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KERATINOLYTIC ACTIVITIES OF *Aspergillus flavus* and *Alternaria tenuissima* ASSOCIATED WITH BIODEGRADATION OF SELECTED ANIMAL WASTES

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ABSTRACT

Fungi associated with the biodegradation of keratinous animal wastes namely:Chicken feathers, cow horns, cow hoofs, goat horns and goat hoofs, were investigated using standard microbiological methods. The species of fungi isolated were *Aspergillus flavus, A. niger, A. fumigatus, Penicillium chrysogenum,, P. italicum, P. oxysporum, Alternaria tenuissima, Alternaria alternate, Articulospora inflate,Pleurothecium recurvatum, Fusarium culmorum, Fusarium oxysporum, Chlamydomyces palmorum,, Cladosporium sphaeropermum, Gonatobotrium apiculatum, Rhizopus stolonifer, Saccharomyces cerevisiae, Trichoderma viride* and *Varicosporium eloedae.* Keratinase screening revealed that *Aspergillusf lavus, Alternaria tenuissima*, and *Fusarium culmorum* were the most active keratin utilizing fungi with mean keratinolytic activity values of 56 U/ml, 52 U/ml and 43 U/ml respectively. Optimum temperature for keratinase production by *Aspergillus flavus* and *Alternaria tenuissima* was recorded at 40°C, while the optimum pH recorded was 8.0 and 8.5 respectively. Large scale production of Keratinase by these fungi will help reduce environmental pollution caused by these animal wastes.

Keywords: Keratinase, Keratinolytic fungi, Animal wastes, Biodegradation.

INTRODUCTION

Keratin a structural component of vertebrates' skin, is found naturally in feathers, hairs, wools, horns and nails of animals, and can constitute waste to the environment in many ways. Keratin is added to the environment through various natural and artificial

or human activities such as shedding and molting of animal appendages, death of animals, poultry by product, abattoir and leather industry. In particular, chicken feather constitute a troublesome waste in large quantities in commercial poultry processing plants and their utilization is of economic value as well as ecological significance in reducing pollution problems (Acda, 2010). Many fungi (yeasts and dermatophytes) grow on human skin, hair, and nails exhibiting ability to utilize and degrade keratin. The degradation of keratin by these organisms are performed by specific protease enzyme called keratinases (All press *et al*., 2002; Gupta and Ramnani. 2006).

Proteolytic enzymes are largely produced in the presence of keratinous substrates such as hair, feather, wool, nail, horn (Gupta and Ramnani, 2006). Abattoir and poultry wastes are major sources of keratin, therefore there is need to examine the fungal flora of selected keratinous wastes from poultry and abattoir for their keratinolytic activity and also determine their ability to utilize keratinous substrates thereby ensuring their breakdown and degradation by these fungi and ultimate reduction in environmental pollution.

MATERIALS AND METHODS

Isolation of Keratinolytic Fungi

Animal keratinous wastes (Chicken feathers, cow and goat horns and hooves) were collected from two abattoirs and a poultry site in Akure, Nigeria. These wastes were cut into fragments, washed extensively with water and detergent and dried in a ventilated oven at 40ºC for 72 hr. The samples were milled by a table top milling machine and passed through a small mesh grid to remove coarse particles. One gram of the powdered sample was dissolved in 10 ml sterile distilled water in a test tube, shaken at 100 rpm for 10 minutes to allow thorough dilution. Serial dilution was done and the resulting suspension (10^{-7}) was used as a source for fungal isolation on agar plates medium containing g/l; agar; 15, $MgSO_4.H_2O$; 0.5, KH_2PO_4; 0.1, $FeSO_4.7H_2O$; 0.01 and $ZnSO_4.7H_2O$; 0.005 and supplemented with 1% animal waste powder as a sole source of nitrogen and carbon (Wawrzkiewicz *et. al*., 2006). Pure fungal isolates obtained were identified by staining with lactophenol cotton blue and observed microscopically (Barnett, 2004).Morphologically different fungal colonies were inoculated onto sterile meal agar from selected keratinous wastes and incubated at 28°C for 5 days. The strain(s) showing high level of clearance were selected and sub-cultured.

Determination of Keratinolytic Activity

Soluble keratin was prepared from white chicken feathers by the modified method of Wawrzkiewicz*et. al.* (2005). Native chicken feathers (10g) in 500 ml of dimethyl sulfoxide were heated in a reflux condenser at 100°C for 2 hr. Soluble Keratin was then precipitated by the addition of cold acetone (1 L) at – 4°C for 2 hr, followed by centrifugation at 10 0000rpm for 10 minutes. The resulting precipitate was washed twice

with distilled water and dried at 40°C in a vacuum dryer. One gram of quantified precipitate was dissolved in 20 ml of 0.05 mol/L NaOH. The pH was adjusted to 8.0 with 0.1 mol/L HCl and the solution was diluted to 200 ml with 0.05mol/L Tris-HCl buffer (pH 8.0). A 0.1 ml aliquot of crude enzymes was diluted in Tris-HCl buffer (0.05mol/L, pH 8.0) and incubated with 1 ml keratin solution at 50°C in a water bath for 10 min. The reaction was stopped by adding 2.0 ml 0.4mol/L trichloroacetic acid (TCA). After centrifugation at 1450rpm for 30 min, the absorbance of the supernatant was determined at 280nm against a control. The control was prepared by incubating the enzyme solution with 2.0ml TCA without the addition of keratin solution. (Gradisar *et.al*., 2005).

Effect of Temperature and pH on Enzyme Activity

The optimum temperature was tested by assaying the keratinolytic activity at different temperatures from 20°C to 70°C in Tris-HCl buffer pH 8. To determine the optimum pH, keratinolytic activity was assayed in the pH range of 4 to 10, using the following 50mM buffers such as citric acid/Na_2HPO_4 for pH 4 to 6, $NaHPO_4$/ Na_2HPO_4 for pH 6 to 8, Tris-HCl for pH 7 to 9 and glycine/NaOH for pH 9 to 10. Two potential and readily available keratinolytic fungi were chosen to further determine the effect of pH, temperature and substrate concentration on keratinase production. The optimum substrate concentration was tested by assaying the keratinolytic activity at various concentrations from 0.2g/20ml to 1g/20ml [Keratin substrate (g)/0.05mol Tris-Hcl buffer (ml).

Degradation of Different Keratinous wastes by the Isolated Microorganisms

To evaluate the biodegradation of different keratinous materials, the keratinous wastes (Chicken feather, turkey feather, nails, Goat horn and hoofs, cow horns and hoof, and hair) were washed extensively with detergent and rinsed with sterile water, sterilized and dried so that they would be free of microorganisms. They were then incubated at room temperature with the crude enzymes produced by *Aspergillus niger* and *Alternaria tenuissima*.

Statistical Analysis of Data

Data obtained were subjected to the general linear model function of statistical package for social sciences (SPSS)and follow up test was done using Duncan multiple range test at $P \leq 0.05$ level of significance.

RESULTS

Nineteen fungi species including *Aspergillus flavus* and *Alternaria tenuissima* were isolated from the various keratinous samples and their percentage occurrence was also recorded as shown in Table 1, while Table 2 shows the list of fungi that have the ability to utilize keratin from the samples. *Aspergillus flavus, Aspergillus niger, Penicillium chrysogenum* and *Penicillium italicum* had the highest distribution of 9.6%. The potential keratinase producers include *Alternaria tenuissima, Fusarium culmorum, Fusarium oxysporum* and

Aspergillus flavus, with approximate mean values of 43mm, 41mm, 39mm and 36mm respectively on a 90mm petri dish. The highest zone of clearance was recorded for *Alternaria tenuissima* isolated from feather meal agar with a mean value of 43.66 mm, while *F. culmorum, F. oxysporum*, and *A. flavus* with mean values of 41.60 mm, 34.60 mm, and 36.00 mm respectively.

Keratinolytic Activities of the Isolated Fungi

Figure 1 shows the keratinolytic activity of the isolated fungi. *Aspergillus flavus* from poultry feather showed the highest affinity to utilize and degrade keratin with a mean value of 55.8U/ml followed by *Alternaria tenuissima* from feather as well with a mean value of 52.6U/ml. *A. flavus, Alt. tenuissima,* and *F. culmorum* were the most active fungi for keratinse production with mean values of 56.8 U/ml, 52.73 U/ml and 37.4 U/ml

Optimization of keratinase Production

For optimization studies, two organisms (*Alternaria tenuissima* and *Aspergillus flavus*) with high keratinolytic activities where picked. The optimum temperature used for the production of keratinase ranged from 20^0C to 60^0C as seen in fig. 2. The optimum temperature for keratinase production was detected at 40^0C for both organisms.

Fig. 3 shows the effect of pH variation ranging from 4.5 to 10 on the isolated fungi. It was observed that the optimum pH for keratinase production was recorded at 8.0 and 8.5 for *Aspergillus flavus* and *Alternaria tenuissima* respectively.

Table 3 shows the degradation studies of the two keratinase producing fungi on six keratinous wastes (turkey feather, chicken feather, nail, hair, horns and hooves from cows and goats).

Fig 4 shows the effect of varying substrate concentration from 0.2 g/20 ml to 1.0 g/20 ml on the tested isolates. It was observed that keratinnolytic activity was at the highest at a low substrate concentration of 0.2g/20ml.

Degradation of keratin substrates was recorded over a period of one month for all the tested keratinous samples except for horn and hoofs from cow and goat.

On the starting day of degradation (day 3), misty appearance was noted with particles of samples in it, while on the completion day of degradation (day 22), misty appearance was only noted.From the degradation studies (table 3), it was observed that all the tested organisms were able to degrade nails and feathers, but degradation was slightly observed on hoofs and horns over a period of one month.

Table 1: Occurrence of Fungal Isolates from selected keratinous wastes

Fungi	CHn	Chf	CF	GHn	Ghf	No.of isolates	Percentage occurrence
Aspergillus flavus	+	+	+	+	+	5	9.60
Aspergillus fumigates	+	+	+	+	–	4	7.60
Aspergillus niger	+	+	+	+	+	5	9.60
Alternaria alternate	–	–	–	–	+	1	1.92
Alternaria tenuissima	+	–	–	–	–	1	1.92
Articulospora inflate	–	+	–	+	+	3	5.76
Chlamydomyces palmorum	–	–	+	–	–	1	1.92
Cladosporium sphaeropermum	+	–	–	–	–	1	1.92
Fusarium culmorum	–	–	+	+	+	3	5.76
Fusarium oxysporum	–	–	+	+	+	3	5.76
Gonatobotrium apiculatum	–	+	–	–	+	2	3.84
Penicilluim chrysogenum	+	+	+	+	+	5	9.60
Penicilluim italicum	+	+	+	+	+	5	9.60
Penicilluim oxysporum	+	+	+	–	–	3	5.76
Pleurothecium recurvatum	–	–	–	+	–	1	1.92
Rhizopus stolonifer	+	+	–	+	+	4	7.60
Saccharomyces cerevisiae	–	–	–	+	–	1	1.92
Trichoderm aviride	+	+	+	–	–	3	5.76
Varicosporium eloedae	–	–	–	–	+	1	1.92
Total						52	100

Key:CHn=Cow horn,Chf = Cow hoof,CF = Chicken feather,GHn = Goat horn, Ghf = Goat hoof.
+ = Present, – = Absent

Table 2: Clear Zone (mm) of Fungal Isolates on Keratinous Meal Agar

Fungi	Cow horn	Cow hoof	Goat horn	Goat hoof	Feather
A. flavus	29.33 ± 0.5^{f}	31.33 ± 0.5^{e}	30.33 ± 0.5^{d}	31.00 ± 1.0^{c}	36.00 ± 1.0^{d}
A. fumigates	17.66 ± 0.5^{d}	12.00 ± 0.0^{b}	17.66 ± 0.5^{c}	–	25.00 ± 1.0^{c}
A.niger	10.33 ± 0.5^{b}	11.00 ± 0.0^{a}	14.00 ± 1.0^{ab}	13.66 ± 1.5^{b}	14.66 ± 1.5^{b}
Alt. alternate	–	–	–	13.66 ± 0.5^{b}	–
Alt.tenuissima	–	–	–	–	43.66 ± 0.5^{f}
Chlamydomycespalmorium	–	–	–	–	12.00 ± 1.0^{a}
F. culmorum	–	–	–	–	41.66 ± 0.5^{e}
F.oxysporum	–	–	39.66 ± 0.5^{e}	39.00 ± 1.0^{d}	–
Gonatobotriumapiculatum	–	18.33 ± 0.5^{d}	–	–	–
P.chrysogenum	12.00 ± 0.0^{c}	14.33 ± 0.5^{c}	13.66 ± 0.5^{a}	–	15.33 ± 0.0^{b}
P. italicum	9.00 ± 1.0^{a}	14.00 ± 0.0^{c}	15.66 ± 0.5^{b}	15.33 ± 1.1^{b}	14.00 ± 0.0^{b}
Pleurotheciumrecuvertum	–	–	13.33 ± 2.3^{a}	–	–
Trichodermaviride	19.66 ± 0.5^{e}	18.00 ± 0.0^{d}	–	–	24.00 ± 0.0^{c}
Varicosporiumeloedae	–	–	–	8.33 ± 0.5^{a}	–

Values are mean of 3 replicates ; Values with the same alphabet in column are not significantly different at $P \leq 0.05$; **Key:** – = organisms are not present.

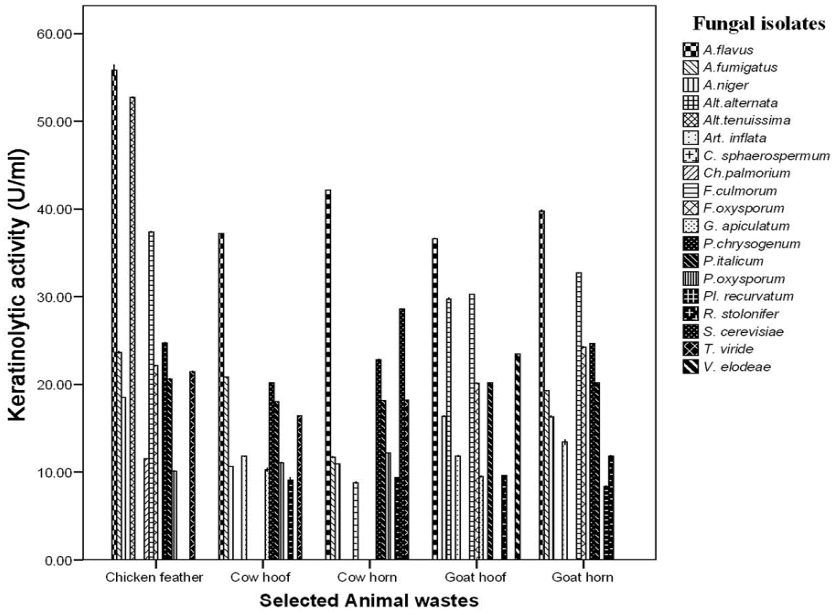

Fig 1: Keratinase Activity of Fungal Isolates

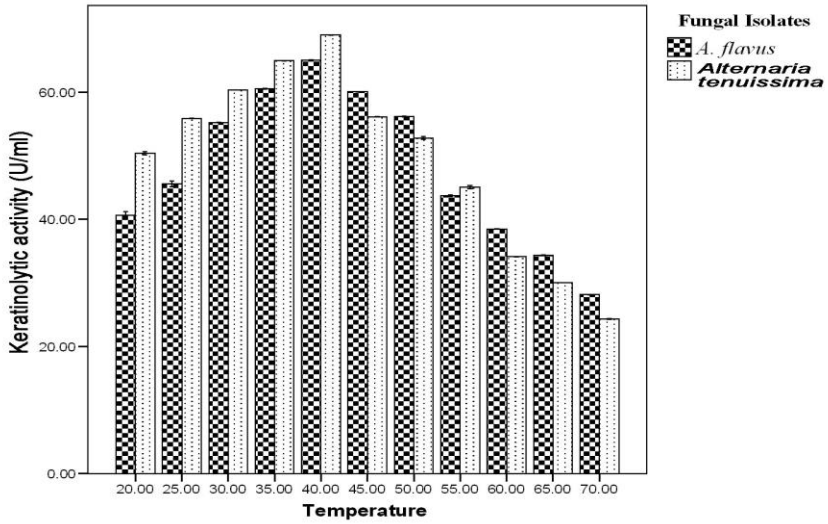

Fig 2: Effect of Temperature (°C) on Keratinase Activity

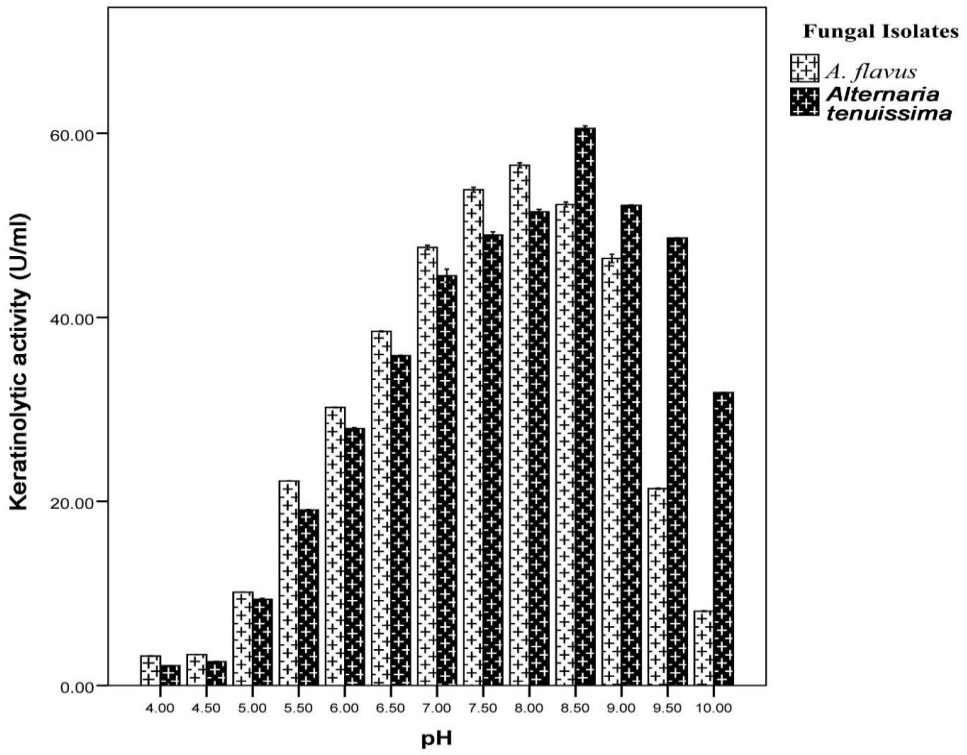

Fig 3: Effect of pH change on Keratinase Activity

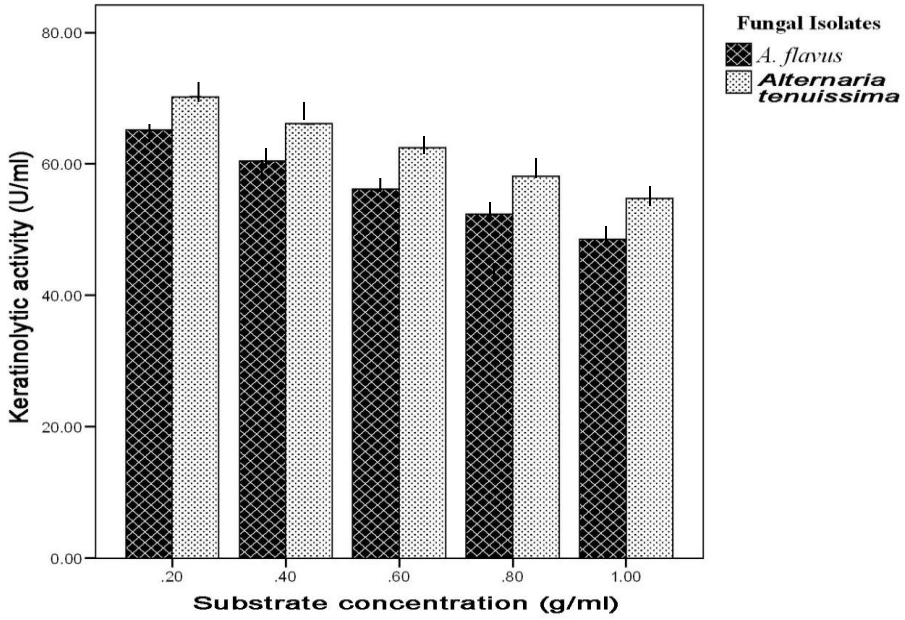

Fig4: Effect of varied substrate concentration on keratinase activity

Table 3: Effect of keratinase from *Aspergillus flavus* and *Alternaria tenuissima* on biodegradable wastes

Keratinous wastes	Starting day of degradation		Completion day of degradation	
	A. flavus	***Alt. tenuissima***	***A. flavus***	***Alt. tenuissima***
Turkey feather	Day 7	Day 3	Day 21	Day 12
Chicken feather	Day 7	Day 3	Day 21	Day 10
Nails	Day 4	Day 6	Day 15	Day 14
Hair	Day 12	Day 10	Day 18	Day 22

DISCUSSION

The result of percentage occurrence in Table 1 showed that *A. flavus, A.niger, P. italicum* and *P. chrysogenum* had the highest occurrence of fungi, which might be due to the fact that they are highly resistant to the harsh environment as well as their wide distribution in different habitats. A clear zone on keratin agar confirmed the ability of the isolates to utilize keratin, hence potential keratinase producers. Comparing the *A. flavus* from the various keratinous waste samples; feather had the highest zone of clearance in respect to *A. flavus* produced by cow and goat horn and hoof meal agar, which showed that feather is the most easily utilized keratin sample by the fungal isolates. The keratinolytic activities shown In Fig.1 conforms with the work of Marcodes *et. al*. (2008), who stated that the evidence of keratinolysis lies on the ability of fungi to release soluble sulphur-containing amino acids and polypeptides into medium in quantities significantly greater than those released by controls.

The optimization studies for keratinase production as seen in figs 2, 3 and 4 were in accordance with the findings of Farag and Hazan, (2004); Riffel and Brandelli (2006) who reported that keratinase producing fungi have different characteristics, such as a broad temperature range of growth. Since these fungi showed broad range of temperature growth, they have ability to produce keratin. An optimum keratin-degrading activity at mesophilic temperatures should be a desirable characteristic because these microorganisms may achieve hydrolysis with reduced energy input. That is, the activation energy needed to catalyse the breakdown of keratinous materials will be reduced. For *Aspergillus flavus* and *Alternaria tenuissima* keratinolytic activity of the culture broth was detectable between 20°C and 60°C showing a maximum activity at 40°C. A sharp drop in keratinolytic activity was observed between 40°C to 60°C. Fungal activity up to 60°C with a peak at 40°C for keratinase is considered worthy of attention as this corresponds to the thermotolerant nature of the

fungi. Some researchers reported similar results for keratinase of *Scopulariopsis breviculis* (Mabrouk 2008), *Streptomyces pactum* (Han *et al*, 2012) and *Chrysosporium keratinophilum* (Lakshmi *et. al.*, 2013).

The effect of pH showed that the keratinolytic activity of the culture broth of *Aspergillus flavus* and *Alternaria tenuissima* were detectable over a wide range of pH values, with optimum at pH 8.0 and 8.5 respectively (Fig 3), independent on the buffer used. Tris-HCl buffer appeared to be the most suitable. The activity declined rapidly over pH 9 and was negligible above 10. Other keratinolytic enzymes have been reported to be active at alkaline pH (Gradisar *et. al.*, 2000; Liang *et. al.*,2011; Mitsuiki *et. al.*, 2002). The result indicates that at higher substrate concentration, repression of keratinase production can take place. This shows that a low concentration of substrate is optimum for yielding maximum enzyme activity. This observation is similar to previous studies which concluded that a low concentration of substrate is optimum for yielding maximum enzyme activity which was evident in this work (Suneetha and Lakshmi, 2005). Degradation occurred for horns and hoof because there was a slight change in the suspension compared to the control that contained no microorganisms. The suspension was misty at the end of one month but coarse particles of hoofs and horn were still clearly seen, these were all evidence of degradation. Other tested samples such as the feather, nails and hair were totally degraded and the solution was totally misty without any trace of the samples in them. This may be due to the fact that disulphide bonds in the hoof and horns are greater than that of the nails and feathers.

For optimization studies, *Alternaria tenuissima* and *Aspergillus flavus* where used. The optimum temperature required for the production of keratinase ranged from 20°C to 60°C as seen in Fig. 2. The optimum temperature for keratinase production was detected at 40°C for both organisms. Fig. 3 shows the effect of pH variation ranging from 4.5 to 10 on the isolated fungi. It was observed that the optimum pH for keratinase production was recorded at 8.0 and 8.5 for *Aspergillus flavus* and *Alternaria tenuissima*, respectively. It can be deduced that both fungi are potential keratin degrader but the most effective for feather and nail degradation was *Alternaria tenuissima*, while *Aspergillus flavus* proved to be more effective in degrading human hair than *Alternaria tenuissima*.

This result conforms to the findings of Lakshmi *et. al.*, (2013) who stated that the lower level of degradation of buffalo horns at the expense of higher release of enzyme might be due to the restricted substrate specificity of the enzyme or removal of some accessory proteins capable of splitting the disulphide bonds present in the keratin molecules during hydrolysis. Keratinolytic microorganisms are of great ecological importance not only in pathogenesis but also in keratin degradation. The degrading enzymes produced by

the organisms are capable of breaking down complex keratinous substrates in nature. Thus, they are responsible for the degradation of keratin wastes in polluted habitat. Therefore, the utilization of these potential keratin degraders will definitely find biotechnological use in various industrial processes involving keratin hydrolysis. It would also solve the problem of poultry and abattoir waste disposal and with limited resources, recycling of keratinous waste would be beneficial financially and environmentally.

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