PRINT ISSN 1119-8362
Electronic ISSN 2659-1499

Full-text Available Online at
https://www.ajol.info/index.php/jasem
https://www.bioline.org.br/ja

J. Appl. Sci. Environ. Manage.
Vol. 29 (1) 205-215 Jan. 2025

Ethanol Production Yield from Cassava Mill Effluent using Starch Hydrolyzing Bacterium (*Bacillus* species) and Glucose Fermenting Yeast (*Saccharomyces* species)

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ABSTRACT: Ethanol yield (EY) from carbohydrate fermentation can be affected by microorganisms involved in the fermentation. Hence, the objective of this paper was to evaluate the ethanol production yield from cassava mill effluent (CME) using a starch hydrolyzing bacterium (*Bacillus* species) and a glucose fermenting yeast (*Saccharomyces* species) with high starch hydrolyzing and glucose fermenting potential using appropriate standard methods. The results obtained revealed that the bacterium with the highest zone of clearance on Starch-Nutrient Agar was *Bacillus infantis*, and the yeast with the highest glucose fermenting potential was *Saccharomyces cerevisiae*. About 50 % reduction in the population of *Bacillus infantis* and *Saccharomyces cerevisiae* occurred beyond 15 % and 25 % ethanol concentration respectively. The highest ethanol concentration (17.2±0.7 %) and yield (47.65±1.92 %) was in CME fermented with *Bacillus-Saccharomyces* inocula (BSI) ratio of 1:2. However, statistical analysis revealed that there was no significant difference (P = 0.135) in the results obtained with the different inocula ratios. There was pH reduction to slightly acidic after fermentation of the CME, and drastic reduction in cyanide concentration by about 99 %. It is concluded that appreciable EY can be obtained from fermentation of CME using *Bacillus infantis* and *Saccharomyces cerevisiae*.

DOI: https://dx.doi.org/10.4314/jasem.v29i1.26

Cite this Article as: PEEKATE, L. P; AMADI, L. O; AMOS, E. (2025). Ethanol Production Yield from Cassava Mill Effluent using Starch Hydrolyzing Bacterium (*Bacillus* species) and Glucose Fermenting Yeast (*Saccharomyces* species). *J. Appl. Sci. Environ. Manage.* 29 (1) 205-215

Dates: Received: 22 October 2024; Revised: 20 November 2024; Accepted: 28 December 2024; Published: 31 January 2025

Keywords: Fermentation; Cassava mill effluent; Ethanol production potential; Ethanol tolerance; Ethanol yield.

Ethanol is an alcohol, a colourless volatile flammable liquid, and has diverse applications in various industries and in the health sector. In the chemical industry, it is used as a solvent and in the synthesis of other chemicals (Strohm, 2014); it is used in the manufacture of drugs, plastics, lacquers, polishes, and cosmetics. Consumer products containing ethanol include alcoholic beverages, perfumes, and sanitizers. In the health sector, ethanol is used as an antiseptic and disinfectant. Fuel mixtures containing higher percentages of ethanol is used in powering vehicles in some countries (Altieri, 2012; Dasgupta and Sidana, 2022; Lu, 2016). Ethanol is produced biologically through fermentation of carbohydrates (Amelio *et al*., 2016; Yang *et al*., 2007). Carbohydrate containing substrates commonly used for large scale ethanol production include Corn and Sugarcane (Alonso-Gómez and Bello-Pérez, 2018; Goettemoeller and Goettemoeller, 2007; RFA, 2010). Corn is food for many household around the world, and is used in the manufacture of some food products. Also, Sugarcane is used in making sugar which is required by many household around the world. The continuous use of Corn, Sugarcane, and

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other food crops in ethanol production could therefore lead to a problem of food shortage for the growing human population in the world. Such problem can be prevented by using Carbohydrate containing substrates that are not used as food or food additives by humans, or by using food wastes or wastes of food industries that contain fermentable carbohydrate. Cassava mill effluent (CME) is a waste of Garri processing factories in Nigeria and some countries in Africa; Garri is a major food eaten by many households in these countries. CME is generated in large amounts during Garri production, and it is made up of appreciable quantity of starch slurry. A review has shown that the physicochemical quality of CME exceeds the limit for effluent discharge onto land and into water as recommended by the Environmental Protection Agency in Nigeria (Izah *et al*., 2018). CME is acidic, having pH of 3.2 to 4.6, and contains about 30 to 70 % carbohydrate, about 1 to 50 mg/L cyanide, and little amounts of nitrate and phosphate (Lawal *et al*., 2019; Nwakaudu *et al*., 2012; Obueh and Odesiri-Eruteyan, 2016). Channelling CME into ethanol production could improve the economy of the country, while putting an end to the discharge of CME into the environment thereby eliminating its negative impact on the environment.

The carbohydrate content in CME is mainly starch. Therefore the starch in CME has to be converted to glucose for onward fermentation into ethanol. Some researchers have achieved this conversion through the use of acid or enzyme hydrolysis (Akponah and Akpomie, 2012; Akponah *et al*., 2013; Umo *et al*., 2013). Use of acid or enzymes in the hydrolysis of carbohydrates to free sugars can be potentially economically demanding for large scale production. There exist microorganisms that can hydrolyze carbohydrates, particularly starch, into glucose. Such microorganisms include *Aspergillus fumigatus*, *Chaetomium globosum*, *Bacillus* species, and *Lactobacillus acidophilus* (Peekate, 2022; Sharma and Shukla, 2008). Glucose, the end product of starch hydrolysis by microorganisms, can in turn be fermented into ethanol by some other microorganisms. Microorganisms that can ferment glucose into ethanol include *Zymomonas mobilis*, *Saccharomyces cerevisiae*, and *Pichia kluyveri* (De-Matos *et al*., 2017; Gumienna *et al*., 2013). The use of combination of starch hydrolyzing and glucose fermenting microorganisms in ethanol production from CME could be potentially economically viable for large scale production.

Ethanol yield of fermentation processes is crucial for industries involved in ethanol production, wine making, and bear brewing. In Microbiology, yield is defined as the quantity of a desired product (antibiotics, enzymes, ethanol, etc) obtained from a fermentation or biotechnological process, often measured relative to the input substrate (Madigan *et al*., 2017). Therefore, ethanol yield can be defined as the amount of ethanol produced per unit carbohydrate (sugars or starch) fermented by microorganisms. Studies (Green *et. al*., 2015; Gutt and Gutt, 2009; Izah and Ohimain, 2015) have revealed that ethanol yield from fermentation of carbohydrates depends on the type of substrate, the substrate concentration, the microorganisms involved in the fermentation, the fermentation conditions, and the duration of the fermentation. The level of ethanol yield from fermentation of wastes containing carbohydrate could therefore be affected by the composition of the microbial consortium involved in the fermentation. Therefore, the objective of this paper is to evaluate the ethanol production yield from cassava mill effluent using a starch hydrolyzing bacterium (*Bacillus* species) and a glucose fermenting yeast (*Saccharomyces* species) with high starch hydrolyzing and glucose fermenting potential respectively.

MATERIALS AND METHODS

Sample collection: Cassava Mill Effluent (CME) and palm wine (PW) were obtained from Cassava Mills and PW tappers, respectively, in Ahoda-West Local Government Area of Rivers State, Nigeria. CME and PW were collected using disinfected 5 L and 500 ml plastic bottles respectively. Collected CME and PW were transported within 24 hours to the Microbiology laboratory of the Rivers State University, Nigeria, and stored at 4 °C in a fridge until required for use.

Physicochemical analysis of the cassava mill effluent: The CME was analyzed for pH, slurry content, glucose concentration, starch concentration, and cyanide concentration. The pH was determined using a calibrated hand held pH meter (Hanna Instruments, Ltd, UK). The probe end of the meter was dipped into about 50 ml of the CME, and the reading taking after about 10 seconds.

Slurry content was determined by placing 50 ml of the CME in a 100 ml capacity measuring cylinder of known weight, and the measuring cylinder and its content allowed for 1 hour for sedimentation to occur. After this duration, the liquid surface was carefully decanted off, and the sediment (slurry) and measuring cylinder weighed. The percentage slurry content was then determined using the equation: (weight of slurry ÷ weight of CME) × 100.

The concentration of glucose in the CME was determined using the Phenol-Sulphuric acid method (Otegbayo, 2019), with slight modification. About 5 ml of the CME was mixed with 5 ml hot ethanol (95 % v/v), and the mixture centrifuged at 4000 rpm for 10 minutes. After centrifugation, the supernatant was decanted into a test tube, and the sediment kept aside for starch analysis. A volume of 9 ml of the supernatant was placed in 250 ml capacity beaker, followed by the addition of 500 µl of phenol solution (5 % w/v), and 2.5 ml conc. Sulphuric acid. The resulting coloured (yellow-orange colour) hot solution was allowed to cool down, and the absorbance of the solution at 490 nm was measured using a 721 VIS Spectrophotometer (Huanghua Faithful Instrument Co. Ltd, China). The absorbance reading was used to extrapolate the glucose concentration from a previously obtained graph of concentrations of glucose in phenol-sulphuric acid solution versus absorbance.

The concentration of starch in the CME was determined through acid hydrolysis and the Phenol-Sulphuric acid method described above. To the sediment kept aside in the determination above, was added 7.5 ml Perchloric acid. The mixture was transferred into a test tube, and allowed for 1 hour at ambient temperatures (29-32 °C) for complete starch hydrolysis to occur. After this duration, the mixture was filtered through a Whatman no. 4 filter paper into 50 ml measuring cylinder, and then the volume made up to 10 ml with distilled water. The phenol solution (500 µl) and conc. Sulphuric acid (2.5 ml) was then added. The resulting yellow-orange coloured hot solution was allowed to cool down, and the absorbance of the solution measured. The absorbance reading was used to extrapolate the amount of glucose obtained due to starch hydrolysis, and the amount expressed as the starch concentration.

The cyanide content in the CME was determined using the alkaline picrate method as described by Eke-emezie *et al* (2022). About 2 ml of the CME was mixed with 2 ml of alkaline picrate solution. The mixture was incubated in a water bath for 10 min at 60 °C. The absorbance of the resulting deep orange coloured solution was then measured at 490 nm using the Spectrophotometer. The cyanide concentration was then extrapolated from previously prepared Hydrogen cyanide concentration versus Absorbance graph.

Isolation of Bacillus species: A volume of 1 ml of the CME was subjected to 10-fold serial dilution to a dilution of 10^{-4} using sterile normal saline. Aliquot of 0.1 ml of the different dilutions were inoculated on plates of Starch Nutrient Agar (SNA) using the spread plate technique. The SNA medium was compounded as follows: starch – 1 %, nutrient broth – 0.5 %, and agar – 1.5 %. Inoculated SNA plates were incubated at 35 °C for 24 hours. After incubation, colonies surrounded by a clearing indicating starch hydrolysis were sub-cultured onto appropriately coded nutrient agar plates so as to obtain pure isolates.

The coded pure isolates were inoculated onto SNA plates using the streak method, and the size of the zone of clearance around the colonial growth after incubation was measured. Also, they were subjected to morphological and biochemical tests peculiar for *Bacillus* species. The tests included Gram staining, motility, catalase, oxidase, indole production, methyl red, Voges-Proskauer, citrate utilization, casein hydrolysis, Lecithinase production, lipase production, and fermentation tests using glucose, lactose, maltose, mannitol, sucrose, xylose, and glycerol. The tests were carried out as described by Peekate (2022). The results of the tests were keyed into the dialogue boxes on the online identification platform of Advance Bacterial Identification Software (ABIS), available at https://www.tgw1916.net/bacteria_abis.html, so as to reveal the identity of the isolates.

The isolate identified through ABIS as a *Bacillus* species and having the highest starch hydrolyzing potential among the identified *Bacillus* species was further subjected to genotypic identification through biomolecular techniques as follows: DNA was extracted from cells of the isolate, and then the 16S rRNA gene on the extracted DNA was amplified using the 27F: 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R: 5'-CGGTTACCTTGTTACGACTT-3' primers. Amplification was carried out through Polymerase Chain Reaction (PCR) in an ABI 9700 Applied Biosystems thermal cycler. Amplified 16S rRNA gene was sequenced using the BigDye Terminator kit and a 3510 ABI sequencer. Obtained nucleotide sequence of the 16S rRNA gene was compared with nucleotide sequences deposited in the National Center for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST).

Isolation of Saccharomyces species: A volume of 0.1 ml of the palm wine, and its 10^{-1} to 10^{-3} dilutions were inoculated on plates of modified potato dextrose agar (MPDA) medium using the spread plate technique. The MPDA medium was compounded as follows: potato infusion – 1 L, glucose – 20 g, K_2HPO_4 – 5 g, agar – 20 g, and phenol red – 0.05 g.

Inoculated MPDA plates were incubated in a candle jar at ambient temperatures (24 – 32 ºC) for 3 days. After incubation, yeast colonies with yellow hue indicating acid production due to glucose fermentation were sub-cultured onto appropriately coded potato dextrose agar (PDA) plates so as to obtain pure yeast isolates. The coded yeasts were inoculated into test tubes of sterile glucose-nutrient broth (1 % each of glucose and nutrient broth) containing Durham tubes for detecting gas production, and incubated at 35 °C for 48 hours. After incubation, the test tubes were checked for gas production and the ethanol concentration (% v/v) in them determined using a Refractometer. Afterwards, the coded yeasts were subjected to phenotypic tests peculiar for non-pathogenic yeasts. The tests included Gram staining catalase, oxidase, indole production, starch hydrolysis, citrate utilization, methyl red, Vogues-Proskauer, and fermentation tests using glucose, maltose, and sucrose. The isolate identified as a *Saccharomyces* species based on the results of the tests, and having the highest ethanol producing potential among the identified *Saccharomyces* species was further subjected to identification through bio-molecular technique. A summary of the technique is as follows: DNA was extracted from cells of the yeast, and then the Internal Transcribed Spacer **(**ITS) region on the extracted DNA was amplified using the ITS1F: 5'-CTTGGTCATTTAGAGGAAGTAA-3' and ITS4: 5'- TCCTCCGCTTATTGATATGC-3' primers. Amplification was carried out through Polymerase Chain Reaction (PCR) in an ABI 9700 Applied Biosystems thermal cycler. Amplified ITS region was sequenced using the BigDye Terminator kit and a 3510 ABI sequencer. Obtained nucleotide sequence of the ITS region was compared with nucleotide sequences deposited in the NCBI database using the BLAST.

*Determination of the ethanol tolerance of the selected isolates***:** Standardized broths of the selected *Bacillus* and *Saccharomyces* isolates were prepared by adjusting the turbidity of 48 hours old broth cultures of the isolates to the turbidity of a freshly prepared 0.5 McFarland standard using sterile normal saline. The standard was prepared as described in the Clinical and Laboratory Standards Institute manual (CLSI, 2012). Turbidity/Absorbance measurement was achieved with the aid of a 721 VIS Spectrophotometer set at 600 nm. A volume of 1 ml of the standardized broth cultures of the selected *Bacillus* and *Saccharomyces* isolates was inoculated into 9 ml of nutrient broths and glucose-nutrient broths respectively containing 0, 5, 10, 15, 20, 25, 30, 35, and 40 % ethanol. Inoculated nutrient broths and glucose-nutrient broths were incubated at ambient temperatures (24 – 32 ºC) for 24 and 48 hours respectively. After incubation, the optical densities at 600 nm of the broths were determined, and used to determine the ethanol tolerance of the isolates.

Modification and fermentation of the Cassava Mill Effluent: About 5 g Nutrient broth powder was added to 1 L CME, and then the pH of the resulting mixture was adjusted to 7.0 using 2.0 M NaOH (aqueous). The modified CME was distributed into 12 conical flasks (150 ml capacity), 97 ml per flask, and sterilized in an autoclave at 121 °C for 15 minutes. After sterilization, the flasks were labeled as follows: control, B:Y(1:1), B:Y(1:2), and B:Y(2:1); all in triplicates. The labeled flasks were inoculated with standardized broth cultures of the selected isolates as shown in Table 1. After inoculation, the flasks were incubated at ambient temperature (24 – 32 ºC) in semi-anaerobic condition for 5 days. On completion of the incubation period, the pH of the fermented media, cyanide and ethanol concentrations in the media were determined. The ethanol concentration (EC) and initial starch concentration (ISC) in the CME were used to calculate the ethanol yield with the aid of the formula, Ethanol yield (%) = $\frac{\text{EC}}{\text{ISC}} \times 100$.

Table 1: Inoculation arrangement for the labeled flasks

Flask	SBS (ml)	SYS (ml)
Control	0	0
B:Y(1:1)	1.5	1.5
B:Y(1:2)	1	2
B:Y(2:1)	2	1

SBS: Standardized Bacillus suspension, SYS: Standardized Saccharomyces suspension

RESULTS AND DISCUSSION

Physicochemical characteristics of the cassava mill effluent: The pH of the cassava mill effluent **(**CME) was 3.85 ± 0.07, slurry content 25.5 ± 2.1 %, glucose concentration 4.7 ± 0.3 mg/ml (0.47 %), starch concentration 361±8 mg/ml (36.1 %), and cyanide concentration was 3.45 ± 0.07 mg/L (0.0003 %). This indicates that the CME was acidic, and it had adequate level of carbohydrate (glucose + starch) content. These physicochemical characteristics fall within the ranges that have been observed in previous studies. Cassava mill effluenthas been shown to be acidic with pH ranging from 3.20 to 4.98 (Akponah *et al*., 2013; Lawal *et al*., 2019; Obueh and Odesiri-Eruteyan, 2016). Carbohydrate content of 34 to 73 % in CME have been recorded in other related studies (Nwakaudu *et al*., 2012; Obueh and Odesiri-Eruteyan, 2016), while cyanide content of 1.3 to 70 mg/L has been observed (Lawal *et al*., 2019; Nwakaudu *et al*., 2012; Obueh and Odesiri-Eruteyan, 2016).

Starch hydrolyzing (SH) potential and identity of isolated SH bacteria: Four distinct groups of colonies with zone of clearance around them on starch-nutrient agar (SNA) were noted and coded as SHB1, SHB2, SHB3, and SHB4. The size of the zone of clearance around their colonial growth after sub-culturing on SNA was 4, 3, 6, and 4 mm respectively. This indicated that isolate SHB3 had the highest starch hydrolyzing potential**.** The result of the morphological and biochemical tests carried out on the starch hydrolyzing **(**SH) bacteria is presented in Table 2. From the Table, it can be seen that all the SH bacteria were identified through ABIS as *Bacillus* species.

Table 2: Morphological and biochemical characteristics of the starch hydrolyzing bacteria

TEST	SHB1	SHB2	SHB3	SHB4
GSR	+	+	+	+
MPL	Rods	Rods	Rods	Rods
CTS	+	+	+	+
OXD	-	-	+	+
MTL	+	+	+	+
INP	-	-	-	-
MTR	-	+	+	-
VPR	+	-	-	-
CTU	-	+	-	+
CSH	-	+	-	-
LCP	-	+	+	+
LIP	-	+	-	+
GLF	A	A	A	A
LTF	-	-	A	-
MLF	A	A	A	A
MNF	A	-	A	A
SUF	A	A	A	-
XYF	-	-	A	-
GRF	A	A	-	-
SB.ABIS (Similarity)	*Bacillus coagulans* (81.7 %)	*Bacillus cereus* (89.8 %)	*Bacillus lentus* (87.6 %)	*Bacillus smithii* (81.7 %)

GSR: Gram stain reaction, MPL: Morphology, CTS: Catalase, OXD: Oxidase, MTL: Motility, INP: Indole production, MTR: Methyl red, VPR: Voges-Proskauer, CTU: Citrate utilization, CSH: Casein hydrolysis, LCP: Lecithinase production, LIP: Lipase production, GLF, LTF, MLF, MNF, SUF, XYF, and GRF: Glucose, Lactose, Maltose, Mannitol, Sucrose, Xylose, and Glycerol fermentation respectively, A: Acid, SB.ABIS: Suspected bacteria as determined using ABIS online tools.

The concentration of extracted DNA from SHB3 (*Bacillus lentus*) which had the highest starch hydrolyzing potential was 8.9 ng/µl. The band of the amplified 16S rRNA gene of the isolate on agarose gel after electrophoresis aligned with the molecular marker at 1500 bp. The nucleotide sequence of the 16S rRNA gene of SHB3 was as follows:

ATGGGAGCTTGCTCCCTGAAGTCAGCGGCGG
ACGGGTGAGTAACACGTGGGCAACCTGCCTG
TAAGACTGGGATAACTTCGGGAAACCGGAGC
TAATACCGGATAATGCACAGCCTCTCATGAG
GCTATGCTGAAAGATGGTTTCGGCTATCACTT
ACAGATGGGCCCGCGGCGCATTAGCTAGTTG
GTGAGGTAACGGCTCACCAAGGCAACGATGC
GTAGCCGACCTGAGAGGGTGATCGGCCACAC
TGGGACTGAGACACGGCCCAGACTCCTACGG
GAGGCAGCAGTAGGGAATCTTCCGCAATGGA
CGAAAGTCTGACGGAGCAACGCCGCGTGAGT
GATGAAGGTTTTCGGATCGTAAAACTCTGTTG
TCAGGGAAGAACAAGTGCCGGAGTCACTGCC
GGCACCTTGACGGTACCTGACCAGAAAGCCA
CGGCTAACTACGTGCCAGCAGCCGCGGTAAT
ACGTAGGTGGCAAGCGTTGTCCGGAATTATT
GGGCGTAAAGCGCGCGCAGGCGGTCTCTTAA
GTCTGATGTGAAAGCCCACGGCTCAACCGTG
GAGGGTCATTGGAAACTGGGGGACTTGAGTG
CAGAAGAGGAAAGTGGAATTCCACGTGTAGC
GGTGAAATGCGTAGAGATGTGGAGGAACACC
AGTGGCGAAGGCGACTTTCTGGTCTGTAACT
GACGCTGAGGCGCGAAAGCGTGGG.

On comparing this nucleotide sequence with nucleotide sequences of 16S rRNA genes of known bacteria in the NCBI database, it was revealed that SHB3 matched with *Bacillus infantis* at 100 %. In a related study (Artha *et al*., 2019), extracellular enzyme-producing bacteria isolated from pond sediment and identified as *Bacillus thuringiensis* , *B. lentus*, *B. sphaericus*, and *Corynebacterium pilosum* were shown to produce hydrolysis zone of 10, 12, 11, and 14 mm respectively on amylum (starch). The bacterium shown to have the highest hydrolysis zone (6 mm) in this study was phenotypically identified as *B. lentus*, which is among theextracellular enzyme-producing bacteria isolated by Artha *et al* (2019) with a hydrolysis zone of 12 mm. The discrepancy in the zone of hydrolysis could be attributed to the starch-agar thickness used in accessing the hydrolysis. *Bacillus*species have been shown in another related study (Nimisha *et al*., 2019) to have the highest starch degrading or amylase activity among starch degrading bacteria. Also, *Bacillus* species appears to be the predominant members of starch degrading bacteria as revealed in the works of Artha *et al* (2019) and Mawa *et al* (2022). The isolation of only *Bacillus* species as the starch degrading bacteria in this study is therefore in agreement with what has been observed.

Glucose fermenting (GF) potential and identity of isolated GF yeasts: Three colonies with yellow hue on MPDA were randomly selected based on differences in size, and they were coded as PYA, PYB, and PYC. All the isolates produced gas in glucose nutrient-broth (GNB), with the highest gas volume in GNB culture of PYA. The ethanol concentrations in GNB culture of PYA, PYB, and PYC after incubation were 0.4, 0.1, and 0.1 % v/v respectively. This indicated that isolate PYA had the

highest glucose fermenting potential**.**The results of the morphological and biochemical tests carried out on the glucose fermenting(GF) yeasts are presented in Table 3. From the Table, it can be seen that the three isolates all appeared as oval cells and were Gram positive. Based on comparison with information in Microbiology literatures (Ali and Khan, 2014; Ghosh, 2011) PYA, PYB, and PYC were identified as *Saccharomyces*, *Pichia*, and *Candida* species respectively.

Table 3: Morphological and biochemical characteristics of the glucose fermenting yeasts

TEST	PYA	PYB	PYC
GSR	+	+	+
MPL	Oval cells	Oval cells	Oval cells
CTS	+	+	+
OXD	-	-	-
IND	-	-	-
MTR	+	-	-
VPR	-	+	-
CTU	+	+	-
STH	+	-	-
GLF	AG	A	A
MLF	AG	A	A
SUF	A	A	A
SID	*Saccharomyces* sp.	*Pichia* sp.	*Candida* sp.

GSR: Gram stain reaction, MPL: Morphology, CTS: Catalase, OXD: Oxidase, INP: Indole production, MTR: Methyl red, VPR: Voges-Proskauer, CTU: Citrate utilization, STH: Starch hydrolysis, GLF, MLF, and SUF: Glucose, Maltose, and Sucrose fermentation respectively, AG: Acid and gas, A: Acid only, SID: Suspected identity.

The concentration of extracted DNA from PYA (*Saccharomyces* sp.) which had the highest glucose fermenting potential among the yeasts was 78.4 ng/µl. The band of the amplified ITS region on the DNA of the isolate on agarose gel after electrophoresis aligned with the molecular marker at 1500 bp. The nucleotide sequence of the ITS region on the DNA of PYA was as follows:

TGGCAAGAGCATGAGAGCTTTTACTGGGCAA
GAAGACAAGAGATGGAGAGTCCAGCCGGGC
CTGCGCTTAAGTGCGCGGTCTTGCTAGGCTTG
TAAGTTTCTTTCTTGCTATTCCAAACGGTGAG
AGATTTCTGTGCTTTTGTTATAGGACAATTAA
AACCGTTTCAATACAACACACTGTGGAGTTTT
CATATCTTTGCAACTTTTTCTTTGGGCATTCG
AGCAATCGGGGCCCAGAGGTAACAAACACA
AACAATTTTATTTATTCATTAAATTTTTGTCA
AAAACAAGAATTTTCGTAACTGGAAATTTTA
AAATATTAAAAACTTTCAACAACGGATCTCT
TGGTTCTCGCATCGATGAAGAACGCAGCGAA
ATGCGATACGTAATGTGAATTGCAGAATTCC
GTGAATCATCGAATCTTTGAACGCACATTGC
GCCCCTTGGTATTCCAGGGGGCATGCCTGTTT
GAGCGTCATTTCCTTCTCAAACATTCTGTTTG
GTAGTGAGTGATACTCTTTGGAGTTAACTTGA
AATTGCTGGCCTTTTCATTGGATGTTTTTTTTC
CAAAGAGAGGTTTCTCTGCGTGCTTGAGGTA
TAATGCAAGTACGGTCGTTTTAGGTTTTACCA
ACTGCGGCTAATCTTTTTTTATACTGAGCGTAT
TGGAACGTTATCGATAAGAAGAGAGCGTCTA
GGCGAACAATGTTCT.

On comparing the nucleotide sequence with nucleotide sequences of ITS region on the DNA of known fungi in the NCBI database, it was revealed that PYA matched with *Saccharomyces cerevisiae* at 100 %.

Ethanol concentrations produced by strains of *Saccharomyces cerevisiae* from fermentation of broths containing glucose of 10 to 25 % w/v have been shown to range from 3.0 to 14.0 % v/v (Brooks, 2008; Thammasittirong *et al*., 2012; Ruyters *et al*., 2015). In this study, 0.4 % v/v ethanol was obtained from fermentation of broth containing 1 % w/v glucose by the *Saccharomyces cerevisiae*isolate. Theoretically, increasing the glucose concentration in the fermentation medium of this yeast by 10 or 25 would imply that ethanol concentration of 4 or 10 % v/v could be obtained. It can therefore be assumed that the *Saccharomyces cerevisiae*isolate has similar glucose fermenting or ethanol producing potential as has been shown for other strains of *Saccharomyces cerevisiae*.

*Ethanol tolerance of the Bacillus and Saccharomyces isolates:*The optical densities (ODs) of standardized broth cultures of *Bacillus infantis* and *Saccharomyces cerevisiae* in 0 to 40 % v/v ethanol after 24 and 48 hours incubation, respectively, is presented in Figure 1. In the Figure, it can be seen that with increase in ethanol concentration, the optical density reduced indicating reduction in population and thus tolerance. Also it can be seen that there was about 50 % reduction in the population of *Bacillus infantis* beyond 15 % ethanol concentration, and about 50 % reduction in the population of *Saccharomyces cerevisiae* beyond 25 % ethanol concentration. This means that with increasing ethanol concentration, *Bacillus infantis* was more affected than *Saccharomyces cerevisiae*. The viability of a strain of *Saccharomyces cerevisiae* has been shown to reduce below 50 % beyond 10 % v/v ethanol concentration (Thammasittirong *et al*., 2012). Suspension cultures of two industrial strains of *Saccharomyces cerevisiae* were found to be highly tolerant to 78.80 g L^{-1} (10 % v/v) ethanol, however their growth ability showed a distinct decrease with increasing

ethanol concentration such that only (1–2) % of the control growth was observed in media containing 118.20 g L^{-1} (15 % v/v) ethanol (Kasavi *et al*., 2012). In another related study (Brooks, 2008), a strain of *Saccharomyces cerevisiae* was shown to tolerate 12 % v/v ethanol.

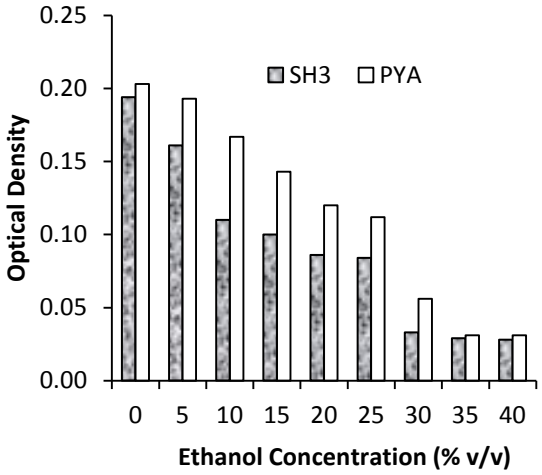

Fig 1: Optical density of broth cultures of SH3 and PYA in ethanol after incubation.
SH3: *Bacillus infantis*, PYA:*Saccharomyces cerevisiae*.

Ethanol at 10 % has been shown to prevent the growth of several *Bacillus* species strains (Rizk *et al*., 1989); some species were killed after 10 - 60 minutes exposure to 25 % ethanol. In other related studies (Abood *et al*., 2021; Ezebuiro *et al*., 2015), one species of *Bacillus* (*B. cereus*) have been shown to be able to grow in medium containing 5 to 6 % v/v ethanol. Due to the relatively higher ethanol concentration observed in this study for reduction in population of *Bacillus infantis* and *Saccharomyces cerevisiae* isolate by about 50 %, it can be inferred that the isolates are more ethanol tolerant than others that has been isolated in previous studies. This could be attributed to the source and strain of the isolate.

Ethanol yield from fermentation of the modified cassava mill effluent: Ethanol concentration and yield, after 5 days of incubation, in the modified cassava mill effluent (MCME) inoculated with the different *Bacillus-Saccharomyces* (B:Y) ratiosis presented in Figure 2. In the Figure, it can be seen that the highest ethanol concentration (17.2±0.7 %) and yield (47.65±1.92 %) was in the fermented MCME which was inoculated with *Bacillus-Saccharomyces* ratio of 1:2. However, Analysis of Variance (ANOVA) of the ethanol concentrations and yields (Table 4) showed that there was no significant difference (P = 0.135) in the results obtained with the different inocula ratios.

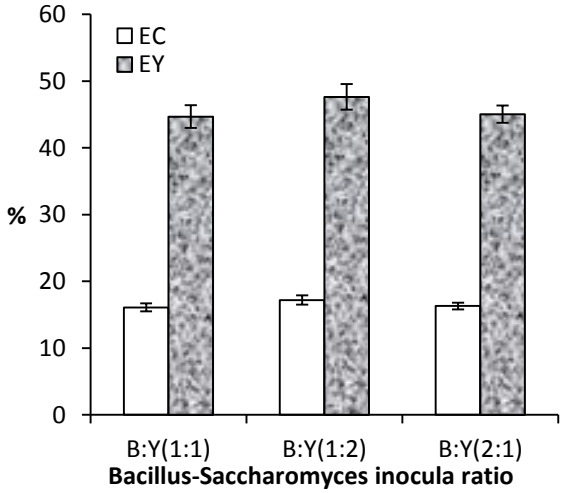

Fig 2: Ethanol concentration (EC) and yield (EY) in the fermented MCME.

Table 4: ANOVA summary of the ethanol concentrations in the fermented MCME

SV	SS	DF	MS	FC	*P-value*	FT at α = 0.05
Between Groups	2.027	2	1.013	2.85	0.135	5.14
Within Groups	2.133	6	0.356			
Total	4.160	8				

Groups: B:Y(1:1, 1:2, and 2:1), SV: Source of variation, SS: Sum of squares, DF: Degree of freedom, MS: Mean square, FC: Calculated F statistics, P: Probability, FT: Tabulated F statistics.

Ethanol yield of 8.3 to 30 % has been obtained from acidic or enzymatic hydrolysis of cassava processing wastes and subsequent fermentation with yeasts (Akponah and Akpomie, 2012; Martinez *et al*., 2018; Umo *et al*., 2012). Akponah and Akpomie (2012) through optimization experimentation obtained ethanol yield of 8.3 % after enzymatic hydrolysis of cassava wastewater and subsequent 24 hours fermentation using *Saccharomyces cerevisiae*. Martinez *et al* (2018) obtained an average ethanol yield of 30 % from enzymatic hydrolysis of cassava processing residues and subsequent 24 hours fermentation with *Saccharomyces cerevisiae* at constant agitation. Umo *et al* (2012) through optimization experiment obtained ethanol yield of 8.69 % after acid hydrolysis of cassava wastewater and subsequent 5 day fermentation using yeast. The ethanol yield obtained in this study was greater than what was obtained in the other studies. This could probably be due to the difference in the method employed in the treatment of the cassava processing wastes. In the other studies the pH of the cassava processing wastes was allowed in its acidic condition, whereas in this study the pH was adjusted to neutrality (7.0) before simultaneous hydrolysis and

fermentation. Also, in this study *Bacillus* having a high starch hydrolyzing ability was added for hydrolysis of starch present in the cassava wastes, whereas in the other studies starch hydrolyzing enzymes or acid was used.

Physicochemical characteristics of the fermented modified cassava mill effluent: The pH of the fermented modified cassava mill effluent **(**MCME) and its cyanide concentration is presented in Table 5. In the Table it can be seen that there was general reduction in pH, with the lowest pH (5.97±0.71) in the fermented MCME which was inoculated with *Bacillus-Saccharomyces*ratio of 1:2. There was also drastic reduction in cyanide concentration from 3.45 mg/L to a range of 0.035 to 0.040 mg/L; representing a reduction of about 99 %.

Table 5: Physicochemical characteristic of the fermented MCME

Inoculum-ratio	pH	CN (mg/L)
Control	6.73±0.15	3.450±0.071
B:Y(1:1)	6.23±0.55	0.040±0.001
B:Y(1:2)	5.97±0.71	0.037±0.002
B:Y(2:1)	6.20±0.26	0.035±0.001

CN: Cyanide concentration, B:Y: Bacterium-yeast ratio.

Literatures on change of physicochemical characteristics during fermentation of cassava mill effluent or wastewater were scarce as of the time of this study. However, cyanide and pH reduction has been shown to occur during fermentation of cassava tubers (Amajor, 2022; Kobawila *et al*., 2005; Obi and Ugwu, 2019). In the work of Amajor (2022), cyanide content reduced from 22.4 to 3.0 mg/100g representing about 87 % decrease, and pH reduced from 5.9 to 3.0 after 6 days fermentation of cassava mash. Kobawila *et al* (2005) and Obi and Ugwu (2019) studied spontaneous fermentation of cassava tubers in water for 4 days, and observed cyanide reduction of about 71 and 68 % respectively. There was also pH reduction from between 6.2 and 7.2 to 3.14 and 3.8. Species of *Lactobacillus* and *Saccharomyces* were among the microorganisms present during the fermentation studied by Kobawila *et al*., (2005), while *Bacillus* and *Saccharomyces* specieswere among the microorganisms present during the fermentation studied by Obi and Ugwu (2019). In a related study (Kandasamy *et al*., 2015), a species of *Bacillus* was observed to reduce the concentration of cyanide in a mineral salts medium containing cyanide and glucose by 63 % after 7 days. This indicates that the use of *Bacillus* in this study greatly assisted in the reduction of cyanide in the MCME. Furthermore, the cyanide reduction observed in this study was greater than what was observed in the other studies. This could be attributed to the small starting concentration of cyanide (3.45±0.07 mg/L) in the cassava mill effluent as compared to starting concentrations of cyanide (equivalence of 92 to 414 mg/L) in the cassava tubers in the other studies. On the other hand, pH reduction of the MCME was not as much as observed in the other studies. This could be due to the treatment of the cassava mill effluent and/or action of the *Bacillus* and *Saccharomyces*. Be that as it may, highest ethanol production by *Saccharomyces cerevisiae* during fermentation of sugars has been shown to occur at pH 4.5 to 5.5 (Narendranath and Power, 2005; Salam*et al*., 2024; Wong and Sanggari, 2014). On the other hand, pH values of 4.0 and below have negative effect on yeast cell growth, fermentation, and thus ethanol production (Bayrock, 2019; Krajang *et al*., 2021; Narendranath and Power, 2005). Therefore the not much reduction in pH of the MCME was beneficial for the ethanol production.

Conclusion: Appreciable ethanol yield can be obtained from fermentation of treated cassava mill effluent using *Bacillus infantis* and *Saccharomyces cerevisiae*. The fermented medium contains very low amount of cyanide and is of moderate acidic pH. Therefore the medium left behind after extraction of the produced ethanol, probably through distillation, can be used for other purposes such as soil enrichment for cultivation of plants. Future research directed towards enhancement of spontaneous fermentation of untreated cassava mill effluent through addition of *Bacillus*, *Saccharomyces*, and a nitrogen/protein source should be carried out to see if higher ethanol yield can be achieved. Higher ethanol yield from this approach would imply that work and cost associated with modification/treatment of cassava mill effluent for ethanol production through fermentation would be circumvented.

Acknowledgment: The authors extend their appreciation to Tertiary Education Trust Fund (TETFUND) Nigeria, (2023/2024 grant), for funding this research.

Declaration of Conflict of Interest: The authors declare no conflict of interest.

Data Availability: Data are available upon request from the corresponding author

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