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Original article

# Amylolytic, lipolytic and proteolytic activity of *Kocuria varians* isolated from fermented African oil bean seed (*Pentaclethra macrophylla*)

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ABSTRACT

*Kocuria/ Micrococcus* species have been implicated as microbial population of fermented African oil bean seed 'ugba', but has never been characterized and its role in the fermentation not known. In this study *Kocuria varians* was isolated from fermented African oil bean seed. The organism could not utilize citrate and was coagulase, methyl red and oxidase negative. It appeared as deep yellow circular, entire, convex colonies without hemolytic reaction. The organism was alkalophilic and moderately halophilic and could utilize a range of substrates as carbon source including soluble starch, bambara nut flour, palm, oil, olive oil and gelatin. The *K. varians* isolate produced extracellular amylase, lipase and protease when grown on various media. Rate of production of these enzymes was dependent on the composition of the growth medium. Ability to produce proteolytic, lipolytic and amylolytic enzymes which are required to hydrolyze the major nutrients in African oil bean seed indicates that it could play a role in nutrient availability to the fermenting flora, or in aroma and flavor qualities of the fermented food.

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## 1. Introduction

Fermented *Pentaclethra macrophylla* seed (African oil bean seed) popularly known as 'ugba' is a delicacy consumed by West Africans especially the natives of Eastern Nigeria. *Pentaclethra macrophylla* Benth is a woody plant belonging to the family Leguminosaea, sub-family Mimosoidae and its seeds contain 35-52% oil, 17-22%

protein and 12-23% carbohydrates (Oboh and Ekperigin, 2004). Considering the high protein content, 'ugba' can serve as a cheap and easily available source of plant protein, and can be used to address the Protein Energy Malnutrition in developing countries. Unfermented seeds contain anti-nutritional components including cyanide, oxalates, saponin, phytic acid and tannins (Enujiugha et al., 2008). . To enhance edibility and nutrient availability, the seeds have to be boiled and fermented. Fermentation is locally done using wild type bacteria strains spontaneously introduced in to the food during processing through water, handler, leave for wrapping etc. Organisms implicated during this process belong to the genera *Bacillus, Staphylococcus, Escherichia, Micrococcus, Kocuria* (formerly same family with Micrococcus), *Leuconostoc, Proteus, Lactobacillus, Corynebacterium* amongst others (Mbata and Orji, 2008). These organisms utilize some of the nutrients inherent in the seed while their metabolic activities detoxify the seed, soften it and also produce flavor and aroma compounds which impact characteristic tastes and flavor. The major problem of 'ugba' remains its short shelf life (4-5 days). A number of studies have been carried out to investigate various methods of preserving this food e.g. salting in combination with various heat treatment regimens (Mbata and Orji, 2008). None of these have shown much success; rather the treatments affect the taste, texture and consistency of this food which in turn affecting its consumer demand.

In an earlier study *Bacillus* species and *Micrococcus roseus* (now *Kocuria rosea*) were especially found to persist during the spoilage of 'ugba', highly alkaline and unfavorable conditions notwithstanding (Nwagu et al., 2010). *Bacillus* species especially *B. megaterium and B. subtilis* (Obeta, 1983; Isu and Njoku, 1997) have been reported as the key organisms responsible for 'ugba' production. This group of organisms is spore forming, possesses capsules and is known to persist in adverse environment. Also *Bacillus* species have been reported to produce a wide range of starch, protein and fat hydrolyzing enzymes active at extreme of pH and temperature; an attribute which would help it to colonize and thrive during the spoilage of 'ugba' irrespective of the high content of ammonia released or the heat generated. On the other hand the role of *Kocuria* species in 'ugba' production and spoilage remains unclear (Mbajunwa et al., 1998; Nwagu et al., 2011). This work was therefore carried out to isolate *Kochuria* sp from 'ugba' and to evaluate some of its properties including the ability to produce amylolytic, lipolytic and proteolytic enzymes. Understanding this may be a key towards the determination of how to curb spoilage since production is generally by local women and no starter culture is used.

## 2. Materials and methods

### 2.1. Sample collection

The 'ugba' samples were purchased from local traders in New Market in Enugu City, Enugu State, Nigeria.

### 2.2. Isolation of Kocuria sp

Fermented 'ugba' samples (10 g each) was homogenized in a tared blender with sterilized cups using 90 ml of sterile 0.1% peptone water as diluents. The homogenates were poured in tubes, plugged with cotton wool and allowed to stand for 5 hr at 30 $^{o}$C. Subsequent decimal dilution ($10^{-2}$,$10^{-3}$, $10^{-4}$, $10^{-5}$ etc.) were made by serially adding 10 ml of solution from proceeding concentration of 90 ml of the diluents. On a Petri dish containing 0.1 ml of the inoculum, 10-15 ml of sterile medium containing nutrient agar and blood agar was poured for the isolation of the bacterium. The plates incubated aerobically at 30 $^{o}$C for 24-48 h. The colonies that developed were isolated and sub-cultured. Pure cultures resulting from the isolation were sub-cultured and preserved on nutrient agar slants at 4 $^{o}$C for identification.

### 2.3. Identification of isolate

The strain was identified by the standard procedures. Cultural characteristics of the isolates on the plates were noted. The motility of the isolates was examined by the hanging drop technique. Gram reactions and cell morphology were examined from heat fixed smears. The microorganisms were identified by physiological and biochemical test including catalase, coagulase, urease and oxidase test, effect of NaCl on growth, and growth on Simmon's citrate agar. Methyl red and Voges Proskauer test were also carried out (Cheesebrough, 2006). Plate incubation was extended for 96 h based on the recommendation that plates be incubated for >48 h for better appreciation of colony pigmentation and to enable the differentiation of Stapylococcal isolates from *Kocuria* isolates by phenotypic characteristics (Sovini et al., 2010).

### 2.4. Determination of amylase activity

#### 2.4.1. Amylase production

Fermentation medium contained ($gL^{-1}$): Peptone, 5.0; Yeast extract, 2.0; Tween-80, 2.0; NaCl, 5.0; $MgSO_4.7H_2O$, 0.3; KCl, 0.15 and carbon source. Based on carbon source ($gL^{-1}$) medium was divided into Multiple carbon source medium (MCSM) (soluble starch, 5; gelatin, 2.5; olive oil, 2.5), Bambara nut medium (bambara nut flour, 15) and soluble starch medium (soluble starch, 10). To prepare the inoculum, agar plug of profuse growth of isolate was inoculated into 100 mL Erlenmeyer flask containing 20 mL of the fermentation medium and incubated for 24 h. A suspension containing $10^5$ of bacterial cells in 1mL solution served as inoculum. The cultures were incubated at 37 $^oC$ in a Gallenkamp orbital incubator at 120 rpm for 96 h. Samples were collected for analysis of biomass and absorbance read using a Spectronic 20 UV Spectrophotometer (OD 600 nm). The extract was centrifuged at 10,000 rpm (25,900×*g*) for 15 min at 4 $^oC$ to remove the microbial cells and suspended particles.

#### 2.4.2. Amylase assay

Amylase activity assay was done as earlier described (Nwagu and Okolo, 2011). Reaction mixture comprised of 0.5 ml of crude enzyme extract and 0.5 mL of 1% (w/v) soluble starch in 0.2 M phosphate buffer, pH 9. It was then incubated for 10 min at 30 $^oC$. Total reducing sugars were determined by dinitrosalicylate method (Miller, 1959).

One unit of amylase (U) was defined as the amount of enzymatic extract that liberated 1mole of reducing sugar as glucose per minute under the assay conditions.

### 2.5. Determination of lipase activity

#### 2.5.1. Lipase production

Fermentation medium contained ($gL^{-1}$) $MgSO_4.7H_2O$, 0.3; KCl, 0.15; NaCl 5; Peptone, 5.0; Yeast extract, 2.0; Lablemco powder, 1.0; Tween-80, 2.0 and the carbon source. Based on the carbon source ($gL^{-1}$) the fermentation medium was divided into three: Olive Oil Medium (olive oil, 10), palm oil medium (palm oil, 10 g), MCSM (Gelatin, 2.5 g soluble starch, 5.0 g and 2.5 g olive oil). A suspension containing $10^5$ of vegetative bacterial cells in 1mL solution served as inoculum. The cultures were incubated at 37 $^oC$ in a Gallenkamp orbital incubator at 120 rpm for 96 h. Samples were collected under sterile condition for determination of biomass concentration (OD 600nm) and enzyme activity. The extract was centrifuged at 10,000 rpm (25,900×*g*) for 15 min at 4 $^oC$ to remove the microbial cells and suspended particles prior to determination of lipase activity.

#### 2.5.2. Lipase assay

Lipase activity was determined by titrimetry using olive oil emulsion which was prepared by mixing 25 mL of olive oil and 75 mL of 7% Arabic gum solution in a homogenizer for 2 min (Dos Prazeres et al., 2006). The reaction mixture containing 5 mL of olive oil emulsion, 4 mL of 50 mM Tris-HCl buffer, pH 9.0, 1 mL of 110 mM $CaCl_2$ and 1 mL of enzyme (5 mg/mL) was incubated at 50 ºC for 30 min under orbital shaking at 160 rpm. The reaction was immediately stopped after the incubation period by the addition of 15 mL acetone:ethanol mixture (1:1 v/v), and the released free fatty acids were titrated with 50 mM NaOH.

One unit (U) of lipase activity was defined as the amount that released 1 mol of fatty acid per min.

### 2.6. Determination of protease activity

#### 2.6.1. Protease production

Fermentation medium contained ($gL^{-1}$) $MgSO_4.7H_2O$, 0.3; KCl, 0.15; NaCl 5; Peptone, 5.0; Yeast extract, 5.0; Lablemco powder, 1.0; Based on carbon source ($gL^{-1}$) the fermentation medium was divided into three gelatin medium (gelatin, 10), glutamine medium (glutamine, 10 g), MCSM (Gelatin, 2.5 g soluble starch, 5.0 g and 2.5 g olive oil). A suspension containing $10^5$ of vegetative bacterial cells in 1mL solution served as inoculum. The cultures were incubated at 37 $^oC$ in a Gallenkamp orbital incubator at 120 rpm for 96 h. Samples were collected under sterile condition for determination of biomass concentration (OD 600 nm) and enzyme activity. The extract was centrifuged at 10,000 rpm (25,900×*g*) for 15 min at 4 $^oC$ to remove the microbial cells and suspended particles prior to determination of protease activity.

#### 2.6.2. Protease assay

Protease activity was determined by the modified method of Kembhavi et al. (1993). Total of 2 ml of reaction mixture containing 1 ml of freshly prepared casein 1% (w/v) dissolved in 0.1 M phosphate buffer pH 9.0 and 0.2 ml of casein in 0.8 ml buffer was pre-incubated at 37 $^{o}$C. The reaction was terminated after 60 min by adding 1 ml of 10% TCA. The reaction mixture was filtered through Whatman No.1 paper and absorbency of the filtrate was determined at 280 nm.

One protease activity was defined as the amount of enzyme required to liberate 1 mg of tyrosine/ml under experimental conditions.

## 3. Results

### 3.1. Identification of the isolate

The isolate was Gram-positive, non-motile, non-encapsulated and non-spore forming. It could not utilize citrate and tested negative to coagulase, methyl red and oxidase but was urease and catalase positive (Table 1). Colonies on blood agar were circular, entire, convex, smooth and deep yellow without hemolytic reaction. Spherical cells < 1.5 µm in diameter arranged in singles, pairs and tetrads were observed during microscopy. The organism was subsequently identified as *Kocuria varians*.

**Table 1**
Characteristics of the *Kocuria varians* isolate.

| **Characteristics** | **Isolate (*Kochuria varians*)** |
|---|---|
| Gram stain reaction | Gram positive cocci |
| Cell arrangement | singles, pairs and tetrads |
| **Colony morphology (on blood agar)** | |
| Size | < 1.5 µm |
| Shape/margin | circular/entire |
| Colour | deep yellow |
| Elevation | slightly convex |
| Density | Glistening |
| Motility | - |
| Encapsulation | - |
| Spore forming | - |
| **Biochemical characteristics** | |
| Catalase | + |
| Coagulase | - |
| Citrate utilization | - |
| Methyl red | - |
| Oxidase | - |
| Urease | - |
| Indole | - |
| **Physiological characteristics** | |
| 25$^{0}$C | + |
| 37$^{0}$C | + |
| pH 5.5 | + |
| pH 7.6 | + |
| pH 9.0 | + |
| 5% NaCl | + |
| 10% NaCl | + |

+, positive; -, negative

### 3.2. Growth and amylase production

*K. varians* isolated from 'ugba' grew well in the soluble starch medium, bambara nut medium and the multiple carbon source medium (MCSM) which contained soluble starch, gelatin and olive oil as shown in Figure 1. During the first 48 h of cultivation, growth of *K. varians* was rapid in the soluble starch medium; irrespective of this, growth peak was observed after 78 h cultivation in all media tested. Figure 2 shows that the least amylase production (20.0 U/ml) by the isolate was observed using the MCSM, while highest amylase production (30.8 U/ml) was attained when soluble starch was used as carbon source. Though the optimum amylase production in bambara nut medium was observed after 48 h cultivation, optimum amylase production in soluble starch medium and MCSM was observed after 58 h cultivation.

### 3.3. Growth and lipase production

The *K. varians* isolate could grow on the various types of media used as seen in Figure 3. Similar growth rate and pattern was observed irrespective of the medium, however while maximum growth as OD at 600 nm was 0.075 and 0.082 when grown in olive oil and palm oil respectively, it was 0.15 for *K. varians* grown in MCSM. There was a rapid production of extracellular lipase in MCSM and highest lipase production of 5.1 U/ml was observed after 30 h (Figure 4). Lipase production was slow in the olive oil and palm oil medium; extracellular lipase concentration gradually increased in the medium reaching the maximum of 3.3 U/ml and 3.4 U/ml respectively after 40 h cultivation.

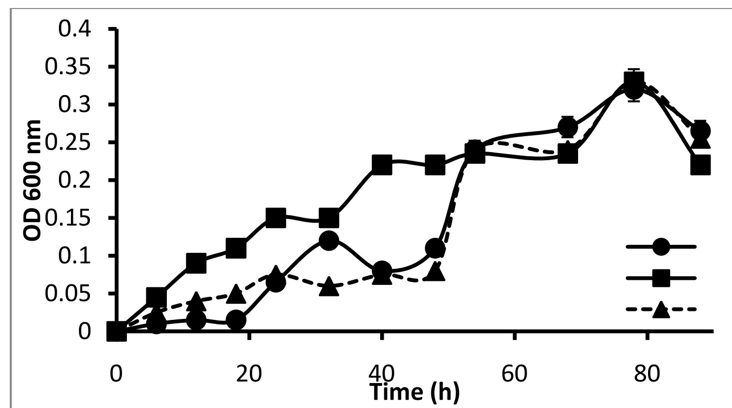


**Fig. 1.** Growth of *Kochuria varians* in amylase production medium (A - soluble starch; B - bambara nut starch; C - soluble starch + gelatin + olive oil).

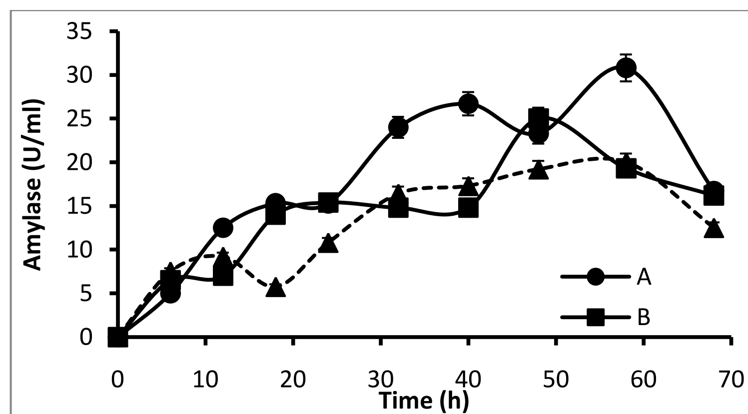


**Fig. 2.** Amylase production from *Kochuria varians* (A - soluble starch; B - bambara nut starch; C - soluble starch + gelatin + olive oil).

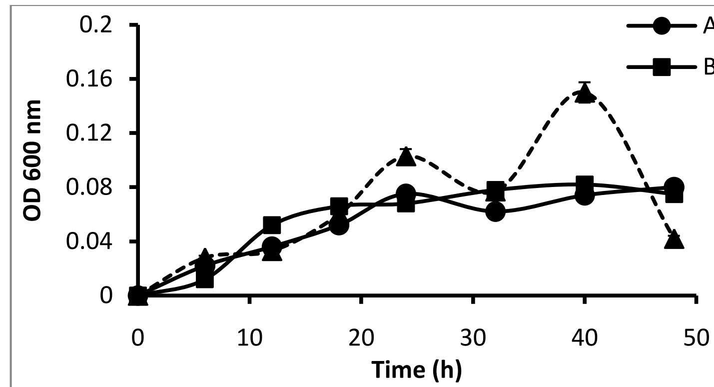


**Fig. 3.** Growth of *Kochuria varians* in lipase production medium (A - olive oil; B - palm oil; C - soluble starch + gelatin + olive oil).

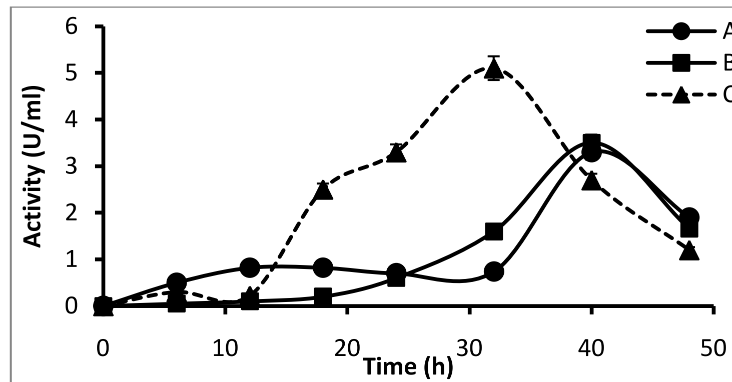


**Fig. 4.** Lipase production from *Kochuria varians* (A - olive oil; B - palm oil; C - soluble starch + gelatin + olive oil).

**3.4. Growth and protease production**

Growth of *K. varians* measured as OD at 600 nm showed maximum growth of 0.8, 1.0 and 1.1 in glutamine medium, gelatin medium, MCSM respectively following 58 h of cultivation (Figure 5). Extracellular protease was detected in the culture fluid irrespective of the media used for cultivation (Figure 6). Protease yield was poor in the glutamine medium; highest protease production of 0.8 U/ml was observed after 58 h cultivations. Optimal yields of 2.0 U/ml and 1.8 U/ml was observed after 58 h of culture in gelatin medium and MCSM.

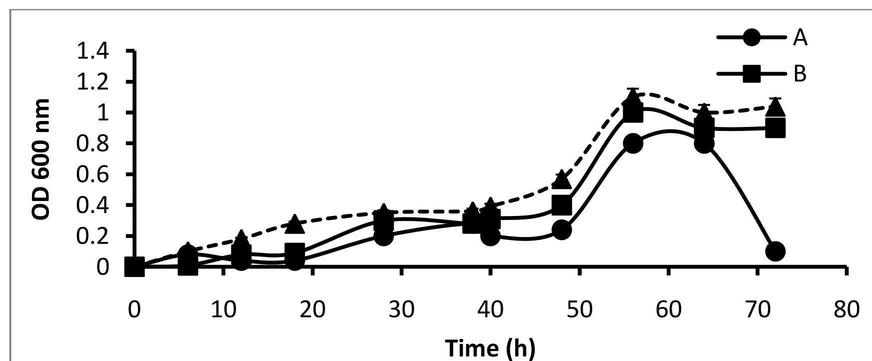


**Fig. 5.** Growth of *Kochuria varians* in protease production medium (A - glutamin; B - gelatine; C -soluble starch + gelatin + olive oil).

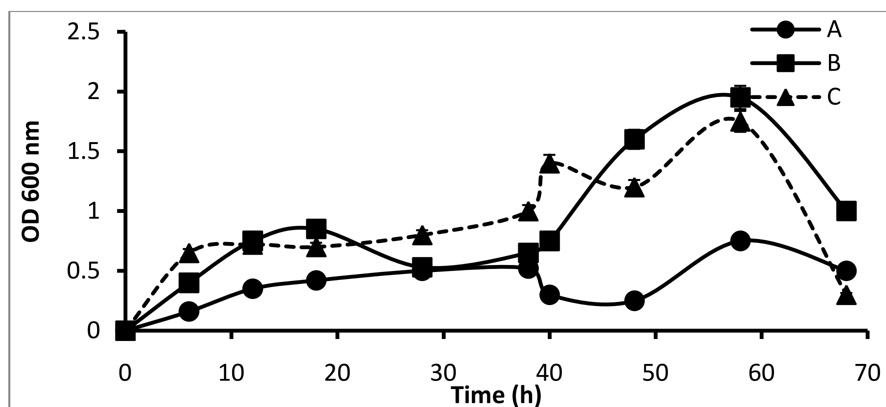


**Fig. 6.** Protease production from *Kochuria varians* (A - glutamin; B - gelatine; C -soluble starch + gelatin + olive oil).

## 4. Discussion

The genus Kocuria was created from the genus Micrococcus and belongs to the family Micrococcaceae, Suborder Micrococcineae, Order Actinomycetales, Class Actinobacteria (Stackebrandt et al., 1995; Takarada et al., 2008). These are gram positive obligate coagulase negative cocci found as skin, mucosa and oropharynx commensals in human and other mammals (Ben-Ami et al., 2003; Tsai et al., 2010) and have been isolated from fermented foods of different origins and culture (Savini et al., 2010; Nwagu et al., 2010; Park et al., 2010a). Similar to the reports associating members of the family Micrococcaceae as part of the microbial population of 'ugba' a source of plant protein, numerous reports exist of the isolation of these organisms from fermented sausages, a rich source of animal protein (Cocolin et al., 2001; Park et al., 2010b). *Kocuria* species are also used as starter cultures for some of these fermented foods (Tremonte et al., 2007). Unlike in fermented sausages where they are attributed to flavor and aroma development as a result of enzyme production, nitrate reduction and acetoin production (Papamanoli et al., 2002), their role in 'ugba' production remains unknown. In the present study the isolated *K. varians* was alkalophilic and moderately halophilic; and was able to utilize soluble starch, bambara flour, olive oil, palm oil, glutamine and gelatin as carbon sources for their growth and metabolic activities. The secretion of amylase (a primary metabolite) was possibly induced in the presence of starch sources which had to be hydrolyzed to simple sugars prior to their assimilation into the cell. Similarly the organism produced lipase needed for the hydrolysis of palm and olive oil, and also proteases required for the utilization of gelatin. Fats and oil, protein and carbohydrates are the major nutrients contained in 'ugba' and the ability to produce their hydrolytic enzymes signifies that the *K. varians* can breakdown these complex polymers to simpler units in order assess nutrients for their growth and metabolism. Microbial activities in fermented foods are the results of complex interactions between various microorganisms including amensalism, commensalism and symbolism (Tremonte et al., 2007). It is possible that when present, *Kochuria* sp in this case *K. varians* increases nutrient availability to other organisms by breaking down some of the complex nutrient to simpler forms; this may accelerate the rate of microbial activities in the fermentation environment which could be channeled towards the proper fermentative pathways or even spoilage depending on the microbial population present. Previous studies involving *K. varians* (reported as *M. varians*) as single culture or in combination with *Alkaligenes viscolatis* gave a poor product but that may be partially because on its own *Alkaligenes viscolatis* is a poor choice for the fermentation (Nwagu et al., 2011). Coagulase negative cocci which include the members of the genus Kocuria are reported to produce flavor and aroma compounds through proteolysis and lipolysis (Tremonte et al., 2007). Our results show that this isolate is capable of producing these enzymes and may therefore contribute to the characteristic flavor and aroma of 'ugba'. Moreover, the ability to produce hydrolytic enzymes enables the organism to favorably compete with other microorganism in its environment. This coupled with the alkalophilic and moderately halophilic nature of some members of this genus including the current one may also explain the persistence of *Kocuria rosea* in spoilt 'ugba' as previously reported (Nwagu et al., 2010; Yamaguchi et al., 2012).

## 5. Conclusion

Though *Kocuria*/ *Micrococcus* is the not the major organism involved in 'ugba' production, attributes of the isolated *K. varians* indicate that it may be involved in the flavor and aroma formation and help to accelerate the rate of fermentation. Therefore further studies are required to determine exactly how these characteristics impact on 'ugba' production and quality when present. Also ability to produce these proteolytic, lipolytic and amylolytic enzymes can also be further studied and conditions of production optimized to obtain high yields given that these enzymes are invaluable in numerous industries and there remains a continuous search for these enzymes from organisms with unique characteristics.

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