

Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

**OPTIMISATION STUDIES OF AN ATOXIGENIC STRAIN OF
PITHOMYCES CHARTARUM IN SUBMERGED FERMENTATION**

GEOFFREY D. LUDEMANN

1999

**A thesis presented in partial fulfillment of the requirements for the
degree of Master of Technology in Biotechnology**

Massey University, Palmerston North, New Zealand

ABSTRACT

The conditions for maximum biomass concentration and propagule production by an atoxigenic strain of *Pithomyces chartarum* were investigated, in shake-flask and submerged fermenter. On the shake-flask scale, a variety of carbon sources, nitrogen sources, growth additives and pH values were studied as medium components and conditions. The dry weight biomass and the rate of substrate utilisation were used to assess the growth of the organism, while the number of colony-forming-units per unit volume was also determined following a standard homogenisation procedure, to monitor organism viability. The organism was shown to be capable of utilising a wide range of nutrient sources. Glucose and casamino acids were the best carbon and nitrogen sources, respectively, while the optimum culture pH value was in the broad range pH 4 to 7. The effect of growth additives was negligible.

The performances of different strains of the organism on the semi-defined medium were compared and two of the three strains tested grew to desirable parameters. The third strain, however, was reluctant to sporulate despite repeated efforts. This non-sporulating strain did produce the greatest biomass concentration however. Following the establishment of a semi-defined medium, a possible production medium was formulated by replacing the nutritional components of the defined medium with low-cost complex substrates. The use of whey permeate was investigated for this role. The results showed that whey permeate alone was a poor medium; however when supplemental carbon sources, such as starch or sucrose, were added, the viability counts and biomass produced were the greatest yet achieved.

Studies of the effect of agitation, aeration and inoculum size were conducted in small-scale submerged fermentation. This technology was demonstrated to be satisfactory for production of *P. chartarum* biomass. In the range of these variables studied, however, there was little effect noted on the viability or biomass concentration produced. Finally, the effect of storage of the harvested biomass on its viability was studied. It was shown that the viability of the organism decreased markedly with storage over a 3-month period, and that the preserving techniques tested had little effect in reversing this trend.

ACKNOWLEDGEMENTS

I would like to sincerely thank my supervisor, Associate Professor Ian Maddox for giving the opportunity to work with him in this study. His constant guidance, supervision and encouragement were greatly appreciated.

I am also grateful to Roger Collin and Agresearch, Ruakura, for their funding for this project and I wish them success in their work with *Pithomyces chartarum*.

I would also like to extend my appreciation and gratitude to all the people who provided the technical and laboratory assistance and answers to my endless barrage of who, what, where, why and how's, namely. Anne-Marie Jackson, Judy Collins, Jon Palmer, Steven Arnold, Mike Sahayam, John Sykes, John Alger, Bruce Collins, Don Maclean and Mike Stevens.

Thanks go also to Dom, Fiona and the numerous other flatmates and friends for their queries, discussions and help during the course of this research.

Last but not least, I would like to express my deepest gratitude to my parents, whose love, encouragement and early morning phone calls to ensure I was up, were greatly appreciated, without which, this would not have been possible.

TABLE OF CONTENTS

ABSTRACT.....	II
AKNOWLEDGEMENTS.....	III
TABLE OF CONTENTS.....	IV
LIST OF FIGURES.....	VIII
LIST OF TABLES.....	X
CHAPTER 1 INTRODUCTION	1
CHAPTER 2 LITERATURE REVIEW	3
2.1 FACIAL ECZEMA.....	3
2.1.1 HISTORY	3
2.1.2 PATHOGENECITY	4
2.2 <i>PITHOMYCES CHARTARUM</i>	7
2.2.1 OCCURRENCE OF <i>PITHOMYCES CHARTARUM</i>	7
2.2.2 MORPHOLOGY	7
2.2.3 PHYSIOLOGY.....	9
2.3 BIOCONTROL FUNGL.....	11
2.3.1 INTRODUCTION	11
2.3.2 BIOCONTROL PRACTICES.....	12
2.3.3 BIOCOMPETITIVE FUNGI	13
2.4 SUBMERGED FERMENTATION OF FUNGI.....	15
2.4.1 PRINCIPLES.....	15
2.4.2 OPTIMISATION STRATEGIES	19
CHAPTER 3 MATERIALS AND METHODS	22

3.1	MATERIALS	22
3.1.1	ORGANISM.....	22
3.1.2	MEDIA	22
3.1.3	CHEMICALS.....	22
3.1.4	ENZYMES.....	23
3.1.5	EQUIPMENT.....	23
3.2	METHODS	24
3.2.1	STOCK CULTURES.....	24
3.2.2	GROWTH OF ORGANISMS	24
3.2.2.1	Shake Flask.....	24
3.2.2.2	Fermenter.....	25
3.2.3	ANALYSIS	26
3.2.3.1	Biomass determination	26
3.2.3.2	Determination of Colony-Forming-Units	27
3.2.3.3	Carbon sources	27
3.2.3.4	Nitrogen sources	28
3.2.3.5	Starch hydrolysis.....	28
CHAPTER 4 OPTIMISATION OF CARBON SOURCE, NITROGEN SOURCE, GROWTH ADDITIVES AND INITIAL CULTURE PH VALUE.....		29
4.1	HOMOGENISATION STANDARDISATION	29
4.2	EFFECT OF CARBON SOURCE	30
4.2.1	INTRODUCTION	30
4.2.2	INITIAL SCREENING EXPERIMENTS.....	31
4.2.3	TIME COURSE STUDIES	31
4.2.3.1	Specific rate of growth	33
4.2.3.2	Substrate utilisation	35
4.2.3.3	Specific rate of substrate utilisation	37
4.2.3.4	Biomass growth yield	37
4.2.3.5	Productivity.....	40
4.2.4	DISCUSSION.....	40
4.3	EFFECT OF NITROGEN SOURCE	41
4.3.1	INTRODUCTION	41
4.3.2	SCREENING EXPERIMENTS	41
4.3.3	DISCUSSION.....	42

4.4	EFFECT OF PH	43
4.4.1	INTRODUCTION	43
4.4.2	OPTIMAL pH	43
4.4.3	DISCUSSION.....	45
4.5	GROWTH FACTORS AND TRACE ELEMENTS	45
4.5.1	INTRODUCTION	45
4.5.2	RESULTS	46
4.5.3	DISCUSSION.....	47
4.6	CONCLUSION	48
 CHAPTER 5 STRAIN CHARACTERISTICS		49
5.1	INTRODUCTION	49
5.2	RESULTS.....	49
5.2.1	QUANTITATIVE ANALYSIS	49
5.2.2	QUALITATIVE OBSERVATIONS.....	51
5.3	DISCUSSION.....	52
5.4	CONCLUSION	53
 CHAPTER 6 WHEY PERMEATE TRIALS		54
6.1	INTRODUCTION	54
6.2	RESULTS.....	55
6.2.1	WHEY+GLUCOSE/CASAMINO ACIDS	55
6.2.2	WHEY+STARCH/SUCROSE	57
6.3	DISCUSSION.....	60
6.4	CONCLUSION	62
 CHAPTER 7 FERMENTER TRIALS.....		63
7.1	INTRODUCTION	63
7.2	RESULTS.....	65

7.3	DISCUSSION.....	67
7.4	CONCLUSION.....	69
CHAPTER 8 STORAGE TRIALS		70
8.1	INTRODUCTION	70
8.2	RESULTS.....	71
8.3	DISCUSSION.....	73
8.4	CONCLUSION	74
CHAPTER 9 CONCLUSIONS AND RECOMMENDATIONS		75
REFERENCES.....		78

LIST OF FIGURES

FIGURE 1 BASE STRUCTURE OF SPORIDESMIN.	3
FIGURE 2 PATHOGENESIS OF FACIAL ECZEMA. SOURCE FACIAL ECZEMA INFORMATION UPDATE, FACIAL ECZEMA RESEARCH GROUP. RUAKURA AGRICULTURAL CENTRE, HAMILTON, NEW ZEALAND.	6
FIGURE 3 PHOTOMICROGRAPH OF SPORES OF <i>P. CHARTARUM</i> (X2400). SOURCE ATHERTON <i>ET AL</i> (1974).	9
FIGURE 4 FORMS OF FUNGAL GROWTH, FROM LEFT TO RIGHT, SINGLE CELL GROWTH, PELLETED GROWTH, AND FILAMENTOUS GROWTH (SOLOMONS, 1975).	16
FIGURE 5 OPTIMISATION STRATEGY FOR PRODUCING MICROBIAL BIOCONTROL AGENTS IN LIQUID CULTURE. SOURCE, JACKSON <i>ET AL</i> (1996).	20
FIGURE 6 A PICTURE OF THE FERMENTATION APPARATUS USED IN THE 1.5-L SCALE.	26
FIGURE 7 EFFECT OF CULTURE HOMOGENISATION ON CFU'S/ML IN SHAKE FLASK CULTURES OF <i>P. CHARTARUM</i> INCUBATED AT 25°C FOR 4 DAYS IN GLUCOSE/YEAST EXTRACT MEDIUM. THE RANGE OF READINGS IS INDICATED.	30
FIGURE 8 DEVELOPMENT OF CFU'S VERSUS TIME WITH GLUCOSE MEDIA.	32
FIGURE 9 DEVELOPMENT OF CFU'S VERSUS TIME WITH LACTOSE MEDIA.	32
FIGURE 10 BIOMASS VERSUS TIME, GLUCOSE MEDIUM.	33
FIGURE 11 BIOMASS VERSUS TIME, LACTOSE MEDIUM.	33
FIGURE 12 LOG DRY WT BIOMASS VERSUS TIME, GLUCOSE.	34
FIGURE 13 LOG DRY WT BIOMASS VERSUS TIME, LACTOSE.	34
FIGURE 14 GLUCOSE CONCENTRATION VERSUS TIME.	36
FIGURE 15 LACTOSE CONCENTRATION VERSUS TIME.	36
FIGURE 16 BIOMASS CONCENTRATION VERSUS GLUCOSE USED.	38
FIGURE 17 BIOMASS CONCENTRATION VERSUS LACTOSE USED.	38
FIGURE 18 pH VERSUS DRY WEIGHT BIOMASS. THE RANGE OF READINGS IS INDICATED.	44
FIGURE 19 pH VERSUS CFU'S/ML. THE RANGE OF READINGS IS INDICATED.	44

FIGURE 20 EFFECT OF GROWTH FACTORS (GF) AND TRACE ELEMENTS (T) ON BIOMASS.	47
FIGURE 21 EFFECT OF GROWTH FACTORS (GF) AND TRACE ELEMENTS (T) ON VIABILITY COUNT	47
FIGURE 22 EFFECT OF STRAIN TYPE ON BIOMASS CONCENTRATION. THE RANGE OF READINGS IS INDICATED..	50
FIGURE 23 EFFECT OF STRAIN TYPE ON VIABILITY COUNT. THE RANGE OF READINGS IS INDICATED	50
FIGURE 24 EFFECT OF pH AND CONCENTRATIONS OF GLUCOSE AND CASAMINO ACIDS IN WHEY PERMEATE ON BIOMASS CONCENTRATION. THE RANGE OF READINGS IS INDICATED.....	56
FIGURE 25 EFFECT OF pH AND CONCENTRATIONS OF GLUCOSE AND CASAMINO ACIDS IN WHEY PERMEATE ON VIABILITY COUNTS. THE POINTS SHOWN ARE FROM SINGLE CFU DETERMINATION ONLY.....	57
FIGURE 26 EFFECT OF WHEY+STARCH OR SUCROSE ON BIOMASS CONCENTRATION. THE RANGE OF READINGS IS INDICATED	58
FIGURE 27 EFFECT OF WHEY + STARCH OR SUCROSE ON VIABILITY. THE RANGE OF READINGS IS INDICATED.	58
FIGURE 28 STARCH USED VERSUS BIOMASS PRODUCED AFTER 5 DAYS INCUBATION.	59
FIGURE 29 BIOMASS CONCENTRATION PRODUCED DURING FERMENTER EXPERIMENTS. DATA SHOWN AFTER 5 DAYS INCUBATION. THE RANGE OF READINGS IS INDICATED	65
FIGURE 30 VIABILITY COUNTS FROM FERMENTER EXPERIMENTS. DATA SHOWN AFTER 5 DAYS INCUBATION.	66
FIGURE 31 AVERAGE GLUCOSE AND NITROGEN CONCENTRATION OVER TIME IN FOUR DIFFERENT FERMENTATION PROTOCOLS.	66

LIST OF TABLES

TABLE 1 EFFECT OF CARBON SOURCE ON GROWTH AND COLONY FORMING ABILITY OF <i>P. CHARTARUM</i>	31
TABLE 2 GROWTH RATES OF <i>P.CHARTARUM</i> ON GLUCOSE AND LACTOSE.	35
TABLE 3 RATES OF SUBSTRATE UTILISATION DURING GROWTH ON GLUCOSE.....	39
TABLE 4 RATES OF SUBSTRATE UTILISATION DURING GROWTH ON LACTOSE.....	39
TABLE 5 GLUCOSE AND LACTOSE PRODUCTIVITY	40
TABLE 6 EFFECT OF NITROGEN SOURCE ON GROWTH AND COLONY-FORMING-ABILITY OF <i>P. CHARTARUM</i> AFTER 6 DAYS INCUBATION.....	42
TABLE 7 CHANGE OF pH WITH TIME.....	45
TABLE 8 STRAIN CHARACTERISTICS NOTED DURING GROWTH ON GLUCOSE/CASAMINO ACIDS MEDIA.	51
TABLE 9 CONCENTRATION OF LACTOSE UTILISED (G/L) BY <i>P.CHARTARUM</i> /.....	56
TABLE 10 CONCENTRATIONS OF GLUCOSE AND LACTOSE UTILISED (G/L) BY <i>P.CHARTARUM</i>	56
TABLE 11 STARCH AND LACTOSE CONCENTRATIONS (G/L) UTILISED BY <i>P.CHARTARUM</i>	59
TABLE 12 SUCROSE AND LACTOSE CONCENTRATIONS (G/L) UTILISED BY <i>P.CHARTARUM</i>	60
TABLE 13 CONDITIONS USED IN FERMENTER STUDIES	64
TABLE 14 COMPARATIVE PERFORMANCE OF <i>P.CHARTARUM</i> IN SHAKE-FLASK AND FERMENTER INCUBATION. FERMENTER DATA SHOWN ARE AVERAGE OF ALL PROTOCOLS.	68
TABLE 15 CONTROL (WATER TREATED) VIABLE UNIT COUNTS.....	71
TABLE 16 LACTOSE TREATMENT VIABLE UNIT COUNTS.....	72
TABLE 17 TREHALOSE TREATMENT VIABLE UNIT COUNTS.....	72

CHAPTER I INTRODUCTION

Pithomyces chartarum is a saprophytic fungus found in New Zealand pastures and which sporulates prolifically in the warm, humid conditions found there during summer and autumn (Brook, 1963). The spores of *P. chartarum* have on their surface a hepatotoxin called sporidesmin, identified as the causal element in facial eczema disease in grazing livestock such as sheep and cattle in New Zealand (Thornton and Sinclair, 1959; Thornton and Ross, 1959; Thornton and Percival, 1959). The disease is characterised by icterus, photosensitisation and in severe cases liver injury causing death. Sub-lethal exposure to sporidesmin leads to production losses, such as lower weight gains and poorer lambing percentages (Smeaton, 1985). The cost of this disease is financially high with estimates put at \$30 million per annum in the livestock industry (Smith and Towers, 1984) to \$63.1-\$126.2 million in the sheep industry alone (N.Z. Meat and Wool Boards Economic Service, 1990).

At present, methods for controlling the disease include dosing animals with zinc salts, pasture management and fungicide treatment of pasture and breeding from rams selected for disease resistance. These methods are rather inefficient by being either labour intensive, expensive or slow to yield benefits (Smith and Towers, 1984; Parle and di Menna, 1978). These factors have provided reason to look for alternative means of controlling the incidence of facial eczema. One promising means is that of biocontrol by biocompetitive exclusion. This approach involves introduction of atoxicogenic fungi to the toxic fungus's environment, which, by competing for the same resources, reduces the toxic fungus numbers. This technique has been used successfully by a number of research groups using other types of fungus (Cotty, 1990; Dorner *et al* 1992). Trials involving *P. chartarum* have been carried out, also with success, where the levels of sporidesmin have been reduced by up to 80% in pastures treated with the atoxic strains (Fitzgerald *et al* 1998).

Armed with the fact that biocompetitive exclusion works in practice in reducing the incidence of facial eczema, work now focuses on finding appropriate conditions to grow *P. chartarum* on a commercial scale. To this end, the present work aimed to develop a submerged fermentation process for fungal biomass production. At present, liquid culture fermentation is the most appropriate method for producing microbial biocontrol agents commercially, as solid substrate methods cannot be easily scaled up (Jackson and Schisler, 1995). There have been numerous publications on determining the best nutritional conditions for fungal biological control agents (Jackson and Schisler, 1995; Jackson, 1997; Stowell *et al* 1989; Papavizas *et al* 1984; Lewis and Papavizas, 1991). They all follow similar strategies i.e. a strategy where optimising biocontrol agent production is based on developing a medium that maximises propagule fitness and propagule yield.

These strategies begin with the development of a defined medium that supports good growth and propagule formation of the biocontrol agent. With a basal medium developed, the nutritional components can be varied and the impact of these changes assessed in terms of propagule yield, fitness and stability with storage. These are important factors to consider during optimisation, as all are required for an effective biocontrol agent. Once a optimised, defined medium has been developed, a production medium can be formulated by replacing the nutritional components of the defined medium with low cost complex substrates.

CHAPTER 2 LITERATURE REVIEW

2.1 FACIAL ECZEMA

2.1.1 HISTORY

Facial eczema (pithomycototoxicosis) is an economically important disease that affects grazing sheep and cattle in the warmer regions of the North Island of New Zealand, particularly in the summer and autumn pastures. The N.Z. Meat and Wool Boards' Economic Service (1990) estimated the cost of facial eczema to sheep production to be \$63.1 - \$126.2 million per annum for the years 1983 – 1988. This was an estimation based on losses in animal performance and did not include any costs involved in prevention of facial eczema. Smith and Towers (1984) estimated that the total cost of facial eczema to all livestock industries for 1981 was \$58.4 million, the break-up of costs being \$35 million sheep, \$0.7 million beef, and dairy \$17 million, associated control measures costing \$4.6 million across all sectors. The incidence of facial eczema from this study indicated 69 % of North Island sheep and beef and 63 % of dairy farms were afflicted. The causative agent of this disease is sporidesmin, a mycotoxin produced by *Pithomyces chartarum*. Sporidesmin was first isolated as a crystalline substance by Synge and White (1959). Further work by Hodges *et al.* (1963) elucidated its structure (Figure 1).

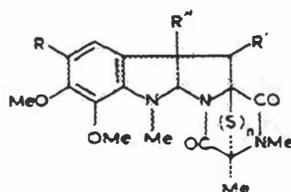


Figure 1 Base structure of sporidesmin.

Observations by Bertaud *et al.* (1963) concluded that the toxin and related sporidesmolides, a family of similarly structured compounds, were produced as crystalline spicules covering the surface of *P.chartarum* spores, and that the formation of these spicules is controlled by the amino acids present in the culture medium. Percival and Thornton (1958) and Thornton and Sinclair (1960) first made the observations of the presence of the fungus and related it to outbreaks of facial eczema, although at that stage the organism was classified as *Sporidesmin bakeri*. Ellis (1960) later reclassified the fungus to be *P. chartarum*.

2.1.2 PATHOGENECITY

Clinical signs occur 6 days or more after access to toxic pastures, the variation depending on the level of pasture contamination. Facial eczema takes its name from the intense itching, oedema, erythema, scaling and exudation affecting primarily unpigmented skin that is not covered by thick wool, particularly on the face. The reason for this is the action of sunlight on the photodynamic phylloerythrin in the skin leading to itching and self-induced trauma. Animals with pigmentation of the skin most often show no evidence of photosensitivity, in these cases there are other indicators of liver damage. Raised levels of the enzyme gamma glutamyl transferase (GGT) are a more sensitive indicator of liver damage than the presence of clinical signs. The GGT test is the best test for sporidesmin toxicity in ruminants and this test forms the basis of facial eczema resistance testing of rams.

Mortimer and Taylor (1962) revealed, in experimental intoxication of sheep with sporidesmin, that the observed pathogenicity of the fungal metabolite was very similar to symptoms of facial eczema. Effects of facial eczema on ewe reproduction and ewe lamb live weights were studied by Smeaton *et al* (1985) and from this study it was concluded that the high levels of GGT were associated with depressed ewe performance. Relationships were drawn between the level of liver damage and the weights of ewes at

mating and weaning. For a modest outbreak of facial eczema causing a rise in GGT of 200 iu/l, the loss in weight of lambs weaned could be as high as 13 %.

Sporidesmin affects ruminants by damaging the liver, which leads to clinical facial eczema. The liver performs a central role in the detoxification and excretion of drugs, chemicals, and plant and mycotoxins. In many intoxications however, the liver is severely affected. The reason for this is that the toxins undergo biotransformation in the liver. In the process of transforming them into excretable metabolites, intermediate radicals are produced which are sometimes much more toxic than the parent compounds. With sporidesmin, tissue damage is caused by the generation of the “active oxygen” compounds superoxide radical (O_2°), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH°). This process of auto-oxidation is catalysed by copper but inhibited by zinc, thus explaining the protective effect of zinc against sporidesmin toxicity. The spores are ingested with pasture. The sporidesmin is released from the spores, and absorbed into the blood, removed by the liver and excreted into the bile. The principal clinical signs of facial eczema in sheep are jaundice, loss of condition and photosensitization. Sporidesmin damages bile ducts and occasionally the surrounding hepatocytes causing obstruction of the biliary system. Accumulation of bile pigments in the tissues (obstructive jaundice) and phylloerythrin in the skin (hepatogenous photosensitization) arise due to this cholestasis. The pathogenesis of facial eczema is illustrated in Figure 2.

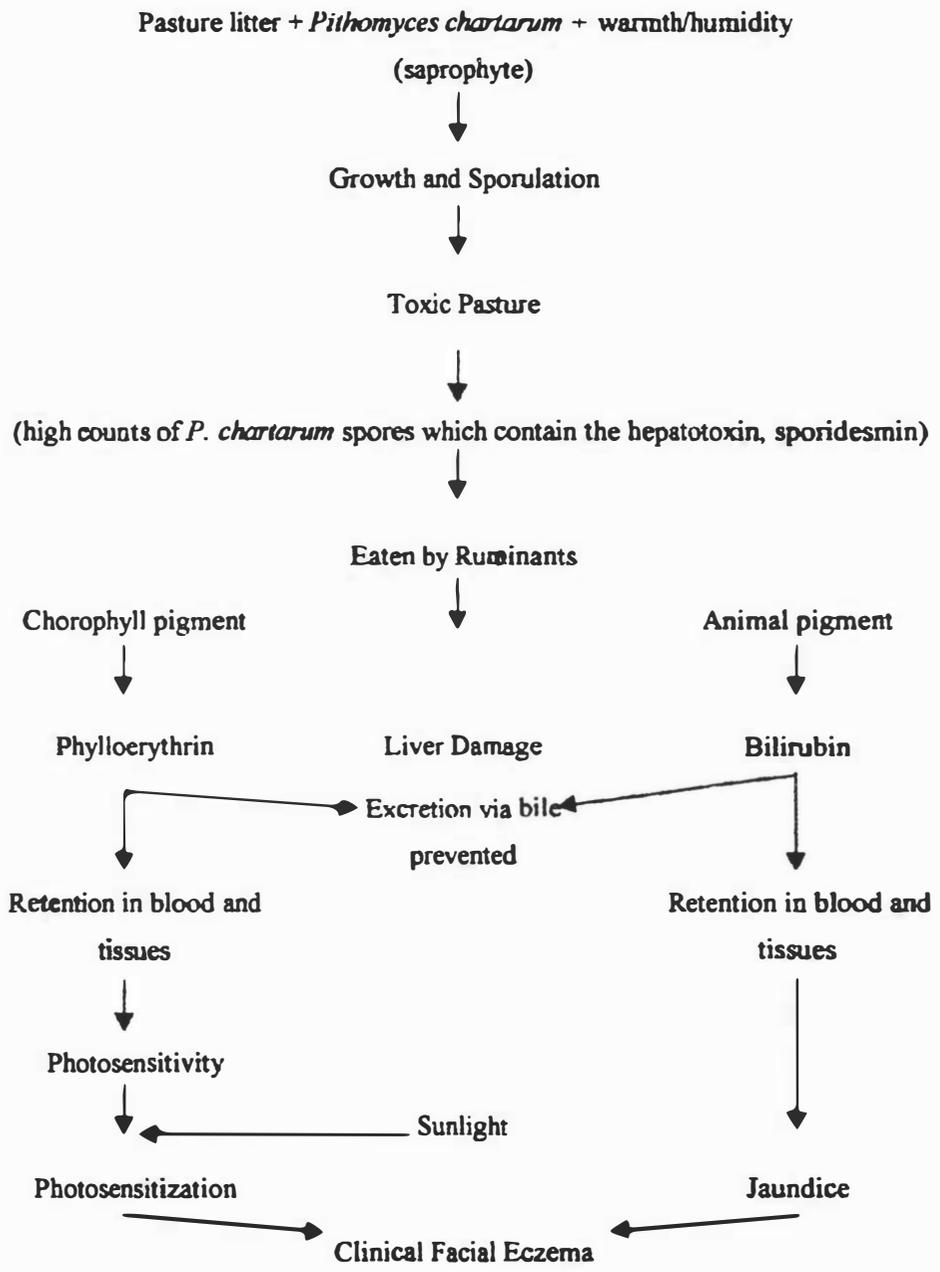


Figure 2 Pathogenesis of facial eczema. Source Facial eczema information update, Facial eczema research group. Ruakura Agricultural Centre, Hamilton, New Zealand.

2.2 PITHOMYCES CHARTARUM

2.2.1 OCCURRENCE OF PITHOMYCES CHARTARUM

The distribution of this fungus was first described by Hughes (1953) and later by Ellis (1960). Originally recorded in West Africa it was also found in a number of warm temperate and sub-tropical countries, including the Philippines, Malaya, Australia, Sudan, Zimbabwe and Trinidad. Ellis expanded the distribution of the species to include Ghana, Jamaica, Mauritius, New Zealand, United States of America and North Italy. Observations made in New Zealand during 1959-61 (Dingley, 1962) have shown this fungus to be a cosmopolitan saprophytic species common in the north of New Zealand during the warm summer months. The humid coastal areas in the Auckland province are particularly dense in the fungus. Many collections have been made in the Hauraki plains, Bay of Plenty and the Waikato. It is also common in Gisborne, Hawkes Bay, Taranaki, and Manawatu. In the South Island its distribution is limited to the northern regions of Nelson and Malborough but has on occasion been found in grass samples from Lincoln, Canterbury, (Dingley, 1962).

2.2.2 MORPHOLOGY

Ellis (1960) and Dingley (1962) reported extensive studies on the morphology of *P. chartarum* and showed it to be an extremely variable species. This variation is dependent on the substrate upon which it grows, and the morphology of *P. chartarum* grown on plant debris differs greatly from that of the fungus grown in the laboratory on a nutrient medium. The fungus grows on plant debris from germinating spores that produce hyaline superficial hyphae. These quickly penetrate the plant tissues and become both intra- and intercellular. This mycelium is only lightly pigmented, but is composed of branched, septate hyphae and sometimes chains of unicellular, intercalary chlamydospores. These superficial hyphae form a dense compacted network of interlocking branches. Conidiophores are formed in

lens-shaped clusters in this superficial mycelium. Mature conidia usually have 3 transverse, sometimes 4, and 2 to 4 longitudinal divisions and vary in length 16-35 x 13-20 μm . These conidia increase in size from summer through winter. Many different studies of this fungus have been performed on numerous media. Dingley (1962) made observations of the morphology of the organism growing on potato dextrose agar and reported as follows:

“Cultures were relatively quick growing, covering the surface of a petri dish in 8 days. Colonies formed were zonate, flat growing, sometimes floccose, hyaline. These became dark as cultures aged owing to the formation of pigmented mycelium, chlamydospores, and conidia. Three distinct types of mycelium were present in cultures:

(i) Light coloured or hyaline, thin walled sparsely septate hyphae 2-4.5 μm diameter, often distinctly verrucose and lightly pigmented. Hyphal loops and coils were common.

(ii) Thin walled, dark brown hyphae, 5-8 μm diameter, usually with numerous transverse septa.

(iii) Echinulate, thin walled, dark brown hyphae 5-8 μm diameter, sometimes with numerous transverse septa; chains of intercalary chlamydospores unicellular, sometimes one septate, up to 12 μm diameter, were formed among this mycelium”.

The relative amounts of these types of mycelium gave each isolate a distinct appearance. Pale floccose cultures consisted of hyphae of type (i), whereas dark cultures with more compact growth consisted of greater quantity of type (ii) and (iii) mycelia. Dingley (1962) also reported that the characteristic manner in which the conidia were liberated suggested that they cannot be regarded as true conidia but are in fact terminal chlamydospores freed

only by the destruction of the hyphae which bear them. Incubation temperatures were reported to play an important role in the type of growth produced. Temperatures $<20^{\circ}\text{C}$ produced hyaline colonies of type (i) mycelium, whereas temperatures $>26^{\circ}\text{C}$ produced dark mycelium of types (ii) and (iii). The spores of *P. chartarum* are distinguishable from superficially similar spores by the frill of tissue left at their base by the separation from the conidiophore (Figure 3), (Atherton *et al* 1974).

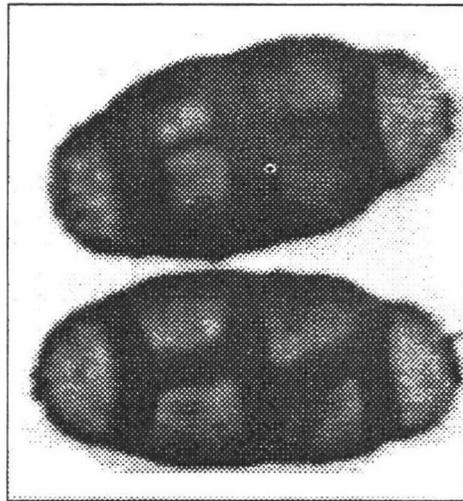


Figure 3 Photomicrograph of spores of *P. chartarum* (x2400). Source Atherton *et al* (1974)

2.2.3 PHYSIOLOGY

P. chartarum has simple nutritional requirements as explored by Ross (1960). The fungus can utilise a variety of carbon and nitrogen sources, as well as various growth factors and trace elements, influencing its growth and development. Thornton and Ross (1959) established that it grew on defined media but that growth was more vigorous on non-defined media. As the morphology of the organism varies with the substrate upon which it grows, so too does the physiology. Studies by Clare and Gumbley (1962), Bertaud *et al* (1963), di Menna *et al* (1970), Davidson and Marbrook (1964) to name a few, all studied the affects of various growth conditions on physiological parameters of *P. chartarum*.

Extensive research by Brook (1963) on the ecology of the fungus in pasture was a significant study in establishing the environment most favoured by the organism and from this suggestions were proposed for pasture and stock management for the prevention of facial eczema outbreaks. Ross (1960) investigated the physiology of the organism with interest in its nutritional needs revealing it to be widely capable. Subsequent research by Dingley (1962) was centred on the occurrence, morphology and taxonomy of the fungus and the effect of temperature and moisture on its growth. Much of the subsequent work by others was concerned with spore germination and spore toxicity. Smith and Crawley (1962) looked at temperature and moisture effects on germination. Clare and Gumbley (1962) found that sporidesmin was broken down by ultraviolet light and water. Marbrook and Mathews (1962) found similar phenomena. Done *et al* (1961) found that there were correlation between the amount of sporidesmin produced and the extent of fungal growth. The production of toxin on laboratory media (Ross and Thornton, 1962) revealed that New Zealand strains produced higher yields of sporidesmin than did strains isolated in other countries. The ability to vary sporidesmin and sporulation by altering environmental conditions, and differences due to culture type, were investigated by di Menna *et al* (1970).

The nutritional studies by Ross (1960) gave valuable data regarding the media and strains of the organism and he reported that growth of three strains occurred between 16°C and 31.5°C with an optimum at 24°C. Temperatures higher than 24°C were observed to be more favourable for the production of spores, with an optimum around 28°C. Growth of *P. chartarum* was observed to occur over a wide range of pH values but there was no well-defined optimum. Growth occurred at pH values as low as 3.1 and as high as 8.6, but greatest growth was in the range above pH 5.0. Neutral liquid media were observed to have an initial slight drop in pH due to the production of acids, but after prolonged incubation there was a pH increase to alkaline values (Ross 1960).

Thornton and Ross (1959) observed that growth of *P. chartarum* when grown on Czapek-Dox and Raulin-Thom media was stimulated by the addition of yeast extract. Work by Ross (1960) found that two of three strains were stimulated by addition of yeast extract to a basal medium. Similar results were obtained by replacing yeast extract with defined

growth factors. These trends were only noticeable in the short-term as upon prolonged incubation the crop yields were all as great in basal medium as in the media containing yeast extract or growth factors, indicating that the effect was on growth rate. Addition of nucleic acids or purines or pyrimidines to basal medium did not increase yields of mycelial felts or growth in general. Addition of trace metals to basal salts-growth medium stimulated growth with a resultant increase in mycelial production. Once again, the trend of slower growth was observed with the synthetic media, but with prolonged growth, crop yields were usually similar.

The organism is capable of utilising a wide range of carbohydrates of varying types such as pentoses, including the D- forms of arabinose, ribose and xylose, hexoses including fructose, galactose, glucose and mannose, along with disaccharides such as sucrose and polysaccharides such as xylan, pectin and starch (Ross, 1960). It was also found that *P. chartarum* could use many inorganic or organic nitrogen compounds for growth. Utilisation of ammonia was found to cause the development of acidity in the medium to values around pH 2.7 after 14 days incubation whereas the usage of nitrates resulted in a medium pH of about 6.6 for the same growth period. Urea and glucosamine were observed to be readily utilised and to support good growth, as was the addition of amino acids. Ross concluded that the fungus has simple growth requirements and a marked ability to utilise a wide variety of substrates for growth.

2.3 BIOCONTROL FUNGI

2.3.1 INTRODUCTION

Plant-borne diseases have been associated with agriculture since its very beginnings. With the expansion and growth of population, increased need for efficient disease-free production of food has become necessary. Intensification and continuous cultivation have resulted in increasing disease pressure. Chemical disease control has been the main

method employed by farmers to prevent the rise of costly production losses. In the 1960s it became apparent that agricultural chemicals were responsible for environmental pollution, were present in the food chain, and were capable of inducing resistant organisms. Eventuating from this, the need to develop alternative disease control measures has become a priority for scientists' worldwide.

2.3.2 BIOCONTROL PRACTICES

Biocontrol can be achieved through practices, which create an environment favourable to antagonists, development of host resistance, stimulation of resident microflora, or through the mass introduction of antagonists, non-pathogenic strains, or other beneficial microorganisms. The approach most investigated in the implementation of biocontrol has been, and continues to be, the introduction of an antagonist preparation to the ecosystem (Papavizas and Lewis, 1981). However early attempts to control diseases by this method were largely unsuccessful, suggesting that inducing biocontrol in this manner would be futile. Many scientists recognised that such an approach was doomed to failure from the start because of the ecological axiom which stated that microbial populations reflect the habitat and that non-conducive habitats will not sustain an introduced microflora. It is currently recognised that environments must be altered to accept biocontrol agents or that formulations must be developed which allow the antagonist to survive, proliferate, become active and establish itself in an alien environment (Lewis and Papavizas, 1991).

Fungi have numerous advantages in their use as living biocontrol agents. The ability of fungi to actively infect and kill the insect or weed host is certainly the most important in direct disease control, but is of less use when considering the nature of biocontrol by competitive exclusion of the pathogen, as is of interest in the present study. Another advantage of fungi is their propagule stability. Fungal spores or sclerotial propagules are generally more stable than bacterial cells. Desiccation tolerance and stability as a dry preparation (shelf life) are absolute requirements for commercial production (Hebbar *et al* 1997). Fermenter-produced chlamydospores of the biocontrol fungi, *Gliocladium virens*

and *Trichoderma* spp. (Papavizas *et al.* 1984) have been formulated into dry alginate pills using various food bases (Lewis and Papvizas, 1985). Various methods can be employed when harvesting the biomass to achieve best efficacy, the work of Pereira and Roberts (1990) providing a good example of some of the treatments in use such as treating biomass with sugar solutions. While the ability or requirement for fungal spores to actively infect their host is their greatest advantage, it also represents one of the major constraints to the commercial use of fungal biocontrol agents. Fungal spores require free moisture during the germination and penetration process. This requirement is a major environmental constraint to effective control under field conditions.

Biological control of plant pests, fungal pathogens and weeds has been used as an alternative to chemical control with varying degrees of success (Hebbar *et al* 1997). The use of fungi as bioherbicides has become a developing area over recent years. Currently, three fungal bioherbicides are registered for use in the United States and Canada, *Colletotrichum gloeosporioides* (Collego®) for the control of northern jointvetch in Arkansas rice, *Phytophthora palmivora* (Devine®) for the control of strangler vine in citrus groves and *Puccinia canaliculata* (Dr. Biosledge®) for control of yellow nutsedge (Charudattan, 1991). BioMal®, a *Colletotrichum gloeosporioides* strain that infects and kills round-leaved mallow is being developed for commercial use in Canada. *C. truncatum* has been identified and is being developed as a biological control agent of the troublesome weed, hemp sesbania (Jackson and Schisler 1995; Jackson *et al*, 1996).

2.3.3 BIOCOMPETITIVE FUNGI

Biocontrol involving the use of fungi as biocompetitive agents has been used successfully in a number of instances. This method of control, called competitive exclusion works, whereby the fungus, added to the environment of the pathogen, proceeds to grow and utilise resources used by the pathogen. The fungi used in this method of biocontrol so far have been atoxic or more desirable strains of the pathogen as these are the most likely organisms to compete for the same resources as the unwanted strains. The reductions of

aflatoxin (a potent fungal carcinogen) contamination in cotton bolls, peanuts and maize (Cotty 1990; Brown *et al.* 1991; Dorner *et al.* 1992) are successful applications of this technique. The study by Dorner *et al.* (1992), in which soil treated with an atoxigenic strain of *Aspergillus parasiticus*, resulted in a reduction in toxins in the soil that persisted for at least two years. The method of competitive exclusion has not been restricted to fungi. Non-pathogenic strains of *Pseudomonas syringae* bacteria were successfully used to exclude ice-nucleation strains and thus prevent frost injury (Lindow 1987).

Most recently, Fitzgerald *et al.* (1998) described the use of atoxigenic *P. chartarum* fungus (the organism which the author is investigating here), in successful biocontrol of the toxic strain, for the prevention of facial eczema. Their research showed that the level of sporidesmin contamination was reduced by up to 80% when treated pasture plots were compared with untreated plots. Spore numbers were also reduced in the trial but to a lesser extent. It was also noted that there was a gradual decline, from 90% to 54%, in the percentage of atoxigenic spores isolated from the treated plots during the trial (a period of 19 weeks). The decline continued over winter and spring so that 15 months following the treatment, only 4% of 145 isolates from the treated plots were atoxigenic. This could be due to significant contamination of the treated plots from the surrounding untreated area, or that the atoxigenic *P. chartarum* were less persistent than the toxigenic wild types. However, these preliminary trials have established that sporidesmin concentrations in pasture can be reduced by application of atoxic isolates of *P. chartarum* early in the summer, pre-empting the sporulation of the resident toxic *P. chartarum*. Further trials of this method are needed to investigate the responses when the trials are conducted under conditions suiting the toxic strain and at higher spore levels.

2.4 SUBMERGED FERMENTATION OF FUNGI

2.4.1 PRINCIPLES

The World's fermentation industry has been producing mycelial biomass for many years, but mainly as a method of obtaining certain desired secondary metabolites. The use of *Penicillium chrysogenum* to produce penicillin, *Aspergillus niger* to produce citric acid and *Rhizopus nigricans* to convert progesterone to 11-hydroxy-progesterone are three major examples of this 'secondary' biomass production, but in no case is the organism itself required (Solomons, 1975). In these cases the biomass can be a considerable nuisance, consuming valuable nutrients, often causing fermenter bulking problems and posing disposal problems when its work is completed. Nonetheless, much of the valuable information obtained regarding the microbiology, biochemistry and biochemical engineering can be applied to the problems of producing mycelial biomass.

Fungal biomass can be cultured from spores or vegetating cells in submerged culture. Depending upon the strain of micro-organism, the nature of the medium and the degree of agitation of the culture fluid, fungi will increase their biomass by vegetative growth forming either hyphal filaments, often with copious branching, or various forms of flocs and pellets of mycelium (Brown, 1988). Provided there is no nutrient limitation cells will grow in exponentially increasing quantities. The growth kinetics and effects of nutrient limitation have been established and these can be used to predict conditions in both batch and continuous culture. The viscous nature of cultures of filamentous fungi gives rise to problems associated with aeration and mixing. Fungal adhesion to bioreactor walls is a problem that should be addressed, particularly so in the small scale. The basic information necessary for the design of bioreactors for the culture of fungi is known and the operating procedures for large-scale equipment are well established (Brown, 1988).

There are three types of mycelial growth observed in submerged cultures, and there is often not a sharp differentiation between them; they are single cell, pelletal and filamentous (Figure 4). Single cell forms are similar to yeast cultures in that the cells are usually spherical or ellipsoidal measuring 5-12 μ m. These forms are not spores but true single cells. They often arise as a result of high shear conditions in a fermenter. The formation of pellets is a balance between inoculum level and type, culture medium and conditions (Camici *et al.*, 1952; Galbraith and Smith, 1969). The growths of pelletal cultures produce a medium that is easily stirred. The increasing diameter of the pellets can cause diffusion problems towards the centre of the pellet with the centre often anaerobic as well as hollow due to autolysis. Interlocking strands of mycelium characterise filamentous growth, which is usually branching, and the culture appears to be 'thick' with an appearance that can most aptly be described as porridge. Most filamentous cultures form pellet-like structures termed 'agglomerates'. These structures are not permanent like pellets and can be seen to form, disperse and reform depending on the local shear rate in the fermenter. The length of the hyphae can vary considerably due to changes in the culture medium and conditions such as pH. With a strain of *Penicillium chrysogenum*, Pirt and Callow (1959) showed that comparatively small changes in pH considerably altered the length of the hyphae.

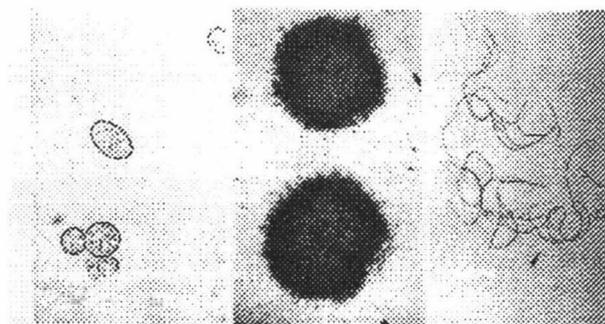


Figure 4 Forms of fungal growth, from left to right, single cell growth, pelleted growth, and filamentous growth (Solomons, 1975).

The influence of the growth form in liquid culture has profound effects on the physico-chemical properties of the medium and organism (Brown, 1988). Changes in morphology can result in a whole range of biochemical changes, some of which reflect the changes of form of the cell. Additional changes arise due simply to the alteration of the physical properties of the culture. The viscosity of the culture influences the mass transfer rates and this in turn can trigger biochemical pathways, which reflect, for example, the degree of anaerobiosis of the culture; also the growth rate of the organism is affected. In single cell cultures the size of the cells means a low viscosity broth not far removed from Newtonian fluid properties and which allows maximum mass transfer rates (Wang *et al* 1979). Pellet growth with sizes varying from 0.1 mm to 1-cm remains as thin Newtonian fluids with what is the approximate equivalent of 'solid' bodies suspended in the fluid. The oxygen transfer to the fluid is high, but diffusion rates into the pellets are minimal (Solomons, 1975). Because pellets become anaerobic in the centre, growth proceeds only at the periphery of the pellet and is therefore cubic in nature (Solomons 1975). Filamentous growth leads to a culture broth predominantly pseudoplastic in nature, that is that the viscosity of the fluid is dependent on the shear rate. As the hyphal length or cell density increases, the apparent viscosity rises, the turbulence of the system falls and the heat and mass transfer rates fall (Solomons 1975, 1980).

There are many areas to be considered when attempting to understand cell culturing and much has been documented on microbial cell cultivation in texts and journals. Pirt (1975) and Stanbury and Whitaker (1984) are good examples of texts on the principles of culturing microorganisms. Growth phases and parameters as well as estimation of biomass need to be established to allow correct decisions on the type of fermentation method to use, be it batch, plug-flow or continuous culture. The growth media conditions are obviously important too. The energy and carbon source requirements and general nutrition need to be identified along with factors such as the aeration and agitation methods, temperature, pH and any other contributing factors to the physico-chemical properties of the culture.

The successful growth of *P. chartarum* in liquid culture has been readily achieved (di Menna *et al.* 1970, Dingley *et al.* 1962, Done *et al.* 1961 and Ross 1960). Observations

by di Menna *et al.* (1970) revealed that while mycelial growth in submerged culture was much greater than in surface culture, the ability of the organism to produce spores was suppressed. Conversely Dingley *et al* (1962) reported that the organism was capable of producing large numbers of spores in both submerged and surface culture although this was only when the growth rate was decreasing.

Dingley *et al* (1962) reported that the differences observed between growth in submerged and in surface culture were similar to those described by Morton (1960) for other organisms. Thus in submerged cultures growth was more rapid with a shorter lag period. Dingley *et al* (1962) reported that breakdown of fungal tissue was observed after four to five days growth in submerged culture. This was evident from microscopical examination, decreased weight of mycelium on harvest and increased nitrogen levels in the spent culture medium. It was proposed that the sporulation which occurred in static surface culture occurred when the medium became nitrogen depleted, and Dingley theorised that sporulation was stimulated by the local accumulation of metabolic products. In a well-mixed submerged culture, however, she suggested that mycelial breakdown occurs before such products can reach sufficiently high concentrations, and such breakdown provides additional nutrients for further mycelial growth, thus preventing sporulation. However there was no experimental evidence for this suggestion and in this aspect, the physiology of spore formation in submerged culture remains obscure. The desire for the production of spores is because of their hardiness in less favourable environmental conditions, and tolerance to post harvest treatments. This 'shelf-life' is an important consideration when producing a biocontrol agent.

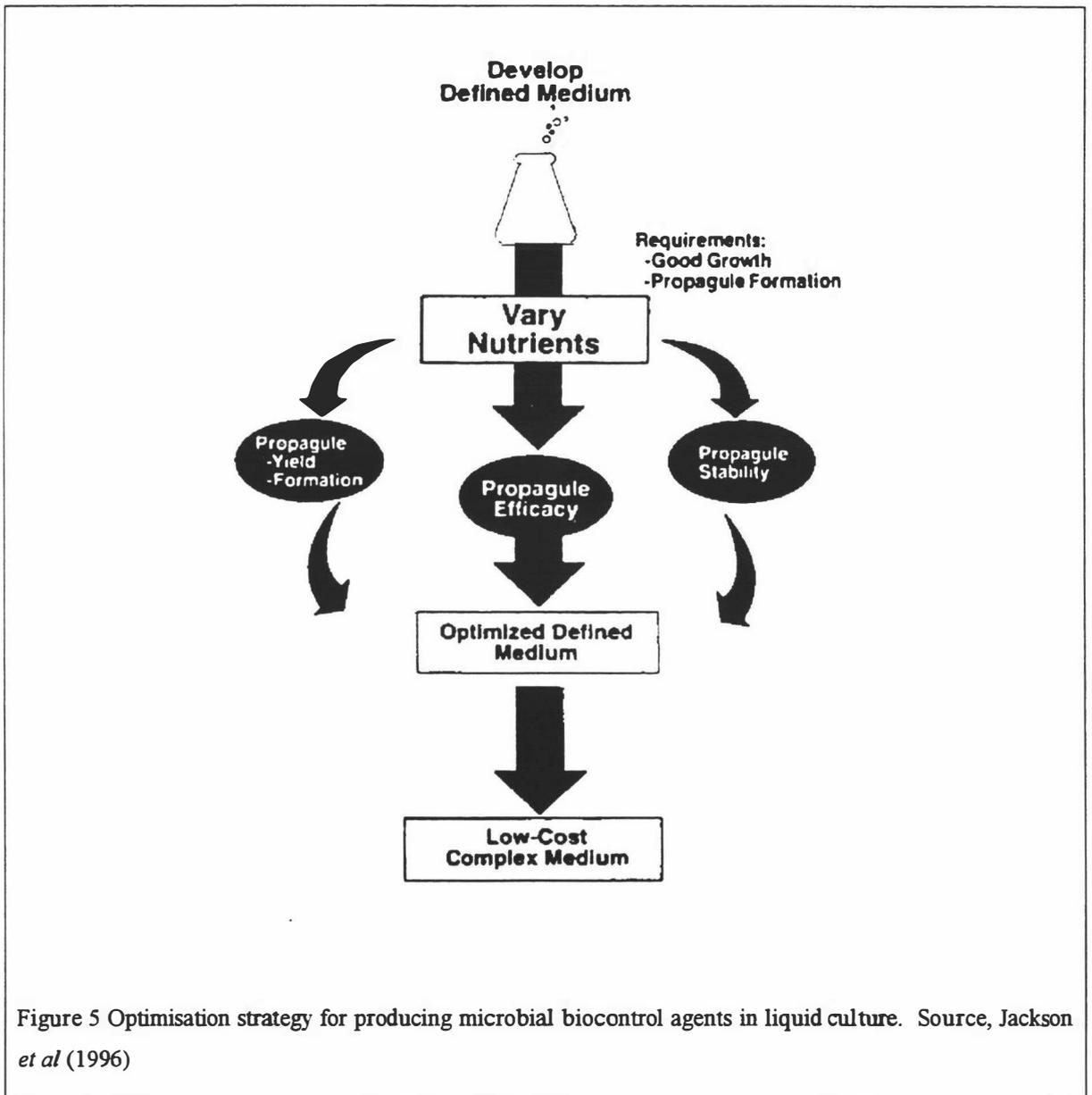
The studies by Ross (1960) on the nutrition of *P. chartarum* revealed a highly versatile organism capable of using many substrates in liquid culture. This is of particular importance when investigating the possibility of the organism for commercial production as it increases the likelihood of finding an economically feasible means of growing it. The production of *P. chartarum* in submerged culture has at this stage been on only the shake-flask scale (media volumes 100-150ml), or on solid-substrate media (Ross 1960; Dingley 1962). The reason for the lack of spores can be attributed to the fungus's environmental

physiology. Fungi can be classified into two categories: (i) Fungi which grow and sporulate within plant tissues, and (ii) Fungi which push conidiophores through the plant surface or grow in the gaseous environment outside the plant. According to a review of literature by Stowell *et al.* (1989) fungi that sporulate outside the plant are reluctant to sporulate in submerged fermentation. In contrast the fungi which sporulate in the aqueous environment present in plant tissues find submerged fermentation conditions suitable. The observed absence of spore formation in submerged culture does not necessarily pose a problem for the successful development of a commercial process, since there is the option of using mycelia as the infective agent. Although most production of biocontrol fungi has concentrated around the formation of conidial structures, there has been successful research on the use of mycelium preparations. The use of entomopathogenic fungi for the control of insect crop pathogens by Rombach *et al* (1986) and Pereira and Roberts (1990) was achieved using mycelial preparations.

2.4.2 OPTIMISATION STRATEGIES

There is considerable literature on strategies for the optimisation of growth conditions for biocontrol microorganisms, and all follow a similar principle (Jackson *et al* 1996; Jackson 1997; Lewis and Papavizas 1991; Stowell *et al* 1989; Hebbar *et al* 1997; Papavizas *et al* 1984; Jackson and Schisler 1995; Jackson *et al* 1991). Typically they are based on maximising propagule yield and propagule fitness as a biocontrol agent. The first step in the optimisation is the development of a defined medium that supports good growth and propagule formation by the potential biocontrol organism. The type of propagule depends on the type of control agent being evaluated, for example, bacterial cells, bacterial or fungal spores, fungal sclerotia, or mycelial fragments (Jackson *et al* 1996). With a basal medium developed, nutritional components of the medium can be varied and the changes in the propagule yield and fitness can be assessed, as well as propagule stability as a dry preparation. All three factors must be considered during optimisation since all are required for an effective biocontrol agent. Nutritional factors such as carbon sources, nitrogen sources, vitamins, trace metals, carbon loading, carbon-to-nitrogen ratio, pH and oxygen levels can all have an influence on growth, propagule formation, and biocontrol efficacy.

With an optimal defined medium developed, the formulation of the production medium can begin. A production medium is one that replaces the nutritional components of the defined medium with low cost, complex substrates. A suitable medium should consist of inexpensive, readily available agricultural by-products with the appropriate nutrient balance. Acceptable materials include molasses, brewer's yeast, corn steep liquor, sulphite waste liquor and cottonseed and soy flours (Lisansky 1985). Use of this directed optimisation strategy (Figure 5) not only aids in the development of production media for that specific biocontrol agent, but also provides nutritional information which will be useful in developing production media for other biocontrol organisms.



A good example of successful optimisation of growth conditions can be seen in the work of Papavizas and Lewis (1989) and Lewis *et al* (1990). Preparations of *Trichoderma* and *Gliocladium* containing chlamydo spores (the hardy survival structures of the fungus) more effectively prevented diseases than preparations containing conidia only. The small-scale fermentation in molasses-brewer's yeast, resulting in abundant chlamydo spore production, gives rise to the possibility of the process being adapted to large-scale industrial fermentation, (Lewis and Papavizas 1991). Hebbar *et al* (1997) investigated the optimisation of spore production (specifically chlamydo spores) in mycoherbicidal strains of *Fusarium oxysporum*; dissolved oxygen and pH were shown to have significant effects. While the total viable counts were significantly higher under high than under low dissolved O₂, the chlamydo spore counts were higher under low than under high dissolved O₂. Also, the percentage of chlamydo spores obtained, as a proportion of total viable, was higher when the fermenter pH was increased than when it was not.

There are advantages of using submerged culture rather than solid substrate fermentation. Potentially, a much cheaper substrate can be used to maintain growth conditions and the ability exists to easily sterilise large volumes of liquid. The scale-up from small-scale trials to production scale will be easier, and there is ready expertise in this area while the technology is readily available. Hence, the development of optimal growing conditions in submerged culture is the first of the challenges on the road to the commercial production of atoxigenic *Pithomyces chartarum* as an agent for the biocontrol of facial eczema.

CHAPTER 3 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 ORGANISM

Three strains of non-sporidesmin producing *Pithomyces chartarum* were obtained from Dr. Roger Collin of the Ruakura Agricultural Research Centre, Hamilton, New Zealand. The three strains were designated RC3, RC13 and RC33. These strains had been selected from 60 isolates of *P. chartarum* that were examined and fell into three distinct groups, a sporidesmin-positive group and two sporidesmin-negative groups. Of these strains, RC3 was used for the optimisation of growth conditions as this was the only sporidesmin-negative strain to fall within a positive group and was thought to be the best strain for biocontrol due to its similarity to sporidesmin producing strains.

3.1.2 MEDIA

Buffered peptone water and yeast extract were obtained from OXOID (Unipath Ltd, Basingstoke, Hampshire, England). Bacteriological agar was obtained from GIBCO BRL (Life Technologies, Ltd, Paisley, UK). Potato dextrose agar was obtained from MERCK (MERCK Industries, Darmstadt, Germany).

3.1.3 CHEMICALS

All chemicals used were of analytical grade. Ferrous sulfate 7-hydrate, lactose, sucrose, galactose, ammonium chloride, ammonium sulfate, urea, thiamine, nicotinic acid,

riboflavin, calcium pantothenate, biotin, para-amino-benzoic acid, zinc sulfate 7-hydrate, manganese chloride 4-hydrate, sodium molybdenum oxide 2-hydrate, sulfuric acid and boric acid were obtained from BDH Laboratory Supplies, (Poole, England). Di-potassium hydrogen phosphate, ammonium nitrate, copper sulfate 5-hydrate were obtained from Ajax Chemicals, (Auburn, NSW, Australia). Xylose, pyridoxine and calcium EDTA were from SIGMA, Sigma Chemical Co., (St Louis, USA). Solubilised starch was from Pauling Industries Ltd., (Avondale, Auckland). Peptone was from OXOID, Unipath Ltd., (Basingstoke, Hampshire, England) Glucose was from GIBCO BRL, Life Technologies, (Paisely, Scotland). Potassium di-hydrogen phosphate was from Scharlau, (Barcelona, Spain). Magnesium sulfate 7-hydrate was from Scientific Supplies Ltd., (Auckland). Casamino acids (Technical) were obtained from DIFCO, (DIFCO Laboratories, Detroit, USA).

3.1.4 ENZYMES

The starch hydrolysing enzymes Termamyl and San Super 240 were obtained from Novo Nordisk, Bagvaerd, Denmark.

3.1.5 EQUIPMENT

The equipment used in the study included: YSI Glucose analyser (Yellow Springs Instrument Co., Yellow Springs, Ohio, USA); High Performance Liquid Chromatograph (HPLC), Waters model 440 absorbance detector and differential refractometer and a model 590 pump unit (Waters Associates Inc., Millipore Corporation, Milford, Massachusetts, USA) connected to a Waters model 740 data module recorder. Buchi 435 digester, Buchi 323 distillation unit, (BUCHI, Switzerland). Mettler DL25 titrator supplied by Watson Victor Ltd, Christchurch, New Zealand. pH meter E520 (Metrohm Herisau, Switzerland). Gallenkamp cooled incubator and a Gallenkamp orbital incubator (England). MSE Microcentaur microcentrifuge (Fisons, England). Waring

blender, model 2057 (Waring products Co., Winsted, Connecticut, USA). Julabo refrigerated circulator, model F10-UC (Julabo, Labortechnik).

3.2 METHODS

3.2.1 STOCK CULTURES

Stock cultures of the organism were cultivated at 25°C on slopes of Potato Dextrose Agar and on Rabbit Chow Agar, consisting of 25g/L rabbit food pellets (ATTC formulation 340), boiled and allowed to stand for a minimum of one hour. The mixture was filtered through cheesecloth, 15g/L of bacteriological agar was added, the mixture was boiled to dissolve the agar, and was then autoclaved at 121°C for 15min. The spores were harvested off the slopes by addition and agitation of sterile water, containing 0.05% Tween 20, to the slope. Spores from Rabbit Chow Agar were used as the inoculum as their harvest proved easier than of spores from PDA slopes

3.2.2 GROWTH OF ORGANISMS

3.2.2.1 Shake Flask

Growth studies were conducted in 100ml of medium contained in 250ml conical flasks, incubated in a rotary incubator operating at 150 rpm and 25°C. The nitrogen and carbon sources and trace elements and growth factors were added to a basal salts solution before autoclaving at 121°C for 15min. The constitution of the basal salts solution was: KH_2PO_4 (0.5g/L), K_2HPO_4 (0.5g/L), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.5g/L), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.01g/L) in 1L RO water. Trace metals and vitamins were included in the initial experiments when various carbon and nitrogen sources

were being evaluated. [This is required to reduce the impact of the vitamins and trace metals which may or may not be present in the complex nitrogen and carbon sources being tested (Jackson, 1997)]. The trace elements were zinc ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4ppm), copper ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.1ppm), manganese ($\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.05ppm) and molybdenum ($\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$, 0.04ppm). Growth factors were thiamine (1ppm), nicotinic acid (1ppm), riboflavin (1ppm), calcium pantothenate (0.5ppm), pyridoxine (0.5ppm), biotin (0.2ppm) and para-amino benzoic acid (0.1ppm). Flasks were inoculated with approximately 1.0×10^6 spores/ml. Flasks were run in replicate and whole-flask samples were withdrawn as appropriate.

3.2.2.2 Fermenter

Batch fermentation experiments were carried out using apparatus as pictured in (Figure 6) and described below. Spore suspensions of 10^3 or 10^4 spores/ml concentration were inoculated into a 2L-fermentation glass jar with a working volume of approximately 1.5L. The fermenter was provided with a polyethylene-polypropylene head with insertion holes for the filtered air sparger and condensing air-outlet, heating coil and temperature probe and sample collection point. Agitation was provided by two 50-mm diameter Rushton type turbines located at 45mm and 9mm from the base of the fermenter, (approximately 2/3 and 1/3 of the depth of the fluid volume).

Upon inoculation, a sample (15ml approx.) was withdrawn to mark zero time, then every 24 hours for 5 days. The samples were withdrawn by positive pressure created by blocking the condenser air-outlet causing culture to be pushed up the sample tube into a collecting bottle. The sample was inspected microscopically for contamination, and Potato Dextrose Agar plates were inoculated to check for contaminant colony growth; culture pH was measured and the sample was stored frozen, to be analysed further for carbon and nitrogen concentrations. After 5

days incubation the fermentation was halted and approximately 200 ml of culture was homogenised as per standard procedure for determining the number of colony-forming-units. The remaining culture was filtered for biomass determination.

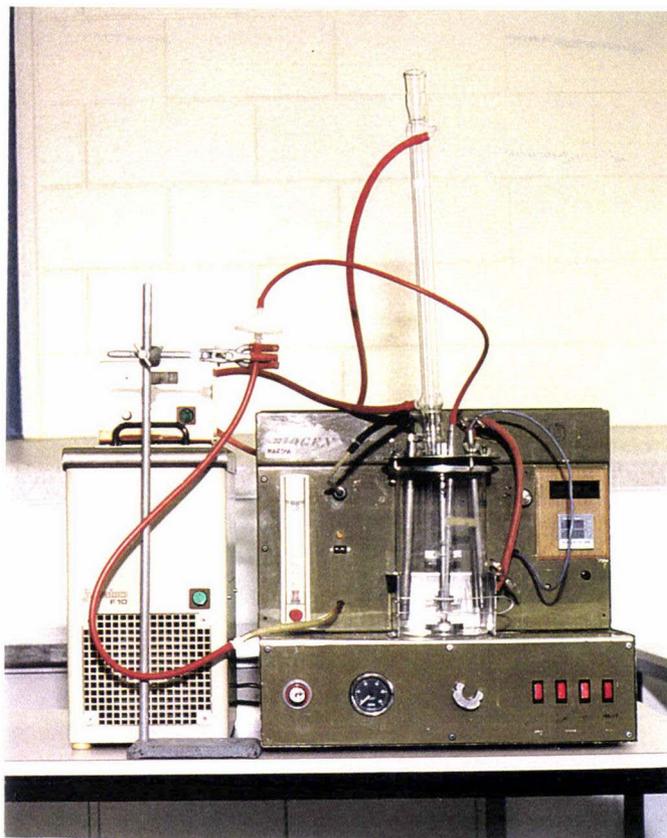


Figure 6 A picture of the fermentation apparatus used in the 1.5-L scale

3.2.3 ANALYSIS

3.2.3.1 Biomass determination

The dry-weight biomass was determined following filtration (Buchner apparatus) of the contents of duplicate flasks on separate preweighed miracloth followed by rinsing with 100ml of RO water to remove any traces of media. The papers

were then dried overnight at 105°C and placed in a dessicator for 24 hours before re-weighing.

3.2.3.2 Determination of Colony-Forming-Units

The number of colony forming units after a standard homogenisation procedure was used to assess the “efficacy” of the culture. The contents of two flasks of culture were blended for 30 seconds at low speed in a Waring blender. Dilutions of this homogenate, using peptone water, were then plated out on Potato Dextrose Agar and incubated for 3 days at 30°C. The resultant colonies were counted.

3.2.3.3 Carbon sources

Carbon substrate concentrations in culture filtrates were analysed by use of a glucose analyser, or for other carbon sources, by high-performance liquid chromatography (HPLC). Enzymatic digestion of starch into glucose, and subsequent analysis, allowed determination of starch concentration. The glucose analyser was calibrated using 2g/L and 5g/L glucose and samples were diluted to the range of the analyser. Duplicate readings were taken from single fermenter samples and single readings from duplicate shake flask samples. HPLC readings were taken from 50µl samples using a Sugar-Pak 90 separating column run at 0.5ml/min and 90°C in a 50mg/L Calcium EDTA mobile phase. Standards for calibration were typically 10 and 30g/L except for analysis of whey where a 50g/L lactose standard was used.

3.2.3.4 Nitrogen sources

Nitrogen substrate concentrations in culture filtrates were determined by the total kjeldahl nitrogen method, except in the instances of measuring nitrates which were done by Soil Science Department, Massey University, Palmerston North. Determining Total Kjeldahl Nitrogen (TKN) involved digestion of samples (10ml) with 13ml conc. H₂SO₄ and two digester tablets under heat for approximately 90 min. Samples were allowed to cool under vacuum for 30 min before distillation. The distillation unit was set to 30ml H₂O, 70ml NaOH, 5 second delay, 5 min distillation time. Boric acid (4%, 30 ml) was added to the distillate flask and the digested sample was distilled. The distillate flask was titrated to determine the nitrogen released as ammonium ion.

3.2.3.5 Starch hydrolysis

Two drops of Termamyl (α -amylase) were added to 10 ml of sample filtrate, held at 90°C for one hour and then cooled to 70°C. Two drops of San Super (amyloglucosidase) were then added and the temperature was held at 70°C for one hour. The glucose concentration was then measured as described above. The starch concentration was calculated as:

$$\text{Starch (g/L)} = \text{glucose (g/L)} \times 0.9.$$

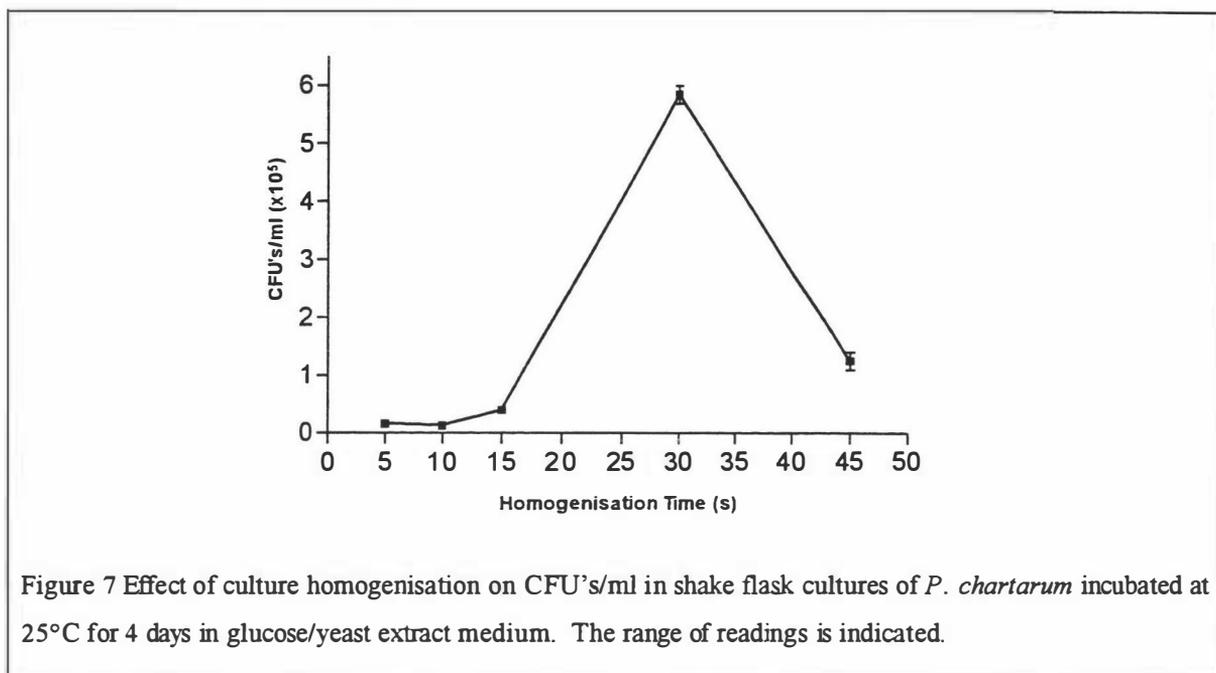
CHAPTER 4 OPTIMISATION OF CARBON SOURCE, NITROGEN SOURCE, GROWTH ADDITIVES AND INITIAL CULTURE PH VALUE

4.1 HOMOGENISATION STANDARDISATION

To develop a standard homogenisation procedure, the organism was grown for 4 days in 100ml of a semi-defined medium of glucose (5g/L) and yeast extract (5g/L). Resultant cultures were then either filtered and the harvested biomass was homogenised in sterile peptone water (5g/L), or the culture was homogenised directly. Homogenisation times of 5, 10, 15, 30 and 45sec were used to determine the degree of biomass homogenisation. Homogenate was plated out at dilutions of 10^{-1} to 10^{-6} in sterile peptone water (5g/L). Homogenisation of the culture resulted in a peak in colony-forming-units at 30s (Figure 7). This suggests that homogenisation separates mycelial fragments into further viable units but that prolonged treatment results in fragmentation into nonviable units.

It should be noted here that the organism was markedly different in floc morphology when grown in the glucose/yeast extract medium for this experiment from the flocs seen in the later experiments with various other media. This has been noted before (Ellis, 1960; Ross, 1960; Dingley, 1962) and was not surprising, but it could have an important effect on the optimal time of homogenisation. This is because different media gave rise to different flocs with differing degrees of fragmentation. From this it can be inferred, and indeed it was seen in some time course studies using different carbon sources, that the number of CFU's formed was higher after longer homogenisation times than after the standard time of 30sec. Nevertheless, the standard homogenisation procedure was adopted. A standard homogenisation protocol was decided to be the contents of two shake-flasks (200ml) added to a sterilised Waring blender for 30s on low speed and the homogenate was plated out in

dilutions up to 10^7 fold on Potato Dextrose Agar. The plates were incubated for 3 days at 30°C before counting the colonies.



4.2 EFFECT OF CARBON SOURCE

4.2.1 INTRODUCTION

Glucose, sucrose, lactose, galactose, xylose, and starch (all 30g/L) were separately tested as carbon sources for the growth of the organism. Casamino acids (3g/L) were used as the nitrogen source as their concentrations are easily measured in culture filtrates (Jackson, 1997). For each carbon source, mycelial biomass was determined after 3 and 5 days incubation, while CFU was determined after 4 days. Carbon and nitrogen concentration and culture pH were measured on days 0,3 and 5. After the initial screening experiment, two carbon sources were selected for a further 14-day time course study with CFU, biomass and substrate concentrations being determined every 2 days.

4.2.2 INITIAL SCREENING EXPERIMENTS

The results (Table 1) of the screening experiments showed that *P. chartarum* is capable of utilising, and producing similar biomass from, a wide range of carbon sources. Their colony-forming-ability was variable, however, with lactose, galactose and xylose yielding lower counts than starch, sucrose and glucose.

Table 1 Effect of carbon source on growth and colony forming ability of *P. chartarum*.

CARBON SOURCE	CFU's/ml	BIOMASS AT 72 HRS (g/L)	BIOMASS AT 120 HRS (g/L)
Glucose	4.3×10^4	6.99	6.98
Galactose	2.0×10^4	6.19	6.70
Lactose	1.5×10^4	6.50	6.03
Starch	5.2×10^4	8.70	7.05
Sucrose	5.0×10^4	6.78	9.36
Xylose	2.4×10^4	4.83	7.27

4.2.3 TIME COURSE STUDIES

The decision was made to use glucose and lactose for the time trial analysis of growth parameters in greater detail. Glucose was selected as it was one of the better utilised carbon sources and was easily assayed, and lactose for its potential low economic cost, being obtainable in whey permeate from the dairy industry. The development of the colony-forming-ability of the organism increased with increasing incubation time, but subsequently decreased. The glucose medium showed this trend up to 144h incubation after which the CFU decreased (Figure 8). It should be noted that the flocs in this medium were substantially larger structures than those used during development of the standard homogenisation procedure, and the biomass appeared not to be homogenised to the same extent. Thus, cultures were subjected to a further 30sec homogenisation, resulting in

higher viable counts. The lactose medium showed a much slower increase in the colony-forming-ability, not peaking until after 240-288h incubation, and again demonstrating the same effect with increased homogenisation time giving higher CFU/ml (Figure 9).

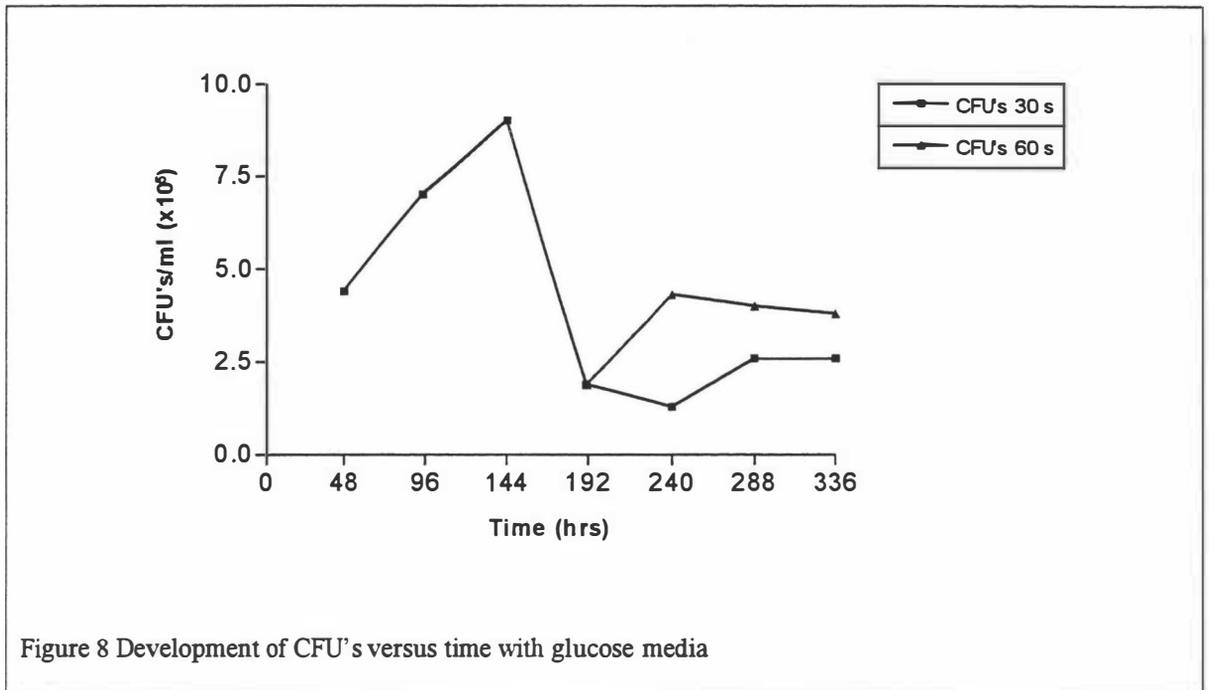


Figure 8 Development of CFU's versus time with glucose media

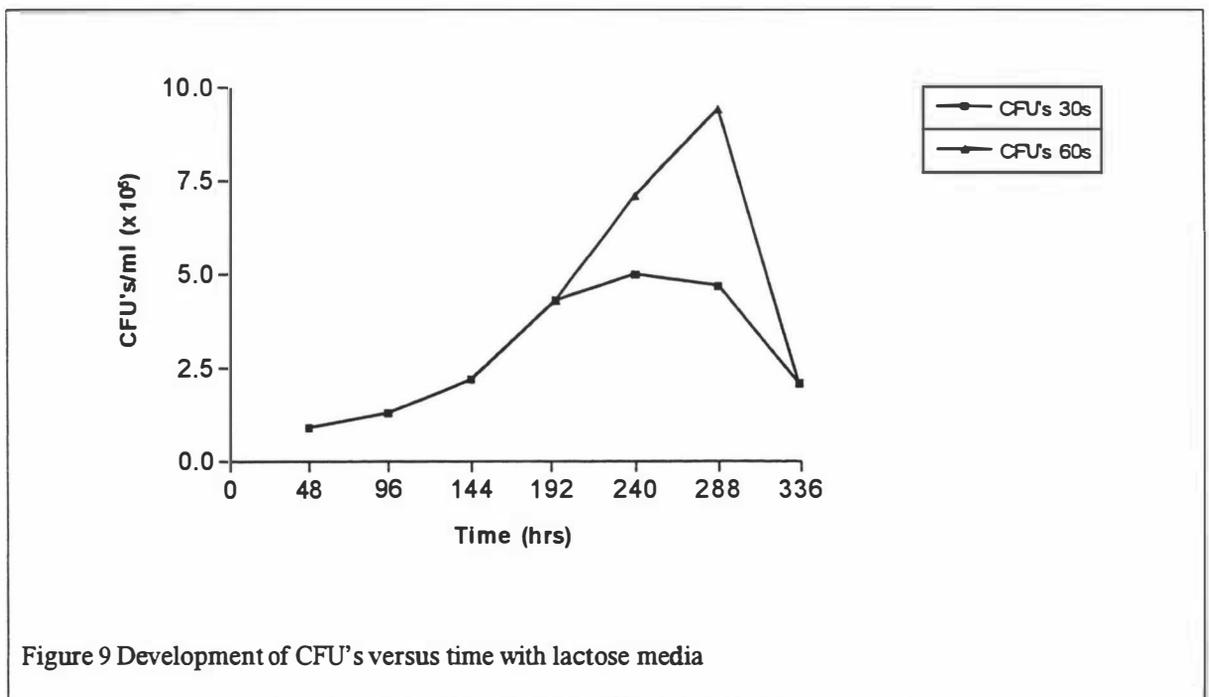
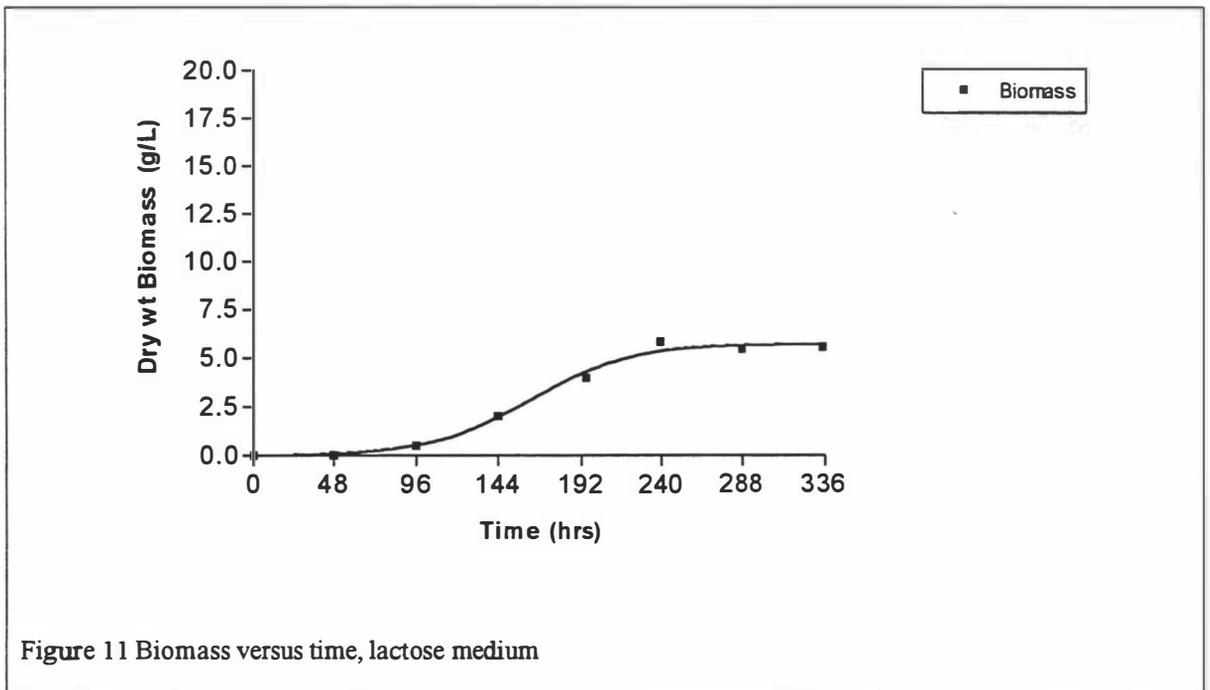
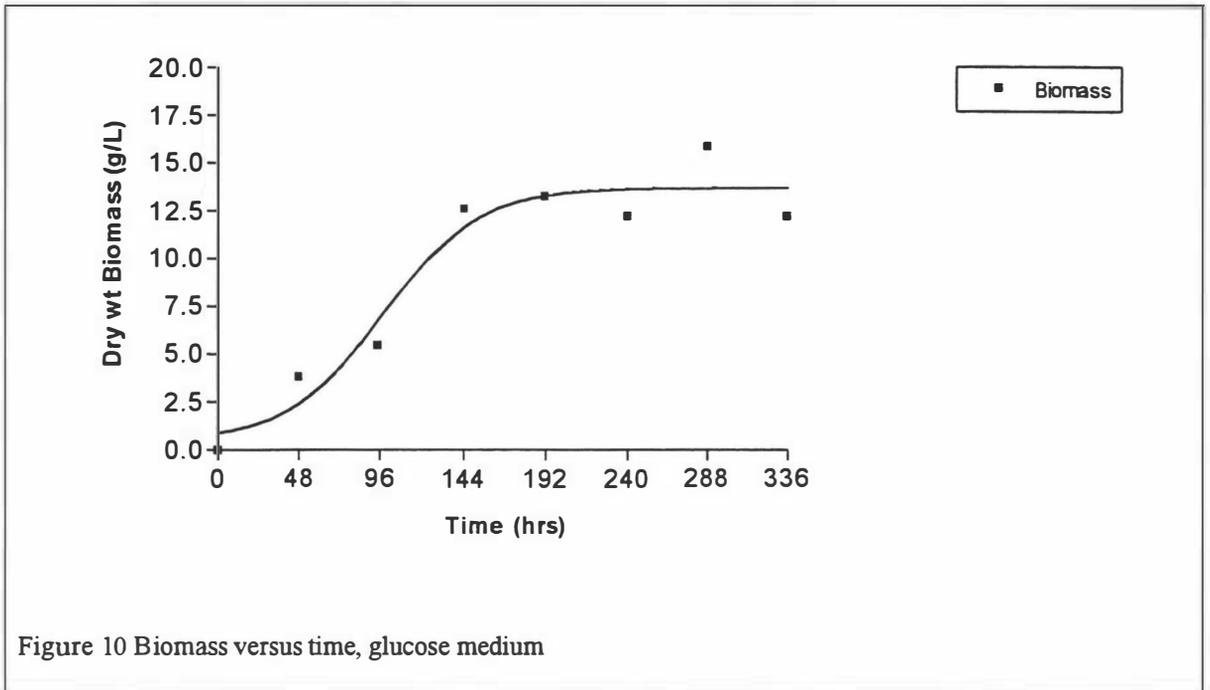


Figure 9 Development of CFU's versus time with lactose media

4.2.3.1 Specific rate of growth

The amount of biomass produced from glucose was over twice that from lactose, with values around 14g/L and 6g/L respectively (Figures 10 and 11).



To determine the specific rate of growth of the organism (Table 2), the logarithm (base 10) of the biomass concentration was plotted against time (Figures 12 and 13), and

$$\text{Specific rate} = \mu = 2.303 \times \text{slope}$$

where the slope was measured during the initial stage of growth ($t \leq 48$ h).

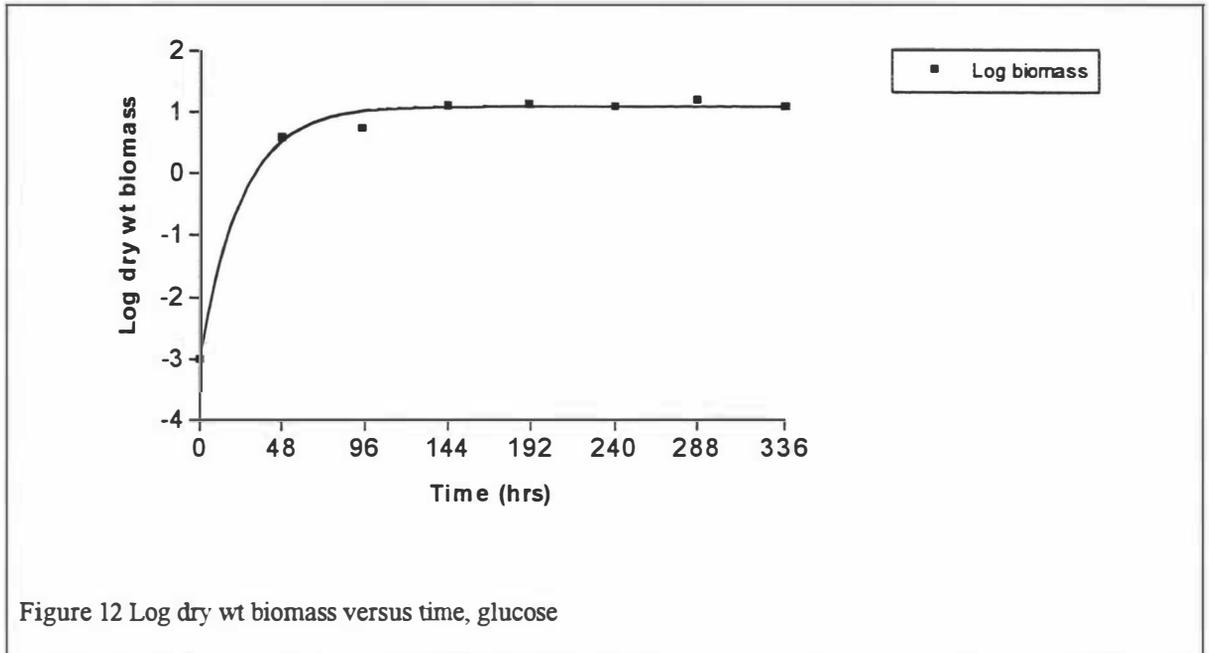


Figure 12 Log dry wt biomass versus time, glucose

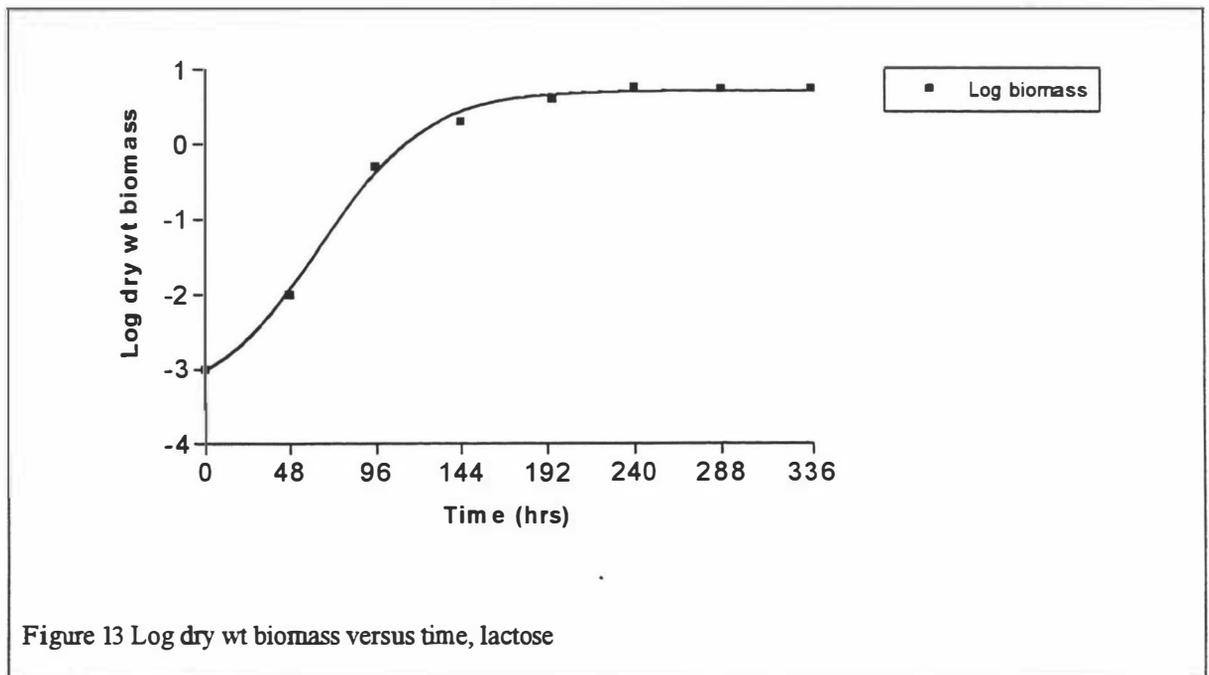


Figure 13 Log dry wt biomass versus time, lactose

Table 2 Growth rates of *P.chartarum* on glucose and lactose.

CARBON SOURCE	SPECIFIC RATE OF GROWTH (h ⁻¹)
Glucose	0.172
Lactose	0.065

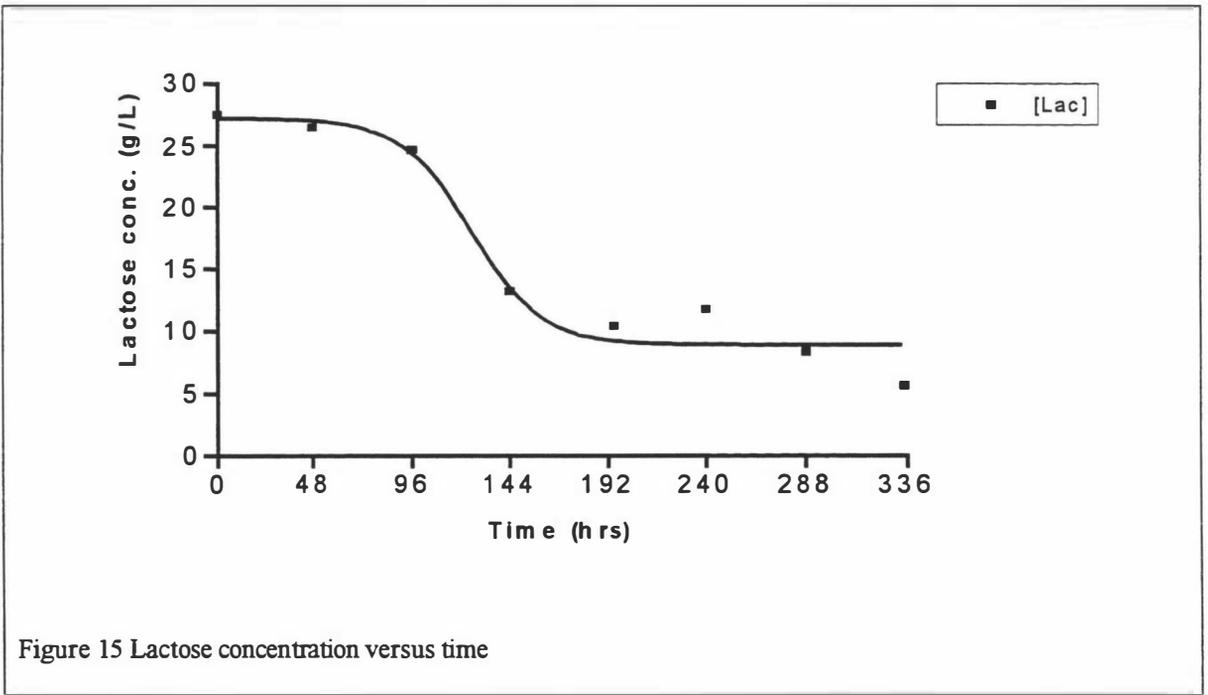
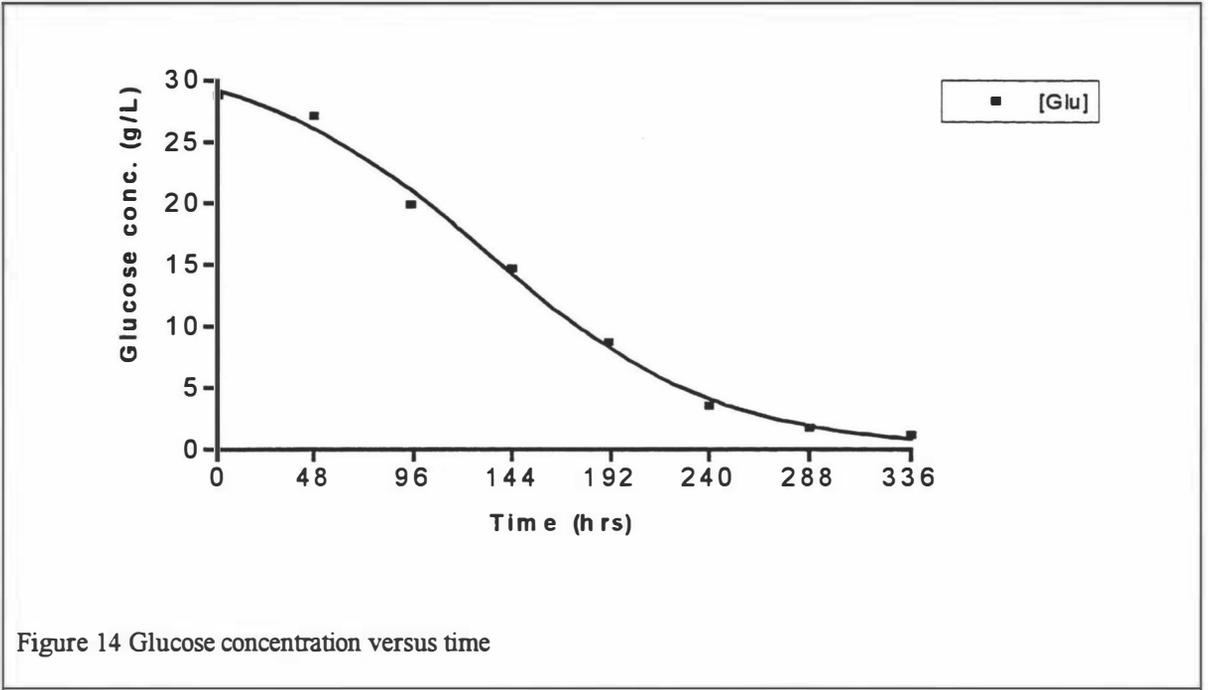
The results showed that the specific growth rate during growth on glucose was more than double that during growth on lactose.

4.2.3.2 Substrate utilisation

To determine the rate of substrate utilisation, (Tables 3 and 4) substrate concentration was plotted against time (Figures 14 and 15) and

$$\text{Rate} = -(\text{Slope})$$

where the slope was measured at every $t = 48$ h.



4.2.3.3 Specific rate of substrate utilisation

The specific rate of substrate utilisation (Tables 3 and 4) was calculated as follows

$$\text{Specific rate of substrate utilisation} = \text{volumetric rate/biomass}$$

where, volumetric rate of utilisation = amount of substrate used/time, and biomass concentration was taken at time t = every 48 h.

4.2.3.4 Biomass growth yield

To calculate the biomass growth yield, $Y_{x/s}$ (Table 3 and 4), the biomass concentration was plotted against the amount of substrate consumed (Figures 16 and 17). From this plot,

$$Y_{x/s} = \text{slope,}$$

where slope was calculated at time t = every 48 h.



Figure 16 Biomass concentration versus glucose used

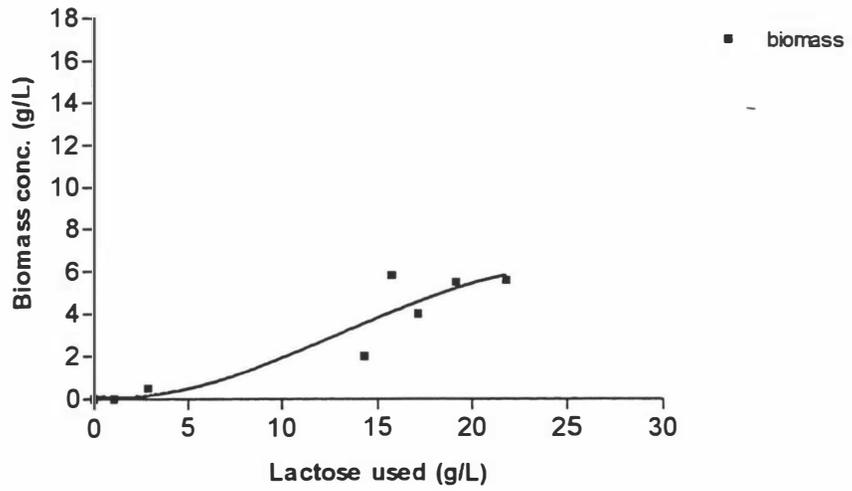


Figure 17 Biomass concentration versus lactose used

Table 3 Rates of substrate utilisation during growth on glucose

TIME (hrs)	VOLUMETRIC RATE OF SUBSTRATE UTILISATION (g/L.h)	SPECIFIC RATE OF SUBSTRATE UTILISATION (g/g _{biomass} .h)	GROWTH YIELD, Y _{X/S}
48	0.083	0.027	1.796
94.5	0.156	0.020	0.876
144	0.115	0.010	0.859
191	0.095	0.007	0.662
240	0.083	0.006	0.554
288	0.021	0.002	0.517
336	N/A	N/A	0.507

Table 4 Rates of substrate utilisation during growth on lactose

TIME (hrs)	VOLUMETRIC RATE OF SUBSTRATE UTILISATION (g/L.h)	SPECIFIC RATE OF SUBSTRATE UTILISATION (g/g _{biomass} .h)	GROWTH YIELD, Y _{X/S}
48	0.048	4.800	0.010
96	0.177	0.340	0.178
144	0.104	0.052	0.140
195	0.031	0.008	0.237
240	0.031	0.006	0.330
288	0.052	0.009	0.300
335	N/A	N/A	0.270

4.2.3.5 Productivity

Two productivity parameters were calculated; Reactor biomass productivity was calculated from

$$\text{Final biomass concentration} / \text{Total time incubated}$$

Reactor substrate productivity was calculated from

$$\text{Total substrate used} / \text{Total time incubated}$$

Table 5 Glucose and lactose productivity

CARBON SOURCE	REACTOR BIOMASS PRODUCTIVITY (g/L.h)	REACTOR SUBSTRATE PRODUCTIVITY (g/L.h)
Glucose	0.042	0.082
Lactose	0.017	0.065

4.2.4 DISCUSSION

The rate data for *P. chartarum* showed that its growth on glucose was superior to that on lactose. Also, the specific growth rate on glucose was over twice that on lactose. The observed volumetric rates of utilisation for the two substrates followed similar patterns with similar values and likewise with the specific rates of substrate utilisation, with the exception of the first 96 h of growth on lactose in which high specific rates were obtained. This is due to the very low amounts of biomass produced combined with inaccuracies in determining lactose concentration. The growth yields however, were markedly different, with the glucose giving higher yields than lactose. It should be noted, however, that the very high initial yield on glucose, with a value of 1.796, can probably be accounted for by the preferential use of casamino acids as a carbon source, as evidenced by following the medium nitrogen concentrations (data not shown).

During these experiments there was no nitrogen-nutrient limitation observed, with nitrogen concentrations falling no lower than 0.4g/L in the time course studies.

4.3 EFFECT OF NITROGEN SOURCE

4.3.1 INTRODUCTION

Nitrogen sources tested were casamino acids, ammonium chloride, ammonium sulphate, urea, ammonium nitrate and peptone (all 5g/L). From the carbon source experiments described in section 4.2, it was estimated that there would be no nitrogen limiting effects at this concentration, as evidenced from the above that there were none when using casamino acids at 3g/L. Glucose (30 g/L) was used as the carbon source. It was decided that the assessment for growth on different nitrogen sources should be conducted after 6 days incubation after observing in the carbon source experiments that there was little increase in biomass after this time. Duplicate biomass and substrate readings were taken.

4.3.2 SCREENING EXPERIMENTS

These experiments showed that the organism is capable of utilising the organic nitrogen sources best of all the sources trialed here (Table 6). Casamino acids were shown to be the best of the organic sources with CFU count and Biomass after 6 days of 5.5×10^4 and 15.9 g/L respectively. Peptone was not far behind with a CFU count of 4.0×10^4 and a biomass of 12.5 g/L. Urea was the best of the non-organic sources with 10.5 g/L dry weight biomass produced and a CFU count of 2.5×10^4 , NH_4NO_3 had a slightly higher CFU count but markedly lower biomass. The minimal growth of the *P. chartarum* on NH_4Cl , NH_4SO_4 and NH_4NO_3 should be viewed with caution as Ross (1960) demonstrated that it was readily capable of utilising nitrate and ammonium salts. A possible reason for the present results could be the formation of hydrogen ions during growth from the metabolism of

these nitrogen sources. The observed drop in pH in these cultures from above pH 5.5 to values of around 2.7 for NH_4Cl and NH_4SO_4 and pH3.9 for NH_4NO_3 supports this suggestion.

Table 6 Effect of nitrogen source on growth and colony-forming-ability of *P. chartarum* after 6 days incubation

NITROGEN SOURCE	CFU'S/ml	BIOMASS (g/L) AFTER 6 DAYS
Casamino acids	5.5×10^4	15.90
NH_4Cl	1.0×10^4	2.40
NH_4SO_4	1.2×10^4	2.20
Urea	2.5×10^4	10.50
NH_4NO_3	2.8×10^4	6.20
Peptone	4.0×10^4	12.50

4.3.3 DISCUSSION

These results show the importance of careful choice of nitrogen source for the medium. The use of inorganic sources should be combined with control of the pH, as growth resulted in acid formation to inhibitory levels. The discovery of urea as a higher yielding nitrogen source holds promise, as this is a substrate readily available and at cheap cost. The casamino acids however, provide the highest yields and this nitrogen source was maintained for the remaining experiments.

4.4 EFFECT OF PH

4.4.1 INTRODUCTION

The optimal initial pH was determined from initial values of culture medium of pH 3,4,6 and 7. The medium used was chosen from the carbon and nitrogen optimisation previously performed, being glucose (30g/L) and casamino acids (3g/L) in basal salts solution with added trace metals and vitamins. The pH optimisation was determined with biomass, substrate concentration and CFU's determined after 3 and 5 days incubation. Duplicate biomass and substrate readings were taken.

4.4.2 OPTIMAL PH

Growth on the glucose/ casamino acids medium was good over a wide range of initial pH values (Figures 18 and 19). This supports previous observations made by Ross (1960). There was an inhibitory effect observed, however, at around pH 3. This occurred in cultures grown at pH 3 and, to a lesser extent, pH 4 after some growth of the organism, resulting in pH drop due to acid formation (Table 7).

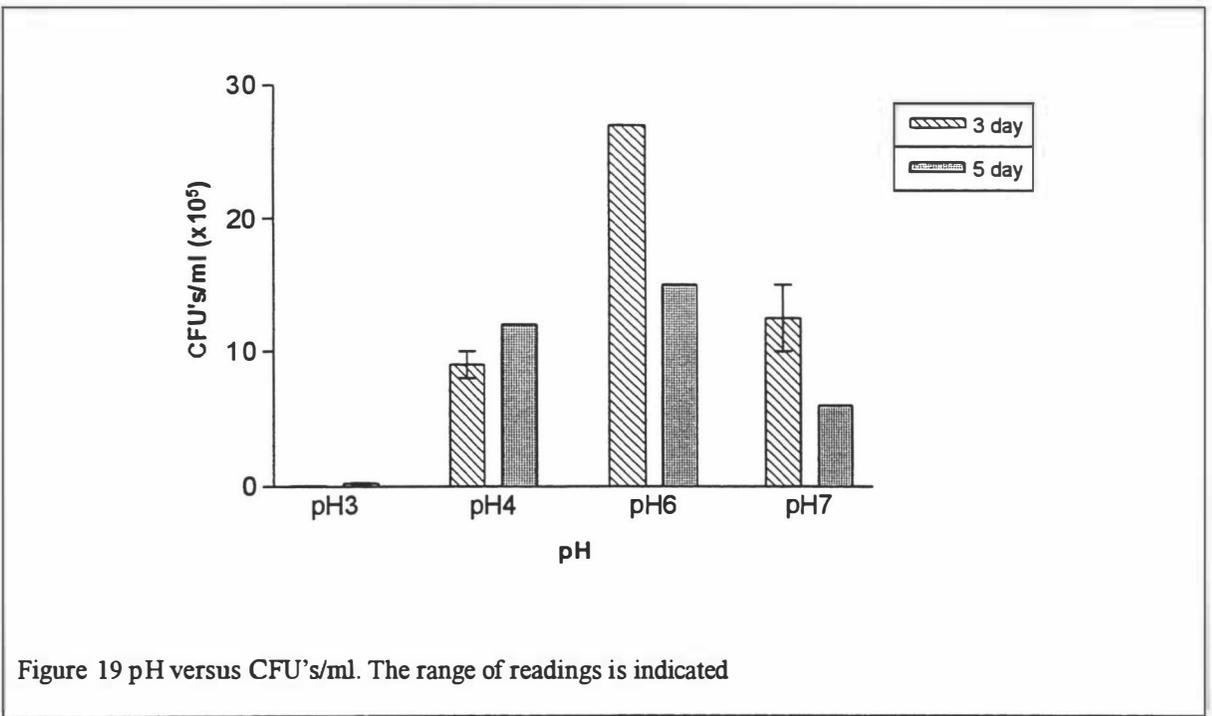
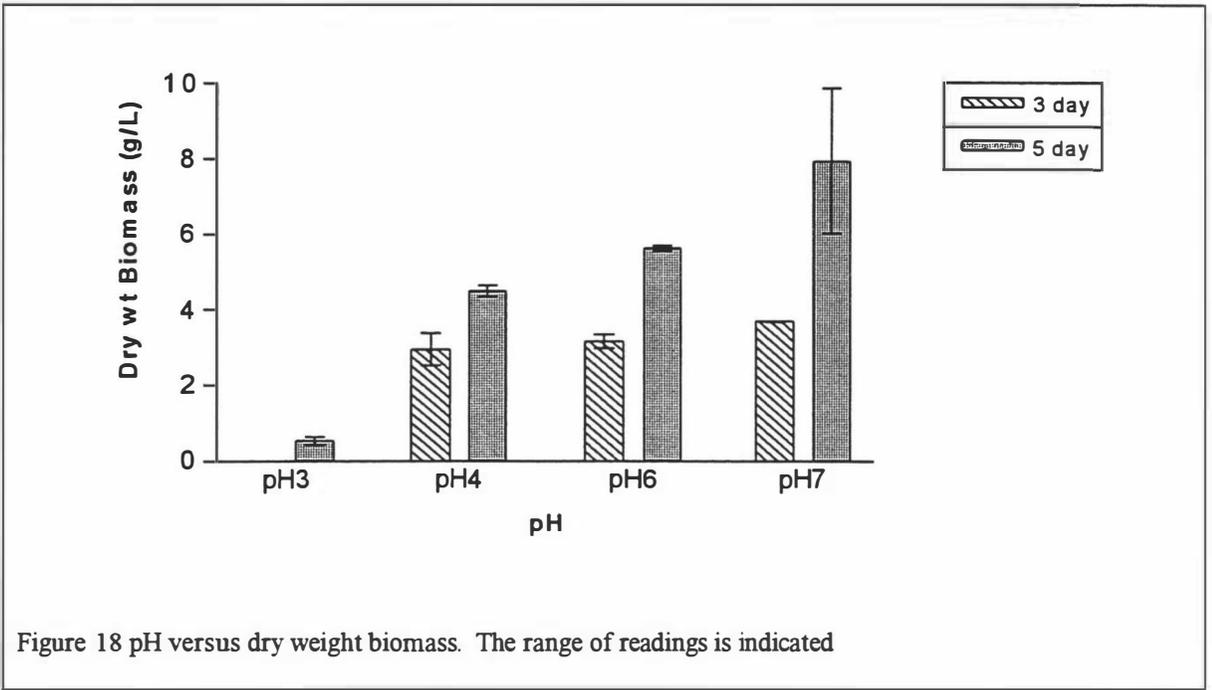


Table 7 Change of pH with time

Initial pH	pH t =3 days	pH t= 5 days
3	2.9	2.7
4	3.5	2.8
6	5.0	4.1
7	6.1	5.9

4.4.3 DISCUSSION

On glucose/casamino acids medium *P.chartarum* was able to grow over a wide range of pH values, with an optimum value of around pH 6. From these results it is evident that the pH value of the medium drops with growth of the organism, and becomes inhibitory at values around 3. Nevertheless a low pH to grow the organism is desirable, as this decreases the possibility of contamination of the culture by bacteria.

4.5 GROWTH FACTORS AND TRACE ELEMENTS

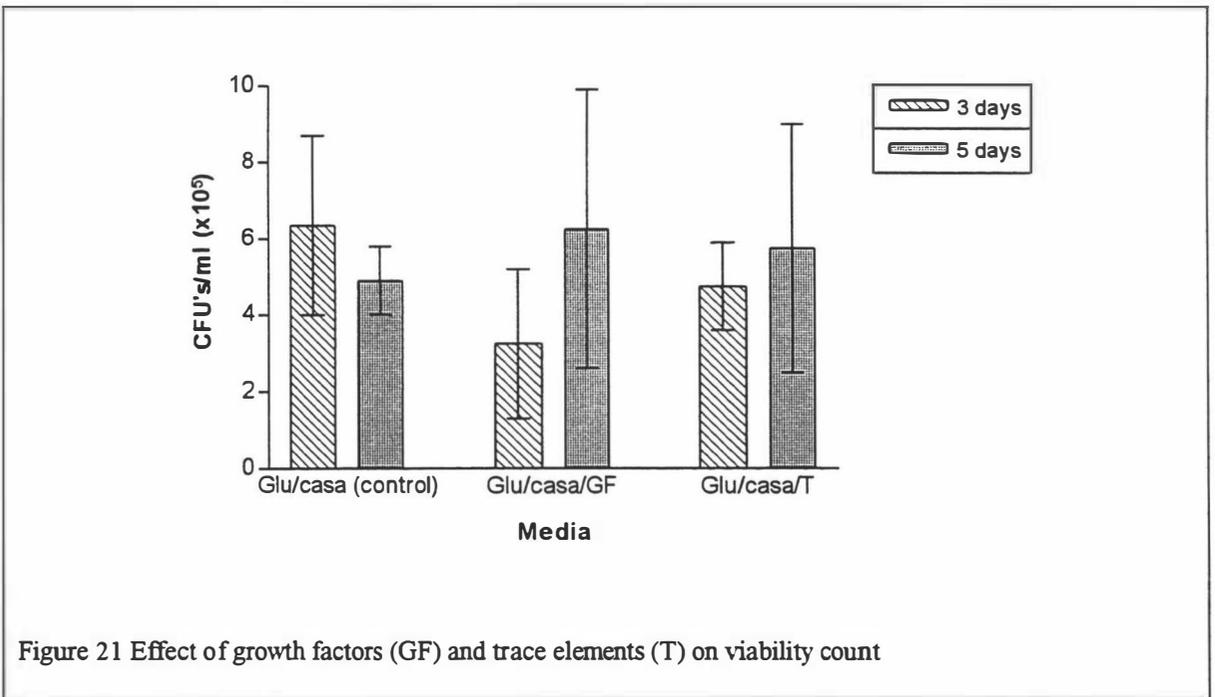
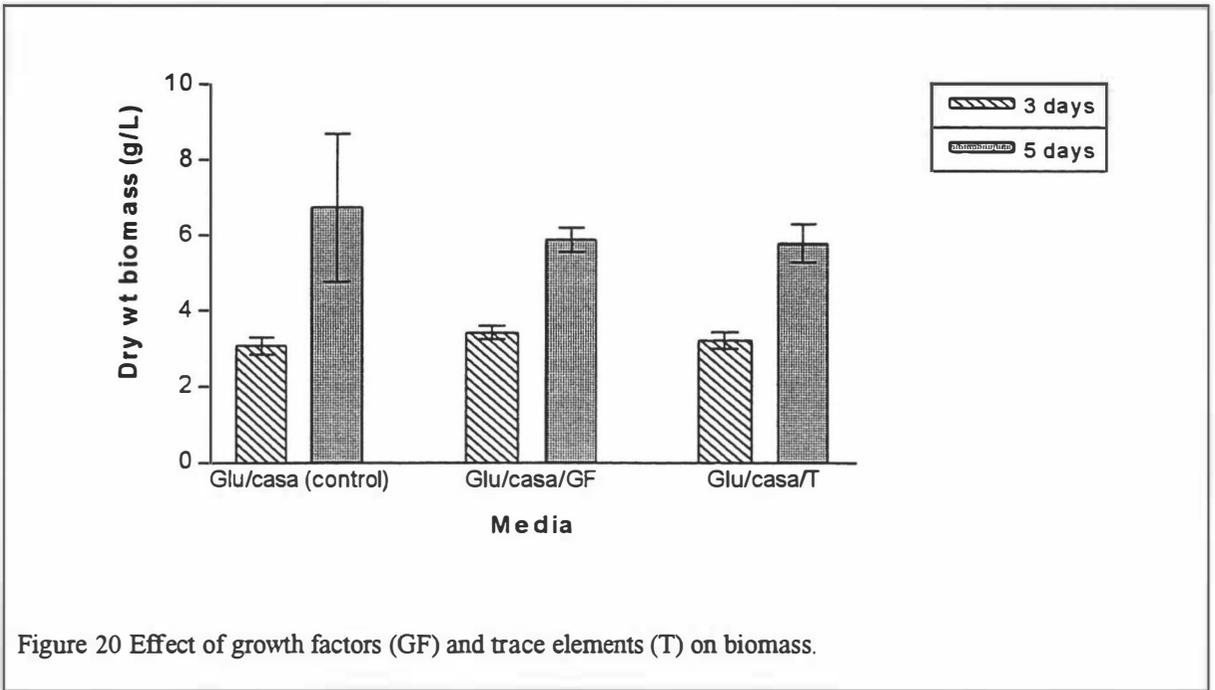
4.5.1 INTRODUCTION

Now that the carbon and nitrogen sources had been identified for maximum biomass concentration and colony forming ability, the role of trace elements and growth factors on the performance of the organism was investigated. Previous literature by Ross (1960) had identified that addition of growth factors resulted in increased rate of growth, but following prolonged incubation, the crop yields of all strains studied were as great in the basal medium as in media containing growth factors. The addition of trace metals to the basal salts-growth factor medium stimulated growth and resulted in increased production of mycelial felts. Sporulation of all strains studied by Ross was increased by the addition

of trace metals, but the sporulation observed on synthetic media was less than that on complex potato-carrot medium. In the present work, the effect of additives to the basal medium on the atoxigenic strain's growth characteristics and colony forming ability was assessed. The media used were; glucose (30g/L) + casamino acids (3g/L) (control), glucose + casamino acids + trace elements, and glucose + casamino acids + growth factors. Biomass, substrate concentration and CFU's were determined after 3 and 5 days incubation. Duplicate biomass and substrate readings were taken. This experiment was repeated, and the average of the results plotted.

4.5.2 RESULTS

There was little difference evident from the three different treatments, on the biomass produced. The glucose/casamino acids control showed similar values after 3 and 5 days growth as the glu/casa + growth factors and the glu/casa + trace metals of around 3g/L and 6g/L respectively. This result was also observed with the colony-forming-ability of the three treatments, all values being between 4 and 6×10^5 CFU's/ml (Figures 20 and 21). The range of readings is indicated.



4.5.3 DISCUSSION

The addition of growth factors or trace elements to the glucose/casamino acid medium had no noticeable effect on either the biomass concentration or the viability count of the

organism. The rate of biomass production was also similar on all three media. Hence it can be assumed that the necessary trace elements for growth are provided in sufficient concentration as impurities in the other medium components, and that the organism has the capability of synthesising its own growth factors.

4.6 CONCLUSION

From the experiments performed so far, the best defined medium for growth of this strain of *P.chartarum* consists of a glucose/casamino acids medium with a slightly acidic pH. The organism is capable of utilising a range of carbon sources; the best of those investigated here would be any of glucose, sucrose and starch.

The organism is capable of using a range of nitrogen sources, the organic sources being the best of these. The need for control of pH when growing the organism on inorganic nitrogen sources is recommended if the use of these for a substrate is to be pursued.

The effects of added growth factors and trace elements on the performance of the organism in terms of viability and biomass were negligible, and this indicates that the glucose and casamino acids provide enough of these components already and that the strain can synthesise its own growth factors.

CHAPTER 5 STRAIN CHARACTERISTICS

5.1 INTRODUCTION

The investigation of the growth of different strains of a biocontrol organism is carried out for two reasons. First, the growth parameters of the different strains will often vary, and therefore each strain needs to be assessed to identify the most productive ones. The second reason is that the greater the genetic pool of the biocontrol agent, the greater the chances of the agent survival and propagation when in use in the field. The growth characteristics of the three different strains supplied by Agresearch Ltd. (designated RC3, RC13 and RC33) were assessed here for their biomass and colony-forming-ability. The medium used consisted of only glucose (30g/L), casamino acids (3g/L) and basal salts after it was decided from previous experiments that there was little effect from the addition of growth factors or trace elements. Flasks for strain RC 13 were inoculated with 4.5×10^4 spores, rather than 1×10^6 , due to very low spore numbers being obtainable from stock cultures. For each strain, biomass, substrate concentration and CFU's were determined after 3 and 5 days incubation. Duplicate biomass and substrate readings were taken.

5.2 RESULTS

5.2.1 QUANTITATIVE ANALYSIS

The three strains produced different amounts of biomass (Figure 22). Strain RC 13 produced the greatest amount of biomass of the three strains with values of 4.1 g/L and 7.6 g/L after 3 and 5 days incubation respectively. The next greatest biomass was produced by strain RC 33 with values of 2.6 and 6.3 g/L. Strain RC 3 had the smallest growth of the strains investigated with only 1.2 g/L after 3 days and 3.8 g/L after 5 days incubation.

The viable unit counts of the strains were all of similar values around 2×10^5 /ml after 3 days and 4×10^5 /ml following 5 days, with the exception of strain RC 33 which had a higher value of 8×10^5 /ml, but this value also had much greater error (Figure 23).

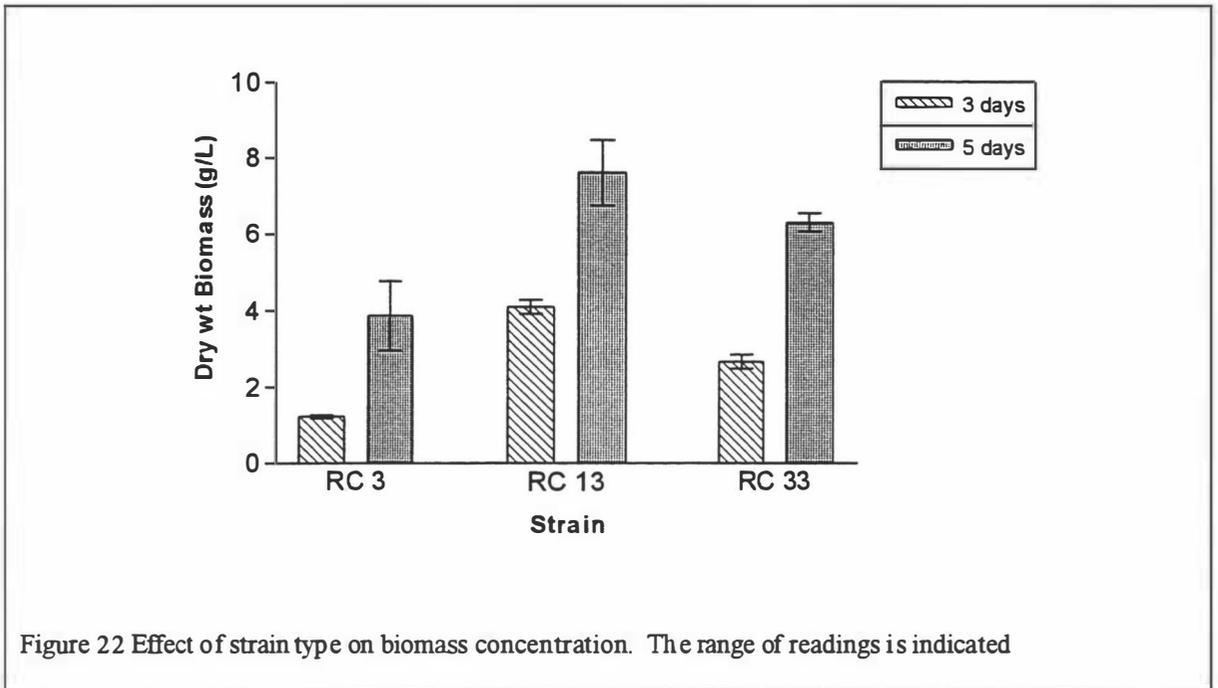


Figure 22 Effect of strain type on biomass concentration. The range of readings is indicated

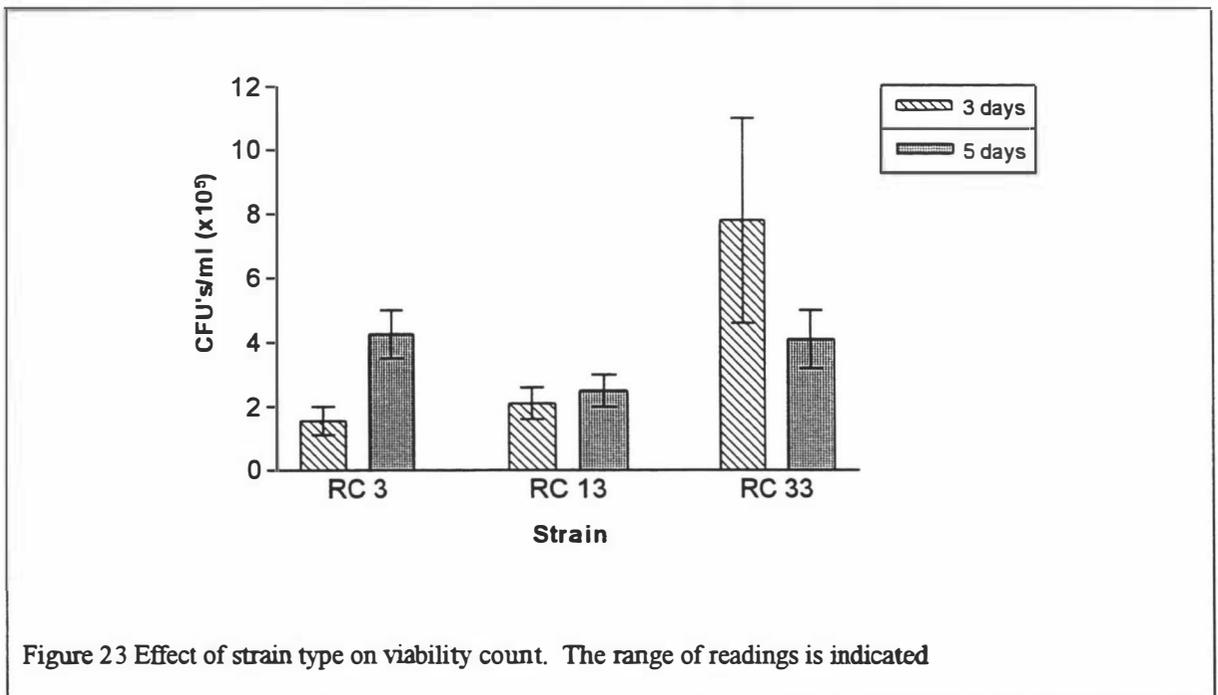


Figure 23 Effect of strain type on viability count. The range of readings is indicated

Comparison of the performance of these strains on the glucose/casamino acids medium with the growth data from the carbon source time course experiment (section 4.2.3) reveals that all three strains had slightly slower growth rates than when grown on glucose/casamino acid medium+growth factors and trace elements. Comparison of the viability counts also showed that they were lower than the counts from that time course experiment with the exception of the three-day colony count for strain RC 33.

5.2.2 QUALITATIVE OBSERVATIONS

The following morphological characteristics of the strains were noted during their growth in shake-flask culture.

Table 8 Strain characteristics noted during growth on glucose/casamino acids media.

STRAIN	3 DAYS INCUBATION	5 DAYS INCUBATION
RC 3	Olive green mycelia, some budding structures present, both terminal and intercalary. Structures not quite resembling chlamydo spores.	Further budding structures evident, but no free spores evident.
RC 13	Large amounts of mycelial growth, white, no pigmentation evident. No budding structures present. Growth was large (8mm diam.) flocs.	Same growth characteristics noted except for increased floc diameter to 10mm.
RC 33	Olive green mycelia, some budding structures present, both terminal and intercalary. Structures not quite resembling chlamydo spores.	Further budding structures evident, but no free spores evident

The spore-like budding growths observed were olive green in pigmentation, had 2-3 transverse divisions and occasionally a longitudinal division, but did not form free spores within the incubation time studied. They seemed to resemble immature chlamydo spores of *P. chartarum*.

5.3 DISCUSSION

The biomass concentration achieved was clearly the greatest in strain RC 13, but this result should be viewed with caution when regarding potential use as a biocontrol agent, due to observations of the fungus during growth. The growth of this strain was devoid of any pigmentation or hyphal budding characteristic of the other strains. This strain also produced large (10mm diameter) flocs during growth, white in colour. This observation could account for the lower colony viability observed, due to absence of the hyphal budding chlamyospore-like structures observed with pigmentation of the other strains. The colonies formed from the viability count could be assumed to be from mycelial fragments alone and did not form spores or pigmentation. This inability to produce spores upon application of homogenate to pasture is a severe limitation to its usefulness in the field. It has been proposed that the reason for this switch to mycelial growth is successive sub-culturing (Roger Collin, personal communication). However the production of sporulating RC 13 from stock cultures proved to be futile also.

The normal dark olive-green pigmentation with associated hyphal budding structures was observed during growth of strain RC 33. The larger amount of growth of RC 33 could also have led to higher viability counts with increased homogenisation of the greater biomass concentration. Strain RC 3, the strain used in the growth studies so far, had the smallest biomass of the three strains, but still produced viable unit counts comparable with strain RC 33. The absence of the pigmentation and hyphal budding in RC 13 could account for the lower viability counts obtained. The presence of these hyphal budding chlamyospore-like structures in shake-flask culture supports previous research by Dingley *et al* (1962) reporting spores being present when the growth rate was decreasing. The typical spores of *P.chartarum* on agar plates had similar morphological characteristics to the hyphal budding structures observed in the liquid medium. These budding formations resembled "immature" *P.chartarum* chlamyospores, exhibiting their septation but with less pigmentation, smaller size (approx. 4/5 the size), and thinner walls.

5.4 CONCLUSION

From these experiments it is recommended that strains RC 3 and RC 33 be used together in the application as a biocontrol agent for facial eczema. The use of both these strains would increase the chances of success in the field, particularly with RC 3 being the sporidesmin-negative strain that fell into a positive group. The use of RC 13 at this stage would seem unlikely until the problem of non-sporulation can be solved to allow the organism to grow beyond its application rate and self-propagate. The use of RC 13 would only have short-term effects due to its inability to sporulate. Whether this would occur on complex media however, i.e. pasture, has yet to be determined.

CHAPTER 6 WHEY PERMEATE TRIALS

6.1 INTRODUCTION

With the development of the optimised semi-defined medium which supported adequate growth and propagule formation of *P.chartarum* in the previous section, attention now turned to the varying of the nutrients in a directed way and the assessment of their impact on growth and viability of the organism. The optimised defined medium served as a nutritional framework from which a production medium could be formulated. In a production medium, the nutritional components of the defined medium are replaced with low-cost, complex substrates. In this section, therefore, the use of whey permeate alone and in association with other carbon sources was investigated as a substrate for strain RC3. The typical composition of lactic casein whey permeate is given by Maddox *et al.* (1993).

Two experiments were performed with whey permeate as the basal medium. The first experiment was run with the addition of glucose at two different concentrations (30g/L and 10g/L) and separately, the addition of casamino acids at two different concentrations (3g/L and 1g/L). In addition whey permeate was tested alone at initial pH values 4.5 and 6, with the latter pH adjusted to this value using NaOH. This was to simulate cheese whey permeate, also obtainable commercially. The whey permeate medium was prepared by dissolving 60g of dried acid whey permeate powder in 1L of RO water, (giving a constitution typical of liquid whey permeate, with a concentration of lactose at 45g/L). After inoculation, biomass, substrate concentration and CFU's were determined after 3 and 5 days incubation. Duplicate biomass and substrate readings were taken. For the second experiment, an investigation of cheaper nutrient sources than glucose and casamino acids was made. Thus starch and sucrose were added separately to whey permeate at concentrations of 5, 10 and 20g/L. Biomass, substrate concentration and CFU's were

determined after 5 days incubation only. Duplicate biomass and substrate readings were taken.

6.2 RESULTS

6.2.1 WHEY+GLUCOSE/CASAMINO ACIDS

The growth and viability counts of *P. chartarum* are shown in figures 24 to 25. Using whey permeate without any supplementation, the biomass concentrations of the organism at initial pH values of 6 and 4.5 were shown to be similar, with values of 2.5 and 3g/L after three days and 2.7 and 3.5g/L after five days, respectively. The viability counts were also similar but as only single CFU readings were taken for this count, their accuracy should be viewed with some caution. The whey+glucose media provided the greatest biomass concentration with values approaching 10g/L; the lower concentration of glucose gave a similar level of biomass production to the high concentration. The viability counts observed when the whey permeate was supplemented with glucose were much higher than had been obtained previously using the glucose/casamino acids media, with values of the magnitude of 3×10^6 CFU's/ml. The whey+casamino acids medium provided some high levels of biomass production particularly with the higher casamino acids concentration, giving values of 4.0 and 7.7g/L after three and five days incubation, respectively. The viability counts on this substrate were again higher than in previous experiments, of the magnitude of between 1 to 2×10^6 CFU's/ml, with the higher concentration of casamino acids having higher values.

The extent of lactose utilisation from the whey permeate at an initial pH value of 6 was greater than that at pH 4.5. The lactose utilisation in the medium containing casamino acids was greatest at the higher concentration (3g/L) (Table 9). When glucose was added to whey permeate, this sugar was well utilised, with virtually all of the 10g/L supplied being used after 3 days incubation thereafter, 5.1g/L of the lactose was utilised. At the

higher glucose concentration in the whey permeate, lactose was utilised while glucose was still present, and to a similar extent (Table 10).

Table 9 Concentration of lactose utilised (g/L) by *P.chartarum*

	WHEY PERMEATE pH 4.5	WHEY PERMEATE pH 6.0	WHEY PERMEATE+ 3g/L CASAMINO ACIDS	WHEY PERMEATE+ 1g/L CASAMINO ACIDS
3 days	-0.5	5.6	0.7	5.1
5 days	7.0	10.2	9.6	6.1

Table 10 Concentrations of glucose and lactose utilised (g/L) by *P.chartarum*.

Carbon source	WHEY PERMEATE+30g/L GLUCOSE		WHEY PERMEATE+10g/l GLUCOSE	
	[Glucose]	[Lactose]	[Glucose]	[Lactose]
3 days	8.0	9.5	9.9	1.3
5 days	12.4	10.5	9.9	5.1

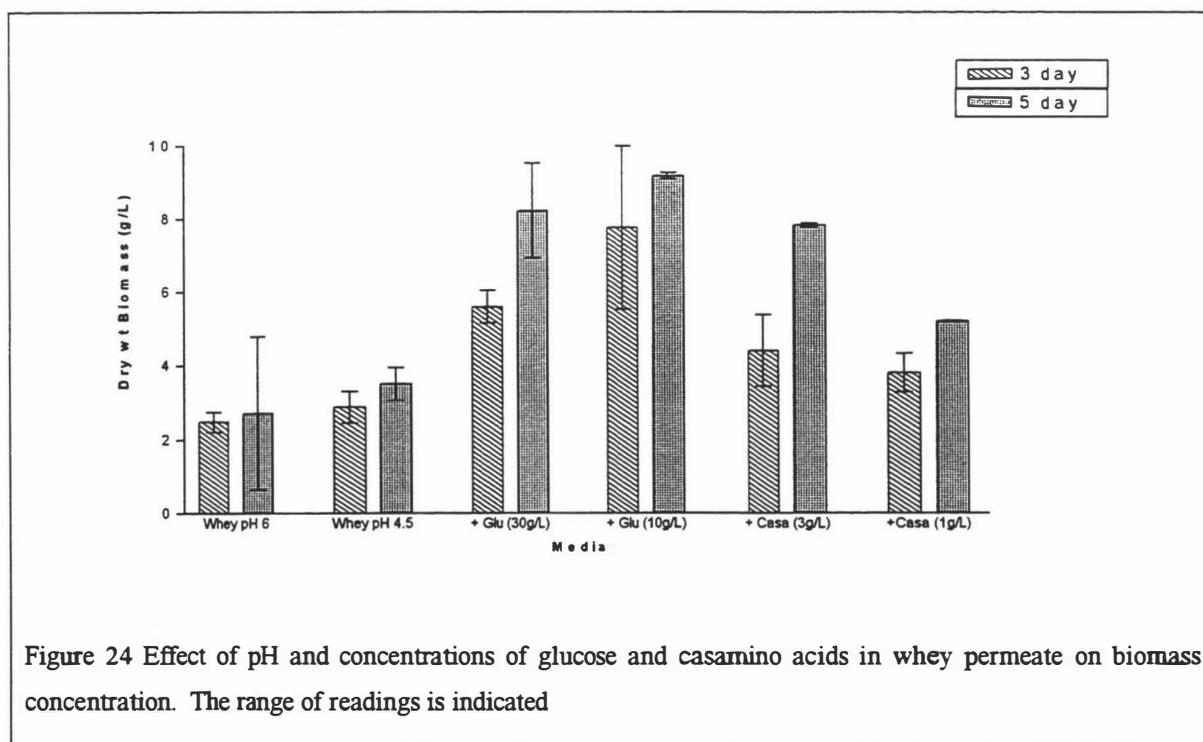


Figure 24 Effect of pH and concentrations of glucose and casamino acids in whey permeate on biomass concentration. The range of readings is indicated

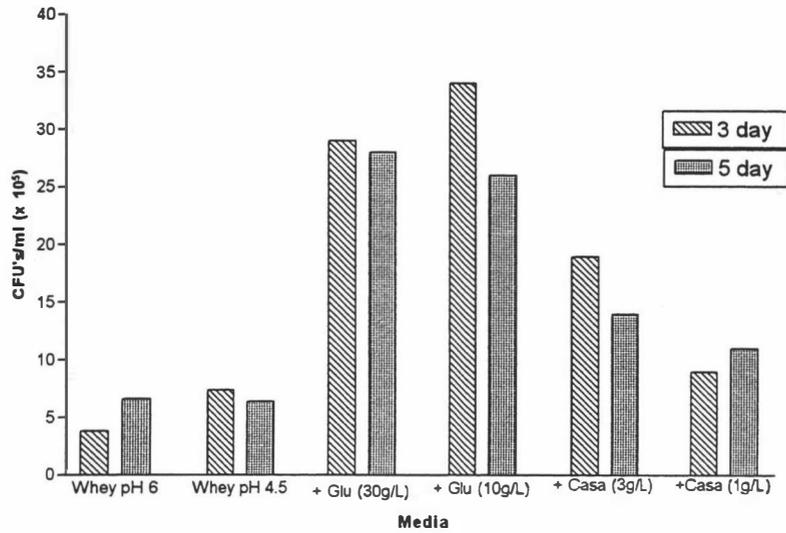


Figure 25 Effect of pH and concentrations of glucose and casamino acids in whey permeate on viability counts. The points shown are from single CFU determination only

6.2.2 WHEY+STARCH/SUCROSE

With the good growth and viability obtained from the studies of growth on whey permeate+glucose and whey permeate+ casamino acids, it was decided to look for cheaper sugar sources. The preliminary carbon source screening experiments (section 4.2.2) had revealed that starch and sucrose supported good biomass production. The biomass concentration and viability counts from the present experiments are given in figures 26 and 27. The biomass produced from the whey permeate supplemented with starch shows an approximate linear relationship, i.e. increasing biomass with increasing initial starch concentration, or more accurately from Fig. 28, the starch used versus biomass produced. The medium containing whey permeate supplemented with 20g/L starch gave 9g/L biomass. Using a sucrose supplement, however, a reverse relationship was seen, with 5g/L sucrose giving a biomass of 11g/L, 10g/L sucrose giving 10.5g/L and 20g/L giving 8.5g/L biomass. The viability counts showed the same trend in the starch medium with 20g/L giving the highest count of 3.5×10^6 CFU's/ml. The viability from sucrose supplementation was maximum at 10g/L with a viability count of 2.5×10^6 . The 20g/L sucrose medium had

the lowest reading of 1×10^6 CFU/ml, but this was still markedly higher than the count from whey permeate alone.

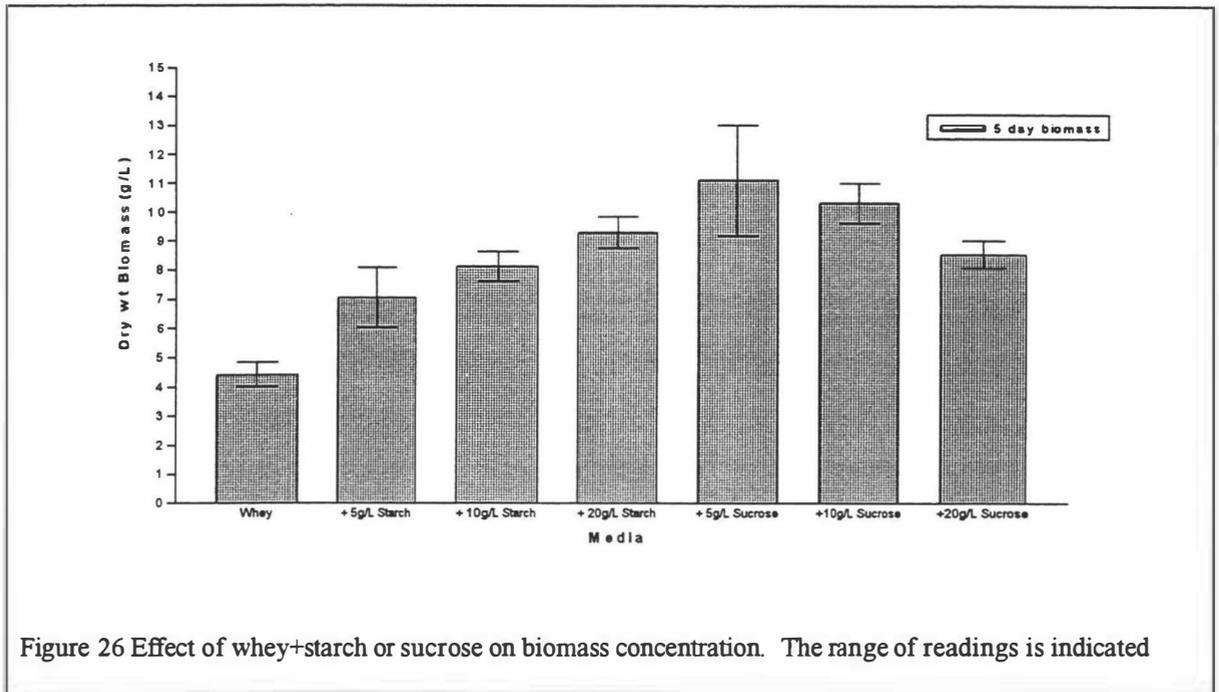


Figure 26 Effect of whey+starch or sucrose on biomass concentration. The range of readings is indicated

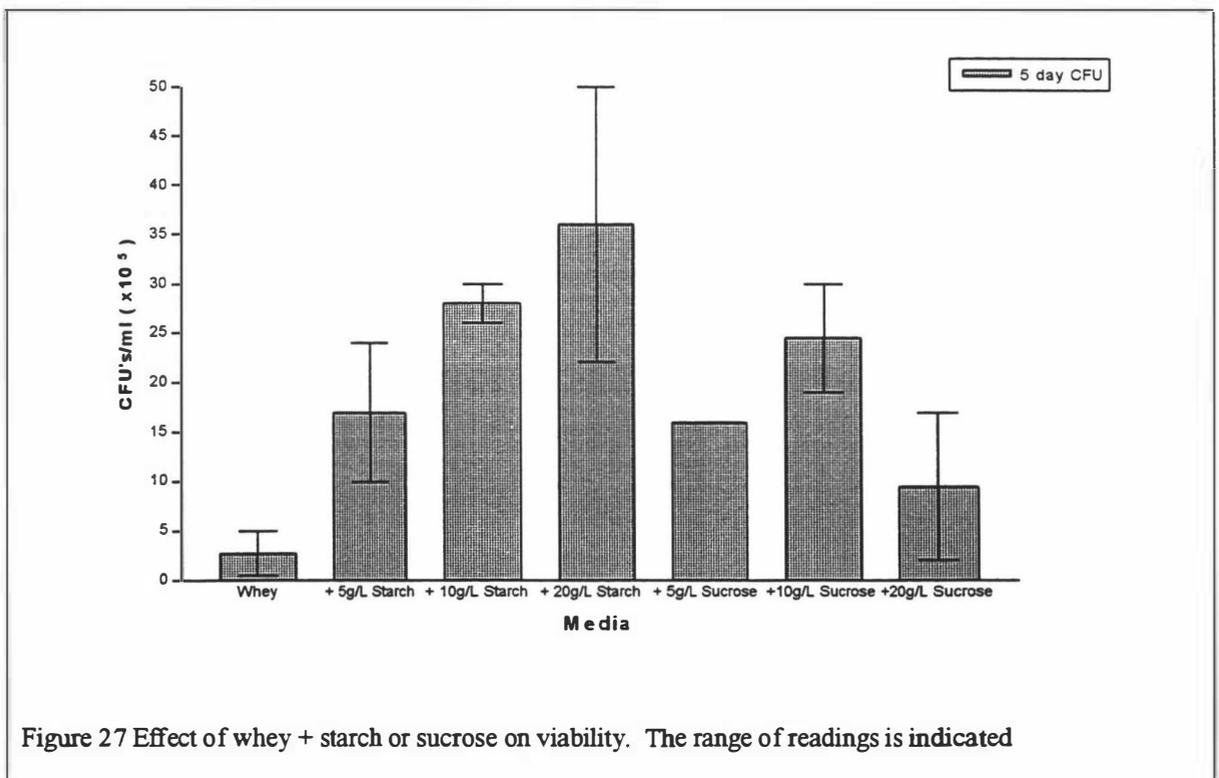


Figure 27 Effect of whey + starch or sucrose on viability. The range of readings is indicated

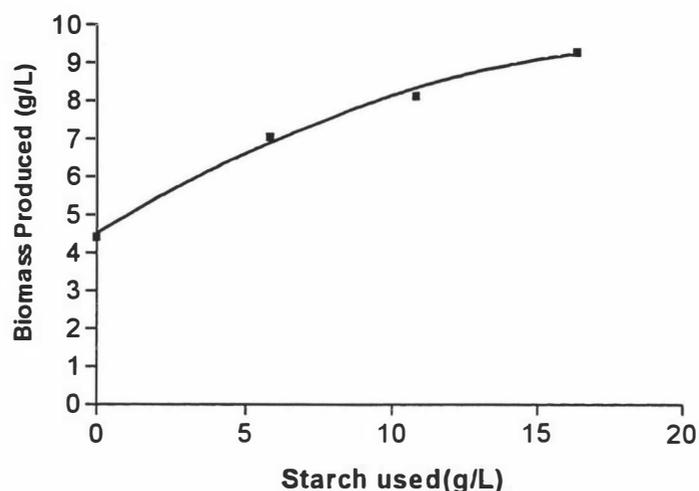


Figure 28 Starch used versus biomass produced after 5 days incubation.

The organism grew well on starch, with virtually all the starch utilised in the lowest concentration medium. The higher concentrations of starch were also the chiefly utilised carbon source, with 8.7g/L and 13.6g/L being used by the organism. The lactose from the whey permeate was used to a lesser extent with values between 0.9 and 2.7g/L for the three media, (Tables 11 and 12). The sucrose supplements were also well utilised, with 4.7, 9.4 and 13.6g/L of sucrose being used from the lowest to highest concentrations. Lactose usage from whey permeate was only found with the 5 and 20g/L sucrose supplementation, using 2.5 and 3.1g/L respectively.

Table 11 Starch and lactose concentrations (g/L) utilised by *P.chartarum*

Carbon source	WHEY PERMEATE	WHEY PERMEATE + 5g/L STARCH		WHEY PERMEATE + 10g/L STARCH		WHEY PERMEATE + 20g/L STARCH	
	[Lactose]	[Starch]	[Lactose]	[Starch]	[Lactose]	[Starch]	[Lactose]
5 days	4.9	4.9	0.9	8.7	2.1	13.6	2.7

Table 12 Sucrose and lactose concentrations (g/L) utilised by *P. chartarum*.

Carbon source	WHEY PERMEATE + 5g/L SUCROSE		WHEY PERMEATE + 10g/L SUCROSE		WHEY PERMEATE + 20g/L SUCROSE	
	[Sucrose]	[Lactose]	[Sucrose]	[Lactose]	[Sucrose]	[Lactose]
5 days	4.7	2.5	9.4	0.0	13.6	3.1

6.3 DISCUSSION

The results obtained for the growth of *P. chartarum* on unsupplemented whey permeate at initial pH 4.5 and 6 were not quite what was expected. The earlier studies (section 4.4) had revealed an optimum initial pH value of around 6 and so the result of better growth and viability at the lower pH value was unexpected. However, the pH value during growth of the organism did not change as markedly (data not shown) with the whey medium as it did with the pH optimisation medium. This could explain the discrepancy between the two experiments, with the whey media having a better buffering capacity than the glucose/casamino acids medium, and thus the pH not dropping to inhibitory levels during growth of the organism.

It had been earlier shown that glucose and casamino acids were readily utilised as carbon and nitrogen sources, respectively, by *P. chartarum*, so the use of whey permeate to supply either of these nutrients was investigated. The performance seen on the whey permeate+glucose and whey permeate+casamino acids revealed that these substrates stimulated the growth compared with whey permeate alone, and, indeed, out-performed the optimised medium used in the earlier experiments. The point that is illustrated by this, is that whey permeate is better than casamino acids during growth on glucose, and whey

permeate is better than glucose during growth on casamino acids. When grown on whey containing casamino acids, the organism possibly utilised the latter as a carbon source. This leads to the idea that whey permeate is an excellent nitrogen source, and so finding a good carbon source to add, since lactose is a poor source, at least in the initial stages of growth, is the next step. The robust nature of the growths formed, and the homogenisation procedure subsequently not being fully optimal, could explain the trend of lower viability counts on the fifth day of incubation compared with the third. The trend of complex media providing more rigorous growth is not a new concept in biotechnology, and has been previously recorded with *P.chartarum* (Ross, 1960, Dingley *et al*, 1962).

The revelation of whey permeate in conjunction with other carbon sources providing the highest biomass and viability count so far, encourages further work on finding cheaper alternatives to glucose and lactose. The use of starch and sucrose gave higher biomass and viability values of all the experiments so far, particularly the high viability counts, being in the area of magnitude 3×10^6 CFU's/ml. The reverse trend observed when using sucrose, with lower concentrations giving higher biomass, can be hypothesised to be due to a biphasic growth characteristic of *P.chartarum*. This is only a theoretical account for this phenomenon and is a minor factor in regard to the optimisation strategy. Further research could be conducted to better understand this. The viability count when the whey was supplemented with 5g/L sucrose was only a single reading and therefore should be viewed with caution. It should also be noted that the biomass did not appear fully homogenised, and so, again, the homogenisation procedure was not optimal and higher viability counts could possibly have been obtained. Despite this, supplemented whey permeate is a sufficiently productive complex medium to warrant further investigation.

6.4 CONCLUSION

The experiments conducted here revealed some very promising data on the medium for which to base commercial production of *Pithomyces chartarum* in submerged fermentation.

Whey permeate on its own is not a very productive medium. However whey permeate when supplemented with an alternative carbon source is an excellent medium for the growth of *P. chartarum*.

The utilisation of whey permeate provided high biomass and viability counts when used in association with starch or sucrose. Either of these would seem to be an ideal medium with which to start small-scale fermenter pilot trials.

CHAPTER 7 FERMENTER TRIALS

7.1 INTRODUCTION

Attention now turned to other fermentation variables, and to growth of the organism in submerged fermentation. Aeration and agitation are two important factors to consider when growing a microorganism in submerged culture. The purposes of aeration and agitation in fermenters are, firstly, to supply microorganisms with oxygen, and secondly, to mix fermentation broths in such a way that a uniform suspension of microbes is achieved and the mass transfer rate any metabolic product is accelerated. Many fermenters are equipped with impellers for the mechanical agitation of broths, to disintegrate air bubbles and to intensify the turbulence of the liquid, but fermenters without mechanical agitators are also used, as in some aerated activated sludge processes (Aiba *et al*, 1973).

Agitation and aeration can be viewed from two aspects; the demand for oxygen by the microbes, and the supply of oxygen from air bubbles to the liquid. Fungal fermentations are generally more viscous than bacterial fermentations and, there can therefore be difficulty in maintaining a supply of oxygen, especially with changes in microbial oxygen demand which are liable to occur with changing dissolved oxygen concentration. The rational design of aerators for Newtonian liquids is possible, but in the case of calculating oxygen transfer to non-Newtonian liquids, such as concentrated mycelial broths, many problems are incurred. Thus it is desirable to establish the effects of dissolved oxygen levels on the growth parameters of the organism.

Another variable that needs to be addressed is the effect of inoculum size. In particular, the effect of this parameter on the morphology of the growth of the organism needs to be established. Smaller inocula may result in there being less competition for the medium resources from each individual propagule, and these spores give resultingly larger

pelletal growths. Hence in the fermenter trials described in the present chapter, the effects of aeration, agitation rate and inoculum size were investigated. To reduce the number of fermenter runs to be made, the aeration rate was maintained constant since in a bench-scale fermenter it is the agitation rate that has the largest influence on the dissolved oxygen concentration. Thus, the aeration of sterile filtered (0.45 μ m) air was kept at a constant rate of approximately 0.5L/min, while the agitation rate was varied between 150rpm and 250rpm. The spore inoculum was also set at two levels, 10³ spores/ml and 10⁴ spores/ml. The trial set-up is given in the table below.

Table 13 Conditions used in fermenter studies

VARIABLE	PROTOCOL 1	PROTOCOL 2	PROTOCOL 3	PROTOCOL 4
Aeration	0.5L/min	0.5L/min	0.5L/min	0.5L/min
Agitation	150rpm	150rpm	250rpm	250rpm
Inoculum	10 ³ spores/ml	10 ⁴ spores/ml	10 ³ spores/ml	10 ⁴ spores/ml

The medium used for this experiment was the glucose/casamino acids medium with added trace elements and growth factors. This medium was used since the experiment was performed before the growth factors and trace elements were found to be excess to requirements in the shake flask studies. The medium was adjusted to pH 4.5 using HCl to provide protection against bacterial contamination, following initial fermentation runs being problematic in this respect. The aeration and agitation rates were decided upon from subjective visual analysis of the sparging air at the various settings. Due to supply problems in obtaining a dissolved oxygen probe, the experiments were performed without it. The intention was that the best protocol, in terms of biomass production and viability counts, would be repeated upon receiving the probe to identify the dissolved oxygen level that produced the best results.

The final fermentation investigated used a medium of whey permeate + sucrose (5g/L) to gain information on the performance of this substrate in a small-scale fermenter with aeration and agitation.

7.2 RESULTS

The concentrations of biomass produced were similar in all four experimental protocols (Figure 29), and were in the range of 4 to 5g/L after 5 days incubation. There were only single readings obtained for protocols 1 and 3 due to bacterial contamination during the latter stages of the fermentation. The viability counts (Figure 30) were somewhat more varied however, with a highest count of an average of 18×10^5 CFU's/ml. On a cautionary note, though, these conditions also provided the largest range in counts (8×10^5 to 28×10^5). These fermentation conditions (protocol 4) were selected as the conditions under which to run the fermentation again, and to measure the dissolved oxygen concentration. Unfortunately technical difficulties with the dissolved oxygen probe resulted in no reliable readings. However, the resulting biomass and viability count in this experiment were of 5.24g/L and 6×10^5 CFU's/ml, respectively.

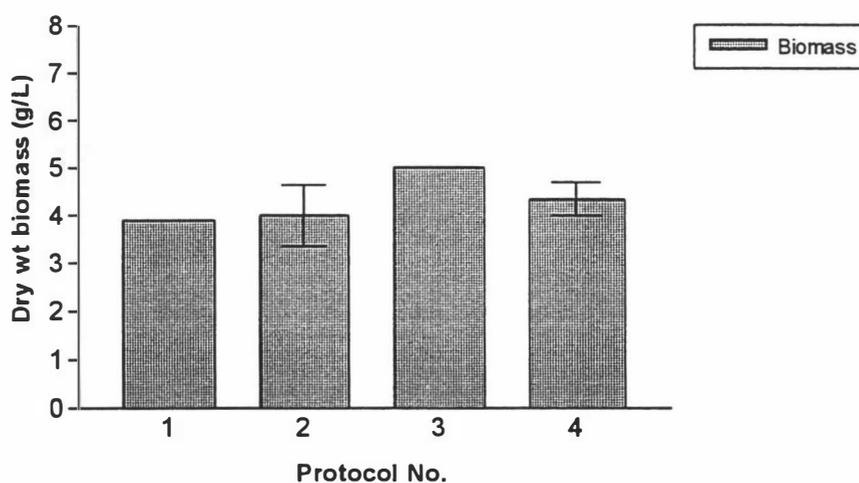


Figure 29 Biomass concentration produced during fermenter experiments. Data shown after 5 days incubation. The range of readings is indicated

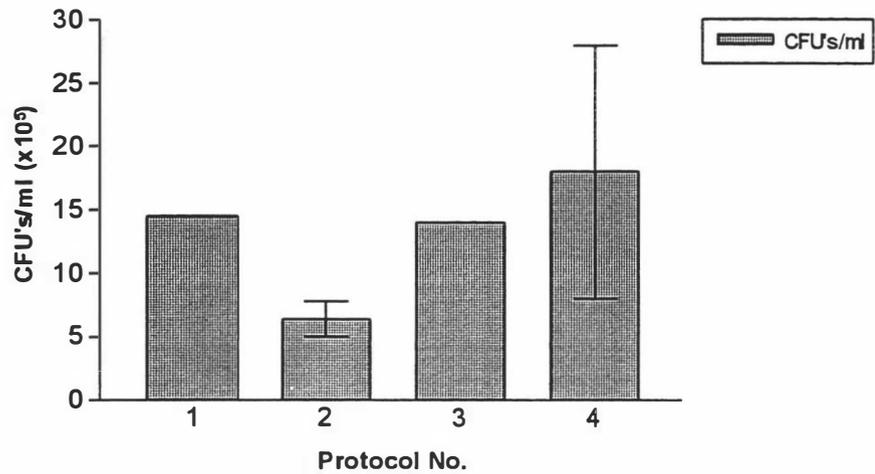


Figure 30 Viability counts from fermenter experiments. Data shown after 5 days incubation

The utilisation of glucose and nitrogen from the fermenter medium is given in figure 31 below, these are the average readings from all the fermentations. The glucose concentration decreased from around 30g/L to approximately 20g/L over the time of fermentation.

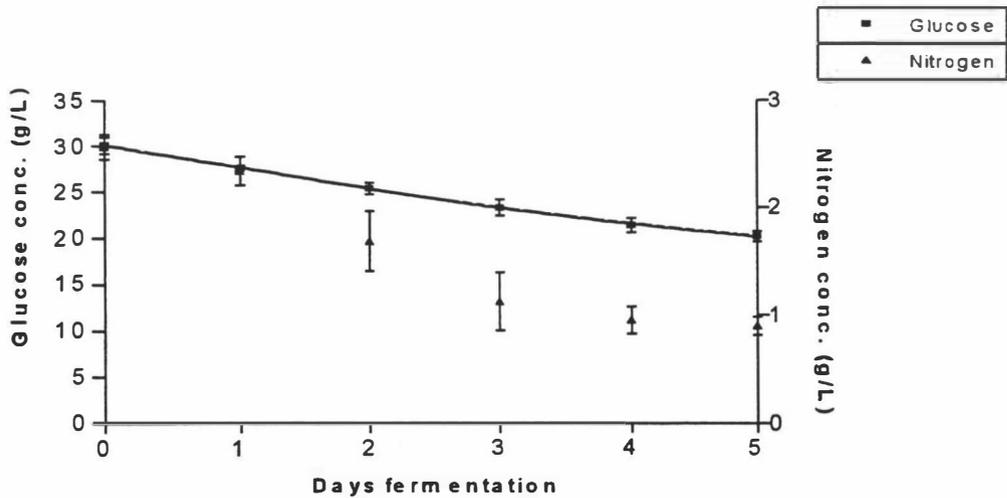


Figure 31 Average glucose and nitrogen concentration over time in four different fermentation protocols.

The experiments conducted with whey permeate+sucrose were unsuccessful due to technical problems with medium foaming and subsequent contamination. The solution to these problems would involve automatic addition of anti foam. Unfortunately, this facility was not available on the equipment being used.

7.3 DISCUSSION

The results of this experiment show that there was little effect of the agitation or inoculum size on the biomass produced during growth of *P. chartarum* in small-scale fermentation, at least in the ranges tested. The effects on viability counts showed no clear trend. The smaller inoculum size gave values of approximately 15×10^5 CFU's/ml while the larger size gave readings of 5×10^5 and 28×10^5 /ml. The higher agitation rate may have been expected to give higher viability counts, but given the wide range of results, this effect is unclear. In terms of finding the optimum conditions for the fermenter, considering both variables together, then the higher agitation speed with higher inoculum size provided the highest biomass concentration and viability count when taking into account the results of the repeated protocol 4. The range of difference between each individual treatment was small and that there was no discernable difference between them. The substrate utilisation data showed little variation in glucose utilisation in any of the experimental protocols. The larger variation in nitrogen utilisation can be accounted for by the inaccuracies of the kjeldahl method for determining nitrogen. The investigation of the effect of inoculum size should perhaps be investigated on a shake flask scale, before switching to a small-scale fermenter.

Comparing the performance of the fermenter with the previous chapter's (section 4.2.3) work (Table 14), we see that much less biomass was produced in the fermenter after 5 days incubation, than was produced in the shake flasks, using the same medium with the exception of the initial pH value being 4.5 in the fermenter. The yield of the organism in shake-flask was twice that in the fermenter. The viability counts, however, were similar or slightly greater in the fermenter.

Table 14 Comparative performance of *P.chartarum* in shake-flask and fermenter incubation. Fermenter data shown are average of all protocols.

PARAMETER	SHAKE FLASK	FERMENTER
Biomass concentration (g/L)	9.0	4.3
Glucose used (g)	11.1	9.9
Yield $Y_{x/s}$	0.8	0.4
Nitrogen used (g)	2.3	1.7
Viability count (CFU's/ml)	8×10^5	12×10^5

There are several possible explanations for the greater biomass concentration and yield exhibited by the shake flask cultured organism. Firstly, the decrease in pH with time (data not shown) to levels approaching pH 3 in the fermenter, a value shown in earlier experiments to be inhibitory, would be one of the more obvious causes for poorer performance. This problem could be solved by either setting the initial pH at the higher value of pH 6.0, as used in shake flask culture, or by using a pH control system to prevent the value decreasing to far below pH 4.5. The reason for operating at pH 4.5 in the fermenter in the present work was to minimise any bacterial contamination. Secondly, the dissolved oxygen level in the fermenter may have not been sufficient for optimal growth of the organism. Thirdly, agitation rates used may have caused too high a local shear rate within the fermenter, breaking up the mycelium as it grew. The similar substrate utilisation values shown in both types of culture, but the less biomass produced in the fermenter, could indicate cell damage and lysis in the fermenter, with resulting lower biomass.

Probably, the culture pH value was the likely reason for the poorer performance in the fermenter culture.

7.4 CONCLUSION

The size of the spore inoculum, in the range 10^3 - 10^4 /ml, had little effect on the biomass produced or the colony-forming-ability of the organism grown in a small-scale fermenter.

The effect of the agitation rate, in the range 150-250rpm, had no major effect on the performance of the organism. However, further work is required to clarify the effect of the dissolved oxygen concentration.

CHAPTER 8 STORAGE TRIALS

8.1 INTRODUCTION

The final studies performed on the optimisation of conditions for the growth of *P.chartarum* were the viability of the organism during storage. The ability of a biocontrol agent to have a reasonable shelf-life is an important criterion in meeting commercial requirements. The use of sugars as preserving agents in the preparation of mycelial and spore biocontrol agents is a common practice. The use of starch and sucrose is an example of treatment of spores or mycelium for protection; the starch acts to 'autoencapsulate' the biocontrol agent (forms a thin film over the material) and the sugar acts as a sticking agent, preventing peeling off after drying. The experiments here were designed to assess the best time of incubation for harvesting of the organism, to assess the effect of storage at room temperature of the harvested biomass on viability, and to assess whether application of sugar solutions to the harvested biomass before storage improved its viability. The harvesting/storage procedure was performed as follows:

The flasks were harvested for each treatment after 4, 6, 8, and 10 days growth. This was to identify the best time of incubation for optimal harvest. The medium used for these experiments was the glucose (30g/L) + casamino acids (3g/L) in basal salts solution with added growth factors and trace elements. At harvest, the contents of two flasks were filtered using sterilised miracloth filter paper and a Buchner funnel under vacuum. This mycelium was rinsed with 100ml sterile RO water to remove residual medium. The miracloth+mycelium was then immersed in a sugar solution (5%) for 30 min, the miracloth and mycelium were then placed back under vacuum until filtrate stopped flowing. The mycelium was then scraped off the miracloth into sterile petri dishes. These dishes were dried under vacuum with silicon desiccant at room temperature for four days. Following the drying procedure, half of the dried mycelium samples were suspended in 200ml sterilised peptone water (5g/L) and homogenised for the CFU determination procedure.

The remaining dried samples were sealed with parafilm in sterile petri dishes and stored at room temperature in the dark for 3 months to await viability measurement. Penicillin G (1580 iu/mg) was added to PDA plates for the viability assessment of stored felts. Ethanol sterilised forceps and spatulas were used throughout the procedure. The sugar treatments that each harvested biomass was subjected to were lactose, trehalose or a water control. Trehalose was chosen as a preserving agent because of its proven use in this regard, while lactose was selected for its cheaper cost, making it a possible choice for commercial application.

8.2 RESULTS

The viability counts from the three different sugar treatments are given in tables 15 to 16 below. Viable counts were obtained after 3 months of storage, but only for those harvested after 6 and 10 days of growth of the organism, with the exception of the 10 day trehalose-treated felt. The counts obtained were in the range of 8×10^2 to 3×10^3 CFU's/ml and were generally lower than those measured immediately after the drying process.

Table 15 Control (water treated) viable unit counts

Days Incubated	4	6	8	10
Storage control	9.5×10^4	1.1×10^3	4.9×10^5	2.5×10^5
3 months storage	0	1.8×10^3	0	1.6×10^3
Observations	These felts were quite hard and dry and were hardly broken down by blending. Moderate contamination evident	These felts were quite hard and dry and were hardly broken down by blending. Moderate contamination evident	Moderate to high bacterial contamination in control, high contamination on storage plates. The stored felts were still quite wet after drying procedure. The wetness of these felts could have allowed bacterial growth, with resultant mycelial lysis	Moderate to high bacterial contamination on control, high contamination on storage plates. The stored felts were still quite wet after drying procedure. The wetness of these felts could have allowed bacterial growth, with resultant mycelial lysis

However it should be noted that for mycelia harvested after 6 days of growth, the viability count after 3-months storage was rather similar to that before storage. The latter count, however was lower than expected.

Table 16 Lactose treatment viable unit counts

Days Incubated	4	6	8	10
Storage control	4.3×10^4	9.5×10^3	3.0×10^5	3.6×10^5
3 months storage	0	3.0×10^3	0	9.9×10^2
Observations	Storage control had moderate bacterial contamination,	Storage control had moderate bacterial contamination,	Storage control had moderate bacterial contamination. High bacterial contamination, high felt moisture content possibly accounts for mycelial lysis in stored felts.	Storage control had moderate bacterial contamination. High bacterial contamination, high felt moisture content possibly accounts for mycelial lysis in stored felts.

Table 17 Trehalose treatment viable unit counts

Days Incubated	4	6	8	10
Storage control	3.6×10^4	9.5×10^3	3.2×10^5	2.4×10^6
3 months storage	0	7.9×10^2	0	0
Observations	Moderate bacterial contamination, <i>P. chartarum</i> colonies from this treatment were smaller than usual in size	No bacterial contamination in storage control	Low contamination in storage control. High bacterial contamination, high felt moisture content possibly accounts for mycelial lysis in stored felts.	Storage control had moderate bacterial contamination, High bacterial contamination, high felt moisture content possibly account for mycelial lysis in stored felts.

8.3 DISCUSSION

The results show that using the harvesting and storage methods detailed above, the viability of *P. chartarum* decreased over a three month period to such an extent that the viable count found after the standard homogenisation procedure was considerably less than that prior to storage. The treatment of the mycelial felts with the sugar solutions had no significant effect on the viability after storage. Nevertheless, it may be suggested that the optimum time of harvesting the mycelium for retention of viability would be either after 6 or 10 days growth. Some observations that were made during these experiments may be beneficial in future work. Some thought should be given to the moisture content of the stored mycelial felts. The harvested felts from shorter incubation times were smaller in biomass, and the drying procedure removed more of the mycelial and medium moisture. This seemed to have two effects. Firstly, the felts obtained after 4 days incubation were dried to a hard, crust-like consistency. These felts were hardly broken up by the homogenisation procedure, giving less fragmented biomass for propagation. Secondly there may have been too much moisture removed from the felts, possibly inactivating or killing the cells/chlamydo spores.

As the incubation time was increased to 6 days, greater biomass was produced, and the drying regime used in this experiment produced a felt, which was still relatively moist, but structurally weak enough to be broken down by homogenisation. The production of still larger amounts of biomass after 8 days incubation produced felts that were still heavy with moisture. This higher moisture content could have allowed heavy growth of contaminating microorganisms that were visible on the viability plates, which may have caused the lysis of *P. chartarum* cells, but there may also have been some autolysis of the fungus itself. The resurrection of viability in felts harvested after 10 days incubation may have been due to the fact that by this stage there was enough growth present of the organism, that the detrimental factors present (lysis by contaminants) were simply overcome by sheer weight of numbers by *P. chartarum*.

8.4 CONCLUSION

The viability of *Pithomyces chartarum* was shown to decrease during storage at room temperature, and the effect of the sugar solutions on maintaining viability was negligible.

The ideal time of harvest of the organism was unclear, and the residual moisture content after the drying procedure could be a crucial factor in obtaining viable units after storage.

Further research needs to be done in perfecting the harvesting procedure to maintain sterility and the harvesting/drying procedure in general.

CHAPTER 9 CONCLUSIONS AND RECOMMENDATIONS

This study has evaluated the growth characteristics and viability of an atoxigenic strain of *P. chartarum* when grown on a wide range of substrates and medium conditions. In particular, critical factors for the development of a biocontrol agent from the growth of this organism in submerged fermentation have been identified.

The initial part of the study involved determining the best carbon sources, nitrogen sources, initial culture pH value and effects of trace metals and growth factors for maximum biomass concentration and viability count. It was found that the organism was able to use a range of carbon sources, of which the best of those tested would be any of glucose, sucrose or starch. Time course studies were conducted using glucose and lactose as carbon sources to identify growth parameters of the organism, during which it was revealed that the performance on glucose was, in general, superior to that on lactose. The utilisation of nitrogen source was also wide ranging, with the best sources being the organic nitrogen sources of casamino acids and peptone. There were indications that inorganic sources could also be effective provided that there was some form of pH control of the medium. This led to defining the optimal pH of the organism, which was found to grow over a wide range of pH values (3-7), with an optimum of around pH 6. The results of adding growth factors and trace metals to the glucose/casamino acids, medium revealed that their effect on the biomass concentration and viability of the organism was negligible. This implied that the glucose/casamino acids medium provided sufficient trace elements and that *P. chartarum* can synthesise its own growth factors. Further work in this area includes such factors as the carbon to nitrogen ratio which is often an important factor in the sporulation of fungi, and the growth of the organism in inorganic nitrogen sources with pH control.

Comparison of the growth parameters of three different atoxigenic strains *P. chartarum* was performed. Of the three strains tested, it was found that RC13, while producing the greatest biomass concentration was unable to sporulate from the viable unit counts, and hence would grow only from mycelial fragments. This phenomenon could effectively eliminate the strain from commercial application, until the problem of non-sporulation can be solved, thus allowing the organism to grow beyond its application rate and self propagate. The strains RC3 and RC33 did not have this problem, however, and their application together as a biocontrol agent is recommended. The use of both strains is recommended as it increases the genetic diversity of the treatment, and its chances of success in the field. Future work to be conducted with these strains includes trials with strains RC13 and RC33 in fermenter culture and in the whey permeate+supplements medium to assess their performance under these conditions.

With the establishment of the optimised semi-defined medium, attention turned to the development of a production medium. Thus, replacement of the nutritional components of the defined medium with low cost complex substrates was investigated. Whey permeate was assessed for its productivity. It was shown that whey permeate on its own is not a very productive medium; however, when it was supplemented with an alternative carbon source of either casamino acids or glucose, the resulting biomass and viability counts were the highest yet obtained. With this information, cheaper carbon supplements of starch and sucrose were tested. The biomass concentration and viability counts obtained were higher still than on whey permeate+glucose or whey permeate+casamino acids. The best medium from these experiments was the whey permeate+sucrose (5g/L), although either of sucrose- or starch-supplemented media should be further investigated in small-scale fermenter trials. Time course studies to investigate the rate of use of the supplements and whey permeate lactose is an aspect which could be further investigated. The use of the whey+starch or sucrose medium has additional promise for the fact that in a production scale, the use of the unused starch, sucrose or lactose could have further uses downstream of the fermenter, to be used in the storage treatment of the harvested biomass.

The effects of aeration, agitation and inoculum size were studied in submerged fermenter culture. Neither the size of the spore inoculum nor the agitation rate, in the ranges investigated, had a major effect on the performance of the organism. However, further work is needed to identify the effect of dissolved oxygen concentration. pH control should also be addressed, as should foaming control when using whey permeate-based media. The effect of inoculum size should be further assessed at a greater range of spore loads.

Finally, the effect of storage on the viability of *P.chartarum* biomass was investigated. The organism was harvested after various periods of growth and subjected to various post-harvest/pre-storage treatments. The results of this investigation showed that the viability of the organism decreased with storage at room temperature, and that treatment with sugar solutions proved to have negligible preserving effect. The optimal time of harvest was unclear, and the residual moisture content after drying could be a crucial factor in obtaining viable units post-storage. Further study is needed in finding a better drying procedure than the one used, or “just-in-time” management techniques should be used to minimise any storage time for the harvested biomass.

To conclude, the objective of this study, which was to determine optimal conditions for the growth of atoxigenic strains of *P.chartarum*, has been achieved to the extent of the factors studied. However, one exception is the dissolved oxygen concentration of the culture, since technical problems with the D.O. probe gave unreliable data. The best carbon source, nitrogen source and pH values were identified; the effect of supplementary growth factors and trace metals was shown to be negligible; and a possible industrial medium based on whey permeate has been identified. The fermenter experiments, while not providing data on dissolved oxygen levels, did provide insights into the performance of the organism in a small-scale fermenter, and confirmed that submerged fermentation is a suitable technology for large scale production of *P.chartarum* biomass.

REFERENCES

- Aiba, S., Humphrey, A. E., and Millis, N. F. (1973). *Biochemical engineering*, (2nd ed.), New York, Academic Press Ltd.
- Atherton, A. G., Brewer, D. and Taylor, A. (1974). *Pithomyces chartarum*: A Fungal parameter in the aetiology of some diseases of domestic animals. In *Mycotoxins*. I. F. H. Purchase (ed.). pp29-69. Elsevier Scientific Publishing Co. Oxford.
- Bertaud, W. S., Morice, I. M., Russell, D. W. and Taylor, A. (1963). The spore surface in *Pithomyces chartarum*. *Journal of General Microbiology* **32**, 385-395.
- Brook, P. J. (1963). Ecology of the fungus *Pithomyces chartarum* (Berk. and Curt.) M. B. Ellis, in pasture in relation to facial eczema disease of sheep. *New Zealand Journal of Agricultural Research*. **6** 147-228.
- Brown, D. E. (1988). The Submerged Culture of Filamentous Fungi. In *Physiology of Industrial Fungi*. D. R. Berry (ed.) pp219-248. Blackwell Scientific Publications. London.
- Brown, R. L., Cotty, P. J. and Cleveland, T. E. (1991). Reduction in aflatoxin content of maize by atoxigenic strains of *Aspergillus flavus*. *Journal of Food Protection*. **54**, 623-626.

- Camici, L., Sermoniti, G. and Chain, E. B. (1952). Observations on *Penicillium chrysogenum* in submerged culture. *Bulletin of the World Health Organization*. **6**, 265-276. Via Solomons, G. L. (1975). Submerged Culture Production of Mycelial Biomass. In *The Filamentous Fungi, Volume 1, Industrial Mycology*. J. E. Smith and D. R. Berry, (eds.) pp249-264. Edward Arnold Publishers Ltd. London.
- Charudattan, R. (1991). The mycoherbicide approach with plant pathogens. In *Microbial Control of Weeds* (ed. D.O. TeBeest), pp. 24-57. Chapman and Hall, New York, U.S.A.
- Clare, N. T. and Gumbey, J. M. (1962). Some factors which may affect the toxicity of spores of *Pithomyces chartarum*. (Berk. and Curt.) M. B. Ellis. Collected from pasture. *New Zealand Journal of Agricultural Research*. **5**, 36-42.
- Cotty, P. J. (1990). Effect of atoxigenic strains of *Aspergillus flavus* on aflatoxin contamination of developing cottonseed. *Plant Disease*. **74**, 233-235.
- Davidson, S. and Marbrook, J. (1964). The effect of temperature on the toxicity of spores of *Pithomyces chartarum*. (Berk. and Curt.) M. B. Ellis. *New Zealand Journal of Agricultural Research*. **8**, 126-130.
- di Menna, M., Cambell, J. and Mortimer, P. H. (1970). Sporidesmin production and sporulation in *Pithomyces chartarum*. *Journal of General Microbiology*. **61**, 87-96.
- Dingley, J. M. (1962). *Pithomyces chartarum*, its occurrence, morphology, and taxonomy, *New Zealand Journal of Agricultural Research*, **5**, 49-61.

- Dingley, J. M., Done, J., Taylor, A. and Russell, D. W. (1962). The production of sporidesmin and sporidesmolides by wild isolates of *Pithomyces chartarum* in surface and submerged culture. *Journal of General Microbiology*. **29**, 127-135.
- Done, J., Mortimer, P. H., Taylor, A and Russell, D. W. (1961). The production of sporidesmin and sporidesmolides by *Pithomyces chartarum*. *Journal of General Microbiology*. **26**, 207-222.
- Dorner, J. W., Cole, R. J. and Blankenship, P. D. (1992). Use of a biocompetitive agent to control preharvest aflatoxin in drought stressed peanuts. *Journal of Food Protection*. **55**, 888-892.
- Ellis, M. B. (1958). *Clasterosporium* and some allied Dematiaceae phragmosporae I *Mycology Papers* **70**, 1-89.
- Ellis, M. B. (1960). Dematiaceous Hyphomycetes. I. *Mycology Papers*. **76** 13-15.
- Facial Eczema Information Update, Facial eczema research group. Ruakura Agricultural Centre, Hamilton, New Zealand (1991) Ministry of Agriculture and Fisheries.
- Fitzgerald, J. M., Collin, R. G. and Towers, N. R. (1998). Biological control of sporidesmin-producing strains of *Pithomyces chartarum* by biocompetitive exclusion. *Letters in Applied Microbiology*. **26**, 17-21.
- 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. Via Solomons, G. L. (1975). Submerged Culture Production of Mycelial Biomass. In *The Filamentous Fungi, Volume 1, Industrial Mycology*. J. E. Smith and D. R. Berry, (eds.) pp249-264. Edward Arnold Publishers Ltd. London.

- Hebbar, K. P., Lumsden, R. D., Poch, S. M. and Lewis, J. A. (1997). Liquid fermentation to produce biomass of mycoherbicide strains of *Fusarium oxysporum*, *Applied Microbiology and Biotechnology*, **48**, 714-719.
- Hodges, R. Donaldson, J.W., Taylor, A. and White, E.P. (1963). Sporidesmin and sporidesmin B. *Chemistry and Industry*, 42-43. Via Butler, W.H.(1975) Mycotoxins in *The Filamentous fungi Volume 1 Industrial Mycology*. J. E. Smith and D. R. Berry, (eds.) pp 320-329. Edward Arnold Publishers Ltd. London.
- Hughes, S. J. (1953). Fungi from the gold coast II *Mycology Papers* **50**, 104. via Dingley, J.M. (1962) *Pithomyces chartarum*, its occurrence, morphology, and taxonomy, *New Zealand Journal of Agricultural Research*, **5**, 49-61.
- Jackson, M. A. and Schisler, D. A. (1995). Liquid culture production of microsclerotia of *Colletotrichum truncatum* for use as bioherbicide propagules. *Mycological Research*, **99**, (7): 879-884.
- Jackson, M. A., Schisler, D. A., Slininger, P. J., Boyette, C. D., Silman, R. W. and Bothast, R. J. (1996). Fermentation strategies for improving the fitness of a bioherbicide. *Weed Technology*, **10**, 645-650.
- Lewis, J. A., Barksdale, T. H., and Papavizas, G. C. (1990). Greenhouse and field studies on the biological control of tomato rot caused by *Rhizoctonia solani*. *Crop protection*, **9**, 8-14.
- Lewis, J.A and Papavizas, G. C. (1991). Biocontrol of plant diseases: the approach for tomorrow. *Crop Protection* **10**, 95-105.

- Lindow, S. E. (1987). Competitive exclusion of epiphytic bacteria by ice-*Pseudomonas syringae*. *Applied Environmental Microbiology*. **53**, 2520-2527. Via Cotty, P. J. (1990). Effect of atoxigenic strains of *Aspergillus flavus* on aflatoxin contamination of developing cottonseed. *Plant Disease*. **74**, 233-235.
- Lisansky, S. G. (1985) Production and commercialisation of pathogens. In: *Biological pest control*, pp. 210-218, N. W. Hussey and N. Scopes (eds.), Blandford Press Ltd., Poole, UK, Via Lewis, J.A and Papavizas, G. C. (1991). Biocontrol of plant diseases: the approach for tomorrow. *Crop Protection* **10**, 95-105.
- Maddox, I. S., Qureshi, N. and Gutierrez, N. A. (1993). Utilisation of whey by Clostridia and Process Technology. In *The Clostridia and biotechnology*, pp344, D. R. Woods (ed.), Butterworth-Heinemann, Boston, USA.
- Marbrook, J. and Matthews, R. E. F. (1962). Loss of sporidesmin from spores of *Pithomyces chartarum* (Berk and Curt.) M. B. Ellis. *New Zealand Journal of Agricultural Research*, **5**, 223-236.
- Mortimer, P. H. and Taylor, A. (1962). The experimental intoxication of sheep with sporidesmin, a metabolic product of *Pithomyces chartarum*. I. Clinical observations and findings at post-mortem examination. *Research in Veterinary Science* **3**, 147-60.
- Morton, A. G. (1960). The induction of sporulation in mould fungi. *Proceeds of the Royal Society B*. London. **153**, 548.
- N.Z. meat and Wool Boards' Economic Service. (1990). *Estimated cost of facial eczema to sheep production*. Paper no. T124. Wellington.

- Papavizas, G. C. and Lewis, J. A. (1989) Effect of *Gliocladium* and *Trichoderma* on damping-off and blight of snapbean caused by *Sclerotium rolfsii*. *Plant Pathology*. **38**, 277-286.
- Papavizas, G.C. and Lewis, J. A. (1981) Induction and augmentation of microbiological antagonists for the control of soilborne plant pathogens. In: *Biological Control in Crop Production*. pp305-322 (ed. G. C. Papavizas). Totowa, New Jersey: Allenheld and Osmun.
- Papavizas, G.C. Dunn, M.T. Lewis, J.A. and Beagle-Ristaino, J. (1984) Liquid fermentation technology for experimental production of biocontrol fungi. *Phytopathology*, **74**, 1171-1175.
- Percival, J. C. and Thornton, R. H. (1958). Relationship between the presence of fungal spores and the test for hepatotoxic grass. *Nature*, London. **182**, 1095-1096.
- Pereira, R. M. and Roberts, D. W. (1990). Dry mycelium preparations of entomopathogenic fungi, *Metarhizium ansopliae* and *Beauveria bassiana*. *Journal of Invertebrate Pathology*, **56**, 39-46.
- Pirt, S. J. (1975). *Principles of microbe and cell cultivation*. Palo Alto, California: Blackwell Scientific Publications Inc.
- Pirt, S. J. and Callow, D. S. (1959). Continuous flow culture of the filamentous mould *Penicillium chrysogenum* and the control of its morphology. *Nature*, London. **184**, 307-310.

- Rombach, M. C., Aguda, R. M., Shepard, B. M., and Roberts, D. W. (1986) Infection of rice brown plant hopper, *Nilaparvata lugens* (Homoptera: Delphacidae), by field application of entomopathogenic Hyphomycetes (Deuteromycotina). *Environmental Entomology*, **15**, 1070-1073.
- Ross, D. J. (1960). A study of the physiology of *Sporidesmin bakeri* syd. 1 Nutrition. *New Zealand Journal of Science* **3**, 15-25.
- Ross, D. J. and Thornton R. H. (1962). A study of the physiology of *Pithomyces chartarum* (Berk. and Curt.) M.B. Ellis. 3. Production of toxin on some laboratory media. *New Zealand Journal of Science*, **5**, 165-183.
- Smeaton, D. C., Hockey, H. U. P. and Towers, N. R. (1985). Effects of facial eczema on ewe reproduction and ewe lamb life weights. *Proceedings of the New Zealand Society of Animal Production* **45**, 133-135.
- Smith, B. L. and Towers, N. R. (1984). Pithomycotoxicosis (facial eczema) in New Zealand and the use of zinc salts for its prevention. Brisbane: Queensland Poisonous Plants Committee.
- Smith, J. D. and Crawley, W. E. (1962). Some aspects of the germination of spores of *Pithomyces chartarum* (Berk. and Curt.) M. B. Ellis. *New Zealand Journal of Agricultural Research*. **5**, 183-187.
- Solomons, G. L. (1975). Submerged Culture Production of Mycelial Biomass. In *The Filamentous Fungi, Volume 1, Industrial Mycology*. J. E. Smith and D. R. Berry, (eds.) pp249-264. Edward Arnold Publishers Ltd. London.

- Solomons, G. L. (1980) Fermenter Design and Fungal Growth. In *Fungal Biotechnology*. J. E. Smith, D. R. Berry and B Kristiansen, (eds) pp55-80. Academic Press Ltd. London.
- Stanbury, P. F. and Whitaker, A. (1984). *Principles of fermentation technology* (1st ed.). Oxford: Pergammon Press Ltd.
- Synge, R. L. M. and White, E. P. (1959). Sporedesmin: a substance from *Sporedesmin bakeri* causing lesions characteristic of facial eczema. *Chemistry and Industry* 1546-7.
- Thornton, R. H. and Ross, D. J. (1959). The isolation and cultivation of some fungi from soils and pastures associated with facial eczema disease of sheep. *New Zealand Journal of Agricultural Research* 2, 1002-1016.
- Thornton, R.H. and Sinclair, D.P.(1960). Some observations on the occurrence of *Sporidesmium bakeri* Syd. and facial eczema disease in the field. *New Zealand Journal of Agricultural Research*. 3, 300-313.
- Wang, D. I. C., Cooney. C. L., Demain. A. L. Dunhill. P., Humphrey, A. and Lilly M. D. (1979). In *Fermentation and enzyme technology*. C. Héden (ed.) pp157-194. John Wiley & Sons, New York.
- Wraight, S. P., Galani-Wraight, S., Carruthers, R. I., and Roberts, D. W. (1986) Field transmission of *Erynia radicans* to *Empoasca* leaf-hoppers in alfalfa following application of a dry mycelial preparation. In *Fundamental and Applied Aspects of Invertebrate Pathology*. R. A. Samson, J. M. Vlcek, and D. Peters, (eds.), p233