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Isolation, Characterisation And Identification Of Some Indigenous Bacteria Species From A Discharged Brewery Wastewater And Soil Sediments Along The Discharge Pathway

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Abstract: ***Samples of wastewater and solid support were obtained from a brewery industry as well as it's discharging tunnel and were analyzed to evaluate its pollution status. Three (3) points were mapped out for sample collections which were sampling points A, B and C, whereas point A is the discharge point, point B and C were points along the effluent discharge tunnel. The samples were collected two times each for rainy and dry seasons in order to assess the impact of each season on the pollution load of the brewery wastewater. Twenty-two (22) bacteria isolates were characterized, identified morphologically, microscopically and biochemically. The following results were obtained: ten (10) Bacillus spp., three (3) E.coli spp., two (2) Klebsiella spp., and one (1) each for S. proteus, Enterobacter spp., Salmonella spp., Bordetella spp., and Pseudomonas spp. respectively. More so, the four predominant isolates found were further characterized molecularly and were identified as: Bacillus cereus, Bordetella pertussis, Lysinibacillus sphaericus strain G39 and Lysinibacillus sphaericus strain TMB2.***

Keywords: biochemically, brewery, isolates, molecularly, morphologically, wastewater, seasons

I. INTRODUCTION

Industrialization has become an important factor to the development of any country's economy through the establishment of plants and factories. However, the wastes or by-products discharged from them are severely disastrous to the environment because it consists of various kinds of contaminants which contaminate the surface water, ground water and soil. The world is faced with problems related to the management of wastewater, due to extensive industrialization, increasing population density and high urbanized societies The effluents generated from domestic and industrial activities constitute the major sources of natural water pollution load. This is a great burden in terms of wastewater management and can consequently lead to a point-source pollution problem, which not only increases treatment cost considerably, but also introduces a wide range of chemical pollutants and microbial contaminants to water sources (Amir et al., 2004).

Large quantities of untreated solid and liquid wastes are discharge indiscriminately into streams and rivers, particularly those flowing through towns, cities and villages (Ademoroti, C.M.A., 2006) and they contain toxic and hazardous materials which settle in rivers as bottom sediments and constitute health hazards to the populations (animals, plants and aquatic habitats) that depend on the water as source of supply (Akaniwor et al., 2007). For instance, in areas where industrial waste effluents are discharged into surface waters, there is

general reduction in the quality of such water and its ability to support aquatic life is equally reduced.

In this way, water is heavily polluted and water, which should be a blessing to life, becomes a carrier of poisons, toxicants and pathogens leading to dreadful diseases that can cause death. Many diseases and premature deaths have broken out as a result of the impact of wastewater discharge to our environment. These pollutants from industrial effluents discharge have been identified as being responsible for some major health and environmental problems such as motor neurone diseases (Adenuga et al., 2006), reproduction disorders ((Mantovani, 1993) and cardiovascular diseases (Clayton 1976).

Brewery industries have been known to cause pollution by discharging effluent into receiving stream, ground water and soil (Olajumoke A. etal, 2010). There are about fifty brewery industries in Nigeria and they all generate wastes waters. This wastewater is sometimes discharged untreated into the environment and they flow into inland water bodies resulting to stench, discoloration and a greasy oily nature of such water bodies (Mombeshora,1981). Production steps in the brewery processes include malt production, wort production and beer production (World Bank Group, 1997). Water consumption for breweries generally ranges from 4-8 cubic meters per cubic meter of beer produced. The pollution discharge from brewery plant effluent comes from the losses in the beer production process and from the clean-in-place (CIP) system located in the brewing house, cellar house and bottling house. The quality of brewery effluent can fluctuate significantly as it depends on various different processes that take place within the brewery. The organic components in brewery effluent are generally easily biodegradable since these mainly consists of sugars, soluble starch, ethanol, volatile fatty acids as well as solids which are mainly spent grains, waste yeast and trub (Driessen et al., 2003). Thus, based on their properties, the constituents of brewery effluent fluid can be classified as: physical, chemical and biological. The physical characteristics of the effluent fluids constitute the total solids content, smell or odour, colour and temperature. The important chemical characteristics of effluent fluid are determined by the pH value, chloride content, nitrogen, fat and grease content, dissolved oxygen, chemical oxygen demand and biochemical oxygen demand. The biological characteristics relate to the various micro-organisms found in effluent fluid, some of which may be pathogenic. However, some of the bacteria present in the brewery wastewater are not harmful for some help to treat the wastewater and reduce the cost of treatment plants (Bhatia, 2005;). Thus, isolation, characterisation and identification of bacteria species are necessary steps towards the application of these indigenous isolates in the bioremediation processes. This is because it can cost-effectively and expeditiously destroy or immobilize contaminants in a manner that protect human health and the Environment. The bioremediation systems in operation today rely on microorganisms native to the contaminated sites, encouraging them to work by supplying them with the optimum levels of nutrients and other chemicals essential for their metabolism.

II. MATERIALS AND METHODS

A. MATERIALS

The materials used in this research work were sourced from Sabon-gari market and National Research Institute for Chemical Technology Laboratories both in Zaria. The equipments used are properties of National Research Institute for Chemical Technology Laboratories, Ahmadu Bello University, Microbiology Laboratory and DNA labs Kaduna all in Kaduna State. The chemicals used for this Research were of analytical quality.

B. SAMPLING POINTS AND COLLECTION

Effluent samples were monitored for rainy and dry seasons. Sampling was carried out for two times each for the two seasons monitored. Sampling was done in July and August for rainy season and in December and January for dry season around mid-day respectively. Three sampling points from the site were mapped out and designated as A, B and C. A is the point of discharge of the effluent from the brewery; B and C are along the effluent discharge tunnel with a distance of 150 meters apart.

Samples of wastewater and solid supports were collected from these stipulated points. Sterilized universal sampling bottles (50mls) were used to collect wastewater samples as well as sterile polythene bags were used to collect soil sediments for microbial analysis. During the sampling, collection was done in replicate with their covers replaced, labeled and kept in the ice pack to retain its original microbial activities. The samples were then transported to the Laboratory immediately and stored in the refrigerator at about 4°C prior to analysis.

C. MICROBIAL ANALYSIS OF THE BREWERY WASTEWATER SAMPLES

a. PREPARATION OF MEDIA FOR MICROBIAL ANALYSIS

Nutient Agar media was prepared by dissolving 28g of the Agar in one litre of distilled water inside a conical flask, then autoclaved at 121^{o}C, 15 pressures per square inch (PSI) for 15mins and cooled at 40^{o}C before plating in a sterilized petri dish.

b. PREPARATION OF THE SAMPLES FOR MICROBIAL ANALYSIS

One (1) ml of wastewater sample was measured from using different sterile pipettes aseptically and individually dispensed into two different sterile test tubes containing 9 ml of sterile distilled water. The test tubes were labelled accordingly. Serial dilution was done for the sample as to reduce the load of organisms in the wastewater sample according to the method of van Soestbergen and Ching (1969). An aliquot (0.1 ml) of each diluent was measured into sterile Petri dishes and overlaid with 15 ml of molten agar using the pour plate method. The plates swirled for homogeneity of the

diluents and the medium was allowed to solidify and then incubated in inverted position. Incubation was carried out at 37°C for 24 hrs.

c. ISOLATION OF PURE CULTURE OF THE BACTERIA ISOLATES

This was done according to the method of Van Soestbergen and Ching, (1969). The 24h bacteria isolates from the effluent samples were sub-cultured on sterile nutrient agar plates to obtain pure cultures. This was done aseptically by taking a loopful of a colony that will be isolated from other colonies onto a solidified nutrient agar plate and streaked over the plate using a sterile inoculating loop. This was to relatively reduce the density of the microbial cells of the given organism from the plate. The plates were incubated at 37°C for 24 hrs in an inverted position.

d. IDENTIFICATION AND DIFFERENTIAL CHARACTERIZATION OF BACTERIA

Gram Staining Procedure

On a sterile slide, one drop of distilled water was added. An inoculating loop was used to pick each colony from the petri dish containing bacterial cultures to create a smear. The smears were fixed using flames from Bunsen burner. Fixed bacterial were subjected to four different reagents in the following order; crystal violet (Primary stain) for 30 seconds, iodine solution (Mordant) for one minutes, alcohol (Decolorizing agent) for 20 seconds and safranin (Counter stain) for 30 seconds and was viewed under the microscope. The bacteria which retained the primary stain was identified as gram positive organism while those that lost the crystal violet and counter stained with safranin (Appeared red) was the gram negative (Aneja, 2001).

Cultural Morphology Of Bacterial Isolates

Isolated colony of each bacteria was observed culturally and morphologically based on their superficial forms (Circular, filamentous and irregular); elevation (Flat, convex and umbonate), margin (Lobate, convex or iregular), and shape (Spiral, rod or cocci) using hand magnifying lens.

e. BIOCHEMICAL IDENTIFICATION OF THE BACTERIAL ISOLATES

Triple Sugar Iron (TSI) Agar Test

Agar slants was prepared in test-tube. Each pure colony of the unidentified bacterium picked with a sterilized wire and stabbed into the butt of the test tube. The colonies were then separately streaked on the slope before stabbing into each butt and allowed to incubate at 30^0c for 24hrs. When only glucose was fermented, the slant turns red (Alkaline) and butt turned yellow (Yellow). When only lactose or sucrose was fermented, so much acid was produced that both slant and butt remained yellow (Acid). Gas production resulted in a cracks or the medium pushed upward. H_2S production was confirmed by the change of medium colour to black along the streak line.

Motility Indole Ornithine Agar

Motility Indole Ornithine (MIO) medium was used to determine motility and indole production. 10ml of medium was dispensed into a test-tube and autoclaved. Each pure colony of the unidentified organism stabbed into the test tube using a sterilized wire and incubated at room temperature for 24hrs. Motility observed as hazy diffuse spreading growths (Swarm) along the stab unlike non motiles, restricted to the stab line. The presence of ornithine decarboxylase was observed when the purple colour of the medium changes to yellow (Brooke *et al*. 2007). For indole production, 2-3 drops of Kovac's reagents (Dimethylaminobenzyaldehyde) was added to the medium in the test tube. A red precipitate at the top of the interface indicated a positive reaction of indole production.

Catalase Test

The principle is used for the detection of catalase enzyme in a bacterial isolate. Few colonies of the test organisms were picked with a platinum loop from nutrient agar plate and then applied in a drop of 10% hydrogen peroxide on a clean slide. The production of gas bubble from the culture indicated positive reaction (Aneja, 2001).

Oxidase Test

A drop of 1% solution of oxidase reagent was added on a piece of filter paper. Few colonies of the test organisms were rubbed onto it. The production of a deep purple colour within 10 seconds indicated a positive reaction (Aneja, 2001).

Citrate Test

The test organisms were inoculated into Simmon's citrate agar slants and incubated at room temperature for 24hrs. Color change from green to blue indicated a positive test while no color change indicated a negative result (Aneja, 2001).

Methyl Red Test

This test was conducted to detect the production of sufficient acid by fermentation of glucose so that pH drops to 4.5. The test organisms were inoculated into glucose phosphate broth and incubated at room temperature for 2-5 days. Five drops of 0.04% solution of methyl red was added and mixed well. A bright red colour indicated a positive result while a yellow colour indicated a negative result (Aneja, 2001).

Urease Test

The ability of an organism to produce urease enzyme was evaluated by this test. The test organisms were separately inoculated on the entire slope of Christensen's medium which contained urea and phenol red indicator. It was incubated at

room temperature and examined after 4hrs overnight. Development of red colour indicated the production of urease enzyme by the organisms (Aneja, 2001).

The Coagulate Test

0.5ml suspension of a 24h broth culture of the bacteria was mixed with 1m1 of anticoagulant containing blood plasma in a test tube. This was incubated at room temperature and positive coagulated bacteria revealed for the formation of fibrin clots in the test-tubes.

f. MOLECULAR ANALYSIS

Isolation Of DNA From Predominant

Organic extraction method was used to obtain DNA from the bacteria isolates. A 24hr culture of the bacteria isolate was scrapped into a 1.5ml tube containing 400µl of lysis buffer and 10µl proteinase K using a wire loop and vortexed. The tube was placed on heat block at 6 ^{o}C for 1hr. 400µl of chloroform was added to the tube and vortexed briefly, then spinned at 10000rpm for 10minutes to separate the phases. The upper layer was carefully pipette into another 1.5ml tube leaving behind the white interphase in the old tube. Equal volume of 100% ethanol and 20µl of 3M sodium acetate was added and mixed by inverting several times and then incubated at 2^{o}C over night. The tube was then spinned in a refrigerated centrifuge at a maximum speed of 30 minutes in the same orientation. The ethanol in the tube was decanted and 400µl of 70% ethanol was added again and spinned at maximum speed for 5minutes @ 4 ^{o}C. This later stage was repeated in order to ensure a proper elimination of the salt present. The ethanol in the tube was removed completely and the tube was left open for 10 minutes as to allow the DNA to dry up. The pellet was resuspended in 20µl of sterile water.

Amplification Of The Four Predominant Bacteria Isolates Genes Using 16S rRNA Polymerase Chain Reaction (PCR)

The principle of PCR consist of an exponential amplification of DNA fragment and it is based on the mechanism of DNA replication invivo. Double stranded DNA is denatured to single stranded DNA, each single strand DNA is annealed by the forward and reverse primers of known sequence and elongated using taq DNA polymerase to produce copies of DNA template.

Polymerase Chain Reaction (PCR) amplification of the bacterial DNA (predominant isolates) was amplified using16S rRNA universal primer set GGACTACAGGTATCTAAT 16S for primer Ribose-1 forward and AGAGTTTGATCCTGG 16S REV primer RIBOSE-2-Reverse (Momba and Kamika, 2013). The genomic DNA, the primers and PCR master mix were added into a PCR tube. The tubes were spinned to collect the droplets. The tubes were then inserted into the PCR machine with the regulation for initial denaturation for 5 min at 94 ^{0}C, 30 cycles of denaturation (1 min at 94 ^{0}C), Annealing (30sec at 55 ^{0}C), extension (30sec at 72 ^{0}C) and final extension (5 min at 72 ^{0}C). The amplified genes were then separated in 1% agarose gel electrophoresis.

Agarose Gel Electrophoresis Of The PCR Products

For 1.5%, 3g of agarose gel solution was subjected in a boiling water bath until agarose completely dissolved. The solution was allowed to cool in a water bath set for 55^{0}C. A gel casting tray was prepared by sealing the ends of gel chamber with a tape and appropriate number of combs was placed in the gel tray. 5µl of ethidium bromide was added to the cooled gel and transferred into gel tray. This was allowed to cool for 30 min at room temperature. The combs were removed, placed in electrophoresis chamber and covered with buffer. The DNA and standard (ladder) were loaded onto the gel and electrophoresis. The DNA bands were visualized using UV lightbox.

Sequencing And Bioinformatics Of The Sequences

This analysis was carried out using the Dye terminator cycle sequencing method with quick start kit from Bechman counter U.S.A. The sequencing reaction was prepared in a 2.0ml tube. All reagents used was kept on ice while preparing the sequencing reactions and were added in the order listed as follows; dH2O 0 - 9.5ul > DNA template 0.5 – 10.0ul > Primers 2.0ul > DTCS Quick start master mix 8.0ul. After the preparation of the sequencing reagents, the sequencing reaction was set up in the PCR machine in the order as follows; for thermal cycling program: 9^{0}C for 20sec, 5^{0}C for 20sec X 30cycle1 and 6^{0}C for 4min. The resuspended samples were transferred to their appropriate wells of the sample plate (PN 609801) and overlaid with one drop of mineral oil from the kit and then loaded into the instrument to run. The genetic sequences generated were subjected to BLAST using the NCBI website (www.ncbi.nlm.nih.gov) for identification.

III. RESULTS OF THE MICROBIAL ANALYSIS ON BREWERY WASTEWATER

A. RESULT OF THE TOTAL BACTERIA COUNT OBTAINED FROM THE BREWERY WASTEWATER

Table 1.0 shows the total bacteria count obtained from the brewery wastewater and solid support. It was discovered from this study that the maximum bacteria count for the month of July was in wastewater at sampling point B i.e. along the wastewater flow tunnel (120cfu) and the least count was observed in the solid support at sampling point B (50cfu). Also, in the month of August, highest and lowest colony count was obtained in wastewater at point B (60cfu) and point A i.e. wastewater discharge point (10cfu) respectively. For the month of August, the highest no of bacteria count was obtained from wastewater and solid support at point C (>300cfu) while the lowest bacteria count was observed in wastewater at point A (30cfu). Lastly, In the month of January, it was observed that the highest number of bacteria count was found in the wastewater and solid support at points

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B and C (>300cfu) and the lowest bacteria count was obtained in wastewater (50cfu)

B. MORPHOLOGICAL CHARACTERIZATION OF THE BACTERIA ISOLATES FROM THE DISCHARGED WASTEWATER AND SOLID SUPPORTS SAMPLES

Table 2.0 shows that Twenty one (22) bacteria organisms were isolated from the discharged wastewater and solid support of the discharged brewery wastewater. Morphological test carried out on them showed variations in their colonial appearances such as shape, color, form and elevation as shown in the table 4.3 below.

C. GRAM STAINING RESULT OF THE BACTERIAL ISOLATES OBTAINED FROM THE BREWERY WASTEWATER STUDIED

The bacteria isolates were gram stained and viewed under the microscope. Table 3.0 shows that there were seventeen (17) Rods (12 gram + and 5 gram -) and five (5) Cocci (4 gram – and 1+).

D. RESULT OF THE BIOCHEMICAL ANALYSIS CARRIED OUT ON THE ISOLATED BACTERIA SPECIES

Table 4.0 shows the various biochemical tests carried out on the bacteria isolates and their probable identities. It was discovered that out of the 22 isolates obtained, ten (10) were *Bacillus spp*., three (3) *E.coli spp.*, two (2) *Klebsiella spp*., and one (1) each for *S. proteus, Enterobacter spp*., *Salmonella spp*., *Bordetella spp.*, and *Pseudomonas spp*. respectively.

E. RESULT OF THE MOLECULAR ANALYSIS ON THE PREDOMINANT ISOLATED BACTERIA SPECIES

a. GENE SEQUENCING FOR THE AMPLIFICATION OF THE FOUR PREDOMINANT BACTERIA ISOLATES GENES USING 16S RRNA POLYMERASE CHAIN REACTION (PCR)

The genes of the four predominant bacteria isolates were sequenced using 16S rRNA PCR and the nucleotide sequences in plate 4.1 were obtained. A total query length of 465, 535, 511 and 270 were obtained for *Bordetella pertussis*, *Bacillus cereus*, *Lysinibacillus sphaericus* strain G39 and *Lysinibacillus sphaericus* strain TMB2

b. AGAROSE GEL ELECTROPHORESIS OF THE PCR PRODUCTS

PCR was carried out on four bacteria isolates. Figure 4.1 shows the picture of the isolate on Agarose Gel Electrophoresis.

Sampling Points	July		August		December		January	
	Wastewater (cfu)	Solid Support (cfu)	Wastewater (cfu)	Solid Support (cfu)	Wastewater (cfu)	Solid Support (cfu)	Wastewater (cfu)	Solid Support (cfu)
A	$1.2X10_6$	$5.0X10_4$	$1.0X10_4$	$2.0X10_8$	$1.5X10^4$	$2.5X10_6$	$3.0X10_8$	$1.0X10_6$
B	$5.5X10_3$	$6.0X10_4$	$6.0X10_2$	$5.0X10_5$	$3.0X10_6$	$7.5X10_6$	$5.0X10_5$	$3.0X10_8$
C	$6.5X10_2$	$5.0X10_5$	$5.0X10_3$	$2.0X10_7$	$3.0X10_7$	$3.3X10_8$	$3.0X10_8$	$3.0X10_6$

Key: cfu= colony forming unit. A= point of discharge of wastewater from the brewery, B= sampling point along the wastewater discharge tunnel (150meteer away from the brewery) and C= sampling point along the wastewater discharge tunnel (150meteer away from sampling point B).

Table 1.0: Total Bacteria Count of the isolates obtained from the brewery wastewater studied

Colonial variables	Isolates Number												
	1 , 2, 4	3	5	6	7	8,9,10, 11,12, 13, 14	15	16	17	18	19	20, 21	22
Shape	Rod	Rod	Rod	Cocci	Rod	Rod	Cocci	Rod	Rod	Cocci	Cocci	Rod	Rod
Color	Yellow	Yellow	Creamy	Pink	Gray	Creamy	Yellow	Yellow	Creamy	Yellow	Yellow	Yellow	Yellow
Form	Irregular	Circular	Circular	Circular	Irregular	Circular	Circular	Circular	Circular	Circular	Circular	Irregular	Circular
Elevation	Flat	Raised	Raised	Raised	Raised	Raised	Raised	Raised	Raised	Raised	Raised	Raised	Raised
Gram staining	+	-	-	-	-	+	+	-	-	-	-	+	-
Probable identity	Bacillus spp.	E.coli spp.	Bordetella spp.	Pseudomonas spp.	K.. pneumoniae spp.	Bacillus spp.	Bacillus spp.	S. proteus	Klebsiella spp.	Enterobacter spp.	Salmonella spp.	E.coli spp.	Enterobacter spp.

Table 2.0: Morphological Characterization of the Bacteria Isolates Obtained from Brewery Discharged Wastewater and Solid supports

Isolate	TSI Agar Test	Indole Test	Motility Test	Oxidase Test	Citrate Test	Methyl Red Test	Voges Proskaur Test	Urease Test	Catalase Test	Coagulase Test	Spore Staining	Probable Organism
1	-	-	-	-	-	-	-	-	+	+	-	*Bacillus spp*
2	-	-	+	-	+	-	+	-	+	+	-	*B. cereus*
3	A/AG	+	+	-	+	+	-	+	-	-	-	*E.coli*
4	-	-	-	-	-	-	-	-	+	+	-	*B. cereus*
5	A/A+H_2S	+	+	+	+	-	-	-	+	-	-	*Bordetella spp.*
6	K/AG	+	+	+	+	-	-	+	-	-	-	*Pseudomonas spp*
7	A/A+H_2S	+	+	-	+	-	-	+	-	-	-	*Klebsiella spp*
8	-	-	-	-	-	-	-	-	+	+	-	*Bacillus spp*
9	-	-	-	-	-	-	-	-	+	+	-	*Bacillus spp*
10	-	-	-	-	-	-	-	-	+	+	-	*Bacillus spp*
11	-	-	-	-	-	-	-	-	+	+	-	*Bacillus spp*
12	-	-	-	-	-	-	-	-	+	+	-	*Bacillus spp*
13	-	-	-	-	-	-	-	-	+	+	-	*Bacillus spp*
14	-	-	-	-	-	-	-	-	+	+	-	*Bacillus spp*
15	-	-	-	-	-	-	-	-	+	+	-	*E.coli*
16	A/AG	+	+	-	+	+	-	+	-	-	-	*S. Proteus*
17	A/AG	+	+	+	+	-	-	+	-	-	-	*Klebsiella spp*
18	A/AG	+	+	-	+	-	-	+	-	-	-	*Enterobacter spp*
19	A/A	-	-	+	-	+	-	+	-	-	-	*Salmonella spp*
20	-	-	-	-	-	-	-	-	+	+	-	*E.coli*
21	-	-	-	-	-	-	-	-	+	+	-	*E. coli*
22	A/A	+	-	+	+	-	-	+	+	-	-	Enterobacter spp.

Key: A/AG- Yellow/yellow with Gas present, A/A- Yellow/Yellow, K/AG- Red/ Yellow with Gas Present, - Negative, + Positive,TSI-Tripple Sugar Iron, Spp-Specie

Table 3.0: Biochemical Characterization of the Bacterial Isolates obtained from Discharged Brewery Wastewater and Solid Support along the Discharging Tunnel

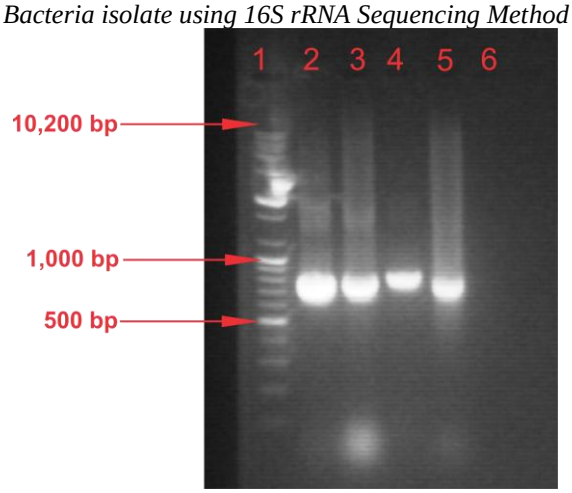
Bordetella pertussis
AAGTCGAGCGAATGGATTAAGAGCTTGCTCTTATGAAGTTAGCGGC
GSMCGKKTKAGTAACACGTGGGTAACCTGCCCATAAGACTGGGAT
AACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACCRC
ATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGA
CCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGC
AACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGAC
TGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCT
TCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGAT
GAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGT
GCTASTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCC
ACGGCTAACTACGTGCCAGCAGCCGCGGWAATACGTAGGTGGCAA
GCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAG

Bacillus cereus
GAACGCAGCACGGACTTCGGCTGKTGGMGAGTGGCGAACGGTGAG
TAATGTATCGAACGTGCCCAGTAGCGRGATAACTACGCGAAAGGG
SCTAATACCGATACGCCCTACGGGGGAAAGCGGGGGACCTTCGGG
CCTCGAACTATTGGAGCGGCCGATATCGATTACTATGGGGATAACG
GTCACCAAGGGACGATCCGAGTGTTGAGAGGACGACCACACACTG
GACTGAGACACGGCCAGACTCCTACGGGAGGCATCCTGGGGAATT
TTGACAATGGGCAACCCTGAGCCAGCCATCCCGCGTGTGCGATGA
AGGCTTCGTTGAAAGCACTTTTGGAGGAAAGAAACGGGCCGTAAT
ACCTGGCGCTAATGACGGCCTGCAAATAAGCCGTAACTACGGCCA
GACCGCGGATACTASGGKGCAAGCGTTAATCGGAATTACTGGGCG
AAAGCGTGCGCAGG

***Lysinibacillus sphaericus* strain G39**
TCGAGCGAACAGAGAAGGAGCTTGCTCCTTTGACGTTAGCGGCGG
MCGGKTGAGTAACACGTGGGCAACCTACCTTATAGTTTGGGATAA
CTCCGGGAAACCGGGGCTAATACCGAATAATCTGTTTCACCTCATG
GTGAAATATTGAAAGACGGTTTCGGCTGTCGCTATAGGATGGGCCC
GCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGAC
GATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGA
GACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCC
ACAATGGGCGAAAGCTGATGGAGCAACGCCGCGTGAGTGAAGAAG
GATTTCGGTTCGTAAAACTCTGTTGTAAGGGAAGAACAAGTACAGT
AGTAACTGGCTGTACCTTGACGGTACCTTATTAGAAAGCCACGGCT
AACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGAAGCGTGTC
CGAATTATTGG

***Lysinibacillus sphaericus* strain TMB2**
GCCCGCGGCGTGCCTATACTGCAAGTCGAGCGTAACAGAGAAGGA
GCTTGCTCCTTTGACGTTAGCGGCGGACGGGTGAGTAACACGTGGG
CAACCTACCTTATAGTTTGGGATAACTCCGGGAAACCGGGGCTAAT
ACCGAATAATCTGTTTCACCTCATGGTGAAATATTGAAAGACGGTT
TCGGCTGTCGCTATAGGATGGGCCCGCGGCGCATTAGCTAGTTGGT
GAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTT

Plate 1.0: Sequenced Nucleotides for the Four Predominant Bacteria isolate using 16S rRNA Sequencing Method



Key: Well 1- DNA Marker (Ladder), Well 2- Bordetella pertussis, well 3- Bacillus thuringiensis, Well 4- Lysinibacillus spp, well 5- Lysinibacillus sphaericus spp and Well 6 - Negative Control

Figure 1.0: Agarose Gel Electrophoresis of PCR Amplification of the Four Predominant Bacteria Isolate

D. BIOINFORMATICS RESULT OF THE NUCLEOTIDE SEQUENCES OF THE FOUR PREDOMINANT ISOLATES

The PCR products (nucleotide sequences) were subjected to BLAST for the confirmation of the identities of the bacteria isolates as seen in Figures 4.2- 4.5 The BLAST results revealed the Bacteria isolates identities as *Bordetella pertussis*, *Bacillus cereus*, *Lysinibacillus sphaericus* strain G39 and *Lysinibacillus sphaericus* strain TMB2B for Isolate 1,2,3 and 4 respectively.

IV. DISCUSSION

A. BACTERIA SPECIES ISOLATED FROM THE BREWERY EFFLUENT

The presence of high counts of bacteria species (morphological, Gram reaction and biochemical tests conducted on the isolates) (Table 4.3a) in the brewery effluent recorded in this study could be attributed to the high nutritional contents of the brewery effluent in terms of carbohydrate, protein (Table 4.1) and minerals that serves as nutrient for the growth of these microorganisms. The findings of this study in terms of the bacterial load of the brewery effluent discharged from the industry was found to be in agreement with a study reported by Rheinheimer, (1991) were he attributed the reason to the introduction of wastewater that contain high amount of organic matter and essential nutrients which eventually brings about changes in the micro flora. This is because the presence of high microbial population in waste water such as brewery effluent and aquatic system is often a reflection of the input of microorganisms from extraneous sources and availability of growth supporting organic matter as earlier reported by Sayler *et al.* (1975). This finding is in accordance to a similar study reported by Kanu and Achi, (2011), were they all reported that brewery effluents are high in carbohydrates; nitrogen contents and the introduction of these organic matter in wastewater, high in essential nutrients can bring about changes in the aquatic microflora present in the receiving water body. The discharged brewery wastewater might have also served as a conduit through which a plethora of viable microorganisms were introduced into the river. Also, the identification and prevalence of fecal coliforms especially *E. coli*, *Enterobacter species*, *Salmonella species* and *Klebsiella species* examined in the brewery effluent samples is alarming as the presence of these bacteria is indicative of fresh fecal contamination of the river from an external source and also the potential presence of pathogens in the effluent. Gerardi and Zimmerman, (2005), also reported a similar finding.

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Description	Max score	Total score	Query cover	E value	Ident	Accession
Bordetella petrii strain SFSA64 16S ribosomal RNA gene, partial sequence	571	571	99%	5e-159	88%	KU744861.1
Uncultured Achromobacter sp. partial 16S rRNA gene, isolate U5T_11323	571	571	99%	5e-159	88%	LT685908.1
Bordetella pertussis strain VITSBSTV04 16S ribosomal RNA gene, partial sequence	571	571	99%	5e-159	88%	KX138518.1
Bordetella trematum strain H044680328 genome assembly, chromosome: 1	571	2858	99%	5e-159	88%	LT546645.1
Bordetella sp. E124 16S ribosomal RNA gene, partial sequence	571	571	99%	5e-159	88%	KR703504.1
Uncultured bacterium clone HF72 16S ribosomal RNA gene, partial sequence	571	571	99%	5e-159	88%	KR188948.1
Bordetella sp. BAB-4401 16S ribosomal RNA gene, partial sequence	571	571	99%	5e-159	88%	KP751929.1
Bordetella sp. BAB-4396 16S ribosomal RNA gene, partial sequence	571	571	99%	5e-159	88%	KM289182.1
Bordetella sp. BAB-4383 16S ribosomal RNA gene, partial sequence	571	571	99%	5e-159	88%	KM289174.1
Uncultured bacterium clone ncm38h07c1 16S ribosomal RNA gene, partial sequence	571	571	99%	5e-159	88%	KF087634.1
Uncultured bacterium clone ncm38d04c1 16S ribosomal RNA gene, partial sequence	571	571	99%	5e-159	88%	KF085789.1
Uncultured bacterium clone ncm21d12c1 16S ribosomal RNA gene, partial sequence	571	571	99%	5e-159	88%	KF085098.1
Uncultured bacterium clone nck79d07c1 16S ribosomal RNA gene, partial sequence	571	571	99%	5e-159	88%	KF082134.1
Uncultured bacterium clone nck78e07c1 16S ribosomal RNA gene, partial sequence	571	571	99%	5e-159	88%	KF081989.1

Figure 2.0: Bioinformatic Result on the Genetic Sequences of Bordetella pertusis Generated from the PCR Analysis of the predmoninant bacteria Isolates from the Discharged Brewery Samples

Description	Max score	Total score	Query cover	E value	Ident	Accession
Bacillus sp. strain 01-1A31 16S ribosomal RNA gene, partial sequence	959	959	100%	0.0	99%	KX688647.1
Bacillus subtilis strain WA 16S ribosomal RNA gene, partial sequence	959	959	100%	0.0	99%	MF001286.1
Bacillus cereus strain S3FP28-IIa 16S ribosomal RNA gene, partial sequence	959	959	100%	0.0	99%	KX641082.1
Bacillus cereus strain C1L, complete genome	959	13077	100%	0.0	99%	CP022445.1
Bacillus sp. strain R13 16S ribosomal RNA gene, partial sequence	959	959	100%	0.0	99%	KX618295.1
Bacillus sp. strain R66 16S ribosomal RNA gene, partial sequence	959	959	100%	0.0	99%	KX618256.1
Bacillus cereus strain 28 16S ribosomal RNA gene, partial sequence	959	959	100%	0.0	99%	MF480467.1
Bacillus sp. strain 12-2-4B 16S ribosomal RNA gene, partial sequence	959	959	100%	0.0	99%	MF480432.1
Bacillus thuringiensis strain c25, complete genome	959	13344	100%	0.0	99%	CP022345.1
Bacillus cereus strain M13, complete genome	959	12468	100%	0.0	99%	CP016360.1
Bacillus cereus strain K8, complete sequence	959	13333	100%	0.0	99%	CP016595.1

Figure 3.0: Bioinformatic Result on the Gene Sequences of Bacillus cereus Generated from the PCR Analysis of the predmoninant bacteria Isolates from the Discharged Brewery Samples

Description	Max score	Total score	Query cover	E value	Ident	Accession
Lysinibacillus sphaericus strain G39 16S ribosomal RNA gene, partial sequence	918	918	100%	0.0	99%	KX344027.1
Lysinibacillus sp. strain G56 16S ribosomal RNA gene, partial sequence	918	918	100%	0.0	99%	KX344012.1
Lysinibacillus sp. strain G50 16S ribosomal RNA gene, partial sequence	918	918	100%	0.0	99%	KX344006.1
Lysinibacillus sphaericus strain PD2 16S ribosomal RNA gene, partial sequence	918	918	100%	0.0	99%	KX343941.1
Lysinibacillus fusiformis strain NBRC 15717(T) 16S ribosomal RNA gene, partial sequence	918	918	100%	0.0	99%	KY328837.1
Lysinibacillus sp. strain WUST-Cr1 16S ribosomal RNA gene, partial sequence	918	918	100%	0.0	99%	KX096881.1
Lysinibacillus sphaericus strain ARD1 16S ribosomal RNA gene, partial sequence	918	918	100%	0.0	99%	KX023224.1
Lysinibacillus sphaericus strain OCC29 16S ribosomal RNA gene, partial sequence	918	918	100%	0.0	99%	KU525327.1
Lysinibacillus fusiformis strain AN-09 16S ribosomal RNA gene, partial sequence	918	918	100%	0.0	99%	KU937310.1
Lysinibacillus fusiformis strain Se15 16S ribosomal RNA gene, partial sequence	918	918	100%	0.0	99%	KX959988.1
Lysinibacillus sphaericus strain VN3-1 16S ribosomal RNA gene, partial sequence	918	918	100%	0.0	99%	KX863497.1
Lysinibacillus sp. strain RR7 16S ribosomal RNA gene, partial sequence	918	918	100%	0.0	99%	KU752895.1
Lysinibacillus fusiformis strain B10 16S ribosomal RNA gene, partial sequence	918	918	100%	0.0	99%	KU953930.1

Figure 4.0: Bioinformatic Result on the Gene Sequences of Lysinibacillus sphaericus strain G39 obtained from the PCR Analysis of the predmoninant bacteria Isolates from the Discharged Brewery Samples

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Lysinibacillus sphaericus strain TMB2 16S ribosomal RNA gene, partial sequence	479	479	98%	2e-131	99%	GU172164.1
Lysinibacillus sphaericus strain E6 16S ribosomal RNA gene, partial sequence	477	477	98%	6e-131	99%	JX286700.1
Lysinibacillus sphaericus strain EI-15 16S ribosomal RNA gene, partial sequence	477	477	98%	6e-131	99%	FJ613543.1
Lysinibacillus sphaericus strain VN3-1 16S ribosomal RNA gene, partial sequence	475	475	97%	2e-130	99%	KX863497.1
Bacterium strain PAH10 16S ribosomal RNA gene, partial sequence	473	473	98%	8e-130	99%	MF278993.1
Lysinibacillus sphaericus strain LSR1 16S ribosomal RNA gene, partial sequence	473	473	98%	8e-130	99%	MF000302.1
Lysinibacillus sphaericus strain SADAAB_31 16S ribosomal RNA gene, partial sequence	473	473	98%	8e-130	99%	KX908032.1
Lysinibacillus sphaericus strain 2362, complete genome	473	5630	98%	8e-130	99%	CP015224.1
Lysinibacillus sphaericus III(3)7, complete genome	473	5630	98%	8e-130	99%	CP014856.1
Lysinibacillus sphaericus strain OT4b.25, complete genome	473	5630	98%	8e-130	99%	CP014643.1
Lysinibacillus sp. C-3-43 16S ribosomal RNA gene, partial sequence	473	473	98%	8e-130	99%	KT583559.1
Bacillus sp. B-3-35 16S ribosomal RNA gene, partial sequence	473	473	98%	8e-130	99%	KT583528.1
Bacillus sp. B-3-30 16S ribosomal RNA gene, partial sequence	473	473	98%	8e-130	99%	KT583523.1

Figure 5.0: Bioinformatic Result of the Gene Sequences of Lysinibacillus sphaericus strain TMB2 obtained from the PCR Analysis of the predmoninant bacteria Isolates from the Discharged Brewery Samples

This trend is also in agreement with an earlier observation by Bello-Osagie and Omoruyi (2012) who reported the isolation of high number of *E. coli* from water samples at the point of discharge of brewery effluent into Ikpoba River.

A worrisome observation noticed by the researchers during the sampling of both the treated effluent and the water sample at the point of discharge was the deliberate contamination of the treated effluent being conveyed within the pipe from the brewery plant at sections close to the river with human feces by certain individuals living around the vicinity of the river bank. This is suggestive of the low level of public hygiene exhibited by people living around the river which is contributing to the fecal pollution alongside the deleterious effects of the brewery wastewater on the receptacle (Ikpoba River).

B. MOLECULAR IDENTIFICATION OF THE FOUR PREDOMINANT ISOLATED BACTERIA SPECIES

The use of 16S rRNA gene sequences to study bacterial phylogeny and taxonomy has been by far the most common housekeeping genetic marker used for a number of reasons. These reasons include: its presence in almost all bacteria, often existing as a multigene family, or operons; the function of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time (evolution); and the 16S rRNA gene (1,500 bp) is large enough for informatics purposes(Patel J.B., 2001). One of the most attractive potential uses of 16S rRNA gene sequence informatics is to provide genus and species identification for isolates that do not fit any recognized biochemical profiles, for strains generating only a "low likelihood" or "acceptable" identification according to commercial systems (Woo, P.C.Y *et al*, 2003). The cumulative results from a limited number of studies to date suggest that 16S rRNA gene sequencing provides genus identification in most cases (>90%) but less so with regard to species (65 to 83%), with from 1 to 14% of the isolates remaining unidentified after testing (Drancourt, M. et al 2000).

The sequencing analysis conducted on the four predominant bacteria isolates revealed their identities up to strain levels which conforms to the report of Woo et al (2003). The conventional approaches (Biochemical and microscopic) identified the predominant isolates 1, 2, 3 and 4 as Bordetella spp, Bacillus cereus, Klebsiella spp and Bacillus spp respectively but the molecular approach identified them as Bordetella pertussis, Bacillus cereus, L. sphaericus G39 and L. sphaericus TMB2 respectively. The variation in the identities of this isolates with this two approaches conforms with the investigation of Fontana et al (2005). This variation is attributed to the fact that biochemical identification of bacteria is on the basis of phenotypic characterization while molecular identification is based on genotypic methods (woese, C.R 1987). 16S rRNA gene sequence analysis can better identify poorly described, rarely isolated or or phenotypically aberrant strains and can lead to the identification of novel pathogens and noncultured bacteria. Thus, it remains the most accurate means of bacteria identification over the conventional approach (Mignard, S. etal 2006).

V. CONCLUSION

Total of 22 bacteria species were isolated from the brewery wastewater comprising of; *Bacillus spp.*, *Lysinibacillus spp.*, *E. coli*, *Enterobacter* species, *Salmonella* species and *Klebsiella* species. Colony counts of the bacteria species were conducted. The four predominate isolated bacteria species with the most counts were subjected to molecular characterization to further confirm their identities (*Bacillus cereus*, *Bordetella pertussis*, *lysinibacillus sphaericus* strain G39, *lysinibacillus sphaericus* strain TMB2).

VI. RECOMMENDATION

Based on the findings of this research, I recommend that these indigenous bacteria isolates should be tested on the wastes from which they were obtained inorder to assess their capabilities in biodegrading the contaminants in them.

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