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CELLULASE AND RELATED ENZYME ACTIVITY IN PROTOPLASTS AND MYCELIUM OF FILAMENTOUS FUNGI

By

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A thesis submitted to the Council for National Academic Awards in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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(i) October 1984 - May 1985
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ABSTRACT

CELLULASE AND RELATED ENZYME ACTIVITY IN PROTOPLASTS AND MYCELIUM OF FILAMENTOUS FUNGI

By Alan Collings, BSc

Production of cellulase, pectinase and xylanase was investigated using culture filtrates from selected mesophilic (Aspergillus niger, Penicillium funiculosum, Penicillium janthinellum, Penicillium ochrochloron, Trichoderma viride), thermotolerant (Aspergillus fumigatus) and thermophilic (Geosmithia emersonii, formerly Talaromyces emersonii; the perfect stage of Penicillium emersonii) fungi. The fungi were grown on Acetobacter xylinum cellulose pellicles and soluble carboxymethyl cellulose and their enzyme complements were characterised under various growth conditions.

The factors affecting the production and release of protoplasts from the closely related A. fumigatus and A. niger and P. ochrochloron and G. emersonii were investigated. Protoplast yields were found to be greatly dependent upon growth temperature, cultural conditions, mycelial age, mycelial concentration, incubation time, incubation temperature and lytic enzyme used. Greatest protoplast yields were obtained after 3 h incubation using 1 mg ml^{-1} Novozym™ 234 in 0.1 M 2-N-[Morpholino] ethane sulphonic acid (MES) buffer, pH 5.0 containing 0.6 M sodium chloride. Supplementation of the incubation medium with 0.03 M calcium chloride enhanced protoplast release in P. ochrochloron but not in the other three test fungi.

Protoplasts from all four fungi were capable of regenerating a cell wall in both liquid and solid regeneration media. Regenerating protoplasts were shown to retain endo-β-1,4-glucanase, exo-β-1,4-glucanase and β-glucosidase activity.

Attempts were made to cross related mesophilic with thermotolerant and/or thermophilic fungi using the technique of protoplast fusion. P. ochrochloron was crossed with G. emersonii and A. niger was crossed with A. fumigatus in an attempt to improve cellulase complement and stability. The test fungi were screened for resistance to a variety of heavy metal salts and commercial fungicides, and mutants resistant to Benlate and Mystox™ fungicides were obtained. These selection markers, together with an auxotrophic mutant of P. ochrochloron, were used to select out protoplast fusion products against parental types in the trial crosses.

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ABBREVIATIONS USED IN THE TEXT

A. aculeatus	*Aspergillus aculeatus*
A. fumigatus	*Aspergillus fumigatus*
ai	active ingredient
A. nidulans	*Aspergillus nidulans*
A. niger	*Aspergillus niger*
A. phoenicis	*Aspergillus phoenicis*
AR	Analar
A. xylinum	*Acetobacter xylinum*
BMC	Ball-milled cellulose
CA	Complete Agar
$CaCl_2$	Calcium chloride
$Ca(NO_3)_2$	Calcium nitrate
C. fimi	*Clostridium fimi*
C. globosum	*Chaetomium globosum*
Chem.	Chemicals
cm	Centimetre
CMC	Carboxymethylcellulose
CMI	Commonwealth Mycological Institute
Co.	Company
C. thermocellum	*Clostridium thermocellum*
C. thermohydrosulfuricum	*Clostridium thermohydrosulfuricum*
C. thermophilum	*Chaetomium thermophilum*
D	Dextro
DNA	Deoxyribonucleic acid
DNS	Dinitrosalicylic acid
EC	Enzyme convention
E. coli	*Escherichia coli*
EDTA	Ethylene diamine tetraacetic acid

g	gramme
G. emersonii	Geosmithia emersonii
GPR	General purpose reagent
h	hour
HEPES	n-2-hydroxyethyl piperazine-N-2 ethane sulphonic acid
IMI	International Mycological Institute
IU	International units of enzyme activity
KCl	Potassium chloride
l	litre
L	Laevo
LR	Laboratory Reagent
M	Molar
MA	Minimal Agar
MES	2-[N-morpholino]-ethane sulphonic acid
mg	milligram
$MgSO_4$	Magnesium sulphate
min	minute
ml	millilitre
mm	millimetre
M. thermophila	Myceliophthora thermophila
mut	mutant
NaCl	Sodium chloride
N. crassa	Neurospora crassa
NH_4CL	Ammonium chloride
nm	nanometre
No.	number
o	ortho

oz	ounce
p	para
PAHBAH	p-hydroxybenzoic acid hydrazide
P. capsulatum	Penicillium capsulatum
P. chrysogenum	Penicillium chrysogenum
P. chrysosporium	Phanerochaete chrysosporium
P. cyclopium	Penicillium cyclopium
P. dupontii	Penicillium dupontii
PEG	Polyethylene glycol
P. expansum	Penicillium expansum
P. funiculosum	Penicillium funiculosum
P. Janthinellum	Penicillium janthinellum
P. ochrochloron	Penicillium ochrochloron
P. purpurescens	Penicillium purpurescens
P. roquefortii	Penicillum roquefortii
P. spinulosum	Penicillium spinulosum
P. wortmani	Penicillium wortmani
RNA	Ribonucleic acid
rpm	revolutions per minute
S. cerevisiae	Saccharomyces cerevisiae
S. commune	Schizophyllum commune
Sp.	species
S. pulverulentum	Sporotrichum pulverulentum
SSF	Simultaneous Saccharification/ Fermentation
S. thermophilum	Sporotrichum thermophilum
T. koningii	Trichoderma koningii
T. reesei	Trichoderma reesei

T. viride	Trichoderma viride
UV	Ultraviolet
v/v	volume to volume ratio
w/v	weight to volume ratio
WT	wild type
x	no result
Z. mobilis	Zymomonas mobilis
α	Alpha
β	Beta
^{o}C	degrees centigrade
%	per cent
lb in^{-2}	pounds per square inch
	micro
μg	microgramme
μm	micrometre
μM	micromole
-	no growth
+	growth

Annual production of cellulosic biomass has been estimated at 4×10^{10} tonnes (Coughlan and Folan, 1979). This presents a valuable resource, which is available in large quantities throughout the globe from municipal, agricultural, industrial and forestry wastes and benefits from being a renewable resource. Increasing interest has been shown towards the feasibility of converting cellulosic biomass into usable glucose (Mandels and Weber, 1969; Nystrom and Andren, 1976; Ladisch, 1979; Lipinsky, 1981). Glucose or other fermentable sugars could then be used in the production of a wide range of compounds including antibiotics, vitamins, solvents such as ethanol, butanol and acetone and other chemicals such as citric acid, ethylene, glycerol, lactic acid and methane (Srinivasan and Han, 1969; Toyama, 1969; Ghose and Pathak, 1973; Allen, 1976; Elder, 1976; Ghose and Ghosh, 1978; Mandels, 1985). Liquid fuels and many chemicals are currently derived from fossil fuels,however, with diminishing supplies of these resources the incentive to fully utilize waste cellulose is receiving added impetus (Tsao, et al., 1978; Lipinsky, 1981). Alternatively, glucose could be used directly as animal feed or as a growth substrate to produce single cell protein for both human and animal consumption (Bellamy, 1974; Coughlan and Folan, 1979). It has been noted that we produce enough food to feed the world but unfortunately lack the will to ensure an equitable distribution of this food (Hall, 1983).

It is important, however, to note the current use of cellulose before considering alternative uses. Cellulose is the principal source of energy in ruminant nutrition. It is also found in building materials, textiles and paper. Moreover, various derivatives of cellulose are used in the manufacture of a diverse range of products

including adhesives, explosives, film, inks, lacquers, plastics and rayon. Agricultural and forestry residues are degraded by cellulolytic microorganisms thereby contributing to soil fertility and preventing soil erosion. Such residues may also be used to generate methane (Detroy, et al., 1980).

Most agricultural residues such as bagasse or corn stover consist largely of cellulose (30-50%), hemicellulose (20-40%) and lignin (5-20%) (Ramadhan, et al., 1976; Dekker and Lindner, 1979; Bisaria and Ghose, 1981). Cellulose is a linear glucose polymer of anhydroglucose units coupled by β-1,4-glycosidic bonds. Aggregation of these glucose chains forms fibrils and these in turn form bundles of fibrils imparting a high degree of crystallinity on the structure of cellulose. Hemicelluloses represent various branched polymers including xylans, pectin and starch. Lignin on the otherhand is a complex three dimensional matrix which is in close association with cellulose and is interpenetrated by hemicellulose (Higuchi, 1982).

Cellulose may be hydrolysed to its monosaccharide component, glucose by acid or as a result of the action of a group of enzymes collectively referred to as "cellulase". Enzymatic saccharification of cellulosic biomass offers certain advantages over acid hydrolysis. These include reaction at moderate temperatures and pressures, efficiency of conversion, purity of the sugar syrup product, uncontaminated by toxic degradation products and potential re-use of the catalyst. In addition, highly toxic waste streams and high neutralization costs may also be avoided (Goldstein, 1976). The hydrolysis of cellulose is hindered by amongst other factors, the crystalline nature of cellulose (Cowling and Kirk, 1976; Fan et

al., 1980) and the presence of lignin (Detroy et al. 1980; Bisaria and Ghose, 1981). These factors impede the hydrolysis of cellulose, both by acid (Ladisch, 1979) and enzymes (Cowling and Brown, 1969). The moisture content of cellulosic materials also affects their susceptibility to enzymatic degradation. Moisture swells the fibres by hydrating the cellulose molecules, causing their fine structure to open up and making the surface more accessible to cellulases and other reagents (Etheridge, 1957). Fungi rather than bacteria are the main degraders of lignocellulosic materials, particularly of wood (Eriksson, 1983). Soft rot and brown rot fungi mainly attack the carbohydrate components while the white rot fungi also degrade the lignin (Eriksson, 1983; Agosin and Odier, 1985). A specific attack on the lignin by white rot fungi probably does not occur (Ander and Eriksson, 1977). Usually lignin and the wood polysaccharides are attacked simultaneously by white rot fungi.

The extent of saccharification of a large variety of substrates has been investigated (Srinivasan and Han, 1969; Toyama and Ogawa, 1975; Andren et al., 1976; Toyama, 1976). Substrates most widely used in characterising cellulase enzymes or cellulolytic micro-organisms are cotton fibre and Avicel. Cotton fibre is highly crystalline with few chain ends, limiting access to enzymes. Avicel on the other hand has up to 30% amorphous regions, varying particle size (5-100 μm diameter) with more chain ends and is thus more easily degraded than cotton (Berghem and Pettersson, 1973). Soluble derivatives of cellulose such as carboxymethylcellulose (CMC) or highly hydrated cellulose that has been swollen in acid or alkali are easily degraded by cell free cellulase enzymes derived from most cellulolytic fungi and bacteria. However, very few microorganisms

are capable of hydrolysing crystalline forms of cellulose to soluble sugars in vitro (Mandels, 1975; Mandels and Andreotti, 1978). Some fungi and bacteria which have been reported to produce high activity cellulase capable of extensively degrading insoluble cellulose to soluble sugars are listed in Table 1.1. Fungal enzymes are generally thought to offer the best possibilities for commercial exploitation (Mandels and Weber, 1969). These organisms grow readily on simple media into which they secrete cellulase enzymes, thereby facilitating isolation. A lot of attention has been attributed to the Trichoderma spp. particularly Trichoderma koningii (Wood,1968; Halliwell and Griffen, 1973; Wood and McCrae, 1978a) Trichoderma reesei (Ryu and Mandels, 1980; Pettersson et al.,1981;Labudova and Farkas, 1983) and Trichoderma viride (Emert et al., 1974; Beldman, et al., 1985). Several Penicillium spp. have been studied for their production of cellulolytic enzymes. These include Penicillium funiculosum (Wood et al., 1980; Joglekar, et al., 1983; Mishra, et al., 1985) and Penicillium janthinellum (Rapp, et al., 1981). Enzymes produced by the Aspergilli are not very efficient in the hydrolysis of native cellulose although Aspergillus fumigatus has been reported capable of degrading cellulose, hemicellulose and lignin (Mills,1973; Crawford and Crawford, 1980; Bisaria and Ghose, 1981; Wase and Raymahasay, 1985). Other fungi capable of degrading crystalline cellulose include Chaetomium thermophilum (Eriksen and Goksøyr, 1977), Geosmithia emersonii (Folan and Coughlan,1978; McHale and Coughlan, 1981; Moloney et al., 1985), Sporotrichum pulverulentum [Phanerochaete chrysosporium] (Eriksson and Pettersson, 1975a,b; Streamer et al., 1975) and Sporotrichum thermophilum [Myceliophthora thermophila] (Coutts and Smith, 1976; Canevascini et al., 1983).

TABLE 1.1 CELLULOLYTIC MICROORGANISMS

FUNGAL ORGANISMS	REFERENCES
Aspergillus sp.	Stewart et al., 1983; Wase and Raymahasay, 1985
Chaetomium sp.	Romanelli et al., 1975; Eriksen and Goksøyr, 1977
Fusarium solani	Wood, 1969; Wood and McCrae, 1977
Geosmithia emersonii	McHale and Coughlan, 1980; Maloney et al., 1985
Penicillium sp.	Joglekar and Karanth, 1984; Mishra et al., 1985
Phanerochaete sp.	Eriksson and Pettersson, 1975a,b; Streamer et al., 1975
Polyporus sp.	Agosin and Odier, 1985; Rouau and Odier, 1986
Sclerotium rolfsii	Sadan et al., 1979
Trichoderma spp.	Labudova and Farkus, 1983; Beldman et al., 1985
BACTERIAL ORGANISMS	**REFERENCES**
Cellvibrio fulvus	Berg, 1975
Cellulomonas sp.	Beguin and Eisen, 1977; Nakamura and Kitamura, 1983
Clostridium thermocellum	Johnson et al., 1982; Lamed et al., 1983
Streptomyces flavogriseus	Kleupfel et al., 1980
Thermomonospora sp.	Ferchak et al., 1980; Meyer and Humphrey, 1982

Reese et al. (1950) proposed a two enzyme system to explain the action of cellulase on crystalline cellulose. A hypothetical, non-hydrolytic C_1 fraction was thought to cause disaggregation of adjacent linear cellulose chains in crystalline cellulose producing a more amorphous cellulose which was then susceptible to attack by the Cx enzymes. Both C_1 and Cx enzymes were thought to be necessary to achieve complete hydrolysis of crystalline cellulose whereas only Cx enzymes were necessary for amorphous cellulose. Many workers now believe that the cellulase enzyme complex is formed from at least three different types of enzymes (Halliwell and Griffen, 1973; Eriksson and Pettersson, 1975a,b; Wood and McCrae, 1978a, 1979; Parry et al., 1983). These enzymes have been identified as exo-β-1,4-glucanase (β-1,4-D-glucan cellobiohydrolase, EC.3.2.1.91), endo-β-1,4-glucanase (β-1,4-D- glucan-4-glucohydrolase, EC.3.2.1.4) and β-glucosidase (β-D-glucoside glucohydrolase, EC.3.2.1.21). All three enzymes are required to achieve the complete hydrolysis of crystalline cellulose and each of the enzymes must be present in the right amounts and under the right conditions (Gritzali and Brown, 1979; Saddler, 1982; Labudova and Farkas, 1983; Heptinstall et al., 1986). However, there is still disagreement as to the specific mechanism of the initiation of attack against crystalline cellulose. Chanczy et al. (1983) isolated an exo-β-1,4-glucanase capable of hydrolytic activity and acting as a swelling factor in disrupting cellulose chains. Wood and McCrae (1978b) also postulated that C_1 may have disruptive and hydrolytic activity. However, most of the evidence points towards the three enzyme system although the possibility of a swelling factor has not been ruled out.

Initial identification of cellulolytic microorganisms usually involves simple screening methods. A widely used technique involves the clearing of opaque or semi-opaque agar medium in a zone adjacent to microbial growth (Rautela and Cowling, 1966; Saddler, 1982). Although measurement of clearance zones is an uncertain criterion for assaying cellulolytic activity (Mandels, 1975; Saddler, 1982) such techniques are useful for preliminary screening. Smith (1977) developed a rapid tube test for detecting fungal cellulase production by monitoring the uncoupling of a dye from dye-bound cellulose powder present in an agar medium, non-cellulolytic species being unable to release the dye. After identification of possible cellulolytic microorganisms, more in-depth studies are required to determine the activity of individual components of culture filtrates. Growth of organisms in liquid media containing cellulose as the sole carbon source may serve to induce the production of cellulase enzymes. Culture filtrates may then be incubated with "model" substrates such as CMC, filter paper, salicin, etc., and hydrolysis monitored by assaying for reducing sugars (Wood and McCrae, 1977; Stewart and Parry, 1981; Rouau and Odier, 1986), total sugar (Dubois et al., 1956) or glucose (Lloyd and Whelan, 1969; Umerzurike, 1971). Other methods include: measurement of viscosity (Wood and McCrae, 1978a; Foda et al., 1984; Rouau and Odier, 1986) and measurement of p-nitrophenol release from p-nitrophenyl-β-D-glucopyranoside (Lachke et al., 1983; Beldman et al., 1985), p-nitrophenyl-β-D-xylopyranoside (Shaker et al., 1984; Kato et al., 1985) and p-nitrophenyl-β-D-cellobioside (Deshpande et al., 1984). More recently 4-methyl-umbelliferyl-β-D-glycosides of glucose, cellobiose, cellotriose and lactose have been used to differentiate endo-β-1,4-glucanase, exo-β-1,4-glucanase and β-glucosidase in crude cellulase (Van Tilbeurgh

and Claeyssens, 1985; Heptinstall et al., 1986). An alternative method involves staining polyacrylamide gels with congo red (Beguin, 1983; Bartley et al., 1984). It is possible to investigate the efficiency of a cellulase system by analysing culture filtrates using thin layer chromatography (Takao et al., 1985) and high performance liquid chromatography (Palmer, 1975; Andren et al., 1976; Mishra et al., 1985) to identify the end products of hydrolysis.

The distinguishing feature of organisms capable of degrading crystalline cellulose is the ability to secrete exo-β-1,4-glucanase (Wood, 1985). Purified forms of this enzyme have been isolated from G. emersonii (McHale and Coughlan, 1982), S.pulverulentum (Eriksson and Pettersson, 1975b), T. koningii (Wood and McCrae, 1978b), T. reesei (Gong et al., 1979; Pettersson et al., 1981) and T. viride (Berghem and Pettersson, 1973; Beldman et al., 1985). Exo-β-1,4-glucanase has been shown in a number of cases to be a cellobiohydrolase and is often referred to as such (Halliwell and Griffen, 1973; Berghem et al., 1975). These enzymes may be characterised by their endwise attack on non-reducing ends of cellulose chains (Wood, 1975; Coughlan and Folan, 1979) producing cellobiose as the primary product (Berghem et al., 1975; Ladisch et al., 1983). Exo-β-1,4-glucanase is active against cellodextrins and acid swollen cellulose but more importantly crystalline cellulose such as Avicel (Berghem and Pettersson, 1973; Takao et al., 1985), cotton (Mandels and Weber, 1969; Takao et al., 1985) and filter paper (Mandels et al., 1976; Shaker et al., 1984). It has been noted that the release of soluble sugars from crystalline substrates depends upon the presence of both endo-β-1,4-glucanase and exo-β-1,4-glucanase (Wood, 1969; Eriksson and Pettersson, 1975b). Exo-β-1,4-glucanase can be assayed in enzyme

mixtures independently of endo-β-1,4-glucanase using MU-cellobiose [β-D-Glcp-(1→4)-β-D-Glcp-7-o-4-methylumbelliferone] as the substrate (Heptinstall et al., 1986). There is no significant hydrolysis of MU-cellobiose by endo-β-1,4-glucanase. Interference by β-glucosidase can be overcome by using D-glucono-1,5-lactone in the assay (Heptinstall et al., 1986). Exo-β-1,4-glucanases are capable of hydrolysis of soluble cellotriose and cellotetraose to yield cellobiose and glucose or cellobiose respectively as products (Hsu et al., 1980). Some workers on the other hand, claim to have purified exo-β-1,4-glucanases, capable of attacking cellobiose (Li et al., 1965; Berghem et al., 1975; Beldman et al., 1985). However, this observation could well be explained by a slight contamination of β-glucosidase (Gong and Tsao, 1979).

Purified forms of endo-β-1,4-glucanase have been isolated from A. fumigatus (Parry et al., 1983), G. emersonii (Moloney et al., 1985), S. pulverulentum (Eriksson and Pettersson, 1975a), T. koningii (Wood and McCrae, 1975, 1978a) and T. viride (Shoemaker and Brown, 1978a,b; Beldman et al., 1985). Endo-β-1,4-glucanases preferentially hydrolyse internal glycosidic linkages. They attack at various sites with varying degrees of randomness (Ladisch et al., 1981, 1983; Parry et al., 1983; Beldman et al., 1985), producing more chain ends at which exo-β-1,4-glucanase may act (Eriksson and Pettersson, 1975b; Wood, 1975). Prolonged action of endo-β-1,4,-glucanase results in a rapid decrease in chain length or viscosity of cellulose and increasing levels of cellobiose and higher cellodextrins with very little reducing sugar (Wood and McCrae, 1979). In contrast to exo-β-1,4-glucanase, endo-β-1,4-glucanase exhibits very little activity towards crystalline cellulose and strong activity

towards amorphous cellulose such as acid-swollen cellulose and CMC (Wood and McCrae, 1978a; Parry et al., 1983; Beldman et al., 1985; Wood, 1985). Nisizawa (1973) demonstrated that the rate of hydrolysis of oligosaccharides decreases with decreasing degree of polymerisation. It has also been shown that they are capable of hydrolysing cellotriose but not cellobiose or p-nitrophenyl-β-D-glucopyranoside (Beldman et al., 1985; Moloney et al., 1985). Other workers have claimed to have isolated an endo-β-1,4-glucanase from T. viride which demonstrates relatively high activity on crystalline cellulose and only moderate activity on CMC (Beldman et al., 1985), whether this is a true endo-β-1,4-glucanase remains to be seen.

β-glucosidase has been isolated from numerous filamentous fungi including A. fumigatus (Rudrick and Elbein, 1975), Aspergillus niger (King and Smibert, 1963; Reese et al., 1968), C. thermophilum (Lusis and Becker, 1973), G. emersonii (McHale and Coughlan, 1982), S. pulverulentum (Deshpande et al, 1978) and S.thermophilum (Meyer and Canevascini, 1981), T. koningii (Wood and McCrae, 1975b), T. viride (Berghem and Pettersson, 1974; Gong et al., 1977; Beldman et al., 1985). P. funiculosum (Joglekar et al., 1983; Lachke et al., 1983) and Aspergillus phoenicis (Sternberg et al., 1977) both produce high levels of extracellular β-glucosidase. Essentially β-glucosidase catalyses the hydrolysis of cellobiose and cellodextrins, formed by the action of endo-β-1,4-glucanase and exo-β-1,4-glucanase enzymes to glucose. However, the physical, chemical and physiological properties of β-glucosidase from different fungi are markedly different (Lusis and Becker, 1973; Deshpande et al., 1978; Meyer and Canevascini, 1981). Various glucosides such as cellobiose, salicin, esculin, p-nitrophenyl-β-D-glucopyranoside and many more have been

investigated (Shewale, 1982). The existence of two types of β-glucosidase has been proposed, one with aryl-β-glucosidase activity and another showing broader activity towards various glucosides (Berghem and Pettersson, 1974; Rudrick and Elbein, 1975; Sternberg et al., 1977; Deshpande et al., 1978; Meyer and Canevascini, 1981). β-glucosidases from Aspergillus aculeatus (Sakamoto et al., 1985), A. phoenicis (Sternberg et al., 1977), T. reesei (Sternberg et al., 1977) and T. viride (Berghem and Pettersson, 1974) are all similar in that they can hydrolyse both aryl-β-glucosides and cellobiose. Aryl β-glucosidases from C. thermophilum (Lusis and Becker, 1973), Schizophyllum commune (Wilson and Niederpruem, 1967) and Stachbotrys atra (Jermyn, 1965) show no activity towards cellobiose. A broad substrate specificity is characteristic of β-glucosidase enzymes and this property has been used to distinguish β-glucosidase from exo-β-1,4-glucanase (Reese et al., 1968). Typically the β-glucosidases of T. koningii have the ability to hydrolyse other β-1,4 linked dimers of glucose, albeit at widely differing rates (Wood and McCrae, 1978a). Sophorose and laminaribiose are better substrates than cellobiose or gentiobiose, and in this respect the β-glucosidases of T. koningii are similar to those of A. fumigatus (Rudrick and Elbein, 1975). The β-glucosidases of A. phoenicis (Sternberg et al., 1977) and A. niger (Reese et al., 1968) differ in that cellobiose is the better substrate. Berg and Pettersson (1977) reported that β-glucosidase in Trichoderma spp. is mainly intracellular and that only about 5% of total β-glucosidase could be detected in culture filtrates. A number of workers have observed increasing β-glucosidase levels with increasing age of culture (Sternberg et al., 1977; Gong et al., 1979). It has been suggested that this may occur due to

autolysis (Gong and Tsao, 1979). In Clostridium thermocellum (Ng and Zeikus, 1981a) and Thermoactinomyces (Hagerdal et al., 1979) β-glucosidase is entirely intracellular.

Fractionation studies on fungal culture filtrates have demonstrated that each of the cellulase enzymes are generally found to exist in multiple forms (Wood and McCrae, 1975; Shoemaker and Brown, 1978a; Wood et al., 1980; Eriksson, 1981; McHale and Coughlan, 1981). However, opinion is divided on the question of whether such multiplicity reflects genetically determined differences in primary sequence, differential glycosylation after translation or proteolytic modification after secretion (Gum and Brown, 1977; Gong and Tsao, 1979; Moloney and Coughlan, 1985). Individual enzymes would differ in molecular weight, amino acid and carbohydrate composition and have differing specific activities with various substrates (Shoemaker and Brown, 1978a,b; Wood and McCrae, 1978a). Most of the cellulase enzyme components are glycoproteins with the exception of the β-glucosidases of T. viride (Berghem and Pettersson, 1974; Gong et al., 1977). Nakayama et al. (1976) suggested that limited proteolysis may partially be responsible for the multiplicity of endo-β-1,4-glucanase in T. viride. Other workers have supported the notion of post-translation modification by protease in the Trichoderma cellulase system (Gong et al., 1979; Gritzali and Brown, 1979). The carbohydrate content of some cellulase enzymes varies between 1-10% (Enari, 1983) although at least one has been reported to contain as much as 50% carbohydrate (McHale and Coughlan, 1981). As a consequence of this, it has been suggested that the four endo β-1,4-glucanases in G.emersonii (Moloney et al., 1985) and the four exo-β-1,4-glucanases in T.viride (Gum and Brown, 1977) represent

differential glycosylation products of a single enzyme. In contrast, the cellulase enzymes produced by T.reesei are thought to represent an inherently complex system consisting of multiple genetically determined forms of each enzyme type (Fagerstam and Pettersson, 1979; Labudova and Farkas, 1983). These various forms are often found to exhibit differences in substrate specificity suggesting that they may have different functions in vivo (Shoemaker and Brown, 1978a,b). Wood et al. (1984) concluded that multiplicity of forms in the cellulase system is not surprising in view of the steric problems confronting these enzymes. However, it is important to appreciate the difficulty in isolating cellulolytic enzymes in a state of physico-chemical homogeneity. The possibility of forming artificial aggregates and the effect of minute quantities of contaminating enzyme should be borne in mind when investigating multiplicity (Montenecourt, 1983). Investigations of immunological relationships and amino acid composition and sequence may give some clarity to this rather complex situation.

Many assume that the mechanism of cellulase action can be explained in terms of sequential action, where the randomly acting endo-β-1,4-glucanase initiates the attack and the new chain ends generated are then hydrolysed by the endwise attacking exo-β-1,4-glucanase (Berghem and Pettersson, 1973; Streamer et al., 1975). This mechanism, however, does not explain why both endo-β-1,4-glucanase and exo-β-1,4-glucanase are individually both capable of degrading amorphous cellulose but not crystalline cellulose. Yet, when they are acting in concert they can hydrolyse highly ordered cellulose with comparative ease (Wood, 1975). Exo-β-1,4-glucanases are generally more strongly adsorbed to insoluble cellulose than the

endo-β-1,4-glucanases and β-glucosidases are only weakly adsorbed (Bisaria and Ghose, 1977; Ladisch et al., 1983). It would seem, however, that only those endo-β-1,4-glucanase and exo-β-1,4-glucanase enzymes which are adsorbed in close proximity are able to carry out hydrolysis (Ghose and Bisaria, 1979). This would suggest that these two enzymes act as an enzyme-enzyme complex on the surface of cellulose chains (Wood and McCrae, 1978a; Fagerstam and Pettersson, 1980; Wood, et al. 1980). One could envisage, exo-β-1,4-glucanase adsorbing to the crystalline cellulase but only becoming active when an endo-β-1,4-glucanase generates a new chain end. This synergism between endo-β-1,4-glucanases and exo-β-1,4-glucanases on crystalline cellulose has been demonstrated in a number of microorganisms (Halliwell and Griffen, 1973; Streamer et al., 1975; Wood and McCrae, 1979; McHale and Coughlan, 1980; Beldman et al., 1985) but unfortunately is not well understood (Wood and McCrae, 1978a; Wood, 1985). Endo-β-1,4-glucanases from one source have been shown to demonstrate `cross-synergism' with exo-β-1,4-glucanases from another source (Wood and McCrae, 1979; Wood and McCrae, 1986). However, other endo-/exo- pairs even from the same source have not shown such synergism (Wood and McCrae, 1986). This may add further support to the idea that isoenzymes have a characteristic and specific role to play in enzymatic hydrolysis. Synergism has also been demonstrated between two immunologically unrelated exo-β-1,4-glucanases of Trichoderma reesei in solubilizing crystalline cellulose (Gritzali and Brown, 1979; Fagerstam and Pettersson, 1980; Pettersson et al., 1981), further supporting the importance of multiple enzyme forms. This type of synergism has also been demonstrated in Penicillium pinophilum (Wood and McCrae, 1986) and T. viride (Beldman et al., 1985). Wood and McCrae (1986) suggested that the two exo-β-1,4-

glucanases may be stereospecific enzymes concerned with the hydrolysis of the two different configurations of non-reducing end groups that would exist in cellulose. To date, no such synergism has yet been reported between individual endo-β-1,4-glucanases. In the absence of exo-β-1,4-glucanases, the cleaved glycosidic links would rapidly reform due to the highly ordered nature of the substrate. On the other hand, in the absence of endo-β-1,4-glucanases, ends for exo-attack would not be available. When acid swollen cellulose is the substrate, purified components show a simple addition of enzyme activity rather than synergism (Streamer et al., 1975). The fact that cellulase enzymes may rapidly be removed from solution to form persistent enzyme-substrate complexes may well explain the low activity of cellulase and the rapid decline in hydrolysis rates (Mandels, 1985). Other suggestions for the decline in hydrolysis rates are substrate depletion, product inhibition, enzyme inactivation or decreased susceptibility of the residual substrate (Mandels, 1985). However, if the cellulase concentration was increased there would be a greater chance of endo-β-1,4-glucanases and exo-β-1,4-glucanases being adsorbed in close proximity. This would result in prolonged rapid hydrolysis and a higher levelling off value (Gilbert and Tsao, 1983; Ryu et al., 1984). It has been suggested that the surface area available for enzyme attack may be a controlling factor (Millet et al., 1979; Ryu and Lee, 1983). Ball-Milling and/or heating of resistant residual substrates has been shown to make the residue more susceptible to enzymatic hydrolysis (Ghose and Kostick, 1969; Mandels and Weber, 1969). Adsorbed enzyme is held very tenaciously and is difficult to remove by washing with water or dilute buffer. The catalytic mechanism for hydrolysis is

not yet clear although it has been suggested that endo-β-1,4-glucanases and exo-β-1,4-glucanases use the same catalytic mechanism for hydrolysis (Wood, 1985).

Generally, the synthesis of cellulase enzymes in microorganisms is regulated by induction and catabolite repression (Nisizawa et al., 1971, 1972: Eriksson and Hamp, 1978; Canevascini et al., 1979; Sternberg and Mandels, 1980). A range of substrates have been reported to induce cellulase synthesis in different organisms. These include cellulose, lactose and a number of other glucosides (Sternberg et al., 1977; Deshpande et al., 1978; Shewale and Sadana, 1978; Gong et al., 1979). Other workers have indicated that cellobiose, at low concentrations may induce cellulase synthesis whereas at high concentrations actually inhibits cellulase synthesis (Berg and Pettersson, 1977; Deshpande et al., 1978). Sophorose does not normally induce cellulase synthesis in filamentous fungi, although it has been reported to induce cellulase synthesis in T. viride (Mandels et al., 1962; Nisizawa et al., 1971; Sternberg and Mandels, 1979). Bacteria are generally more responsive to sophorose induction (Yamane et al., 1970; Stewart and Leatherwood, 1976). When cellobiose or sophorose are the inducing substrates very little cellulase is usually observed in the culture filtrates indicating that these substrates are taken into the cell and then hydrolysed to glucose by intracellular or cell-bound β-glucosidases (Wilson and Niederpruem, 1967; Lusis and Becker, 1973; Deshpande et al., 1978; Gong et al., 1979; Sternberg and Mandels, 1979). Cellulose is the universal inducer of cellulase synthesis, however, the actual mechanism of induction by insoluble cellulose is open to debate. A constitutive "basal synthesis" of cellulase enzymes has

been proposed in a number of organisms (Mandels and Reese, 1960; Eriksson and Hamp, 1978; Gong et al., 1979). In this way, small amounts of cellulase are released by the cells into the growth medium. These enzymes then hydrolyse the cellulose, thus providing a limited amount of cellobiose which may then induce cellulase synthesis and release into the culture medium. Other workers have proposed that physical contact must occur between the fungus and the inducing substrate for induction to occur (Berg and Pettersson, 1977; Binder and Ghose, 1978). This suggests that there may be recognition sites on the surface of the cell which trigger induction.

In addition to catabolite repression, the cellulase enzymes themselves have been shown to be susceptible to end product inhibition (Halliwell and Griffen, 1973; Ghose, 1977; Folan and Coughlan, 1979; Ladisch et al., 1981, 1983). Cellobiose has been shown to inhibit exo-β-1,4-glucanase (Halliwell and Griffen, 1973; Berghem et al., 1975; Hsu et al., 1980; Moloney et al., 1985) and endo-β-1,4-glucanase (Halliwell and Griffen, 1973; Wood and McCrae, 1975; Ladisch et al., 1980). A number of workers have observed that exo-β-1,4-glucanases are more strongly inhibited by cellobiose than the endo-β-1,4-glucanases (Gong and Tsao, 1979; Ladisch et al., 1983; Moloney et al., 1985). Cellobiose inhibition has also been shown to be greater with crystalline than amorphous substrates (Ryu et al., 1982). Glucose also inhibits exo-β-1,4-glucanase but to a lesser extent (Hsu et al., 1980). β-glucosidase plays an important role in saccharification of cellulose by removing cellobiose from solution and thus preventing inhibition of endo-β-1,4-glucanase and exo-β-1,4-glucanase (Halliwell, 1975; Sternberg, 1976a; Gong et

al., 1979; Mandels et al., 1981). However, the β-glucosidases are subject to product inhibition by glucose which accumulates as hydrolysis proceeds (Sternberg, 1976a; Gong et al., 1977; Bisset and Sternberg, 1978). Thus at a critical glucose concentration, β-glucosidase enzymes will be inhibited and as a consequence cellobiose levels will rise and thus switch off endo-β-1,4-glucanase and exo-β-1,4-glucanase activity and subsequently cellulose hydrolysis. Nature provided T. reesei with β-glucosidase adequate to support its growth on cellulose, but inadequate for use in a hydrolysis reactor where glucose concentrations quickly reach 10% or more. This problem may be alleviated by adding supplementary β-glucosidase from another source such as A. phoenicis (Sternberg et al., 1977; Bisset and Sternberg, 1978; Allen and Sternberg, 1980; Allen and Andreotti, 1981). Other inhibitors of β-glucosidase include gluconolactone and nojirimycin (Reese and Parrish, 1971; Wood and McCrae, 1982; Van Tilbeurgh and Claeyssens, 1985). Inhibition may be competitive depending on the enzyme source and the inhibitor (Emert et al. 1974; Sternberg et al., 1977; Deshpande et al., 1978).

S. pulverulentum possesses several mechanisms to prevent enzyme inhibition by cellobiose. This organism produces two β-glucosidases, cellobiose oxidase and cellobiose-quinone oxidoreductase (Westermark and Eriksson, 1974, 1975; Ayers et al., 1978; Deshpande et al., 1978; Eriksson, 1981). The latter is important in cellulose as well as in lignin degradation. Its function is to reduce phenoxy radicals and quinones formed by the action of phenol oxidases on degradation products from lignin (Westermark and Eriksson, 1974; Eriksson,

1983). Cellobiose quinone oxidoreductase has also been reported to be produced by S. thermophilum (Canevascini et al., 1979; Coudray et al., 1982). An oxidative enzyme, cellobiose oxidase, which oxidizes cellobiose and higher cellodextrins to their corresponding aldonic acids, has been isolated from culture filtrates of S. pulverulentum (Ayers et al., 1978; Eriksson, 1981). It has been suggested that this enzyme may also be present in T. koningii (Wood and McCrae, 1978b) and T. reesei (Eriksson, 1975). Gluconolactone, a competitive inhibitor of β-glucosidase is produced by oxidation of glucose by the enzyme, glucose oxidase. How glucose oxidase is induced in S. pulverulentum is not fully known. It may be that one of its functions is to regulate the glucose level by preventing β-glucosidase enzymes from splitting cellobiose or cellobionic acid (Deshpande et al., 1978).

Numerous bacteria and protozoa produce endo-β-1,4-glucanase but only a few have been reported to produce exo-β-1,4-glucanase (Chang and Thayer, 1977). Cytophaga and Thermoactinomyces have both been reported to secrete both endo-β-1,4-glucanase and exo-β-1,4-glucanase (Chang and Thayer, 1977; Hagerdal et al., 1979). The anaerobic bacterium, Clostridium thermocellum has been shown to secrete a large quantity of extracellular cellulase that effects extensive degradation of crystalline cellulose (Ng et al., 1977; Shinmyo et al., 1979; Ng and Zeikus, 1981b; Johnson et al., 1982; Lamed et al., 1983). Such cellulase activity differs considerably from that of T. reesei (Ng and Zeikus, 1981a). The endo-β-1,4-glucanase/ exo-β-1,4-glucanase activity ratio is higher and there is no extracellular β-glucosidase and β-xylosidase activity. Cellobiose and xylobiose

are mainly formed in the prolonged hydrolysis of microcrystalline cellulose or xylan. In C. thermocellum, it has been suggested that cellodextrins may be transported into the cells where they can be converted to glucose-1-phosphate and glucose by intracellular cellodextrin phosphorylase and cellobiose phosphorylase (Eriksson, 1983). An advantage of C. thermocellum and Thermomonospora sp. is the thermal stability of the cellulases produced (Ng et al., 1977; Zeikus, 1979; Hagerdal et al., 1980; Moreira et al., 1981). Bacterial cellulases also tend to be less sensitive to end product inhibition (Choudhury et al., 1980; Ng and Zeikus, 1981a; Sonnleitner, 1983). Although C. thermocellum may grow more rapidly than fungi on derived or native forms of cellulose, it has yet failed to show extracellular cellulolytic activities comparable with that of Trichoderma (Ng and Zeikus, 1981a). However, efficient mutants of C. thermocellum (Shinmyo et al., 1979; Garcia-Martinez et al., 1980) and Thermomonospora sp. (Meyer and Humphrey, 1982) have been developed.

Of the many organisms examined for the production of cellulase enzymes, Trichoderma reesei has emerged as one with clear advantages and potential. This organism produces high levels of extracellular enzymes and is capable of hydrolysis of highly ordered cellulose (Ryu and Mandels, 1980; Labudova and Farkas, 1983). However, the disadvantages of the Trichoderma system are that it (i) has low specific activity, (ii) suffers from end product inhibition and/or catabolite repression, (iii) shows no digestion of lignin, and (iv) is rapidly inactivated at high temperatures. Cellobiose is the major product of saccharification (Montenecourt and Eveleigh, 1977a; Ryu and Mandels, 1980) due to the relatively low level of β-glucosidase

in culture filtrates. Supplementary β-glucosidase from other sources including A. aculeatus (Murao et al., 1979) and A. phoenicis (Sternberg et al., 1977; Bisset and Sternberg, 1978) has been shown to increase the rate and extent of cellulose hydrolysis (Berghem et al., 1975; Sternberg et al., 1977; Allen and Sternberg, 1980). Since the substrate for β-glucosidase is soluble, the enzyme can be immobilized, recovered and reused (Ghose and Kostick, 1970; Srinivasan and Bumm, 1974; Bisset and Sternberg, 1978).

The success of the enzymatic process depends very much on improving the economics of a currently expensive process. In this context, several recent assessments of this process have shown that the major cost is the production of cellulase (Ryu et al., 1979; Ryu and Mandels, 1980; Spano et al., 1980; Mandels et al., 1981). Therefore, a great deal of research has been directed towards improving the activity and productivity of the cellulase. These objectives include (i) optimisation of fermentation conditions to increase enzyme yields, (ii) search for more efficient and economical pretreatments, (iii) advances in process technology, (iv) increased stability, and (v) search for new strains and improvement of existing strains to increase resistance to end product inhibition and/or catabolite repression and to further the utilization of lignin and pentoses (Mandels, 1985).

The optimisation of conditions for cellulose hydrolysis involves strain selection and optimisation of media composition and growth conditions (Mandels et al., 1981; Tangnu et al., 1981; Saddler, 1982; Shaker et al., 1984). A number of substrates have been investigated for suitability. Solka Floc and Avicel are too expensive and so it is desirable to use lignocellulosic raw materials

(Blanch and Wilke, 1983). The action of cellulase on cellulosic biomass depends greatly on the origin of the biomass, its composition, the synergistic action with other classes of enzymes (laccase, pectinases, phenol oxidases and xylanases) and the susceptibility of the substrate to enzymatic attack (Ladisch et al., 1983). Particle size, degree of crystallinity and the extent of lignification all affect the susceptibility of the substrate to enzymatic hydrolysis (Fan et al., 1980). Paper mill pulps and wastes are most susceptible to enzymatic degradation due to their high cellulose content and the chemical and physical treatments they undergo during the paper making process.

Physical pretreatments such as grinding, milling or Shearing (Han et al., 1978; Millet et al., 1979), chemical pretreatments (Tsao et al., 1978; Fan et al., 1981) or a combination of the two (Detroy et al., 1980), have been shown to increase the rate and extent of hydrolysis. Other pretreatments including steam, radiation and microbial treatments (Kirk and Moore, 1972; Nesse et al., 1977; Han et al., 1981) have been used. Ball-milling, though expensive has been shown to be very effective in reducing the crystallinity of the cellulose, increasing the surface area and bulk density and therefore allowing higher substrate concentrations (Mandels et al., 1974). As explained earlier, cellulases are adsorbed very strongly onto the cellulosic substrate (Mandels, 1975), sometimes by as much as 90% (Reese, 1982). Due to the high cost of cellulase production, enzyme recovery and re-use is obviously very important. Methods used to recover the enzymes from residual substrates include:- acetone precipitation, addition of urea, counter-current adsorption of enzyme onto fresh substrate, ultrafiltration and simple washing (Mitra and

Wilke, 1975; Blanch and Wilke, 1983). Surfactants such as Triton X 100 and Tween 80, at low concentration have been reported to increase cellulase production in a number of organisms (Folan and Coughlan, 1978; Castonon and Wilke, 1981; Tangnu et al., 1981; Lachke et al., 1986). This phenomenon has been attributed to an increase in the permeability of the cell membrane, therefore allowing more rapid secretion of the enzyme (Sternberg, 1976b; Blanch and Wilke, 1983). However, these surfactants may also cause foaming problems in stirred vessels.

Cellulases may be isolated and used to effect cellulose hydrolysis in separate saccharification reactions. Glucose syrups of 10-15% have been produced by hydrolysis of pure cellulose (Mandels and Weber, 1969; Mandels et al., 1971) and milled newspaper (Mandels, 1975) using T. reesei cellulase. Continuous production of cellulase enzymes is a route for increasing productivity. This may be done in a two stage process in which the first stage is operated at conditions optimum for cell growth and the second stage is optimised for enzyme production (Blanch and Wilke, 1983). In order to obtain high concentrations of sugar it is necessary to operate the hydrolysis stage with high solid loadings and subsequently high enzyme activities are required (Blanch and Wilke, 1983). Production of soluble sugars may then be followed by fermentation (Ghose and Ghosh, 1978; Gong et al., 1981a), yielding a variety of products (Ladisch et al., 1983). There are a number of microorganisms that are able to produce ethanol from soluble sugars. The majority of fermentation alcohol produced today employs the yeast, Saccharomyces cerevisiae or its related species. Unfortunately, S. cerevisiae is unable to utilize the pentoses that result from the hydrolysis of

lignocelluloses (Avgerinos and Wang, 1980; Ladisch and Tsao, 1986). It is possible however, to add glucose isomerase which would convert D-xylose to D-xylulose which could then be converted to ethanol (Gong et al., 1981b).

An attractive alternative involves a process in which cellulase production, cellulose hydrolysis and ethanol production are all carried out simultaneously in a single stage process (Cooney et al., 1978; Avgerinos and Wang, 1980; Herrero and Gomez, 1980). The simultaneous saccharification/fermentation (SSF) of cellulose to ethanol alleviates the problem of end product inhibition (Blotkamp et al., 1978). Glucose does not accumulate in this system but rather is fermented to ethanol immediately following saccharification. The result is an increase in yield of 25% or more when compared with the separate processes of saccharification and fermentation. Zymomonas sp. may be preferred to S. cerevisiae because of the advantages of anaerobic growth which include ease of operation and faster growth rates (Bringer et al., 1984; Spangler and Emert, 1986). However, since the optimum temperature for cellulose hydrolysis by T. reesei cellulase is 50°C (Saddler et al., 1983) it would be advantageous to utilize a temperature tolerant ethanol producer. Candida brassicae may therefore be preferred to S. cerevisiae and Z. mobilis due to its ability to produce ethanol efficiently at high temperatures (Spangler and Emert, 1986). C. thermocellum is a very useful organism due to its ability to convert cellulose to ethanol (Ng et al., 1977; Weimer and Zeikus, 1977; Herrero and Gomez, 1980). However, unlike other alcohol producing microbes such as yeasts, C. thermocellum is strongly inhibited by relatively low concentrations (5 g l^{-1}) of ethanol. An ethanol resistant mutant of this organism has been

isolated (Herrero and Gomez, 1980) which is inhibited by 20 g l^{-1} ethanol. The anaerobic degradation of cellulose by bacteria is limited by the accumulation of inhibitory fermentation products and by the low titre of extracellular cellulase (Mandels, 1981; Wolin and Miller, 1983). Toxic fermentation end products can be avoided by growing the primary hydrolytic decomposer in the presence of a secondary organism such as a methanogen resulting in an increase in the extent of cellulose utilization (Weimer and Zeikus, 1977; Wolin and Miller, 1983; Zeikus, 1983). To this end, C. thermocellum has been used in co-culture with C. thermohydrosulfuricum (Ng et al.,1981),C. thermosaccharolyticum (Wang et al., 1979; Avgerinos et al., 1981), and Methanobacterium thermoautotrophicum (Weimer and Zeikus, 1977) increasing the variety of suitable substrates and ethanol yields (Ng et al., 1981). There are, however, a number of drawbacks and limitations (Linko et al., 1983). Consequently, a lot of work needs to be done before this process becomes economically feasible.

The best known cellulases such as those from P. funiculosum (Wood et al., 1980; Mishra et al., 1985),S. pulverulentum (Eriksson, 1975; Eriksson and Pettersson, 1975a,b) and the Trichoderma spp. (Wood and McCrae, 1975; Ryu and Mandels, 1980; Beldman et al., 1985) are usually stable at temperatures around 50°C. Unfortunately, at this temperature, enzyme reactors are frequently contaminated by microorganisms which utilize the sugars liberated by the hydrolytic reactions. A number of workers have therefore examined the possibility of using thermophilic bacteria and fungi (Table 1.1) as a source of thermally stable cellulases. Use of such organisms would facilitate the use of higher temperatures,

therefore reducing the likelihood of contamination. Other advantages of thermally stable cellulases would be (i) a reduction in cooling and operating costs and (ii) a reduction in the time required to achieve maximum enzyme production (Liu et al., 1984).

Mutation and selection programmes have been used in an attempt to improve the yield and efficiency of the cellulase complex in G. emersonii (Moloney et al., 1983), P. funiculosum (Pavusek et al., 1980; Joglekar and Karanth, 1984; Lachke et al., 1986) and T. reesei (Montenecourt and Eveleigh, 1977b, 1979; Mishra et al., 1982). Mutants with enhanced cellulase production have been obtained from these fungi,however, these are often unstable and suffer from delayed growth and sporulation (Joglekar and Karanth, 1984; Lachke et al., 1986).

Fungal species do not normally exchange genetic material by natural means. However, the introduction of new methodologies such as protoplast fusion and transformation, including gene cloning and recombinant DNA techniques has opened up the horizons for the genetic manipulation of microorganisms. Such techniques could therefore be applied to the field of cellulose hydrolysis in an attempt to improve the efficiency of the enzymatic process.

Transformation is the term given to the transfer of a heritable character by a naked DNA molecule (Mishra, 1985). This technique is well established in bacteria (Hotchkiss, 1977) and yeasts (Beggs, 1978; Hinnen et al., 1978). Transformation systems have also been developed in the fungi, Aspergillus nidulans (Ballance et al., 1983; Tilburn et al., 1983; Yelton et al., 1984) and Neurospora crassa (Mishra et al., 1973; Case et al., 1979; Case, 1981; Schweizer

et al., 1981). In this process, fungal cells may be treated with total DNA isolated from the donor strain (Mishra et al., 1973). Alternatively, fungal cells may be treated with a DNA preparation that has been previously cloned and amplified in Escherichia coli. Both methods depend upon the presence of suitable selectable characters such as drug resistance, allowing the isolation of transformants by complementation of a defective function in the recipient cells. In molecular cloning, eukaryotic DNA is broken down into small fragments by hydrodynamic shearing or by partial digestion with a restriction endonuclease. The DNA fragments are then inserted into a suitable vector which is then used to transfect a suitable organism. A number of vectors have been used in the cloning of fungal genes. These include bacteriophage (Murray and Murray, 1974), cosmid (Ish-Horowicz and Burke, 1981), eukaryotic plasmids (Gunge, 1983) and prokaryotic plasmids (Sinsheimer, 1977; Bolivar and Backman, 1979). Gene libraries are usually constructed in E. coli as a collection of individual bacterial colonies, harbouring recombinant plasmids containing foreign DNA as segments (Clarke and Carbon, 1978). Other workers have used yeast cells as the recipient organism (Fink et al., 1978; Hinnen and Meychak, 1981). Some cellulase genes have been successfully cloned. Endo-β-1,4-glucanase genes from Bacillus subtilis (Koide et al., 1986), Cellulomonas fimi (Whittle et al., 1982; Gilkes et al., 1984;) and C. thermocellum (Cornet et al., 1983; Millet et al., 1985) have been successfully cloned in E. coli. Other workers have demonstrated the expression in S. cerevisiae of cloned endo-β-1,4-glucanases from B. subtilis (Hincliffe and Box, 1984), C. fimi (Skipper et al., 1985) and C. thermocellum (Sacco et al., 1984). Obviously, a lot of work has yet to be done on the cloning of cellulase genes before an organism could be constructed

for use in cellulose hydrolysis. Fungal cellulase genes could be cloned but, to date, no reports have been published. There are a number of problems in cloning fungal genes. Fungal organisms are eukaryotic and their genome normally contains introns which would not be processed effectively in prokaryotic organisms. Additionally, many fungal enzymes are glycoproteins and E. coli is unable to glycosylate protein. Recently Shoemaker et al. (1983) reported the isolation of a complete gene for exo-β-1,4-glucanase. However, there are still a lot of problems to unravel in the expression of fungal genes in bacteria and yeasts. Due to the complexity of the cellulase enzyme system, gene cloning may well take some time before any real success is achieved. Clearly, success will depend on the development of new and improved vector and gene transfer systems.

In recent years, liposomes have been successfully used as vehicles for introducing genetic material into both eukaryotic and prokaryotic cells. After encapsulation of RNA (Ostro et al., 1977), chromosomes (Mukherjee et al., 1978) and viruses (Fraley et al., 1980) into liposomes, the phospholipid membranes of the vesicles were induced to fuse with mammalian cells resulting in the transfer of liposome contents into the recipient cells. With similar procedures, nucleic acids (Rouze et al., 1983; Lurquin, 1984) and plant viruses (Fukunaga et al., 1981; Nagata et al., 1981) have been introduced into plant cells. Increased transformation rates have been achieved in bacteria (Makins and Holt, 1981), fungi (Radford et al., 1981) and yeast (Ahn and Pack, 1985) using liposome-mediated transformation. Recent developments involve the use of protoplasts. Liposomes are taken up by protoplasts by endocytosis in the presence of polyethylene glycol (PEG) and calcium chloride (Tilburn et al.,

1983; Nagata, 1984). Other workers have demonstrated the transfer of isolated nuclei into S. cerevisiae protoplasts (Becher et al., 1982; Ferenczy and Pesti, 1982).

An alternative approach to transformation involves the use of protoplast fusion. Fusion of protoplasts may occur spontaneously (Lopez-Belmonte et al., 1966) or as a result of centrifugation (Ferenczy et al., 1974). Binding and Weber (1974) reported that seawater or calcium ions, at high pH, induced protoplast fusion. However, it was only after the introduction of PEG as a fusion agent that this technique became the focus of any major interest. Kao and Michayluk (1974) demonstrated that this agent was highly effective in inducing fusion of plant protoplasts. Large random aggregates form in the presence of PEG, but when this agent is removed, the aggregates dissociate. The presence of calcium ions has been shown to increase the efficiency of PEG in fusing fungal protoplasts (Anne and Peberdy, 1975; Ferenczy et al., 1976). This combination has been shown to be equally effective in fusing protoplasts from bacteria (Hopwood et al., 1977; Fodor et al., 1978) and yeasts (Svoboda, 1978; Whittaker and Leach,1978). Another approach to achieving cell fusion involves a technique based on the combined action of dielectrophoresis and the electrical breakdown of cell membranes (Zimmermann and Vienken, 1982, 1984). Electrical fusion has been successful in plants (Zimmermann and Scheurich, 1981; Watts and King, 1984; Bates et al., 1985) and yeasts (Halfmann et al., 1983). Unfortunately, there have been no published data regarding the electrical fusion of fungal protoplasts. It should be noted that whatever the method used, protoplast fusion is an aid to cell fusion and not necessarily an aid to nuclear fusion, although indirectly the frequency of nuclear fusion may be

enhanced in a fusion mixture (Ball, 1985).

In protoplast fusion, a situation similar to the sexual reproductive process occurs, in which the whole genomes of the two parental types are brought together into one cell. Nuclear fusion (karyogamy) may then occur producing diploids or polyploids, which may or may not be stable. Recombinant colonies may then arise from these "hybrids" as spontaneous events or following exposure to haploidizing agents such as benomyl and p-fluorophenylalanine. This technique provides a means of breeding in fungi which are not capable of sexual reproduction and thus provides opportunities for industrial breeding. Intraspecies hybrids have been obtained in bacteria (Hopwood et al., 1977) and fungi (Ferenczy et al., 1975; Anne and Peberdy, 1976; Wilson et al., 1982). Interspecies crosses, on the otherhand, offer great potential in the genetic manipulation of industrial organisms. Hybridization between different species provides the possibility of an enhancement of product titre or the generation of novel metabolites, arising from the interaction of different pathways (Anne and Peberdy, 1985). Interspecies hybrids have been reported in bacteria (Godfrey et al., 1978), fungi (Anne et al., 1976; Ferenczy, 1976) and yeasts (Whittaker and Leach, 1978; Sipiczki, 1979). Intergeneric hybrids have been reported in yeasts (Provost et al., 1978; Svoboda, 1980; Groves and Oliver, 1984; Perez et al., 1984). Generally, fusion products from interspecies crosses between distantly related species and intergeneric crosses show a marked predominance of one of the partners, indicating a preferential loss of chromosomes (Peberdy et al., 1977; Anne and Eyssen, 1978).

Fusion products have been selected in most instances through nutritional complementation of auxotrophic mutants (Anne and Peberdy, 1975, 1976; Ferenczy et al., 1977; Kevei and Peberdy, 1977). This technique, although useful in classical genetics, is extremely time consuming and the resulting mutants often have reduced growth rates and lowered biochemical activity. The application of unselected markers such as colony morphology or metabolite production (Chang et al., 1982) requires much more effort to select recombinant progeny. Other workers have used drug resistance markers such as resistance to acriflavine (Peberdy and Bradshaw, 1982) or oligomycin (Croft et al., 1980; Anne, 1982).

Attempts to increase the fusion frequency have largely been restricted to bacteria. Protoplasts of one or both of the parental types are rendered non-viable by treatment with antibiotic (Levi et al., 1977), heat (Fodor et al., 1978) or UV-irradiation (Hopwood and Wright, 1981) prior to fusion. To date, no reports have been published regarding the use of these "dead donor" techniques in the fusion of filamentous fungi, although an increased fusion frequency has been obtained in P. chrysogenum using UV-irradiation (Anne unpublished results).

The success of protoplast fusion and transformation experiments depends upon the generation of protoplasts in vast quantities (Grachek and Emert, 1984; Hou and Jong, 1985). Therefore, the optimum conditions for the isolation of protoplasts need to be determined for each of the species under investigation (Davis, 1985).

Protoplasts have been isolated from all four major taxonomic groups of fungi (Strunk, 1970; Villanueva and Garcia-Acha, 1971; Peberdy, 1972) including A. fumigatus (Archer, 1977; Ferenczy et al., 1977; Hearn et al., 1980). A. niger (Musilkova and Fencl, 1968; Davis et al., 1977; Thomas et al., 1979), Penicillium ochrochloron (Gadd and White, 1985) and T. reesei QM 9414 (Grachek and Emert, 1984; Kolar et al., 1985) using a variety of methods.

Initially protoplasts were obtained by exerting mechanical pressure upon cells (Necas, 1955). Protoplasts have been produced from Penicillium glaucum by cultivation on thickened cell-free guinea pig serum (Meinecke, 1960). The digestive juices of the snail, Helix pomatia, available commercially as β-glucuronidase has also proved effective in producing protoplasts from yeasts (Eddy and Williamson, 1957; Deutch and Parry, 1974) and filamentous fungi (Bachmann and Bonner, 1959; Musilkova and Fencl, 1968; Thomas et al., 1979). In filamentous fungi, the most effective method of obtaining protoplasts involves the digestion of the cell wall by lytic enzymes of microbial origin.

The effectiveness of lytic enzyme preparations depends on the composition and structure of the fungal cell wall. Chemically, the fungal cell wall is composed of 80-90% polysaccharides with the remainder consisting of protein and lipids (Bartnicki-Garcia, 1968) although there are some deviations from this general rule (Shifrine and Phaff, 1958). It should also be noted that the fungal cell wall is not a homogeneous structure but an orientated, inter-connected series of different polymers (Hunsley and Burnett, 1970; Trinci, 1978). A number of enzymes have been reported to be important in determining protoplast release from filamentous fungi. These include

cellulase, chitinase, α-1,3-glucanase, β-1,3-glucanase, laminarinase, lipase and protease (Sietsma et al., 1968; de Vries and Wessels, 1973; Anne et al., 1974; Hamlyn et al., 1981).

During their autolytic phase of growth, filamentous fungi produce lytic enzymes which can catalyse the degradation of cell wall polysaccharides (Reyes and Lahoz, 1977). Protoplasts have been obtained from Neurospora crassa (Reyes and Lahoz, 1976), Aspergillus nidulans (Isaac and Gokhale, 1982) and some other fungi (Reyes et al., 1984) using autolytic enzymes isolated from the same fungus. Extracellular crude enzyme preparations from Micromonospora sp. (Gascon et al., 1965a,b; Benitez et al., 1975) and Streptomyces sp. (Gibson and Peberdy, 1972; Anne et al., 1974; Laborda et al., 1974) have been used effectively to isolate protoplasts from a number of filamentous fungi. Other fungi have been used for lytic enzyme preparation including Penicillium sp. (Musilkova et al., 1969; Benitez et al., 1975) and T. viride (Peberdy and Isaac, 1976; Hearn et al., 1980; Hou and Jong, 1985). A number of inducing substrates have been used to promote the synthesis of lytic enzymes including crude mycelial preparations (Gibson and Peberdy, 1972; Anne et al., 1974), purified cell walls (de Vries and Wessels, 1972; Davis et al., 1977) and polysaccharides such as chitin and laminarin (Laborda et al., 1974; Peberdy and Isaac, 1976). The effectiveness of an enzyme preparation depends on the inducing substrate and the conditions of preparation. However, the laboratory production of suitable enzyme mixtures is time consuming and sometimes unreliable (Peberdy, 1978; Isaac and Peberdy, 1979). Such procedures have generally been superseded by the use of commercially available enzyme mixtures (Hamlyn et al., 1981).

In addition to their use in protoplast fusion and transformation, protoplasts are useful in the isolation of DNA (Morris, 1978) and organelles (Longley et al., 1968). They have also been used to study aflatoxin synthesis (Dutton and Anderson, 1978), antibiotic production (Duncan and Newton, 1970; Fawcett et al., 1974), enzyme secretion (Kolar et al., 1985), protein synthesis (Isaac and Peberdy, 1985) and steroid transformation (Dlugonski et al., 1984). Other workers have used protoplasts to study the biosynthesis and function of the cell wall (Strunk, 1970; Villanueva and Garcia-Acha, 1971; Archer, 1977; Necas and Svoboda, 1985).

The aims of this thesis will be to investigate: (i) Cellulase and related enzyme production in a number of filamentous fungi using both protoplast and mycelial cultures; (ii) the conditions affecting protoplast release in a number of filamentous fungi; (iii) the prospects for intraspecies and interspecies hybridisation within the genera Aspergillus and Penicillium with a view to increasing enzyme production and enzyme stability.

2.1 ORGANISMS

(a) Acetobacter xylinum (8426) was obtained from the National Collection of Marine Bacteria and Industrial Bacteria, Torry Research Station, Aberdeen, Scotland.

(b) Aspergillus fumigatus (Fresenius, IMI 226 070); Chaetomium globosum (Kunze ex. steudel, IMI 45 550); Geosmithia emersonii (Stolk. Pitt., IMI 146 499); Penicillium funiculosum (Thom, IMI 61 383); Penicillium janthinellum (Biourge, IMI 90 838); Penicillium ochrochloron (Biourge, IMI 61 271) Sporotrichum pulverulentum (Phanerochaete chrysosporium, Novobranova, IMI 174 727) and Sporotrichum thermophilum (Myceliophthora thermophila, Apinis, IMI 145 135) were obtained from the Commonwealth Mycological Institute (CMI), Kew, Surrey, England.

(c) Chaetomium thermophilum was isolated from soil by overlaying soil particles with Eggins and Pugh Basal medium (Table 2.1) containing 1%(^{w}/v) ball-milled cellulose (BMC) and 0.2%(^{w}/v) glucose. Rose bengal (0.035g l^{-1}) was included in the medium to prevent the growth of bacteria. Plates were incubated for two days at 48°C to encourage the growth of thermophilic fungi. C.thermophilum was isolated free from other thermophiles by successive replating onto fresh agar plates. Further purification was achieved by preparing a dilution series of a spore suspension in sterile saline solution, 0.9%(^{w}/v) to cover the range 1 x 10^{3}-1 x 10^{10} spores ml^{-1}. Duplicate 0.5ml aliquots of each dilution were spread onto fresh agar plates of the same medium and incubated at 48°C. The dilution which produced a single, fast growing colony was selected and used to

inoculate fresh medium contained in 1oz McCartney bottles. These were maintained as stock cultures of the strain.

(d) Penicillium dupontii was isolated from soil using the same method as for C. thermophilum but using Eggins and Pugh Basal medium (Table 2.1) containing 1%(w/v) glucose and Rose bengal (0.035g l^{-1}).

(e) Aspergillus niger (University of Strathclyde strain) and Trichoderma viride were provided by Dr. B. Davis, Sheffield City Polytechnic.

2.2 GROWTH OF ORGANISMS

2.2.1 A. xylinum

Stock cultures of A. xylinum were produced by growing the bacterium in Universal bottles on agar slopes of growth medium described in Table 2.2. Cultures were incubated at 30°C for two days after which they were removed and stored at room temperature.

2.2.2 Mesophilic and thermophilic fungi

The mesophilic fungi (A. niger, C. globosum, P. funiculosum, P. janthinellum and P. ochrochloron) and the thermophilic fungi (G. emersonii and P. dupontii) were all maintained on Eggins and Pugh Basal medium (Table 2.1) containing 1%(w/v) glucose. Another mesophile, T. viride was maintained on agar slopes of the medium described in Table 2.3. The mesophile, S. pulverulentum and the thermophiles, C. thermophilum and S. thermophilum were all maintained on Eggins and Pugh Basal medium (Table 2.1) containing 1%(w/v) BMC.

TABLE 2.1. EGGINS AND PUGH BASAL MEDIUM

Ammonium sulphate	0.5 g
L-Asparagine	1.0 g
Potassium dihydrogen orthophosphate (stock solution: 100 g l^{-1})	10.0 ml
Potassium chloride	0.5 g
Magnesium sulphate	0.2 g
Calcium chloride	0.1 g
Yeast extract	0.5 g
Agar, No. 3	20.0 g
Distilled water	1.0 l

The above constituents were mixed although potassium dihydrogen orthophosphate was dissolved separately. After adjusting the pH to 5.5 with 1 M sodium hydroxide, the medium was autoclaved at 15 lb in^{-2} and 121°C for 15 min. For liquid cultures agar was omitted and Rose bengal (0.035 g l^{-1}) was added in cellulase screening methods as described in the text (Section 3.2).

TABLE 2.2. ACETOBACTER XYLINUM GROWTH MEDIUM

Glucose	20.0 g
Bacteriological peptone	5.0 g
Yeast extract	5.0 g
Citric acid	1.2 g
Disodium hydrogen orthophosphate	5.7 g
Agar, No. 1	20.0 g
Distilled water	1.0 l

The above constituents were mixed and after the pH was adjusted to 6.0 with 1M Sodium hydroxide the medium was autoclaved at 15 lb in^{-2} and 121°C for 15 min.

TABLE 2.3. TRICHODERMA VIRIDE GROWTH MEDIUM

Ammonium sulphate	1.4 g
Urea	0.3 g
Glucose	3.0 g
Glycerol	6.0 ml
Potassium dihydrogen orthophosphate (stock solution: 100 g l^{-1})	10.0 ml
Mycological peptone	1.0 g
Magnesium sulphate	0.3 g
Agar, No. 3	20.0 g
Distilled water	1.0 l

The above constituents were mixed although potassium dihydrogen orthophosphate was dissolved separately. After adjusting the pH to 5.5 with 1 M sodium hydroxide, the medium was autoclaved at 15 lb in^{-2} and 121°C for 15 min. For liquid cultures agar was omitted and glucose was substituted with powdered fungal cell walls in the preparation of the lytic enzymes (Section 2.5).

Mesophilic and thermophilic fungi were incubated at 30°C and 48°C respectively. A. fumigatus, a thermotolerant fungus, was maintained on Eggins and Pugh Basal medium (Table 2.1) containing 1%(W/v) glucose and incubated at 44°C. After 5-7days, dense sporulation was evident. Cultures were removed and stored at 4°C until required for use. The thermophiles, however, were found to be sensitive to cold storage and were therefore stored at room temperature.

Auxotrophic mutants of A. niger and P. ochrochloron were maintained on slopes of complete medium (Table 2.4). Both these mutants required a longer incubation time before sporulation occurred. Fungicide mutants of A. fumigatus and A. niger were maintained on Eggins and Pugh Basal medium (Table 2.1) containing 1%(W/v) glucose and the appropriate fungicide.

All organisms were sub-cultured by aseptic transfer every 8-10 weeks and checked for contamination every two months.

Conidia were obtained for inoculation of liquid media by growing the fungi on plates containing the appropriate growth medium. About 15-20 plates were inoculated with a few spores from a stock slope and then incubated at the appropriate temperature for 7-10 days. The conidia were harvested by covering the plates with a sterile solution of Triton X 100 (1.0ml l^{-1}) and then dislodging the spores using a sterile glass rod. Conidial suspensions were pooled and then filtered through sterilised glass wool and cheesecloth to remove hyphae and other debris. The filtrate was centrifuged at 4000 rpm for 10min. After washing the pellet three times with sterile distilled water it was resuspended in 5-10ml sterile distilled

TABLE 2.4 COMPLETE MEDIUM

Glucose	10.0g
Mycological peptone	5.0g
Yeast extract	1.0g
Ferrous sulphate	0.01g
Magnesium sulphate	0.2g
Potassium chloride	0.5g
Calcium chloride	0.1g
Potassium dihydrogen orthophosphate (stock solution: 100g l^{-1})	10.0ml
Agar No 3	20.0g
Distilled water	1.0 litre

The above constituents were mixed although potassium dihydrogen orthophosphate was dissolved separately. After adjusting the pH to 5.5 with 1.0M sodium hydroxide the medium was autoclaved at 15lb in^{-2} and 121°C for 15min. For liquid cultures agar was omitted and Triton X 100 (1.0ml) was added when isolating auxotrophic mutants to prevent the merging of colonies.

water. Spore suspensions were shaken on a Stuart flask shaker to break up clumps of spores, counted on a haemocytometer and stored at 4°C until required for use (spores of the thermophilic organisms were stored at room temperature).

Mycelia for protoplast experiments and culture filtrates for enzyme production were prepared by inoculating 200ml of growth medium, contained in 500ml Erlenmeyer flasks with 1 x 10^6 spores ml^{-1} of culture medium. These were then incubated in an orbital shaker at 200rpm at the appropriate temperature for the chosen length of time.

2.3 PRODUCTION OF CELLULOSE PELLICLES

Stock cultures of A. xylinum were used to inoculate the liquid growth medium (Table 2.2) contained in sterile petri-dishes, half to three quarters full. After incubation at 28°C for 48h the organism was transferred into fresh medium by taking 2.0ml of the liquid phase. The first surface pellicles (membranes) produced were discarded. Incubation of the fresh medium at 28°C for 48h resulted in the formation of crystalline cellulose pellicles growing on the surface of the medium which were then harvested and washed in running tap water. The pellicles were then immersed for 1-2days in 5%(w/v) sodium hydroxide, after which they were removed and washed with distilled water. They were then immersed in dilute hydrochloric acid to remove traces of alkali. After 2h, the pellicles were removed and washed thoroughly in tap water and then in distilled water, prior to use.

These surface pellicles consist mainly of cellulose microfibrils and are highly crystalline, lack a non-cellulosic matrix and are not consolidated into the bacterial cell wall. The bacterium showed very rapid growth, the time required for the formation of a pellicle of about 2mm thickness being 48h. Only a few microorganisms are able to develop in this acid medium and no contamination was evident. A wet membrane of 9cm diameter and 2mm thickness had a weight of approximately 5g. After drying, the same membrane had a thickness of only 0.03mm and weighed approximately 0.11g. Wet membranes were used in subsequent experiments.

2.4 PROTOPLAST PRODUCTION AND ISOLATION

Mycelia, cultivated as described in section 2.2 were harvested by vacuum filtration using a No. 1 sintered glass filter funnel, washed twice and finally resuspended in an incubation medium consisting of 0.1M 2-[N-morphilino] ethane sulphonic acid (MES), pH 5.5 and 0.6M sodium chloride. Calcium chloride, 0.03M was included in the incubation medium when isolating protoplasts from P. ochrochloron. When protoplasts were required in very high numbers, mycelium was harvested by centrifugation at 10,000rpm for 40min. using a 6 x 500ml rotor in a Beckman model No. J2 21.

Mycelial suspensions (10.0ml) were then incubated with Novozym^{TM} 234 at a final concentration of 1-5mg ml^{-1} (depending on the batch activity) contained in Universal bottles at 30°C in an orbital platform shaking incubator at 200rpm. When protoplasts were required in very high numbers, mycelial suspensions, 200ml were incubated with Novozym^{TM} 234 in 500ml Erlenmeyer flasks

After an appropriate time of incubation, protoplasts were isolated by gravity filtration through a No. 1 sintered glass filter funnel. The residue remaining was resuspended in the incubation medium and refiltered. Filtrates were then combined and centrifuged at 2,500rpm for 5min. After this time they were washed three times and finally resuspended in the incubation medium. Larger volumes were first filtered through cheesecloth to remove mycelial debris before filtering through the sintered glass filter funnel.

Dry weights of all mycelial suspensions were determined by taking 5.0-50.0ml of each culture and filtering through a pre-weighed filter paper (Whatman No. 1, 9.0cm) and then washing twice with distilled water before placing in an oven at 80°C for 24h. Samples were then cooled to room temperature by placing in a desiccator for 20min before weighing.

Protoplast numbers were determined using an improved Neubauer counting chamber, observed using phase contrast microscopy (x 40 objective). Two counts were made for each sample and all incubations were carried out in triplicate. Results were thus expressed as the mean number of protoplasts ± 1 standard deviation per ml mycelium or per milligram (mg) dry weight of mycelium.

NOTE Care should be taken when using Novozym™ 234 as this enzyme may cause irritation of the skin and the mucous membranes. Persons susceptible to hay fever or asthma should take extra precautions. Weighing and other manipulations should be carried out in a closed system. Where open handling cannot be avoided, the skin, eyes and respiratory passages must be protected.

2.5 PRODUCTION OF LYTIC ENZYMES

Cells of P. dupontii were produced in the form of mycelia, grown in Eggins and Pugh Basal medium (Table 2.1) containing 1%(^{w}/v) glucose, in 500ml Erlenmeyer flasks. The above medium, 200ml was inoculated with 2 x 10^{6} spores ml^{-1} of culture medium and then incubated at 48°C in an orbital platform shaking incubator at 200 rpm. After 5-7days, the mycelia were harvested by centrifugation at 10,000rpm for 40min using a 6 x 500ml rotor in a Beckman model J2 21 centrifuge. After washing the mycelia with distilled water, mycelial suspensions were autoclaved at 15lb in^{-2} and 121°C for 15min. Batches were stored at -20°C until sufficient material was available, after which the autoclaved cells were freeze dried.

Dried cells were ground into a fine powder using a mortar and pestle. Addition of 100ml distilled water was followed by centrifugation at 4000rpm for 10min. After discarding the supernatent the residue was washed three times with distilled water to remove the soluble portions of cytoplasm. The cell wall material was then suspended in 200ml Tris-Maleate buffer (0.01M; pH7.5) and incubated with 10mg non-specific protease for 12h to digest any protein. The incubate was centrifuged at 4000rpm for 10min and the residue washed three times with 50ml distilled water. This protein-free material was then suspended in chloroform/ethanol [1:1(^{v}/v)] for 1h to remove any lipid material. Again this was centrifuged at 4000rpm for 10min and the residue washed successively with 100ml ethanol/water [1:1(^{v}/v)] and twice with distilled water. The material was then freeze-dried and stored desiccated at -20°C until required for use.

Trichoderma viride was cultivated on the growth medium described in Table 2.3 in which glucose was replaced with the powdered P. dupontii cell walls. Erlenmeyer flasks (500ml) containing 200ml of the above medium were inoculated with 2 x 10^6 spores ml^{-1} culture medium and incubated at 30°C in an orbital platform shaking incubator at 200rpm. After 5-7days, mycelium and fine polysaccharide granules were removed by centrifugation at 4,000 rpm for 10min. Crude lytic enzymes were obtained by carefully raising the culture filtrate to 75% saturation with ammonium sulphate. The pH was maintained at 4.5-6.5 for 12h to allow precipitation. The precipitate was dissolved in 20-50ml cold distilled water and this was then dialysed against several changes of distilled water over 24h. Insoluble material was removed by centrifugation at 15,000rpm for 15min. The enzyme was then freeze-dried and stored desiccated at -20°C until required for use.

2.6 TOTAL REDUCING SUGAR ASSAYS

Total reducing sugars released from various cellulose substrates as a result of enzyme hydrolysis were quantitatively assessed using the dinitrosalicylic acid (DNS) method (Miller, 1959) and the p-hydroxybenzoic acid hydrazide (PAHBAH) method (Hurst et al., 1977). As can be seen from the calibration curves for D-galacturonic acid (Figure 2.1), D-glucose (Figure 2.2) and D-xylose (Figure 2.3), the DNS method is less sensitive and therefore less accurate than the PAHBAH method.

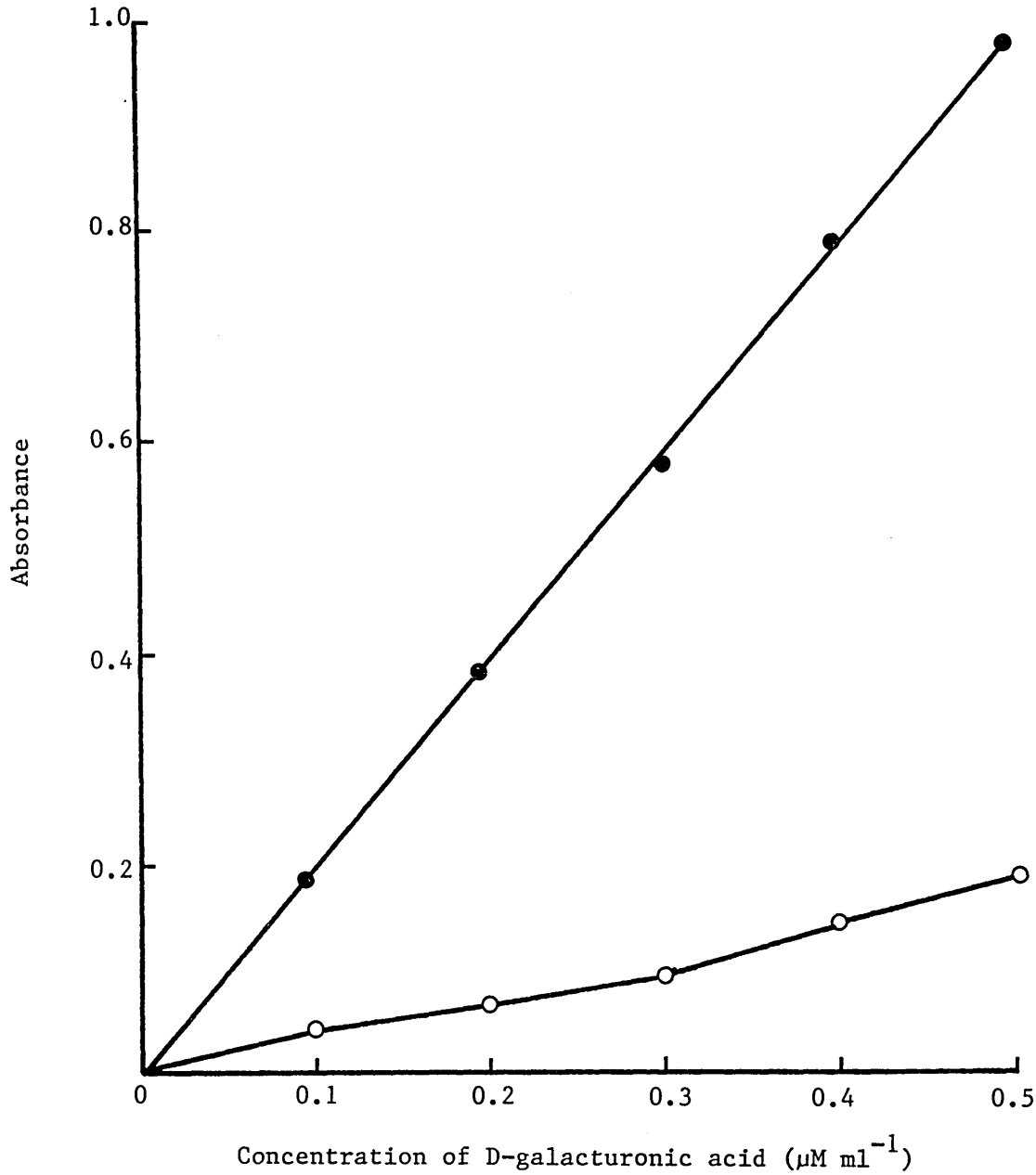

FIGURE 2.1 Standard calibration curve for D-galacturonic acid. 0.6 ml D-galacturonic acid standards (0 - 0.5 μM ml^{-1}) in 0.1 M sodium acetate buffer, pH 5.0 were developed with DNS (O) and PAHBAH (●) reagent as described in Section 2.6.

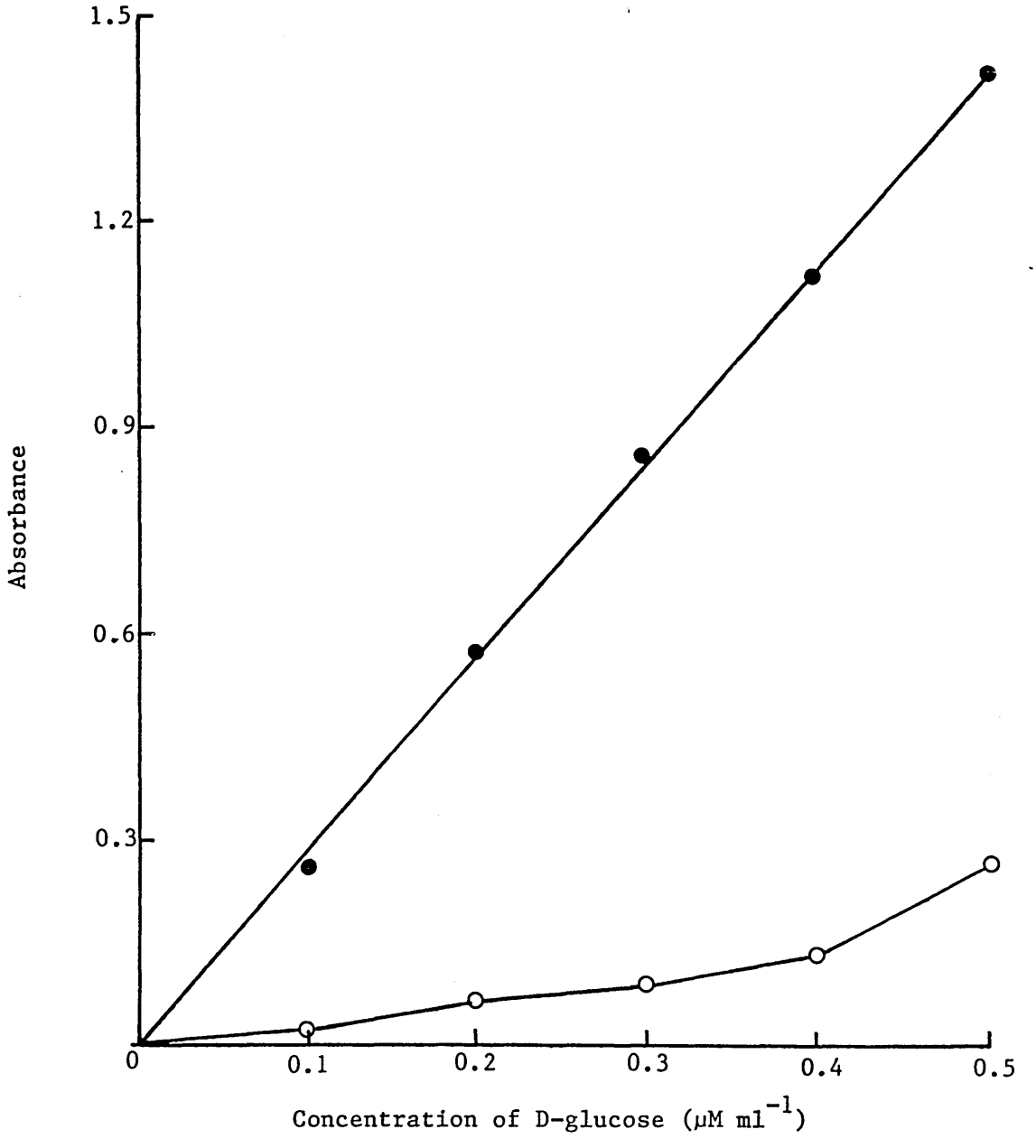

FIGURE 2.2. Standard calibration curve for D-glucose. 0.6 ml D-glucose standards (0 - 0.5 μM ml^{-1}) in 0.1 M sodium acetate buffer, pH 5.0 were developed with DNS (O) and PAHBAH (●) reagent as described in Section 2.6.

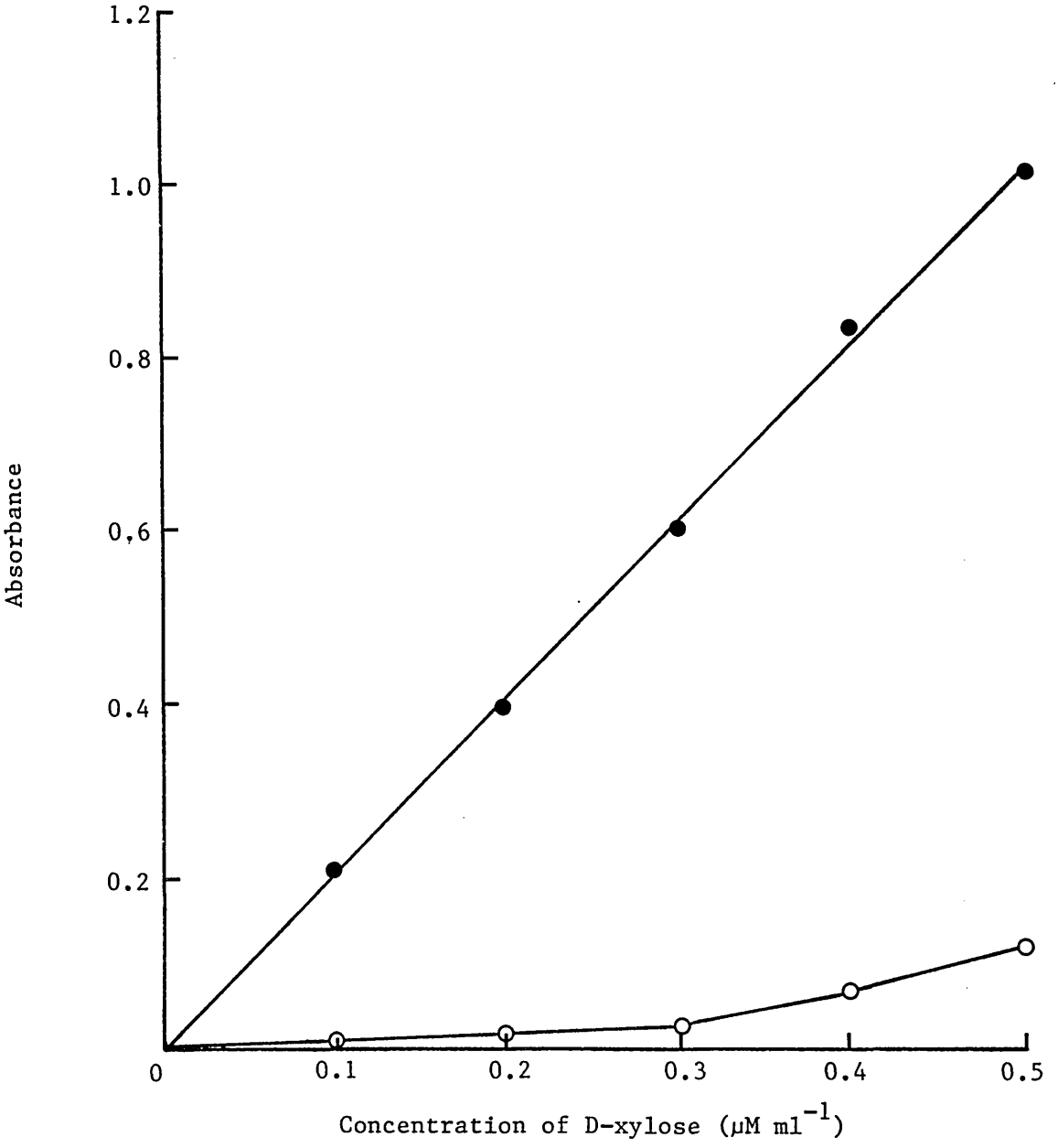

FIGURE 2.3. Standard calibration curve for D-xylose. 0.6 ml D-xylose standards (0 - 0.5 μM ml^{-1}) in 0.1 M sodium acetate buffer, pH 5.0 were developed with DNS (O) and PAHBAH (●) reagent as described in Section 2.6.

2.6.1 **DNS assay**

To 0.6ml of enzyme incubation mixture, 3.0ml of DNS reagent (Table 2.5) were added to terminate the reaction and the mixture boiled for 10min in a water bath to allow colour development. After cooling to 20°C, the absorbance was measured at 550nm against a distilled water blank.

2.6.2 **PAHBAH assay**

To 0.6ml of enzyme incubation mixture, 5.0ml of PAHBAH reagent (Table 2.6) were added to terminate the reaction and the mixture boiled for 10min in a water bath to allow colour development. After cooling to 20°C, the absorbance was measured at 420nm against a distilled water blank.

2.7 **PROTEIN ASSAY**

The protein content of culture filtrates was determined using the method of Ohnishi and Barr (1978). This involves a combination of the methods of Lowry *et al*. (1951) and Gornall *et al*. (1949) producing a stable colour complex, enabling many samples to be developed at once and aiding reproducibility.

To 0.8ml of the sample, 3.2ml of BS7 reagent (Biuret reagent diluted 1:7 with 2.3%(^{w}/v) sodium carbonate) were added. The solutions were mixed and left for 10min at room temperature. After this period, 0.1ml Folin and Ciocalteau phenol reagent was added and quickly mixed in. Samples were incubated for a further 20min at

TABLE 2.5. PREPARATION OF DNS REAGENT

3,5-dinitrosalicylic acid	10.6 g
Sodium hydroxide	19.8 g
Distilled water	1416.0 ml
The above components were mixed until dissolved and then the following were added:	
Sodium potassium tartrate	306.0 g
Phenol	7.6 ml
Sodium metabisulphite	8.3 g

3.0 ml of the reaction mixture was used to stop the enzyme reaction and then boiled to allow colour development as described in Section 2.6.1.

TABLE 2.6. PREPARATION OF PAHBAH REAGENT

Sodium hydroxide	5.0 M; 5.0 ml
Trisodium citrate	0.5 M; 5.0 ml
Sodium sulphite	1.0 M; 5.0 ml
Calcium chloride	0.2 M; 5.0 ml

The above components were added in the order shown and mixed until dissolved. To this mixture, 1.0 g p-hydroxybenzoic acid hydrazide (PAHBAH) was added. After this had dissolved, the solution was diluted to 100 ml with distilled water.

5.0 ml of the reaction mixture was used to stop the enzyme reaction and then boiled to allow colour development as described in Section 2.6.2.

NB. It is essential that the reagent is prepared immediately before use and maintained at 0°C.

room temperature to allow further colour development and the absorbance measured at 600nm against a distilled water blank. Stock BS7 reagent was stable at 20°C for several months. A standard calibration curve for protein was constructed (Figure 2.4) using bovine serum albumin (Fraction V) as the standard, dissolved in 0.1M sodium acetate buffer, pH5.0. An equivalent volume, 0.8ml of each standard was used and the above procedure was followed.

2.8 ENZYME ASSAYS

A number of assays have been selected to screen the test organisms for the ability to secrete a number of important enzymes. The majority of these assays depend upon the hydrolysis of "model" substrates and assaying for reducing sugars released after incubation under various conditions (Stewart and Parry, 1981; Rouau and Odier, 1986).

Control samples were incubated for zero minutes and were included to provide an assessment of reducing sugars present before enzyme activity was measured. Reagent blanks were also included in which 0.1M sodium acetate buffer, pH5.0 replaced the enzyme/ supernatent. All assays were carried out in triplicate and results expressed as mean $\pm$ 1 standard deviation. Enzyme activity was reported in milli International Units (milli-IU) which represents µmoles reducing sugars released per ml per min under the assay conditions.

(2.8.1) Filter paper activity

Filter paper was used as the assay substrate in an attempt to

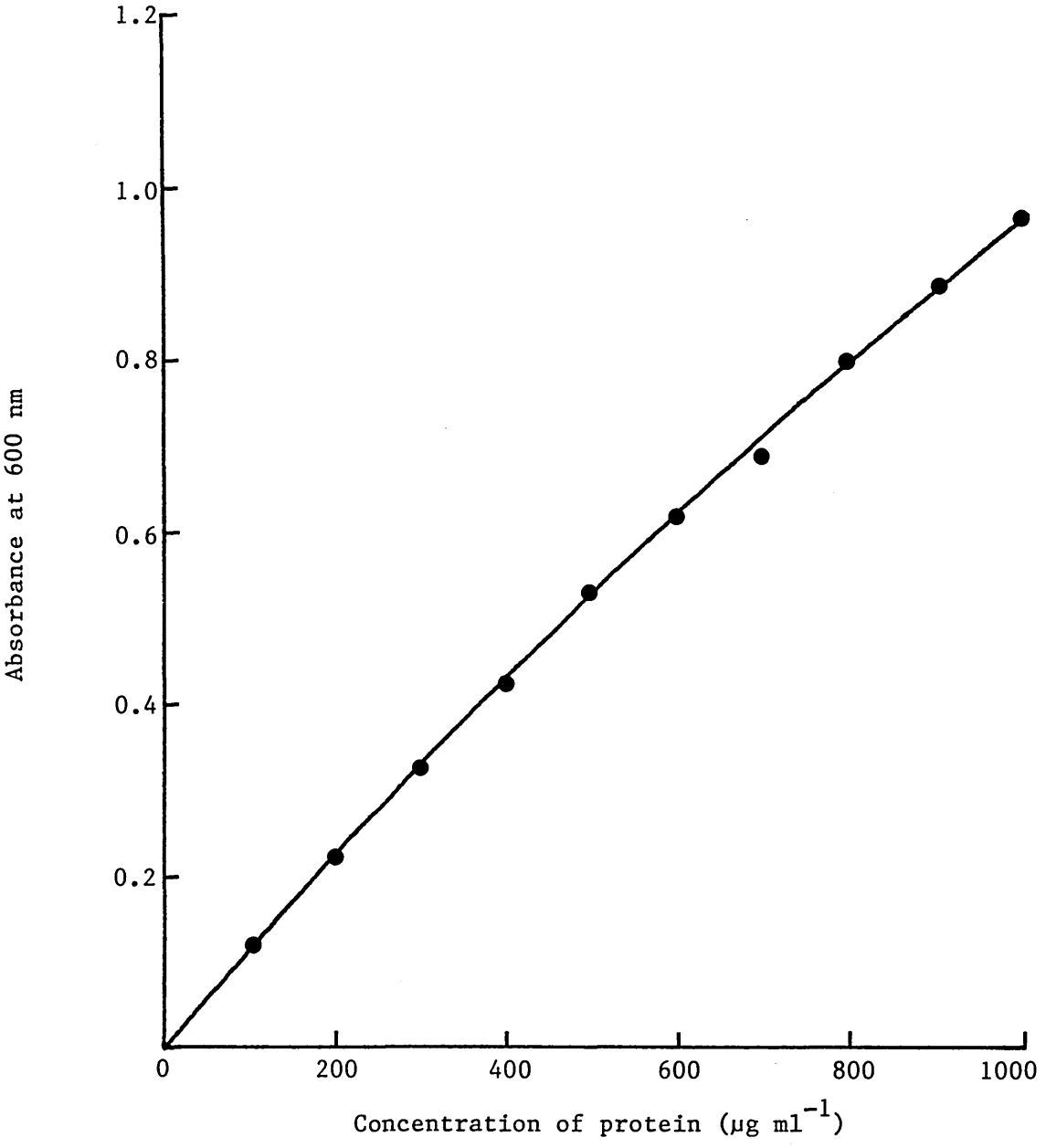

FIGURE 2.4. Standard calibration curve for protein. 0.8 ml bovine serum albumin (Fraction V) standards (0 - 1000 μg ml^{-1}) in 0.1 M sodium acetate buffer, pH 5.0 were developed using the method of Ohnishi and Barr (1978) as described in Section 2.7.

determine the effect of cellulase enzymes on a crystalline form of cellulose.

Discs of Whatman No. 1 filter paper, 25.0mg were added to 0.5 ml sodium acetate buffer (0.1M; pH5.0) and incubated with 0.1ml enzyme/supernatent for 60min at 50°C. The reaction was terminated and time allowed for colour development with PAHBAH Reagent (Section 2.6.2).

2.8.2 Other enzymes

A number of substrates were used at a concentrations of 1%(w/v) to measure the activity of several enzymes in culture filtrates. The substrates, 0.5ml dissolved in 0.1M sodium acetate buffer,pH5.0 were incubated with 0.1ml enzyme/supernatant at 50°C for varying lengths of time. After the appropriate incubation time had lapsed, enzyme activity was terminated and time allowed for colour development with PAHBAH reagent (Section 2.6.2). Conditions for the enzyme assays are summarised in Table 2.7.

2.8.3 p-Nitrophenyl-β-D-glucopyranoside activity

As explained earlier β-D-glucosidase activity may take a number of different forms and this assay was included to monitor the presence of aryl-β-D-glucosidase activity in culture filtrates. In this assay, the release of p-nitrophenol from p-nitrophenyl-β-D-glucopyranoside was monitored (Lachke *et al.*, 1983; Beldman *et al.*, 1985).

TABLE 2.7. CONDITIONS FOR ENZYME ASSAYS

ENZYME	EC NUMBER	SUBSTRATE	INCUBATION TIME (min)
Endo-β-1,4-glucanase	3.2.1.4	Carboxymethylcellulose	60
Endo-β-1,3-xylanase	3.2.1.32	Oat xylan	15
Endo-β-1,4-xylanase	3.2.1.8	Xylan purum	15
Exo-β-1,4-xylosidase	3.2.1.37	o-Nitrophenyl-β-D-xylopyranoside	15
β-Glucosidase	3.2.1.21	Cellobiose/Salicin	30
Laminarinase	3.2.1.6	Laminarin	30
Pectinase	3.2.1.15	Polygalacturonic acid	15

The substrate, 2.0ml p-nitrophenyl-β-D-glucopyranoside (pNPG) (1mmol l^{-1}) in sodium acetate buffer (0.1M; pH5.0) was incubated with 0.1ml enzyme/supernatant at 40°C. After 30min incubation 10.0ml sodium carbonate solution (0.1M) were added to terminate the reaction and allow colour development. Activity was reported as milli-IU or μmoles p-nitrophenol released per min under the assay conditions. A standard curve for p-nitrophenol was constructed (Figure 2.5).

2.9 RESISTANCE OF FUNGAL ORGANISMS TO ANTIFUNGAL AGENTS

Selective resistance to antifungal agents was investigated by incorporating heavy metal salts and commercial fungicides into Eggins and Pugh Basal medium (Table 2.1) containing 1%(w/v) glucose. Agar plates were inoculated with a spore inoculum of each of the test organisms. These were then incubated at the appropriate growth temperature and growth measured as colony diameter, in millimetres (mm) after five days incubation.

2.9.1 Resistance to metal salts

A range of metal salts (Table 2.8) were incorporated into the growth medium at concentrations of 1.0×10^{-4}–1.0×10^{-3}M.

2.9.2 Resistance to commercial fungicides

A number of commercial fungicides (Table 2.9) were incorporated into the growth medium at concentrations of 0.0001-1.0%(w/v).

TABLE 2.8. METAL SALTS USED IN RESISTANCE STUDIES

METAL SALT	FORMULA	ELEMENT OF INTEREST
Cobaltous chloride	$CoCl_2 . 6H_2O$	Co
Cupric chloride	$CuCl_2 . 2H_2O$	Cu
Ferric chloride	$FeCl_3 . 6H_2O$	Fe
Lead nitrate	$Pb(NO_3)_2$	Pb
Magnesium chloride	$MgCl_2.6H_2O$	Mg
Manganous chloride	$MnCl_2.4H_2O$	Mn
Mercuric chloride	$HgCl_2$	Hg
Nickel chloride	$NiCl_2$	Ni
Zinc chloride	$ZnCl_2$	Zn

TABLE 2.9. FUNGICIDES USED IN RESISTANCE STUDIES

FUNGICIDE	CONSTITUTION
Benlate	50% (W/v) Benomyl
Captafol Sanspor	48% ai
Captan	83% ai
Chlorothalonil Daconil	75% ai
Impact	-
Maneb	80% ai
Mystox 8	-
Mystox ELC	-
Mystox G	-
Mystox LPL	100% (W/v)
Mystox WFA	-
Quintozene Botrilex	-
Thiabendazole Mertect	40% ai
Thiram	75% ai

ai = active ingredient; - = not given

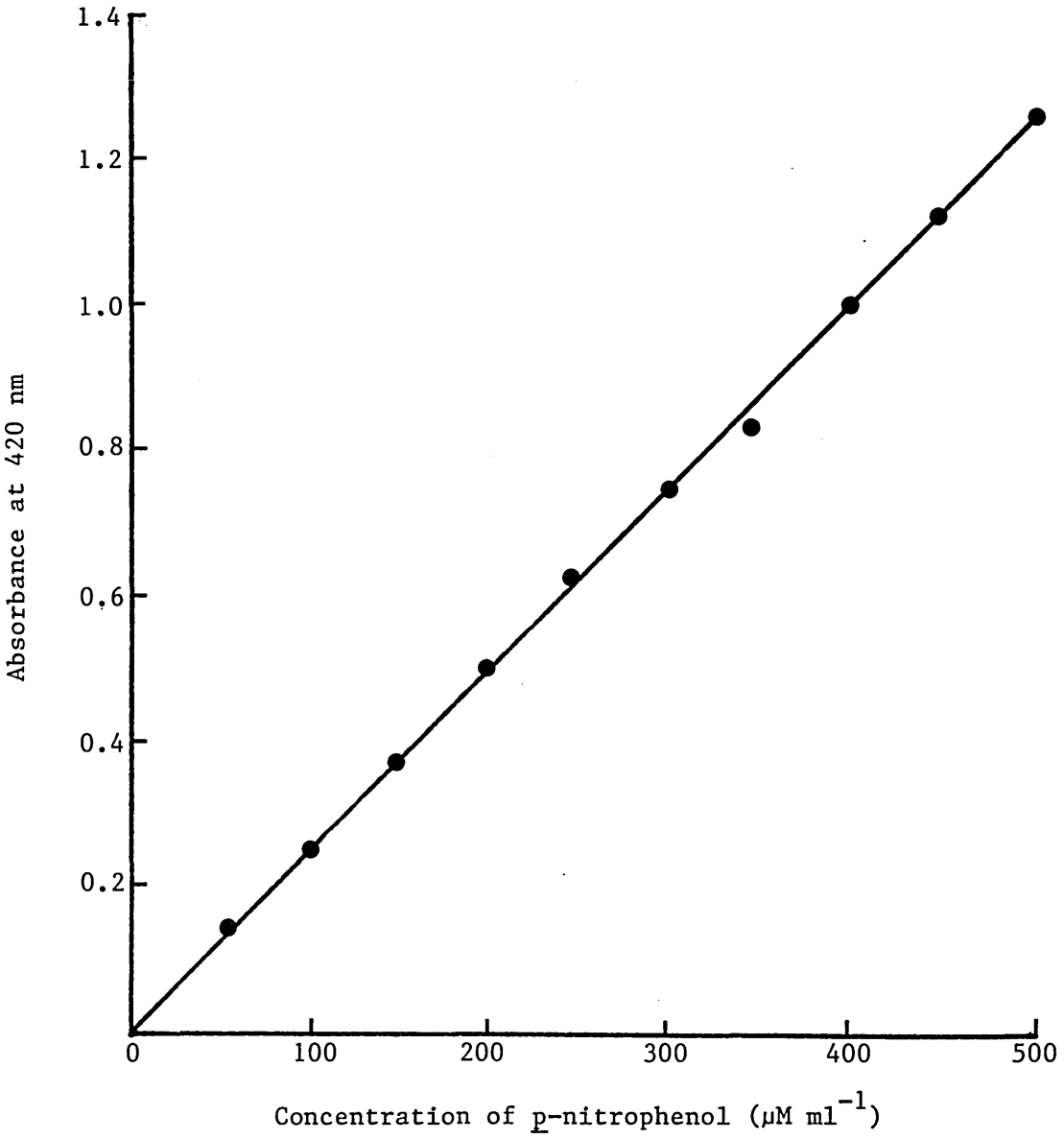

FIGURE 2.5. Standard calibration curve for p-nitrophenol. 2.1 ml p-nitrophenol standards (0 - 500 μM ml^{-1}) in 0.1 M sodium acetate buffer, pH 5.0 were developed using the method described in Section 2.8.3.

2.10 MUTAGENESIS

To further increase the selection parameters available in fusion studies, a number of mutagenesis experiments were carried out.

2.10.1 UV-Irradiation of P.ochrochloron and A. niger

P. ochrochloron (5.6×10^8 spores ml^{-1}) and A. niger (1.6×10^8 spores ml^{-1}) spores were placed in a sterile UV cabinet, 7cm away from the light source. The spores were irradiated for 3h with light of 257nm. At half-hour intervals, 1.0ml of the irradiated spore suspensions were transferred and serially diluted with sterile distilled water over the range 1.0×10^{-1}-1.0×10^{-9} spores ml^{-1}. Each dilution, 1.0ml was then placed onto complete agar (Table 2.4) plates containing Triton X 100 (1.0ml l^{-1}). After incubation at 30°C for 48h, colonies were counted on each plate. Semi-log plots were constructed for P. ochrochloron (Figure 2.6) and A. niger (Figure 2.7) demonstrating the kill rate with response to irradiation time. The percentage surviving spores were then calculated for the irradiated spore suspensions of P. ochrochloron (Table 2.10) and A. niger (Table 2.11). Spore suspensions representing 0.1-2.0% survival were then plated out onto 20-30 plates of complete agar (Table 2.4). After incubation at 30°C for 48h, colonies were picked off and transferred onto complete agar (Table 2.4) and minimal agar (Table 2.12) plates.

2.10.2 Fungicide mutagenesis

A number of antifungal agents were incorporated into Eggins and Pugh Basal medium (Table 2.1) containing 1%(w/v) glucose at relatively high concentrations (Table 2.13). Agar plates containing

TABLE 2.10. PERCENTAGE SURVIVAL OF IRRADIATED SPORES OF P. OCHROCHLORON

UV IRRADIATION TIME (h)	VIABLE SPORE CONCENTRATION (spores ml^{-1})	% SURVIVORS
0.0	5.6×10^8	100.0
0.5	7.9×10^7	14.1
1.0	3.2×10^7	5.7
1.5	1.0×10^7	1.8
2.0	5.6×10^5	0.1
2.5	2.0×10^5	0.04
3.0	6.3×10^4	0.01

TABLE 2.11. PERCENTAGE SURVIVAL OF IRRADIATED SPORES OF A. NIGER

UV IRRADIATION TIME (h)	VIABLE SPORE CONCENTRATION (spores ml^{-1})	% SURVIVORS
0.0	1.6×10^8	100.0
0.5	7.9×10^7	49.4
1.0	4.0×10^7	25.0
1.5	3.0×10^7	18.6
2.0	1.5×10^7	9.5
2.5	7.9×10^6	4.9
3.0	5.6×10^6	3.5
3.5	4.4×10^6	2.8
4.0	2.0×10^6	1.3

TABLE 2.12. MINIMAL AGAR

Glucose	10.0 g
Potassium chloride	0.5 g
Magnesium sulphate	0.2 g
Calcium chloride	0.1 g
Sodium nitrate	3.0 g
Potassium dihydrogen orthophosphate (stock solution: 100 g l^{-1})	10.0 ml
Trace metals (stock stolution: Ferrous sulphate, 1 g l^{-1}; Zinc sulphate, 1 g l^{-1}; Copper sulphate, 0.5 g l^{-1})	10.0 ml
Agar, No. 1	20.0 g
Distilled water	1.0 l

Potassium dihydrogen orthophosphate and trace metals were dissolved separately. After adjusting the pH to 5.5 with Hydrochloric acid or Sodium hydroxide, the mixture was autoclaved at 15 lb in^{-2} and 121°C for 15 min.

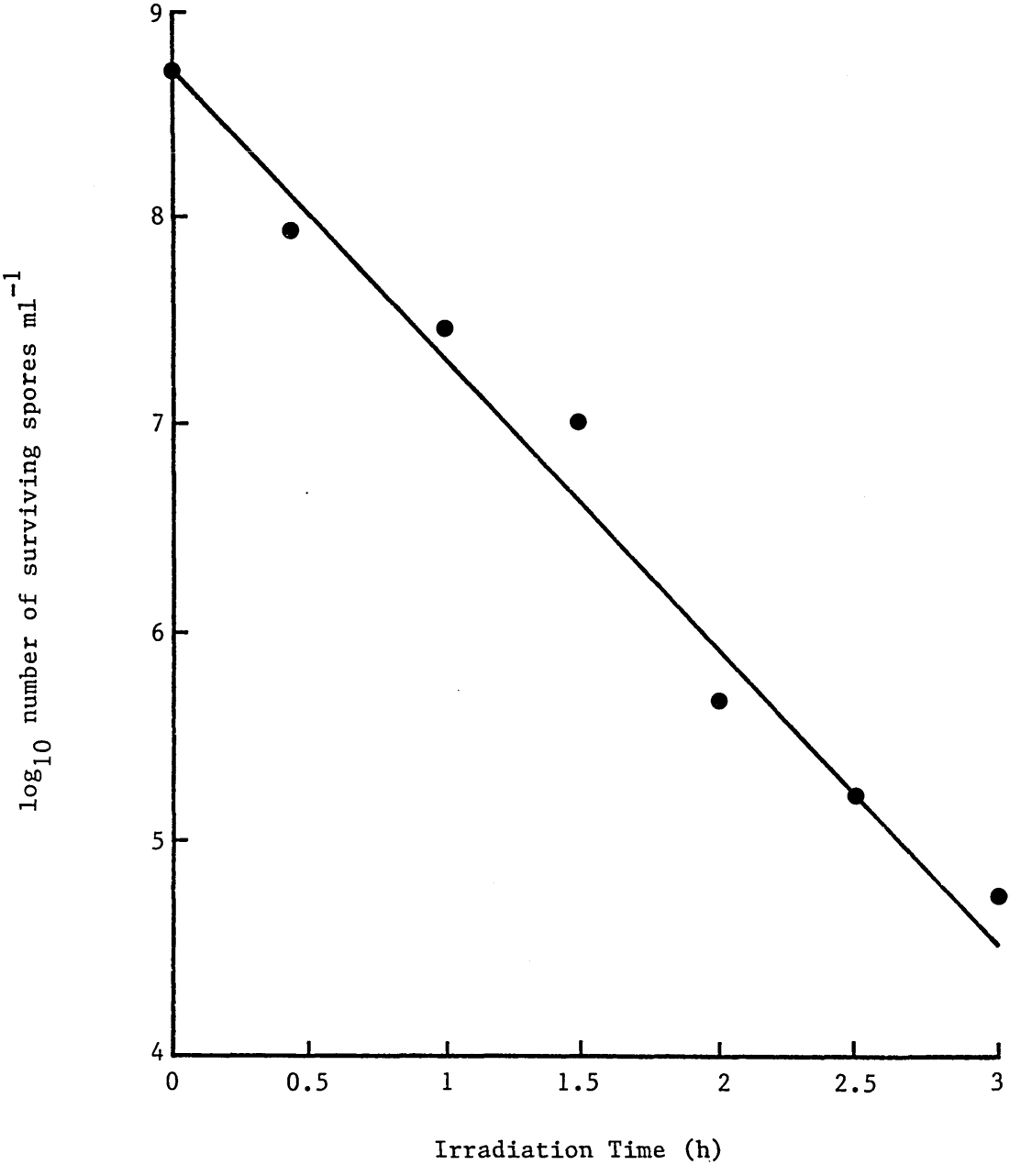

FIGURE 2.6. UV-irradiation of P. ochrochloron. Spores (5.6×10^8 spores ml^{-1}) were irradiated with light of 257 nm for 0 - 3 h. The number of surviving spores were determined as detailed in Section 2.10.1.

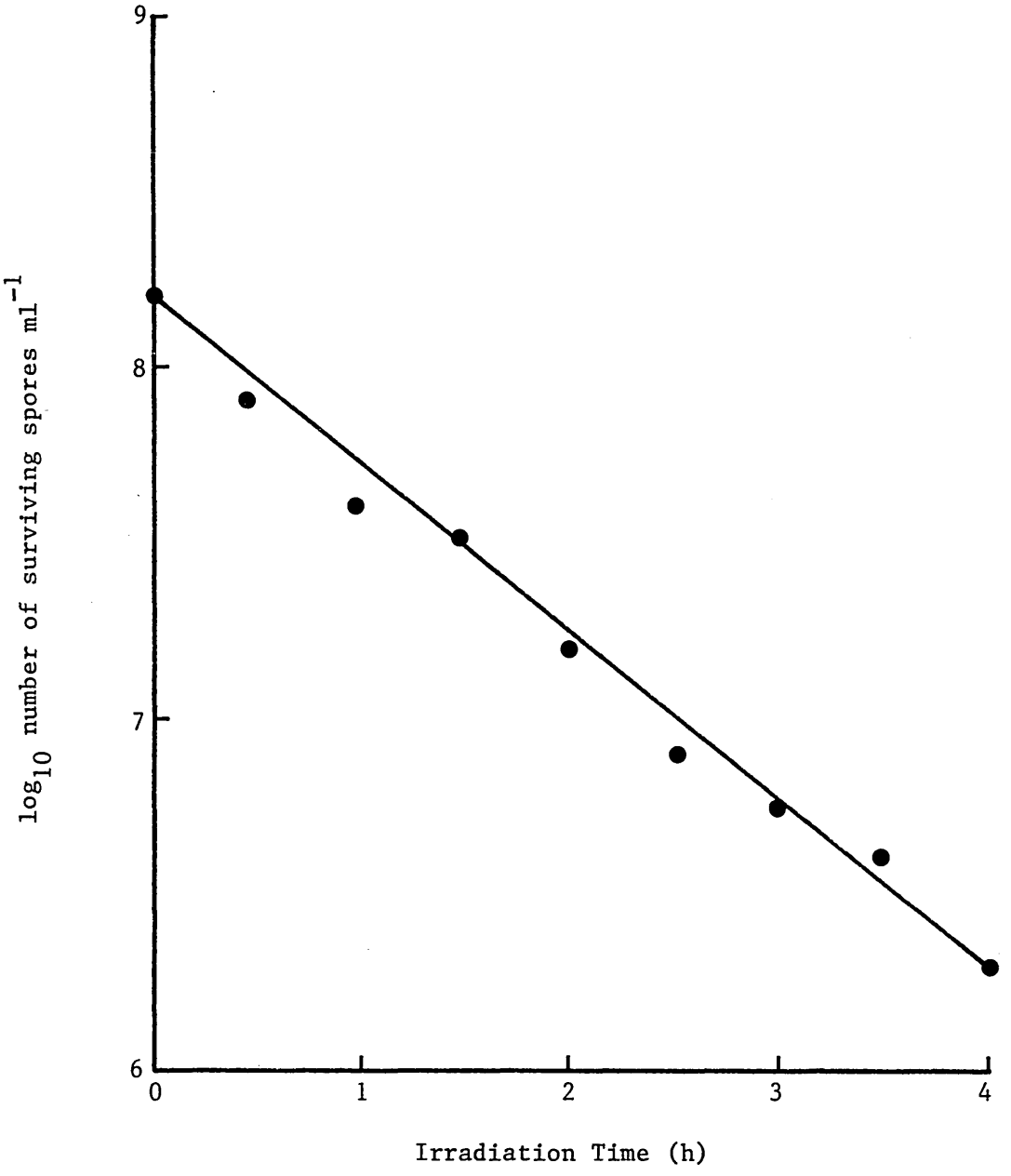

FIGURE 2.7. UV-irradiation of A. niger. Spores (1.6×10^8 spores ml^{-1}) were irradiated with light of 257 nm for 0 - 3 h. The number of surviving spores were determined as detailed in Section 2.10.1.

the above growth medium were inoculated with a spore inoculum of the test organisms, these were then incubated at the appropriate growth temperature (Section 2.2). After 5-7days, any colonies were picked off and plated out onto fresh medium. Fungicide mutants were maintained on Eggins and Pugh Basal medium (Table 2.1) containing 1% (w/v) glucose and the appropriate fungicide.

2.11 PROTOPLAST FUSION STUDIES

Attempts were made to fuse protoplasts from a number of the test organisms namely P. ochrochloron with G. emersonii and A.fumigatus with A. niger.

Protoplasts were isolated from the two organisms to be fused using the techniques detailed in Section 2.4 and the optimum conditions for isolation as determined in Section 4. After isolation, protoplasts (1.0×10^9-1.0×10^{10}) from the two species were mixed and the resultant suspension was centrifuged at 2,500rpm for 10min. The pellet was then resuspended in prewarmed incubation medium (30°C) containing Polyethylene glycol 4000 [30%(w/v)], calcium chloride (0.01-0.10M) and glycine (0.05M) at a final pH of 8.6. After incubation for 2-30min at 30°C, the incubation medium was removed by centrifugation at 2,500rpm for 10min. The resultant pellet was washed three times with MES buffer (0.1M; pH5.0) containing 0.6M sodium chloride and then finally resuspended in 10.0ml of the same buffer. Regeneration of the protoplasts was then allowed by plating out onto selected regeneration plates of three types, each containing 0.6M sodium chloride. The first type of plate (A) allows growth of only one of the parental types and is selective against the other. The second type of regeneration plate

TABLE 2.13. ANTIFUNGAL AGENTS USED IN MUTAGENESIS

FUNGICIDE /METAL SALT	CONCENTRATION [% (w/v)]
Benlate	0.01 0.001
Cupric chloride	0.001 M
Impact	0.1 0.05
Mercuric chloride	0.001 M
Merolan	1.0 0.05
Mystox 8	0.1
Mystox G	0.01
Mystox WFA	0.1 0.05
Thiabendazole	0.01
Thiram	1.0

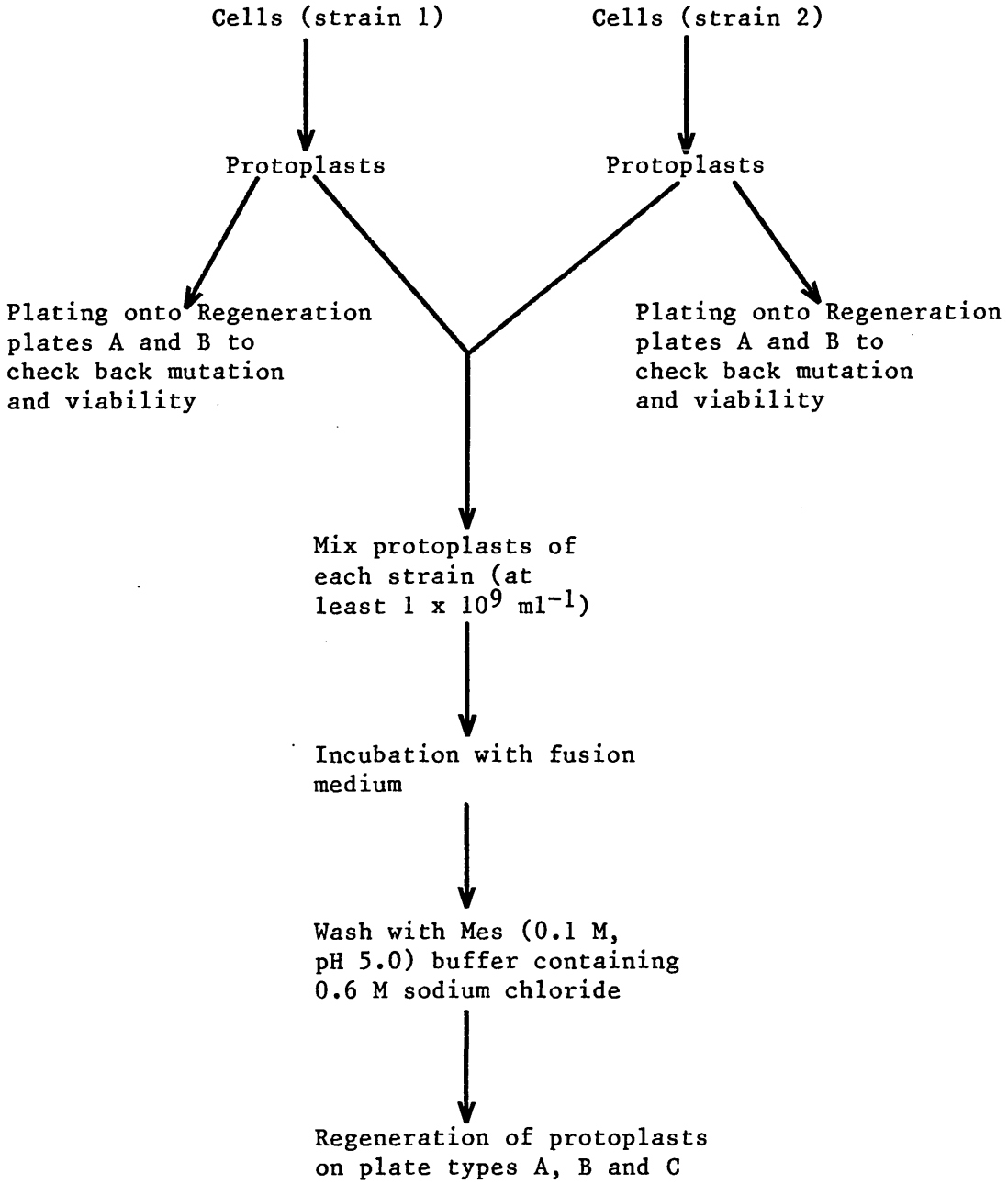

FIGURE 2.8. Schematic diagram for protoplast isolation and fusion

(B) allows growth of both of the parental types and may be used to check the viability of the protoplasts. Finally the third type of regeneration plate (C) does not support growth of either of the parental types and may therefore be used to identify any fusion products. All the regeneration plates were then incubated for 5-7days at 30°C, 37°C, 44°C and 48°C to isolate any possible recombinants which may have new optimum growth temperatures. A schematic diagram (Figure 2.8) has been included to summarise the procedures of protoplast isolation and fusion.

2.12 MATERIALS AND SUPPLIERS

2.12.1 Materials

(a)	Agar No. 1, L11	Oxoid
	Agar No. 3, L13	Oxoid
	Ammonium sulphate, AR	BDH Chem.
	L-Asparagine	BDH Chem.
	Bacteriological peptone, L37	Oxoid
	Bovine serum albumin, fraction V	Sigma Chem. Co.
	Calcium chloride (dihydrate), AR	BDH Chem.
	Carboxymethylcellulose	Sigma Chem. Co.
	Cellobiose, Biochemical grade	BDH Chem.
	Cellulase, Practical grade Type 2	Sigma Chem. Co.
	Chitinase	Sigma Chem. Co.
	Citric acid, monohydrate	Sigma Chem. Co.
	Cobalt chloride, hexahydrate	Sigma Chem. Co.
	Cupric chloride, dihydrate	Sigma Chem. Co.
	Cupric sulphate, AR	BDH Chem.
	3,5-Dinitrosalicylic acid (DNS)	Sigma Chem. Co.

Ethylenediaminetetraacetic acid, tetrasodium salt (EDTA)	Sigma Chem. Co.
Ferric chloride, anhydrous	Sigma Chem. Co.
Ferrous sulphate, AR	BDH Chem.
Folin and Ciocalteau phenol reagent	Sigma Chem. Co.
D-Galacturonic acid	Sigma Chem. Co.
D-Glucose, AR	BDH Chem.
β-Glucuronidase, Type H2	Sigma Chem. Co.
Glycerol	Sigma Chem. Co.
p-hydroxybenzoic acid hydrazide (PAHBAH)	Sigma Chem. Co.
n-2-hydroxyethylpiperazine-N-2-ethane sulphonic acid (HEPES)	Sigma Chem. Co.
Laminarin, crude	Sigma Chem. Co.
Lead nitrate, AR	BDH Chem.
Magnesium chloride, AR	FSA Lab. Supplies
Magnesium sulphate, AR	BDH Chem.
Manganous chloride, SLR	FSA Lab. Supplies
Mercuric chloride, AR	BDH Chem.
2-[N-Morphilino] ethane sulphonic acid (MES)	Sigma Chem. Co.
Mycological peptone, L40	Oxoid
Nickel chloride, hexahydrate	Sigma Chem. Co.
p-nitrophenol, LR	FSA Lab. Supplies
o-nitrophenyl-β-D-xylopyranoside	Sigma Chem. Co.
p-nitrophenyl-β-D-glucopyranoside	Sigma Chem. Co.
Phenol, SLR	FSA Lab. Supplies
Potassium chloride, AR	BDH Chem.
Potassium dihydrogen orthophosphate, AR	BDH Chem.
Polyethylene glycol 4000 (Biochemical grade)	BDH Chem.
Polygalacturonic acid, sodium salt	Sigma Chem. Co.

Protease, purified, Type XIV	Sigma Chem. Co.
Rose bengal	BDH Chem.
Salicin	BDH Chem.
Sodium acetate (trihydrate)	BDH Chem.
Sodium carbonate, anhydrous	Sigma Chem. Co.
Sodium chloride, AR	Sigma Chem. Co.
Tri sodium citrate, AR	BDH Chem.
di Sodium hydrogen orthophosphate, dodecahydrate, GPR	BDH Chem.
Sodium hydroxide, AR	BDH Chem.
Sodium metabisulphate, AR	BDH Chem.
Sodium nitrate, AR	BDH Chem.
Sodium potassium tartrate, tetrahydrate	Sigma Chem. Co.
Sodium sulphite, anhydrous, GPR	BDH Chem.
Triton X 100	BDH Chem.
Urea, AR	BDH Chem.
Xylan, oat	Sigma Chem. Co.
Xylan, purum	Fluka
D-Xylose	Sigma Chem. Co.
Yeast extract, L21	Oxoid
Zinc chloride, SLR	FSA Lab. Supplies
Zinc sulphate, AR	BDH Chem.

(b) L-amino acids were obtained from Sigma Chem. Co.

(c) Commercial fungicides were kindly supplied by Imperial Chemical Industries PLC, Jealotts Hill Research Station, Bracknell, Berkshire, RG12 6EY, England and Mystox reagents were obtained from Catomance Ltd, 89/96 Bridge Road East, Welwyn Garden City,

Hertfordshire, AL7 1SW, England.

(d) Novozym $^{TM}234$ was purchased from Novo Industri A/S, Novo Alle, DK-2880 Bagsvaerd, Denmark.

2.12.2 ADDRESSES OF SUPPLIERS

(a). BDH Chemicals Ltd., Broom Road, Parkstone, Poole BH12 4NN, England.

(b). Fluka, Fluorochem Ltd., Peakdale Road, Glossop, Derbyshire, SK13 9XE

(c). FSA Laboratory Supplies, Bishop Meadow Road, Loughborough, Leicestershire, LE11 ORG, England.

(d). Oxoid Ltd., Wade Road, Basingstoke, Hampshire, RG24 OPW, England.

(e). Sigma Chemical Company Ltd., Fancy Road, Poole, Dorset, BH17 7NH, England.

In order to achieve the complete hydrolysis of pure crystalline cellulose to soluble sugars, three enzymes are required, namely endo-β-1,4-glucanase [EC.3.2.1.4], exo-β-1,4-glucanase [EC.3.2.1.91] and β-glucosidase [EC.3.2.1.21] (Gritzali and Brown, 1979; Saddler, 1982; Labudova and Farkas, 1983). Two types of β-glucosidase may be observed, one with activity towards aryl-β-glucosides and another towards cellobiose (Sternberg, et al., 1977; Shewale, 1982). Surface pellicles obtained from liquid cultures of A. xylinum (Section 2.3) were used as the growth substrate because of their pure crystalline cellulosic matrix (White, 1982). However, in many cellulosic wastes, cellulose is associated with other polysaccharides (xylans and pectin) and with polyphenols (lignins and tannins). Consequently, the enzymes responsible for their hydrolysis are also important in the saccharification of such wastes. Four enzymes, endo-β-1,3-xylanase, endo-β-1,4-xylanase, exo-α-1,4-xylosidase and exo-β-1,4-xylosidase have been implicated in the hydrolysis of xylans (Dekker and Richards, 1976).

3.1 FACTORS AFFECTING ENZYME ACTIVITY

A number of characterisation experiments were conducted, initially with commercial A. niger cellulase and then with culture filtrates from A. fumigatus to investigate the suitability of the assays chosen and the factors affecting enzyme activity. Commercial cellulase was dissolved in 0.1M sodium acetate buffer, pH5.0 and culture filtrates were produced by growing the organism in Eggins and Pugh Basal Medium (Table 2.1) containing A. xylinum cellulose pellicles and 0.2%(w/v) glucose as described in Section 2.2. After incubation for seven days at 44°C, cultures were harvested and

centrifuged at 4000rpm for 10min to remove mycelia and insoluble materials. The supernatant was then used in characterisation experiments. All dilutions of culture filtrates were carried out using 0.1M sodium acetate buffer, pH5.0.

3.1.1 **Effect of pH on enzyme activity**

The effect of pH on endo-β-1,4-glucanase activity was determined using a commercial cellulase, purified from A. niger (Figure 3.1). Although active over the pH range tested, optimal endo-β-1,4-glucanase activity was clearly defined between pH4.5-5.0. A slightly lower value, pH3.8-4.0 was reported by Hurst et al. (1977) using the same assay and another commercial A. niger cellulase (Type 2). These workers, however, had further purified this preparation and identified the isolated enzyme as endo-β-1,4-glucanase. This slight difference in pH optima could be explained by impurities in the crude cellulase preparation. No further experiments were conducted to investigate this phenomenon.

Figure 3.2 demonstrates the effect of pH on endo-β-1,4-glucanase, β-glucosidase and endo-β-1,3-xylanase activity of a culture filtrate from A. fumigatus. Optimal endo-β-1,4-glucanase activity was again clearly defined in the pH range 4.5-5.5 and agrees well with the reported value of 4.8, obtained by Stewart and Parry (1981) using a culture filtrate of A. fumigatus. Other workers have obtained widely differing values for the same enzyme obtained from G. emersonii. Folan and Coughlan (1978) reported an optimum of 4.2 which differs significantly from that reported by Moloney et al. (1985) of 5.5-5.8. This discrepancy could be explained by the fact that the former used a crude culture filtrate whereas the latter used

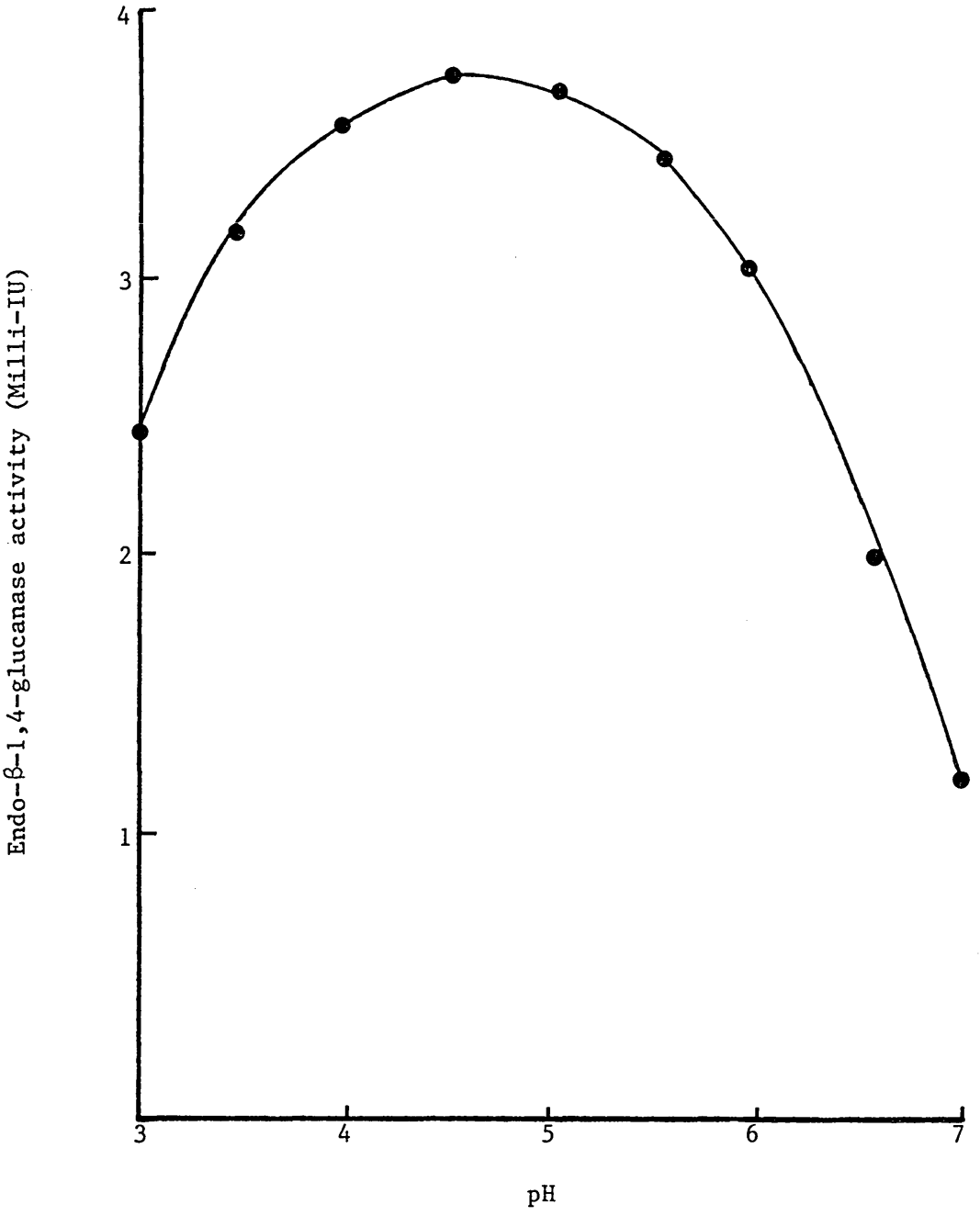

FIGURE 3.1. Effect of pH on the activity of endo-β-1,4-glucanase. Commercial A. niger cellulase, 50 μg ml^{-1} in 0.1 M sodium acetate buffer was incubated at 50°C with 1% (w/v) CMC in the same buffer (Section 2.8.2). Reducing sugars were determined after 0 and 60 min incubation using the PAHBAH method (Section 2.6.2).

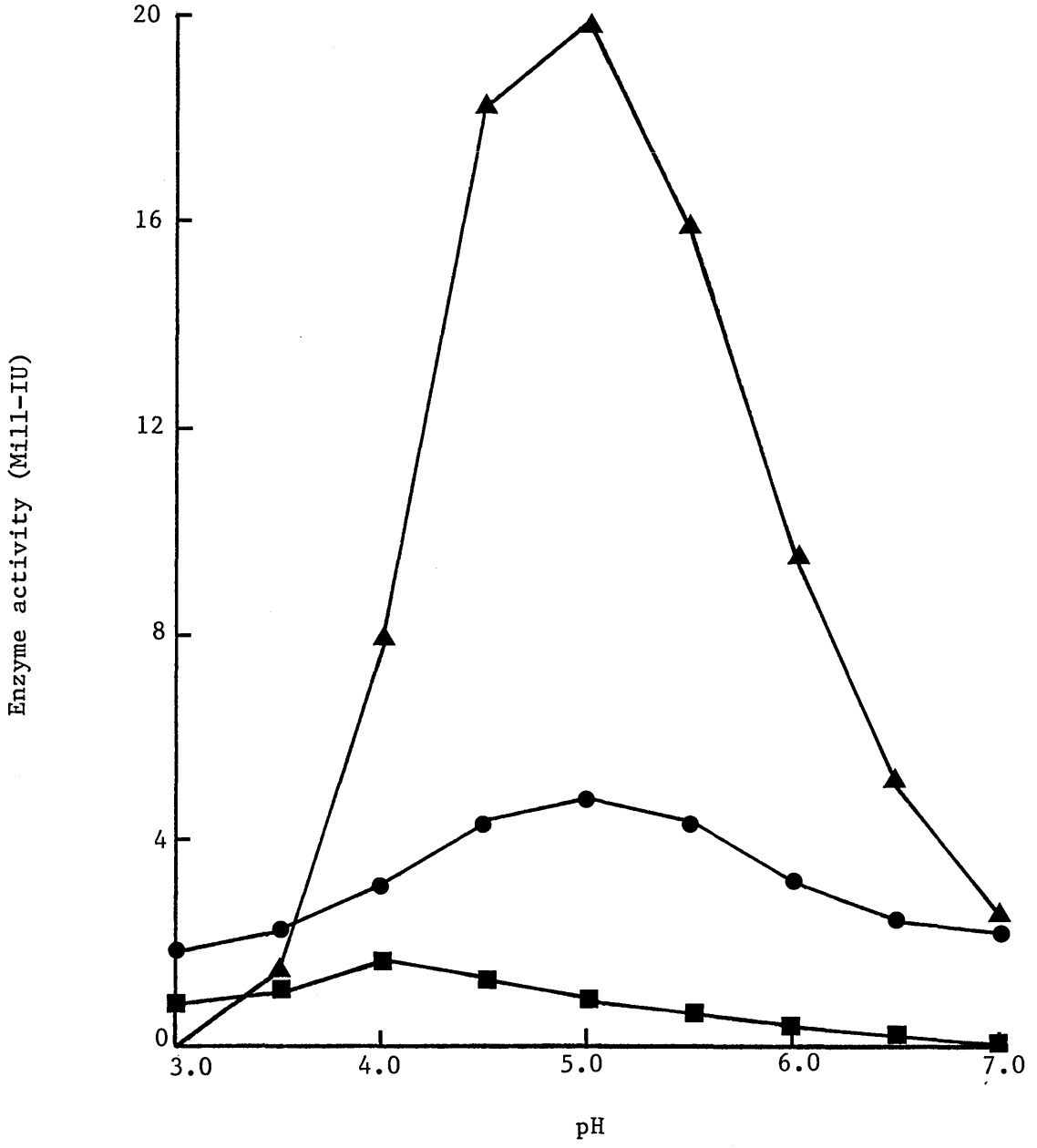

FIGURE 3.2. Effect of pH on the activity of a culture filtrate from A. fumigatus grown on A. xylinum cellulose pellicles. A supernatant from a 7 day culture of A. fumigatus was used to assess the effect of pH on endo-β-1,4-glucanase (●), β-glucosidase (■) and endo-β-1,3-xylanase (▲) activity (Section 2.8.2). Reducing sugars were determined after 0 and 60 min incubation using the PAHBAH method (Section 2.6.2).

a purified endo-β-1,4-glucanase. A possible explanation for these results is that impurities in the crude culture filtrate could have interfered with endo-β-1,4-glucanase activity. Optimum β-glucosidase activity was observed at pH4.0 using the culture filtrate of A. fumigatus (Figure 3.2). Similar values have been reported for the same enzyme from crude culture filtrates of A. phoenicis (Sternberg et al., 1977) and purified β-glucosidase enzymes from A. aculeatus (Sakamoto et al., 1985) and S. pulverulentum (Deshpande et al., 1978). Slightly higher values, ranging from 4.75-6.3 have been reported for the same enzyme from crude culture filtrates of T. reesei (Durand et al., 1984) and purified β-glucosidase enzymes from S. thermophilum (Meyer and Canevascini, 1981) and T. viride (Ladisch et al., 1977). Optimal endo-β-1,3-xylanase activity was observed at pH4.5-5.5 using the culture filtrate of A. fumigatus. This value agrees well with those obtained from other fungi including A. niger, P. janthinellum and T. viride (Dekker and Richards, 1976). Similar optima of 4.8 and 5.0 have been reported for a purified exo-β-1,4-glucanase obtained from T. viride (Berghem et al., 1975) and a crude culture filtrate of T. reesei (Durand et al., 1984) respectively. Lower values of pH3.8-4.8 have been observed for pectinase activity in crude culture filtrates from A. niger (Hara et al., 1984) and T. reesei (Durand et al., 1984). All further characterisation experiments and investigations of enzyme production were carried out at pH5.0.

3.1.2 **Effect of enzyme concentration**

Since widely differing levels of cellulase activity may be encountered in culture filtrates, it is important that the assay used should be sensitive enough to detect the lowest levels present and

yet not saturate at higher levels. In order to determine the sensitivity of the assay, a range of dilutions of the commercial cellulase were incubated with 1%(w/v) CMC in 0.1M sodium acetate buffer, pH5.0 for differing periods of time. Figure 3.3 shows that reducing sugar increases linearly with enzyme concentration up to 50μg ml^{-1}. At concentrations greater than 50μg ml^{-1}, linearity was lost due to saturation of the PAHBAH developing reagent. Consequently, when high concentrations of reducing sugars were produced, assays were repeated using suitable dilutions of the supernatant. Figure 3.4 shows the effect of incubation time on endo-β-1,4-glucanase, β-glucosidase and endo-β-1,3-xylanase of a culture filtrate of A. fumigatus grown on A. xylinum cellulose pellicles. All three enzymes demonstrated a linear increase in activity with incubation time. Figure 3.5 clearly shows the linear relationship between enzyme activity and enzyme concentration using commercial A. niger cellulase.

3.1.3 **Effect of temperature on enzyme activity**

The effect of temperature on endo-β-1,4-glucanase activity was determined using commercial A. niger cellulase (Figure 3.6). Optimal enzyme activity was observed between 50-60°C with a rapid loss of activity at temperatures above 70°C. Further experiments using a culture filtrate of A. fumigatus, grown on A. xylinum cellulose pellicles, demonstrated a broad optimum of 50-70°C for endo-β-1,4-glucanase activity. These results agree well with those reported by Stewart and Parry (1981) for the same enzyme and again with a culture filtrate of A. fumigatus. Higher optima ranging from 65-80°C have been reported for the same enzyme both in crude culture filtrates (Folan and Coughlan, 1978) and purified preparations (Moloney et al.,

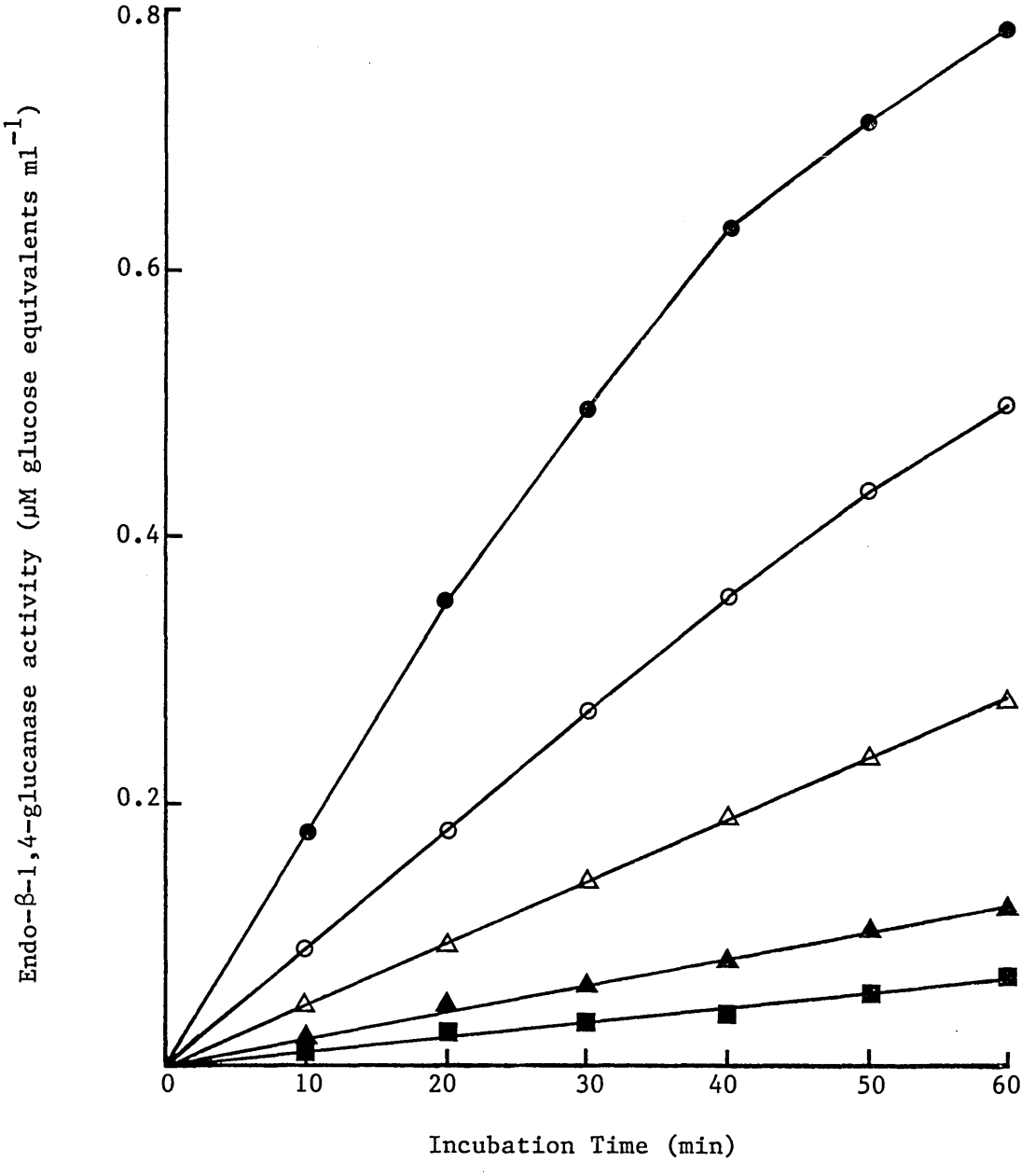

FIGURE 3.3. Effect of incubation time on reducing sugar production from CMC. Commercial A. niger cellulase was incubated at 50°C with 1% (W/v) CMC in 0.1 M sodium acetate buffer, pH 5.0 (Section 2.8.2). Reducing sugars were determined after 0 - 60 min incubation using the PAHBAH method (Section 2.6.2). The concentrations of cellulase used were:-

10 μg ml^{-1} (■), 20 μg ml^{-1} (▲), 50 μg ml^{-1} (△), 100 μg ml^{-1} (O) and 200 μg ml^{-1} (●).

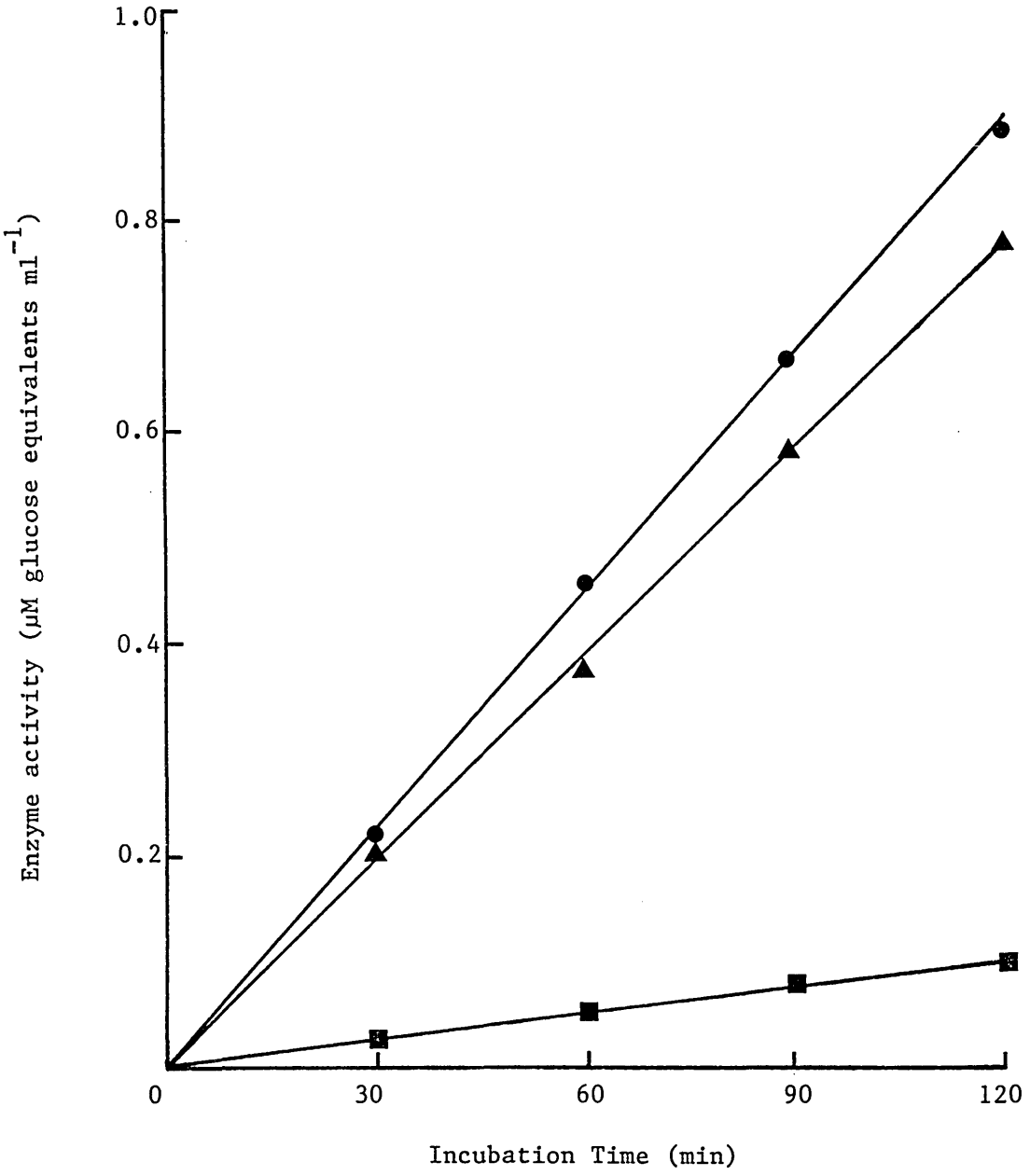

FIGURE 3.4. Effect of incubation time on the activity of a culture filtrate from A. fumigatus grown on A. xylinum cellulose pellicles. A supernatant from a 7 day culture of A. fumigatus was used to assess the effect of incubation time on endo-β-1,4-glucanase (●), β-glucosidase (■) and endo-β-1,3-xylanase (▲) activity. After 0 - 120 min incubation of the supernatant with the appropriate substrate (Section 2.8.2) at 50°C, reducing sugars were determined using the PAHBAH method (Section 2.6.2).

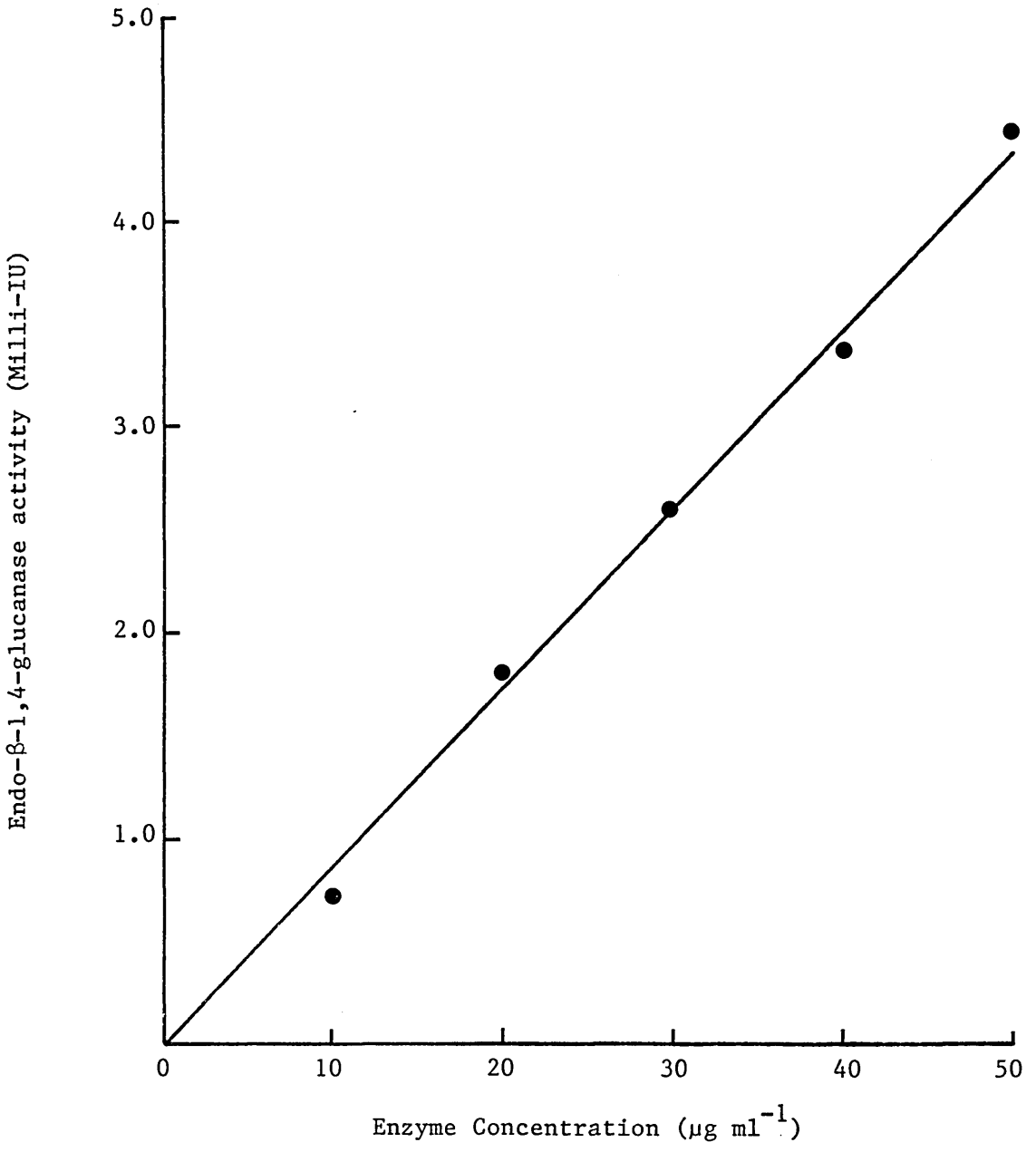

FIGURE 3.5. Effect of enzyme concentration on the activity of endo-β-1,4-glucanase. Commercial *A. niger* cellulase, 0.5 μg ml^{-1} in 0.1 M sodium acetate buffer, pH 5.0 was incubated at 50°C with 1% (W/v) CMC in the same buffer (Section 2.8.2). Reducing sugars were determined after 0 and 60 min incubation using the PAHBAH method (Section 2.6.2).

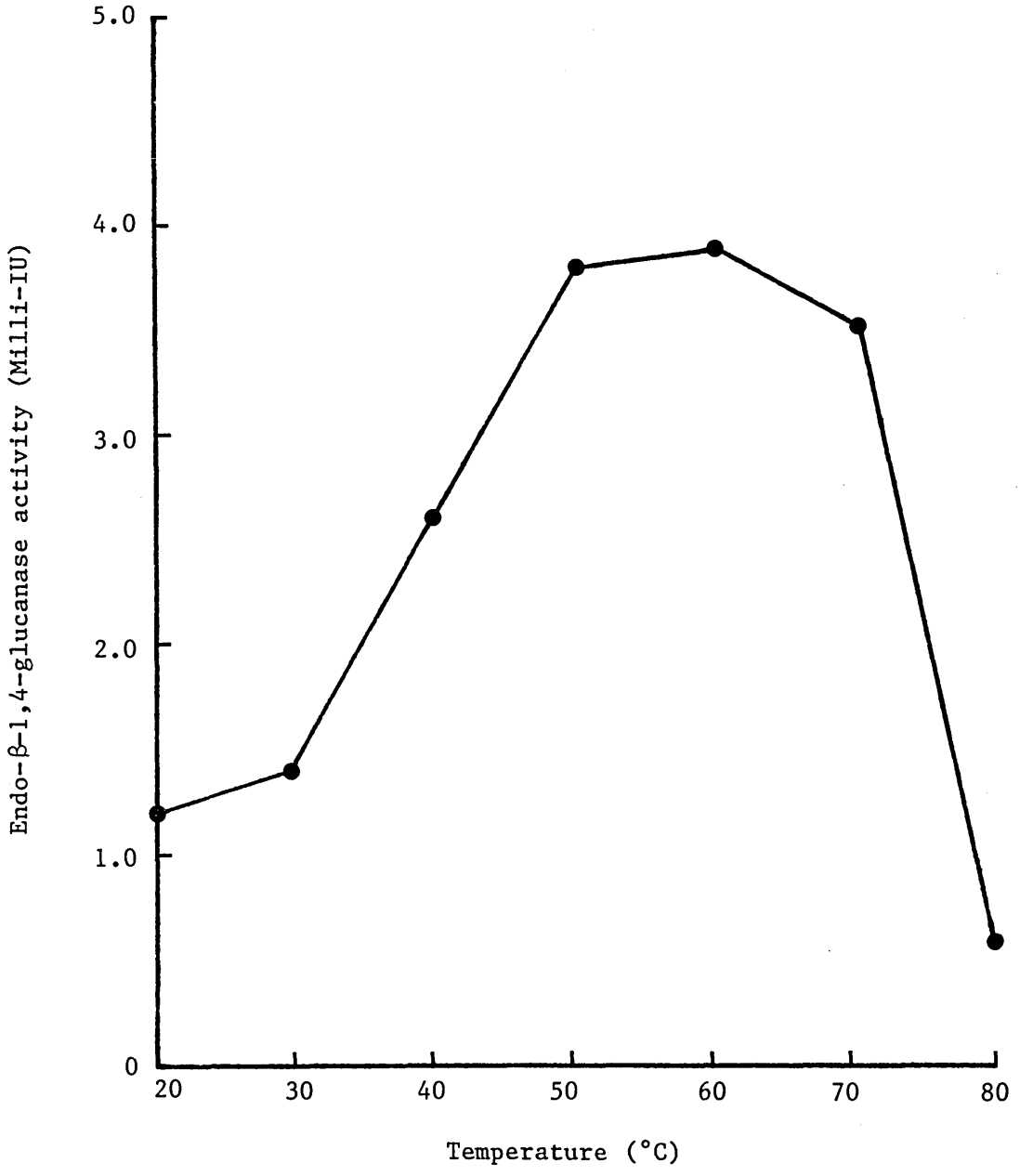

FIGURE 3.6. Effect of incubation temperature on the activity of endo-β-1,4-glucanase. Commercial A. niger cellulase, 50 μg ml^{-1} in 0.1 M sodium acetate buffer, pH 5.0 was incubated at 20 - 80°C with 1% (W/v) CMC in the same buffer (Section 2.8.2). Reducing sugars were determined after 0 and 60 min incubation using the PAHBAH method (Section 2.6.2).

1985), indicating some thermal stability of the enzymes produced by this fungus. Endo-β-1,3-xylanase in the culture filtrate from A. fumigatus shows a well defined optimum at 55-65°C, with a rapid loss of activity at temperatures exceeding 70°C (Figure 3.7). Lower values of 50°C and 55°C have been reported for the same enzyme derived from A. niger (Dekker and Richards, 1976) and T. reesei (Durand et al., 1984) respectively. β-glucosidase in the culture filtrate of A. fumigatus shows optimum activity in the range 55-65°C (Figure 3.7). These results agree well with those obtained for the same enzyme from A. aculeatus (Sakamoto et al., 1985), S. thermophilum (Meyer and Canevascini, 1981) and T. reesei (Durand et al., 1984). A slightly higher optimum of 70°C has been reported for G. emersonii (Liu et al., 1984). Other workers have observed optimum exo-β-1,4-glucanase activity at 55-60°C using culture filtrates of A. fumigatus (Stewart and Parry, 1981) and T. reesei (Durand et al., 1984). The optimum temperature has been determined for a number of enzymes, from a number of species of fungi but there are few reports regarding the thermal stability of these enzymes. Some workers have observed a rapid loss of activity with prolonged incubation at the temperature giving optimal activity (Ladisch et al., 1977).

3.2 ENZYME PRODUCTION

Initially, experiments were conducted to investigate the cellulolytic activity of a number of fungal species using the method of Rautela and Cowling (1966). These results demonstrated the superior cellulolytic activity of S. pulverulentum (Table 3.1). However, for reasons explained later in the text, this organism was

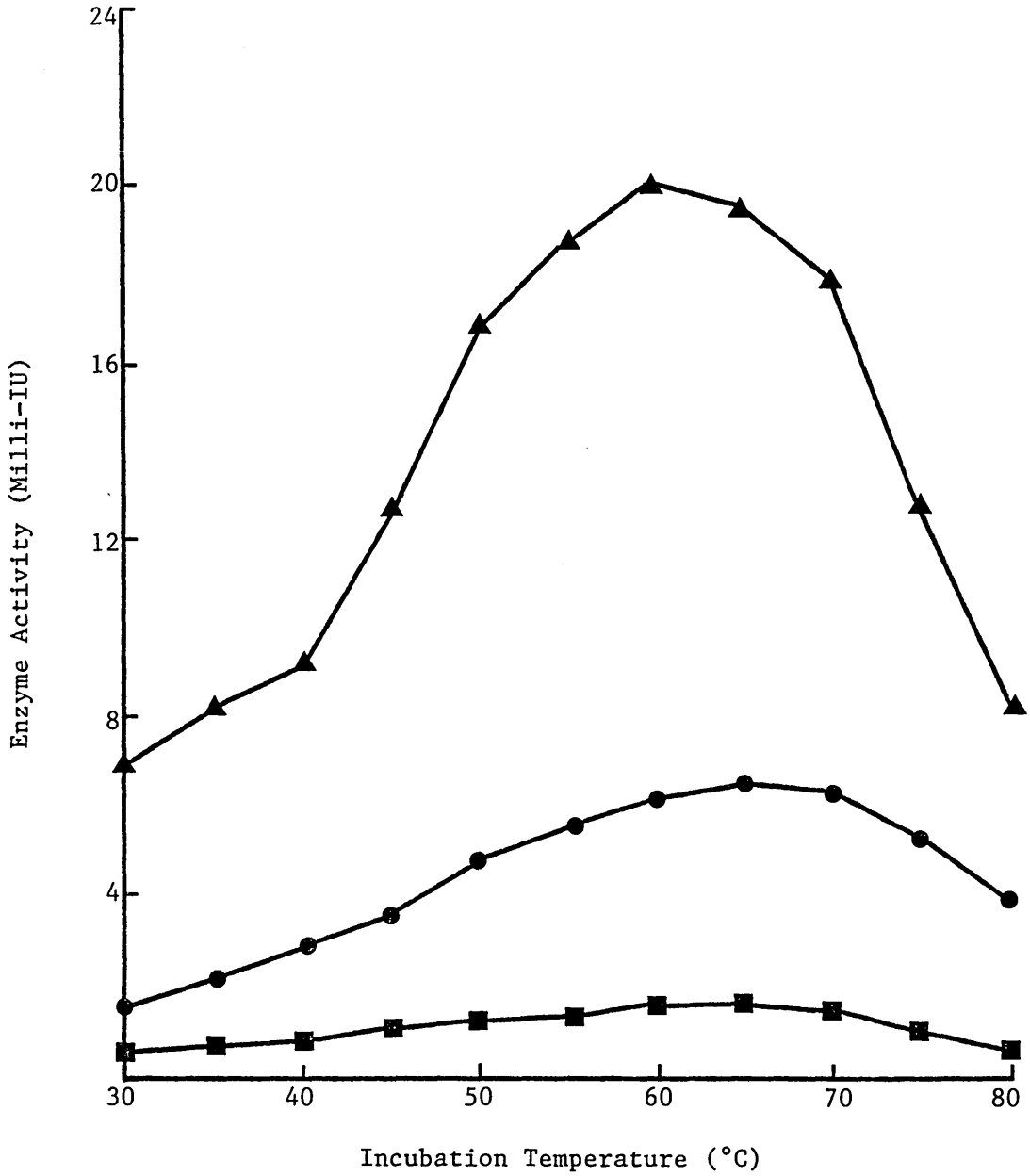

FIGURE 3.7. Effect of incubation temperature on the activity of a culture filtrate from A. fumigatus grown on A. xylinum cellulose pellicles. A supernatant from a 7 day culture of A. fumigatus was incubated with the appropriate substrate at 30 - 80°C in order to determine the effect of temperature on endo-β-1,4-glucanase (●), β-glucosidase (■) and endo-β-1,3-xylanase (▲) activity (Section 2.8.2). Reducing sugars were determined after 0 and 60 min incubation using the PAHBAH method (Section 2.6.2).

TABLE 3.1 SCREENING OF FUNGAL ORGANISMS FOR CELLULOLYTIC ACTIVITY

ORGANISMS	DEPTH OF CLEARING (mm)	
	30°C	48°C
A. fumigatus	2 ± 0.1	2 ± 0.2
A. niger	0 ± 0.0	0 ± 0.0
G. emersonii	0 ± 0.0	2 ± 0.1
P. dupontii	0 ± 0.0	0 ± 0.0
P. funiculosum	6 ± 0.8	0 ± 0.0
P. janthinellum	3 ± 0.2	0 ± 0.0
P. ochrochloron	3 ± 0.1	0 ± 0.0
S. pulverulentum	22 ± 1.4	0 ± 0.0
T. viride	3 ± 0.3	0 ± 0.0

Boiling tubes containing Eggins and Pugh Basal Medium (Table 2.1) 1% (W/v) BMC and Rose Bengal (0.035 g l^{-1}) were inoculated with equal amounts of mycelia from a number of fungal organisms. After incubation at 30°C and 48°C for 14 days, cellulolytic activity was estimated by measuring the depth of clearing. Results were expressed as mean depth of clearing ± 1 standard deviation.

unsuitable for use in current work and therefore, was not investigated further. Other organisms showing cellulolytic activity were A. fumigatus, G. emersonii, P. funiculosum, P. janthinellum, P. ochrochloron and T. viride (Table 3.1). A number of Penicillium species were also screened for endo-β-1,4-glucanase activity (Table 3.2). High levels of endo-β-1,4-glucanase activity were observed in the culture filtrates of P. funiculosum, P. janthinellum and P. ochrochloron in comparison to the other Penicillium species tested. Owing to time limitations, only these three Penicillium, species were used in further investigations, together with A. fumigatus, A. niger, G. emersonii and T. viride. Culture filtrates of these seven fungi were assayed for endo-β-1,4-glucanase, exo-β-1,4-glucanase, β-glucosidase (Section 2.8) and soluble protein (Section 2.7). Two assays were carried out for β-glucosidase, one using p-nitrophenyl-β-D-glucopyranoside and the other using salicin. Both assays are specific for aryl-β-glucosidase (Berghem and Pettersson, 1973; Sternberg et al., 1977; Bisaria and Ghose, 1981; Sakamoto et al., 1985). A third assay in which the release of reducing sugars from cellobiose was monitored using the PAHBAH method (Section 2.6.2) was found to be unsuitable due to chemical interference during the assay procedure. Other workers have monitored the release of glucose from cellobiose using the glucose oxidase method (Bucht and Eriksson, 1969), but this method was not used in current investigations.

All seven fungi were shown to produce endo-β-1,4-glucanase, with A. niger, G. emersonii, P. janthinellum and T. viride producing very high levels of this enzyme when grown on CMC (Table 3.3). These results agree well with those reported by other workers. High endo-β-1,4-glucanase activity has been observed in culture filtrates of

TABLE 3.2 ENDO-β-1,4-GLUCANASE PRODUCTION BY VARIOUS PENICILLIUM SPECIES

PENICILLIUM SPECIES	DRY WEIGHT OF CULTURE (mg ml^{-1})	ENDO- -1,4-GLUCANASE ACTIVITY EC.3.2.1.4 (Milli-IU)
P. capsulatum	2.23	0.2 ± 0.04
P. cyclopium	3.13	0.8 ± 0.08
P. expansum	1.37	0.4 ± 0.05
P. funiculosum	3.42	13.2 ± 0.23
P. janthinellum	4.12	12.4 ± 0.32
P. ochrochloron	2.92	13.9 ± 0.17
P. purpurescens	3.32	1.4 ± 0.07
P. roquefortii	2.79	0.7 ± 0.08
P. spinulosum	2.67	0.2 ± 0.03
P. wortmani	2.28	0.3 ± 0.02

Organisms were cultivated in Eggins and Pugh Basal Medium (Table 2.1) containing 1% (w/v) CMC and 0.2% (w/v) glucose for seven days at 30°C. Culture filtrates were incubated at 50°C with 1% (w/v) CMC in 0.1 M sodium acetate buffer, pH 5.0 (Section 2.8). Reducing sugars were determined after 0 and 60 min incubation using the PAHBAH method (Section 2.6.2).

TABLE 3.3 ENZYME PRODUCTION BY SEVERAL FUNGI WHEN CULTIVATED ON CMC

ORGANISM	SALICIN	CMC	FILTER PAPER	p-NITROPHENYL-β-D-GLUCOPYRANOSIDE	PROTEIN
	β-GLUCOSIDASE EC.3.2.1.21 (MILLI-IU)	ENDO-β-1,4-GLUCANASE EC.3.2.1.4 (MILLI-IU)	EXO-β-1,4-GLUCANASE EC.3.2.1.91 (MILLI-IU)	β-GLUCOSIDASE EC.3.2.1.21 (MILLI-IU)	($\mu g\ ml^{-1}$)
A. fumigatus (WT)	9.1 ± 0.36	10.7 ± 0.42	0.28 ± 0.009	4.5 ± 0.19	456 ± 8.1
A. niger (WT)	51.4 ± 0.41	67.8 ± 0.35	0.16 ± 0.028	10.9 ± 0.27	532 ± 9.7
G. emersonii	14.7 ± 0.33	49.1 ± 0.49	1.43 ± 0.019	1.70 ± 0.11	487 ± 8.6
P. funiculosum	5.3 ± 0.12	13.2 ± 0.23	0.37 ± 0.016	2.8 ± 0.13	236 ± 2.5
P. janthinellum	21.7 ± 0.48	38.0 ± 3.18	0.73 ± 0.029	6.7 ± 0.17	501 ± 7.4
P. ochrochloron (WT)	5.2 ± 0.10	8.4 ± 0.25	0.23 ± 0.010	2.4 ± 0.14	632 ± 8.3
T. viride	5.9 ± 0.51	84.8 ± 7.62	2.57 ± 0.037	4.7 ± 0.37	537 ± 7.0

WT = Wild Type

Flasks containing Eggins and Pugh Basal medium (Table 2.1), 1% (w/v) CMC and 0.2% (w/v) glucose were inoculated with 2×10^6 spores ml^{-1}. After incubation for seven days at the appropriate growth temperature (Section 2.2), the culture were harvested and the supernatants assayed for various enzyme activities (Section 2.8) and soluble protein (Section 2.7).

A. niger (Okada, 1985) and T. viride (Mandels, 1975; Saddler, 1982). Good yields have also been obtained from A. fumigatus (Parry et al., 1983; Shaker et al., 1984), G. emersonii (Liu et al., 1984), P. funiculosum (Bastawde et al., 1977; Rao et al., 1983) and P. janthinellum (Rao et al., 1986).

Exo-β-1,4-glucanase activity was observed in culture filtrates of all seven fungi when cultivated on CMC with G. emersonii and T. viride producing the highest yields and A. niger and P. ochrochloron producing low levels of this enzyme (Table 3.3). Similar results have been obtained from A. fumigatus (Stewart and Parry, 1981; Shaker et al., 1984), G. emersonii (Liu et al., 1984), P. funiculosum (Lachke et al., 1986), P. janthinellum (Rao et al., 1986) and T. viride (Mandels, 1975; Saddler, 1982).

All seven fungi were shown to produce aryl-β-glucosidase enzymes as shown by the production of reducing sugars from salicin and p-nitrophenol from p-nitrophenyl-β-D-glucopyranoside (Table 3.3). Culture filtrates of A. niger and P. janthinellum demonstrated the highest aryl-β-glucosidase activity. Comparison of these results with those reported by other workers is almost impossible due to the numerous variations in assay procedures and the substrate specificity of β-glucosidases (Deshpande et al., 1978; Meyer and Canevascini, 1981).

Higher activities of endo-β-1,4-glucanase, exo-β-1,4-glucanase and β-glucosidase were observed in culture filtrates of P. funiculosum and T. viride when grown on A. xylinum cellulose pellicles (Table 3.4). These results indicate that insoluble forms of cellulose may be better substrates for inducing cellulase

TABLE 3.4 ENZYME PRODUCTION BY SEVERAL FUNGI WHEN CULTIVATED ON A. XYLINUM CELLULOSE PELLICLES

ORGANISM	SALICIN	CMC	FILTER PAPER	p-NITROPHENYL-β-D-GLUCOPYRANOSIDE	PROTEIN
	β-GLUCOSIDASE EC.3.2.1.21 (MILLI-IU)	ENDO-β-1,4-GLUCANASE EC.3.2.1.4 (MILLI-IU)	EXO-β-1,4-GLUCANASE EC.3.2.1.91 (MILLI-IU)	β-GLUCOSIDASE EC.3.2.1.21 (MILLI-IU)	($\mu g\ ml^{-1}$)
A. fumigatus (WT)	0.4 ± 0.04	6.7 ± 0.18	0.16 ± 0.013	0.6 ± 0.11	362 ± 5.3
A. niger (WT)	20.3 ± 0.49	3.5 ± 0.14	0.06 ± 0.017	4.7 ± 0.12	151 ± 6.7
G. emersonii	3.0 ± 0.14	20.2 ± 0.37	0.52 ± 0.034	0.3 ± 0.04	438 ± 6.8
P. funiculosum	7.4 ± 0.67	41.2 ± 3.87	1.97 ± 0.026	3.8 ± 0.03	277 ± 6.1
P. janthinellum	19.8 ± 0.79	30.7 ± 2.14	0.82 ± 0.35	5.2 ± 0.14	185 ± 8.5
P. ochrochloron (WT)	4.2 ± 0.06	2.9 ± 0.09	0.19 ± 0.032	1.2 ± 0.03	349 ± 4.6
T. viride	7.9 ± 0.58	159.5 ± 7.98	3.87 ± 0.019	6.5 ± 0.13	585 ± 7.0

WT = Wild Type

Flasks containing Eggins and Pugh Basal medium (Table 2.1), A. xylinum cellulose pellicles and 0.2% (w/v) glucose were inoculated with 2×10^6 spores ml^{-1}. After incubation for seven days at the appropriate growth temperature (Section 2.2), the cultures were harvested and the supernatants assayed for various enzyme activities (Section 2.8) and soluble protein (Section 2.7).

synthesis in these two organisms than soluble forms of cellulose such as CMC. In contrast, lower yields of these three enzymes were obtained from the other five fungi, when cultivated on A. xylinum cellulose pellicles (Table 3.4) indicating that soluble forms of cellulose would appear to be better substrates for inducing cellulase synthesis in these fungi. A dramatic decrease in enzyme production was observed in A. niger when insoluble, highly crystalline cellulose was the growth substrate (Table 3.4). Soluble protein in culture filtrates obtained from A. niger, P. janthinellum and P. ochrochloron was reduced dramatically when insoluble cellulose was the growth substrate, indicating a decrease in extracellular enzyme production. A slight reduction in soluble protein was also observed in culture filtrates of A. fumigatus and G. emersonii. In contrast, soluble protein in culture filtrates of P. funiculosum and T. viride was slightly increased when these fungi were cultivated on insoluble cellulose.

Endo-β-1,4-glucanase production by five test fungi was monitored over seven days in Eggins and Pugh Basal medium containing 0.2%(W/v) glucose and 1%(W/v) CMC (Figure 3.8) and A. xylinum cellulose pellicles (Figure 3.9). Comparison of the two graphs shows clearly the superior cellulolytic ability of T. viride when cultivated on highly crystalline cellulose. As indicated earlier CMC proved to be a better substrate for A. fumigatus, A. niger, G. emersonii and P. ochrochloron. This soluble form of cellulose induced endo-β-1,4-glucanase production in these fungi after less than 24h in contrast to 48h when A. xylinum cellulose pellicles were the growth substrate.

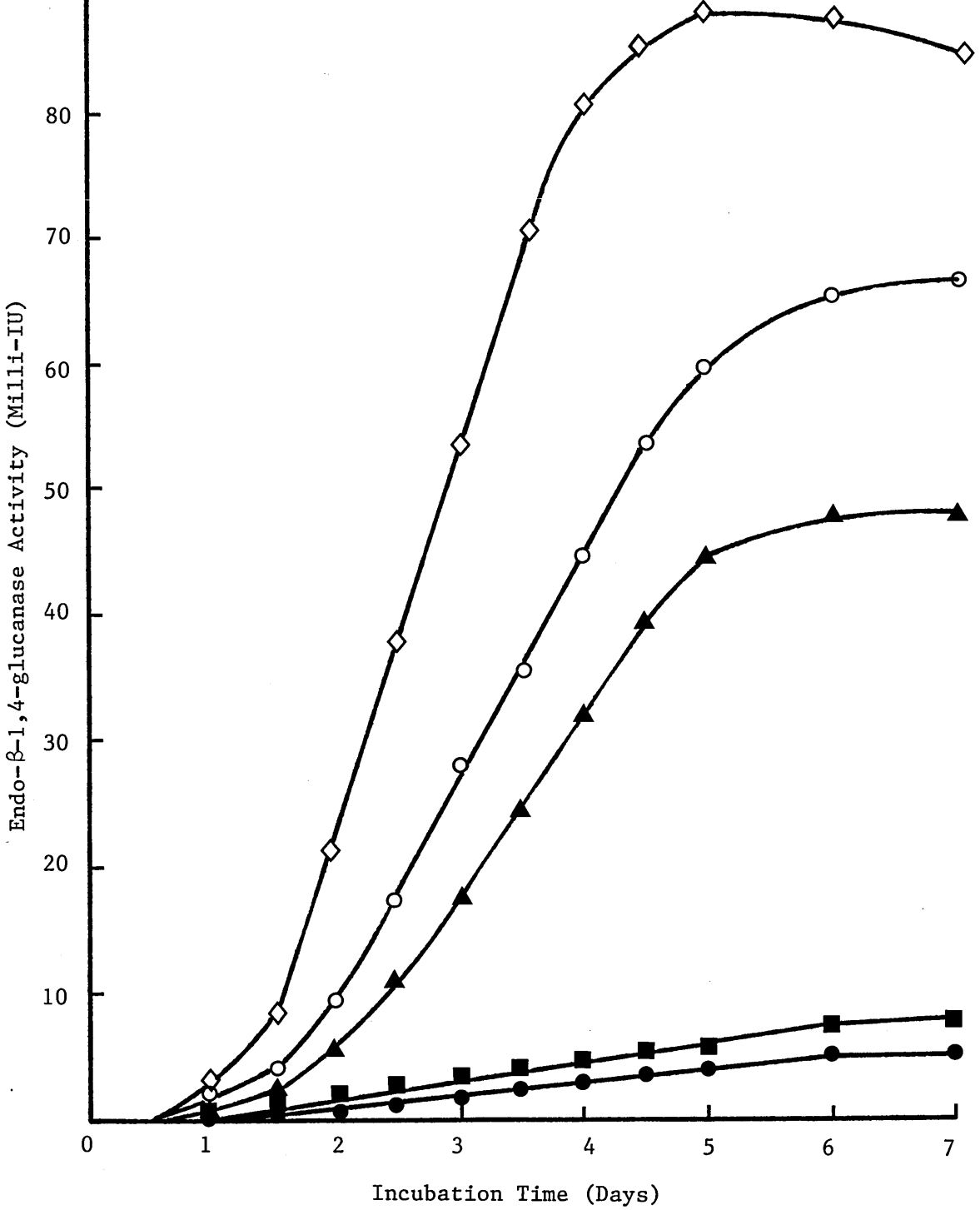

FIGURE 3.8. Endo-β-1,4-glucanase production by A. fumigatus (■), A. niger (0), G. emersonii (▲), P. ochrochloron (●) and T. viride (◇) when grown on CMC. Supernatants from 0 - 7 day cultures were assayed for endo-β-1,4-glucanase activity (Section 2.8.2). Reducing sugars were determined after 0 and 60 min incubation using the PAHBAH method (Section 2.6.2).

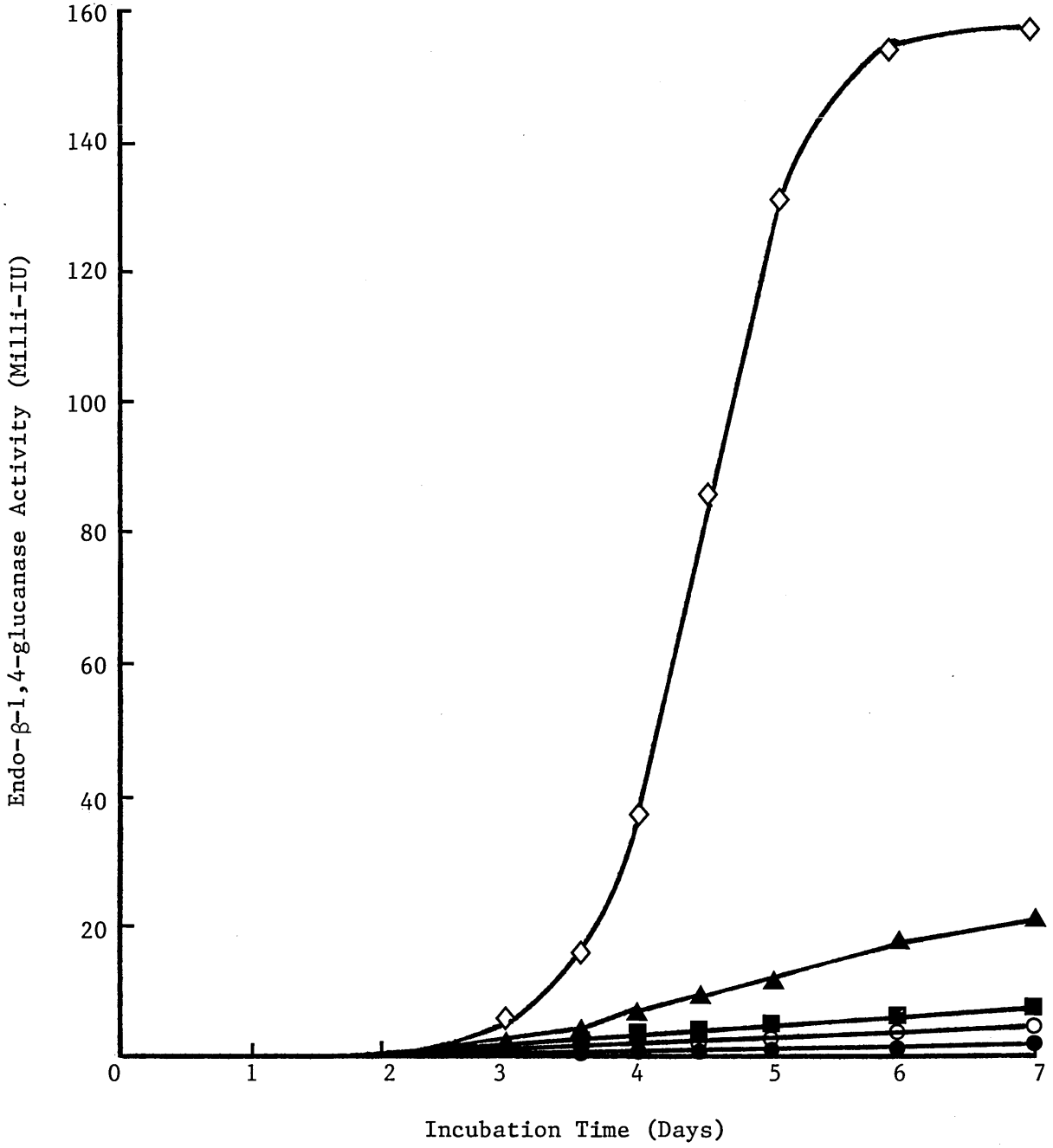

FIGURE 3.9. Endo-β-1,4-glucanase production by A. fumigatus (■), A. niger (○), G. emersonii (▲), P. ochrochloron (●) and T. viride (◇) when grown on A. xylinum cellulose pellicles. Supernatants from 0 - 7 day cultures were assayed for endo-β-1,4-glucanase activity (Section 2.8.2). Reducing sugars were determined after 0 and 60 min incubation using the PAHBAH method (Section 2.6.2).

For reasons explained later in the text, further investigations concentrated on the production of enzymes from A. fumigatus, A. niger, G. emersonii and P. ochrochloron. Culture filtrates of these four fungi were obtained after growth on A. xylinum cellulose pellicles and assayed for various enzyme activities (Table 3.5). High activities of endo-β-1,3-xylanase and endo-β-1,4-xylanase were observed in culture filtrates of all four fungi tested. Other workers have reported similar findings (Dekker and Richards, 1976; Wase and Raymahasay, 1985; Lachke et al., 1986). However, no exo-β-1,4-xylosidase was detected in culture filtrates of A. niger and G. emersonii and only low activity was observed in culture filtrates of A. fumigatus and P. ochrochloron (Table 3.5). In contrast to these results, Oguntimein and Reilly (1980) detected the presence of exo-β-1,4-xylosidase in a culture filtrate of A. niger. It is possible that the substrate used in these investigations was incapable of inducing synthesis of this enzyme in these fungi and therefore these findings should not be taken as conclusive. Further, low levels of pectinase activity were observed in culture filtrates of A. fumigatus, A. niger and P. ochrochloron and no activity was observed in G. emersonii (Table 3.5). Laminarinase activity was observed in culture filtrates of all four fungi although only low levels were detected in A. fumigatus (Table 3.5). However, the major interest in this investigation was the group of enzymes responsible for the hydrolysis of pure crystalline cellulose. Further, one of the aims of this thesis was to increase enzyme production, particularly those responsible for the breakdown of crystalline cellulose by crossing mesophilic and thermophilic fungi using protoplast fusion. Hybrids

TABLE 3.5 ENZYME PRODUCTION BY FOUR TEST ORGANISMS

ENZYME	EC. NO.	ENZYME ACTIVITY (MILLI-IU)			
		A. FUMIGATUS (WT)	A. NIGER (WT)	G. EMERSONII (WT)	P. OCHROCHLORON (WT)
Endo-β-1,4-glucanase	3.2.1.4	6.3 ± 0.47	2.8 ± 0.21	10.5 ± 0.29	8.7 ± 0.36
Laminarinase	3.2.1.6	0.4 ± 0.11	2.7 ± 0.38	3.5 ± 0.38	6.2 ± 1.59
Endo-β-1,4-xylanase	3.2.1.8	16.0 ± 0.96	18.5 ± 2.01	17.3 ± 1.11	14.0 ± 1.06
Pectinase	3.2.1.15	0.3 ± 0.52	0.3 ± 0.20	0.0 ± 0.00	0.3 ± 0.12
β-glucosidase	3.2.1.21	0.5 ± 0.24	15.0 ± 0.65	1.5 ± 0.14	6.8 ± 0.13
Endo-β-1,3-xylanase	3.2.1.32	13.0 ± 0.26	12.4 ± 0.29	9.3 ± 0.57	8.9 ± 0.67
Exo-β-1,4-xylosidase	3.2.1.37	3.0 ± 1.34	0.0 ± 0.00	0.0 ± 0.00	0.3 ± 0.12
Exo-β-1,4-glucanase	3.2.1.91	0.34 ± 0.091	0.04 ± 0.006	0.38 ± 0.43	0.22 ± 0.067

WT = Wild Type

Flasks containing Eggins and Pugh Basal Medium (Table 2.1), A. xylinum cellulose pellicles and 0.2% (w/v) glucose were inoculated with 2 x 10^6 spores ml^{-1}. After incubation for seven days at the appropriate growth temperature (Section 2.2), the cultures were harvested and the supernatants assayed for various enzyme activities (Section 2.8)

could then be screened for enzyme complement and thermal stability of the enzymes produced, these results could then be compared with those obtained for the parental types.

Mutagenesis was induced in some of the parental types in order to obtain suitable genetic markers for use in fusion studies. However, such treatments may have affected enzyme production and experiments were therefore conducted to investigate this possibility. Enzyme production was altered in a UV-mutant of P. ochrochloron (Mutant 42) with increased endo-β-1,4-glucanase and lower aryl-β-glucosidase activities, when grown on CMC (Table 3.6) and A. xylinum cellulose pellicles (Table 3.7). A dramatic increase in soluble protein was also observed in the culture filtrates of this UV-mutant when grown both on CMC (Table 3.6) and A. xylinum cellulose pellicles (Table 3.7). There was, however, no alteration in enzyme production by a fungicide resistant mutant of A. niger, although a slight increase in endo-β-1,4-glucanase activity was observed. A slight decrease in the yield of all three enzymes was observed in the culture filtrates of a fungicide resistant mutant of A. fumigatus when grown on CMC (Table 3.6) and A. xylinum cellulose pellicles (Table 3.7).

TABLE 3.6 COMPARISON OF ENZYME PRODUCTION BY MUTANT AND WILD TYPE ORGANISMS WHEN CULTIVATED ON CMC

ORGANISM	SALICIN	CMC	FILTER PAPER	P-NITROPHENYL-β-D-GLUCOPYRANOSIDE	PROTEIN ($\mu g\ ml^{-1}$)
	β-GLUCOSIDASE EC.3.2.1.21 (MILLI-IU)	ENDO-β-1,4-GLUCANASE EC.3.2.1.4 (MILLI-IU)	EXO-β-1,4-GLUCANASE EC.3.2.1.91 (MILLI-IU)	β-GLUCOSIDASE EC.3.2.1.21 EC.3.2.1.21	
A. fumigatus (MUT)	8.2 ± 0.29	9.5 ± 0.20	0.20 ± 0.003	4.2 ± 0.14	420 ± 9.5
A. fumigatus (WT)	10.1 ± 0.42	11.9 ± 0.17	0.69 ± 0.002	4.7 ± 0.37	363 ± 8.4
A. niger (MUT)	56.9 ± 0.39	80.4 ± 0.41	1.69 ± 0.022	12.8 ± 0.46	536 ± 9.9
A. niger (WT)	58.6 ± 0.26	69.8 ± 0.20	1.44 ± 0.020	12.7 ± 0.34	587 ± 7.8
P. ochrochloron (MUT)	0.5 ± 0.07	3.0 ± 0.22	0.20 ± 0.008	0.0 ± 0.00	2490 ± 10.4
P. ochrochloron (WT)	6.8 ± 0.13	10.7 ± 0.36	0.26 ± 0.015	1.9 ± 0.25	537 ± 6.1

MUT = Mutant; WT = Wild Type

Flasks containing Eggins and Pugh Basal Medium (Table 2.1), 1% (w/v) CMC and 0.2% (w/v) glucose were inoculated with 2×10^6 spores ml^{-1}. After incubation for seven days at the appropriate growth temperature (Section 2.2), the cultures were harvested and the supernatants assayed for various enzyme activities (Section 2.8) and soluble protein (Section 2.7). Eggins and Pugh Basal Medium was replaced by Complete Medium (Table 2.4) when cultivating P. ochrochloron (Mut).

TABLE 3.7 COMPARISON OF ENZYME PRODUCTION BY MUTANT AND WILD TYPE ORGANISMS WHEN CULTIVATED ON A. XYLINUM CELLULOSE PELLICLES

ORGANISM	SALICIN	CMC	FILTER PAPER	P-NITROPHENYL-β-D-GLUCOPYRANOSIDE	PROTEIN ($\mu g\ ml^{-1}$)
	β-GLUCOSIDASE EC.3.2.1.21 (MILLI-IU)	ENDO-β-1,4-GLUCANASE EC.3.2.1.4 (MILLI-IU)	EXO-β-1,4-GLUCANASE EC.3.2.1.91 (MILLI-IU)	β-GLUCOSIDASE EC.3.2.1.21 EC.3.2.1.21	
A. fumigatus (MUT)	0.6 ± 0.25	4.2 ± 0.12	0.05 ± 0.017	0.6 ± 0.12	401 ± 3.5
A. fumigatus (WT)	1.3 ± 0.14	6.2 ± 0.16	0.12 ± 0.022	0.6 ± 0.05	237 ± 7.3
A. niger (MUT)	22.5 ± 0.23	1.8 ± 0.07	0.03 ± 0.011	5.5 ± 0.14	162 ± 4.3
A. niger (WT)	18.4 ± 0.59	3.0 ± 0.23	0.07 ± 0.025	4.1 ± 0.13	142 ± 9.8
P. ochrochloron (MUT)	0.2 ± 0.14	1.8 ± 0.08	0.17 ± 0.027	0.0 ± 0.00	1605 ± 14.1
P. ochrochloron (WT)	2.9 ± 0.09	3.5 ± 0.13	0.24 ± 0.043	0.3 ± 0.08	300 ± 2.9

MUT = Mutant; WT = Wild Type

Flasks containing Eggins and Pugh Basal Medium (Table 2.1), A. xylinum cellulose pellicles and 0.2% (w/v) glucose were inoculated with 2×10^6 spores ml^{-1}. After incubation for seven days at the appropriate growth temperature (Section 2.2), the cultures were harvested and the supernatants assayed for various enzyme activities (Section 2.8) and soluble protein (Section 2.7) Eggins and Pugh Basal Medium was replaced by Complete Medium (Table 2.4) when cultivating P. ochrochloron (MUT),

Protoplast yields from filamentous fungi are affected by a number of factors. These include: enzyme type and concentration, incubation conditions, mycelial age and concentration, osmoticum and pH (Peberdy et al., 1976; Hamlyn et al., 1981; Davis, 1985; Hou and Jong, 1985). Davis (1985) indicated that there are no set conditions for protoplast isolation and that the optimum conditions must be determined for each organism under investigation. A number of characterisation experiments were consequently carried out on the selected species to determine the most suitable isolation conditions for protoplast production on a larger scale, for use in enzyme, fusion and regeneration studies.

The criteria used for protoplast identification were those stipulated by Villanueva and Garcia-Acha (1972). Protoplasts are usually spherical and are always osmotically sensitive due to the absence of the rigid cell wall. Protoplasts may be differentiated from spores by their differences in phase characteristics and the fact that they readily burst in water. Sometimes protoplast-like structures may be isolated which still have cell wall residues attached and these are referred to as sphaeroplasts. The status of protoplasts may be confirmed using fluorescence (Peberdy and Buckley, 1973; Anne et al., 1974) or electron microscopic techniques (Davis, 1985) but these are time-consuming and not practicable on a routine basis.

4.1 ENZYME EFFECTS ON PROTOPLAST RELEASE

Due to variations in fungal cell wall structure and composition (Bartnicki-Garcia, 1968; Reiss, 1983; Farkas, 1985), each lytic enzyme will have a characteristic effect on different organisms.

Hamlyn et al. (1981) investigated the mycolytic activities of a number of commercial enzymes. These workers found that Novozym^{TM} 234, a multi-component enzyme preparation, produced by submerged cultivation of T. viride, was effective in producing protoplasts from both yeasts (Stephen and Nasim, 1981) and filamentous fungi, including Aspergillus sp. and Penicillium chrysogenum (Hamlyn et al., 1981). This preparation is reported to have high activities of chitinase, α-1,3-glucanase, β-1,3-glucanase, laminarinase, protease and xylanase. Hamlyn et al. (1981) also indicated that protoplast yields may be improved by supplementation of Novozym^{TM} 234 with other commercial enzymes.

Novozym^{TM} 234 produced good yields of protoplasts from A. fumigatus, A. niger, G. emersonii and P. ochrochloron (Table 4.1). This enzyme used at a concentration of 1mg ml^{-1} produced more protoplasts from A. fumigatus and A. niger than a combination of chitinase (1unit ml^{-1}),β-glucuronidase (0.1ml ml^{-1}) and protease (20units ml^{-1}).

Supplementation of Novozym^{TM} 234 with chitinase markedly increased protoplast yields from all four organisms (Table 4.1). The importance of chitinase in protoplast production has been observed in other fungi (de Vries and Wessels, 1973; Davis et al., 1977; Thomas et al., 1979) and is necessary owing to the presence of chitin in the majority of filamentous fungal cell walls (Bartnicki-Garcia, 1968; Reiss, 1983; Farkas, 1985). Chitin has been observed as an inner layer in fungal cell walls, covered with protein and a glycoprotein reticulum (Hunsley and Kay, 1976; Van der Valk et al., 1977; Trinci, 1978). This observation could explain why chitinase, when used on its own, is ineffective in producing protoplasts from filamentous

TABLE 4.1 EFFECT OF VARIOUS ENZYME COMBINATIONS ON PROTOPLAST RELEASE FROM A. FUMIGATUS, A. NIGER, G. EMERSONII AND P. OCHROCHLORON

LYTIC ENZYME(S)	PROTOPLASTS RELEASED (X 10^7 ml^{-1} MYCELIUM)			
	A. FUMIGATUS	A. NIGER	G. EMERSONII	P. OCHROCHLORON
Novozym^{TM}234	0.64 ± 0.05	0.29 ± 0.02	1.26 ± 0.08	0.74 ± 0.05
Novozym^{TM}234/chitinase	1.26 ± 0.12	1.20 ± 0.09	3.72 ± 0.13	2.09 ± 0.11
Novozym^{TM}234/chitinase/protease	1.08 ± 0.05	0.63 ± 0.04	2.31 ± 0.11	6.33 ± 0.14
Novozym^{TM}234/protease	1.05 ± 0.06	0.29 ± 0.02	0.94 ± 0.07	1.31 ± 0.05
Novozym^{TM}234/cellulase CP/cereflo	2.18 ± 0.16	4.11 ± 0.19	2.01 ± 0.14	6.85 ± 0.17
Novozym^{TM}234/cellulase CP	1.23 ± 0.08	4.21 ± 0.14	1.58 ± 0.13	6.83 ± 0.22
Novozym^{TM}234/cereflo	0.82 ± 0.09	0.56 ± 0.03	2.07 ± 0.15	2.45 ± 0.09
β-glucuronidase/chitinase/protease	0.16 ± 0.02	0.09 ± 0.01	3.33 ± 0.13	1.09 ± 0.07

Mycelia of optimum age (Figure 4.7) were harvested, washed and resuspended in incubation medium (Section 2.4). 1.0 ml aliquots of mycelial suspensions were incubated with various lytic enzymes at 30°C. After 2 h, protoplast numbers were assessed using an improved Neubauer counting chamber. Lytic enzymes used were cellulase CP (5 mg), cereflo (0.1 ml), chitinase (1 unit), β-glucuronidase (0.1 ml), Novozym^{TM}234 (1 mg) and protease (20 units) all in 1.0 ml mycelium.

fungi, but with other enzymes promotes protoplast release from the same fungi (Thomas et al., 1979). Thomas (1981) indicated that protoplast yields from A. niger increased proportionally with increasing chitinase concentration (up to 10units of enzyme ml^{-1} mycelium), further indicating the importance of chitinase in increasing protoplast yields. However, commercial preparations of this enzyme were considered to be too expensive and consequently it was not used to produce protoplasts for further studies.

The commercial enzyme, cellulase CP, produced by P.funiculosum, was used in combination with Novozym^{TM} 234 to further investigate protoplast release from the test organisms. This combination resulted in a dramatic increase in protoplast yields from A. niger and P. ochrochloron and a slight increase in yields from A. fumigatus and G. emersonii (Table 4.1). Cellulase has also been shown to be important in the formation of protoplasts from Pythium sp. (Sietsma et al., 1968) and other filamentous fungi (Hamlyn et al., 1981). The importance of cellulase in determining protoplast release from these fungi is clear in that higher protoplast yields may be obtained. However, the solubility of cellulase CP was poor, preventing the isolation of protoplasts, free from enzyme and mycelial debris. This enzyme was therefore also left out in the production of protoplasts for further studies.

Protein exists in fungal cell walls in the form of protein-polysaccharide complexes in which the proportion of carbohydrate varies in different organisms (Hunsley and Burnett, 1970; Trinci, 1978; Farkas, 1985). The protein content of fungal cell walls has been reported to be about 3-10% (Ruiz-Herrera, 1967; Bull, 1970;

Bainbridge et al., 1979). Experiments were therefore conducted to determine whether protease was important in effecting protoplast release from the test organisms. This enzyme, when used in combination with Novozym™ 234 and other enzymes did not increase protoplast yields from the test organisms to any great extent (Table 4.1). Protease had no effect on protoplast release from A. niger and actually reduced protoplast yields from G. emersonii (Table 4.1). This enzyme was, therefore, also left out when producing protoplasts for use in further studies.

Detergents have also been shown to increase protoplast yields from filamentous fungi (Sietsma and de Boer, 1973) and therefore experiments were carried out to investigate this effect. Cereflo increased protoplast yields from all four organisms when used in combination with Novozym™ 234 (Table 4.1). Surprisingly, Cereflo when added to a combination of Novozym™ 234 and cellulase CP did not result in any further increase in the number of protoplasts obtained from A. niger and P. ochrochloron (Table 4.1). This may indicate that maximum protoplast yields were obtained from these fungi using Novozym™ 234 and Cellulase CP. Very little information is available regarding the effect of detergents on membrane stability and protoplast physiology and so Cereflo was not used for the production of protoplasts for use in further studies.

Three different batches of Novozym™ 234 were used throughout the work carried out in this laboratory. Consequently, the optimum concentration had to be determined for each batch. Figure 4.1 demonstrates the effect of Novozym™ 234 concentration on the release of protoplasts from the four test organisms. For this batch, the optimum concentration of Novozym™ 234 for protoplast release from

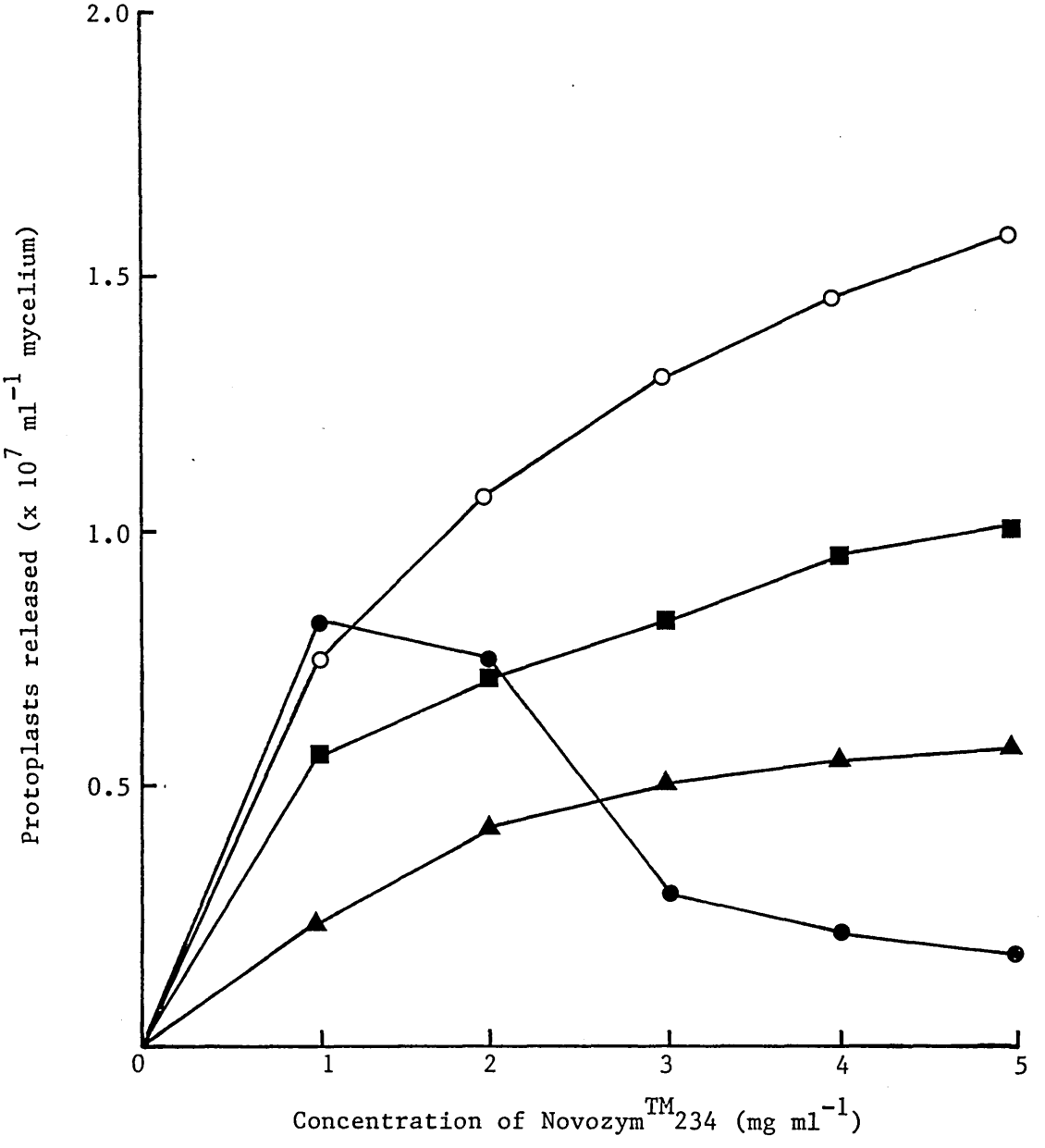

FIGURE 4.1. Effect of Novozym^{TM}234 concentration on the release of protoplasts from A. fumigatus (■), A. niger (▲), G. emersonii (○) and P. ochrochloron (●). Mycelia of optimum age (Figure 4.7) were harvested, washed and resuspended in incubation medium (Section 2.4). After incubation at 30°C with 1 - 5 mg ml^{-1} Novozym^{TM}234 for 2 h, protoplast numbers were assessed using an improved Neubauer counting chamber.

the four test organisms was 1mg ml^{-1} (Figure 4.1). Higher concentrations did result in a slight increase in the number of protoplasts released from A. niger, G. emersonii and P. ochrochloron (Figure 4.1). Protoplasts produced using high concentrations of Novozym^{TM} 234 showed signs of degeneration and the number of protoplasts obtained from P. ochrochloron decreased significantly (Figure 4.1). Considering all the information regarding the effect of enzyme type and concentration, it was decided to use Novozym^{TM} 234 only, at a concentration of 1mg ml^{-1}, in the production of protoplasts for further studies.

The time of incubation with the lytic enzyme(s) is also a very important parameter in determining protoplast release. It is not known how prolonged incubation will affect the physiological nature of the protoplasts produced. Protoplast release with respect to incubation time has been well documented (Gibson and Peberdy, 1972; Anne et al., 1974) and the situation was no different in the test organisms used in this study. After 10-15min, hyphal tips appeared swollen and protoplasts emerged from the hyphal tips or in their close vicinity, through ruptures or pores in the cell wall caused by the action of lytic enzymes. Later, protoplasts emerged from the more mature hyphal regions. The proportion of distally released protoplasts increased with time. After 4-5h incubation, the mycelium was almost completely transformed into protoplasts leaving mycelial debris and empty walls. This differential digestion of hyphal walls may be explained by differences in the structure and complexity of the wall regions (Burnett, 1979). Early released protoplasts originating from hyphal tips were small, spherical bodies showing dense cytoplasm with few vacuoles, if any. In contrast, protoplasts

appearing after prolonged incubation were much larger than those observed initially with a diffuse, highly vacuolated and granular cytoplasm. Grove and Bracker (1970) demonstrated that the distribution of organelles differs in the apex and distal regions of hyphae. Consequently, protoplasts produced from the fungal mycelium will be heterogeneous in their organelle constitution and possibly their properties also (Peberdy, 1976). Further heterogeneity in filamentous fungi has been observed with respect to the number of nuclei in protoplasts (Peberdy, 1976). In unicellular organisms such as yeasts, the protoplasts produced are more homogeneous with respect to organelle constitution.

All four organisms used in this study demonstrated a proportional increase in protoplast numbers after incubation with Novozym^{TM} 234 for 0-3h (Figure 4.2). Prolonged incubation with high concentrations of lytic enzymes resulted in the degeneration of protoplasts. Gabriel (1968) observed that protoplasts obtained after prolonged incubation with lytic enzymes showed impaired regeneration. Other workers have pointed out that protoplasts to be used in cellular studies should be obtained in the shortest possible time (de Vries and Wessels, 1973; Peberdy *et al*., 1977). The protoplasts obtained after incubation with 1mg ml^{-1} Novozym^{TM} 234 for 2h showed no signs of degeneration and were subsequently used in further studies.

4.2 EFFECT OF THE INCUBATION MEDIUM ON PROTOPLAST RELEASE

A wide range of osmotic stabilizers including inorganic salts, sugars and sugar alcohols have been used to stabilise protoplasts released from fungi (Sietsma and de Boer, 1973; Peberdy *et al*.,

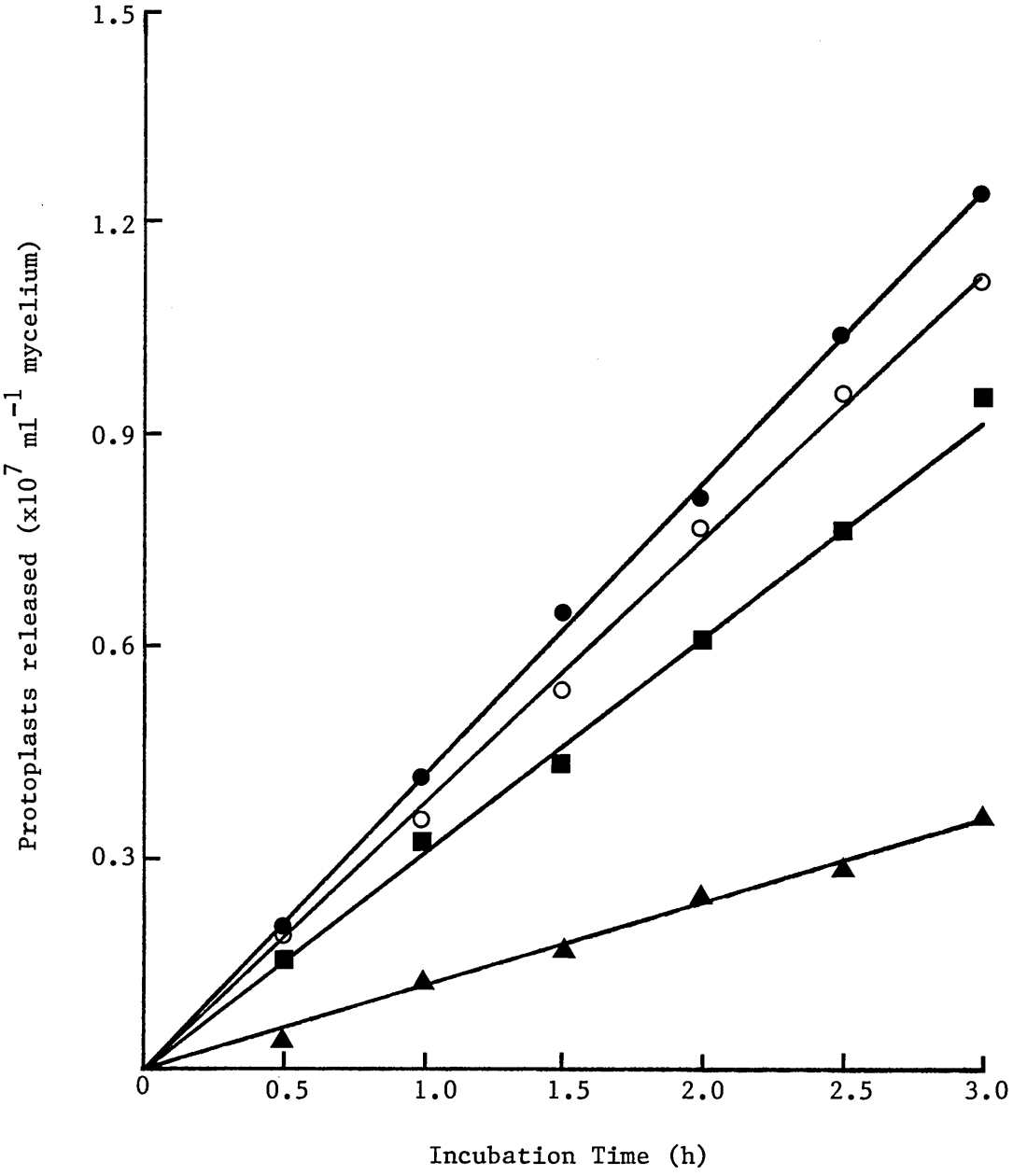

FIGURE 4.2. Effect of incubation time on the release of protoplasts from A. fumigatus (■), A. niger (▲), G. emersonii (○) and P. ochrochloron (●). Mycelia of optimum age (Figure 4.7) were harvested, washed and resuspended in incubation medium (Section 2.4). After incubation at 30°C with 1 mg ml^{-1} Novozym^{TM}234 for 0 - 3 h, protoplast numbers were assessed using an improved Neubauer counting chamber.

1976; Davis, 1985). The inorganic salts, ammonium chloride, magnesium sulphate and potassium chloride have been shown to be most effective in the isolation of protoplasts from filamentous fungi (de Vries and Wessels, 1972; Peberdy et al., 1976; Thomas et al., 1979). In contrast, sugars and sugar alcohols are generally used with yeasts (Eddy and Williamson, 1957; Gabriel, 1968; Stephen and Nasim, 1981). Other workers have observed that protoplasts isolated in magnesium sulphate are highly vacuolated which facilitates the isolation of protoplasts, free from mycelial debris as they float on the supernatant after centrifugation (de Vries and Wessels, 1972; Peberdy and Isaac, 1976). The effect of four inorganic salts; ammonium chloride, magnesium sulphate, potassium chloride and sodium chloride on the production of protoplasts from the four test organisms was investigated. magnesium sulphate was either a poor osmoticum or it prevented protoplast release. Alternatively it may have had a detrimental effect on the protoplasts produced since this inorganic salt produced the smallest number of protoplasts from all four fungi (Table 4.2). The best yields of protoplasts from these fungi were achieved using sodium chloride as the osmoticum (Table 4.2) and consequently this was used as the osmoticum in the production of protoplasts from the test organisms for use in further studies.

In addition to the type of osmoticum, the concentration of osmoticum has also been shown to be an important factor in determining protoplast release (de Vries and Wessels, 1972; Anne et al., 1974; Peberdy et al., 1976). When monovalent or divalent ions are used the optimum concentration is usually 0.5-0.7M (Anne et al., 1974; Peberdy et al., 1976; Thomas and Davis, 1980). This was confirmed when sodium chloride was used to isolate protoplasts from

TABLE 4.2 EFFECT OF VARIOUS OSMOTICA ON THE RELEASE OF PROTOPLASTS FROM A. FUMIGATUS, A. NIGER, G. EMERSONII AND P. OCHROCHLORON

OSMOTICUM	PROTOPLASTS RELEASED (x 10^7 ml^{-1} MYCELIUM)			
	A. FUMIGATUS	A. NIGER	G. EMERSONII	P. OCHROCHLORON
0.6 M NaCl	0.99 ± 0.09	0.38 ± 0.04	1.60 ± 0.12	1.07 ± 0.09
0.6 M KCl	0.57 ± 0.07	0.10 ± 0.01	0.91 ± 0.11	0.27 ± 0.02
0.6 M NH_4Cl	0.18 ± 0.02	0.09 ± 0.01	0.54 ± 0.07	0.23 ± 0.03
0.6 M $MgSO_4$	0.16 ± 0.02	0.04 ± 0.01	0.42 ± 0.04	0.15 ± 0.01
0.6 M NaCl + 0.1 M $CaCl_2$	0.34 ± 0.05	0.10 ± 0.02	0.44 ± 0.05	2.80 ± 0.14
0.6 M KCl + 0.1 M $CaCl_2$	0.54 ± 0.06	0.11 ± 0.02	0.41 ± 0.02	2.84 ± 0.19
0.6 M KCl + 0.1 M $Ca(NO_3)_2$	0.60 ± 0.05	0.09 ± 0.02	0.36 ± 0.04	2.35 ± 0.09

Mycelia of optimum age (Figure 4.7) were harvested, washed and resuspended in MES buffer (0.1 M; pH 5.5) containing various inorganic salts. After incubation at 30°C with 1 mg ml^{-1} Novozym^{TM}234 for 2 h, protoplast numbers were assessed using an Improved Neubauer Counting Chamber.

P. ochrochloron (Figure 4.3). The optimum concentration was determined to be 0.6M. Similar results were obtained with A. fumigatus and A. niger. Higher concentrations have been reported in other systems (Anne et al., 1974; Hamlyn et al., 1981; Lynch et al., 1985).

Some workers have demonstrated an enhancement in protoplast production from yeast (Necas, 1955) and filamentous fungi, including A. fumigatus and A. niger (Musilkova and Fencl, 1966; Thomas and Davis, 1980) when the osmoticum was supplemented with calcium chloride. Protoplast release from the four test organisms was examined using potassium chloride and sodium chloride with and without calcium chloride. Only P. ochrochloron showed any enhancement in protoplast yields with calcium chloride (Table 4.2). In contrast to the results obtained by Musilkova and Fencl (1966) and Thomas and Davis (1980), protoplast yields from A. fumigatus and A. niger were not increased when the osmoticum was supplemented with calcium chloride. The only parameter that was different in these experiments was the lytic enzymes used. The above workers used β-glucuronidase rather than Novozym^{TM} 234. Thomas and Davis (1980) linked this increase in activity with chitinase. More information is needed to link this enhancement to a particular enzyme or group of enzymes. The optimum concentration of calcium chloride for protoplast release from P. ochrochloron was determined as 0.03M (Figure 4.4) which is close to the value reported for A. fumigatus (Thomas and Davis, 1980).

Numerous buffers have been used to maintain the pH of the incubation medium. These include: citrate phosphate, HEPES, MES, phosphate and sodium citrate (Anne et al., 1974; Peberdy et al.,

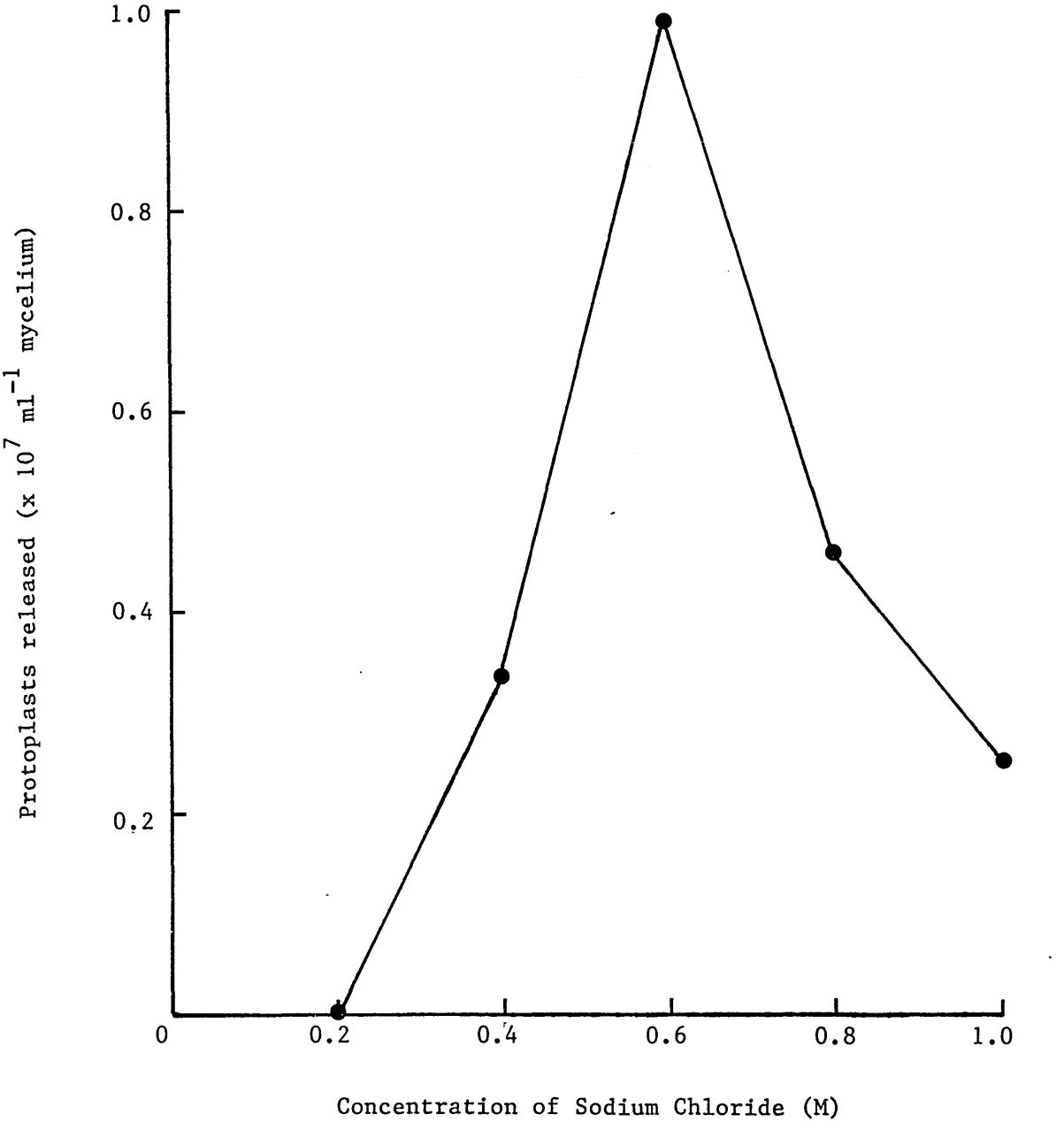

FIGURE 4.3. Effect of sodium chloride concentration on the release of protoplasts from P. ochrochloron. Mycelia of optimum age (Figure 4.7) were harvested, washed and resuspended in MES buffer (0.1 M; pH 5.5) containing 0 -1.0 M sodium chloride. After incubation at 30°C with 1 mg ml^{-1} Novozym^{TM}234 for 2 h, protoplast numbers were assessed using an improved Neubauer counting chamber.

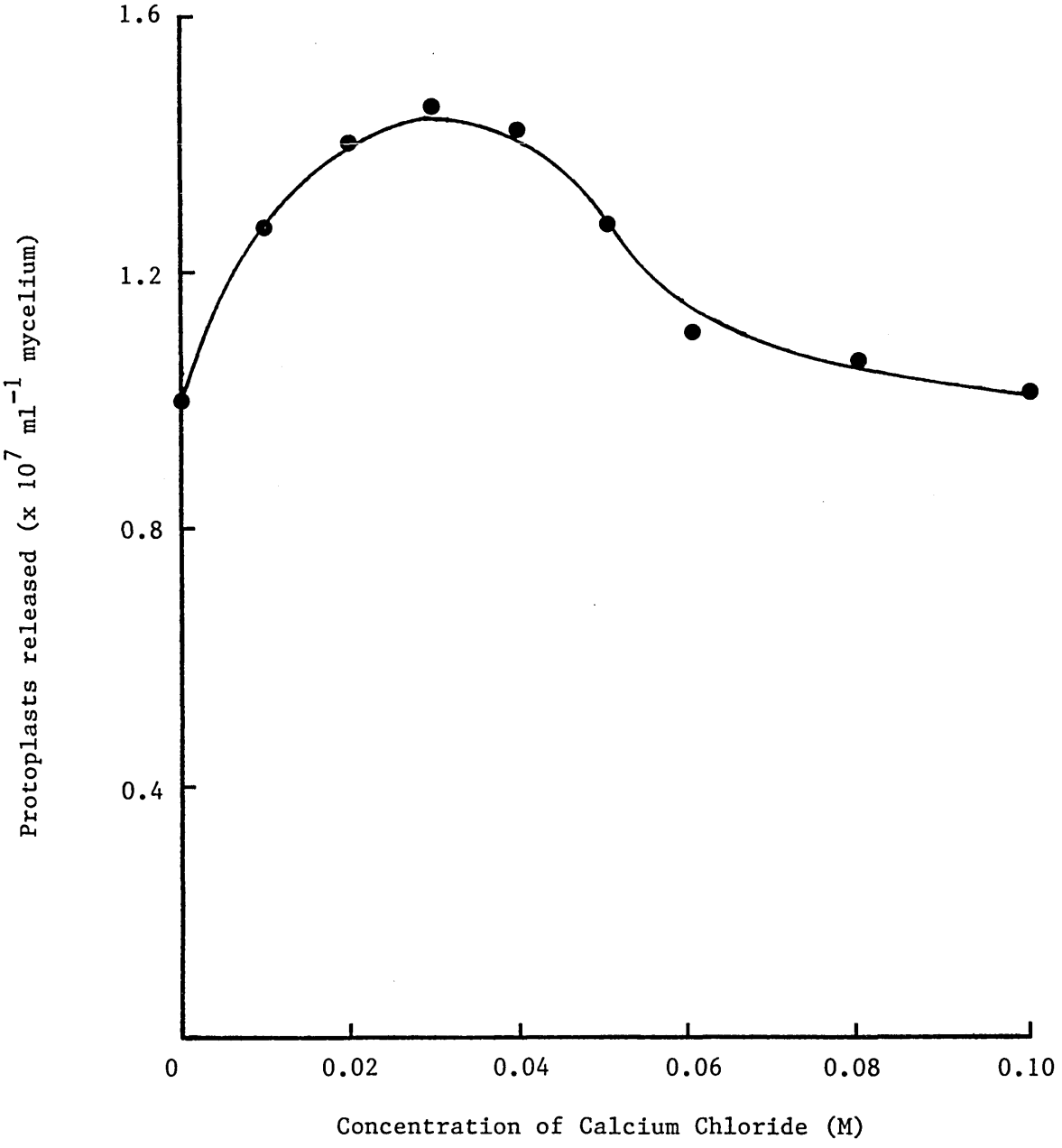

FIGURE 4.4. Effect of calcium chloride concentration on the release of protoplasts from P. ochrochloron. Mycelia of optimum age (Figure 4.7) were harvested, washed and resuspended in MES buffer (0.1 M; pH 5.5) containing 0.6 M sodium chloride and 0 - 0.1 M calcium chloride. After incubation at 30°C with 1 mg ml^{-1} Novozym^{TM}234 for 2 h, protoplast numbers were assessed using an improved Neubauer counting chamber.

1976; Thomas and Davis, 1980; Hamlyn et al., 1981; Stephen and Nasim, 1981) at concentrations ranging from 0.05-0.2M. Phosphate buffers have been reported to interfere with osmotic stabilizers (de Vries and Wessels, 1972) and enzyme activity (Gascon et al., 1965b). Protoplast yields from the four test fungi were generally better using MES than HEPES buffer (Table 4.3) and 0.1M was more effective than 0.05M (Table 4.3). Concentrations higher than 0.1M were not necessary.

The pH of the incubation medium has also been shown to affect protoplast yields (Anne et al., 1974; Peberdy et al., 1976). Optima for protoplast release have been reported to be in the range 5.5-6.0 for a number of fungi (de Vries and Wessels, 1972; Anne et al., 1974; Peberdy et al., 1976; Hou and Jong, 1985) including A. fumigatus and A. niger (Thomas and Davis, 1980). The situation in P. ochrochloron is no different with an optimum for protoplast release in the range 5.0-6.0 (Figure 4.5). This value was the same for both HEPES and MES buffers. Figure 4.5 also confirms the superiority of MES over HEPES in producing protoplasts from P. ochrochloron. Very few or no protoplasts were isolated from this organism outside the pH range, 3.0-8.0 (Figure 4.5). Little or no enzyme activity was observed at low pH and at higher pH, protoplasts lysed on emergence. Similar effects were observed by Eyssen (1977) and Thomas (1981). In Candida utilis, protoplast isolation has been shown to be equally as good between pH5.5-8.8 with lytic enzymes produced from Micromonospora suggesting the presence of two or more enzymes having different optimum pH values (Gascon et al., 1965b).

TABLE 4.3 EFFECT OF BUFFER TYPE AND CONCENTRATION ON THE RELEASE OF PROTOPLASTS FROM A. FUMIGATUS, A. NIGER, G. EMERSONII AND P. OCHROCHLORON

BUFFER (M)	PROTOPLASTS RELEASED ($\times 10^7$ ml^{-1} MYCELIUM)			
	A. FUMIGATUS	A. NIGER	G. EMERSONII	P. OCHROCHLORON
MES 0.05	0.38 ± 0.06	0.32 ± 0.04	0.65 ± 0.08	0.48 ± 0.05
MES 0.10	0.85 ± 0.09	0.33 ± 0.06	1.39 ± 0.12	1.19 ± 0.09
HEPES 0.05	0.53 ± 0.07	0.04 ± 0.01	1.34 ± 0.10	0.27 ± 0.02
HEPES 0.10	0.61 ± 0.07	0.09 ± 0.02	0.81 ± 0.07	0.47 ± 0.04

Mycelia of optimum age (Figure 4.7) were harvested, washed and resuspended in MES or HEPES buffer (pH 5.5) containing 0.6 M sodium chloride. After incubation at 30°C with 1 mg ml^{-1} Novozym^{TM}234 for 2 h, protoplast numbers were assessed using an improved Neubauer Counting Chamber.

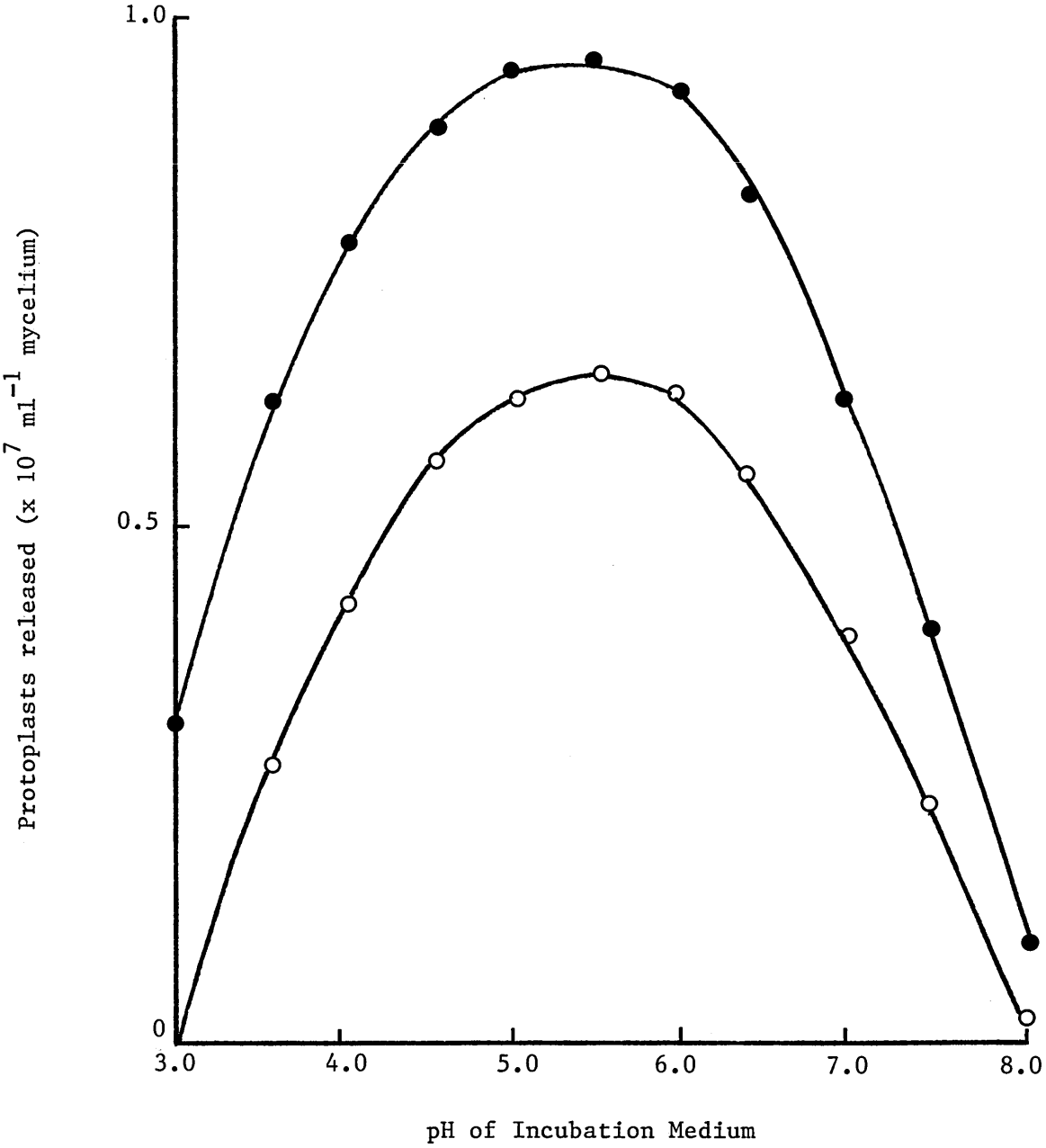

FIGURE 4.5. Effect of pH on the release of protoplasts from P. ochrochloron. Mycelia of optimum age (Figure 4.7) were harvested, washed and resuspended in HEPES (O) and MES (●) buffers both at 0.1 M and containing 0.6 M sodium chloride. After incubation at 30°C with 1 mg ml^{-1} Novozym^{TM}234 for 2 h, protoplast numbers were assessed using an improved Neubauer counting chamber.

The incubation medium used to isolate protoplasts for use in further studies contained 0.1M MES and 0.6M sodium chloride, adjusted to a final pH of 5.5 with 1.0M sodium hydroxide. When producing protoplasts from P. ochrochloron 0.03M calcium chloride was included.

4.3 EFFECT OF INCUBATION TEMPERATURE ON PROTOPLAST RELEASE

The incubation temperature has also been shown to affect protoplast yields from filamentous fungi (Musilkova and Fencl, 1968; Thomas, 1981). Optimum protoplast production from G. emersonii and P. ochrochloron was achieved at 30°C (Figure 4.6). A slightly higher optimum of 35°C was required for A. fumigatus and A. niger (Figure 4.6). These results are in agreement with those obtained by Musilkova and Fencl (1968) and Thomas and Davis (1980). The manufacturers quote that optimum activity of Novozym^{TM} 234 occurs at 50°C and yet protoplast numbers from all four fungi were drastically reduced at 45-50°C (Figure 4.6.). It would seem that high temperatures affect membrane stability resulting in the lysis of protoplasts. Such an occurrence in protoplasts from A. fumigatus and G. emersonii is surprising in view of the ability of these organisms to withstand and grow within such a temperature range. It would seem that the cell wall protects the membranes of these organisms from high temperatures. Low temperatures have also been reported to affect membrane stability (Kovac and Subik, 1970).

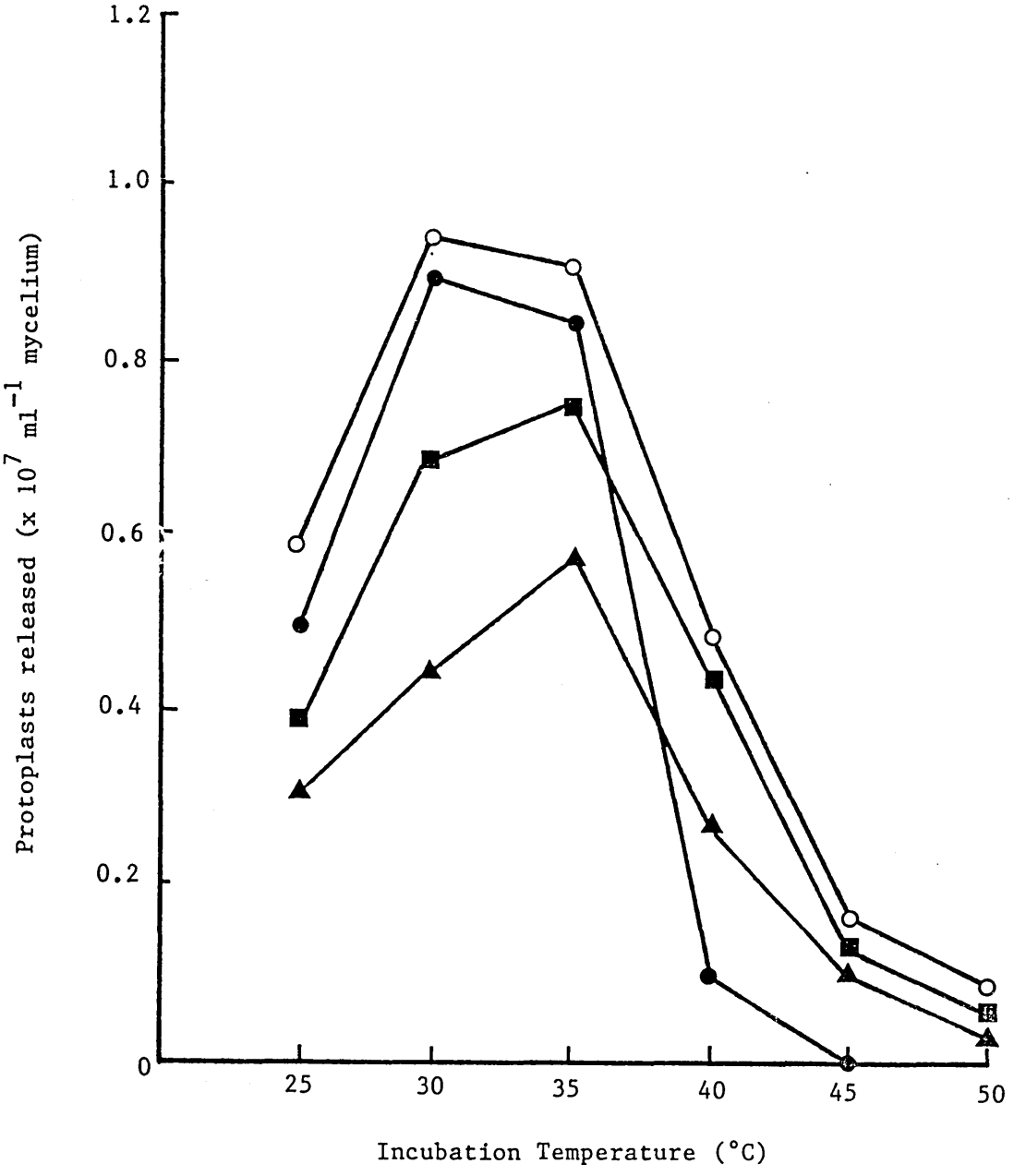

FIGURE 4.6. Effect of incubation temperature on the production of protoplasts from A. fumigatus (■), A. niger (▲), G. emersonii (O) and P. ochrochloron (●). Mycelia of optimum age (Figure 4.7) were harvested, washed and resuspended in incubation medium (Section 2.4). After incubation at 25 - 50°C with 1 mg ml^{-1} Novozym^{TM}234 for 2 h, protoplast numbers were assessed using an improved Neubauer counting chamber.

4.4 EFFECT OF MYCELIAL AGE AND CONCENTRATION ON PROTOPLAST RELEASE

In general, protoplast yields are usually greater from mycelium in the exponential growth phase (Benitez et al., 1975; Peberdy et al., 1976). Other workers have obtained optimum yields from 10h mycelium (Coudray and Canevascini, 1980). Optimum protoplast yields from G. emersonii and P. ochrochloron were observed after 16h and 20h respectively (Figure 4.7). No protoplasts were obtained from 20h mycelium of G. emersonii (Figure 4.7). Maximum protoplast yields from A. fumigatus and A. niger were observed later in the exponential growth phase (28h) and few or no protoplasts were observed from 16h mycelium of these Aspergillus spp. (Figure 4.7). No protoplasts were isolated from 48h mycelium of any of the organisms investigated. This resistance of older mycelium to lytic digestion has been observed by other workers (de Vries and Wessels, 1972; Peberdy et al., 1976; Schwencke et al., 1977). It is not clear why mycelium yields far fewer protoplasts in the late exponential growth phase and in the stationary phase. Bartnicki-Garcia and Lippman (1972) suggested that the concentrations of endogenous wall-building enzymes during the exponential growth phase may be high and these may complement the effect of the exogenous lytic enzymes on the cell wall, so giving greater yields during this phase of growth. Other workers have suggested that changes in wall structure may be responsible for reduced protoplast yields. For example, deposition of α-1,3-glucan as an outer wall layer in older mycelium has been observed in A. nidulans (Zonnerveld, 1972) and S. commune (de Vries and Wessels, 1972). In contrast, high yields of protoplasts have been obtained from cultures well into the stationary phase in P. chrysogenum (Fawcett et al., 1973) and S. commune (de Vries and

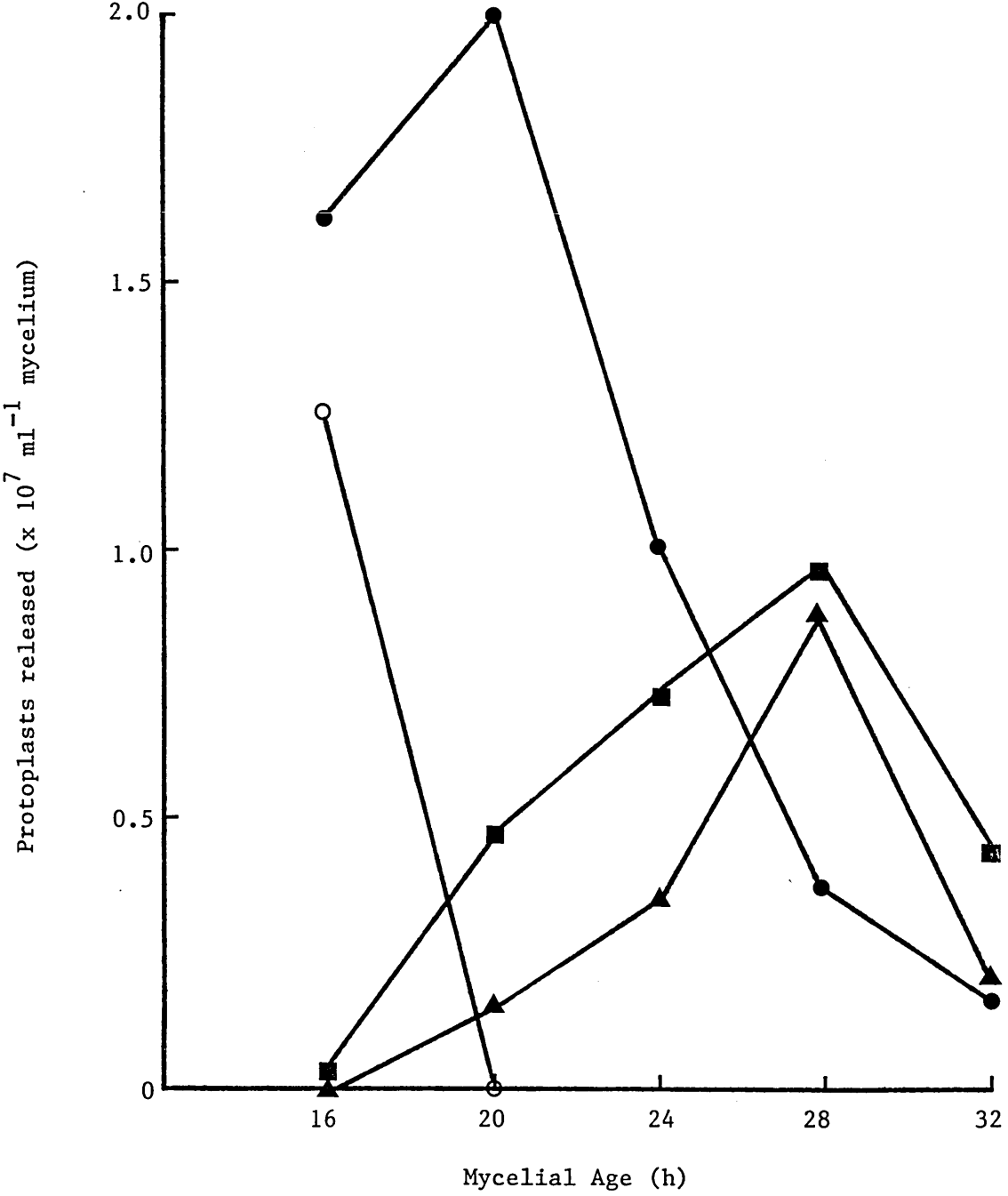

FIGURE 4.7. Effect of mycelial age on the production of protoplasts from A. fumigatus (■), A. niger (▲), G. emersonii (○) and P. ochrochloron (●). Mycelia at various stages of growth were harvested, washed and resuspended in incubation medium (Section 2.4). After incubation at 30°C with 1 mg ml^{-1} Novozym™234 for 2 h, protoplast numbers were assessed using an improved Neubauer counting chamber.

Wessels, 1972). The four organisms were harvested after 16h (*G. emersonii*), 20h (*P. ochrochloron*) and 28h (*A. fumigatus* and *A. niger*) and used to produce protoplasts for use in further studies.

Protoplast numbers from all four fungi increased proportionally with mycelial concentrations up to 12mg ml^{-1} (Figure 4.8). Optimal mycelial concentrations of 30-40mg ml^{-1} have been reported for *Aspergillus* spp. (Peberdy *et al.*, 1976; Thomas, 1981). However, at such high concentrations it is difficult to isolate the protoplasts due to the large quantity of undigested mycelium remaining. Consequently, mycelial concentrations of 8-10mg ml^{-1} were used when producing protoplasts for use in further studies.

4.5 EFFECT OF CULTURAL CONDITIONS ON PROTOPLAST ISOLATION

The nature of the growth medium and cultural conditions have also been shown to be of prime importance in considering protoplast isolation (Davis, 1985). Maximum yields of protoplasts from *A. niger* and *P. ochrochloron* were obtained from mycelium grown at 30°C and 25°C respectively (Figure 4.9). No protoplasts were isolated from *P. ochrochloron* mycelium grown at temperatures exceeding 40°C (Figure 4.9). In contrast, optimum protoplast numbers obtained from *G. emersonii* were achieved using mycelium cultivated at 45°C (Figure 4.9). No protoplasts were isolated from *G. emersonii* mycelium cultivated at temperatures below 30°C. One interesting observation with *A. fumigatus*, was that optimal protoplast production was attained from mycelium grown at 30°C, even though the maximum temperature for growth is 37°C. For further studies, the organisms were cultivated at 25°C (*P. ochrochloron*), 30°C (*A. fumigatus* and *A. niger*) and 45°C (*G. emersonii*).

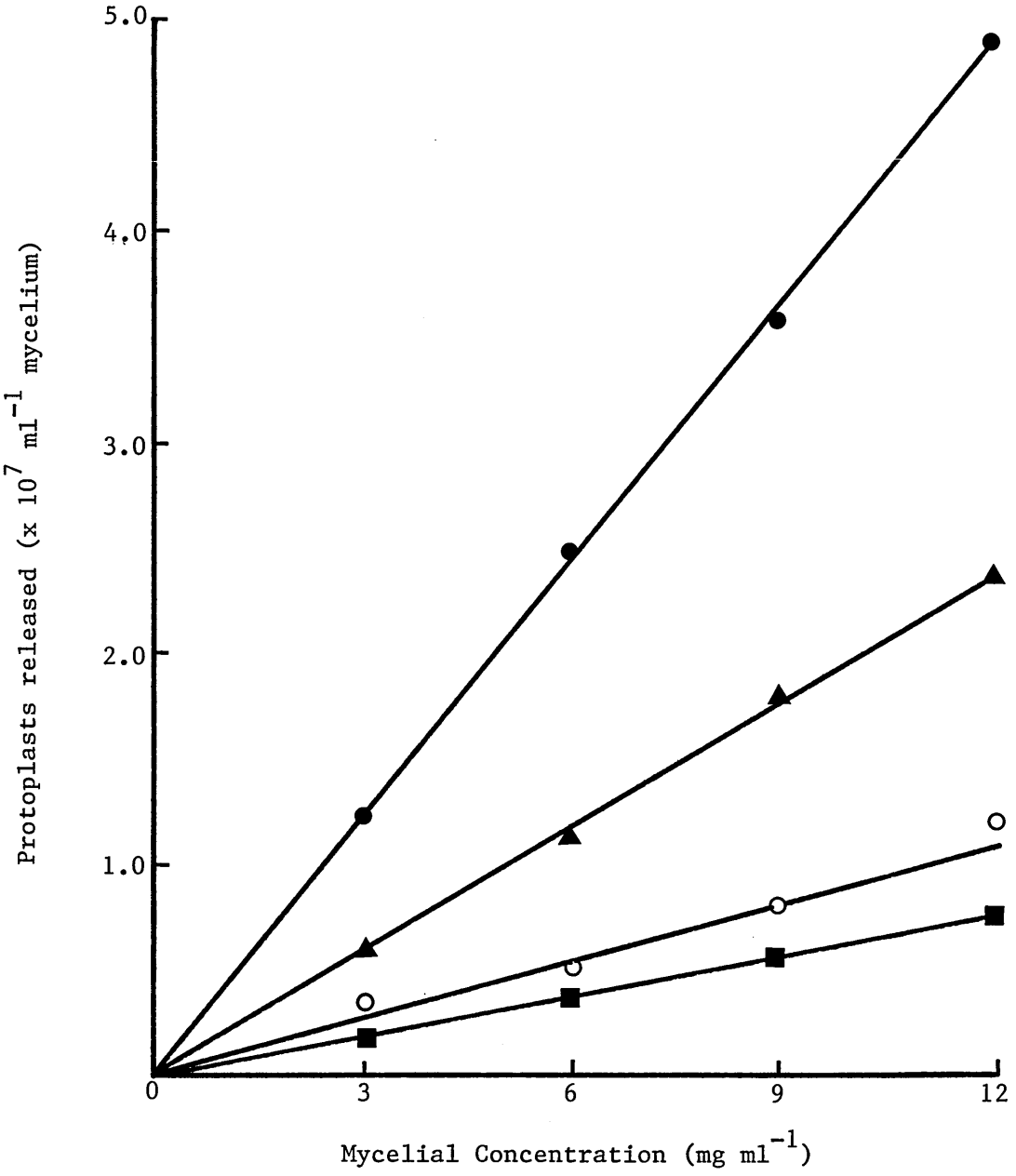

FIGURE 4.8 Effect of mycelial concentration on the production of protoplasts from A. fumigatus (■), A. niger (▲), G. emersonii (O) and P. ochrochloron (●). Mycelia at various concentrations were harvested, washed and resuspended in incubation buffer (Section 2.4). After incubation at 30°C with 1 mg ml^{-1} Novozym^{TM}234 for 2 h, protoplast numbers were assessed using an improved Neubauer counting chamber.

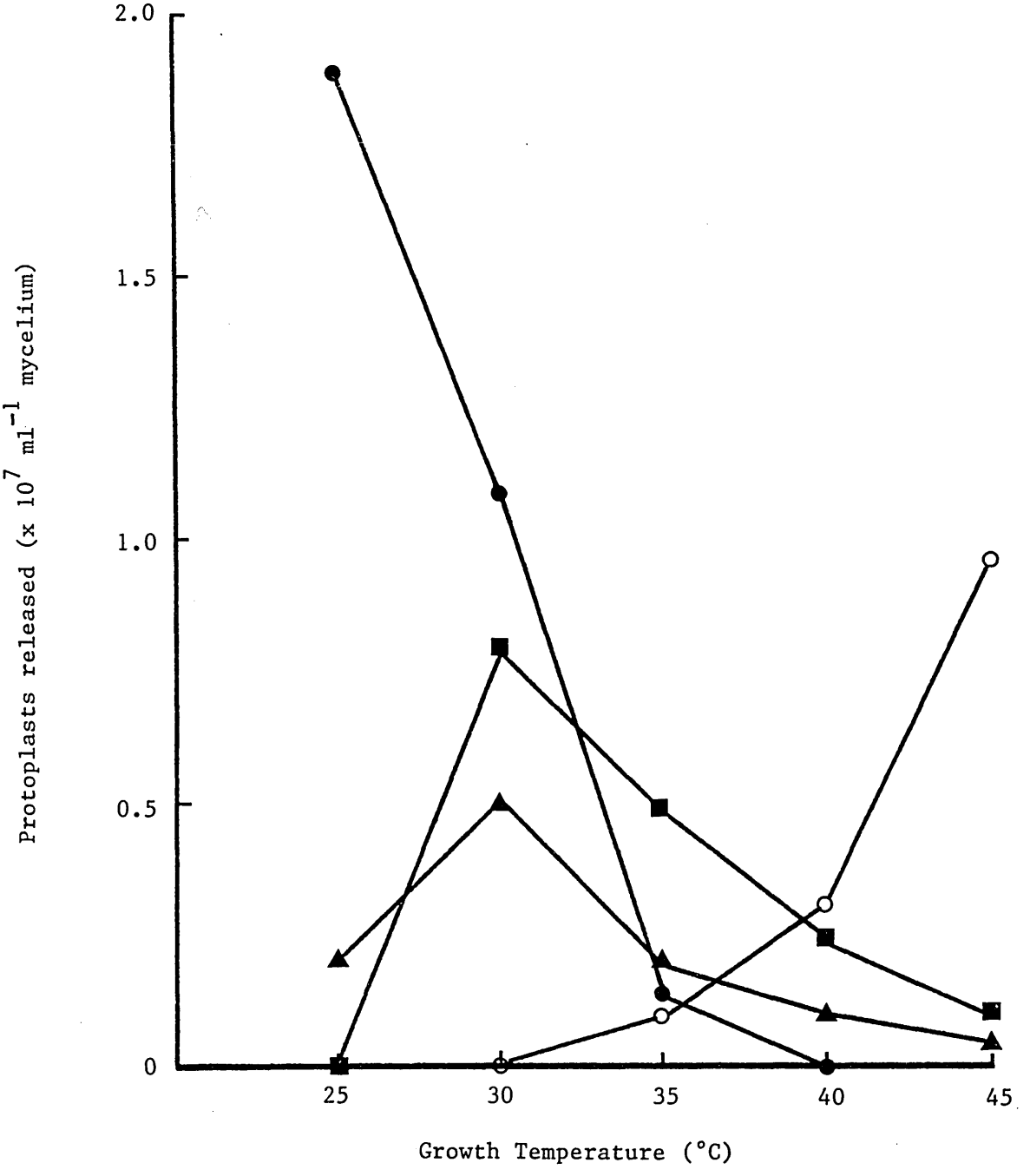

FIGURE 4.9. Effect of growth temperature on the production of protoplasts from A. fumigatus (■), A. niger (▲), G. emersonii (○) and P. ochrochloron (●). Mycelia cultivated at various temperatures (25 - 45°C) were harvested, washed and resuspended in incubation medium (Section 2.4). After incubation at 30°C with 1 mg ml^{-1} Novozym™234 for 2 h, protoplast numbers were assessed using an improved Neubauer counting chamber.

4.6 PROTOPLASTS FROM OTHER SPECIES

As stated earlier (in the Introduction) one of the aims of this work was to attempt to cross mesophilic and thermophilic filamentous fungi with a view to increasing the thermal stability of cellulase enzymes. A number of mesophilic and thermophilic fungi were screened for cellulolytic activity and protoplast production.

Initial experiments were carried out on Chaetomium spp. and Sporotrichum spp. because these two genera provided mesophilic and thermophilic cellulolytic fungi. Although protoplasts were obtained from S. thermophilum (Figure 4.10) none were isolated from S. pulverulentum. No protoplasts were isolated from C. thermophilum and only low yields were obtained from C. globosum. Further studies were then carried out to investigate the possible use of Penicillia in the knowledge that this genus contained a thermophilic fungus (P. dupontii) and some mesophilic cellulolytic species. In these studies, protoplasts were produced from three mesophilic cellulolytic species: P. funiculosum, P. janthinellum and P. ochrochloron (Figure 4.10). The latter oganism produced good yields of protoplasts whereas P. funiculosum and P. janthinellum, under the same conditions, did not produce sufficient numbers to warrant further investigation with the lytic enzyme system used (Figure 4.10). The thermophilic fungus, P. dupontii, was screened for protoplast production with a view to using these protoplasts in a cross with protoplasts from P. ochrochloron. Unfortunately, no protoplasts were isolated from P. dupontii under these conditions; this organism was subjected to further investigation.

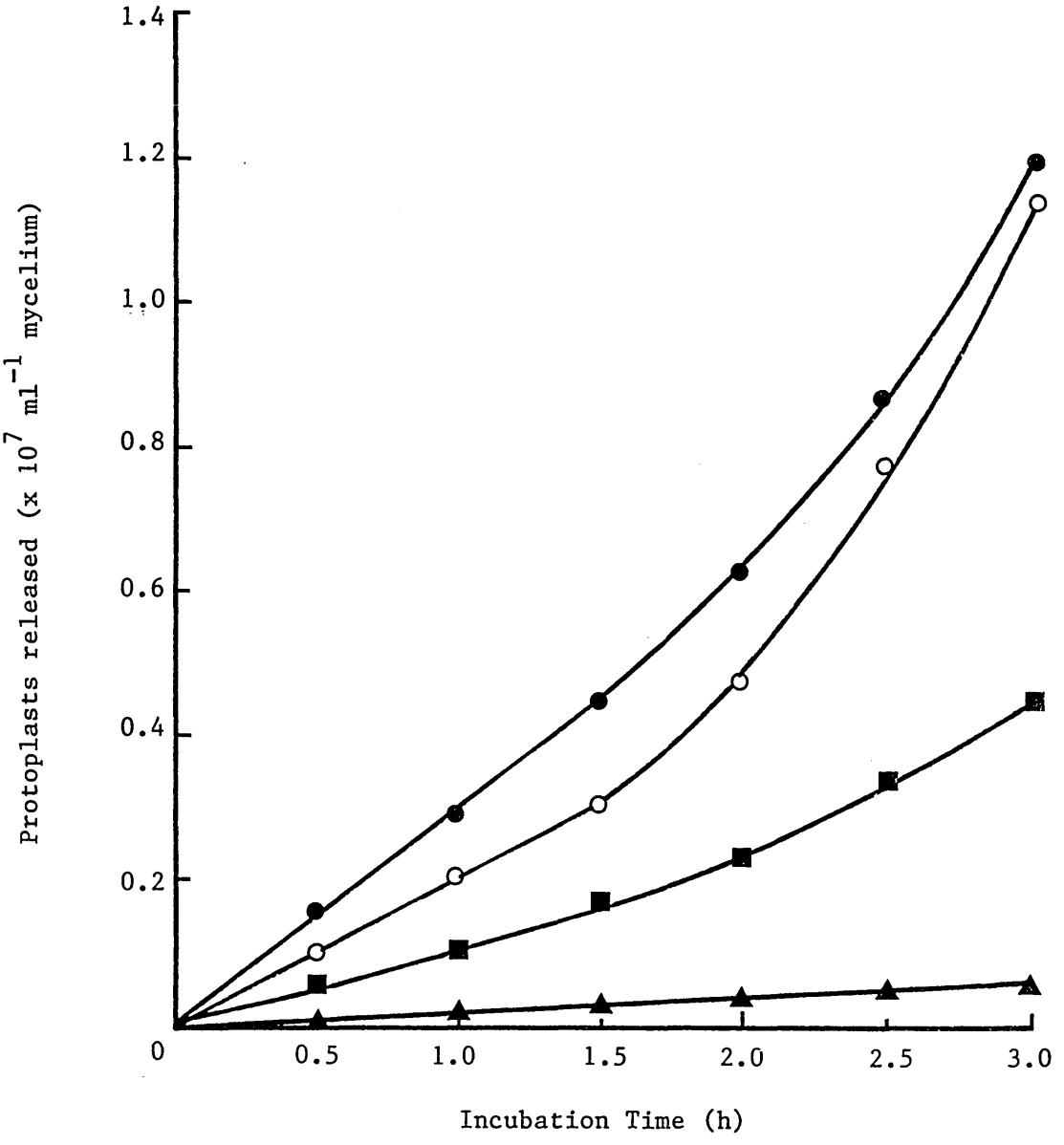

FIGURE 4.10. Release of protoplasts from P. funiculosum (▲), P. janthinellum (■), P. ochrochloron (●) and S. thermophilum (○). 24 h mycelia were harvested, washed and resuspended in incubation medium (Section 2.4). After incubation at 30°C with 1 mg ml^{-1} Novozym^{TM}234 for 0-3 h, protoplast numbers were assessed using an improved Neubauer counting chamber.

A number of lytic enzymes were used, alone and in various combinations. These included: Cellulase CP, chitinase, β-glucuronidase, lipase, Novozym^{TM} 234 and protease. However, no protoplasts were isolated from P. dupontii mycelium using these enzymes. Numerous variations in the incubation medium were tried including the use of MES, HEPES and phosphate buffers with numerous osmotica at concentrations ranging from 0-1.2M again with no success. No protoplasts were isolated from P. dupontii when the pH of the incubation medium was varied to cover the range pH3.0-8.0. Mycelium was incubated at 25-50°C with 1,2 and 5mg ml^{-1} Novozym^{TM} 234 for 0-4h, with and without shaking, again with no success. Some workers have reported that pretreatment of mycelium with sulphydyl and thiol compounds may increase protoplast release from yeasts (Sommer and Lewis, 1971) and filamentous fungi (Fawcett et al., 1973; Hamlyn et al., 1981). However, this technique also proved unsuccessful, as did the inclusion of the detergent Cereflo, which had increased protoplast yields in the four other fungi investigated (Table 4.1). Some workers have managed to isolate protoplasts from spores (Bachmann and Bonner, 1959; Laborda et al., 1974; Moore and Peberdy, 1976). Unfortunately, this technique also proved unsuccessful. As explained earlier (in the Introduction), extracellular crude enzymes isolated from species such as T. viride have proved successful in producing protoplasts from other fungal species (Gibson and Peberdy, 1972; Anne et al., 1974; Peberdy and Isaac, 1976; Hearn et al., 1980). T. viride was cultivated in a medium containing dried P. dupontii mycelium. After 5-7days, the culture filtrate was purified (Section 2.5) and this crude preparation was used as the source of lytic enzymes. Protoplasts were produced from P. dupontii using this technique but unfortunately

not in sufficient numbers for fusion studies. More work is, therefore, needed to perfect this technique. This organism was therefore dropped from further investigation and replaced with G. emersonii [formerly Talaromyces emersonii, the perfect stage of Penicillium emersonii] (Benjamin, 1955).

4.7 PROTOPLAST REGENERATION

When protoplasts are placed in a suitable osmotically stabilised growth medium, some will revert to typical mycelial growth in a process known as reversion. The percentage of protoplasts reverting to normal mycelium is termed the reversion frequency. These values vary between 10-100% depending on the organism and the cultural conditions (Garcia-Acha et al., 1966; Peberdy and Gibson, 1971; Anne et al., 1974). Low values may be explained by the absence of a nucleus or a fault in the regeneration mechanism (Necas and Svoboda, 1985). The type of growth and wall regeneration is determined by the concentration of the osmoticum and the cultural conditions under which regeneration takes place (Garcia-Acha et al., 1966; Peberdy and Gibson, 1971).

The process of reversion may be observed in both liquid and solid media. Eggins and Pugh Basal Medium (Table 2.1) containing 1% (w/v) glucose and 0.6M sodium chloride was inoculated with 1×10^5 protoplasts ml^{-1} of each type of organism. Samples were removed and examined under the light microscope. These protoplast suspensions were incubated in an orbital platform shaking incubator at 200rpm and at the appropriate growth temperature (Section 2.2). Regeneration was observed after 6-8h in protoplasts from all four species of fungi. These results are in agreement with observations by Anne et

al. (1974) and Hou and Jong (1985) on protoplasts of P. chrysogenum and Penicillium digitatum respectively. No regeneration was observed in any of the test fungi when protoplasts were first incubated in water. Three types of regeneration are normally observed in fungal protoplasts (Garcia-Acha et al., 1966; Anne et al., 1974; Peberdy, 1978). These are described as (i) formation of chains of yeast-like cells; (ii) formation of germ tubes in one or more directions; and (iii) formation of convoluted hyphae devoid of septa which usually burst on prolonged incubation. The second type of regeneration was most commonly observed in the test fungi although the other types were also observed.

Regeneration of protoplasts was also observed on agar plates. Protoplast suspensions from all four test organisms were placed onto agar plates of Eggins and Pugh Basal medium (Table 2.1) containing 1%(^{w}/v) glucose and 0.6M sodium chloride. After 48h incubation at the appropriate growth temperature (Section 2.2), colonies developed which were morphologically identical to those of the parental type. Sporulation of all four fungal types was observed after 5-7days incubation.

Further regeneration studies were carried out in liquid media to assess the capability of protoplasts to retain enzyme activity. Very little activity was observed in the regeneration media after 12h, however all three types of enzyme activity, endo-β-1,4-glucanase, exo-β-1,4-glucanase and β-glucosidase were detected after seven days regeneration. Endo-β-1,4-glucanase activity was observed in regenerated protoplasts of all four test fungi with CMC being the best substrate for inducing synthesis of this enzyme. Very little exo-β-1,4-glucanase activity was observed in regenerated protoplasts

of any of the four test fungi with CMC and glucose being the best inducers for synthesis of this enzyme. In contrast, β-glucosidase activity in regenerated protoplasts was dependent on the inducing substrate and the species. A. fumigatus and P. ochrochloron showed optimal β-glucosidase activity after regeneration on soluble substrates with very little being produced on insoluble forms of cellulose (Tables 4.4 and 4.5 respectively). β-glucosidase activity was greatest in both these fungi when they were regenerated on glucose medium, indicating that β-glucosidase is produced constitutively in these fungi. Furthermore, both A. fumigatus and P. ochrochloron demonstrated β-glucosidase activity when protoplasts were regenerated in medium lacking a growth substrate. In A. niger high levels of β-glucosidase activity were obtained after regeneration in media containing both soluble and insoluble substrates (Table 4.6). Maximum β-glucosidase activity in A. niger protoplasts was observed after regeneration in CMC and considerably less activity was observed after regeneration in medium without a carbon substrate. In G. emersonii, maximum β-glucosidase activity was observed after regeneration in CMC medium and very little or no activity was observed in the other four media (Table 4.7).

TABLE 4.4 ENZYME PRODUCTION BY REGENERATED PROTOPLASTS OF A. FUMIGATUS (MUT)

CARBON SUBSTRATE(S)	SALICIN	CMC	FILTER PAPER	P-NITROPHENYL-β-D-GLUCOPYRANOSIDE	PROTEIN ($\mu g\ ml^{-1}$)
	β-GLUCOSIDASE 3.2.1.21 (Milli-IU)	ENDO-β-1,4-GLUCANASE 3.2.1.4 (Milli-IU)	EXO-β-1,4-GLUCANASE EC.3.2.1.91 (Milli-IU)	β-GLUCOSIDASE EC.3.2.1.21 (Milli-IU)	
1% (w/v) CMC + 0.2% (w/v) glucose	9.1 ± 1.25	15.2 ± 0.29	0.33 ± 0.06	4.2 ± 0.26	380 ± 3.5
Pellicles + 0.2% (w/v) glucose	0.5 ± 0.19	3.5 ± 0.09	0.00 ± 0.002	0.7 ± 0.18	971 ± 9.4
1% (w/v) glucose	50.1 ± 0.76	9.6 ± 0.17	0.24 ± 0.004	13.0 ± 0.43	986 ± 8.9
No carbon substrate	9.7 ± 0.24	2.4 ± 0.05	0.00 ± 0.001	2.7 ± 0.09	303 ± 2.7
1% (w/v) BMC + 0.2% (w/v) glucose	0.7 ± 0.38	3.9 ± 0.07	0.00 ± 0.002	0.4 ± 0.08	907 ± 8.6

mut = mutant

Flasks containing Eggins and Pugh Basal medium (Table 2.1) and various carbon substrates were inoculated with 2×10^6 protoplasts ml^{-1}. After incubation for seven days at 44°C, the cultures were harvested and the supernatants assayed for various enzyme activities (section 2.8) and soluble protein (section 2.7).

TABLE 4.5 ENZYME PRODUCTION BY REGENERATED PROTOPLASTS OF P. OCHROCHLORON (MUT)

CARBON SUBSTRATE(S)	SALICIN	CMC	FILTER PAPER	P-NITROPHENYL-β-D-GLUCOPYRANOSIDE	PROTEIN ($\mu g\ ml^{-1}$)
	β-GLUCOSIDASE EC.3.2.1.21 (Milli-IU)	ENDO-β-1,4 GLUCANASE EC.3.2.1.4 (Milli-IU)	EXO-β-1,4-GLUCANASE EC.3.2.1.91 (Milli-IU)	β-GLUCOSIDASE EC.3.2.1.21 (Milli-IU)	
1% (w/v) CMC + 0.2% (w/v) glucose	0.6 ± 0.14	8.0 ± 0.42	0.01 ± 0.002	0.2 ± 0.07	2180 ± 16.0
Pellicles + 0.2% (w/v) glucose	0.3 ± 0.07	3.8 ± 0.05	0.02 ± 0.003	0.1 ± 0.01	1503 ± 5.0
1% (w/v) glucose	3.4 ± 0.30	5.8 ± 0.15	0.08 ± 0.004	0.9 ± 0.03	1127 ± 14.4
No carbon substrate	0.7 ± 0.13	8.2 ± 0.03	0.03 ± 0.004	0.2 ± 0.05	1181 ± 10.2
1% (w/v) BMC + 0.2% (w/v) glucose	0.4 ± 0.13	4.1 ± 0.10	0.02 ± 0.005	0.1 ± 0.03	1480 ± 8.7

mut = mutant

Flasks containing complete medium (Table 2.4), 0.0 - 1.0% (w/v) glucose and various carbon substrates were inoculated with 2×10^6 protoplasts ml^{-1}. After incubation for seven days at 30°C, the cultures were harvested and the supernatants assayed for various enzyme activities (section 2.8) and soluble protein (section 2.7).

TABLE 4.6 ENZYME PRODUCTION BY REGENERATED PROTOPLASTS OF A. NIGER (MUT)

CARBON SUBSTRATE(S)	SALICIN	CMC	FILTER PAPER	P-NITROPHENYL-β-D-GLUCOPYRANOSIDE	PROTEIN (μg ml^{-1})
	β-GLUCOSIDASE EC.3.2.1.21 (Milli-IU)	ENDO-β-1,4-GLUCANASE EC.3.2.1.4 (Milli-IU)	EXO-β-1,4-GLUCANASE EC.3.2.1.91 (Milli-IU)	β-GLUCOSIDASE EC.3.2.1.21 (Milli-IU)	
1% (w/v) CMC + 0.2% (w/v) glucose	61.7 ± 1.72	80.4 ± 0.63	0.21 ± 0.007	14.4 ± 0.08	105 ± 2.4
Pellicles + 0.2% (w/v) glucose	23.3 ± 0.98	1.8 ± 0.11	0.00 ± 0.002	6.0 ± 0.12	349 ± 3.2
1% (w/v) glucose	45.1 ± 1.14	2.4 ± 0.14	0.39 ± 0.011	18.9 ± 0.08	932 ± 4.7
No carbon substrate	7.0 ± 0.32	0.0 ± 0.03	0.00 ± 0.001	2.1 ± 0.07	166 ± 1.8
1% (w/v) BMC + 0.2% (w/v) glucose	27.8 ± 0.75	1.2 ± 0.06	0.00 ± 0.003	10.1 ± 0.02	207 ± 1.9

mut = mutant

Flasks containing Eggins and Pugh Basal medium (Table 2.1) and various carbon substrates were inoculated with 2×10^6 protoplasts ml^{-1}. After incubation for seven days at 30°C, the cultures were harvested and the supernatant assayed for various enzyme activities (section 2.8) and soluble protein (section 2.7).

TABLE 4.7 ENZYME PRODUCTION BY REGENERATED PROTOPLASTS OF G. EMERSONII

CARBON SUBSTRATE(S)	SALICIN	CMC	FILTER PAPER	P-NITROPHENYL-β-D-GLUCOPYRANOSIDE	PROTEIN ($\mu g\ ml^{-1}$)
	β-GLUCOSIDASE EC.3.2.1.21 (Milli-IU)	ENDO-β-1,4-GLUCANASE EC.3.2.1.4 (Milli-IU)	EXO-β-1,4-GLUCANASE EC.3.2.1.91 (Milli-IU)	β-GLUCOSIDASE EC.3.2.1.21 (Milli-IU)	
1% (w/v) CMC + 0.2% (w/v) glucose	41.2 ± 2.93	91.5 ± 0.58	0.02 ± 0.006	2.4 ± 0.09	695 ± 11.9
Pellicles + 0.2% (w/v) glucose	2.3 ± 0.13	0.9 ± 0.07	0.00 ± 0.004	0.8 ± 0.07	433 ± 8.5
1%(w/v) glucose	4.2 ± 0.17	2.0 ± 0.08	0.01 ± 0.002	1.9 ± 0.03	605 ± 10.2
No carbon substrate	0.0 ± 0.10	1.2 ± 0.05	0.00 ± 0.003	0.2 ± 0.01	334 ± 2.5
1% (w/v) BMC + 0.2% (w/v) glucose	1.4 ± 0.13	0.9 ± 0.10	0.00 ± 0.003	0.6 ± 0.03	482 ± 5.1

Flasks containing Eggins and Pugh Basal medium (Table 2.1) and various carbon substrates were inoculated with 2×10^6 protoplasts ml^{-1}. After incubation for seven days at 30°C, the cultures were harvested and the supernatants assayed for various enzyme activities (section 2.8) and soluble protein (section 2.7).

The aim of this work was to attempt to cross thermotolerant/ thermophilic species with mesophilic species using the technique of protoplast fusion, to try and improve enzyme production. Two trial crosses, A. fumigatus with A. niger and P. ochrochloron with either G. emersonii or P. dupontii were proposed. However, before a cross between two fungal species or strains may be attempted, it is essential that a method of detecting and recovering fusion products is available. Selection markers are therefore required that would prevent growth of parental types but allow growth of "hybrid" colonies. Many workers in this field have employed the use of nutritional complementation of auxotrophic mutants in selecting fusion products (Anne and Peberdy, 1975,1976; Ferenczy et al., 1977; Kevei and Peberdy, 1977). This technique, although useful in classical genetics is extremely time consuming and the resulting mutants often have reduced growth rates and lowered biochemical activity. The application of unselected markers such as colony morphology or metabolite production (Chang et al., 1982) requires much more effort to select recombinant progeny. Other workers have used drug resistance markers such as resistance to acriflavine (Peberdy and Bradshaw, 1982) or oligomycin (Croft et al., 1980; Anne, 1982). There have been no published reports regarding the use of commercial fungicides and heavy metals in selecting fusion products. It was, therefore, decided to investigate the possible use of these anti-fungal compounds as genetic markers in the two trial crosses.

5.1 RESISTANCE TO COMMERCIAL FUNGICIDES AND METAL SALTS

The five fungi of interest, A. fumigatus, A. niger,

G. emersonii, P. dupontii and P. ochrochloron were screened for resistance to a number of commercial fungicides and metal salts as described in Section 2.9. It is clear from Table 5.1, that the commercial fungicides have widely differing effects on the fungi tested. The fungicides, Benlate, Mystox G, Mystox 8 and Thiabendazole were very effective in preventing growth of all five fungi, whereas Captafol, Captan and Quintozene were not so effective. This table also shows that the two thermophiles, G. emersonii and P. dupontii were more sensitive to fungicidal action than the other three fungi. Whether this reflects a temperature effect on increasing fungicidal action or sensitivity of thermophiles to fungicides is not clear. It should be noted, however, that the thermotolerant fungus, A. fumigatus was grown at 44°C and yet this organism was no more sensitive to fungicidal action than the two mesophiles, A. niger and P. ochrochloron. The fungicide, Thiram at a concentration of 0.5%(w/v) prevented the growth of A. fumigatus whereas A. niger was capable of growth at this concentration (Table 5.1). This difference in resistance could be used to select against A. fumigatus in a cross with A. niger. Other fungicides such as Chlorothalonil [0.01%(w/v)], Impact [0.001%(w/v)], Merolan [0.1% (w/v)], Mystox ELC [0.1%(w/v)], Mystox LPL [0.1%(w/v)], Mystox 8 [0.01%(w/v)] and Thiram [0.5%(w/v)] were shown to prevent the growth of G. emersonii and P. dupontii but not P. ochrochloron (Table 5.1). These fungicides could therefore be used to select against the thermophiles, G. emersonii and P. dupontii in a cross with P. ochrochloron. Of the nine metal salts screened, only cupric chloride and mercuric chloride demonstrated any selective anti-fungal activity (Table 5.2). In the cross between A. fumigatus and A. niger, a combination of cupric chloride (1×10^{-3}M) and mercuric chloride

TABLE 5.1 RESISTANCE OF FUNGAL ORGANISMS TO COMMERCIAL FUNGICIDES

FUNGICIDE	CONCENTRATION [% (w/v)]	GROWTH OF ORGANISM (mm)				
		A.FUMIGATUS	A.NIGER	G.EMERSONII	P.DUPONTII	P.OCHROCHLORON
Benlate	1.0	-	-	-	-	-
	0.1	-	-	-	-	-
	0.01	-	-	-	-	-
	0.001	-	-	-	-	-
	0.0001	35	40	-	-	-
Captafol	1.0	x	x	x	x	x
	0.5	15	14	12	20	14
	0.1	33	31	32	36	27
Captan	1.0	x	x	x	x	x
	0.1	x	x	x	x	x
	0.01	54	46	45	30	36
Chlorothalonil	1.0	13	9	-	-	4
	0.1	23	26	-	-	9
	0.01	29	32	-	-	32
Impact	1.0	-	-	-	-	-
	0.1	-	-	-	-	-
	0.01	23	13	-	-	13
	0.001	29	39	-	-	24
Merolan	1.0	-	-	-	-	-
	0.1	48	31	-	-	11
	0.01	53	57	42	37	15
Mystox ELC	1.0	10	33	-	-	13
	0.1	41	48	-	-	16
	0.01	57	50	11	-	26
Mystox G	1.0	-	-	-	-	-
	0.1	-	-	-	-	-
	0.01	-	-	-	-	-
	0.001	34	29	9	2	24
Mystox LPL	1.0	32	26	-	-	24
	0.1	50	27	-	-	24
	0.01	51	45	19	34	28
Mystox WFA	1.0	-	-	-	-	-
	0.1	-	-	-	-	-
	0.01	29	9	35	30	13
Mystox 8	1.0	x	x	x	x	x
	0.1	-	-	-	-	-
	0.01	-	-	-	-	16
Quintozene	1.0	42	9	-	6	24
	0.1	49	26	6	13	27
	0.01	50	37	17	38	28
Thiabendazole	1.0	-	-	-	-	-
	0.1	-	-	-	-	-
	0.01	-	2	-	-	-
Thiram	1.0	-	-	-	-	-
	0.5	-	18	-	-	4
	0.1	20	37	13	6	27
	0.01	29	51	35	36	33

x = no result; - = no growth

Various commercial fungicides were incorporated into Eggins and Pugh Basal medium (Table 2.1) containing 1% (w/v) glucose. Plates were inoculated with a spore inoculum and then incubated at the appropriate growth temperature (section 2.2). After five days incubation, growth was measured and expressed as colony diameter.

TABLE 5.2 RESISTANCE OF FUNGAL ORGANISMS TO METAL SALTS

CONCENTRATION OF METAL SALT (M)	GROWTH OF ORGANISM (mm)				
	A.FUMIGATUS	A.NIGER	G.EMERSONII	P.DUPONTII	P.OCHROCHLORON
Cobaltous chloride					
1×10^{-3}	20	21	15	26	19
5×10^{-4}	42	37	25	34	19
1×10^{-4}	51	40	27	35	19
Cupric chloride					
1×10^{-3}	-	24	-	-	8
5×10^{-4}	12	25	-	-	16
1×10^{-4}	32	27	22	29	25
Ferric chloride					
1×10^{-3}	42	37	40	36	33
5×10^{-4}	45	39	44	39	34
1×10^{-4}	47	42	44	39	34
Lead nitrate					
1×10^{-3}	45	35	42	40	34
1×10^{-4}	45	40	43	40	36
1×10^{-4}	47	44	44	40	36
Magnesium chloride					
1×10^{-3}	43	39	39	41	35
5×10^{-4}	44	41	46	46	36
1×10^{-4}	47	42	50	47	36
Manganous chloride					
1×10^{-3}	44	36	45	38	37
5×10^{-4}	49	39	47	39	38
1×10^{-4}	54	40	47	42	39
Mercuric chloride					
1×10^{-3}	9	-	-	-	-
5×10^{-4}	21	-	-	-	13
1×10^{-4}	27	42	25	33	27
Nickel chloride					
1×10^{-3}	43	11	44	38	12
5×10^{-4}	45	13	45	39	22
1×10^{-4}	46	26	45	39	38
Zinc chloride					
1×10^{-3}	42	31	27	27	20
5×10^{-4}	44	34	37	34	26
1×10^{-4}	44	38	44	39	33

- = no growth

Various metal salts were incorporated into Eggins and Pugh Basal medium (Table 2.1) containing 1% (w/v) glucose. Plates were inoculated with a spore inoculum and then incubated at the appropriate growth temperature (section 2.2). After five days incubation, growth was measured and expressed as colony diameter.

(5×10^{-4}M) could be used to select against the two parental types, allowing the isolation of fusion products. In the other cross between P. ochrochloron and G. emersonii or P. dupontii, the thermophiles were more sensitive to cupric chloride and mercuric chloride than P. ochrochloron (Table 5.2). These two metal salts at a concentration of 5×10^{-4}M could also be used to select against the thermophiles in the appropriate crosses.

5.2 MUTAGENESIS OF P. OCHROCHLORON AND A. NIGER

In the cross between P. ochrochloron and G. emersonii or P. dupontii, numerous selection markers have been identified to select against the thermophiles but none against the mesophile, P. ochrochloron. One alternative would have been to screen other fungicides or antibiotics in an attempt to identify a suitable marker against P. ochrochloron. However, this procedure could have been as time consuming as isolating an auxotrophic mutant of this organism and with no guarantee of success. It was, therefore, decided to take the latter approach and risk any changes in enzyme profile and sporulation.

A spore suspension of P. ochrochloron was irradiated with UV-light of 257nm for 0-4h, as described in Section 2.10.1. Developing colonies from a spore suspension representing 0.1% and 1.8% survivors (Table 2.10) were sub-cultured onto complete agar (CA) [Table 2.4] and minimal agar (MA) [Table 2.12] plates to identify mutant colonies. Five mutant colonies, numbered 42, 254, 283, 292 and 294 were selected for further characterisation studies. These mutants were screened for amino acid requirement using specified groups of amino acids (Table 5.3). Colonies 283 and 292 were

TABLE 5.3 GROWTH OF P. OCHROCHLORON MUTANTS ON SPECIFIED GROUPS OF AMINO ACIDS

MUTANT	GROWTH OF MUTANTS (mm)						
	MA	AMINO ACID GROUPS					CA
		I	II	III	IV	V	
42	-	-	-	-	20	-	39
254	-	32	-	4	29	32	37
283	2	34	34	32	34	34	37
292	3	32	31	28	29	31	41
294	-	-	-	-	4	5	28

- = no growth

Specified groups of amino acids (I - V) were incorporated into minimal agar (MA) [Table 2.12] plates. These plates together with complete agar (CA) [Table 2.4] and MA [Table 2.12] plates were inoculated with five mutants of P. ochrochloron. After incubation at 30°C for five days, growth was measured and expressed as colony diameter. The amino acid groups were as follows:
I. phenylalanine, tryptophan and tyrosine;
II. histidine, isoleucine, leucine and lysine;
III. arginine, glutamate and proline; IV. aspartate, cysteine, methionine and valine; V. alanine, glycine, serine and threonine.

rejected because they were capable of some growth, although very small, on MA. Mutant 42 was selected for further studies because of its greater growth than mutant 294 and of an apparent block late in the amino acid synthetic pathway (Table 5.3). This mutant was capable of growth on MA only when it was supplemented with group IV amino acids (Table 5.3). When the group IV amino acids were added individually to MA, mutant 42 was shown to be capable of growth only when cysteine or methionine were present (Table 5.4). Examination of Figure 5.1 shows that the block in the amino acid synthetic pathway occurred after aspartate. Mutant 42 showed no growth on isoleucine, lysine or threonine (Table 5.3) and so the block was before these amino acids in the amino acid synthetic pathway. Experiments were conducted to see if the block occurred after homoserine (Table 5.5). This table clearly shows that the block occurred after homoserine and indicates the requirement of the mutant for sulphur containing amino acids such as cysteine, cystine or methionine. Mutant 42 was therefore used in subsequent experiments on fusion studies with G. emersonii and P. dupontii.

Although antifungal agents have been identified for selection against A. fumigatus and A. niger (Section 5.1), these chemicals affected the growth and sporulation of the organisms. As a consequence of this observation, further markers were sought. An auxotrophic mutant of A. niger was isolated using the same technique as that described for P. ochrochloron. Developing colonies from a spore suspension representing 1.3% survivors (Table 2.11) were sub-cultured onto CA (Table 2.4) and MA (Table 2.12) plates to identify mutant colonies. Only one mutant was isolated after 4h irradiation, indicating that A. niger may have a greater resistance to UV-

TABLE 5.4 GROWTH OF MUTANT 42, WILD TYPE P.OCHROCHLORON AND G. EMERSONII ON GROUP IV AMINO ACIDS

GROUP IV AMINO ACIDS	GROWTH OF ORGANISMS (mm)		
	MUTANT 42	P. OCHROCHLORON	G. EMERSONII
ASPARTATE	-	42	86
CYSTEINE	18	45	85
METHIONINE	17	44	85
VALINE	-	42	85

The group IV amino acids were incorporated into minimal agar (MA) [Table 2.12]. These plates were then inoculated with a spore inoculum of the wild type organisms, G. emersonii and P. ochrochloron and the auxotrophic mutant of P. ochrochloron (Mutant 42). After five days incubation at the appropriate growth temperature (Section 2.2), growth was measured and expressed as colony diameter.

FIGURE 5.1 SYNTHESIS OF AMINO ACIDS

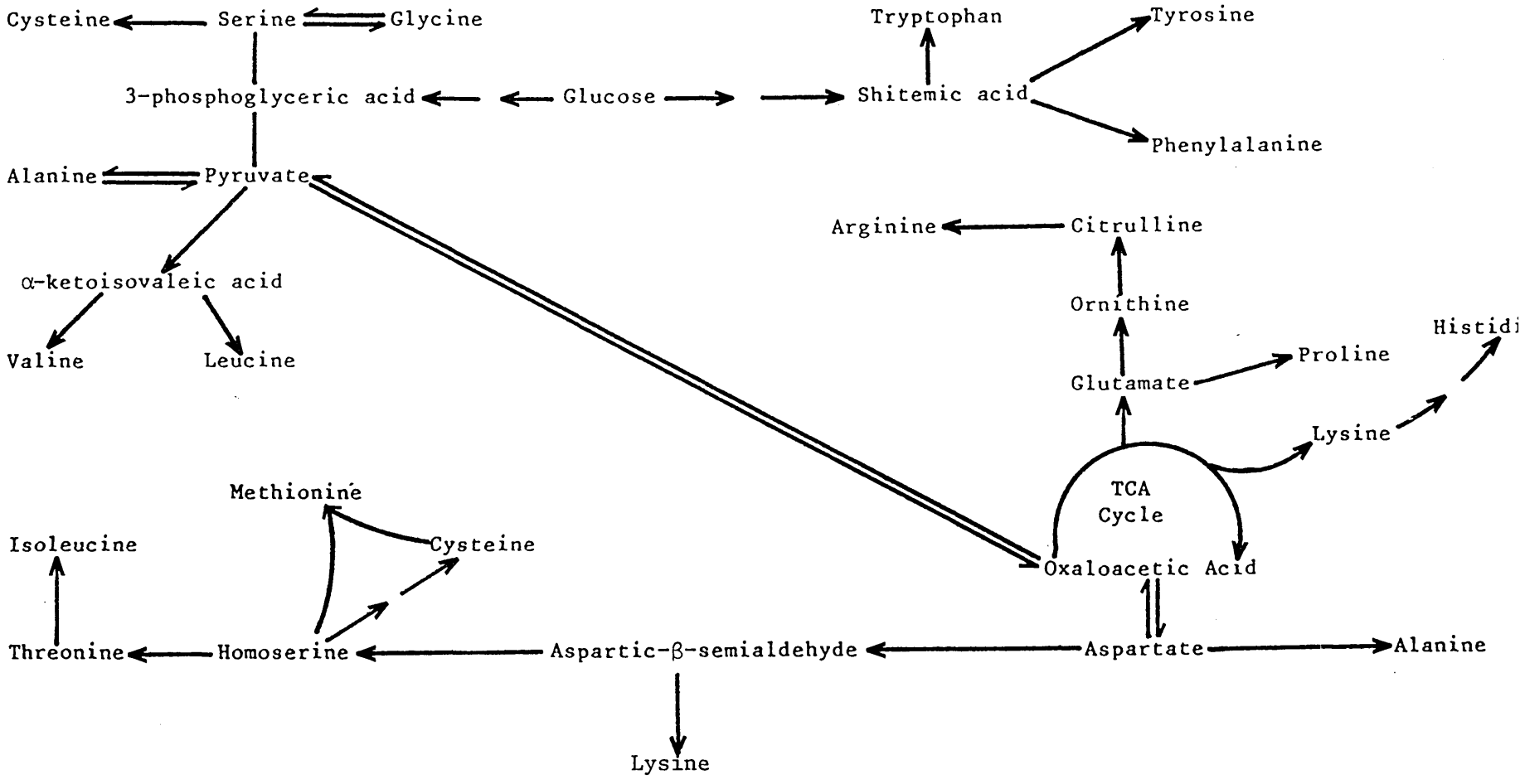

TABLE 5.5 GROWTH OF MUTANT 42 ON SULPHUR CONTAINING AMINO ACIDS

GROWTH MEDIUM	GROWTH OF MUTANT 42 (mm)
MA (minimal agar)	-
MA plus amino acids (with the exception of cystein, cystine and methionine)	-
MA + cysteine	18
MA + cystine	18
MA + homoserine	-
MA + methionine	17

- = No growth

A number of amino acids or their precursors were incorporated into minimal agar (MA) [Table 2.12] plates. These plates were then inoculated with a spore inoculum of the auxotrophic mutant of P. ochrochloron (Mutant 42). After five days incubation at 30°C, growth was measured and expressed as colony diameter.

irradiation than P. ochrochloron under the same conditions (Section 2.10.1). Further characterisation experiments were being carried out on this mutant and attempts were being made to isolate an auxotrophic mutant of A. fumigatus when an alternative method of isolating genetic markers was suggested (J. F. Peberdy, Personal Communication). This technique involved the use of fungicides as inducing agents for mutagenesis and benefits from being less time consuming than isolating auxotrophic mutants and has little effect on enzyme production (Table 3.6).

5.3 FUNGICIDE MUTAGENESIS

Agar plates containing growth medium and various anti-fungal agents, at high concentration were inoculated with high density spore suspensions of A. fumigatus and A. niger, as described in Section 2.10.2. A mutant of A. niger, resistant to Benlate at 0.01%(W/v) [Table 5.6] and a mutant of A. fumigatus, resistant to Mystox 8 at 0.01%(W/v) [Table 5.7] were isolated. These mutants were stable and could, therefore, be used as selection markers in a cross between these two fungi.

5.4 PROTOPLAST FUSION

Protoplasts were isolated from the two organisms to be fused using the technique described in Section 2.4 and the optimum conditions for isolation as determined in Section 4.

Initially a test cross was attempted between a Benlate resistant mutant and a wild type strain of A. niger to investigate the possibility of using the dead donor technique as described by

TABLE 5.6 GROWTH OF A. NIGER (WILD TYPE AND MUTANT) ON BENLATE

BENLATE % (W/v)	GROWTH OF A. NIGER (mm)	
	WILD TYPE	MUTANT
0.01	-	60
0.001	-	86

- = no growth

The fungicide, Benlate was incorporated into Eggins and Pugh Basal Medium (Table 2.1) containing 1% (W/v) glucose. Plates were inoculated with spores from the wild type and mutant A. niger. After incubation at 30°C for five days, growth was measured and expressed as colony diameter.

TABLE 5.7 GROWTH OF A. FUMIGATUS (WILD TYPE AND MUTANT) ON MYSTOX 8

MYSTOX 8 % (W/v)	GROWTH OF A. FUMIGATUS (mm)	
	WILD TYPE	MUTANT
0.01	-	24
0.005	-	27

- = no growth

The fungicide, Mystox 8 was incorporated into Eggins and Pugh Basal Medium (Table 2.1) containing 1% (W/v) glucose. Plates were inoculated with spores from the wild type and mutant A. fumigatus. After incubation at 44°C for five days, growth was measured and expressed as colony diameter.

Fodor et al. (1978). Protoplast suspensions (1 x 10^8 ml^{-1}) of the resistant strain were incubated at 50-65°C for 0-3h. Samples (0.5ml) were taken at half-hour intervals and plated onto Eggins and Pugh Basal medium (Table 2.1) containing 1%(^{w}/v) glucose and 0.6M sodium chloride, to determine the viability of the heat-treated protoplasts. Protoplasts incubated at 50°C remained viable after 3h, whereas, those incubated at 60°C were rendered non-viable after 30min. A slightly prolonged incubation (1h) was required to render protoplasts non-viable when incubated at 55°C. An attempt was made to fuse protoplasts of the wild type strain with heat treated protoplasts of the resistant strain using the conditions described by Anne and Peberdy (1975) and Ferenczy et al. (1976). Growth on suitable selective plates would then indicate that the gene for resistance had been transferred from the non-viable resistant protoplasts to viable protoplasts which lack the resistance gene. A protoplast suspension (1 x 10^9 ml^{-1}) of the resistant strain was incubated for 1h at 55°C and then mixed with an equal number of untreated protoplasts from the wild type strain. After mixing, the resulting protoplast suspension was centrifuged at 2,500rpm for 10min. The pellet was then resuspended in 1.0ml pre-warmed fusion medium (30°C) consisting of 30%(^{w}/v) polyethyleneglycol (PEG) 4000, 0.01M calcium chloride and 0.05M glycine, at a final pH of 8.5. After incubation at 30°C for 2min, the mixture was diluted with 30-40ml of protoplast incubation medium (Section 2.4). The suspension was mixed and then centrifuged at 2,500rpm for 10min. The pellet was washed three times with 30-40ml of the protoplast incubation medium and then finally resuspended in 5.0ml of the same medium. Undiluted, 1:10 and 1:100 dilutions of the protoplast mixture were then plated out onto two types of regeneration medium. Both types consisted of Eggins and

Pugh basal medium (Table 2.1), 1.0%(W/v) glucose and 0.6 M sodium chloride. However, one type of plate contained 0.01%(W/v) Benlate. Individual protoplast suspensions (1 x 10^9 ml^{-1}) of both strains were also plated out onto the two types of plate, as controls. All the regeneration plates were incubated at 30°C for 5-7 days. Protoplasts of the wild type strain demonstrated growth on the glucose regeneration medium but no growth was observed on the same medium containing 0.01%(W/v) Benlate. In contrast, protoplasts of the resistant strain demonstrated growth on both types of regeneration medium. Although the fusion mixture did show growth on the glucose regeneration medium, no growth was observed on the same medium containing 0.01%(W/v) Benlate. It may be concluded from these results that transfer of genetic material has not taken place. A number of variations were carried out in an attempt to achieve protoplast fusion in the test cross. PEG 4000 was used at concentrations of 5, 10, 15, 20, 30, 40 and 60%(W/v) and calcium chloride was used at 0.0, 0.01, 0.05 and 0.1M. The pH of the fusion medium was set at 4.5, 5.5, 6.5, 7.5, 8.5 and 9.5 with and without glycine. However, no fusion products were isolated after 1, 2, 5, 10, 20 and 30min incubations with these fusion media. Owing to these difficulties, the dead donor technique was dropped in favour of the more conventional method of protoplast fusion (Peberdy, 1980).

A number of genetic markers were available for the two crosses of interest, A. fumigatus with A. niger (Table 5.8) and G. emersonii with P. ochrochloron (Table 5.9). P. dupontii was not used in fusion studies because of the problems in isolating protoplasts from this fungus (Section 4.6). The best markers for the cross between A. fumigatus and A. niger were obtained from the fungicide resistant

TABLE 5.8 GENETIC MARKERS FOR THE CROSS BETWEEN A. FUMIGATUS AND A. NIGER

FUNGICIDE/ METAL SALT	CONCENTRATION	GROWTH OF PARENTAL TYPES (mm)	
		A. FUMIGATUS (MUT)	A. NIGER (MUT)
BENLATE	0.01% (W/v)	-	60
	0.001% (W/v)	-	86
CUPRIC CHLORIDE	0.001 M	-	26
MERCURIC CHLORIDE	0.0005 M	18	-
MYSTOX 8	0.01% (W/v)	24	-
	0.005% (W/v)	27	-
THIRAM	0.5% (W/v)	-	25

mut = mutant; - = no growth

The above fungicides and metal salts were incorporated into Eggins and Pugh Basal Medium (Table 2.1) containing 1% (W/v) glucose. Agar plates containing these genetic markers were inoculated with a spore inoculum from the two mutants. After incubation at the appropriate growth temperature (Section 2.2) for five days, growth was measured and expressed as colony diameter.

TABLE 5.9 GENETIC MARKERS FOR THE CROSS BETWEEN G. EMERSONII AND P. OCHROCHLORON (MUT)

FUNGICIDE/ METAL SALT	CONCENTRATION	GROWN OF PARENTAL TYPES (mm)	
		G. EMERSONII (WT)	P. OCHROCHLORON (MUT)
Chlorothalonil	0.01% (W/v)	-	32
Cupric Chloride	0.0005 M	-	16
Impact	0.001% (W/v)	-	24
Mercuric Chloride	0.0005 M	-	13
Merolan	0.1% (W/v)	-	11
Minimal Agar		87	-
Mystox ELC	0.1% (W/v)	-	16
Mystox LPL	0.1% (W/v)	-	24
Mystox 8	0.01% (W/v)	-	16
Quintozene	1.0% (W/v)	-	24
Thiram	0.25% (W/v)	-	7

mut = mutant; - = no growth

The above fungicides and metal salts were incorporated into complete agar (Table 2.4) and minimal agar (Table 2.12). Agar plates containing these genetic markers were inoculated with a spore inoculum from G. emersonii and the auxotrophic mutant of P. ochrochloron (mutant 42). After incubation at the appropriate growth temperature (Section 2.2) for five days, growth was measured and expressed as colony diameter.

mutants of these strains. Table 5.8 shows that a combination of 0.01%(W/v) Benlate and 0.01%(W/v) Mystox 8 could be used to select against the two parental types, if the two mutant strains were used. (Plates 1 and 2) Other markers were available, such as 0.001M Cupric chloride, 0.0005M Mercuric chloride and 0.5%(W/v) Thiram (Table 5.8) but these chemicals caused reduced sporulation and may have affected enzyme production. Previous experiments had shown that enzyme yields in the two fungicide resistant mutants were only slightly altered (Table 3.6) and both mutants showed normal sporulation although slightly delayed in comparison with the wild type strains. These mutant strains and the two fungicides, Benlate and Mystox 8 were therefore used in fusion experiments. In the other cross, no anti-fungal agents were identified which could have been used to select against P. ochrochloron and so an auxotrophic mutant (Mutant 42) of this fungus was used in fusion experiments. Selection against this mutant could be achieved using MA containing 0.6M sodium chloride, as the mutant would be incapable of growth on this regeneration medium (Plate 3). Enzyme production in this mutant was reduced significantly, particularly with respect to endo-β-1,4-glucanase and β-glucosidase production as a result of UV-irradiation (Table 3.6). However, as this marker was the only one available for this organism, it was decided to use this mutant in fusion experiments with G. emersonii. In contrast, numerous anti-fungal agents could be used to select against G. emersonii (Table 5.9). No experiments were conducted to investigate the effect of these anti-fungal agents on enzyme production. Control plates (4 and 5) are included to show growth of all four test organisms on complete medium and glucose medium respectively.

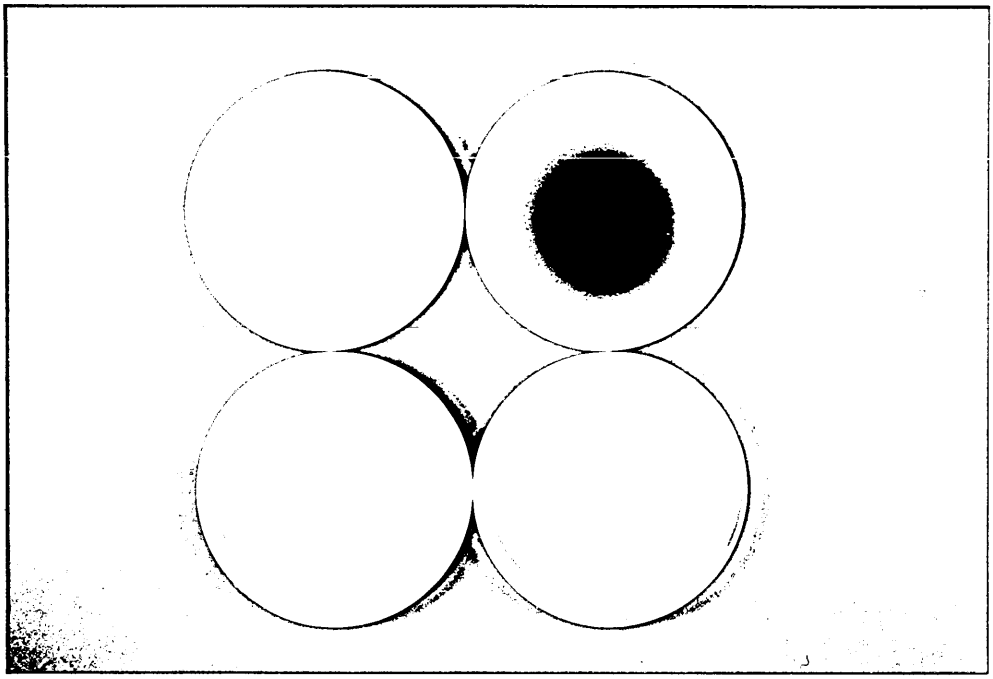

PLATE 1

Eggins and Pugh Basal medium containing 1% (W/v) glucose and 0.01% (W/v) Benlate. Top left: P. ochrochloron; top right: A. niger (mutant); bottom left: G. emersonii; bottom right: A. fumigatus

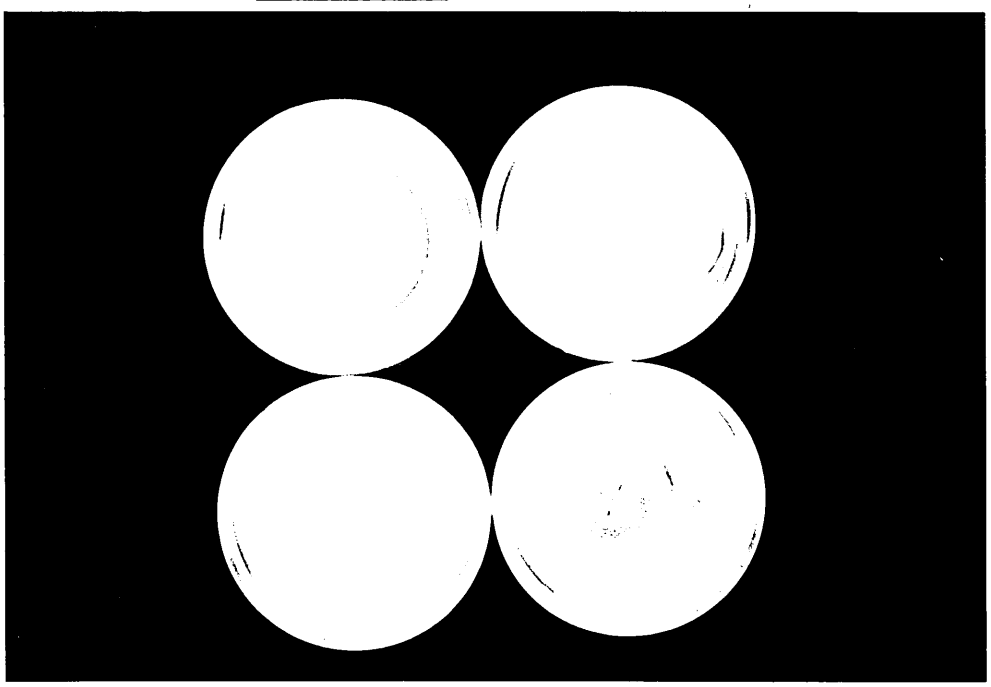

PLATE 2

Eggins and Pugh Basal medium containing 1%(W/v) glucose and 0.01% (W/v) Mystox 8. Top left: P. ochrochloron; top right: A. niger (mutant); bottom left: G. emersonii; bottom right: A. fumigatus (mutant).

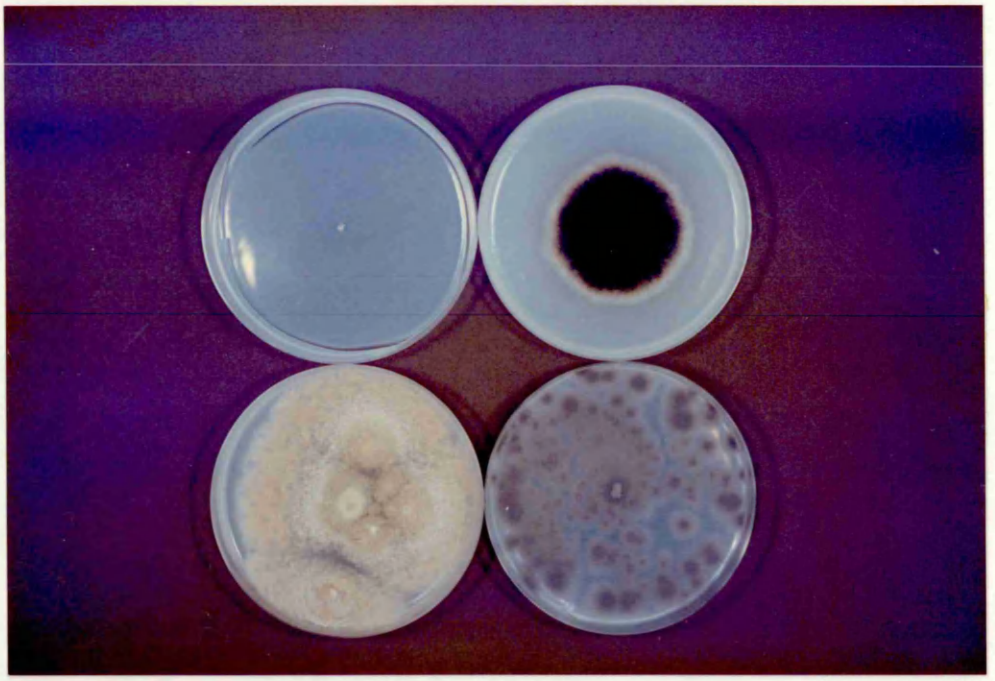

PLATE 3

Minimal medium. Top left: *P. ochrochloron* (mutant); top right: *A. niger*; bottom left: *G. emersonii*; bottom right: *A. fumigatus*.

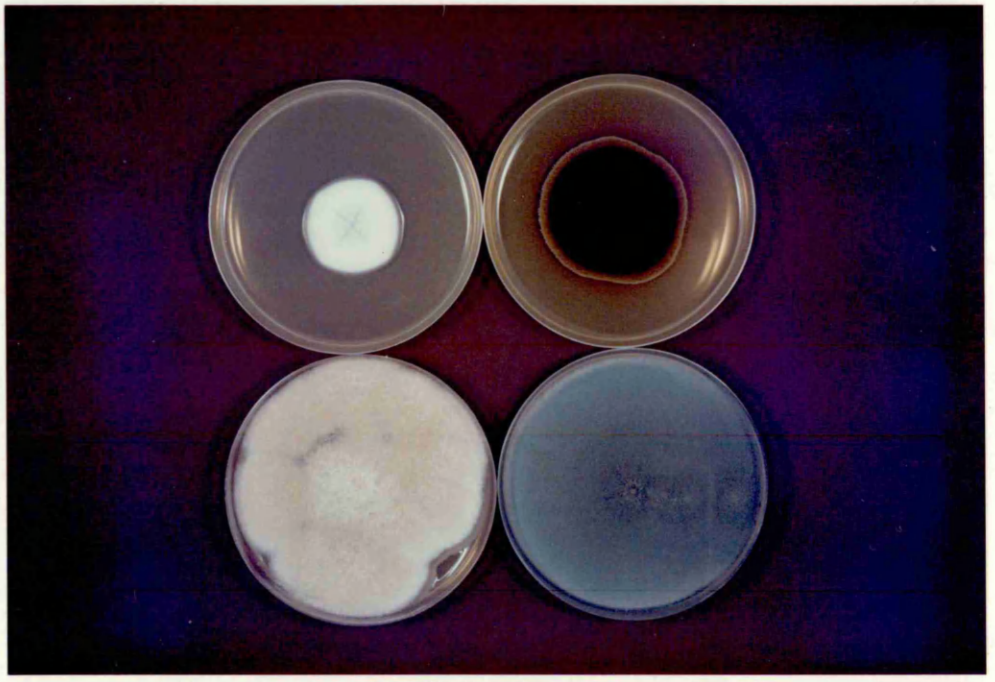

PLATE 4

Complete medium. Top left: *P. ochrochloron* (mutant); top right: *A. niger*; bottom left: *G. emersonii*; bottom right: *A. fumigatus*.

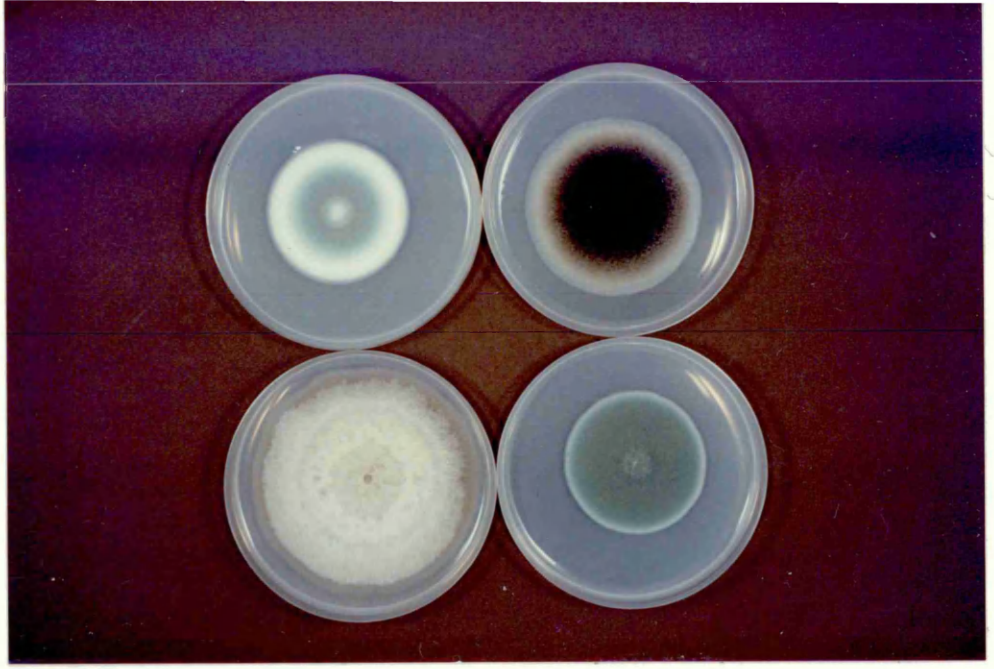

PLATE 5

Eggins and Pugh Basal medium (Table 2.1) containing 1% (W/v) glucose. Top left: *P. ochrochloron* (mutant); top right: *A. niger*; bottom left: *G. emersonii*; bottom right *A. fumigatus*.

Protoplasts of the two species to be crossed were mixed in equal quantities (1×10^9-1×10^{10} ml^{-1}) and fusion was attempted using the same technique described for the test cross. Fusion mixtures were then plated out onto three types of regeneration plate, each of which contained 0.6M sodium chloride. One type of plate (A) allowed growth of one of the parental types and yet was selective against the other. The second type of regeneration plate (B) allowed growth of the other parental type and prevented growth of the first parental type. A third type of regeneration plate (C) did not support growth of either of the parental types and could be used to isolate any possible fusion products. For a summary of fusion procedures refer to Figure 2.8. In the cross between A. fumigatus and A. niger, Benlate at 0.01%(W/v) and 0.001%(W/v) was used to check the viability of A. niger (mutant) protoplasts and Mystox 8 at 0.01%(W/v) and 0.005%(W/v) was used to check the viability of A. fumigatus (mutant) protoplasts (Table 5.10). Fusion plates containing a combination of the two fungicides, Benlate and Mystox 8 were used to allow regeneration of any fusion products. Regeneration plates were incubated at 30°C, 37°C and 44°C for 5-7days. Unfortunately, no fusion products were isolated under the conditions tested. In the other cross between G. emersonii and P. ochrochloron, CA plates were used to determine the viability of the parental type protoplasts. Other plates were included to check for back mutations (Table 5.11). When CA was supplemented with 0.001%(W/v) Mystox 8 or 0.001%(W/v) Impact, protoplasts of P. ochrochloron (Mutant 42) were capable of growth whereas those from G. emersonii were not. The situation was reversed when protoplasts from the two parental types were plated out onto MA. Plates containing minimal agar and 0.001%(W/v) Impact or 0.001%(W/v) Mystox 8 were used to allow

TABLE 5.10 SELECTION MEDIA USED IN THE CROSS BETWEEN A. FUMIGATUS (MUT) AND A. NIGER (MUT)

SELECTION MEDIUM	GROWTH OF PARENTAL TYPES	
	A. FUMIGATUS (MUT)	A. NIGER (MUT)
Benlate [0.001% (W/v)]	-	+++
Benlate [0.01% (W/v)]	-	++
Mystox 8 [0.005% (W/v)]	++	-
Mystox 8 [0.01% (W/v)]	++	-
Benlate [0.001% (W/v)] + Mystox 8 [0.001% (W/v)]	-	-
Benlate [0.01% (W/v)] + Mystox 8 [0.01% (W/v)]	-	-

MUT = mutant

The above fungicides were incorporated into Eggins and Pugh Basal Medium (Table 2.1) containing 1% (W/v) glucose and 0.6 M sodium chloride. Agar plates of these selection media were inoculated with 1.0×10^9 and 1.0×10^8 protoplasts ml^{-1} of the two parental types, A. fumigatus and A. niger. After incubation at the appropriate growth temperature (Section 2.2) for five days, growth was assessed and indicated as - (no growth) or + (growth).

TABLE 5.11 SELECTION MEDIA USED IN THE CROSS BETWEEN G. EMERSONII AND P. OCHROCHLORON (MUT)

SELECTION MEDIUM	GROWTH OF PARENTAL TYPES (mm)	
	G. EMERSONII	P. OCHROCHLORON (MUT)
CA (Complete Agar)	+++	+++
CA + Mystox 8 [0.001% (W/v)]	-	++
CA + Impact (0.001% (W/v)]	-	++
MA (Minimal Agar)	+++	-
MA + Mystox 8 [0.001% (W/v)]	-	-
MA + Impact [0.001% (W/v)]	-	-

MUT = mutant

The above two fungicides were incorporated into Complete Agar (CA) [Table 2.4] and minimal agar (MA) [Table 2.12]. Agar plates of these media together with CA (Table 2.4) and MA (Table 2.12). These plates all containing 0.6 M sodium chloride, were inoculated with 1.0×10^9 and 1.0×19^8 protoplasts ml^{-1} of the two parental types, G. emersonii and P. ochrochloron. After incubation at the appropriate growth temperature (Section 2.2) for five days, growth was assessed and indicated as - (no growth) or + (growth).

regeneration of any fusion products. Regeneration plates were incubated at 30°C, 37°C and 47°C for 5-7days. However, no fusion products were isolated from this cross under the conditions tested, even when protoplast suspensions of 1 x 10^{10} ml^{-1} were used. The fusion parameters were varied, as in the test cross, in an attempt to achieve protoplast fusion in the two trial crosses, unfortunately with no success.

Other workers have successfully crossed closely related species of Aspergillus (Ferenczy, 1976; Kevei and Peberdy, 1977, 1979) and Penicillium (Anne and Peberdy, 1976; Anne and Eyssen, 1978). Heterokaryons were produced, from which spores of both the complementing parents could be isolated. Prolonged incubation of these heterokaryons under selective conditions resulted in the growth and development of vigorously growing sectors which were phenotypically different from either parent. These colonies having nuclei derived from different species may be referred to as "hybrids" or "alloploids" (Rieger et al., 1976). Heterokaryons have been obtained from crosses between less related species of Aspergillus (Ferenczy, 1976; Ferenczy et al., 1977) and Penicillium (Anne et al., 1976). These workers reported that spores formed from the heterokaryons were predominantly of one parental type, when selection was relaxed. Fusion frequencies have been reported between 0.03-4.0% for crosses between closely related species and intraspecies crosses within the Penicillium genus (Anne and Peberdy, 1976; Anne, 1977). Lower values have been reported in crosses between less related species of Aspergillus (Ferenczy, 1976) or species of yeast (Provost et al., 1978; Sipiczki, 1979), perhaps indicating a higher degree of compatibility between Penicillium species. The fact that no fusion

products were isolated from the trial crosses, A. fumigatus with A. niger and P. ochrochloron with G. emersonii seems to indicate that the differences between thermotolerant/thermophilic and mesophilic organisms are too great to allow somatic or nuclear fusion under the conditions used.

Cellulosic wastes normally consist of crystalline cellulose in association with hemicelluloses and lignin. All three components must be utilised if cellulose hydrolysis is to become cost effective. Although only the enzymatic hydrolysis of cellulose is discussed in this thesis, it should be noted that the alternative process of acid hydrolysis may also be considered in any prospective industrial production of glucose from cellulosic wastes. As discussed earlier, both processes suffer from a number of drawbacks, and improvements are required before they become commercially viable.

The enzymatic process is complicated by the fact that at least three different types of activity are required for the hydrolysis of crystalline cellulose: endo-β-1,4-glucanase, exo-β-1,4-glucanase and β-glucosidase. All three enzymes have been isolated from culture filtrates of G. emersonii (McHale and Coughlan, 1982; Moloney et al., 1985), S. pulverulentum (Eriksson and Pettersson, 1975a,b; Deshpande et al., 1978), T. koningii (Pettersson et al., 1981) and T. viride (Beldman et al., 1985). Experiments were conducted to investigate the production of these three enzymes in a number of fungal species. Out of seven fungi tested P. funiculosum and T. viride showed the greatest degradation of crystalline cellulose. Culture filtrates of these two fungi demonstrated all three types of activity when grown on A. xylinum cellulose pellicles (Table 3.4) and CMC (Table 3.3). In contrast, the other five fungi tested (A. fumigatus, A. niger, G. emersonii, P. janthinellum and P. ochrochloron) were less efficient in degrading crystalline cellulose. Due to time limitations, no detailed experiments were carried out on S. pulverulentum, T. koningii and T. reesei.

Rapid secretion of all three types of cellulase enzyme into the surrounding medium is required for an organism to be useful in the industrial production of cellulases. Insoluble substrates, such as A. xylinum cellulose pellicles are usually more efficient in inducing the secretion of cellulase enzymes, particularly endo-β-1,4-glucanase and exo-β-1,4-glucanase than soluble substrates such as CMC. This was the situation with P. funiculosum and T. viride; whereas A. fumigatus, A. niger, G. emersonii and P. ochrochloron showed higher extracellular enzyme activity when grown on CMC. P. janthinellum, on the other hand, demonstrated reasonable activities of all three enzymes and production was the same in filtrates from cultures grown on CMC (Table 3.3) and A. xylinum cellulose pellicles (Table 3.4). A. niger demonstrated high endo-β-1,4-glucanase and β-glucosidase activity when grown on CMC (Table 3.3) and much less activity when grown on A. xylinum cellulose pellicles (Table 3.4). Only low levels of β-glucosidase were observed in culture filtrates of T. viride, in comparison to A. niger and P. janthinellum which produced much higher levels. Many workers have reported the presence of intracellular and wall-bound β-glucosidases in fungi, including T. viride (Gong et al., 1979; Sternberg and Mandels, 1979) and bacteria (Hagerdal et al., 1979; Ng and Zeikus, 1981a). Although beneficial to these organisms, this situation is undesirable in the commercial production of cellulase and the hydrolysis of cellulose to soluble sugars. No experiments were conducted to investigate the other types of β-glucosidase activity which are thought to exist (Shewale, 1982). In addition to cellulose a number of other substrates have been reported to induce cellulase synthesis (Sternberg et al., 1977; Shewale and Sadana, 1978; Gong et al., 1979), Due to time limitations no experiments were conducted to investigate this

phenomenon. Endo-β-1,4-glucanase activity was evident in culture filtrates after 48h in five fungi tested when grown on A. xylinum cellulose pellicles (Figure 3.9). This lag time was reduced to less than 24h in the same fungi when CMC was the growth substrate (Figure 3.8), indicating that induction of cellulase biosynthesis and secretion was slower with insoluble substrates.

Maximal endo-β-1,4-glucanase activity was observed after five days in culture filtrates of five fungi grown on both CMC (Figure 3.8) and A. xylinum cellulose pellicles (Figure 3.9). Many workers have observed a decline in hydrolysis rates with time after prolonged incubation (Mandels, 1985), This observation could be explained by substrate depletion, product inhibition (cellobiose and/or glucose) or enzyme inactivation. When native cellulose is used as the growth substrate the more accessible and susceptible portions of cellulose are more readily degraded, leaving a residual substrate which has decreased susceptibility to enzyme attack. Ball-milling of these residual substrates has been shown to restore enzyme susceptibility (Ghose and Kostick, 1969). Cellulase enzymes adsorb to the substrate as a prerequisite for hydrolysis (Wood et al., 1980). Exo-β-1,4-glucanase shows the greatest adsorption, endo-β-1,4-glucanase slightly less and β-glucosidase very little adsorption to crystalline cellulose (Ladisch et al., 1983). It has been suggested that endo-β-1,4-glucanase and exo-β-1,4-glucanase form an enzyme-enzyme complex on the surface of cellulose polymers (Fagerstam and Pettersson, 1980; Wood et al., 1980). This hypothesis could explain the reported synergistic relationship between these two enzymes in the hydrolysis of crystalline cellulose (Streamer et al., 1975; Wood and McCrae, 1979; Beldman et al., 1985). It would seem that

only those endo-β-1,4-glucanase and exo-β-1,4-glucanases adsorbed in close proximity would be able to carry out hydrolysis and return to solution.

It is thought that a large proportion of cellulase adsorbs to the substrate to form persistent enzyme/substrate complexes (Wood et al., 1980). This may also contribute to a decline in hydrolysis rates and explain the low specific activity of cellulase enzymes. Higher concentrations of endo-β-1,4-glucanases and exo-β-1,4-glucanases are therefore required to increase the effectiveness of the enzymatic degradation of native cellulose. It is appreciated, however, that this situation is probably more complex since synergism has also been reported between multiple forms of exo-β-1,4-glucanase (Fagerstam and Pettersson, 1980; Wood and McCrae, 1986). Further complication arises as a result of the observation that some endo/exo pairs, even from the same fungi, do not show any such synergism (Wood and McCrae, 1986). This seems to indicate that multiple forms, whether genetically determined or, as a result of proteolytic modification have a specific role to play in cellulose hydrolysis. β-glucosidase is also required to remove cellobiose from culture media (Gong et al., 1979; Mandels et al., 1981), therefore preventing inhibition of endo-β-1,4-glucanase (Halliwell and Griffen, 1973; Ladisch et al.,1980) and exo-β-1,4-glucanase (Hsu et al., 1980; Moloney et al., 1985). All three enzyme types are, therefore, required for the hydrolysis of crystalline cellulose to soluble sugars. Unfortunately, the end product of cellulose hydrolysis, glucose, inhibits β-glucosidase (Gong et al., 1977; Bisset and Sternberg, 1978). This end product inhibition of the cellulase complex significantly reduces the maximum attainable yield of reducing sugars from crystalline

cellulose. The same enzymes are also subject to catabolite repression (Nisizawa et al., 1971). New strains are therefore required which are less susceptible to end product inhibition and/or catabolite repression.

Low concentrations of glucose [0.2%(W/v)] were shown to reduce the lag time and increase enzyme production in the organisms tested. Since no enzyme activity was observed until the glucose was utilized it would seem that supplementary glucose allowed an increase in mycelial mass at a faster rate, rather than inducing enzyme production directly. However, addition of glucose may be undesirable in a commercial situation and it may be worth investigating other waste products which contain readily available sugar.

A number of developments are required to make the enzymatic hydrolysis of cellulose to soluble sugars a cost effective process. These include: (i) improvements in the pretreatment of substrates in order to increase the susceptibility of cellulose to hydrolysis; (ii) a method to encourage the desorption of cellulase enzymes from the substrate, which would allow the recovery and reuse of those enzymes; (iii) process developments in the form of, either continuous reactors, or simultaneous saccharification fermentation processes.

The Trichoderma system is generally accepted as being the best available for the hydrolysis of crystalline cellulose. However, this system suffers from a number of limitations, such as the low specific activity and the susceptibility to end product inhibition and/or catabolite repression. Additionally, Trichoderma spp. are unable to degrade lignin and therefore when lignocellulosic wastes are used the

lignin remains in the residual substrate. The white rot fungi, particularly S. pulverulentum are notable in their degradation of lignin (Westermark and Eriksson, 1974; Eriksson, 1983; Agosin and Odier, 1985). Unfortunately, although S. pulverulentum is capable of extensive degradation of crystalline cellulose, only low levels of extracellular cellulase may be obtained (Eriksson, 1983). Improvements in the secretion of cellulase enzymes from this fungus would be most beneficial in the hydrolysis of lignocellulosic wastes. In addition, pectinases and xylanases are also important in the hydrolysis of cellulosic wastes due to the association of crystalline cellulose with hemicelluloses. High activities of endo-β-1,3-xylanase and endo-β-1,4-xylanase were observed in culture filtrates of A. fumigatus, A. niger, G. emersonii and P. ochrochloron, whereas only low activities of exo-β-1,4-xylosidase and pectinase were observed in the same culture filtrates. More information is required on the production of these enzymes from other fungi. It may be that synthesis of these enzymes was not induced under the conditions tested and therefore further experiments should be conducted using more diverse forms of cellulose such as straw and spent grains. Another disadvantage of the Trichoderma system is that these enzymes are rapidly inactivated at high temperatures and so reactors have to be run at 45-50°C. At this temperature, reactors are readily contaminated by other organisms and therefore increased thermal stability of the cellulase and related enzymes would facilitate the use of higher temperatures.

Attempts were made to cross mesophilic and thermophilic fungi using the technique of protoplast fusion, with a view to increasing the thermal stability of the enzymes produced. Two trial crosses

were set up: A. fumigatus with A. niger and G. emersonii with P. ochrochloron. A number of possibilities may arise from the genetic recombination of these fungi such as increased enzyme production and resistance to end product inhibition and/or catabolite repression. Furthermore, the time required for maximum enzyme production may also be reduced since thermophilic organisms usually have faster growth rates. A number of genetic markers were obtained for the two crosses using an auxotrophic mutant, two fungicide resistant mutants and using natural resistance to heavy metals and fungicides. An auxotrophic mutant of P. ochrochloron was shown to have reduced and delayed sporulation and lower aryl-β-glucosidase activity than the wild type strain. Unfortunately, no success was achieved in either of the two trial crosses using the technique of protoplast fusion. This lack of success could be attributed to incompatibility of the parental types. Other workers have reported that isolation of viable heterokaryons and stable hybrids depends on species relatedness and somtic or nuclear compatibility (Peberdy et al., 1977; Anne, 1983). Further, Ball (1982) suggested that protoplast fusion may be optimally useful in crosses between closely related species. Protoplast fusion may therefore have a role in determining the genetic homology that exists between strains (Hamlyn et al., 1985). A number of developments have been made in gene cloning such as the isolation of "gene banks" for cellulase and the use of yeast plasmids. Introduction of these genes into yeast plasmids and subsequent fusion with protoplasts may be a more effective method of improving industrially important strains. Ahn and Pack (1985) reported that fusion of protoplasts with liposome-encapsulated plasmids may be more effective in introducing genetic material into yeast cells than by using naked plasmids.

The results presented in this thesis show that the differences between the selected mesophilic and thermophilic fungi may be too great to allow protoplast fusion by methods currently available. Although successful in bacteria and in an intraspecies cross of P. chrysogenum, the dead donor technique was found to be unsuitable for an intraspecies cross of A. niger. This work emphasises that each organism and strain of organism needs to be carefully characterised with respect to protoplast production and enzyme complement before attempting to produce "hybrids" with improved enzyme activity and stability. Further work is required to improve protoplast fusion, selection of "hybrids" and screening of recombinant products.

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