Probing Yeast Fermented Palm Wine Interactions with the Surface of its Plastic Container

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ABSTRACT

The internal surface sections of polyethylene terephthalate (PET) plastic bottles of the same make used for storage of yeast fermented palm wine or still water were compared in order to determine if the palm wine drink caused any corrosion or distortion to the plastic surface. Surfaces of triplicate sections from different parts of both plastic bottles were examined using scanning electron microscopy (SEM) and energy dispersive spectroscopy. The yeasts that attached on the palm wine bottle were identified by performing restriction fragment length polymorphism analysis of the ITS1-5.8SrDNA-ITS2 genes using HaeIII restriction endonuclease. Viable yeast species of Candida ethanolica and Saccharomyces cerevisiae remained attached on plastic bottle internal surface for up to three weeks after the contents were discarded. No pitting or gradual destruction of the plastic surface was observed and the constituent elements of the PET bottles, carbon and oxygen were detected from both samples whereas only the palm wine bottle showed the element potassium and chlorine. The source of chlorine, the major component of polyvinyl chloride plastic found in the bottle used for palm wine storage will need to be verified in the palm wine supply chain.

Keywords: Yeasts; palm wine; plastic containers; scanning electron microscopy; energy dispersion x-ray; surface corrosion

INTRODUCTION

Palm wine is a popular local drink from palm tree saps around the world [1] and a source of novel organisms [2, 3]. In Nigeria, the drink is consumed in many regions because it is believed to have several health benefits and it is not uncommon to see nursing mothers who consume the fresh drink possibly due to the anecdotal evidence that the drink increases the flow of breast milk. The drink is sometimes stored and transported to markets in 50-200 L polyvinyl chloride (PVC) based plastic containers and then sold in used beer bottles or polyethylene terephthalate (PET) bottled water containers sourced from retail outlets. Even though there is the likelihood of introduction of extraneous materials and bacteria and potential leaching of the plastic composites into the palm wine, retail sellers of palm wine reuse plastic bottled water containers because they are cheap and convenient. It is not unusual for palm wine to come into contact with up to five different plastic containers before it is consumed. First during tapping of the drink, plastic containers are used for palm wine (sap) collection from the tree. Then the palm wine is transported to the market with (20-200 L) plastic containers and the buyer will normally transfer the drink to his own plastic container. Before selling to consumers, plastic jugs may be used to pour the drink into reusable plastic water bottles. Different plastic materials are available [4] and they include PVC, PET, high density polyethylene (HDPE), low density polyethylene (LDPE), polypropylene (PP), and polystyrene (PS). The vast majority of containers used for bottling water in Nigeria are made from PET, while the containers lids may be of different material e.g. polystyrene. There are different types of bottled water on sale and it is common knowledge that some bottle materials could be made from a combination of one or more type of plastic.
Monomers and additives of plastics can migrate into water during bottle manufacturing, water filling or storage [5]. Compounds that can leach into the drink include phthalates commonly used as plasticizers in the manufacturing of flexible polyvinyl chloride containers. They are believed to be reproductive toxicants and endocrine-disrupting chemical products [6]. There is a lot of debate concerning leaching of products from PET container into the stored product and storage of water and soft drinks are the most widely studied with virtually no studies on leaching of plastics components into fermented palm wine. Most reports show that there are very little leaching within acceptable standards. A review [7] of genotoxic and estrogenic activities in PET-bottled water has been carried out. The authors surveyed toxicological studies with contradictory results that may be explained by the wide variety of analytical methods, bioassays and exposure conditions employed. An investigation [8] on the effect of temperature on the release of intentionally and non-intentionally added substances from PET bottles into water has also been performed. Temperature and the presence of CO$_2$ increased the release of formaldehyde, acetaldehyde and antimony. Also Ceretti et al. [9] evaluated the potential migration of genotoxic compounds into mineral water stored in PET bottles by an integrated chemical-biological approach using short-term toxicity-genotoxicity tests and chemical analysis and found the absence of toxic compounds migrating from PET regardless of time and conditions of storage. In the investigator’s conclusion, bottle materials were not associated with the genotoxic properties of the water and the genotoxic effects detected in bottled water may be related to the characteristics of the water’s minerals and CO$_2$ content.

The characteristics of yeast fermentation products of palm wine may predispose the drink to leaching of materials from the plastic containers. This is because during fermentation, the drink generates known reactive compounds like ethanol, lactic and acetic acid [10, 11]. Also the pH of the drink becomes more acidic as fermentation progresses [12] and acidic liquids have the capability of reacting easily with other compounds and may cause corrosion. Studies on fungal colonization of plasticized PVC (pPVC) have been performed. It was found that Aureobasidium pullulans was the principal colonizing fungus, establishing itself on the pPVC between 25 and 40 weeks of exposure [13]. Also in the study, a group of yeasts and yeast-like fungi, including Rhodotorula aurantiaca and Kluyveromyces spp., established themselves on the pPVC much later (after 80 weeks of exposure). Furthermore, it was reported that microbial succession may occur during the colonization of pPVC and that A. pullulans is critical to the establishment of a microbial community on pPVC. The success of A. pullulans in colonizing pPVC in situ was attributed to a combination of several factors. These included desiccation and high temperatures tolerance and production of extracellular polysaccharides that could facilitate permanent adhesion to surfaces.

In a recent study [14], non-products of yeast fermentation that included the plastic monomer styrene, were detected which indicated that yeast fermentation of palm wine may cause corrosion of its plastic container. Therefore the aim of this work was to closely examine the interactions of yeast fermented palm wine with the surface of its container when plastic material is used for storage and determine if there is any effect of the palm wine on the surface properties of the plastic container.

**MATERIALS AND METHODS**

**Samples**

A pair of table water (still) in a PET bottle with polystyrene cap and palm wine in a re-used PET bottle of the same brand as the water sample was purchased in Eleme, Rivers State, Nigeria. The palm wine and water were discarded on the day of purchase and bottles were left at room temperature with screw caps on (approximately 25 °C) for 3 weeks to allow for dispatch to the laboratory from Nigeria to England after which the internal surfaces of the bottles (3 replicate sections) were analyzed using SEM and energy dispersive x-ray spectroscopy (EDX). Yeast isolates were identified by the analysis of the ITS1-5.8SrDNA-ITS2 genes [15]. Isolates previously sequenced [14] and a reference yeast strain NCYC 1406 were used as controls (Table 1).

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<td>Saccharomyces cerevisiae</td>
<td>*</td>
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<td>5</td>
<td>NCYC 1406</td>
<td>Saccharomyces cerevisiae</td>
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**SEM and EDX analysis**

Sections were taken from top, middle and base or bottom of bottle (Fig. 1). Sections were cut from the bottles using sterile scissors and tweezers to avoid contact with hands and mounted onto carbon stubs without any fixing or
pretreatment (Fig. 1D) and then coated once with gold to a thickness of approximately 900 Angstroms using a sputter coater (Quorum Technologies, United Kingdom; 95638-25). The SEM images were acquired for all sections using a scanning electron microscope (Jeol JSM 6490LV, Jeol, United Kingdom) immediately after the gold spotting. After the SEM was performed, the equipment was switched to EDX mode and elemental spectra were captured using Oxford Instruments Inca Micro analysis software version 4.08. Spectra were obtained for higher SEM magnifications (x 5000) at room temp of 26.1°C and relative humidity of 10%. Elements obtained were used for a search on the yeast metabolome data base [16].

![Figure 1: Preparation of inner surfaces of palm wine container for SEM and EDX analysis (a) top bottle part of water container used (b) middle part (c) base or bottom part (d) cut sections from plastic bottles mounted on carbon stubs before spotting with gold](image)

**Identification of yeast strains**

After sections were taken for SEM imaging, swabs were also taken very near where the sections were made specifically from the top, middle and bottom of the containers and broken off in a universal bottle containing 20 ml of Yeast Peptone Dextrose broth (Oxoid). This was incubated for 24 h after which a loop full of the cell culture was streaked on Rose Bengal Chloramphenicol Agar (CM0549; Oxoid, Basingstoke, UK) prepared with the supplement (SR0078; Oxoid) to get single colonies. Two isolates each were selected for identification from the three different areas of the bottle swabbed. Selected isolates were Gram stained and viewed under the microscope to confirm yeast morphology and then stored in -20 °C. Sequenced strains of palm wine yeast from a previous study and reference wine yeast NCYC 1406 were used as control. Control strains used for the study are shown in table 1.

Species identification were performed after PCR amplification of the ITS1-5.8SrDNA-ITS2 regions by using HaeIII restriction endonuclease (Promega, Madison, USA) for restriction fragment length polymorphism (RFLP) analysis. The PCR reaction was carried out using 0.5 µM of primers ITS1 (5’TCCGTAGGTGAACCTGCGG3’) and ITS4 (5’ TCCTCCGCTTATTGATATGC 3’) under the conditions used previously [14]. This consisted of initial denaturation at 95 °C for 5 min; 35 cycles of denaturing at 94 °C for 1 min, annealing at 55.5 °C for 2 min and extension at 72 °C for 2 min; and a final extension at 72 °C for 10 min. The HaeIII restriction enzyme (0.5µl) was added to 25 µl of unpurified amplified PCR fragments, after which the mixture was incubated for 3 hours at 37 °C and examined in 3% agarose gel.
RESULTS

SEM imaging of plastic surfaces
The use of SEM by many workers to visualize the topography and composition of surfaces is due to the detailed information that is normally generated. Mixed microbial cells with different morphology were observed after SEM analysis of cut sections and some cells consistent with yeast morphology were found in all the cut sections of the bottle used to store palm wine (Fig 2; panels A, C, E), whereas no microbial cells were observed for all the sections cut from the bottle that contained water (Fig 2; panels B, D, F). Cells from the base (Fig. 2A) of the palm wine bottle were clumped together whereas cells from the middle part (Fig. 2C) were less clumped up. From the top part of the bottle, cells that attached on the palm wine bottles were well separated and distinct (Fig. 2E). Signs of swelling, surface corrosion, distortion, pitting or significant destruction of the plastic surface as a result of the chemical reaction of the plastic was not observed.

Elemental analysis of container’s surface
The EDX analysis is normally used together with SEM and generates qualitative and quantitative information. The results of the EDX analysis carried out showed that only the elements carbon, oxygen and the artifact gold (Au) were found in all the cut sections of the plastic that contained water (Fig. 3; panels B, D, F). The same elements

Figure 2: Scanning electron micrographs of the internal surface of plastic bottles that contained palm wine (panels A, C, E) and water (panels D, B, F). Images were taken at x1000 magnification for all sections. The settings were: Accelerating voltage (AccVolt) = 15kV, spot size = 40 μm, Vac mode = High Vacuum, working distance (WD) =10 mm. Images were taken at x1000 magnification.
were observed for the bottle that contained palm wine but in addition, the section from the top part (Fig 3; panel A) and the base of the bottle (Fig 3; panel E) showed the presence of potassium. Only the section from the middle part of the bottle showed the presence of potassium and chlorine together (Fig 3; panel C).

Figure 3 EDX analysis of plastic surfaces: internal surface of plastic bottles that contained palm wine (panels A, C, E) and water (panels D, B, F). Analysis was carried out using Oxford instruments Inca Micro analysis software version 4.08 at room temp 26.1°C relative humidity of 10%. The X-axis for all panels represents the minimum emission voltage (keV).

Yeasts from plastic container surface
From the SEM micrographs, it was evident that yeast cells responsible for palm wine fermentation were present on the surface of the plastic container used for palm wine storage. Preliminary identification carried out for 6 presumptive isolates (serial number 6-11; Table 2) from the palm wine bottle showed that the cells were consistent with yeast morphology (Fig. 4) in that they were about 5 µm in diameter, ovoid, and some cells were seen to be budding. Five out of the six isolates analyzed were *S. cerevisiae*. However, one strain of *Candida ethanolica* was found in the base of the bottle used for palm wine storage.
The *S. cerevisiae* isolates had the same PCR amplicon and restriction profile consisting of 4 bands (Fig. 5) with their corresponding reference control strain *S. cerevisiae* NCYC 1406 and *S. cerevisiae* HG425326. The only *C. ethanolica* strain identified also had the same PCR amplicon and restriction profile (2 bands) with control *C. ethanolica* HG425332. The approximate bands produced by the ITS1-5.8S rDNA-ITS2 gene amplicon and the restriction profile of all strains tested after fragmenting with *Hae*III enzyme is shown in Table 2.
The elements carbon and oxygen are found in the structure of PET [24] and it explains why the elements occurred in the top of a fermented product. Elements detected on plastic surface metabolized by microorganisms; activation energy is required to initiate the incorporation of oxygen atoms into the outlined [17, 18, and 19]. It was pointed out that for plastics to reach sufficiently low molecular weight to be interaction of palm wine yeasts with plastics surfaces. However, the process of plastic degradation has been reaction of palm wine with plastic surface. The lack of swelling of the sample bottle containing palm wine indicates that the palm wine purchased was well fermented and the residual sugar of the palm wine film on the bottle surface after the palm wine was discarded was not enough to rapidly increase yeast fermentation and production of CO₂ big enough to cause swelling of the bottles. Well fermented palm wine is normally in the stage three of palm wine fermentation when ethanol production has reduced [1].

The lack of pitting which is synonymous with corrosion of surfaces observed in this study may be because the contact of palm wine with the container surface was not long enough. There is lack of data in literature on the interaction of palm wine yeasts with plastics surfaces. However, the process of plastic degradation has been outlined [17, 18, and 19]. It was pointed out that for plastics to reach sufficiently low molecular weight to be metabolized by microorganisms; activation energy is required to initiate the incorporation of oxygen atoms into the polymer which causes the plastic to become brittle over a long period.

The viability of yeasts recovered from the surface of the plastic three weeks after the palm wine was discarded indicates that the yeasts isolated attached on the plastic surface under nutrient stress. The ability of yeasts to attach to plastics and form mats has been demonstrated by others [20]. It has been explained that adhesion is conferred by a class of special cell wall adhesinproteins and that tandem repeats within adhesin genes trigger recombination events with the formation of novel adhesins, thereby offering yeasts an endless reservoir of adhesion properties [21]. Clumping of cells was highest at the base of the palm wine bottle and explains why the drink has been described as a heavy suspension of yeasts and bacteria in fermenting palm sap [22]. The aggregation found at the base of the bottle confirms that palm wine contains mainly bottom sedimenting yeasts. The life cycle of yeasts has been described [23] and it consists of 4 phases of lag period, growth phase, fermentation and sedimentation. The description added that during sedimentation, yeasts prepare for dormancy by producing glycogen and collect at the bottom or float at the top of a fermented product.

**Elements detected on plastic surface**

The elements carbon and oxygen are found in the structure of PET [24] and it explains why the elements occurred in the containers used for both storage of palm wine or water. In other to confirm if other elements found in the plastic container used for palm wine storage are associated with secondary yeast metabolism, a search for potassium and chlorine in the yeast metabolome database [16] was carried out and it was found that both elements were present in baker’s yeast. Potassium is listed as being necessary for the function of all living cells while chlorine is documented in the database as an essential electrolyte responsible for maintaining acid/base balance and regulates fluid in and out of cells. Another source of chlorine could be PVC based plastic containers used to store the product in the drink’s supply chain. When it is considered that the PVC material is about 56% chlorine [25], it may be that the chlorine detected may be from chlorides on the alternating carbon centers present in the plastic’s linear polymer microstructure which broke off as a result of reaction with the palm wine. Since the container used for water did not show the presence of chlorine, the plastic material appears to be the source. A wider study of plastics from the palm wine distribution chain to evaluate action of yeasts on plastics will be beneficial.

**Yeast recovery from plastic surface**

To identify yeasts attached on the plastic surface, isolation was carried out with Rose Bengal Chloramphenicol Agar because according to the manufacturers, the media has a neutral pH and the chloramphenicol present is used as a selective agent to suppress the growth of bacteria. Preliminary identification was carried out by RFLP analysis of

### DISCUSSION

**Reaction of palm wine with plastic surface**

The lack of swelling of the sample bottle containing palm wine indicates that the palm wine purchased was well fermented and the residual sugar of the palm wine film on the bottle surface after the palm wine was discarded was not enough to rapidly increase yeast fermentation and production of CO₂ big enough to cause swelling of the bottles. Well fermented palm wine is normally in the stage three of palm wine fermentation when ethanol production has reduced [1].

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ITS1-5.8S rDNA-ITS2 region. The method is used by many workers as a first step in yeast identification because it is a quick method for routine identification and the profile of one restriction enzyme is enough for identification of some yeast species [15]. For S. cerevisiae in particular, digestion with HaeIII endonuclease would normally yield a profile containing 4 bands. The yeasts S. cerevisiae and Candida sp. has been identified in palm wine by other workers [26, 27] and the isolates appear to be potential candidates for yeast attachment studies in low water environment.

CONCLUSION

The presence of the element chlorine, a monomer of PVC suggests dissociation from the surface of the plastic bottle used for palm wine storage and will need to be verified to determine whether the chlorine found is mainly from yeast cells or plastics from the supply chain. The yeast species of Candida ethanolica and Saccharomyces cerevisiae attached on plastic bottle internal surface and were viable upon recovery. Other factors like sensory quality of palm wine, water quality after storage and bottles formulations from different manufacturers should be taken into account in future studies.

Acknowledgments

We acknowledge Eminate Ltd, Sutton Bonington, United Kingdom for help with SEM analysis

REFERENCES

[2] LI Ouoba; DS Nielsen; A Anyogu; C Kando; B Diawara; L Jespersen; JP Sutherland. International Journal of Systematic Bacteriology, 2015, 65, 3576-3579
[16] T Jewison; C Knox; V Neveu; Y Djombou; AC Guo; J Lee; P Liu; R Mandal; R Krishnamurthy; I Sinelnikov; M Wilson; DS Wishart. Nucleic Acids Research 2012, 40, D815-D820.
[27] M Stringini; F Comitini; M Taccari; M Ciani. Food Microbiology, 2009, 26, 415-420.