--	--
15	4.16	3.75	3.95	3.75	2.50	3.12	2.10	2.60	2.35	--	--	--
16	24.58	24.58	24.58	20.00	20.00	20.00	4.20	5.20	5.05	2.47	2.86	2.66
17	37.50	37.50	37.50	23.75	24.37	24.37	6.00	5.50	5.75	2.74	2.88	2.78

* Each value in experiments I and II is the average of three determinations.