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ISOLATION, CHARACTERIZATION AND OPTIMIZATION OF THERMOSTABLE METALLO-KERATINOLYTIC PROTEASE FROM HUMAN HAIR-DEGRADING *ASPERGILLUS NIGER* STRAIN ND44

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Keywords:

Aspergillus niger strain **(ND44)** Keratin, Keratinase, Co-factors, Optimum pH, Optimum Temperature, 16SrRNA gene amplification, Phylogenetic tree, DNA Sequence

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ABSTRACT: Keratinases of microbial origin are a group of protease enzymes with specific characteristic feature of proteolytic cleavage of keratin resulting in the generation of soluble peptides and free amino acids. They play important roles in conversion of world richest proteinous solid-waste'' keratinaceous waste'' into value-added products. This current research work aimed at isolation, purification, characterization and optimization of keratinase isolate from *Aspergillus niger* Strain (ND44) obtained from human hair macerated in salt medium, for its enzymatic properties in the degradation of trichocytic α-keratin(a fibrous structural protein that resist most proteases degradation based on its stabilized intra-molecular disulphide and hydrogen bonding), while gene sequencing and phylogenetic tree gave expanded details on the structural identity of the isolated *Aspergillus niger* Strain (ND44). High keratinolytic activity exhibited by this microbial keratinase at high temperature up to 90ºC confirms it as a hyperthermophilic enzyme, with non-optimums pH peaks at 5.5 and 9.00that spread across both acidic and alkaline regions. The optimum pH, temperature and best co-factors for this human hair keratinase activities obtained via quantification of liberated soluble peptides/proteins using Lowry method; and liberated free amino acids from keratin using Ninhydrin methods were found to be at pH {(12.5) and (12.5)}, temperature {(90 °C) and (60 °C)} and co-factors {($Ni^{2+}$and Co^{2+}) and ($Cu^{2+}$and Ni^{2+})} respectively. The optima percentage degree of human hair degradation and incubation period were found to be 60% in 14 days. The molecular size of Keratinase produced by *Aspergillus niger* strain (ND44) was about the size of 600bp (0.6Kb).

INTRODUCTION: Quantitatively, after absorption excess amino acids are channelled towards the generation of ammonium (NH_4^+) via deamination process followed by generation of (ATP, H_2O and CO_2) viaglucogenic/glycogenic and ketogenic amino acids catabolic pathways in a well fed state.

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However, this deamination process significantly increases during fasting and starvation [9, 21, 38]. It may look strange to say that another major excretory pathway for amino acids from animal is *via* trichocytic keratin, an intermediate filament and the most abundant macromolecular component of hairs, furs, feathers, nails, peaks and horns [55]. Trichocytic α-keratin, the most abundant macromolecular component of human hair, is a cytoskeletal coiled-coil structural protein [27]. Keratin-associated protein, which infiltrate the space between the keratin intermediate filaments, plays a role in the organization of the keratin

intermediate filaments, (IF) into macrofibrils [25]. It also determines the physical qualities of a fully-keratinised hair fibre which includes; its pliability, toughness, hardness, cross linking of one filament to another, durability, resistant to proteolytic enzymatic attacks, thermal stability and insoluble in both polar and non-polar solvents [10]. These physical qualities are biochemically possible because of hydrophobic interactions of coiled-coil structure, intra- and intermolecular hydrogen bonding, and a high degree of cross-linking by disulfide bonds. Most research works in literature on microbial degradation of keratin focused more on feathers the world's most abundant source of keratin, with little attention on human hair the second most abundant source of keratin [23].

The number of hair follicles in an average individual's head is approximately between 80,000 and 120,000, but the entire body has approximately 5 million unaltered hair follicles that remains constant all through one's life time, since only the soles of the feet, palms of the hands and red part of lips lack hair follicles. Hair density, which is the number of hair per square inch, is approximately within the range of 1000-1300(130-250 hairs/cm^2) (28 kg hair weight/person). The diverse nature of human hair with myriad colours and textures has varieties of functions which includes; to protect humans especially the head from adverse environmental factors such as UV radiation from the sun, dust and debris; it also helps to regulate our body temperature *via* promoting evaporation of sweet and serves as insulator protecting the skull from extreme cool; it aids in the sensational detection *via* nerve cells surrounding the hair follicles; it plays an important psychological role in our identity. Pubic hair serves in maintaining optimal genital temperature, friction reduction during sex and disease prevention [44].

Barbing and hairstylist salons are globally increasing rapidly especially in the urban cities in proportion to both urban migration and global population growth. Based on the geometric increase inhuman population in urban cities, shaved human hairs from barbing salons and hairstylist shops constitute one of the crucial metropolitan ''keratinaceous wastes'' aside from feathers, horns, hooves, and nails that pose challenge to waste management in urban cities, especially in third world countries [26]. Bioremediation of keratinaceous wastes via biodegradation of keratin by keratinases are of essences, since huge amount of these wastes accumulate as part of sewage at the bottom of aquatic systems. The current world population of 7.6 billion is expected to reach 8.6, 9.8, 11.2 billion in 2030, 2050 and 2100respectively, according to a new United Nations report being launched recently (World Population Prospects; 2017). This information is an essential guide to policies aimed at achieving sustainable development. Keratinophilic fungi produce sulphide for sulphitolysis. However, during this process, the disulfide bond of cystine, which functions as a site for redox reactions and mechanical linkage is broken down, creating room for proteolytic enzymes released by the fungi to cleave the peptide bonds of the keratin [40].

In this degradation process, the products released are cysteine, sulphocysteine, cysteine acid, cystine, and inorganic sulfate, and the detection of these products in the culture media indicate the presence of true keratinophilic fungi (Kornikłowicz-Kowalska and Bohacz, 2011). Fungi that do not show this behaviour during degradation are considered non-keratinophilic fungi. Keratinophilic fungi are predominantly anthropophilic (human loving) or zoophilic (animal loving) (Spiewak and Szostak 2000). Moreover, many geophilic keratinophilic fungi have been isolated from contaminated soil samples due to accumulation of keratin wastes in the soils.

Proteases (proteolytic enzymes or proteinases) are one of the most valuable industrial enzymes, produced commercially and used in bioremediation, effluent treatments, food industry, pharmaceuticals, protein recovery or solubilisation, and leather and dairy industries (Anwar and Saleemuddin, 1998; Abdelwahed *et al*., 2014). Today many top brands in the detergent industries use proteases as a crucial intermediate/safe alternative to substitute dangerous chemicals like caustic soda (Nascimento and Martins, 2006; Gupta *et al*., 2002). *Tinea capitis*, ananthropophilic keratinophilic fungal infection, considered to be a form of superficial mycosis or dermatophytosis, a good example of dermatophytes that affects children scalp, hair follicles and often hair shafts, but grows well in warm moist areas like dead

tissues of the hair, nails and outer skin layers (Weitzman and Summer bell, 1995). People who are infected with *Tinea capitis* may experience a localized area of scaling, bald patches on the head, itching, pus filled bumps and hair loss because the secreted keratinases catalyse the degradation of keratin the only structural protein in hair shaft leading to hair loss. This provide another source of keratinase enzyme for further investigation. However, in November, 2023, as part of our ongoing experimental research work at Brain Phosphorylationship Scientific Solution Services, 5th Floor, Right Wing, Number 9 Ogui Road, Enugu, Enugu State, Nigeria, on bioremediation and biodegradation of human hair keratin, we discovered that a fungus co-habited human hair macerated in salt medium with non-pathogenic keratinolytic bacteria *Alcaligenes faecolis* Strains AIR10 and TPB18 with NCBI accession numbers of MG835355.1. and MW475277.1 respectively (Ezenwali, 2022). The above observation serves as the bases for this current research work aimed at isolation, purification, characterization and optimization of keratinase isolate from the above fungus (*Aspergillus niger* Strain (ND44)), for its enzymatic properties in the degradation of trichocytic α-keratin, while gene sequencing and phylogenetic tree gave expanded details on the structural identity of the *Aspergillus niger* Strain (ND44).

MATERIALS AND METHODS:

Materials:

Equipment: Ultraviolet-Spectrophotometer, Scanning Electron Microscopy (SEM), Ohaus Precision Weighing Balance, Digital Orbital Shaking incubator, Table Incubator, Digital pH-Meter, Autoclave, Centrifuge, Dialysis bag, Scissor, Colony counter and Microscopy. This scientific research work was conducted with calibrated equipment in the Brain Phosphorylationship Scientific Solution Services, 5th Floor, Right Wing, Number 9 Ogui Road, Enugu, Enugu State, Nigeria.

Reagents: Phosphate buffers, Dipotassium Phosphate K_2HPO_4, Ammonium Chloride (NH_4Cl), Monopotassium Phosphate KH_2PO_4, Yeast extract media, Sodium Carbonate ($NaCO_3$), Magnesium Sulphate ($MgSO_4$), Sodium Chloride (NaCl), Ethanol, Methanol, Chloroform, Nutrient Agar, Bovine serum albumin, Trichloroacetic acid (TCA), Peptone, Sucrose, Na-azide, Potassium chloride (KCl), Ammonium Sulphate $\{(NH_4)_2SO_4\}$, Ninhydrin (2,2-dihydroxyindane-1,3-dione), Hydrochloric Acid (HCl), Cysteine and FolinCiocalteau phenol reagent for protein asaay. These reagents were of analytical grades.

Hair Sample: The shaved human hairs used in this research were sourced from local barbing salon in Enugu, Nigeria.

Methods:

Keratin Substrate Preparation: Shaved human hair used in this research work were mainly from different male individuals, since100% of customers who visits Toscana barbing salon for shaving were mainly men. The shaved hairs were sourced from a waste binat Toscana barbing salon located at number 14 Moore House Street, Ogui, Enugu North, Enugu State, Nigeria. These samples were first washed with liquid detergent followed by rinsing with distilled water, and then finally dried at room temperature for 48 hours. Using sharp scissor, the sizes of the hairs were reduced to approximate 1-3 mm in length. A specific volume (1000 ml) of fat-soluble solvent {chloroform/methanol (1:1 v/v)} was used to defat 500g of hair sample at room temperature using shaking incubator for 10h (200 rev min^{-1}). These were pulled together after defatting and rewashed with distilled water again and dried at 70ºC for 12 h and used as substrate in our culture media.

Microbial Culture for Growth of Keratinolytic Fungi: A known volume (1000 mL), but well sterilized salt medium containing (K_2HPO_4 300 mg, KH_2PO_4 400 mg, NaCl 500 mg, $MgSO_4$ 100 mg, Yeast extract 100 mg, NH_4Cl 400 mg, in 1L distilled/deionized water with pH 9.00 maintained with phosphate buffer) was allowed to attend normal room temperature of 25°C. A known quantity (10g) of soil contaminated human hair collected from a waste bin at Toscana barbing salon was macerated with the sterilized salt medium in an air tight plastic container and left undisturbed for two months **Fig. 1AB.**

Preparation of Pure Culture Media for Isolation of Keratinolytic Fungi: A known volume (250 mL), but well sterilized (at 121°C for 15 min)

nutrient agar-salt medium containing ($mgdL^{-1}$: K_2HPO_4 180, NaCl 10, $MgSO_4$ $7H_2O$ 20, $FeSO_4$ $7H_2O$ 1.00, NH_4Cl 400, Chloramphenicol and Nutrient Agar, in 1L distilled/deionized water with pH 9 maintained with phosphate buffer) was poured into 5 different petri dishes and were allowed to attend normal room temperature of 25^oC. However, after flaming and cooling of wire loop, white fungal growth patches on the lumps of hair samples from **Fig. 1** were streaked on these petri dishes and were incubated at 37^oC for 72 h, to obtain two different colonies **Fig. 2**. Moreover, the 2 identified colonies were separately transferred into 2 differently labelled-sterilized and cooled250 ml conical flasks containing each, 100 ml of salt medium ($mgdL^{-1}$: $K_2HPO_4$180, NaCl 10, $MgSO_4$ $7H_2O$ 20, $FeSO_4 7H_2O$ 1.0, NH_4Cl40 and Defatted Human hair 1.0) with pH 9 maintained with phosphate buffer. For multiplication of these microbial cells these samples were incubated at 37^oC using shaking incubator for 4 days (200 rev min^{-1}). The two colonies were subjected to confirmatory keratinase activity tests stated here. The isolate-1 that gave positive reactions to the confirmatory activity tests was selected for identification via biochemical, gram staining techniques, and physiological and morphological characterizations.

Confirmatory Keratinase Activity Test:

Quantification of Free Amino Acids Concentration Using Ninhydrin Method: Based on the principle that 2 molecules of ninhydrin (2, 2-dihydroxyindane-1, 3-Dione) react with free amino acid to produce a Ruhemann's purple (Moore and Stein, 1954), one millilitre (1ml) each of the clear supernatants of the sample and the standard were properly mixed using a vortex mixer with 1 mL of ninhydrin reagent (8% w/v of Ninhydrin in acetone) in separate test tubes. Both the sample and the standard were covered with aluminium foils and incubated at 90^oC for 20 min. However, the development of purple or crimson colour confirms the presence of free alpha-amino acids.

Production of Keratinase Enzymes: Different 1000 ml conical flasks containing both sterilized salt medium (500 mL) with pH 9 and homogenized defatted human hair (50g) each were inoculated with isolate-1and incubated using shaking incubator (200 rev min^{-1}) at 37^oC. After 2 days of incubation, crude enzymes were extracted from the culture media via centrifugation at 4,000 rpm for 10 min at 4°C. The supernatants obtained were termed the crude enzyme extracts. These were combined and stored in a freezer for further precipitation and determination of keratinase activity.

Purification of the Crude Enzyme:

Partial Purification of the Crude Keratinase with $(NH_4)_2SO_4$: Different but known percentage saturating concentrations of $(NH_4)_2SO_4$ (20.00, 30.00, 40.00, 50.00, 60.00, 70.00, 80.00, 90.00 and 100.00 %) solutions were prepared by dissolving the following corresponding crystals of $(NH_4)_2SO_4$ salt (0.106, 0.164, 0.226, 0.291, 0.361, 0.436, 0.516, 0.603 and 0.697 g) each in 1 mL of distilled/deionized water with constant stirring using magnetic stirrer until all the crystals dissolved. A known volume (0.5 mL) of the crude keratinase extract was added to each of the test tubes (percentage saturating concentrations of $(NH_4)_2SO_4$) in ice bags to determine the one that gave the highest precipitation. However, after total desolation the solutions were incubated for one hour at room temperature. Separation of the precipitated enzymes was carried out via centrifugation (4000 rpm) for 30 minutes at 4°C. The percentage $(NH_4)_2SO_4$ salt saturation concentration that gave the highest precipitation and maximum keratinase activity was considered best for mass precipitation of the enzyme.

Dialysis: The precipitated enzyme was dialyzed against the buffer (10 mM phosphate buffer, pH 7.5) for 48 h with intermittent replacement of the buffer solution at 4°C, to increase its percentage purity. The dialysis (bag) membrane used has a flat width of 10 mm (0.4 in), a diameter of 6 mm and capacity ~10 mL/ft. Both Lowry's and Ninhydrin methods were used to estimate the soluble proteins, free amino acid and keratinase activities using the dialyzed sample as detailed in this work.

Isoelectric Point Determination: The isoelectric point (pI) the isolated enzyme, which the pH at which its net charge is zero was assayed. A known volume (1 mL) of the dialyzed enzyme solution and 1 mL of different phosphate buffer solutions pH 2.00, 3.00, 4.00, 5.00, 6.00, 7.00, 8.00, 9.00, 10.00, 11.00, 12.50 and 13.00were added/mixed

separately in 12 clean, but dried test tubes. These were properly mixed with a vortex mixer and the varied degrees of turbidity at 0.00, 10.00, and 30.00 minutes were measured and recorded. The pH of the test tube with the highest turbidity deposits was considered the isoelectric point of the enzyme.

Genotypic/Molecular Characterization:

DNA Extraction: For DNA extraction Zr fungal DNA miniprep a product of Zymo Research was used in this analysis. Both bacterial cells broth (2mL) and 750µL of Lysis solution were added to a ZR Bashing TM Lysis Tube. This was secured in a bead fitted with a 2 mL tube holder assembly, processed at maximum speed for 5 minutes and further centrifuged in a micro-centrifuge at 10,000g for 1 minute. The supernatant (400 µL) from above was transferred to a Zymo-SpinTM IV Spin Filter (orange top) in a collection tube and spin at 7,000g for 1 minute. The resultant filtrate was mixed with 1200 µL of Fungal DNA binding Buffer in a collection tube. An aliquot (800 µL) from this mixture was transferred to a Zymo-SpinTM IIC Column in a collection tube and centrifuge at 10,000g for 1 minute. The flow-through from the collection tube was discarded. DNA Pre-Wash Buffer (200 µL) was added to the Zymo-Spin TM IIC Column in a fresh collection tube and spin at 10,000g for 1 minute, followed by the addition of 500 µLFungal DNA Wash Buffer to the Zymo-SpinTM IIC Column and further centrifuged at 10,000g for 1 minute. The whole contents in Zymo-SpinTM IIC Column was transferred to a clean 1.5 ml microcentrifuge tube and was mixed with 100 µL (35 µL minimum) DNA Elution Buffer directly to the column matrix and centrifuge at 10,000g for 30 seconds to elute the DNA.

PCR Assay:

Internal Transcribed Spacer (ITS) Gene Amplification of the Fungal Isolate: The PCR mix is made up of 12.5µL of Taq 2X Master Mix from New EnglandBiolabs (M0270); 1µL each of 10µM forward (ITS 1: TCC GTA GGT GAA CCT GCG G) and reverse primer (ITS4 TCCTCCGCTTATTGATATGS); 2µL of DNA template and then made up with 8.5µL Nuclease free water.

Cycling Conditions for the Internal Transcribed Spacer (ITS) Gene. Initial denaturation at 94°C for 5 min, followed by 36 cycles of denaturation at 4°C for 30 sec, annealing at 50°C for 30 sec and elongation at 72°C for 45 sec. Followed by a final elongation step at 72°C for 7 minutes and hold temperature at 10°C.

Sequencing: Genetic Analyzer 3130xl sequencer from Applied Biosystems was used to sequence the amplified fragments using manufacturers' manual. Big Dye terminator v3.1 cycle sequencing kit was the sequencing kit used in this analysis. Bio-Edit software and MEGA X were used for all genetic analysis done.

Phylogenetic Analysis: To establish evolutionary relationships of *A. niger* strains ND4, a mould classified within the *Nigri* section of the *Aspergillus* genus and *Aspergillaceae* family. The complete DNA sequence of *A. niger* strains ND44species available in GenBank was used. The evolutionary history was inferred using the UPGMA method. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The resulting phylogenetic trees were drawn in Molecular Evolutionary Genetics Analysis (MEGA) version 6.0 [35].

Quantitative Determination of Keratinase Activity:

Determination of Soluble Peptides/Proteins Concentration, using Lowry Method: Two different 10 ml test tubes containing homogenized 1.00 g defatted and sterilized human hair (with sizes approximately ≤ 0.5 mm), phosphate buffer (2 mL, pH 12.5) and 10 µL of isolated enzyme solution each were incubated in a shaking incubator 200 rpm at 37^{o}C. The reaction was terminated after incubation, by heating the test tubes in a boiling water bath for 5 min and later allowed equilibrated with the room temperature. Undigested keratins were separated via centrifugation at 3,000 rpm for 10 min at 4°C. The supernatants obtained were combined and stored in a freezer for determination of keratinase activity. The obtained clear supernatants were used to quantify the soluble peptides and free amino acidslevels. The sample supernatant (1 mL) and the different concentrations of standard bovine serum albumin (mg/mL; 0.20, 0.40, 0.60, 0.80, 1.00 and 1.20) were each mixed

with 5.00 mL of 4.20% $NaCO_3$ and 0.50 ml of FolinCiocalteau phenol reagent. The sample and the standard reaction mixtures were kept in an ice bags for 15 for proper precipitation to occur and the insoluble precipitates were removed by centrifugation at 1000 rpm for 10 min. The absorbance of these supernatants of the reaction mixtures were read at 660nm. A control assay without the isolated enzyme solution in the reaction mixture was prepared and used as the blank in all the spectrophotometric measurements.

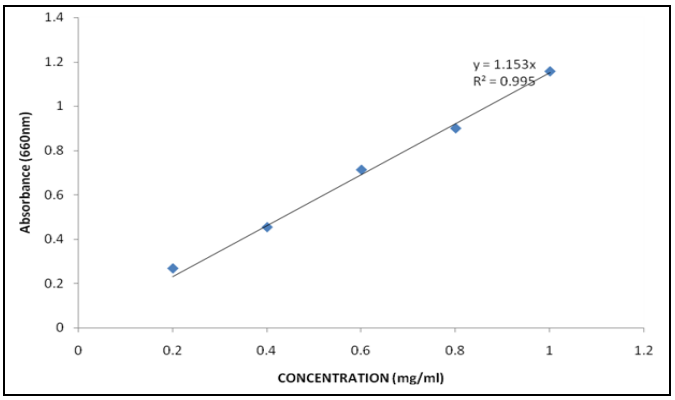

FIG. 1: BOVINE SERUM ALBUMIN STANDARD CURVE FOR PROTEIN CONCENTRATION DETERMINATION USING LOWRY METHOD

Quantitative Determination of Free Alpha-Amino Acids Concentration Using Ninhydrin Method: The principle of this ninhydrin assay method is based on the fact that 2 molecules of ninhydrin (2, 2-dihydroxyindane-1, 3-dione) react with a free alpha-amino acid to produce a deep purple or blue colour known as Ruhemann's purple (Moore and Stein, 1954). An aliquot (1 mL) from each of the clear supernatants of the sample and the various concentrations (2000, 1000, 500, and 250 µg mL^{-1}) of amino acid (cysteine) solutions were separately mixed with 1 mL of ninhydrin reagent (8% w/v of Ninhydrin in acetone) and 4 ml of diluent solvent in separate test tubes, using phosphate buffer solution to maintained the pH at 5.5. Finally both the sample and the standard were properly mixed using a vortex mixer, covered with aluminium foils and incubated at 90^{o}C for 20 min. However, after equilibrating with the room temperature, one millilitre of 98% ethanol was added and mixed properly. The absorbance of both sample and standards were measurement at 570 nm. The concentration of free amino acids in the sample was determined via extrapolation from the standard graph of Cysteine.

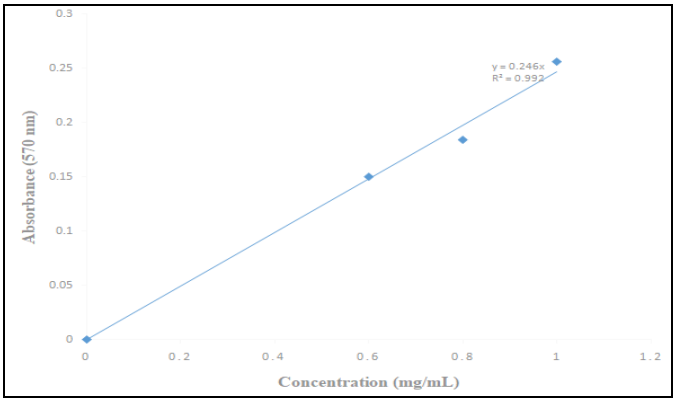

FIG. 2: STANDARD CURVE FOR CYSTEINE CONCENTRATION DETERMINATION USING NINHYDRINE METHOD

Keratinase Activity Assay Using Direct Absorbance at 280 nm: The dialyzed enzyme obtained from dialysis were diluted with phosphate buffer solution in the ratio of 1: 4 (200 µL enzyme extract: 800 µL phosphate buffer solution of pH 12.5). Two millilitres of the diluted dialyzed enzyme were added to homogenize 20.00 mg defatted and sterilized human hair and the solution incubated for 1 hour at 37 ° C, after which the solution was cooled in ice bags-water mixture for 10 min. The absorbance of the solution was measured using a UV-spectrophotometer at 280 nm [28].

Characterization/ Optimization of Keratinase Production:

Percentage Degradation of Human Hair (PDHH) by Keratinase from *A. niger* Strains ND44 within a Period of 14 days using Nickel and Copper as Co-factors: A known quantity (2.00g) of defatted human hairs was mixed with 2 mL of phosphate buffer (pH 12.5), 2 µL of isolated enzyme solution and two drops of 0.5M $NiCl_2$ or 0.5M $CuSO_4$ solutions in two sets (n and c) of triplicate test tubes each as follows{(A_{n1}, A_{n2}, A_{n3}: A_{c1}, A_{c2}, A_{c3}); (B_{n1}, B_{n2}, B_{n3}; B_{c1}, B_{c2}, B_{c3}); (C_{n1}, C_{n2}, C_{n3}: C_{c1}, C_{c2}, C_{c3}); (D_{n1}, D_{n2}, D_{n3}: D_{c1}, D_{c2}, D_{c3}); (E_{n1}, E_{n2}, E_{n3}: E_{c1}, E_{c2}, E_{c3}); (F_{n1}, F_{n2}, F_{n3}: F_{c1}, F_{c2}, F_{c3}) and (G_{n1}, G_{n2}, G_{n3}: G_{c1}, G_{c2}, G_{c3})}, where n represents samples with nickel and c represents samples with copper and were kept in an digital orbital shaking incubator at 37^{o}C for 14 days at 200 rpm (operational only during the day time). Moreover, each set of triplicates for each of the co-factors were analyse devery other day (2nd, 4th, 6th, 8th, 10th, 12thand 14th day). The catalytic activities of the keratinases in each triplicate sample (n_1, n_2,

n_3: c_1, c_2, c_3) to be analysed on that particular day were terminated by heating them in a boiling water bath for 10 min. The samples were centrifuged at 2000 rpm for 10 min to obtain the supernatants used for analysis, after cooling to room temperature. Percentage degradation of human hair (PDHH) was calculated using the formula below;

$$PDHH = \{(IWHH- TFPP)/ IWHH\} \times 100$$

Where, IWHH is the initial weight of human hair used.

TFPP = (Total concentrations of free alpha-amino acids, soluble peptides and proteins).

Both Ninhydrin and Lowry methods were used to determining the concentrations of free alpha-amino acid, soluble proteins, and soluble proteins in the supernatants of the reaction media every other day. The absorbance of the supernatants was measured using a UV-spectrophotometer at 280 nm.

Effect of pH on Keratinase: The enzymatic activities of keratinase at pH (2, 4, 5.5, 7, 9, 10, and 12.5) were assayed using0.02g of defatted human hair in 2 mL of salt solution and 2 mL of the following buffer solutions; 0.1 M citrate phosphate buffer for pH (2.00 - 5.50); 0.1 M sodium phosphate buffer for pH (7.00); and 0.1 M carbonate bicarbonate buffer for pH (9.00 - 14.00). Immediately after incubation, the enzyme solutions were centrifuged at 4,000 rpm at 4ºC and 1mL aliquot of each supernatants taken for keratinase activities assay using Lowry and Ninhydrin methods; and direct absorbance at 280 nm.

Effect of Temperature on the Activity of Keratinase: The enzymatic activity of keratinase was examined within the temperature ranges of 0, 4, 25, 37, 60, and 90ºC with 2 mL of salt solution and defatted human hair (0.02g) as substrate at constant pH of 12.5.

Immediately, after the incubation period, the enzyme solutions were centrifuged at 4,000 rpm at 4ºC and 1mL aliquots of the supernatant each were taken and the keratinase activity was assayed using Lowry and Ninhydrin methods; and direct absorbance at 280 nm.

Effect of Bio-Metals on the Activities of Keratinase: The enzymatic activity of keratinase was assayed in the presence of 0.1 mL of salt solutions of these bio-metals (Zn^{2+}, Ca^{2+}, Mg^{2+}, Fe^{2+}, Ni^{2+}, Cu^{2+}, Co^{2+}, Al^{3+}, Hg^{2+} and Pb^{2+})with 0.1 M sodium phosphate buffer- pH 12.5 and defatted human hair (0.02g) as substrate for 1 hr.

After the incubation period, the enzyme solutions were centrifuged at 4,000 rpm at 4ºC and 1mL aliquots of the clear supernatant each were taken and the keratinase activity was assayed using Lowry and Ninhydrin methods; and direct absorbance at 280 nm.

Chapter Three
RESULTS:

FIG. 3A: WHITE FUNGAL GROWTH PATCHES ON THE LUMPS OF HAIR SAMPLES INCUBATION AT 25ºC IN A SALT MEDIUM AT PH 9 FOR 2 MONTHS

FIG. 3B: WHITE FUNGAL GROWTH PATCHES ON THE LUMPS OF HAIR SAMPLES INCUBATION AT 25ºC IN A SALT MEDIUM AT PH 9 FOR 2 MONTHS

FIG. 4: POTATO DEXTROSE AGAR MEDIUM CONTAINING DIFFERENT COLONIES WITHIN 72 HR OF INCUBATION AT 37ºC

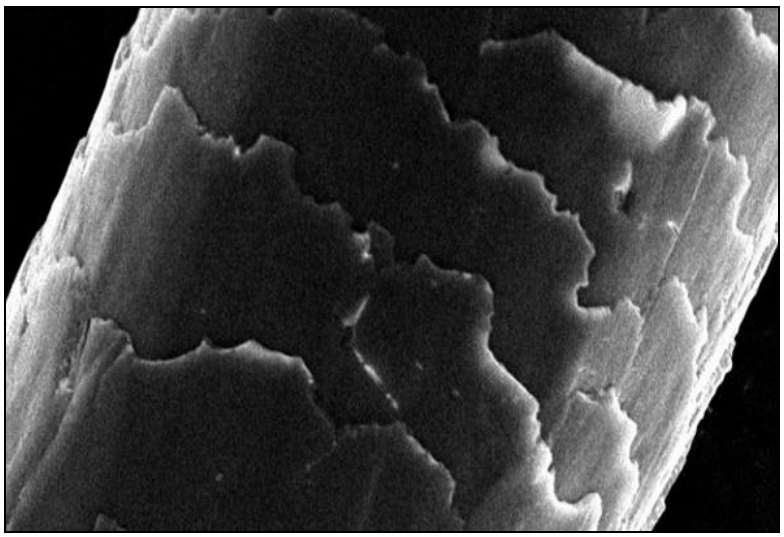

FIG. 5: SCANNING ELECTRON MICROSCOPY OF CONTROL, INTACT HUMAN HAIR. SCALE BAR: 10 MM

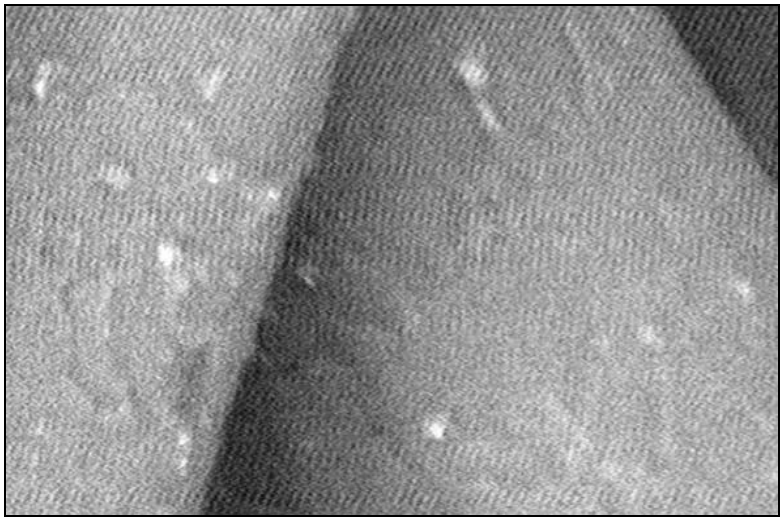

FIG. 6: SCANNING ELECTRON MICROSCOPY OF DEFATTED HUMAN HAIR. DEFATTED HUMAN HAIR AFTER 14 DAYS OF DEGRADATION WITH EXTRACELLULAR KERATINASE ENZYME ISOLATED FROM *A. NIGER* STRAIN ND44. SCALE BAR: 10 MM.

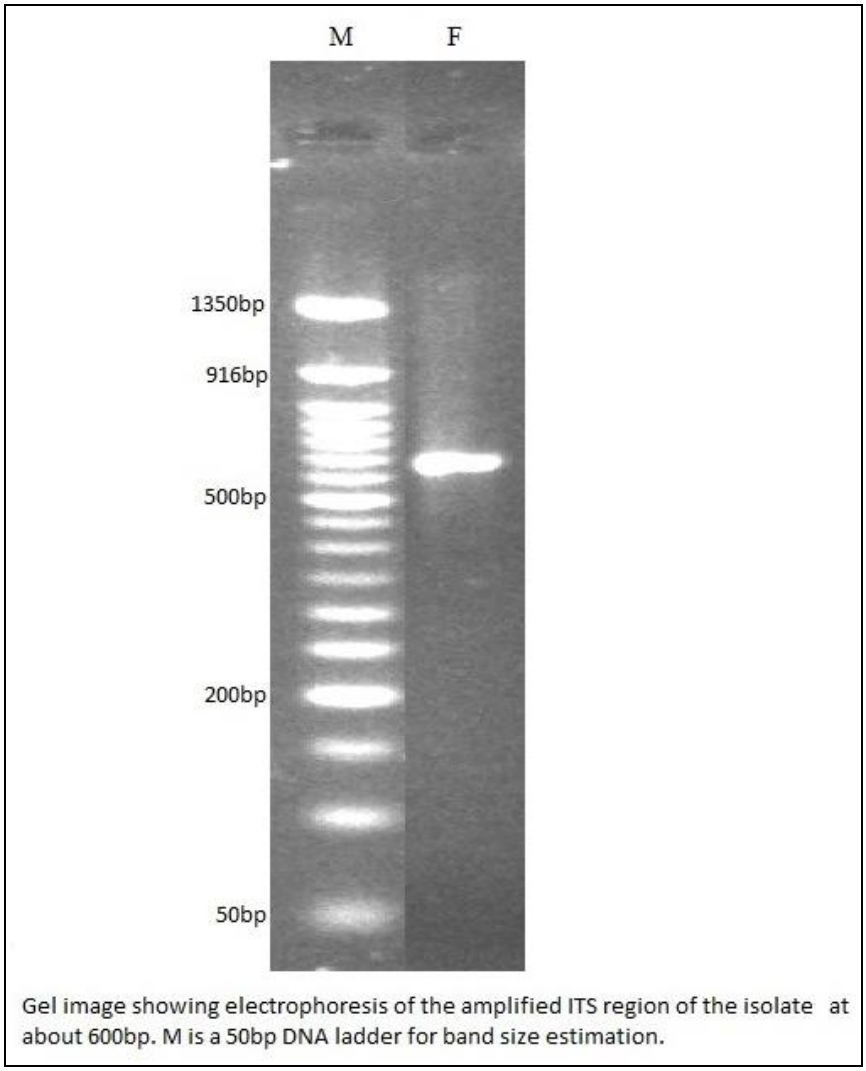

FIG. 7: *A. NIGER* STRAIN ND44 GENE (16SRRNA) DETECTION USING ENDPOINT PCR

The fungal isolate has 98.25% Pairwise Identity to *Aspergillus niger* strain ND44 accession number MG659638. The e value is 0 while the isolate sequence is as shown below:

GGGGGGGGTCTTTGGGACACCTCCCATCCGTGTCTATTGTACCCTGTTGCTTCGG
CGGGCCCGCGCTTGTGACGGCGGAAAACGCTCACCTGAGACGGAGGGTGAACCT
AAAAAAATGTAAACGGGAGTGGATTTATTAATCAAACGGTAAAAATTTTTAAAA
AAAGGAATCTTTTTGTTTCCGGGCCCCCCAAAAAAAAAACCCCCGGAAAAAACG
GAAAAAACAAAAAGGGAAATTGGGAAAAATTTCAGGGAAAAACCCCCGAGTTT
TTTTTAAAAAAAAATTTTGGGCCCCCCCGGGGTATTTTCGGGGGGGGAATCCCTT
TTCCCAAAACCCAATTTTGGGGCCCCTTAAAACCCCGTTTTTTTTTTTGGGAAAG
GATACTTTTTTTTGGGGGGGGAAAGGCCCCAAAAAAGAAGGGGGGGGGGCCCCC
GGGCCAAAAACCTCGGGGGGGGAATGGGGGGTTTTTTAAAAAAAGTTTTTAAAAA
AAATAGGCCCCCCCCCCCCCCAATTTTTTTTAAAAAAATTTTTTTTTTCGGGGGGG
ACACCCCCCAAAAAAAGGGAAGGAAACCCCCCGGGGGTTTTTGGCTTTTTTTGG
GGGGGGGGAAAAAAAATGGGGGGCGAAGACACTTTTACACCCCTTATGTTTGTG
GTGAAAGAACAGGGGGAGGGAGGGGGGGGGTGGAGAGAGGGTTAGGGTAAGA

FIG. 8: THE FUNGAL ISOLATE-1 HAS 98.25% PAIRWISE IDENTITY TO *ASPERGILLUS NIGER* STRAIN ND44 ACCESSION NUMBER MG659638

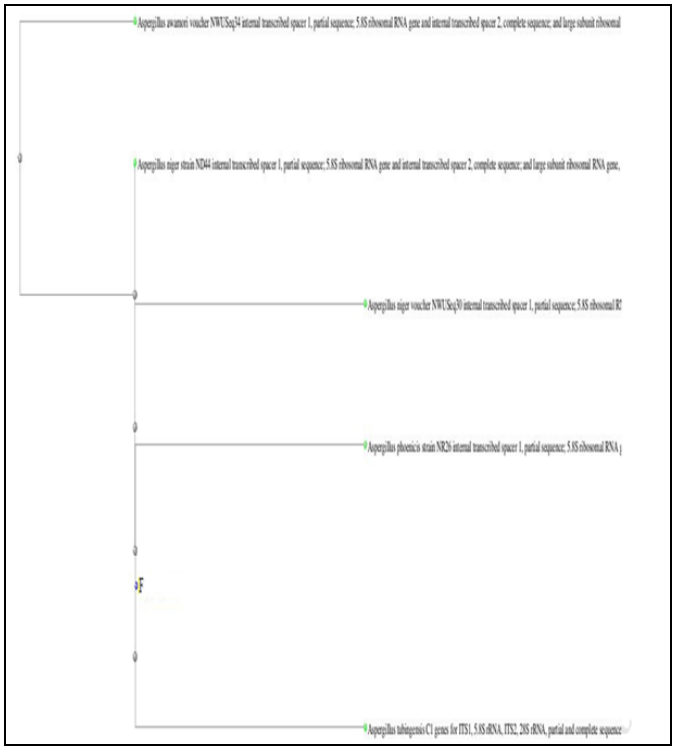

FIG. 9: PHYLOGENETIC TREES INFERRED FROM 4 NUCLEOTIDE SEQUENCES. THE PHYLOGENETIC ANALYSES WERE CONDUCTED WITH A MAXIMUM COMPOSITE LIKELIHOOD (ML) AND ARE IN THE UNITS OF THE NUMBER OF BASE SUBSTITUTIONS PER SITE. THE NUMBERS IN FRONT OF THE SPECIES ARE GENBANK ACCESSION NUMBERS

Sequencing: The fungal isolate has 98.25% Pairwise Identity to *A. niger* strain ND44 accession number MG659638. The e value is zero while the isolate sequence is as shown in **Fig. 9.**

Isolation and Identification *A. niger* Strain ND44: Isolation and molecular identification of one fungal isolate; *A. niger* strain ND44 accession number MG659638. This was isolated from soil contaminated with shaved human hairs, collected from a dumping site at Toscana barbing salon. Isolation was made possible because of its degrading activities on keratin and observed easy growth in salt medium with human hair as the only carbon and nitrogen source. Its identifications were based on cell morphology, physiological characteristics, biochemical and molecular analyses. Isolate-1 gave positive reactions to spore staining test. The 16S rDNA sequence analysis of *A. niger* strain (ND44) with NCBI accession number (MG659638) showed (98.52% homology) high sequence similarity with *A. niger* sequence deposited in the GenBank

Effect of Metal Ions on Keratinase Activity: Two different assay methods were used to assay for the effect of metal ions (co-factors) on keratinolytic degradation of human hair keratin. The best metal ions for keratinase degradation of human hair keratin to soluble protein concentrations as determined by FolinCiocalteau phenol reagent method were Ni^{2+} and Co^{2+} **Fig. 10**, while the best metal ions for liberation of free amino acids as determined by the Ninhydrin method were Cu^{2+} and {Ni^{2+} and Mg^{2+}} **Fig. 11**.

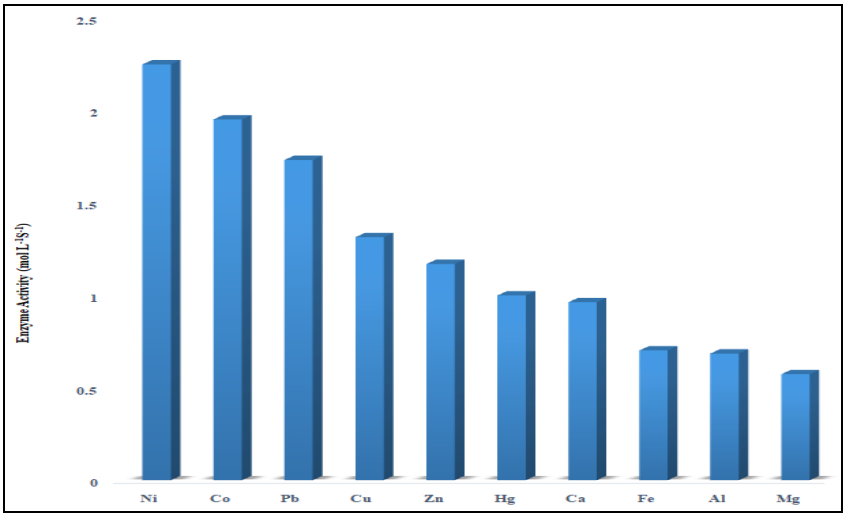

FIG. 10: DETERMINATION OF THE EFFECTS OF DIFFERENT METAL IONS ON KERATINASE ACTIVITY USING LOWRY METHOD

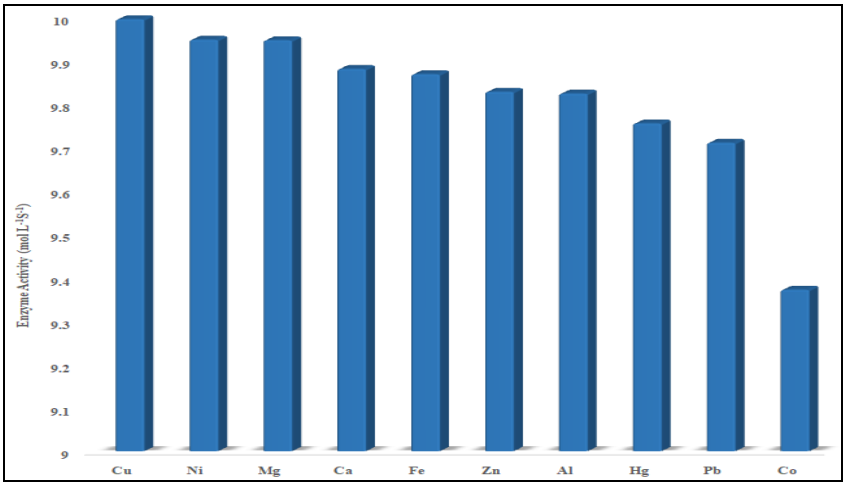

FIG. 11: THE EFFECTS OF METAL IONS ON KERATINASE ACTIVITY USING NINHYDRIN METHOD

Effect of pH on Keratinase Activity: Two different assay methods were implored to investigate the effect of change in pH on the catalytic activities of keratinase in degradation of human hair keratin samples. The keratinase showed optimal catalytic efficiency at pH 12.5, although we recorded steady increase in enzymatic activities within pH 4 and 9, before shape drop in enzymatic activity from pH 9 -10. Beyond pH 10.0, enzyme activity steadily increases with increase in alkalinity, until it got to pH 12.5. **Fig. 12** and **13**).

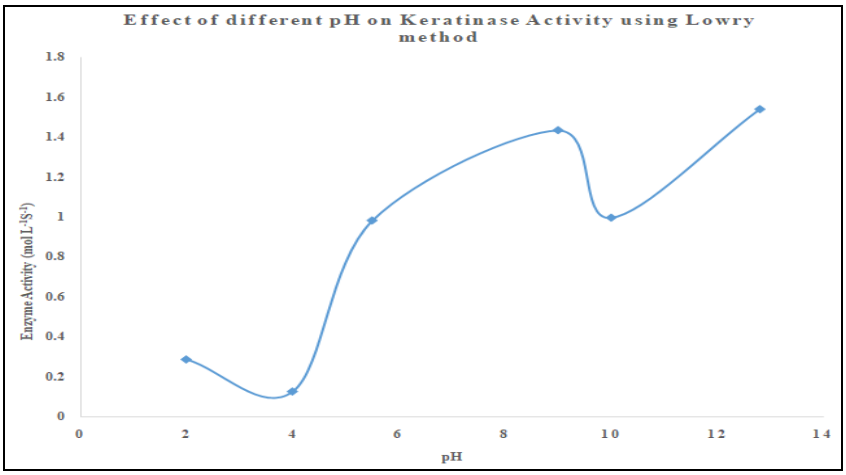

FIG. 12: THE EFFECT OF VARIED PH ON KERATINASE ACTIVITIES AT 37 °C USING LOWRY METHOD

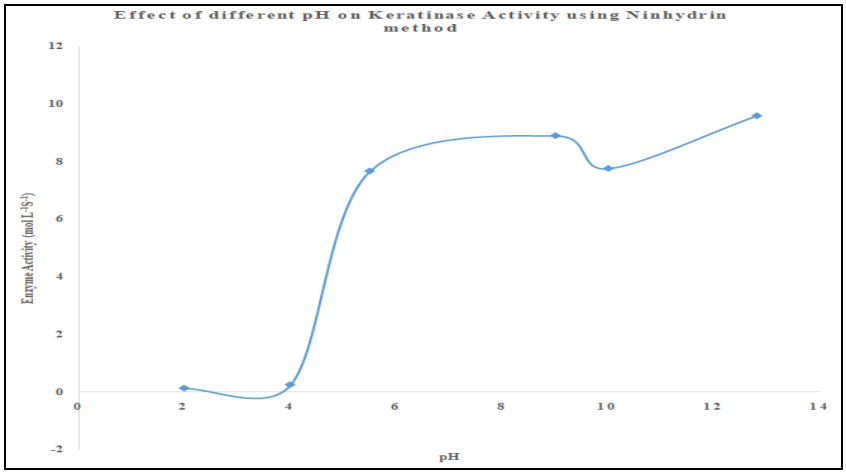

FIG. 13: THE EFFECT OF VARIED PH ON KERATINASE ACTIVITIES AT 37 °C USING NINHYDRIN METHOD

Effect of Temperature on Keratinase Activity: Two different assay methods were used to investigate the effect of varied temperatures on catabolic breakdown of human hair keratins by keratinase enzyme. Our obtained optimum temperature for degradation of human hair keratin to soluble protein as determined by FolinCiocalteau phenol reagent method was 90ºC, with another major but not optimum peak, at 37 ºC **Fig. 14**. Moreover, the optimum temperature for liberation of free amino acids as determined by Ninhydrin method were between 37 and 60ºC as seen in **Fig. 15.**

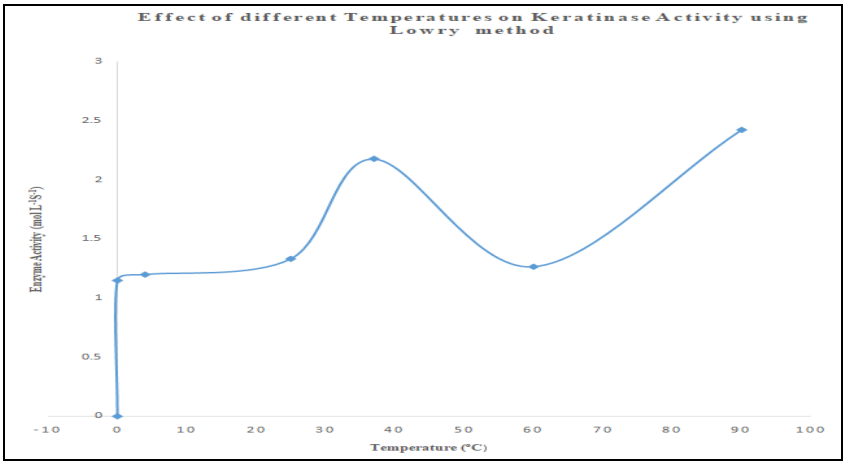

FIG. 14: VARIED EFFECT OF TEMPERATURE ON KERATINASE ACTIVITIES USING LOWRY METHOD AFTER INCUBATION FOR 1 HR WITH HUMAN HAIR KERATIN

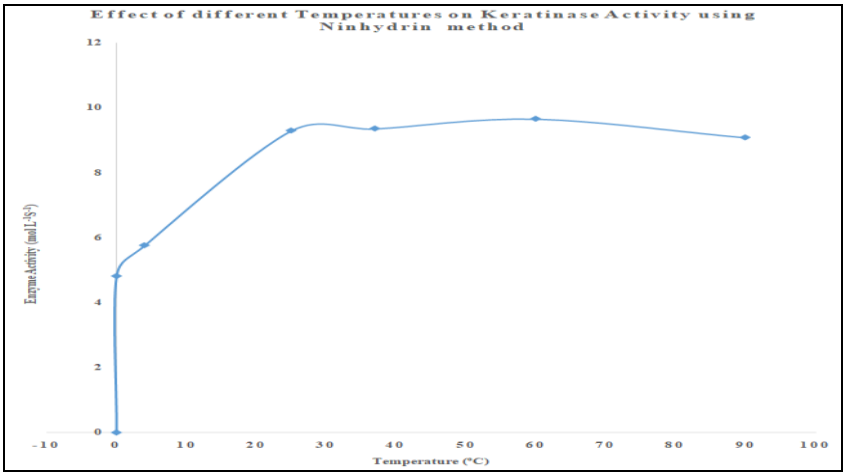

FIG. 15: VARIED EFFECT OF TEMPERATURE ON KERATINASE ACTIVITIES USING NINHYDRIN METHOD AFTER INCUBATION FOR 1 HR WITH HUMAN HAIR KERATIN

Percentage Degradation of Human Hair by Keratinase Enzyme from *A. niger* Strain ND44 for Fourteen Days: The concentrations of soluble proteins, free amino acids and aromatic amino acids as determined by the FolinCiocalteau phenol reagent method, Ninhydrin method and absorbance of UV-light at 280 nm, obtained from the degradation of our human hair in cultured medium were {Cu:2.2142 (12^{th} day); Ni:2.4337 (14^{th} day)}, {Cu:10.3968 (14^{th} day); Ni:10.3117 (14^{th} day)} and {Cu:2.7133 (12^{th} day); Ni:2.7021 (12^{th} day)} respectively. The observed degradation peak on 14^{th} day for ninhydrin test indicates that the maximum production of free amino acids occurred on 14^{th} day. Our data further depict that the maximum concentration of liberated soluble proteins occurred on 12^{th} day for Cupper and on 14^{th} day for Nickel, but further indicate that liberated free amino acids from the keratin substrate were significantly far greater that the concentrations of the liberated soluble proteins.

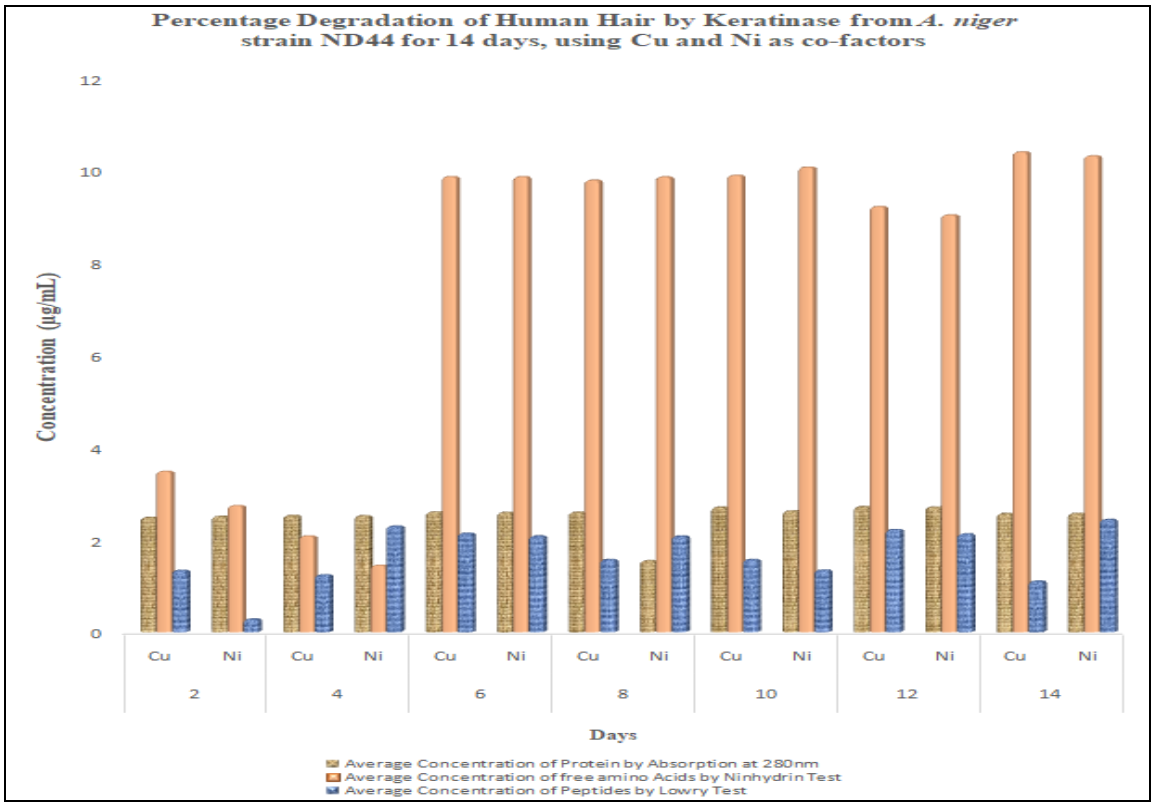

FIG. 16: DEGRADATION OF HUMAN HAIR BY KERATINASE ENZYME FROM *A. NIGER* STRAIN ND44 FOR A PERIOD OF 14 DAYS, USING CU^{2+} AND NI^{2+} AS CO-FACTORS

DISCUSSION: In this current research work, synthesized and secreted keratinase from an isolated keratinophilic fungus was aimed for characterization and optimization. Most research works in literature on degradation of keratin by microbes focused more on biodegradation of poultry feathers, the world's most abundant source of keratin, but not much attention has been given to biodegradation of human hair the second most abundant source of keratin by keratinophilic fungi knowing fully well that there might be an intrinsic molecular differences in the two keratins and on the catalytic mechanisms of the different keratinases involved (Ezenwali, 2022). However, an in-depth scientific based knowledge on Keratinase enzyme from *A. niger* strain ND44and its enzymatic mechanism of action on human hair keratin, its production, and optimization needs to be expanded in order to facilitate its industrial usage in waste recycling management and future biotechnological applications feasible.

Fig. 1AB, show white fungal growth patches on lumps of shaved human hair samples incubated in a salt medium at room temperature for 2 months. These are eukaryotic, non-vascular, non-motile, heterotrophic, spores forming, saprophytic, filamentous fungus. The microscopic observation of the colonies on potato dextrose agar at room temperature were initially white, but with time it

changes to light yellow. The sequence analysis of 16S rDNA showed high sequence identity to one subspecies of *A. niger* strainND44 (98.25% homology) for the isolate-1. The sequence was compared with NCBI accession number MG659638 of GenBank. The phylogenetic tree showed that isolate-1 is *A.niger.*

The observed crimson colour from Ninhydrin test method indicates that predominate free alpha-amino acids in the solution is a semi essential proteinogenic amino acid ‘’Cysteine’’.

Biodegradation of human hair keratins were carried out at varying pH, temperature and in presence of different metal ions. The optimum pH, temperature and the best co-factors for human hair keratinase activities using Lowry and Ninhydrin methods were found to be at pH {12.5}, temperature of {(90 °C) and (60 °C)} and co-factors of {(Ni^{2+}andCo^{2+}) and (Cu^{2+}amd Ni^{2+})} respectively. The observed dual temperature peaks of {37 °C and 90 °C} and {37 °C and 60 °C} identified under Ninhydrin and Lowry methods respectively in this present research work may be attributed to the fact that keratinase degradation of the hair engrosses the mutual characteristic actions of two enzymes: disulfide reductase and keratinase, an indication of a multi-enzyme catalyzed reaction.

Since, other enzymes, particularly disulfide reductases, also play a key role in keratin degradation as they catalyze the breakage of disulfide bonds for better keratinase catalysis [49]. Keratinases are serine and metalloproteases, and their active sites are formed by several conserved residues [36].

Our observation that the co-factor (copper, nickel and cobalt) increased the enzymatic activity of this isolated keratinase is in agreement with the report of Meng, *et al*., who discovered that Li^{+}, Mg^{2+} and Co^{2+} enhances the enzymatic activity of keratinase DeokerA from *Deinococcus gobiensis* for Feather degradation [40]. Recently, eco-friendly biodegradation methods/processes are fast replacing many conventional chemical processes capable of generating large amount of sulfide and other toxic chemicals, as keratinases are fast gaining larger applications in many industries. The observed significant reduction in enzymatic activity at acidic pH should be attributed to the ionization state of the side chains of the amino acid residues at either one or both the catalytic cleft and the binding site at acidic pH, which do not favour keratinase-keratin complex formation and bond breakage/new bond formation [7].

Our current observation regarding increased enzymatic activities of keratinase enzyme from *A. niger* strain ND44 at high temperature of 90 ºC is consistent with both our previous study Ezenwali, 2022 and that of Li, 2021 that established that keratinases are heat-stable enzymes. Enzymes that exhibit maximum activities at temperature >80 ºCare classified as hyperthermophilic enzymes by Vieille and Zeikus, 2001. Our isolated keratinase enzyme exhibits maximum activity at an elevated temperature of 90 ºC and can thus be regarded as hyperthermoactive, which is also in total agreement with Keratinase (KerBAN) activity a thermoactive metallo-keratinase from *Bacillus* sp. NFH5 [32].

Based on our oral interview with undertakers, it was observed that the only part of human body system that continue to grow in a formalin preserved dead body some months after death is the human hair. Biosynthesis of hairs strive on dead body cells, utilizing all the protein components from decomposing dead cells to synthesize keratin the major component of hair. This may however, suggested that human hair is a ‘’Pseudo-independent cell’’ that may have during the course of evolution lost some of its features when it first came in contact with human cells in a symbiotic association.

CONCLUSION: The identity of cosmopolitan microscopic filamentous keratinolytic fungus used in this research work was confirmed as *Aspergillus niger* strain ND44, which strives well on human hair keratin as the major source of nitrogen and carbon with the help of its secreted extracellular keratinase. This isolated hyperthermophilic metallo-keratinase showed optimal catalytic efficiency at pH, temperature and co-factor (12.5, 90°C and Ni^{2+}) and (12.5, 90°C and Cu^{2+}) using Lowry and Ninhydrin methods respectively. Molecular identification of keratinase-coding gene justified the keratinolytic potential demonstrated by the isolate.

Hence, the future study in this research should aim at analysing human hair hydrolysates to identify and quantify the quality of the liberated amino acids/soluble peptides which invariably depends on the amount of essential amino acids (EAA) and the prospect of the amino acids/soluble peptides hydrolysate in livestock dietary feed formulation.

Recommendation: We recommend necessary financial support/research grant for the above proposed future studies on the hydrolysates to facilitate its use in livestock dietary feed formulation, since most livestock farmers in Nigeria have been pushed out of business because of high cost of feeds. However, based on the fact that keratinolytic enzymes/proteolytic enzymes play crucial ecological role in degradation of keratin the most abundant macromolecular component of the world richest proteinous solid-waste ''keratinaceous waste'' (feathers, hairs, wools, nails, horns etc.) leading to/resulting in circulation of nitrogen in nature. We strongly advocate/recommend for slight modification of ''Nitrogen Cycle'' to include both keratins and keratinolytic enzymes as key players in this cycle.

''The New Nitrogen Cycle'':

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Declaration of Competing Interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CONFLICTS OF INTEREST: The authors declare no conflict of interest.

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