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Research Article

Cassava Wastewater and Solid Waste Leachate as Cyanogenic Substrates for the Growth of Nitrile and Linamarin-Utilizing Bacteria

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ABSTRACT

The direct discharge of wastewaters containing cyanogenic compounds poses severe health hazards, hence this study aimed to establish the biodegradative potential of nitrile and linamarin utilizing bacterial strains in the degradation of cyanogens in cassava wastewaters (CWW) and solid waste leachates (SWL). Glutaronitrile-utilizing bacterial strains (*Bacillus* sp. strain WOD8 KX774193 and *Corynebacterium* sp. strains WOIS2 KX774194) were isolated from solid waste leachates while linamarin-utilizing bacteria strains (*Bacillus pumilus* strain WOB3 KX774195 and *Bacillus pumilus* strain WOB7 KX774196) were isolated from cassava wastewaters. They were identified on the basis of morphological and biochemical characteristics, microscopic and 16S rRNA gene sequencing. Microbial growth assessment coupled with pH changes were performed under aerobic batch conditions. Growth was evaluated at intervals (2 days) by the intensity of turbidity (O.D. _{600 nm}) in CWW and SWL media. The doubling times of strains WOD8 and WOIS2 when grown on CWW and SWL (without supplementing mineral salts medium) were 12.83 and 10.83 d (specific growth rate, μ : 0.054 and 0.064 d⁻¹) and 20.38 and 17.77 d (μ : 0.034 and 0.039 d⁻¹) respectively. Also, strains WOD8 and WOIS2 grew on supplemented CWW and SWL with doubling times of 10.04 and 9.9 d (μ : 0.069 and 0.070 d⁻¹) and 16.12 and 16.12 d (μ : 0.043 and 0.043 d⁻¹) respectively. Similarly, the doubling times of strains WOB3 and WOB7 when grown on CWW and SWL (without supplementing mineral salts medium) were 8.25 and 7.53 d (μ : 0.084 and 0.092 d⁻¹) and 8.66 and 9.90 d (μ : 0.080 and 0.070 d⁻¹) respectively. Whereas, the same strains had doubling times of 6.30 and 5.78 d (μ : 0.11 and 0.12 d⁻¹) and 6.30 and 9.24 d (μ : 0.11 and 0.075 d⁻¹) respectively when grown on supplemented CWW and SWL. It would appear that CWW has the highest potential as a natural growth substrate than SWL, and its use for biomass production may contribute to a reduction in the overall environmental impact generated by discarding cyanogenic residues.

Keywords: Cassava wastewater, solid waste leachate, bacterial strains, doubling times, specific growth rates, cyanogenic

Introduction

The cyanide molecule can exist in the environment as organic nitriles (R-CN) or as cyanogenic glucosides found in plants like cassava. Cyanide is also discharged into the environment due to coal combustion, refining of petroleum, iron and steel production, burning of plastics, incineration of

household waste, combustion of fossil fuels, and cigarette smoking [1]. An effort of government is to boost cassava production to turn the cassava sector in Nigeria into a major player in local and international industries. Wastes generated by cassava processing pose serious environmental pollution problems [2]. The direct discharge of

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wastewaters containing these nitriles and other cyanogenic compounds poses severe health hazards as most of them are highly toxic while some are mutagenic and carcinogenic in nature. The release of cyanide molecules (nitriles) into water bodies results in letting cyanide, which persists in the soil or surface water causing severe environmental pollution and hence requires detoxification. Cassava production has increased over time and Nigeria is one of the largest producers of cassava as raw material for starch and ethanol production [3, 4]. This implies a release of cyanogenic glucosides into the environment and there is a need to address this challenge. In Nigeria, there is virtually little or no official data relating to the harmful effects of toxic wastes on the environment. Biological approaches have been explored in the past to bring down the level of cyanide and nitriles in the environment. It has been found that certain microorganisms are able to detoxify cyanide from wastes by the formation of organic or inorganic acids (protons), by oxidation and reduction reactions [5, 6]. A series of organic acids are formed by bacterial (as well as fungal) metabolism resulting in organic acidolysis, complex and chelate formation [7, 8]. Cyanide is formed as a secondary metabolite. Unfortunately, many industrial processes produce cyanide as a by-product and large quantities of it must be disposed of. Chemical methods of cyanide degradation are expensive; there have been many cases reported of cyanide pollution and the dangerous practice of "dumping" cyanide wastes. Blumer and Haas [9] reported that its formation has an advantage for the organism by inhibiting competing microorganisms. In certain ecosystems, cyanogenic microorganisms represent a large part (up to 50%) of the soil microbial community [10]. However, cyanide is formed at early stationary phase during growth only within a short time period [11]. A large amount of cyanide formation can be generated only in certain growth media under specific conditions [12, 13]. In this connection, this study aims to increase the knowledge base of bioremediation potentials of the nitrile and linamarin-degrading organisms as well as their biodegradation of environmental nitrile-contaminants.

Material and Methods

Microorganisms and inoculum development

Nitrile and linamarin-utilizing bacterial strains

were isolated and identified as previously described [14, 15]. They were identified on the basis of morphological and biochemical characteristics, light microscopy and 16S rRNA gene sequencing. The bacterial isolates are *Bacillus* sp. strain WOD8 KX774193, *Corynebacterium* sp. strain WOIS2 KX774194, *Bacillus pumilus* strain WOB3 KX774195 and *Bacillus pumilus* strain WOB7 KX774196. In previous laboratory studies, these glutaronitrile utilizing strains and linamarin utilizing strains showed abilities to utilize cyanogenic compounds for growth. The isolates were re-suscitated using nutrient agar and pre-enrichment was carried out using Luria-Bertani broth.

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) in cassava wastewater (CWW) (20% v/v) and solid waste leachate (SWL) (50% v/v) of cyanogenic substrates as sole carbon sources for a period of 12 days. The mineral salts medium (MSM) (100 mL, pH 7.2) used for supplementation of each natural cyanogenic substrate contained; K₂HPO₄ 2.5; KH₂PO₄ 2.0; MgSO₄·7H₂O 0.5; MnSO₄·4H₂O 0.1; CaCl₂·2H₂O 0.06; FeSO₄·7H₂O 0.1; Na₂MoO₇·2H₂O 0.006. The pH of the medium was adjusted to 7.2. The cyanogenic CWW and SWL were tested for their ability to support the growth of the nitrile and linamarin-utilizing bacterial species. The culture fluids were sampled at an interval of 24 h and were tested for cyanogenic glucoside utilization and hydrogen cyanide (HCN) production over a period of 12 days. Controls (uninoculated cyanogenic medium with and without supplementing mineral salts medium, inoculated cyanogenic medium with and without supplementing mineral salts medium) were put in place and monitored to rule out contamination. Two sets of controls (the uninoculated cyanogenic medium with and without supplementing mineral salts medium and the inoculated cyanogenic medium with and without supplementing mineral salts) were put in place to monitor the extent of growth of each isolate and to rule out contamination of the setup. The extent of growth was determined by the turbidity of the growth medium compared to the controls. Bacterial growth was determined spectrophotometrically (Beckman coulter, DUR 800, Beckman coulter Inc. Fullerton, CA) at 600 nm.

Hydrolysis of cyanogen was measured in terms of estimation of HCN spectrophotometrically at 540 nm [16]. The release of HCN is the indicator of cyanide cleavage which simultaneously increases the pH of the medium, which was also measured throughout the experiment.

Linamarin extraction

Linamarin was extracted as previously described by Ogunyemi *et al.* [17] and this was used for hydrogen cyanide (HCN) assay by test organisms.

HCN assay

Culture filtrates obtained at intervals (48 h) from each of the culture flasks were centrifuged ($10,000 \times g$, 4°C , 10 min). The supernatants were disposed of (cell debris) and were used for the HCN assay. HCN was measured as described earlier (16) by monitoring the production of hydrocyanic acid (HCN) using a UV-visible spectrophotometer (Thermoscientific TM Spectronic 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 and the reaction was halted 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.

Results and Discussion

Growth profiles of test organisms on cassava wastewaters and solid waste leachates

The test organisms studied utilized cyanogens in CWW and SWL as substrate for growth. Doubling times of strains WOD8, WOIS2, WOB3 and WOB7 and their specific growth rates are presented in Table 1 while the time-course growth profiles are shown in Figures 1 and 2. The doubling times of strains WOD8 and WOIS2 when grown on CWW and SWL (without supplementing MSM) were 12.83 and 10.83 d (specific growth rate, μ : 0.054 and 0.064 d^{-1}) and 20.38 and 17.77 d (μ : 0.034 and 0.039 d^{-1}) respectively. Also, strains WOD8 and WOIS2 grew on supplemented CWW and SWL with doubling times of 10.04 and 9.9 d (μ : 0.069 and 0.070 d^{-1}) and 16.12

and 16.12 d (μ : 0.043 and 0.043 d^{-1}) respectively. Similarly, the doubling times of strains WOB3 and WOB7 when grown on CWW and SWL (without supplementing MSM) were 8.25 and 7.53 d (μ : 0.084 and 0.092 d^{-1}) and 8.66 and 9.90 d (μ : 0.080 and 0.070 d^{-1}) respectively. Whereas, the same strains had doubling times of 6.30 and 5.78 (μ : 0.11 and 0.12 d^{-1}) and 6.30 and 9.24 (μ : 0.11 and 0.075 d^{-1}) respectively when grown on supplemented CWW and SWL. Abiona *et al.* [18] stated that effluent from cassava had high cyanide content and was nutritive enough to support microbial growth, which agrees with this work. Mirzadeh *et al.* [19] reported cyanide utilization occurred mainly during the exponential phase of growth and the highest growth rate (1.23×10^8) was obtained on day 4 of the incubation time by strain C. *Bacillus* species have been reported to biodegrade cyanide to non-toxic end-products by using cyanide as a nitrogen source [20, 21]. Castric and Conn [22] reported the metabolism of cyanide by *Bacillus* and proposed the presence of a metabolic pathway involving the condensation of serine with cyanide to form betacyanoalanine followed by the hydrolysis of the latter to asparagine and then to aspartic acid. The growth of *Pseudomonas* in a cyanide medium requires that cyanide be enzymatically converted to ammonia which is then readily assimilated into cellular nitrogen [23, 24]. Maniyam *et al.* [25] reported that the cyanide biodegradation ability of *Rhodococcus* UKMP-5M was greatly affected by the presence of organic nutrients in the medium. In this study wastewater fortified with mineral salts medium promoted the highest growth rate of the bacteria species which simultaneously assisted complete biodegradation. Our findings corroborate with reports by Maniyam *et al.* [25] that observed the effect of presence of salts and glucose on microbial growth.

The change in pH

Continuous monitoring of pH revealed a slight increase in pH from 7.2 to 7.56 and 7.2 to 7.62 of the CWW medium (with and without supplementing MSM), respectively, inoculated with strain WOD8, (Figure 3). While a progressive increase in the pH from 7.2 to 8.34 and 7.2 to 8.88, respectively as observed with SWL medium (with and without supplementing MSM), when inoculated with the same strain (Figure 3). Also, strain WOIS2 when cultured on CWW medium (with

Table 1. Growth potentials of linamarin-utilizing bacteria grown on cyanogenic cassava wastewaters and solid waste leachates supplemented with or without mineral salts medium

Isolate	Substrate							
	CWW		CWW+MSM		SWL		SWL+MSM	
	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
WOD8	0.054	12.83	0.069	10.04	0.034	20.38	0.043	16.12
WOIS2	0.064	10.83	0.070	9.9	0.039	17.77	0.043	16.12

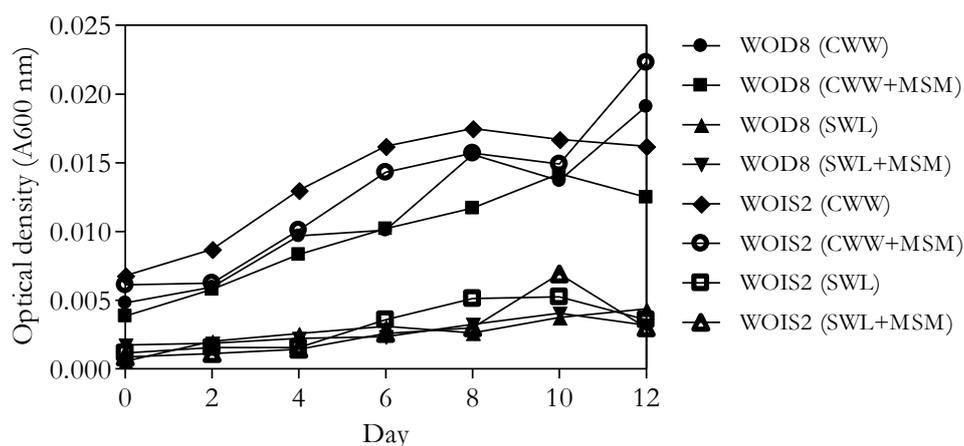


Figure 1. Growth profile of nitrile-utilizing bacteria grown on cassava wastewaters and solid waste leachates. WOD8: *Bacillus* sp. KX774193; WOIS2: *Corynebacterium* sp. KX774194; CWW: Cassava wastewater (without supplementing mineral salts medium); CWW+MSM: Cassava wastewater plus mineral salts medium (with supplementing mineral salts medium); SWL: Solid waste leachate (without supplementing mineral salts medium); SWL+MSM: Solid waste leachate plus mineral salts medium (with supplementing mineral salts medium)

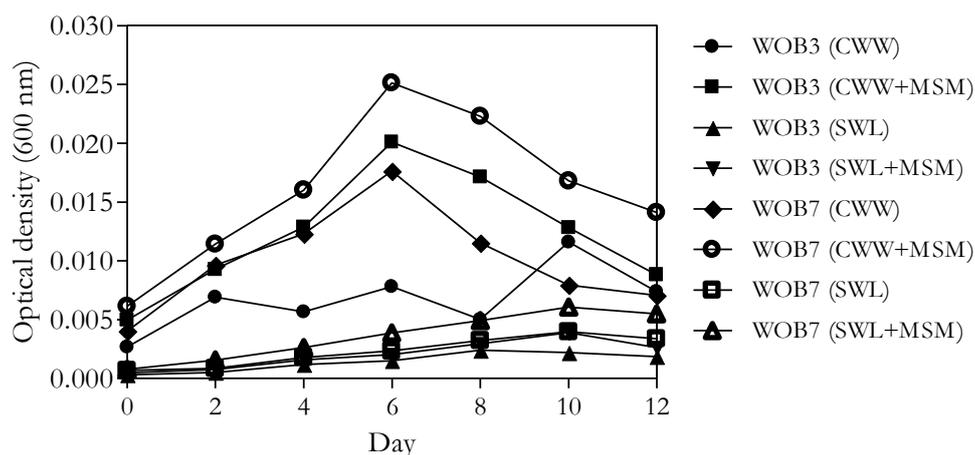


Figure 2. Growth profile of linamarin-utilizing bacteria grown on cassava wastewaters and solid waste leachates (with or without mineral salts medium). WOB3: *B. pumilus* KX774195; WOB7: *B. pumilus* KX774196; CWW: Cassava wastewater (without supplementing mineral salts medium); CWW+MSM: Cassava wastewater plus mineral salts medium (with supplementing mineral salts medium); SWL: Solid waste leachate (without supplementing mineral salts medium); SWL+MSM: Solid waste leachate plus mineral salts medium (with supplementing mineral salts medium)

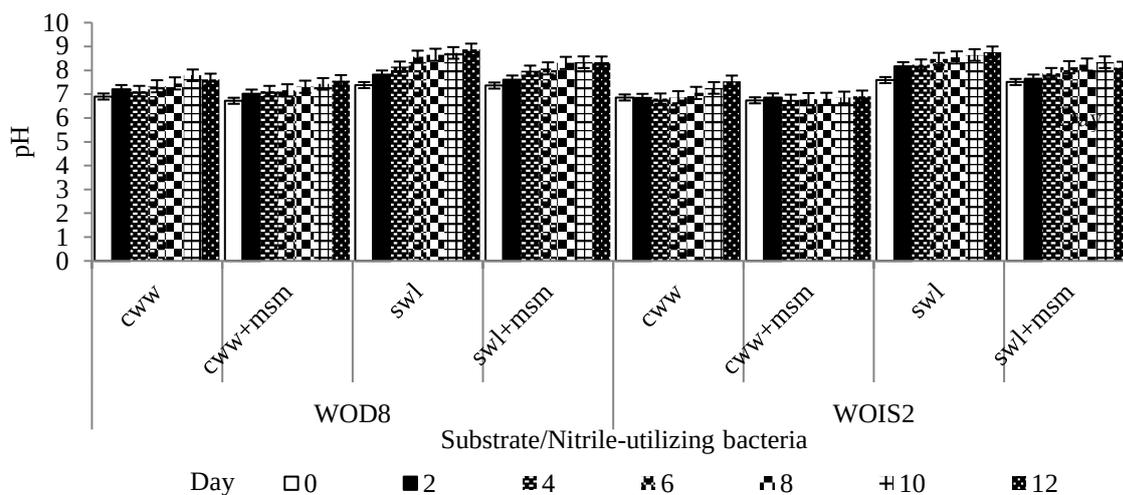


Figure 3. pH changes of culture fluid of nitrile-utilizing bacteria on cassava wastewaters and solid waste leachates. WOD8: *Bacillus* sp. KX774193; WOIS2: *Corynebacterium* sp. KX774194; CWW: Cassava wastewater (without supplementing mineral salts medium); CWW+MSM: Cassava wastewater plus mineral salts medium (with supplementing mineral salts medium); SWL: Solid waste leachate (without supplementing mineral salts medium); SWL+MSM: Solid waste leachate plus mineral salts medium (with supplementing mineral salts medium)

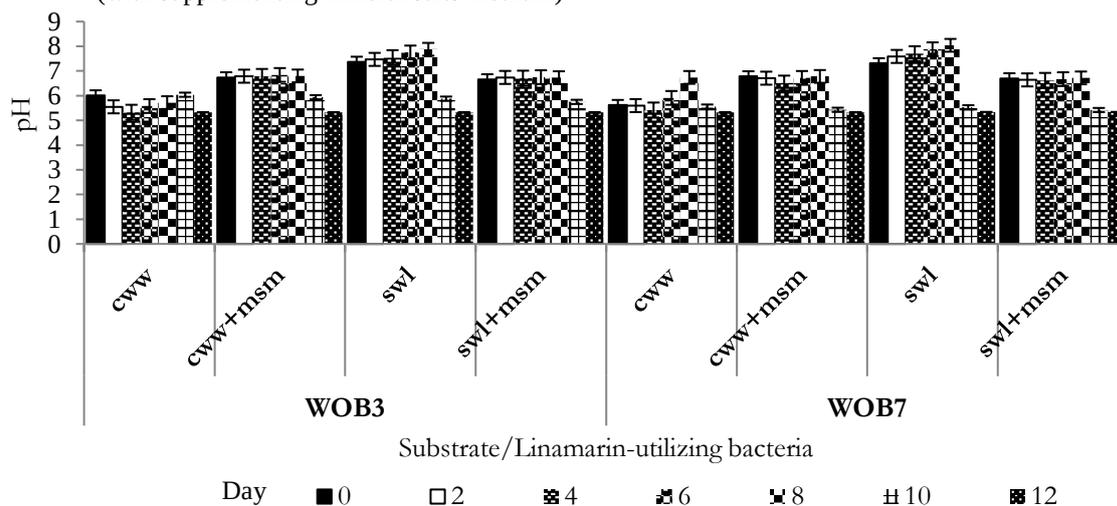


Figure 4. pH changes of culture fluid of linamarin-utilizing bacteria on cassava wastewaters and solid waste leachates. WOB3: *B. pumilus* KX774195; WOB7: *B. pumilus* KX774196; CWW: Cassava wastewater (without supplementing mineral salts medium); CWW+MSM: Cassava wastewater plus mineral salts medium (with supplementing mineral salts medium); SWL: Solid waste leachate (without supplementing mineral salts medium); SWL+MSM: Solid waste leachate plus mineral salts medium (with supplementing mineral salts medium)

and without supplementing MSM), the pH of the culture medium slightly dropped from 7.2 to 6.91 and increased from 7.2 to 7.54, respectively. Whereas in SWL (with and without supplementing MSM) cultured 7.2 to 8.12 and 7.2 to 8.76, respectively, was noted (Figure 3). Similarly, in CWW medium (with and without supplementing MSM) inoculated with strain WOB3, the pH of the

culture medium slightly dropped from 7.2–5.33 in both cases, respectively (Figure 4). While in the SWL medium (with and without supplementing MSM) inoculated with the same strain, the pH slightly declined from 7.2 to 5.32 in both cases, respectively (Figure 4). Whereas in CWW medium (with and without supplementing MSM) grown with WOB7, it appeared to be a slight drop

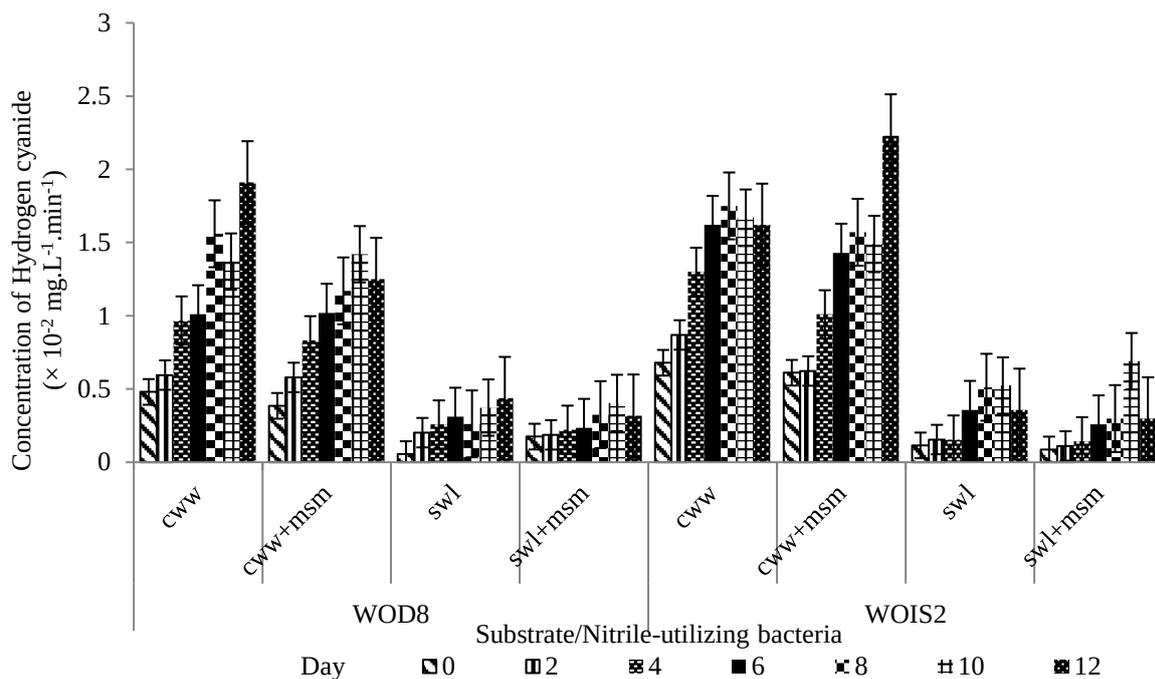


Figure 5. Hydrogen cyanide production by nitrile-utilizing bacteria when grown on cassava wastewaters and solid waste leachates. CWW: Cassava wastewater (without supplementing mineral salts medium); CWW+MSM: Cassava wastewater plus mineral salts medium (with supplementing mineral salts medium); SWL: Solid waste leachate (without supplementing mineral salts medium); SWL+MSM: Solid waste leachate plus mineral salts medium (with supplementing mineral salts medium)

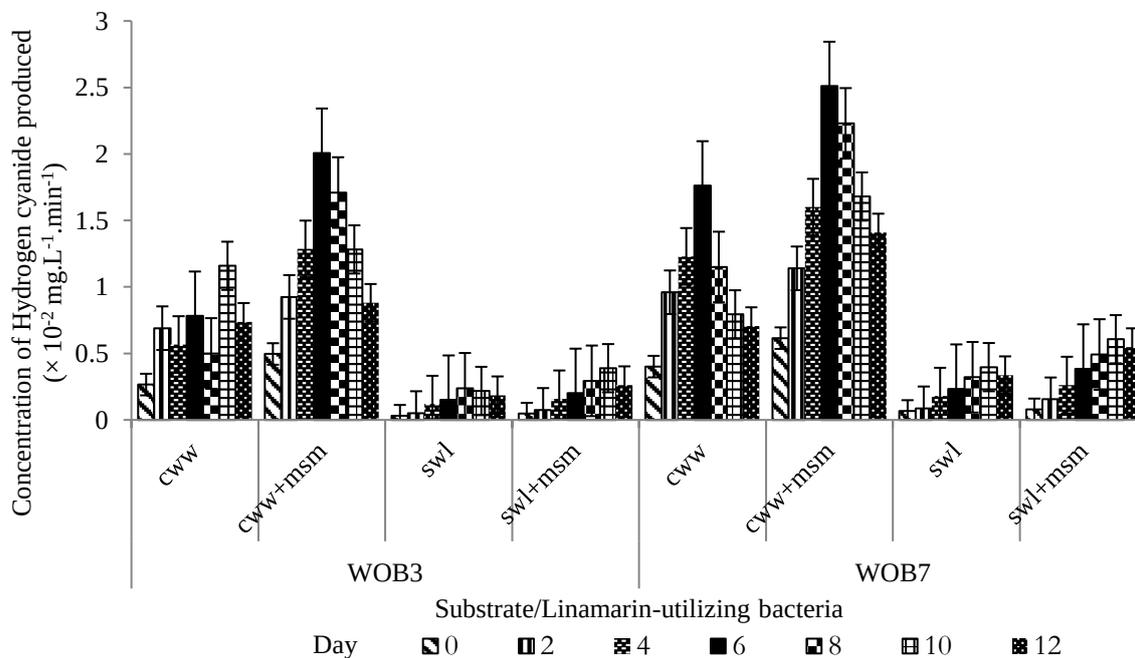


Figure 6. Hydrogen cyanide production by linamarin-utilizing bacteria when grown on cassava wastewaters and solid waste leachates. CWW: Cassava wastewater (without supplementing mineral salts medium); CWW+MSM: Cassava wastewater plus mineral salts medium (with supplementing mineral salts medium); SWL: Solid waste leachate (without supplementing mineral salts medium); SWL+MSM: Solid waste leachate plus mineral salts medium (with supplementing mineral salts medium)

in pH from 7.2 to 5.33 and 7.2 to 5.32, respectively (Figure 4). While the growth of the same strain on SWL (with and without MSM), the pH slightly dropped from 7.2 to 5.34 in both cases, respectively (Figure 4).

Cassava wastes can be degraded with microorganisms under acidic conditions [26]); however, the process is time-consuming and requires technical know-how. A report had that some microorganisms are able to degrade cyanide at neutral or acidic conditions, but under this condition, a high concentration of cyanide evaporates as hydrocyanic acid (HCN), a weak acid with a pKa value of 9.2 [27]. This was in line with the linamarin-utilizing bacteria which degraded cyanogens in the wastewaters at acidic ranges.

Hydrocyanic acid production by the test organisms

Bacillus sp. strains WOD8 and *Corynebacterium* sp. strain WOIS2 had accumulated 1.91×10^{-2} and 1.75×10^{-2} mg.L⁻¹.min⁻¹ of hydrogen cyanide (HCN) on CWW (without supplementing MSM) whereas 1.42×10^{-2} mg.L⁻¹.min⁻¹ and 2.23×10^{-2} mg.L⁻¹.min⁻¹ of hydrogen cyanide (HCN) were obtained on supplemented CWW (Figure 5). While the same strains when grown on SWL (without supplementing MSM), produced 4.37×10^{-3} mg.L⁻¹.min⁻¹ and 5.24×10^{-3} mg.L⁻¹.min⁻¹ of HCN whereas 4.05×10^{-3} and 6.9×10^{-3} mg.L⁻¹.min⁻¹ of HCN had accumulated by the same strains on supplemented SWL (Figure 5). Similarly, strains WOB3 and WOB7 had produced 1.116×10^{-2} mg.L⁻¹.min⁻¹ and 1.76×10^{-2} mg.L⁻¹.min⁻¹ HCN when grown on CWW (without supplementing MSM) whereas the same strain liberated 1.29×10^{-2} mg.L⁻¹.min⁻¹ and 2.51×10^{-2} mg.L⁻¹.min⁻¹ HCN on supplemented CWW (Figure 6). While strains WOB3 and WOB7 accumulated 2.38×10^{-3} mg.L⁻¹.min⁻¹ and 3.97×10^{-3} mg.L⁻¹.min⁻¹ HCN on SWL (without supplementing MSM) whereas the same strains gave 3.89×10^{-3} mg.L⁻¹.min⁻¹ and 6.07×10^{-3} mg.L⁻¹.min⁻¹ HCN on supplemented SWL (Figure 6). Mirizadeh *et al.* [19] found that cyanide degradation had reached a maximum level of 96% during the exponential phase. On a general note, nitrile-utilizing bacterial strains had declined in hydrogen cyanide production when the CWW and SWL media were supplemented with mineral salts medium. Unlike in the case of linamarin-utilizing bacterial strains, there was an increase in the

production of hydrogen cyanide in supplemented media. Murugan *et al.* [31] reported that *B. subtilis* was able to grow and produce hydrogen cyanide (HCN) in addition to ammonia during cyanogenic glycoside utilization.

In most of the recent studies, researchers carried out on cyanide removal using different bacteria under different conditions. Most of the bacteria were mesophilic while optimal temperature and pH ranged were about 25 – 30°C and 5.5 to 11.5 respectively [30]. Ezzi and Lynch [33] studied cyanide removal at a concentration of 2,000 ppm and successfully removed total cyanide at 90 days. Other researchers investigated the effect of lower amounts of cyanide. Naveen *et al.* [34] found that 83% removal of cyanide with an initial concentration of 150 ppm at 120 h. Özel *et al.* [35] investigated the use of fungi for cyanide removal and found that by using *S. commune*, *P. arcularis*, and *G. lucidum* at pH 10.5 for 42 h, cyanide degradation was almost 100%, but the initial cyanide concentration was just 25 ppm. It showed that by increasing cyanide concentration up to 200 ppm, the removal efficiency decreased to less than 50%.

Conclusion

In this study, it was established that the test organisms grew on both cassava wastewaters and solid wastewaters containing cyanogenic glucosides and used as natural substrates or as cheap alternative carbon sources. Therefore, nitrile and linamarin-utilizing bacterial species are prospective seed candidates for the bioremediation of polluted sites and in the treatment of waste effluents containing cyanogenic compounds.

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