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Keratinolytic Activity of Five *Aspergillus* Species Isolated from Poultry Farming Soil in Korea

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Various soil samples were collected from twenty-four areas of ten different poultry farms in Korea and screened for prevalence of keratinolytic fungi. Fourteen species of feather-associated fungi belonging to ten genera *Acremonium*, *Alternaria*, *Aspergillus*, *Cladosporium*, *Curvularia*, *Fusarium*, *Monascus*, *Mucor*, *Penicillum*, and *Verticillium* isolated from poultry soils were grown on keratin medium. Especially, *Aspergillus* spp. populations associated with the soil sample is 1×10^5 cfu/g. *A. flavus*, *A. fumigatus*, *A. niger*, *A. nidulans*, and *A. terreus* could utilize keratin of chicken feather and degrade it, producing sulphydryl-containing compounds detected as keratinase, cysteine and total proteins. Keratinolytic activities of five *Aspergillus* species also changed the pH of the medium more alkaline than those that were less keratinolytic.

KEYWORDS: Feathers, Keratinase, Keratinophilic fungi, *Aspergillus* spp.

Feather, which account for 5~7% of the total weight of mature chickens, are produced in huge quantities as waste by-product at commercial poultry processing plants. Every year more than 20,000 tons of feathers are produced as waste by poultry farming (Vogt and Stute, 1975). Keratin is a major component of feathers. A distinctive feather of keratin is its relatively high sulfur content due to the presence of sulfur containing amino acids viz. cystiene, cysteine and methionine. Thus, the disulfide bonds are considered to be responsible for the stability of keratin and its resistance to enzymatic degradation (Kunert, 1989). Nevertheless, feathers do not accumulate in nature, since structure of keratin and parasitic fungi (Bahuguna and Kushwaha, 1989; Rajak *et al.*, 1992; Safranek and Goos, 1982). Surveys of keratinophilic fungi inhabiting soil (Kushwaha, 1981), air (Marchiso *et al.*, 1981), and sewage sludge (Tawfik and Rawa, 2001) have been carried out. The fungus belonging to the genus *Aspergillus*, found ubiquitously in nature, is an opportunistic airborne pathogen affecting humans, birds, other animals and plants. It accounts for a variety of respiratory disorders and severe invasive infections (Santos *et al.*, 1996). These fungi have been characterized as a producer of several proteolytic enzymes, which have been reported to be responsible for the key events involved in the physiology of *Aspergillus* spp. (Gradišar *et al.*, 2000). Fungal generated keratinolytic follows particular growth and morphological patterns correspondent with particular substrate attack, involving mechanical and enzymatic action. Keratinolytic fungi exhibit two methods of mechanical invasion to the keratinized substrate, surface erosion and radical bore through. These colonizing methods are readily visible with enzymatic degradation and are a consistent substrate fingerprint of keratinolytic (Fusconi and Filipello, 1991). In this work, five species belonging to the genus *Aspergillus* were isolated from soils of poultry farms and were assessed for production of cystiene, total proteins, keratinase and change in pH of medium.

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Materials and Methods

Samples. *Aspergillus* species were obtained from soil collected from poultry farms. A total thirty-nine samples were collected from twenty-four different sites of ten different poultry farms of Korea, during 2001~2002. At each location, 50 g of soil were collected from the superficial layer, at a depth of 3~5 cm. Each sample was placed in a sterile plastic bag, transported to the laboratory in a chilled box, and analyzed on the day of collection.

Isolation, identification and culture of fungi. For total fungal counts, 10 g soil sample was blended in 100 m*l* of sterile water containing Tween 20 (0.01%) and the dilution was plated on dichlroran-rose bangal chloramphenicol medium with streptomycin and chlorotetracycline (Beuchat, 1992). Plates were incubated at 25°C for 5 days. Plates that contained 15 to 150 colonies were used for counting and the results were expressed as CFU per gram of sample (Mislivec *et al.*, 1979). For the isolation of *Aspergillus* spp., samples were incubated at 28°C for 5 days on plates containing a modified dichloran-rose bengal medium (Horn and Domer, 1998; Horn *et al.*, 1994) and Czapek Dox Agar with 10% lactic acid. The Pleatelia *Aspergillus* Kit (Bio-Rad, cat. N. 62797) was used to identify the circulating galactomanan of *Aspergillus* spp. The kits were kindly supplied by the Manufacturer and

the tests were performed according to the Manufacturers instructions. The test is based on monoclonal antibody (EB-A2), which recognizes the β-D-galactofuranoside side chain of the galactomannan molecule. Feather-meal agar plates containing feather meal, 10 g; NaCl, 0.5 g; K_2HPO_4, 0.3 g; KH_2PO_4, 0.4 g; agar 15 g, and deionized water, 1,000 m*l* were used to screen fungi exhibiting keratinolytic activity. Keratinase activity of the fungus was detected as a clear zone around the colony after incubation for 3 days at 30°C. The diameter of the clear zone was measured to quantify activity, preliminarily.

Preparation of keratin substrate. Chicken feather was washed four times with chloroform-methanol (1:1, v/v), finally with glass-distilled water and than dried in air. Pieces of feather about 1 cm length were weighed into portions of 500 mg and sterilized by tyndallization at 80°C for 30 min on three successive days with intermediate incubation at 28°C.

Preparation of medium for inoculation. Basal medium was prepared for five *Aspergillus* species, as follows: Glucose, 0.9 g; $MgSO_4 \cdot 7H_2O$, 0.025 g; $CaCl_2$, 0.025 g, $FeSO_4 \cdot 7H_2O$, 0.015 g, $ZnSO_4 \cdot 7H_2O$, 0.005 g; distilled water, 1,000 m*l*; pH 7.8. Erlenmeyer flasks containing 50 m*l* of sterilized basal medium supplemented with 500 mg of pre-sterilized feather as a keratin source were inoculated and incubated at 28°C and 120 rpm for 40 days. Flasks containing the medium with a disc of agar without the fungus served as control. For each species, four test flasks and one control set were maintained. For inoculation, inoculum discs (8 mm diameter) were obtained from the periphery of activity growing 7-day-old subcultures of *Aspergillus* species.

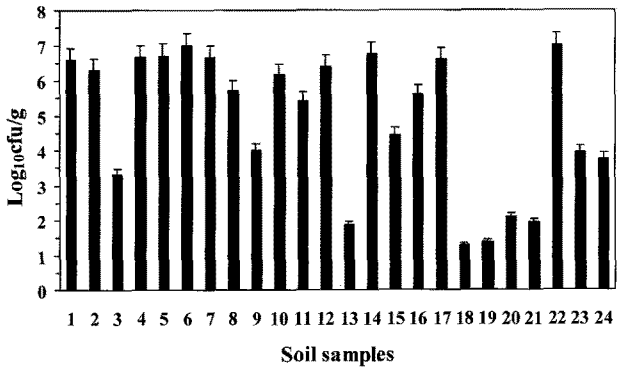

Fig. 1. Log_{10} of total count (cfu/g) of fungi in soil samples collected from poultry farms.

Enzyme activity. At the end of the growth period, the fungal mat and feather were separated from culture medium by filtering through whatman filter paper NO. 42. The culture filtrate from four test flasks was pooled, centrifuges at 5,000 rpm for 5 min and the supernatant was assayed for change in alkalinity, release of cystiene, protein and keratinase. Estimation of cystiene was done by the method of Ramakrishna *et al.* (1979). Total proteins were estimated following the method of Bradford (1976) using bovine serum albumin as standard and extracellular keratinase was measured as per the method of Yu *et al.* (1968).

Results and Discussion

Distribution and identification of keratinolytic fungi. Thirty-four soils (87%) were polluted with keratinolytic fungi, and *Aspergillus* was isolated from twenty-seven samples (69%). Densities (cfu/g) of total fungi varied among

Table 1. Percentage frequency of keratinophilic fungi isolated from poultry farming soil

Isolated species	Percent frequency[a]									
	1[b]	2	3	4	5	6	7	8	9	10
Acremonium cerealis	–	–	12.7	10.6	–	13.0	15.0	4.0	–	9.3
Alternaria alternata	–	–	–	14.8	–	–	15.0	6.0	–	15.6
Aspergillus flavus	23.3	20.0	12.7	18.5	18.1	–	15.0	–	13.7	15.6
Aspergillus fumigatus	20.0	15.0	14.0	17.7	–	–	22.0	13.6	17.2	–
Aspergillus niger	–	17.5	10.6	10.9	13.6	21.7	–	22.2	17.2	–
Aspergillus nidulans	6.4	17.5	15.8	10.6	8.2	–	–	–	10.0	–
Aspergillus terreus	10.0	15.0	10.6	–	5.8	13.0	18.0	–	10.3	–
Cladosporium cladosporioides	20.0	12.5	–	8.5	22.7	–	–	22.2	–	–
Curvularia lunata	10.3	–	10.6	–	13.6	10.6	15.0	-	10.6	18.3
Fusarium moniliforme	10.0	–	–	4.2	–	13.0	–	18.5	7.0	15.6
Monascus ruber	–	–	–	4.2	–	7.0	–	3.5	–	15.6
Mucor hiemalis	–	–	13.0	–	18.0	5.0	–	–	–	4.2
Penicillum chrysogenum	–	2.5	–	–	–	8.6	–	5.0	6.8	
Verticillium albo-atrum	–	–	–	–	–	7.1	–	5.0	7.2	5.8

[a]% Frequency = (Number of isolated of a keratinolytic fungus/Total number of fungi)×100.
[b]1. Ansung; 2. Pyungtaek; 3. Hongcheon; 4. Heongseong; 5. Goesan; 6. Chungju; 7. Jeonju; 8. Andong; 9. Ulsan; 10. Kimhae.

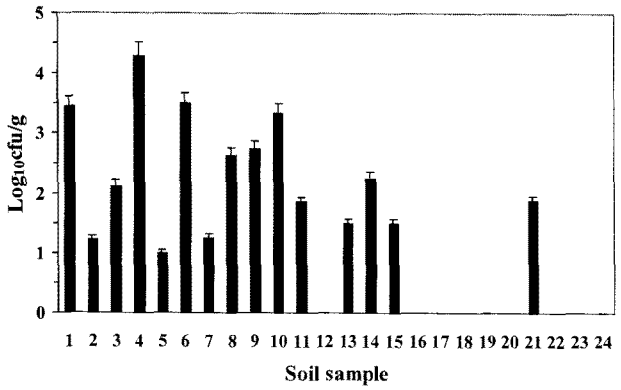

Fig. 2. Log_{10} of total count (cfu/g) of *Aspergillus* species in soil samples collected from poultry farms.

soil samples (Fig. 1). In the results of identification of the fungi according to their macro- and micro-morphological characteristics in monographs (Ellis, 1971; Booth, 1977; Domsch *et al.*, 1980), fourteen species of feather-associated fungi belonging to ten genera *Acremonium*, *Alternaria*, *Aspergillus*, *Cladosporium*, *Curvularia*, *Fusarium*, *Monascus*, *Mucor*, *Penicillum*, and *Verticillium* were recovered and assessed for their keratinolytic ability (Table 1). Although some of the fungi isolated are unusual soil fungi, the presence of keratin in the soil may promote their distribution and growth. The populations of *Aspergillus* spp. also varied considerably among samples (Fig. 2), and the difference in the mean *Aspergillus* spp. populations associated with the soil sample is 1×10^5 cfu/g. Only 12.5% of soil samples had no detectable *Aspergillus* species. After identification of *Aspergillus* spp. with using Pleatelia Aspergillus Kit (Bio-Rad, cat. N. 62797), three isolates of *A. flavus*, six of *A. fumigatus*, six of *A. niger*, six of *A. nidulans*, and nine of *A. terreus* exhibited keratinolytic activity.

Change in alkalinity. Change in the pH of medium towards alkalinity was noted after the release of cysteine, keratinase and proteins by the five *Aspergillus* species. Observation showed that the fungi, which have strong keratinolytic ability, changed the culture medium more alkaline than those were less keratinolytic (Fig. 3D). It is suggested that the basis of keratinolysis is the high level of deamination, which renders the medium alkaline (Yu *et al.*, 1968). Although deamination and alkalization of the medium surely play a role in keratinolysis, they cannot alone cause important substrate denaturation (Kunert, 1995).

Keratinolytic activity of five *Aspergillus* species. Five the test fungi viz. *Aspergillus flavus*, *A. fumigatus*, *A. niger*, *A. nidulans*, and *A. terreus*, could grow on chicken feather and degrade it, releasing sulphydryl-containing

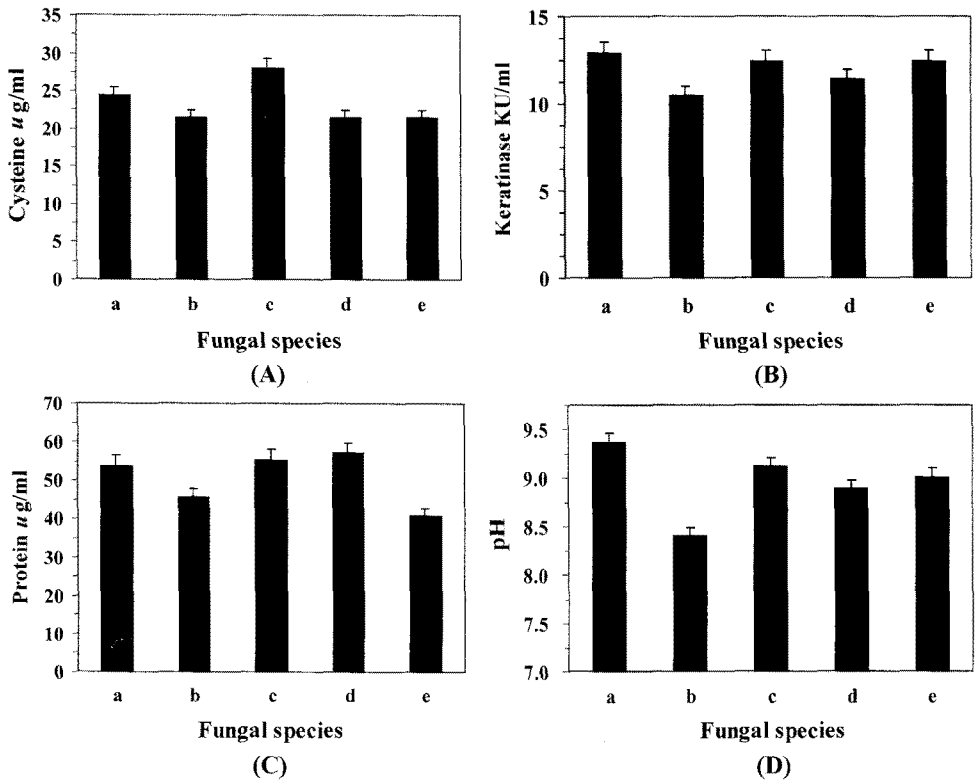

Fig. 3. Keratinolytic activity of four *Aspergillus* species on feather after 40 days of incubation at 28°C and 150 rpm. (A), Release of cysteine; (B), Release of keratinase; (C), Release of protein; (D), Change of pH. Initial pH is 7.8. Fungal species, a, *Aspergillus flavus*; b, *A. fumigatus*; c, *A. niger*; d, *A. nidulans*; e, *A. terreus*.

compounds detected as cysteine (Fig. 3A), keratinase (Fig. 3B) and total proteins (Fig. 3C). In fact, 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 releases by controls (Weary *et al.*, 1965). These amino acids may enter the protein pool of the fungus, thereby enhancing its growth and simultaneous keratinase production (Kaul and Sumbali, 1999).

The most distinctive character of keratin is its high cysteine content. The disulphide linkage between the amino acids renders the keratin molecule more resistant to enzyme digestion. The five isolates showed fairly good amounts of cysteine in the culture medium. Maximum cysteine was released in the basal medium supplied with 500 mg of pre-sterilized chicken feather as a keratin source by *Aspergillus niger* (28 μg/ml) followed by *A. flavus* (24 μg/ml), *A. fumigatus*, *A. nidulans* and *A. terreus* (21 μg/ml). Kunert (1973) reported the release of cysteine by *Trichophyton rubrun* and *Microsporium gypseum* growing on wool and hair, respectively. Hasija *et al.* (1990) isolated thirty keratinophilic fungi for their ability to degrade human scalp hair and found that most of them could release cysteine. Determining and comparing the protein release into the medium also monitored the degradation of chicken feather. The *Aspergillus* species produced variable amounts of total protein (Fig. 3C). *Aspergillus nidulans*, *A. niger*, and *A. flavus* produced 57 μg/ml, 55.3 μg/ml, and 53.7 μg/ml protein at the end of the experimental period, respectively. Malviya *et al.* (1992) have reported that similar values of protein release after prolonged incubation. In the investigation of keratinolytic activity measured in keratinase units (KU), five fungal isolates secreted considerable amounts of keratinase (Fig. 3B), which is known to be responsible for hydrolysis of keratin in nature. Higher (12.9 KU/ml) and lower keratinase activity (10.4 KU/ml) was recoded by *Aspergillus flavus*, and *A. fumigatus*, respectively. But the differences were not significant. Santos *et al.* (1996) have investigated that *A. fumigatus* was useful for the microbial conversion of keratinous waste and *A. flavus* has been selected as a prospective producer of a keratinolytic enzyme (Gradišar *et al.*, 2000). Mahgoub (1973) described a case of black grain mycetoma in human caused by *Curvularia lunata*. In fact, keratinase activity of keratinophilic fungi is considered to be one of the most important traits correlated with their capacity to infect and cause pathogenic syndromes in diverse kinds of living organisms.

In conclusion, keratinolytic fungi are of great ecological interest not only in pathogenesis but also in keratin degradation. The degradative enzymes produced by *Aspergillus* spp. are capable of breaking down complex keratinous substrates in nature, and thus are responsible for the biodegradation of keratinized structure in polluted habitats.

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