

Relationship between Mycelial Morphology, Cell Wall
Composition and Product Formation of
Rhizopus arrhizus

Thesis

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by

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DECLARATION

This is to certify that the research presented in this thesis is entirely the work of Mary Morrin, unless otherwise stated.

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ABSTRACT

Pelleting of hyphae of Rhizopus arrhizus commences 9-10 h after inoculation, and not at a pregermination stage as for other fungi. The anionic polymers carboxymethylcellulose (CMC) and Carbopol-934 prevent pelleting, causing dispersal of growth of most species of Rhizopus, including R. arrhizus, when used as media supplements. The non-ionic polymer methylcellulose also dispersed growth, but less effectively. The specific growth rate of mycelia in control (unsupplemented) and in polymer-supplemented media was similar until 24 h, when growth became restricted in control medium. Polymer supplementation promoted increased biomass production, but in the case of Carbopol, this increase may be partly due to the tightly bound polymer, as indicated by scanning electron microscopy and biomass studies, particularly at low pH's. Hyphal extension zones and branch points of mycelia from Carbopol-supplemented media at low pH did not fluoresce when treated with Calcofluor White, unlike those from control, CMC- or Carbopol-supplemented media at higher pH values.

The cell walls of dispersed mycelia from viscous media had a higher concentration of hexosamines (34-35%) and a lower concentration of protein (7-6%) compared to the cell walls of pelleted (24% hexosamines/18% protein) or less finely dispersed mycelia (23% hexosamines/13% protein). Results from acid/alkali extraction indicate that a greater proportion of glucuronan is held in non-glucosamine linkages in walls from pelleted than from dispersed mycelia. The analysis of the chitin component of the cell walls by X-ray diffraction demonstrated less crystallinity in the cell walls isolated from Carbopol-supplemented media compared to the other cell wall types examined.

The presence of solid or semi-solid medium components were necessary to promote production of glucoamylase by R. arrhizus. The presence of Carbopol inhibited glucoamylase production. It was also shown to inhibit fumaric acid production when mycelia were grown in its presence and when used in the biotransformation media using pre-grown mycelia. Dispersed filamentous mycelia from CMC-supplemented medium produced the highest yields of fumaric acid in batch culture and in biotransformation studies. However in pH controlled fermenters, where higher aeration/agitation rates prevented densely-packed pelleting, the control mycelia produced the highest yields. Dispersed mycelia from CMC-supplemented medium also had the highest specific capacity to convert progesterone to 11 α -hydroxyprogesterone. 48 h congealed mycelia from control medium manifested a low metabolic rate and poor rates of biotransformation.

CONTENTS

Section		Page
1.	INTRODUCTION	1
1.1	THE GENUS <u>RHIZOPUS</u>	1
1.2	FUNGAL GROWTH MORPHOLOGY	3
1.3	PELLET FORMATION	8
1.4	HYPHAL GROWTH	12
1.5	THE FUNGAL CELL WALL	14
1.5.1	The Zygomycete Cell Wall	15
1.5.2	Analysis of Hyphal Walls	16
1.5.3	Analysis of <u>Rhizopus</u> Cell Wall	18
1.5.4	Architecture of the Hyphal Wall	20
1.5.6	Polymer Assembly and Hyphal Growth	21
1.5.7	Cell Wall Composition and Mycelial Morphology	24
1.6	MYCELIAL MORPHOLOGY, GROWTH AND PRODUCT FORMATION	26
1.6.1	Production of Fumaric Acid by <u>R.arrhizus</u>	29
1.6.2	Production of Glucoamylase by <u>R.arrhizus</u>	32
1.6.3	Biotransformation of Progesterone to 11 α -Hydroxyprogesterone by <u>R.arrhizus</u>	34
1.7	THIS THESIS	38
2.	MATERIALS AND METHODS	39
2.1	CHEMICALS	39
2.2	FUNGAL CULTURE	39
2.2.1	Source of Strain	39

2.2.2	Culture Maintenance	39
2.2.3	Detection of Contamination	40
2.3	SPORE INOCULUM PREPARATION	40
2.4	SHAKE-FLASK CULTIVATION	40
2.5	BIOMASS DETERMINATION USING DRY WEIGHT ANALYSIS	40
2.6	SURFACE CULTURE CULTIVATION	41
2.7	FERMENTER CULTIVATION	42
2.8	MYCELIAL TREATMENT PROCEDURES	42
2.8.1	Radiolabelling of Cell Walls	42
2.8.2	Ruthenium Red Staining of Mycelia	42
2.9	PRODUCT RECOVERY	43
2.9.1	Recovery of Glucoamylase or Fumaric Acid	43
2.9.2	Recovery of Progesterone and 11 α -Hydroxy- progesterone	43
2.10	BIOTRANSFORMATION PROTOCOL	44
2.10.1	Biotransformation of Glucose to Fumaric Acid	44
2.10.2	Biotransformation of Progesterone to 11 α - Hydroxyprogesterone	44
2.11	FERMENTATION MEDIA	44
2.11.1	Control Medium: Peptone/Glucose Medium	44
2.11.2	CMC Medium: CMC-Supplemented Medium	44
2.11.3	CP-4.0/CP-5.5 Medium: Carbopol- Supplemented Media	45
2.11.4	Glucose-CSL-Salt Medium: Defined Medium	45
2.11.5	Cellulase-Pretreated Methylcellulose- Supplemented Medium	45
2.12	STERILISATION PROCEDURES	45

2.13	CELL DISRUPTION TECHNIQUES	45
2.14	ISOLATION OF CELL WALLS	46
2.15	HYDROLYSIS PROTOCOL OF CELL WALLS	47
2.15.1	Hydrolysis for Hexosamine Analysis (Amberlite Hydrolysis)	47
2.15.2	Hydrolysis for Neutral Sugar Analysis	47
2.16	FRACTIONATION OF CELL WALLS	48
2.16.1	Nitrous Acid Extraction	48
2.16.2	Potassium Hydroxide Extraction	48
2.16.3	Enzymatic Hydrolysis of Cell Walls	48
2.17	ANALYTICAL PROCEDURES	50
2.17.1	Macroscopic Analysis	50
2.17.2	Microscopic Analytical Procedures	50
2.17.2.1	Phase Contrast Microscopy	50
2.17.2.2	Fluorescence Microscopy	50
2.17.2.3	Scanning Electron Microscopy	51
2.17.2.4	Transmission Electron Microscopy	51
2.17.3	Estimation of Hyphal Growth Unit (HGU) in Submerged Culture	52
2.17.4	Estimation of Hyphal Extension Rate on Solid Media	52
2.17.5	X-ray Diffraction Analysis	52
2.17.6	Infrared Spectroscopic Analysis of Cell Walls	53
2.17.7	Chromatographic Analysis	53
2.17.7.1	Thin Layer Chromatography	53
2.17.7.2	High Performance Liquid Chromatography	53

2.17.7.2.1	Fumaric acid estimation	54
2.17.7.2.2	Progesterone/11 α -hydroxyprogesterone estimation	54
2.18	CHEMICAL ANALYSIS	55
2.18.1	Estimation of Reducing Sugars	55
2.18.2	Estimation of Neutral Sugars	56
2.18.3	Estimation of Uronic Acids	56
2.18.4	Estimation of Hexosamines	57
2.18.5	Estimation of Protein	57
2.18.6	Estimation of Inorganic and Total Phosphate	58
2.18.7	Estimation of Amino Acids	
2.18.8	Estimation of Ash Content	59
2.19	RADIOACTIVITY ANALYSIS PROCEDURE	59
2.20	ENZYMATIC ANALYSIS	59
2.20.1	Estimation of Glucose	59
2.20.2	Estimation of Glucoamylase Activity	60
2.21	ROUTINE MEASUREMENTS	61
3.	RESULTS	62
3.1	Effect of Polymer Supplement on Morphology of a Range of <u>Rhizopus</u> species	62
3.1.1	Comparison of Morphology of Four Fungal Genera	63
3.1.3	The Effect of Time of Addition of Polymer Supplements on Morphology	65
3.1.4	Effect of Methylcellulose on Morphology	68
3.1.5	Investigation of Effect of Vegetative Inocula on Morphology in Polymer-Supplemented Media	70

3.1.6	Summary	73
3.2	EFFECT OF POLYMER SUPPLEMENTS ON HYPHAL GROWTH AND BIOMASS PRODUCTION	74
3.2.1	Photomicrographic Monitoring of Hyphal Development in Polymer-supplemented Media	74
3.2.2	Measurement of Hyphal Growth Unit (HGU)	77
3.2.3	Effect of Polymers on Biomass Production by <u>R. arrhizus</u>	79
3.2.4	Effect of Polymers on the Specific Growth Rate, μ , of <u>R. arrhizus</u>	81
3.2.5	Effect of Polymers on Hyphal Extension Rate on Solid Media	81
3.2.6	Interaction of Polymers with Hyphae of <u>R. arrhizus</u>	85
3.2.8	Examination of Carbopol-grown Mycelia using Scanning Electron Microscopy	92
3.2.9	Staining of Polymer-grown Mycelia with Ruthenium Red	95
3.2.10	Summary	98
3.3	INVESTIGATION OF CELL WALL COMPOSITION OF DIFFERENT MYCELIAL FORMS OF <u>R. ARRHIZUS</u>	98
3.3.1	Analysis of Cell walls by Infrared Spectroscopy	99
3.3.2	Enzymatic Hydrolysis of Cell Walls of <u>R. arrhizus</u>	99
3.3.3	Chemical Composition of Cell wall	102
3.3.4	Acid/Alkali Extraction of Cell Walls	105
3.3.5	Investigation of Skeletal Wall Components	108

3.3.6	Surface Examination using Transmission Electron Microscopy	110
3.3.7	Labelling of Hyphal Walls using N-(¹⁴ C-acetyl)-glucosamine	112
3.3.7.1.	Solubilisation of N-acetyl glucosamine using Nitrous Acid Extraction	112
3.3.7.2	Solubilisation of N-acetyl glucosamine using Alkali Extraction	114
3.3.8	Summary	115
3.4	INVESTIGATION OF RELATIONSHIP BETWEEN MYCELIAL MORPHOLOGY AND PRODUCT FORMATION OF <u>R. ARRHZUS</u>	117
3.4.1.	Glucoamylase Production and Mycelial Morphology	117
3.4.1.1.	Glucoamylase Production by <u>R. arrhizus</u> in Supplemented Control Medium	117
3.4.1.2	Production of Glucoamylase by <u>R. arrhizus</u> in Surface Culture	118
3.4.1.2.1	Effect of Moisture Level on Glucoamylase Production in Surface Culture	122
3.4.1.2.2	Effect of Medium Composition on Production of Glucoamylase in Surface Culture	122
3.4.1.2.3	Investigation of effect of CMC and Carbopol on Glucoamylase Production in Surface Culture	125
3.4.1.3	Investigation of Glucoamylase Production by Different Mycelial Forms	125
3.4.1.4	Comparison of Glucoamylase Yields in Surface and Submerged Culture	128

3.4.1.5	Appraisal of Results	128
3.4.2	Relationship between Fungal Growth, Morphology and Fumaric Acid Production	129
3.4.2.1	Production of Fumaric Acid in Shake Flask Cultures	129
3.4.2.2	Production of Fumaric Acid in Fermenters	132
3.4.2.3	Biotransformation of Glucose to Fumaric Acid by Different Mycelial Forms	137
3.4.2.4	Summary	139
3.4.3	Biotransformation of Progesterone to 11 α -Hydroxyprogesterone by Different Morphological Forms of <u>R. arrhizus</u>	141
3.4.3.1	Summary	144
4.	DISCUSSION	145
5.	REFERENCES	165
6.	APPENDIX I	i
	APPENDIX II	ii
	APPENDIX III	v

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1. INTRODUCTION

1.1 THE GENUS RHIZOPUS

Members of the genus Rhizopus belong to the group Zygomycotina, class Zygomycetes, according to the classification system of Ainsworth (1973). These fungi, along with another group, the Mastigomycotina, are usually referred to as "lower fungi" due to some properties which are considered to be primitive. These include the mode of formation of asexual non motile spores; cytoplasmic cleavage within a cell called the sporangium; a lack of cross walls or septa in their hyphae and the inability of their vegetative hyphae to anastomose or fuse with one another at points of contact. Higher fungi such as Aspergillus or Penicillium (group Deuteromycotina) form asexual spores called conidia in various ways directly from the hypha, have simple septa in their mycelia and generally have the ability to anastomose.

Sexual reproduction in the Zygomycetes occurs when two fertile branches, from hyphae of two mating types, form gametangia upon meeting. These gametangia fuse, with development of a diploid resting spore called the zygospore. After meiosis, this germinates to form a hypha or sporangium.

Rhizopus species are common soil saprophytes but generally have a very limited ability to degrade plant polymers such as cellulose. They colonise damp organic matter very readily, such as bread, jam, leather, etc., They grow with extreme rapidity on most media surfaces, spreading widely by means of their stolons, completely filling the culture tube with dense cottony masses of mycelium.

Their growth in submerged culture is equally rapid once there is a sufficient level of agitation/aeration. Spores are black in colour and can vary from 5-15µm in length, depending on the species.

The hyphae of members of the genus Rhizopus contain cellular organelles which are common to most fungi, such as nuclei, mitochondria, ribosomes, lipid droplets and endoplasmic reticulum, but centrioles are absent. The cell wall is composed of interwoven microfibrils embedded in an amorphous matrix containing heteropolysaccharides together with protein. The microfibrillar component consists of a chitin-chitosan matrix, characteristic of the Zygomycetes, whereas chitin and cellulose or other glucans are the skeletal components in most other taxonomic groups (Bartnicki-Garcia, 1968).

Many Rhizopus species are of importance economically. They have been used for centuries in traditional oriental fermentations, such as Tempeh production, where dehulled soybean grits are inoculated with spores of Rhizopus oligosporus and grown in shallow trays, leading to the production of a solid cake of grits and mycelia (Wood and Yong Fook Min, 1975). A strain of Rhizopus delemar was used in the production of alcohol in the Amylo process in the late 19th century and in the 1930's where grain starches were first saccharified by inoculation of the fungus and then addition of yeast converted the sugars to alcohol (Miall, 1975). The use of Rhizopus nigricans to make fumaric acid was patented in 1943 by workers from Merck & Co. Inc. and Pfizer & Co. Inc. (Lockwood, 1975). Peterson and Murray (1952) from the Upjohn Co. first observed that a strain of Rhizopus arrhizus could convert progesterone to 11α-hydroxyprogesterone, a

step which allowed the bypass of complex chemical synthetic procedures in the manufacture of cortisone. This drastically reduced the cost of production, making corticosteroids available for common usage.

1.2 FUNGAL GROWTH MORPHOLOGY

Filamentous fungi exhibit a diverse range of morphologies when grown in submerged culture, varying from discrete tightly packed pellets to the filamentous growth form where the hyphae are homogeneously distributed throughout the growth medium. Many factors have been cited in the literature as affecting the morphology, either at a metabolic level or by controlling the physical environment. In quite a few cases, it is not possible to state why a particular component or environmental condition results in a certain morphology, as variation is observed between fungi, making interpretation difficult.

Levels of metal ions and chelating agents have been shown to affect growth morphology. One of the most frequently studied fungi is Aspergillus niger, due to its involvement in citric acid production. Growth in the form of discrete, hard pellets was favoured for citric acid production (Clark, 1962). Metal complexing agents such as EDTA or ferrocyanide were used to control the morphology in this optimum form for citric acid production, indicating a role played by metal ions in morphology determination (Choudhary & Pirt, 1965). Manganese deficiency is particularly important in citric acid accumulation (Clark et al., 1966). Its main role appears to be in the disruption of protein turnover leading to increased intracellular NH_4^+ pool which prevents inhibition of phosphofructokinase by citrate (Kubicek

et al., 1979). This causes increased flux through glycolysis. An altered cell wall composition is associated with bulbous hyphae formed in this citric acid producing medium (Kisser et al., 1980). Increased chitin and reduced β -glucan levels were found in the bulbous hyphae, alterations which are also associated with cycloheximide-induced morphology alterations. Increased concentrations of fructose-6-phosphate and glutamine (precursors of chitin synthesis) have been found in manganese deficient hyphae (Kubicek et al., 1979), perhaps leading to increased metabolic flux in the direction of chitin synthesis. Thus, impairment of protein turnover may account for citrate accumulation and also the morphology expressed during acid accumulation. Concentrations of divalent cations above trace level have been shown to cause pelleting of Rhizopus mycelia (Byrne & Ward 1987). This has led to suggestions that pelleting may be analogous to yeast flocculation where divalent cations act by bridging cells through negative charges on the cell surface (Stewart and Goring, 1976). Fungal biomass has been shown to absorb metal ions from solution (de Rome & Gadd, 1987; Gadd & White 1989; Tsezos, 1983). The carboxyl, phosphate and other functional groups on the cell surface have been suggested as being involved in metal complexation in R. arrhizus (Tobin et al., 1984). The ability of anionic polymers to absorb divalent cations was linked to their role in dispersal of fungal growth whereby metals, which may otherwise have given rise to pelleted growth, were taken up by the polymers, thereby allowing dispersed growth (Byrne & Ward, 1987). However, the process of pelleting is more complex than one which arises solely as a result of metal ion bridges, as A. niger grows as pellets in media containing metal chelators, leaving few free ions in solution (Choudhary & Pirt, 1965).

Polymeric substances have been implicated in influencing growth morphology (Byrne & Ward, 1987; Elmayergi et al., 1973; Takahashi et al., 1960; Trinci, 1983). The increase in medium viscosity due to polymer supplementation has been suggested to act by keeping the developing spores/hyphae physically separated and promoting dispersed growth (Takahashi et al., 1960). But high viscosity is not the only reason dispersed growth is obtained, as seen when a polymer-supplemented medium of low viscosity gave rise to dispersed growth of R. arrhizus (Byrne & Ward, 1987). When Mortierella vinaceae was cultured in viscous media, pelleted growth was obtained (Kobayashi & Suzuki, 1972). Anionic polymers and copolymers of acrylic acid were shown to yield the most significant improvement in metabolic rate in a range of polymers tested for dispersal of growth of A. niger (Elmayergi et al., 1973). One polymer, Carbopol-934 (carboxypolymethylene), gave a 200% increase in respiration rate when used as a supplement. The properties of this polymer change with pH. At pH 4.0 or lower, the polymer backbone is poorly ionised and tightly coiled, whereas if the pH is increased, the backbone unwinds due to ionisation of the carboxyl groups on the polymer, conferring substantial viscosity on the solution (B.F. Goodrich, 1986). Pellet growth is initiated in A. niger when spores agglutinate and so dispersal of growth was attributed to electrostatic repulsion between the negatively charged spores and the ionised carboxyl groups of Carbopol, thus inhibiting spore agglutination and pellet formation. A thin film of polymer surrounding the clusters of hyphae was observed and was suggested to be a reservoir of potassium ions, as the simultaneous disappearance of potassium ions and polymer was observed from the culture medium during the active stage of growth (Elmayergi & Moo-Young, 1973). The increased growth and respiration rate might

be attributed to this polymeric layer where the potassium ions may combine with carboxyl groups and thus aid diffusion through the dense mycelia. In a range of polymers investigated, only anionic polymers were shown to influence the degree of flocculation of spores of A. niger prior to germination (Elmayergi, 1975). Another anionic acrylic resin, Junlon, influenced morphology of A. niger in a similar manner to Carbopol, giving dispersed growth (Trinci, 1983).

The level of spore inoculum in submerged culture is a critical factor in determining morphology of certain genera. A threshold level of spores of approximately 10^5 cells/ml is necessary to ensure filamentous growth of Penicillium chrysogenum with lower concentrations leading to pelleted growth (Camici et al., 1952). A similar observation was reported for Aspergillus oryzae (Calam, 1976). These fungi are thought to develop in this manner because at low conidial concentrations each conidium or cluster develops radially, uninfluenced by other conidia and generally consists of one main hypha, giving filamentous growth. This threshold concentration did not hold true for Aspergillus flavus where cultures with conidial suspensions in excess of 4×10^6 cells/ml grew in a pelleted manner (Testi-Camposano, 1959). If the spores of a certain fungal species have a tendency to aggregate then it would seem logical that pellet formation would occur as a result of this aggregation, regardless of concentration.

The effect of medium pH on growth morphology has been suggested as being in terms of alterations in cell wall or surface chemistry, although no detailed analysis providing evidence has been carried out. The production of shorter, thicker hyphae as the pH was

increased from 6.0 to 7.4 during continuous culture of P. chrysogenum was attributed to changes in cell wall structure involving loss of rigidity and inability to resist osmotic pressure (Pirt & Callow, 1959). The influence of pH on spore-wall chemistry was implicated when growth of A. niger at pH values below 2.3 was filamentous whereas at higher values, spore agglutination occurred leading to pellet formation (Galbraith and Smith, 1969). Surface charge was not considered to be a major factor in spore agglutination as mutual repulsion of spores due to charge would be at a minimum under the conditions in which least agglutination occurred. This observation is confirmed by an electrophoretic study on spores of A. niger which showed that there was no net charge on spores at pH 2.0 and yet no agglutination took place (Seviour & Read, 1985). pH may influence morphology at the control level of enzymic activity involved in wall assembly and hyphal extension.

Morphology can be controlled by physical parameters also such as aeration or agitation level of the medium. Agitation usually limits pellet formation in a medium by break-up or prevention of pellet initiation, as with A. niger (Choudhary & Pirt, 1965). Elmayergi et al. (1973) found that the number of spores per spore agglomerate of A. niger was inversely proportional to the power input of the fermenter. At high agitation rates, shearing forces can damage mycelia, as seen with increased autolysis in hyphae of P. chrysogenum (van Suijdam & Metz, 1981). Physiology and wall composition play a role in determining response to agitation as aseptate species such as Rhizopus spp. and Mucor spp. became pelleted under intense agitation (Dion & Kaushal, 1959). This might be a protective response to high shear rate. Increasing the aeration rate in cultures of Morchella

hortensis causes growth to change from a restricted pelleted form to filamentous, due in part to the elimination of oxygen starvation and allowing recommencement of normal growth (Litchfield et al., 1963).

Suspended particles in solution can affect morphology. Maize solids have facilitated growth of Aspergillus species in a dispersed form (Smiley et al., 1967). Maize supplemented media have also caused R. arrhizus to grow in a dispersed morphology in media which normally promoted pelleted growth (Byrne, 1985). The maize particles may be acting as growth centres for the developing hyphae preventing their clumping together to give pellets.

In many studies on morphology, different fungal genera react differently to a given set of growth conditions, as seen in one study of 150 species using three types of media (Burkholder & Sinnott, 1945). It is not always easy to interpret effects on morphology, particularly those involving media constituents where different results are obtained with different species. An example of this is the substitution of ammonium sulphate for corn steep liquor in growth of P. chrysogenum which changed the morphology from filamentous to pelleted (Pirt & Callow, 1959) whereas increasing the ammonium sulphate concentration of Aureobasidium pullulans media gave filamentous growth (Seviour et al., 1984). These random observations in the literature are merely an indication of the delicate balance that exists between basic physiological functions, as controlled by nutritive factors, and growth morphology.

1.3 PELLET FORMATION

Pellet formation has been classified into the coagulating type and

the non-coagulating type (Takahashi & Yamada, 1960). In coagulating pellet formation, the spores agglutinate in the early stages of germination and as development proceeds, clumps of germinating spores aggregate to form pellets. The development of pellets in growth of A. niger follows the coagulative pattern. At pH 4.5, spore agglutination occurred but as the pH was lowered, the incidence of large clumps decreased until pH 2.1 was reached, where the spores remained single or in small clumps (Galbraith & Smith, 1969). The lower pH is likely to influence the metabolic processes which lead to agglutination. This process of spore agglutination has been inhibited in A. niger cultivation when the anionic polymers Carbopol-934 or Junlon were added to the medium (Elmayergi & Moo-Young 1973; Trinci, 1983; van Suijdam et al., 1980). The inhibition of coagulation is attributed to an electrostatic repulsion induced amongst the negatively charged spores and the carboxyl groups on the polymer backbone.

A similar process of spore agglutination leading to pellet formation was observed in growth of P. chrysogenum (Trinci, 1970). The germinating clusters of spores trap other ungerminated or partially germinated spores in their developing hyphae thus forming the nucleus of a large pellet (Burkholder & Sinnott, 1945; Galbraith & Smith, 1969).

In non-coagulating type pellet formation, one spore gives rise to one pellet. This process is not as commonly observed as the coagulating type. The germinating spore initially develops a filamentous germ tube but as growth proceeds this hypha becomes highly branched and intertwined, leading to pellet formation (Burkholder & Sinnott,

1945). It is likely that a highly branched hypha would react with other hyphae, and so pellet formation from a single hypha is not common.

Many pellet types have been reported in the literature. Testi-Camposano (1959) described three types obtained upon varying the concentration of conidia in the growth medium of A. flavus. These included a "fluffy" type (compact centre with loose boundary), a "compact-smooth type" (compact centre and boundary with smooth periphery) and a "hollow-smooth" type (smooth boundary but hollow centre due to autolysis). The growth of A. niger in citric acid fermentation falls into the "hollow-smooth" category, as it commenced as small pellets but as the fermentation continued, growth at the periphery became dense and autolysis occurred at the centre (Clark, 1962). The extent of autolysis decreased at higher oxygen tensions, indicating the role played by oxygen starvation in the autolysis of the mycelia at the centre of the pellet. Evidence to support peripheral growth only in pellets of A. niger was presented in trials showing uptake of ^{32}P -phosphate by 3 day old pellets only in the outer regions (Yanagita and Kogane, 1963). Nutrient limitation was demonstrated by arthrospore formation in pellets of Pencillium urticae. Arthrospore formation was also noted in cultures of Mucor rouxii under anaerobic conditions (Bartnicki-Garcia and Nickerson, 1962), suggesting that oxygen is the primary limiting nutrient in the P. urticae pellets.

When hyphae are growing in a dispersed form, each mycelium is exposed to the nutrients in the medium and so all the mycelia can contribute to the growth of the fungus. This gives rise to exponential growth.

But when growth is in the compact pelleted form, a cube-root plot defines growth more accurately (Marshall & Alexander, 1960; Pirt, 1966). It is unlikely that once a pellet exceeds a certain diameter, unrestricted growth of the whole pellet could be maintained. Eventually, only a peripheral shell would have sufficient substrate to maintain exponential growth. By approximating to say that the internal portion of the pellet is not growing at all:

$$\frac{dx}{dt} = \mu x_p \quad (1)$$

where x_p is the mass of the peripheral growth zone and μ the specific growth rate, and

$$\begin{aligned} \frac{dr}{dt} &= \mu w \\ r &= \mu w t + r_0 \end{aligned} \quad (2)$$

where r is the radius of the pellet and w is the depth of the peripheral growth zone and is a constant for each growth limiting substrate concentration. The mass of a pellet of density d is given by:

$$x = \frac{4}{3} r^3 \pi d$$

substituting for r in equation (2) gives

$$x^{1/3} = \left(\frac{3}{4\pi d} \right)^{-1/3} \mu w t + x_0^{1/3}$$

$(3/4\pi d)^{-1/3}$ is a constant equal to 0.73 if the pellet density is assumed to be 0.1g dry weight cm^{-3} . Once r is greater than w , the growth of a pellet is predicted to follow a cube-root pattern. When r is smaller than w , exponential growth will occur.

1.4 HYPHAL GROWTH

If the maximum specific growth rate of a fungus is to be determined in batch culture, growth must be in the filamentous form or in very small pellets to allow exponential growth, or a close approximation, to proceed. All of the fungal mass must contribute equally to growth, although this is rarely the case. It is more usual to see exponential growth in batch culture for approximately five doublings but it can be prolonged indefinitely in continuous culture (Righelato, 1979). The specific growth rate, μ , as described by Trinci (1973) is a measure of the exponential rate of growth i.e.

$$x = \frac{\ln 2}{T_d}$$

where T_d is the doubling time. Exponential growth of a series of Neurospora crassa mutants in batch culture continued while growth was filamentous, but after 20h growth the hyphal fragments aggregated and growth entered the stationary phase (Trinci, 1973). During exponential growth of Geotrichum candidum on solid media, it was observed that a unit of hyphal growth which consisted of a hyphal tip with a constant mean length of hypha, was duplicated as exponential growth proceeded (Caldwell & Trinci, 1973). The growth unit was defined as

$$(G) \text{ Hyphal Growth Unit} = \frac{\text{Total hyphal length } (\mu\text{m})}{\text{number of hyphal tips}}$$

At any given time, some hyphae in a mycelium will be extending at the linear rate characteristic of the organism and the conditions, while the remainder will be accelerating towards this rate (Trinci, 1974). Close similarity existed between the growth parameters of undifferentiated mycelia in submerged and solid media during

exponential growth (Steele & Trinci, 1975). The measured value of the hyphal growth unit of mycelia growing in submerged culture and on solid media were similar. The hyphal length specific growth rates determined on solid media were greater by approximately 50% than the biomass specific growth rates obtained in submerged culture, indicating a faster doubling time for hyphal length compared to mycelial biomass. Hyphal diameter was similar in mycelia cultured in both ways. The mean hyphal extension rate (E) was a function of the length of the mycelial hyphal growth unit (G) and the organism's specific growth rate (μ)

$$E = G\mu$$

The ease of measurement of such growth parameters as hyphal growth unit or specific growth rate is affected when working with submerged cultivation of the organism of interest. When using solid media, an individual hypha may be monitored and its progress recorded using time lapse photography as shown by Trinci (1974) in his kinetic study of five different fungal genera. The specific growth rate may be determined by biomass measurements as long as the medium being used lends itself to filtration through fine mesh filter paper. A significant error is introduced into hyphal growth unit measurements when a different sample has to be taken from the culture flask at every sampling point. Even though all of the hyphae may be growing at the same rate there will probably be some differences in the stage of development of the hyphae examined at each sampling point. This could lead to an inaccurate assessment of the length of the hyphal growth unit.

In an exponential culture, the specific rate of branch formation is identical with the specific growth rate of the culture (Caldwell & Trinci, 1973; Trinci, 1974). In their study on branch initiation in Aspergillus nidulans, Katz et al. (1972) found that the average number of branches per unit length of hypha was different for the three different growth rates used and increased proportionately with the growth rate. They concluded that a new branch or tip is formed when the capacity of the mycelium to extend exceeds that of the existing tips. Studies with P. chrysogenum suggest that branching frequency rather than extension rate is the prime variant with growth rate (Morrison & Righelato, 1974). However in a study on a different strain of P. chrysogenum, Metz (1976) found that the hyphal growth unit remained unchanged as the total length of hypha (i.e. specific growth rate) increased. A theory for regulation of branch initiation was proposed by Trinci (1978). He suggested that the cytoplasmic components needed for primary wall extension (such as vesicles, precursors of wall polymers, enzymes for synthesis or lysis of wall polymers during growth or branching) are produced at a constant rate throughout the mycelium. They are transported towards the tips of hyphae and if they happen to fuse with the rigidified wall of the hypha instead of going to the tip, a branch is initiated. The specific rate of production of these cytoplasmic components will be the same as the specific growth rate of the mycelium.

1.5 THE FUNGAL CELL WALL

The fungal cell wall is a complex structure composed mainly of polysaccharides and their chemical complexes with protein. These components are arranged as a fabric of interwoven microfibrils embedded in or cemented by amorphous matrix materials. Their

structural architecture has been compared to such man-made composites as glass fibre reinforced plastic (Rosenberger, 1976). Chitin, cellulose and more rarely chitosan are the microfibrillar substances whereas protein and polysaccharides such as glucans, mannans, galactans or heteropolysaccharides constitute the amorphous component of most types of fungi (Bartnicki-Garcia, 1968).

1.5.1 The Zygomycete Cell Wall

The analysis of cell walls of different taxonomic groups has revealed a close correlation between the chemical composition of the walls and the major taxonomic grouping compiled from morphological criteria (Bartnicki-Garcia, 1968). One of the eight taxonomic groupings described was the chitin-chitosan based wall of the Zygomycetes. Chitin is usually considered as a homopolymer of N-acetyl glucosamine but even in crystalline chitin a number of non-acetylated glucosamine residues may occur. Chitosan is a homopolymer of glucosamine (deacetylated chitin) and so, in the walls of Zygomycetes there may be a continuous range of polymers from chitin to chitosan varying in their degree of acetylation (Wessels, 1986).

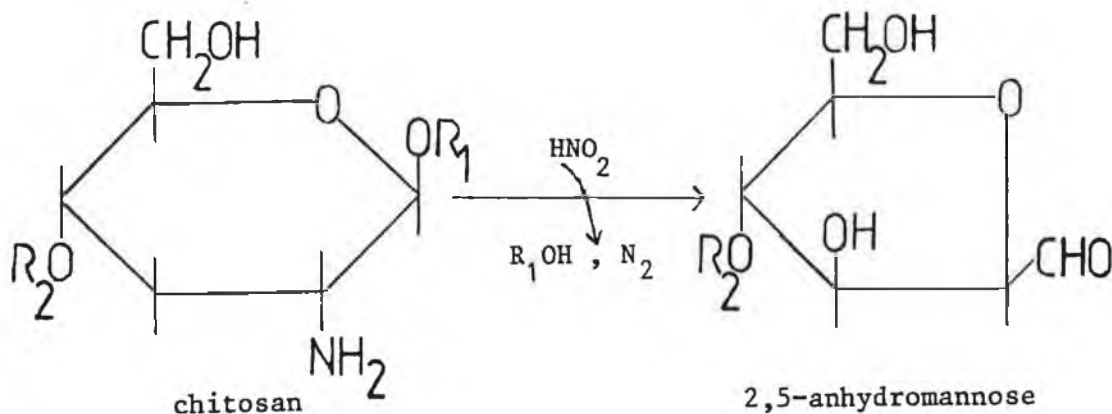
The cationic nature of chitosan or partially deacetylated forms of chitin is thought to be balanced by the presence of anionic polymers such as inorganic polyphosphate and glucuronans in the Zygomycete wall (Bartnicki-Garcia and Reyes, 1968 a, b; Datema et al., 1977 a, b). Fucose, mannose, galactose and glucuronic acid constitute the heteroglucuronan component of the wall (Bartnicki-Garcia & Nickerson, 1962; Bartnicki-Garcia & Reyes, 1968 a, b; Crook & Johnson, 1962; Datema et al., 1977 a, b; Dow et al., 1983). The Zygomycetes are the exception as glucose is absent from their cell walls.

Glucans with β -1,3- and β -1,6- linkages occur in the walls of all other fungi (Bartnicki-Garcia, 1968; Rosenberger, 1976; Wessels & Sietsma, 1981).

1.5.2 Analysis of Hyphal Walls

The different components of the hyphal wall may be analysed initially based on their solubilisation in acid or alkali. As a general rule, the skeletal components tend to be alkali insoluble, whereas the amorphous components are usually soluble in alkali (Wessels, 1986). Separation of the polysaccharide constituents is usually achieved through a fractionation procedure and the resultant hydrolysates may be characterised by methods such as chromatography or specific assays. Consecutive extractions with alkali and acid are usually necessary to reveal the chitin component of the wall, which can be identified using chemical analysis, infra red spectroscopy or X-ray diffraction analysis (Wessels & Sietsma, 1981).

Extraction with nitrous acid is a useful tool when analysing the Zygomycete cell wall as it only affects deamination and depolymerisation if the polymer carries free amino groups (Datema *et al.*, 1977b):



A pure polymer of N-acetylglucosamine would not be affected. In the investigation of cell wall composition of Mucor mucedo, nitrous acid extraction solubilised three fractions from the wall: one which contained N-acetylglucosamine mixed with glucosamine; one which became nitrous acid soluble after treatment with pronase or alkali and a third which consisted of a homopolymer of N-acetylglucosamine (Datema et al., 1977 a). Chitosan has been identified in the walls of other Zygomycetes, such as Phycomyces blakesleeanus (Kreger, 1954) and M. rouxii (Bartnicki-Garcia & Nickerson, 1962; Bartnicki-Garcia & Reyes, 1968a). Unlike chitin, it can be extracted from the wall with dilute acid.

In the alkali soluble fractions of the walls of Zygomycetes, heteropolysaccharides containing fucose, mannose, galactose and glucuronic acid are present (Ballesta & Alexander, 1971; Bartnicki-Garcia & Reyes, 1968a,b; Datema et al., 1977a; Miyazaki and Irino, 1971). The heteroglucuronan from M. rouxii (mucoran) contained fucose, mannose and glucuronic acid in a 2:3:5 ratio (Bartnicki-Garcia & Lindberg, 1972). A homopolymer of glucuronic acid (mucoric acid) was solubilised by alkali after acid extraction. Even though mucoran and mucoric acid were isolated as two distinct polysaccharides, the possibility that they may have been derived from a single polysaccharide has been considered (Bartnicki-Garcia & Reyes, 1968a). This was proven to be the case with the heteroglucuronan of M. mucedo (Datema et al., 1977a). The heteroglucuronan solubilised after treatment with nitrous acid contained fucose, mannose, galactose and glucuronic acid in a molar ration of 5:1:1:6. By treatment with 1M HCl at 100°C, this was partly converted into a crystalline glucuronan, containing only

glucuronic acid, with the properties of mucoric acid. This indicates that in M. mucedo mucoric acid arises by partial acid hydrolysis of the heteroglucuronan and subsequent crystallisation of the homopolymeric segments containing glucuronic acid.

1.5.3 Analysis of Rhizopus Cell Wall

Very few detailed analyses of Rhizopus cell wall have been carried out. A polysaccharide composed of fucose, mannose, galactose and glucuronic acid in a molar ratio of 11:1:3:10 has been identified in the walls of R. nigricans using alkali extraction followed by column chromatography (Miyazaki & Irino, 1971). The alkali-soluble but water-insoluble fragment of the cell wall contained uronic acid, glucosamine and amino acids with small amounts of fucose, mannose and galactose. 53% of the dry weight of the wall was deemed to be chitin, as identified using infrared spectroscopy.

Acid-extracted, dried mycelial preparations of some Rhizopus species contained fucose, mannose, galactose and some glucose in a general study of fungal polysaccharides (Martin & Adams, 1956). The discovery of glucose is questionable in view of the lack of glucan-type polymers in Zygomycete walls, and may have arisen as a contaminant from the medium.

The structure of the Rhizopus cell wall has been investigated using lytic enzymes (Tominaga and Tsujisaka, 1981). When the cell wall was digested with chitinase, chitosanase and protease individually and in combination, the degree of lysis observed using turbidity measurements was 40%, 60%, 30% and 90%, respectively. Examination of the treated walls by scanning electron microscopy showed that

protease caused partial surface deterioration but that chitosanase incubation resulted in exposure of the interwoven microfibrillar layer. The cell wall appeared to collapse upon incubation with protease and chitosanase. The fraction remaining insoluble after protease and chitosanase incubation was composed of N-acetylglucosamine, as determined by chromatography of the acid hydrolysate, and also incubation of the fraction with chitinase. When the sugar content of each of the enzyme-treated solubilised fractions was analysed, 60-70% of the total hexosamine was present in the chitosanase or the chitosanase and protease-treated solutions. Even in the protease treated fraction, 16% of the total hexosamine was found. These results were thought to imply the existence of an indistinct layer structure in the Rhizopus cell wall with chitin, chitosan and protein each being exposed to lytic enzyme action on the wall surface. The authors proposed that the cell wall of R. delemar consists of chitin fibers which are cemented by chitosan and protein or peptides scattered in the cell wall in a mosaic manner.

The regeneration of the cell wall of protoplasts of R. nigricans has been studied (Gabriel, 1968, 1984). A structurally incomplete but firm wall is synthesised when protoplasts are incubated in the presence of snail gastric juice (Gabriel, 1968). A fibrillar net on the surface of the protoplast is formed concomitantly with a decrease in snail enzyme activity. This net is not destroyed by either prolonged incubation with undiluted snail juice or by boiling with 2% HCl, thus excluding the possibility that chitosan is the basic unit, but suggesting that it is most probably chitin (Gabriel, 1984).

1.5.4 Architecture of the Hyphal Wall

Electron microscopic observations combined with the use of specific enzymic or chemical extractions have been used to reveal the molecular architecture of the hyphal wall (Bartnicki-Garcia, 1973; Hunsley & Burnett, 1970; Mahadevan & Tatum, 1967; Manocha & Colvin, 1967; Tominaga & Tsujisaka, 1981; van der Valk & Wessels, 1976; Vermeulen & Wessels, 1984; Wessels et al., 1972). Based on their analysis of a series of hyphal walls using sequential treatments with enzymes such as pronase, laminarinase, cellulase and chitinase, along with chemical treatments, Hunsley & Burnett (1970) have modelled the vegetative hyphal wall as a coaxially layered structure. In N. crassa these layers are proposed as being an outermost layer of mixed α - and β -glucans, merging into a glycoprotein reticulum which becomes increasingly rich in protein which in turn merges into a distinct layer of protein, and finally, an innermost chitinous region in which chitin microfibrils are embedded in proteinaceous material. Wessels and Sietsma (1981) have suggested that a model, wherein the wall components are more closely associated with each other and form essentially one layer with some components accumulating at the outside, might be a more realistic description.

Extensive studies have been carried out on the cell wall of Schizophyllum commune supporting such a structural arrangement of the wall components (Sietsma & Wessels, 1977; van der Valk et al., 1977; Wessels et al., 1972). A water-soluble gel-like β -1,3/ β -1,6- glucan and an alkali soluble α -1,3- glucan (S-glucan) accumulate at the outside of a layer which contains the alkali insoluble chitin - glucan complex. The branched glucans are thought to be attached to chitin chains through their reducing ends via amino acids. Sietsma

and Wessels (1979) suggest that these substituted chitin chains are hydrogen bonded to microfibrillar chitin. Evidence to support the existence of a linkage between β -glucan chains and chitin became known when it was observed that the glucan chains became soluble in water or alkali after specific depolymerisation of (acetyl) glucosamine-containing polymers. Little is known about the location of polymers in the Zygomycete cell wall. It is postulated by Wessels (1986) that the chitin microfibrils coated with partially deacetylated chitin chains (chitosan) are held in a rigid structure by their ionic interactions with the anionic polymers (glucuronans and polyphosphates).

1.5.6 Polymer Assembly and Hyphal Growth

Vegetative hyphae increase in length as a result of tip extension. The tapered region of the tip involved in this growth is called the extension zone. Secondary wall growth occurs distal to the extension zone and thus the wall increases in thickness with distance from the tip, eventually forming the mature, fully grown wall of the hypha (Gooday & Trinci, 1980; Trinci & Collinge, 1975). The newly-synthesised wall at the apex is thin and smooth and composed of either chitin or cellulose microfibrils whereas, in older portions, the primary wall is covered with secondary layers composed of amorphous matrix material (Farkas, 1979; Trinci, 1978). Primary wall growth always involves the fusion of vesicles with the tip and, in hyphae that are growing linearly, vesicles are present at the apex to the exclusion of most other types of organelles (Collinge & Trinci, 1974; Gooday, 1971; Syrop, 1973). These vesicles, formed from endomembrane systems, are assumed to carry wall precursors and or/enzymes required for the insertion of these precursors into the

existing wall (Bartnicki-Garcia, 1973).

The wall at the hyphal apex has been regarded as a rigid entity where growth is possible only by the maintenance of a delicate balance between lysis and synthesis of wall components (Bartnicki-Garcia, 1973). The model for wall growth proposes that:-

1. High turgor pressure forces broken microfibrils away from each other before synthesising enzymes can insert new microfibrils or extend the broken ones;
2. Amorphous wall material is delivered to the wall in vesicles and forced in between the fibrillar network by turgor pressure;
3. The individual polymers or hydrolytic enzymes are excreted at the apex where the vesicles that package these components fuse with the plasma membrane.

Hyphal extension is said to be dependent on the rate of supply of vesicles to the wall (Trinci & Collinge, 1975). An alternative wall growth model is proposed to be a 2-stage process, without the involvement of autolysins (Wessels & Sietsma, 1981). These steps are:-

1. Deposition of the individual polymers outside the plasma membrane (either by synthesis on the plasma membrane or by extrusion through vesicles that fuse with the plasma membrane).
2. Extracellular modification of the individual polymers, such as partial crystallisation or mutual interactions, including β -glucan cross linking between chitin microfibrils, thus changing the mechanical properties of the wall from a viscoelastic fluid to a rigid composite.

Rigidification of the wall was alternatively suggested to occur by the interlacing and aggregation of microfibrils with a decrease in elasticity during maturation due to the increase in the diameter, number and packaging of microfibrils in the wall (Burnett, 1979).

Autoradiographic studies have shown that growing hyphae incorporate ³H-N-acetylglucosamine almost exclusively at the tip (Katz & Rosenberger, 1971). This polarity of synthesis is lost when extension growth is halted by cycloheximide (which inhibits protein synthesis) or by an osmotic shock. The authors suggest that the supply of primers or substrates necessary for synthesis to the tip may have been disorganised due to the treatments. Chitin synthesis proceeds by the transfer of N-acetylglucosamine from UDP-N-acetylglucosamine into chitin, catalysed by chitin synthase (Gooday & Trinci, 1980). Chitin synthase is found in a zymogenic form in microvesicles called chitosomes (Bartnicki-Garcia et al., 1978). By proteolytic digestion, chitosomes are activated in vitro and then synthesise crystalline chitin from UDP-N-acetylglucosamine, apparently one microfibril per chitosome. Thus the chitosomes are the transport vesicles for the inactive enzyme to the plasma membrane.

Araki & Ito (1975) proposed that chitosan (β -1,4- polymer of nonacetylated glucosamine) is made from chitin by enzymic deacetylation but their evidence was inconclusive since the deacetylase they isolated from M. rouxii was not effective against native chitin, but only against glycol chitin, so chitosan synthesis in vitro was not achieved. Davis and Bartnicki-Garcia (1984a,b) have

developed a model system for chitosan in vivo synthesis based on the tandem action of chitin synthase and chitin deacetylase. They found that chitin deacetylase from M. rouxii can deacetylate chitin efficiently if it is allowed to act on chitin chains as they are being formed, i.e. nascent chitin. Neither enzyme alone catalysed significant chitosan formation. Addition of chitinase to the chitosan synthesising mixtures reduced chitosan formation, whereas the enzyme was not active against biosynthesised chitosan. They suggested that deacetylation occurred after a chitin chain is formed, i.e. that chitin is a precursor of chitosan.

Calvo-Mendez & Ruiz-Herrera (1987) have reported that a membrane-bound deacetylase is likely to be regulating the proportion of deacetylated product being synthesised in M. rouxii. The two different polymers, one containing only acetylated glucosamine moieties, i.e. chitin, and another containing a high proportion of deacetylated units (chitosan) are possibly formed in vitro and possibly in vivo. It is hypothesised that the membrane bound deacetylase initiates the deacetylation of a proportion of nascent chitin chains which may then be recognised by an extracellular soluble deacetylase responsible for their further deacetylation.

1.5.7 Cell Wall Composition and Mycelial Morphology

Cell wall differentiation has been equated with morphological differentiation (Bartnicki-Garcia, 1968, 1973; Farkas, 1979; Wessels, 1986). However, the relationship between structure and function in the fungal cell wall is not yet clear.

The composition of the cell walls of filamentous and yeast-like forms of the dimorphic fungus M. rouxii has been investigated (Bartnicki-García & Nickerson, 1962). Chitin, chitosan and phosphate were found in similar amounts in both morphological types. Major quantitative differences were found in the protein, purine-pyrimidine and particularly the mannose content of the walls, all of which were higher in the yeast type walls. Mannose was 5.6 times more abundant in walls of yeast like cells, and thus was indicative of a morphogenic role.

The relationship between cell wall composition of the wild type and colonial mutant of N. crassa has been investigated (de Terra & Tatum, 1961, 1963; Mahadevan & Tatum, 1965). Colonial growth, characterised by highly branched, dense colonies, is induced by the addition of L-sorbose to the growth medium. A large increase in the ratio of glucosamine: glucose was observed in the wall of the mutant compared to the wild type. Colonial growth was also induced by addition of snail digestive juice, an agent known to alter the structure of the cell wall by digesting at least one of the major components (glucan). It was suggested that colonial growth may result from a weakening of the cell wall (de Terra & Tatum, 1961). L-Sorbose had little effect on the specific growth rate of N. crassa in batch culture, but caused a dramatic decrease in the colony radial growth rate in solid medium, due to the reduction in the peripheral growth zone width (Trinci & Collinge, 1973). L-Sorbose may upset the balance between synthesis and cleavage of glucan polymers resulting in wall softening, as it inhibits the activity of β -1,3- glucan synthetase (Mishra & Tatum, 1972.) Bisaria et al. (1986) observed the release of β -glucosidase from the walls of Trichoderma reesei treated with L-sorbose, and a

similar increase in the glucosamine:glucose ratio of the wall, as for the colonial mutant of N. crassa. A temperature sensitive mutant of A. nidulans, which forms swollen hyphae, has been shown to have chitin-deficient walls (Katz & Rosenberger, 1970).

Omission of manganese from the growth medium of A. niger resulted in the formation of squat, bulbous hyphae, whose cell walls had increased chitin and reduced β -glucan content, along with reduction in galactose-containing polymers (Kisser et al., 1980). Addition of copper or cycloheximide had a similar effect on morphology and cell wall composition. The effect on wall composition was considered to be due to inhibition of protein synthesis, leading to inhibition of glycoprotein turnover with loss of hyphal polarity and concomitant increased branching and chitin synthesis. There has been little evidence to support the presence of Mn^{2+} -dependent wall biosynthetic enzymes in A. niger, and reduced β -glucan was probably due to high turnover rates of the various synthases. Increased metabolic flux in the direction of chitin synthesis and higher precursor concentrations might account for increased chitin concentration. Cycloheximide caused a loss in hyphal polarity with increased chitin incorporation and branching along the length of the hypha in A. nidulans (Katz & Rosenberger, 1971). These alterations are initiated at the metabolic level but their net effect is apparent in the cell wall composition and ultimately in the mycelial morphology.

1.6 MYCELIAL MORPHOLOGY, GROWTH AND PRODUCT FORMATION

The growth kinetics of mycelia in submerged culture are influenced by morphology in two ways; by determining the rheology of the culture

and through diffusion limitations of substrate assimilation within the mycelial body (Righelato, 1979). Cultures containing approximately spherical microbial bodies such as pelleted mycelia, exhibit low viscosities and more or less Newtonian flow behaviour (viscosity unaffected by shear rate). Mycelia growing in long filaments cause high viscosities and their cultures exhibit pseudoplastic flow behaviour (viscosity varies with shear rate). A growing suspension may thus be heterogeneous with respect to the mass transfer of substrates and products. The diffusion limitations which accompany pelleted growth, generally leading to autolysis at the centre of the pellet are well documented (Clark, 1962; Pirt, 1966; Yanagita & Kogane, 1963). Filamentous cultures can quickly become oxygen limited also, due to the high viscosity and low diffusion rates (Banks, 1977).

The production of citric acid by A. niger is one of the few processes where pelleted growth is promoted rather than filamentous, for optimum yields (Lockwood, 1975). A dense, compact round, pelleted morphology gave optimum yields of citric acid, whereas soft, filamentous pellets formed when the medium was lacking in ferrocyanide and produced little citric acid (Clark, 1962). These alterations in yield and morphology are due to changes in the trace metal concentration, particularly manganese (Section 1.2), (Kubicek & Rohr, 1977). The effect of inoculum type and agitation rate on citric acid yield from A. niger were investigated (Gomez et al., 1988). The highest yield was obtained from media which had been inoculated with a preculture of pellets and grown at 1000 rpm, giving rise to small, compact, smooth pellets. The fermentation advantages of such a system were low culture viscosity, improved mixing and

aeration conditions and lower oxygen consumption than in cultures composed of filamentous mycelia. In contrast, improved citric acid yields were obtained with filamentous mycelia in media supplemented with non-ionic surface-active agents (Takahashi et al., 1965).

Penicillin has traditionally been produced in stirred fermenters by mycelia growing in a filamentous or pulp-like mode (Metz & Kossen, 1977). When P. chrysogenum was grown in a variety of morphological forms ranging from dense pellets to filaments (obtained by varying spore inoculum concentration), the penicillin yield increased from 500 units/ml in the pellet form to 5000 units/ml in filamentous cultures (Calam, 1976). In an attempt to reduce the operating cost of stirred tank fermenters for penicillin production, where a very high specific power input is necessary due to the highly viscous growth obtained, an investigation was carried out using tower loop reactors with mycelia growing in the pelleted form (Konig et al., 1982). Morphology was maintained as small pellets by employing definite impeller speeds in the subculture along with definite inoculum amounts and substrate concentrations in the main culture. The specific penicillin yield was higher, under these conditions, with regard to power input, biomass, and consumed substrate than in the stirred tank reactors with a viscous, filamentous morphology.

The production of exocellular polysaccharide by Aureobasidium pullulans was seen to vary as the growth morphology in batch culture underwent a series of changes, from pelleted to dispersed.

Increasing the ammonium sulphate concentrations caused the mycelia to grow in a filamentous form with a significant reduction in polysaccharide production (Seviour et al., 1984).

The variations in product formation that accompany alterations in mycelial morphology are very often the final result of changes that were initiated at the metabolic level of activity. Thus the morphological growth form of mycelia in submerged culture may have a profound affect on product formation, but the changes are indirectly related to the morphology and primarily a result of altered metabolism.

1.6.1 Production of Fumaric Acid by R. arrhizus

R. arrhizus has been qualitatively shown to produce a range of organic acids, as identified using thin layer chromatography (Thompson & Rountree, 1982). Fumaric acid was once made commercially by R. oryzae but has now been replaced on the market by fumaric acid made by the catalytic oxidation of benzene (Lockwood, 1975). Traditionally the organic acid is produced in a well-aerated and agitated fermentation of glucose or invert sugar solutions, in the presence of suspended CaCO_3 . The insoluble calcium fumarate formed is redissolved by heating, allowing removal of the fermentation solids by filtration. Upon cooling, the calcium fumarate recrystallises and the free acid is recovered by acidulation using an excess of sulphuric acid.

Rhodes et al. (1959, 1962) have studied the production of fumaric acid by R. arrhizus in batch culture and 20-litre fermenters. CaCO_3 was used to neutralise the acid as it was produced and the fermentation media heated to effect its solubilisation, as described above. The organic acids produced (some malic and succinic detected also) were separated on an anionic exchange resin and the acid measured by titration with NaOH. The fermentation was run at pH 6.0

and 33°C in a defined medium, where growth of the mycelia was finely dispersed. Yields of 67% of the sugar supplied (glucose) were obtained, with lower yields if sodium or potassium hydroxide were used to neutralise the fumarate instead of CaCO₃. If a mould inoculum of heavy vegetative growth was used, pelleted growth was obtained along with a slower fermentation. In an earlier study by Foster & Waksman (1939), zinc was seen to have a profound effect on growth and fumaric acid production by Rhizopus. In the absence of added zinc, yields of over 50% conversion of glucose to fumaric acid were obtained with relatively slow and poor growth. When 12 ppm zinc was added to the medium the glucose was utilised three times more efficiently with much higher biomass yields and lower fumaric acid production. In contrast to zinc, added iron tended to depress growth but the fumaric acid production was not affected. The function of zinc in the fermentation was interpreted as catalyzing a more complete oxidation of the glucose with a consequential increase in efficiency of energy utilisation by the fungus.

Tween surfactants and vegetable oils have been shown to increase the rate of fumaric acid accumulation in R. arrhizus (Goldberg and Stieglitz, 1985). Addition of 0.5-1.0 mg/l of fatty acid esters (Tweens or vegetable oils) to a medium with excess CaCO₃ increased the rate of fumaric acid production (g/l/hr) by 20%. The authors suggested that the fatty acids exerted their effect by being incorporated into the fungal membrane and altering membrane permeability, thereby resulting in a more rapid excretion of fumaric acid.

The very high molar yields (moles of fumarate per mole of glucose utilised) of 144%, obtained by Rhodes et al. (1962), have prompted investigations into the biochemical aspects of the accumulation of the acid in R. arrhizus fermentation. In earlier studies using ^{14}C -ethanol, Foster et al. (1949) had suggested that fumarate synthesis involved a C_2 plus C_2 condensation (i.e. condensation of 2 moles of ethanol). Because the yield of fumarate from ethanol was so high, the TCA cycle was excluded as being the sole mechanism in the accumulation of the acid. With the subsequent discovery of the glyoxylate bypass, Kornberg (1959) pointed out that Foster's results were consistent with this pathway and suggested that fumarate accumulation took place via this route. But the discovery that the key enzyme in the glyoxylate pathway, isocitrate lyase, was strongly repressed in high-glucose media, which is necessary for fumarate production, ruled out any significant role being played by this pathway in fumaric acid production (Romano et al., 1967). An alternative mechanism involving a C_3 (pyruvate) plus C_1 carbon dioxide fixation was suggested, where pyruvate carboxylase in conjunction with reactions of the TCA cycle led to fumarate accumulation. A study on the subcellular localisation and regulatory properties of pyruvate carboxylase in R. arrhizus has indicated that fumaric acid accumulation can occur by a completely cytosolic pathway. This involves the conversion of pyruvate to fumarate by the combined activities of pyruvate carboxylase, malate dehydrogenase (decarboxylating) and fumarase (Osmani and Scrutton, 1985). The high molar yield of fumarate (greater than 100%) obtained with R. arrhizus can be explained in terms of the carboxylation of pyruvate and the operation of the reductive reactions of the TCA cycle (Kenealy et al., 1986).

1.6.2 Production of Glucoamylase by R. arrhizus

Glucoamylase (E.C. 3.2.1.3. 1,4- α -D-glucan glucohydrolase) is an exo-acting enzyme that cleaves β -D-glucose from the non reducing ends of amylose, amylopectin and glycogen by hydrolysing α -1,4 linkages in a consecutive manner. It also hydrolyses α -1,6 and α -1,3 linkages, although at a much slower rate than α -1,4 linkages (Fogarty, 1983). The pH optima of glucoamylases are generally in the range 4.5 - 5.0, with temperature optima between 40 - 60°C. All fungal glucoamylases are glycoproteins with 5-20% carbohydrate. Glucose, glucosamine, mannose and galactose are the usual sugars found in these glycoproteins. Methionine, tryptophan and cysteine are the amino acid which are common to most glucoamylases. The glucoamylase produced by Endomycopsis bispora has been shown to be subject to catabolite repression by starch, maltose, glucose and glycerol (Wang et al., 1979).

A number of fungi are known to produce glucoamylase with strains of Aspergillus in the forefront. The annual commercial production is about 300 tonnes of pure enzyme and very high yields are essential if the process is to be viable (Kristiansen & Bu'lock, 1980). A. niger has been genetically manipulated to produce up to 20g/l enzyme from a single glucoamylase gene copy (Ward, 1989). Glucoamylase is primarily used in hydrolysis of starch replacing the acid hydrolysis procedure which had previously been used in the starch industry. It is used in conjunction with α -amylase to produce 'high conversion' syrups in the confectionary industry and also in the brewing industry to degrade dextrans.

In the Western world, the preferred mode of cultivation for fungal enzyme production is submerged using stirred tank fermenters. Surface cultivation is still popular in the East (Blain, 1975). A. niger, A. oryzae, Aspergillus awamori or Aspergillus foetidus are all used for glucoamylase production in fermenters, using maize as the raw material (Kristiansen & Bu'lock, 1980). A Japanese surface culture process with Rhizopus has been developed for commercial production (Underkofler, 1969).

Glucoamylase has been reported to be produced by R. delemar, Rhizopus javanicus and Rhizopus niveus (Fogarty, 1983). There are many more reports in the literature concerning the characterisation of glucoamylase produced by Rhizopus compared to those describing the production protocol. The glucoamylase from A. niger and R. delemar have been investigated, and observed to contain about 13% carbohydrate with molecular weights of about 100,000 (Pazur & Okada, 1967). When the activities of three glucoamylase enzymes from Aspergillus, Rhizopus and Endomyces species were compared, the pH optima were 4.0, 4.8 and 4.8 respectively and the temperature optima were 60, 55 and 55°C respectively. Thus, the Aspergillus enzyme is more favourable for commercial purposes, where the lower pH and higher temperature help prevent microbial contamination during long incubation periods (Underkofler et al., 1965).

The glucoamylase from Rhizopus sp. was observed to hydrolyse soluble waxy corn starch completely. When the enzyme was fractionated using DEAE-cellulose chromatography, glucoamylase I exhibited strong debranching properties and actively degraded raw starch, whereas glucoamylase II was weak both in debranching activity and in

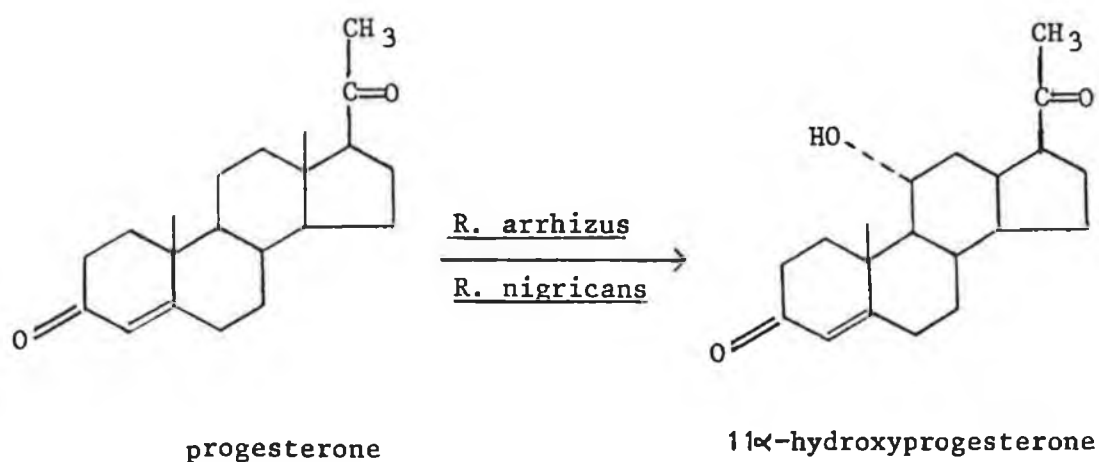
hydrolysing raw starch (Ueda & Kano, 1975).

Complex, semi-solid media components appear to be most efficient at inducing glucoamylase activity. In a range of fifteen media investigated for production of glucoamylase by A. awamori, optimum yields were obtained from a medium consisting of 20% whole ground corn (Attia & Ali, 1973). Production of glucoamylase was observed in media containing 1% rice bran in liquid culture by Rhizopus spp. whereas no production was seen in media containing 10% raw cassava starch or 2% glucose (Nishise et al., 1988). The productivity in the basal liquid medium was improved 6-fold by pretreating the rice bran with n-hexane and inclusion of a gauze support in the liquid medium. Comparable enzyme yields (on a units/g solids basis) were obtained in the turpentine-supplemented solid media and the hexane-treated liquid media with gauze support. The gauze acted as a support for the growing mycelia yielding good growth with only slight pellet formation. The hexane treatment may have extracted substances such as fatty acids, which were shown to inhibit glucoamylase production. Thus complex carbohydrates, such as maize or bran, give better glucoamylase production, with semi solid liquid media aiding growth of the fungus and hence leading to higher enzyme activities.

1.6.3 Biotransformation of Progesterone to 11 α -Hydroxyprogesterone by R. arrhizus

The anti-inflammatory activity of corticosteroids depends on the oxygen function in C-11 of the steroid structure. The numerous steroid compounds which occur in nature do not possess this function and the chemical process of shifting the oxygen function from C-12 to C-11 alone involves 9 steps, thus making cortisone synthesis very

expensive and very complex (Sedlacek, 1988). But research carried out at the Upjohn Company in the 1950's revolutionised the process with the discovery that selected microorganisms could carry out the 11- α -hydroxylation of progesterone in a one-step process (Peterson et al., 1952). The 11 α -hydroxyprogesterone structure can be easily changed into cortisone and hydrocortisone, thereby significantly reducing the cost of manufacture of corticosteroids and increasing their common application.



R. arrhizus was used initially in the biotransformation, but R. nigricans later proved to be more efficient. The biotransformation protocol involved a 24 h growth period followed by a 24 h transformation period. This simple system gave conversion yields of 80-90%, with up to 2% each of a dihydroxyprogesterone and a 11 α -hydroxyallopregnane-3,20-dione as side products. Aspergillus ochraceus was identified as being very efficient at carrying out the biotransformation also (Weaver et al., 1960). A concentration of

5g/l progesterone was found to give optimum yields of 11 α -hydroxyprogesterone (>90%) with minimum yield of side products using A. ochraceus (Somal & Chopra, 1985).

Enzymes involved in steroid biotransformation have been shown to be inducible (Sedlaczek, 1988). If hydroxylation using R. nigricans is monitored in the presence of cycloheximide, mycelia which have been pretreated with progesterone show some hydroxylation activity, whereas those which have not been pretreated do not hydroxylate progesterone (Breskvar & Hudnik-Plevnik, 1978). The hydroxylase of R. nigricans was unstable, as harvesting and washing of mycelia on removal from a fermenter resulted in a loss of up to 90% of the activity observed in the fermentation (Hanisch *et al.*, 1980). Maximum induction occurred in flasks with 0.5 g/l progesterone added. Oxygen was also seen to have a significant effect on the amount of hydroxylase synthesised, with a maximum obtained at a dissolved oxygen tension (DOT) of 10% of air saturation. Cytochrome P-450 has been implicated in the hydroxylase system, and this low DOT optima is a property of cytochrome systems (Breskvar & Hudnik-Plevnik, 1977). Carbon monoxide was shown to inhibit the hydroxylation, with reversal of inhibition by irradiation with light at 450 nm, an inhibition specific for cytochrome P-450 mediated reactions. The requirement for the reducing power of NADPH in the cytochrome P-450 system is satisfied by the addition of glucose to the biotransformation medium. This allows generation of NADPH during sugar metabolism (Kolot, 1982). Periodate has been used successfully to regenerate the 11- α -hydroxylase of R. nigricans and can thus substitute for NADPH by directly oxidising the terminal oxidase of the electron transport chain (Hanisch & Dunnill, 1980).

The use of organic solvents to increase stability of cells and reactants in bi-phasic systems of progesterone biotransformation using A. ochraceus has been investigated (Ceen et al., 1987). While some organic solvents show high solubility for steroids, the hydroxylating ability of A. ochraceus is destroyed within ten minutes of contact, believed to be due to a detrimental effect on cellular lipids. Natural oils, mainly oleic acid, maintained hydroxylase activity for longer periods, but separation and assay of steroids in oils proved difficult. If the development of a biphasic organic/aqueous system is to be successful, a partially polar organic solvent with limited interaction with cellular lipids must be utilised.

R. nigricans has been immobilised in gels composed of polyacrylamide, alginate and agar respectively, with maintenance of hydroxylase activity in the latter two types of gel (Maddox et al., 1981). Polyacrylamide was thought to have a toxic effect on the cells as the cells immobilised in the other two matrices retained many metabolic functions, with hyphal growth occurring within the gels. Glucose was an essential component of the incubation solution, indicating the absolute requirement for NADPH in the hydroxylase system. Activity rates were limited by the solubility and diffusion of the steroid within the gels.

Thus the mechanism by which R. nigricans or R. arrhizus hydroxylate progesterone has been well documented, but little attention has been given to the mycelial cells themselves and how the biotransformation is influenced by their metabolic/morphological state.

1.7 THIS THESIS

This thesis describes the investigation of the relationship between mycelial morphology, cell wall composition and product formation in R. arrhizus. The effect of controlling morphology on parameters of growth such as biomass production, specific growth rate or length of hyphal growth unit is assessed. The possibility that the different mycelial morphologies, as controlled by anionic polymers, are accompanied by changes in cell wall composition or physical properties is analysed. Finally, the ultimate effect of these morphological variations on product formation is described.

2. MATERIALS AND METHODS

2.1 CHEMICALS

Chemicals used in assays and other biochemical analyses were of Analar or Analytical grade. Those used for laboratory cultures were of General Purpose Reagent grade and supplied by British Drug House, England or Toronto, Canada. Biochemicals were obtained from Sigma Ltd., London, England or St. Louis, Mo., U.S.A., or Aldrich Ltd., Gillingham, England or Milwaukee, Wn, U.S.A. Soya peptone and glucose (food grade) were obtained from Biocon Ltd., Ireland. Carbopol-934 was supplied by B.F. Goodrich Ltd., U.S.A. HPLC grade acetonitrile was obtained from Caledon Ltd., Canada. The glucose GOD-PAP kit was obtained from Boehringer Mannheim GmbH, Germany. Water used for media preparation and assays was distilled and deionised. Milli-Q grade water was used for HPLC solvents and sample preparations.

2.2 FUNGAL CULTURE

2.2.1 Source of Strain

The culture of Rhizopus arrhizus 2582 was obtained from the Northern Regional Research Laboratory, U.S.A. All other cultures used in the preliminary investigations were obtained from the same source or from the American Type Culture Collection, U.S.A. (A.T.C.C.), as stated in the text.

2.2.2 Culture Maintenance

Stock cultures were maintained on yeast-malt extract agar slopes, subcultured monthly and stored at 4°C. Cultures were incubated at 30°C and sporulated after 3-4 days. Yeast-malt extract agar contained: yeast extract, 4g/l; malt extract, 10g/l; glucose,

4g/l; Technical Agar, 20g/l; pH 7.0.

2.2.3 Detection of Contamination

Bacterial contamination of stock cultures, spore suspensions or fermentation samples was detected by plating samples onto Nutrient Agar containing 0.1ml/l cycloheximide and incubating overnight at 30°C.

2.3 SPORE INOCULUM PREPARATION

R. arrhizus 2582 and other cultures were cultured on yeast-malt extract agar, 20 ml in a 250 ml conical flask, for 4 days at 30°C. Spores were washed from the culture with 20 ml of Triton - X 100 (0.01% v/v) with 1 mm glass beads under sterile conditions. This produced a black spore suspension, free from mycelia. Spores were counted using a phase-contrast microscope and a Hawksley counting chamber. The suspension was adjusted to give $1-5 \times 10^7$ spores/ml. Suspensions were unaffected by storage at 4°C for up to one month.

2.4 SHAKE-FLASK CULTIVATION

Media were dispersed in 100 ml aliquots in 250 ml conical flasks unless otherwise stated. Incubation was conducted at 30°C on an L.H. Engineering 2-tier orbital shaker (model MK 11/111) at 150 rpm with a displacement of 50 mm or on a Labline model 3590 shaker table at 150 rpm.

2.5 BIOMASS DETERMINATION USING DRY WEIGHT ANALYSIS

Filter papers (Whatman No 1, 9 cm dia.) were dried at 105°C to constant weight in glass petri dishes. After cooling under vacuum,

the papers were weighed to 4 decimal places. Biomass was harvested from flask cultures onto the filter papers on a Buchner funnel if the media components were soluble and onto a fine mesh nylon cloth when the medium was highly viscous or contained partially soluble components. The mycelia were washed using three culture volumes of distilled water. When using nylon cloth, the washed mycelia were quantitatively transferred to the dried and weighed filter paper. The filters were then dried at 105°C to a constant weight, cooled and weighed as before. Dry weight was determined by difference. Controls consisted of water or media, but generally gave negligible yields. Good reproducibility was obtained among duplicates.

2.6 SURFACE CULTURE CULTIVATION

All surface culture trials were carried out in 250 ml conical flasks. Solid media components were added to the flasks and sterilised for 20 minutes at 15 p.s.i. and 121°C. A specified volume of sterilised water was then dispersed aseptically over the surface of the media components, followed by 1 ml spore inoculum (containing $1-5 \times 10^7$ spores). Flasks were incubated in a stationary position at 30°C for the period indicated in the text. After the required growth period, 100 ml of 1% (w/v) NaCl solution was added to the flask, the contents dispersed and incubated at 150 rpm at 30°C for 1 h. The solid components were then removed by filtration through Whatman No 1 (9 cm diameter) filter paper and the extract used for assay.

2.7 FERMENTER CULTIVATION

Fermenter trials were carried out in 20 l in situ sterilisable laboratory fermenters (C.F. series, Chemap, Switzerland), with a final operating volume of 16 l. Media were sterilised in situ with steam and maintained at 15 p.s.i. and 121°C for 30 min. Fermenters were inoculated with a 10% (v/v) 12 h culture prepared in the same medium in shake flasks. Fermenter temperature was controlled at 30°C. Fermenters containing non viscous media were agitated at 400 rpm with an aeration rate of 0.3 l/l/min. These conditions maintained dissolved O₂ concentration at greater than 70% saturation. Viscous media required greater agitation/aeration rates, as stated in the results section. pH was maintained at 4.0 or 5.5 by automatic addition of 4M HCl or NaOH. A mechanical foam breaking device (Fundaf foam, Chemap, Switzerland) was used for fermenter trials containing the polymers, as these media supplements caused substantial foaming.

2.8 MYCELIAL TREATMENT PROCEDURES

2.8.1 Radiolabelling of Cell Walls

N-[¹⁴C-acetyl]-D-glucosamine (DuPont Canada Inc.), labelled in the methyl group, was used for radiolabelling of cell walls. Mycelia were grown in 400 ml volumes of media, to which 2.5 µCi were added after 12 h and 24 h growth under standard conditions. Growth proceeded for a further 1 h to allow incorporation of the label into the cell wall. Mycelia were then harvested, washed and cell walls isolated as described in Section 2.14.

2.8.2 Ruthenium Red Staining of Mycelia

Samples of mycelium were taken from shake flask cultures after 24 h

growth and washed with sodium acetate buffer (Appendix I). A 0.3% (w/v) solution of Ruthenium Red was diluted 1:1 with acetate buffer. The washed mycelium was suspended in approximately 1 ml of the diluted Ruthenium Red solution and left for 5 min. The mycelium was then sampled, wet-mounted and examined using light microscopy.

2.9 PRODUCT RECOVERY

2.9.1 Recovery of Glucoamylase or Fumaric Acid

Mycelia were separated from supernatant by filtration through Whatman No 1 (9cm) filter papers, or through nylon cloth when polymer-supplemented media were used. The supernatant was diluted appropriately and used directly for estimation of glucoamylase (by DNS method) or fumaric acid content (by HPLC). Supernatants containing insoluble Carbopol residues were centrifuged for 10 min, 1000xg to remove the polymer particles before assaying for the product in the particle-free supernatant.

2.9.2 Recovery of Progesterone and 11 α -Hydroxyprogesterone

After the 24 h biotransformation period, the whole broth (50 ml) was extracted using 2 x 50 ml dichloromethane. The residual extracted mycelia were then broken using a bead beater (Biospec Products, OK., U.S.A.) for 3 x 1 min cycles, with temperature maintained at 4°C, and then extracted using 2 x 40 ml dichloromethane. Combined whole broth extracts and the combined broken mycelial extracts were each concentrated using rotary evaporation by a Buchi Rotavapor R110 model and allowed to dry over P₂O₅. Dried extracts were resuspended in 10 ml acetonitrile and assayed using HPLC.

2.10 BIOTRANSFORMATION PROTOCOL

2.10.1 Biotransformation of Glucose to Fumaric Acid

Biomass from shake flask cultures (100 ml) was harvested at 24 h or 48 h by filtration through nylon cloth, washed with distilled water and resuspended in 50 ml of 2% (w/v) glucose solution. pH was set at 4.0 or 5.5 as indicated by addition of 0.1M HCl or NaOH.

Biotransformation reactions were carried out at 30°C on an orbital shaker at 150 rpm. The pH was monitored at 30 min intervals and readjusted back to 4.0 or 5.5 by addition of 0.1M NaOH. 2 ml samples were withdrawn every hour for analysis.

2.10.2 Biotransformation of Progesterone to 11 α - Hydroxyprogesterone

Mycelia were harvested aseptically after a 24 h or 48 h incubation period, washed with water and re-inoculated into 50 ml of 10 mM sodium phosphate buffer, pH 6.5. The buffer contained 50 mg progesterone, slurried in 2 ml ethanol. The biotransformation was carried out on an orbital shaker set at 150 rpm and 30°C for 24 h.

2.11 FERMENTATION MEDIA

2.11.1 Control Medium: Peptone/glucose medium

Soya peptone, 5 g/l; glucose, 20 g/l; pH 5.5

Developed by Byrne (1985).

2.11.2 CMC Medium: CMC-Supplemented Medium

Soya peptone, 5 g/l; glucose, 20 g/l;

carboxymethylcellulose 20 g/l; pH 5.5

Developed by Byrne (1985).

2.11.3 CP-4.0/CP-5.5 Medium: Carbopol-Supplemented Medium

CP-4.0 medium: Soya peptone, 5 g/l; glucose, 20 g/l;

Carbopol-934, 3 g/l; pH 4.0

CP-5.5 medium: Soya peptone, 5 g/l; glucose, 20 g/l;

Carbopol-934, 3 g/l: pH 5.5.

Developed by Byrne (1985).

2.11.4 Glucose-CSL-Salts Medium: Defined Medium

KH_2PO_2 , 0.4 g/l; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4 g/l; ZnSO_4

$7\text{H}_2\text{O}$, 0.044 g/l; iron tartrate, 0.01 g/l; corn-steep

liquor, 0.5 ml/l; $(\text{NH}_4)_2\text{SO}_4$, 4 g/l; glucose, 20 g/l.

Developed by Rhodes et al. (1959).

2.11.5 Cellulase Pretreated Methylcellulose Supplemented Medium

Soya peptone, 5 g/l; glucose, 20 g/l; methylcellulose, concentration as stated in Results section. 1 ml of a 1% (v/v) solution of Celluclast T (Novo Inc., U.S.A.) in sodium acetate buffer (pH 5.5) was added to 100 ml of the medium and incubated for 1 h at 55°C, prior to autoclaving.

2.12 STERILISATION PROCEDURES

Media were sterilised in flasks at 121°C and 15 p.s.i. for 15-20 min. Fermenter media were sterilised by steam injection.

2.13 CELL DISRUPTION TECHNIQUES

Two techniques were used in the course of the work for breaking the fungal mycelia: the French cell for cell wall isolation work and the bead beater (Biospec Products, OK., U.S.A.) for extraction of cell contents during progesterone biotransformation studies.

The French cell (2 x 25 cm) was operated at a temperature of 4-8°C. Mycelia, suspended in 10 mM sodium phosphate buffer, pH 6.5 (Appendix I) were forced under pressure through an aperture. The broken mycelia were collected and passed through the French cell two more times. The degree of disruption was investigated after each passage through the French cell by preparation of a wet-mounted slide and examination using a Nikon Optiphot phase contrast microscope at 400x magnification.

The disruption chamber of the bead beater was kept at 4-8°C by immersion in ice. The chamber was half filled with glass beads (0.5 mm diameter) and then filled to the top with a suspension of the mycelia in 10 mM sodium phosphate buffer, pH 6.5. 4 x 1 min cycles were sufficient to cause cell breakage of greater than 90% of the mycelia, as estimated using phase contrast microscopy at 400x magnification.

2.14 ISOLATION OF CELL WALLS

Cell walls were isolated from the disrupted cell wall suspension using centrifugation (10 min, 1000xg). (A refrigerated Heraeus Christ Minifuge 2 centrifuge was used). The cell wall preparation was washed approximately 10 times (or until free from cytoplasmic contamination when examined microscopically) with 10 mM sodium phosphate buffer, pH 6.5 (Appendix I). One detergent wash step was included, using 1% (v/v) Nonidet P40. Sonication was used to resuspend between washes. The washed cell walls were lyophilised using an Edwards EF4 Modulyo freeze drier.

The dried cell walls were lipid extracted with petroleum ether (40-60°C) for 16 h and then with chloroform: methanol (1/2, v/v) for 8 h, using a Soxhlett apparatus.

The defatted walls were washed four times in distilled water and lyophilised.

2.15 HYDROLYSIS PROTOCOL OF CELL WALLS

2.15.1 Hydrolysis for Hexosamine Analysis (Amberlite Hydrolysis).

A gentle acid hydrolysis procedure using Amberlite IR-120 resin was employed for analysis of hexosamines. Approximately 5 mg cell wall material was suspended in 0.5 M HCl for 1 h at 100°C under N₂.

The residue was isolated and resuspended in 1 ml 6M HCl for 1 h at 100°C under N₂. This supernatant was combined with that from

the 0.5M HCl hydrolysis and evaporated to dryness over

P₂O₅/KOH. The dry residue was resuspended in 5 ml of 0.25 M

HCl and added to tubes containing Amberlite IR-120 (H⁺) cation

exchange resin. After incubation for 24 h at 100°C under N₂,

the resin was eluted with 2M HCl. The eluate was dried by rotary

evaporation and taken up in approximately 5 ml water. This

suspension was dried in vacuo over P₂O₅/KOH. The final residue

was resuspended in 1 ml H₂O and stored until use at -20°C.

2.15.2 Hydrolysis for Neutral Sugar Analysis

Cell walls were hydrolysed in 1M H₂SO₄ for 8 h at 100°C under

N₂. The supernatant was neutralised using Ba(OH)₂ and solid

BaCO₃. After centrifugation (10 min, 1000xg) to remove

suspended solids, the supernatant was analysed for neutral sugar content.

2.16 FRACTIONATION OF HYPHAL WALLS

Two fractionation procedures were used for cell walls; extraction using nitrous acid as described by Datema et al. (1977a) and extraction using potassium hydroxide.

2.16.1 Nitrous Acid Extraction

1.5 ml of a freshly prepared 2M NaNO₂ solution and 0.5 ml 2M HCl were added to a 1 ml suspension of cell walls (containing less than 20 mg) in distilled water. The screw-capped tubes were closed and left at room temperature for 1.5 h, with periodic mixing. The tubes were then opened and nitrogen bubbled through the suspension for 30 min. The insoluble and soluble fractions were then separated by centrifugation (10 min. 1000xg) and the residue washed four times with distilled water. The insoluble residue was then lyophilised.

2.16.2 Potassium Hydroxide Extraction

Cell walls were suspended in 1M KOH and incubated at 60°C for 20 min. After cooling, the residue was isolated and washed five times in distilled water using centrifugation (10 min, 1000xg). The residue was then lyophilised.

2.16.3 Enzymatic Hydrolysis of Cell Walls

Four different enzymes were used to investigate hydrolysis of cell walls. Their names, source and optimum working conditions are as follows:-

<u>Enzyme</u>	<u>Source</u>	<u>Optimum Conditions</u>	<u>Buffer Used</u>
β -Glucuronidase	<u>Helix pomatia</u> , Type H-2 (Sigma)	pH 5.0, 37°C	50 mM sodium acetate
Celluclast T	<u>T. viride</u> , (Novo)	pH 5.5, 55°C	" "
Protease	<u>B. subtilis</u> , Type XVI (Sigma)	pH 7.5, 37°C	10 mM sodium phosphate
Chitinase	<u>S. griseus</u> (Sigma)	pH 6.0, 25°C	" "

For individual tests using of the protease, celluclast T or β -glucuronidase, 1/500 dilutions were made in the appropriate buffers and 1 ml added to a 3 ml suspension of each cell wall sample. After incubation for 18 h at the appropriate optimum temperature, the residue and supernatant were separated by centrifugation (10 min, 1000xg), and the supernatant analysed.

For the combined enzymatic analysis using protease, chitinase and β -glucuronidase, 1/200 dilutions of the enzymes in 10 mM sodium phosphate buffer, pH 6.2 were made and 1 ml of the mixture added to a 3 ml suspension of each cell wall sample in the same buffer. Incubation was at 30°C for 18 h. The supernatant was analysed for released wall components after separation by centrifugation.

Controls were run with each enzyme treatment by incubating the cell wall samples with buffer only, and analysing these supernatants along with those from the tests.

2.17 ANALYTICAL PROCEDURES

2.17.1 Macroscopic Analysis(Fungal Growth Morphology)

Macroscopic morphology was estimated visually and categorised according to this key:

- D - Dispersed, filamentous hyphae, generally filling medium
- CD - Clumped dispersed; mycelia which were initially dispersed in the medium but became clumped and aggregate slightly, not quite filling the medium.
- CC - Clumped congealed growth; growth tightly packed into one mass, not filling medium
- P - Pelleted, with mycelia forming dense regular growth centres; pellets can clump together or can become clumped coalesced at later stages of growth.

2.17.2 Microscopic Analytical Procedures

2.17.2.1 Phase Contrast Microscopy:

Wet-mount mycelial samples were prepared and examined using an Optiphot phase contrast microscope (Nikon). Photomicrographs were made using Kodak Panatomic-X 32 ASA black-white film with a Microflex AFX-11A light meter (Nikon). Measurements were made using an eye piece micrometer graduated using a standard graduation slide.

2.17.2.2 Fluorescence Microscopy:

An Optiphot epifluorescence microscope (Nikon) equipped with a high pressure mercury vapour lamp was used for examination of fluorescent samples. A violet excitation filter IF 395-425 was employed. Hyphae were stained by treatment of an aqueous mycelial

suspension with 0.1% (w/v) Calcofluor White M2R (Sigma, Mo., USA).

2.17.2.3 Scanning Electron Microscopy:

Samples were prepared for scanning electron microscopy using two methods.

- (i) 10 mM sodium phosphate buffer, pH 6.5, was used throughout this preparative procedure. Mycelial samples were fixed in 2.5% glutaraldehyde in buffer for 1 h, washed in buffer, postfixed for 1 h in 1% osmium tetroxide in buffer, washed and dehydrated in an acetone series (20, 50, 70, 90, 100% (v/v) acetone) The samples were then critical-point dried, sputter-coated with gold and examined in a Hitachi S570 Scanning Electron Microscope.

- (ii) Samples were alternatively prepared using a cryogenic system. Mycelia were taken directly from culture, washed and frozen in liquid nitrogen in an Emscope SP 2000 unit. After etching at -90°C for 20 minutes, the samples were sputter-coated with gold and examined while frozen in an ISI DS 130 Scanning Electron Microscope.

2.17.2.4 Transmission electron microscopy:

Cell wall samples were prepared for examination by transmission electron microscopy by placing droplets of sample suspension on pioloform/carbon coated grids. After allowing to dry, these were shadowed at an angle of 30° with platinum/carbon. A Philips EM 300 electron microscope operating at 60 kV was used for examination.

2.17.3 Estimation of Hyphal Growth Unit (HGU) in Submerged Media

Mycelia growing in submerged culture under standard conditions were sampled 8, 12 and 24 h after inoculation. Wet-mount slides were prepared of each sample and examined using phase contrast microscopy. An eye piece micrometer, calibrated with a standard graduation slide, was used for measurements of hyphal length. A minimum of 10 hyphae, with measurement of total hyphal length and total number of tips associated with that hypha, were counted for each sample. The hyphal growth unit (HGU) for each sample was then estimated using the following formula:-

$$\text{Hyphal Growth Unit (HGU)} = \frac{\text{Total length of hypha } (\mu\text{m})}{\text{Number of tips}}$$

2.17.4 Estimation of Hyphal Extension Rate on Solid Media

Control, CMC, CP-4.0 and CP-5.5 media were supplemented with 2% (w/v) Technical Agar for estimation of growth rate on solid media. 15 ml of each type of medium was dispensed into petri dishes, to maintain constant depth of agar. 10^5 cells were inoculated onto a plate of control medium and incubated at 30°C for 24 h. Plugs of agar with mycelial growth, measuring 3 mm in diameter were then excised from the plate and placed onto plates containing the range of media used in the analysis, with the culture side touching the agar. These plates were then incubated for 12 h at 30°C. The rate of hyphal extension was then measured every 2 h between 12 and 24 h growth, by following growth along a diameter line across the plate.

2.17.5 X-ray Diffraction Analysis

X-ray diffraction patterns were recorded as described by Sietsma and Wessels (1977), using a flat film technique. Samples were

packed into plastic specimen discs and mounted on the X-ray collimator. Nickel-filtered copper radiation was used with optimum conditions for the X-ray tube determined as 38 kV and 23 mA with an exposure time of 30 min. The specimen to film distance was 40 mm. Radial density tracings were made using a Bio-Rad Model 1650 Transmittance/Reflectance Scanning Densitometer.

2.17.6 Infrared Spectroscopic Analysis of Cell Walls

Infrared spectra of cell wall samples were recorded using the KBr disc method. 1-5 mg of dried cell wall material were mixed thoroughly using a mortar and pestle with potassium bromide powder. When mixing was complete, a flat disc was made and mounted on the specimen holder. The spectra were read using a Perkin Elmer 983 G infrared spectrophotometer.

2.17.7 Chromatographic Analysis

Thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) were used during the course of the work.

2.17.7.1 Thin Layer Chromatography:

Samples were applied to Whatman precoated aluminium-backed silica plates (activated at 100°C for 1 h) in 10µl volumes. The solvent system used for detection of sugars was ethyl acetate: pyridine: acetic acid: water (5:5:1:3) with visualisation by p-anisidine reagent (Merck reagent, supplied by B.D.H. Ltd., Toronto, Canada).

2.17.7.2 High Performance Liquid Chromatography (HPLC):

A Waters Association System (Millipore, MA, USA) was used for all HPLC procedures. The system incorporated a Model 501 pump, U6K

injector, Model 484 tunable absorbance detector, a radial compression module 8 x 10 and a M 745 data module. The column was a Waters Radial pak cartridge 10 x 1 cm, packed with Resolve C₁₈-bonded silica (5 μ). A Guard pak pre-column module was used. This instrumentation and column were used for both fumaric acid and progesterone/11 α -hydroxyprogesterone determination. Samples were prepared using Millex (Millipore) filters. Solvents were degassed by filtration through Durapore (Millipore) filters and stirring under vacuum for 10 min.

2.17.7.2.1 Fumaric acid estimation: Fumaric acid solutions in the range 100-500 ppm were prepared in Milli Q water and were used to calibrate the instrument. The working parameters for fumaric acid estimation were:-

1. Mobile phase: 0.5% (w/v) ammonium sulphate at pH 2.4
2. Flow rate : 2 ml/min
3. Chart speed : 0.5 cm/min
4. Detector : Model 484 tunable absorbance detector at
215 nm
5. Injection
volume : 20 μ l

The retention time for fumaric acid was 4.40 min. A chromatogram for fumaric acid is attached in Appendix II.

2.17.7.2.2 Progesterone/11 α -hydroxyprogesterone estimation:

Solutions containing either progesterone or 11 α -hydroxyprogesterone or combinations of the two substances were prepared in HPLC-grade acetonitrile and used to calibrate the instrument. The working parameters for progesterone/11 α -hydroxyprogesterone estimation

were:-

1. Mobile phase: 70:30 acetonitrile:water (Milli Q)
2. Flow rate : 2 ml/min
3. Chart speed : 0.5 cm/min
4. Detector : Model 484 tunable absorbance detector at
254 nm
5. Injection
volume : 20 μ l

The retention time for progesterone was 5.47 min and for 11α -hydroxyprogesterone was 3.48 min. A chromatogram for the two compounds is attached in Appendix II.

2.18 CHEMICAL ANALYSIS

2.18.1 Estimation of Reducing Sugars

Reducing sugars were estimated using the Dinitrosalicylic Acid (DNS) method (Miller, 1959). Results were expressed as reducing equivalents g/l using glucose as standard.

D.N.S. Reagent

3,5-dinitrosalicylic acid, 10 g/l; potassium sodium tartarate, 300 g/l; sodium hydroxide, 16 g/l were dissolved in 600 ml distilled water by heating but without boiling. The solution was cooled and diluted to 1 litre.

A standard curve was prepared using a range of glucose solutions (0.1-1.5 g/l). 1 ml samples were added to 1 ml water and 2 ml DNS reagent. The tubes were placed in a boiling water bath for 10 min and then cooled. 10 ml water was then added to each tube, the contents mixed and absorbance read at 540 nm. The blank solutions consisted of 2 ml water + 2 ml DNS reagent.

2.18.2 Estimation of Neutral Sugars

Neutral sugars were estimated using the Anthrone method (Fairbairn, 1953). A range of mannose solutions (20-200 µg/ml) were used as standards.

Anthrone reagent

0.1% (w/v) anthrone in 72% H₂SO₄ (250 mls H₂O/750 mls 96% H₂SO₄).

Procedure

5 ml anthrone reagent was added to 1 ml of sample, in an ice bath. The tubes were mixed while keeping cool in ice. After exactly 10 min. in a boiling water bath, the tubes were cooled to room temperature. The absorbance was read at 617 nm. The blank solution consisted of 1 ml distilled water and 5 ml anthrone reagent.

2.18.3 Estimation of Uronic Acids

Uronic acids were estimated using the Carbazole method (Bitter and Muir, 1962). Glucuronolactone was used as standard, in the range 4-40 µg/ml.

Reagents

0.025M sodium tetraborate.10H₂O in 96% H₂SO₄.

0.125% Carbazole in ethanol or methanol.

Procedure

1 ml sample was carefully layered onto the surface of 5 ml sodium tetraborate reagent which had been cooled on ice. The contents

were mixed gently, keeping the temperature below 4°C. After exactly 10 min in a boiling water bath, the tubes were cooled and 0.2 ml carbazole reagent added. After mixing, the tubes were incubated for 15 min in a boiling water bath. The tubes were then cooled to room temperature and their absorbance read at 530 nm. The substrate blank consisted of 1 ml distilled water instead of sample.

2.18.4 Estimation of Hexosamines

Hexosamines were estimated by the procedure of Rondle & Morgan (1955). Glucosamine-HCl was used as standard, in the range 5-90 µg/ml.

Reagents

Ehrlichs reagent: 0.8 g p-dimethylaminobenzaldehyde + 30 ml 36% HCl + 30 ml 95% ethanol.

Acetylacetone reagent: 1.0 ml acetylacetone in 50 ml 0.25 M Na₂CO₃.

Procedure

1 ml sample and 1 ml acetylacetone reagent were mixed in screw capped tubes and boiled for 20 min. After cooling, 5 ml ethanol and 1 ml Ehrlichs reagent were added to the tubes. The tubes were mixed and incubated at 65°C for 10 min. After cooling to room temperature, the absorbance was read at 530 nm. 1 ml distilled water was used instead of sample as the substrate blank.

2.18.5 Estimation of Protein

Protein was estimated using the Bradford method (Bradford, 1976).

Bovine serum albumin (BSA) was used as standard in the range 0-100 μ g. The assay is based on the principle that the absorbance maximum of the dye Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs.

Reagents

Dye reagent concentrate, containing dye, phosphoric acid and methanol (Bio-Rad Ltd., England). The concentrate was diluted 1:4 with distilled water for use.

Procedure

0.1 ml sample was mixed with 5 ml diluted dye reagent. After mixing, the tubes were left to stand for a period of 5-60 min. The absorbance was determined at 595 nm. Distilled water was used as substrate blank.

2.18.6 Estimation of Inorganic and Total Phosphate

Inorganic and total phosphate were estimated by the method of Ames (1966). Na_2HPO_4 or Na_3PO_4 were used as standard in the range 0-50 μ g/ml.

Reagents

1. 10% (w/v) solution of ascorbic acid
2. 0.42% (w/v) ammonium molybdate.4 H_2O in 1N H_2SO_4

Mix 1 part 1 with 6 parts 2 for final reagent.

Ashing reagent: 10% $\text{Mg}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ in 95% ethanol

Procedure

10-100 μ l of sample were mixed with 30 μ l $\text{Mg}(\text{NO}_3)_2$ solution and shaken to dryness over a strong flame until the brown fumes disappeared. 300 μ l of 0.5M HCl were added to each tube after cooling. Tubes were then covered and boiled for 10 min. After cooling, 0.7 ml of the ammonium molybdate mixture was added and the tubes incubated at 45°C/200 min or 37°C/60 min. The absorbance was then read at 820 nm. Blanks contained water instead of sample.

2.18.7 Estimation of Amino Acids

Amino acids were estimated using a Beckman 120 B Amino acid analyser. 200 μ l aliquots from the Amberlite hydrolysates (Section 2.15.1) used for preparation of samples for hexosamine analysis were applied to the Dowex columns in the Amino acid analyser.

2.18.8 Estimation of Ash Content

The ash content of the cell wall samples was estimated using a Temco type 1400 furnace at 600°C. Known weights of sample were placed on predried and preweighed crucibles, and ashed until constant weight was obtained.

2.19 RADIOACTIVITY ANALYSIS PROCEDURE

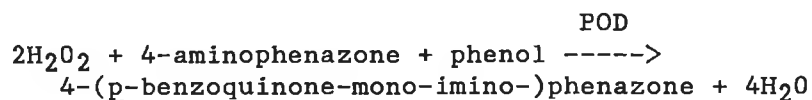
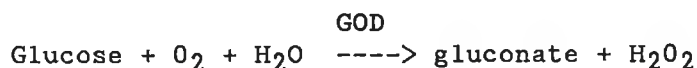
1 ml samples were mixed with 4 ml Beckman Ready Safe Liquid Scintillation Cocktail in Beckman Mini Q vials and counted in a Beckman LS 1701 scintillation counter.

2.20 ENZYMATIC ANALYSIS

2.20.1 Estimation of Glucose

Glucose was measured using an enzymatic colourimetric method based

on Trinder (1969). A diagnostic kit (Boehringer Mannheim GmbH, Germany) was used. The principle of the assay is



GOD = glucose oxidase

POD = peroxidase

Reagents

1. Phosphate buffer, 100 mmol/l, pH 7.0; GOD > 18 μ l
POD > 1.1 u/ml; 4-aminophenazone, 0.77 mmol/l.
2. Phenol, 11 mmol/l

One bottle of reagent 1 was dissolved in 200 ml distilled water, and 1 bottle of reagent 2 added.

Procedure

0.2 ml sample was added to 2 ml reagent mixture and incubated at 20-25°C in the dark for 35-60 min. The absorbance was read at 510 nm, using a water blank to zero the spectrometer.

2.20.2 Estimation of Glucoamylase Activity

Glucoamylase activity was determined by production of glucose reducing equivalents from soluble starch at 40°C, pH 4.2. 1 unit glucoamylase liberates 1 mg reducing sugar equivalents per minute. Reducing sugars were determined by the DNS method (Section 2.18.1).

Reagents

Enzyme: Cell free supernatants or standard enzyme were diluted in sodium acetate buffer, pH 4.2 (Appendix I).

Substrate: 1 g soluble starch was slurried in approximately 50 ml distilled H₂O, brought to the boil to dissolve and cooled. 5.6 ml sodium acetate buffer was added and made up to 100 ml with distilled H₂O.

Procedure

1 ml substrate was allowed to equilibrate at 40°C before addition of 1 ml sample. After exactly 10 min reaction time, 2 ml DNS was added to stop the reaction. Tubes were then boiled for 10 min, cooled, and 10 ml distilled H₂O added. Absorbance was read at 540 nm. The substrate blank contained 1 ml sodium acetate buffer instead of sample. 2 ml DNS was added before addition of 1 ml sample to the enzyme blank.

2.21 ROUTINE MEASUREMENTS

pH was measured using a Philips PW 9420 pH meter. Spectrophotometric measurements were conducted on a Pye-Unicam SP6-550 U.V./vis spectrophotometer or on a LKB Ultraspec II 4050 U.V./vis spectrophotometer with a 1 cm light path.

Balances used routinely included a Sartorius 1219 MP electronic balance (600 g ± 0.01g) and Precisa 80A electronic balance (30g ± 0.0001g).

Centrifugation was carried out using a bench-top Heraeus Christ model 600, a refrigerated Heraeus Christ Minifuge 2 and a DuPont Instruments Sorvall RC-5B Refrigerated Superspeed centrifuge.

3. RESULTS

3.1 PRELIMINARY INVESTIGATIONS

The effect of medium constituents on fungal morphology has been reported by many authors (Whitaker & Long, 1973; Metz & Kossen, 1977; Byrne 1985). There was variation between fungal species and fungal genera in the morphology observed upon addition of different substances to the growth media (Burkholder & Sinnott, 1945; Pirt & Callow, 1959; Seviour et al., 1984). Byrne (1985) investigated the effect of a range of medium constituents on the morphology of R. arrhizus 1526. Polymeric supplements were found to cause dispersed growth of R. arrhizus, and brought about the reversal of the pelleted morphology caused by the addition of metal cations.

The aim of this section of work was to determine whether these polymers affected the morphology of a range of Rhizopus species and fungal genera in a similar manner. Also, the mode of action of the polymers is investigated by determining the stage of development of the hyphae at which the polymers are effective, and by studying the role played by polymer properties such as charge and viscosity.

3.1.1 Effect of Polymer Supplements on Morphology of a Range of Rhizopus species

The effect of polymer supplements on the morphology of a range of Rhizopus species was investigated. CaCO₃ was used as a buffering agent in one series of samples to determine whether culture pH was a factor in the control of mycelial morphology. The range of Rhizopus species was cultured in control medium containing various supplements, with and without CaCO₃, which were previously shown to

affect morphology. The flasks were inoculated and incubated under standard conditions (Section 2.4). The pH and growth morphology were recorded after 48 h.

The results are presented in Table 3.1.

When CaCO₃ was added to flasks containing sodium alginate, insoluble calcium alginate gels were formed and existed as large clumps in the medium. As a result of this, large mycelial clumps were suspended in the medium. This series of flasks was omitted from the trial because of the difficulties this caused in assigning the growth morphology. Pellets formed in media containing CaCO₃ had a white appearance, suggesting that the CaCO₃ particles may be used as growth centres for the developing mycelia.

The control medium without any supplements resulted in pelleted morphology with all of the species used, both with and without CaCO₃ added (except for R. stolonifer). The presence of the polymer supplements caused growth to be dispersed or clumped dispersed (Section 2.17.1). When CaCO₃ was included with the polymers, growth became pelleted in nearly all of the species. The pH of media containing CaCO₃ was between 6.2 and 7.6 whereas media without CaCO₃ showed a drop in pH in the range 2.6-4.7. The lowest pH's were observed in media without any polymer supplements. Some species such as R. stolonifer ATCC 90609, R. oryzae ATCC 83711 and R. oryzae ATCC 40564 did not grow well in some of the media used.

3.1.2 Comparison of Morphology of Four Fungal Genera

The variation in morphology between four fungal genera in complex

TABLE 3.1: Comparison of growth morphology and media pH of a range of Rhizopus species

<u>Medium Supplement g/l</u>				<u>R. arrhizus</u>			<u>R. arrhizus</u>		<u>R. oligosporus</u>		<u>R. thailandensis</u>		<u>R. stolonifer</u>		<u>R. oryzae</u>		<u>R. oryzae</u>		
				NRRL 1526			NRRL 2582		NRRL 2710		ATCC 20344		ATCC 90609		ATCC 83711		ATCC 40564		
Sodium	Alginate	CMC	Carbopol	CaCO ₃	pH _i	pH	Morph.	pH	Morph	pH	Morph.	pH	Morph.	pH	Morph.	pH	Morph.	pH	Morph.
-	-	-	-	-	5.5	2.8	P#	2.9	P	2.9	P	2.8	P	4.1	CD	2.9	P	2.6	P
20.0	-	-	-	-	5.6	3.2	D	3.2	D	3.4	D	3.5	D	4.7	D	3.7	D	3.5	D
-	20.0	-	-	-	5.6	3.5	D	3.4	D	3.7	D	3.7	CD	5.1	D	3.8	CD	3.7	CD
-	-	3.0	-	-	5.5	3.6	D	3.7	D	3.9	D	3.8	CD	4.8	CD	3.9	C	3.9	CD
-	-	-	10.0	6.5	6.6	P	6.8	P	6.8	P	7.0	P	6.4	PG	7.2	PG	7.3	PG	
-	20.0	-	10.0	6.6	6.5	P	7.0	P	6.3	P	6.7	P	6.7	PG	7.1	PG	7.6	C	
-	-	3.0	10.0	6.2	6.1	P	6.8	P	6.2	P	7.1	P	6.2	PG	6.9	PG	7.3	PG	

P= Pelleted Growth; D= Dispersed Growth; CD= Clumped Dispersed Growth; PG= Poor Growth; C= Clumped, Congealed Growth

Flasks were inoculated and incubated under standard conditions. The pH and growth morphology were recorded after 48 h.

See Section 2.17.1 for morphology descriptions.

media was investigated. The four genera used were R. arrhizus NRRL 2582, A. niger NRRL 3, T. reesei NRRL 11460 and P. chrysogenum NRRL 807. Control medium, with supplements previously shown to affect morphology (Byrne, 1985), was used. Inoculation and growth were carried out under standard conditions (Section 2.4). Culture pH and growth morphologies observed after 48 h are presented in Table 3.2

Morphology varied with genus and with supplement. In control medium, (i.e. no supplements) morphology was either pelleted or clumped. All four genera grew in a dispersed form in Carbopol-supplemented media. T. reesei was the only species to grow dispersed in MgSO₄-supplemented media. The addition of sodium alginate to MgSO₄-supplemented media caused the morphology to change from pelleted to dispersed except in the case of P. chrysogenum where the morphology remained pelleted. The pH decreased in all cases over the 48 h incubation period.

3.1.3 The Effect of Time of Addition of Polymer Supplements on Morphology of R. arrhizus

Carbopol-934 has been reported to prevent agglutination of spores of A. niger prior to germination, leading to dispersed growth (Elmayergi et al., 1973). The effect of the time of addition of Carbopol-934 and CMC, which also disperses growth, on the morphology of R. arrhizus was investigated. CMC (2% (w/v)) and Carbopol- 934 (0.3% (w/v)) were each added aseptically to the cultures of R. arrhizus in control medium at various stages in the first 12 h growth. The growth morphologies observed after 48 h growth under standard conditions are presented in Table 3.3.

TABLE 3.2: Comparison of growth morphology and media pH of four fungal genera

Sodium Alginate	Medium Supplements g/l			<u>R.arrhizus</u> NRRL 2582		<u>A.niger</u> NRRL 3		<u>T.reesei</u> NRRL 11460		<u>P.chrysogenum</u> NRRL 807	
	Carbopol	MgSO ₄	pH _i	pH	Morph.	pH	Morph.	pH	Morph.	pH	Morph.
-	-	-	5.6	2.5	P	1.7	P	6.2	CC	3.5	P
20.0	-	-	5.7	3.2	D	2.6	D	5.1	D	4.2	P
-	3.0	-	5.7	3.6	D	3.2	D	3.9	D	4.4	D
-	-	1.25mM	5.7	2.3	P	1.6	P	4.2	D	3.7	P
20.0	-	1.25mM	5.7	3.1	D	2.4	D	4.8	D	4.2	P
-	3.0	1.25mM	5.7	2.6	D	1.8	D	3.7	D	4.3	D

P = Pelleted Growth; CC = Clumped, Congealed Growth; D = Dispersed Growth

Flasks were inoculated and incubated under standard conditions. The pH and growth morphology were recorded after 48 h.

See Section 2.17.1 for morphology descriptions.

TABLE 3.3 Effect of addition time of Carbopol-934 and CMC on morphology of R. arrhizus

Addition Time	Morphology Observed	
	Carbopol-934	Carboxymethylcellulose (CMC)
Hrs		
0	Dispersed	Dispersed
3	Dispersed	Dispersed
5	Dispersed	Dispersed
6	Clumped dispersed	Dispersed
7	Clumped dispersed	Dispersed
8)	Dispersed with some growth
)	Centres
10)	Dispersed with many growth
)	Centres
12)	Dispersed with many growth
)	Centres
Control	Clumped Pellets	Clumped Pellets

The polymer supplements were added to the control media at the indicated times. The mycelial morphology was recorded after 48 h growth under standard conditions.

See Section 2.17.1 for morphology description.

Germ tube emergence commences at approximately 5 h after inoculation. Addition of CMC or Carbopol prior to this event results in a dispersed mycelial morphology at 48 h. A less dispersed growth form is observed if Carbopol is added around the time of germ tube emergence. When Carbopol is added between 8-10 h, mycelia have already begun to pellet and remain pelleted. Addition of CMC between 8-12 h caused a change in growth form from pelleted to an intermediate form where dispersed mycelia appear to have grown from the original pellet, indicating the stronger influence of CMC on morphology compared to Carbopol.

3.1.4 Effect of Methylcellulose on Morphology

The anionic carboxyl groups of the CMC polymer have been implicated in dispersal of growth by causing ionic repulsion between hyphae, thus leading to dispersed growth (Byrne & Ward, 1987). Byrne (1985) observed that a concentration of 10g/l methylcellulose, a nonionic polymer, resulted in dispersed growth in a defined medium. In this experiment the effect of methylcellulose concentration in control medium on morphology was investigated. A series of flasks with concentrations of methylcellulose ranging from 0.25 - 3% (w/v) was inoculated with R. arrhizus spores and grown under standard conditions. The morphology and pH recorded after 24 h are presented in Table 3.4

In order to obtain completely dispersed growth, methylcellulose must be present at a concentration of 1% (w/v) or greater. The requirement of greater than 1% methylcellulose in the medium to induce completely dispersed growth indicates a possible role played by viscosity in dispersal of growth. To investigate this

TABLE 3.4: Effect of methylcellulose on morphology of R. arrhizus

Methylcellulose g/l	pH _i	Morphology	pH
-	6.06	Clumped Pellets	2.99
2.5	5.81	Clumped Pellets	3.10
5.0	5.83	Clumped Dispersed	3.25
10.0	5.96	Dispersed	2.89
20.0	6.08	Dispersed	2.79
30.0	6.15	Dispersed	2.75

Flasks containing control medium supplemented with methylcellulose were inoculated and incubated under standard conditions. The pH and morphology were recorded after 24 h.

See Section 2.17.1 for morphology description.

possibility, a series of samples of methylcellulose-supplemented control media were pretreated with a cellulase enzyme for 1 h/55°C/pH 5.6 (Section 2.11.5). The viscosity was measured before and after the enzyme treatment with a Brookfield Digital Viscometer. The treated media samples were autoclaved and inoculated with R. arrhizus spores. Morphology and pH values of 24 h cultures are presented in Table 3.5.

Reduction in viscosity induced by cellulase treatment caused the morphology to change to partially dispersed from the totally dispersed morphology obtained with untreated, viscous methylcellulose-supplemented media. This indicates that a more viscous medium aids dispersal of the hyphae but is not necessary in prevention of pelleting.

3.1.5 Investigation of Effect of Vegetative Inocula on Morphology in Polymer-Supplemented Media

CMC or methylcellulose in control medium have been shown to cause dispersal of growth when inoculated with spores of R. arrhizus. The ability of these supplements to disperse pregrown pelleted inocula was investigated. 20 ml culture from control media were sampled at various stages of growth and re-inoculated into CMC- or methylcellulose-supplemented media. Flasks were cultured for 48 h and morphology was then recorded (Table 3.6).

Unpelleted inocula, i.e. at 6 or 9 h, promoted a clumped dispersed morphology in both CMC- and methylcellulose-supplemented media. A pelleted inoculum (12 h) initially remained pelleted in CMC-supplemented medium, but became dispersed after 48 h

TABLE 3.5: Effect on morphology of cellulase pretreatment of methylcellulose

Methylcellulose g/l	<u>Viscosity (cP)</u>		Morphology	pH
	Before Cellulase Treatment	After Cellulase Treatment		
10	32	5	Clumped Dispersed	3.05
15	67	9	Clumped Dispersed	3.19
20	202	13	Clumped Dispersed	3.25

The viscosity of the methylcellulose-supplemented control media was measured before and after cellulase treatment. Flasks were then inoculated and incubated under standard conditions for 24 h.

TABLE 3.6: Relationship between inoculum age/morphology and morphology resulting in 48 h polymer supplemented media

Inoculum Age	Inoculum Morphology	Morphology after 48h in:	
		CMC Medium	Methylcellulose Medium
6h	Germinating	Clumped Dispersed	Clumped Dispersed
9h	Developing hyphae	Clumped Dispersed	Clumped Dispersed
12h	Pelleted	Dispersed	Clumped Dispersed
24h	Pelleted, clumped	Pelleted	Pelleted

Aliquots were taken from control medium at the indicated times and inoculated into CMC- or Methylcellulose-supplemented media. Flasks were then cultured for 48 h under standard conditions.

See Section 2.17.1 for morphology descriptions.

incubation. The equivalent inoculum in methylcellulose-supplemented medium promoted partially dispersed growth at both 24 and 48 h after inoculation. A 24 h old pelleted inoculum remained pelleted after growth in CMC or methylcellulose-supplemented media. A 9 h old inoculum, where pelleting has just commenced, became partially dispersed when grown in CMC or methylcellulose-supplemented media. Thus it is possible to reverse the morphology when mycelia are still actively growing, i.e. at 12 h, but not so at later stages, i.e. 24 h.

20ml samples were taken at 0, 6, 9, 12 and 24 h from CMC supplemented medium and re-inoculated into control medium supplemented with 1.25 mM MgSO₄. The resultant growth was poor and irregular with clumps of mycelia suspended in the medium. Morphology was thus indeterminable.

3.1.6 Summary

Polymer supplements caused dispersal of growth of most species of Rhizopus. CaCO₃ reversed this effect, possibly by acting as growth centres for the developing mycelia and promoting pellet formation. Carbopol dispersed the growth of the four fungal genera studied, but other medium supplements had varying effects, indicating the possible role of cell wall composition or fungal physiology in determination of morphology.

CMC supplementation had a strong effect on morphology, causing the reversal of morphology in a 12 h old pelleted inoculum. But the possible mode of action of charge and viscosity on growth dispersal

is unclear, since dispersed growth was obtained using a cellulase-pretreated methylcellulose supplement.

3.2 EFFECT OF POLYMER SUPPLEMENTS ON HYPHAL GROWTH AND BIOMASS PRODUCTION

In this section the effect of the polymer supplements on growth parameters including biomass production, the specific growth rate (μ) and the hyphal growth unit (HGU) of R. arrhizus in complex media is assessed. The effect of the polymers on the rate of hyphal extension on solid media is also investigated. Attempts were made to gain a greater understanding of the specific interaction between the polymers and mycelia through the use of scanning electron microscopy and the uptake of the fluorescent dye Calcofluor White M2R.

3.2.1 Photomicrographic Monitoring of Hyphal Development in Polymer-Supplemented Media

Control media supplemented with 2% (w/v) CMC (CMC medium) or 0.3% (w/v) Carbopol-934 (CP-5.5 medium) were inoculated with spores of R. arrhizus and incubated under standard conditions. Because the physical properties of the Carbopol polymer are affected by pH (Section 1.2), flasks of Carbopol-supplemented media with initial pH (pH_i) of 4.0 (CP-4.0 medium) were also inoculated. Cultures were sampled hourly from 5-12 h after inoculation and at 24 h. Mycelial samples were examined microscopically and photographs taken of representative stages of growth (Figure 3.1 and 3.2).

Germination started at approximately 5 h after inoculation in all media except CP-4.0 media where it occurred about 1 h later.

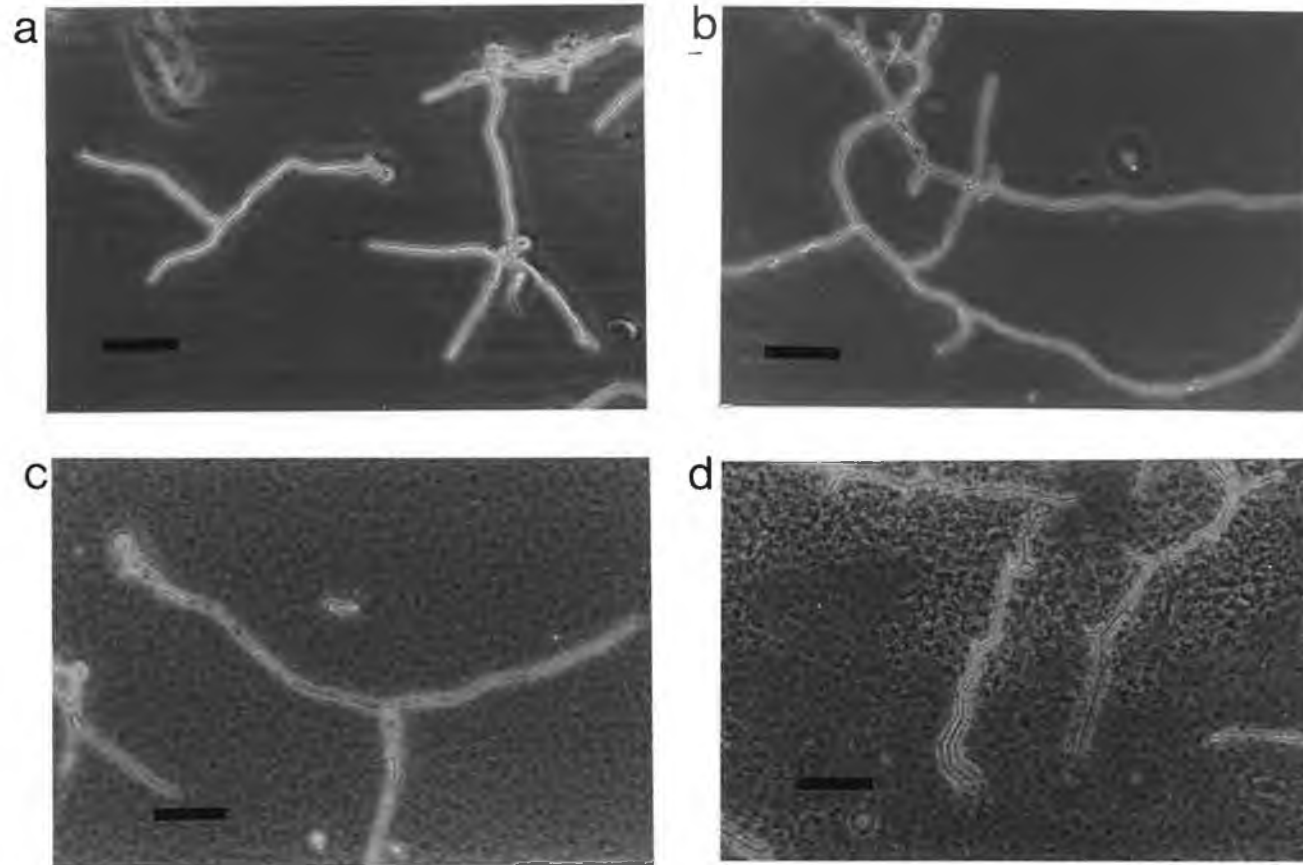


FIG. 3.1 Development of hyphae in first 12 h after inoculation (Phase contrast microscopy)

A. Hyphae in control medium - 9 h (Hyphae begin to pellet after this stage, leaving few free in the medium)

B. Hyphae in CMC medium - 12 h

C. Hyphae in CP-5.5 medium - 10 h

D. Hyphae in CP-4.0 medium - 11 h

Bar = 100 μ m

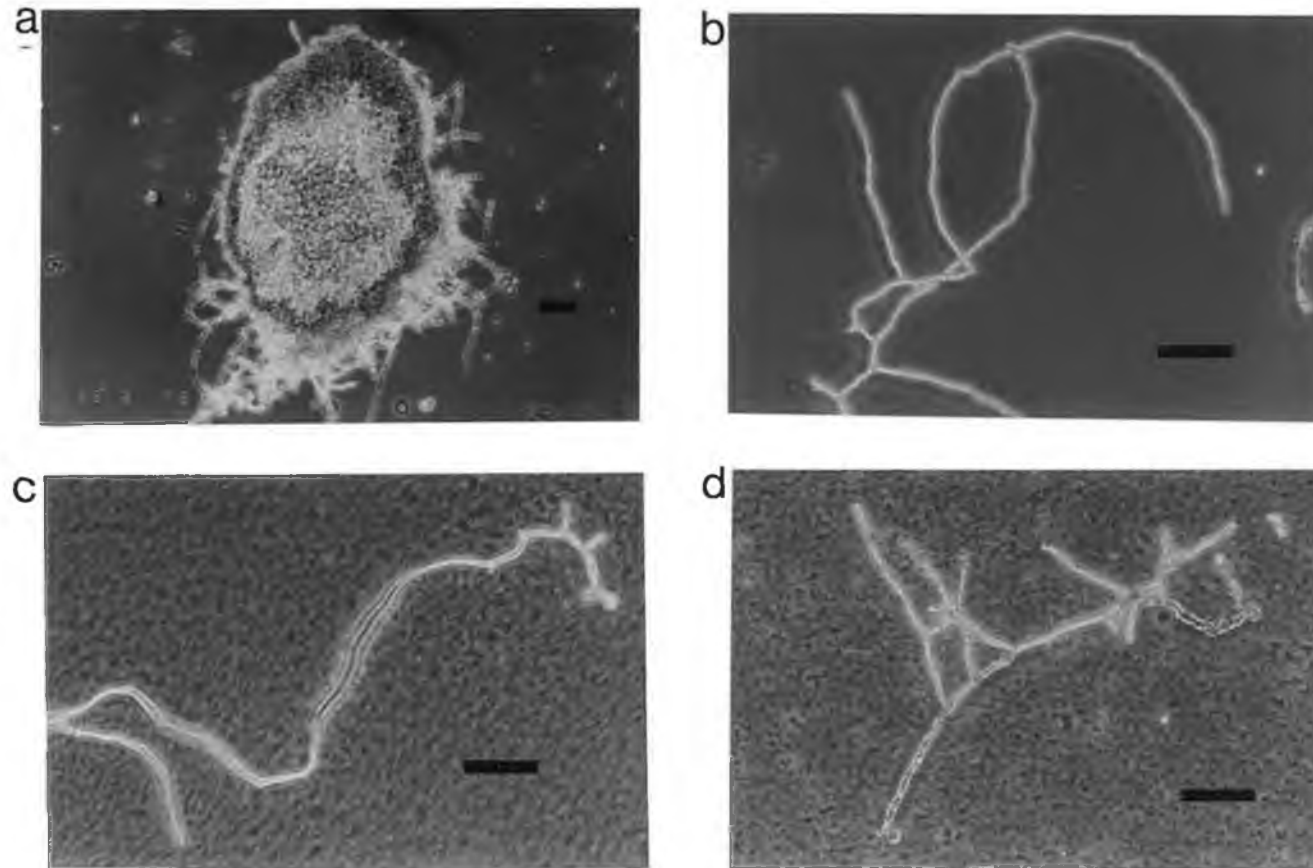


FIG. 3.2 Hyphal morphology after 24 h growth (Phase contrast microscopy)

A. Control medium
B. CMC medium

C. CP-5.5 medium
D. CP-4.0

Bar = 100 μ m

Branch initiation started at the 7-8 h stage with less advanced germ tube elongation occurring in the CP-4.0 sample. The control mycelia began to pellet at 9-10 h. After 12 h growth, free mycelia were difficult to detect in this medium. Prior to pelleting, the control mycelia exhibited a growth pattern similar to that of CMC and CP-5.5 media. These hyphae were long and tapering with infrequent branching. The hyphae in the CP-4.0 medium were shorter and more highly branched. At 24 h growth, the mycelia from CMC and CP-5.5 media were still longer and less highly branched than those from CP-4.0 medium.

3.2.2 Measurement of Hyphal Growth Unit (HGU)

Measurements were taken of the total hyphal length and the number of tips associated with the hypha, of a selected number of samples after different periods of growth. The hyphal growth unit (HGU) data (Section 2.17.3) are presented in Table 3.7.

The HGU data on their own do not give an accurate assessment of the fungal growth pattern, particularly when working with samples from submerged culture. Because a different sample is taken at every growth point, the method has a much higher error margin associated with it than if growth of a single hypha was being followed on an agar plate. The values for the standard deviations are indicative of this problem. A written commentary and possibly photographic evidence are necessary to describe fully the growth pattern of the fungus. For instance, the HGU value for the CP-4.0 sample at 8 h is higher than the other three. It might be assumed from this that the mycelia growing in this sample are much longer with fewer tips associated with it than the others, whereas the value is really

TABLE 3.7: Calculation of Hyphal Growth Unit for R. arrhizus grown in control medium with polymer supplements

Supplement	pH _i	8 h	12 h	24 h
-	5.5	140 ± 44	110 ± 24	Pelleted
CMC	5.5	90 ± 16	173 ± 59	146 ± 28
Carbopol-934	5.5	130 ± 25	160 ± 45	170 ± 39
Carbopol-934	4.0	160 ± 34	83 ± 19	110 ± 19

The data shown are the means of not less than six measurements, ± the standard deviation.

Cultures were sampled after 8, 12 and 24 h growth under standard conditions and the hyphal growth unit determined.

All data fall within a 95% confidence interval.

higher due to the slower germination rate and less advanced stage of growth of these mycelia.

3.2.3 Effect of Polymers on Biomass Production by *R. arrhizus*

A growth curve of *R. arrhizus* in polymer-supplemented media was constructed using biomass production data. Four samples of media including control, CMC, CP-4.0 and CP-5.5 media were inoculated and incubated under standard conditions. After 12, 24, 36 and 48 h growth, cultures were sampled and biomass dry weights and culture pH's determined. Results are presented in Figure 3.3

The four samples followed a similar rate of growth up until 18h. Then growth in the control sample began to level off, as the mycelia had become tightly clumped, leading to restricted growth. The biomass from the dispersed samples continued to increase but at different rates. The mycelia from CP-4.0 media attained the highest biomass. The presence of the polymers cause a buffering effect in the media, as seen when comparing the pH profile of the control with those of the polymer-supplemented media.

The biomass data for CP-4.0 media is called into doubt due to the insoluble nature of the polymer at this pH. This is discussed more fully in Section 3.2.6.

Growth may become restricted in the control medium after 24 h as a result in part of the low medium pH. But this possibility is investigated in Section 3.4.2.2 where pH control is used.

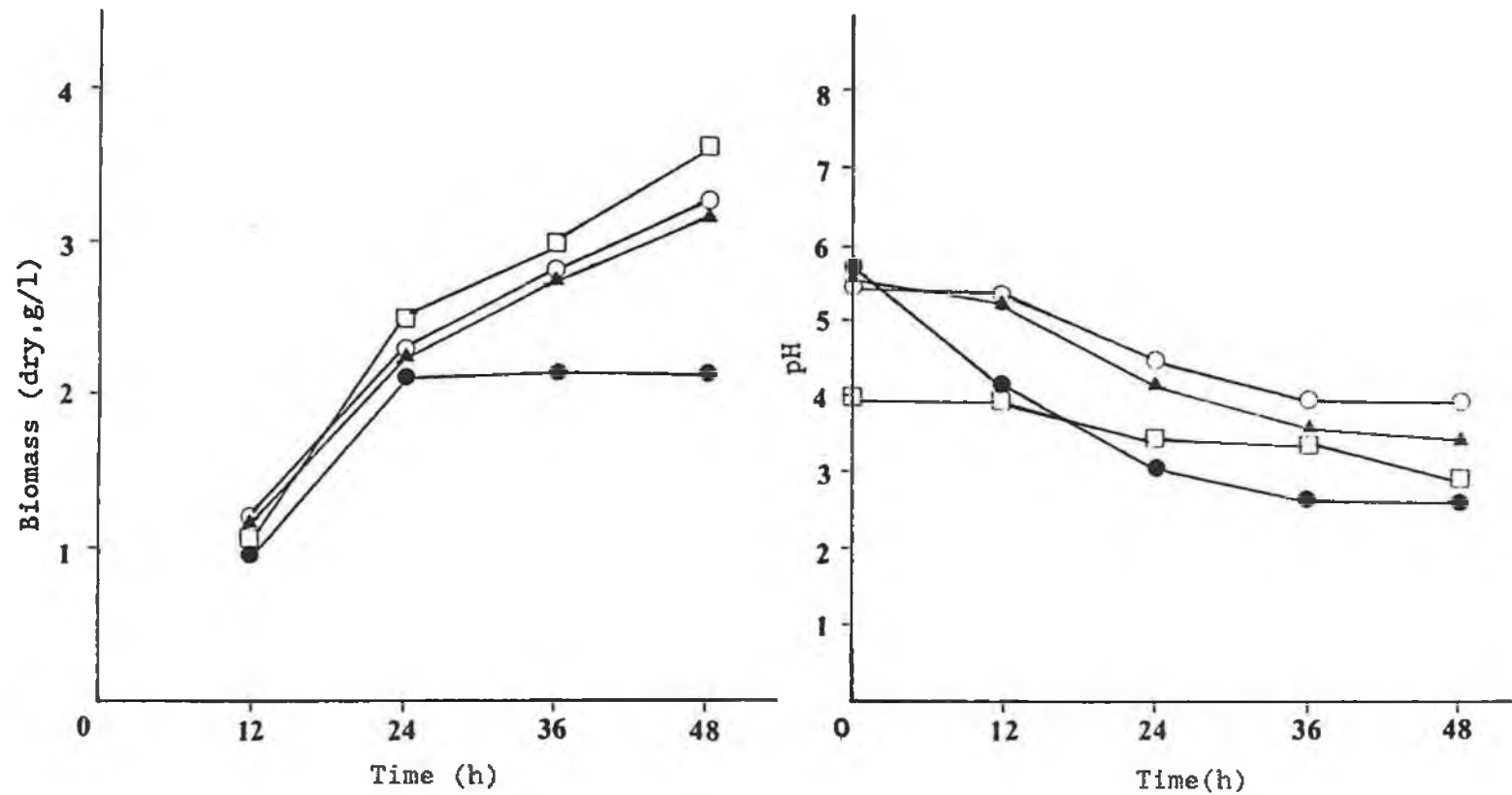


FIG. 3.3 Patterns of Biomass production and medium pH profile during growth in shake flask cultures.

- , control medium
- ▲, CMC medium
- , CP-5.5 medium
- , CP-4.0 medium

3.2.4 Effect of Polymers on the Specific Growth Rate, μ , of R. arrhizus

The specific growth rate (μ) of R. arrhizus in control and polymer-supplemented media was estimated from the doubling time, T_d , by the method of Trinci (1973) (Section 1.4). Mycelia were harvested after 8, 10, 12, 18 and 24 h growth, their dry weights determined and a semi-log plot of biomass vs. time drawn (Figure 3.4). The specific growth rates, μ , at 12 and 24 h estimated from this data are presented in Table 3.8.

Specific growth rates were similar in each of the four cultures at 12h. By 24h, the production of biomass had decreased in each of the four cultures (as shown in Fig. 3.3), with a concomitant decrease in specific growth rate.

3.2.5 Effect of Polymers on Hyphal Extension Rate on Solid Media

Polymer supplements have been shown to affect the hyphal extension rate in submerged culture (Section 3.2.2). Their effect on the extension rate on solid media was investigated. Control medium supplemented with 2% (w/v) CMC, 0.3% (w/v) Carbopol ($pH_1 = 4.0$) and 0.3% (w/v) Carbopol ($pH_1 = 5.5$) were used, with 2% (w/v) Technical Agar added. Blocks of agar from a 24 h solid culture of R. arrhizus on control medium were inoculated onto the range of plates and incubated at 30°C. The plot of the hyphal extension rate over a 12 h period is shown in Figure 3.5

The fastest extension rate was observed on control plates, with CMC- supplemented medium marginally slower. Carbopol-supplemented

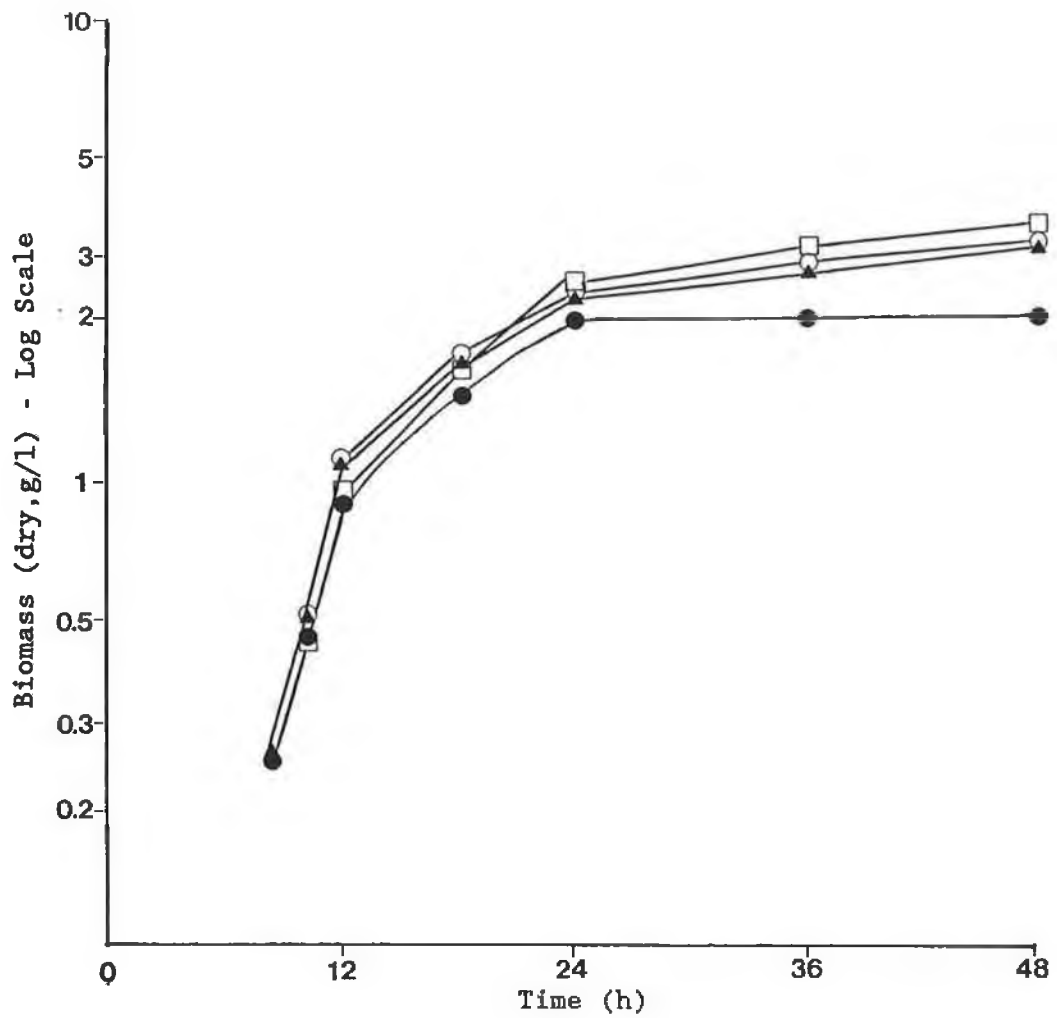


FIG. 3.4 Semi-log plot of Biomass production for estimation of μ , the specific growth rate.

- , Control medium
- ▲, CMC medium
- , CP-5.5 medium
- , CP-4.0 medium

TABLE 3.8: The specific growth rate (μ) of *R. arrhizus* in control and polymer-supplemented media.

Medium	Specific growth rate (μ), h^{-1}	
	μ up to 12h	μ from 18 - 24h
Control	0.28 \pm 0.03	0.12 \pm 0.03
CMC	0.28 \pm 0.03	0.14 \pm 0.03
CP-4.0	0.28 \pm 0.04	0.15 \pm 0.03
CP-5.5	0.28 \pm 0.03	0.14 \pm 0.03

Data are the means of four analyses, \pm standard deviation

Cultures were grown under standard conditions for 12 or 24 h, their dry weights determined and a semi-log plot of biomass vs. time drawn. The specific growth rate, μ , was estimated from the doubling time, T_d .

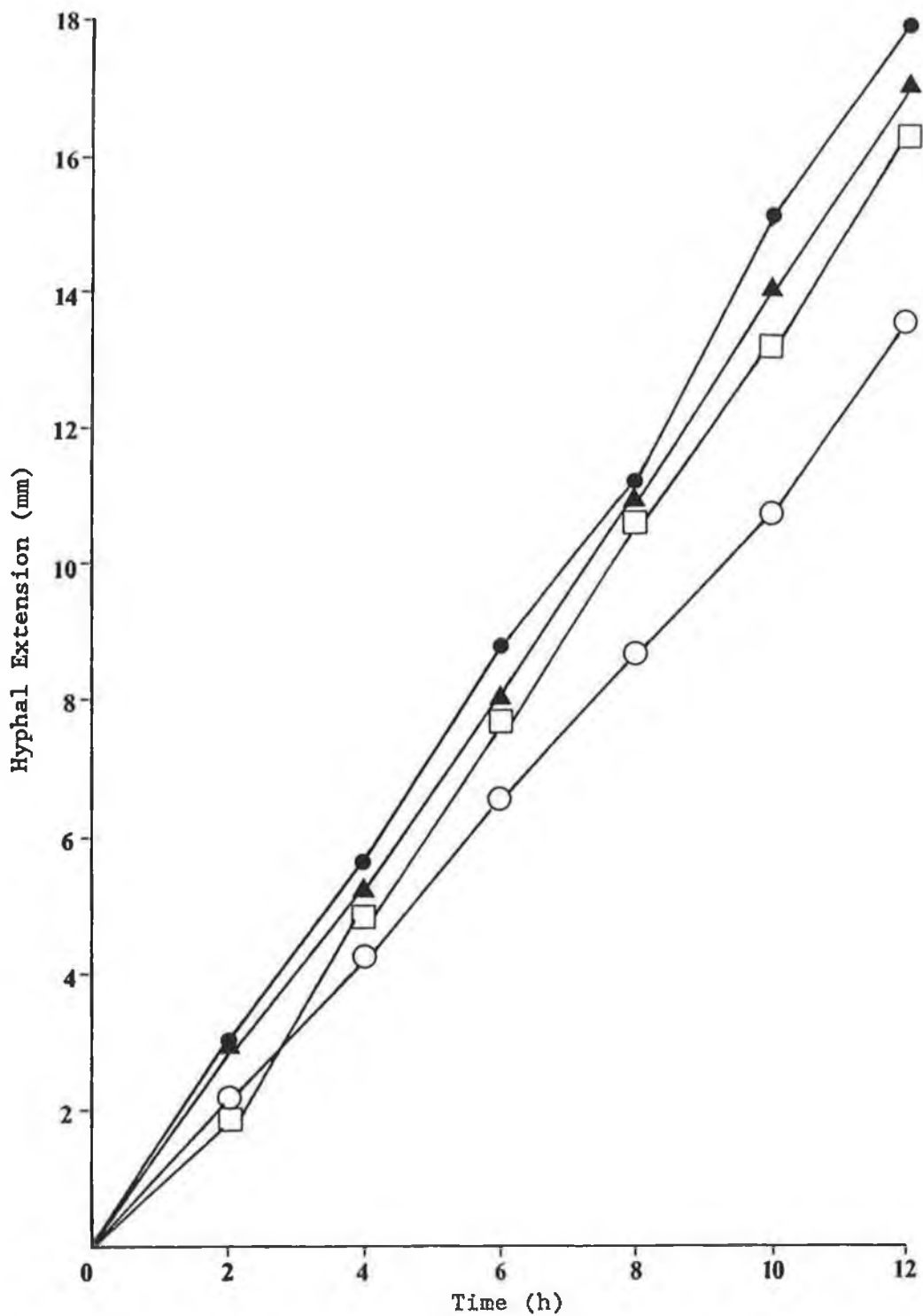


FIG. 3.5 Rates of hyphal extension on solid media.

- , Control medium
- ▲, CMC-supplemented medium
- , CP-5.5-supplemented medium
- , CP-4.0-supplemented medium

media had an even slower extension rate. The medium supplemented with Carbopol at $\text{pH}_i = 5.5$ had the lowest extension rate at 13.7 mm over the 12 h period. This may be due to the solubilisation and thus more homogeneous mixing of the Carbopol in this medium, which possibly leads to a greater effect on the growth rate compared with the medium with Carbopol at pH 4.0, where the insoluble polymer particles are not uniformly mixed in the agar medium. No major differences in branching pattern were observed between the samples (Figure 3.6).

3.2.6 Interaction of Polymers with Hyphae of *R. arrhizus*

Both CMC and Carbopol cause dispersal of growth of *R. arrhizus*. However, the properties of Carbopol vary considerably with pH (Section 1.2), unlike CMC. The solubility of the polymer (and the resultant viscosity of the medium) are influenced by the pH. The increased biomass obtained with Carbopol-supplemented media may be due in part to an interaction between the mycelia and the polymer. The specific effects of Carbopol and pH on mycelial development and morphology were evaluated. The fluorescent brightener Calcofluor White M2R, known to interact with extension zones and branch points (Gull and Trinci, 1974), was used to investigate the effect of Carbopol on extension zone/branch initials in developing mycelia.

Shake flask cultures containing control media with and without Carbopol were adjusted to pH 3.0, 4.0, 5.0 and 6.0, inoculated with spores and incubated under standard conditions for 10 h. Mycelia were then harvested, washed and treated with Calcofluor White. The results are presented in Table 3.9.

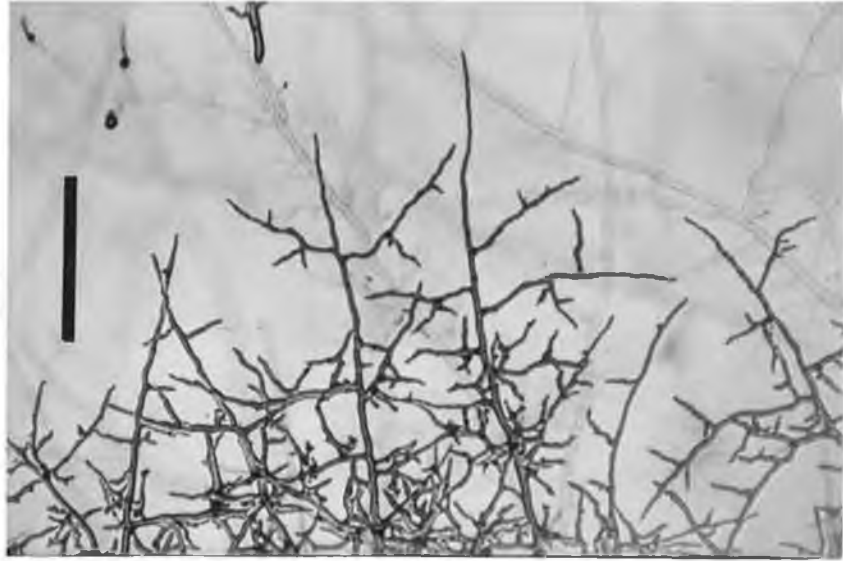


FIG. 3.6 Light micrograph showing representative branching pattern found in either of the four media used for monitoring hyphal development on solid media.

Bar = 500 μm .

TABLE 3.9: Effect of culture pH and Carbopol on mycelial development and observed fluorescence of R. arrhizus after 10 h incubation

Sample source	pH _i	Morphological development (total hyphal length)	Fluorescence
Control medium	3.0	Germination initiated (hyphae 50-100 µm)	++
	4.0	More developed hyphae (200-400 µm)	++
	5.0	Hyphae beginning to pellet (600-800 µm)	++
	6.0	Hyphae beginning to pellet (800-1000 µm)	++
Control medium plus Carbopol-934	3.0	Germination beginning (50 µm)	-
	4.0	Short hyphae with branch initiation (400 µm)	-
	5.0	Long tapering hyphae (700-1000 µm) sparsely branched)	+
	6.0	Similar to pH 5.0	++

++ Fluorescence as for control; + hyphae visible, but only slightly fluorescent; - hyphae not visible or barely, with minor background fluorescence

Flasks were inoculated and incubated under standard conditions for 10 h. Cultures were sampled and the medium pH, morphological development and hyphal fluorescence assessed.

After a 6 h incubation period, cultures having an initial pH of 5.0 and 6.0 had germinated whereas the cultures having an initial pH of 3.0 and 4.0 had not. In Carbopol containing media, the mycelia grown at lower pH values had a greater number of branch initiation points than those at higher initial pH values. Photomicrographs of mycelia from Carbopol-containing cultures, having initial pH values of 4.0 and 6.0, after a 10 h incubation, are illustrated in Figure 3.7. Hyphal development was more extensive at the higher initial pH values with mycelium tending to form pellets at an initial pH of 5.0 and 6.0 in the absence of Carbopol. There is no significant difference in the extent of branch initiation in mycelia in the absence of Carbopol at all four pH values. The extent of branch initiation was intermediate between that of mycelia at low initial pH and high initial pH in the presence of Carbopol.

In control samples at initial pH values of 3.0 and 4.0, stained with Calcofluor, the entire hypha fluoresced slightly with very bright fluorescence at the extension zone and branch points. In the corresponding media at initial pH of 3.0 and 4.0 containing Carbopol, the only fluorescence observed was that of the insoluble polymer particles binding Calcofluor, both in the background and also where clumps had gathered on the hyphae. When these hyphae were washed to remove the Carbopol particles, only very weak hyphal fluorescence was observed, with the mycelia barely visible under uv illumination. Mycelia from media having an initial pH of 5.0 and 6.0 with and without Carbopol fluoresced slightly throughout with strong fluorescence at extension zones and branch points. Mycelia from CMC media showed characteristic fluorescence at the apex and

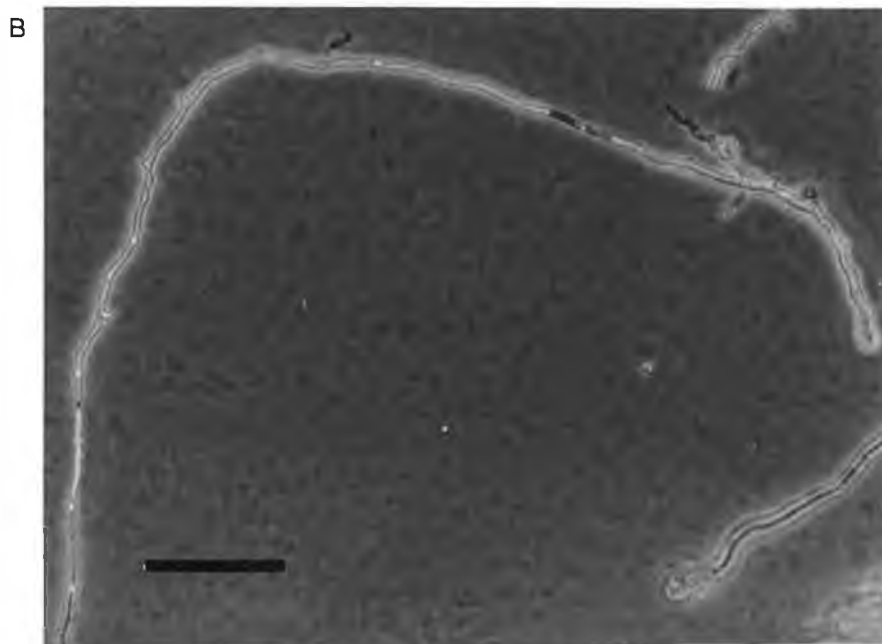
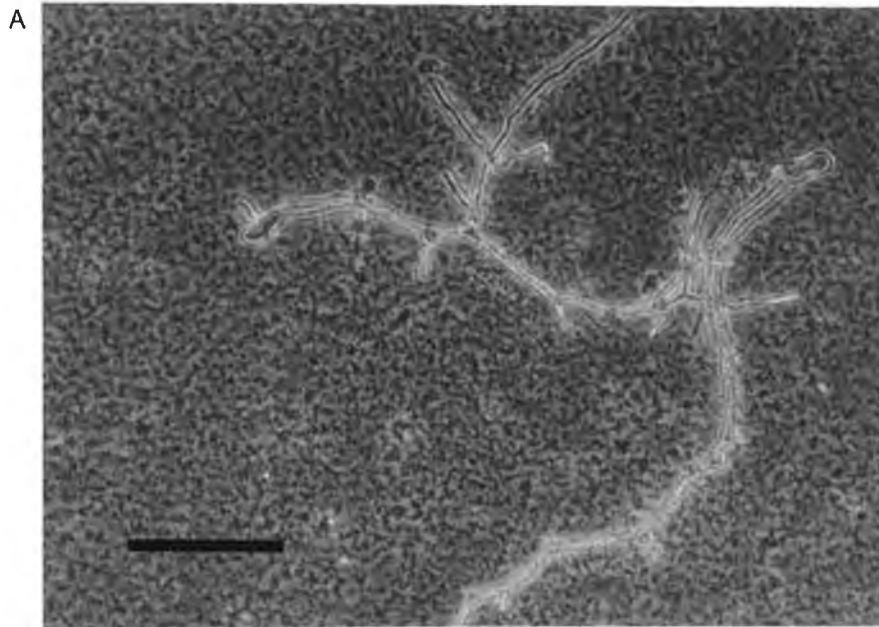


FIG. 3.7 Mycelia from Carbopol containing cultures at pH 4.0(A) and pH 6.0(B) after 10h cultivation.

Bar = 100 μ m.

branch points at all pH's. Figure 3.8a illustrates a fluorescent photomicrograph of hyphae recovered from Carbopol-containing media, pH 4.0. Normal fluorescence of extension zones and branch points when stained with Calcofluor is illustrated in Figure 3.8b.

When Calcofluor was added to a solution of Carbopol at pH 4.0, the insoluble particles of polymer were observed to fluoresce dimly. Carbopol is soluble in aqueous solutions at pH 6.0 and no fluorescence was observed. This confirms the ability of insoluble, weakly ionized polymer to bind Calcofluor.

These results suggest that mycelia, grown in Carbopol at the lower pH values, have undergone a compositional alteration in hyphal components binding Calcofluor or that Carbopol blocked Calcofluor binding by forming a layer over the hypha. An additional possibility was that at low pH values, Calcofluor preferentially binds to insoluble Carbopol particles, leaving none of the marker available to bind to mycelium. Since similar patterns of fluorescence can be obtained by suspension and shaking of grown mycelia in solutions at pH 3.0, 4.0, 5.0, 6.0 for 30 min, with and without Carbopol, compositional alteration of hyphae during growth in Carbopol is not needed to effect the change in fluorescent patterns.

3.2.7 Binding of Carbopol by Mycelia

To investigate the hypothesis that Carbopol binds to mycelia at low pH, equal amounts of mycelia, grown in the absence of Carbopol, were suspended in 100 ml aqueous solutions containing 0.3% (w/v) Carbopol at pH 3.0, 4.0, 5.0 and 6.0 and shaken for 30 min.

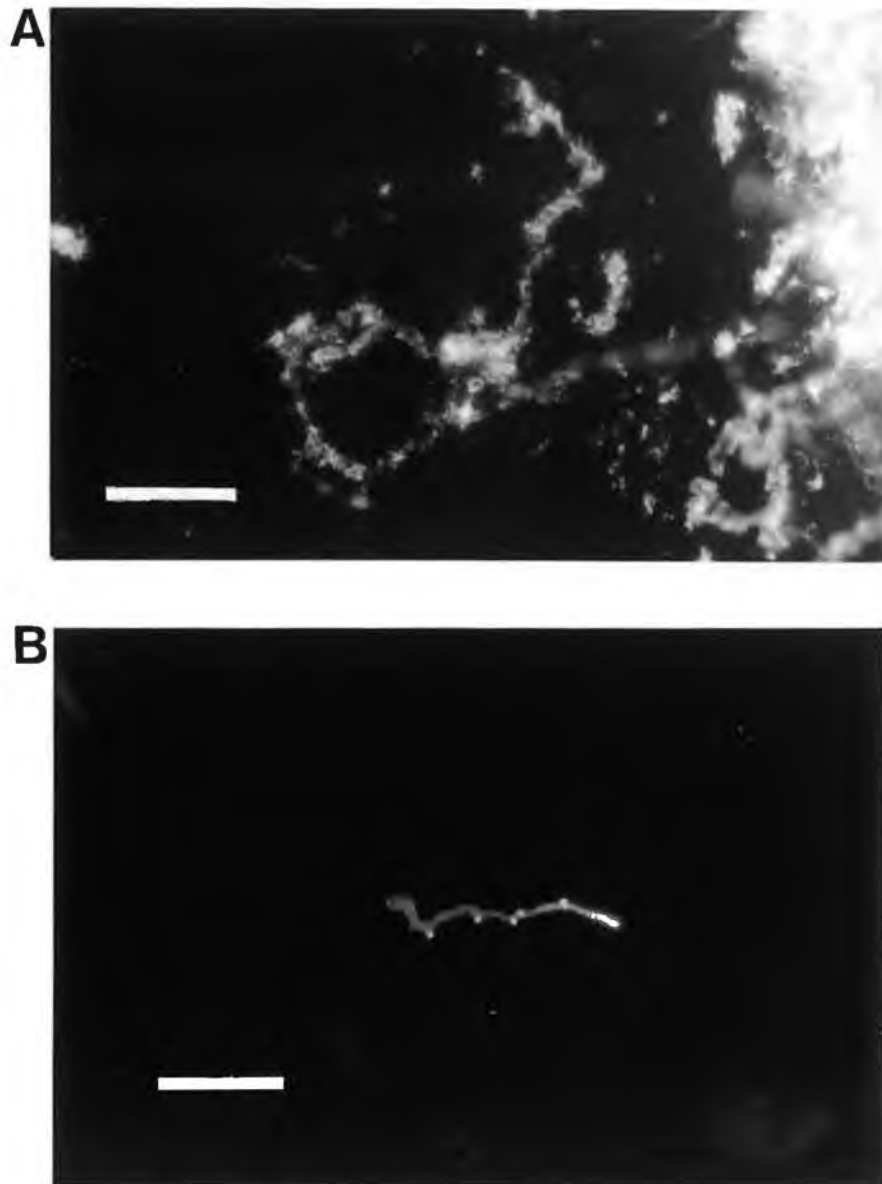


FIG. 3.8 Fluorescence observed after staining of mycelial samples with Calcofluor White M2R.

- A. Mycelia from Carbopol-supplemented media at pH 4.0, showing fluorescence of associated insoluble Carbopol particles.
- B. Normal fluorescence pattern of mycelia after staining with Calcofluor White M2R, with visualisation of extension zone and branch points.

Bar = 100 μ m.

Control mycelial samples were suspended in aqueous solution containing no Carbopol. Mycelia were then harvested by filtration. The mycelium was washed once by resuspension and shaking in 100 ml distilled water and then refiltered. In order to wash with two and three volumes of distilled water, this process was repeated once and twice respectively. Dry weights of the washed mycelial preparations were determined and their ability to bind Calcofluor White was evaluated. The results are presented in Table 3.10.

At all pH values, washing decreased biomass dry weight although in the case of pH 3.0 and 4.0 biomass did not drop to the level of the control. In the latter cases, washing resulted in improved fluorescence, but not at the level of the control. At pH 5.0, repeated washing did decrease dry weight to a level similar to that of the control, again with improved fluorescence but, nevertheless, the washed mycelia did not manifest the characteristic bright fluorescence of the control. While this latter observation appears inconsistent, good agreement was obtained between the duplicate biomass data.

3.2.8 Examination of Carbopol-grown Mycelia using Scanning Electron Microscopy

Mycelia, grown with and without Carbopol at an initial pH of 4.0 for 24 h, were recovered by filtration, washed and examined using scanning electron microscopy. Electron micrographs of specimens prepared using standard glutaraldehyde/osmium tetroxide fixation techniques, as described in Section 2.17.1.3, are illustrated in Figure 3.9

TABLE 3.10: Effect of extent of washing of mycelia of R. arrhizus on dry weight and observed fluorescence after treatment with Calcoflour White

	Wash 1		Wash 2		Wash 3	
	Dry wt. mg/100 ml	Fluor- escence	Dry wt. mg/100 ml	Fluor- escence	Dry wt. mg/100ml	Fluor- escence
Control	219	++	208	++	210	++
pH 3.0	249	-	229	+	230	+
pH 4.0	305	-	231	+	222	+
pH 5.0	247	+	208	+	205	+
pH 6.0	258	+	213	++	211	++

The results are the mean of duplicate analyses.

Mycelia isolated from control medium were suspended in Carbopol solutions of pH 3.0, 4.0, 5.0 and 6.0 and shaken for 30 min. Mycelial dry weight and extent of fluorescence were determined after different degrees of washing.

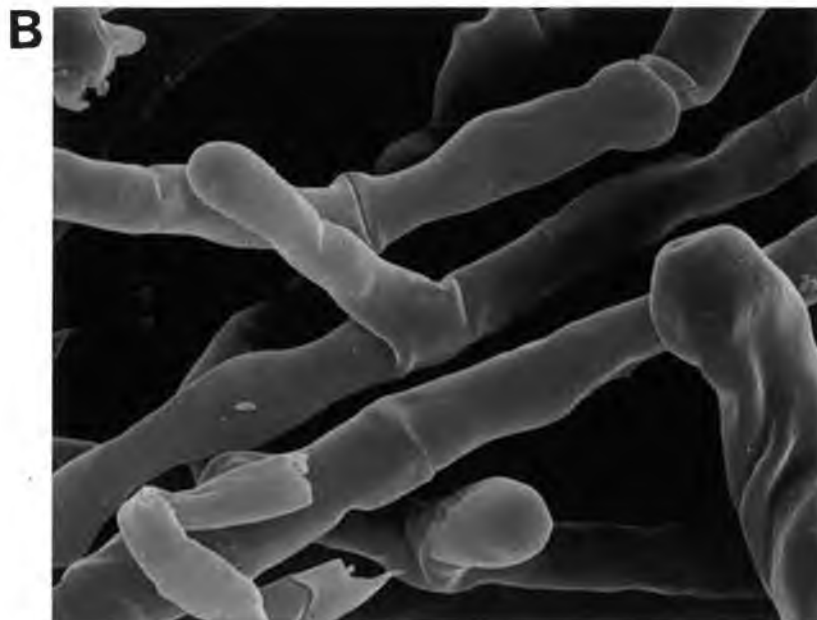
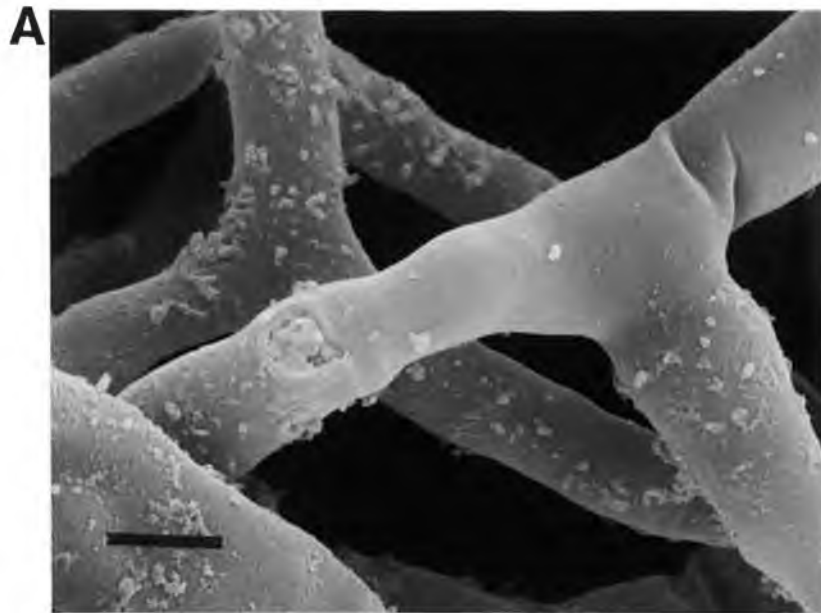


FIG. 3.9

Scanning electron micrographs of mycelia prepared using glutaraldehyde/osmium tetroxide fixation of specimen.

A. Mycelia grown in Carbopol-supplemented medium at pH 4.0 with insoluble particles adhering to mycelia.

B. Mycelia grown in control medium at pH 4.0.

Bar = 5 μ m

Clumps of insoluble material were seen to be associated with hyphae taken from the Carbopol-containing media. Due to the possibility that the adhering material might be an artifact caused by the extensive fixation procedure required, a cryogenic preparative technique, using no chemical fixation, was also employed for comparative purposes. Photomicrographs of samples prepared using cryogenic techniques are illustrated in Figure 3.10

A web-like matrix was associated with hyphae from Carbopol media but not in the control. This matrix is the result of partial removal of water from the specimen by etching, leaving the insoluble material adhering to the hyphae. While this is an unrealistic image of the mycelial matrix as it exists in the media, it supports the conclusion drawn from Figure 3.9 that insoluble material appears to be associated with the mycelia. The matrix was also seen to survive thorough washing. Washed mycelia, taken from Carbopol containing media at pH 6.0 had much less matrix material.

3.2.9 Staining of Polymer-grown Mycelia with Ruthenium Red

Ruthenium Red is a positively-charged, high molecular weight compound used to stain anionic materials. Mycelia grown in control, CMC, CP-4.0 and CP-5.5 media were harvested after a growth period of 24 h, washed and stained with ruthenium red as described in Section 2.8.2. The stained samples were wet mounted and examined using phase contrast microscopy. The photomicrographs are shown in Figure 3.11

Mycelia from CP-4.0 medium were seen to have clumps of the red dye adhering at random points to the mycelia. Mycelia taken from

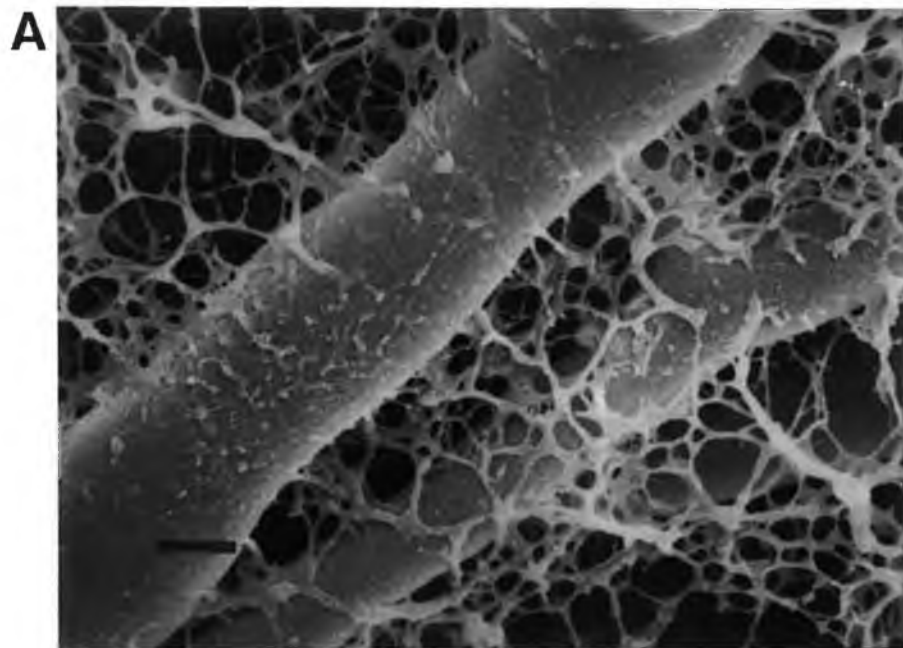


FIG. 3.10 Scanning electron micrographs showing mycelia prepared using cryogenic techniques.

A. Mycelia grown in Carbopol-supplemented medium at pH 4.0, with web-like matrix remaining after the water in the specimen has been etched off.

B. Mycelia grown in control medium at pH 4.0.

Bar = 3 μ m.

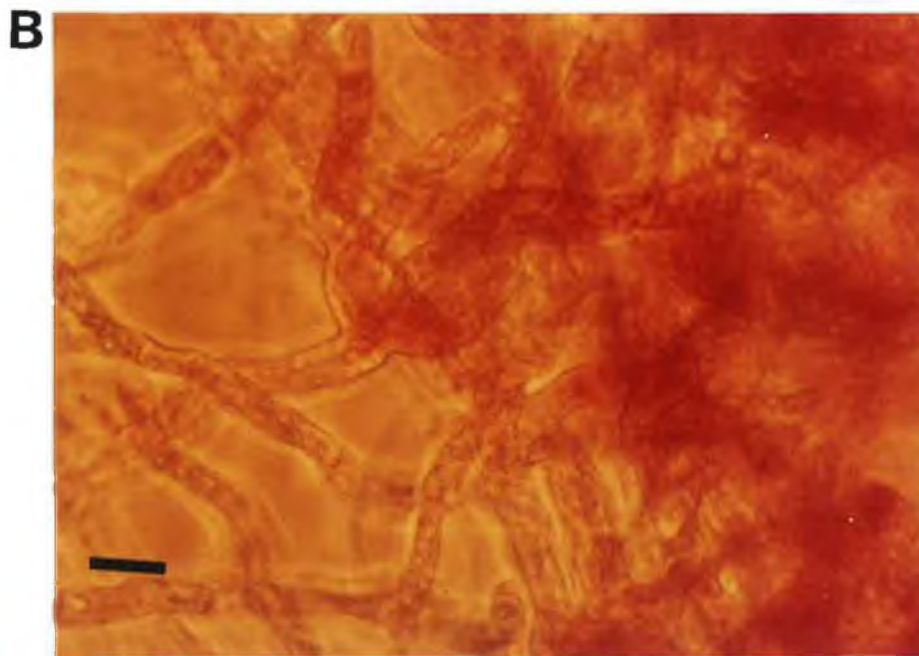
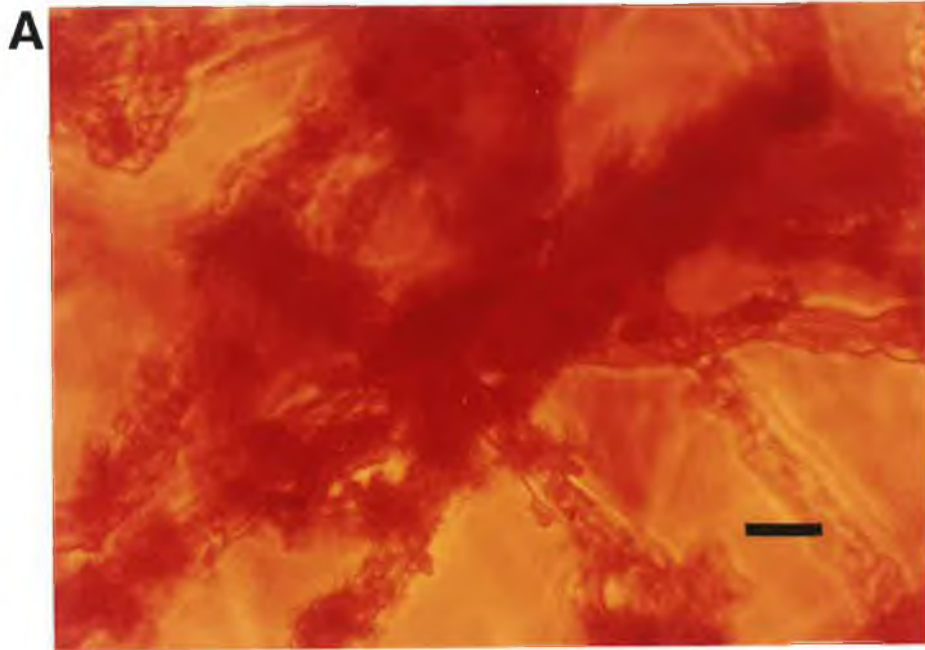


FIG. 3.11 Mycelia from (A) Carbopol-supplemented media and (B) control medium, stained with Ruthenium Red (Light microscopy).

Bar = 10 μ m

CP-5.5 medium did not have such widespread staining, but clumps of dye were present in places. Mycelia from control medium and CMC medium did not demonstrate any specific staining. The random staining of the Carbopol-grown mycelia may be due to interaction of the dye with the anionic carboxyl groups of the polymer adhering to the mycelia.

3.2.10 Summary

Pelleting commences at 9-10 h after inoculation with R. arrhizus and not at a pregermination stage as for other fungi (Elmayergi et al., 1973; Galbraith and Smith, 1969). The specific growth rate of mycelia in control and in polymer-supplemented media was similar, until growth became restricted in control medium.

Polymer supplementation promoted increased biomass production, but in the case of Carbopol, this increase may be partly due to the tightly bound polymer, as indicated by scanning electron microscopy or biomass studies, particularly at low pH's. Hyphal extension zones and branch points of mycelia from Carbopol-supplemented media at low pH did not fluoresce when treated with Calcofluor white, unlike those from control or CMC medium.

3.3 INVESTIGATION OF CELL WALL COMPOSITION OF DIFFERENT MYCELIAL FORMS OF R. ARRHZUS

Changes in cell wall composition associated with changes in morphology have been observed in several species of fungi (Section 1.5.7). The possibility that the different mycelial forms of R. arrhizus observed in polymer-supplemented media were accompanied by changes in bulk cell wall chemical composition or physical

properties was investigated. Chemical composition was analysed using specific assays for cell wall components in combination with acid/alkali extraction. Analysis of the microfibrillar chitin/chitosan component was performed using X-ray diffraction analysis and uptake of N [¹⁴C-acetyl]-glucosamine studies. Gross surface physical differences were investigated using transmission electron microscopy.

3.3.1 Analysis of Cell Walls by Infrared Spectroscopy

The detection of major structural differences between the four cell wall samples was carried out using infrared spectroscopy.

The infrared spectrum of each of the cell walls isolated from control, CMC, CP-4 and CP 5.5 media was recorded using the KBr disc method (Section 2.17.6). A representative spectrum can be seen in Figure 3.12. A summary of the absorption bands obtained and what groups they represent is shown in Table 3.11.

No major structural differences were observed between the spectra of the four cell wall samples.

3.3.2 Enzymatic Hydrolysis of Cell Wall of R. arrhizus

The 24 h cell walls isolated from control, CMC, CP-4.0 and CP-5.5 media were each incubated with a range of enzymes in order to establish whether compositional differences in the walls gave rise to differences in susceptibility to the enzymes.

A protease (Type XVI, B. subtilis), cellulase (celluclast T, T. viride) and β -glucuronidase (EC 3.2.1.31 Type H-2, Helix pomatia)

100

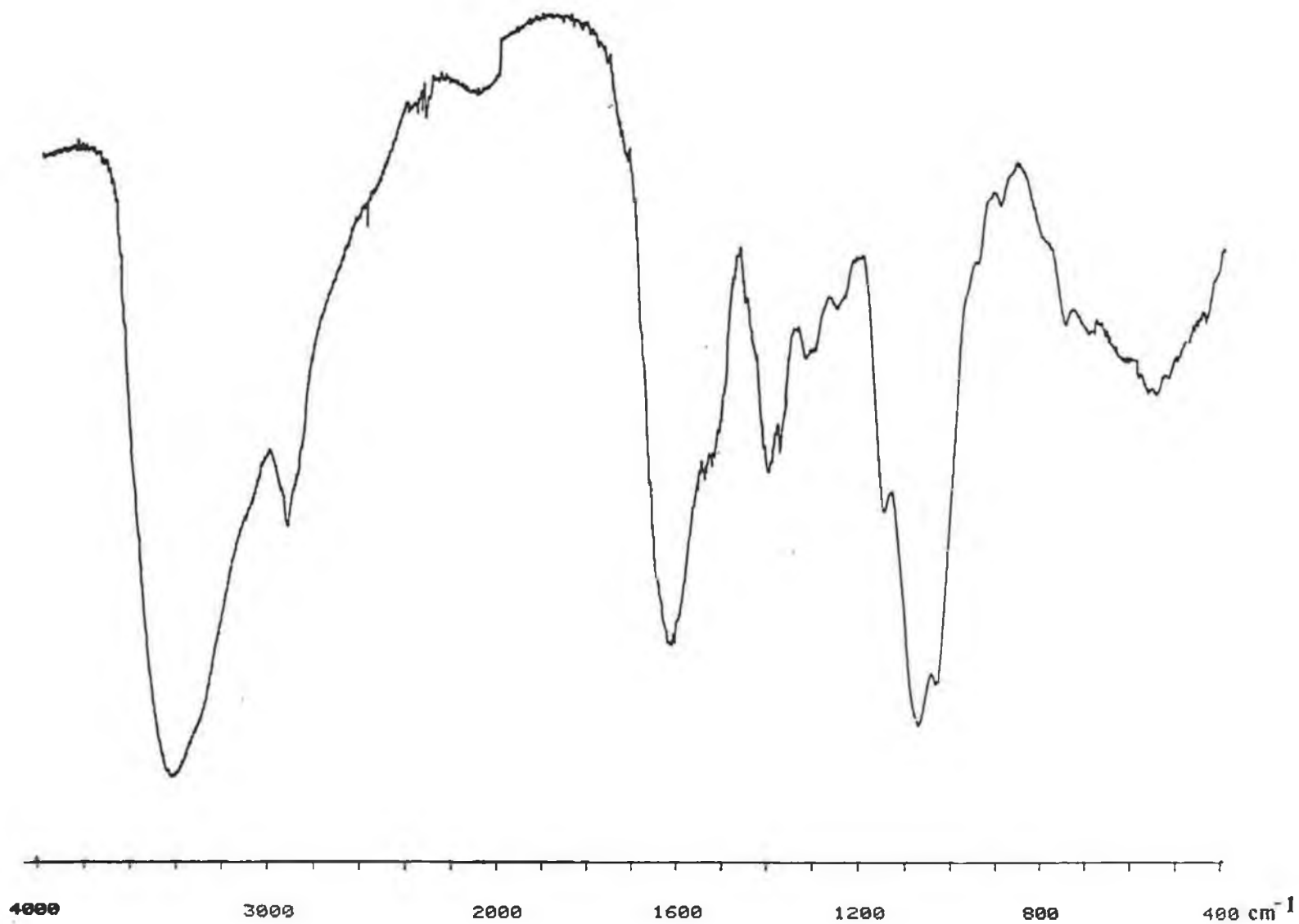


FIG. 3.12 Infrared spectrum of native fungal cell wall of R. arrhizus
The sample was prepared using the KBr disc method.

TABLE 3.11: Major absorption bands found in cell wall of R. arrhizus

Frequency (cm ⁻¹)	Group represented
3400	O-H stretch, broadened by hydrogen-bonding
2900	Aliphatic C-H stretch in C-CH ₃ or C-CH ₂
1650	C=O stretch of amide; also N-H of NH or NH ₂ group
1550	Aromatic C-C stretch
1400	C-H stretch in C-CH ₃
1340 - 1250	C-N stretch in ArNH ₂ or ArNHR
1100	C-O stretch in aliphatic ether

The infrared spectra of the cell wall samples isolated from control, CMC, CP-5.5 and CP-4.0 media were obtained using the KBr disc method.

were each incubated separately under the appropriate optimum conditions with samples of cell wall isolated from the four media (See section 2.16.3 for conditons). But analysis of the mixture supernatants for neutral sugars (anthrone reaction), uronic acids (carbazole method) or protein (Bradford reagent) failed to reveal any solubilised wall components. A combination of enzymes was then used in order to evaluate whether three enzymes working together would be more effective in breaking down the wall. The three enzymes were the protease and β -glucuronidase as described above, and a chitinase (Sigma). This mixture of enzymes was incubated using optimum conditions as described in Section 2.16.3 for 18 h with each of the cell wall samples. The supernatants were then assayed for neutral sugars and uronic acids. The results are presented in Table 3.12.

A similar amount of material was released from each type of cell wall, but this amount was very small. Enzymatic degradation is not an efficient means of investigating the structure of R. arrhizus 2582 cell wall.

3.3.3 Chemical Composition of Cell Wall

The bulk chemical composition of the four cell wall samples was investigated. Mycelia were harvested from each medium after 12 and 24 h, their cell walls isolated and their chemical analysis carried out (Table 3.13).

In the case of the control, CMC and CP-5.5, these analyses accounted for 77-91% of the cell wall. In the case of CP-4.0, the percentage of cell wall accounted for was significantly lower

TABLE 3.12: Breakdown of fungal cell wall by incubation with Protease/Chitinase/ β -glucuronidase mixture

Cell Wall Isolated from:	% Dry Wt. Cell Wall Released as	
	Neutral Sugars	Uronic Acids
Control	3	3
CMC	3	2
CP-4.0	3	4
CP-5.5	4	3

The cell wall samples were each incubated for 18 h in the protease/chitinase/ β -glucuronidase mixture at 30°C/pH 6.2. The supernatant was then analysed for released neutral sugars or uronic acids.

TABLE 3.13: Chemical composition of 12 and 24 h old hyphal walls of R. arrhizus

Component	Cell walls isolated from:							
	Control		CP-4.0		CMC		CP-5.5	
	24h	12h	24h	12h	24h	12h	24h	12h
Neutral sugars	13 [*] ±0.7	12±0.9	11±0.8	8±1.2	16±1.1	13±1.0	15±1.0	14±0.6
Uronic acids	19±1.5	20±1.3	16±1.4	14±1.3	22±1.2	14±1.1	22±1.4	22±1.7
Protein	18±1.1	15±1.8	13±2.1	16±1.5	7±1.4	9±1.3	6±1.6	8±1.1
Phosphate	7±1.3	5±0.9	4±1.1	4±1.2	5±1.9	4±1.6	2±0.5	3±0.8
Hexosamines	24±1.3	23±2.1	23±0.9	21±2.3	34±2.0	31±2.5	35±1.2	34±2.4
Ash	9±2.9	6±1.7	7±1.8	7±2.5	7±2.5	6±2.2	6±2.7	5±1.9
Total %	90	81	76	70	91	77	86	86

* All data are as a percentage of dried cell wall.

Neutral sugars, uronic acids and phosphates were determined in untreated hyphal wall; protein was determined in the alkali-soluble portion; hexosamines were determined in Amberlite hydrolysate (See Materials and Methods).

All data presented are the mean of four individual cell wall analyses, ± the standard error.

(70-73%). The concentration of hexosamines in the long, sparsely branched mycelia from CMC and CP-5.5 media was substantially higher than that seen in the pelleted or more highly branched dispersed mycelia from the control and CP-4.0 media respectively. The protein content of the latter mycelia was considerably higher than that from CMC and CP-5.5 media. The 24 h hyphal control sample had a particularly high protein content of 18%. The uronic acid content of cell walls from CMC medium at 12h was significantly lower than that of the 24 h walls, i.e. 14% vs 22%. The data from the amino acid analysis of the four samples is presented in Table 3.14. The overall proportion of each type of amino acid is similar in all four cell wall types, with a high concentration of the acidic aspartic and glutamic acids being noted in each.

The neutral sugar and uronic acid component of the hyphal wall samples was determined qualitatively using thin layer chromatography to separate the components solubilised using mild H_2SO_4 hydrolysis (Section 2.15.2). Fucose, mannose, galactose and glucuronic acid were observed in each of the 24 h old cell walls from the 4 different media.

3.3.4 Acid/Alkali Extraction of Cell Walls

Acid and alkali extraction were used to gain greater insight into the manner in which the components are linked in the walls. The isolated cell walls from mycelia recovered after a 24 h cultivation were extracted with nitrous acid and potassium hydroxide as described in Section 2.16. The insoluble residues were analysed for neutral sugars and uronic acids. The results of an analysis using these extraction procedures are presented in Table 3.15.

TABLE 3.14: Amino acid composition of hyphal cell walls of R. arrhizus.

Cell walls isolated from:				
Amino Acid	Control	CP-4	CMC	CP-5.5
Aspartic acid	4.0	2.4	1.5	0.8
Threonine	1.4	1.1	0.8	0.5
Serine	1.6	1.2	0.8	0.5
Glutamic acid	3.1	2.2	1.0	0.7
Glycine	1.3	1.1	0.5	0.4
Alanine	1.6	1.3	0.7	0.5
Valine	1.3	1.0	0.4	0.0
Isoleucine	1.6	0.7	0.4	0.3
Leucine	2.0	1.1	0.6	0.3
Totals	17.9	12.1	6.7	4.0

All data are as percentage of dried cell wall.

The cell wall samples were hydrolysed using the Amberlite hydrolysis protocol (Section 2.15.1). 200 µl aliquots of the hydrolysate were then applied to the Amino Acid analyser.

TABLE 3.15: Extraction of hyphal cell walls

Extraction		HNO ₂	KOH	KOH of HNO ₂ Residue
Cell wall residue after extraction* (% of original dried cell wall)				
Control		38±1.0	49±2.5	14±2.2
CP-4		36±2.1	48±2.8	11±2.8
CMC		25±1.7	62±3.4	11±3.1
CP-5.5		25±2.2	65±3.1	14±2.7
Composition of residue** (% of original dried cell wall)				
Control	Neutral sugars	2.0±0.7	6.0±1.5	0.7±0.3
	Uronic acids	4.0±1.0	7.0±1.6	0.3±0.2
CP-4	Neutral sugars	2.0±0.8	5.0±1.5	0.7±0.3
	Uronic acids	3.0±1.1	7.0±1.2	0.4±0.2
CMC	Neutral sugars	2.0±1.0	6.0±1.6	1.0±0.5
	Uronic acids	5.5±1.4	6.0±1.5	0.4±0.2
CP-5.5	Neutral sugars	2.0±1.0	7.0±1.9	0.8±0.3
	Uronic acids	5.0±1.1	8.0±2.0	0.5±0.2

*Mean of four determinations ± the standard error.

**Mean of two determinations ± the standard error.

Cell wall samples were extracted using HNO₂ or KOH. The neutral sugar and uronic acid content of the residues were determined.

A greater proportion of the wall constituents was released from cell walls from CMC and CP-5.5 using nitrous acid extraction, than from cell walls from the control and CP-4.0. This would be expected considering the higher hexosamine content of these two types of wall. However, the composition of residues remaining after nitrous acid extraction of each of the four wall types was similar. In contrast, cell walls from the control and CP-4.0 had a higher proportion of potassium hydroxide soluble components than those from CMC and CP-5.5. The results from the two extractions suggest that a greater proportion of glucuronan is held in non-glucosamine linkages in cell walls from the control and CP-4.0 media compared to those from CMC and CP-5.5 media.

3.3.5 Investigation of Skeletal Wall Components

Skeletal wall components such as chitin can be identified using X-ray diffraction due to their crystalline nature. Native cell walls were extracted with nitrous acid, followed by potassium hydroxide and their X-ray diffraction patterns recorded. The radial density tracings are demonstrated in Figure 3.13

In cell walls from the control and CMC media, which were both grown in the absence of Carbopol, the characteristic reflections of chitin became sharper as the non-crystalline matrix components of the wall were removed by the acid and alkali extractions. However, these characteristic reflections were not very sharp in the cell walls isolated from CP-5.5 medium and were particularly weak in the cell walls isolated from CP-4.0 medium.

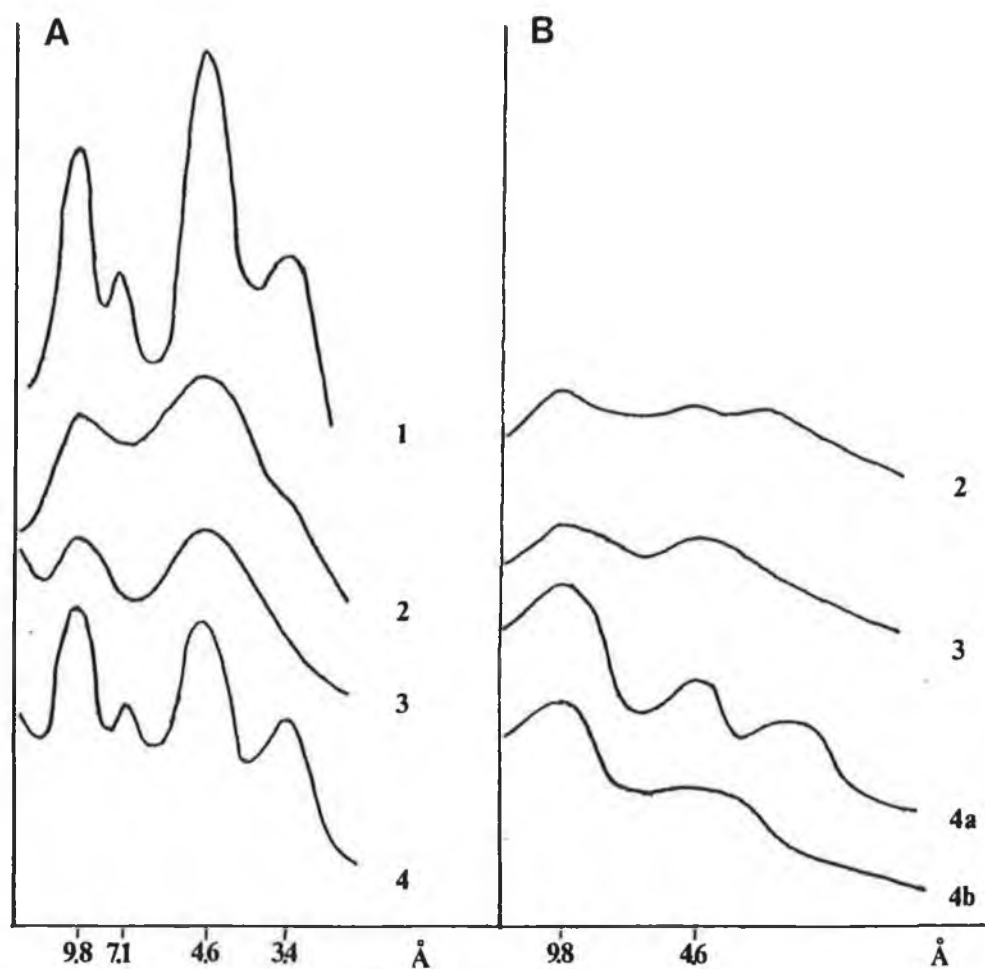


FIG. 3.13 Radial density tracings from X-ray powder diagrams.

- A. Cell walls isolated from control or CMC media.
 B. Cell walls isolated from CP-4.0 or CP-5.5 media.
1. Chitin (crustacean).
 2. Native cell wall.
 3. Residue after nitrous acid extraction of cell walls.
 4. Residue after alkali extraction of nitrous acid residue.
 - 4a. Residue as for 4 but cell walls isolated from CP-5.5.
 - 4b. Residue as for 4, but cell walls isolated from CP-4.0.

3.3.6 Surface Examination using Transmission Electron

Microscopy

Surface characteristics of native cell walls and cell wall residues after nitrous acid and potassium hydroxide extraction were examined using transmission electron microscopy following heavy metal shadowing of the specimens. Transmission electron micrographs of platinum/carbon coated cell wall samples are shown in Figure 3.14.

The surface texture of native cell walls from CMC medium (Figure 3.14b) was more rugged, more amorphous than that of the other three samples which had a smoother, slightly granular appearance (Figure 3.14a). This effect was seen repeatedly, with every batch of walls examined, and thus is not just a random occurrence, such as the inside wall being exposed instead of the outer wall.

The microfibrillar network is more clearly exposed in the nitrous acid residue of walls from control or CMC media compared to those from Carbopol-supplemented media (Fig. 3.14c and d).

Transmission electron micrographs of the residue remaining after alkali-extraction of nitrous acid residue of the cell walls are shown in Figure 3.14e and f. Clearly exposed interweaving microfibrils of varying width and orientation are revealed in cell walls from the control and CMC (Figure 3.14e). The microfibrils from cell walls from CP-4.0 and CP-5.5 are similar except that the bundles are less organised and intact, especially in cell walls from CP-4.0 (Figure 3.14f). The tightly interwoven microfibrils appear to have been loosened by the extraction procedures.

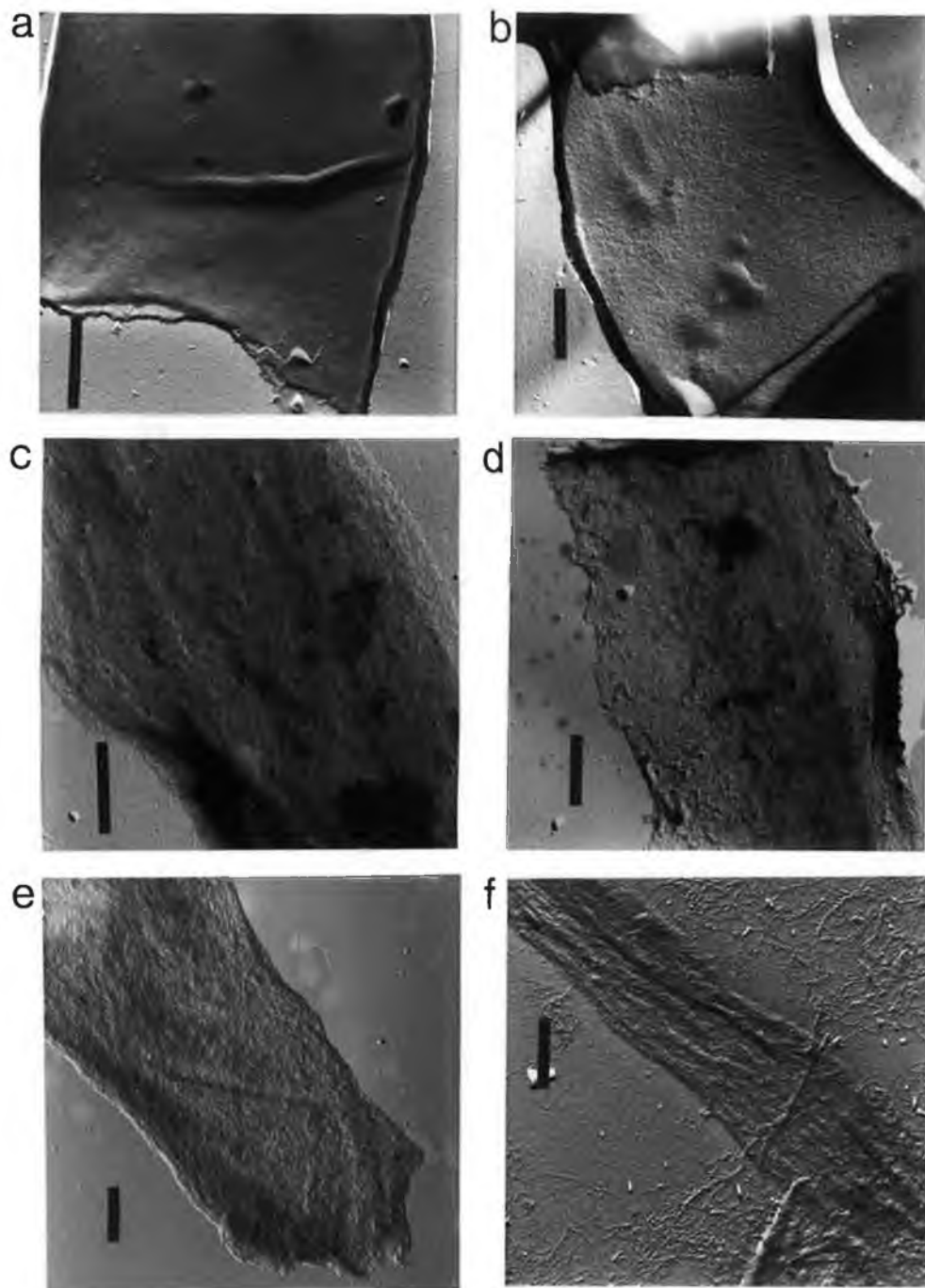


FIG. 3.14 Transmission electron micrographs showing cell walls shadowed with Pt/C at 30°.

- A. Native cell wall isolated from either control, CP-4.0 or CP-5.5 media.
- B. Native cell wall isolated from CMC medium.
- C. Residue remaining after HNO_2 extraction of cell walls from control or CMC media.
- D. Residue as for C, but for cell walls from CP-4.0 or CP-5.5 media.
- E. Residue remaining after alkali extraction of HNO_2 residue of cell walls from control or CMC media.
- F. Residue as for E, but for cell walls from CP-4.0 or CP-5.5 media.

Bar = 500 μm

3.3.7 Labelling of Hyphal Walls using N-[¹⁴C-acetyl]-glucosamine

Incorporation of N-[¹⁴C-acetyl]-glucosamine into the wall and subsequent extraction with nitrous acid allows a comparison between the amount of 'radioactivity' solubilised and that which remains insoluble for the different cell wall samples. The radioactivity in the soluble portion represents the N-acetylglucosamine which is linked to nitrous-acid susceptible glucosamine homopolymers via the reducing end. There is the possibility that some heavily deacetylated chitin residues, which still retain a few labelled acetyl groups, may also be solubilised. The insoluble radioactive fraction represents a homopolymer of N-acetylglucosamine, i.e. chitin. Thus, the distribution of the radioactivity between the soluble and insoluble fractions allows an assessment of the proportion of chitosan:chitin in the four cell wall samples. The possible relevance of the ratio of chitosan:chitin to the mycelial morphology may then be assessed.

3.3.7.1 Solubilisation of N-acetylglucosamine using Nitrous acid

Extraction:

12 and 24 h old cultures of R. arrhizus in control, CMC, CP-4.0 and CP-5.5 media were pulse-labelled for 1 h with N-[¹⁴C-acetyl]-glucosamine (for conditions see Section 2.8.1). The mycelia were then harvested, washed and their cell walls isolated. A nitrous acid extraction was carried out on duplicate samples from each of the four samples. The amount of radioactivity in the insoluble and soluble portions was estimated. The results are presented in Table 3.16.

TABLE 3.16: Cell wall total label content and the percentages
in the nitrous acid-insoluble fractions

Medium	Cell wall total label content - dpm/mg		Label content in nitrous acid- insoluble fractions % of total	
	12h	24h	12h	24h
Control	7576 ± 477	3549 ± 105	59 ± 3	70 ± 3
CMC	10214 ± 385	3529 ± 320	57 ± 2	67 ± 5
CP-4.0	4254 ± 86	2265 ± 93	55 ± 2	64 ± 4
CP-5.5	7088 ± 391	2412 ± 136	55 ± 4	66 ± 3

The data are the means of three batches of cell wall ± standard deviation.

Mycelia from the four media were pulse-labelled for 1 h with N-(¹⁴C-acetyl)-glucosamine, their cell walls isolated and a nitrous acid extraction carried out. The total label content of the cell wall samples and the label content in the nitrous acid residues were estimated.

A much higher concentration of radioactivity was found in the 12 h cell walls compared to the 24 h walls, probably due to the higher specific growth rate of the mycelium at 12 h. Total cell wall label content was markedly higher in 12 h mycelia from CMC medium than in the control or Carbopol supplemented media. After 24 h, walls from Carbopol-supplemented media contained substantially less label than walls from the control or CMC media.

Walls from CP-4.0 or CP-5.5 media have a slightly lower percentage of radioactivity remaining insoluble after nitrous acid extraction, suggesting a possibly higher chitosan:chitin ratio in these cell walls.

The percentage radioactivity remaining insoluble after nitrous acid extraction in 12 h walls is much lower than that at 24 h walls, indicating that there is a greater amount of chitosan type polymer or short chain chitin polymer, linked to the chitosan and solubilised when it is depolymerised, in 12 h walls compared to 24 h walls.

3.3.7.2 Solubilisation of N-acetyl-glucosamine using Alkali

Extraction:

Extraction with potassium hydroxide causes the partial solubilisation of a glucuronan containing the neutral sugars and uronic acid present in the wall. The possibility that N- ^{14}C -acetyl]-glucosamine labelled material is linked to the glucuronan and thus released upon treatment with alkali, as with M. mucedo (Datema et al., 1977b), was investigated. Labelled 12 and 24 h cell walls were extracted with 1M KOH (Section 2.16.2) and the

amount of radioactivity present in the insoluble and soluble fractions assessed. The results are presented in Table 3.17.

Alkali extraction does not solubilise any significant amount of labelled material from the cell walls. This is as expected, as the linkages found in chitin/chitosan polymers are not susceptible to alkali. The small amount of radioactivity found in the soluble fraction represents labelled material that was released in the washing process.

3.3.8 Summary

The cell walls of dispersed mycelia from the more viscous media, i.e. CMC and CP-5.5, were seen to have a higher concentration of hexosamines (34-35%) and a lower concentration of protein (6-7%) compared to the cell walls of pelleted or less finely dispersed mycelia i.e. control or CP-4.0, respectively. Results from acid/alkali extraction indicated that a greater proportion of glucuronan is held in non-glucosamine linkages in walls from pelleted than from dispersed mycelia. The analysis of the chitin component of the cell walls by X-ray diffraction demonstrated less crystallinity in the cell walls isolated from Carbopol-containing media compared to the other cell wall types examined. These cell walls also had a lower concentration of N-[¹⁴C-acetyl]-glucosamine after labelling compared to control or CMC cell walls, suggesting a difference in the chitin: chitosan ratio between these walls.

TABLE 3.17: Solubilisation of N-(¹⁴C-acetyl)-glucosamine using IM KOH

Cell walls isolated from medium:	Label content in alkali-insoluble fractions (% of total)	
	12h	24h
Control	90 ± 3	87 ± 3
CMC	90 ± 2	92 ± 4
CP-4.0	91 ± 4	89 ± 4
CP-5.5	92 ± 3	94 ± 4

The data are the means of three analyses ± standard deviation.

Mycelia from the four media were pulse-labelled for 1 h with N-(¹⁴C-acetyl)-glucosamine, their cell walls isolated and a KOH extraction carried out. The label content in the KOH residues was determined.

3.4 INVESTIGATION OF RELATIONSHIP BETWEEN MYCELIAL MORPHOLOGY
AND PRODUCT FORMATION OF R. ARRHZUS

The relationship between mycelial morphology of R. arrhizus and product formation is investigated in this section.

The products/processes which are studied are:

- (i) glucoamylase
- (ii) fumaric acid
- (iii) biotransformation of progesterone to 11 α -hydroxy-progesterone

The effect of the mycelial growth form on the rate of production/ biotransformation of each of these substances was studied.

3.4.1 Glucoamylase Production and Mycelial Morphology

In order to investigate the relationship between morphology and glucoamylase production, preliminary studies on production of glucoamylase by R. arrhizus were carried out. Production using shake flask cultures, surface culture and vegetatively-inoculated cultures was assessed.

3.4.1.1 Glucoamylase Production by R. arrhizus in Supplemented Control Medium:

The effect of supplements to the standard control medium on production of glucoamylase by R. arrhizus was investigated. Glucose and starch were used as carbohydrate source with and without CaCO₃ incorporated as a buffering agent. Anionic polymers which affect morphology were used as supplements. Inoculation and growth were carried out under standard conditions

for 72 h. The results are presented in Table 3.18

Negligible levels of glucoamylase were observed in all cases. Consequently a wider range of medium components were tested. Production of glucoamylase was monitored at 24 h intervals over a 7 day incubation. For most combinations, maximum activity was observed after 168 h. The results are presented in Table 3.19.

The results indicate that substantially higher levels of glucoamylase activity are produced in media containing 5-10% maize.

The patterns of production of glucoamylase in media containing maize (5%), starch (5%) and glucose (5.5%), with bacteriological peptone (2%) as nitrogen source are presented in Figure 3.15.

The mycelia grew in a clumped, entangled manner in most of the complex media, with a slightly more dispersed growth form in media with insoluble maize as carbohydrate source. Thus no definite morphology could be ascribed to these samples. Therefore, difficulties were encountered in relating fungal morphology and glucoamylase production.

3.4.1.2 Production of Glucoamylase by R. arrhizus in Surface Culture:

Given the observation that maize containing media (semi-solid) resulted in production of substantially higher levels of enzyme than more soluble carbohydrate containing media, this line of investigation was extended to investigate enzyme production in surface culture. This study is particularly relevant with

TABLE 3.18: Glucoamylase production by R. arrhizus in supplemented control media

	Medium Supplements (g/l)					pH _i	pH/72h	Glucoamylase (units/ml)	Morphology	
	Glucose	Starch	CaCO ₃	Sodium Alginate	CMC					Carbopol
	20.0	-	-	-	-	-	5.9	2.4	0.07	Pelleted
	20.0	-	-	20.0	-	-	6.0	3.0	0.03	Dispersed
	20.0	-	-	-	20.0	-	6.1	3.4	0.08	Dispersed
	20.0	-	-	-	-	3.0	4.0	2.4	0.02	Clumped, Dispersed
	20.0	-	10.0	-	-	-	6.1	6.8	0.02	Pelleted
	20.0	-	10.0	-	20.0	-	6.5	6.7	0.11	Dispersed
	20.0	-	10.0	-	-	3.0	6.4	6.6	0.09	Pelleted
	-	10.0	-	-	-	-	7.5	8.6	0.05	Pelleted
	-	10.0	-	20.0	-	-	4.5	3.9	0.03	Dispersed
	-	10.0	-	-	20.0	-	7.4	6.3	0.09	Dispersed
	-	10.0	-	-	-	3.0	4.2	4.4	0.10	Clumped, Congealed
	-	10.0	10.0	-	-	-	7.0	8.6	0.12	Poor Growth
	-	10.0	10.0	-	20.0	-	7.5	8.1	0.01	Dispersed
	-	10.0	10.0	-	-	3.0	4.4	4.5	0.03	Clumped, Congealed

119

The results are the means of duplicate analyses.

Flasks were inoculated and incubated under standard conditions for 72 h. The morphology, pH and glucoamylase activity were then determined in each culture supernatant.

See Section 2.17.1 for morphology descriptions.

TABLE 3.19: Glucoamylase production by R. arrhizus in a range of media

	Medium Supplements (g/l)					pH _i	pH/168 h	Glucoamylase (Units/ml)	
	Máize	Starch	Glucose	Corn Steep Powder	Yeast Extract				Bactopeptone
15	-	-	-	10	-	-	5.7	7.2	0.55
50	-	-	-	10	-	-	5.8	5.8	0.82
100	-	-	-	10	-	-	5.8	9.3	0.80
50	-	-	-	20	-	-	5.4	7.5	0.61
100	-	-	-	20	-	-	5.9	4.6	0.65
20	-	-	-	-	20	-	5.9	8.7	0.38
50	-	-	-	-	20	-	5.9	7.9	1.02
20	-	-	-	-	-	20	6.3	8.3	0.34
50	-	-	-	-	-	20	6.2	6.9	0.88
-	-	20	-	20	-	-	5.8	6.7	0.16
-	-	55	-	20	-	-	5.5	4.2	0.16
-	-	20	-	-	20	-	5.8	7.8	0.06
-	-	55	-	-	20	-	5.7	7.0	0.07
-	-	20	-	-	-	20	5.8	7.5	0.16
-	-	55	-	-	-	20	5.5	3.9	0.08
-	20	-	-	-	-	20	6.5	8.2	0.03
-	50	-	-	-	-	20	6.1	4.2	0.05

120

Flasks were inoculated and incubated under standard conditions. The pH and glucoamylase activity were determined after 168 h.

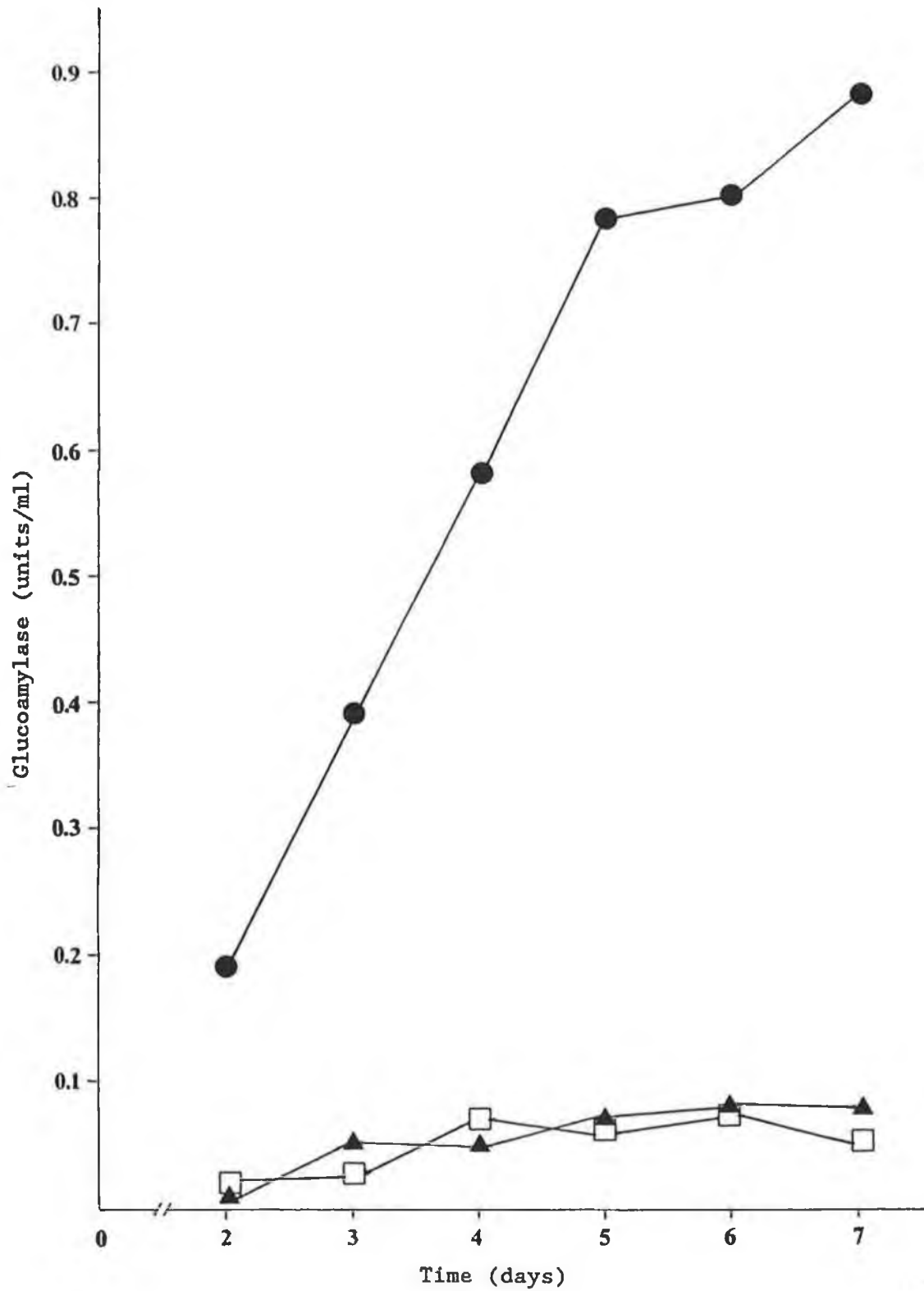


FIG. 3.15 Patterns of glucoamylase production in media containing bacteriological peptone with varying carbohydrate source.

- , Maize (5%)
- ▲, Glucose (5.5%)
- , Starch (5%)

R. arrhizus which grows very vigorously on agar and other solid surfaces and has been used traditionally in surface culture in Eastern fermentation processes (Wood and Yong Fook Min, 1975).

3.4.1.2.1 Effect of Moisture Level on Glucoamylase Production in Surface Culture: 250 ml erlenmeyer flasks containing bran (5g) and maize (5g) were supplemented with various volumes of distilled water. Flasks were inoculated with R. arrhizus (10^7 cells), incubated without shaking at 30°C (Section 2.6) and assayed for glucoamylase activity. The results are presented in Table 3.20.

The optimum volume of distilled water for production of glucoamylase was 7 mls. Glucoamylase activity increased steadily over the 7 day incubation period for this series of flasks, but was erratic for the other volumes used.

3.4.1.2.2 Effect of Medium Composition on Production of Glucoamylase in Surface Culture: The effect of medium composition on glucoamylase production in surface culture was investigated. Each flask contained 10 g total medium solids and 7 ml water. Glucoamylase activities after 72 h are presented in Table 3.21.

Optimal enzyme activity was observed with bran alone. Glucose supplementation appeared to depress activity. With maize-bran combinations, enzyme activity decreased with increasing maize content. Observations on growth suggest some correlation between level of growth and enzyme production.

TABLE 3.20: Effect of moisture content and incubation time on glucoamylase production in surface culture

Volume of H ₂ O in Medium (mls)	Glucoamylase (Units/g medium solids)				
	1 Day	2 Days	3 Days	5 Days	7 Days
3	0.7	1.2	1.4	1.0	4.1
5	2.3	5.4	18.0	14.0	12.0
7	2.2	19.5	21.0	22.0	30.0
10	3.0	16.0	12.4	11.5	24.0
20	3.1	8.5	5.0	12.4	14.8
30	3.7	7.1	3.7	4.2	8.3

Flasks containing bran (5 g) and maize (5 g) were supplemented with various volumes of distilled water, inoculated with 10^7 cells and incubated without shaking at 30°C. After various growth periods, flask contents were extracted and assayed for glucoamylase activity.

TABLE 3.21: Effect of medium composition on production of glucoamylase in surface culture

	Media composition (g)					Units Glucoamylase/g Solids	pH	Description of Growth	
	Bran	Maize	Starch	Glucose	Bactopeptone				Yeast Extract
	10	-	-	-	-	-	33.0	6.01	Good; sporulated
	8	-	-	-	2	-	23.0	6.98	Poor
	8	-	-	-	-	2	12.7	6.79	Poor
	8	-	2	-	-	-	16.6	5.45	Fair; sporulated
124	8	-	-	2	-	-	4.2	4.60	Poor
	7.5	2.5	-	-	-	-	28.2	5.64	Good; sporulated
	5	5	-	-	-	-	22.4	4.82	Good; beginning to sporulate
	4	4	-	-	-	2	29.5	6.52	Poor
	4	4	-	2	-	-	3.6	3.88	Some growth
	2.5	7.5	-	-	-	-	12.8	4.21	Good; beginning to sporulate
	-	10	-	-	-	-	3.5	3.68	Fair; sporulated

Flasks containing the solid medium components were inoculated with 10^7 cells and incubated at 30°C without shaking. Flask contents were extracted after 72 h and the pH and glucoamylase activity measured.

3.4.1.2.3 Investigation of effect of CMC and Carbopol on Glucoamylase Production in Surface Culture: The effect of CMC and Carbopol on glucoamylase production in media containing bran (10g) and distilled water (7ml) was investigated. CMC and Carbopol polymers were added at concentrations of 2% and 0.3% respectively to the water component. Inoculated flasks were incubated for 48, 72 and 96 h and glucoamylase activity determined. The results are presented in Table 3.22.

Similar vigorous growth was observed in all flasks and mycelia appeared alike when examined microscopically. Substantial differences in glucoamylase activity were not observed. It was noted that the fumaric acid content of the enzyme extracts after 72h growth were 46, 109 and 57 mg/l for the control, CMC and Carbopol-supplemented media, respectively. These values do not constitute any significant production of fumaric acid.

3.4.1.3 Investigation of Glucoamylase Production by Different Mycelial Forms:

In the enzyme production experiments described above, the effect of environmental conditions on growth and enzyme production cannot be separated. Attempts were made to separate the growth and enzyme production stages to try to relate rate of enzyme production to growth morphology. Mycelia having different morphological forms were recovered from 24 h cultures, grown under standard conditions in control, CMC, CP-4 and CP-5.5 media (Section 2.4), washed with distilled water and resuspended in 2% (w/v) bran solutions, supplemented with CMC (2%, w/v) or Carbopol (0.3%, w/v). Samples were incubated for 24 and 48 h and enzyme activities were

TABLE 3.22: Effect of CMC/Carbopol on glucoamylase production in surface culture

Supplement	Glucoamylase (Units/g medium solids)		
	48h	72h	96h
-	19	50	72
CMC	11	43	64
Carbopol	17	55	67

Flasks containing bran (10 g) and distilled water (7 mls) supplemented with 2% (w/v) CMC or 0.3% (w/v) Carbopol were inoculated with 10^7 cells and incubated at 30°C without shaking. The flask contents were extracted after 48, 72 or 96 h and assayed for glucoamylase activity.

TABLE 3.23: Effect of polymer supplements on glucoamylase production

Medium Supplement	Inoculum Mycelia isolated from:	24 h		48 h	
		pH	Glucoamylase (units/ml)	pH	Glucoamylase (units/ml)
-	Control	4.4	0.19	6.5	0.32
-	CMC	3.9	0.30	6.5	0.41
-	CP-4.0	5.8	0.17	6.4	0.26
-	CP-5.5	5.6	0.14	6.7	0.23
CMC	Control	5.5	0.15	7.1	0.48
CMC	CMC	5.2	0.30	7.2	0.72
CMC	CP-4.0	6.0	0.11	6.6	0.24
CMC	CP-5.5	5.7	0.11	6.8	0.24
Carbopol	Control	4.3	0	4.4	0
Carbopol	CMC	4.3	0	4.6	0
Carbopol	CP-4.0	4.6	0	4.3	0
Carbopol	CP-5.5	4.6	0	4.3	0

2% (w/v) bran solutions were inoculated with 24 h mycelia from control, CMC, CP-4.0 or CP-5.5 media and incubated for 24 and 48 h at 150 rpm/30°C. The pH and glucoamylase activity of the supernatants were then determined.

determined. The results are presented in Table 3.23.

Supplementation of bran solution with CMC caused increased production of glucoamylase after 48 h from control and CMC mycelia, but not from Carbopol-grown mycelia. The presence of Carbopol in the bran solution completely inhibited glucoamylase production, even though growth occurred over the 48 h incubation period. This might be due to the prevention of excretion of the enzyme caused by the adherence of Carbopol to the mycelia. Dispersed filamentous mycelia from CMC medium showed the highest glucoamylase production.

3.4.1.4 Comparison of Glucoamylase Yields in Surface and Submerged Culture:

The data from glucoamylase production in submerged culture must be converted from units/100 mls media to units/g solids in order to provide a basis for comparison of the data from submerged and surface culture. The data from spore inoculated media are all lower than those obtained with surface culture. However, the yields obtained with vegetatively inoculated media are more comparable, i.e. approximately 20 units/g solids glucoamylase produced after 48 h incubation of CMC-grown mycelia in 2 g Bran/100mls (Table 3.23). Thus the use of complex nutritive sources are essential for production of glucoamylase, making control of morphology difficult.

3.4.1.5 Appraisal of results:

The highest glucoamylase activities produced by R. arrhizus were observed when solid or semi-solid medium components were used. In

media where soluble medium components were used and so growth morphology easier to control and describe, very poor enzyme activities were recorded. Even though dispersed mycelia from CMC medium produced highest glucoamylase activities in trials using pregrown mycelia, the yields were so low that their significance is questionable. The presence of Carbopol in the bran solution completely inhibited enzyme production. No firm conclusion can be drawn regarding the relationship between morphology type and glucoamylase production.

3.4.2 Relationship between Fungal Growth, Morphology and Fumaric Acid Production

The effect of mycelial morphology, controlled by polymer supplements, on production of fumaric acid was investigated. The rate of production in batch culture and in laboratory fermenters using control medium supplemented with CMC or Carbopol, was assessed, along with other growth parameters such as pH, biomass and reducing sugar level. Because of the substantial variation in biomass production observed in these trials, the ability of biomass recovered from different medium types to convert glucose to fumaric acid was investigated in biotransformation experiments.

3.4.2.1 Production of Fumaric acid in Shake flask Cultures:

A preliminary investigation of the relative ability of R. arrhizus to produce fumaric acid in shake flask cultures in control and glucose-CSL-salts media was carried out. Each medium was supplemented with CMC and Carbopol to modify morphology. Calcium carbonate was added to one set of cultures as a buffering agent. Cultures were analysed after a 48 h incubation for growth

morphology, biomass content, pH, residual glucose and fumaric acid content. To ensure that fumaric acid did not bind to any of the media supplements, solutions of fumaric acid of known concentration were mixed with solutions of CMC, Carbopol and CaCO_3 , respectively and the fumaric acid content estimated. No uptake was detected. The results are presented in Table 3.24

It should be noted that each of the unsupplemented media, ie without additives, resulted in a pelleted morphology. Supplementation of these media with CMC or Carbopol resulted in production of dispersed or clumped dispersed growth. In some cases, growth morphology was modified by addition of calcium carbonate. Except in the case of the unsupplemented controls, glucose was essentially depleted at 48 h. Calcium carbonate contributed a significant buffering capacity to the cultures. Ranges of culture pH values in the absence and presence of calcium carbonate were 2.1-4.1 and 4.6-7.3 respectively. In all cases, medium supplements increased biomass production. Especially noticeable was the higher biomass level and congealed mass morphology observed in media supplemented with both Carbopol and calcium carbonate. The method of biomass determination normally allows the finely suspended calcium carbonate particles to be washed from the biomass. Although the possibility exists that some of these particles remain trapped in the congealed mycelium, it has not been possible to demonstrate this either microscopically or by adjusting the pH to solubilise calcium carbonate.

In the glucose-CSL-salts cultures, very little fumaric acid was produced. In contrast, fumaric acid levels ranged from 0.24-5.36

TABLE 3.24: Production of fumaric acid by *R. arrhizus* in supplemented control and Glucose-CSL-Salts media

Medium	Medium Supplements (g/l)			Growth morphology	Culture Analysis at 48 h			
	CMC	Carbopol	CaCO ₃		pH	Glucose (g/l)	Biomass (g/l)	Fumaric Acid (g/l)
Control	-	-	-	Clumped pellets	2.9	8.0	2.03	1.78
CMC	20	-	-	Dispersed	3.4	0.0	3.09	5.36
CP-5.5	-	3	-	Clumped dispersed	4.0	0.3	3.51	0.92
CP-4.0	-	3	-	Clumped dispersed	3.0	1.5	3.63	0.90
Control	-	-	3	Clumped pellets	4.6	0.4	3.43	2.62
CMC	20	-	3	Clumped dispersed	6.3	0.2	3.33	1.14
CP-5.5	-	3	3	Congeaed mass	6.3	0.4	6.49	0.24
CP-4.0	-	3	3	Congeaed mass	4.6	0.2	6.84	0.78
Glucose-	-	-	-	Pellets (2 mm dia.)	2.1	6.0	1.70	0.02
CSL-salts	20	-	-	Dispersed	4.1	0.6	3.29	0.02
	-	3	-	Clumped dispersed	2.7	0.2	2.54	0.03
	-	-	3	Clumped dispersed	7.3	0.2	2.20	0.12
	20	-	3	Dispersed	6.4	0.6	2.74	0.14
	-	3	3	Clumped dispersed	6.3	0.2	7.14	0.10

131

Flasks were inoculated and incubated under standard conditions. The culture pH, morphology, glucose content, fumaric acid content and biomass were determined after 48 h growth.

See Section 2.17.1 for morphology descriptions.

in control cultures and varied with medium supplement. The control medium, with and without calcium carbonate supplementation, produced 1.78 and 2.62 g/l fumaric acid respectively.

Supplementation of this medium with CMC significantly increased the fumaric acid yield to 5.36 g/l. A reduced yield of fumaric acid (0.92 g/l) was observed when the control medium was supplemented with Carbopol although these values were higher than the maximum product concentrations observed in glucose CSL-salts-media.

Buffering of CMC and Carbopol-supplemented media with calcium carbonate diminished fumaric acid yield. The patterns of fumaric acid production in the different media are presented in Figures 3.16 and 3.17.

3.4.2.2 Production of Fumaric Acid in Fermenters:

In order to eliminate the variation in pH during the fermentation, the effect of Carbopol and CMC on fumaric acid production was investigated in fermenters controlled at pH 5.5 and 4.0. Media containing polymers at pH 5.5 were quite viscous and required an agitation rate of 500 rpm compared to 400 rpm in control medium. In CMC medium the aeration rate was varied between 0.3-0.6 l/l/min after 12 h to maintain dissolved oxygen saturation level at greater than 70%. Fermentation patterns and other observations are presented in Figures 3.18 and 3.19.

The morphology of the control, appearing as fluffy growth centres, approximately 1 cm in diameter, uniformly distributed throughout the vessel, contrasted to the clumped pellets observed in this medium in shake flasks. Dispersed and clumped dispersed morphologies were observed in fermenters supplemented with CMC and

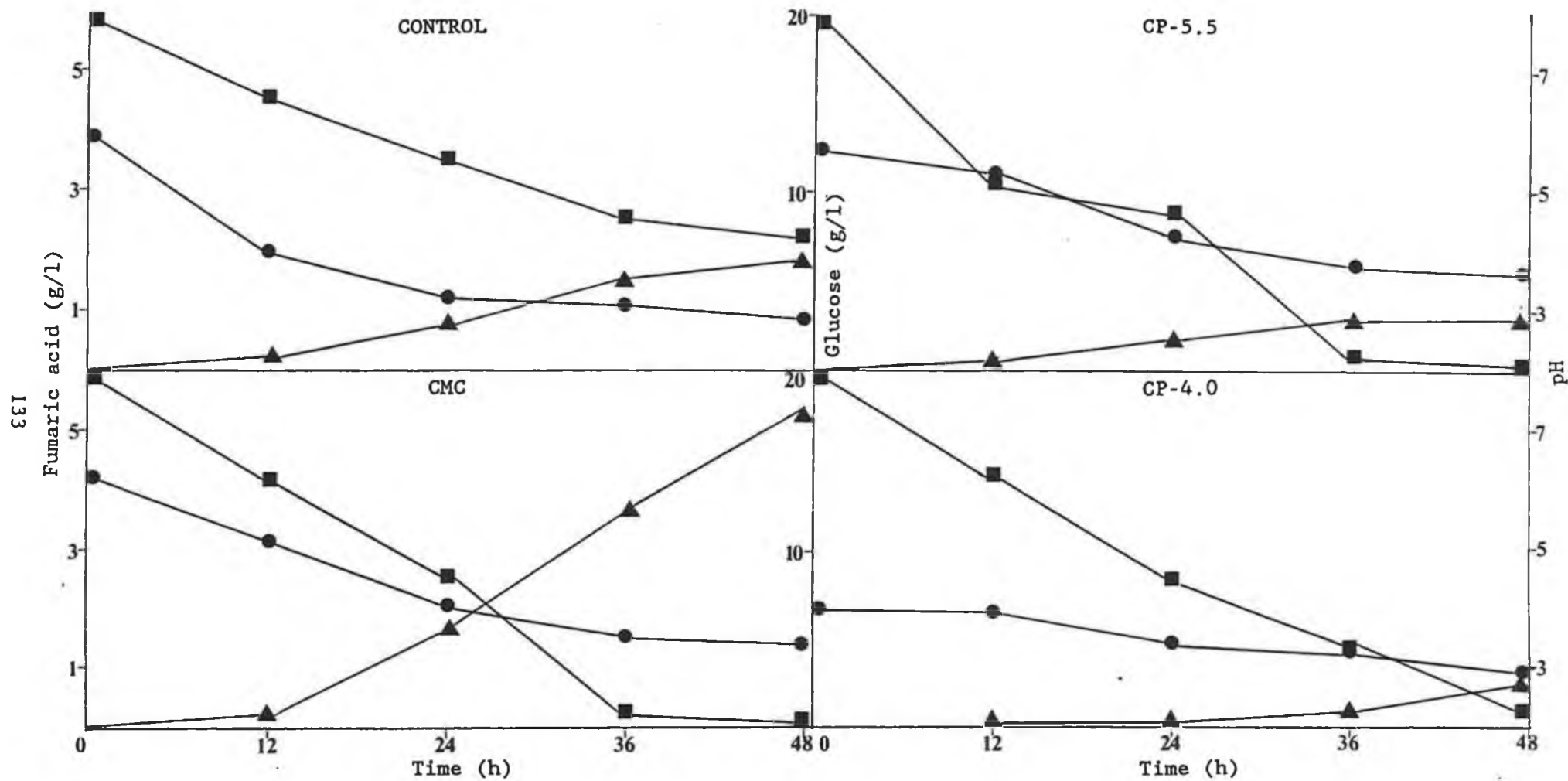


FIG.3.16 Patterns of production of fumaric acid in control, CMC, CP-5.5 and CP-4.0 media in shake flask cultures.

▲, Fumaric acid ■, Glucose ●, pH

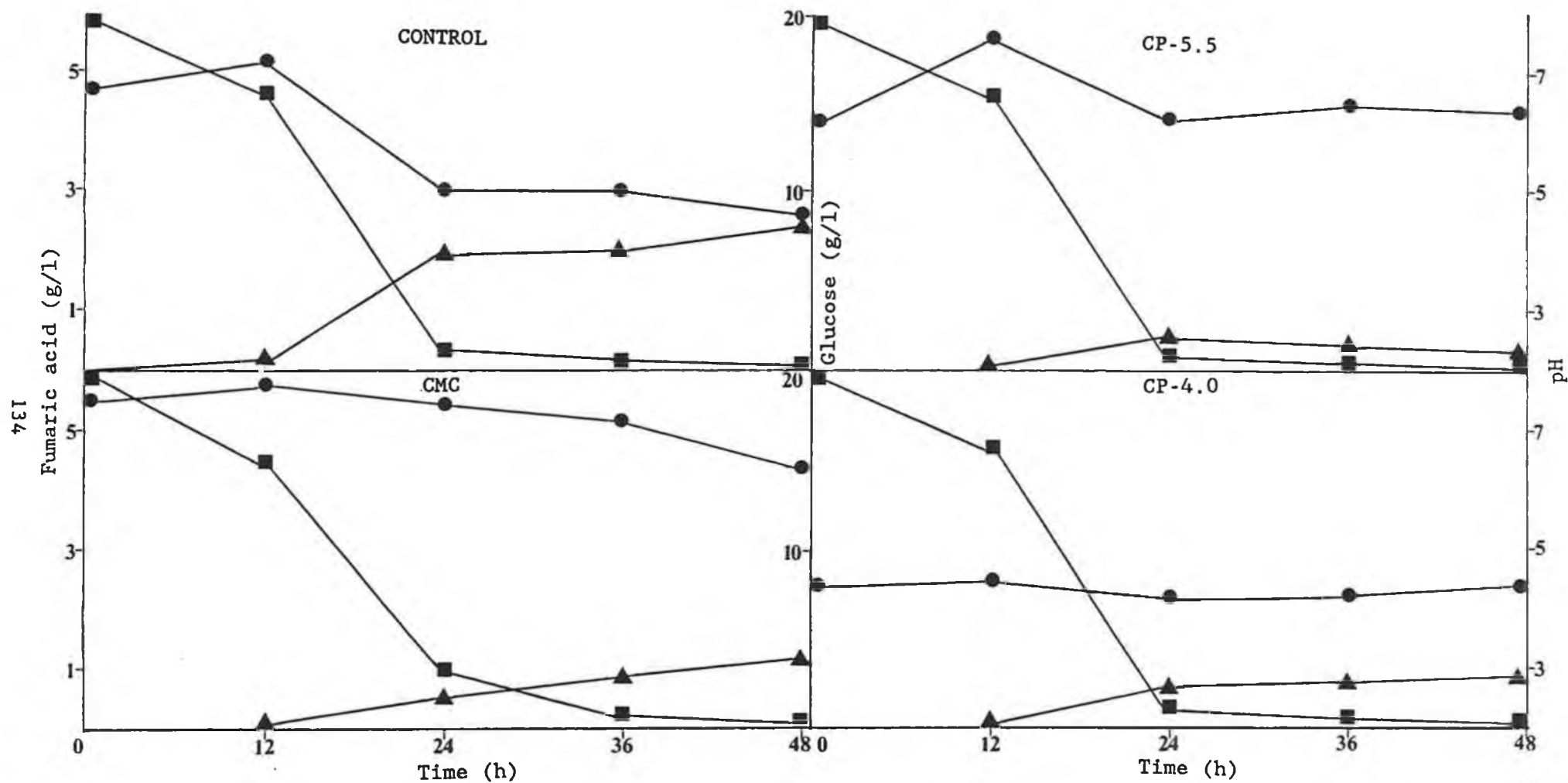


FIG.3.17 Patterns of production of fumaric acid in control, CMC, CP-5.5 and CP-4.0 media supplemented with 1% CaCO_3 , in shake flask cultures.

▲, Fumaric acid

■, Glucose

●, pH

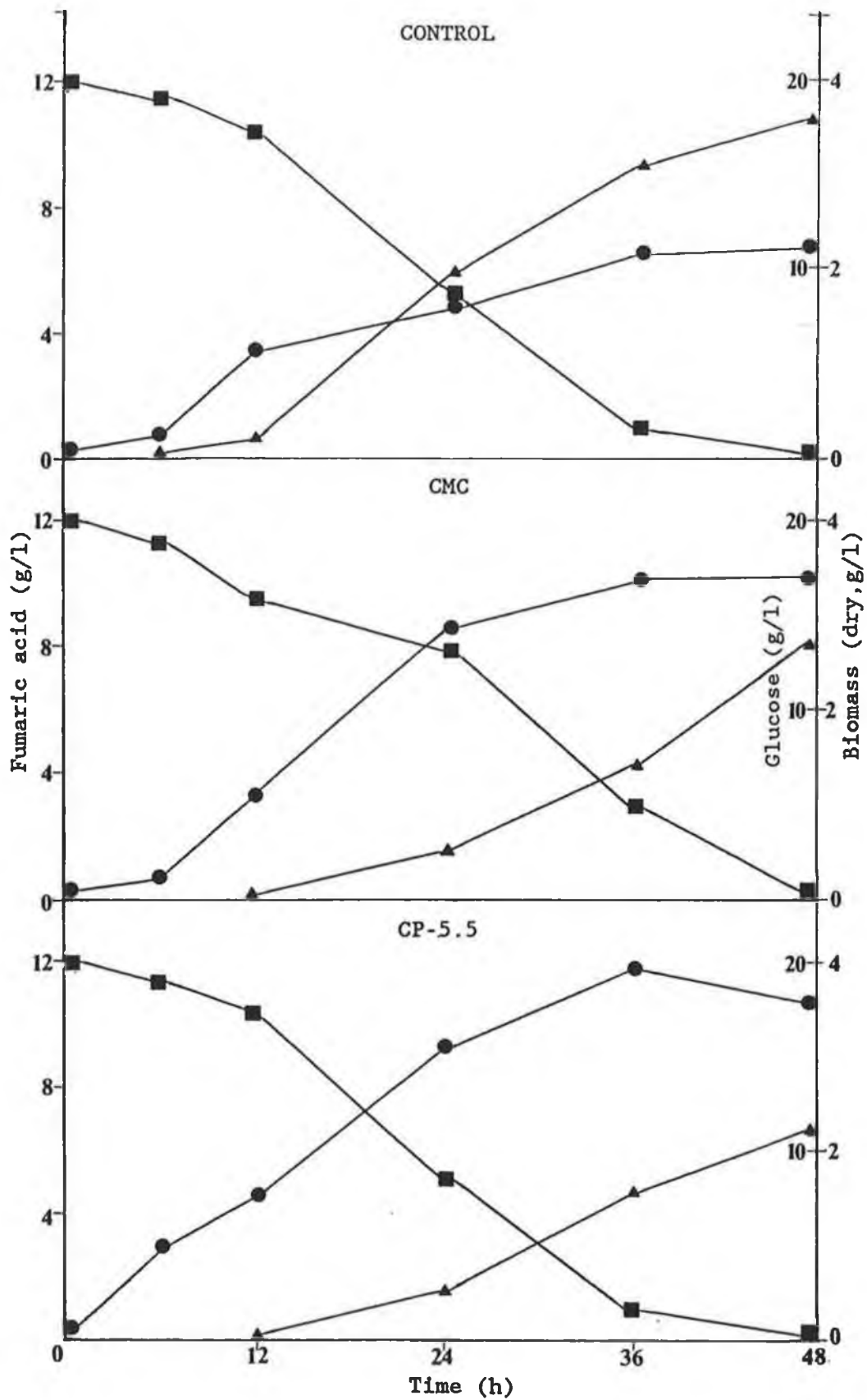


FIG. 3.18 Production of fumaric acid in laboratory fermenters in control, CMC and CP-5.5 media with pH controlled at 5.5.

▲, Fumaric acid
 ■, Glucose
 ●, Biomass

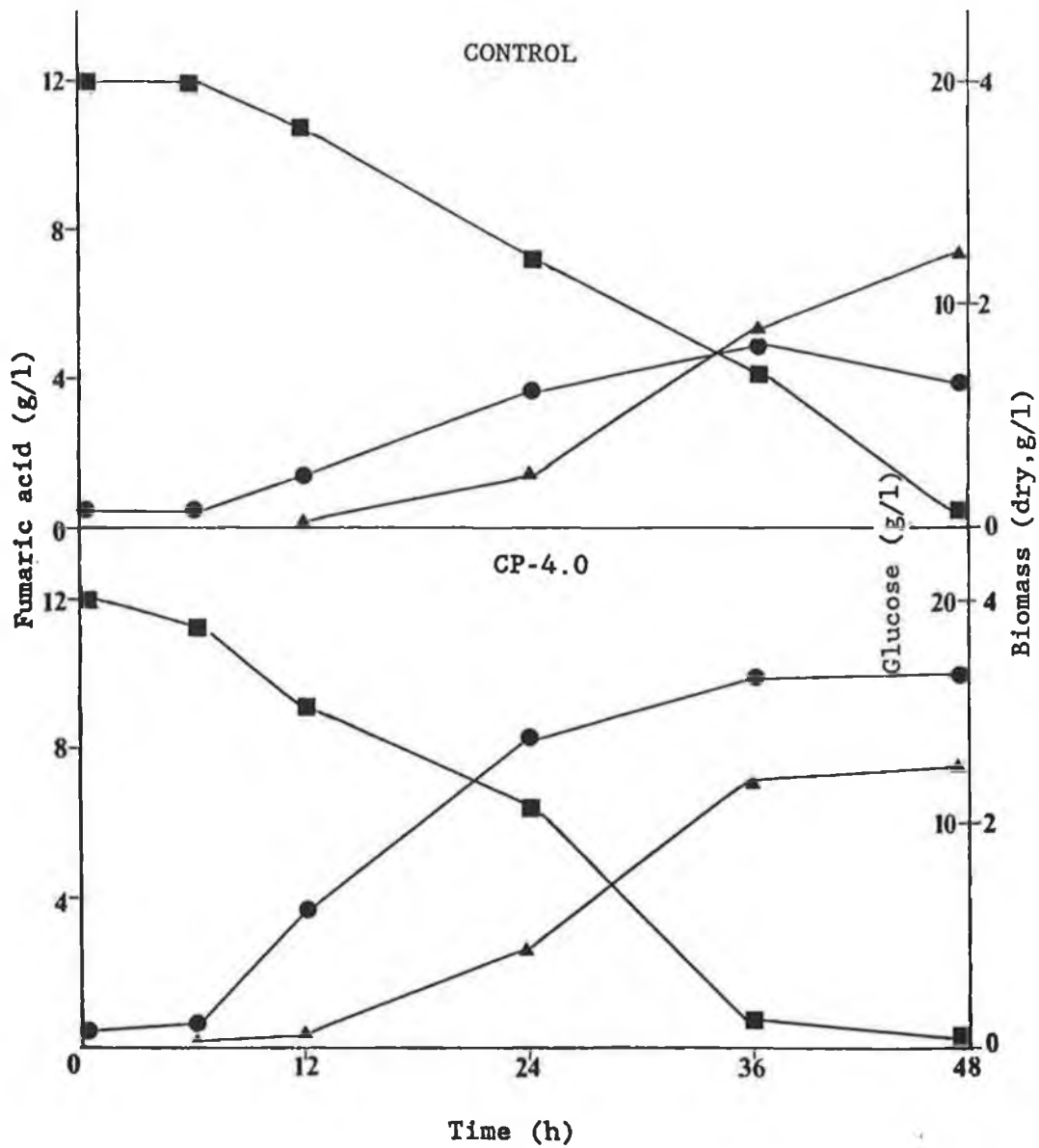


FIG. 3.19 Production of fumaric acid in laboratory fermenters in control and CP-4.0 media with pH controlled at 4.0.

- ▲, Fumaric acid
- , Glucose
- , Biomass

Carbopol respectively, similar to the morphologies observed in shake flasks. The fermentation patterns indicate that CMC addition resulted in increased biomass production but at the expense of fumaric acid. Fumaric acid concentration produced in the control medium in fermenters was approximately double the best value achieved (with CMC medium) in shake flasks.

Final biomass and fumaric acid concentrations are approximately 40% higher at pH 5.5 than at pH 4.0 in control medium. The yields of biomass and fumaric acid were similar in Carbopol-supplemented media at pH 5.5 and 4.0.

3.4.2.3 Biotransformation of Glucose to Fumaric Acid by Different Mycelial Forms:

Because of the substantial variation in biomass production observed in these fermenter trials, the ability of biomass recovered from different medium types to convert glucose to fumaric acid was investigated in biotransformation experiments. Mycelia grown in control, CMC, CP-4.0 and CP-5.5 media for 24 h was harvested, washed and resuspended in 2% glucose solution in shake flasks. Carbopol (0.3%) was also added to mycelia recovered from control medium to test its effect on fumarate formation when mycelia have not been grown in its presence. Flasks were incubated at 30°C/150 rpm for 6h. Initial biotransformation pH was set at pH 4.0 or 5.5 and was monitored and readjusted back to these values at 30 min intervals. The biotransformation patterns are presented in Figure 3.20.

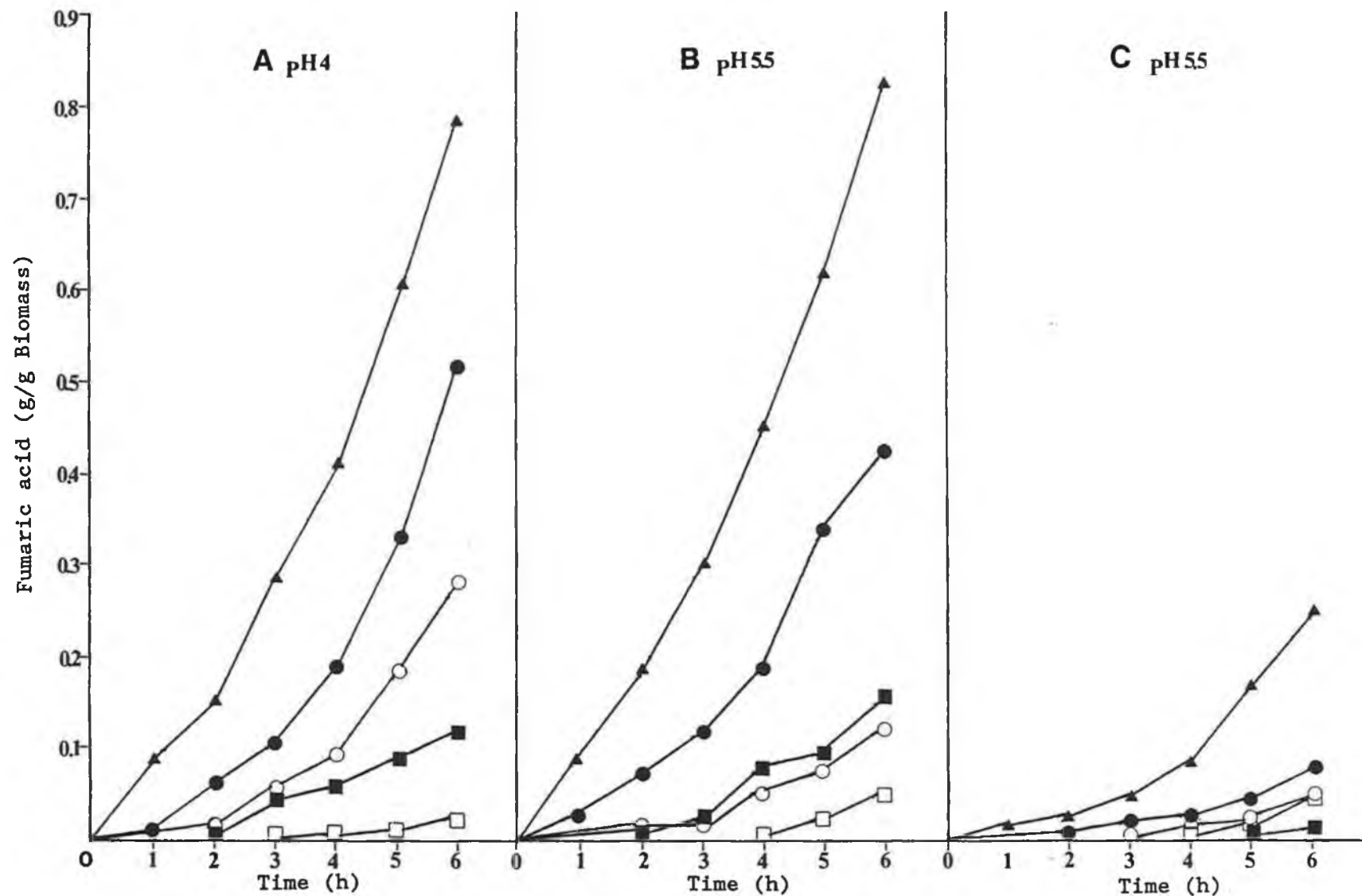


FIG 3.20 Patterns of biotransformation of glucose to fumaric acid by 24 h old mycelium (A, B) at pH 4 and 5.5 and by 48 h old mycelium (C) at pH 5.5.

Mycelia isolated from: Control medium (●), CMC medium (▲), CP-5.5 medium (○), CP-4.0 medium (□), Control medium but Carbopol added for biotransformation (■).

The results clearly demonstrate that mycelia recovered from CMC medium had a substantially higher biotransformation capacity than mycelia from the control medium. Mycelia recovered from Carbopol-supplemented media had a significantly reduced fumaric acid production capacity. Addition of Carbopol at the biotransformation stage to mycelia recovered from the control medium also dramatically retarded fumaric acid production.

The effect of Carbopol concentration on capacity of dispersed biomass, recovered after 24 h under standard conditions from CMC medium, to convert glucose to fumaric acid was also investigated. The results are presented in Figure 3.21. Increasing Carbopol concentration resulted in decreased production of fumaric acid. 0.1% Carbopol had a greater inhibitory effect on fumaric acid production at pH 5.5 than at pH 4.0. Fumaric acid levels observed in the presence of 0.3% and 0.6% Carbopol were similar.

3.4.2.4 Summary:

CMC medium with dispersed filamentous mycelia gave the highest yields of fumaric acid in flask culture but in pH controlled fermenters where aeration was probably more efficient, the control mycelia gave the highest yields. Dispersed mycelia isolated from CMC medium converted a much higher rate of glucose to fumaric acid than the other mycelial forms isolated. The morphological form promoted by CMC supplementation is most efficient at fumaric acid production.

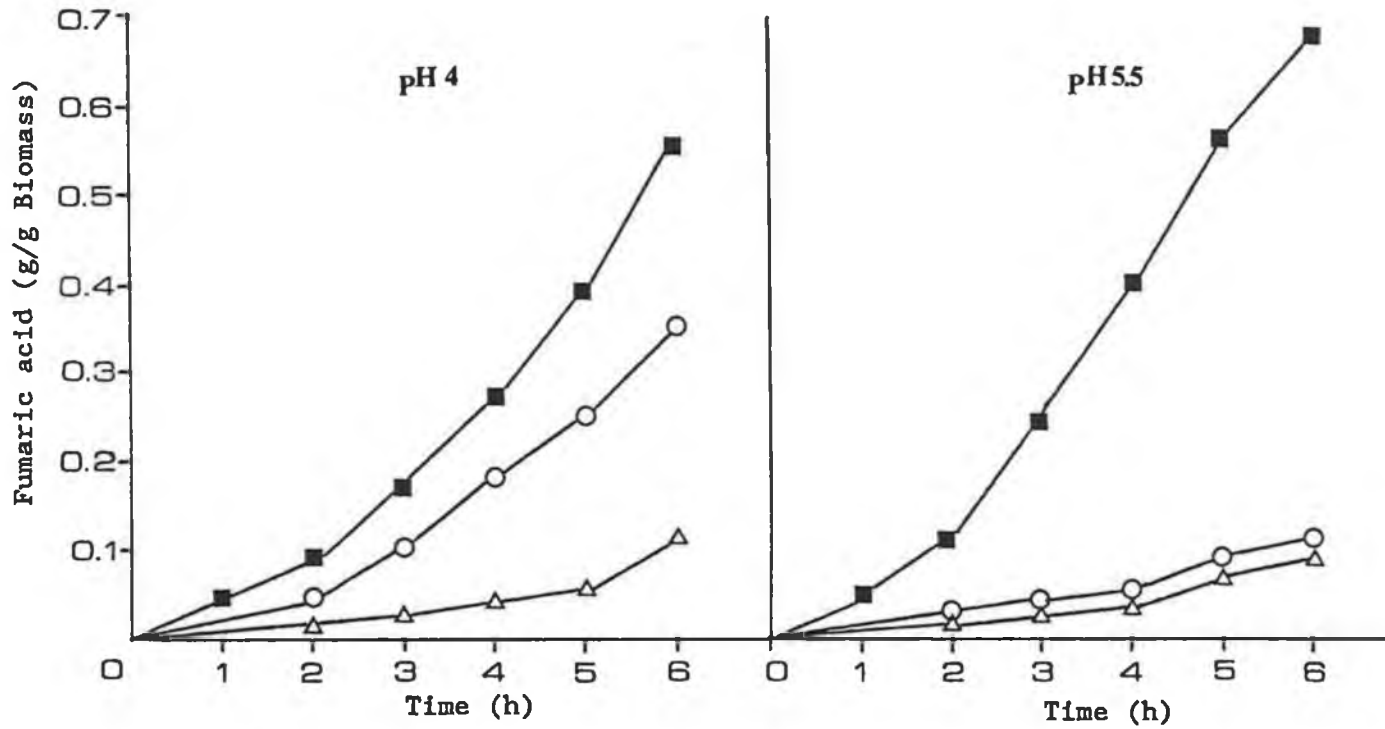


FIG.3.21 Effect of Carbopol concentration on biotransformation of glucose to fumaric acid by CMC-grown mycelium (dispersed).

Carbopol concentration (g/l): ■, 0; ○, 1; △, 3.

3.4.3 Biotransformation of Progesterone to 11 α -Hydroxy-
progesterone by Different Morphological Forms of
R. arrhizus:

In this section, the conversion rate of the four types of mycelia used previously, i.e. mycelia isolated from control, CMC, CP-4.0 and CP-5.5 media, are compared. The effect of mycelium age and presence of glucose on conversion are investigated.

Mycelia were grown in shake flask cultures in control, CMC, CP-5.5, and CP-4.0 media for 24 or 48 h under standard conditions. Mycelia were recovered, washed and resuspended in the biotransformation medium either in the presence or the absence of 1% (w/v) glucose and incubated for 24 h. 11 α -hydroxyprogesterone and glucose concentrations were measured after the 24 h incubation. The results are presented in Table 3.25

Higher levels of biomass were produced in media supplemented with CMC or Carbopol than in the control. In all cases except the 48 h control, addition of glucose to the biotransformation medium substantially enhanced 11 α -hydroxyprogesterone production.

In glucose-supplemented biotransformation media, maximum % conversion and amount of product formed per unit weight of biomass was observed with 48 h mycelia recovered from CMC medium. Total extracted product formed by the 24 h control (19.2 mg) and 24h CMC-grown mycelium (23 mg) were only 60% and 72% respectively of the value for 48 h CMC-grown mycelium. However, in terms of mg product formed per mg biomass, values for the 24 h control (0.101 mg) and 24 h CMC-grown mycelium (0.106 mg) were 86% and 91%

TABLE 3.25: Biotransformation of progesterone to 11 α -hydroxyprogesterone using different morphological forms

Mycelia isolated from	<u>Biomass production</u>		<u>Biotransformation</u>			
	Growth period h	Biomass recovered mg	11 α -hydroxy progesterone		Conversion %	Final glucose mg/ml
			Total mg extracted	mg formed per mg biomass		
Control Medium	24	191	a) 8.2	0.043	21	-
			b) 19.2	0.101	63	0.43
	48	240	a) 8.9	0.037	23	-
			b) 3.4	0.014	10	6.9
CMC medium	24	220	a) 4.0	0.018	11	-
			b) 23.0	0.106	71	2.4
	48	275	a) 12.2	0.044	36	-
			b) 32.1	0.117	88	1.2
CP-4.0 medium	24	233	a) 2.7	0.012	7	-
			b) 9.7	0.042	44	1.2
	48	303	a) 5.1	0.022	16	-
			b) 20.0	0.066	66	0.44
CP-5.5 medium	24	225	a) 4.4	0.020	13	-
			b) 12.0	0.053	43	0.15
	48	271	a) 6.2	0.023	19	-
			b) 20.1	0.074	57	0.59

a) No glucose added for biotransformation.

b) 1% glucose added.

24 and 48 h mycelia isolated from control, CMC, CP-4.0 and CP-5.5 media were inoculated into the biotransformation media, with and without glucose. After 24 h, the % conversion and glucose content were estimated.

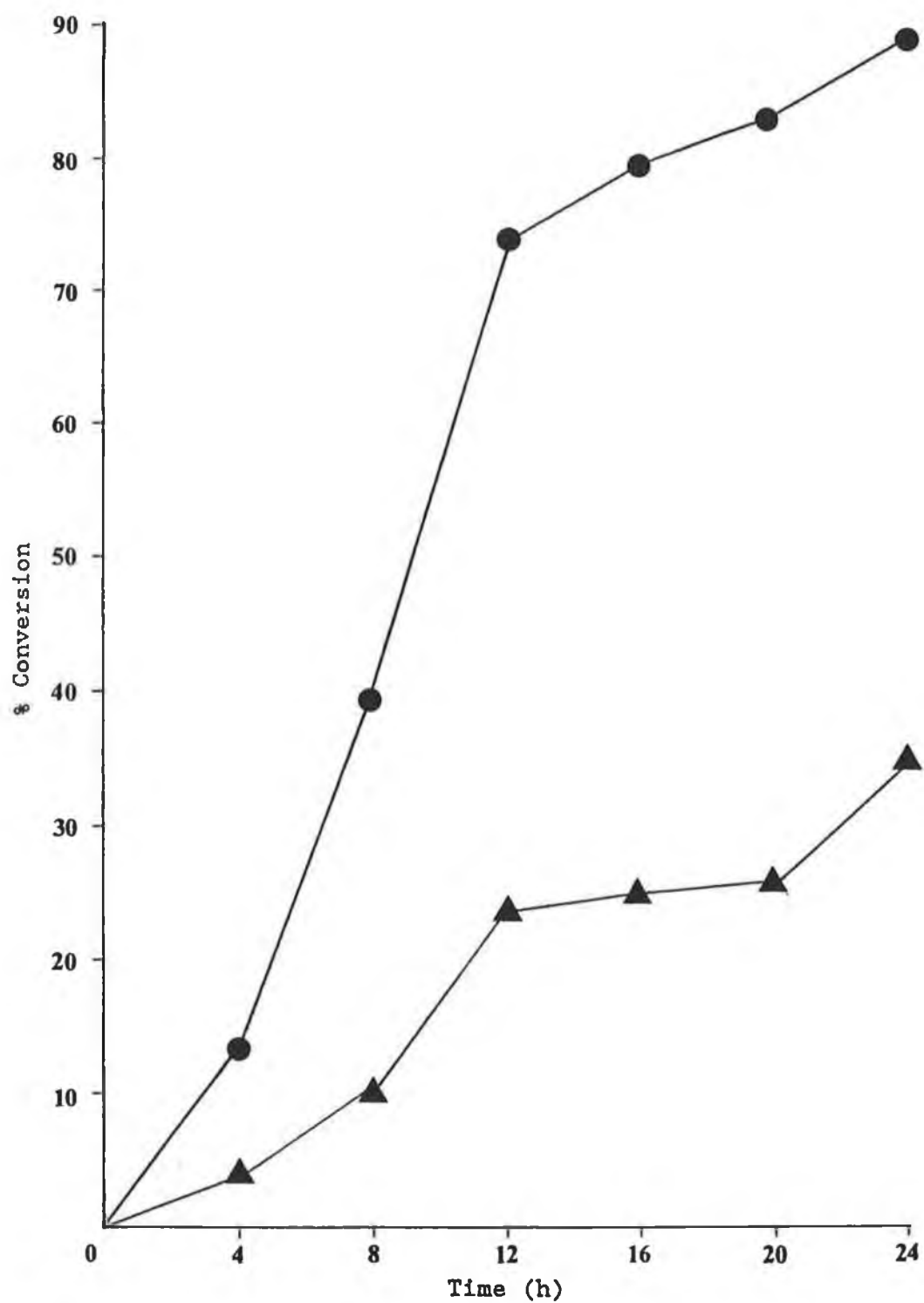


FIG. 3.22 Patterns of production of 11 α -hydroxyprogesterone by CMC-grown mycelia.

●, glucose present
▲, glucose absent

respectively of that of 48 h CMC-grown mycelium. Although Carbopol-supplemented media increased mycelial biomass, product formed per unit weight of mycelia was approximately 66% of the values for the 24 h control and the CMC-grown mycelia. The 48 h control mycelium, which produced little 11 α -hydroxyprogesterone, manifested reduced glucose metabolism during the biotransformation.

The patterns of 11 α -hydroxyprogesterone production, by 48 h old CMC-grown mycelia, in the presence and absence of glucose, during the biotransformation are illustrated in Figure 3.22. High rates of conversion are observed during the first 12 h in the presence of glucose.

3.4.3.1 Summary:

Dispersed mycelia from CMC medium had an increased specific capacity to biotransform progesterone to 11 α -hydroxyprogesterone compared to pelleted mycelia from control medium or clumped dispersed mycelia from Carbopol-supplemented media. 48 h congealed mycelia from control medium had a low metabolic rate and showed very low rates of biotransformation. Even though mycelia from Carbopol media utilised glucose more efficiently than mycelia from CMC media, the % conversion of progesterone to 11 α -hydroxyprogesterone was lower.

4. DISCUSSION

The growth morphology of R. arrhizus is determined in the first 8-10h of growth. Developing hyphae of R. arrhizus in control medium begin to form entangled mycelial pellets at approximately 4h after germ tube emergence (i.e. approximately 9h after inoculation). There is no evidence of spore aggregation as with A. niger (Galbraith & Smith, 1969; Elmayergi, 1975) or P. chrysogenum (Trinci, 1970). The pellets formed in cultures of these species are comprised of clumps of ungerminated spores and spores with emerging germ tubes, which can 'trap' other hyphae or spores within the entangled mass. During continuous-flow culture of P. chrysogenum, pellets were initiated when developed hyphae began to agglutinate upon increasing the pH (Pirt & Callow, 1959). The presence of either of the polymers carboxymethylcellulose (CMC) or Carbopol in the medium prevents pellet formation in R. arrhizus. It has previously been observed that polymers and copolymers of acrylic acid (such as Carbopol) yielded the most significant improvement in metabolic activity of A. niger when a range of polymer supplements were tested (Elmayergi et al., 1973). It has been suggested that anionic polymers such as Carbopol (Elmayergi et al., 1973; van Suijdam et al., 1980) or Junlon (Trinci, 1983) prevent pellet formation in cultures of A. niger by inhibiting spore aggregation due to electrostatic repulsion.

In the period prior to commencement of pelleting, the hyphae in control medium develop at a similar rate of extension and branch initiation as those in CMC or CP-5.5 media, where pH is comparable at 5.5 in the first 12 h of growth. Germ tube emergence occurs 1 h later in CP-4.0 medium compared to the other three media, and an

increased rate of branch initiation is evident at 9-10 h growth. The nutritional requirements for germination of R. arrhizus have been studied (Ekundayo & Carlile, 1964; Ekundayo, 1966) but no reference to optimum pH was made. However, a more recent study (Medwid & Grant, 1984) has shown that optimal conditions for germination of R. oligosporus in defined medium are 42°C and pH 4.0. When R. arrhizus was grown at pH 4.0 in the presence and absence of Carbopol, germ-tube emergence was delayed compared to cultures at pH 5.0 and 6.0 (Section 3.2.6). Elmayergi et al. (1973) reported that Carbopol, or any of the other supplements studied, had no effect on the time period of germination in cultures with an initial pH of 5.0. This would suggest that the delay in germ tube emergence in CP-4.0 medium is a result of the lower pH in this medium compared to the other three media.

The hyphae in control, CMC and CP5.5 media are all in a similar pH environment and yet only those in the control medium begin to aggregate. Byrne & Ward (1987) observed pelleting of mycelia of R. arrhizus in media with pH_i ranging from 3-7. This is in contrast to observations of other workers where pH was seen to have a profound effect on morphology. Growth of A. niger at pH 4.0 or 4.5 resulted in pellets whereas at pH 2.0 or 2.3, growth was filamentous (Galbraith & Smith, 1969). Seviour et al. (1984) reported a similar observation for Aureobasidium pullulans where filamentous growth was observed at lower pH's but when the pH was increased to 6.5, growth was almost entirely unicellular. But pH does not appear to be the main influential factor in determining whether pelleting occurs in R. arrhizus. The effect of pH has been interpreted as being on the metabolic events which lead to the changes in spore wall structure

responsible for agglutination (Galbraith & Smith, 1969). As spore agglutination is not a factor in pelleting of R. arrhizus, pH would not play such an influential role.

The presence of the anionic polymer chains in CMC and CP-5.5 media confers substantial viscosity upon the solutions. Growth remains partially dispersed in cellulase-pretreated methylcellulose-supplemented medium, even though the polymer is uncharged and the solution non viscous. Therefore the maintenance of disperse hyphae is not simply a physical phenomenon which can be attributed to a viscous environment, although this possibly plays some part in keeping developing hyphae separate, as seen with the dispersed growth obtained using A. niger and P. chrysogenum in cultures of high viscosity (Takahashi & Yamada, 1960). An increase in culture viscosity using 1% (w/v) CMC as a medium supplement caused the pellet size of Mortierella vinacea to increase from 0.68 to 0.94 mm (Kobayashi & Suzuki, 1972). The addition of PVP (polyvinylpyrrolidone) to cultures of P. chrysogenum resulted in increased medium viscosity and dispersed growth (Moo-Young et al., 1969). The majority of the studies carried out on viscosity and morphology refer to the rheological implications of the growth form and not to how the viscosity influences the growth form (Bull & Trinci, 1977; Solomons, 1975, 1983). The Elmayergi group (1973, 1975) believe that the electrostatic properties of anionic polymers such as Carbopol or Reten (polyacrylamide) are the major influential factor in morphology determination and not the increase in medium viscosity that they give rise to.

Growth is more finely dispersed in CMC and CP-5.5 media than in CP-4.0 medium, which contains the weakly ionised (low net charge) Carbopol polymer at pH 4.0, or in methylcellulose-supplemented medium. The ability of these anionic polymers to interact with divalent cations has been reported (Byrne & Ward, 1987). Both CMC and Carbopol show marked absorption of Mg^{2+} ions, with slight absorption observed in methylcellulose solutions. The role of divalent cations in the process of pelleting must be considered. The phenomenon which bears a close similarity is that of yeast flocculation. Studies have shown this process to be absolutely dependent upon the presence of Ca^{2+} (Mill, 1964), but Mg^{2+} and Mn^{2+} can also act as inducers (Lyons & Hough, 1970; Stewart & Goring, 1976). The hypothesis that the divalent cations act by bridging cells via negative groups on the cell surface was questioned by the failure to reveal any significant difference in total uptake of Ca^{2+} by flocculent and non-flocculent cultures (Stewart et al., 1975). When the composition of walls from a flocculent and non-flocculent strain of Saccharomyces uvarum were compared, the walls of the flocculent cells had a higher mannose/glucose molar ratio, a lower percentage of protein and Ca^{2+} and a higher percentage of K^+ (Amri et al., 1982). A mannan layer essential for flocculation, which is comprised of a proteinaceous, lectin-like compound which binds to the α -mannan carbohydrates of adjoining cells via Ca^{2+} ions, has been isolated (Miki et al., 1982a). Flocculation may be controlled by the dominant FL01 gene which governs the expression of the Ca^{2+} -dependent lectin-like compound (Miki et al., 1982b). Thus, information about the nature of the interaction in flocculation is abundant. The unicellular growth form aids charge investigations based on electrophoretic techniques (Fisher, 1975), whereas only fungal

spores can be investigated in this manner (Fisher and Richmond, 1969; Somers and Fisher, 1967) and not the mycelia. The heterogeneous nature of the fungal mycelium, be it in pelleted or filamentous form, hampers resuspension of harvested mycelia but if a homogeneous suspension could be attained, metal ion uptake trials such as that of Stewart et al. (1975) using radio-labelled Ca^{2+} ions, could be carried out.

The work of Choudhary & Pirt (1965) suggests that metal ions influence morphology. The presence of EDTA or other metal chelators caused growth to change from large pellets with a filamentous periphery to discrete, smooth pellets. The dramatic effect of Mn^{2+} ions on the morphology and citric acid yield of A. niger, where low concentrations can drastically reduce yields and change pelleted growth to filamentous, has been well documented (Clark et al., 1966; Kissler et al., 1980; Kubicek et al., 1979). A range of other divalent cations, including Mg^{2+} and Ca^{2+} , had no effect on the morphology or citric acid yield (Clark et al., 1966). The concentration of Ca^{2+} has been found to be critical in some morphogenic studies. The presence of 9mM Ca^{2+} in the medium of Penicillium cyclopium caused an increase in lateral branching, followed by the appearance of phialides and spores (Ugalde and Pitt, 1983). When media contained less than 1mM Ca^{2+} , extension of N. crassa was impaired, and stubby, bulbous cells formed (Schmid & Harold, 1988). It was suggested that a cytoplasmic Ca^{2+} gradient may be necessary to ensure dominance of the apex during polarised extension.

The ability of R. arrhizus biomass to absorb metal ions is well documented (deRome & Gadd, 1987; Shumate & Strandberg, 1985; Tobin et al., 1984; Tsezos, 1983). R. arrhizus absorbed a variety of metal cations, including Mn^{2+} , Zn^{2+} and Cu^{2+} , at pH 4.0 and absorption was suggested to occur by a complexation mechanism with sites on the biomass containing carboxylate, phosphate or other functional groups (Tobin et al., 1984). The optimum pH for biosorption of thorium by R. arrhizus was found to be pH 4.0 with indications that the metal was co-ordinating with the nitrogen of the chitin cell wall network (Tsezos and Volesky, 1982). Uptake was reduced at pH 2.0, an observation that was confirmed in a more recent study (Gadd & White, 1989). These workers found that uptake of thorium was similar for all biomass concentrations of R. arrhizus used, and that the presence of divalent cations such as Mg^{2+} and Ca^{2+} did not inhibit uptake. The adsorption of copper by R. arrhizus was seen to be a more complex process than in other species studied, as it followed the Brunauer-Emmett-Teller (BET) isotherm for multilayer adsorption, compared to the Freundlich or Langmuir isotherms (single layer adsorption) for the other species (deRome & Gadd, 1987). Again, uptake of copper was decreased at low pH. Mowll & Gadd (1984) have postulated that cation uptake is a biphasic system which involves an initial rapid binding of cations to negatively charged sites of the wall followed by a slower, metabolism-dependent uptake into the cytoplasm. They found that yeast-like or mycelial cells of Aureobasidium pullulans manifested a six-times lower rate of initial adsorption of cadmium compared to chlamydo spores.

In species where spore agglutination gives rise to pelleted growth, it has been suggested that a similar mechanism to that proposed for

yeast flocculation, involving divalent cationic bridge formation, may be in operation (Seviour & Read, 1985). But electrophoretic studies on spores of A. niger have shown that clumping occurs at pH 3.5 and 4.5 where contribution to mobility of carboxyl groups is low, and that no agglutination occurs at pH 2.0 where the net charge is nil (Seviour & Read, 1985). Galbraith & Smith (1969) have deduced that surface charge is not a factor in pellet formation of A. niger from electrophoretic data showing no agglutination under conditions of low charge, similar to Seviour & Read's findings. Thus the process of pelleting is possibly much more complex than cationic-bridge formation. This is particularly so with a species like R. arrhizus where pelleting is initiated after germ tube emergence and not at the spore stage. The studies on mycelial uptake of divalent cations and the ability of the polymers to absorb cations would suggest a hypothesis for pelleting involving aggregation of hyphae mediated by ionic bridges, either via charged surface groups or divalent cations. The delay in the onset of pelleting until 9-10 h after inoculation with R. arrhizus would imply that hyphal aggregation can only commence after a certain stage of development has been reached, perhaps when a specific proportion of ionic components such as carboxylate or phosphate groups are present in the extending germ tube. In polymer-supplemented media, this aggregation is prevented due to viscosity, metal-ion uptake or electrostatic repulsion. Pelleting may be partially as a result of entanglement of developing hyphae, which is prevented in viscous media.

Polymer supplementation ultimately results in increased biomass production after 48 h growth. The difference in biomass production is only beginning to become noticeable at the 24 h stage where the

polymer-supplemented media show approximately 10% higher biomass yields than the control medium compared to the three polymer-supplemented media. The pH of the control medium also drops to below 3.0 in this period. However, Sorenson & Hesseltine (1966) have shown that the biomass yield from cultures of R. oligosporus is unaffected by media pH in the range 2.6 - 5.5. Comparison of pelleted and filamentous cultures of A. niger at pH 4.5 and 2.1 respectively show almost identical biomass yields and rate of glucose uptake (Galbraith & Smith, 1969). So a disturbance in metabolic functions due to the low environmental pH does not seem to be the main factor in contributing to the decreased biomass yield in the control medium.

The limitations on diffusion of nutrients within a pelleted mycelial mass have been described previously (Phillips, 1966; Pirt, 1966; Yanagita & Kogane, 1963). Oxygen is the substrate that is most commonly found to be limiting because its solubility in water is low and it is required in substantial quantities (Righelato, 1979; Righelato et al., 1968). High dissolved oxygen concentrations are necessary to avoid oxygen limitation in pellets of A. niger (Kobayashi et al., 1973). Schugerl et al. (1983) report decreased yields of penicillin from large pellets compared to small ones, as oxygen transfer to the centre of the large pellets is limited. They report studies on oxygen pressure within pellets of P. chrysogenum and deduce that the dissolved oxygen concentration is reduced below the critical value in the centre of pellets with a diameter larger than 400 μm . This has profound consequences on cell growth and product formation. It is possible therefore, that limitations on oxygen transfer within the pelleted mycelial mass in the control

medium would contribute to a decrease in biomass yield in the 24-48 h growth period.

Before the hyphae become very tightly packed, the specific growth rate, rate of glucose utilisation and production of biomass proceeds at a similar rate in the control medium as the dispersed mycelia in the polymer-supplemented media. Elmayergi et al. (1973) report an increased rate of glucose utilisation in media supplemented with anionic polymers. They report increased specific growth rates and dry weights in Carbopol-supplemented media, but at 24 h in R. arrhizus cultivation, control and Carbopol media had similar dry weight yields. Thus the polymers do not appear to cause any increase in the specific growth rate of the fungus, but act by promoting a growth form where unrestricted growth may proceed for longer periods. The decrease in the rate of biomass production in the polymer-supplemented media coincides with a depletion of glucose in the media.

The hyphae in CP-4.0 medium grow at the same specific growth rate as those in CMC or CP-5.5 media, but have a lower hyphal growth unit (HGU) length, due to the greater rate of branch initiation. L-sorbose has a similar but more pronounced effect on the growth of N. crassa, where the spatial distribution of biomass is affected, but not its rate of production (Trinci, 1973). Because branching frequency is not increased in CP-5.5 medium, the pH is the more probable influential factor in branch initiation, and not the Carbopol polymer itself. Mishra & Tatum (1972) have observed an inhibition of polysaccharide synthetases, namely glucan synthetase, in sorbose-grown cultures of N. crassa and propose that this leads to a

"weakening" of the cell wall which manifests itself in an increased branching frequency. A higher concentration of autolytic enzymes was found to be associated with walls from a branching mutant of N. crassa compared to the wild type (Mahadevan & Mahadkar, 1970). In a more general hypothesis on branch initiation, Trinci (1979) proposes that a higher frequency of branching can arise either from an increased rate of fusion of lysins with the subapical wall or alternatively an increased supply of vesicles which cannot be incorporated into the apex quickly enough and instead are deposited subapically. A mathematical model has been derived which is capable of predicting changes in number and position of branches on the basis of changes in vesicle and nuclear concentration (Prosser & Trinci, 1979). It has been suggested that the electro-potential gradient within the hypha may generate sufficient current to drive the vesicles by electrophoretic forces (Farkas, 1979; Trinci, 1978). An inward-moving electrical current has been reported in Achyla bisexualis which precedes branching and predicts the branch site (Kropf et al., 1983). Proton pumps have been implicated in exchange of K^+ and H^+ in N. crassa (Slayman & Slayman, 1962). These pumps have been proposed as acting as a regulatory mechanism for the internal pH of cells (Raven & Smith, 1973). The internal potential is seen to shift towards zero with increasing H^+ concentration (Slayman, 1970). Perhaps a lower environmental pH, as in CP-4.0 medium, may disturb the electro-potential gradient, causing decreased electrophoresis of vesicles towards the apex, leading to more frequent branch initiation. However, the hypha itself must maintain ionic balance, so if it takes up a cation it must exchange this for a similarly charged ion. There is evidence to support a substantial passive permeation of H^+ ions (Raven & Smith, 1973; Slayman, 1970),

and in media of low pH, the proton pump would be driven to cause an efflux of protons to restore equilibrium (Burnett, 1976).

Hyphal development takes the same course in CMC and CP-5.5 media where the polymer supplement is highly charged and the solution viscous. More specifically, the composition of the cell walls of mycelia isolated from CMC and CP-5.5 media are practically equal, whereas those from control and CP-4.0 media show similar trends in total protein and hexosamine concentration. Protein content is particularly affected in mycelia from CMC or CP-5.5 media where values of 6 and 7% are obtained compared to 18 and 13% respectively, in control or CP-4.0 media. Elevated hexosamine levels were observed in cell walls of stubby, bulbous hyphae from Mn^{2+} -deficient media of A. niger (Kisser et al., 1980). Influence on protein synthesis was considered to be important as cycloheximide induced a similar type of morphology and increased chitin content (Kubicek et al., 1979). These authors reported increased cellular levels of fructose-6-phosphate and glutamine in manganese-deficient hyphae and suggest that these elevated precursor concentrations might account for increased synthesis of chitin. Manganese deficiency impairs protein turnover, leading to elevated levels of NH_4^+ , which counteracts inhibition of phosphofructokinase by citrate (Kisser et al., 1980).

Cycloheximide addition caused depolarisation of chitin synthesis and induced wall thickening in A. nidulans (Katz & Rosenberger, 1971; Sternlicht et al., 1973). It has been observed previously that inhibition of protein synthesis usually results in a concomitant increase in skeletal polysaccharides (Elorza et al., 1976; Farkas, 1979). Farkas (1979) postulates that the inhibition of protein

synthesis may lead to the depletion of some metabolically unstable proteinaceous component which usually inhibits the activity of polysaccharide synthases. A higher concentration of the active protease, which activates the zymogenic form of chitin synthase, was found in the mycelial form of M. rouxii, which had a higher chitin content compared to the yeast-like form (Ruiz-Herrera & Bartnicki-Garcia, 1976). Thus it would appear both from the literature and from the cell wall content of R. arrhizus that protein and hexosamine synthesis are interlinked, but the exact mechanism is as yet unclear.

The stability of the protein synthesising system of A. nidulans was reported to be high, with no effect on its efficiency over a pH range of 3.1-6.9 (Bull & Trinci, 1977). The higher protein content of cell walls from control and CP-4.0 media does not seem to be a function of medium pH, as 12 h walls from control medium, where pH was comparable to CMC or CP-5.5 media, have already attained the high protein content seen in 24 h walls. The anionic polymers present in CMC and CP-5.5 media have been shown to bind divalent cations (Byrne & Ward, 1987). Divalent cations can have profound effects on wall integrity (Bartnicki-Garcia & Lippman, 1972; Dow & Rubery, 1975; Schmid & Harold, 1988). The presence of 0.01-0.075 M solutions of Mg^{2+} , Mn^{2+} or Ca^{2+} caused swelling of the hyphal apex of M. rouxii and was considered to be a consequence of disturbing the delicate balance between loosening or rigidification of the wall at the apex (Bartnicki-Garcia & Lippman, 1972). Dow and Rubery (1975) interpreted the antagonistic effect on bursting of hyphal tips exposed to H^+ or Ca^{2+} as being due to a modification of the physical properties of matrix polymers, such as mucoran. They suggest that H^+

ions cause the wall matrix to become less viscous while Ca^{2+} ions cause the wall matrix to become more viscous and hence more rigid. Thus the relative concentrations of divalent cations may affect wall integrity and composition.

The other major differences in cell wall properties relate to whether mycelia were grown in the presence or in the absence of Carbopol. These include differences in X-ray diffraction patterns observed, in the appearance of chitin ghosts, in interaction with Calcofluor White and in the concentration of radioactivity in cell walls after uptake of N-(^{14}C -acetyl)-glucosamine. The radial density tracings obtained from the X-ray diffraction patterns of the four cell wall samples suggest a decrease in crystallinity in the chitin in cell walls from Carbopol-supplemented media. Mycelia from CP-4.0 medium do not fluoresce when treated with Calcofluor, whereas those grown in CP-5.5, control or CMC media all manifest bright fluorescence at the apex and branch points. Calcofluor specifically interacts with nascent chitin chains, preventing crystallisation (Elorza *et al.*, 1983; Herth, 1980; Vermeulen & Wessels, 1986). The adherence of Carbopol to mycelia at low pH appears to block binding of Calcofluor. An ionic attraction between chitin and Carbopol might account for the ability of Carbopol to prevent fluorescence at lower pH values, and if the interaction was similar to that with Calcofluor, then crystallinity would be reduced. The characteristic reflections of chitin are more pronounced in walls from CP-5.5 medium where fluorescence is obtained with Calcofluor than those from CP-4.0 medium, where they are very indistinct. However, these results must be interpreted with caution, since removal of wall material surrounding the chitin by chemical treatment may interfere with

crystallinity (Sietsma and Wessels, 1979).

Cell walls from Carbopol-supplemented media at 24 h had a lower concentration of radioactivity compared to walls from control or CMC medium. This is not a result of a difference in the rate of incorporation, as similar concentrations of residual 'cold' N-acetylglucosamine remained after addition to the four samples under the same conditions as those used in the radiolabelling trial. The lower concentration may be due to an increased rate of deacetylation of the labelled molecule in these cells, in the manner proposed for chitosan synthesis by Davis & Bartnicki-Garcia(1984a). This suggests a higher chitosan:chitin ratio in walls from mycelia grown in Carbopol-supplemented media. However, the possibility exists that a portion of the released acetyl groups would not be removed upon washing of the walls, but instead would be incorporated into the wall at an alternative site. This would imply that some of the radioactivity measured might represent material labelled by secondary incorporation of the released acetyl groups.

The chitin 'ghosts' i.e. residue remaining after alkali extraction of nitrous acid residue, of walls from control and CMC media are more intact and retain the original hyphal shape compared to those from Carbopol-supplemented media. No obvious differences in fibril orientation or dimensions were observed between the four samples. Microfibrils from four dimorphic fungi were shorter than those from Coprinus cinereus or M. mucedo and it was suggested that they might be more suited to the alternate construction of both spherical (yeast) and tubular (hyphal) cell types by the same organism (Gow & Gooday, 1983). There is a possibility that microfibrils may appear

differently after chemical extraction to the manner in which they actually exist in the wall (Sietsma & Wessels, 1979; van der Valk et al., 1977). However, the differences observed in the Carbopol-grown cell walls were reproducible and possibly represent actual structural and configurational differences in the chitin/chitosan component in vivo.

When monitoring the production of fumaric acid, 11 α -hydroxyprogesterone or glucoamylase, the optimum yield in each case was obtained using dispersed, filamentous mycelia from CMC medium. Different yields were obtained from the four media in shake flask trials. After 24 h growth, mycelia in control medium became tightly pelleted giving decreased glucose consumption and biomass production profiles. Decreased aeration and diffusion of nutrients is known to cause restricted growth when mycelia become tightly packed into pelleted structures (Kobayashi et al., 1973). Even when the pelleted mycelia are removed from the low pH environment of the shake flask, as in the biotransformation trials, production of fumaric acid is still at a much lower rate than CMC-grown mycelia. Thus the restrictions on diffusion within the clumped mycelial mass may cause a reduction in glucose consumption, biomass production and fumarate accumulation.

In contrast to shake flask trials, fluffy growth centres were formed instead of clumped, entangled pellets in control medium in pH controlled fermenters and high fumaric acid levels obtained. It has been demonstrated that high shear forces can reduce clumping of mycelia (Metz & Kossen, 1977; Ziegler et al., 1980). The increased aeration efficiency of fermenters compared to shake flasks also

contributes to reduced clumping of mycelia with subsequently higher oxygen transfer rates within the mycelial mass (Carilli et al., 1961; Gomez et al., 1988). Increasing the aeration rate caused production of citric acid to rise from 30 to 48 g/l, with no effect on the morphology (Gomez et al., 1988). High agitation rates caused P. chrysogenum to grow as short, branched hyphae compared to long hyphae produced at low agitation rates (Dion et al., 1954; van Suijdam & Metz, 1981). The increased aeration efficiency and higher shear forces of impeller stirred fermenters are probably responsible for the shift in morphology in control medium with a subsequent enhancement of metabolic activity.

The biomass production, glucose consumption and growth rate profiles of dispersed mycelia from CMC and CP-5.5 media are alike, and yet dramatic differences in fumaric acid yields are observed in shake flask, fermenter and biotransformation trials. The biotransformation experiments confirmed that the presence of Carbopol reduced fumaric acid production. The adherence of the Carbopol polymer to the mycelia, as indicated using scanning electron microscopy, may influence cell metabolism or cell permeability to substrates and products, thereby retarding fumaric acid production or secretion. Elmayergi et al. (1973) showed that Carbopol enhanced respiration rate of A. niger by as much as 200%. Pyruvate carboxylation and the reductive reactions of the tricarboxylic acid cycle have been implicated in the accumulation of fumaric acid by R. arrhizus (Kenealy et al., 1986; Osmani & Scrutton, 1985). Carbopol may, therefore, act by increasing respiratory activity in R. arrhizus with consequentially accelerated oxidation of tricarboxylic acid intermediates at the expense of fumarate production.

The extra biomass produced in CMC medium in fermenters compared to the control medium was probably at the expense of fumarate production. Hyphal extension could proceed at a less restricted rate in CMC medium compared to the growth centres present in control medium, allowing increased biomass production with lower accumulation of fumaric acid. Zinc, added to the growth medium of R. nigricans, has been shown to alter the physiology of the fungus, promote more complete glucose metabolism and rapid growth with less efficient fumarate production (Foster & Waksman, 1939). Wegener & Romano (1963) have shown that addition of zinc to cultures of R. nigricans results in an immediate increase in RNA, followed by a corresponding increase in protein and cell mass. The quantity of zinc is regarded as being critical for secondary metabolic processes in fungi and actinomycetes where it is thought to function in enzyme regulation prior to, during and following transcription (Weinberg, 1970, 1982).

The more active metabolic state of the dispersed mycelia from CMC and Carbopol-supplemented media is also reflected in the higher conversion rates of progesterone to 11α -hydroxyprogesterone. While higher % conversions were observed in biotransformations utilising CMC grown mycelia than in control, data on the amount of product formed per unit biomass indicate that part of the increased conversion was a result of the higher biomass produced. Consequently, different morphological forms produced in 24 h control (pelleted) and CMC (filamentous) media show similar 11α -hydroxyprogesterone formation efficiency. A low dissolved oxygen tension (DOT) optimum was found to favour maximum hydroxylase activity in R. nigricans (Hanisch et al., 1980). The DOT for a maximum hydroxylation rate was much higher than for enzyme synthesis so that it was preferable to

increase the DOT after induction was complete.

The low level of glucose consumption and 11α -hydroxyprogesterone production by 48 h control mycelia compared to 24 h control suggests that the older mycelium had practically ceased metabolic activity. There is a specific requirement for NADPH as a cofactor in the functioning of the mono oxygenase and cytochrome P-450 involved in the hydroxylase system (Breskvar & Hudnik-Plevnik, 1977). Low DOT optima are a property of various cytochromes in other microbial systems (Harrison, 1972). Maddox *et al.* (1981) reported that a decrease in the cell's general metabolic activity would lead to a decrease in the steroid hydroxylase activity, as NADPH regeneration is involved in the hydroxylase system. These authors see the role of glucose in the system as serving as an energy source, and not having any direct effect on the enzyme. The regeneration of the cofactor is seen as an important advantage of using immobilised whole cells over immobilised enzymes (Somerville & Mason, 1979). NADPH is generated from the pentose phosphate pathway, a route which normally accounts for only 30-40% of the carbon flux, the predominant pathway being the Embden-Meyerhof (Bull & Trinci, 1977).

With glucoamylase production an increased production capacity is demonstrated by dispersed mycelia from CMC medium. The presence of CMC in the bran medium used was seen to induce higher levels of glucoamylase activity in control and CMC-grown mycelia. This induction effect has been reported previously for α -amylase production by *Rhizopus* species (Takahara *et al.*, 1965). It was more pronounced at 48 h, indicating that growth of the mycelial inoculum may have occurred with greater enzyme activity. The presence of

Carbopol repressed glucoamylase production in all four types of mycelia. This inhibitory effect, as seen also with fumaric acid production in biotransformation trials, may be a result of the polymer coating the mycelia, preventing excretion of the enzyme or alternatively, interacting with the enzyme in solution and repressing activity. A mutation affecting cell wall composition in N. crassa also altered secretion of a range of extracellular enzymes produced by the fungus (Gratzner, 1972).

Soluble medium components, such as glucose or starch, did not promote glucoamylase production in submerged culture whereas higher yields of over 1 unit/ml were obtained with more complex sources such as maize. Nishise et al. (1988) noted that glucose repressed glucoamylase production in liquid culture whereas rice bran promoted reasonable activity. The difficulties in attaining satisfactory levels of production with Rhizopus strains in submerged culture have been noted previously, whereas a commercial process using ground corn and steep liquor for production of glucoamylase by A. foetidus in submerged culture has been reported (Underkofler, 1969). The traditional and preferred use of Rhizopus in surface culture fermentation (Miall, 1975) was borne out by the increased yields of glucoamylase obtained using a solid bran medium. Rice bran, supplemented with 0.1% turpentine oil, has recently been shown to give increased glucoamylase activity in surface culture (Nishise et al., 1988).

Thus, dispersed filamentous mycelia from CMC medium produced optimum yields of fumaric acid, 11 α -hydroxyprogesterone and glucoamylase. Mycelia from control medium produced highest yields when agglutination of hyphae/pellets was limited by high

aeration/agitation rates in fermenter cultivation. This suggests that the relative metabolic activity of the mycelium, as determined by the morphology, is the major influencing factor in product formation.

5. REFERENCES

- Ainsworth, G.C. (1973). Introduction and keys to the higher taxa. In *The Fungi: An Advanced Treatise*, volume 4a, pp 1-7. Edited by G.C. Ainsworth, K.F. Sparrow and A.S. Sussman. New York: Academic Press.
- Ames, B.N. (1966). Assay of inorganic phosphate, total phosphate and phosphatases. In *Methods in Enzymology*, volume 8, pp 115-118. Edited by S.P. Colowick and N.O. Kaplan. New York and London: Academic Press.
- Amri, M.A., Bonaly, R., Duteurtre, B and Moll, M. (1982). Yeast Flocculation: Influence of nutritional factors on cell wall composition. *Journal of General Microbiology* 128, 2001-2009.
- Araki, Y. and Ito, E. (1975). A pathway of chitosan formation in *Mucor rouxii*. *European Journal of Biochemistry* 55, 71-78.
- Attia, R.M. and Ali, S.A. (1973). Production of glucoamylase by *Aspergillus awamori*. *Agricultural Research Review (Cairo)* 52, 201-206.
- Ballesta, J.P.G., and Alexander, M. (1971). Resistance of *Zygorhynchus* species to lysis. *Journal of Bacteriology* 107, 938-945.
- Banks, G.T. (1977). Aeration of mould and streptomycete culture fluids. In *Topics in Enzyme and Fermentation Biotechnology*, Volume 1, pp 73-110. Edited by A. Wiseman. Chichester: Ellis Horwood.
- Bartnicki-Garcia, S. (1968). Cell wall chemistry, morphogenesis and taxonomy of fungi. *Annual Review of Microbiology* 22, 87-108.
- Bartnicki-Garcia, S. (1973). Fundamental aspects of hyphal morphogenesis. *Symposium No. 23 of the Society for General Microbiology* 23, 245-267.
- Bartnicki-Garcia, S., Bracker, C.E., Reyes, E. and Ruiz-Herrera, J. (1978). Isolation of chitosomes from taxonomically diverse fungi and synthesis of chitin microfibrils *in vitro*. *Experimental Mycology* 2, 173-192.
- Bartnicki-Garcia, S. and Lindberg, B. (1972). Partial characterisation of mucroan: the glucuromannan component. *Carbohydrate Research* 23, 75-85.
- Bartnicki-Garcia, S. and Lippman, E. (1972). The bursting tendency of hyphal tips of fungi: Presumptive evidence for a delicate balance between wall synthesis and wall lysis in apical growth. *Journal of General Microbiology* 73, 487-500.
- Bartnicki-Garcia, S. and Nickerson, W.J. (1962). Nutrition, growth and morphogenesis of *Mucor rouxii*. *Journal of Bacteriology* 84, 841-857.

- Bartnicki-García, S. and Reyes, E. (1968a). Chemical composition of the sporangiophore walls of Mucor rouxii. Biochimica et Biophysica Acta 165, 32-42.
- Bartnicki-García, S. and Reyes, E. (1968b). Polyuronides in the cell walls of Mucor rouxii. Biochimica et Biophysica Acta 170, 54-62.
- Bisaria, V.S., Nanda, M. and Ghose, T.K. (1986). Effect of L-sorbose on the release of β -glucosidase by Trichoderma reesei QM 9414. Journal of General Microbiology 132, 973-978.
- Bitter, T. and Muir, H.M. (1962). A modified uronic acid carbazole reaction. Analytical Biochemistry 4, 330-334.
- Blain, J.A. (1975). Industrial enzyme production. In The Filamentous Fungi, volume 1, pp 193-211. Edited by J.E. Smith & D.R. Berry. London: Edward Arnold.
- Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. Analytical Biochemistry 72, 248-254.
- Breskvar, K. and Hudnik-Plevnik, T. (1977). A possible role of cytochrome P-450 in hydroxylation of progesterone by Rhizopus nigricans. Biochemical and Biophysical Research Communications 74, 1192-1198.
- Breskvar, K. and Hudnik-Plevnik, T. (1978). Inducibility of progesterone hydroxylating enzymes in Rhizopus nigricans. Journal of Steroid Biochemistry 9, 131-134.
- Bull, A.T. and Trinci, A.P.J. (1977). The physiology and metabolic control of fungal growth. Advances in Microbial Physiology 15, 1-84.
- Burkholder, P.R. & Sinnott, E.W. (1945). Morphogenesis of fungus colonies in submerged shaken cultures. American Journal of Botany 32, 424-431.
- Burnett, J.H. (1976). Transport processes in fungi. In Fundamentals of Mycology, pp 249-301. London: Edward Arnold.
- Burnett, J.H. (1979). Aspects of the structure and growth of hyphal walls. In Fungal Walls and Hyphal growth, pp 1-25. Edited by J.H. Burnett & A.P.J. Trinci. Cambridge University Press.
- Byrne, G.S. (1985). Growth and production of lipase by Rhizopus arrhizus in submerged culture. Ph.D. Thesis, National Institute for Higher Education, Dublin, Ireland.
- Byrne, G.S. and Ward, O.P. (1987). Effect of polymers on pelleting of Rhizopus arrhizus. Transactions of the British Mycological Society 89, 367-371.

- Calam, C.T. (1976). Starting investigational and production cultures. *Process Biochemistry* 11, 7-12.
- Caldwell, I.Y., and Trinci, A.P.J. (1973). The growth unit of the mould Geotrichum candidum. *Archiv fur Mikrobiologie* 88, 1-10.
- Calvo-Mendez, C. and Ruiz-Herrera, J. (1987). Biosynthesis of chitosan in membrane fractions from Mucor rouxii by the concerted action of chitin synthetase and a particulate deacetylase. *Experimental Mycology* 11, 128-140.
- Camici, L., Sermonti, G. and Chain, E.B. (1952). Observations on Penicillium chrysogenum in submerged culture. *Bulletin of the World Health Organisation* 6, 265-276.
- Carilli, A., Chain, E.B., Gaulandi, G. and Morisi, G. (1961). Aeration studies III. Continuous measurements of dissolved oxygen during fermentations in large fermenters. *Science Reports, 1st Super Sanita* 1, 177-189.
- Ceen, E.G., Herrmann, J.P.R. and Dunnill, P. (1987). Solvent damage during cell free catalysis and its avoidance: Studies of 11 α -hydroxylation of progesterone by Aspergillus ochraceus. *Enzyme and Microbial Technology* 9, 365-368.
- Choudhary, Q.A. & Pirt, S.J. (1965). Metal-complexing agents as metal buffers in media for the growth of Aspergillus niger. *Journal of General Microbiology* 41, 99-107.
- Clark, D.S. (1962). Submerged citric acid fermentation of ferrocyanide-treated beet molasses: morphology of pellets of Aspergillus niger. *Canadian Journal of Microbiology* 8, 133-136.
- Clark, D.S., Ito, K. and Haristu, H. (1966). Effect of manganese and other heavy metals on submerged citric acid fermentation of molasses. *Biotechnology and Bioengineering* 8, 465-471.
- Collinge, A.J. and Trinci, A.P.J. (1974). Hyphal tips of wild-type and spreading colonial mutants of Neurospora crassa. *Archives of Microbiology* 99, 353-368.
- Crook, E.M. and Johnson, I.R. (1962). The qualitative analysis of the cell walls of selected species of fungi. *Biochemical Journal* 83, 325-331.
- Datema, R., van den Ende, H. and Wessels, J.G.H. (1977a). The hyphal wall of Mucor mucedo: 1. Polyanionic Polymers. *European Journal of Biochemistry* 80, 611-619.
- Datema, R., Wessels, J.G.H. and Van den Ende, H. (1977b). The hyphal wall of Mucor mucedo: 2. Hexosamine-containing polymers. *European Journal of Biochemistry* 80, 621-626.
- Davis, L.L. and Bartnicki-Garcia, S. (1984a). The co-ordination of chitosan and chitin synthesis in Mucor rouxii. *Journal of General Microbiology* 130, 2095-2102.

- Davis, L.L. and Bartnicki-Garcia, S. (1984b). Chitosan synthesis by the tandem action of chitin synthetase and chitin deacetylase from Mucor rouxii. Biochemistry 23, 1065-1073.
- De Rome, L. and Gadd, G.M. (1987). Copper adsorption by Rhizopus arrhizus, Cladosporium resinae and Penicillium italicum. Applied Microbiology and Biotechnology 26, 84-90.
- DeTerra, N. and Tatum, E.L. (1961). Colonial growth of Neurospora. Science 134, 1066-1068.
- DeTerra, N. and Tatum, E.L. (1963). A relationship between cell wall structure and colonial growth in Neurospora crassa. American Journal of Botany 50, 669-677.
- Dion, W.M., Carilli, A., Sermonti, G. and Chain, E.B. (1954). The effect of mechanical agitation on the morphology of Penicillium chrysogenum in stirred fermenters. Bulletin of the World Health Organisation 17, 187-205.
- Dion, W.M. and Kaushal, R. (1959). The effect of mechanical agitation on the morphology of some common fungi grown in submerged culture. Selected scientific papers from the Instituto Superiore di Sanita 2, 357-369.
- Dow, J.M., Barnall, D.W. and Villa, V.D. (1983). Two distinct classes of polyuronide from the cell walls of a dimorphic fungus, Mucor rouxii. Journal of Bacteriology 155, 1088-1093.
- Dow, J.M. and Rubery, P.H. (1975). Hyphal tip bursting in Mucor rouxii: Antagonistic effects of calcium ions and acid. Journal of General Microbiology 91, 425-428.
- Ekundayo, J.A. (1966). Further studies on germination of sporangiospores of Rhizopus arrhizus. Journal of General Microbiology 42, 283-291.
- Ekundayo, J.A. and Carlile, M.J. (1964). The germination of sporangiospores of Rhizopus arrhizus; spore swelling and germ-tube emergence. Journal of Genral Microbiology 35, 261-269.
- Elmayergi, H. (1975). Mechanisms of pellet formation of Aspergillus niger with an additive. Journal of Fermentation Technology 53, 722-729.
- Elmayergi, H. and Moo-Young, M. (1973). Effects of a polymer additive on mass transfer into mold pellets. Biotechnology and Bioengineering Symposium No. 4, 507-512.
- Elmayergi, H., Scharer, J.M. and Moo-Young, M. (1973). Effect of polymer additives on fermentation parameters in a culture of A. niger. Biotechnology and Bioengineering 15, 845-859.

- Elorza, M.V., Lostau, C.M., Villaneuva, J.R. & Sentandreu, R. (1976). Cell wall synthesis regulation in Saccharomyces cerevisiae. Effect of RNA and protein inhibition. Biochimica et Biophysica Acta 454, 263-272.
- Elorza, M.V., Rico, H. and Sentandreu, R. (1983). Calcofluor White alters the assembly of chitin fibrils in Saccharomyces cerevisiae and Candida albicans cells. Journal of General Microbiology 129, 1577-1582.
- Fairbairn, N.J. (1953). A modified anthrone reagent. Chemistry and Industry (London) 72, 86.
- Farkas, V. (1979). Biosynthesis of cell walls of fungi. Microbiological Reviews 43, 117-144.
- Fisher, D.J. (1975). Flocculation - some observations on the surface changes of yeast cells. Journal of the Institute of Brewing 81, 107-110.
- Fisher, D.J. and Richmond, D.V. (1969). The electrokinetic properties of some fungal spores. Journal of General Microbiology 57, 51-60.
- Fogarty, W.M. (1983). Microbial amylases. In Microbial Enzymes & Biotechnology, pp 1-92. Edited by W.M. Fogarty. London & New York: Applied Science Publishers.
- Foster, J.W., Carson, S.F., Anthony, D.S., Davis, J.B., Jefferson, W.E. and Long, M.V. (1949). Aerobic formation of fumaric acid in the mold Rhizopus nigricans: synthesis by direct C-2 condensation. Proceedings of the National Academy of Sciences 35, 663-672.
- Foster, J.W. and Waksman, S.A. (1939). The specific effect of zinc and other heavy metals on growth and fumaric acid production by Rhizopus. Journal of Bacteriology 37, 599-617.
- Gabriel, M. (1968). Formation and regeneration of protoplasts in the mold Rhizopus nigricans. Folia Microbiologica 13, 231-234.
- Gabriel, M. (1984). Inhibition of the cell wall regeneration and reversion in protoplasts of Rhizopus nigricans. Folia Microbiologica 29, 365-374.
- Gadd, G.M. and White, C. (1989). Removal of thorium from simulated acid process streams by fungal biomass. Biotechnology and Bioengineering 33, 592-597.
- Galbraith, J.C. and Smith, J.E. (1969). Filamentous growth of Aspergillus niger in submerged shake culture. Transactions of the British Mycological Society 52, 237-246.
- Goldberg, I. and Stieglitz, B. (1985). Improved rate of fumaric acid production by tweens and vegetable oils in Rhizopus arrhizus. Biotechnology and Bioengineering 27, 1067-1069.

- Gomez, R., Schnabel, I. and Garrido, J. (1988). Pellet growth and citric acid yield of Aspergillus niger 110. Enzyme and Microbial Technology 10, 188-191.
- Gooday, G.W. (1971). An autoradiographic study of hyphal growth of some fungi. Journal of General Microbiology 67, 125-133.
- Gooday, G.W. and Trinci, A.P.J. (1980). Wall structure and biosynthesis in fungi. In The Eukaryotic Microbial Cell, pp 207-251. Edited by G.W. Gooday, D. Lloyd and A.P.J. Trinci. Cambridge University Press.
- Goodrich, B.F. (1986). In Carbopol water soluble resins. Cleveland, U.S.A.: B.F. Goodrich.
- Gow, N.A.R. and Gooday, G.W. (1983). Ultrastructure of chitin in hyphae of Candida albicans and other dimorphic and mycelial fungi. Protoplasma 115, 52-58.
- Gratzner, H.G. (1972). Cell wall alterations associated with the hyperproduction of extracellular enzymes in Neurospora crassa. Journal of Bacteriology 111, 443-446.
- Gull, K. and Trinci, A.P.J. (1974). Detection of areas of wall differentiation in fungi using fluorescent staining. Archives of Microbiology 96, 53-57.
- Hanisch, W.H. and Dunnill, P. (1980). Regeneration of the progesterone 11 α -hydroxylase of Rhizopus nigricans by the use of periodate. Biotechnology Letters 2, 123-126.
- Hanisch, W.H., Dunnill, P. and Lilly, M.D. (1980). Optimisation of the production of progesterone 11 α -hydroxylase by Rhizopus nigricans. Biotechnology and Bioengineering 22, 555-570.
- Harrison, D.E.F. (1972). Physiological effects of dissolved oxygen tension and redox potential on growing populations of microorganisms. Journal of Applied Chemistry & Biotechnology 22, 417-440.
- Herth, W. (1980). Calcofluor White and Congo Red inhibit chitin microfibril assembly of Poteroiochromonas: Evidence for a gap between polymerisation and microfibril formation. Journal of Cell Biology 87, 442-450.
- Hunsley, D. and Burnett, J.M. (1970). The ultrastructural architecture of the walls of some hyphal fungi. Journal of General Microbiology 62, 203-218.
- Katz, D., Goldstein, D. and Rosenberger, R.F. (1972). Model for branch initiation in Aspergillus nidulans based on measurements of growth parameters. Journal of Bacteriology 109, 1097-1100.

- Katz, D. and Rosenberger, R.F. (1970). A mutation in Aspergillus nidulans producing hyphal walls which lack chitin. Biochimica et Biophysica Acta 208, 452-460.
- Katz, D. and Rosenberger, R.F. (1971). Hyphal wall synthesis in Aspergillus nidulans: Effect of protein synthesis inhibition and osmotic shock on chitin insertion and morphogenesis. Journal of Bacteriology 105, 184-190.
- Kenealy, W., Zaady, E., du Preez, C., Stieglitz, B. and Goldberg, I. (1986). Biochemical aspects of fumaric acid accumulation by Rhizopus arrhizus. Applied and Environmental Microbiology 52, 128-133.
- Kisser, M., Kubicek, C.P. and Rohr, M. (1980). Influence of manganese on morphology and cell wall composition of Aspergillus niger during citric acid fermentation. Archives of Microbiology 126, 26-33.
- Kobayashi, H. and Suzuki, H. (1972). Studies on the decomposition of raffinose by α -galactosidase of mold II. Formation of mold pellet and its enzyme activity. Journal of Fermentation Technology 50, 625-632.
- Kobayashi, T., van Dedem, G. and Moo-Young, M. (1973). Oxygen transfer into mycelial pellets. Biotechnology and Bioengineering 15, 27-45.
- Kolot, F.B. (1982). Microbial catalysts for steroid transformations - Part 1. Process Biochemistry 17, 12-18.
- Konig, B., Schugerl, K. and Seewald, C. (1982). Penicillin fermentation in tower-loop reactors. Biotechnology and Bioengineering 24, 259-280.
- Kornberg, H.L. (1959). Aspects of terminal respiration in microorganisms. Annual Review of Microbiology 13, 49-78.
- Kreger, D.R. (1954). Observations on cell walls of yeasts and some other fungi by X-ray diffraction and solubility tests. Biochimica et Biophysica Acta 13, 1-9.
- Kristiansen, B. and Bu'lock, J.D. (1980). Developments in industrial biotechnology. In Fungal Biotechnology, pp 203-223. Edited by J.E. Smith, D.R. Berry and B. Kristiansen. London: Academic Press.
- Kropf, D.L., Lupa, M.D.A., Caldwell, J.H. & Harold, F.M. (1983). Cell polarity: Endogenous ion currents precede and predict branching in the water mold Achlya. Science 220, 1385-1387.
- Kubicek, C.P., Hampel, W. and Rohr, M. (1979). Manganese deficiency leads to elevated amino acid pools in citric acid accumulating Aspergillus niger. Archives of Microbiology 123, 73-79.

- Kubicek, C.P. and Rohr, M. (1977). The influence of manganese on enzyme synthesis and citric acid accumulation by *Aspergillus niger*. *Journal of Applied Microbiology and Biotechnology* 4, 167-175.
- Litchfield, J.H., Overbeck, R.C. & Davidson, R.S. (1963). Factors affecting the growth of morel mushroom mycelium in submerged culture. *Journal of Agricultural and Food Chemistry* 11, 158-163.
- Lockwood, L.B. (1975). Organic acid production. In *The Filamentous Fungi*, volume I, pp 140-157. Edited by J.E. Smith and D.R. Berry. London: Edward Arnold.
- Lyons, T.P. and Hough, J.S. (1970). Flocculation of brewers yeast. *Journal of the Institute of Brewing* 76, 564-571.
- Maddox, I.S., Dunnill, P. and Lilly, M.D. (1981). Use of immobilised cells of *Rhizopus nigricans* for the α -hydroxylation of progesterone. *Biotechnology and Bioengineering* 23, 345-354.
- Mahadevan, P.R. and Mahadkar, U.R. (1970). Role of enzymes in growth and morphology of *Neurospora crassa*: Cell-wall bound enzymes and their possible role in branching. *Journal of Bacteriology* 101, 941-947.
- Mahadevan, P.R. and Tatum, E.L. (1965). Relationship of the major constituents of the *Neurospora crassa* cell wall to wild-type and colonial morphology. *Journal of Bacteriology* 90, 1073-1081.
- Mahadevan, P.R. and Tatum, E.L. (1967). Localisation of structural polymers in the cell wall of *Neurospora crassa*. *Journal of Cell Biology* 35, 295-302.
- Manocha, M.S. and Colvin, J.R. (1967). Structure and composition of the cell wall of *Neurospora crassa*. *Journal of Bacteriology* 94, 202-212.
- Marshall, K.C. and Alexander, M. (1960). Growth characteristics of fungi and actinomycetes. *Journal of Bacteriology* 80, 412-416.
- Martin, S.M. and Adams, G.A. (1956). A survey of fungal polysaccharides. *Canadian Journal of Microbiology* 34, 715-721.
- Medwid, R.D. and Grant, D.W. (1984). Germination of *Rhizopus oligosporus* sporangiospores. *Applied and Environmental Microbiology* 48, 1067-1071.
- Metz, B. (1976). From pulp to pellet. Ph.D. Thesis, Technical University of Delft, The Netherlands.
- Metz, B. and Kossen, N.W.F. (1977). Biotechnology review: The growth of molds in the form of pellets - a literature review. *Biotechnology and Bioengineering* 19, 781-790.

- Miall, L.M. (1975). Historical development of the fungal fermentation industry.
In *The Filamentous Fungi*, volume 1, chapter 6, pp 104-121. Edited by J.E. Smith and D.R. Berry, London: Edward Arnold.
- Miki, B.L.A., Hung Poon, J., James, A.P. and Seligy, V.L. (1982a). Possible mechanisms for flocculation interactions governed by gene FLO-1 in *Saccharomyces cerevisiae*.
Journal of Bacteriology 150, 878-889.
- Miki, B.L.A., Hung Poon, N. and Seligi, V.L. (1982b). Repression and induction of flocculation interactions in *Saccharomyces cerevisiae*.
Journal of Bacteriology 150, 890-899.
- Mill, P.J. (1964). The nature of the interactions between flocculent cells in the flocculation of *Saccharomyces cerevisiae*.
Journal of General Microbiology 35, 61-68.
- Miller, G.L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugars.
Analytical Chemistry 31, 426-428.
- Mishra, N.C. and Tatum, E.L. (1972). Effect of L-sorbose on polysaccharide synthases of *Neurospora crassa*.
Proceedings of the National Academy of Sciences (USA) 69, 313-317.
- Miyazaki, T. and Irino, T. (1971). Studies on fungal polysaccharides IX. The acidic polysaccharide from the cell wall of *Rhizopus nigricans*.
Chemical and Pharmacological Bulletin 19, 2545-2550.
- Moo-Young, M., Hirose, T. and Geiger, K.H. (1969). The rheological effects of substrate-additives on fermentation yields.
Biotechnology and Bioengineering 11, 725-730.
- Morrison, K.B. and Righelato, R.C. (1974). The relationship between hyphal branching, specific growth rate and colony radial growth rate in *Penicillium chrysogenum*.
Journal of General Microbiology 81, 517-520.
- Mowll, J.L. and Gadd, G.M. (1984). Cadmium uptake by *Aureobasidium pullulans*.
Journal of General Microbiology 130, 279-284.
- Nishise, H., Fiyi, A., Ueno, M., Vongsuvanlert, V. and Tani, Y. (1988). Production of raw cassava starch-digestive glucoamylase by *Rhizopus* sp. in liquid culture.
Journal of Fermentation Technology 66, 397-402.
- Osmani, S.A. and Scrutton, M.C. (1985). The sub-cellular localisation and regulatory properties of pyruvate carboxylase from *Rhizopus arrhizus*.
European Journal of Biochemistry 147, 119-128.
- Pazur, J.H. and Okada, S. (1967). Properties of the glucoamylase from *Rhizopus delemar*.
Carbohydrate Research 4, 371-379.

- Peterson, D.H. and Murray, H.C. (1952). Microbiological oxygenation of steroids at carbon 11.
Journal of the American Chemical Society 74, 1871-1872.
- Peterson, D.H., Murray, H.C., Epstein, S.H., Reineke, L.M., Weintraub, A., Meister, P.D. and Leigh, H.M. (1952). Microbiological transformations of steroids-1. Introduction of oxygen at carbon-11 of progesterone.
Journal of the American Chemical Society 74, 5933-5936.
- Phillips, D.H. (1966). Oxygen transfer into mycelial pellets.
Biotechnology and Bioengineering 8, 456-460.
- Pirt, S.J. (1966). A theory of the mode of growth of fungi in the form of pellets in submerged culture.
Proceedings of the Royal Society B, 166, 369-373.
- Pirt, S.J. and Callow, D.S. (1959). Continuous-flow culture of the filamentous mould Penicillium chrysogenum and the control of its morphology.
Nature 184, 307-310.
- Prosser, J.I. and Trinci, A.P.J. (1979). A model for hyphal growth and branching.
Journal of General Microbiology 111, 153-164.
- Raven, J.A. and Smith, F.A. (1973). The regulation of intracellular pH as a fundamental biological process.
In Ion Transport in Plants, pp 271-278. Edited by W.P. Anderson, London and New York: Academic Press
- Rhodes, R.A., Moyer, A.J., Smith, M.L. and Kelley, S.E., (1959). Production of fumaric acid by Rhizopus arrhizus.
Applied Microbiology 7, 74-80.
- Rhodes, R.A., Lagoda, A.A., Misenheimer, T.J., Smith, M.L., Anderson, R.F. and Jackson, R.W. (1962). Production of fumaric acid in 20-litre fermenters.
Applied Microbiology 10, 9-15.
- Righelato, R.C. (1979). The kinetics of mycelial growth.
In Fungal Walls and Hyphal Growth, pp 385-401. Edited by J.H. Burnett and A.P.J. Trinci. Cambridge University Press.
- Righelato, R.C., Trinci, A.P.J., Pirt, S.J. and Peat, A. (1968). The influence of maintenance energy and growth rate on the metabolic activity, morphology and conidiation of Penicillium chrysogenum.
Journal of General Microbiology 50, 399-412.
- Romano, A.H., Bright, M.M. and Scott, W.E. (1967). Mechanism of fumaric acid accumulation in Rhizopus nigricans.
Journal of Bacteriology 93, 600-604.
- Rondle, C.J.M. and Morgan, W.T.J. (1955). The determination of glucosamine and galactosamine.
Biochemical Journal 61, 586-589.

- Rosenberger, R.F. (1976). The cell wall.
In *The Filamentous fungi*, volume 2, pp 328-344. Edited by J.E. Smith and D.R. Berry. London: Edward Arnold.
- Ruiz-Herrera, J. and Bartnicki-Garcia, S. (1976). Proteolytic activation and inactivation of chitin synthase from Mucor rouxii. *Journal of General Microbiology* 97, 241-249.
- Schmid, J. and Harold, F.M. (1988). Dual roles for calcium ions in apical growth of Neurospora crassa. *Journal of General Microbiology* 134, 2623-2631.
- Schugerl, K., Wittler, R. and Lorenz, T. (1983). The use of molds in pellet form.
Trends in Biotechnology 1, 120-123.
- Sedlacek, L. (1988). Biotransformations of steroids.
CRC Critical Reviews in Biotechnology 7, 187-236.
- Seviour, R.J., Kristiansen, B. and Harvey, L. (1984). Morphology of Aureobasidium pullulans during polysaccharide elaboration.
Transactions of the British Mycological Society 82, 350-356.
- Seviour, R.J. and Read, M.A. (1985). Electrophoretic mobility of conidia of Aspergillus niger and the role of their surface properties in pelleting.
Transactions of the British Mycological Society 84, 745-747.
- Shumate, S.E. and Strandberg, G.W. (1985). Accumulation of metals by microbial cells.
In *Comprehensive Biotechnology*, volume 4, pp 235-247. Edited by C.W. Robinson and J.A. Howell. Oxford: Pergamon Press.
- Sietsma, J.H. & Wessels, J.G.H. (1977). Chemical analysis of the hyphal wall of Schizophyllum commune.
Biochimica et Biophysica Acta 496, 225-239.
- Sietsma, J.H. and Wessels, J.G.H. (1979). Evidence for covalent linkages between chitin and β -glucan in a fungal wall.
Journal of General Microbiology 114, 99-108.
- Slayman, C.L. (1970). Movement of ions and electrogenesis in microorganisms.
American Zoologist 10, 377-392.
- Slayman, C.L. and Slayman, C.W. (1962). Measurement of membrane potentials in Neurospora.
Science 136, 876-877.
- Smiley, K.L., Cadmus, M.C. & Liepins, P. (1967). Biosynthesis of D-mannitol from D-glucose by Aspergillus candidus.
Biotechnology and Bioengineering 9, 365-374.
- Solomons, G.L. (1975). Submerged culture production of mycelial biomass.
In *The Filamentous Fungi*, volume 1, pp 249-264. Edited by J.E. Smith and D.R. Berry. London: Edward Arnold.

- Solomons, G.L. (1983). Production of biomass by filamentous fungi. In *Comprehensive Biotechnology*, volume 3, pp 483-505. Edited by H.W. Blanch, S. Drew and D.I.C. Wang. Oxford: Pergamon Press.
- Somal, P. and Chopra, C.L. (1985). Microbial conversion of steroids III: 11 α -hydroxylation by fungal mycelium. *Applied Microbiology and Biotechnology* 21, 267-269.
- Somers, E. and Fisher, D.J. (1967). Effect of dodine acetate on the electrophoretic mobility of *Neurospora crassa* conidia. *Journal of General Microbiology* 48, 147-154.
- Somerville, H.J. and Mason, J.R. (1979). The physiology of aerobic bacteria in immobilised systems. *Biochemical Society Transactions* 7, 85-88.
- Sorenson, W.G. and Hesseltine, C.W. (1966). Carbon and nitrogen utilisation by *Rhizopus oligosporus*. *Mycologia* 58, 681-689.
- Steele, G.C. and Trinci, A.P.J. (1975). Morphology and growth kinetics of hyphae of differentiated and undifferentiated mycelia of *Neurospora crassa*. *Journal of General Microbiology* 91, 362-368.
- Sternlicht, E., Katz, D. and Rosenberger, R.F. (1973). Subapical wall synthesis and wall thickening induced by cycloheximide in hyphae of *Aspergillus nidulans*. *Journal of Bacteriology* 114, 819-823.
- Stewart, G.G., Russell, I. and Garrison, I.F. (1975). Some considerations of the flocculation characteristics of ale and lager yeast strains. *Journal of the Institute of Brewing* 81, 248-257.
- Stewart, G.G. and Goring, T.E. (1976). Effect of some monovalent and divalent metal ions on the flocculation of brewers yeast strains. *Journal of the Institute of Brewing* 82, 341-342.
- Suijdam van, J.C. & Metz, B. (1981). Influence of engineering variables upon the morphology of filamentous molds. *Biotechnology & Bioengineering* 23, 111-118.
- Suijdam van, J.C., Kossen, N.W.F. and Paul, P.G. (1980). An inoculum technique for the production of fungal pellets. *European Journal of Applied Microbiology and Biotechnology* 10, 221-221.
- Syrop, M. (1973). The ultrastructure of the growing regions of aerial hyphae of *Rhizopus sexualis* (Smith) Callen. *Protoplasma* 76, 309-314.
- Takahara, Y., Oote, S. and Aikawa, T. (1965). Effect of CMC on amylase formation in *Rhizopus* species. *Journal of Fermentation Technology* 43, 896-900.
- Takahashi, J., Hidaka, H. and Yamada, K. (1965). Effect of mycelial forms on citric acid fermentation in submerged mold culture. *Agricultural and Biological Chemistry* 29, 331-336.

- Takahashi, J., Kosano, Y. and Yamada, K. (1960). Effects of the culture fluid viscosity on the mycelial forms of molds. *Journal of the Agricultural and Chemical Society (Japan)* 34, 440-442.
- Takahashi, J. and Yamada, K. (1960). Studies on the effect of some physical conditions on the submerged mold culture. III. Relations between the morphological forms of molds and the viscosity of mycelial suspensions. *Journal of the Agricultural and Chemical Society (Japan)* 34, 100-103.
- Testi-Camposano, A. (1959). Morphological observations on the growth of Aspergillus flavus (Link) in shake flasks. *Selected Scientific Papers from the Instituto Superiore di Sanita* 2, 448-453.
- Thompson, D.P. and Rountree, H.A. (1982). The free organic acid composition of Rhizopus and Mucor species. *Indian Phytopathology* 35, 616-618.
- Tobin, J.M., Cooper, D.G. and Neufeld, R.J. (1984). Uptake of metal ions by Rhizopus arrhizus biomass. *Applied and Environmental Microbiology* 47, 821-824.
- Tominaga, Y. and Tsujisaka, Y. (1981). Investigation of the structure of Rhizopus cell wall with lytic enzymes. *Agricultural and Biological Chemistry* 45, 1569-1575.
- Trinci, A.P.J. (1970). Kinetics of the growth of mycelial pellets of Aspergillus nidulans. *Archiv fur Mikrobiologie* 73, 353-367.
- Trinci, A.P.J. (1973). Growth of wild type and spreading colonial mutants of Neurospora crassa in batch culture and on agar medium. *Archiv fur Mikrobiologie* 91, 113-126.
- Trinci, A.P.J. (1974). A study of the kinetics of hyphal extension and branch initiation of fungal mycelia. *Journal of General Microbiology* 81, 225-236.
- Trinci, A.P.J. (1978). Wall and hyphal growth. *Scientific Progress, Oxford* 65, 75-99.
- Trinci, A.P.J. (1979). The duplication cycle and branching in fungi. In *Fungal Walls and Hyphal Growth*, pp 319-358. Edited by J.H. Burnett & A.P.J. Trinci. Cambridge University Press.
- Trinci, A.P.J., (1983). Effect of Junlon on morphology of Aspergillus niger and its use in making turbidity measurements of fungal growth. *Transactions of the British Mycological Society* 81, 408-412.
- Trinci, A.P.J. and Collinge, A. (1973). Influence of L-sorbose on the growth and morphology of Neurospora crassa. *Journal of General Microbiology* 78, 179-192.

- Trinci, A.P.J. and Collinge, A. (1975).
Hyphal wall growth in Neurospora crassa and Geotrichum candidum.
Journal of General Microbiology 91, 355-361.
- Trinder, P. (1969). Determination of blood glucose using an
oxidase-peroxidase system with a non-carcinogenic chromagen.
Journal of Clinical Pathology 22, 158-161.
- Tsezos, M. (1983). The role of chitin in uranium adsorption by R. arrhizus.
Biotechnology and Bioengineering 25, 2025-2040.
- Tsezos, M. and Volesky, B. (1982). The mechanism of uranium
bisorption by Rhizopus arrhizus.
Biotechnology and Bioengineering 24, 385-401.
- Ueda, S. and Kano, S. (1975). Multiple forms of glucoamylase of
Rhizopus species.
Die Starke 27, 123-128.
- Ugalde, U. and Pitt, D. (1983). Morphology and calcium-induced
conidiation of Penicillium cyclopium in submerged culture.
Transactions of the British Mycological Society 80, 319-325.
- Underkofler, L.A. (1969). Development of a commercial enzyme process:
glucoamylase.
In Cellulases and their applications, pp 343-358. Edited by R.F.
Gould. Washington: American Chemical Society.
- Underkofler, L.A., Denault, L.J. & Hore, E.F. (1965). Enzymes in the
starch industry.
Die Starke 17, 179-184.
- van der Valk, P., Marchant, R. and Wessels, J.G.H. (1977).
Ultrastructural localisation of polysaccharides in the wall and
septum of the basidiomycete Schizophyllum commune.
Experimental Mycology 1, 69-82.
- van der Valk, P. and Wessels, J.G.H. (1976). Ultrastructure and
localisation of wall polymers during regeneration and reversion of
protoplasts of Schizophyllum commune.
Protoplasma 90, 65-87.
- Vermeulen, C.A. & Wessels, J.G.H. 1986. Chitin biosynthesis by a
fungal membrane preparation: Evidence for a transient non-crystalline
state of chitin.
European Journal of Biochemistry 158, 411-415.
- Wang, D.I.C., Cooney, C.L., Demain, A.L., Dunnill, P., Humphrey, A.E.
& Lilly, D.D. (1979). Regulation of enzyme production.
In Fermentation and Enzyme Technology, pp 46-56. New York: John
Wiley.
- Ward, O.P. (1989).
In Fermentation Biotechnology, Principles, Processes and Products.
Milton-Keynes: Open University Press.
- Weaver, E.A., Kennedy, H.E. and Wall, M.E. (1960). Effect of
concentration on the microbiological hydroxylation of progesterone.
Applied Microbiology 8, 345-348.

- Wegener, W.S. & Ramano, A.H. (1963). Zinc stimulation of RNA and protein synthesis in Rhizopus nigricans.
Science 142, 1669-1670.
- Weinberg, E.D. (1970). Role of trace metals in biosynthesis of secondary metabolites.
Advances in Microbial Physiology 4, 1-44.
- Weinberg, E.D. (1982). Biosynthesis of microbial metabolites-regulation by mineral elements and temperature.
In Overproduction of Microbial Products, pp 181-210. Edited by V. Krumphanzl, B., Sikyta and Z. Vanek. London: Academic Press.
- Wessels, J.G.H. (1986). Cell wall synthesis in apical hyphal growth.
International Review of Cytology 104, 37-79.
- Wessels, J.G.H., Kreger, D.R., Marchant, R., Regensburg, B.A. and deVries, O.M.H. (1972). Chemical and morphological characterisation of the hyphal wall surface of the basidiomycete Schizophyllum commune.
Biochimica et Biophysica Acta 273, 346-358.
- Wessels, J.G.H. and Sietsma, J.H. (1981). Significance of linkages between chitin and β -glucan in fungal walls.
In Microbiology, pp 232-234. Edited by D. Schlessinger. Washington D.C.: American Society for Microbiology.
- Whitaker, A. and Long, P.A. (1973). Fungal pelleting.
Process Biochemistry 8, 27-31.
- Wood, B.J.B. and Yong Fook Min. (1975). Oriental Food Fermentations.
In The Filamentous Fungi, volume 1, pp 265-280. Edited by J.E. Smith and D.R. Berry. London: Edward Arnold.
- Yanagita, T. & Kogane, F. (1963). Cytochemical and physiological differentiation of mold pellets.
Journal of General and Applied Microbiology 9, 179-187.
- Ziegler, H., Dunn, I.J. and Bourne, J.R. (1980). Oxygen transfer and mycelial growth in a tubular loop fermenter.
Biotechnology and Bioengineering 22, 1613-1635.

6. APPENDIX I

The following buffers were used as diluents.

1. Na₂HPO₄ - NaH₂PO₄ buffer, pH 6.0 - 7.5 (0.01M)

0.01M solutions of Na₂HPO₄.2H₂O (1.78 g/l) and of NaH₂PO₄.2H₂O (1.56 g/l) are made in distilled water and mixed in the following proportions:

Buffer pH	Na ₂ HPO ₄ .2H ₂ O (ml)	NaH ₂ PO ₄ .2H ₂ O (ml)
6.0	6.2	43.8
6.2	9.3	40.7
6.5	15.7	34.3
7.5	42.0	8.0

2. Sodium acetate - acetic acid buffer, pH 4.2-5.5 (0.05M)

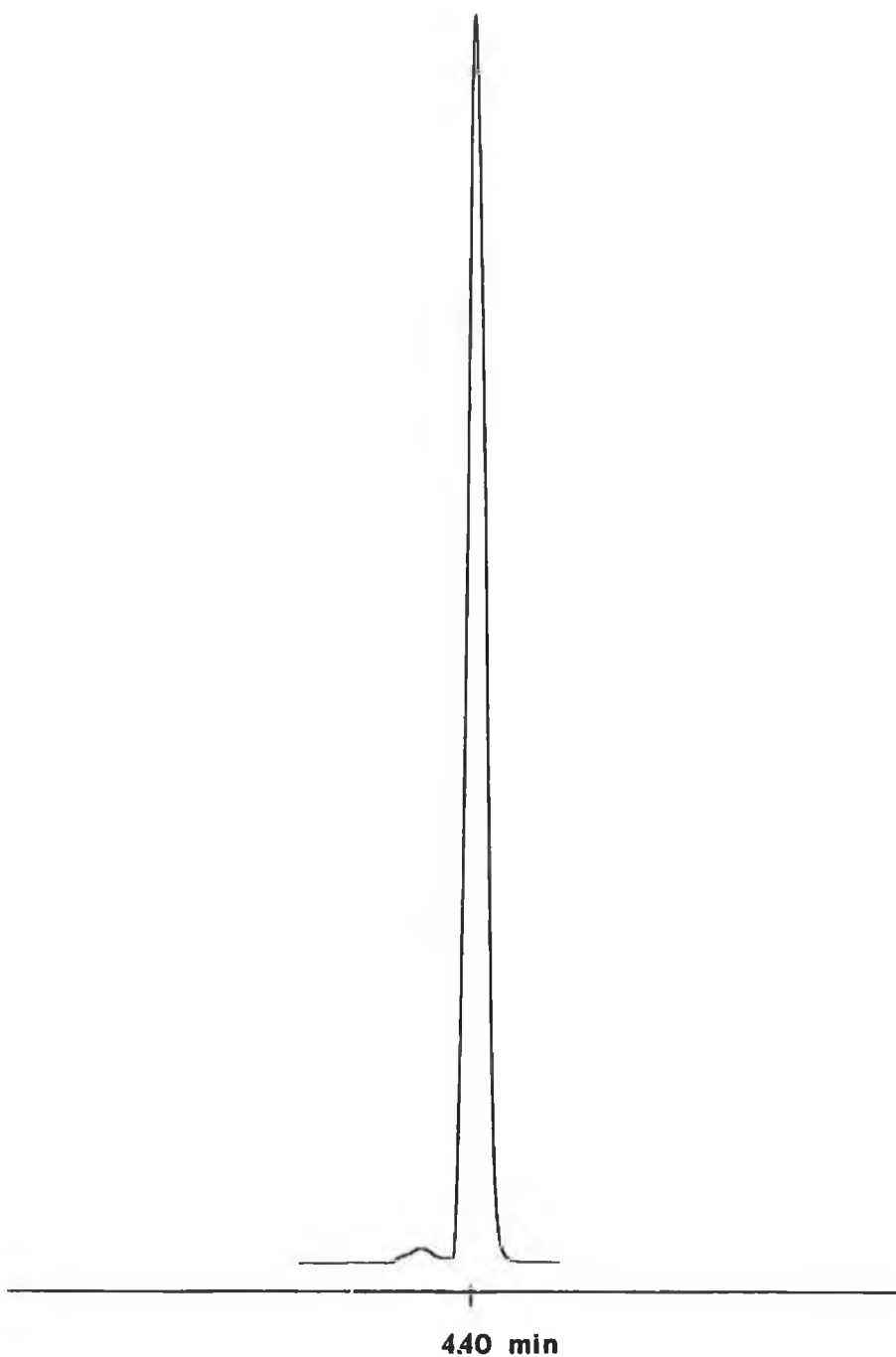
0.05M solutions of sodium acetate (4.10 g/l) and of acetic acid (3.0 g/l) are made in distilled water and mixed in the following proportions:

Buffer pH	Sodium acetate (ml)	Acetic acid (ml)
4.2	19.0	31.0
5.0	29.5	20.5
5.5	34.6	15.4

6. APPENDIX II

Chromatograms

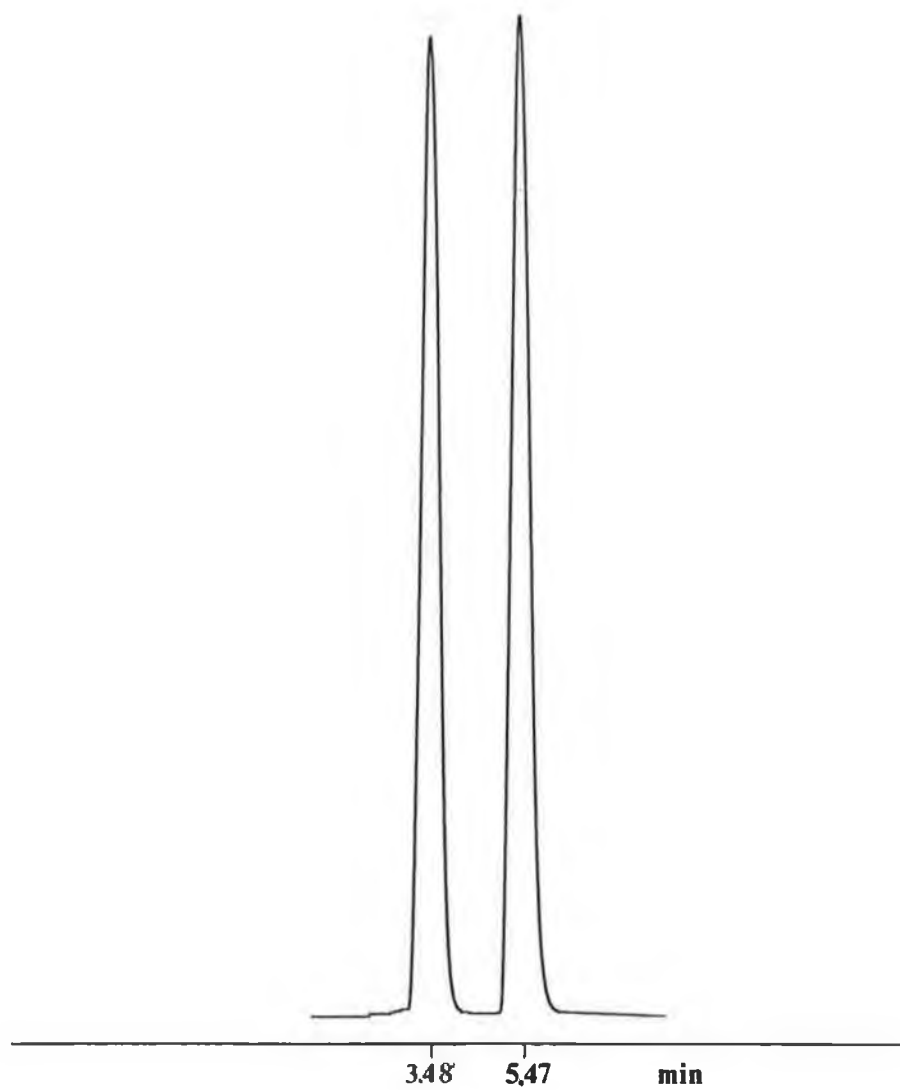
Sample chromatograms of fumaric acid and of progesterone and 11 α -hydroxyprogesterone are presented. These were obtained using a Waters Radial pak cartridge 10 x 1 cm, packed with Resolve C₁₈-bonded silica (5 μ) (Section 2.17.7.2).



Chromatogram 6.1. 100 ppm fumaric acid separated on a Resolve (5 μ) radial pak-cartridge, 10 x 1 cm.

Flow rate: 2 ml/min.

Eluent: 0.5% (w/v) ammonium sulphate, pH 2.4.



Chromatogram 6.2. 100 ppm progesterone and 11 α -hydroxyprogesterone separated on a Resolve 5(μ) radial-pak cartridge, 10 x 1 cm.

Flow rate: 2 ml/min.

Eluent: 70:30 acetonitrile:water

Research Communications

Studies on the interaction of Carbopol-934 with hyphae of Rhizopus arrhizus.

M. Morrin and O.P. Ward; Mycological Research (1989); 92(3), 265-272.

Investigation of cell wall composition of different mycelial forms of Rhizopus arrhizus.

M. Morrin and O.P. Ward; Mycological Research (1989); In Press.

Biotransformation of Progesterone to 11 α -Hydroxyprogesterone by different morphological forms of Rhizopus arrhizus.

M. Morrin and O.P. Ward; Biotech. Letters (1989); 11(5), 319-324.

Relationship between fungal growth, morphology and fumaric acid production by Rhizopus arrhizus.

M. Morrin and O.P. Ward; Accepted for publication in Mycological Research.

Effect of media composition on growth morphology of Rhizopus arrhizus.

M. Morrin and O.P. Ward; Proceedings of 103rd Ordinary Meeting of the Society for General Microbiology (at Warwick University, England), p14, 1985.

Studies on mycelial structure and cell wall composition of different morphological forms of Rhizopus arrhizus.

M. Morrin and O.P. Ward; Proceedings at 113th Ordinary Meeting of the Society for General Microbiology (at Cambridge University, England), p12, 1989.

Influence of fungal morphology on product formation by Rhizopus arrhizus.

Ward, O.P., Morrin, M. and Byrne, G.S; Proceedings of the Annual General Meeting of the Canadian Society for Microbiology (at Laval, Quebec, Canada, July), 1989.

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