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Degradation of Polycyclic Aromatic Hydrocarbons and Aroma Oxidation of Fermented Locust Bean Seeds 'Iru' by Laccase Enzyme Produced by *Kluyveromyces dobzhanskii* DW1 and *Pichia manshurica* DW2

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Authors' contributions

This work was carried out in collaboration between all authors. Authors OAA and WSM proposed, designed and supervised the study while authors ESA and SKO performed the experiment. Authors SKO and ESA wrote the draft manuscript while author WSM wrote the final version of the manuscript. All the authors read and approved the final manuscript.

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ABSTRACT

Laccases (EC.1.10.3.2 are multi-phenolics and non-phenolic oxidizing enzymes) have received much attention in their use for environmental bioremediation, food quality and functionality improvement. This work was aimed at degrading polycyclic aromatic hydrocarbons and aroma oxidation of fermented locust beans seeds 'iru' by laccase produced by yeast strains. *Kluyveromyces dobzhanskii* DW1 and *Pichia manshurica* DW2 were the yeast strains employed for the production of laccase enzyme. The enzymes were used in the crude, partially purified and agar-gel immobilized forms. The percentage laccase immobilization of *Kluyveromyces dobzhanskii* DW1

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and *Pichia manshurica* DW2 were 65.2% and 73.1% respectively. The PAHs degraded were Naphthalene and Phenanthrene which were degraded by 50.1%, 23.3%, 18.2% and 65.0%, 54.4%, 39.1% respectively by the crude, partially purified and immobilized laccases from *Kluyveromyces dobzhanskii* DW1 while the following degradation percentages; 43.6%, 14.6%, 37.1% and 62.5%, 40.4%, 60.1% for the respective compounds were obtained for *Pichia manshurica* DW2 laccase. The use of the crude, partially purified and immobilized laccases from *Kluyveromyces dobzhanskii* DW1 and *Pichia manshurica* DW2 resulted in aroma oxidation by 78.6%, 51.4%, 31.4% and 62.8%, 57.2%, 45.7% respectively. Chemical analysis of flavour-oxidized 'iru' by crude laccase from *Kluyveromyces dobzhanskii* DW1 showed an increase in protein and tocopherol contents by 9.38% and 30.46% respectively. From this study, the crude laccase from strains worked best in the degradation of Naphthalene and Phenanthrene as well as the aroma flavour oxidation of 'iru' which confers economic benefit to the laccase as additional cost of purification and immobilization of enzyme is not required.

Keywords: Lacccase; degradation; aroma oxidation; PHAs; Iru.

1. INTRODUCTION

Many of the currently used oxidation methods are not economically or environmentally satisfactory, because they produce unwanted side reactions and the oxidants or reaction catalysts are often harmful. Hence, enzymatic oxidation is a potential alternative to chemical methods, because enzymes are very specific and efficient catalysts, and are ecologically sustainable. Also, with the increasing demand for application of enzymes in industry due to the need to overcome high consumption of energy and raw-materials as well as increased environmental concerns over disposal of chemicals into landfills, water or air during chemical processing. Hence, enzyme applications have expanded to various fields, including the chemical, fuel, food, agricultural, paper, textile and cosmetic industrial sectors [1]. One of such enzymes of great importance is Laccase. Laccases are currently seen as very interesting enzymes for industrial oxidation reactions, because they are capable of oxidizing a wide variety of both phenolic and non-phenolic substrates.

Laccases (EC 1.10.3.2) are multi-copper containing oxidoreductases mainly obtained from bacteria and fungi and have the ability to oxidise a wide variety of substrate both phenolic and non-phenolic [2]. They are blue copper oxidases or blue copper proteins that are characterised by four catalytic copper atoms [3] with the four copper ions of laccase classified into 3 types referred to as Type 1 (T1), Type 2 (T2), and Type 3 (T3) [4]

Laccase was first discovered in the sap of the Japanese lacquer tree *Rhus vernicifera*, and its characteristic as a metal containing oxidase was described by Bertrand in 1985 [5]. Since then, laccases have also been discovered in various basidiomycetous and ascomycetous fungi and so far fungal laccases have accounted for the most important group of multicopper oxidases (MCOs) with respect to the number and extent of characterization [5].

Various Polycyclic Aromatic Hydrocarbons, which closely correlate to the 16 compounds selected by the Environmental Protection Agency (USA) and other national institutions as compounds of toxicological relevance were removed by a Laccase Mediator System (LMS). Polycyclic Aromatic Hydrocarbons that were removed included acenaphtylene, anthracene, benzo(α)pyrene, acenaphthene, fluoranthene, pyrene, benzo(α)anthracene, chrysene, benzo(β)fluoranthene, benzo(α)fluoranthene and perylene [6]. PAH quinones were formed to differing degrees as oxidation products.

There has been an increased concern on the quality improvement of food condiments to increase general acceptability with growing concern over environmental impact of the continuous disposal of recalcitrant pollutants into the environment; the presence of such compounds pose a serious risk to humans and the ecosystem because they are toxic and carcinogenic and their presence in aquatic ecosystem reduces oxygen level as a result of reduced amount of sunlight for photosynthetic organisms.

Bioremediation is a pollution control technology that uses biological methods to catalyse the transformation of various polluted chemicals to less toxic intermediates. Polycyclic aromatic

hydrocarbons (PAHs) are an important type of persistent organic pollutants (POPs) that are ubiquitous, bioaccumulative and toxic in the environment whose clean-up requires our immediate attention [7]. The unwanted oxidation of foods is damaging on its quality with some giving off–odours. Hence, laccase treatment may lead to may lead to new functionality, quality improvement, or cost reduction [2].

The high cost of enzyme production, low stability and low production yield are main constraints for application of laccases in industrial processes [8, 9]. Such aforementioned constraints have brought about immobilization of enzymes which is now an important facet of biotechnology. Immobilized enzyme can be more effective in industrial applications because they can be easily recovered and reused any day any time and are more stable than purified enzyme [9,10]. Entrapment of enzymes on agar gel is one of the means of immobilizing enzymes and it is very effective. Agar (agar-agar) is an agarose polysaccharide which is acid stable and has a strong gelling ability with no great significance in protein reactivity [11]. Moreover, the cost of this material is low when compared with other materials commonly used for immobilization.

This investigation was focused on the industrial applications of the laccase produced from yeast strain using the crude, purified and the immobilized laccase enzymes.

2. MATERIALS AND METHODS

2.1 Microorganisms and Culture Conditions

Yeast strains *Kluyveromyces dobzhanskii* DW1 and *Pichia manshurica* DW2 were obtained from the culture collection of the previous experiments [12] in the Department of Microbiology, Faculty of Science, University of Ibadan, Ibadan, Nigeria. Stock cultures were stored in Glucose Yeast Extract Agar slants in sterile McCartney bottles and were kept in the refrigerator at 4ºC.

2.2 Chemicals and Reagents

ABTS was purchased from Sigma-Aldrich, USA. Hydroquinone and Tannic acid were obtained from Plus Chemical, India. Acetaminophen and Catechol were obtained from May and Baker, England. Phenanthrene and Naphthalene were purchased from Tokyo Chemical Industry Co., Ltd. (Japan). African locust bean seeds were purchased at Bodija market, Ibadan, Nigeria few hours after fermentation was completed.

2.3 Laccase Production, Purification and Assay

The liquid medium containing rice bran (10g/l) (*Kluyveromyces dobzhanskii* DW1), cane bagasse (10 g/l) (*Pichia manshurica* DW2), $NaNO_3$ (3 g/l), $CuSO_4$ (0.2 g/l), $FeSO_4$ (0.05 g/l), $MgSO_4.7H_2O$ (0.5 g/l), $ZnSO_4$ (0.001 g/l), Na_2HPO_4 (0.2 g/l), $MnSO_4$ (0.05 g/l), $CaCl_2$ (0.01 g/l), and KCl (0.1 g/l) at pH (6.0). The liquid medium was sterilized by autoclaving at 121°C for 15 minutes. After cooling of the sterilized medium, then, 1ml of yeast extract broth with actively growing *Kluyveromyces dobzhanskii* DW1 and *Pichia manshurica* DW2 isolates containing (3.1×10^9) CFU and (9.8×10^9) CFU respectively were aseptically inoculated separately into a 250 ml Erlenmeyer flask containing 50 ml of the liquid medium. Cultures were incubated at 30℃ and 35℃ for *Kluyveromyces dobzhanskii* DW1 and *Pichia manshurica* DW2 isolates respectively in a shaker incubator at 120 rpm to ensure aerobic conditions. After 14 days of incubation the cells were removed by filtration through filter paper (Whatman no.1). The clear supernatant was stored at 4°C and used for purification. The laccase purification was performed according to the method of Ding et al. [13].

Laccase activity was determined spectrophotometrically by measuring the oxidation of 0.02 M 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) at 30°C according to the method of Mongkolthanaruk et al. [14] for the crude and purified enzyme while the method of Faramarzi and Forootanfar [15] was used for the immobilized laccase. The ABTS was the substrate and absorbance increase in assay mixture was monitored at 420 nm (ε_{420}=36.0 $mM^{-1}cM^{-1}$) and the enzyme activity was expressed in an international units (U) defined as the moles of substrate converted per unit time = rate x reaction volume. Protein concentration was determined by the method of Lowry et al. [16] with bovine serum albumin as a standard.

2.4 Immobilization of Laccase

2.4.1 Entrapment in agar gel

The enzyme was immobilized according to the method of Om and Nivedita [11]. A 4.0% agar

solution was prepared in 25 mM sodium acetate buffer (pH 6.0) by warming at 50℃. After cooling down to room temperature, a 1.0 ml enzyme (containing 0.017 mg (DW1) and 0.020 mg (DW2) protein/ml) was mixed with 9.0 ml agar solution (the total volume of matrix and enzyme mixture being 10 ml) and immediately casted on preassembled petri dishes. After solidification at room temperature, the gel was cut into a small size of 5 x 5 mm to make beads of immobilized enzyme. The beads were stored in 25mM sodium acetate buffer (pH 6.0) and at 4℃ for further use. The percentage immobilization was then calculated.

3. INDUSTRIAL APPLICATIONS OF THE CRUDE, PURIFIED AND IMMOBILIZED LACASE ENZYME

3.1 Degradation of Polycyclic Aromatic Hydrocarbons

The degradation experiment was done according to the method of Hadibarata and Tachibana [17]. The sample contains mixture of two PAH compounds (Naphthalene and Phenathrene) with individual concentration of 10 mg/l. The PAH mixture was dissolved in acetone (1 ml) and after the acetone had become completely volatile, the PAH was made into a solution which consisted of 50mM of sodium acetate buffer pH 5.5, 1 ml of the enzyme (for crude and purified) and 0.5g (5tablets) of the immobilized enzyme. The PAH solution 2ml of each experimental set-up (crude, purified and immobilized), was then incubated for 7 days at 30℃ using a rotary shaker incubator 150 rpm. No enzyme was added in the control experiment. The degradation samples were centrifuged at 1000 rpm for 1minute and were characterized by Gas Chromatography-Mass Spectrometry (GC-MS) and compared with standard PAHs.

3.2 Polycyclic Aromatic Hydrocarbons (PAHs) GC-MS Analysis

Gas Chromatography-Mass Spectrometry (GC-MS, Shimadzu QP-5050). GC-MS was performed with a column 30 m in length and 0.25 mm in diameter, and a helium pressure of 100kPa. The temperature was initially 80℃, held for 2 minutes, raised from 80℃ to 200℃ at 20℃ min^{-1}, then to 260℃ at 7.5℃ min^{-1}, and held for 4 minutes. The flow rate was 1.5 ml min^{-1}, interface temperature was 260℃, and injection volume was 1 µl [17].

3.3 Aroma Oxidation of Fermented Locust Bean Seeds 'Iru'

This was done by incubating freshly fermented *Parkia biglobosa* seed (4.0 g) in 9 ml of sterilized citrate phosphate buffer (pH 6.0) and 1 ml of (crude and purified) laccase enzyme was added to the mixture while 0.5 g (5 tablets) of immobilized enzyme was used. However, no enzyme was added to the control experiment. The mixture was incubated at 30°C and 35°C for *Kluyveromyces dobzhanskii* DW1 and *Pichia manshurica* DW2 respectively in a shaker incubator at 120 rpm for 3 days. After 72 hours of incubation with the laccase enzymes (crude, free and immobilized), the seeds were subjected to sensory evaluation for aroma oxidation.

3.4 Sensory Evaluation of Aroma Oxidation of 'Iru'

Sensory evaluation of each aroma oxidized iru was carried out by a 10 man panelists who were regular consumers of 'iru'. The panelists were asked to indicate the oxidation of the aroma using the oxidized flavour scale as described by Oliveira [18]. The descriptive terms and their ratings were as follows: not noticeable (7), trace (6), faint (5), mild (4), moderate (3), strong (2), very strong (1).

3.5 Proximate Analysis of Oxidized and Non-oxidized 'Iru'

'Iru' samples were analysed chemically according to the official methods of analysis described by the Association of Official Analytical Chemist (A.O.A.C.) [19]. All analyses were carried out in duplicate.

3.6 Statistical Analysis

The statistical analyses of the above experiments were carried out using Statistical Package for the Social Sciences (SPSS) software (version 16.0). All the above experiments were carried out in duplicates and their significance level was analysed using the SPSS software.

4. RESULTS AND DISCUSSION

The laccase was purified using the Ding et al. [13] procedure. The laccase enzyme was immobilized on agar gel and Table 1 shows Percentage immobilization of Laccase enzyme of both *Kluveromyces dobzhanskii* DW1 and *Pichia manshurica* DW2 which shows that

immobilization of enzyme reduced its activity in comparison to free enzyme (100%). The activity immobilization yield of laccase was 73.1% for *Pichia manshurica* DW2 and 65.2% for *Kluyveromyces dobzhanskii* DW1.

4.1 Degradation of Naphthalene and Phenanthrene by Laccase Enzymes

The percentage degradation of Polycyclic Aromatic Hydrocarbon (PAH) compounds by Laccase enzyme of *Kluyveromyces dobzhanskii* DW1 is shown in Table 2. The crude, partially purified and immobilized laccase enzymes of *Kluyveromyces dobzhanskii* DW1 were used to degrade two PAHs; Naphthalene and Phenanthrene. It was generally observed that *Kluyveromyces dobzhanskii* DW1 degraded Phenanthrene better than Naphthalene. Also, the crude laccase enzyme degraded the PAHs best with percentage degradation of 50.1% for Naphthalene and 65% for Phenanthrene, followed by the purified and the immobilized enzyme degraded least.

Table 3 shows the percentage degradation of Polycyclic Aromatic Hydrocarbon (PAH) compounds by laccase enzyme of *Pichia manshurica* DW2. Also, the crude, partially purified and immobilized laccase enzymes of *Pichia manshurica* DW2 were used to degrade two PAHs; Naphthalene and Phenanthrene. It was also observed that *Pichia manshurica* DW2 degraded Phenanthrene better than Naphthalene with the crude laccase enzyme degrading the PAHs best with percentage degradation of 43.6% for Naphthalene and 62.5% for Phenanthrene, followed by the immobilized and the crude enzyme degraded least.

Fig. 1 shows the Gas Chromatography-Mass Spectroscopy (GC-MS) Chromatogram of both Naphthalene and Phenanthrene that was not treated with laccase enzyme acting as the control experiment. The figure shows the peaks and the retention time which helped in the identification of the compound. It was observed that Naphthalene was eluted at 11.004 minutes and Phenanthrene was eluted at 19.593 minutes.

Table 1. Percentage immobilization of laccase enzyme of *Kluyveromyces dobzhanskii* DW1 and *Pichia manshurica* DW2

Laccase enzyme	Total enzyme activity in immobilized gel (U/ml)	Total enzyme activity in soluble partially purified enzyme (U/ml)	% Immobilization
Kluyveromyces dobzhanskii	0.015	0.023	65.2
Pichia manshurica	0.019	0.026	73.1

Table 2. Percentage degradation of Polycyclic Aromatic Hydrocarbons by laccase enzymes of *Kluyveromyces dobzhanskii* DW1

Polycyclic aromatic hydrocarbon (PAH) compound	Enzyme treatments	% degradation
Naphthalene	Crude	50.1
	Purified	23.3
	Immobilized	18.2
Phenanthrene	Crude	65.0
	Purified	54.4
	Immobilized	39.1

Table 3. Percentage degradation of polycyclic aromatic hydrocarbons by laccase enzymes of *Pichia manshurica* DW2

Polycyclic aromatic hydrocarbon (PAH) compound	Enzyme treatments	% degradation
Naphthalene	Crude	43.6
	Purified	14.6
	Immobilized	37.1
Phenanthrene	Crude	62.5
	Purified	40.4
	Immobilized	60.1

Similarly, Fig. 2 shows the Gas Chromatography-Mass Spectroscopy (GC-MS) Chromatogram of both Naphthalene and Phenanthrene that was degraded by crude laccase enzyme of *Kluyveromyces dobzhanskii*. The figure shows the peaks and the retention time with Naphthalene being eluted at 10.910 minutes and Phenanthrene was eluted at 19.189 minutes.

Furthermore, Fig. 3 shows the Gas Chromatography-Mass Spectroscopy (GC-MS) Chromatogram of both Naphthalene and Phenanthrene that was degraded by the purified laccase enzyme of *Kluyveromyces dobzhanskii*. The figure shows the peaks and the retention time with Naphthalene being eluted at 10.987 minutes and Phenanthrene was eluted at 19.296 minutes.

Fig. 4 also shows the Gas Chromatography-Mass Spectroscopy (GC-MS) Chromatogram of both Naphthalene and Phenanthrene that was degraded by the immobilized laccase enzyme of *Kluyveromyces dobzhanskii*. The figure shows the peaks and the retention time with Naphthalene being eluted at 11.016 minutes and Phenanthrene eluted at 19.528 minutes.

Fig. 5 shows the Gas Chromatography-Mass Spectroscopy (GC-MS) Chromatogram of both Naphthalene and Phenanthrene that was degraded by crude laccase enzyme of *Pichia manshurica* DW2. The figure shows the peaks and the retention time with Naphthalene being eluted at 10.928 minutes and Phenanthrene was eluted at 19.196 minutes.

Furthermore, Fig. 6 shows the Gas Chromatography-Mass Spectroscopy (GC-MS) Chromatogram of both Naphthalene and Phenanthrene that was degraded by the purified laccase enzyme of *Pichia manshurica* DW2. The figure shows the peaks and the retention time with Naphthalene being eluted at 10.994 minutes and Phenanthrene was eluted at 19.531 minutes.

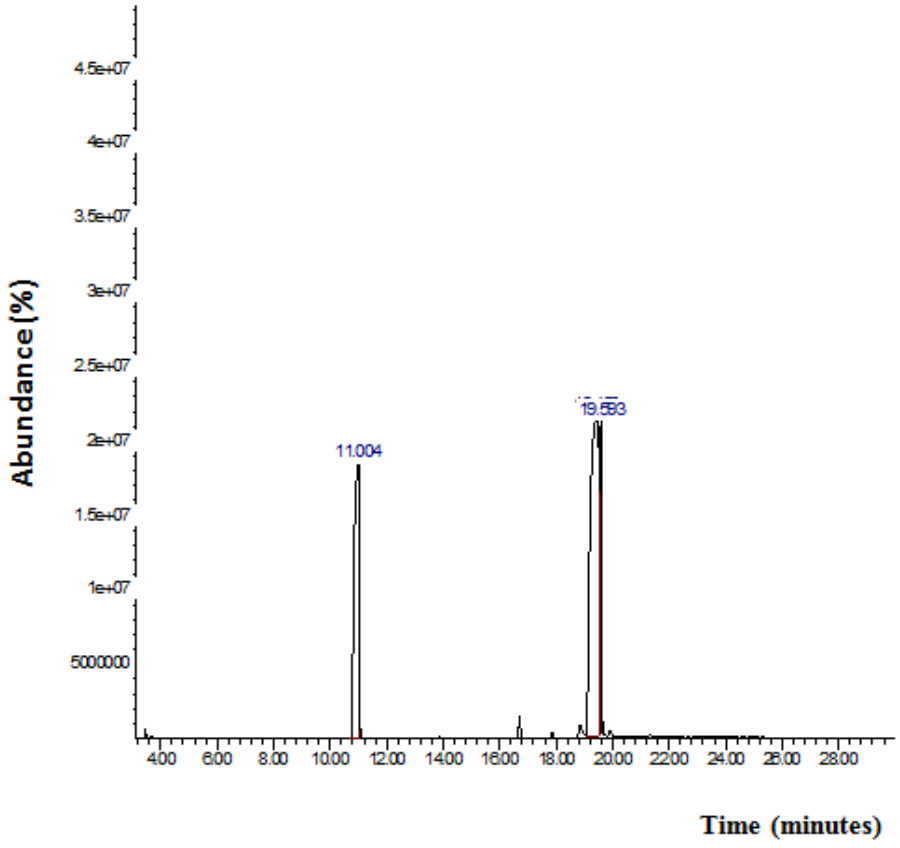

Fig. 1. GC-MS chromatogram showing the peaks and retention time of Naphthalene and Phenanthrene of the Control experiment (without any laccase enzyme)

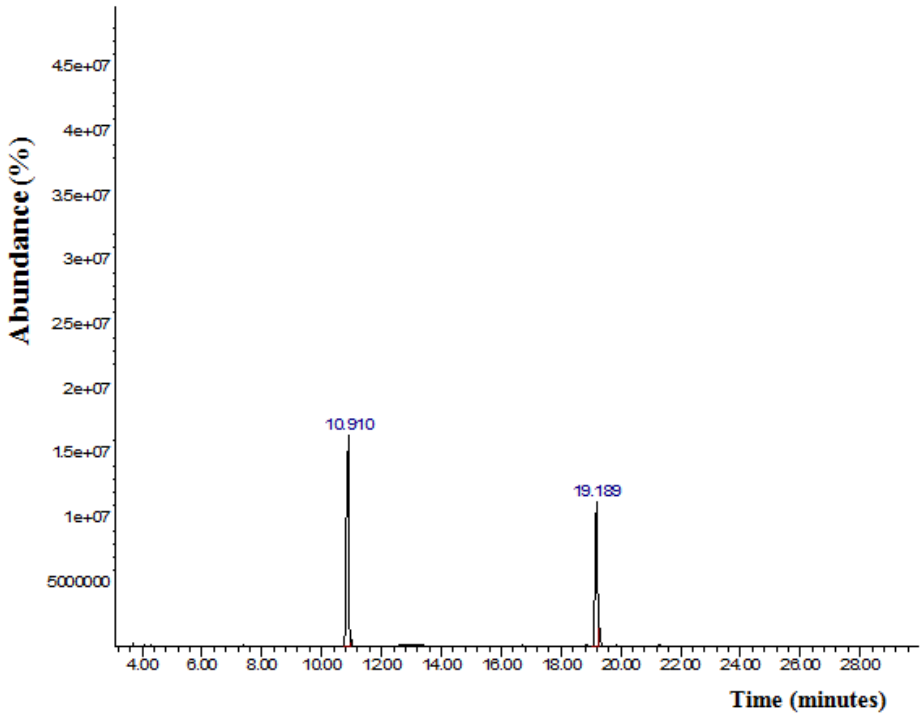

Fig. 2. GC-MS chromatogram showing the peaks and retention time of Naphthalene and Phenanthrene by the Crude laccase enzyme of *Kluyveromyces dobzhanskii* DW1

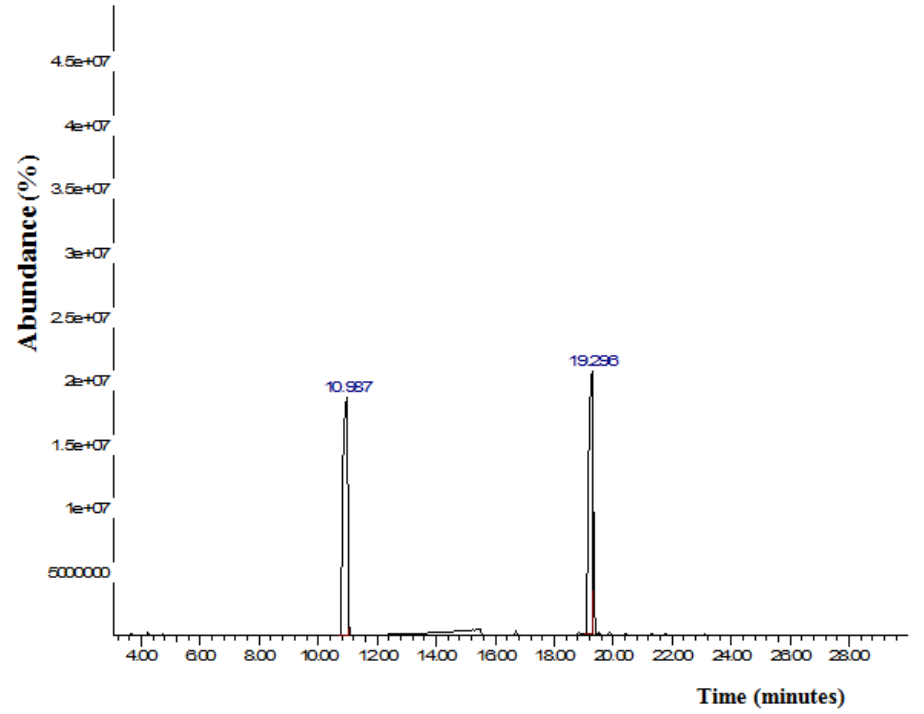

Fig. 3. GC-MS chromatogram showing the peaks and retention time of Naphthalene and Phenanthrene by the Purified laccase enzyme of *Kluyveromyces dobzhanskii* DW1

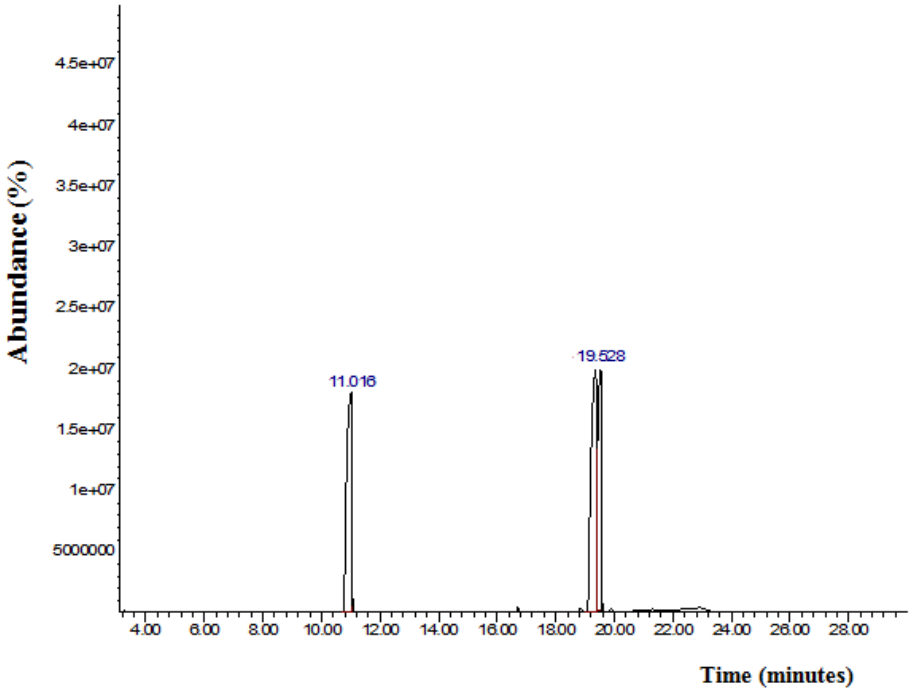

Fig. 4. GC-MS chromatogram showing the peaks and retention time of Naphthalene and Phenanthrene by the Immobilized laccase enzyme of *Kluyveromyces dobzhanskii* DW1

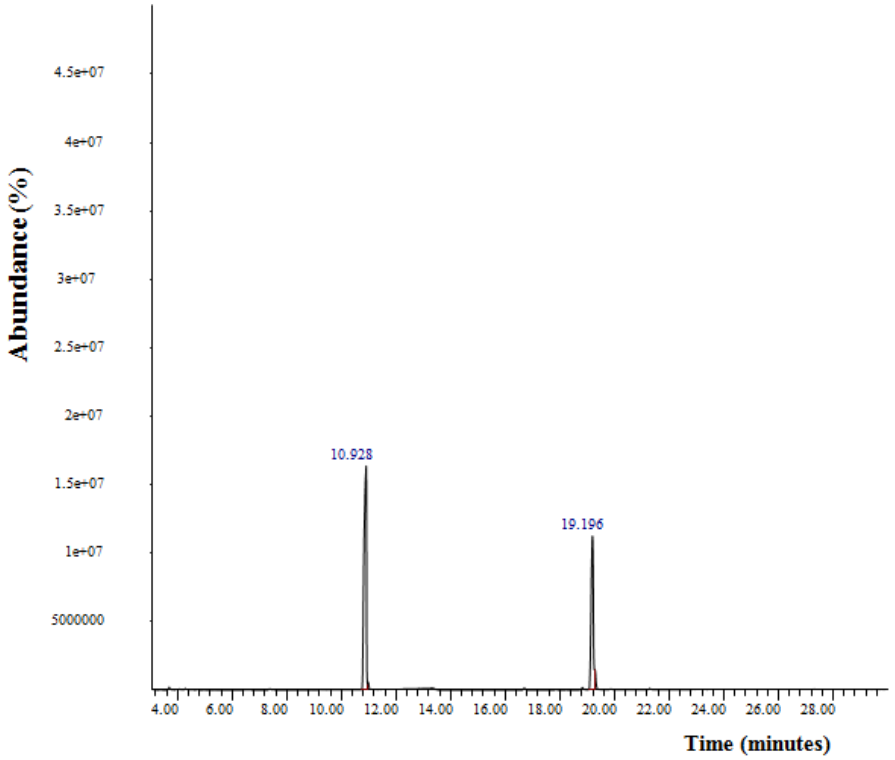

Fig. 5. GC-MS chromatogram showing the peaks and retention time of Naphthalene and Phenanthrene by the Crude laccase enzyme of *Pichia manshurica* DW2

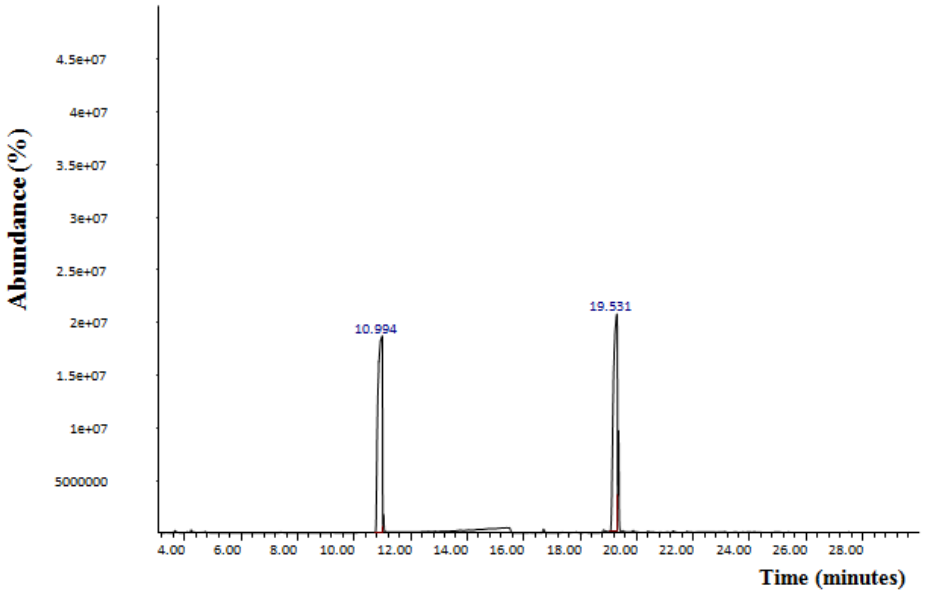

Fig. 6. GC-MS chromatogram showing the peaks and retention time of Naphthalene and Phenanthrene by the Purified laccase enzyme of *Pichia manshurica* DW2

Fig. 7 also shows the Gas Chromatography-Mass Spectroscopy (GC-MS) Chromatogram of both Naphthalene and Phenanthrene that was degraded by the immobilized laccase enzyme of *Pichia manshurica* DW2. The figure shows the peaks and the retention time with Naphthalene being eluted at 10.942 minutes and Phenanthrene eluted at 19.365 minutes.

In the degradation of polycyclic aromatic hydrocarbons; Naphthalene and Phenanthrene by different forms of laccase enzymes of *Kluyveromyces dobzhanskii* strain DW1 and *Pichia manshurica* strain DW2, it was observed that phenanthrene was more degraded than naphthalene. Degradation efficiencies of naphthalene by the crude, partially purified and immobilized laccase enzymes of *Kluyveromyces dobzhanskii* DW1 were 50.1%, 23.3% and 18.2% respectively. While the degradation efficiencies of phenanthrene by the crude, purified and immobilized laccase enzymes of the same yeast strain were 65.0%, 54.4%, and 39.1% respectively. Similarly, the same trend of degradation efficiencies were observed by the forms of laccase enzymes produced by *Pichia manshurica* DW2 with the degradation efficiencies of naphthalene by the crude, purified and immobilized laccase enzymes being 43.6%, 14.6% and 37.1% respectively. The degradation efficiencies of phenanthrene by the crude, partially purified and immobilized laccase enzymes of the same yeast strain; *Pichia manshurica* DW2 were 62.5%, 40.4%, and 60.1% respectively.

From the above results in Tables 2 and 3 dissimilar trends of naphthalene and phenanthrene degradation was recorded by the purified and immobilized laccase enzymes of both *Kluyveromyces dobzhanskii* DW1and *Pichia manshurica* DW2 with the immobilized laccase enzyme of *Pichia manshurica* DW2 degrading both naphthalene and phenanthrene better the purified laccase. While the purified laccase enzyme of *Kluyveromyces dobzhanskii* DW1 degraded both naphthalene and phenanthrene better the immobilized laccase. This was probably owing to the higher immobilization percentage (73.08%) of laccase enzymes in *Pichia manshurica* DW2 as compared to an immobilization percentage of (65.2%) for laccase enzyme of *Kluyveromyces dobzhanskii* DW1.

4.2 Aroma Oxidation of Iru by Laccase Enzymes

Table 4 shows the percentage aroma oxidation achieved by 'iru' treated with the crude, purified and immobilized laccase enzymes of *Kluyveromyces dobzhanskii* DW1 and *Pichia manshurica* DW2. After sensory evaluation for aroma oxidation of Iru, It was observed that the crude laccase enzymes of both isolates oxidized

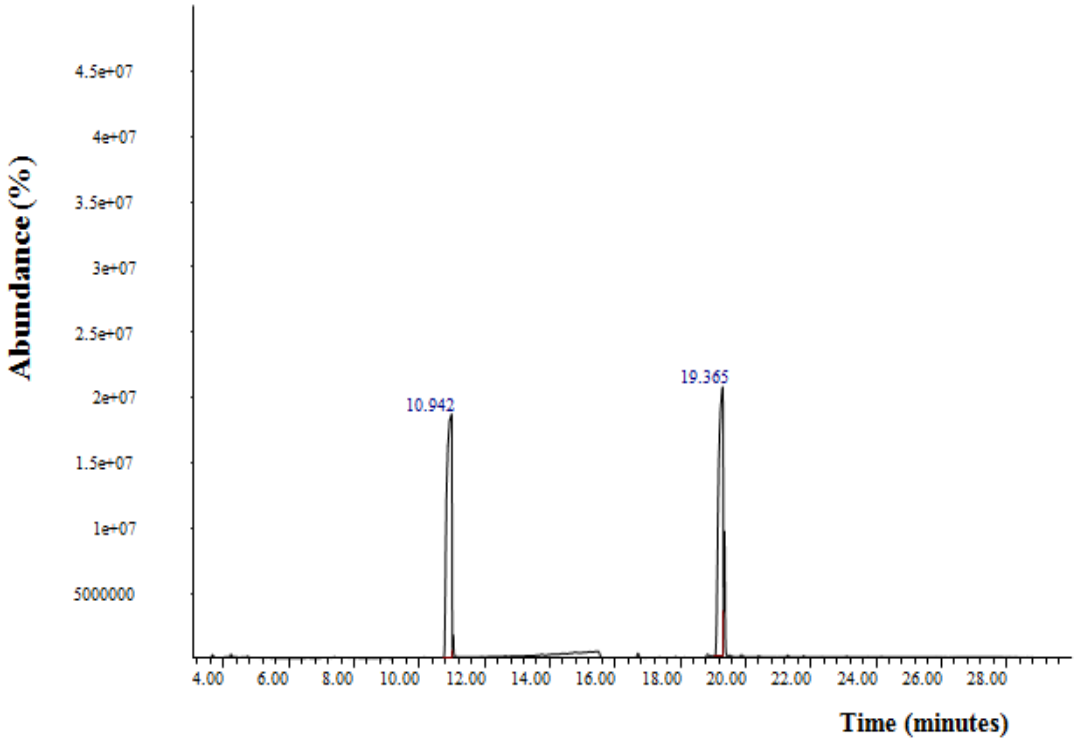

Fig. 7. GC-MS chromatogram showing the peaks and retention time of Naphthalene and Phenanthrene by the immobilied laccase enzyme of *Pichia manshurica* DW2

the flavour of iru most followed by the partially Purified and then the immobilized. The crude laccase enzymes of *Kluyveromyces dobzhanskii* DW1 and *Pichia manshurica* DW2 oxidized the flavour of iru by 78.6% and 62.8% respectively with the purified laccase enzymes of both yeast strain oxidizing the flavour of iru by 51.4% and 57.2% respectively. Likewise, the immobilized laccase enzymes of both oxidized the flavour of iru by 31.4% and 45.7% respectively.

The result of the chemical analysis of the best flavour-oxidized 'iru' (Dw1 laccase enzyme treated iru) is as shown in Table 5. The enzyme treated iru (Cr1) had higher percentage moisture, crude protein, crude fat and vitamin E (tocopherol) contents compared to the non-oxidized 'iru'. The flavour-oxidized 'iru' (Cr1) showed an increase of 6.07%, 9.38%, 21.35%, and 30.46% increase in moisture, protein, fat and tocopherol contents respectively. Also, there was 24.45%, 25.82%, 26.46%, 10.15%, 27.39% and 12.4% decrease in crude fibre, ash, carbohydrate, ascorbic acid, acid value and iodine respectively.

Table 4. Percentage aroma oxidation of 'iru' by laccase enzymes of *Kluyveromyces dobzhanskii* DW1 and *Pichia manshurica* DW2

Laccase enzyme	Enzyme treatments	Experimental code	% flavour oxidation
K. dobzhanskii DW1	Crude	Cr1	78.6
	Purified	Pr1	51.4
	Immobilized	Im1	31.4
Pichia manshurica DW2	Crude	Cr2	62.8
	Purified	Pr2	57.2
	Immobilized	Im2	45.7

Values are average of sensory evaluation of 10 panelists

Table 5. Result of chemical analysis on Flavour-oxidized 'iru' by the crude laccase enzyme of *Kluyveromyces dobzhanskii* DW1 and untreated 'iru'

Sample parameters	Untreated 'iru' (C0)	Treated 'iru' (Cr1)	% increase	% decrease
% Moisture	44.66±0.02	47.37±0.02	6.07	–––
% Crude protein	39.88±0.13	43.62±0.08	9.38	–––
% Crude fat	19.91±0.42	24.16±0.01	21.35	–––
% Crude fibre	6.87±0.14	5.19±0.03	–––	24.45
% Ash	4.57±0.28	3.39±0.21	–––	25.82
% Carbohydrate	22.60±0.28	16.62±0.04	–––	26.46
Ascorbic acid mg/100 g	28.67±0.28	25.76±0.02	–––	10.15
Acid value	2.41±0.42	1.75±0.01	–––	27.39
Iodine (mg/kg)	7.50±0.28	6.57±0.04	–––	12.4
Tocopherol (mg/100 g)	11.82±0.5	15.42±0.04	30.46	–––

Values are average of duplicate readings ± standard deviation.
Key: C0 - Untreated 'iru' without enzyme (control)
Cr1 - Treated 'iru' with the crude laccase enzyme of Kluyveromyces dobzhanskii strain DW1

With the highest percentage oxidized flavour of 'iru' being 78.6% achieved by the crude laccase enzyme of *Kluyveromyces dobzhanskii* DW1, suggesting that some of the aroma volatiles are gone and these volatile compounds as reported by Ouoba et al. [20] could be aldehydes, pyrazines, alcohols, ketones, esters, alkanes, alkenes, acids, benzenes, phenols, amines, pyridines, sulfur compounds and furans. The moisture content of the flavour-oxidized 'iru' by the laccase enzyme of *Kluyveromyces dobzhanskii* strain DW1 increased by 6.07% from 44.66% in the unoxidized flavour (control) to 47.37% in the flavour oxidized 'iru'. This was probably due to the soaking of the 'iru' in buffered solution containing the laccase enzyme, which was in agreement with the report of Omafuvbe et al. [21] where the moisture content of African locust bean seed increased from 51.9% to 56.7% as a result of soaking the locust bean seed in water.

The laccase enzyme treatment of the 'iru' increased the protein content by 9.38% which is indicative that consumers of such 'iru' will have enough protein in their food. The increase in crude protein from 39.88% in the unoxidized flavour (control) to 43.62% in the flavour oxidized 'iru' was of higher value as when compared with 35.0% value reported by Alabi [22] and 34.3%, by Obizoba [23]. From Table 5, the percentage increase in crude fat (21.35%) and crude protein (9.38%) by the laccase enzyme of DW1 strain was probably due to the reduction in the content of ash (25.82), crude fibre (24.45%) and carbohydrate (26.46%).

The laccase enzyme treatment of the 'iru' brought about a huge decrease in the acid value (27.39%) but there was a small decrease in ascorbic acid and iodine contents, 10.15% and 12.4% respectively.

The total Tocopherol (vitamin E) for the oxidized 'iru' increased by 30.46% making the 'iru' oxidized by laccase enzyme a very good source of vitamin E. The total tocopherol content of seasoning salts used in recent times as a replacement for this local condiment ranged between 0.6 mg/100 g (Doyin cube), 1.3 mg/100 g (Royco cube) and 1.7 mg/100 g (Knoor cube) [21]. Rural communities and low-income families use 'iru' liberally often as a substitute for meat and thereby consume a fair amount of this condiment on a daily basis. Vitamin E has a lot of benefits which include: Protecting cells from free radical damage, decreasing risk markers associated with certain cancers, oxidation of Low-Density Lipoprotein cholesterol (a risk factor associated with development of atherosclerosis), protection against the risk of cataract formation and vitamin E also helps in immune responses.

The ability of the laccase enzyme produced by isolates *Kluyveromyces dobzhanskii* DW1 and *Pichia manshurica* DW2 was employed in this study to degrade PAHs (Naphthalene and Phenanthrene). Higher degrading ability of the laccase enzyme from the two yeast isolates was recorded in Phenanthrene with the crude enzyme. Laccase enzymes (crude, purified and immobilised) of *Kluyveromyces dobzhanskii* DW1 degraded the PAHs better, thus chosen for aroma oxidation of *iru*, which resulted in higher nutrient and vitamin E (Tocopherol) contents when compared with some seasonings/ condiments.

5. CONCLUSION

In conclusion, crude laccase have a potential to be used in the degradation or bioremediation of PAH in the environment and also in the flavor oxidation of *iru* in order to achieve better nutritional health and environment. Across these applications, the crude laccase of both organisms worked best which makes its application cheaper and affordable. Therefore, further studies should be performed on the purity of the laccase enzyme and its efficiency on the degrading of PAH outside the laboratory environment.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Maciel MJM, Silva ACE, Ribeiro HCT. Industrial and biotechnological applications of ligninolytic enzymes of the basidiomycota: A review. Electron Journal of Biotechnology. 2010;13:1-12.
2. Kunamneni A, Plou Fransico J, Ballesteros A, Alcalde M. Laccases and their applications: A patent review. Recent patents on Biotechnology. 2008;2(1): 10-24.
3. Alcalde M. Laccases: Biological functions, molecular structure and industrial applications. Industrial Enzymes: Structure, Function and Applications. 2007;461-476.
4. Shleev SV, Khan IG, Morozova OV, Mazhugo YM, Khalunina AS, Yaropolov AI. Phenylpyrazolones, novel oxidoreductase redox mediators for degradation of xenobiotics. Applied Biochemistry and Microbiology. 2004;40:140–145.
5. Giardina P, Faraco V, Pezzella C, Piscitelli A, Vanhulle S, Sannia G. Laccases: A never-ending story. Cellular and Molecular Life Sciences. 2010;67:369–385.
6. Johannes C, Majcherczyk A, Hutterman A. Oxidation of acenaphtalene and acenaphthylene by laccase of *Trametes versicolor* in a laccase- mediator system. Journal of Biotechnology. 1998;61: 151-156.
7. Gao D, Liang H, Du L, Chen J. Biodegradation of polycyclic aromatic hydrocarbons (PAHs) by white rot-fungus *Pseudotrametes gibbosa* isolated from the boreal forest in Northeast China. African Journal of Biotechnology. 2010;9(41): 6888-6893.
8. Salis A, Pisano M, Monduzzi M, Solinas V, Enrico Sanjust E. Laccase from *Pleurotus sajor-caju* on functionalised SBA-15 mesoporous silica: Immobilisation and use for the oxidation of phenolic compounds. Journal of Molecular Catalysis B. 2009;58: 175–180.
9. Ping W, Xuerong F, Li C, Qiang W, Aihui Z. Decolorization of reactive dyes by laccase immobilized in alginate/gelatin blent with PEG. Journal of Environmental Science. 2008;20:1519–1522.
10. Forde J, Tully E, Vakurov A, Gibson TD, Millner P, Ofagain C. Chemical modification and immobilisation of laccase from *Trametes hirsuta* and from *Myceliophthora thermophila*. Enzyme Microbial Technology. 2010;46:430-437.
11. Om P, Nivedita J. Immobilization of a thermostable-amylase on agarose and agar matrices and its application in starch stain removal. World Applied Sciences Journal. 2011;13(3):572-577.
12. Wakil Sherifah M, Salawu Kehinde O, Eyiolawi Seun A, Onilude Abiodun A. Production, characterization and purification of laccase by yeasts isolated from ligninolytic soil. Journal of Pure and Applied Microbiology; 2016 (in press).
13. Ding Z, Peng L, Chen Y, Zhang L, Gu Z, Shi G, Zhang K. Production and characterization of thermostable laccase from the mushroom, *Ganoderma lucidum*, using submerged fermentation. African Journal of Microbiology Research. 2012; 6(6):1147-1157.
14. Mongkolthanaruk W, Tongbopit S, Bhoonobtong A. Independent behaviour of bacterial laccases to inducers and metal ions during production and activity. African Journal of Biotechnology. 2012;11(39): 9391-9398.
15. Faramarzi MA, Forootanfar H. Biosynthesis and characterization of gold nanoparticles produced by laccase from *Paraconiothyrium variabile*. Colloids and Surfaces B. 2011;87:23–27.

16. Lowry OH, Rosebrough NJ, Farr AL, Randall PJ. Protein measurement with the folin, phenol reagent. Journal of Biological Chemistry. 1951;193:265-275.
17. Hadibarata T, Tachibana S. Bioremediation of phenanthrene, chrysene, and Benzo [a] pyrene by fungi screened from nature. ITB Journal of Science A. 2009;41(2):88-97.
18. Oliveira A. Sensory evaluation of foods, FITC - SFOS University of Alaska, FISH 261; 2011.
19. AOAC, Association of Official Analytical Chemists. official methods of analysis 18th edition, AOAC, Arlington, USA; 2005.
20. Ouoba LII, Diawara B, Annan NT, Poll L, Jakobsen M. Volatile compounds of Soumbala, a fermented African locust bean (*Parkia biglobosa*) food condiment. Journal of Applied Microbiology. 2005;99: 1413–1421.
21. Omafuvbe BO, Falade OS, Osuntogun BA, Adewusi SRA. Chemical and biochemical changes in African locust bean (*Parkia biglobosa*) and melon (*Citrullus vulgaris*) seeds during fermentation to condiments. Pakistan Journal of Nutrition. 2004;3(3): 140-145.
22. Alabi DA. *Parkia biglobosa* an endangered species. International Conference Proceedings on Lost Crops and Trees in Africa. 1993;3:265-285.
23. Obizoba IC. Fermentation of African locust bean. Text on nutrional quality of plant fruits. 1998;160-198.

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