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ORIGINAL RESEARCH

Hydrolysis of fish waste using fruit wastes of *Ananas comosus* and *Carica papaya* for the formulation of liquid fertilizers

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Abstract

Purpose Fish waste is a protein-rich source that can be used as a value-added product in the formulation of organic liquid fertilizers. This study carried out to investigate the utilizing potential of fruit wastes of *Ananas comosus* and *Carica papaya* that contain bromelain and papain as major proteases in order to hydrolyze fish waste proteins to fulfill plant nitrogen requirements.

Method Proteases were extracted from *A. comosus* (leaves, crown, unripe fruit pulp, ripe fruit peels and pulp) and *C. papaya* (leaves, ripe fruit peels and unripe fruit peels). The optimum temperature and pH for the enzymatic activities were determined. Four liquid organic fertilizers were produced with hydrolyzed fish waste that enriched by adding *Gliricidia sepium, Chromolaena odorata, Tithonia diversifolia*, *Mikania scandens* and coconut husk-ash. Fertilizers were tested on the growth of *Basella alba* comparing with a standard fertilizer.

Results *Ananas comosus* ripe fruit peels and the mixture of *(A. comosus + C. papaya)* showed the highest enzyme activities (0.33±0.02 and 0.36±0.01 U mL^{-1} enzyme respectively) at 55 ^{o}C and 70 ^{o}C. The optimum pH for all the studied extracts was 7.5 at 37 ^{o}C. The highest plant fresh and dry weights were recorded in the foliar-applied fertilizer produced by hydrolyzing the fish waste using *A. comosus* and *C. papaya,* showing no significant differences to the standard fertilizer.

Conclusion Hydrolysis of fish waste using the fruit wastes of *A. comosus* and *C. papaya* could be effectively used as an organic fertilizer for the growth of *B. alba* that leads towards sustainable waste management.

Keywords *Ananas comosus, Carica papaya,* Fish wastes, Plant-derived proteases, Organic liquid fertilizers

Introduction

Proteolytic enzymes are widely used in the hydrolysis of different protein substrates. Since ancient times, different fruit extracts were used to tenderize hard textured meat or fish flesh; however, it is now deliberately and more systematically practiced using plant, animal or microbial-derived proteolytic enzymes, i.e., papain, bromelain, ficin, pepsin, trypsin, pancreatin and alkalase (Islam and Molinar-Toribio 2013; Himonidase et al. 2011) to hydrolyze different collagen materials, i.e., fish scale, animal bones, leather waste, horns and feathers (Ekram and Prasetyo 2016; Damrongsakkul et al. 2007). Among them, papain is a major plant-derived endolytic cysteine protease that can be extracted from latex, fruits, leaves and roots of *Carica papaya* to degrade proteins into short-chain peptides, amino acid esters and amide links (Lambri et al. 2014; Vishal et al. 2013). Bromelain is another plant-derived cysteine protease from *Ananas comosus* that catalyzes the hydrolytic cleavages of the internal peptide bonds of the proteins (Ramalingam et al. 2012; Upadhyay et al. 2011).

Large amounts of *C. papaya* and *A. comosus* ripe fruit peels are generated as a by-product during the production of jams, jellies and cordials (Pathak et al. 2018; Lakshminarasimaiah et al. 2014; Thomas et al. 2008). Those fruit wastes are widely available in market complexes and agro-industrial yards as agro-waste produced during the harvesting, processing or post-har-

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vest handling. Most of these fruit wastes are dumped by landfilling or incinerate due to the high costs of transportation and the limitation of available lands for disposal (Zainuddin et al. 2014). Hence, it is greatly important to study beneficial utilization methods of these fruit wastes towards sustainable and economical organic waste management to lower their negative impacts on the environment.

Moreover, fish waste that contains a higher amount of proteins and many other macro and micronutrients can be converted into value-added products (Dahama 1997). Central fish market located in Colombo, Sri Lanka generates about 4 MT of fish waste daily and it is mainly used for the production of fish meal. However, the majority of other fish processing plants and small-scale fish markets throughout the country are underutilized these nutrient-rich fish waste (Ariyawansa and Arachchi 2013). According to the available literature, few attempts have been made to utilize fish waste as a nutrient-rich organic waste in formulating organic fertilizers (Kim 2011) and fewer studies have utilized fish waste after hydrolyzing with commercially available protease enzymes (Himonides et al. 2011). Himonides et al. (2011) stated that papain from papaya latex and bromelain from pineapple stem recorded the highest hydrolyzing activity of the fish frames. Moreover, Abdulazeez et al. (2013) also revealed the great potential and higher effectiveness of digestion of fish protein using papain (1%) at 37 ^{o}C within 6 hours. However, the main drawback of enzymatic hydrolysis is the high cost of commercial enzymes that create economic difficulties in large scale fish waste hydrolysis (Wisuthiphaet and Kongruang 2015).

Therefore, the crude proteases remain in fruit wastes could be used as a cheap alternative in hydrolyzing fish waste proteins in order to convert nitrogen in soluble and available forms to fulfill plant nitrogen requirements in agricultural purposes. *Basella alba* L. is one of the most consumed leafy vegetables in Sri Lanka and extensively grown due to its higher nutrient values. As most of the leafy vegetables, *B. alba* also required higher amount of nitrogen for its vegetative growth. As a novel approach, the present study was carried out to study the utilizing potential of proteases from fruit waste of *C. papaya* and *A. comosus* for the hydrolysis of fish waste in order to formulate organic liquid fertilizers for the cultivation of *B. alba*. Moreover, this study was further aimed to determine their optimum pH and temperature conditions for digestion of fish waste.

Materials and methods

Preparation of the crude bromelain and papain enzymatic extracts from different plant components of *A. comosus* and *C. papaya*

The pineapple (*A. comosus*) and papaw (*C. papaya*) plant components, i.e., leaves, unripe fruits and ripe fruits were collected from a small-scale plantation at Gampaha, Sri Lanka. The plant components were washed, air-dried and their different parts (i.e. leaves, crown, peels and pulp) were manually separated. Protease enzyme solutions, which contain papain in major, were extracted from the different components of *C. papaya,* i.e., ripe fruits peels, unripe fruits peels, leaves, ripe fruit peels + leaves in 1:1 ratio (w/w) by mixing 10 g from each. Based on the experimental data, ripe fruit peels and leaves were mixed in 1:1 (w/w) to enhance the protease content as *C. papaya* leaves contained comparatively higher amount of proteases. Those were cut into small pieces and ground using a ceramic mortar and pestle with 40 mL of distilled water (1:4 w/w). The latex of *C. papaya* was collected from the cuttings of mature green papaya fruits skins and the milky latex was dried at 55 ^{o}C and powdered using a dry mortar and pestle. Dried powder of *C. papaya* latex (10 g) was dissolved in 40 mL of distilled water. All the extracts were filtered through muslin clothes and centrifuged at 5,000 rpm for 15 minutes to obtain clear supernatant rich with crude papain enzyme. They were then refrigerated at 4 ^{o}C until the analysis. Similarly, crude bromelain enzyme-rich solutions were extracted from the different components of *A. comosus*, i.e., ripe fruits peels, unripe fruits peels, crown, leaves, ripe fruits peels + crown in 1:1 ratio (w/w) by mixing 10 g from each following the same procedure.

Qualitative determination of the presence of proteolytic enzymes in different components of *A. comosus* and *C. papaya*

The presence of the proteolytic enzymes in the prepared enzyme solutions was tested, using casein as the substrate (Andrademahecha et al. 2011). Ten milliliters of fresh-milk of 20% (v/v) was used as a casein substrate and the pH of the milk solution was adjusted with dilute acetic acid (0.01%). Prepared enzyme solutions, 0.50 mL of each was added to the milk samples separately and maintained at 37 ^{o}C in a water bath and observed until the first sign of coagulation. The control was carried out incubating a sample with 0.50 mL of distilled water without any enzyme solution.

Effect of proteolytic enzymes activity of *A. comosus* and *C. papaya* on protein degradation of Tuna fish and chicken

The uniform-sized chunks of Tuna fish and chicken breast (2 x 2 x 2 cm) were taken and immersed separately in 15 mL of aqueous protease enzyme extracted from different components of *C. papaya,* i.e., ripe fruits peels, unripe fruits peels and leaves and *A. comosus* ripe fruits peels, unripe fruit peels and crown with (1:4 w/w) distilled water. As a negative control, 15 mL of distilled water was used. The tenderness of the samples was measured at 2, 4, 24 and 48 hours using a penetrometer (FT 011, QA Supplies, Italy) by gently pressing against the digested flesh blocks until it indicated a constant value.

Quantitative determination of the proteolytic activity of bromelain and papain in *A. comosus* and *C. papaya* following the universal protease enzyme assay

The volumes of 0.05, 0.10, 0.20, 0.40, and 0.50 mL were measured from the stock solution of L-tyrosine (1.1 mM) and top-up to 2.00 mL with distilled water in order to make a concentration series of 0.055, 0.111, 0.221, 0.442 and 0.553 µM. A blank solution was prepared without the addition of tyrosine. Five milliliters of sodium carbonate (500 mM) and 1 mL of the Folin's phenol reagents (0.5 M) were added to all standards and the blank. Absorbance measurements were performed at a wavelength of 660 nm using a spectrophotometer (Analytik Jena SPEKOL-2000).

Determination of protease enzyme activity on different temperatures and pH

Casein solution (0.65% w/v) was prepared using 50 Mm potassium phosphate buffer (pH 7.5) and 5.00 mL of casein solution was added to each set of four replicates. All the test tubes were allowed to equilibrate in a water bath at different temperature conditions (37, 55, 70 and 90 ^{o}C) for 5 minutes. One milliliter of each enzyme sample was added to three of the test samples except the blank. Those were mixed by swirling and incubated at the given different temperatures (37, 55, 70 and 90 ^{o}C) exactly for 10 minutes. Trichloro-acetic acid (TCA) (5.00 mL) was added to each and filtered the mixtures. Two milliliters of the filtrates were mixed with 5.00 mL of Na_2CO_3 and 1.00 mL of Folin's reagent and incubated for 30 minutes at different temperatures (37, 55, 70 and 90 ^{o}C). Absorbance measurements were performed at a wavelength of 660 nm using a spectrophotometer (Analytik Jena SPEKOL-2000). Similarly, the pH profile was determined following the proteolytic activity assay as given in the section 3.8.1.4.2 using a series of potassium phosphate buffers (50Mm) at different pH values (2.5, 4.0, 5.5, 7.5, and 9.0) at 37 ^{o}C. Absorbance measurements were performed at a wavelength of 660 nm using a spectrophotometer (Analytik Jena SPEKOL-2000) and the activities of the enzymes were calculated (Cupp-Enyard 2008).

Determination of hydrolyzed protein amount of fish waste using bromelain and papain from *A. comosus* and *C. papaya* (Pedrol and Tamayo 2001)

Ten grams of chicken egg white lyophilized powder that contained lysozyme was dissolved in 10 mL of phosphate buffer solution (pH 7.0) to prepare the standard protein series. The standard protein 0, 20, 40, 60, 80 and 100 µL were pipetted to set up three replications each of six different concentrations and 100, 80, 60, 40, 20 and 0 µL of phosphate buffer were added to make the final volume 100 µL. The blank was prepared by pipetting 100 µL of phosphate buffer without any protein solution. Coomassie Brilliant Blue G 250 reagent (6.00 mL) was pipetted into each tube and mixed using a vortex mixer and absorbance was measured using a spectrophotometer (Analytik Jena Spekol - 2000) at the wavelength of 595 nm. Similarly, fish waste powder (10.00 g) was dissolved in 50.00 mL of distilled water and the resultants were filtered. Filtrates (2.50 mL) were taken and 0.50 mL of extracted enzyme solutions were added separately with three replicates. The enzyme-substrate mixtures were incubated for 1, 2, 5, 24 and 48 hours at 37 ^{o}C. At the end of the incubation periods, those mixtures were heated up to 90 °C to terminate the enzyme reactions and 100 µL were pipetted and mixed with 6.00 mL of Coomassie Brilliant Blue G-250 indicator. A blank solution was prepared by adding 2.50 mL of distilled water in 0.5 mL of each enzyme solution without adding the fish waste and it was used as the reference and absorbance values were measured at the wavelength of 595 nm. Hydrolyzed protein content was determined by subtracting the initial protein content from the respective hydrolyzed sample.

Preparation of liquid fertilizers

The liquid organic fertilizers were formulated by hydrolyzing fish waste proteins (1:3 w/v) using crude bromelain extracted from *A. comosus* (fruit peels + crown), crude papain extracted from *C. papaya* (ripe fruits peels + leaves) and the mixture of both enzymes (1:1w/w) for 48 hrs. Crude enzymes were prepared by extracting 1:1:5 w/w/v of each plant component in distilled water. Thereafter, hydrolyzed fish waste samples were added separately with two months decomposed organic materials consisting with plant materials (i.e. *Gliricidia sepium, Chromolaena odorata, Tithonia diversifolia* and *Mikania scandens*): topsoil: coconut husk ash: distilled water in 2:2:2:2:0.2: 0.05:5 ratios on a weight basis to formulate four different fertilizer combinations, i.e., FB1 (fish waste hydrolyzed with papain + decomposed organic materials), FB2 (fish waste hydrolyzed with bromelain + decomposed organic materials), FB3 (fish waste hydrolyzed with papain and bromelain + decomposed organic materials) and FB4 (decomposed organic materials + un-hydrolyzed fish waste). The chemical characteristics of liquid organic fertilizers are presented in Table 1.

Table 1 Chemical characteristics of the liquid fertilizers

	FB1	FB2	FB3	FB4	Commercial fertilizer (M)
Macronutrient contents (%)					
Total N	0.49	0.38	0.35	0.30	0.24
P	0.05	0.01	0.03	0.08	0.01
K	0.34	0.29	0.25	0.24	0.36
Ca	0.26	0.09	0.11	0.15	0.03
Mg	0.04	0.03	0.03	0.05	0.03
Organic C	0.74	0.81	0.65	0.55	2.9
C/N	1.51	2.13	1.86	1.83	12.08
Micronutrient contents (mg L^{-1})					
Cu	0.9	0	0.8	0.8	0.33
Mn	3.5	3.63	3.26	5.56	0.46
Zn	13.36	1.77	15.5	23.80	3.86
Chemical properties					
pH	7.1	7.09	7.04	6.86	7.90
EC (mS cm^{-1})	17.2	16.57	17.7	14.03	22.34

Plant material and experimental design

Seeds of *Basella alba* were obtained from the Department of Agriculture, Sri Lanka. A pot experiment was executed under a plant house at the University of Kelaniya, Sri Lanka (6° 58'26.4'' N, 79° 54'54.72'' E) between April – Sept. 2016. The seeds were allowed to germinate initially in a soil tray filled with sieved and solarized garden soil in the nursery stage. The soil was a sandy clay-loam with a pH (1:5 in H_2O) of 6.43, an organic matter content of 2.60% and a total N of 0.13%. Three weeks after seedling establishment, each healthy seedling with uniform-sized was transplanted separately in one plant per plastic pot (16 cm diameter and 13 cm height) filled with 2 kg of sieved and solarized garden soil. Pots were placed in a completely randomized design. This resulted in a total of five treatments (control; FB1, FB2, FB3 and FB4 and the commercially available liquid fertilizer [M]) and each treatment contains two groups (foliar and soil applications of fertilizers) with a total of 72 pots (6 replicates x 6 treatments x 2 fertilizing methods).

Foliar and soil applications of fertilizers

All treatments were started after one week of transplant. For foliar application, 5 mL of diluted (1:50) liquid fertilizers, i.e., FB1, FB2, FB3 and FB4 were sprayed on foliar parts of each plant twice a week for a period of three months. Five milliliters of diluted (1:50) commercially available seaweed liquid organic fertilizer (M) was sprayed as the positive control and the plants in negative controls (C) were sprayed with the same volumes of distilled water (5 mL). A manual sprinkler was used for foliar application. Similarly, the soil application of liquid fertilizers was carried out as another parallel pot experiment and five milliliters of diluted liquid fertilizer solution was added to each pot. The plants were watered every other day with 5 mL of distilled water to keep the soil moist.

Basella alba vegetative growth parameters

The total number of leaves was assessed for all plants during cultivation and plant shoot height was recorded at the end of three months' treatment period. Similarly, mature leaves were randomly selected from each treatment and leaf area was determined using a leaf area meter and plant fresh and dry weights also determined after three months of treatment.

Statistical analysis

Data were analyzed statistically using analysis of variance (ANOVA, $p<0.05$) followed by Tukey's pairwise multiple comparisons to determine the significant differences between the treatments using IBM SPSS software package (SPSS version 20 for Windows).

Results and discussion

Fish wastes are easily available, cheap and nutrient-rich organic waste that produces in large quantities causing environmental issues in discarding due to its unpleasant odor. Fish waste contains a large amount of proteins with many other macro and micronutrients; therefore, it can be utilized as a soil amendment or organic fertilizer in agriculture (Ghaly et al. 2013, Dahama 1997) lowering their adverse impacts. The high protein content presents in the fish waste can be efficiently converted into easily available nitrogen source for plant growth, by hydrolyzing with plant derived-proteolytic enzymes. Therefore, different components of *C. papaya* and *A. comosus* were checked as protease enzyme-rich plant-based materials in order to hydrolyze fish waste. Among them, priority was given to identify the possibility of utilizing fruit waste, i.e., fruit peels, crown and other leftovers for converting into agriculturally valuable resources by reducing, reusing and recycling organic wastes as these wastes create significant environmental problems during the post-harvest handling and processing (Jirapornvaree et al. 2017; Zainuddin et al. 2014).

Sustainable utilization of these fruit wastes in hydrolyzing fish waste would be beneficial in organic waste management and nutrient cycling, thereby enhance the easily available nitrogen content making it more suitable for the formulation of organic liquid fertilizers.

Qualitative determination of the presence of proteolytic enzymes in different components of *A. comosus* and *C. papaya*

All the crude protease enzymes extracted from *A. comosus* (leaves, crown, immature fruit pulp, ripe fruit peels and ripe fruit pulp) and *C. papaya* (leaves and ripe fruit peels) showed positive results for the casein hydrolysis test by forming coagulation of milk. However, time taken for appearing the coagulation was varied based on the amount of the proteolytic enzymes contained in different plant components of *C. papaya* and *A. comosus.* Among them, *C. papaya* latex exhibited an immediate response of milk coagulation soon after the addition of the enzyme as it contained the highest amount of enzymes among all the different parts of *C. papaya* checked. These findings were in accordance with Chaiwut et al. (2007). They stated that dried latex contained the highest concentrations of papain among different components of *C. papaya* and papain is commercially manufactured from the cuttings of green *C. papaya* fruits (Amri and Mamboya 2012). Moreover, the latex, the milky fluid oozes out of the cuttings of green *C. papaya* is important for protecting plants against insects, especially lepidopteran larvae and polyphagous pests (Konno et al. 2004).

Therefore, in this study, *C. papaya* latex was used as the standard to compare the protease activities of different plant components used. Moreover, *C. papaya* leaf extract, *A. comosus* fruit peels and rest of other extracts showed the milk coagulation within 10-15 minutes after the addition of crude enzyme extracts forming a comparatively lower amount of coagulation compared to the *C. papaya* latex. As explained by Chaiwut et al. (2007), the variability of the casein hydrolysis could be due to the different amounts of crude enzymes present in different plant components.

Tenderization of Tuna fish and chicken breast samples using crude papain and bromelain enzymes

As shown in Fig. 1A, the tenderness of the Tuna fish block has varied over time with respect to proteases from different plant components of *C. papaya* and *A. comosus*. Among them, fish samples dipped separately in *A. comosus* immature fruit peels, leaves and *C. papaya* unripe fruit peels for 2 hrs showed significantly higher ($p<0.05$) degree of tenderness (0.56±0.02, 0.56± 0.02 and 0.60 ±0.00 N). Leaves of *C. papaya* showed significantly the highest degree of tenderness of the fish block after 4 hours of hydrolysis. At 48 hours of the incubation period, *C. papaya* unripe fruit peels showed the highest degree of tenderness (0.20±0.02 N) followed by *C. papaya* ripe fruit peels, leaves and immature fruit peels of *A. comosus* (0.22±0.00, 0.23±0.02 and 0.22±0.01 N) compared to the control (0.38±0.03 N). Unripe fruit peels of *C. papaya* showed the highest degree of tenderness as it contained a comparatively higher amount of latex (Islam and Molinar-Toribio 2013). However, the unripe fruit peels of *C. papaya* were not selected in this study as they lower the edible fruit yield indirectly, by damaging the green fruits. However, pickle manufacturing industry, where large amount of green *C. papaya* fruits peels is discarded (Chaiwut et al. 2007), shows a great potential of utilizing in this manner. Moreover, papain showed the effectiveness over the tenderization of Tuna fish, as it disorganizes and disintegrates the muscular collagen structural elements by loosening and disrupting of the inter-molecule bonds. These findings were consistent with the opinion of Gokoglu et al. (2016) who stated that papain showed a significantly higher tenderization of squid muscles than bromelain. Evidently, Wisuthiphaet and Kongruang (2015) and Abdulazeez et al. (2013) also revealed the successful hydrolysis of the marine fish flesh and skin of *Scomberomorus commerson*, respectively using papain enzymes. In addition to the hydrolysis of flesh or skin of fish in different studies, Himonides et al. (2011) revealed the potential of hydrolyzing fish frames using plant-derived papain and bromelain enzymes.

When considering the tenderness of chicken breast block, *A. comosus* unripe fruit peels showed the highest enzymatic hydrolysis (0.49±0.01and 0.45±0.00 N) after 2 hrs and 4 hrs respectively followed by *C. papaya* unripe fruit peels, ripe fruit peels and leaves (Fig. 1B). The control sample without any enzyme showed the lowest degree of tenderness (0.67±0.07 N and 0.66±0.01 N) respectively after 2 hrs and 4 hrs of enzymatic hydrolysis (Fig. 1B). Similarly, Ketnawa and Rawdkuen (2011) and Doneva et al. (2015) stated that bromelain extracted from *A. comosus* fruit peels increased the tenderness by increasing the soluble peptide content while reducing the water-holding capacity, shear force values, toughness and firmness of chicken, beef, turkey meat and squid samples. Based on the microscopic observations, they further revealed that the disintegration and degradation of muscle fibers create inter-fibrillar spaces or voids between fibers and disrupt the inter-muscular connective tissues due to the activity of the bromelain enzyme. Moreover, both chicken and fish samples treated for 24 hours retained their colour and fresh appearance similar to the control, and samples treated for 48 hours showed faded colour with mucous surfaces due to the comparatively higher muscle fiber degradation and deformation. It proved that higher soaking time with the enzyme resulted in a higher tenderization effect. However, at the end of 48 hours Thereafter *A. comosus* unripe fruit peels, *C. papaya* unripe fruit peels, ripe fruit peels and leaves showed significantly the highest degree of tenderness ($p>0.05$) of the chicken samples (0.26±0.03, 0.32±0.02, 0.30±0.01 and 0.30±0.01 N), respectively. Similarly, the unripe fruit peels of *C. papaya* were not selected in-this study and priorities were given to the ripe fruit peels and other potential plant components. Moreover, studying the tenderization effect on both Tuna fish and chicken breasts samples which soaked in different plant based proteases allowed to exhibit their potential on protein degradation. Therefore, this could be applied directly on the degradation of protean-rich fish or other animal waste, left-overs or by-products effectively lowering their unpleasant odor.

According to statistical analysis, there was a significant difference ($p<0.05$) in the tenderness of both the chicken breast and Tuna fish blocks separately when the effect of treatments, time and the interaction of treatment x time were considered (Table 2).

Effect of temperature on the proteolytic activity of bromelain and papain extracted from *A. comosus* and *C. papaya*

Temperature plays an important role in relation to enzyme activities and their stability. Among the bromelain rich proteases extracted from different components

Table 2 ANOVA results for the effect of different treatments and time on the tenderness of different protein sources (Tuna fish and chicken breast)

Factor	d.f.	F-value	P-value
Tenderness of chicken breast blocks			
A (time)	3	222.142	0.000
B (treatment)	6	27.922	0.000
A x B (time x treatment)	18	2.500	0.005
Tenderness of Tuna fish blocks			
A (time)	3	435.995	0.000
B (treatment)	6	24.881	0.000
A x B (time x treatment)	18	4.641	0.000

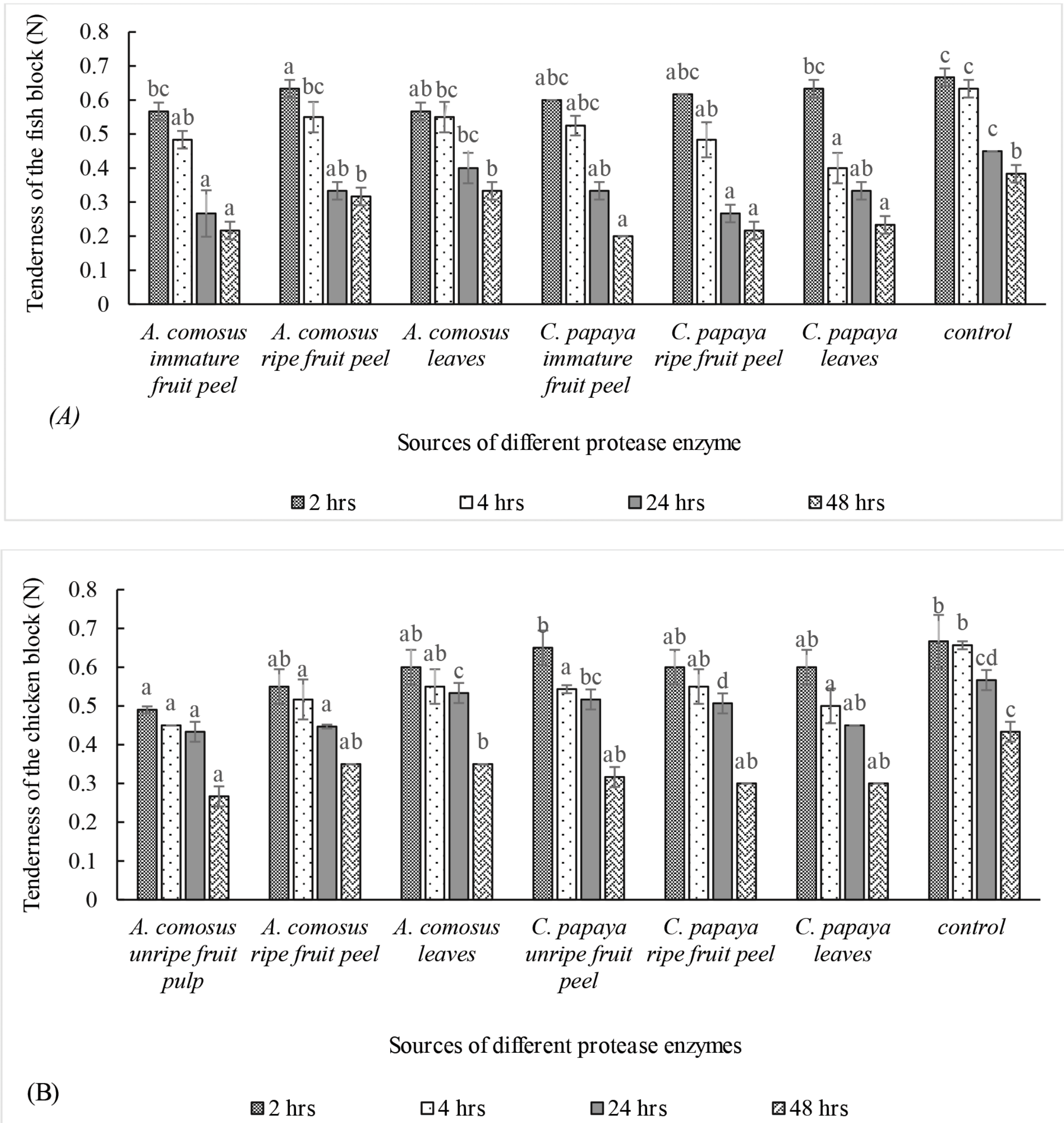

Fig. 1 Tenderness of (A) Tuna fish and (B) chicken breast blocks after dipping in crude papain and bromelain rich plant protease enzymes over time

Means sharing a common letter(s) in each treatment at a given time are not significantly different by Tukey's multiple comparison test (p<0.05). Each data point represent the mean of three replicates± standard deviation.

of *A. comosus* mature fruit peels and pulp have shown significantly the highest bromelain rich proteases enzyme activities (0.33 ± 0.02 and 0.27 ± 0.02 U mL^{-1} enzyme) than that of other studied parts respectively at 55 ^{o}C (Fig. 2). It also indicated that the enzyme activities had decreased with increasing temperature after 70 ^{o}C. Crown, leaves and unripe fruit pulp of *A. comosus* and 1:1 (w/w) mixture of *A. comosus* (crown + ripe fruit peels) + *C. papaya* (ripe fruit peels + leaves) showed the highest enzyme activities (0.23 ± 0.01, 0.16 ± 0.02, 0.23 ± 0.01, 0.36 ± 0.01 U mL^{-1} enzyme respectively) at 70 ^{o}C and protease activities of all tested extracts showed a significant decrease of their activities beyond 70 ^{o}C until 90 ^{o}C due to the thermal enzyme denaturation. These results were found to be in agreement with those of Miranda et al. (2016) who reported that all the bromelain extracts showed the optimum temperatures above 50 ^{o}C and among them, the bromelain from pulp and peels showed the highest activities at temperatures ranging from 60 ^{o}C to 90 ^{o}C.

Effect of pH on the proteolytic activity of bromelain and papain extracted from *A. comosus* and *C. papaya*

The pH values also play an important role in relation to enzyme activities and their stability. Small pH changes affect different ionization degrees in the biomolecules, increase the affinity between the active site and the sub-

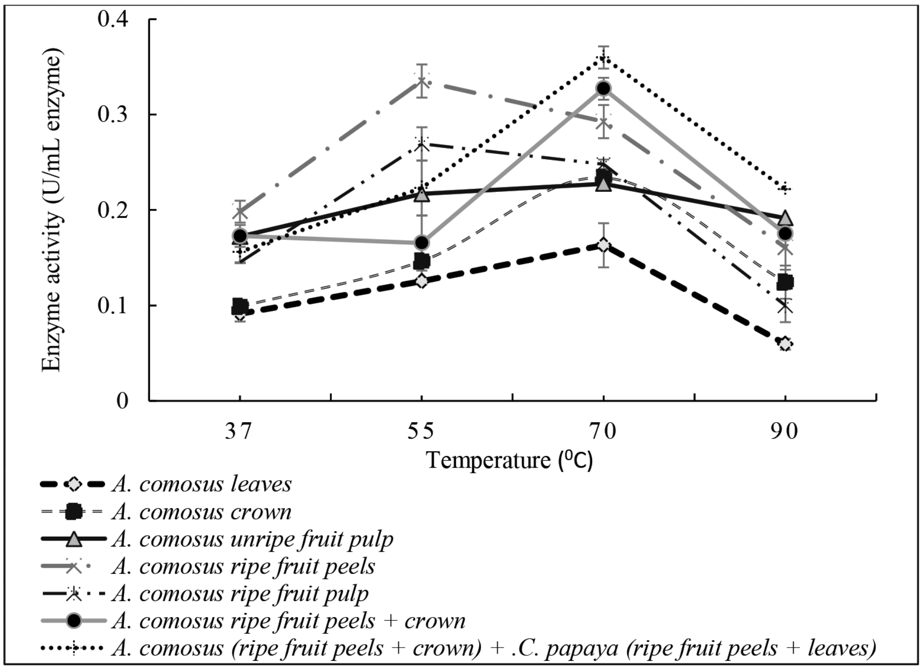

Fig. 2 Protease enzyme activities at different temperatures of bromelain and papain rich extracts from different components of *A. comosus* and *C. papaya*

Each data point represents the mean of four replicates ± standard deviation.

strate through conformational changes and cause enzyme denaturation (Miranda et al. 2016). As shown in Fig. 3, the pH in this study was ranged from 4.0 to 9.0 at 37 ^{o}C and the optimum pH to obtain the highest activity of bromelain extracted from different components of *A. comosus* was at pH 7.5 and this was in accordance with Corzo et al. (2012). However, the leaves and ripe fruit pulp of *A. comosus* and 1:1 (w/w) mixture of *A. comosus* (crown + ripe fruit peels) + *C. papaya* (ripe fruit peels + leaves) presented another low peak at pH 4. This is in agreement with the results of Bala et al. (2012), who recorded an optimum pH range of 3 - 8 for bromelain. Ripe fruit peels of *A. comosus* showed the highest protease activity (0.34 ± 0.02 U mL^{-1} enzyme) whereas the leaves of *A. comosus* showed the least (0.13 ± 0.02 U mL^{-1} enzyme) in pH 7.5 at 37 ^{o}C. This is in agreement with the results of other authors, who have reported the optimum pH for bromelain from the *A. comosus* fruit between pH 3 to 8 (Bala et al. 2012) and at pH 7.7 (Corzo et al. 2012). Moreover, Calkins and Sullivan (2007) recorded that the bromelain contained the optimum temperature at 65 - 75 ^{o}C and optimum pH at

5.0 - 6.0 whereas it has an active range between 50 - 80 °C and pH 4 - 7.

In addition to temperature and pH, the protease activity is affected by the presence of metal ions, method of enzyme extraction, i.e., hydraulic pressing, precipitation methods, etc. and their varieties, age and the plant part from which the enzymes were extracted and the environmental factors, i.e., soil, light amount and the climate affected during the plant growth (Agrahari and Sharma 2014; Silvestre et al. 2012). However, in this experiment, both crude aqueous extracts of *A. comosus* and *C. papaya* were simply extracted with water and were not further concentrated or purified. Therefore, it may have resulted in a comparatively lower amount of bromelain and papain enzymes respectively with some other proteases present in those plant materials. Moreover, *A. comosus* contains bromelain as the major protease in addition to a minor amount of ananain (Hale et al. 2005) and *C. papaya* contains papain as the major with other proteases, i.e., chymopapain, glycyl endopeptidase and caricain (Chaiwut et al. 2007).

The protein content of the bromelain and papain extracted from different components of *A. comosus* and *C. papaya*

Table 3 indicates the protein content of the bromelain and papain extracted from different components of *A. comosus* and *C. papaya.* Considering the papain enzymes extracted from the different plant components of *C. papaya,* latex has shown significantly the highest protein content (1.443±0.010 mg/g) followed by leaves (0.801±0.039 mg/g). With respect to bromelain, *A. comosus* ripe fruit peel and crown showed the highest protein contents (0.134 ±0.003 mg/g); however, the combinations of *A. comosus* (ripe fruit peels + crown) + *C. papaya* (ripe fruit peels + leaves) possessed significantly the highest ($p<0.05$) protein content (0.187 ±0.001 mg/g). Overall, *C. papaya* contained significantly higher amount of papain enzymes and its proteolytic activity compared to the amount of bromelain enzyme and its proteolytic activity of *A. comosus*.

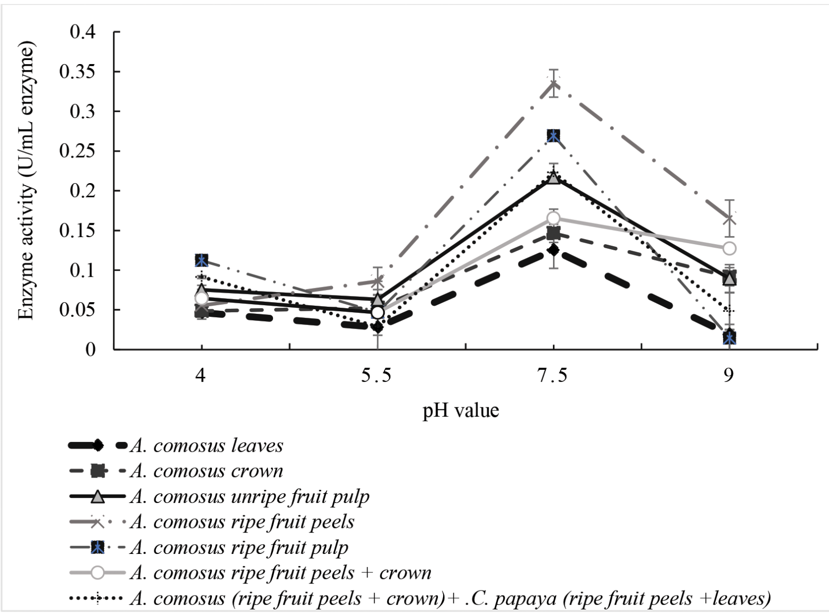

Fig. 3 Protease enzyme activities at different pH of bromelain rich extracts from different components of *A. comosus* and papain rich extracts from different components of *C. papaya* at 37 °C

Each data point represents the mean of four replicates ± standard deviation.

Determination of hydrolyzed protein amount of bromelain and papain extracted from different components of *C. papaya* and *A. comosus* over different incubation time

Among the different plant components of bromelain enzymes extracted from the *A. comosus,* leaves showed significantly the highest ($p<0.05$) hydrolyzed protein amount at 5 hrs (0.32±0.22) followed by the combi-

Table 3 Protein contents of the bromelain and papain extracted from different components of *A. comosus* and *C. papaya*

Sample		Protein content (mg/g)
C. papaya	Latex	1.443 (±0.010)[d]
	Leaves	0.801 (±0.039)[c]
	Unripe fruit peel	0.250 (±0.021)[b]
	Ripe fruit peel	0.217 (±0.002)[b]
	Ripe fruit pulp	0.154 (±0.000)[a]
	Ripe fruit pulp + peel	0.175 (±0.008)[a]
A. comosus	Leaves	0.029 (±0.000)[a]
	Crown	0.059 (±0.000)[a]
	Immature fruit pulp	0.092 (±0.003)[b]
	Ripe fruit peel	0.102 (±0.002)[b]
	Ripe fruit pulp	0.109 (±0.001)[b]
	Ripe fruit peel + crown	0.134 (±0.003)[c]
	A. comosus (ripe fruit peel + crown) + *C. papaya* (ripe fruit peel + leaves)	0.187 (±0.001)[d]

Mean values sharing a common letter(s) in each treatment is not significantly different by Tukey's multiple comparison te st ($p<0.05$). Each data point represents the mean of three replicates ± standard deviations.

nation of both *C. papaya* and *A. comosus* (0.29±0.17) and hydrolyzed protein amount has lowered at 24 hrs of incubation (Fig. 4). However, it has again increased after 48 hrs incubation showing the highest for bromelain extracted from the crown (0.28±0.15). At the end of 48 hrs incubation, immature fruit fresh showed the lowest hydrolyzed protein amount (0.17±0.02).

According to the results obtained, considerable meat degradation was shown by the plant-derived proteases within 48 hrs. Therefore, it showed the great potential of digesting other protein-rich organic waste, i.e., leftovers of meat processing industries including skins, guts, heads, tails and bones of pork, beef, poultry and fish wastes from the fish markets. This efficient enzymatic degradation of the rotting leftovers of animal wastes from meat industries within a short period of time would help to reduce the bad odor facilitating efficient degradation. This practice lowers the adverse environmental impacts and provides a great solution to urban organic waste management. Moreover, the utilization of hydrolyzed proteins from different organic wastes as organic fertilizers showed promising results in agricultural purposes (Nurdiawati et al. 2019; Gioseffi et al. 2012). Similarly, the production of liquid poultry feather-derived protein hydrolysate by hydrothermal treatments recorded up to 90% reduction of the volume of solid waste and showed higher growth performances for the production of *Vigna radiata* (Nurdiawati et al. 2019).

Effect of formulated liquid organic fertilizers on the growth of *Basella alba*

With the emerging concern for environmental protection and sustainability, organic fertilizers have become an eco-friendly alternative to chemical fertilizers (Hernandez et al. 2019; Lim et al. 2015). The multifaceted environmental impacts associated with chemical fertilizers could be mitigated by the adoption of organic fertilizers. Among diverse groups of organic fertilizers, liquid organic fertilizers can be applied on to foliage, into the soil or both (Wang et al 2019; Canfora et al. 2015), providing more homogeneous and soluble nutrients in easily available forms for effective nutrient uptake and plant growth. As indicated in Table 1, all three fertilizers enriched with fish waste hydrolysates (FB1, FB2 and FB3) contained a significantly higher ($p<0.05$) amount of nitrogen contents than the fertilizer with un-hydrolyzed fish waste (FB4). Among them, the organic liquid fertilizers formulated with hydrolyzed-fish waste using fruit wastes of *C. papaya* (FB1) showed the highest nitrogen content (0.49%) followed

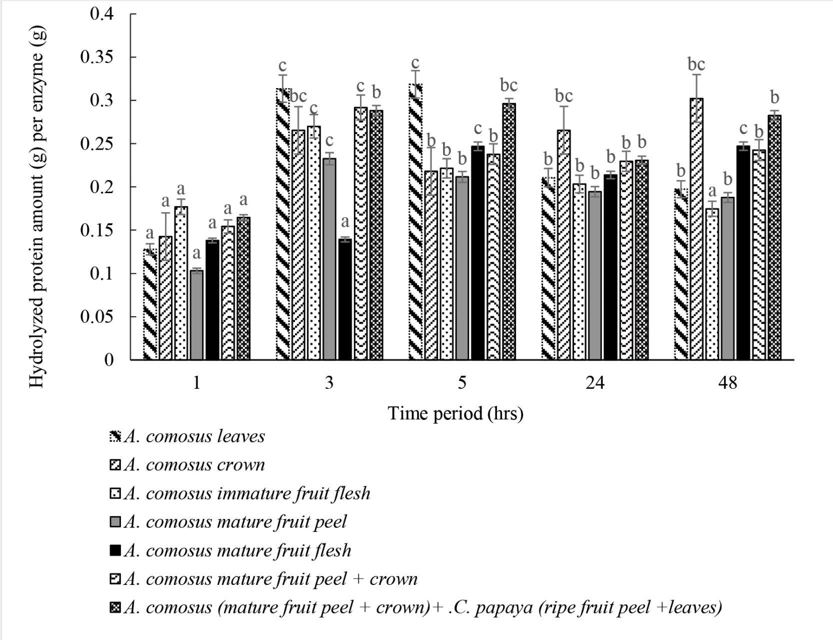

Fig. 4 Amount of hydrolyzed protein from fish waste mixed with crude papain and bromelain over different incubation time periods at 37 °C

Each data point represents the mean of four replicates ± standard deviation.

by the fish waste hydrolyzed with the fruit wastes of *A. comosus* (FB2) and the mixture of both fruits wastes (FB3) (0.38% and 0.35%, respectively). Similarly, Nurdiawati et al. (2019) revealed that hydrolysis of nitrogen-rich poultry feather produced amino acids and simple, water-soluble nitrogen compounds that can be easily available for the plants. Gioseffi et al. (2012) also revealed that the vegetable plant residues of tomatoes and cauliflowers hydrolyzed with papain enzyme has increased the nitrogen contents of the organic liquid fertilizers. Moreover, the fertilizers were enriched by adding the easily available nutrient-rich plants, i.e,. the fresh leaves and immature shoots of problematic invasive plants of *Chromolaena odorata, Tithonia diversifolia* and *Mikania scandens* mixed with the green manure *Gliricidia sepium* in order to obtain many other macro and micronutrients (Olabode et al. 2007). Coconut husk ash was incorporated as the main potassium source and topsoil with decaying litter was used as an inoculant of the favorable microbes to accelerate the degradation of organic materials throughout the preparation of liquid organic fertilizers. Moreover, all the formulated liquid fertilizers showed lower C/N ratio as most of the organic substartes have degraded during the decomposition stages (Phibunwatthanawong and Riddech 2019) and the remaining fibrous carbon materials also removed during the filteration steps.

According to the results of the foliar-applied liquid fertilizers, significantly the highest number of leaves and shoot height of *B. alba* were observed in the FB1 fertilizer which the fish waste was hydrolyzed by *C. papaya* (18.0±0.6 and 29.3±0.6 cm) followed by FB3 fertilizer which the fish waste was hydrolyzed by a mixture of *C. papaya* and *A. comosus* (17.2±0.8 and 29.7±1.0 cm), whereas the control possessed the minimum (11±0.6 and 17.5±1.2 cm) (Table 4 A). All the fertilizers that contained hydrolyzed fish waste, i.e., FB1, FB2 and FB3 showed significantly the higher leaf area and plant dry weights of *B. alba* than the un-hydrolyzed fish waste containing FB4 fertilizer due to the presence of higher nitrogen contents showing no significant differences to the commercial fertilizer (M).

Similarly, Shormin and Kibria (2018) also revealed that higher nitrogen supply through fertilizers significantly enhances the plant height, the number of leaves, fresh and dry weights of *B. alba* due to the increased cell division and enlargement. The highest fresh weight of plants was recorded in the foliar-applied FB3 fertilizer and the standard (55.9±1.1 and 56.3±0.8 g respectively) showing no significant differences followed by the FB1 fertilizer (55.2±1.2 g), whereas the control possessed the lowest (42.0±1.1 g). Similarly, Akanbi et al. (2007)

stated foliar fertilization enhances the net photosynthesis rates in plants, thereby increase leaf area, nutrient and dry matter contents.

Moreover, soil application of liquid fertilizers recorded significantly lower overall growth performances of *B. alba* compared to the foliar application showing its higher effectiveness over nutrient uptake through the foliage (Table 4 B). Among the soil-applied fertilizers, formulated FB1 and FB3 fertilizers and the commercial liquid fertilizer (M) recorded the significantly highest average number of leaves per plant of *B. alba* (17.0±0.8, 16.0±1.0 and 17.0±1.0 respectively) showing no significant differences among them, whereas the control recorded the lowest (10.0±0.5). Similarly, formulated FB1 and FB3 fertilizers showed significantly the highest shoot height (27.2±1.4 and 27.5±0.8 cm) followed by the commercial liquid fertilizer (M) (25.6±0.7 cm), whereas the control possessed the lowest (17.3±0.5 cm). Considering the leaf area as a growth parameter, the commercial liquid fertilizer (M) recorded the significantly the highest (69.9±1.3 cm^2) followed by FB3, FB2 and FB1 (68.4±0.5, 66.9±2.5 and 64.9±0.8 cm^2 respectively).

Significantly the highest plant fresh weights were recorded in the FB2, FB3 fertilizers and the commercial liquid fertilizer (M) (56.1±1.1, 55.6±0.6 and 56.9±1.4 g respectively) followed by the FB1 and FB4 fertilizers (53.3±0.8 and 52.8±1.8 g). Moreover, no significant differences were recorded among the plant dry weights of all the fertilizer treatments except the control. Furthermore, the highest growth performances shown by FB3 fertilizer combination where the fish waste is digested with fruit wastes of both *C. papaya* and *A. comosus* could be due to the presence of plant growth promoting hormones and vitamins that are beneficial for the plant growth (Kosit 2011). Proper management and utilization of organic wastes in this manner prevent nutrient losses through runoff and leaching. Overall, this study showed the potential of applying liquid fertilizers in both foliar and soil applications for the growth of *B. alba*, whereas the foliar application showed the best performances than the soil application. Similarly, Netpae (2012) also revealed that foliar application of liquid fertilizers enhances the fresh mass, plant height and number of leaves of *Brassica alboglabra* than the soil application.

Liquid fertilizers developed in this study were formulated using a simple methodology that can be followed by the home gardeners easily. Therefore, liquid fertilizers were formulated at room temperature (37 ^{o}C), without providing the optimum temperature or pH conditions for the hydrolysis of fish waste. However, hydrolysis of fish waste at their optimum conditions would enhance their further hydrolysis and release an even higher amount of nitrogen and other nutrients that required for plant growth.

Table 4A Effect of foliar applied liquid organic fertilizers on the growth of *Basella alba* L.

Foliar application of fertilizers					
Fertilizers	No. of leaves per plant	Shoot height (cm)	Leaf area (cm^2)	Plant fresh weight (g)	Plant dry weight (g)
FB1	18±0.6^{d}	29.3±0.6^{de}	71.2±1.8^{c}	55.2±1.2^{cd}	4.7±0.0^{c}
FB2	16±0.8^{c}	27.6±1.1^{cd}	69.4±2.4^{c}	53.9±1.2^{bc}	4.5±0.1^{c}
FB3	17±0.8^{cd}	29.7±1.0^{e}	71.2±1.8^{c}	55.9±1.1^{d}	4.7±0.1^{c}
FB4	15±0.6^{b}	23.8±1.9^{b}	63.9±0.5^{b}	52.1±1.2^{b}	4.3±0.0^{b}
C	11±0.6^{a}	17.5±1.2^{a}	53.7±1.1^{a}	42.0±1.1^{a}	3.5±0.1^{a}
M	18±0.5^{cd}	26.9±0.4^{c}	71.4±1.1^{c}	56.3±0.8^{d}	4.7±0.1^{c}

Each data point represents the mean of six replicates ± standard deviation. Means sharing the same letter in each row are not significantly different at p<0.05 by Tukey's multiple comparison test. (FB1: fish waste hydrolyzed with papain + decomposed organic materials; FB2: fish waste hydrolyzed with bromelain + decomposed organic materials; FB3: fish waste hydrolyzed with papain and bromelain + decomposed organic materials; FB4: decomposed organic materials + un-hydrolyzed fish waste; C: negative control and M: commercial liquid fertilizer).

Table 4B Effect of soil applied liquid organic fertilizers on the growth of *Basella alba* L.

Soil application of fertilizers					
Fertilizers	No. of leaves per plant	Shoot height (cm)	Leaf area (cm^2)	Plant fresh weight (g)	Plant dry weight (g)
FB1	17±0.8^{c}	27.2±1.4^{de}	64.9±0.8^{c}	53.3±0.8^{b}	4.5±0.1^{b}
FB2	16±0.6^{bc}	24.3±0.6^{c}	66.9±2.5^{cd}	56.1±1.1^{c}	4.5±0.1^{b}
FB3	16±1.0^{c}	27.5±0.8^{de}	68.4±0.5^{de}	55.6±0.6^{c}	4.6±0.1^{b}
FB4	15±0.5^{b}	20.9±1.1^{b}	62.1±1.8^{b}	52.8±1.8^{b}	4.4±0.1^{b}
C	10±0.5^{a}	17.3±0.5^{a}	53.6±0.8^{a}	41.9±1.0^{a}	3.4±0.2^{a}
M	17±1.0^{c}	25.6±0.7^{cd}	69.9±1.3^{e}	56.9±1.4^{c}	4.6±0.1^{b}

Each data point represents the mean of six replicates ± standard deviation. Means sharing the same letter in each row are not significantly different at $p<0.05$ by Tukey's multiple comparison test. (FB1: fish waste hydrolyzed with papain + decomposed organic materials; FB2: fish waste hydrolyzed with bromelain + decomposed organic materials; FB3: fish waste hydrolyzed with papain and bromelain + decomposed organic materials; FB4: decomposed organic materials + un-hydrolyzed fish waste; C: negative control and M: commercial liquid fertilizer).

Conclusion

All the tested *A. comosus* and *C. papaya* plant waste components showed considerable proteolytic activities due to the presence of major protease enzymes of bromelain and papain, respectively. The optimum pH values for all the studied extracts were 7.5 at 37 °C and the optimum temperature was at 55 - 70 °C. The highest proteolytic activity resulted in *A. comosus* crown and the combination of both *A. comosus* (crown + ripe fruit peels) + *C. papaya* (ripe fruit peels + leaves). Therefore, hydrolysis of fish waste proteins using fruit waste of *A. comosus* and *C. papaya* enhanced the available nitrogen content and enriched the organic liquid fertilizers for the growth of *B. alba*. Further, this study opens avenues for reducing, reusing and recycling organic wastes (i.e., fish wastes and fruit wastes) in agriculture through sustainable waste management.

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Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest associated with this study.

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