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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 10^3 to 5.0x10^6 while the bacteria count ranged from 4.08 x 10^2 to 9.0x 10^8. 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 170% and 200% for day 14 and day 28 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*

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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 lipophillic 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, myocarditis, pneumoconiosis, candidiasis and mucormycosis [15]. Reports also show that respiratory tract infections, septicemia 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

secrete 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 MacCakney 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 also kept in tightly sealed bottles without sterilization served as control for the experiment.

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 (H_20_2) was placed on a clean glass slide. A sterile wire loop was used to pick the organism and mixed with the drop of (H_20_2) 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 on the Simmon citrate agar and incubator 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 demonstarte bacteria that ferment carbohydrates with the production of acetyl methyl carbinol (CH_3-CO-CHOH.CH_3). 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 aerugenosa*, *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

Identification tests					
Gram reaction	-	+	-	+	+
Morphological	Cocci	Straight rod	Straight	Spherical	Cocci
Motility	±	+	+	-	+
Biochemical Tests					
Oxidase	±	+	+	-	+
Catalase	±	+	+	+	-
Indole	-	+	-		+
Citrate	+	+	-		+
Glucose	-	+	+	-	+
Lactose	-	-	-	+	-
Mannitol	-	-	-	-	+
Urease	-	+		+	
H_2S	-	+	+	ND	
Nitrate	+	-	+		+
Voges Proskauer (V- P) Test	-	+		+	+
Methyl Red	-	-	-	ND	
Coagulase	-	-	-	+	+
Oxygen requirement					+
Tellurite			-		
ISOLATES	*Pseudomonas aerugenosa*	*Bacillus subtilis*	*Enterococcus aerogenes*	*Staphylococcus saprophyticus*	*Micrococcus varians*

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 Pictomicrograph captures of their individual matured spores.

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

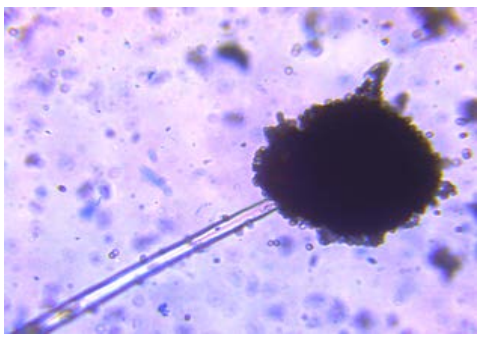

Plate 1b. Long stalk holding spore head of *Aspergillus niger aggregate* under magnification of x40

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

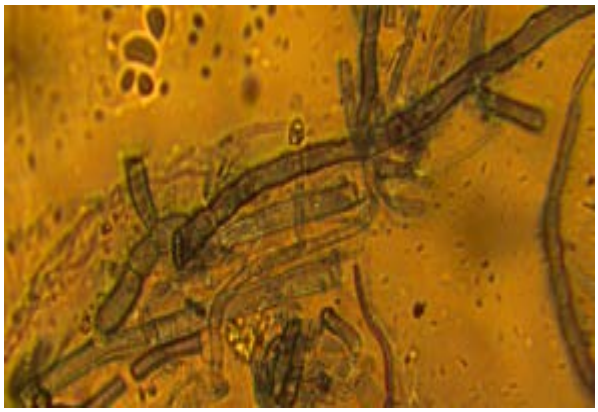

Plate 2b. Pictomigrograph of *Cochliobolus* sp. (IMI No. 503811)

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

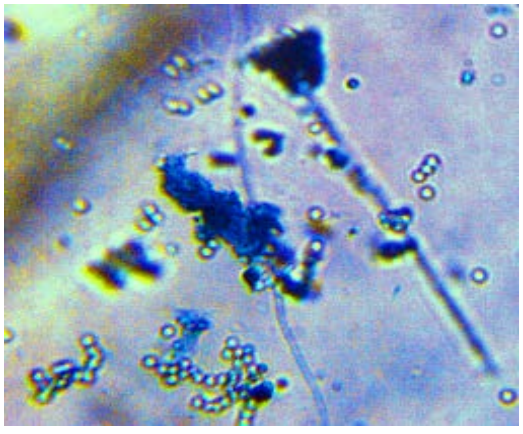

Plate 3b. *Penicillium citrinum* (IMI No: under magnification of x40

Table 2. Microscopic morphology and cultural characteristics of fungi isolated from unsterilized freshly milled palm oil

Fungi Isolates	Microscopic Morphology	Cultural characteristics
Aspergillus niger aggregate (IMI number 503810),	Presence of septate hyphae; long and smooth conidiophores, long unbranded sporangiospores with large, round head.	Dark brown powdery mycelium
Cochliobolus sp. (IMI number 503811),	Conidia are cylindrical or slightly curved with or without a prominent hilum, Conidia contains three or more transverse septa and one of the central cells being larger and darker.	Colonies are fast growing with wooly texture. Color is grey and gradually turns brown gray on the surface. while the reverse side is black.
Penicillium citrinum (IMI number 503812)	Presence of septate and fruity mycelium and branched conidiophores.	A rapid dark green coloured growth with the edge surrounded by whitish margin was observed on the surface of the organism and pale yellow on the reverse side.
Meyerozyma guilliermondii (IMI number 503813).	Microscopic morphology shows spherical to subspherical budding yeast-like cells or blastoconidia.	Colonies are flat, moist, smooth, and cream to yellow in color on Sabouraud dextrose agar and yeast-like in appearance.

Table 3. shows results for changes in biochemical parameters tested during the period of storage of the sterilized and unsterilized crude palm oil

Day	Sample	PV(meq02/kg)	An V	FFA(%)	DOBI	CAROTENE(ppm)
0	Initial	5.37±0.03	Nd	6.48±0.05	3.99±0.01	1056±8.00
14	SS	11.65±0.03	4.05±0.05	7.43±0.04	2.02±0.14	832.23±22.88
	US	11.08±0.26	1.5±0.00	7.50±0.35	3.23±0.40	944.00±16.09
28	SS	16.03±0.03	6.15±0.07	8.30±0.30	1.98±0.11	797.00±4.30
	US	13.82±0.04	2.05±0.25	8.94±0.27	3.18±0.14	946.44±22.88

Key: PV- Peroxide Value, AnV- Anisidine value, FFA- Free fatty Acid, DOBI-Deterioration of bleachability index, SS – Sterilized sample, US- Unsterilized sample, Nd- Not detected

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 10^3 for the fungi population and 2.30 x 10^2 for bacteria population to $2.40x10^6$ and $4.0x10^8$ after 14 days respectively. At the end of the 28 day period, the bacteria population in the unsterilized sample had significantly increased to a count of 9.0x 10^8 and $4.3x10^4$ for fungi while the sterilized sample remained without any fungal or bacterial contamination. The fungi isolated in this work is in close similarity with the work of [15] who isolated *Aspergililus niger*, *A. flavus*, *A. fumigatus*, *Candida species*, *Mucor species and Penicillium species* from crude palm oil collected from eight smallholder oil palm processing facilities in Elele, Niger Delta, Nigeria.

From the results of the experiments carried out, it was observed that the microbial load of the crude palm oil samples at the 28 day period was above the minimum standard microbiological population of 10^4 cfu/ ml as stipulated by Nigerian agency for food and drug Administration (NAFDAC). The microbial isolates obtained in this work was also above the maximum allowable number of isolates (2) also set by NAFDAC [17]. The presence of some pathogens such as *Aspergillus* sp. and *Penicillium* sp. may raise possible cause for concern as some species of this fungi have been implicated in the production of mycotoxins in food and feed products [25,30]. Most mycotoxins are relatively heat-stable within the conventional food processing temperature range (80–121°C), therefore, little or no destruction occurs under normal cooking conditions, such as boiling and frying, or even following pasteurization [25]. The bacteria, *Bacillus* sp., *Pseudomonas* sp. and *Staphylococcus* sp. are lipase producing organisms associated with pathogenicity. *Pseudomonas* sp. was first identified by Griffith *et al*., (1981) as causing spoilage of dairy products and fats containing food. The production of the spores of this bacterium makes the organism dormant and highly resistant to lethal effect of boiling, dry heating and ultra violet radiation [11]. Studies have also shown that *P. aeruginosa* is an opportunistic pathogen which can cause severe attack on patients especially those have different kind of problems in their immune system [21] [8]. This bacterium can adapt itself in different kind of environmental condition and can survive and replicate in minimum nutritional conditions such as in drinking water containing traces of organic compounds. The bacterium has several virulence factors like extracellular toxins [1] and proteases [31] which adapt to specific host tissues and cause infection. *Enterobacter* sp. can be found in the environment and water, their presence in the palm oil samples might be due to contamination from the environment or water. Some strains of *Enterobacter* sp. are opportunistic pathogens and are involved in respiratory tract infection and occasionally cause septicaemia 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 (aspergilloma, osteomyelitis), pulmonary infections and clinical allergies (allergic bronchopulmonary aspergillosis, rhinitis, Farmers's 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 flavogenic 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 fungi 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/O_2) and unsterilized (11.08 meq/O_2) samples when compared with the initial value (5.37 meq/O_2) 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/O_2) and unsterilized (13.82 meq/O_2) 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 hydroperoxide 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 Palm oil can be bleached [20,32]. The value is a numerical ratio of the UV absorbance of the sample at 446 nm to the absorbance at 269 nm. Hence, it is interplay between the amounts of carotenes present in the crude palm oil to the amount of secondary oxidation products present in the oil [28]. The higher the DOBI value, the easier it is to bleach the oil through heat and absorptive cleansing bleaching. There was a slight drop in DOBI values of unsterilized sample from the initial value of 3.99 for day 0 to 3.23 and 3.18 for days 14 and 28 respectively. This 0.19% and 20.3% decrease in DOBI values may be due to natural deterioration reactions associated with natural products like palm oil [10]. However, more drastic changes were observed in sterilized samples where values dropped to as low as 2.02 in day 14 and 1.98 in day 28 representing a 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.

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