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EXTRACTION, PARTIAL PURIFICATION AND CHARACTERIZATION OF BROMELAIN ENZYME FROM PINEAPPLE (*ANANAS COMOSUS*)

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ABSTRACT

Pineapples as a fruit have effective juice and a vibrant tropical flavour that balances the tastes of sweet and tart. Bromelain is a multiple mixture of substances that can be extracted from the stem and core fruit of the pineapple. Acetone fractional precipitation of bromelain from pineapple peel its characterization after the recovery process was studied completely. The natural source Pineapples have been initially screened and used to produce Bromelain, a protease enzyme. Extract of bromelain was homogenized by processing pineapple's peel and pulp using pre-cooled buffer at specific pH for screening process. Acetone fractional precipitation studies were performed under refrigerating condition. Bromelain was characterized before and after precipitation to determine its optimal pH, temperature and buffer. Results showed that bromelain was precipitated successfully in the 65% acetone fraction and yielded over than 85-90 % of enzyme recovery. Bromelain enzyme was finally characterized with molecular weight of 66kilodaltons. These results showed that bromelain recovery with acetone fractional precipitation is a viable and easier process, in which results in a good quality enzyme for industrial applications.

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INTRODUCTION

Enzymes are macromolecular biological catalyst. They are responsible for thousands of metabolic processes that sustain life [1]. Enzymology is the study of enzyme and enzyme catalyzed reaction. The comprehensive study of an enzyme involves investigation of:

- Its molecular structure (i.e. 1°, 2°, 3° and 4° structure).
- Protein properties (isoelectric point, electrophoretic mobility, pH, temperature, stability and spectroscopic properties).
- Enzyme property (specificity and reversibility; kinetic).
- Thermodynamic (activation free energy and entropies energy).
- Active site (Number, molecular nature of site and the mechanism of catalyzed involved).
- Biological properties (cellular location, isoenzymic forms and metabolic relevance of the reaction promoted).

Bromelain is a chief protease enzymes found in pineapple plant (*Ananas comosus*) [1]. It has been known chemically since 1876 [2] and was identified for the first time by Marcano in 1891 [3]. The investigation and isolation of bromelain has been started since 1894 [4]. It was first therapeutically supplemented in the year 1957. Sulfhydryl proteolytic enzymes are the chief constituents of bromelain [2] [5]. Bromelain is abundant in stem and fruit of pineapple plant and it can also be isolated in small amount from pineapple waste such as core, leaves, peel etc. [6]

Pineapple plant also contains minor quantities of other proteinases like ananain and comosain [7] but bromelain is regarded as a primary and extensively investigated component [8]. The reason of being such valuable is due to its miraculous utilization as phytomedical compound [9]. Pineapple [*Ananas comosus* (L.) Merr. Family: Bromeliaceae] is one of the most important and commercial fruit crops in the world. It is known as the queen of fruits due to its excellent flavour and taste. Pineapple is the third most important tropical fruit in the world after Banana and Citrus. Pineapples are consumed or served fresh, cooked, juiced and can be preserved. This fruit is highly perishable and seasonal. Mature fruit contains 14% of sugar; a protein digesting enzyme, bromelain, and good amount of citric acid, malic acid, vitamin A and B. Pineapple juice's composition varies depending on geography, season, process and time of harvest. Its balance of sugar and acid contributes to the fruit's refreshing flavour. The U.S. National Library of Medicine lists bromelain as a proteolytic digestive enzyme. When taken with meals, bromelain aids in the digestion of proteins, working to break proteins down into amino acids.

Nutritional Value

Pineapple is a wonderful tropical fruit having exceptional juiciness, vibrant tropical flavor and immense health benefits. Pineapple contains considerable amount of calcium, potassium, vitamin C, carbohydrates, crude fibre, water and different minerals that is good for the digestive system and helps in maintaining ideal weight and balanced nutrition. Half a cup of pineapple juice provides 50 percent of an adult's daily recommended amount of vitamin C. Bromelain supplements are particularly popular among athletes for treating all sorts of physical aches and injuries. [6]. The reason of being such valuable is due to its miraculous utilization as phytomedical compound [9]. Bromelain displays antiedematous, fibrinolytic, anticancer, anti-inflammatory, antibiotic, anticoagulative and antithrombotic functions [9]. Hence, the study was to produce the enzyme from the testing source by screening process, partially purify by precipitation and dialysis and to characterize the proteins present in it by electrophoresis and also to study the respective applications of the enzyme.

MATERIALS AND METHODS

Collection and Processing process

The pineapple (*Ananas comosus*) were been collected from local market, Chennai. The samples were washed, peeled and rinsed with tap water and distilled water to remove any dust particles repeatedly. It was then kept in the refrigerator for experimental studies.

Extraction process

10g of the sample was homogenized in 0.2M phosphate buffer ($_p$H-7) (cooling condition) (1:1 ratio). The filtrate was centrifuged at 10,000 rpm for 15 minutes. The supernatant was stored and used as enzyme source.

Qualitative assay for Screening of protease production

Water agar medium (half strength) is to be supplemented with proteinous substrate (1% gelatin, casein and skimmed milk) for the assay of proteolytic enzyme was prepared and autoclaved at 121^0C for 15minutes. Different volumes (25µl, 50µl, 75µl and 100µl) of the supernatant were loaded in the wells and 25µl of phosphate buffer was used as control. The plates were incubated for 24 hours at room temperature. After 24hours of incubation, the plates were flooded with (indicator) mercuric chloride ($HgCl_{2)}$ solution for 5 – 10 minutes. Protease production was visualized by a translucent zone around the wells. Protease activity for the supernatant can be assayed quantitatively according to the method of [11]. One unit of protease activity is defined as the amount of enzyme required to liberate 1µmol.tyrosine/ml/min.

Estimation of protein (Bradford method)

The protein is usually determined according to the method of [12]. 1ml of the enzyme source (supernatant) was mixed with 5ml of CBB-dye solution (coomassive Brilliant Blue-G250). The mixture was mixed well and incubated for 5 minutes at room temperature. Simultaneously, control without the enzyme source and with 5ml of CBB-dye solution was maintained. The OD of the solution was measured at 595nm in a spectrophotometer and compared with Bovine Albumin Serum (BSA) to determine the protein content of the sample.

Optimization of enzyme production under different parameters

Based on the screening results between the two samples, potent source was optimized on the basis of pH and temperature used and the enzyme going to be produced. The different parameters are;

- Effect of buffer
- Effect of pH
- Effect of temperature

In the optimization of enzyme production, a minimal production will be selected and variable parameter will be incorporated. The supernatant was tested for various parameters such as buffer, pH and incubation period and also the supernatant was estimated for enzyme activity and protein estimation. The parameters, at which maximum enzyme activity found, was selected as optimum for the production of the respective enzyme.

Effect of buffer on enzyme activity

To determine optimal buffer, the selected potent source was been homogenized with different buffers such as Sodium acetate buffer (0.1M) and Sodium citrate buffer (0.1M) range. The supernatant was filtered and centrifuged at 10,000 rpm for 15minutes. The supernatant was subjected to quantitative assay for enzyme activity.

Effect of pH on enzyme activity

To determine optimal pH, the selected potent source was been homogenized with the optimized buffer under different pH ranges from 4-6. The supernatant was filtered and centrifuged at 10,000 rpm for 15minutes. The supernatant was subjected to quantitative assay for enzyme activity.

Effect of temperature on enzyme activity

To determine optimal temperature, the selected potent source was homogenized with the optimized buffer and pH and the supernatant was filtered, centrifuged at 10,000 rpm for 15minutes and treated with varying temperatures ranging from room temperature, refrigerator, incubator and water bath. Then, the supernatant was subjected to quantitative assay for enzyme activity.

Bulk Production for the optimized parameter

Based on the optimized buffer, pH and temperature, the respective source was chosen, weighed and homogenized with specific buffer using mortar and pestle. It was then filtered. The filtrate was centrifuged at 10,000 rpm for 15 minutes. The supernatant was stored in the refrigerator and used as enzyme source for partial purification.

Precipitation technique by solvent - Acetone precipitation

Analyses of proteins play an important role in modern biology. Thousands of different proteins dictate the whole cellular processes. In order to analyze the number and kind of proteins at any given time during cell development, the proteins should be precipitated quantitatively.

Precipitation of proteins using organic solvent is done usually at 0^0C or subzero temperature in order to minimize denaturation of proteins especially enzymes by the solvents. Hence the solvent is cooled overnight at -20^0C and added slowly with constant stirring to an ice – cold solution of the enzyme in cool condition. 65% acetone precipitation was preferred for precipitating the proteins.

The enzyme source was transferred to clean beaker and the magnetic pellet was dropped inside the beaker and was placed on the magnetic stirrer. While the sample is stirring, required amount of solvent was slowly added until desired saturation level attains. After the total amount of solvent was added, the mixture was incubated over night to ensure complete precipitation. Then the sample was centrifuged 8000rpm for 15 minutes. The supernatant was carefully removed and the pellet was dissolved in sodium phosphate buffer and stored in a refrigerator for purification process. The precipitated sample was subjected to quantitative assay for enzyme activity and also for application studies.

Application studies – Anti-browning activity, Anti-bacterial activity

(1) Anti-browning activity:

Fresh red apples were purchased from local market and were washed and peeled to remove the outer layer. Apples were crushed using the kitchen blender; the resulting juice was filtered using muslin cloth. The juice was transferred into new eppendorf tubes containing anti-browning agents. Only apple juice was taken as a control. Samples were incubated at room temperature for 1hr and reading for each sample was taken at 420nm for every 10minutes. [13]

The percentage of inhibition was calculated using values coming from control, test and anti-browning agents.

Inhibition % = A (420 nm) control – A (420 nm) test* 100 / A (420 nm) control.

(2) Anti-bacterial activity:

Well diffusion assay

Nutrient agar was preferred and 24 hours growing bacterial cultures [14] *Staphylococcus aureus* (Gram positive), *Micrococcus luteus* (Gram positive), *Proteus vulgaris* (Gram negative), *Bacillus subtilis* (Gram negative), *Klebsiella pneumoniae* (Gram negative), *Schigella flexneri* (Gram negative) and *Escherichia coli* (Gram negative) were used as test pathogens. Four different concentrations (25µl, 50µl, 75µl and 100µl) of the precipitated sample were loaded in the wells. Tetracycline was used as the standard. The plates were then incubated at 37ºC for 24hours. After incubation the inhibition diameter was measured.

Dialysis process

Dialysis is a process, by which small molecules are selectively removed from a sample containing of both small and large molecules. Dialysis is a usually performed using a special type of membrane known as semi permeable membrane. The semi permeable membrane allows small molecules to pass freely through, holding the large molecules inside. These membranes are essentially made up of cellulose derivatives (cellulose acetate or cellulose nitrate). Dialysis is routinely used to remove small molecules from protein sample.

Characterization of proteins

The protease enzyme was characterized by Sodium Dodecyl sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE) according the method of [15].

RESULTS AND DISCUSSION

Collection, Processing and Extraction process

The *Ananas comosus* was been collected and the processing steps such as cleaning in tap water and distilled water were performed in order to remove the unwanted dust materials (fig.1).The *Ananas comosus* was been packed in sterile polythene bags, labeled and stored which was needed for further studies.The *Ananas comosus* was homogenized with phosphate buffer (($_p$H-7) using mortar and pestle. The *Ananas comosus* were homogenized since the cellular content will be released from the samples. Further, the filtrate was centrifuged so as to use the supernatant as the enzymatic source.

Pulp of *Ananas comosus* **Peel of *Ananas comosus***

Fig.1: Collection of sample materials.

Qualitative assay (Preliminary plate assay)

The present study was conducted to produce and characterize bromelain, a protease enzyme from pulp and peel of *Ananas comosus*. Primary screening for protease producing samples was tested on water agar medium with three different substrates (Gelatin, Casein and Skimmed milk), based on the zone formation due to protease hydrolysis. Gelatin was selected as substrate because the zone of clearance was observed, whereas, casein exhibited zone of clearance in lower amount when compared to gelatin and skimmed milk did not exhibit the zone of clearance. Totally, two species of samples showing protease activity were screened. Among the two samples, the peel extract (*Ananas comosus*) as gelatin – substrate was selected based on the zone of clearance as 19mm.

Table 1: Screening of samples under three different substrate - *Ananas comosus.*

S.No	Sample Volume (µl)	Peel extract of *Ananas comosus*			Pulp extract of *Ananas comosus*		
		Casein	Skimmed milk	Gelatin	Casein	Skimmed milk	Gelatin
1	25	Nil	10 mm	12 mm	Nil	10 mm	11 mm
2	50	Nil	11 mm	15 mm	Nil	10 mm	12 mm
3	75	Nil	13 mm	17 mm	Nil	11 mm	14 mm
4	100	Nil	14 mm	19 mm	Nil	12 mm	17 mm

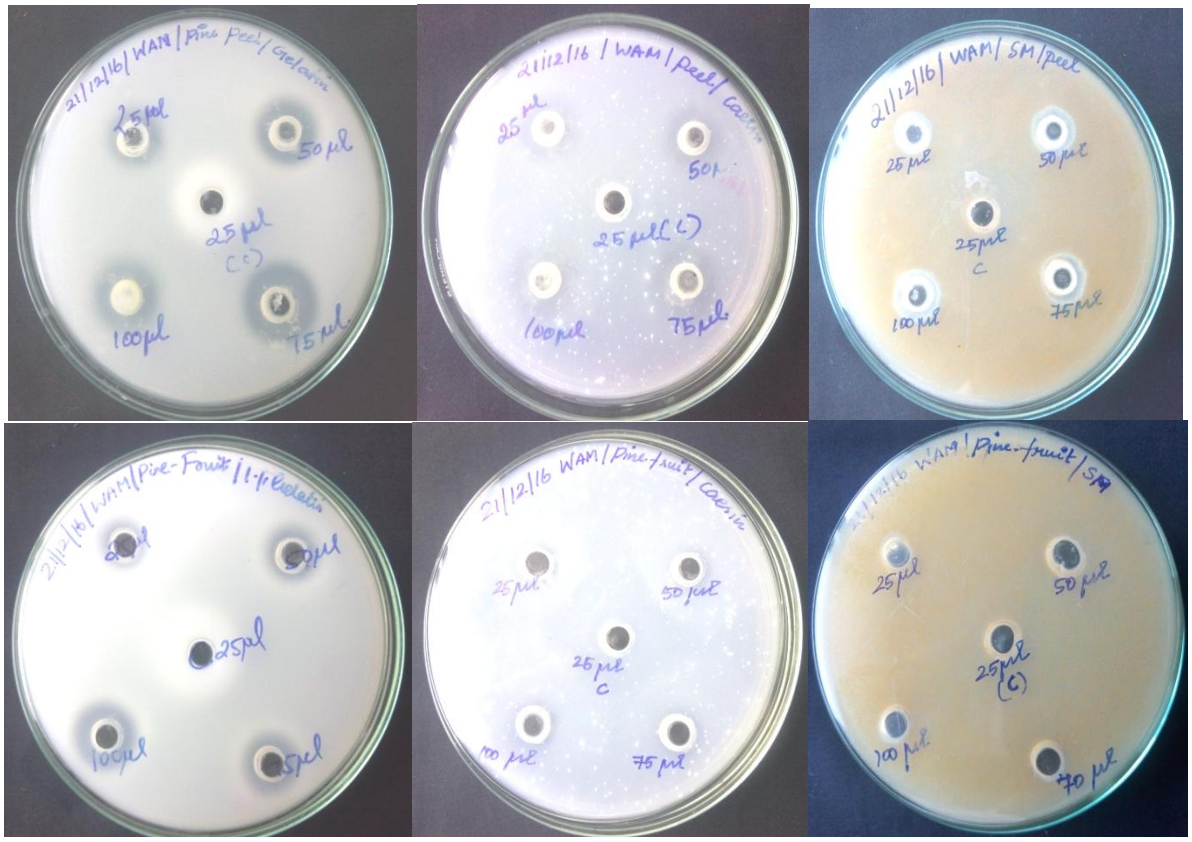

Fig.2: Screening of samples for protease production-Gelatin, Casein and Skimmed milk as substrate.

Comparitively, *Ananas comosus* had better results for the peel extract when compared to pulp extract. Since gelatin was selected as substrate, it was tested for enzyme production (plate assay) for other buffers such as Sodium acetate buffer and Sodium citrate buffer.

Table 2: Screening of Peel extract of *Ananas comosus* for specific buffers.

S.No	Sample Volume (µl)	Peel extract of *Ananas comosus* (Gelatin – substrate)	
		Sodium acetate buffer	Sodium citrate buffer
1	25	17mm	17mm
2	50	17mm	18 mm
3	75	18mm	19 mm
4	100	20mm	22 mm

Based on the results obtained from plate assay, sodium citrate buffer was selected for further experiments, since the zone of clearance was found to be 22mm when compared to other two buffers relatively.

Optimization of enzyme production under different parameters

Based on the results obtained from screening-plate assay, peel extract of *Ananas comosus* was chosen considering gelatin as the substrate. The sample was optimized on the basis of buffer, pH and temperature used and the enzyme going to be produced. The different parameters are;

- Effect of buffer
- Effect of pH
- Effect of temperature

Effect of buffer on enzyme activity

For optimization studies, phosphate buffer (0.2M), sodium citrate and sodium acetate buffer (0.1M) was prepared, selected for peel extract of *Ananas comosus* and the enzyme was quantified as 917.01 EU/ml for phosphate buffer, 1097.1 EU/ml for sodium acetate buffer. The enzyme activity was 1518.6 EU/ml for sodium citrate buffer and was found to be higher when quantified. Hence, sodium citrate was selected to study the effect of pH and temperature on enzyme activity.

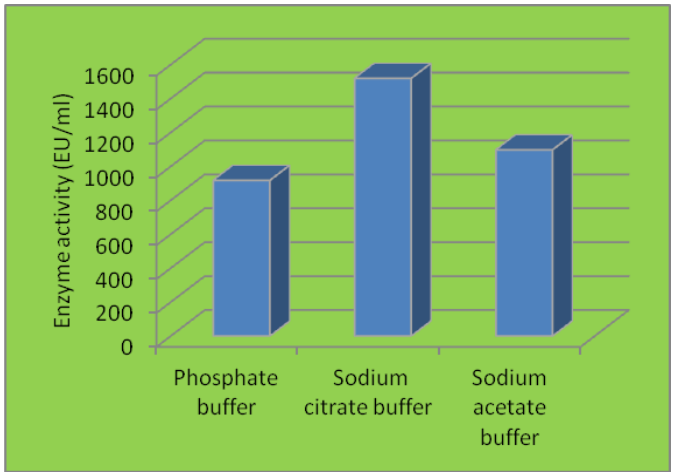

Fig.A: Effect of three different buffers on enzyme activity parameter.

Effect of pH on enzyme activity

Sodium citrate buffer (0.1M) was selected to study the effect of pH in the varying range of 4.0 – 6.0 on enzyme activity. The Sodium citrate buffer was prepared separately in the selective range of pH – 4, 4.2, 4.4, 4.6, 4.8, 5.0, 5.2, 5.4, 5.6, 5.8, 6.0.The peel extract of *Ananas comosus* was homogenized, filtered and centrifuged and the sample was determined for the enzyme activity quantitatively.

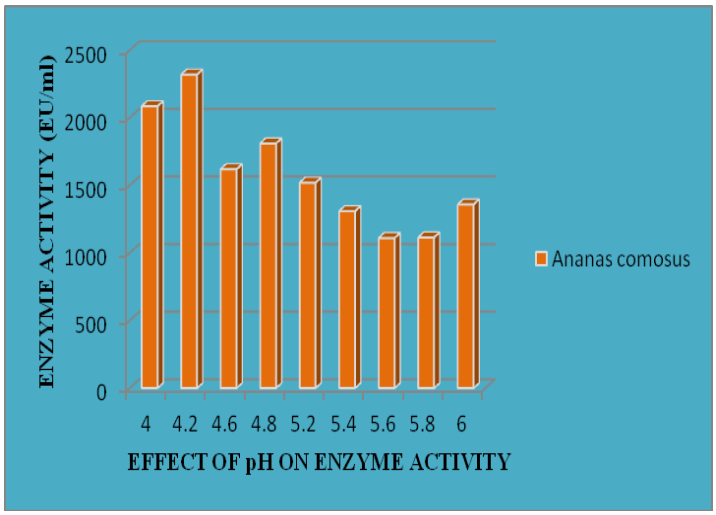

Fig. B: Effect of pH on enzyme activity.

From the fig.B, the effect of different pH on the activity of the enzyme can be differentiated. At pH 4.2, the enzyme showed highest activity which indicates the optimum pH of the bromelain enzyme.

Effect of temperature on enzyme activity

The peel extract of *Ananas comosus* was homogenized, filtered and centrifuged and the sample was determined for the enzyme activity quantitatively under varying temperatures.The activity of protease enzyme being produced at different temperatures was observed for the optimized pH condition. At cooling temperature, the enzyme shows highest activity which indicates the optimum temperature of the enzyme.

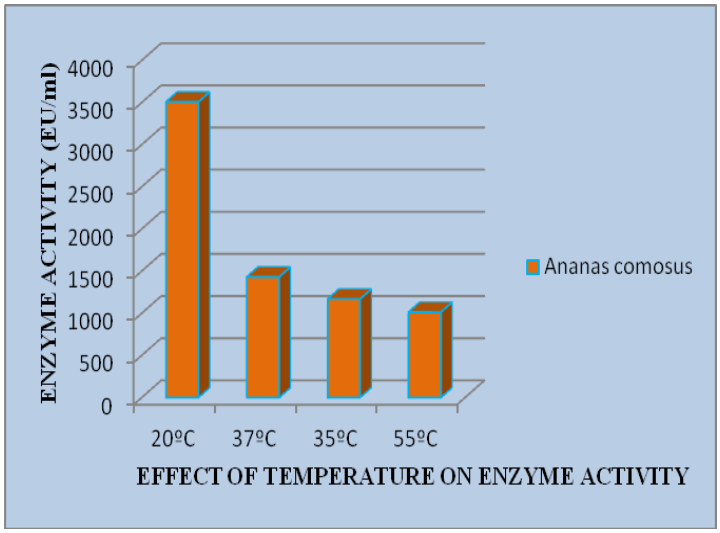

Fig. C: Effect of temperature on enzyme activity.

From the optimization studies, the enzyme activity wasfound to be higher in sodium citrate buffer (0.1M) pH 4.2 and temperature at -20^{0}C. Based on the optimized result, the *Ananas comosus* was subjected to bulk production for precipitation and partial purification techniques.

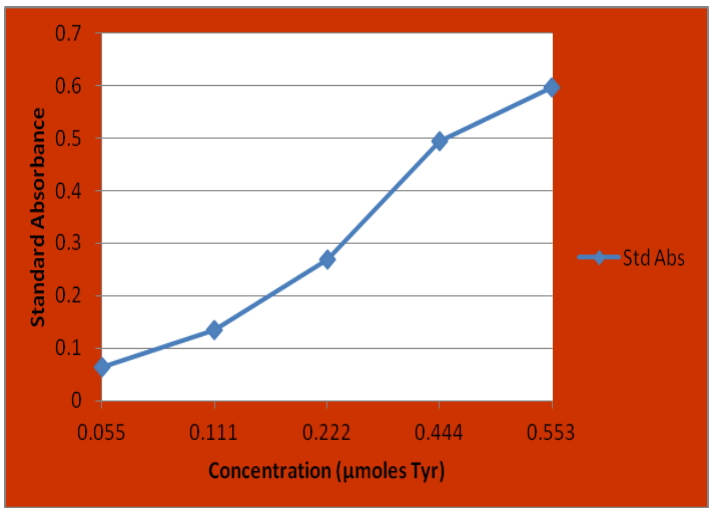

Fig. D: Standard Tyrosine – Enzyme activity.

Bulk Production for the optimized parameter

The *Ananas comosus* was weighed and homogenized with optimized pH 4.2 and temperature -20^{0}C using pre-chilled sodium citrate (0.1M) buffer with the help of mortar and pestle. It was then filtered. The filtrate was centrifuged at 10,000 rpm for 15 minutes. Based on the optimized condition, the plate assay was done using gelatin as substrate. The supernatant was stored in the refrigerator and used as enzyme source for partial purification.

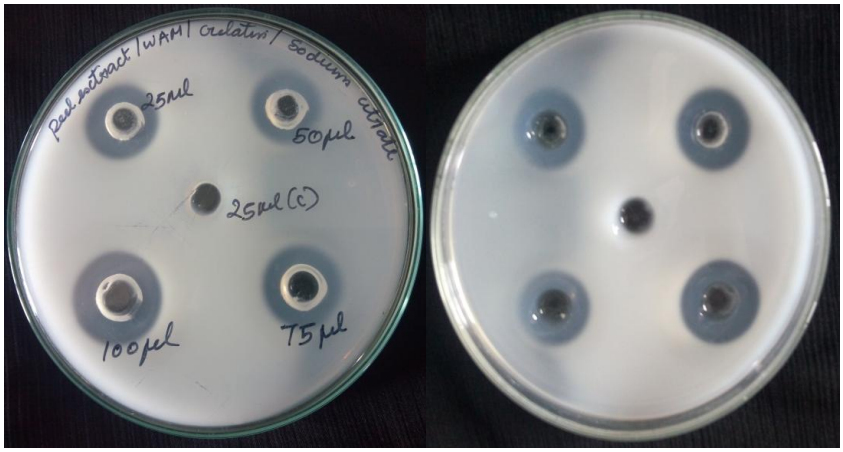

Fig.3: Plate assay for the optimized parameter.

Precipitation technique by solvent - Acetone precipitation

Precipitation of proteins using organic solvent is done usually at 0^0C or subzero temperature in order to minimize denaturation of proteins especially enzymes by the solvents. Hence the solvent is cooled overnight at -20^0C and added slowly with constant stirring to an ice – cold solution of the enzyme in cool condition. 65% acetone precipitation was preferred for precipitating the proteins.

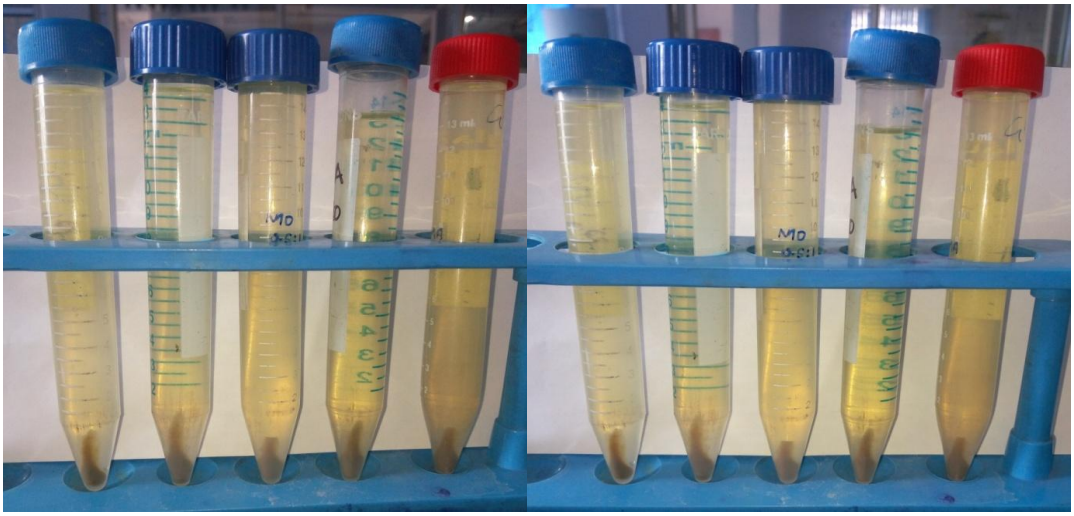

Fig.4: Partial purification of protease enzyme by 65% acetone precipitation.

Estimation of protein by Bradford method

Simultaneously the amount of protein present in the peel extract of *Ananas comosus* was also estimated by Bradford method and the values are compared with BSA and were represented graphically.

Table 3: Estimation of Protein content for three different buffers.

Parameter	Protein content(mg/ml)
Phosphate buffer	8.16
Sodium citrate buffer	17.7
Sodium acetate buffer	15.18

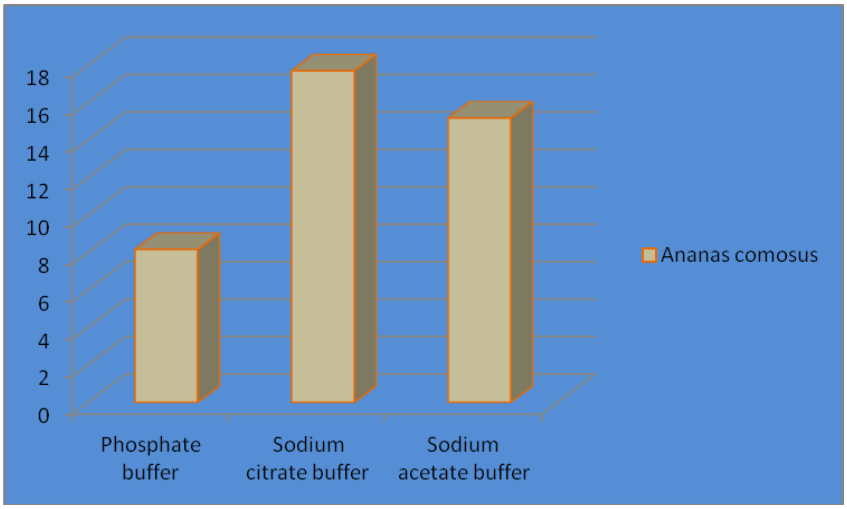

Fig. E: Estimation of protein by Bradford method – Three buffers.

Table 4: Protein content for optimized pH and temperature.

Parameter	Protein content(mg/ml)
Optimized Ph	118.86
Optimized temperature	122.04

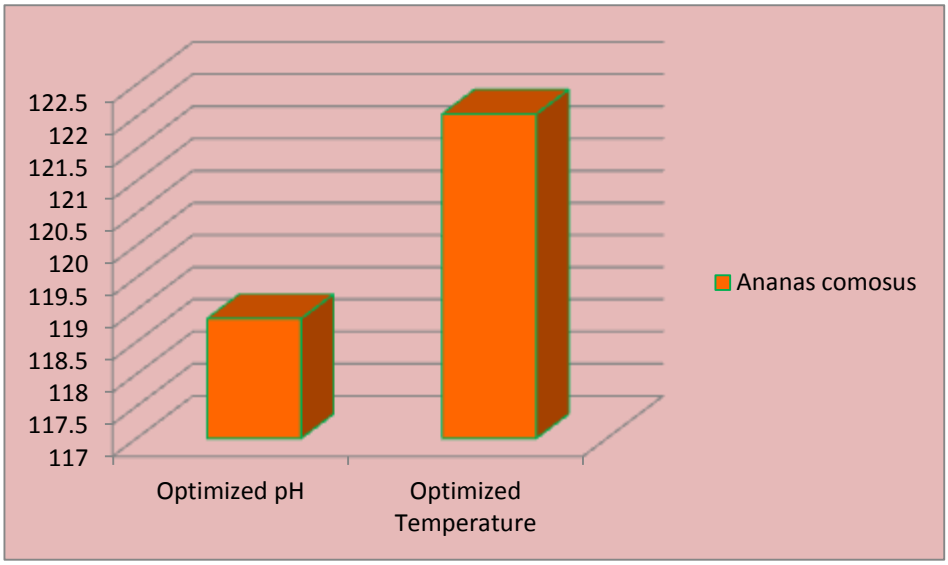

Fig. F: Estimation of protein by Bradford method – optimized pH and temperature.

Table 5: Protein content for precipitated sample and dialyzed sample.

Parameter	Protein content(mg/ml)
Precipitated sample	13.92
Dialyzed sample	6.18

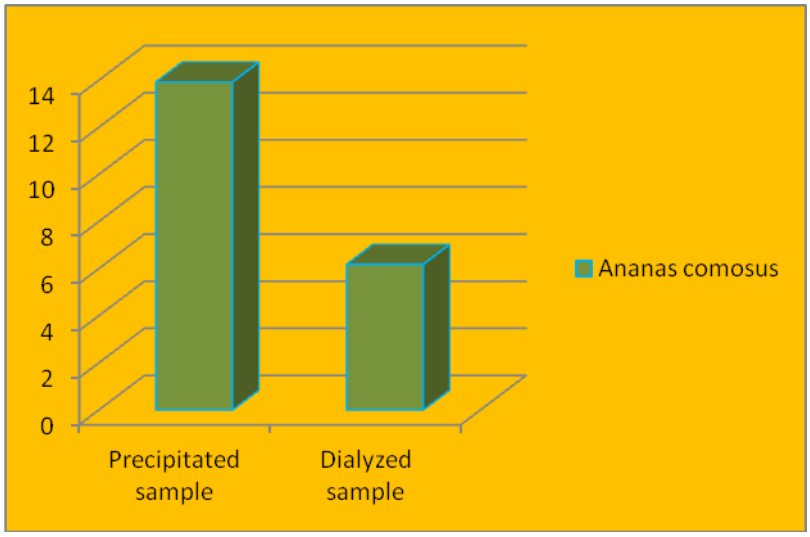

Fig. G: Estimation of protein by Bradford method – Precipitated sample and dialyzed sample.

The amount of protein was found to be 17.7 mg/ml for peel extract of *Ananas comosus* for sodium citrate buffer and significantly low for phosphate buffer and sodium acetate buffer. The protein content was estimated for the optimized conditions such as pH and temperature and it was found to be 118.86 mg/ml for pH and 122.04 mg/ml for temperature. From precipitation and partial purification studies, the amount of protein was found to be 13.92 mg/ml (precipitation) and 6.18 mg/ml (dialysis) for peel extract of *Ananas comosus*.

Application studies – Anti-browning activity, Anti-bacterial activity

(1) Anti-browning activity:

The study showed that bromelain is a moderate anti-browning agent when compared with some of the available commercial anti-browning agents. All of the commercially available anti-browning agents were taken at a concentration of 0.1gm/100 ml (0.1%). Amongst the anti-browning agents taken for study ascorbic acid is the most effective followed by oxalic, citric acid, acetic acid and moderate was bromelain. The bromelain extracted from the pineapple peel was significantly effective in preventing browning. This can be explained by the fact that protein content in the fruit peel was found out to be more, which directly correlates it to the amount of protease present (i.e.) amount of protease is more in the fruit peel extract.

Table 6:Anti-browning activity.

Anti-browning agents	Percentage of inhibition at 420nm		
	20 minutes	40 minutes	60 minutes
Test sample	38.77	53.65	58.61
Ascorbic acid	39.58	57.32	63.39
Acetic acid	20.26	41.32	48.45
Citric acid	23.76	45.04	51.81
Oxalic acid	31.58	51.68	59.11

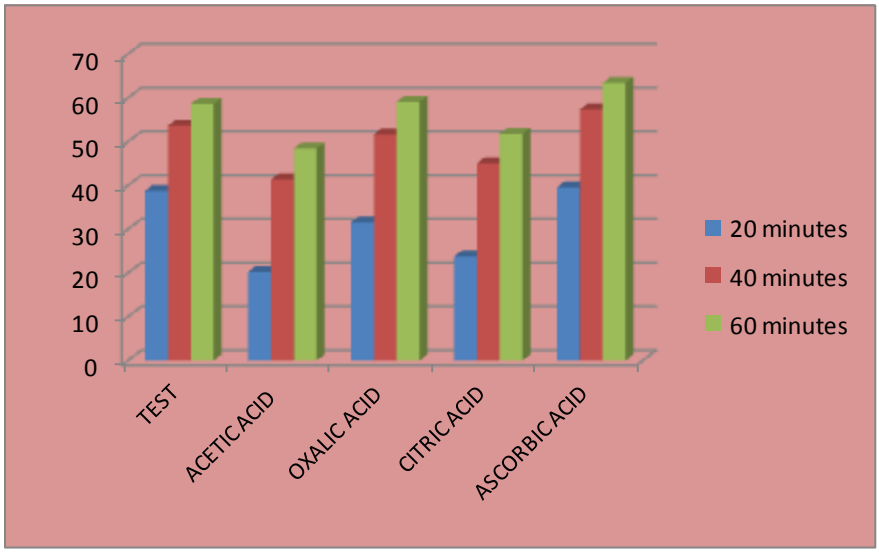

Fig. H: Anti-browning activity.

Although commercial anti-browning agents are widely used, some are being restricted and banned by FAO such as sulfites. Even with many beneficial effects there are several negative attributes associated with sulfite use which has led to decreased consumer acceptance. In particular, sulfites can induce severe allergic reactions or even anaphylactic shock in a proportion of the asthmatic population. Hence there is a need for natural anti-browning agents which can be used in the food industry.

(2) Anti-bacterial activity:

The peel extract of *Ananas comosus* was tested for antibacterial activity against bacterial pathogens, the inhibition effect was less observed for all the pathogens. Among them, *Escherichia coli* had maximum inhibition effect at the concentration 1000µg/ml exhibiting the zone of 15mm whereas for *Proteus vulgaris* the inhibitory zone was 13mm, and there was no inhibitory activity for the other tested pathogens.

Dialysis process

The precipitated sample was dialyzed in order to remove excess salt and other small impurities estimation of proteins, where it could pass through the dialysis membrane leaving the protein behind. The activity of the enzyme as well as the protein content was also been estimated.The time required to accomplish dialysis is determined by factors that affect the rate of diffusion of a molecule. Because heat affects the thermodynamics of molecules, increasing temperature speeds diffusion. Therefore, dialysis will proceed faster at room temperature than at 4°C. In selecting the most appropriate temperature, it is important to take into account the thermal stability of the molecule of interest. The rate of diffusion is also directly proportional to the concentration of a molecule, while inversely proportional to its molecular weight. As the concentration of a molecule increases, so does the probability that one of those molecules will contact the dialysis membrane and then diffuse across to the other side. However, as a molecule's molecular weight increases, the rate of movement in solution decreases along with the chance of diffusion through the membrane - even if it the molecule is small enough to pass through the pores.

Fig.5: Dialysis process under sodium citrate buffer (0.1M) condition.

The enzyme activity was quantified for precipitated sample and dialyzed sample and the effectiveness was comparatively observed and proved to be in higher purity after dialysis process.

Table 7: Enzyme activity for precipitated sample and dialyzed sample.

Parameter	Enzyme activity(EU/ml)
Precipitated sample	647.9
Dialyzed sample	329.1

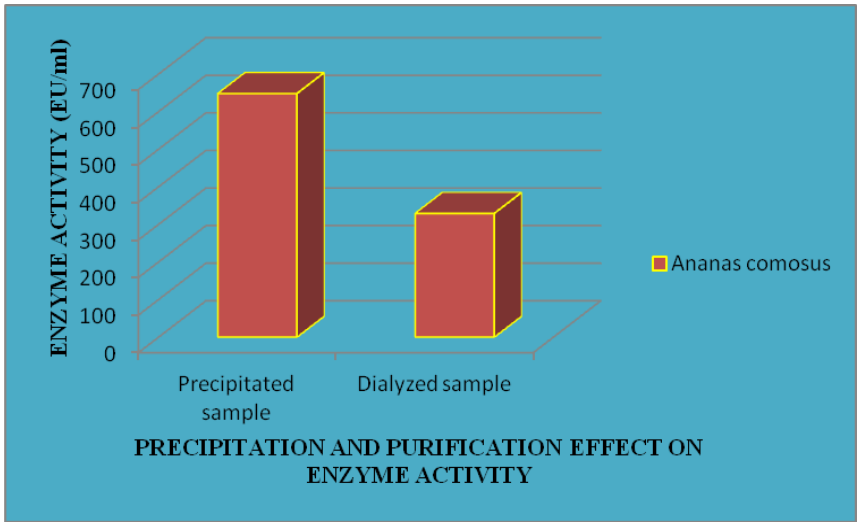

Fig. I: Effect of precipitated and dialyzed samples on Enzyme activity.

The enzyme activity wasfound to be higher as 647.9 EU/ml for precipitated sample and comparatively as 329.1 EU/ml for dialyzed sample.

Characterization of proteins

The molecular weight of the purified sample was determined by 10% SDS-PAGE. From fig.6, the purified sample (i.e., after dialysis) was loaded in lane 3, in lane 1and 2, the precipitated sample was loaded and in lane 4 the protein marker was loaded as comparatively. The molecular weight of the bromelain, a protease enzyme was approximately 66kilodaltons.

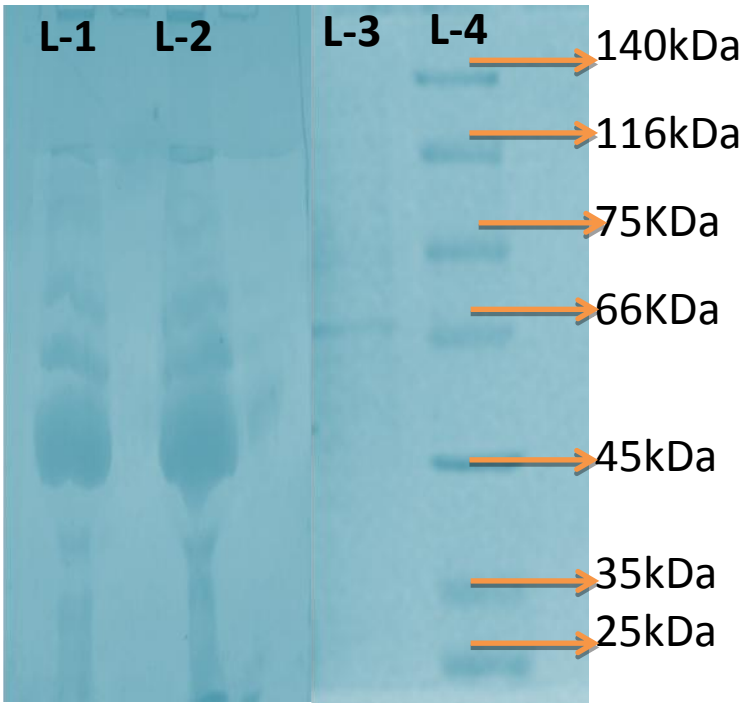

Fig.6: Characterization of proteins by SDS-PAGE.

CONCLUSION

As the study was aimed at understanding the effectiveness of bromelain as a potential anti-browning agent we utilized the commercially available anti-browning agents for comparison. As observed peel bromelain at a concentration of 0.1% has the potential to be a better agent on comparison with ascorbic acid and but its potential use for long time usage has not been clearly proved. Hence a further better option would be to use for commercial agents that should not be any hazardous, deleterious effects on the food and the human system. However further studies about increase in bromelain yield, high purification and for bromelain could be helpful to identify and better analyze the potency and use of bromelain as a natural anti-browning agent in the food industry. Also, the bromelain enzyme which is highly purified shall be designed as a drug for treating several diseases such as ulcer, arthritis, slowing down blood clotting, cancer, etc. Based on the evaluation from the clinical trials, the PK and PD (Pharmacokinetics and Pharmacodynamics) the enzyme can be converted as a drug followed by FDA (Food and Drug Administration) approval, practicing and marketing as directed for various treatments.

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AUTHOR'S STATEMENTS

The author's declare no conflict of interest.

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