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CHARACTERIZATION OF LINAMARIN-UTILIZING BACTERIAL STRAINS ASSOCIATED WITH DETOXIFICATION OF CYANOGENS IN WASTE EFFLUENTS

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ABSTRACT

The cyanide molecule is one of the dominant pollutants in the environment. This study aimed to isolate and identify the bacterial strains capable of utilizing linamarin and to assess their roles in the detoxification of cyanogenic substances in waste effluents in Lagos, Nigeria. Two bacterial strains, *Bacillus pumilus* strain WOB3 KX774195 and *Bacillus pumilus* strain WOB7 KX774196 were isolated from cassava wastewater samples by standard microbiological procedure. They were identified on the basis of morphological and biochemical characteristics and 16S rRNA gene sequencing. Microbial growth assessment was performed in triplicates under aerobic batch conditions. The generation times of strains WOB3 and WOB7 were 40.71 and 10.88 d; with specific growth rates of 0.025 and 0.064 d^{-1} respectively on linamarin. Strain WOB3 had maximum growth of 0.552 (OD_{600nm}) by day 12 at pH 6.2. Likewise, strain WOB7 recorded optimum growth of 1.276 ($OD_{600\ nm}$) by day 10 at pH 6.4. The maximum values obtained for linamarase activities by WOB3 and WOB7 were 3.3 x 10^{-2} $mgmL^{-1}min^{-1}$ and 7.61 x 10^{-2} $mgmL^{-1}min^{-1}$ respectively. The results from this study suggest that the bacterial isolates possess degradative capacities, which could be deployed in the bioremediation of cassava processing wastes.

Key words: Cyanide molecule, Detoxification, *Bacillus pumilus*, Linamarase, Linamarin, Waste Effluent, Bioremediation

INTRODUCTION

The roots of cassava (*Manihot esculenta* Crantz) form the staple diet of over 500 million people in developing countries (Bhat *et al*., 2012; Golob *et al*., 2012). However, they have a high level of toxicity as they contain large amounts of two cyanoglucosides, linamarin (96%) and lotaustralin (4%) (Uyoh *et al*., 2007; Jorgensen *et al*., 2011). High utilization of processed cassava products has increased the environmental pollution associated with the disposal of effluents. The highly offensive odour emanating from the fermenting effluent calls for regulation in the discharge of waste generated (Adewoye *et al*., 2005; Akani *et al*., 2006). Numerous human disorders such as development of goitre, tropical ataxic neuropathy, paralysis and death are associated with consumption of improperly processed cassava (Oluwole *et al*., 2000; Ernesto *et al*., 2002; Siritunga and Sayre, 2003). Cassava is normally processed before consumption as a means of detoxification, preservation and modification (Oyewole, 2001, Umeh and Odibo, 2013). The discharge of effluent into water bodies such as streams and rivers pose health hazard and endanger fish and other aquatic organisms (Oti, 2002; Oboh and Akindahunsi, 2003; Oboh, 2004). Disposal of agricultural by-products such as cyanogenic waste from cassava processing activities is a concern in Nigeria. There is a considerable high level of pollution arising from the discharge of effluents on the water system; hence the need for proper treatment before discharge and conversion of these cassava wastes into biosorbents that can remove toxic and valuable metals from the effluent. Chemical oxidation methods such as the addition

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of sodium hypochlorite and hydrogen peroxide are currently used to remediate cyanide contaminated wastewaters with attendant high reagent costs associated with the use of these chemical techniques. Also, complete breakdown of some cyanide complexes may not be achieved (Yanase *et al*., 2000). The cyanide elimination from industrial wastewaters by biological approach has proven to be cost effective and environmentally friendly and acceptable method (Akcil *et al*., 2003; Siriantapiboon and Chuamkaew, 2007). Bacteria and fungi are examples of microorganisms that can metabolize cyanide to mineral form (non-toxic end products) using the cyanide as the sole nitrogen and carbon sources under aerobic and anaerobic settings. At concentrations higher than 1 mM, organisms which include species of *Bacillus* and *Klebsiella* are resistant to cyanide (Kao *et al*., 2003; Ebbs, 2004). The present investigation describes the isolation and characterization of two tropical bacterial species capable of utilizing linamarin and a variety of other cyanogenic molecules with the objective of evaluating capabilities for uses in remediation of cyanide-contaminated cassava mill wastewater dumping site.

MATERIAL AND METHODS

Collection of samples for physicochemical analysis and isolation of bacteria

For the physicochemical analysis and isolation of bacterial cultures, cassava wastewater from a cassava processing factory was collected from the disposing pond at location, Odogunyan (Co-ordinates: N6°39'39.40236"E3°30'33.19883") in sterile sample bottles, carefully labelled and stored in the refrigerator at 4°C, before processing within 24 h. Figure 1 shows the satellite view of cassava factory site and sampling points.

Determination of physicochemical parameters of cassava waste water samples

The waste water samples were analyzed for a number of physicochemical properties including chemical oxygen demand (COD), total solids (TS), total alkalinity (TA), total dissolved solids (TDS, biochemical oxygen demand (BOD), hardness, electrical conductivity (EC), sulphate (SO_4), nitrate (NO_3), phosphates (PO_4) and cyanide (CN) were determined according to standard analytical methods (APHA, 1985; Ademoroti, 1996). Fourteen

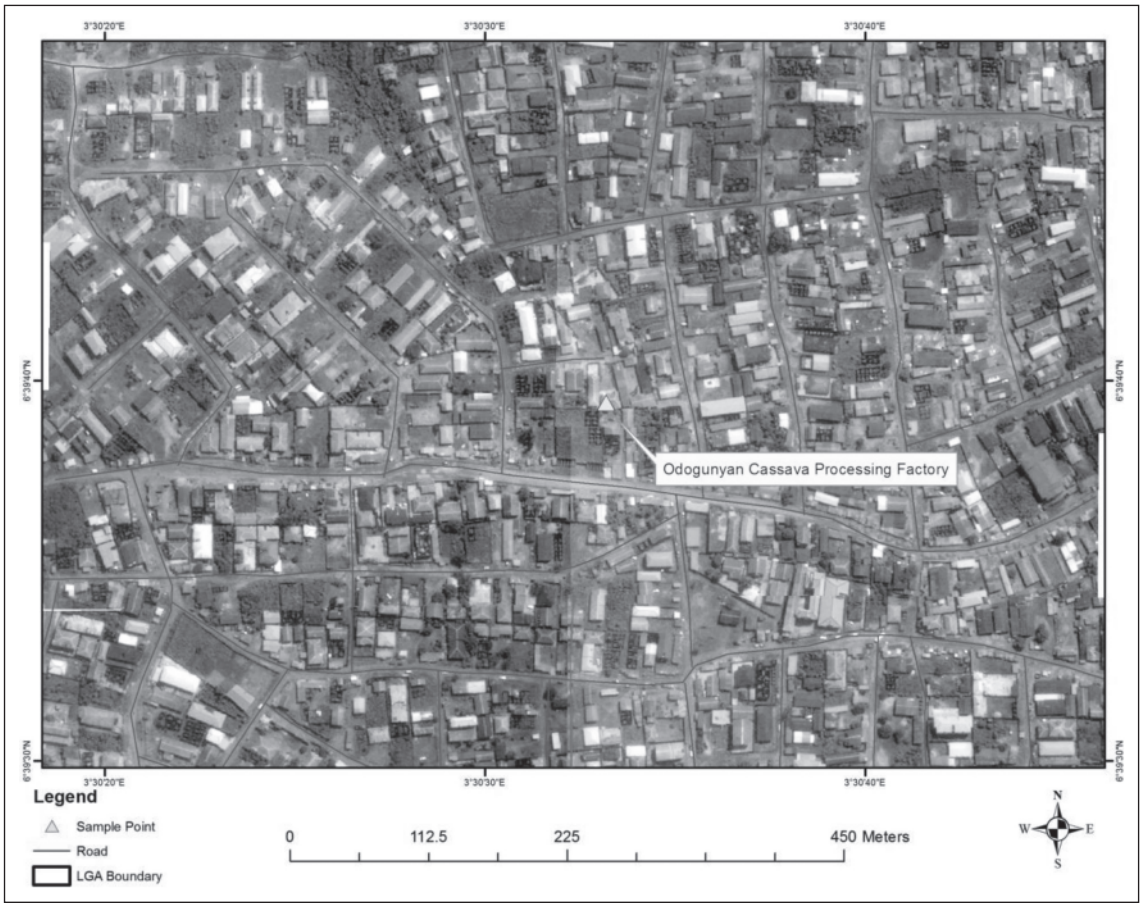


Fig. 1. Satelite image of Odogunyan of showing the location of cassava processing factory in Lagos State, Nigeria.

heavy metals (including nickel (Ni), cadmium (Cd), chromium (Cr), copper (Cu), mercury (Hg), zinc (Zn), silver (Ag) and cobalt (CO), were determined in the wastewater samples according to standard analytical methods (APHA *et al*., 2005; Ademoroti, 1996). Wastewater pH was measured electrometrically with Orion 3 Star bench top pH meter (Thermoscientific, USA).

Isolation of linamarin-utilizing bacteria from cassava waste water

The method described by Ahaotu *et al*. (2011) was used for isolation of bacteria. The samples were serially diluted and 0.1mL was plated out on the surface of the solidified nutrient agar media (Fluka, Sigma-Aldrich, USA). Plates were incubated at 37°C for 24-48 h. The microorganisms isolated were sub-cultured by repeated streaking until pure cultures were obtained.

Identification of linamarase producing Organisms

Two out of the eight (8) isolates were screened having higher linamarase activities and they were further characterized. The screened isolates were then identified using appropriate biochemical tests, cultural and morphological characteristics (Holt *et al*., 1994; Cowan and Steel, 1985) and 16S rRNA gene sequences, and were stored on agar slants of media used for their isolation at 4°C until needed. The screened isolates were also used for further tests.

Cultural and morphological characteristics

The samples used for microscopy were young cultures of 24 h. The subculturing was done on fresh nutrient agar plate and smear made and then Gram-stained. They were examined for Gram reaction and characteristics using a Hitachi S-3500N model compound microscope (ThermoNaran, Hitachi technologies, America Inc.).

Biochemical characteristics

Catalase reaction, oxidase test, urease test, indole test, citrate utilization, nitrate reduction test, methyl-red-Vogues Proskaeur reaction and sugar fermentation were studied as described by Lanyi (1987). Pure cultures of bacterial isolates were identified according to the identification scheme of Bergey's manual of determinative bacteriology (Holt *et al*., 1994).

VITEK identification system

The available identification system was applied to the isolates obtained and the assays were performed according to the manufacturer's recommendations (bioMérieux, Inc.). VITEK 2 fluorescent GP card (Gram positive card) assays, identifications not categorized as excellent or very good according to the instructions of the manufacturer were doubtful; and any result with excellent or very good identification of species other than *Bacillus pumilus* strains WOB3 and WOB7 were subjected to 16S rRNA gene sequencing when accurate identification was of concern.

Molecular characterization of test Organisms

DNA extraction and PCR amplification

The genomic DNA of the strains was extracted and precipitated following the standard protocol for bacterial genomic DNA preparations using bacteria DNA purification kit (Jena Bioscience, Germany). Ribosomal DNA (16S rDNA) was amplified by PCR (94°C for 5 min, 30 cycles consisting of 94°C for 30 s, 55°C for 30 s, 72°C for 90 s followed by a terminal incubation at 72°C for 10 min) using universal 16S rDNA forward 27F (5'- AGA GTT TGA TCM TGG CTC AG-3') and reverse 1492R (5'-GGT TAC CTT GTT ACG ACT T-3') primers (Martin-Laurent *et al*., 2001; Walcher *et al*., 2013). Polymerase chain reaction-amplified DNA segments were separated by electrophoresis in a 1.5% agarose gel, using 100 bp DNA marker (Promega, USA) as DNA standard, Millipore water (blank) was used as negative control and ethidium bromide was used as the stain. Gel was run for 80 min at 100 V. The amplified products were observed under the Kodak fluorescent imaging equipment, model IS 4000R (Kodak image station, care stream molecular imaging health Inc. Rochester, NY, USA.).

Sequencing and phylogenetic analysis

PCR amplified products were purified and nucleotide sequences were determined with an automated sequencing apparatus (ABI PRISM 377, PE Biosystems Inc.). The 16S rDNA sequence of the strains were compared to sequences in public databases with the BLAST search program on the NCBI website (http://www.ncbi.nlm.nih.gov/) to find closely related bacterial 16S rDNA gene sequences. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 (Tamura *et al*., 2007). Neighbor-Joining method was used to construct the phylogenetic tree. The generated sequences for strains WOB3 and WOB7 were deposited in the National Centre for Biotechnology Information (NCBI) nucleotide sequence database under accession number KX774195 and KX774196.

Growth and substrate utilization studies

A forty-eight hour old culture (1.0 mL) of each pure isolate was grown in a 250 mL conical flask at 30°C with agitation (150 rpm) on cassava effluent (20% v/v) as a sole carbon source. The pH of the medium was adjusted to 7.2. The cassava effluent was tested for their abilities to support growth of the linamarin-utilizing bacterial species. The culture

fluids were sampled at an interval of 48 h and were tested for linamarase activities over a period of 12 days. Controls (the uninoculated H_2O containing cassava effluent medium: linamarin and the inoculated H_2O without cassava effluent: linamarin) were put in place and monitored to rule out contamination. The extent of growth was determined by measuring the turbidity of the growth medium compared to the controls. Bacterial growth was determined spectrophotometrically (Thermoscientific™ Spectonic GENESYS 8, Thermofisher Scientific, USA) at 600 nm. The breakdown of the linamarin in the substrates was measured in terms of estimation of hydrocyanic acid (HCN) release spectrophotometrically at 540 nm (Thermoscientific™ Spectonic GENESYS 8, Thermofisher Scientific, USA).

Linamarase assay

Culture filtrates obtained at intervals (48 h) from each of the culture flasks were centrifuged (10,000 × g, 4°C, 10 min). The supernatants were used as the source of enzymes. Linamarase activity was measured as described earlier (Oyewole e*t al*., 2001) by monitoring the production of hydrocyanic acid (HCN) using a UV-visible spectrophotometer (Thermoscientific™ Spectonic GENESYS 8, Thermofisher Scientific, USA) at 540 nm for 15 min. The reaction mixture (3.0 ml) contained: culture supernatant (1.0 ml), linamarin (1.0 ml) and 1.0 ml phosphate buffer (0.2M, pH 7.2). The reaction mixture was incubated at 37°C. The reaction was terminated by the addition of 1.0 mL of 0.1M sodium carbonate (Na_2CO_3). A standard curve enabled the conversion of the absorbance obtained to the quantity of HCN released from linamarin. One unit of enzyme activity was defined as 1.0 mM of linamarin oxidized per min.

Statistical analysis

Mean generation times (T_d) and specific growth rates (μ) of the isolates on linamarin was calculated using non-linear regression of growth curves for the period when growth rates were maximal using Prism version 5.0 (Graphpad software, San Diego, CA). Also, data generated for the physico-chemical properties and chemical characteristics were expressed as mean ± standard deviation using the excel software.

RESULTS AND DISCUSSION

Physicochemical characteristics of cassava waste waters

The concentrations of heavy metals obtained from the cassava waste water are also presented in Table 1. Of all the elements and ions measured, the average concentration of these metals at the site are 53.59±5.27 $mgL^{-1}$for potassium, 29.50±4.2 mgL^{-1} for sodium, 51.50±5.7 mgL^{-1} for calcium, 31.70±4.3 mgL^{-1} for magnesium, 17.97±2.515 mgL^{-1} for aluminum, 0.502±0.114 mgL^{-1} for cadmium, 4941±1259 mgL^{-1} for chloride, 0.904±0.035 mgL^{-1} for manganese, 4.004±0.373 mgL^{-1} for iron and 1.374±0.087 mgL^{-1} for zinc while average concentrations of 161.0±9.0 mgL^{-1} for nitrate and 118.6±9.3 mgL^{-1} for sulphate were obtained while 18.4±0.3.3 mgL^{-1} was recorded for phosphate. Interestingly, cyanide anion, which is of considerable interest was detectable at a concentration of 12.49±1.14 mgL^{-1}. In this study, the concentration of cyanide ion obtained was significantly higher than the maximum permissible levels recommended by WHO (0.07 mg/l) (Table 1). Based on these findings, cassava mill waste discharged from the cassava factory may pose serious hazard to animal, human and aquatic life.

Table 1. Physico-chemical properties and chemical characteristics of cassava wastewater samples collected from cassava factory site

Parameters	Value/ Observation	WHO Standard limit*
Colour	White	Unobjectionable
pH	4.01±0.12	6.5–9.5
Appearance	Cloudy	Unobjectionable
Odour	Objectionable	Unobjectionable
Conductivity (μScm^{-1})	11602±620	1200
TS (mgL^{-1})	14810±286	1500
TDS (mgL^{-1})	9240±472	2000
Alkalinity (mgL^{-1})	BDL	100
Hardness (mgL^{-1})	1300±176	500
BOD (mgL^{-1})	155±21	50
COD (mgL^{-1})	224±12	1000
K^+ (mgL^{-1})	53.59±5.27	–
Na^+ (mgL^{-1})	29.50±4.2	–
Ca^{2+} (mgL^{-1})	51.50±5.7	–
Mg^{2+} (mgL^{-1})	31.70±4.3	20
NO_3^- (mgL^{-1})	161±9.0	50
PO_4^{2-} (mgL^{-1})	18.4±3.3	–
SO_4^{2-} (mgL^{-1})	118.6±9.3	500
CN^{-1} (mgL^{-1})	12.49±1.14	0.07
Cl^{-1} (mgL^{-1})	4941±1259	250
Al^{3+} (mgL^{-1})	17.971±2.515	0.2
Ag^+ (mgL^{-1})	BDL	–
Cu^{2+} (mgL^{-1})	0.162±0.019	2.0
Cr^{2+} (mgL^{-1})	1.423±0.467	0.05
Cd^+ (mgL^{-1})	0.502±0.114	0.003
Pb^{2+} (mgL^{-1})	BDL	0.01
Mn^{2+} (mgL^{-1})	0.904±0.035	0.4
Fe^{2+} (mgL^{-1})	4.004±0.373	3.0
Zn^{2+} (mgL^{-1})	1.374±0.087	3.0

CWW – Cassava wastewater, TS – Total solid, TDS – Total dissolved solid, BOD – Biochemical oxygen demand, COD – Chemical oxygen demand, BDL – Below detectable level, ± – Standard deviation, WHO – World Health Organization, * – Source (Institute of public Analysts of Nigeria, IPAN, 2005).

Cultural, morphological, biochemical and genotypic characterization of bacterial isolates

In this study, two linamarin-utilizing *Bacillus pumilus* strains were isolated from cassava waste-water discharged into the environment by standard microbiological procedures. The morphological, physiological and biochemical characteristics of the strains WOB3 and WOB7 are summarized in Table 2. Both strains WOB3 and WOB7 are Gram-positive. They are motile, spore formers and coccoid shaped. Full sequences (1500bp (Figure 2) of the 16S rRNA gene of both strains have been registered in GenBank with accession numbers KX774195 and KX774196 respectively. A homology search showed that the 16S rRNA gene sequences had a high level of identities (95-100%) with similar strains in the GenBank data base. Evidences from 16S rRNA gene sequence analyses as summarized (Table 3) showed that the 16S rRNA genes of strains WOB3 had 99% similarity to *Bacillus pumilus* HPC460 DQ460034 and *Bacillus* sp. BAB-572 KU728634 respectively, while the 16S rRNA genes of strains WOB7 are similar to those of *Bacillus* sp. 1026B6 12AMannit KU644503 and *Bacillus pumilus* MB-P8 HM022734 by 99%. The phylogenetic tree which was constructed for comparison of the sequences of some bacterial strains in the GenBank indicated that both strains WOB3 and WOB7 belong to the genus *Bacillus* (Figure 3). The phylogenetic tree showed three distinct clusters; *Bacillus* group that are closely related but are likely to have evolved from same ancestors. Their characteristics were similar to those previously reported by Maughan and Van der Auwera (2011). Sequence analysis of 16S rRNA gene has been used by authors for successful identification of linamarase producing bacteria strains. Essers *et al*. (1995) reported a *Bacillus* sp. strain with capability to utilize linamarin (to 1% of initial concentration) isolated from Ugandan domestic fermented cassava. Murugan *et al.* (2012) reported the isolation of cyanogenic glucoside utilizing indigenous bacterium from cyanide rich cassava peel waste. According to Ugwuanyi *et al*. (2007), confirmed the potentials of *Bacillus* genus bacteria, including *Bacillus coagulans*, *Bacillus licheniformis* and *Bacillus stearothermophilus*, to degrade linamarin.

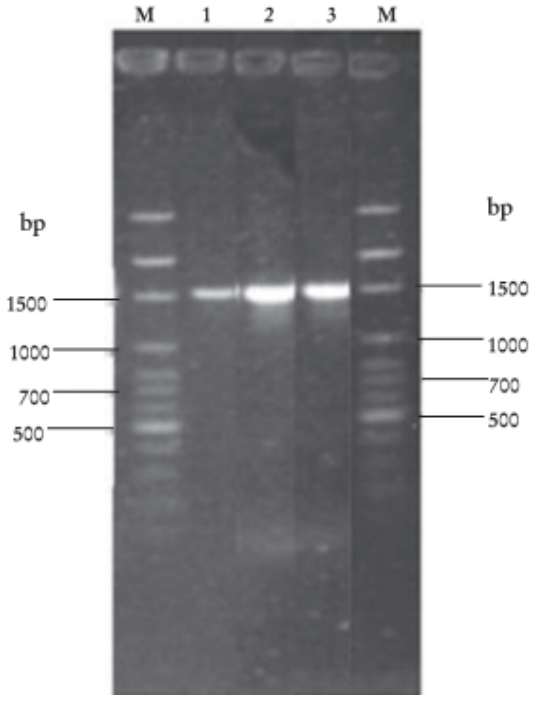
Growth of strains WOB3 and WOB7 in cassava waste water

In this study, the doubling times of strains WOB3 and WOB7 were 40.71 and 10.88 d; with specific growth rates of 0.017 and 0.064 d^{-1} respectively on linamarin (Table 4). Growth patterns of organisms are graphically shown in Figure 4 & Figure 5. They exhibited exponential growth patterns in the first 2 days. The highest growth exhibited by *Bacillus pumilus* strain WOB3 on

Table 2. Morphological, physiological and biochemical characteristics of test strains

Characteristics	Isolates	
	WOB3	WOB7
Gram reaction	+	+
Shape	R	R
Colour	C	C
Motility	+	+
Growth	+	+
Catalase	+	+
Oxidase	+	-
Urease	+	+
H2S	+	+
Indole	+	+
Citrate	+	+
MR	–	–
VP	–	–
Glucose	+	+
Lactose	–	+
Arabinose	+	+
Mannitol	+	+
Maltose	+	+

Putative Identification *Bacillus pumilus* strain WOB3 *Bacillus pumilus* strain WOB7.
+ – Positive reaction; – negative reaction; C – Creamy, R – Rod shaped.



Key: Lane 1 & 5: Molecular weight marker.
Lane 2: Positive control (*E. coli* HB 101).
Lane 3: 16S rRNA gene fragment of strain WOB3.
Lane 4: 16S rRNA gene fragment of strain WOB7.

Fig. 2. Gel showing PCR products of about 1500bp using universal 16S rDNA forward 27F and reverse 1492R primers.

Table 3. Genotypic identities of linamarin-utilizing bacterial isolates from amplified sequences of 16S rRNA gene fragment of genomic DNA

Bacteria Strain	Tentative Identity	GenBank Accession number	Closest strain	% Identity	GenBank Accession number
WOB3	*Bacillus pumilus*	KX774195	*Bacillus pumilus* HPC460	99	DQ460034
WOB7	*Bacillus pumilus*	KX774196	*Bacillus* sp. 1026B6 write 12AMannit	99	KU644503

B3, *Bacillus pumilus* strain WOB3; B7, *Bacillus pumilus* strain WOB7.

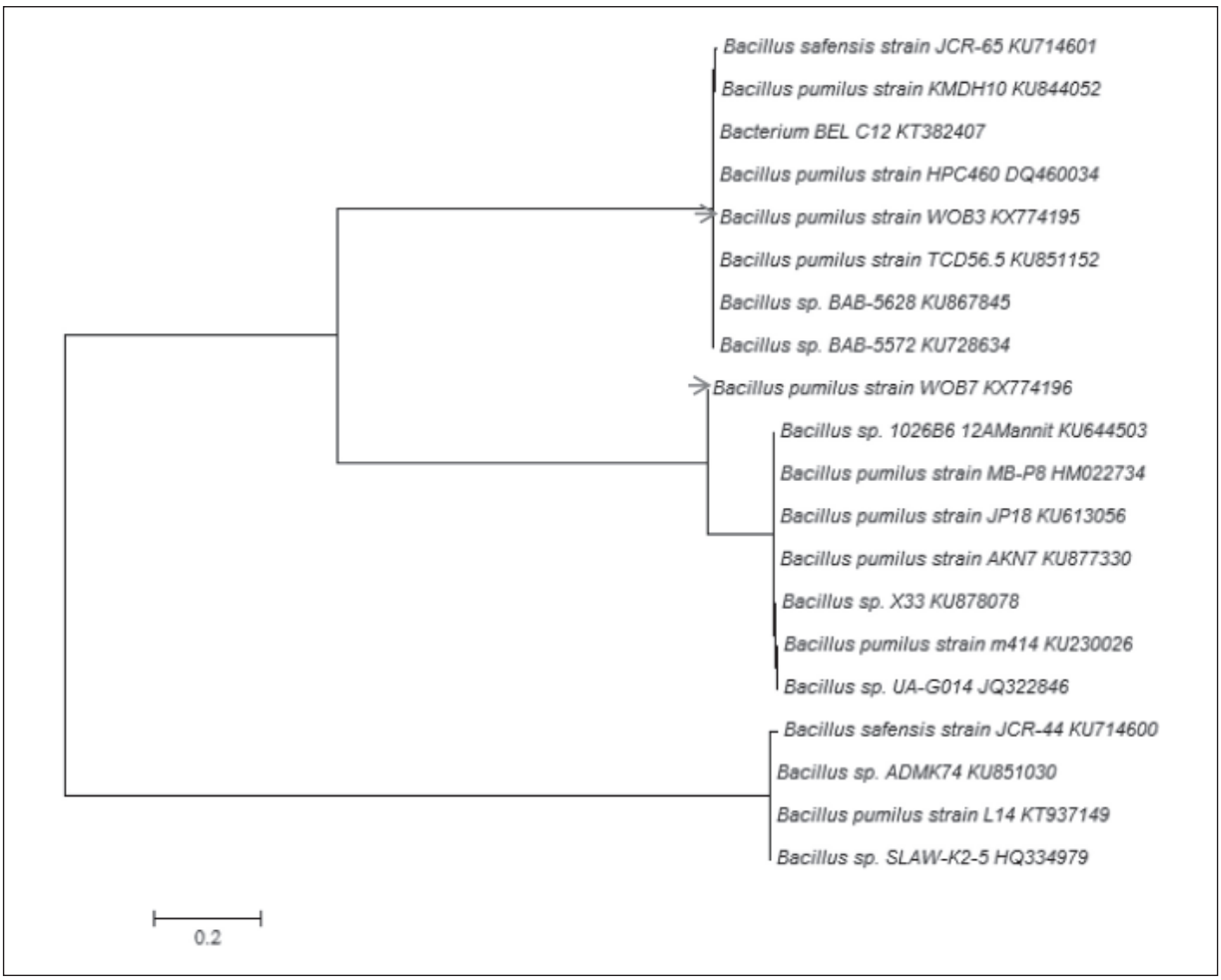


Fig. 3. Phylogenetic tree (neighbour-joining method) showing genetic relationship between strains WOB3 and WOB7 and other related reference microorganisms based on the 16S rRNA gene sequence analysis. Species names are followed by the accession numbers of their 16S rRNA gene sequences. The numbers at branching points or nodes refer to bootstrap values, based on 20 re-samplings.

Table 4. Growth kinetics' data for utilization of linamarin in liquid cultures by *Bacillus* species

Organisms	Cassava effluent	
	$\mu(d^{-1})$	T_d (d)
WOB3	0.017	40.71
WOB7	0.064	10.88

μ – Specific growth rate, T_d – Doubling time.

linamarin in cassava effluent was 0.552 ($O.D_{600\ nm}$) whereas *Bacillus pumilus* strain WOB7 recorded highest growth of 1.276 ($O.D_{600\ nm}$) on linamarin. Apart from growing in Cassava effluent containing linmarin the test organisms grew on the solid waste leachates. The ability of microorganisms to utilize linamarin as a source of energy that may be used to generate cellular biomass was reported by Vasconcellos *et al*. (2009). *Bacillus licheniformis* (isolate2_2) and *Rhodotorula glutinis* (isolate L1)

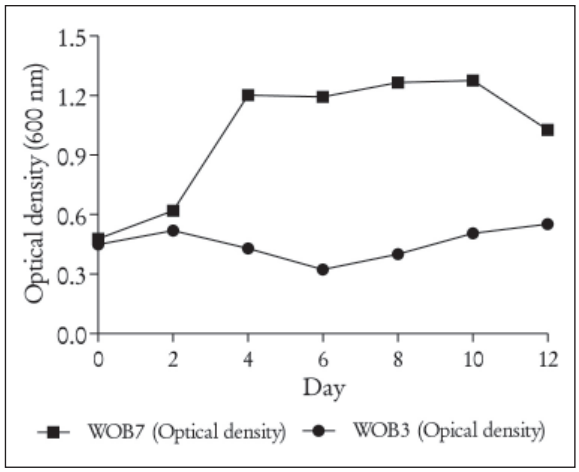


Fig. 4. Growth patterns of *Bacillus pumilus* strain WOB3 and *Bacillus pumilus* strain WOB7 in cassava effluent medium.

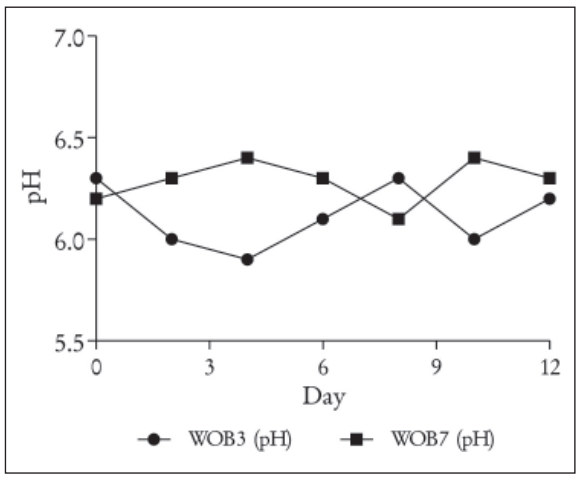


Fig. 5. *Bacillus pumilus* strain WOB3 and *Bacillus pumilus* strain WOB7's pH changes in the cassava effluent medium.

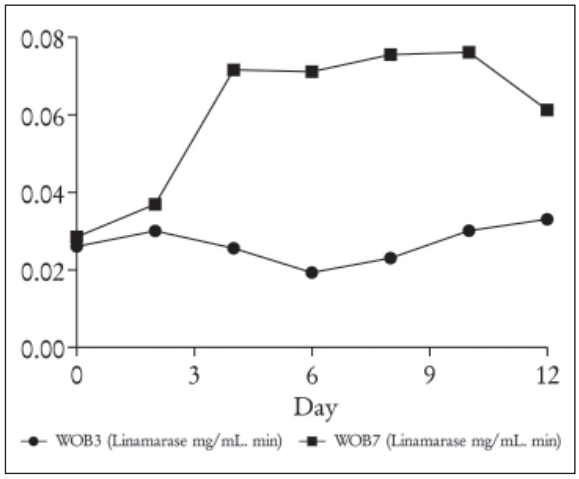


Fig. 6. Linamarase activities of *Bacillus pumilus* strain WOB3 and *Bacillus pumilus* strain WOB7 in cassava effluent medium.

had optimum optical densities of 0.08 and 0.14 nm when the absorbance was measured at a wavelength of 605 nm.

Changes in pH

The decrease in the level of hydrocyanic acid observed during cassava mash fermentation has been attributed to the degradation of cyanogenic glucosides to cyanohydrins which at lower pH becomes hydrolysed to form HCN (Tetchi *et al*., 2012). The release of HCN is the indicator of cyanide cleavage which simultaneously increases the pH of the medium. In this study, continuous pH monitoring revealed that there was a decrease in pH of the media containing cassava effluent in the experimental flasks inoculated with strains WOB3 and WOB7 (Figure 5). With culture fluid of WOB3 containing cassava effluent, the pH of 7.2 dropped to 6.0 within 0-2 days, thereafter fluctuated within 6.0 and 6.3 and eventually dropped to 6.2 on day 12. While with WOB7, the culture fluid containing cassava effluent, the pH of 7.2 dropped to 6.3 at 0-2 days, and remained around the range of 6.1 and 6.4 and later dropped to 6.3 on day 12 (Figure 5).

Enzyme detection and assay

In this study, maximum values obtained for linamarase activities by *Bacillus pumilus* strain WOB3 and *Bacillus pumilus* strain WOB7 were 3.3 x 10^{-2} $mgmL^{-1}min^{-1}$ (12 day) and 7.61 x 10^{-2} $mgmL^{-1}min^{-1}$ (10 day) respectively. In similar study, Ahaotu *et al.* (2011) isolated an array of linamarase-producing bacteria from cassava waste water and assessed their linamarase activities. They found that the linamarase activities of the isolates ranged from 0.0416 µmol/ml/nmol (*Lactobacillus plantarum*) to 0.2618 µmol/ml/nmol (*Leuconostoc cremoris*).

CONCLUSION

The results showed the isolation of two bacterial strains with biodegradation potentials to mineralize cyanoglucoside linamarin. *Bacillus pumilus* strains WOB3 and WOB7 grew well in cassava effluent as well in solid leachates as judged by the time course growth studies in this work. Therefore, these strains may be promising for the remediation of sites contaminated with cyanogens.

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