Indian Journal of Environmental Sciences 22(2), 2018, pp.68-75
Green Earth Foundation
ISSN 0971-8958

KERATIN DEGRADATION POTENTIAL OF BACTERIA AND FUNGI ISOLATED FROM CHICKEN FEATHER WASTE DUMPING SITE

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ABSTRACT

Four species of keratinolytic bacteria and 6 species of fungi were isolated from the feather dumping site soil at Teppakulam (9.9104° N Latitude and 78.1482° E Longitude), Madurai, India. The bacteria were identified based on their morphological and biochemical characteristics whereas fungi were identified based on morphological examination. Isolates were initially screened for growth, protein concentration and keratinase activity. One preeminent bacterial and fungal strain namely; *Serratia marcescens* and *Aspergillus niger* respectively were chosen and subjected to pH, temperature, media and substrate optimization. The optimum temperature and pH for *S. marcescens* growth were 58 °C and 9.5 and for *A. niger* were 32 °C and 9.0 respectively. Media with 1.0% feather concentration increased the growth of both bacteria and fungi. Of the different substrates analyzed, maximum growth of bacteria was obtained with casein and of fungi in bovine serum albumin. When provided the optimum conditions, *A. niger* was more potent than *S. marcescens* in feather degradation.

Key words: Feather, Degradation, Keratinase, *Serratia marcescens, Aspergillus niger*

INTRODUCTION

Every year, millions of tons of feathers are produced as waste by-product by the poultry processing plants, all over the world (De-azeredo et al. 2006). These feather wastes make a serious problem to the environment by polluting the land as well as water and provide habitat for harmful pathogens (Mariana et al. 2008, Lateef et al. 2015). Feathers are composed of over 90% protein, the main component being keratin, a mechanically durable protein, highly cross-linked with disulphide and other bonds (Kumar et al. 2011, Tamilkani et al. 2017). This feather waste is a very good source of protein and amino acids that could be used for various biotechnological applications (Swetlana and Jain 2010, Kani et al. 2012). The traditional way to degrade feathers such as alkali hydrolysis and steam pressure cooking consume large amount of energy may destroy certain amino acids and decrease the quality of protein (Riffel and Brandelli 2006, Anitha and Eswari 2012). Biodegradation of feathers by keratinase from microorganisms may provide a viable alternative (Cheng et al. 2008). Keratinase is an efficient proteolytic enzyme that could hydrolyze insoluble keratin, also has the capacity to act on compact substrates better than other comparable proteolytic enzymes that distinguishes keratinase from the other protease and peptidases.

At present, keratinolytic microorganisms and their enzymes have become a subject of substantial scientific interest (Nadier et al. 2008, Matsui et al. 2009). Keratinase production by microorganisms has been influenced by number of factors, such as temperature, pH, and the nature of the carbon and nitrogen source present in the medium (Cortezi et al. 2008). Keratin utilization has been reported in a few microorganisms including non-filamentous and filamentous bacteria, water mould and filamentous fungi (Scott and Untereriner 2004). These bacterial strains produce keratinase which selectively degrade the beta-keratin found in feathers which in turn help in their growth and maintenance (Savitha et al. 2007). Many fungi especially those belonging to the class fungi imperfective have high keratinolytic activity.

The keratinolytic microorganisms and technologies developed for feather degradation not only remove waste feathers efficiently from the nature but also have important applications in food and leather industries, manufacturing of textiles, biodegradable film and cosmetics and nitrogen fertilizer for plants (Onifade et al. 1998, Gessesse et al. 2002, Suntornusk and Suntomsuk 2003). Based on the above mentioned facts, it seems to be essential to isolate and characterize novel keratinolytic microbes and to evaluate various physical parameters in order to achieve complete and efficient disintegration of poultry feathers. The aim of this work is to

degrade the poultry feather wastes (insoluble protein) to soluble protein.

MATERIALS AND METHODS

Preparation of chicken feather powder

Poultry feathers were collected from a slaughter house at Alanganallur (10.0478° N Latitude and 78.0894° E Longitude), Madurai District, India, washed well with water and detergent, cut into small fragments, dried in a ventilated oven at 40 °C for 72 hrs. After that, the feathers were milled and passed through a small mesh grid to remove coarse particles. This feather powder was used for further studies.

Isolation and characterization of keratin hydrolyzing microorganisms

Soil samples were collected from the feather dumping area at Teppakulam (9.9104° N Latitude and 78.1482° E Longitude), Madurai, brought to the laboratory in sterile container and serially diluted following standard protocol (Xu et al. 2009). For bacterial isolates, diluted soil samples (10^{-2}, 10^{-4}, 10^{-6} and 10^{-8}) were plated on feather meal agar plate (0.5 g/L NH_4Cl; 0.5 g/L NaCl; 0.3 g/L K_2HPO_4; 0.4 g/L KH_2PO_4; 0.1 g/L $MgCl.6H_2O$; 10 g/L feather powder, 15 g/L agar) and for fungal isolation, samples were inoculated on agar plates, which contain 15g/L agar; 0.5g/L; $MgSO_4.H_2O$; 0.1g/L KH_2PO_4; 0.01g/L $FeSO_4.7H_2O$; and 0.005g/L $ZnSO_4.7H_2O$; the pH was adjusted to 7.5. The plates were incubated at 37 °C. The isolated colonies were streaked on agar slants for further characterization. The isolates of bacteria were compared and identified according to Bergey's Manual of Systematic Bacteriology (Sneath et al. 1986, Brenner et al. 2004) and fungi were identified according to Domsch et al. (2007).

Preliminary screening of keratinolytic microorganisms

The isolated bacterial colonies were grown on feather substituted broth and growth was determined by reading the absorbance at 620 nm against blank. The fungal growth was measured following colony diameter method. The sample filtrate was assayed at regular intervals (24 hrs) for soluble protein content and keratinase activity. Protein content of the cell free supernatant was determined following the Biuret method with bovine serum albumin as standard protein. From the four isolates of bacteria and six isolates of fungi, one was chosen to be screened for the degradation of feather wastes and for increased enzyme production, respectively.

Effect of pH and temperature on microbial growth

The ability of the isolates to degrade native keratin substrate was investigated. The assay consisted of feather powder suspended in 100mL of minimal medium (pH 6.5), sterilized at 121° C for 15 min. For studying the effect of pH and temperature, overnight cultures of bacteria were inoculated into the broth containing minimal medium substituted with feather and incubated for 120 hrs at different temperatures and pH viz., 28°C, 38°C, 48°C, 58°C and 68°C and 6.5, 7.5, 8.5, 9.5 and 10.5 respectively. The controls were maintained without inoculums for each category having six replicates. At every 24 hrs interval, the bacterial growth was estimated by measuring the absorbance at 620nm.

The fungi were inoculated on to plates containing minimal media substituted with feather and exposed to various temperature and pH of 27°C, 32°C, 37°C, 42°C and 47°C and 6, 7, 8, 9 and 10 respectively and their growth was measured following colony diameter method. Their respective controls (without inoculums) were maintained with six replicates for each category.

Effect of feather concentration and substrates on microbial growth

The experiments were performed in 250mL Erlenmeyer flasks containing minimal media substituted with feather at different concentrations (0.1, 0.5, 1.0, 1.5 and 2.0%). To study effect of different substrates on growth of bacteria, medium was externally supplemented separately with 0.5% each of casein, bovine serum albumin, feather, peptone, and gelatin. Each of the above said flasks were inoculated with the isolates and incubated at 37°C with 120 rpm for 120 hrs. The growth of bacteria was determined by measuring the absorbance at 620 nm. Similar constituents were taken for fungi also but the experiments were performed in solid media and their growth was determined by colony diameter method.

Enzyme production by the keratinolytic microorganisms

A single colony from the agar plate was aseptically transferred to 100mL nutrient broth in 250mL Erlenmeyer flask. The flask was incubated overnight at 37°C in an orbital shaker (150rpm). To induce enzyme production, 5mL of the seed culture was transferred to 95mL of enzyme

producing broth in a 250mL Erlenmeyer flask. The feather degrading enzyme producing broth contained minimal medium supplemented with 1.0% feather corresponding to 10 g/L (initial pH 8.5). The inoculated flasks were incubated at 40°C. Crude enzyme was collected after 120 hrs of incubation by centrifuging at 5000rpm for 20 min. The supernatant was preserved at 4°C and assayed for keratinolytic activity and soluble protein concentration (Riffel and Brandelli 2006).

Assay for keratinolytic activity

Keratinase activity was assayed by the modified method of Gradisar et al. (2005). 20mg of chicken feather powder were suspended in 3.8mL of 100mL Tris-HCl buffer (pH 7.8) to which 300µl of the culture filtrate (enzyme source) was added. The reaction mixture was incubated at 37°C for 1 hrs. After incubation, the assay mixture was dipped into ice cold water for 10 min and the remaining feathers were filtered out. The absorbance of the clear mixture was measured at 280nm using UV spectrophotometer. The keratinase activity was expressed as one unit of the enzyme corresponding to an increase in the absorbance value 0.01/ hrs.

Optimum conditions for effective feather degradation

The bacterial isolate was investigated in basal medium containing 1% feather powder and media enriched with casein at 58°C (pH 9.5) for 120 hrs. For the fungal isolate, the optimum conditions were provided such as 1% feather powder and BSA at 32°C and pH 9 for 10 days. The protein concentration was estimated by Biuret method and keratinase activity by keratinolytic assay for both bacterial and fungal isolates.

RESULTS

Isolation and characterization of feather degrading microbes

Soil samples were collected from feather dumping area at Teppakulam, Madurai for isolation of keratinolytic bacteria by serial dilution method. After being incubated for 48 hrs, the plate containing feather meal agar showed the growth of four bacteria and six fungi respectively.

Identification and screening of keratinolytic microbes

Based on the morphological, physiological and biochemical characterization and in agreement with the features described in Bergey's Manual of Systematic Bacteriology, bacterial isolates were identified to be *Streptomyces sp., Bacillus sp., Micrococcus sp.* and *Serratia sp.* respectively.

The bacteria were also screened for keratinase production based on their growth, protein concentration and keratinase activity. Among the four isolates, *Serratia sp.* formed the clear zone, produced maximum protein and exerted high keratinase activity than the other three isolates (Fig. 1) and was identified to be *Serratia marcescens*.

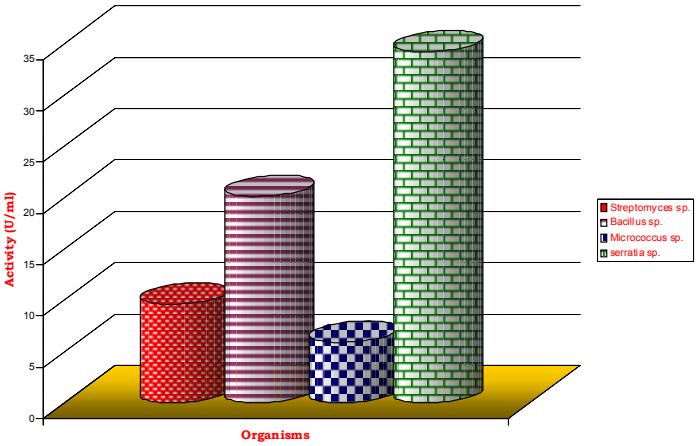

Fig. 1. Keratinase activity of the feather degrading bacterial isolates

The feather degrading fungi isolated from soil on agar medium substituted with feather meal were identified as one species each of *Alternaria, Nicrospora, Cladosporium,* and *Rhizopus* and two of *Aspergillus*. The selected isolates were also quantitatively tested in submerged culture for protein concentration and keratinolytic activity. *Aspergillus niger* achieved significantly higher growth, protein concentration, keratinase activity and also maximum extra cellular keratinase production (Fig. 2).

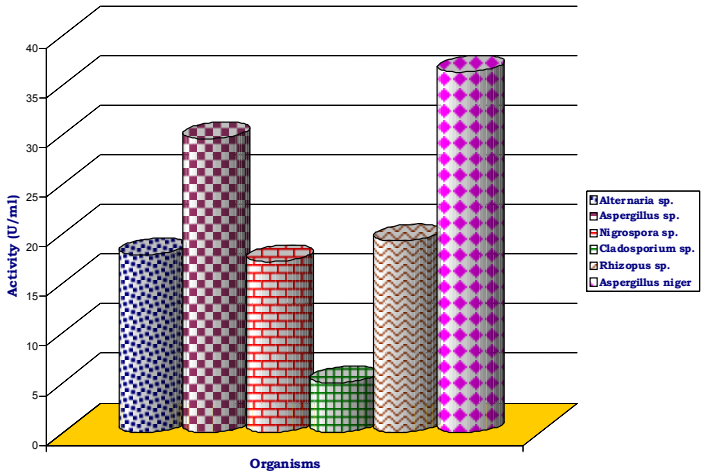

Fig. 2. Keratinase activity of the feather degrading fungal species

Influence of pH and temperature on microbial growth

Keratin was used as substrate for pH and temperature optimization. The effect of the reaction temperature on the growth was investigated using optical density method for bacteria and colony diameter method for fungi. The maximum growth of *S. marcescens* was observed at pH 9.5 (Fig. 3) and 58 °C (Fig. 4).

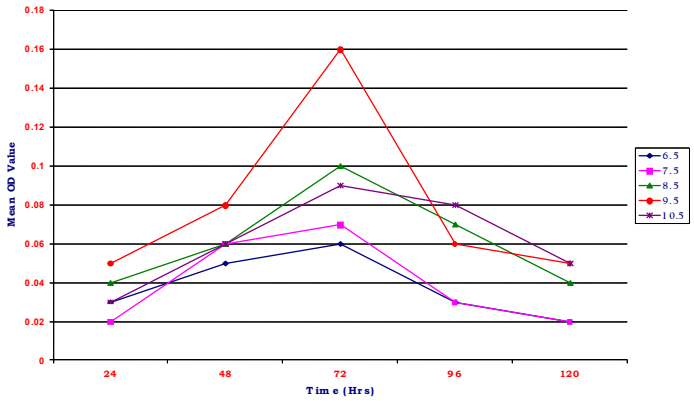

Fig. 3. Effect of pH on the growth of *Serratia marcescens*

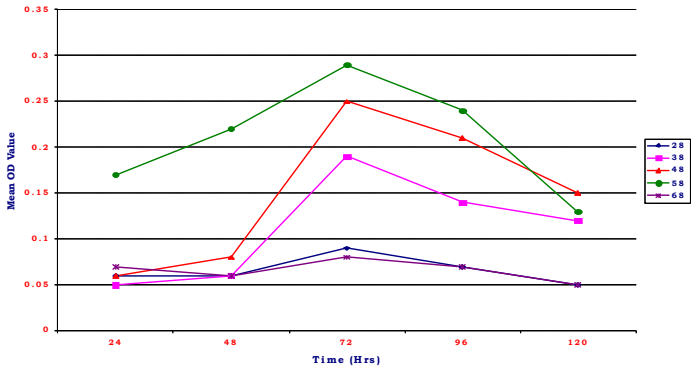

Fig. 4. Effect of temperature on the growth of *Serratia marcescens*

Aspergillus niger attained maximum growth in alkaline condition at pH 9 (Fig. 5). The different tested temperature, fungal growth was higher at 32 °C (Fig. 6).

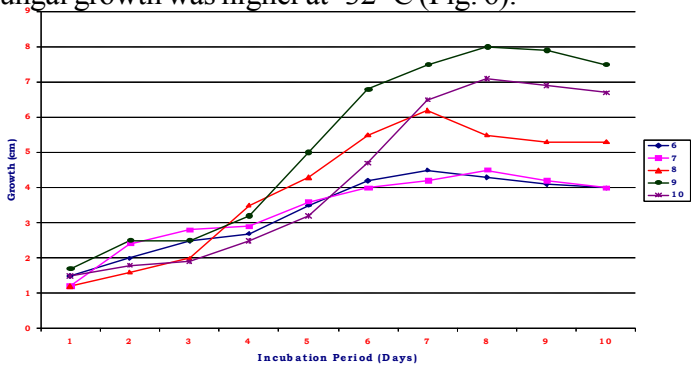

Fig. 5. Effect of pH on the growth of the isolated feather degrading fungi *Aspergillus niger*

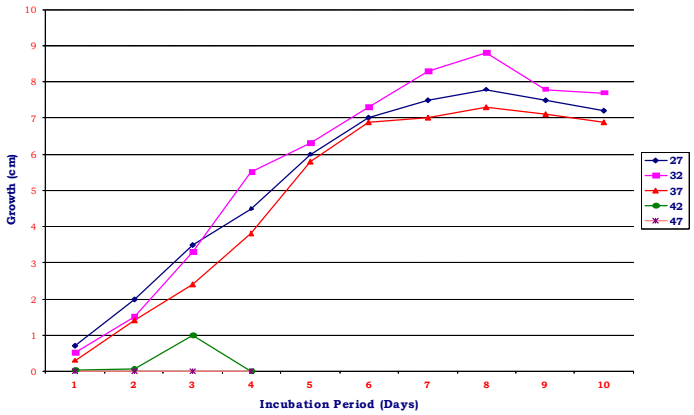

Fig. 6. Effect of temperature on the growth of *Aspergillus niger*

Influence of feather concentration and substrates on microbial growth

The effects of different feather concentrations and substrates on the growth of *S. marcescens* and *A. niger* were investigated and the results are presented in Fig. 7, 8, 9 and 10. Medium containing 1.0% feather concentration was better for growth than that of other feather concentrations and casein was the suitable substrate for growth of bacteria and BSA for growth of fungi.

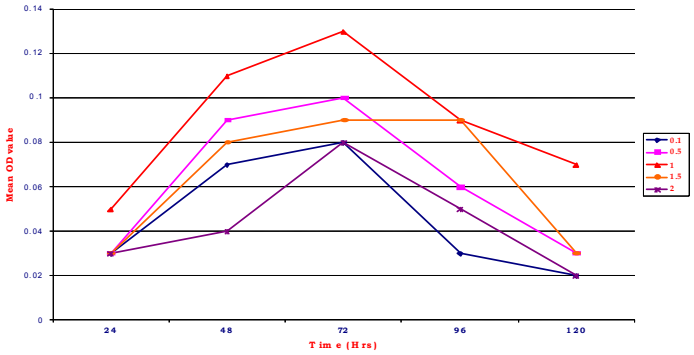

Fig. 7. Effect of feather concentration on the growth of *Serratia marcescens*

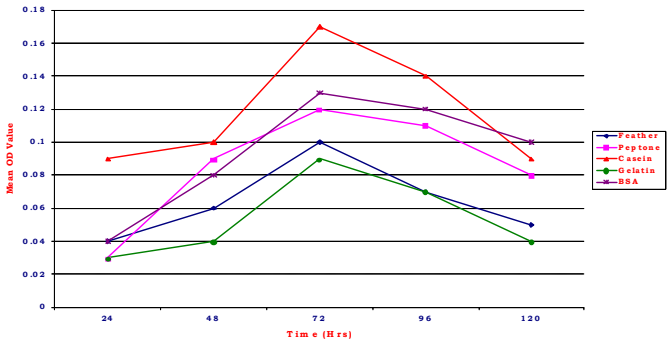

Fig. 8. Effect of substrates on the growth of *Serratia marcescens*

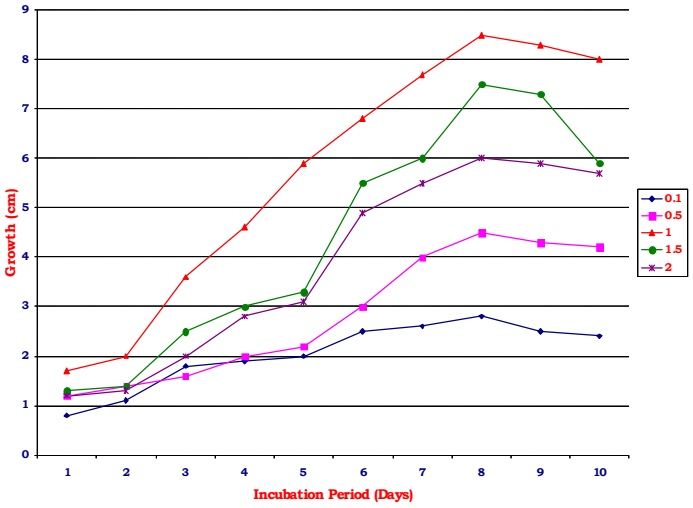

Fig. 9. Effect of feather concentrations on the growth of *Aspergillus niger*

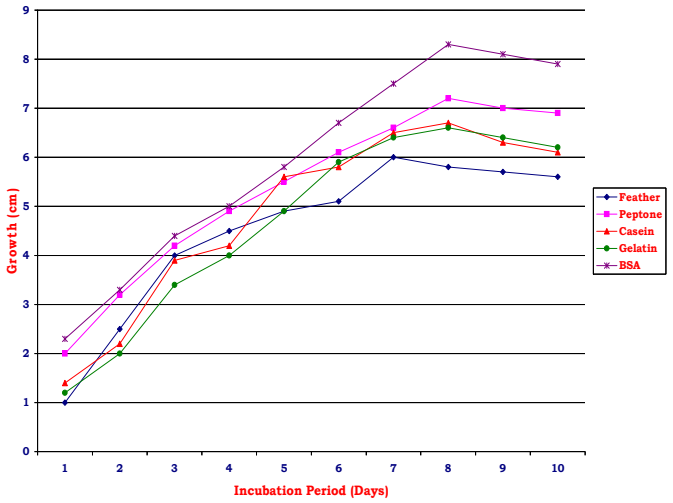

Fig. 10. Effect of substrate on the growth of *Aspergillus niger*

Keratinolytic activity by *S. marcescens* and *A. niger*

Based on the above results, the bacteria was cultured for 120 hrs in the basal medium containing 1.0% feather and enriched with casein at 58 °C (pH 9.5). The optimum conditions such as 1.0% feather, enriched with BSA at 32°C and pH 9 for 10 days were provided to the fungi for effective feather degradation. The keratinolytic activity was higer (40.1 U/mL) for *S. marcescens* and in 216 hours (45 U/mL) for *A. niger* respectively (Table 1 and 2) which was also evidenced from the increased protein concentration during that time. The keratinolytic activity in fungi has taken longer duration to confirm the keratinase enzyme production.

Table 1. Keratinase activity and protein production by *Serratia marcescens* at optimized culture conditions

S. No.	Time (hours)	Protein concentration (mg/mL)	Keratinase activity (U/mL)
1	24	0.0394	20.9
2	48	0.0787	33.7
3	72	0.1679	40.1
4	96	0.1579	34.2
5	120	0.0394	20.1

Table 2. Keratinase activity and protein production by *Aspergillus niger* at optimized culture conditions

S. No	Days	Protein concentration (mg/mL)	Keratinase activity (U/ mL)
1	1	0.1315	23.1
2	2	0.1716	25.1
3	3	0.1974	29.1
4	4	0.2631	33.2
5	5	0.3239	36.3
6	6	0.4605	37.6
7	7	0.5263	40.1
8	8	0.6263	41.2
9	9	0.6445	45.0
10	10	0.4210	38.3

DISCUSSION

Chicken feathers are naturally resistant to degradation by common microbial proteases; so, several million tons of feathers generated annually by the poultry industry leads to troublesome environmental pollution and wastage of protein (Gousterova et al. 2005, Grazziotin et al. 2006, Wei et al. 2017). Keratinase enzyme produced by certain microbes can hydrolyse keratin (insoluble material) into soluble amino acids that has various applications (Onifade et al. 1998, Tork et al. 2010, Gurav et al. 2016). In the present study, an attempt has been made to isolate, identify and optimize both bacterial and fungal strains, able to completely degrade keratin rich feathers into soluble utilizable material. Based on the

results of preliminary identification tests, bacterial isolates were *Sterptomyces* sp.*, Bacillus* sp.*, Micrococcuss* sp. and *Serratia* sp. respectively. *Bacillus* species are ubiquitous microorganisms, which can grow on natural media without any special requirements and are capable of producing keratinolytic proteases (Zerdani et al. 2004, Suntornsuk et al. 2005, Cheng et al. 2009, Jin et al. 2017). Kim et al. (2001) have isolated similar type of keratinolytic bacteria belonging to *Streptomyces* sp. from the soil. In this study, a new genus of feather degrading bacteria *Micrococcus* sp. has been isolated, which has not been previously reported. Degradation of chicken feathers by a proteolytic enzyme produced by *Serratia* species could find application in numerous waste management programs, such as in conversion of the large amount of chicken feather waste generated from poultry into highly digestible animal feed (Anushuman et al. 2009, Iruolaje et al. 2016).

Growth of bacterial isolates was determined to access the nutritional need for the strain and their capacity to use the nitrogen and other nutrients from the feather during protein hydrolysis (Lange et al. 2016). pH 9.5 was found to be optimal for high growth and growth was found to be active between the neutral to alkaline ranges of pH. The growth showed broad temperature specificity with a maximum growth being observed at 58^{o}C. The growth of the bacteria was higher in the temperature range of 38 - 58^{o}C than at the normal assay temperature 37^{o}C (Lee et al. 2002, Gessesse et al. 2002).

In the present study, six feather degrading fungi were isolated from the soil. The identification of these fungal isolates based on colony morphology and microscopic appearance revealed one species each of *Alternaria*, *Nicrospora*, *Cladosporium, Rhizopus* and two of *Aspergillus*. Domsch et al. (2007) reported that *Aspergillus niger* was the most prevalent keratinophilic fungus and also dominant species isolated from 51 soil samples collected from farm lands and poultries. The growth of the culture of *A. niger* was deductible over a wide range of pH values, with an optimum at pH 9.

The amount of catabolic degradative products such as protein and keratinase production along with the simultaneous increase in pH was an indication of feather degradation (Wang and Liao 2014). Keratinolytic enzymes have been reported to be active at alkaline pH, the accumulation of ammonium ions as the products of deamination increase pH for fermentation with increasing keratinolytic activity (Gradisar et al. 2000, Mitsiki et al. 2004). In the present study, when the concentration of feather increased above 1.0%, the growth of both bacteria and fungi and protein concentration of the medium decreased because it prevented aeration ~~to~~ of the culture. Many keratinase are capable of hydrolyzing a broad range of soluble and insoluble proteins. Soluble proteins are easily degraded by microorganisms hence casein and BSA are suitable substrate for bacteria and fungi, respectively. It was evident from the results, that the optimum conditions increased the protein concentration and keratinase activity of *S. marcescens* and *A. niger.*

In this present investigation, compared to *S. marcescens*, maximum protein concentration and keratinase activity was observed in *A. niger*, because keratinolytic fungi have the unique ability to degrade keratinous substrates completely (Kushwaha and Pallavi 2008). Keratinolytic fungi exhibit two methods of mechanical invasion to the keratinized substrate, surface erosion and radical bore through (Jeong 2003). Nutritional enhancement can also be achieved by hydrolysis of raw feathers using keratinolytic fungi hence they have the potential to be inducted in the feather waste management programme.

CONCLUSION

Keratinolytic microbes are of great ecological interest not only in pathogenesis but also in keratin degradation. The degradative enzymes produced by *Aspergillus* sp. are capable of breaking down complex keratinous substrates in nature, and thus responsible for the biodegradation of keratinized structure in polluted habitats. The selected isolate, *Aspergillus niger* was able to grow and display keratinolytic activity in raw feather. This would be beneficial for the utilization of these residues and the isolates present potential bio-technological use in processes involving keratin hydrolysis. Novel keratinase will have tremendous potential in the meat processing, leather manufacturing, detergent, cosmetics and vaccines for dermatophytosis. Based on the above mentioned facts, recycling of keratin wastes would be beneficial financially and environmentally.

ACKNOWLEDGEMENTS

The authors are thankful to the authorities of Thiagarajar College, Madurai for providing the necessary infrastructure facilities to carry out the work. This study was supported financially by University Grants Commission funding (MRP-MAJOR-ZOOL-2013-24210 [943-573/2014(SR)]).

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