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## Purification and Properties of *Wickerhanomyces Anomalous* Keratinase and Its Prospective Application in Poultry Feed Industries.

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### Abstract

The pollution resulting from growing livestock wastes are posing significant threat to human health and the environment. Animal wastes emanating from cow horns, hooves and feathers contain keratin which can only be degraded by keratinolytic microorganisms. This study was carried out using cow hooves as substrate for the production of keratinase from fungus identified as *Wickerhomyces anomalous* 9 (18S rDNA gene sequencing) isolated from soil rich hooves using basal salt agar medium and potato dextrose agar. Keratinase activity of the isolate was assessed using skim milk agar and the enzyme was produced by solid state fermentation. The crude enzyme was purified using ammonium sulphate precipitation, ion-exchange and gel-filtration chromatography. The specific activity of the enzyme was 0.29 U/mg with a yield of 45% and a 7.25 purification fold. The optimal pH and temperature of the enzyme were 8.0 and 60°C respectively. The enzyme was observed to be thermo-stable at 50°C for 30 minutes. The kinetics revealed that the  $V_{max}$  was 0.384U/min with  $K_m$  86.95mg/ml. The native molecular weight of the enzyme was found to be 34KDa. There was significance differences at 95% confidence with poultry feed treated with *Wickerhanomyces anomalous* keratinase in moisture, ash content, crude fibre, crude fat, nitrogen content, crude protein and carbohydrate compare to the untreated feed. This results suggest an environment-friendly approach for biodegradation of cow hooves wastes for production of keratinases, animal waste management as well as a promising tool for chicken feed additives.

**Keywords:** Keratinase, *Wickerhomyces anomalous*, Chromatography, Purification, Cow hooves

### Introduction

Abattoir wastes often contain solid waste biomass that comprises of bones, hooves horns, undigested ingest, and occasional aborted fetus, while the liquid waste includes blood, urine, water, dissolved solids, and gut contents. Most of this solid waste biomass that is being dumped in the slaughterhouses contains a high level of keratin. Keratins are insoluble fibrous proteins found in

hair, wool, feather, nail, horns, and other epithelial covering, with rich beta helical coils that are linked through cysteine bridges (Selvan & Vishnuprya, 2012; Tanabe, Okitsu, & Yamauchi, 2004; Reichi, Borelli, & Geerling, 2011). Animal wastes are on the upward trajectory and, would continue to

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increase as the world's burgeoning population needs sustenance. These wastes have led to the accumulation of byproducts either as waste or secondary products with recyclable value (Baweja, Tiwari, Singh, Nain & Shukla, 2016). A very large amount of animal-derived keratinaceous wastes such as feathers, skin, hair, bristles, horns, hooves, claws, nails, beaks, reptilian osteoderm, and fish teeth and slime are generated annually (Huang, Łęzyk, Herbst, Busk & Lange, 2020). The stock of cattle worldwide is the largest among livestock and meat products; they have been the fastest component of the global agriculture and food industry. Of the world stock, India (30.70%) has the most extensive cattle inventory in the world in 2018, followed by Brazil (24.72%) and United States (9.56%) (USDA, 2020). These keratins are biologically insoluble, fibrous, recalcitrant and biochemically rigid molecules which are resistant to degradation by most common proteolytic enzymes (Barman, Zohora, Das, Mowla, Banu, Salimullah & Hashem, 2017)

Keratin is an insoluble protein which is resistant to degradation by peptidases, such as trypsin, pepsin and papain (Zaghloal, Embaby & Elmahdy, 2011). The protein chains are packed tightly either in  $\alpha$ -helix ( $\alpha$ -Keratin) or in  $\beta$ -sheet ( $\beta$ -Keratins) structures which fold into final 3-dimensional form (Kim, 2007). Keratin is a sulfur-containing, insoluble, fibrous protein and the main constituent of skin, hair, nails, hooves, horns, scales, claws, and teeth. It is synthesized by keratinocytes and is resistant to degradation by general proteases (Wang, Yang, McKittrick & Meyer, 2015). Keratins are classified as  $\alpha$ -keratin and  $\beta$ -keratin, based on the major secondary structural elements of polypeptides,  $\alpha$ -helices or pleated  $\beta$ -sheets, respectively (Fontoura, Daroit, Correa, Meira, Mosquera & Brandelli, 2014). The conformational orientations of the cysteine residues and interactions of hydrophobic groups confer mechanical stability to the polymer against biotic and abiotic factors; thus, a major contributor for the recalcitrance of keratin to decomposition (Ma, Qiao, Hou & Yang, 2016). In food and feed supplements, keratinase-treated feather is increasingly seen as a viable source of dietary protein, as the enzyme-treated final product

preserved good nutritional value. Keratinases are expected to develop a significant total global demand comparable to other commercial proteases. Diverse class of keratinase have been isolated from various microbial populations, such as bacteria (Fang, Zhang, Liu & Jiang, 2014; Pereira, Lopes, Petry, da Costa Medina, & Brandelli, 2014) actinomycetes and fungi (Gafar, Khayat, Ahmad, Yasid & Shukor, 2020).

Microbial keratinases (EC 3.4.21/24/99.11), which is a member of proteolytic enzymes, is a group predominantly associated with keratin hydrolysis. Increasing attention is focused on keratinolytic microorganisms and microbial keratinases due to their potential application in the bioconversion of keratin-rich wastes generated from meat (particularly poultry) and leather industries, through the development of ecologically safe and economically feasible processes. Additionally, keratinases are postulated for utilization in food, feed, detergent, leather, and biomedical industries (Bach, Sant'Anna, Daroit, Corrêa, Segalin & Brandellia, 2012; Nnolima, Okoha & Nwodoa, 2020). The present study reports the biodegradation of cow hooves by isolated *Wickerhomyces anomalus strain 9* (characterized by 18S rDNA gene sequencing) for keratinase production as well as the biotechnological potential of the keratinolytic enzyme as chicken feed supplements.

## Materials and Methods

### Reagents and Apparatus

All other reagents were of high analytical grade and used without further purification. The cow hooves used were obtained locally, ground to powder using mechanical grinder, sieved and dissolved in the assay buffer. The hooves were collected from South-Western part of Nigeria. Apparatus used include, mortar and pestle, weighing balance (Mettler PN1210), pH meter (PHS-25), Centrifuge (Helmreasinn 80-2) and UV/VIS Spectrophotometer (Bosch 752N).

### Isolation and Molecular Identification of Organism

One gram (1.0 g) of the scrapes samples of soil-rich hooves was obtained and suspended into 9.0ml of distilled water. The samples were serially diluted up to  $10^8$  cfu/ml and 0.2 ml of each sample was

plated unto compounded basal salt medium (g/L: NaNO<sub>3</sub>, 2; NaCl, 2; K<sub>2</sub>HPO<sub>4</sub>, 2; MgSO<sub>4</sub>, 0.5; FeSO<sub>4</sub>, 0.01; CaCO<sub>3</sub>, 0.1; agar-agar, 2; feather, horn or hooves powder, 2) by spread plate technique (Lateef et al., 2014). The plates were incubated for three to five days at 37°C. Distinct colonies were selected and sub-cultured on potato dextrose agar plates and incubated for growth at 37°C for 5 days for fungi. The pure cultures were obtained and stored on agar slants at 4°C until needed. Milk agar medium was used for the screening of keratinolytic organisms (Riffel & Brandelli, 2006). A clear zone formation on the milk agar plates after 5 days was observed. Only keratinolytic organisms showing clear zone formation around their growth on the milk agar medium were picked for identification. Genomic fungal DNA obtained was used for PCR reaction. ITS (ITS 1 and 4) primers were used to amplify the conserved regions in fungi. After PCR amplification, 10.0 µL products were resolved by 1.5% agarose gel electrophoresis. The amplicons were sent for sequencing on commercial basis. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura & Nei, 1993). Evolutionary analyses were conducted using Mega7 software as described by Kumar et al. 2016.

### Enzyme Extraction

Hooves were washed extensively with tap water; after that, they were oven-dried; and chopped into pieces and milled into powdery size and kept at room temperature (30 ± 2°C) (Lateef et al., 2014). The hooves powder was then used for solid-state fermentation. 2.0 g hooves powder were mixed with 2.0 gram of agar-agar in 250 ml Erlenmeyer flasks. After sterilization, fermentation media were inoculated with 10ml of the inoculum (spore suspension) and incubated at 37 °C for 5 to 9 days. The culture basic salt media were harvested on the fifth, seventh and ninth day of incubation. This was to enable maximal enzyme activity. The cultures were filtered using Whatman 40 and Millipore 0.2µm. to obtain the crude enzyme.

### Keratinase Assay and Protein Concentration Determination

The enzyme activity of the crude extract was measured as described by Thys, Lucas, Riffel, Heeb, & Brandell, 2004. The assay mixture contained 500 µL enzyme solution and 750 µL 0.1

M phosphate buffer pH 7.0 (containing 50 mg/mL of azocasein). The mixture was incubated at 30 °C for 10 min, followed by addition of 400 µL of 10% (w/v) trichloroacetic acid (TCA) and kept standing at 10 °C for 10 minutes. The final mixture was centrifuge at 4,000 rpm for 15 mins, and 800 µL of the supernatant was added to 200 µL of 1.8 M NaOH. The absorbance was read at 420 nm.

Bradford method (1976) was used to measure the protein concentration of the enzyme using bovine serum albumin (BSA) as standard, where the protein absorbance was interpolated from a standard protein curve. The reaction mixture consists of 100 µl of the enzyme solution and 1.0 ml of Bradford reagent (Bradford, 1979). The absorbance was read at 460nm and 595nm. The standard protein solutions at concentration ranging from 100 to 1000 µg/mL were prepared in triplicate. Each standard contains 100µL of standard albumin and 1ml of Bradford reagent solution which were allowed to stand at room temperature for 10 minutes. The corresponding blank was prepared by replacing the 100µL standard with 100µL deionized water. The absorbance be read at 460nm and 595nm, and the average absorbance was recorded, and the standard curve also generated.

All the assay were carried out in triplicate and the results were presented as mean value with standard error of mean (SEM).

### Purification of Enzyme

The crude extract was purified using 80% ammonium sulphate salt. To the supernatant obtained from the crude extract after centrifugation was added 56 g/100 ml of solid ammonium sulphate with occasional stirring for one hour and the mixture was left overnight in the refrigerator. This was followed by centrifugation at 10,000 rpm for 10 min. the supernatant was discarded and the resulting pellet dissolved in minimal amount of 0.5 mM phosphate buffer (pH 7.8). The dissolved sample was dialyzed against 0.5 mM phosphate buffer (pH 7.8) for 8 hours. The ammonium sulphate precipitate was dialysed against several changes of 0.2 M phosphate buffer, pH 7.5, for 18 h. The dialysate was centrifuged at 4000 rpm for 30 minutes to remove insoluble materials, and the

supernatant was assayed for keratinolytic activity and protein concentration of the dialysate was also determined. CM Sephadex cation exchanger resin was used. A column (1.5 x 10 cm) of Sephadex C-50 was packed and equilibrated with 0.2 M phosphate buffer, pH 7.5. The dialyzed protein from the preceding step was then layered on the column. The column was first washed with 0.2 M phosphate buffer, pH 7.5 to remove unbound proteins, followed by elution with 0.1 M NaCl in 0.2 M phosphate buffer, pH 7.5. Fractions of 2.0 ml were collected from the column that was maintained at a flow rate of 30 ml per hour. Keratinase activity and protein concentration of the fractions were monitored. The active fractions were pooled and used for further experiment. The fraction from the above step was used for gel filtration chromatography on Sephadex G-100 column that have been equilibrated with 0.2 M phosphate buffer (pH 7.8). Elution was conducted at a flow rate of 15ml/h, and 3ml fractions were collected. The significant keratinase peaks were detected, and the fractions containing these peaks were pooled.

#### **Determination of native and of subunit molecular weight**

The native molecular weight of the enzyme was determined using Sephadex G-100 column (2.5 x 90 cm). The standard proteins were bovine serum albumin ( $M_r$  66000; 5 mg/ml), ovalbumin ( $M_r$  45000; 5 mg/ml), trypsinogen ( $M_r$  29,000; 5 mg/ml), creatinine phosphokinase (88,000; 5mg/ml) and  $\gamma$ -globulin, (150,000; 5 mg/ml). The subunit molecular weight of the purified keratinase was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Weber and Osborn (1975) in a 10% gel, using the phosphate buffer system (pH 7.2). The standard proteins that were used for the calibration of the gel are bovine serum albumin (66,000 Da), ovalbumin (45,000 Da), Pepsin (35,000 Da), trypsin (24,000 Da) and lysozyme (14,000 Da), which were denatured by mixing with SDS and heating for 3 minutes. Then stacking was achieved and the voltage was increased to 100 volts to allow separation of the proteins in the resolving gel

#### **Determination of kinetic parameters**

The kinetic parameters ( $K_m$  and  $V_{max}$ ) of the enzyme were determined by varying concentrations of protein and measuring the initial reaction velocities ( $\mu\text{mol}$  of casein/min) at 40°C for 1 hr. Casein (10 mg) was prepared by weighing and dissolving 1 g of casein in 10 ml 50 mM Tris buffer pH 8. The reaction mixture contained varied concentration between 0.1 ml and 1.0 ml of protein solution, and proteolytic activity was determined. Plots of the reciprocal of initial reaction velocity (1/V) versus reciprocal of the varied substrates 1/(S) was made according to Lineweaver & Burk (1934).

#### **Determination of Optimum Temperature and pH**

The optimal temperature for the purified enzyme activity was investigated by assaying the enzyme at different temperature from 30-100 °C in 50 m M Tris-HCl buffer (pH 8.0). To determine the optimal pH, keratinolytic activity was assayed using varying buffers of different pH: 50 mM of citrate (pH 3-5); phosphate (6 - 8) and borate (pH 9 - 11) (Thys, et al., 2004)

#### **Effects of Salts and Other Compounds**

The effect of various salts on the activity of keratinase was examined at 1 and 5 mM final concentration in a typical keratinase assay. The cations used were sodium chloride (NaCl), potassium chloride (KCl), Zinc chloride ( $\text{ZnCl}_2$ ) and the other compounds includes Ethylenediamine tetracetic acid (EDTA), 2-mecaptoethanol and urea.

#### **Substrate Specificity**

The substrate specificity of the enzyme was determined by using various soluble protein. Casein, Ovalbumin and Bovine serum albumin in a typical keratinase assay mixture and the activity expressed as percentage residual activity of the enzyme.

#### **Proximate Analysis of Chicken Feed treated with purified keratinase**

Proximate analysis of chicken feed was carried out after treating with purified enzyme. 5.0 g of the chicken feed was added to 1.0ml of the purified

fungal keratinase. The mixture was allowed to stay overnight (for about 24hrs) in the refrigerator. The procedure of the Association of Official Analytical Chemistry (A.O.A.C., 2006) was used in the determination.

### Statistical analysis

All experiments were performed in triplicate and the results were presented as mean value with standard error of mean (SEM) while the proximate analysis data were further subjected to student t-test (2-tailed) with level of significance at 95% confidence (P-Value).

## Results

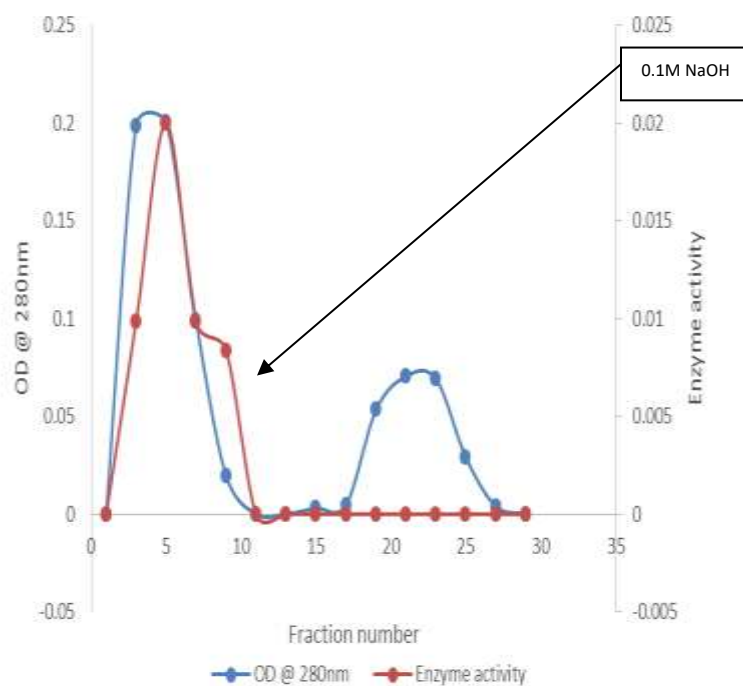
### Isolation and Molecular Characterization of Keratinolytic Fungi by 18s Rdna Gene Sequencing

In the present study, keratinolytic fungal was isolated from scrapes of soil-rich hooves samples. After incubating for 5-7 days at 35 °C, fungal growth was observed on 5<sup>th</sup> day. The proteolytic activity of the fungus isolate was confirmed by using skim milk agar. A clear zone was formed around the colony on the milk agar media after 5 days of incubation which indicated the proteolytic nature of the isolate. Molecular characterization of the fungal isolate was carried out by 18S rDNA gene sequencing and identified on the basis of phylogenetic analysis. Based on the phylogenetic analysis with the closest homolog sequences the isolate belongs to *Wickerhomyces anomalus* species strain 9. The identified isolate was designated as *Wickerhomyces anomalus* species strain 9. The nucleotide sequence of the isolate was deposited in GenBank database with the accession number MT431381.1.

### Production and preparation of keratinase

Hooves powder was used for solid-state fermentation. 2.0 g hooves powder was mixed with 2.0 g of agar-agar in 100 ml Erlenmeyer flasks. After sterilization, fermentation media were inoculated with 10ml of the inoculum and incubated at 37 °C for 5 to 9 days. The culture basic salt media were harvested on the fifth, seventh and

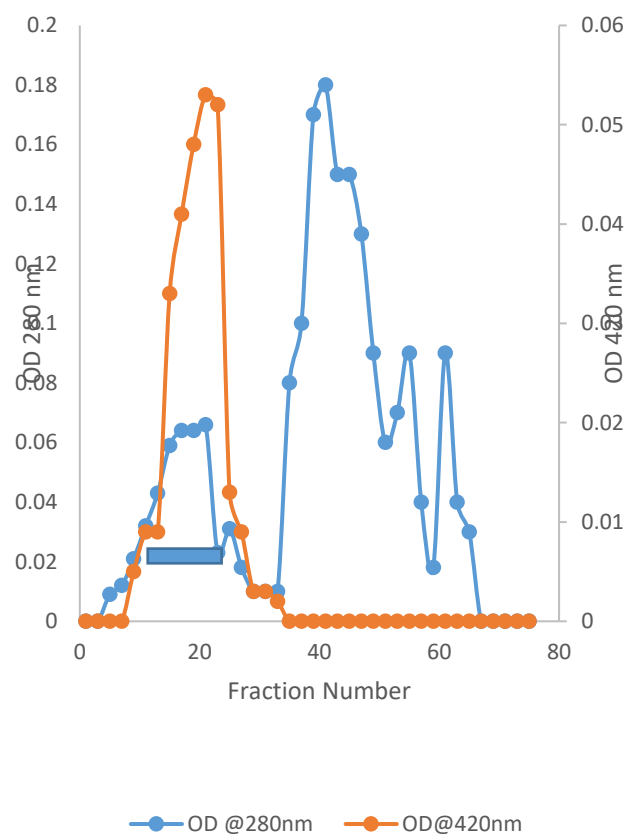
ninth day of incubation. This was to enable maximal enzyme activity. The culture was filtered using Whatman 40 and Millipore 0.2µm. The filtrate was a crude enzyme extract which was used for further studies (Couri et al., 2000). *Wickerhomyces anomalus* species strain 9 was harvested after extracellular protein concentration peaked (7<sup>th</sup> day of incubation) The keratinase was purified to homogeneity following ammonium sulphate precipitation, ion exchange and affinity chromatography as determined by SDS- PAGE. The summary of the purification procedure for keratinase from soil-rich hooves sample is shown in Table 1. The elution profile of the enzyme using ion-exchange chromatography on CM-Sephadex C-25 is shown in Figure 1 while the elution profile of the enzyme using affinity chromatography is shown in Figure 2. The native molecular weight of the keratinase was estimated at 34 KDa, while the subunit molecular weight was 31 kDa (Figure.3).



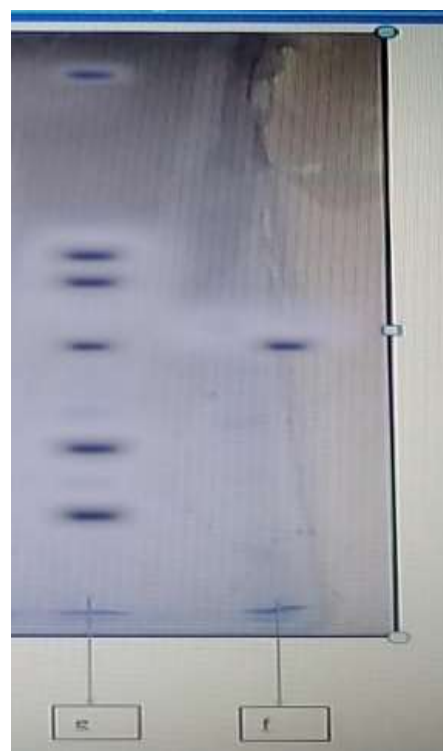
**Figure 1:** CM Sephadex ion-exchange chromatography elution profile of the *Wickerhomyces anomalus* keratinase obtained from cow hooves. Fractions of 2ml were collected from the column Protein profile OD was measured at 595 nm. Activity profile OD was measured at 540 nm.

**Table 1. Purification of *Wickerhanomyces anomalus* 9 keratinase from cow hooves**

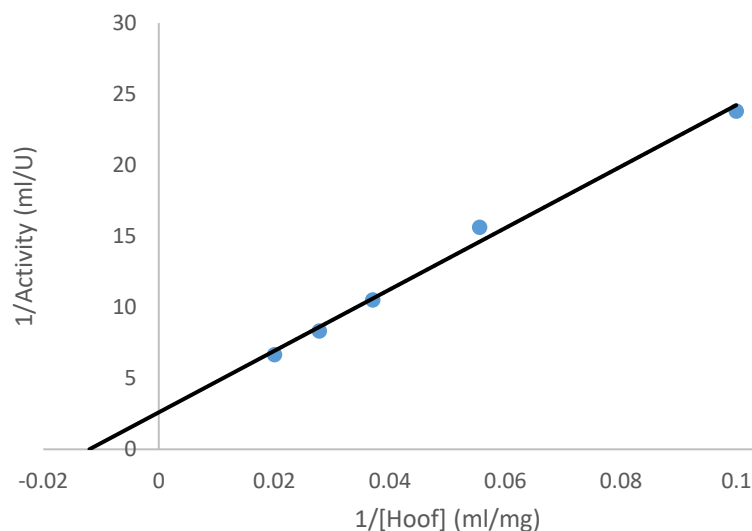
Purification Step	Total	Total	Specific Activity	%
	Activity (U)	Protein (mg)	(U/mg)	Yield
Crude Extract	7.25	150.23	0.04	100
80 % Ammonium Sulphate	6.05	51.07	0.11	83
CM- Sephadex C-50	5.09	20.70	0.24	70
Sephadex G 100 Gel Filtration	3.24	11.17	0.29	45



**Figure 2: Sephadex G-100 gel filtration chromatography elution profile of the *Wickerhanomyces anomalus* Keratinase from Horn. Fractions of 2ml were collected from the column. Activity profile (OD 540 nm) Protein profile (OD 595 nm). Pooled fractions of the enzyme for further analysis**



**Figure 3: SDS-PAGE of purified keratinase. Gel e contains the standard protein markers:bovine serum albumin ( 66000 Da), ovalbumin (45000 Da), trypsinogen (29,000 Da), creatinine phosphokinase (88,000 Da) and  $\gamma$ -globulin, (150,000 Da). While gel f is the keratinase enzyme**

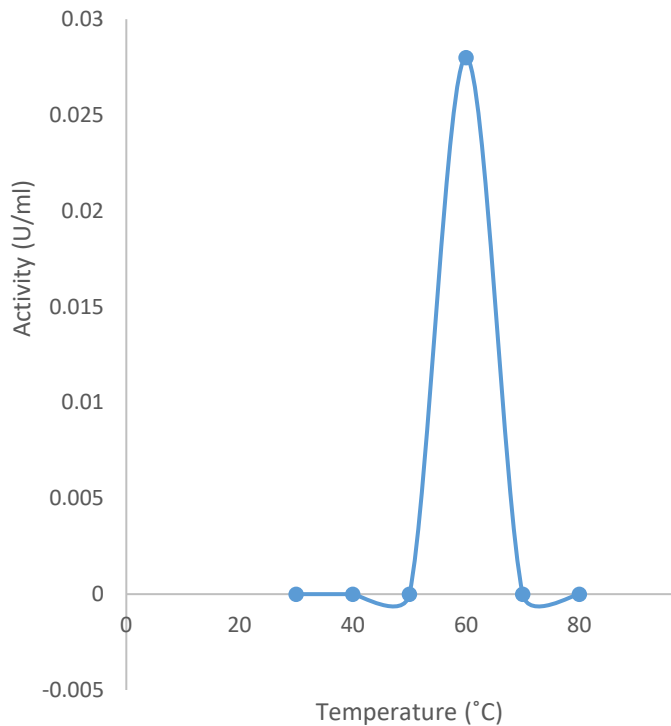


**Figure 4: Lineweaver-Burk plot for the determination of kinetic parameters. The concentration of keratinase used varied between 0.1-1.0mg/ml**

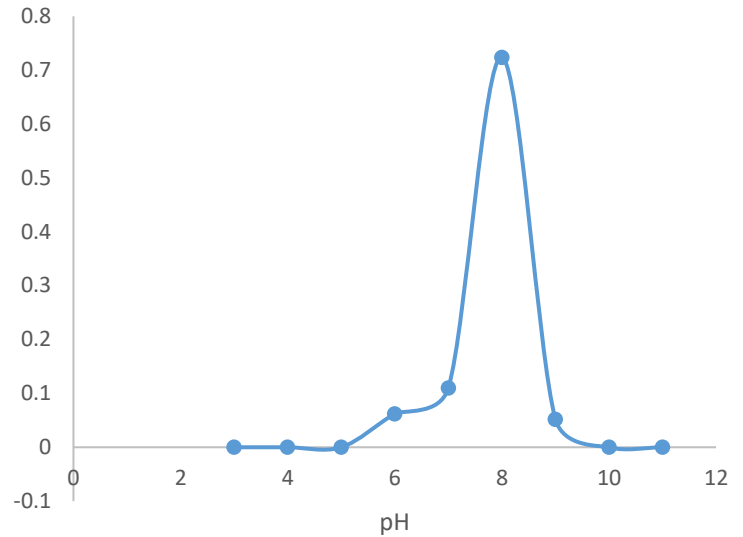
**Enzyme characterization**

The optimum temperature and the pH for the keratinolytic activity was observed at 60°C (Figure 5 ) and 8 (Figure 6 ) respectively. The pH value shows an alkaline enzyme. The effect of various metals (Chloride salts) show that the metals affected the enzyme in a rather inhibitory manner especially NaCl at different concentrations (Figure 7). The keratinase activity was inhibited by β-mercaptoethano, ethylenediaminetetraacetate (EDTA) and Urea at different concentrations. 1.0 mM β-mercaptoethanol did not inhibit the enzyme activity while 10.0 mM β-mercaptoethanol inhibited enzyme activity. Similar result was observed by EDTA, while Urea at both concentrations of 1.0 and 10.0 mM inhibited keratinase activity (Figure 8).

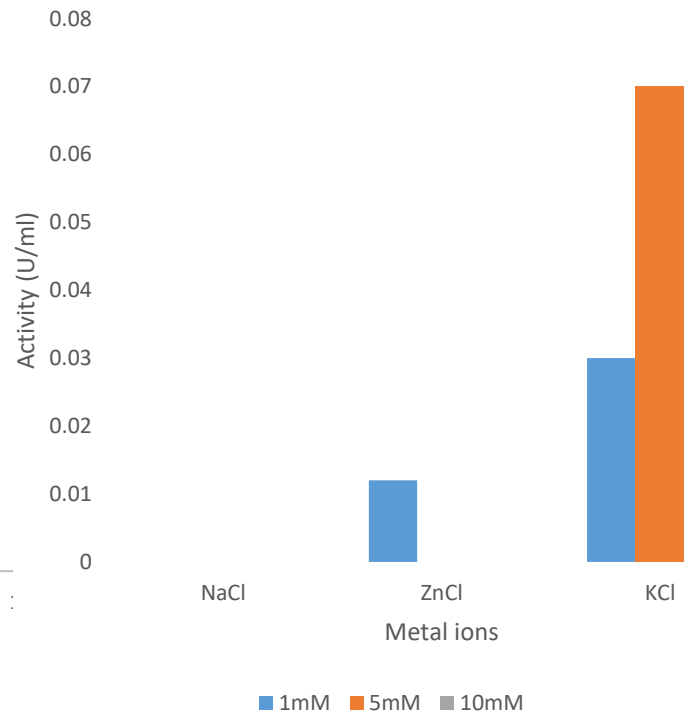
The substrate specificity of the purified enzyme showed highest activity against casein even though this activity was considerably low. The enzyme did not show any enzymatic reaction towards ovalbumin and BSA.



**Figure 5: Effect of temperature on *Wickerhamomyces anomalus* keratinase from cow hooves substrate.**



**Figure 6: Effect of pH on the activity of keratinase produced by *Wickerhamomyces anomalus* from hooves substrate**



**Figure 7: Effects of salts on the activity of *Wickerhamomyces anomalus* keratinase from hooves substrate**

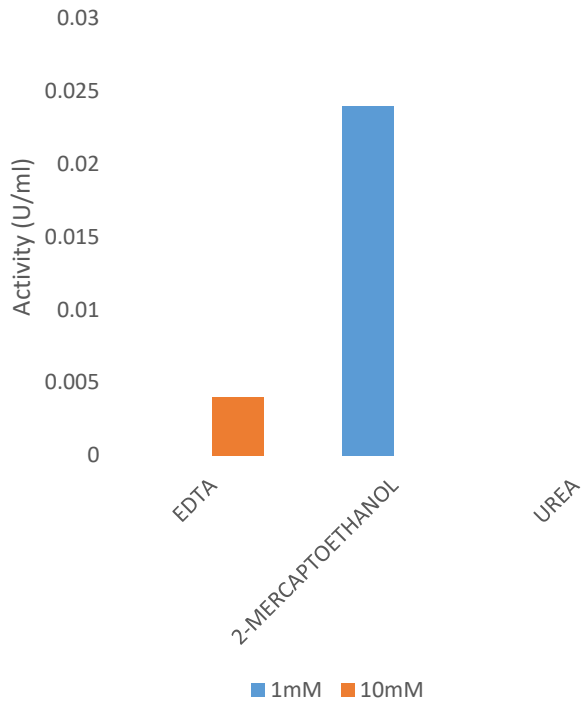
### Biotechnological potential of *Wickerhamomyces anomalus* 9 keratinases as chicken feed supplement

The biotechnological potential of the keratinase obtained from this study on proximate analysis of chicken feed treated with *Wickerhamomyces anomalus* 9 keratinases showed an increase in the amount of percentage total protein in the feed and also the quality of the feed in totality as shown in Table 2. The % ash content was 30.79 while for crude protein was higher (13.38%) to the control. This may be due to the activity of treatment of the chicken feed with enzyme which synthesize amino acid. The percentage nitrogen content is high in the treated feed with fungi keratinase (2.14%) as shown in Table 2.

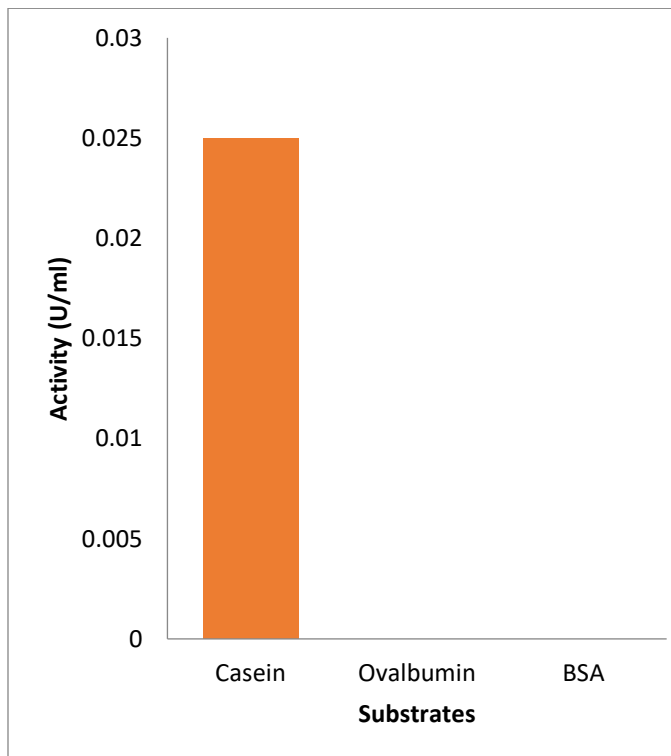
### Discussion

The purification of keratinase from the *W. anomalus* 9 was achieved using a procedure that included 80% ammonium sulphate precipitation, ion exchange chromatography on CM-Sephadex C-50 and gel filtration using Sephadex G-100 (Table 1). The procedures were similar to those used by some researchers for isolating the keratinase enzyme from other microbial sources. Gladisar et al. (2005) used a procedure that involve lyophilization ammonium sulfate and hydrophobic interaction chromatography for fungal keratinolytic proteases. while Ali et al. (2011) used ammonium sulphate (50 and 70%) and acetone precipitation and DEAE Sephadex A-25 column chromatography to purify serine keratinase from *Aspergillus oryzae*. The procedures used in this study yielded purified keratinase with 7.5 fold increase in specific activity of the enzyme with percentage recovery of 45. A feather degrading fungus, *Aspergillus flavus* strain K-03 was purified using 80% ammonium sulfate saturation, gel filtration in Sephadex G-100 and ion exchange chromatography on DEAE-Sephadex A-50, yielded 32.36% with 11.53-fold (Kim, 2007).

The kinetic parameters of keratinase from *Wickerhamomyces anomalus* 9 had a  $K_m$  value of 86.95 mg/ml and a  $V_{max}$  of 0.38 U/min . Other similar results have been reported with other keratinases, Silveira et al. (2008) reported a  $K_m$



**Figure 8: Effect of some inhibitors on the activity of *Wickerhamomyces anomalus* keratinase**



**Figure 9: Substrate specificity of the purified keratinase substrates activity on Caesin, Ovalbumin and Bovine Serum Albumin**

**Table 2: Proximate Analysis Results for Chicken Feed Treated with *Wickerhamomyces anomalus* Keratinases**

samples	%Moisture Content	%Ash Content	%Crude Fibre	%Crude Fat	% Nitrogen Content	%Crude Protein	% Carbohydrate
Control	0.93	34.41	33.19	5.7	1.52	9.51	15.27
Wickerhamomyces anomalus Keratinase Chicken Feed	0.91	30.79	32.65	5.11	2.14	13.38	15.02

value of 0.75 mg/ml and a  $V_{max}$  of 59.5 U/min working with keratinase from *Chryseobacterium* sp. Strain kr6. Pawar et al., (2018) reported the kinetic parameter value of 0.61 mg/ml( $K_m$ ) for a thermostable keratinase from *Bacillus altitudinis* RBDV1. The optimum pH of *Wickerhamomyces anomalus* keratinase was found to be 8.0. Similar result was reported for keratinases of *Arthrobacter* sp., *Fusarium solani*, *Cladosporium cladosporioides* and *Trichoderma viride* (Sapna, Ashish & Kahita., 2014; Nwadiaro, Ogbonna, Wuyep & Adekojo, 2015). The optimal temperature for keratinase activity from *Wickerhamomyces anomalus* was found to be 60°C. Similar results have been reported for keratinases from other sources. Godbole et al., (1999) reported an optimum temperature of 50°C from fungal strain. Anto et al. (2006) obtained an optimum temperature of 55°C for keratinase enzyme in *Aspergillus* sp HA-2strains and it is greater than the reported by Sapna et al., (2014) that have an optimum temperature of 24-32°C from *Fusarium solani*, *Aspergillus niger*, *Penicillium chrysogenum*, *Trichoderma harzianum* respectively but it is lower than the one reported by Adefisoye & Sakariyau (2018) that have an optimum temperature of 60°C from *Aspergillus niger*.

In this study, the keratinase enzyme was slightly inhibited by EDTA,  $Na^+$  and  $Zn^{2+}$ . This result was similar to the work reported by Ali et al., (2011), while Cai, et al., (2011) found that ethylene diamine tetraacetic acid (EDTA) had positive effect on the keratinase activity. The lack of an inhibitory effect of metalloproteinase inhibitor (EDTA) on Keratinolytic activity might have been caused by a competition between the excess of metals present at non-active sites (Han et al., 2012). Vigneshwaran et

al., (2010) reported that, keratinolytic activity of *Bacillus licheniformis* was completely inhibited by EDTA and the metal ions  $Zn^{2+}$  and  $Mg^{2+}$  enhance the keratinolytic activity, but  $Cu^{2+}$  and  $Hg^{2+}$  inhibited the enzyme. According to substrate specificity, the keratinase from *W. anomalus* shows activity on the soluble proteins (casein, bovine serum albumen and ovalbumin as soluble proteins as well as insoluble substrates such as feather meal, feather keratin, horn keratin, hoove keratin. Many keratinases are capable of hydrolyzing a broad range of soluble and insoluble proteins. Reports of keratinase from *Chryseobacterium* sp. Strain kr6 showed that the enzyme hydrolyzed chicken nails (Silveira et al., 2010). The keratinase from *W. anomalus* has a broad range of substrates specificity and could degrade substrates containing both  $\alpha$  and  $\beta$  keratins.

In this study, the gel filtration on sephadex G-100 for *W. anomalus* resulted in a molecular weight 34 kDa. The subunit molecular weights estimate of the fungi keratinases were found to be 31kDa. This is suggestive that the enzymes from this fungal keratinases are monomeric enzymes. Other researchers have reported similar monomeric keratinase enzymes working with different fungi. Kim (2007) worked with *A. flavus* strain K-03 used Sodium dodecyl sulfate polyacrylamide gel electrophoresis and zymograms with purified keratinase from *A. flavus* strain K-03 and obtained a monomeric enzyme with 31kDa molecular weight. In general, a survey showed that the molecular weight of keratinases falls between 16 kDa to 440 kDa.

Further study on the biotechnological potential of the keratinase obtained from this study on proximate analysis of chicken feed treated with *Wickerhamomyces anomalus* 9 keratinase showed

an increase in the amount of percentage total protein in the feed and also increases the quality of the feed as showed in Table 2. The % ash content for *Wickerhanomyces anomalus* keratinase was 30.79 while the crude protein was 13.38%. This may be due to the activity of treatment of the chicken feed with enzyme which synthesizes amino acid. Therefore, the nutritional components of chicken feeds fortified with *Wickerhanomyces anomalus* keratinase are boosted. The report of Wang et al., (2011) on dietary keratinase supplementation showed improve immune response, weight gain, nutrient digestibility, intestinal morphology and ecology in growing and nursery pigs. This study also shows that the use of keratinase is advantageous as it avoids the possibility of exposure of the users to organisms that could be pathogenic (Isiaka & Agbaje, 2016). Avdiyuk & Varbanets (2019) reported the successful application of the enzyme for bioconversion of keratin wastes to animal feed and nitrogenous fertilizer, as well as in leather, textile, detergent, cosmetic, pharmaceutical industries. Keratinases have been usefully applied in agricultural, pharmaceutical, leather and textile processes as well as within environmentally friendly waste management solutions (Purchase, 2016). Furthermore, microbial keratinases from fungi and bacteria have been reported to be useful in different industries such as agriculture (use for making organic fertilizers); feed industry (a good source of feed protein); and cosmetics and medicine industry (Sahni et al., 2015). This finding is also similar to previous research stating that tilapia fed with 20% fish meal substitution had significantly higher total protein in their muscle compared to tilapia fed commercial feeds. In addition, *Oreochromis niloticus* fed 20% fish meal substitution had also minimum lipid content (Al-Ghanim et al., 2017). In contrast, another study revealed no significant effects on crude protein and fat of Juvenile tilapia fed poultry instead of fish meal (Yones & Metwalli, 2016).

Diverse groups of microorganisms are able to produce keratinases and more are being discovered every year. In this present findings, the characterization and optimum conditions for production of *W. anomalus* 9 keratinase were

determined which is an essential step for the production of adequate amounts for application in research of feed and also highly effective in the management of keratinous wastes through a non-polluting process.

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