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IN VITRO EVALUATION OF FUNGICIDAL ACTIVITY OF BROMELAIN FROM *ANANAS COMOSUS* AGAINST FUNGAL PHYTOPATHOGENS INFECTING *MUSA PARADISIACA*

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ABSTRACT

Bromelain from *Ananas comosus* is a mixture of proteolytic enzymes which are cysteine protease inhibitors containing thiol endopeptidases employed as a phytomedical compound. The present study focussed on extraction and characterization of bromelain and to determine its protein concentration and protease enzyme activity. The effect of temperature and pH on the extracted enzyme was also determined. The extracted enzyme was determined for its molecular weight by SDS and characterized for the presence of their functional groups by FT-IR spectroscopy. The purified bromelain of stem showed highest concentration of protein with maximum protease activity when compared to that of fruit bromelain and on characterization by FT-IR was found to contain thiol containing amino groups. The extracted and characterized bromelain was treated against isolated fungal pathogens of *Musa paradisiaca*. The isolated bromelain was assessed for their enzyme activity and protein concentration *in vitro* against the isolated fungal spores and determined for its activity. The determined two-way ANOVA indicated that there were significant differences at different concentrations after incubation at varying time period and the effective concentration and time were determined through Turkey post-hoc analysis which showed stem bromelain possess more effective antifungal activity *in vitro* against fungal phytopathogens of *Musa* paradisiaca.

Keywords: Bromelain, SDS-PAGE, FT-IR, *Musa paradisiaca,* Fungal phytopathogens, *In vitro* assessment.

1. INTRODUCTION

Bananas (Musa spp.) represent the fourth most significant staple fruit with a worldwide production of over one hundred million metric tons and rank initially among all sorts of fruits created (FAO 2015). It is important to develop wilt disease tolerant cultivars of banana using novel biotechnological approaches, since it quenches both food supplement and financial gain in developing countries worldwide. Phyto-pathogenic fungi are the most common reason for communicable disease in plants and crops and need the continued use of chemical fungicides for management moreover to meet world food desires. However, perennial use of venturesome agrochemicals for management of flora diseases has many drawbacks. Among them square measure their lack of specificity, the augmented incidence of resistance development upon prolonged application and therefore the adverse impact on human health and setting. Indeed, negative long-run effects on human health and setting have resulted in deregistration of some vital fungicides [1-3].

Plants have evolved a classy innate system to intercept incursive pathogens. The plant defence response involves a spread of mechanisms like the fast generation of reactive Oxygen species (ROS), the induction of the supposed hypertensive response (HR) and production of little molecules (phytoalexins) and proteins with antimicrobial activity. The induction of genes secret writing endo-proteases with totally different chemical action mechanisms, particularly Metallo-, serine-, aspartic- or amino acid endo-proteases, in response to infective agent infection has been delineated [4].

Amino acid proteases are noted to be concerned in several aspects of plant physiology and development, together with senescence, embryogenesis, flower development and response to varied kinds of environmental stress. Different works have incontestable the involvement of amino acid proteases within the

management of programmed necrobiosis or time unit in several plant species. On a different hand, the medicine action of plant amino acid proteases has long been recognized. Papain, the primary amino acid proteolytic enzyme to be characterized in papaya latex, is getting used as a possible drug for microorganism and flora diseases in medicine analysis [5-7].

Bromelain is that the collective name of connected chemical action enzymes found within the tissues of species belonging to family Bromeliaceous, of that pineapple is that the best noted. Two distinct kinds of pineapple bromelain recognized: Stem bromelain and fruit bromelain. Besides enzyme, bromelain is most generally used proteolytic enzyme with the therapeutic application. Pure stem bromelain possesses antifungal activity against agronomically vital crops. The aptitude of bromelain to inhibit flora growth is said to its chemical action activity [8-9].

Keeping in mind the utilities of the plant extact the current work is aimed in stretching its utility towards antifungal effect. The work mainly involves extraction, characterization and quantification of bromelain. The work also deals with analyzing its purity and its efficacy as antifungal activity agent.

2. MATERIAL AND METHODS

2.1. Isolation of fungus and characterization

The plantlets of *Musa paradisiaca* were used for study which was obtained from Spic Agro Biotech Centre, Coimbatore and infected portions of leaves and pseudostem were carefully selected and surface sterilized with 1% Sodium hypochlorite followed by distilled water for 2-3 times at an interval of 1 min. The surface sterilized samples each of 1x1 dimensions were inoculated on PDA with antibiotic and incubated for 96 hrs at 27°C for fungal growth.

2.2. Isolation of pure cultures

The respective single spores of fungal cultures grown on PDA were transferred to PDA after complete growth, sub cultured and maintained at 27°C for 3 to 5 days. The pure isolate after growth was maintained on Potato Dextrose broth at 27°C for 3-5days for revival of cells and stored for further use.

2.3. Morphological characterization of fungus

Fungal structures such as conidia and conidiogenous cells obtained from respective cultures were used to enable specific identification. Mycelia were mounted in 0.01% lacto phenol cotton blue and observed under light microscope at 40X magnification. The fungi were identified based on their mycelia and spores characteristics. Monosporic isolates cultured on PDA were used to determine the growth rate and aspects of colony were recorded.

2.4. Isolation of fungal genomic DNA

The genomic DNA was extracted from five to seven days old fungal cultures grown in Potato Dextrose Broth. The fungal mass obtained from pure culture broth was collected in a sterile tube. About 2ml of broth culture was transferred to fresh sterile tube and freezed at 4°C overnight. The overnight freezed contents were centrifuged at 13,000 rpm for 25mins. To the pellet added 500µl of lysis buffer and homogenization of fungal mass was done twice with a tissue homogenizer for 2-3 mins. The resulting fungal tissue homogenate was centrifuged at 10,000 rpm for 10 mins and the supernatant was transferred to a fresh microcentrifuge tube. To the supernatant added 100µl of 3M potassium acetate, centrifuged the contents at 10k rpm for 10 mins. To the mixture added, 2µl of RNase and 2µl of Proteinase K (Papain) and incubated at 37°C for 15 mins. After this treatment, equal volume of phenol: chloroform: Isoamyl alcohol (5:4.8:0.2) was added and mixed well, followed by centrifugation at 13,000 rpm for 10 min for 2-3 times to remove proteins and cell debris. The upper aqueous layer was taken carefully in a fresh microcentrifuge tube and then equal volume of 100% ethanol was added and incubated at -20°C for 30 min, allowing precipitation and centrifuged at 12,000 rpm for 10 min to pellet down the DNA. To the pellet, added 150µl of isopropyl alcohol, incubated at -20°C for 15 min and centrifuged at 12,000 rpm for 10mins. To the pellet, added 300µl of IPA: 70% ethanol (1:1), incubated at -20°C overnight. The contents were centrifuged at 12,000 rpm for 20 min and washed thrice in 70% ethanol and centrifuged at 12,000 rpm for 5 min. The DNA pellets were air dried and dissolved in 1X TE buffer and stored at 4°C.

2.5. Quality and quantity determination of DNA

The quantity of extracted DNA was determined by measuring the absorbance at 260nm, 280nm and 320nm using UV spectrophotometer. The quality of extracted DNA was accessed on 0.8% agarose gel followed by staining with ethidium bromide. The molecular weight of isolated DNA was compared with 1kb DNA ladder by visualizing under UV Tran illuminator.

2.6. Extraction and characterization of bromelain

2.6.1. Sample collection

The samples used for study are the fruit pulp and the stem of *Ananas comosus,* (pineapple), which was obtained from the Green house maintained at PSG College of Arts & Science, Coimbatore and authenticated for their identity from Botanical Survey of India (BSI), TNAU, Coimbatore.

2.6.2. Sample preparation

The fruit pulp and stem of *Ananas comosus* were used in the present study. The samples were rinsed well under running tap water to remove impurities and surface sterilized with 0.05% Tween 20 followed by rinsing in distilled water for 3-5 times to remove environmental debris from the sample.

2.6.3. Crude extract preparation

About 80g of samples respectively were weighed and rinsed with distilled water. The samples were homogenized with 100ml of 1M Sodium acetate buffer (pH 7) and made into paste. The respective contents were filtered through a muslin cloth (10-20µ micron) and centrifuged at 6000 rpm for 20 min at 4°C. The supernatant was collected in respective tubes, added 0.6g Benzoic acid and stored at 4°C. This was used as crude extract for assays.

2.6.4. Ammonium sulphate precipitation

Ammonium sulphate precipitation has been widely used to precipitate proteins in a partially purified form [10]. Ammonium sulphate is the most commonly used salt as it is cheap and sufficiently soluble and is an effective way to produce substantial amount of proteases [11]. The precipitation was carried out using 120 ml of crude extract of pulp and stem extract respectively. Precipitation was carried out at 50% saturation by addition of ammonium sulphate salt from saturation table under constant stirring for 1hr using a magnetic stirrer and allowed for attainment of equilibrium between dissolved and aggregate proteins for 24hrs at 4°C. The salt enriched solution was then subjected to centrifugation at 8,000 rpm for 20 min at 4°C. The precipitated pellet of respective samples were collected separately and dissolved in 1M sodium acetate buffer. The samples were subjected to further salt precipitation at 60% saturation with the previously obtained precipitated samples and allowed for aggregation of proteins for 24hrs at 4°C with constant stirring. The further saturated salt enriched solution was centrifuges at 8,000 rpm for 15 min at 4°C and the pellet so obtained is dissolved in 0.1M sodium acetate buffer and stored for further use.

2.6.5. Dialysis

The dialysis membrane with molecular cut off of 10,000-12,000KDa was purchased from Precision pharming. The membrane was subjected to activation by pre-treatment of the dialysis membrane in 2% sodium bicarbonate solution for 10 mins followed by treatment in boiling water bat 37°C for 10 mins and treatment with 1M sodium acetate buffer for 10 mins. The pre-treatment was repeated for 3-4 times and the membrane was soaked in 0.1M sodium acetate buffer overnight. After 24hrs the membrane was subjected to activation by repeating the pre-treatment for 2-3 times. Into 500ml of 0.01M sodium acetate buffer, the enzyme sample obtained after precipitation was transferred into the dialysis bag and tagged without any air bubbles. The respective salt precipitated pellet dissolved in 0.1M sodium acetate buffer was dialysed against 0.01M sodium acetate buffer for 48hrs at 4°C. The dialysed sample so obtained is centrifuged at 8,000 rpm for 10 mins at 4°C and the pellet is dissolved in 0.1M sodium acetate buffer (pH 6.8-7). This recovered dialysed sample is subject to second dialysis as described above and stored at 4°C.

2.6.6. Thin layer chromatography (TLC)

Thin Layer Chromatography (TLC) is based on solid-liquid adsorption where stationary phase is solid adsorbent silica gel coated on clean, dry, glass-Figs. Slurry of silica adsorbent was prepared with distilled and coated on to the applicator. The slurry was allowed to dry at room temperature and heat activated at 110°C for 30-60 mins. Mobile phase was prepared as described above and allowed to saturate. About 5µl of crude extracts of stem and fruit pulp were loaded on to the silica adsorbent respectively and placed in the mobile phase chamber. The TLC chamber was allowed to remain undisturbed, when the solvent front has reached three quarters, the TLC Fig was removed and the position of solvent front was marked and allowed to dry. The developer solution was sprayed and incubated until colour develops and Rf value was calculated with the distance travelled by the solute and solvent. The amino acid of unknown was identified with the obtained R_f value by comparing with the standard known value.

R_f = Distance travelled by solute / Distance travelled by solvent

2.6.7. Estimation of protein-lowry's method

The protein concentration of crude, salt precipitated pellet and dialysed samples were determined by Lowry's method with BSA as standard (Stock: 1mg/1ml; Working: 1ml stock made up to 10 ml with 1M sodium acetate buffer - 100µg/1ml) by measuring optical density at 660nm. The concentration of unknown samples were estimated using the calibrated graph.

2.6.8. Estimation of protease enzyme

The protease enzyme concentration was determined by modified method of Lowry's method with tyrosine (Stock: 1mg/1ml; Working: 1ml stock made up to 10 ml with 1M sodium acetate buffer - 100µg/1ml) as standard and 1.5% casein as substrate by measuring the optical density at 660nm. The protease concentration of unknown samples were estimated using calibrated graph. One unit of protease activity is defined as the amount of enzyme required to catalyse the formation of 1µmol of standard per minute at 37°C (pH 6.8). The specific activity was calculated as the relation between the enzymatic activity and protein concentration (U/mg of protein).

2.6.9. Gelatin Digestion Unit (GDU) Assay

One gelatin digestion unit can be defined as the amount of enzyme that will hydrolyse 1mg of amino nitrogen from gelatin after 20 mins of digestion at pH 4.5 at 45°C. Prior to this assay, the enzymes were allowed to stand for 60 mins at 4°C.

Gelatin + H_2O_2 ⟶ Amino acids + Oligopeptides

A 5% gelatin serves as a substrate which is equilibrated to 45°C followed by addition of test enzyme and control enzyme respectively and 3% hydrogen peroxide by incubating at 45°C for 20mins. Titrated against 0.05N NaOH, pH adjusted to 6.9 and incubated at room temperature for 2-3 mins. Added 37% formaldehyde to all the tubes and pH adjusted to 7.2 and optical density reading measured at 660nm. The GDU can be calculated using the formula,

GDU/mg = (T-B) x 14 x Normality of NaOH x 1000/ Conc. of enzyme

2.6.10. Determination of molecular weight by SDS-PAGE

SDS-PAGE was performed with dialysed extract enzyme. About 20µl of extracted samples were dissolved in 500µl of protein extraction buffer and vortex for 30-60secs. Centrifuge the contents at 10,000 rpm for 10 min at 4°C. The supernatant (100µl) was collected and made up to 500µl with 398µl sample buffer and 2µl ß-mercaptoethanol. Vortexed the contents gently. Prior to loading, the samples were placed in boiling water bath at 37°C for 8-10 mins. The samples were loaded onto 12% resolving gel and 5% stacking gel with medium rage protein molecular marker and subjected to electrophoresis for 3-5 hrs at 50V.

2.6.11. Staining and destaining

The SDS-PAGE gel was carefully placed in staining solution overnight at 37°C with constant agitation. The stained gel was subjected to destaining with constant agitation in distilled water for 2-3 times followed by placing the gel immersed in destaining solution with constant agitation for 3-4 hrs at 37°C or until staining solution disappears. The obtained respective bands were observed under white Trans-illuminator and photographed.

2.6.12. Activity staining

Activity staining for bromelain was done by casein gel electrophoresis. The completely destained gel was carefully placed in 2% Casein substrate solution for 45 min at 4°C. After incubation, the 2% casein substrate gel was subjected to continuous agitation in freshly prepared 2% casein substrate solution for 30-45 min at 37°C. Development of clear band indicate the presence or absence of bromelain.

2.7. Determination of optimum pH

The optimum pH of bromelain was determined by modified method of estimation as defined by Lowry's method of estimation. Sodium acetate buffer of varying pH (2, 4, 6, 8, 10 and 12) were used to determine the optimum pH and optical density reading was measured at 660nm. The optimum pH was determined using calibrated graph.

2.8. Determination of optimum temperature

The optimum temperature of bromelain was determined by modified method of Lowry's estimation by incubating the unknown samples at varying pH (4°C, 20°C, 40°C, 60°C, 80°C and 100°C). The optical density reading was measured at 660 nm. The optimum temperature was determined using calibrated graph.

2.9. Fourier Transform Infra-Red spectroscopy (FTIR)

FTIR is useful for identification of organic molecular groups and compounds. The functional groups, side

chains and cross-links involved in the compound, give rise to characteristic vibrational frequencies in the infra-red range by absorbing the light in the infra-red region of the electromagnetic spectrum. FTIR absorption spectra of the purified protease (Bromelain) were obtained. The spectrum was scanned from 400 to 4000 cm^{-1}, at a resolution of 7 cm^{-1} and the data was analysed by using PE-GRAMS/32 1600 software [12].

2.10. Assessment of antifungal activity of bromelain

2.10.1. In vitro proteolytic activity

The proteolytic activity of bromelain was assayed in 50% PDB, the media used in *in vitro* antifungal assay to determine if the proteolytic activity was found to be affected in presence of bromelain in PDB. The reaction mixture contained enzyme solution with 1% casein as substrate in 50% PDB. The mixture was incubated at 37°C for 20 min and the reaction was stopped by addition of 10% TCA. The activity was determined by following the Anson's method (Anson 1938) and the absorbance was read at 440nm. Varying concentrations of tryptophan was used as standard and the concentration of unknown protein *in vitro* was estimated using the calibrated graph. One unit of protease activity is defined as the amount of enzyme required to produce an absorbance change of one unit (1 cm light path) per minute for conditions under which assay is carried out.

2.10.2. In vitro protein estimation

The protein concentration of the extracts was determined by Lowry's method of protein estimation. The reaction mixture contained 50% PDB with extracts. The protein concentration was compared with standard tryptophan of varying concentration. The optical density reading was read at 660nm and the concentration of unknown protein *in vitro* was estimated from the calibrated graph.

2.11. Antifungal activity of *Ananas comosus* extracts on fungal isolates

The crude, salt precipitated pellet and dialysed sample of fruit pulp and stem extracts of *Ananas comosus* were assessed for their antifungal property by agar well diffusion method. The test specimens (isolated fungus) of 7-10 days culture maintained on PDB were swabbed with sterile cotton on PDA Figs. Wells were made with gel puncture and 25µl of extracts were loaded onto the wells. A positive control was maintained with commercially available fungicide - 0.1% Dazomet. The Figs were incubated at 28°C for 3-5 days. The incubated Figs were examined for the interruption of growth over the inoculum. The size of the clear zone was measured to evaluate the inhibitory action of the extracts.

2.12. Antifungal activity of *Ananas comosus* extract at varying concentrations

The dialysed sample of fruit pulp and stem extracts of *Ananas comosus* were assessed for their antifungal property by agar well diffusion method at varying concentrations. A 0.1% Dazomet of varying concentration was maintained as positive control. The effective concentration at which bromelain possess fungicidal property can be determined. Concentrations of 100%, 75%, 50% and 25% were prepared and used for assay. The test specimens (isolated fungus) of 7-10 days culture maintained on PDB were swabbed with sterile cotton on PDA Figs. Wells were made with gel puncture and 25µl of extracts of varying concentrations were loaded onto the wells. A positive control was maintained with commercially available fungicide of varying concentration - 0.1% Dazomet. The Figs were incubated at 28°C for 3-5 days. The incubated Figs were examined for the interruption of growth over the inoculum. The size of the clear zone was measured to evaluate the inhibitory action of the extracts.

2.13. Assessment of *In vitro* antifungal activity

2.13.1. Preparation of fungal spore suspension

The fungal spore suspension was prepared from fungal culture maintained on PDA for 12-15 days at 28°C. The spores of 12-15 days cultures were collected by removal of mycelia with 0.01% Tween 20 and collection of spores with sterile distilled water. The mycelia were removed and discarded. The spores collected were made up to 50ml and stored.

2.13.2. In vitro antifungal activity

The *in vitro* antifungal activity was determined by broth microdilution assay as described. The assay mixture contained 40µl of spore suspension made up to 170µl with 50% PDB and Bromelain and positive control (at varying concentrations). About 40µl spore suspension served as a control and sodium acetate buffer as blank and stem and fruit bromelain as test sample and added 10µl leupeptin to all the wells and incubated at 28°C. The fungal growth and inhibiting activity was monitored for 3-5 consecutive days by measuring the optical density at 492nm. The OD reading obtained were analysed to identify if there is any significant variance for each day at

varying concentrations using ANOVA (Analysis Of Variance). If there is any significant variance the least and maximum possessed fungicidal activity were calculated for their percentage of fungicidal activity.

Percentage of fungicidal activity = {(Control - Test)/ Control} x 100

2.14. Analysis of Variance (ANOVA)

The fungicidal activity was calculated using statistical technique, two-way ANOVA to test whether the means of more than two quantitative populations are equal. The determined values indicate if there is any significant difference in the variance both within and between the samples. The variance indicates the significant difference within the concentration of test sample supplemented and between the days of fungicidal activity.

2.15. Turkey *post hoc* analysis

Turkey's honestly significant difference (HSD) post hoc test is used to analyses data that were not specified in ANOVA which is used in conjugation to find means that are significantly different from each other. This can be used to assess the fungicidal activity at specific day to determine the effective concentration.

3. RESULTS

3.1. Isolation and characterization of fungus

3.1.1. Isolation of fungus

The infected leaves and pseudo stem which showed symptoms of wilting and black streak diseases were inoculated on PDA and incubated at 28°C for 4 days and checked for the growth of fungal isolates. There was a characteristic growth of mycelia, carbon black spores and pigment producing mycelial fungus (Fig. 1A &B).

3.1.2. Isolation of pure fungal colonies

Pure isolates were obtained by specific selection of fungal culture grown on PDA of respective sample followed by inoculation on PDB for 5-6 days at 27°C and identified for their morphology after sporulation. Three distinct fungal colonies were obtained which showed varied pattern of growth such as carbon black spore producing mycelia, pigment producing mycelial fungi and colony forming fungi on macroscopic observation (Fig. 1C, D & E).

3.1.3. Morphological characterization

The pure isolates were observed under light microscope at 40X magnification by lacto phenol cotton blue staining. The pure isolates were observed for their morphology such as conidia, hyphae, macrospore, microspore and chlamydospore each of which showed distinct characteristics on PDA (Fig 2).

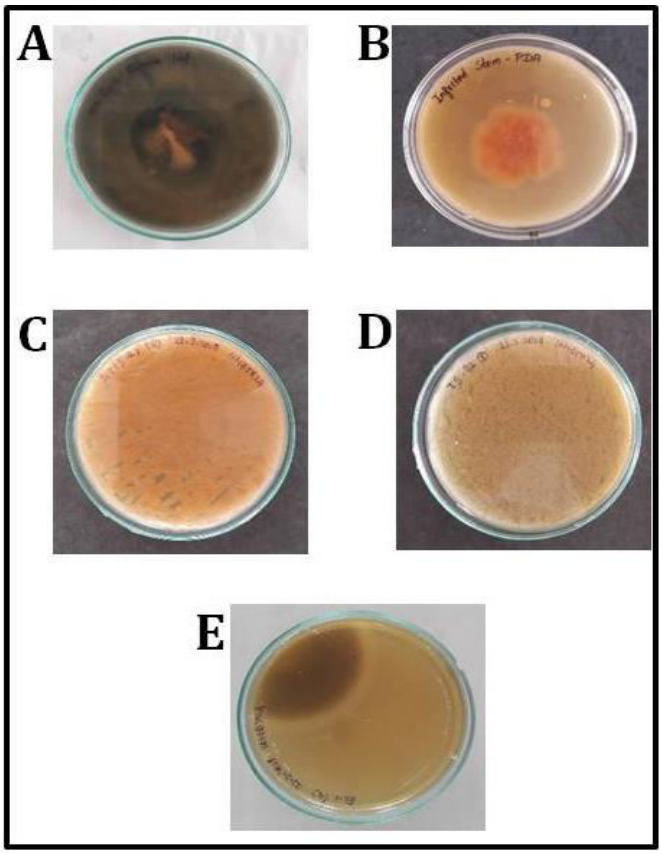
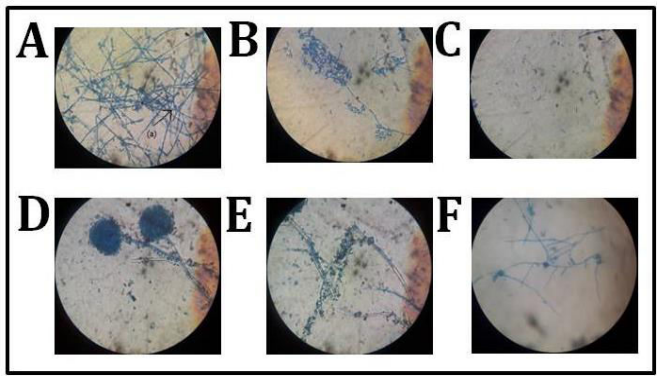


Fig. 1: Isolation of fungus from *Musa paradisiacal* (A) Infected banana leaf (B) Infected banana pseudo stem (C) *Fusarium oxysporum* (D) Fungal isolate (E) *Aspergillus niger*



Fusarium oxysporum–(A) Single terminal chlamydospore (B) False head monophiles (C) Microconidia and macroconidia Aspergillus niger- (A) Conidia and conidiophore (B) Hyphae (C) Immature chlamydospore and hyphae

Fig. 2: Microscopic view of fungal isolates at 40x magnification

3.2. Molecular characterization

3.2.1. Quantity analysis-UV-spectrometry

The quantity of isolated DNA were determined by UV-Spectrometry at Abs (260nm, 280nm and 320nm). The ratio of respective isolates was found to be around and nearer to 1, which indicates that the isolated DNA contains traces of contaminants. The concentration of isolated DNA were determined and tabulated (Table 1).

The total yield of genomic DNA of respective fungal isolates were found to be around 110-365ng/µl. Pure preparation of DNA is known to have a ratio about 1.8-2, which in some cases can have slight variations due to presence of phenolic contents or interactions due to protein. On quantitative determination the ratio of respective fungal isolates were found to be ≤1, which indicates that the isolated DNA contains protein/ phenolic contaminants which can be identified by qualitative analysis on 0.8% agarose gel.

Table 1: Quantity determination of fungal genomic DNA

S. No	Sample	Ratio	Concentration (ng/µl)
1.	*Fusarium oxysporum*	1.001	110
2.	*Nigrospora spp.,*	1.003	365
3.	*Aspergillus niger*	0.952	200

3.3. Quality analysis-agarose gel electrophoresis

The proposed method yielded good quality of DNA from fungal isolates on agarose gel electrophoresis, which on comparison with standard 1 kb DNA maker was evident that the fungal isolate contained high molecular weight DNA. The molecular weight of isolated fungus (*Fusarium oxysporum,* and *Aspergillus niger*) was found to be 8 kb (Fig. 3).

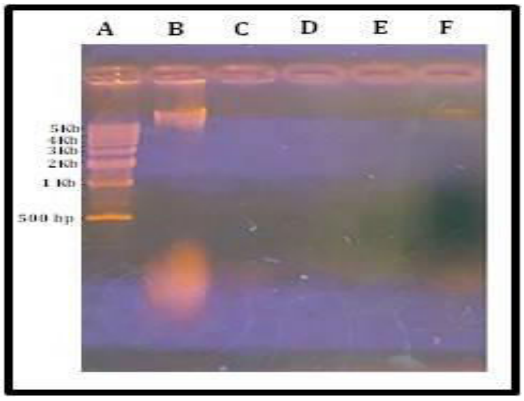


Fig. 3: Bands of DNA on 0.8% agarose gel (a) DNA marker (b) *Fusarium oxysporum* (c) *Nigrospora sp.,*(f) *Aspergillus niger*

The isolated DNA can be purified further by removal of phenolic contents for further analysis by PCR amplification and sequencing.

3.4. Extraction and characterization of bromelain

3.4.1. Thin Layer Chromatography (TLC)

The R_f value was calculated from the distance travelled by the solute through the mobile phase solvent. The R_f values of unknown samples (a) fruit pulp - 0.41 and (b) stem-0.40 on comparison with standard amino acid table were found to be similar to that of Cysteine which shows the extracted sample contains Cysteine (Fig. 4). The R_f value of fruit pulp and stem extract, on comparison with standard TLC table of amino acids were found to be Cysteine ($R_f = 0.4$).

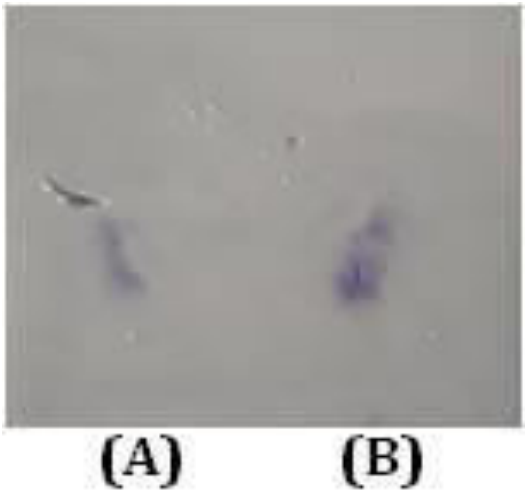


Fig. 4: TLC of *Ananus comosus* (a) fruit pulp (b) stem extract

3.4.2. Estimation of Protein-Lowry's method

The protein concentration of unknown samples of fruit pulp and stem of *Ananas comosus* was determined by Lowry's method by comparison with a standard protein BSA. The standard graph (Fig. 5A) was used to identify the concentration of protein present in the unknown samples of crude extracts, Ammonium sulphate precipitated sample and dialysed sample. The concentration of protein was found to be high in dialysed samples of fruit pulp and stem of *Ananas comosus* (Fig. 5A). The concentration of protein was found to be high in dialysed sample of stem than fruit pulp with crude extract containing less amount of protein concentration due to interfering impurities.

3.4.3. Estimation of protease enzyme activity

The enzyme activity of fruit pulp and stem extract of *Ananas comosus* was determined by modified method of Lowry's method by comparison with a standard amino acid Tyrosine. The standard graph (Fig 5B) was used to identify the enzyme activity of unknown samples of crude extracts, ammonium sulphate precipitate sample and dialysed sample. The enzyme activity was found to be high in dialysed samples of fruit pulp and stem of *Ananas comosus* (Fig 5B).The enzyme activity was found to be high in dialysed sample of stem than fruit pulp with crude extract containing less amount of activity due to interfering impurities.

3.5. Enzyme activity, Specific activity, Purification factor and Yield of enzyme

The enzyme activity, specific activity, purification fold and yield percentage was calculated from the extracted samples and tabulated (Table 1). The enzyme activity was found to be high in dialysed sample of fruit pulp and stem with highest yield of 91.21% for fruit pulp and 76.84% for stem. The protein purification fold was found to be high in dialysed sample of fruit pulp, 1.69 and stem, 1.55.

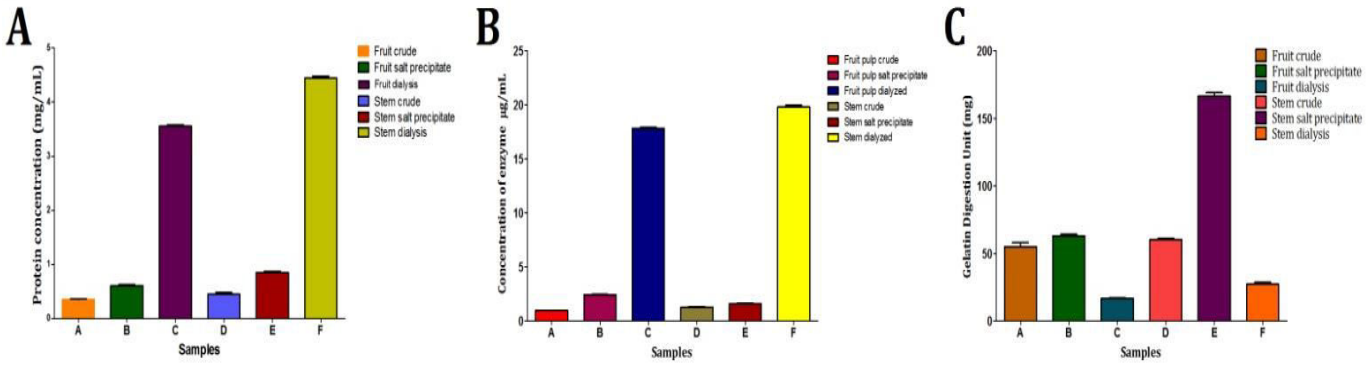


Fig. 5: (A) Estimation of (A) protein concentration (B) Protease enzyme activity and (C) GDU in fruit pulp and stem of *Ananas comosus*

Table 1: Enzyme activity, Specific activity, Purification Factor and Yield of enzyme

Sample	Test sample	Enzyme activity (U/ml)	Specific activity (µmol/ml/min)	Purification fold	Yield %
FRUIT	Crude	0.161	0.488	1	100
	Amm. SO_4 Pellet	0.404	0.662	1.36	37.64
	Dialysis	2.937	0.823	1.69	91.21
STEM	Crude	0.212	0.471	1	100
	Amm. SO_4 Pellet	0.265	0.323	0.69	18.75
	Dialysis	3.258	0.732	1.55	76.84

3.6. Gelatin Digestion Unit (GDU)

The GDU assay indicates the amount of free nitrogen released due to enzyme activity on binding with substrate. The GDU was found to be high in Ammonium sulphate salt precipitated sample of fruit pulp and stem extract due to presence of nitrogen containing ammonium group (Fig. 5C).

3.7. Determination of optimum pH of enzyme

The enzyme stability was determined by subjecting the extracted enzyme to varied pH. The fruit pulp and stem extract showed activity up to a range of pH 8 after which the activity declined. The extracts were found to stable at a pH range of 6-8 indicating the stable pH of the enzyme (Fig. 6A). The activity of enzyme declined at a pH range from 10-12 which showed that the extracts were unstable above pH 10.

3.8. Determination of optimum temperature

The stability of extracts were determined by incubating the extracts at varying temperatures of 4°, 20°, 60°, 80° and 100°. The extracts exhibited activity up to 60°C, after which there was a drastic decline in the activity which indicates that the extracts are stable up to 60°C (Fig 6-B).

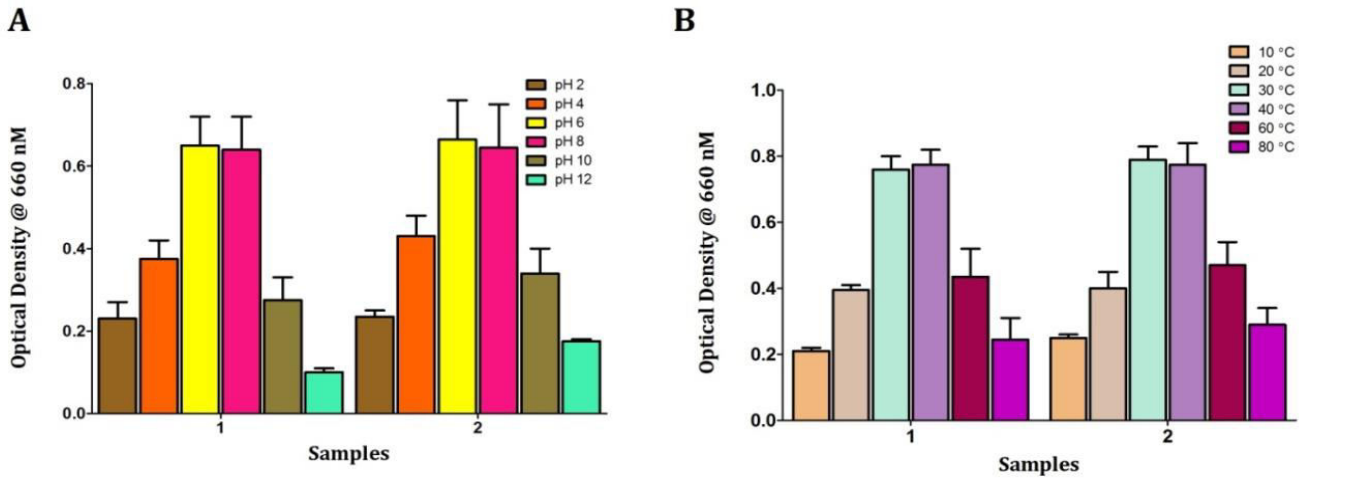


Fig. 6: Determination of optimum (A) pH and (B) Temperature of Bromelain from *Ananas comosus*

3.9. Molecular weight determination by SDS-PAGE

The SDS-PAGE (Fig. 7) showed that the molecular weight of fruit pulp and stem extracts of partially purified dialysed sample were of molecular range 29 kDa of that of Molecular marker. Therefore the molecular weight of fruit pulp and stem were found to be 29,000 Da which is similar to that of Bromelain.

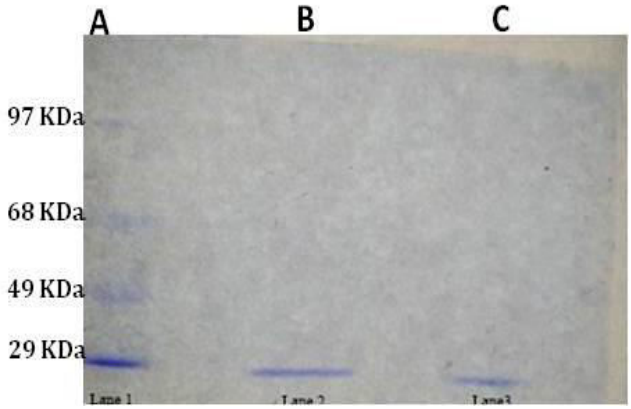
Bromelain, from pineapple, has a molecular weight of range 24 kDa to 39 kDa. The fruit pulp and stem (Lane 2&3) of pineapple was used to as unknown to determine the molecular weight of unknown protein. On SDS-PAGE, the samples were compared with molecular marker of medium range (94 kDa) to determine the molecular weight. The fruit pulp and stem extract had an intense band around 29 kDa, indicating that the molecular weight of unknown protein as 29 kDa which is bromelain.

3.10. Activity staining

The SDS-PAGE gel was subjected to activity staining to determine the presence/absence of Bromelain. The dark and intense bands on activity staining with 2% Casein substrate indicated formation of clear and intense band indicating the presence of bromelain (Fig. 7).

3.11. Characterization of functional groups of bromelain by FT-IR

The FT-IR was used to characterize the potential functional group and vibrational interactions in bromelain and compared with blank (Sodium acetate buffer). The functional group common to fruit pulp and stem were S-H stretch and secondary amide NH_2 stretch indicating the presence of functional group of cysteine which is a thiol peptidase (Fig. 8).



(a) Protein molecular weight marker (b) Stem bromelain (c) fruit bromelain

Fig. 7: SDS-PAGE gel after activity staining by casein gel electrophoresis with 2% casein substrate

Table 2: FT-IR spectra peak of Sodium acetate buffer

Wavelength (cm^{-1})	Functional group	Peak characterization
1276	Aliphatic C-N	Stretching
1407	Azo compound N=N	Stretching
1539	Secondary Amide N-H C-N	Bending Stretching
1647	Primary Amide NH_2	Bending

Table 3: FT-IR spectra peak of Fruit pulp of *Ananas comosus*

Wavelength (cm^{-1})	Functional groups	Peak characteristics
1215	Aromatic C-O	Stretching
1377	Aliphatic nitro compound NO_2	Symmetric Stretching
1539	Secondary Amide N-H C-N	Bending Stretching
1639	Primary amide NH_2	Bending
1745	C-H	Stretching
2958	S-H	Stretching
3390	Secondary amide N-H	Stretching

Table 4: FT-IR spectra peak of stem extract of *Ananascomosus*

Wavelength (cm-1)	Functional groups	Peak characteristics
1137	Amines N-C	Stretching
1276	Aliphatic C-N	Stretching
1431	Azo compound N=N	Stretching
1508, 1547	Secondary Amide N-H C-N	Bending Stretching
1639	Primary amide NH_2	Bending
2360, 2951	S-H	Stretching
3375	Secondary amide N-H	Stretching

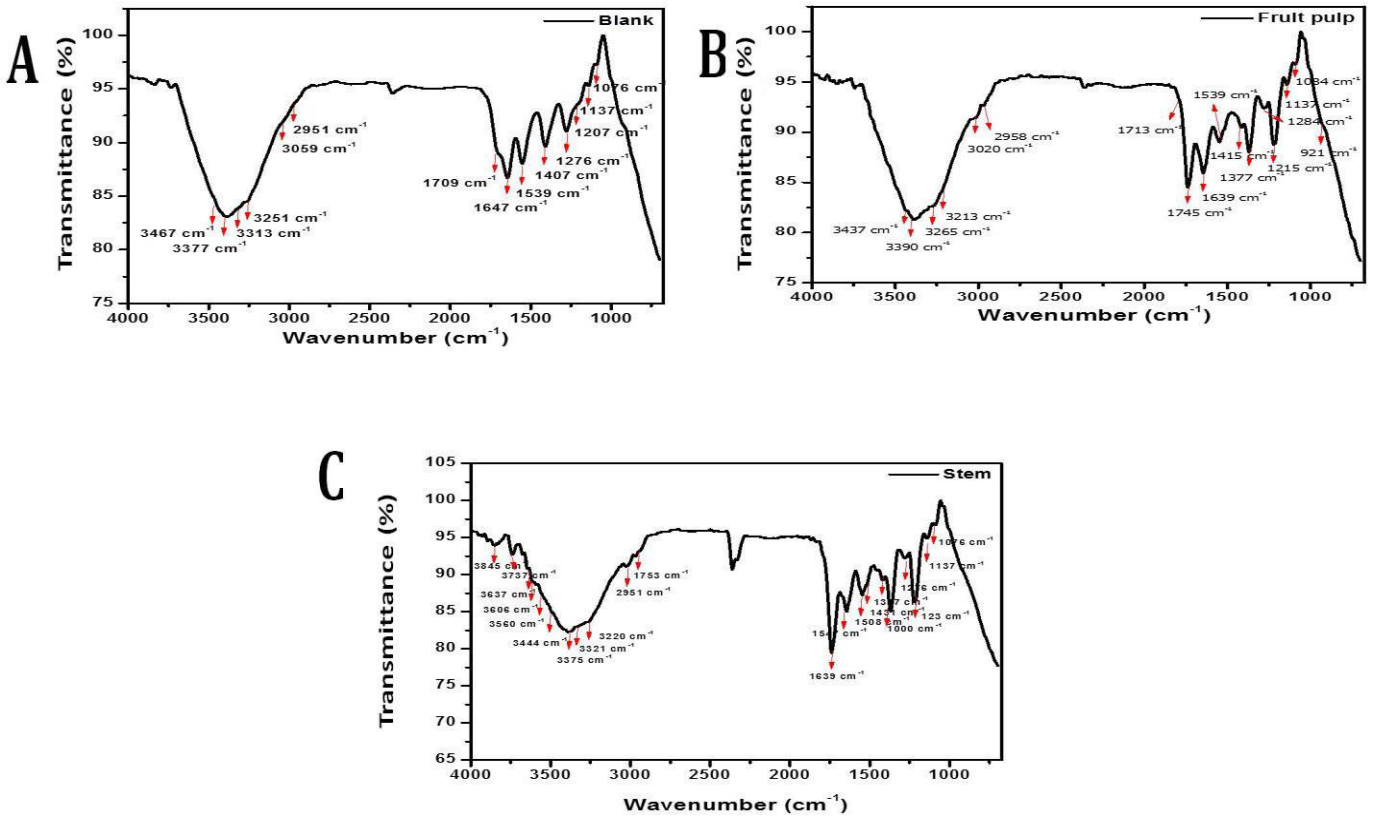


Fig. 8: FT-IR spectroscopy of (A) Blank-Sodium acetate buffer (B) Fruit pulp and (C) Stem extract of *Ananas comosus*

3.12. *In vitro* antifungal activity of bromelain against the fungal phytopathogens

3.12.1. Antifungal susceptibility test-Agar well diffusion method

The fruit pulp and stem extracts of crude sample, Ammonium sulphate precipitation (pellet) and dialysed sample were assessed for their antifungal activity by agar well diffusion method. The extent of zone of inhibition indicate the antifungal activity of each of the extracts. The antifungal activity was tested against *Fusarium oxysporum, Aspergilus niger and Nigrospora spp.,* and. 0.1% Dazomet, a commercially available fungicide was maintained as a positive control (Fig 8-10). The dialysed sample of stem extract containing bromelain and crude extracts possessed antifungal activity against *Fusarium oxysporum, Aspergillus niger* and *Nigrospora spp.,*. The extent of zone of inhibition against the isolated plant phytopathogens are tabulated (Table 5).

3.12.2. In vitro estimation of protein

The protein concentration of fruit pulp and stem extracts (Fig. 9A) were estimated *in vitro* with std. BSA. The so obtained concentration was compared with previously estimated protein concentration to determine if 50% PDB possess any inhibitory action. On comparison, it was observed that they possess inhibitory mechanism when supplemented in PDB medium.

3.12.3. In vitro Protease Enzyme activity

The protease enzyme activity of fruit pulp and stem extracts (Fig 9B) were estimated *in vitro* with std. tyrosine. The soobtained concentration was compared with previously estimated protein concentration to determine if 50% PDB possess any inhibitory action. On comparison, it was known that they possess inhibitory mechanism when supplemented in PDB medium.

Table 5: Antifungal susceptibility test – agar well diffusion method – zone of inhibition

Sample	Test sample	*F.oxysporum* (Fig. 10)	*A.niger* (Fig.12)	*Fungal isolate* (Fig.11)
	Crude	0.00	0.9	1.6
Fruit	**Amm.SO_4 pellet**	0.00	0.00	0.00
	Dialysis	1.9	0.9	1.5
	Crude	0.00	1.2	2.0
Stem	**Amm.SO_4 pellet**	1.1	0.00	0.9
	Dialysis	1.8	0.9	1.7
Positive control	**Dazomet**	2.5	0.00	0.7
Blank	**Bufffer**	0.7	0.7	1.5

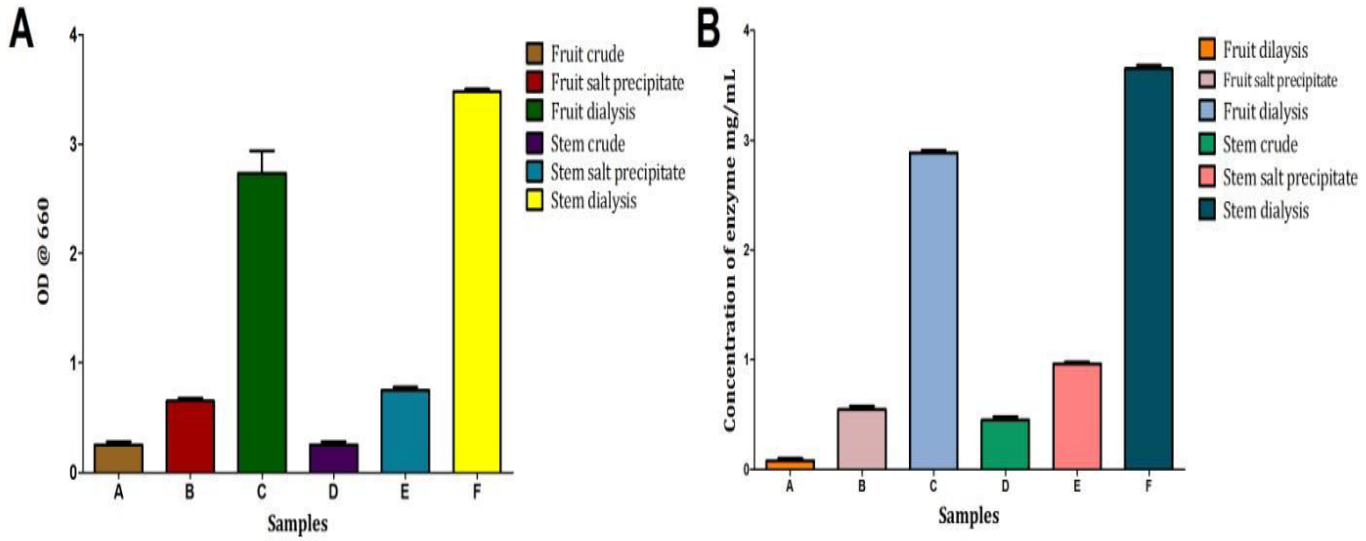


Fig. 9: *In vitro* estimation of (A) Protein and (B) Protease enzyme activity in fruit pulp and stem of *Ananas comosus*

3.13. Comparative analysis on protein concentration and protease enzyme activity of lowry's assay and *in vitro* assay of fruit pulp and stem extracts of *Ananas comosus*

The protein concentration and enzyme activity of fruit pulp and stem extracts were compared to determine if PDB possess any inhibitory action. On comparison of *In vitro* proteolytic enzyme activity with protease assay, the enzyme activity was found to be less in *in vitro* proteolytic assay which indicates that PDB possess inhibitory action toward bromelain when incorporated into the medium. To overcome this, protease inhibitor specific for cysteine proteases are added which preserve protein integrity after membrane lysis.

3.14. *In vitro* antifungal assay

The antifungal assay was carried out in varying concentration to determine the effective fungicidal property of bromelain by Broth micro dilution assay. The fungicidal activity of fruit pulp and stem bromelain with buffer as negative control and dazomet as positive control was assessed *in vitro* against *Fusarium oxysporum, Aspergillus niger* and *Nigrospora spp.*, (Tables 7-9).

Table 6: Enzyme activity, specific activity, purification factor and yield of enzyme

Sample	Test sample	Enzyme activity (U/ml)	Specific activity (µmol/ml/min)	Purification fold	Yield %
	Crude	0.013	0.062	1	100
FRUIT	**Amm. SO_4 Pellet**	0.089	0.146	2.36	4.56
	Dialysis	0.477	0.161	2.6	73.39
	Crude	0.075	0.3	1	100
STEM	**Amm. SO_4 Pellet**	0.161	0.227	0.76	1.43
	Dialysis	0.604	0.174	0.58	16.11

Table 7(a): *In vitro* fungicidal activity against *Fusarium oxysporum* (after 24 hrs)

	% of fungicidal activity					
Sample	20µl	40µl	60µl	80µl	100µl	120µl
Buffer	95.65	93.41	92.58	91.7	90.97	86.91
Fruit pulp	89.16	87.74	85.01	82.76	75.54	58.79
Stem	87.40	82.81	74.12	74.51	54.69	20.56
Dazomet	73.83	37.89	28.96	10.74	5.27	1.17

Table 7(b): *In vitro* fungicidal activity against *Fusarium oxysporum* (after 120 hrs)

	% of fungicidal activity					
Sample	20µl	40µl	60µl	80µl	100µl	120µl
Buffer	99.76	99.36	99.24	99.20	99.08	98.96
Fruit pulp	99.64	99.36	98.80	98.68	98.44	98.36
Stem	99.80	98.32	97.84	96.42	96.16	99.08
Dazomet	99.08	99.40	98.40	96.72	96.24	95.60

Table 8(a): *In vitro* fungicidal activity against *Aspergillus niger* (after 24 hrs)

	% of fungicidal activity					
Sample	20µl	40µl	60µl	80µl	100µl	120µl
Buffer	96.55	95.56	94.62	94.1	92.51	91.38
Fruit pulp	92.95	91.42	88.27	88.17	87.58	74.08
Stem	97.19	88.81	84.67	84.67	64.47	20.89
Dazomet	99.16	46.08	28.98	12.46	6.26	1.01

Table 8(b) *In vitro* fungicidal activity against *Aspergillus niger* (after 120 hrs)

	% of fungicidal activity					
Sample	20µl	40µl	60µl	80µl	100µl	120µl
Buffer	99.68	97.65	97.36	97.21	96.5	96.3
Fruit pulp	95.53	94.34	92.98	92.15	91.87	81.69
Stem	96.45	95.45	94.53	93.86	87.00	41.92
Dazomet	91.51	80.22	76.47	70.73	64.4	98.47

Table 9(a): *In vitro* fungicidal activity against *Nigrospora spp.,* (after 24 hrs)

	% of fungicidal activity					
Sample	20µl	40µl	60µl	80µl	100µl	120µl
Buffer	97.28	96.80	96.52	96.23	95.61	52.31
Fruit pulp	96.95	96.61	96.28	96.14	95.422	46.63
Stem	74.68	70.2	68.19	62.28	59.42	43.92
Dazomet	44.35	27.94	87.73	7.53	4.72	3.2

Table 9(b): *In vitro* fungicidal activity against *Nigrospora spp.,* (after 120 hrs)

	% of fungicidal activity					
Sample	20µl	40µl	60µl	80µl	100µl	120µl
Buffer	99.96	99.51	99.25	99.22	99.17	9910
Fruit pulp	99.81	99.25	98.58	98.54	98.47	98.13
Stem	99.93	99.81	98.39	97.42	97.09	95.29
Dazomet	99.85	99.78	97.94	97.94	96.73	95.32

3.15. Analysis of Variance

The significant concentration of test samples at varying concentrations for varying days were determined by two-way ANOVA and Turkey post hoc test. The variance was determined at 0.05% significance which showed that the test samples possessed significant variance between each column (Concentration) and rows (days). There was a significant difference between and within the samples for each day wherein maximum fungicidal activity occurred between the samples which indicates that the extract has possessed inhibitory activity against *Nigrospora spp., Aspergillus niger* and *Fusarium oxysporum.*

3.16. Turkey *post hoc* test

The Turkey *post hoc* test was used to determine the significant difference between each sample number within each concentration for multiple comparison. The variance as determined by two-way ANOVA indicates that if the samples possess variance at 0.05% significance and if possessed they are analysed by multiple comparison of post hoc analysis. This determines the variance within each groups, between each samples and significance among each samples (Tables 10-12).

Table 10: Turkey *post hoc* analysis of *Aspergillus niger*

S. NO	Sample	Concentration (µl)	Effective after (hrs)
1.	**Buffer**	120	72 -120+
2.	**Fruit pulp**	120	72 -120+
3.	**Stem extract**	120	120+
4.	**Dazomet**	120	120+

Table 11: Turkey *post hoc* analysis of *Fusarium oxysporum*

S. NO	Sample	Concentration (µl)	Effective after (hrs)
1.	**Buffer**	100 120	72 -120+ 24-120+
2.	**Fruit pulp**	100-120	24 -120+
3.	**Stem extract**	120	72-120+
4.	**Dazomet**	40-120	24-120

Table 12: Turkey *post hoc* analysis of *Nigrospra spp.,*

S. NO	Sample	Concentration (µl)	Effective after (hrs)
1.	**Buffer**	120	24-120+
2.	**Fruit pulp**	120	24 -120+
3.	**Stem extract**	20-80 100 120	24-120+ 120+ 72-120+
4.	**Dazomet**	20-120	24-120+

4. CONCLUSION

Bromelain, has been widely used as a novel therapeutic substance due to its diverse proteolytic mechanism of action which still is unknown. The use of this cysteine protease as a fungicidal agent against plant pathogens has not been much known. The *in vitro* evaluation study herein shows that there is a significant difference in the growth of fungal pathogens infecting *Musa paradisiaca* after 3 days of supplementation of bromelain at a concentration range of 100-120µl which contains nearly 0.5mg of protein indicating that bromelain possess inhibitory action against *Fusarium oxysporum, Aspergillus niger* and *Nigrospora spp.,*. The highest fungicidal activity

was exhibited after 5 days on supplementation of bromelain, which indicates that bromelain from *Ananas comosus* can be used as a substitute for chemical fungicides in order to retain the natural environmental conditions and overcome the hazards caused due to chemical fungicides.

Conflict of Interest

All the authors involved in designing and execution of this work are given due recognition and have no conflicts in composing and submission of the article.

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