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In vitro Degradation of Extracted Cassava Linamarin by *Bacillus* Species Isolated from Cassava wastewater

Ogunyemi, A.K[1], Samuel, T.A[2], Buraimoh, O.M[1], Amund, O.O[1], Ilori, M.O[1], Alagbada, B.C[3], Olumuyiwa, E.O[4], Odetunde, S. K[5] and Akinrodoye, F[2]

1- Department of Microbiology, University of Lagos, Akoka, Lagos-Nigeria
2- Department of Biochemistry, College of Medicine, University of Lagos, Idi-Araba, Lagos-Nigeria
3- Department of Chemical Sciences, Yaba College of Technology, Yaba, Lagos-Nigeria
4- Department of Biological Sciences, College of Natural and Applied Sciences, Bells University of Technology, Ota, Ogun- Nigeria.
5- Department of Science Laboratory Technology, Microbiology Unit, Lagos State Polytechnic, Ikorodu, Lagos-Nigeria
*Correspondence Author: waleogunyemi2002 @yahoo.com

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ABSTRACT

Linamarin is the most abundant cyanoglucoside present in cassava cells and may generate the equivalent amount of hydrocyanic acid. This study was aimed to assess degradative capacities of *Bacillus pumilus* strain WOB3 and WOB7on linamarin. The test organisms for linamarase activity were identified on the basis of phenotype, biochemical properties and 16S rDNA gene sequencing as: *Bacillus pumilus* strain WOB3 KX774195 and *Bacillus pumilus* strain WOB7 KX774196. Growth studies showed that the strains grew in all the substrates tested. The doubling times of *Bacillus pumilus* strain WOB3 and *Bacillus pumilus* strain WOB7 were 8.25 d and 7.53 d on cassava wastewater (CWW), 6.30 d and 5.78 d on supplemented cassava wastewater (CWW), 8.66 d and 9.90 d on solid waste leachates (SWL) and 6.30 d and 9.24 d on supplemented solid waste leachates (SWL) respectively; with specific growth rates of 0.084 d^{-1} and 0.092 d^{-1} on CWW, 0.11 d^{-1} and 0.12 d^{-1} on supplemented CWW, 0.080 d^{-1} and 0.070 d^{-1} on SWL and 0.11 d^{-1} and 0.075 d^{-1} on supplemented SWL respectively. High-performance liquid chromatographic analysis studies revealed that linamarin degradation by the strains followed a linamarase pathway involving carbon dioxide (CO_2) and hydrocyanic acid (HCN) as metabolic intermediates. Based on HPLC analysis, linamarin residual concentration at day 12 by the strains WOB3 and WOB7 was 26.73 mgL^{-1}(19.79 %) and 29.79 mgL^{-1} (21.92 %). These novel features make the bacteria suitable candidates for in-situ application on sites contaminated with cassava processing wastes.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) roots and leaves contain high concentrations of linamarin (alphahydroxyisobutyronitrile- beta-*D*-glucopyranoside) and lotaustralin (methyl-linamarin) (Vasconcellos *et al*., 2009). Linamarin is the most abundant cyanoglucoside present in cassava cells (Conn, 1969) and may generate the equivalent to 0.2-100 mg of HCN per 100 g of fresh cassava following cellular lysis (Bradbury *et al*., 1991).

Microorganisms can grow on substrates containing cyanides by anaerobic metabolism, or by using an aerobic respiration chain as an alternative pathway (Cereda *et al*., 1981). In both pathways, HCN is eliminated from the substrate, and converted into a non-toxic product (Jensen and Abdel-Ghafar, 1979).

The current global concern with environmental problems has made the biological detoxification of cyanide an attractive alternative to chemical detoxification procedures (Meyers *et al*., 1991). Biological detoxification methods may be both cheaper and more environmentally acceptable than chemical methods. Most attempts to develop biological processes for the detoxification of cyanide-containing effluents from industry have concentrated on cyanide degrading fungi (Knowles and Bunch, 1986). This enzymatic cyanide-removing property can be exploited for the detoxification of cyanide-rich cassava wastewater and industrial residues. These residues currently cause serious environmental problems in many cassava flour producing plants in Nigeria, one of the largest producers worldwide, and in many African, Latin American and Asian countries (Romero *et al*., 2002). However, none has achieved complete detoxification (Blanshard *et al*., 1994) and in spite of the availability of several processing techniques, cyanide exposure from cassava diets prevails (Ernesto *et al*., 2000). Cassava roots can be industrially applied for obtaining starch and flour. However, cassava industries generate some undesirable sub-products, such as solid residues and a liquid effluent named manipueira, which may represent a major disposal problem due to the high organic charge and toxic potential, resulting from the presence of cyanoglucosides (Cereda, 2001). Cyanoglucosides are secondary metabolites produced by several plant species (Conn, 1994) used in animal and human diets, such as apple, bamboo shoot, cassava, cherry, lima bean, maize, oat, peach, papaya, sorghum and wheat (Pitcher *et al*., 1989). These compounds are dispersed throughout the plant organs, mostly in non-edible parts (Jones, 1978), but may become concentrated in edible roots and leaves, as in the case of cassava. Microorganisms can grow on substrates containing cyanides by anaerobic metabolism, or by using an aerobic respiration chain as an alternative pathway (Cereda *et al*., 1981). In both pathways, HCN is eliminated from the substrate, and converted into a non-toxic product (Jensen and Abdel-Ghafar, 1979). This enzymatic cyanide-removing property can be exploited for the detoxification of cyanide-rich cassava wastewater and industrial residues. These residues currently cause serious environmental problems in many cassava flour producing plants in Brazil, the largest producer worldwide, and in many African, Latin American and Asian countries (Romero *et al*., 2002), where cassava products are an important input for human diet. Manipueira is rich in potassium, nitrogen, magnesium, phosphorous, calcium, sulfur and iron, presenting a great potential as an agronomic fertilizer. It contains cyanoglucosides, which explains the application as nematicide and insecticide (Palmisano *et al*., 2000). The study intent was to evaluate the potentials of two bacterial strains in bioremediative clean-up of the polluted environment and elucidate the degradation pathway of linamarin contained in the waste products.

MATERIALS AND METHODS

Microorganisms and Inoculum Development:

Linamarin-utilizing bacterial strains were isolated and identified as previously described by Ahaotu *et al*. (2013). They were identified on the basis of morphological and biochemical characteristics and 16S rRNA gene sequencing. The bacterial isolates are *Bacillus pumulis* strain WOB3 KX774195 and *Bacillus pumilus* strain WOB7 KX774196. In previous laboratory studies, these strains have demonstrated substantial capabilities to utilize linamarin for growth.

They also showed varied abilities to utilize other natural substrates such as solid waste leachate. The isolates were resuscitated using nutrient agar and pre-enrichment was carried using Luria-Bertani broth.

Extraction of Linamarin from Cassava Tuber:

Extracts were obtained from the same cultivars of cassava growing in the cassava plant farm at Lagos State Polytechnic, Ikorodu, Lagos. Preliminary extractions were carried out using cassava roots in phosphoric acid organic solvents as described by King and Bradbury (1995), but the final aqueous method used was as follows: A 800 g sample of very young cassava root was cut up with stainless knife and scissors and immediately ground in a glass pestle and mortar with 80 mL of 0.1M phosphoric acid. A further 80 mL of 0.1 M phosphoric acid were added with further grinding and the pasty solution was sieved through a cloth which was squeezed. The pink-coloured, cloudy solution was then centrifuged and the clear, supernatant liquid removed with a Pasteur pipette. This solution, which also contained linamarase (inactivated in the 0.1M phosphoric acid) and linamarin, was stored frozen in a deep freezer cabinet at 20 °C.

Bacterial Growth Studies on Cassava Wastewater (CWW) and Solid Waste Leachates (SWL):

Growth study of the microbial strains was performed under aerobic batch conditions. Turbidity method was used for the growth study of the strains. Organisms were grown in Erlenmeyer flasks (250 mL) containing CWW (20 $\%^{v}/_{v}$; 100 mL; pH 7.2). The medium containing linamarin as substrate as sole carbon source was autoclaved at 121 °C and was inoculated with 1.0 mL of inoculum. Apart from CWW (CWW), the bacterial isolates also grew on (SWL). Growth was evaluated at intervals (2 days) by the intensity of turbidity ($O.D_{600\ nm}$) in CWW medium. A positive control flask contained the inoculum but without the substrate while the negative control contained the substrate but without the inoculum. The growth cultures were set up in duplicates. The culture fluid (5.0 mL) from the two sets of flasks was harvested every 2 days for determination of optical density and pH change.

Substrate Utilization by Test Organisms:

Similarly, for linamarin degradation studies, organisms were grown in Erlenmeyer flasks (500 mL) containing minimal salts medium (250 mL; pH 7.2) with extracted linamarin (0.5 %w/v) and fortified with 1.0 mL of trace elements solution (Obayori *et al*., 2009). This was autoclaved, allowed to cool before inoculation with 2.0 mL of the inoculum and incubation was done on a rotary shaker (28 ^{o}C, 150 rpm) for 12 days. The culture fluid (25.0 mL) from the two sets of flasks was harvested every 2 days for high-performance liquid chromatographic analysis to determine residual linamarin and detect other possible metabolites in the culture fluids.

Analysis of Growth Kinetic Data:

Mean generation times (T_d) and the specific growth rates (μ) of the bacterial strains on linamarin as carbon source were computed with non-linear regression of growth curves using Graph pad software prism version 6. Briefly, an "XY "data table was created. Time (days) was entered into "X", and response (cell optical density and pH) into "Y"- axis respectively. Analysis was carried out by clicking "analyse", choosing the non-linear regression panel of exponential equations and exponential growth respectively. The parameter Y0 (value at time zero) was constrained to a constant value by going to the constrain tab of the non-linear regression dialog, setting the drop down next to Y0 to "constant equal to". The values were entered. The model Y=Y0*Expo (k*X) was used; where Y0 is the Y value when X (time) is zero, K is the rate constant, expressed in reciprocal of the X-axis time units. Doubling time was computed as ln 2/K (Buraimoh *et al*., 2017).

High-Performance Liquid Chromatographic Analysis (HPLC):

Determination of the residual linamarin content in the media during the period of incubation was carried out using high-

performance liquid chromatography (Schimadzu Nexera MX, Japan) with a flame ionization detector. Instrument operating conditions were as follows: an μbandapak C-18 glass column pack with internal diameter of 4.6 mm and length of 100 mm, a detector UV (254 nm, diode array detector), pump pressure of 15 mpa, with mobile phase of acetonitrile and water (75:25 v/v) at a flow rate of 1.0 mL/min. A standard profile was first obtained by injecting 10 ul of the linamarin into the HPLC and a chromatogram was generated to serve as a calibration window with which the test sample was analyzed, after which 20.0 mL of the growth culture was extracted with 20 mL acetonitrile, and then concentrated to 5.0 mL (each test sample) from which 1 μL was injected into the HPLC column and an equivalent chromatogram was generated to detect the metabolites of biodegradation. The peak areas of the standard and test sample chromatograms were compared and were used to calculate the concentration of the sample, given by the formula:

$$\text{Concentration of Linamarin} = \frac{\text{Total peak of sample} \times \text{Concentration of standard}}{\text{Peak area of standard}}$$

RESULTS

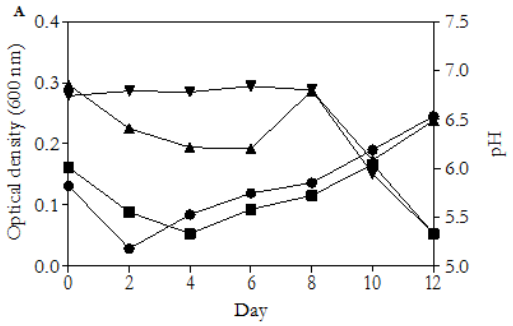
Growth of *Bacillus pumilus* (strains WOB3 and WOB7) on Cassava Wastewater and Solid Waste Leachates:

Table 1 shows doubling times and specific growth rates of test organisms on CWW (supplemented with or without MS). While Fig. 1 indicates the growth profiles of the test organisms on CWW only and supplemented CWW. When grown on CWW, strain WOB7 had higher specific growth rate of 0.092 d^{-1} with doubling time of 7.53 d than strain WOB3 (0.084 d^{-1}, 8.25 d). An increase in specific growth rates by strains WOB3 and WOB7 was observed when grown on CWW supplemented with MS and specific growth rates of 0.11 and 0.12 d^{-1} were recorded respectively. Apart from using linamarin in CWW, the test organisms also grew on SWL only and when supplemented with MS.

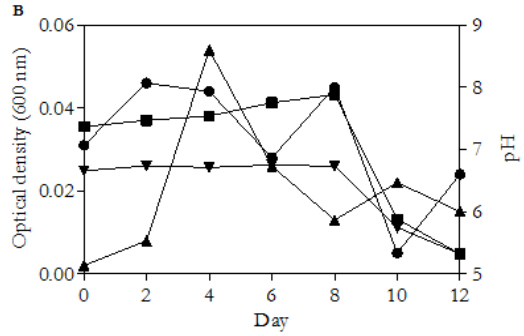
Table 1: Growth potentials of linamarin-utilizing bacteria grown on cassava wastewater and solid waste leachates (supplemented with or without mineral salts)

Isolates	Substrates							
	CWW		CWW+MS		SWL		SWL+MS	
	μ (d^{-1})	T_d (d)	μ (d^{-1})	T_d (d)	μ (d^{-1})	T_d (d)	μ (d^{-1})	T_d (d)
WOB3	0.084	8.25	0.11	6.30	0.080	8.66	0.11	6.30
WOB7	0.092	7.53	0.12	5.78	0.070	9.90	0.075	9.24

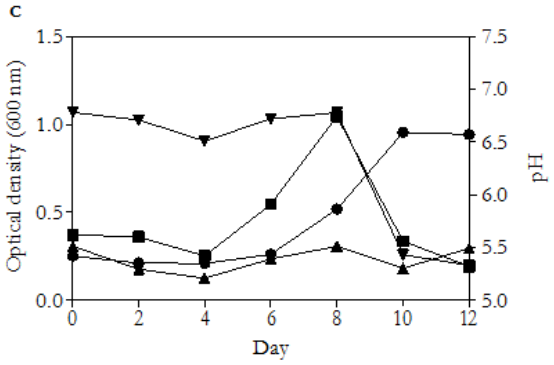
CWW=Cassava wastewater only; CWW+MS=Cassava wastewater plus mineral salts, SWL=Solid waste leachates only, SWL+MS=Solid waste leachates plus mineral salts, μ-Specific growth rate, T_d-Doubling time, d-Day



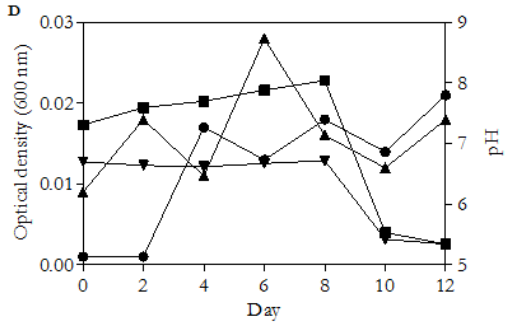
A-Growth of *Bacillus pumilus* strain WOB3 on cassava wastewater only (without mineral salts) and supplemented cassava wastewater (with mineral salts).



B-Growth of *Bacillus pumilus* strain WOB3 on solid waste leachates only (without mineral salts) and supplemented solid waste leachates (with mineral salts).



C- Growth of *Bacillus pumilus* strain WOB7 on cassava wastewater only (without mineral salts) and supplemented cassava wastewater (with mineral salts)



D-Growth of *Bacillus pumilus* strain WOB7 on solid waste leachates only (without mineral salts) and supplemented solid waste leachates (with mineral salts)

Fig. 1: Growth profiles and pH changes in the culture fluids of *Bacillus pumilus* strain WOB3 and *Bacillus pumilus* strain WOB7

●-Optical density (substrate only);▲- Optical density (supplemented substrate); ■ -pH changes (substrate only); ▼ -pH changes (supplemented substrate)

The Change in pH:

Generally, there were dramatic changes in pH of the culture fluids which tends towards acidic direction from neutral as shown in Table 2. Continuous monitoring of pH revealed a drop in pH (7.2–5.33) of the CWW medium with or without MS, in both cases respectively, when inoculated with WOB3 strain. There is little or no difference in the pH of the culture medium using the same strain on SWL with or without MS (7.2-5.32). When *Bacillus pumilus* strain WOB7 was grown on CWW medium with or without MS, the pH of the culture medium was 7.2–5.32 and 7.2-5.33, respectively. Similarly, in SWL medium with or without mineral salts, the pH of the culture medium was 7.2–5.34, in both cases respectively (Fig. 1).

Table 2: pH changes in the culture fluids having test organisms grown on cassava wastewater and solid waste leachates (supplemented with or without mineral salts)

Days	Organisms							
	WOB3				WOB7			
	CWW	CWW+MS	SWL	SWL+MS	CWW	CWW+MS	SWL	SWL+MS
0	6.01	6.74	7.37	6.66	5.62	6.78	7.31	6.70
2	5.55	6.79	7.47	6.74	5.60	6.71	7.59	6.64
4	5.33	6.78	7.54	6.71	5.42	6.51	7.70	6.62
6	5.58	6.84	7.75	6.75	5.91	6.72	7.88	6.67
8	5.72	6.80	7.88	6.73	6.74	6.78	8.04	6.72
10	6.04	5.94	5.88	5.75	5.56	5.43	5.53	5.42
12	5.33	5.33	5.32	5.32	5.32	5.33	5.34	5.34

CWW=Cassava wastewater only; CWW+MS=Cassava wastewater plus mineral salts; SWL=Solid waste leachates only; SWL+MS=Solid waste leachates plus mineral salts

Extraction of Linamarin from Cassava Tuber:

The amount of linamarin in the pink solution produced from crushed root was assayed using 25.0 mL aliquots and a calculation gave the amount of linamarin present in the root. The linamarin content of very young root of cultivars was 137.18 mg/L. The linamarin solution from of this cultivar was concentrated enough to be used for biodegradative study.

HPLC Analysis of the Products of Degradation of Linamarin by *Bacillus pumilus*:

The degradation of linamarin and formation of other possible metabolites were analyzed by high-performance liquid chromatography (HPLC). The strains WOB3 and WOB7 were able to degrade 80.21 and 78.08 % linamarin, after 12 days respectively. The results of HPLC analysis showed that strain WOB3 had degraded linamarin from 135.07 to 26.73 mg/L while 6.01-8.71 mg/L of hydrocyanic acid (HCN) and 0.70-12.25 %v carbon dioxide (CO_2) had accumulated as possible metabolites whereas strain WOB7 degraded of 135.90 to 29.79 mg/L with formation of 2.77-8.73 mg/L of hydrocyanic acid (HCN) and 3.95-9.32 %v carbon dioxide (CO_2) (Fig. 2). There was a dramatic decrease in the concentration of linamarin as clearly indicated in the reduction of the peaks after 12 days of incubation for the two bacterial species (Fig. 3). The retention times of the authentic compounds and the metabolites detected are given in Table 3. In the control experiment, the production of hydrocyanic acid and carbon dioxide was not observed in culture flasks devoid of linamarin as well as in uninoculated flasks containing linamarin. This confirmed the utilization of linamarin from the formation of these metabolites as a consequence of hydrolysis of linamarin by *Bacillus pumilus* strain WOB3 and *Bacillus pumilus* strain WOB7. On the whole, the utilization of linamarin by these strains

indicates that these organisms are promising candidates for the removal of cyanogenic wastes from contaminated wastewater. Also, the results of HPLC analysis showed that the two bacterial strains, *Bacillus pumilus* strain WOB3 and *Bacillus pumilus* strain WOB7 converted the linamarin into hydrocyanic acid, carbon dioxide and acetone (Fig. 4).

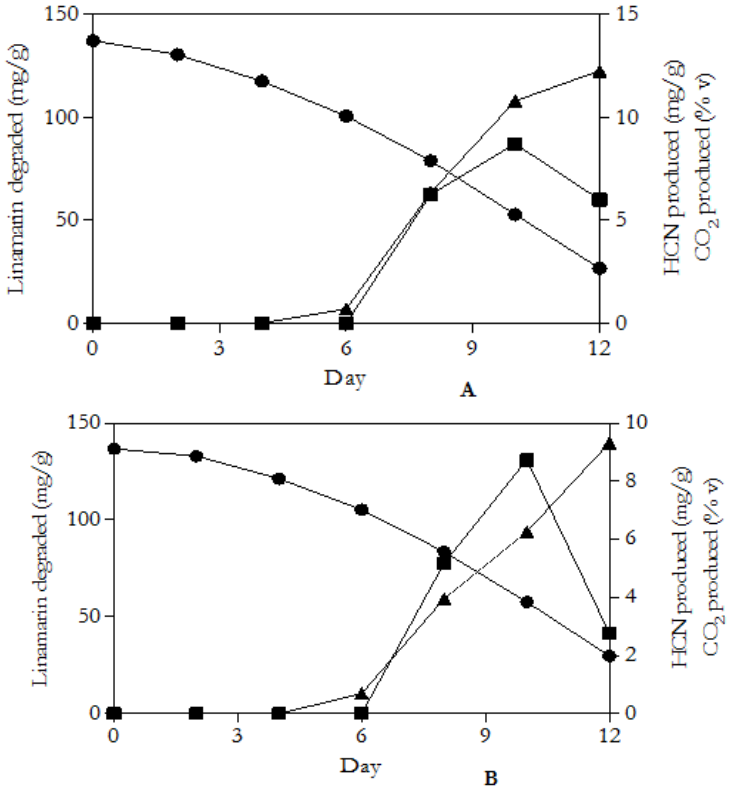
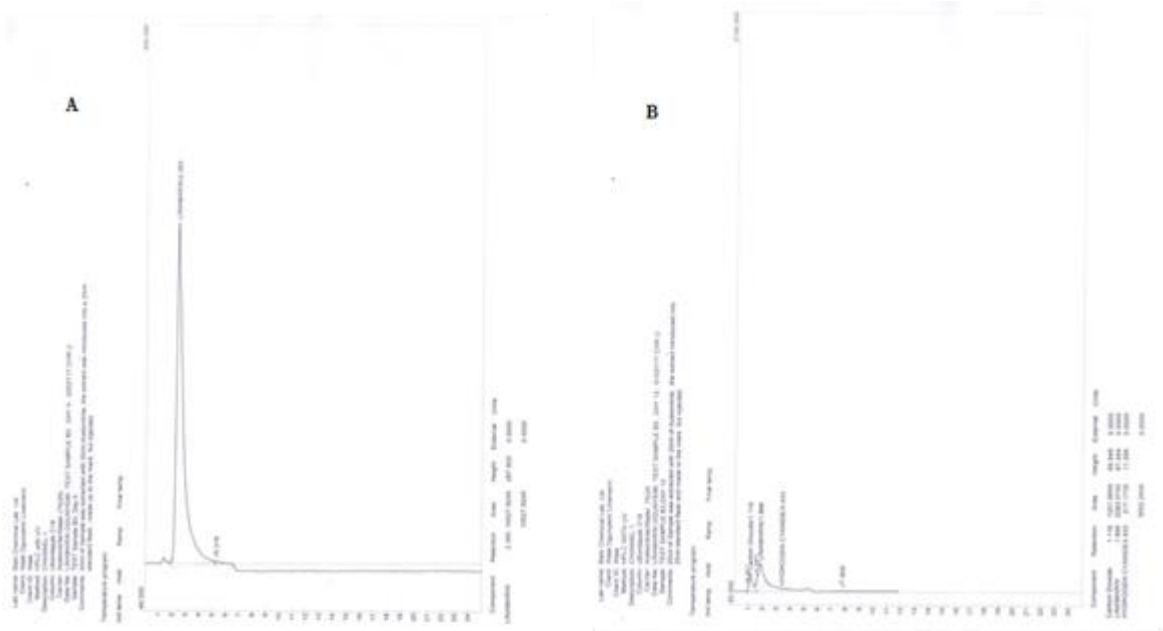


Fig. 2: Degradation of Linamarin and other possible product formation by *Bacillus pumilus* strains WOB3 (A) and *Bacillus pumilus* strain WOB7 (B);●-linamarin; ■-HCN-Hydrogen cyanide; ▲-CO_2-Carbon dioxide



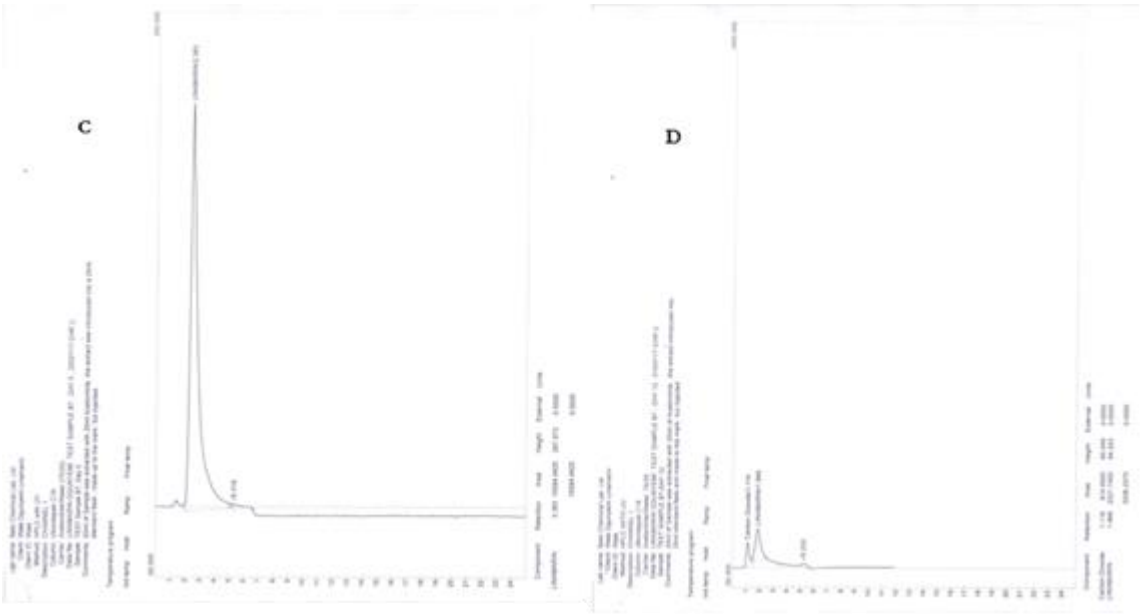


Fig. 3: HPLC analysis of linamarin degradation products by *Bacillus pumilus strain* WOB3 (A-day 0 and B-day 12) and *Bacillus pumilus* strain WOB7 (C-day 0 and D-day 12) after different incubation times.

Table 3: High-performance liquid chromatographic analysis of authentic standard compounds as compared to peaks of metabolites detected in culture filtrates

S.I. NO.	Compounds	WOB3 Retention times (min) of		WOB7 Retention times (min) of	
		Authentic standards	Metabolites	Authentic standards	Metabolites
1	Linamarin	2.383	1.866	2.383	1.866
2	Hydrocyanic acid (HCN)	3.566	3.433	3.766	3.533
3	Carbon dioxide (CO_2)	1.383	1.116	1.200	1.116

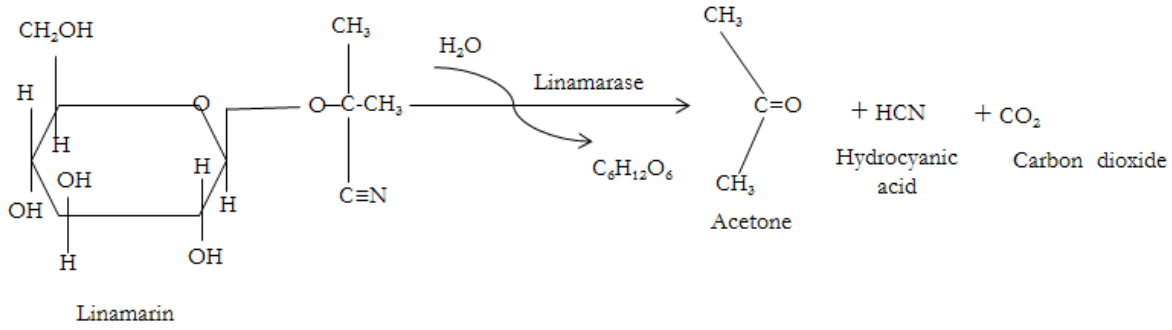


Fig. 4: Proposed catabolic routes of Linamarin degradation by *Bacillus pumilus* strain WOB3 and *Bacillus pumilus* strain WOB7

DISCUSSION

Some studies on cyanoglucoside degradation by *Candida tropicalis* and *Candida utilis*, exist in literature but these studies did not report the degradation rates (Legras *et al*., 1990; Fagbemi and Ijah, 2006; Vasconcellos *et al*., 2009). To the best of our knowledge, this was first time where other products of linamarin degradation were detected using high performance liquid chromatography (HPLC). Essers *et al*. (1995) reported that several fungi and bacteria were isolated from Ugandan domestic fermented cassava, which released HCN from linamarin in defined growth media. They further found out that in 72 h, a *Bacillus* sp. decreased the linamarin to 1 % of initial concentrations while *Mucor racemosus*, *Rhizopus oryzae* and *R. stolonifer* decreased it to 7 and 30 %,

respectively whereas *Neurospora sitophila* and *Geotrichum candidum* hardly degraded the linamarin. Ugwuanyi *et al*. (2007) described *Bacillus* species including *Bacillus coagulans*, *Bacillus licheniformis* and *Bacillus stearothermophilus*, able to degrade linamarin. The use of HPLC for the quantification of the microbial linamarin degradation and other metabolites (HCN and CO_2) was an innovation introduced in the present work. The results obtained herein revealed the potentials of two *Bacillus pumilus* strains with high capacities for biodegrading the cyanoglucoside linamarin. In this study, at the end of incubation period of day 12, the strain WOB3 was able to degrade linamarin from an initial concentration of 137.18 to final concentration of 26.73 mg/L (80.52 %) while strain WOB7 was able to degrade linamarin from concentration of 135.90 mg/L to final concentration of 29.79 mg/L (78.08 %). In similar study by Vasconcellos *et al*. (2009), the test organism was able to degrade linamarin from an initial concentration of 7.47 mg/mL to final concentration of 2.16 mg/mL (71 %) after seven days. Murugan *et al*. (2012) reported the utilization of cyanogenic glycoside by *Bacillus subtilis* KM05 which went through assimilatory degradation with the release of hydrogen cyanide and ammonia. Also, the formation of HCN and release of ammonia as an end product evidenced the assimilatory degradation of linamarin. Kobawila *et al*. (2005) reported reduction of the cyanide content during fermentation of cassava roots and leaves to produce bikedi and ntoba mbodi, two food products from Congo. The cyanide content decreases during the fermentation of cassava roots and leaves by more than 70 % through the activities of the bacterial produced linamarase, allowing the hydrolysis of cyanogenic glucosides. Certain lactic bacteria present in the environment of fermentation are resistant to the strong cyanide concentrations of between 200 and 800 ppm. Vasconcelos *et al*. (1990) observed that the degradation of cyanogenic compounds during the fermentation of cassava, leads to the accumulation of free cyanide, which can reach 200 ppm in the fermenting medium. The linamarase produced by the cassava lactic acid bacteria, notably *Leuconostoc mesenteroides* and *Lactococcus lactis*, and the endogenous linamarase contribute to the process of detoxification. Besides, hydrolysis of cyanogenic glucosides (Louembe *et al*., 1997; Kobawila *et al*., 2003; Vaconcelos *et al*., 1990; Okafor and Ejiofor, 1986) takes place in acid environment (pH 3.8) during lactic fermentation of cassava roots as well as in basic environment (pH 8.5) during alkaline fermentation of the cassava leaves. Forty *Bacillus* spp. including *Bacillus subtilis* (20), *Bacillus licheniformis* (11), *Bacillus sonorensis* (7), *Bacillus cereus* (2) isolated from acid fermented primary starters, were screened by Abban *et al*. (2012) for their ability to hydrolyze and to grow on linamarin at pH levels below 5.0. Abban *et al*. (2012) found that *B. licheniformis* and *B. sonorensis* isolates grew and degraded cyanogens at pH 5.0 in a HCl environment, while two *B. cereus* isolates used in the study showed no breakdown reaction under all conditions tested. The decrease of pH during the fermentation of cassava roots results from the production of organic acids by lactic acid bacteria, which constitute the dominant microflora (Malonga *et al*., 1993; Malonga *et al*., 1996). The alkaline pH during the fermentation of cassava leaves could be due to amines produced by *Bacillus* (Louembe *et al*., 2003). In this study, similar findings were obtained in the culture fluid during the incubation period. Certain strains of *Bacillus*, notably *Bacillus pumilus*, have the capacity to use cyanhydric acid for their nutrition (Knowles, 1976; Skowronski *et al*., 1969). They can thus contribute to the reduction of the cyanide content in the medium of fermentation. The alkaline pH would facilitate reduction of the cyanogenic glucosides content because cyanohydrin acetone, produced by the hydrolysis of linamarin, is cleaved spontaneously when pH is above 5.0 or by the action of hydroxynitril

lyase to give acetone and cyanhydric acid (Nartley, 1968; Conn, 1969; Cooke *et al*., 1978; Formunyam *et al*., 1985).

CONCLUSION

The results obtained in this study revealed the high abilities of two *Bacillus pumilus* strains for biodegrading the cyanoglucoside linamarin. The use of such microorganisms in plants´ treatment for cassava industries, which are widely distributed in Nigeria, may offer a great potential for detoxification of cassava wastewaters for future application in fertirrigation.

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