Isolation of Food Pathogens From Freshly Milled Palm Oil and the Effect of Sterilization on Oil Quality Parameters

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Abstract The isolation and identification of food pathogens from freshly milled palm oil as well as the effect of steam sterilization on some quality parameters of palm oil was evaluated. Microbial isolations and quality parameters were carried out at day 0, day 14 and day 28. Biochemical parameters such as Peroxide value, Anisidine value, Free fatty acid, Deterioration of bleachibility index (DOBI) and Carotene value was analyzed in the same samples using the digital Palmoiltester. The most frequently isolated bacteria from the unsterilized samples were Pseudomonas aerugenosa, Bacillus subtilis, Enterococcus aerogenes, Staphylococcus saprophyticus and Micrococcus varians while the most frequently isolated fungal species were Aspergillus niger aggregate (IMI number 503810), Cochliobolus sp. (anamorphic state: Curvularia) (IMI number 503811), Penicillium citrinum (IMI number 503812) and a yeast, Meyerozyma guilliermondii (IMI number 503813). The fungal count in the unsterilized samples from day 0 to day 28 was in the range of 2.17 x 10³ to 5.0x10⁶ while the bacteria count ranged from 4.08 x 10² to 9.0x 10⁸. The sterilized sample showed no microbial contamination throughout the 28 day storage. However, sterilization caused significant changes when compared with unsterilized sample as thus; significant (p<0.05) increases in peroxide value of up to 5.14% and 15.99% after the 14th and 28th day respectively, significant (p<0.05) increases in anisidine value of up to 5.14% and 15.99% after the 14th and 28th day respectively, significant (p<0.05) decreases in carotene content of up to 11.84% and 15.79% for day 14 and day 28 respectively, significant (p<0.05) decreases in DOBI value of up to 37.46% and 37.73% for day 14 and day 28 respectively and no significant (p>0.05) changes in free fatty acids.

Keywords: palm oil, pathogens, sterilization, biochemical parameters, Palmoiltester


1. Introduction

Palm oil contributes substantially to the global total vegetable oils accounting for about 34 percent of the global annual production and more than 60 percent of the world’s export [5,14]. It is extracted from palm fruit [2] from the oil palm tree which thrives well in tropical climate areas [14]. In Nigeria, about 80% of palm oil destined for consumption are produced by small scale processors [3,4]. In addition to using locally contrived equipment, most of these processors carry out the practices in unhygienic environments with no standard operating conditions. The common practice involves the exposure of hot extracted palm oil in large uncovered drums for a minimum period of twenty four hours in order to allow the oil to cool [24] thus exposing the oil to possible food pathogens. Most times, these drums are refilled with palm oil without prior washing hence providing room for cross contamination of batches [25]. Crude palm oil (CPO) is vulnerable to microbial attack [12] and it has been found to support the growth of lipophilic microbes [31]. Some microorganisms found in CPO often lead to deterioration in their biochemical quality besides the rancidity, acidity, bitterness, soapiness and other off flavor they cause [31]. Hence, the presence or absence of microorganisms can be considered as quality determinants of palm oil. The microbial quality of CPO is essential because of the adverse role played by most lipophilic micro-organisms in human and animal health. It has been reported that lipophilic microorganisms such as Candida species and Mucor species respectively that flourish in CPO could cause diseases such as cerebral aspergillosis, endophthalmitis, meningitis, osteomelitis, endocarditis, myocaridis, pneumonia, sepsis, candidiasis and mucormycosis [15]. Reports also show that respiratory tract infections, septicaemia and meningitis could be caused by Enterobacter species that flourish in Crude Palm Oil [27]. Some of these organisms have been implicated as food pathogens as they have the ability to...
secreted toxic secondary products in the oil and some of
which have been found to be heat stable even at cooking
and frying temperatures [26]. In 1984, Abalaka, detected
aflatoxins B1, B2, G1 and G2 in crude groundnut oil and
crude cottonseed oil with G1 in crude palm oil collected
from three palm oil processing factories in Nigeria.
Though CPO used for cooking is subjected to heat which
may kill all the microorganisms that could invade the CPO,
it has been reported that many individuals still consume
CPO raw especially in rural areas [26].
Several methods have been adopted in reducing or
eliminating microbes in foods. One of such methods is the
process of steam sterilization. This is a technique used to
prolong the shelf life of foods by killing all the
microorganisms and it generally involves heating the food
products using steam at temperatures between 110-121°C
for about 15 to 20 minutes while some canned products
are however heated for up to one hour. This work was
carried out to assess the effect of steam sterilization on the
microbial quality and biochemical quality parameters of
fresh palm oil collected in a local oil mill in Edo state,
Nigeria.

2. Materials and Methods

Freshly processed palm oil was allowed to cool in open
storage drums for twenty four hours (day 0) and then
collected using sterile flasks which were carefully covered
with sterile foil paper. The oil was then divided into two
groups; the first group was kept in tightly sealed
MacKean bottles and then steam sterilized using a
Gallenkamp autoclave at temperature of 121°C, pressure
15 psi for 15 minutes, while the other group which was
however heated for up to one hour. This work was
carried out to assess the effect of steam sterilization on the
microbial quality and biochemical quality parameters of
fresh palm oil collected in a local oil mill in Edo state,
Nigeria.

2.1. Media Preparation

All isolation media used in this study were prepared
according to the manufacturer’s instructions.

2.2. Bacteria Isolation

A serial dilution was performed by adding 1ml of the
oil sample in 9 ml of sterile distilled water already
emulsified with 10% v/v of tween 80 solution which
served as an emulsifying agent. Aliquots of the
suspensions were transferred carefully into sterile Petri
dishes in a sterile laminar flow chamber with the aid of a
sterile pipette. Already prepared 10ml of Oxoid nutrient
agar for isolation of bacteria [9] was then poured over
plates (the pour plate method) containing the samples and
swirled gently to allow for proper mixing of the sample
and the medium. The plates were transferred to an
incubator with temperature set at 30 ±2°C for a 24 hour
period.

2.3. Test Methods for Bacteria Identification

2.3.1 Gram Staining

A smear of each bacterial isolate was made and fixed
on a sterile glass slide by briefly passing it through a
flame. The primary dye (crystal violet) was used to stain
the bacteria isolates and allowed to stay for a minute,
before flooding it with water. Iodine was then added and
subsequently washed with sterile distilled water after a
minute. It was decolourized by alcohol for a few seconds
before counterstained with safranin solution for 30
seconds. The slides were allowed to air dry; a drop of
immersion oil was then added and viewed with a
magnification of x100 under a light microscope. Bacterial
cells that retained the colour of the primary dye were
grouped as gram positive, while those that retain the
secondary dye as gram negative.

2.3.2. Catalase Test

This test was performed on slides. A drop of 3%
hydrogen peroxide (H2O2) was placed on a clean glass
slide. A sterile wire loop was used to pick the organism
and mixed with the drop of (H2O2) on the slide and
observed for production of gas bubbles, which is an
indication of a positive reaction.

2.3.3. Starch Hydrolysis

Starch agar was used for this test. After sterilization, the
starch agar was allowed to cool to about 45°C before
dispensing 20 ml portion into sterile Petri dishes
appropriately labelled for the test organisms. The plates
were aseptically inoculated with test organisms by
streaking across the surface of the medium. They were
then incubated at room temperature for 3-5 days. At the
end of the incubation, the plates were flooded with
Lugol’s iodine solution. Hydrolysis of starch was
indicated by clear zones appearing around the colonies of
the organisms. Unhydrolysed starch gave blue black
colouration with Lugol’s iodine.

2.3.4. Citrate Utilization Test

The ability of bacteria isolates to utilize citrate as a sole
source of carbon and energy for growth and an ammonium
salt as a sole source of nitrate was investigated using this
test. Simmon citrate agar was prepared according to
manufacturer’s instructions. On cooling, 5 ml of the
medium was dispensed into respective test tubes and the
test organisms was inoculated using this
medium. The plates were allowed to cool to about 37°C for
24-48 hrs. The development of deep blue
colour gave an indication of a positive reaction. A
green colour indicated that the isolate was citrate negative.

2.3.5. Voges Proskauer (V- P) Test

This test was used to demonstrarte bacteria that ferment
carbohydrates with the production of acetyl methyl
carbino (CH3-CO-CHOH.CH3). This compound
is oxidized during the test to diacetyl which reacts with a
guanido group under alkaline condition to give a pink
colour. The bacteria culture of the test organism was
inoculated into 2 ml of sterile glucose phosphate peptone
water and incubated at 37°C for 48 hrs. 1 ml of 40% KOH
and 3 ml of 5% alcoholic alpha-naphthol (Barritts reagent)
was then added. It was then shaken and observed for
colour formation. A pink colour within 2-5 minutes
indicated a positive result.

2.3.6. Spore Formation

A heat fixed smear of the organisms were prepared on a
sterile slide and malachite green solution was added to it
and then steamed for five to ten minutes without allowing
the stain from drying out. The slide was then washed with cold water, followed by the counterstaining with Safranin solution for 15 seconds. It was washed with water, blotted dried and examined under the microscope with oil immersion objective of x100. Spore stained green and bacteria cells stained red.

**2.3.7. Oxidase Test**

The culture of the each isolate was streaked onto the dry surface of a nutrient agar plate and incubated at optimum growth conditions until a reasonable growth was obtained. The reagent (1% aqueous solution of tetramethyl-p-phenylenediamine hydrogen chloride) was poured over the surface of the agar growth. Oxidase positive colonies developed a pink colour which turned successively dark-red purple and black within 10-30 minutes.

**2.3.8. Strict Anaerobes**

A loop full of the test organism was inoculated into a test tube of sterile water and 0.1 ml was taken out with the aid of pipette into a sterile Petri dish. Nutrient agar was introduced and swirled properly. It was then allowed to solidify before the addition of oil immersion to cut off oxygen supply. The presence of growth revealed the organism is strict anaerobe.

**2.3.9. Glucose Fermentation Test**

Glucose broth was prepared by adding 0.5% glucose into nutrient broth. 20ml of nutrient broth was poured at 45°C into sterile test tube and allowed to cool. The organism was stabbed once into the broth through the surface, Durhams tube was inverted in the broth to detect gas production, and the tubes were incubator at 37°C. Positive result indicated a growth with gas production and a colour change from red to pink.

**2.3.10. Lactose fermentation Test**

MacConkey broth was prepared by adding 0.5% lactose into nutrient broth. 20 ml of nutrient broth was poured when cooled at 45°C into sterile test tube and allowed further cooling. The organism was stabbed once into the broth through the surface, Durham tube was inverted in the broth to detect gas production, and the tubes were incubator at 37°C. Positive result indicates a growth with gas.

**2.3.11. TSA+7.5% NaCl at 55°C**

Tryptic agar was prepared according to manufacturer’s instruction by dissolving 6 grams of agar in 500 ml distilled water in an Erlenmeyer flask. It was heated to dissolve and allow for proper mixing properly. 15 grams of TSA was then weighed and added to the mixture, heated and stirred. The mixture was autoclaved at 121°C for 15 minutes. It was allowed to cool to about 50°C before 7.5% NaCl was added. The agar was allowed to gel and the organism streaked on it. A colour change from the initial yellow amber indicated a positive result.

**2.4. Fungal Isolation**

This was carried out by using appropriate dilutions of the oil and plated using the pour plate method into sterile Petri dishes and 10 ml of prepared Potato dextrose agar was poured over each plate. The fungal types and counts were analyzed in triplicates at day 0, day 14 and 28 days of storage. The fungal isolates were identified macroscopically and microscopically and further sent to Common Wealth Agricultural Bureaux International (CABI), Surrey, UK for further identification and characterization as well as assignment of ascension numbers.

**2.5. Analysis of Biochemical Quality Parameters**

All biochemical quality parameters such as Free fatty acid (FFA) values, Peroxide values, Anisidine values, Carotene content and Deterioration of Bleachability index, (DOBI) were analyzed in duplicates during the test period using the digital Metalab Palmoiltester with model number 225001/0038 procured from CDR Group, Italy.

**2.6. Statistical Analysis**

Results presented are means of replicates and values subjected to statistical analysis using SPSS 17.

**3. Results**

The initial microbial analysis at day 0 (24 hours of exposure in the open drum) revealed the presence of Aspergillus niger, Meyerozyma guilliermondii, Cochliobolus sp. (anamorphic state: Curvularia). The analysis from the unsterilized samples from day 14 and day 28 consisted of a total of four fungal isolates (as shown in plates 1a-3b) and five bacterial isolates as shown in Table 1. These isolates were consistently present in all the replicates of the unsterilized crude palm oil. The bacterial isolates were identified based on morphological characteristics, gram staining reactions, biochemical reactions and cell types (Table 1). For each fungus isolate identified in the unsterilized oil samples at CABI, Surrey, UK, a unique CABI reference number (IMI number) was ascribed after identification by processing using ITS rDNA sequencing analysis. Following sequencing, identification was undertaken by comparing the sequences obtained with those available at the European Molecular Biology Laboratory (EMBL) database via the European Bioinformatics Institute (EBI) and where appropriate, the Central Bureau voor Schimmel cultures (CBS) yeast database. All procedures were validated and processing undertaken in accordance with CABI’s in-house methods for filamentous fungi and yeasts. The results of the bacteria analysis revealed that the following isolates were present; Pseudomonas aeruginosa, Bacillus subtilis, Enterococcus aerogenes, Staphylococcus saprophyticus and Micrococcus varians. The four fungal isolates consistently isolated from day 0, day 14 and days 28 were identified as; Aspergillus niger aggregate (IMI number 503810), Cochliobolus sp. (anamorphic state: Curvularia) (IMI number 503811), Penicillium citrinum (IMI number 503812) and Meyerozyma guilliermondii (yeast) (IMI number 503813). The spores of each isolate as shown on plate 1-3 were captured with a 9MP Amscope digital Motic camera attached to a light microscope. It was observed that the sterilized sample contained no microorganisms; however, the sterilization had a
detrimental effect on the biochemical parameters of the oil as shown in Table 3.

**Table 1. Characteristics of bacteria isolates from unsterilized palm oil**

<table>
<thead>
<tr>
<th>Identification tests</th>
<th>Gram reaction</th>
<th>Motility</th>
<th>Biochemical Tests</th>
<th>Oxidase</th>
<th>Catalase</th>
<th>Indole</th>
<th>Citrate</th>
<th>Glucose</th>
<th>Lactose</th>
<th>Mannitol</th>
<th>Urease</th>
<th>H₂S</th>
<th>Nitrate</th>
<th>Voges Proskauer (V-P) Test</th>
<th>Methyl Red</th>
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<td>ISOLATE</td>
<td>Pseudomonas aeruginosa</td>
<td>Bactillus subtilis</td>
<td>Enterococcus aerogenes</td>
<td>Staphylococcus saprophyticus</td>
<td>Micrococcus varians</td>
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Key: - Negative to the test, + positive to the test, ND- not detected

The plates below show the growth pattern of the fungi on PDA medium as well as the Pictomicograph captures of their individual matured spores.

**Plate 1a.** *Aspergillus niger* aggregate (IMI No.503810) grown on PDA (IMI No.503810)

**Plate 1b.** Long stalk holding spore head of *Aspergillus niger* aggregate under magnification of x40

**Plate 2a.** *Cochliobolus* sp. (IMI No. 503811) grown on PDA

**Plate 2b.** Pictomicograph of *Cochliobolus* sp. (IMI No. 503811)

**Plate 3a.** *Penicillium citrinum* (IMI No: 503812) grown on PDA medium

**Plate 3b.** *Penicillium citrinum* (IMI No: under magnification of x40
**4. Discussion**

The results of the unsterilized oil used as control revealed that there was a significant (p<0.05) increase in the microbial population from the initial count of 2.10 x 10^7 for the fungi population and 2.30 x 10^4 for bacteria population to 2.40 x 10^8 and 4.0 x 10^4 after 14 days respectively. At the end of the 28 day period, the bacteria population in the sterilized sample had significantly increased to a count of 9.0 x 10^8 and 4.3 x 10^4 for fungi population in the unsterilized sample had significantly increased to a count of 2.10 x 10^7 and 4.3 x 10^4 for fungi population as compared with the initial count of 2.10 x 10^7 and 4.3 x 10^4. The microbial population from the initial count of 2.10 x 10^7 and 4.3 x 10^4 increased to 2.10 x 10^8 and 4.3 x 10^4 respectively. At the end of the 28 day period, the bacteria population in the sterilized sample had significantly increased to a count of 9.0 x 10^8 and 4.3 x 10^4 for fungi population as compared with the initial count of 2.10 x 10^7 and 4.3 x 10^4.

Aspergillus niger aggregate (IMI number 503810), Cochliobolus sp. (IMI number 503811), and Penicillium citrinum (IMI number 503812) were isolated from the crude palm oil. Aspergillus niger aggregate is a common contaminant of food, while Cochliobolus sp. and Penicillium citrinum are opportunistic pathogens and are involved in respiratory tract infection and occasionally cause septicemia and meningitis [27].

Members of the Aspergillus niger aggregate have been implicated in human and animal infections including superficial and local infections (cutaneous infections, otomycosis, tracheobronchitis), infections associated with damaged tissue (aspergillosis, osteomyelitis), pulmonary infections and clinical allergies (allergic bronchopulmonary aspergillosis, rhinitis, Farmers’ lung) [19]. Members of this genus are assigned to hazard group 2 by the Advisory Committee on Dangerous Pathogens' (ACDP) (UK). The yeast, M. guilliermondii has also been isolated from human and environmental sources, including insects, air and trees. They are flavo- yeasts known for the production of flavor compounds in fermented foods [34]. M. guilliermondii has been reported for the efficient production of isoflavone aglycone which is a widely known bioactive compound and also known for its various health promoting functions [18]. This organism has also been known for the overproduction of vitamin B2 (riboflavin) [6]. M. guilliermondii has been shown to exhibit great potential in the biological control of fungal responsible for post-harvest spoilage of fruits and vegetables [7,22,35]. This yeast has however been categorized as hazard group 1 organisms by ACDP (UK) and are not known to be pathogenic to man. The bacteria,
Bacillus sp., Pseudomonas sp. and Staphylococcus sp. are lipase producing organisms associated with pathogen city.

Peroxide value (PV) is an indication of early events during oxidative rancidity as it measures the amount of peroxides and/or hydroperoxides formed during oxidation. There was a significant (P<0.05) increase in both sterilized (11.65 meq/O₂) and unsterilized (11.08 meq/O₂) samples when compared with the initial value (5.37 meq/O₂) with the value of sterilized sample slightly (5.14%) higher than the unsterilized at day 14. However, at day 28, a larger difference in PV between sterilized (16.03 meq/O₂) and unsterilized (13.82 meq/O₂) was observed with a 15.99% increase in sterilized sample when compared with the unsterilized. This may partly be due to the fact that sterilization might have initiated the induction stage of the process of auto-oxidation [10] which became evident on day 28. The peroxides or hydroperoxides formed in the primary oxidation of oil are high unstable intermediates and are readily decomposed into various secondary products such as ketones, aldehydes etc. [10]. These secondary products are measured using Anisidine value (10). Sterilization appears to have a direct effect in the formation of these products as significant (P<0.05) increase was observed in sterilized samples after day 14 when compared to unsterilized samples. Similar pattern was observed in day 28 however, further significant (P<0.05) increase was observed in day 28 of sterilized sample than day 14 of sterilized sample. Sterilization had no significant effect on palm oil samples. Though, there was a significant (P<0.05) increase in free fatty acid content from day 0 to day 14 of both sterilized and unsterilized samples, there was no significant (P>0.05) difference between the two samples. The carotene content showed a significant (P>0.05) decrease after 14 weeks from the initial in both sterilized and unsterilized samples however, the sterilized sample showed greater decrease in carotene when compared with the unsterilized after 14 days and 28 days. The Deterioration of Bleachability Index (DOBI) is a quality parameter developed specifically for carotene-containing oils like palm oil [16]. It is used as a guide as to how easily a sample of crude absorbance at 269 nm. Hence, it is interplay between the ratio of the UV absorbance of the sample at 446 nm to the lipase producing organisms associated with pathogen city.

Amount of secondary oxidat ion products present in the oil through heat and absorptive cleansing bleaching. [28]. The higher the DOBI value, the easier it is to bleach 49.4% and 50.4% decrease respectively.

5. Conclusion

The process of steam sterilization has generally been accepted as a method of eliminating harmful microorganisms in food, however its effects on the food quality especially bulk oils have not been evaluated. In this study, though steam sterilization process clearly eliminated all micro organisms from the oil, it however had adverse effect on its chemical qualities as it increased both primary and secondary products of oxidation. This was observed by significant (P<0.05) increase in both peroxide and anisidine values after sterilization of the oils. Also, important phytonutrients such as carotene was also reduced and the oil lost its refine ability as evident in the significant (P<0.05) decrease in the DOBI values. Hence, the process of steam sterilization should not be recommended in preserving palm oil instead other methods such as: Flash pasteurization and microfiltration should be exploited.

Acknowledgement

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References


