Isolation and Screening of Linamarase Producing Microorganisms for Detoxification of Cyanide in Cassava

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Abstract: This research focuses on isolation and screening of linamarase producing microorganisms for detoxification of cyanide in cassava tubers. Linamarase is the enzyme that breaks down the toxic substance, linamarin (cyanogenic glucoside) in cassava. A total of six microorganisms were isolated and screened. Out of the six isolates, two were able to grow in a medium containing potassium cyanide (KCN) solution (800 ppm) namely *Priestia flexa* and *Pichia kudriavzevii*. These were the isolates that gave optical density (OD) readings ≥0.4 after 2 days of incubation. In conclusion, this research provides a useful information on the choice of these microorganisms for linamarase production for detoxification of cyanide in cassava tubers.
Key word: Cassava, cyanide, detoxification, linamarase, screening

INTRODUCTION

Cassava 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). Being a nutritionally strategic famine crop, cassava could support food security in areas of low rainfall. Mature roots are able to survive for a long time in the absence of moisture and still retain its nutritional value. Cassava has become a preferred crop in countries where HIV/AIDS is prevalent because it requires low input of labour, cash, and time (Topouzis, 2003). 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 glucoside. The sweet cassava is considered as not having much cyanide. In local classification of cassava, some varieties are regarded as "sweet" (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 *et al.,* 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 *et al.,* 2011). Therefore according to Osuntokun (1994) long-term consumption of small amounts of cyanide can cause severe health problems such as tropical neuropathy. Alitubeera *et al.* (2019) reported an outbreak of cyanide poisoning involving 98 persons in Uganda in 2017 in which two death cases occurred. Linamarase is the enzyme that detoxifies cyanide in cassava tubers. This enzyme is produced by microorganisms, therefore, microorganisms are important in the fermentation process. Selection of the linamarase producing microorganisms is based on their fast growth in media containing 800 mg/L potassium cyanide solution (Ogbonnaya, 2015). This research is focused on isolation and screening of these microorganisms for production of linamarase and its ability to detoxify cyanide content in cassava tubers.

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.

Media: The media that were used for the growth and isolation of microorganisms included the following: potato dextrose extract agar (PDA) for isolating the fungi (yeasts and moulds), while nutrient agar and De Man Rogosa Sharpe (MRS) agar was used for isolation of bacteria.

***Isolation of microorganisms from fermenting cassava tubers*:** One milliliter of fermenting cassava liquor was withdrawn with a sterile pipette into a 9 ml 0.1% peptone water diluents after stirring with a sterile glass rod. Then using a sterile pipette, 0.1 ml of the mixture was withdrawn aseptically and inoculated on agar plates in three replicates. The plates were incubated aerobically at 27^{o}C for 2-4 days for fungi; aerobically at 27^{o}C for 24 hours for bacteria. The isolates was subcultured to obtain pure cultures. Then the pure cultures were stored on agar slants in Bijou bottles (Ogbonnaya, 2015).

***Identification of microorganisms*:** Pure bacterial cultures were used for identification. Morphological, biochemical and physiological characteristics of each isolate was used for its identification. The following biochemical tests were carried out namely gram staining reaction, oxidase test, catalase test, indole test, methyl red test, Voges Proskauer test, motility test, citrate utilization test, sucrose, maltose, starch hydrolysis, urease, glucose and lactose tests. Bergey's Manual of determinative Bacteriology (Holt *et al*., 1994) was used to identify the bacterial isolates. Pure cultures of fungal isolates stored on PDA slants at 4^{o}C was used for fungi identification studies. The morphological and cultural characteristics of the isolates formed the basis of their identification. The taxonomic schemes for fungi was used to identify the fungal isolates (Frazier and Westhoff, 2009; Ray and Ryan, 2003).

***Molecular identification - fungal and bacterial genomic DNA extraction*:** Extraction was done using a ZR fungal/bacterial DNA mini prep extraction kit supplied by Inqaba South Africa. A heavy growth of the pure culture of the fungal isolates was suspended in 200 microliters of isotonic buffer into a ZR Bashing Bead Lysis tubes, 750 microliters of lysis solution was added to the tube. The tubes were secured in a bead beater fitted with a 2ml tube holder assembly and processed at maximum speed for 5 minutes. The ZR bashing bead lysis tube were centrifuged at 10,000x*g* for 1 minute. Four hundred (400) microliters of supernatant were transferred to a Zymo-Spin IV spin Filter (orange top) in a collection tube and centrifuged at 7000 x*g* for 1 minute. One thousand two hundred (1200) microliters of fungal/bacterial DNA binding buffer were added to the filtrate in the collection tubes bringing the final volume to 1600 microliters, 800 microliters were then transferred to a Zymo-Spin IIC column in a collection tube and centrifuged at 10,000x*g* for 1 minute, the flow through was discarded from the collection tube. The remaining volume was transferred to the same Zymo-spin and spun. Two hundred (200) microliters of the DNA Pre-Was buffer was added to the Zymo-spin IIC in a new collection tube and spun at 10,000x*g* for 1 minute followed by the addition of 500 microliters of fungal/bacterial DNA Wash Buffer and centrifuged at 10,000x*g* for 1 minute.The Zymo-spin IIC column was transferred to a clean 1.5 microliters centrifuge tube, 100 microliters of DNA elution buffer were added to the column matrix and centrifuged at 10,000x*g* for 30 seconds to elute the DNA. The ultra-pure DNA was then stored at -20 degree for other downstream reaction (www.dataphyte.com).

DNA quantification

The extracted genomic DNA was quantified using the Nanodrop 1000 spectrophotometer. The software of the

equipment was lunched by double clicking on the Nanodrop icon. The equipment was initialized with 2 ul of sterile distilled water and blanked using normal saline. Two microlitre of the extracted DNA was loaded onto the lower pedestal, the upper pedestal was brought down to contact the extracted DNA on the lower pedestal. The DNA concentration was measured by clicking on the "measure" button (www.dataphyte.com).

16S rRNA amplification

The 16s RRNA region of the rRNA genes of the isolates were amplified using the 27F: 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R: 5'-CGGTTACCTTGTTACGACTT-3' primers on a ABI 9700 Applied Biosystems thermal cycler at a final volume of 50 microlitres for 35 cycles. The PCR mix included: the X2 Dream taq Master mix supplied by Inqaba, South Africa (taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4M and the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; annealing, 52ºC for 30 seconds; extension, 72ºC for 30 seconds for 35 cycles and final extension, 72ºC for 5 minutes. The product was resolved on a 1% agarose gel at 120V for 15 minutes and visualized on a UV transilluminator (Rolph *et al.*, 2001).

***Internal transcribed spacer (ITS) amplification*:** The ITS region of the rRNA genes of the isolates were amplified using the ITS1F: 5'-CTTGGTCATTTAGAGGAAGTAA-3' and ITS4: 5'- TCCTCCGCTTATTGATATGC-3, primers on a ABI 9700 Applied Biosystems thermal cycler at a final volume of 50 microlitres for 35 cycles. The PCR mix included: the X2 Dream Taq Master mix supplied by Inqaba, South Africa (Taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4M and the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; annealing, 53ºC for 30 seconds; extension, 72ºC for 30 seconds for 35 cycles and final extension, 72ºC for 5 minutes. The product was resolved on a 1% agarose gel at 120V for 15 minutes and visualized on a UV transilluminator by modified method of Rolph *et al*. (2001).

***Sequencing of 16SRNA and ITS*:**

Sequencing was done using the BigDye Terminator kit on a 3510 ABI sequencer by Inqaba Biotechnological, Pretoria South Africa. The sequencing was done at a final volume of 10ul, the components included 0.25 ul BigDye® terminator v1.1/v3.1, 2.25ul of 5 x BigDye sequencing buffer, 10uM Primer PCR primer, and 2-10ng PCR template per 100bp. The sequencing conditions were as follows 32 cycles of 96°C for 10s, 55°C for 5s and 60°C for 4min by modified method of Rolph *et al*. (2001).

Phylogenetic analysis: Obtained sequences were edited using the bioinformatics algorithm Trace edit, similar sequences were downloaded from the National Center for Biotechnology Information (NCBI) data base using BLASTN. These sequences were aligned using ClustalX. The evolutionary history was inferred using the Neighbor-Joining method in MEGA 6.0 (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 500 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the taxa analysed. The evolutionary distances were computed using the Jukes-Cantor method (Jukes and Cantor, 1969).

Screening the isolates for their resistance to cyanide: Five millilitres of screening medium (yeasts extract, 0.5% (w/v); peptone 0.7% (w/v), glucose 2% (w/v) in 100 ml of distilled water) was placed in test tubes and the test tubes were autoclaved at 121 ºC for 15 minutes. Potassium cyanide (KCN) solution was sterilized and then, aliquots (0.1 ml) of potassium cyanide (KCN) solution (800 ppm) was placed in each test tube containing the screening medium. A loopful of each bacterial and fungal isolate was inoculated into each test tube. The test tubes was incubated at room temperature (27±2 ºC) for 2 days and 10-fold serial dilution was made. The sensitivity or resistance of each isolate to cyanide was

monitored with a spectrophotometer at 600 nm against distilled water blank. Then observation of isolates that gave optical density (OD) readings ≥0.4 after 2 days of of prepared Mueller

RESULTS AND DISCUSSION

The following microrganisms were isolated from fermenting cassava: bacteria included *Priestia flexa*, *Bacillus* sp, *Lactobacillus* sp, *Escherichia* sp and one yeast *Pichia kudriavzevii* and one mould *Aspergillus niger*. A total of six microorganisms were isolated and identified. Among them, *Bacillus* sp was the most abundant fermenting microorganism. The results are presented in tables 1 and 2 the results of the molecular identification are shown in figure 1 to 4. For the bacteria, the obtained 16s rRNA sequence from the isolate produced an exact match during the megablast search for highly similar sequences from the NCBI non-redundant nucleotide (nr/nt) database. The 16S rRNA of the isolates showed a percentage similarity to other species at 100%. The evolutionary distances computed using the Jukes-Cantor method were in agreement with the phylogenetic placement of the 16S rRNA of the isolates within the *Priestia* sp and revealed a closely relatedness to *Priestia flexa* (Figure 1). For the yeast, the obtained ITS sequence from the isolate produced an exact match during the megablast search for highly similar sequences from the NCBI non-redundant nucleotide (nr/nt) database. The internal transcribed spacer of the isolate showed a percentage similarity to other species at 100%. The evolutionary distances computed using the Jukes-Cantor method were in agreement with the phylogenetic placement of the ITS of the isolate within the *Pichia* sp and revealed a closely relatedness to *Pichia kudriavzevii* (Figure 2). Fig. 3 shows Agarose gel electrophoresis of the 16s rRNA of the bacterial isolates, lanes 1 and 2 showing the amplified fragment at 1500bp while lane 2 represents the 100bp ladder for *Priestia flexa*. Figure 4 shows the agarose gel electrophoresis showing the amplified internal transcribed spacer of the fungal isolate. Lane 1 and 2 represent the ITS band at 600bp while lane L represents the 500bp molecular ladder for *Pichia kudriavzevii*. The organisms identified in this research are similar to those isolated by other researchers which include *Lactobacillus plantarum, L. brevis, L.coprophillus, L. Mesenteroides,L. acidophilus,Bacillus subtilis* for fufu production (Oyedeji *et al.*, 2013; Braide *et al.*, 2018) and those employed in the fermentation of cassava for garri production such as *Lactobacillus plantarum, L. fermentum* and *B. subtilis* (Kostinek *et al.*, 2005; Onyango, Bley, Raddatz & Henle, 2004; Braide *et al.*, 2018). The result also corresponds to the report that the identified microorganisms in the fermented pulp juice were *Aspergillus niger, Aspergillus flavus* and *Lactobacillus* spp (Naa-Ayikailey *et al.*, 2010).

Table 1: Biochemical characteristics and probable identities of bacterial isolates

Isolates	Gram Reaction	Cell Morphology	Catalase	Oxidase	Urease	Citrate	Methyl red	Voges proskauer	Slant	Butt	H_2S	Gas	Indole	Motility	Lactose		Maltose		Glucose		Sucrose		Probable Organism
ISO 1	+	Rod	+	+	+	+	+	-	B	A	+	-	-	-	-	-	-	-	A	G	-	-	*Priestia* sp
ISO 2	+	Rod	+	-	-	+	-	+	A	A	-	-	-	+	A	G	A	G	A	G	-	-	*Bacillus* sp
ISO 3	+	Rod	+	-	-	+	-	+	A	A	-	-	-	+	A	G	A	G	A	G	A	G	*Bacillus* sp
ISO 4	+	Rod	+	-	-	-	+	-	A	A	-	-	-	-	A	G	A	-	A	-	A	-	*Lactobacillus* sp
ISO 5	_	Rod	+	-	+	-	+	-	A	A	-	+	+	-	A	G	A	G	A	G	A	G	*Escherichia* sp

Key: + = Positive, - = Negative, A = Acid, B = Base/Alkaline, G = Gas

Table 2: Cultural, cellular and probable identities of fungal isolates

Isolate	Cultural characteristics	Cell morphology	Probable fungi
ISO 5	Shiny smooth creamy colony	Oval yeast cells with single septate hyphae and conidia	*Pichia* sp
ISO 6	Black surface with white border and cream reverse	Septate hyphae with long smooth conidiophores and rough dark conidia	*Aspergillus niger*

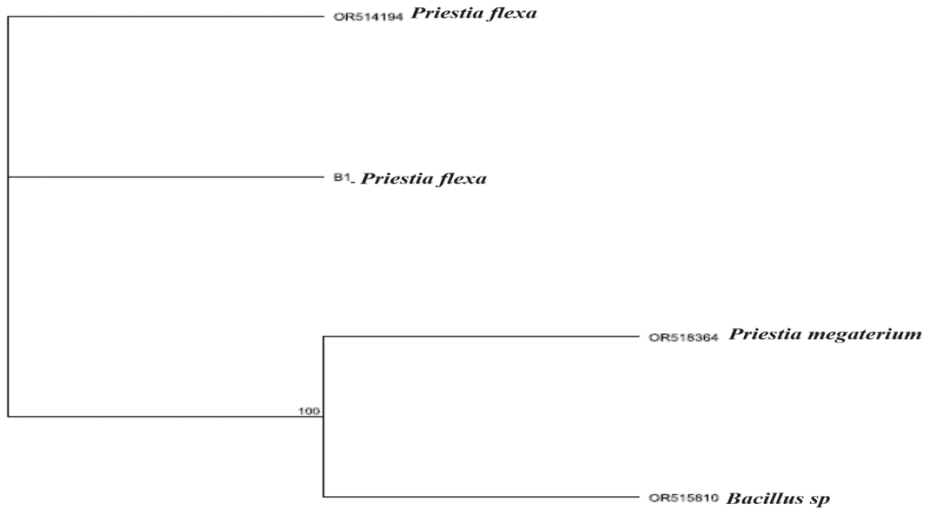

Figure 1: Phylogenetic tree showing the evolutionary distance between the bacterial isolates

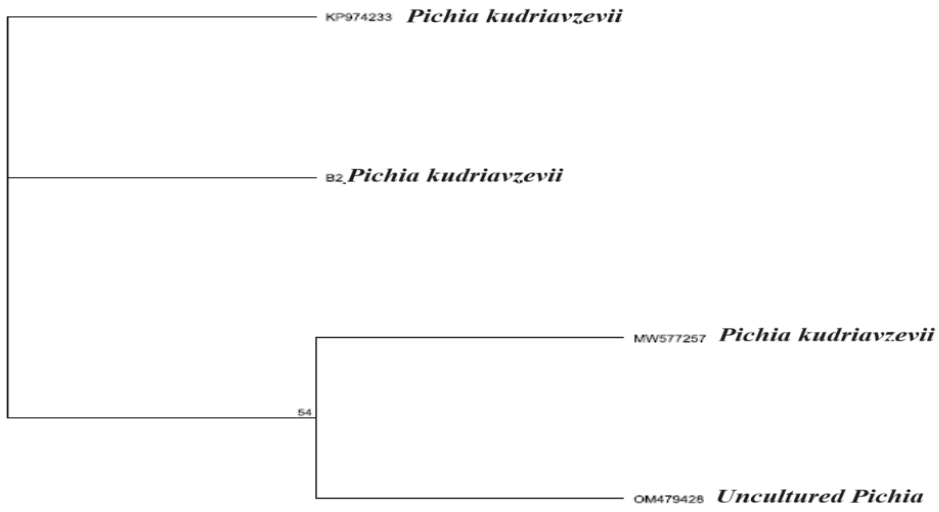

Figure 2: Phylogenetic tree showing the evolutionary distance between the fungal isolates

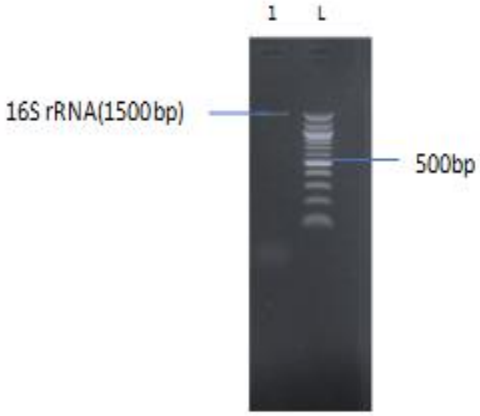

Figure 3: Agarose gel electrophoresis of the 16s rRNA of the bacterial isolates, lanes 1 and 2 showing the amplified fragment at 1500bp while lane 2 represents the 100bp ladder

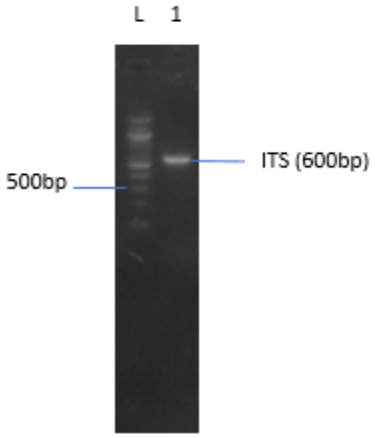

Figure 4: Agarose gel electrophoresis showing the amplified internal transcribed spacer (ITS) of the fungal isolate. Lane 1 and 2 represent the internal transcribed spacer (ITS) band at 600bp while lane L represents the 500bp molecular ladder

Table 3: Optical density values for different isolates at 600 nm

Isolate		Optical density at 600 nm
1	*Priestia flexa*	0.408
2	*Bacillus* sp	0.236
3	*Bacillus* sp	0.234
4	*Lactobacillus* sp	0.245
5	*Escherichia* sp	0.214
6	*Pichia kudriavzevii*	0.408
7	*Aspergillus niger*	0.087

CONCLUSION

From this present research work, *Priestia flexa* and *Pichia kudriavzevii* have been revealed as linamarase producing microorganisms. These microorganisms can be applied by cassava processors and other researchers for the purpose of detoxifying cyanide content in cassava in order to make it safer for human consumption.

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