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Molecular Identfication of *Bacillus pumilus* BY 16S rRNA from Abattoir Wastewater

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Abstract. Abattoir is one of the most pronounced but yet ignored sources of highly recalcitrant wastewater that pose many environmental challenges and cause harm to mankind. However, little was reported on the molecular identification of bacterial committee from the abattoir wastewater. This study aimed at isolation and molecular identification of most abundant bacterium species from wastewater of abattoir. Wastewater sample was collected, isolate and identify the most abundant bacterium by gram staining and microscopy. Genomic DNA (gDNA) was extracted from the isolated bacterium and conducted 16S rRNA PCR amplification. Multiple sequence and phylogenetic relationship among other 8 *Bacillus* species and 1 outgroup were inferred by comparing the sequence data sets from NBCI. The biochemical identification analyses indicated that the bacterium is gram positive with *Bacillus* shape and cream-yellow colour. 16S rRNA and bioinformatics analyses demonstrated that the most abundant isolated bacterium is *Bacillus pumilus* with 99% identification. Moreover, the phylogenetic tree analysis discovered that the identified bacterium from this study is more closely related to *Bacillus* species as they share the same clade. The sequence is partial as compared with the other deposited sequences in the GeneBank. This study provides an insight on the microbial species of Nigeria abattoir wastewater which was identified using molecular approach. As well, gave a clue for potential treatment of the wastewater.

Key words: 16S rRNA, *Bacillus pumilus*, Abattoir, Wastewater, Nigeira

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INTRODUCTION

Abattoir/slaughterhouse industry is a major supplier of a source of meat and contribute to so many environment problem. In abattoir, wastewater and solid waste are generated which later released to the environment (Ikekwem et al., 2017). The demand for meat really increases the release of wastewater from slaughter houses that has significant impacts on the environment toward the spread of microbes (Businge, Kagoya, Omara, & Angiro, 2021). The negative effect of abattoir waste on the quality of both surface and ground water is alarming in Nigeria and other part of the world. Waste produces from abattoir is more than 90% water and less than 10% solid which comprises animal tissues, clog blood and animal pieces. Abattoir operation generate huge amount of sewage which contains suspended solids, organic matter, phosphorus and nitrogen compounds in high concentration (Ikekwem et al., 2017). However, the waste products are released directly into the environment without undergoing any treatment in many areas (Okey-Onyesolu, Onukwuli, Ejimofor, & Okoye, 2020).

Microbial contamination can equally affect the quality of fresh meat, shorten its shelf-life and cause economic losses and health hazards. (Adegbola, Adewoye, & Abosede, 2012), reported on the evidence of water related illness caused by microbial contamination as a result of pollution from abattoir wastewater. In such

wastewater condition, oxygen continue to deplete as an electron acceptor, prompting denitrifying bacteria to reduce available nitrate into gaseous nitrogen which later enters the atmosphere resulting to environmental hazard. Likewise, methanogens (anaerobic archaea), may produce overweening methane at higher rate than methanotrophs (aerobic methane oxidizing bacteria) could cope with, thus contributing to global warming and green house effects. Leaching into groundwater is a major part of the concern, especially due to the recalcitrant nature of some abattoir contaminants (Iroha et al., 2016).

The most important issue in abattoir and meat-processing industries is maintenance of proper hygiene and adequate sanitary conditions. The isolation of bacteria from animal tissues usually involves series of extraction, purification steps and also consumes a lot of resource and time (Karray, Alonazi, Horchani, & Ben Bacha, 2021). Therefore, intermittent microbial analysis is necessary to determine the nature of the contamination and kind bacterium responsible in order to provide maintenance to the meat and ensure safe public health. Little was reported on the quality analysis of abattoir wastewater, as well as identification of bacterial community to molecular level. This study was carried out to isolate and identify the most abundant bacterium species using molecular approach from abattoir wastewater collected from Potiskum main abattoir, Nigeria.

METHODS

Collection of Wastewater Sample and Bacterial Isolation

Final discharged wastewater from Potiskum main abattoir was aseptically collected in a sterile bottle at 7am. The sample was immediately transferred to laboratory and stored at 4ºC immediately prior analysis. Different media were used for the isolation of the bacterium from the wastewater. The media were prepared and sterlized as instructed by the manufacturers; Nutrient agar (International Diagnostic Group, UK), McConkey agar, No.3 (Oxoid, UK) and Eosin methylene blue (EMB) agar (Fisher Scientific, USA).

A drop of the wastewater sample was spread on the different agar plates and incubated at 28ºC overnight for bacterial growth. Sub-culturing was continued until pure culture of most abundant colony was obtained and grown to observed morphological features. A clean grease-free slides were obtained, and a drop of sterile water was put, then emulsified the pure colony on the different slides by making a thin smears. Gram staining was carried out using Lugol's iodine, 95% acetone and flooded with Safranin which served as counter stain. Microscopy was carried out at different objective lens under oil immersion.

Bacterium Genomic DNA Extraction and Assessment of its Quantity and Quality

Genomic DNA (gDNA) was extracted from pure colony of most abundant bacterium using Promega mini-kit following the manufacturer's instruction. For concentration and purify of the gDNA; spectrophotometric absorbance at A_{230}/A_{260} nm was observed using Nanodrop^{TM} 1000 spectrophotometer. While the quality of the gDNA was determined using agarose gel electrophoresis on 1 % (w/v) agarose in 1 X TAE buffer and post stained using Ethidium Bromide. The gel was run at 70V and 425A for 30 minutes, then visualized under the UV light using Gel documentation unit.

16S rRNA Polymerase Chain Reaction (PCR) and Amplicon Sequencing

PCR method employed was 16S rRNA gene as target using universal primer for identifying the unknown bacterium as described by Alikunhi, Batang, AlJahdali, Aziz, and Al-Suwailem (2017). The thermocycling condition involved pre-denaturation at 94 ºC for 5 min, followed by 30 cycles: denaturation for 60 sec at 94 ºC, annealing for 60 sec at 52 ºC and extension for 2 min at 72 ºC, then final extension at 72 ºC for 5 min and cooling at 4 ºC. The amplicon quality was analyzed by electrophoresis (70 V, 425 A and 45 min) on 1.2 % (w/v) agarose gel, post stained with Ethidium Bromide. The gel bands were sent for purification and sequencing.

Analysis of 16S rRNA Gene Sequence

Different bioinformatics tools were used for the analyses (Kumar, Stecher, & Tamura, 2016; Mohammed, Deba, Abubakar, Muhammed, & Abdullahi). BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for identification and similarity checking was employed. 16S rRNA sequence from the present study with 10 others from GenBank (http://www.ncbi.nlm.nih.gov/) including 8 *Baccilus pumilus*, 1 *Baccilus safensis* and single out group was selected from multiple sequence alignment. The multi-sequence alignment was performed using Clustal-X (Xu et al., 2012).

Meanwhile, a phylogenetic tree of the sequences was constructed using Molecular Evolutionary Genetics Analysis (MEGA X) and multiple alignment too (Kumar et al., 2016). The MEGA-X tree was generated by maximum-likelihood method with distance matrix of 1000 bootstrap trials (Lv et al., 2019).

RESULTS AND DISCUSSION

Isolation of Most Abundant Bacterium

This study investigates the most abundant bacterium from Potiskum' main abattoir wastewater by isolation, gram stain identification and molecular approach. Most abundant colony was identified from Nutrient agar plate containing abattoir wastewater and chosen for further analysis. The gram staining analysis resulted that the bacterium is gram positive. The morphological characteristics analysis of the isolates includes rod-shaped, flat with raised margin, glistering surface and cream-yellow colour. Similar was reported by (Karray et al., 2021) on the isolation of gram positive bacteria from abattoir wastewater. The predominant bacterium species isolated and identified from the sample of the abattoirs wastewater was found to be common to most abattoirs industries.

The microbe isolated from the abattoir can thus be attributed to the personal hygiene and improper sanitary condition of the environments and the animals slaughtered. Such unhygienic practice and improper handling of meat would lead to spread of microbes and invariably affect the nutritive value as meat supplied from the abattoir. The usual practice by some workers through washing the carcass with the same water in which intestines and offal had been washed was considered as one of the predominant reasons for increased in microbial counts of the carcasses. Other researchers identified diverse microbes from from abattoir wastewater including *E. coli*, *Bacillus* species and *Staphylococcus aureus* (Ikekwem et al., 2017; Karray et al., 2021).

Furthermore, presence of such pathogenic microbes on protein source can cause a lot of disease such as diarrhea, hepatitis, dysentery, throat infection and at times fatal food poisoning. This signifies public health importance; hence, the slaughter house workers should be enlightened on hygienic slaughtering system, handling and processing of abattoir products. Proper waste disposal practice and also advised to maintain strict healthful condition at all costs.

Molecular Characterization of *Baccilus pumilus* Isolated from Abattoir Wastewater

DNA and RNA are the two core components of central dogma of molecular biology (Morange, 2009). High-quality and quantity gDNA is the most important prerequisite in nucleic acids downstream application (Zhang et al., 2012). The quality of the gDNA was validated on agarose gel which showed clear band, whereas optimum concentration at 1,219.2±09.63 and purity 1.94±0.07 was obtained by the spectrophotometric analysis. The integrity analysis involving concentration, purity and band determination indicated that the gDNA is contamination-free and can be used for further experiment. Molecular characterization by PCR resulted in clear band showing 16S rRNA amplicon band (Figure 1). Lane M shows the standard DNA size marker ladder, while Lane A indicate the 1,500bp of the 16S rRNA amplified (PCR amplicon band).

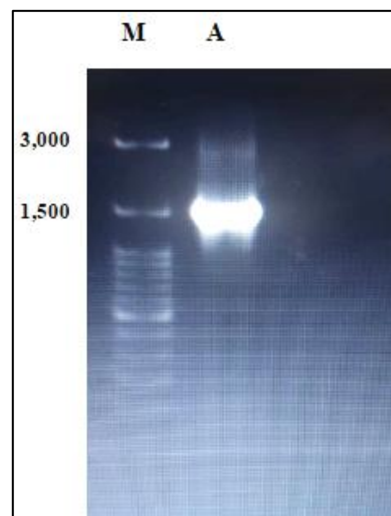

Figure 1. Agarose gel electrophoresis showing the 16S rRNA gene amplicon band of the isolated and most abundant bacterium from abattoir wastewater

The electrophoresis result showed that the 16S rRNA gene was well separated and aligned to the 1,500 bp of the DNA ladder. This indicates that the amplified gene fragment has a size about ± 1,500 bp which is above and more amplified than that of (Ayantunji, Omole, Olojo, & Awojobi, 2020) which appeared at 1,000 bp. Thus, it can be concluded that the amplification process of 16S rRNA gene was successfully performed.

16S rRNA has been used and chosen as bacterial molecular identification method for reasons including its distribution in all bacteria and function that has never change (Muyzer, De Waal, & Uitterlinden, 1993). The gene is large enough to reach the size of 1,500 bp. This molecular approach has led to the standard procedure for determining the phylogenetic relationship among bacteria.

Bioinformatics Analyses of the Gene Sequence

Analysis of the nucleotide sequence of the bacterial isolate (gene sequencing) illustrated that the test bacterium from the present study (YGY abattoir) gave similar sequence which has 99% identity to *Baccilus pumilus* strains available on the Genbank database. The bacterium isolate was therefore deduced as *Bacillus pumilus*. The BLAST significant sequence alignments and percentage identification is shown in Figure 2. The analysis further revealed that the sequence is partial is it contains 1,234 nucleotides (Li et al., 2018).

The alignment and phylogenetic tree construction were examined using different sequence analyzing tools. The 16S rRNA sequence obtained (YGY abattoir) was analyzed for multiple sequence alignment with 10 other 16S rRNA gene sequences from GeneBank. The similarity index of the multi-sequencing analysis indicated that *Bacillus* species are closely conserved (Figure 3). Hence, this conservation among the different strains may be due to their close evolutionary trend. Rare was reported on the sequence alignment of 16S rRNA gene from *Bacillus* species towards determining how conserved they are or their active region(s) (Dorcas & Pindi, 2016; Li et al., 2018).

Furthermore, a phylogenetic tree was constructed through the maximum likelihood method. This was to identify and investigate the evolutionary relationships between the diverse strains and single outgroup (Mohammed, Abd Samad, & Rahmat, 2019). The relationship of all the strains is represented in phylogram (Figure 4). They were classified into two main groups using the consensus tree. The first group comprises *Bacillus species*, while the second group contains the outgroup. *Bacillus pumilus* strains make same clade with *Bacillus safensis*. The phylogeny revealed that YGY abattoir 16S rRNA was clustered with *Bacillus pumilus* UBT3 strains showing their close relations (Figure 4). Consequently, the results implies that YGY abattoir is *Bacillus pumilus* but partial sequence.

Higher population of bacteria discovered at the early stage of the study is possibly due to level of contamination in the wastewater environment. The type, quantity or concentration of the effluent and the level of toxicity are very important. Optimum level of contamination of the abattoir wastewater revealed by many authors confirmed the dangers associated with discharging untreated wastewater to the environment. Thus, the need for proper disposal, think of using the indigenous bacteria for adequate treatment to ensure decontamination remains of paramount important. In fact, sustainability in meat supply should be given

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Bacillus pumilus strain O8 16S ribosomal RNA gene, partial sequence	Bacillus pumilus	1596	1596	99%	0.0	98.99%	1431	KM596793.1
Bacillus pumilus strain MCM B-884 16S ribosomal RNA gene, partial sequence	Bacillus pumilus	1592	1592	99%	0.0	99.10%	1395	JN701186.1
Uncultured Bacillus sp. clone Filt.149 16S ribosomal RNA gene, partial sequence	uncultured Bacillus sp.	1591	1591	99%	0.0	98.99%	1515	HM152736.1
Bacillus sp. (in: Bacteria) strain TuzS2.1 16S ribosomal RNA gene, partial sequence	Bacillus sp. (in: Bacteria)	1589	1589	99%	0.0	98.99%	1384	MH458834.1
Bacillus pumilus strain UBT3 16S ribosomal RNA gene, partial sequence	Bacillus pumilus	1589	1589	99%	0.0	98.99%	1536	KR780583.1

Figure 2. BLASTn analysis for identification of nucleotide sequence. The sequence indicates 99% identity with Bacillus pumilu strains.

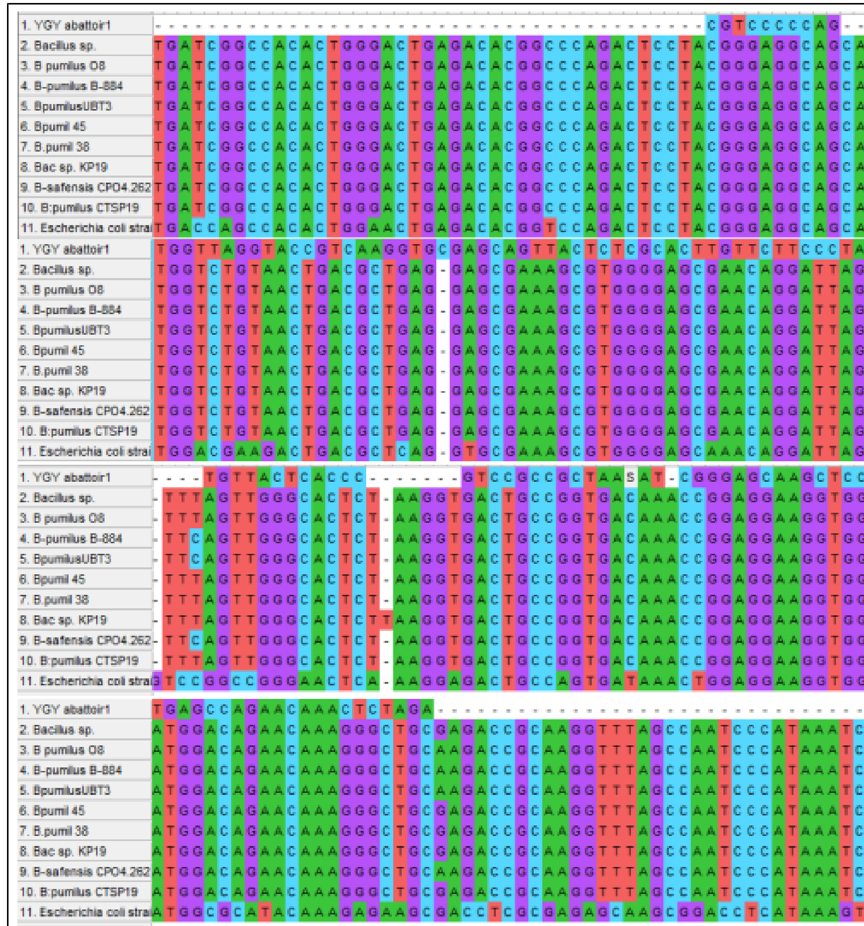

Figure 3. Multiple sequence alignment of the 16S rRNA gene sequence of the bacterium isolate with that of the other bacterial strains and out group

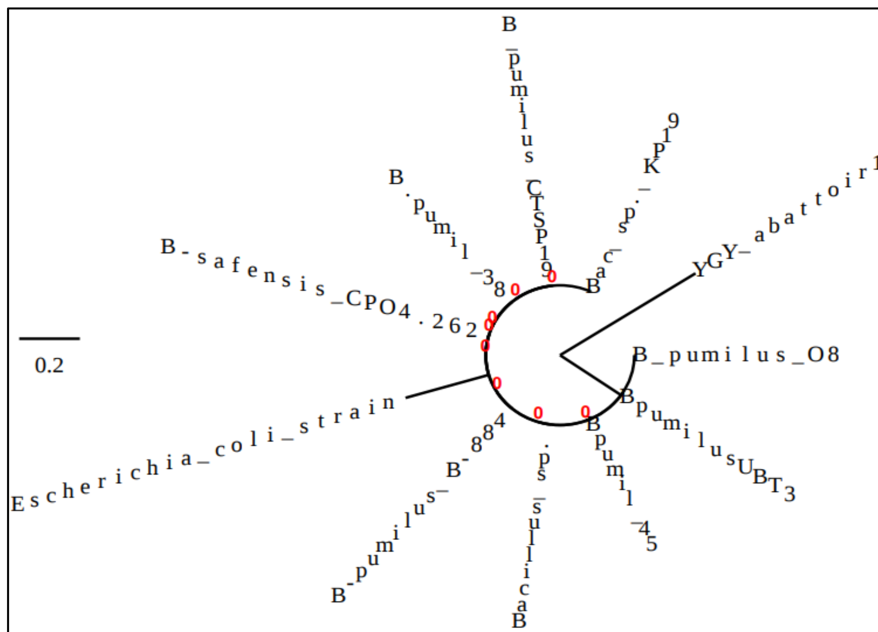

Figure 4. The phylogenetic tree showing the molecular evolutionary relationship between the different bacterial strains

priority of place since it intertwines with public health (Ikekwem et al., 2017). In fact, this was the first to report on the microbial community in abattoir wastewater from Northeastern Nigeria, particularly, Potiskum in Yobe state. It has provided an insight on the possible microbial species of Nigeria abattoir wastewater which was identified up to molecular level. As well, gave a clue for potential treatment of the wastewater both bio-remediation and phyto-remediation (Ogbomida, Kubeyinje, & Ezemonye, 2016). Determining such important information would help the closer communities to take necessary measures toward preventing the society from waterborne diseases and other wastewater related environmental pollution (IJah, 2016).

CONCLUSION

In this study, most abundant bacterium species was successfully and purely isolated from the abattoir wastewater. The bacterium was found to be gram positive *Bacillus species.* This was deduced with the morphological features as rod-shaped, flat with raised margin and cream-yellow colour. Molecular characterization by PCR of the 16S rRNA showed 1,500bp. nBLAST analysis of the isolates' nucleotide gave similar sequence which has 99% identity to *Baccilus pumilus*, while the similarity index based on multi-sequencing analysis indicated that *Bacillus* species are conserved. Moreover, the relationship of all the strains is represented in the phylogram which were classified into two main groups: *Bacillus* strains and out group. Further research should be carried out to determine other pathogenic microbes in such wastewater and also identify microbes that can equally be used for bioremediation of the wastewater.

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