Some Optimize Parameters of Proteolytic Bacteria for *Shigella* spp. And *Enterobacter* spp. from Papaya Fruit (*Carica papaya* L.)

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Abstract

The papaya fruit *Carica papaya* L. samples were collected from Naung Hnit Pin Village, Hmawbi Township, and Yangon Region to screen for protease producing bacteria. Morphological and biochemical characterization of the two selected strains were performed and identified as GC-3 belong to *Shigella* spp. and RC-10 belong to *Enterobacter* spp. according to Bergey's Manual of Systematic Bacteriology (nine editions,2000). In the detection of protease enzyme activity, the release of tyrosine was used to prepare the standard curve, in which the amount of tyrosine product in the X-axis and the enzyme activity was detected with absorbance at 275nm in Y-axis. In the reaction mixture, casein was used as a substrate. The effect of optimum enzyme producing parameters like temperature (47°C for GC-3 and 57°C for RC-10), reaction time (20min for GC-3 and RC-10), fermentation period (2days for GC-3 and RC-10), aeration (100ml/250ml for GC-3 and RC-10), size of inoculums (1% for GC-3 and 1.5% for RC-10), carbon sources (sucrose for GC-3 and RC-10) and nitrogen sources (yeast extract for GC-3 and RC-10).The present experiment also investigated the effect of optimum pH, which was pH 7 for GC-3 and pH 8 for RC-10 in the reaction mixture on the activity. The estimation of amino acids that appear in the reaction mixture was done by TLC using seven different amino acids as standard. It was found that five amino acids were detected in *Shigella* spp. and three amino acids were detected in *Enterobacter* spp. In the present study it was concluded that the protease activity of *Shigella* spp. was more pronounced than that of *Enterobacter* spp.

Keywords: Papaya fruit, *Carica papaya* L., Protease enzyme, *Shigella* spp. and *Enterobacter* spp.

Introduction

Enzymes are biological catalysts that allow chemical reactions to occur in living organisms at ambient conditions. One group of enzymes that has been studied extensively is proteases or proteolytic enzymes due to their wide variety of applications in various fields. Protease (Peptidase or Proteinase) is an enzyme that hydrolyses the peptide bonds that link amino acids together in the polypeptide chain forming the protein. Proteases are widely distributed in all living organisms: in plants (papaya, pineapple and kiwi), in animals and mainly in microbes (bacteria, fungi and viruses) (Muhammed *et al*., 2015 and Saranaraj *et al*., 2015).

Protease production mainly requires the appropriate substrates. There are many substrates used for protease production, which include skim milk, peptone and casein (Banik and Prakash, 2004). Many factors can influence the catalytic activity of an enzyme, presumably by affecting the enzyme's shape or ionization state. Included among these factors are pH, temperature, reaction time, fermentation period, aeration, size of inoculums, carbon sources and nitrogen sources (Sawhney and Singh, 2000).

A molecule made up of amino acids that are needed for the body to function properly. Proteins are the basis of body structures such as skin and hair and of substances such as enzymes and antibodies. Proteins have high molecular weights and therefore contain a large number of amino acids linked together. The buildings are blocks of proteins, only 20 of the naturally occurring amino acids are commonly found (Das,1982).

One of the most important groups of enzymes in the food industry is proteolytic enzymes. They have multiple applications in the food industry as meat tenderizers, for chill proofing of beer, in the manufacture of cheeses, and in bread making where they modify gluten

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properties (Hewitt *et al*., 2000 and Knight, 1980). Proteolytic enzymes help to digest proteins. Plant extracts with a high content of proteolytic enzymes have been used for years in traditional medicine.

Materials and Methods

Collection of fruit samples

In the present research, the samples of healthy papaya fruits used in the screening of bacteria were collected from Naung Hnit Pin Village, Hmawbi Township, and Yangon Region. The collected samples were identified.

Identification and characterization of protease enzyme (Holt *et al*., 2000)

The protease producing strains were identified based on morphological and biochemical characterization methods.

Production of protease enzymes (Wiseman, 1975 and Oser, 1976)

The selected bacteria were maintained regularly on respective agar slants and used in this work. For protease production, it contained (g/L); casein 20g, dextrose 10g, peptone 10g, magnesium sulfate 0.2g, potassium dihydrogen phosphate 0.5g, sodium chloride 2g and calcium chloride 0.02 g. The pH was adjusted to 6.8 ± 7.0 with 1% NaOH and this broth medium was sterilized by autoclaving at 121°C (15 psi) for 15 min. The above medium was inoculated with a loopful of bacteria and incubated at room temperature in a rotary shaker operated at 200 rpm for two days. At the end of the fermentation period the contents were centrifuged at 3000 rpm for 30 min at room temperature, and the cell free supernatant was used as crude enzyme for enzyme assay.

Determination of wavelength of maximum absorption of tyrosine (Breed, 1975)

An extract amount of 5% TCA solution used as reagent blank was placed in the reference cell whereas standard tyrosine solution was placed in the sample cell of a UV - visible spectrophotometer and the spectra was scanned between 250 to 300 nm. The absorbance was plotted against different wavelengths.

Calibration curve for standard tyrosine (Pelczarand Reid, 1972)

5% TCA solution was used as a reagent blank. The absorbance of six different concentrations of standard tyrosine was measured at 275nm against the blank solution with a UV-visible spectrophotometer. The absorbance was plotted against the concentration of tyrosine to give a calibration curve.

Procedure of enzyme assay (Colowick and Kaplan, 1955)

A proteolytic activity assay, using casein as the substrate was performed according to the protocol described by Ramakrishna and Bndit, 1988 with some modification. The reaction mixture contained 2ml of pH 7, 2% casein solution and 1ml of pH 7, 10% enzyme solution. The mixture was shaken well and incubated at 37°C for 30 minutes. After that, 4.5 ml of 5% TCA solution was added to inhibit the enzyme reaction at room temperature. The mixture was allowed to stand for 20 minutes. Then, the filtrate was collected by centrifugation at 3000 rpm for 30 min. The filtrate was read at 275 nm against a reagent blank prepared in the same manner by a UV-visible spectrophotometer. One unit (U) of proteolytic enzyme activity in the present study was defined as the amount of enzyme that liberated one mM of tyrosine per ml per minute from casein under specified assay conditions.

Effect of pH on protease enzyme activity (Wiseman, 1975)

A 1.25ml of 10% enzyme solution was reacted with 2.5ml of 2% casein solution in reaction mixture of various pH values 4, 5, 6, 7, 8, 9, and 10 at 37°C for 30 min and the enzyme activity was assay as described above.

Effect of temperature on protease enzyme activity (Hawk *et al.*, 1965)

The influence of different temperatures on protease enzyme activity was determined by holding the reaction mixture at various temperatures of 27, 37, 47, 57, 67, 77, and 87°C for 30 minutes during the standard enzyme assay as described above.

Effect of reaction time on protease enzyme activity (Joslyn *et al.*, 1970)

The influence of different reaction times on protease enzyme activity was determined by holding the reaction mixture for 10, 20, 30, 40, 50, 60and 90 minutes during the standard enzyme assay as described above.

Effect of fermentation period on protease enzyme activity (Naik *et al*., 2013)

In this experiment, each enzyme production medium was incubated at various fermentation periods of 1, 2, 3, 4, 5, 6 and 7 days respectively. The detection of enzyme activities was subjected to the standard enzyme procedure described earlier.

Effect of aeration on protease enzyme activity (Naik *et al.*, 2013)

Aeration conduction with respect to volume of enzyme production media such as (25, 50,100, 150 and 200) ml in 250 ml conical flasks respectively and then spend of agitation from static to 150rpm, respectively in separate flasks. At the end of the incubation period, the cell free culture filtrate was obtained and used as an enzyme source. The detection of enzyme activities by using the standard enzyme assay described earlier.

Effect of size of inoculums on protease enzyme activity (Tiwari *et al*., 2015)

The enzyme production medium at pH 7 was incubated with a selected bacterial strain. The broth was incubated with different volumes of strains such as (0.5, 1, 1.5, 2 and 2.5) ml in 100ml of culture media. At the end of the incubation period the cell-free culture filtrate was obtained as an enzyme assay by centrifuging at 3000rpm for 30 minutes.

Effect of carbon sources on protease enzyme production (Prabhakaran *et al*., 2015)

To find optimum carbon source for enzyme production, 1% of five carbon sources such as glucose, fructose, lactose, sucrose and maltose, were supplemented to respective broth medium and incubated at room temperature for 48 hrs. The enzyme activity was assayed in the culture supernatant.

Effect of nitrogen sources on protease enzyme production (Prabhakaran *et al*., 2015)

To optimize the nitrogen sources for enzyme production, 1% of five nitrogen sources such as urea, yeast extract, malt extract, $NaNO_3$ and KNO_3 were added to respective broth medium and incubated at room temperature for 48 hrs. The enzyme activity was assayed in the culture supernatant.

Estimation of amino acid from reaction mixture of selected bacteria by Thin Layer Chromatography (TLC) (Stahl, 1969 and Buzarbarus, 2000)

Preparation of the TLC plate

Silicagel GF_{254} absorbent 40 g was dissolved in 80 ml of distilled water in a beaker by stirring with a glass rod till uniformity in consistency and freedom from air-bubbles were

achieved. And then the slurry of silicagel was poured onto the layer of chromatographic glass plate. The TLC plate was adjusted using a spreader with a thickness of 0.25mm, the plate was air dried for 15min and then placed in an oven at 110°C for one hour.

Preparation of the chromatographic tank and solvent system

The glass tank was prepared by partially lining the walls of the developing glass tank containing the solvent system, Phenol: DW (4:1)$^{v}/_{v}$, which was dipped 1cm in the solvent system.

Spotting of a sample

The samples were spotted on the plate at a distance of 1cm from the lower edge of the plate. The sample solution and standard amino acid were spotted with the help of the glass capillary tubes and then the solvents were evaporated for about 15minutes at room temperature before inserting the plate into the developing glass tank.

Procedure of chromatogram

The chromatogram was developed by placing the TLC plate vertically in the developing tank which had been pre-saturated with the above mentioned solvent system for 1hr before insertion of the plate. The depth of insertion of a plate in the solvent was approximately 1 cm.

Results

In the present investigation, the green and ripe fruits of *Carica papaya* L. were used for the isolation of bacteria by using the serial dilution of the pour plate method at room temperature for three days. All isolated bacteria were maintained in pure culture by repeated streaking on their respective agar plates as shown in Figure 2. Then, they were sub-cultured on the agar slants for further investigation.

In this investigation, all isolated bacterial strains were screened for protease producing ability on 1% skim milk agar by the streak plate method. The clear zone formation around the bacterial growth was identified as the positive protease produced which may be due to hydrolysis of casein.

In above investigation, two bacterial strains GC-3 and RC-10 showed the highest proteolytic activity as shown in Figure 3. Therefore, the efficient protease producing strains GC-3 and RC-10 were selected for further experimental studies and biochemical tests. According to Bergey's Mannual of Determinative Bacteriology (ninth edition, 2000), *Shigella* spp. has characteristics similar to GC-3 and *Enterobacter* spp. identical to RC-10 as shown in Figure 4 and Table 1 and 2 .

The UV spectra of standard tyrosine were scanned between 250 to 300 nm at different concentrations and λ_{max} was found at 275nm. Casein was used as the substrate in all experiment studies of enzyme catalyzed reactions in this research. The products of the bacterial protease catalyzed reaction contain other amino acids beside tyrosine, but in this research, the absorbance were determined from the bacterial protease catalyzed reaction were interpreted in terms of standard tyrosine which was constructed for this purpose and the standard comparison method was used for the calculation of the results as shown in Figure 5 and 6.

pH played an important role in the enzyme's production. In the present study, the pH optimum of protease activity in a reaction mixture was studied at different pH ranging from 4 to 10. The highest protease production was observed at pH 7 for *Shigella* spp. and whereas an enhanced protease production was observed at pH 8 for *Enterobacter* spp. below and above

that pH the enzyme activity was found to be decreased. The obtained results are presented Figure 7.

Temperature also plays an important role in the activation and inactivation of enzymes. In the present study, the effect of temperature on protease production was studied at various temperatures ranging from 27 to 87°C. The protease activity is relatively stable in the temperature range of 47°C for *Shigella* spp. and 57°C for *Enterobacter* spp. The enzyme activity was found to be decreased above and below those temperatures for both strains. The present investigation showed that *Shigella* spp. and *Enterobacter* spp. produced maximum protease at 47 and 57°C as shown in Figure 8.

Reaction time is also a major factor in the determination of enzyme activity. The optimum temperature for protease activity increases inversely with the period of reaction time. In this research, the protease activity was studied for reaction time ranging from 10 to 90 minutes. Above and below the reaction time of enzyme activity was found to be decreased by 20 minutes for *Shigella* spp. and *Enterobacter* spp. at room temperature. The obtained results are presented in Figure 9.

Different fermentation time durations had an effect on both the growth and protease production carried out with time durations from 1 to 7 days. Maximum enzyme production is observed at 2 days' incubation for *Shigella* spp. and *Enterobacter* spp. as shown in Figure 10. The above and below incubation periods resulted in a gradual decline in the enzyme production.

In general, it is well known that all organism and microbes vary in their aeration requirements. Aeration affected both the growth rate and protease productions. They were carried out with an aeration rate rating of 25-200ml/250 ml conical flask. Maximum protease production was observed in a 100 ml/250 ml conical flask as shown in Figure 11.

Different inoculums concentration was affected protease production. In the present study, the effect of inoculum size on protease production was studied with different inoculums concentration from 0.5 to 2.5% production medium. The optimum inoculums size of the bacterial isolate for protease production was observed at 1% inoculums for *Shigella* spp. and 1.5% inoculums for *Enterobacter* spp. The less protease production in small inoculums size of 0.5 ml may be due to an insufficient number of bacteria, which would have led to a reduced amount of secreted protease and a decrease even though luxurious growth was observed in higher inoculums of 2.5 ml, as shown in Figure 12.

The effects of 1% different carbon sources such as glucose, fructose, lactose, sucrose and maltose on enzyme activity were studied. Maximum protease production was observed in both *Shigella* spp. and *Enterobacter* spp. when sucrose was supplemented as a carbon source. The least enzyme activity was observed in lactose and maltose for *Shigella* spp. and *Enterobacter* spp. as shown in Figure 13.

In the present investigation, 1% of nitrogen sources such as urea, yeast extract, malt extract, $NaNO_3$ and KNO_3 were added in the medium for determining protease activity. Among the different nitrogen sources tested maximum protease production was observed in yeast extract for *Shigella* spp. and *Enterobacter* spp. The addition of $NaNO_3$ in the medium decreased the enzyme production in both species as shown in Figure 14.

In the present investigation, the estimation of amino acid content in protease enzyme extract from *Shigella* spp. and *Enterobacter* spp. was done using TLC. When developed spots with phenol:distilled water(4:1)$^{v}/_{v}$ solvent system the chromatogram showed at least five amino acid spots were clearly seen in *Shigella* spp. and three spots were clearly seen in *Enterobacter* spp. The R_f values of *Shigella* spp. in the plate are 0.14, 0.24, 0.35, 0.38 and 0.70

respectively and the R_f values of *Enterobacter* spp. in the plate are 0.14, 0.24 and 0.89 respectively. Both species of R_f values were compared with the R_f value of standard amino acids such as asparatic acid 0.15, glutamic acid 0.25, serine 0.36, glycine 0.40, alanine 0.54, histidine 0.69 and proline 0.91 respectively. There were also one or two unidentified spots of amino acids because of the lack of other standard amino acids as shown in Figure 15.

Green fruit

Ripe fruit

Fig. 1 Fruit of *Caricapapaya* L.

GC-3

RC-10

Fig. 2 Pure cultures of isolated strains from green and ripe

GC-3

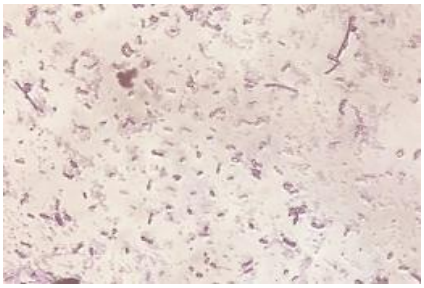
RC-10

Fig. 3 Screening of bacteria from green and ripe papaya fruit for proteolytic activity on 1% skim milk agar

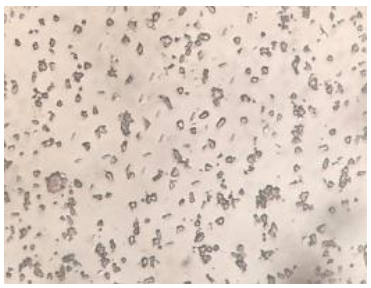
Table 1 Morphological characteristic of two selected bacteria

Isolated Strain	Color	Form	Margin	Elevation	Cell Shape	Gram-staining	Endospore Staining	Motility
GC-3	Cream	Irregular	Undulate	Flat	Rod	-	-	-
RC-10	White	Circular	Entire	Flat	Rod	-	-	+

\+ = positive / present / motile - = negative / absent / non-motile



GC-3



RC-10

Figure 4 Cell morphology of two selected bacteria (micrograph x 400)

Table 2 Biochemical characteristic of two selected bacteria

No	Biochemical Tests	Selected Strains	
		GC-3	RC-10
1	Oxygen requirement	Facultativeanaerobic	FacultativeAnaerobic
2	Oxidase	-	-
3	Catalase	+	+
4	Nitrate reduction	+	+
5	Citrate utilization	-	+
6	Starch hydrolysis	-	-
7	Vogesproskauer	+	-
8	Indole	+	-
9	Ammonium Salt tolerance	+	+
10	Phenylananine production	-	-
11	Gelatin liquefaction	-	-
12	Hydrogen Sulfide Production	-	-
13	Methyl red test	+	-
14(a)	Fermentation of carbohydrate Glucose Fermentation	+	+
	Glucose gas	+	+
(b)	Fructose fermentation	+	+
	Fructose gas	+	+
(c)	Lactose fermentation	-	+
	Lactose gas	-	+
(d)	Sucrose fermentation	-	+
	Sucrose gas	-	+
(e)	Maltose fermentation	+	+
	Maltose gas	+	+
(f)	Mannitol fermentation	+	+
	Mannitolgas	+	+
15(a)	Fermentation of Nitrogen Source of Urea	-	-
	Urea gas	-	-
(b)	Yeast extract fermentation	-	-
	Yeast extract gas	-	-
(c)	Malt extract fermentation	-	+
	Malt extract gas	-	+
(d)	$NaNO_3$ fermentation	-	-
	$NaNO_3$ gas	-	-
(e)	KNO_3 fermentation	-	-
	KNO_3 gas	-	-

+ = positive/present/hydrolysis, - =negative/absent/non-hydrolysis ,+ - = moderate/present or absent

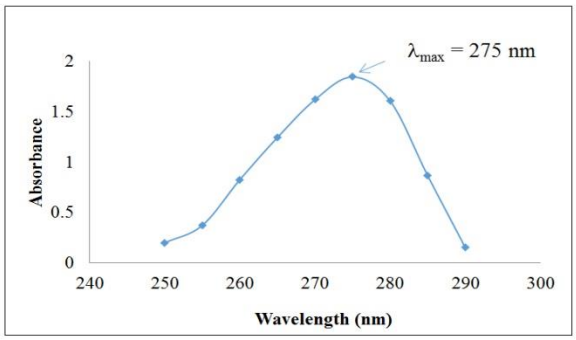


Fig. 5 Plot of relationship between absorbance and wavelength of tyrosine solution

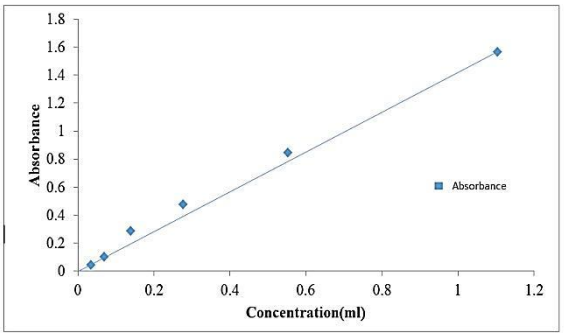


Fig. 6 Plot of absorbance as a function of standard tyrosine concentration

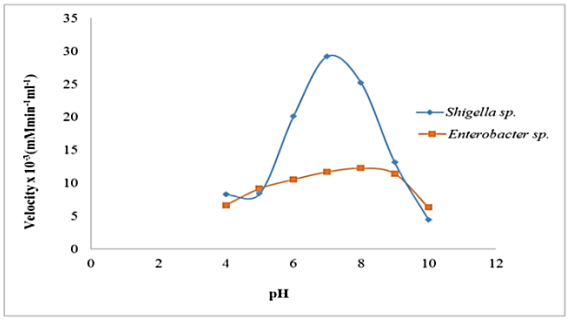


Fig. 7 Plot of velocity as a function of pH of *Shigella* spp. and *Enterobacter* spp.

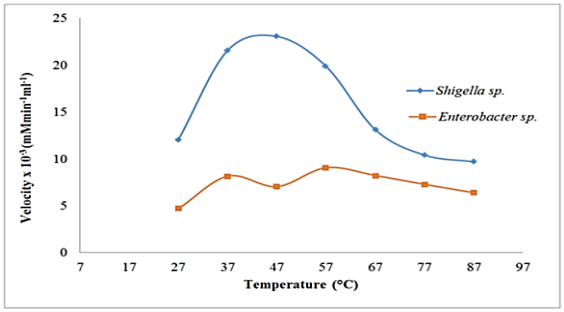


Fig. 8 Plot of velocity as a function of temperature for *Shigella* spp. and *Enterobacter* spp.

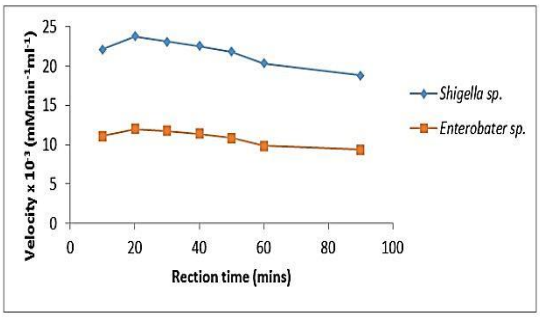


Fig. 9 Plot of velocity as a function of reaction time of *Shigella* spp. and *Enterobacter* spp.

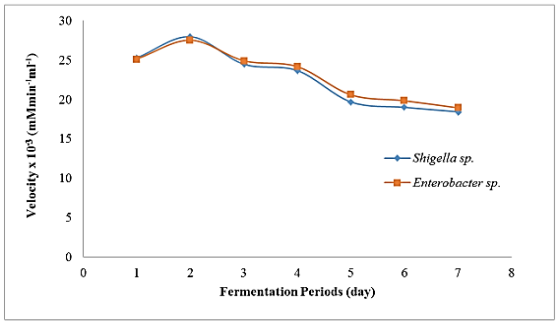


Fig. 10 Plot of velocity as a function of fermentation period of *Shigella* spp.and *Enterobacter* spp.

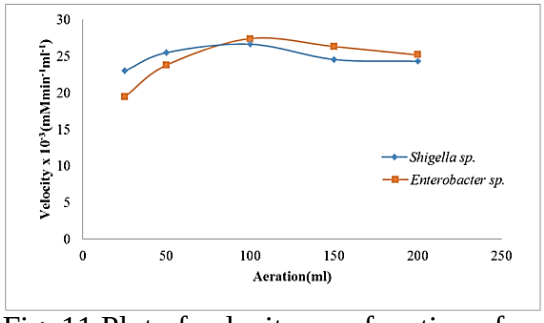


Fig. 11 Plot of velocity as a function of aeration of *Shigella* spp. and *Enterobacter* spp.

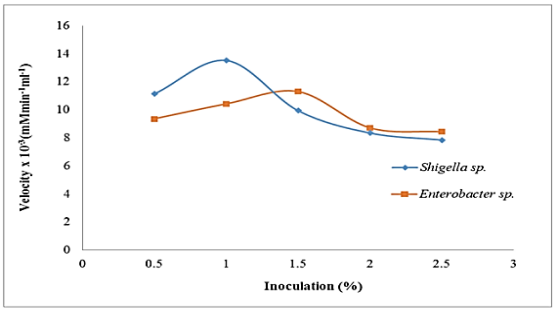


Fig. 12 Plot of velocity as a function of inoculation size of *Shigella* spp.and *Enterobacter* spp.

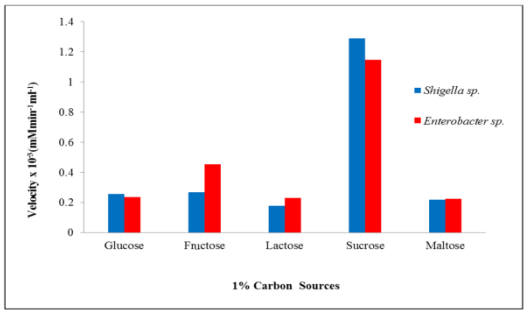


Fig. 13 Plot of velocity as a function of carbon sources of *Shigella* spp. and *Enterobacter* spp.

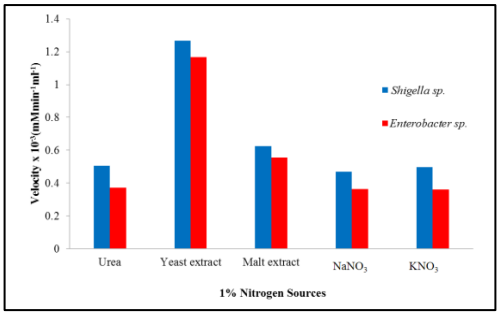


Fig. 14 Plot of velocity as a function of nitrogen sources of *Shigella* spp. and *Enterobacter* spp.

Standard Amino Acids	**R_f value of standard amino Acids**	**Name of Sample**
1=Aspartic acid	Aspartic acid = 0.15	S1= *Shigella* spp.
2=Glutamic acid	Glutamic acid = 0.25	S2= *Enterobacter* spp.
3=Serine	Serine = 0.36	
4=Glycine	Glycine = 0.40	
5=Alanine	Alanine = 0.54	
6=Histidine	Histidine = 0.69	
7=Proline	Proline = 0.91	

Fig. 15 Estimation of amino acid content in a reaction mixture by *Shigella* spp. and *Enterobacter* spp.

Discussion and Conclusion

In the present study, protease enzyme from green and ripe papaya fruits was collected from Naung Hnit Pin Village, Hmawbi Township, and Yangon Region. The green and ripe papaya fruit showed a clear zone on a 1% Skim Milk Agar (SMA) plate for the estimation of preliminary protease producing bacteria as shown in Figure 3. Only two isolated bacteria designated as GC-3 and RC-10 showed maximum proteolytic activity and then the strains as shown in Figure 2.In 2015, Thanika *et al.,* had reported that proteolytic enzyme was a mixture of enzymes found naturally in the extract of stem, leaves and fruits of the plant *Carica papaya* L.GC-3 and RC-10 were identified by five morphological characters and fifteen biochemical tests proved that two selected bacteria may be genus of *Shigella* spp. for GC-3 and

Enterobacter spp. for RC-10 according to Bergey's Manual of Determinative Bacteriology (ninth edition, 2000) as shown in Figure 4 and Table 1and2.These results of the present identification have already been reported by Roberts *et al*., 1999 and Almalki *et al*., 2017.

The enzyme activity of the culture supernatant was determined by measuring the amount of tyrosine released from casein. Therefore, casein was used as a substrate (Pelczar and Reid, 1972).In the present study, the amino acid tyrosine was used to construct the standard curve using 275 nm as shown in Figure 5 and 6. Enzyme properties such as reaction mixture of pH, temperature, reaction time, fermentation period, aeration, size of inoculums, carbon sources and nitrogen sources which were determined by using spectroscopic method (Wisemon,1975 and Oser,1976).

In the present data *Shigella* spp. clearly pointed out that optimum pH was 7, the optimum temperature was 47°C and the reaction period was 20 minutes. Although *Enterobacter* spp. clearly showed optimum pH was 8, optimum temperature was 57°C and reaction period was 20 minutes as shown in Figure 7, 8 and 9.The present observations agree with the results of kumar *et al*., 2014.

During the fermentation period, aeration and size of inoculum, *Shigella* spp. clearly showed 2 days, 100 ml and 1ml respectively and *Enterobacter* spp. exhibited 2 days, 100ml and 1.5 ml respectively as shown in Figure 10, 11 and 12. These results are in accordance with the findings of Tiwari *et al.,* 2015.

To find out suitable carbon sources such as glucose, fructose, lactose, sucrose and maltose, both genus prominently as shown in Figure 13.The present observations agree with the results of Naik *et al*., 2013. In the case of suitable nitrogen sources such as urea, yeast extract, malt extract, $NaNO_3$ and KNO_3, only yeast extract is clearly exhibited in both isolated genus as shown in Figure 14.The result of the present study agree with those reported by Feroz *et al*., 2013.

The hydrolysate by protease isolated from *Shigella* spp. was found to contain five amino acids such as asparatic acid, glutamic acid, serine, glycine and histidine and the R_f values of amino acids observed in the plate were 0.14, 0.24, 0.35, 0.38 and 0.89 respectively. Similarly, in the hydrolysate of protease isolated from *Enterobacter* spp., there were three amino acids such as asparatic acid, glutamic acid and proline and the R_f values of amino acids observed in the plate are 0.14, 0.24 and 0.89 as shown in Figure 15. The R_f values of all amino acids were shown to be in agreement with the R_f values of standard amino acids. In the result of present study, the amino acid from reaction product as shown in Figure 15 by TLC agreed to those reports by Mello, 2017. One of the main results from the present study was that the protease enzyme producing microorganisms *Shigella* spp. and *Enterobacte*r spp. can thrive in papaya and produce some kind of protease enzyme. It is also assumed that the wide use of *Carica papaya* L. fruit may be based on two facts, (1) plant enzyme easily digests the milk protein by having papaya fruit tissue and (2) the easy digestibility also depends on microbial enzyme produced by bacteria in the fruit.

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References

Almalki, M.A., Rakesh, V. and Ponnuswamy, V., 2017.Production and Characterization of Halo Tolerant Alkaline Protease of *Enterobacteraerogenes* Strain ZB and its Application.

Banik, R.M., Prakash, M., 2004. Laundry Detergent Compatibility of the alkaline protease from Bacillus cereus. Microbiology Res 159: 135-140.

Breed, R.S., 1957. Bergey's Manual of Determinative Bacteriology. The Williams and Wilkins Co. New York. 507.

Buzarbarus., 2000. A Text Book of Practical Plant Chemistry. S. Chand & Company Ltd.

Colowick, S.P. and Kaplan, N.O., 1955. Methods in Enzymology Academic Press Inc. New York. 1:33.

Das, D., 1982. Biochemistry. Academic Pub., Inc., New Delhi, 125-135.

Feroz, K., 2013. New Microbial Proteases in Leather and Detergent Industries.Innov. Res. Chem. 1:1,1-6.

Hawk, P.B., Oser, B.L. and Summerson, W.H., 1965.Practical Physiology Chemistry. McGraw-Hill Book Co. Inc. New York. 1333.

Hewitt, H.H., Whittle, S., Lopez. S.A., Bailey.E.Y, Weaver.S.R.,2000. Topical Uses of Papaya in Chronic Skin Ulcer Therapy in Jamaica.West Indian Medicinal Journal.32-33.

Holt, J. G., Noel, R.,krieg Peter, H.A., SneathJomes, T., Staley, T., 2000.Bergey's Manual of Determinative Bacteriology. 9th Ed. Williams &wilkins. New York, Hong Kong, Tokyo.

Joslyn, M.A., 1970. Methods in Food Analysis. Academic Press Inc. New York. 737.

Knight, R.j.,1980. Origin and World Importance of Tropical and Subtropical Fruit Crops.Inc:Nazy S, Shaun SE (Eds) Tropical and Subtropical Fruits. Composition, Properties and Uses.AVI Publishing, Connecticut, USA.1-120.

Kumar, D.J .,Rakshitha .R., Vidhya M.A., Jennifer . P.S, Prasad.S., 2014.Production, Optimiation and Characteriation of FibrinolyticEnyme b Bacillus subtilis RJAS19. Pak J Biol Sci. 17:529-534.

Mello, J.P.F.D., 2017. The Handbook of Microbial Metabolism of Amino acids. Formerly of SAC, University of Edinburgh King's Buildings Campus, Edinburgh, UK.

Muhammed, Y.H. I., 2015.Isolation of protease production microorganism from food waste.Department of Biochemistry, Hajja University, Hajja, Yemen. Email: issayasser 16@ gmail.com.

Naik, L.S., Dr. Kopuri, A.P.S. and Prof. Ch. Venkata, R.D., 2013. Isolation and Biochemical Characterization of Protease Isolated from *Bacillussp*. SVN12. International Journal of Research in Pure and Applied Microbiology.ISSN 2277-3843.

Oser, B.L. (Editor)., 1976. Hawk's Physiological Chemistry. Tata MagGraw-Hill Book Co., Ltd. New Delhi. 129.

Pelczar, M.J. and Reid, R.D., 1972. Microbiology. McGraw- Hill Book Co. Inc. New York. 10.

Prabhakaran, V[1]., Soma, P.A. and Blessy, T., T.S1.,2015. Screening and Production of Protease Enzyme from Marine Microorganisms and Its Industrial Application.

Roberts, D.P., P.D. Dery, J. Yucel and J.S. Buyer., 1999.Importance of PFKA for rapid growth of *Enterobactercloaceae* during colonization of crop seeds.Applied and Environmental Microbiology.Vol.66. no.1. (87-91).

Saranraj, P., and Kumar, M.M., 2015. Commercial Production and Application of Bacterial Alkaline Protease: An Overview. Department of Biochemistry, Sacred Heart College (Autonomous), Tirupattur, Tamil Nadu, India. Assistant professor of Microbiology, Department of Biochemistry, Sacred Heart College (Autonomous), Tirupattur, Tamil Nadu, India.

Sawhney, S.K. and Singh, R., 2000.Introductory Practical Biochemistry.Narosa Publishing House, New Delhi,88-92.

Stahl, Egon (Ed.)., 1969. Thin Layer Chromatography, A Laboratory Handbook, Academic Press in, New York.

Thanika, V.P. and Shaleesha, A.S., 2015.Studies on the Effect of the Different Modes of Extraction on Enzyme Activity of Proteolytic Enzymes from the Peels of Three Fruit Samples Carica Papaya (papaya), AnanasComosus (Pine Applie) and ActinidiaDeliciosa (Kiwi). Department of Biotechnology,

JeppiaarEngineering College, Chennai and Biochemical characterization of protease isolated from *Bacillus* sp. SVN12.Department of Biochemistry, Osmania University, Hyderabad,India – 500007.ISSN 2277-3843.

Tiwari.O.N., Thyam, B.D., Kangions, S.D., 2015. Isolation and Optimization of Alkaline Protease Producing Bacteria from Undisturbed Soil of NE-region of India Falling Under Indo-Burma Biodiversity Hotspots. Vol. III. (04), pp.028-031, Journal & Applied Biology and Biotechnology.

Wiseman, A., 1975. Handbook of Enzyme Biotechnology.John Wiley and Sons.Inc. Sydney.