REPORT OF CDR PROJECT ON PHYTOPATHOGENIC ENZYMES AND THEIR SPECIFIC INHIBITORS

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CONSTRUCTION OF THE LABORATORY

Central Department of Chemistry, Tribhuvan University, Kathmandu, Nepal has alloted a separate lab space for the project. The lab covers approximately an area of 25 sq. meter. The lab has been renovated and made fit for biological research. The lab has its own tap water and electricity facillities. This is the first research laboratory related to biochemistry and biotechnology to be established in the university campus.

SUMMARY OF THE EQUIPMENTS

The lab has acquired basic equipments for biochemical research. At present, the lab has following equipments in running condition.

1. Spectrophotometer
2. pH meter
3. Horizontal laminar flow cabinet
4. Balance
5. Centrifuge
6. Autoclave
7. Incubator
8. Oven
9. Constant temperature water bath shaker
10. Flasks shaker
11. Refrigerator
12. Deep freeze (-18°C)
13. Distilling set
14. Deioniser

RESEARCH REPORT

Introduction

Phytopathogenic fungi are known to cause tissue maceration and soft rot diseases in post harvest fruits and vegetables. The fungus invades the fruits or vegetables and secretes pectinolytic enzymes. 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 - linked rhamnopyranose. These pectins are the substrates of pectinolytic enzymes. There are two main groups of pectinolytic enzymes that attack pectic substances - esterases and depolymerases. The esterase splits 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 hydrolases or lyases. After invading the fruits and vegetables, in the course of their multiplication, they secreat pectin hydrolyzing

polygalacturonase resulting in the separation of cells. This brings about extensive tissue maceration that ultimately causes soft rot diseases in the fruits and vegetables (Fig. 1).

The spoilage of foods and vegetables by micro organisms brings about huge economic loss in both developed as well as developing countries. Synthetic fungicides have been used to control such diseases. Excessive use of fungicides have resulted various problems. Among them, three important are development of pest resistance, non-selective destruction of microbes and environmental concerns.[1]

One of the problems for the growers is that synthetic chemical fungicides gradually loss their effectiveness. The sensitive pest populations are easily destroyed by fungicides but resistance builds up in the resistant population. Resistant individuals may breed even more resistant off-springs.

Fungicides are in general not specific to a particular target organism. Many other useful micro organisms are also affected and may have negative impact on the ecosystem.

Fungicides by nature are toxic. These substances may accumulate in the soil and inhibit the plant growth. Fungicide residues remain in the soil and river system and finally may end up in the food chain. They poison animals as well as human beings.

One way to reduce these concerns of fungicides will be to search and develop product/s that will inhibit phytopathogenic enzymes without poisoning the invading pathogens. In this regard, search for specific microbial inhibitors of fungal pectinolytic enzymes could be considered as a novel approach. This approach is modelled after Umezawa's novel approach of the development of a new class of microbial products that have acted as specific inhibitors of eukaryotic enzymes[2]. The efforts of the Umezawa group have led to the development of several therapeutic agents.

In this report, we describe the results of our experiments

- in the production of crude polygalacturonase from *Botrytis cinerea*, *Geotrichum candidum*, *Aspergillus niger*, *Aspergillus fumigatus* and *Aspergillus flavus*.
- in enzymatic characterization of these enzyme preparations
- in the search for natural inhibitors of these phytopathogens as well as phytopathogenic enzymes.

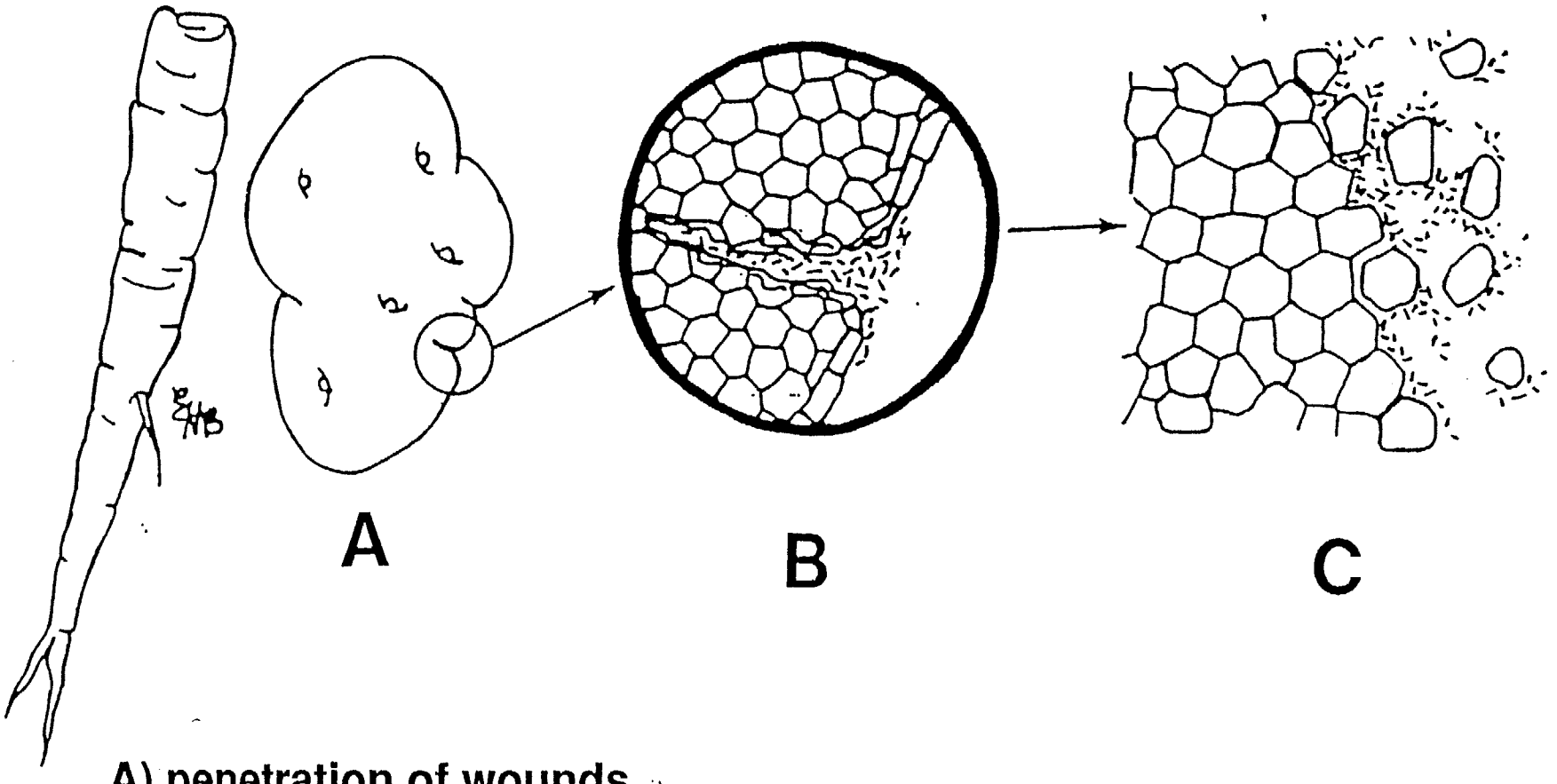
Materials and methods

Microbial Strains

Pure stains of *Botrytis cinerea*, *Geotrichum candidum* and *Streptomyces satsumaensis* were obtained from Professor D. Gutnick, Department of Microbiology, Tel Aviv University, Isreal. Strains of *Aspergillus niger*, *Aspergillus flavus* and *Aspergillus fumigatus* were gifts from Dr.A.P. Sharma, Central Department of Microbiology, Tribhuvan University, Kirtipur, Kathmandu, Nepal.

Fig. 1

Sequence of events in the soft rot of vegetables



A) penetration of wounds
B) multiplication of microorganisms in the wound, and
C) seperation of cells by enzymatic action of the microorganisms

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 days. Different dilutions of this extract was transferred to MY solid plates (malt 0.01% and yeast extract 0.04%). The plates were incubated at 30° C for 3-4 days.

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_2 HPO_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}).

Growth condition

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 30^0 C for 3-4 days. This inoculum form the starter medium was transferred to same YGP medium (10% volume). Samples were taken at various time for biomass and enzyme activity.

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 30^0C for 2-3 days. This inoculum form the starter medium was transferred to same gucose free general culture (10% volume). Samples were taken at various times for biomass and enzyme.

MSG medium

A starter MSG medium containing mono sodium glutamate 2.5g/l, glycerol 10g/1,K_2HPO_4, $3H_20$ 1 g/l, $MgS0_4$ $7H_20$ 0.5g/l, 1 ml of $FeS0_47H_20$ (1%) and 2 ml of trace metal solution consisting of $CuS0_4$ $5H_20$ 0.1%, $ZnS0_4$ 0.1%and $MnS0_4$ 0.1% were prepared and a small agar disc (~5mm) of *Streptomyces satsumaensis* was added. It was inclubated at 30^0 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.

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.

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 ($NH_4)_2$ SO_4 or by cold acetone. 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. The precipitate was dissolved in 0.1M sodium acetate buffer (pH 4.2). DNS assay and protein measurement on crude enzyme preparation revealed increased specific activity.

Polygalacturonase assay

Reducing groups determination (DNS) assay[3]

Polygalacturonase activity was determined by measuring the amount of realased reducing groups by pectin hydrolysis with 3,5-dinitrosalicyclic acid (DNS) reagent. Mono-D-galacturonic acid was taken as standard. The assay system contained 3-4 tube containing 0.1 ml, 0.2 ml, 0.3 ml, 0.4 ml of crude enzyme solution and 0.1 M sodium acetate buffer pH 4.2 added upto each tube to make volume of 1ml and 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^{0}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 taht amount of enzyme which catalyzed the realease of 1 mg of mono galacturonic acid per hr.

Cup-plate assay [4]

Pactin (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. Cups (~5 mm) 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 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.

Viscosity loss measurement

In a typical experiment, 8ml of 0.5% pectin in 0.1M acetate buffer (pH4.2) with 2ml of crude enzyme was taken. Viscosity of 2ml of enzyme plus 8ml of acetate buffer was assigned 100 percent viscosity loss.

Protein determination

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

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 citrate buffer. Solutiions of pH 6.2 to 7.2 were prepared from 0.1M 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.

Phosphate determination

Polyphosphate was determined as acid labile phosphate and was calculated as the difference in phosphate concentration before and after hydrolysis in presence of boiling HCl (1N, 20minutes). The phophate determining reagent was prepared in the following way : 0.52% ammonium molybdate, 0.46% ascorbic acid, 0.01 % antimonyl potassium tartarate dissolved in 2.5N H_2SO_4. After twenty minutes of mixing with test samples, absorbance was read at 660nm.

Tissue maceration assay[5]

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. Discs 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 discs were incubated at water bath shaker at 30° C. Polygalacturonase was added at different concentrations from 0.5 U/ml to 4 U/ml. Enzyme mediated maceration was expressed as the net turbidity measured at 540 mm.

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

Inhibitor activity assay

Two methods were used for inhibition studies.

The inhibitory activity was assayed by measuring the residual polygalacturonase activity after the enzyme was preincubated 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 5-30 minutes at 30°C. A control sample without inhibitor solution was included in parallel with test sample. Percent inhibition of polygalacturonase activity was calculated from the equation A-B/B x 100% when A and B are values of galacturonic acid (expressed in absorbancy at 575mm) in the absence and in the presence of inhibitor respectively.

Cup-plate assay 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 waer (1:1v/v). Inhibition was determined by comparing with control (enzyme only) in a semi transparent halozone that was formed.

Inhibitory study on fungal grouth.

Standard inoculum containing about 10^5 colony forming units/ml was determined by standard plate count method. This standard inoculum (about 10^5 colonies/ml) of the fungus together with various concentrations of inhibitors m YGP media were incubated at 30° C for a week. For each inhibitor tube, there was a negative control(without a inhibitor) and a positive control (with a fungicide e.g. Betrafen solution). After a week, the growth of the fungus was observed.

Mycelial growth inhibition test

On 5mm thick MY plates, 100µl of 48-72 hours old a liquid culture of test microorganism was sprayed. This was covered with approximately 10 ml of PDA at 40° C. A 3-5 mm diameter mycelial disc of sporulation *B.cinerea* was placed in the middle of the plate and incubated at 30° C for seven days. A PDA plate without test microorganism served as a control.

Studies on *Botrytis cinerea*

The soft rot disease caused by *Botrytis cinerea* is a major world wide problem for post harvest fruits and vegetables[6] . Nepal is no exception for this disease[7].

Production of crude polygalacturonase

In glucose free medium incubated at 30°C, polygalacturonase production was found to be optimal at 48 hour of growth of *B. cinerea* (Table 1 Fig 2) and precipitation with cold acetone, specific activity of this enzyme preparation increased from 1700 to 2700 (Table 2)

Table : 1

Production of polygalacturonase
by *B. cinerea* in glucose free medium

Time (hr)	Growth (Measured at 600 nm)	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 : 2

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 centrifugation	400	17	6800	10	4	1700
66% Acetone precipitation	60	36	2160	13.3	0.798	2706

Characterization of crude polygalacturonase

Dingle's 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 3, Fig 3). The optimum pH for polygalacturonase activity was pH 4.2 (Table 4, Fig 4)

Table : 3

Influence of temperature on enzyme stability

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

Table : 4

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

Velocity of reducing group liberation by polygalacturonase was linear up to 16 unit/ml of enzyme concentration (Table 5, Fig 5) and up to 20 minute of incubation time (Table 6, Fig 6)

Table : 5

Influence of enzyme concentration on enzyme activity

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

Table : 6

Influence of incubation time on enzyme activity

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

Tissue maceration studies

Enzyme mediated weight loss of cucumber discs was observed (Table 7, Fig 7). The weight loss in found to be dependent upon enzyme concentration.

Table : 7

Cucumber discs maceration
influence of enzyme concentration

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

With potato, carrot and turnip discs, tissue maceration was slow. However, after 21 hour of incubation, tissue maceration was observed (Tables 8,9 and 10 ; Figs 8,9, and 10.)

Table : 8

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 : 9

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 : 10

Turnip 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	0.00	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

Inhibition studies

Screening of microbial population for inhibitory action against *B. cinerea.*

In order to isolate potential microbial populations whose liquid cultures would be expected to act as the inhibitors of *B. cinerea* polygalacturonase, soils were collected from the following places,

i. Rampur, Chitwan from a farm of chick peas infested with *B.cinerea.*

ii. From a traditional Nepali farmer's mud store house where vegetables such as onions, potatoes etc. are kept for a period of time, Bhaktapur.

iii. From a home made cow dung/rice husk compost.

iv. From Tribhuvan University, Kirtipur Campus.

v. From Pokhara area.

vi. From a place called Bhotang (Alt. 12000 ft.)

After repeated transfer of cultures, the following pure microbial populations were obtained.

From Rampur, Chitwan

RC-1, RC-2, RC-3, RC-4, RC-5, RC-6, RC-7 (tentatively unidentified as *Micrococus* sp), RC-8, RC-9, RC-10 (tentatively identified as *Staphylococcus sp.*), RC-11, RC-12, RC-13. All these pure strains were obtained from MY plates.

Form a traditional Nepali farmer's mud store house, Bhaktapur

BC-1, BC-2, BC-3, BC-4 and BC-5. All these pure strains were obtained from MY plates.

From home made cow dung/rice husk compost

CC-1 (tentatively identified as *Bacillus sp.*), CC-2 (tentatively identified as *Bacillus sp.*), CC-3 (identified as *Aspergillus flavus*), CC-4, CC-5 and CC-6. CC-1 and CC-2 were obtained from NA plates; CC-3 was obtained from PDA plate. CC-4, CC-5 and CC-6 were obtained from MY plates.

From Tribhuvan University, Kirtipur Campus.

TC-1, TC-2 and TC-3. All these strains were obtained from MY plates.

From Pokhara area

PC-1, PC-2, PC-3, PC-4, PC-5, PC-6 and PC-7. All these strains were obtained from MY plates.

From Bhotang area.

BBC-1 (tentatively identified as *Penicillin sp*). It was obtained from MY plates.

Inhibition studies of liquid culture broth of microbial isolates against *B. cinerea* polygalacturonase.

All these isolated microbial populations were grown in appropriate liquid cultures. On repeated experiment, only isolates CC-7 and BBC-1 were shown to exhibit feeble inhibitory activity.

In case of BBC-1, a 14 days old liquid culture in one experiment showed up to 15% inhibition. Works are directed towards purification and selective precipitation of inhibitory materials.

Inhibition studies of mycelial growth of *B. cinerea* by microbial isolates on agar plates .

All these microbial isolates were tested against the mycelial growth of *B. cinerea*. Eight isolated microbial populations were found to inhibit mycelial growth of *B. cinerea* on PDA plate (Table 11)

Table : 11

Effect of microbial isolates
on mycelial growth (on PDA) of *B. cinerea*.

Microbial isolates	Colony diameter after seven days at 30^0 C (mm)	
	Colony diameter without microbial isolate (Control)mm	Colony diameter with microbial isolate mm
RC-7	39	18
RC-10	39	22
CC-1	42	20
CC-2	42	16
CC-3	42	25
CC-4	30	20
CC-5	30	17
BCC-1	47	27

Inhibitory effect of metal ions on polygalacturonase activity

Table 12 shows inhibitory effect of some ions on polygalacturonase preparation obtained from *B. cinerea*

Table : 12

Inhibition effect of some meal ions
on polygalacturonase activity

Chemicals	Remaining activity %
$BaCl_2$	86
$CoCl_2$	110
$FeSO_4$	100
$HgCl_2$	80
$MnCl_2$	203
KCl	100
$NiCl_2$	100
$(CH_3 COO)_2 Pb$	100
$ZnCl_2$	90
EDTA	100
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.

Plant extracts as inhibitors

Seventeen different plants containing medicinal properties were purchased from the local market. Methanolic extracts of these plant materials were prepared. The name of plants and the parts of them used in this study are given below.

Name of plants	Parts of the plants used
Acorus calamus	Stem
Allium sativum	Bulb
Areca catechu	Fruit
Azadiracha indica	Stems and leaves
Cumin cyaminum	Seed
Cinnamomum zeylanicum	Bark
Cinnamomum tamala	Leaves
Curcuma long linn	Root
Ferula narthex	Resin
Glycerhia glabra	Stem
Myristica fragrans	Fruit
Nardostachys jatamasi	Whole plant
Swertia chirata	Whole plant
Sizygium aromatica	Fruit
Terminalia belarica	Fruit
Terminalia chebila	Fruit
Zanthoxylum aromatum	Fruit

Cup plate Method

In this method only *Areca catechu* extract was able to inhibit the target enzyme activity. (Table 13, Fig. 11). *Areca catechu* extract (250 μg/ml) showed 50% inhibition of the enzyme activity.

Table : 13

Inhibition of *B.cinerea* polygalacturonase as measured by cup plate method.

Inhibitor concentration (μg/ml)	% Inhibition
250	50
333	73
500	83

DNS Method

In DNS method also only *Areca catechu* inhibited the activity of target enzyme (Table 14, Fig. 12). *A. catechu* extract (400 μg/ml) showed 74% inhibition of the enzyme activity. It is at present not clear which compounds present in *A. catechu* inhibited the enzyme preparation. It has been already reported that tannins act as inhibitors for pectinase from *Fusarium oxysporum, F. lycopersici* III[8] . Because tannins are chief constituents of *A. catechu*[9] it is believed that tannins of *A. catechu* might be responsible for the inhibition.

Table 14

Inhibition measured by DNS Method

Inhibitor concentration (μg/ml)	% Inhibition
100	28
200	63
400	74
600	85

Inhibition study on fungal growth

Out of 17 plant extracts used to inhibit the fungal growth in YGP media, only *Acorus calamus, Sizygium aromatica, Cinnamomum zeylanicum and Glycerrhia glabra* retarded the fungal growth (Table No. 15, Fig. 13).

Table : 15

Fungal growth inhibition by plant extracts.

Plant extracts	Concentration of inhibitor		
	2500 μg/ml	1250 μg/ml	625μg/ml
Acorus calamus	No growth	No growth	Negligible growth
Sizygium aromatica	No growth	No growth	Negligible growth
Cinnamomum zeylanicum	No growth	No growth	Negligible growth
Glycerrhia glabra	No growth	No growth	Negligible growth

Effect of water extracts of cowdung / rice husk compost on *Botrytis cinerea*

It has been reported that water extract of composted manure-straw mixture made by incubating in water for 3-18 days inhibited conidial germination on glass slides and reduced mycelial growth of *B. cinerea* on agar[10]. Keeping this in mind, we prepared cow dung/rice husk compost and conducted experiments on the effects of their water extracts on mycelial growth of *B. cinerea* as well as on polygulacturonase produced by *B. cinerea*. Compost was prepared in the following way : fresh cow dung (~5kg), rice husk (~2kg) and husk ash (~ 4kg) were thoroughly mixed and tap water (1 liter) was added to the mixture. The mixture was wrapped up in a jute bag and kept in a open place. Occasionally the mixture was thoroughly mixed. In about 2-3 months, compost was prepared.

After grounding the compost, 100g of compost was mixed with 500ml of tap water and incubated at 30°C. After 3,5,6,12 and 18 days of incubation, this mixture was filtered through cheese cloth and filtrate was used as compost extract.

Agar (1.5g) was mixed with tap water (100ml) and autoclaved. Autoclaved extract was cooled to 40°-45°C and mixed with 1:1 v/v of incubated compost extract and poured into the petridish to give 5mm depth. It was covered with 10 ml of PDA. Mycelial disc of *B.cinerea* (3mm) was placed on the middle of the plate and incubated at 30°C for 72 hours. A PDA plate containing 3 mm of mycelial disc of *B. cinerea* served as positive control and a PDA plate containing 0.1% Betrafen solution (fungicide) served as a negative control.

Effect of water extracts of compost against *B. cinerea* polygalacturonase

All 3,5,8,12 and 18 days incubated water extracts were tested against *B. cinerea* polygalacturonase by DNS method and cupplate methods. Inhibitory activity was not detected in these water extracts.

Effect of water extracts of compost against mycelial growth of *B cinerea*

All 3,5,8, 12 and 18 days incubated water extracts retarted the mycelial growth of *B. cinerea* on PDA plate. (Table No. 16)

Table : 16

Effect of compost extract
on mycelial growth (on PDA) of *B. cinerea*

Compost extract	Colory diameter after 72 hours incubation at 30°C (mm)	
	Colony diameter without compost extract (control)mm	Colony diameter with compost extract mm
3 - day extract	22	11
5 - day extract	24	8
8 - day extract	23	8
12 - day extract	24	8
18 - day extract	24	8

We also estimated the number of microbial populations on the compost/water extracts. (Table No 17)

Table : 17

No of days	No of colonies per ml of extract		
	WYA	PDA	NA
3	220×10^6	32×10^3	50×10^6
5	2.2×10^6	0.8×10^3	20 X 10
8	25×10^8	50×10^3	uncountable
12	12×10^8	8×10^3	70×10^6
18	30×10^7	19×10^7	33×10^{11}

From those compost extracts., five pure microbial population CC-1, CC-2, CC-3, CC-4, and CC-5 that retard the mycelial growth of *B.cinerea* were isolated.

Liquid culture broth of S. satsumaensis as an inhibitor

S.satsumaensis was grown on MSG medium and its culture both was tested for inhibitory action against polygalacturonase preparation from *B. cinerea* . Although feeble inhibitory activity seemed to have detected in the preliminary experiments, repeated experiments established that inhibitory activity could not be produced in this liquid culture. In addition, *S. satsumaensis* was cultured in the following liquid media: (a) Glucose, yeast extract, malt extract, water (b) asparagine, glycerel, K_2HPO_4, Mg SO_4, $7H_2O$, $FeSO_4$, $7H_2O$, and traces of $CuSO_4$,$5H_2O$,$ZnSO_4$ and $CoSO_4$ (c) arginine,glycerol, K_2HPO_4,$MgSO_4.7H_2O$, $FeSO_4$. $7H_2O$ and traces of $CuSO_4$. $5H_2O$, Zn SO_4 and $CoSO_4$ (d) peptone water. In these liquid cultures also, inhibitory activity could not be established. It is interesting to note that polyphosphate production in these cultures also could not be detected.

Studies on *Geotrichum candidum*

Production of crude polygalacturonase

Geotrichum candidum, a citrus 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 18,19)

Optimum production of polygalacturonase 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.

Table : 18

Growth and production of polygalacturonase
by *Geotrichum candidum* in YGP medium

Time (Hour)	Growth (600nm)	Vol. of culture broth (ml)	Unit/ml	Protein µg/ml	Total unit	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 : 19

Growth and production of polygalacturonase
by *G. candidum* in glucose free medium

Time (hour)	Growth (600nm)	units/ml	Protein µg/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 20)

Table : 20

Partial purification of polygalacturonase
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	8.1×10^3	6753.0

Assay of crude polaygalcturonase

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 21) Viscosity loss of pectin solution by the action of enzyme indicated degradation of pectin into smaller fragment of lower molecular weight.

Table: 21

Viscosity loss measurement

Time in minute	% Viscosity loss
5	10.8
15	18.9
25	27.0
35	37.8
60	50.0
85	62.1
110	70.9

Cup plate assay

Plates were developed with concentrated hydrochloric acid and water (1:1), Semitransparent zones formed around the cups. This indicated hydrolysis of substrate by polygalaturonase. 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. 22)

Table : 22

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 with orange peels. However, onset of maceration 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. 23)

Table : 23

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 polygalacturonase activity was lost at 50°C. Enzyme was completely deactivated at 70°C. (Table No. 24)

Table : 24

Influence of temperature on enzyme stability

Temp °C	0	5	12	20	25	30	40	50	60	70
% 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 where as pH 2.2 had 20.8% activity. At pH 5.2, 6.2, and 7.2 activities were 62.0%, 41.6% respectively. (Table No. 25)

Table : 25

Influence of pH enzyme stability

pH	2.2	3.2	4.2	5.2	6.2	7.2
Activity(%)	20.8	66.6	100.0	62.0	41.6	16.6

Influence of incubation time on activity

The reducing group liberation by polygalacturonase 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 polygalacturonase was linear upto 30 minute of incubation time. (Table No 26)

Table : 26

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 16 U/ml ofenzyme concentration. (Table No.27)

Table : 27

Influence of enzyme concentration on enzyme activity

Units/ml	2	4	8	12	20	24
Abs. at 575nm	0.16	0.33	0.58	1.1	1.3	1.5

Studies on *Aspergillus niger*

Production of crude polygalacturonase

Aspergillus niger was grown in two liquid culture media i,e YGP and glucose free media. The enzyme production was measured at various intervals of time. In YGP medium, the optimum production of polygalacturonase was observed at 48 hours after inoculation from starter (Table No 28) while in glucose free medium the production of polygalacturonase could not be detected.

Table : 28

Enzyme activity in YGP medium

Time (hours)	Vol. of culture broth (ml)	Units/ml	Total units
0	275	0.000	0.00
24	260	0.066	17.16
48	245	0.081	19.85
72	230	0.058	13.34

Precipitation with 66% cold acetone brought about 6 fold increase of specific activity of crude polygalacturonase (Table No 29).

Table 29

Partial purification of polygalacturonase
from *Aspergillus niger*

Purification Step	Enzyme (U/ml)	Protein (mg/ml)	Specific Activities
Cell free broth	0.9416	0.0022	428
66 % acetone precipitation	14.3	0.0055	2600

Experiments with cup-plate method also showed that the optimum production of crude enzyme was at 48 hours after inoculation in YGP medium.

Assay of partially purified polygalacturonase

Vescosity loss measurement

Viscosity loss measurement of 0.5% pectin solution in sodium acetate buffer (0.1M, pH 4.2) using 2 ml of the crude enzyme was carried out. The viscosity loss were measured at various intervals of time. Viscosity loss of pectin solution was due to the enzyme mediated degradation of pectin into small fragments. (Table No 30)

Table : 30

Viscosity loss measurement
(Room temperature 21°C)

Time in minutes	% viscosity loss
5	26
15	48
25	56
35	60
60	70
85	75
110	80

Cup-plate method

Enzyme activity was also measured with the help of cup-plate method. Enzymes from 0.125 U/ml to 1 U/m lwere taken and relationship between diameter of halozone and enzyme concentration was linear. (Table No. 31)

Table : 31

Cup-plate assay

Units/cup	0.125	0.25	0.50	1.00
Diameter mm	13	14	16	20

Tissue maceration assay

The tissue maceration assay of polygalacturonase produced by *A. niger* was studied on potato, carrot, turnip, cucumber discs and fresh orange peels. Substantial maceration was obtained with cucumber slices only. Preliminary experiments showed that the onset of maceration was slow, only overnight incubation at 30°C produced maceration. (Table 32)

Table : 32

Tissue maceration oncucumber slices

Units/ml	Absorbance (540 nm)
1	0.01
3	0.05
5	0.07
10	0.21

Characterisation of partially purified polygalacturonase

Influence of pH on enzyme activity

The optimal pH for polygalacturonase activity was found to be 4.2 (Table No. 33)

Table : 33

Influence of pH on enzyme activity

Units/ml	% Activity
2.2	35
3.2	50
4.2	100
5.2	20
6.2	15
7.2	10

Influence of temperature on enzyme activity

The enzyme was stable in the temperature range from 0°C to 30°C. More than 60% activity was lost temperature 40°C. At 70°C, the enzyme was completely deactivated. (Table No. 34)

Table : 34

Influence of temperature on enzyme activity

Temperature (°C)	% Activity
0	100
5	100
10	100
20	100
30	100
35	86
40	66
50	2
60	2
70	0

Influence of incubation time on enzyme activity

The reducing group liberation by polygalacturonase increased with increment of incubation time. The plot of μ mole reducing groups/ml against incubation time illustrated that velocity of reducing group liberation by polygalaturonase was linear upto 15 minutes of incubation time. (Table No. 35)

Table : 35

Influence of incubation time on enzyme activity

Time (min)	μ Mole reducing groups/ml
5	2.1
10	2.4
15	2.6
20	2.7
30	2.8
60	2.9
120	2.9

Influence of enzyme concentration on enzyme activity

The influence of enzyme concentration on enzyme activity was studied upto enzyme 12 units/ml, and it was found that the enzyme activity was dependent upon the concentration of enzyme. The plot of enzyme concentration against absorbance (λ575 nm) illustrated that the relationship between enzyme activity and enzyme-concentration was linear upto 8 units/ml of enzyme concentration. (Table No. 36)

Table : 36

Influence of enzyme concentration on enzyme activity

Units/ml	Absorbance (575 nm)
1	0.12
2	0.22
4	0.46
6	0.69
8	0.92
12	0.98

Inhibition studies

Inhibition study on enzyme activity

Medicinal plants as inhibitors

DNS-assay

Eighteeen medicinal plants *Acorus calmus, Allium sativum, Areca catechu, Azadirachta indica, Cumin cyaminum, Cinnamomum tamala, Cinnamomum zeylanicum, Curcuma longa, Euphorbia thymifolia, Ferula narthex, Glycyrrhira glabra, Myristica fragrans, Nardostachys jatamasi, Swertia chirata, Sizygium aromatica, Terminalia belarica, Terminalia chebula* and *Zanthozylum armatum* methanolic extracts were tested against this polygalacturonase preparation. Out of eighteen herbal extracts, only *Areca catechu* extract showed inhibitory activity against the target enzyme. (Table37)

Table : 37

Inhibition study by DNS method

Inhibitor Concentration	Inhibition Percentage
50 µg/ml	23
80 µg/ml	34
100 µg/ml	42

Cup-plate method

In the cup-plate method also, same observation as in DNS method was found. No other plant extracts except *Areca catechu* inhibited the target enzyme in cup-plate inhibition study also. (Table No. 38)

Table : 38

Inhibition study
by cup-plate method .

Inhibitor Concentration µg/ml	% Inhibition
100	16
150	23
200	30
300	44

Use of metal ions as inhibitor

DNS-method

Out of 11 metal ions considered, two compounds $NiCl_2$ and $CoCl_2$ were found to show inhibition towards target enzyme. Different concentration i.e 5ml mole, 10 m mole and 15 m mole of inhibitors were used for 2 unit enzyme . The result obtained from DNS assay showed that $NiCl_2$ had complete inhibition (100%) even in 5 m mole concentration. $CoCl_2$ had had 50% inhibition in 5 m mole concentration and 85% inhibition in 15 m mole concentration. Percentage inhibition was find to be dependent upon concentration of $CoCl_2$ taken. (Table No. 39)

Table :39

Inhibitory activity of $NiCl_2$ and $CoCl_2$ on polygalaturonase by DNS-method

Candidates	Concentration of inhibitor (m mole)	% activity
$NiCl_2$	5	100
	10	100
	15	100
$CoCl_2$	5	50
	10	75
	15	85

Cup-plate method

On cup-plate also, only $NiCl_2$ and $CoCl_2$ were found to show inhibitory activities towards the target enzyme. $NiCl_2$ was found to have more inhibition than $CoCl_2$ toward the same unit of enzyme. (Table No. 40)

Table : 40

Inhibitory activities of $NiCl_2$ and $CoCl_2$ on enzyme as assayed by cup-plate method

Candidates	Extract concentration μ mole	Diameter (mm)
$CoCl_2$	1.5	18
	3	15
$ColCl_2$	1.5	19
	3	17

Out of seventeen different medicinal plant extracts, only *Cumin cyamminum*, *Cinnamomum zeylanicum*, *Myristica fragrans* and *Sizygium aromatica* retarded the growth of fungus. (Table No. 41)

Table : 41

Retardation of fungal growth

	2500 µg/ml	1250 µg/ml	625 µg/ml
Cumin cyaminum	No growth	No growth	Negligible growth
Cinnamomum zeylanicum	No growth	No growth	Negligible growth
Myristica fragrans	No growth	No growth	Negligible growth
Sizygium aromatica	No growth	No growth	Negligible growth

Studies on *Aspergillus fumigatus*

Production of crude polygalacturonase

The fungus *A. fumigatus* was grown in two liquid media viz yeast glucose pectin and glucose free media. Samples were taken out from both the media at various time intervals for enzyme activity study. The optimum production of polygalacturonase was observed at 72 hours of incubation in both the media. (Table No. 42,43)

Table : 42

Polygalacturonase production by
Aspergillus fumigatus in YGP Medium

Time Hour	Unit/ml	Total Unit
0	0.00	0.00
24	3.37	891.13
48	4.70	1149.22
72	10.05	2311.86
96	5.36	1152.60

Table : 43

Polygalacturonase production by *A. fumigatus*
in glucose free medium

Time Hour	Unit/ml	Total Unit
0	0.00	0.06
24	2.70	696.90
48	4.02	985.05
72	8.71	2003.60
96	3.35	720.40

Crude enzyme was obtained by precipitation of cell free broth from glucose free medium first with 20% cold acetone (v/v) followed by 66% cold acetone (v/v). No precipitation was obtained from the cell free broth of YGP medium. (Table No 44)

Table : 44

Partial purification of polygalacturonase form *A. fumigatus*.

Purification Step	Enzyme (U/ml)	Protein (mg/ml)	Specific activity
Cell free broth	8.02	0.0190	421.85
66% acetone precipitation	58.4	0.0186	2138.80

There was about 7 times increase in specific activity of polygalacturonase after 66% acetone precipitation.

Assay on partially purified enzyme

Viscosity loss measurement

The loss in viscosity of 0.5% pectin solution in 0.1 M sodium acetate buffer (pH 4.2) mixed with 8 units/ml enzyme, with time, is due to the pectin degradation by the enzyme. About 70% viscosity loss was recorded at 120 minutes (Table No. 45)

Table : 45

Viscosity Loss Measurement

Time in Minutes	% Viscostiy Loss
5	11.76
10	23.52
15	29.40
25	35.00
35	47.05
50	52.00
60	58.00
80	64.00
120	70.00

Cup plate assay

The relationship between the enzyme concentration and semitransparent zones i.e. diameter of the ring (including cup) was round to be linear. The formation of the zones is due to the pectin hydrolysis by the enzyme (Table No. 46)

Table : 46

Cup Plate Assay

Units of Enzyme/Cup	Diameter of Cups (mm)
0.125	6
0.25	8
0.50	10
1.00	13

Tissue maceration assay

Tissue maceration assay of the enzyme produced by *A. fumigatus* was done on potato, zucchini, orange peels, cucumber and carrot.Only feeble enzyme mediated maceration was observed only with orange peels, zucchini slices and cucumber slices but not with potato and carrot discs. One possible reason for this is, maceration pattern of potato tuber, carrot tissue may be quiet different from cucumber, zucchini etc. because of the difference in compactness of the two tissues and the differences in the chemical make up of the middle lamella of the cell walls. When cucumber slices were incubated with different concentration of enzyme, the maceration was found to be increased with increase in enzyme concentration. However, the onset of maceration was slow, only slight maceration was observed even after incubation for 24 hrs (Table No. 47). Considerable amount of maceration was observed only after 72 hours of incubation. This delayed maceration may be governed by two factors (a) the diffusion of enzyme preparation into the tissue samples is slow (b) longer time is required for the hydrolysis or degradation of a 1,4 bonds of pectic substances in the middle lamella of plant cell walls.

Table : 47

Tissue Maceration on Cucumber Slices

Concentration of enzyme U/ml	Absorbance 540 nm
1	0.01
3	0.02
5	0.03
10	0.05

Characterization of polygalacturonase

Influence of incubation time on polygalacturonase activity

There was increase in enzyme activity with the increase of incubation time. When a plot of μ mole reducing group liberation upto 30 minutes of incubation time was drawn, linear relationship was observed upto 30 minutes of incubation time (Table No. 48)

Table : 48

Influence of incubation time
on polygalacturonase activity

Incubation Time (min.)	μ Mole Reducing Group/ml
5	1.21
10	1.26
15	1.31
20	1.36
30	1.43
60	1.47
120	1.47

Influence of pH on polygalacturonase activity

The enzyme was found to be stable in the pH range 3.2 to 6.2. However, maximum activity of the enzyme was observed at pH 4.2 . (Table No. 49)

Table : 49

Influence of pH on polygacturonase activity

pH	% Activity
2.2	15
3.2	46
4.2	100
5.2	69
6.2	54
7.2	23

Influence of enzyme concentration on polygalcturonase activity

In this study, enzyme concentration ranging from 1 unit/ml to 10 unit/ml was taken and the linear relationship of enzyme concentration ad its activity was observed upto 8 unit/ml of enzyme concentration. (Table No. 50)

Table : 50

Influence of enzyme concentration
on polygalacturonase activity

Concentration of enzyme (unit/ml	Absorbance (λ 575 nm)
1	0.36
2	0.56
3	0.75
4	0.90
5	1.00
8	1.50
10	1.50

Influence of temperature on polygalacturonase activity

Influence of temperature on enzyme activity was studied by incubating the enzyme on different temperature range i.e. 0^0C-100^0C. The enzyme was stable upto 30^0C (Table 51).

Table: 51

Influence of temperature
on polygalacturonase activity

Temperature (^{0}C)	% Activity
0	100
5	100
10	100
15	100
20	100
25	100
30	100
35	95
40	89
50	56
60	48
70	30
80	24
100	0

Inhibition study

Plant extracts as inhibitor

- Out of 17 plant extracts used, none was able to inhibit polygalancturonase activity produced by *A. fumigatus*. It is interesting to note that when polygalacturonase from *Botrytis cinerea* and *Aspergillus niger* were tested against *Areca catechu* extract, substantial inhibitions were obtained. As there is difference in genus and species of the fungi, the patterns of enzyme produced by the organisms may differ. The enzyme produced by *A. fumigatus* may have different composition, structure and active sites from the enzyme produced by *B. cinerea* and *A. niger*.

Metal ions as inhibitor

Out of 12 metal ions, only Hg_2Cl_2 was able to show considerable inhibition of target enzyme activity. Different concentrations of Hg_2Cl_2 solution (1M) i.e. 5 μg/ml, 10μg/ml and 15μg/ml were used for 1 unit enzyme. The result obtained from DNS method showed that Hg_2Cl_2 had complete inhibition in 10μg/ml concentration (Table 52). However, inhibition of enzyme activity by Hg_2Cl_2 solution was not observed from cup plate method . It has been reported that metal ions such as Hg, Cd, and the metalloid like As are strong chelation agents for thiol groups. Consequently they are efficient enzyme inhibitors.

Table : 52

Inhibiotory effect of Hg_2Cl_2 solution on polygalacturonase by DNS method.

Inhibitor used	Concentration of Inhibitor (μg/ml)	% Inhibition
Hg_2Cl_2	5	67
	10	100
	15	100

Studies on *Aspergillus flavus*

Aspergillus flavus was cultured in glucose free medium and maximum production of polygalacturonase was found after 120 hours of incubation at 30°C. This enzyme preparation was stable in 0°-40°C range and at 80°C, the enzyme was deactivated. The optimum pH for polygalacturonase activity was 4.2. However, in other pH range also i.e. 3.2; 5.2 and even at 6.2, it showed substantial activity. Velocity of reducing group liberation by polygalacturonase was linear up to 8 unit/ml and up to 20 minutes of incubation time.

Preliminary experiments have suggested that *Areca catechu* extract inhibits this polygalacturonase preparation also.

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SCIENTIFIC COLLABORATION

On the onset of the project, Professor David Gutnick visited Nepal lab in July-Aug, 1992. He sat down with Nepali collaborators, planned for the construction of the lab and finalized the order of required eqipments and chemicals. The future works for the project was also discussed. In Oct 1992, Dr. Mohan B. Gewali visited Professor David Gutnick's and Professor Yair Aharonowitz's laboratories at Tel Aviv University for about three

eks. Dr. Gewali spent most of his time learning microbiological techniques and ethodologies related to proposed research of phytopathogenic enzymes in Professor Gutnick's and Professor Aharonowitz's labs. The future plan for works to be done in Nepal was finalized. By the time of Dr. Gewali's return from Isreal, Nepali lab was fit for starting and sustaining viable researches on phytopathogenic enzymes. Technology was already transfered from a Isreali lab to a Nepali lab. Initially Nepali lab suffered setbacks in many of their early experiments. Professor Yair Aharonowitz visited Nepali lab in March-April 1993. Many outstanding problems were thoroughly discussed and remedies were sought. There after, Nepali lab seemed to have been standarized and experiments started to work. In Sept-Oct, 1993, Professor Gutnick visited Nepali lab. The results obtained in Nepali lab were carefully evaluated and future direction was chalked out. Some viable experiments suitable to Nepali lab conditions were devised. In Oct. 1994, Dr. Gewali visited Israeli labs. Dr Gewali brought with him an interim progress report of Nepali lab. The Israeli team sat down with Dr. Gewali and evaluated the report. Future direction for on going research was suggested. Dr. Gewali had fruitful discussion with faculty members of Tel Aviv University and Volcani Institute and observed their research programs. Dr Gewali also participated in deparmental seminar and other activities at Tel Aviv University.

After the project was launched, a Central Department of Microbiology has been established in the university campus. Dr Gewali and his team has a good working contact and collaboration with this newly established department.

RESEARCH PERSONNEL

Besides the principal investigators, four master level students were involved in the project. One student is still working for Ph. D. degree.

RESEARCH PRESENTATION

The findings of the research works were presented in the form papers and posters in different national and international symposiums and workshops. They are as follows.

Paper presentation

1. Gewali M.B.; Thapa, S.; Baral, A.; Bhattarai, S. and Shrestha, J. K.; Second National Conference on Science and Technology, Royal Nepal Academy of Science and Technology, Kathmandu, Nepal, June 8-11, 1994.

   "Studies on Polygalacturonase from *Botrytis cinerea*."

Paper presentation

2. Gewali, M.B.; Baral, A.; Thapa, S. ; Bhattarai, S. and Shrestha, J.K. ; Second National Conference on Science and Technology, Royal Nepal Academy of Science and Technology, Kathmandu, Nepal, June 8-11, 1994.

   "Polygalacturonase Production from *Geotrichum candidum*".

Poster presentation

3. Gewali, M.B.; International Conference on Current Progress in Medicinal and Aromatic Plant Research, Calcutta, India, Dec. 30, 1994-Jan 1,1995.

   " Inhibition Studies of Medicinal Plant Extracts on Polygalacturonase from *Botrytis cinerea*".

Paper presentation and demonstration

4. Gewali, M.B.; Report of Regional Workshop on Phytochemistry of High Altitude Medicinal Plants, Kathmandu, Nepal, Aug. 21-25, 1995.

   "Inhibitors for Phytopathogenic Enzymes"

Poster presentation

5. Banskota, A.H. ; Manandhar, S. ; Thapa, S. and Gewali, M.B. ; International Symposium on Trends in Microbiology, Dec. 5-8, 1995, Bose Institute, Calcutta, India.

   "Study of Natural and Microbial Inhibitors of Pectinolytic Enzymes from *Botrytis cinerea* and *Aspergillus niger*"

Invited lecture presentation.

6. Gewali; M.B. Proceedings of the 4th International Symposium on Traditional

   Medicines in Toyama, Japan(1995) 219-227

   " Studies on Inhibition of Medical Plant Extracts on Polygalacturonase from *Botrytis cinerea."*

LIST AND NAME OF DISSERTATIONS

The following is the list and names of dissertations undertaken during the tenure of the project.

1. Tentative Ph. D. dissertation title

   "Search of Natural Inhibitors for Phytopathogenic Enzymes".

2. Master dissertation title (completed 1994)

   "Biochemical Studies on Polygalactomase from *Geotrichum candidum.*"

3. Master dissertation title (completed 1995)

   "Biochemical Studies on Polygalacturonase from *Aspergillus niger.*"

4. Master dissertation title (completed 1996)

   " Biochemical Studies on Polygalacturonase from *Botrytis cinerea and Aspergillus fumigatus.*"

5. Master dissertation title (to be completed in 1997)

   " Biochemical Studies on Polygalacturonase from *Aspergillus flavus.*"

Unofficial
Translation

ISRAEL BULLETIN
OCTOBER, 1992

RESEARCH GRANT AWARDED TO ISRAELI AND NEPALI SCIENTISTS

Professor David Gutnick of Tel Aviv University, Israel and Dr. Mohan B. Gewali of Trubhuvan University, Nepal have recently been jointly awarded a CDR research grant.

The theme of the joint project is research and develop specific inhibitors of phytopathogenic enzymes.

The objective of the research project to establish and develop microbial remedies of post harvest disease. Such natural substances are expected to destroy plant disease causing fungal enzymes.

A Short Introduction of Professor Gutnick

Dr. David Gutnick is a Professor of Microbiology at Tel Aviv University. Born in 1939 at Los Angeles, USA. Professor Gutnick obtained Ph.D. from University of Southern California, Los Angeles, USA. He has served as a Visiting Scientist/Visiting Professor in a number of premier scientific institutes such as National Institute of Health, USA ; Amsterdam University, Holland ; Boston Biomedical Research Lab, USA ; Chicago University, USA ; Mc Gill University, Canada ; Institute for Biotechnology and Genetic Engineering, Mexico and Chinese Academy of Sciences, China. He is in the editorial board of several international journals of repute. To his credit are a large number of research articles, research grants and patents.

A Short Introduction of Dr. Mohan B. Gewali

Dr. Mohan B. Gewali is a reader (Associate Professor) at Central Department of Chemistry, Tribhuvan University, Kathmandu, Nepal. Born in 1951, Dr. Gewali obtained Ph.D. from Washington State University, USA in 1982. He served as a Visiting Scientist at Toyama Medical and Pharmaceutical University, Japan from 1987 to 1989. In 1990, Dr. Gewali obtained Young Scientist Award from Royal Nepal Academy of Science and Technology.

Unofficial
Translation

TRIBHUVAN UNIVERSITY BULLETIN
Vol 28, No. 1 Asar 23, 2052 (July , 1995)

RESEARCH NEWS

PROGRESS ON STUDIES OF PLANT-DISEASES

Fungi and other microbes cause soft rot diseases in the fruits and vegetables. There are some ways to control them. Associate Professor Dr. Mohan B. Gewali states that these methods are not complete in themselves.

According to Dr. Gewali who works in the Central Department of Chemistry of Tribhuvan University, fungi such *as Botrytis cinerea, Geotrichum candidum, Aspergillus niger* and many others produce an enzyme called pectinolytic enzyme that dissolves plant cells cementing substances, pectins, causing soft not disease in the fruits and vegetables.

To cure such plant disease, the fruits and vegetables are sprayed with the poisonous fungicides. The shelf life of the fruits and vegetables are increased by keeping them in big cold storages. Some times the pathogens are distroyed by the applications of U.V. light and other treatments. According to Dr. Gewali, these controlling methods are either environmentally not friendly or are very expensive from the point of view of developing countries. Keeping this in mind, Dr. Gewali is researching natural substances that can destroy the disease causing enzyme without killing the invading fungi.

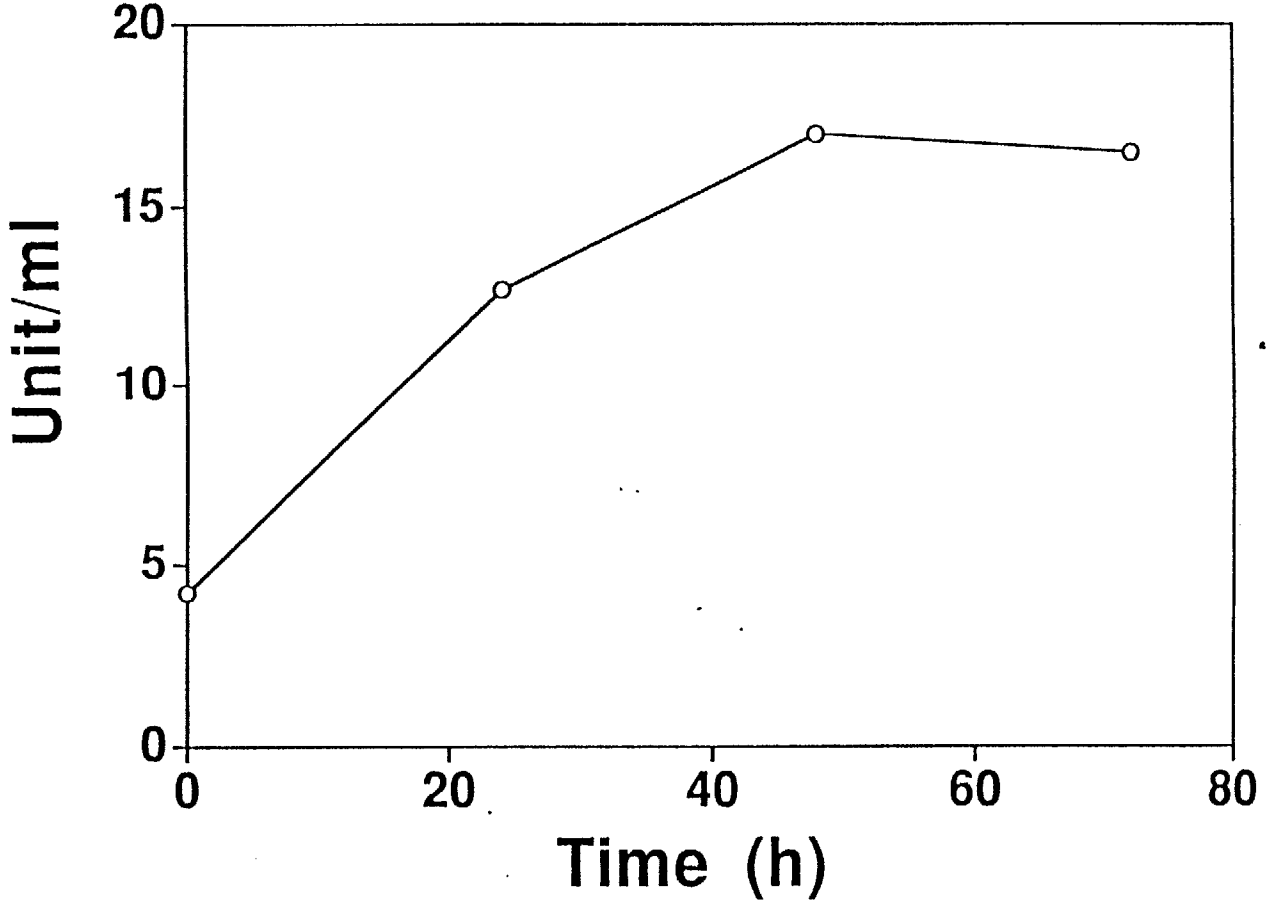
Some encouraging signs have already been emerged out from this research. They include isolation and characterization of disease causing enzymes from different fungal sources, identification of polyphosphate for these phytopathogenic enzymes, isolation of some microbial populations that check the growth of disease causing fungi and screening of liquid cultures of these microbial isolates for inhibitory action upon phytopathogenic enzymes.

For this research project, Dr. Gewali heads a group of researchers and students.

Annex I

Fig. 2

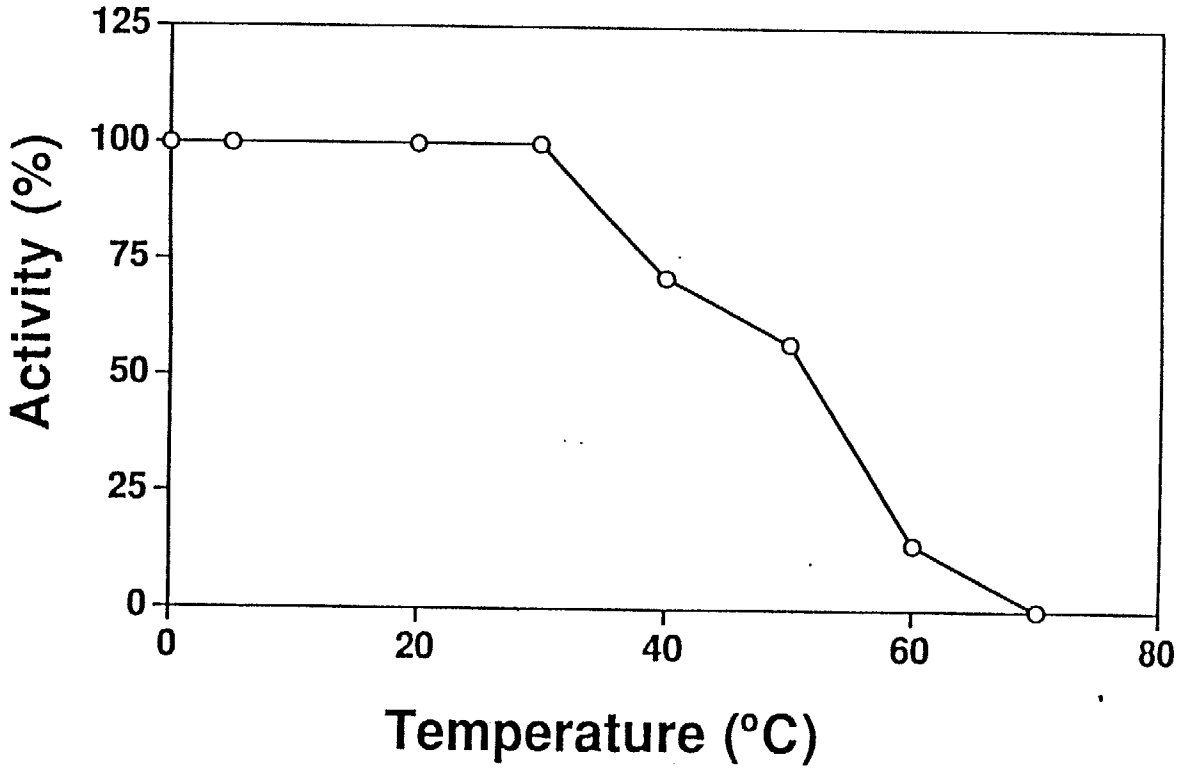
Production of Polygalacturonase by *Botrytis cinerea* in Glucose Free Medium



Annex II

Fig. 3

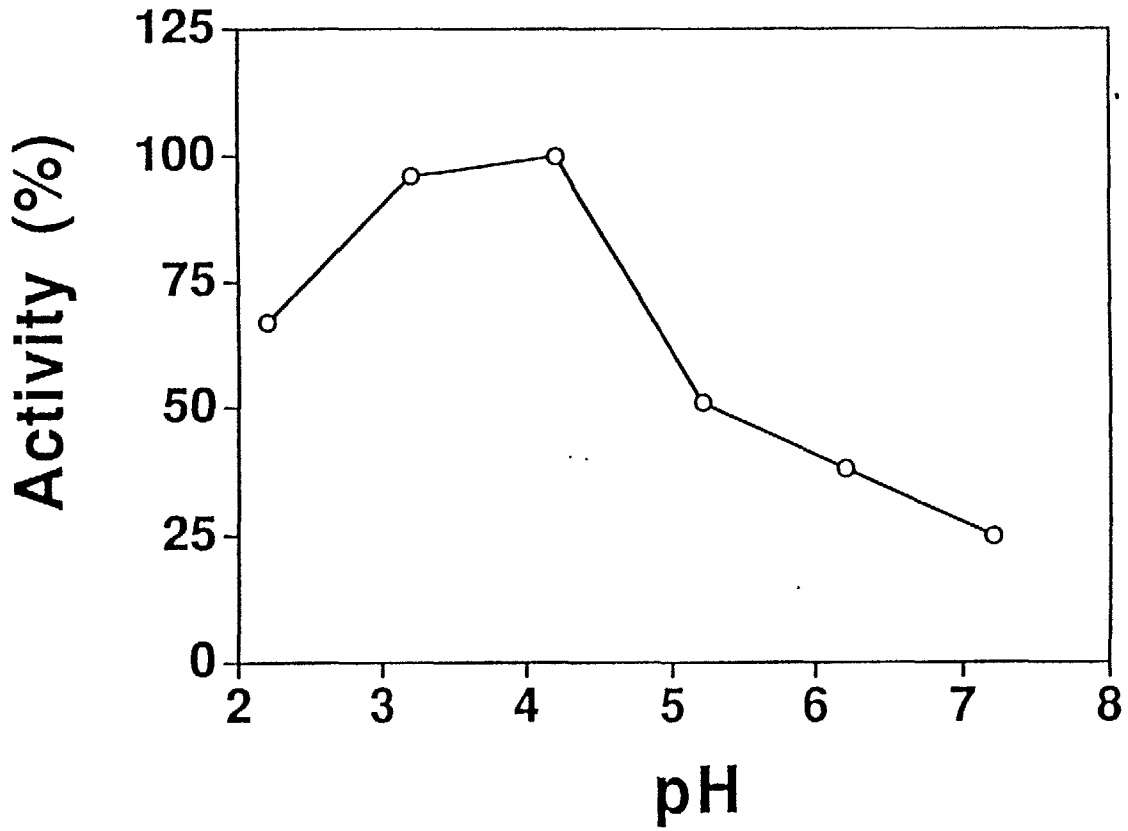
Influence of temperature on enzyme activity



Annex III

Fig. 4

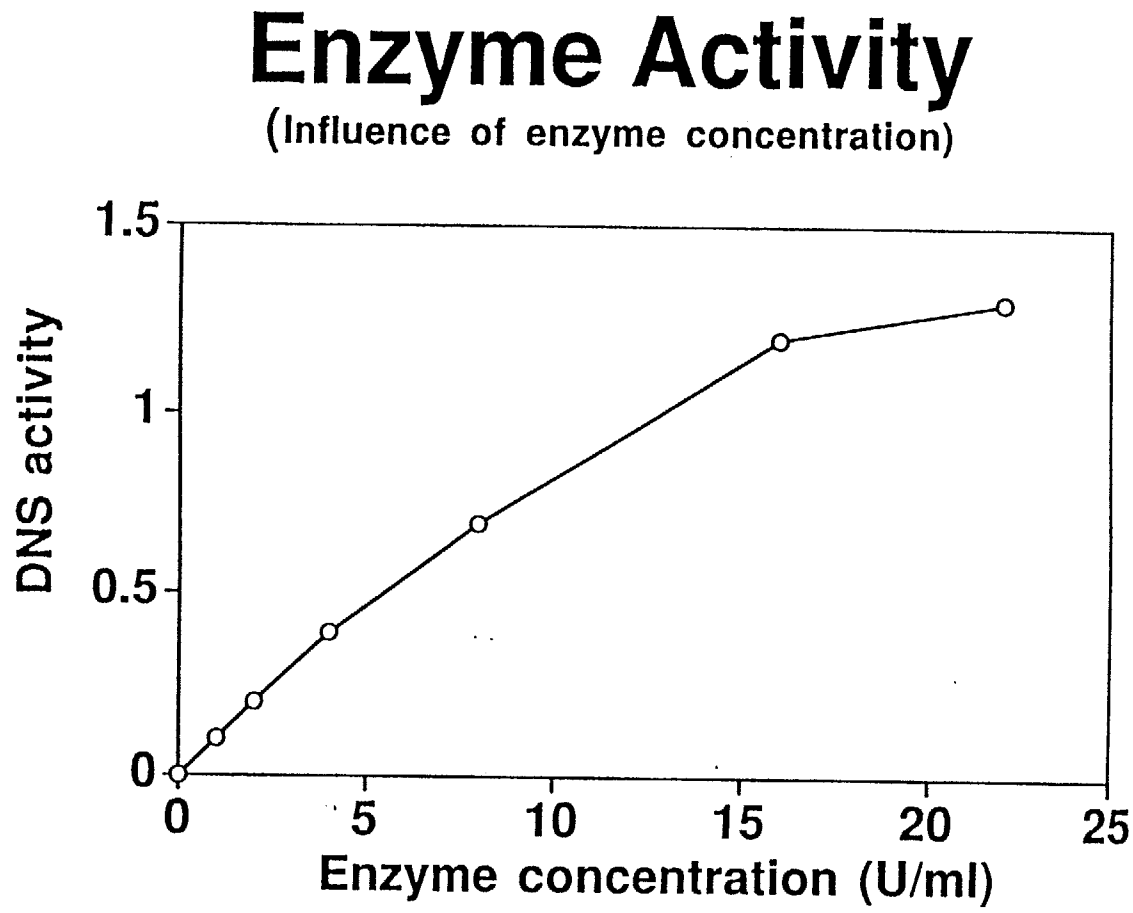
Influence of pH on enzyme activity



38

Annex IV

Fig. 5

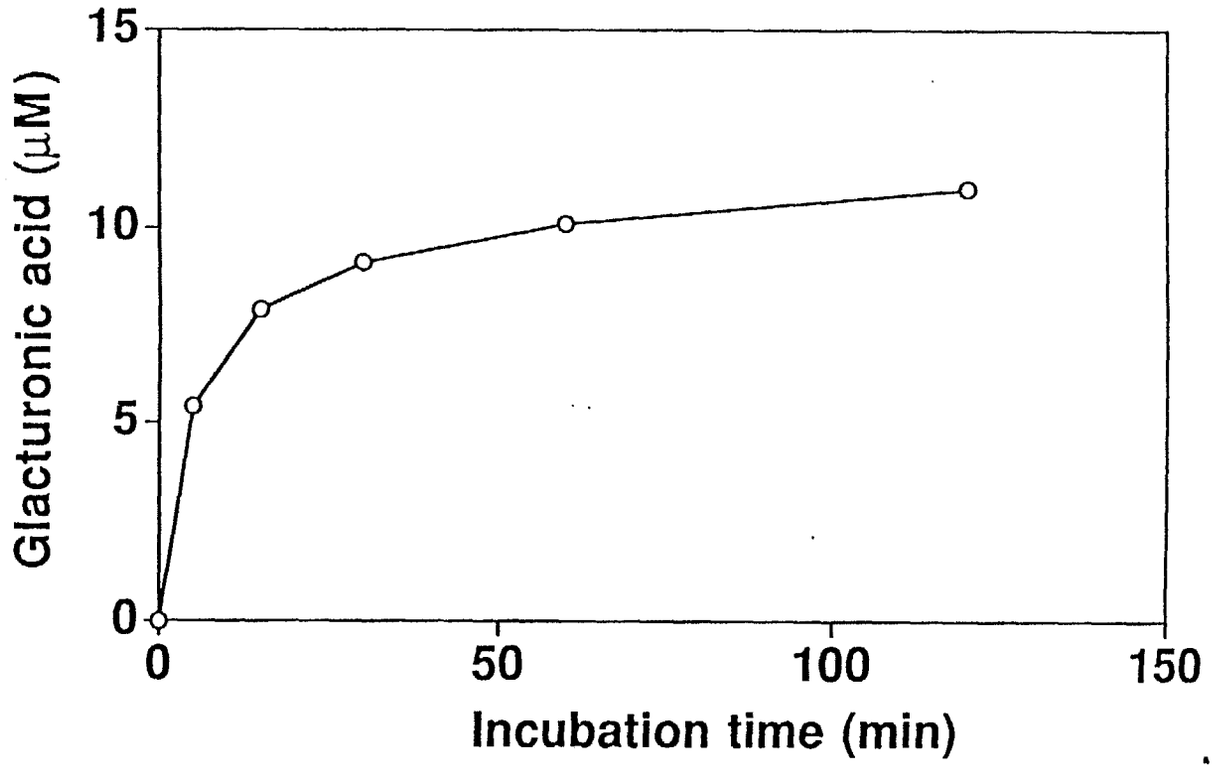


39

Annex V

Fig. 6

Enzyme Activity

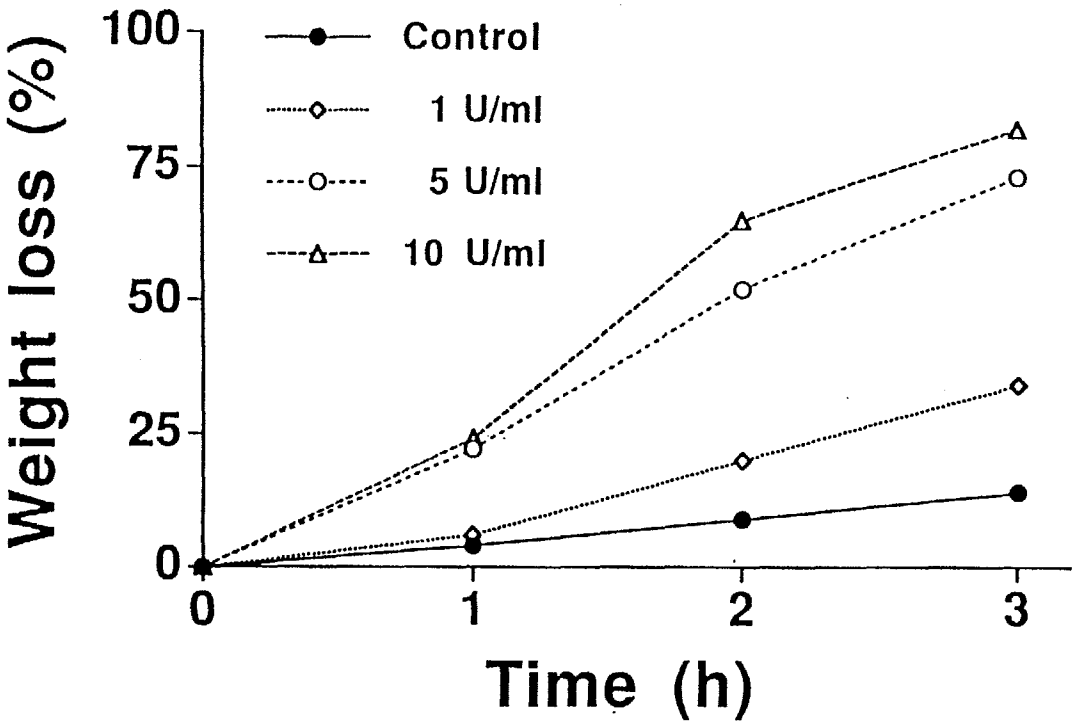


Annex VI

Fig. 7

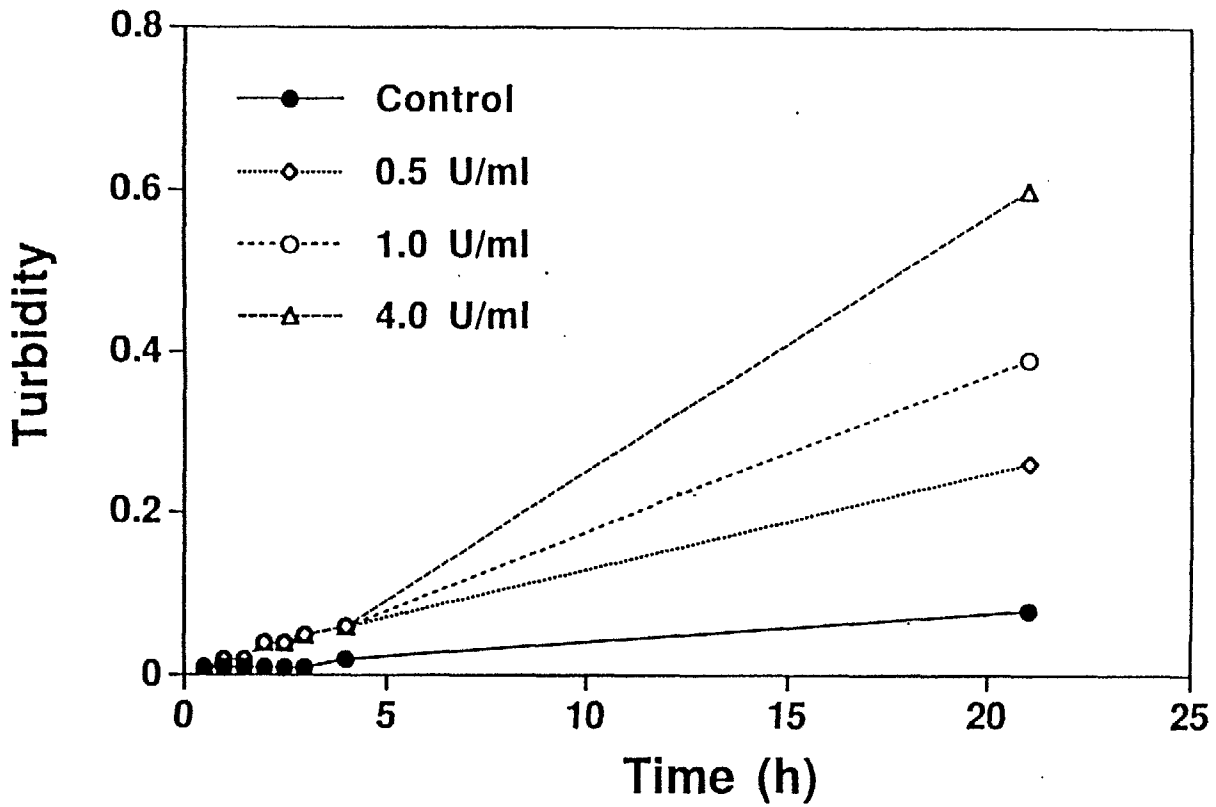
Cucumber maceration

(Influence of enzyme concentration)



POTATO DISCS MACERATION

(Influence of Enzyme concentration)



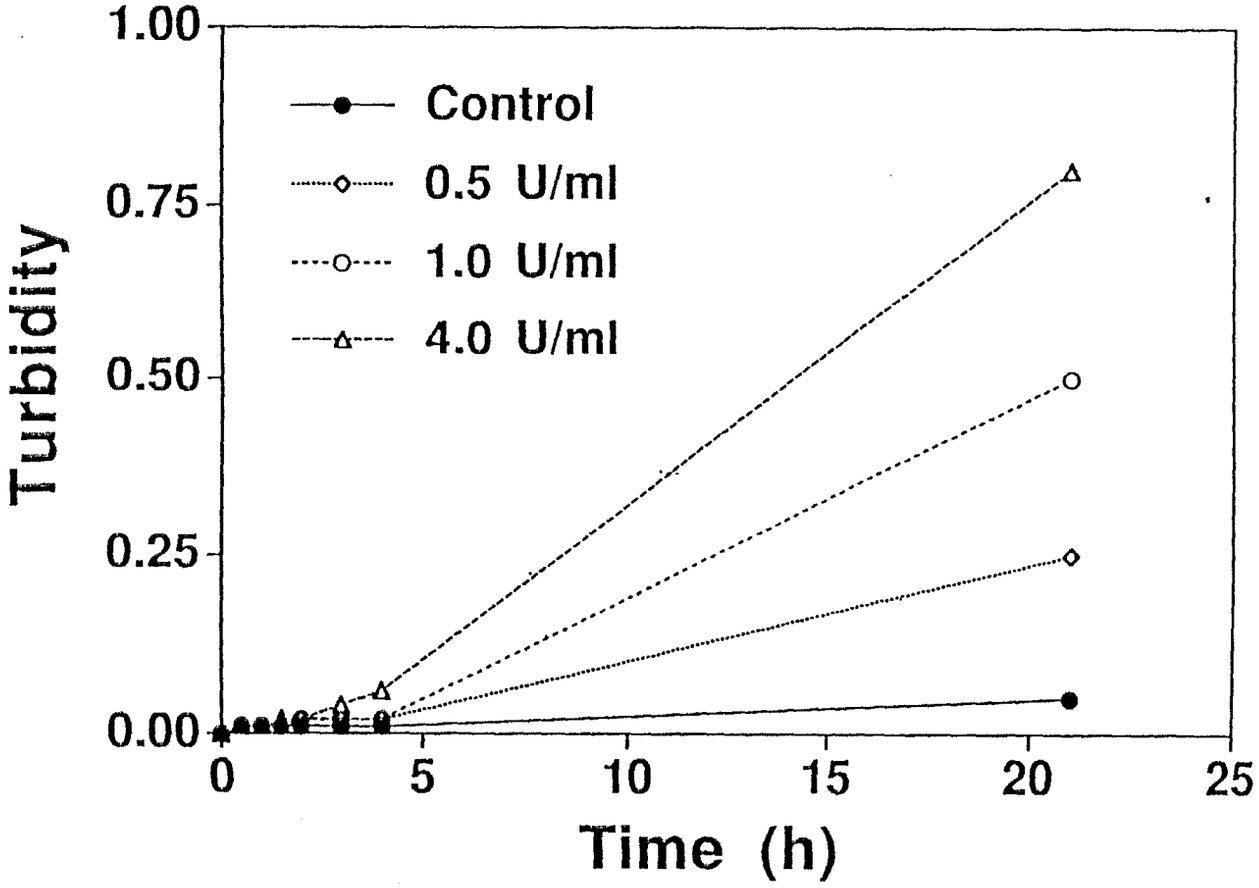
Turbidity was measured at 540 nm

Annex VIII

Fig. 9

Carrot Discs Maceration

(Influence of enzyme concentration)

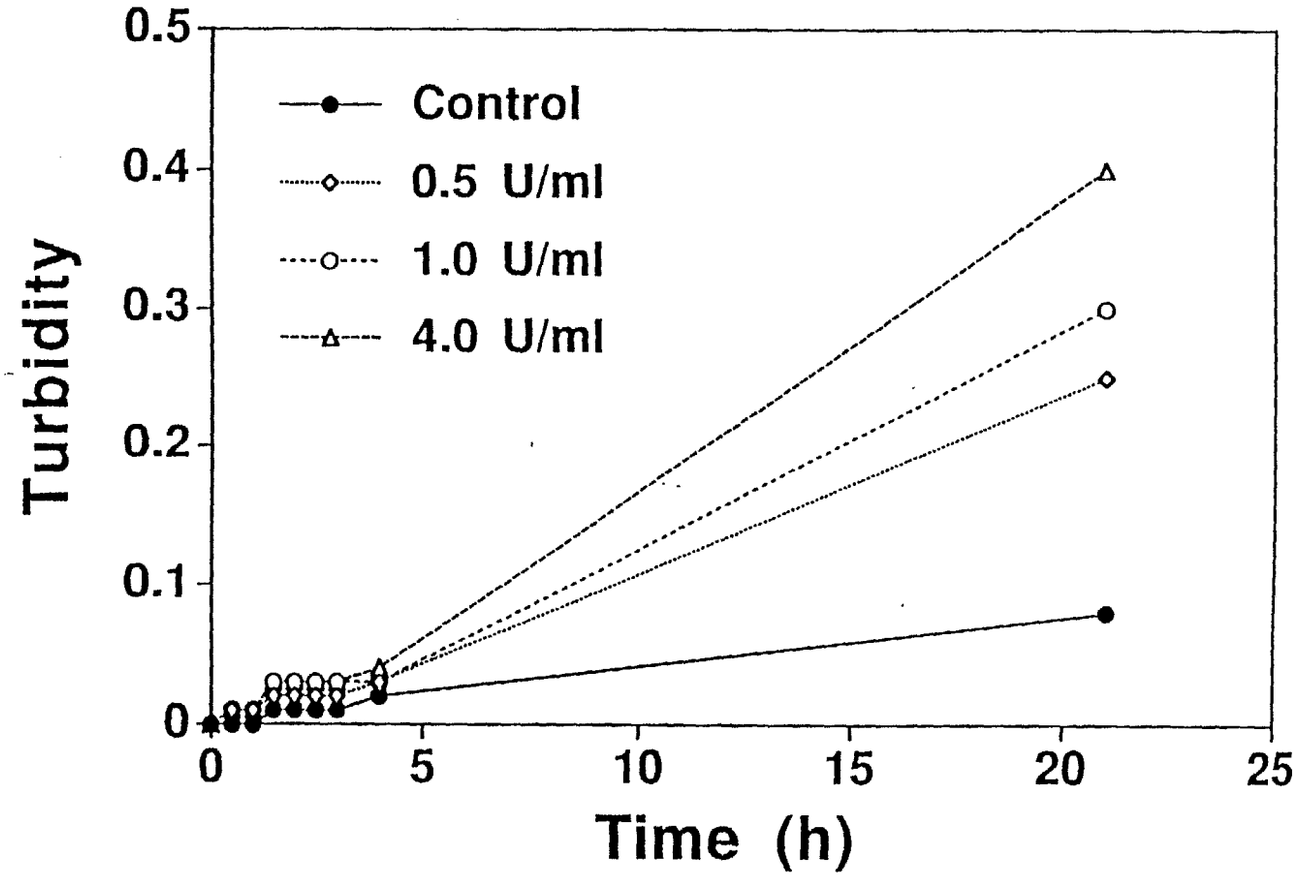


Turbidity was measured at 540 nm

Fig. 10

Turnip Discs Maceration

(Influence of Enzyme Concentration)

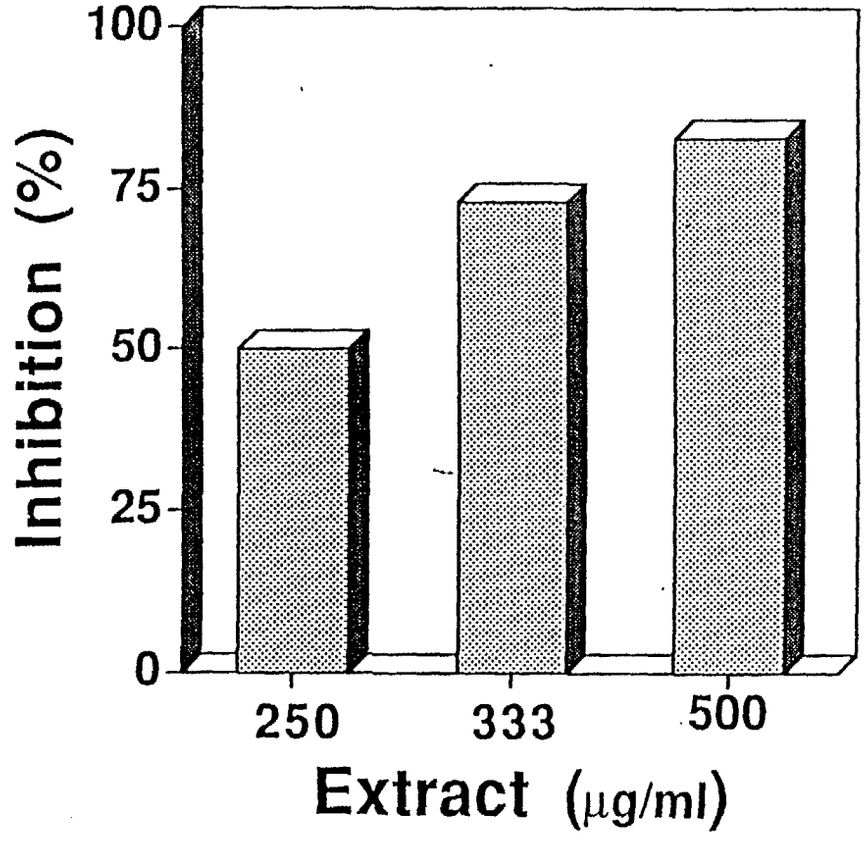


Turbidity was measured at 540 nm

Annex X

Fig. 11

Inhibitory activity of *Areca catechu* extract by cup-plate method

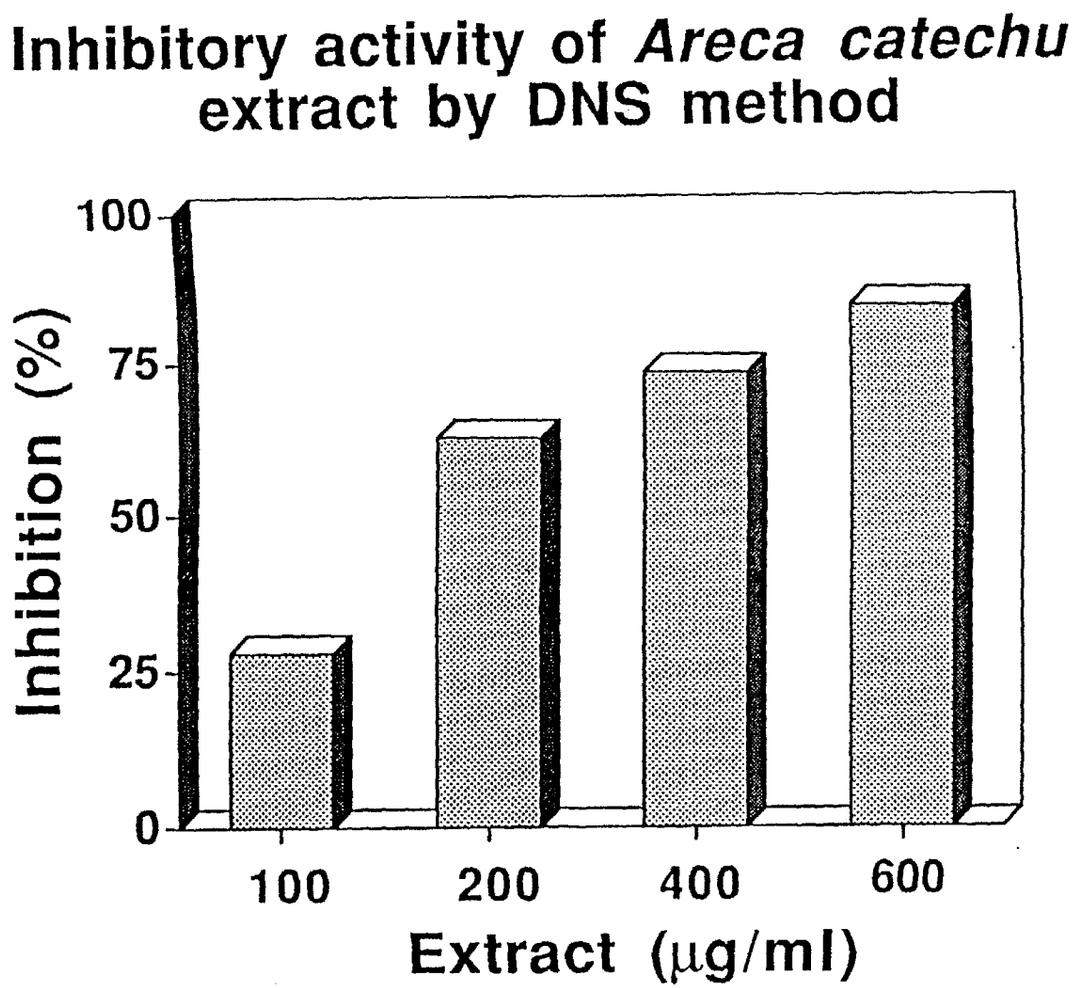


415

Annex XI

Fig. 12

46



Annex XII

Fig. 13

Inhibition of fungal growth

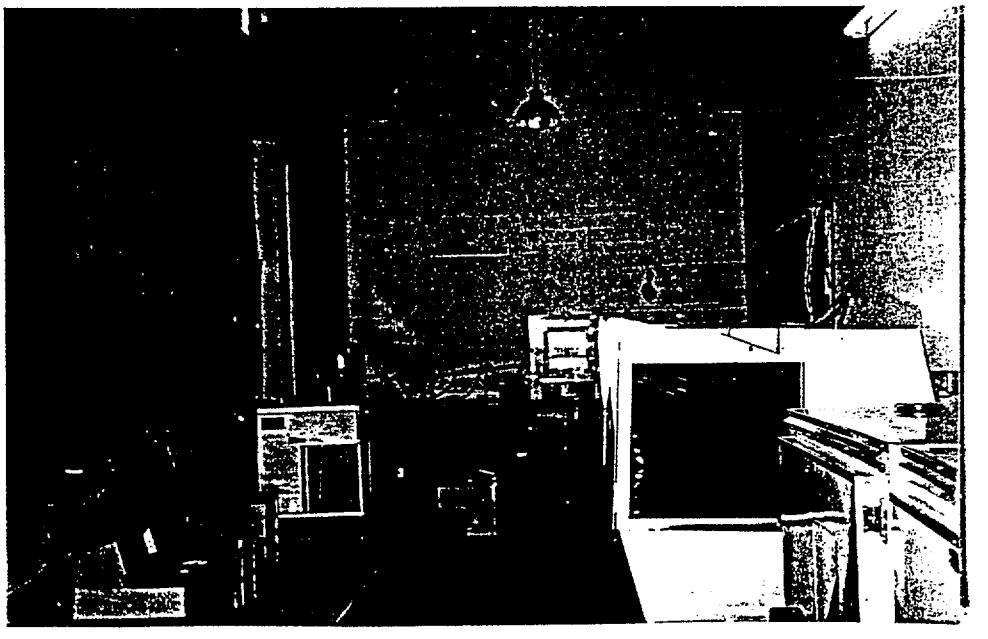
Plant name	Concentration (mg/ml)		
	2.5	1.25	0.625*
Acorus calamus	-	-	±
Sizygium aromatica	-	-	±
Cinnamomum zeylanicum	-	-	±
Glycyrrhiza glabra	-	-	±

- : no growth; ± : negligible growth

Annex XIII

BEST AVAILABLE DOCUMENT

Annex XIII



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