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Optimization, Isolation and Characterization of a Keratinolytic Protease from Human Hair-Degrading Bacterium Alcaligenes Faecalis Strain TPB18

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***Abstract*— Microbial keratinases are a group of highly specific proteases that catalyze the hydrolytic cleavage of peptide bonds in keratin, a structural fibrous polypeptide/protein highly resistant to most proteolytic degradation/attack because of its complex bonding structure stabilized by disulfide and hydrogen bonds, an attribute that also made it one of the most world environmental richest proteinous solid waste (keratinaceous waste). In this present research, an extracellular keratinase identified by genomics, proteomics, and biochemical methods was isolated from soil Alcaligenes faecalis (TPB18), purified and then applied in the degradation of defatted human hair. The keratinase activities were determined by measuring the concentrations of both free amino acids and soluble peptides/proteins using Ninhydrin and Lowry methods respectively. This heat-stable keratinase exhibited keratinolytic activities at temperatures ranging from 0 to 90oC. The optimum pH, temperature, and the best co-factors for this isolated human hair keratinase activity using Lowry and Ninhydrin methods were found to be at pH {(12.5) and (5.5, 9, 10)}, temperature of {(37 °C) and (37-60 °C)} and co-factors of {(Co2+, Al3+, Hg2+) and (Co2+, Hg2+, Cu2+)} respectively. The optimal incubation time and the percentage degree of human hair degradation were found to be 85% on day 5. The phylogenetic tree identified two out of the four isolates, isolate-3 and -4 with 98.72% and 96.01% pairwise identities to Alcaligenes faecalis strain AIR10 and TPB18 with NCBI accession numbers of MG835355.1 and MW475277.1 respectively. An end-point polymerase chain reaction gel image test showed 16SrRNA gene amplification for both Alcaligenes faecalis (TPB18) and (AIR10) at about 1.5kb**

***Index Terms*— Alcaligenes faecalis strain TPB18 and AIR10, Keratin, Keratinase, Co-factors, Optimum pH, Optimum Temperature, 16SrRNA gene amplification, Phylogenetic tree, DNA Sequence**.

I. INTRODUCTION

Alcaligenes faecalis, a flagellated alkaline-tolerant gram-negative rod-shaped aerobic bacterium that belongs to the family of Alcaligenaceae, in the order of Burkholderiales, is commonly found in water, soil, and our environment but specifically in the intestinal tracts of vertebrates, decaying materials and wounds of hospitalized patients with compromised immune systems [1, 2]. In association with humans [3]. A. faecalis a non-pathogenic bacterium, serves as an opportunistic pathogen that triggers local infections such as peritonitis, meningitis, otitis media, and appendicitis. Although, research work by Rafi et al (2017) marks Alcaligenes strain as a prospective eco-friendly bacterium with numerous benefits including sustainable environmental clean-up via bioremediation of various environmental pollutants [3]. Pahua-Ramos et al (2017) on the biodegradation of chicken feathers by Co-culture of A. faecalis and Brevundimonas reported the potential exploitation of keratinase in total degradation of feathers [4]. Paul (2007) reported that Alcaligenes are capable of stimulating plant growth via the production of growth regulators: auxins, cytokinins, gibberellins, or ethylene or abscisic acid, and they have been reported to degrade pesticides and hydrocarbons, both alkanes and polyaromatic compounds [5]. Human hair keratin, a trichocytic α-keratin is an intermediate filament, biochemically described as a cytoskeletal coiled-coil structural protein with a high degree of disulfide cross-link, is the most abundant macromolecular component of human hairs. These supercoiled fibrous keratin molecules are highly stable, resistant to most proteolytic enzymatic attacks, and insoluble in both polar and non-polar solvents basically because of hydrophobic interactions of coiled-coil structure, intra- and intermolecular hydrogen bonding, and a high degree of cross-linking by disulfide bonds which is responsible for thermally stability and stringency of keratin in human hair. Because of the extraordinary concentration of human population in the cities, shaved human hair from barbing saloons and hairstylist shops are considered one of the major metropolitan keratin pollutants that may lead to massive local pollution and environmental degradation. Due to the environmental and health problems posed by decaying human hair and hair dust generated from human hair dumped in an open place, the city population in developing countries are being affected with the problems of tuberculosis and respiratory-related infections [6]. Human hair fibre accumulation and other pollutants, in the form of waste heaps, solid waste, or waste streams may be responsible for the unnecessary health complications and illnesses witnessed among many who live in city slums. Enzymatic degradation of the hair engrosses the mutual characteristic actions of two enzymes, disulfide reductase and keratinase [7].

Apart from being one of the most widely generated soil/water wastes, it stays in the dumpsites/waste streams for long occupying large volumes of space due to slow degradation. Over time, leachate from these dumpsites increases the

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nitrogen concentrations in the water bodies, causing problems of eutrophication. The microbial degradation of the lipid constituents of the hair protective cuticle results in a foul odour and creates a breeding ground for pathogens [8]. Rapid decomposition of keratinous waste via incineration and solubilization methods specifically geared towards increasing its solubility by subjecting it to high pressure and temperature destroys some of the thermolabile amino acids and finally produces hazardous compounds that pose both health and ecological treaties to both man and his environment. However, specialized keratinolytic enzymes produced by some microorganisms (fungi and bacteria) can efficiently and effectively degrade keratinous waste [9, 10]. Many research works have implicated the following genera of bacteria as keratinolytic bacteria capable of degrading feathers: Bacillus subtilis AMR [11, 12], Strain Chryseobacterium sp. kr6 shown to be a useful microbial agent in the hydrolysis of poultry feathers and de-hairing of bovine pelts [13], Kocuria rosea [14], Xanthomonas maltophilia Strain [15], Pseudomonas aeruginosa SU-1 [8], Pseudomonas microphilus and Leuconostoc [16], Scopulariopsis brevicaulis (Sacc.) [17]) Stenotrophomonas [18] and actinomycetes [19]. A. faecalis strain TPB18 with NCBI accession number MW475277.1.was isolated in November 2020 from soil contaminated with human hair at Toscan Barbing saloon, No 14 More House Street, Ogui, Enugu North Local Government Area, Enugu State in the South-Eastern region of Nigeria and its genome was sequenced as a part of the on-going project on biodegradation of human hair keratin and application of its by-products in biosorption of toxic heavy metals from the contaminated aquatic system [20] and in novel poultry feed formulation, since consumption of (fish, poultry, and eggs) fed with growth hormone or genetically modified chickens may increase the risk of prostate cancer [21].

Human hair wastes used in this study were collected from local barbers' shops in Enugu Nigeria. All reagents used in this work were of analytical grades. The research was mainly carried out with standard equipment in the Brain Phosphorylationship Scientific Solution Services, 5th Floor, Right Wing, Number 9 Ogui Road, Enugu, Enugu State, Nigeria.

II. Methods

Preparation of hair substrate

Human hair wastes from different male individuals were collected from local barbers' shops. The human hair samples were mixed, washed with liquid detergent, rinsed several times with distilled water, and then left to dry at room temperature (25°C). The hair was cut to an approximate length of 1-3 mm using scissors. Five hundred grams of this human hair was defatted with 1000 ml of chloroform/methanol (1:1 v/v) at room temperature for 1 h with agitation (200 rev min^{-1}). The filtrate was pulled together and concentrated by evaporating the solvent using rotary evaporator, thereby leaving behind the extracted lipid. The extracted lipid was further characterized. Afterward, hair was washed with distilled water and dried at 70°C for 12 h. This was used as substrate in culture medium.

Microbial culture of keratinolytic bacteria

Human hair were collected from a temporal dumping site/waste bin from a local barbing saloon named Toscana at number 14 Moore House Street, Ogui, Enugu North, Enugu State, Nigeria. A well-calibrated 1000 ml conical flask containing 1000 ml of salt medium (K_2HPO_4 0.3g, KH_2PO_4 0.4g, NaCl 0.5g, $MgSO_4$ 0.1g, Yeast extract 0.1 g, NH_4CL 0.4 g, in 1000 ml distilled H_2O maintained within the pH range of 9 with phosphate buffer) was sterilized in an autoclave at 121°C for 15 min. The salt medium was allowed to cool to room temperature after sterilization and then inoculated with 10 g of the human hair as the only carbon source sample collected from the barber's shop contaminated with soil. This was finally incubated in an orbital shaking incubator at 37 °C for 72 h at 200 rpm. However, at the end of the incubation, aliquots of this culture were streaked on the same medium containing agar (20 gL^{-1}) to obtain different colonies within 24 hr of incubation at 37 °C. The individual colonies were sub-cultured into different Petri dishes to obtain pure cultures (plate 2). However, the four colonies were separately transferred into different labelled 250 ml conical flasks containing 150 ml of sterilized yeast extract medium (gL^{-1}: yeast extract, 5; peptone, 5; sucrose, 20; and KCl, 20) and phosphate buffer solution with pH 9. These samples were incubated at 37°C in an orbital shaking incubator at 200 rpm for 4 days, for the multiplication of microbial cells. The isolate that showed the best growth and keratin degradation were selected and all were identified by biochemical, gram staining techniques, and physiological and morphological characterizations.

Isolation and identification of bacterial isolates

Plate growths were noticed after 24 hours of incubation, the isolates were then sub-cultured on fresh media plates until pure isolates were observed. The pure cultures of isolates were stocked into MacCartney bottles. The isolates were identified based on their morphological appearance, gram staining test, and biochemical characteristics.

Genotypic/Molecular Characterization

DNA extraction

DNA extraction was carried out using Zr fungal/bacterial DNA miniprep (Manufactured by Zymo Research). A known volume of bacterial cells broth (2mL) was added to a ZR BashingTM Lysis Tube, followed by the addition of 750µL Lysis solution to the tube. This was secured in a bead fitted with a 2 mL tube holder assembly and processed at maximum speed for > 5 minutes. The ZR BashingTM Lysis Tube was centrifuged in a micro-centrifuge at 10,000g for 1 minute. A known volume of the supernatant (400 µL) was transferred to a Zymo-SpinTM IV Spin Filter (orange top) in a collection tube and centrifuge at 7,000g for 1 minute. The resultant filtrate was mixed with 1200 µL of Fungal/Bacterial DNA binding Buffer in the 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. 200 µL DNA Pre-Wash Buffer was added to the Zymo-Spin TM IIC Column in a new collection tube and centrifuge at 10,000g for 1 minute and this was followed by the addition of 500 µL Fungal/Bacterial DNA Wash Buffer to

the Zymo-SpinTM IIC Column and centrifuge at 10,000g for 1 minute. Zymo-SpinTM IIC Column was transferred to a clean 1.5 ml microcentrifuge tube and were 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

Two isolates were identified using the partial sequencing of the intergenic region of the rDNA. An ITS region was selected as a target sequence for PCR using universal primers 27 (5'-AGAGTTTGATCMTGGCTCAG-3') as a forward primer and 1525 (5'-AAGGAGGTGWTCCARCCGCA -3') as a reverse primer. A PCR using the thermocycler

(Eppendorf, Hamburg, Germany) was performed with the following parameters: an initial denaturation of 5min at 95 °C, 30 cycles of 30s at 95 °C for denaturation, 30s at 46 °C for annealing, 80s at 72 °C for extension, and a final extension of 7 min at 72 °C, according to Horisawa and Sakuma, 2009, with slight modifications. All PCR products were stored at -20 °C until analyzed. Aliquots of PCR products were examined after electrophoresis on 0.9% agarose gels [22]

Sequencing

The amplified fragments were sequenced using a Genetic Analyzer 3130xl sequencer from Applied Biosystems using manufacturers' manual while the sequencing kit used was that of Big Dye terminator v3.1 cycle sequencing kit. Bio-Edit software and MEGA X were used for all genetic analysis.

Phylogenetic Analysis

To establish evolutionary relationships between *A. faecalis* strains AIR10 and *A. faecalis* strains TPB18 DNA sequence within the family *Alcaligenaceae*, the complete DNA sequence of *A. faecalis* strains AIR10 and *A. faecalis* strains TPB18 and 2 other species available in GenBank were used. The evolutionary history was inferred using the UPGMA method. The optimal tree with the sum of branch length = 0.34552324 is shown. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. This analysis involved 4 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1133 positions in the final dataset. The resulting phylogenetic trees were drawn in Molecular Evolutionary Genetics Analysis (MEGA) version 6.0 [23].

Multiplication of *A. faecalis* strains TPB18 and AIR10 growths

The isolated bacteria (*A. faecalis* strains TPB18 and AIR10) with the best degradation capacities were propagated in different yeast extract media at 28 ºC with shaking (200 rev min^{-1}) for 12 days to obtain masses of *A. faecalis* cells. The cells were harvested by centrifugation, washed thrice (2000 rpm at 20 min) with saline solution (NaCl 9.0 gL^{-1}), and used for keratinase production.

Optimization of keratinase production

Inoculum of 10^8 cells ml^{-1} of each species were introduced into two different sets of five samples each of oven-dried defatted human hair medium (dried DHHM: (100) gL^{-1} w⁄v human hair in 500 ml of ($Na_2HPO_4.7H_2O$ and KH_2PO_4) phosphate buffer at pH 9.0 and hair medium supplemented with 0.01% yeast extract (HMY). Moreover, 1 ml of 1% sodium sulphite was added. Control hair samples were incubated in phosphate buffers without inocula/enzyme. These samples were incubated at 37 ^oC in an orbital shaking incubator at 200 rpm for 12 days, each set of the reactions were terminated by the addition of 5 ml of 10% TCA. Then the cultures were centrifuged at (3000 rpm for 10 min). The culture supernatants were used for daily keratinase and protein analyses. Soluble peptides were quantified by the Lowry method after each day of enzyme production, while the residual undigested defatted human hair in both test samples and control samples were used for analysis.

Scanning electron microscopy (SEM)

Defatted and partly degraded human hair test samples and a control sample from the above-cultured media were oven-dried at 40 ^oC and scanned with SEM for observation of hair degradation. Hair samples were put on stubs and gold sputtered. Images were collected on a JEOL JSM 5310 scanning electron microscope operating at 15 kV.

Production of keratinase

Sterilized salt medium (500 mL) containing homogenized 50 g defatted human hair was inoculated with isolate-4 (***A. faecalis* strain TPB18**) in 1000 ml conical flasks. After 24 h incubation, crude enzyme was extracted from the culture medium and centrifuged at 4,000 rpm for 10 min at 4 °C. The supernatants obtained were termed the crude enzyme extract for the measurement of keratinase activity and were stored in the freezer.

Purification of enzyme

Ammonium sulphate precipitation

The partial purification of crude extract of keratinase was done by gradual precipitation with $(NH_4)_2SO_4$. Different percentage (%) saturation concentration of $(NH_4)_2SO_4$ (20, 30, 40, 50, 60, 70, 80, 90 and 100 %) solutions were prepared by gradually dissolving their 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) in 1 mL with constant stirring using magnetic stirrer to determine the percentage (%) saturation that gave the highest precipitation. The treatments were carried out in ice bags. The solutions were left to stand for one hour after dissolution. The precipitated enzymes were separated by centrifugation at 4000 rpm for 30 minutes at 4 °C. The keratinase assay and protein estimation was performed using the individual precipitate. 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

Precipitated enzyme solution was dialyzed against the same buffer (10 mM phosphate buffer, pH 7.5) for two days with intermittent replacement/changing of the buffer solution, at 4 °C by using a dialysis membrane with flat width

of 10 mm (0.4 in), a diameter of 6 mm when full and capacity ~10 mL/ft. The keratinase assay and protein estimation was performed using the dialyzed sample as detailed in this work.

Determination of Isoelectric point

The isoelectric point is the pH at which the net charge of a protein is zero and fails to move when placed in an electric field. At the isoelectric pH (pI), the solubility of protein is minimal, thus causing the protein to precipitate. First, 12 clean and dry tubes were prepared, followed by the addition of 1 mL of dialyzed enzyme solution and 1 mL phosphate buffer solution each of different pH 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12.5 and 13 in each test tubes. The test tubes were shaken and the degree of turbidity at 0, 10, and 30 minutes were recorded. The pH of the test tube with the highest turbidity deposits is the isoelectric point of the enzyme.

Ion-Exchange Chromatography

The crude concentrated dialyzed enzyme from ***A. faecalis*** **strain TPB18** was used at this stage. A known quantity (3.00g) of glass wool was introduced into the neck region of the column and an appropriate amount of CM-cellulose ($\cong 5.00\ g$) was weighed out and dissolved in a beaker containing 200 ml of distilled water and 1 mL of (0.1%) Na-azide. The gel was allowed to swell in the dark overnight. Three columns A, B, and C were set up, with column B and column-C set on top of column-A. The swollen stationary phase (CM-cellulose) resins were slowly added into column-A which is half-filled with phosphate buffer solution {pH = optimum pH of enzyme} but with constant agitation of the column for proper packing and the tap slightly open. After loading the resin, a known volume (300 ml of {10mM of HCl}) from Column-C were allowed to flow into the main column-A for activation of the resin. For full activation of the resins, the pH of the effluent from column-A must be the same as the pH of the HCl in column C. However, elution buffer solution, from column-B (0.5M potassium phosphate buffer {pH = optimum pH of that enzyme}) was used to equilibrate the resin. The equilibration was continued until the effluent from column-A was the same pH as the buffer solution B. The level of buffer solution was maintained at 4 cm above the stationary phase at all times. Before enzyme loading, the enzyme was concentrated and centrifuged to remove colloidal materials that could clog the resin. The crude concentrated dialyzed enzyme solution (50 ml) was loaded on top of the resin in column-A. The enzymes were allowed to permeate the resin while keeping the tap slightly open. The unbound proteins and unwanted enzymes were eluted with phosphate buffer-B. Finally, to elute the bound enzymes from the resins, solution-D (0.5M of sodium chloride) from an elevated conical flask-D was allowed to flow into conical flask-B containing 300 ml of phosphate buffer-B constantly stirred with a magnetic stirrer and connected to column-A. The flow rate from solution-D into conical flask-B and from conical flask-B into the main column was the same (0.4 ml/min). The elution continued until the effluent from column-A confirms the presence of our enzyme. Fifty (50) fractions of 5 ml each were collected. Fractions were screened for enzyme activities using Ninhydrin and Lowry methods to determine the concentration of free amino acids and soluble peptides/proteins respectively. The absorbance of the fractions were measured using a UV-spectrophotometer at 280 nm wavelength [24].

Keratinase activity

Determination of soluble peptides/proteins concentration, using Lowry method

A known quantity (1.00 g) of a defatted human hair (with a size approximately ≤ 0.5 mm) was mixed with 2 mL of phosphate buffer (pH 12.5) and 10 µL of enzyme solution in test tubes. The solutions were incubated for 30 min at 37 ^{o}C. After incubation, the reaction was terminated by heating the test tubes in a boiling water bath for 5 min. After cooling, the undigested keratin was removed by centrifugation at 3000 rpm for 10 min. One millilitre (1 mL) of the supernatant, standard bovine serum albumin (0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 mg/mL) were mixed with 5 mL of 4.2% $NaCO_3$ and 0.5 ml of Folin Ciocalteau phenol reagent. The reaction mixtures were precipitated by standing in ice for 15 min and the insoluble precipitates were removed by centrifugation at 1000 rpm for 10 min. The absorbance of the supernatants of the reaction mixtures were read at 660 nm. A control assay without the enzyme in the reaction mixture was prepared and used as the blank in all the spectrophotometric measurements.

Quantitative estimation of free amino acid concentration using ninhydrin method

This assay is based on the fact that two molecules of ninhydrin (2, 2-dihydroxyindane-1, 3-dione) react with a free alpha-amino acid to produce a deep purple or blue color known as Ruhemann's purple [25]. A known volume (1ml) each of the clear supernatant ''SoluSDHY'' and 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. The pH of the reactions was maintained at 5.5 by phosphate buffer solution. These were properly mixed using a vortex machine, after which the test tubes were covered with aluminum foils, followed by incubation at 90 ^{o}C for 20 min. However, after cooling to room temperature, one milliliter (1 mL) of absolute ethanol was added and properly mixed, followed by a measurement of absorbance at 570 nm. The concentrations of amino acids in the samples were determined via extrapolation from the standard graph of Cysteine.

Keratinase assay using direct absorbance method

The purified enzyme fractions obtained from ion-exchange chromatography were mixed with a buffer solution in the ratio of 1: 4 (200 µL enzyme extract: 800 µL phosphate buffer solution of pH 12.5) were added to 20 mg of defatted human hair. The solutions were incubated for 1 hour at 37 ° C. After incubation, the solutions were cooled in ice water for 10 min. The absorbance of the solutions were measured using a UV-spectrophotometer at 280 nm wavelength [24].

Characterization of keratinase enzyme

Determination of Degree of human hair degradation (DHHD-1(%) and DHHD-2) by Keratinase from *A. faecalis* strain TPB18 for a specific period

A known quantity (2.00g) of a defatted human hair (with a size approximately ≤ 0.5 mm) were mixed with 2 mL of

phosphate buffer (pH 12.5) and 2 µL of enzyme solution in ten sets of triplicates of test tubes labeled (**A_1, A_2, A_3; B_1, B_2, B_3; C_1, C_2, C_3; D_1, D_2, D_3; E_1, E_2, E_3; F_1, F_2, F_3; G_1, G_2, G_3; H_1, H_2, H_3; I_1, I_2, I_3 and J_1, J_2, J_3**) and kept in an orbital shaking incubator at 37 ^oC for 19 days at 200 rpm (operational only during the day time). However, each set of triplicates were analyzed at two days intervals (**1st, 3rd, 5th, 7th, 9th, 11th, 13th, 15th, 17th, and 19th day**). On each day of analysis, the enzymatic actions in the triplicate samples to be analyzed were terminated by heating them in a boiling water bath for 5 min. After cooling the samples, to room temperature, the undigested keratin fibres were removed by centrifugation at 2000 rpm for 10 min to determine DHHD-1(%), while the supernatants were used to determine DHHD-2 via the enzyme assays. The DHHD-1(%) and DHHD-2 were calculated using different procedures. For DHHD-1(%), the residual human hair was washed, dried, and scaled to calculate DHHD-1 (%) [20] (Ezenwali, 2022) using the following equation [26](Kim, 2001);

DHHD-1 (%) = (TWHH-RWHH) × 100/TWHH. Where WHH is the total initial weight of human hair and RWHH is the residual weight of human hair.

However, the second DHHD-2 was calculated by measuring/determining the concentrations of free amino acids, soluble peptides, and soluble proteins, the by-products of keratin degradation in the supernatants of the reaction media at three days intervals, using Ninhydrin and Lowry methods. The absorbance of the supernatants were measured using a UV-spectrophotometer at 280 nm wavelength [24].

Effect of pH on the activity of keratinase

The activity of keratinase was examined within the pH ranges of 2, 4, 5.5, 7, 9, 10, and 12.5 using the following buffer solutions of 0.1 M citrate phosphate buffer- pH, (2.00 - 5.50); 0.1 M sodium phosphate buffer- pH, (7.00); 0.1 M carbonate bicarbonate buffer pH (9.00 - 14.00) with defatted human hair as substrate. After the incubation period, the enzyme solutions were centrifuged at 4,000 rpm at 4 ºC and 1mL aliquots of the supernatant each were taken and the keratinase activity was assayed using Lowry, Ninhydrin methods and direct absorbance at 280 nm.

Effect of temperature on the activity of keratinase

The activity of **keratinase** was examined within the temperature ranges of 0, 4, 25, 37, 60, and 90 ºC with defatted human hair as substrates. After the incubation period, the enzyme solutions were centrifuged at 4,000 rpm at 4 ºC and 1mL aliquots of the supernatant each were taken and the keratinase activity was assayed using Lowry, Ninhydrin methods and direct absorbance at 280 nm.

Effect of metals on the activity of keratinase

The enzymatic activity of **keratinase** was assayed in the presence of various metals (Zn^{2+}, Ca^{2+}, Mg^{2+}, Fe^{2+}, Ni^{2+}, Cu^{2+}, Co^{2+}, Al^{3+}, Hg^{2+} and Pb^{2+}) with defatted human hair as substrate. The effect of these various co-factors on keratinase activity was determined by incubating the keratinase in 0.1 M sodium phosphate buffer- pH 12.5 for 1hr. After the incubation period, the enzyme solutions were centrifuged at 4,000 rpm at 4 ºC and 1mL aliquots of the supernatant each were taken and the keratinase activity was assayed using Lowry, Ninhydrin methods and direct absorbance at 280 nm.

III. Results

Screening for keratinolytic bacteria

In order to isolate the keratinolytic bacteria from an aliquot of sterilized salt medium containing contaminated human hair that has been incubated in an orbital shaking incubator at 37 ^oC for 72 h at 200 rpm. Several wire loops full of the medium were inoculated into a nutrient agar medium and incubated at 37 °C. **Plates 1** represents the cultured petri dish with four different colonies obtained from the salt medium. **Plate 2** represents the pure cultures (isolates) of the different four colonies sub-cultured from plate 1.

Keratinase production by *A. faecalis* strains TPB18 and AIR10 for a Period of 12 days

Both keratinases and liberated peptides/polypeptides from keratin were detected as soluble peptides/polypeptides by Lowry methods. Cultivation of *A. faecalis*, strains TPB18 and AIR10 in hair medium supplemented with 0.01% yeast extract (HMY) for 12 days indicates that the highest significant difference in keratinase production between *A. faecalis*, strains TPB18 and AIR10 occurred on the 4th day of production (**Figure 8**).

DNA extraction

PCR Assay

Sequencing

Isolate 3 has 98.72% Pairwise Identity to *A. faecalis* strain AIR10 with NCBI accession number MG835355.1. The e value is 0 while the isolate sequence is as shown in **figure 3**. Isolate 4 has 96.01% Pairwise Identity to *A. faecalis* strain TPB18 with NCBI accession number MW475277.1. The e value is 0 while the isolate sequence is as shown in **figure 4.**

Isolation and Identification *A. faecalis* strains

Isolation of four different isolates and molecular identification of only two; *A. faecalis* strain AIR10 with NCBI accession number MG835355.1 and *A. faecalis* strain TPB18 with NCBI accession number MW475277.1, the two motile gram-negative rods, catalase and oxidase positive bacteria (**Table 1**). These were isolated from hair saloon waste dumping site based on the degradation of human keratin and growth in a minimal medium with human hair as the only carbon and nitrogen source. These identifications were based on cell morphology, physiological characteristics, biochemical and molecular analyses. Only isolates-2 and -4 gave positive reactions to spore staining and indole tests. The 16S rDNA sequence analysis of two *A. faecalis*, strains (AIR10 and TPB18) with NCBI accession numbers (MG835355.1 and MW475277.1) showed (98.72% and 96.01% homology) high sequence similarities respectively with other *A. faecalis* sequences deposited in the GenBank (**Figures 1, 2 and 5**).

Effect of Metal ions on keratinase activity

The purification process to identify the extracellular keratinase in *A. faecalis* strain TPB18 is outlined in materials and methods under enzyme purification. The effect of metal

ions (co-factors) on keratinolytic degradation of human hair keratin samples were studied using two different assay methods. The best metal ion for keratinase degradation of human hair keratin sample to soluble protein concentrations as determined by the folin-phenol reagent method was Co^{2+} (Figure 9), while the best metal ions for liberation of free amino acids as determined by the Ninhydrin method were Co^{2+} and Hg^{2+} (**Figure 10**).

Effect of temperature on keratinase activity

The effect of temperature on keratinolytic breakdown of human hair keratin samples were studied at varying temperature, using two different assay methods. The optimum temperature for human hair keratin sample degradation to soluble protein concentrations as determined by the folin-phenol reagent method was 37 ºC (**Figure 11**) , while the optimum temperature for liberation of free amino acids as determined by the Ninhydrin method was (37-60 ºC) as seen in **figure 12**.

Effect of pH on keratinase activity

The effect of change in pH on the activities of keratinase in biodegradation of human hair keratin samples were studied, using two different assay methods. The optimum pH for human hair keratin sample degradation to soluble protein concentrations as determined by the folin-phenol reagent method was 12.5 (**Figure 13**), while the optimum pH for liberation of free amino acids as determined by the Ninhydrin method was 5.5 but with plateau within pH of 7.0, 9.0, 10.0, and 12.5 (**Figure 14**).

Degradation of Human Hair by Keratinase enzyme from *A. faecalis* strain TPB18 for Nineteen days

The concentration of soluble proteins and aromatic amino acids determined by the Folin phenol reagent method and by ultraviolet light spectrophotometry at 280 nm respectively as derived from hair degradation in our cultured medium were greatest on 19^{th}, followed by 9^{th} days of analysis (**Figure 15**). The observed degradation peak on 9^{th} day in this study indicates that the maximum production of free amino acids occurred on the 9^{th} day according to Ninhydrin method. Our data further depict that the concentration of liberated free amino acids from the keratin substrate were significantly far greater that the concentrations of the liberated soluble proteins.

IV. Discussion

In this current research, identified keratinolytic bacterium was utilized in the production of human hair degrading keratinase which was characterized based on some biotechnological properties. Gram staining and morphological investigations indicated that the two most active strains isolates of *A. faecalis*, are flagellated alkaline-tolerant gram-negative rod-shaped aerobic bacteria that belong to the family of *Alcaligenaceae*, in the order of *Burkholderiales*, while the biochemical tests affirm their positive reactions to catalase, oxidase, motility, and methyl red tests, except isolate-4 that gave negative reaction to methyl red tests. Only isolates-2 and 4 gave positive reactions to spore staining and indole tests. The sequence analysis of 16S rDNA showed high sequence identity to two subspecies of *A. faecalis*, strains AIR10 and TPB18 (98.72% and 96.01% homology) for the isolate-3 and 4 respectively. The sequences were compared with NCBI accession numbers MG835355.1 and MW475277.1 of GenBank.

Most research works in the literature on microbial keratin degradation focused 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 knowing fully well that there might be an intrinsic molecular difference in the two keratins and too on the catalytic mechanisms of the two different keratinases involved. A clear understanding of the above-mentioned facts will throw more light on the differences in isolation methods, purification procedures, genotypic/molecular characterization, and optimization values. However, a better understanding of how human hair keratinase acts towards its specific substrate would facilitate the generation of a new technique targeted toward the utilization of this isolated keratinase in human hair keratinous waste recycling and future biotechnological applications.

Biodegradation of human hair keratins were carried out at varying pH, temperature and in the presence of different metal ions. The optimum pH and temperature; and the best co-factors for human hair keratinase activity using Lowry and Ninhydrin methods were found to be at pH {(12.5) and (5.5, 9, 10)}, temperature of {(37 °C) and (37-60 °C)} and co-factors of {(Co^{2+}, Al^{2+}, Hg^{2+}) and (Co^{2+}, Hg^{2+}, Cu^{2+})} respectively. The observed dual pH peaks of acidity (5.5) and alkalinity (9 and10) identified under Ninhydrin method 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 [7].

Keratinases are serine and metalloproteases, and their active sites are formed by several conserved residues [27]. Our observation that the co-factor (cobalt) exhibited the maximum activity of the newly isolated keratinase is in total agreement with the report of Li, 2021, who stated that the active site of FisCP a keratinase enzyme from *Fervidobacterium islandicum* AW-1 contains several amino acids (His253, Glu254, His257, and Glu283) and a Co^{2+} ion which is important for coordination of the substrate [27]. Recently, keratinases are gaining larger applications in many industries as eco-friendly methods/processes that are fast replacing many conventional chemical processes that can generate a large amount of sulfide and other toxic chemicals in both aquatic environment and atmosphere. Moreover, as part of our ongoing research work on the application of keratinase via the use of its value-added by-products as a supplement in poultry feed formulation, it is possible to

recycle these building blocks of keratin, thereby circumventing high endogenic energy required by organisms in the biosynthesis of these vital micro molecules (non-essential amino acids). However, these organisms deploy the simple salvage pathways that require little endogenic energy for the recycling of these monomers needed for the biosynthesis of protein. This faster biosynthetic pathway of endogenous proteins in the body cells culminates in faster growth/development of the organism. Our results regarding increased enzymatic activities of keratinase at temperatures of 0, 4, and 90 ºC is consistent with previous studies [28] that established that keratinase is a heat-stable enzyme.

The observed difference in the trend of curves between the concentration of soluble proteins and aromatic amino acids determined by the Folin phenol reagent method and by ultraviolet light spectrophotometry at 280 nm respectively, may be accounted for by the liberated soluble peptides/polypeptides from human keratin.

V. Conclusions

In this study, a purified keratinase enzyme extracted from soil isolates of *A. faecalis* strain TPB18 was used for the degradation of human hair. The optimal conditions were determined as pHs (12.5 and 5.5), temperature range of (37-60 ^{o}C), and incubation time (5 days). The percentage degree of human hair degradation (DHHD) by *A. faecalis* strain TPB18 was found to be 60% on day 5 of human hair degradation. Keratinase produced by the organism was about the size of 5.6kd.

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Acknowledgments

Conflicts of Interest

The authors declare no conflict of interest.

Abbreviations

(-SH) Sulfhydryl

dried DHHM dried defatted human hair medium

HMY hair medium supplemented with 0.01% yeast extract

InsoluPPDK insoluble peptides, and partially degraded keratins

SoluSDHY' clear supernatant (soluble peptides, and free amino acids)

SEM Scanning electron microscopy

DHHD-1(%) Percentage Degree of human hair degradation

DHHD-2 Degree of human hair degradation

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Figure legends

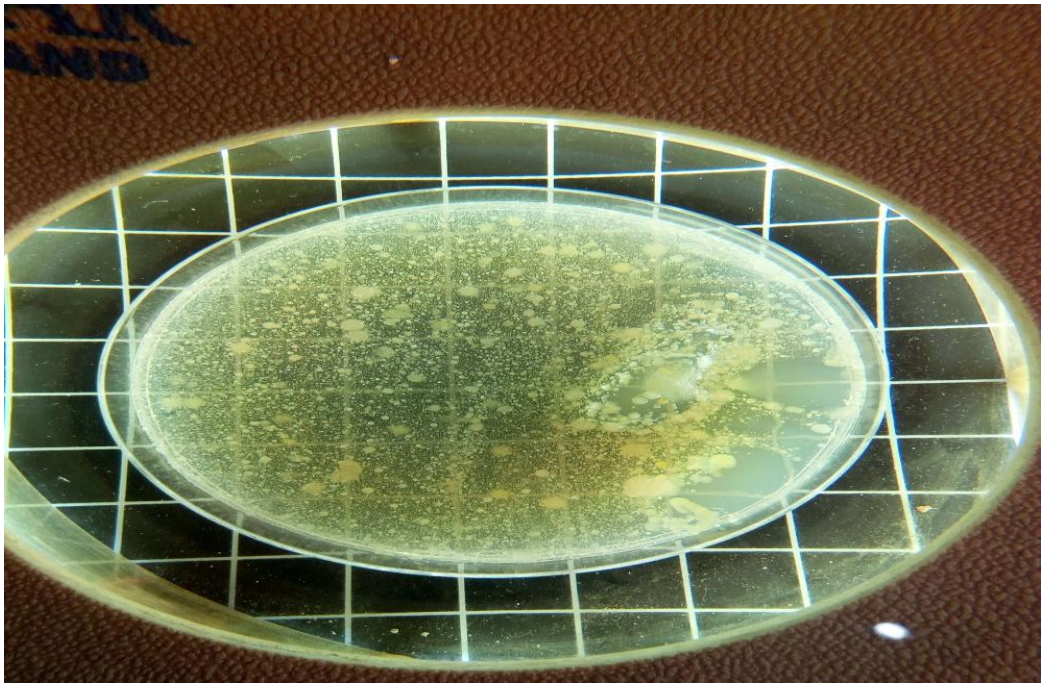


Plate-1: Agar medium containing different colonies within 24 hr of incubation at 37 ^{o}C

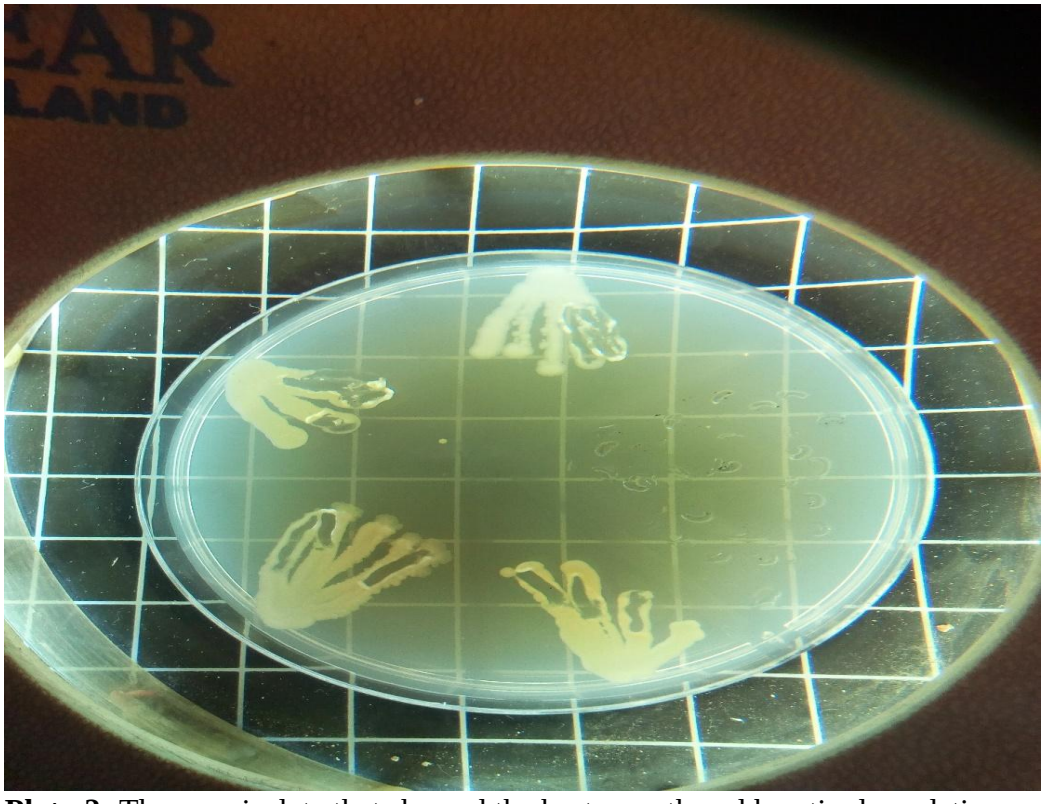


Plate-2: The pure isolate that showed the best growth and keratin degradation

Table-1: Biochemical Tests on the four isolated *A. faecalis* strains`

Sample	Gram Staining	Spore Staining	Catalase Test	Motility Test	Methyl Red Test	Indole Test	Oxidase Test
Isolate-1	- Rod	-	+	+	+	-	+
Isolate-2	- Rod	+	+	+	+	+	+
Isolate-3	- Rod	-	+	+	+	-	+
Isolate-4	- Rod	+	+	+	-	+	+

Figure 1: Gel image showing genomic DNA extracted from isolates

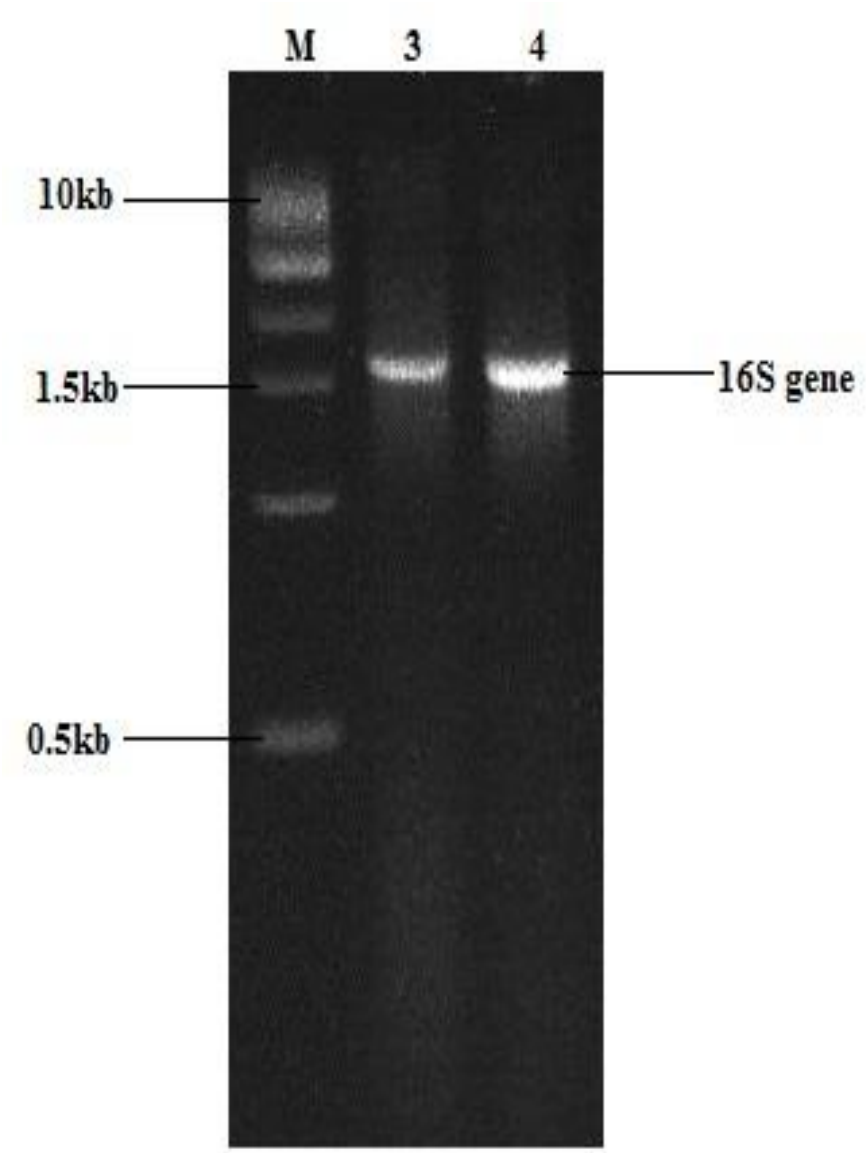


Figure 2: Gel image showing 16SrRNA gene for *A. faecalis* strains AIR10 and TPB18 (Lanes 3 and 4 respectively) at about 1.5 kb. M is 10 kb DNA ladder NEB

ACGAGTAACTCTAGAGACTGCCATCGCCCCGGGCCAGGATACCCTCTAAAGGGTTGGCCGCGGCCC
CGGGCGATGATCCCCCCTTCAGGGGGGGCCACCCCCCCGGCTCCTCGAAGCCGGAGTGCCGCATAC
ACCCTACGGGCGCAGGGGGAAATTAACTCCTCTTGTATTGGAGCCGGGGCGGGGGTCACAACGCAG
AAAATTTGGTCCCGGGCCCATCGCACGATCCACCCTCCTTTTGAGAGAGAAAGCACCGGCTAACTA
CGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGTG
TGTAGGCGGTTCGGAAAGAAAGATGTGAAATCCCAGGGCTCAACCTTGGAACTGCATTTTTAACTG
CCGAGCTAGAGTATGTCAGAGGGGGGTAGAATTCCACGTGTAGCAGTGAAATGCGTAGATATGTGG
AGGAATACCGATGGCGAAGGCAGCCCCCTGGGATAATACTGACGCTCAGACACGAAAGCGTGGGG
AGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGTCAACTAGCTGTTGGGGCCGT
TAGGCCTTAGTAGCGCAGCTAACGCGTGTGTCTGGAGGATACCGATAGCGAAGGCGGCCCCGCTGG
AATAATACTGATGCTCACACACGCAGCCTGGGGTGCAAAGCAGGATTAGTATACCCTGGTAGTCCG
GGCCCCTAAACGACGATCACCTATCTGTTGCGTCGTAGGCATTTTTAGCCTCACCAACGCAATTATA
GTTGCCCCCCTGGGGAGTACGGTCGCCAGAATAAATTCAAAGGCAATGAGGGAGACCCGCCATACC
AGTGCATGATTCGATACATTCGAAAGCCACCGGTACA

Figure 3: Isolate 3 with 98.72% Pairwise Identity to *A. faecalis* strain AIR10 with NCBI accession number MG835355.1.

GGGGACGTCATAGAGCTGATCATGGCTCAGGTGCGGTTGGAACACTTCTTTATAGTTTGCCAGGGCT
CGACGGGTGAGTAATATATCTCTTCGTGCCCTGTACCGGGGGATAACTACTCGAAAGAGTGGCTAA

TACCGCATACGCCCTACGGGGGAAAGGGGGGGATCGCAAGACCTCTCACTATTGGAGCGGCCGATA
TCGGATTAGCTAGTTGGTGGGGTAAAGGCTCACCAAGGCAACGATCCGTAGCTGGTTTGAGAGGAC
GACCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTG
GACAATGGGGGAAACCCTGATCCAGCCATCCCGCGTGTATGATGAAGGCCTTCGGGTTGTAAAGTA
CTTTTGGCAGAGAAGAAAAGGTATCCCCTAATACGGGATACTGCTGACGGTATCTGCAGAATAAGC
ACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTAATCGGAATTACTGG
GCGTAAAGCGTGTGTAGGCGGTTCGGAAAGAAAGATGTGAAATCCCAGGGCTCAACCTTGGAACTG
CATTTTTAACTGCCGAGCTAGAGTATGTCAGAGGGGGGTAGAATTCCACGTGTAGCAGTGAAATGC
GTAGATATGTGGAGGATACTGATGGCGAAGGCAGCCCCTGGGATATACTGACGCTCAGACACGAA
GCGTGGGGAGCAACAGGATTAGATACCTTGTAGTCCACGCCTAACGATGCCACTAGCTTTGGGCGT
TAGCTTTGTAGCCCGCAACGTGAGTGACCGCTTGGAGTACGTCGAGTACTCAGGATGGGACCGGCA
CGGGGATTGTATTCGAGCACGAAAACCTACCC

Figure 4: Isolate 4 with 96.01% Pairwise Identity to *A. faecalis* strain TPB18 with NCBI accession number MW475277.1.

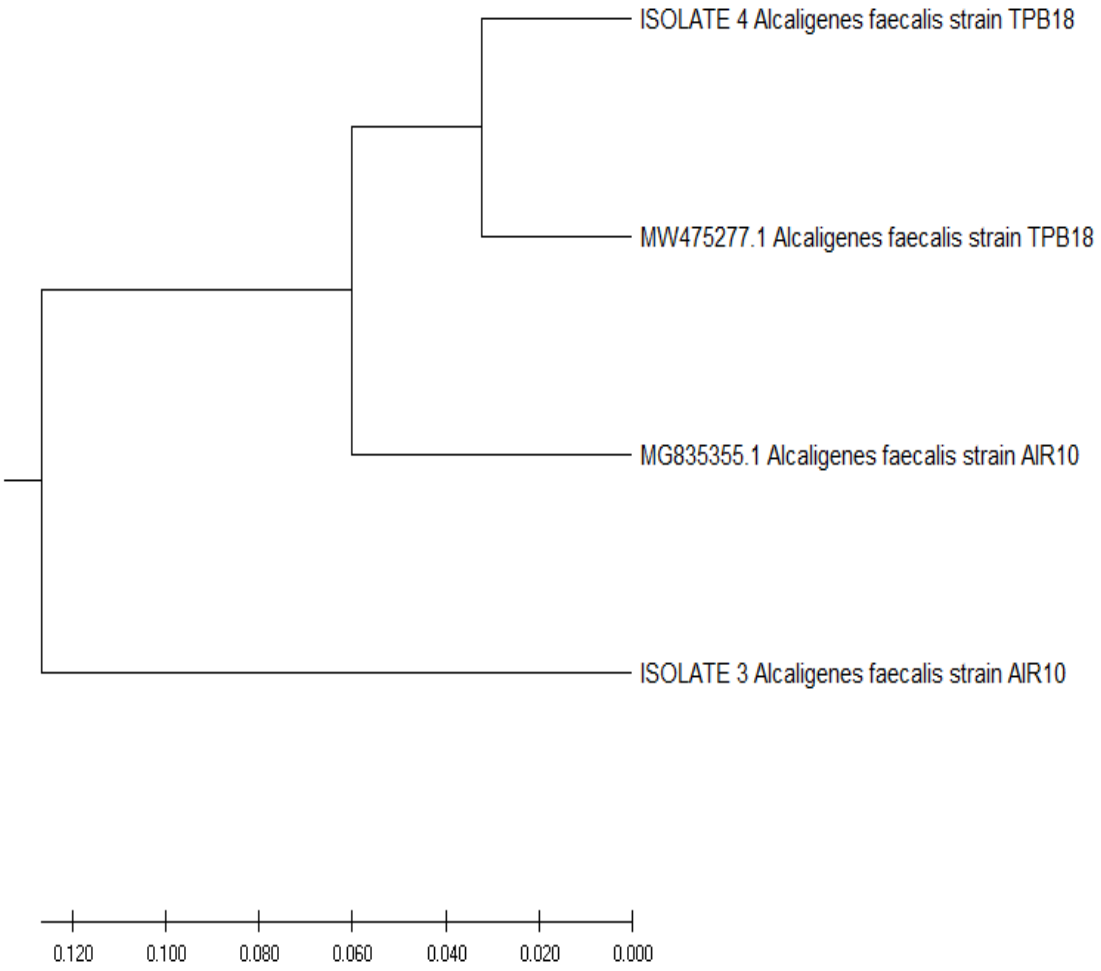


Figure 5: Phylogenetic trees inferred from 4 nucleotide sequences. The phylogenetic analyses were conducted with a maximum composite likelihood (ML) and are in the units of the number of base substitutions per site. The numbers in front of the species are GenBank accession numbers

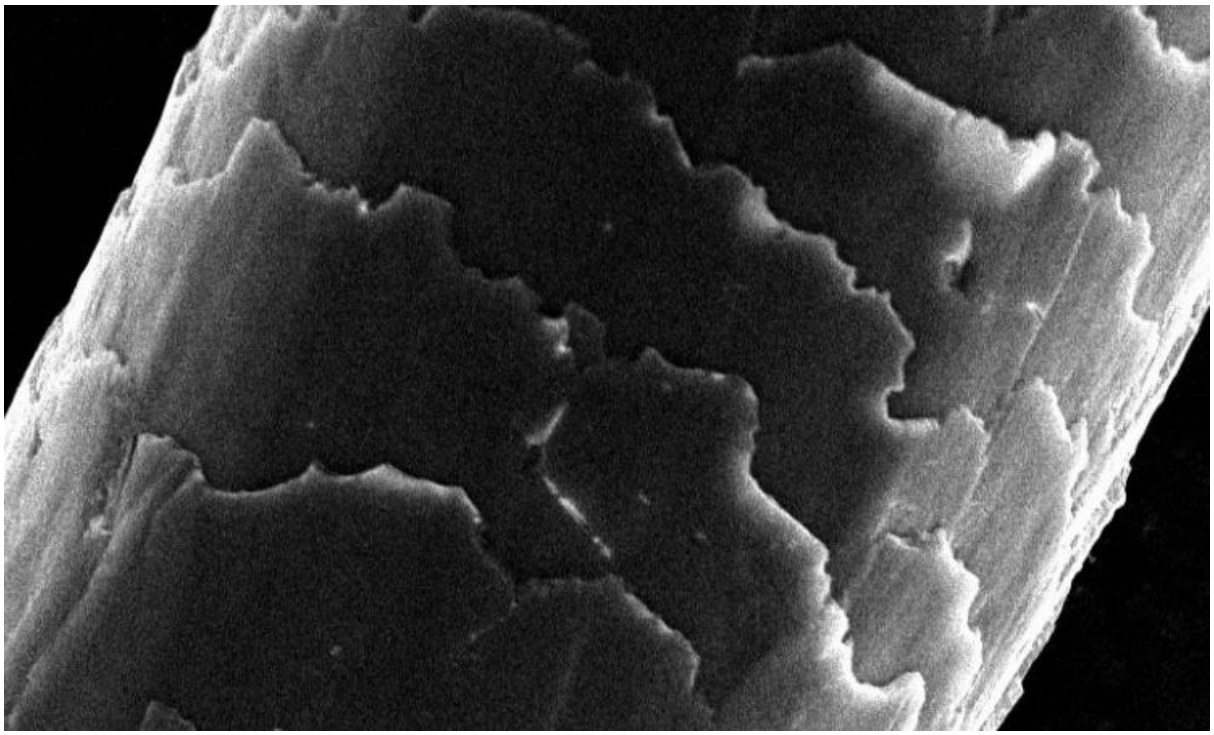


Figure 6: Scanning electron microscopy of control, intact human hair. Scale bar: 10 µm.

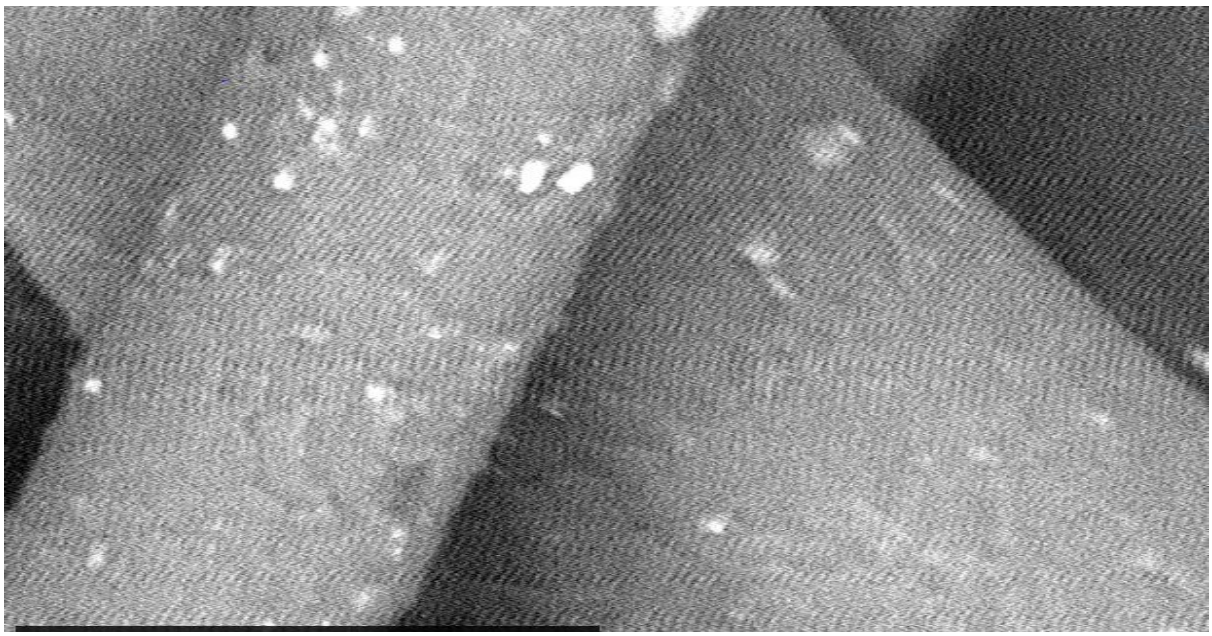


Figure 7: Scanning electron microscopy of defatted human hair after 12 days of degradation with *A. faecalis* strain TPB18. Scale bar: 10 µm.

Scanning electron microscopy (SEM)

Figures 6 and **7** represent the scanning electron microscopy of intact human hair and defatted human hair after 12 days of degradation with *A. faecalis* strain TPB18 respectively.

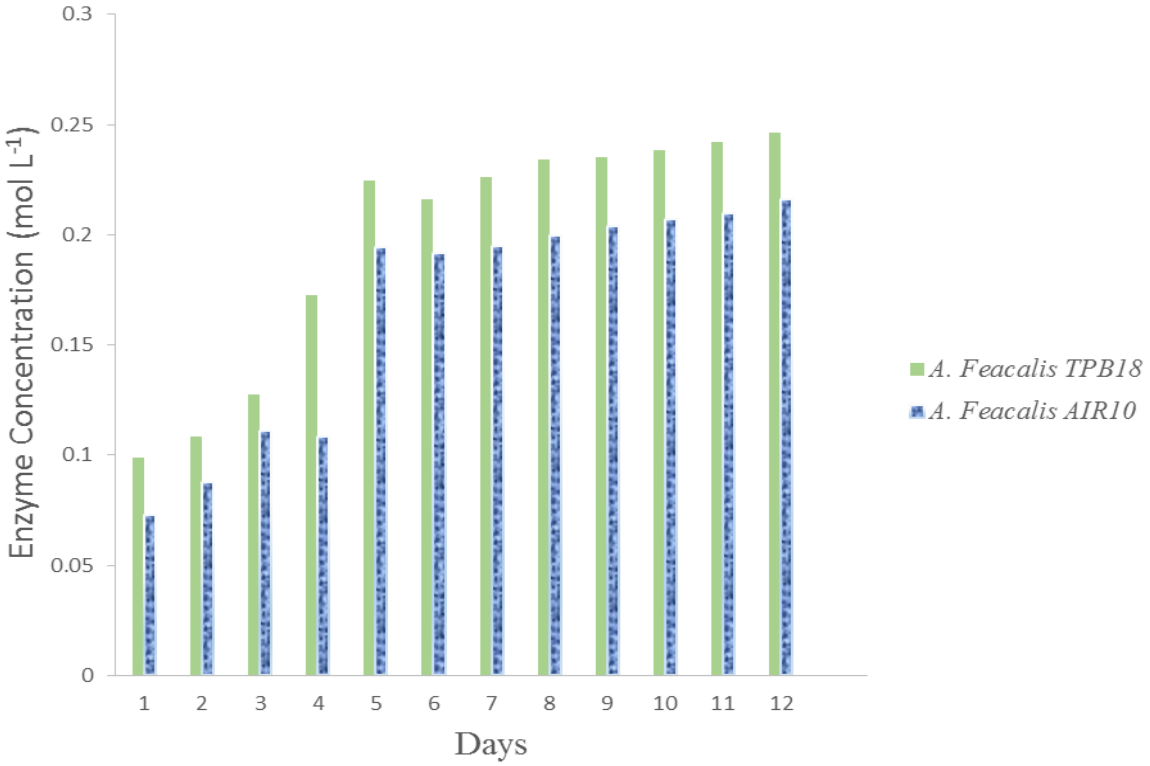


Figure 8: Keratinase Production by *A. faecalis* strains TPB18 and AIR10 for a Period of 12 days

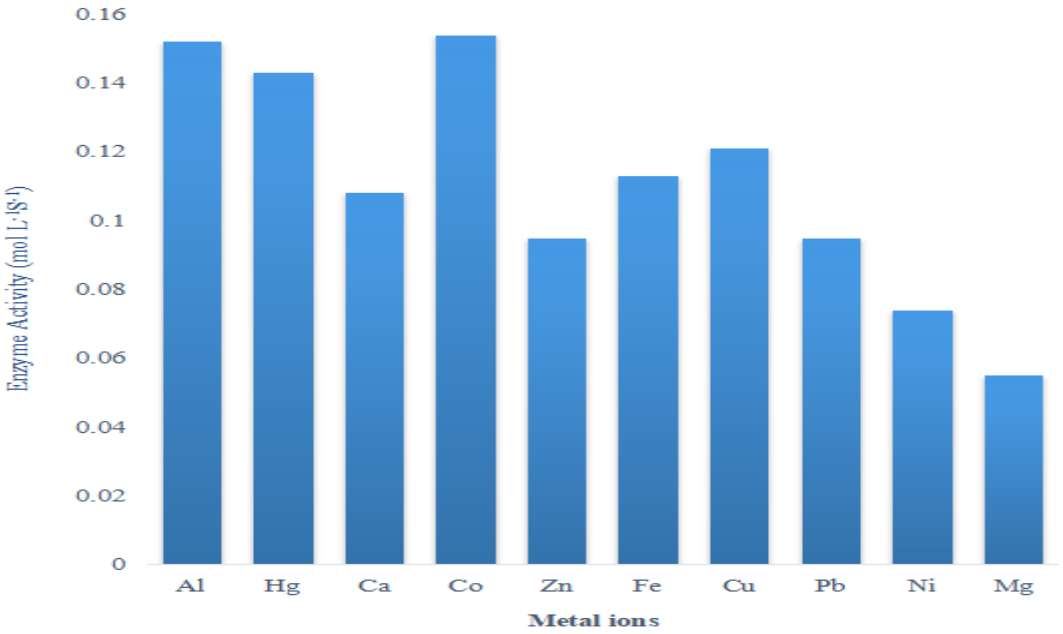


Figure 9: The Effect of metal ions on the keratinase activity using Lowry method

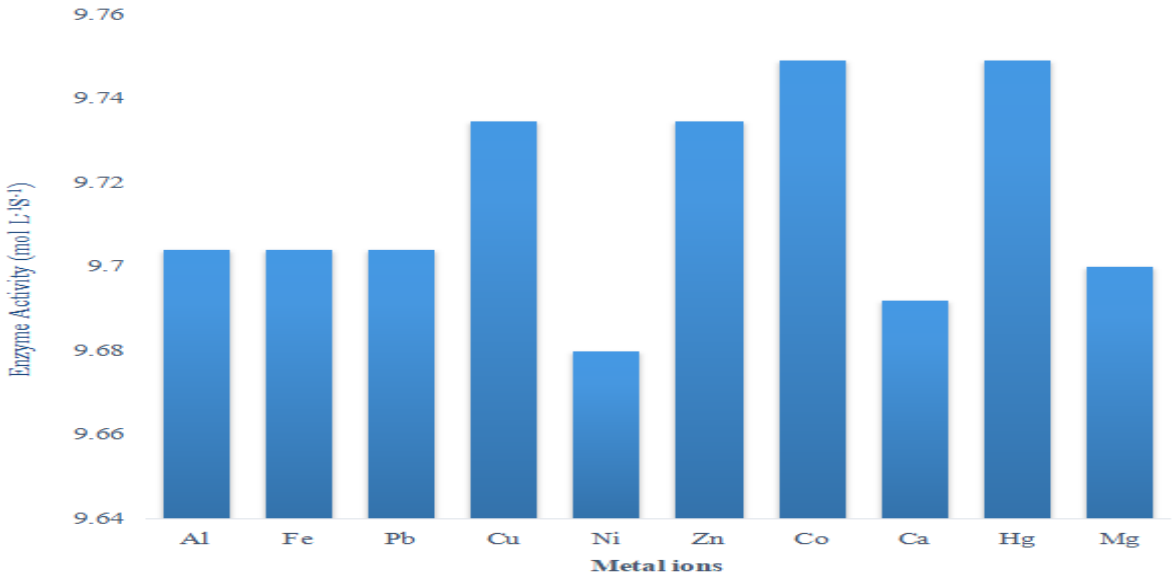


Figure 10: Effect of metal ions on keratinase activity using Ninhydrin Method

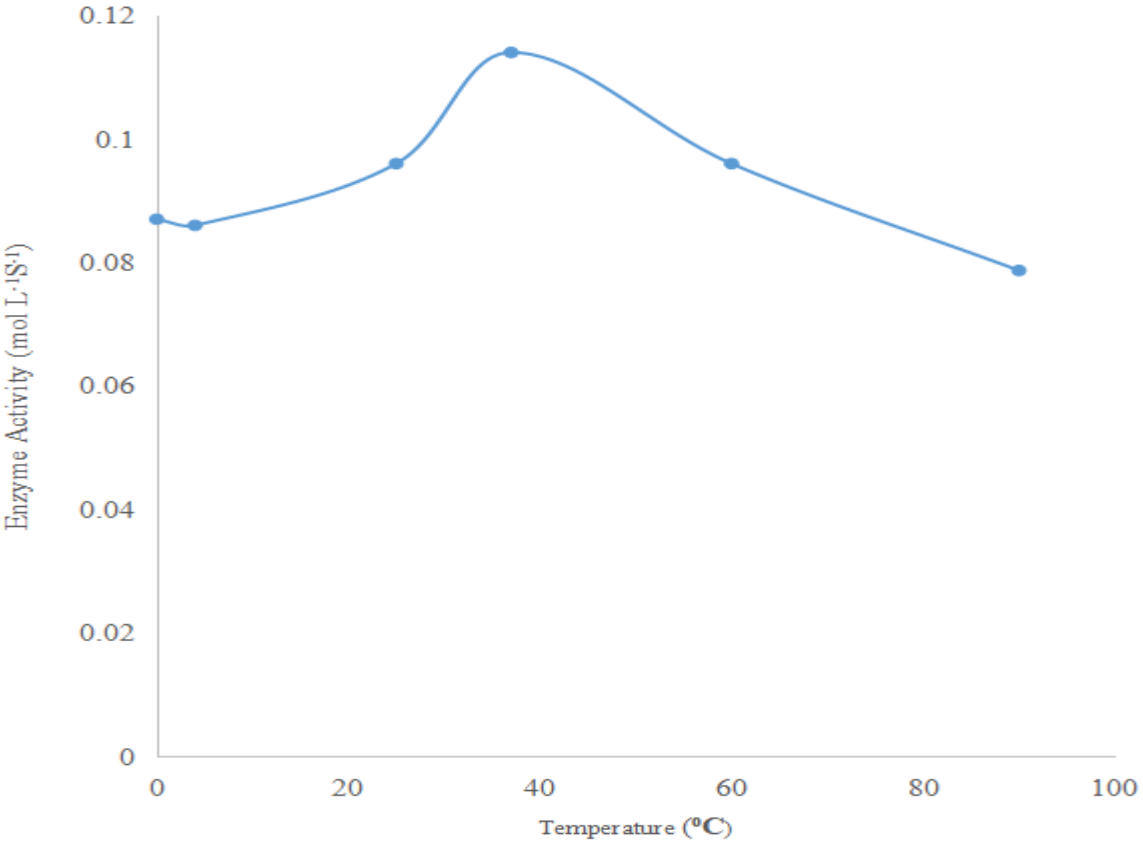


Figure 11: The optimum temperature of Keratinase activity was detected using Lowry Method at different temperatures 0–90 °C for 1 h with human hair keratin as substrate.

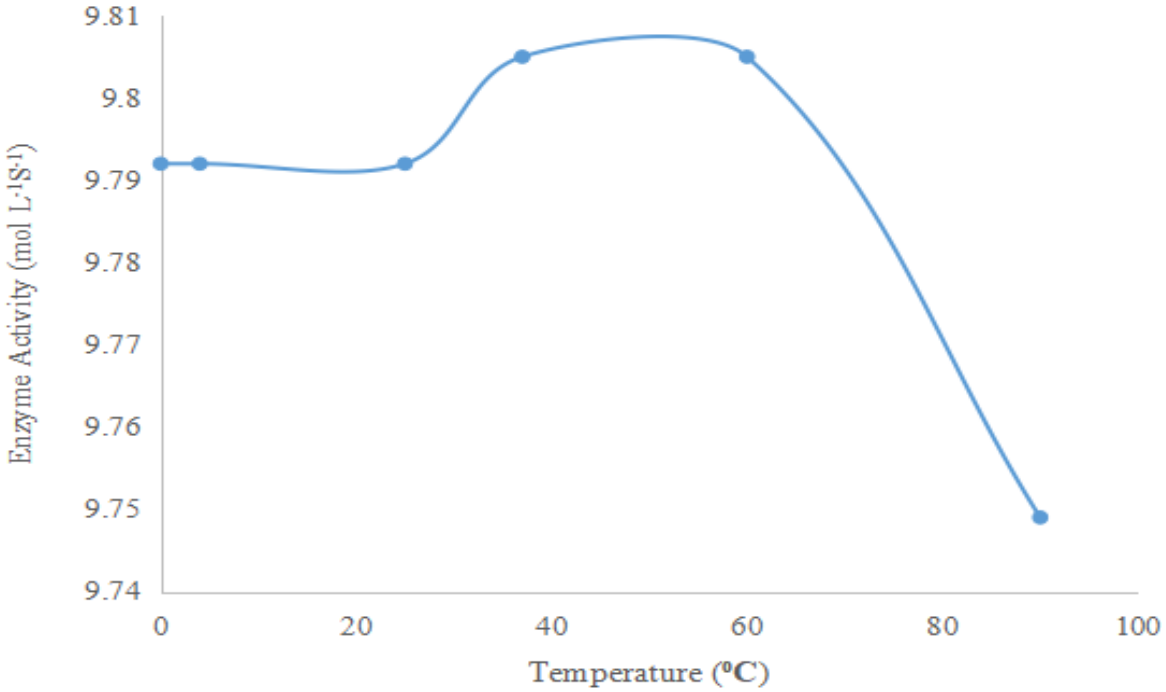


Figure 12: The optimum temperature of Keratinase activity was detected using Ninhydrin Method at different temperatures 0–90 °C for 1 h with human hair keratin as substrate.

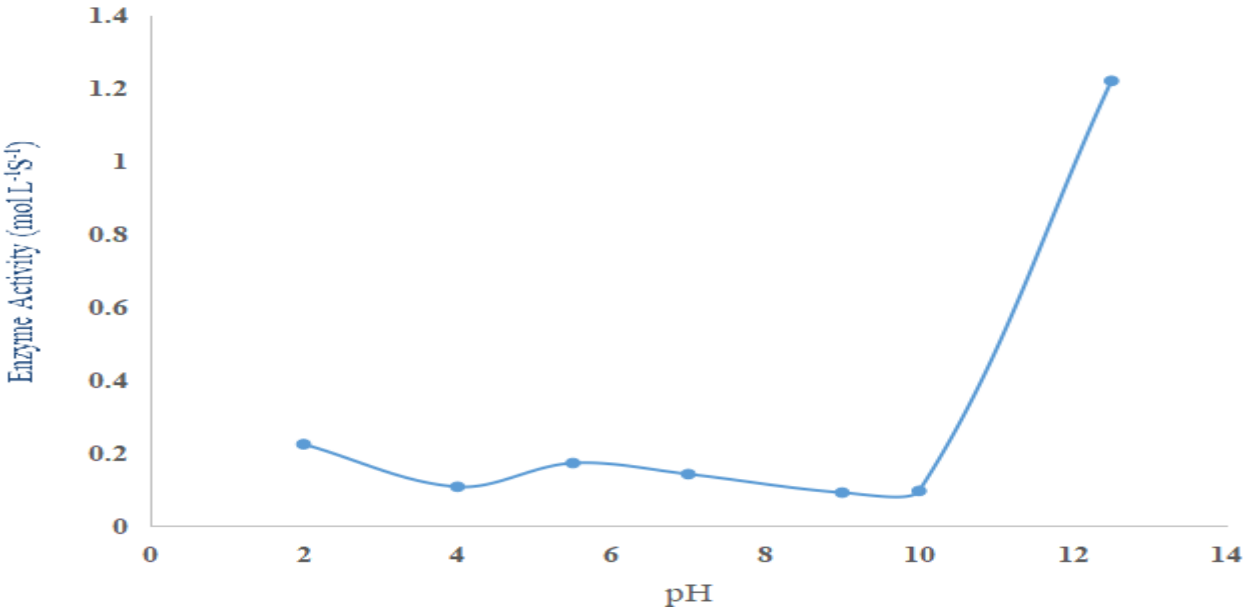


Figure 13: The optimum pH of keratinase activity on human hair keratin was determined at 37 °C in various pH buffers (pH 2~12.5) using Lowry method.

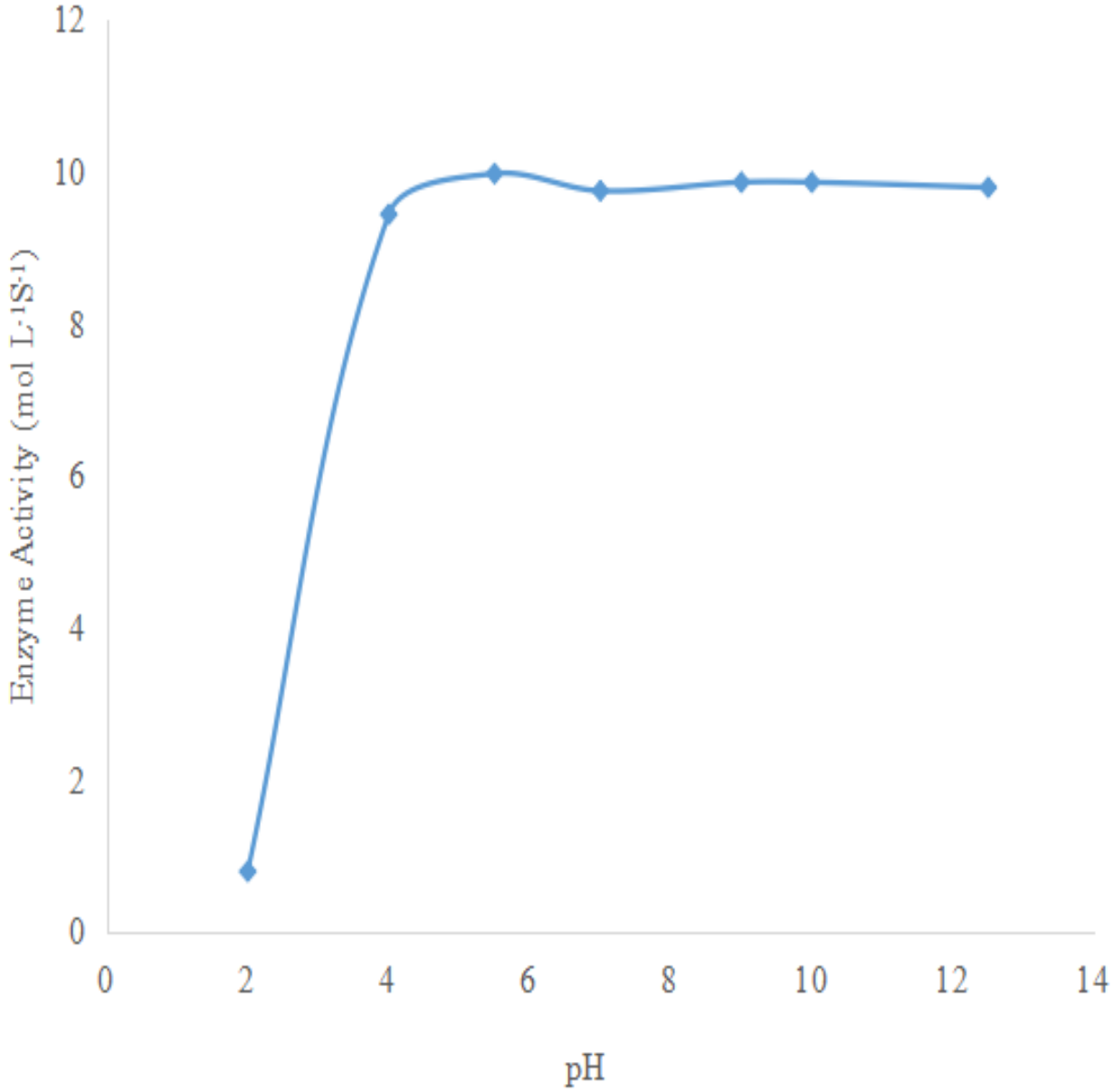


Figure 14: The optimum pH of keratinase activity on human hair keratin was determined at 37 °C in various pH buffers (pH 2~12.5) using Ninhydrin method.

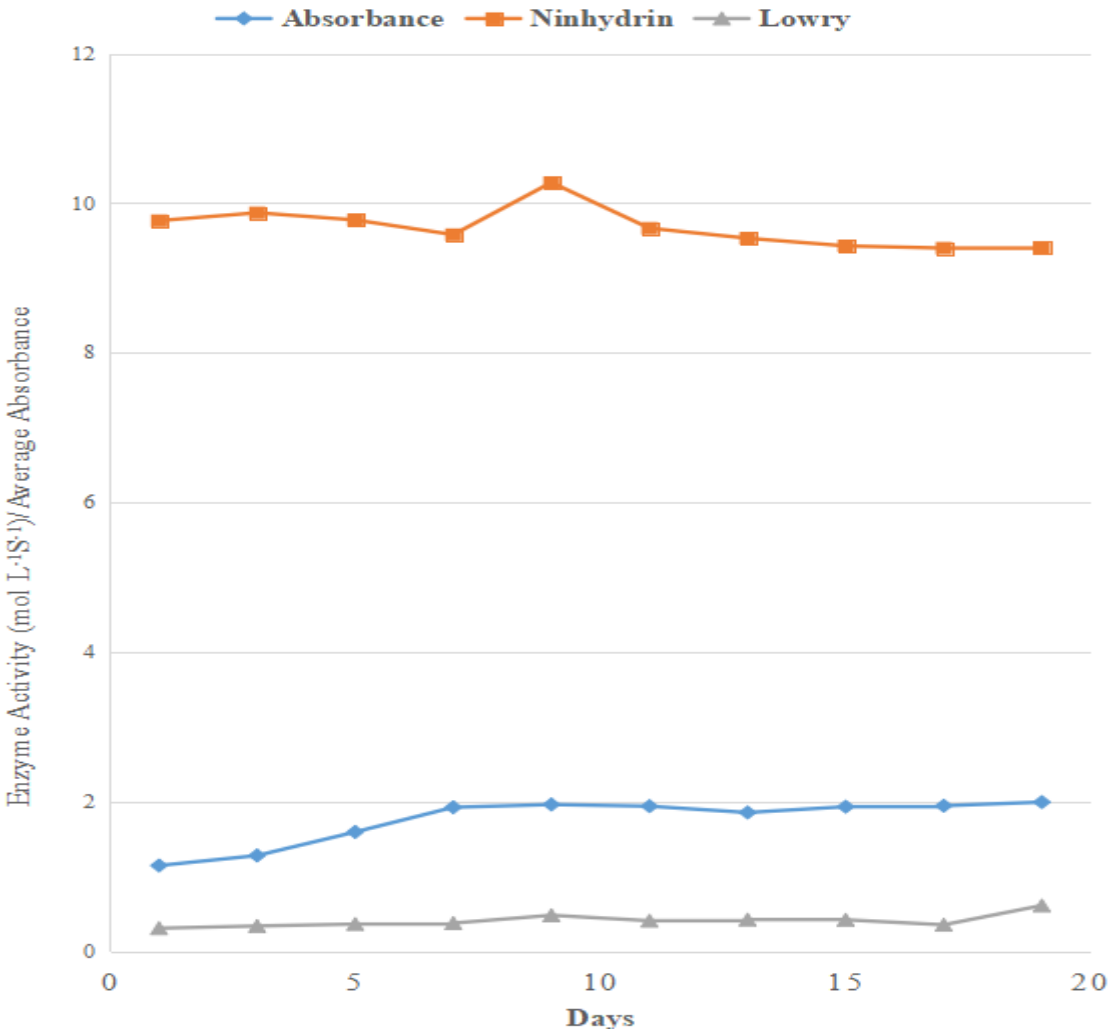
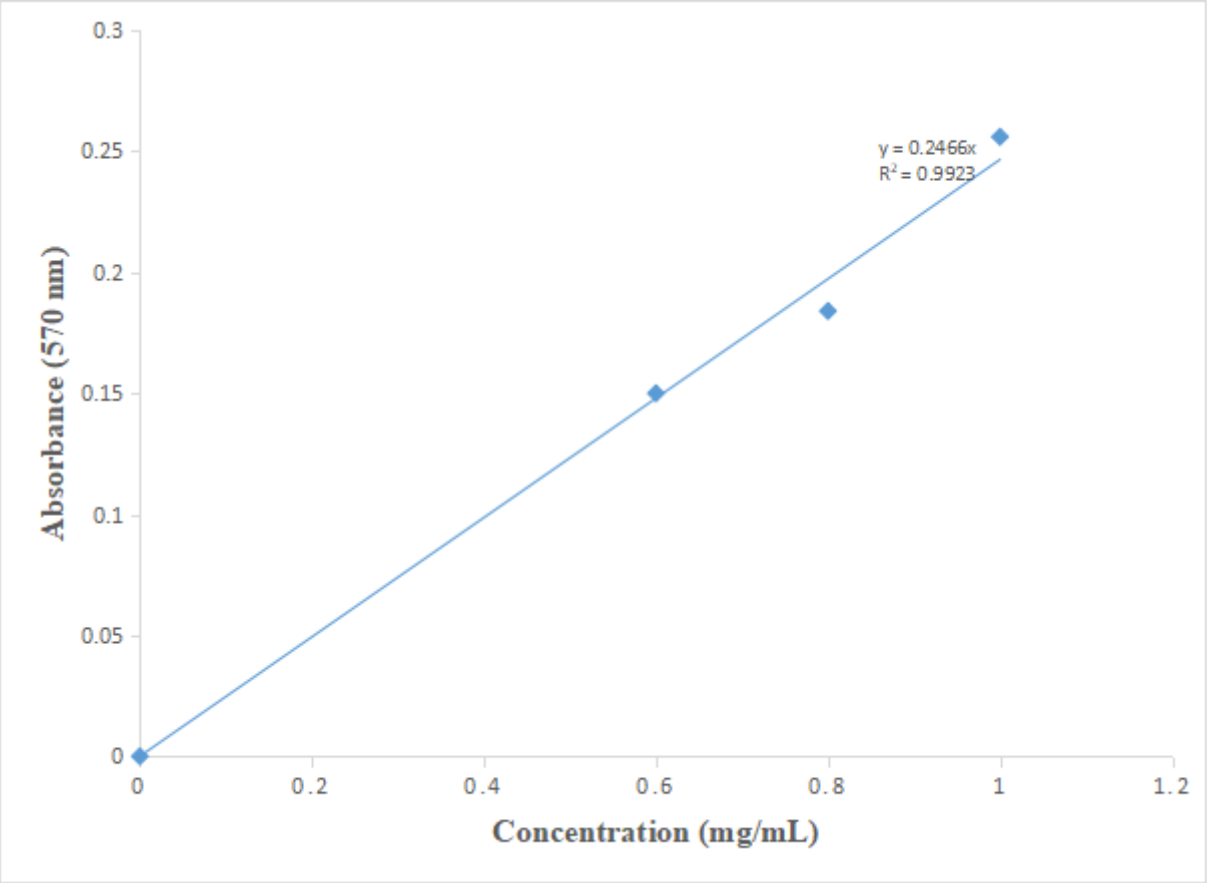
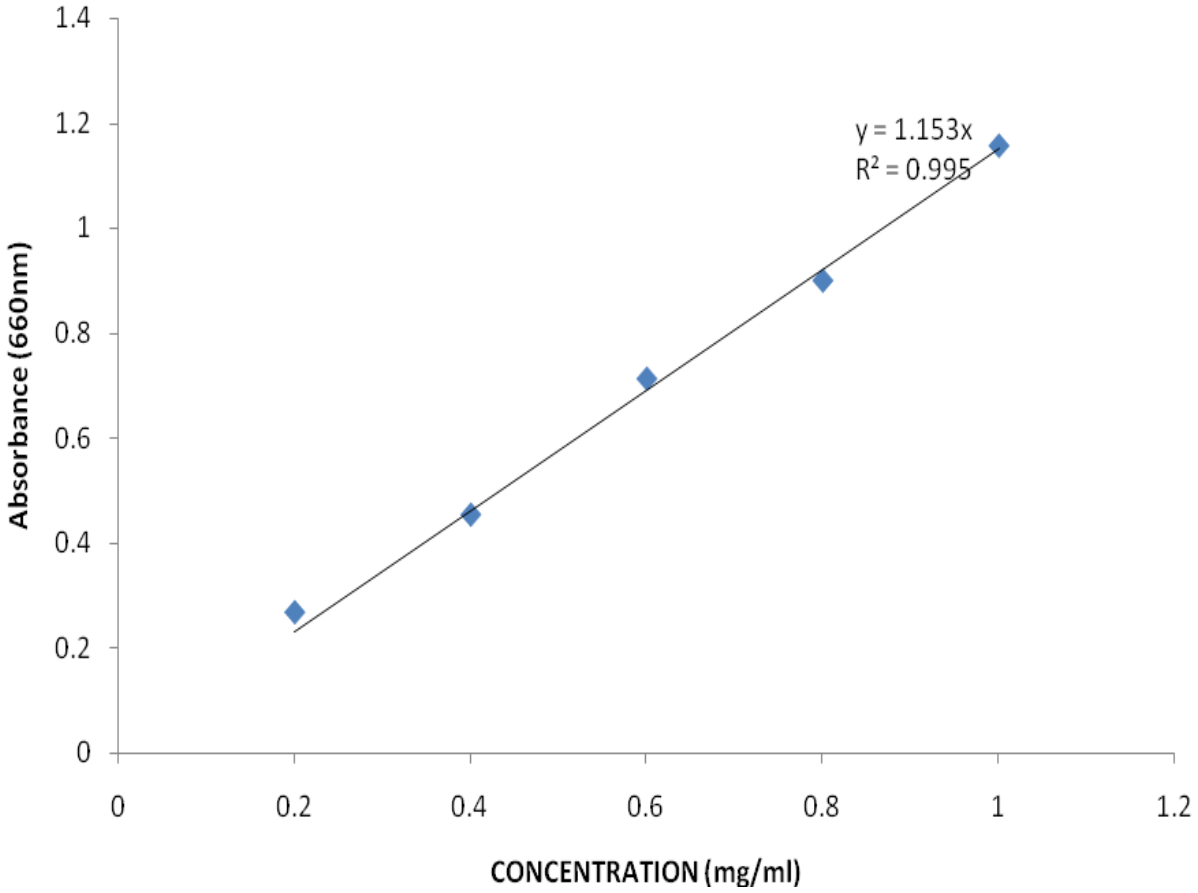


Figure 15: The Measure of Degradation of Human Hair by Keratinase from ***A. faecalis*** **strain TPB18** for Nineteen days determined from the supernatants of the degraded media

Appendices



Appendix 1: Standard curve for cysteine concentration determination using Ninhydrin method.



Appendix 2: Bovine Serum Albumin standard curve for protein concentration determination using Lowry method.