South Asian Journal of Research in Microbiology

10(4): 18-28, 2021; Article no.SAJRM.73854
ISSN: 2582-1989

Isolation and Identification of Bacteria and Fungi from Cassavamill Effluent in Afikpo, Ebonyi State Nigeria

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/SAJRM/2021/v10i430235
Editor(s):
(1) Dr. Ana Claudia Coelho, University of Tras-os-Montes and Alto Douro, Portugal.
Reviewers:
(1) Timothy Omara, Moi University, Kenya.
(2) Chuah Cheng Hock, University of Malaya, Malaysia.
Complete Peer review History: https://www.sdiarticle4.com/review-history/73854

Original Research Article

Received 09 July 2021
Accepted 19 September 2021
Published 25 September 2021

ABSTRACT

Wastes from cassava processing mills are usually discharged uncontrollably into the environment where they cause serious environmental challenges. This research work was undertaken to investigate the effects of cassava mill effluent on the microbiological quality of soils and to determine the effects of the polluted soils on the growth of plants. Fresh cassava mill effluent and unpolluted soil samples were collected and analyzed microbiologically. The soil sample was polluted with the cassava mill effluent and the polluted soil was also subjected to microbiological analyses for 31days. Germination and growth of pumpkin plants were also investigated using soil samples polluted with different concentrations of the cassava mill effluent. The microbial population of the effluent showed presence of *Staphylococcus aureus, Bacillus* spp*, Streptococcus* spp*, Pseudomonas aeruginosa, Aspergillus* spp*, and Candida* spp. The bacterial count was $1.4x10^4$cfu/ml while the fungal count was $1.1x10^3$cfu/ml. The results of the analyses of the unpolluted soil and the effluent polluted soil samples showed that the effluent had noticeable

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effects on the physicochemical and microbiological properties of the soil. The total microbial count increased from $2.2x10^5$ cfu/g to $4.6x10^5$cfu/g for bacteria and $1.6x10^4$cfu/g to $3.3x10^5$cfu/g for fungi. The polluted soil showed higher diversity of micro-organisms. The isolated organisms were: *Staphylococcus aureus, Bacillus* spp*, Streptococcus* spp*, Pseudomonas aeruginosa, Micrococcus* spp*, Proteus* spp*, Enterobacter* spp*, Aspergillus* spp*, Candida* spp*, Penicillium* spp*, and Rhizopus* spp*.* The polluted soil also, at higher concentrations of the effluent inhibited the germination of plants. This research revealed that effluents from cassava processing mills have significant effects on the microbiological properties of the surrounding soils and also affected the growth of plants on these soils negatively. It therefore suggested that this effluent should be treated properly before disposal to the environment.

Keywords: Cassava mill; effluent; microbiology; bacteria; Fungi.

1. INTRODUCTION

Like rice and maize, cassava (*Manihot esculenta*) which belongs to the Euphorbiaceae family is a major staple food in Africa especially Nigeria [1]. It contains laticifers and produces latex. The cassava plant is said to originate in Northeast Brazil with an additional Centre of origin in Central America, from these Centers, the crop spread to several parts of the world including Africa, Asia and America especially in the tropical zones. Cassava is a typical food security crop, it is one of the most vital food crops consumed in developing countries especially in tropical areas [2]. Its cultivation and processing into useful products such as gari and fufu. Cassava is an annual crop that is propagated by stem and harvested between 7 – 13 months after planting depending on variety [3]. In Nigeria several varieties of cassava are grown, but the two major cultivars cultivated are sweet and bitter variety, the bitter variety has its Cyanogenic glucoside distributed throughout the tuber and in high concentration while the sweet variety has low Cyanogenic glucoside, mainly in the peel of the tuber. The fresh/pulp of the sweet variety therefore has low Cyanogenic glucoside [4]. However, the growing environmental conditions could influence the Cyanogenic glucoside concentrations of each variety. Some farmers harvest cassava after 2 - 3 years of planting depending on their income [5]. Cassava thrives well in warm, moist climate but it can also tolerate harsh environmental conditions [6].

Cassava effluent is a source of land pollution, if discharged into the environment before proper treatment [7]. Pollution of our environment occurs when waste water discharged from cassava processing are allowed to spread slowly into the soil or flow into streams or when cassava roots are fermented in surface water like ponds and streams, upstream of drinking water sources [8]. Effluent is normally discharged beyond the "factory" wall to roadside ditches or fields and allowed to flow freely, or sometimes settles in shallow depressions, in places where traditional processing is practiced. Eventually this effluent will soaked into the soil or flow into streams [9]. The effluent include the milky colloid pressed out of the fresh tuber paste, the latex, the wash water, etc. These wastes are automatically discharged into the surrounding environment causing pollution .Starch processors in Colombia, usually return the waste water directly to streams and other surface water sources [10]. According to Oboh [11] who discussed broadly on the effluents obtained from the processing of cassava into various end products cassava effluent on the environment and realized that the effluent had negative effects on water, soil, air, domestic animals and plants. In Nigeria, cassava farming and processing into useful food items is a major source of livelihood to several families especially in rural areas.

Igbinosa and Igiehon [12] found that the total bacteria (*Lactobacillus planetarium*, *Pseudomonas aeruginosa*, *Bacillus* spp and Viltro spp.) obtained from the contaminated soil with cassava waste water was more than that in the soil without contaminant [13]. According to Igbinosa and Igiehon [12] organisms isolated during the fermentation of cassava tubers, as practised for “fufu” production included *Bacillus subtilus, Pseudomonas alealigenes, Lactobacillus planetarium, Leuconostic mensenteriodes and Pseudomonas aeruginosa.* The effluent from cassava greatly affected the activities of the microorganisms in the polluted soil and the soil became more acidic in nature [14]. The cassava effluent has great influence on the chemical properties of the soil. Studies by Okpamen et al. [15] using fresh cassava processing effluent and aged effluent obtained from a cassava processing mill in Ekpoma, Edo

State showed increase in the level of pH, organic carbon, phosphorus, sodium, potassium and decrease in calcium magnesium and nitrogen in the soil after treatment with the effluent. The increase in pH and sodium may be due to the higher calcium and magnesium components of the effluent used [16]. There was no marked difference on the level of exchangeable acidity and particle sizes.

In cassava producing countries like Nigeria and Ghana, wide adoption of high-yielding varieties and better pest management have resulted in a sharp rise in production [17]. The clusters of small and medium-sized cassava processors in Afikpo Ebonyi State, Eastern Nigeria generate income and improve the household economy by adding value to the cassava roots harvested. These clustered and seasonal processing activities tend to generate more waste water effluent than can be utilized, converted or managed. Also, the directives by the Federal Government of Nigeria on the use of at least 10% of cassava flour as partial substitution for wheat flour in bread making boosted output in cassava production and utilization. The introduction of cassava bread into the Nigerian market which is gaining widespread acceptance will continue to generate tremendous cassava processing waste effluent from the industrial processing of cassava into flour [18].

2. MATERIALS AND METHODS

2.1 Microbiological Analysis

The bacterial and fungal counts of the samples (cassava mill effluent, unpolluted soil and polluted soil) were determined using the pour plate technique. For the soil samples, 1g of the soil was measured into 9ml of the sterile water contained in a test tube and allowed to stand for ten minutes. 1ml of the mixture was serially diluted using tenfold serial dilution. In the case of the cassava mill effluent sample, 1ml of the effluent was measured into 9ml of sterile water and the mixture was serially diluted and in this case also tenfold serial dilution was conducted.1ml of the serially diluted samples was aseptically plated out for total viable counts of bacteria on Nutrient Agar and MacConkey Agar, and total fungal counts on Sabouraud's Dextrose Agar. After inoculation of the sterile media aseptically, they were incubated at 37^{o}C for 24 hours for the bacteriological media and at 27^{o}C for 48 hours for the mycological media. At the end of the incubation periods, the colonies were observed and counted, using the colony counter and the counts expressed as colony forming units per millilitre (cfu/ml). Representatives of the different purified colonies were subjected to various cultural, morphological and biochemical analyses. Identification of the bacterial isolates was based on Bergey's Manual of Determinative Bacteriology and the fungal isolates was through wet mount method of Yarrow [19] using Lactophenol cotton blue staining techniques and the use of colour atlas.

2.2 Identification of Bacterial Isolates

2.2.1 Gram Staining Technique

In this staining technique, a loopful of the bacteria culture was smeared on a glass slide to make a thin film using a drop of distilled water to emulsify it. The film was fixed by heating over a bunsen burner flame. The fixed smear was covered with crystal violet stain for 60 seconds and rapidly washed off with water. The smear was then covered with Lugol's iodine for 60 seconds and washed off with water. The smear was decolourized with acetone alcohol and washed off after 10 seconds. The smear was finally flooded with Safranin for 2 minutes and washed off with clean water. The back of the slide was then wiped and placed in a draining rack for the smear to dry before it was viewed with oil immersion (X100) objective lens of the microscope [20]. This staining technique is carried out to detect the Gram positive or Gram negative bacteria. Gram positive bacteria appear purple in colour while the Gram negative bacteria appear pinkish in colour [20].

2.2.2 Motility Test

The semi-solid agar method adopted from Cappuccino and Sherman [21] was used. A straight needle was touched on the young growing culture on the agar medium. The inoculum was stabbed into the centre of a semi-solid agar deep. This inoculated agar deep was incubated at 37^{o}C and examined daily for 7 days. Bacterial motility was evident by diffuse zone of growth extending out from the line of inoculation. Some of the organisms grow throughout the entire medium, whereas others show small areas that grow out from the line of inoculation. Growths that are confined to the stab-line, with sharply defined margins and leaving the surrounding medium clearly transparent indicate a negative result.

2.2.3 Spore Staining Technique

A smear of each of the bacterial isolates was made on a clean grease-free glass slide and fixed by air drying. The smear was then covered with malachite green and placed over steam for 5 minutes while topping the slide with more malachite green when it gets dried up. At the end of the 5 minutes, the smear was washed off with clean water and counter stained with safranin for 2 minutes and washed off with clean water. The smear was then allowed to dry before it was viewed with oil immersion (X100) objective lens [22] Spore positive slide gave a co-appearance of pink and green colours while negative slide gave only pinkish colouration.

2.2.4 Catalase Test

Catalase is an intracellular enzyme which catalyses the breakdown of hydrogen peroxide (H_2O_2) to oxygen and water. In this test, 2ml of 5% hydrogen peroxide solution was measured into several test tubes for each of the bacterial isolates. Using a sterile wooden stick, each colony of the bacterial isolates was immersed in each of the hydrogen peroxide solution in the test tubes. Active bubbling within 10 seconds was an indication of a positive result while none was an indication of a negative result [22]. This test was useful for quick separation of catalase positive staphylococci from catalase negative streptococci.

2.2.5 Coagulase Test

This test can be done in two ways, the slide method and the tube method. In this work, the slide method was used. A denser suspension of the culture was made on a clean glass slide using distilled water. A loopful of plasma was added to the suspension and gently mixed properly. In a positive result, clumping of the organism occurs within 5 – 10 seconds. Absence of clumping indicates a negative result [22].

2.2.6 Citrate Utilization Test

Citrate media contain citrate as the only carbon source. They also contain a pH indicator known as bromothymol blue. If the organism can utilize this carbon source, it grows and produces an alkaline by-product which gives rise to a change in the colour of the medium from green to blue.In this test, each of the test organisms was inoculated into sterile agar slopes of Simmon's citrate agar in each case using stab inoculation technique. The inoculated agar slope was then incubated at 37^{o}C for 24 hours. A bright blue colouration indicates a positive result while none was an indication of a negative result [22].

2.2.7 Oxidase Test

Oxidase test determines the ability of the organism to oxidize aromatic amines to coloured products. This can be used as a differential test for many organisms. In this test, a drop of the oxidase reagent (1% tetramethyl-n-phenylene diamine hydrochloride) was added to a suspension of the colony in a test tube. The development of a deep purple –blue colouration indicates a positive result while none was an indication of a negative result [22].

2.2.8 Indole Test

The test organisms were suspended in 3ml sterile peptone preparation in sterile test tubes and incubated at 37^{o}C for 48 hours after which 0.5ml of KOVAC's reagent was added and shaken gently. A red colouration in the surface layer within 10 minutes was an indication of a positive result while none was an indication of a negative result. This test indicates the capacity of an organism to breakdown a compound called Indole, off the amino acid called Tryptophan [22].

2.2.9 Methyl Red Test

This test indicates a type of glucose fermentation in which large amount of mixed acids accumulate in the medium. The pH of the medium is lowered to about 4.2 so that the methyl red dye remain red when added to the medium (tube containing the test organism) [22].

2.2.10 Voges Proskaeur Test

The test determines if the end product of glucose fermentation is a neutral metabolite called Acetyl Methyl Carbinol. This substance reacts with Barits reagent to form pink to red colour in the medium. If the organism is negative, the tube remains yellow [22].

2.2.11 Sugar Fermentation Test

Glucose, Maltose, Lactose, Sucrose, Fructose and Galactose were the sugars used. Each sugar was dispensed as 2% solution in tubes containing peptone water medium and phenol indicator. Durham's tubes were meticulously dropped into each tube in an inverted position

before they are sterilized. The sterilized medium in the tubes were aseptically inoculated with the test organisms and incubated aerobically at 37°C for 24 hours with other similar uninoculated tubes serving as control. After the incubation period, the results were the change in colour of the indicator with the medium from pink to yellow indicating acid formation and the presence of air bubbles in the inverted Durham's tubes indicates gas production. Absence of this two changes indicates a negative result [22].

2.3 Identification of the Fungal Isolates

2.3.1 Lactophenol cotton blue staining technique

Each of the fungal isolates was separately collected with a sterile wooden stick and teased out on a drop of Lactophenol cotton blue stain and potassium hydroxide solution on a clean glass slide. The wet mount preparation was then viewed under the microscope for branched and unbranched hyphae [22].

2.4 Statistical Data Analysis

Statistical analysis of data was based on the procedure outlined by Dike [23] for Chi-square analysis. The data were analysed at 5% level of significance. The statistical tool was used to test the relationship between observed frequencies and expected frequencies, and it is denoted as

$$X^2 = \sum \frac{(O - E)^2}{E}$$

Where O = Observed frequency
E = Expected frequency
X^2 = Calculated value of Chi-square.

$$\text{Expected frequency, E, } = \frac{RT \times CT}{GT}$$

Where RT = Row total
CT = Column total
GT = Grand total

Degree of freedom (DF) = (R – I) (C – I)

Where R = Number of rows
C = Number of columns

3. RESULTS

The results of the microbiological analysis of the cassava mill effluent are presented in Table 1. The total microbial counts in the effluent were 1.4 x 10^4 cfu/ml for bacteria and 1.1 x 10^3 cfu/ml for fungi. The following organisms were isolated from the effluent: *Staphylococcus aureus, Bacillus spp, Streptococcus spp, Pseudomonas aeruginosa, Aspergillus spp* and *Candida spp.*

The results of the microbiological analysis of the unpolluted soil sample are presented in Table 2. The microbial counts obtained were 2.2 x10^5cfu/g for bacteria and 1.6 x 10^4cfu/g for fungi. The isolated micro – organisms were: *Staphylococcus aureus, Bacillus spp, Micrococcus spp, Streptococcus spp, Pseudomonas aeruginosa, Candida spp and Penicillum spp*

Table 1. Microbiological Analysis of the Cassava Mill Effluent

Effluent	Bacteria	Fungi
Microbial count (cfu/ml)	1.4 x 10^4	1.1 x 10^3
Isolated organisms	*Staphylococcus aureus* *Bacillus spp* *Streptococcus spp* *Pseudomonas aeruginosa*	*Aspergillus spp* *Candida spp*

Table 2. Microbiological Analysis of the Unpolluted Soil Sample

Unpolluted Soil Sample	Bacteria	Fungi
Microbial count (cfu/g)	2.2 x 10^5	1.6 x 10^4
Isolated organisms	*Staphylococcus aureus* *Bacillus spp* *Micrococcus spp* *Streptococcus spp* *Pseudomonas aeruginosa*	*Candidia spp* *Penicillum spp*

Table 3. Microbiological Analysis of the Cassava Mill Effluent Polluted Soil Sample

Polluted Soil Sample	Bacteria	Fungi
Microbial count (cfu/g)	4.6 x 10^5	3.3 x 10^5
Isolated organisms	*Staphylococcus aureus* *Bacillus spp* *Pseudomonas aeruginosa* *Proteus spp* *Streptococcus spp* *Enterobacter spp*	*Aspergillus spp* *Rhizopus spp* *Candida spp* *Penicillum spp*

The results of the microbiological analysis of the soil sample polluted with the cassava mill effluent are presented in Table 3. The microbial counts obtained were 4.6x 10^5 cfu/g for bacteria and 3.3 x 10^5 cfu/g for fungi. The following bacteria were isolated: *Staphylococcus aureus, Bacillus spp, Pseudomonas aeruginosa, Proteus spp, Streptococcus spp* and *Enterobacter spp.* The isolated fungi were: *Aspergillus spp, Rhizopus spp, Candida spp* and *Penicillum spp.*

Table 5 shows the identification procedure of the fungal isolates. The identification of the fungal isolates was based on their macroscopic appearance on the culture media and their microscopic characteristics under the microscope using lactophenol cotton blue stain.

4. DISCUSSION

The following bacteria were isolated from the cassava mill effluent: *Staphylococcus aureus, Bacillus* spp*, Streptococcus* spp and *Pseudomonas aeruginosa* (Table 1). Most of them had been reported earlier in similar work. Iyakndue *et al*. [24] observed some of them in Palm oil mill effluent. Some are natural saprophytes like Bacillus, others like Staphylococcus and Enterobacter species could occur due to human activities [25]. These organisms were those which could utilize the effluent. Iyakndue *et al*., 2017stated that effluent from cassava was nutritive enough to support microbial growth, which agree with this work.

The isolated fungi were *Aspergillus* sppand *Candida* spp*.* Some of these micro-organisms have earlier been isolated by Obueh and Odesiri-Eruteyan [26] from cassava mill effluent. Table 1 also showed the microbial populations of the effluent. The bacterial and fungal counts were 1.4 x 10^4cfu/ml and 1.1 x 10^3cfu/ml respectively. According the reports of Uzochukwu*et al.* [27] whom reported that the acidic nature of cassava mill effluent favours the growth of the microbial isolates because these organisms are acid tolerants. According to Agbo et al. [28] about Eleven species of fungi were equally isolated which also include *Penicillium* sp., *Mucor* sp., *Candida* sp., *Saccharomyces* sp., *Aspergillus* sp., and *Rhizopus* sp. These isolated bacterial and fungal species were equally isolated by Oghenejoboh [29]. These microbial isolates were more in number in the cassava effluent impacted soil sites than in the un-impacted soil and in the cassava effluent.

The presence of these micro-organisms in the effluent could be attributed to the presence of some organic compounds. According to Uzochukwu *et al.* [27] cassava mill effluent contains fermentable sugars, celluose and starch. These may account for the high microbial counts and diversity recorded from the effluent.

The microbial load of the cassava mill effluent polluted soil was higher than those obtained from the unpolluted soil. Also, there was increase in the number of micro – organisms due to the pollution of the soil with cassava mill effluent. This general increase in the microbial load and diversity in the polluted soil sample when compared to the unpolluted soil sample can be attributed to some factors. It is possible that as mineralization of organic materials in the soil continued, multiplication of responsible organisms increased. This increased number of organisms may have been associated with increases in some soil properties (pH, organic carbon and total nitrogen) of soils treated with cassava mill effluent [30]

Table 4. Cultural, Morphological and Biochemical Characteristics of the Bacterial Isolates

Colonial Characteristics	Staining Reaction and Arrangements	Oxidase Test	Motility Test	Indole Test	Spore Stain	Catalase Test	Coagulase Test	Citrate Test	Methyl Red Test	Voges Proskauer	Sugar Fermentation						Possible Bacteria
											GL	M	L	S	F	GA	
Golden Yellow and Pinkish Coloured raised colonies	Gram Positive cocci in clusters	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	*Staphylococcus aureus*
Pale Flat Non-Mucoid Colonies With rough edges	Gram Positive rods with rounded ends	-	+	-	+	+	-	+	-	+	+	+	-	+	ND	-	*Bacillus* spp
Greenish Pigmented Colonies, and Irregular Surface with entire edge	Gram Negative slightly curved rods	+	+	-	-	+	-	+	-	-	+	-	-	-	+	-	*Pseudomonas aeruginasa*
Small Pinkish Coloured colonies	Gram Positive cocci in chains	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	*Streptococcus* spp
Milkish enlarged non-mucoid colonies	Gram Positive cocci in tetrads and clusters	+	-	-	-	+	+	+	-	-	+	+	-	-	ND	+	*Micrococcus* spp

Pinkish raised	Gram negative	-	+	-	-	+	-	+	+	-	+	-	-	-	ND
ND*Proteus*spp															
Mucoid colonies	rods														
Creamy colonies	Gram negative														
with even edges	rods in singles	-	+	-	-	+	-	+	-	+	+	+	-	+	ND
ND*Enterobacter*															
	and chains														
spp															

Key : - = Negative; + = Positive; ND = Not Determined; GL = Glucose; M = Maltose; L = Lactose; S = Sucrose; F = Fructose; GA = Galactose

Table 5. Identification of the Fungal Isolates

Macroscopic Appearance on SDA	Microscopic Characteristics	Possible Fungi
Whitish brown – like Cottony Colonies with greenish centre	Septate hyphae with conidia bearing sterigmata	*Aspergillus* spp
Whitish broom – like Cottony colonies	Non – seplate hyphae with terminal spores	*Rhizopus* spp
Creamy raised non-mucoid Colonies	Budded yeast cells	*Candida* spp
Whitish green filamentous Cottony colonies	Septate hyphae with Conidiophores bearing the conidiospores	*Pennicillum* spp

Table 3 showed that the microbial counts obtained from the cassava mill effluent polluted soil sample were 4.6 x 10^5cfu/g for bacteria and 3.3 x 10^5cfu/g for fungi. The isolated bacteria from the polluted soil sample include: *Staphylococcus aureus, Bacillus* spp*, Pseudomonas* spp*, Proteus* spp*, Streptococcus and Enterobacter* spp*. Aspergillus* spp*, Rhizopus* spp*, Candida* spp and *Penicillum*spp were the isolated fungi. M*icrococcus* spp which was originally present in the unpolluted soil, was absent in the polluted soil. Many factors could be responsible for this development. They may have been predated upon by other organisms or possibly with time the effluent may have inhibited their growth. The inhibition of *Serratia macroscens* by cassava mill effluent had earlier been reported [30].

The Cultural, Morphological and Biochemical Characteristics used for the identification of the bacterial isolates are presented in Table 4. Identification of the bacterial isolates was based on Bergey's Manual of Determinative Bacteriology. These microbes may possess or have acquired the genetic attributes that enable them to survive in such acidic environment. This ability to degrade cyanide has been reported to be widely distributed in natural ecosystems and have enzymatic systems that can be broadly described as oxidative, hydrolytic, and substitution/transfer in nature [31]. In addition, the high organic matter and organic carbon contents of the mill effluent may have contributed to the proliferation of these aerobic microorganisms as reported by [32]. The aerobic bacterial counts observed at the surface and subsurface were significantly higher than those of the deeper samples. This could be attributed to the continuous sinking and accumulation of the effluent known to be high in cyanogenic glycosides content. Such great negative effects led to the greatest loss of microbes in the soil thus causing soil infertility for agricultural products as reported by [33].

The fungal counts obtained at the surface were statistically higher than those of the subsurface and deeper samples. This could equally be attributed to the deleterious effects of the effluent on the microbes as the concentration increases with depth. The stratification in diversity and number of these organisms may also be associated with such factors as the gradient or regime in the availability of nutrients in the soil, biological and physicochemical factors of the soil and soil intrinsic and extrinsic parameters [34].

5. CONCLUSION

Nigeria is the largest cassava producing nation. Cassava cultivation and processing is a major source of livelihood to several families especially in rural area. This study reviews the impacts of cassava mill effluent in Afikpo Ebonyi State Nigeria and found that it causes air, soil and water pollution, and toxicological responses in human. There is the need for treatment of cassava mill effluent before discharge and/ or utilization through biotechnological advancement. This study therefore revealed adverse environmental effects of cassava mill effluent on soil biological parameters. Again, it also calls for serious rehabilitation, if the soil will be used for agricultural and other purposes as the factors important in soil health are negatively affected. The results obtained indicated that similar organisms were observed in each environment. The microbial loads as well as the fungal counts were affected by the cassava mill effluent at the various depths. It is hereby recommended that the government of Ebonyi State and Nigeria at large should set aside, a large expanse of land away from residential areas where cassava processing milling machines should be located just as we have mechanic villages where mechanic workshops are located in some of our

cities today. Those involved in the processing of cassava and the cassava farmers do not understand the implication of the dangers posed by cassava effluent because of inadequate awareness. Enlightenment campaigns by the government and agricultural extension workers, on detoxifying cassava effluent in accordance with standard and appropriate methods of disposal of both solids and cassava waste water are recommended for save and healthy environments. More research work should be done on the effects of cassava mill effluent on the surrounding soils, crops its effect on human health.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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