FINAL REPORT

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SPECIFIC INHIBITORS OF PECTOLYTIC ENZYMES FOR CONTROL OF FUNGAL PLANT DISEASES

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TABLE OF CONTENTS

EXECUTIVE SUMMARY
A. Research Objectives....3
B. Research Accomplishments - Tel-Aviv....4
C. Research Accomplishments - Kathmandu....4
D. Progress Report-Kathmandu....11
E. Description of Project Impact....12
F. Strengthening of Developing Country Institutions....39
G. Final Year Activities....42
List of Figures....43
Table 1....44
Table 2....45
H. Final Report - Kathmandu....46

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EXECUTIVE SUMMARY

The purpose of this project is to carry out a joint research program in collaboration with Prof. Mohan B. Gewali of the Central Dept. of Chemistry, Tribhuvan University, Nepal which we anticipate will lead to the development of new, inexpensive products for application in control of various post harvest and other infectious crop diseases in agriculture. The current project is based on our prior results demonstrating that specific compounds of bacterial origin may control the spread of various plant pathogens by inhibiting those fungal enzymes which destroy the plant tissue. As a target enzyme we chose the polygaluronase(s) from the highly ubiquitous pathogenic fungus *Botrytis cinerea*.

We report here our progress in using biological and synthetic inhibitors, consisting primarily of inorganic polyphosphate to 1. inhibit the target enzyme in an *in-vitro* enzymatic assay, and 2. inhibit the onset of the disease in semi *in-vivo* models incorporating either enzyme -mediated or fungal spore-mediated tissue maceration. Strong inhibition was found in all cases including the growth of *B. cinerea* itself on polypectate as a sole source of carbon. In addition, inhibition was observed with polyphosphates from a variety of sources. Efficacy was shown to depend on polyphosphate chain length. Progress was also made in understanding the mode of PP production in inhibitor producing strains. To this end ^{31}P NMR spectroscopy was used to follow the fate of both intra- and extracellular polyphosphate as well as the production of heretofore unidentified phosphorylated compounds in the cells. Other physiological observations include an effect both of carbon source as well as inorganic phosphate on extracellular inhibitor production. In addition, we have begun Phase II of this project including field testing at the level of green house experiments and larger scale crop storage facilities. We hope to present early findings in this area in subsequent reports.

Also included in this report is a detailed summation of the work of the Nepali investigators who have made remarkable progress in developing their newly constructed laboratory to: 1. develop techniques to produce and assay inhibitor in the laboratory; and 2. initiate screening for the isolation of new strains. It should be stressed that this is the first research laboratory of its kind to be established in the Chemistry Department of Tribhuvan University. Last year, Prof. Gewali received an award given by the King of Nepal as one of Nepal's outstanding young scientists.

A. Research Objectives

Our overall aim in this project is to develop a general approach based on specific pathogenic enzyme inhibition for the control of a variety of plant. Our overall strategy includes: 1. the isolation of pathogenic target enzymes, 2. the isolation of products of microbial origin capable of inhibiting the target polygalacturonase known to play a key role in development of the disease, 3. elucidation of the structure, specificity and mode of action of the inhibitor, 4. development of semi-in-vivo assays for evaluation of product efficacy, and 5. development of systems for the production of such inhibitors in large scale for product testing under storage conditions.

The results presented in this report outline our progress in items 1,2, and 5 and shed new light on item 3 as well.

B. Research Accomplishments --Tel-Aviv

1. Background.

The experimental basis for our study is based on the fact that certain fungal and bacterial plant diseases are caused by the elaboration of tissue hydrolyzing enzymes which frequently play a key role in the pathogenic process of penetration, infection and tissue maceration. Our working hypothesis suggests that it might be possible to intervene in the disease process, by specifically inhibiting these pathogenic enzymes without poisoning the pathogen. In this report we describe our progress in the isolation and characterization of inhibitors isolated from different microbial

sources. These compounds exert specific inhibition of a pathogenic endoplygalacturonase isolated from the pathogen, *Botrytis cinerea*.

Currently, we are using cell free extracts prepared from both *Streptomyces satsumaensis* and another natural streptomycete isolate, A50; both producers of endopoly-galacturonase inhibitor. This material was shown to consist primarily of polyphosphate (PP). One unit of the inhibitor activity is defined as that amount of material which inhibits the activity of a standard amount of enzyme by 50%. Typical inhibitor levels in shake flask fermentations are generally between 200-500 units per ml of cell culture broth. In addition to the enzyme inhibition test, a semi in vivo assay was also developed. The method depends on the ability of the inhibitor to prevent fruit tissue maceration normally brought about by a fungal spore or mycelium-associated polygalacturonase (PG). The method involves immersing disks of cucumbers in the presence of either isolated PG or in the presence of spores of the pectinase-producing fungus. A series of experiments was carried out in order (a) to understand the dynamics of growth and inhibitor production by the streptomycete culture, (b) to isolate microbial polyphosphates from a variety of sources other than streptomycetes and to test their ability to inhibit the target endopolygalacturonase, (c) to determine the size of natural and synthetic polyphosphate required for enzyme inhibition, and (d) to study the specificity of inhibition to other enzyme activities of similar nature. In this report we are also presenting results of experiments employing the synthetic polyphosphate as substrate.

2. *Results*

Polyphosphate composition of the inhibitor and NMR studies.

The polygalacturonase inhibitor was found to consist of more than 98% by weight acid-labile phosphate. The polyphosphate content of a *S. sp.* A-50 culture was followed in both intra- and extracellular fractions using ^{31}P-NMR spectroscopy (Fig. 1-5). It can be seen that polyphosphate was present in cells of 2-day cultures (Fig. 2) and to a lesser extent in cells of 4-day cultures (Fig. 3). In contrast, polyphosphate was found only in the cell-free broth of 4- day cultures (Fig. 5), but not in the broth of the younger, 2-day cultures. It should be noted, however, that there may be a slight amount of polyphosphate in the medium which is not detectable by NMR. Moreover, an unknown material present in the A-50 cells was also found in the broth of both early (2-day) and late (4-day) cultures.

Polyphosphate production in S. sp. A-50.

The NMR results shown above illustrated the fact that polyphosphate accumulates first as an intracellular product during the first two days and is primarily released into the medium as the cells approach stationary phase (Fig. 6). In these experiments the cells were grown on glycerol as carbon source using glutamate as a nitrogen source. As illustrated in Fig. 7 , extracellular polyphosphate was also produced when the cells were grown on either glycerol or on glucose as carbon sources. In the latter case, however, polyphosphate release was delayed by 24 hours, suggesting the possibility that glucose may be causing a delay in the development of a polyphosphate export system. This is consistent with the finding that the amount of

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intracellular polyphosphate in glucose-grown cells is not significantly lower than that in cells grown on glycerol. Similar differences were observed between glycerol and maltose grown cells (not shown).

In addition to the effect of carbon source, polyphosphate production was found to increase with increasing concentrations of inorganic phosphate (Pi) in the medium (Fig. 8). Optimum extracellular inhibitor production was at 4mM Pi, while at higher concentrations, polyphosphate production decreased. No effect on cell biomass was observed within this concentration range. When cells were grown on 4mM Pi about 40% of the total added inorganic phosphate was recoverable as acid-labile Pi in polyphosphate.

Inhibition of PG from Botrytis cinerea by various polyphosphates.

As shown in Fig. 9 polyphosphate preparations from several different sources can be used to inhibit the target phytopathogenic PG preparations from *B. cinerea*. At inhibitor concentrations between 10 and 500 μmoles Pi equivalents per ml, inhibitory activity of commercial polyphosphate (Sigma) consisting of an average chain length of 92 inorganic phosphate units, did not vary significantly from that of biological polyphosphate preparations from *S. sp.* A50 and *Colletotrichum gloeosporiodes*. Gel filtration experiments suggest that these latter preparations possess an average chain length of between 75 and 100 phosphate residues. In this regard it was of interest to determine the effect of polyhphosphate chain length preparations. As shown in Fig. 10, inhibitory activity is highly dependent on polyphosphate chain length. For example, P-75, with an average chain length of 92 phosphate residues showed a 50% inhibition in the standard assay at a concentration of 15μg/ml, while similar levels of inhibition with shorter chain polyphosphates of

42 and 22 phosphate residues required concentrations of 45 and 61 μg/ml respectively. In terms of the number of molecules required for 50% inhibition, the P45 had 14% of the inhibitory activity seen with P75, while P25 had less than 5%. No soluble fractions of polyphosphate larger than P-75 were commercially available, although preliminary evidence suggests that a higher molecular weight polyphosphate from the yeast *Saccharomyces cerevisiae* is even more active than P-75 (not shown). Consistent with these findings are the results in the Table 1 which demonstrate the sensitivity of two polyphosphate inhibitors to alkaline phosphatase. It can be seen that cleavage resulting in a 50% breakdown of the polyphosphate, completely abolished the inhibitory activity.

The tissue maceration assay.

Polygalacturonase(PG) inhibition using inhibitor preparations from biological sources and/or synthetic polyphosphate was analysed in an assay more closely resembling the pathogenic effect of *B. cinerea.* The basis of this assay is the PG-mediated hydrolysis of pectin normally present in the tissues of cucumber fruit or other plant tissues. It has been shown that accompanying such hydrolysis is an increase in turbidity in the suspension resulting from polygalacturonase-catalyzed tissue maceration. The kinetics of such maceration mediated by the PG of *B.cinerea* was studied in two experimental systems. In the first system the soluble PG from *B. cinerea* was used to catalyze the pathogenic reaction. In the second system the cucumber disk suspension was infected with viable spores of the pathogen.

In order to test the effect of polyphosphate on tissue maceration, increasing concentrations of PP were added to cucumber disks in the

presence of different enzyme concentrations. Maceration was assayed 5 (Fig. 11) and 24 hrs (Fig 12) after enzyme addition. The results shown in these two figures indicate that the onset of maceration was delayed to a large extent at enzyme concentrations of 1.0 U/ml and was inhibited almost completely at inhibitor concentration of 2.5 mg/ml when enzyme concentration did not accede 0.5 U/ml. When enzyme concentrations were higher than this, onset of maceration was delayed for several hrs and then proceeded at a much slower rate.

Inhibition of spore-mediated maceration.

As illustrated in Fig 13, tissue maceration can also be catalyzed by the spores of *B. cinerea* and inhibited by the polyphosphate inhibitor. In this experiment $4.2X10^5$ spores per milliliter were used to initiate maceration. At 24 hrs. In the presence of 1.0 mg/ml PP the turbidity was about 20%of the control. By 44 hrs after infection there was an increase in turbidity and the effect was diminished.

Growth inhibition of B. cinerea on polypectate..

The inhibitory effect of polyphosphate on PG was also when *B.cinerea* was grown on polypectate as sole carbon source, since growth is dependent on hydrolysis of the pectate. If the PG normally produced by the culture is inhibited by polyphosphate then growth should be inhibited. As shown in Fig. 14 and Table 2, biomass development of the phytopathogen was severely inhibited in the presence of increasing concentrations of PP. In addition, the added polyphosphate appeared not be destroyed during the course of the experiment and retained its ability to inhibit both PG activity using the DNS assay as well as tissue maceration.

C. Research Accomplishments -- Kathmandu

During the course of this project an ongoing research program has been initiated within the main campus at Central Department of Chemistry and expanded to include the Central Department of Microbiology at Tribhuvan University of Nepal under the leadership of Prof. Mohan P. Gewali. During his recent visit to the laboratory in October, 1994, Prof. Gewali brought with him a detailed report summarizing the research activities in his laboratory. This report from Nepal is included in this report in its entirety.

PROGRESS REPORT

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INTRODUCTION

Phytopathogenic fungi are known to cause tissue maceration and soft rot diseases in post harvest fruits and vegetables. The extracellular polygalacturonase produced by these phytopathogens is responsible for causing tissue maceration. The substrate for these pectolytic enzymes are pectic substances. In the plant cell, the pectic substances are the primary constituents of the middle lamella and are composed of high molecular weight polymer consisting of a backbone of α-4 linked D-galactopyranose interspersed with 1, 2 - α - linked rhamnopyranose. There are two main groups of pectolytic enzymes that attack pectin substances - esterases and depolymerases. The esterase split the methyl ester group of esterified poly - D - galacturonic acids and is referred to as pectin esterase or pectin methylesterase. The depolymerizing enzymes are either hydrolyses or lyases. Pectolytic enzymes act upon the pectin substances and bring about tissue maceration resulting in the soft rot diseases.

The commonly used methods to control post harvest diseases such as applications of fungicides, cold storage, UV radiation treatment suffer from various constrains such as high cost, health hazard, mutation etc. A new approach to control these post harvest diseases would be to find out specific microbial inhibitors of fungal pectolytic enzymes. The advantage of using inhibitors of microbial origin lies on the fact that they are natural, biodegradable, affordable and would be expected to produce less health hazard. This new approach is modeled after Umezawa's novel approach to the development of a new class of microbial products that have acted as specific inhibitors of eukaryotic enzymes. (1) The efforts of the Umezawa group have led to the development of a relatively large number of therapeutic agents.

The soft rot disease caused by Botrytis cinerea is a major world wide problem for post harvest fruits and vegetables. (2) Nepal is no exception for this disease. (3)

In this report, we describe our progress

- in the production of crude polyglacturonase from Botrytis cinerea and Geotrichum candidum
- in enzymatic characterization of both of these enzyme preparations
- in tissue maceration of different fruits and vegetables
- in studies on microbial and plant inhibitors of these crude enzymes

MATERIALS AND METHODS

1. MICROBIAL STRAINS

1.1 Pure strains of Botrytis cinerea, Geotrichum candidum and Streptomyces satsumaensis were obtained from Professor D. Gutnick, Department of Microbiology, Tel Aviv University, Israel

1.2 **Isolation of actinomycetes from the soil** : soil samples were collected from different locations. Soil sample (1 gm) was mixed with 10 gm of calcium carbonate and grounded with a mortar and pestle. Insect wings in form of chitin were added to this mixture. Finely powdered mixture was transferred to a petridish and incubated at 30°C for 7-9 day. Different dilutions of this extract was transferred to MY solid plates (malt 0.1% and yeast extract 0.04%). The plates were incubated at 30°C for 3-4 days.

1.3 **Isolation of microbial population from compost extract** : Actinomycetes population were isolated by surface plating 0.1 ml of compost extract on water yeast agar (WYA) containing (g l^{-1}) : yeast extract 0.25; K_2HPO_4 0.5, Betrafen 0.05 and agar 18g. Bacterial population were isolated on nutrient agar (NA) containing Betrafen solution (50 mg l^{-1}). Fungal populations were isolated by surface plating 0.1 ml of compost extract on potato dextrose agar (PDA) containing chloramphenicol (0.32g l^{-1}).

2. GROWTH CONDITION

2.1 **YGP medium** : A starter YGP (0.1% yeast extract, 0.1% glucose and 0.1% pectin) medium was prepared in 50 ml of double distilled water. A small agar plate disc (~5mm) was cut from a PDA plate containing sporulating fungus and transferred to the starting medium. The flask was kept in a water bath shaker at 30°C for 3-4 days. This inoculum from the starter medium was transferred to same YGP medium (10% volume). Samples were taken at various time for biomass and enzyme activity.

2.2 **Glucose free medium** : A small agar disc (~5mm) was cut from PDA plate containing sporolating fungus and was transferred to a glucose free starter medium containing 0.5g pectin, 0.5g $NaNO_3$, 0.25g KH_2PO_4, 0.12g $MgSO_4$, $7H_2O$, 0.05g $CaCl_2$ and traces of $FeCl_3$ in 50 ml of double distilled water. The flask was kept in water bath shaker at 30°C for 2-3 days. This inoculum from the starter medium was transferred to same glucose free general culture (10% volume). Samples were taken at various times for biomass and enzyme.

2.3 MSG medium : A starter MSG medium containing mono sodium glutamate 2.5 g/l, glycerol 10g/l, $K_2HPO_4.3H_2O$ 1 g/l, $MgSO_4.7H_2O$ 0.5 g/l, 1 ml of $FeSO_4.7H_2O$ (1%) and 2 ml of trace metal solution consisting of $CuSO_4.5H_2O$ 0.1%, $ZnSO_4$ O.1% and $MnSO_4$ 0.1% were prepared and a small agar disc (~5mm) of Streptomyces satsumensis was added. It was incubated at 30°C for 3-5 days in a water bath shaker. This inoculum from starter medium was added to same MSG medium. Samples were taken at various time for inhibitory activity.

2.4 Growth study : Growth of fungus in culture medium was studied in terms of turbidity of solutions. For turbidity measurement, culture samples were taken at various interval of time and turbidity was measured at 600mm using distilled water as blank.

3. PREPARATION OF CRUDE ENZYME

Mycelia of the broth was removed by centrifugation followed by filtration on Whatman I filter paper. Protein was precipitated either by adding $(NH_4)_2SO_4$ or by cold acetone. Precipitation with $(NH_4)_2SO_4$ was not satisfactory. Cold acetone was added to the clarified supernatant (cell free broth) to 20% (by volume) concentration and precipitate was centrifuged off. The supernatant was adjusted with acetone to 66% (V/V) and centrifuged for ten minutes. The precipitate was dissolved in 0.1 M sodium acetate buffer (pH4.2). DNS assay and protein measurement on crude enzyme preparation revealed increased specific activity.

4. POLYGALACTURONASE ASSAY

4.1 **Reducing groups determination (DNS assay) (4)** : Polygalacturonase activity was determined by measuring the amount of released reducing groups by pectin hydrolysis with 3,5- dinitrosalycilic acid (DNS) reagent. Mono - D- galacturonic acid was taken as standard. The assay system contain 3-4 tube containing 0.1 ml, 0.2 ml, 0.3 ml, 0.4 of crude enzyme solution and 0.1 M sodium acetate buffer pH 4.2 was added upto each tube. 0.5 ml of 0.5% pectin dissolved in 0.1 M sodium acetate buffer pH 4.2 was added in each tube and incubated at 30°C. After 5 minute, 4 ml of DNS reagent (NaOH 1%, DNS 1%, phenol 0.2%, sodium sulfite 0.05%) was added and boiled for 15 minutes. After cooling, absorbance was measured at 575 mm. Reaction mixture also contained boiled enzyme which was used as control.

One unit of enzyme was that amount of enzyme which catalyzed the release of 1 mg of mono glacturonic acid per hr.

4.2 **Cup-plate assay (5)** : Pectin (2.5g), agar (lg) and ammonium oxalate (1.25g) were mixed with 125 ml of 0.1 M sodium acetate buffer pH 4.2 and autoclaved. After cooling, gel was poured on petridish to give 5 mm thick layer. Cup (7mm) were cut with the help of a cork borer. Different units of enzyme from 0.125 to 1 were added into the cup and incubated for 18 hours at 30°C. At least 4 cups were taken for each unit of enzyme preparation. The plates were

14

flooded with mixture of concentrated. HCl. and distilled water (1:1V/V). Enzyme activity was indicated by a semi transparent halo zone that was formed.

4.3 **Viscosity loss measurement :** In a typical experiment, 8ml of 0.5% pectin in 0.1 M acetate buffer (pH4.2) with 2ml of crude enzyme was taken. Viscosity loss was monitored by using a Viscometer at room temperature. The Viscosity of 2ml of enzyme plus 8ml of acetate buffer was assigned 100 percent viscosity loss.

4.4 **Protein determination :** Protein was measured according to procedure of Bradford using bovine serum albumin as a standard.

5. ENZYMATIC PROPERTIES OF CRUDE POLYGACTURONASE

The pH dependence of enzyme activity of polygalacturonase was followed in buffers of pH ranging from 2.2 to 7.2 Polygalacturonase was preincubated in different pH and pectin substrate of the same pH was added. Usual DNS assay was performed. Solutious of pH 2.2 to 5.2 were prepared from 0.1 M sodium citric buffer. Solutions of pH 6.2 to 7.2 were prepared from 0.1 M potassium dihydrogen phosphate buffer.

Temperature stability of the enzyme was checked by preincubation of polygalacturonase in different temperature range 10-70°C followed by incubation with pectin substrate at 30°C. DNS assay was performed.

Dependence of enzyme activity on incubation time, substrate concentration and enzyme concentration were carried out with DNS assay.

6. TISSUE MACERATION ASSAY (6)

6.1 **For potato, carrot and turnip discs :** They were locally purchased and gently wiped with a moist sponge to remove dirt. They were soaked for 15 minutes in hypochlorite solution in order to destroy contaminating micro organisms and washed several times with water. Disc of about 6mm were excised at random with cork borer to a depth of 5 mm. The discs were rinsed twice with 0.1 M sodium acetate buffer pH. 4.2 and blotted with filter paper before use. In an assay vial containing 5 ml of acetate buffer were incubated at water bath shaker at 30°C. Polygalacturonase was added at different concentrations from 0.5 U/ml to 4U/ml. Enzyme mediated maceration was expressed a the net turbidity measured at 540 mm.

6.2 **For cucumber discs :** Green Cucumber fruits were washed thoroughly and sliced perpendicular to their long axis into several pieces. Cylinders of white pericarp tissue were removed by a 6 mm diameter cork borer. These cylinders were sliced into 6 mm long section using a blade. Sections(5 slices) were stored in distilled water for 1 hour and were gently blotted and incubated in enzyme solution at 30°C. After incubation, cucumber slices were placed on a sieve and loosened cell were removed by spraying water. After putting the slices in

distilled water for about half an hour, the slices were blotted and weighed. Net weight loss was determined by correcting for weight changes for slices incubated in identically prepared boiled solutions of enzyme or solutions containing no enzyme.

7. INHIBITOR ACTIVITY ASSAY

Two methods were used for inhibition studies.

7.1 The inhibitory activity was assayed by measuring the residual polygalacturonase activity after the enzyme was pre incubated with inhibitor solution for fifteen minute at 30°C. The assay system contained 1-2 unit of enzyme with different concentration of inhibitor solution and 0.1 M sodium acetate buffer pH 4.2 up to 0.5 ml which was incubated with 0.5 ml of 0.5% pectin dissolved in 0.1 M acetate buffer pH 4.2 for 5 minutes at 30°C. A control sample without inhibitor solution was incubated in parallel with test sample. Percent inhibition of polygalacturonase activity was calculated from the equation $\frac{A - B}{A} X\ 100\ \%$ when A and B are values of glacturonic acid (expressed in absorbancy at 575mm) in the absence and in the presence of inhibitor respectively.

7.2 Cup-plate essay also used for inhibition studies. In the cup, a mixture of enzyme and the potential inhibitor (1:2v/v) were added and incubated for 18 hour at 30°C. The plates were flooded with a mixture of concentrated HCl and distilled water (1:1v/v). Inhibition was determined by comparing with control (enzyme only) in a semi transparent halozone that was formed.

RESULTS AND DISCUSSION

This section is divided into two parts. First part describes our works on polygalacturonase from. Geotrichum candidum. Second part is devoted to our works on Botrytis cinerea polygalacturonase.

Part I

Geotrichum candidum ,a citrus rot pathogen, was grown in two liquid media viz. YGP and glucose free medium. The fungal growth and production of enzyme were measured at various interval of time. The trend of fungal growth and enzyme production matched each other in both liquid culture media. (Table 1,2,)

Optimum production of polygalacturonase (PG) was observed at different time periods. In YGP medium, enzyme production was maximum at 72 hour after inoculation from starter. Optimum production of enzyme was reduced to 48 hours in glucose free medium. Enzyme was harvested at the time of optimum production. Enzyme in crude form was obtained by precipitation of centrifuged broth first with 20% cold acetone (v/v) followed by 66% cold acetone. This enzyme served as target enzyme of study of kinetics and inhibition.

Table No:-1

Growth and production of PG by Geotrichum candidum in YGP medium

Time (Hour)	Growth (600)	Vol. of culture broth (ml)	Units/ml	Protein µg/ml	Total units	Total protein (mg)	Specific activity (U/mg)
0	0.16	275	6.8	12.2	1870.0	3.3	556.6
24	0.6	265	8.8	39.0	2332.0	10.3	226.4
48	0.8	225	44.1	57.0	11245.5	14.5	775.5
72	0.7	245	52.6	34.1	12887.0	8.3	1552.6
96	0.68	235	40.7	39.0	9564.5	9.2	1039.6

Table No:-2

Growth and Production of PG by G. candidum in glucose free medium

Time (hour)	Growth (600)	units/ml	Protein mg/ml	Specific activity (U/mg)
0	0.54	5.1		
5	0.75	6.3		
24	1.2	26.7		
48	1.3	39.5		
72	1.2	22.9	39.0	587.2

Precipitation with 66% cold acetone brought about ten fold increase of specific activity of partially purified polygalacturonase. (Table No 3)

Table:-3

Partial purification of PG with cold acetone from glucose free medium

Before precipitation			After 66% cold acetone precipitation		
Units/ml	Protein (mg/ml)	Specific activity (U/mg)	Units/ml	Protein (mg/ml)	Specific activity (U/mg)
22.9	0.039	587.2	54.7	$8.1X10^{-3}$	6753.0

Assay of Crude Polygalcturonase:

Viscosity Loss Measurement:

Viscosity loss measurement of 0.5% pectin solution in 0.1M acetate buffer was carried out using 4.5 U/ml of enzyme at 17°C. About 71% of viscosity loss was recorded at 110 minutes (Table No. 4.) Viscosity loss of pectin solution by the action of enzyme indicated degradation of pectin into smaller fragment of lower molecular weight.

Table No:- 4

Viscosity loss measurement

S.N.	Time in minute	% viscosity loss
1	5	10.8
2	15	18.9
3	25	27.0
4	35	37.8
5	60	50.0
6	85	62.1
7	110	70.9

Dingl's Cup Plate Assay:

Plates were developed with concentrated hydrochloric acid and water (1:1). Semi-transparent zones formed around the cups. This indicated hydrolysis of substrate by PG. Plots of enzyme unit Vs diameter of ring (including cup) depicted that relationship between the diameter of ring and enzyme concentration was linear (Table No. 5)

Table No:5

Dingle's cup plate assay.

Units	0.125	0.25	0.5	1
Diameter (mm)	13	14	16	20

Tissue Maceration Assay:

Enzyme mediated maceration of plant tissue was observed in our experiment with orange peels. However onset of maceration was delayed and it was only after 22 hours we could observe considerable maceration,. Maceration was also found to be dependent upon the enzyme concentration (Table No. 6; Fig I)

Table No:-6
Orange peel maceration assay

Time \ Units/ml	Control	5	10	20
0 min	0	0	0	0
30 min.	0.01	0.02	0.03	0.04
90 min	0.01	0.04	0.06	0.07
150 min.	0.02	0.07	0.10	0.12
210 min.	0.02	0.10	0.134	0.15
22 hr.	0.04	0.40	0.50	0.60

Characterization of Crude Polygalacturonase:

Effect of Temperature on Stability:

Enzyme was stable in temperature range 0-30°C. More than 50% of PG activity was lost at 50°C. and 86% at 60°C. Enzyme was completely deactivated at 70°C. (Table No. 7)

Table No:-7

Influence of temperature on enzyme stability

Temp	0°C	5°C	12°C	20°C	25°C	30°C	40°C	50°C	60°C	70°C
%Act	100	100	100	100	100	100	92	57	14	0

Effect of pH on Stability :

At pH 4.2 enzyme showed the highest activity. pH 3.2 retained 66.6% of activity whereas pH 2.2 had 20.8% activity. At pH 5.2, 6.2 and 7.2 activities were 62.0%, 41.6%, 16.6% respectively. (Table No. 8)

Table No:- 8

Influence of pH on enzyme stability

pH	2.2	3.2	4.2	5.2	6.2	7.2
% Act	20,8	66.6	100.0	62.0	41.6	16.6

Influence of incubation time on activity

The reducing group liberation by PG increased with increment of incubation time. The plot of μ mole reducing groups/ml versus incubation time illustrated that velocity of reducing group liberation by PG was linear upto 30 minute of incubation time. (Table No 9)

Table No:-9

Influence of incubation time on enzyme activity

Time (min)	5	10	15	20	30	60	120
μmole reducing group/ml	0.41	0.72	1.0	1.36	1.5	1.9	2.0

Influence of Enzyme concentration on activity

Results obtained indicated that activity of enzyme was dependent upon the concentration of enzyme. The relationship between activity and enzyme concentration was linear upto 16U/ml of enzyme concentration. (Table No. 10)

Table No:-10

Influence of enzyme concentration on enzyme activity

Units/ml	2	4	8	12	16	20	24
Abs 575	0.16	0.33	0.58	0.85	1.1	1.3	1.5

Influence of Substrate Concentration on Enzyme activity

Plot of 0.001 mole reducing groups per 30 minutes versus pectin concentration depicted that velocity of reducing group liberation by PG was linear upto 1% pectin concentration (Fig II). Above 1% pectin concentration, enzyme showed typical saturation pattern. From Linewever Burk plot, Vm and Km were found to be 0.0120m mole reducing group per 30 min. and 0.48 (w/v) respectively (Fig III).

Table No:-11

Influence of substrate concentration on enzyme activity

Pectin Concentration (%)	0.001 m mole reducing group/30min (v)	10/v
0.1	2.1	4.76
0.3	4.2	2.38
0.5	5.7	1.75
0.8	7.8	1.28
1.0	9.0	1.11
2.0	10.2	0.98
3.0	10.8	0.93

Inhibition Study:

Inhibition studies were attempted on mercuric chloride, EDTA, iodoacetic acid upto concentration of 1 mg/ml. But no conclusive inhibition was observed with these chemical. But solvent DMSO did produce inhibition. The inhibition with 100µl, 150µl and 200µl of dimethyl sulphoxide were 15.4%, 28.9%, 46.2% respectively. (Table No. 12)

Table No :-12

Inhibition study on dimethyl sulphoxide

Vol of DMSO	Abs. 575	Remaining (%) activity	Percent inhibition
0 µl	0.52	100	0
100 µl	0.44	84.6	15.4
150 µl	0.37	71.1	28.9
200 µl	0.28	53.8	46.2

Part II

In glucose free medium incubated at 30°C, polygalacturonase production was found to be optimal at 48 hour of growth of B cinerea (Table 13) and precipitation with cold acetone, specific activity of this enzyme preparation increased from 1700 to2700 (Table 14)

Table 13

Production of polygalacturonase by B. cinerea in glucose free medium

Time (hr)	Growth (Measured at 600 mm)	Volume (ml)	Unit/ml.	Total Unit
0	0.75	275	4.2	1155
24	1	265	12.7	3365
48	1.50	255	17	4335
72	1.50	245	16.5	4042

Table 14

Partial purification of crude enzyme

Purification Step	Volume (ml)	Unit/ml	Total Unit	Protein (µg/ml)	Total Protein (mg)	Specific activity
Cell free broth after centifugation	400	17	6800	10	4	1700
66% acetone precipitation	60	36	2160	13.3	0.798	2706

Dingle' cup-plate assay of this polygalacturonse preparation revealed the linear relationship between halo ring and concentration of enzyme. Enzyme was found to be stable in the temperature range 0-30°C and at 70°C, it was completely deactivated(Table 15) The optimum pH for polygalacturonase activity was pH 4.2 (Table 16)

Table 15

Influence of temperature on enzyme activity

Temperature C	0	5	20	30	40	50	60	70
Activity (%)	100	100	100	100	71	57	14	0

Table 16

Influence of pH on enzyme stability

pH	2.2	3.2	4.2	5.2	6.2	7.2
Activity (%)	67	96	100	51	38	25

Volocity of reducing group liberation by polygalacturonase was linear up to 16 Unit/ml of enzyme concentration (Table 17, fig IV) and up to 20 minute of incubation time (Table 18, Fig V)

Table 17

Influence of enzyme concentration on enzyme activity

Enzyme concentration unit/ml	DNS activity (at 575 nm)
1	0.10
2	0.20
4	0.39
8	0.69
16	1.20
22	1.30

Table 18
Influence of incubation time on enzyme activity

Incubation time	μ mole of galacturonic acid released
5	5.4
15	7.9
30	9.1
60	10.1
120	11.0

21

Tissue maceration Studies

Enzyme mediated weight loss of cucumber discs was observed (Table 19). The weight loss is found to be dependent upon enzyme concentration.

Table 19

Cucumber discs maceration influence of enzyme concentration

Time (hour)	1				2				3			
	No. enzyme	1 U/ ml	5 U/ ml	10 U/ ml	No enzyme	1U/ ml	5 U/ ml	10 U/ ml	No enzyme	1U/ ml	5U/ ml	10U/ ml
Weight loss (%)	4	6	22	24	9	20	52	65	14	34	73	82

With potato, carrot and turnip discs, tissue mceration was slow. However, after 21 hour of incubation, tissue maceration was observed (Tables 20,21 and 22; Figs VI, VII and VIII)

Table 20

POTATO DISCS MACERATION

Influence of enzyme concentration

Turbidity measured at 540 nm

Time (Hrs)	No enzyme	Unit of enzyme		
		0.5 U/ml	1U/ml	4U/ml
0.5	0.01	0.01	0.01	0.01
1.0	0.01	0.02	0.02	0.02
1.5	0.01	0.02	0.02	0.02
2.0	0.01	0.02	0.02	0.02
2.5	0.01	0.04	0.04	0.04
3.0	0.01	0.04	0.04	0.04
4.0	0.02	0.06	0.06	0.06
21.0	0.08	0.26	0.39	0.60

Table 21

CARROT DISCS MACERATION

Influence of enzyme concentration

Turbidity measured at 540 nm

Time (Hrs)	No enzyme	Unit of enzyme		
		0.5 U/ml	1U/ml	4U/ml
0.50	0.01	0.01	0.01	0.01
1.00	0.01	0.01	0.01	0.01
1.50	0.01	0.01	0.01	0.02
2.00	0.01	0.02	0.02	0.02
3.00	0.01	0.02	0.02	0.04
4.00	0.01	0.02	0.02	0.06
21.00	0.05	0.25	0.50	0.80

Table 22

TURNIP DISCS MACERATION

Turbidity measured at 540 nm

Time (Hrs.)	No enzyme	Unit of enzyme		
		0.5 U/ml	1U/ml	4U/ml
0.00	0.00	0.00	0.00	0.00
0.50	0.00	0.01	0.01	0.01
1.00	0.00	0.01	0.01	0.01
1.50	0.01	0.02	0.03	0.03
2.00	0.01	0.02	0.03	0.03
2.50	0.01	0.02	0.03	0.03
3.00	0.01	0.02	0.03	0.03
4.00	0.01	0.03	0.03	0.04
21.00	0.08	0.25	0.30	0.40

Inhibitory action of liquid culture extracts of Streptomyces satsumaensis

Streptomyces satsumaensis was grown in MSG liquid culture medium at 30°C. In some cases, feeble inhibitory activity as well as polyphosphate concentration in 3-4 days old liquid cultures were detected. No concrete conclusion could be arrived. Recently, we have started using arginine in place of sodium glutamate as nitrogen source, we are getting encouraging results. further work is in progress.

Table 23

Inhibitor effect of some metal ions on polygalacturonase activity

Chemicals	Remaining activity %	
1. $BaCl_2$	86	
2. $CoCl_2$	110	
3. $FeSO_4$	100	
4. $HgCl_2$	80	
5. $MnCl_2$	203	
6. KCl	100	
8. $NiCl_2$	100	
9. $(CH_3 COO)_2 Pb$	100	
10. $ZnCl_2$	90	
11. EDTA	100	
12. Urea	80	

The enzyme (2U/ml) was preincubated with 5m mole of inhibitors at 30°C for 30 minutes and then, pectin solution was added.

Inhibitory action of methanolic extracts of Kathmandu home herbal remedies

Eighteen Kathmandu home herbal remedies i.e. Acorus calamus, Allium sativum, Areca catechu, Azadirachta indica, Cumin cyaminum, Cinnamomum tamala, Cinnamomum zevlanicum, Curcuma longa, Euphorbia thymifolia, Ferula narthex, Glycyrrhira glabra, Myristica fragrans, Nardostachys jatamasi, Swertia chirata, Sizygium aromatica, Terminalia belarica, Terminalia chebula and Zanthoxylum armatum methanolic extracts were tested against this polygalacturonase preparation. Out of eighteen herbal extracts, only Areca catechuextract showed inhibitory activity against the target enzyme.
(Tables 24, 25)

Table 24

Inhibition assay by Cup-plate method using Areca catechu extract as an inhibitor

Inhibitor cencentration	Inhibition percentage
250 µg/ml	50
333 µg/ml	73
500 µg/ml	83

Table 25

Inhibition study by DNS method

Inhibitor concentration	Inhibition percentage
100 µg/ml	28
200 µg/ml	63
400 µg/ml	74
600 µg/ml	85

Inhibitory action of microbial isolates

Microbial samples were isolated from different locations of Kirtipur campus, Tribhuvan University and from different places in Pokhara, Nepal. Seven pure isolates namely MG-1, MG-2, MG-3, MG-4, MG-5, MG-6 and MG-7 were grown in MSG liquid cultures and were subjected to inhibitory studies by both cup-plate method as well as DNS method. None of them was found to depict inhibitoryaction except MG-7. Even in the case of latter sample, inhibition could be observed on the 5th and 6th days of inoculation with cupplate assay. No inhibition was observed with DNS. assay possibly because of simultaneous production of reducing sugars in the liquid culture. Further work is in progress.

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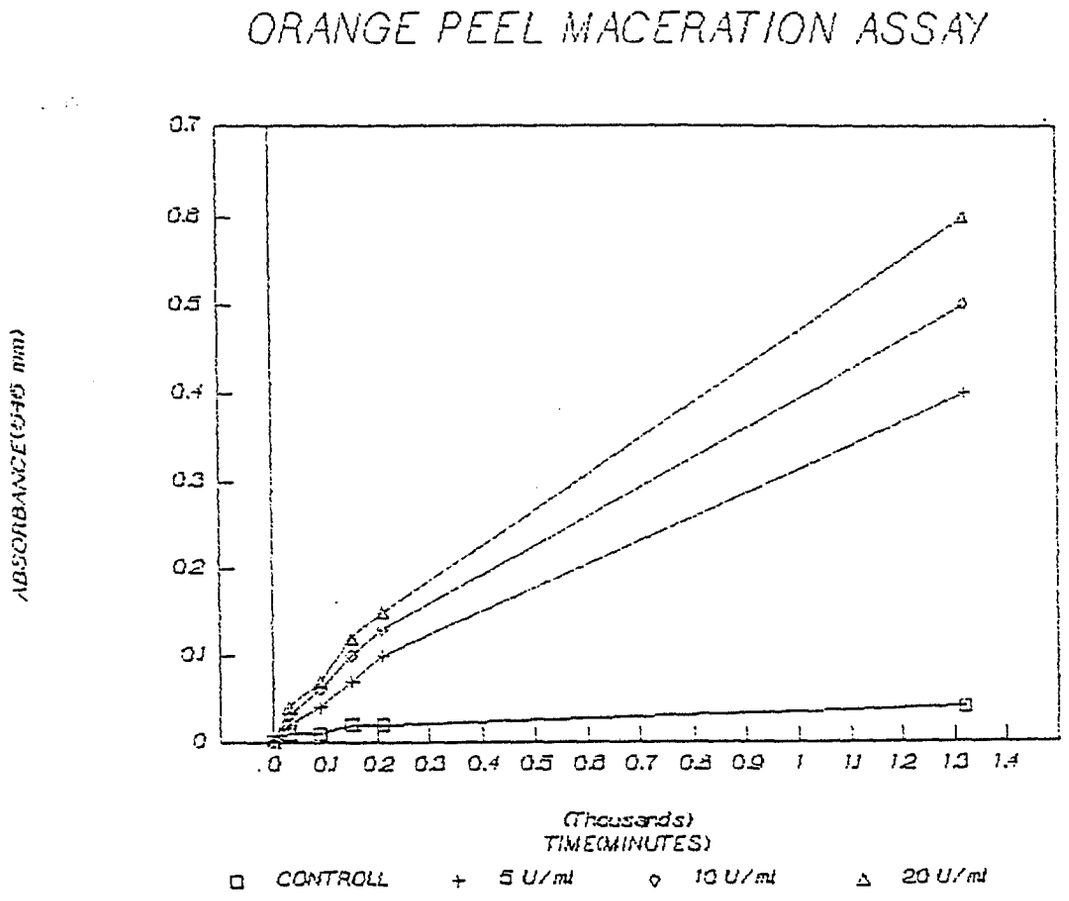
6. Bateman, D.F., 1968. Netherland Journal of Plant Pathology, 74 (Supplement 1) : 67 -80.

Fig I


ORANGE PEEL MACERATION ASSAY
ABSORBANCE(545 nm)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1 1.1 1.2 1.3 1.4
(Thousands)
TIME(MINUTES)
CONTROLL
5 U/ml
10 U/ml
20 U/ml

30

Fig II

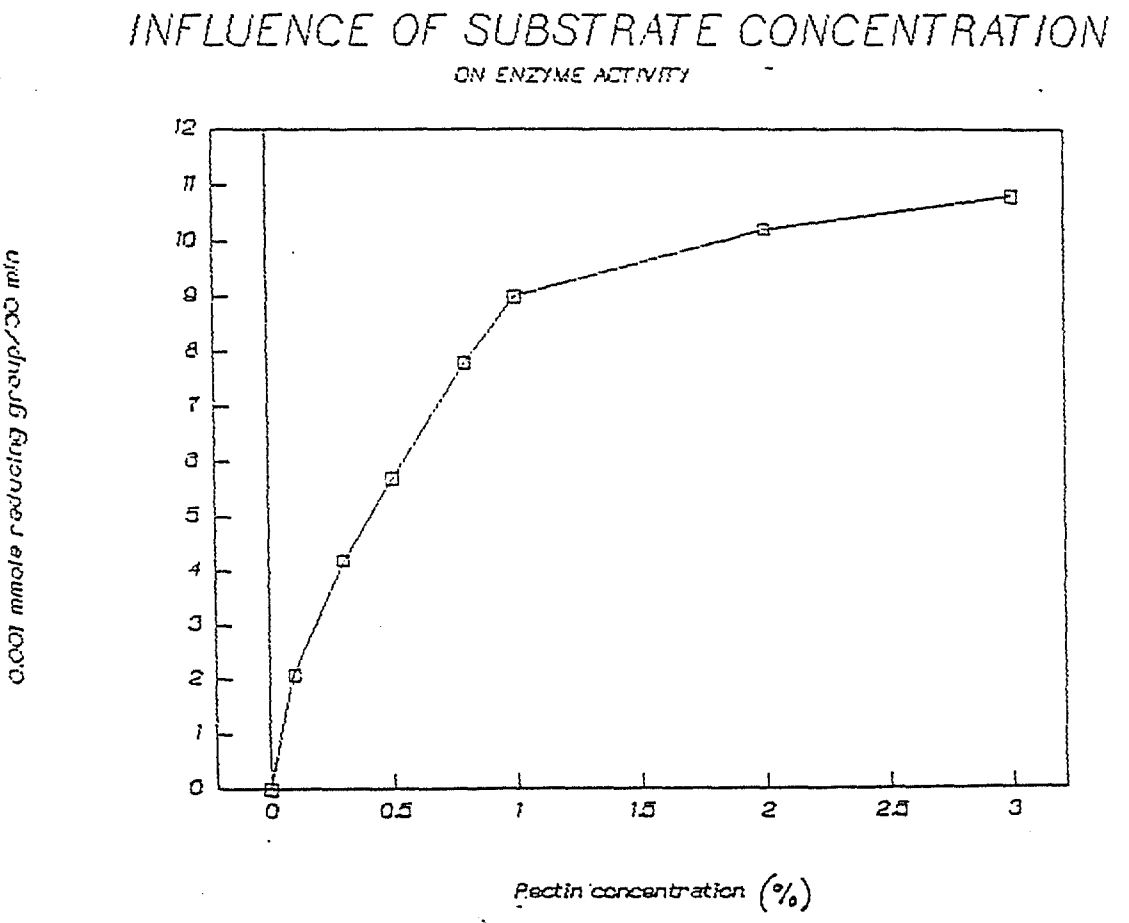
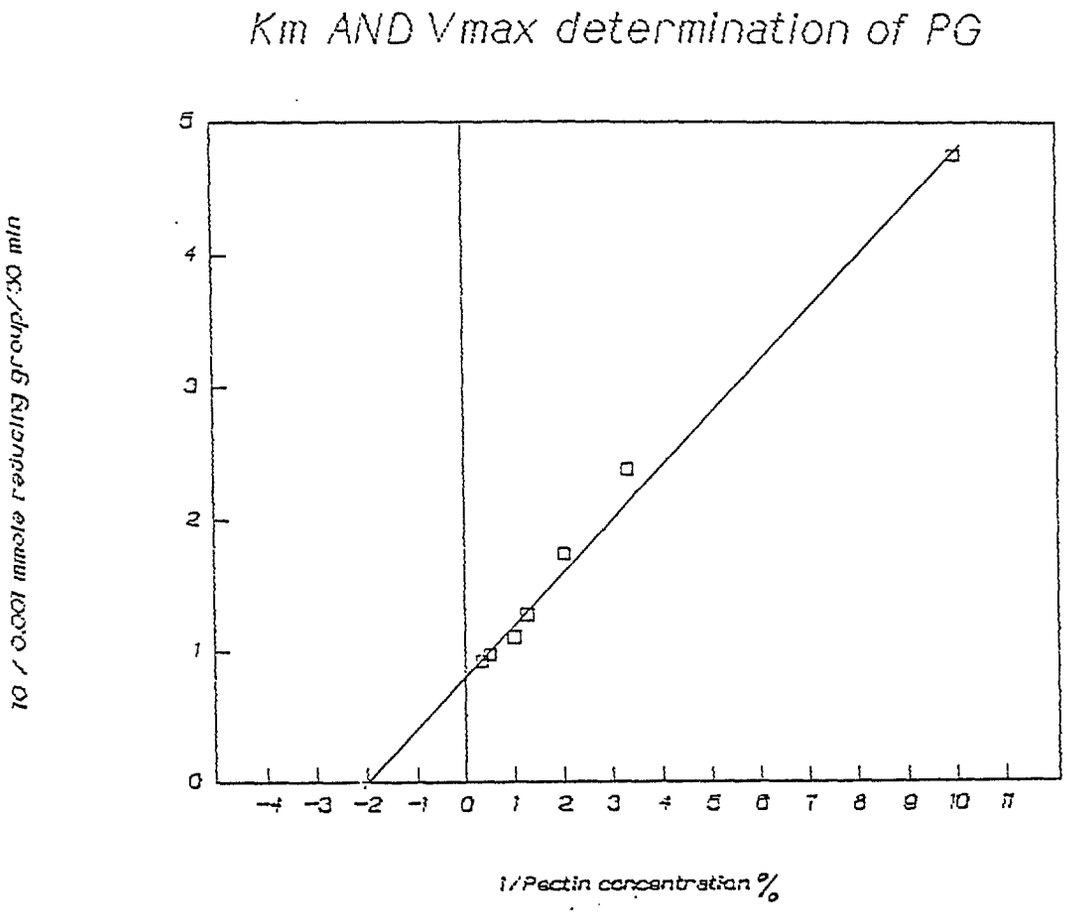

INFLUENCE OF SUBSTRATE CONCENTRATION
ON ENZYME ACTIVITY
0,001 mmole reducing group/30 min
12
11
10
9
8
7
6
5
4
3
2
1
0
0
0.5
1
1.5
2
2.5
3
Pectin concentration (%)

Fig III


Km AND Vmax determination of PG
10 / 0.001 mmole reducing group/30 min
1/Pectin concentration %
0
1
2
3
4
5
-4
-3
-2
-1
0
1
2
3
4
5
6
7
8
9
10
11

32

Fig IV

ENZYME ACTIVITY

Influence of enzyme concentration

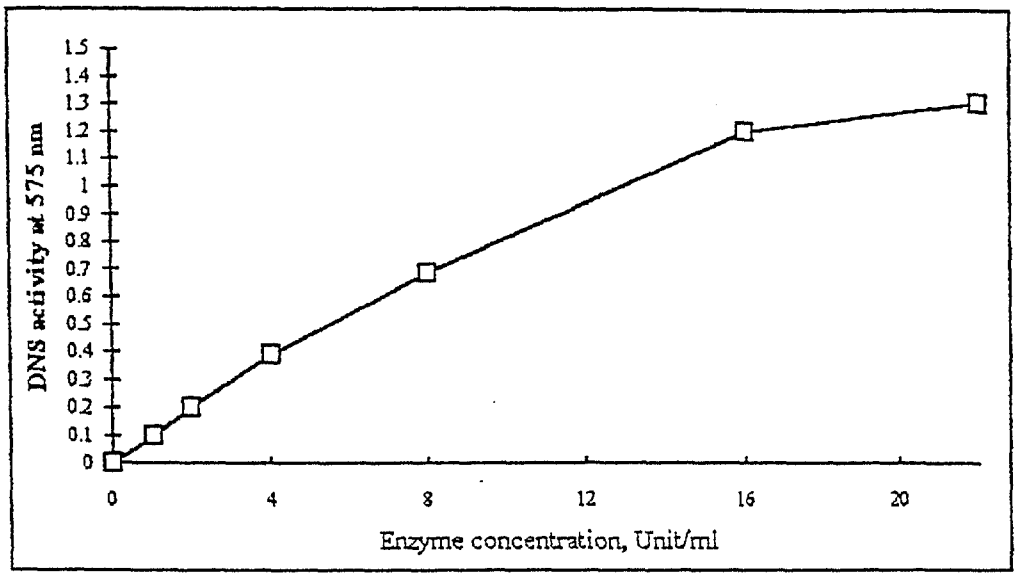


Fig 4

ENZYME ACTIVITY

Influence of Incubation time

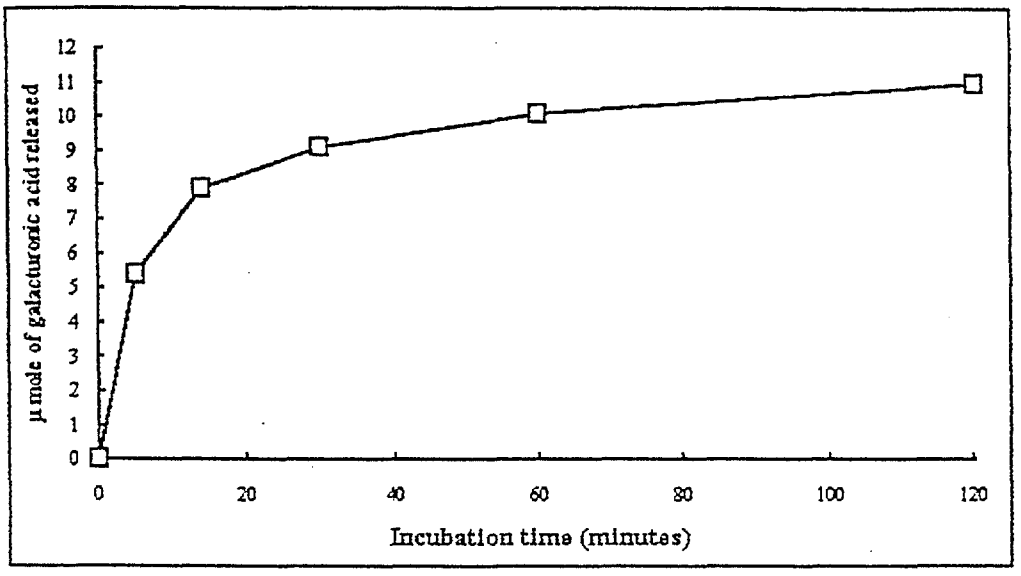
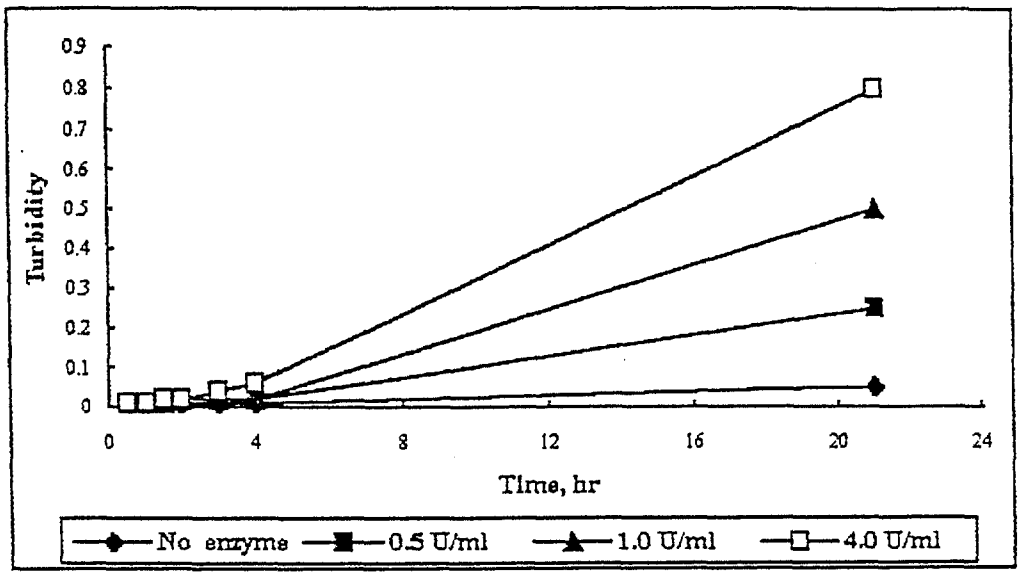


Fig VI

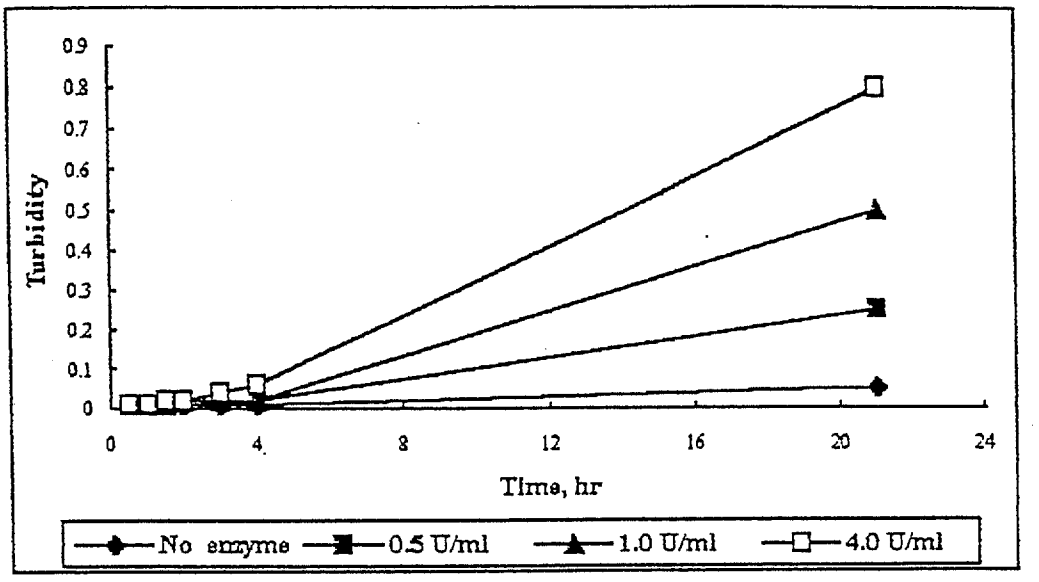
POTATO DISCS MACERATION

Influence of enzyme conc. (OD at 540 nm)



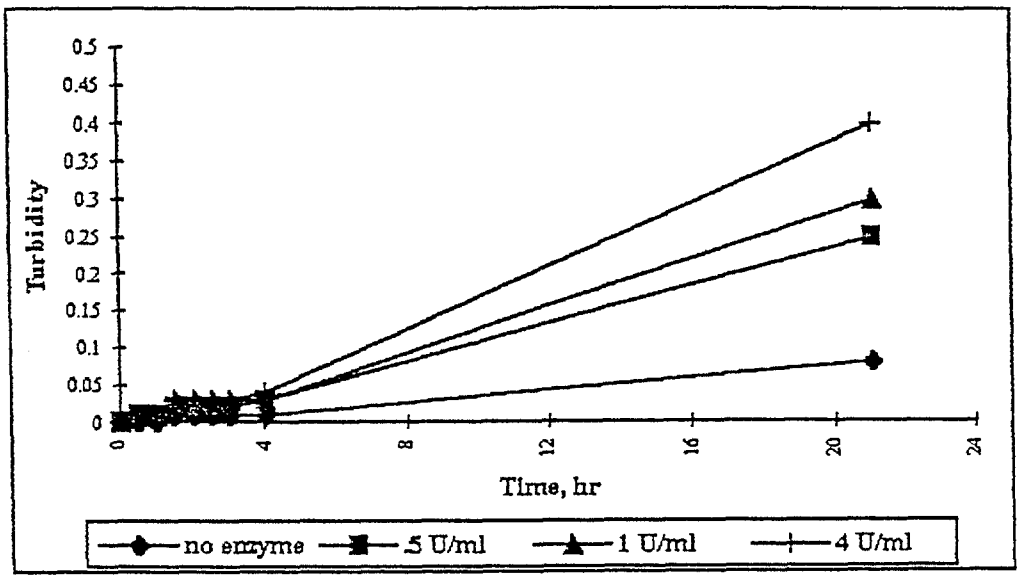
CARROT DISCS MACERATION

Influence of enzyme conc. (OD at 540 nm)



TURNIP DISCS MACERATION

Influence of enzyme conc. (OD at 540 nm)



D. Scientific Impact of Collaboration.

Since the inception of this project a number of groups have initiated strong programs in various aspects of polyphosphate metabolism. For example, the laboratory of Prof. Arthur Kornberg in Stanford is now almost totally devoted to studying the enzymology, molecular genetics and metabolic implications of polyphosphate synthesis and breakdown. In this regard, one the unique features of the Streptomycetes used in this project lies in the fact that the polyphosphate produced is exported to the growth media, while in all other reported cases polyphosphate accumulates only within the cells. Understanding these results and the basis of polyphosphate export are likely to have have important implications for the potential use of polyphosphate preparations either within microbial cells, or as isolated fermentation products as inexpensive, convenient and environmentally friendly products in phytopathogen control in agriculture. We are particularly excited about the recent progress in the maceration assays with *Botrytis cinerea* since this phytopathogen is considered to be responsible for over 25% of crop damage throughout the world.

In addition, polyphosphates may find new uses in the development of biofiltration and chelation systems for the biological removal of heavy metals from various water and soil environments. In addition, the production of intracellular polyphosphate is currently the method of choice in sewage treatment systems for the efficient removal of inorganic phosphate from waste waters. Understanding the behavior of these new strains may shed light on

how to stabilize these polyphosphate producing systems to ensure efficient phosphate removal.

In terms of the scientific collaboration, the results offer potential new modes of screening for target organisms which may produce elevated levels of phytopathogenic enzyme inhibitors. This will allow the Nepalese chemists and microbiologists to take advantage of the biodiversity in their own country to isolate specific strains and novel activities. In addition, Prof. Gewali's training in natural products chemistry and NMR analysis will enable his groups to carry out some of the simply NMR experiments on polyphosphate production, breakdown and export. This is likely to be possible in the near future, since Prof. Gewali will be responsible for the functioning of the new NMR machine which has been ordered for Tribhuvan University.

E. Description of the Project Impact

We anticipate that the by the time the project is complete we will be able to evaluate number of biological products for their ability to control either post-harvest and/or infectious phytopathogenic diseases in various industrial and rural settings. This should also pave the way for production of novel formulations employing various polyphosphate preparations as one of the active ingredients. A positive result in any of these directions might lead to the eventual development of new agricultural products which could be manufactured and applied regionally throughout the third world.

F. Strengthening of Developing Country Institutions

As already reported, the Vice Chancellor Tribhuvan University has donated about 120 square meters of laboratory space in order to carry out the CDR project. This laboratory has been equipped for all standard biochemical and microbiological experimentation. This makes it one of the very few laboratories of its kind in Nepal. During the past year, Prof. Gewali has initiated and expanded the collaboration to include members of the Central Department of Microbiology in Tribhuvan University. In addition, to staffing with research students, the combination of both laboratories is sufficiently well equipped to run a number of advanced laboratory courses within the University framework. These facilities will also allow the investigators to expand their activities to include the screening program. In addition, they will provide the University with the necessary capability to train advanced students in the areas of Applied Microbiology, Microbial Physiology and various aspects of Biotechnology. They will also enable other well trained faculty members in the Nepali departments to expand their research activities.

G. Research Activities for the Final Year

Research Area	Activities	Location
Botrytis cinerea	Maceration assays: 1. Inhibition of multienzyme systems 2. Testing of novel formulations 3. Effects of environmental conditions on maceration Infectivity assays	Tel-Aviv Kathmandu
Enzyme inhibition	1. Specificity 2. Kinetics 3. New inhibitors 4. Purified enzyme, crude enzyme, spores 5. Zymogram analysis	Tel-Aviv Kathmandu
Screening for new products		Kathmandu
Polyphosphate metabolism and production (see enclosed manuscript)	1. Enzymes for synthesis and breakdown 2. NMR analysis of intra- and intercellular polyphosphate 3. Identification of new phosphorylated compound 4. Pi and P-P transport	Tel-Aviv

List of Figures

Fig. 1	^{31}P NMR of growth medium.
Fig. 2	^{31}P NMR of 2-day cells
Fig. 3	^{31}P NMR of 2-day broth
Fig. 4	^{31}P NMR of 4-day cells
Fig. 5	^{31}P NMR of 4-day broth
Fig. 6	Growth and production of polyphosphate
Fig. 7	Production of polyphosphate on glucose and glycerol
Fig. 8	Effect of inorganic phosphate on extracellular polyphosphate production
Fig. 9	Inhibition of PG by polyphosphates
Fig. 10	Effect of polyphosphate chain length on inhibition of PG
Fig. 11	Inibition of *Botrytis cinerea* PG in a semi-*in-vivo* system; Five hours
Fig. 12	Inibition of *Botrytis cinerea* PG in a semi-*in-vivo* system; Twenty two hours
Fig. 13	Inibition of *Botrytis cinerea* PG in a semi-*in-vivo* system Inhibition of maceration
Fig. 14	Inibition of *Botrytis cinerea* growth by polyphosphate.

42

Exp 4

9156 medium

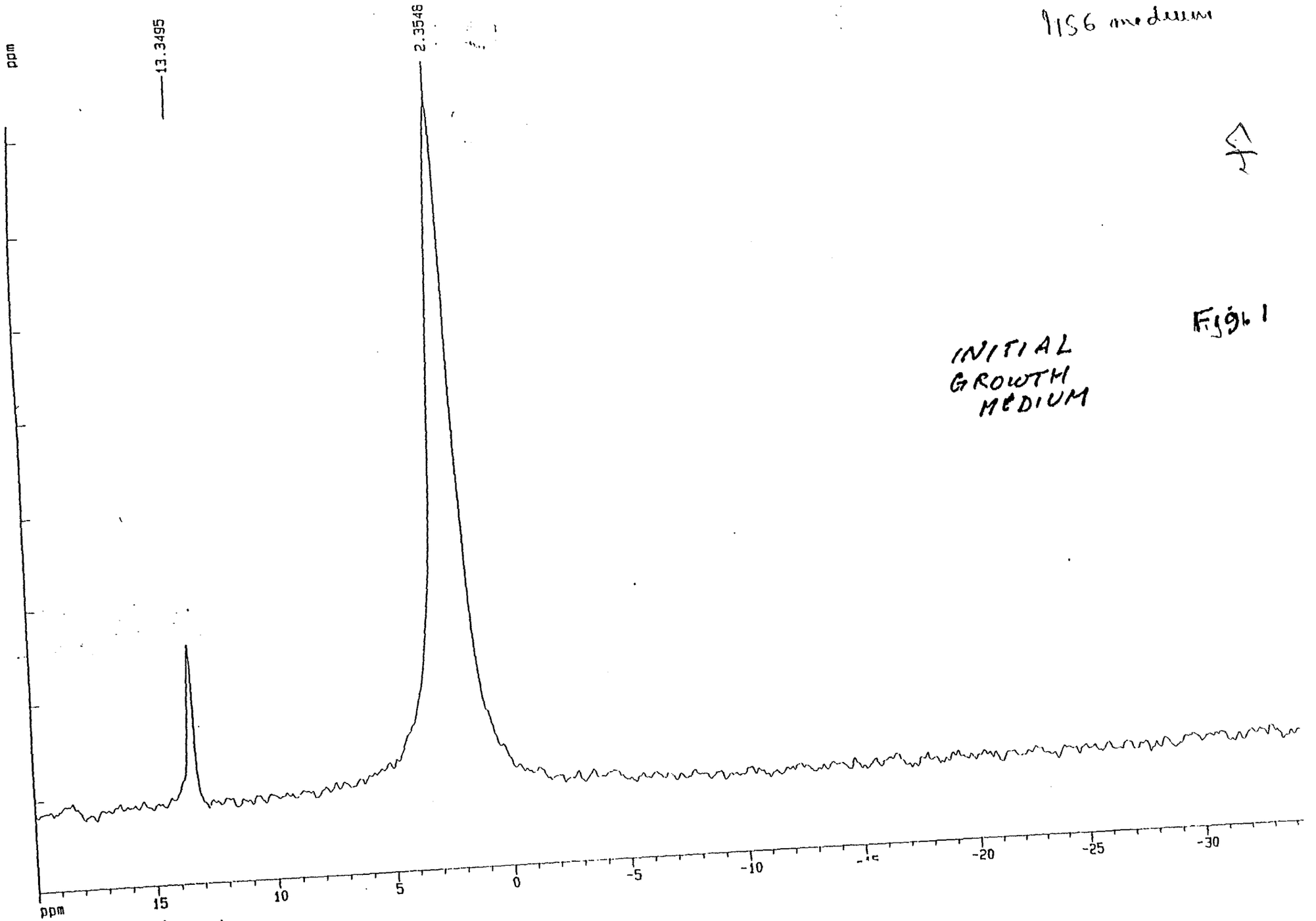
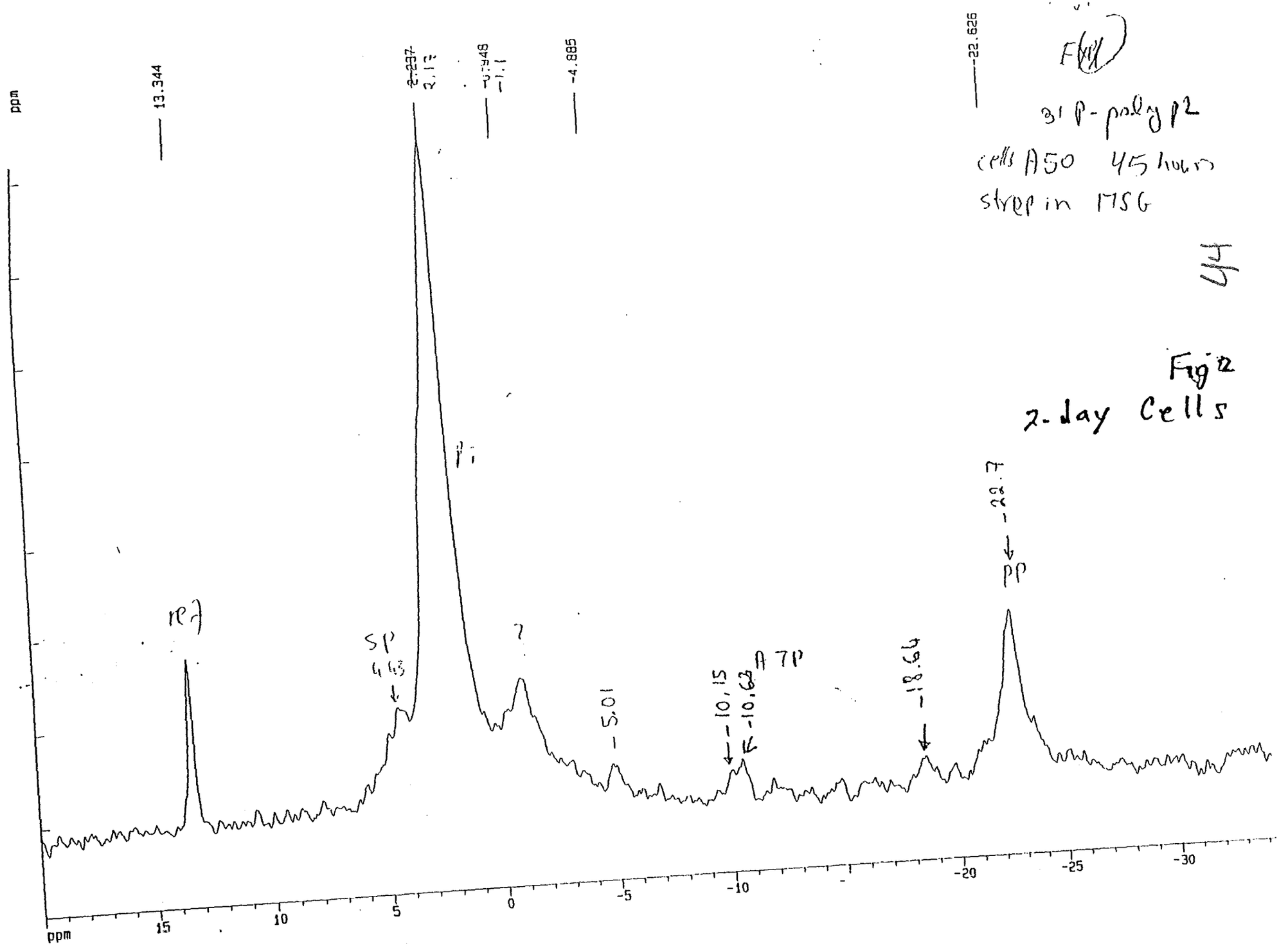


Fig 6.1

INITIAL GROWTH MEDIUM



31P-poly P2
cells A50 45 hours
strep in MSG

44

Fig 12
2-day Cells

(3)

A50 sup
45 hrs
1756

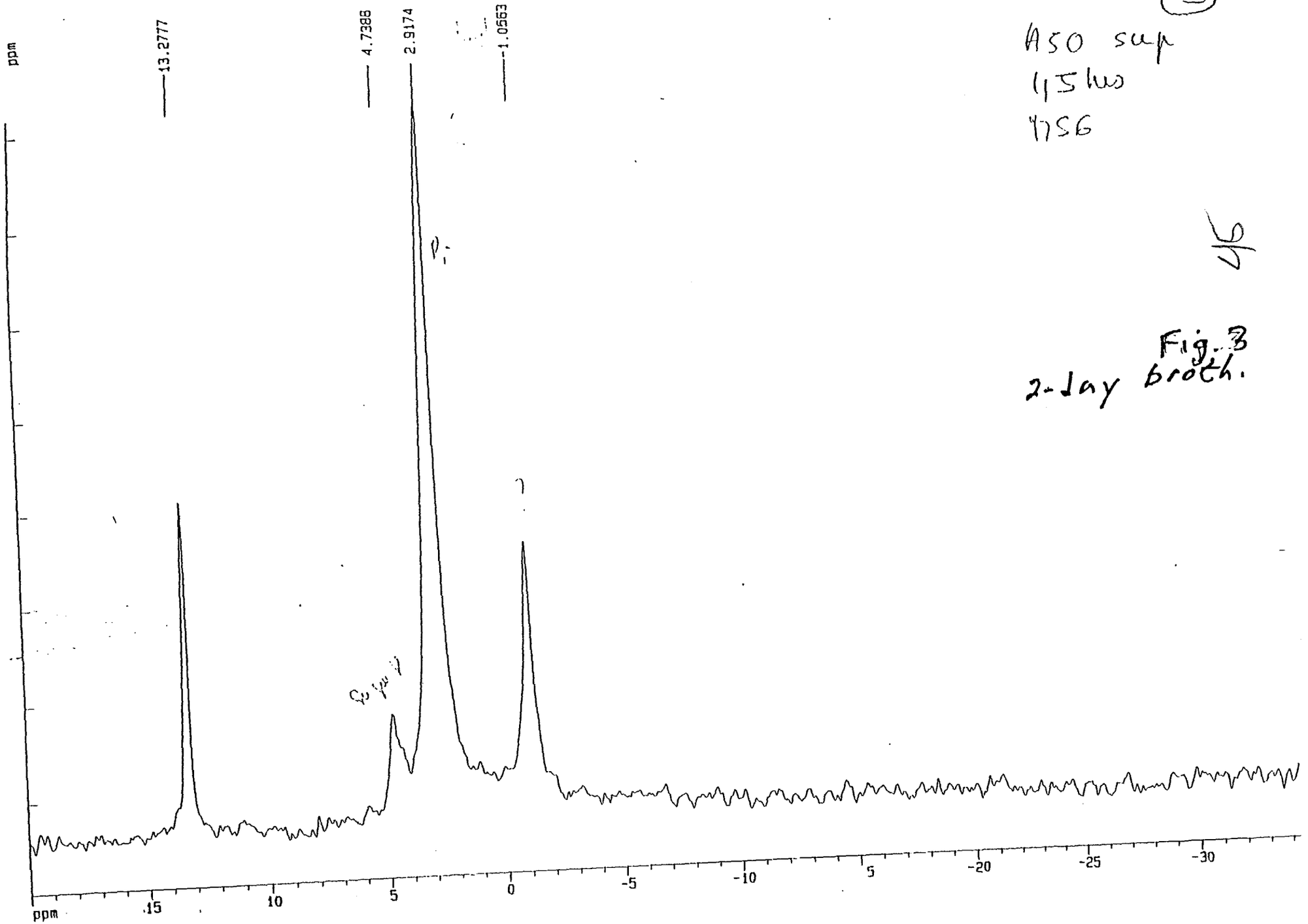


Fig. 3
2-day broth.

1150 cells
NS6 4 days
PPi exp 4

4b

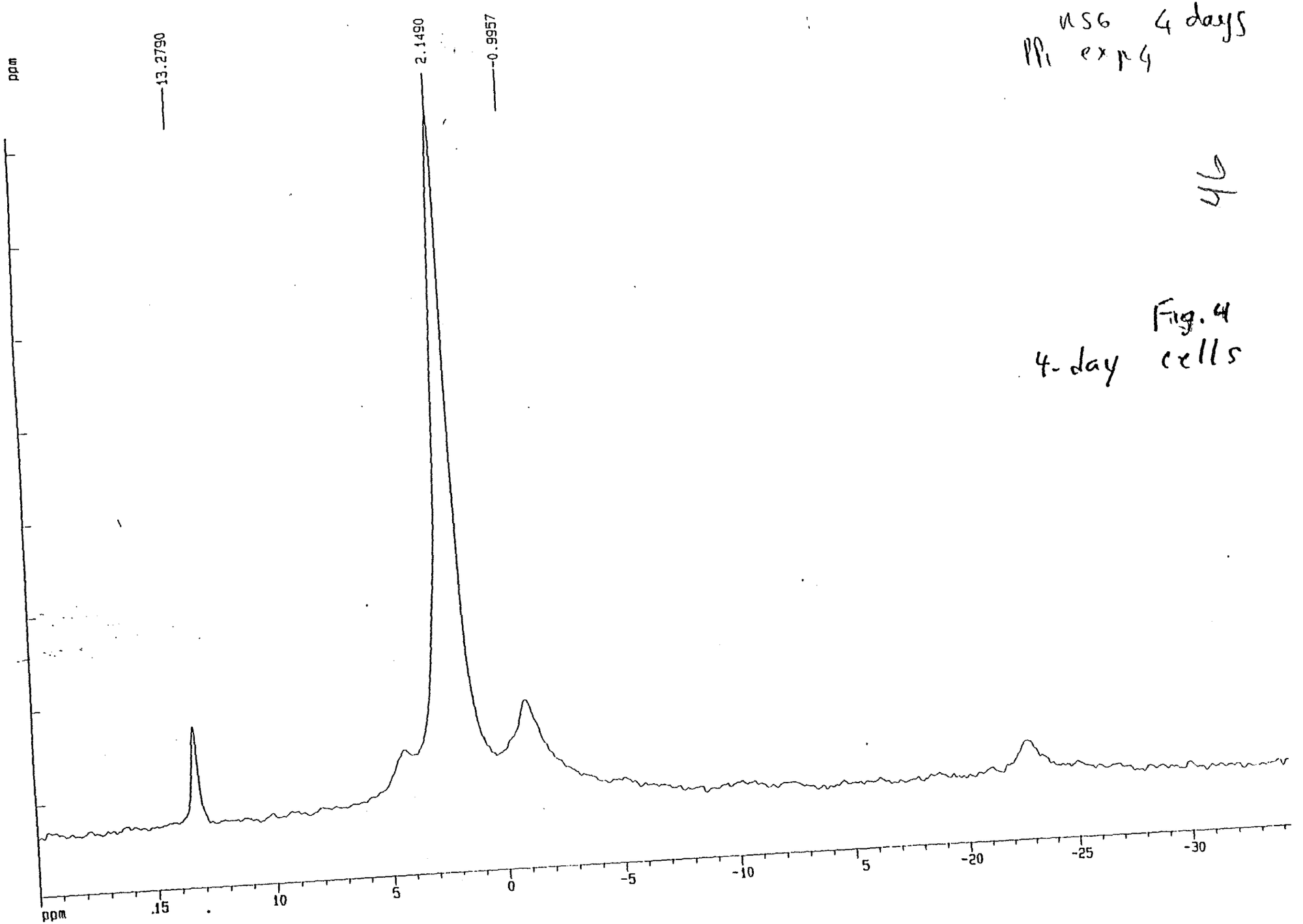
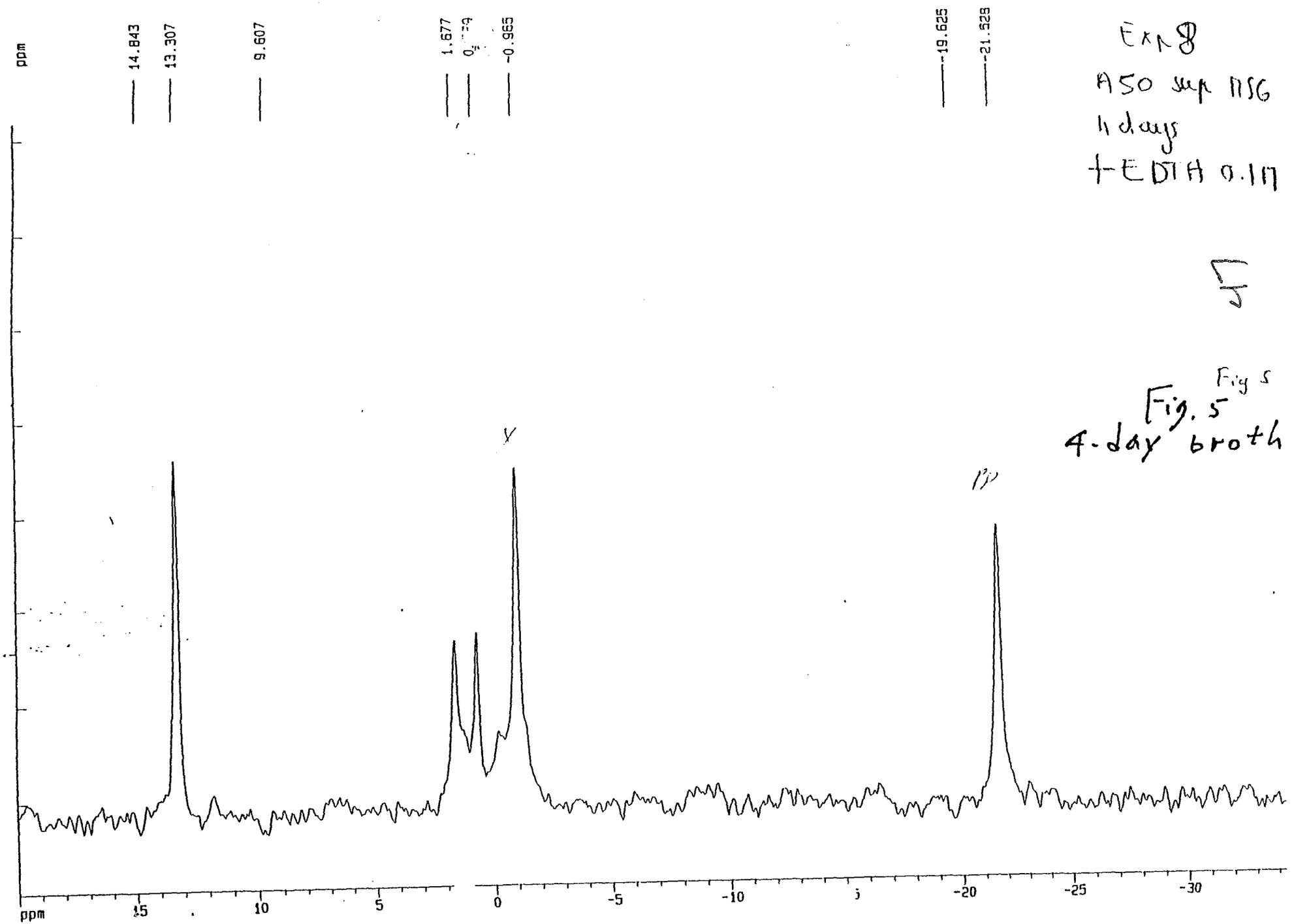


Fig. 4
4-day cells

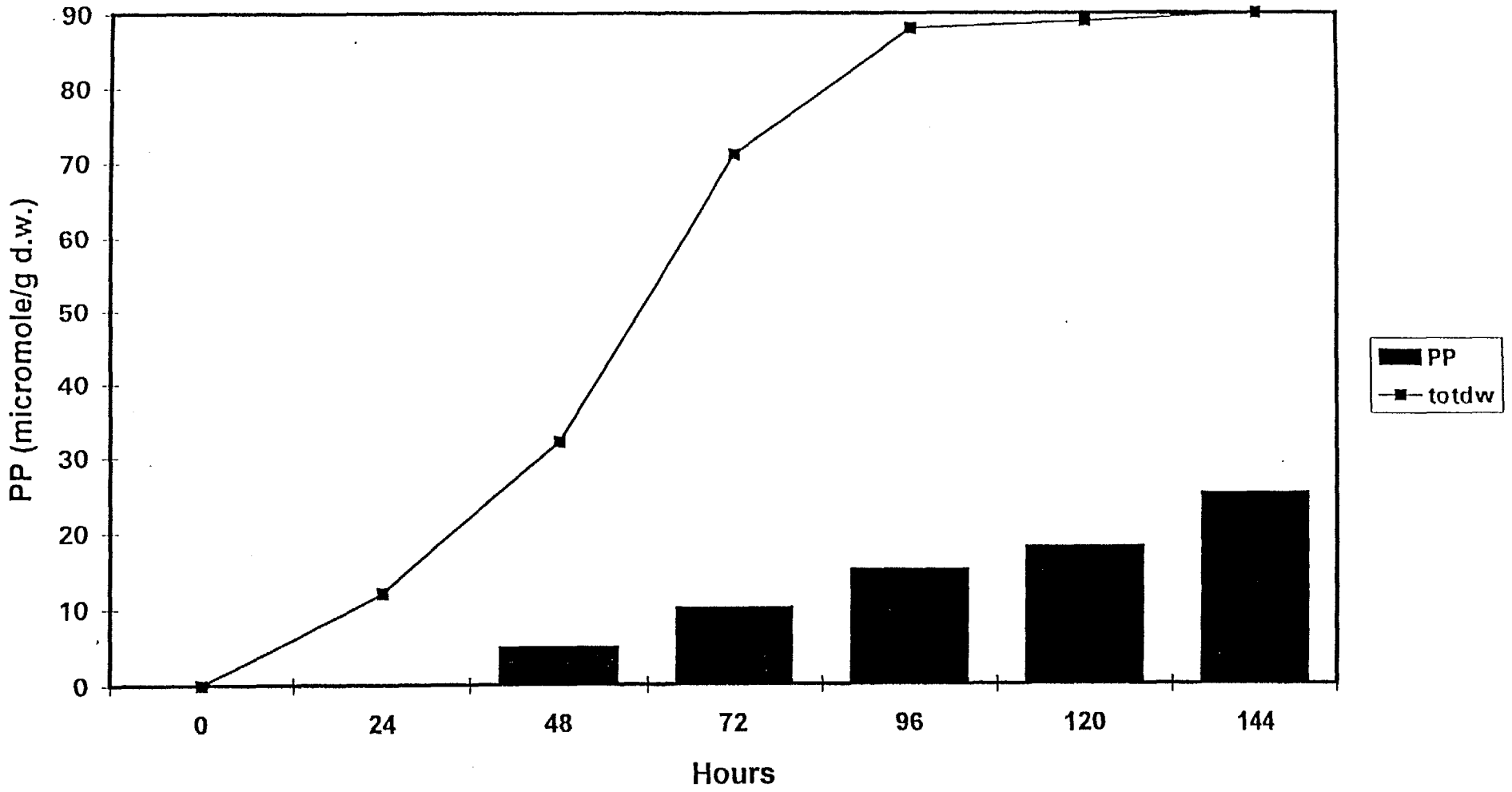


Ex 8
A50 sup MSG
4 days
+EDTA 0.1M

5

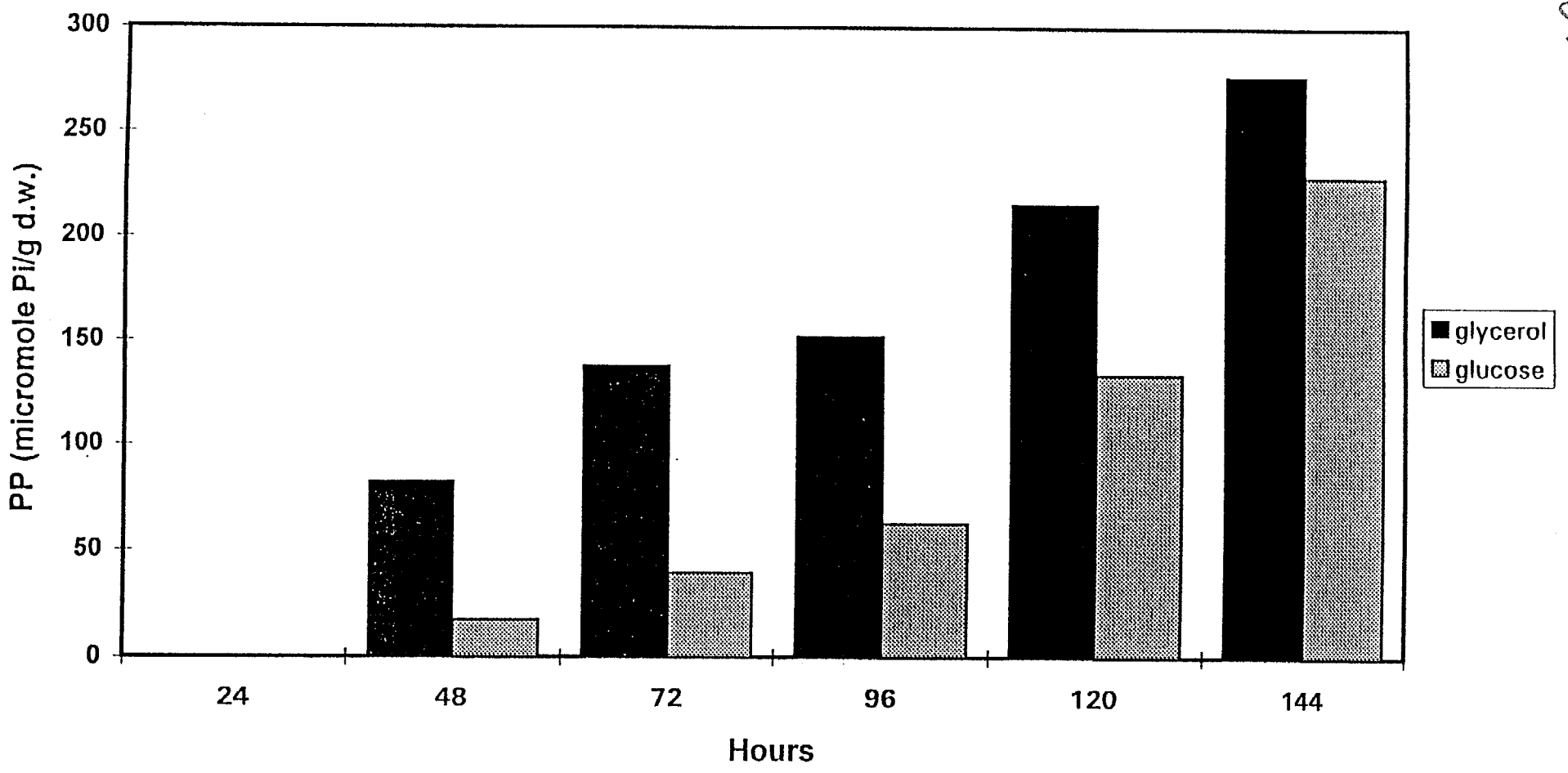
Fig 5
Fig. 5
4-day broth

Fig. 6 Growth and production of polyphosphate



48

Fig. 7 Production of polyphosphate on glucose and glycerol



49

Fig. 8 Effect of inorganic phosphate on extracellular polyphosphate production

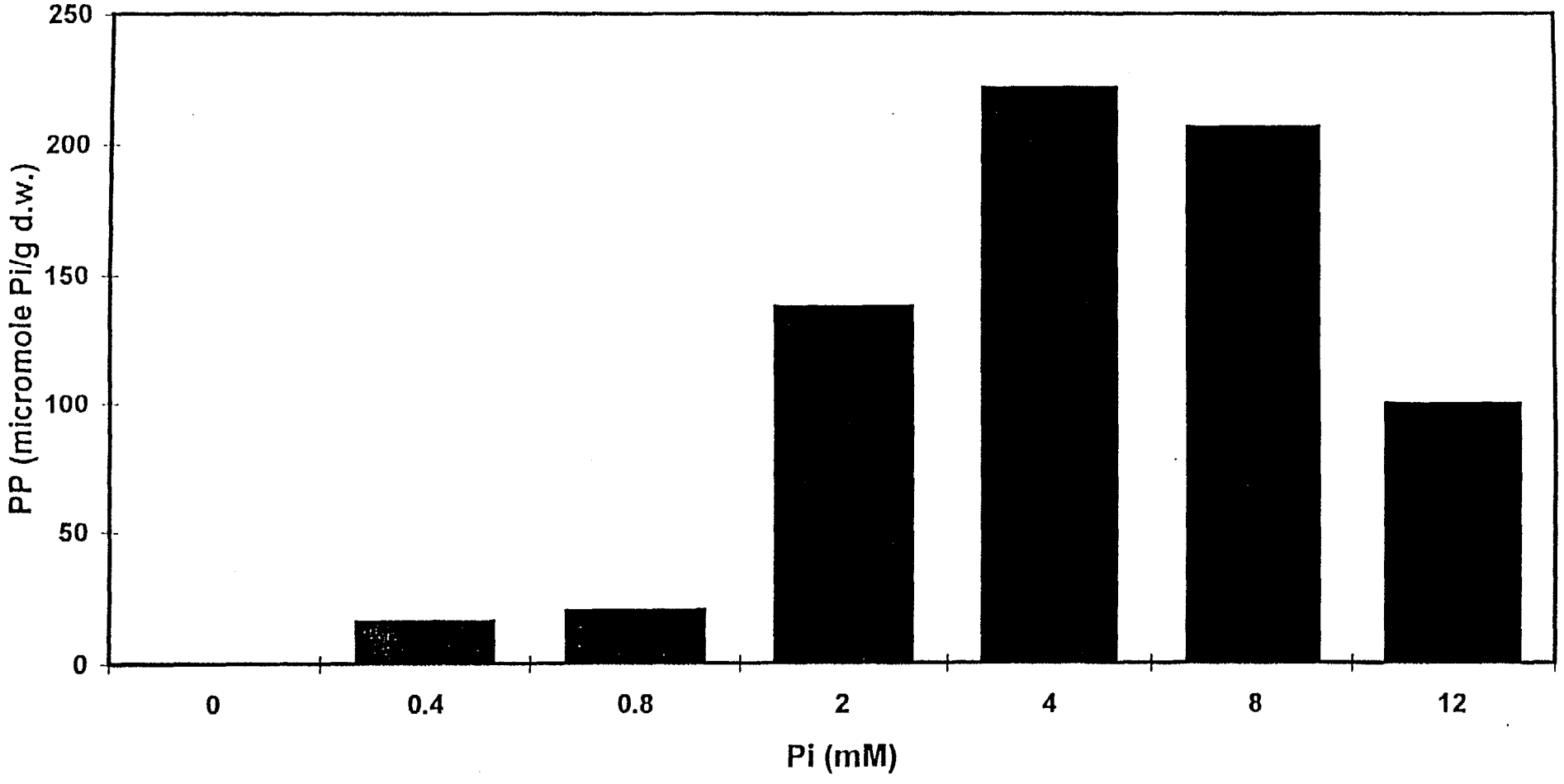


Fig. 9 Inhibition of PG by polyphosphates

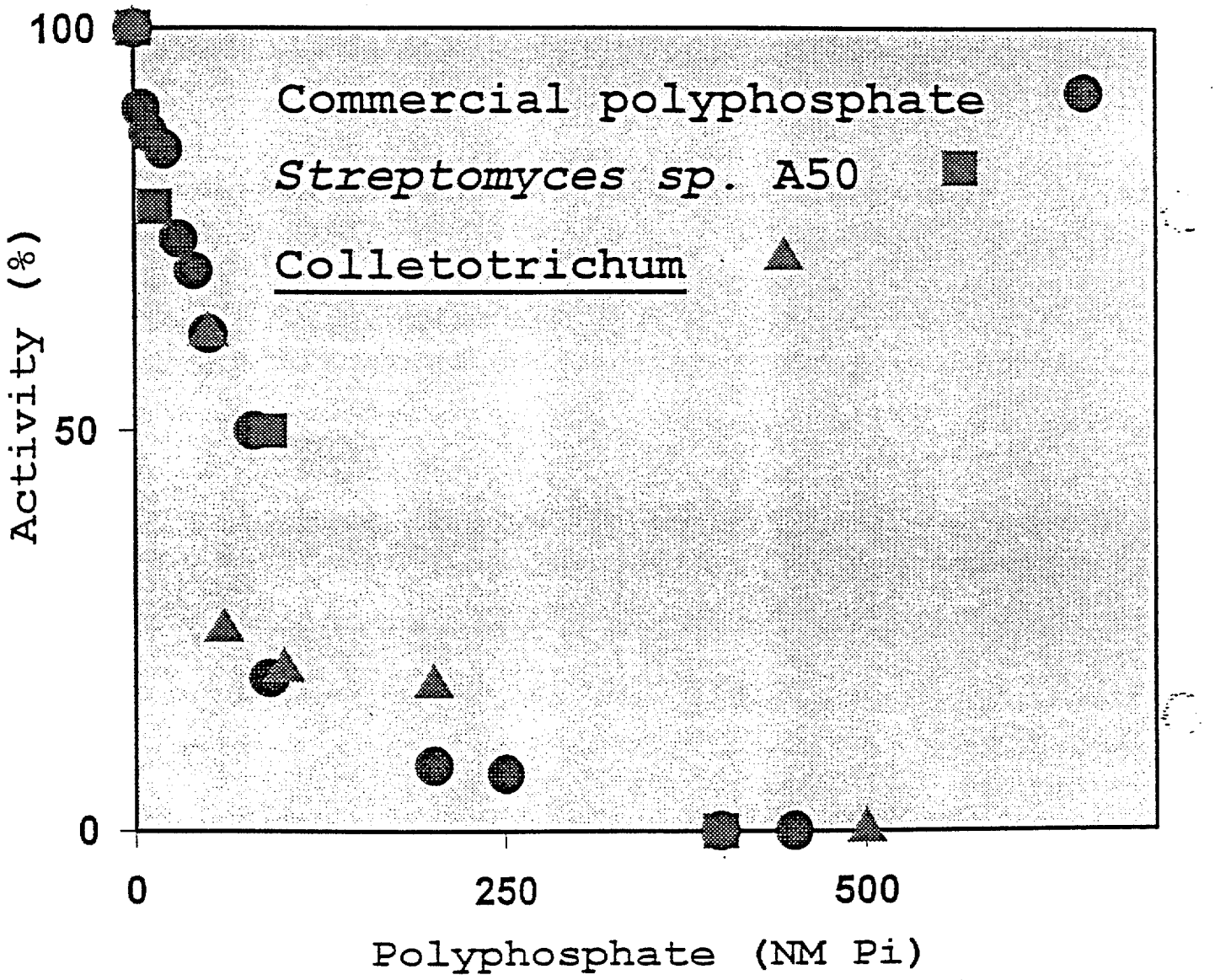
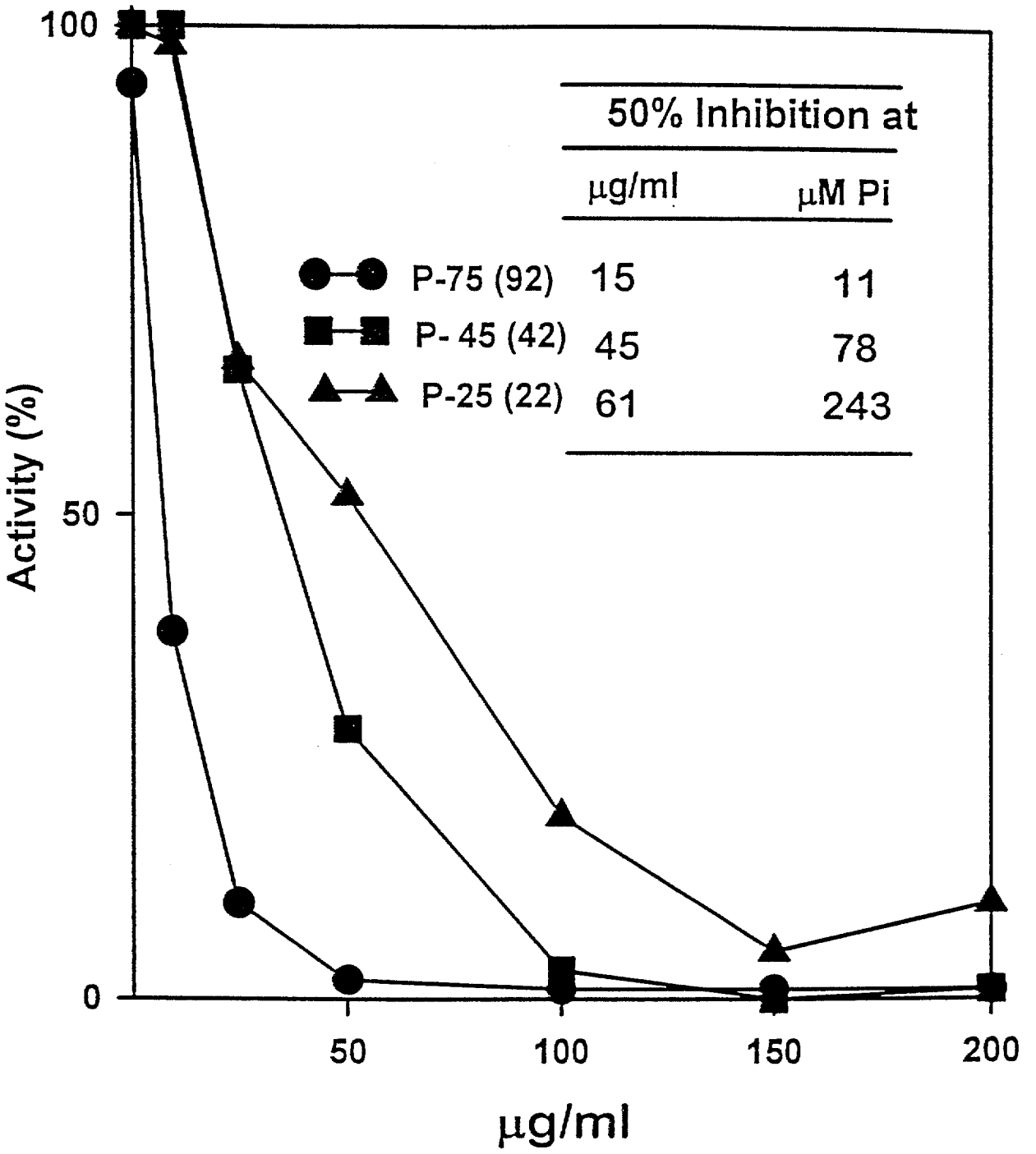


Fig. 10 Effect of polyphosphate chain length on inhibition of PG



52

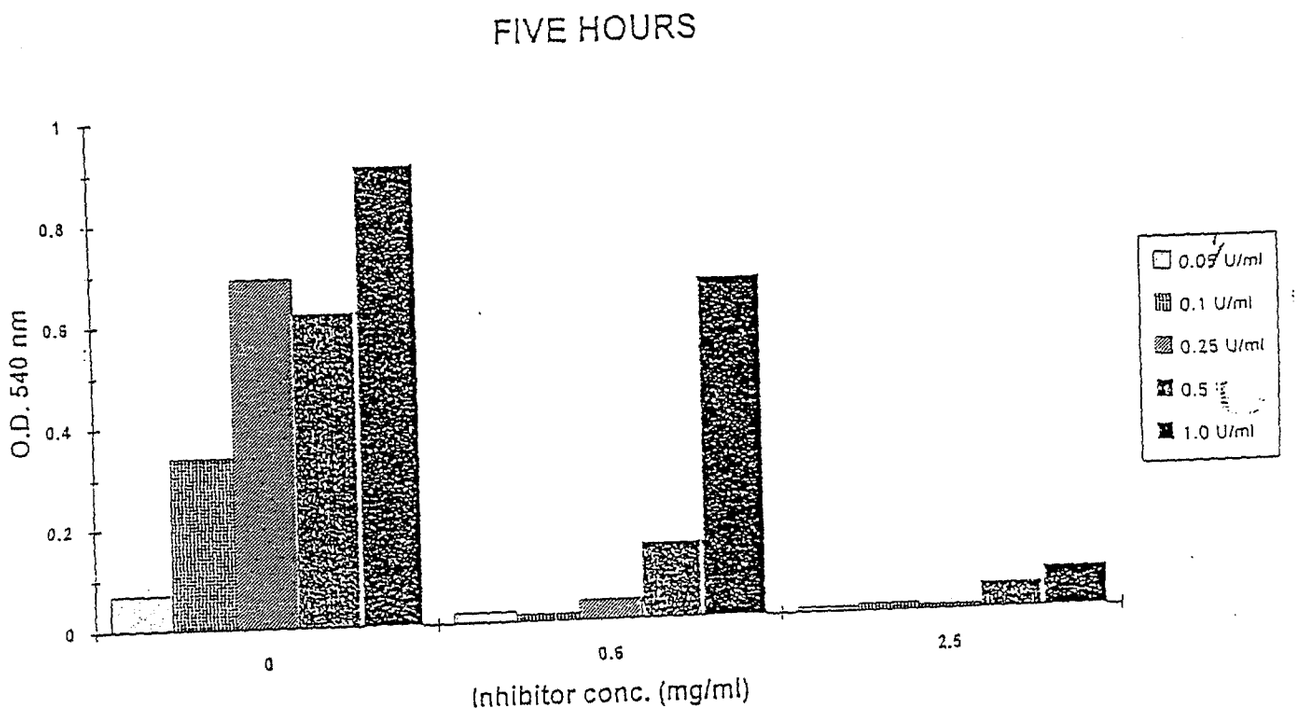


Fig 11. Inhibition of *Botrytis cinerea* PG in a semi in vivo system. Cucumber disks were incubated at 30°C in the presence of crude enzyme (0.01 to 1.0 U/ml) alone or in the presence of polyphosphates (0.6 to 2.0 mg/ml). Turbidity was measured at 540 nm after a short incubation time (5 hrs).

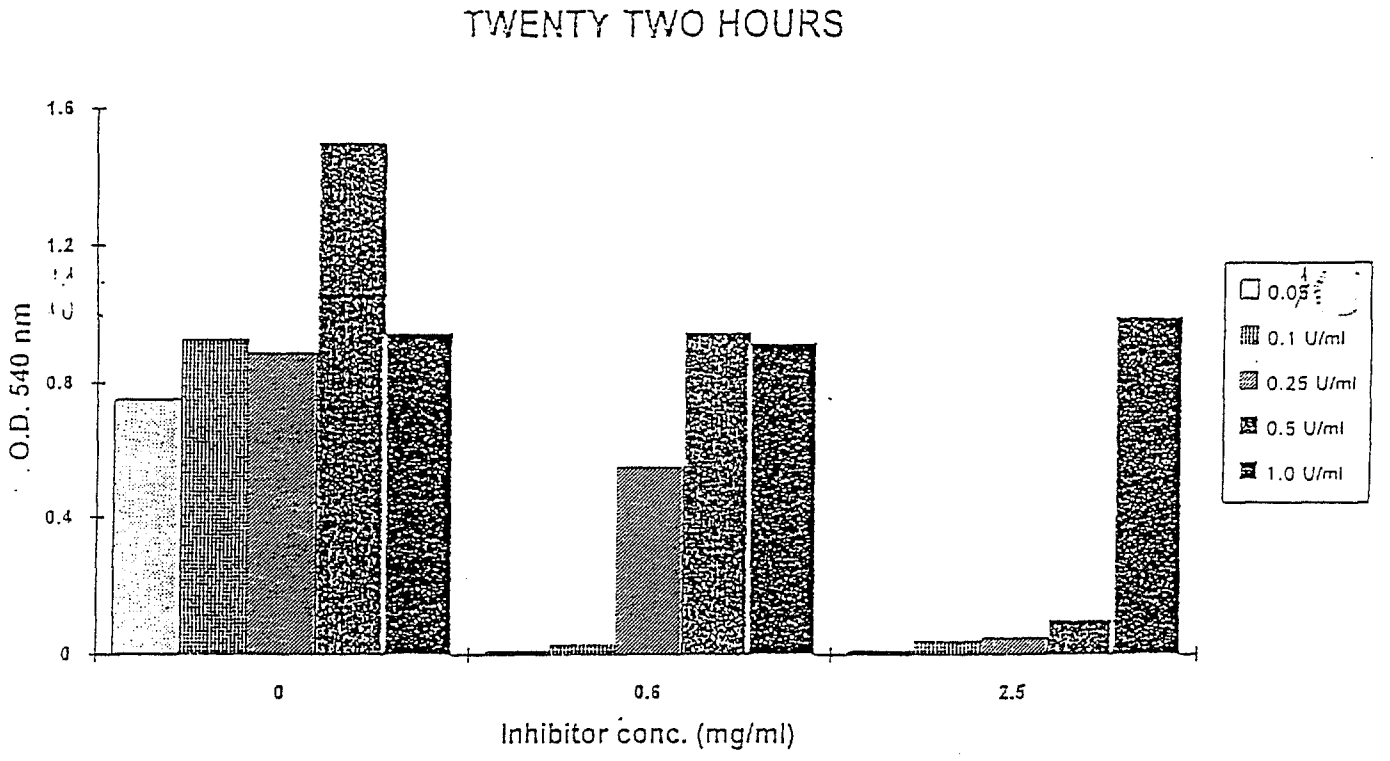


Fig 12. Inhibition of *Botrytis cinerea* PG in a semi in vivo system. Cucumber disks were incubated at 30°C in the presence of crude enzyme (0.01 to 1.0 U/ml) alone or in the presence of polyphosphates (0.6 to 2.0 mg/ml). Turbidity was measured at 540 nm after a long incubation time (20 hrs).

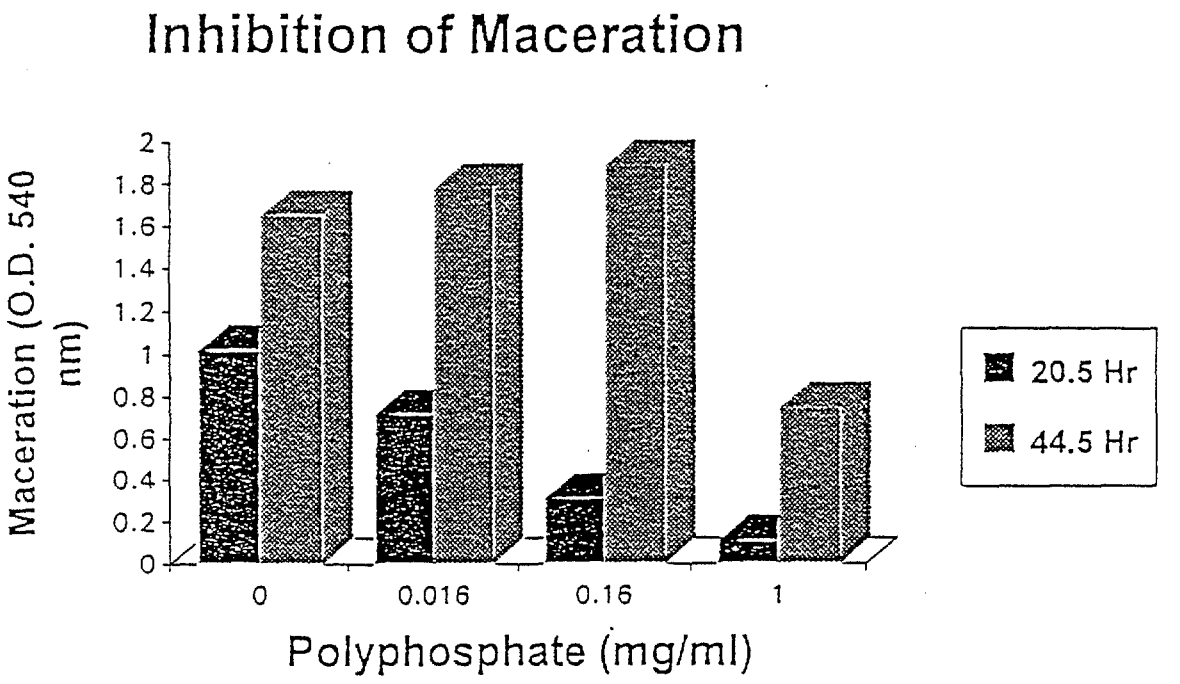


Fig 13. Inhibition of *Botrytis cinerea* PG in a semi in vivo system. Cucumber disks were incubated at 30°C in the presence of fungal spores (4.2X105 spores/ml) alone or in the presence of polyphosphates (0.0.016 to 1.0 mg/ml). Turbidity was measured at 540 nm after 20.5 or 44.5 hrs incubation time.

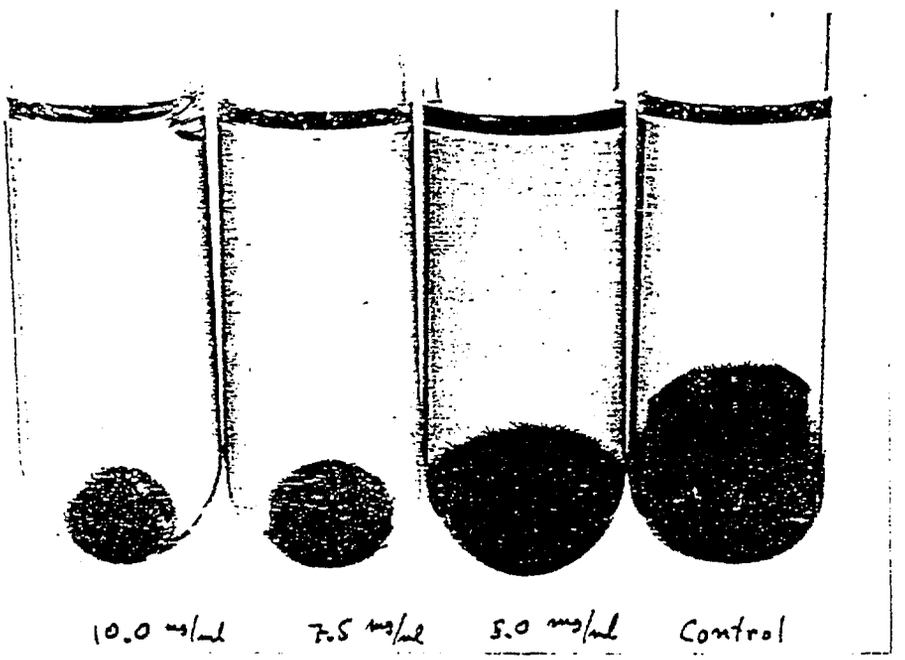


Fig 14. Inhibition of *Botrytis cinerea* growth by polyphosphate.
B.cinerea culture was grown in liquid culture with polypectate as sole carbon source. Polyphosphate was added at three different concentrations and growth was compared to a control culture to which no polyphosphate was added.

Table 1. Effect of alkaline phosphatase on polyphosphate-mediated inhibitor activity.

Inhibitor Preparation	% Breakdown of Inhibitor	%Residual PG Activity
None	–	100
P-75	0	3
P-75+alkaline phosphatase	52	101
A-50	0	<1
A-50 + alkaline phosphatase	43	89

Polygalacturonase (2.4U/ml) was preincubated with 3mg/ml inhibitor. Alkaline phosphatase (40 U, calf-intestine, Boehringer) was for 2h at 37C. Inhibitor breakdown was estimated from the amount of inorganic phosphate released during the treatment.

Table 2. Growth inhibition of *B. cinerea*.

Inhibitor (mg/ml)	Dry weight (mg)	Polyphosphate[1] (miliequivalents Pi)	DNS reaction (% activity)[2]	Maceration[3] (A_{540})
Control	20.2	0	100	0.86
5.0	10.6	29	0	0.04
7.5	5.6	57	16	0.03
10.0	4.3	82	0	0.02

Spores of *B. cinerea* were grown on polypectate in the presence of the indicated concentrations of P-75 to yield the indicated dry weight. At the end of the growth period, the cell free broth was separated and assayed for: 1. residual polyposphate, 2. effect on PG activity (DNS reaction), and 3. inhibitory effect in a standard cucumber maceration assay.