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The Bacteriolytic Enzymes of *Gliomastix murorum* var. *felina*

A thesis
submitted in fulfilment of the
requirements for the Degree
of
Master of Applied Science (Biochemistry)
by Lesley Rhodes
Lincoln College, 1987

ABSTRACT

(i) Extracellular bacteriolytic activity was obtained when *Gliomastix murorum* var. *felina* was grown on heat-killed *Bacillus subtilis* cells (2.5 mg ml^{-1}) suspended in a defined salts solution, buffered at pH5 or at pH7. Maximal yields were measured when shake flask cultures were incubated in light at 30°C for 10 days. Lytic supernatants were designated E_1 and E_3 (fungus grown at pH5, supernatant optimally active at pH3.6 and pH8.0 respectively) and E_2 (fungus grown at pH7.0, supernatant optimally active at pH7.5).

E_1 was investigated further and found to be inducible and to be repressed by glucose addition.

(ii) E_1 was purified by ammonium sulphate precipitation and gel filtration on a Sephadex G-75 column. The pH optimum was 3.4 (ionic strength 0.05) and the molecular weight was estimated by gel filtration as 17000. The mode of action of the bacteriolytic enzyme was that of a β-N-acetylmuramidase. Specific activity was increased 7-fold (from 61.4 units mg^{-1} protein to 448 units mg^{-1} protein).

THE BACTERIOLYTIC ENZYMES OF
GLIOMASTIX MURORUM ver. *FELINA*

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BACKGROUND

The work presented in this thesis arose directly from a study of the growth of fungi on heat-killed bacterial cells as sole C, N and P source. The study was initiated by Dr. W.D. Grant, Cawthron Institute, Nelson as a result of earlier work with Agaricus bisporus, in which it was shown that that fungus could utilise bacteria as sole nutrient source (Fermor & Wood, 1981; Fermor, 1983), and that in A.bisporus the fungal attack on the bacteria was mediated by a bacteriolytic enzyme (Grant et al, 1984).

Of the fungi studied (in a project with which I was involved as a technician), most grew on bacterial cells. In general the fungi produced bacteriolytic activity either when grown at pH5, in which case the activity was optimal at low pH, or when grown at pH7, when the optimal pH for activity was neutral to alkaline (Grant et al, 1986).

Gliomastix sp. was of particular interest because it produced both the low pH and the neutral to alkaline activities when grown at pH5, but also produced the neutral to alkaline activity when grown at pH7. None of the other fungi studied produced bacteriolytic activities at more than one pH.

Work has continued with selected fungi producing activity at one pH only (Dr. Grant, personal communication), and so this thesis was based on Gliomastix sp, which, producing as it does different enzymes at different pHs, must have a more complex array of enzymatic controls.

Continuation of this line of research as the basis of a Masterate in Applied Science was made possible by the approval of Lincoln College for this thesis to be completed at the Cawthron Institute, and by the Cawthron's decision to provide laboratory space, material and support funds.

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Bacteriolytic Enzymes

Bacteriolytic enzymes catalyse the hydrolysis of the insoluble and protective cell wall peptidoglycan of bacteria (Fig. 1A).

The known bacteriolytic enzymes are:

(i) N-acetylmuramidases (Fig. 2) - glycosidases exhibiting lysozyme-like activity, hydrolysing the linear sequence of N-acetylmuramyl, β (1-4) N-acetylglucosamine bonds in wall glycans to liberate free reducing groups of N-acetylmuramic acid (Fig. 1B).

(ii) N-acetylglucosaminidases (Fig. 3), which liberate the free reducing groups of N-acetylglucosamine (Fig. 1C).

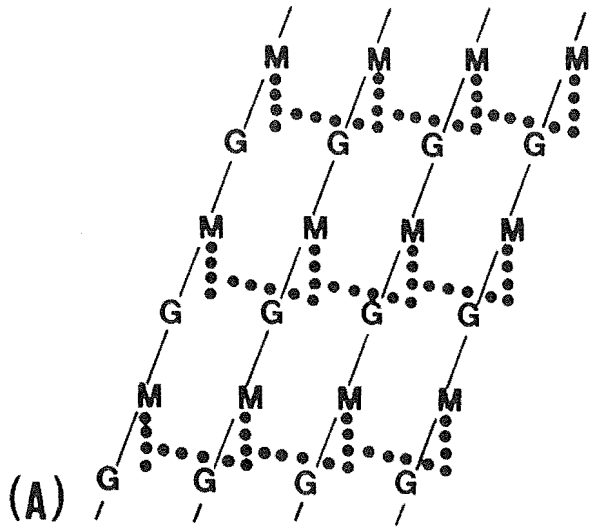
(iii) N-acetylmuramyl-L-alanine-amidases. These enzymes hydrolyse the bond between the peptide and glycan chains (Fig. 4).

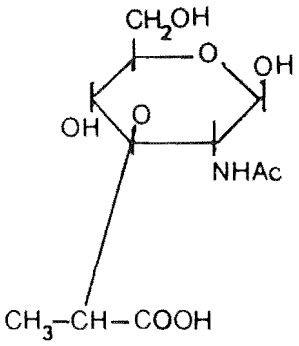
(iv) endopeptidases, which hydrolyse bonds within the peptides or their cross linkages (Fig. 5).

As an illustration of this range of specificities Streptomyces globisporus produces mutanolysin, a lytic enzyme preparation consisting of muramidases and amidases, which causes rapid solubilisation of Streptococcus mutans cell walls accompanied by the liberation of reducing sugars and free amino groups (Yokogawa, 1975). Mutanolysin also lyses the living cells of Streptococcus mutans, Streptococcus salivarius, Lactobacillus acidophilus and Actinomyces viscosus, all cariogenic bacteria (Yokogawa et al 1974).

1.2 N-acetylmuramidases (muramidases)

The bacteriolytic enzyme lysozyme (Lz) (a muramidase) was named by Fleming (1922) following his discovery that a drop of diluted nasal mucous caused complete disappearance of intact cocci in a few minutes at 37^{o}C. "Lyso"





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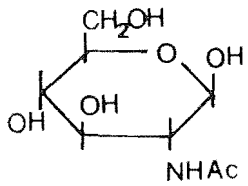


(C)

Fig. 1: (A) DIAGRAM OF PEPTIDOGLYCAN MONOLAYER
(G represents N-acetyl glucosamine, M represents N-acetylmuramic acid, vertical dots represent peptide subunits and horizontal dots represent cross-linking peptide bridges)
(B) β-N-ACETYLMURAMIC ACID
(C) β-N-ACETYLGLUCOSAMINE

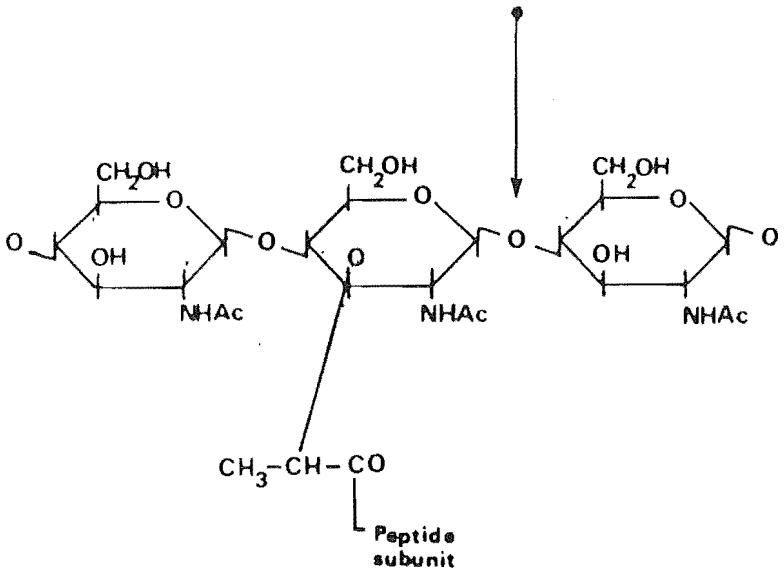


Fig. 2: PORTION OF PEPTIDOGLYCAN STRAND SHOWING SITE OF ACTION OF ENDO-N-ACETYLMURAMIDASE

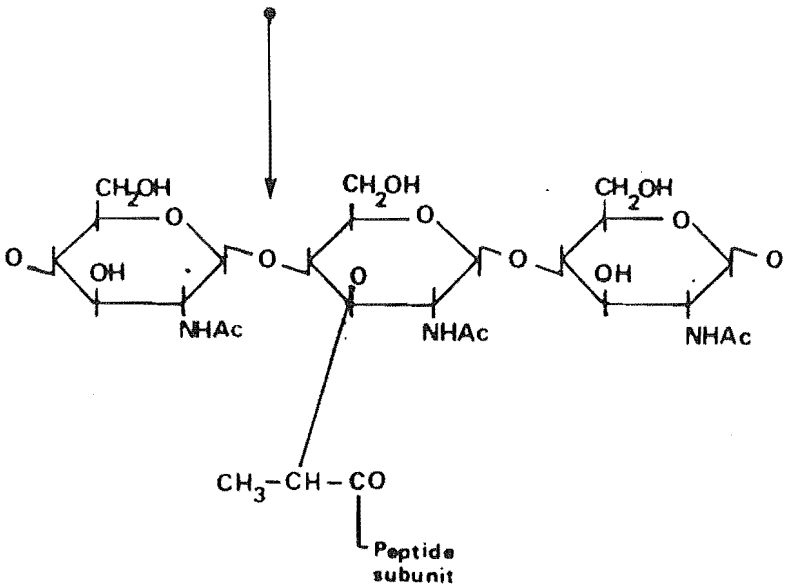


Fig. 3: SITE OF ACTION OF ENDO-N-ACETYLGLUCOSAMINIDASE

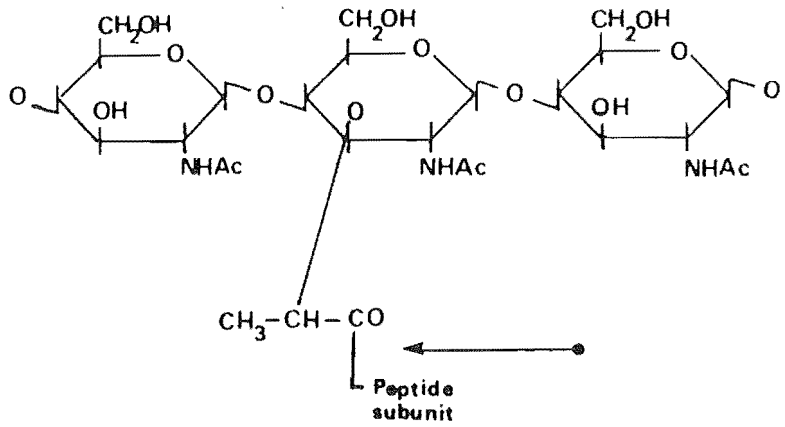


Fig. 4: SITE OF ACTION OF N-ACETYLMURAMYL-L-ALANINE AMIDASE

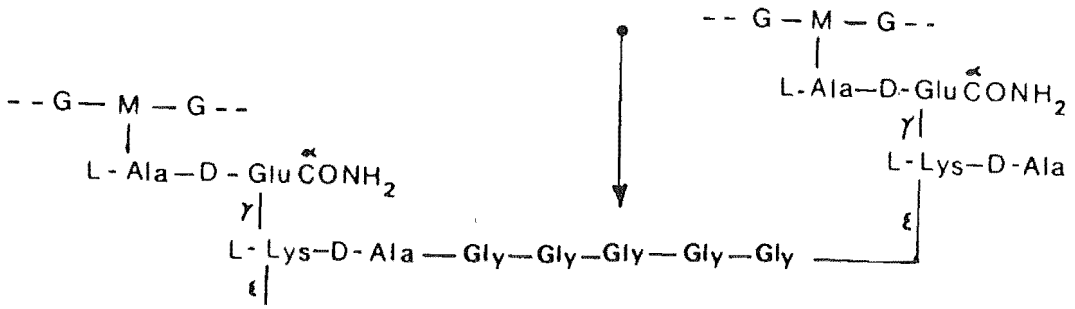


Fig. 5: SEGMENT OF PEPTIDOGLYCAN FROM STAPHYLOCOCCUS AUREUS CELL WALL SHOWING SITE OF ACTION OF ENDOPEPTIDASES
(Most gram positive bacteria have a short chain linking the tetrapeptides or have a direct linkage)

referred to the enzyme's capacity to lyse bacteria and "zyme" to its enzymatic properties. Micrococcus luteus was particularly susceptible to lysozyme and was originally named M.lysodeikticus, or a displayer of lysis ("deikticus" means able to show) (Stryer, 1975).

Occurrence: Ubiquitous in its distribution, Lz has been isolated from most living systems, from human secretions (Fleming, 1922), avian egg white (e.g. hen egg white lysozyme or HEWL) (Fleming, 1922), ruminant stomachs (Dobson et al, 1984), trout gastric extracts (Lindsay, 1984), bivalve styles (McHenery & Birkbeck, 1982), insects (Hoffman & Van Regenmortel, 1984), and protozoans (Drozanski, 1969; Vick et al, 1979) to rubber tree latex (Tata et al, 1983), filamentous fungi (Hash, 1963; Grant et al 1984) and slime moulds (Chapman & Coote, 1983).

Mode of Action: Lz causes dissolution of the living cells of Micrococcus luteus (M.lysodeikticus) (Fleming, 1922) by acting specifically on the fabric of the cell wall (Salton, 1952). The point of cleavage of the glycan backbone by Lz is between the N-acetylmuramic acid and the N-acetylglucosamine residues (see 1:1).

Function: Lz has a protective role for those organisms producing it. It is present in saliva (Fleming, 1922) and has a recognised role in the regulation of oral microflora, inhibiting bacterial growth and mediating bacterial aggregation (Laible and Germaine, 1982).

A role in bacterial cell growth and division is postulated since most bacteria produce autolysins (see 1:1). The type of lytic activity varies amongst the different bacteria, but in the bacterium Streptococcus faecium muramidases are secreted through the plasma membrane in latent form (Kawamura & Shockman, 1983). Lz of S.faecium is a glycoprotein, rare in prokaryotes. The carbohydrate moiety might have a recognition role in S.faecium, (allowing proteases to activate the enzyme by selective hydrolysis), and might help maintain the three dimensional conformation of the enzyme in the extracellular environment. It is possibly responsible for the high binding affinity of Lz to substrate (Kawamura & Shockman, 1983).

A nutritional role is now accepted for Lz. Bivalve Lz (McHenery et al 1979) and ruminant stomach Lz (Dobson et al, 1984) are digestive enzymes. Ruminant stomach Lz is adapted to an acid environment, having a lower pH optimum for lytic activity than HEWL. Many protozoans produce Lz. Extracts of soil amoebae lyse several species and strains of Bacillus, Micrococcus and Staphylococcus which are resistant to HEWL (Upadhyay, 1968) and an N-acetyl muramidase obtained from a Limax amoeba degrades the partially Lz-resistant peptidoglycan of the gram-negative Proteus mirabilis (Katz et al, 1971). A ciliate, Tetrahymena pyriformis, feeds on bacteria, and produces a "lysosomal hydrolase" that degrades Lz-resistant Streptococcal cell walls (Vick et al, 1979).

pH Optima and Ionic Strength: HEWL is optimally active at pH8.0 against live cells of Streptococcus faecalis (Carvalho et al, 1984), as is Streptomyces griseus Lz against live cells of Staphylococcus aureus: against S.aureus cell walls, however, Streptomyces griseus Lz is optimally active at pH6.5 (Ward and Perkins, 1968). Streptomyces rutgersensis Lz has an optimal pH of 6.0 against whole cells (Hayashi et al, 1981) and cell walls of Streptococcus faecalis (Hayashi et al, 1984), whereas the optimum pH for lysis of peptidoglycan is 3.5, enzyme activity being observed even at pH1.3 (Hayashi et al, 1984). Tetrahymena Lz is optimal at pH3-4 against streptococcal walls (Vick et al, 1979). Ruminant Lz has an optimum pH of 5 at physiological ionic strength (0.1), which supports the hypothesis of a nutritional role for this enzyme. (In the anterior part of the ruminant stomach the pH is about 6, decreasing posteriorly (Dobson et al, 1984).)

Clearly muramidases have different pH optima depending on their source and their substrate. The ionic strength of the buffer used is also critical. For example Mytilus edulis (a bivalve), produces Lz optimally active at pH7 at low ionic strength (0.011) but optimal at pH4.6 when ionic strength is 0.05 (McHenery and Birkbeck, 1979). The Lz of Hartmannella glebae, optimal at pH8.0, is highly active when ionic strength increases initially but once an optimum is passed activity decreases until there is no activity at 0.1 (Chung et al, 1969).

There is an initial increase in the activity of HEWL towards cell suspensions of M.luteus when ionic strength is initially increased, followed by inhibition as ionic strength is further increased, this effect being most marked at higher pH

values (Davies et al, 1969). Activity of six human and four avian Lzs on M.luteus increases as pH is raised and as ionic strength increases for all Lzs except goose egg white (GEWL) (Saint-Blanchard et al, 1970). GEWL is optimal at low pH (3.8 and 5.25) and at a higher ionic strength than the other Lzs tested, underlining the different conditions necessary for different muramidases to achieve maximal activity.

Activity Against Live Bacteria: Supernatant from the centrifuged nasal secretions of a cold sufferer causes inhibition of growth of live M. luteus cells suspended in agar, the zone of inhibition extending 1 cm beyond the limits of the supernatant (Fleming 1922). A 1 ml liquid suspension of live cocci disappears completely in a few minutes at 37°C when a drop of diluted nasal mucus is added to it.

Streptomyces griseus Lz lyses freeze-dried cells of Staphylococcus aureus (Ward and Perkins, 1968) and S.griseus strains isolated from soils and sewer produce lytic enzymes active against live cariogenic streptococci (Yokogawa et al, 1972).

Constitutivity/Inducibility: A muramidase isolated from insect haemolymph is induced by the injection of Pseudomonas aeruginosa into the insect (Powning and Davidson, 1973, 1976), but little work has been reported on the induction of Lz in other organisms.

Ghuysen (1968) has postulated a role in cell wall development for bacterial muramidases in those bacteria producing them. If this hypothesis is correct the muramidases would be constitutive, but their production strictly reguleted to prevent wall degradation.

Uses: Lz plays an important role in studies of bacterial wall structure, the preparation of type specific antigens, the retrieval of plasmid DNA (Horinouchi et al, 1977) and the release of protoplasts from live bacteria (Kawata et al, 1983). HEWL is used as a preservative in a variety of foods (Hayashi et al, 1984).

Conclusion: Bacteriolytic enzymes, in particular Lz, have an ubiquitons distribution and varied range of functions.

Lz might have an as yet undetermined but significant metabolic function, its bacteriolytic activities being secondary (Raghunathan, 1985).

1.3 Chalaropsis Lysozyme

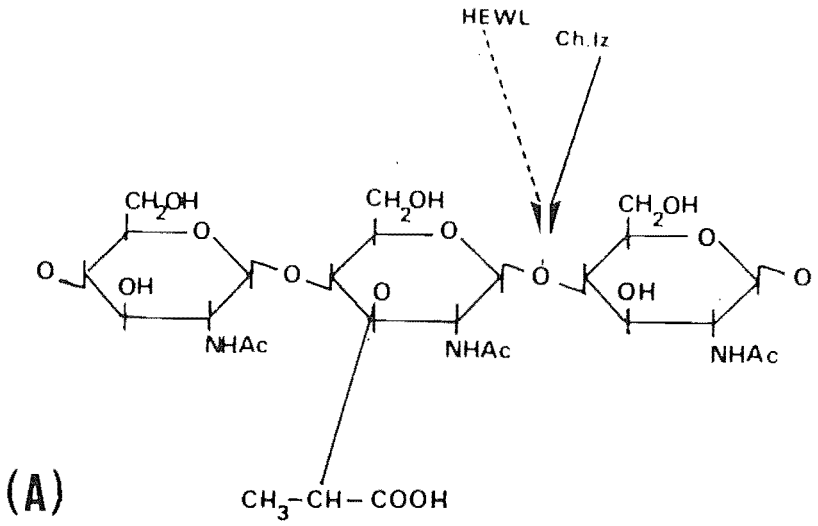
J.H. Hash (1963) published the first report on the production of bacteriolytic enzymes by a filamentous fungus, that fungus being an unidentified Chalaropsis species.

Production: Hash states that Chalaropsis Lysozyme (Ch. Lz) is constitutive, and that levels of lytic activity attained when the fungus is grown on a complex medium of glucose, peptone, and molasses, at concentrations of 10, 5 and 20g L^{-1} respectively, are no higher than when the fungus is grown on bacterial cell walls (Hash, 1963; Hash 1974).

Structure: The Chalaropsis bacteriolytic enzyme has been purified and crystallised (Hash & Rothlauf, 1967). Despite comparable muramidase action, HEWL and Ch.Lz are totally dissimilar proteins structurally (Mitchell & Hash, 1969). The two enzymes share no sequence homologies yet utilise the same amino acid residues in their active sites, i.e. aspartic acid-6 and glutamic acid-33 in Ch.Lz; aspartic acid-52 and glutamic acid-35 in HEWL, suggesting parallel evolution (Wai-Kuo & Hash, 1971; Fouche & Hash, 1978).

Mode of Action: Ch.Lz lyses live cells of Staphylococcus aureus, Streptococcus faecalis, Corynebacterium diptheriae, Bacillus subtilis, Sarcina lutea and Micrococcus luteus, exhibiting optimal activity at pH5.6 (Hash, 1963). Ch.Lz is an N,O-diacetylmuramidase. It cleaves the same glycosidic bond as lysozyme, i.e. the β-1,4 bond at C_1 of N-acetylmuramic acid but, unlike HEWL, Ch.Lz lyses in the presence of O-acetyl groups at C_6 of N-acetylmuramic acid residues (Hash & Rothlauf, 1967) (Fig. 6).

HEWL has a molecular weight of 14,388 (Barman, 1969) and is a more compact enzyme than the larger Ch.Lz (23,385 m.wt) (compactness has been related to the



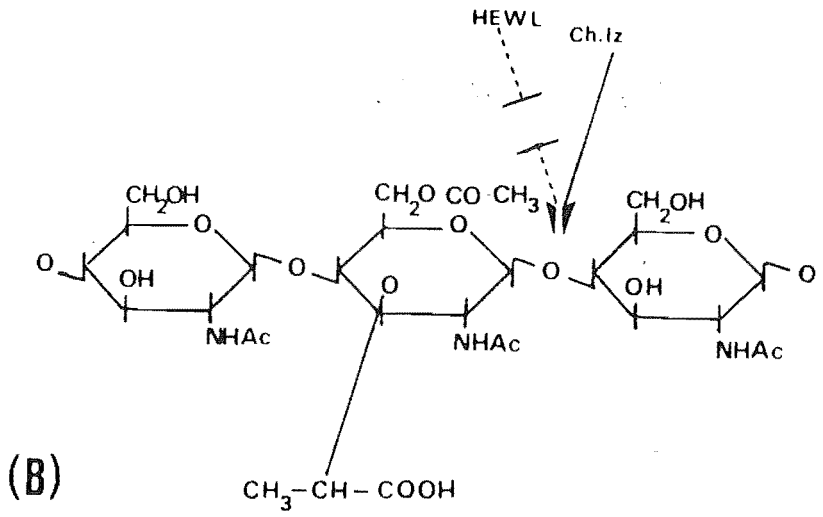


Fig. 6: ACTION OF HEWL AND CHALAROPSIS LYSOZYME AGAINST A PORTION OF PEPTIDOGLYCAN OF (A) MICROCOCCUS LUTEUS AND (B) STAPHYLOCOCCUS AUREUS (which has N, O -diacetylmuramic acid residues)

number of disulphide bonds, HEWL having four and Ch.Lz one) (Wai-Kuo Shih & Hash, 1971). It is possible that the 6-O-acetylation of muramic acid prevents its entry into the deep cleft of the compact HEWL. The N,O- diacetylmuramic acid residue therefore fails to reach the active site carboxyl groups of the enzyme (Fouche & Hash, 1978). Ch Lz has been shown to have similar specificities to Streptomyces albus Lz (the "32 enzyme") which also cleaves in the presence of O-acetyl groups (Tipper et al, 1964).

Again unlike HEWL, Ch.Lz is not a chitinase and might require the 3-O substitution on N-acetylmuramic acid residues to function enzymatically (Hash & Rothlauf, 1967).

Enzymes (such as chitinases and cellulases) acting on an insoluble substrate, generally depend on the amount of surface area exposed to establish rates of reaction, but Ch.Lz depends on the concentration of insoluble substrate rather than on surface area, possibly due to the fibrous nature of cell wall peptidoglycan (Fouche & Hash, 1978).

Enzyme Inhibition: When enzyme activity is assayed lytic activity of Ch.Lz is greatly diminished by the presence of buffers, their removal by dialysis restoring full activity. The optimum pH for lytic activity against Staphylococcus aureus cells with purified Ch.Lz is 5.6 when ionic strength is constant at 0.01. When pH is constant at 5.6 lysis is greatest at low ionic strength suggesting that the decrease in lytic activity in the presence of buffers is a function of ionic strength.

Over long periods comparable lysis occurs at higher ionic strength suggesting that ionic strength affects the rate of lysis (or assay system) but not the enzyme itself. Buffer ions might prevent cells from lysing, even if bonds are cleaved, by conferring stability on the lyophilised S.aureus cells (Hash, 1963).

Uses: As S.aureus bears the 6-O-acetyl group on muramic acid that confers resistance to HEWL, Ch.Lz is a useful tool for establishing the chemical structure of the staphylococcal cell wall and in obtaining S.aureus protoplasts, thus extending the lytic spectrum available with HEWL.

1.4 Other Fungal Bacteriolytic Enzymes

Filamentous Fungi: Following Hash's work with Chalaropsis sp., filamentous fungus production of bacteriolytic enzymes is next alluded to in a paper by Bohlool and Schmidt (1973) in which photographic evidence is given of rhizobial cells, tagged with fluorescent antibodies, being lysed by an unknown soil fungus.

Agaricus bisporus and a range of other compost fungi (encompassing Basidiomycetes, Ascomycetes, Fungi Imperfecti and Phycomycetes) grow on Bacillus subtilis cells as sole carbon, nitrogen and phosphorus source (Fermor and Wood, 1981). A range of filamentous fungi grown on both gram positive and gram negative bacterial cells did not produce muramidase activity in those fungal culture supernatants analysed for it i.e. A.bisporus, Pleurotus ostreatus, Neurospora tetrasperma and Aspergillus niger (Fermor, 1983).

Further work with A.bisporus (Grant et al, 1984) indicates that during growth of the fungus in liquid culture more than 80% of the muramic acid and diaminopimelic acid residues of B.subtilis cell walls disappears from the microbial biomass and appears in the supernatant as soluble glycopeptides. Bacteriolytic enzymes can be obtained from the culture supernatants by ion exchange chromatography and the main enzyme is a β-N-acetylmuramidase.

The earlier failure by Fermor (1983) to detect muramidase activity might be due to the high concentrations of bacteria used, with maximal binding of the enzyme to the substrate (Grant et al, 1984). Another possible reason is that whole cells of M.luteus are used in the assays to detect lytic activity, and fungal bacteriolytic enzymes are only slightly active against whole cells under usual in vitro conditions (Grant, unpublished). When the muramidase is assayed against bacterial cell walls most of the muramic acid residues which are present in the walls are accounted for by release of reducing groups during hydrolysis (Grant et al, 1984).

Recent work with a range of fungi isolated from the rhizoplane of clover growing in Manawatu pasture soils further demonstrates the production of bacteriolytic enzymes by fungi. The enzymes detected are of two distinct types, (i) those

optimal at acid pH and in which degradation of walls is accompanied by the appearance of reducing groups from the glycosyl linkages in the peptidoglycan (i.e. muramidases, glucosaminidases, or both) and (ii) enzymes optimal at neutral to alkaline pH, with free amino groups being released during wall hydrolysis (Grant et al, 1986).

Slime Moulds: Amoebae of the cellular slime mould Dictyostelium discoideum, when grown in submerged cultures on Escherichia coli, produce both a muramidase and an N-acetyl-D-glucosamimidase. Proteases and lipases aid the degradation of the bacterial cells. These enzymes have an optimum of pH3, which suggests a lysosomal origin (Braun et al, 1972).

The true slime mould Physarum polycephalum also grows on different strains of bacteria (Chapman & Coote, 1983) and generally utilises gram negative bacteria as a food source more readily than gram positive species. β-N-acetylhexosamimidase activity might act in conjunction with proteases, phospholipases, and other hydrolases, enabling rapid degradation, intracellularly, of bacterial cells. Culture supernatant of P.polycephalum exhibits a Lz-like activity against whole cell suspensions, and degrades cell wall preparations of both B.subtilis and M.luteus (Chapman and Coote, 1983).

1.5 Purification Procedures

Many procedures for the purification of lysozymes have been published. These include:

1.5.1 Ion-Exchange Chromatography: Cation-exchange resins are commonly used in the purification of bacteriolytic enzymes, for example those from B.subtilis (Del Rio & Berkeley, 1976), Streptomyces rutgersensis (Hayashi et al, 1981), Hartmannella glebae (Upadhyay et al, 1977), Streptococcus lactis (Mullan & Crawford, 1985) and mutanolysin (Yokogawa et al, 1974).

1.5.2 Affinity Chromatography: Carboxymethyl chitin, used as an adsorbent for Lz proves useful in the purification of Lz by affinity chromatography (Imoto et al, 1968). Chitin-coated cellulose (c/c) is an alternative adsorbent of Lz-like enzymes and is stable, reusable and easy to handle when used as an

insoluble substrate for column chromatography. Preparation of c/c from alkali chitin (Imoto et al, 1968; Yamada & Imoto 1981) is described by Imoto & Yagishita (1973(a)) and the method is used successfully to purify Lz (Yamasaki et al, 1976, 1979). C/c shows specificity for HEWL (which is adsorbed firmly at pH8 in 1M NaCl and desorbed with 0.1M acetic acid), and gives a good flow rate when packed in a column. (Imoto & Yagishita, 1973(a)). Specificity of the c/c is reaffirmed by the isolation of a Lz-like enzyme from yam. This enzyme is found to be nearly pure following the above procedure, being eluted as a single peak when run through a Sephadex G-75 column (Imoto & Yagishita, 1973(b)).

1.5.3 Affinity Separation: Fuchs et al (1986) outline a method of partial purification of crude chitinase which is a modification of the affinity chromatography procedure.

Crude chitinase is mixed with colloidal chitin (equal volume) at $4^{o}C$ and, following binding (nearly 100% in 15 minutes), is centrifuged, washed and suspended in buffer then incubated overnight at $30^{o}C$. The enzyme solubilises the chitin, and is thus released. Particulate matter is removed by centrifugation and chitin degradation products by dialysis, the enzyme being concentrated by ultrafiltration. The procedure could be adapted to bacteriolytic enzymes by using cell walls in place of chitin.

1.5.4 Gel Filtration: Fernandez-Sousa et al (1978) decribe a rapid one-step method of gel filtration chromatography on agarose columns to selectively purify HEWL. 100% recovery of pure active Lz is achieved.

Partial purification of a Tetrahymena bacteriolytic enzyme is achieved by ammonium sulphate precipitation followed by gel filtration on a Sephadex G-75 column. A single active peak is obtained with 52% recovery. Following ultrafiltation of the active fractions the concentrate is applied to a CM-cellulose column with 69% recovery and a high degree of purification. The Tetrahymena bacteriolytic enzyme is optimally active at pH3.4 (Vick et al, 1979).

1.5.5 Purification of Chalaropsis Lysozyme: Primary concentration of lytic enzymes from large volumes of filtrate is achieved by direct adsorption of the basic proteins on to weak cation-exchange resins (Amberlite CG-50).

Lytic enzymes are eluted from the resin with ammonium acetate (4°C, pH5), precipitated with ammonium sulphate and impurities removed by passage of the preparation through a DEAE-cellulose column (pH5.0) followed by chromatography on a Sephadex G-25 column (pH5.0) (Hash, 1963, 1974; Hash & Rothlauf, 1967).

1.6 Gliomastix Murorum var Felina

[Nature and Aims of Investigation]

Gliomastix murorum, a fungus isolated from the rhizoplane of white clover growing in pasture soil produces both an enzyme optimal at acid pH (when the fungus is grown in a liquid culture of B.subtilis cells at pH5) and an enzyme optimal at neutral to alkaline pH (when the fungus is grown at pH7), (Grant et al, 1986).

G.murorum appears to be unique in producing both enzyme types, other fungi examined producing one enzyme type or the other. This feature has made G. murorum particularly interesting for further study, as complex control mechanisms must operate to allow this "dual" enzyme production. G.murorum exhibits relatively rapid growth in liquid culture and so makes an ideal model system for the study of fungal bacteriolysis.

The investigation can be divided into five main areas:

(i) Experimental trials to confirm production of at least two bacteriolytic enzymes by G.murorum and to define optimal growth conditions for production of these enzymes.

(ii) Definitive measurements of enzyme production and growth of the fungus at pH5 and pH7.

(iii) Determination of the inducible or constitutive nature of the enzymes.

(iv) Purification of at least one of the enyzmes.

(v) Determination of the pH optimum and mode of action of the purified enzyme against isolated, purified bacterial cell walls.

[see Appendix II: Taxonomy and Morphology]

CHAPTER 2: MATERIALS AND METHODS

PART A: GLIOMASTIX SPECIES: GROWTH AND BACTERIOLYTIC ENZYME PRODUCTION

2.1 Organisms. The fungus Gliomastix sp was obtained as an isolate from the rhizoplane of white clover growing in Manawatu pature soil. It was provided by Dr. R.A. Skipp and identified as Gliomastix murorum var. felina by Dr. P.M. Kirk (see Appendix II).

Gliomastix spp PDDCC 1080, 1071 and 3857 were obtained from Plant Diseases Division Culture Collection, D.S.I.R. Mt. Albert, Auckland (see Appendix II).

Mycelium from each fungal species was cryopreserved in a solution containing tryptic soy broth (Gibco) (3% w/v) and glycerol (15% w/v) sterilised at 15lb 15min. The broth mixture (0.3ml plus 0.6 ml sterile water) was added to each cryopreservation tube. Fungal samples were added aseptically and tubes were stored at -80^{o}c (Gherna, 1981).

The Bacillus subtilis strain used was B.subtilis 168, (spores dried on silica gel), obtained from Prof. J. Mandelstam, Microbiology Unit, University of Oxford.

2.2 Growth of Fungi

2.2.1 Solid Culture Media

G.murorum was grown on malt extract agar (MEA) and 1/6th strength Czapek-Dox agar (Cz-Dx).

The Gliomastix species PDDCC 1080, 1071 and 3857 were grown on potato dextrose agar (PDA), MEA and nutrient agar (NA).

B.subtilis agar medium: B.subtilis plates were prepared as for B.subtilis liquid culture medium (see 2.2.2) but with 2% bacteriological agar added.

Soil agar medium: Garden loam was finely sieved and soil particles suspended in water adjusted to pH5. 2% bacteriological agar was added, and the medium autoclaved, 15lb 15min.

2.2.2 Liquid Culture Media

B.subtilis medium: heat-killed B.subtilis cells (2.5 mg ml^{-1}) were suspended in a basal salts medium containing $MgSO_4$, 1mM; H_3BO_3, 46 μM; $MnCl_2$, 9μM; $ZnCl_2$, 0.73 μM; $CuSO_4$, 0.32 μM; $(NH_4)_6$ Mo_7O_{24}, 0.032 μM (Grant et al, 1986) buffered with 0.05 M-KH phthalate buffer at pH3.3, 0.05 M-MES at pH5, 0.05 M-TES at pH7 or 0.05 M-Tris at pH8.5.

Glucose medium: The glucose/MES/salts medium consisted of

Solution A:	KH_2PO_4	1.0 g
	NH_4NO_3	1.0 g
	$MgSO_4.7H_2O$	0.5 g

dissolved in 200 ml dist. H_2O plus 10 ml of a trace elements solution (to give a final concentration of H_3BO_3, 46 μM; $MnCl_2$, 9 μM; $ZnCl_2$, 0.73 μM; $CuSO_4$, 0.32 μM; $(NH_4)_6$ Mo_7O_{24}, 0.032 μM (Grant et al, 1986)).

Solution B: 500 ml of 0.1M MES/NaOH buffer, pH5.
Solutions A & B were mixed, pH adjusted and volume taken up to 800 ml, then autoclaved in 80 ml lots (121°C 15 mins).

Solution C: 20% glucose sterilised (10lb 15mins) in 10 ml lots

Solution D: 2.5g L^{-1} $CaCl_2$ (sterilised at 15lb 15mins). The final medium was obtained by combining 80 ml solutions A + B with 10 ml C and 10 ml D to give a final glucose concentration of 2%.

2.2.3 Growth of Fungus in Liquid Medium

Erlenmeyer flasks (250 ml) containing 20 ml medium were fitted with non-absorbent cotton wool bungs, autoclaved at 121°C for 15 minutes and inoculated when cool with either

(i) 3mm x 3mm peripheral mycelial segments from a 1 week old agar culture or

(ii) 1ml of mycelium from a 6 day old liquid culture. Mycelium, which tended to form a solid mass in liquid culture, was transferred aseptically to a sterile bottle, containing 20 ml sterile mineral salts and 4mm glass beads, for breakage into mycelial fragments. The bottle was handshaken until mycelium appeared uniformly dispersed and then 1ml transferred to culture flask.

2.2.4 Preparation of Inoculum for B.subtilis Liquid Cultures

The fungus was maintained on MEA plates and sub-cultured on to B.subtilis plates (see 2.2.1) (at appropriate pH) one week prior to use as inoculum.

2.2.5 Harvesting of Fungal Supernatant

The contents of the culture flasks were centrifuged (2800g, 5°C, 15min) and the resulting supernatants were filtered (0.45 μm) and frozen. Pellets were freeze-dried if dry mycelial weights were required.

2.3 Preparation of Cells and Cell Walls of Bacillus subtilis

2.3.1 Preparation of B.subtilis cells

B.subtilis 168 spores were inoculated on to a NA plate and incubated at 37°C for 24 hours. 2 x 10 ml of sterile nutrient broth (NB) (Oxoid CM1) in 20 ml bottles were inoculated from the plate and incubated (static) at 25°C overnight; 2 x 100 ml sterile NB (in 500 ml Erlenmeyer flasks) were each inoculated with the 10 ml cultures and incubated on a rotary shaker (100 r.p.m.) at 25°C for 8 hours.

2 x 1L sterile NB (in 5L Erlenmeyer flasks) were each inoculated with a 100 ml culture and shaken 100 rpm) at 25°C for 15 hrs (overnight). NA plates were then streaked with culture samples to check for purity. The 1L cultures were then used to inoculate 2 x 12 L sterile NB in 14L vessels. This gradual scale up was a convenient means of ensuring an inoculum of required optical density and volume was ready at the beginning of a working day. 10 drops of anti-foam (Bevaloid 5901, Bevaloid Chemicals) were added to each vessel. Vessels were incubated at 37°C in a New Brunswick Model CFS-314 stir-tank fermenter, agitated at 200 rpm and aerated at 1.5 volumes per volume per min (v/v/m).

Samples were taken 1/2 hourly and optical density at 600 nm measured. When O.D. 600 nm was 0.9 to 1.0, the fermenters were stopped, and the culture was pumped through narrow tubing embedded in ice and collected in cooled 5L flasks.

The chilled cultures were centrifuged (Beckman Model J-21B Centrifuge) at 4000g., 5°C, 20 minutes. Pellets were combined, resuspended in cold water and recentrifuged. This water wash was repeated once, supernatants being discarded. The final pellet was resuspended in 200 ml cold water and boiled for 20 minutes. When cool the heat-killed bacteria were spun down as before, washed in water and freeze-dried over NaOH pellets and H_2SO_4 in a vacuum desiccator.

2.3.2 Preparation of B.subtilis Cell Walls

B.subtilis cells grown and harvested as above (2.3.1) were finally suspended in 150 ml cold saline (0.9% (w/v)). The cell suspension (25 ml) plus 25 ml of acid washed Ballotini beads (0.2 mm) were chilled on ice in homogeniser bottles then shaken for 1 minute at 4000 rpm in a Braun MSK cell homogeniser, cooled with 2 second blasts of solid CO_2 every 15 seconds. Shaking was repeated twice, than cells were checked microscopically. If >1% whole cells remained, further shaking was given. The contents of the Braun bottles were filtered through a coarse-grade sintered funnel and beads were washed with cold saline. The combined filtrates were centrifuged (19000 g, 5°C, 20 min). The pellet was resuspended in cold saline and spun (200 g, 5°C, 5 min) and the pellet, which was largely unbroken cells and debris, discarded. The supernatant was respun (200 g for 5 min, then 11000g for 15 min, 5°C) and all but the "basal" pellet resuspended in M KCL, spun (11000g, 5°C, 15 min) then washed 10 times in cold water, centrifuging after each wash then resuspending the pellet.

The final pellet (following washes) was resuspended in water, boiled for 20 minutes, cooled, respun and washed once, then freeze-dried.

B.subtilis peptidoglycan preparation was a gift from Dr. W.D. Grant.

2.3.3 Removal of Free Amino Groups from B.subtilis Cell Walls

Cell walls (2mg) (2.3.2) were suspended in 3 ml water (held on ice). Sodium nitrite (0.1g) and glacial acetic acid (0.15 ml) were added, held at $0^{o}C$ for 2 hours. The cell walls were collected by centrifugation (11000g, $0^{o}C$, 10min), the pellet then being washed 3x in 10ml water (Fordham & Gilvarg, 1974).

2.4 Analytical Procedures and Assays

2.4.1 "Durham Tube" Assay of Bacteriolytic Activity

A semi-quantitative assay of the bacteriolytic activity of culture supernatants was carried out by incubating 50 µl of supernatant with 15 µl cell walls (10 mg $ml^{-1}H_2O$) and 30 µl appropriate buffer in 25 x 5 mm tubes (Durham tubes) at $37^{o}C$. Buffers were 0.1 M acetate, pH3.7, 0.1M MES, pH6 or 0.1 M TES, pH8. After 24 h, changes in turbidity due to wall lysis were visually assessed (Grant et al, 1986) (Plate 1).

2.4.2 Quantitative Assay of Bacteriolytic Activity

The decrease in optical density (Δ O.D.^{600nm} h^{-1} using 10 mm path length micro-cuvettes in a Beckman Acta CII spectrophotometer) was measured when 100 µl of culture supernatant or enzyme preparation was mixed with 60 µl of an aqueous suspension of B.subtilis cell walls (10 mg ml^{-1}) and 250 µl appropriate buffer, and all incubated at $30^{o}C$.

Basal salts medium (see 2.2.2) was used for controls (Grant et al, 1986).

N.B. One unit is defined as the change in OD^{600nm} per hour x10 under conditions of the standard assay outlined above.

2.4.3 Glucose Analysis

Glucose contents of culture fluids were determined as follows:

Glucose reagent: 3ml 1% (w/v) aqueous phenol
3ml Tekit enzyme reagent (Fermcozyme)
and 94ml Solvent.

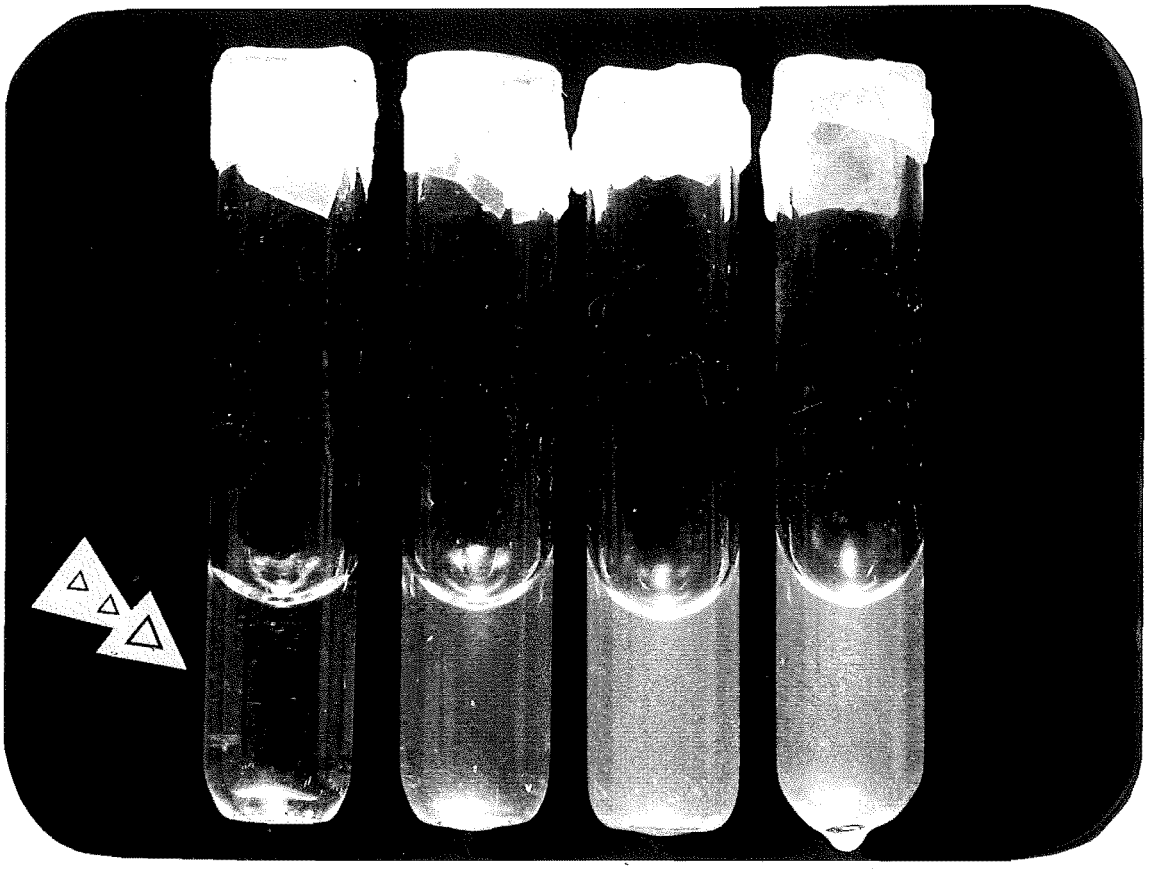


PLATE 1: SEMIQUANTITATIVE ASSAY TO ASSESS APPROXIMATE BACTERIOLYTIC ACTIVITY OF SUPERNATANT WITH BUFFERED CELL WALLS OF B.SUBTILIS AS SUBSTRATE

The assay was carried out in Durham tubes containing: 15 µl of 10 mg ml^{-1} cell wall suspension (in water), 30 µl of appropriate buffer, and 50 µl of culture supernatant or 50 µl of salts medium for control. Incubation was at 25°C for 24 hours and the tubes were examined visually for turbidity. Clearing indicated activity (see arrow on plate).

Solvent comprised Na_2HPO_4 (20g) NaN_3 (2g), 4-aminophenazone (0.6g) and water to 2 litres.

Glucose reagent (2ml) was added to aliquots of culture fluids in duplicate, mixed, and heated at 37°C for 10 minutes. Standards (10-50ug D-glucose) and blanks were treated in the same way.

Readings of optical density were taken at 515nm on the spectrophotometer (PYE/UNICAM SP-6) against a reagent blank. Glucose content of the samples was obtained from the standard curve.

2.4.4 Analysis of Mycelial Ergosterol Content

The mycelial pellet from liquid cultures was washed with water, respun, then freeze-dried and weighed. The dried mycelium was mixed with 40 ml cold methanol in a 200 ml beaker (held on ice) and homogenised with an Ultraturrax homogeniser for 2 x 30 seconds.

After 30 min the homogenisation was repeated and then the contents of the beaker filtered (Whatman GFC) and washed 3x with 10 ml methanol. The filtrate (made up to 70 ml with methanol) was refluxed for 30 min with 20 ml ethanol and 8g KOH (plus anti-bumping granules) in 250 ml round bottomed flasks. After cooling, the flask contents were transferred to 250 ml separating funnels, 25 ml of water was added, and ergosterol was extracted with 65 ml dist. hexane. The aqueous layer was re-extracted with a further 65 ml of hexane and finally with a further 30 ml. The hexane fractions were combined then reduced by rotary evaporation to dryness. Residues were stored at 4°C in the dark (Seitz et al, 1977, 1979; Matcham et al, 1985).

Residues were brought to room temperature in the dark, dissolved in 3 ml hot methanol and transferred to 5 ml volumetric flasks. When cool the solutions were brought to 5 ml with methanol, mixed, and filtered through 0.2 µm teflon filters (Gelman Acrodisc CR) into high pressure liquid chromatography (HPLC) vials. Standards were also filtered and analysis was carried out by HPLC using an Alltech 30 cm x 4.6 m, 5 µm column; the mobile phase was methanol containing 0.5% H_2O and 0.1% glacial acetic acid; the flow rate was 1.8 ml min^{-1}; the injection volume was 20 µl; the detector was set at 282 nm. These conditions were adapted from Seitz et al (1979).

PART B: ENZYME PURIFICATION AND CHARACTERISATION

2.5 Affinity Chromatography (Imoto et al, 1968; Yamada & Imoto, 1981)

Preparation of Chitin Coated Cellulose (c/c):
Powdered chitin (2.5g; from crab shells, Sigma Practical Grade) was suspended in 50 ml 42% (w/v) NaOH and kept under vacuum at 20°C for 4 hours and mixed occassionally. The alkali-chitin was then filtered (glass fibre filter paper) through a Buchner funnel, washed with 42% NaOH and pressed to remove excess NaOH. The chitin was then vigorously mixed with >30g finely crushed ice (frozen distilled water) for 20 minutes. The resultant viscous solution was diluted to 40 ml, 5g α-cellulose powder (Sigma Chemical Co.) was added and mixed thoroughly. 80 ml cold water was then added gradually (over 1 hour), followed by 300 ml 2 M acetic acid. The mixture was filtered and washed with water and kept as a 100 ml suspension at 4°C with 0.02% NaN_3 added.

Preparation of Column: Both c/c and the starter buffer (at a pH optimal for lytic activity) were degassed under vacuum. The column (10mm diameter) was then filled with the c/c and equilibrated by flushing through with the buffer. Fungal culture supernatant was added at the top of the column, drained into the bed under grevity and left to stand for 30 minutes then buffer run through and fractions collected.

The elution buffer (at a pH at which lytic activity, and therefore binding, would not occur) was then run through the column and all fractions assayed for bacteriolytic activity by Durham tube assay.

2.6 Cation-Exchange Chromatography

The column (10 cm x 0.5 cm) was packed with washed resin (Zeokarb 226), flushed through with 2M NaOH and washed with water until it reached neutral pH. the column was then flushed through with 2N HCl, again washed with water until it regained neutral pH and then transferred to 4°C. Fungal culture supernatant was added to the top of the column which wes then eluted with water followed by an elution buffer. All fractions were semi-quantitatively assayed (Durham tube assay) for bacteriolytic activity.

2.7 Ammonium Sulphate Precipitation (Dawson et al, 1969)

Fungal culture supernatant, concentrated two-fold by ultrafiltration (YM-10 Amicon membrane filter), was magnetically stirred at 4°C as ammonium sulphate crystals were added. The solution (at a known ammonium sulphate concentration) was left to stand overnight, unstirred, and any resultant precipitate separated by centrifugation (20000g, 2°C, 30 minutes). The pellet was then dissolved in 100 ml 0.1M MES buffer, pH5, (held on ice), then concentrated to 5-10ml by ultrafiltration (YM-10 membrane filter) to remove ammonium sulphate, and quantitatively assayed for bacteriolytic activity.

2.8 Gel Filtration

The column (2.5 cm diameter) was packed with a degassed slurry of Sephadex G-75 in pH7 sodium phosphate buffer (0.02 M) which was the mobile phase. The final gel bed height was 84 cm.

The void and total included volumes were measured by running blue dextran and glucose through the column (6.0 ml of 1% (w/v) glucose and 0.2% blue dextran made up in the buffer) using a 3-way valve and syringe. Fractions (100 x 3 ml) were collected and assessed visually for blue dextran, then measured for optical density at 620 nm on the spectrophotometer. The elution volume of glucose was established by assaying the fractions with glucose oxidase (see 2.4.3).

2.9 Identification of Reducing End Groups of Cell Wall Digest

2.9.1 Conversion of Reducing End Groups to Alcohols

Cell wall digest, obtained by incubating B.subtilis cell walls with E_1 in buffer overnight was treated as follows:

(a) A 0.1M solution of $NaBH_4$ was prepared with cooled boiled distilled H_2O. 1M HOAc was added to 2 ml of this until effervescence stopped, i.e. $NaBH_4$ was inactivated.

(b) The sample (digest) was brought to neutrality with M NH_4OH and divided equally into 2 acid washed tubes, then dried over silica gel under vacuum.

(c) A control, 200 µl of a 25 µmol ml^{-1} glucosamine solution, was added to 500 µl pH3.4 formate buffer (ionic str = 0.05), neutralised with M NH_4OH, divided into 2 acid washed tubes and dried as above.

(d) To one of the digest tubes and one of the glucosamine tubes (after drying) was added 200 µl of active $NaBH_4$. Inactivated $NaBH_4$ (200 µl) was added to the remaining tubes. Tubes were shaken, sealed and left at room temperature for 3 hours. Concentrated HCl (100 µl) wes added to each tube, then each tube was flushed with N_2, tightly stoppered, heated at 100°C for 4 hours and then dried down, redissolved in 100 µl of water and re-dried in vacuo over NaOH and silica gel to remove HCl. The drying was repeated 2x. The samples were then dissolved in 200 µl water, sealed, end kept on ice (Ghuysen et al, 1966).

2.9.2 Chromatography of Acid Hydrolysates of $NaBH_4$- Treated Cell Wall Digests

Chromatography on Whatman paper, No. 3mm was either (a) in the descending direction only, using Solvent A, i.e. butan-1-ol: pyridine: glecial acetic acid: water (60:40:3:30) or (b) two directional. In the latter case papers were run in the descending direction in Solvent B, i.e. butan-1-ol: glacial acetic acid: water (3:1:1) then, following drying, the papers were turned through 90 degrees and rerun in the descending direction in solvent C, i.e. pyridine:water (4:1) (Ghuysen et al, 1966). Papers were dried thoroughly in air, stained with a ninhydrin reagent (2.10.4) then kept in the dark over H_2SO_4 for several days prior to analysis for amino acids and amino sugars (see 2:10:4).

2.10 Analytical Procedures and Assays

2.10.1 Reducing Sugar Assay (Park & Johnson, 1949)

Solutions:

1. $K_3Fe(CN)_6$ (0.5 g L^{-1})
2. CO_3-CN reagent (Na_2CO_3, 5.3 g L^{-1} + KCN, 0.65 g L^{-1})
3. Ferric-SLS reagent ($FeNH_4(SO_4)_2$-$6H_2O$, 1.5g L^{-1} + Na lauryl sulphate, 1g L^{-1}, made up in 0.025M H_2SO_4)

Glucosamine was used as the standard. 2-10µg were each made up to 1 ml with water. Samples of 50 µl were also made up to 1 ml with water. To each 1 ml standard or sample 1 ml of Sol^{n} 1. and 1 ml Sol^{n} 2. was added, mixed and heated to 100^{o}C for 15 minutes. After cooling, 5ml of Sol^{n} 3. was added to each tube, mixed and left at room temperature for 15 mins. The O.D. ^{690nm} was then read against a reagent blank. (PYE/UNICAM SP-6 Spectrophotometer).

2.10.2 Amino Group Assay (Ghuysen et al, 1966)

Reagents: 1. 100 mM 1-fluoro 2-4 dinitrobenzene (FDNB) in 95% ethanol (weighed out as 18.6 mg ml^{-1}).
2. 1.67% $Na_2B_4O_7 . 10H_2O$
3. 2M HCl
4. 10 mM Glycine (standard)

Samples of 10 mM glycine, (10, 20, 30, 40 and 50 µl) were each brought up to 300 µl in acid washed tubes. 300 µl of each sample was assayed. To each tube 300 µl of $Na_2B_4O_7$ was added, then 100 µl FDNB. Tubes were vortexed, heated at 60^{o}C for 30 minutes, cooled and then 2.5 ml 2M HCl added to each tube. The optical density was read at 420 nm against a reagent blank.

2.10.3 Protein Estimation

A: (Lowry et al, 1951)

Solutions: 1. 2% (w/v) Na_2CO_3 in 0.1 N NaOH
2. 1% (w/v) $CuSO_4.5H_2O$
3. 2% (w/v) NaK Tartrate
4. 1mg ml^{-1} Bovine Serum Albumin (Standard) (BSA)
5. 1:2 aqueous dilution of Folin-Ciocalteau's Phenol Reagent.

1ml of Solution 3. was added to 100 ml of Solution 1.
1ml of Solution 2. was then added and mixed to make reagent A.

0.05, 0.10, 0.15 and 0.20 ml of BSA standard were each brought up to 0.5 ml with dist. H_2O. 0.1ml of the experimental samples were similarly treated (in duplicate).

5ml of reagent A was added to each tube and mixed thoroughly, then left to stand for 10 mins. 0.5 ml of Solution 5. was then added to each tube, mixed, and heated at 37^{o}C for 1 hour in a waterbath. Optical density was read at 750 nm against a reagent blank.

B: (Bradford, 1976)

Reagent: 100 mg Coomassie Brilliant Blue G-250 dissolvd in 95% ethanol. 100 ml 85% (w/v) phosphoric acid was added to the dye and the solution diluted to 1 litre with water. Standards were 10, 30, 50, 70, 100 μl of 1mg ml^{-1} bovine serum albumin, each brought up to 100 μl with 0.02M sodium phosphate buffer, pH7.

100 μl of each sample was taken and 5 ml of reagent added to each (acid washed) tube. Tubes were vortexed, stood for 2 minutes, then the optical density read at 595 mn against a reagent blank. (Tubes were read within 1 hour (as colour fades) and plastic cuvettes were used to overcome the staining that occurs in glass.)

2.10.4 Analysis of Amino Acids and Amino Sugars Following Chromatography

Following chromatography in the appropriate solvent, papers were dipped in a Ninhydrin developer, made up as follows:

100 mg cadmium acetate was dissolved in 5 ml glacial acetic acid plus 10 ml dist. H_2O. This solution was combined with 1g Ninhydrin dissolved in 100 ml AR Acetone.

Following development, the chromatographic spots (see 2.9.2) were cut out and eluted in tubes with 5 ml of methanol, then held in the dark (shaken every 15 minutes) for 1 hour. The optical density of the eluate was then read at O.D. 500 ^{nm} (Heilmann et al, 1957).

CHAPTER 3: RESULTS

PART A: OPTIMISATION OF GROWTH CONDITIONS TO PRODUCE MAXIMAL BACTERIOLYTIC ACTIVITY

3.1 General Introduction

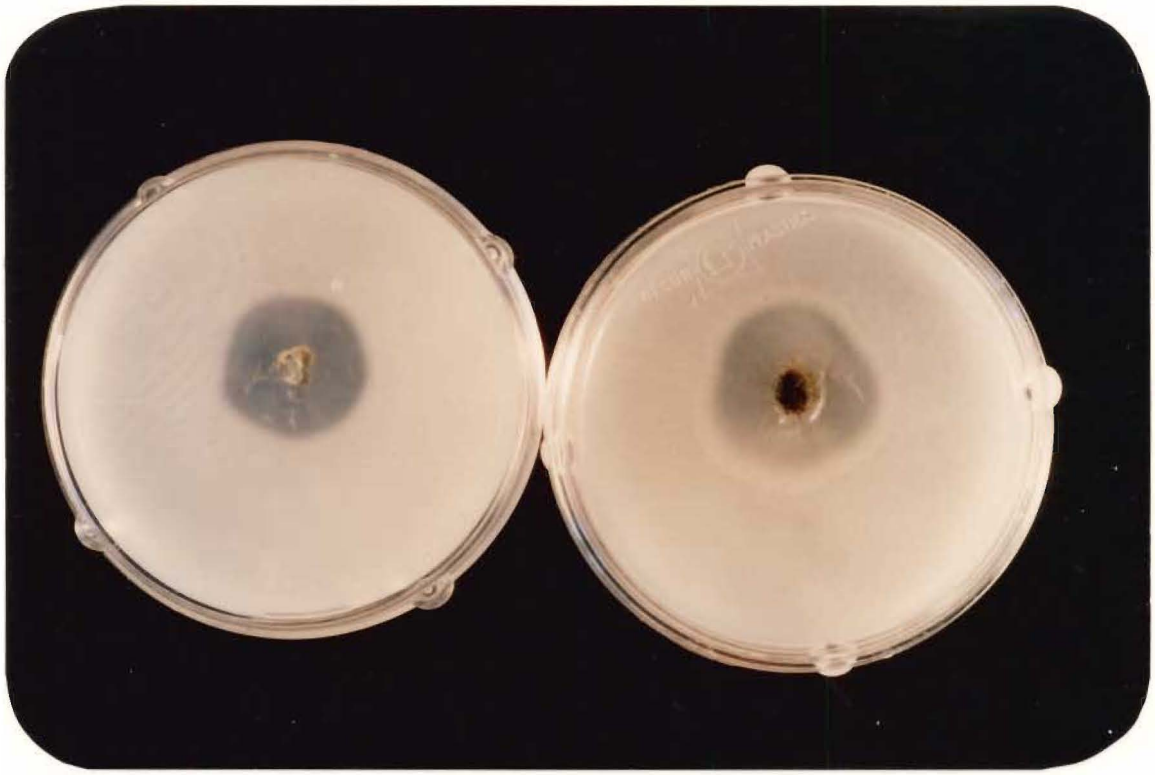
An investigation into a wide range of filamentous fungi from pasture soils, compost and garden loam was undertaken (Grant et al, 1986) and results indicated that bacteriolytic enzymes were produced in four of the five classes of fungi examined and that half of those fungi produced either an enzyme with a low pH optimum when grown at pH5, or an anzyme with neutral to alkaline pH optimum when grown at pH7, in a B.subtilis liquid culture. Gliomastix murorum was unique in producing both enzyme types (Plate 2, A & B). Activity was also detacted at an alkaline pH when G.murorum was grown at pH5 (Table 1).

In the work which follows E_1 refers to bacteriolytic activity of supernatants from G.murorum cultured at pH5 and optimal at pH3.6; E_2 refers to bacteriolytic activity of supernatants from the fungus cultured at pH7 and optimal at pH7.5; E_3 refers to bacteriolytic activity of supernatants from the fungus cultured at pH5 and optimal at pH8.0.

3.1.1 Growth Form of Gliomastix (Plates 3-9)

On solid medium (MEA) Gliomastix murorum produced a fluffy mycelial growth form giving rise to hyphal ropes whilst on a solid B.subtilis medium a delicate non-aerial mycelium was formed. Pigmented conidia developed in both cases. In liquid media the growth form varied markedly depending on the environmentel conditions. In non-agitated B.subtilis medium a loose mat of mycelium formed, whilst in agitated cultures (160 strokes min^{-1}) a dense "lump" of mycelium formed (pH5 or 7, 25°C or 30°C). In glucose medium, small pellets were formed. (Gliomastix spp PDDCC 1080, 1071 and 3857, see Appendix II.)

A



B

PLATE 2, A & B: GLIOMASTIX MURORUM GROWN ON B.SUBTILIS CELLS AT pH5 (l.) AND pH7 (r.)

Clearing of agar was due to bacteriolytic enzyme activity (A) 6-day culture, (B) 14-day culture.

TABLE 1: Production of Bacteriolytic Activity by *Gliomastix* spp.

Bacteriolytic activity was estimated by the Durham tube semi-quantitative assay (2.4.1).

GROWTH pH	DAY OF GROWTH	*GLIOMASTIX MURORUM* ASSAY pH 3.7	6.0	8.0	*GLIOMASTIX* SP. 1080 ASSAY pH 3.7	6.0	8.0
5.0	4	+++	–	–	–	–	–
7.0		–	–	–	–	–	–
8.5		–	–	–	–	–	–
5.0	6	++++	–	–	–	–	–
7.0		–	–	–	–	–	–
8.5		–	–	–	–	–	–
5.0	8	++++	+	++	–	–	–
7.0		–	–	++	–	++	–
8.5		–	–	–	–	–	–
5.0	11	++++	+	++++	–	–	–
7.0		–	++	+++	–	++	++
8.5		–	+	++	–	–	++
5.0	14	+++	–	++	+	++	++
7.0		–	–	–	–	++	++
8.5		–	–	+	–	+	+

++++ total clearing of cell wall suspensions by culture supernatants in 24h

+++ >50% clearing (as above)

++ <50% clearing "

\+ slight clearing "

– no activity

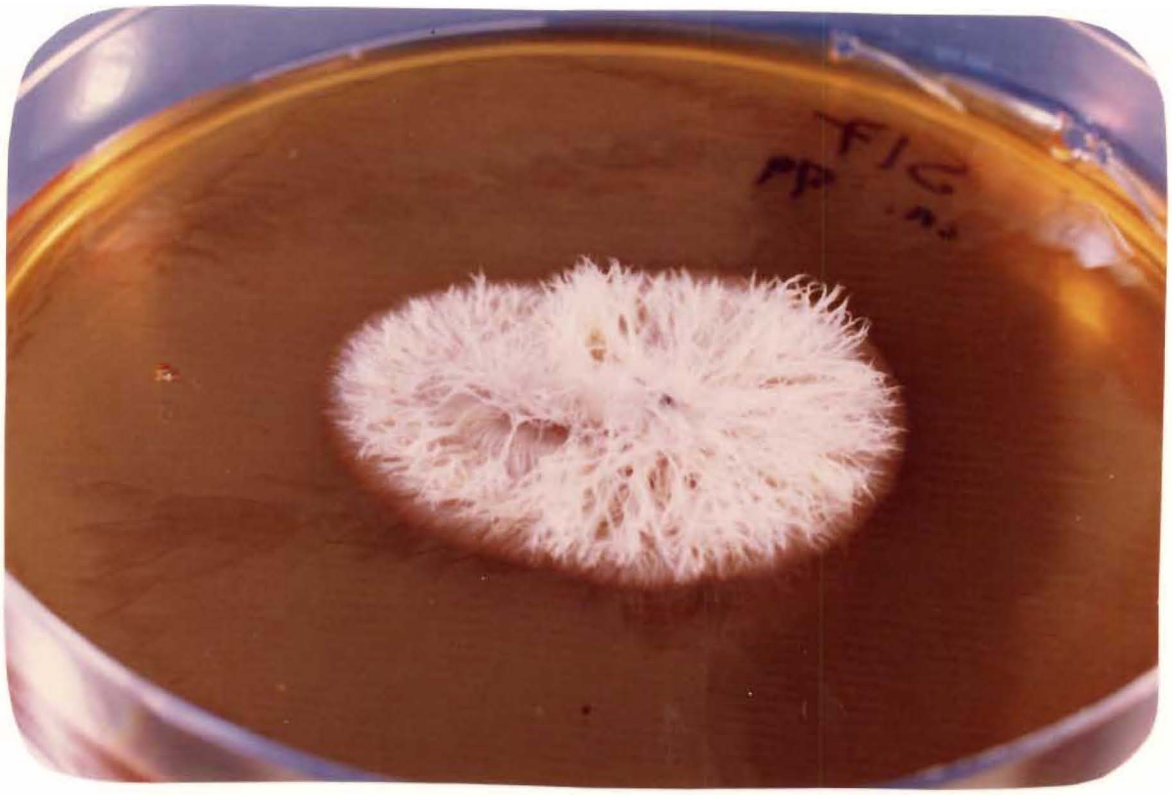


PLATE 3: **GLIOMASTIX MURORUM var. FELINA**

1-week culture grown on MEA at 25°C showing aggregation of hyphae into ropes.

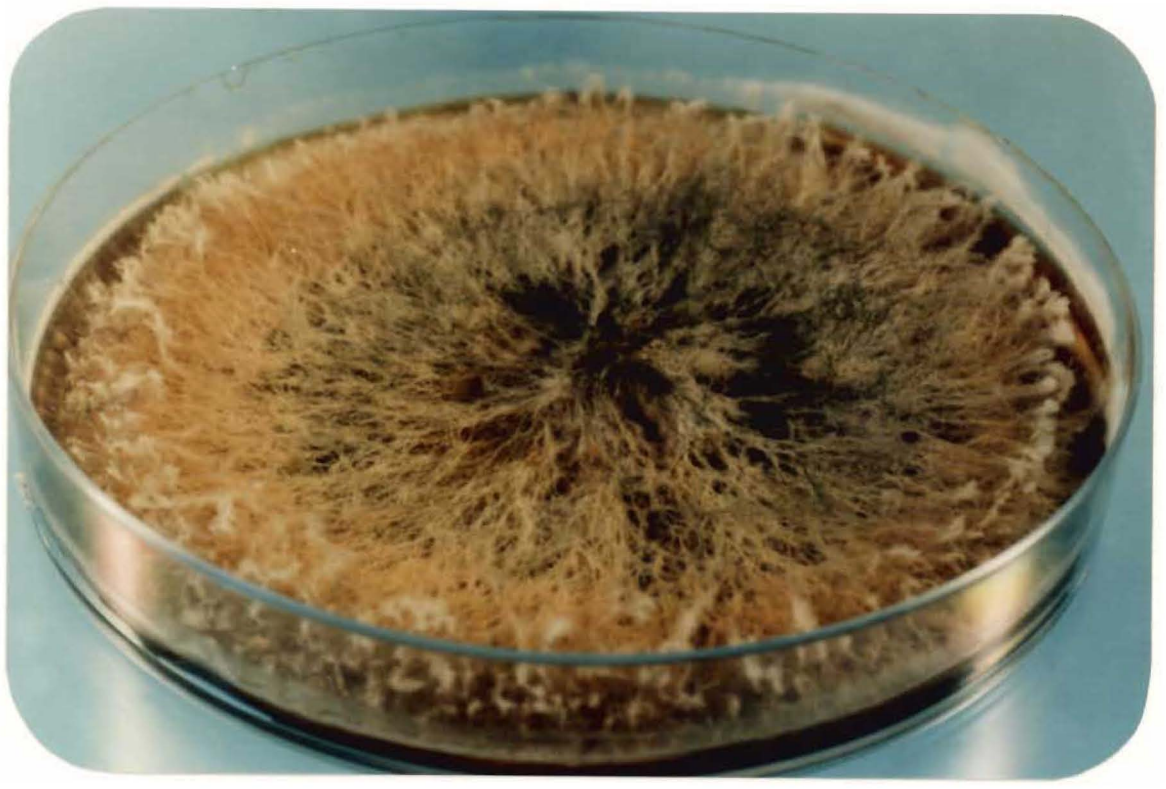


PLATE 4: **GLIOMASTIX MURORUM var. FELINA**

3-week culture grown on MEA at 25°C appearing black due to the sporulation.

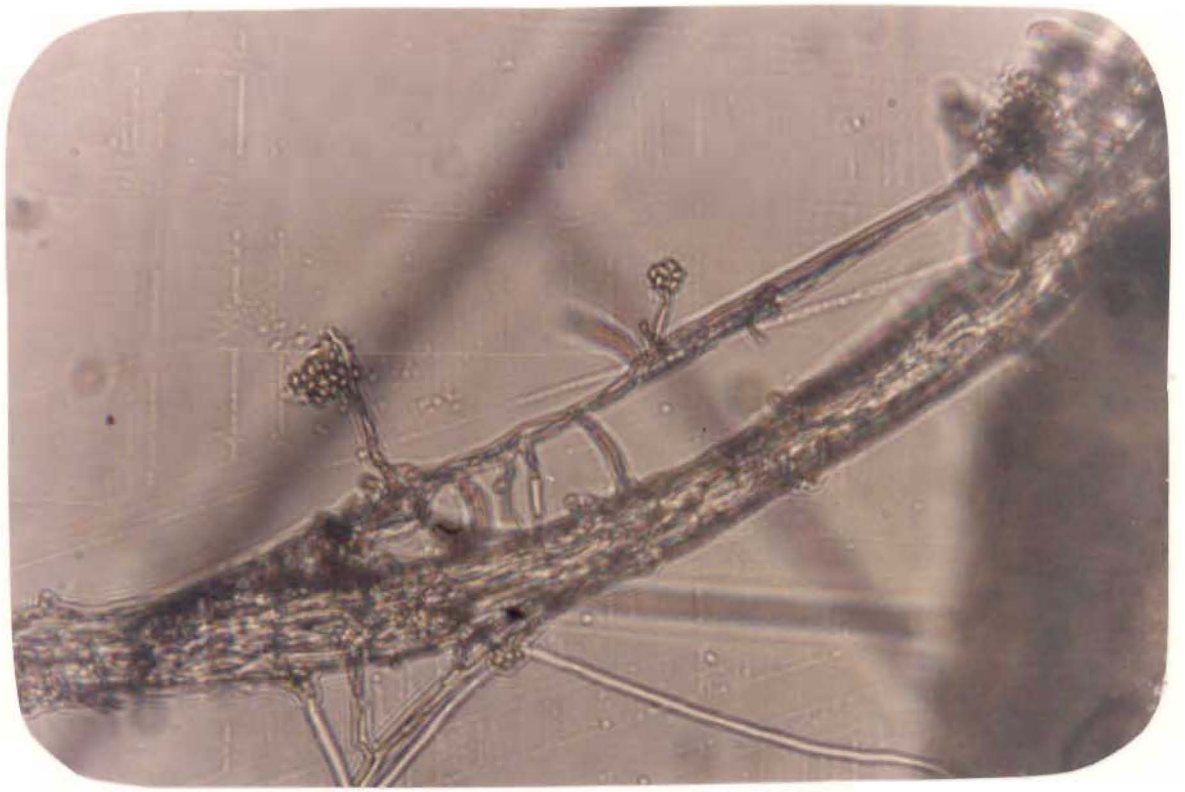


PLATE 5: GLOEOID MASSES OF CONIDIA OF GLIOMASTIX MURORUM var. FELINA

Characteristic coalescing spore drops on a 2-week culture grown on MEA at 25°C (500 x magnification).

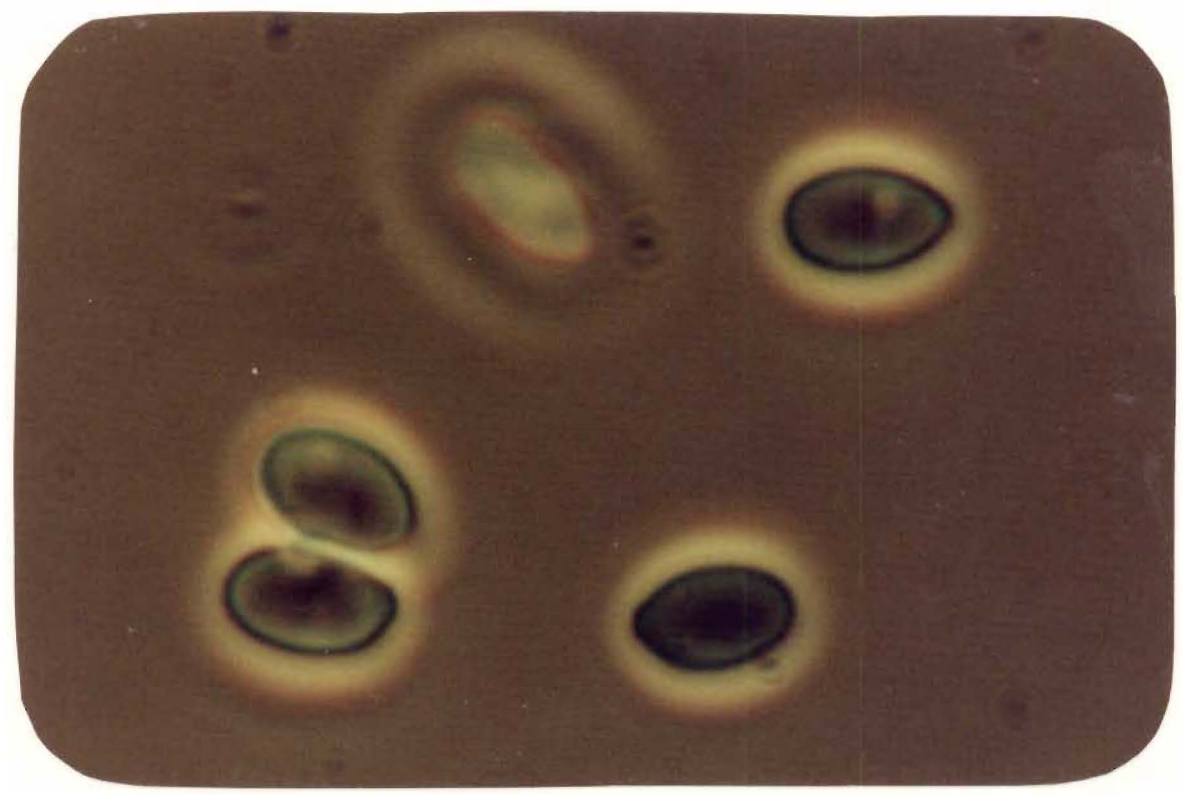
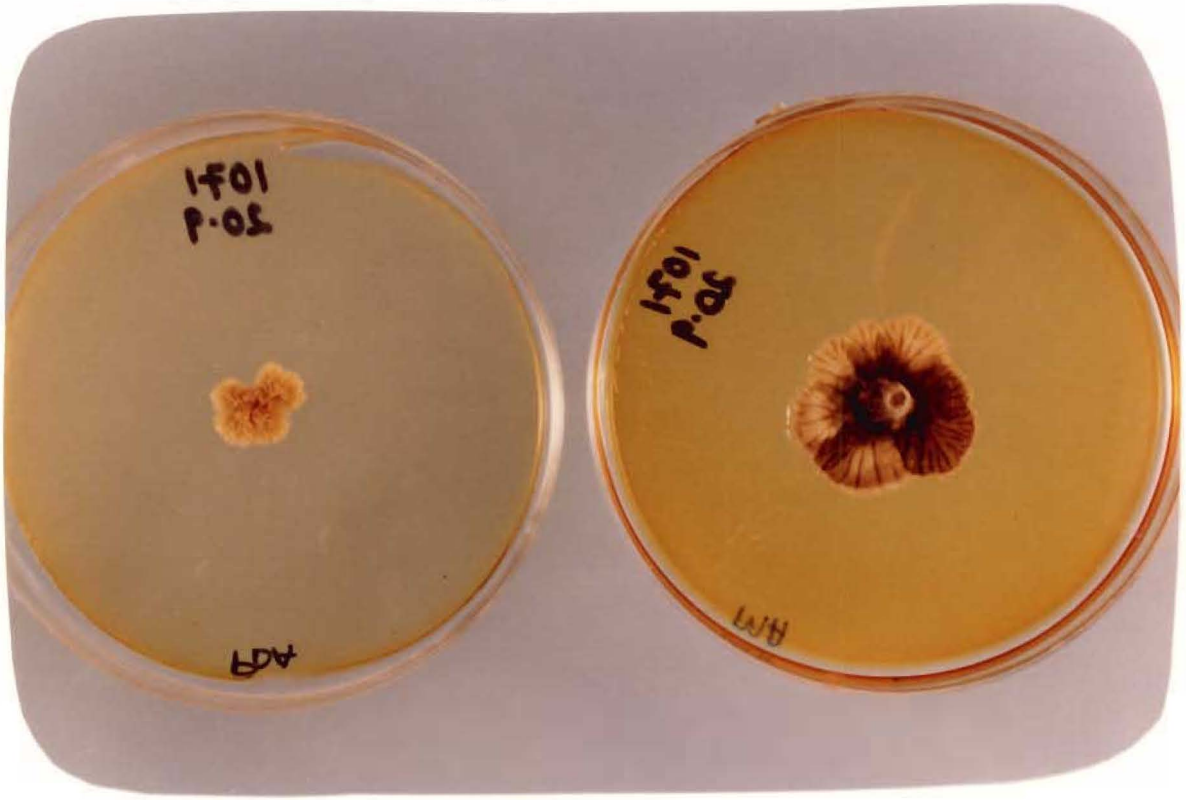


PLATE 6: CONIDIA OF GLIOMASTIX MURORUM var. FELINA

Smooth, non-septate, pigmented, ovoid phialospores, 4.0 x 2.6 u (5000 x magnification).



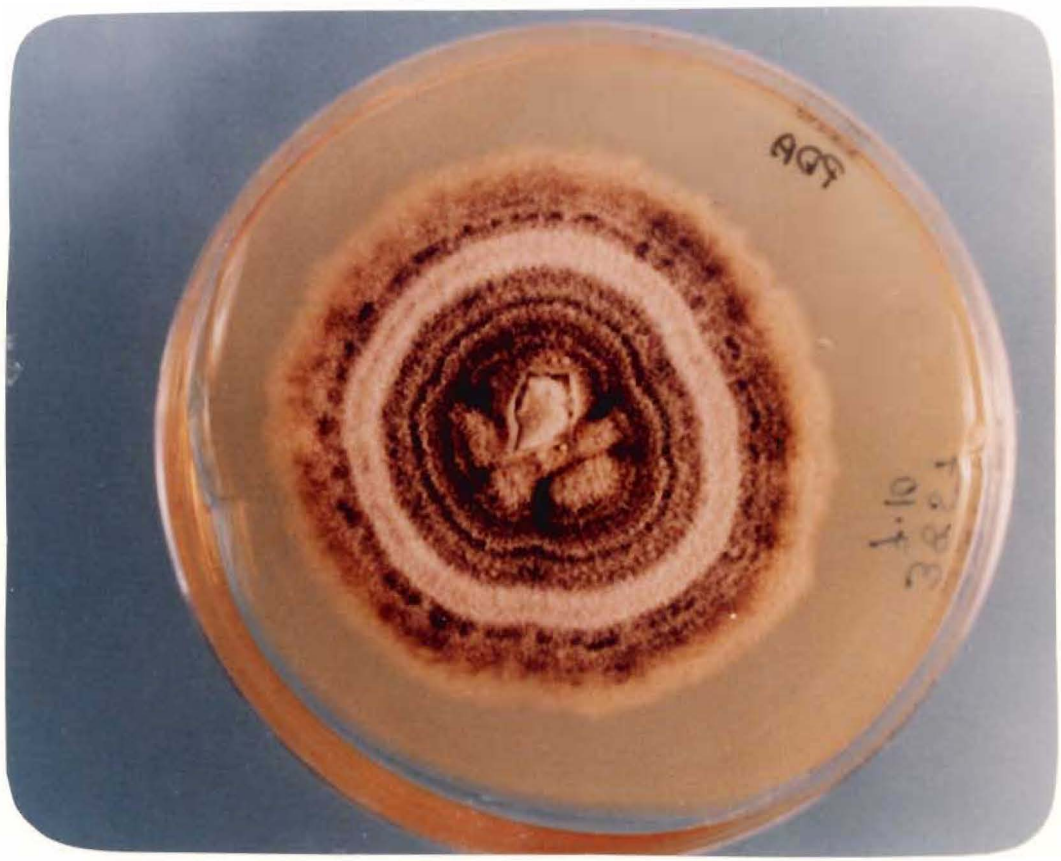


PLATE 7: (above) GLIOMASTIX SP. 1071*

3-week culture on MEA (l) and PDA (r).

PLATE 8: (below) GLIOMASTIX SP. 3857*

3-week culture on MEA.

Supernatants from G. spp. 1071 and 3857 grown on B.subtilis cells showed no bacteriolytic enzyme activity when assayed against bacterial cell walls.

*PDDCC catalogue number.

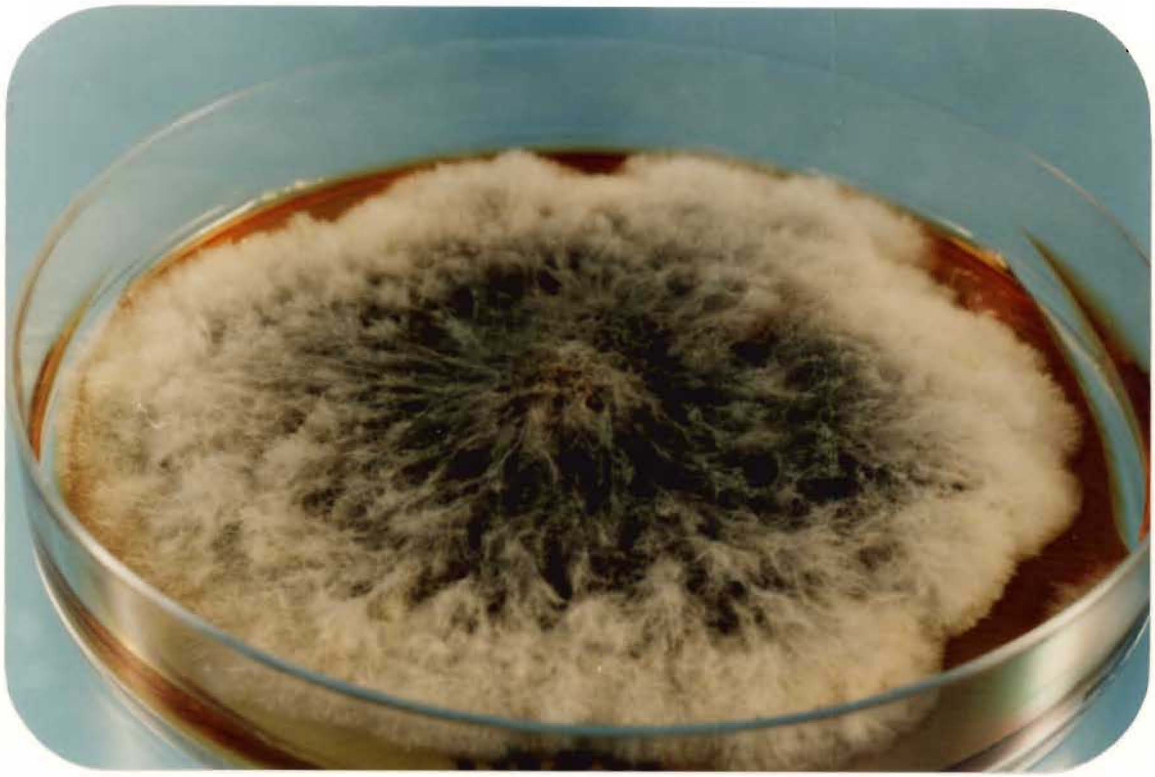


PLATE 9: GLIOMASTIX SP. 1080*

Supernatant from this species grown on B.subtilis cells exhibited low bacteriolytic activity in the higher pH range when assayed against bacterial cell walls.

*PDDCC catalogue number.

3.1.2 Bacteriolytic Activity Produced by *Gliomastix* Species

Gliomastix murorum produced E_1, E_2 and E_3 on *B.subtilis* liquid medium depending on pH (see 3.1, Plate 2, Table 1).

Gliomastix spp PDDCC 1071 and 3857 produced no detectable activity in *B.subtilis* liquid culture at pH5, 7, and 8.5, whilst 1080 produced some activity at all three pHs (Table 1).

None of the fungi investigated grew at pH3.3.

3.1.3 Types of *B.subtilis* Cell Degradation by *Gliomastix* Species

Two types of bacterial cell degradation occurred, when *Gliomastix* spp. were grown on *B.subtilis* cells in liquid culture. These were detected visually as a clearing of the initially turbid culture fluid in the culture flasks and were differentiated microscopically as:

(i) cytolysis, which resulted in empty cell walls, or "ghost" cells, the bacterial cytoplasm having been degraded and

(ii) bacteriolysis, in which a gradual disintegration of the entire bacterial cell was begun with an initial wall collapse, continued with the break-down of the wall into fragments, and completed with the final disappearance of microscopically detectable degradation products (Table 2).

3.2 pH and Temperature Optima for Assaying for Bacteriolytic Activity

3.2.1 pH:

The pH optima of culture supernatants were determined by quantitatively assaying over the pH range 2.7 - 9.1 using formate, acetate, MES, TES and TRIS buffers (see Appendix I) at an ionic strength of 0.05 (Long, 1961).

Culture supernatants were obtained from the fungus grown in *B.subtilis* liquid medium (agitated at 30°C, at both pH5 and pH7, and harvested on days 8 and 11 of

TABLE 2: Types of *B.subtilis* Cell Degradation by *Gliomastix* Species

Results were determined by microscopic examination of cell walls in the culture media.

SPECIES	GROWTH PH	FINAL PH	CYTOLYSIS	BACTERIOLYSIS
	5.0	5.8	–	+
G.murorum	7.0	7.3	+	+
	8.5	8.3	+	+
	5.0	5.8	–	+
*G.*sp 1080	7.0	7.5	–	+
	8.5	8.3	–	+
	5.0	5.5	–	–
*G.*sp 1071	7.0	7.3	+[a]	–
	8.5	8.4	+[a]	–
	5.0	5.5	–	–
*G.*sp 3857	7.0	7.4	+[a]	–
	8.5	8.5	+[a]	–

[a] cells remained in chains; – no degradation; + degradation

growth for each pH. It was noted that E_1 displayed linear, whilst E_2 exhibited non-linear, kinetics when quantitatively assayed (Fig. 7).

The pH optimum for E_1 was confirmed at 3.6; the pH optimum for E_2 was 7.8. (Fig. 8A and B). There was no demonstrable peak of activity for E_3.

3.2.2 Temperature:

Rates of Δ O.D.^{600nm} for E_1 were maximal at the maximum assay temperature tested of 35^{o}C with a decrease in activity at 30^{o}C and again at 25^{o}C (Fig. 9).

3.3 Determination of Growth Conditions for Production of Maximal Bacteriolytic Activity

As E_3 could not be detected by quantitative assay (3:2:1) the following investigations included E_1 and E_2 only.

3.3.1 Temperature

E_1 was most active when the fungus was grown at 30^{o}C and activity occurred earlier in cultures grown at 30^{o}C than at 25^{o}C. E_2 exhibited little change in activity between 30^{o}C and 25^{o}C (Fig. 10 A & B). At 16^{o}C activity was lower, and appeared later, than at 25^{o}C or 30^{o}C; at 35^{o}C neither mycelial growth nor enzyme production occurred at either pH.

3.3.2 Agitation

Agitation gave rise to a marked increase in activity of E_1 and some increase of E_2 (Fig. 10 A & B).

A comparison between reciprocal shaking (160 strokes minute^{-1}) and rotary shaking (110 r.p.m) of E_1 (25^{o}C, light, pH5) showed that greater activity occurred when using the former, as assessed by "Durham" tube assay.

The effect of increase in temperature plus agitation appeared to be synergistic for E_1; the activity when agitated at 30^{o}C was 5-fold that at 25^{o}C non-agitated. This experiment was repeated and confirmed.

Fig. 7: QUANTITATIVE ASSAY OF BACTERIOLYTIC ACTIVITY

The drop in OD^{600nm} is measured over time intervals under standard conditions, i.e. 250 µl buffer (pH 3.8 formate for E_1; pH 7.5 Tris for E_2), 60 µl cell wall suspension (10 mg ml^{-1}), and 100 µl fungal culture supernatant, all incubated at 30°C.

The enzyme optimal at pH 7.5 displays non-linear kinetics.

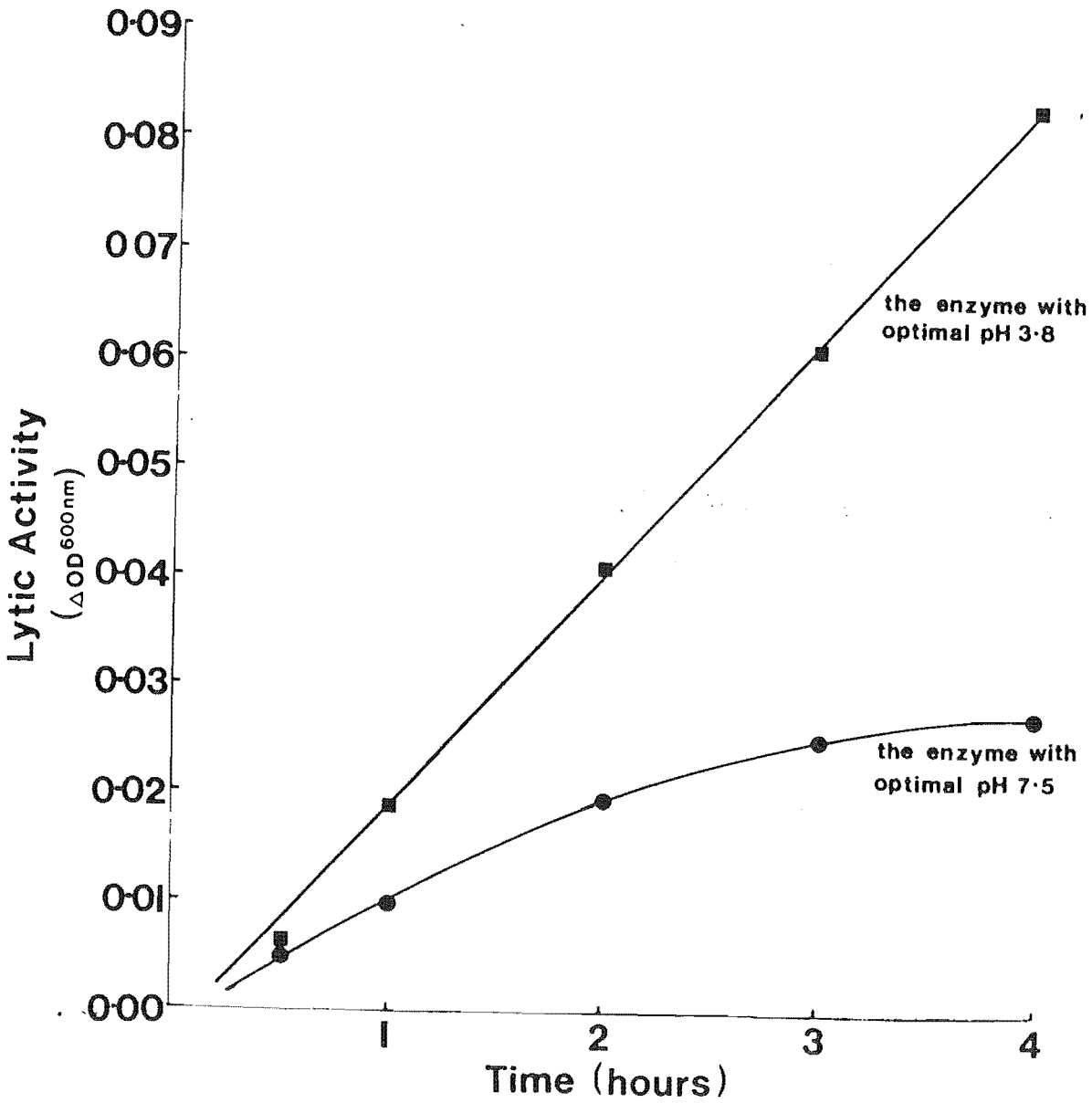
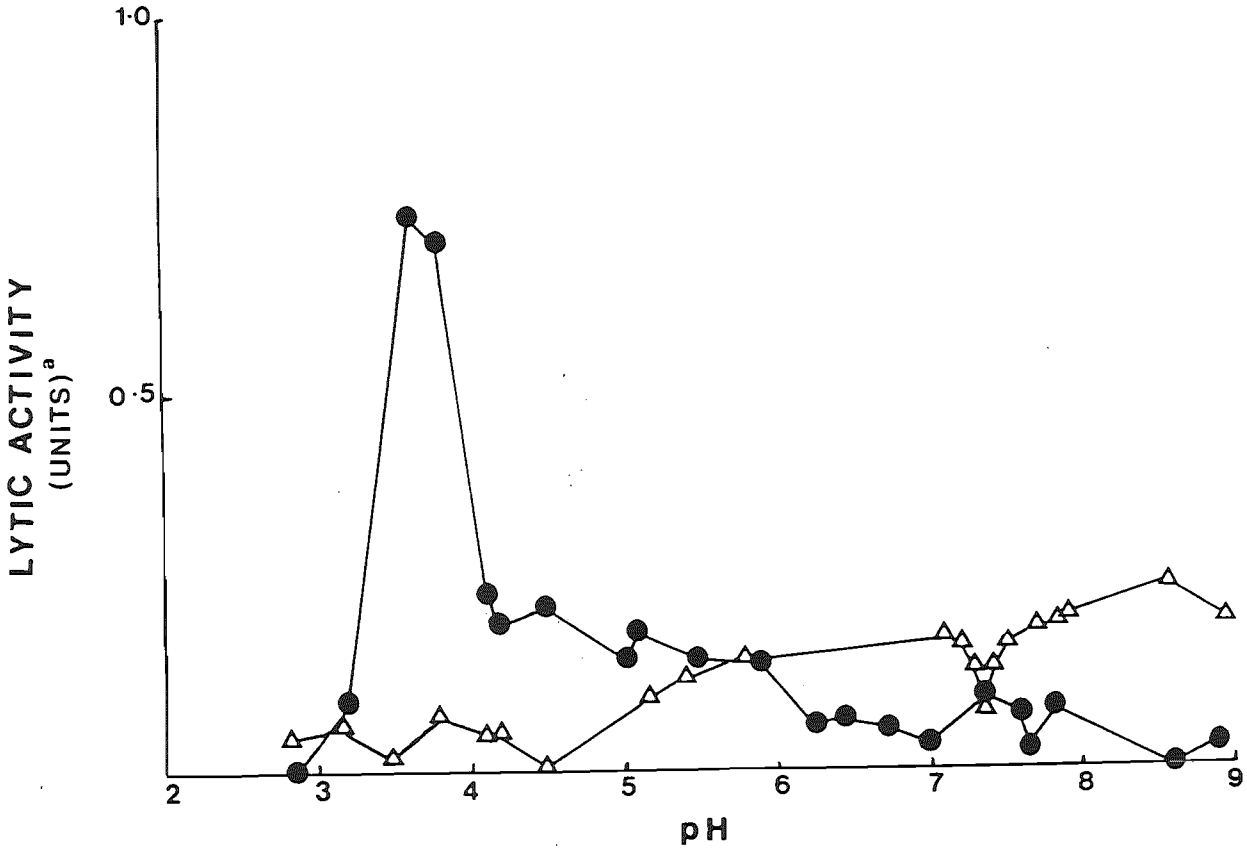


Fig. 8A: DETERMINATION OF THE pH OPTIMUM FOR E_1 [●] AND E_2 [△]

Culture supernatants (day 8) were assayed quantitatively in a range of buffers of constant ionic strength (I = 0.05). pH 2.7 - 4.0 were formate, pH 4.4 - 5.8 were acetate and pH 7.0 -9.1 were Tris buffers.



[a] One unit is defined as the change in OD^{600nm} per hour x10 under condition of the standard assay (2:4:2).

Fig. 8B: DETERMINATION OF THE pH OPTIMUM FOR E_1 (●) AND E_2 (△)

Culture supernatants (day 11) were assayed quantitatively in a range of buffers of constant ionic strength (I = 0.05). pH 2.7 - 4.0 were formate, pH 4.4 - 5.8 were acetate and pH 7.0 - 9.1 were Tris buffers.

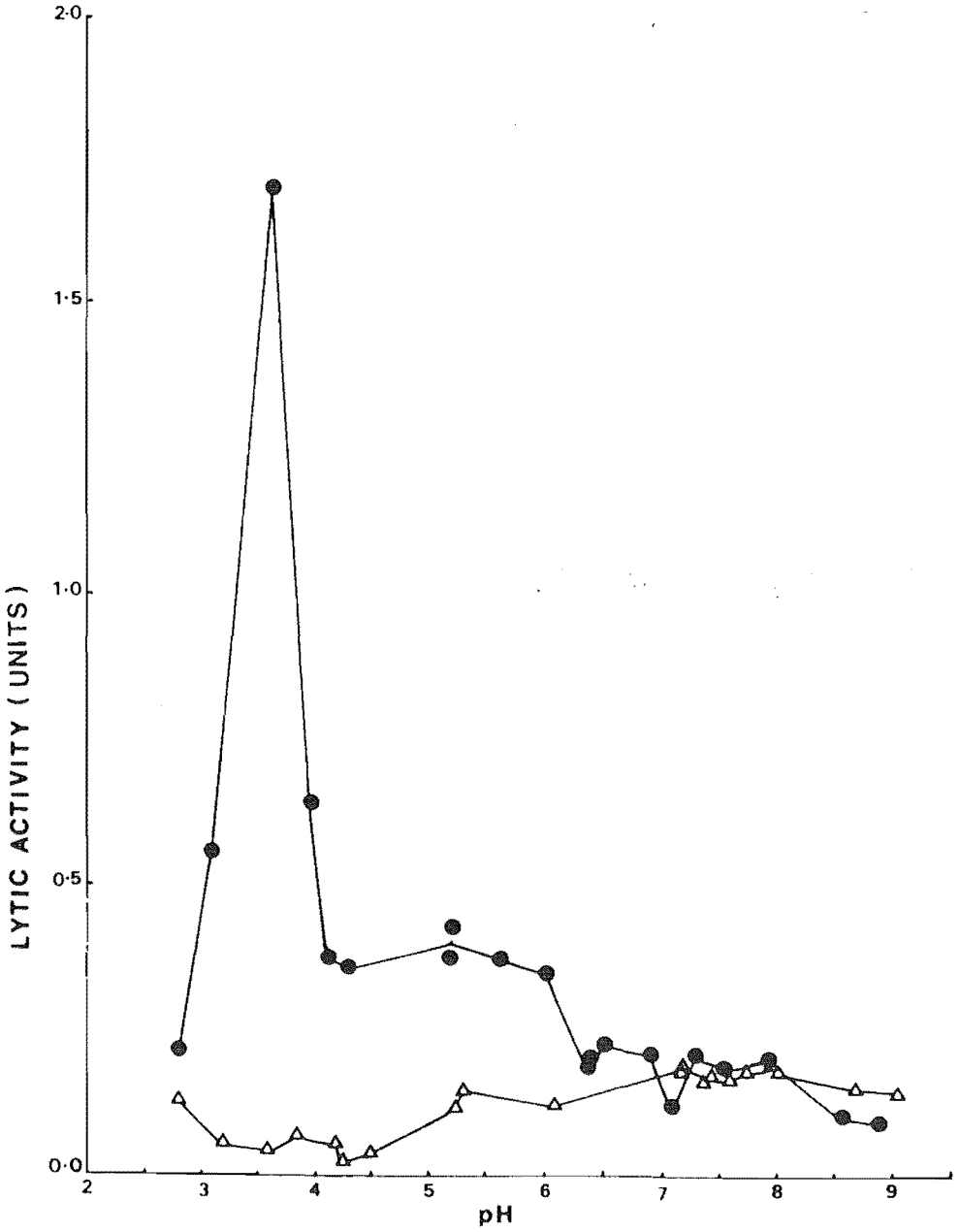


Fig. 9: EFFECT OF TEMPERATURE ON QUANTITATIVE BACTERIOLYTIC ASSAY OF E_1

Time course of lysis of cell walls carried out under otherwise standard conditions (see 2.4.2) at 25°C (◇), 30°C (◆) and 35°C (△).

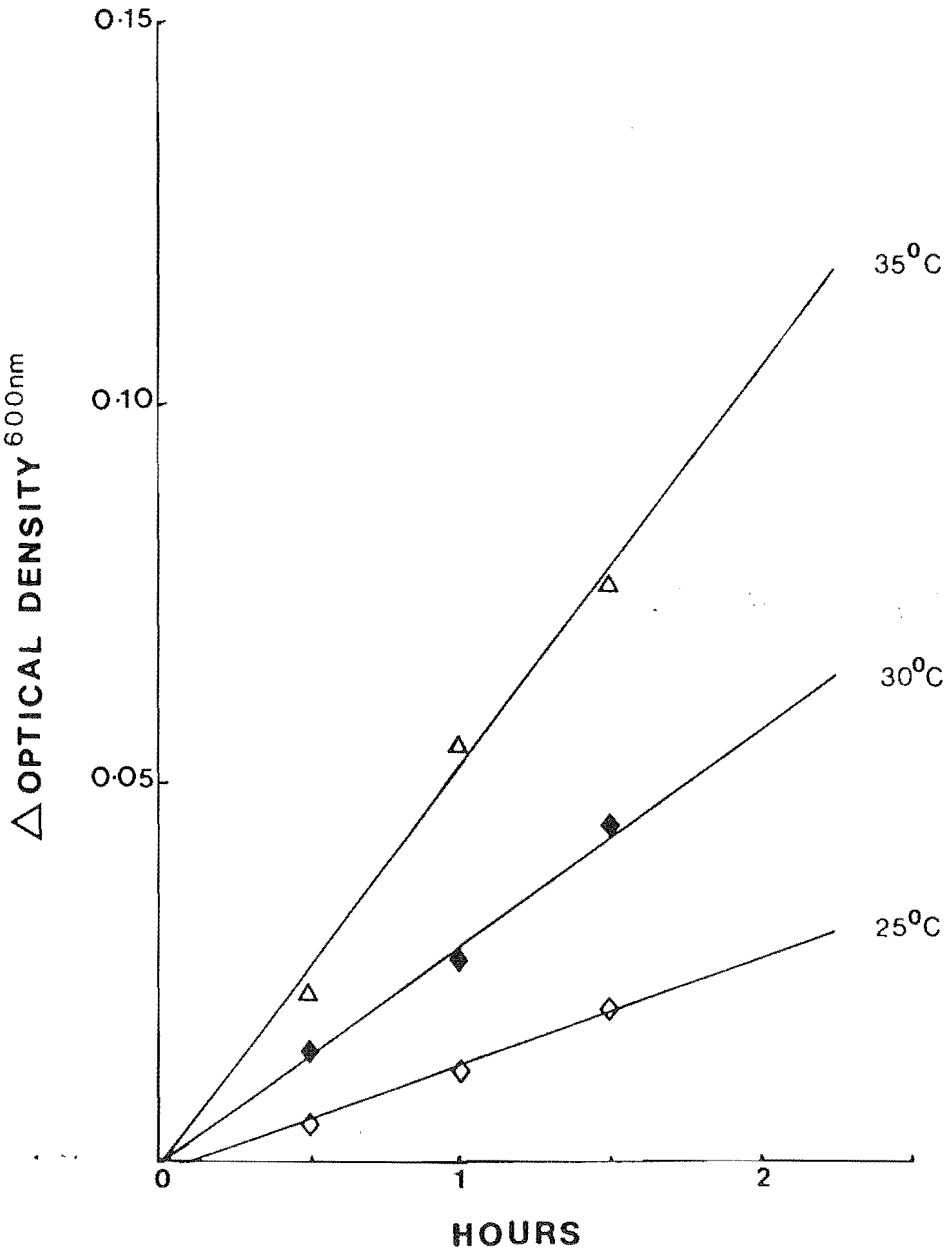
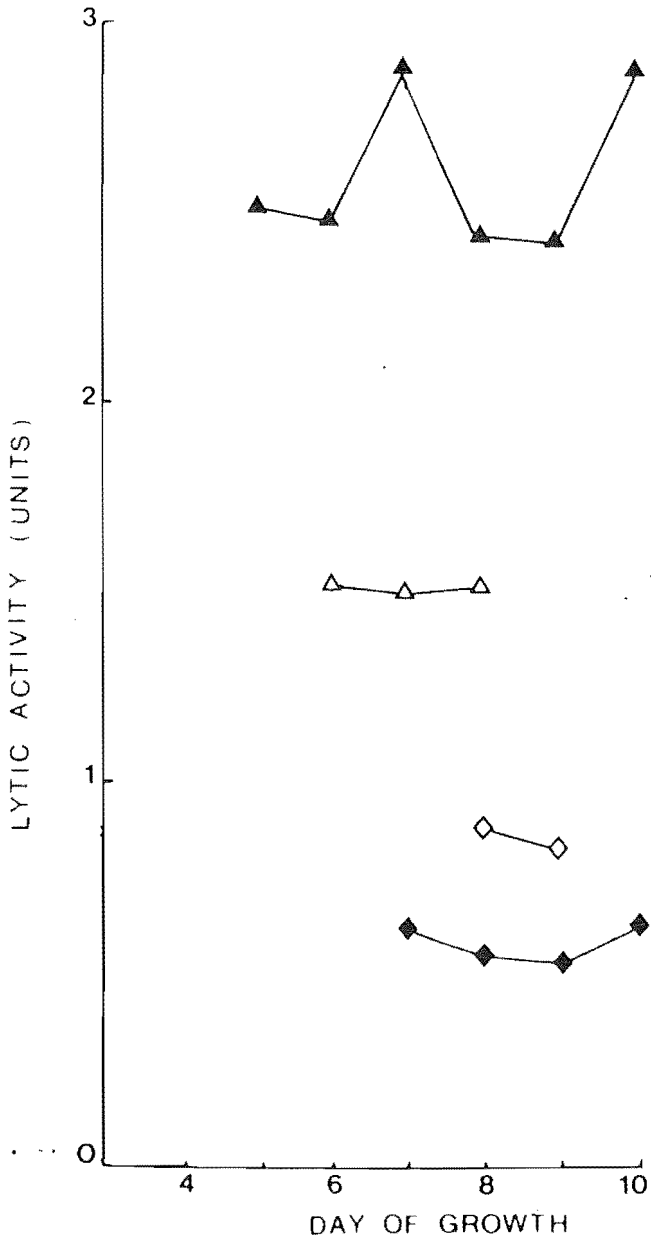


Fig. 10A: ACTIVITY OF E_1 WHEN PRODUCED UNDER VARIOUS GROWTH CONDITIONS OVER A 10-DAY GROWTH PERIOD

Supernatants from the entire growth period for each set of conditions were initially assayed semiquantitatively, then quantitative assays carried out on the days of maximal production indicated.

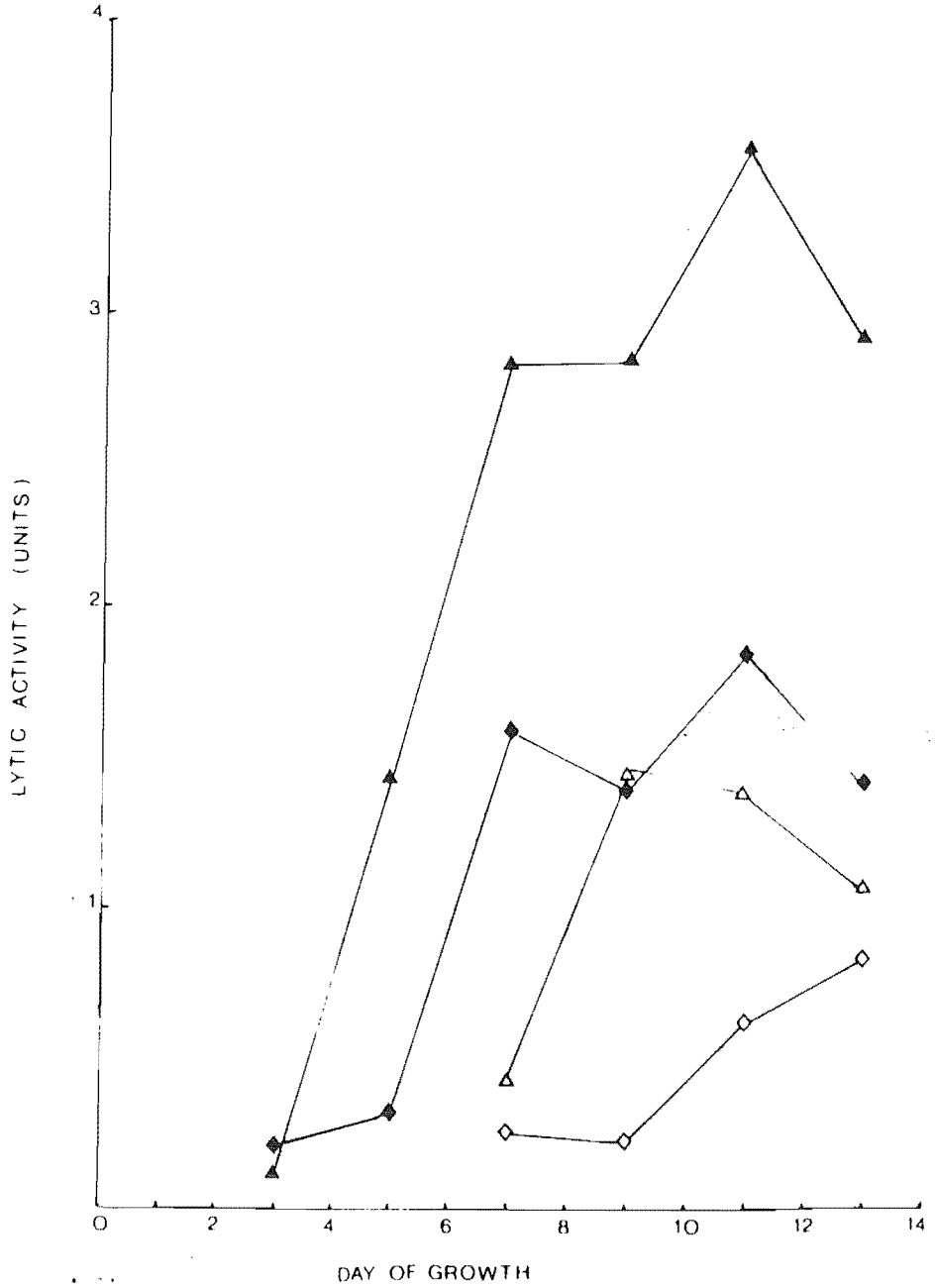


Growth conditions (all at pH5 with B.subtilis substrate concentration of 2.5 mg ml^{-1}):

- ▲ 30°C, agitated
- △ 30°C, non-agitated
- ◆ 25°C, agitated
- ◇ 25°C, non-agitated

Fig. 10B: ACTIVITY OF E_1 WHEN PRODUCED UNDER VARIOUS GROWTH CONDITIONS OVER A 10-DAY GROWTH PERIOD (repeat of A)

Supernatants from the entire growth period for each set of conditions were initially assayed semiquantitatively, then quantitative assays carried out on the days of maximal production indicated.



Growth conditions (all at pH5 with B.subtilis concentration of 2.5 mg ml^{-1}).

▲ 30°C, agitated
△ 30°C, non-agitated
◆ 25°C, agitated
◇ 25°C, non-agitated

3.3.3 Matrix

When Ballotini beads were included in the B.subtilis liquid medium, in an effort to more closely replicate the particulate soil environment, activity was reduced (as indicated by "Durham" tube assay).

3.3.4 Light

When the fungus was grown in continuous light it produced twice the activity of E_1 than was produced under the same conditions in constant darkness. E_2 was not investigated (Fig. 11).

3.3.5 Substrate Concentration

The fungus was grown on a range of B.subtilis cell concentrations (0.5, 1.0, 2.0, 2.5, 5.0 and 10.0 mg ml^{-1}). The optimal concentration for E_1 production was 2.5 mg mg^{-1} (Fig. 12). Maximal production of E_2 occurred at a substrate concentration of 5.0 mg ml^{-1}, although the differences in activity between the various concentrations were slight (Fig. 13).

At 10 mg ml^{-1}, E_1 was active at day 6 of growth, but by day 8 activity had declined markedly, suggesting the presence of inhibitory substances or proteases. No such decline occurred for E_2 at any of the substrate concantrations.

3.3.6 Production of Fungal Inoculum

When Gliomastix was maintained on a B.subtilis agar medium prior to inoculation into liquid B.subtilis medium (pH5) a rate of 4.46 units was obtained for maximal E_1 activity as opposed to 1.76 and 2.94 when maintenance prior to inoculation was on 1/6th strength Czapek dox (Cz.dx) and MEA respectively (Fig. 14).

Liquid culture supernatants inoculated from B.subtilis plates several weeks later (after several transfers) showed a marked decline in activity. A variety of regimes for inoculum production were tried to assess the most productive:

Fig. 11: EFFECT OF LIGHT ON PRODUCTION OF E_1 BY GLIOMASTIX

Time courses of lysis of bacterial cell walls by supernatants of Gliomastix grown in the dark [△] or in the light [▲].

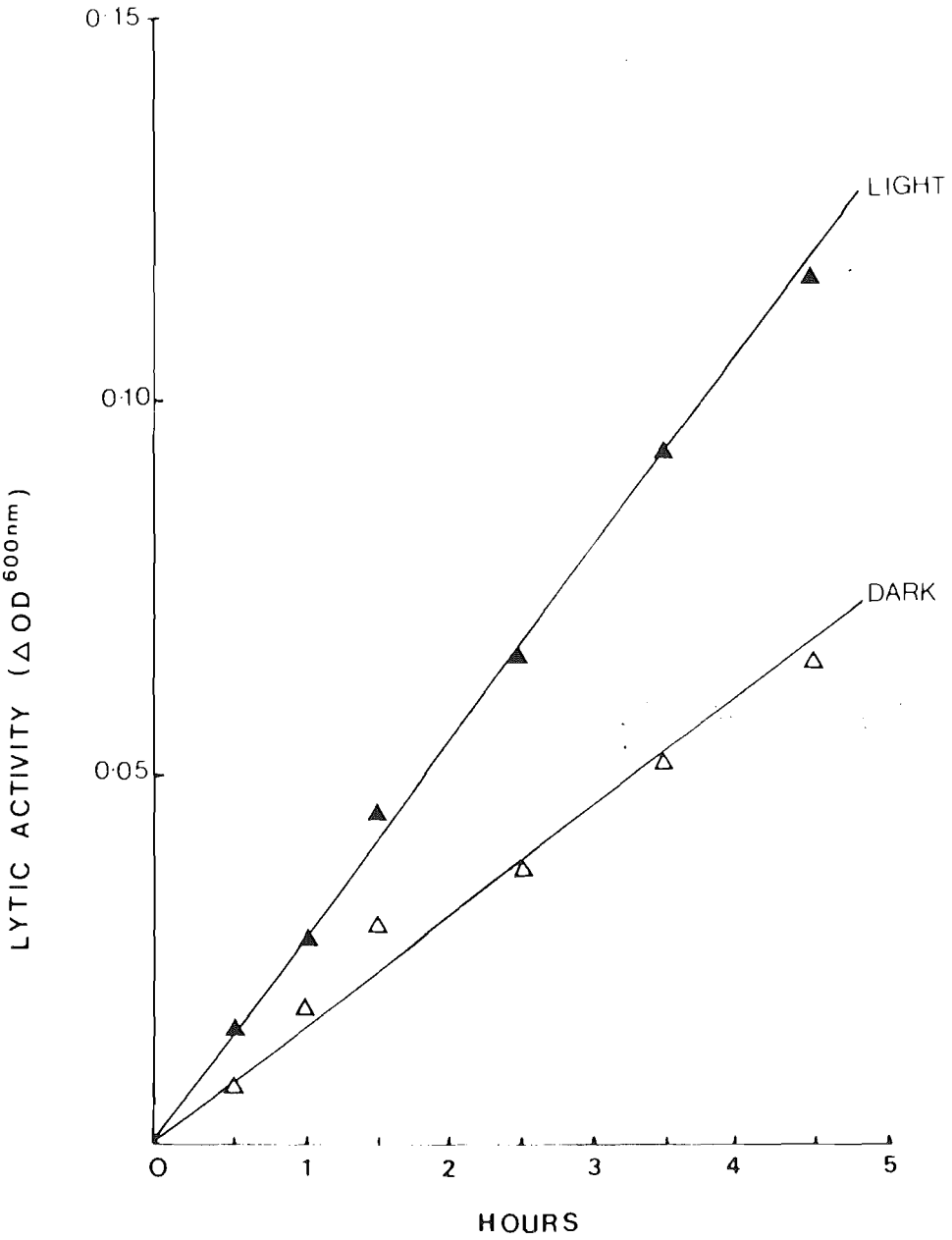
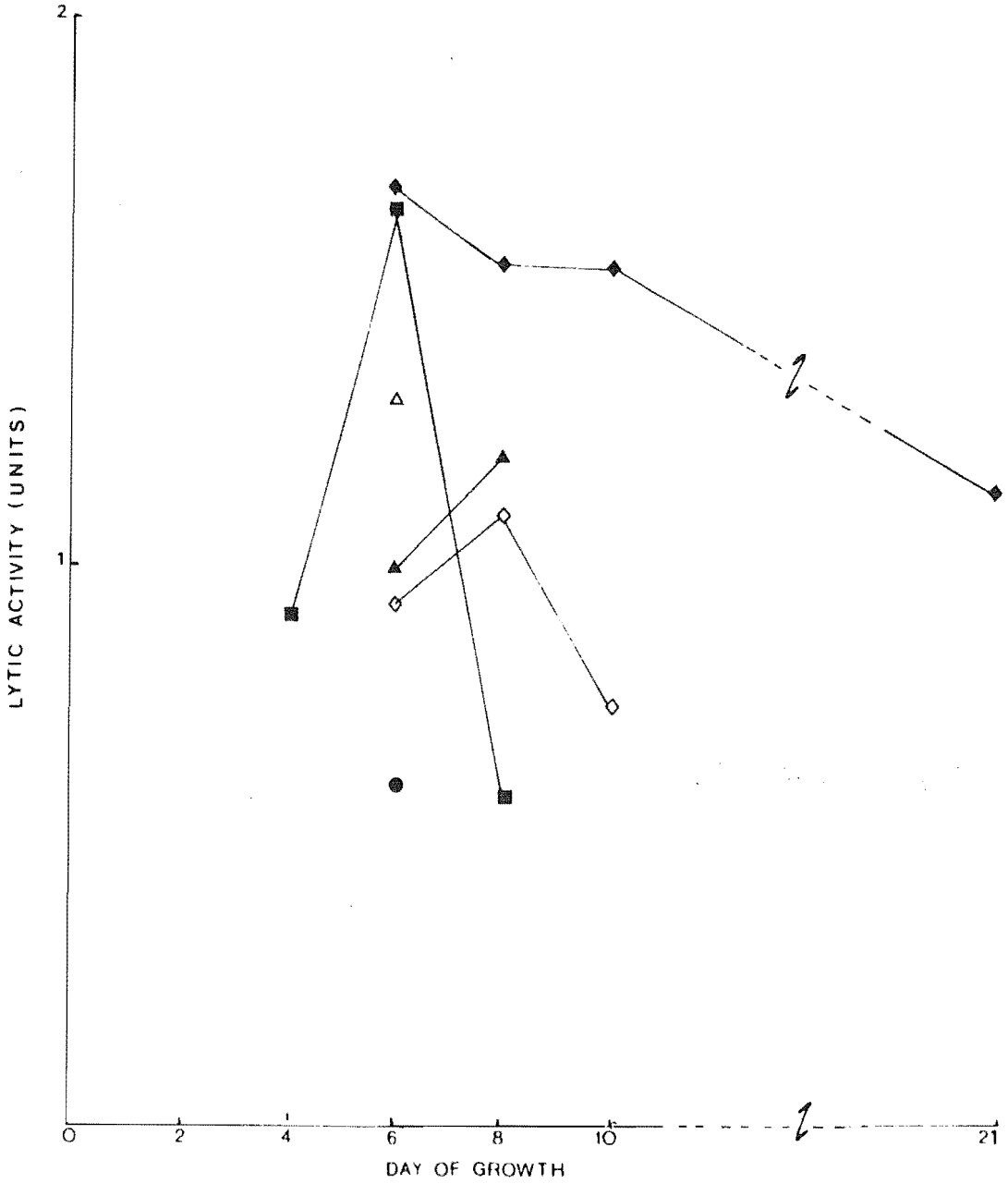


Fig. 12: DETERMINATION OF OPTIMAL B.SUBTILIS CONCENTRATION FOR MAXIMUM E_1 PRODUCTION BY GLIOMASTIX

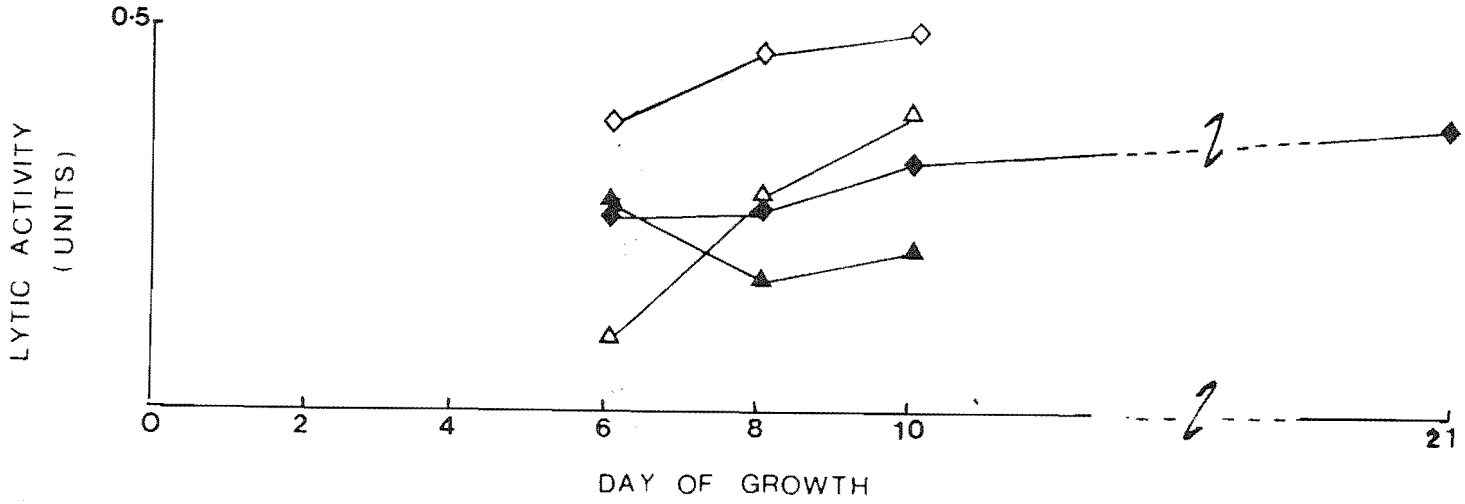
Semiquantitative assays of all samples (taken every 2 days) allowed selection of supernatants with greatest activity. These were then assayed quantitatively.



● B.subtilis conc. of 0.5 mg ml^{-1}
▲ " " " 1.0 " "
△ " " " 2.0 " "
◆ " " " 2.5 " "
◇ " " " 5.0 " "
■ " " " 10.0 " "

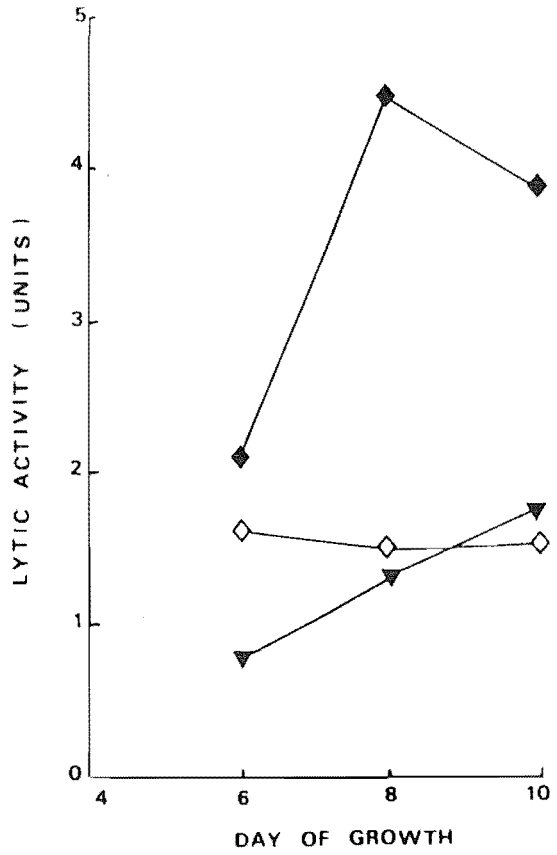
Fig. 13: DETERMINATION OF OPTIMAL B.SUBTILIS CONCENTRATION FOR MAXIMUM E_2 PRODUCTION BY GLIOMASTIX

Semiquantitative assays of all samples (taken every 2 days) allowed selection of supernatants with greatest activity. These were then assayed quantitatively.



▲ B.subtilis conc. of 1.0 mg ml^{-1}
△ " " " 2.0 " "
◆ " " " 2.5 " "
◇ " " " 5.0 " "

Fig. 14: INVESTIGATION OF GROWTH CONDITIONS OF GLIOMASTIX INOCULUM TO OBTAIN MAXIMUM E_1 PRODUCTION



◆ Fungus grown on B.subtilis agar prior to growth in liquid B.subtilis medium
◇ Fungus grown on Malt extract agar prior to growth in liquid B.subtilis medium
▼ Fungus grown on Czapek Dox agar prior to growth in liquid B.subtilis medium

(i) Grown (1 to 2 weeks) on MEA - B.subtilis (1 week) - B.subtilis liquid culture

(ii) Grown (1 to 2 weeks) on NB - B.subtilis (1 week) - B.subtilis liquid culture

(iii) Grown (1 to 2 weeks) on 1/6 Cz.dx - B.subtilis (1 week)-B.subtilis liquid culture

(iv) Grown (1 to 2 weeks) on MEA - B.subtilis liquid culture

(v) Grown (1 to 2 weeks) on B.subtilis - B.subtilis liquid culture.

(Fig. 15)

As maximum activity was still lower than that obtained initially, cryopreserved mycelium from the original isolate was cultured on MEA, sub-cultured for one week on B.subtilis and used as the inoculum for liquid cultures in subsequent experiments (Fig. 16).

3.4 Growth Experiments to Determine Specific Activity of E_1 and E_2

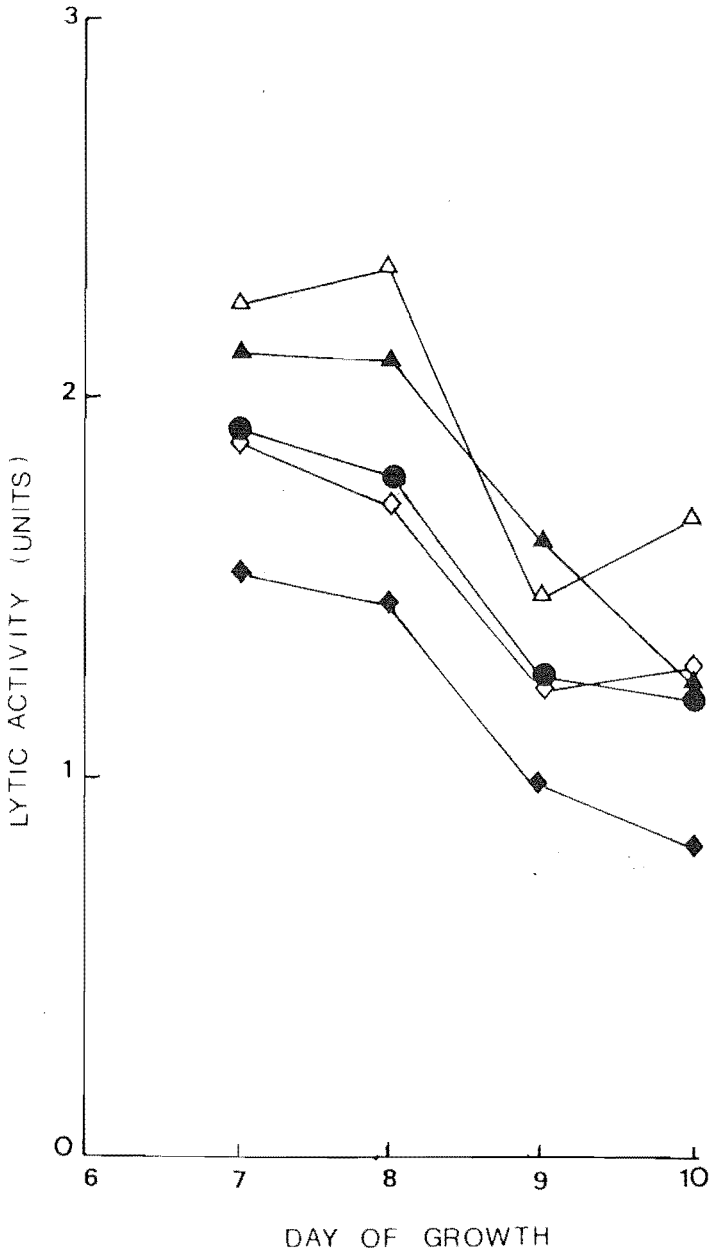
3.4.1 Ergosterol as a Measure of Fungal Biomass

Erogsterol was produced by Gliomastix, as shown by thin layer chromatography of mycelial extract on a silica gel plate (see procedure 2.4.4). The extract, run with an ergosterol standard and examined under ultra violet light, had an R_F of 0.31 (Fig. 17).

3.4.2 Measurement of Growth on Glucose Substrate

Gliomastix was grown in 25 ml glucose medium (1% final concentration) under optimal conditions and duplicate flasks (250 ml Ehrlenmeyer) were harvested daily. Dry mycelial weights were obtained following harvesting and freeze-drying of the fungus, and then ergosterol was extracted from the mycelium and analysed by HPLC (see 2.4.4) (Table 3).

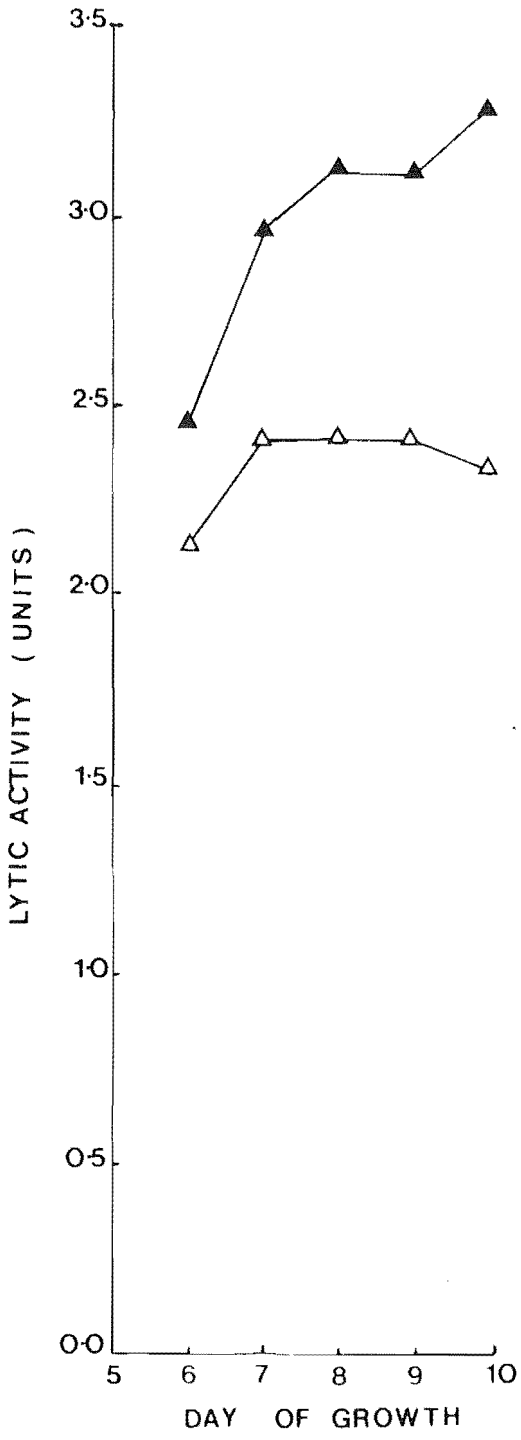
Fig. 15: OPTIMISATION OF GROWTH CONDITIONS OF GLIOMASTIX INOCULUM TO OBTAIN MAXIMUM E_1 PRODUCTION



△ Fungus grown on Malt extract agar 1 wk then B.subtilis agar 1 wk prior to use as inoculum
● Fungus grown on Nutrient broth 1 wk then B.subtilis agar 1 wk prior to use as inoculum
▲ Fungus grown on Czapek Dox agar 1 wk then B.subtilis agar 1 wk prior to use as inoculum
◇ Fungus grown on malt extract agar 1 wk prior to use as inoculum
◆ Fungus grown on B.subtilis agar 1 wk prior to use as inoculum

Fig. 16: PRODUCTION OF E_1 USING ORIGINAL ISOLATE OF GLIOMASTIX (FOLLOWING CRYOPRESERVATION)

Fungus was grown on Malt extract agar for 1 wk then B.subtilis agar for 1 wk prior to use as inoculum.



▲ Assay pH 3.4
△ Assay pH 3.6

Fig. 17: CHROMATOGRAM OF MYCELIAL EXTRACT* FROM GLIOMASTIX AND SCHIZOPHYLLUM COMMUNE RUN ALONGSIDE ERGOSTEROL STANDARDS

Fungal mycelium was harvested from 100 ml Sabarouds medium (day 7) and freeze-dried. The ergosterol was then extracted in methanol*, rotary evaporated to dryness and dissolved in 1 ml methanol. Thin layer chromatography on a silica gel plate was carried out with 20 µl of each mycelial extract and 10 µl and 20 µl of erogsterol standard (1 mg ml^{-1}, ergosterol being dissolved in methanol). The solvent used was diethyl ether and cyclahexane, 1:1, and the plate was examined under UV light.

*ref: Grant & West, 1986.

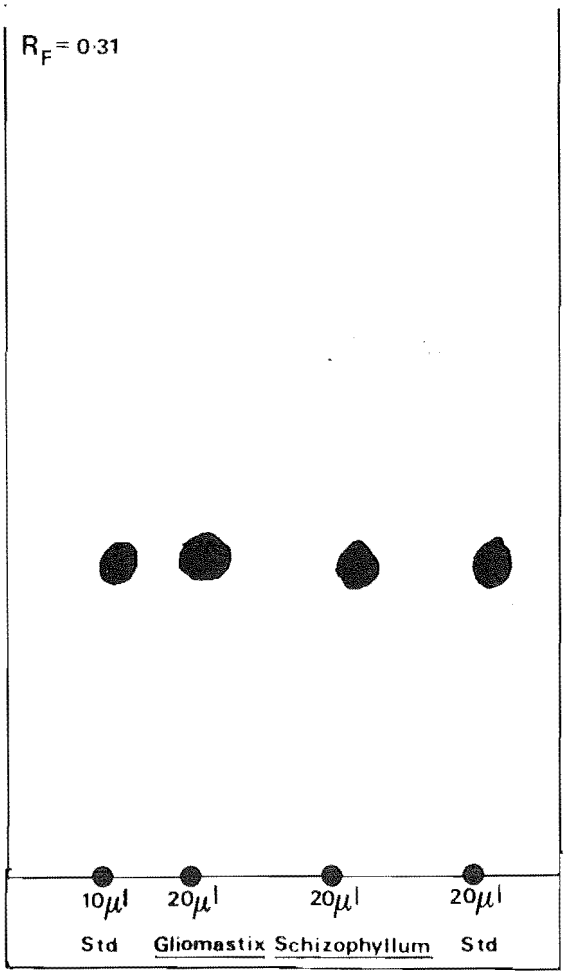


TABLE 3: Measurement of Growth of *Gliomastix* on Glucose

Culture flasks each contained 25 ml glucose medium (1% final conc.).

DAY OF GROWTH	MYCELIAL DRY WT (mg)	ERGOSTEROL (μg)
2	4.3	32.60
	4.3	20.40
3	13.4	63.10
	13.3	41.45
4	51.8	184.30
	73.7	251.55
5	115.5	287.25
	72.2	149.75
6	127.3	279.75
	98.0	302.20
7	214.6	503.90
	197.1	512.55

The linear regression equation, $y = \beta_0 + \beta_1 x$
was calculated from the known data (using the method of least squares) as
$y = 28.055 + 2.33x$

Thus (mycelial dry wt) $= \frac{y - 28.055}{2.33}$

(where y = estimated dependent variable
β_0 = y intercept
β_1 = slope of straight line and
x = independent variable)

The regression coefficient was 0.9807.

3.4.3 Measurement of Growth on B.subtilis Medium, pH5

Gliomastix was grown on 25 ml B.subtilis liquid medium (pH5) (in 250 ml flasks) and duplicates harvested daily. Dry weights of mycelium plus insoluble substrate were obtained following harvesting and freeze-drying and then ergosterol was extracted and analysed using HPLC (as for 3.4.2).

Using the linear regression equation calculated previously (3.4.2) estimated dry mycelial weights were obtained. These estimated weights were, from the 4th day of growth, higher than the actual dry weights obtained from mycelium plus bacterial substrate (Table 4).

3.4.4 Measurements of Growth on B.subtilis, pH7

As for 3.4.3.

Estimated dry weights were again (from the 4th day of growth) higher than actual dry weights obtained from mycelium plus bacterial substrate (Table 5).

3.4.5 Bacteriolytic Activity per Ergosterol (µg)

Bacteriolytic activity was quantitatively measured for each culture harvested (see 3.4.2, 3 + 4) and then related to ergosterol measurements.

TABLE 4: Measurement of Growth of Gliomastix on *B.subtilis* Cells, pH 5

Culture flasks each contained 25 ml *B.subtilis* (2.5 mg ml^{-1}) liquid medium.

DAY OF GROWTH	MYCELIAL DRY WT (mg)+	ERGOSTEROL (µg)	ESTIMATED MYCELIAL DRY WT (mg)#
2	48.9	31.54	1.50
3	39.5	61.68	14.46
4	40.4	82.85	23.56
"	35.3	118.91	39.06
5	38.7	119.44	39.29
"	31.3	147.92	51.53
6	35.4	129.68	43.69
"	28.9	130.57	44.08
7	31.3	170.33	61.17
8	28.7	159.38	56.46
"	26.0	160.60	56.99
9	28.5	199.25	73.60
"	27.4	138.39	47.44
10	24.0	160.89	57.11
"	24.6	134.07	45.57
11	23.8	96.19	29.29

+ including bacterial substrate

calculated using linear regression equation

TABLE 5: Measurement of Growth of *Gliomastix* on *B.subtilis* Cells, pH7

Culture flasks each contained 25 ml *B.subtilis* (2.5 mg ml^{-1}) liquid medium.

DAY OF GROWTH	MYCELIAL DRY WT (mg)+	ERGOSTEROL (µg)	ESTIMATED MYCELIAL DRY WT (mg)#
2	31.9	5.90	-9.51
"	30.6	5.90	-9.51
3	28.2	75.40	20.32
4	28.5	136.40	46.50
"	35.6	156.70	55.21
5	30.3	120.30	39.59
"	40.9	152.55	53.43
6	102.1	191.15	70.00
"	29.8	105.10	33.07
7	26.0	136.90	46.72
"	28.8	147.80	51.39
8	29.2	145.95	50.60
"	29.1	129.30	43.45
9	33.1	152.25	53.30
"	31.6	151.15	52.83
11	28.1	147.65	51.33
12	39.3	167.30	59.76
"	68.0	160.50	56.84

\+ including bacterial substrate

\# calculated using linear regression equation

Activity was not expressed in terms of dry mycelial weight (mg) because of the discrepancies noted between estimated and actual dry weights (3.4.3 and 4) (Figs. 18, 19 & 20).

3.5 Constitutive Nature of E_1

Gliomastix was grown on glucose-based media (pH5) under various environmental conditions and culture supernatants were assayed for bacteriolytic activity. Where activity was detected it was very low when compared with the B.subtilis grown controls (Table 6, Fig. 21).

A maximum dry mycelial weight of 214.6 mg was recorded for the fungus grown on glucose on day 7 (Table 3) whereas the combined dry mycelial plus residual substrate weight for the B.subtilis grown fungus (day 7) was 31.3 mg (Table 4). This indicated that the specific activity for the bacterial-grown cultures was very much higher than appeared.

3.5.1 Effect of Glucose on Previously Induced Bacteriolytic Activity

Gliomastix was grown on B.subtilis cells under optimal growth conditions and 2.5% sterile glucose (final concentration) added aseptically to growing cultures (i.e. 3 ml of 20% glucose) on days 3, 4 and 7 of growth. Water (3 ml) was added to a control flask on day 3. Culture supernatant (0.4 ml) was sampled daily until day 11 and assayed quantitatively.

Results indicate a decrease in bacteriolytic activity following addition of glucose to the culture supernatants (Fig. 22).

3.6 The E_3 Enzyme

Bacteriolytic activity was detected at both pH3.8 and pH8.0, but not at pH6.0 in the initial semiquantitative assays carried out on supernatant from liquid G.murorum cultures grown on B.subtilis cells at pH5. (The inoculum for these cultures was taken from an MEA plate). The results suggested that two enzymes, one active at an acid and one at a neutral to alkaline pH, were present, but this was not substantiated by a quantitative assay. One month later the experiment was repeated with activity only being detected at pH3.8. (Table 7).

Fig. 18: MYCELIAL DRY WEIGHT AND ERGOSTEROL CONTENT OF, AND BACTERIOLYTIC ENZYME PRODUCTION BY, GLIOMASTIX GROWN ON GLUCOSE AT pH5

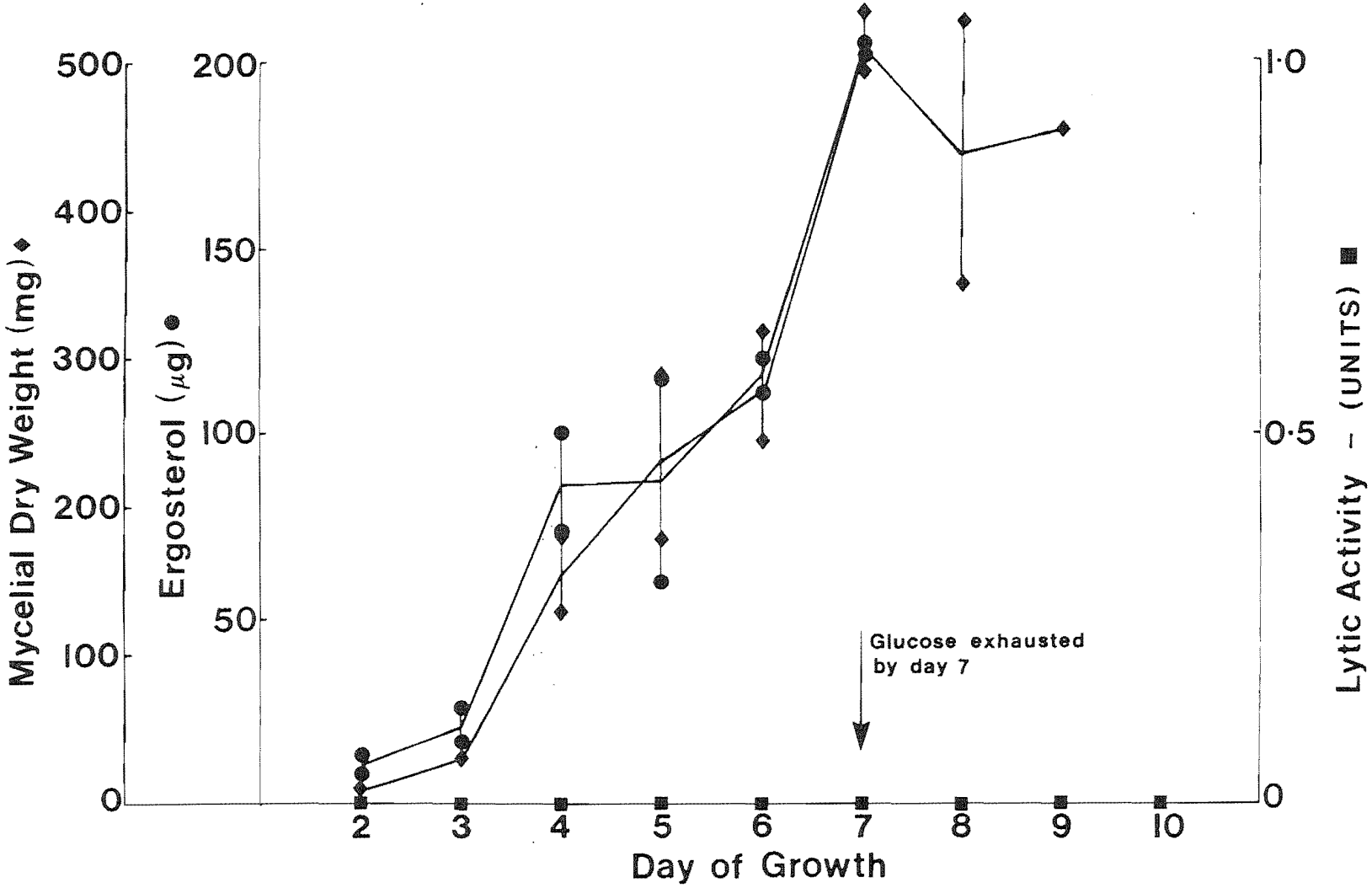


Fig. 19: **GROWTH OF, AND BACTERIOLYTIC ENZYME PRODUCTION BY, <u>GLIOMASTIX</u> ON BACTERIAL CELLS AT pH5**

Cultures were harvested daily in duplicate, mycelial dry weights obtained and ergosterol content extracted. Supernatants were brought to 30 ml (dist. water) and assayed for bacteriolytic activity.

Growth was estimated by measurement of the ergosterol content of the biomass (difficulties encountered are outlined in the Discussion).

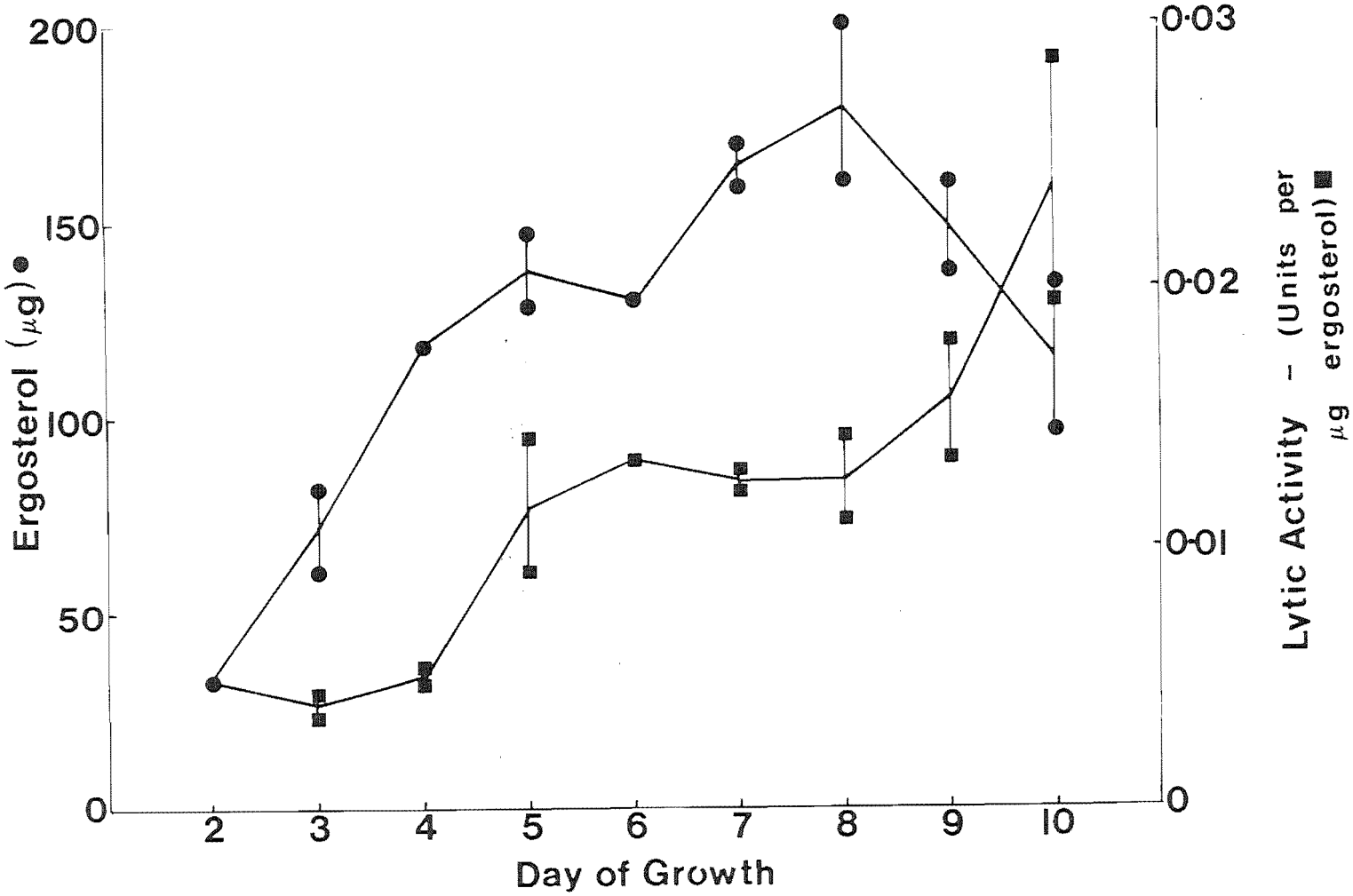


Fig. 20: **GROWTH OF, AND BACTERIOLYTIC ENZYME PRODUCTION BY, <u>GLIOMASTIX</u> ON BACTERIAL CELLS AT pH7**

Cultures were harvested daily in duplicate, mycelial dry weights obtained and ergosterol content extracted. Supernatants were brought to 30 ml (dist. water) and assayed for bacteriolytic activity.

Growth was estimated by measurement of the ergosterol content of the biomass (difficulties encountered are outlined in the Discussion).

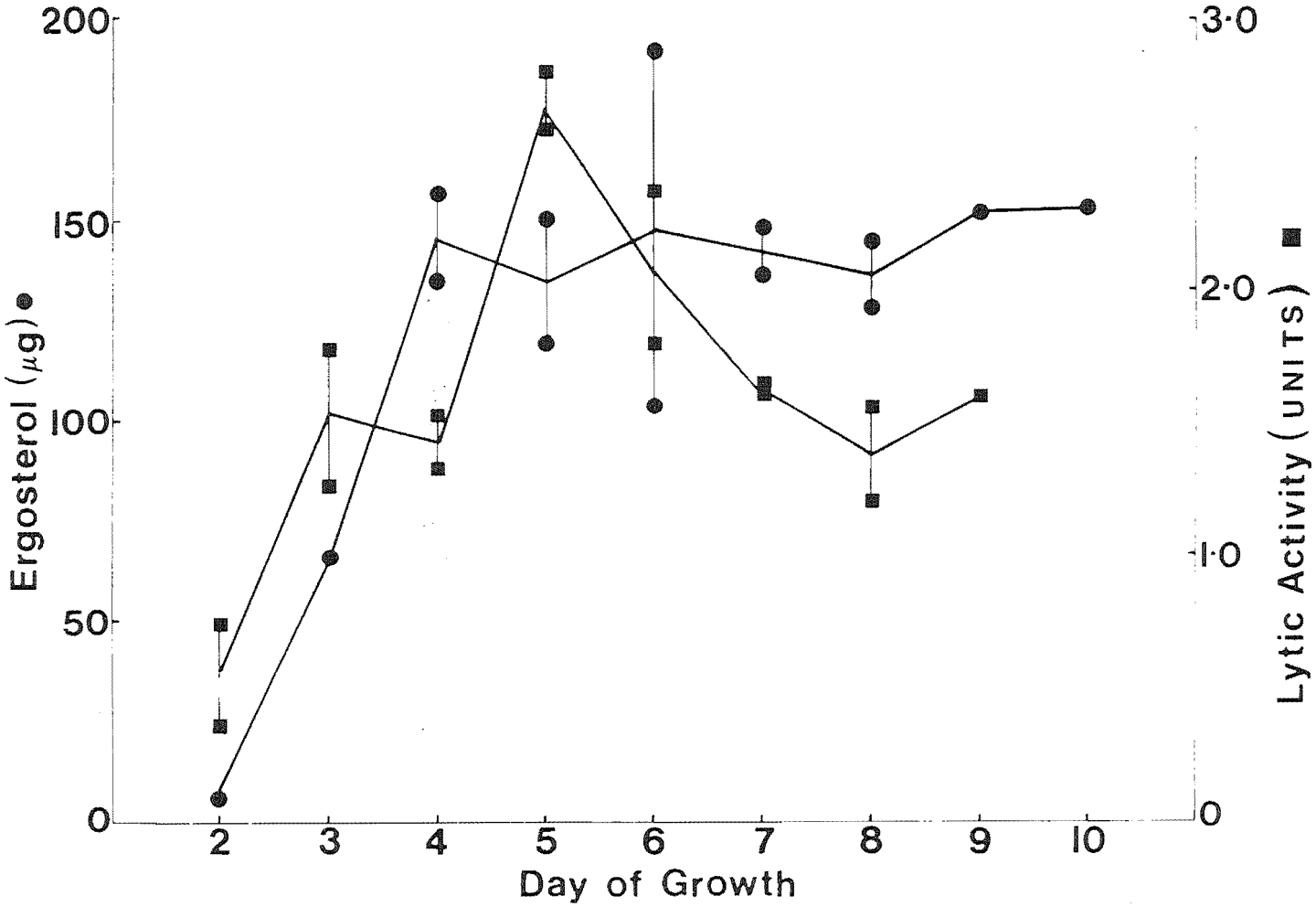


TABLE 6: Production of Bacteriolytic Activity During Growth of *Gliomastix* on Different Concentrations of Glucose

Activity in 20 ml culture supernatants was measured by Durham tube assay of 0.2 ml samples.

DAY OF GROWTH	STATIONARY CULTURES 25°C			AGITATED CULTURES 30°C			
	2% GLUCOSE	0.5% GLUCOSE	CONTROL[a]	2% GLUCOSE	1% GLUCOSE	0.2% GLUCOSE	CONTROL[a]
3	–	–	–	–	–	–	–
5	–	–	+	–	–	–[b]	+++
7	–	–[b]	+++	–	–[b]	–	+++
9	–	–	+++	+	–	–	+++
11	–	–	+++	+[b]	–	+	+++
14	–	–	+++	++			+++

[a] glucose substrate replaced by *B.subtilis* cells (2.5 mg mg^{-1})

[b] glucose exhaustion, determined by glucose assay

+++ total clearing of cell wall suspensions by culture supernatants in 24 h

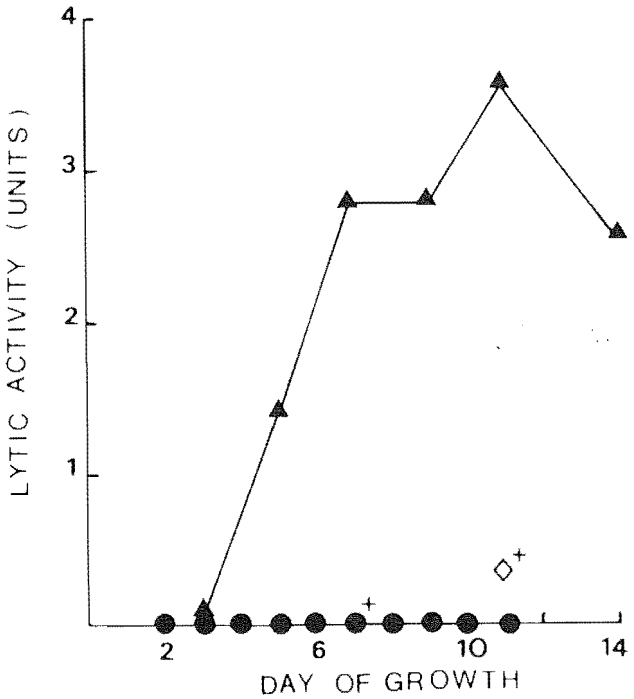
++ >50% clearing (as above)

+ <50% clearing (as above)

– no activity

Fig. 21: BACTERIOLYTIC ACTIVITY OF GLIOMASTIX GROWN ON GLUCOSE

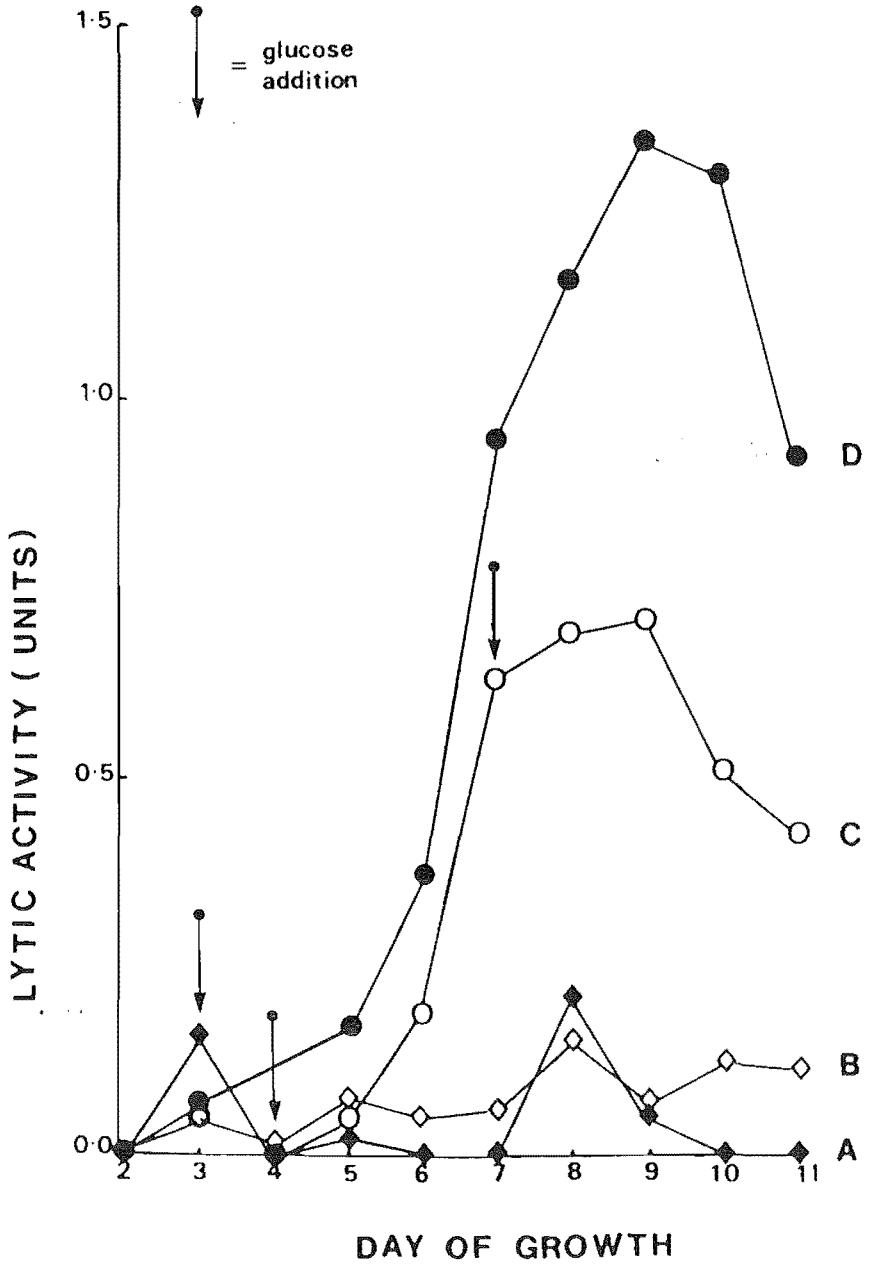
Bacteriolytic activity was measured quantitatively.



▲ Supernatant from Gliomastix grown under optimal conditions on B.subtilis medium, pH5.
● Supernatant from Gliomastix grown under optimal conditions on 1% glucose medium, pH5.
◇ Supernatant from Gliomastix grown under optimal conditions on 2% glucose medium, pH5.
+ glucose exhaustion.

Fig. 22: EFFECT OF GLUCOSE ADDITION ON GLIOMASTIX CULTURES PRODUCING BACTERIOLYTIC ACTIVITY

Four cultures were grown on B.subtilis medium. Glucose to 3% final concentration was added to the individual cultures on day 3 (A, ◆), day 4 (B, ◇) and day 7 (C, O) of growth. A control (D, ●) had no glucose addition. Lytic activity of culture supernatants was measured daily.



ABLE 7: Determination of E_3 Activity

acteriolytic activity was estimated by Durham tube assay.
ungal supernatant was obtained for assaying from B.subtilis liquid cultures noculated from (A) MEA plates of the original G.murorum isolate; (B) MEA plates ifter several transfers; (C) B.subtilis plates; (B) Soil agar plates.

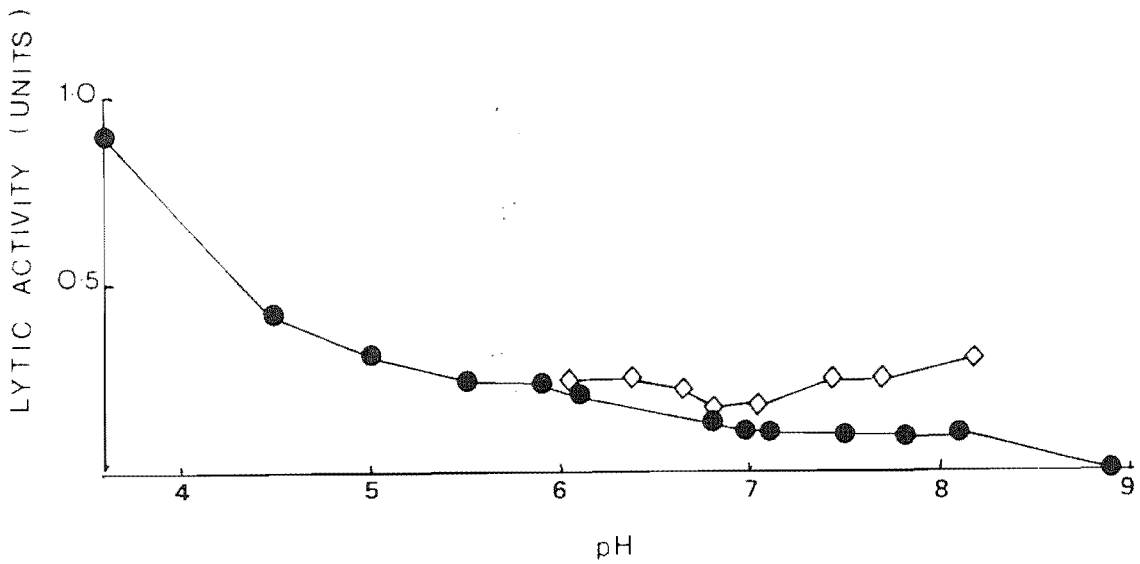
)AY OF	ASSAY pH 3.8				6.0				8.0			
;ROWTH	A	B	C	D	A	B	C	D	A	B	C	D
4	++	+++	+++		-	-	-	-	-	-	-	-
6	+++	+++	+++	+++	-	-	-	-	-	-	++	++
8	+++	+++	+++	+++	+	-	++	++	++	-	++	++
9		+++	+++	+++			++	++			+++	++
11	+++	+++			+	-			+++	-		
14	++	+++			-	-			++	-		

+++ Total clearing of cell wall suspensions by culture supernatants in 24 h
++ >50% clearing (as above)
+ <50% clearing (as above)
- No activity.

Gliomastix was then subcultured for six weeks on (a) B.subtilis cells suspended in agar at pH5 and (b) a soil impregnated agar medium at pH5 (see 2.2.1), then grown in B.subtilis liquid culture as before, supernatants being semi-quantitatively assayed (Table 7). A quantitative assay of supernatant from the culture inoculated from (a) showed a slight increase in activity between pH7.05 and 8.2, "E_3" exhibiting linear kinetics (Fig. 23). Despite the increases in activity gained, the rate of activity was still extremely low in comparison with E_1 and E_2 and further efforts to characterise E_3 were abandoned.

Fig. 23: DETERMINATION OF pH OPTIMUM FOR E_3

Bacterial activity of culture supernatant inoculated from the original isolate of Gliomastix (●) and of supernatant inoculated from B.subtilis agar (◇) was assayed quantitatively in a range of buffers of constant ionic strength (I = 0.05). pH 3.6 - 4.0 were formate, pH 4.4 - 5.8 were acetate and pH 7.0 - 9.1 were Tris buffers.



PART B : PURIFICATION PROCEDURES

3.7 Affinity Chromatography (E_1 and E_2)

Chitin coated cellulose (c/c) was prepared and 10 ml packed into a 10 mm diameter column. The column was flushed through with "starter" buffer, crude supernatant (2 ml unless stated otherwise) was applied, and the column was left to stand for 20 minutes. The eluent was then run through the column (0.5 ml min^{-1}).

All fractions collected were semi-quantitatively assayed (Table 8). No binding occurred.

To check that the lack of binding was not due to a poor c/c preparation, 0.2 ml Schizophyllum commune bacteriolytic enzyme (which was known to bind to c/c) was combined with c/c (1 ml) and pH 3.0 formic buffer (ionic strength 0.05), and held at 20°C for 20 minutes, shaken regularly. The c/c was then pelleted by centrifugation (8000g, 5°C, 5 minutes).

No activity was detected in the supernatant by "Durham" tube assay, indicating that the enzyme had bound.

1M NaCl was included with the "starter" buffer in one run through the column but was found, by "Durham" tube assay, to be inhibitory to E_1 and its use was discontinued.

The effect of low pH on the fungal culture supernatant was also assessed; pH2.5 was inhibitory to E_1 (Table 9).

From the above results it appeared that if E_1 bound at all it did so at pH3.6, but was not eluted under the pH conditions applied. E_2 did not bind.

Table 8: Results of Affinity Chromatography of Bacteriolytic Supernatant

Following application of E_1 or E_2 to the column, starter buffer was run through, then eluent. Fractions collected were assayed for bacteriolytic activity ("Durham" tube assay).

EXPT.	TEMP.	STARTER BUFFER (ionic strength 0.05)	S/N	FRACTIONS COLLECTED	ELUENT (ionic strength 0.05 unless stated)	FRACTIONS COLLECTED	FRACTIONS IN WHICH LYTIC ACTIVITY DETECTED
1	20°C	pH2.5 formate	E_1; 2 ml	10 x 3 ml Nos 1–10	pH8.0 phosphate (0.1M)	8 x 3 ml Nos 11–19	None[a]
					pH8.0 phosphate + M NaCl	8 x 3 ml Nos 20–27	None[b]
2	20°C	pH3.0 formate	E_1; 2 ml	10 x 3 ml Nos 1–10			No. 9
3	4°C	pH3.6 formate	E_1; 2 ml	10 x 3 ml Nos 1–10	pH7.0 Tris	8 x 3 ml Nos 11–18	None
					pH9.1 Tris	8 x 3 ml Nos 19–26	None
4	4°C	pH 3.6 formate	E_1; 0.5 ml	15 x 1 ml Nos 1–15	pH 8.0 Tris	15 x 1 ml Nos 16–30	Nos 5 & 6
5	4°C	pH7.5 Tris	E_2; 1 ml	15 x 1 ml Nos 1–15	pH4.0 formate	15 x 1 ml Nos 16–30	Nos 11 & 12

[a] low pH effect – see Table 9.

[b] NaCl also found to be inhibitory to E_1.

Table 9: Effect of Low pH on E_1 During Affinity Chromatography

*Bacteriolytic activity was estimated by Durham tube assay.

TREATMENT (formate buffers at ionic strength 0.05 unless molarity stated)	ACTIVITY*
A (control) E_1 (0.25 ml) + pH3.6 buffer (0.25ml) held 10 mins; pH3.6 buffer (2M; 0.5ml) added	++
B E_1 " + pH2.5 " " " " " " " " " "	–
C E_1 " + pH3.0 " " " " " " " " (.2M; 0.5ml) "	+++

+++ total clearing of cell wall suspensions by culture S/N in 24h.

++ slight clearing of cell wall suspensions by culture S/N in 24h.

– no activity

3.8 Affinity Separation of E_1

B.subtilis cell walls and peptidoglycan were used as substrates for the "batch"-type separation process. Cell walls (10 mg ml^{-1} Aq) and peptidoglycan (10 mg ml^{-1} Aq) (0.5ml) were each suspended in 0.5 ml pH3.6 formate buffer (ionic strength 0.05), at 1 mg ml^{-1} concentration. E_1 (0.5 ml) was combined with each substrate and stirred gently for 1 hour at 0^oC, then centrifuged (11740g, 0^oC, 15 minutes). Supernatants were assayed semi-quantitatively. Pellets were resuspendad in 0.5 ml salts medium (see 2.2.2) and 0.5 ml pH3.6 buffer, incubated at 37^oC. Results suggested that E_1 failed to bind (Teble 10). The slight clearing of resuspended pellet (Table 10, (e)) was probably due to a small amount of enzyme solution remaining with the pellat after centrifugation. (Fuchs at al, 1986).

3.9 Cation-Exchange Chromatography of E_1

Hash (1963) used Amberlite CG-50-H^+ resin during purification of the fungal enzyme Chaloropsis lysozyme (Ch.lz). Zeo Karb 226 is a resin with very similar properties to CG-50 and was used in this experiment.

A 10 x 0.5 cm column of resin was flushed with 2M NaOH then washed with water until a neutral pH was recorded. The column was then flushed through with N HCl and again washed with water till the pH was neutral, then transferred to 4^oC.

E_1 (9ml) was applied to the column, water (18 ml) run through and all the fractions (1ml) collectad were semi-quantitatively assayed. (Rate of flow was 1ml min^{-1}). No binding occurrad. The pH of E_1 was then lowered to pH3.6 from 5.3 using pH2.7 formate buffer, ionic strength 0.05.

E_1 (6 ml) was then applied to the column, water washed through, and fractions assayed as before. Binding appeared to have occurred as no lytic activity was detected. The column was then eluted sequentially with 24 ml each of pH4, 5 and 7 ammonium acetate buffer (0.5 M). None of the fractions assayed had bacteriolytic activity; E_1 was not eluted.

Table 10: Affinity Separation of E_1

Bacteriolytic activity was measured by Durham tube assay.

TREATMENT		Assay of Supernatants	Resuspended Pellets
(a) (control)	0.5 mls buffer + 0.5 mls E_1	++	
(b)	0.5 mls peptidoglycan + 0.5 mls E_1	++	-
(c)	0.5 mls walls + 0.5 mls E_1	++	-
Repeated expt. with E_1 conc. x 6 by ultrafiltration			
(d) (control)	0.5 mls buffer + 0.5 mls E_1	+++	
(e)	0.5 mls peptidoglycan + 0.5 mls E_1	+++	-
(f)	0.5 mls walls + 0.5 mls E_1	+++	++

+++ total clearing of cell wall suspensions by culture S/Ns in 24 h.
++ slight clearing of cell wall suspensions by culture S/Ns in 24 h.
- no activity

3.10 Ammonium Sulphate Precipitation

E_1 (120 ml) was concentrated to 50 ml in an ultrafiltration cell (YM-10 membrane filter) and bacteriolytic activity was measured as 3.07 units. Over 1 hour 10.9g of ammonium sulphate ($(NH_4)_2 SO_4$) was added to E_1, which was held on a magnetic stirrer at 4°C. The solution (35% $(NH_4)_2SO_4$ saturation) was held at 4°C overnight but, as no precipitation occurred, a further 10.9 g of $(NH_4)_2SO_4$ was added as before to bring the solution to 70% saturation. After 16 hours a precipitate (A) had formed, and this was pelleted by centrifugation (18000g, 2°C, 30 minutes).

The pellet was "washed", i.e. dissolved in 100 ml pH 5 MES buffer (0.1M) then concentrated (YM-10 membrane filter) to 7.6 ml to remove $(NH_4)_2SO_4$ crystals. The supernatant was again stirred at 4°C and a further 14.75g $(NH_4)_2SO_4$ added to bring the solution to saturation. A precipitate (B) was formed and this was pelleted and "washed" in MES buffer as before, the final solution being 7.5 ml.

The resuspended pellets and the final supernatant (also "washed" free of $(NH_4)_2SO_4$ crystals and concentrated to 5ml) were all assayed quantitatively for bacteriolytic activity. Protein was measured, using the Lowry procedure (Lowry et al, 1951), at 25 mg for the original supernatent, 0.95 mg for precipitate (A), 0.56 mg for precipitate (B) and 0.25 mg for the final supernatant. (Table 11).

The specific activity for the original supernatant was determined at 61.4 units per mg protein and the redissolved pellet, obtained from 70% $(NH_4)_2SO_4$ precipitation, was determined at 448 units per mg protein. The total protein loss was 96.2% and the increase in specific activity was >7-fold.

3.11 Gel Filtration

The void and included fractions were determined for the Sephadex G-75 column (using blue Dextran and glucose), at fractions 54 and 139 respectively (Fig. 24). (For column details see 2.8).

Table 11: The Percentage of Bacteriolytic Activity and Protein Recovered During $(NH_4)_2SO_4$ Precipitation of E_1.

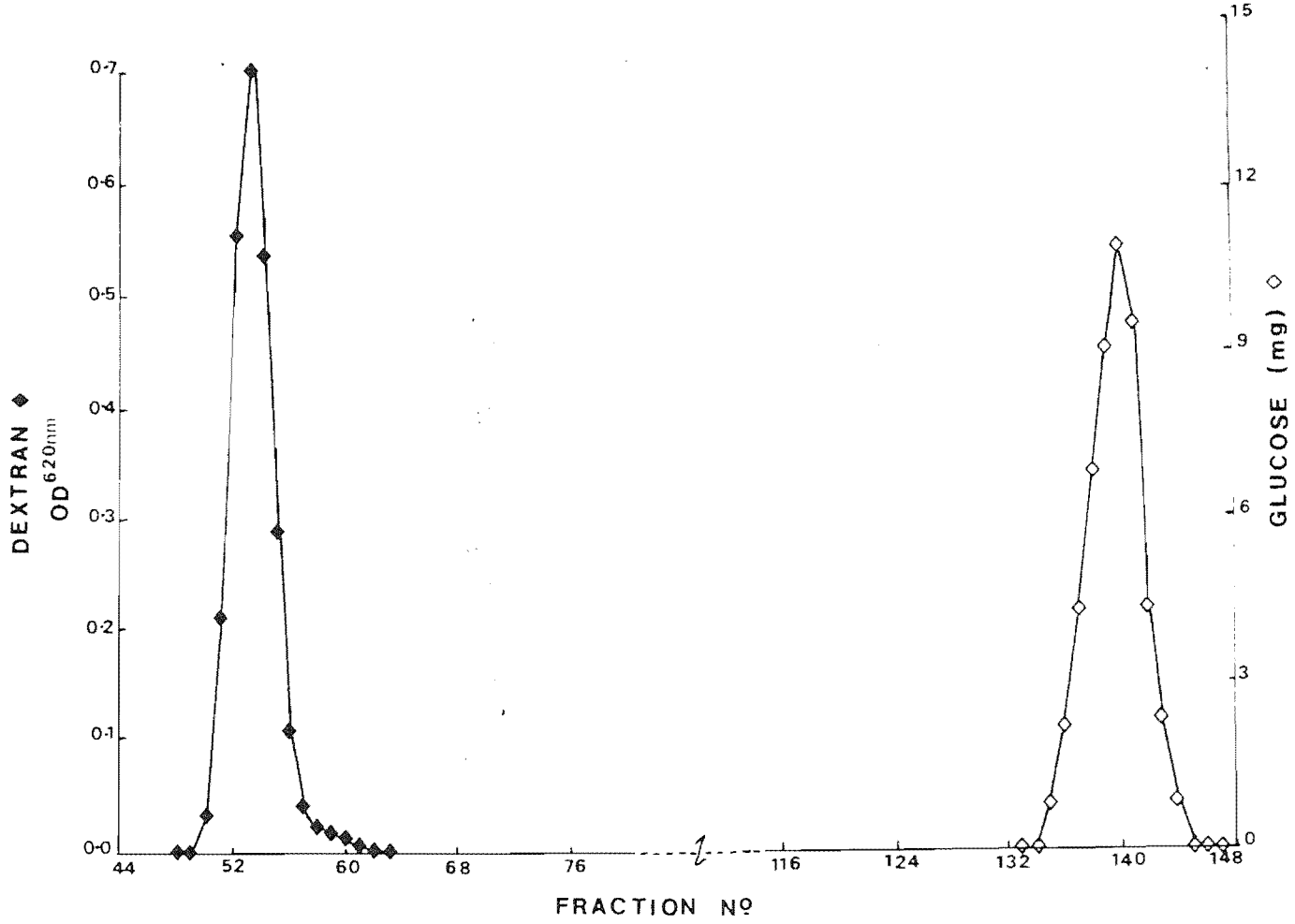
Bacteriolytic activity was measured by quantitative assay and protein (mg) estimated by Lowry method (Lowry et al, 1951).

$(NH_4)_2SO_4$ conc. (%)	Fraction obtained	Activity (%)	Protein (%)
0	E_1 (original S/N)	100	100
70	Precipitate A[a]	27.7	3.8
100	Precipitate B[a]	5.9	2.3
100	Final S/N[a]	0	1

[a]$(NH_4)_2SO_4$ was removed by dissolving in excess pH5 MES buffer (0.1 M) then concentrating by repeated ultrafiltration (YM-10 membrane).

Fig. 24: DETERMINATION OF VOID AND TOTAL INCLUDED VOLUMES OF A SEPHADEX G-75 COLUMN

Blue dextran (0.2%) and glucose (1% w/v) made up to 6 ml in buffer were applied to the column (84cm x 2.5 cm), then eluted with 0.02M sodium phosphate buffer, pH7. Flow rate was 42 ml hr^{-1} and 150 x 3.1 ml fractions were collected. Fractions containing Blue dextran were determined by measuring $O.D._{620nm}$ and those containing glucose by glucose assay (2.4.3). The void and total included volumes equalled 167.4 ml and 434 ml respectively.



Following $(NH_4)_2SO_4$ precipitation E_1 (6ml) was applied to the column, sodium phosphate buffer, pH7.0 (0.02M) was run through the column and 120 x 3 ml fractions were collected in acid washed tubes. Tubes were selected for quantitative assaying on the basis of results from "Durham" tube assays. Fractions which showed activity (Fig. 25) were pooled, concentrated in an ultrafiltration cell (YM-10 membrane), diluted with pH5 MES buffer (0.1M) and reconcentrated to 9.5 ml to remove phosphate buffer, then assayed. The rate of activity ($\Delta O.D.^{600nm}\ hr^{-1}$ x 10) for E_1 when applied to the column was 1.82, and for the pooled and "washed" frections recovered from the column was 0.80 indicating a loss of activity of 66%.

3.12 Effect of BSA on Activity Loss of Partially Purified E_1

The bacteriolytic activity of partially purified E_1 was found to decrease over time. Bovine serum albumin (B.S.A.) prevented this "dropping off" in activity (Kawamura and Shockman, 1983). Phenylmethyl sulfonylfluoride (PMSF), a protease inhibitor, had no effect. Neither dithiothreitol (DTT), which would have prevented possible oxidation of sulphide groups on the enzyme, nor (ethylenediaminetetra) tetra acetic acid (EDTA), which would have combatted the effects of heavy metals on the enzyme, had any effect (Fig. 26 & 27).

3.13 pH Optimum of Partially Purified E_1

The pH optimum (for method see 3.2.1) was determined at 3.4 (Fig. 28).

3.14 Estimation of Molecular Weight

The Sephadex G-75 column was set up as for gel filtration of the fungal supernatant (2.8) but 3 ml of protein were applied to the column as follows: 1mg ribonuclease (bovine pancreas), 1mg α-chymotrypsinogen A, 1mg ovalbumin, 1mg bovine serum albumin all dissolved in 2ml of purified E_1 plus 1 ml 0.02M sodium phosphate buffer (pH7).

Fig. 25: BACTERIOLYTIC ENZYME ACTIVITIES IN FRACTIONS FROM SEPHADEX G-75 CHROMATOGRAPHY OF E_1

The pellet formed, following precipitation of Gliomastix culture supernatant with 70% $(NH_4)_2SO_4$, was dissolved in 0.1M MES buffer, pH5 (7 ml). This solution (6ml) was applied to a Sephadex G-75 column (84 cm x 2.5 cm) and then eluted with 0.02M $NaHPO_4$ buffer, pH7. The flow rate was 42 ml hr^{-1} and 120 x 3.1 ml fractions were collected.

Fractions were semiquantitatively assayed for bacteriolytic activity. Activity was present in fractions 75-92 and these were quantitatively assayed. No ectivity occurred in the other fractions.

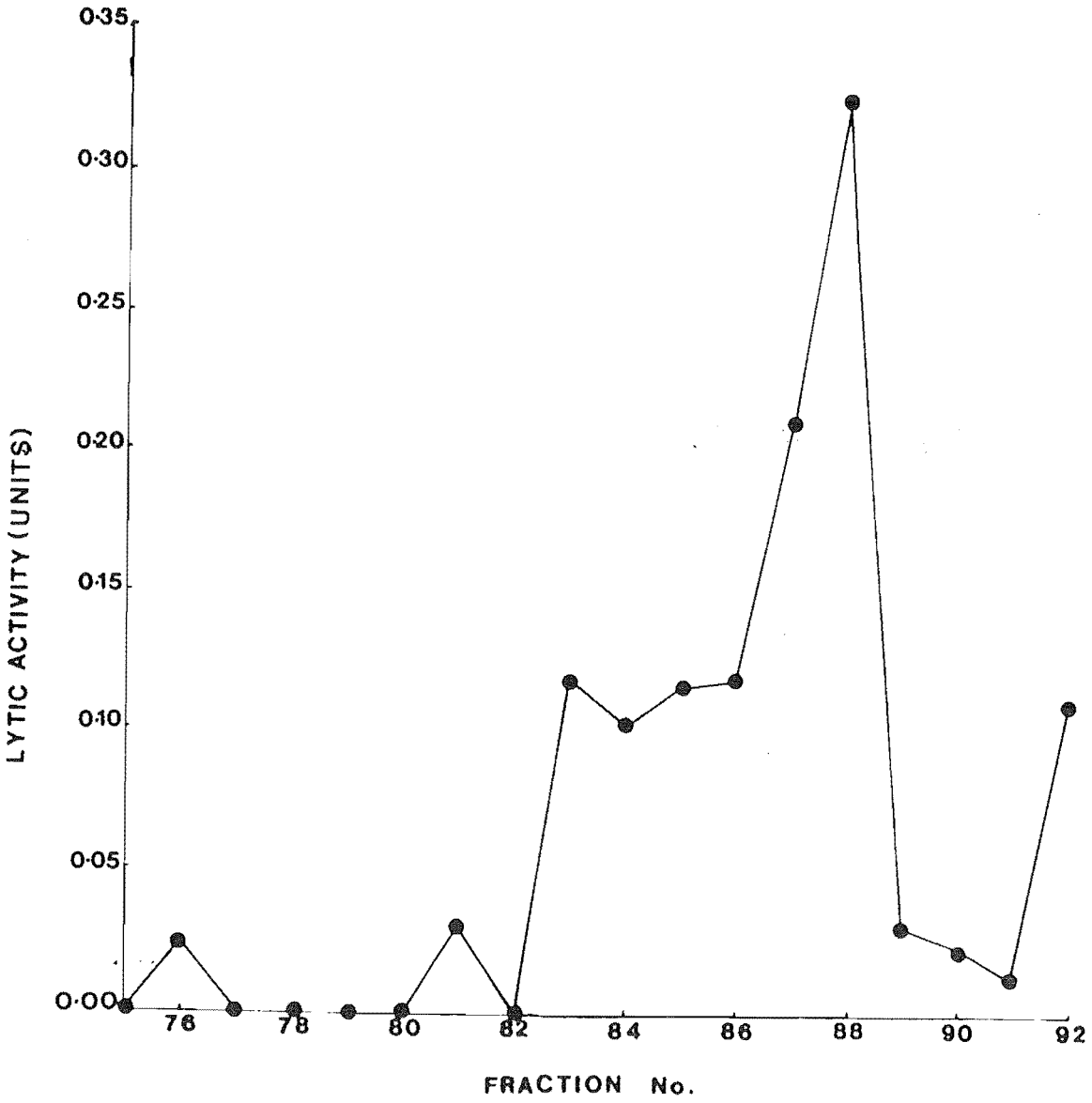


Fig. 26: STABILITY OF PARTIALLY PURIFIED E_1: EFFECT OF BSA, PMSF and DTT ADDITIONS

The assay, to determine bacteriolytic activity, was run under standard conditions (2.4.2) but with 190 µl buffer and 60 µl additions. The additions were buffer (E_1, ▼), BSA at 0.3 mg ml^{-1} final concentration (BSA, ●), PMSF at 0.1 mM final concentration (PMSF, ▽) and DTT at 1 mM final concentration (DTT, ◆).

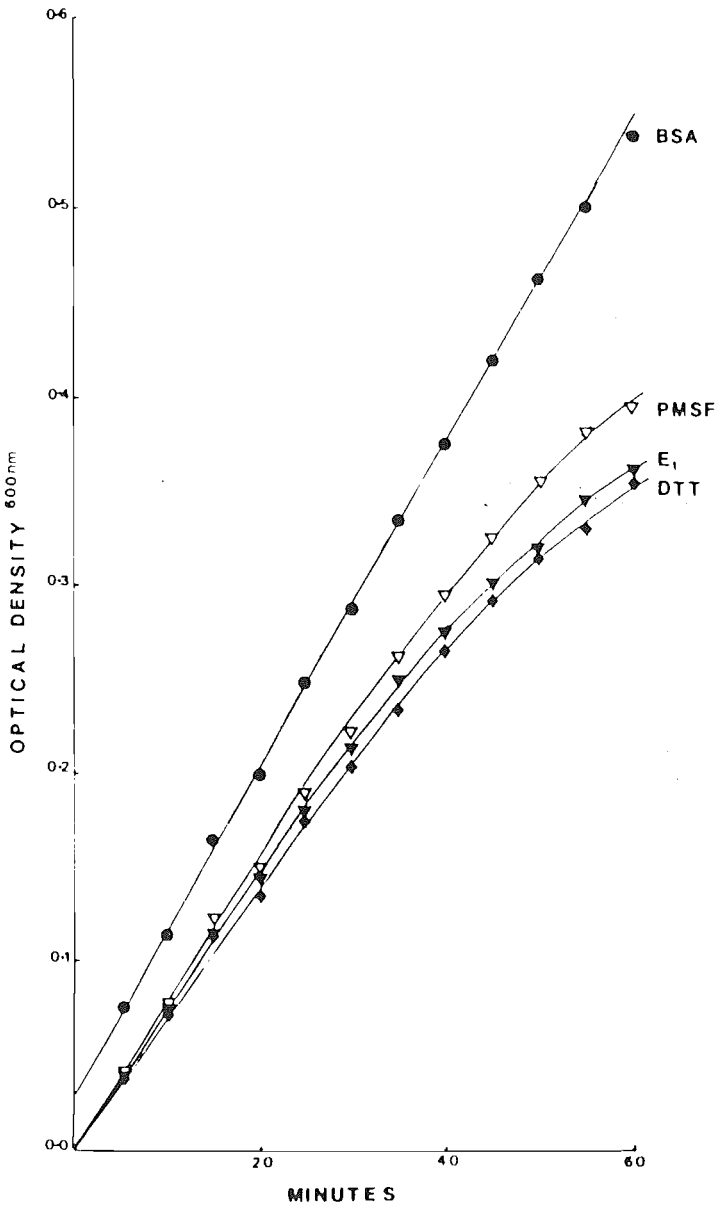


Fig. 27: STABILITY OF PARTIALLY PURIFIED E_1: EFFECT OF BSA and EDTA

The assay, to determine bacteriolytic activity, was run under standard conditions (2.4.2) but with 190 µl buffer and 60 µl additions. The additions were buffer (E_1, ▼), BSA at 0.3 mg ml^{-1} final concentration (BSA, ●) and EDTA at 1.9 mg ml^{-1} final concentration (EDTA, ◇).

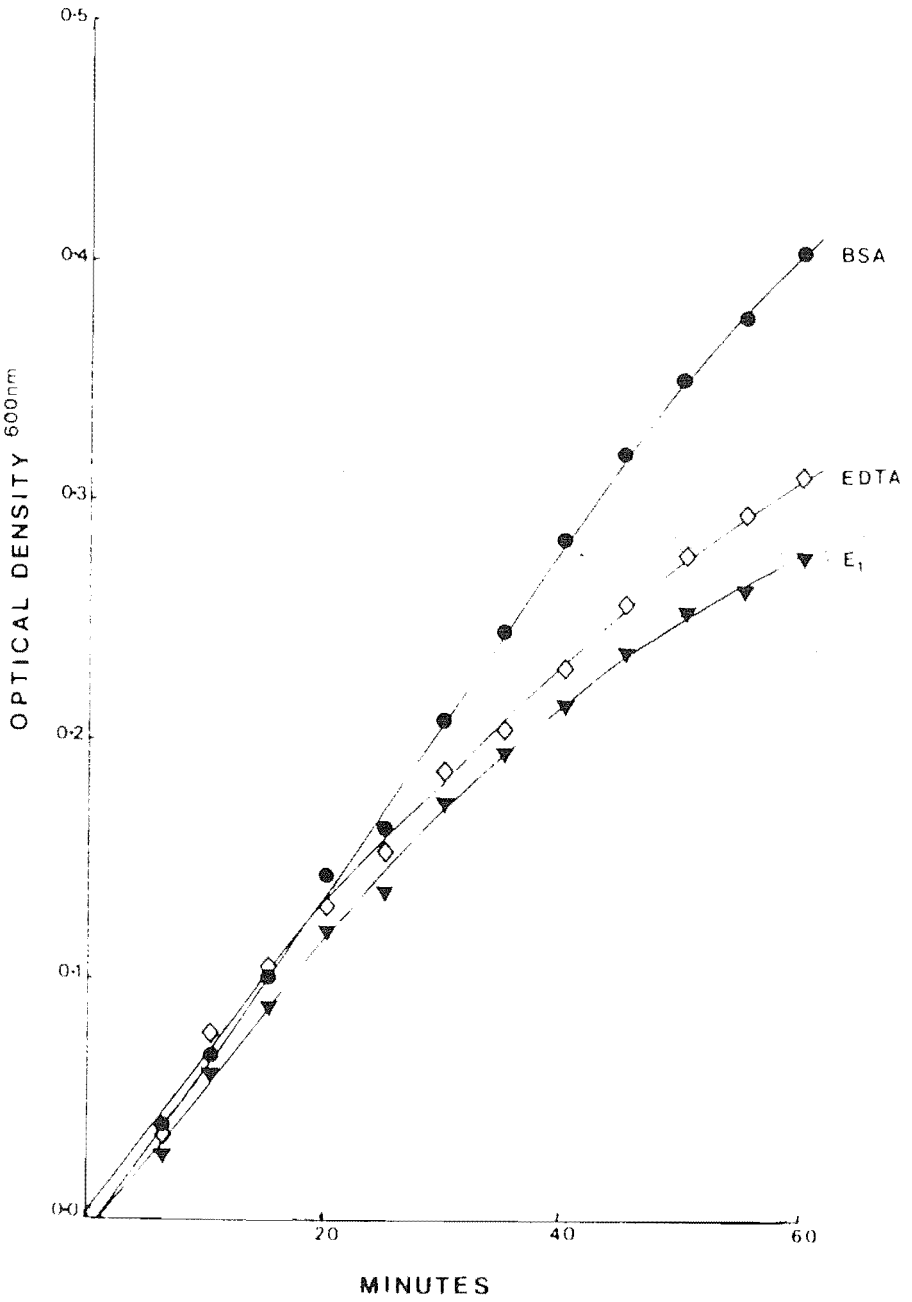
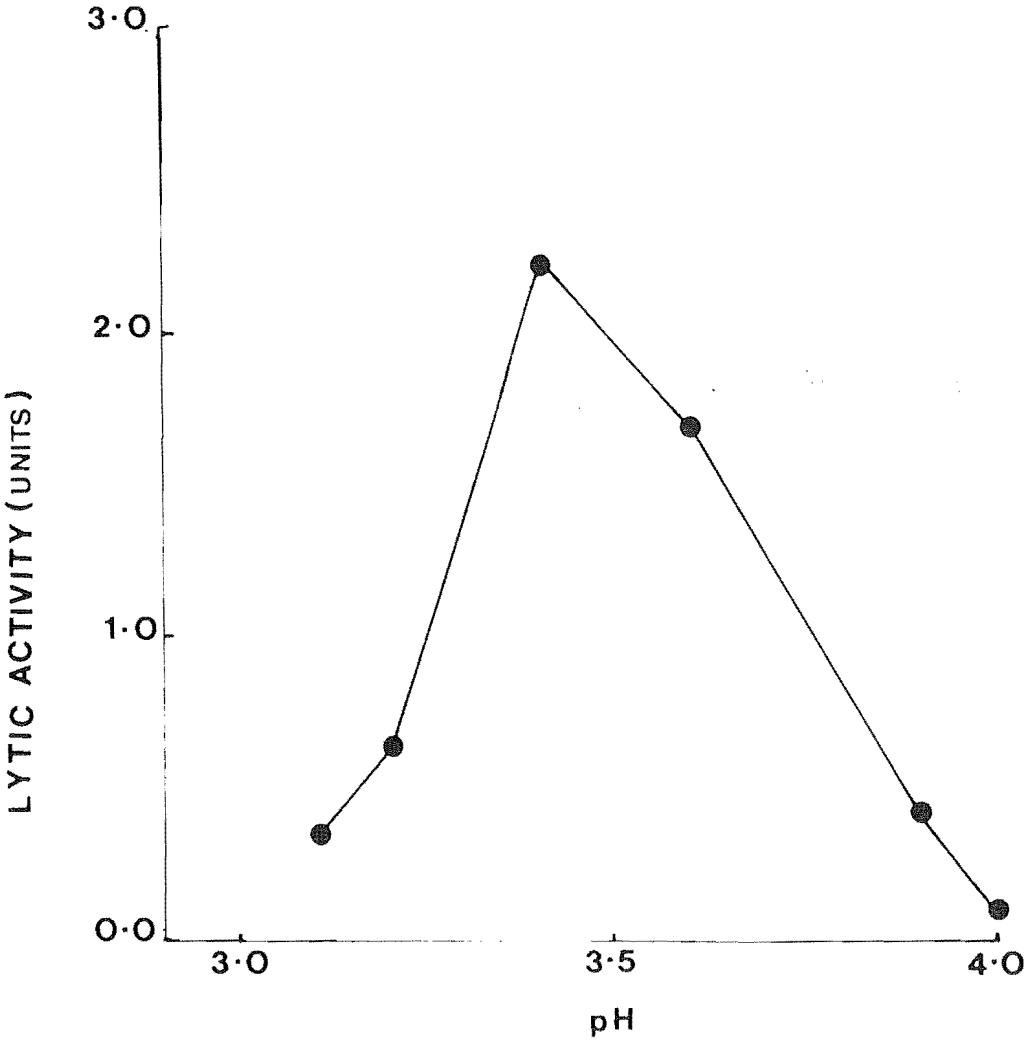


Fig. 28: DETERMINATION OF pH OPTIMUM FOR E_1 AFTER PARTIAL PURIFICATION BY AMMONIUM SULPHATE PRECIPITATION AND GEL FILTRATION

Following gel filtration, fractions with bacteriolytic activity (as determined by quantitative assay) were pooled and concentrated 6-fold by ultrafiltration (YM10 AMICON filter). The partially purified E_1 was then quantitatively assayed in a range of buffers of constant ionic strength (I = 0.05). pH 2.7 - 4.0 were formate and pH 4.35 - 4.8 were acetate buffers.



The elution fractions were quantitatively assayed for bacteriolytic activity (Fig. 29) and assayed for protein content (Bradford, 1976) (Fig. 30). The molecular weight of E_1 was calculated against the known weights of the other proteins by plotting molecular weight against the elution volume from column (Andrews, 1964, 1965).

The molecular weight for E_1 was 17000 (Fig. 31).

3.15 Mode of Action of E_1

3.15.1 Amino Group Assay of Cell Wall Digest

Initially this experiment was carried out using digest obtained following degradation of untreated B.subtilis cell walls by E_1 but the high background level of amino groups from the cell walls made the assay results inconclusive. The experiment was repeated using nitrite-treated (and therefore amino-group free) walls (see 2.3.3).

To obtain the digest tubes were set up as in Table 12.

Samples (300µl) were taken and frozen immediately after the addition of E_1 (or basal salts) and the optical density read at 600^{nm} on the spectrophotometer. The tubes were incubated overnight ($30^{o}C$) then samples again taken and $O.D.^{600nm}$ read.

An amino assay, lower limit of detection 25 nmol, (Ghuysan et al, 1966) (2.10.2) was carried out on the samples taken and no amino groups were detected. The amount of releasable amino groups (alanine) per mg of cell walls has been estimated as 0.3 µmol (Hughes et al, 1968). Under the conditions used (Table 12) the 300µl samples would have contained 0.19 µmol of releasable amino groups. The results indicated that there was no increase in free amino groups following degradation of nitrite-treated cell walls by E_1.

Fig. 29: ELUTION PEAK FOR E_1 ON FRACTIONATION BY GEL FILTRATION ON SEPHADEX G-75

A semiquantitative assay showed that all active fractions were between 75 and 95. These fractions were assayed quantitatively.

The void volume was determined with Blue-Dextran (Fig. 24).

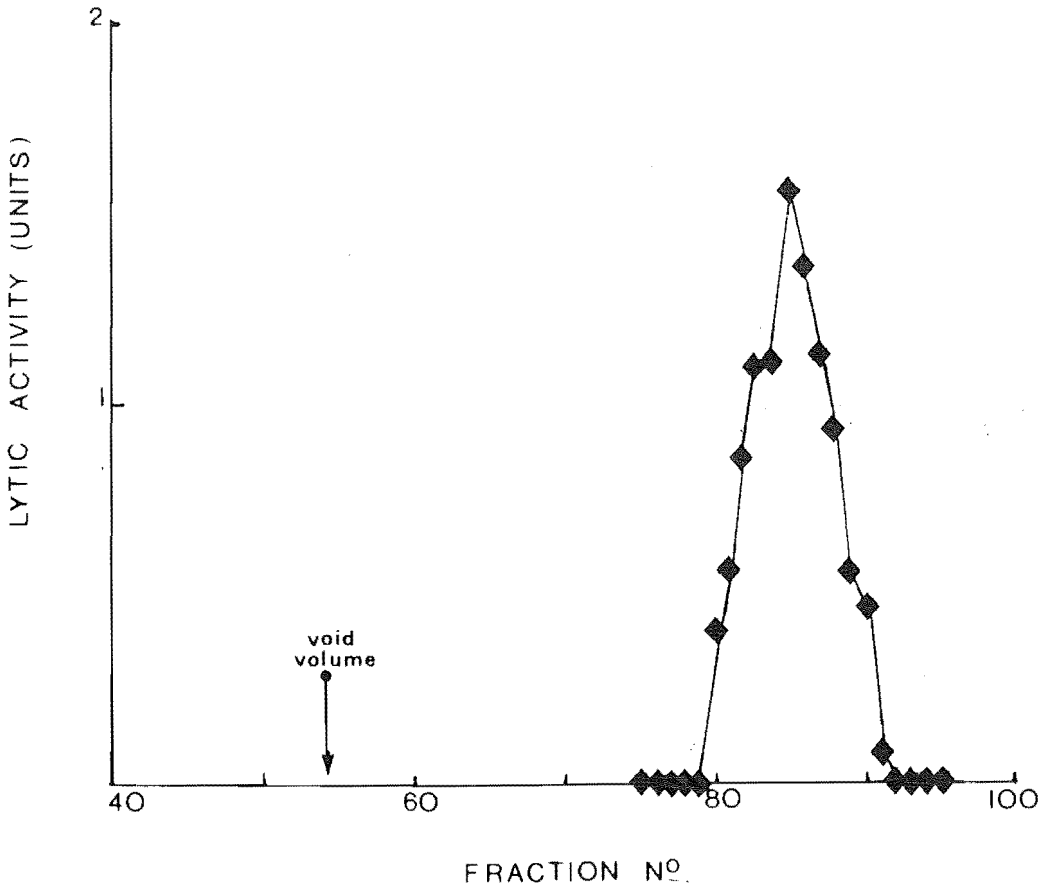


Fig. 30: ELUTION PROFILE OF PROTEINS (bovine serum albumin, ovalbumin, α-chymotrypsinogen and ribonuclease) ON SEPHADEX G-75

The protein mixture ((i) 1mg of each protein dissolved in 2ml elution buffer, ◆ ; (ii) 5 mg of each protein dissolved in 3 ml elution buffer, ◇) was applied to the column then eluted with 0.02 M sodium phosphate buffer, pH 7.0. The flow rate was 42 ml hr^{-1} and 100 x 3.1 ml fractions were collected. OD_{575nm} readings were obtained from protein assays (Bradford, 1976).

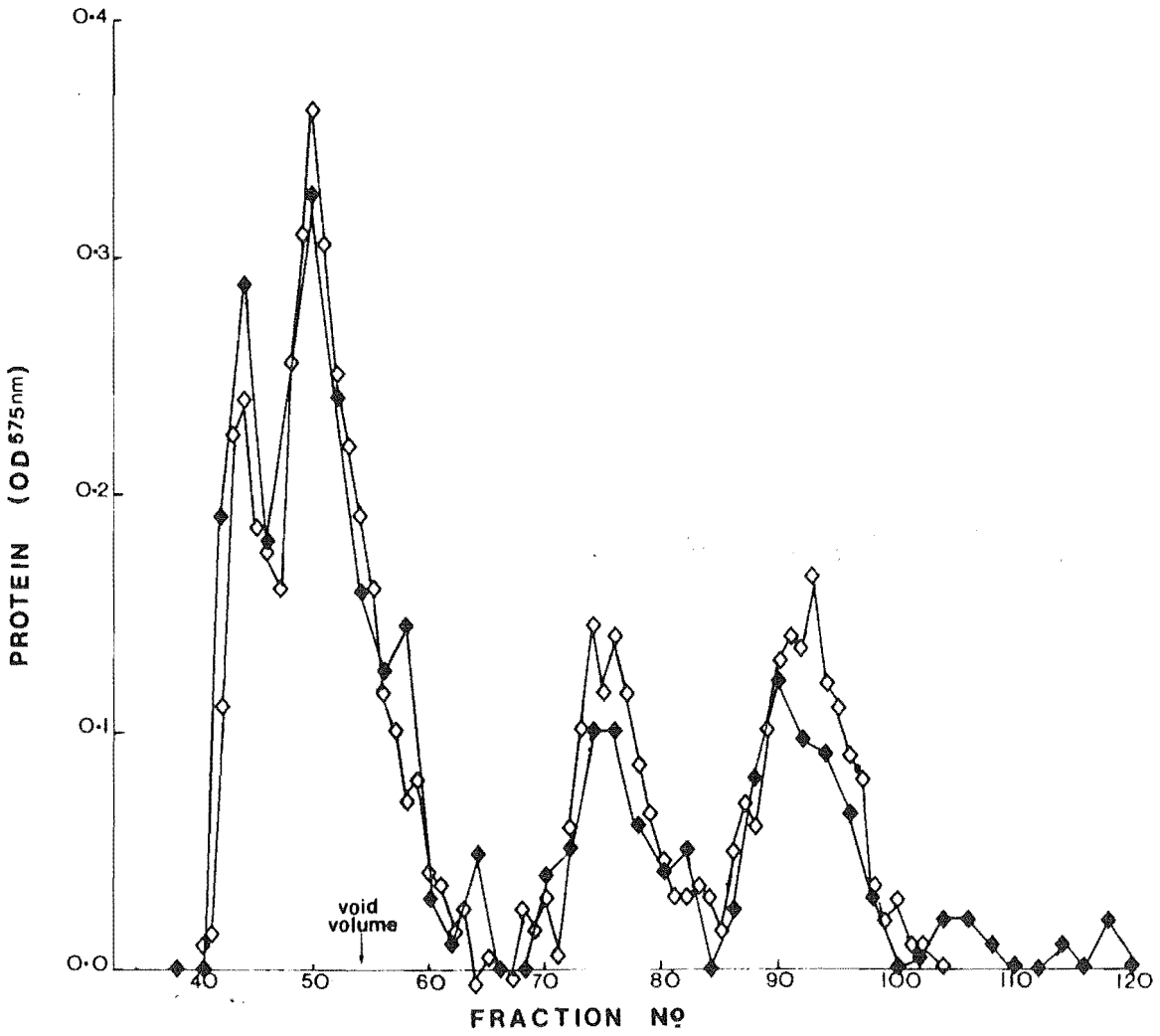


Fig. 31: DETERMINATION OF THE MOLECULAR WEIGHT OF E_1 BY GEL FILTRATION ON SEPHADEX G-75

First run ● ;Second run ◇ .

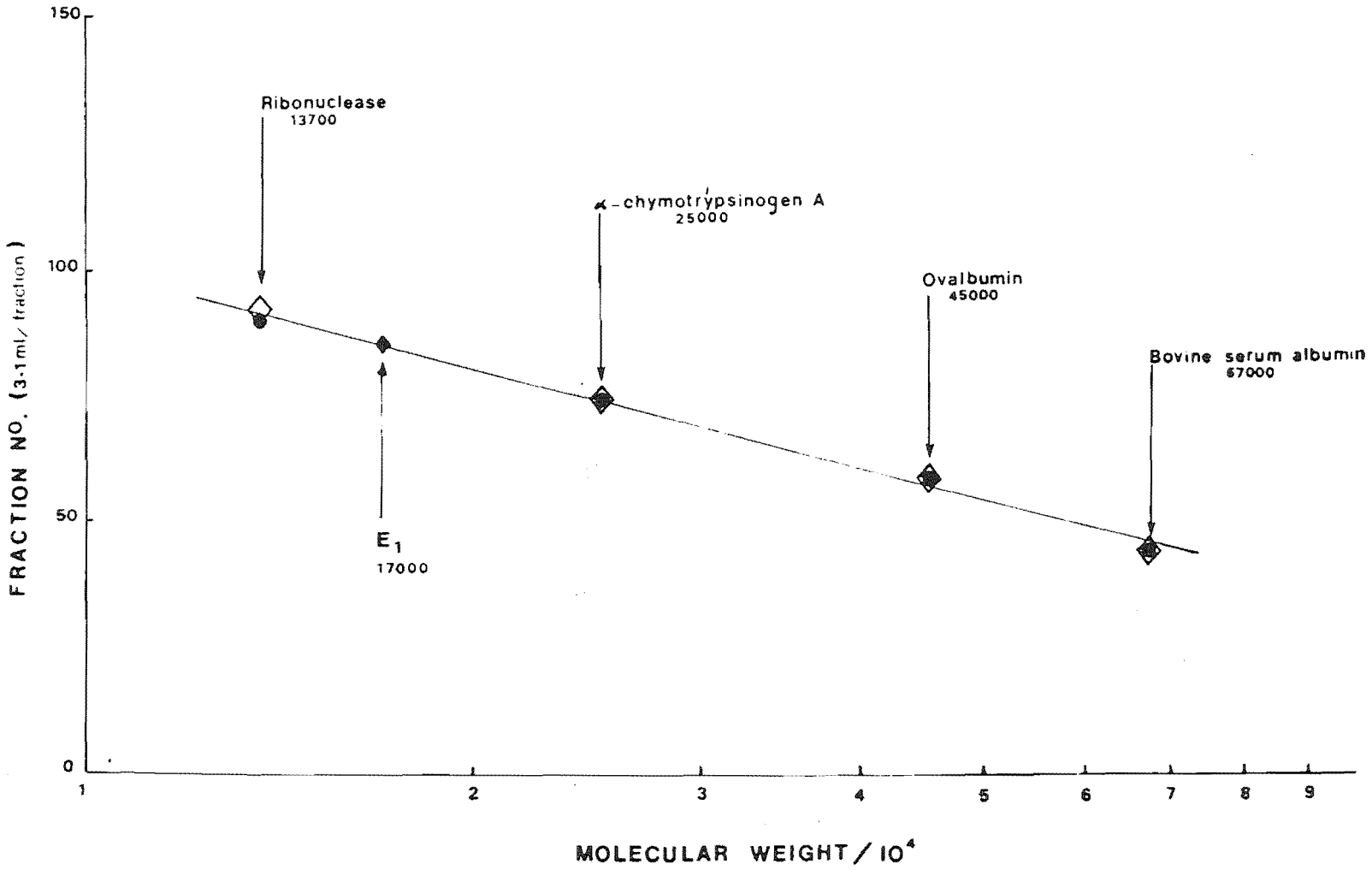


Table 12: Assay of E_1 Against Cell Walls

Tubes were incubated at 30°C

Assay Components (µl)	Tubes	1	2	3
pH 3.4 formate buffer		1000	1000	1000
walls (10mg.ml^{-1}): NO_2^--treated		400	400	–
water		–	–	400
basal salts medium*		–	500	–
E_1*		500	–	500

*Immediately after addition of E_1 (or salts) samples (200 µl) were taken and frozen, and the O.D.^{600nm} read. Samples (200 µl) were again taken when walls were totally degraded.

3.15.2 Reducing Group Assay of Cell Wall Digest

B.subtilis cell walls were degraded by E_1 over time (3.15.1), samples being taken, and optical density at 600nm read, at regular intervals. Samples were then analysed for reducing sugars (Park & Johnson, 1949). A gradual drop in optical density in the samples taken over the time course was correlated with a gradual increase in reducing groups in those samples (Fig. 32). Of 0.6 μmoles of potentially available reducing end groups per mg of cell wall (Hughes et al, 1968), 0.38 μmoles per mg of wall were released, or 60% of available reducing end groups.

The reducing group assay was repeated using nitrite-treated walls (samples were those obtained in 3.15.1). Based on the standard curve obtained with 10mM glucosamine samples, reducing sugars were shown to be released.

3.15.3 Paper Chromatography of Cell Wall Digest

Cell wall digest was obtained by incubating cell walls (300 μl of walls (10μg ml^{-1} aqueous solution) suspended in pH3.4 formate buffer, ionic strength 0.05) and buffer (500 μl as above) with E_1 (450 μl) at 30°C for 14 hours. A drop of toluene was added to the incubation tube to prevent microbial growth.

Both glucosamine (200 μl of 25 μmol glucosamine-HCl standard) and the digest (200 μl) were treated with $NaBH_4$ then hydrolysed with acid (2:9:1) as follows:

Sample 1 = glucosamine treated with inactivated $NaBH_4$
2 = " " " active "
3 = cell wall digest treated with inactivated $NaBH_4$
4 = " " " " " active "

(i) Single-direction chromatography using Solvent A (2:9:2) was not completely satisfactory because of the presence of salts (Fig. 33). Sample 1 showed a glucosamine spot and sample 2 a glucosaminitol spot, confirming that $NaBH_4$ had acted on the reducing end groups and converted these to the alcohol. The muramic acid spot in Sample 4 was noticeably less intense than in Sample 3. No muramitol spot was detected. Glucosamine (but not glucosaminitol) was found in

Fig. 32: INCREASE IN REDUCING GROUPS AND DECREASE IN OPTICAL DENSITY (600nm) DURING HYDROLYSIS OF BACTERIAL CELL WALLS BY E_1

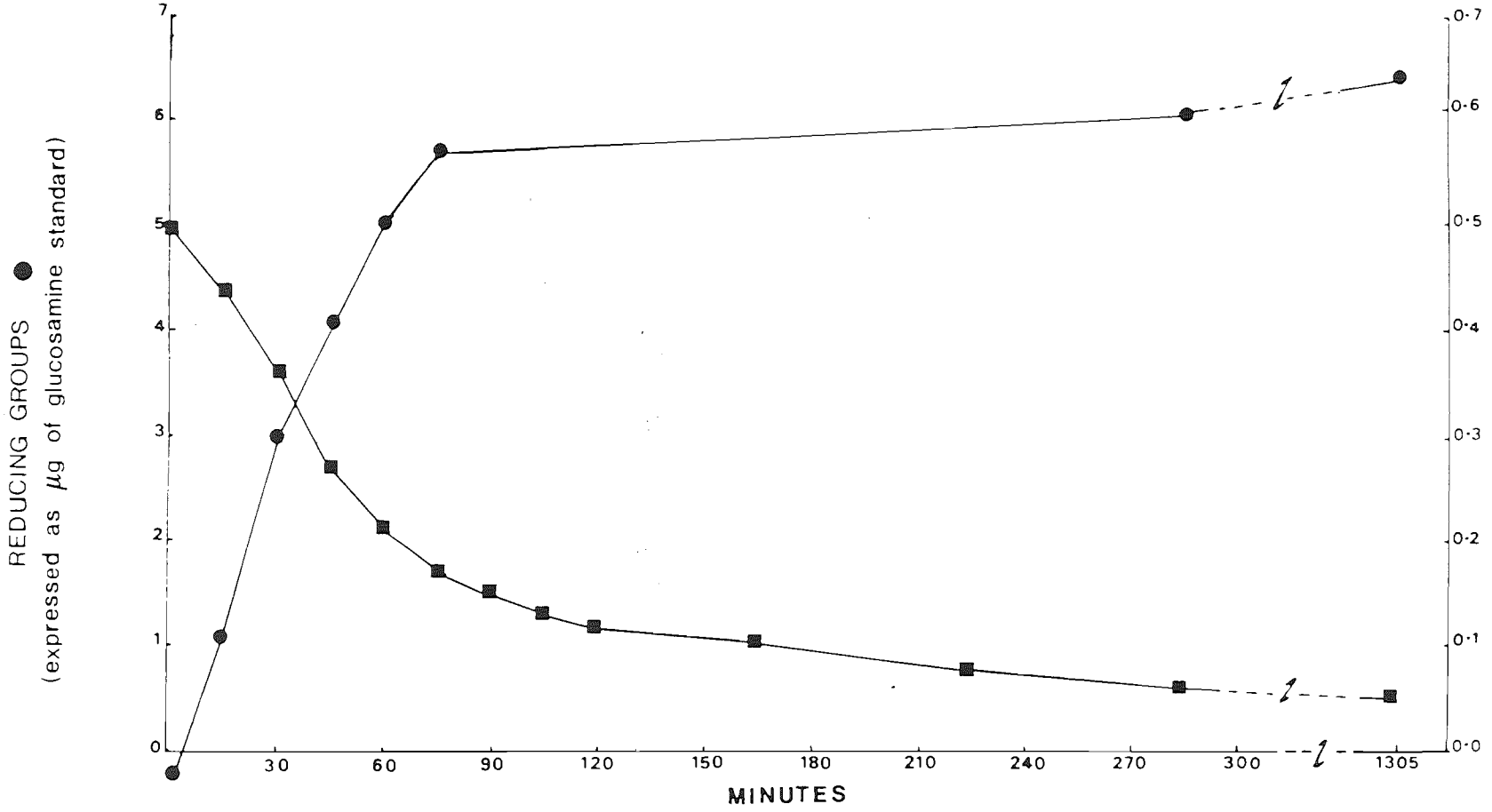
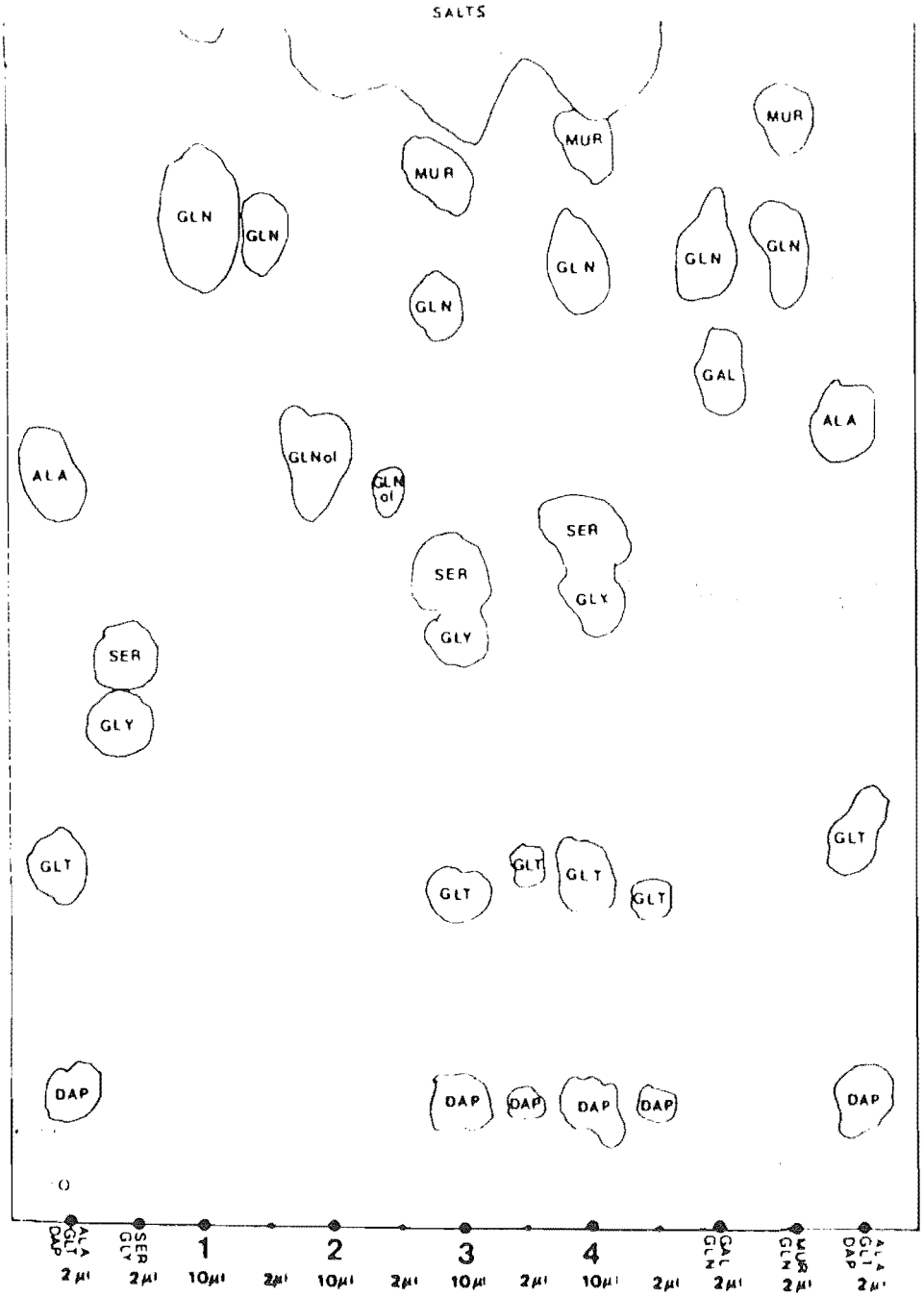


Fig. 33: CHROMATOGRAM OF CELL WALL DIGEST

Glucosamine (200 µl of 25 µmol ml^{-1} glucosamine-HCl solution) and cell wall digest (200 µl) were each treated with active, and a further 200 µl with inactivated, $NaBH_4$ (2.9.1). Acid treatment and drying followed and the treated glucosemine and digest were then each dissolved in 200 µl distilled water. 20 µl and 10 µl of each were then run on Whatmen 3 mm chromatography paper. Standards (2 µl of each) were also run on the paper: alanine (ALA) (21.14 µmol ml^{-1}), glutamic acid (GLT) (21.8 µmol ml^{-1}) diaminopimelic acid (DAP) (20.29 µmol ml^{-1}), L-serine (SER) (21.26 µmol ml^{-1}), glycine (GLY) (19.97 µmol ml^{-1}), galactosamine (GAL) (25 µmol ml^{-1}), glucosamine (GLN) (25 µmol ml^{-1} glucosamine-HCl) and muramic acid (MUR) (5.5 mg ml^{-1}).

The solvent was butan-1-ol, pyridine, glacial acetic acid and distilled water (60: 40: 3: 30) and spots were developed with a Ninhydrin reagent.



1 Glucosamine treated with inactivated $NaBH_4$
2 " " " active "
3 Cell wall digest treated with inactivated $NaBH_4$
4 " " " " " active "
GLNol = glucosaminitol.

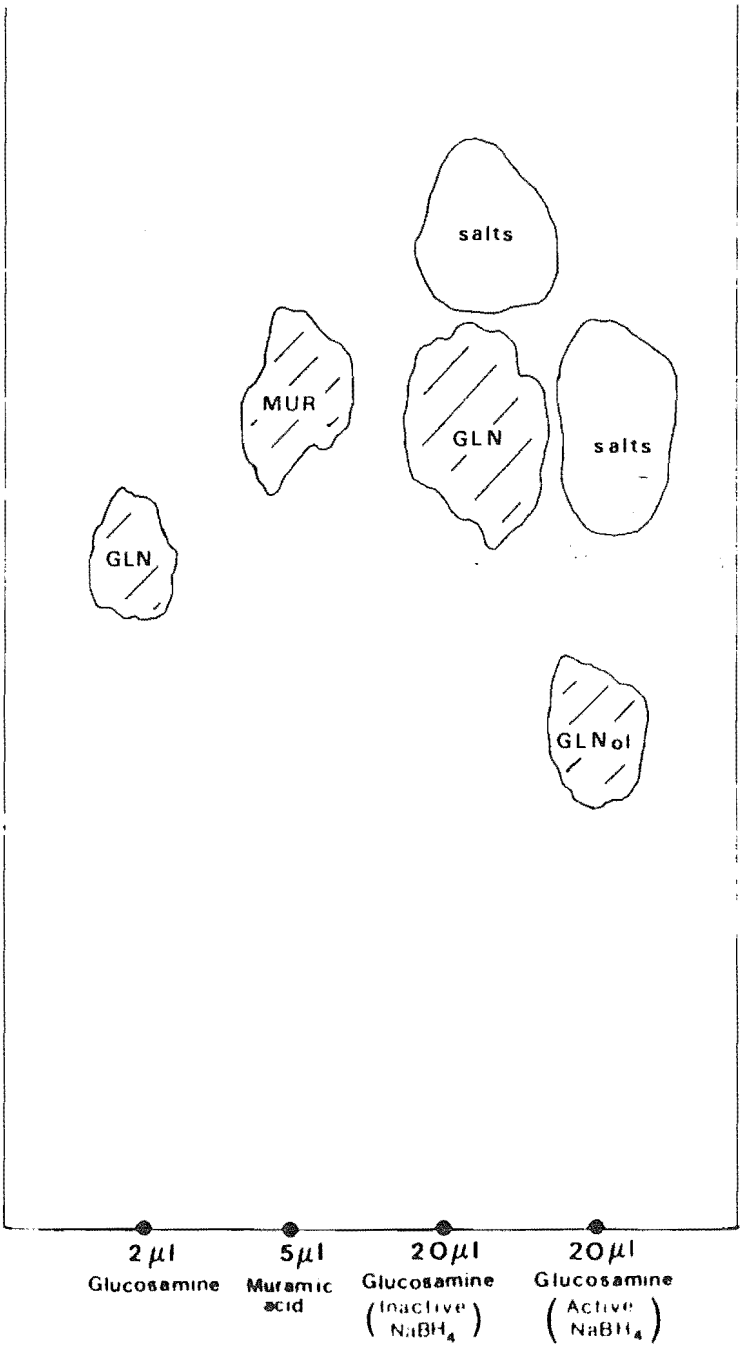
both Samples 3 and 4.

Single-direction chromatography of Samples 1 and 2, with glucosamine and muramic acid standards run alongside, confirmed that salt spots interferred with the movement of the reducing sugars on the paper (Fig. 34).

(ii) Two-directional chromatography of Samples 3 and 4 was carried out (on individual papers and in duplicate) in the descending direction in Solvent B (2.9.2). Following drying, the papers were turned 90 degrees and rerun in the descending direction in Solvent C (2.9.2) (Ghuysen et al, 1966). Papers were then dried and analysed for amino sugars (2:10:4). The $NaBH_4$ treatment resulted in a 53% loss of muramic acid end groups but only a 12% loss of glucosamine end groups.

Fig. 34: CHROMATOGRAM OF GLUCOSAMINE TREATED WITH ACTIVE AND INACTIVATED $NaBH_4$

Glucosamine (GLN) (200 µl of 25 µmol ml^{-1} glucosamine-HCl solution) was treated with inactive $NaBH_4$ (2.9.1). Acid treatment and drying followed and the glucosamine was then dissolved in 200 µl of distilled water and 20 µl was run on Whatman 3 mm chromatography paper. A glucosamine standard (25 µmol ml^{-1} glucosamine-HCl) and a muramic acid (MUR) standard (5.5 mg ml^{-1}) were also run. The solvent was butan-1-ol, pyridine, glacial acetic acid and distilled water (60: 40: 3: 30) and spots were developed with a Ninhydrin reagent.



(GLNol = Glucosaminitol)

CHAPTER 4: DISCUSSION AND CONCLUSIONS

PART A: PRODUCTION OF BACTERIOLYTIC ENZYMES

(i) Production of Extracellular Lytic Activity

Gliomastix murorum var. felina was selected for investigation because it was considered unique amongst filamentous fungi in producing two different types of bacteriolytic enzyme activity depending on growth pH (Grant et al, 1986).* This "dual" enzyme production was confirmed for G.murorum. Other Gliomastix species were investigated but only G.sp. 1081 exhibited bacteriolytic activity, and then only at acid pH.

Growth conditions were optimised to maximise production of extracellular bacteriolytic activities from G.murorum. The maximum activity obtainable decreased as the number of transfers of the fungus increased. The original activity of E_3 diminished after successive transfers and was not regained despite efforts to induce it. Further investigations involved only E_1 and E_2.

Yokogawa et al (1973) reported that only 18% of the original lytic activity of a Streptomyces globisporus strain was detected after four successive in vitro transfers. Inclusion of streptococcal cells in the medium boosted bacteriolytic enzyme production in the short term, but activity again decreased over several successive transfers.

Production of E_1 (and to a small degree E_3) was increased by growth on either a B.subtilis cell based medium or a sterile soil based medium. Maintenance of enyme activity in the fungus was most successful when it was

*[E_1: optimally active at acid pH when the fungus was grown at pH5.0.
E_2 & E_3: optimally active at alkaline pH when the fungus was grown at pH7.0 and pH5.0 respectively.]

grown on MEA plates followed by a week's growth on an agar-based B.subtilis medium (at appropriate pH) prior to inoculation. The decrease in lytic activity production over time in vitro was thus overcome in the short term, and will be reassessed in the long term. Cryopreservation of mycelium from the original isolate has ensured preservation of material capable of producing the original levels of lytic activity.

Rates of lytic activity obtained from E_2 were approximately 10% of rates obtained from E_1. To characterise E_2 further a regime of induction on B.subtilis cells followed by bulk production and concentration of the enzyme, either by ultrafiltration or ammonium sulphate precipitation, would be desirable. A scale-up to fermenter production of the bacteriolytic enzymes of Gliomastix is being undertaken at the Cawthron Institute by Dr. A. Broderick.

(ii) Measurement of Specific Activities of Extracellular Bacteriolytic Enzymes

Measurement of specific activities of extracellular bacteriolytic enzymes produced by Gliomastix in a B.subtilis liquid culture was hampered by the insoluble nature of the bacteria. Simple dry weight measurements would have included unused bacterial substrate. Dry weights of mycelium were therefore obtained from fungus grown on glucose, and the level of ergosterol in that mycelium determined. [Ergosterol, a lipid present in fungal membranes but not present in bacteria, has been shown to be a "valuable biomass indicator" [Grant and West, 1986]]. On the basis of those measurements, a linear regression equation was obtained and mycelial dry weights from B.subtilis based cultures were estimated from this equation. These figures were approximately double the actual dry weights obtained directly from the fungal cultures (i.e. the residual bacterial substrate as well as the mycelium).

Possible explanations for this anomaly are

(i) that the fungus produced longer, thinner hyphae on the insoluble substrate than on the soluble glucose. The surface area of the fungal plasma membrane, and thus the ergosterol levels, would increase in conjunction with an increase in the surface area of the fungal hyphae.

The phenomenon of variations occurring in hyphal length and diameter depending on the nutritional status of the substrate has been reported (J. Schnurer and K. Paustian, abstract, Fourth International Symposium on Microbial Ecology, Ljubljana, Yugoslavia, Aug. 24-29, 1986, (B5)).

(ii) that as the fungus produces extracellular bacteriolytic activity when grown on the insoluble substrate the lytic enzymes would probably be packaged in lysosomes (via the Golgi apparatus) prior to secretion from the hyphae (de Duve, 1983). Lysosomal membranes could vastly increase the total membrane area, and thus the levels of ergosterol, in such cultures. Glucose, on the other hand, would be transported directly across the membrane and into the fungal cytoplasm without the requirement for secretory machinery. Ergosterol levels in glucose based cultures would thus be inaccurate as a means of estimating dry mycelial weights in bacterial based cultures.

One solution to this difficulty would be to grow the fungus on a soluble protein substrate. Extracellular proteases would be required to provide breakdown products for transportation across the fungal membrane. The intracellular lysosomal packaging of these proteases would give ergosterol levels more comparable to those in the bacteria grown hyphae, while the soluble nature of the protein substrate would allow direct weight determination.

Preliminary investigations into this problem have been made, but further work is required to confirm whether either of these hypotheses are tenable. To confirm the hypothesis that increased hyphal length occurred on a bacterial substrate measurements of hyphal lengths and diameters would need to be made. As ergosterol has proved a useful tool in fungal biomass measurements (Seitz et al, 1979; Grant & West, 1986) this area of enquiry will be pursued.

(iii) The Constitutivity/Inducibility of E_1

Hash (1974) has stated that *Chalaropsis* lysozyme is constitutive. It is not a chitinase (as is HEWL) and so problems of "self-degradation" of cell-wall chitin are avoided.

A wide range of fungi have been shown to produce bacteriolytic enzymes (Grant et al, 1986) and inducibility of these enzymes *in vivo* would appear advantageous. Constitutively produced lytic enzymes would face a succession of "hazards" including adsorption to soil particles and denaturation by proteases in soils (Burns, 1983). Constitutive production would also be unfavourable energetically for the organism. Burns (1983) has postulated that induction of enzymes following the production of breek-down products by basal levels of the enzyme would conserve energy. Enzymes released from moribund cells could also fulfil a "scout" or "trigger" function for the living fungal population.

Immobilised enzyme (soil colloid-bacteriolytic enzyme complex) interactions with bacterial cells is seen as possible, but dependent on enzyme stability.

Burn's hypotheses give an insight into the factors governing the activities of an extracellular enzyme in soil and raise questions as to the advantages of the constitutivity of the lytic enzyme to *Chalaropsis* sp.

The *Gliomastix* bacteriolytic enzyme was not constitutive. *Gliomastix* required the presence of *B.subtilis* cells in the growth medium before bacteriolytic activity was induced. (An investigation into the inductive agent(s) of *B.subtilis* is being carried out at the Cawthron Institute by Dr. W.D. Grant).

The wide-spread ability of fungi to produce extracellular bacterial wall hydrolases capable of dagrading a range of bacteria and to utilise the degradation products as a nutrient source, points to a previously unsuspected role of fungi in the cycling of nutrients in the soil and in soil fertility (Grant et al, 1986).

PART B: PURIFICATION AND MODES OF ACTION OF BACTERIOLYTIC ENZYMES

Once maximal bacteriolytic enzyme production under optimal growth conditions had been achieved for Gliomastix murorum, purification and characterisation of one of the enzymes was undertaken. Experimental work centred on E_1 because of its greater rate of lytic activity.

Affinity chromatography utilising chitin-coated cellulose was unsuccessful. E_1 appeared to bind weakly at pH3.6 at $4^{o}C$, but not at room temperature. E_1 was not eluted under the given pH conditions, whilst E_2 did not bind. Gliomastix murorum is strongly cellulolytic (Williams & Pugh, 1971) and moderately chitinolytic (Jackson, 1965). If basal levels of either cellulase or chitinase were present in the culture supernatant then some degradation of the column on application of the supernatant is possible, with consequent prevention of binding of the lytic enzyme(s). The binding of E_1 detected at pH3.6 in the cold could indicate that cellulases and/or chitinases were relatively inactive at the low temperature whereas binding of the lytic enzymes was probably less dependent on temperature.

Affinity Separation differed from the bacteriolytic assays of E_1 only in that low temperatures ($0^{o}C$ for 1 hour) and centrifugation were involved, yet no binding of E_1 to the bacterial cell wall substrate occurred. A low temperature effect on the binding capabilities of E_1 is unlikely, although if the enzyme occurred in an inactive form it might heve required a temperature dependent activation by a second (possibly wall-bound) substance.

Agitation is known to cause inactivation of some fungal cellulases (Basu & Pal, 1956). Whilst agitation of growing Gliomastix cultures and centrifugation during harvesting (2800g) occurred without apparent detriment to E_1, the more vigorous agitation of centrifugation during the affinity separation experiment (11740g) might have caused temporary inactivation and loss of binding of the enzyme.

Whilst this hypothesis is open to criticism a more reasonable explanation for the failure of E_1 to bind to the affinity substrate has yet to be elucidated.

Cation-exchange chromatography on Sephadex G-75 resin wes also unsuccessful, E_1 apparently binding at pH3.4, but remaining bound despite a range of elution buffers being run through the column. No further efforts were made to elute the enzyme as ammonium sulphate precipitation and gel filtration offered a viable alternative.

Partial purification of E_1 was obtained by $(NH_4)_2SO_4$ precipitation followed by gel filtration on a Sephadex G-75 column. There was a 7-fold increase in specific activity (from 61.4 units mg^{-1} protein to 448 units mg^{-1} protein) but a >90% loss of protein during the precipitation. Possible explanations for this loss are that

(i) proteins, including some of the bacteriolytic enzyme (there was a drop in total bacteriolytic activity), were bound to the ultrafiltration membrane during concentration of the precipitate (which was dissolved in MES buffer to remove $(NH_4)_2SO_4$);

(ii) the assay method used (Lowry et al, 1951) was inaccurate. The Lowry procedure is subject to interference by a number of compounds (Bradford, 1976) including phenols (Peterson, 1979). Brown colouration of the original culture fluid might well have been caused by phenols which could have been included in the original protein determination. Following ultrafiltration a brown substance coated the membrane. If the substance was phenolic, this would account for the apparent large loss of protein experienced.

Gel filtration on the Sephadex G-75 column was also used to obtain a molecular weight for E_1. This was determined as 17,000.

The partially purified enzyme lost some activity on thawing and lost further activity over a time course to assess rate of activity on the cell wall substrate. Addition of E.D.T.A., a metal-chelator, to the assay mixture had no effect on this loss of activity, and so the inhibitor was unlikely to have been a heavy metal. Addition of dithiothreitol (D.T.T.), which prevents oxidation of sulphydryl groups in enzymes, had no effect either.

If the inhibitor was a protease then it was neither metal-chelator sensitive nor a serine-protease; neither E.D.T.A. nor phenylmethyl sulfonylfluoride (P.M.S.F.), a serine-protease inhibitor, prevented loss of E_1 activity. The inhibitor could have fallen into the thiol or acid protease groupings (Loffler, 1986).

The instability was overcome by the addition of bovine serum albumin (B.S.A.), which lends credence to the view that the inhibitor was a protease. B.S.A. could have "buffered" the proteolytic effect on E_1 by being degraded itself. If the inhibitor was an enzyme-poison rather than a protease then B.S.A. could have had a similar "buffering" effect by absorbing the impact of the poison on the enzyme.

The mode of action of E_1 was shown to be that of a hexosaminidase and not an amidase nor a peptidase. A reducing group assay carried out on cell walls prior to and following degradation by E_1 showed an increase of 60% in reducing end groups. Of 0.6 μmoles of potentially available reducing groups per mg of cell wall (Hughes et al, 1968), 0.38 μmoles per mg of wall were released.

The amino group assay (Ghuysen et al, 1966) was able to detect a minimum amount of amino groups of approximately 25 μmoles. The cell wall digest sample contained 190 μmoles of potentially releasable L-alanine amino groups (3.15.1), but no release of amino groups was detected. If E_1 was a peptidase an even greater amount of potentially releasable amino groups could have been expected from any or all of the peptide linkages in the peptidoglycan. A reducing group assay carried out on the same wall digest as the amino assay, to confirm that cell wall degradation had taken place, showed that an increase in reducing groups had occurred.

Single-direction paper chromatography of both glucosamine and the E_1 digest (each treated with both active and inactivated $NaBH_4$) demonstrated that whilst glucosamine residues were converted to glucosaminitol by active $NaBH_4$ no glucosaminitol appeared when the E_1 digest was similarly treated. No muramitol appeared either, but the muramic acid spot did decrease visually in comparison with the digest treated with inactivated $NaBH_4$. This suggested that muramic acid residues with free reducing groups had resulted from cell wall degradation

by E_1, and that these reducing end groups might have been converted to muramitol following the $NaBH_4$ treatment. This view was supported by the analytical results of the two-directional chromatography (3.10.3). Tha 53% loss of muramic acid reducing end groups, when compared with the 12% loss of glucosamine reducing end groups indicated that E_1 was a muramidase, albeit with low glucosaminidase activity. The reducing group assay carried out on bacterial cell walls prior to and at completion of degradation showed that 60% of potentially releasable reducing groups were in fact released. The total percentage of reducing groups released during chromatography was only 32.5%. Unfortunately, as E_1 lost activity over a time course following partial purification, not all preparations of digest went to completion.

A similar purification and detection of mode of action of E_2, not undertaken in this study, would further characterise the bacteriolytic capabilities of Gliomastix.

CONCLUSIONS

(i) The production of at least two bacteriolytic enzymes by Gliomastix murorum was confirmed, and the optimisation of growth conditions for maximal enzyme production was achieved. Optimal conditions were a concentration of B.subtilis cells of 2.5 mg ml^{-1} (suspended in a defined salts solution and appropriate buffer) in shake flask cultures incubated in light at $30^{o}C$ for 10 days.

(ii) Measurements of specific activity of the extracellular lytic activities of the fungus (by conversion of ergosterol to estimated dry weight) were complicated by the high ergosterol levels obtained when the fungus was grown on a bacterial substrate instead of soluble glucose. Suggested reasons for this anomaly were a the development of longer and thinner hyphae, with consequent greater membrane content, on the insoluble substrate and/or b the production of lysosomes to "package" the extracellular enzymes prior to secretion, which would also increase the amount of membrane, and thus ergosterol levels, present.

(iii) E_1 was not constitutive and was induced by the presence of B.subtilis cells in the medium.

(iv) E_1 was partially purified by ammonium sulphate precipitation and gel filtration, but was then found to be unstable. Addition of B.S.A. to the enzyme solution prevented loss of lytic activity. The inhibitor was probably a protease.

(v) The pH optimum of E_1 (when partially purified) was 3.4 and the mode of action that of a muramidase. Molecular weight was estimated as 17000 and specific activity was increased 7-fold (from 61.4 units mg^{-1} protein to 448 units mg^{-1} protein).

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APPENDIX I - Abbreviations

Term	Abbreviation or Symbol
Buffers	
Sodium formate	formate
Tris(hydroxymethyl) aminomethane	Tris
Media	
Czapek-Dox	Cz-dx
Malt Extract Agar	MEA
Nutrient Agar	NA
Nutrient Broth	NB
Potato Dextrose Agar	PDA
Chemicals	
Bovine serum albumin	BSA
Ethylenediaminetetra-acetic acid	EDTA
1-Fluoro 2-4 dinitrobenzene	FDNB
Phenylmethyl sulfonylfluoride	PMSF
Sodium borohydride	$NaBH_4$
Other	
Bacillus subtilis	*B.subtilis*
Chalaropsis lysozyme	Ch.Lz
Chitin-coated cellulose	C/C
Hen egg white lysozyme	HEWL
High pressure liquid chromatography	HPLC
Lysozyme	lz
Optical density	O.D.

APPENDIX: II

Taxonomic and Morphological Description of Fungal Species Investigated

1. Gliomastix murorum var. felina
2. Gliomastix spp. 1071, 1080, 3857

1. GLIOMASTIX MURORUM var felina was obtained as an isolate from the rhizoplane of clover growing in pasture soil in the Manawatu and was provided by Dr. R.A. Skipp, Plant Diseases Division, D.S.I.R., Palmerston North.

The identification was made by Dr. P.M. Kirk, Commonwealth Mycological Institute, Kew, Surrey, England, as follows:

"Gliomastix murorum (Corda) S. Hughes var. felina (Marchal) S. Hughes. Your isolate practically indistinguishable from IMI 107709 isolated from grassland soil, Canterbury, New Zealand and referred to this species/variety by Dickinson in Mycological Papers, No. 115".

G.murorum v. felina is common in soil, having a world-wide distribution. It appears tolerant of all but acid peats and has been differentiated from G.murorum var. murorum on the basis of conidial measurements (Hammill, 1981). G.murorum v. felina forms aerial ropes in culture, grows well on D-arabinose (unlike many fungi) and prefers L-arabinose to glucose, utilises mannose, cellobiose and starch and various forms of cellulose. On cellulose agar the fungus exhibits cellulolytic activity. Optimum temperature for cellulolysis is 29^{o}C, declining rapidly to 35^{o}C. Optimum pH is 9. Chitinase activity is exhibited. (Williams & Pugh, 1971) (Plates 3,4,5, & 6).

G.murorum v. felina is a destructive parasite of Ophiobolus graminis. (syn: Gaeumannomyces graminis). No antibiotic production has been reported.

Description: see (Dickinson, 1968).

Classification: Mycota

Fungi Imperfecti

Hyphomycete

Phialospora

Gliomastix Gueg (Barron, 1972)

Mycota

Deuteromycete

Moniliales

Dematiacease

Gliomastix Gueg (Barnett, 1955)

2. Three Gliomastix spp. were obtained as freeze dried cultures from the culture collection of Plant Diseases Division, D.S.I.R., Auckland. They were listed as:

1071 Bambusa sp. - bamboo, Mt Albert, Ak., Mar 1962 F.J. Morton H122 (Plate 7)

3857 Soil. Havelock North. H.B. J.B. Taylor NZ 158 (Plate 8)

1080 Timber. North Shore, Ak, Jul 1962. F.J. Morton H147 (Plate 9)

(N.Z.D.S.I.R., P.D.D., 1983)