CHARACTERIZATION OF CYANIDE DEGRADING AMYLOLYTIC BACILLUS SPECIES ISOLATED FROM CASSAVA EFFLUENT

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Abstract
Cassava tubers are excellent source of carbohydrates which are cultivated extensively as a staple food in tropical regions. It is of high economic value and nutrient, however the presence of high cyanide content constitutes a major concern among cassava processors and consumers. Therefore, there is need to search for an efficient method to reduce cyanide content present to the recommended amount for human consumption. Thus, this research was designed to characterize bacteria isolated from cassava effluent with the ability to effectively hydrolyse starch and reduce cyanide present in cassava. Isolation and identification of cyanide degrading-amylolytic bacteria from cassava effluent was carried out using the culture dependent, phenotypic and molecular methods respectively. Growth in minimum medium, cyanide reduction efficiency, production of ammonia, linamarase and amylase activities, growth at different temperatures and pH were monitored spectrophotometrically. Eighteen Bacillus species were isolated, only four were non pathogenic and simultaneously utilized cyanide and hydrolyzed starch. The two isolates with the highest cyanide utilizing and starch hydrolyzing abilities were identified as Bacillus pumilus CN1 and Bacillus megaterium CN3. These Bacillus species grew well in minimum medium with an optimum growth of 0.676 and 1.200 at 72 hours respectively. They also exhibited cyanide removal efficiency of 98% and 99% respectively at 500ppm KCN at 96 hours of incubation. They produced considerable quantities of linamarase and amylase at optimum temperature of 37^0C and pH 8.0. In conclusion, the two isolates could be used as potential starter cultures in cassava fermentation for the production of cassava based foods.

Key words: Cyanide, Linamarase, Amylase, Bacillus spp. Cassava, Effluent.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is a root crop consisting of starchy deposit which is a source of calories for more than 500 million people worldwide (FAO, 2000). It provides about 60% of the daily calorific requirements of Africa and Central America. It has been rated as the third most important food crop after rice and maize in the developing countries (Kobawila *et al.*, 2005). Cassava is resistant to drought, pest, disease, and produces high yield under unfavourable environmental conditions (Edward *et al.,* 2012). Nigeria is one of the leading producers of cassava in the world in excess of about 26 million tons (Onwuka and Ogbogu, 2007; Okpako *et al.,* 2008) and over 95% of the harvested cassava is processed into foods for humans. All morphological parts of the plant are valuable, the leaves are used for making soup and feed for livestock, while the stems are used for cultivation, mushroom production and cooking as firewood. In addition, the tuber is eaten fresh or processed into flour and can be used for the production of bio-fuel and industrial starch while the young leaves are consumed particularly in Africa because of its high protein content (McMahon *et al.,* 1995; Achidi *et al.,* 2005; Lebot, 2009) Nutritionally cassava tubers are made up 85-90% carbohydrate, 1.3% protein and high level of cyanide in the form of cyanogenic glucosides, linamarine and lotaustraline, which constitutes a major safety problem among cassava processors and consumers (Dunstan *et al.,* 1996; Oyewole and Afolami, 2001; Nwabueze and Odunsi, 2007; Owuamanam *et al.,* 2010). Long term consumption of substantial amount of cyanide has been implicated in the etiopathogenesis of serious health problems such as tropical ataxic

neuropathy, Konzo- aspastic paraparesis of the leg, fibrocalculous pancreatic diabetes (FCPD), glucose intolerance coupled with iodine deficiency which leads to goitre and cretinism had been reported (Mathangi *et al.*, 2000; Lambien *et al.*, 2004; Kaewkannetra *et al.*, 2009; Bandna, 2012). The toxicity of cyanide is achieved by attaching itself to cytochrome oxidase an important iron containing enzymes required for aerobic respiration in the living cells. The traditionally developed methods of processing cassava such as cooking, sun-drying, oven-drying and roasting have been reported to be inefficient in the removal of cyanogens present to safe level recommended for human consumption (Maopoog, 1989; Owuamanam *et al.,* 2010; Murugan *et al.,* 2012). In this study attempt was made to isolate bacteria from cassava effluent and characterize them for use as potential starter cultures in the fermentation/production of cassava based products.

MATERIALS AND METHODS
Collection of samples
Cassava effluent was collected from Orogun cassava processing plant located at Water corporation bus stop opposite University of Ibadan Nigeria in sterile container and transported to the Applied food microbiology laboratory University of Ibadan for subsequent analysis.

Isolation of bacteria from cassava effluent
The sample was serially diluted by transferring 10 ml into 90 ml of sterile distilled water and 0.1 ml of 10^{-4} dilution was inoculated into nutrient agar in sterile Petri dish using pour plate method of Harrigan and McCance (1966). Incubation was carried out at 37^0C for 24 hours. Representative colonies were selected and streaked to obtain pure isolates which were maintained on nutrient agar slants at -4^0C for further use.

Safety Evaluation
Gelatinase activity
This was carried out using the method described by Smith and Goodner (1959).
DNase

The DNAse agar (Oxoid, Wesel, Germany) was used to carry out this test. Spot inoculation of the test isolates was done and incubated at 37^0C for 4days. HCl (1M) was poured on the agar and a zone of clearance around the bacterial growth indicated a positive result (Deighton *et al.,* 1988).

Heamolysis
A colony of each of the bacterial isolates was inoculated onto freshly prepared blood agar plates and incubated at 37^0C for 48 hours. Halos around the colonies indicated alpha-haemolysis or clear beta-heamolysis. The isolates that were not pathogenic or with safety properties were used for further study (Akinjogunla *et al.*, 2014)

Isolation of cyanide-degrading bacteria.
The isolation procedure was carried out as described by Perumal *et al*. (2013). The two isolates with the highest cyanide degrading abilities were selected for further study.

Screening for Amylase producing bacteria
This was done according to the method described Thippeswamy *et al.* (2006). The two isolates with the highest cyanide degrading-amylolytic abilities were selected for further study.

Identification Procedure
The two isolates with the highest cyanide-degrading amylolytic activities were identified using phenotypic and molecular methods The Phenotypic methods considered the following parameters such as Gram staining, Spore stain, Indole test, Starch hydrolysis, Gelatin hydrolysis,Urease, Methyl red Motility, Catalase, Casein hydrolysis, Citrate, Oxidase and Sugar fermentation

Molecular identification
DNA extraction and PCR amplification of bacterial 16S rDNA were carried out using the methods described by Gomaa and Montaz (2006) while sequencing was done according to the method described by Hengstmann *et al* .(1999)

Phylogenetic analysis
Phylogenetic tree was generated by performing distance matrix analysis using the NT system. Database search and comparisons were done with the BLAST search using the National

Center for Biotechnology Information (NCBI) database.

Growth of isolates in minimal media

This was done according to the method described Perumal *et al.* (2013). The pure bacterial isolates were grown in minimal medium (M9) which comprises of K_2HPO_4 (1.00g), KH_2PO_4 (3.40g), KCl (0.50g), $MgSO_4$ (0.50g), $FeSO_4$ (0.01g), Glucose (1%) and KCN in 1L of distilled water, pH adjusted to 7.0 and incubated in water bath set at 30^0C for 96hours. Samples were collected at 24 hours intervals and optical density values were read at 600 nm using UV spectrophotometer.

Cyanide degradation assay

Preparation of bacterial cell suspension

This was carried out according to the method of Atta *et al.*(2008)

Determination of cyanide concentration

The method Fisher and Brown (1952) was employed to determine cyanide concentration.

Determination of ammonia release

This was carried out according to the method described by Adjei and Ohta (1999).

Determination of linamarase activity

This was carried out according to the method described by Okafor and Ejiofor (1985)

Amylase enzyme extraction

The selected isolates were incubated at 37° C for 24 hours in 50 ml of 8% (w/v) starch medium in 250 ml Erlenmeyer flasks, placed in a shaker incubator at 120 rpm. The extracellular enzyme solutions were obtained by centrifugation at 5000 rpm for 20 minutes using a high speed centrifuge. The supernatant obtained was collected and used for enzyme assay.

Amylase Assay

Amylase activity was carried out according to the method described by Bertrand *et al*. (2004).

GrowthatdifferentpH

The two organisms were grown in the previously described composed medium with pH adjusted to 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0. Incubation was carried out at 30^0C for 48 hours and growth was determined using a spectrophotometer.

Growth at different Temperature

The two organisms were cultivated separately in the previously described composed medium and incubated at 25^0C, 30^0C, 35^0C and 37^0C for 48 hours and growth was determined using a spectrophotometer.

RESULTS AND DISCUSSION

The result of the safety assessment of the bacterial isolates is shown in Table 1. All the eighteen bacterial isolates showed negative reactions to haemolysis, Dnase and gelatinase tests. The result of screening for cyanide degrading- amylolytic bacteria is shown in table 2. All the isolates utilised cyanide but only isolates CN1, CN3, CN4 and CN6 were potential amylase producers which is evident by exhibiting different zones of starch hydrolysis of 18, 15, 05 and 08mm respectively.

The results of phenotypic and 16S rRNA identification methods of CNI and CN2 are shown in tables 3a, 3b, figures 1a and 1b respectively. The phenotypic identification showed that the two isolates are *B. pumilus and B.megaterium* based on the biochemical characteristics and with reference to Bergey's manual of systematic bacteriology while16S rRNA confirmed their Identities and phylogeny of the isolates CN1 and CN3 as *Bacillus pumilus* SAFR-032 and *Bacillus megaterium* QM B1551 respectively. The strains showed maximum similarity ratio towards *Bacillus pumilus* SAFR-032 (99%) accession number NC_074977.1 and a similarity of 99% identity with the sequence of 16S rRNA of *Bacillus megaterium* QM B1551 accession number NC_074290.1 respectively using NCBI data base and hence the isolates were named as *B. pumilus* CN1 and *B. megaterium* CN3. The phylogenetic tree was calculated using the neighbour-joining algorithm.

Table 1. Safety assessment of the bacterial isolates

Isolates code	Heamolysis test	DNase test	Gelatinase test
CN1	-	-	-
CN2	-	-	-
CN3	-	-	-
CN4	-	-	-
CN5	-	-	-
CN6	-	-	-
CN7	-	-	-
CN8	-	-	-
CN9	-	-	-
CN10	-	-	-
CN11	-	-	-
CN12	-	-	-
CN13	-	-	-
CN14	-	-	-
CN15	-	-	-
CN16	-	-	-
CN17	-	-	-
CN18	-	-	-

Table 2. Screening for cyanide degrading- amylolytic bacteria.

Isolate CN1 code	Cyanide utilization	Zone of hydrolysis(mm)
CN1	+	18
CN2	+	-
CN3	+	15
CN4	+	05
CN5	+	-
CN6	+	08
CN7	+	15
CN8	+	-
CN9	+	-
CN10	+	-
CN11	+	-
CN12	+	-
CN13	+	-
CN14	+	-
CN15	+	-
CN16	+	-
CN17	+	-
CN18	+	-

Table 3a. Morphological, biochemical and physiological properties of cyanide-degrading bacteria isolated from cassava effluent

Isolate codes	Colony morphology	Cell morphology	Catalase test	Gram stain	Spore stain	Vp	Oxidase	Growth at 45°C	Methyl red	6.5 NaCl	Motility	Indole	Argine	Casein	Starch	Gelatin	Urease	Propable Identity
CN1	Small, white	Rod	+	+	+	-	-	+	+	+	+	-	-	+	+	-	-	*Bacillus pumilus*
CN3	Large, white	Rod	+	+	+	-	-	+	+	+	+	-	-	+	+	-	+	*Bacillus megaterium*

Table 3b. Sugar fermentation pattern of CN1 and CN3

Isolate codes	Glucose	Fructose	Sucrose	Maltose	Mannose	Galactose	Xylose	Arabinose	Inositol	Inulin	Sorbitol	Lactose	Raffinose	Mannitol	Propable Identity
CN1	+	+	+	+	+	-	+	+	-	-		-	+	+	*Bacillus pumilus*
CN3	+	+	+	+	+	-	+	+	-	-		-	+	+	*Bacillus megaterium*
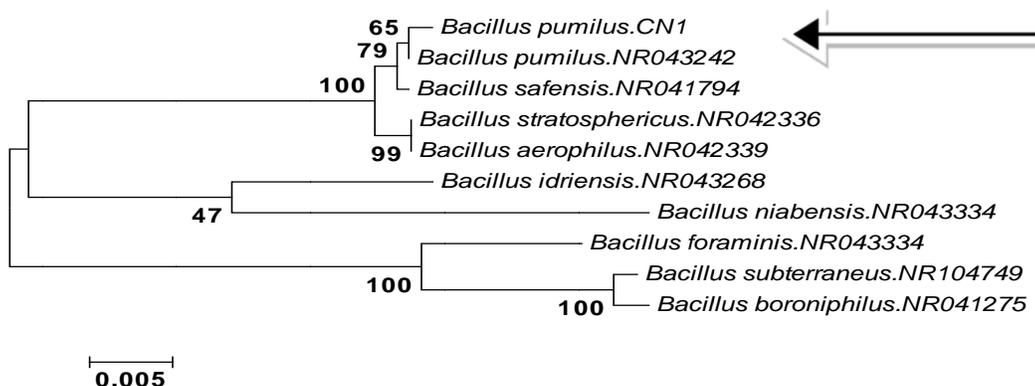
Figure 1a. Phylogenetic showing the evolutionary relationship of *B. pumilus* CN1 to closely related sequences obtained from the GenBank using MEGA 5.

❖ Multiple alignments of the sequences corresponding to the 16S rRNA of the studied isolate were carried out followed by neighbour joining clustering. Bootstrap values expressed as percentages of 1000 replications.
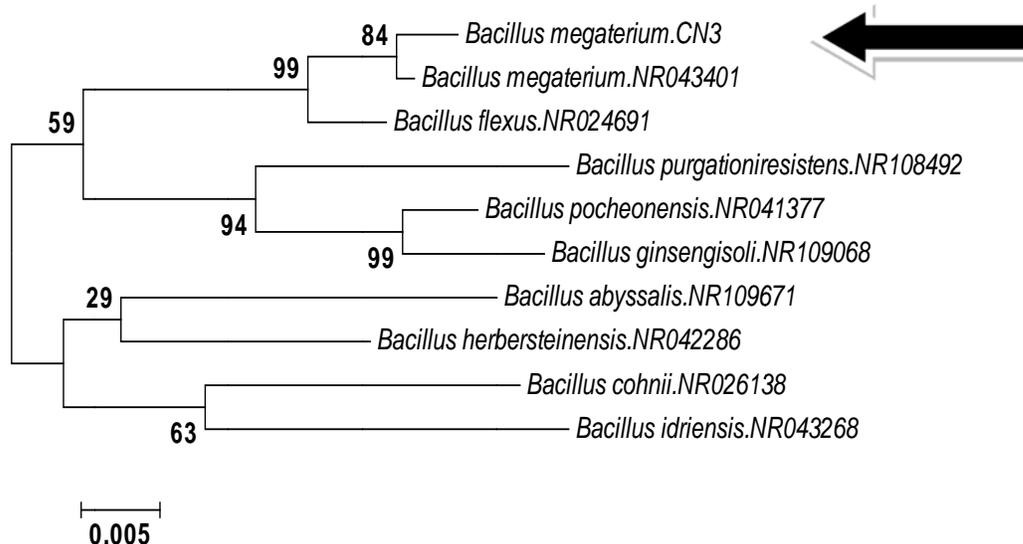
Figure 1b: Phylogenetic showing the evolutionary relationship of *B. megaterium* CN3 to closely related sequences obtained from the GenBank using MEGA 5.

❖ Multiple alignments of the sequences corresponding to the 16S rRNA of the studied isolate were carried out followed by neighbour joining clustering. Bootstrap values expressed as percentages of 1000 replication

The growth pattern of *B. pumilus* CN1 and *B. megaterium* CN3 in minimal medium is shown in Figure 2. The two microorganisms grew well in minimum medium with *B. pumilus* CN1 showing an optimum growth of 0.676 at 72 hours which decreased to 0.532 at 96 hours while *B. megaterium* CN3 had a maximum growth of 1.200 at 72 hours which decreased to 0.960 at 96 hours.

The results of Cyanide removal efficiency (Cyanide tolerance) for *B. pumilus* CN1 and *B. megaterium* CN3 at 96 hours of incubation in minimal medium with KCN (25, 50, 150 and 500ppm) as the sole nitrogen source are shown in Figures 3a, 3b, 3c, 3d. It was observed that at 24 hours period of incubation *B. pumilus* CN1 recorded 70% removal while *B. megaterium* showed 77% removal efficiency. However, at 96 hours of incubation *B. Pumilus and B. megaterium* recorded 96.8% and 98% removal efficiencies respectively (Fig 3a). *B.*

pumilus showed removal efficiency of 71% after 24 hours which increased to 97% at 96 hours of incubation while *B. megaterium* recorded 79% and 98% removal efficiencies respectively at 24 and 96 hours of incubation respectively (Fig 3b). Figure 3c shows the removal efficiency at 150 ppm. It was also noted that *B. pumilus* exhibited 87% removal efficiency at 24hr which increased to 97% removal efficiency at 96 hours while 89% and 99% were recorded for *B. megaterium* at 24 and 96 hours of incubation periods respectively. The result for removal efficiency of *B. pumilus* CN1 and *B. megaterium*CN3 at 500ppm is shown in Figure 3d. *B. pumilus* CN1 was able to remove 86% of KCN present while *B. megaterium* CN3 was able to remove 88% at 24 hours of incubation. 98% and 99% removal efficiency was recorded for *B. pumilus* CN1 and *B. megaterium*CN3 respectively at 96 hours of incubation
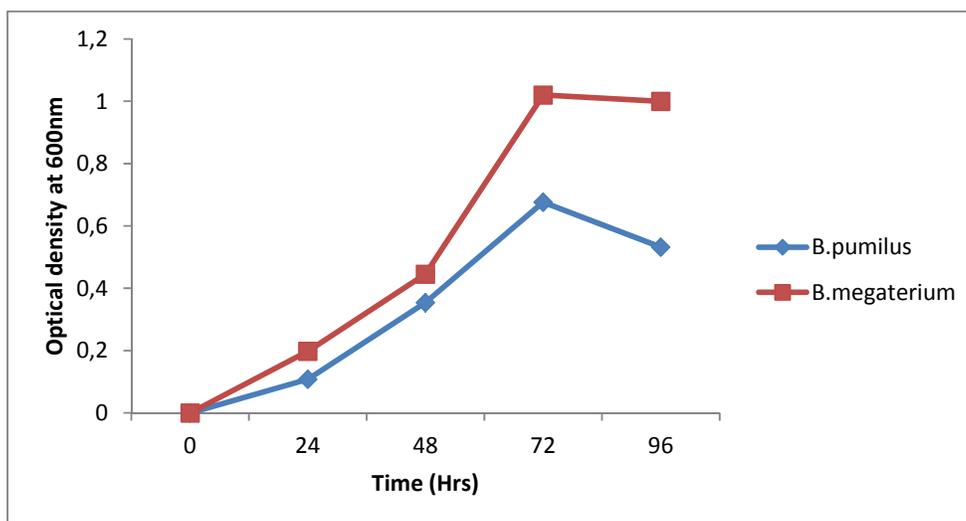
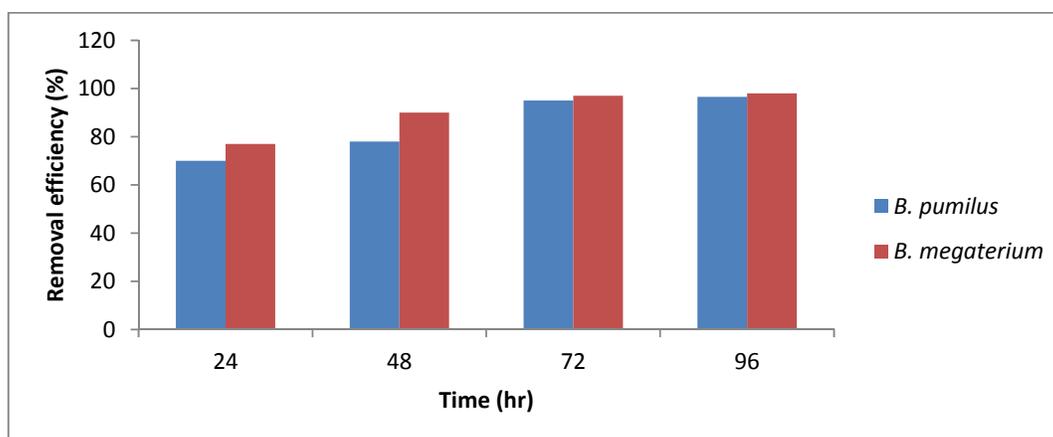**Figure 2**: Growth pattern of *B. pumilus* CN1 and *B. megaterium* CN3 in minimal medium

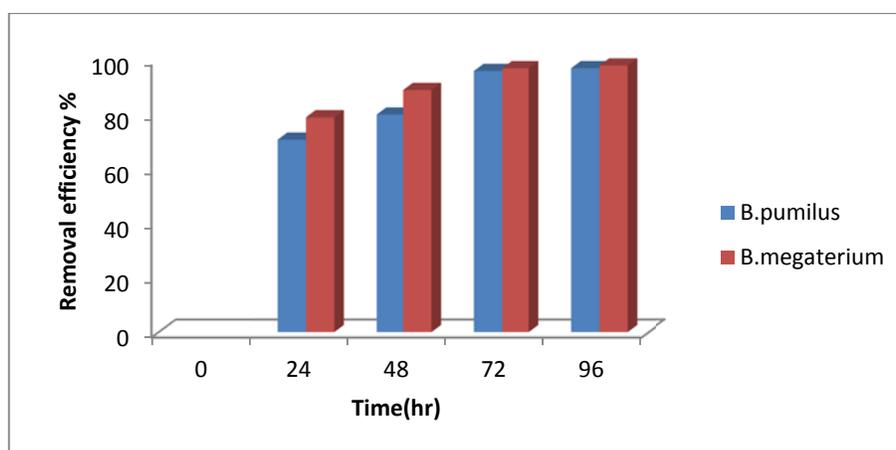**Figure 3a:** Cyanide removal efficiency for *B. pumilus* CN1 and *B. megaterium* CN3 at 25ppm

Figure 3b: Cyanide removal efficiency of *B. pumilus* CN1 and *B. megaterium* CN3 at 50ppm
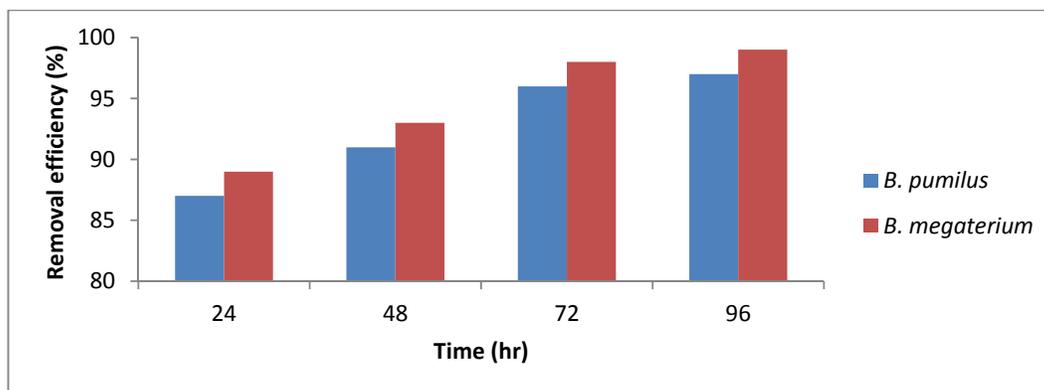
Figure 3c: Cyanide removal efficiency of *B. pumilus* CN1 and *B. megaterium* CN3 at 150ppm
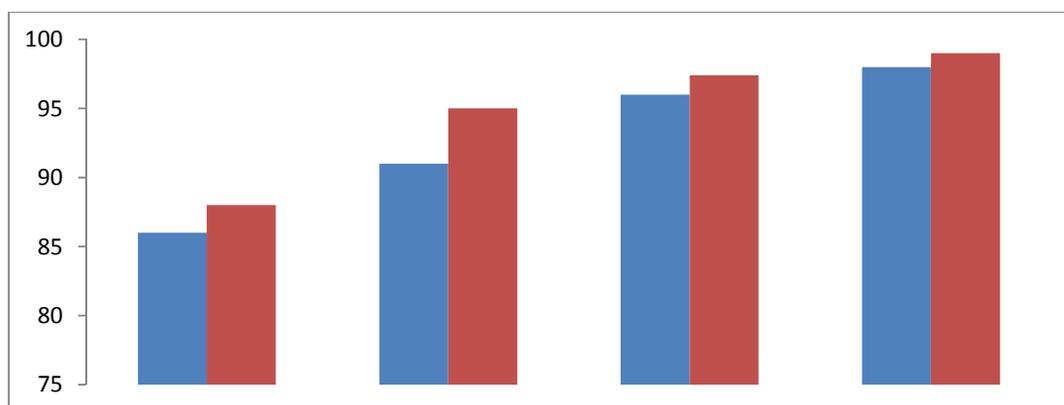

Figure 3d: Cyanide removal efficiency of *B. pumilus* CN1 and *B. Megaterium* CN3 at 500ppm

The quantities of ammonia released during cyanide degradation by *B. pumilus* CN1 *and B. megaterium* CN3 is depicted in figure 4. The quantity of ammonium produced by *B.megaterium* at 24 and 96 hours was 0.134 and 1.298 respectively while *B. pumilus* produced 0.089 and 1.012 at 24 and 96 hours respectively. Figure 5 shows the linamarase activities of *B. pumilus* CN1 *and B. megaterium.* It was observed that the two species of Bacillus had linamarase activities with *B. megaterium* CN3 producing the higher activity of 7.2 µmol/ml at 72 hours which decreased to 6.3 µmol/ml at 96 hours while *B.pumilus* produced an activity of 6.4 µmol/ml at 72 hours which decreased to 5.2 at 96h µmol/ml.

The amylase activities of two Bacillus species are presented in Figure 6. It was revealed that two Bacillus species had amylase activities with *B. megaterium* producing its optimum activity of 6.2µmol/ml at 72 hours which decreased ton4.7 µmol/ml at 96 hours while *B.pumilus* produced its optimum of 5.8 µmol/ml at 72 hours which decreased to 4.2 µmol/ml at 96 hours.c

The result of growth pattern of the two Bacillus species at different temperatures is shown in figure 7. *B. megaterium and B. pumilus* produced their optimum growth of 0.39 and 0.35 respectively at 37 ^{0}C.

Figure 8 shows the growth pattern of the two Bacillus species at different pH. *B. pumilus* and *B. megaterium* had their maximum growth of 0.58 and 0.51 respectively at pH 8.0.
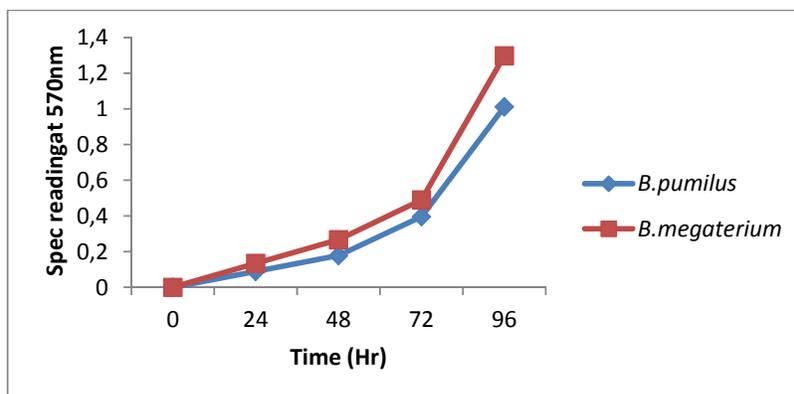
Figure 4. Ammonia release during cyanide degradation by *B. pumilus* CN1 *and B. megaterium* CN3.
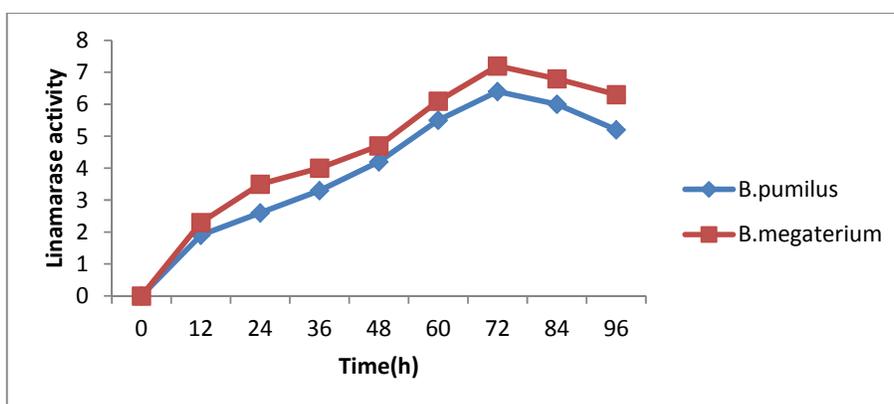

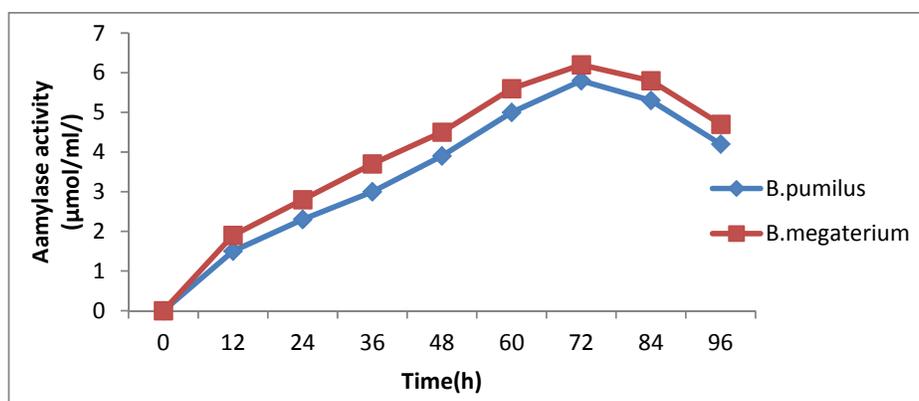**Figure 5:** Linamarase activities of *B. pumilus* CN1 *and B. megaterium*

Figure 6: Amylase activities of *B. pumilus* CN1 *and B. megaterium* CN3
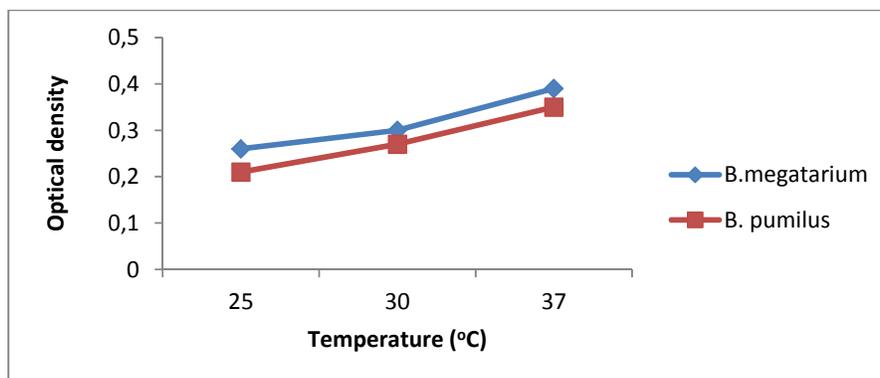
Figure 7: Growth Pattern of *B. pumilus* CN1 and *B. megaterium* at different Temperatures
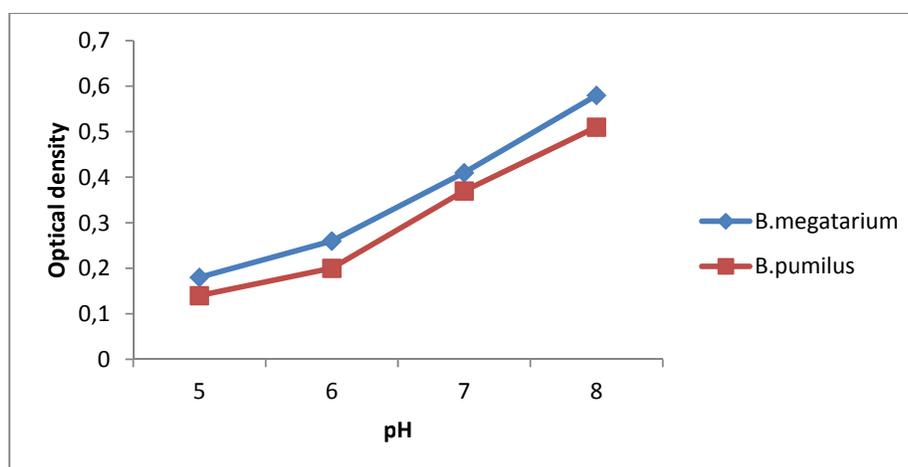

Figure 8: Growth Pattern of *B. pumilus* CN1 and *B. megaterium* at different pH

Discussion

The isolated bacteria from cassava effluent were identified using phenotypic and molecular methods as *Bacillus pumilus* and *Bacillus megaterium.* Full and partial 16S rRNA gene sequencing methods has been reported as a more objective, accurate, and reliable method for bacterial identification. In addition it possesses the capability of confirming taxonomic relationships among bacteria (Pretti *et al*., 2005) and identifying phenotypically aberrant microorganisms.

The isolation of Bacillus species from cassava effluent had earlier been reported by Dash *et al.*(2009) ; Dursun *et al.*(2009) ; Kumar *et al.*(2013); Wu *et al.*(2013). The survival of B*acillus* species in cyanide contaminated environment is due to their ability to grow and tolerate cyanide toxicity. There is the possibility of these microorganisms making use of the cyanide in this environment as a source of nitrogen for growth and metabolism. Perumal *et al.* (2013) had earlier reported the isolation of two *Bacillus* species in a minimal medium containing cyanide as the only nitrogen source which survived higher concentration of cyanide and grew in 6000mg/L and 7000mg/L of KCN, while Skowronski & Strobel (1969) isolated a strain of *Bacillus pumilus* which could survive in solutions containing 2.5 M KCN and grew in media containing KCN (2600mg CN) In addition, Sartry (1986) reported the isolation of some cyanide-metabolizing bacteria such as *Bacillus megaterium, B. pumilus, B. cereus,*

and *B. Stearothermophilus* from cassava effluent.

The ability of an organism to hydrolyse starch is an indication of a potential amylase producer. The two Bacillus species selected in this study were highly amylolytic which is evident from the wide zone of clearance they produced. This observation was similar to the earlier report of Anto *et al.* (2006).

The tolerance and degradative ability of the organisms were monitored by quantifying growth, cyanide degradation and ammonia released. The production of large quantity of ammonia coupled with the growth of the organisms showed that the organisms can tolerate high level of cyanide. Fisher e*t al.* (2000) had earlier reported similar finding. In this study it was observed that the quantity of ammonia released increased as the time of degradation increased or removal efficiency increased. This occurrence is in agreement with previous submissions which reported that free cyanide disappearance was accompanied by a concomitant increase in ammonia as one of the end-products of aerobic and anaerobic degradation of cyanide. The ability of an organism to metabolize cyanide is dependent on factors such as possession of a biodegradable pathway to convert cyanide into an assimilative product ($NH4^+$), cyanide resistance mechanism and a system for taking up Fe^{3+} from the medium. The presence of these pathways in organisms confers on them the ability to be used in the development of biotechnologies to degrade cyanide compounds or convert cyanide into ammonium (Ebbs, 2004; Luque-Almagro *et al*., 2005). In addition the degradation of cyanogenic glycosides contains a series of steps, such as the splitting of the sugar moieties from the aglycone/cyanohydrin by β-glycosidases, followed by the degradation of the cyanohydrin into a carbonyl compound and hydrogen cyanide (Vetter, 2000). It is evident from this study that both *B. pumilus* CN1 and *B. megaterium* CN3 were able to degrade cyanide and produce ammonia and this is in tandem with the earlier report of Peruma *et al.* (2013).

The safety assessment revealed the suitability of the bacteria as starter culture for food fermentation. The use of pathogenic organisms as starter cultures in food fermentation can lead to public health challenge for consumers. Linamarase and amylase activity are important factors in the selection of organism for cyanide degradation. The bacterial isolates had high amylase activity which is responsible for their starch hydrolysing activity. This observation is in conformity with earlier reports of Oyewole and Odunfa, (1990) ; Kimaryo *et al.* (2000); Anto *et al.* (2006) which confirmed the presence amylases in microorganisms. Pandey *et al*. (2000) described amylases as extracellular enzymes that randomly cleave the 1, 4-a-D-glucosidic linkages between adjacent glucose units in the linear amylose chain. *Bacillus* species had been reported as the most important sources of amylase and have been employed for amylase (enzyme) production in laboratory study and industrially (Anto *et al*., 2006). The result of amylase activity reported in this work is in conformity with the previous findings of Thippeswamy, *et al*. (2006); Rasooli *et al*. (2008); Oyeleke *et al*.(2010); Sethi *et al* (2013).

In this study, it is evident that there was considerable linamarase production. The two Bacillus spp. produced linamarase which is an indication of their ability to degrade cyanide. This observation corroborates the earlier submissions of Oyewole and Odunfa, (1990), Kimaryo *et al.* (2000), Cumbara *et al.* (2007) which emphasised the significance of linamarase enzyme in cyanide degradation. In addition, Okafor and Ejiofor (1985); Oyewole and Odunfa (1990); Kimaryo *et al.* (2000); Ahaotu *et al.* (2013) reported that linamarase producing microorganisms are useful in the liberation of cyanide from grated cassava during *gari* production. The optimum growth of the two organisms was seen at high pH which is an indication that they were alkaline in nature and could be suitable for alkaline fermentation. This observation is in conformity with the report of Maegala *et al*. (2011).

Temperature is an essential factor for growth and metabolism of microorganisms (Maegala *et al*., 2012). The temperature of natural

fermentation of cassava falls within the growth temperature range of the *Bacillus* spp. therefore, it can be inferred that they could be employed for the fermentation of cassava for the production of cassava based food products.

CONCLUSION

From the empirical results obtained from this study it could be concluded that the two *Baciilus* spp. demonstrated high cyanide degrading and starch hydrolysing abilities. Hence, they could be used as potential starter cultures in the fermentation of cassava and production of cassava based products with reduced cyanide and starch contents.

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