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Activity and Stability of Linamarase from Cassava (*Manihot Esculenta* Crantz) Leaves and its Ability to Hydrolyze Linamarin

Maherawati
Student, Doctoral Programme of Food Science, Faculty of Agricultural Technology,
Gadjah Mada University Yogyakarta, Indonesia
Dr. Tyas Utami
Lecturer, Department of Food and Agricultural Product Technology,
Faculty of Agricultural Technology, Universitas Gadjah Mada, Yogyakarta, Indonesia
Yudi Pranoto
Professor, Department of Food and Agricultural Product Technology,
Faculty of Agricultural Technology, Universitas Gadjah Mada, Yogyakarta, Indonesia
Dr. M. Nur Cahyanto
Lecturer, Department of Food and Agricultural Product Technology,
Faculty of Agricultural Technology, Universitas Gadjah Mada, Yogyakarta, Indonesia

Abstract:
Linamarase from cassava leaves was studied its activity, stability, and ability to degrade linamarin. Linamarase was extracted from young cassava leaves using 0.2 M phosphate buffer pH 6.0. Linamarin as a substrate for linamarase was extracted from young cassava leaves using 0.1 M hydrogen chloride (HCl). The activity of crude linamarase was examined at various pH (4.0 to 7.0), and temperatures (30 to 75°C). Stability of crude linamarase at various temperatures was conducted at 40 to 70°C for 240 minutes. The activity of crude linamarase was 306.13mU/mL. Yields of linamarase and linamarin were 244.08mU/g leaf and 338.50µg hydrogen cyanide (HCN) eq./g leaf respectively. The enzyme showed the highest activity at pH 5.5 and temperature 55°C. Crude linamarase showed good stability at temperature of 40, 50, and 60°C for 240 minutes with the remaining activity more than 90%. Hydrolysis of linamarin using crude linamarase resulted in the release of more than 90% HCN within 30 minutes incubation. Linamarase extracted from young leaves cassava has potent as an exogenous enzyme to hydrolyze linamarin from cassava.

Keywords: *endogenous linamarase, activity, stability, linamarin, cassava leaves.*

1. Introduction

Cassava (*Manihot esculenta* Crantz) is an important source of calories and the staple food of about eight hundred million people in the world, especially in developing countries. Grow in more than 90 countries, it ranks as the 4th supplier of energy after rice, sugar, and corn (Santana *et al*., 2002; Sornyotha *et al*., 2007;). Almost all of cassava tissues contain large amount of cyanogenic glucosides with major compound of linamarin (95%) and a small amount of lotaustralin (Montacnag *et al*., 2009). When cassava plant tissues are damaged linamarin are hydrolyzed by endogenous linamarase to liberate glucose and acetone cyanohydrin compounds. Acetone cyanohydrin decomposes spontaneously under neutral and alkaline conditions (pH> 5) and temperature above 30°C to liberate volatile hydrogen cyanide or free cyanide (White *et al*., 1998; BeMiller and Whistler, 2009). Linamarin and linamarase naturally occurring in cassava and be part of a biological system for defense against predators.

Food products containing nonhydrolized linamarin can cause health problems due to linamarin can be hydrolyzed in the body releasing cyanide (Mkpong *et al*., 1990). Various health disorders are associated with the consumption of cassava which contain residual cyanogens, such as hyperthyroism, tropical ataxic neuropathy, and konzo (White *et al*., 1998). Although cassava is an important food, it contains potentially toxic cyanogenic glucosides. Therefore, reducing its toxicity is crucial.The presence of these toxic compound in cassava and its products showed that endogenous linamarase cannot remove all the cyanide from cassava roots. Though the role of linamarase on linamarin catabolism in plants and their effects on the levels of linamarin in cassava roots are not yet fully known, but linamarase believed to have an important role in the reduction of linamarin during processing (Nambisan, 2011).

Many authors introduced linamarase from various fungi and bacteria to degrade linamarin (Essers *et al*., 1995; Yeoh *et al*., 1995; Petruccioli *et al*., 1999; Ugwuanyi *et al*., 2007). These microorganisms should be grown in appropriate media and condition for linamarase production, followed by recovery and purification of the enzyme. Endogenous linamarase extracted from parts of cassava

plant might be used as exogenous linamarase to degrade linamarin in cassava by introducing the enzyme into cassava or cassava processing.

The distribution of linamarase activity varies between different organs and tissues of the same cassava plants. Very high linamarase activity was observed in stem, root peel, and in young emerging leaves but the enzyme activity was decreased in mature and old leaves. Root parenchyma contained the lowest enzymes levels (Nambisan and Sundaresan, 1994). Leaves may have 3-50-fold higher of linamarase activity than do roots (McMahon *et al*., 1995). The linamarase activity on leaves of low cyanogenic cassava has a very high peak of activity in young leaves (third from apex) of 8-month-old plants, whereas in high cyanogenic cassava, the activity of young leaves remained very low (Santana *et al*., 2002).

Mkpong *et al.* (1990) investigated the properties of the cassava linamarase in leaves using immunofluorescent labeling and indicated that linamarase was localized either in the inner cell wall or the plasma lemma of leaf tissue. There are a number of different linamarase isozymes present in cassava. There are at least three difference isozymes of 65 kD enzyme in cassava leaves which can be distinguished on the basis of their isoelectric points. The enzyme is capable of hydrolyzing a number of β-glycosides in addition to linamarin. The activity of linamarase was higher in tissues containing higher concentration of linamarin indicating a higher requirement of enzyme in tissues which had high cyanide potential (Nambisan and Sundaresan, 1994).

Young cassava leaves potentially as both linamarin and linamarase sources. It is necessary to know this potential sources which used as a source of exogenous enzymes that can be added to the processing of cassava to reduce levels of cyanide in cassava food products. Therefore, the aims of this work were to study the activity and stability of linamarase extracted from young cassava leaves, and to investigate the ability of this enzyme to degrade linamarin. The information obtain will be useful for the application of cassava leaves linamarase as exogenous enzyme for reduction of linamarin in cassava, by addition of this enzyme into cassava or cassava processing.

2. Materials and Method

2.1. Materials

Cassava leaves "Ketan" variety were obtained from local farmer in Warungboto, Umbulharjo, Yogyakarta, Indonesia. The young leaves of 6-8 months old cassava plant were collected by picking up the third leaf from the apex. All chemicals used were products of Merck (Darmstadt, Germany).

2.2. Extraction of linamarase and linamarin from cassava leaves

Cassava leaves (50 g) were cut by scissorsand ground using mortar with 50 ml chilled phosphate buffer (0.2 M pH 6.0), and sonicated using Sibata Ultrasonic Cleaner SU-2TH (25 watt 34 Khz) for 5 min. The homogenate was filtered through a Whatman paper No. 4 to remove the insoluble material and then the filtrate was centrifuged at 8000*xg* for 20 min at 4°C (Eppendorf Centrifuge 5417R). Supernatant was collected as crude linamarase, and determined its activity and volume. Crude linamarase was stored at 4°C until further use.

The method of extraction of linamarin from cassava leaves was derived from Haque and Bradbury (2004). The amount of 10 g of young leaves of cassava were cut and ground with 20 mL of 0.1 M HCl then filtered and squeezed through a cloth. The pink-colored solution was centrifuged at 1000x*g* for 10 minutes. Supernatant was taken as linamarin solution, and measured the linamarin concentration. This solution was stored frozen at 4°C. Linamarin solution was used as a substrate for linamarase activity assay.

2.3. Determination of linamarase activity

Linamarase activity was assayed using the method of Nwokoro and Anya (2011) with some modification. The amount of HCN liberated from hydrolysis of linamarin by linamarase was determined by the method of William and Edwards (1980). Crude linamarase solution (0.5 mL) in 0.2 M phosphate buffer pH 6.5 was added into 0.5 mL of linamarin solution in the tube. The mixture was incubated at 50°C for 20 minutes. Reaction was terminated by adding 1 mL of 4% TCA. The reaction mixture then was added with 1 mL of 2% KOH and 5 mL of picric acid solution and placed in boiling water for 15 seconds. Picric acid solution consists of mixture of picric acid, Na_2CO_3, and H_2O in the ratio 1: 5: 200 (v/w/v) respectively. Aquadest were added in total volume 10 mL and the absorbance was quantified spectrophotometrically at 510 nm (Shimadzu Spectronic 200). Under the assay conditions, one unit of enzyme activity (U) was defined as the amount of enzyme that released 1 µmol of HCN per minute.

2.4. Determination of linamarin content

The amount of linamarin in solution extracted from young cassava leaves was assayed using Picrate Paper Kit (Haque and Bradbury, 2004). Aliquots of 100 µL linamarin solution in a small plastic bottle was added with 0.5 mL of distilled water, and then followed by addition of linamarase enzyme paper. A picrate paper was placed in the bottle, which was closed with a screw cap and left at 30°C overnight. The brownish picrate paper was removed from the bottle and immersed in 5.0 mL water for 30 minutes and the absorbance of the solution measured at 510 nm using spectrophotometer. Linamarin content was expressed in equivalent HCN. The cyanide content in ppm, was obtained by calculation using KCN standard curve.

2.5. Determination of enzyme activity and stability at different pH and temperature

Enzyme activity at different pH was evaluated by preparing linamarin solution at various pH ranging from 4.0 to 7.0. The appropriate pH was obtained using the following buffer solution: acetate buffer (pH 4.0; 4.5; 5.0; and 5.5) and phosphate buffer (pH 6.0; 6.5 and 7.0). The pH was determined using a glass electrode pH meter. The reaction mixture contained 0.5 mL of crude linamarase and 0.5

mL of linamarin solution at various pH were incubated at 50°C for 20 min. The reaction was stopped by the addition of 2 mL trichloroacetic acid. The enzyme activities were spectrophotometrically tested.
The effect of temperature on linamarase activity was determined by incubating the reaction mixtures of 0.5 mL crude enzyme and 0.5 mL linamarin solution at various temperatures ranging from 30-75°Cat pH 6.0 for 20 minutes. The reaction was stopped by addition of 2 mL trichloroacetic acid, and measured the enzyme activity.
The thermal stability of crude linamarase was evaluated by incubating crude linamarase solution at 40, 50, 60, and 70°C for 0, 30, 60, 120, 180, and 240 minutes. After a specified incubation time, the sample was taken, and enzyme assay was conducted at 50°C for 20 minutes. The reaction was stopped by addition of 2 mL trichloroacetic acid and measured the remaining enzyme activity.

2.6. Hydrolysis linamarin by crude linamarase
The ability of crude linamarase to hydrolyze linamarin was determined using linamarin solution from cassava leaves as substrates. The reaction mixtures of 0.5 mL crude enzyme and 0.5 mL linamarin solution were incubated at 50°C for various time. The reaction was stopped by addition of 2 mL 4% trichloroacetic acid. Samples were taken at certain times. HCN content showed linamarin hydrolysis by crude linamarase.

3. Results and Discussion

3.1. Extraction of linamarase and linamarin from cassava leaves
Linamarase was extracted from 50 g young cassava leaves using 0.2 M phosphate buffer pH 6.0 resulted 39.5±3.50mL crude enzyme which has enzyme activity 306.13±32.02 mU/mL. The yield of linamarase extraction was 244.08±46.73mU/g leaf. Nambisan (1999) reported that partial purified linamarase from leave had the activity of 3±1 U/25 g leaf or 0.12 U/g leaf. Crude linamarase then was examined its activity at various pH and temperature, and its thermal stability.
The yield of linamarin extracted from young cassava leaves was 186.40±22.48 µgHCN eq./mL or 338.50±27.04 µgHCN eq./g leaf fresh weight. This results in accordance with Haque and Bradbury (2004) that showed yields of linamarin from very young cassava leaves of different varieties were range between 180-630 µgHCNeq. /g leaf. Young emerging cassava leaves was chosen because it contained more linamarin than other parts of cassava plant. Linamarin solution subsequently used as a substrate for determine the enzyme activity and stability.

3.2. Effect of pH on the crude linamarase activity
The activity of crude linamarase was assayed at various pH (4.0-7.0) at 50°C. The results showed that crude enzyme linamarase had an optimum pH of 5.5 (Figure 1). The enzyme activity decreased as the pH of reaction mixture decrease or increase from pH 5.5. At pH 4.0, the remaining enzyme activity was about 70%, however when the pH increased to pH 7.0, the remaining enzyme activity dropped to 55%. Effect of pH on the enzyme activity associated with ionization prototropic group in the active site of an enzyme. The role of the active group prototropic include maintaining a balance conformation of the active site of an enzyme, substrate binding, and conversion of the substrate into a product. Change of pH may change the ionization prototropic group in the active site, thus affect the enzyme activity. Enzyme from different sources might have different pH profile for activity.

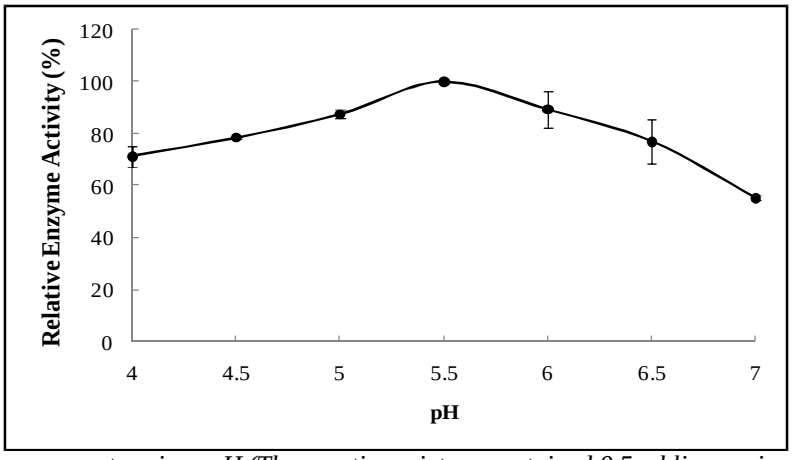

Figure 1: Activity of crude linamarase at various pH (The reaction mixture contained 0.5 ml linamarin of 18.6 µg/ml and 0.5 ml crude linamarase of 30.6 mU/ml; enzyme assay was conducted at 50°C, 20 minutes at various pH)

Linamarase from *Mucor circinelloides* also had the optimum pH 5.5 (Petruciolli *et al*., 1999), meanwhile Yeoh *et al*. (1995) reported that the optima pH for linamarase activity from *Aspergillus nidulans*, *Penicillium funiculosum,* and *Fusarium oxysporum* were 5.1; 4,8; and 4.2 respectively. Linamarase from *Lactobacillus delbrueckii* also had a lower optimum pH at 4.5 suggesting a possible the use of this enzyme in detoxification processes based on lactic acid fermentation (Nwokoro and Anya, 2011). Linamarase from *Bacillus stearothermophilus* and *Bacillus coagulans* showed slightly alkaline pH optima (pH 8.0). However, the activity dropped markedly beyond pH 8.0, and at pH 5.0 the enzyme activity was less than 20% (Ugwuanyi *et al*., 2007).

Information of linamarase activity profile at various pH is very useful in the application of this enzyme to hydrolyze linamarin. This study showed that linamarase extracted from young cassava leaves had optimum pH of 5.5, and had good performance at pH 5.0 – 6.0. The optimum pH of this enzyme was similar to the pH of cassava slurry that between 5.4-5.8. Therefore, this enzyme can be applied to hydrolyze linamarin in cassava processing.

3.3. *Effect of temperature on the activity and stability of crude linamarase*

The hydrolytic activity of crude linamarase was investigated over a temperature ranges from 30 to 75°Cat pH 6. The activity of the enzyme gradually increased as the temperature increase from 30°C to 55°C, but decreased significantly at higher temperature. The optimum temperature of crude enzyme linamarase was determined at 55°C (Figure 2). The optimum temperature of crude linamarase in this study was in accordance with the previous study by Mkpong *et al*. (1999), but higher than linamarase from microorganisms such as *Mucor circinelloides* which has an optimum temperature at 40 °C and *Lactobacillus delbrueckii* at 50°C (Petruciolli *et al*., 1999; Nwokoro and Anya, 2011).

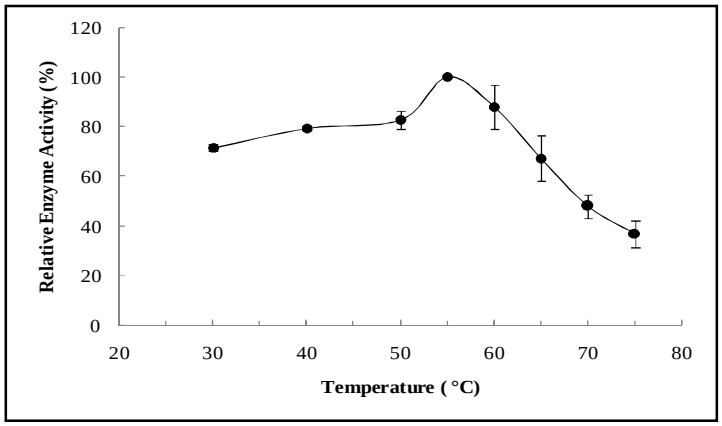

Figure 2: Activity of crude linamarase at various temperature (the reaction mixture contained 0.5 ml linamarin of 18.6 µg/ml and 0.5 ml crude linamarase of 30.6 mU/ml; enzyme assay was conducted at pH 6.0, 20 minutes at various temperature)

Temperature is an important factor that influence the activity of an enzyme. Each enzyme has a specific optimum temperature for its activity that dependent on the strengthof amino acids as building blocks of the enzyme. Increasing temperatures cause an increase in kinetic energy of the enzyme but the three-dimensional structure of enzyme is thermo-sensitive and becomes destabilized at high temperature causing denaturation (Bisswanger, 2014).

The enzyme activity was quite stable at temperature of 30 to 50°C with the remain activity in the range of 71-82%, while at temperatures above 60°C the enzyme activity decreased sharply, and only 36% enzyme activity remained at 75°C. Based on these results, enzyme linamarase cannot work at high temperature, so the use of this enzyme preferably at a temperature below 60°C.

Information about the stability of the enzyme is required for the further usage of the enzyme. Stability of enzymes is a function of temperature, pH, ionic strength, the presence of the substrate, the concentration of enzymes and other proteins in the system, incubation time, and the presence of activator and inhibitor. The stability of the enzyme at a specific temperature is required as consideration for applying the enzyme at a temperature of the system for a certain period.

Crude linamarase was most stable at temperature between 40, 50, and 60°C with activity remained 91-97% (Figure 3), while linamarase activity sharply decrease as the temperature increased beyond 70°C with the remaining was only 24% for 240 minutes' incubation. Therefore, use crude linamarase to hydrolysis linamarin on the processing of cassava should be carried out at a temperature below 70°C.

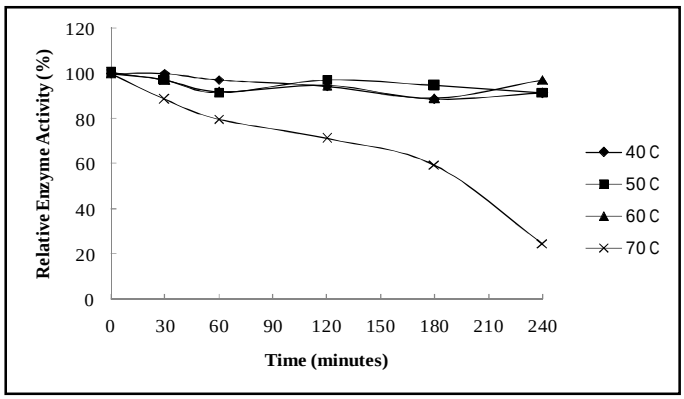

Figure 3: Thermal stability of crude linamarase at various temperature (The reaction mixture contained 0.5 ml linamarin of 18.6 µg/ml and 0.5 ml crude linamarase of 30.6 mU/ml; enzyme assay was conducted at pH 6.0, 20 minutes at various temperature)

3.4. Hydrolysis of linamarin by crude linamarase

Crude linamarase was studied its ability to hydrolyse linamarin. The hydrolyses of linamarin enzymatically was conducted at 50°C, pH 6.0 for 30 minutes. The free HCN released during hydrolyses proses was measured. The result showed that HCN released increase rapidly within 5 minutes of producing HCN of 110.67±41.50 µg/mL. HCN released continued to increased, and after 30 minutes' incubation HCN released was reached to 179.92±34.58 µg/mL. This was equal to the removal of 93% linamarin. Sornyotha *et al.* (2010) showed that the linamarin exhibited the fastest removal within 5 minutes of incubation (71.6% removal of linamarin, 0.3 mg of residual linamarin/g of tissues) and then increased slightly until 90.3% of linamarin was removed from cassava parenchyma at 90 min incubation. This result indicated that crude linamarase can hydrolyze linamarin and produce HCN. Thus, can be used to reduce linamarin content in the system. Further studied should be done to examine the ability of linamarase from young cassava leaves to hydrolyze linamarin in cassava.

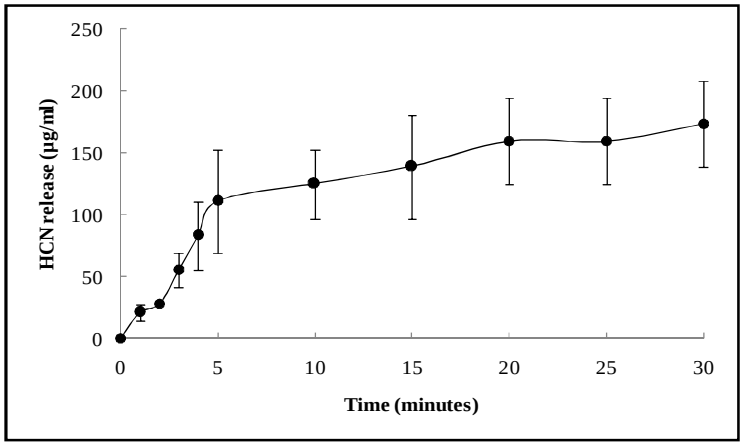

Figure 4: Hydrolysis of linamarin by crude linamarase (hydrolysis reaction was conducted at pH 6.0 and 50 °C)

4. Conclusion

This study concluded that cassava leaves have potential as a source of linamarase. Crude linamarase were successfully extracted from cassava leaves using phosphate buffer 0.2 M pH 6.0 and had activity 306.13 mU/mL with yield of 244.08 µg/g fresh leaf. Extraction of linamarin from young cassava leaves produced linamarin of 186.40 µg/mL with yield 338.5 µg/g leaf fresh weight. Linamarase had optimum pH and temperature of 5.5 and at 55°C respectively. This enzyme was stable at 40, 50, and 60°C for 240 minutes, but at 70°C the remaining enzyme activity was only 36%. The enzyme has the ability to hydrolyse linamarin and produce HCN. Hydrolysis of linamarin using crude linamarase resulted in the release of more than 90% HCN within 30 minutes' incubation at 50°C and pH 6.0. Further study is needed to examine the use of crude linamarase extracted from young cassava leaves as an exogenous enzyme to hydrolyze linamarin in cassava processing.

5. References

i. BeMiller, J. and Whistler, R. (2009). Starch: chemistry and technology. 3rd edn. New York: Academic Press.
ii. Bisswanger, H. (2014). Enzyme assays. Prespective in Science, 1, 41-55.
iii. Essers, A. J. A., Bennik, M. H. J. and Nout, M. J. R. (1995). Mechanisms of increased linamarin degradation during solid-substrate fermentation of cassava. World Journal of Microbiology and Biotechnology, 11, 266-270.
iv. Haque, M.R. and Bradbury, J. H. (2004). Preparation of linamarin from cassava leaves for use in a cassava cyanide kit. Food Chemistry, 85, 27-29.
v. McMahon, J.M., W.L.B. White, dan R.T. Sayre. (1995). Cyanogenesis in cassava (*Manihot esculenta* Crantz). Journal of Experimental Botany, 46 (288), 731-741
vi. Mkpong, O.E., Yan, H., Chism, G. and Sayre, R.T. (1990). Purification, characterization, and locatization of linamarase in cassava. Plant Physiology, 93, 176-181.
vii. Montacnag, J.A., Davis, C.R. and Tanumihardjo, S.A. (2009). Processing techniques to reduce toxicity and antinutrients of cassava for use as a staple food. Comprehensive Reviews in Food Science and Food Safety, 8, 17-27.
viii. Nambisan, B. and Sundaresan, S. (1994). Distribution of linamarin and its metabolizing enzymes in cassava tissues. Journal of Science, Food and Agriculture, 66, 503-507.
ix. Nambisan, B. (1999). Cassava latex as a source of linamarase for determination of linamarin. Journal of Agricultural Food Chemistry, 47, 372-373.
x. Nambisan, B. (2011). Strategies for elimination of cyanogens from cassava for reducing toxicity and improve food safety. Food and Chemical Toxicology, 49, 690-693.
xi. Nwokoro, O., and Anya, F.O. (2011). Linamarase enzyme from *Lactobacillus delbrueckii* NRL B-763: purification and some properties of a β-glucosidase. Journal of Mexican Chemical Society, 55(4), 246-250.
xii. Petruccioli, M.,Brimer, L., Cicalini, A.R.and Federici, F. (1999). The linamarase of *Mucor circinelloides* LU M40 and its detoxifying activity on cassava. Journal of Applied Microbiology, 86, 302-310.

xiii. Santana, M.A., Vasquez, V., Matehus, J. and Aldao, R.R. (2002). Linamarase expression in cassava cultivars with roots of low- and high-cyanide content. Plant Physiology, 129, 1686-1694.
xiv. Sornyotha, S., Kyu, K.L.and Ratanakhanokchai, K. (2007). Purification and detection of linamarin from cassava root cortex by high performance liquid chromatography. Food Chemistry, 104, 1750-1754.
xv. Sornyotha, S., Kyu, K.L. and Ratanakhanokchai, K. (2010). An efficient treatment for detoxification process of cassava starch by plant cell wall-degrading enzymes. Journal of Bioscience and Bioengineering, 109(1), 9-14.
xvi. Ugwuanyi, OJ. O., Harvey, L. M. and McNeil, B. (2007). Linamarase activities in *Bacillus* spp, responsible for thermopihilic aerobic digestion of agricultural waste for animal nutrition. Waste Management, 27, 1501-1508.
xvii. White, W.L.B., Arias-Garzon,D.I., McMahon,J.M. and Sayre,R.T. (1998). Cyanogenesis in cassava. Plant Physiology, 116, 1219-1225.
xviii. Williams H.J. and Edwards, T.G.(1980). Estimation of cyanide with alkaline picrate. Journal of Science, Food and Agriculture, 31, 15-22.
xix. Yeoh, H. H., Tan, T.K. and Loh, C.M. (1995). Sources of fungal linamarase. World Journal of Microbiology and Biotechnology, 11, 678-680.