



Isolation and screening of bacteria associated with fermented cassava peels for linamarase production

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ABSTRACT

This study was aimed at isolating bacteria associated with fermented cassava peels as well as screening the isolated bacteria for linamarase production. Primary screening for linamarase production by bacterial isolates was determined on modified de Mann Rogosa and Sharpe agar (MRS). Bacteria were isolated on MRS incubated at room temperature for 72 h and identified by standard microbiological methods. Screening of bacteria for linamarase activity was determined on MRS medium inside the test tube while the enzyme activities were determined spectrophotometrically. Six of the bacteria isolated from the fermented cassava peels were identified as *Lactobacillus plantarum*, *Bacillus subtilis*, *Bacillus megatarum*, *Lactobacillus fermentum*, *Lactobacillus bulgaricus*, *Lactobacillus casei* and *Lactobacillus delbrueckii*. The screened bacteria produced linamarase on MRS containing paranitrophenyl β -D glucosides (1%) indicated by development of black spot around the perimeter on injection. The bacterial isolates were grown under different cultural conditions for linamarase production. The highest linamarase activities observed was 94.01 U/ml from *L. plantarum*. Linamarase activities observed were between 8.01 and 94.01 U/ml, respectively. The optimum pH and temperature for linamarase production was obtained at 45°C and pH 7.0 while the optimum inoculum concentration was at 0.8%, respectively. This study shows the ability of bacterial isolates (from cassava peel) to produce linamarase that can be explored in food industry.

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INTRODUCTION

Linamarase (EC 3.2.1.21) is a linamarin degrading enzyme that breaks down the glycolytic bond between the chiral carbons attached to the nitrile group of linamarin structure into acetocyanohydrin. Linamarase an enzyme found in many plants including cassava and in microorganisms converts the cyanide containing compounds into acetone cyanohydrins, which spontaneously decomposes to hydrogen cyanide (Kasi et al., 2012). The secretion of extracellular linamarase during fermentation of cassava peels could be responsible for the hydrolysis of linamarin that present in cassava or cassava peels. The maceration or cutting of cassava peels increased the

surface area of the substrates bringing in close contact between linamarin and linamarase, which could enhance the further degradation of linamarin into acetone and hydrogen cyanide which are easily soluble in water (Ogbonnaya and Florence, 2011). Linamarin can only be broken under high pressure, high temperature and use of mineral acids, while its enzymatic break occurs easily (Cereda and Mattos, 1996). Hence, removal or conversion of these antinutrient cyanogenic glycosides would be possible through target specific enzymatic conversion if the methods of application are standardized. The degradation of linamarin by linamarase produced from cassava itself could be more efficient on the inoculation of the cassava peels by the microorganisms involved in its fermentation that are capable of secreting extracellular linamarase (Ogbonnaya and Florence, 2011).

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Table 1. The parameters used for optimization studies.

Parameters	Conditions/Range
Temperature	4, 23, 37, 45, 50, 60, 70, 80 and 90°C
pH	3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0
Substrate concentration	0.5, 1.0, 2.0, 2.2.0, 3.0, 3.5, 4.0, 4.5, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0 g
Inoculum concentration	0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 µl

Linamarase could be of importance in food processing industry, agricultural sector and in waste management. Therefore, this present study focus on the optimization of process parameters for the production of linamarase from bacterial associated with the fermentation of cassava peels.

MATERIALS AND METHODS

Source of samples

Fresh cassava peels obtained from cassava tuber were collected from Teaching and Research Farm, the Federal University of Technology, Akure, Nigeria.

Isolation of microorganisms from cassava peels

Pour plate technique was used for the isolation of *L. plantarum* from the fermented cassava peels using MRS under anaerobic condition. The well grown bacterial isolates were purified to obtain pure culture by repeated streaking on the bacteriological medium. The pure cultures on slants were stored in the refrigerator at 4°C. Different biochemical tests were carried out and the isolates were identified using Bergey's Manual of Determinative Bacteriology.

Linamarase production and assay

Production of linamarase enzyme

The bacterial isolates were screened for linamarase production under solid state fermentation. Enzyme production was performed in 250 ml Erlenmeyer flask containing 1000 ml of enzyme producing medium. The basal cassava mineral salt medium (g/L) for linamarase production contain: 22% cassava peel flour, 0.2% MgSO₄.7H₂O, 0.2% CaCl₂, 0.2% NH₄NO₃, 1% KH₂PO₄ and 1% inoculums, the medium was adjusted to pH 6.8 using NaOH (Ogundu et al., 2014). The flasks were incubated at 37°C for 24 h on a rotary shaker at 200 rpm. The crude enzyme was prepared by adding 10-fold (v/w) 0.1 M phosphate buffer (pH 6.8) and shaking (180 rpm)

at 37°C for 15 min. The solid materials and bacterial cells were separated by centrifugation at 6000 rpm for 15 min at 4°C. The clear supernatant was used for enzyme assays and soluble protein determination.

Assay of linamarase enzyme

Linamarase assay was carried out according to the method of Ugwuanyi et al. (2007). The reaction mixture contained 1 ml of (1%) para nitrophenyl β-D glucoside prepared in 0.1 M potassium sulphate pH 6.0 and 1 ml of supernatant. The control and experimented tube were incubated at 37°C for 15 min. Both the experimental and control tubes were removed from the water bath and the reaction was terminated by addition of 2 ml of borate buffer per tube (pH 9.0). The tubes were observed for colour development and were cooled rapidly. The optical density was measured at 540 nm. One unit of linamarase activity was defined as amount of enzyme producing 1 micromole of p-nitrophenol per minute under the experiment conditions.

Effect of incubation time, incubation temperature, pH, substrate and inoculum concentrations

The solid state fermentation (SSF) experiment was carried out to determine the incubation time (day) required for the optimum linamarase production. The flask containing the mixture of fermentation media, cells and solid substrate was incubated at 37°C. The linamarase enzyme was extracted every hour for one and half day (33 h). The optimum time of incubation was then applied to study the effect of incubation temperature, pH, substrate and inoculum concentrations.

The different incubation temperatures, pH, substrate and inoculum concentrations were the parameters to be optimized and their concentrations and range are listed in Table 1.

Protein determination

The amount of protein liberated in the fermentation media was evaluated according to the method designed by

Table 2. Screening of bacteria strains of *L. plantarum* for linamarase production in submerged state fermentation.

Isolate	Enzyme activity (mg/ml)	Protein content ($\mu\text{mol/mg}$)
<i>L. plantarum</i>	94.01 ^g \pm 0.01	1.39 ^a \pm 0.00
<i>B. megatarum</i>	68.01 ^d \pm 0.02	11.11 ^d \pm 0.01
<i>L. fermentum</i>	62.01 ^c \pm 0.01	11.39 ^f \pm 0.01
<i>L. bulgaricus</i>	80.01 ^f \pm 0.02	11.58 ^g \pm 0.01
<i>B. subtilis</i>	73.00 ^e \pm 0.01	8.78 ^c \pm 0.01
<i>L. delbrueckii</i>	8.01 ^a \pm 0.02	11.30 ^e \pm 0.01
<i>L. casei</i>	18.00 ^b \pm 0.01	8.66 ^b \pm 0.01

Means of the same superscript letters along the same column are not significantly different ($p < 0.05$).

Lowry et al. (1951) using Bovine Serum Albumin as a standard.

RESULTS AND DISCUSSION

Screening of bacterial isolates for linamarase production

The production of linamarase by microorganisms in the fermentation media depend on the availability of suitable substrate and the utilization of the substrate by the microorganisms. All the different species of *Lactobacillus* and *Bacillus* sp. produce extracellular linamarase in the fermentation medium although with difference in their enzyme activity. *L. plantarum* produce the highest specific enzyme activity (67.63 ± 0.01) while lowest was recorded for *L. delbrueckii* (8.01 ± 0.02). The protein content ranged from 1.39 ± 0.00 to 11.58 ± 0.01 with the highest protein content lied on isolate *L. bulgaricus* (Table 2). The variation exhibited by the *L. plantarum* for linamarase production might be attributed to the source of isolation and slight variation in their genetic make-up. Variation in protein content generated by each isolates could be attributed to production of varieties of enzymes (amylases, xylanases and protease) aside the enzyme examined in this present study (Arotupin and Olaniyi, 2013). Linamarase activity has been reported in a variety of bacterial strains (Offiong et al., 1990) but few data are available on linamarase activity of *L. plantarum*.

Effect of incubation time on linamarase production

The time course for the production of linamarase by *L. plantarum* in the solid SSF process using cassava peels flour as the substrate is depicted in Figure 1. Linamarase activity increased during the growth phase of the culture and the optimum incubation time was reached at 22 h. α -Linamarase production declined after 22 h and reached

the minimum level after 33 h.

Effect of pH on linamarase production

The effect of initial pH (3.0-9.0) on the culture for the biosynthesis of linamarase by *L. plantarum* was studied (Figure 2). The linamarase activity increased with increase in pH until optimum activity 779 U/ml was reached at pH 7.0. Further increase beyond this results in reduction in enzyme activity. Bodade et al. (2010) had reported change in pH might result in morphological changes of the microorganisms, decrease in enzyme secretion thereby affecting the enzyme activity.

Effect of incubation temperature on linamarase production

The effect of incubation temperature for enzyme production could provide the information on whether the microorganism used is mesophilic or thermophilic. Figure 3 shows the highest enzyme activity of 726 U/ml at 45°C. The enzyme activity dropped considerably at 90°C due to enzyme denaturation which led to degradation of its activity. The result obtained from this study was lower to the finding of Offiong et al. (1990) and Yeoh et al. (1995) who reported 55 and 60°C optimum temperatures for linamarase production by *Penicillium funiculosum* and *Aspergillus nidulans*.

Effect of inoculum concentration on linamarase production

The effect of different inoculum concentration (0.1 to 1.0%) on linamarase biosynthesis was monitored (Figure 4). The maximum linamarase yield was observed when the cultured medium was provided with 0.8% inoculum.

An increase in inoculum size ensures increased

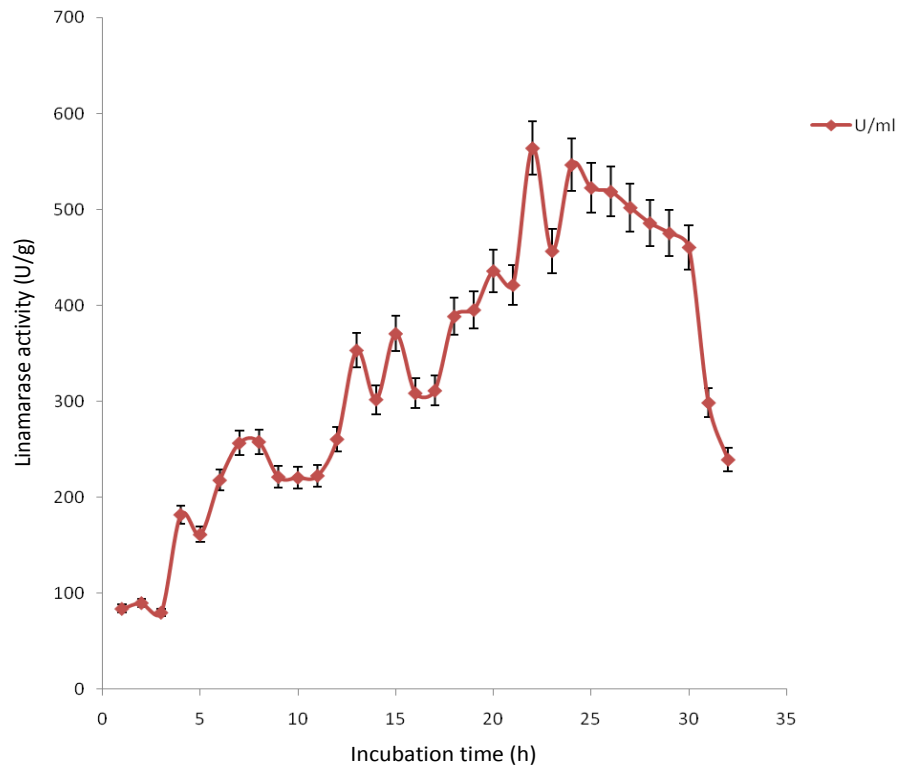


Figure 1. Effect of incubation time on linamarase production.

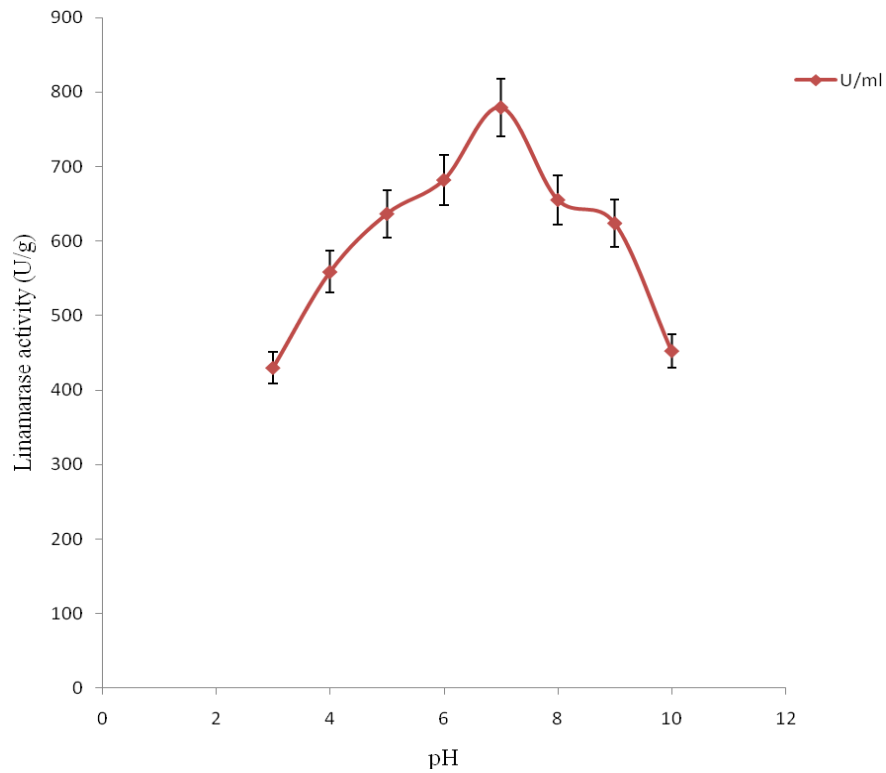


Figure 2. Effect of pH on linamarase production.

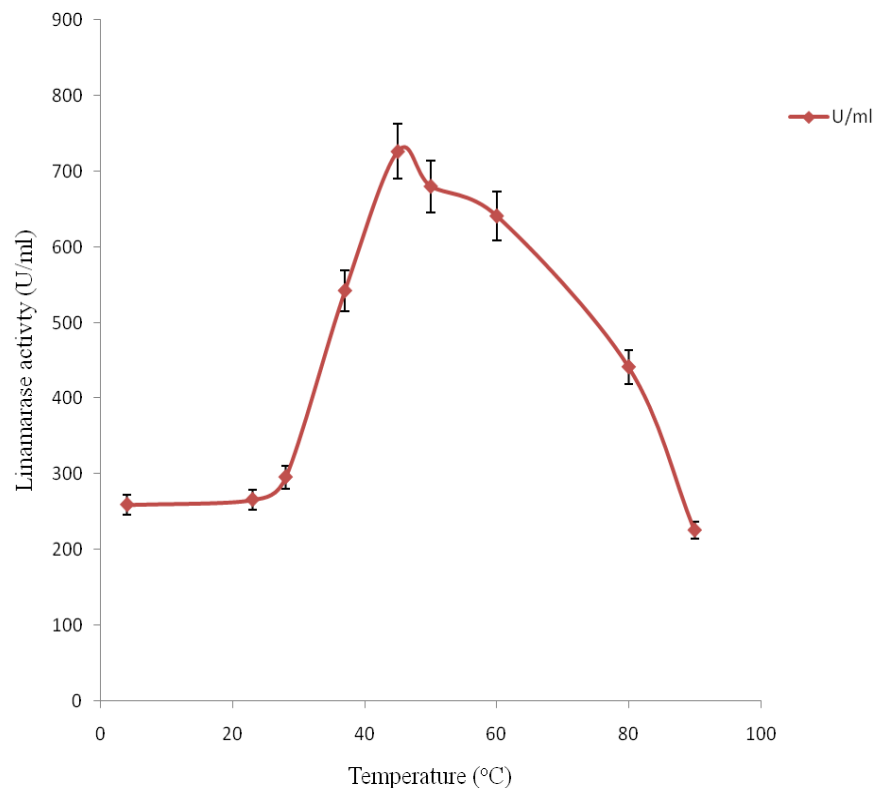


Figure 3. Effect of incubation temperatures on linamarase production.

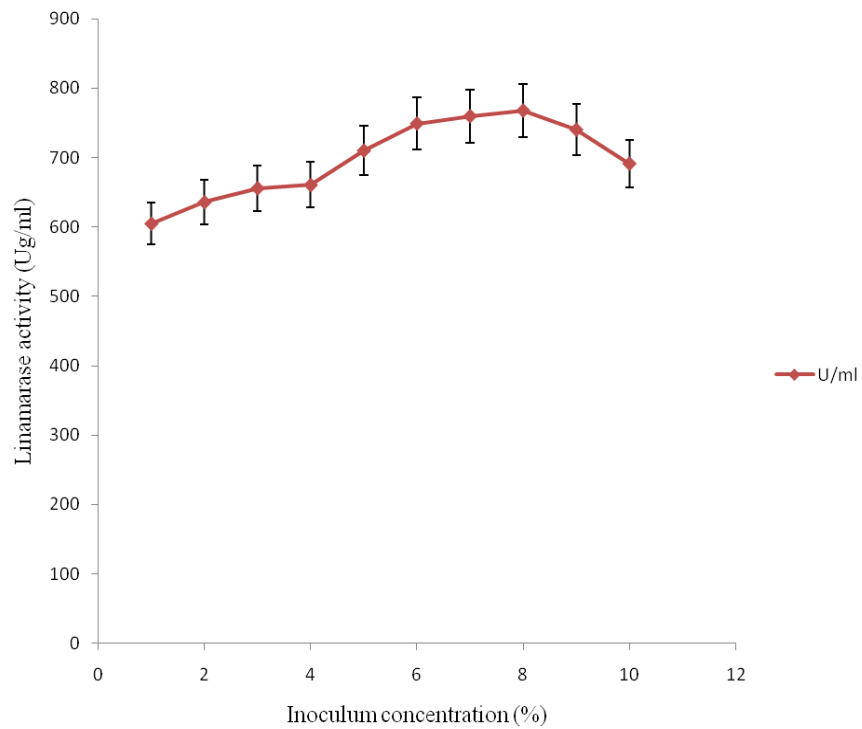


Figure 4. Effect of inoculum concentration on the production of linamarase.

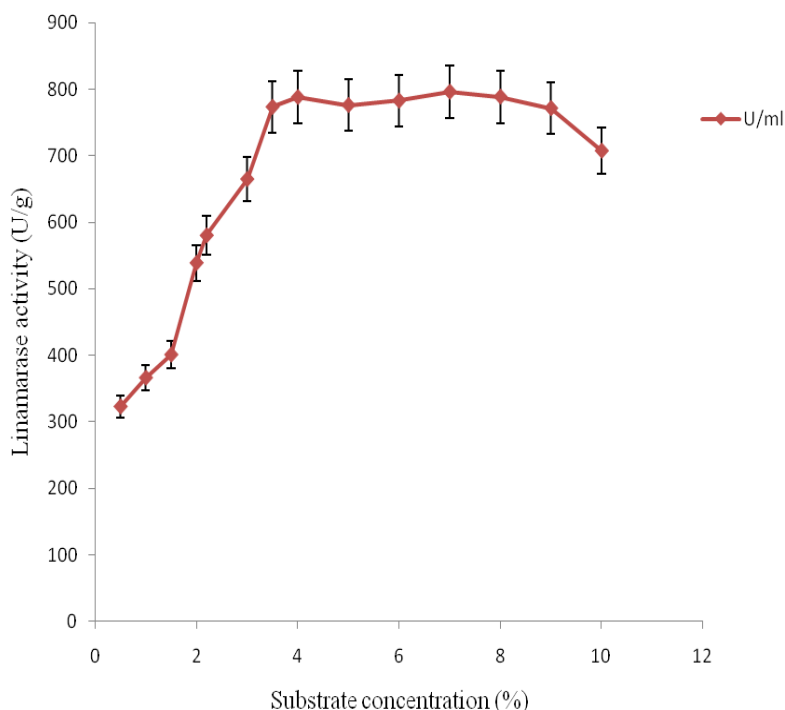


Figure 5. Effect of substrate concentration on linamarase production.

linamarase by *L. plantarum* up to 0.8%. The reduction in enzyme activity beyond optimum might be due to increased competition for carbon and nutrients, which might lead to exhaustion of nutrients. Lower inoculum size might also resulted in lesser number of cells in the production medium, which might require longer time for the microbial cells to grow for utilization of the substrate for desired products formation (Kashyap et al., 2002). Iqbal et al. (2010) had reported that lower inoculum size controls and shortened the microbial lag phase.

Effect of substrate concentration on linamarase production

Substrate selection for enzyme production depends upon several factors, mainly relating to cost and availability. Different agricultural wastes have been used as a suitable substrate for enzymes production. The optima substrate concentration for linamarase production was obtained at 7% (v/w). The enzyme activity was relatively stable between 4% (788 $\mu\text{mol/ml}$) and 6% (782 $\mu\text{mol/ml}$) (Figure 5). An increase in the substrate concentration beyond optimum might resulted in decrease in enzyme activities (Irshad et al., 2013).

Conclusion

From this present research work, linamarase production

has been examined by *L. plantarum* isolated from fermented cassava peels. It was revealed that *L. plantarum* has the ability to produce linamarase under different cultural conditions such temperature, pH, substrate and inoculum concentration using cassava peels. Therefore, the use of this enzyme in breaking down of linamarin to a desirable level will be more useful in food industry.

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