

Isolation and Characterization of Keratinase-Producing Bacteria from Poultry Waste

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(Received: 25 December 2010; accepted: 21 February 2011)

A total of ten isolates were obtained from rotted feathers and soils from a local poultry farm waste at Bodija in Ibadan and screened for proteolytic activity and feather degradation. Selected isolates were identified as *Bacillus licheniformis* (E1), *Bacillus subtilis* A (D1), *Bacillus subtilis* B (D1W), *Bacillus cereus* (B1), *Pseudomonas aeruginosa* (E2) and *Pseudomonas putida* (B2). *B. licheniformis* (E1) gave highest feather degradation rate of 47% while the lowest degradation rate of 25% was observed with *B. subtilis* B (D1W). The effect of incubation time, pH and agitation rate on keratinase production was determined using feather powder as substrate. *B. licheniformis* (E1) had the highest keratinolytic activity of 25.336U/ml and *B. subtilis* B (D1W) had the lowest keratinolytic activity of 10.604U/ml at incubation time of 72hrs, pH 7.2 at 200rpm. The effect of other keratin substrates on keratinase production using chicken nails, wool and hair as substrates at pH 6.8 and 7.2 was determined. All the substrates induced keratinase production and the highest production was observed with feathers (25.336U/ml) followed by wool (22.5U/ml) and then hair (20.04U/ml). Keratinase production by nails was the least with the value of 6.43U/ml. The isolated bacteria were capable of degrading other keratin-containing materials including feathers, hair, wool and nail. The isolated *B. licheniformis* with the highest keratinolytic activity could be a potential organism for degradation and utilization of keratin.

Key words: Keratinases, Feather degradation, Proteolytic activity, Keratin substrates.

Keratin is a family of fibrous structural proteins, the monomers assemble into bundles to form intermediate filaments, which are tough and insoluble and form strong unmineralized tissues found in reptiles, birds, amphibians, and mammals. They are rivaled as biological materials in toughness only by chitin (Hickman *et al.*, 2003). There are various types of keratins within a single bioform. The α -Keratins in the hair (including wool), horns nails, claws and hooves of animals, and the harder β -keratins found in nails and in the

scales and claws of reptiles, their shells (chelonians, such as tortoise, turtle, terrapin), and in the feathers, beaks, claws of birds and quills of porcupines. These keratins (β -keratins) are formed primarily in beta sheets. However, beta sheets are also found in β -keratins (Kreplak *et al.*, 2004). In addition to intramolecular and intermolecular hydrogen bonds, keratins have large amounts of the sulfur-containing amino acid cysteine required for the disulfide bridges that confer additional strength and rigidity by permanent, thermally-stable crosslinking (Lee *et al.*, 2002).

Keratinases are proteolytic enzymes that catalyse the cleavage of keratin (Saunders *et al.*, 2007). Microbial keratinases have become biotechnologically important since they target the hydrolysis of highly rigid, strongly cross-linked

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structural polypeptide “Keratin” which is recalcitrant to the commonly known proteolytic enzymes. These enzymes (Keratinases) are largely produced in the presence of keratinous substances in the form of hair, feathers, wool, horn etc. (Parry *et al.*, 1977; Bragulla and Hermann, 2009). Bacterial strains are known which are capable of producing keratinase. This enzyme makes it possible for the bacteria to obtain carbon, sulfur and energy for their growth and maintenance from the degradation of keratin (Suzuki *et al.*, 2006). Previous studies have isolated keratin producers of the genera *Microsporium* (Mignon *et al.*, 1998; Takiuchi *et al.*, 1984), *Trichophyton* (Qin *et al.*, 1992; Tsuiboi *et al.*, 1989), *Bacillus* (Cheng *et al.*, 1995; Han and Damodaran, 1998, Lee *et al.*, 2002) *Streptomyces* (Bockle *et al.*, 1995; Mukhopadhyay and Chandia, 1990), *Aspergillus* (Friedrich *et al.*, 1999) and *Doratomyces* (Gradisar *et al.*, 2000).

Feathers are composed of over 90% protein (Onifade *et al.*, 1998) and produced in large amount as a waste by poultry processing worldwide. Accumulation of feather will lead to feather protein wastage (Onifade *et al.*, 1998; Gousterova *et al.*, 2005). For keratinase production, different keratin-containing materials such as feathers and wool can be used as substrates (Gupta and Ramnani, 2006), although, other keratin-containing materials such as Nails and hair may serve as potential substrates for keratinase production. The present research therefore is carried out to isolate keratinase-producing bacteria from poultry feather waste, determine the effect of cultural conditions on keratinase production and feather degradation and to determine the effect of other substrates on keratinase production.

MATERIAL AND METHODS

Collection of samples

Rotted feather samples and soil were collected from a local poultry plant's waste site at Bodija in Ibadan.

Preparation of feather culture media

The basic medium used for the isolation and cultivation of the feather-degrading microorganisms contained the following constituents (g/L); NaCl (0.5), KH_2PO_4 (0.7), K_2HPO_4 (1.4), MgSO_4 (0.1), and feathers (10), pH 7.2. Feather agar medium containing the basic

medium and 20 g/L of agar was used for screening the microorganisms in plates (Cai *et al.*, 2008).

Isolation of keratinolytic organisms

Ten grams (10g) of rotted feather samples and soils collected from a local poultry plant's waste site at Bodija in Ibadan were shaken in 100ml 0.85% (w/v) NaCl solution in 500ml Erlenmeyer flasks for 30 minutes, and 1ml of the suspensions were inoculated on feather agar medium ((g/L); NaCl (0.5), KH_2PO_4 (0.7), K_2HPO_4 (1.4), MgSO_4 (0.1), and feathers¹⁰), pH 7.2 using the pour plate technique, followed by cultivation at 37°C for 48 hours. Well-grown single colonies were isolated and purified by streak-plating onto new feather agar plates. The largest single colony on the plate was isolated and inoculated into feather (broth) medium-containing flasks and shaken at 200rpm, 37°C for 3 days. The feathers in the flasks were observed for degradation by purified isolates which were designated and maintained on Luria-Bertani (LB) agar slants (containing peptone 1% (w/v), yeast extract 0.3% (w/v), NaCl 0.5% (w/v), pH 7.2) at 4°C for further work.

Screening for Proteolytic Activity

Test for proteolytic activity was carried out using skimmed milk agar as described by Harrigan and McCance (1966). The agar was inoculated with the isolates and incubated for 2 days at 37°C. At the end of incubation, clear zones around inoculum indicated were measured and recorded. Isolates with distinct clear zones were selected for further work and were identified according to *Bergey's Manual of Systematic Bacteriology* (Sneath *et al.*, 1986).

Preparation of keratin solution

Keratinolytic activity was measured with soluble keratin (0.5%, w/v) as substrate. Soluble keratin was prepared from white chicken feathers by a modified method of Wawrzkievicz *et al.* (1987). Native chicken feathers (10g) in 500ml of dimethyl sulfoxide were heated at 100°C for 2hrs. Soluble keratin was then precipitated by addition of cold acetone (1L) at freezing temperature for 6 h, followed by centrifugation at 10 000×g for 10 min. The resulting precipitate was washed twice with distilled water and dried in an oven dryer. One gram of quantified precipitate was dissolved in 20ml of 0.05mol/L NaOH. The pH was adjusted to 8.0 with 0.1 mol/L Tris and 0.1mol/L HCl and the solution was diluted to 200ml with 0.05mol/L Tris-HCl buffer (pH 8.0).

Determination of keratinolytic activity

The keratinolytic activity was assayed as follows: 1.0 ml of crude enzyme properly diluted in Tris-HCl buffer (0.05mol/L, pH 8.0) was incubated with 1ml keratin solution at 50°C in a water bath for 10min, and the reaction was stopped by the addition of 2.0ml of 0.4mol/L trichloroacetic acid (TCA). After centrifugation at 1450×g for 30min, 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. One unit (U/ml) of keratinolytic activity was defined as an increase of corrected absorbance of 280nm (A_{280}) (Gradisar *et al.*, 2005) with the control for 0.01 per minute under the conditions described above and calculated by the following equation:

$$U = 4 \times n \times A_{280} / (0.01 \times 10)$$

Where;

n = dilution rate

4 = final reaction volume (4ml)

10 = incubation time (min).

Substrate preparation

Local chicken feathers were thoroughly washed with water, oven-dried at 40°C for 24hrs and milled to powder. After sieving to remove undesirable strands, the feather powder was packed in clean sample bags and kept at 30±2°C until use.

Effect of Different incubation time on keratinase production and residual hydrolysates

This was carried out on each isolate using the method of Cai *et al.* (2008). Young culture of the isolate was inoculated in McCartney bottles containing 10ml of feather medium and was incubated at 30±2°C on a shaker at 200rpm for 24hrs. This was repeated for each isolate at incubation time of 48, 72 and 96hrs. The residual hydrolysates were removed by centrifugation at 1450×g for 30min and dried for their determination. The weight of the residual hydrolysates was determined by subtracting the weight of the filter paper from the weight of the filter paper and residue. The cell free supernatant was analyzed for keratinase activity.

The Effect of pH variation on keratinase production and residual hydrolysates

The effect of different pH on keratinase production was carried out on each isolate using

the method of Cai *et al.* (2008). McCartney bottles containing 10ml of feather medium were inoculated with young culture of isolates. Cultivation was performed at 200rpm at 28±2°C for 96hrs at different pH of 6.0, 6.4, 6.8, 7.2 and 8.0 (pH was maintained using phosphate buffer). The keratinase activity and residual hydrolysates were determined after incubation.

Effect of Agitation Rate on keratinase production

This was carried out on each isolate using the method of Cai *et al.* (2008). Young culture of the isolate was inoculated in McCartney bottles containing 10ml of feather medium and was incubated at 30±2°C on a shaker at 200rpm for 24hrs. This was repeated for each isolate at different agitation rates of 100, 150, 200, 250 rpm at pH of 6.8 and 7.2 for each speed. The keratinase activity and residual hydrolysates were determined after incubation.

Effect of different substrates on keratinase production

The effect of different substrates on keratinase production was determined by the method of Cai *et al.* (2008). One percent (w/v) hair, wool, chicken nail and feather powder were used as sole source of carbon and nitrogen for keratinase production. Cultivation was performed at 200rpm at 30±2°C for 24h, 48h, and 72h at pH of 6.8 and 7.2 each.

RESULTS

A total of 10 microorganisms were isolated and were subjected to feather degradation and

Table 1. Feather degrading and proteolytic abilities of isolates

Isolates	Feather degradation	Clear zone on skimmed milk (mm)
B2	++	15
B4	++	21
B5	-	-
D1W	+++	17
D1	++	21
D2	+	-
E1	+++	35
E2	+++	28
E3	++	-
F1	-	-

- = No degradation, + = Partial degradation
 ++ = Moderate degradation +++ = Complete degradation

Table 2. Effect of pH on keratinase production and residual hydrolysates.

Isolates	B 2		E 1		D 1		D 1 W		B 4		E 2	
	Keratinolytic activity (U/ml)	Residual hydrolysates (g)	Keratinolytic activity (U/ml)	Residual hydrolysates (g)	Keratinolytic activity (U/ml)	Residual hydrolysates (g)	Keratinolytic activity (U/ml)	Residual hydrolysates (g)	Keratinolytic activity (U/ml)	Residual hydrolysates (g)	Keratinolytic activity (U/ml)	Residual hydrolysates (g)
pH 6.0	24	1.398 ^m	0.100 ^a	0.100 ^a	6.826 ^m	0.100 ^a	6.272 ^a	0.098 ^a	3.002 ^a	0.100 ^a	0.164 ^j	0.100 ^a
	48	1.531 ^m	0.100 ^a	0.095 ^{bc}	7.296 ^l	0.095 ^{bc}	6.115 ^r	0.095 ^b	6.532 ^{kl}	0.093 ^c	0.394 ^j	0.100 ^a
	72	1.526 ^m	0.100 ^a	0.093 ^c	7.259 ^l	0.095 ^{bc}	7.333 ^j	0.094 ^{bc}	6.443 ^{kl}	0.092 ^c	0.386 ^j	0.100 ^a
	96	1.624 ^m	0.100 ^a	0.092 ^c	7.232 ^l	0.094 ^c	7.610 ^s	0.094 ^{bc}	6.135 ^m	0.092 ^c	0.248 ^j	0.100 ^a
pH 6.4	24	3.923 ^l	0.099 ^a	0.099 ^a	7.239 ^l	0.100 ^a	6.460 ^p	0.100 ^a	5.007	0.100 ^a	10.094 ⁱ	0.100 ^a
	48	6.488 ^h	0.092 ^b	0.087 ^d	8.440 ⁱ	0.094 ^c	7.470 ^h	0.091 ^c	6.833 ⁱ	0.095 ^b	15.623 ^d	0.093 ^b
	72	5.526 ^j	0.087 ^c	0.084 ^d	10.496 ^f	0.093 ^c	9.236 ^e	0.091 ^c	6.252 ^j	0.095 ^b	14.600 ^e	0.083 ^{de}
	96	4.644 ^k	0.087 ^c	0.079 ^f	9.960 ^h	0.093 ^c	9.410 ^e	0.087 ^d	7.239 ^h	0.093 ^c	11.280 ^h	0.079 ^f
pH 6.8	24	10.827 ^d	0.098 ^a	0.100 ^a	10.236 ^g	0.099 ^a	6.648 ^m	0.100 ^a	5.391 ^p	0.100 ^a	15.412 ^d	0.097 ^a
	48	15.526 ^b	0.082 ^{de}	0.081 ^{ef}	10.462 ^f	0.092 ^{cd}	6.484 ^o	0.096 ^b	8.364 ^e	0.095 ^b	21.644 ^a	0.075 ^g
	72	17.687 ^a	0.079 ^e	0.067 ^g	12.001 ^d	0.089 ^d	9.439 ^b	0.090 ^c	8.265 ^f	0.091 ^c	20.632 ^b	0.073 ^g
	96	17.393 ^a	0.079 ^e	0.066 ^g	12.623 ^c	0.086 ^e	7.042 ⁱ	0.087 ^d	7.910 ^g	0.087 ^d	20.720 ^b	0.073 ^g
pH 7.2	24	10.488 ^e	0.098 ^a	0.098 ^{ab}	10.976	0.100 ^a	6.540 ⁿ	0.098 ^a	5.280 ^o	0.097 ^a	13.012 ^g	0.100 ^a
	48	14.888 ^c	0.083 ^d	0.079 ^f	11.88 ^d	0.092 ^{cd}	7.392 ⁱ	0.092 ^c	12.644 ^a	0.085 ^e	18.484 ^c	0.081 ^e
	72	7.008 ^g	0.079 ^e	0.053 ⁱ	17.248 ^b	0.086 ^e	10.604 ^a	0.088 ^d	9.600 ^b	0.084 ^e	20.528 ^b	0.076 ^g
	96	5.520 ⁱ	0.079 ^e	0.062 ^h	17.456 ^a	0.081 ^f	8.040 ^f	0.088 ^d	8.956 ^d	0.084 ^e	14.496 ^c	0.073 ^g
pH 8.0	24	9.210 ^f	0.100 ^a	0.100 ^a	6.692 ^m	0.100 ^a	5.431 ^s	0.100 ^a	5.431 ⁿ	0.098 ^a	12.830 ^g	0.099 ^a
	48	10.327 ^e	0.093 ^b	0.095 ^{bc}	6.323 ^m	0.095 ^{bc}	6.629 ^m	0.095 ^b	6.629 ^j	0.095 ^b	13.073 ^g	0.089 ^c
	72	6.078 ⁱ	0.093 ^b	0.082 ^{ef}	8.277 ⁱ	0.095 ^{bc}	9.313 ^d	0.094 ^{bc}	9.313 ^c	0.091 ^{cd}	15.655 ^d	0.085 ^d
	96	4.081 ⁱ	0.092 ^b	0.081 ^{ef}	7.866 ^l	0.095 ^{bc}	7.141 ^k	0.091 ^c	7.141 ^h	0.090 ^{cd}	14.006 ^f	0.085 ^d

Means with the same letter in the same column are not significantly different (P<0.05) using Duncan's multiple range test

Table 3. Effect of shaking speed on keratinase activity of the isolates

Agitation (rpm)	pH	Keratinolytic activity (U/ml) rate Isolates							
		E1	D1	D1W	B4	E2	B2		
100	6.8	20.081±0.022	10.098±0.016	8.648±0.074	7.488±0.032	18.272±0.059	15.326±0.064		
	7.2	24.085±0.005	15.264±0.047	10.06±0.042	8.52±0.095	18.081±0.035	6.095±0.005		
150	6.8	23.764±0.000	12.623±0.054	9.012±0.160	7.821±0.043	19.627±0.078	17.765±0.024		
	7.2	25.127±0.003	17.823±0.07	10.274±0.014	9.893±0.387	20.735±0.075	7.239±0.047		
200	6.8	24.06±0.035	12.007±0.08	9.439±0.019	8.265±0.04	20.632±0.085	17.687±0.06		
	7.2	25.336±0.004	17.248±0.05	10.604±0.049	9.6±0.190	20.528±0.063	7.008±0.078		
250	6.8	23.876±0.082	11.626±0.07	9.722±0.086	7.856±0.072	20.926±0.071	17.221±0.043		
	7.2	25.326±0.054	16.023±0.07	10.029±0.049	9.786±0.102	19.98±0.045	6.876±0.056		

Each value is a duplicate determination of mean±SEM (n=2)

proteolytic activity test. Six strains of keratinase-producing bacteria showing elevated feather degradation and proteolytic activity (Table 1) were selected for further studies. They were characterized into five species belonging to two genera and are identified as *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Bacillus licheniformis*, *Bacillus subtilis* and *Bacillus cereus* based on their cultural, morphological, biochemical and physiological properties. It was observed that aeration (aerobic growth) supported the growth of isolates and feather degradation. From table 1, profuse degradation is observed in three of the isolates; E2 (*Pseudomonas aeruginosa*), E1 (*Bacillus licheniformis*) and D1 (*Bacillus subtilis*) of which E1 has the highest proteolytic activity (35mm) followed by E2 (*Pseudomonas aeruginosa*) (28mm), while B2 (*Pseudomonas putida*) has the least proteolytic activity with a clearing zone of 15mm.

Fig. 1 shows the time course production of keratinase by the isolates. The figure shows that all six isolates produced keratinase within the first 24hrs of cultivation. Isolate E1 (*Bacillus licheniformis*) showed highest keratinolytic activity of 25.3U/ml followed by E2 (*Pseudomonas aeruginosa*) with an activity of 21.644 U/ml. The lowest keratinase activity was recorded in isolate D1W (*B. subtilis* B) with a unit of 10.6 U/ml. Isolates B2 and B4 had their highest enzyme activity at 48hrs of incubation, after which a sharp decline in activity was observed for B2. At 72hrs of cultivation, E1, E2 and D1W are shown to have their optimum enzyme production. D1 (*Bacillus subtilis* A) shows highest keratinolytic activity at 96hrs of cultivation with a slight increase in keratinolytic activity from 17.248 to 17.456U/ml.

The effect of pH on keratinolytic activity is shown in table 2. All the isolates had their least keratinolytic activity at pH 6.0. Isolate E1 showed highest activity of 25.336U/ml compared to other isolates, this optimum activity was reached at pH 7.2, which was also the optimum pH for D1 (17.456U/ml), D1W(10.604U/ml) and B4 (12.644U/ml). Isolates E2 and B2 show optimum keratinolytic activity at pH 6.8 after which there was a decline as pH increased to 8.0. The result showed that both pH and incubation time has significant difference on all the isolates except for isolates E2 and B2 at pH 6.0. The result of the residual hydrolysates shows

that the least feather hydrolysates matched the highest keratinolytic activity for each of the isolate. *B. licheniformis* (E1) with highest optimum keratinolytic activity showed the least residual hydrolysates of 0.053g. *B. subtilis* B (D1W) recorded the highest value for residual hydrolysates of 0.088g at its optimum keratinolytic activity. The table also shows that the feather

hydrolysates reduce as pH increases, although statistically, there is no significant reduction in feather hydrolysates for all isolates (from pH 6.0 to 8.0) at 24hrs. For isolate E2 and B2 there is no significant reduction in residual hydrolysates for pH 6.8-7.2 at 72hrs. So also for isolate D1W (at pH 6.8-7.2) For each pH, residual hydrolysates reduce as incubation time increases.

Table 4. Feather degradation levels for isolates during optimum keratinase production

Isolates	Optimum production condition			Feather reduction (%)
	pH	Incubation time(h)	Shaking speed (rpm)	
E1(<i>B.licheniformis</i>)	7.2	72	200	47
E2 (<i>P. aeruginosa</i>)	6.8	48	250	43
B4 (<i>P. putida</i>)	6.8	72	150	39
D1w (<i>B. subtilis</i> A1)	7.2	72	200	32
B4 (<i>B. cereus</i>)	7.2	48	150	27
D1(<i>B. subtilis</i> B)	7.2	96	150	25

From table 3 all the isolates show that keratinolytic activity increases with increase in agitation rate. Isolates E1 and D1W show a steady increase in enzyme activity from 100rpm through to 200rpm at both pH 6.8 and 7.2. Isolates D1 and B4 also showed a steady increase in activity as the shaking speed increases from 100 to 200rpm (at pH 6.8) after which there was a decline in activity as the shaking speed increases to 200rpm while their activity at pH 7.2 was noted to be highest at 150rpm.

Generally, *P. putida* (B2) (17.765 ± 0.024), *B. subtilis* A (D1) (17.823 ± 0.07) and *B. cereus* (B4) (9.893 ± 0.387) showed optimum keratinase activity at a shaking speed of 150rpm, *B. licheniformis* (E1) (25.336 ± 0.004), *B. subtilis* (B) (10.604 ± 0.049) showed optimum keratinase activity at a shaking speed of 200rpm, while E2 (*P. aeruginosa*) (20.926 ± 0.071) showed optimum keratinase production at an agitation rate of 250 rpm. The results show significant increase in keratinolytic

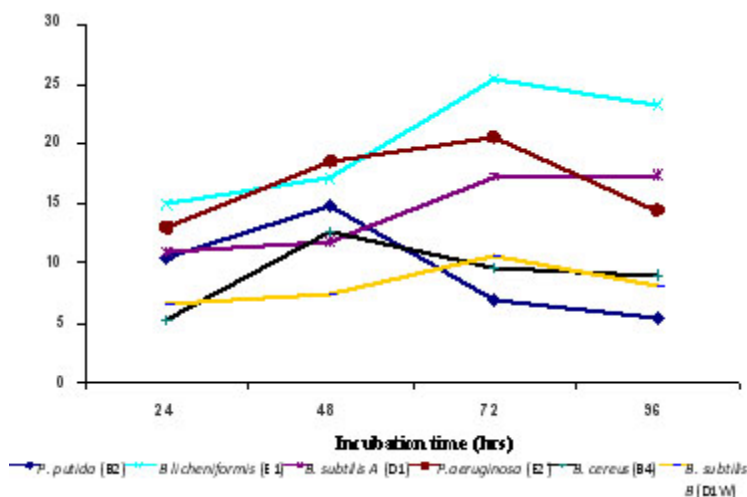


Fig. 1. Time course production of keratinase for the isolates

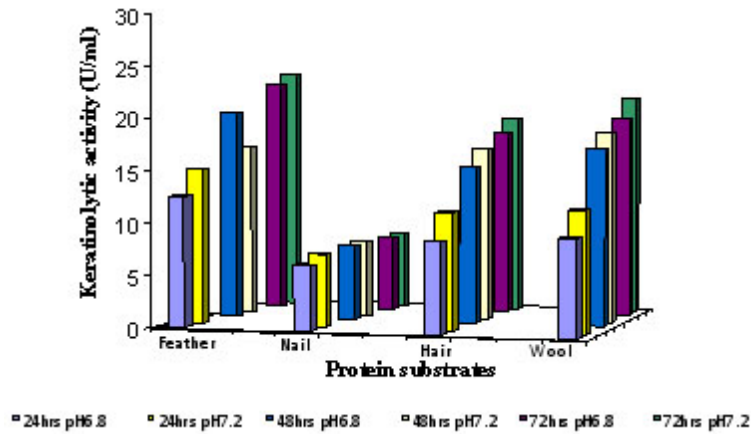


Fig. 2. Keratinase production by *B. licheniformis* (E1) on different substrate

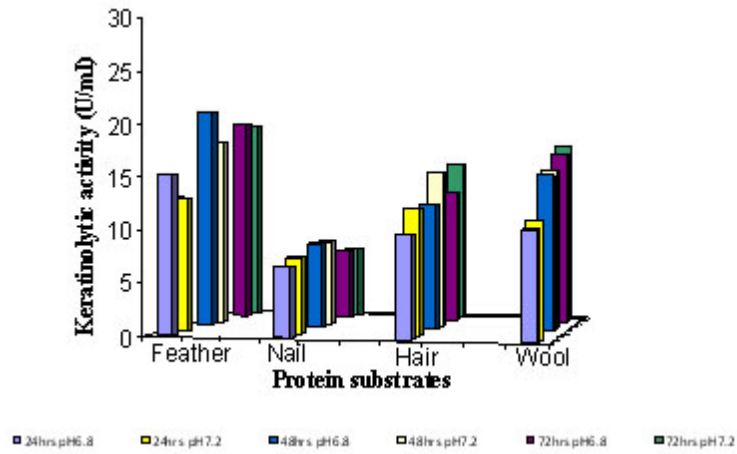


Fig. 3. Keratinase production by *P. aeruginosa* (E2) on different substrates

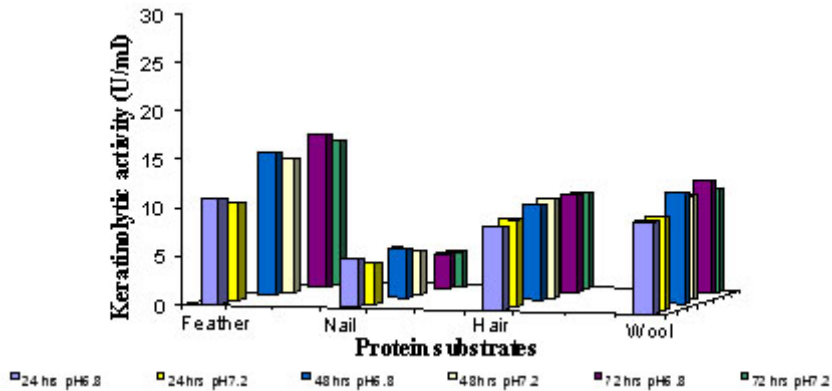


Fig. 4. Keratinase production by *P. putida* (B2) on different substrates

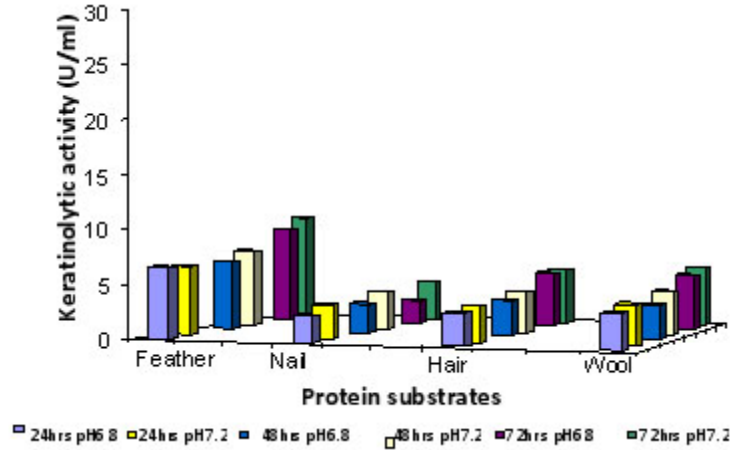


Fig. 5. Keratinase production by *B. subtilis* (B) (D1W) on different substrates

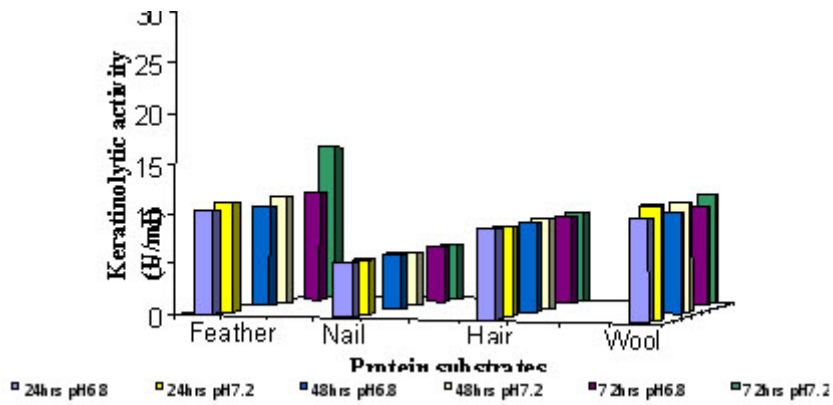


Fig. 6. Keratinase production by *B. subtilis* (A) (D1) on different substrates

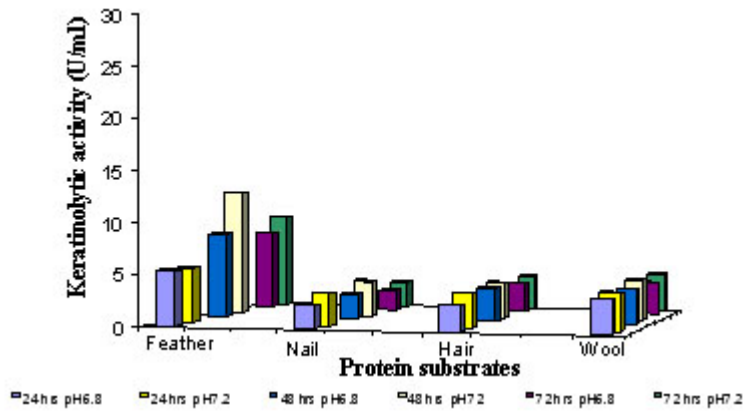


Fig. 7. Keratinase production by *B. cereus* (B4) on different substrates

activity as pH increases from 6.0 to 6.8 for isolates B2 and E2 while activity increases significantly from pH 6.0 to 7.2 for isolates E1, D1, D1W and B4.

Feather hydrolysates results were used to obtain the degree of degradation of feather by isolates (Table 4). Feather reduction level by E1 (*B. licheniformis*) was observed to be highest at (47%) an optimum production condition of pH, incubation time and shaking speed of 7.2, 72hrs and 200rpm respectively. This degradation level is followed by a 43% feather degradation level by E2 (*P. aeruginosa*) at optimum production of condition of pH 6.8, shaking speed of 250rpm for 48hrs. The least feather reduction level of 25% was observed in *B. subtilis* (B) (Isolate D1W) at an optimum condition of pH 7.2, 96hrs incubation time and 150rpm shaking speed.

The result of the effect of different keratinolytic substrate on keratinase production by *Bacillus licheniformis* (E1) is shown on figure 2. From the figure, production of keratinase is highest using feather as substrates followed by wool, then hair, while nail gave the lowest keratinase production. The figure also shows that keratinolytic activity increases with increase in cultivation time for all the substrates. Higher keratinolytic activity was recorded at pH 7.2 for all the other keratinolytic substrates except for feather (pH 6.8).

Figure 3 shows keratinase production by *P. aeruginosa* (E2) on different substrates. The figure shows that E2 produces least keratinolytic activity with nails as substrates and highest with feathers followed by wool and then hair. The figure also shows that nail produced highest keratinolytic activity at 48hrs of cultivation while activity was higher for hair and wool at 72hrs of cultivation

Keratinase production by isolate B2 (*P. putida*) on different substrates is shown in figure 4. Only nail is shown to have highest keratinase production at 48hrs while other substrates show increase in production of keratinase as incubation time increases. Nails produced least keratinolytic activity and Wool produced highest activity after feather substrates.

The result of the effect of different substrates on keratinase production by isolate D1W (*B. subtilis* B) (Fig. 5). Generally, keratinolytic activity of isolate D1W increases with increase in cultivation time for all four substrates used, though

low activity was recorded for all substrates. Feathers produced highest keratinolytic activity while nails gave the lowest keratinolytic activity. The figure also shows that keratinolytic activity increases with increase in cultivation time.

Keratinase production by isolate D1 (*B. subtilis* A) using different substrates (Fig. 6). For the different substrates, keratinase production increases with incubation time. Keratinolytic activity is higher at pH 7.2 than at pH 6.8 for all the substrates. The figure also shows that nail produces least activity while feather shows highest enzyme activity followed by wool, then hair.

DISCUSSION

Six isolates from poultry waste has been shown to degrade feather keratin by using feathers as a primary source of energy, carbon and nitrogen. Previous literatures have documented the isolation of similar keratinase-producing strains of *Bacillus licheniformis*, *B. subtilis*, *B. pumilis*, *B. cereus*, *B. halodurans*, *B. pseudofornis* (Williams *et al.*, 1990; Takami *et al.*, 1999; Rozs *et al.*, 2001; Kim *et al.*, 2001; Gessesse *et al.*, 2003; El-Refai *et al.*, 2005), *Pseudomonas sp.* (Brandelli and Riffel, 2006) and fermentation studies concerning their respective keratinase production. They also reported maximum growth under aerobic conditions as would be expected of the families *Bacillaceae* and *Pseudomonadaceae* (Williams *et al.*, 1990). Most *Pseudomonas* species are found in soil, water and most man-made environment through the world; they thrive not only in normal atmosphere, but also with little oxygen and thus colonize many natural and artificial environments (Balcht and Raymond, 1994). One explanation for the presence of a keratin-degrading organism in poultry waste according to Joshi *et al.* (2008) may be that the bacterium is indigenous to the chicken gut; however, it is more likely that it is indigenous to the environment in which poultry excreta are collected. This environment also contains feather and the isolate may have adapted to utilize this substrate.

Keratinases do not only hydrolyze keratin but have the ability to hydrolyze a wide range of soluble and insoluble protein substrates as observed for keratinases of *Bacillus licheniformis* (Lin *et al.*, 1992) and *Doratomyces microsporium*

(Fredrich and Antranikianern, 1996), this serves as a basis for the proteolytic hydrolysis of casein in the proteolytic activity test of this study. Proteolytic activity test shows that *Pseudomonas putida* (B2) gave the lowest proteolytic activity as indicated by a 15mm clear zone on skimmed milk agar. This value is slightly lower than that obtained by *Bacillus subtilis* B (D1W) (17mm) and *B. cereus* (B4) (21mm) indicating that *Bacillus subtilis* B (D1W) and *B. cereus* (B4) have a higher proteolytic activity than *Pseudomonas putida* (B2). However, the keratinolytic activity assay shows that *Pseudomonas putida* (B2) with lowest proteolytic activity recorded a higher keratinolytic activity of 14.888U/ml than those of *B. subtilis* B (D1W) (10.604U/ml) and *B. cereus* (12.644U/ml). A similar observation was made by Brandelli and Riffel (2006) where a *Bacillus* species (isolate Kr 10) was shown to have a larger clearing zone than another keratinolytic strain of *Chryseobacterium* sp. (Kr6) on milk agar but showed a far lesser keratinolytic activity (of more than 20 units) than *Chryseobacterium* sp.

This observation may be due to the fact that keratinase-producing bacteria also produce one or more other broadly specific bacterial proteases such as alcalase, produced by *B. licheniformis*, pronase (produced by keratinase-producing *Streptomyces*) papain, alcalase, elastase and trypsin (Brandelli, 2005). Therefore, the proteolytic activity test did not only show the ability of the keratinase enzyme from isolates to hydrolyze casein, but might have included the activities of other proteolytic enzymes synthesized along side with keratinase by isolates.

The screening of the bacteria isolated from a poultry industry waste resulted in the selection of feather degrading strains, it was observed that aerobic growth by the isolates on feathers, with feathers serving as its primary source of carbon, nitrogen, and energy resulted in nearly complete feather degradation by one of the isolates; E1 (*B. licheniformis*) after 72hrs of cultivation at $30 \pm 2^\circ\text{C}$. It showed an activity of 25.3 U/ml which is lower when compared to the *Bacillus licheniformis* (PW) strain with optimum keratinolytic activity of 50U/ml (Joshi *et al.*, 2008) observed at the 12th day (288th hour) of incubation and the *B. licheniformis* (PWD-1) strain (Shih *et al.*, 1992) with optimum keratinolytic activity of

30.50U/ml. The demonstration of keratinase enzymes by *B. licheniformis* 511 (Matkeviciene *et al.*, 2009) showed a keratinolytic activity of 82U/ml after 48hrs of cultivation. The result is similar very similar to that of *Bacillus licheniformis* (FK-28) (Dakrong and Worapot, 2001) which was observed to yield optimum keratinolytic activity of 26U/ml on the third day (72hr) of cultivation at 37°C . The variation in the cultivation time for maximum keratinolytic activity by the same species of organism may be as a result of variety in strains and differences in other parameters such as enzyme treatment and purification and the type of substrates used in the determination of keratinolytic activity of the enzymes.

All strains of selected bacteria produced maximum level of keratinase under different cultivation time, majority of the isolates had optimum keratinolytic activity between 48 and 72 hrs of incubation, except for isolate D1 (*B. subtilis* A) which had a non-significant increase after 72hrs of cultivation. Matkeviciene *et al.* (2009) discovered that the keratinase activity of all the *Bacilli* he worked with reduced drastically after 72hrs. During the submerged cultivation of *Bacillus* KD-N2 (Cai *et al.*, 2008) maximum keratinolytic activity was achieved at about 30hrs, after which activity started to decrease. Although a study by Joshi *et al.* (2008) gave a maximum keratinolytic activity after 11 days of cultivation. The two *Pseudomonas* species produced highest keratinolytic activity of all the isolates at 48hrs after which the activity of *Pseudomonas putida* sharply reduced to the least at 72 hours. The highest keratinolytic activity (21.644U/ml) of *P. aeruginosa* was recorded at time which is quite low compared to about 48U/ml recorded for a *Pseudomonas* species examined by Brandelli and Riffel (2006). *Bacillus licheniformis* gave the highest keratinolytic activity of 25.3U/ml, a result similar to that obtained from the work carried out on *B. subtilis* by Cai *et al.* (2008) with an activity of 24.3U/ml, while in contrast with the isolated *B. subtilis* (B) in this work with a relatively low keratinolytic activity.

The result also shows that as initial pH is increased from 6.0 to 7.2 keratinolytic activity increased. *Bacillus* sp. showed maximum enzyme production between pH 6.8 and 7.2, as would be expected for optimum enzyme production of the

member of the family *Bacillaceae* (Williams *et al.*, 1990). The two *Pseudomonas* species identified had little or no growth at initial pH of 6.0 which is expected for most of the family *Pseudomonaceae* (Williams *et al.*, 1990), hence, little or no keratinase production at pH 6.0. Keratinolytic activity was then noticed to reduce for most isolates as initial pH increased from 7.2 to 8.0.

This same trend for keratinolytic activity with respect to pH was maintained with increase in cultivation time for 24hrs through to 96hrs, although at 24hrs and 48hrs *Pseudomonas aeruginosa* (E2) showed overall highest keratinolytic activity after which *B. licheniformis* emerged as highest at 72 and 96hrs. In another study on *Bacillus*, maximum keratinase production was obtained when initial pH of medium was 7.5. Poor growth was obtained when initial pHs were at 4.0, 6.0 and 9.0 (Atalo and Gashe, 1993). They also found the optimum pH of keratinase production by *Bacillus sp.* P-001A at pH 9.5. Cheng *et al.* (1995) reported highest keratinase production by *B. licheniformis* PWD-1 at an initial pH of 8.7.

The feather hydrolysate is expected to decrease as keratinase production by the isolates increases. As enzymes are produced, the digestion and solubility of the feather increases as described by Cai *et al.* (2008) in the electron micrography studies of the degradation of feathers, silk and hair during submerged cultivation process, leading to reduction in the mass of the substrate residue. Similar reduction in residual hydrolysate was obtained in this study. No significant reduction in mass of substrate residue was obtained for isolates B2 (*P. putida*) and E2 (*P. aeruginosa*) at pH 6 for the whole incubation period because the keratinolytic activity for that pH did not show any significant increase for both isolates.

Optimum shaking speed for keratinase-producing isolates was found to be between 150-200 rpm, this optimum range is found to be quite consistent when compared to other experiments in the past. The shaking speed at 150rpm yielded maximum keratinase production by *Bacillus sp.* Fk-28 (Dakrong and Warapot, 2001). Generally, increased shaking speed provided high oxygen transfer rate supporting cell growth. However, high shaking speed of about 250rpm gave good bacteria growth but low keratinase production possibly because too high dissolved oxygen and too much

shear stress repressed keratinase synthesis and excretion. Perlman (1969) reported that at low shaking speed of 100rpm, bacterial cells and substrates were not well mixed with heterogeneous distribution and lower oxygen dissolved result in lower keratinase production.

Both α -keratin (from hair and wool) and β -keratin (from feathers and nails) can be utilized as substrates for the inducement of keratinase production. From this work, it was observed that among all the keratin-containing substrates, feather was mostly utilized, followed by wool, hair and then nail, a result similar to that was obtained by Cai *et al.* (2008). Among all seven proteins used as substrates for keratinase activity, feather was highest followed by wool, before hair. Block (1958) stated that keratins differ in resistance towards enzymes and in sulfur content. Investigations on the composition of the keratins have indicated that they may be divided into two classes; eukeratins and pseudokeratins (Buckle *et al.*, 1997). It was stated that those keratin which are chemically similar to cattle horn (K α s) such as nails be named eukeratins. Eukeratins are insoluble keratins, resistant to enzymatic digestion which yields histidine, lysine, and Arginine in the molecular ratio of approximately 1:4:12. Pseudokeratins (such as wool) which do not yield histidine, lysine and Arginine in the molecular ratio of approximately 1:4:12 in general do not show the same resistance towards enzymatic digestion as do the eukeratins. From the categorization given above each of the substrates used in this study can be classified as either eukeratins or pseudokeratins: This may serve as one of the factors that determined the varying keratinolytic activity produced by the different keratin protein substrates.

Two Gram-negative organisms (E2; *Pseudomonas aeruginosa* and B2; *P. putida*) were obtained in this study. Although the works on keratinase from Gram-positive bacteria has been reported by many works (Buckle and Muller, 1997; Korkmaz *et al.*, 2004), there are few informations on keratinolytic proteases produced by Gram-negative bacteria (Allpress *et al.*, 2002; Lucas *et al.*, 2003). Of the two *Pseudomonas* species, *Pseudomonas aeruginosa* (E2) was able to compete favourably with other *Bacillus* species from this study (with activity of 21.644U/ml) emerging as the isolate with the highest

keratinolytic activity following *Bacillus licheniformis* (E1) (25.3U/ml). Brandelli and Riffel (2006) were able to isolate a *Pseudomonas* species as one of the three Gram-negative organisms isolated from feather waste.

Feathers contain mostly β -keratin, and as was described in other works, *Bacillus* species mostly produce β -keratin degrading keratinases (Korkmaz *et al.*, 2004; Hoq *et al.*, 2005). In past works *B. subtilis* have shown to produce better keratinolytic activity than *B. licheniformis* (Cai *et al.*, 2008; Matikeviciene *et al.*, 2009), in this work, *B. subtilis* and *B. licheniformis* strains demonstrates a different pattern; *B. licheniformis* showed higher keratinolytic activity than other *Bacillus* species.

CONCLUSION

In conclusion, six feather-degrading bacteria were isolated from poultry waste and the optimum conditions for keratinase synthesis by these isolates were determined, which is an essential step for the production of adequate amounts for application in research.

A pH of 7.2 favours the production of keratinase in most of the isolates. The optimum incubation time ranged from 48hr to 96hr and the optimum shaking speed of 150-250rpm is optimum for keratinase production

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