Screening, optimization and antimicrobial activity of Bromelain from *Ananas comosus*

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Abstract: **Bromelain is a major protease, isolated from pineapple *(Ananas comosus)*. Bromelain is accumulated in the entire plant to different extent and properties depending on its source. In the present study, Bromelain was extracted from all parts of pineapple using sodium citrate buffer. Bromelain was filtered, centrifuged and used for further studies. After the determination of protease activity and protein content, the Core and Pulp extract of *A.comosus* was chosen using gelatin as the substrate. The samples were optimized on the basis of pH, temperature, Substrate concentration and etc. After optimization, the Bromelain was tested for antibacterial activity against bacterial pathogens. Among them, Pulp bromelain had maximum inhibition effect on *Bacillus subtilis, Klebsiella pneumonia* and Core bromelain had maximum inhibitory effect on *Bacillus subtilis, Proteus vulgaris, Schigella flexneri* and *Escherichia coli* and there was no inhibitory activity for the other tested pathogens.**

Keywords: ***Ananas comosus*, Bromelain, pine apple, Antimicrobial activity, Optimization.**

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I. INTRODUCTION

Pineapples widely grow in tropical countries may be cultivated from a crown cutting of the fruit, possibly flowering in 20–24 months and fruiting in the following six months. Pineapple does not ripen significantly post-harvest. Pineapples are consumed fresh, cooked, juiced, and preserved and are found in a wide array of cuisines. In addition to consumption, in the Philippines, the pineapple leaves are used to produce the textile fibre pina employed as a component of wallpaper and furnishings, among other uses. Pineapple (*Ananas comosus*) is one of the tropical plants that have been used as traditional medicines from a long time. It was originated from tropical South America and was discovered by Europeans **[1]**.

A protease is also termed as peptidase or proteinase is any enzyme that performs proteolysis activity by hydrolysis of the peptide bonds that link amino acids together in the polypeptide chain forming the protein. Proteases have evolved multiple times, and different classes of protease can perform the same reaction by completely different catalytic mechanisms **[2].** Proteases can be found in animals, plants, bacteria, archea and viruses. Papaya and pineapple are two of the richest plant sources, as attested by their traditional use as natural "tenderizers" for meat. Papain and Bromelain are the respective names for the proteolytic enzymes found in these fruits **[3].**

Bromelain has been used widely in food, medical, pharmaceutical and cosmetic industries and other industries as well. In the food industry, it is used for meat tenderization, grain protein solubilisation, beer clarification, baking cookies and protein hydrolysate production **[4].** It was studied that pineapple juice was an effective enzymatic browning inhibitor in fresh apple slices and several important medical applications **[5], [6].**

II. MATERIALS AND METHODS

1. Collection : Fresh pineapple was 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.

2. Extraction : 10g of the sample was weighed and homogenized with 0.1M of Sodium Citrate buffer (pH 5) (cooling condition) (1:1 ratio) using mortar and pestle, it was then filtered. The filtrate was centrifuged at 10,000 rpm for 15 minutes. The supernatant was stored and used as enzyme source.

3. Qualitative Protease assay : 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°C for 15minutes. The plates were allowed to solidify and then, 5 wells (8mm diameter) were made by using a sterile cork borer. The 4 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 24h at room temperature. After 24h of incubation, the plates were flooded with mercuric chloride indicator solution (concentrated HCl-20 ml, Distilled water-80 ml and $HgCl_2$-15g) for 5 – 10 minutes. Protease production was visualized by a

translucent zone around the wells. The zone of clearance were observed and measured. Based on the results produced, one test material will be selected for further studies **[7].**

4. Estimation of protein: 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 **[8].**

5. Determination of Protease activity

An assay mixture was prepared by mixing 0.5ml of 1% gelatin with 0.5ml of cell free culture filtrate (enzyme source). The mixture was incubated at 37°C for 60 minutes. 1ml of 10% trichloroacetic acid (TCA) was added to the reaction mixture. The reaction mixture was centrifuged at 10,000rpm for 15 minutes and the supernatant was collected. To 0.5ml of supernatant, 2.5ml of alkaline solution (2.9% Na_2CO_3 and 0.3N NaOH) and 0.75ml Folin phenol reagent (1ml of reagent diluted with 3ml of double distilled water before use was added and incubated at room temperature). After 20 minutes, the absorbance of the solution was measured at 650nm in a spectrophotometer. Simultaneously, control without the enzyme source was maintained. One unit of protease activity is defined as the amount of enzyme required to liberate 1µmol of tyrosine/ml/min/mg of protein was expressed **[9].**

6. Optimization of Bromelain: To determine optimal pH, temperature, substrate concentration, activators, the selected potent source with different paprametrs were set according to varying pH (3.0 to 6.2), temperature (-20°C to 55°C), substrate concentration (0.1% to1.0%) and in the presence of activators magnesium chloride and calcium chloride (0.01 to 0.10g) was subjected to the determination of enzyme activity [**7].**

7. Antibacterial activity: Nutrient agar (Peptone-5g; Yeast extract-3g; Nacl-5g; Agar-30g; Distilled water - 1000ml; pH - 7.2) was prepared and poured in the sterile Petri dishes and allowed to solidify. 24h growing bacterial cultures includes Gram positive (*Staphylococcus aureus, Micrococcus luteus*) and Gram negative (*Proteus vulgaris, Bacillus subtilis, Klebsiella pneumonia, Schigella flexneri and Escherichia coli*) were swabbed on it. Then, 5 wells (8mm diameter) were created on nutrient agar with well puncture. The four different concentrations (25µl, 50µl, 75µl and 100µl) of the precipitated sample were loaded in the wells. Remaining well is used for the Control. The plates were then incubated at 37ºC for 24hours. After incubation the inhibition diameter was measured.

III. RESULTS AND DISCUSSION

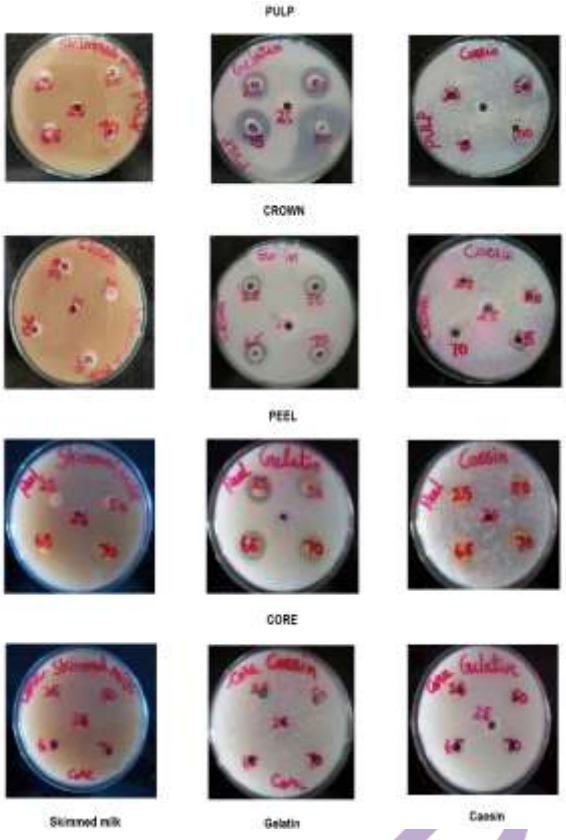
1. Collection and Extraction of Bromelain: The Crown extract of pineapple were green in colour, this may be due to the presence of chlorophyll. The Pulp and Core extract were yellow in colour, this may be due to the presence of carotenoids and xanthophylls. The peel extract were brown in colour, which may be due to the presence of melanin. On extraction, various volume of crude sample were obtained, Pulp - 25ml, Core - 23ml, Peel - 19ml and Crown - 16ml. The difference in volume of extraction obtained may be due to the amount of water content, fibre and other components present in *A.comosus*. The chemical composition of pineapple it has also to be mentioned the presence of bromelain enzyme and phenol compound.

2. Qualitative Protease assay: The sample of pulp, peel, crown, core extract of *A. comosus* was tested for the presence of protease and to detect the suitable substrate. 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. The more suitable substrate was selected based on the zone of clearance observed. Casein exhibited zone of clearance in lower amount when compared to gelatin and skimmed milk did not exhibit the zone of clearance. On comparison of all four samples (Pulp, Core, Peel and Crown), the pulp extract showed highest zone of clearance (28mm) when compared to the zone of clearance obtained by Peel (22mm), Crown (17mm), Core(13mm). From the result obtained, it was concluded that the selected four samples has protease activity and gelain is considered as the most suitable substrate (**Table 1, and Figure 1). A** study reported that alkaline protease activity of 141 test fungi was determined using 0.5% casein as protein substrate on solid Reese media **[10].**

Table 1: Qualitative Protease Assay

Substrate	Control	Zone of Inhobition (mm)															
		Pulp (µl)				Core (µl)				Peel (µl)				Crown (µl)			
		25	50	65	70	25	50	65	70	25	50	65	70	25	50	65	70
Caesin	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	17	19	21	22	Nil	Nil	Nil	Nil
Skimmed milk	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	12	14	15	16	Nil	Nil	Nil	Nil
Gelatin	Nil	14	20	22	28	10	11	12	13	18	19	20	22	12	13	15	17

Figure 1: Qualitative Protease Assay



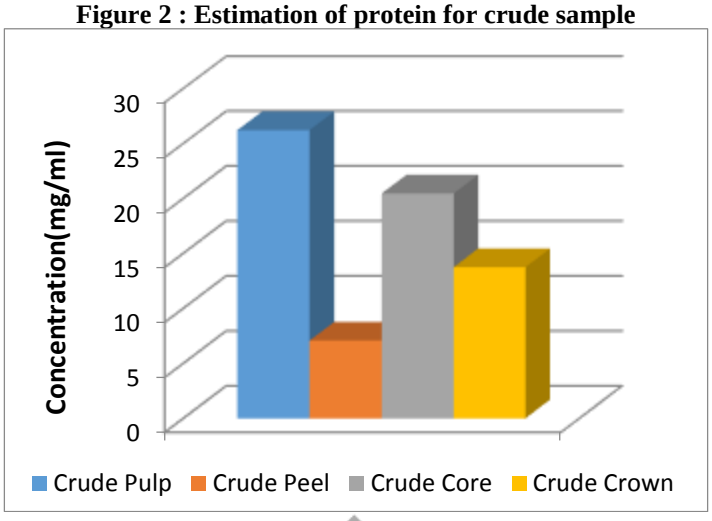
3. Determination of Protease Activity : The results showed that the crude pulp sample had higher activity. Followed by it, the peel sample had higher activity than the core and crown sample **(Table 2).** Previous study reported that the activity of crude Bromelain enzyme from fruit was found to be 4.71 U/mL of enzyme and from peel was found to be 4.52 U/mL **[11]**. Another report says that the enzyme assay was conducted to determine the activity of the crude extracts and it was found to be the highest in the leaves followed by the peel, stem and was lowest in the pulp**[12].**

Table 2: Determination of Protease activity

Sample	Protease assay (EU/ml)
Pulp	2802.7
Peel	2016.1
Core	1268.9
Crown	281.5

4. Estimation of protein for crude sample : The protein content of the crude pulp sample was found to be 26.22mg/ml. Next to pulp the crude core sample had higher protein content, which shows 20.46mg/ml. The quantity of protein found in crude crown sample had 13.74mg/ml. The least amount of protein was identified in the crude peel sample 7.08mg/ml. Comparatively within all the selected parts of *A.comosus* the pulp and core were rich in protein content may be reflect in the level of enzymes. **(Figure 2).** Extracts concluded that on quantitative estimation by Lowry's method and the concentration of the enzyme in the crude extracts of the peel, pulp, leaves and stem was found to be 11.4 mg/ml, 9 mg/ml, 2.08 mg/ml and 6.2 mg/ml respectively. Hence the concentration of the enzyme in the crude extracts was the highest in the peel and lowest in the leaves.

Figure 2 : Estimation of protein for crude sample



5. Optimization of Bromelain : The sample was optimized on the basis of pH, temperature, Substrate concentration and etc., and the enzyme activity is determined. The highest Enzyme activity was obtained at pH 4.4 for pulp and at pH 3.8 for Core **(Table: 3).** The optimum pH of pulp and core bromelain differs, because bromelain from one part of pineapple possess different biochemical properties and composition as compared to other bromelain **[13]** and contains a variegated blend of different thiol-endopeptidases. In their study, Studies reported that on analyzing two varieties of *A.comosus*, described that high enzyme activity was observed in pH ranging from 6.5 to 8.0, and the maximum activity was near pH 7.0. reported that the bromelain from crown leaf showed maximum activity at pH 6.0, where as the fruit pulp showed maximum activity at pH 8.0. **[14], [15].**

Table 3: Effect of pH on Bromelain activity

pH	Enzyme activity (EU/ml)	
	Pulp	Core
3.0	1954.0	1583.5
3.2	2366.0	1299.9
3.4	2216.9	1958.2
3.6	1831.9	1796.7
3.8	1858.8	1991.3
4.0	2281.1	1792.6
4.2	1918.8	1757.4
4.4	2394.9	1660.1
4.6	1633.2	1817.4
4.8	2262.5	1538.0
5.0	1978.9	445.0
5.2	1331.0	1186.1
5.4	1627.0	1171.6
5.6	1598.0	1320.6
5.8	1564.9	1372.4
6.0	1229.5	1260.6
6.2	1720.1	1312.3

With the optimum pH the pulp and core of *A.comosus* was determined under different temperatures such as -20°C, 35°C, 37°C to 55°C. From the result, it was concluded that the maximum was observed at 55°C for pulp and at 37°C for core. The optimum temperature of pulp and core bromelain differs, because bromelain from one part of pineapple possess different biochemical properties and composition as compared to other bromelain (Pavan *et al.*, 2012) and contains a variegated blend of different thiol-endopeptidases **(Table 4. 5 and Figure 3).** Previous research on the optimum pH for SBM ranges from 6-7 and its optimum temperature ranges from 50-60ºC **[16], [17]**. Another study says that the optimum pH range for FBM is 3-8 and optimum temperature ranges from 37-70ºC **[18], [19].** Reserch findings the optimum temperature of enzyme activity was to reported as 50 - 60°C **[20]**. Early studies confirmed that the optimum temperature for the protease activity was 60°C. The activity gradually declined at temperature beyond 60°C. and similar result of temperature optimum of 60°C for protease derived from *Bacillus sp* BZI-2. Has the maximal activity was found at 30°C **[21].**

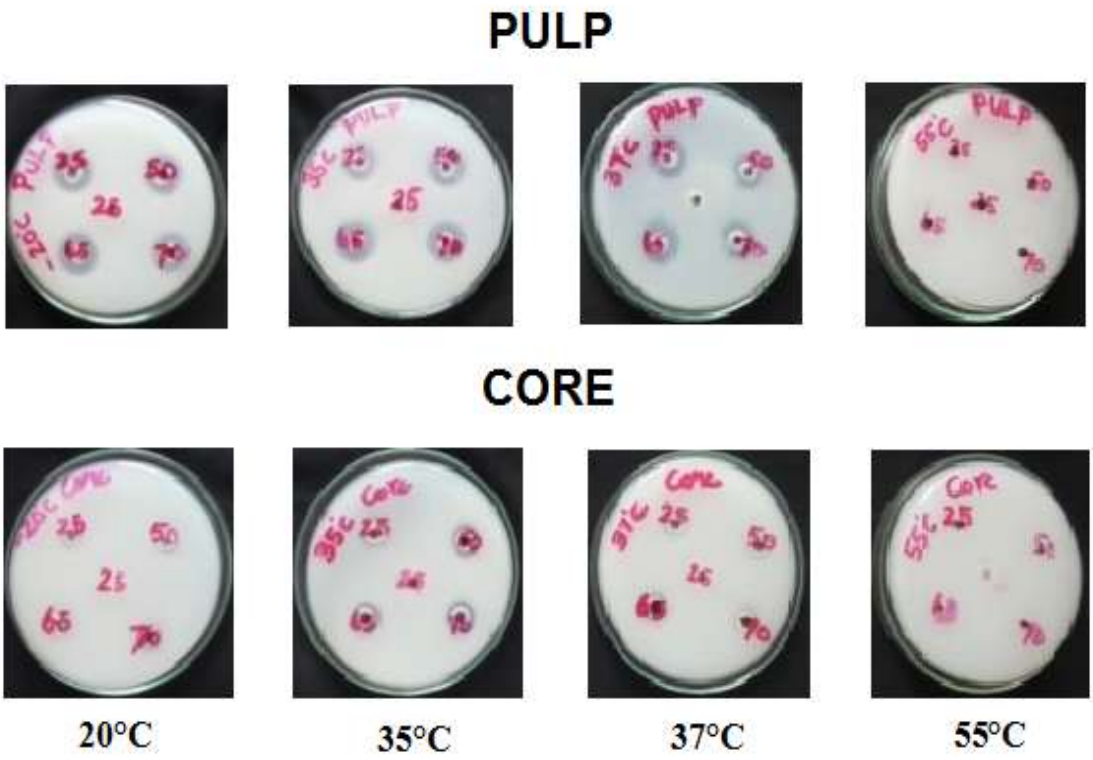
Table 4: Effect of temperature on Bromelain activity

Temperature	Control	Zone of Inhibition (mm)							
		25µl		50µl		65µl		70µl	
		Pulp	Core	Pulp	Core	Pulp	Core	Pulp	Core
-20°C	Nil	16	11	17	12	19	12	20	13
35°C	Nil	15	13	17	14	18	15	19	16
37°C	Nil	18	14	16	15	19	16	17	18
55°C	Nil	14	10	18	10	20	12	21	13

Table 5: Effect of temperature on Bromelain activity

Temperature	Enzyme activity (EU/ml)	
	Pulp	Core
-20°C	1616.6	815.5
35°C	1900.2	1289.6
37°C	2475.7	2467.4
55°C	2864.8	1682.9

Figure 3: Effect of temperature



The activity of Pulp and core extract of *A.scomosus* of optimum pH (Pulp-4.4 and core-3.8) was determined at different substrate concentration ranging from 0.1% to 1%. From the result obtained, it was concluded that the higher activity was found at the substrate concentration of 1% for both the pulp and core bromelain. **Table 6** shows the effect of substrate concentration on bromelain activity. Ram Kumar Pundir and his groups reported that the various concentrations of substrate (casein) ranging from 0.5 to 3.5 % with respect to its optimum time were observed. It was concluded that the rate of reaction declined if the substrate concentration was more than 1% for *A. niger* protease **[22]**. The declination in the rate of reaction after optimum concentration could be due to the alteration of enzyme substrate concentration ratio.

Table 6: Effect of substrate concentration on Bromelain activity

Substrate Concentration	Enzyme activity (EU/ml)	
	Pulp	Core
0.1%	2053.4	1935.4
0.3%	2016.1	1422.0
0.5%	1790.5	1295.8
0.7%	1451.0	1415.8
0.9%	1689.1	1612.5
1%	2394.9	1991.3

The activity of pulp and core bromelain was determined for activity with different activators such as $MgCl_2$ and $CaCl_2$ of various concentrations ranging from 0.01 to 0.10g. From the result, it was observed that the effect of $CaCl_2$ on pulp and core bromelain increases as the amount of $CaCl_2$ increases. As of $MgCl_2$, the activity doesn't increases as the amount of $MgCl_2$ increases. But the higher activity of both pulp and core bromelain was observed at 0.10g concentration of $MgCl_2$.The results of bromelain activity on effect of the activators are depicted in the **Table 7.** Tsuchiya *et al.*,(1987) reported that the proteases isolated from *Cephalosporium sp.* was inhibited by Hg^{2+}, Mn^{2+} , Cu^{2+}, Ca^{2+} and these same ions were found to inhibit the activity of the alkaline proteases secreted by Bacillus polymyxa **[23]**. Studies reported that Mg^{2+} activated the alkaline protease produced by *Aspergillus sp.* **[24], [25]**.

Table 7: Effect of activators on bromelain activity

Concentration (g)	Enzyme Activity (EU/ml)			
	PULP		CORE	
	$MgCl_2$	$CaCl_2$	$MgCl_2$	$CaCl_2$
0.02	364.3	683.1	1513.1	560.9
0.04	461.6	1121.9	685.17	1281.3
0.06	1643.5	1552.5	1399.3	2314.2
0.08	937.7	2293.5	1196.4	2693.0
0.10	2138.3	2753.1	2883.5	3359.6

6. Antibacterial Assay : The core and pulp bromelain extract of *A.comosus* was tested for antibacterial activity against bacterial pathogens, the inhibition effect was observed for many pathogens. Among them, pulp bromelain had maximum inhibition effect on *Bacillus subtilis, Klebsiella pneumonia* exhibiting the zone of 16mm and core bromelain had maximum inhibitory effect on *Bacillus subtilis, Proteus vulgaris, Schigella flexneri and Escherichia coli* exhibiting the inhibitory zone of 22mm, 14mm, 24mm and 17mm respectively and there was no inhibitory activity for the other tested pathogens. **(Table 8. Figure 4 and 5).** Bansode (2013) reported that fresh pineapple fruit had antimicrobial effect against *E. coli* (6mm zone of inhibition by agar well diffusion method) **[26]**. Hanan (2013) reported the effectiveness of bromelain at concentrations of 1-4 mg/ml in reducing *E. coli* populations at 5°C, 25°C, and 35°C **[27]**. The gram positive bacteria, *B. subtilis* and *S. pyogenes* were resistant to both crude bromelain as well as the standard bromelain. This finding corroborates the works of **Sparso (2002)** who concluded that bromelain is more efficient against gram negative than gram positive bacteria **[28]**.

Table 8: Anti bacterial activity for Pulp and Core Bromelain

Micro organisms	Zone of Inhibition (mm)								
	Control	Core				Pulp			
		25µl	50µl	65µl	70µl	25µl	50µl	65µl	70µl
M.luteus	-	-	-	-	-	-	-	-	-
K.Pneumonia	-	-	-	-	-	12	14	15	16
S.aureus	-	-	-	-	-	-	-	-	-
P.Vulgaris	-	19	20	21	22	-	-	-	-
E.Coli	-	-	11	12	14	-	-	-	-
S.flexneri	-	18	20	22	24	-	-	-	-
B.subtilis	-	13	15	16	17	16	18	20	21

Figure 4: Antibacterial activity of Pulp Bromelain

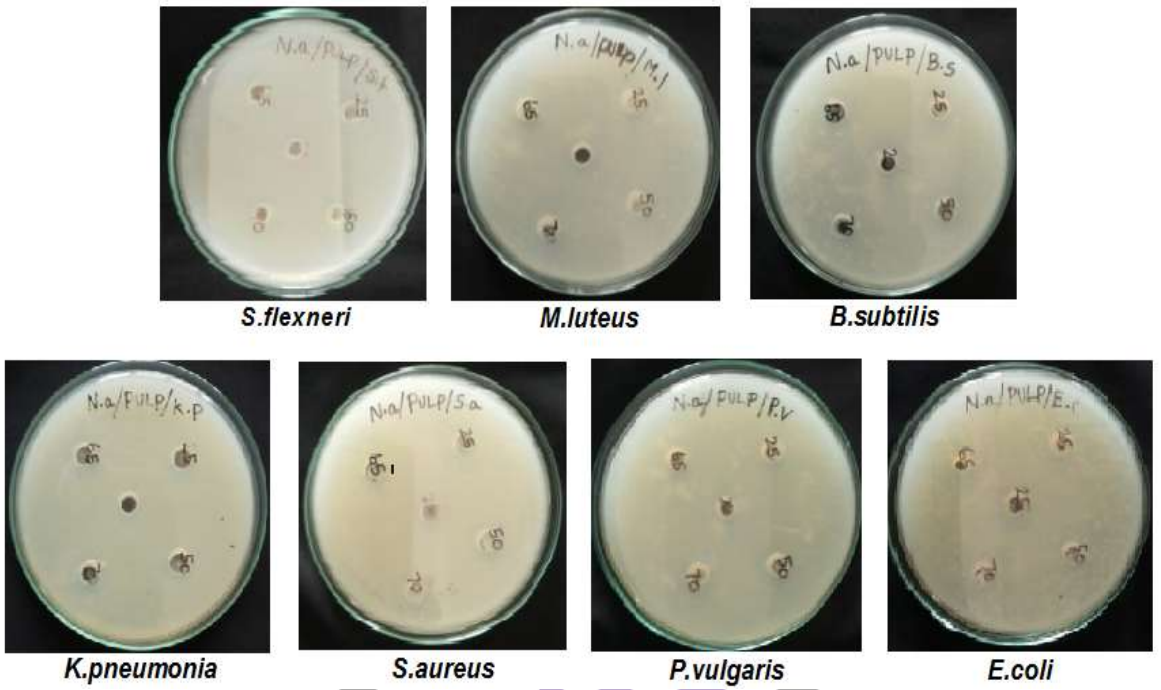
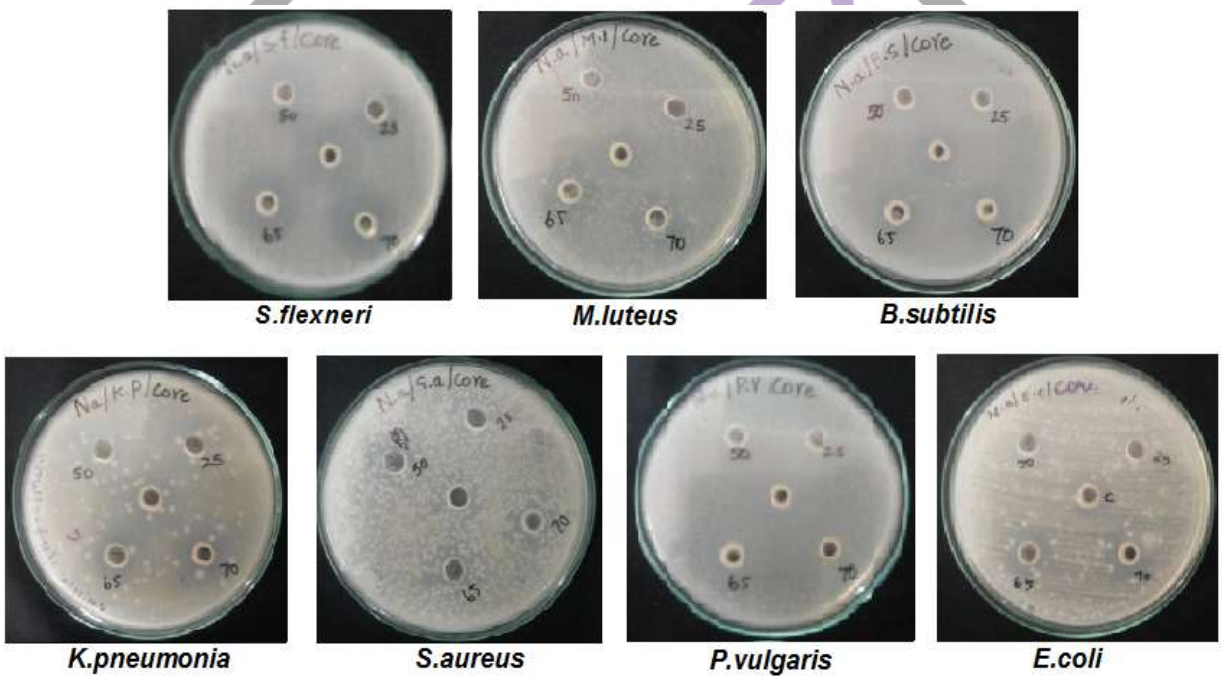


Figure 5: Antibacterial cativity of Core Bromelain



IV. SUMMARY AND CONCLUSION

Protease is one of the major group of an enzyme that plays the important role in the regulation and nutritional value in the living system. Due to the diverse application field of protease enzyme, they are in great demand and are being produced in high amount. Protease can be used in the detergents, food industries, cheese production, meat processing, the medical field so the demand is also increasing day by day. In recent times protease accounts for the 60% of the total enzyme consumed in the market.

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