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Optimization of Linamarase Production by *Priestia Flexa* for Detoxification of Cyanide in Cassava

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ABSTRACT

Box- Behnken design method under Response Surface Methodology was used for the design of the experiment for the optimization of cultural parameters for the production of linamarase by *Priestia flexa* using cassava as the substrate. The conditions optimized were pH, temperature, moisture content, period of fermentation and inoculum size. In many parts of the world, cassava (*Manihot esculenta*) is one of the most widely cultivated crops and is an important source of carbohydrates. However, it can be toxic when consumed in large amounts due to its cyanogenic glycoside content. The goal of this research is to assess the optimum conditions for linamarase production by *Priestia flexa* as a way to reduce the cyanide content of cassava. The results of the study showed that linamarase production was significant at the pH 6, temperature of 50 ºC, moisture content of 64.4 %, fermentation time of 24 hours and inoculum size of 20 %. Linamarase concentration (40.5 µg/g) was produced by *Priestia flexa* at these optimum conditions. The experimental linamarase concentration (40.5 µg/g) was lower than the concentration of 45.1 µg/g predicted by the design. In conclusion, this research demonstrates the potential of linamarase production by *Priestia flexa* for cyanide detoxification of cassava.

Key words: Cassava, cyanide, fermentation, inoculum size, linamarase, moisture content, optimization, pH, *Priestia flexa*, temperature.

1.0 INTRODUCTION

1.1 Background Information

Cassava (*Manihot esculenta*) is the third largest source of carbohydrates food in the tropics, after rice and maize. It is also called mandioca, manioc, yuca, or tapioca. It is a major staple root crop in many tropical and subtropical developing countries, especially in West Africa. Grown in more than 90 countries, it ranks as the 6th most important source of energy in human diets on a worldwide basis and as the 4th supplier of energy after rice, sugar, and corn/maize (Heuberger, 2005). Researchers have developed several processing methods for cassava with the aim of reducing their toxicity and at the same time convert the highly perishable roots to products which can be regarded as being more stable products. Fermentation, sun drying, soaking and followed by drying or roasting have been reported as the processes (Irtwange & Achimba, 2009). The two distinct types of cassava are the sweet cassava (*Manihot dulcis*) and the bitter cassava (*Manihot esculenta*). The bitter cassava is associated with high level of cyanogenic glycoside. The sweet cassava is considered as not having much cyanide. In local classification of cassava, some varieties are regarded as "sweet" (i.e., non-poisonous). This has resulted to the complacency among consumers to apply the simple treatments in order to reduce cyanide levels in tubers before they are consumed. Therefore, lack of awareness of potential dangers of cyanide poisoning is the reason why raw cassava tubers are consumed (Cornelius, Robert, Gaymary, James & Sakurani, 2019). This is because research has shown that in certain regions particularly in East Africa even those cassava cultivars which have been considered are a source of disaster to humans (Mburu, Njue & Sauda, 2011). Therefore according to Osuntokun, (1994) long-term consumption of small amounts of cyanide can cause severe health problems such as tropical neuropathy. Alitubeera, Eyu, Benon, Alex & Bao-Ping (2019) reported an out break of cyanide poisoning involving 98 persons in Uganda in 2017 in which two death cases occurred. Insufficient processing can also result to high cyanide exposure and this causes severe diseases such as *konzo* (Jorgensen, Bak, Busk, Sorensen, Olsen, Puonti-Kaerlas & Moller**,** 2005). The presence of this anti- nutrient, linamarin is reduced through hydrolysis by linamarase in the cassava. Several processing methods have been adopted to reduce the toxicity of cassava roots and simultaneously transform the highly perishable roots to more stable products. These include sun drying, soaking and fermentation followed by drying or roasting (Irtwange & Achimba, 2009). Cassava cultivars with a low cyanide potential have been generated by traditional breeders, but they have not succeeded in providing cassava cultivars totally devoid of cyanogenic glycosides (Ngudi, Kuo & Lambien, 2003). In cassava, the major cyanogenic glycoside is linamarin. Also present in a small amount is lotaustralin (methyl linamarin). The enzyme linamarase is also present. Linamarin is catalyzed by linamarase which rapidly hydrolyses it to glucose and acetone cyanohydrin. It also hydrolyses lotaustralin to a related cyanohydrin and glucose. Acetone cyanohydrin decomposes to acetone and hydrogen cyanide under neutral conditions (Food Standards Australia New Zealand, 2005). In some tropical countries where cassava is consumed a major staple food product, it is difficult to assay the quantity of the cyanide in cassava because the facilities needed to perform the assay procedure are not readily available and getting an accurate method of analysis is another area of difficulty.

Linamarase enzyme is specific for the degradation of cyanogenic glycosides. It works best at its optimum pH and temperature. The microorganisms that produce linamarase require adequate amount of moisture to produce sufficient quantity of the enzyme. For a food product to be acceptable, it is expected be nutritious, wholesome, pure and safe (Irtwange & Achimba, 2009). Furthermore, the amount of microorganisms present in the substrate determines the rate of production of the cyanogenic glycosidase. The duration of fermentation is also very important because if it is too short, it leads to incomplete detoxification process. This will result in a product that is potentially toxic to consumers. On the other hand, if the duration of fermentation is too long, the acidity will increase and the result is that the product will have a strong sour taste and it will possess a poor texture (Azam-Ali, Judge, Fellows & Battcock**,** 2003). For the maximum production of this enzyme and its activity, certain conditions must be optimized such as temperature and pH, moisture and inoculum size and duration of fermentation . When the linamarase level is high, cassava can be adequately detoxified thereby making the product less harmful and more useful.

MATERIALS AND METHODS

Sample collection

Cassava samples (TMS-9800581) were randomly bought from farms in Port Harcourt. The tubers were peeled and then washed to obtain the parenchyma. The parenchyma were cut into several portions, which were grinded using a grinding machine to obtain ground parenchyma tissue. At the laboratory, the grinded tubers were preserved in a refrigerator (at 4^{O}C) until analysis. The blended samples were divided into several portions and each portion was kept in sterile containers and stored in the refrigerator throughout the analysis.

The microorganism, *Priestia flexa,* was obtained from previous researches.

Experimental design

A Box-Behnken Design method under Response Surface Methodology was used for the experimental design, and the design was done using Minitab version 17.0. Three levels of pH (4, 5 and 6), three levels of moisture (60%, 70%, and 80%), three levels of inoculums size (10%, 15% and 20%), three levels of Temperature (4^0C, 27^0C and 50^0C) and three levels of fermentation period (24hrs, 48hrs and 72hrs) were substituted into the experimental design interface of the software.

Table 1. Box-Behnken Experimental Design

Factors	**Levels**		
	-1	0	1
pH	4	5	6
Moisture, %	60	70	80
Inoculum size, %	10	15	20
Temperature ^{0}C	4	27	50
Fermentation period, Hrs	24	48	72

Factors: 5 Replicates: 1

Base runs: 92 Total runs: 92 for two isolates

Base blocks: 1 Total blocks: 1

Center points: 6

Standardization of inoculum for fermentation

The pure isolates were standardized by growing in nutrient medium and diluting to obtain to obtain inoculum of $1.5x10^8$ cfu/ml. This inoculum has 0.5 McFarland standard. The inoculum was thereafter used to inoculate the cassava pulp for fermentation purpose.

Extraction of Fermented Cassava Fluid

Five grammes of fermented cassava were dissolved in 50 mls of distilled water. The mixture was allowed for one hour and was then filtered using Whatman number one filter paper. The filtrate was used for enzyme extraction (Onwuka, 2018).

Enzyme Extraction From the Filtrate

The minimal medium for the assay consists of NaCl 0.3%; $(NH_4)_2SO_4$ 0.1%; KH_2PO_4 0.05%; $MgSO_4$ 0.02%; Lactose 2% and $CaCl_2$ 0.02%. One hundred milliliters of the minimal medium in a 250ml Erlenmeyer flask was sterilized. Ten milliliters of the filtrate was transferred into the minimal medium and centrifuged at 2500rpm for 5 minutes. The supernatant obtained was used for enzyme assay (Ilesanmi et al., 2020).

Determination of Enzyme Concentration

Linamarase activity assay was performed by determining the HCN liberated from KCN as follows: 0.5ml of enzyme solution in phosphate buffer (6.5) in screw cap tubes was added to 0.5ml of potassium cyanide (KCN) and incubated at 28°C for 20 minutes. Two milliliters of 2% potassium hydroxide (KOH) and 1 ml of sodium alkaline picrate were added to the reaction mixture. The red colour developed was read at 540nm in a spectrophotometer (model BC300, China) under the above conditions. One unit of linamarase activity was defined as the amount of the enzyme that released 1µg HCN under assay conditions (Ilesanmi et al., 2020).

I**nfluence of different pH on Linamarase Production by Microorganisms**

Effect of pH on linamarase production by microorganisms was investigated by adjusting the pH of cassava to 4.0, 5.0 and 6.0 using hydrochloric acid (HCl) and potassium hydroxide (KOH) (Nwokoro & Onyebuchi, 2011). The different values of pH was investigated with different values of temperature, moisture content, inoculum sizes and fermentation periods according to Box- Behnken design. Extraction of fermented cassava fluid, enzyme extraction from the filtrate and determination of enzyme concentration were performed as described above (Onwuka, 2018; Ilesanmi et al., 2020).

Influence of Different Temperatures on Linamarase Production by Microorganisms

Effect of temperature on linamarase production by microorganisms was investigated as follows: The isolate that gave OD readings ≥0.4 after 2 days of incubation during the screening test was used to inoculate the cassava samples in containers. The samples were incubated separately at 4 ºC, 27 ºC, and 50 ºC. The different values of temperature was investigated with different values of pH, moisture content, inoculum sizes and fermentation periods according to Box- Behnken design. Extraction of fermented cassava fluid, enzyme extraction from the filtrate and determination of enzyme concentration were performed as described above (Onwuka, 2018; Ilesanmi et al., 2020).

Determination of the Effect of Moisture Content on Linamarase Production by Microorganisms

The moisture content of cassava samples were determined by oven drying method described by Chryssoua et al. (2018). The cassava samples were dried in the oven to a constant mass and the moisture content determined using the equation

$$\%H_20 = \frac{(M_0\text{-}M_1)}{M_0} X100$$

Where M_0 is the mass in grams of the cassava paste before oven drying and M_1 is the mass in grams of the cassava paste after oven drying and the moisture content of the cassava paste was 60%. Then the moisture content of cassava samples were adjusted to 70% and 80%. The inoculum of the isolates that gave optical density readings equal or greater than 0.4 (≥0.4) after 2 days of incubation during the screening test were used to inoculate the cassava samples in containers. The different values of moisture content were investigated with different values of pH, temperature, inoculum sizes and fermentation periods according to Box- Behnken design. Extraction of fermented cassava fluid, enzyme extraction from the filtrate and determination of enzyme concentration were performed as described above (Onwuka, 2018; Ilesanmi et al., 2020).

Determination of the Effect of Inoculum Size on Linamarase Production by Microorganisms

The effect of inoculum size on linamarase production by microorganisms was studied by inoculating cassava samples with inoculum sizes of 10% (i.e 10 mls of inoculum in 100 g of cassava pulp), 15% (i.e 15 mls of inoculum in 100 g of cassava pulp) and 20% (i.e 20mls of inoculum in 100g of cassava pulp) separately. The inoculum of the isolates that gave optical density readings equal or greater than 0.4 (≥0.4) after 2 days of incubation during the screening test were used to inoculate the cassava samples in containers. The different values of inoculum sizes were investigated with different values of pH, temperature, moisture content and fermentation periods according to Box- Behnken design. Extraction of fermented cassava fluid, enzyme extraction from the filtrate and determination of enzyme concentration were performed as described above (Onwuka, 2018; Ilesanmi et al., 2020).

Determination of the Effect of incubation or fermentation period on Linamarase Production by Microorganisms

The effect of incubation or fermentation period linamarase production by microorganisms was determined by incubating cassava samples inoculated with inoculum of the isolates that gave optical density readings equal or greater than 0.4 (≥0.4) after 2 days of incubation during the screening test. The different values of fermentation periods were investigated with different values of pH, temperature, moisture content and inoculum sizes according to Box- Behnken design. Extraction of fermented cassava fluid, enzyme extraction from the filtrate and determination of enzyme concentration were performed as described above (Onwuka, 2018; Ilesanmi et al., 2020).

Enzyme (Linamarase) purification

Enzyme supernatant fluid was brought to 40% saturation by adding 22.9g of solid $(NH_4)_2SO_4$ to 100 ml of the crude enzyme fluid .This was followed by centrifugation at 2515xg for 15mins .Acetone 40%v/v was added with further centrifugation. The supernatant was applied to carboxylmethyl cellulose (CMC) column equilibrated with 0.2m phosphate buffer and eluted with a linear NaCL gradient (0.8m). Fractions were collected and precipitated with cold acetone. The precipitate was collected by centrifugation and re-suspended in the same buffer (Ogbonanya & Onyebuchi, 2011)

RESULTS AND DISCUSSION

Table 2 shows the linamarase concentration from *Priestia flexa* for different experimental runs involving the following parameters namely pH, moisture content (%), inoculum size (%), temperature (ºC) and period of fermentation (hours). Run 14 had the maximum concentration of 50.0 µg/g at pH 6, moisture content of 70 %, inoculum size of 15 %, temperature of 50 ºC and period of fermentation of 48 hours while run 6 had the least linamarase concentration of 0.0 µg/g at pH 5, moisture content of 60 %, inoculum size of 15 %, temperature of 4ºC and period of fermentation of 24 hours. In table 3 the low value of S (7.65) and R^2 value of 57.7% also suggest the data fits the model. There was interaction between different variables but the analysis of variance showed that the interactions were not significant ($p>0.05$). Each factor or variable affected the production of linamarase but only fermentation time had significant effect on the concentration of linamarase produced with *Priestia flexa* ($p<0.05$). The application of *Priestia flexa* in other areas of biotecnology has been reported. It has been revealed as a polyhydroxybutyrate (PHB) synthesizing bacterial species found in the mangrove ecosystem. It is used to develop eco-friendly and biodegradable plastics because biopolymers have been thoroughly researched as a potential replacement for plastics. The medium optimized with glucose demonstrated increased cell growth as well as maximum PHB yield (Chathalingath, Kingsley & Gunasekar, 2023). Yet no report has been given about its application in the production of cassava cyanogenic glycosidase (linamarase). The result of this research has revealed that when proper cultural conditions such pH, moisture content (%), inoculum size (%), temperature (ºC) and period of fermentation (hours) are created, *Priestia flexa* has the ability to release linamarase for the breakdown of cyanogenic glycosides such as linamarin. The major contribution of food fermentation is the release of the enzyme linamarase from plants tissues. This enzyme is involved in the breakdown of the linamarin and lotaustralin (cyanogenic glycosides) of cassava, which releases hydrogen cyanide and thus detoxifies the product. Awua et al. (1997) in a research made an observation of which revealed that all yeasts and moulds identified in traditional cassava dough inocula exhibited linamarase activities and were therefore capable of degrading cyanogenic glycosides. Several microorganisms have been reported to possess the abilty to produce linamarase. For instance, Guyot et al. (1998) reported that *L. plantarum* lowers the HCN content of cassava because of its ability to produce linamarase which can hydrolyze linamarin (a cyanogenic glucoside). Ahaotu, Ogueke, Owuamanam, Ahaotu & Nwosu (2011) reported that *Alcaligenes faecalis*, *Lactobacillus plantarum*, *Bacillus subtilis* and *Leuconostoc cremoris* were the microorganisms isolated from cassava waste water. Among them, only *Lactobacillus plantarum* and *Leuconostoc cremoris* had linamarase activity observed for them. Howevever, the linamarase production potential of the microorganism, *Priestia flexa* has not been reported.

Table 2: Linamarase concentration from *Priestia flexa* for different experimental runs

Runs Order	pH	Moisture content (%)	Inoculum size (%)	Temperature (ºC)	Period of fermentation (hours)	Mean Linamarase concentration for *Priestia flexa* (µg/g)
1	5	70	10	27	72	8.5
2	5	60	20	27	48	4.8
3	5	60	20	27	24	11.2
4	6	60	15	27	48	2.0
5	5	70	20	4	48	2.5
6	5	60	15	4	48	0.0
7	4	70	15	50	48	8.5
8	5	80	10	27	48	2.5
9	4	80	15	27	48	5.3
10	5	70	15	27	48	0.7
11	5	70	15	4	24	18.5
12	5	60	10	27	48	3.8
13	5	60	15	27	72	8.5
14	6	70	15	50	48	50.0
15	4	70	15	27	72	6.3
16	5	70	15	27	48	5.0
17	6	70	20	27	48	6.3
18	6	70	15	27	72	7.0

19	5	70	20	50	48	14.5
20	5	70	10	50	48	3.8
21	5	70	15	27	48	0.7
22	5	70	20	27	24	25.5
23	5	80	15	50	48	7.8
24	5	70	15	27	48	1.5
25	5	70	4	4	48	6.0
26	6	70	10	27	48	4.5
27	4	60	15	27	48	3.8
28	5	80	15	4	48	6.3
29	5	80	15	27	24	25.5
30	5	70	15	27	48	13.0
31	5	80	15	27	72	6.3
32	5	70	15	27	48	4.3
33	6	70	15	27	24	21.0
34	5	70	20	27	72	6.5
35	5	70	20	27	72	8.5
36	5	70	10	27	24	14.0
37	5	70	15	50	24	18.5
38	5	70	15	4	72	6.5
39	4	70	10	27	48	11.0
40	4	70	10	27	48	1.0
41	6	70	15	4	48	16.0
42	5	60	15	50	48	1.0
43	4	70	15	27	24	12.0
44	5	80	20	27	48	8.5
45	4	70	20	27	48	6.0
46	6	80	15	27	48	6.5

TABLE 3 Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
7.65118	57.78%	24.00%	0.00%

Interaction Between Variables on The Enzyme Production by *Priestia flexa*

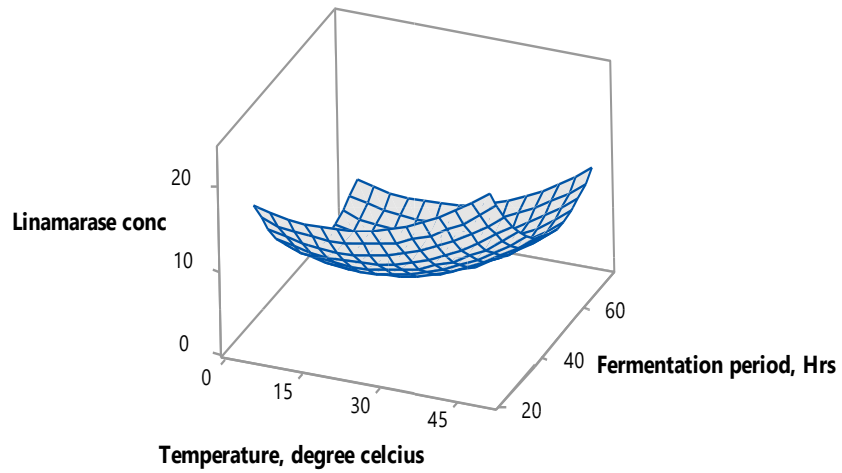

Fig 1: Surface Plot of Linamarase concentration for *Priestia flexa* vs Fermentation period and temperature

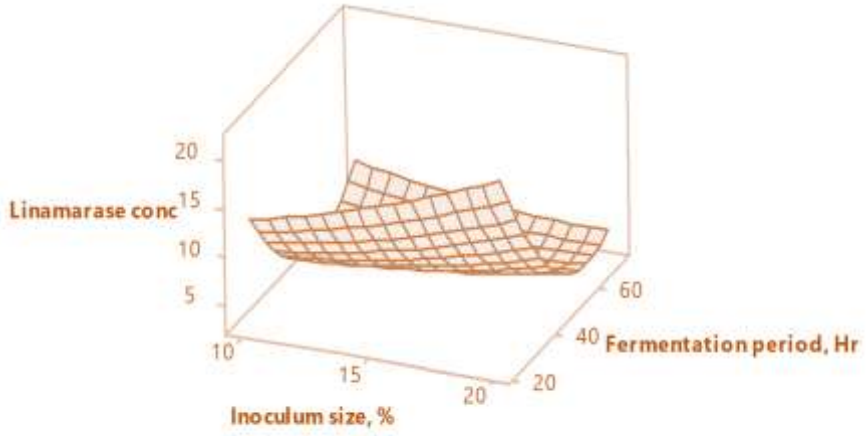

Fig 2: Surface Plot of Linamarase concentration for *Priestia flexa* vs Fermentation period and inoculums size

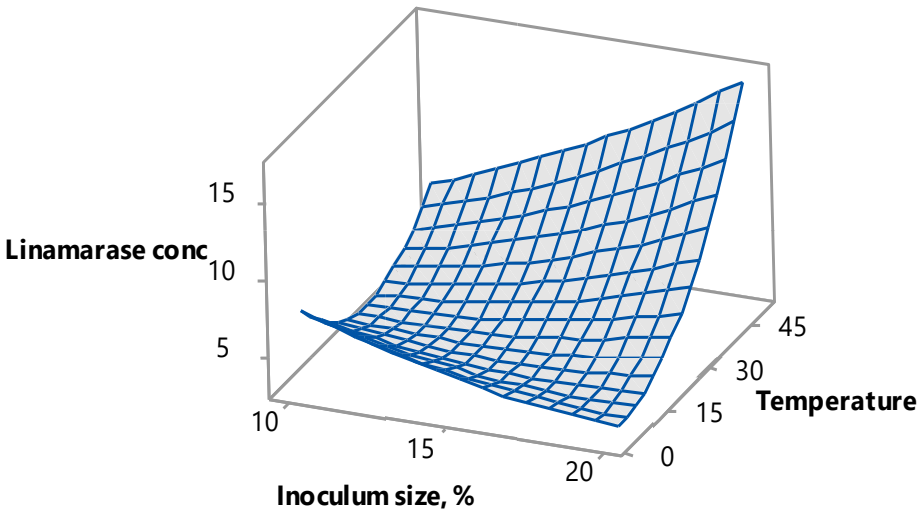

Fig 3: Surface Plot of Linamarase concentration for *Priestia flexa* vs temperature and inoculum size

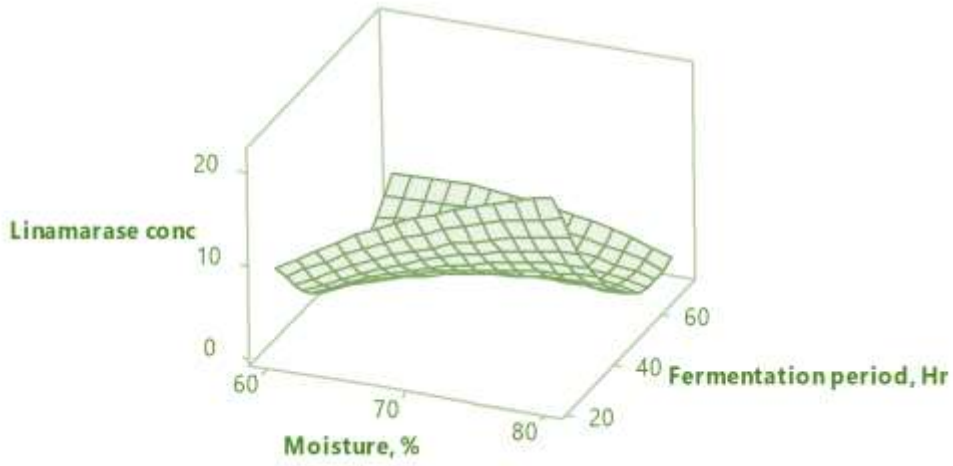

Fig 4: Surface Plot of Linamarase concentration for *Priestia flexa* vs fermentation period and moisture

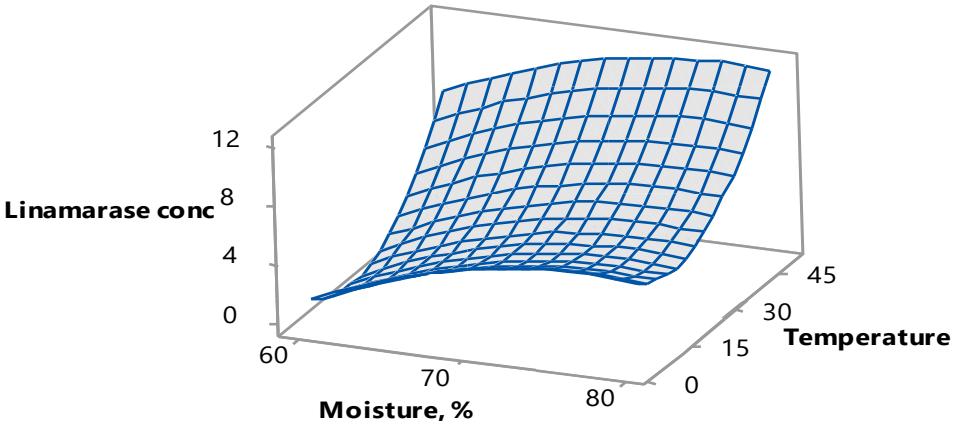

Fig 5: Surface Plot of Linamarase concentration for *Priestia flexa* vs temperature and moisture

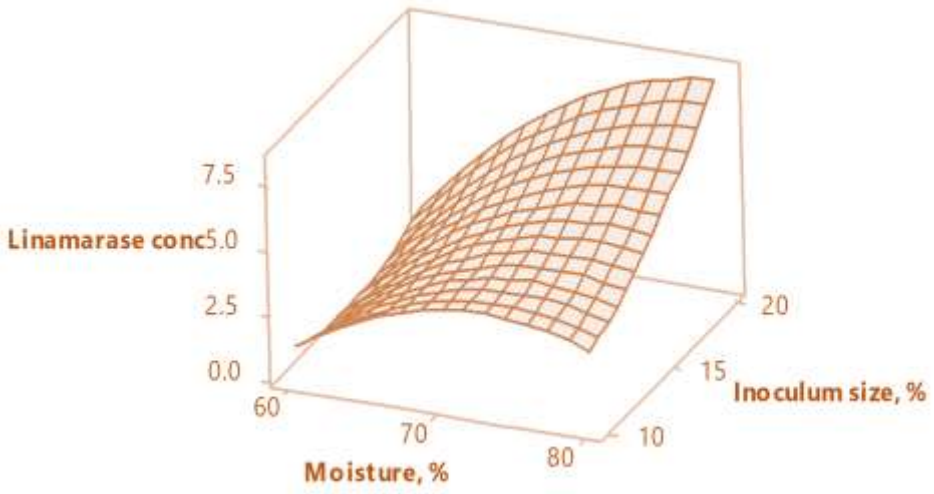

Fig 6: Surface Plot of Linamarase concentration for *Priestia flexa* vs inoculums size and moisture

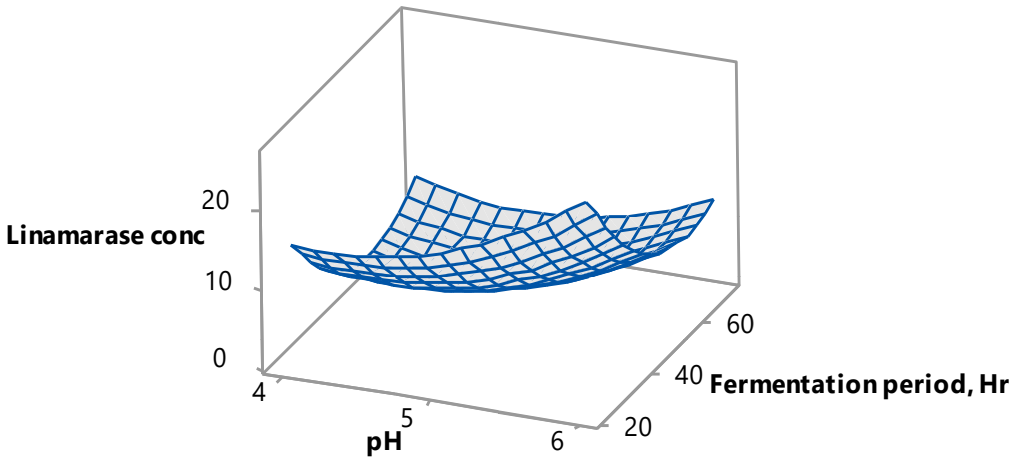

Fig 7: Surface Plot of Linamarase concentration for *Priestia flexa* vs Fermentation period and pH

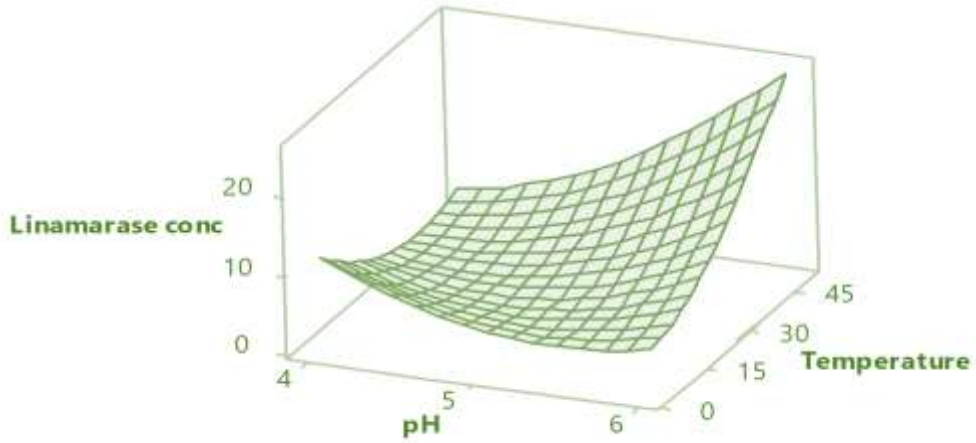

Fig 8: Surface Plot of Linamarase concentration for *Priestia flexa* vs Temperature and pH

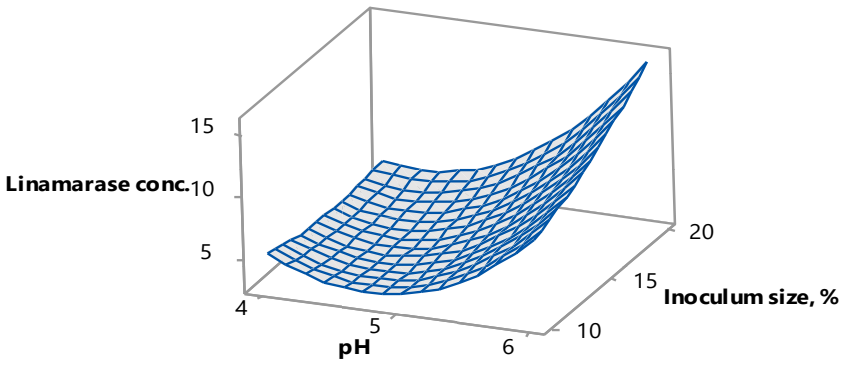

Fig 9: Surface Plot of Linamarase concentration for *Priestia flexa* vs Inoculum size and pH

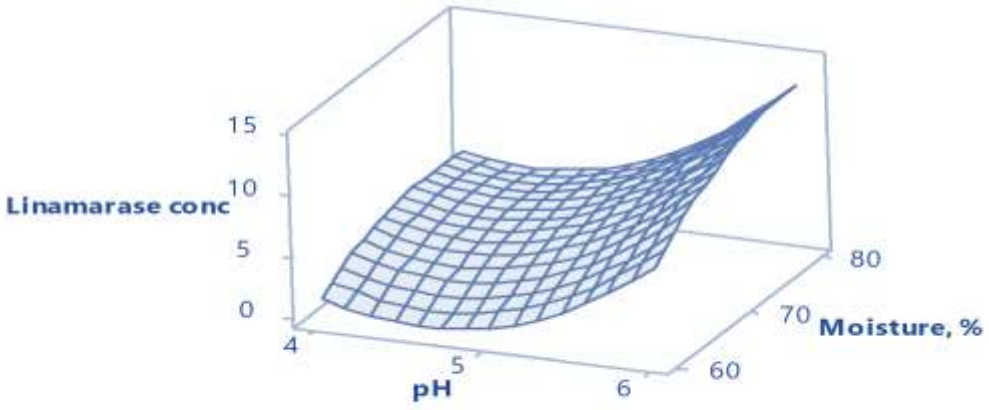

Fig 10: Surface Plot of Linamarase concentration for *Priestia flexa* vs Moisture, %, pH

The optimization plot with goal to predict the maximum enzyme production is shown in figure 4.23. It revealed that at optimum conditions of 64.4 % moisture, 20.0 % inoculums size, 6.0 pH, 24 hrs fermentation time and 50 ºC the highest concentration of enzyme is produced as shown in figure 11. Table 4 shows the comparison of the predicted linamarase concentration with the experimental concentration. It was revealed that the experimental concentration 40.5 µg/g was lower than the predicted linamarase concentration of 45.1 µg/g.

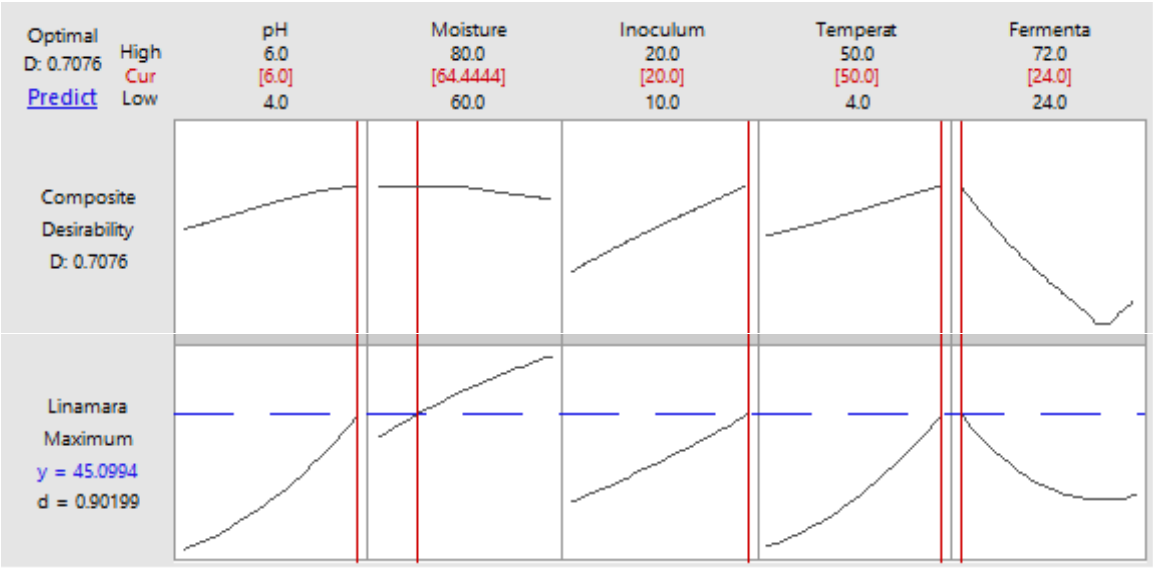

Fig. 11: Response Optimization Plot of Linamarase Enzyme Concentration for *Priestia flexa*.

Table 4 Comparison of predicted optimum value and experimental value for linamarase production by *Priestia flexa*.

pH	Moisture content (%)	Inoculum Size (%)	Temperature (ºC)	Period of fermentation (Hours)	Predicted linamarase	Experimental linamarase (µg/g)
6	64.4	20	50	24	45.1	40.5

Conclusion

From this present research work, linamarase production by *Priestia flexa*.has been optimized by adjusting fermentation conditions such as temperature, pH, moisture content, and inoculum size and fermentation period. The optimum conditions for the production of linamarase and breakdown of linamarin are pH 6, 50 ºC temperature, 64.4% moisture content, 20 % inoculum size and 24 hours. It was revealed that at optimum conditions, *Priestia flexa* has the ability to produce high amount of linamarase which leads to significant breakdown cyanogenic glycosides in cassava. This will make cassava and its products to be more useful in food industry and to be safer for consumers.

References

Ahaotu, I., Ogueke, C. C., Owuamanam, C. I., Ahaotu, N. N. & Nwosu, J. N. (2011). Use of bacteria that produce linamarase in cassava processing for gari. *Nig. J. of food, 29(2) 6.*

Albert, L., Klanarong, S. & Tzou-chi, H. (2005). Proximate composition, mineral contents, hydrogen cyanide and phytic acid of 5 cassava genotypes. *Food chem., 92 (4), 615-620.*

Alitubeera, P., Eyu, P., Benon K., Alex R. & Bao-Ping, Z. (2019). Morbidity and Mortality Weekly Report (MMWR). *Centers for Disease Control and Prevention, 68(13), 308–31.*

Azam-Ali, S., Judge, E., Fellows P., & Battcock, M., (2003). Small-scale food processing. A directory of equipment and methods. *ITDG Publishing. 2, 256.*

Chathalingath, N., Kingsley, J. S. & Gunasekar A. (2023). Biosynthesis and biodegradation of poly (3-hydroxybutyrate) from *Priestia flexa*; A promising mangrove halophyte towards the development of sustainable eco-friendly bioplastics.*Microbiological Research 267, 5129–5135*

Chryssoua, K., Stassinopouloua, M., & Lampia, E. (2018). Calculation of the Expanded Uncertainty of the Measurements of the Moisture Content of a Lot of Paper Samples Oven Dried at 105°C. *Res. & Rev: J. of Chem. 7(3), 1-13.*

Cornelius B. M., Robert A. M., Gaymary G. B., James R. M. and Sakurani T. B. (2019). Cassava Varieties and People's Perception on Cyanide Poisoning in Selected Regions of Tanzania. *J. of Agric. Stud. 7(4), 180*

Food Standards Australia New Zealand (FSANZ). (2005). Cyanogenic glycosides in cassava and bamboo shoots a human health risk assessment. *Technical Report Series. 28, 6-18.*

Guyot, J. M. (1998). Lactobacillus manihotivorans species. nov, a new starch-hydrolysing lactic acid bacterium isolated during cassava sour starch fermentation. International Journal of System Bacteriology 48: 1101-1109.

Heuberger, C. (2005). Cyanide content of cassava and fermented products with focus on attiéké and attiéké garba. *Dissertation submitted to the Swiss Federal Institute of Technology, Zurich, for the degree of Doctor of Nat. Sci. No. 16247.*

Ilesanmi, F. F., Nafsat, K. B., & Olumide, S. F. (2020). Efect of cultural conditions on the growth and linamarase production by a local species of Lactobacillus fermentum isolated from cassava efuent. *Bull Natl Res Cent. 44,185*

Irtwange, S. & Achimba, O. (2009). Effect of the Duration of Fermentation on the Quality of Gari. *Current Research Journal of Biological Sciences 1(3), 150-154.*

Jorgensen, K., Bak, S., Busk, P., Sorensen, C., Olsen, C., Puonti-Kaerlas, J. & Moller, B. (2005). Cassava plants with a depleted cyanogenic glucoside content in leaves and tubers. *Plant*

Mburu, F., Njue, W., Sauda, S. (2011). Fatal cassava food poisoning in Eastern Province. *Kenya Cassava Cyanide Disease and Neurolathyrism Network (CCDN), 18, 1-2.*

Ngudi, D., Kuo, Y. & Lambien, F. (2003).Cassava cyanogens and free amino acids in raw and cooked leaves. *Food Chem.Toxicol. 41, 1193-1197.*

Nwokoro O. & Onyebuchi A. (2011). Linamarase Enzyme from Lactobacillus delbrueckii NRRL B-763: Purifcation and Some Properties of a β-Glucosidase. *J. Mex Chem Soc 55(4):246–250*

Onwuka, G. (2018). Food analysis and instrumentation. *Department of Food Science and Technology, Michael Opara University of Agriculture, Umudike 2, 370-380.*

Osuntokun, B. (1994). Chronic cyanide intoxication of dietary origin and a degenerative neuropathy in Nigerians. *Acta Hortic. 375, 311– 21.*