THE BREAKDOWN OF PLANT CELL BIOMASS BY FUNGI

ELIZABETH ANNE WHITEHEAD

A thesis submitted for the
degree of
Doctor of Philosophy

ASTON UNIVERSITY
August 1987

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SUMMARY

Three species of fungi Sporotrichum thermophile, Botrytis cinerea and Trichoderma viride were assessed for their ability to utilize a variety of plant cell substrates (Catharanthus roseus, Daucus carota, re-autoclaved C. roseus, re-autoclaved D. carota and methanol extracted C. roseus) which preliminary studies had indicated contained the necessary nutrients for fungal growth. Incubated in a suitable manner all three fungal species were able to grow on C. roseus and D. carota plant cell biomass in addition to material which had undergone methanol extraction or a re-autoclaving process to remove soluble components. Fungal biomass yields were markedly influenced by substrate, with each fungal species demonstrating a preference for particular plant cell material. Incubation conditions i.e. static or shaken and temperature also proved important.

Release of glucose (i.e. values higher than Day 0) promoted by fungal breakdown of plant cell biomass was only noted with methanol extracted, re-autoclaved C. roseus and re-autoclaved D. carota material. A re-autoclaved substrate was also generally associated with high fungal C_1, C_x, B-glucosidase and endo-polygalacturonase activity. In addition for each enzyme highest values were usually obtained from a particular fungal species. Buffering cultures at pH 3 or 5 further influenced enzyme activity, however in a majority of cases when flasks were unbuffered and the pH rose naturally to alkaline values higher enzyme activity was recorded. Likewise Tween 80 addition had only a limited beneficial effect.

Finally filtrates containing glucose produced both from the re-autoclaving process and through fungal activity on plant cell biomass were utilized for Fusarium oxysporum, Saccharomyces cerevisiae and C. roseus plant cell culture. Although reasonable fungal biomass was obtained the use of such filtrates proved unsuitable for plant cell growth.

Key words:
Fungi, plant cell biomass, biomass breakdown, C_1, C_x, B-glucosidase and endo-polygalacturonase activity.

To Mum, Dad and Helen.

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LIST OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF PHOTOGRAPHS

LIST OF APPENDICES

CHAPTER ONE

GENERAL INTRODUCTION

Secondary metabolites produced by plants are still an important source of chemical and medicinal compounds despite advances in organic chemistry. The main industrial applications of such compounds are as pharmaceuticals and agents in food flavouring or perfumery. Klein, (1960) and Puhan and Martin, (1971) proposed that many secondary metabolites produced by intact plants could be synthesized using plant cell culture techniques. While Nickell (1962) described the basic technology involved in the large scale culture of such cells.

Progress towards industrial scale production of plant cell compounds has been slow, and at present only rare or very expensive chemicals can be produced on a commercial basis. However, waste material which exists after extraction of the desired product potentially contains cellulose, hemicellulose, protein and pectic substances, thus offering a source of biodegradable material which could be utilized to produce single cell protein, enzymes or an upgraded animal feed. Also through microbial breakdown nutrients contained in this waste could be recycled back into plant cell culture thus making the process more economically viable.

Many other examples of biodegradable waste materials exist, however, because of its renewable nature and the vast quantities available lignocellulosic wastes have attracted particular interest.

These wastes consist of mainly cellulose, hemicellulose and lignin (Table 1:1). In addition, smaller quantities of other components may also be present e.g., starch, pectin, protein and minerals.

TABLE 1:1 COMPOSITION OF VARIOUS LIGNOCELLULOSIC MATERIALS

	CELLULOSE	HEMICELLULOSE	LIGNIN
	%	%	%
Hardwoods	40-55	24-40	18-25
Softwoods	45-50	23-35	25-35
Grasses eg bamboo rice, sugarcane	25-40	25-50	10-30
Newspaper	40-55	25-40	18-30
Municipal refuse	76	-	-
Wheatstraw	30.5	28.4	18.0
Oat hulls	34	30	13

Data compiled from Cowling and Kirk (1976), Rosenberg, Obrist and Stohs (1979) Brown (1983)

Utilization of such wastes have centered on acidic or enzymatic hydrolysis to produce glucose (a starting point for numerable fermentations) or conversion via a controlled microbial incubation to form single cell protein (SCP) or an upgraded animal feed.

Acid hydrolysis of lignocellulose is a well established technique and the process is usually performed in two stages. The first step involves contact with dilute acid at low temperatures to extract the hemicellulose fraction (mostly as pentoses) for xylitol or furfural production. A more concentrated acid and higher temperatures are then employed to convert cellulose to glucose. However, unlike enzymes, acid acts as a non specific catalyst thus the sugar syrup may be contaminated by side reaction products.

Although enzymes may be more specific, two major obstacles prohibit effective utilization of lignocellulosic residues. These are a) crystalline structure, which may also affect the rate of acid hydrolysis and b) the lignin barrier, which also restricts microbial access (Cowling and Brown, 1969). However, by use of various pretreatments, the reactivity of lignocellulosics can be increased by disrupting crystalline structure and removing the lignin barrier. Methods include, milling, alkali, SO_2 or steam treatment, and the use of solvents or swelling agents (Millett, Baker and Satter, 1976; Dunlap, Thompson and Chiang, 1976).

The range of lignocellulosic wastes and their utilization by fungi can be divided into:-

Agricultural Wastes

e.g., straw, rice and other hulls, almond and other shells, corn cobs, bagasse.

Strehler (1985) estimated UK (1983) straw production to be approximately 21 million tonnes per annum. A large proportion of this being surplus to requirements is usually burnt. As a result more potentially suitable ways of using such agrowaste have been investigated. Examples include (i) production of mushrooms - Chang and Hayes, 1978; Hayes and Lim, 1979; Rajarathnam, Wankhede and Bano, 1987 (wheat and rice straw). In addition spent mushroom compost is an ideal soil conditioner. Other agricultural wastes have also been developed as a substrate for mushroom culture. These include sugar cane rubbish; (Kneebone and Mason, 1972; Hu, Song, Liu and Peng, 1974) and ground corn cobs (Kalberer, 1974) (ii) production of ruminant feed - straw has been upgraded through incubation with Coprinus cinereus (Burrows, Seal and Eggins, 1979), Schizophyllum commune (Latham, 1979) and Trichoderma viride (with acid pretreatment, Han and Anderson, 1975). Such cultivations result in a product with an improved protein content and increased digestibility. Recently Rao, Mishra, Keskar and Srinivasan (1985) have used Neurospora crassa to directly ferment bagasse and straw to ethanol. While Manomani and Sreekantiah (1987) have produced citric acid from bagasse using A.niger.

T.viride was also used by Rosenberg, Obrist and Stohs (1979) to upgrade oathulls (pretreated with acid). While Stubblefield, Shotwell, Hesseltine, Smith and Hall (1967) used this substrate for

growth and aflatoxin production from Aspergillus flavus.

Food Processing Wastes

e.g., fruit or vegetable peels, trimmings and pulps, spoilt fruit.

Cooper (1976) gives comprehensive details of fruit and vegetable wastes and their estimated crude fibre content (an unknown amount of which is cellulose). Such wastes also contain starches, sugars, pectins, vitamins and minerals. Imrie and Righelato (1976) classified the above into (i) solid and semi-solid and ii) low concentration wastes such as process and canning waste water which represent a pollution hazard. This latter group generally contain non cellulosic carbohydrate mixtures which may be fermented producing SCP with a resulting decrease in effluent BOD (Church, Nash and Brosz, 1971; Church, Erickson and Widmer, 1973; Chung and Meyers, 1979).

Submerged and solid substrate fermentation methods have been investigated with respect to cellulose containing wastes (Davy, 1981; Karapina and Okuyan, 1982; Sengupta, Naskar and Jana, 1984). Using apple pomace, Hang, Lee, Woodams and Cooley (1981) produced 43g ethyl alcohol per kg substrate using Saccharomyces cerevisiae. While the fibrous residues from sweet potatoes have been used for citric acid production by Aspergillus niger (Lockwood, 1975). Hofsten (1976) inoculated fruit and vegetable peels, presscakes, Wheat and rice brans or polishings with Sporotrichum pulverulentum. All proved to be suitable substrates for mycoprotein production provided a nitrogen source was added.

However, Kirsop and Hilton (1981) suggested it would be unlikely that production of biomass will find general application as a method for utilizing food industry waste. This is because any biomass produced (which is also true of other ligno-cellulosic wastes) must be acceptable to regulatory authorities as a component of diet even if animal feed is to be produced. This necessitates sterilization of wastes to prevent contamination by unwanted microorganisms, and checks to ensure purity of any end product, thus rendering the process uneconomic.

Forestry/Timber Wastes

e.g., brush, chip, bark, sawdust, paper mill fines

All stages of wood processing produce ligno-cellulosic waste, from logging through primary and secondary manufacture (Stone 1976). Such residues however can again be used for mushroom cultivation. Low value logs (Leatham, 1982), sawdust (Sengupta, Naskar and Jana, 1984) and tree bark (Imbernon, Delmas, Laborde and Poitou, 1974) have all been used for production of Lentinus edodes, Termitomyces clypeatus and Pleurotus ostreatus respectively. This latter substrate was also investigated by Daugulis and Bone (1978) for production of mycoprotein using Phanerochaete chrysosporium, with yields of 1500 mg protein/litre from maple achieved.

Sawdust has been used by Toyama (1976), to produce cellulases, and has also been upgraded for use as a ruminant feed through solid substrate fermentations (Pamment, Robinson, Hilton and Moo-Young, 1978; Zadrazil, 1980). Kurtzman (1979) suggests that although technical problems do exist with solid substrate cultivations (in

relation to scaling up and incubation time), if edible mushrooms are produced in conjunction with animal feed the whole process becomes more commercially viable due to their high retail value.

The ability of certain fungal species to hydrolyze wood has been exploited by a number of workers to treat pulp/paper industry waste. Ek and Eriksson (1980) used S.pulverulentum to convert waste water, from fibreboard manufacture, into a protein rich product which in addition to being a satisfactory ruminant feed (Thomke, Rundgren and Eriksson, 1980) promoted substantial reductions in the effluent BOD and COD.

Municipal Waste

Domestic refuse is a mixed or multiple component waste which is more difficult to reclaim due to the time and cost involved in separating organic material from non bio-degradable plastic, metal etc (Fulbrook, Barnes, Bennett, Eggins and Seal, 1973). As a consequence little work has been reported, although Rogers, Coleman, Spino, Purcell and Scarpino (1972) investigated fungal (Aspergillus fumigatus) protein production on ground refuse, while Harsh, Bisht and Upreti (1981) cultivated Pleurotus ostreatus on tea leaves and wastepaper obtained from household rubbish. Using a solid substrate fermentation, Barnes, Eggins and Smith (1972) found it possible to upgrade newspaper (using Sporotrichum thermophile), increasing the protein content from 0 to 6.5% in 6 days. Likewise Updegraff (1971) obtained a 5.6% protein yield through submerged fermentation of Myrothecium verrucaria on ball milled newspaper. Cell free enzyme solutions (derived from T.viride) have also been used to hydrolyze

newspaper to produce glucose ultimately for ethanol production (Wilke and Mitra, 1975, Brown and Fitzpatrick, 1976). However Grethlein (1978) on comparing the economics of acid and enzymatic hydrolosis found the former to be much cheaper. Although development of mutants with enhanced cellulase productivity and stability may achieve parity of costs in the future.

All the aforementioned examples show how through biodegradation waste substrates can be utilized to produce valuable commodities. As noted earlier plant cell culture produces biodegradable waste which is at present discarded. Therefore the aim of this study is to assess the potential of such plant cell biomass as a substrate for fungal growth particularly of Sporotrichum thermophile, Botrytis cinerea and Trichoderma viride. To determine optimum cultural conditions and investigate the activity of any hydrolytic enzymes produced during fungal growth on such plant cell material. In addition, the possibility of recycling nutrients released by fungal breakdown of plant cell biomass into further plant cell culture or single cell protein production is investigated. Two species of plant cells were utilised as substrates in this study, Catharanthus roseus (L) G. Don and Daucus carota (L). These were chosen because such lines have attracted widespread scientific interest and lend themselves to mass cell culture techniques ensuring a readily available source of waste plant cell biomass. Likewise the above species of fungi were selected as they have already been shown by Smith, Armstrong and Fowler (1985) to be capable of degrading plant cell biomass produced by suspension cell culture techniques.

CHAPTER TWO

PLANT CELL CULTURE AND COMPOSITION

INTRODUCTION

Plants are capable of synthesizing a vast array of biochemicals, many of which find use in the food and pharmaceutical industries, eg codeine (analgesic) - Papaver somniferum, Thaumatin (non nutritive sweetener) - Thaumatococcus daniellii, Camptothecin (antileukaemic agent) - Camptotheca acuminata.

Although a part of today's biotechnology, the beginnings of plant tissue culture date back to the late 1890's. Haberlandt (1902, available in translation Krikorian and Berguam, 1969) was probably the first person to take a major initiative in this area. His experiments involved using single cells isolated from the palisade tissue of leaves, pith parenchyma, the epidermis and epidermal hairs of various plants. These he maintained in a viable state in simple nutrient solutions, although at this stage he did not observe cell division.

Very little progress was made following Haberlandts paper, until White (1934) pioneered the work on root culture. At this time he reported the establishment of an actively growing clone of tomato roots. In the same year, Gautheret (1934) reported that pieces of cambium removed under aseptic conditions from a number of trees continued to proliferate for some months if placed on the surface of a nutrient medium. Further work by Gautheret (1937, 1938) and White (1937) on the composition of culture media led to the establishment in 1939 of the basic techniques of tissue culture still used today.

Muir, Hildebrandt and Riker (1954) reported that if fragments of

callus from Tagetes erecta or Nicotiana tabacum were transferred to liquid medium and the medium agitated, the callus fragments break up to give a suspension of single cells and cell aggregates. This suspension can then be propagated by means of subculture.

Within only two years, the possibility of using cell cultures as a system for natural product synthesis was recognised and detailed by Routien and Nickell (1956).

The 1970's marked the development of large scale propagation of plant cells. Use of mass cultivation was initiated by the Japanese who grew tobacco cells in stirred tank reactors, and by 1983 production of shikonin and berberine were on stream (Curtin, 1983). The future of plant tissue culture has been considered by Zenk (1978). He stated that "The only criterion which will govern the introduction of these new techniques into industry is the economic aspect. Only if the plant product under consideration can be produced industrially (by cell culture techniques) at a price equal to or cheaper than the field produced goods will the technique of mass cell culture advance." Utilisation of waste material remaining after extraction of desired plant products may thus improve the economics of such processes.

With this in mind the initial step was to determine as far as possible the chemical composition of the plant cells used in this work, ie Catharanthus roseus (L) G.Don and Daucus carota (L) and to assess their potential as a substrate for fungal growth.

MATERIALS AND METHODS

1. MAINTENANCE OF CATHARANTHUS ROSEUS CALLUS CULTURE

The initial callus culture was kindly donated by the Wolfson Institute of Biotechnology, Sheffield University. This was maintained on B5 medium (Gamborg, Miller and Ojima, 1968) supplemented with 1mg/litre 2, 4-dichlorophenoxyacetic acid, 0.1 mg/litre Kinetin, 1% Agar ($N^{O}1$, Oxoid) at pH 5.8 25°C. (Appendix 2.1) Subculturing occurred every 2 - 4 weeks. See Photograph 1.

2. INITIATION OF PRIMARY CELL SUSPENSION

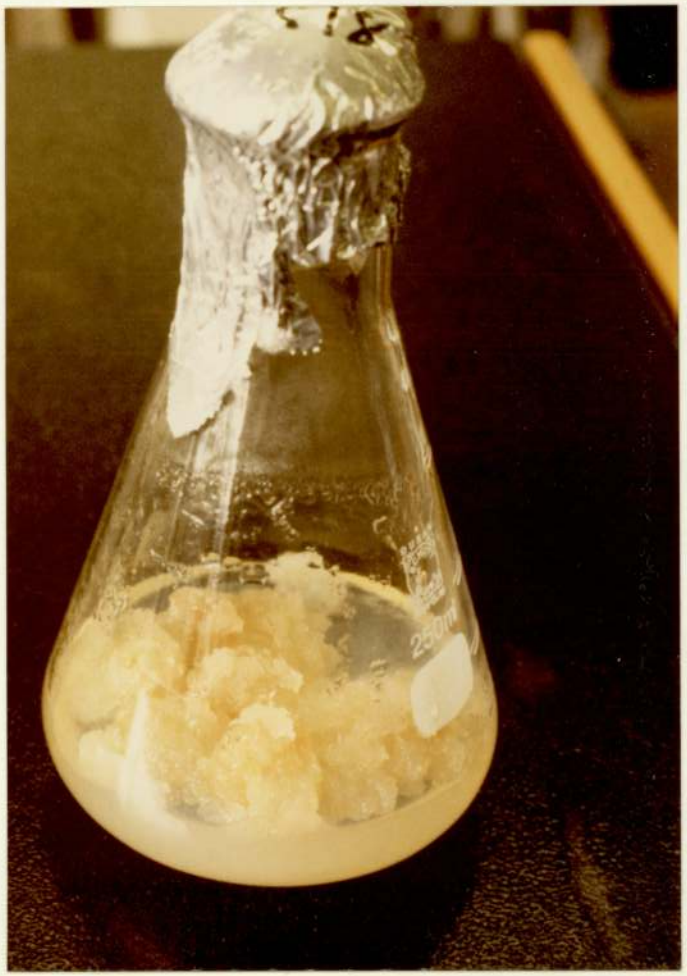
A piece of callus culture was placed into a 250 ml Erlenmeyer flask containing 100 ml of sterile B5 Medium. When placed on an orbital shaker the friable callus broke up and the resulting suspension could then be routinely subcultured. A Gilson pipetman was used to transfer 20 mls of mature suspension to 100 mls of fresh aseptic B5 Medium. Surplus cells were filtered off, washed with 10mM TES (Tris - (hydroxymethyl) - methyl - 2 - aminoethanesulphuric acid) buffer (pH 7.0) and dried at 40°C. These cells were ground in a pestle and mortar then stored at 4°C. See Photograph 3. (cells were subcultured every 10 days.)

3. PLANT CELL GROWTH CURVE

Over a 14 day period of growth three flasks of cells were removed every other day. After filtering their wet and dry weights were determined. This data was then plotted on a graph of time/weight

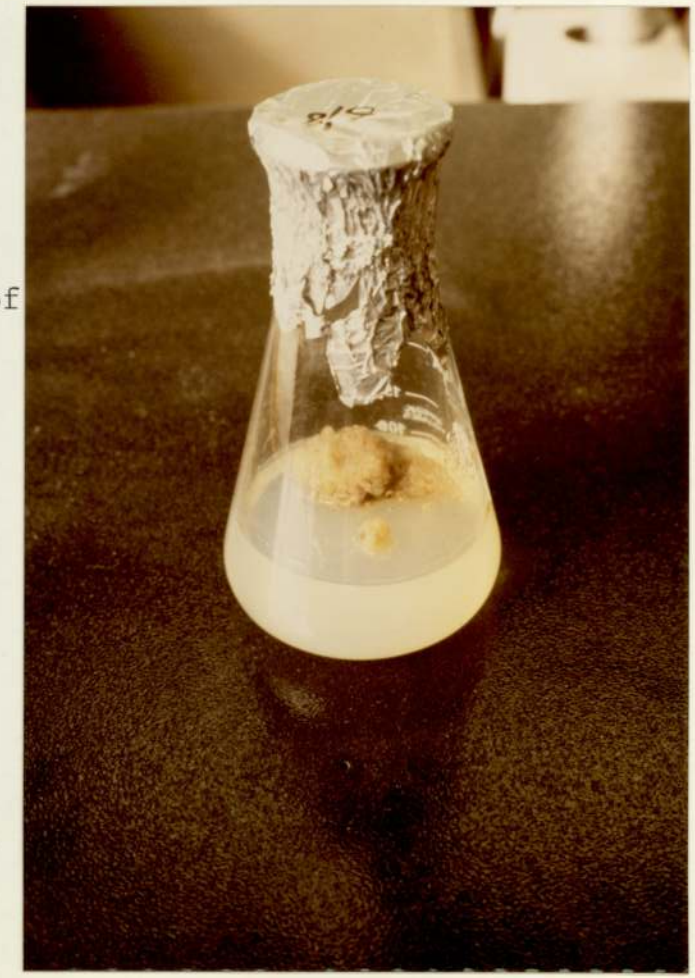
PHOTOGRAPH ONE

A callus culture of Catharanthus roseus.



PHOTOGRAPH TWO

A callus culture of Daucus carota.



PHOTOGRAPH THREE

Cells from a suspension culture of Catharanthus roseus.

4. ISOLATION OF EXPLANTS, ESTABLISHMENT AND MAINTENANCE OF CARROT CALLUS (DAUCUS CAROTA)

After surface sterilization of a carrot using 20% (v/v) Sodium Hypochlorite, a series of transverse slices 1 mm in thickness were cut from the carrot root. From each slice, explants 4 mm x 4 mm square were cut in such a way so that each piece contained parts of phloem, cambium and xylem. Each square was placed in a culture tube containing MDAUC (Reinert and Yeoman, 1982) agar (Appendix 2:1). These were then incubated in the dark at 25°C. After 4 weeks explants had formed substantial callus tissue (see Photograph 2) which was subcultured and maintained as in section (1.) Initiation of a primary cell suspension culture of D carota cells was also carried out as in section 2 using B5 medium. (cells were subcultured every 14 days.)

5. COMPOSITION OF PLANT CELLS - PLANT CELL SIZE

Cells were measured using a microscope eye piece graticule, calibrated from a stage micrometer.

6. CELLULOSE CONTENT

This was determined using the method of Updegraff (1969). To a known weight of dried plant cell material contained in a boiling tube, 3 ml of acetic-nitric reagent (150 ml 80% acetic acid + 15 ml concentrated nitric acid) was added. The tube was placed in a boiling water bath for 30 minutes. After this time, 10 ml of distilled water was added and the tube centrifuged for 5 minutes at 2000 rpm. The supernatant after decanting was discarded. 10 ml 67% H_2SO_4 (v/v) was added and the tube left to stand for 1 hour. After dilution by a factor of 20, 1 ml of

this dilution was placed in a fresh boiling tube and 4 ml distilled water added. This tube was cooled in an ice bath before 10 ml cold anthrone reagent (0.2g anthrone (Sigma) in 100 ml concentrated H_2SO_4, prepared fresh daily) was layered on top. After mixing, the tube was boiled for 16 minutes cooled and read at 610nm against a reagent blank. A standard curve of ug cellulose against OD 610nm was also constructed (see Appendix 2:2). The percentage cellulose composition of the two plant cell types was determined by comparison with the afore mentioned standard curve.

7. CRUDE PROTEIN CONTENT - MICROKJELDAHL METHOD

A known weight of dried plant cell material was placed in a microkjeldahl flask, to it was added 150 mg catalyst mixture (32 parts A.R. potassium sulphate, 5 parts A.R. copper sulphate, 1 part selenium powder) and 0.1 ml of concentrated H_2SO_4. The flask was heated for 20 minutes and allowed to cool. The contents of the flask were then distilled with 10 ml 40% NaOH and the ammonia released absorbed into 25 ml saturated boric acid (plus a few drops of screened indicator, 0.1% Bromocresol green in 95% ethanol). This mixture was then titrated against 0.01 M HCl. It was assumed that 1 ml of 0.01 M HCl is equivalent to 0.14 mg of nitrogen. Crude protein (%) = % nitrogen x 6.25.

8. SOLUBLE CARBOHYDRATE CONTENT

Known weights of dried samples were refluxed for 15 minutes with 3 changes of 80% ethanol. These changes were pooled and reduced to 2 ml using a rotary evaporator. Extracts were shaken

with "Amberlite" IR 120 and IR45 resins, (BDH) to remove amino acids, and left for a minimum of 30 minutes. After this time, extracts were dried down completely in pear shaped flasks. These extracts were then silylated with a combination of hexamethyldisilazane (HMDS, Sigma) and trimethylchlorosilane (TMCS, Sigma) in anhydrous pyridine (BDH). After 18 hours the resulting carbohydrate (TMS) derivatives were analysed using 1.5 metre columns of 3% SE 30 (BDH) on chromosorb WHP (80 - 100, Phase Separation) with a "Pye-Unicam 304" gas chromatograph. Known standards were used for the indentification and quantification of carbohydrates present in each extract.

9. STARCH CONTENT

To 250 mg dried plant cell material in a test tube was added 200 mg fine sand with 5 ml water. The contents were mixed and placed in a boiling water bath for 15 minutes. After cooling to 30°C, 5 ml 60% perchloric acid was added, and the tube allowed to stand for 20 minutes. After this period, the contents were transferred to a 100 ml volumetric flask and diluted to volume. From this a 1 ml aliquot was transferred into a 50 ml volumetric. At the same time a standard solution (1 ml = 1 mg starch) was prepared (in order to construct a standard curve). From this aliquots (0.1 ml - 2.5 ml) containing from 0.1 mg to 2.5 mg starch were also transferred into 50 ml volumetrics. From this point standards and samples were treated in the same way. A few drops of phenol red indicator (0.1% (w/v) in industrial spirit) were added to each volumetric with 1M NaOH until the solution turned red. Sufficient acetic acid (10% v/v) was added to destroy the colour

plus a further 2.5 ml. To this, additions of 0.5 ml (10%, w/v) potassium iodide and 5.0 ml (0.0125 M) potassium iodate were made, shaken and diluted to volume. Aliquots were then measured at 680 nm against a reagent blank. Starch content was calculated from the afore mentioned standard curve. (See Appendix 2:3).

10. VITAMIN ASSAY

Specific organisms were used which give a growth response appropriate to the level of vitamin present in the sample. These organisms were:-

Bacillus coagulans (NCIB 88701) - Folic acid
Proteus mirabilis (NCIB 10466) - Pantothenic acid
Micrococcus sodonensis (NClB 8854) - Biotin
Lactobacillus plantarum (NCIB 8960) - Nicotinic acid
Lactobacillus fermenti (NCIB 8961) - Thiamine
Lactobacillus casei Var rhamnosus (NCIB 8651) - Riboflavin
Neurospora sitophila (CMI 21944) - Pyridoxine

To test for the vitamin, a plant cell agar (PCA) was prepared. In which a 1% concentration of plant cells in distilled water was steamed for 1 hour, the plant cells then filtered off (through muslin) and 'Ionagar' (Difco) to a level of 2% added. A water agar control (2% (w/v) 'Ionagar' in distilled water) was also prepared. Both agars were then autoclaved at 115°C for 20 minutes (10 psi). Cooled to 50°C and poured into petri dishes. The surface of both agars was then streaked with the test bacterium or plug of agar (N. sitophila), and incubated at 37°C for the bacteria, 25°C for N. sitophila. Ascorbic acid content was determined chemically, with the first step involving the standardisation of ascorbic acid. A solution

of 40 mg ascorbic acid in 100 ml, 10% (v/v) acetic acid was made. From this 5 ml was diluted to 100 ml with 10% (v/v) acetic acid. The amount of this latter solution needed to decolourise 0.5 ml 2,6 - dichlorophenol indophenol (40 mg dye in 100 ml water) was resolved. The filtrate from 0.5 g dried plant cell material in 20 ml distilled water shaken for 1 hour was then analysed. The amount of filtrate needed to decolourise 0.5 ml dye was determined and from the standardisation procedure, ascorbic acid content of the filtrate was calculated.

11. AMINO-ACID ANALYSIS

This was carried out on known weights of material by hydrolysing samples in 2 ml 6 M HCl in sealed tubes at 110°C for 24 hours. The resulting hydrolysate was neutralised with 5.3 ml 2M NaOH and diluted with 25 ml distilled water. After centrifugation, aliquots of hydrolysate were loaded onto a 'Locarte' amino acid analyser and separated using a sodium citrate buffer system. Identification and quantification of unknown amino acids was by comparison to known standards.

N.B. All assays were carried out on plant cells subcultured as in Sections 2 and 4.

RESULTS

Growth curves of C.roseus cells in suspension culture for wet and dry weight production over 14 days are given in Figures 2:1 and 2:2, with maximum dry and wet weight values occurring on Day 5 and Day 7 respectively. Using such curves the doubling time for C.roseus cells is calculated to be approximately 2.5 days.

The basic composition of C.roseus and D.carota plant cells is outlined in Tables 2:1 - 2:3. C.roseus cells show a higher percentage content of cellulose and crude protein compared to D.carota cells. Whereas the latter plant cells have significantly higher amounts of all the individual soluble carbohydrates except sucrose, with mannitol and arabitol levels similar to those of C.roseus.

Only C.roseus was tested for starch quantitatively as the analysis procedure proved unsuitable for use with D.carota cells (this was also the case for ascorbic acid). Although addition of iodine to D.carota cells on a microscope slide gave a positive result.

Results in Table 2:4 indicate the presence in C.roseus cells of 7 vitamins, although only ascorbic acid was assayed using a quantitative method.

Amino acid analysis of C.roseus and D.carota plant cell material (Table 2:5) indicates that for an individual plant cell substrate marked differences exist between the amounts of each amino acid

FIGURE 2:1 C.roseus plant cell growth curve-wet weight.

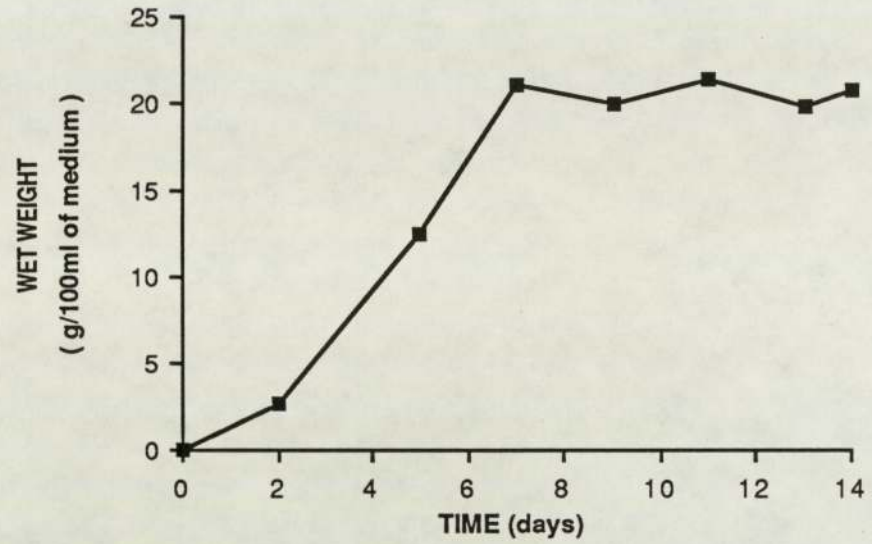


FIGURE 2:2 C.roseus plant cell growth curve-dry weight.

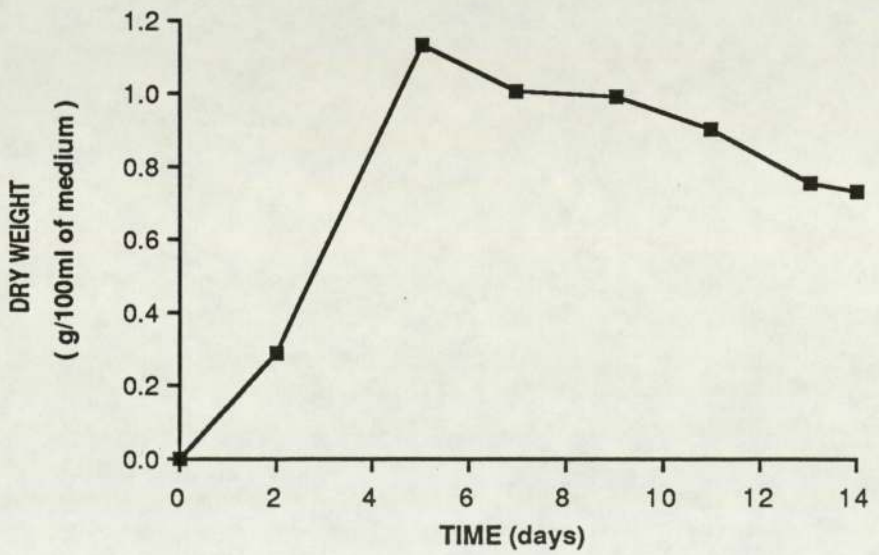


TABLE 2:1 PLANT CELL SIZE

SPECIES	SIZE (um)
C.ROSEUS	70 - 240 (57.3)* X 40 - 50 (4.26)
D.CAROTA	Mainly large clumps of cells

* Standard deviation

TABLE 2:2 PLANT CELL COMPOSITION

	C. ROSEUS	D.CAROTA
CELLULOSE CONTENT (%)	6-9	5-7
CRUDE PROTEIN CONTENT (%)	10.76	6.77
STARCH CONTENT (%)	3-4	present

TABLE 2:3 PLANT CELL SOLUBLE CARBOHYDRATE CONTENT

SOLUBLE CARBOHYDRATE (ug/mg dry wt)		
	C.ROSEUS	D.CAROTA
ARABITOL+	0.011 (2.77)*	0.028 (7.2)
FRUCTOSE	0.081 (5.17)	3.43 (2.12)
α GLUCOSE	0.076 (7.20)	1.48 (0.907)
MANNITOL+	0.278 (0.67)	0.19 (0.31)
β GLUCOSE	0.145 (0.104)	2.2 (1.32)
SUCROSE	0.627 (0.606)	0.195 (0.37)
TREHALOSE	0.42 (0.383)	1.62 (1.61)

* Standard Deviation

Each value is the mean of 21 samples

+ Student's 't' test result = not significant difference between the two plant cell species

TABLE 2:4

C.ROSEUS PLANT CELL VITAMIN CONTENT

ORGANISM	VITAMIN ASSAYED FOR	GROWTH ON PCA	GROWTH ON WA
BACILLUS COAGULANS	Folic Acid	✓	X
PROTEUS MIRABILIS	Pantothenic Acid	✓	X
MICROCOCCUS SODONENSIS	Biotin	✓	X
LACTOBACILLUS PLANTARUM	Nicotinic acid	✓ *	✓
L. FERMENTI	Thiamine	✓	X
L. CASEI VAR RHAMNOSUS	Riboflavin	✓	X
N. SITOPHILA	Pyridoxine	✓ *	✓
ASCORBIC ACID	0.658 mg/g dry wt of C. roseus Cells		

PCA = plant cell agar
WA = water agar
* growth response greater than on WA

TABLE 2:5

AMINO ACID PROFILE OF PLANT CELL MATERIAL

* AMINO ACIDS (n mol/mg dry weight) IN PLANT CELL MATERIAL

	C.ROSEUS	D.CAROTA
L-ASPARTATE	161.2	119.5
L-THREONINE	87.9	62.2
L-SERINE	109.2	75.5
L-GLUTAMINE	175.9	101.1
L-PROLINE	59.0	57.7
GLYCINE	161.0	110.3
L-ALANINE	170.8	89.2
L-VALINE	81.3	66.8
L-METHIONINE	20.9	17.8
L-ISOLEUCINE	81.7	54.6
L-LEUCINE	125.3	88.8
L-TYROSINE	46.1	29.6
L-PHENYLALANINE	60.7	29.1
L-HISTIDINE	33.8	23.4
L-LYSINE	100.9	63.8
L-ARGININE	80.0	40.8

* Average of 3 samples

present. In addition when the amino acid profiles of both substrates are compared there is a significant difference. For all amino acids, higher amounts are present in C.roseus compared to D.carota plant cell material. In particular levels of glutamine, alanine, phenylalanine and arginine in C.roseus are nearly twice those of D.carota.

DISCUSSION

A number of differences exist between microbial and plant cells, which in turn influences their large scale cultivation. One such difference is the slow growth rate exhibited by plant cells, for example in this study C.roseus cells had a doubling time of 2.5 days. Whereas Maynard-Smith (1969) reported for E.coli (at 37°C) a value of 20 minutes. Plant cells are also much larger and have a tendency to grow as aggregates. In many cases a range of cell sizes and shapes have been documented. Steward, Mapes and Smith (1958) reported in carrot suspension cultures the occurrence of spherical cells (50 - 100 um diameter), giant cells (100 - 300 um) and very elongated cells. Although there is diversity of size and shape cells of suspension cultures, including those of C.roseus used in this study, generally remain parenchymatous i.e., living, thin walled and vacuolated (see Photograph 3).

Plant cell suspension cultures have provided an important tool in understanding the composition of plant cell walls (Talmadge, Keegstra, Bauer and Albersheim, 1973). A particular advantage of suspension cultured cells compared to those obtained from whole plant sources is the absence of a secondary cell wall. In addition, the relative abundance of suspension cells ensures workers a supply of homogeneous primary cell wall material devoid of any problems associated with removal of a secondary cell wall. Much of the primary cell wall is composed of cellulose. Talmadge, Keegstra, Bauer and Albersheim, (1973) using the method of Updegraff (1969) calculated sycamore cells to be composed of 23% cellulose. Whereas Lamport (1965) using chemical extraction procedures on the same type

of cells reported a value of 27%. Both these figures are over twice those found with C.roseus (6 - 9%) and D.carota (5 - 7%) in this work. However, these values are closer to ones reported by Burke, Kaufman, McNeil and Albersheim (1974) for oat (10%), wheat (14%) and rice (11%) cells in suspension culture. Work by Talmadge, Keegstra, Bauer and Albersheim (1973) and Bauer, Talmadge, Keegstra and Albersheim, (1973) has also shown suspension cultured plant cell wall material to contain both hemicellulose and pectic substances.

The perennial world food shortage and a need for reliable food supplies during war time promoted military research into plant tissue culture (Byrne and Koch, 1961). Matthern (1962) reported the composition of an unnamed plant cell type to be 18% fat, 12% carbohydrate and 35% protein. This latter value for protein content is 3-5 times greater than was found for C.roseus or D.carota in this study. However, this could be a consequence of assay method, media or species difference.

Such a species difference is also illustrated in this study by comparing amino acid profiles of C.roseus and D.carota plant cell material. In all cases higher amounts of individual amino acids (particularly glutamine, alanine, phenylalanine and arginine) were present in C.roseus compared to D.carota cells. Differences may also occur due to cultural conditions. For example, Wickremasinghe, Swain and Goldstein (1963) found in limited air cell cultures from sycamore, bean and rose showed a marked increase in the amount of free amino acids compared to tissue grown with free access to air. Although in this study both plant cell species were treated in the same way. Gamborg and Finlayson (1969) also noted plant cells

produced by cell culture techniques contained higher levels of essential amino acids compared to seed material.

Space research has also supported plant cell work. Vasil and Hildebrandt (1966) suggested plant tissue culture could provide a rich source of fresh vegetable food, essential vitamins and minerals on long space flights. However, plant tissue culture in this situation would only be viable if the tissue cultured were capable of de novo synthesis of such metabolites

In this study presence of ascorbic acid in C.roseus plant cells was estimated quantitatively. De novo synthesis seems to be indicated as this vitamin was not a component of the culture medium. Routien and Nickell (1956) have also reported ascorbic acid synthesis from a plant gall culture, however the level of vitamin produced (150 ug/g dry weight) is less than from C.roseus in this study. In addition Dravnieks, Skoog and Burris (1969) found thiamine was synthesized by a tobacco callus culture when activated by cytokinin.

Although in this study the de novo synthesis of folic acid, pantothenic acid, biotin and riboflavin is indicated. A number of factors may affect the results when using microbiological techniques. In general, micro-organisms require growth factors such as vitamins at or below micromolar levels therefore any contamination in the media, can have a profound effect on the outcome of an assay. This is indicated in Table 2:4 by unexpected positive results on control agar with the test organisms for nicotinic acid and pyridoxine. Other factors such as pH, temperature or salt

concentration can also affect a test organism's requirement for the vitamin under assay (Lilly and Barnett, 1947; Barnett and Lilly, 1948).

Although de novo synthesis of vitamins by C.roseus cells may be indicated in this study, the rate of synthesis may be too slow to support rapid cell growth. Thus even though Carew and Krueger (1977) suggest C.roseus has a zero vitamin requirement for thiamine, nicotinic acid, pyridoxine and pantothenic acid, all but the latter are routinely added to C.roseus culture medium. In many cases these are added to ensure a deficiency does not occur due to, as noted above, a low synthesis rate, as a result of cell leakage, or during transfer to fresh medium.

Another important component of culture media is the carbon source, which in turn can influence plant cell growth and biomass yield. In general sucrose or glucose are used, although Fowler (1982) has tested a wide range of alternative carbohydrates for their ability to support the growth of C.roseus cultures. These include starch, maltose, galactose and lactose, however only maltose was found to promote biomass yields approaching those of glucose or sucrose. Biomass yield is also dependent on the incubation period. This is illustrated by Figure 2:2 in which maximum dry weight production occurred at Day 5 and decreased there after. This decrease may indicate nutrient exhaustion had occurred with cells utilizing their own reserves towards the end of the experimental period. Wet weight (Figure 2:1) would be less affected due to extra water retained to maintain cell turgor. The percentage carbon converted to biomass is also important as it indicates the efficiency

of nutrient utilization. Fowler (1982) reported carbon conversion values of 60% for C.roseus cultures compared to 35 - 45% from this work. Whereas for other micro-organisms values of 44% A.niger on glucose (Burnett, 1968) and E.coli on glucose 14.33% (Stouthamer, 1968) have been reported. However, such values may be improved by altering the concentration of carbohydrate used or the incubation time.

Work on fungal nutrition indicates a major requirement for organic carbon compounds usually in the form of carbohydrates. In addition, fungi need a source of nitrogen, minerals, vitamins and other growth factors. Therefore results from this study which indicate C.roseus and D.carota plant cell material is composed of primarily cellulose, starch, soluble carbohydrate including sucrose and glucose, protein, amino acids and possibly a variety of vitamins suggest plant cell biomass could provide a suitable substrate for fungal growth. In turn such fungal growth offers a means of utilizing waste plant cell biomass for the production of potentially useful substances.

CHAPTER THREE

GROWTH OF THREE SPECIES OF FUNGI ON PLANT CELL BIOMASS - STATIC CULTURE

INTRODUCTION

The three fungal species used in this study were Sporotrichum thermophile Apinis, Botrytis cinerea Pers. ex fr. and Trichoderma viride Pers. ex S.F. Gray aggr. The first species (a thermophile) has been isolated (Semeniuck and Carmichael, 1966; Tansey, 1971) from self heating wood chip piles, and shown by Romanelli, Houston and Barnett (1975) to exhibit considerable cellulolytic activity. In addition Coutts and Smith (1976) found S.thermophile produced equivalent quantities of cellulase to mesophiles but in a markedly shorter period of time. A further advantage of using thermophilic species suggested by these workers is the high incubation temperatures used in their cultivation may limit the number of contaminants, thus allowing the use of relatively unsophisticated equipment for large scale fermentations.

B.cinerea is a common plant pathogen attacking vegetable, fruit, ornamental flowers and field crops. Infection by this species is associated with a rapid breakdown of plant tissue and work by Verhoeff (1978) and Verhoeff, Liem, Scheffer and Surya (1983) has shown B.cinerea is capable of producing both pectinolytic and cellulolytic enzymes.

The final species, T.viride is a ubiquitous soil fungus, and has been extensively investigated since the 1950's when Siu and Reese (1953) found it to be a highly active producer of cellulolytic enzymes. Since that time several mutant strains have been obtained through u.v radiation or chemical treatment which show enhanced cellulase production, and have been recognised as demonstrating

potential for the economic breakdown of waste cellulose (Mandels Hontz and Nystrom, 1974; Ryu and Mandels, 1980).

In this study two species of plant cells were used C.roseus and D.carota. Cells also underwent methanol extraction and re-autoclaving to determine whether a reduction in the more readily available components present in plant cell biomass had any adverse effect on fungal growth. During the 18 - 21 day incubation period a number of factors including fungal growth, glucose concentration, pH, and composition of the resulting fungal biomass were measured in order to assess the potential of these three species of fungi to degrade plant cell substrates.

MATERIALS AND METHODS

1. PREPARATION OF FLASKS FOR INOCULATION

Dried cells were added to water (20 mls) in a 100 ml Erlenmeyer flask at a concentration of 1% (w/v). These were then autoclaved at 115°c, 20 minutes (10 psi). The type of cells used were:-

i. Methanol extracted plant cell material from the Wolfson Institute of Biotechnology

ii. C.roseus plant cells

iii. D.carota plant cells

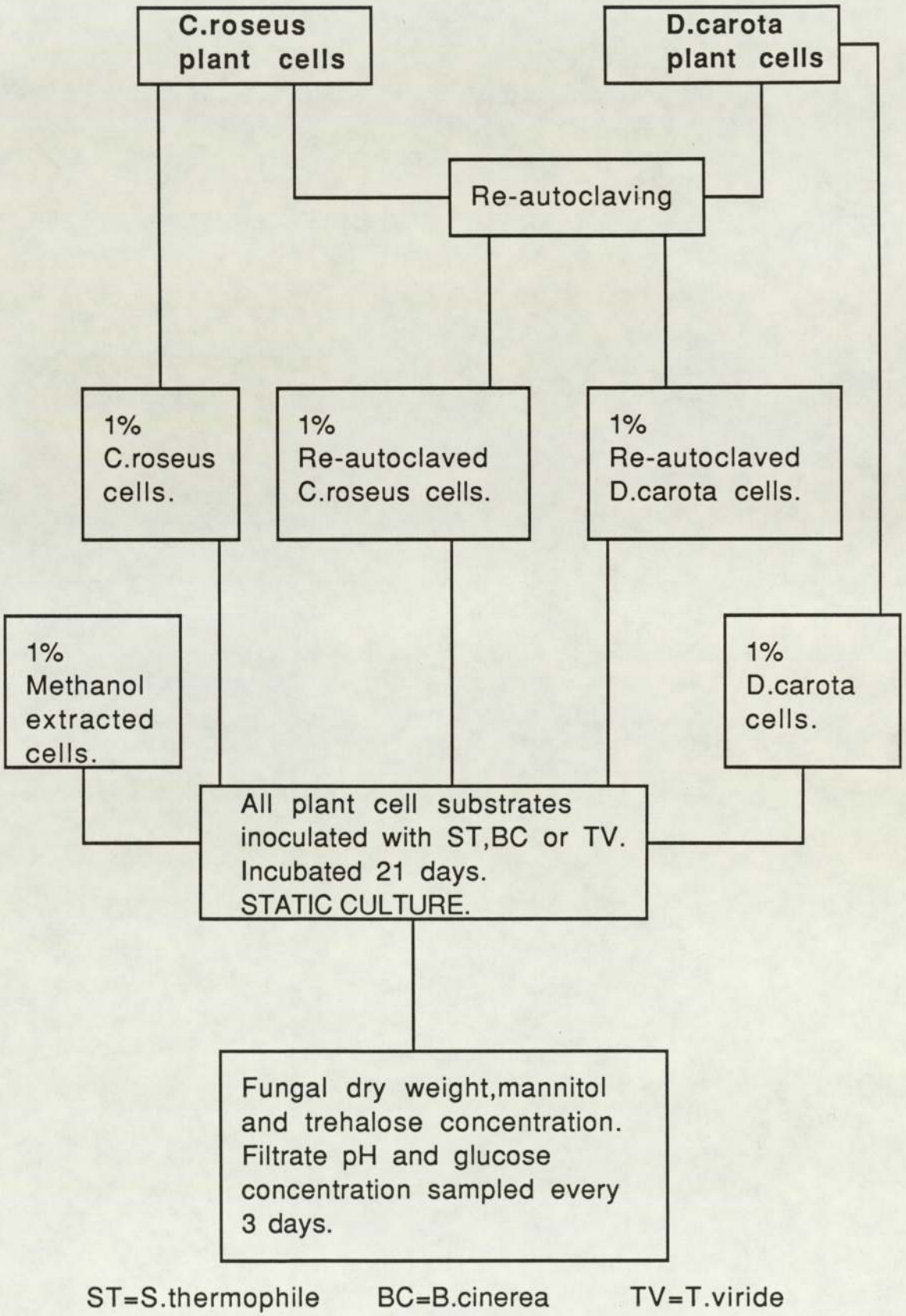
iv. Re-autoclaved plant cells of (ii) and (iii).

Re-autoclaved cells were prepared by making up 1% concentrations of cells as above. After autoclaving these cells were filtered, dried and used again to make a 1% concentration. After a second autoclaving these cells and those of (i) - (iii) were inoculated with spores from cultures (maintained on Malt Extract Agar (1.5%) Oxoid) (Appendix 3:1) of B.cinerea, T.viride and S.thermophile Obtained from the Aston Culture Collection. The first two species were incubated at 25°C while S.thermophile was maintained at 40°C. Uninoculated flasks were also prepared to act as controls. (See Figure 3:1).

2. GROWTH

This was measured in individual species over 18 - 21 days (values determined every 3 days) by removal of fungal mycelium from Erlenmeyer flasks onto pre-weighed dried Whatman No 1 filter papers. After drying to a constant value at 60°C dry

FIGURE 3:1 Flow diagram of the procedure used to investigate fungal growth on plant cell biomass -static culture.



weight was determined. Where no mycelium was present or could not be successfully removed from the cell residue, a value for the fungal/plant cell combination was determined.

3. PH OF THE FILTRATE

After removal of the fungal mycelium any remaining plant cell material was filtered off and the pH of this filtrate determined using a Pye-unicam pH meter.

4. REDUCING SUGAR CONCENTRATION OF THE FILTRATE

This was assessed using the method of Nelson (1944), to 1ml of filtrate, was added 1ml of copper reagent (Appendix 3:1). After boiling for 20 minutes, the solution was cooled before addition of 1ml arsenomolybdate reagent (BDH). Optical density of the resulting blue colour was estimated at 660 nm on a spectrophotometer against a reagent blank and the concentration of glucose determined by comparison to a standard curve of D-glucose concentration against optical density (Appendix 3:2).

5. SOLUBLE CARBOHYDRATES

These were determined in the manner outlined in Chapter 2.

6. CELLULOSE CONTENT OF RE-AUTOCLAVED PLANT CELL MATERIAL

This was determined by the method of Updegraff (1969). See Chapter 2.

7. STATISTICAL ANALYSIS

Analysis of variance was determined using a 3 x 6 (7) factorial split plot analysis in a randomised design.

RESULTS

From Figure 3:2a - 3:2e (data given in Appendix 3:4 - 3:8) it is evident that all three fungal species are able to grow on all plant cell types without nutrient supplementation. Within such a pattern individual species demonstrated significantly different modes of biomass production (determined as dry weight) which in turn is markedly influenced by the differing plant cell substrates. The amount of plant cell material converted to fungal biomass varied from 14.7% (S.thermophile on methanol extracted) to 51% (T.viride on D.carota cells) with each species producing their greatest conversion value on a different plant cell type. However both S.thermophile and B.cinerea gave an average value for all 5 substrates of 28%, with T.viride producing an average conversion value of 37%.

Although the fungi under investigation did not release significant amounts of D-glucose with C.roseus and D.carota cells (Figure 3:3b, c) these same fungal species when grown on other plant cell material released considerable quantities of D-glucose (Figure 3:3a, d, e) with maximum production occurring within 3 - 6 days of inoculation. The highest recorded amount of D-glucose was produced by B.cinerea grown on re-autoclaved C.roseus cells with a value 560% greater than that of the control.

Except for some minor differences, Figure 3:4 (a - e) shows the pattern of pH change whatever combination of cell type or fungal species employed is similar. An initial sharp rise occurs over the first 3 - 6 days followed by a more gradual change over the remaining incubation period. Amongst discrepancies to such a pattern however

FIGURE 3:2 (a-e) Fungal dry weight production on five plant cell substrates-static culture.

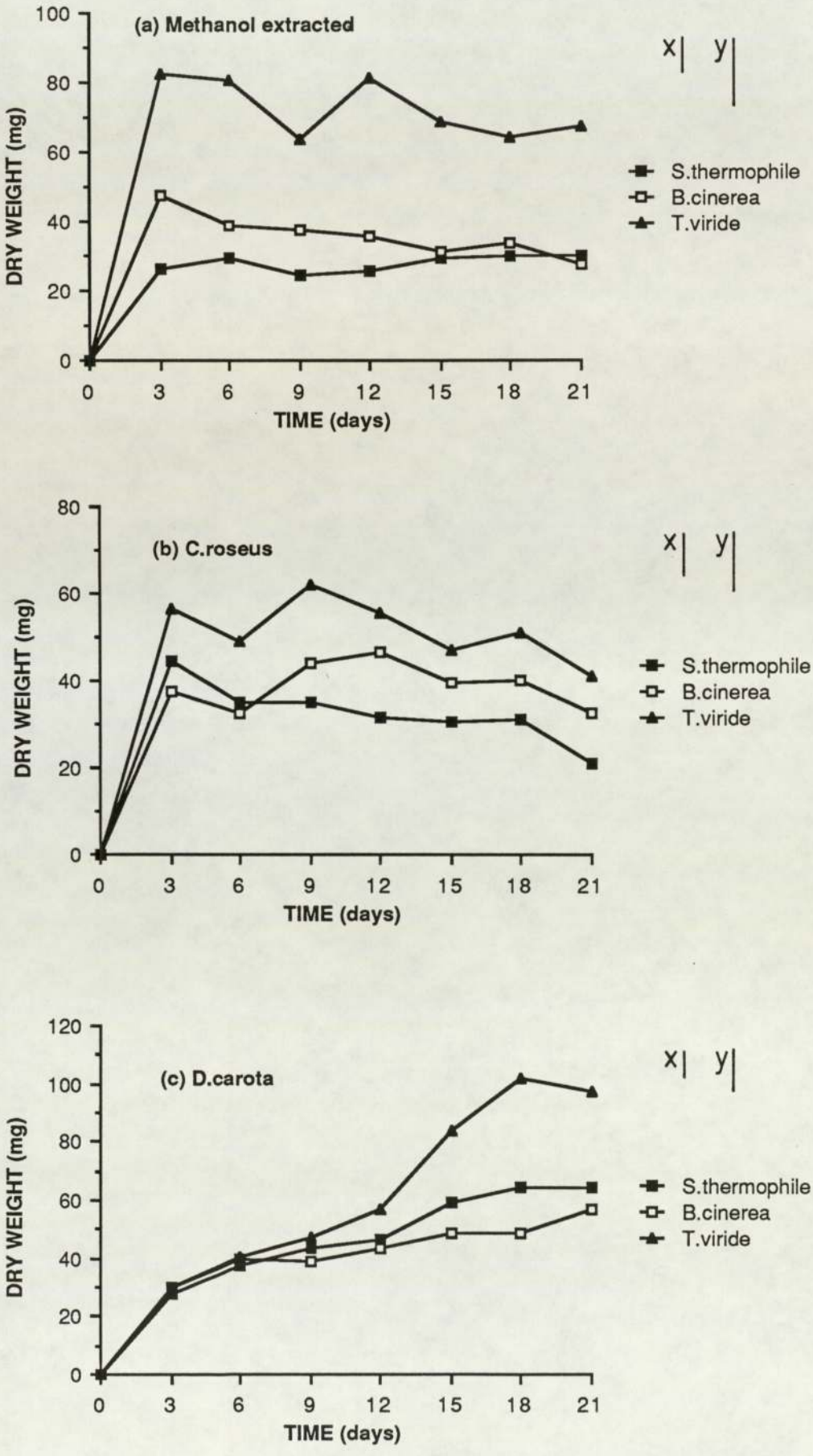
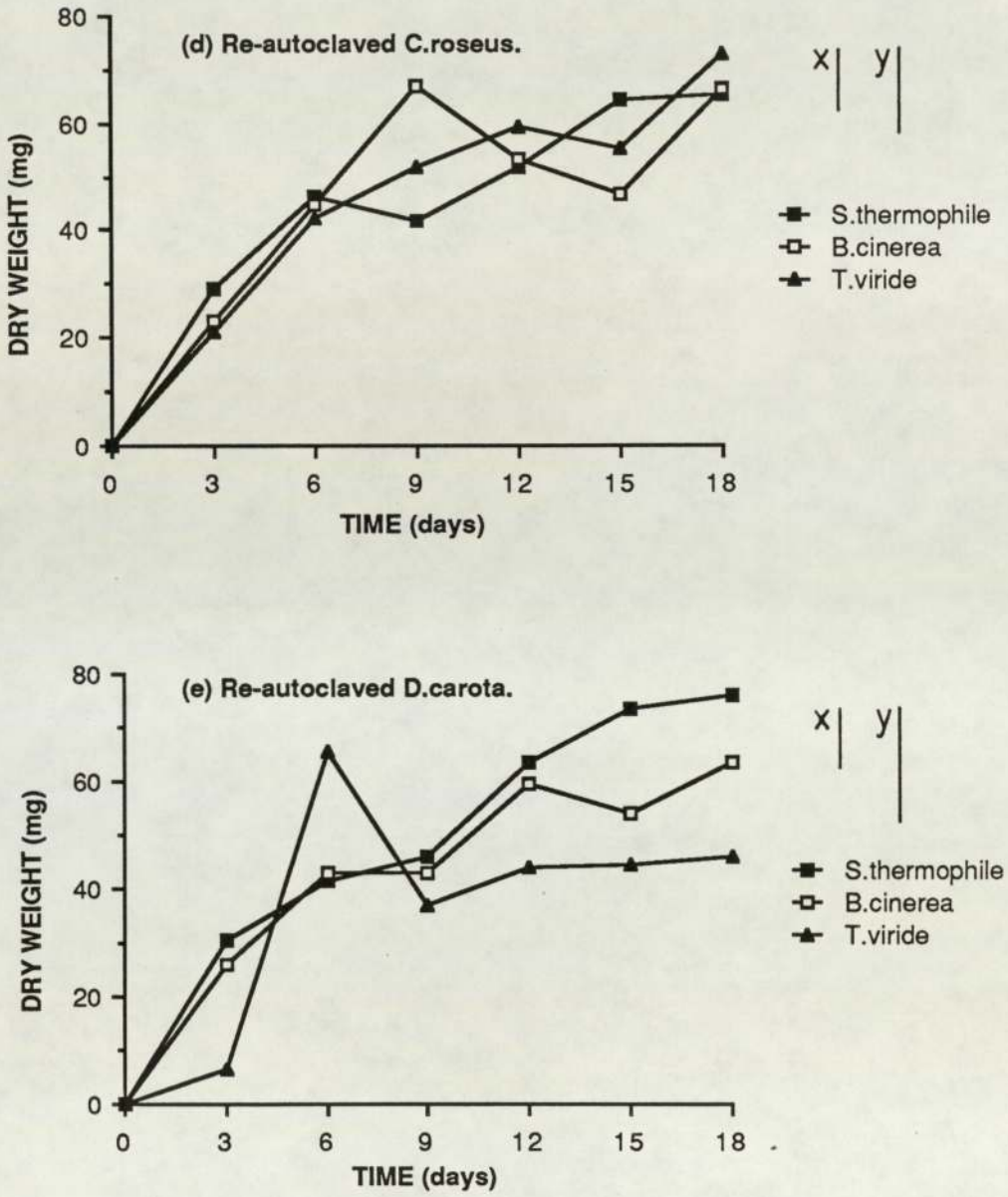


FIGURE 3:2 (continued)



X=95% confidence limits for differences within a treatment and Y=95% confidence limits between two treatments at the same or different times.

FIGURE 3:3 (a-e) Glucose release from five plant cell substrates-static culture.

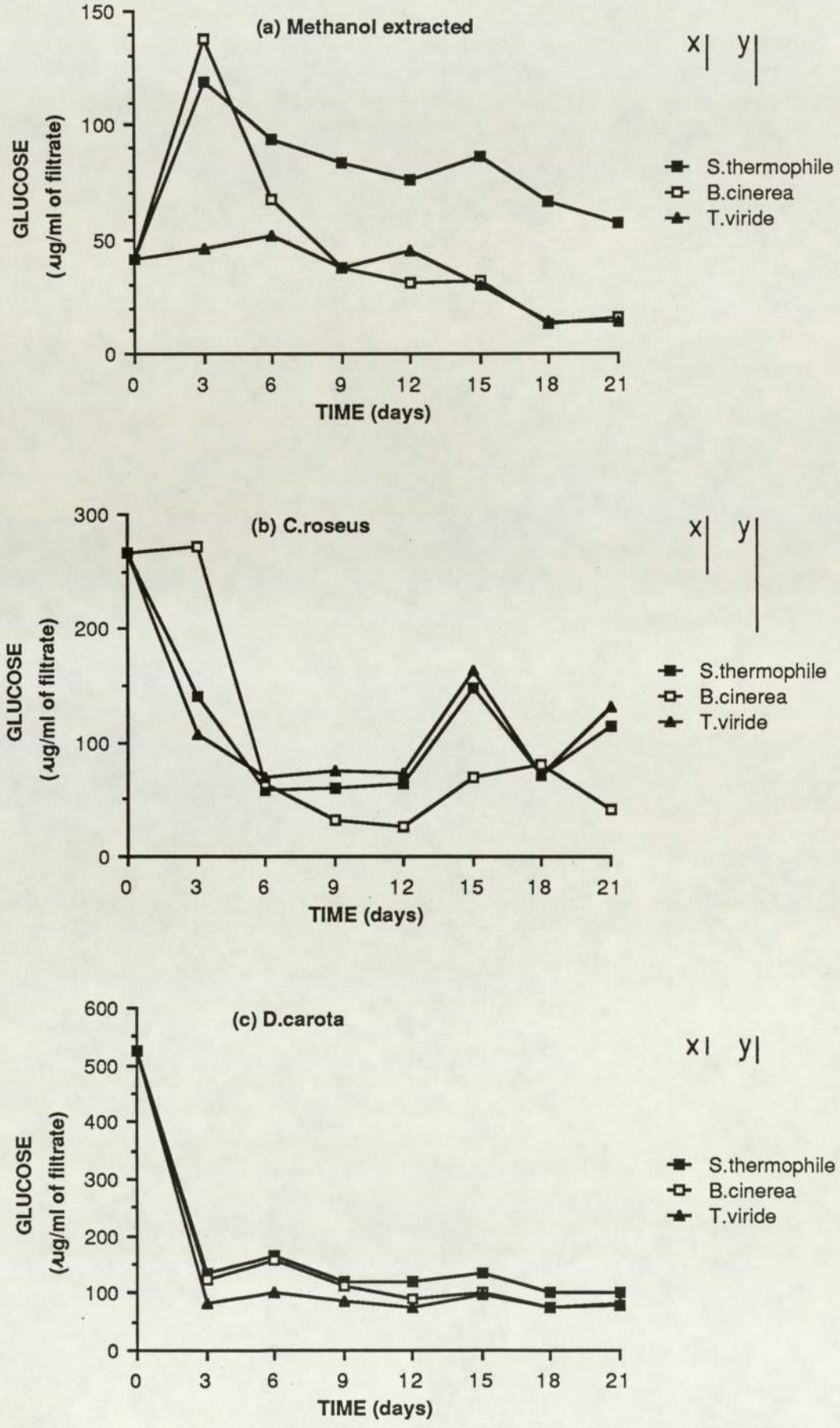
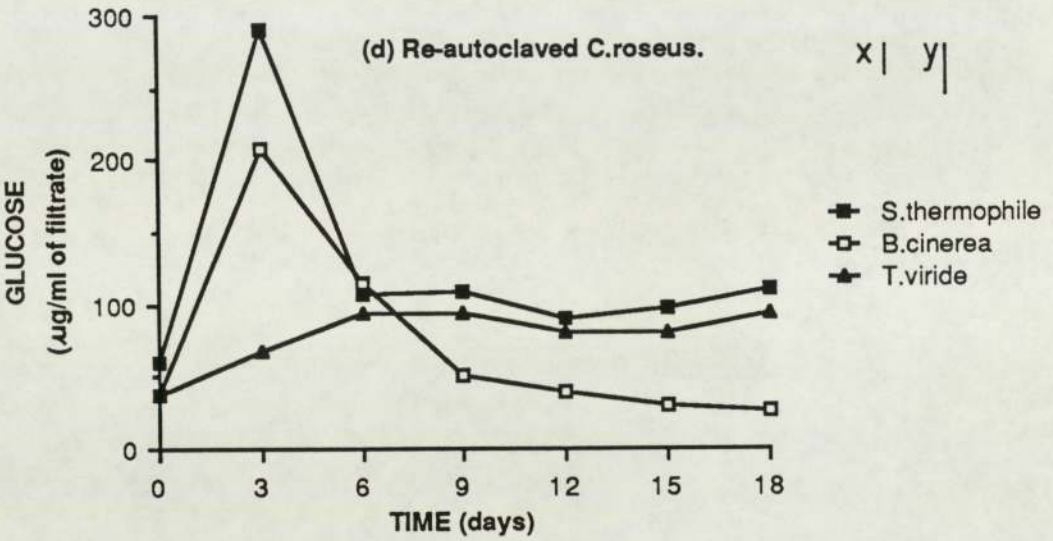
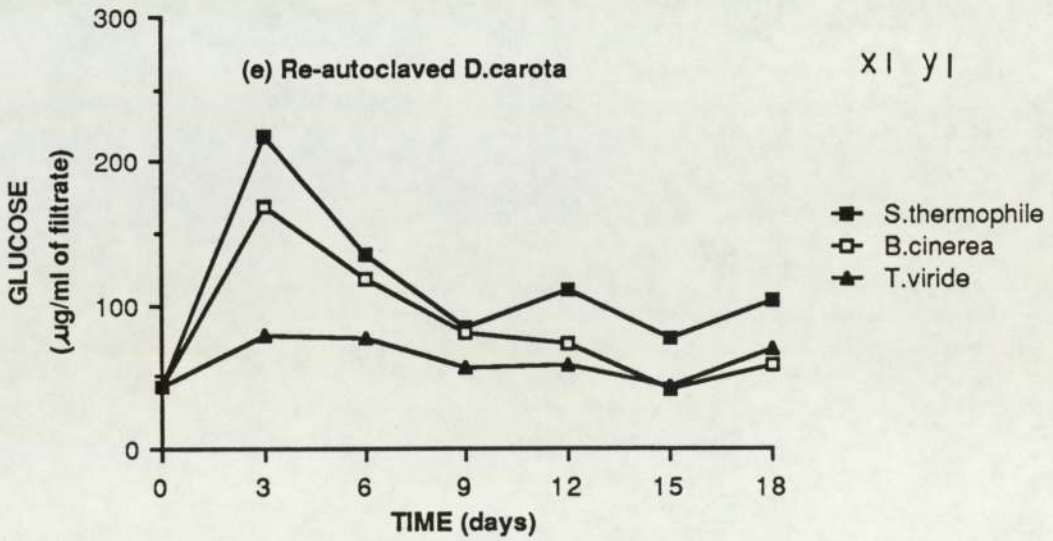


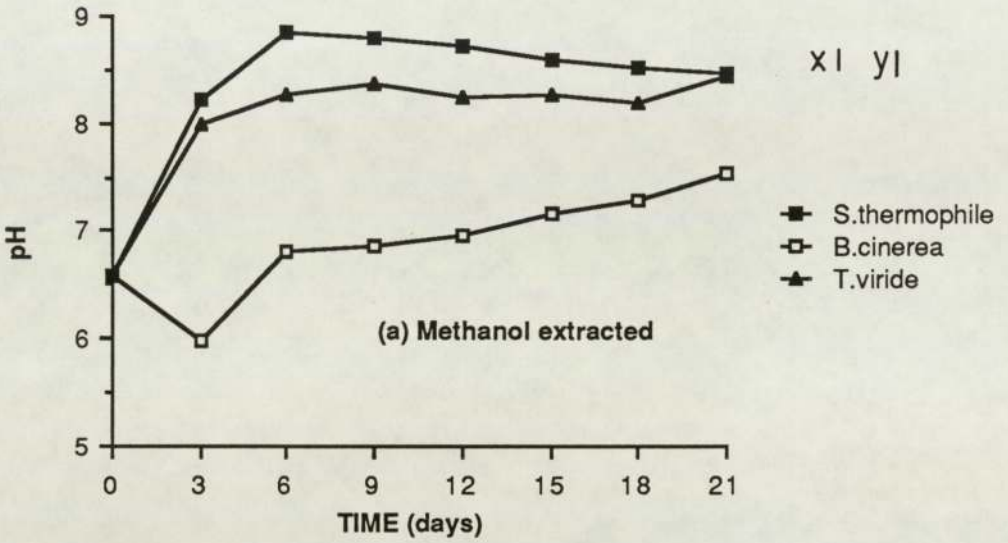
FIGURE 3:3 (continued)

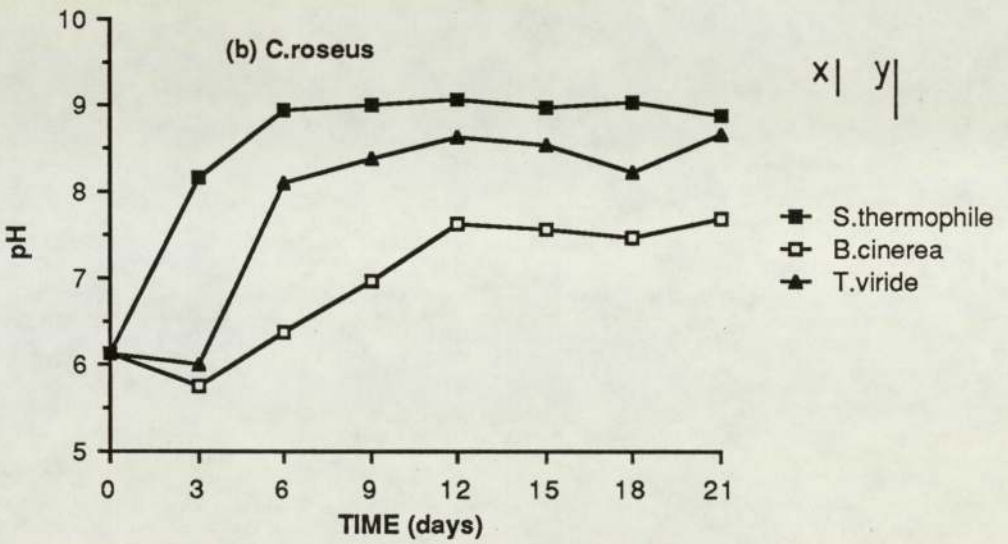




X=95% confidence limits for differences within a treatment and Y=95% confidence limits between two treatments at the same or different times.

FIGURE 3:4 (a-e) pH of the culture filtrate -static culture.





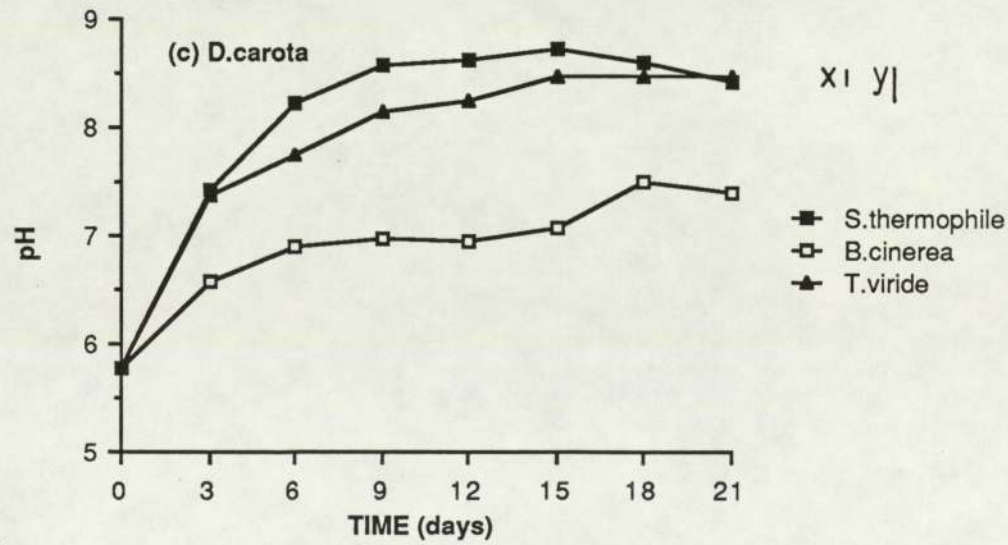
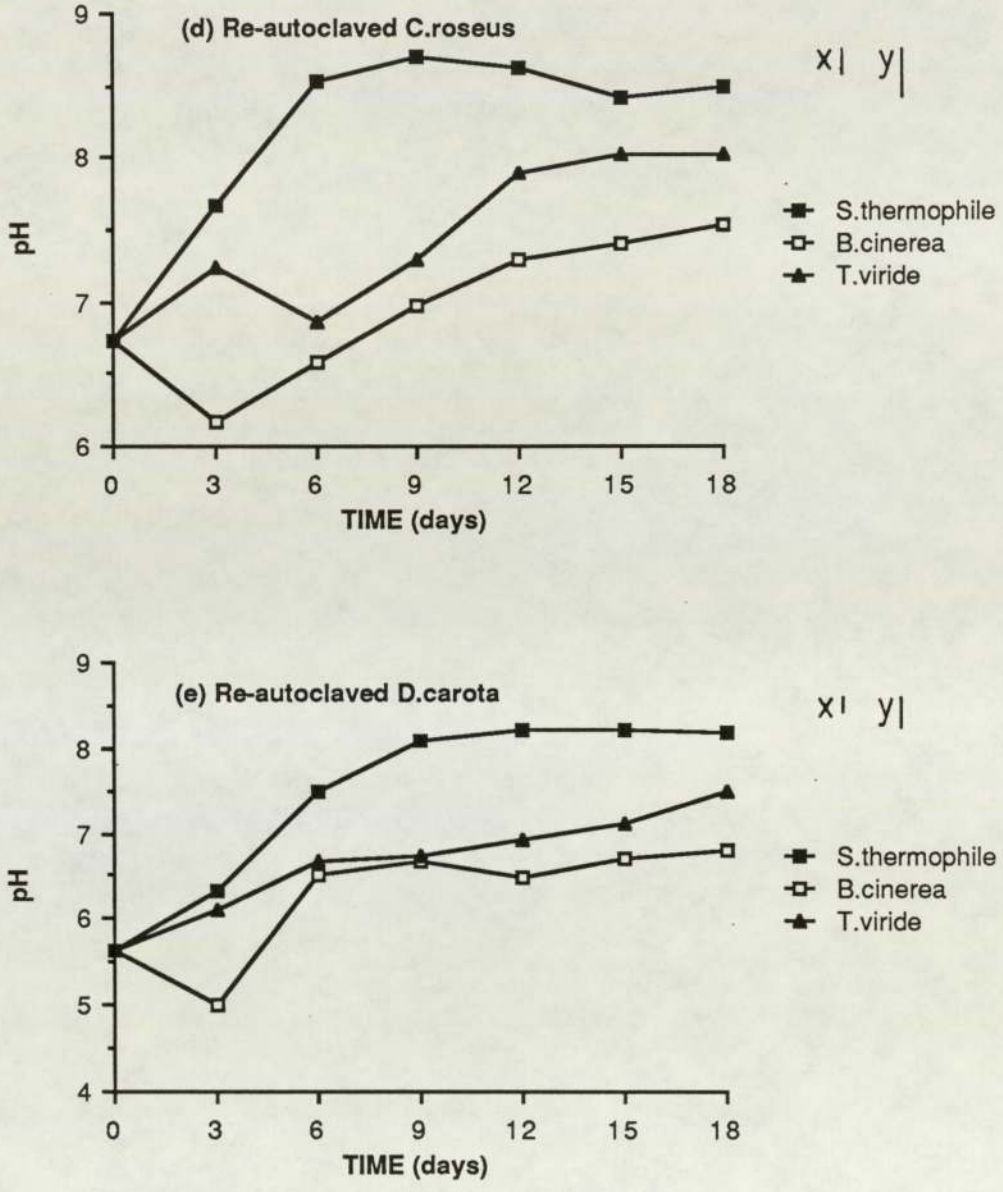


FIGURE 3:4 (continued)



X=95% confidence limits for differences within a treatment and Y=95% confidence limits between two treatments at the same or different times.

FIGURE 3:5 (a-c) Concentration of mannitol in fungi grown on three plant cell substrates-static culture.

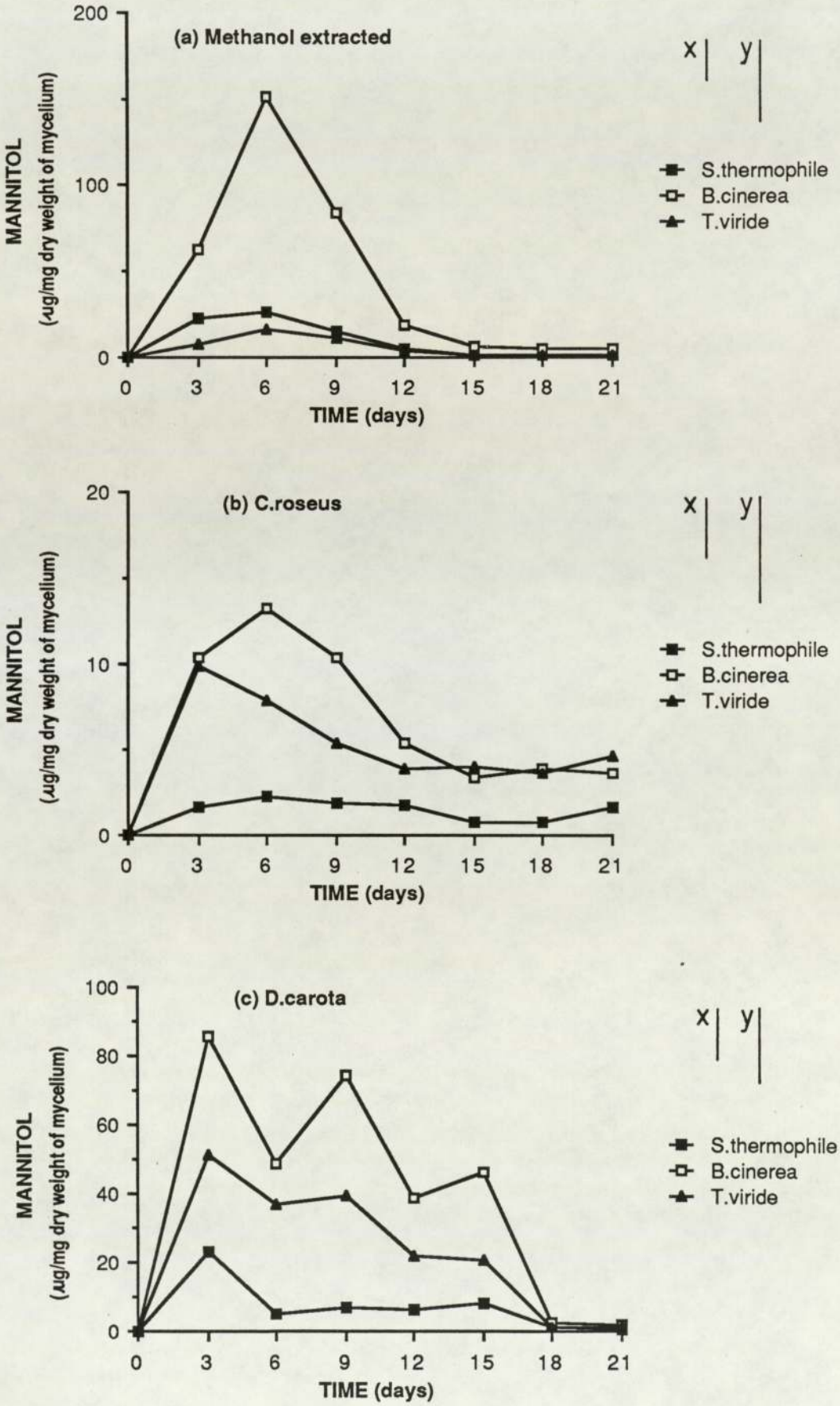
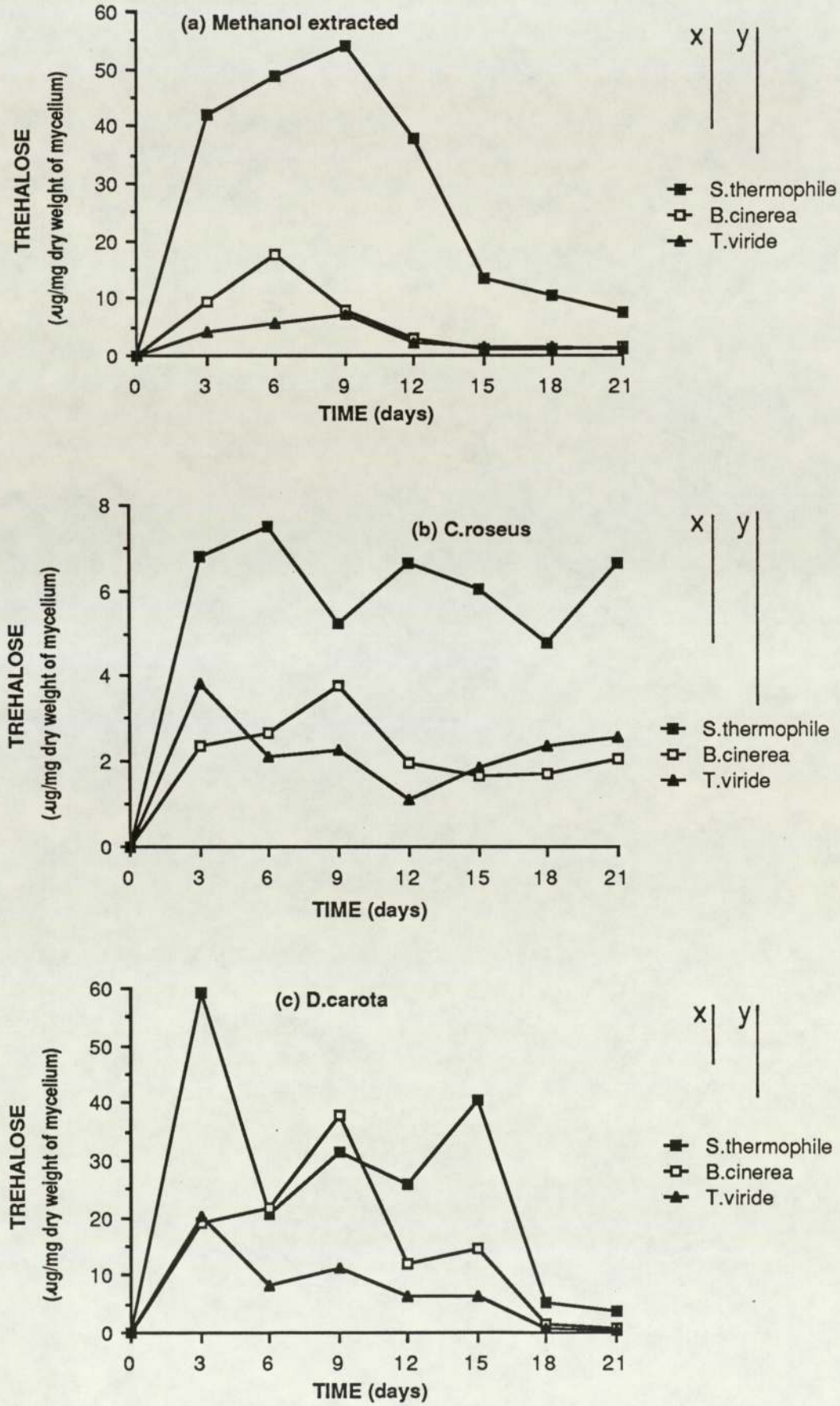


FIGURE 3:6 (a-c) Concentration of trehalose in fungi grown on three plant cell substrates-static culture



is an initial decrease in pH at Day 3 supported by B.cinerea when grown on a majority of substrates. Also S.thermophile which generates the greatest increase in pH departs from such a pattern with a gentle fall in pH after approximately 18 days.

In common with the reported changes in free glucose and pH outlined above, incubating plant cell biomass with fungi also influences the soluble carbohydrate present. On all 3 types of plant cells tested (methanol extracted, C.roseus and D.carota cells), B.cinerea produced greatest amounts of the sugar alcohol mannitol (Figure 3:5 a - c), conversely S.thermophile produced the most trehalose (Figure 3:6 a - c). Maximum production occurred early on in the experiment, within 3 - 9 days of inoculation. In all cases except S.thermophile on C.roseus and D.carota cells, the levels of mannitol found were of a greater magnitude than for trehalose.

Finally, in addition to data from Chapter 2 the percentage cellulose in re-autoclaved C.roseus (11.25) and re-autoclaved D.carota (10.5) was determined and found in both cases to be greater than for C.roseus (6-9) or D.carota (5-7) plant cell substrates.

DISCUSSION

Although these results would seem to indicate all five plant cell substrates tested are capable of sustaining fungal growth without the need for nutrient supplementation, a distinction can be made between plant cell type with respect to glucose produced. Thus only methanol extracted and re-autoclaved cells released quantities of glucose which exceeded that of the controls when incubated with fungi. Glucose and any other sugar produced in this way are of particular value as they may be further utilized e.g., by yeast fermentation to SCP or ethanol (Cysewski and Wilke, 1976; Toyama and Ogawa, 1977). In all other cases reducing sugar readings for the entire incubation period were less than those of Day 0.

This release of glucose may be accounted for by the different percentages of cellulose present in re-autoclaved (and methanol extracted) compared to C.roseus or D.carota cells. The re-autoclaving process is likely to remove soluble components from plant cell biomass. Thus leaving (for fungal utilization) a greater percentage of the dry weight as insoluble polysaccharide compared to ordinary cells.

Another factor is the initial level of glucose present (i.e., Time 0 values) which for example with D.carota cells was over ten times greater in ordinary compared to re-autoclaved cells. Such high levels of glucose may have resulted in catabolite repression (Magasanik, 1961), reducing the synthesis of cellulase enzymes (which in turn breakdown plant cell biomass releasing glucose). Ryu and Mandels (1980) suggested most cellulase is released after residual

reducing sugar concentration falls below 0.1 mg/ml. In these experiments using C.roseus and D.carota cells the initial level of glucose was > 0.2 and > 0.5 mg/ml respectively. Whereas in re-autoclaved samples this value was approximately 0.05 mg/ml.

Mandels, Sternberg and Andreotti (1975) suggested progress in cellulose utilization may be indicated by following changes in media pH. In this study with all fungal species and plant cell substrates, pH rose throughout the incubation period (with B.cinerea this may have been preceded by an initial decrease). However, Mandels, Sternberg and Andreotti (1975) found T.reesei QM 9414 to be a vigorous acid producer when grown on a medium containing purified cellulose and ammonium sulphate as nitrogen source (Trichoderma cannot use nitrates) with pH falling from 5 to 3 within 2 days (on uptake of NH_3 there is a concomitant accumulation of H^+ in the medium). In contrast, when Knapp and Legg (1986) cultured T.reesei QM 9123 (parent of QM 9414) on the above medium with Wheat straw (also contains hemicellulose, lignin, pectin) as the major carbon source, pH rose from 5 to 7.5 within 7 days. Coutts and Smith (1976) also found with S.thermophile a similar decrease in pH (to that of QM 9414) when ammonium salts were used in a medium containing Solka Floc. However, if replaced by sodium nitrate, pH rose to 7 and remained alkaline throughout the incubation period. Thus the pH profile obtained can not only vary between species but for an individual species can be influenced by both purity of cellulose and nitrogen source.

The ability of plant cell biomass to sustain the growth of fungi suggests it may be used to produce SCP or a combined mycelial/plant

cell biomass material with potential as an animal foodstuff. Enhanced yields could be obtained by pairing individual plant cell substrates and fungal species to give maximum biomass conversion values i.e., S.thermophile on re-autoclaved D.carota, B.cinerea on re-autoclaved C.roseus and T.viride on D.carota. It must however be noted, the high dry weight production values of T.viride may be due to its mode of growth on plant cells as the other two species grew at the plant cell-liquid interface and could be easily lifted off. Whereas T.viride grew as an integrated plant cell-mycelial mat which even after cleaning probably still contained plant cell material so adding to dry weight values.

A number of studies have investigated production and use of fungal mycelium in animal diets. Gray and Staff (1967) estimated the calorific value of Botrytis, Sporotrichum and Trichoderma to be 4.16, 4.29 and 4.5 kcal/g respectively (pure vegetable oil at 8.84). While Smith, Palmer and Reade (1975) assessed a number of filamentous fungi including T.viride as protein sources for rats and pigs. All fungal species tested showed similar essential amino acid profiles. Neither rats or pigs showed any ill effects or loss of appetite when fungi were added to test diets. However, when the fungal amino acid profile was compared to the requirements of growing animals they were found to be deficient in the sulphur containing amino acids. Also both Chaetomium cellulolyticum (up to 40%, Moo-Young, Chahal, Swan and Robinson, 1977) and Sporotrichum pulverulentum (Thomke, Rundgren and Eriksson, 1980) have been used as diet protein replacements with no adverse pathological reactions. However, in the latter case, mycoprotein was found to be inferior to more conventional dietary components e.g., soybean oil meal. In addition many cellulosic

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wastes e.g., straw and sawdust have little nutritional value as animal feeds. However their dietary value can be upgraded through a controlled fungal hydrolysis, with the final product (a combination of substrate and mycelium) having both an increased protein content and improved digestibility. For example, Han and Anderson (1975) reported a 3 and 5 fold increase in crude protein and fat respectively through action of T.viride on pretreated rye grass straw.

In all processes involving microorganisms the cultural conditions employed are very important. In the next chapter the effect of shaking on fungal breakdown of plant cell biomass is investigated for comparison with data outlined in this chapter.

CHAPTER FOUR

GROWTH OF THREE SPECIES OF FUNGI ON PLANT CELL BIOMASS - SHAKE CULTURE

INTRODUCTION

Shake flask techniques were developed by Kluyver and Perquin (1933) primarily for the submerged culture of fungi. Since that time shake flasks have become a routine tool for laboratory studies involving aerobic microbial processes. As a consequence of the relative ease of carrying out a large number of tests at one time, such tasks as primary screening, development of media or strain selection are nearly always done in shake cultures before fermenter studies are carried out.

Agitation provided by shaking is required to suspend cells and nutrients evenly through the medium, aid heat transfer and to render nutrients available to the cells. It is also needed to facilitate transfer of oxygen to the liquid, thus agitation allows growth of aerobic microorganisms to high densities.

In the laboratory rotary shakers are generally used as their action avoids throwing organisms out of the medium resulting in adhesion to the walls above the liquid level. An advantage particularly marked with filamentous fungal cultures. Gaden (1962) suggested aeration in shake flasks appears to take place primarily by absorption of oxygen in the thin film of fluid deposited on the flask wall. He also suggested a marked improvement in aeration efficiency could result by breaking up these films with an independent baffling surface. However such baffles can cause foaming which is in turn detrimental to oxygen absorption.

In large scale cultivations of micro organisms using fermenters,

oxygen is supplied by passing air through the vessel, with impellers facilitating mixing. In Tower fermenters however, air is introduced at the bottom and rising air bubbles are used to mix the medium as no mechanical agitation is employed. As with other environmental parameters, the regulation of many biochemical pathways is influenced by oxygen levels. However maximum aeration is not necessarily the main objective as over aeration may prove detrimental to the fermentation. Foster (1949) found excess aeration had an adverse effect on the accumulation of gallic acid produced by Aspergillus niger. Similarly, Jensen and Shu (1961) reported high aeration resulted in reduced production of lysine in yeast (S.cerevisiae) although too little air reduced both cell yield and lysine accumulation. Reasons for the deleterious effects of high aeration on microorganisms are not as yet fully understood. However Kristiansen and Chamberlain (1983) have suggested that formation of free radicals may cause cell damage or at higher aeration rates CO_2 gas (which is often desirable for good growth) is forced out of the culture medium.

In this study, the use of shake culture was assessed for its effect on fungal dry weight production, and filtrate pH. In addition filtrate glucose levels were monitored and by comparison with data obtained during static culture (Chapter 3) optimum cultural conditions for fungal breakdown of plant cell biomass are elucidated.

MATERIALS AND METHODS

1. PREPARATION OF FLASKS FOR INOCULATION

Dried cells were added to water in a 100 ml Erlenmeyer flask at a concentration of 1% (w/v). These were then autoclaved at 115°c, 20 minutes (10 psi). The type of cells used were:-

(i) C.roseus plant cells

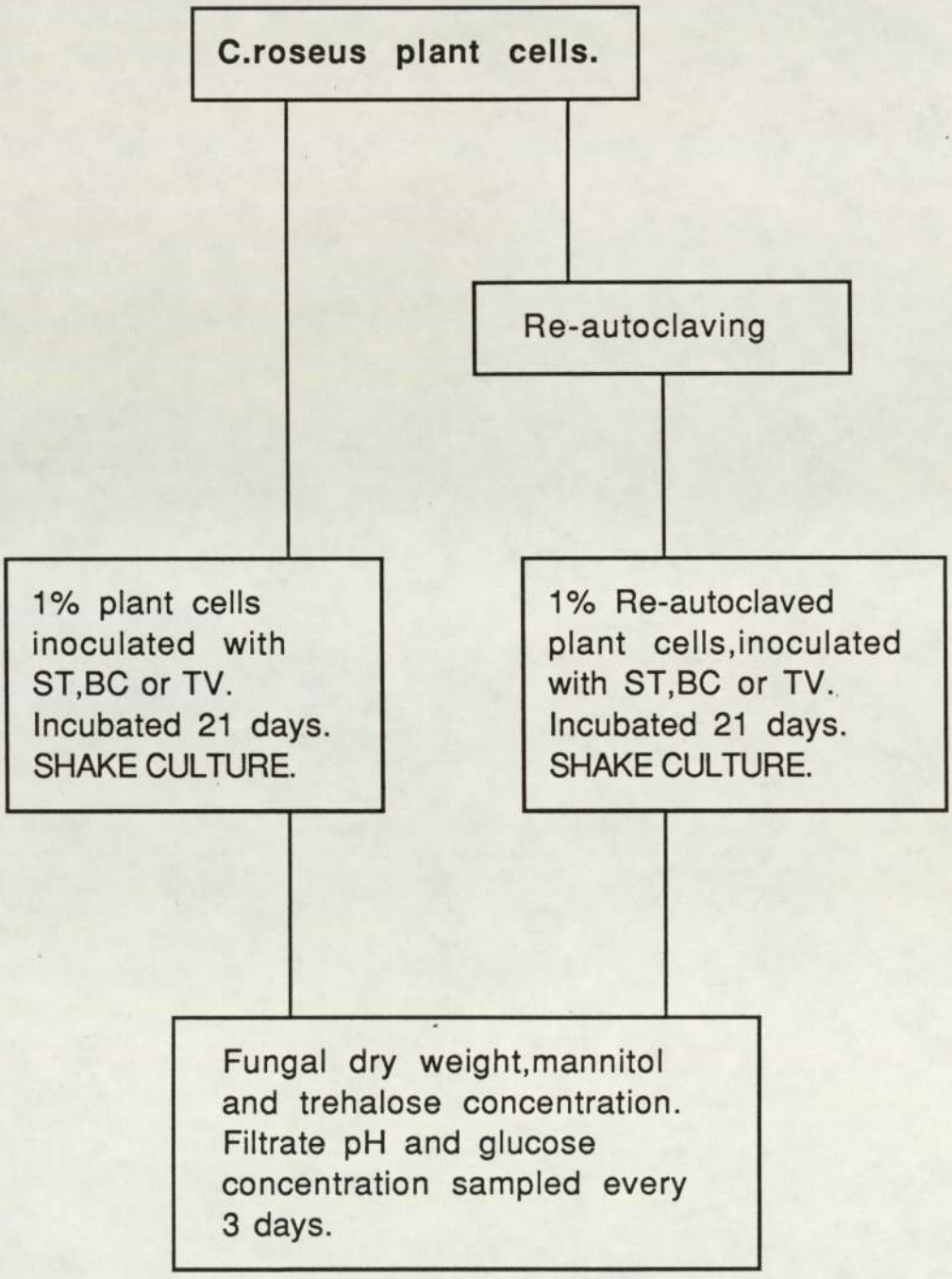
(ii) Re-autoclaved C.roseus plant cells

Re-autoclaved cells were prepared by making up 1% concentrations of cells as above. After autoclaving these cells were filtered, dried and used again to make a 1% concentration. After a second autoclaving these cells and those of (i) were inoculated with spores from cultures (maintained on malt extract agar) of B.cinerea, T.viride and S.thermophile. Once inoculated, flasks were incubated on a LKB orbital shaker at 100 r.p.m at either 25°c (B.cinerea, T.viride) or 40°c (S.thermophile). Uninoculated flasks were also prepared to act as controls. See Figure 4:1.

2. GROWTH

This was measured in individual species over 18 - 21 days (values determined every 3 days) by removal of fungal mycelium from Erlenmeyer flasks onto dry pre-weighed Whatman No 1 filter papers. After drying to a constant value dry weight was determined.

FIGURE 4:1 Flow diagram of the procedure used to investigate fungal growth on plant cell biomass -shake culture.



ST=S.thermophile BC=B.cinerea TV=T.viride

3. PH OF THE MEDIUM

After removal of the fungal mycelium any remaining plant cell material was filtered off. The pH of this filtrate was determined using a Pye-unicam pH meter.

4. REDUCING SUGAR CONCENTRATION OF THE FILTRATE

This was assessed using the method of Nelson (1944), to 1ml of filtrate, 1ml of copper reagent was added (see Appendix 3:1). After boiling for 20 minutes, the solution was cooled before addition of 1ml arsenomolybdate reagent (BDH). Optical density of the resulting blue colour was estimated at 660 nm on a spectrophotometer against a reagent blank and the concentration of glucose determined by comparison to a standard curve. (Appendix 3:2).

5. SOLUBLE CARBOHYDRATES

These were determined in the manner outlined in Chapter 2.

6. STATISTICAL ANALYSIS

Analysis of variance was determined using a 3 x 6 (7) factorial split plot analysis in a randomised design.

RESULTS

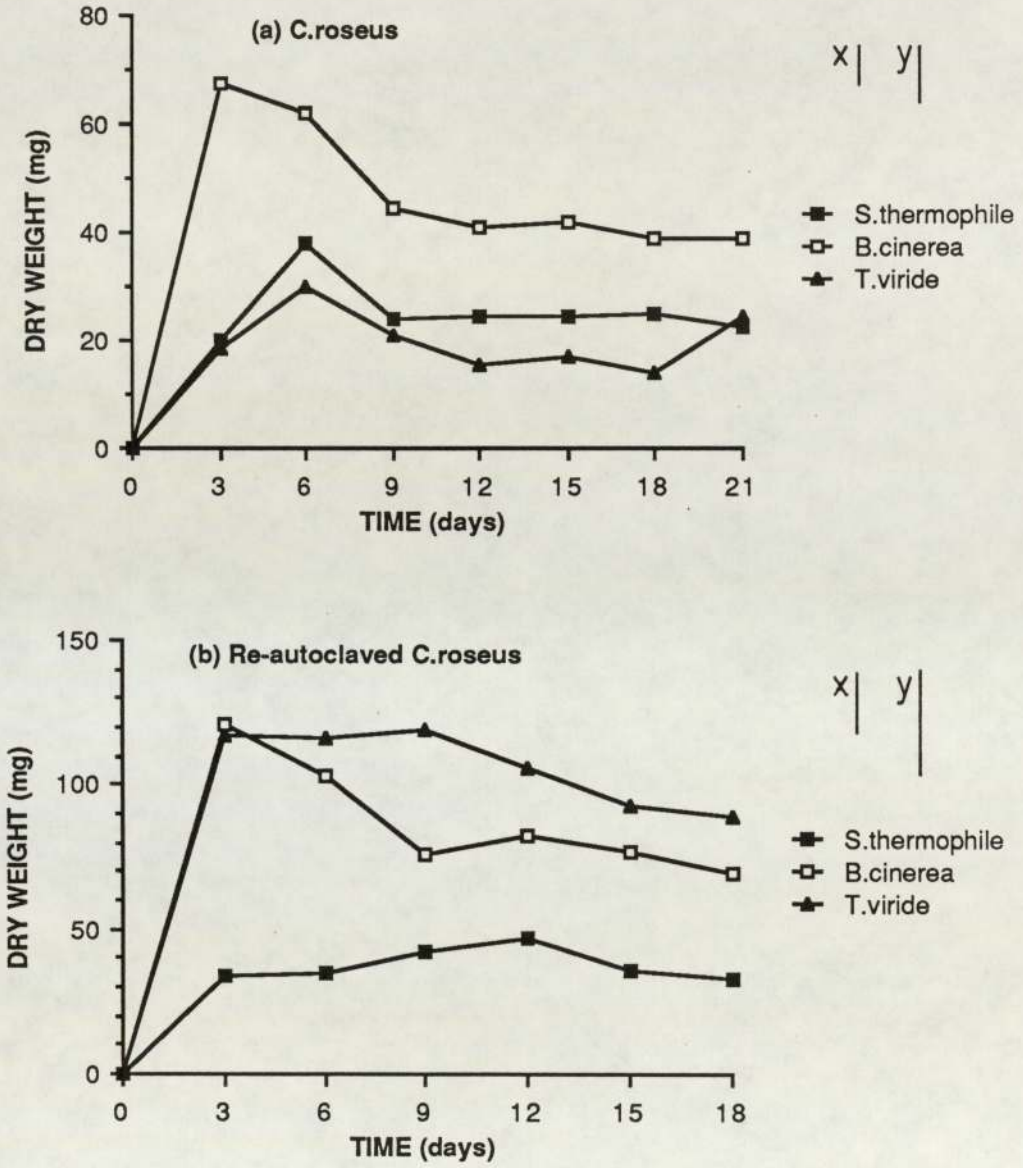
Figure 4:2a - b (data given in Appendix 4:1 - 4:5) indicates that shake cultures of C.roseus and re-autoclaved C.roseus plant cells are able to support fungal growth. However individual species showed significantly different patterns of biomass production (determined as dry weight), which in turn is influenced by plant cell substrate. The amount of plant cell material converted by S.thermophile to fungal biomass is very similar between both plant cell types (approximately 20%). Whereas B.cinerea produced nearly twice as much biomass on re-autoclaved C.roseus cells (60.26%) compared with C.roseus cells (32.84%). T.viride however, (14.88%) gave the lowest conversion value of the three species (on C.roseus cells).

On C.roseus plant cells, the three fungi did not release significant amounts of glucose (Figure 4:3a). However these same fungi when grown on re-autoclaved C.roseus cells (Figure 4:3b) released 100 - 200% more glucose than controls.

Figure 4:4a - b shows the pattern of pH change between species and plant cell type to be similar. An initial sharp rise in pH between Days 3 - 6 is followed by a more gradual change over the remaining experimental period. However B.cinerea on re-autoclaved C.roseus cells exhibits a drop in pH at Day 3. Also on this substrate a slight decrease in pH at Day 18 is exhibited by all three species.

Incubating plant cell biomass with fungi also affects the

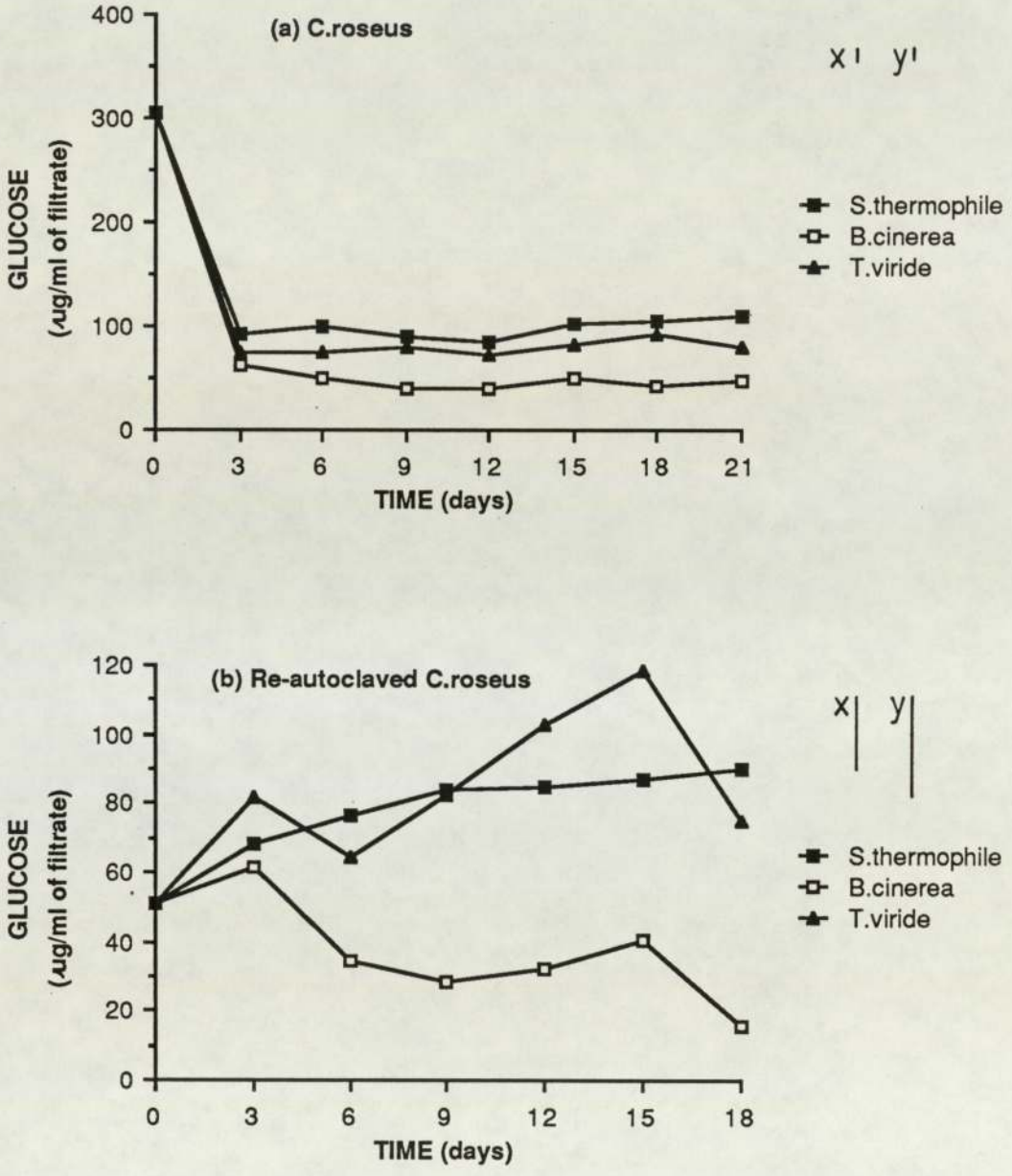
FIGURE 4:2 (a-b) Fungal dry weight production on two plant cell substrates-shake culture.



For T.viride dry weight=plant cell+fungal biomass

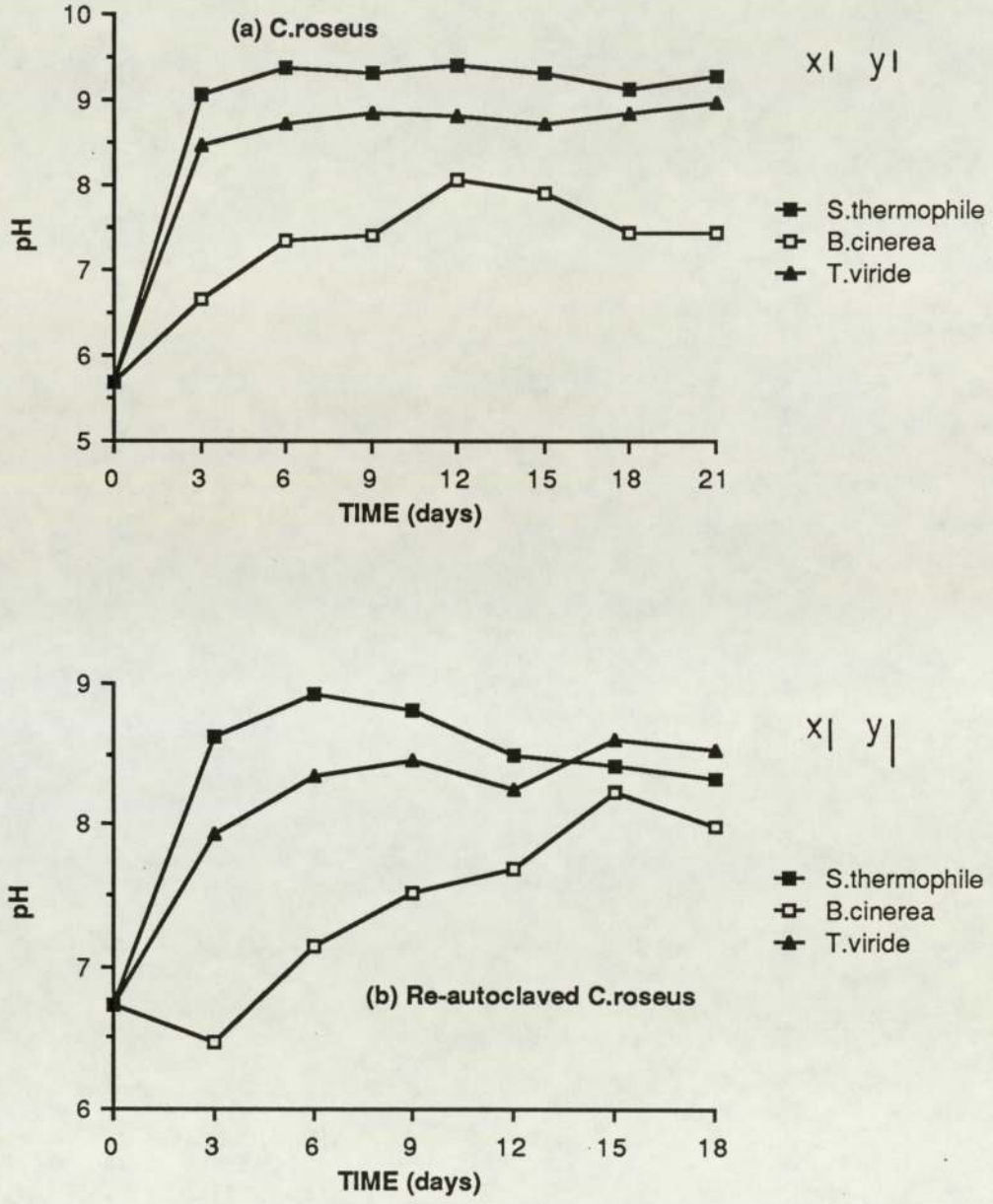
X=95% confidence limits for differences within a treatment and Y=95% confidence limits between two treatments at the same or different times.

FIGURE 4:3 (a-b) Glucose release from two plant cell substrates -shake culture.



X=95% confidence limits for differences within a treatment and Y=95% confidence limits between two treatments at the same or different times.

FIGURE 4:4 (a-b) pH of the culture filtrate -shake culture



X=95% confidence limits for differences within a treatment and Y=95% confidence limits between two treatments at the same or different times.

FIGURE 4:5 Concentration of mannitol in fungi grown on plant cell biomass-shake culture.

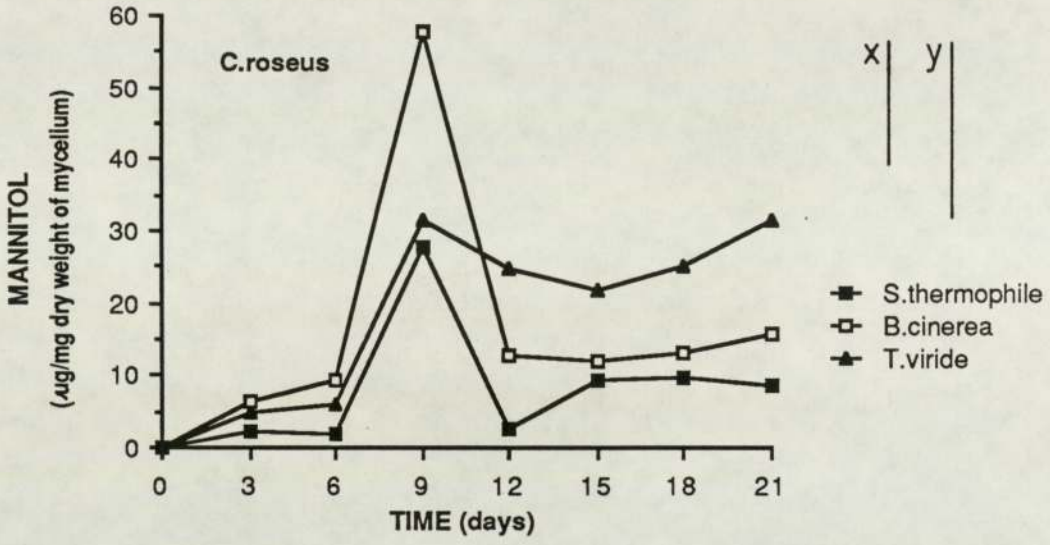
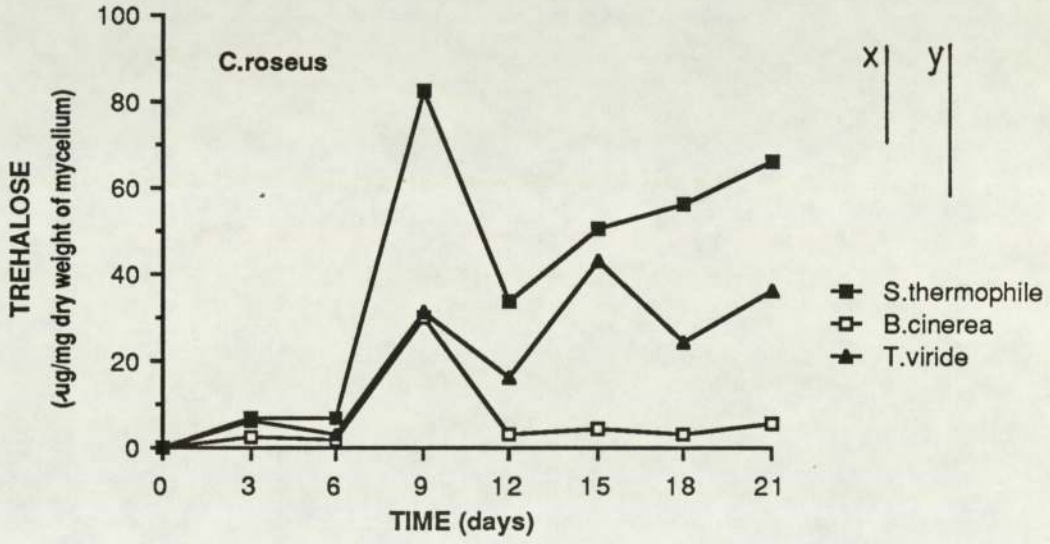


FIGURE 4:6 Concentration of trehalose in fungi grown on plant cell biomass-shake culture.



soluble carbohydrate present. Using C.roseus cells only B.cinerea produced the greatest amount of mannitol (Figure 4:5) whereas S.thermophile exhibited the highest level of trehalose (Figure 4:6). In addition for all fungi except B.cinerea the level of trehalose produced is greater than for mannitol.

DISCUSSION

Agitation of cultures aids transport of nutrients and oxygen to the single organism and the dispersion of metabolic products or heat away from it. However, by which ever method employed, forces are generated which can affect microbial cultures in a number of ways. Such forces can for example influence morphology. In this study, cultures of B.cinerea tended to form pellets on shaking. Whitaker and Long (1973) have reviewed various aspects of fungal pelleting, formation of which can also be influenced by medium composition, pH, size of inoculum and type of culture vessel. The development of a particular morphological form may also be important for product formation, e.g., pellet growth is desired in the production of citric acid (Metz and Kossen 1977).

Agitated cultures of fungi have been found to release intracellular material to the surrounding medium. Tanaka, Takahashi and Ueda (1975); Tanaka, Mizuguchi and Ueda (1975) reported agitation of mycelial suspensions including Mucor javanicus and Rhizopus javanicus resulted in leakage of RNA related nucleotides, although no visible damage to the mycelium occurred. Composition of these nucleotides was the same regardless of culture apparatus (jar fermenter or shake flask). However rate of leakage was dependent on agitator speed (increasing at higher speeds). Using these above observations, a method for estimating the intensity of agitation shock on mycelia was proposed. When tested, data showed the shock in a jar fermenter to be 2 - 3 times that for a flask culture. Further work by Tanaka and Ueda (1975) found biomass production by Mucor javanicus was inhibited with increasing agitation. Investigations

showed the growth rate of this fungus to be dependent on the ratio between synthesis of high molecular weight nucleotides and leakage of low molecular weight nucleotides. With higher agitation speeds leading to leakage in excess of synthesis rates. In this study, dry weight production by T.viride on C.roseus plant cells was particularly affected by shaking (compared to static culture). In contrast, higher values (on shaking) were recorded for B.cinerea on both C.roseus and re-autoclaved C.roseus cells, whereas for S.thermophile there was no real difference. These findings might therefore reflect the susceptibility of these species towards nucleotide leakage, which is in turn related to mycelial strength (Tanaka, Mizuguchi and Ueda, 1975).

In contrast, Ujocova, Fencl, Musilkova and Seichert (1980) using Aspergillus niger S59 found lower leakage of nucleotides at higher stirring speeds. A possible reason for this was high agitation produced a morphologically compact, thick walled, highly septated mycelium, whereas at lower speeds long, thin, sporadically branching filaments with few septa were found. Increased dry weight production from B.cinerea on shaking in this study may also be related to the above finding. As the pellet form is a compact structure and may therefore be less susceptible to forces generated through shaking. Whereas the diffuse mycelium of T.viride may be much weaker.

Agitation has also been shown to affect microbial metabolism. Konig, Seewald and Schugerl (1981) studied the effect of stirring speed on growth and penicillin production from Penicillium chrysogenum. Results indicated at high stirring speed although maximum biomass levels were achieved, penicillin concentrations

remained low. Ujocova, Fencl, Musilkova and Seichert (1980) have also shown production of citric acid from A niger is highly dependent on agitator speed.

In this study agitation of fungi results in the accumulation and subsequent mobilization of reserve carbohydrates such as trehalose and mannitol. However by comparing levels of mannitol and trehalose detected in fungi growing on the same plant cell substrate but under static or shake conditions, differences in both the levels recorded and pattern of production with respect to time are found. Thus providing a further indication of the effect that incubation condition has on fungal metabolism. In all three fungi higher levels of both trehalose and mannitol were noted under shake conditions. Although only B.cinerea during both static and shake culture complied with the findings of Lewis and Smith (1967) in which maximum mannitol levels were generally found to exceed those of trehalose.

Some similarities do exist between data derived from static and shake cultures e.g., the pH profiles are very similar. Although taken in its entirety data from Chapters 3 and 4 indicates that in general for maximum fungal dry weight production and glucose release (products which may be utilized as SCP or as a starting point for further fermentations) static conditions and a re-autoclaved substrate are advantageous. The only exception being B.cinerea for which shake culture results in maximum dry weight production.

CHAPTER FIVE

FUNGAL C_1, C_x, B-GLUCOSIDASE AND ENDO-POLYGALACTURONASE ACTIVITY DURING BREAKDOWN OF PLANT CELL BIOMASS

INTRODUCTION

Production of some enzymes is referred to as constitutive implying that enzyme is formed regardless of environmental conditions. Other enzymes are termed inducible. In most inducible enzyme systems an inducer enters the cell either by active transport or diffusion. Once inside, such entities interact with repressor proteins causing derepression resulting in enzyme biosynthesis (Jacob and Monod, 1961).

Pectinases are in general inducible although examples of constitutive production have been reported (Ayers, Papavizas and Lumsden, 1969). These enzymes attack pectic material of the middle lemallae and primary cell walls with the most common pectinase produced by fungi being an endo-polygalacturonase (EC 3.2.1.15). Cellulases are also inducible enzymes; known inducers include cellulose, cellulose derivatives, cellobiose, sophorose and lactose. However as cellulose is insoluble and thus cannot enter a cell, it has been proposed by Mandels and Reese (1960) that a soluble product is the actual inducer. Their hypothesis suggests a small amount of cellulase is produced constitutively, which solubilizes cellulose thus providing a limited amount of cellobiose for further induction. Gong, Ladisch and Tsao (1979) produced direct evidence for this indicating that Trichoderma reesei secretes trace amounts of cellulase even under conditions of starvation.

The role of an inducer is complex. Mandels and Reese, (1960) found at low concentrations cellobiose is a potent inducer, while at high cellobiose levels cellulase synthesis is inhibited. Inducers

are also influenced by other environmental factors such as pH (Mandels, Sternberg and Andreotti, 1975, Romanelli, Houston and Barnett, 1975). Biosynthesis of cellulase is also repressed by glucose or other rapidly metabolised carbon sources. A phenomenon termed catabolite repression by Magasanik, (1961). Mandels, (1975) also found, even in the presence of inducers, cellulase synthesis can be strongly inhibited by glucose. Likewise pectinases are also subject to this form of repression.

Enzymes which make up the cellulase complex are:-

i) endo-B-1, 4-glucanase (EC 3.2.1.4), commonly known as CMCase or C_x cellulase

ii) exo-B-1, 4-glucanase (EC.3.2.1.91), commonly known as Avicelase or C_1 cellulase

iii) B - 1, 4-glucosidase (EC. 3.2.1.21)

In most mycelial fungi the above enzymes are extracellular. Therefore use of culture filtrates to study enzyme production is widely accepted and has been used in this study to investigate fungal enzyme activity during growth on plant cell biomass.

MATERIALS AND METHODS

1. PREPARATION OF FLASKS FOR INOCULATION

Flasks were prepared and inoculated as in Chapter 3. The type of plant cells and incubation regime used were:-

i) C.roseus - static culture

ii) C.roseus - shake culture

iii) Re-autoclaved C.roseus - static culture

iv) Re-autoclaved C.roseus - shake culture

v) Re-autoclaved D.carota - static culture

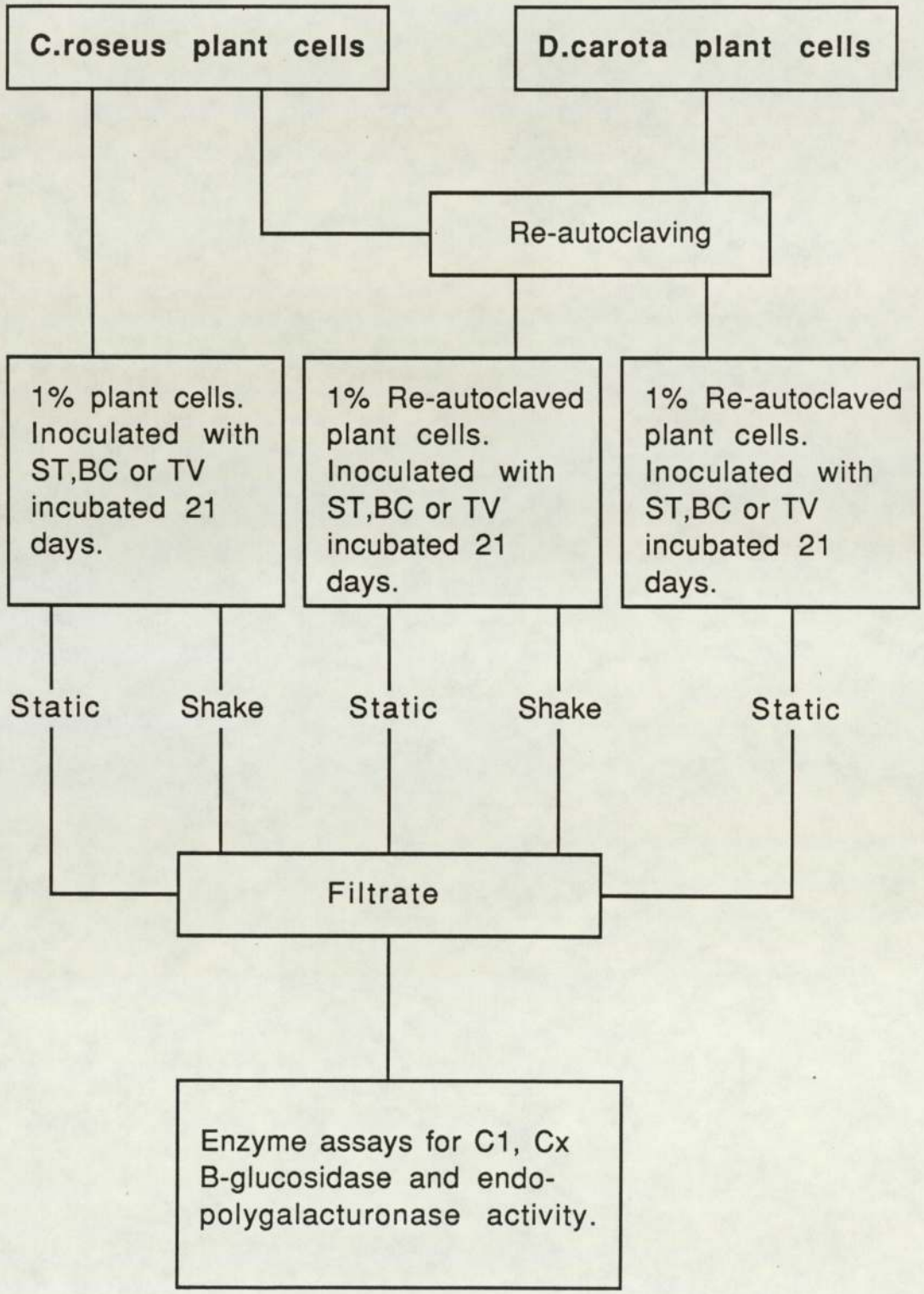
Substrates (iii - v) were prepared by autoclaving a 1% concentration of plant cells in distilled water in a similar manner to (i) and (ii) filtering off the biomass and after drying, re-autoclaving with distilled water at the above cell concentration. See Figure 5:1.

2. C_1 ENZYME ACTIVITY (Mandels, Hontz and Nystrom, 1974)

After the required length of incubation, contents of individual flasks were filtered (using a Whatman No 1 filter paper) and the filtrates used in this and subsequent assays for enzyme activity. An enzyme control was prepared in each case using the appropriate amount of filtrate boiled for 15 minutes.

5 ml of each filtrate (adjusted to pH 4.8 with 0.1 M HCl) and 250 mg absorbent cotton wool were incubated in a water bath (with shaking) at 50°c for 24 hours. The amount of reducing

FIGURE 5:1 Flow diagram of the procedure used to investigate fungal enzyme activity during growth on plant cell biomass.



ST=S.thermophile BC=B.cinerea TV=T.viride

sugar liberated (D-glucose) was measured using the method of Nelson (1944; see Chapter 3). Enzyme activity is expressed as ug glucose released/ml of filtrate.

3. Cx ENZYME ACTIVITY (Mandels, Hontz and Nystrom, 1974)

0.5 ml of each filtrate was mixed with 0.5 ml 1% carboxymethyl cellulose (Fisons) in 0.1M citrate buffer (pH 4.8, Appendix 5:1). After 30 minutes incubation (with shaking) at 50°c, 3 ml of DNSA reagent (Appendix 5:1) was added and tubes boiled for 5 minutes. Optical density was estimated at 550 nm against a reagent blank. The concentration of D-glucose was determined by comparison with a standard curve (Appendix 5:2). Enzyme activity is expressed as ug glucose released/ml of filtrate.

4. B-GLUCOSIDASE ACTIVITY (Shewale and Sadana, 1978)

0.2 ml of each filtrate was mixed with 1.8 ml P-nitrophenyl -B-D-glucopyranoside (BDH; 1mg/ml) in 0.1M citrate buffer pH 4.5 (Appendix 5:1). After incubation at 70°c for 30 minutes (with shaking) 2 ml of 1M sodium carbonate was added. Optical density of the resulting yellow colour was estimated at 410 nm against a citrate buffer blank. The concentration of nitrophenol released was determined by comparison to a standard curve (Appendix 5:3). Enzyme activity is expressed as ug nitrophenol released/ml of filtrate.

5. ENDO-POLYGALACTURONASE ACTIVITY (Kurian and Stelzig, 1979)

1 ml of each filtrate was mixed with 1.7 ml of 0.25% polygalacturonic acid (BDH) in 0.05M sodium citrate (pH 5).

After incubation (with shaking) at 30°c for 10 minutes, D-glucose release was measured using the method of Nelson (1944). Enzyme activity is expressed as ug glucose released/ml of filtrate.

6. STATISTICAL ANALYSIS

Analysis of variance was determined using a 3 x 6 (7) factorial split plot analysis in a randomised design.

RESULTS

Each of the three fungal species produced all four enzymes whatever culture conditions or plant cell type was utilized (Figures 5:2 (a - e) - 5:5 (a - e), Appendix 5:4 - 5:7). Within such an overall pattern a degree of variation may be observed with individual enzyme activity differing between species and with time.

Table 5:1 demonstrates that with few exceptions average enzyme activity values (calculated from the 18 - 21 day incubation period) are 100 - 400% higher under static compared to shake conditions for both C.roseus and re-autoclaved C.roseus plant cells. However the first peak in enzyme activity generally occurs earlier in shake cultures in contrast to their static counterparts. In addition, irrespective of culture mode (shaken/static), average enzyme activity levels are usually higher on re-autoclaved C.roseus compared to C.roseus plant cells. Furthermore, with re-autoclaved D.carota plant cells (static culture) C_x enzyme activity of all three fungal species is noticeably higher than with any other plant cell substrate or culture condition (static or shaken, see Table 5:1).

The four enzyme types do not appear to be produced in any marked temporal sequence by the fungi employed. However, Table 5:2 indicates on all substrates the maximum value for C_1 and endo-polygalacturonase activity occurs not later than 12 days (exceptions being T.viride and B.cinerea on re-autoclaved D.carota). Whereas for C_x and B glucosidase activity this period is extended to 18 days. For each individual enzyme the maximum activity found is associated with a particular fungal species (see Table 5:2). Thus for C_x, endo-polygalacturonase, C_1 and B-glucosidase, S.thermophile, B.cinerea and

FIGURE 5:2 (a-e) Fungal C_1 enzyme activity during growth on plant cell biomass.

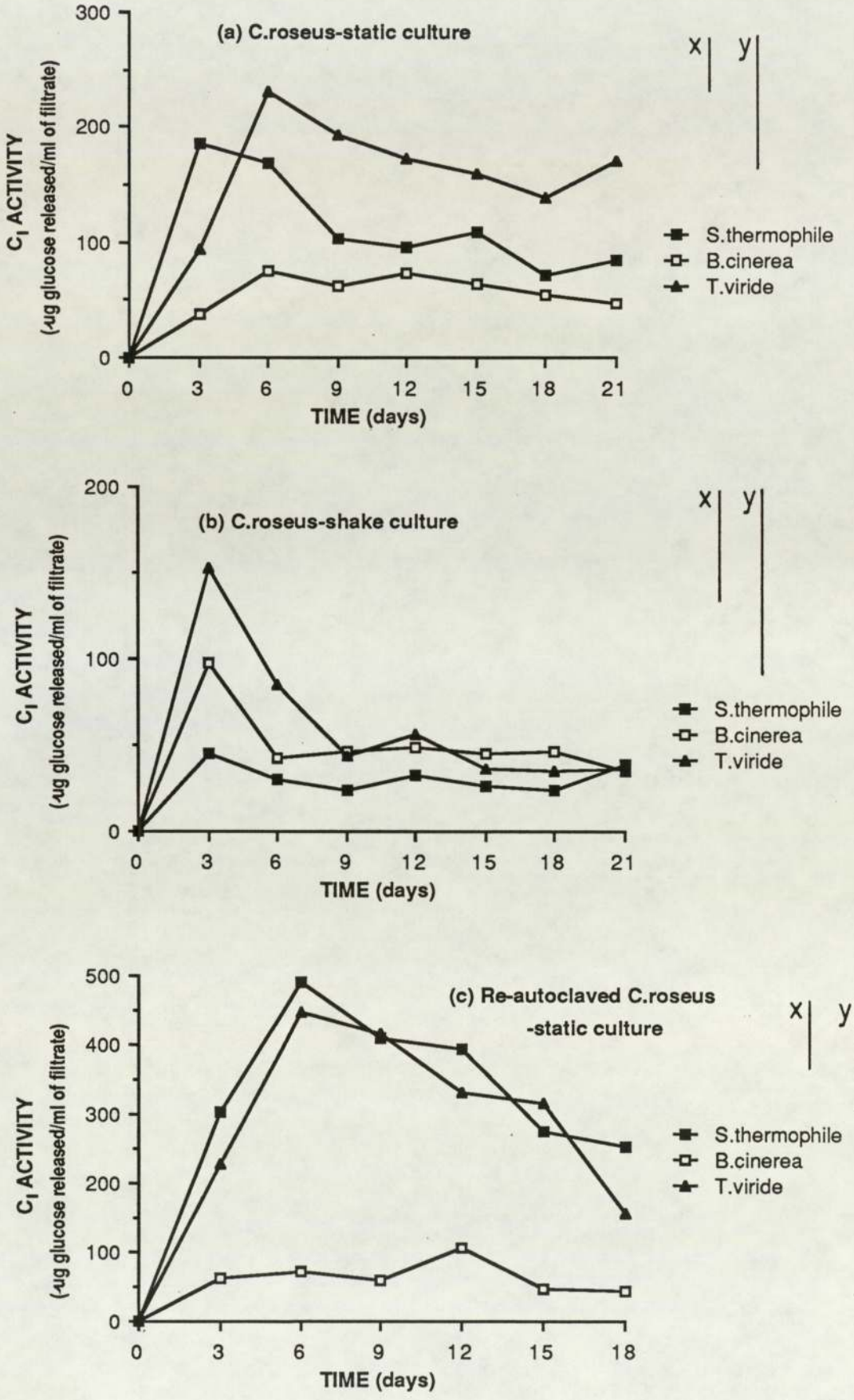
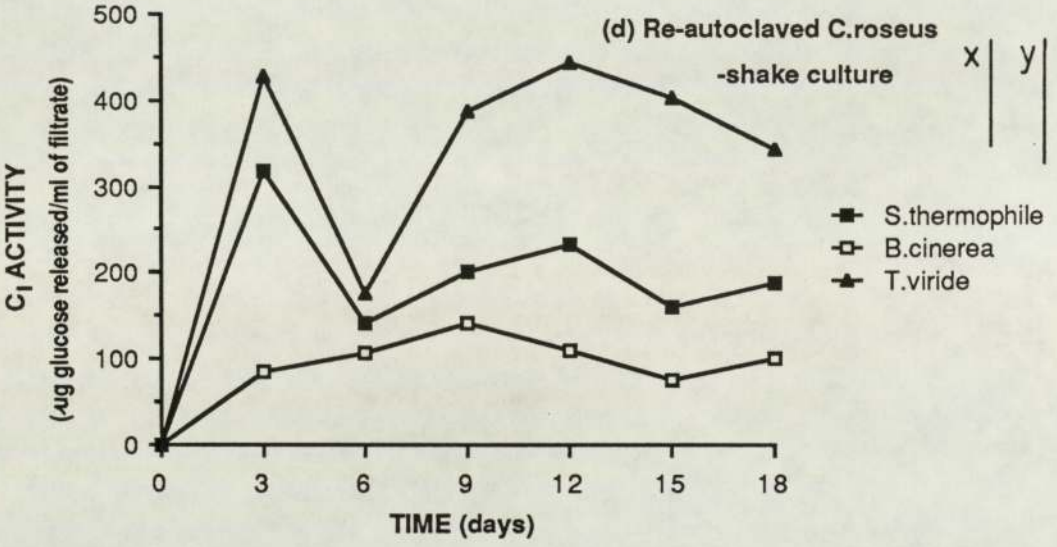
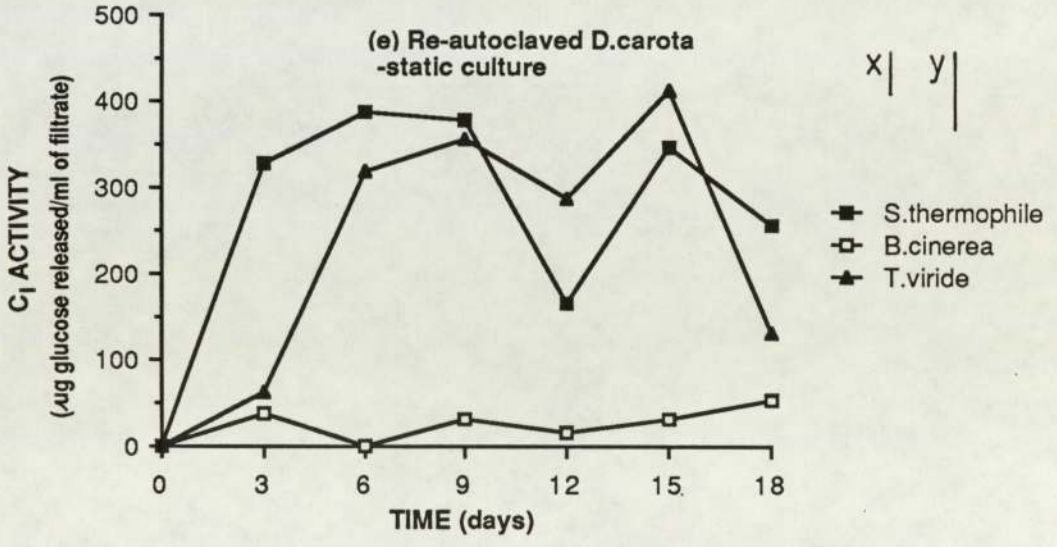


FIGURE 5:2 (continued).





X=95% confidence limits for differences within a treatment and Y=95% confidence limits between two treatments at the same or different times.

FIGURE 5:3 (a-e) Fungal Cx enzyme activity during growth on plant cell biomass.

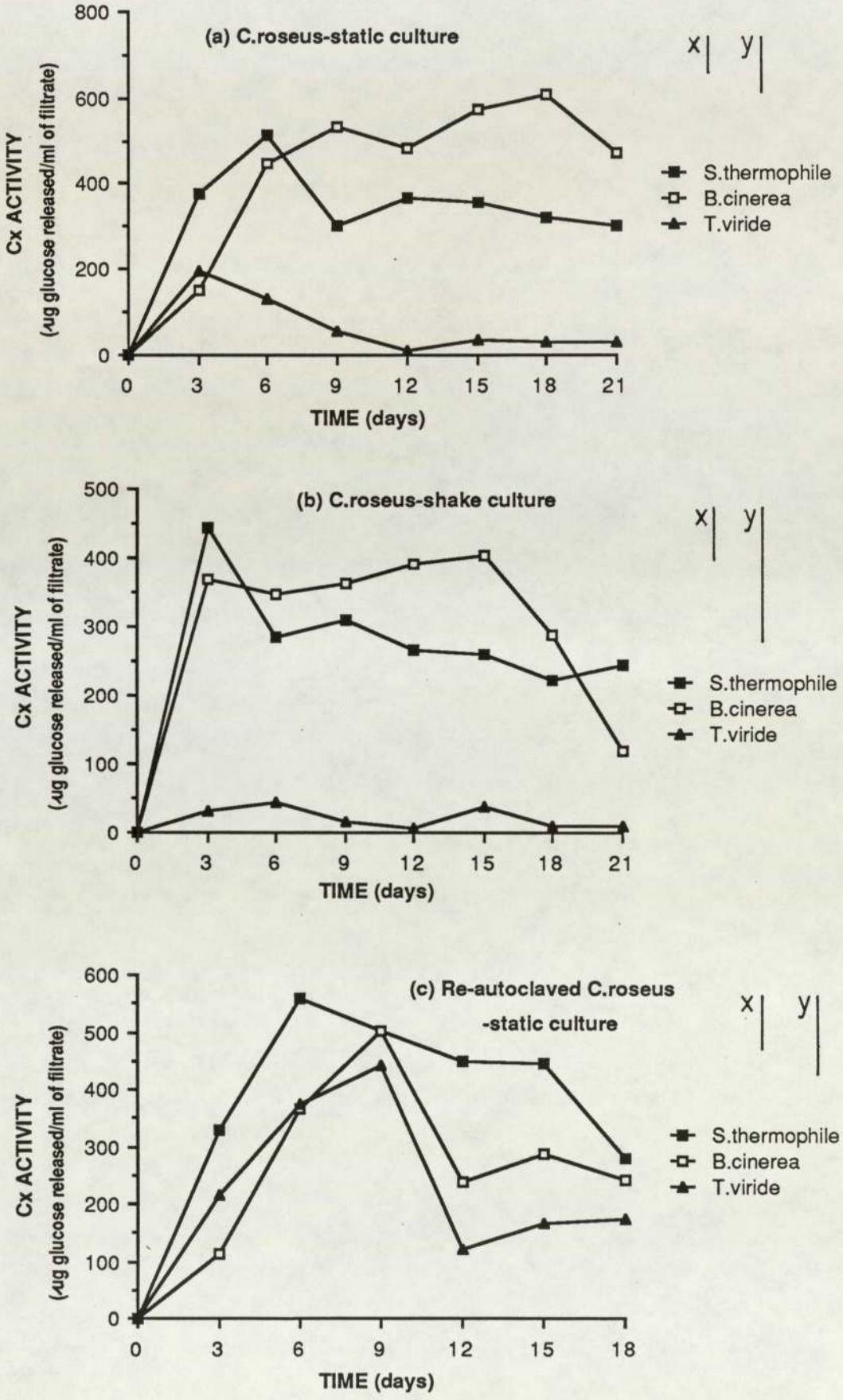
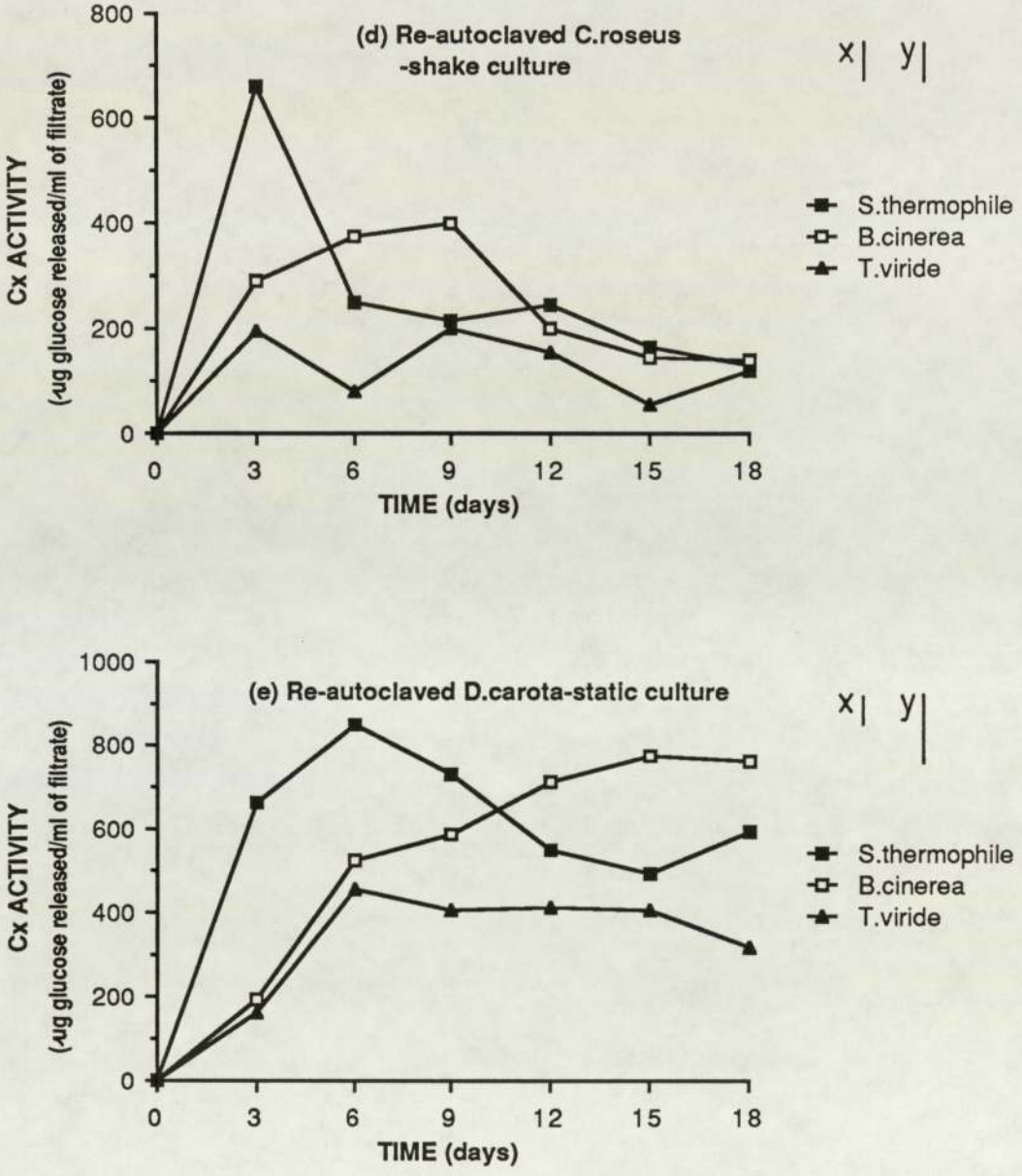
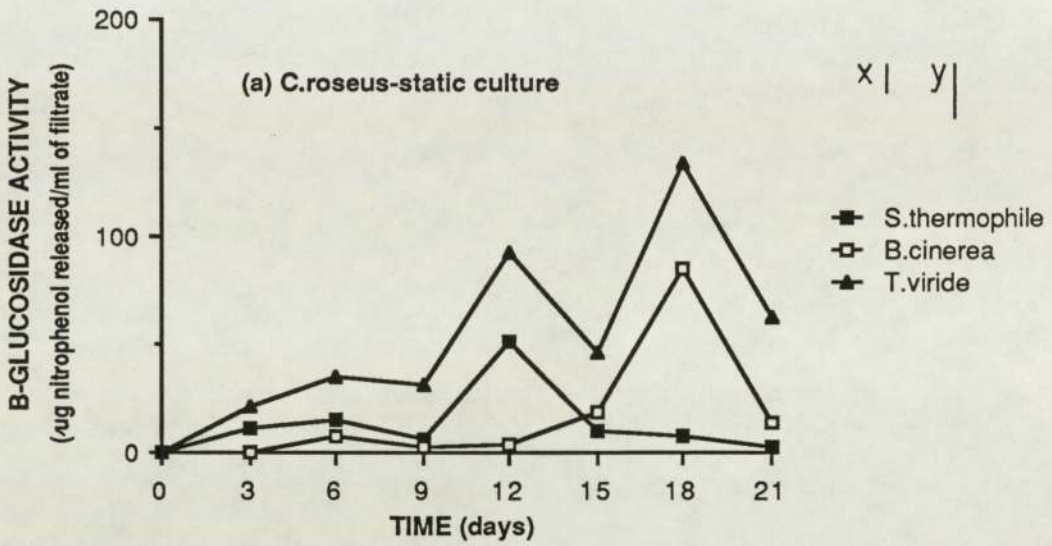


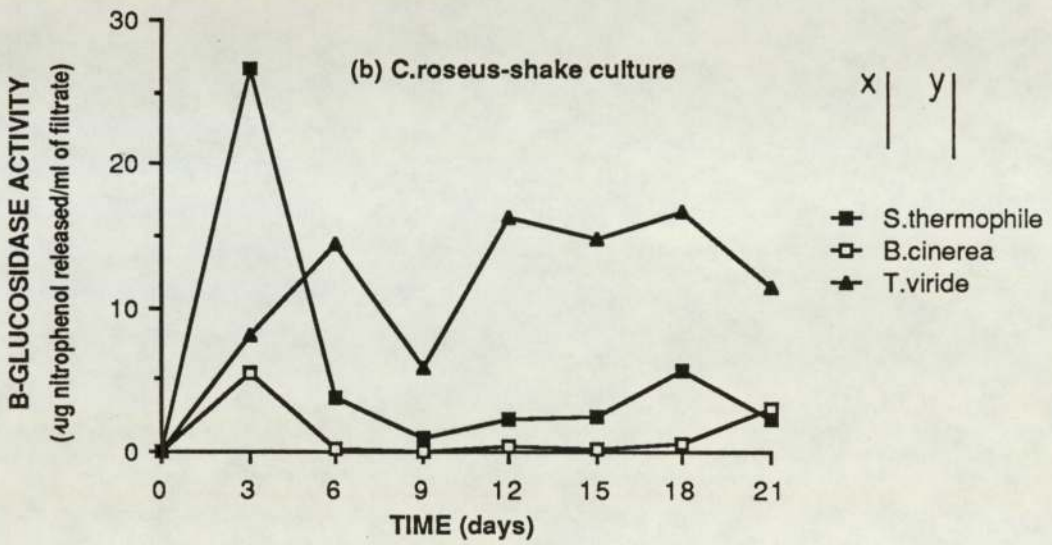
FIGURE 5:3 (continued).



X=95% confidence limits for differences within a treatment and Y=95% confidence limits between two treatments at the same or different times.

FIGURE 5:4 (a-e) Fungal B-glucosidase activity during growth on plant cell biomass.





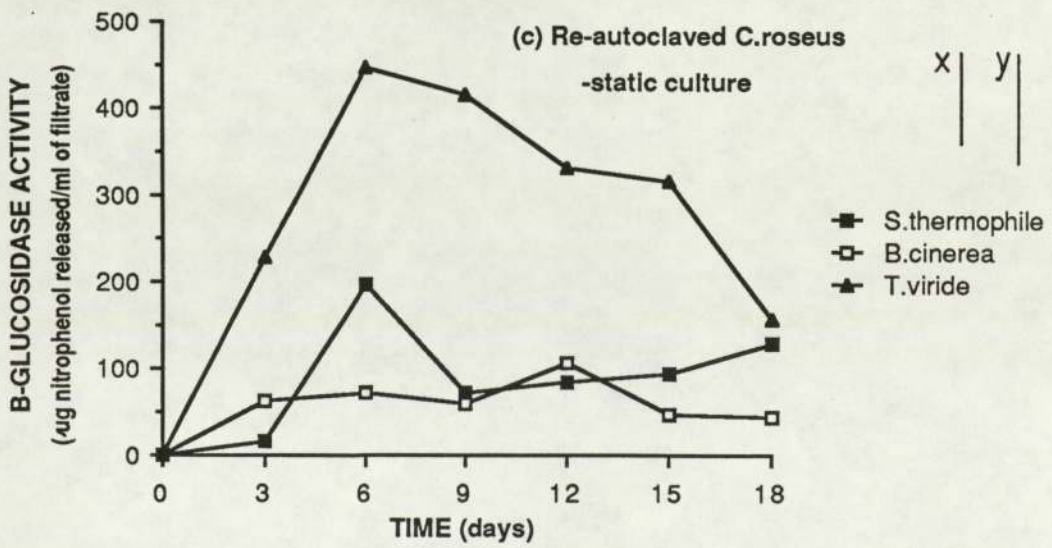
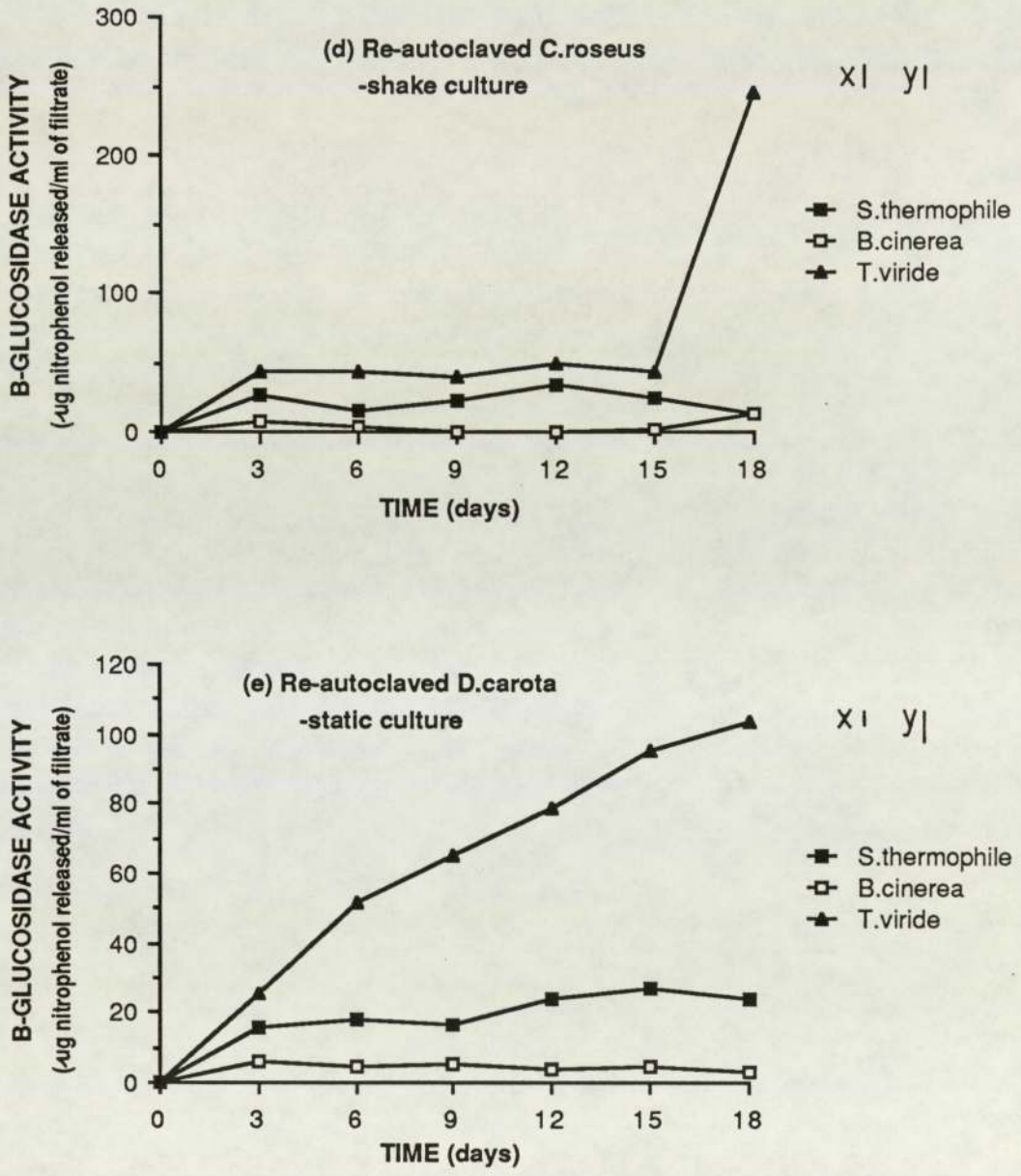


FIGURE 5:4 (continued).



X=95% confidence limits for differences within a treatment and Y=95% confidence limits between two treatments at the same or different times.

FIGURE 5:5 (a-e) Fungal endo-polygalacturonase activity during growth on plant cell biomass.

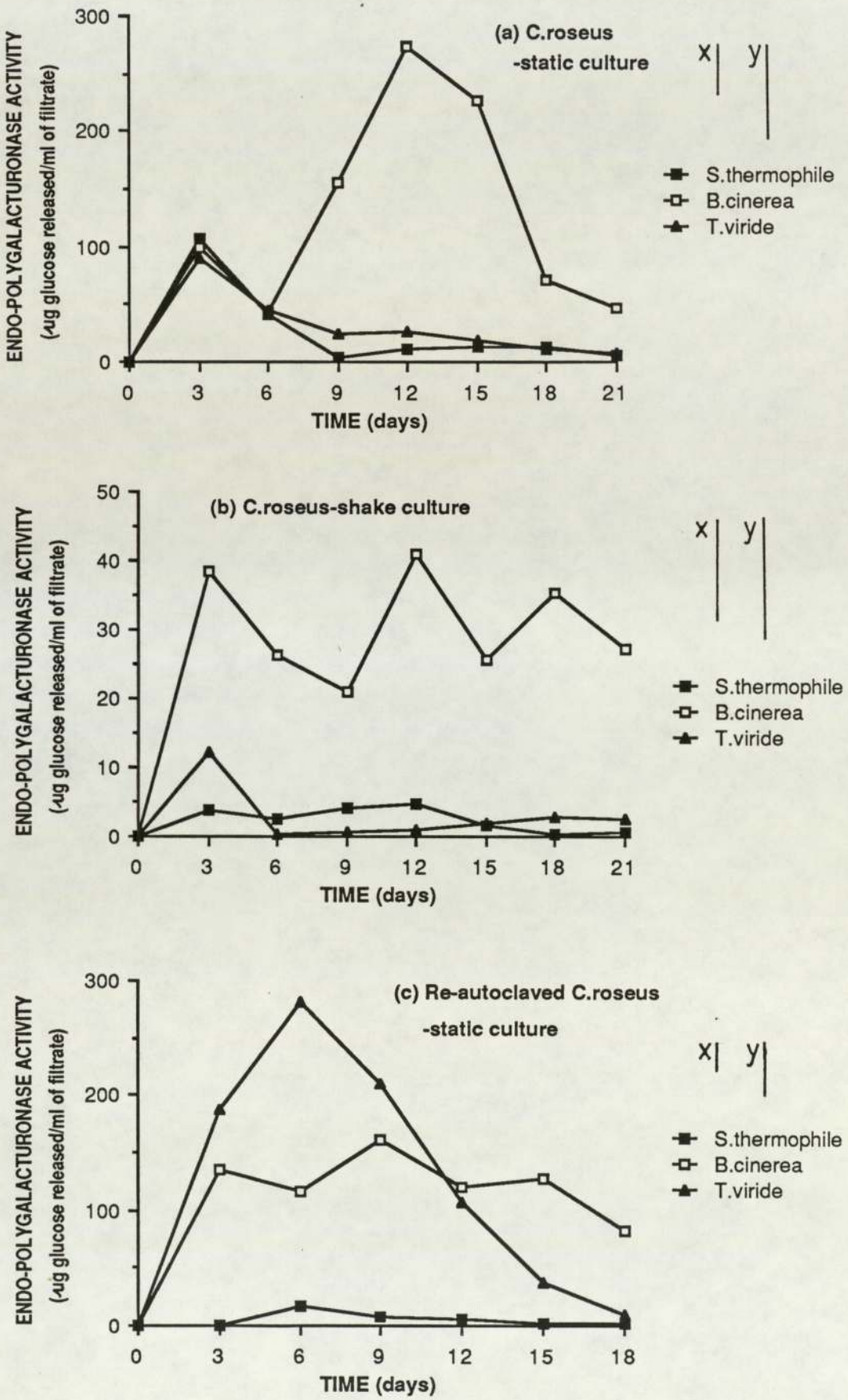
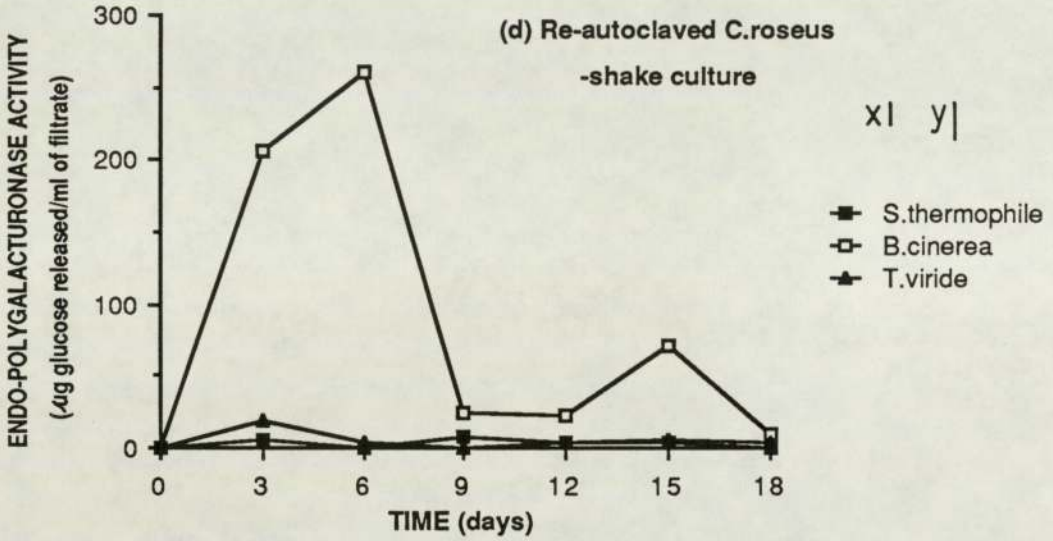
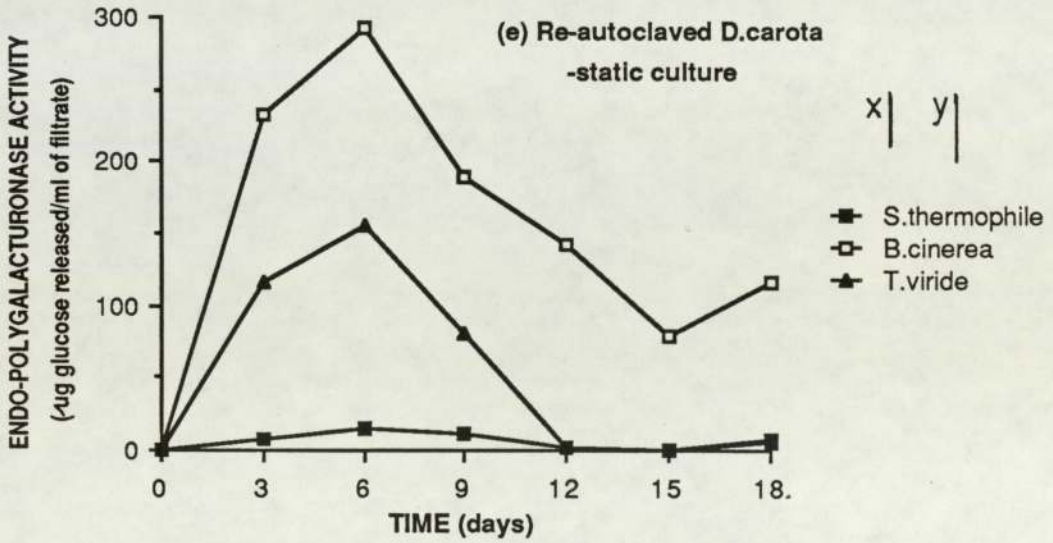


FIGURE 5:5 (continued).





X=95% confidence limits for differences within a treatment and Y=95% confidence limits between two treatments at the same or different times.

TABLE 5:1 AVERAGE ENZYME ACTIVITY PRODUCED UNDER VARIOUS CULTURAL CONDITIONS

		AVERAGE ENZYME ACTIVITY				
		C.ROSEUS		RE-AUTOCLAVED C.ROSEUS		RE-AUTOCLAVED D.CAROTA
		static	shaken	static	shaken	static
C_1	ST	116.7	31.4	354.3	207.3	310.5
	BC	59.1	51.8	65.5	103.4	28.4
	TV	165.6	63.6	315.2	363.1	261.8
C_x	ST	362.3	288.1	427.5	277.5	645.5
	BC	468.3	325.2	291.4	258.1	593.9
	TV	69.8	21.9	247.5	133.8	359.5
BG	ST	15.1	6.2	98.4	22.8	20.9
	BC	18.8	1.4	65.5	4.3	4.4
	TV	60.2	12.6	315.2	77.4	70.0
PG	ST	28.1	2.5	5.8	3.2	7.0
	BC	131.2	30.7	123.9	99.4	175.5
	TV	31.9	3.0	138.8	5.7	60.0

In Table 5:1 and 5:2

C_1 = C_1 enzyme activity

C_x = C_x enzyme activity

BG = B-glucosidase activity

PG = endo-polygalacturonase activity

ST = S.thermophile

BC = B.cinerea

TV = T.viride

For Units see Materials and Methods Section.

TABLE 5:2 MAXIMUM ENZYME ACTIVITY PRODUCED UNDER VARIOUS CULTURAL CONDITIONS

		MAXIMUM ENZYME ACTIVITY				
		C.ROSEUS		RE-AUTOCLAVED C.ROSEUS		RE-AUTOCLAVED D.CAROTA
		static	shaken	static	shaken	static
C_1	ST	185.4 (3)	45.3 (3)	492.0 (6)	318.6 (3)	387.0 (6)
	BC	75.9 (6)	97.5 (3)	106.8 (12)	142.2 (9)	54.6 (18)
	TV	231.0 (6)	152.4 (3)	445.8 (6)	442.8 (12)	412.2 (15)
C_x	ST	515.0 (6)	443.3 (3)	558.3 (6)	661.7 (3)	848.3 (6)
	BC	610.0 (18)	401.7 (15)	503.3 (9)	398.3 (9)	777.0 (15)
	TV	195.0 (3)	45.0 (6)	440.0 (9)	201.7 (9)	453.3 (6)
BG	ST	51.7 (12)	26.2 (3)	196.8 (6)	33.47 (12)	27.0 (15)
	BC	85.3 (18)	5.5 (3)	106.8 (12)	13.1 (18)	6.0 (3)
	TV	134.0 (18)	16.7 (18)	445.8 (6)	246.1 (18)	103.3 (18)
PG	ST	107.4 (3)	4.8 (12)	16.2 (6)	6.9 (9)	14.4 (6)
	BC	274.5 (12)	40.8 (12)	160.8 (9)	261.0 (6)	292.2 (6)
	TV	90.0 (3)	12.3 (3)	280.8 (6)	18.6 (3)	155.4 (6)

Figures in parentheses are the day on which the activity occurred.

T.viride respectively are the greatest producers. These values in each case are recorded on all but one of the plant cell substrates/culture mode utilized. A similar pattern may again be observed for average enzyme activity values (Table 5:1). Only minor exceptions disrupt such a pattern associating individual species and enzyme classes, in particular two species demonstrating highest average C_1 or C_x activity interchange depending on substrate type.

DISCUSSION

Although agitation of cultures is frequently employed results from this work indicate that shaking has an adverse effect on average enzyme activity. Unless stated enzyme activity refers to average values from Table 5:1 as results in this form give a better picture of activity over the whole experimental period, rather than maximum activity which is transient, and where a high value may be followed by a steep decline in activity.

The lower enzyme activity observed in this study may be caused by forces generated through agitation damaging the mycelium and disrupting enzyme synthesis. Such a negative effect on both growth and enzyme production was noted by Hecht, Schugerl and Scheiding (1983) using Chaetomium cellulolyticum. Wase, McManamey, Raymahasay and Vaid (1985) also found high agitation rates ruptured Aspergillus fumigatus mycelium liberating intracellular material resulting in decreased enzyme activity. In addition, an as yet unidentified intracellular protein (not a protease) was released which was found to be a cellulase inhibitor. Once enzymes are secreted into the culture medium they are also subject to inactivation as a result of forces generated during shaking or through surface denaturation (Asakura, Adachi and Schwartz, 1978; Reese, 1980; Reese and Ryu, 1980). In the latter case molecules exposed at an air-liquid interface spread over bubble surfaces resulting in unfolding of protein molecules with subsequent denaturation. Such inactivation was first reported by Basu and Pal (1956) using culture filtrates from cellulolytic fungi in which agitation resulted in decreased hydrolysis of both Walseth and sodium carboxymethyl cellulose, with

activity of the C_x enzyme being most affected. Likewise Halliwell (1961) using Myrothecium verrucaria also observed loss in C_x activity (72%) with shaking. In this work activity of B-glucosidase from B.cinerea and endo-polygalacturonase of T.viride on both C.roseus and re-autoclaved C.roseus plant cells were the most profoundly affected by shaking. As exampled by Table 5:1 which demonstrates that static culture of both C.roseus and re-autoclaved C.roseus promotes 13.4 and 15.2 times respectively more B-glucosidase activity from B.cinerea compared to shake cultures of the same species. Other cellulase enzymes have also been shown to be affected by agitation for example Reese, 1980, Reese and Mandels, 1980, Reese and Ryu, 1980 using Trichoderma reesei enzyme preparations attributed a decrease in hydrolysis during agitation to inactivation of the C_1 enzyme. In contrast, Sakata, Ooshima and Harano (1985) have suggested that shaking enhances the hydrolysis of crystalline cellulose by increasing adsorption between the C_1 enzyme and substrate. Similarly, Table 5:1 shows that on re-autoclaved C.roseus plant cells, B.cinerea and T.viride demonstrated a 1 - 1.5 fold increase in C_1 activity on shaking, however this result was not found with any other plant cell substrate.

Techniques have been developed to increase enzyme stability. These have been outlined by Reese and Mandels (1980) and include 1) Use of biocides to prevent enzyme inactivation by contaminating organisms and 2) Addition of protein e.g., bovine serum albumen (foreign protein reactants act on BSA and spare the enzyme). In the case of Wase, McManamey, Raymahasay and Vaid (1985) enzyme inactivation was reversed by use of inorganic protein absorbents e.g., alumina. Reese (1980) found inactivation of T.reesei

cellulases by shaking was prevented by addition of surfactants and related materials. Highly effective agents were flurocarbon surfactants and polyethylene glycols. Such results and techniques outlined by Reese and Mandels (1980) may provide a way of preventing the deactivation of enzymes which occurred in this work as a result of shaking. However the effect of surfactant on enzyme stability has been investigated further in the next chapter.

Another of the marked contrasts observed in this study is that enzyme activity differed between C.roseus and re-autoclaved C.roseus plant cell substrates, with higher activity generally resulting from the latter substrate. This may occur because the re-autoclaving process removes soluble components from plant cell biomass, leaving a greater percentage of the dry weight as insoluble poly-saccharide (for fungal utilization) in re-autoclaved C.roseus (11.25) compared to C.roseus cells (6 - 9). A further factor is catabolite repression which is defined by Magasanik (1961) as the mechanism by which organisms decrease their rate of synthesis of catabolic enzymes when catabolites accumulate to excess in cells. Such repression has been noted for T.viride (Nisizawa, Suzuki and Nisizawa, 1972) and S.thermophile (Canevascini, Coudray, Rey, Southgate and Meier, 1979). In this study, data in previous chapters indicate a higher initial concentration of glucose is found in the filtrate from C.roseus compared to re-autoclaved C.roseus plant cells (Figure 3:3). Such high glucose levels from C.roseus cells may therefore cause reduced enzyme synthesis as a result of catabolite repression. However as fungal growth proceeds, glucose is utilized and when levels fall below a critical value enzyme repression ceases. Whereas in re-autoclaved C.roseus cells fungal growth will rapidly deplete the low

levels of glucose present (Figure 3:3) thus stimulating enzyme synthesis. Enzymatic hydrolysis of the substrate releases glucose which initially accumulates, however with fungal growth glucose levels rapidly decrease initiating further enzyme synthesis. Eventually on both C.roseus and re-autoclaved C.roseus cells, glucose levels decline stabilizing at about 100 ug/ml. Over the latter stages of the incubation period enzyme activity declines as plant cell biomass is depleted, although even on substrate exhaustion enzyme activity may still be recorded as synthesized enzymes may remain viable within the culture filtrate for an as yet unknown time.

Enzyme activity also differed between the three fungal species, with in general one species producing higher levels (on a majority of substrates) of a particular enzyme compared to the other two species i.e., S.thermophile can be associated with C_x enzyme, B.cinerea and endo-polygalacturonase, T.viride with C_1 enzyme and B-glucosidase. Therefore the possibility exists of a mixed culture of fungi, leading to a more complete breakdown of plant cell biomass. However, the combination of fungi used (especially for C_1 and C_x activity) would be dependent on substrate. This consideration is supported by a number of reports. Selby (1968) and Wood (1975) have shown cross synergism between fungi. Using purified cellulase enzymes from Fusarium solani and Trichoderma koningii, Wood (1975) found the C_1 component of F.solani acted almost as effectively on cotton when mixed with T.koningii C_x as it did with its own C_x fraction. There was also a potentiation in activity towards cotton fibre when C1 from T.koningii was added to crude culture filtrates exhibiting C_1 and C_x activity. Duff Cooper and Fuller (1986) also used mixed cultures of T.reesei Rut C30 and Aspergillus phoenicis to

produce a cellulase complex which showed enhanced activity against cellulose. Initial rates of glucose production were improved up to two fold over cellulase from T.reesei Rut C30 alone.

The highest endo-polygalacturonase activity was exhibited by B.cinerea (i.e., both maximum and average activity values on all substrates except re-autoclaved C.roseus-static). This could be an expected result due to the pathogenic nature of this species. Such enzymes are of particular value in solubilizing pectic substances and in turn rapidly disrupting plant cell walls with their associated membranes during the infection process. T.viride and S.thermophile in contrast are generally considered to be saprophytes or possibly secondary invading organisms of diseased plant material and are not "true" pathogens, a consequence possibly of their stronger cellulolytic potential.

The importance of endo-polygalacturonase to efficient breakdown of plant cell biomass has been shown by Bauer, Talmadge, Keegstra and Albersheim (1973). Using cultured Sycamore plant cells they found a purified cellulase released 10 - 15% of wall polysaccharides if plant cells were pretreated with endo-polygalacturonase, whereas only 1% was released if cellulase alone was used. English, Jurale and Albersheim (1971) using cells isolated from bean plants as carbon source found sequential production of fungal enzymes from Colletotrichum lindemuthianum to be pectinase, cellulase and B-glucosidase. This pattern of enzyme release may therefore reflect the relative accessibility of various substrates in plant cell walls. Thus such observations and the results outlined above indicate that temporal use of the three fungal species from this

study in the order B.cinerea, then S.thermophile and T.viride may bring about a more efficient utilization of plant cell biomass.

CHAPTER SIX

THE EFFECT OF INCUBATION TEMPERATURE, pH AND TWEEN 80 ADDITION ON FUNGAL GROWTH AND ENZYME ACTIVITY

INTRODUCTION

For the enzymatic hydrolysis of cellulose to be commercially viable, a supply of cellulase which is both consistently high in quality yet inexpensive is required. Wilke Yang and Stocker (1976) have calculated that enzyme production accounts for 60% of the total processing costs involved in waste paper hydrolysis. Therefore to ensure cost effective cellulase production optimum process conditions need to be determined, of these temperature and pH are amongst the most important.

Temperature is clearly a major factor influencing fungal growth, development and metabolic activity. Fungi can be assigned to one of three groups depending on their cardinal growth temperatures, which are inturn determined by individual minimum, optimum and maximum values. B.cinerea and T.viride are mesophiles (optimum temperature 20 - 30°C, growth ceases above 40°C or below 5°C) whereas S.thermophile as the name implies is thermophilic (minimum at or above 20°C, maximum at or above 50°C, optimum in the upper half of this range). The third group not represented in this study are psychrophiles and favour lower temperatures.

However these optima and ranges are valid only under specified conditions of time, medium and method of measurement. For example, Fries (1953) found Coprinus fimetarius grew poorly at 44°C because of the failure of methionine biosynthesis to keep pace with other processes, whereas if exogenous methionine was supplied, growth at elevated temperatures was normal. Fries also found the optimum temperature for radial growth in Sclerotinia fructicola was affected

by pH. Temperature can affect physico-chemical characteristics of the environment, so that a complexity of factors influenced by but distinct from temperature may also be involved in alterations to cell structure and metabolism. For example, Sumner, Morgan and Evans (1969) have suggested in some instances growth limitation or metabolic changes in fungi at supraoptimal temperatures may be related to a decreased amount of dissolved oxygen in the culture medium resulting from the effect of temperature on gas solubility.

The effect of temperature on enzymes is also very important. As with other proteins denaturation can occur under extreme conditions and in microorganisms inactivation of a single enzyme may lead to thermal death. In general, extracellular enzymes because they function outside the cell usually have greater stability characteristics with respect to chemical and physical changes. Whereas intracellular enzymes only remain stable as long as the integrity of the cell is maintained.

Enzymes are also influenced by pH, often having specific pH optima. Activity can be restricted to quite a narrow range with denaturation occurring under unfavourable conditions. Several other factors are also affected by pH, including membrane permeability (Brown and Halstead, 1975) and dissociation of molecules into ions. Hence a fungus may be unable to take up essential nutrients at a certain pH or it may encounter toxic levels of certain compounds depending on whether these are most toxic in their dissociated or undissociated form. (Deacon, 1980).

Culture pH can also effect the growth habit of a fungus as Pirt

and Callow (1959) reported that increasing the pH of a steady state culture of Penicillium chrysogenum above pH 6.0 caused a corresponding decrease in hyphal length. Also Whitaker and Long (1973) suggested the tendency for fungal cultures to form pellets increases as the pH value is raised. Additionally, the metabolic activity of a fungus may inturn alter the environmental pH, by absorption of anions and production of ammonia from nitrogenous compounds or formation of organic acids and absorption of cations. (Cochrane, 1958).

In addition to temperature and pH, a further factor which has been found to improve cellulase production is the use of surfactants (Mandels and Weber, 1969) particularly polyoxyethylene sorbitan monooleate (Tween) although as yet their mode of action is not fully understood.

In this study changes in pH of the growth medium, presence of Tween 80, and temperature of incubation were investigated for their effect on fungal enzyme activity. The pH values chosen were those found by Sternberg (1976) to have influenced the production of cellulases of T.viride and Tween 80 was added at a level which is routinely incorporated into the growth medium for cellulase production (Reese and Maguire, 1970).

MATERIALS AND METHODS

1. EFFECT OF INCUBATION TEMPERATURE

(a) PREPARATION AND INOCULATION OF FLASKS (Figure 6:1)

Flasks were prepared and inoculated as in Chapter 3, using *C.roseus* plant cells. The incubation temperatures investigated were:-

(i) *S.thermophile*: 30, 35, 40, 45, 50°C

(ii) *B.cinerea*, *T.viride*: 15, 20, 25, 30, 35°C

(b) DRY WEIGHT PRODUCTION

See Chapter 2.

(c) ENZYME ASSAYS

See Chapter 5.

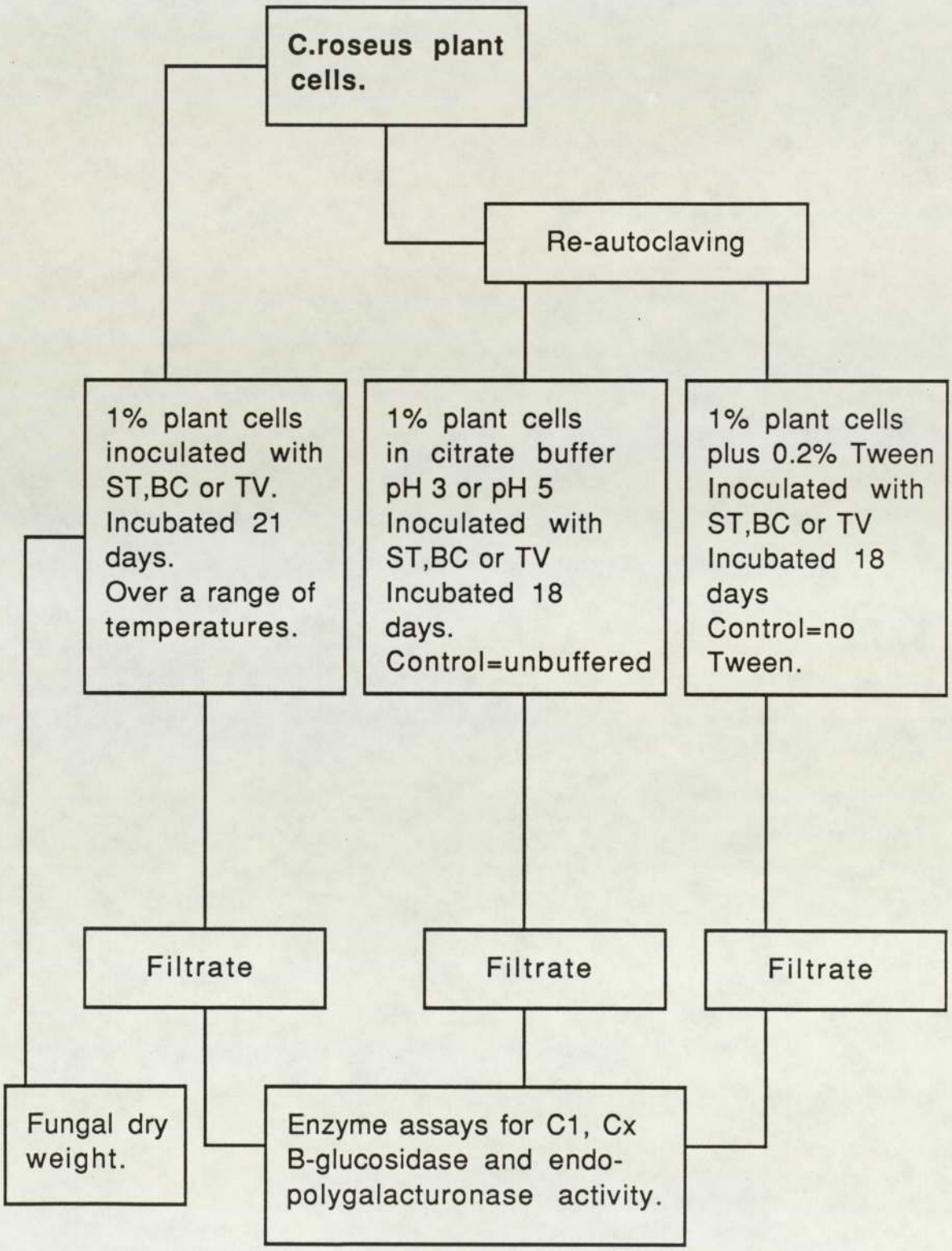
2. EFFECT OF INCUBATION pH

(a) PREPARATION AND INOCULATION OF FLASKS

Flasks of re-autoclaved *C.roseus* plant cells were prepared as in Chapter 3, with 0.1M citrate buffer (pH 3 or pH 5) being substituted for distilled water (Appendix 6:1).

A set of unbuffered flasks were used as a control. All flasks were inoculated and incubated as in Chapter 3. Citrate buffer was used as it covered both pH values employed, also Dighe, Patel and Rao (1985) suggested

FIGURE 6:1 Flow diagram of the procedure used to investigate the effect of incubation temperature,pH and Tween 80 on fungal enzyme activity.



phosphate has a deleterious effect on the DNSA reagent used in C_x enzyme assays.

(b) ENZYME ASSAYS

See Chapter 5.

3. EFFECT OF TWEEN 80

(a) PREPARATION AND INOCULATION OF FLASKS

Flasks of re-autoclaved C.roseus plant cells were prepared as in Chapter 3, with the addition of 0.2% Tween 80 (BDH). A set of Tween free flasks were used as a control.

(b) ENZYME ASSAYS

See Chapter 5.

4. STATISTICAL ANALYSIS (of all 3 experiments)

Analysis of variance was determined using a 3 x 6 (7) factorial split plot analysis in a randomised design.

RESULTS

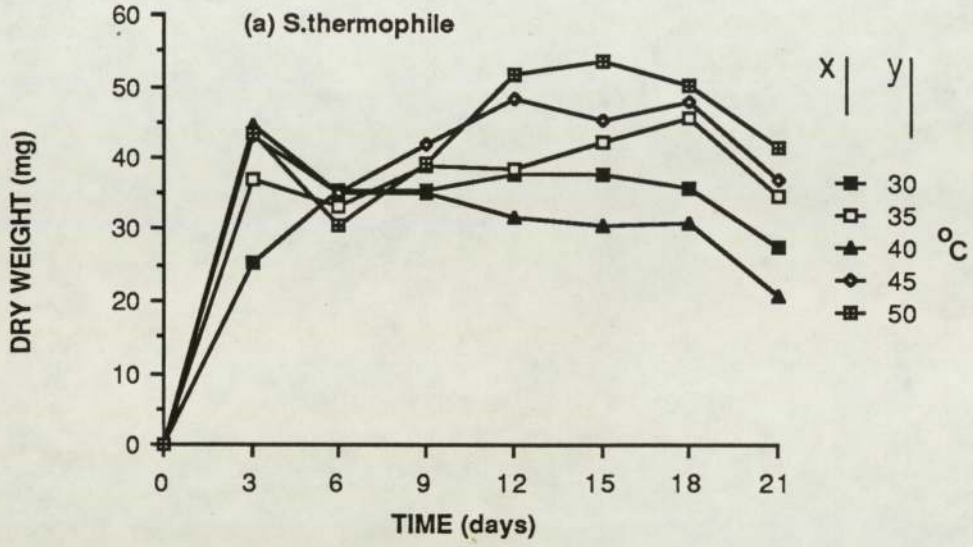
For all 3 species, dry weight and enzyme activity values (recorded over the whole incubation period) are significantly different between the five incubation temperatures.

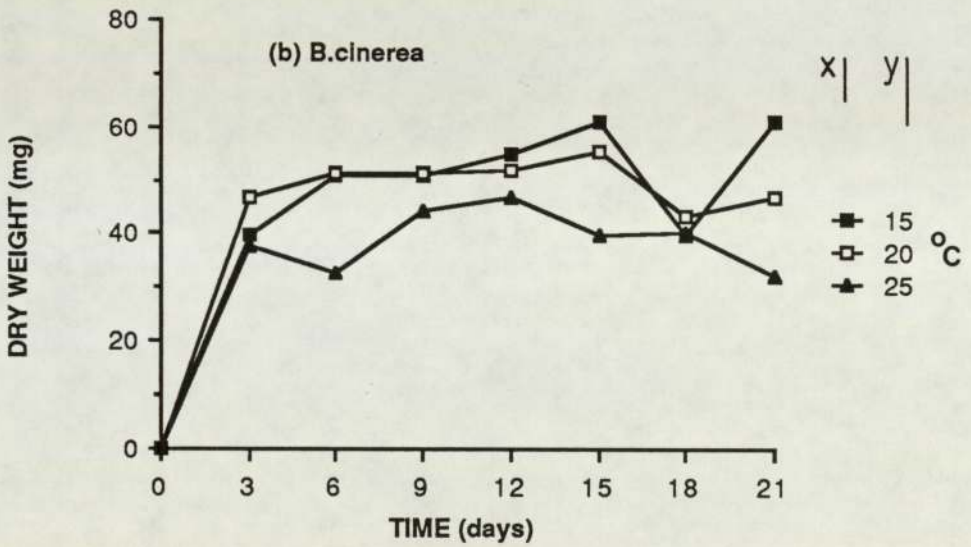
However, although maximum dry weight production occurs at 50°C for S.thermophile and 15°C for both B.cinerea and T.viride (Figure 6:2 a - c), highest average and maximum activity recorded for each enzyme does not necessarily occur at these temperatures (Data are given in Appendix 6:2 a - e). For S.thermophile derived enzyme activity (Figures 6:3a - 6:6a) highest average activity values for all enzymes are found between 30°C and 45°C (Table 6:1) whereas maximum activity is greatest over a slightly higher temperature range i.e., 40 - 50°C (Table 6:2). Within this pattern the most notable difference are for C_1 and C_x activity e.g., highest value for maximum and average C_x activity is at 45° and 30° respectively.

B.cinerea failed to produce any mycelium at 30 or 35°C (Figure 6:2b) although enzyme activity particularly endo-polygalacturonase and C_1 was evident (Figures 6:3b - 6:6b). For all enzymes from this species highest average and maximum activity occur between 15 and 25°C. A similar temperature range (15 - 20°) covers all the highest average and maximum enzyme activities exhibited by T.viride (Figures 6:2c - 6:6c).

Overall the highest maximum and average activities recorded for each enzyme can be assigned to a particular species i.e., S.thermophile : C_x, B.cinerea : endo-polygalacturonase, T.viride : C_1

FIGURE 6:2 (a-c) The effect of incubation temperature on fungal dry weight production.





No growth was recorded for this species at 30 and 35 c

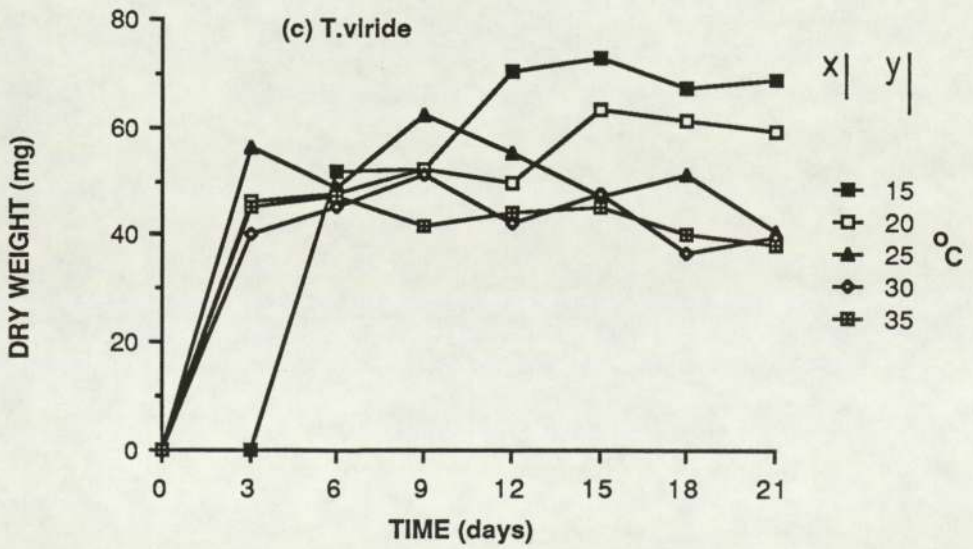


FIGURE 6:3 (a-c) The effect of incubation temperature on fungal C_1 enzyme activity.

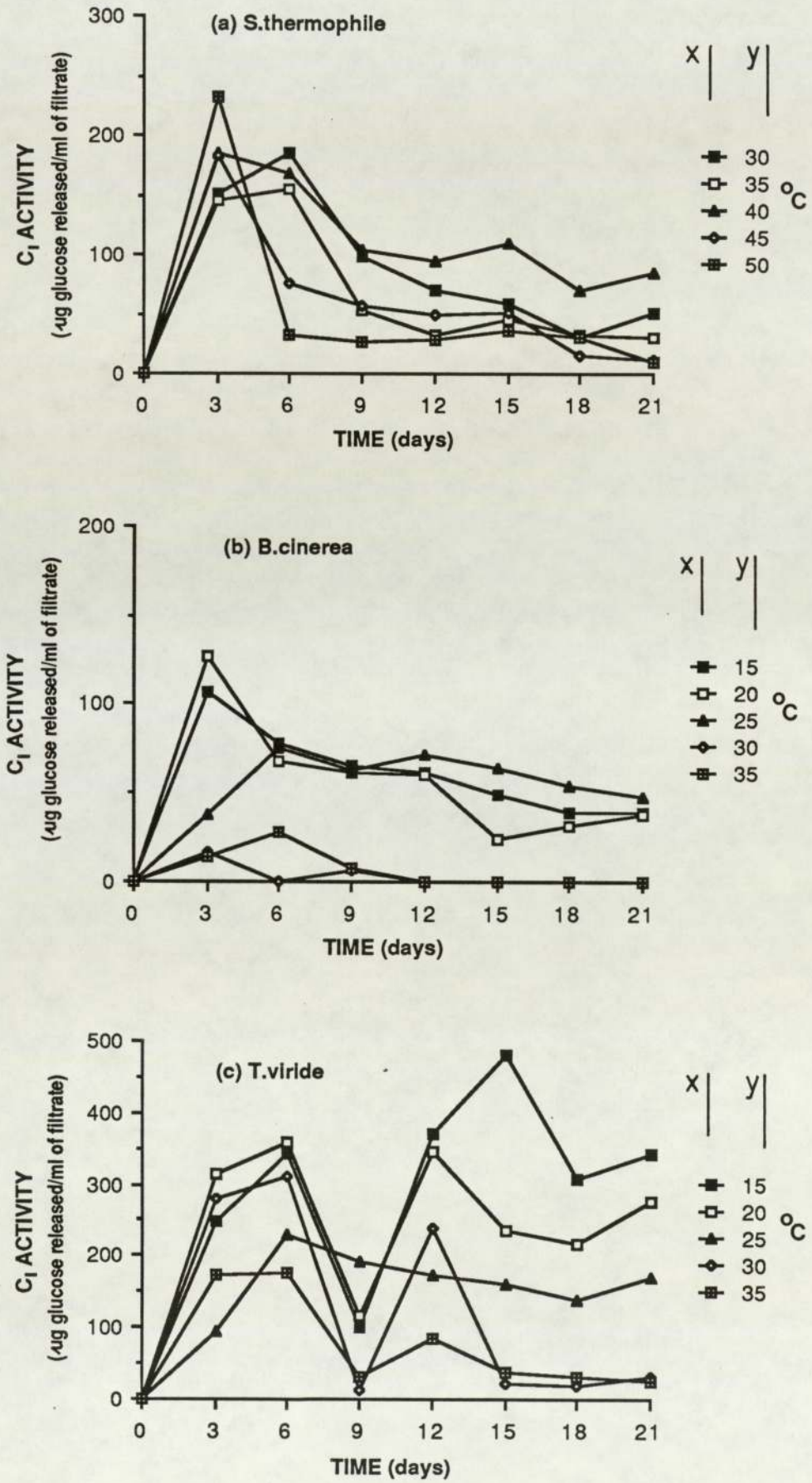


FIGURE 6:4 (a-c) The effect of incubation temperature on fungal Cx enzyme activity.

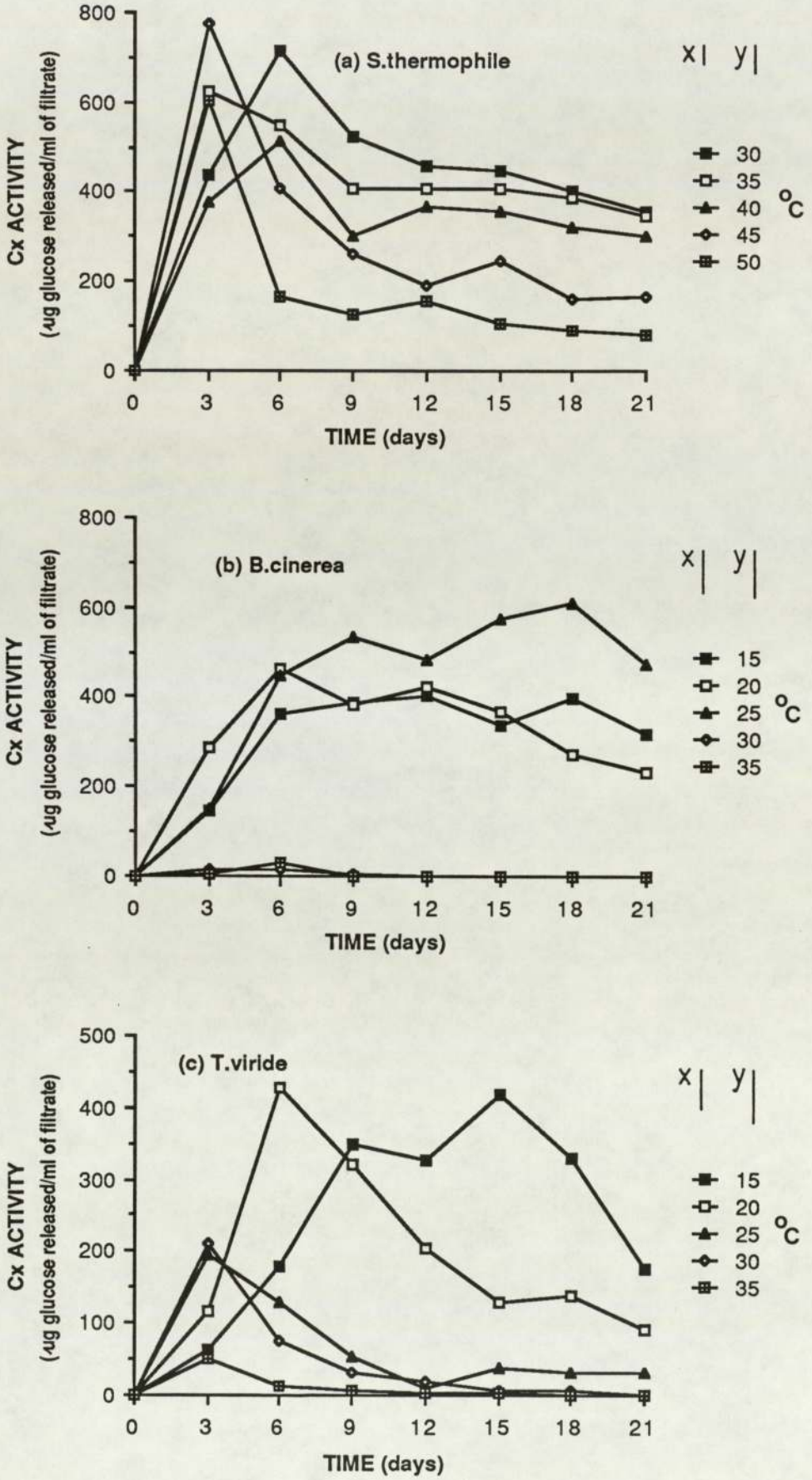


FIGURE 6:5 (a-c) The effect of incubation temperature on fungal B-glucosidase activity.

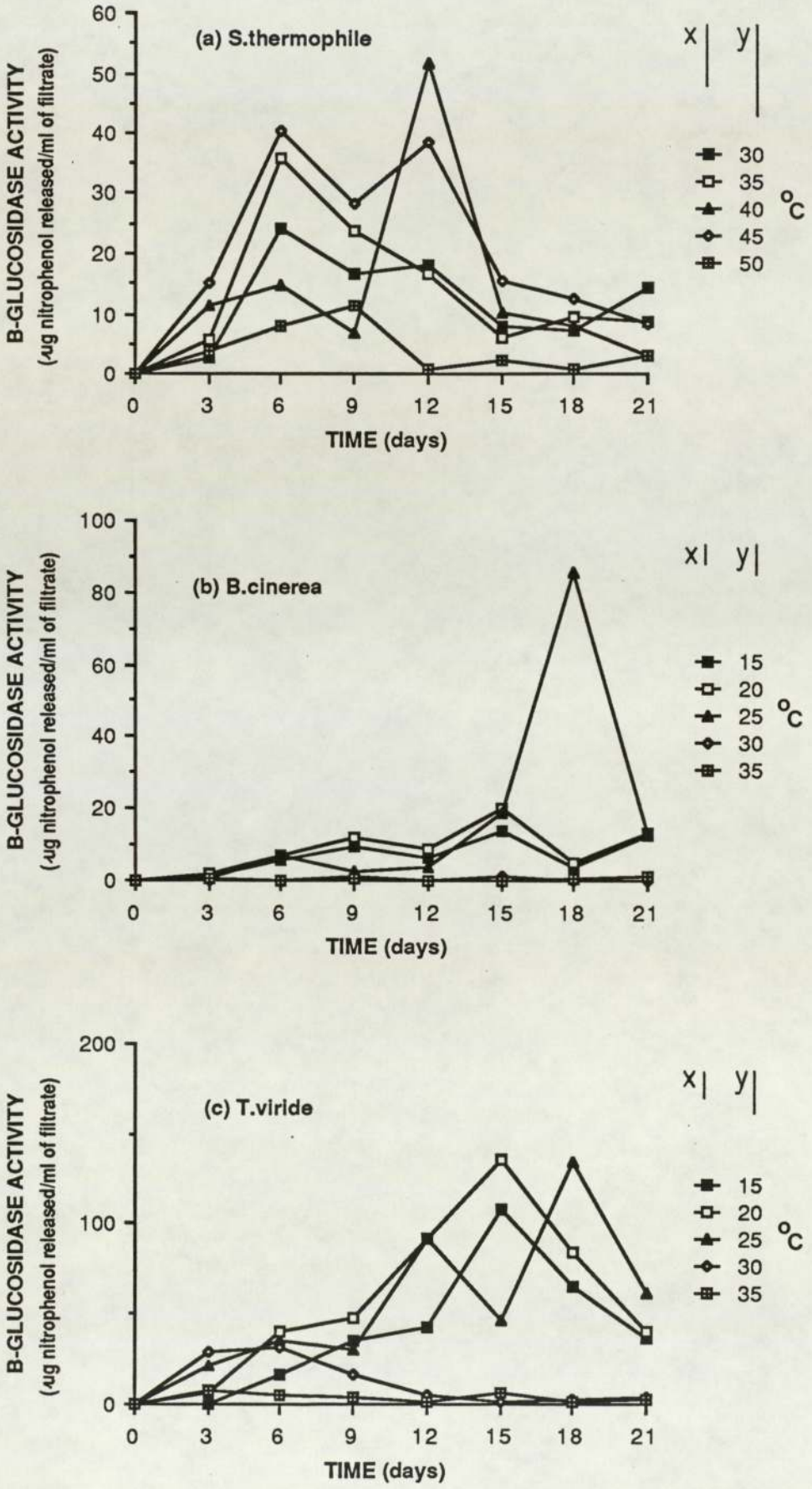


FIGURE 6:6 (a-c) The effect of incubation temperature on fungal endo-polygalacturonase activity.

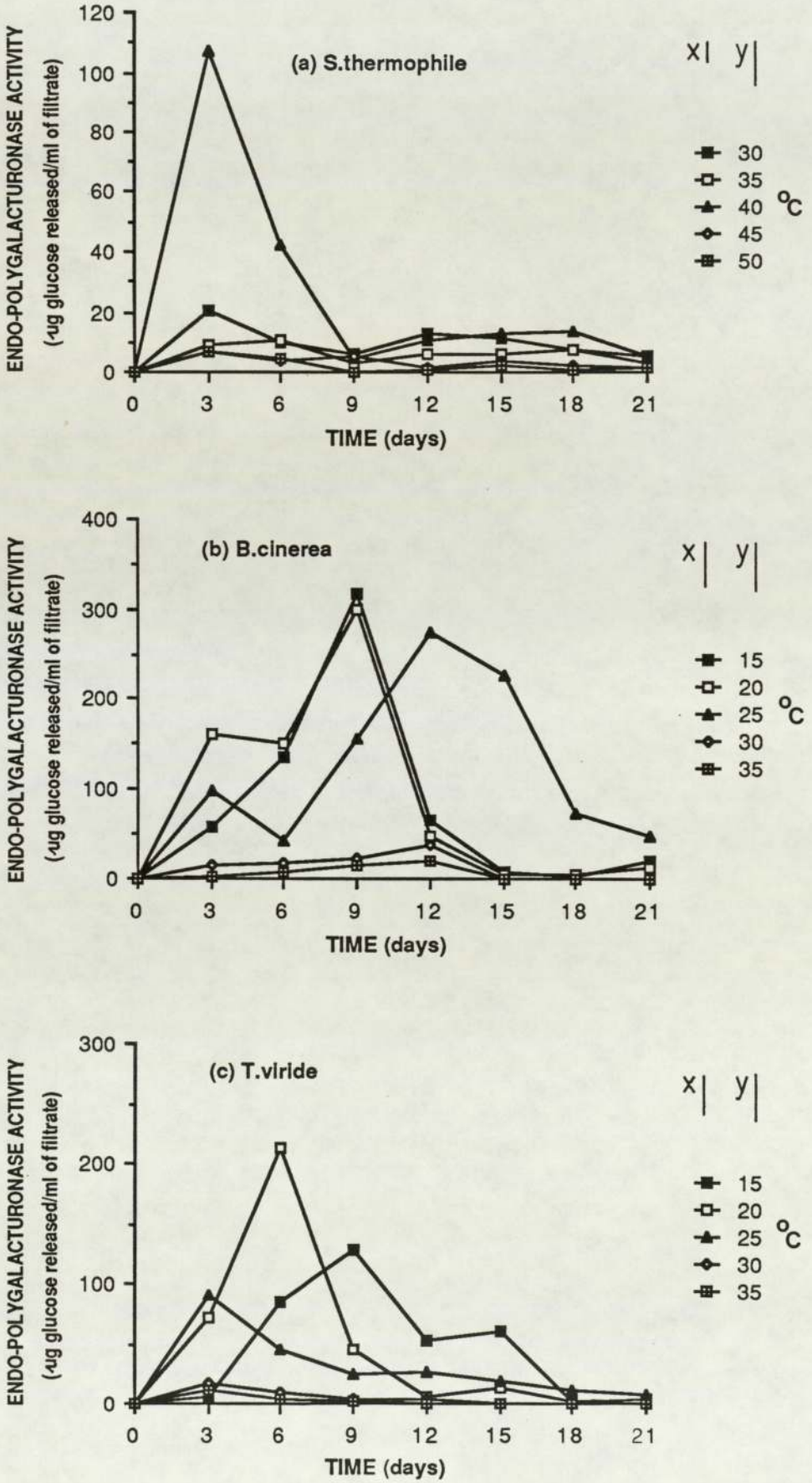


TABLE 6:1 THE EFFECT OF INCUBATION TEMPERATURE ON ENZYME ACTIVITY - AVERAGE VALUES

	AVERAGE ENZYME ACTIVITY				
	TEMP	S.THERMOPHILE	TEMP	B.CINEREA	T.VIRIDE
	°C		°C		
C_1	30	91.9	15	62.5	313.6
	35	70.5	20	58.4	266.9
	40	116.7	25	59.1	165.6
	45	63.1	30	3.3	131.1
	50	56.3	35	7.02	79.8
C_x	30	479.0	15	336.3	263.3
	35	446.7	20	345.7	203.8
	40	362.3	25	468.3	69.7
	45	315.4	30	4.7	49.7
	50	190.0	35	5.0	10.2
BG	30	12.9	15	7.5	43.4
	35	15.1	20	9.6	63.6
	40	15.0	25	18.8	60.2
	45	22.6	30	0.4	12.3
	50	4.1	35	0.4	3.9
PG	30	10.4	15	86.8	48.0
	35	6.3	20	96.8	49.8
	40	28.1	25	131.0	31.9
	45	3.4	30	12.8	5.0
	50	2.3	35	6.5	2.7

In this and subsequent tables

C_1 = C_1 enzyme activity
C_x = C_x enzyme activity
BG = B-glucosidase activity
PG = Endo-polygalacturonase activity

For units see Materials and Methods section

TABLE 6:2 THE EFFECT OF INCUBATION TEMPERATURE ON ENZYME ACTIVITY - MAXIMUM VALUES

	MAXIMUM ENZYME ACTIVITY					
	TEMP	S.THERMOPHILE	TEMP	B.CINEREA		T.VIRIDE
	°C		°C			
C1	30	185.4 (6)	15	107.4	(3)	481.5 (15)
	35	155.4 (6)	20	127.2	(3)	360.0 (6)
	40	185.4 (3)	25	75.9	(6)	231.0 (6)
	45	183.6 (3)	30	16.8	(3)	311.4 (6)
	50	231.6 (3)	35	28.2	(6)	177.6 (6)
C_x	30	716.7 (6)	15	405.0	(12)	416.7 (15)
	35	621.7 (3)	20	463.3	(6)	428.3 (6)
	40	515.0 (6)	25	610.0	(18)	195.0 (3)
	45	773.3 (3)	30	13.3	(3)	210.0 (3)
	50	603.3 (3)	35	28.3	(6)	50.0 (3)
BG	30	24.0 (6)	15	13.7	(15)	107.6 (15)
	35	35.8 (6)	20	20.2	(15)	135.3 (15)
	40	51.7 (12)	25	85.3	(18)	134.0 (18)
	45	40.4 (6)	30	1.3	(9)	31.0 (6)
	50	11.2 (9)	35	0.6	(18)	7.4 (3)
PG	30	20.6 (3)	15	318.0	(9)	128.4 (9)
	35	10.3 (6)	20	299.4	(9)	212.4 (6)
	40	107.4 (3)	25	274.5	(12)	90.0 (3)
	45	6.4 (3)	30	36.4	(12)	16.8 (3)
	50	6.5 (3)	35	20.4	(12)	11.4 (3)

Figure in parentheses is the day on which the activity occurred.

and B glucosidase with these results supporting those observed in Chapter 5.

When the effect of culture pH was investigated, all three species demonstrated enzyme activity in both buffered and unbuffered cultures with the exception of S.thermophile (pH 3) where no C_x or endo-polygalacturonase activity was recorded (Figures 6:7 - 6:10, Appendix 6:3 a - d). In addition, B.cinerea shows a lag period of 6 - 9 days before C_x activity may be noted at both pH 3 and 5. Results in Tables 6:3, 6:4 indicate that for S.thermophile values for all four enzyme activities are greater at pH5 than 3, whereas the reverse case applies for B.cinerea and T.viride (the only exception being B-glucosidase (pH5) from T.viride). However, in a majority of cases, higher values are recorded from all three species with unbuffered conditions. The only exceptions are for S.thermophile (endo-polygalacturonase at pH5), B.cinerea (C_1 at pH3), T.viride (C_x at pH3) in which no significant difference exists between unbuffered and pH controlled cultures.

Addition of Tween 80 appears to have a beneficial effect on C_x activity (average and maximum) from S.thermophile and B.cinerea with a four fold increase recorded (Tables 6:5, 6:6, Figures 6:11 - 6:14), and use of this adjunct also increased maximum C_1 activity from B.cinerea. In other cases its use had no effect on enzyme activity e.g., C_1 and C_x from T.viride (Appendix 6:4a - d). Furthermore, in some cases addition of Tween 80, would seem detrimental to enzyme activity e.g., B glucosidase from S.thermophile and B.cinerea, endo-polygalacturonase activity from B.cinerea and T.viride (Table 6:5).

FIGURE 6:7 (a-c) The effect of incubation pH on fungal C_1 enzyme activity.

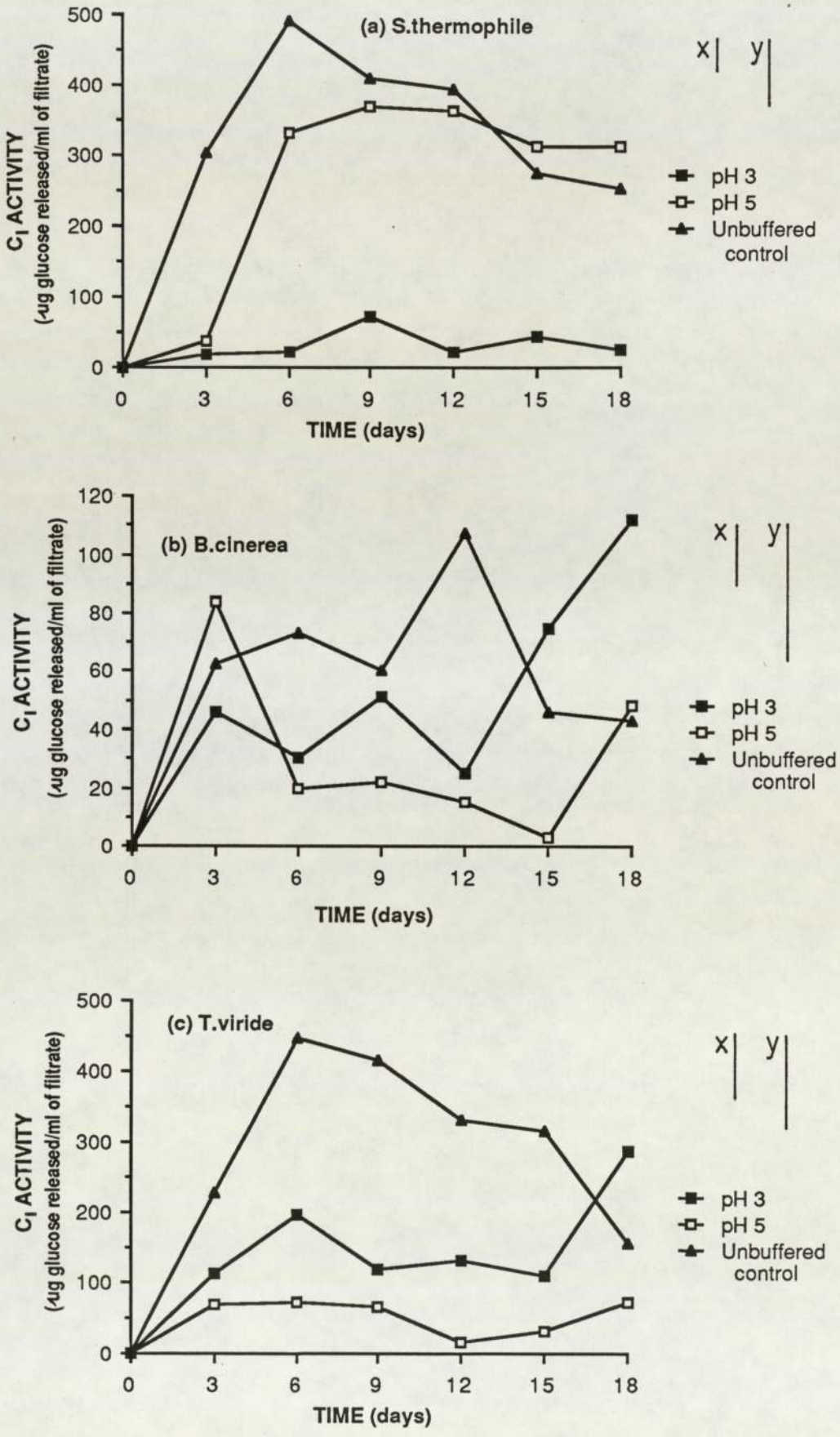


FIGURE 6:8 (a-c) The effect of incubation pH on fungal Cx enzyme activity.

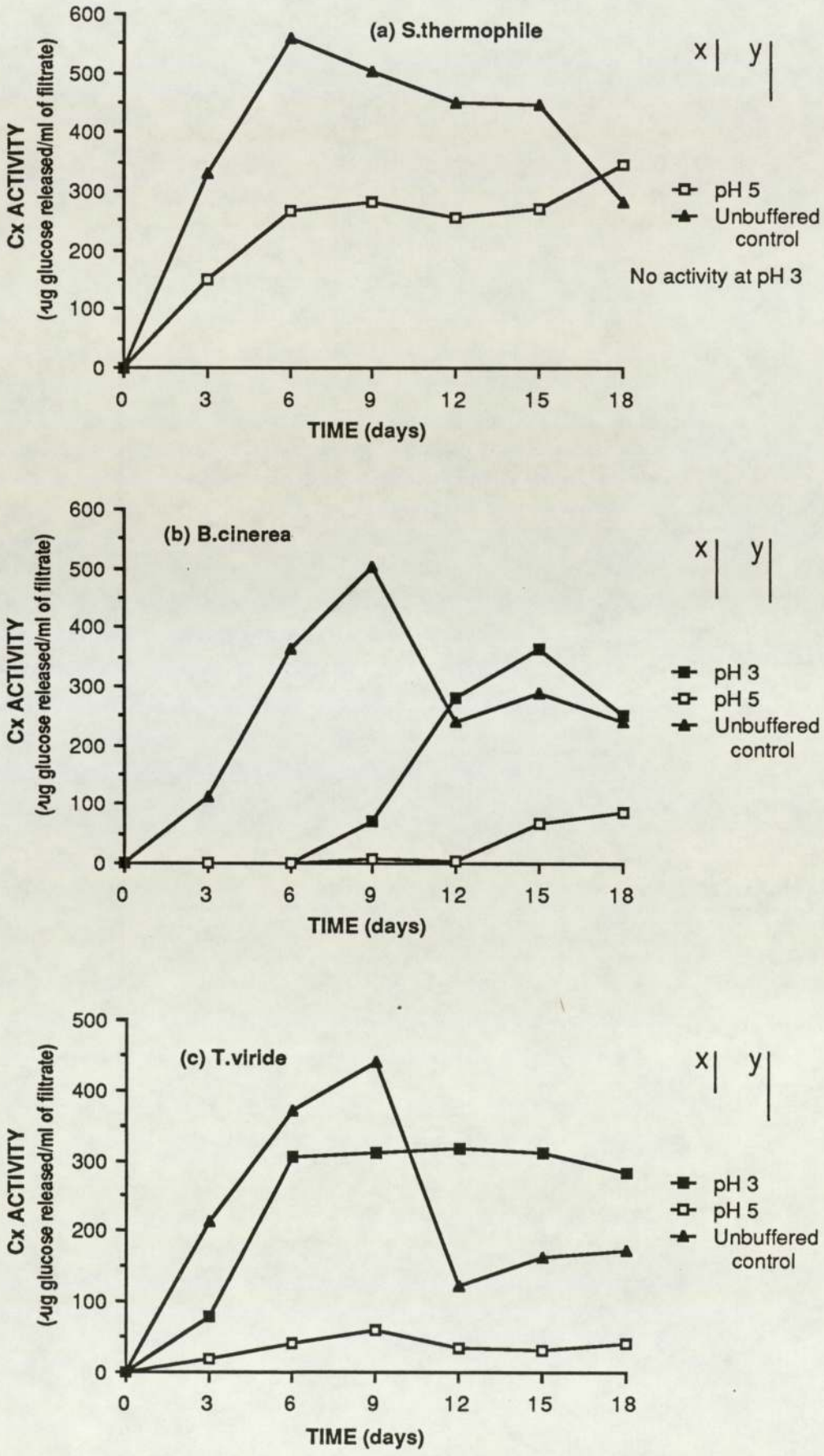


FIGURE 6:9 (a-c) The effect of incubation pH on fungal B-glucosidase activity.

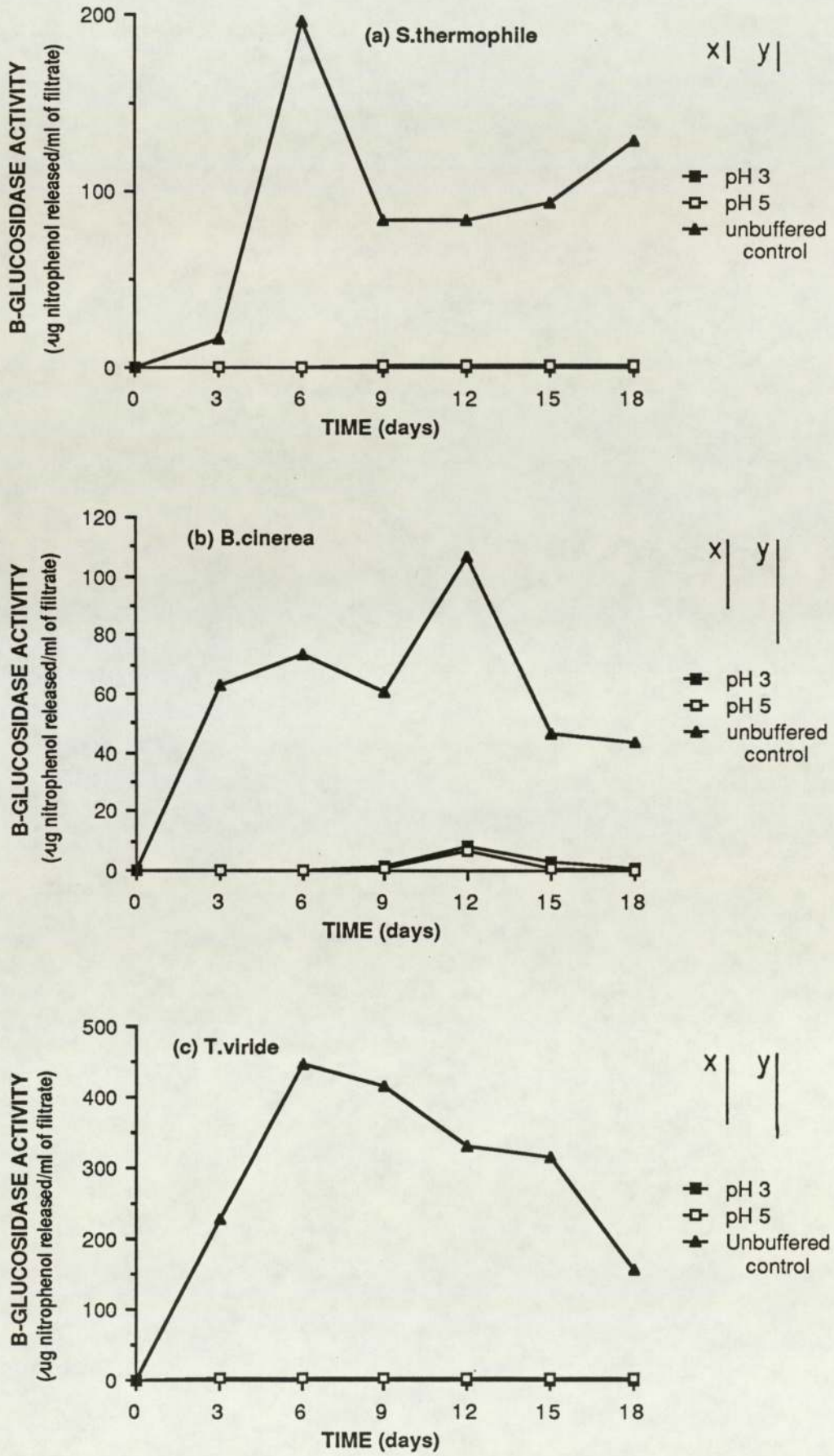


FIGURE 6:10 (a-c) The effect of incubation pH on fungal endo-polygalacturonase activity.

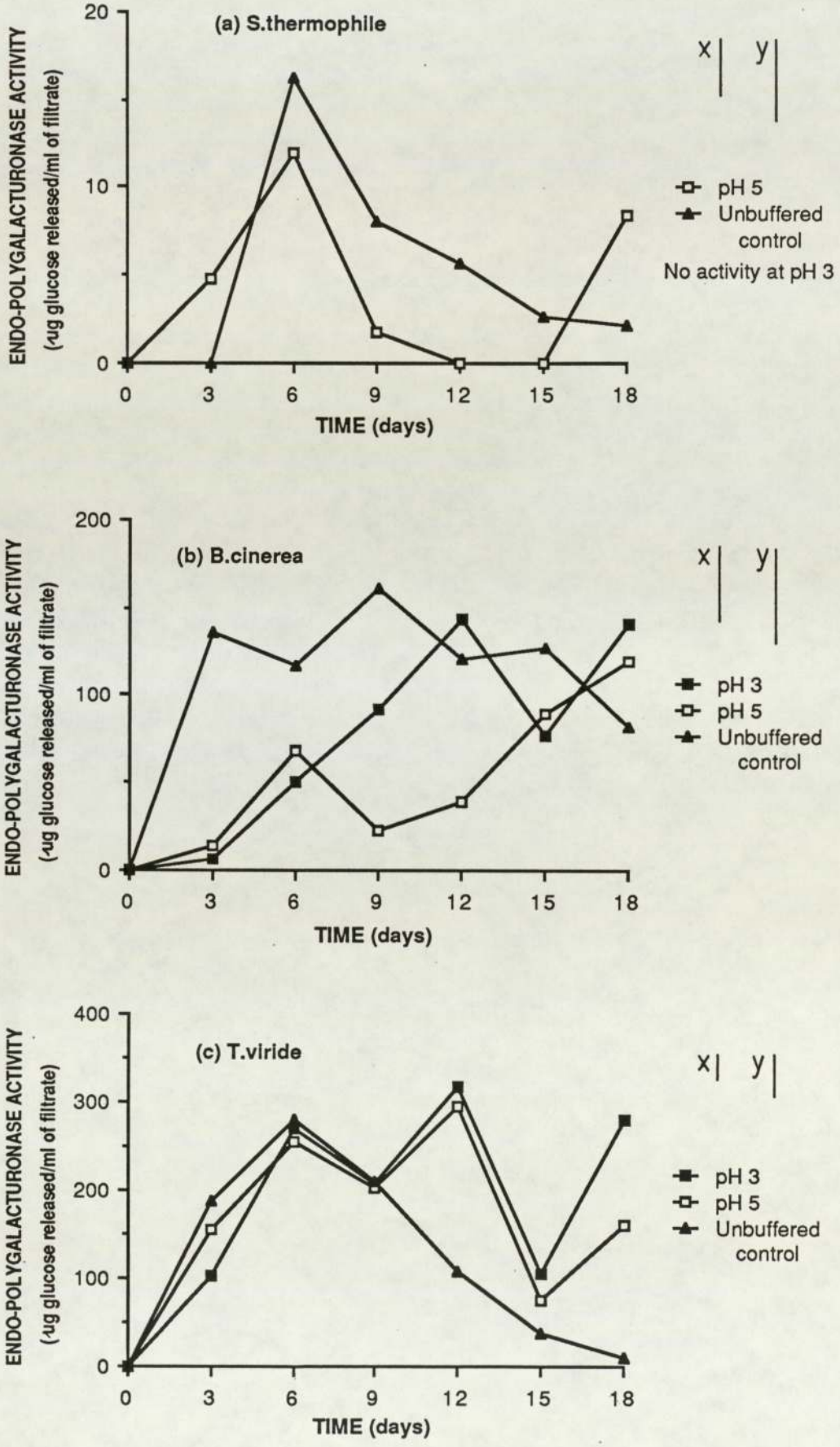


TABLE 6:3 THE EFFECT OF INCUBATION pH ON ENZYME ACTIVITY - AVERAGE VALUES

AVERAGE ENZYME ACTIVITY				
	pH	S.THERMOPHILE	B.CINEREA	T.VIRIDE
C_1	3	34.3	56.5	159.2
	5	287.5	31.9	53.9
	C	354.3	65.5	315.2
C_x	3	0.0	161.1	268.8
	5	259.9	27.8	37.5
	C	427.5	291.4	247.5
BG	3	0.1	2.3	1.7
	5	0.7	1.5	2.9
	C	98.4	65.5	315.2
PG	3	0.0	84.7	213.2
	5	4.5	58.7	190.7
	C	5.8	123.9	138.8

C = Control i.e., unbuffered cultures

TABLE 6:4 THE EFFECT OF INCUBATION pH ON ENZYME ACTIVITY - MAXIMUM VALUES

MAXIMUM ENZYME ACTIVITY

	pH	S.THERMOPHILE	B.CINEREA	T.VIRIDE
C_1	3	72.6 (9)	111.6 (18)	288.6 (18)
	5	368.4 (9)	83.4 (3)	72.0 (6)
	C	492.0 (6)	106.8 (12)	445.8 (6)
C_x	3	0.0	363.3 (15)	316.7 (12)
	5	345.0 (18)	86.7 (18)	60.0 (9)
	C	558.3 (6)	503.3 (9)	440.0 (9)
BG	3	0.2 (12)	8.5 (12)	3.3 (3)
	5	1.1 (18)	6.9 (12)	4.1 (18)
	C	196.8 (6)	106.8 (12)	445.8 (6)
PG	3	0.0	142.8 (12)	317.4 (12)
	5	12.0 (6)	119.4 (18)	295.8 (12)
	C	16.2 (6)	160.8 (9)	280.8 (6)

Figure in parentheses is the day on which the activity occurred.

FIGURE 6:11 (a-c) The effect of Tween 80 (0.2%) on fungal C_1 enzyme activity.

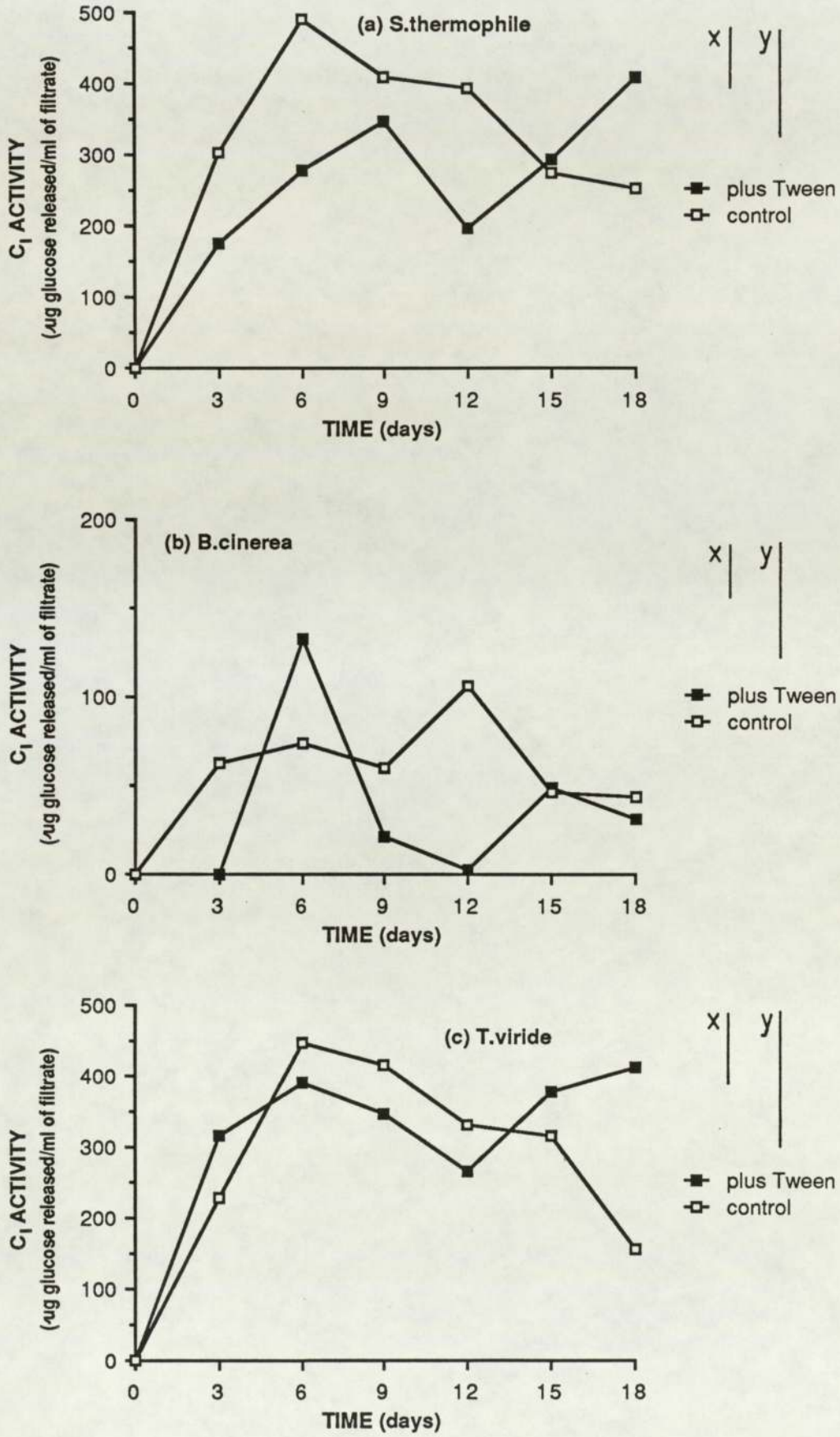


FIGURE 6:12 (a-c) The effect of Tween 80 (0.2%) on fungal Cx enzyme activity

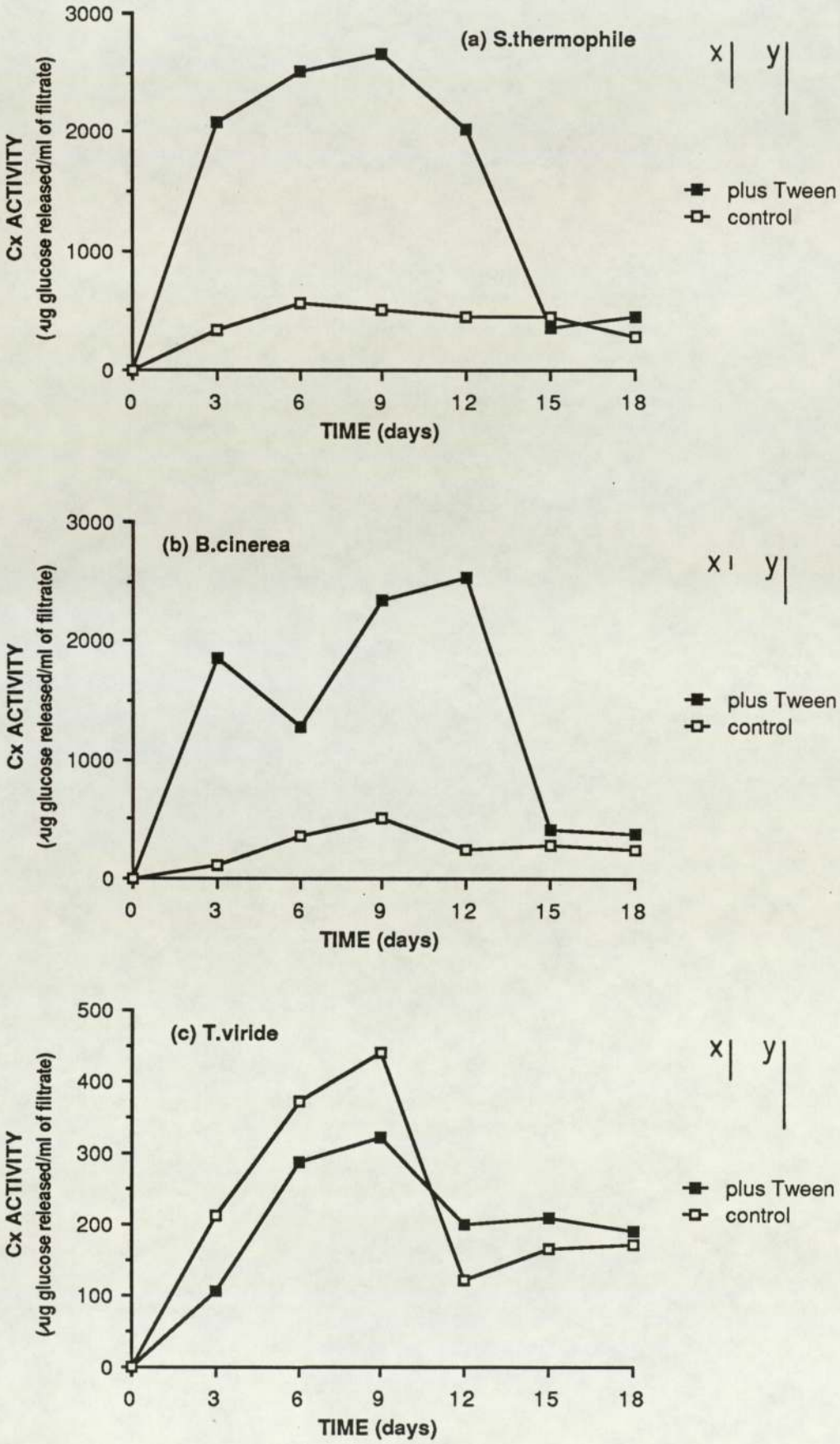


FIGURE 6:13 (a-c) The effect of Tween 80 (0.2%) on fungal B-glucosidase activity.

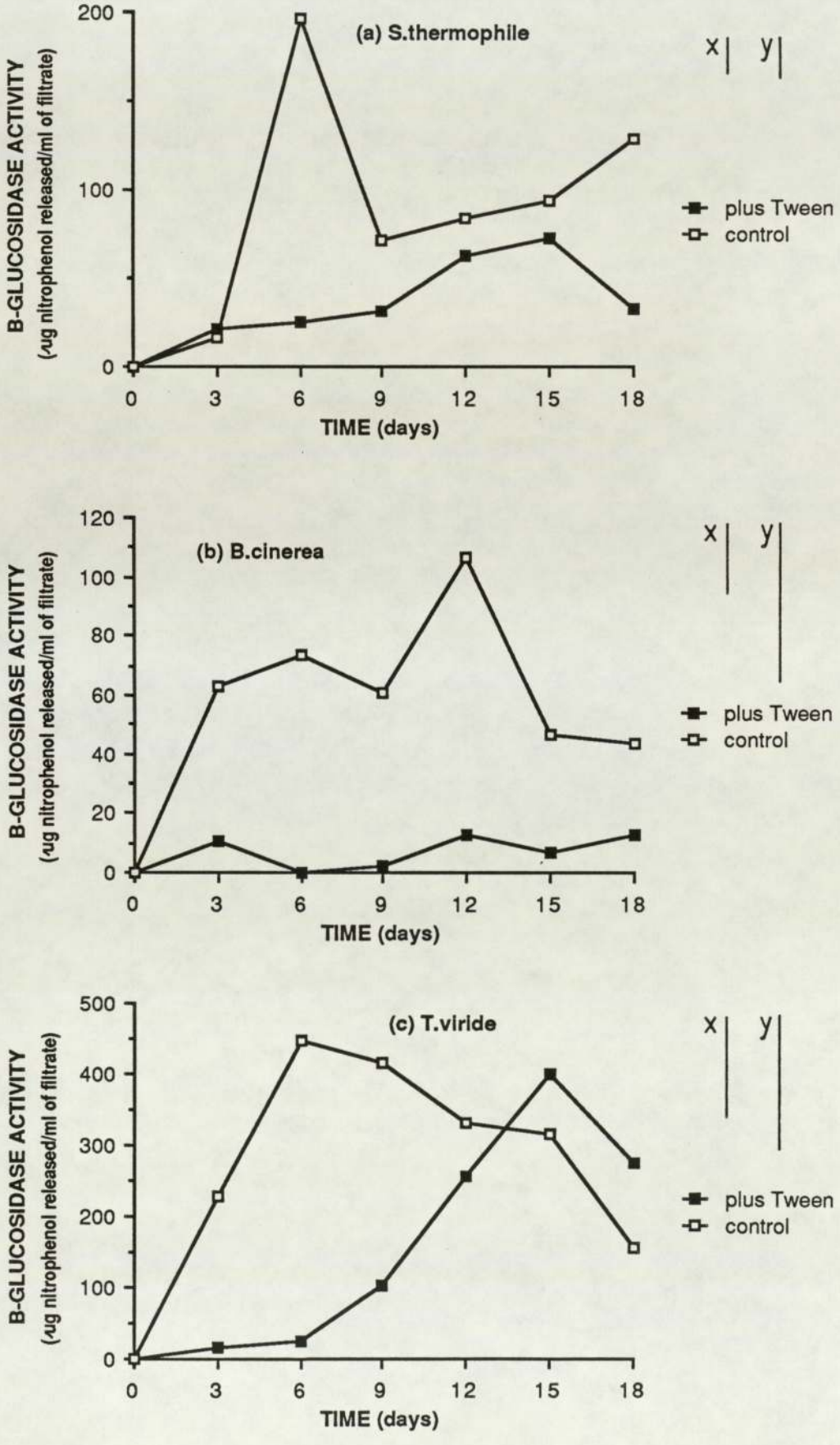


FIGURE 6:14 (a-c) The effect of Tween 80 (0.2%) on fungal endo-polygalacturonase activity.

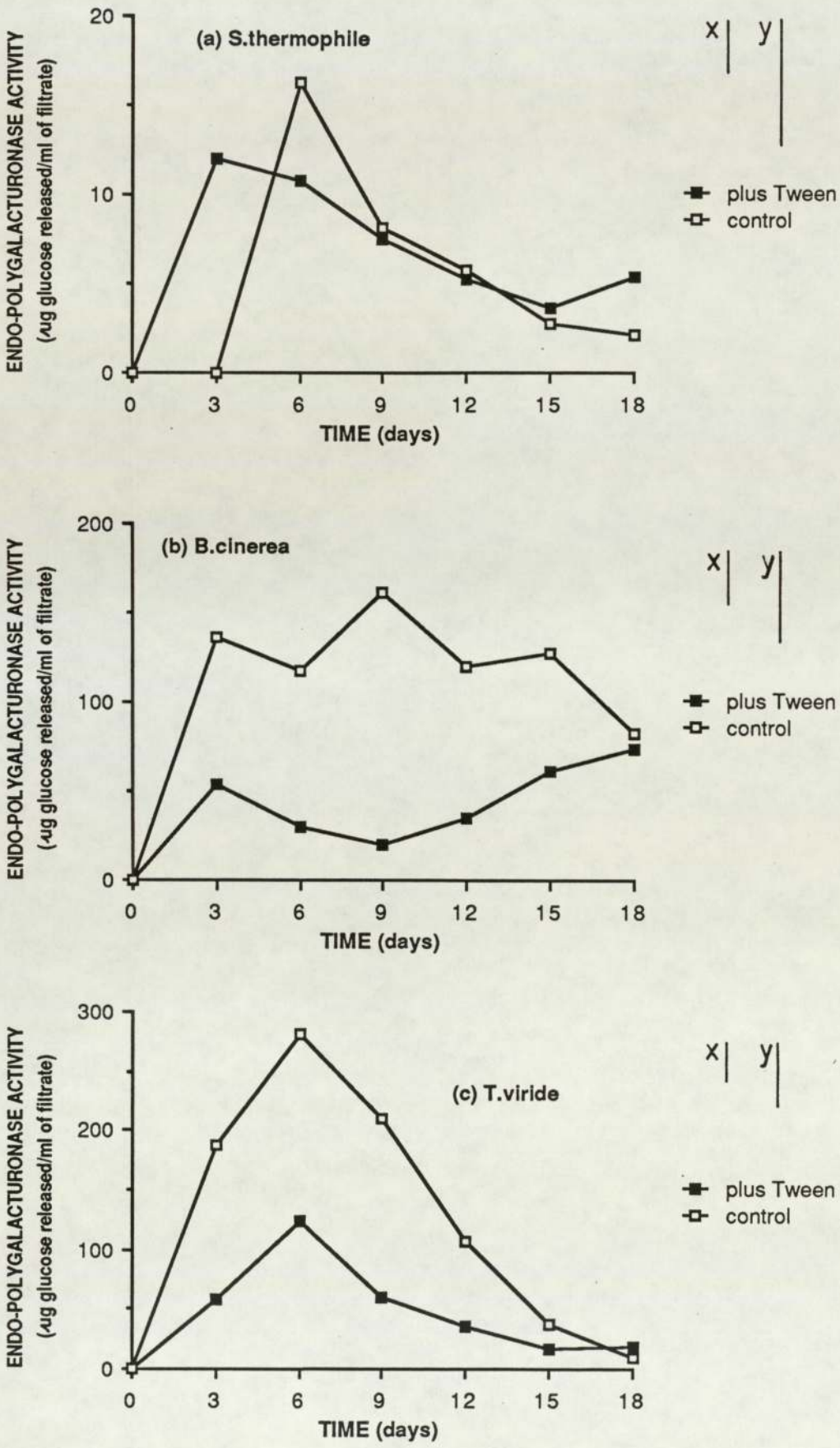


TABLE 6:5 THE EFFECT OF TWEEN 80 ADDITION ON ENZYME ACTIVITY - AVERAGE VALUES

AVERAGE ENZYME ACTIVITY

		S.THERMOPHILE	B.CINEREA	T.VIRIDE
C_1	TWEEN	283.2	39.3	351.3
	C	354.3	65.5	315.2
C_x	TWEEN	1682.2	1272.2	219.1
	C	427.5	291.4	247.5
BG	TWEEN	40.9	7.5	179.4
	C	98.4	65.5	315.2
PG	TWEEN	7.4	45.5	52.0
	C	5.8	123.9	138.8

TABLE 6:6 THE EFFECT OF TWEEN 80 ADDITION ON ENZYME ACTIVITY - MAXIMUM VALUES

MAXIMUM ENZYME ACTIVITY

		S.THERMOPHILE	B.CINEREA	T.VIRIDE
C_1	TWEEN	409.2 (18)	132.0 (6)	412.2 (18)
	C	492.0 (6)	106.8 (12)	445.8 (6)
C_x	TWEEN	2660.0 (9)	2523.3 (12)	323.3 (9)
	C	558.3 (6)	503.3 (9)	440.0 (9)
BG	TWEEN	72.8 (15)	12.8 (18)	400.9 (15)
	C	196.8 (6)	106.8 (12)	445.8 (6)
PG	TWEEN	12.0 (3)	73.2 (18)	123.0 (6)
	C	16.2 (6)	160.8 (9)	280.8 (6)

C = Control i.e., no Tween addition

Figure in parentheses is the day on which the activity occurred

DISCUSSION

Results presented in the previous section indicate the amount of biomass produced by each fungal species is dependent on temperature. However filtrates with high enzyme activity are not necessarily associated with cultures giving maximum dry weight production.

Using T.viride QM9414, Mandels, Sternberg and Andreotti (1975) established that the optimum conditions for cell growth (30°C) and cellulase production (25°C) may be distinctly different with cellulase production stimulated by less rapid fungal growth at lower temperatures.

In this study, S.thermophile appears to follow the above pattern i.e., highest average enzyme activities were produced at a lower temperature than maximum dry weight. Whereas for B.cinerea and T.viride the reverse was true, highest average enzyme activities occurred at or above the temperature promoting maximum dry weight production. In addition, Tables 6:1 and 6:2 indicate that average and maximum activity for a given enzyme do not necessarily occur at the same temperature. Maximum activity and the time taken to promote such a value is of importance if a filtrate with high hydrolytic potential is required. Whereas an average value gives an indication of enzyme activity over the whole cultural period and how (along with Figures 6:3 - 6:6) such activity is sustained over a prolonged period at a defined temperature.

The rate of enzyme synthesis by fungi from different thermal

groups has also been investigated. Coutts and Smith (1976) when comparing enzyme production of a mesophile and thermophile grown on identical media found yields (based on reducing sugar produced from 1 ml of filtrate incubated with filter paper for 1 hour) produced by T.viride in 14 - 18 days could be matched by S.thermophile in 3 - 4 days. In contrast, data from this study (Appendix 6:2) demonstrates that only C_x and endo-polygalacturonase activity could be said to follow the pattern predicted by Coutts and Smith (1976). Although as the values compared were all recorded on Day 3 (the 1st sampling time) enzyme activity for the preceding days would have to be elucidated before results in this study were confirmed to coincide with those of the above authors.

The greater deleterious effect of temperature on fungal growth compared to enzyme activity is highlighted in this present work by B.cinerea. No mycelial growth was apparent at 30°C or 35°C although there was evidence of enzyme activity in the filtrate. Verhoeff (1978) found leachates from ungerminated conidia of B.cinerea to contain endo-polygalacturonase and later studies of Verhoeff, Liem, Scheffer and Surya (1983) indicated the presence of cellulolytic enzymes in conidia. Such enzymes may therefore exude into the surrounding medium prior to germination, yet remain active at temperatures which do not support growth, as seen in this present work.

The second factor investigated in this study was pH. In buffered cultures higher enzyme activity occurred at pH 3 compared to pH 5 for B.cinerea and T.viride (with the exception of B glucosidase from T.viride). However, only levels (at pH3) of C_1 and C_x for

B.cinerea and T.viride respectively were comparable to those exhibited by unbuffered cultures. Sternberg (1976) working with T.viride QM9414 in shake flask cultures containing cellulose (Solka Floc) found as with T.viride above; if citrate buffer (0.01lM) was incorporated into the growth medium to prevent the pH from falling below 5, B-glucosidase activity was increased 10 fold. Whereas production of other components of the cellulase complex were greater in unbuffered medium, where pH fell naturally to pH 2.8.

In contrast for S.thermophile (in this study) the reverse case applies (i.e., higher activity at pH5 than 3). Most notable with this species is an absence of any C_x or endo-polygalacturonase activity at pH3, although levels of this latter enzyme at pH5 are similar to these in unbuffered cultures. Coutts and Smith (1976) also found greater yields of C_1 and C_x were produced by S.thermophile under alkaline conditions compared to media at pH3. The beneficial effect of these alkaline conditions may they proposed result from its adverse effect on fungal growth rate. With a decline in metabolic activity as a consequence of increased pH stimulating cellulase production to maintain adequate growth.

Similar findings were reported by Andreotti, Mandels and Roche (1977). Using T.viride QM9414, they found an inverse relationship between growth and enzyme production which in turn was related to pH. They concluded that as the mycelial yield (based on protein levels) decreased the percentage of biomass diverted to other products including enzymes increased, and this increase was steeper at low pH levels. Thus higher cellulase production resulted from cultures in which the pH was not allowed to go below 3.5 after a

natural fall compared to cultures controlled at pH 5. However, as in this study, if cultures were controlled at pH 3.5 from the time of inoculation fungal growth and enzyme production were markedly retarded. Work by Knapp and Legg (1986) further suggests that enzyme activity is not only affected by culture pH but also by growth substrate. As on investigating cellulase production by T.ressei in fermenters with pH control (from Day 0), they found that when wheat straw was used as the major carbon source cellulase production was similar over a range of pH values (pH 3-6). However with more refined substrates e.g., Solka Floc the enzyme activity recorded was very dependent on pH.

As previously noted for a majority of cases, results in this study showed higher enzyme activity from unbuffered cultures (in which results from Chapter 3 indicate pH rose to 8 - 9 for S.thermophile and T.viride or pH 6 - 7 with B.cinerea) compared to those with pH control. These findings suggest that although citrate may be metabolized by fungi, citrate buffer (citric acid and sodium citrate) may have an adverse effect perhaps due to its molarity (although the level was that used in enzyme assays). However, as data also indicates that pH control does affect enzyme activity hence for example higher values at pH 3 than 5 for B.cinerea and T.viride, further work is needed to clarify the role played by pH. Areas for investigation might include, use of different buffering systems thus allowing pH control at values similar to those found in unbuffered cultures, or varying the time from which pH is controlled.

Cellulase production may also be affected by surfactants. Reese and Maguire (1970) found incorporation of Tween 80 into culture media

resulted in a two fold increase in cellulase yield from T.viride. However in this study addition of Tween 80 has only a limited effect on enzyme activity, with significant increases (four fold) recorded for C_x production from S.thermophile and B.cinerea. In other cases addition of this adjunct caused a decrease in activity. The reasons for enhancement by Tween 80 is not as yet fully understood. Possible mechanisms were outlined by Shewale (1982) but would not explain the selective beneficial effect this surfactant had in the present work. Therefore in the overall degradation/recycling process (static culture) investigated in this study its use would not initially be recommended. Although it may be more appropriate to shake conditions as Reese (1980) found inactivation of enzymes due to agitation was prevented by Tween 80 addition.

Results in this chapter therefore suggest that if fungal biomass were the desired product improvements in maximum dry weight values on C.roseus cells for all three fungi can be obtained through selective use of incubation temperature (i.e., S.thermophile 50°C, B.cinerea and T.viride 15°). Although such temperatures may not be conducive to enzyme production. Data in Chapter 5 implies that in the main a re-autoclaved substrate and careful selection of incubation conditions (static or shaken) are beneficial for enzyme activity. Therefore by further investigating optimum incubation temperature for each fungal species on the different plant cell substrates used in Chapter 5 still further increases in enzyme activity or fungal biomass production may be possible.

CHAPTER SEVEN

UTILIZATION OF GLUCOSE PRODUCED BY THE RE-AUTOCLAVING PROCESS AND THROUGH FUNGAL BREAKDOWN OF PLANT CELL BIOMASS FOR FUNGAL AND PLANT CELL CULTURE

INTRODUCTION

The conversion of cellulose to D-glucose via enzymatic hydrolysis can be a forerunner of many applications in food preparation, fermentation technology and fuel production. In addition to its value as a food sweetener, glucose may be used as a starting material for nearly all fermentation products made at present such as organic acids (e.g., acetic, lactic, citric), amino acids, vitamins, antibiotics and a number of bacterial and fungal polysaccharides e.g., xanthan, pullulan and scleroglucan. (Edwards, 1975). Such polysaccharides find applications in foods, cosmetics and pharmaceuticals as viscosity additives. In addition xanthan is used in the oil and mining industry as a "drilling mud" or lubricant while Shipman and Fan (1978) have produced pullulan derived plastics.

Glucose is also a readily utilized substrate for single cell protein (SCP) production, which may be comprised of algae, bacteria, fungi or yeasts. Except for algae, SCP can be produced independently of climate, season or arable area and due to the high growth rates exhibited by microorganisms, biomass can be obtained in a much shorter time compared to conventional plant or animal foods. SCP may be used both in animal and human nutrition, although the high nucleic acid content of SCP, means that it must undergo processing before it is acceptable for human consumption. However, Myco (fungal) protein (Edelman, Fewell and Solomans, 1984) can be found in certain food products for example pies which are currently marketed.

Bioconversion of cellulose to alcohol (ethanol) via glucose has also been investigated as an alternative energy source i.e., cars run

on a mixture of ethanol and petrol. For example Cysewski and Wilke (1976) have produced ethanol via a two step process in which cellulose (Newspaper) was hydrolyzed to glucose using an enzyme rich broth from T.viride QM 9414 (mycelium/enzyme separated by filtration) followed by fermentation to ethanol using Saccharomyces cerevisiae. Seventy percent of the glucose was converted to alcohol with the remaining 30% used to produce SCP (Candida utilis). Andren and Nystrom (1976) and Allen (1976) further suggested that the overall process could be made more economical by recycling fungal (T.viride) biomass (as a source of protein) back into enzyme production. In addition this mycelial by product may be incorporated into wood fibre paper without a deleterious effect on paper strength (Johnson and Carlson, 1978).

The above two stage process has since been superceded by single operation methods. Savarese and Young (1978) have reported a procedure by which enzymic saccharification of cellulose is coupled with yeast fermentation (of resultant glucose) to ethanol in the same reactor. Direct fermentation of cellulose to alcohol by using mixed (Avgerinos and Wang, 1983) or mono (Gong, Maun and Tsao, 1981; Deshpande, Keskar, Mishra and Rao, 1986) cultures have also been reported. Separation of ethanol from the fermented hydrolysate is achieved by distillation, after filtration to remove biomass (which can be used as a food supplement). While the remaining non volatile liquid (stillage) which consists of residual sugars, ethanol, waxes, fats, fibres and mineral salts; may be used as a fertilizer, inoculated with yeast for SCP or digested anaerobically to produce methane, (Serovich, 1986). Ethanol itself can form a substrate in SCP production with McAbee (1974) reporting work (on a commercial

scale) by Japanese companies in this area. It is also a key raw material in the manufacture of drugs, plasticizers, perfumes, plastics and cosmetics. (Bisaria and Ghose, 1981).

In this work ways of utilizing glucose produced by 1) the plant cell re-autoclaving process and 2) fungal growth on plant cell biomass, were investigated as a means of recycling nutrients back into plant cell culture and for production of single cell protein.

MATERIALS AND METHODS

As it was anticipated that the filtrate produced from re-autoclaving cells would contain a mixed carbohydrate profile, C.roseus plant cells were routinely subcultured onto B5 medium (Appendix 2:1) in which the carbohydrate content was comprised of equal amounts of glucose and sucrose. Thus enabling C.roseus cells to be conditioned to utilize such a media before their use in the experiments outlined below.

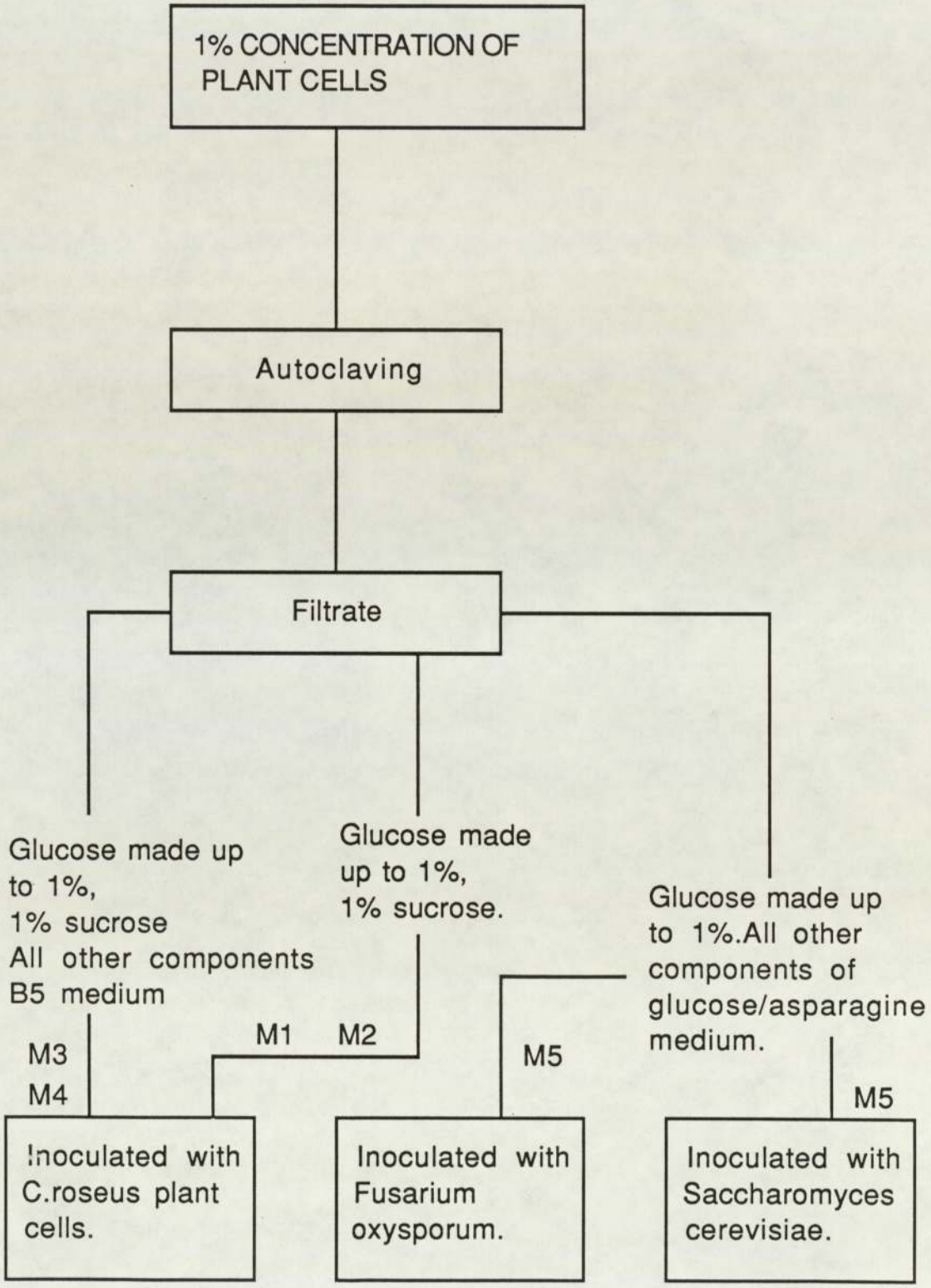
1. PLANT CELL FILTRATE USED FOR PLANT CELL CULTURE (Figure 7:1a)

Plant cells (C.roseus or D.carota) were combined with distilled water at a 1% concentration. After autoclaving, solids were removed by filtering through muslin (these were then used as re-autoclaved plant cells as indicated in previous chapters). Glucose in the filtrate was determined by the Nelson method (see Chapter 2) and Filtrates produced in this manner then used to make media 1 - 4 (see Table 7:1) for C.roseus plant cell culture. After 14 days growth plant cells were filtered off, dried overnight at 40°C and weighed.

2. PLANT CELL FILTRATE USED FOR FUNGAL CULTURE (Figure 7:1a)

C.roseus plant cells were combined with distilled water at a 1% concentration. After autoclaving, solids were removed and glucose in the filtrate determined. This filtrate was used to make medium 5 (Table 7:2). After autoclaving, 100 ml Erlenmeyer flasks containing 20 mls medium 5 were inoculated with (i) a plug of Fusarium oxysporum grown on malt extract agar (Appendix 3:1) or (ii) a drop of Saccharomyces cerevisiae from a suspension of cells in peptone water (Appendix 7:1). Dry weight production was

FIGURE 7:1a Flow diagram of the procedure used to investigate C.roseus and fungal biomass production on filtrate produced during the re-autoclaving process.



M=Medium (see TABLE 7:1, 7:2)

TABLE 7:1

MEDIA FOR PLANT CELL GROWTH CONTAINING FILTRATES FROM THE RE-AUTOCLAVING PROCESS

Medium (No)	Composition
1	C.roseus plant cell filtrate (from re-autoclaving process) with glucose level made up to 1% 1% sucrose pH 5.8
2	D.carota plant cell filtrate (from re-autoclaving process) with glucose level made up to 1% 1% sucrose pH 5.8
3	C.roseus plant cell filtrate (from re-autoclaving process) with glucose level made up to 1% 1% sucrose All other components of B5 medium (Appendix 2:1) pH 5.8
4	D.carota plant cell filtrate (from re-autoclaving process) with glucose level made up to 1% 1% sucrose All other components of B5 medium pH 5.8
Control for experiments utilizing media 1 - 4	B5 medium with carbohydrate content composed of 1% glucose and 1% sucrose

TABLE 7:2

MEDIA FOR PLANT CELL AND FUNGAL GROWTH CONTAINING FILTRATES FROM EITHER THE RE-AUTOCLAVING PROCESS OR FUNGAL BREAKDOWN OF PLANT CELL BIOMASS

Medium (No)	Composition
5	C.roseus plant cell filtrate (from re-autoclaving process) with glucose level made up to 1% All other components of Glucose/Asparagine medium (Appendix 7:1)
6	Filtrate from the breakdown of re-autoclaved C.roseus plant cells by S.thermophile, B.cinerea or T.viride. Glucose level made up to 1% 1% sucrose pH 5.8
7	Filtrate from the breakdown of re-autoclaved C.roseus plant cells by S.thermophile, B.cinerea or T.viride glucose level made up to 1% 1% sucrose All other components of B5 medium pH 5.8
8	Filtrate from the breakdown of re-autoclaved C.roseus plant cells by B.cinerea. Glucose level made up to 1%. All other components of Glucose/Asparagine medium.

Control used with 5 + 8 = Glucose/Asparagine medium
Control used with 6 + 7 = as for media 1 - 4

determined for F.oxysporum every 3 days for 15 days using dried pre-weighed Whatman No 1 filter papers (less dry weight of MEA plug) and for S.cerevisiae every 2 days for 8 days using dried pre-weighed Whatman glass fibre filter papers (GF/B).

3. FILTRATE FROM FUNGAL GROWTH ON PLANT CELL BIOMASS FOR PLANT CELL CULTURE (Figure 7:1b)

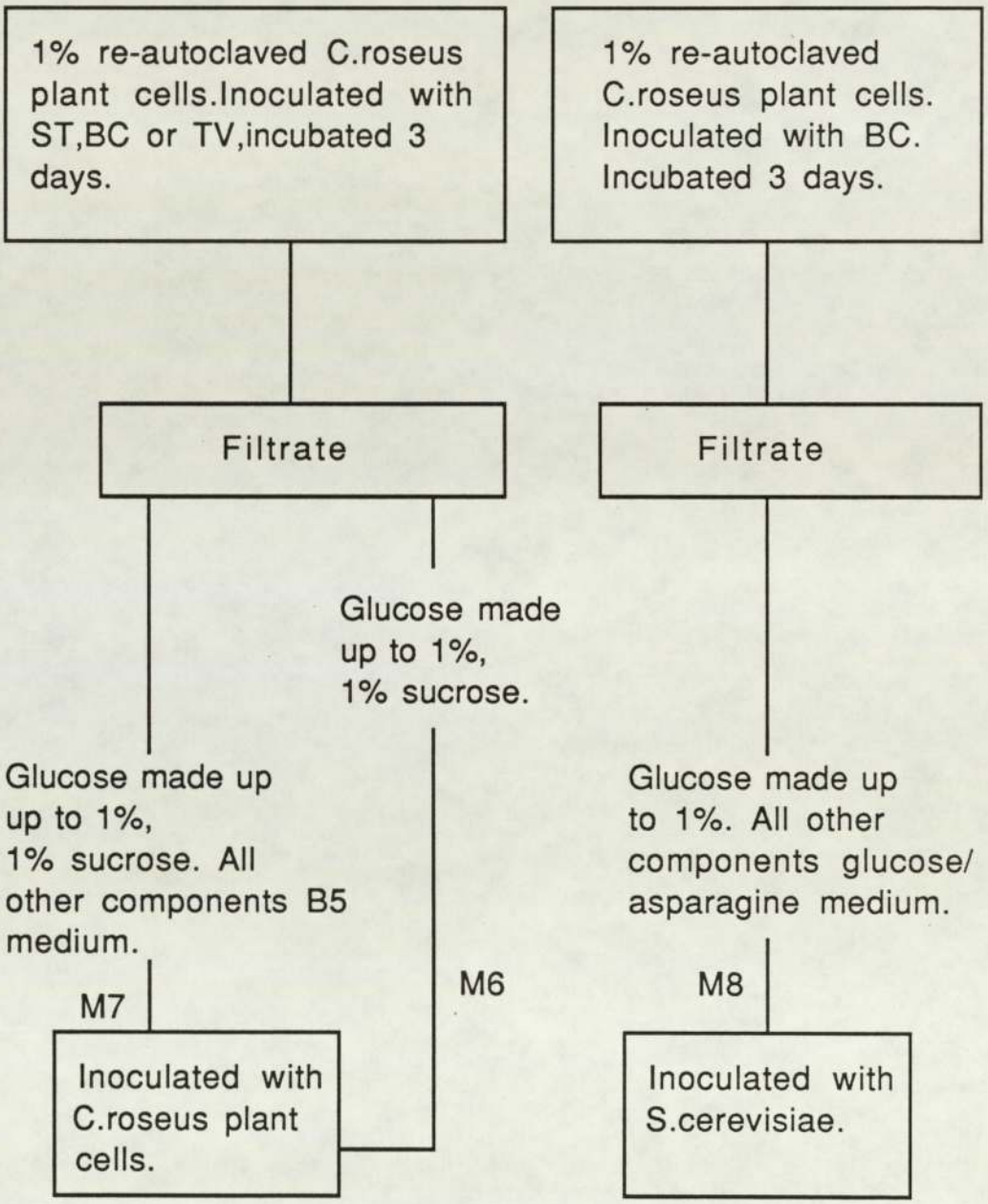
Re-autoclaved C.roseus plant cells were combined with distilled water at a 1% concentration, autoclaved and inoculated with either S.thermophile, B.cinerea or T.viride. After 3 days growth, mycelium was filtered off and the supernatants glucose level determined. This filtrate was used to make either medium 6 or medium 7 (Table 7:2) for C.roseus plant cell culture. After 14 days growth, plant cells were filtered off dried overnight at 40°C and weighed.

4. FILTRATE FROM FUNGAL GROWTH (B.cinerea) ON PLANT CELL BIOMASS FOR FUNGAL CULTURE (Figure 7:1b)

Only B.cinerea was used in this experiment as it generally promoted the highest release of glucose from plant cell breakdown.

A 1% concentration of re-autoclaved C.roseus cells were inoculated with B.cinerea. After 3 days growth, mycelium was filtered off and glucose levels determined for the filtrate. This filtrate was used to make medium 8 (Table 7:2). After autoclaving, 100 ml Erlenmeyer flasks containing 20 ml medium 8 were inoculated with a drop of S.cerevisiae from a suspension of cells in peptone water. Dry weight production was determined every 2 days for 10 days using dried pre-weighed Whatman glass fibre filter papers.

FIGURE 7:1b Flow diagram of procedures used to investigate C.roseus and S.cerevisiae production on filtrate from fungal breakdown of plant cell biomass.



M=Medium (see TABLE 7:2)

5. STATISTICAL ANALYSIS

Analysis of variance was determined on data from sections 2 and 4 using a 2 x 4 (5) factorial split plot analysis in a randomised design.

RESULTS

Data obtained from these experiments shows that when filtrate obtained from re-autoclaving C.roseus cells is incorporated into media (1 and 3) for C.roseus plant cell culture, similar dry weight values result (Table 7:3). Thus indicating addition of other nutrients to plant cell filtrate (medium 3) is unnecessary as autoclaving must break down plant cell material, liberating adequate amounts of such components (medium 1). However, in both the above media higher plant cell dry weight values were obtained with the control. Most interesting is the reduced growth experienced with medium 3 compared to its control, as these two media are essentially the same except for the presence of plant cell filtrate in medium 3.

Using media 2 and 4 containing filtrate from re-autoclaving D.carota plant cells, less C.roseus plant cell growth results from medium 4 (includes additional nutrients) than medium 2 (Table 7:3). Additions of such nutrients to medium (4) may add to those already in the filtrate thus producing toxic levels. As with C.roseus derived media (1 and 3), less C.roseus cells were produced on media 2 and 4 compared with their respective controls. This difference may also indicate D.carota plant cells contain metabolites e.g., anthocyanin which are released through autoclaving and thus incorporate novel substances into media 2 and 4 used for C.roseus plant cell culture (No D.carota plant cells were available at the time to grow a media 2 and 4).

Dry weight production of S.cerevisiae on medium 5 (C.roseus plant cell filtrate produced through re-autoclaving) is very similar

TABLE 7:3

PLANT CELL GROWTH ON MEDIA 1 - 4

DRY WEIGHT (g) OF C.ROSEUS PLANT CELLS/LITRE	
MEDIUM	CONTROL
(1) 7.45	11.34
(2) 8.83	11.21
(3) 7.97	12.31
(4) 6.82	11.21

TABLE 7:4

PLANT CELL GROWTH ON MEDIA 6 AND 7

DRY WEIGHT (g) OF C.ROSEUS PLANT CELLS/LITRE		
MEDIUM		CONTROL
(6) 5.86	Using	11.16
(7) 7.20	B.cinerea	11.76

C.roseus cells failed to grow on media containing the filtrate of plant cells inoculated with S.thermophile or T.viride

FIGURE 7:2a Growth of S.cerevisiae on Medium 5

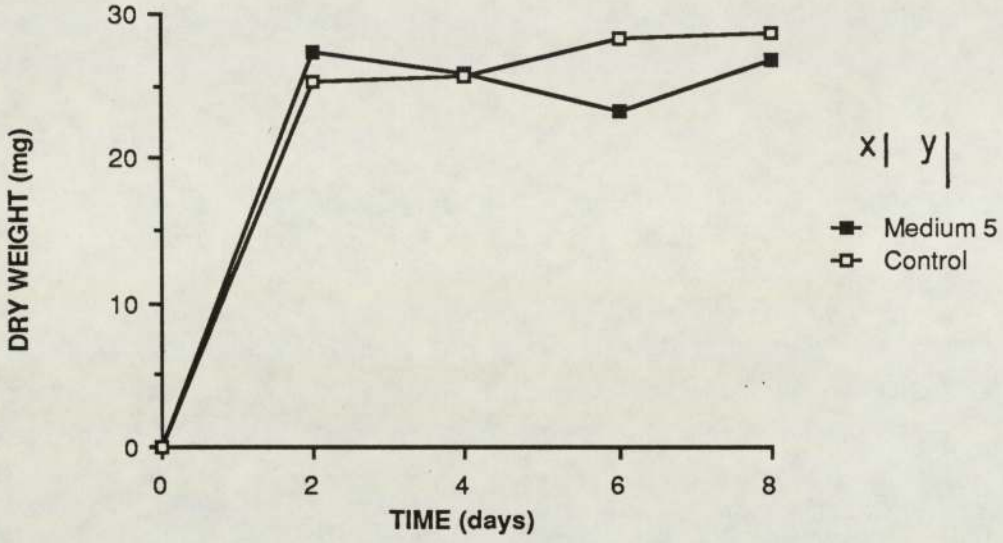


FIGURE 7:2b Growth of F.oxysporum on Medium 5

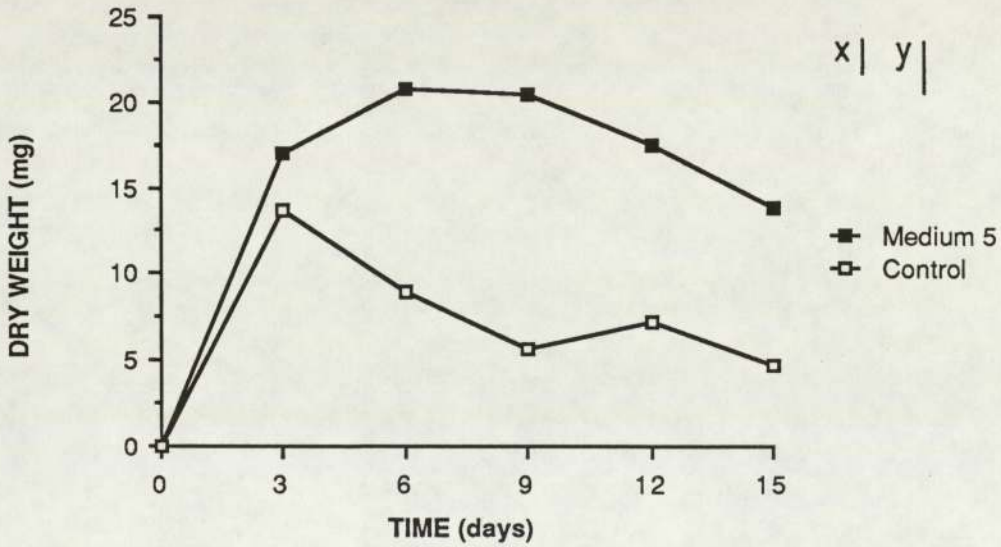
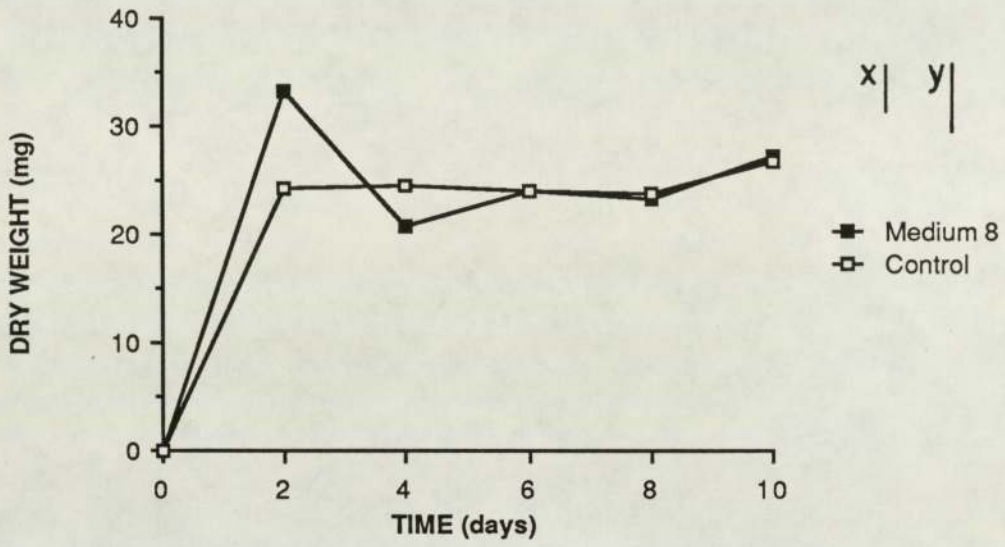


FIGURE 7:3 Growth of S.cerevisiae on Medium 8.



X=95% confidence limits for differences within a treatment and Y=95% confidence limits between two treatments at the same or different times.

to the control (Figure 7:2a, Appendix 7:2). In contrast there is a significant difference between growth of F.oxysporum on this medium (5) and its control (Figure 7:2b, Appendix 7:2). However this may in part be due to additional carbohydrate e.g., sugar alcohols, or non reducing sugar present in medium 5 (derived from the re-autoclaving filtrate) which cannot be assayed for by the Nelson method (reducing sugars only).

Results in Table 7:4 suggest during fungal growth on plant cell biomass, nutrients are depleted hence on medium 6 (filtrate plus carbohydrate only) less plant cell growth occured compared to the control. However, even on addition of metabolites (medium 7) plant cell yield is still less than the control even though medium 7 and the control are essentially the same. Thus suggesting some factor produced by B.cinerea may be responsible for this reduced growth. C.roseus plant cells also failed to grow on medium containing S.thermophile or T.viride filtrates which may indicate these species also produce a thermostable plant cell "toxin" (Table 7:4).

Figure 7:3 (Appendix 7:2) indicates dry weight yields of S.cerevisiae on medium 8 (containing filtrate from B.cinerea breakdown of plant cells) is similar to the control. Therefore any factor produced by B.cinerea appears to be plant cell specific.

In media 1 - 5, 4% of the total amount of glucose was provided by filtrates produced during the re-autoclaving process. However in the remaining media, although fungal breakdown of re-autoclaved C.roseus cells had produced a 400% increase in filtrate glucose levels, this provided only 1 - 2% of the total glucose used in media 6 - 8.

DISCUSSION

Results from Table 7:3 indicate that C.roseus plant cell growth is reduced on medium 3 compared to the control (B5 medium, 1% glucose, 1% sucrose) even though both have essentially the same composition (medium 3 contains C.roseus filtrate). This suggests a thermostable "toxic factor" (e.g., plant cell wall constituent or metabolite) is released from C.roseus biomass during the re-autoclaving process and is therefore present in the filtrate incorporated into medium 3.

Other examples of biochemical factors affecting plant cell growth have been reported, including metabolites secreted into the culture medium during active cell growth (such factors may also be present in cells on harvesting, and released when autoclaved as above). Woodruffe, Anthony and Street (1970) studying cultures of excised wheat roots found they released a self inhibitory substance which could be absorbed onto activated charcoal. The inhibitor although not identified was not thought to be an auxin or indole. Also using root cultures (Tomato), Winter and Street (1963) found staled culture media contained many organic metabolites including phenolic substances e.g., salicylic acid which proved inhibitory to root growth. Amino acids have also been shown in certain cases to inhibit plant cell growth. Using tobacco cell cultures Matsumoto, Okunishi and Noguchi (1976) and Koiwai, Tanno and Noguchi (1970) have reported inhibition through addition of such components to culture media. In the latter case, glycine, aspartic acid, asparagine and tyrosine where found to inhibit cell growth by 25 - 30%. Soybean plant cells cultured on B5 medium by Gamborg (1970) were similarly

inhibited on addition of a range of amino acids. Results in Table 7:3 also indicate reduced C.roseus plant cell growth on medium (4) containing filtrate from D.carota cells. The reasons for this may in part be due to those outlined above. However by using the filtrate produced from re-autoclaving D.carota cells in medium (4), C.roseus plant cells would be introduced to novel compounds e.g., anthocyanin present only in or to a larger extent in the former species. Likewise, Steward and Caplin (1952) reported onion bulb extracts to be inhibitory to carrot tissue. Whereas Barker (1970) investigating the influence of metabolites produced by explants e.g., carrot, lettuce, potato on other tissue cultures, found where two or more species were grown sequentially on the same agar a stimulatory effect was generally observed.

In contrast to the results outlined above, when C.roseus plant cell filtrate was incorporated into medium (5) and inoculated with S.cerevisiae or F.oxysporum no deleterious effects were noted (Figure 7:2a, b). In the latter case growth was superior to the control, however an unknown amount of non reducing sugar may have been supplied to medium (5) in the plant cell filtrate, and therefore contributed to this result.

When the filtrate produced from fungal (B.cinerea) activity on re-autoclaved plant cell biomass was incorporated into medium (7), C.roseus plant cell growth was reduced (although all other nutrients were provided). Whereas filtrates resulting from S.thermophile or T.viride activity proved totally inhibitory (Table 7:4). This therefore suggests the presence in filtrates used in medium 7 of a heat stable fungal factor which is detrimental to plant cell

growth. Trichoderma species have been shown to produce a number of volatile and non volatile antibiotic substances (Dennis and Webster, 1971a, 1971b), also a range of toxic compounds including Trichotoxin A (Hou, Ciegler and Hessletine, 1972), which are generally fungistatic, and although not tested on plant cells in suspension culture may well prove equally phytostatic. Whereas no investigations appear to have been conducted with S.thermophile, known metabolic products from B.cinerea which inhibit growth of many common fungi and gram positive bacteria include organic acids e.g., oxalic (Gentile, 1954) and antibiotics 'botrycine' (Ribereau-Gayon, Peynaud, Lafourcade and Charpentie, 1955) 'botrydial' (Fehlhaber, Giepel, Mercker, Tschesche and Welmar, 1974). Aksenova (1962) has also investigated a toxin produced by B.cinerea which causes grey rot in cabbages. Such fungal toxins may act on cell membranes, altering permeability and thus affecting the cells osmotic potential, enzymatic activity may also be disrupted through inhibition or the chelating properties of some toxins. However, whatever the nature of these substances produced by S.thermophile, B.cinerea or T.viride, Figure 7:3 would seem to indicate that they are less fungistatic and more plant cell specific, as growth of S.cerevisiae in medium (8) containing filtrate from B.cinerea activity on plant cell biomass was not significantly different from the control.

The reduction in plant cell growth experienced in media containing both filtrate produced during the re-autoclaving process and after fungal breakdown of plant cell biomass may also suggest more exacting growth conditions are needed to culture C.roseus plant cells compared to fungi (i.e., F.oxysporum, S.cerevisiae). Even slight changes in media composition (e.g., by addition of filtrates)

appear to affect plant cells to a much larger degree which is inturn reflected by a reduced biomass yield.

Calculated from current prices glucose provided by filtrates produced during the re-autoclaving process and from fungal breakdown of plant cell biomass represent a 0.304 p/l and 0.152 p/l saving in media costs respectively. This amount may appear small but as plant cell biomass is usually treated as a waste product any saving is important. In addition, any SCP produced from such filtrates will also be of value. However, these experiments also indicate that although filtrates can be used for the growth of F.oxysporum or S.cerevisiae they are unsuitable for plant cell growth in an unamended form. Factors in such filtrates which affect plant cell growth must first be identified and subsequently removed before these filtrates could form part of a recycling process in plant cell culture.

CHAPTER EIGHT

GENERAL DISCUSSION

The initial aim of this study was to assess the ability of plant cell biomass to support fungal growth. In order to provide a source of readily available material, suspension cultures of C.roseus and D.carota plant cells were established on B5 medium (Gamborg, Miller and Ojima, 1968). Subsequently through a series of assay techniques the general composition of these plant cells was investigated.

Cellulose content was determined using a method outlined by Updegraff (1969), however values of 5 - 7% and 6 - 9% for D.carota and C.roseus cells respectively were much smaller than reported for suspension cultured sycamore cells by Talmadge, Keegstra, Bauer and Albersheim (1973) but nearer to percentages calculated by Burke, Kaufman, McNeil and Albersheim (1974) for oat, wheat and rice cells cultured in a similar manner. Likewise, crude protein levels for C.roseus and D.carota cells (10.7 and 6.7% respectively) were lower than data reported by Matthern (1962) using an unnamed plant cell type, but comparable values have been found for rice (8.7%) and sweet clover (6.7%) by Gamborg and Finlayson (1969). However for both cellulose and crude protein the above differences may be media, assay method or species related.

Amino acid analysis of C.roseus and D.carota material reveals that whereas both substrates have similar overall amino acid profiles, for an individual amino acid (particularly glutamine, alanine, phenylalanine and arginine) higher amounts are found in C.roseus compared to D.carota cells. In contrast, for a range of soluble carbohydrates the reverse case applies with significantly higher amounts detected in D.carota plant cells. Biochemical and microbiological assay procedures established in C.roseus material the

presence of a variety of vitamins, with de novo synthesis of ascorbic acid, folic acid, pantothenic acid, biotin and riboflavin indicated. Although using microbiological techniques other factors such as pH, temperature or salt concentration may affect the outcome of an assay.

The composition of plant cell material outlined above therefore suggested such a substrate could provide all the basic nutrients needed to sustain fungal growth. On investigation this was confirmed as all three fungi, S. thermophile, B. cinerea and T. viride were able to grow on each plant cell substrate (C. roseus, D. carota, re-autoclaved C. roseus, D. carota, and methanol extracted C. roseus) without nutrient addition whatever culture conditions were employed. However, the amount of fungal biomass produced varied between species, and for each species with plant cell substrate, culture conditions (static or shaken) and incubation temperature.

Fungal growth, particularly for S. thermophile and T.viride, was reduced when shake conditions were employed, whereas such a mode of culture proved beneficial for B. cinerea. Shaking has been shown by Tanaka, Takahashi and Ueda (1975) to cause leakage of intracellular material (RNA related nucleotides) from fungal mycelium, with the amount of leakage dependent on agitator speed. Ajcova, Fencl, Muslikova and Seichert (1980) however, found the opposite with a lower leakage of intracellular material at higher stirring speeds using Aspergillus niger S59 and it was suggested by Musilkova, Ujcova, Placek, Fencl and Seichert (1981) that this may be because high agitation of A. niger S59 leads to a morphologically compact and strong mycelium which is more resistant to the forces generated

during shaking. In this study, B.cinerea tended to form pellets on shaking thus perhaps affording some protection as indicated above. Such a growth form in conjunction with the increased aeration provided by agitated cultures may therefore have contributed to the higher biomass yields reported for this species.

Incubation temperature also influenced the amount of fungal biomass produced on C.roseus plant cells, with maximum yields obtained at 50°c for S.thermophile and 15°c for both B.cinerea and T.viride. In addition, biomass yields are also markedly influenced by substrate, with species demonstrating a preference for particular plant cell material i.e., S.thermophile : re-autoclaved D.carota (static), B.cinerea : re-autoclaved C.roseus (shake) and T.viride : D.carota (static). Such combinations promoting high yields are important if SCP (mycoprotein) production is the primary objective.

A number of studies have investigated the role of fungal biomass in animal diets. Smith, Palmer and Reade (1975) reported mycelium including that of T.viride could be fed to rats or pigs without any adverse effects, but the amino acid profile of such mycelium was found to be deficient in sulphur containing amino acids essential for actively growing animals. Inclusion of SCP in human diets has been reviewed (Lovland, Harper and Frey, 1976), although it has long been recognised that due to a high nucleic acid level such material must be processed before it can be incorporated into products for human consumption. At the present time some examples of commercially available products containing mycoprotein do exist e.g., Fusarium graminearum has been developed by Rank, Hovis MacDougal for use as a meat analogue in pies and pasties (Edelman, Fewell and Solomons,

1984).

Five plant cell substrates were utilized in this study, however only methanol extracted (static culture), re-autoclaved C.roseus (static and shake) and re-autoclaved D.carota (static) when incubated with the three fungal species released significant amounts of glucose (i.e., values higher than Day 0). A particularly marked increase was noted with a combination of re-autoclaved C.roseus cells and B.cinerea where, after 3 days a glucose level 5.6 times higher than Day 0 was detected in the filtrate. Levels were also generally lower and the pattern of release different in shake compared to static cultures. Glucose and simple carbohydrates liberated in this way could be used as a starting point for a whole range of fermentation processes e.g., for the manufacture of amino acids, vitamins, edible organic acids, antibiotics, plant growth stimulants, ethanol, exopolysaccharides and SCP. In this study, glucose released during the re-autoclaving process and by fungal breakdown of plant cell biomass was employed for the growth of S.cerevisiae and Fusarium oxysporum, with resultant yields equal to or greater than controls. However, such carbohydrate could not be recycled into plant cell culture due to the presence of unknown factors possibly of both plant cell and fungal origin (particularly S.thermophile and T.viride) which precluded plant cell growth. Fungal species such as T.viride and B.cinerea have been reported to produce antibiotic substances and toxins (Dennis and Webster, 1971, Aksenova, 1962) which although only tested on other fungi and bacteria may prove equally toxic to plant cells.

Analysis of fungal enzyme activity (C_1, C_x, B-glucosidase, endo-

polygalacturonase) in culture filtrates revealed significant differences between the three species, also for an individual species activity varied with plant cell substrate and culture conditions. Cellulase enzyme preparations are already used in some industrial processes e.g., extraction of agar-agar from seaweed, mushroom softening and garlic processing in the food industry, and as an aid in oil extraction from vegetables (Wiseman, 1975). Maximum enzyme activity data obtained in this work would therefore be of value in developing the use of culture filtrates in the above processes.

Consideration of average values demonstrates that enzyme activity from all three species was with few exceptions higher from cultures under static compared to shake conditions. As noted earlier, shaking causes the release of intracellular material which in turn may disrupt fungal metabolism including enzyme synthesis. Also once released into a culture filtrate enzymes can be inactivated due to the effects of agitation. Such an observation has been reported by Reese and Ryu (1980), Reese (1980) and Mukataka, Tada and Takahashi (1983). Conversely Sakata, Ooshima and Harano (1985) suggested shaking was beneficial to the saccharification of cellulose by enhancing adsorption of C_1 cellulase. Prevention of such enzyme inactivation has been achieved by Reese (1980) through use of fluro-carbon surfactants and high molecular weight polyethylene glycols. Tanaka, Mizuguchi and Ueda (1975) also found that on increasing the viscosity of a medium fungal nucleotide release was reduced.

Enzyme activity in this study was in the main higher when re-autoclaved plant cell material was used, which may be due to the higher percentage cellulose found in such substrates. In addition a

higher level of glucose was present at Day 0 in C.roseus and D.carota cells compared to re-autoclaved material. This high level of glucose may have initially promoted catabolite repression in fungi growing on such cells thus reducing enzyme synthesis. Whereas in re-autoclaved material low levels of glucose present at Day 0 would be quickly utilized thus encouraging enzyme synthesis. These enzymes subsequently act on plant cell biomass releasing glucose which as previously noted could form the basis of a number of fermentations. However, as incubation proceeds and fungal biomass increases any glucose liberated is quickly metabolised and therefore does not accumulate. In contrast one of the major problems facing the economic saccharification of cellulosic wastes using cell free enzyme solutions is accumulation of glucose (Mandels, Dorval and Medeiros, 1978). This is because inhibition of B-glucosidase by glucose mediates a build up of cellobiose which in turn inhibits other members of the cellulase complex (Berghem, Pettersson and Axio-Fredrickson, 1975; Ladisch Gong and Tsao, 1977). Such a problem is particularly acute when using T.reesei cellulase preparations as this species produces only low levels of B-glucosidase (Mandels and Weber, 1969). Addition of supplementary B-glucosidase from for example Aspergillus phoenicis (Bissett and Sternberg, 1978) has been found to alleviate this problem. Other solutions preventing a build up of glucose include simultaneous conversion to either ethanol (Cysewski and Wilke, 1976; Viikari, Nyberg and Linko, 1981) or fructose (Woodward and Arnold, 1981).

Application of mixed cultures may improve the utilization of plant cell biomass, as Peitersen (1975) produced both cellulase and protein from a mixed culture of T.viride and either S.cerevisiae or

Candida utilis on straw. Also, results in this study indicate for each enzyme highest activities can be assigned to a particular species i.e., S.thermophile - C_x, B.cinerea - endo-polygalacturonase, T.viride - C_1 and B-glucosidase. Thus by growing such species together a culture filtrate with increased hydrolytic potential may be produced as was found by Duff, Cooper and Fuller (1986) using a combination of T.reesei and A.phoenicis. Such improvements are important as Wilke, Yang and Stockar (1976) have estimated that 60% of the costs involved in the hydrolysis of newspaper are related to enzyme production. Therefore ways of improving enzyme synthesis have been extensively investigated.

One such method is the development of mutant strains, particularly from Trichoderma species. Mandels, Weber and Parizek (1971) irradiated conidia of T.reesei QM 6a (wild type) producing a mutant (QM9123) with an enzyme yield twice that of the parent. While more recent work has reported mutant strains which show resistance to catabolite repression (Montenecourt and Eveleigh, 1979). Enzyme production using such strains can also be improved through the promotion of favourable culture conditions. Mandels, Sternberg and Andreotti (1975) using T.reesei QM 9414 first suggested the temperature for optimum growth ($30^{o}c$) and maximum cellulase production ($25^{o}c$) are not necessarily the same. In this study such a pattern was also generally noted. Further work by Andreotti, Mandels and Roche (1977) reported highest cellulase yields (with T.reesei QM 9414) resulted from conditions in which pH was controlled at 3.5 after a natural fall. Although if cultures were controlled at this value from the time of inoculation, enzyme production was markedly retarded. Likewise in this study where the pH was maintained at pH 3

or 5 (from Day 0) enzyme activity was in a majority of cases lower compared to unbuffered controls. Further improvements have been achieved by Mukhopadhyay and Malik (1980) using T .reesei QM 9414 in which a combination of the above parameters i.e., pH cycling (between 3 and 5.2) commencing 40 hours after inoculation and temperature profiling resulted in a 16% improvement in cellulase yields.

Addition of protein supplements including recycling spent mycelium (Andren and Nystrom, 1976) has been reported to be beneficial to enzyme production (Mandels and Weber, 1969). Such adjuncts e.g., proteose peptone, soyflour have also resulted in shorter lag times and faster mycelial growth. However, if used in amounts above 10% of the cellulose levels, enzyme synthesis may be repressed (Mandels, Sternberg and Andreotti, 1975). Usage of such substrates was further suggested by Mandels and Weber (1969) to enhance the effect of Tween 80 on enzyme production. Reese (1968) first noted the stimulatory effect associated with this adjunct however on its addition to cultures in this study any benefit was limited and therefore would not initially be recommended.

Many cellulosic wastes contain significant amounts of hemicellulose, for this reason Bisaria and Ghose (1981) and Chahal, Ishaque, Brouillard, Chornet, Overend, Jaulin and Bouchard (1987) have suggested that unless the hemicellulose fraction can be properly utilized, enzymatic hydrolysis of waste biomass based on cellulose utilization alone has little prospect of becoming economically viable. Although hemicellulose hydrolyzing enzymes have been studied (Dekker and Richards, 1976) their mode of action, kinetics and other properties are not as yet fully understood. Substantial interest has

centered on xylanases which are produced by a variety of microorganisms including T.viride (Siwinska and Galas, 1977; Dekker, 1983). Hydrolysis of hemicellulose produces a hydrolysate which contains mainly xylose together with minor quantities of other sugars such as arabinose, mannose, galactose and their uronic acids. Xylose can be used for example as a source of furfural, xylitol (a sweetener) or converted by yeasts e.g., Pachysolen tannophilus to ethanol (Slininger Bothast, Van Cauwenberge and Kurtzman, 1982). However, since hemicellulose is easily hydrolyzed by dilute acid at low temperatures, the use of hemicellulases for sugar production has aroused little commercial interest.

Pectic substances are also present in the primary cell walls of suspension cultured cells (Talmadge, Keegstra, Bauer and Albersheim, 1973). In this study B.cinerea in particular produced high levels of endo-polygalacturonase activity with preparations of such enzymes already having established uses in industry (Wiseman, 1975) e.g., in the extraction and clarification of fruit juices, and in olive or citrus oil recovery.

Other valuable enzymes may also be produced as a result of fungal activity on plant cell biomass and this could form the basis of future work. In addition, purification of the enzymes investigated in this present work using column chromatography would be required to elucidate their exact nature. Existing data indicates the cellulase complex is composed of three basic enzymes i.e., C_1, C_x and B-glucosidase, however multiple forms of each of these may exist. Thus Beldman, Searle-Van Leeuwen, Rombouts and Voragen (1985) reported a crude filtrate from T.viride to be comprised of 6 endo-glucanases

(C_x), 3 exo-glucanases (C_1) and a B-glucosidase, likewise using S.thermophile Mayer and Canevascini (1981), and Canevascini, Fracheboud and Meier (1983) have isolated 3 endo-glucanases, 1 exo-glucanase and 2 B-glucosidases. Once such enzymes have been separated and purified their temperature and pH optima can be established as these may be quite different from conditions under which enzyme synthesis occurs. Also their stability under such environments is important.

At present, the amounts of waste plant cell biomass available in the UK are small. Fowler (1985) estimated this to be 200 - 300 kg per year, but should plant cell biotechnology develop greater amounts will become available. Results from this study support those of Smith, Armstrong and Fowler (1985) indicating that plant cell biomass can support substantial fungal growth and may in turn have potential for mycoprotein production. Glucose released during fungal breakdown of plant cell material could also be further utilized, although results suggest recycling it back into plant cell culture is not feasible. Filtrates resulting from such fungal action exhibit C_1, C_x, B-glucosidase and endo-polygalacturonase activity, with preparations of such enzymes already used in industrial processes and for the saccharification of other cellulosic wastes as demonstrated above. However in all cases careful selection of fungal species, plant cell substrate and cultural conditions are essential to maximize formation of the desired product.

APPENDICES

APPENDIX 2:1 PLANT CELL CULTURE MEDIA

B5 MEDIUM (Gamborg et al 1968)

FORMULA (PER LITRE)

$MgSO_4.7H_2O$	250 mg
$NaH_2PO_4.2H_2O$	150 mg
$CaCl_2.2H_2O$	150 mg
KNO_3	2500 mg
$(NH_4)_2SO_4$	134 mg
EDTA (Na)	37.3 mg
$FeSO_4.7H_2O$	27.8 mg
$MnSO_4.4H_2O$	10 mg
KI	0.75 mg
$CoCl_2.6H_2O$	0.025 mg
$ZnSO_4.7H_2O$	2.0 mg
$CuSO_4.5H_2O$	0.025 mg
H_3BO_3	3 mg
$Na_2MoO_4.2H_2O$	0.025 mg
Myo-inositol	100 mg
Thiamine HCl	10 mg
Pyridoxine HCl	1 mg
Nicotinic acid	1 mg
2,4-dichlorophenoxyacetic acid	1 mg
Kinetin	0.1 mg
Sucrose	20 g
	pH 5.8

For callus cultures, the above plus 10 g/litre agar ($N^{o}1$, Oxoid) pH 5.8.

METHOD

Suspend in 1 litre of distilled water. Sterilise by autoclaving at 115°C, 20 mins (10 psi)

APPENDIX 2:2 (continued)

MDAUC MEDIUM (Reinert and Yeoman, 1982)

FORMULA (PER LITRE)

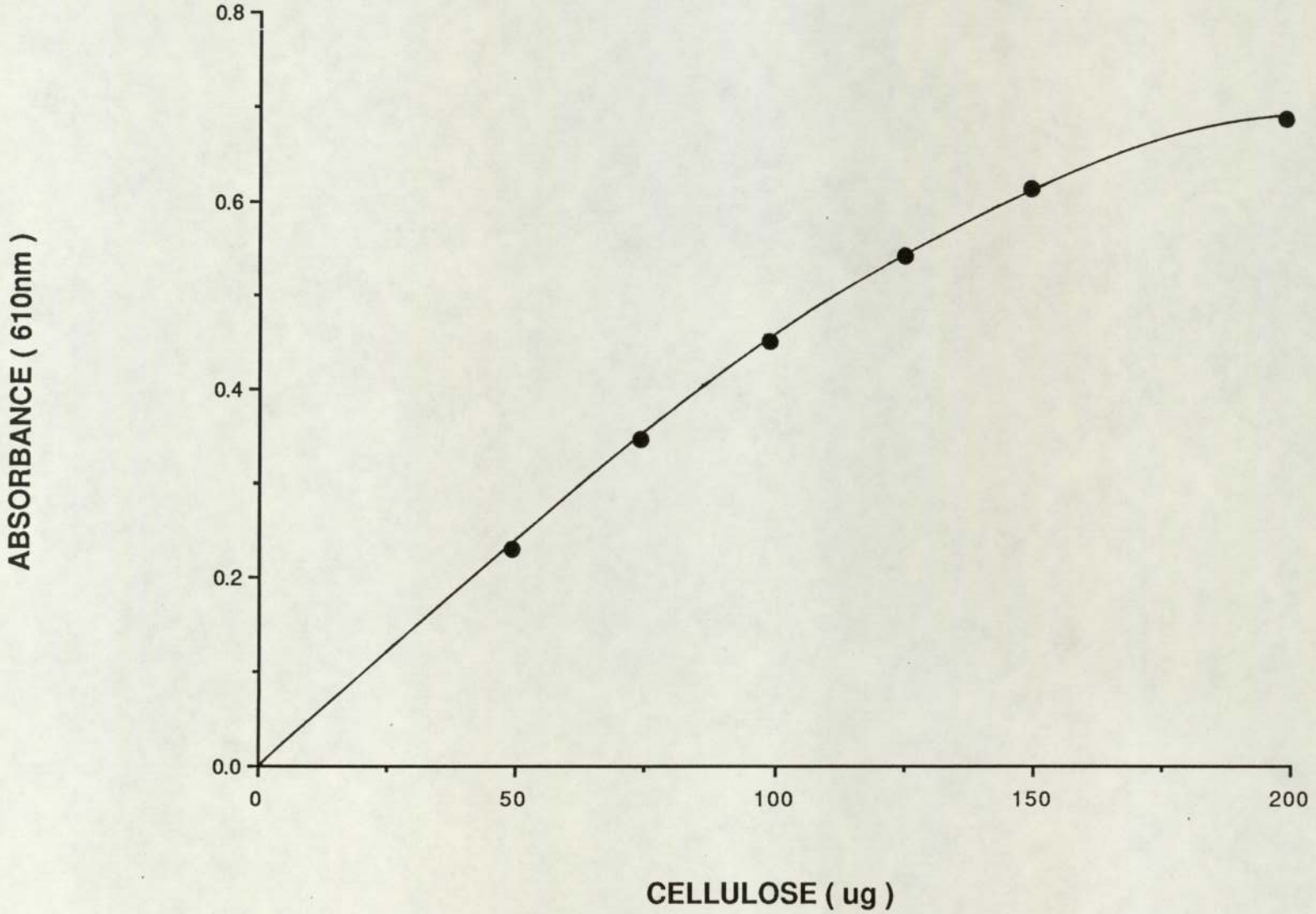
$CaCl_2.2H_2O$	440 mg
$FeSO_4.7H_2O$	27.8 mg
KH_2PO_4	170 mg
KNO_3	1900 mg
$MgSO_4.7H_2O$	370 mg
EDTA (Na)	37.3 mg
NH_4NO_3	1650 mg
$COCl_2.6H_2O$	0.025 mg
$CuSO_4.5H_2O$	0.025 mg
H_3BO_3	6.2 mg
KI	0.83 mg
$MnSO_4.4H_2O$	22.3 mg
$Na_2MoO_4.2H_2O$	0.25 mg
$ZnSO_4.7H_2O$	8.6 mg
Nicotinic acid	0.5 mg
Pyridoxine HCl	0.1 mg
Thiamine HCl	0.1 mg
Glycine	3 mg
2,4-dichlorophenoxyacetic acid	0.05 mg
Sucrose	20 g
	pH 5.8

For callus cultures, the above plus 10 g/litre agar ($N^0$1 Oxoid) pH 5.8

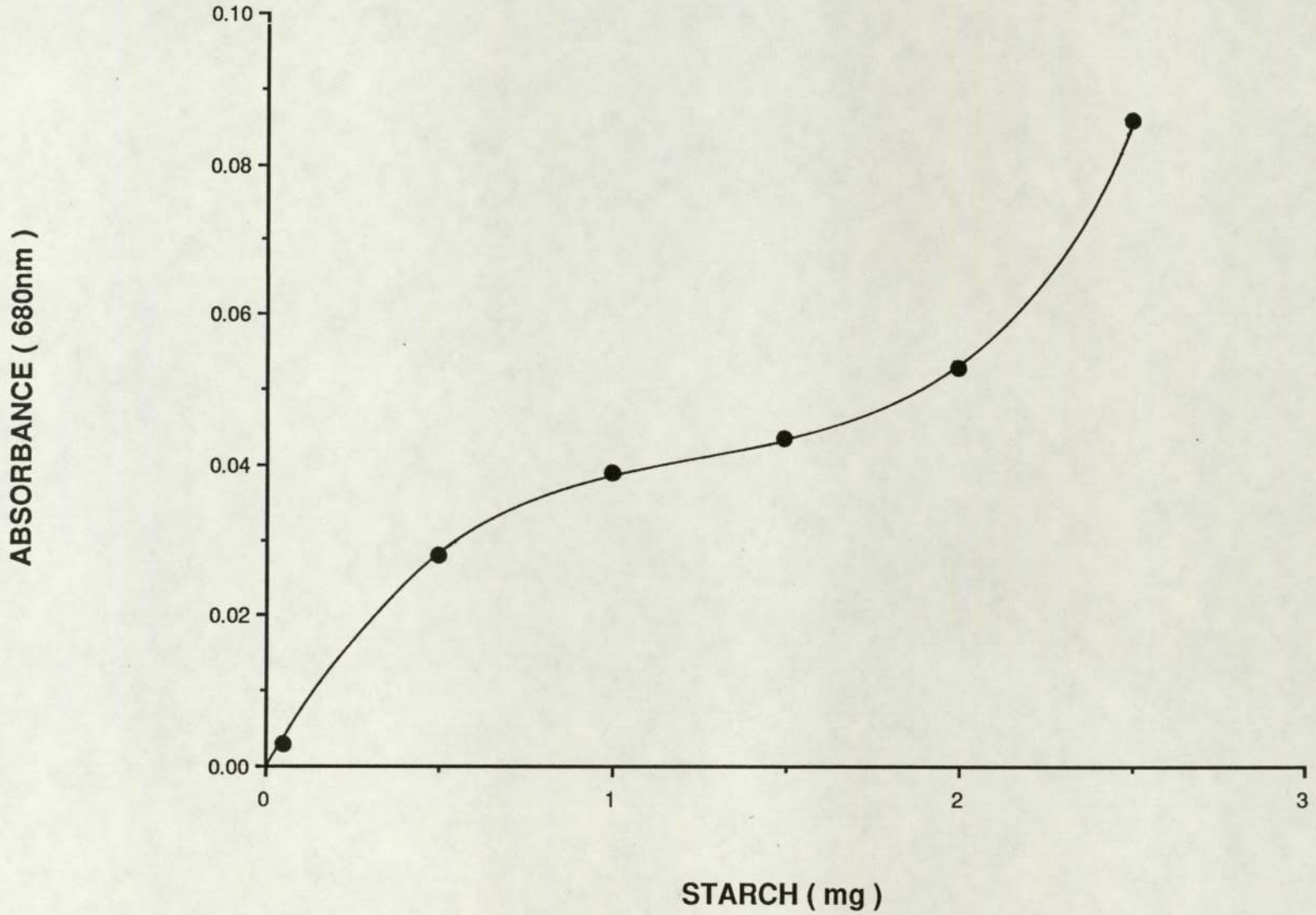
METHOD

Suspend in 1 litre of distilled water sterilise by autoclaving at 115^{0}C, 20 mins (10 psi)

APPENDIX 2:2 Cellulose standard curve.



APPENDIX 2:3 Starch standard curve.



APPENDIX 2:4

AMINO ACID PROFILE OF PLANT CELL MATERIAL

AMINO ACIDS (n mol/mg dry weight) IN C.ROSEUS PLANT CELL MATERIAL

	1	2	3
L-ASPARTATE	169.1	148.1	166.6
L-THREONINE	93.4	75.6	94.8
L-SERINE	119.9	97.2	110.6
L-GLUTAMINE	181.8	163.5	182.4
L-PROLINE	57.5	50.0	69.4
GLYCINE	159.0	157.4	166.6
L-ALANINE	186.8	160.4	165.2
L-VALINE	88.3	72.5	83.3
L-METHIONINE	21.4	18.5	22.9
L-ISOLEUCINE	88.3	66.3	90.5
L-LEUCINE	131.3	111.1	133.6
L-TYROSINE	47.9	43.2	47.4
L-PHENYLALANINE	59.3	54.0	68.9
L-HISTIDINE	36.6	27.7	37.3
L-LYSINE	101.0	95.6	106.3
L-ARGININE	69.4	80.24	90.5

APPENDIX 2:4 (continued)

AMINO ACIDS (n mol/mg dry weight) IN D.CAROTA PLANT CELL MATERIAL

	1	2	3
L-ASPARTATE	118.9	134.3	105.4
L-THREONINE	60.9	67.1	58.7
L-SERINE	77.7	78.1	70.7
L-GLUTAMINE	89.9	115.6	97.8
L-PROLINE	57.2	60.1	55.9
GLYCINE	112.8	120.3	97.8
L-ALANINE	92.9	85.9	88.8
L-VALINE	64.0	71.8	64.7
L-METHIONINE	15.2	23.4	15.0
L-ISOLEUCINE	54.8	60.9	48.1
L-LEUCINE	85.3	98.4	82.8
L-TYROSINE	28.9	32.8	27.1
L-PHENYLALANINE	13.7	7.5	36.1
L-HISTIDINE	21.3	29.6	19.5
L-LYSINE	64.0	68.7	58.7
L-ARGININE	39.6	48.4	34.6

STATISTICAL ANALYSIS

F Ratio		Df	Significance
MA	18.78	1,4	*
MI	58.13	15,60	* * *
A x B	5.62	15,60	* * *

MA = Major factor (plant cell substrate)
MI = Minor factor (amino acid)
A x B = Interaction (MA x MI)

APPENDIX 3:1 MEDIA AND REAGENTS

REDUCING SUGAR CONCENTRATION (NELSON, 1944)

COPPER REAGENT A

Na_2CO_3	25 g
Potassium Sodium Tartrate	25 g
$NaHCO_3$	20 g
Na_2SO_4	200 g
distilled water	1 litre

COPPER REAGENT B

15% copper sulphate plus 1 or 2 drops concentrated sulphuric acid.

METHOD

1 ml of material, 1 ml of copper reagent (25 parts reagent A to 1 part reagent B). Boil for 20 minutes. When cool add 1 ml arsenomolybdate reagent.

Read at 660 nm against a reagent blank.

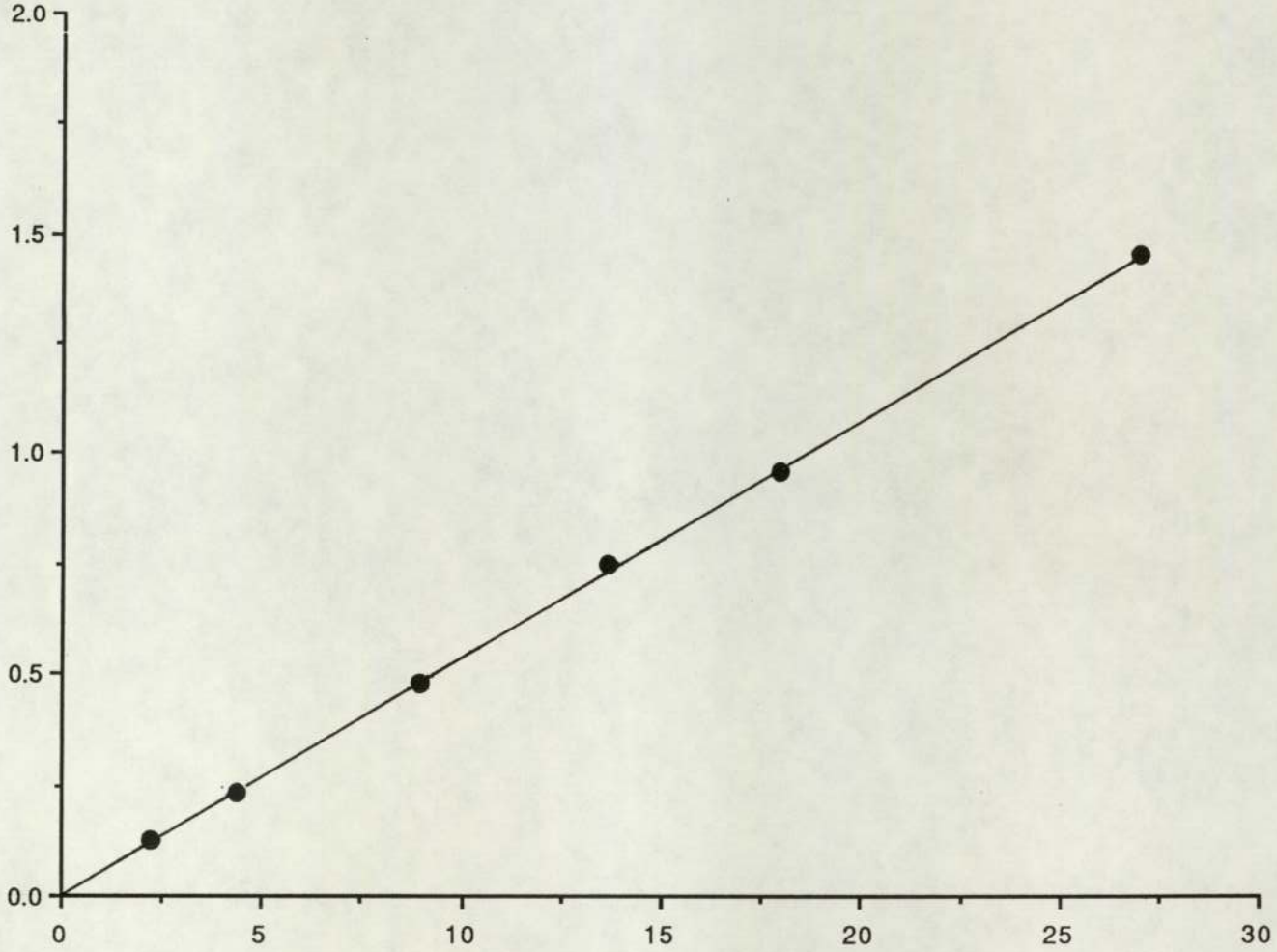
MALT EXTRACT AGAR (MEA)

FORMULA (PER LITRE)

Malt Extract	30 g
Mycological Peptone	5g
Agar $N^0 1$ (Oxoid)	15 g

Suspend 50 g in 1 litre of distilled water. Stir to dissolve. Sterilise by autoclaving at 121^0C, 15 mins (15 psi).

APPENDIX 3:2 Glucose standard curve.



APPENDIX 3:3

STATISTICAL NOTATION

The following notation is used throughout.

PROBABILITY (p)		Symbol and Interpretation
$p > 0.05$		NS ie Not Significant
$p < 0.05$	*	Significant
$p < 0.01$	**	Very Significant
$p < 0.001$	***	Highly Significant

APPENDIX 3:4

FUNGAL DRY WEIGHT PRODUCTION ON PLANT CELL BIOMASS - STATIC CULTURE

(1) METHANOL EXTRACTED CELLS

SPECIES	TIME (Days)	DRY WEIGHT OF MYCELIUM (mg)			
		1	2	3	$\bar{x}$
S.THERMOPHILE	3	32.10	16.10	31.45	26.55
	6	28.20	29.65	30.10	29.32
	9	24.85	23.15	24.75	24.25
	12	28.50	24.50	23.35	25.45
	15	32.50	31.70	23.40	29.20
	18	29.50	28.05	32.00	29.85
	21	26.05	31.30	33.25	30.20
B.CINEREA	3	44.50	49.95	48.30	47.58
	6	44.65	36.45	36.05	39.05
	9	31.95	39.45	41.65	37.68
	12	33.70	36.50	36.25	35.48
	15	32.60	27.70	33.35	31.22
	18	27.00	36.25	38.20	33.82
	21	28.20	24.75	29.40	27.45
T.VIRIDE	3	85.20	77.75	83.75	82.23
	6	85.10	87.20	69.40	80.57
	9	54.25	76.90	59.50	63.55
	12	88.30	72.35	82.30	80.98
	15	73.20	62.40	70.45	68.68
	18	72.45	51.30	68.60	64.12
	21	78.45	67.90	55.45	67.27

$\bar{x}$ = The mean of the three replicates

FUNGAL DRY WEIGHT PRODUCTION ON PLANT CELL BIOMASS - STATIC CULTURE

(1) METHANOL EXTRACTED PLANT CELLS

STATISTICAL ANALYSIS

Summary Table for analysis of variance using a 3 x 7 factorial split plot analysis in a randomised design.

VARIABLE (VAR)	SUMS OF SQUARES (SS)	DEGREES OF FREEDOM (DF)	MEAN SQUARE (MS)
MA.F	23727.8958	2	11863.9479
MP ERROR	244.487	6	40.747833
MI.F	964.750458	6	160.791743
A x B	1151.24081	12	95.9367345
SP ERROR	1459.07306	36	40.5298072

F RATIO (MAF) =	291.155308	***
F RATIO (MI.F) =	3.96724668	**
F RATIO (A x B) =	2.36706615	*

MA = Major Factor = Fungal Species
MI = Minor Factor = Time
A x B = Interaction of the two factors

The above notation is used throughout.

APPENDIX 3:4 (continued)

FUNGAL DRY WEIGHT PRODUCTION ON PLANT CELL BIOMASS - STATIC CULTURE

(2) C.ROSEUS PLANT CELLS

SPECIES	TIME (Days)	DRY WEIGHT OF MYCELIUM (mg)			
		1	2	3	$\bar{x}$
S.THERMOPHILE	3	42.00	43.15	47.95	44.36
	6	35.85	36.05	33.10	35.00
	9	23.20	35.80	45.95	34.98
	12	35.15	20.10	39.35	31.53
	15	32.35	40.75	18.05	30.38
	18	32.40	39.70	21.10	31.08
	21	15.15	29.20	18.25	20.86
B.CINEREA	3	46.15	43.50	23.20	37.62
	6	31.20	37.80	29.10	32.70
	9	32.75	48.05	57.80	44.20
	12	41.95	49.25	48.50	46.56
	15	38.45	37.30	43.05	39.60
	18	37.80	42.80	39.75	40.12
	21	35.15	31.25	30.85	32.42
T.VIRIDE	3	49.95	63.50	56.35	56.60
	6	49.20	47.85	49.95	49.00
	9	52.35	61.35	72.85	62.18
	12	58.45	53.25	54.45	55.38
	15	48.30	43.80	49.45	47.18
	18	46.45	52.85	54.35	51.22
	21	42.35	41.05	39.15	40.85

STATISTICAL ANALYSIS SUMMARY TABLE

F Ratio		Df	Significance
MA	64.53	2,6	* * *
MI	5.13	6,36	* * *
A x B	1.09	12,36	NS

APPENDIX 3:4 (continued)

FUNGAL DRY WEIGHT PRODUCTION ON PLANT CELL BIOMASS - STATIC CULTURE

(3) D.CAROTA PLANT CELLS

SPECIES	TIME (Days)	DRY WEIGHT OF MYCELIUM (mg)			
		1	2	3	$\bar{x}$
S.THERMOPHILE	3	26.25	32.55	24.85	27.88
	6	39.00	36.30	37.90	37.73
	9	39.65	50.35	39.70	43.23
	12	41.85	50.05	47.10	46.30
	15	68.15	54.70	54.00	58.95
	18	67.40	59.85	67.20	64.82
	21	62.20	63.85	67.65	64.57
B.CINEREA	3	31.65	29.90	27.60	29.72
	6	37.30	39.95	42.80	40.02
	9	41.80	35.55	39.10	38.82
	12	37.95	40.85	50.95	43.25
	15	43.10	54.75	48.85	48.90
	18	40.10	44.70	60.55	48.45
	21	52.40	56.55	61.55	56.83
T.VIRIDE	3	31.50	26.80	32.15	30.15
	6	38.90	38.10	44.55	40.52
	9	48.45	42.85	50.45	47.25
	12	46.80	50.20	74.15	57.05
	15	83.30	84.95	84.60	84.30
	18	99.90	107.30	98.65	101.95
	21	102.55	96.20	94.40	97.72

STATISTICAL ANALYSIS SUMMARY TABLE

F Ratio		Df	Significance
MA	64.33	2,6	* * *
MI	81.82	6,36	* * *
A x B	11.34	12,36	* * *

APPENDIX 3:4 (continued)

FUNGAL DRY WEIGHT PRODUCTION ON PLANT CELL BIOMASS - STATIC CULTURE

(4) RE-AUTOCLAVED C.ROSEUS PLANT CELLS

SPECIES	TIME (Days)	DRY WEIGHT OF MYCELIUM (mg)			
		1	2	3	$\bar{x}$
S.THERMOPHILE	3	32.20	28.20	26.75	29.05
	6	40.00	38.60	59.95	46.20
	9	38.15	43.50	44.05	41.90
	12	49.60	54.45	52.00	52.02
	15	61.15	64.85	67.35	64.45
	18	68.70	62.95	64.20	65.28
B.CINEREA	3	28.95	15.50	24.65	23.03
	6	43.00	38.70	53.10	44.93
	9	65.10	73.25	62.70	67.02
	12	40.65	58.65	61.15	53.48
	15	44.25	47.05	49.25	46.85
	18	63.00	65.30	70.80	66.37
T.VIRIDE	3	17.30	19.95	25.65	20.97
	6	34.45	43.85	48.30	42.20
	9	46.60	50.80	58.50	51.97
	12	58.85	68.10	51.20	59.38
	15	63.65	44.25	57.65	55.18
	18	80.05	71.00	68.35	73.13

STATISTICAL ANALYSIS SUMMARY TABLE

F Ratio		Df	Significance
MA	6.61	2,6	*
MI	42.98	5,30	* * *
A x B	3.83	10,30	* *

APPENDIX 3:4 (continued)

FUNGAL DRY WEIGHT PRODUCTION ON PLANT CELL BIOMASS - STATIC CULTURE

(5) RE-AUTOCLAVED D.CAROTA PLANT CELLS

SPECIES	TIME (Days)	DRY WEIGHT OF MYCELIUM (mg)			
		1	2	3	$\bar{x}$
S.THERMOPHILE	3	29.55	31.65	30.50	30.60
	6	39.90	39.70	45.15	41.60
	9	42.75	52.45	42.80	46.00
	12	63.65	58.50	68.00	63.40
	15	71.30	77.20	71.25	73.30
	18	78.80	66.65	83.25	76.23
B.CINEREA	3	29.75	26.20	22.05	26.00
	6	42.25	47.75	39.45	43.15
	9	46.25	40.35	42.75	43.12
	12	50.80	69.15	57.80	59.30
	15	57.50	51.10	52.65	53.75
	18	51.95	67.20	72.00	63.70
T.VIRIDE	3	7.40	6.25	5.20	6.28
	6	61.55	61.60	73.40	65.50
	9	32.30	17.95	60.75	37.00
	12	42.90	37.50	50.85	43.75
	15	45.15	45.95	42.10	44.40
	18	44.25	44.85	48.65	45.92

STATISTICAL ANALYSIS SUMMARY TABLE

F Ratio		Df	Significance
MA	12.53	2,6	* *
MI	40.18	5,30	* * *
A x B	7.25	10,30	* * *

APPENDIX 3:5

D-GLUCOSE RELEASE FROM PLANT CELL BIOMASS - STATIC CULTURE

(1) METHANOL EXTRACTED PLANT CELLS

SPECIES	TIME (Days)	D-GLUCOSE CONCENTRATION (ug/ml)			
		1	2	3	$\bar{x}$
S.THERMOPHILE	3	115.2	133.2	108.9	119.1
	6	88.2	91.8	100.8	93.6
	9	99.0	74.7	76.5	83.4
	12	79.2	74.7	72.9	75.6
	15	116.1	80.4	62.8	86.4
	18	68.4	71.1	60.3	66.6
	21	52.2	64.8	54.0	57.0
B.CINEREA	3	132.3	140.4	140.4	137.7
	6	71.1	54.0	76.5	67.2
	9	27.0	51.3	35.1	37.8
	12	35.1	29.7	28.8	31.2
	15	39.4	28.8	26.1	31.4
	18	17.6	7.7	12.9	12.7
	21	22.6	12.0	13.3	15.9
T.VIRIDE	3	51.3	40.5	45.9	45.9
	6	44.1	57.6	54.0	51.9
	9	43.2	33.3	36.9	37.8
	12	44.1	42.3	47.7	44.7
	15	31.1	25.5	34.0	30.2
	18	12.9	16.3	14.2	14.4
	21	18.3	23.7	21.0	21.0

Control = 41.55

STATISTICAL ANALYSIS SUMMARY TABLE

F Ratio		Df	Significance
MA	142.56	2,6	* * *
MI	63.32	3,36	* * *
AXB	12.84	12,36	* * *

APPENDIX 3:5 (continued)

D-GLUCOSE RELEASE FROM PLANT CELL BIOMASS - STATIC CULTURE

(2) C.ROSEUS PLANT CELLS

SPECIES	TIME (Days)	GLUCOSE CONCENTRATION (ug/ml) 1	2	3	$\bar{x}$
S.THERMOPHILE	3	139.5	142.2	142.2	141.3
	6	70.2	69.3	33.1	57.5
	9	77.4	52.2	52.2	60.6
	12	79.2	59.4	52.2	63.6
	15	172.4	131.4	138.9	147.5
	18	88.2	49.5	75.6	71.1
	21	124.2	108.0	111.2	114.4
B.CINEREA	3	410.4	288.9	118.8	272.7
	6	84.6	51.3	57.6	64.5
	9	32.7	32.4	32.4	32.5
	12	40.5	20.7	18.0	26.4
	15	90.0	63.0	57.6	70.2
	18	75.9	80.2	86.4	80.8
	21	52.9	32.2	36.5	40.5
T.VIRIDE	3	117.0	75.8	126.9	106.5
	6	78.3	65.7	63.0	69.0
	9	74.7	75.6	75.6	75.3
	12	74.7	68.4	74.7	72.6
	15	172.4	156.6	159.1	162.7
	18	74.7	69.3	71.1	71.7
	21	137.8	122.7	133.5	131.3

Control = 266.4

STATISTICAL ANALYSIS SUMMARY TABLE

F Ratio		Df	Significance
MA	0.52		NS
MI	16.96		* * *
A x B	6.39		* * *

APPENDIX 3:5 (continued)

D-GLUCOSE RELEASE FROM PLANT CELL BIOMASS - STATIC CULTURE

(3) D.CAROTA PLANT CELLS

SPECIES	TIME (Days)	D-GLUCOSE CONCENTRATION (ug/ml) 1	2	3	$\bar{x}$
S.THERMOPHILE	3	133.2	135.0	136.8	135.0
	6	214.2	138.6	138.6	163.8
	9	133.2	111.6	118.8	121.2
	12	129.6	111.6	115.2	118.8
	15	153.0	133.2	118.8	135.0
	18	95.4	102.6	102.6	100.2
	21	99.0	106.2	99.0	101.4
B.CINEREA	3	158.4	106.2	106.2	123.6
	6	210.6	135.0	126.0	157.2
	9	129.6	108.0	104.4	114.0
	12	91.8	95.4	77.4	88.2
	15	111.6	102.6	91.8	102.0
	18	68.4	84.6	68.4	73.8
	21	72.0	84.6	81.0	79.2
T.VIRIDE	3	77.4	77.4	88.2	81.0
	6	113.4	102.6	91.8	102.6
	9	77.4	91.8	91.8	87.0
	12	75.6	81.0	70.2	75.6
	15	95.4	95.4	104.4	98.4
	18	81.0	70.2	75.6	75.6
	21	77.4	75.6	91.8	81.6

Control = 523.2

STATISTICAL ANALYSIS SUMMARY TABLE

F Ratio		Df	Significance
MA	11.77	2,6	* *
MI	14.40	6,36	* * *
A x B	1.76	12,36	NS

APPENDIX 3:5 (continued)

D-GLUCOSE RELEASE FROM PLANT CELL BIOMASS - STATIC CULTURE

(4) RE-AUTOCLAVED C.ROSEUS PLANT CELLS

SPECIES	TIME (Days)	D-GLUCOSE CONCENTRATION (ug/ml)			
		1	2	3	$\bar{x}$
S.THERMOPHILE	3	268.2	282.6	318.6	289.8
	6	106.2	102.6	109.8	106.2
	9	111.6	111.6	100.8	108.0
	12	90.0	88.2	91.8	90.0
	15	100.8	97.2	95.4	97.8
	18	111.6	115.2	105.3	110.7
B.CINEREA	3	226.8	165.6	230.4	207.6
	6	115.2	102.6	126.0	114.6
	9	52.2	45.0	52.2	49.8
	12	59.4	32.4	28.8	40.2
	15	38.7	24.3	26.1	29.7
	18	26.1	26.1	26.1	26.1
T.VIRIDE	3	64.8	70.2	70.2	68.4
	6	91.8	95.4	95.4	94.2
	9	88.2	88.2	106.2	94.2
	12	77.4	75.6	88.2	80.4
	15	78.3	80.1	85.5	81.3
	18	93.6	93.6	93.6	93.6

Control for S.thermophile = 60.0
Control for B.cinerea, T.viride = 36.9

STATISTICAL ANALYSIS SUMMARY TABLE

F Ratio		Df	Significance
MA	59.94	2,6	* * *
MI	145.82	5,30	* * *
A x B	55.73	10,30	* * *

APPENDIX 3:5 (continued)

D-GLUCOSE RELEASE FROM PLANT CELL BIOMASS - STATIC CULTURE

(5) RE-AUTOCLAVED D.CAROTA PLANT CELLS

SPECIES	TIME (Days)	D-GLUCOSE CONCENTRATION (ug/ml)			
		1	2	3	$\bar{x}$
S.THERMOPHILE	3	214.2	207.0	228.6	216.6
	6	133.2	136.8	136.8	135.6
	9	88.2	91.8	75.6	85.2
	12	118.8	102.6	109.8	110.4
	15	63.0	81.0	84.6	76.2
	18	108.0	100.8	102.6	103.8
B.CINEREA	3	163.8	169.2	172.8	168.6
	6	122.4	113.4	118.8	118.2
	9	81.0	84.6	77.4	81.0
	12	64.8	72.0	84.6	73.8
	15	43.2	39.6	39.6	40.8
	18	54.0	55.8	63.0	57.6
T.VIRIDE	3	75.6	77.4	81.0	78.0
	6	75.6	75.6	77.4	76.2
	9	57.6	55.8	55.8	56.4
	12	57.6	57.6	61.2	58.8
	15	46.8	46.8	34.2	42.6
	18	68.4	64.8	75.6	69.6

Control = 42.3

STATISTICAL ANALYSIS SUMMARY TABLE

F Ratio		Df	Significance
MA	893.91	2,6	* * *
MI	269.49	5,30	* * *
A x B	39.37	10,30	* * *

APPENDIX 3:6

FILTRATE - CHANGE IN PH WITH TIME - STATIC CULTURE

(1) METHANOL EXTRACTED PLANT CELLS

SPECIES	TIME (Days)	pH			
		1	2	3	$\bar{x}$
S.THERMOPHILE	3	8.20	8.20	8.23	8.21
	6	8.83	8.82	8.92	8.86
	9	8.68	8.87	8.85	8.80
	12	8.69	8.72	8.76	8.72
	15	8.63	8.62	8.57	8.61
	18	8.32	8.59	8.69	8.53
	21	8.42	8.58	8.44	8.48
B.CINEREA	3	6.06	6.00	5.86	5.97
	6	6.82	6.83	6.82	6.82
	9	6.90	6.89	6.82	6.87
	12	6.91	7.01	6.92	6.95
	15	7.11	7.16	7.24	7.17
	18	7.20	7.44	7.22	7.29
	21	7.39	7.53	7.68	7.53
T.VIRIDE	3	7.97	8.03	7.99	7.99
	6	8.24	8.29	8.27	8.27
	9	8.27	8.32	8.50	8.36
	12	8.29	8.24	8.20	8.24
	15	8.27	8.34	8.24	8.28
	18	8.33	7.95	8.29	8.19
	21	8.41	8.47	8.48	8.45

Control = 6.59

STATISTICAL ANALYSIS SUMMARY TABLE

F Ratio		Df	Significance
MA	1520.94	2,6	* * *
MI	65.21	6,36	* * *
A x B	24.55	12,36	* * *

APPENDIX 3:6 (continued)

FILTRATE - CHANGE IN PH WITH TIME - STATIC CULTURE

(2) C.ROSEUS PLANT CELLS

SPECIES	TIME (Days)	pH			
		1	2	3	$\bar{x}$
S.THERMOPHILE	3	8.20	8.18	8.15	8.17
	6	8.91	8.93	8.97	8.94
	9	8.94	9.05	9.02	9.00
	12	8.92	9.12	9.14	9.06
	15	8.98	9.02	8.94	8.98
	18	9.04	9.10	8.95	9.03
	21	8.82	8.87	8.94	8.88
B.CINEREA	3	5.60	5.90	5.80	5.76
	6	6.54	7.05	5.49	6.36
	9	6.56	7.10	7.27	6.98
	12	7.12	7.89	7.91	7.64
	15	7.42	7.48	7.78	7.56
	18	7.25	7.58	7.60	7.48
	21	7.55	7.69	7.80	7.68
T.VIRIDE	3	6.05	6.23	5.76	6.01
	6	8.07	8.10	8.12	8.09
	9	8.36	8.42	8.41	8.39
	12	8.61	8.61	8.62	8.61
	15	8.51	8.52	8.55	8.52
	18	7.25	8.72	8.71	8.23
	21	8.65	8.69	8.67	8.67

Control = 6.11

STATISTICAL ANALYSIS SUMMARY TABLE

F Ratio		Df	Significance
MA	132.83	2,6	* * *
MI	43.34	6,36	* * *
A x B	5.56	12,36	* * *

APPENDIX 3:6 (continued)

FILTRATE-CHANGE IN PH WITH TIME - STATIC CULTURE

(3) D.CAROTA PLANT CELLS

SPECIES	TIME (Days)	pH			
		1	2	3	$\bar{x}$
S.THERMOPHILE	3	7.36	7.34	7.60	7.43
	6	8.00	8.33	8.33	8.22
	9	8.52	8.58	8.62	8.57
	12	8.59	8.65	8.65	8.63
	15	8.65	8.73	8.81	8.73
	18	8.51	8.65	8.65	8.60
	21	8.20	8.60	8.50	8.43
B.CINEREA	3	6.37	6.67	6.70	6.58
	6	6.90	6.86	6.95	6.90
	9	6.98	6.95	6.98	6.97
	12	6.95	6.93	7.00	6.96
	15	7.08	7.13	7.02	7.08
	18	7.48	7.56	7.50	7.51
	21	7.50	7.30	7.30	7.40
T.VIRIDE	3	7.39	7.37	7.37	7.38
	6	7.72	7.77	7.85	7.76
	9	8.11	8.20	8.15	8.15
	12	8.21	8.28	8.28	8.26
	15	8.47	8.48	8.50	8.48
	18	8.45	8.49	8.49	8.48
	21	8.40	8.50	8.50	8.47

Control = 5.77

STATISTICAL ANALYSIS SUMMARY TABLE

F Ratio		Df	Significance
MA	401.92	2,6	* * *
MI	206.93	3,36	* * *
A x B	16.33	12,36	* * *

APPENDIX 3:6 (continued)

FILTRATE-CHANGE IN PH WITH TIME - STATIC CULTURE

(4) RE-AUTOCLAVED C.ROSEUS PLANT CELLS

SPECIES	TIME (Days)	pH			
		1	2	3	$\bar{x}$
S.THERMOPHILE	3	7.65	7.70	7.65	7.67
	6	8.60	8.50	8.50	8.53
	9	8.70	8.70	8.70	8.70
	12	8.65	8.60	8.60	8.62
	15	8.40	8.45	8.40	8.42
	18	8.50	8.50	8.50	8.50
B.CINEREA	3	6.12	6.18	6.17	6.16
	6	6.60	6.60	6.55	6.58
	9	6.90	7.00	7.00	6.97
	12	7.10	7.40	7.40	7.30
	15	7.30	7.40	7.50	7.40
	18	7.40	7.55	7.65	7.53
T.VIRIDE	3	7.16	7.25	7.31	7.24
	6	6.80	6.90	6.90	6.87
	9	7.25	7.30	7.35	7.30
	12	7.65	8.10	7.95	7.90
	15	8.15	8.05	7.90	8.03
	18	8.00	8.00	8.05	8.02

Control = 6.74

STATISTICAL ANALYSIS SUMMARY TABLE

F Ratio		Df	Significance
MA	532.69	2,6	* * *
MI	226.71	5,30	* * *
A x B	42.10	10,30	* * *

APPENDIX 3:6 (continued)

FILTRATE-CHANGE IN PH WITH TIME - STATIC CULTURE

(5) RE-AUTOCLAVED D.CAROTA PLANT CELLS

SPECIES	TIME (Days)	pH			
		1	2	3	$\bar{x}$
S.THERMOPHILE	3	6.20	6.30	6.50	6.33
	6	7.35	7.50	7.60	7.48
	9	8.00	8.20	8.10	8.10
	12	8.20	8.10	8.30	8.20
	15	8.10	8.30	8.25	8.22
	18	8.20	8.15	8.15	8.17
B.CINEREA	3	5.00	5.05	5.00	5.02
	6	6.50	6.50	6.50	6.50
	9	6.75	6.60	6.65	6.67
	12	6.40	6.50	6.55	6.48
	15	6.70	6.70	6.70	6.70
	18	6.80	6.80	6.80	6.80
T.VIRIDE	3	6.00	6.10	6.20	6.10
	6	6.70	6.70	6.60	6.67
	9	6.65	6.75	6.80	6.73
	12	6.90	6.95	6.95	6.93
	15	7.00	7.20	7.15	7.12
	18	7.50	7.50	7.50	7.50

Control = 5.63

STATISTICAL ANALYSIS SUMMARY TABLE

F Ratio		Df	Significance
MA	642.61	2,6	* * *
MI	686.59	5,30	* * *
A x B	38.96	10,30	* * *

APPENDIX 3:7

CHANGES IN THE LEVEL OF FUNGAL MANNITOL WITH TIME - STATIC CULTURE

(1) METHANOL EXTRACTED PLANT CELLS

SPECIES	TIME (Days)	MANNITOL (ug/mg dry weight of mycelium)			
		1	2	3	$\bar{x}$
S.THERMOPHILE	3	19.76	10.30	37.05	22.37
	6	37.83	15.48	24.43	25.91
	9	16.55	20.07	6.59	14.40
	12	5.73	5.93	2.23	4.63
	15	1.57	1.06	1.15	1.26
	18	0.61	1.55	0.80	0.99
	21	0.72	0.72	0.75	0.73
B.CINEREA	3	30.76	109.25	47.47	62.49
	6	132.72	156.73	164.02	151.16
	9	37.39	111.85	100.74	83.33
	12	35.63	11.21	8.53	18.46
	15	5.26	9.06	5.05	6.46
	18	6.79	5.12	4.39	5.44
	21	7.01	2.76	4.97	4.92
T.VIRIDE	3	5.57	1.66	15.22	7.49
	6	20.26	19.11	10.48	16.62
	9	17.02	8.67	8.82	11.51
	12	3.35	5.79	1.98	3.71
	15	1.46	1.25	2.08	1.6
	18	1.83	1.425	1.66	1.64
	21	0.99	1.46	1.22	1.23

STATISTICAL ANALYSIS SUMMARY TABLE

F Ratio		Df	Significance
MA	38.81	2,6	* * *
MI	26.45	6,36	* * *
A x B	11.90	12,36	* * *

APPENDIX 3:7 (continued)

CHANGES IN THE LEVEL OF FUNGAL MANNITOL WITH TIME - STATIC CULTURE

(2) C.ROSEUS PLANT CELLS

SPECIES	TIME (Days)	MANNITOL (ug/mg dry weight of mycelium) 1	2	3	$\bar{x}$
S.THERMOPHILE	3	3.18	0.64	1.23	1.68
	6	1.60	2.64	2.37	2.20
	9	2.57	1.47	1.44	1.82
	12	1.34	1.91	1.88	1.71
	15	0.92	0.86	0.64	0.81
	18	0.68	0.68	0.89	0.75
	21	3.06	0.36	1.46	1.62
B.CINEREA	3	13.06	7.35	10.87	10.43
	6	13.14	13.67	12.86	13.22
	9	15.43	9.28	6.38	10.36
	12	4.78	4.37	6.84	5.33
	15	2.33	3.65	4.19	3.39
	18	2.99	4.05	4.49	3.85
	21	4.91	2.99	2.87	3.59
T.VIRIDE	3	19.55	5.40	4.65	9.87
	6	7.40	8.41	7.98	7.93
	9	6.70	4.85	4.39	5.32
	12	3.48	4.02	4.10	3.87
	15	4.16	4.22	3.67	4.02
	18	3.13	3.65	4.20	3.66
	21	6.01	3.61	4.08	4.57

STATISTICAL ANALYSIS SUMMARY TABLE

F Ratio		Df	Significance
MA	27.96	2,6	* * *
MI	8.19	6,36	* * *
A x B	2.35	12,36	*

APPENDIX 3:7 (continued)

CHANGES IN THE LEVEL OF FUNGAL MANNITOL WITH TIME - STATIC CULTURE

(3) D.CAROTA PLANT CELLS

SPECIES	TIME (Days)	MANNITOL (ug/mg dry weight of mycelium)			
		1	2	3	$\bar{x}$
S.THERMOPHILE	3	15.55	24.58	30.08	23.40
	6	5.69	4.25	5.11	5.02
	9	8.75	5.30	7.41	7.15
	12	8.21	4.47	5.39	6.02
	15	5.52	9.76	8.41	7.89
	18	1.21	0.85	0.76	0.94
	21	0.67	0.51	0.51	0.56
B.CINEREA	3	52.89	111.72	93.11	85.91
	6	58.07	38.28	49.48	48.61
	9	65.91	74.85	81.90	74.22
	12	55.16	28.13	33.41	38.90
	15	31.53	54.06	53.73	46.44
	18	2.43	3.31	2.58	2.77
	21	1.61	1.53	1.60	1.58
T.VIRIDE	3	29.17	58.51	65.91	51.20
	6	41.79	40.94	28.23	36.99
	9	37.85	39.74	40.75	39.45
	12	28.31	22.11	15.47	22.0
	15	13.91	18.82	28.93	20.55
	18	1.49	1.62	1.54	1.55
	21	1.54	1.07	1.56	1.39

STATISTICAL ANALYSIS SUMMARY TABLE

F Ratio		Df	Significance
MA	134.47	2,6	* * *
MI	31.14	6,36	* * *
A x B	5.13	12,36	* * *

APPENDIX 3:8

CHANGES IN THE LEVEL OF FUNGAL TREHALOSE WITH TIME - STATIC CULTURE

(1) METHANOL EXTRACTED PLANT CELLS

SPECIES	TIME (Days)	TREHALOSE (ug/mg dry weight of mycelium)			
		1	2	3	$\bar{x}$
S.THERMOPHILE	3	22.37	27.55	75.86	41.93
	6	44.98	61.75	38.96	48.57
	9	55.61	49.39	56.47	53.83
	12	48.68	65.22	0.00	37.97
	15	15.02	15.37	9.74	13.38
	18	7.82	15.57	8.35	10.58
	21	9.17	8.93	4.83	7.65
B.CINEREA	3	6.46	8.31	12.92	9.23
	6	16.36	21.59	14.85	17.60
	9	9.01	10.51	4.59	8.04
	12	8.63	0.00	0.00	2.88
	15	1.17	1.41	0.51	1.04
	18	0.88	2.45	0.00	1.12
	21	3.67	0.00	0.75	1.48
T.VIRIDE	3	0.45	3.29	8.17	3.98
	6	3.89	7.96	5.36	5.74
	9	9.02	7.53	4.54	7.03
	12	3.32	3.81	0.00	2.38
	15	2.40	0.80	0.84	1.34
	18	2.37	1.70	0.64	1.58
	21	1.04	1.35	1.20	1.20

STATISTICAL ANALYSIS SUMMARY TABLE

F Ratio		Df	Significance
MA	133.27	2,6	* * *
MI	5.84	6,36	* * *
A x B	2.26	12,36	*

APPENDIX 3:8 (Continued)

CHANGES IN THE LEVEL OF FUNGAL TREHALOSE WITH TIME - STATIC CULTURE

(2) C.ROSEUS PLANT CELLS

SPECIES	TIME (Days)	TREHALOSE (ug/mg dry weight of mycelium)			
		1	2	3	$\bar{x}$
S.THERMOPHILE	3	12.84	1.86	5.68	6.79
	6	6.01	9.33	7.18	7.50
	9	8.68	3.19	3.86	5.24
	12	5.03	9.89	4.98	6.64
	15	6.97	4.36	6.80	6.05
	18	4.56	4.32	5.48	4.79
	21	9.30	2.09	8.52	6.64
B.CINEREA	3	2.01	1.03	4.03	2.36
	6	2.94	3.02	2.01	2.66
	9	4.44	3.63	3.30	3.79
	12	1.39	2.36	2.09	1.95
	15	1.89	1.49	1.65	1.68
	18	1.63	1.08	2.36	1.69
	21	2.02	1.46	2.65	2.04
T.VIRIDE	3	8.53	1.28	1.70	3.84
	6	1.55	2.68	2.03	2.09
	9	2.51	2.20	2.06	2.26
	12	0.90	1.37	1.10	1.12
	15	1.32	2.55	1.71	1.86
	18	2.48	2.00	2.66	2.38
	21	3.29	2.18	2.25	2.58

STATISTICAL ANALYSIS SUMMARY TABLE

F Ratio		Df	Significance
MA	21.75	2,6	* *
MI	0.54	6,36	NS
A x B	0.48	12,36	NS

APPENDIX 3:8 (continued)

CHANGES IN THE LEVEL OF FUNGAL TREHALOSE WITH TIME - STATIC CULTURE

(3) D.CAROTA PLANT CELLS

SPECIES	TIME (Days)	TREHALOSE (ug/mg dry weight of mycelium)			
		1	2	3	$\bar{x}$
S.THERMOPHILE	3	45.40	59.81	72.72	59.31
	6	16.57	20.56	24.70	20.61
	9	29.96	31.96	32.24	31.40
	12	39.60	17.80	19.94	25.78
	15	23.61	50.46	47.60	40.56
	18	5.40	4.58	5.68	5.22
	21	5.02	2.92	3.65	3.86
B.CINEREA	3	11.91	21.37	24.62	19.30
	6	19.61	16.95	28.73	21.76
	9	32.11	38.42	43.58	38.04
	12	19.80	7.44	8.27	11.84
	15	8.12	21.10	14.91	14.71
	18	2.17	1.65	0.75	1.52
	21	0.97	0.46	0.65	0.69
T.VIRIDE	3	11.13	28.66	21.07	20.29
	6	9.47	10.06	5.19	8.24
	9	12.31	9.98	11.60	11.30
	12	10.52	4.50	3.47	6.20
	15	3.74	6.21	8.70	6.22
	18	0.75	0.89	0.83	0.82
	21	0.40	0.35	0.58	0.44

STATISTICAL ANALYSIS SUMMARY TABLE

F Ratio		Df	Significance
MA	63.16	2,6	* * *
MI	27.88	6,36	* * *
A x B	6.39	12,36	* * *

APPENDIX 4:1

FUNGAL DRY WEIGHT PRODUCTION ON PLANT CELL BIOMASS - SHAKE CULTURE

(1) C.ROSEUS PLANT CELLS

SPECIES	TIME (Days)	DRY WEIGHT OF MYCELIUM (mg)			
		1	2	3	$\bar{x}$
S.THERMOPHILE	3	19.30	21.00	19.75	20.02
	6	45.85	26.20	41.60	37.88
	9	29.45	17.20	25.70	24.12
	12	24.90	30.65	17.85	24.47
	15	32.35	18.15	22.55	24.35
	18	20.25	25.45	29.10	24.93
	21	23.85	22.95	21.25	22.68
B.CINEREA	3	65.95	75.30	61.75	67.67
	6	61.90	61.70	61.65	61.75
	9	44.25	45.45	43.80	44.50
	12	39.35	41.95	42.40	41.23
	15	40.50	42.70	43.10	42.10
	18	38.30	39.60	39.45	39.12
	21	39.20	40.35	38.20	39.25
T.VIRIDE	3	16.70	18.65	20.75	18.70
	6	30.20	30.35	28.75	29.77
	9	21.45	18.55	23.75	21.25
	12	14.70	15.85	15.50	15.35
	15	16.70	18.45	16.00	17.05
	18	13.00	15.70	13.65	14.12
	21	22.80	23.85	27.20	24.62

STATISTICAL ANALYSIS SUMMARY TABLE

F Ratio		Df	Significance
MA	229.28	2,6	* * *
MI	21.59	6,36	* * *
A x B	8.65	12,36	* * *

APPENDIX 4:1 (continued)

FUNGAL DRY WEIGHT PRODUCTION ON PLANT CELL BIOMASS - SHAKE CULTURE

(2) RE AUTOCLAVED C.ROSEUS PLANT CELLS

SPECIES	TIME (Days)	DRY WEIGHT OF MYCELIUM (mg)			
		1	2	3	$\bar{x}$
S.THERMOPHILE	3	32.90	30.60	38.20	33.90
	6	39.65	30.20	35.15	35.00
	9	39.80	38.00	48.75	42.18
	12	40.05	50.00	49.75	46.60
	15	20.00	40.25	36.90	35.38
	18	34.90	32.00	32.35	33.08
B.CINEREA	3	129.50	131.65	100.40	120.52
	6	103.95	101.25	103.70	102.97
	9	89.20	66.80	71.95	75.98
	12	83.15	83.05	81.95	82.72
	15	68.50	79.50	82.70	76.90
	18	59.45	76.20	73.40	69.70
T.VIRIDE*	3	78.80	133.65	138.70	117.05
	6	109.45	127.95	111.00	116.13
	9	128.35	97.75	130.20	118.77
	12	95.55	110.25	111.75	105.85
	15	96.50	88.70	92.70	92.63
	18	55.25	95.95	115.45	88.90

* contents of the whole flask filtered

STATISTICAL ANALYSIS SUMMARY TABLE

F Ratio		Df	Significance
MA	80.34	2,6	* * *
MI	5.44	5,30	* * *
A x B	2.64	10,30	*

APPENDIX 4:2

D-GLUCOSE RELEASE FROM PLANT CELL BIOMASS - SHAKE CULTURE

(1) C.ROSEUS PLANT CELLS

SPECIES	TIME (Days)	D-GLUCOSE CONCENTRATION (ug/ml) 1	2	3	$\bar{x}$
S.THERMOPHILE	3	99.0	84.6	91.8	91.8
	6	102.6	95.4	102.6	100.2
	9	91.8	91.8	88.2	90.6
	12	81.0	84.6	90.0	85.2
	15	91.8	106.2	106.2	101.4
	18	91.8	111.6	115.2	106.2
	21	115.2	108.0	108.0	110.4
B.CINEREA	3	64.8	61.2	59.4	61.8
	6	55.8	48.6	48.6	51.0
	9	40.5	42.3	40.5	41.1
	12	45.9	36.9	36.9	39.9
	15	48.6	48.6	55.8	51.0
	18	41.4	37.8	45.0	41.4
	21	41.4	52.2	52.2	48.6
T.VIRIDE	3	70.2	75.6	77.4	74.4
	6	70.2	73.8	84.6	76.2
	9	79.2	78.3	79.2	78.9
	12	74.7	72.9	71.1	72.9
	15	75.6	84.6	84.6	81.6
	18	95.4	91.8	91.8	93.0
	21	81.0	77.4	81.0	79.8

Control = 306

STATISTICAL ANALYSIS SUMMARY TABLE

F Ratio		Df	Significance
MA	714.27	2,6	* * *
MI	8.55	6,36	* * *
A x B	5.59	12,36	* * *

APPENDIX 4:2 (continued)

D-GLUCOSE RELEASE FROM PLANT CELL BIOMASS - SHAKE CULTURE

(2) RE-AUTOCLAVED C.ROSEUS PLANT CELLS

SPECIES	TIME (Days)	D-GLUCOSE CONCENTRATION (ug/ml) 1	2	3	$\bar{x}$
S. THERMOPHILE	3	66.6	66.1	71.1	68.1
	6	68.4	80.1	81.0	76.5
	9	84.6	86.4	81.0	84.0
	12	81.0	91.8	81.0	84.6
	15	84.2	88.4	88.7	87.1
	18	86.4	95.4	88.2	90.0
B.CINEREA	3	61.2	61.2	61.2	61.2
	6	32.4	36.0	36.0	34.8
	9	27.9	28.8	28.8	28.5
	12	32.4	32.4	32.2	32.3
	15	52.2	21.6	48.6	40.8
	18	11.7	19.8	16.2	15.9
T.VIRIDE	3	118.8	68.4	57.6	81.6
	6	48.6	72.0	72.0	64.2
	9	59.4	101.7	85.5	82.2
	12	107.1	90.0	110.7	102.6
	15	115.2	115.2	126.0	118.8
	18	69.3	99.0	57.6	75.3

Control = 51

STATISTICAL ANALYSIS SUMMARY TABLE

F Ratio		Df	Significance
MA	303.90	2,6	* * *
MI	4.05	5,30	* *
A x B	3.87	10,30	* *

APPENDIX 4:3

FILTRATE - CHANGE IN PH WITH TIME - SHAKE CULTURE

(1) C.ROSEUS PLANT CELLS

SPECIES	TIME (Days)	pH			
		1	2	3	$\bar{x}$
S.THERMOPHILE	3	9.00	9.20	9.00	9.07
	6	9.50	9.30	9.30	9.37
	9	9.30	9.30	9.30	9.30
	12	9.45	9.40	9.35	9.40
	15	9.30	9.30	9.25	9.30
	18	9.15	9.15	9.10	9.13
	21	9.25	9.15	9.40	9.27
B.CINEREA	3	6.70	6.60	6.70	6.67
	6	7.15	7.30	7.60	7.35
	9	7.75	7.30	7.15	7.40
	12	8.30	7.60	8.30	8.07
	15	7.60	8.10	8.00	7.90
	18	7.35	7.55	7.40	7.43
	21	7.50	7.45	7.40	7.45
T.VIRIDE	3	8.40	8.50	8.50	8.47
	6	8.70	8.75	8.75	8.73
	9	8.80	8.85	8.85	8.83
	12	8.80	8.80	8.85	8.82
	15	8.70	8.70	8.75	8.72
	18	8.75	8.90	8.85	8.83
	21	8.95	9.00	9.00	8.98

Control = 5.70

STATISTICAL ANALYSIS SUMMARY TABLE

F Ratio		Df	Significance
MA	2019.22	2,6	* * *
MI	17.43	6,36	* * *
A x B	6.30	12,36	* * *

APPENDIX 4:3 (continued)

FILTRATE-CHANGE IN PH WITH TIME - SHAKE CULTURE

(2) RE-AUTOCLAVED C.ROSEUS PLANT CELLS

SPECIES	TIME (Days)	pH			
		1	2	3	$\bar{x}$
S.THERMOPHILE	3	8.63	8.62	8.64	8.63
	6	9.00	8.88	8.92	8.93
	9	8.81	8.78	8.85	8.81
	12	8.40	8.57	8.54	8.50
	15	8.41	8.41	8.42	8.41
	18	8.28	8.36	8.31	8.32
B.CINEREA	3	6.42	6.42	6.58	6.47
	6	7.24	7.20	7.02	7.15
	9	7.59	7.51	7.47	7.52
	12	7.74	7.67	7.66	7.69
	15	8.65	8.05	8.03	8.24
	18	7.81	8.05	8.10	7.99
T.VIRIDE	3	7.66	8.08	8.07	7.94
	6	8.41	8.39	8.26	8.35
	9	8.56	8.40	8.41	8.46
	12	8.10	8.38	8.27	8.25
	15	8.65	8.60	8.54	8.60
	18	8.54	8.51	8.55	8.53

Control = 6.73

STATISTICAL ANALYSIS SUMMARY TABLE

F Ratio		Df	Significance
MA	647.79	2,6	* * *
MI	33.54	5,30	* * *
A x B	27.79	10,30	* * *

APPENDIX 4:4

CHANGES IN THE LEVEL OF FUNGAL MANNITOL WITH TIME - SHAKE CULTURE

(1) C.ROSEUS PLANT CELLS

SPECIES	TIME (Days)	MANNITOL (ug/mg dry weight of mycelium) 1	2	3	$\bar{x}$
S.THERMOPHILE	3	1.49	2.60	2.30	2.13
	6	2.85	2.08	1.12	2.02
	9	17.56	46.48	19.1	27.71
	12	1.97	3.28	2.22	2.49
	15	7.75	10.36	9.49	9.20
	18	8.23	14.28	6.24	9.58
	21	14.88	5.74	4.83	8.48
B.CINEREA	3	6.28	6.43	6.01	6.24
	6	8.97	9.30	9.85	9.37
	9	66.28	52.93	53.62	57.61
	12	2.36	28.15	7.96	12.82
	15	6.32	17.63	11.97	11.97
	18	13.30	9.06	17.68	13.31
	21	6.57	22.51	18.33	15.80
T.VIRIDE	3	6.15	4.73	4.22	5.03
	6	6.07	6.69	5.37	6.04
	9	25.27	38.54	31.11	31.64
	12	10.18	57.93	5.86	24.66
	15	28.13	12.64	24.36	21.71
	18	11.44	30.32	44.07	25.28
	21	25.69	26.54	42.26	31.5

STATISTICAL ANALYSIS SUMMARY TABLE

F Ratio		Df	Significance
MA	10.29	2,6	* *
MI	9.60	6,36	* * *
A x B	1.87	12,36	NS

APPENDIX 4:5

CHANGES IN THE LEVEL OF FUNGAL TREHALOSE WITH TIME - SHAKE CULTURE

(1) C.ROSEUS PLANT CELLS

SPECIES	TIME (Days)	TREHALOSE (ug/mg dry weight of Mycelium			
		1	2	3	$\bar{x}$
S.THERMOPHILE	3	7.65	7.18	6.66	7.16
	6	6.85	9.89	3.92	6.89
	9	79.01	107.86	60.96	82.61
	12	19.23	31.96	50.87	34.02
	15	59.42	41.74	50.30	50.50
	18	86.17	51.12	30.69	55.99
	21	108.54	52.68	38.44	66.55
B.CINEREA	3	2.82	2.52	2.87	2.74
	6	2.33	1.57	2.36	2.09
	9	33.29	26.71	29.51	29.84
	12	3.54	4.27	1.71	3.17
	15	1.79	4.82	5.68	4.10
	18	4.28	2.05	2.86	3.06
	21	8.72	4.67	3.04	5.48
T.VIRIDE	3	8.22	6.14	4.11	6.16
	6	2.87	3.19	2.68	2.91
	9	22.76	36.83	34.10	31.23
	12	12.39	28.62	6.99	16.00
	15	67.83	21.65	39.15	42.89
	18	7.48	27.34	37.60	24.14
	21	19.14	45.41	43.50	36.02

STATISTICAL ANALYSIS SUMMARY TABLE

F Ratio		Df	Significance
MA	34.57	2,6	* * *
MI	11.69	6,36	* * *
A x B	2.73	12,36	* *

APPENDIX 5:1 BUFFERS AND REAGENTS

CITRATE BUFFER

Stock Solutions

A : 0.1 M solution of citric acid
B : 0.1 M solution of sodium citrate

x ml of A, y ml of B, diluted to a total of 100 ml.

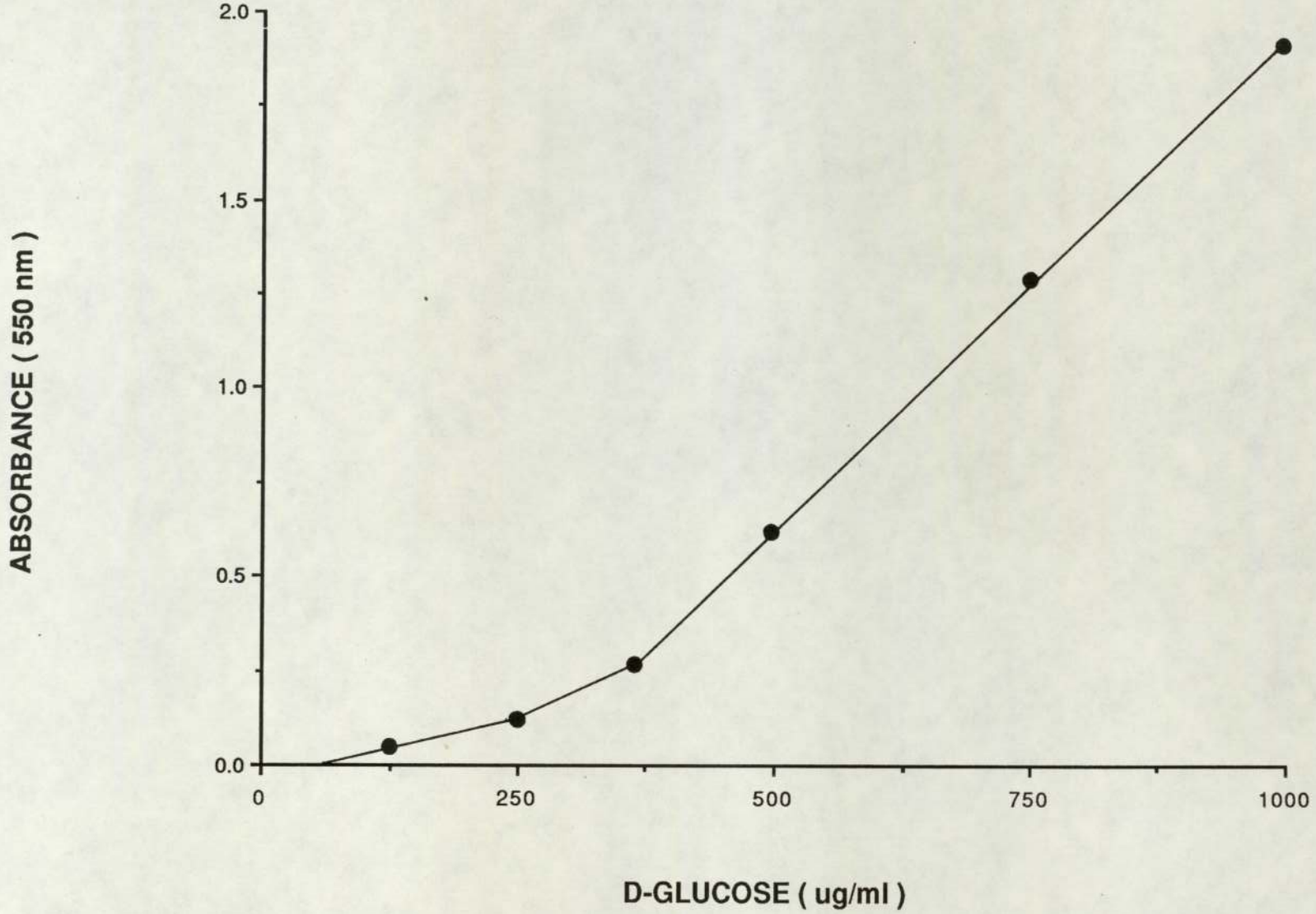
x	y	pH
26.75	23.25	4.5
23.00	27.00	4.8

DNSA REAGENT

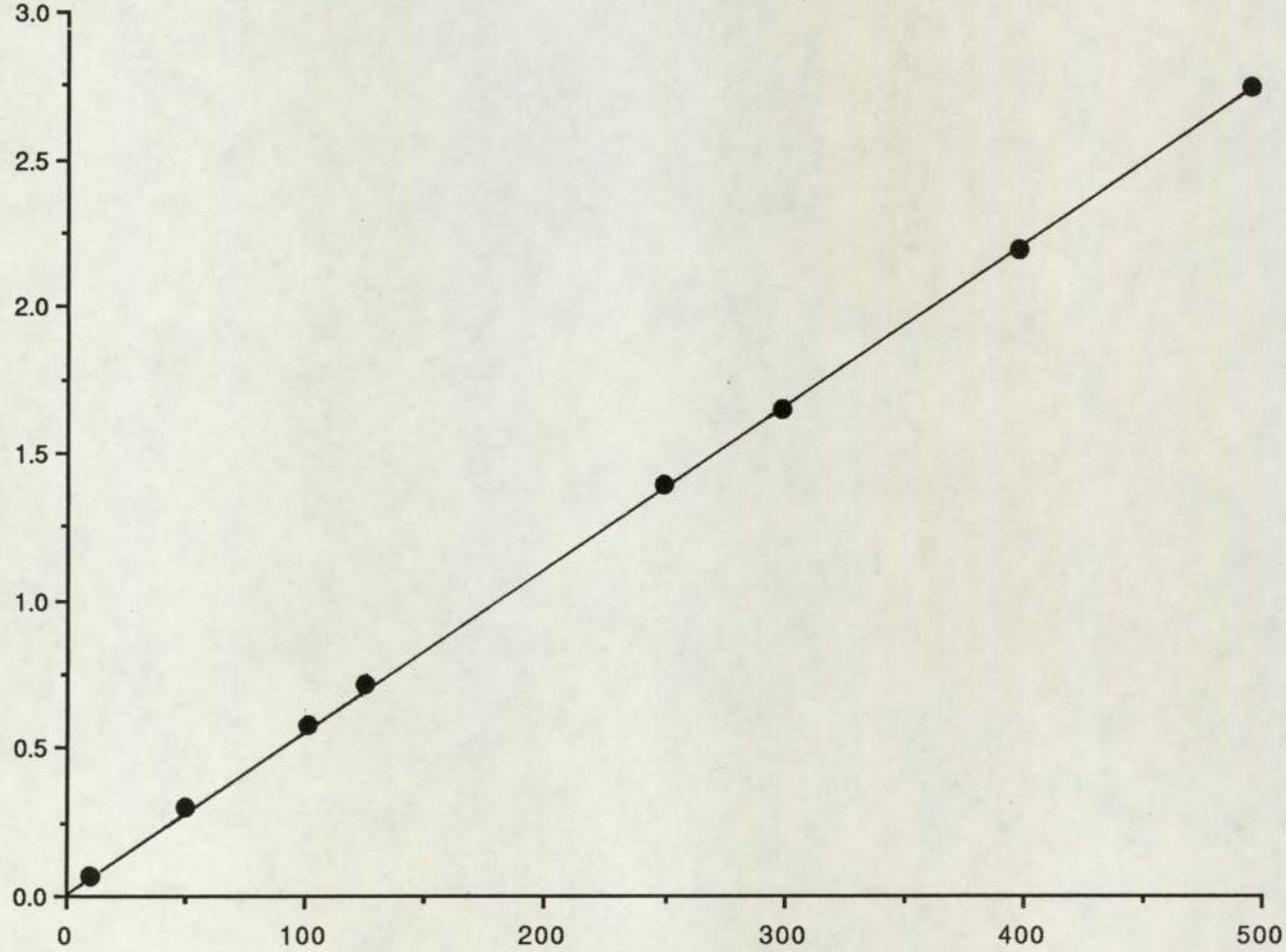
FORMULA (PER 100 mL)

3, 5 Dinitrosalicylic acid	1g
phenol	0.2g
$Na_2 (SO_4)$	0.05 g
NaOH	1g
Sodium potassium tartrate	20 g
distilled water	100 ml

APPENDIX 5:2 Glucose standard curve (DNSA method).



APPENDIX 5:3 Nitrophenol standard curve.



APPENDIX 5:4

FUNGAL C_1 ENZYME ACTIVITY ON PLANT CELL BIOMASS

(1) C.ROSEUS PLANT CELLS - STATIC CULTURE

SPECIES	TIME (Days)	C_1 ACTIVITY (ug D-glucose released/ml filtrate) 1	2	3	$\bar{x}$
S.THERMOPHILE	3	244.8	105.3	206.1	185.4
	6	189.0	167.4	149.4	168.6
	9	102.6	114.3	92.7	103.2
	12	139.5	99.0	46.8	95.1
	15	145.8	74.7	106.2	108.9
	18	68.4	54.0	89.1	70.5
	21	56.7	111.6	87.3	85.2
B.CINEREA	3	63.0	43.2	7.2	37.8
	6	76.5	79.2	72.0	75.9
	9	86.4	59.4	41.4	62.4
	12	99.0	56.7	61.2	72.3
	15	71.1	70.2	51.3	64.2
	18	66.6	46.8	48.6	54.0
	21	47.2	46.8	47.7	47.2
T. VIRIDE	3	47.7	107.1	126.9	93.9
	6	260.1	273.6	159.3	231.0
	9	267.3	192.6	117.9	192.6
	12	240.3	181.8	96.3	172.8
	15	193.5	117.9	168.3	159.9
	18	180.0	147.6	88.2	138.6
	21	208.8	138.6	164.7	170.7

STATISTICAL ANALYSIS SUMMARY TABLE

F Ratio		DF	Significance
MA	16.02	2,6	* * *
MI	3.54	6,36	* *
A x B	2.90	12,36	* *

APPENDIX 5:4 (continued)

FUNGAL C_1 ENZYME ACTIVITY ON PLANT CELL BIOMASS

(2) C.ROSEUS PLANT CELLS - SHAKE CULTURE

SPECIES	TIME (Days)	C_1 ACTIVITY (ug D-glucose released/ml filtrate) 1	2	3	$\bar{x}$
S.THERMOPHILE	3	105.3	24.3	6.3	45.3
	6	77.4	0.0	12.6	30.0
	9	34.2	27.0	9.9	23.7
	12	40.5	25.2	30.6	32.1
	15	33.3	15.3	31.5	26.7
	18	29.7	0.0	41.4	23.7
	21	48.6	27.9	38.7	38.4
B.CINEREA	3	62.1	163.8	66.6	97.5
	6	78.3	49.5	0.0	42.6
	9	48.6	45.0	46.8	46.8
	12	50.4	43.2	52.2	48.6
	15	45.9	46.8	44.1	45.6
	18	54.9	16.2	69.3	46.8
	21	35.1	35.1	35.1	35.1
T.VIRIDE	3	249.3	162.0	45.9	152.4
	6	143.1	75.6	36.9	85.2
	9	33.3	54.0	44.1	43.8
	12	96.3	44.1	27.9	56.1
	15	67.5	25.2	17.1	36.6
	18	54.9	49.5	0.0	34.8
	21	48.6	29.7	31.5	36.6

STATISTICAL ANALYSIS SUMMARY TABLE

F Ratio		Df	Significance
MA	1.73	2,6	NS
MI	2.51	6,36	*
A x B	1.23	12,36	NS

APPENDIX 5:4 (continued)

FUNGAL C_1 ENZYME ACTIVITY ON PLANT CELLS BIOMASS

(3) RE-AUTOCLAVED C.ROSEUS PLANT CELLS - STATIC CULTURE

SPECIES	TIME (Days)	C_1 ACTIVITY (ug D-glucose released/ml of filtrate) 1	2	3	$\bar{x}$
S.THERMOPHILE	3	275.4	297.0	340.2	304.2
	6	460.8	491.4	523.8	492.0
	9	374.4	435.6	415.8	408.6
	12	347.4	423.0	412.2	394.2
	15	165.6	381.6	275.4	274.2
	18	248.4	261.0	248.4	252.6
B.CINEREA	3	75.6	88.2	25.2	63.0
	6	68.4	73.8	77.4	73.2
	9	54.0	66.6	61.2	60.6
	12	122.4	122.4	75.6	106.8
	15	46.8	68.4	23.4	46.2
	18	46.8	39.6	43.2	43.2
T.VIRIDE	3	225.0	226.8	230.4	227.4
	6	451.8	448.2	437.4	445.8
	9	428.4	419.4	401.4	416.4
	12	428.4	183.6	381.6	331.2
	15	91.8	415.8	435.6	314.4
	18	176.4	145.8	145.8	156.0

STATISTICAL ANALYSIS SUMMARY TABLE

F Ratio		Df	Significance
MA	111.82	2,6	* * *
MI	10.60	5,30	* * *
A x B	2.42	10,30	*

APPENDIX 5:4 (continued)

FUNGAL C_1 ENZYME ACTIVITY ON PLANT CELL BIOMASS

(4) RE-AUTOCLAVED C.ROSEUS PLANT CELLS - SHAKE CULTURE

SPECIES	TIME (Days)	C_1 ACTIVITY (ug D-glucose released/ml filtrate) 1	2	3	$\bar{x}$
S.THERMOPHILE	3	311.4	322.2	3222.2	318.6
	6	153.0	133.2	135.0	140.4
	9	208.8	207.0	190.8	202.2
	12	237.6	210.0	248.4	232.2
	15	153.0	144.0	187.2	161.4
	18	180.0	181.8	205.2	189.0
B.CINEREA	3	88.2	88.2	77.4	84.6
	6	113.4	97.2	111.6	107.4
	9	135.0	140.4	151.2	142.2
	12	113.4	109.8	106.2	109.8
	15	55.8	72.0	97.2	75.0
	18	99.0	102.6	102.6	101.4
T.VIRIDE	3	502.2	561.6	210.6	428.4
	6	153.0	198.0	175.5	175.5
	9	192.6	484.2	486.0	387.6
	12	550.8	315.0	462.6	442.8
	15	378.8	378.0	450.0	402.0
	18	307.8	369.0	349.2	342.0

STATISTICAL ANALYSIS SUMMARY TABLE

F Ratio		Df	Significance
MA	343.38	2,6	* * *
MI	3.91	5,30	* *
A x B	1.98	10.30	NS

APPENDIX 5:4 (continued)

FUNGAL C_1 ENZYME ACTIVITY ON PLANT CELL BIOMASS

(5) RE-AUTOCLAVED D.CAROTA PLANT CELLS - STATIC CULTURE

SPECIES	TIME (Days)	C_1 ACTIVITY (ug D-glucose released/ml filtrate) 1	2	3	$\bar{x}$
S.THERMOPHILE	3	320.4	343.8	320.4	328.2
	6	385.2	392.4	383.4	387.0
	9	354.6	388.8	392.4	378.6
	12	192.6	163.8	142.2	166.2
	15	376.2	307.8	356.4	346.8
	18	279.0	268.2	221.4	256.2
B.CINEREA	3	23.4	50.4	37.8	37.2
	6	3.6	0.0	0.0	1.2
	9	50.4	23.4	18.0	30.6
	12	14.4	14.4	14.4	14.4
	15	43.2	23.4	30.6	32.4
	18	50.4	63.0	50.4	54.6
T.VIRIDE	3	64.8	54.0	68.4	62.4
	6	298.8	334.8	322.2	318.6
	9	354.6	365.4	351.0	357.0
	12	192.6	365.4	307.8	288.6
	15	399.6	450.0	387.0	412.2
	18	109.8	160.2	126.0	132.0

STATISTICAL ANALYSIS SUMMARY TABLE

F Ratio		Df	Significance
MA	278.73	2,6	* * *
MI	45.72	5,30	* * *
A x B	37.66	10,30	* * *

APPENDIX 5:5

FUNGAL C_x ENZYME ACTIVITY ON PLANT CELL BIOMASS

(1) C.ROSEUS PLANT CELLS - STATIC CULTURE

SPECIES	TIME (Days)	C_x ACTIVITY (ug D-glucose released/ml filtrate) 1	2	3	$\bar{x}$
S.THERMOPHILE	3	360.0	390.0	385.0	378.3
	6	585.0	460.0	500.0	515.0
	9	285.0	340.0	280.0	301.6
	12	390.0	405.0	300.0	365.0
	15	370.0	395.0	305.0	356.7
	18	260.0	340.0	360.0	320.0
	21	260.0	315.0	325.0	300.0
B.CINEREA	3	110.0	245.0	105.0	153.3
	6	425.0	480.0	445.0	450.0
	9	420.0	525.0	655.0	533.3
	12	410.0	525.0	515.0	483.3
	15	670.0	480.0	570.0	573.3
	18	690.0	490.0	650.0	610.0
	21	515.0	430.0	480.0	475.0
T.VIRIDE	3	170.0	205.0	210.0	195.0
	6	135.0	110.0	145.0	130.0
	9	90.0	50.0	25.0	55.0
	12	20.0	0	5.0	8.3
	15	35.0	45.0	30.0	36.7
	18	15.0	40.0	40.0	31.7
	21	40.0	35.0	20.0	31.7

STATISTICAL ANALYSIS SUMMARY TABLE

F Ratio		Df	Significance
MA	725.66	2,6	* * *
MI	4.30	6,36	* *
A x B	12.36	12,36	* * *

APPENXIX 5:5 (continued)

FUNGAL C_x ENZYME ACTIVITY ON PLANT CELL BIOMASS

(2) C.ROSEUS PLANT CELLS - SHAKE CULTURE

SPECIES	TIME (Days)	C_x ACTIVITY (ug D-glucose released/ml filtrate) 1	2	3	$\bar{x}$
S.THERMOPHILE	3	445.0	450.0	435.0	443.3
	6	280.0	290.0	285.0	285.0
	9	315.0	295.0	315.0	308.3
	12	240.0	275.0	280.0	265.0
	15	235.0	230.0	315.0	260.0
	18	210.0	225.0	230.0	221.7
	21	220.0	240.0	270.0	243.3
B.CINEREA	3	390.0	345.0	370.0	368.3
	6	395.0	450.0	195.0	346.7
	9	465.0	455.0	179.0	363.3
	12	380.0	415.0	375.0	390.0
	15	420.0	425.0	360.0	401.7
	18	445.0	280.0	140.0	288.3
	21	205.0	85.0	65.0	118.3
T.VIRIDE	3	5.0	10.0	80.0	31.6
	6	5.0	105.0	25.0	45.0
	9	20.0	15.0	15.0	16.7
	12	0.0	0.0	20.0	6.7
	15	20.0	25.0	65.0	36.7
	18	25.0	5.0	5.0	8.3
	21	0.0	0.0	25.0	8.3

STATISTICAL ANALYSIS SUMMARY TABLE

F Ratio		Df	Significance
MA	40.02	2,6	* * *
MI	9.33	6,36	* * *
A x B	4.78	12,36	* * *

APPENDIX 5:5 (continued)

FUNGAL C_x ENZYME ACTIVITY ON PLANT CELL BIOMASS

(3) RE-AUTOCLAVED C.ROSEUS PLANT CELLS - STATIC CULTURE

SPECIES	TIME (Days)	C_x ACTIVITY (ug D-glucose released/ml filtrate) 1	2	3	$\bar{x}$
S.THERMOPHILE	3	340.0	315.0	335.0	330.0
	6	510.0	540.0	625.0	558.3
	9	475.0	505.0	530.0	503.3
	12	425.0	475.0	445.0	448.3
	15	430.0	360.0	545.0	445.0
	18	295.0	295.0	250.0	280.0
B.CINEREA	3	150.0	90.0	97.0	112.3
	6	375.0	395.0	325.0	365.0
	9	475.0	495.0	540.0	503.3
	12	215.0	275.0	225.0	238.3
	15	340.0	285.0	240.0	288.3
	18	265.0	190.0	270.0	241.7
T.VIRIDE	3	205.0	195.0	240.0	213.3
	6	360.0	400.0	355.0	371.7
	9	315.0	460.0	545.0	440.0
	12	125.0	100.0	140.0	121.7
	15	145.0	180.0	170.0	165.0
	18	185.0	140.0	195.0	173.3

STATISTICAL ANALYSIS SUMMARY TABLE

F Ratio		Df	Significance
MA	58.66	2,6	* * *
MI	50.89	5,30	* * *
A x B	6.36	10,30	* * *

APPENDIX 5:5 (continued)

FUNGAL C_x ENZYME ACTIVITY ON PLANT CELL BIOMASS

(4) RE-AUTOCLAVED C.ROSEUS PLANT CELLS - SHAKE CULTURE

SPECIES	TIME (Days)	C_x ACTIVITY (ug D-glucose released/ml filtrate) 1	2	3	$\bar{x}$
S.THERMOPHILE	3	670.0	655.0	660.0	661.7
	6	250.0	240.0	255.0	248.3
	9	190.0	225.0	225.0	213.3
	12	250.0	215.0	275.0	246.7
	15	170.0	140.0	190.0	166.7
	18	180.0	80.0	125.0	128.3
B.CINEREA	3	290.0	320.0	260.0	290.0
	6	370.0	355.0	400.0	375.0
	9	345.0	410.0	440.0	398.3
	12	210.0	160.0	230.0	200.0
	15	135.0	140.0	160.0	145.0
	18	210.0	100.0	110.0	140.0
T.VIRIDE	3	200.0	185.0	195.0	193.3
	6	75.0	80.0	85.0	80.0
	9	210.0	210.0	185.0	201.7
	12	180.0	120.0	170.0	156.7
	15	60.0	50.0	50.0	53.3
	18	105.0	125.0	125.0	118.3

STATISTICAL ANALYSIS SUMMARY TABLE

F Ratio		Df	Significance
MA	145.49	2,6	* * *
MI	99.83	5,30	* * *
A x B	45.48	10,30	* * *

APPENDIX 5:5 (continued)

FUNGAL C_x ENZYME ACTIVITY ON PLANT CELL BIOMASS

(5) RE-AUTOCLAVED D.CAROTA PLANT CELLS - STATIC CULTURE

SPECIES	TIME (Days)	C_x ACTIVITY (ug D-glucose released/ml filtrate) 1	2	3	$\bar{x}$
S.THERMOPHILE	3	645.0	610.0	735.0	663.3
	6	825.0	890.0	830.0	848.3
	9	730.0	725.0	730.0	728.3
	12	450.0	640.0	555.0	548.3
	15	465.0	470.0	545.0	493.3
	18	565.0	575.0	635.0	591.7
B.CINEREA	3	190.0	195.0	200.0	195.0
	6	565.0	465.0	550.0	526.7
	9	655.0	570.0	530.0	585.0
	12	745.0	620.0	780.0	715.0
	15	800.0	775.0	755.0	777.0
	18	745.0	760.0	790.0	765.0
T.VIRIDE	3	160.0	155.0	180.0	165.0
	6	465.0	420.0	475.0	453.3
	9	450.0	420.0	345.0	405.0
	12	490.0	405.0	340.0	412.0
	15	425.0	390.0	395.0	403.3
	18	320.0	295.0	340.0	318.3

STATISTICAL ANALYSIS SUMMARY TABLE

F Ratio		Df	Significance
MA	99.90	2,6	* * *
MI	40.17	5,30	* * *
A x B	35.04	10,30	* * *

APPENDIX 5:6

FUNGAL B-GLUCOSIDASE ACTIVITY ON PLANT CELL BIOMASS

(1) C.ROSEUS PLANT CELLS - STATIC CULTURE

SPECIES	TIME (Days)	B-GLUCOSIDASE ACTIVITY (ug nitrophenol released/ml filtrate) 1	2	3	$\bar{x}$
S.THERMOPHILE	3	16.0	16.0	2.0	11.3
	6	6.0	20.0	18.0	14.7
	9	2.0	8.0	1.0	6.7
	12	82.0	43.0	30.0	51.7
	15	11.0	12.0	8.0	10.3
	18	6.0	8.0	9.7	7.9
	21	6.0	1.0	1.6	2.8
B.CINEREA	3	0.5	0.0	1.0	0.5
	6	10.0	4.0	7.0	7.0
	9	4.0	4.0	0.0	2.7
	12	2.0	2.0	8.0	4.0
	15	10.0	30.0	16.0	18.7
	18	86.0	82.0	88.0	85.3
	21	16.0	8.0	16.0	13.3
T.VIRIDE	3	34.0	16.0	14.0	21.3
	6	38.0	32.0	36.0	35.3
	9	28.0	36.0	28.0	30.7
	12	108.0	62.0	106.0	92.0
	15	54.0	38.0	46.0	46.0
	18	134.0	130.0	138.0	134.0
	21	54.0	60.0	72.0	62.0

STATISTICAL ANALYSIS SUMMARY TABLE

F Ratio		Df	Significance
MA	118.07	2,6	* * *
MI	51.35	6,36	* * *
A x B	18.39	12,36	* * *

APPENDIX 5:6

FUNGAL B-GLUCOSIDASE ACTIVITY ON PLANT CELL BIOMASS

(2) C.ROSEUS PLANT CELLS - SHAKE CULTURE

SPECIES	TIME (Days)	B-GLUCOSIDASE ACTIVITY (ug nitrophenol released/ml filtrate) 1	2	3	$\bar{x}$
S.THERMOPHILE	3	32.4	25.6	20.8	26.7
	6	0.8	4.0	6.6	3.8
	9	1.4	0.0	1.2	0.9
	12	4.2	2.0	0.8	2.3
	15	2.4	2.6	2.4	2.5
	18	6.4	4.6	6.0	5.7
	21	3.2	1.6	1.8	2.2
B.CINEREA	3	5.6	3.6	7.4	5.5
	6	0.4	0.0	0.0	0.1
	9	0.0	0.0	0.0	0.0
	12	0.4	0.6	0.0	0.3
	15	0.4	0.3	0.1	0.3
	18	1.2	0.4	0.0	0.5
	21	0.0	5.0	3.8	2.9
T.VIRIDE	3	12.6	3.8	8.0	8.1
	6	13.4	10.8	19.2	14.5
	9	5.8	7.2	4.4	5.8
	12	19.8	16.8	12.6	16.4
	15	13.8	11.2	19.6	14.9
	18	16.6	14.6	19.0	16.7
	21	10.6	10.0	13.8	11.5

STATISTICAL ANALYSIS SUMMARY TABLE

F Ratio		Df	Significance
MA	76.00	2,6	* * *
MI	16.03	6,36	* * *
A x B	16.49	12,36	* * *

APPENDIX 5:6 (continued)

FUNGAL B-GLUCOSIDASE ACTIVITY ON PLANT CELL BIOMASS

(3) RE-AUTOCLAVED C.ROSEUS PLANT CELLS - STATIC CULTURE

SPECIES	TIME (Days)	B-GLUCOSIDASE ACTIVITY (ug nitrophenol released/ml filtrate) 1	2	3	$\bar{x}$
S.THERMOPHILE	3	16.0	17.0	14.3	15.8
	6	194.5	188.0	208.0	196.8
	9	71.2	75.6	65.6	70.8
	12	89.2	64.8	98.4	84.1
	15	101.6	91.2	89.3	94.0
	18	128.2	136.3	121.6	128.7
B.CINEREA	3	75.6	88.2	25.2	63.0
	6	68.4	73.8	77.4	73.2
	9	54.0	66.6	61.2	60.6
	12	122.4	122.4	75.6	106.8
	15	46.8	68.4	23.4	46.2
	18	46.8	39.6	43.2	43.2
T.VIRIDE	3	225.0	226.8	230.4	227.4
	6	451.8	448.2	437.4	445.8
	9	428.4	419.4	401.4	416.4
	12	428.4	183.6	381.6	331.2
	15	91.8	415.8	435.6	314.4
	18	176.4	145.8	145.8	156.0

STATISTICAL ANALYSIS SUMMARY TABLE

F Ratio		Df	Significance
MA	275.08	2,6	* * *
MI	6.43	5,30	* * *
A x B	3.58	10,30	* *

APPENDIX 5:6 (continued)

FUNGAL B-GLUCOSIDASE ACTIVITY ON PLANT CELL BIOMASS

(4) RE-AUTOCLAVED C.ROSEUS PLANT CELLS - SHAKE CULTURE

SPECIES	TIME (Days)	B-GLUCOSIDASE ACTIVITY (ug nitrophenol released/ml filtrate) 1	2	3	$\bar{x}$
S.THERMOPHILE	3	29.3	26.9	21.7	25.9
	6	15.6	18.6	13.6	15.9
	9	20.8	23.8	24.4	23.0
	12	34.3	30.4	35.7	33.4
	15	32.0	17.2	23.8	24.3
	18	14.2	12.6	15.3	14.0
B.CINEREA	3	6.9	7.9	7.2	7.3
	6	3.2	5.0	4.4	4.2
	9	0.0	0.0	0.0	0.0
	12	0.0	0.0	0.0	0.0
	15	1.6	1.4	1.4	1.4
	18	19.1	9.8	10.4	13.1
T.VIRIDE	3	41.6	52.6	35.6	43.3
	6	37.2	37.2	54.8	43.1
	9	40.5	40.2	36.6	39.1
	12	49.0	48.0	53.0	50.0
	15	41.4	43.3	44.2	42.9
	18	268.0	231.2	239.1	246.1

STATISTICAL ANALYSIS SUMMARY TABLE

F Ratio		Df	Significance
MA	1871.2	2,6	* * *
MI	160.91	5,30	* * *
A x B	161.72	10,30	* * *

APPENDIX 5:6 (continued)

FUNGAL B-GLUCOSIDASE ACTIVITY ON PLANT CELL BIOMASS

(5) RE-AUTOCLAVED D.CAROTA PLANT CELLS - STATIC CULTURE

SPECIES	TIME (Days)	B-GLUCOSIDASE ACTIVITY (ug nitrophenol released/ml filtrate) 1	2	3	$\bar{x}$
S.THERMOPHILE	3	16.0	13.0	18.0	15.7
	6	18.0	20.0	16.0	18.0
	9	16.0	16.0	18.0	16.7
	12	23.0	24.0	25.0	24.0
	15	27.0	26.0	28.0	27.0
	18	28.0	20.0	25.0	24.3
B.CINEREA	3	9.0	6.0	3.0	6.0
	6	5.0	5.0	3.0	4.3
	9	5.0	6.0	5.0	5.3
	12	4.0	5.0	3.0	4.0
	15	5.0	3.0	5.0	4.3
	18	2.0	3.0	3.0	2.7
T.VIRIDE	3	27.0	25.0	25.0	25.7
	6	61.0	51.0	43.0	51.7
	9	63.0	70.0	62.0	65.0
	12	80.0	79.0	78.0	79.0
	15	96.0	96.0	94.0	95.3
	18	105.0	98.0	107.0	103.3

STATISTICAL ANALYSIS SUMMARY TABLE

F Ratio		Df	Significance
MA	2039.98	2,6	* * *
MI	111.70	5,30	* * *
A x B	82.79	10,30	* * *

APPENDIX 5:7

FUNGAL ENDO-POLYGALACTURONASE ACTIVITY ON PLANT CELL BIOMASS

(1) C.ROSEUS PLANT CELLS - STATIC CULTURE

SPECIES	TIME (Days)	ENDO-POLYGALACTURONASE ACTIVITY (ug D-glucose released/ml filtrate) 1	2	3	$\bar{x}$
S.THERMOPHILE	3	115.2	103.5	103.5	107.4
	6	47.7	39.6	38.7	42.0
	9	0.0	2.7	10.8	4.5
	12	15.3	11.7	5.4	10.8
	15	13.5	10.8	15.3	13.2
	18	15.3	13.5	12.6	13.8
	21	1.8	9.9	3.6	5.1
B.CINEREA	3	105.3	115.2	79.2	99.9
	6	45.9	43.2	42.3	43.8
	9	180.9	147.6	135.9	154.8
	12	311.4	354.6	157.5	274.5
	15	236.7	239.4	202.5	226.2
	18	48.6	70.2	97.2	72.0
	21	44.1	47.7	49.5	47.1
T.VIRIDE	3	86.4	93.6	90.0	90.0
	6	52.2	40.5	42.3	45.0
	9	29.7	26.1	18.9	24.9
	12	19.8	27.0	29.7	25.5
	15	17.1	16.2	24.3	19.2
	18	9.0	7.2	17.1	11.1
	21	5.4	5.4	11.7	7.5

STATISTICAL ANALYSIS SUMMARY TABLE

F Ratio		Df	Significance
MA	81.34	2,6	* * *
MI	17.44	6,36	* * *
A x B	18.51	12,36	* * *

APPENDIX 5:7

FUNGAL ENDO-POLYGALACTURONASE ACTIVITY ON PLANT CELL BIOMASS

(2) C.ROSEUS PLANT CELLS - SHAKE CULTURE

SPECIES	TIME (Days)	ENDO-POLYGALACTURONASE ACTIVITY (ug D-glucose released/ml filtrate) 1	2	3	$\bar{x}$
S.THERMOPHILE	3	5.4	2.7	2.7	3.6
	6	3.6	2.7	0.9	2.4
	9	3.6	5.4	3.6	4.2
	12	3.6	9.0	1.8	4.8
	15	3.6	0.0	1.8	1.6
	18	0.0	0.9	0.0	0.3
	21	0.0	0.0	1.8	0.6
B.CINEREA	3	68.4	24.3	22.3	38.3
	6	14.4	45.0	19.5	26.3
	9	23.4	19.8	19.8	21.0
	12	48.6	37.8	36.0	40.8
	15	28.8	23.4	24.3	25.5
	18	26.1	21.6	58.5	35.4
	21	45.0	12.6	24.3	27.3
T.VIRIDE	3	9.9	14.4	12.6	12.3
	6	0.9	0.0	0.0	0.3
	9	1.8	0.0	0.0	0.6
	12	2.7	0.0	0.0	0.9
	15	1.8	0.9	2.7	1.8
	18	2.2	1.7	4.5	2.8
	21	1.8	5.4	0.0	2.4

STATISTICAL ANALYSIS SUMMARY TABLE

F Ratio		Df	Significance
MA	86.20	2,6	* * *
MI	1.33	6,36	NS
A x B	0.67	12,36	NS

APPENDIX 5:7 (continued)

FUNGAL ENDO-POLYGALACTURONASE ACTIVITY ON PLANT CELL BIOMASS

(3) RE-AUTOCLAVED C.ROSEUS PLANT CELLS - STATIC CULTURE

SPECIES	TIME (Days)	ENDO-POLYGALACTURONASE ACTIVITY (ug D-glucose released/ml filtrate) 1	2	3	$\bar{x}$
S.THERMOPHILE	3	0.0	0.0	0.0	0.0
	6	18.0	12.6	18.0	16.2
	9	11.7	9.0	3.6	8.1
	12	4.5	8.1	4.5	5.7
	15	4.5	2.7	0.9	2.7
	18	1.8	1.8	2.7	2.1
B.CINEREA	3	104.4	176.4	126.9	135.9
	6	115.2	118.8	117.0	117.0
	9	171.0	156.6	154.8	160.8
	12	115.2	138.6	108.0	120.6
	15	146.7	110.7	123.3	126.9
	18	92.7	82.8	71.1	82.2
T.VIRIDE	3	174.6	158.4	230.4	187.8
	6	259.2	282.6	300.6	280.8
	9	201.6	207.0	219.6	209.4
	12	124.2	93.6	104.4	107.4
	15	38.7	44.1	32.4	38.4
	18	8.1	9.0	9.9	9.0

STATISTICAL ANALYSIS SUMMARY TABLE

F Ratio		Df	Significance
MA	351.07	2,6	* * *
MI	65.55	5,30	* * *
A x B	41.27	10,30	* * *

APPENDIX 5:7 (continued)

FUNGAL ENDO-POLYGALACTURONASE ACTIVITY ON PLANT CELL BIOMASS

(4) RE-AUTOCLAVED C.ROSEUS PLANT CELLS - SHAKE CULTURE

SPECIES	TIME (Days)	ENDO-POLYGALACTURONASE ACTIVITY (ug D-glucose released/ml filtrate) 1	2	3	$\bar{x}$
S.THERMOPHILE	3	4.5	6.3	5.4	5.4
	6	0.0	0.0	0.0	0.0
	9	9.0	4.5	7.2	6.9
	12	3.6	3.6	3.6	3.6
	15	2.7	3.6	3.6	3.3
	18	0.0	0.0	0.0	0.0
B.CINEREA	3	178.2	234.0	208.8	207.0
	6	268.2	253.8	261.0	261.0
	9	29.7	21.6	23.4	24.9
	12	23.4	22.5	23.4	23.1
	15	66.6	73.8	75.6	72.0
	18	6.3	9.0	10.8	8.7
T.VIRIDE	3	18.0	23.4	14.4	18.6
	6	3.6	3.6	5.4	4.2
	9	0.0	0.0	1.8	0.6
	12	3.6	1.8	4.5	3.3
	15	0.0	5.4	9.0	4.8
	18	3.6	2.7	2.7	3.0

STATISTICAL ANALYSIS SUMMARY TABLE

F Ratio		Df	Significance
MA	1938.80	2,6	* * *
MI	218.60	5,30	* * *
A x B	200.51	10,30	* * *

APPENDIX 5:7 (continued)

FUNGAL ENDO-POLYGALACTURONASE ACTIVITY ON PLANT CELLBIOMASS

(5) RE-AUTOCLAVED D.CAROTA PLANT CELLS - STATIC CULTURE

SPECIES	TIME (Days)	ENDO-POLYGALACTURONASE ACTIVITY (ug D-glucose released/ml filtrate) 1	2	3	$\bar{x}$
S.THERMOPHILE	3	3.6	3.6	12.6	6.6
	6	10.8	19.8	12.6	14.4
	9	9.0	12.6	12.6	11.4
	12	5.4	0.0	1.8	2.4
	15	0.0	0.0	0.0	0.0
	18	5.4	3.6	12.6	7.2
B.CINEREA	3	226.8	243.0	230.4	233.4
	6	273.6	311.4	291.6	292.2
	9	181.8	208.8	176.4	189.0
	12	149.4	88.2	190.8	142.8
	15	102.6	77.4	57.6	79.2
	19	126.0	108.0	115.2	116.4
T.VIRIDE	3	117.0	113.4	120.6	117.0
	6	135.0	165.6	165.6	155.4
	9	82.8	82.8	75.6	80.4
	12	1.8	1.8	3.6	2.4
	15	0.0	0.0	0.0	0.0
	18	7.2	3.6	3.6	4.8

STATISTICAL ANALYSIS SUMMARY TABLE

F Ratio		Df	Significance
MA	5004.66	2,6	* * *
MI	77.07	5,30	* * *
A x B	17.33	10,30	* * *

APPENDIX 5:7 (continued)

Analysis of variance using a 2 x 6 (7) split plot analysis in a randomised design was used to determine differences between enzyme activities recorded for an individual species on two different plant cell substrates. Significant differences occurred in all cases except

1. C.roseus (static) Vs C.roseus (shake)

   B.cinerea - C_1 activity

2. re-autoclaved C.roseus (static) Vs re-autoclaved C.roseus (shake).

   All significant differences.

3. C.roseus (static) Vs re-autoclaved C.roseus (static).

   B.cinerea - C_1 and endo-polygalacturonase activity.

4) C.roseus (shake) Vs re-autoclaved C.roseus (shake).

   S.thermophile - C_x and endo-polygalacturonase activity.

   B.cinerea - C_x activity.

APPENDIX 6:1 REAGENTS

CITRATE BUFFER

STOCK SOLUTIONS

A: 0.1M Solution of citric acid

B: 0.1M Solution of sodium citrate

METHOD

x ml of A, y ml of B, diluted to a total of 100 ml.

x	y	pH
46.5	3.5	3.0
20.5	29.5	5.0

APPENDIX 6:2a

THE EFFECT OF INCUBATION TEMPERATURE ON FUNGAL DRY WEIGHT PRODUCTION

S.THERMOPHILE

TIME (Days)		DRY WEIGHT (mg) 30°	35°	40°	45°	50°C
3	i.	26.40	34.65	42.00	41.70	44.30
	ii.	23.85	34.85	43.15	44.15	42.05
	iii.	25.40	41.05	47.95	43.20	44.35
	$\bar{x}$	25.22	36.85	44.36	43.02	43.57
6	i.	33.40	32.25	35.85	35.15	29.90
	ii.	33.30	33.70	36.05	34.85	29.70
	iii.	34.00	33.25	33.10	35.05	32.10
	$\bar{x}$	35.60	33.10	35.00	35.02	30.60
9	i.	40.90	38.45	23.20	42.05	38.90
	ii.	32.35	41.55	35.80	41.15	39.50
	iii.	33.20	36.95	45.95	42.00	39.65
	$\bar{x}$	35.50	38.98	34.98	41.73	39.35
12	i.	39.00	44.90	35.15	48.60	50.70
	ii.	37.70	40.10	20.10	47.95	55.55
	iii.	36.80	30.15	39.35	48.65	49.00
	$\bar{x}$	37.83	38.38	31.53	48.40	51.75
15	i.	41.60	37.95	32.35	51.30	52.20
	ii.	36.00	41.75	40.75	50.25	52.45
	iii.	35.10	46.75	18.05	34.20	55.55
	$\bar{x}$	37.60	42.15	30.38	45.25	53.40
18	i.	34.40	42.20	32.40	47.60	51.10
	ii.	37.05	48.60	39.70	49.70	48.45
	iii.	36.65	46.20	21.10	46.50	50.60
	$\bar{x}$	36.03	45.70	31.06	47.90	50.05
21	i.	25.15	29.15	15.15	43.60	39.20
	ii.	28.80	38.00	29.20	22.80	40.10
	iii.	28.95	37.20	18.25	44.10	45.55
	$\bar{x}$	27.60	34.80	20.86	36.80	41.60

STATISTICAL ANALYSIS SUMMARY TABLE

F Ratio		Df	Significance
MA	41.65	4,10	* * *
MI	8.06	6,60	* * *
A x B	2.60	24,60	* *

In this and subsequent tables

MA = Major Factor = Temperature

MI = Minor Factor = Time

A x B = Interaction between MA and MI

APPENDIX 6:2a (continued)

THE EFFECT OF INCUBATION TEMPERATURE ON FUNGAL DRY WEIGHT PRODUCTION

B.CINEREA

TIME (Days)		DRY WEIGHT (mg) 15°	20°	25°	30°	35°C
3	i.	41.10	50.65	46.15	-	-
	ii.	41.05	43.45	43.50	-	-
	iii.	37.15	46.23	23.20	-	-
	$\bar{x}$	39.80	46.80	37.61	-	-
6	i.	49.70	48.95	31.20	-	-
	ii.	50.50	53.75	37.80	-	-
	iii.	52.65	51.90	29.10	-	-
	$\bar{x}$	50.95	51.53	32.70	-	-
9	i.	50.55	46.30	32.75	-	-
	ii.	52.15	52.40	48.05	-	-
	iii.	50.15	55.05	51.80	-	-
	$\bar{x}$	50.95	51.25	44.20	-	-
12	i.	55.05	50.75	41.95	-	-
	ii.	56.75	50.90	49.25	-	-
	iii.	52.50	53.30	48.50	-	-
	$\bar{x}$	54.80	51.65	46.56	-	-
15	i.	58.20	55.75	38.45	-	-
	ii.	60.95	54.60	37.30	-	-
	iii.	62.90	56.15	43.05	-	-
	$\bar{x}$	60.68	55.50	39.60	-	-
18	i.	54.15	43.45	37.80	-	-
	ii.	53.70	43.15	42.80	-	-
	iii.	10.80	42.85	39.75	-	-
	$\bar{x}$	39.55	43.15	40.11	-	-
21	i.	56.00	47.85	35.15	-	-
	ii.	59.65	46.90	31.25	-	-
	iii.	66.55	45.85	30.85	-	-
	$\bar{x}$	60.73	46.87	32.41	-	-

- No Growth

STATISTICAL ANALYSIS SUMMARY TABLE

F Ratio		Df	Significance
MF	693.85	4,10	* * *
MI	3.32	6,60	* *
A x B	2.13	24,60	* *

APPENDIX 6:2a (continued)

THE EFFECT OF INCUBATION TEMPERATURE ON FUNGAL DRY WEIGHT PRODUCTION

T.VIRIDE

TIME (Days)		DRY WEIGHT (mg) 15°	20°	25°	30°	35°C
3	i.	-	48.05	49.95	40.30	37.30
	ii.	-	45.55	63.50	35.65	47.65
	iii.	-	46.00	56.35	44.60	50.25
	$\bar{x}$	-	46.53	56.60	40.20	45.10
6	i.	51.60	46.80	49.20	51.10	47.75
	ii.	52.40	54.30	47.85	49.75	51.00
	iii.	50.85	42.10	49.95	35.65	43.15
	$\bar{x}$	51.62	47.73	49.00	45.50	47.30
9	i.	51.70	45.50	52.35	53.00	42.00
	ii.	44.40	56.80	61.35	55.75	41.65
	iii.	61.15	53.95	72.85	45.75	41.50
	$\bar{x}$	52.42	52.08	62.18	51.50	41.72
12	i.	67.30	48.60	58.45	42.65	41.85
	ii.	64.25	50.60	53.25	39.00	46.15
	iii.	79.35	49.40	54.45	45.40	45.55
	$\bar{x}$	70.30	49.60	55.38	42.35	44.52
15	i.	70.05	57.70	48.30	39.85	44.35
	ii.	71.75	65.95	43.80	51.45	45.90
	iii.	76.45	66.45	49.45	51.45	45.95
	$\bar{x}$	72.75	63.37	47.18	47.58	45.40
18	i.	64.55	62.45	46.45	38.60	43.15
	ii.	63.85	59.75	52.85	32.45	41.70
	iii.	73.65	62.05	54.35	39.50	35.90
	$\bar{x}$	67.35	61.42	51.21	36.85	40.25
21	i.	72.75	64.50	42.35	36.55	35.40
	ii.	66.85	56.30	41.05	35.40	38.40
	iii.	67.30	56.65	39.15	47.55	40.60
	$\bar{x}$	68.97	59.15	40.85	39.83	38.13

- No Growth

STATISTICAL ANALYSIS SUMMARY TABLE

F Ratio		Df	Significance
MF	25.18	4,10	* * *
MI	21.07	6,60	* * *
A x B	20.50	24,60	* * *

APPENDIX 6:2b

THE EFFECT OF INCUBATION TEMPERATURE ON FUNGAL C_1 ENZYME ACTIVITY

S.THERMOPHILE

TIME (Days)		C_1 ENZYME ACTIVITY (ug glucose released/ ml of filtrate) 30°	35°	40°	45°	50°C
3	i.	138.6	154.8	244.8	153.0	259.2
	ii.	156.6	106.2	105.3	257.4	205.2
	iii.	156.6	176.4	206.1	140.4	230.4
	$\bar{x}$	150.6	145.8	185.4	183.6	231.6
6	i.	221.4	145.4	189.0	91.8	25.2
	ii.	194.4	144.0	167.4	81.0	30.6
	iii.	140.4	172.8	149.4	54.0	39.6
	$\bar{x}$	185.4	155.4	168.6	75.6	31.8
9	i.	66.6	34.2	102.6	50.4	23.4
	ii.	115.2	72.0	114.3	41.4	14.4
	iii.	109.8	54.0	92.7	77.4	41.4
	$\bar{x}$	97.2	53.4	103.2	56.4	26.4
12	i.	77.4	39.6	139.5	27.0	48.6
	ii.	50.4	36.0	99.0	50.4	9.0
	iii.	82.8	23.4	46.8	68.4	27.0
	$\bar{x}$	70.2	33.0	95.1	48.6	28.2
15	i.	61.2	41.4	145.8	57.6	41.4
	ii.	57.6	41.4	74.7	66.6	34.2
	iii.	57.6	54.0	106.2	30.6	34.2
	$\bar{x}$	58.8	45.6	108.9	51.6	36.6
18	i.	18.0	19.8	68.4	27.0	30.6
	ii.	28.8	54.0	54.0	10.8	27.0
	iii.	43.2	19.8	89.1	9.0	30.6
	$\bar{x}$	30.0	31.2	70.5	15.6	29.4
21	i.	52.2	32.4	86.7	7.2	9.0
	ii.	50.4	28.8	111.6	16.2	9.0
	iii.	52.2	27.0	87.3	12.6	12.6
	$\bar{x}$	51.6	29.4	85.2	10.8	10.2

STATISTICAL ANALYSIS SUMMARY TABLE

F Ratio		Df	Significance
MA	18.11	4,10	* * *
MI	67.47	6,60	* * *
A x B	4.43	24,60	* * *

APPENDIX 6:2b (continued)

THE EFFECT OF INCUBATION TEMPERATURE ON FUNGAL C_1 ENZYME ACTIVITY

B.CINEREA

TIME (Days)		C_1 ENZYME ACTIVITY (ug glucose released/ ml of filtrate) 15°	20°	25°	30°	35°C
3	i.	154.8	172.8	63.0	12.6	10.8
	ii.	79.2	113.4	43.2	21.6	10.8
	iii.	88.2	95.4	7.2	16.2	19.8
	$\bar{x}$	107.4	127.2	37.8	16.8	13.8
6	i.	64.8	61.2	76.5	0.0	37.8
	ii.	102.6	54.0	79.2	0.0	16.2
	iii.	64.8	90.0	72.0	0.0	30.6
	$\bar{x}$	77.4	68.4	75.9	0.0	28.2
9	i.	57.6	57.6	86.4	0.0	0.0
	ii.	68.4	68.4	59.4	0.0	3.6
	iii.	72.0	57.6	41.4	19.8	18.0
	$\bar{x}$	66.0	61.2	62.4	6.6	7.2
12	i.	64.8	59.4	99.0	0.0	0.0
	ii.	54.0	59.4	56.7	0.0	0.0
	iii.	64.8	61.2	61.2	0.0	0.0
	$\bar{x}$	61.2	60.0	72.3	0.0	0.0
15	i.	63.0	24.3	71.1	0.0	0.0
	ii.	26.1	25.2	70.2	0.0	0.0
	iii.	57.6	20.7	51.3	0.0	0.0
	$\bar{x}$	48.9	23.4	64.2	0.0	0.0
18	i.	34.2	30.6	66.6	0.0	0.0
	ii.	45.0	37.8	46.8	0.0	0.0
	iii.	36.0	25.2	48.6	0.0	0.0
	$\bar{x}$	38.4	31.2	54.0	0.0	0.0
21	i.	12.6	34.2	47.25	0.0	0.0
	ii.	46.8	27.0	46.8	0.0	0.0
	iii.	55.8	52.2	47.7	0.0	0.0
	$\bar{x}$	38.4	37.8	47.25	0.0	0.0

STATISTICAL ANALYSIS SUMMARY TABLE

	F Ratio	Df	Significance
MA	35.33	4,10	* * *
MI	7.05	6,60	* * *
A x B	3.48	24,60	* * *

APPENDIX 6:2b (continued)

THE EFFECT OF INCUBATION TEMPERATURE ON FUNGAL C_1 ENZYME ACTIVITY

T.VIRIDE

TIME (Days)		C_1 ENZYME ACTIVITY (ug glucose released/ ml of filtrate) 15°	20°	25°	30°	35°C
3	i.	261.0	365.4	47.7	275.4	210.6
	ii.	275.4	358.2	107.1	284.4	162.0
	iii.	208.8	331.2	126.9	284.4	145.8
	$\bar{x}$	248.4	315.6	93.9	281.4	172.8
6	i.	297.0	410.4	260.1	365.4	140.0
	ii.	378.0	412.2	273.6	293.4	140.0
	iii.	351.0	257.4	159.3	275.4	252.0
	$\bar{x}$	342.0	360.0	231.0	311.4	177.6
9	i.	99.0	97.2	267.3	3.6	18.0
	ii.	120.6	117.0	192.6	12.6	34.2
	iii.	82.8	133.2	117.9	18.0	37.8
	$\bar{x}$	100.8	115.8	192.6	11.4	30.0
12	i.	405.0	349.2	240.3	253.8	102.6
	ii.	396.0	345.6	181.8	234.0	81.0
	iii.	316.8	347.3	96.3	230.4	68.4
	$\bar{x}$	372.6	347.4	172.8	239.4	84.0
15	i.	581.4	311.4	193.5	23.4	38.7
	ii.	429.3	214.2	117.9	12.6	29.7
	iii.	433.8	177.3	168.3	27.9	43.2
	$\bar{x}$	481.5	234.3	159.9	21.3	37.2
18	i	196.2	288.0	180.0	30.6	32.4
	ii.	388.8	239.4	147.6	16.2	30.6
	iii.	338.4	126.0	88.2	12.6	28.8
	$\bar{x}$	307.8	217.8	138.6	19.8	30.6
21	i.	360.0	255.6	208.8	27.0	34.2
	ii.	381.6	234.0	138.6	34.2	25.2
	iii.	284.4	342.0	164.7	37.8	19.8
	$\bar{x}$	342.0	277.2	170.7	33.0	26.4

STATISTICAL ANALYSIS SUMMARY TABLE

F Ratio		Df	Significance
MA	55.54	4,10	* * *
MI	31.78	6,60	* * *
A x B	9.58	24,60	* * *

APPENDIX 6:2c

THE EFFECT OF INCUBATION TEMPERATURE ON FUNGAL Cx ENZYME ACTIVITY

S.THERMOPHILE

TIME (Days)		Cx ENZYME ACTIVITY (ug glucose released/ ml of filtrate) 30°	35°	40°	45°	50°C
3	i.	430	665	360	725	610
	ii.	425	610	390	840	545
	iii.	465	590	385	755	655
	$\bar{x}$	440	622	378	773	603
6	i.	690	480	585	430	190
	ii.	690	565	460	460	170
	iii.	770	600	500	335	140
	$\bar{x}$	717	548	515	408	167
9	i.	525	435	285	220	140
	ii.	510	390	340	280	115
	iii.	540	395	280	280	120
	$\bar{x}$	525	407	302	260	125
12	i.	445	415	390	195	150
	ii.	470	405	405	215	165
	iii.	465	410	300	165	160
	$\bar{x}$	460	410	365	192	158
15	i.	450	405	370	275	110
	ii.	455	425	395	200	105
	iii.	440	395	305	260	105
	$\bar{x}$	448	408	357	245	107
18	i.	400	380	260	190	100
	ii.	400	355	340	155	85
	iii.	415	420	360	140	85
	$\bar{x}$	405	385	320	162	90
21	i.	330	315	260	160	75
	ii.	400	355	315	150	90
	iii.	345	370	325	195	75
	$\bar{x}$	358	347	300	168	80

STATISTICAL ANALYSIS SUMMARY TABLE

F Ratio		Df	Significance
MA	334.39	4,10	* * *
MI	148.19	6,60	* * *
A x B	25.69	24,60	* * *

APPENDIX 6:2c (continued)

THE EFFECT OF INCUBATION TEMPERATURE ON FUNGAL Cx ENZYME ACTIVITY

B.CINEREA

TIME (Days)		Cx ENZYME ACTIVITY (ug glucose released/ ml of filtrate) 15°	20°	25°	30°	35°C
3	i.	135	320	110	10	5
	ii.	170	300	245	20	5
	iii.	140	245	105	10	10
	$\bar{x}$	148	288	153	13	7
6	i.	375	375	425	15	55
	ii.	335	535	480	10	20
	iii.	380	480	445	15	10
	$\bar{x}$	363	463	450	13	28
9	i.	410	360	420	5	0
	ii.	420	435	525	5	0
	iii.	335	345	655	10	0
	$\bar{x}$	388	380	533	7	0
12	i.	410	470	410	0	0
	ii.	385	370	525	0	0
	iii.	420	425	515	0	0
	$\bar{x}$	405	422	483	0	0
15	i.	350	340	670	0	0
	ii.	345	425	480	0	0
	iii.	319	330	470	0	0
	$\bar{x}$	338	365	573	0	0
18	i.	405	190	690	0	0
	ii.	385	315	490	0	0
	iii.	400	305	650	0	0
	$\bar{x}$	397	270	610	0	0
21	i.	295	235	515	0	0
	ii.	300	210	430	0	0
	iii.	350	250	480	0	0
	$\bar{x}$	315	232	475	0	0

STATISTICAL ANALYSIS SUMMARY TABLE

F Ratio		Df	Significance
MA	1108.33	4,10	* * *
MI	16.97	6,60	* * *
A x B	7.13	24,60	* * *

APPENDIX 6:2c (continued)

THE EFFECT OF INCUBATION TEMPERATURE ON FUNGAL Cx ENZYME ACTIVITY

T.VIRIDE

TIME (Days)		Cx ENZYME ACTIVITY (ug glucose released/ ml of filtrate) 15°	20°	25°	30°	35°C
3	i.	65	130	170	150	85
	ii.	75	105	205	305	20
	iii.	50	110	210	175	45
	$\bar{x}$	63	115	195	210	50
6	i.	175	500	135	135	10
	ii.	220	400	110	60	5
	iii.	145	385	145	30	20
	$\bar{x}$	180	428	130	75	12
9	i.	300	320	90	30	10
	ii.	435	320	50	35	0
	iii.	315	320	25	30	10
	$\bar{x}$	350	320	55	32	7
12	i.	305	230	20	55	0
	ii.	340	180	0	0	0
	iii.	335	200	5	0	5
	$\bar{x}$	327	205	8	18	2
15	i.	395	120	35	0	0
	ii.	465	160	45	20	0
	iii.	390	110	30	0	5
	$\bar{x}$	417	130	37	7	2
18	i.	325	165	15	20	0
	ii.	305	115	40	0	0
	iii.	365	135	40	0	0
	$\bar{x}$	332	138	32	7	0
21	i.	285	100	40	0	0
	ii.	120	80	35	0	0
	iii.	120	90	20	0	0
	$\bar{x}$	175	90	32	0	0

STATISTICAL ANALYSIS SUMMARY TABLE

F Ratio		Df	Significance
MA	210.86	4,10	* * *
MI	15.85	6,60	* * *
A x B	23,70	24,60	* * *

APPENDIX 6:2d (continued)

THE EFFECT OF INCUBATION TEMPERATURE ON FUNGAL B-GLUCOSIDASE ACTIVITY

S.THERMOPHILE

TIME (Days)		B-GLUCOSIDASE ACTIVITY (ug nitrophenol released/ml of filtrate) 30°	35°	40°	45°	50°C
3	i.	3.2	7.2	16.0	18.4	4.8
	ii.	1.4	4.8	16.0	13.8	2.6
	iii.	3.2	5.0	2.0	13.2	3.4
	$\bar{x}$	2.6	5.7	11.3	15.1	3.6
6	i.	19.0	46.2	6.0	45.2	8.6
	ii.	27.0	24.2	20.0	34.2	5.4
	iii.	26.0	37.0	18.0	41.8	10.0
	$\bar{x}$	24.0	35.8	14.7	40.4	8.0
9	i.	18.2	21.3	2.0	34.2	6.2
	ii.	15.6	30.1	8.0	19.6	21.4
	iii.	16.2	20.0	10.0	31.0	6.0
	$\bar{x}$	16.7	23.8	6.7	28.3	11.2
12	i.	20.8	13.8	82.0	42.8	0.4
	ii.	16.4	15.2	43.0	29.9	1.2
	iii.	17.4	20.4	30.0	43.0	0.4
	$\bar{x}$	18.2	16.5	51.7	38.6	0.7
15	i.	7.3	5.6	11.0	12.4	2.6
	ii.	10.2	5.5	12.0	21.0	1.8
	iii.	6.0	7.4	8.0	13.3	1.9
	$\bar{x}$	7.8	6.2	10.3	15.6	2.1
18	i.	2.6	7.0	6.0	8.3	0.2
	ii.	7.2	13.3	8.0	6.8	0.6
	iii.	11.3	8.0	9.7	21.7	1.2
	$\bar{x}$	7.0	9.4	7.9	12.3	0.7
21	i.	20.8	7.6	6.0	7.8	2.8
	ii.	9.5	8.0	1.0	11.6	5.0
	iii.	12.5	10.1	1.6	5.6	1.5
	$\bar{x}$	14.3	8.6	2.8	8.3	3.1

STATISTICAL ANALYSIS SUMMARY TABLE

F Ratio		Df	Significance
MA	30.12	4,10	* * *
MI	22.53	6,60	* * *
A x B	5.09	24,60	* * *

APPENDIX 6:2d (continued)

THE EFFECT OF INCUBATION TEMPERATURE ON FUNGAL B-GLUCOSIDASE ACTIVITY

B.CINEREA

TIME (Days)		B-GLUCOSIDASE ACTIVITY (ug nitrophenol released/ml of filtrate) 15°	20°	25°	30°	35°C
3	i.	0.9	0.0	0.5	0.0	0.0
	ii.	0.4	4.3	0.0	0.0	1.4
	iii.	1.6	0.6	1.0	0.6	0.0
	$\bar{x}$	0.9	1.6	0.5	0.2	0.5
6	i.	5.6	6.6	10.0	0.0	0.0
	ii.	6.2	5.7	4.0	0.0	0.0
	iii.	4.7	8.3	7.0	0.0	0.0
	$\bar{x}$	5.5	6.9	7.0	0.0	0.0
9	i.	12.1	8.1	4.0	0.0	0.0
	ii.	10.8	14.6	4.0	1.9	0.0
	iii.	6.2	12.5	0.0	2.0	1.3
	$\bar{x}$	9.7	11.7	2.6	1.3	0.4
12	i.	10.3	7.2	2.0	0.0	0.0
	ii.	5.4	6.9	2.0	0.0	0.0
	iii.	3.3	12.9	8.0	0.0	0.0
	$\bar{x}$	6.3	9.0	4.0	0.0	0.0
15	i.	13.6	27.9	10.0	1.5	0.0
	ii.	11.7	14.3	30.0	0.1	0.1
	iii.	15.8	18.5	16.0	1.7	0.0
	$\bar{x}$	13.7	20.2	18.7	1.1	0.03
18	i.	0.3	2.2	86.0	0.0	0.0
	ii.	3.0	8.0	82.0	0.0	0.0
	iii.	8.0	4.1	88.0	0.0	1.9
	$\bar{x}$	3.8	4.8	85.3	0.0	0.6
21	i.	15.2	17.2	16.0	0.0	0.0
	ii.	13.5	7.8	8.0	0.0	4.4
	iii.	9.8	14.6	16.0	0.0	0.0
	$\bar{x}$	12.8	13.2	13.3	0.0	1.5

STATISTICAL ANALYSIS SUMMARY TABLE

F Ratio		Df	Significance
MA	573.87	4,10	* * *
MI	50.50	6,60	* * *
A x B	54.20	24,60	* * *

APPENDIX 6:2d (continued)

THE EFFECT OF INCUBATION TEMPERATURE ON FUNGAL B-GLUCOSIDASE ACTIVITY

T.VIRIDE

TIME (Days)		B-GLUCOSIDASE ACTIVITY (ug nitrophenol released/ml of filtrate) 15°	20°	25°	30°	35°C
3	i.	0.0	1.3	34.0	24.6	4.2
	ii.	0.2	7.0	16.0	30.2	7.2
	iii.	0.0	8.8	14.0	30.8	10.8
	$\bar{x}$	0.07	5.7	21.3	28.5	7.4
6	i.	14.8	39.6	38.0	31.6	4.5
	ii.	16.6	41.8	32.0	27.4	7.4
	iii.	16.0	41.1	36.0	34.0	3.6
	$\bar{x}$	15.8	40.8	35.3	31.0	5.2
9	i.	46.6	45.4	28.0	6.4	2.6
	ii.	25.8	42.0	36.0	24.6	5.2
	iii.	34.4	56.0	28.0	16.3	2.0
	$\bar{x}$	35.6	47.8	30.7	15.8	3.3
12	i.	27.6	100.2	108.0	7.2	4.5
	ii.	54.9	82.4	62.0	0.8	0.8
	iii.	46.0	91.3	106.0	5.5	0.1
	$\bar{x}$	42.8	91.3	92.0	4.5	1.8
15	i.	131.8	136.0	54.0	0.0	6.0
	ii.	106.0	159.4	38.0	0.4	5.6
	iii.	85.0	110.6	46.0	2.2	5.4
	$\bar{x}$	107.6	135.3	46.0	0.8	5.7
18	i.	65.8	88.0	134.0	2.0	0.8
	ii.	69.8	79.8	130.0	2.1	1.4
	iii.	59.6	84.2	138.0	1.5	0.9
	$\bar{x}$	65.1	84.0	134.0	1.9	1.0
21	i.	39.7	42.8	54.0	3.6	4.6
	ii.	35.5	41.8	60.0	3.6	1.4
	iii.	35.2	36.8	72.0	3.8	2.7
	$\bar{x}$	36.8	40.5	62.0	3.7	2.9

STATISTICAL ANALYSIS SUMMARY TABLE

	F Ratio	Df	Significance
MA	214.60	4,10	* * *
MI	58.04	6,60	* * *
A x B	30.29	24,60	* * *

APPENDIX 6:2e

THE EFFECT OF INCUBATION TEMPERATURE ON FUNGAL ENDO-POLYGALACTURONASE ACTIVITY

S.THERMOPHILE

TIME (Days)		ENDO-POLYGALACTURONASE ACTIVITY (ug glucose released ml of filtrate) 30°	35°	40°	45°	50°C
3	i.	28.44	12.96	115.20	8.82	7.02
	ii.	12.60	8.28	103.50	1.08	6.66
	iii.	20.88	6.12	103.50	9.54	5.94
	$\bar{x}$	20.64	9.12	107.40	6.48	6.54
6	i.	6.30	17.10	47.70	4.50	0.90
	ii.	10.80	10.80	39.60	4.50	6.30
	iii.	12.60	2.70	38.70	2.70	5.40
	$\bar{x}$	9.90	10.20	42.00	3.90	4.20
9	i.	3.60	0.90	0.00	9.00	0.00
	ii.	9.00	3.60	2.70	1.62	0.00
	iii.	4.50	3.60	10.80	5.40	0.00
	$\bar{x}$	5.70	2.70	4.50	5.34	0.00
12	i.	18.90	8.10	15.30	1.80	0.90
	ii.	6.30	3.60	11.70	0.90	0.90
	iii.	14.40	5.40	5.40	0.90	0.90
	$\bar{x}$	13.20	5.70	10.80	1.20	0.90
15	i.	11.16	7.74	13.50	6.30	3.06
	ii.	9.54	3.96	10.80	2.70	2.34
	iii.	12.42	5.76	15.30	2.70	1.98
	$\bar{x}$	11.04	5.82	13.20	3.90	2.46
18	i.	7.20	11.70	15.30	4.50	0.00
	ii.	9.00	9.90	13.50	0.00	0.00
	iii.	5.40	1.80	12.60	1.80	2.70
	$\bar{x}$	7.20	7.80	13.80	2.10	0.90
21	i.	5.40	4.50	1.80	1.80	1.80
	ii.	3.60	1.80	9.90	0.90	0.00
	iii.	6.30	1.80	3.60	0.90	1.80
	$\bar{x}$	5.10	2.70	5.10	1.20	1.20

STATISTICAL ANALYSIS SUMMARY TABLE

F Ratio		Df	Significance
MA	115.19	4,10	* * *
MI	117.27	6,60	* * *
A x B	63.80	24,60	* * *

APPENDIX 6:2e (continued)

THE EFFECT OF INCUBATION TEMPERATURE ON FUNGAL ENDO-POLYGALACTURONASE ACTIVITY

B.CINEREA

TIME (Days)		ENDO-POLYGALACTURONASE ACTIVITY (ug glucose released/ml of filtrate) 15°	20°	25°	30°	35°C
3	i.	61.2	174.6	105.3	28.8	0.0
	ii.	57.6	172.8	115.2	5.4	3.6
	iii.	55.8	133.2	79.2	9.0	3.6
	$\bar{x}$	58.2	160.2	99.0	14.4	2.4
6	i.	138.6	189.0	45.9	14.4	0.0
	ii.	153.0	131.4	43.2	7.2	18.0
	iii.	115.2	131.4	42.3	30.6	7.2
	$\bar{x}$	135.6	150.6	43.8	17.4	8.4
9	i.	318.6	300.6	180.9	27.0	12.9
	ii.	338.4	277.2	147.6	22.6	14.9
	iii.	297.0	320.4	135.9	15.3	14.5
	$\bar{x}$	318.0	299.4	154.8	21.6	14.1
12	i.	75.6	48.6	311.4	30.6	43.2
	ii.	64.8	46.8	354.6	34.2	10.8
	iii.	57.6	46.8	157.5	45.0	7.2
	$\bar{x}$	66.0	47.4	274.5	36.6	20.4
15	i.	5.4	5.4	236.7	0.0	0.0
	ii.	7.2	2.7	239.4	0.0	0.0
	iii.	6.3	4.5	202.5	0.0	0.0
	$\bar{x}$	6.3	4.2	226.2	0.0	0.0
18	i.	7.2	0.0	48.6	0.0	0.0
	ii.	1.8	5.4	70.2	0.0	0.0
	iii.	0.0	6.3	97.2	0.0	0.0
	$\bar{x}$	3.0	3.9	72.0	0.0	0.0
21	i.	25.2	18.0	44.1	0.0	0.0
	ii.	18.0	9.0	47.7	0.0	0.0
	iii.	18.0	9.0	49.5	0.0	0.0
	$\bar{x}$	20.4	12.0	47.1	0.0	0.0

STATISTICAL ANALYSIS SUMMARY TABLE

F Ratio		Df	Significance
MA	40.74	4,10	* * *
MI	122.00	6,60	* * *
A x B	21.95	24,60	* * *

APPENDIX 6:2e (continued)

THE EFFECT OF INCUBATION TEMPERATURE ON FUNGAL ENDO-POLYGALACTURONASE ACTIVITY

T.VIRIDE

TIME (Days)		ENDO-POLGALACTURONASE ACTIVITY (ug glucose released/ml of filtrate) 15°	20°	25°	30°	35°C
3	i.	3.6	81.0	86.4	3.6	7.2
	ii.	7.2	66.6	93.6	23.4	21.6
	iii.	3.6	70.2	90.0	23.4	5.4
	$\bar{x}$	4.8	72.6	90.0	16.8	11.4
6	i.	82.8	169.2	52.2	30.6	3.6
	ii.	122.4	273.6	40.5	0.0	0.0
	iii.	46.8	194.4	42.3	0.0	9.0
	$\bar{x}$	84.0	212.4	45.0	10.2	4.2
9	i.	147.6	54.9	29.7	3.6	0.0
	ii.	74.7	30.6	26.1	3.6	8.1
	iii.	162.9	48.6	18.9	1.8	0.0
	$\bar{x}$	128.4	44.7	24.9	3.0	2.7
12	i.	65.7	4.5	19.8	5.4	0.0
	ii.	42.3	9.9	27.0	3.6	0.0
	iii.	52.2	8.0	29.7	2.7	0.0
	$\bar{x}$	53.4	12.2	25.5	3.9	0.0
15	i.	77.4	9.0	17.1	1.8	0.0
	ii.	63.9	13.5	16.2	0.0	0.0
	iii.	40.5	16.2	24.3	0.0	0.0
	$\bar{x}$	60.6	12.9	19.2	0.6	0.0
18	i.	1.8	1.8	9.0	0.0	0.0
	ii.	1.8	1.8	7.2	0.0	0.0
	iii.	1.8	0.9	17.1	1.8	1.8
	$\bar{x}$	1.8	1.5	11.1	0.6	0.6
21	i.	3.6	0.0	5.4	0.0	0.0
	ii.	3.6	0.0	5.4	0.0	0.0
	iii.	3.6	0.0	11.7	0.0	0.0
	$\bar{x}$	3.6	0.0	7.5	0.0	0.0

STATISTICAL ANALYSIS SUMMARY TABLE

F Ratio		Df	Significance
MA	112.99	4,10	* * *
MI	35.68	6,60	* * *
A x B	17.73	24,60	* * *

APPENDIX 6.3a

THE EFFECT OF INCUBATION pH ON FUNGAL C_1 ENZYME ACTIVITY

		C_1 ACTIVITY (ug glucose released/ml of filtrate) TIME (Days)					
		3	6	9	12	15	18
ST	i.	27.0	25.2	79.2	21.6	57.6	27.0
pH 3.0	ii.	18.0	10.8	68.4	27.0	25.2	25.2
	iii.	10.8	27.0	70.2	19.8	50.4	27.0
	$\bar{x}$	18.6	21.0	72.6	22.8	44.4	26.4
pH 5.0	i.	36.0	324.0	385.2	336.6	311.4	282.6
	ii.	41.4	347.4	318.6	376.2	309.6	358.2
	iii.	37.8	320.4	401.4	374.4	313.2	300.6
	$\bar{x}$	38.4	330.6	368.4	362.4	311.4	313.8
NO pH	i.	275.4	460.8	374.4	347.4	165.6	248.4
CONTROL	ii.	297.0	491.4	435.6	423.0	381.6	261.0
	iii.	340.2	523.8	415.8	412.2	275.4	248.4
	$\bar{x}$	304.2	492.0	408.6	394.2	274.2	252.6
BC	i.	43.2	28.8	72.0	27.0	63.0	88.2
pH 3.0	ii.	23.4	28.8	37.8	23.4	82.8	109.8
	iii.	72.0	32.4	45.0	23.4	79.2	136.8
	$\bar{x}$	46.2	30.0	51.6	24.6	75.0	111.6
pH 5.0	i.	72.0	23.4	23.4	9.0	0.9	43.2
	ii.	100.8	18.0	23.4	9.0	3.6	55.8
	iii.	77.4	18.0	19.8	27.0	3.6	46.8
	$\bar{x}$	83.4	19.8	22.2	15.0	2.7	48.6
NO pH	i.	75.6	68.4	54.0	122.4	46.8	46.8
CONTROL	ii.	88.2	73.8	66.6	122.4	68.4	39.6
	iii.	25.2	77.4	61.2	75.6	23.4	43.2
	$\bar{x}$	63.0	73.2	60.6	106.8	46.2	43.2
TV	i.	95.4	178.2	108.0	139.5	79.2	190.8
pH 3.0	ii.	115.2	189.0	118.8	153.9	118.8	338.4
	iii.	122.4	226.8	126.0	100.8	127.8	336.6
	$\bar{x}$	111.0	198.0	117.6	131.4	108.6	288.6
pH 5.0	i.	73.8	75.6	73.8	17.1	35.1	81.0
	ii.	63.0	68.4	50.4	20.7	39.6	57.6
	iii.	66.6	72.0	70.2	8.1	19.8	77.4
	$\bar{x}$	67.8	72.0	64.8	15.3	31.5	72.0
NO pH	i.	225.0	451.8	428.4	428.4	91.8	176.4
CONTROL	ii.	226.8	448.2	419.4	183.6	415.8	145.8
	iii.	230.4	437.4	401.4	381.6	435.6	145.8
	$\bar{x}$	227.4	445.8	416.4	331.2	314.4	156.0

ST = S.THERMOPHILE, BC = B.CINEREA, TV = T.VIRIDE

STATISTICAL ANALYSIS SUMMARY TABLE

Species	F Ratio		Df	Significance
S.THERMOPHILE	MA	175.91	2,6	* * *
	MI	42.86	5,30	* * *
	A x B	22.97	10,30	* * *
B.CINEREA	MA	11.51	2,6	* *
	MI	6.15	5,30	* * *
	A x B	14.96	10,30	* * *
T.VIRIDE	MA	154.08	2,6	* * *
	MI	3.22	5,30	*
	A x B	5.22	10,30	* * *

In this and subsequent tables

MA = Major factor = pH
MI = Minor factor = TIME
A x B = Interaction between MA and MI
No pH CONTROL = Unbuffered cultures

APPENDIX 6.3b

THE EFFECT OF INCUBATION pH ON FUNGAL C_x ENZYME ACTIVITY

		C_x ENZYME ACTIVITY (ug glucose released/ml of filtrate TIME (Days)					
		3	6	9	12	15	18
ST	i.	0.0	0.0	0.0	0.0	0.0	0.0
pH 3.0	ii.	0.0	0.0	0.0	0.0	0.0	0.0
	iii.	0.0	0.0	0.0	0.0	0.0	0.0
	$\bar{x}$	0.0	0.0	0.0	0.0	0.0	0.0
pH 5.0	i.	165	300	290	285	255	280
	ii.	85	225	270	240	245	265
	iii.	195	270	280	235	305	390
	$\bar{x}$	148	265	280	253	268	345
NO pH	i.	340	510	475	425	430	295
CONTROL	ii.	315	540	505	475	360	295
	iii.	335	625	530	445	545	250
	$\bar{x}$	330	558	503	448	445	280
BC	i.	0.0	0.0	70	300	370	100
pH 3.0	ii.	0.0	0.0	55	215	425	325
	iii.	0.0	0.0	85	330	295	330
	$\bar{x}$	0.0	0.0	70	282	363	252
pH 5.0	i.	0.0	0.0	15	5	65	65
	ii.	0.0	0.0	5	5	100	125
	iii.	0.0	0.0	5	5	35	70
	$\bar{x}$	0.0	0.0	8	5	67	87
NO pH	i.	150	375	475	215	340	265
CONTROL	ii.	90	395	495	275	285	190
	iii.	97	325	540	225	240	270
	$\bar{x}$	112	365	503	238	288	242
TV	i.	95	230	295	315	300	275
pH 3.0	ii.	65	310	310	325	350	360
	iii.	80	375	330	310	285	215
	$\bar{x}$	80	305	312	317	312	283
pH 5.0	i.	25	60	60	35	20	55
	ii.	15	25	45	35	45	30
	iii.	15	40	75	30	30	35
	$\bar{x}$	18	42	60	33	32	40
NO pH	i.	205	360	315	125	145	185
CONTROL	ii.	195	400	460	100	180	140
	iii.	240	355	545	140	170	195
	$\bar{x}$	213	372	440	122	165	173

ST = S.THERMOPHILE, BC = B.CINEREA, TV = T.VIRIDE

STATISTICAL ANALYSIS SUMMARY TABLE

Species	F Ratio	Df		Significance
S.THERMOPHILE	MA	421.71	2,6	* * *
	MI	12.37	5,30	* * *
	A x B	12.12	10,30	* * *
B.CINEREA	MA	281.76	2,6	* * *
	MI	20.56	5,30	* * *
	A x B	19.39	10,30	* * *
T.VIRIDE	MA	145.87	2,6	* * *
	MI	20.97	5,30	* * *
	A x B	13.61	10,30	* * *

APPENDIX 6.3c

THE EFFECT OF INCUBATION pH ON FUNGAL B-GLUCOSIDASE ACTIVITY

		B-GLUCOSIDASE ACTIVITY (ug nitrophenol released/ ml of filtrate) TIME (Days)					
		3	6	9	12	15	18
ST	i.	0.15	0.00	0.05	0.10	0.50	0.00
pH 3.0	ii.	0.10	0.15	0.10	0.05	0.00	0.00
	iii.	0.15	0.00	0.10	0.40	0.00	0.00
	$\bar{x}$	0.13	0.05	0.08	0.18	0.17	0.00
pH 5.0	i.	0.40	0.05	0.55	0.85	0.75	0.80
	ii.	0.65	0.20	0.20	0.85	1.05	1.20
	iii.	0.55	0.05	0.55	0.95	1.10	1.15
	$\bar{x}$	0.53	0.10	0.43	0.88	0.97	1.05
NO pH	i.	16.00	194.50	71.20	89.20	101.60	128.20
CONTROL	ii.	17.00	188.00	75.60	64.80	91.20	136.30
	iii.	14.30	208.00	65.60	98.40	89.30	121.60
	$\bar{x}$	15.80	196.80	70.80	84.13	94.03	128.70
BC	i.	0.00	0.25	1.35	8.20	2.50	0.60
pH 3.0	ii.	0.10	0.20	1.30	8.70	2.45	1.05
	iii.	0.25	0.15	1.05	8.75	3.00	1.15
	$\bar{x}$	0.12	0.20	1.23	8.55	2.65	0.93
pH 5.0	i.	0.15	0.20	0.50	6.80	0.95	0.35
	ii.	0.15	0.05	0.55	6.95	1.30	0.35
	iii.	0.05	0.25	0.75	6.95	0.60	0.35
	$\bar{x}$	0.12	0.17	0.60	6.90	0.95	0.35
NO pH	i.	75.60	68.40	54.00	122.40	46.80	46.80
CONTROL	ii.	88.20	73.80	66.60	122.40	68.40	39.60
	iii.	25.20	77.40	61.20	75.60	23.40	43.20
	$\bar{x}$	63.00	73.20	60.60	106.80	46.20	43.20
TV	i.	3.55	3.25	1.70	1.60	0.20	0.15
pH 3.0	ii.	3.65	2.95	1.55	1.40	0.40	0.20
	iii.	2.80	2.65	1.61	1.55	0.90	0.55
	$\bar{x}$	3.33	2.95	1.62	1.52	0.50	0.30
pH 5.0	i.	2.60	4.70	1.16	1.80	1.90	4.50
	ii.	2.60	3.55	2.70	2.45	2.45	4.20
	iii.	3.05	3.55	3.10	1.85	2.45	3.65
	$\bar{x}$	2.75	3.93	2.32	2.03	2.67	4.12
NO pH	i.	225.00	451.80	428.40	428.40	91.80	176.40
CONTROL	ii.	226.80	448.20	419.40	183.60	415.80	145.80
	iii.	230.40	437.40	401.40	381.60	435.60	145.80
	$\bar{x}$	227.40	445.80	416.40	331.20	314.40	156.00

ST = S.THERMOPHILE, BC = B.CINEREA, TV = T.VIRIDE

STATISTICAL ANALYSIS SUMMARY TABLE

Species	F Ratio		Df	Significance
S.THERMOPHILE	MA	4518.16	2,6	* * *
	MI	111.65	5,30	* * *
	A x B	112.43	10,30	* * *
B.CINEREA	MA	70.66	2,6	* * *
	MI	8.41	5,30	* * *
	A x B	4.69	10,30	* * *
T.VIRIDE	MA	692.57	2,6	* * *
	MI	3.49	5,30	*
	A x B	3.48	10,30	* *

APPENDIX 6.3d

THE EFFECT OF INCUBATION pH ON FUNGAL ENDO-POLYGALACTURONASE ACTIVITY

		ENDO-POLYGALACTURONASE ACTIVITY(ug glucose released/ml of filtrate) TIME (Days)					
		3	6	9	12	15	18
ST	i.	0.0	0.0	0.0	0.0	0.0	0.0
pH 3.0	ii.	0.0	0.0	0.0	0.0	0.0	0.0
	iii.	0.0	0.0	0.0	0.0	0.0	0.0
	$\bar{x}$	0.0	0.0	0.0	0.0	0.0	0.0
pH 5.0	i.	7.2	12.6	1.8	0.0	0.0	9.6
	ii.	5.4	12.6	0.0	0.0	0.0	3.6
	iii.	1.8	10.8	3.6	0.0	0.0	12.6
	$\bar{x}$	4.8	12.0	1.8	0.0	0.0	8.4
NO pH	i.	0.0	18.0	11.7	4.5	4.5	1.8
CONTROL	ii.	0.0	12.6	9.0	8.1	2.7	1.8
	iii.	0.0	18.0	3.6	4.5	0.9	2.7
	$\bar{x}$	0.0	16.2	8.1	5.7	2.7	2.1
BC	i.	7.2	59.4	126.0	100.8	79.2	139.5
pH 3.0	ii.	1.8	64.8	75.6	162.0	57.6	165.6
	iii.	9.0	27.0	75.6	165.6	91.8	117.0
	$\bar{x}$	6.0	50.4	92.4	142.8	76.2	140.7
pH 5.0	i.	10.8	126.0	30.6	41.4	81.0	120.6
	ii.	18.0	28.8	19.8	37.8	106.2	113.4
	iii.	12.6	50.4	16.2	37.8	81.0	124.2
	$\bar{x}$	13.8	68.4	22.2	39.0	89.4	119.4
NO pH	i.	104.4	115.2	171.0	115.2	146.7	92.7
CONTROL	ii.	176.4	118.8	156.6	138.6	110.7	82.8
	iii.	126.9	117.0	154.8	108.0	123.3	71.1
	$\bar{x}$	135.9	117.0	160.8	120.6	126.9	82.2
TV	i.	108.0	253.8	212.4	316.8	111.6	283.5
pH 3.0	ii.	104.4	271.8	205.2	325.8	104.4	289.8
	iii.	93.6	282.6	203.4	309.6	95.4	265.5
	$\bar{x}$	102.0	269.4	207.0	317.4	103.8	279.6
pH 5.0	i.	136.8	257.4	198.0	282.6	41.4	144.9
	ii.	162.0	244.8	196.2	302.4	99.0	144.9
	iii.	169.2	264.6	210.6	302.4	82.8	193.5
	$\bar{x}$	156.0	255.6	201.6	295.8	74.4	161.1
NO pH	i.	174.6	259.2	201.6	124.2	38.7	8.1
CONTROL	ii.	158.4	282.6	207.0	93.6	44.1	9.0
	iii.	230.4	300.6	219.6	104.4	32.4	9.9
	$\bar{x}$	187.8	280.8	209.4	107.4	38.4	9.0

ST = S.THERMOPHILE, BC = B.CINEREA, TV = T.VIRIDE

STATISTICAL ANALYSIS SUMMARY TABLE

Species	F Ratio		Df	Significance
S.THERMOPHILE	MA	57.59	2,6	* * *
	MI	22.12	5,30	* * *
	A x B	11.31	10,30	* * *
B.CINEREA	MA	74.31	2,6	* * *
	MI	8.09	5,30	* * *
	A x B	10.86	10,30	* * *
T.VIRIDE	MA	45.25	2,6	* * *
	MI	209.02	5,30	* * *
	A x B	70.97	10,30	* * *

APPENDIX 6:4a

THE EFFECT OF TWEEN 80 ON FUNGAL C_1 ENZYME ACTIVITY

		C_1 ENZYME ACTIVITY (ug glucose released/ml of filtrate) TIME (Days)					
		3	6	9	12	15	18
ST	i.	275.4	460.8	374.4	347.4	165.6	248.4
	ii.	297.0	491.4	435.6	423.0	381.6	261.0
	iii.	340.2	523.8	415.8	412.2	275.4	248.4
	$\bar{x}$	304.2	492.0	408.6	394.2	274.2	252.6
ST + TWEEN	i.	163.8	257.4	381.6	212.4	223.2	444.6
	ii.	176.4	235.8	340.2	225.0	322.1	376.2
	iii.	189.0	342.0	318.6	149.4	333.0	406.8
	$\bar{x}$	176.4	278.4	346.8	195.6	292.8	409.2
BC	i.	75.6	68.4	54.0	122.4	46.8	46.8
	ii.	88.2	73.8	66.6	122.4	68.4	39.6
	iii.	25.2	77.4	61.2	75.6	23.4	43.2
	$\bar{x}$	63.0	73.2	60.6	106.8	46.2	43.2
BC + TWEEN	i.	0.0	153.0	19.8	1.8	72.0	32.4
	ii.	0.0	117.0	16.2	1.8	39.6	25.2
	iii.	0.0	126.0	28.8	2.7	36.0	36.0
	$\bar{x}$	0.0	132.0	21.6	2.1	49.2	31.2
TV	i.	225.0	451.8	428.4	428.4	91.8	176.4
	ii.	226.8	448.2	419.4	183.6	415.8	145.8
	iii.	230.4	437.4	401.4	381.6	435.6	145.8
	$\bar{x}$	227.4	445.8	416.4	331.2	314.4	156.0
TV + TWEEN	i.	295.2	369.0	279.0	239.4	379.8	381.6
	ii.	322.2	435.6	360.0	289.8	385.2	421.2
	iii.	329.4	363.6	399.6	268.2	370.8	433.8
	$\bar{x}$	315.6	389.4	346.2	265.8	378.6	412.2

STATISTICAL ANALYSIS SUMMARY TABLE

Species	F Ratio		Df	Significance
S.THERMOPHILE	MA	10.73	1,4	*
	MI	10.22	5,20	* * *
	A x B	15.93	5,20	* * *
B.CINEREA	MA	9.48	1,4	*
	MI	20.66	5,20	* * *
	A x B	24.66	5,20	* * *
T.VIRIDE	MA	3.92	1,4	NS
	MI	3.65	5,20	*
	A x B	4.40	5,20	* *

In this and subsequent tables:

MA = Major Factor = Presence of TWEEN
MI = Minor Factor = TIME
A x B = Interaction between MA and MI

APPENDIX 6:4b

THE EFFECT OF TWEEN 80 ON FUNGAL C_x ENZYME ACTIVITY

		C_x ENZYME ACTIVITY (ug glucose released/ml of filtrate TIME (Days)					
		3	6	9	12	15	18
ST	i.	340	510	475	425	430	295
	i.	315	540	505	475	360	295
	iii.	335	625	530	445	545	250
	$\bar{x}$	330	558	503	448	445	280
ST + TWEEN	i.	2160	2575	2490	1175	430	415
	ii.	2045	2500	2550	2480	300	450
	iii.	2045	2480	2940	2430	345	470
	$\bar{x}$	2083	2518	2660	2028	358	445
BC	i.	150	375	475	215	340	265
	ii.	90	395	495	275	285	190
	iii.	97	325	540	225	240	270
	$\bar{x}$	112	365	503	238	288	242
BC + TWEEN	i.	1890	1700	2495	2570	445	395
	ii.	1875	1100	2290	2520	405	330
	iii.	1815	1050	2255	2480	375	375
	$\bar{x}$	1860	1283	2347	2523	408	367
TV	i.	205	360	315	125	145	185
	ii.	195	400	460	100	180	140
	iii.	240	355	545	140	170	195
	$\bar{x}$	213	372	440	122	165	173
TV + TWEEN	i.	90	280	325	205	215	195
	ii.	110	320	325	200	230	180
	iii.	115	265	320	195	180	195
	$\bar{x}$	105	288	323	200	208	190

STATISTICAL ANALYSIS SUMMARY TABLE

Species	F Ratio		Df	Significance
S.THERMOPHILE	MA	284.66	1,4	* * *
	MI	33.15	5,20	* * *
	A x B	26.16	5,20	* * *
B.CINEREA	MA	385.30	1,4	* * *
	MI	143.58	5,20	* * *
	A x B	130.83	5,20	* * *
T. VIRIDE	MA	3.28	1,4	NS
	MI	41.31	5,20	* * *
	A x B	7.86	5,20	* * *

APPENDIX 6:4c

THE EFFECT OF TWEEN 80 ON FUNGAL B-GLUCOSIDASE ACTIVITY

		B-GLUCOSIDASE ACTIVITY (ug nitrophenol released/ ml of filtrate) TIME (Days)					
		3	6	9	12	15	18
ST	i.	16.0	194.5	71.2	89.2	101.6	128.2
	ii.	17.0	188.0	75.6	64.8	91.2	136.3
	iii.	14.3	208.0	65.6	98.4	89.3	121.6
	$\bar{x}$	15.8	196.8	70.8	84.1	94.0	128.7
ST + TWEEN	i.	15.2	33.2	30.2	56.8	68.9	26.0
	ii.	25.1	27.6	32.0	63.7	72.5	35.0
	iii.	24.5	14.2	30.6	66.0	77.2	38.3
	$\bar{x}$	21.6	25.0	30.9	62.1	72.9	33.1
BC	i.	75.6	68.4	54.0	122.4	46.8	46.8
	ii.	88.2	73.8	66.6	122.4	68.4	39.6
	iii.	25.2	77.4	61.2	75.6	23.4	43.2
	$\bar{x}$	63.0	73.2	60.6	106.8	46.2	43.2
BC + TWEEN	i.	11.0	0.0	4.6	12.7	9.4	11.8
	ii.	12.1	0.0	1.0	14.8	1.6	14.0
	iii.	8.0	0.0	1.8	10.4	9.1	12.8
	$\bar{x}$	10.4	0.0	2.5	12.6	6.7	12.9
TV	i.	225.0	451.8	428.4	428.4	91.8	176.4
	ii.	226.8	448.2	419.4	183.6	415.8	145.8
	iii.	230.4	437.4	401.4	381.6	435.6	145.8
	$\bar{x}$	227.4	445.8	416.4	331.2	314.4	156.0
TV + TWEEN	i.	22.4	31.6	68.2	199.4	439.4	290.6
	ii.	16.8	27.8	99.8	298.4	457.3	328.0
	iii.	10.8	16.4	144.1	267.2	306.0	204.8
	$\bar{x}$	16.7	25.3	104.0	255.0	400.9	274.5

STATISTICAL ANALYSIS SUMMARY TABLE

Species	F Ratio		Df	Significance
S.THERMOPHILE	MA	862.86	1,4	* * *
	MI	89.29	5,20	* * *
	A x B	96.00	5,20	* * *
B.CINEREA	MA	58.60	1,4	* *
	MI	6.21	5,2	* *
	A x B	5.64	5,20	* *
T.VIRIDE	MA	56.65	1,4	* *
	MI	5.88	5,20	* *
	A x B	11.07	5,20	* * *

APPENDIX 6:4d

THE EFFECT OF TWEEN 80 ON FUNGAL ENDO-POLYGALACTURONASE ACTIVITY

		ENDO-POLYGALACTURONASE ACTIVITY (ug glucose released/ ml of filtrate) TIME (Days)					
		3	6	9	12	15	18
ST	i.	0.0	18.0	11.7	4.5	4.5	1.8
	ii.	0.0	12.6	9.0	8.1	2.7	1.8
	iii.	0.0	18.0	3.6	4.5	0.9	2.7
	$\bar{x}$	0.0	16.2	8.1	5.7	2.7	2.1
ST + TWEEN	i.	14.4	5.4	7.4	5.0	1.8	5.4
	ii.	12.6	18.0	7.1	5.4	5.4	4.5
	iii.	9.0	9.0	8.0	5.2	3.6	6.3
	$\bar{x}$	12.0	10.8	7.5	5.2	3.6	5.4
BC	i.	104.4	115.2	171.0	115.2	146.7	92.7
	ii.	176.4	118.8	156.6	138.6	110.7	82.8
	iii.	126.9	117.0	154.8	108.0	123.3	71.1
	$\bar{x}$	135.9	117.0	160.8	120.6	126.9	82.8
BC + TWEEN	i.	93.6	27.0	20.8	34.9	68.4	37.8
	ii.	23.4	18.0	21.0	34.2	72.0	88.2
	iii.	45.0	43.2	18.8	36.5	43.2	93.6
	$\bar{x}$	54.0	29.4	20.2	35.2	61.2	73.2
TV	i.	174.6	259.2	201.6	124.2	38.7	8.1
	ii.	158.4	282.6	207.0	93.6	44.1	9.0
	iii.	230.4	300.6	219.6	104.4	32.4	9.9
	$\bar{x}$	187.8	280.8	209.4	107.4	38.4	9.0
TV + TWEEN	i.	45.0	124.2	59.8	34.8	13.5	18.0
	ii.	46.8	151.2	59.8	35.0	22.5	22.5
	iii.	81.0	93.6	63.1	35.5	14.4	15.3
	$\bar{x}$	57.6	123.0	60.9	35.1	16.8	18.6

STATISTICAL ANALYSIS SUMMARY TABLE

Species	F Ratio		Df	Significance
S.THERMOPHILE	MA	3.34	1,4	NS
	MI	11.39	5,20	* * *
	A x B	6.83	5,20	* * *
B.CINEREA	MA	345.57	1,4	* * *
	MI	1.21	5,20	NS
	A x B	6.02	5,20	* *
T.VIRIDE	MA	221.83	1,4	* * *
	MI	105.86	5,20	* * *
	A x B	25.40	5,20	* * *

APPENDIX 7:1 MEDIA

GLUCOSE/ASPARAGINE MEDIUM

FORMULA	g/litre
Glucose	10.00
asparagine	1.00
yeast extract	0.50
K_2HPO_4	0.50
$MgSO_4.7H_2O$	0.50
$FeSO_4.7H_2O$	0.01

pH 7.0

METHOD

Add ingredients to 1 litre of distilled water. Stir to dissolve. Sterilise by autoclaving at 115°C for 20 minutes.

PEPTONE WATER

FORMULA	g/litre
peptone	10
sodium chloride	5

METHOD

Add ingredients to litre of distilled water. Mix well and distribute into final containers. Sterilize by autoclaving at 121°C for 15 minutes.

APPENDIX 7:2 GROWTH OF F.OXYSPORUM ON MEDIUM 5 VS CONTROL

	TIME (Days)	F.OXYSPORUM DRY WEIGHT (mg)			
		1	2	3	$\bar{x}$
MEDIUM 5	3	16.265	16.615	18.015	16.965
	6	22.315	19.815	19.915	20.680
	9	20.265	20.915	20.215	20.465
	12	16.120	18.470	17.870	17.490
	15	12.715	13.615	15.215	13.850
CONTROL		1	2	3	$\bar{x}$
	3	14.865	11.115	15.115	13.698
	6	9.815	8.415	8.765	8.998
	9	5.515	6.365	5.315	5.732
	12	7.365	6.365	8.015	7.250
	15	4.515	4.065	5.565	4.715

STATISTICAL ANALYSIS

F Ratio		Df	Significance
MA	460.55	1,4	* * *
MI	27.34	4,16	* * *
A x B	21.11	4,16	* * *

APPENDIX 7:2 GROWTH OF S.CEREVISIAE ON MEDIUM 5 VS CONTROL

	TIME (Days)	S.CEREVISIAE DRY WEIGHT (mg)			
		2	4	6	8
MEDIUM 5	1	29.03	25.38	23.83	29.88
	2	26.38	26.88	23.23	26.03
	3	29.23	26.13	23.83	26.08
	4	24.58	27.78	23.13	26.28
	5	27.28	24.03	23.03	26.28
	$\bar{x}$	27.30	25.94	23.21	26.89
CONTROL		2	4	6	8
	1	25.90	29.60	27.15	32.95
	2	26.35	25.60	26.90	27.90
	3	25.50	24.85	33.85	27.40
	4	25.30	24.95	26.05	27.90
	5	23.95	23.00	27.35	27.25
	$\bar{x}$	25.40	25.60	28.26	28.68

STATISTICAL ANALYSIS

	F Ratio	Df	Significance
MA	2.43	1,8	NS
MI	2.72	3,24	NS
A x B	6.63	3,24	* *

APPENDIX 7:2 GROWTH OF S.CEREVISIAE ON MEDIUM 8 VS CONTROL

	TIME (Days)	S.CEREVISIAE DRY WEIGHT (mg)				
		2	4	6	8	10
MEDIUM 8	1	28.55	19.60	20.85	24.90	25.70
	2	30.00	20.95	36.40	23.40	20.40
	3	34.55	23.30	13.55	25.50	30.65
	4	34.80	18.60	21.20	21.10	37.20
	5	37.85	21.85	28.15	21.45	22.25
	$\bar{x}$	33.15	20.86	24.03	23.27	27.24
CONTROL		2	4	6	8	10
	1	23.30	26.65	23.25	23.25	27.90
	2	23.85	25.30	24.85	24.95	26.30
	3	26.05	24.15	24.50	24.20	26.85
	4	19.80	22.95	24.30	22.25	26.45
	5	28.60	23.10	23.70	23.85	26.50
	$\bar{x}$	24.32	24.43	24.12	23.70	26.80

STATISTICAL ANALYSIS

F Ratio		Df	Significance
MA	2.82	1,8	NS
MI	3.58	4,32	*
A x B	2.89	4,32	*

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