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Isolation, Screening and Selection of Efficient Feather Degrading Bacteria

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ABSTRACT

Feathers are produced in large amount as a waste by poultry product processing plants; it reaches millions of tons per year worldwide. They can be degraded by keratinolytic bacteria. Keratinolytic enzymes have important utilities in biotechnological processes involving keratin-containing wastes from poultry and leather industries, through the development of non-polluting processes. Till date most of the purified keratinase cannot completely degrade keratin, their exact nature and uniqueness for keratinolysis is still not clear, so there is a requirement to isolate new sources of microbial keratinases to meet the industrial demand. The aim of this study was to select keratin-degrading bacteria and to study their possibility to degrade chicken feathers. Three bacteria isolates from 32 isolates were highly efficiency for produced extracellularly keratinolytic in feather meal media. The three isolates were identified as *Bacillus subtilis, Enterobacter cloacae* and *Aeromones* media-like DNA group 5A, respectively. Maximum keratinase activity of isolate PE2 was found at 72 hrs, isolate PW11 at 96 hrs and isolate PW12 at 120 hrs. The optimum condition for the fermentation production of PE2 keratinase was achieved at substrate concentration of 2.0 %, pH 8.0 and 50 ^{o}C over a fermentation time of 72 h. While, PW11 and PW12 the optimum condition for the fermentation production of keratinase was achieved at substrate concentration of 2.0 %, pH 8.5 and 40 ^{O}C over a fermentation time of 96 h.

Key words Feather degradation, keratinolytic bacteria, *Bacillus subtilis, Enterobacter cloacae* and *Aeromones* sp.

Introduction

Worldwide poultry processing plants produce millions of tons of feathers as a waste product annually, which consists of approximately 90% keratin. Feathers represent 5-7% of the total weight of mature chickens. Compared with other keratin resource for production of keratinase, feather causes serious environmental problem all over the world. These feathers constitute a sizable waste disposal problem. Feathers hydrolyzed by mechanical or chemical treatment can be converted to feedstuffs, fertilizers, glues and foils or used for the production of amino acids and peptides. Keratinolytic enzymes have important utilities in biotechnological processes involving keratin-containing wastes from poultry and leather industries, through the development of non-polluting processes. Keratinases are produced only in the presence of keratin containing substrates. Microbial keratinases have become biotechnologically important since they target the hydrolysis of highly rigid, strongly cross-linked structural polypeptide "keratin".

Keratins are grouped into hard keratins (feather, hair, hoof and nail) and soft keratins (skin and callus) according to sulphur content (Gupta and Ramnani, 2006). These proteins belonging to the scleropeptides group are compounds that are extremely resistant to the action of physical, chemical and biological agents. One of the main characteristics of keratins is that they have high mechanical stability and resistance to proteolytic degradation, which depends on the disulfide and hydrogen bonds, salt linkages and other crosslinkings (Hoq et al., 2005). Therefore, keratinous material is water insoluble and extremely resistant to degradation by common proteolytic enzymes such as trypsin, papain and pepsin (Gradišar et al., 2005).

Utilizing poultry feathers as a fermentation substrate in conjunction with keratin-degrading microorganism or enzymatic biodegradation may be a better alternative to improve nutritional value of poultry feathers and reduce environmental waste.

Keratinolytic enzymes have important utilities in biotechnological processes involving keratin-containing wastes from poultry and leather industries, through the development of non-polluting processes. After hydrolysis, the feathers can be converted to feedstuffs, fertilizers, glues, films and as the source of rare amino acids, such as serine, cysteine and proline (Cao et al., 2009).

Given the potential uses of the thermostable keratinase and ever increasing demand for waste disposal of feather there is a need for discovery of new strains of bacteria and also for the development of low cost industrial medium formulations. Studies on medium optimization for protease production are useful techniques for multifactor experiments and they should be less time consuming and capable of detecting the true optimum factor. Conventional methods for optimization of protease parameters are extremely time-consuming and

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expensive. Designing an appropriate fermentation medium is of critical importance because medium composition influences product concentration, yield, and volumetric productivity.

Medium optimization is very important to maximize the yield and productivity and also minimizes the production cost (El-Bendary, et al., 2007, Fakhfakh-Zouari et al. 2009). Many authors have used Response surface methodology to predict the specific activity of the enzyme in study. This method has been successfully applied for media optimization in different fermentation processes (Qasim et al., 2003, Senthilkumar, et al, 2005, and Himabindu, et al., 2006) as well as for establishing the conditions of enzymatic hydrolysis (Adinarayana and Suren, 2005).

The aim of this study was to select keratin-degrading bacteria and to study their optimization of environment condition for possibility to degrade chicken feathers.

Materials and Methods

Isolation, purification and reservation of feather degrading bacteria from different sample sources

To isolate potential feather degrading bacteria, environmental samples were collected from primary effluent and poultry/animal wastes. The samples were diluted and plated out in feather meal agar (FMA) plates composed of agar and 10 % commercial feather meal made up in Minimum Growth Medium (MGM). The Minimum Growth Medium (phosphate basal solution) was composed of (in g/l): NaCl, 0.5; KH_2PO_4, 0.7; K_2HPO_4, 1.4; $MgSO_4.7H_2O$, 0.1; pH 7. The plates were incubated at 30°C, 37°C and 50°C in a incubator and sampled at 24, 48 and 72 h. Single colonies were passaged twice on FMA plates to obtain purified microbial strains. The purified isolates were maintained in nutrient agar slants and stored at +4 °C.

Screening and selection of feather degrading bacteria

MGM (10 ml) supplemented with 1.0 % commercial feather meal was autoclaved and inoculated with a loopful (approximately 20 µl) of single colony grown on nutrient agar plate (preculture) and incubated at 250 rpm for 16 h. One ml of the preculture was inoculated into 50 ml of growth medium (main culture) and incubated for 24 h at pH 8. Crude enzyme extract was recovered by centrifugation of the culture medium at 5000 *g* for 20 min and the supernatant collected and ultra-filtered under vacuum through the 0.45 µm sterile membrane. The microbial cells and the crude enzyme extracts were used to selection for high efficiency microorganisms degradation of feather.

Selection of keratinolytic bacteria by feather degrading culture

Active isolates having keratinolytic activity were screened qualitatively for their keratinolytic activities using MGM supplemented with 1.0 % commercial feather meal. Seed culture of the high efficiency microorganisms degradation of feather were prepared in 500 ml Erlenmeyer conical flask containing 200 ml of the culture media that was maintained at 30°C at 160 rpm in shaken culture medium with a continuous incubation periods for 15 days. The selected isolates were cultural on feather meal agar plate and incubated at 30 ^{O}C for 72 hrs and the clear zones was determined. The bacterial isolates has visually degraded feather were selected for further investigation in this study.

Identification of selected bacterial isolates

The bacteria were identified using Bergey's manual of determinative bacteriology based on the morphological, physiological and biochemical tests (Brenner et al., 2004) as well as Molecular characterization based on 16S rRNA gene (Thompson et al., 1994).

Optimization of the culture medium for growth and keratinase production
1. Selection of media

A loop-full of 48h-old single colony of each strain was transferred from a fresh FMA plate into 25 mL in three media, Basal feather media ((g/L): glucose, 20.0; chicken feather, 10.0; corn steep flour, 2; K_2HPO_4, (1.0; $MgSO_4 \cdot 7H_2O$, 0.1 and pH 7.5), Feather meal media1 ((g/L): NaCl, 0.5; K_2HPO_4, 0.3; KH_2PO_4, 0.4 and Feather, 10; pH 7.5), and Feather meal2 media 2 (NH_4Cl, 0.5; NaCl, 0.5; K_2HPO_4, 0.3; KH_2PO_4, 0.4; $MgCl_2.6H_2O$, 0.1; Yeast extract, 0.1 and Feather, 10; pH 7.5) at 30°C at 160rpm for 5 days, they were used for keratinase production. After five days of incubation, the crude culture broth was centrifuged (10,000g, 4 ^{O}C, 30

min) and cell free supernatant was used to determination of keratinase activity. Medium showing maximum activity was used for the optimization growth and keratinase producing strains.

2. Optimization of cultural parameters of growth and keratinase production

The investigated parameters were carried out in FMA's medium (without agar) as follows: different concentrations of feather (5, 10, 15, 20 and 25 g/l), different initial pH (6.5, 7.5, 8.0 and 8.5), inorganic nitrogen sources (serial concentration of sulfate ammonia and sodium nitrate in the range of 0 to 15 g/l), different concentration of corn steep flour (0, 2, 7, 12 and 20 g/l) and different substrate (chicken feather, pigeon feather, hair and nail). The uninoculated autoclaved medium with feather substrate was incubated under similar conditions to serve as the control.

After five days of incubation, the residual feathers were harvested from the fermentation media by filtering it over Whatman filter paper 3 to determination of degree of feather degradation (DFD). The crude culture broth was centrifuged (10,000g, 4 ^{O}C, 30 min) and the supernatant was collected to be analyzed for keratinase activity and soluble protein Content (SPC).

Determination of Keratinase production

Keratinase activity was measured according to Grzywnowicz et al. (1989). The reaction mixture contained 1.0mL of the culture supernatant diluted five times and 1.5mL of 0.67% (w/v) keratin suspension in phosphate buffer 0.1M, pH 7.4. After 1 hour of incubation at 37◦C the reaction was interrupted by the addition of 1ml trichloroacetic acid 10% and placed in a refrigerator at 4◦C for 30 minutes. An enzyme control was prepared by the addition of 1ml trichloroacetic before incubation. The reaction mixture was centrifuged (2000 g/10 min) and read at 280nm in a spectrophotometer. One unit of keratinase activity (U) was defined as the amount of enzyme producing an absorbance change of 0.01 units (A595). The control experiments were (a) reaction mixture without crude keratinase (enzyme activity control) and (b) reaction mixture without keratin azure substrate (crude extract absorbance control).

Determination of soluble protein Content (SPC)

The total protein contents of the samples were determined according to the method described by Lowry (Lowry et al., 1951); using albumin bovine serum as the standard. Readings were carried out in a spectrophotometer at 660 nm.

Determination of degree of feather degradation (DFD)

Residual feathers were harvested from the fermentation media by filtering it over Whatman filter paper 3. The harvested feathers were kept in hot air oven at 50°C until weight stabilized to constant value. The difference between the weight of residual feather obtained from the control and that of inoculated media has been used as measure of feather degradation.

Statistical analysis

All the experiments were done in triplicate; the average of triplicate determinations was used to represent the result. Values are given as means ± SD. for triplicate samples. Data were analyzed by analysis of variance (ANOVA) and the means were compared with Duncan's Multiple Range Test at $p<0.05$ level.

Results and Discussion

Isolation and Selection Feather Degradation Bacteria

Feather degrading bacteria isolates were specifically screened and selected by measuring the activity of their keratinases. Results of keratinase activities of 32 isolates as evaluated by keratinase assay showed that isolate number PE2 (from primary effluent), PW11 and PW12 (from poultry wastes) demonstrated the highest mean keratinase activity (Fig.1), and hence were selected for further study.

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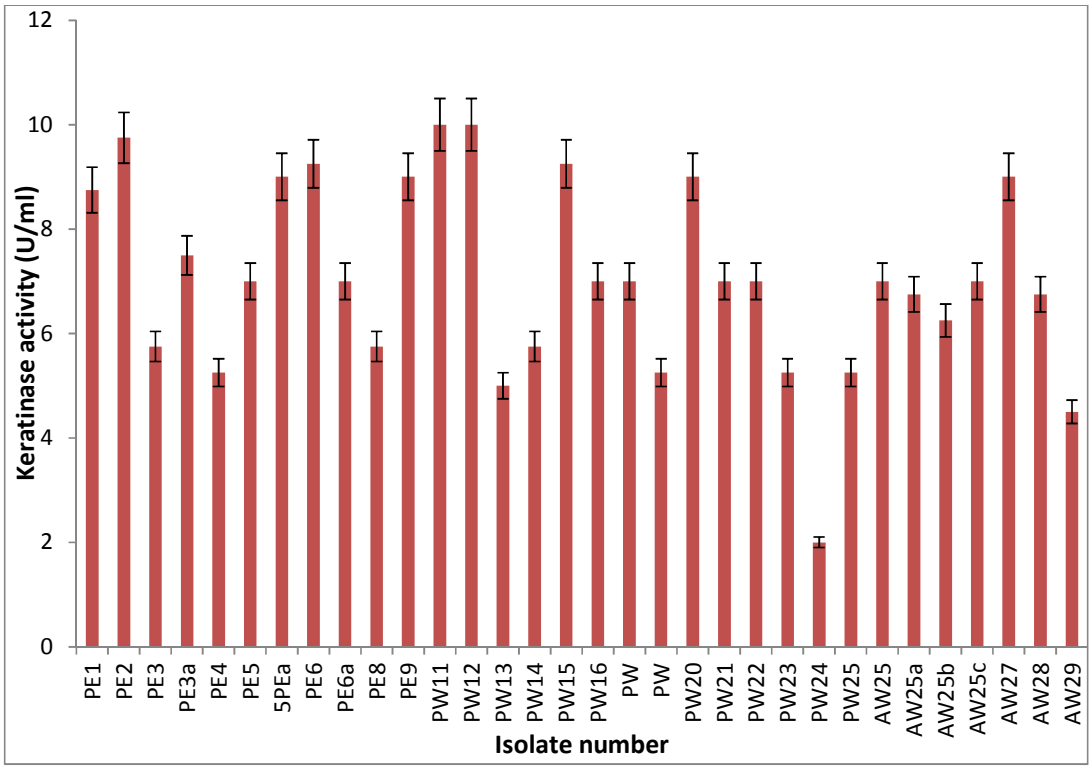
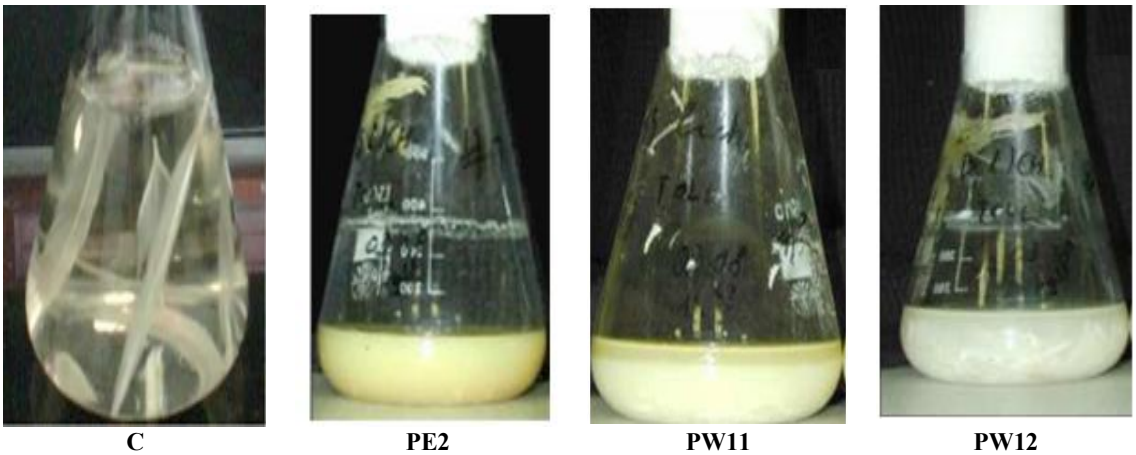


Fig. 1: Keratinase activity (Mean ± SD, n = 3, each data point) for 32 microbial isolates tested

Selection of Feather Degradation Bacteria by Feather Degrading Culture

Complete decomposition of chicken feather was observed within 72 hours in cause of isolate PE2. But in cause of isolates PW11 and PW12, the feathers were fully degraded within 96 hrs of incubation with the selected isolates, respectively (Fig. 2). Therefore, the isolates PE2, PW11 and PW12 were selected as they produced clear zones on incubation at 30ºC for 72hrs suggesting the presence of keratinolytic activity (Fig. 3).



C **PE2** **PW11** **PW12**

Fig. 2: Degradation of chicken feathers by the selected bacterial strain isolated.(C) Feather control without the bacterial strain, (PE2, PW11 and PW12) selected bacteria

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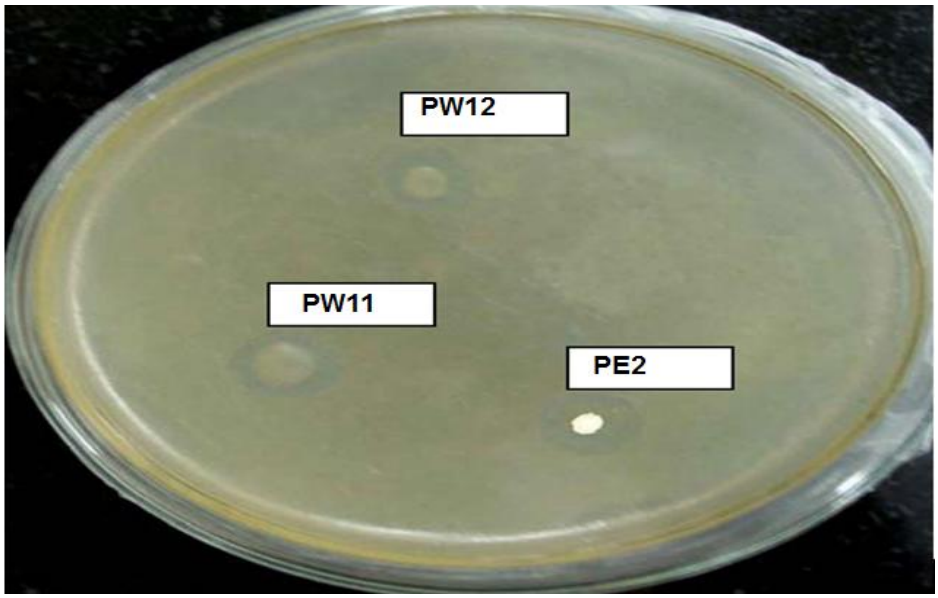

Fig. 3: Production of clear zones in feather meal agar plates by selection keratinolytic bacteria

Identification of Selected Bacterial Isolates

A number of morphological, physiological and biochemical properties were used to identify classify and characterize bacterial isolates. Morphological characteristic and cultural characteristic of the three isolates were identified as:

Isolate PE2 was found to be a Gram positive rod bacterium displaying a number of characteristics matching the *Bacillus* species, Isolate PW11 was found to be a Gram negative rod bacterium displaying a number of characteristics matching the *Enterobacter* and isolate PW12 was to be a Gram negative short rod bacterium displaying a number of characteristics matching the *Aeromonas* as listed in the Bergey's Manual of Determinative Bacteriology (Holt, 1993).

The three isolates showed different capability in growth on feather meal medium. Based on the sequence analysis of 16S rRNA (Fig 4), isolates PE2 and PW11 had 100% homology in 16S rRNA sequence to *Bacillus subtilis* (PE2) and *Enterobacter cloacae* (PW11), respectively and isolate 12 had 100% homology to *Aeromonas* media culture-collection HAMBI (PW12).

Optimization of cultural and environmental conditions

Selection of media

The maximum keratinase activity of each isolate culture was seen on the 120hrs (5^{th} day) of incubation in Basal feather media (Fig. 5). Relatively the microbial culture could produce 2.53 times more keratinase in Basal feather media than all medium tested (Table 1). Table 6 showed that total protein content, yield, specific activity of keratinase from microorganisms grown in the three different enriched Feather media. The resulted show that ammonium sulphate fraction of the crude cell free supernatant which was seeded with the culture inoculum showed 1.5 fold purification for keratinase (Table 1).

Optimization of the concentration of feather

The amount of keratinase production was depended on the initial concentration of feather in the medium and affects the extent of feather degradation and keratinase activity. It was observed that 20 g/l feather had the highest keratinase production as well as the highest SPC and DFD (Fig. 6). Keratinase produced at 5 g/l feather was seven times less than that of 20 g/l feather, while increasing the feather concentration to 25 g/l feather showed substrate repression on the keratinase production, which leads to the decrease in SPC and DFD.

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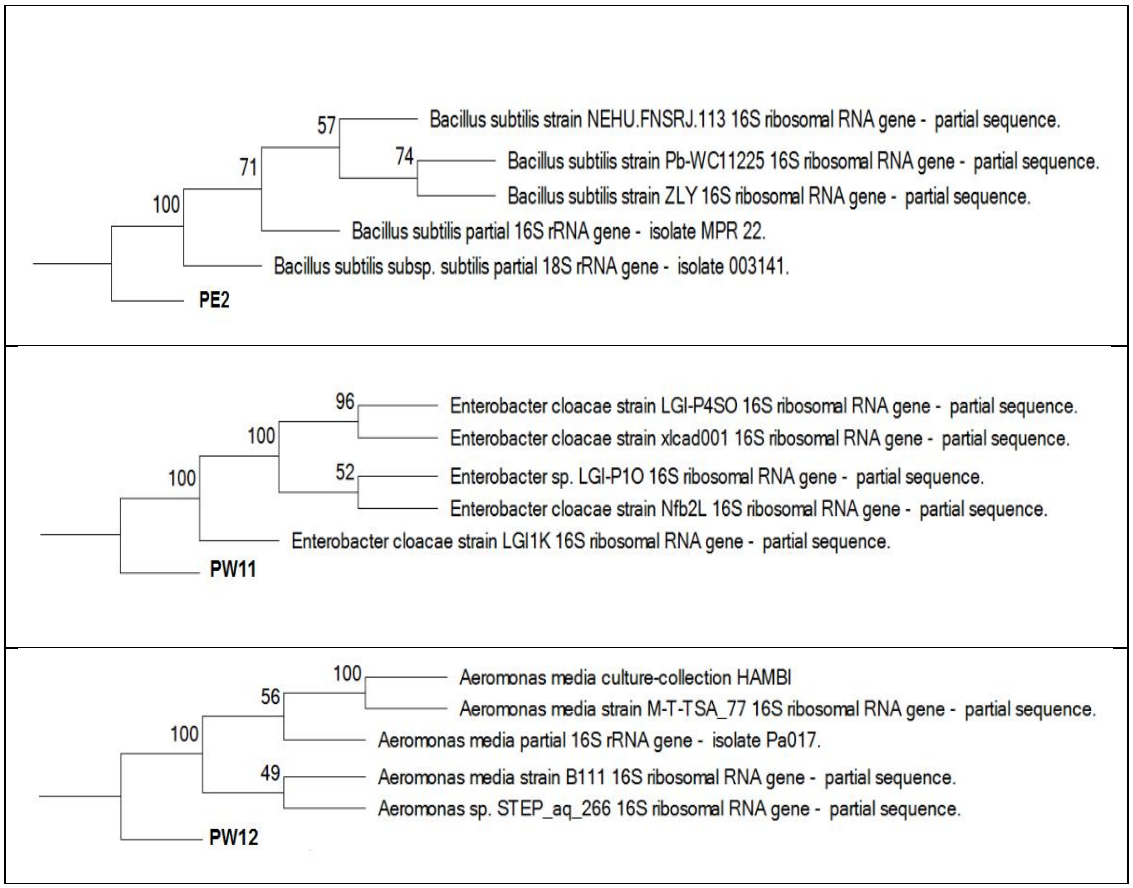

Fig. 4: Phylogenetic tree of bacterial isolates (PE2, PW11 andPW12) and relationship among the selected strains based on sequencing analysis and the most closely related bacterial species

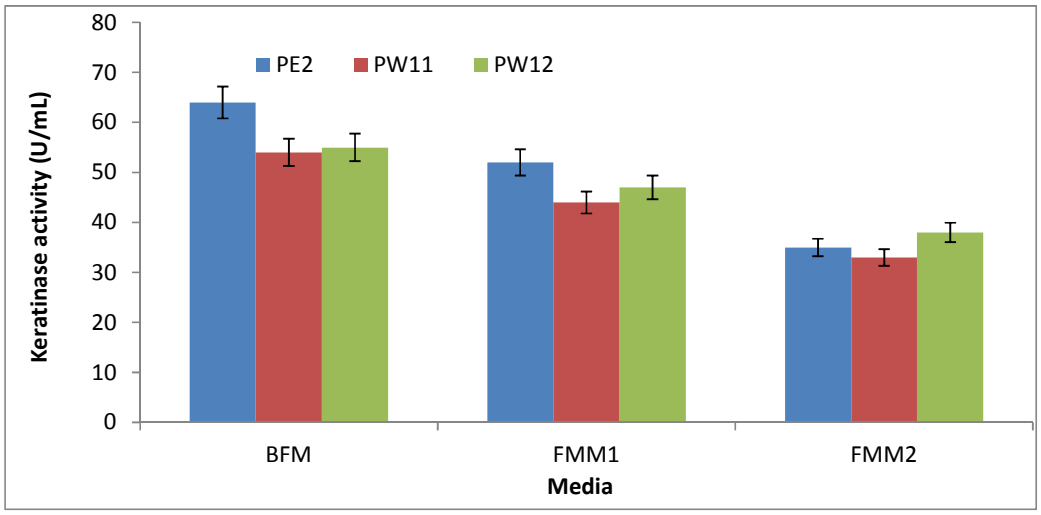

Fig. 5: Keratinase activity produced by the three selected feather degrading bacteria after 5 days

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Table 1: Total protein content, yield, specific activity of keratinase from microorganisms grown in the different enriched Feather media

Purification step	Protein (mg)	Total activity (U[a])	Specific activity (U/mg)	Yield (%)	Purification (Fold)
	Bacillus subtilis				
Basal feather media (0-80% A.S.)	279.5	33.900	121.28	294.78	1.463
Feather meal media 1 (0-80% A.S.)	63	4.650	73.80	40.80	0.890
Feather meal media 2 (0-80% A.S.)	217.5	23.900	110.138	207.82	1.328
	Enterobacter cloacae				
Basal feather media (0-80% A.S.)	198	30.51	119.30	281.11	1.351
Feather meal media 1 (0-80% A.S.)	59	4.347	69.67	38.24	0.728
Feather meal media 2 (0-80% A.S.)	204	21.386	104.942	197.57	1.112
	Aeromonas media culture-collection				
Basal feather media (0-80% A.S.)	191	28.67	112.82	267.94	1.220
Feather meal media 1 (0-80% A.S.)	55	4.026	64.91	37.29	0.648
Feather meal media 2 (0-80% A.S.)	198	20.184	100.893	185.49	1.077

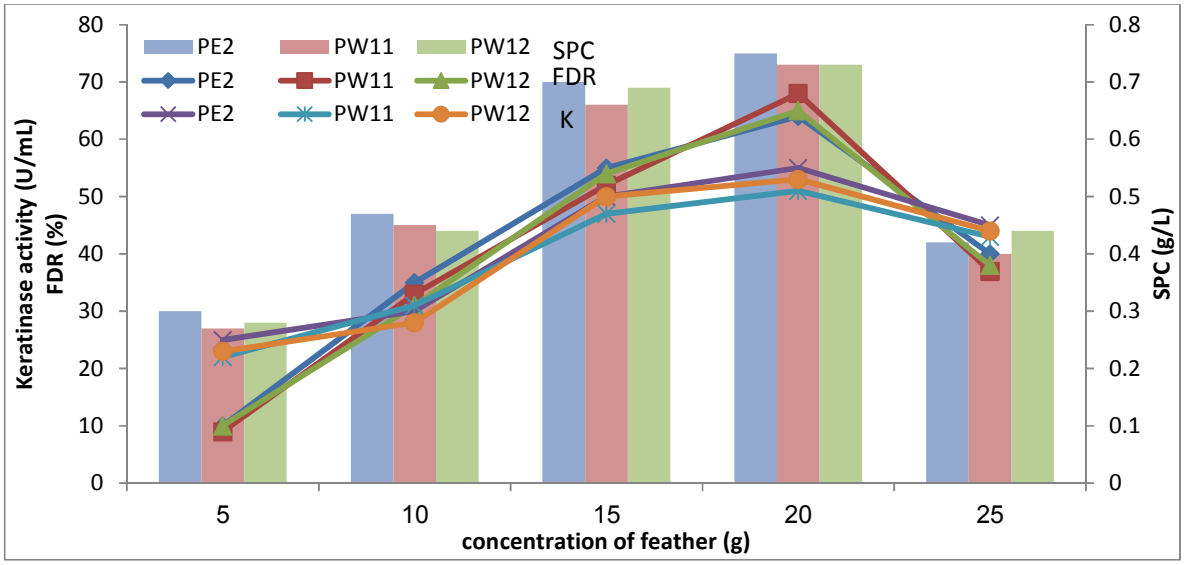

Fig. 6: Effect of initial concentration of feather on keratinase production, SPC and DFD

Optimization of initial pH of culture medium

The initial pH of the culture medium greatly affected the keratinase production, SPC and DFD; initial pH 8.0 was evidently optimal for keratinase production for PE2 (Table 8). While PW11 and PW12 the initial pH 8.5 was evidently optimal for keratinase production. Therefore, it is reasonable to say that the highest keratinase production occur in the basic circumstance at pH 8.0 that helps the keratin dissolve into the culture medium.

Optimization of inorganic nitrogen sources in culture medium

As the two fast nitrogen sources, ammonia and nitrate salts were added into the media. Keratinase activity, SPC and DFD were hardly detected and dramatically decreased in comparison with the control, suggesting that neither ammonia sulfate nor sodium nitrate at any concentrations exerted a positive effect on keratinase production (Table 3). On the contrary, the addition of fast nitrogen source (that is, ammonia salt and nitrate salt) restricted the induction of keratinase. Therefore, it is necessary to exclude any ammonia salt and nitrate salt in the medium for the production of keratinase by all isolates studies.

Table 2: Effect of initial pH of culture medium on keratinase production, DFD and SPC

pH	Keratinase activity (U/mL)	DFD (%)	SPC (%)
	Bacillus subtilis		
6.5	8.3±0.52^{c*}	7.9±0.42^{d}	0.21±0.16^{d}
7.5	26.1±0.56^{b}	35.4±0.52^{c}	0.35±0.22^{c}
8.0	35.7±0.68^{a}	42.3±0.66^{a}	0.73±0.18^{a}
8.5	23.7±0.71^{b}	29.6±0.67^{b}	0.60±0.20^{b}
	Enterobacter cloacae		
6.5	7.7±0.48^{d}	7.0±0.37^{d}	0.18±0.14^{d}
7.5	24.7±0.53^{b}	33.9±0.50^{b}	0.26±0.16^{c}
8.0	22.6±68^{c}	28.9±045^{c}	0.49±0.12^{b}
8.5	33.5±0.63^{a}	41.5±0.94^{a}	0.62±0.21^{a}
	Aeromonas		
6.5	8.0±50^{c}	6.9±0.37^{d}	0.22±0.17^{c}
7.5	25.3±0.54^{a}	34.7±0.51^{b}	0.27±0.17^{c}
8.0	22.9±0.44^{b}	30.1±0.47^{c}	0.50±0.12^{b}
8.5	25.0±0.75^{a}	41.9±0.95^{a}	0.69±0.23^{a}

** Figures in the same column followed by the same letters are not significantly different (p> 0.05) based on Duncan's multiple range test*

Table 3: Optimization of different inorganic nitrogen on keratinase activity and SPC

N source	Control	$(NH_4)_2 SO_4$			$NaNO_3$		
	0	5	10	15	5	10	15
	Keratinase activity (U/mL)						
B. subtilis	35.1±0.68^{a}	10.5±0.36^{d}	8.2±0.51^{e}	7.9±0.47^{e}	17.4±0.49^{b}	12.1 ±0.63^{c}	9.3±0.72^{d}
E. cloacae	33.7±0.65a	9.8±0.34d	7.9±0.49e	7.5±0.45e	16.7±0.47b	11.4±0.59c	8.8±0.68d
Aeromonas	34.9±0.68a	10.0±034d	8.1±0.50e	7.6±0.45e	17.1±0.48b	11.8±0.61c	9.2±0.71d
	SPC (g/L)						
B. subtilis	0.71±0.17a	0.10±0.04c	0	0	0.11±0.04c	0.22±0.18c	0.40±0.11b
E. cloacae	0.65±0.16a	0.09±0.04d	0	0	0.08±0.03d	0.20±0.16c	0.38±0.11b
Aeromonas	0.70±0.17a	0.10±0.04d	0	0	0.09±0.036d	0.21±0.17c	0.41±0.11b

Figures in the same column followed by the same letters are not significantly different (p> 0.05) based on Duncan's multiple range test

Optimization of the concentration of corn steep flour in culture medium

The results presented in (Fig. 7) demonstrated that 7 g/l of corn steep flour gave the highest keratinase activity. Additionally, the SPC displayed similar trend as the enzyme activity.

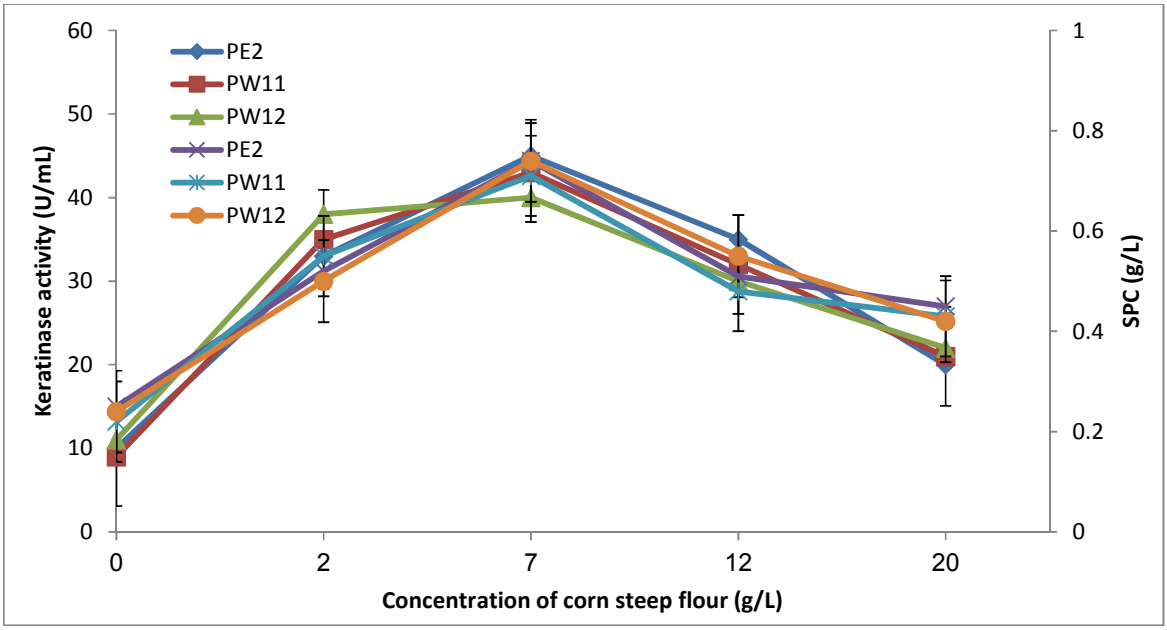

Fig. 7: Effect of concentration of corn steep flour in cultural media on Keratinase activity and SPC

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Effects of different substrates in culture medium

Various substrates like (chicken feather, pigeon feather, hair and nail) were evaluated for the production of enzyme (Fig. 8). All isolated strains (PE2, PW11 and PW12) were grown in four nutrient sources and produced keratinase. The maximum yield of keratinase was seen in basal media supplemented with chicken feathers as compared to other substrate like pigeon feather, hair and nail, followed by the pigeon feather. The isolate PW11 and PW12 shown that highly efficiency degradation of hair and nail compared to PE2 (Fig. 8).

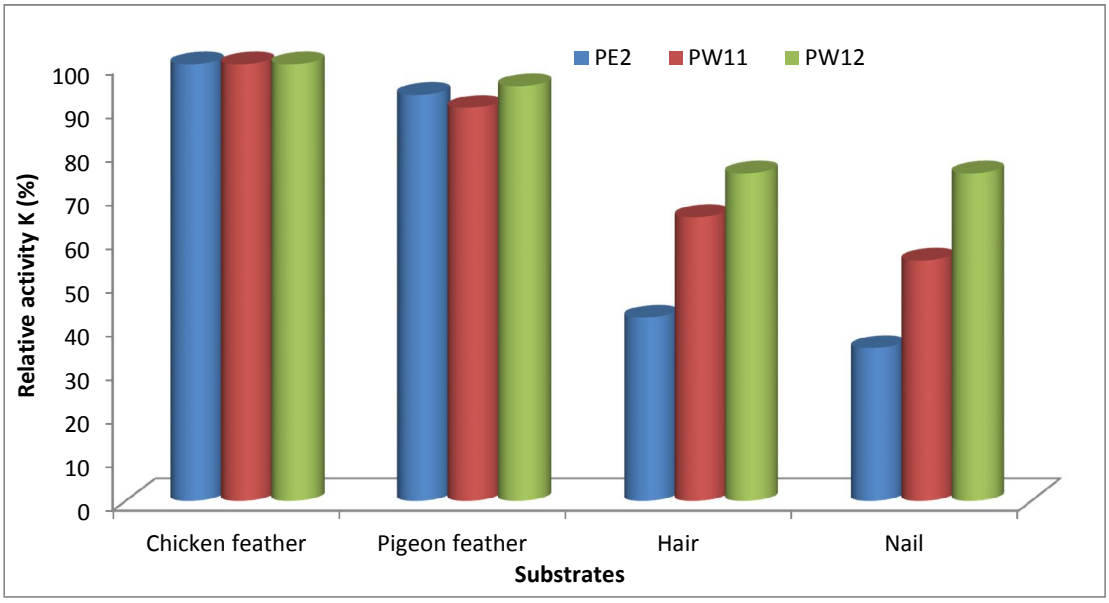

Fig. 8: The relative activity of keratinases in presence of different substrates.

Discussion

In this study, the feather degradation microorganisms has the capacity to producing keratinase was investigated. Microorganisms were isolated from farmyard wastes and primary effluent known to be rich sources of microorganisms and extracellular keratinase (Semple *et al*, 2001; Tork *et al*, 2010). feather degrading microorganisms utilizes feather keratin as a source of carbon and energy for growth and survival, potential keratin degrading microorganisms could be isolated on feather meal agar as have been previously reported (Tapia and Simoes, 2008). A total of thirty-two microbial isolates were selected and differentiated based on their ability to grow on feather meal agar and their distinctive characteristic features. These isolates were screened for keratinolytic activity by the keratinase assay. Three isolates identified as isolates number PE2, PW11 and PW12 demonstrated the highest keratinase activity were selected for further study.

The selected isolate number PE2, PW11 and PW12 were identified by number of morphological, physiological and biochemical properties were used as well as the basis of sequence analysis of 16S rRNA. Based on the morphological, cultural characteristic and phylogenetic tree, isolates PE2 and PW11 had 100% homology in 16S rRNA sequence to *Bacillus subtilis* (PE2) and *Enterobacter cloacae* (PW11), respectively and isolate 12 had 100% homology to *Aeromones* media-like DNA group 5A (PW12) (Fig. 4). The interplay of various factors such as temperature, pH, substrate concentration, the nature and composition of carbon and nitrogen sources and the condition of inoculants, influences cell growth and survival, and the levels of keratinase synthesis and secretion in a microbial culture (Singh *et al*, 1975; North, 1982). Optimization of these factors results in optimum conditions of microbial growth and synthesis of bioproducts.

The results are in accordance with some previously reported keratinases which belonged to the family of alkaline protease (Altalo and Gashe, 1993; Cheng et al., 1995), indicating that the keratinase produced by *Bacillus* species might be most active in neutral or basic conditions. However, the keratinase started to decrease when the initial pH reached 8.5 which inhibit the growth of many of *Bacillus* family.

As the two fast nitrogen sources, ammonia and nitrate salts were added into the media. The addition of fast nitrogen source (that is, ammonia salt and nitrate salt) restricted the induction of keratinase. Therefore, it is

necessary to exclude any ammonia salt and nitrate salt in the medium for the production of keratinase by all isolates studies. This result is in agreement with that of previous study (Cai and Zheng, 2009).

Optimization of the concentration of corn steep flour in culture medium was 7 g/l gave the highest keratinase activity. These results were confirming the presumption of Chen et al. (2002) that keratinase was induced to breakdown the feather into soluble protein to support cell growth.

Keratin substrates such as feather meal and chicken feather induces keratinase production (Brandelli and Riffel, 2005; Chao *et al*, 2007; Anbu *et al*, 2007). Similarly, the maximum yield of keratinase was seen in basal media supplemented with chicken feathers as compared to other substrate like pigeon feather, hair and nail. Also complete degradation of the pigeon feather and chicken feather was seen.

Generally, the optimum keratinase production was achieved at feather meal substrate concentration of 2.0 % for culture growing at pH 7 and 37 ^{O}C. Increasing the substrate concentration to 2.5% resulted in reduced levels of keratinase production similar to previously reported repression of keratinase production at high substrate concentrations (Riffel *et al*, 2003; Brandelli and Riffel, 2005; Cai *et al*, 2008; Lin and Yin, 2010). Growth substrates constitutes around 30-40 % of total production cost of enzymes (Kumar and Parrack, 2003). Therefore, the use of low cost and readily available keratin substrates such as feather meal or chicken feather will enable production of PE2, PW11 and PW12 keratinase at industrially relevant scale. Currently, billions of tonnes of chicken feather waste is produced annually (American Chemical Society, 2011), making it a potentially important raw material. In addition, the use of feather waste as growth substrates for keratinase production will invariably serve as an efficient way to manage the large amount of feather wastes produced in the poultry industry.

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