# PRODUCTION OF A THERMOSTABLE ALKALINE PROTEASE FROM ALKALOPHILIC *KOCURIA VARIANS* GROWN ON VARIOUS AGRICULTURAL WASTES

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The ability of *Kocuria varians* to grow and produce protease when utilizing various local wastes was studied. Impact of cultivation pH on growth and enzyme production was also evaluated. Cassava waste combined with bambara nut waste (1:1) gave the best protease yield. Maximum enzyme production was attained when production medium was adjusted to pH 9. Highest protease concentration in the culture fluid was recorded at 20 h during the exponential phase of growth. The enzyme was optimally active and stable at 80 °C. Optimum pH for protease activity was at 11 with optimal stability at the alkaline range (pH 7–11) after incubation for 1 h. The enzyme was inhibited by EDTA, $Hg^{2+}$, and $Zn^{2+}$, but not by $Pb^{2+}$, and was slightly stimulated by $Cu^{2+}$. The properties of this protease make it a promising candidate for further studies and possible applications in processes involving extreme conditions of pH and temperature.

**Keywords:** protease, *Kocuria* sp, agricultural wastes, alkalophilic, thermophilic

Proteases are enzymes that catalyze the hydrolysis of proteins. Biotechnological application of proteases cuts across various fields including medical, biopharmaceutical, industrial, food, textile, and environmental remediation. Microbial proteases are preferred to plant and animal sources due to their high specificity, genetic diversity, catalytic efficiency, ease of production and manipulation to achieve specific needs (Ghorbel-Frikha et al., 2005).

Proteases account for over 60% of the industrial enzyme market and recent advances indicate its increasing importance (Rao et al., 1998). There is therefore need for continuous search to discover diverse microbial strains that can utilize cheap and easily available materials to produce large quantities of enzymes suitable for application; especially in industries, where extreme reaction conditions may apply, such as detergent industries, leather processing, chemical industries as well as waste treatment (Tremacoldi et al., 2007).

Cassava is a popular starchy staple widely cultivated in Asia, Africa, and Latin America (Howeler & Fahrney, 2008). Bambara nut originates from West Africa and is found in tropical regions of America, Asia, and Australia; it is also cultivated in Southern Africa, especially Zimbabwe (Heuzé & Tran, 2012). There are various methods of processing these foods during which by-products rich in nutrients, including carbon and nitrogen molecules, are obtained (Ukpabi et al., 2008). Traditionally some of these by-products are utilized as animal and bird feeds, while most are discarded as wastes (Onyimonyi & Okeke, 2007). Considering that these agricultural wastes possess low economic value and constitute environmental problems due to improper disposal, more researches should be targeted towards their conversion to value added products.

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In this investigation, we reported the production of an alkalophilic protease from a *Kocuria varians* strain newly isolated from fermented African oil bean seed, grown on agricultural wastes, including cassava and bambara nut waste.

## 1. Materials and methods

### 1.1. Substrate and chemicals

Cassava (*Mannihot esculenta*) wastes were collected from local cassava processing mill in Enugu State, Nigeria and promptly dried. To prepare bambara nut (*Voandzeia subterennea*) waste, whole seeds were split in an attrition mill to remove the shells, followed by winnowing to remove loosened testa. Seeds were then milled gradually into fine flour and sieved. The chaff (offal) was collected as waste (Fig. 1).

Casein was purchased from Sigma USA. All reagents were of analytical grade.



*Fig. 1A.* An illustration of the unpeeled cassava tuber (a), peeled and washed cassava tuber (b), and by-product from soaked, fermented, and sieved cassava (c) prior to drying and milling

### 1.2. Microorganism

*Kocuria varians* strain was isolated in our laboratory from fermented African oil bean (*Pentaclethra macrophylla*) seed (submitted for publication). The pre-inoculum was prepared by transferring a loopful of culture from nutrient agar slants to 250 ml Erlenmeyer flasks containing 50 ml of peptone water and incubating at 30 °C in an orbital shaker at 120 r.p.m. for 12 h. The resulting active culture at a concentration $4.16 \times 10^3$ CFU ml$^{-1}$ was used as inoculum.

*Fig. 1B.* Whole bambara nut *(Voandzeia subterennea)* seeds (a), chaff (offal) from crushed, winnowed, milled, and sieved seeds (b)

### 1.3. Protease production

The production of protease was carried out as previously reported by JOHNVESLY et al. (2002). The medium contained (g l⁻¹): $NaNO_3$, 5.0; $K_2HPO_4$, 5.0; $MgSO_4 \cdot 7H_2O$, 0.6; $CaCl_2 \cdot H_2O$, 0.6; agro-industrial waste, 10.0. The $Na_2CO_3$ (10 g l⁻¹) was separately sterilized and mixed with the rest of the medium after bringing it to room temperature. The pH was adjusted to 7.5 with NaOH and the medium was sterilized by autoclaving at 121 °C for 15 min. The medium (50 ml in 250 ml Erlenmeyer flasks) was inoculated and incubated at 30 °C in an orbital shaker at 150 r.p.m. Triplicate flasks were withdrawn at regular intervals, and the turbidity of the cultures was determined by measuring the increase in optical density at 660 nm using a UV-mini 1240 spectrophotometer from SHIMADZU. Before the assay, the cells were separated by centrifugation at 15 000 *g* for 10 min at 4 °C, and the clear supernatant was used as the crude enzyme.

### 1.4. Optimization of protease production

*1.4.1. Effect of different agricultural wastes on protease production.* Different agricultural wastes including cow blood meal, fermented cassava wastes, potato, yam and plantain peels, fish meal, and corn waste (1% each) was used for protease production.

To further improve yield, the concentrate of the waste that elicited the second highest protease production was used as a supplement (0.25–2%) to the major carbon source (the waste that elicited the highest protease production).

### 1.5. Properties of the enzyme

The cell free supernatant was precipitated with 80% ammonium sulphate and the pellet was dissolved in a small amount of 50 mM sodium phosphate buffer (pH 9) and dialyzed extensively against the same buffer. The concentrated enzyme was used for further studies.

*1.5.1. Effect of pH and temperature on the activity and stability of the protease.* The optimum pH of the protease was determined with casein 1% (w/v) as substrate dissolved in different buffers (citrate phosphate pH 5–6, sodium phosphate pH 7–9, and glycine-NaOH,

pH 10–12). The pH stability of the protease was determined by pre-incubating the enzyme in different buffers for 2 h at 45 °C.

To determine the optimum temperature, the reaction mixture was prepared in a buffer of the optimum pH and incubated at different temperatures from 30 to 100 °C for 20 min. To determine the temperature stability, the enzyme was kept at various temperatures (30–100 °C) for 60 min each.

*1.5.2. The effect of some metal ions and EDTA on the protease activity.* The enzyme was pre-incubated with 10 mM concentration of each of $Pb^{2+}$, $Hg^{2+}$, $Cu^{2+}$, $Zn^{2+}$ and EDTA at 35 °C. After 60 min pre-incubation the reaction was initiated by the addition of 1.0 ml casein 1% (w/v) and kept at 40 °C for 20 min.

*1.5.3. Protease assay.* Alkaline protease activity was determined by the modified method of KEMBHAVI and co-workers (1993). Total of 2 ml of reaction mixture containing 1 ml of casein 1% (w/v) dissolved in 0.1 M sodium phosphate buffer pH 9.0 and 1 ml of protease solution was pre-incubated at 40 °C. The reaction was terminated after 20 min by adding 2 ml of 10% TCA. The reaction mixture was filtered through Whatman No.1 paper and absorbance of the filtrate was determined at 280 nm.

One unit of alkaline protease activity was defined as the amount of enzyme required to liberate 1 mg of tyrosine $ml^{-1}$ under experimental conditions.

## 2. Results and discussion

### 2.1. Results

*2.1.1. Growth and protease production using various agro-industrial wastes.* K. varians was grown on different agro-industrial wastes to determine the best substrate for protease production. Figure 2 shows that cassava waste gave the highest protease yield of 175.2 U $ml^{-1}$ followed by bambara nut waste (138.8 U $ml^{-1}$) and potato waste (111.4 U $ml^{-1}$).
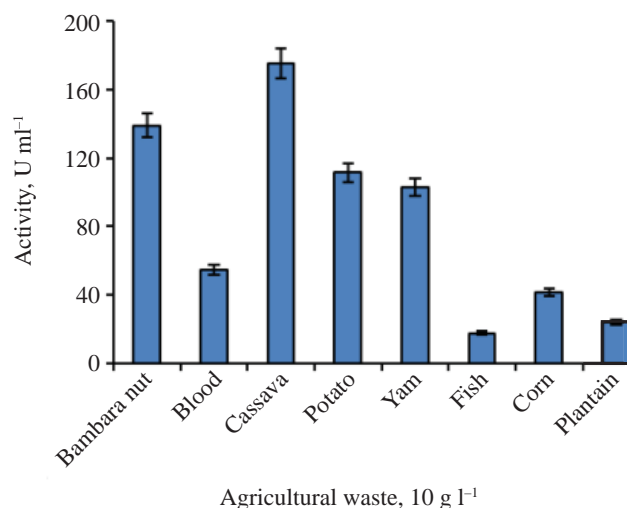


*Fig. 2.* Protease production from *Kocuria varians* grown on agro-industrial wastes

*2.1.2. Effect of supplementing cassava waste with bambara nut waste*. Growth of *K. varians* and protease production gradually increased when the cassava waste was supplemented with the second best substrate (bambara nut waste) as shown in Figure 3. Maximal growth was 1.58 OD 660 nm when 1% cassava waste and 1% bambara nut waste was used. Enzyme produced was 248.4 U ml⁻¹ whereas using only cassava waste as carbon source protease production was 172.6 U ml⁻¹.
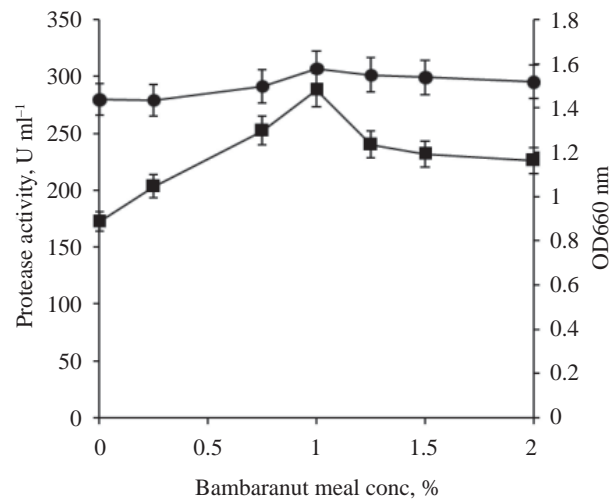


*Fig. 3.* Effect of supplementing cassava waste with bambara nut waste on growth and protease production.
Cassava waste (1%) was used for all samples (●: growth at OD 660 nm, ■: protease activity)

*2.1.3. Influence of cultivation period on bacterial growth and protease production.* Maximum growth of 3.96 OD 660 nm was observed after 24 h cultivation, and growth gradually declined from 24–36 h, followed by rapid cell death. Extracellular protease (52.01 U ml⁻¹) was detected in the culture medium after 4 h cultivation. Extracellular protease production gradually increased with increase in cell growth reaching a peak of 276.41 U ml⁻¹ after 20 h cultivation (Fig. 4).

*2.1.4. Effect of pH on the activity and stability of the protease*. The protease from the *K. varians* strain was poorly active at low pH and only 22.0% activity was recorded at pH 4 (Fig. 5). At the neutral pH 7 protease activity had increased to 28.4% and at pH 9, 74.2% activity was observed. Optimum activity was at pH 11, however, more than 80% protease activity was observed at the alkaline range of 9–12.

*2.1.5. Effect of temperature on the activity and stability of the protease*. As shown in Figure 6, the protease had low activity at 30–40 °C. Optimum temperature for enzyme activity was observed to be at 80 °C. However, the enzyme was most active in the range 70–100 °C. The protease was temperature stable and retained over 45% of its activity at the range 30–100 °C.
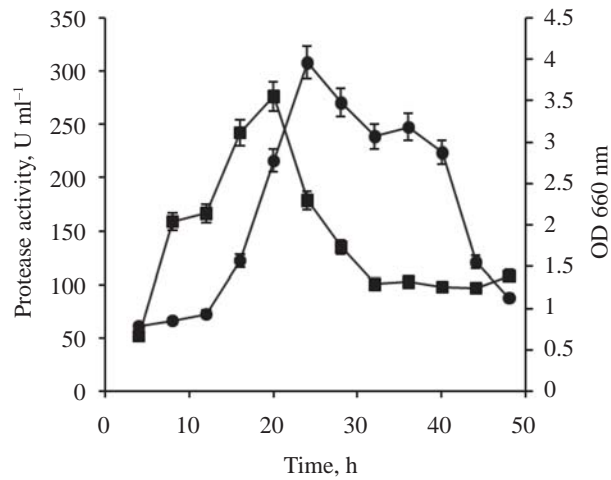
Fig. 4. Time course of growth and protease production from *K. varians*,
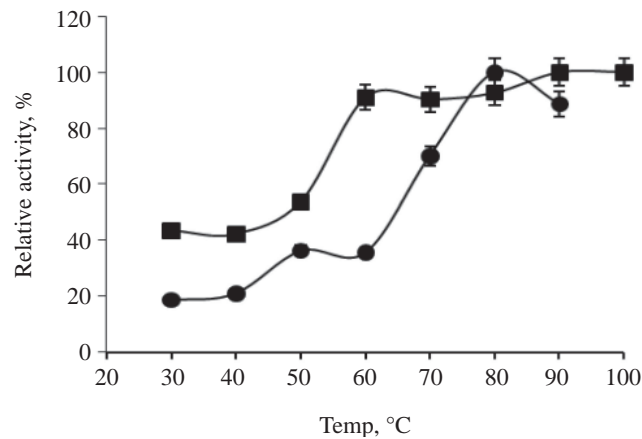(●: growth at OD 660 nm, ■: protease activity)



Fig. 5. Influence of pH on the activity and stability of the protease
(●: enzyme activity, ■: enzyme stability)

*2.1.6. Effect of some heavy metals and EDTA.* Following the incubation of protease in the presence of each of these agents, $Pb^{2+}$ resulted 101.9% residual activity showing that it had little or no effect on protease activity; whereas $Cu^{2+}$ led to 17.1% increase in protease activity (Fig. 7). EDTA had the most inhibitory effect on the protease, 43.4% enzyme activity was lost.

## 2.2. Discussion

Growth and protease production by *K. varians* was influenced by the type of agricultural wastes. Agricultural wastes such as mustard seed cake and pigeon pea have been reported as good substrates for protease production by different microorganisms (EL HADJ-ALI et al., 2010). Though 1% cassava waste gave the highest protease yield, combining both cassava
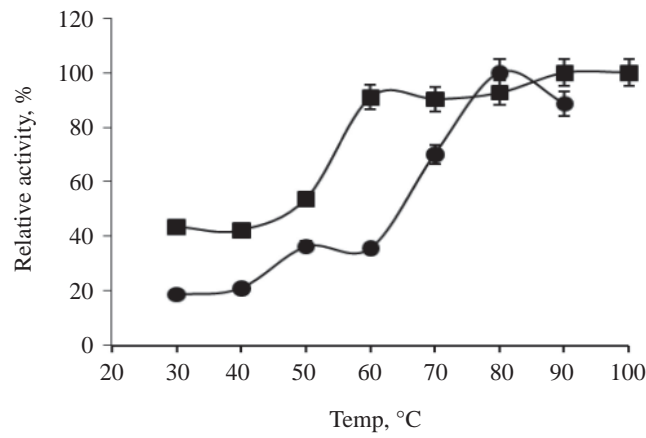
*Fig. 6.* Influence of temperature on the activity and stability of the protease
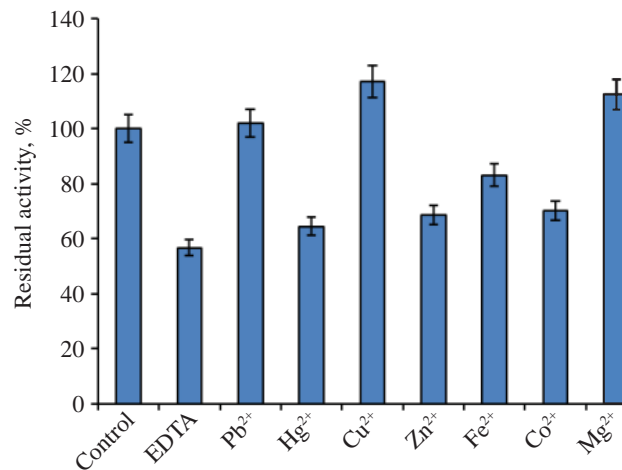(●: enzyme activity, ■: enzyme stability)



*Fig. 7.* Effect of metal ions and EDTA on the activity of the protease

waste and bambara nut waste led to increased protease production by *K. varians*; but had very low effect on the cell growth. It is interesting that the rate of protease production increased without a corresponding increase in cell growth. During microbial production of a desired metabolite it is essential to balance the trophophase-idiophase relationship for optimal yield and this may be achieved by maintaining the correct type and proportion of the required nutrient. Using the wrong nutrient or inappropriate concentrations may prolong the trophophase and negatively affect the idiophase impacting adversely on enzyme production by the isolate. According to a report by Joo and co-workers (2004), the microbial growth medium accounts for approximately 30–40% of the production cost of industrial enzymes; therefore to serve as a potential candidate for large scale enzyme production, a microorganism should be able to produce high amounts of the desired extracellular enzyme, while utilizing cheap and easily available substrates (AHMED & ABDEL-FATTAH, 2010). The above results

indicate that the protease is an inducible enzyme, rather than constitutive (KANCHANA & PADMAVATHY, 2010); and its production can be regulated by utilizing easily available agricultural wastes that would normally constitute environmental nuisance and could cause eutrophication in the water bodies. Protease production by this organism was optimum after 20 h cultivation during the late exponential phase. There was a rapid drop in the level of protease in the culture fluid after 20 h cultivation, which is attributed to cessation of enzyme synthesis coupled with rapid deactivation of the existing enzyme. The protease produced by the bacteria is thermophilic and thermostable. Another interesting feature of this protease is its activity and stability at pH 11. Few organisms are reported to have produced protease with optimal activity above pH 11 and these were from *Bacillus* strains; KHAN and co-workers (2011) recently reported the production of a protease from *Bacillus subtilis* isolated from wastes, which exhibited optimal activity at pH 11.5. Majority of thermophilic and thermostable proteases so far reported belong to the genus *Bacillus* (SALEM et al., 2009), to the best of our knowledge this is the first report on the production of such a protease from *Kocuria* sp. The properties of the protease by *Kocuria varians* are unique in comparison to other documented proteases by *Kocuria*/*Micrococcus* strains. Recently, MANIKANDAN and co-workers (2011) reported protease production from a halophilic, alkalophilic *Micrococcus* sp. VKMM 037 isolated from an effluent of the caustic soda industry. Protease was active between 20–60 °C and pH values 4–12 with an optimum at pH 10. The properties of the *K. varians* protease indicate the production of a thermostable enzyme under mesophilic conditions. This is economically viable considering that thermostable proteases are in high demand for use in numerous industrial applications where cost and profit dictate production processes (HAJJI et al., 2008). Moreover, the activity and stability of this enzyme under extreme conditions of pH and temperature are good characteristics required in biotechnological application of proteolytic enzymes. Though heavy metal ions such as $Cu^{2+}$ and $Pb^{2+}$ have been reported to inhibit the activity of alkaline protease (NASCIMENTO & MARTINS, 2004), this was not the case for the protease in this study. Our results indicate that this protease may find application in the analytical, leather, and detergent industries. However, more work is needed to confirm this.

*Table 1.* Proximate analysis composition of fermented cassava and bambara nut waste

| Parameters | Fermented cassava waste | Bambara nut waste |
|---|---|---|
| | Value (g/100 g) | |
| Protein | 1.31 | 12.79 |
| Moisture | 13.17 | 7.93 |
| Ash | 3.15 | 2.76 |
| Fibre | 3.62 | 3.37 |
| Fats | 2.11 | 4.14 |
| Carbohydrate | 76.64 | 69.01 |

### 3. Conclusion

*K. varians* was able to grow and produce high quantities of protease on cheap and easily available renewable resources. The ability to utilize wastes for the generation of valuable metabolites will minimize production cost and environment issues that arise from improper waste disposal and eutrophication. From the above results, protease produced from *K. varians*

possesses unique qualities, which may be invaluable in processes where alkalophilic thermostable proteolytic enzymes are needed. However, further studies are required to fully determine the properties of the purified enzyme.

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