Growth and Extracellular Enzyme Production of *Micrococcus* Species from Fermented "Ugba"

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Abstract: Growth and extracellular enzyme production by *Micrococcus* species from fermented "ugba" was conducted in Abuja, Nigeria. A total of twenty samples of "Ugba" were collected from four different markets at Gwagwalada area, Abuja and screened for the presence of *Micrococcus* spp by using Spread Plate Technique. A total of 12 *Micrococcus* isolates were observed with *Micrococcus luteus* recorded the highest with 5 (41.67 %), followed by *Micrococcus roseus* with 4(33.33 %) and the least isolate recorded was *Micrococcus lysae* with 3(25 %) out of the total isolates. The results of the primary screening of the bacteria strains by palm oil agar Plate method shows that *Micrococcus luteus* recorded 32.00 ± 4.67 mm, followed by *Micrococcus lysae* with 28.67 ± 2.44 mm and *Micrococcus roseus* recorded 22.33 ± 6.78 mm. The quantities of the lipase enzyme produced by the *Micrococcus* spp also shows that *Micrococcus roseus* recorded the highest value of 14.03 ± 2.10 mm, followed by *Micrococcus luteus* with 12.20 ± 1.00 mm and *Micrococcus lysae* recorded 10.51 ± 2.00 mm. The growth and extracellular enzyme produced by *Micrococcus* spp from fermented "Ugba" were not significantly different at P = 0.05 level of significant. Proper handling of "Ugba" during production or product pasteurization after production may be necessary to kill *Micrococcus* sp in ugba and halt the enzyme production which participates in spoilage.
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1. Introduction

Fermented products remain of interest since they do not require refrigeration during distribution and storage. The traditional condiments have not attained commercial status due to the very short shelf life, objectionable packaging materials, stickiness and the characteristic putrid odor (Arogba *et al.*, 1995). Moreover, fermented condiments often have a stigma attached to them they are often considered as food for the poor (Zhangwe, 2003). As with any other fermentation process, the understanding of the microbial ecology of vegetable fermentation requires the knowledge of the fermentation substrates i.e. the seeds of the various plants as well as the products obtained (Odunka, 1989; Achi, 1992). "Ugba" also called "Ukpaka" is a popular food delicacy in Nigeria especially among Igbo ethnic group. It is rich in protein and is obtained by a solid state fermentation of the seed of African oil bean tree (*Pentaclethra macrophylla* Benth) (Isu and Ofuya, 2000). It is essential food item from various traditional ceremonies where it is mixed with slices of boiled stock fish (ugba and okpoloko). The natural fermentation of the seed which at present is still done at the house-hold level, renders the production nutritious, palatable and non-toxic (Enujiugha, 2002). Its production, like many African fermented foods depends, entirely on mixed fermentation by microorganism from diverse source. Some of these seeds have been exploited as soup bases such as Gbegiri from *Vigna unguicuata* (Akanbi, 1992). *Pentaclethra macrophylla* Benth is a large woody plant abundant in the rain forest areas of west and central Africa. It's origin in Nigeria is believed to be around 1937 (Ladipo, 1987); where it is found in the South Nigeria, (Mbajunwa *et al.*, 1998). "Ugba" Pentaclethra macrophylla Benth belongs to the Family Leguminosae and sub-family microsoideae. "Ugba" seeds are irregular and oval, they are flat, black and hard pods. It is composed of oil, protein and small amounts of carbohydrate (Obeta, 1982). Production of Ugba is still on age old traditional family in the rural area. Fermented African oil bean seed (ugba), has a high rate of susceptibility to microbial spoilage and therefore has a very short shelf life of 1-2 weeks (Enujiugha and Akanbi, 2005). Manifestation of food spoilage are many and vary typically resulting in an off smell, colour, taste and texture. Reports by Nwagu *et al*. (2010) indicated that *micrococcus* species do not play an active role during microbial fermentation of Ugba. However, further work on spoilage association of ugba by Nwagu *et al.* (2010) showed that the population of *Micrococcus* sp increased with increase in keeping time of ugba. This indicates the ability of *micrococcus* to thrive in the alkalophilic environment while constituting as a spoilage organism of ugba. This may be attributed to the ability of microorganism to produce lipase, lipase, or protease able to utilize protein, carbohydrate or lipid content of ugba as source of nutrition (Njoku *et al.*, 1990). Lipase is

defined as glycerol ester hydrolases (EC3.1.1.3) hydrolyzing tri-di and mono-glycerides present at oil-water interface. Some lipases are also able to catalyze esterification, trans-esterification and enantioselective hydrolysis reaction (Nine *et al.*, 2001; Nwanjo and Onwuiri, 2004). The interest in microbial lipase production has increased in the last decades, because of its large potential in a wide range of industrial applications and additives in food processing (flavour modification). The aim of this study was to assess the growth and extracellular enzyme production of *Micrococcus* spp from fermented "Ugba".

2. Materials And Methods

2.1 Study Area

This research work was carried out at the Laboratory of the Department of Biology, School of Sciences, Federal Capital Territory College of Education, Zuba-Gwagwalada, Abuja, Nigeria.

2.2 Sample collection

A total of twenty (20) Ugba samples were collected randomly with four (4) samples each from five (5) different markets in Gwagwalada Area Council of F.C.T-Abuja. Samples were collected from Tungamaje, Zuba market, Mandalla market and Gwagwalada market. At each location, about 10 g of Ugba were collected from the market women using sterile plastic containers and brought to the Laboratory of the Department of Biology, Federal Capital Territory College of Education, Zuba- Gwagwalada for the isolation of *Micrococcus* spp using the method of Nwagu (2010) with some modifications.

2.3 Preparation and sterilization of media

Nutrient agar (Himedia M001-500G) and Palm oil agar were used in this study and they were prepared according to the manufacturer's instructions thus, 28 g of nutrient agar was dissolved in 1000 ml of sterile water and then sterilized using the autoclave at 121ºC and pressure of 15 Pascal for 15 minutes, while Palm oil agar was prepared by adding 2% (5 ml) of palm oil with 1.5% (3.75g) of agar in 250ml of sterile distilled water and then boiled for 30 minutes. Nutrient agar was used for the isolation and maintenance of pure cultures of *Micrococcus* sp while the Palm oil agar was used for the screening of lipolytic activivty (Nwagu *et al*., 2010).

2.4 Isolation of *Micrococcus* spp from Ugba

The bacteria were isolated from Ugba using Spread Plate Technique. One gram (1 g) of ugba sample was dissolved in 10 ml of sterilized distilled water. The suspension was diluted up to 10^5 and then inoculated on already prepared Nutrient agar plates. The inoculated plates were incubated at the temperature of 37 ^{0}C for 24 hours. Colony developments were observed after incubation period (Campbell and Plant, 1987).

2.5 Preparation of pure cultures of fungal isolates

The young bacterial colonies were aseptically picked up and transferred to fresh sterile nutrient agar plates to obtain pure cultures. The pure cultures on Nutrient agar plates were also kept in agar slant and grown at 37 ^{0}C for 24 hours and stored in the refrigerator at 4^0 C until required for further use.

2.6 Identification of *Micrococcus* sp

Isolates obtained were characterized and identified on the basis of their biochemical tests as well as sugar fermentation test, Gram staining reaction and morphological assessment that is, macroscopic and microscopic features. Among the characteristics used includes: colonial characteristics such as size, surface appearance, texture and colour of the colonies. The biochemical characteristics used include; Catalse test, Coagulase test, Indole test, Citrate utilization test, methyl red and voges proskauer test (Cheesbrugh, 2000).

2.7 Screening of *Micrococcus* spp for Lipase production

Primary screening was done by Palm oil agar Plate method. The isolated bacteria were inoculated on the agar plates amended with 2 % of palm oil with 1.5% of agar. The plates were incubated at 37 ^{0}C for 24 hours. The clear zones around the colonies were observed. The plates that showed a maximum hydrolysis halo on the medium was selected for further investigations.

2.8 Determination of Lipase potentiality of bacterial isolates

A basal medium consisting of palm oil; 2%, peptone; 0.2%, ammonium sulphate; 0.3%, potassium dihydrogen phosphate; 0.1%, magnesium sulphate heptahydrate; 0.03% and calcium chloride; 0.03% (w/v) was sterilized and employed as the substrate for the isolated *Micrococcus* spp (Doddamar and Ninneka, 2001; Bayonmi, 2007). Bacteria suspensions containing 10^5 cells/ml of 24 hours old culture of each isolate was aseptically introduced into each medium. Cultures were incubated at 37 ^{0}C for 24 hours.

2.9 Extraction of Lipase enzyme

Two milliliter of 0.1 M phosphate buffer (pH-6.5) was added to cultures, the mixtures were agitated for 30 min at 19^{0}C and 140 rpm on a rotary shaker. The mixtures (Cultures) were centrifuged at 4000 rpm for 10 min. The supernatant was used as the crude enzyme preparation.

2.10 Assay of Lipase enzyme

Lipolytic products in the supernatant were determined by reading absorbance at 280 nm against basal medium using UV-Spectrophotometer (JENWAY 6305). An increase of 0.100 in the absorbance was considered as equivalent to 1 unit of AU (amylase unit).

2.11 Plate assay

The Plate assay was performed using agar plates amended with palm oil. The agar plates were amended with 2% of palm oil and 1.5% of agar. After the agar solidification, about 10 mm diameter of well was cut out aseptically using a 6 mm cork borer. The well was filled with the culture filtrate and incubated at 40^0C for 72 hours and was observed for zones of hydrolysis around the wells. The negative control was maintained by adding sterile water in the separate well. All the examinations were replicated thrice.

2.12 Statistical analysis

The results obtained were analyzed using one-way ANOVA and the F- test statistic at P = 0.05

3. Results

3.1 *Micrococcus* spp from "Ugba"

The Table below shows the isolation rate of *Micrococcus* spp from "Ugba". *Micrococcus luteus* recorded the highest with 5 (41.67 %), followed by *Micrococcus roseus* with 4(33.33 %) and the least isolate was *Micrococcus lysae* with 3(25 %) of the total isolates.

Table 1: Isolation rate of *Micrococcus* spp from "Ugba"

Isolation Rate, Number (%)					
Bacterial Isolates	**TU (n=5)**	**ZM (n=5)**	**GM (n=5)**	**MM (n=5)**	**Total (n=20)**
Micrococcus luteus	2(40)	1(20)	0(0)	2(40)	5(41.67)
Micrococcus roseus	1(20)	2(20)	1(20)	0(0)	4(33.33)
Micrococcus lysae	1(20)	0(0)	1(20)	1(20)	3(25.00)
Total	4(33.33)	3(25)	2(16.67)	3(25)	12(100)

Keys: TU= Tungamaje, ZM= Zuba market, GM= Gwagwalada matrket, MM= Mandalla market

3.2 Preliminary screening and plate assay of enzyme lipase

The results for the primary screening of the bacteria strains by palm oil agar Plate method as described in the materials and methods is shown in Figure 1 below. *Micrococcus luteus* recorded 32.00 ± 4.67 mm, followed by *Micrococcus lysae* with 28.67 ± 2.44 mm and *Micrococcus roseus* recorded 22.33 ± 6.78 mm. The result for the determination of the lipase activity of the crude enzyme of the strains of *Micrococcus* spp cultivated in basal medium as described in materials and methods. lipolytic activity of the culture filtrates (Crude enzyme) were confirmed on palm oil agar plates and the zone diameter is shown in Figure 1.

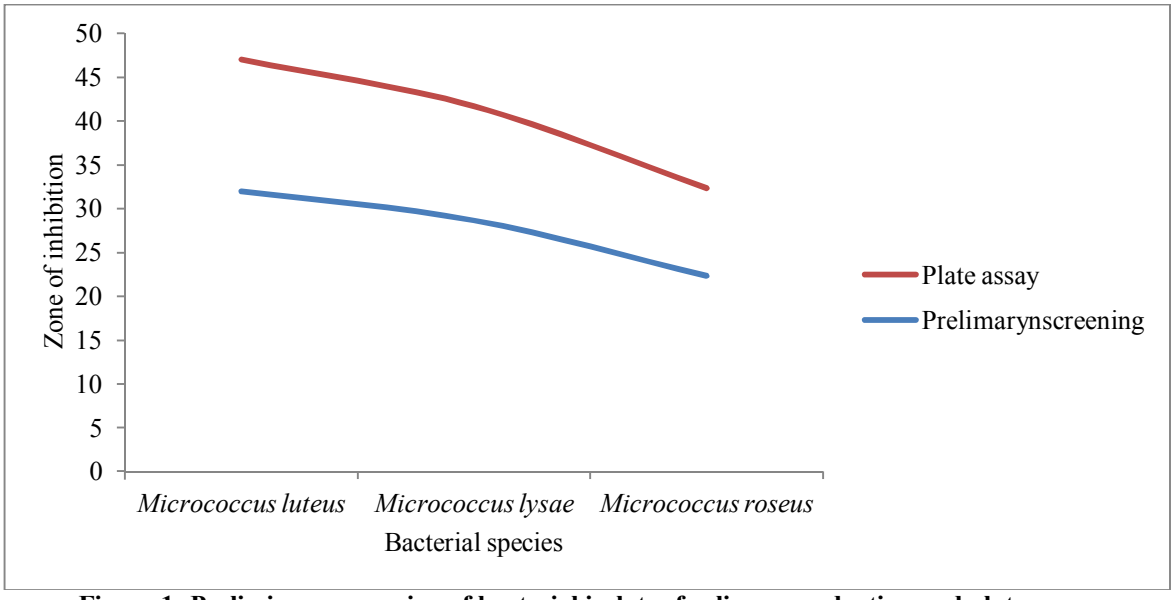

Figure 1: Preliminary screening of bacterial isolates for lipase production and plate assay

3.3 Lipase activities

The quantities of the lipase enzyme produced by the *Micrococcus* spp in the basal medium were measured using UV-Spectrophotometer (JENWAY 6305) and the result is shown in Table 2.

Table 2: Lipase activities of the *Micrococcus* spp isolated from "Ugba"

Bacterial Species	Lipase Activity (U/mL)	Optimum Temp(°C)	Optimum Time (days)
Micrococcus luteus	12.20±1.00	37	7
Micrococcus lysae	10.51±2.00	37	7
Micrococcus roseus	14.03±2.10	37	7

Each value represents Mean ± Standard Deviation of three independent determinations

4. Discussion

The microorganism isolated from the fermented African Oil bean seed (Ugba) in this study agrees with already known organism isolated from previous studies on the microbiology study of Ugba like those of Mbata and Orji, (2008). The isolation rates of *Micrococcus* spp from "Ugba" are as follows: *Micrococcus luteus* was the most frequently isolated species which recorded the highest value of 5 (41.67 %), followed by *Micrococcus roseus* with 4 (33.33 %) and the least isolate was *Micrococcus lysae* with 3(25 %) of the total isolates (Table 1). Bacteria involved in the fermentation are probably introduced through the air, water, utensil, banana leaves or by handling during preparatory stages. This is because the initial step of boiling for 16 – 18 hours would kill most of the natural microbial floral of the seeds (Mbata and Orji, 2008). This study shows that *Micrococcus* was able to produce lipase and therefore could utilize the oil content of "Ugba'. The primary screening of the *Micrococcus* strains by palm oil agar shows that *Micrococcus luteus* recorded 32.00 ± 4.67 mm, followed by *Micrococcus lysae* with 28.67 ± 2.44 mm and *Micrococcus roseus* recorded 22.33 ± 6.78 mm. This also agrees with Njoku *et al*. (1990) which reported the highly alkalophilic environment encountered during post fermentation of "Ugba". The ability to produce this enzyme will enable the successful colonization of this microorganism when introduced in the food, and will enable it to multiply and establish itself as a spoilage association. The determination of the lipase activity of the crude enzyme of the strains of *Micrococcus* spp cultivated in basal medium and lipolytic activity of the culture filtrates (Crude enzyme) were confirmed on palm oil agar plates and the zone diameter are as follows: *Micrococcus luteus* recorded 15.00 ± 1.00 mm, followed by *Micrococcus lysae* with 13.00 ± 1.00 mm and *Micrococcus roseus* recorded 10.00 ± 0.50 mm (Figure 1). *Micrococcus* species have been reported to produce enzymes like lipases, this is in agreement with the work done by Tsekova *et al*. (1993). The quantities of the lipase enzyme produced by the *Micrococcus* spp also shows that *Micrococcus roseus* recorded the highest value of 14.03 ± 2.10 mm, followed by *Micrococcus luteus* with 12.20 ± 1.00 mm and *Micrococcus lysae* recorded 10.51 ± 2.00 mm. African oil bean are largely composed of oil, protein and relatively small amount of carbohydrate (Enujiugha *et al*., 2005); the ability to breakdown these components of the seed is an important characteristics of organism able to ferment and persist till the seed spoils. This explains why *Micrococcus* spp which are known to breakdown oil, were prominent throughout the experiment.

5. Conclusion

Micrococcus isolated from ugba was confirmed as a very good producer of extracellular enzyme (lipase). These bacteria involved in the fermentation were probably introduced through the air, water, utensil, banana leaves or by handling during preparatory stages. This is because the initial step of boiling for 16 to 18 hours would kill most of the natural microbial floral of the seeds (Mbata and Orji, 2008). Proper handling of "Ugba" during production or product pasteurization after production may be necessary to kill *Micrococcus* sp in ugba and halt the enzyme production which participates in spoilage. More work is required to further characterize the lipase produced and to determine its usability in biotechnological processes.

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